CLINICAL IMPLICATIONS OF IMMUNE CELL INFILTRATION IN

VULVAR INTRAEPITHELIAL NEOPLASIA

EDITH VAN ESCH

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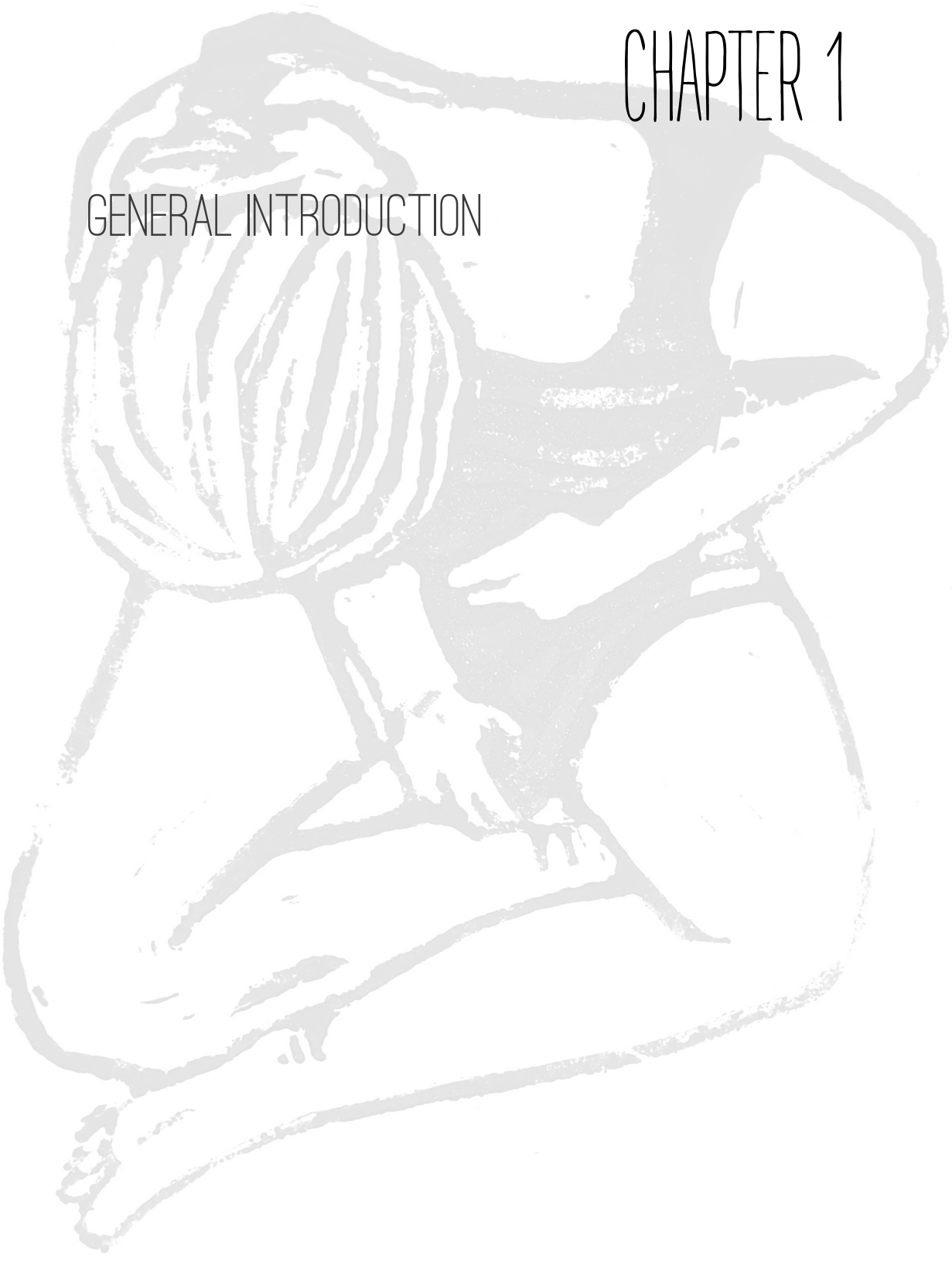
CHAPTER 1

GENERAL INTRODUCTION



In the last two decades a clear role of the immune system in tumor biology has been established for a wide variety of tumors. All types of immune cells can infiltrate both the tumor stroma and epithelium and often it is the balance between these cells that is associated with disease outcome[1]. The importance of the immune system in both rejecting and promoting tumors is stressed by the fact that the updated hallmarks of cancer, as defined by Hanahan and Weinberg in 2011[2], now also comprise avoidance of immune destruction and tumor promoting inflammation. Furthermore, the exploitation of tumor immunity to treat tumors, also named 'immunotherapy' was appointed as the breakthrough of the year in 2013[3]. Currently, many different immunotherapeutic approaches are being developed or combined as new treatments for cancer.

This thesis is dedicated to studies on the role of the immune system in the development of human papilloma virus (HPV) induced (pre-)malignancies of the vulva. It has been widely established that the immune system has an important role in protection against HPV and that it fails to protect a percentage of patients against the development of HPV-induced neoplasia. Immunotherapeutic approaches to treat this disease are being tested, but are successful in only a certain percentage of patients. Following an introduction on the immune system, a summary on the interaction between the immune system and HPV is given. In addition, the development and treatment of vulvar intraepithelial neoplasia (VIN) is discussed. Despite previous studies, our current knowledge on the immune response in VIN lesions is deemed insufficient to understand why patients respond or fail to respond to immune based therapies. The aim of this thesis was to increase this knowledge by the studies that are outlined at the end of this chapter.

1.1. Immune system

The immune system protects us from potential dangerous environmental factors as invasion of viruses, bacteria, fungi or parasites but also from the development of cancers. Different types of immune cells originate from the bone marrow where pluripotent stem cells divide and differentiate into progenitor cells that form the lymphoid, myeloid and erythrocyte/megakaryocyte lineages respectively[4]. The lymphoid lineage develops into B-cells, T cells and natural killer (NK) cells. The myeloid lineage develops into granulocytes (neutrophils, eosinophils and basophils), mast cells and monocytes which differentiate into dendritic cells and macrophages in tissues. The erythrocyte/megakaryocyte lineage forms erythrocytes and platelets[4]. The immune system is divided into two types of immune responses to pathogens; the non-specific innate immune response and the antigen-specific adaptive immune response[4]. A close collaboration between the innate and adaptive immune response

is required for an optimal response to threats and is mediated by a complex interaction of different cell types, cytokines and chemokines.

1.1.1. The innate immune response

The innate immune system is the inherited first line defence against a pathogen that responds immediately upon encounter. The innate immune system activates adaptive immune responses, regulates inflammation, mediates in immune homeostasis, is non-specific as it has no antigen specificity and does not produce an immunological memory to threats[4,5]. The main components are the physical epithelial barriers formed by the skin and mucosal surfaces, phagocytic neutrophils, monocytes and macrophages, dendritic cells, NK cells and the complement system[4].

The innate immune system recognizes pathogens through Pathogen Recognition Receptors (PRRs) present on and in different cells of the innate immune system and epithelial cells. The PRR recognize the molecular components of pathogens called pathogen-associated molecular patterns (PAMPs)[6]. PRRs are divided into two groups; the secreted and circulating PRRs and the transmembrane and intracellular signal transducing receptors. The first group includes antimicrobial peptides, collectins, lectins and pentraxins and these PRRs induce direct lysis of the pathogen, enhance phagocytosis and support the transmembrane PRRs[5]. The latter group of transmembrane and intracellular-transducing PRRs are plasma membrane bound and intracellular Toll-like receptors (TLRs), C-type lectin receptors, Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and RIG-1 like receptors (RLRs)[5]. Membrane bound PPRs are expressed by a variety of innate immune cells and on antigen presenting cells (APCs). In antiviral responses TLRs are essential (TLR 3, 4, 7, 8 and 9) since they trigger a pro-inflammatory response after activation of the NF-kB pathway[5] and TLR-agonists (e.g. TLR-4) can be used to restore immunogenicity in tumors lacking the TLR ligand[7,8].

In response to local factors, CD14+ myeloid cells can differentiate into APCs[9]. APCs process the antigens and present components on the cell surface to activate T cells in the lymph nodes. Three different professional APCs are known; macrophages, dendritic cells (DCs or Langerhans cells (LCs) as they are called when residing in the epidermis) and B lymphocytes[5]. Monocytes are the systemic circulating precursors of macrophages. Macrophages are present in infected tissues and are the first line defence of the innate immune system to eliminate extracellular pathogens by release of cytokines, chemokines and inflammatory mediators that activate other immune cells, create a state of local inflammation and recruit neutrophils and monocytes to the site of infection[4]. Macrophages are also attracted to developing cancers where they can be roughly categorized into tumor suppressive type 1 (M1) or tumor promoting type 2 (M2) macrophages[9].

DCs ingest, process and present antigens which are subsequently shown to T cells in order to activate adaptive immunity or in case of self-antigens to maintain tolerance[5]. The outcome of such an interaction depends on the maturation status of DCs as well as their functional phenotype. In order to combat viruses and cancers, DCs need to mature and produce IL-12. Maturation of DCs is reflected by the production of pro-inflammatory cytokines, the increased expression of MHC-class I and II, as well as the co-stimulatory molecules (e.g. CD80 and CD86). DCs are considered to be the most potent cells to activate the adaptive immune system[5] (Fig. 1).

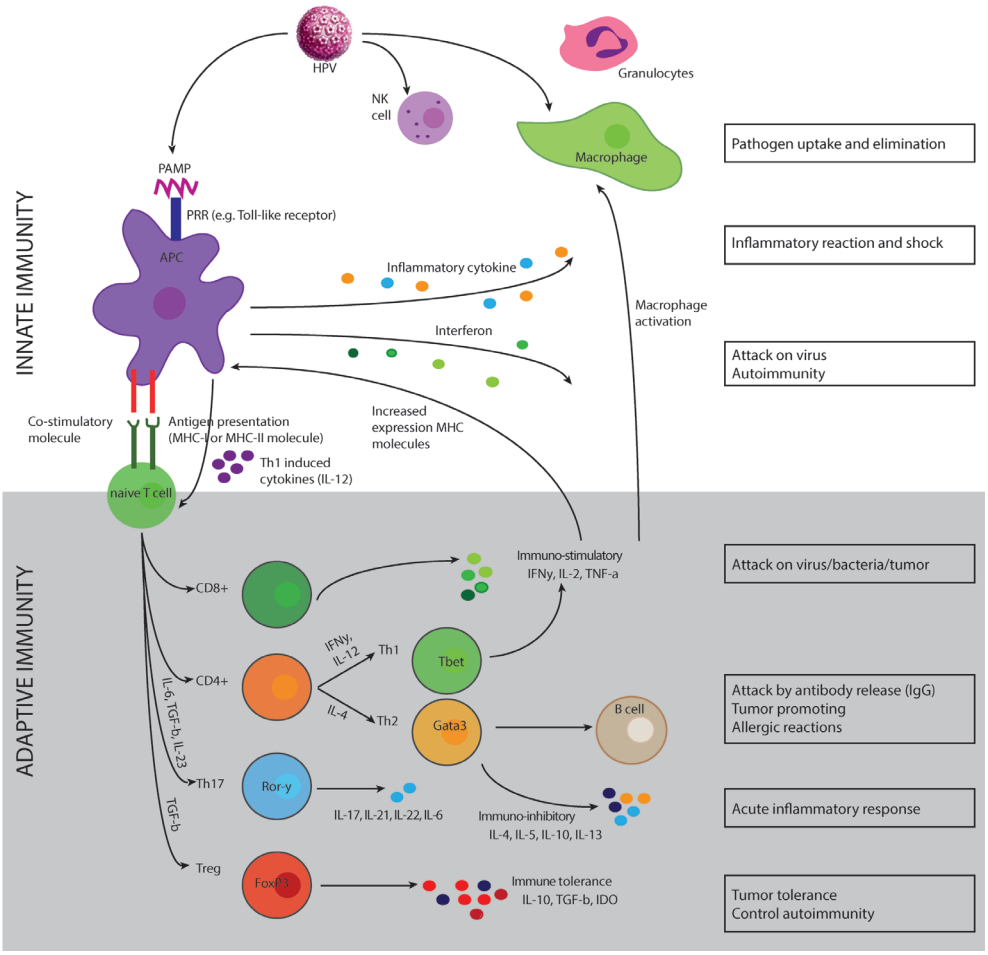


Figure 1 The innate and adaptive immuneresponse and T cell differentiation
Modified from O'Shea (Science 2010), Zou (Nature 2010) and http://jonlieffmd.com

NK cells are activated by cytokines and inflammatory mediators produced by macrophages and neutrophils and recognize and lyse virus infected or tumor cells. NK cells recognize healthy host cells from pathogens through killer immunoglobulin-like receptors (KIRs), which recognize major-histocompatibility (MHC) class I molecules expressed on healthy cells[5]. Activated NKs induce apoptosis and secrete cytokines to promote innate immunity and contribute to adaptive immune responses.

1.1.2. The adaptive immune response

The adaptive immune response is a highly specific immune response mediated by T cells and B cells, which is slow in onset but acquires a memory function and is essential in control of pathogens and transformed cells[10]. B cells produce antibodies (Ig) to neutralize pathogens and prevent an infection. Depending on the different environmental factors in the microenvironment, APCs will become activated, differentiate and migrate to the local lymphoid tissues to present antigens to naïve T cells after which effector and memory T-cell phenotypes develop. Naïve T cells circulate in the blood and lymph nodes. T cells can recognizes small fragments (peptides) of antigens presented in the context of MHC class I or MCH class II molecules via their T-cell receptor (TCR). MHC class I is expressed on the majority of somatic cells and presents peptides of intra-cellular antigens to CD8+ cytotoxic T cells (CTL). CTLs are pivotal to control and kill infected cells or induce tumor cell lysis via their production of IFNγ, TNFα and granzymes. MHC class II molecules are expressed by immune cells, in particular APCs, but also by epithelial cells in inflamed tissue. MHC class II presents processed extracellular antigens to CD4+ T cells. CD4+ T cells are essential in regulating the immune response and are categorized upon their function and profile of secreted cytokines. Type 1 Th (Th1) helper cells are characterized by their production of the pro-inflammatory cytokines IFNγ, TNFα and IL-2 and the expression of the transcription factor Tbet. Th1 responses aid the induction, maintenance and effector function of CD8+ CTL, as well as support the polarisation and activation of M1 macrophages. Type 2 Th (Th2) helper cells can secrete the type 2 cytokines IL-4, IL-5, IL-10 and by Gata3 expression. They are on the other hand known for their immune inhibitory T-cell responses by Tregs (Th3) and TGF-β. Moreover they are well known to induce humoral immune responses, the activation of B cells and thereby the formation of Ig antibodies. More recently Th17 subset of CD4+ T cells were added to the phenotypes characterised by IL-17 production however their role as pro-inflammatory or anti-inflammatory T cells is still under debate as they have been isolated in several autoimmune diseases and have been isolated in different human cancers[11]. The final subtype of CD4+ T cells are regulatory T cells known for they key role in maintenance of peripheral tolerance to tumors by secretion of suppressive cytokines as IL-10 and TGF-β and a number of mechanisms to suppress other T cells or APC. Tregs express high CD25 and transcription factor FoxP3. The differentiation of T cells is influenced by different cytokine production of the APCs (e.g. DCs) and the co-stimulatory signals provided.

1.2. HPV and immunity

HPV in the etiology of cervical cancer was first proposed by Harold zur Hausen in the 1970s[12-14]. In 1982, HPV-DNA was detected in VIN for the first time[12,15]. HPV is the most common sexually transmitted pathogen worldwide and is a DNA virus that infects the basal cells of squamous epithelium when the epithelial surface is disrupted through minor damage of the genital mucosa allowing access of the virus to the basal cells of the epithelium[16-18]. Over 100 types of HPV are identified which are subdivided into low risk (non-oncogenic; *e.g.* lr-HPV 6 and 11) and high risk (hr)HPV (oncogenic; *e.g.* hr-HPV 16 and 18)[19]. The life time risk of acquiring an HPV infection is estimated at 80%, 40% of female adolescents become infected at least once with a hrHPV and in the majority of cases the infection is asymptomatic and is cleared within two years[17,20-22]. Persistent infections develop only in less than 10% of the infected women and are causally related to the development of intraepithelial neoplasia of the cervix (cervical intraepithelial neoplasia (CIN)), vagina (vaginal intraepithelial neoplasia (VAIN)), anus (anal intraepithelial neoplasia (AIN)) and/or vulva (VIN) and their subsequent progression to invasive squamous carcinoma [17,20,21,23,24]. The risk factors associated with HPV-induced disease are the life time number of sexual partners, smoking and the use of oral contraceptives[25,26].

The early viral oncoproteins of HPV (E1, E2, E4-E7) are the key factors in progression of HPV-induced disease and have different and synergistic functions in the maintenance, replication and progression of a potential HPV associated lesion[16,18,19,27]. E6 and E7 are the pivotal players and are consistently expressed in the basal layers of the epithelium in malignant tissue resulting in enhanced cell proliferation and subsequent viral genome replication[16]. In the suprabasal layers viral replication takes place and E1, E2 and E5 are expressed while in the most superficial layers, the late structural proteins L1 and L2 encapsulate newly made viral particles which are released to the epithelial surface[16]. Since there is no cytopathic phase or systemic viraemia in a HPV infection, the potential exposure of HPV to the immune system is reduced and causes delay in the activation of the immune system. The high incidence of persistent HPV infections and subsequent HPV-related malignancies in immunosuppressed individuals indicate towards the key role of the adaptive cellular immune system in protection against HPV-induced lesions[28,29]. In addition, only a small fraction of infected non-immunosuppressed subjects develop progressing epithelial lesions or cancer[17,18,20]. Both the innate and the adaptive immune system play an essential role in the protection against HPV.

1.2.1. Innate immunity to HPV

Keratinocytes form the first line of defence against HPV and express PRRs (TLR9 in differentiated keratinocytes and TLRs 1-3, TLR 5, TLR6, TLR10, RIG-1, protein kinase R and MDA5 irrespective of the differentiation state) to recognise PAMPs of the HPV virus. Binding

of viral components to the all sorts of PRRs during early stages of viral infection leads to direct NF-kB activation which results in the upregulation of pro-inflammatory cytokines but only activation by RLRs and some TLRs results in activation of interferon regulatory factors and the production of antiviral type I interferons[30-37].

These pro-inflammatory cytokines and chemokines subsequently influence the migration and function of APCs, with Langerhans cells and dermal DCs as main representatives in the skin as well as migration of T cells[38,39]. The wide variety of PRRs present in human keratinocytes reflects their ability to respond to different classes of pathogens and HPV infected keratinocytes should be able to sense the presence of HPV[31,33,40]. However persistent HPV infections reflect a failure of the immune system to control the infection and several immune escape mechanisms of HPV are present[27]. Although several mechanisms how HPV evades the innate immune system have been described, the exact role of HPV is undetermined and many details still remain unknown.

The relatively long time to induce spontaneous clearance of HPV in 12-18 months reflects the ability of HPV to efficiently evade the immune system[20]. To start as a result of intracellular shedding of HPV in the basal cells of the epithelia, HPV is able to reduce the exposure to the immune system in absence of a systemic viraemia revealing a delayed activation of the immune system. Moreover HPV infected keratinocytes display suppressed signalling to NF-kB downstream of the PRR, IFNγ and TNFR pathways [31,41-44]. This results in a lower production of antivirals such as type I IFNs, pro-inflammatory and chemotactic cytokines which will lead to a decreased attraction and activation of APC and T cells. The up regulation of UCHL-1 is an example of how hrHPV interferes with the signalling of PPRs and hampers cytokine production and activation of the immune system[37].

Furthermore HPV is able to reduce the antigen-presentation to T cells as the L2 protein suppresses functional and phenotypic maturation of LCs[44,45] and in HPV infected epithelia a lower number of LCs is described[46,47]. This latter is probably due to diminished chemokine production and lack of cell migration and it has been shown that HPV affects the CD40 signalling resulting in less chemo attractants and failure to enhance immune cell migration[48].

One can conclude that HPV hampers the activation of the adaptive immune system by interference with function and migration of APCs present in the epithelial and is able to induce T-cell tolerance by non-properly activated APCs in the absence of pro-inflammatory signals[42].

1.2.2. Humoral immunity to HPV

The majority of HPV infected patients fail to induce immunoglobulin-G (IgG) antibodies to the HPV viral capsids L1 and L2 considering the large proportion of 30-50% of patients who never seroconvert [49,50]. During an HPV infection these IgG levels are low and these IgG antibodies to L1 and L2 can be detected approximately 6 months after an HPV infection[49,50].

The presence of these IgG L1 and L2 antibodies do not protect against a re-infection and they neither clear HPV-induced lesions[51-55]. In patients HPV-induced cancer IgG antibodies reactive to E6 and E7 are induced with seroconversion in 20% of FIGO stage I patients to more than 50% in FIGO stage III[56,57]. In early stage disease IgG E6 more frequently where IgG E7 is observed in patients after viral clearance although IgG E6 and E7 levels have no effect on prognosis at the stage of cancer[56-58].

1.2.3. Cellular immunity to HPV

Adaptive HPV specific CD4+ and CD8+ type 1 T-cell responses to the early oncoproteins are essential components of the immune response in the protection against the development and progression of HPV induced disease[54,59-61]. In a group of healthy subjects approximately 60% of cases display a strong Th1 T-cell reactivity against the early oncoproteins E2, E6 and E7[60,62]. The presence of both lesion infiltration and circulating HPV-specific CD4+ and CD8+ T cells is related to spontaneous regression and clearance of HPV-induced lesions whereas this type of immunity is weak or lacking in patients with progressive HPV-induced diseases[53,63-69]. In one third of patients with CIN or cervical cancer HPV-specific immunity to E6 and E7 is detected however these T cells are generally not able to produce IFNγ and consist of either Th2, non-polarised T cells or Tregs[53,69-71].

The importance of the Th1 HPV-specific immune response to the early HPV oncoproteins is supported by the data from clinical trials where regression of HPV 16-induced uVIN lesions is related to a strong and broad CD4+ Th1 HPV specific T-cell response[54,72-76]. At the time of spontaneous regression of HPV-infected genital warts, the lesions are infiltrated with CD8+ cytotoxic T-cells, CD4+ T-cells and macrophages[77]. The HPV specific T-cell responses to L1 on the other hand have no clinical significance as they are found the majority of both healthy controls and patients and these responses induced by preventive vaccination with L1 particles are not able to clear established infections[54,78].

The mechanisms underlying a failure to properly activate HPV-specific T cells or the tolerization of an already induced effector response are gradually unravelled. In high grade lesions or tumors HPV-specific effector cells may be suppressed by regulatory T cells (Tregs) as they are strongly associated with the prognosis of HPV induced disease and are known to actively inhibit the proliferation and cytokine production of activated CD4+, Th1 and CD8+ T cells[79,80]. Moreover, T-cell function can be influenced by the expression of inhibitory molecules, including CTLA-4, PD1, NKG2a and TIM3 by activated T cells which after cognate with their ligands, e.g. PD-L1 and Galactin-9 may result in suppression of their effector function, including the migration of T cells to the lesions[81-85]. Furthermore, transformed cells may resist an immune attack by CD8+ cytotoxic HPV specific T cell via the down regulation of HLA class I molecules which is frequently observed in HPV induced neoplasia [80,86,87]. Moreover expression of the non-classical HLA molecules (-E, -G and MICA) in HPV-induced

neoplasia may play a role in inhibition of the host immune response by induction of Th2 cytokines and hampering of CD8+ T-cell activity as well[41].

1.3. Vulvar Intraepithelial Neoplasia

VIN is a chronic premalignant skin disorder of the vulva associated with high recurrence rates after standard treatment[12]. Since 2004 VIN lesions are classified into usual type VIN (uVIN), historically called VIN 2 and 3, and differentiated type VIN (dVIN) according to the International Society for the Study of Vulvovaginal Disease (ISSVD)[12,88]. Condylomata acuminata (in old nomenclature VIN 1 lesions) are in general exophytic benign tumors mainly induced by low risk HPV infections HPV 6 and 11[89]. uVIN and dVIN differ completely from each other with respect to etiology, malignant potential and treatment and should therefore be distinguished as different entities of vulvar disease[12,90].

1.3.1 Differentiated VIN

dVIN is associated with chronic dystrophies of the vulva like lichen sclerosus and lichen planus. It occurs mainly in older women and its incidence accounts for <2- 5% of all VIN lesions[12,88,91]. dVIN is associated with invasive vulvar squamous cell carcinoma and its malignant potential is estimated 5.6 fold higher than uVIN[12,91,92]. dVIN has a high degree of cellular differentiation and the absence of widespread architectural disarray, no nuclear pleomorphism and no diffuse nuclear atypia[12,92,93].The histopathological features of dVIN are much more subtle compared to uVIN and its therefore frequently confused with a benign dermatosis or epithelial hyperplasia and there is a high interobserver variability[12,92,93]. The incidence of dVIN was shown to increase nine fold over the last decades from 0.013 to 0.121:100.000 women. Moreover, 32.8% of women previously diagnosed with dVIN developed a vulvar squamous cell carcinoma in a median time of 22.8 months[91].

1.3.2. Usual VIN

uVIN is caused by a persistent HPV infection. In the majority of the cases (90%), high risk type HPV16 is detected[12,94-98]. In old nomenclature uVIN is also referred to as Bowen's disease, classic VIN, undifferentiated VIN, erythroplasia of Queyret and squamous cell hyperplasia with atypia[89,99]. Worldwide uVIN, established at 2:100.000 women, is increasing in incidence reflecting the increase in HPV infections in young women 35-40 years of age[17,20,91,100,101]. The incidence is higher in Caucasian women than among black, Asian or Hispanic women and the highest peak of reported uVIN cases is between 40-49 years of age which reveals an age-adjusted incidence of 5:100.000 women[12,102-104]. The diagnosis of uVIN is related to the risk factors of HPV and include smoking, a history of genital herpes, condylomata and to

immunocompromised patients by either HIV infection or transplantation patients[12,105].
uVIN often causes severe and long-lasting complaints of pruritus, pain and sexual dysfunction in the majority of the patients[12,88]. The clinical features of uVIN are highly variable; lesions are uni- or multifocal and can differ in size, number, color and shape[12,102]. The surrounding skin or mucosa is often unaffected and the location of uVIN is often around the introitus and involving the labia minora although the whole vulvar and (peri)anal skin can be affected[12,102]. Importantly uVIN is associated with multicentric disease affecting cervix, vagina and/or anus in 22-71% of uVIN patients highlighting the importance of regular gynaecological examination and performing cervical cytology[102,106,107].
The malignant potential of uVIN is estimated at 3-4% after treatment and 9% without treatment within 1 to 8 years[12,108]. In relation to the age of diagnosis, the malignant potential is established to 2.7% for women younger than 29 years in a median time of 50 months and of 8.5% for women older than 75 years in a median time of 25 months[91,102]. Because of its low spontaneous regression rate of estimated 1.5% and often severe symptoms, treatment of uVIN is often warranted[12,108-110].

1.3.2.1. Histopathological features of uVIN

uVIN histologically resembles CIN and other HPV-associated intraepithelial neoplasias and is diagnosed and characterised by proliferation of atypical basal cells[93]. The characteristic appearance of uVIN is recognizable already at low magnification due to obvious cytological and architectural abnormalities[99]. The epithelium is characterised by epithelial thickening and surface hyperkeratosis and/or parakeratosis and is composed of a closely packed population of undifferentiated cells with enlarged hyperchromatic nuclei with mitotic and apoptic activity and loss of cell maturation[89,93,99,102]. Moreover below the basal membrane lymphocyte infiltrate characterises the dermis[89,93,99,102]. Due to the enlarged nuclei of atypical cells the histologic feature of uVIN presents as crowded cells with large dark nuclei and a small amount of eosinophilic cytoplasm[89,102] (Fig. 2a). Depending on the level of the atypical proliferating cells respectively above the lower two-thirds of the epithelium the uVIN lesion is classified as either VIN 2 or VIN 3, nowadays high grade uVIN lesions[89]. uVIN is divided into warty and basaloid types based on the architecture and appearance of the intraepithelial lesions but these subtypes are only part of a spectrum of uVIN with overlapping morphology and there is little need in dividing into these subtypes[89,99]. In case of cell invasion through the basal membrane into the dermis either a microinvasive (<1mm) or macroinvasive (>1mm) squamous cell carcinoma is diagnosed. The differentiation, however, especially between uVIN and early microinvasive carcinoma may be difficult. Invasion is suggested by the following features: irregularity in the size and shape of nests, cytoplasmic eosinophilia of the invading cells and desmoplastic stroma[93].

Additional immunohistochemistry by p16, Ki67 (Fig. 2b+2c) or p53 may be of help in the determination of a high grade uVIN lesion. p16 is a surrogate marker for HPV which nuclei and cytoplasmic staining correlated with a high-risk HPV infection because the early oncoprotein E7 inactivates the retinoblastoma protein (pRb) resulting in overexpression of p16 in HPV infected epithelia[18,89,93,99,111]. p16 is a tumor suppressor protein that inhibits the cyclin-dependent kinases which phosphorylate the pRb, responsible for maintenance of the cell cycle and apoptosis[18]. Differentiated VIN is usually p16 negative[99]. Furthermore staining for Ki67 a proliferation marker was shown to be well correlated with the presence of HPV DNA and can help in the distinction of the level and type of VIN lesions with positive staining throughout the epithelial thickness in case of high grade uVIN[89,99]. Staining for the overexpression of mutated p53 aids the detection of dVIN as 90% of the dVIN cases express mutated p53, whereas this is generally absent in uVIN cases due to the expression of the oncoprotein E6[89,93,99].

1.3.2.2. Therapies in uVIN

Treatments for uVIN are aimed at both relief of symptoms and prevention of progression into (micro-) invasive lesions. Expectative management by close follow up of the uVIN lesions is advocated in case of no complaints and no suspicion of malignant disease[112]. Nowadays conventional surgical treatments as surgery or laser ablation are increasingly replaced by immunotherapy as imiquimod (Aldara®), both in standardised and experimental settings, with promising clinical responses however in a notable number of patients these therapies fail to induce clinical responses[72,74,113-115].

1.3.2.2.1. Surgical treatment of uVIN

Surgical treatment options are: cold knife surgery, laser CO2 therapy or loop electrosurgical procedure (LEEP)[116]. Local excision by cold knife surgery aims to remove all visible lesions and is still performed as the therapy of choice. If positive excision borders predict the development of recurrent disease is still under debate although they do not predict development of invasive disease and extensive surgeries do therefore not guarantee a cure and should be avoided[12,102,108-110,117-119]. A 5 mm peripheral margin is generally accepted for the management of local resection of uVIN and a resection up to 4 mm in depth is recommended however in mucosal areas the resection depth does not need to exceed 1 mm since there are no sebaceous glands in this epithelial area[102,120]. Recurrence rates range from 20-40% and recurrences are associated with multifocal disease and smoking[102,118,119,121]. The median time to recurrence is 22-44 months[102].

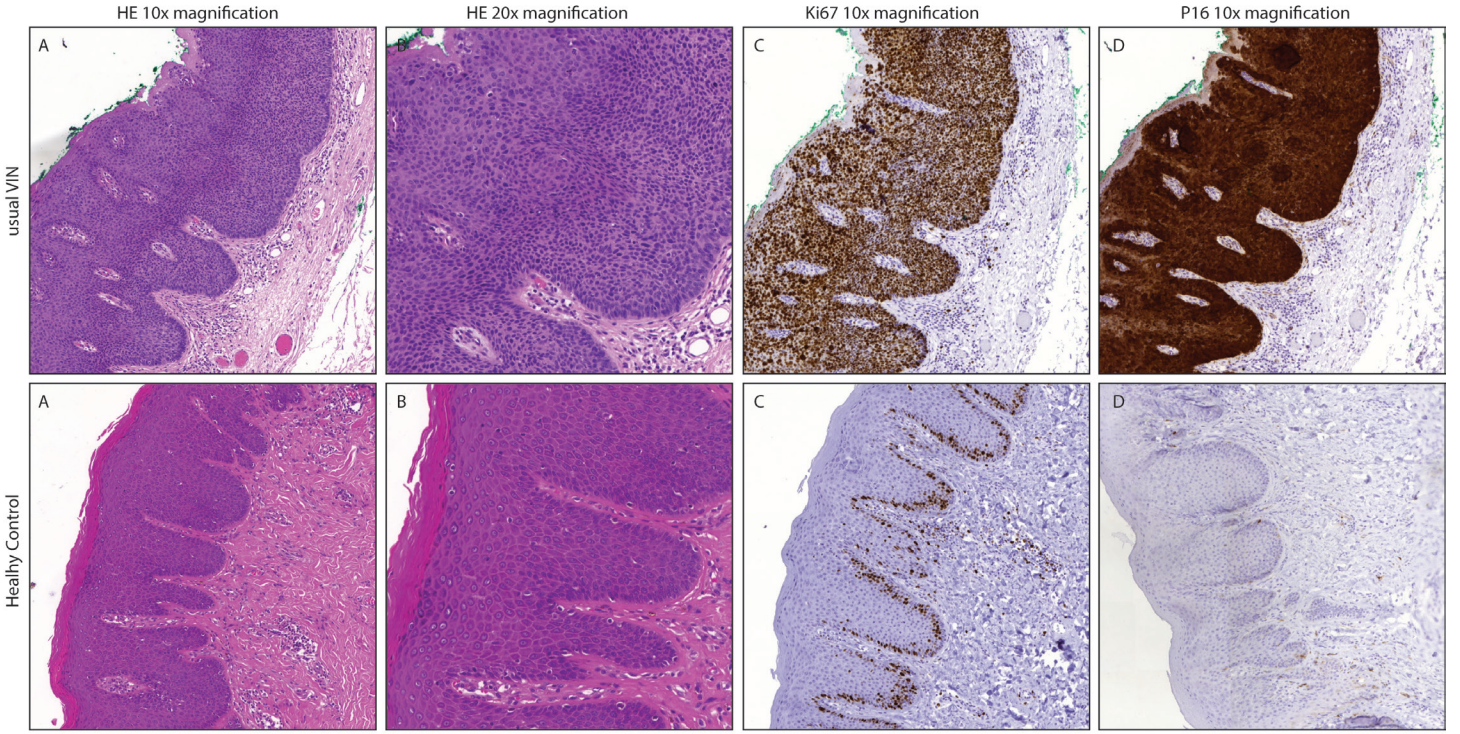


Figure 2 Histopathology of uVIN lesions and healthy control tissue

A: HE staining in 10x magnification, B: HE staining in 20x magnification, C: examples of Ki67 and in D: P16 immunohistochemistry in the diagnosis of HPV induced uVIN

Laser therapy is a destructive technique that preserves normal vulvar anatomy and function resulting in good cosmetic and functional results. The response rate and prevention of development of invasive vulvar cancer are warranted by this therapy even in wide multifocal lesions[12,102,122]. The disadvantage of this method is that due to the tissue destruction no tissue for histological examination is available. Therefore, prior to surgery several biopsies should be performed in order to exclude (micro) invasion of the uVIN lesion as much as possible[12,102]. In the thickest part of the lesion the (micro) invasion is most likely and biopsies should be concentrated here[102]. The complete response rate of laser surgery is 75% and recurrence rates are comparable to excision. The reported shorter recurrence-free survival after laser therapy is probably a reflection of a higher proportion of multifocal lesions treated with laser vaporisation rather than a reflection of therapy failure[102,116,119,121,123].
By LEEP a wide local excision of the uVIN lesion can be performed with the advantage that the resected tissue is available for histologic examination[102]. The recurrence rate of LEEP is estimated at 20% at 48 months follow up[124].

1.3.2.2.2. Immunotherapies in treatment of uVIN
Regarding the potential disfiguring interventions of conventional therapies related to psychosexual problems, several medical and immunotherapies are nowadays replacing conventional therapies in both standardised (imiquimod) and experimental settings[12,108,121,125]. However to date none of these therapies are approved for VIN treatment by the FDA (Food and Drug Adminstration)[102]. Immunotherapy is promising, however, some patients are refractory to therapy[72,74,113-115].

Imiquimod
Imiquimod is an immune modifier and induces T-cell activation by activation of macrophages and pro-inflammatory cytokine release and has antiviral and antitumor activity[12,126,127]. Topical application of imiquimod crème preserves the anatomy and function of the vulva and by inhibition of viral replication, the causative agent HPV is targeted[12]. Imiquimod induces viral clearance, normalisation of immune cell infiltrate and a high number of clinical responses ranging from complete responses of 35-81% and partial responses in 10-46% of uVIN lesions with low recurrence rates in long term follow up[113,128,129]. The side effects as local inflammatory reaction, itching, burning and flu-like symptoms are common and can be treated with NSAIDs without interference with imiquimod therapy[130]. Responses to imiquimod are associated with pre-existing HPV-specific T-cell responses whereas imiquimod did not induce or enhance the HPV-specific T-cell response[54,74,115].

Photodynamic Therapy (PDT)

Photodynamic therapy induces oxygen-induced cell death by use of a tumor-localizing photosensitizer, 5-aminolevulinic acid (ALA), in combination with non-thermal light[102,131]. PDT results in direct tumor destruction and reduces destruction of normal surrounding tissue and activates the local immune system by activation of APCs, recruitment of effector cells and activation of tumor specific immunity[132,133]. Responses range from 40-60% and similar efficacy and recurrences were found in PDT compared to laser and local excision[121,134-136]. Advantages of PDT are minimal tissue destruction, preservation of normal anatomy and short healing time[121].

Therapeutic vaccination

Preventive HPV vaccines are aimed at the induction of Ig antibodies and prevent HPV infection and subsequent lower genital tract neoplasia[137]. In contrast, therapeutic vaccines aim to induce or reinforce HPV specific CD4+ and CD8+ T-cell responses and destroy HPV-infected cells[138]. Several types of therapeutic HPV vaccines have been developed showing different rates of immunogenicity and clinical success[138,139].

Two recombinant vaccines; TA-HPV encoding HPV 16 and 18 E6 and E7 and TA-CIN consisting of HPV 16 L2E6E7 fusion proteins were used either single doses or booster vaccinations in VIN patients. Despite clinical responses there was no obvious correlation with the vaccine induced immune response neither after a three booster regimen with TA-CIN[140-142]. After vaccination the antibody and T-cell responses to L1 were stimulated although they were not sufficient to clear the lesions and clinical responses were low[143]. Reinforcement of HPV specific T-cell response by therapeutic vaccination has shown promising clinical responses, especially when combined with imiquimod[72,74,114]. After 8 weeks of topical imiquimod application followed by three doses of TA-CIN in a 4 week interval, complete regression percentage was seen in 68%[74]. Importantly, an increased CD4+ and CD8+ T cell infiltration in the lesions was associated with a favourable clinical outcome[74].

A synthetic long peptide (SLP) vaccine composed of HPV16 E6 and E7 peptides induced strong HPV-specific immune responses associated with clinical responses in high grade VIN patients. In patients with larger lesions, the HPV-specific immune response was lower compared to the small lesions and there were more vaccine induced Tregs in these patients[72,144]. The correlation between clinical effectiveness and immune responses is confirmed in a second vaccination trial where the HPV-16 SLP vaccine for HPV 16 positive uVIN was combined with imiquimod at the vaccination site[145].

1.3.2.2.3. Antiviral therapies

Cidofovir has antiviral activity and may induce apoptosis of the HPV infected cells[102]. In a small cohort 25% of patients showed a complete response after therapy with 1% cidofovir[146]. Reported side effects are ulceration at application site and pilous areas were refractory to therapy[146].

In HIV positive patients topical cidofovir 1% was efficient in short term treatment with 15% complete responses, 26% partial responses and 21% stable disease of high grade peri-anal intraepithelial neoplasia (PAIN) and VIN[147]. Moreover side effects of pain, burning, irritation and ulceration of skin were acceptable[147]. In a recent randomised controlled trial cidofovir 1% was compared to imiquimod 5% revealing comparable results of approximately 45% complete responses, 87% treatment adherence and 40% of adverse events. Thus in addition to imiquimod, cidofovir also forms an active, safe and feasible alternative to surgery in treatment of uVIN[148].

Topical IFNα has been used in uVIN with a clinical response of 67% although the local side effects and high costs ensured that this therapy was no longer used for uVIN therapy[149]. A different form of IFNα, pegylated-IFNα, is now available with different pharmacokinetic and chemical properties as well as less side effects. Pegylated-IFNα potentially is of interest to treat uVIN since IFNα is known to play an important role in activation of durable anti tumor responses because IFNα drives the generation of IFNγ producing CD4+ and CD8+ T cells and promotes the proliferation and survival of T cells[150-152].

1.4. Immune responses in uVIN

Where cellular immune responses against HPV protect against the development and progression of HPV-induced disease, these responses are either weak or absent in most patients with uVIN[54,59-61]. A lack of proper chemokine signal for DCs to migrate towards the lymph node and present HPV to naïve T cells is an explanation for the lack or insufficient HPV-specific immune response[153]. Systemic HPV-16 specific Th1 T-cell responses are detected in up to 50% of patients with HPV 16 induced high grade VIN. These responses are not associated with spontaneous regression of the uVIN lesions, however, these pre-existing responses are suggested to positively correlate with clinical efficacy of immunotherapy[54,61,74,115,140]. Treatment failure on the other hand, is related to absence of HPV specific immunity and the high number of recurrences in uVIN after conventional treatment may as well be related to absent or weak HPV specific immune responses[72,74,115].

The local immune response in the microenvironment of uVIN is characterised by an immune active dermis and immunosuppressive epidermis since the epidermis of uVIN is characterised by a decreased number of CD8+ T cells and increase of immature DCs and

LCs while the dermis displays an influx of mature DCs, NK cells and both CD4+ and CD8+ T cells[154,155]. Moreover, some uVIN lesions are infiltrated by high numbers of Tregs[74,113,115,154,156]. Clinical response to immunotherapy in uVIN is associated with normalisation of immune cell counts and an increase in intralesional CD8+ T cells as well to low numbers of Tregs and lower numbers of macrophages[74,115,156,157]. In addition, HLA class I expression was present in clinical responders to PDT and there was an increase in infiltrating CD8+ T cells after therapy in these lesions[157]. Failure to respond to immunotherapy may be related to a local immunosuppressive microenvironment since clinical responders were highly infiltrated with DC/LCs, CD4+ and CD8+ T cells before vaccination [74,113-115,154,156]. Despite these suggestions however knowledge on the uVIN microenvironment is limited.

1.5. Outline thesis

With the studies presented in this thesis we aimed to gain more basic knowledge about the pivotal role of both the local and systemic immune responses in uVIN as a prelude to understand non-responsiveness to immunotherapy and to optimize the use of immunotherapeutic approaches for HPV-associated anogenital disease. Therefore we studied the local and systemic immune responses in uVIN patients extensively. In *Chapter 2* a detailed review of the literature on innate and adaptive immune responses and potential causes for failure of immunotherapy in uVIN patients is given. In *Chapter 3* we studied the potential clinical prognostic factors of recurrence and progression in uVIN as this allows us better to estimate the impact of the immunological responses observed in this patient group.

The role of T cells in the protection and regression of uVIN lesions indicate the importance of the infiltration in HPV-infected/transformed cells. HLA downregulation might allow premalignant lesions as uVIN to escape immune surveillance and to progress into invasive cancer. In *Chapter 4* the alterations of both classical and non-classical HLA molecules in uVIN patients were studied. We show that (partial) loss of HLA is not hard-wired (e.g. genetically caused) but soft-wired as it can be restored for instance by exposure to IFNγ.

In *Chapter 5* we focus on the expression of a number of known immune inhibitory molecules which may interfere with the function of lesion-infiltrating immune cells. We analysed the expression of IDO (Indoleamine 2,3-dioxygenase) and Galectins 1, -3 and -9 within the tumor microenvironment and the expression of inhibitory molecules PD1, TIM3 and NKG2a on T cells in relation to immune infiltration and clinical outcome.

Subsequently in *Chapter 6* we describe the infiltration of uVIN by different types of myeloid cells as determined by the expression of CD14+, CD33+ and CD163+ and their association with clinical outcome of uVIN lesions, which is subsequently followed by integrated analysis of both the T cell and myeloid cell infiltrates in relation to recurrence free survival.

Finally, the data of our first vaccination trial in patients with uVIN suggested that there might be a difference in the patient's capacity to respond to the first vaccination[144]. A strong response to the first vaccine dose is associated with complete clinical success. We hypothesized that there may be differences in the immune status of the treated patients which can explain their reactivity to vaccination. In *Chapter 7* we analysed the expression of inhibitor receptors on T-cells (TIM3, PD1, NKG2a, CD94 and CTLA4) and macrophages in blood of clinical responders and non-responders to estimate potential biomarkers involved in recurrence or progression of uVIN lesions. Furthermore in *Chapter 7* we focus on signal transduction activators of transcription (STAT) phosphorylation in different immune cells after stimulation with cytokines and immunomodulatory agents as IFNα and GM-CSF.
The general discussion *Chapter 8* gives an overview of the findings presented in this thesis and focuses on future prospective.

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CHAPTER 2

TREATMENT FAILURE IN PATIENTS WITH HPV 16-INDUCED VULVAR INTRAEPITHELIAL NEOPLASIA: UNDERSTANDING DIFFERENT CLINICAL RESPONSES TO IMMUNOTHERAPY

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Abstract

A failure of the immune system to launch a strong and effective immune response to high risk HPV is related to viral persistence and the development of anogenital (pre)malignant lesions such as vulvar intraepithelial neoplasia (VIN). Different forms of immunotherapy, aimed at overcoming the inertia of the immune system, have been developed and met with clinical success. Unfortunately these, in principal, successful therapeutic approaches also fail to induce clinical responses in a substantial number of cases. In this review we summarize the traits of the immune response to HPV in healthy individuals and in patients with HPV induced neoplasia. We discuss the potential mechanisms involved in the escape of HPV-induced lesions from the immune system and indicate gaps in our knowledge. Finally, the interaction between the immune system and VIN is discussed with a special focus on the different forms of immunotherapy applied to treat VIN and the potential causes of therapy failure. We conclude that there are a number of pre-existing conditions that determine the patient's responsiveness to immunotherapy and that an immunotherapeutic strategy in which different aspects of immune failure are attacked by complementary approaches will improve the clinical response rate.

Keywords: VIN, immunotherapy, immune modulation, vaccination, HPV, immune escape, regulatory T-cells, macrophages

Introduction

Vulvar intraepithelial neoplasia (VIN) is a chronic vulvar skin disease with malignant potential that often causes severe and long-lasting complaints of pruritis, pain and sexual dysfunction.[1] In 2004, VIN lesions were classified according to the International Society for the Study of Vulvovaginal Disease into; usual type VIN (uVIN), historically called VIN 2 and 3, and differentiated type VIN (dVIN).[1,2] The incidence of dVIN accounts for less than 5% of all VIN lesions, occurs in older women, and is associated with chronic dystrophies of the vulva such as lichen sclerosus and lichen planus.[1-3] dVIN has a high malignant potential (5.6 fold compared to uVIN).[1,3] UVIN is caused by a persistent human papilloma virus (HPV) infection, in particular HPV type 16, which is present in over 90% of cases.[1,4,5] The incidence of uVIN, approximately two per 100,000 women, is increasing worldwide and is related to the increase of HPV infections in young women.[3,6-9] UVIN occurs predominantly in younger women (peak incidence 40 years), tends to be multifocal in 60% of patients and is correlated with smoking.[1,2] Progression rates to malignancy of uVIN are estimated at 3-4% after treatment and 9% without treatment in 1–8 years, whereas spontaneous regression of VIN occurs in less than 1.5%.[10,11] Treatments are therefore aimed at both relief of symptoms and prevention of progression into (micro-)invasive lesions. Conventional surgical treatment is often disfiguring, mutilating and suboptimal, as reflected by the high recurrence rates of 20-40% and physical and psychological morbidities.[10,12-14]

HPV is a DNA virus that infects the basal cells of the genital epithelia, in particular the squamous epithelium, and is the most common sexually transmitted pathogen worldwide.[9,15] Over 100 types of HPV are identified, which are subdivided into low risk (non-oncogenic; *e.g.* lr-HPV 6 and 11) and high risk HPV (oncogenic; *e.g.* hr-HPV 16 and 18)[16] Approximately 60% of young women are infected with either an hr-HPV (40%) or lr-HPV (20%) within the5-year period after they become sexually active, while the lifetime risk of acquiring an HPV infection is estimated at 80%.[8,9,17,18] In most cases the infection is asymptomatic and is cleared within 1 year.[8,17] Persistent infections only develop in less than 10% of the infected women and are causally related to the development of intraepithelial neoplasia of the cervix (CIN), vagina (VAIN), anus (AIN) and/or vulva (VIN) and their subsequent progression to invasive squamous- or adeno-carcinoma.[8,9,17,19,20] Multicentric disease affecting the cervix, vagina and/or anus have been described in 22–71% of VIN patients.[21,22] The risk factors associated with HPV-induced disease are the lifetime number of sexual partners, smoking (as it results in a decreased local immune response), and the use of oral contraceptives (of which the estrogens may increase cellular proliferation via an effect on the early oncoproteins of HPV).[23,24]

The early viral oncoproteins of HPV (E1, E2 and E4–E7) are the key factors in progression of HPV induced disease because they have different and synergistic functions in the

maintenance, replication and progression of a potential HPV associated lesion.[15,16,25,26] These early oncoproteins influence several signal transduction pathways such as the cell proliferation pathway due to inactivation of cell cycle arrest protein $p16^{INK4A}$, the TNF-α and IFN pathways.[26] The pivotal players E6 and E7 are constitutively expressed in malignant tissue and their expression results in enhanced cell proliferation and subsequent viral genome replication.[15] E6 downregulates p53 and expression of the proapoptotic protein BAK, leading to resistance to apoptosis and increased chromosomal instability.[25] E7 binds to and degrades the retinoblastoma susceptibility protein (pRB), which leads to apoptosis. By transcriptional activation of the cyclin A and cyclin E genes, E7 regulates cell proliferation and downregulates $p16^{INK4A}$, which can counteract the function of E6.[25] The E1 protein is essential in HPV replication where the E2 protein acts both as a replication and transcription activator.[16] E2 represses the viral promoter of E6 and E7 during early stages of infection.[27] Carcinogenic progression is accompanied by integration of the viral genome into the host cell DNA which disrupts E1 and E2 function and enables upregulation of E6 and E7 expression.[27] E5 appears to be important in the early course of infection by stimulating cell growth and preventing apoptosis following DNA damage.[25]

The high prevalence of hr-HPV infection in uVIN has lead to the suggestion that therapy should aim for the immunological eradication of virus-infected cells. Different types of local and systemic forms of immunotherapy have already been described with encouraging clinical results; in a number of trials almost half of the treated patients had durable complete lesion regression.[28-33] Notwithstanding these successes, these therapeutic approaches also fail to induce clinical responses in a substantial number of cases. The aim of this review is to provide insight into the nature of HPV-induced disease, to indicate the gaps in our knowledge of the interaction between uVIN and the immune system and to identify the possible causes of immunotherapy failure as a guide to optimize the immunotherapy of uVIN.

Immunity to HPV infection

Infection with HPV occurs when the epithelial surface is disrupted through minor damage of the genital mucosa, thereby allowing access to the basal cells of the epithelium.[25] Here, the early proteins E6 and E7 are expressed.[15] In the suprabasal layers, E1, E2 and E5 are expressed and viral replication takes place.[15] In the most superficial layers, newly made viral DNA is encapsulated by the late structural proteins L1 and L2 and the new virions are released by wear and tear of the epithelial surface.[15] The absence of a cytopathic phase or systemic viraemia reduces the potential exposure of HPV to the immune system and causes delay in the activation of the immune system. This is not absolute as over 80% of the HPV infections are controlled within 2 years after infection.[8,17,34] There is strong evidence that both the innate and the adaptive arms of the immune system play a role in the protection against HPV as will be described in detail below.

Innate immunity

The innate immune system acts as the first line of defense against invading viruses. Keratinocytes express pathogen recognition receptors (PRRs), including the membrane bound toll-like receptors (TLR), the cytoplasmic NOD-like receptors (NLR) and RNA helicase retinoic-acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (MDA5).[35-39] The latter two recognize double-stranded viral RNA in the cytoplasm and are constitutively expressed in human keratinocytes.[36-38] The TLR family (TLR 1-10) recognizes different molecular patterns; TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA found during viral replication, whereas TLR9 recognizes unmethylated CpG motifs common in viral DNA.[35,36,40] TLR3 is expressed in undifferentiated keratinocytes while the expression of TLR9 in undifferentiated keratinocytes is debated or present at very low levels.[38-40] Both TLR3 and TLR9 are capable of regulating proinflammatory responses, whereas TLR7 and TLR8 were not functionally expressed in undifferentiated keratinocytes.[38-40] However, human keratinocytes are able to upregulate TLR7 in response to stimulation with poly I:C, which is a strong agonist for TLR3, RIG-I and MDA5, suggesting that under inflammatory conditions the keratinocytes may become responsive to immune-modifying TLR7 agonists.[37] Binding of viral components to these receptors during early stages of viral infection leads to direct NF-kappa-B activation, which results in upregulation of pro-inflammatory cytokines, and/or activation of type I interferon (IFN) response genes, including transcription factors IRF3 and IRF7 regulating the production of antiviral and pro-inflammatory cytokines (e.g. GM-CSF, IL-1β, TNF-α, IL-10, IL-12, MIP3α).[35-40] Proliferative cytokines and chemokines influence the migration and function of antigen presenting cells (APCs), with Langerhans cells (LCs) and dermal dendritic cells (DCs) being their main representatives in the skin.[41,42] This wide variety of PRRs present in human keratinocytes reflects their ability to respond to different classes of pathogens and HPV infected keratinocytes should be able to detect the presence of HPV genomic DNA directly via TLR9 or indirectly via RIG-I.[36,38]

Cellular immunity

The important role of the immune system in protection against HPV-induced lesions is demonstrated by the high incidence of persistent HPV infections and subsequent HPV-related malignancies in immunosuppressed individuals.[43,44] On the other hand, only a minority of infected non-immunosuppressed subjects develop progressing epithelial lesions or cancer.[8,9,17-20,45] Composite data indicate the importance of CD4+ T-cells in the control of HPV-induced disease as more severe lesions are observed in HIV+ patients with low numbers of circulating CD4+ T-cells.[45,46] In addition, the increase in CD4 cell count after anti-retroviral treatment correlates with the regression of HPV-induced CIN lesions in HIV+ patients.[46] Activation of the adaptive immune response is dependent on cross-presented

viral antigens by activated LCs and DCs. Depending on the different environmental cues in the microenvironment; the APC will adopt a certain state of differentiation and migrate to the local lymphoid tissues to present antigens to naïve T-cells. Depending on the status of the APC, as reflected by the levels of co-stimulatory or inhibitory molecules and its cytokine production (e.g. IL-12 or IL-10), a T-cell response will be induced that can consist of different types of CD8+ T-cells, CD4+ helper T-cells (Th-cells) and regulatory T-cells (Tregs).[47,48] At the time of spontaneous regression of HPV-infected genital warts, the lesions are infiltrated with CD8+ cytotoxic T-cells (CTL), CD4+ T-cells and macrophages.[49] Approximately 60% of the healthy subjects display a directly ex-vivo detectable type 1 (i.e. IFNγ associated) T-cell reactivity against the early oncoproteins E2, E6 and E7.[50,51] Spontaneous regression and clearance of HPV-induced lesions is associated with the presence of both lesion infiltration and circulating CD4+ and CD8+ T-cells directed against the early oncoproteins whereas this type of immunity is weak or lacking in patients with progressive HPV-induced diseases.[45,52-58] These data indicate that type 1 T-cell responses to the HPV 16 early proteins play an important role in the protection against persistent HPV infection.This notion is sustained by the data obtained from clinical trials in which the full regression of HPV 16-induced high-grade vulvar lesions is strongly associated with the presence of a proliferative or type 1 HPV-specific T-cell response prior to the treatment.[30,59] Moreover, clinical regression after immunotherapy by vaccination is associated with the strength (i.e. breadth and magnitude) of the vaccine-induced proliferative and/or IFNγ-associated HPV-specific T-cell response.[29,31,60,61] Notably, a directly ex-vivo detectable type 1 T-cell reactivity against the late structural antigen L1 is not only found in healthy subjects but also in the majority of patients with HPV-induced disease.[55] Furthermore, strong type 1 L1-specific T-cell responses are induced by vaccination with L1 virus like particles (VLPs), yet these vaccinations are not able to induce clearance of established infections.[55,62] Together these observations indicate that the response against L1 is not essential for T-cell mediated protection once a person is infected by hr-HPV.

Humoral immunity

Antibodies to the HPV viral capsids L1 and L2 can be detected from approximately 6 months post infection although 30-50% of patients with persistent infections never seroconvert.[63,64] Immunoglobulin-G (IgG) seroconversion rates appear to be higher among women with persistent infection over a long period of time.[65] In general, IgG antibody responses to HPV L1 and L2 are weak during infection (i.e. at low levels) and do not protect against re-infection with the same HPV type or clear HPV-induced lesions as discussed above.[66-68] However, the induction of high levels of antibodies to the virus capsid protein – via prophylactic vaccination with VLPs – prevents viral infection very efficiently and has led to the introduction of two commercial vaccines (Gardasil® and Cervarix®).[69-71] The

prophylactic vaccine protection times depend on follow-up in the clinical trials and range from 4 to 9 years.[69-71] However, no accelerated clearance of existing viral infections has been observed despite these high antibody levels.[62] Antibodies reactive to E6 and E7 are also frequently found in patients with HPV-induced cancer and their induction appears to be dependent on the clinical stage of disease, with approximately 20% of seroconverters at FIGO stage I up to more than 50% in stage III.[72,73] Particularly in early stage disease, the antibody response to HPV 16 E6 is more frequently found compared to E7.[72,73] While it does not appear to affect prognosis at the stage of cancer, positive humoral reactivity to E7 was observed in patients who had cleared the viral infection rather than patients with persistent infection.[74] Although these antibodies are not expected to exert any direct effect on infected or transformed cells, their presence indicates active priming of an underlying T-cell response. Notably, HPV-specific T-cell responses are also found more frequently in cervical cancer patients with more advanced stages of disease.[75]

Immunogenetics

Besides environmental and lifestyle factors, host genetic factors are likely to play a role in persistence and appearance of HPV-induced neoplasia. The antigen processing machinery (APM) and human leukocyte antigen (HLA) class I molecules are key in the presentation of antigenic peptides to CD8+ T cells and, therefore, are important in the destruction of virally infected or transformed cells.[76,77] Defects in the APM and HLA molecules thus may contribute to viral escape, persistence and ultimately induce malignancies. From an array of 13 non-synonymous coding single nucleotide polymorphisms (SNPs) in the *LMP2*, *LMP7*, *TAP1*, *TAP2*, and *ERAP1* genes, the allele distributions at the *LMP7-145*, *TAP2-651*, *ERAP1-12,* and *ERAP1-730* loci differed significantly between cases and controls with the major allele at the *LMP7* and *TAP2* loci and the minor allele at both *ERAP1* loci associated with increased risk for cervical carcinoma.[76,77]

Over 800 different HLA class I and class II alleles have been defined and it is possible that some HLA molecules may be more or less suitable to present HPV-derived peptides and as such influence the ability to clear an HPV infection or HPV-induced neoplasia.[78] Indeed, the susceptibility or resistance to HPV infection and HPV-induced lesions has been associated to particular HLA alleles, albeit that many of these findings were not consistent across different populations.[79] A protective effect of HLA class II DRB1*13/DBQ1*0603 alleles is the most consistently found association, although the effect was only significant in 47% of studies.[79] HLA-DRB1*07 and DRB1*15/DQB1*0602 have been associated with an increased risk of HPV-induced cervical neoplasia in The Netherlands and we confirmed that HLA-DRB1*07 was overrepresented in Dutch patients with HPV 16-positive cervical cancer whereas HLA-DRB1*13 was underexpressed in patients with cervical cancer compared to controls.[75,80] A large (>500 cases and >500 controls) study on co-occuring alleles revealed that of the 137

allele combinations present in >5% of women with squamous cell carcinoma of the cervix, 30 were significantly associated with an increased risk, with all but one including DQB1*0301.[81] Among the six co-expressed alleles that were associated with a decreased risk, four comprised DQB1*02.[81] The particular associations between disease and HLA class II alleles but not HLA class I alleles gain extra weight through the detection of predominant HLA class II –restricted CD4+ T-cell responses over HLA class I-restricted CD8 T-cell responses in healthy individuals and patients.[61,82,83] Furthermore a study of 49 candidate immune response and DNA repair genes revealed that a SNP in the innate immune gene IRF3 was associated with increased HPV persistence.[84] Reports on the influence of particular SNPs in the IL-10 gene are debated and those described to influence the production of the immunosuppressive cytokine TGFβ did not differ between cases and controls.[85-87] A SNP in the chemokine receptor 2 – which binds the macrophage recruiting chemokine MCP-1 – was associated with a decreased risk for cervical cancer.[86] SNPs in the promoter region of TNFγ or the receptor of IL-4 were associated with increased risk for cervical cancer.[86,88]
Other potential genetic factors involved in the progression of HPV-induced neoplasia may comprise genetic differences in the genes of the innate immune response (e.g. PRR pathways, activation of transcription factors), genes of the antigen presenting pathway, genes involved in APC activation and migration, or genes involved in T-cell migration and/or differentiation and in chemo- or cytokine production. For example, WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelocathexis) syndrome is a rare congenital immunodeficiency disorder characterized by high susceptibility to HPV infection and is associated with autosomal dominant heterozygous mutations in the gene for the CXCR4 chemokine receptor.[89] WHIM is characterized by the marked reduction of circulating naïve T cells. T cells bearing this mutated chemokine receptor display an increased migratory response to CXCL12. It has been suggested that the increased migratory response results in the capture of these cells in the bone marrow [89], removing them from the periphery and as such potentially precluding their response to HPV.

Immune escape in HPV-induced disease - lessons from cervical neoplasias

Persistent viral infections reflect a failure of the host's immune system to control infection where several immune escape mechanisms of HPV are present [Box 1] (reviewed in [26,90]). While viral clearance or regression is associated with the presence of circulating CD4+ and CD8+ T-cells, viral persistence corresponds with a weak or absent early antigen specific T-cell response.[45,53,55,58,75] The systemic HPV-specific immunity to E6 and E7 is detected in approximately one third of patients with CIN or cervical cancer. The T-cell responses detected are generally not associated with the capacity to produce IFNy and can consist of Th2 cells, non-polarized T-cells, or even regulatory T-cells.[45,56,58,59,75,82,91,92] Remarkably, CD4+ and CD8+ T-cell responses in tumor and tumor draining lymph nodes in cervical cancer

are broad and aimed at both E6 and E7. When stimulated ex-vivo, they produce only low amounts of IFNy, but in the presence of APC-stimulating compounds cytokine production increases.[82,83] Interestingly, in cervical cancer patients, deep infiltration of the tumor within the normal tissue correlates with the presence of circulating HPV-specific T-cells and a better survival of patients.[75] Cumulatively, these studies suggest that the T-cells are locally not sufficiently stimulated and may even be suppressed.

Impaired antigen presentation and activation of innate immunity

Initial infection by HPV causes a cascade of viral gene expression, replication of the viral genome and enhanced cell proliferation.[26] One of the major sensors of DNA viruses is TLR9. Expression of TLR9 is either lacking or at very low levels in the undifferentiated basal cell layer of the squamous epithelia.[38] Furthermore, infection of keratinocytes with recombinant retroviruses expressing the HPV 16 E6 and E7 oncoproteins inhibits TLR9 transcription and facilitates functional loss of TLR9-regulated pathways.[93] This indicates that infected keratinocytes are not able to signal via TLR9, however, other viral PRRs might be employed.[38] Despite the presence of these other intracellular PRRs which allow infected cells to attract the immune system, the mean clearance time of HPV is 12-18 months, indicating that HPV still manages to delay or escape recognition and immune activation.[8,17] HPV does not affect the expression of different virus-sensing PRR, but genome wide expression profiling studies have demonstrated that the presence of HPV was associated with downregulation of components of the antigen presenting pathway, the inflammasome, the production of antivirals such as type I interferons, pro-inflammatory and chemotactic cytokines and activated pathogen receptors. Notably, many of the downregulated genes are found in a network that is strongly interconnected by IL-1β, a crucial cytokine to activate adaptive immunity.[38] HPV+ keratinocytes were also shown to respond less well to interferon stimulation.[94,95] This concurs with the observation that interferon-inducible genes are downregulated via inhibition of the JAK-STAT activation response pathway and downregulation of the active STAT 1 (i.e. phosphorylated or pSTAT-1), which is the primary regulator of the interferon response.[38,96]

In addition, HPV might also hamper activation of the adaptive immune system by regulating the function and migration of antigen presenting cells present in the epithelia. When viral particles are taken up by LC this does not necessarily result in an antiviral response. The structural L2 protein of HPV is able to suppress phenotypic and functional maturation of LCs and therefore can limit adequate antigen presentation to T-cells.[97,98] Furthermore, the number of LCs is reduced in HPV infected lesions compared to normal tissue.[99-101] In one of our studies the number of LCs varied extensively in cervical cancer and was related to increased migration under the influence of TNF-α.[41] The lower number of LCs is thus probably a result of lower production of chemokines and a subsequent lack of attraction of

precursor cells to replenish LC migrated out of the tissue.[42] Last but not least, the absence of pro-inflammatory signals in HPV-infected epithelia can result in inappropriately activated APCs and upon cognate interaction with T-cells they will induce T-cell tolerance.(reviewed in [102])

Alterations in Human Leukocyte Antigen (HLA) expression

Cervical cancer patients in whom circulating HPV-specific T-cells are detected display a longer survival after chemoradiotherapy than patients without a detectable T-cell response.[75] These T-cells may contribute to the antitumor response or reflect an ongoing CD8+ T-cell sustained tumor-specific immune response as they are only found in patients with HLA class I positive tumors.[103] Downregulation of HLA class I (HLA- A, -B and -C) is frequently observed in cervical neoplasia and may result in an escape of the tumor cells from cytotoxic T-cell attack.[80,104] Defects in HLA class I expression are caused by a variety of mechanisms, including loss of heterozygosity at chromosome 6p, β2-m or HLA class I mutations, defective expression and function of components of the antigen processing machinery (APM) and/or due to lack of IFNy expression.(reviewed in [105])[80,104,106] The HPV-encoded E5 and E7 proteins have also been implicated in downregulation of HLA class I.[26] Downregulation of HLA-A is associated with worse survival of patients with cancer.[107] Induction of HLA class II molecules is observed in the majority of cancers and can be mediated by cytokines such as IFNy.[108] HLA-II expression (HLA-DR, -DQ and -DP) was observed in 67% of CIN I, 58% of CIN II, 93% of CIN III and in 75% of cervical cancers.[108] In each histological category HLA-DR was most commonly expressed and HLA-DQ least commonly expressed.[108] Interestingly, analysis of CD4+ HPV 16- and 18- specific tumor infiltrating T-cells (TILs) revealed that the vast majority (>80%) of CD4+ T-cells were restricted to HLA-DQ or –DP and not to HLA-DR, suggesting that immune escape at the HLA-DQ-restricted CD4+ T-cell level may have occurred.[82] This would also agree with the protective effect of some HLA-DR/DQ combinations found to be associated with protection against HPV-induced cancer.[79] The expression of HLA-class II molecules might contribute to a successful response since activated CD4+ Th1 cells in the tumor environment enhance the recruitment, proliferation and effector function of CD8+ T-cells.[109] However, it is still unclear if the expression of HLA class II by tumor cells can also hamper the immune response and may favor tumor outgrowth as HLA class II may also induce tumor promoting signaling by rendering the CD4+ T-cells anergic after cognate interactions in the presence of immunoinhibitory cytokines and lack of co-stimulatory molecules as CD40 or by activating/inducing Tregs.[110]

Next to the classical HLA class I and II molecules, cervical tumor cells have been reported to express HLA-G. (Figure 1)[111-113] This non-classical HLA type plays an important role in tumor-driven immune escape as it inhibits the function of natural killer (NK) cells, T-lymphocytes and APCs through direct binding of inhibitory receptors immunoglobulin-like

transcripts ILT-2 and ILT-4 and the killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4).[111] The expression of HLA-G in cervical lesions is associated with progression from pre-malignant to malignant lesions and may play a role in inhibiting effective host immune responses by inducing Th2 cytokines.[111,112]

Another non-classical HLA type is HLA-E, which by engagement of the inhibitory CD94/NKG2A receptor expressed by NK and CTLs hampers the activity of these cells in the tumor.(Figure 1)[114] While NK-cells are not frequently observed, CTLs are detected in the tumors of many patients.[107,115] Notably, up to 50% of the CD8+ TILs expressed the inhibiting CD94/NKG2A receptor, whereas CD4+ TILs hardly expressed this receptor.[114] HLA-E is overexpressed in more than 80% of cervical tumors.[114]

A third non-classical HLA molecule is the MHC class I chain-related molecule A (MICA), which is expressed on normal epithelium but is weak or absent in ~60% of cervical cancer cases.(Figure 1)[107] MICA interacts with the stimulating NKG2D receptor on both CD8+ T-cells and NK-cells and enhances the effector function of these cell types.[107,116] A low expression or absence of MICA was shown to be associated with worse survival when analyzed in the context of the ratio between CD8+ T-cells and Tregs and the expression of HLA-A.[107] Downregulated expression of MICA, in addition to weak expression of HLA-A, may surpass the threshold for the infiltrating $CD8^+$ T-cells to exert their tumoricidal function.

Induction of regulatory T-cells

There is a strong correlation between the ratios of CD4+, CD8+ and tumor-infiltrating Tregs and the prognosis of HPV-induced disease.[75,107,117,118] CD4+ Tregs are shown to inhibit the proliferation and cytokine production of activated naïve CD4+ T-cells and Th1 cells and are also able to prevent the activation of CTLs by preventing the expression of the IL-2 receptor alpha (CD25) and inhibiting IL-2 production.[117] Tregs influence several other pathways to suppress the anti-tumor response, including induction of suppressive macrophages, upregulation of IL-10, induction of indoleamine 2,3-dioxygenase (IDO)-positive APCs and TGFβ production.(reviewed in [117]) In cervical dysplasia, Tregs appear to be attracted by CXCL12, a ligand of CXCR4.[119] Importantly, part of the regulatory T cell repertoire comprises HPV-specific Tregs that recognize the same antigens as HPV-specific effector cells.[117] Upon cognate interaction with the HLA class II-positive tumor cells these CD4+ Tregs become activated and can suppress other immune cells within the lesions and tumors.[58,91] Furthermore, as these antigens are also used for therapeutic vaccination strategies, vaccination may result in the expansion of HPV-specific Tregs and subsequently cause the anti-tumor response to fail.[117,118]

Inhibition of T-cell function or infiltration

Exhaustion of CD4+ and CD8+ T-cells during viral infection or malignancies has been associated with expression of the co-inhibitory molecules cytotoxic T-lymphocyte antigen-4 (CTLA-4), program death-1 (PD-1), T-cell immunoglobin mucin-3 (TIM-3) and B- and T-lymphocyte attenuator (BTLA).(Figure1)[120-123] The interaction between PD-1 receptor expressed by effector or Tregs, and program death ligand 1 (PD-L1(B7-H1)) and/or PD-L2 (B7-DC) results in the induction of apoptosis, anergy or exhaustion of effector T-cells.[124-126] Approximately half of the tumor-infiltrating T-cells in cervical cancer are PD-1 positive. PD-L1, however, is only occasionally expressed by cervical cancer cells. Interestingly, patients with PD-L1 positive tumors, infiltrated with relatively high numbers of CD4+FoxP3+ Tregs, show a better survival than patients with relatively high numbers of infiltrating Tregs, but negative for PD-L1.The impairment of the PD-1 positive Tregs by the PD-L1 expressing tumor cells may potentially result in a survival benefit.[126]

The ligand for TIM-3 is Galectin 9 (Gal-9). Their interaction results in a decreased Th1 and CTL immunity by inducing apoptosis of Th1 cells as well as by inhibiting the function of CTLs and Th1 cells.[120,122,127] A decreased Gal-9 expression is inversely associated with malignant potential or differentiation of cervical cancer.[128] Gal-1 and Gal-3, however, have also been implied in the inhibition of T-cell responses and their expression is increased during the progression of HPV-induced neoplasia.[129,130]

Another molecule that may hamper the immune response is the cell surface glycoprotein CD200 (OX-2). This protein can be expressed by many types of human cancers.[131-134] Co-cultures of CD200-expressing, but not CD200-negative, tumor cells suppressed the production of Th1 cytokine by T-cells, the cytolytic activity of CTL and the IFNγ response of NK-cells.[131,132] In the transplantable EMT6 mouse breast cancer model, the neutralization of CD200 led to a decreased tumor growth and an increased number of cytotoxic anti-tumor immune cells in the tumor draining lymph node.[133] Tumor-cell expressed CD200 also hampers the function of tumor-associated APCs.[134,135]

Finally, T-cells can also be physically hampered to infiltrate the lesions. We found that a high expression of versican – one of the extracellular matrix components produced by stromal cells - in the stroma was associated with a low number of tumor-infiltrating T-cells and in particular a low number of CD8-positive T-cells.[136] In addition, a study of the expression of the mucosal homing receptor, α(4)β(7) surface integrin, on T-cells and its ligand mucosal addressin cell adhesion molecule-1 (Madcam-1) on vascular endothelial cells in cervical tissue revealed that the ability of α(4)β(7)(+) CD8(+) T-cells to gain access to cervical epithelium strongly depended on the expression of Madcam-1, which was absent in lesions of which the dysplastic epithelium was not infiltrated by T-cells.[137]

Microenvironment

The local microenvironment may also play a role in HPV-induced lesions. The expression of cytokines such as IL-10 and TGF-β, the increase in tumor-associated macrophages, Tregs and IDO-expressing APCs can all help to suppress local immunity. (Figure 1)[138-142] TGF-β is overexpressed in CIN and cervical cancer.[143,144] It prevents T-cell infiltration into tumors, inhibits T-cell activation and mediates Treg-induced immunosuppression.[145] The immunoregulatory enzyme IDO was found to be expressed in high grade CIN and cervical cancer, and is particularly expressed by IL-10-producing stromal myeloid cells. Diffuse expression in cervical cancer was correlated with an unfavorable outcome.[140,146,147]

Macrophages exist in many flavors ranging from a tumor-rejecting phenotype (M1 type) to the well known tumor promoting macrophages (M2 type). Monocytes recruited to lesions or tumors can differentiate towards M1 or M2 types depending on the local milieu. The M2 macrophages mediate direct effects on tumor growth, vascularisation and local immunosuppression.(reviewed in [148]) Furthermore, they produce cytokines and chemokines resulting in alteration of the phenotype and function of local DCs and the modulation of T-cell responses. The differentiation towards M2 macrophages can be the direct result of tumor cells producing prostaglandin E2 (PGE2) and IL-6. Blocking the tumor-expressed cyclooxygenase-2 (COX-2), and thereby the production of PGE2, as well as IL-6 restores the normal differentiation of monocytes to DCs.[149] Expression of COX-2 is upregulated following overexpression of E5 in cervical carcinoma cell lines.[90] The expression of COX-2 by cervical tumors is associated with a poor response to chemotherapy.[150]

Interestingly, macrophages display plasticity in their differentiation, allowing them to switch from one type to another type depending on the local milieu. Tumor infiltrated Th1 cells can stimulate a tumor rejecting environment by switching M2 tumor promoting macrophages into activated M1 tumor-rejecting macrophages via CD40–CD40 ligand interactions and the production of IFNy.[149] Another cell type reported to play a role in the suppression of immune responses are myeloid-derived suppressor cells (MDSCs). These cells are able to directly inhibit T-cell responses and promote tumor progression.[151] However, the role of both tumor associated macrophages and MDSCs in the clinical outcome of HPV-induced disease is still unclear.

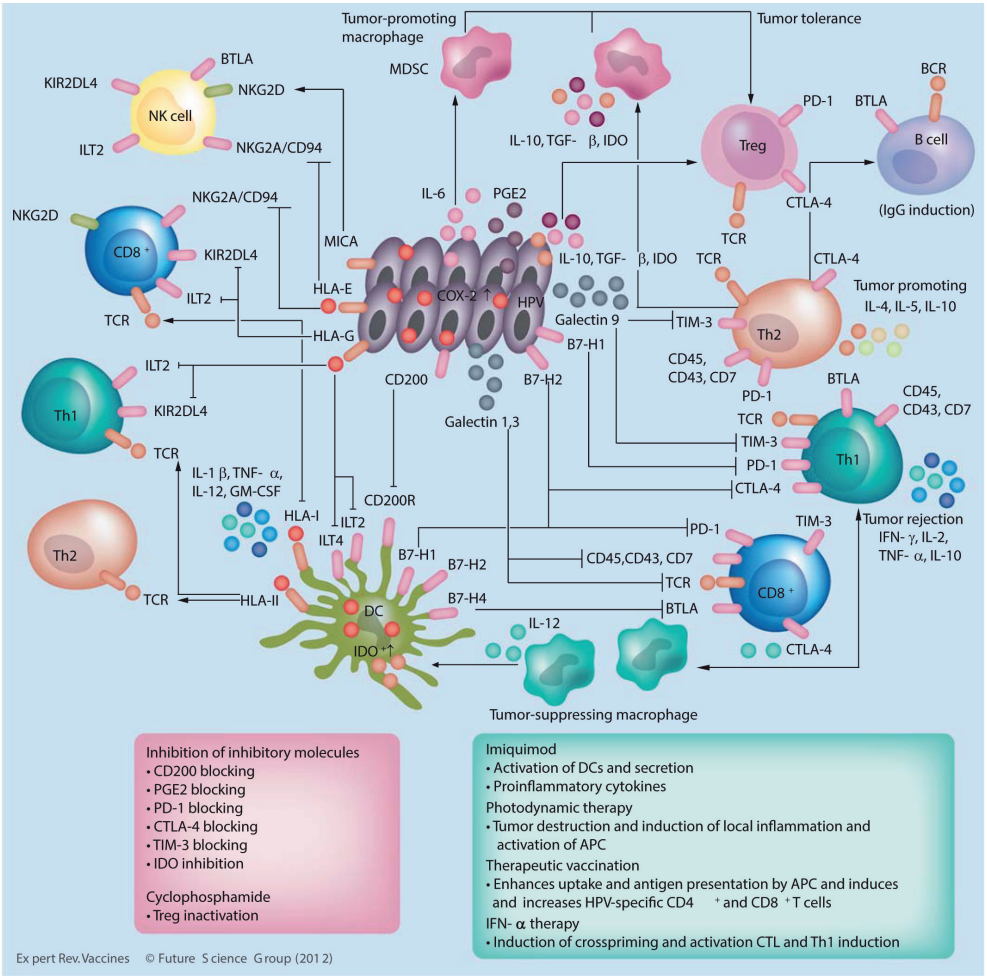

Figure 1 Immune escapes mechanisms and possible solutions

depicts all potentially involved co-inhibitory receptors, ligands and HLA expression and their role in the suppression of the immune system; TIM-3 and Galectin 9 (induction of apoptosis Th1 cells and inhibition of CTL and Th1 function), CD200R and CD200 (suppress function of Th1, CTL and NK-cells), PD-1 and B7-H1 (PD-L1) (induction of apoptosis, anergy or exhaustion of effector T-cells), CTLA-4 and B7-H1/H2 (inhibition of T-cell function), BTLA and B7-H4 (inhibition of T-cell proliferation, cytokine production, CTL function and memory cell generation), Galectin 1 and TCR, CD45, CD43, CD7, pre-BCR (induction of T-cell apoptosis, inhibition of T-cell function, Treg-mediated immune suppression and inhibition of B-cell signaling and activation), Galectin 3 and TCR, CD45, CD43, CD7 (induction of T-cell apoptosis and inhibition of T-cell function, alternative activation of macrophages), HLA-G and ILT2, ILT4 and KIR2DL4 (inhibition of T-cell function, CTL lysis, induction of tolerant APC), HLA-E and NKG2A/CD94 (inhibition of tumor cell lysis by NK-cells and CTLs), MICA and NKG2D (enhances the function of NK and CTLs). Moreover potential immunostimulating (green) and inhibition or blocking of inhibitory factors (red) to overcome some immune escape mechanisms are depicted which are of potential benefit and might improve the immunotherapy for HPV-induced neoplasia.

Immunity to HPV in vulvar intraepithelial neoplasia

VIN lesions are histologically characterized by an increased infiltration with CD4+ T-cells, macrophages but not LCs or CD8+ T-cells when compared to normal vulvar tissue.[152-155] Low-grade VIN has relatively higher numbers of CD8+ T-cells than CD4+ T-cells.[153,155] A recent study on a large series of patients and controls confirm these results with respect to the infiltration of the dermis, but also suggests that there is an increase in mature DC and plasmacytoid DC in the dermis.[152] In the epidermis, however, the number of LCs, immature DC, plasmacytoid DC and CD8+ T-cells seems to be slightly reduced.[152] The gene signature of VIN generally reflected an ongoing immune response and revealed a strong downregulation of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), which is a negative regulator of DC maturation and function.[156] The slightly reduced number of LCs in high grade VIN can be the result of enhanced emigration via downregulation of E-cadherin and LC activation by TNF-α and IL-1β, but it may also reflect a decreased immigration of precursors due to a lower MIP-3α production by keratinocytes.[100,157] Interestingly in non-HPV-related dVIN, a number of LCs were found, suggesting that HPV induces LC activation and migration.[158] The increase of mature DCs in the dermis of VIN appears to indicate that persistent HPV infection does lead to maturation of DCs, making it most likely that disturbed immigration is the culprit in the inaccurate initiation of a strong adaptive immune response, leaving only a weak dermal influx with CD4+, CD8+ and Tregs.[152,153,156,159] Depending on the study, up to half of patients with a HPV 16-induced high grade VIN lesion display a directly detectable ex vivo HPV 16-specific IFNγ-associated type 1 T-cell response against E2, E6 and/or E7.[59,160,161] The presence of such HPV 16-specific Th1 responses is generally not associated with regression of VIN, although the clinical efficacy of treatment with imiquimod cream or electrocoagulation of the lesion is associated with the presence of such a pre-existing HPV 16-specific IFNγ-associated T-cell response.[30,31,54,59] The absence of HPV 16-specific immunity is associated with treatment failure and may contribute to the high number of recurrences after treatment.[29-31,162] The critical role of a strong and broad systemic and local CD4+ and CD8+ HPV 16-specific proinflammatory T-cell response in order to clear usual VIN is demonstrated by therapeutic vaccination studies.[29,31,60,161,163-166] HPV 16 L1-specific serological responses are detected in the great majority of VIN patients but are not related to clinical outcome and is associated with a risk of developing VIN.[59,162,167] NK cells are sporadically found in the epidermis but were found to be more than doubled in numbers in the dermis of VIN lesions compared with healthy women[152] and may reflect co-infiltration with dermal effector T cells and Tregs. Furthermore, in VIN, the intensity of COX-2 expression analyzed by immunohistochemistry is not correlated with the degree of vulvar dysplasia.[168]

Similar to the other HPV-induced anogenital diseases, people carrying the HLA-DRB1*13 and -DQB1*05 alleles are associated with a decreased risk of HPV 16-induced high grade VIN or vulvar cancer, whereas HLA-DQB1*03 is strongly associated with an increased risk.[169] Downregulation of HLA class I was found in nine out of 11 vulvar carcinomas (82% total loss), irrespective if they were HPV-induced or not.[155] In these 11 carcinomas HLA-class II expression was not upregulated.[155] In VIN, 19% total loss of HLA-class I was observed both in dVIN and uVIN. In addition, in 21% of usual VIN biopsies HLA-class I was found to be downregulated.[155] Whilst there are many mechanisms leading to changes in HLA expression[105], which part is still reversible via IFNγ remains to be established, particularly since the presence of an HPV-specific IFNγ-associated T-cell response is correlated with regression of lesions.

In vulvar neoplasia both Gal-1 and Gal-3 were shown to be upregulated from normal vulvar tissue to high grade VIN and vulvar carcinoma.[170] The expression of Gal-1 on macrophages adjacent to the neoplastic cells became stronger with the increase in disease severity, whereas the neoplastic cells stained negative or weak for Gal-1.[129,170] Gal-3 staining was observed mostly in the epithelial cells but was also found in endothelial cells and macrophages. The vast majority of VIN cases did not express Gal-3. However, in approximately 60% of the vulvar carcinomas, Gal-3 expression by the cancer cells was moderate or strong.[171] The potential role of these and the other co-inhibitory factors discussed above in the T-cell response to HPV-induced VIN need to be determined.

Immunotherapy and potential causes of failure in VIN

A successful immunotherapeutic approach probably requires resetting many parameters in the immune response to HPV. Therefore, immunotherapy for VIN must be aimed at adequate priming of T-cells, altering the balance between effectors and Tregs, increasing the homing of T-cells to the lesion, preventing exhaustion of the immune response, and overcoming inhibition by creating an immune stimulatory environment that allows the immune system to work.[172] At present, a number of immunotherapeutic approaches have been tested with varying degrees of clinical success. Although some of these approaches showed some clinical efficacy in patients with VIN, a substantial number of cases failed to display a clinical response to immunotherapy. The potential causes of which will be discussed below.

Topical immunotherapy

In the early 1990s, 21 patients were treated with topical IFNγ with or without nonoxynol-9. Nine patients displayed a complete response for at least 1 year and overall 67% of the patients showed an objective clinical response. However, due to serious local side effects and high costs this therapy is no longer pursued.[173,174] More recent data showed that IFNα plays an important role in the activation of long-lasting anti-tumor responses.[175] IFNα drives

the generation of IFNγ-producing Th-cells and CTL as well as promoting the proliferation and survival of T-cells.[176] The use of pegylated IFNα may overcome the side effects previously observed in IFNα therapy of VIN patients because it differs in pharmacokinetic and chemical properties and is associated with fewer side effects compared to the topically used one.[177]

Imiquimod (Aldara®) is a topically applied immune response modifier that acts by binding to TLR7 resulting in activation of NF-kB, which is followed by secretion of multiple pro-inflammatory cytokines such as TNF-α, type 1 IFNs, IL-12 and activation of DCs.[178,179] Topical imiquimod has been used to treat HPV-induced high grade VIN lesions and resulted in viral clearance, normalization of immune cell infiltrate and clinical responses even in long-term follow up.[28,32,33,180] Complete regressions were observed in 26–100% of patients whereas 0–60% displayed partial regression and 0–37% experienced recurrence.[180] In two randomized controlled trials with imiquimod as treatment of high grade VIN, the first trial demonstrated complete regression in 81% of the 21 patients and partial regression in 10%, whereas the other trial reported a complete regression in 35% and partial response in 46% of the 26 patients.[28,32] Treatment in general is well tolerated, however local side effects of inflammation and burning are common, but can safely and successfully be treated with Non-Steroidal Anti-Inflammatory Drugs (NSAIDs).[28,32,181] Regression of the lesions in the last study was associated with a pre-existing IFNγ-associated HPV 16-specific CD4+ T cell response and afterwards with normalization of the lesional immune cell infiltrate.[59,159] Imiquimod did not induce or enhance the HPV 16-specific CD4+ T-cell response.[59] Non-responsiveness corresponded to the local attraction of macrophages and the presence of Tregs.[31,159] Notably, HPV 16-specific Tregs comprise both FoxP3+ and FoxP3- suppressor cells and are characterized by the production of both IFNγ and IL-10.[91] It can be envisaged that they also play a role in VIN lesions as they are found in high grade premalignant lesions of the cervix.[58]

Photodynamic therapy (PDT) in combination with topically applied 5-aminolevulinic acid (ALA) is a relatively new treatment regimen for VIN that exploits the interaction between a tumor-localizing photosensitizer and non-thermal light to induce oxidation reactions, which lead to tissue necrosis.[182] ALA-based PDT is particularly attractive as this drug is activated in rapidly growing cells, thereby reducing damage to the normal surrounding tissue.[182] PDT results in direct tumor destruction as well as induction of local inflammation resulting in the activation of APCs, recruitment of effector cells and subsequently the activation of tumor-specific immunity and development of immune memory.[183,184] PDT can also have an immunosuppressive effect, but this is prevented by reducing the rate of light delivery.[185] Clinical responses of VIN to PDT vary widely ranging from 20 to 60% of complete histological responses and in 52–89% of the patients it resulted in symptom relief.(reviewed in [186]) Curative responses were more frequently observed in unifocal lower grade VIN and

non HPV-associated VIN as well as in pigmented and hyperkeratinic VIN lesions.[155,187] Moreover, clinical responders retained the expression of HLA-class I and displayed a treatment-associated increase in the numbers of infiltrating CD8+ lymphocytes. In contrast, non-responders showed loss of HLA-class I and low numbers of infiltrated immune cells. [155,187]

Advantages of PDT are a short healing time, minimal tissue destruction and preservation of the normal anatomy of the vulva. However, recurrence rates are high (48.7%) and do not differ significantly from conservative treatments with CO2 laser and surgical excision. [12] In an attempt to increase the immune infiltration and response to PDT of HPV-induced VIN, a combination therapy of imiquimod and subsequent photodynamic therapy was given. The overall response rate was 65%, with 20% complete responders and 40% partial responders after 1 year.[30] Indeed, imiquimod treatment resulted in an increased CD8+ T-cell infiltration in the group of treated patients, but no differences were found between non-responders and responders within this treated group. Non-responders demonstrated relatively stronger infiltration with FoxP3+ T-cells after imiquimod. Clinical responders displayed a stronger HPV-specific proliferative T-cell response.[30] Together, these studies indicate that topical immunotherapy, particularly imiquimod, changes the microenvironment to allow more immune cells to infiltrate into the lesions. When these T-cells display the correct phenotype, as potentially found in patients with circulating HPV-specific T-cells, this infiltration may result in clinical responses. By contrast, when the immune cells display an immune suppressive phenotype (Tregs, M2 macrophages) the patients will not successfully react to the therapy.

Systemic immunotherapy by vaccination

Therapeutic vaccines aim to reinforce the HPV-specific IFNγ-associated CD4+ and CD8+ T-cell responses. As previously reviewed, different types of therapeutic vaccines have been developed and tested in Phase I/II clinical trials in an attempt to eliminate HPV 16-associated disease. The types of vaccines comprise recombinant viral vectors, peptides, fusion proteins, DNA, antigen-pulsed DCs and virus-like particles.(reviewed in [188]) Although some vaccines showed high immunogenicity, vaccination has led to limited clinical successes in HPV induced diseases. Part of it can be explained through the failure of some vaccines to induce a strong and broad HPV 16-specific CD4+ and CD8+ T-cell response.(reviewed in [188])

Different vaccine formulations have been tested in patients with HPV-induced VIN. In general, these vaccines were shown to be safe and immunogenic in most cases. A live recombinant HPV vaccine expressing the HPV 16 and HPV 18 E6 and E7 genes (TA-HPV) was tested in 12 women with high grade VIN or VAIN of up to 15-years duration in a single dose injection. [161] A total of 42% of the patients showed at least a 50% reduction in total lesion size and in one patient a complete regression of the lesion was achieved.[161] The vaccine induced

an increased IFNγ-associated HPV-specific T-cell response in six patients, who showed a concomitant clinical response.[161] However, in four women no vaccine-induced T-cell response was observed while two of them showed a >50% reduction in lesions size.[161] This vaccine was also tested in 18 women diagnosed with VIN that had persisted for 6-months to 17-years. Eight patients showed an increase in the HPV E7-specific IFNγ response, of which four displayed a weak response. One patient showed a complete clinical response and seven others a partial response. There was no obvious correlation with the vaccine-induced response, probably because only the response to two well defined HLA-A*0201 restricted CTL epitopes was measured. E6/E7-specific proliferative responses were measured in 50% of the patients.[164] Interestingly, a comparison of the local immune infiltrate revealed that the lesions of the group of clinical responders were, on average, highly infiltrated with DC/LCs, CD4+ and CD8+ T-cells before vaccination. There was no difference in the number of CD68+ macrophages between the two groups. Notably, the numbers of VIN-infiltrating CD4+ and CD8+ T-cells did increase in the group of non-responders after vaccination, albeit not to the level of the clinical responders.[164] This indicates that the capacity of the immune cells to infiltrate the lesion represents another hurdle to be overcome.

Another vaccine formulation consisting of HPV 16 L2E6E7 fusion protein (TA-CIN), which was given to ten women diagnosed with high grade VIN as three booster vaccinations after they were primed with TA-HPV 7–15 months earlier.[165] All patients displayed a proliferative response to the L2E6E7 fusion protein, but it is not clear how often this response was made against the L2 component. IFNγ-associated CD8+ T-cell responses to two of the HLA-*0201 restricted E7 epitopes were detected in three patients. One patient showed a complete response and one a partial response (>50% reduction in lesion size). There was no correlation found between the outcome of the immune assays and clinical reactivity.[165,166] Preclinical studies on heterologous prime-boost immunizations with TA-CIN and TA-HPV showed that the best vaccination protocol would consist of priming with TA-CIN to focus the immune response toward the oncoproteins, and boosting with TA-HPV to increase the magnitude of the E6/E7-specific response. Therefore, a group of 27 patients with HPV 16+ VIN 3 and two patients with VAIN 3 was vaccinated with three doses of TA-CIN at four week intervals followed by a single boost of TA-HPV. This resulted in one complete response and five partial responses.[166,189] Analysis of the HPV 16 E6/E7-specific IFNγ-associated T-cell response using pools of overlapping peptides of E6 and E7 was performed in 25 patients, three of which showed a pre-existing response to E6. In nine patients, the vaccine-induced IFNγ-associated T-cell response was detected after vaccination, including two of the patients with a pre-existing immune response. The responses were mainly focused at the E6 protein and not at E7. Two patients showed a strong IFNγ-associated T-cell response. Of the six clinical responders, five patients showed an E6-specific IFNγ response, including the complete responder.[166] Notably, the combination of TA-CIN and TA-HPV – although

a strong combination in mouse models - resulted in no advantage over a single TA-HPV vaccination in patients with VIN.[166,189]

Peptide vaccines are attractive because they are well tolerated in humans, relatively inexpensive to produce and easy to design. A vaccine consisting of synthetic long peptides (SLP), spanning the complete amino acid sequence of the two oncogenic proteins E6 and E7 of HPV 16, was safe and highly immunogenic in patients with cervical cancer as reflected by the strong IFNγ-associated HPV 16-specific CD4+ and CD8+ T-cell responses detected. [190,191] In a Phase II trial, 20 patients with high grade HPV 16+ VIN were vaccinated three to four times at 3 week intervals.[29] After 12 months follow-up, a clinical response rate of 79% and a complete and durable regression of the lesion in 47% of the patients was reported. Patients with a good clinical response displayed significantly smaller lesions at study entry than those who did not.[29] Characteristically, patients with a smaller VIN3 lesion displayed a strong and broad IFNγ-associated HPV-specific CD4+ T-cell response to HPV 16 E6 and E7. There was no difference between patients with small or larger lesions in the immune response to recall antigens. The patients with a smaller lesion displayed a distinct peak in the amount of cytokines produced by peripheral blood mononuclear cell (PBMCs) isolated after the first vaccination, suggesting that HPV 16-specific immune responsiveness is already predetermined. By contrast, in patients with larger lesions, higher frequencies of vaccine-enhanced HPV 16-specific Tregs were observed.[29,60] At present in our institute, a Phase II randomized trial in patients with HPV 16 positive high grade VIN is almost completed in which imiquimod is applied to site where the HPV 16 E6/E7-SLP vaccine is injected in an attempt to improve the Th1 polarization of the responding T-cells and as such clinical responses. In a peptide-based vaccine in patients with melanoma, imiquimod appeared to enhance the immunological response to the vaccine.[192] As a response to the observation that non-responders to PDT exhibited a loss of HLA-class I, a pilot study was performed on the available pretreatment biopsies of the HPV 16-SLP vaccinated patients. This revealed that (partial) loss of HLA-class I in the VIN lesions could be observed before vaccination across non and clinical responders, indicating that (partial) loss of HLA does not always prevent successful outcome of vaccination.[unpublished results] The expression of HLA class I may potentially be restored by the presence of IFNγ produced by HPV-specific CD4+ T-cells that recognize their epitope in HLA-class II, with as a result that the recruited CTL once again can exert their cytotoxic function.[109] Alternatively, CD4+ T-cells have a direct antiproliferative effect on HLA class II positive cells of the lesion.

Combined local and systemic immunotherapy

Clearly, there are a number of immunotherapeutic strategies that are promising for the treatment of VIN, but are not able to induce complete regressions of the lesions in every single patient. Based on the finding that clinical responsiveness to vaccination is associated

with the extent of the pre-treatment immune infiltrate in high grade VIN[164] and that this infiltrate can be enhanced by the use of imiquimod[30], a vaccine trial was designed in which imiquimod treatment for 8 weeks was followed by three intramuscular doses of TA-CIN at 4-week intervals.[31] A total of 19 women with (1–20 years of) high grade VIN were treated. After treatment with imiquimod, six patients showed a complete regression and this increased to 11 women after vaccination with TA-CIN. After one year of follow-up, 12 of the 19 treated women displayed a complete regression.[31] The group of clinical responders showed a stronger E6- and E7-specific proliferative response than non-responders. Imiquimod treatment was expected to raise the numbers of lesion infiltrating immune cells. Indeed a small but significant increase in the numbers of CD8+ T-cells was detected. After vaccination, the group of responders showed an increased CD4+ and CD8+ T-cell infiltration. By contrast, the group of non-responders showed an increase in the number of lesion-infiltrated Tregs.[31] In comparison to their previous study where imiquimod was followed by PDT, not only the number clinical responders was much higher, but also the numbers of infiltrating CD4+ and CD8+ T-cells in the lesions did not return to pre-imiquimod levels as was seen with PDT[30], suggesting that the vaccine-induced HPV 16-specific T-cell response mediated this effect. However, similarly to what was observed after HPV 16-SLP vaccination, TA-CIN vaccination resulted in the undesirable side effect of enhanced the number of Tregs in the group of non-responders.[31,60]

Conclusion and Future

Overall, one can conclude that clinical complete regression of HPV-induced disease can be obtained if the numbers of HPV-specific CD4+ and CD8+ T-cells are strongly enhanced and the lesions are (preconditioned to) allow immune cell infiltration. Clearly, a number of pre-existing conditions (e.g. lesion size, presence of Tregs, lack of immune infiltration, lack of HPV-specific IFNγ-producing T-cells, number of infiltrated macrophages, lack of HLA class I expression) may determine the patient's responsiveness to immunotherapy. Other factors that may influence responsiveness to therapy, such as the type of infiltrating macrophages (M1 or M2), the presence of MDSCs, the expression of Madcam or the expression of inhibitory molecules, which are all known to influence the immune response in other HPV-induced or chronic viral diseases, still need to be addressed. The consistent observation that non-responders display enhanced numbers of Tregs after therapy suggests that new modalities should be sought that alter the balance between Tregs and IFNγ-producing effectors in favor of the latter. Tregs are known to suppress the induction of the IFNγ-producing Th1 cells that are required to mobilize and sustain CD8+ T-cells to the site of disease.[109,193,194]

Expert commentary

VIN is the first HPV-induced disease in which real immunotherapeutic clinical successes have been achieved. In comparison to CIN and cervical cancer, VIN is, however, less well studied with respect to its interaction with the immune system. Most studies on the immune response to HPV in uVIN have been performed in clinical trials. On the one hand, this allows immune parameters to be studied in the context of therapy-induced outcome and as such may reveal parameters associated with success or failure. On the other hand, such studies are generally focused on the mechanism of action of the therapy and therefore do not consider other immune factors. Furthermore, most studies are performed in small cohorts of patients which limits the value of findings or significantly obscures potentially important findings. Although it is acceptable to translate findings from other HPV-induced diseases to the study of VIN, they do need to be confirmed preferably in larger cohorts with known clinical follow-up. Gaps in the knowledge of the interaction of HPV and the immune system in VIN include the absence or limited studies on (non-classical) HLA expression, the presence of co-inhibitory molecules or the presence of local inhibitory microenvironmental factors as macrophages and cytokines. Considering that VIN does respond to immunotherapy, in-depth studies should be performed to fully understand why it works, as well as to understand what we need to circumvent in patients who would otherwise not respond for immunotherapy of HPV-induced tumors to be successful. Based on what is already known, we think that HPV-induced VIN is an immunologically active disease as reflected by marked infiltration with T-cells and the presence of IFNγ-associated T-cell responses, when compared, for instance, to HPV-induced CIN. Importantly, while evidence accumulates that HPV-specific T-cells play a role in all HPV-induced diseases, the results of the vaccine trials definitely show that HPV-specific T-cells play a role in the control and regression of VIN. Moreover, these trials show that even high grade HPV-induced lesions can undergo immune-driven regression. However, when HLA expression is lost, infiltration with Tregs and macrophages, or the *per* individual differences in immune infiltration is considered, VIN is similar to the other HPV-induced high grade lesions or cancers. It is therefore likely that the development of new treatment options of this premalignant lesion will follow the same route as that for cancer. Here, one must consider applying immunotherapeutic vaccines in the adjuvant setting but may also include the use of low dose chemotherapy to obtain certain immunological effects such as Treg depletion. For instance, a single dose of cyclophosphamide improved IFNγ-associated T-cell responsiveness to vaccination in ovarian cancer.[195] In view of what has been found already, it is highly likely that a combination of immunotherapeutic strategies is required to increase success rates in the treatment of VIN.

Five-year view

At present the successes of different (combined) immunotherapies in the treatment of VIN are encouraging and unprecedented. However, non- or partial treatment responders are also being reported and this will spark studies that focus on unraveling the mechanisms underlying the differences in clinical responsiveness. Interestingly, there are several roads that lead to clinical success. These – in our opinion – complementary immune strategies all target different aspects of immune failure. We expect that combinations of these strategies will be tested. To the end, this will lead to either patient selection in cases where there is no strategy to overcome an immunological problem or to a number of combinations of immunotherapeutic strategies that together may solve the problems. One may consider, for instance, blocking the co-inhibitory molecules by antibodies, depletion of Tregs before therapy, depletion or re-differentiation of macrophages, increasing T-cell homing by the induction of local inflammation and the use of IFNα to polarize Th1/CTL responses. Combination of imiquimod, vaccines and IFNα are the most likely to be tested within the near future.

Key-issues

- Usual type VIN is a chronic premalignant disease caused by a persistent oncogenic HPV 16 infection in 90% of cases.
- Successful treatment of HPV-induced VIN is associated with an enhanced and broad HPV 16-specific CD4+ and CD8+ T-cell response against the oncoproteins E6 and E7.
- Several types of immunotherapeutic approaches have met clinical success in high grade VIN although a notable number of patients fail to respond to these immunotherapeutic strategies.
- Knowledge of the interaction between VIN and the immune system is limited.
- Immune escape mechanisms that play a role in VIN are loss of HLA, immune infiltration with Tregs and macrophages, or the lack of infiltrating CTLs.
- Studies on the role of inhibitory molecules on T-cell function and the reversibility of HLA loss in VIN are needed.
- Combinations of immunotherapies targeting different aspects of the failing immune response may overcome immune escape and enhance clinical response rates.

Box 1 Potential causes of immunotherapy failure and how to correct this (Figure 1)

Lack of a robust CD4+ and CD8+ HPV-specific T cell response.
The accumulated data show that a strong and broad IFNγ-associated HPV-specific T-cell response is required for complete regression of the VIN lesion. Although some patients spontaneously develop a respectable HPV-specific immune response, many do not. Immunotherapeutic strategies that do not comprise vaccination may fail in patients either by lacking HPV-specific immunity or by developing a dominant Th2 response.

- *Lack of HPV-specific immunity*: The immune system should come into contact with sufficient amounts of antigen for sustained periods to become activated. The first choice would be to vaccinate patients with a highly immunogenic vaccine that induces both CD4+ and CD8+ T-cell responses. Alternatively, one could use lesion destructive therapies that are known to result in the activation of T-cells such as PDT or cryoablation.
- *Wrong polarization of HPV-specific immunity*: Stimulation of the immune system with E6 and E7 antigens (delivered to the APC via natural mechanisms, vaccines or destructive therapies) to induce a strong Th1/CTL polarization requires optimal activation of APC. TLR 3 and 9 agonists are known to appropriately stimulate human APC and could be injected either locally or at the site of vaccination. Alternatively, one could use pegylated IFNγ as this is known to prevent Th2 responses and to drive Th1/CTL responses.

Inhibition of T-cell function and infiltration.
As a group, VIN lesions are well infiltrated with CD4+ and CD8+ T-cells, however, this varies a great deal between individuals. Despite strong infiltration with T-cells, they are incapable of inducing the spontaneous regression of VIN lesions. Immunotherapeutic strategies aiming to induce HPV-specific immunity have a better response rate in patients displaying considerable immune infiltration before treatment, except when there are high numbers of regulatory T-cells present.

- *Regulatory T-cells*: Non responsiveness to therapy is associated with the presence as well as enhancement of regulatory T cell numbers. Part of the regulatory T-cell population is HPV-specific. In the immunotherapeutic strategies that aim to induce HPV-specific immunity, an increase in circulating and local regulatory T-cells is observed in clinically non-responding patients. Preferably, modalities to deplete or disarm regulatory T-cells should be included in the immunotherapeutic strategy. A number of different methods have been tried without much success. So far, low dose cyclophosphamide treatment has produced consistent results, suggesting that low dose chemotherapy should be considered as a treatment option. Since it is not the number of regulatory T-cells *per se* but the balance between regulatory T-cells and effectors that is important one could try to use adjuvants that alter the balance between these two populations in favor of the effector cells.
- *Expression of co-inhibitory molecules:* Overall there is little data on the expression of co-inhibitory molecules by T-cells, immune cells in the microenvironment or the epithelial cells in patients with VIN. Clearly, the increase in severity of VIN lesions is associated with an increased expression of galectin-1 and galectin-3 in VIN. These galectins are known to disrupt T-cell function and to induce apoptosis in melanoma. Similarly, the interaction between T-cell-expressed TIM3 and galectin-9 disrupts T-cell function. The use of galectin inhibitors or antibodies to TIM-3 may alleviate inhibition. The role of other inhibitory molecules has not been studied in VIN but extrapolation of what is known from other HPV-induced diseases and other types of cancers, suggest that PD1 and CTLA-4 may play a role. For the latter molecule a clinically effective blocking antibody has been approved for melanoma, whereas for PD-1, several clinical grade antibodies are being developed. These might be added to the immunotherapeutic strategy. Potentially, CD200/CD200R interaction may also play a role. An antibody to CD200 has been tested in the treatment of B-cell leukemia (ClinicalTrials.gov Identifier: NCT00648739)

- *Inhibition T-cell infiltration:* A lack of Madcam-1 expression on the vasculature of CIN lesions was associated with a failure of T-cells to infiltrate the lesions and this may also explain why in a number of patients VIN lesions are not infiltrated by T-cells or not reactive to therapy. Madcam-1 is upregulated by TNFα, which may explain why the use of topical imiquimod can enhance immune infiltration of VIN lesions.

Microenvironmental factors.

- *Lesion associated macrophages:* VIN lesions display an increased infiltration with macrophages and non-responsiveness to imiquimod treatment corresponded with a local attraction of higher numbers of macrophages, suggesting that the macrophages detected display a tumor-promoting M2 profile and not the tumor-rejecting M1 profile, but this is yet to be confirmed. In HPV-induced cervical cancer, IL-10 producing M2 macrophages can be induced by tumor produced PGE2 and IL-6. COX-2 is variably expressed in VIN[168], but the expression of IL-6 is unknown. If M2 macrophages are involved, treatment could consist of IL-6R blocking antibodies (*e.g.* Tocilizumab) as used in rheumatoid arthritis and COX inhibitors. Alternatively, it has been shown that the interaction between Th1 cells and M2 macrophages switch the latter to activated IL-12 producing M1 macrophages. This suggests that if an immunotherapeutic approach results in enough lesion infiltrating Th1 cells, there might even be a benefit from the macrophages as they help to change the microenvironment to become more favorable.
- *IDO*: The expression of IDO is found in high-grade CIN and cervical cancer, where it is associated with clinical outcome. The IDO inhibitor 1-methyltrypthophan may be utilized if IDO plays a role in VIN.
- *MDSC*: myeloid-derived suppressor cells in many cancers can suppress the infiltrated effector T- cells by various mechanisms, although their exact role remains to be determined.

The expression of classical and non-classical HLA molecules.

- *Loss of HLA-class I (-A,-B,-C):* Downregulation of HLA-class I molecules may hamper the efficacy of HPV 16-specific CD8+ T-cells to exert their function. If this downregulation is irreversible meaning that there are genetic alterations which cannot be restored one may deselect such patients for immunotherapy. If the downregulation is reversible, meaning that it can be restored by IFN, then an increased infiltration of the lesion with Th1 cells should suffice to restore HLA expression. Alternatively, a local injection with pegylated IFNα or IFNγ during the treatment may help to promote HLA expression.
- *HLA-G:* In CIN lesions the expression of this molecule is associated with progression and the induction of Th2 responses. No intervention options are yet available.
- *HLA-E:* This molecule is expressed by the majority of cervical cancer where it can bind to the inhibiting CD94/NKG2A receptor expressed by up to 50% of the tumor-infiltrating CD8+ T-cells. No blocking antibodies have been developed to prevent this interaction to occur.
- *MICA:* This molecule binds to the co-stimulatory molecule NKG2D expressed by CD8+ T-cells. Downregulation of this molecule by cervical cancer cells is associated with lower patient survival. MICA is known to be upregulated by TNFα, suggesting that a local pro-inflammatory reaction may rescue MICA expression, but also by gamma-radiation.[196]

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CHAPTER 3

CLINICAL CHARACTERISTICS ASSOCIATED WITH DEVELOPMENT OF RECURRENCE AND PROGRESSION IN USUAL-TYPE VULVAR INTRAEPITHELIAL NEOPLASIA

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Abstract

Objective

To identify clinical characteristics associated with recurrence and progression in patients with uVIN which may function as prognostic factors and aid the treatment of patients with HPV-related disease of the genital tract.

Methods

A retrospective chart review was performed in 73 patients with uVIN treated in the LUMC between 1990 and 2012. All medical records were reviewed for demographics, treatment type, pathology reports and recurrence and progression rates.

Results

Mean age of diagnosis was 43 years and uVIN was symptomatic in 60.1% of patients. Median follow up time was 49 months. High risk HPV was found in 86.3% of patients. Smoking was reported in 76.8% of patients. 11 of 73 patients were immune compromised. Multicentric HPV-related disease of the cervix or vagina was reported in 75.3% of patients. Recurrences were diagnosed in 50.7% of patients after first treatment type that consisted of excision (45.2%), laser (34.2%), imiquimod (8.2%) and combination of excision and laser (12.3%). Higher recurrence rates were only correlated with multifocality of uVIN lesions. Excision, imiquimod therapy, and unifocal lesions showed an increased recurrence free survival. HPV type, smoking, multicentric disease, use of topical steroids and positive surgical borders were not related to a shorter recurrence free survival. Progression into vulvar carcinoma occurred in 11 (15.1%) of patients, 4 of whom were immune compromised. These patients showed a shorter progression free survival of 54 months vs. 71.5 months.

Conclusion

There are no clinical characteristics that form prognostic factors in uVIN, except for multifocality of lesions, that is correlated with a higher recurrence rate. Furthermore, progression of uVIN to carcinoma was accelerated and increased in immune compromised patients, suggesting that studies of local immunity in uVIN may reveal potential prognostic factors, as well as aid the development of new treatment modalities.

Introduction

Vulvar intraepithelial neoplasia (VIN) is a chronic premalignant skin disease that can be classified into usual type VIN (uVIN), (old nomenclature VIN 2 /3), and differentiated type VIN (dVIN)[1]. dVIN occurs in postmenopausal women and is associated with lichen sclerosus whereas uVIN, accounting for 90% of VIN cases, is caused by a persistent human papilloma virus (HPV) infection, mainly high risk HPV type 16[2,3]. uVIN has an increasing incidence worldwide in young women possibly due to the increase of HPV infections[4,5]. HPV is among the most common sexually transmitted diseases, with a lifetime risk of infection of 80%[5]. HPV-induced disease of the lower genital tract and its outcome are influenced by the host immune response, smoking, and immunosuppression[6-8]. It has been known for long that cellular immune responses against HPV protect against the development and progression of HPV-induced disease[9,10]. In the majority of uVIN patients HPV specific responses are either weak or absent[11,12]. Spontaneous regression is low and estimated at 1.5% of cases[2,13,14]. uVIN has a malignant potential in 3% of treated and 9% of untreated patients in 1-8 years [2,13,15]. The malignant potential of uVIN in immune compromised patients is 50-fold higher in comparison to the general population[16].

Patients with uVIN often suffer from severe and often long-lasting complaints of pruritis, pain and sexual dysfunction. Standard treatment for uVIN has been for a long time surgical (local excision and laser treatment). The aim of treatment is relief of symptoms, exclusion and reduction of the risk on invasive disease, and restoration of normal anatomy. If symptoms lack, expectative management of uVIN by close follow up visits can be advocated[17]. Conventional treatments are well known to be complicated by high recurrence rates, disfigurement of vulvar anatomy and impact on psychosexual function[18,19]. At present, a shift from conventional surgical therapies towards (experimental) immunotherapies such as the topical agent imiquimod (Aldara®, 3M Pharmaceuticals, St Paul,MN), photodynamic therapy, and therapeutic vaccination is visible with the aim to induce clearance of HPV, preserve vulvar anatomy and reduce psychosexual morbity[2]. Responsiveness to imiquimod treatment is associated with pre-existing HPV specific T-cell responses[11,20,21]. Reinforcement of the HPV-specific T-cell response by therapeutic vaccination has shown promising clinical responses of established HPV induced anogenital neoplasia, especially when vaccination was combined with imiquimod[20,22,23]. Despite these promising clinical results a notable number of patients are not able to respond to these immunotherapies[20,22,24]. There is a need to identify factors associated with patients who are at risk of non-responsiveness to (immuno)therapy, developing recurrences and progression into vulvar carcinoma. These predictors may assist in choice of therapy alternative to the complexity and limited possibilities of surgery[25]. We therefore performed a retrospective study in a single centre cohort to determine possible risk factors associated with the development of recurrences and progression into invasive carcinoma in patients with uVIN.

Materials and Methods

A retrospective chart review was performed of 136 patients with uVIN who were treated in our institution between 1990 and July 2012. This study was approved by the medical ethic committee of the Leiden University Medical Center. Patients participating in our vaccination trials were excluded from this study[22]. Patients with micro- (n=9) or macroinvasive vulvar carcinoma (n=16) at first diagnosis and missing demographic baseline information (n=38) were also excluded from analysis which revealed a group of 73 patients with uVIN. Demographic information, treatment type, pathology reports and clinical outcomes were reported. Recurrent disease was defined as diagnosis of uVIN after successful treatment without residual disease. Residual disease was defined as the presence of visible lesions immediately after therapy. Histological examination of margins of excision specimens were analyzed by an experienced pathologist. History of concomitant disease defined as abnormal cervical cytology, CIN, VAIN or cervical cancer was documented. Progressive disease was defined as the development of a (micro-) invasive carcinoma and consists of both occult carcinomas in a persistent lesion as well as development of a carcinoma in the follow up after therapy.

All uVIN samples included in this study were typed for HPV on the first lesion of uVIN by HPV16 PCR with a HPV16 specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotypine *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV16 negativity[26,27].

In data analysis the statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL) was used. Group comparisons of demographic characteristics were performed by Pearson's Chi-square test or Mann-Whitney test. Univariate analysis by Cox-regression was used for comparison of recurrence-free survival analysis (RFS) and progression free survival (PFS). RFS thereafter was analyzed by Cox proportional hazards regression in respect of smoking status, lesion type, age, treatment modality and concomitant disease. RFS was measured from date of first treatment until the date of recurrence or last follow up whereas PFS was measured from date of diagnosis until date of invasive disease.

Results

The mean age at diagnosis of uVIN was 43 years (range 19-84 years) with a median FU time of 49 months. All patient characteristics are shown in *(table 1)*. The majority of patients (60.3%) presented with complaints of pruritis, pain or discomfort at time of diagnosis. Lesions were multifocal in 43.8% and unifocal in 52.1% of the patients. Of the included patients, 65.8% were current smokers at diagnosis of uVIN, 11% former smokers, 13.7% non-smokers and from 4.1% patients smoking state was unknown. Eleven patients used immunosuppressive medication; 4 with autoimmune disease (Crohn's disease, rheumatic arthritis and systemic lupus erythematosus), 6 allograft recipients and 1 HIV positive patient. Topical corticosteroids were used by 40.9% of patients during one or more periods in the follow up. Multicentric disease, determined by cytological or histological diagnosed cervical or vaginal dysplasia (CIN and/or VAIN), was present in 75.3% of patients during follow up and most patients (83.6%) developed uVIN after diagnosis of cervical dysplasia in a median time of 65 months (range -51 to 335 months) whereas only a small number of patients developed CIN after uVIN *(table 1*).

First therapy in the treatment of uVIN (*table 2*) was surgical excision in 33 patients (45.2%), laser therapy in 25 patients (34.2%), laser and excision in 9 (12.3%) and imiquimod in 6 (8.2%) of patients after a median time of 2 months after diagnosis in a range of 0-158 months. More unifocal lesions were treated with excision (21 vs. 10, *ns*) whereas patients treated with combination of laser and excision often had multifocal lesions (2 vs. 6, *ns*) (*table 2*). A total of 70 excisions were performed in 50 patients of which 71.4% had histological positive margins. Similarly, in 61.5% of the combined laser and excision therapies (n=13) the excisions borders were considered positive for uVIN. Residual disease was found in 34 patients (46.6%) which resulted in the use of adjuvant therapy in 30 patients. Residual lesions occurred in 15.7% after excision, in 22.9% after laser therapies, in 57.1% after imiquimod treatment, and in 30.8% of 13 combined laser and excision interventions.

Table 1: Patient Characteristics (N=73)

Characteristic	Value (n)
Lesion histology	
VIN 2	17 (23.3%)
VIN 3	56 (76.7%)
Age at diagnosis (years)	
Mean	44.78
Median	43.0
SD	14.92
Range	19-84
Body mass index (kg/m^2)	
<18.5	5 (6.8%)
18.5-25	31 (42.5%)
>25	29 (39.7%)
Unknown	7 (9.6%)
Lesion type	
Unifocal	38 (52.1%)
Multifocal	30 (43.8%)
Complaints at diagnosis	44 (60.1%)
None	17 (23.3%)
Pruritis	14 (19.2%)
Pain/discomfort	6 (8.2%)
Pruritis and pain/discomfort	23 (31.5%)
Dyspareunia	1 (1.4%)
Unknown	12 (16.4%)
Smoking status	
Current	48 (65.8%)
Former	8 (11%)
Never	10 (13.7%)
Unknown	3 (4.1%)
HPV type	
Negative	5 (6.8%)
16	48 (65.8%)
33	7 (9.6%)
16 + 33	1 (1.4%)
Multiple hrHPV (e.g. 33,31,51,44)	3 (4.1%)
18	1 (1.4%)
6	2 (2.7%)
73	1 (1.4%)
Not tested	5 (6.8%)
Topical use corticosteroids	
Yes	28 (38.4%)
No	45 (61.6%)
Immunosuppressive medication	11 (15.1%)
HIV	1 (1.5%)
Allograft recipient	6 (8.2%)
Autoimmune disease	4 (5.4%)

Concomitant disease	55 (75.3%)
None	7 (9.6%)
Abnormal cervical cytology (pap 2/3a)	18 (24.7%)
CIN	31 (42.5%)
CIN + VAIN	4 (5.5%)
Cervical Carcinoma	2 (2.7%)
Follow-up time (months)	
Median	49.0
Mean	77.1
SD	79.5
Range	0-307

Table 2: Primary treatment and lesion type (N=)

	Excision	Laser	Aldara	Laser and Excision	Chi^2
Unifocal	21 (67.7%)	13 (52%)	2 (33.3%)	2 (25%)	
Multifocal	10 (32.2%)	12 (48%)	4 (66.7%)	6 (75%)	
Total	31	25	6	8	0.105

Recurrences

After first therapy, 37 patients (50.7%) developed recurrent lesions in just more than one year (median 14 months; range 1-168) *(table 3)*. Twelve patients had 1 recurrence (16.4%), 16 patients had 2 recurrences (21.9%), 4 patients 3 recurrences (5.5%), and in 5 patients (6.8%) 4 or more recurrences of uVIN were diagnosed. At time of diagnosis 76.5% of patients presented with symptoms, 11% had no complaints and of 5 patients it was not known. Of the patients with recurrent disease, 16 had prior excision (47%), 14 laser therapy (41.1%), 1 was treated with imiquimod (2.9%) and 6 patients were treated with laser and excision (17.6%). Recurrent disease was associated with multifocal lesions (p=0.008) in a univariate analysis irrespective of time to recurrence *(table 3*). Median time to recurrence was not significantly longer in patients with negative borders (n=2) compared to patients with positive borders (n=12) (79 vs. 30 months, p=0.189). Patients who were treated by excision however, had a significant longer median time until recurrence of 41.5 months, laser therapy of 7.5 months, imiquimod of 13 months, and laser and excision of 6 months (p=0.032).

Table 3: Factors associated with recurrence and progression

	Recurrence (N (%))	No recurrence (N (%))	Chi² (p=)	Cox (p=)	Progression (N (%))	No progression (N (%))	Chi² (p=)	Cox (p=)
First treatment modality (n=73)			0.320	**0.019**			0.302	0.200
Excision	16 (48.8%)	17 (51.5%)			4 (12.1%)	29 (87.9%)		
Laser ablation	14 (56%)	11 (44%)			4 (16%)	21 (84%)		
Imiquimod	5 (83.3%)	1 (16.7%)			0	6 (100%)		
Excision + Laser	6 (66.7%)	3 (33.3%)			3 (33.3%)	6 (66.7%)		
Surgical borders (n=63)			0.667	0.383			0.083	0.326
Positive	12 (41.3%)	17 (58.6%)			5 (17.2%)	24 (82.8%)		
Negative	2 (50%)	2 (50%)			0	4 (100%)		
Not applicable (laser/imiquimod)	16 (53.3%)	14 (46.7%)			4 (13.3%)	26 (86.7%)		
Unknown	7 (70%)	3 (30%)			2 (20%)	8 (80%)		
Age (years) (n=73)			**0.049**	0.682			0.479	0.100
Mean (SD)	41.41 (13.04)	48.25 (16.08)			41.82 (15.02)	45.31 (14.97)		
Smoking (n=69)			0.468	0.083			0.281	0.142
Yes	25 (52.4%)	23 (47.9%)			9 (18.8%)	39 (81.2%)		
No	10 (47.6%)	11 (52.4%)			2 (9.5%)	19 (90.5%)		
Lesion type (n=70)			**0.008**	**0.006**			0.093	0.950
Unifocal	13 (34.2%)	25 (65.8%)			3 (7.9%)	35 (92.1%)		
Multifocal	21 (65.6%)	11 (34.4%)			7 (21.9%)	25 (78.1%)		
Lesion size cm² (n=33)			0.875	0.546			0.181	0.492
Mean	6.41 (7.49)	7.02 (11.11)			13.18 (18.35)	5.96 (8.50)		
Complaints at diagnosis (n=61)			0.567	0.877			0.215	0.823
Yes	20 (45.5%)	24 (54.5%)			8 (18.2%)	36 (81.8%)		
No	8 (47.1%)	9 (52.9%)			1 (5.9%)	17 (94.1%)		
BMI (n=65)			0.806	0.286			0.520	0.814
</= 25	20 (57.1%)	16 (53.3%)			5 (45.5%)	31 (57.4%)		
>25	15 (42.9%)	14 (46.7%)			6 (54.5%)	23 (42.6%)		
Immunocompromised (n=73)			0.480	0.490			0.211	0.091
Yes	5 (45.5%)	6 (54.5%)			3 (27.3%)	8 (72.7%)		
No	32 (51.6%)	30 (48.4%)			8 (12.9%)	54 (87.1%)		
Concomitant disease (n=62)			0.610	0.319			0.734	0.621
Yes	30 (54.5%)	25 (45.5%)			8 (14.5%)	47 (85.5%)		
No	4 (57.1%)	3 (42.9%)			1 (14.3%)	6 (85.7%)		
HPV type (n=58)			0.492	0.752			0.553	0.300
16	27 (56.2%)	21 (43.8%)			8 (16.7%)	40 (83.3%)		
33	5 (50%)	5 (50%)			2 (20%)	8 (80%)		

Considering time to recurrence, a univariate Cox regression analysis of recurrence free survival was performed. RFS after diagnosis was associated with multifocal disease (p=0.006, HR 2.77, 95% CI 1.30-5.89). RFS after first therapy in univariate Cox regression analysis revealed a longer recurrence free survival between different treatment modalities in favor of excision and imiquimod therapy (p=0.010, HR 5.5, 95% CI 1.94-15.63 (laser and excision compared to excision)). RFS was not associated with immunosuppressive medication (p=0.490), smoking (p=0.083), positive margins (p=0.383), concomitant disease (p=0.319), local corticosteroid use (p=0.613), or HPV type (p=0.752). Multifocal disease remained an independent prognostic factor for a decreased RFS in multivariate Cox analysis corrected for multifocal disease, smoking, HPV state, immune compromised patients and BMI (p=0.027) whereas excision as first therapy was no longer associated with a longer recurrence free survival (p=0.142) (*figure 1A+1B)*.

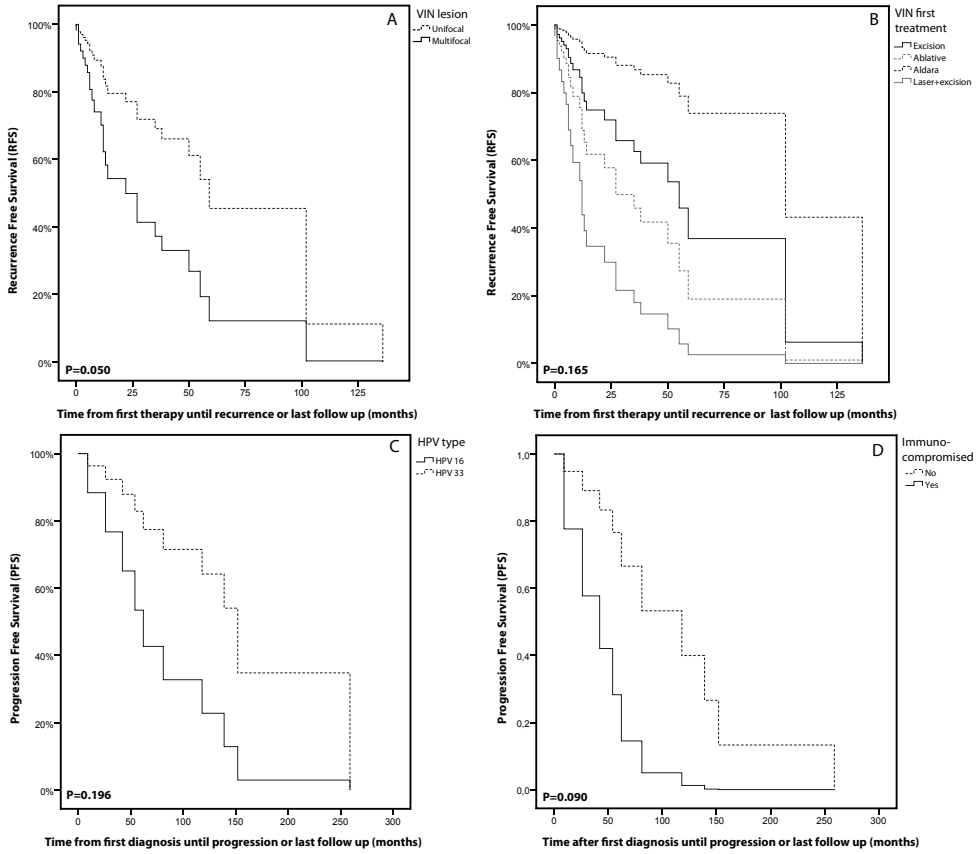

Figure 1

A+B Multivariate Cox-based adjusted curves of recurrence free survival (RFS) of multifocal VIN lesions and first treatment type corrected for smoking, HPV type, first treatment modality, BMI, immunocompromised patients and uVIN lesion type **C+D**: Univariate Cox regression progression free survival curves (PFS) for HPV type and immunocompromised patients

Progressive disease

In 11 patients (15.1%) an invasive carcinoma developed during follow up with a median of 71.5 months after first diagnosis (range 9-259 months) *(table 3).* Progression of uVIN into carcinoma of a persistent lesion occurred in 2 patients (2.7%), in 9 patients a carcinoma was diagnosed after successful therapy of uVIN without residual lesions. In 7 patients a micro invasive carcinoma (≤1mm invasion) and in 4 patients a macro invasive carcinoma (>1mm invasion) was diagnosed. 4 of 11 patients were immune compromised with a median time of progression of 54 months (range 9-62 months). Type of first treatment showed no differences in progression free survival in univariate Cox analysis (p=0.200). HPV type 16 compared to 33 was not associated with progression of uVIN into invasive carcinoma (p=0.300) and despite the shorter time to progression in immune compromised patients this resulted not in a significant shorter PFS (p=0.091, HR 4.744, 95% CI 0.78-28.85) in univariate Cox analyses *(figure 1C+1D*).

Discussion

In this study, higher recurrence rates for uVIN were only correlated with multifocality of lesions. First treatment by excision or imiquimod therapy as well as unifocal lesions were associated with increased recurrence free survival. Immune compromised patients showed a trend towards a shorter progression free survival. HPV type, smoking, multicentric disease, use of topical steroids, and positive surgical borders were not associated with a shorter recurrence free survival.

After standard treatment, recurrence rates for uVIN are high, and most recurrences occur within 3 years of follow-up[13,15,28-30]. This was confirmed in our study. Higher recurrences rates have previously been associated with positive surgical margins and multifocal disease; although other studies showed that positive surgical margins were not associated with recurrent disease [13,15,29,30]. In addition, the margin status of resected uVIN lesions has not shown to be associated with progression to invasive cancer [13,31]. In our study, positive surgical margins were not associated with recurrent disease: 12 of 29 patients with positive margins developed recurrent disease, whereas 17 patients did not (41.3% vs. 58.6%, respectively) *(table 3).* Histological positive margins can be regarded as minimal residual disease, a condition where the immune system might be more effective in comparison to situations with large tumor burden. For example, results from our vaccination trial in patients with HPV16-positive uVIN have shown that smaller lesions were more likely to regress in response to vaccine-induced HPV16-specific immunity [22,32]. In addition, surgical removal of primary tumors was shown to restore cell-mediated immune responses even in the presence of metastatic disease[33]. These data suggest that in some patients, surgical

removal of lesions or reduction of lesion size can lead to the restoration of adaptive immune responses which may prevent recurrent disease.

Patients treated with excision were found to have lower recurrence rates compared to patients treated with laser or combined laser and excision in our cohort. This finding might be explained by the fact that patients with large lesions and/ or multifocal disease were selected for combination therapy with excision and laser (our study: 2 unifocal vs. 6 multifocal lesions). Recently, the same observation was done by Wallbillich et al., who showed an association between a decreased RFS and treatment of excision and laser[30]. Another study by Brown et al showed that after local excision followed by laser treatment of the excision margins, the recurrence rate was decreased in patients with uVIN[34]. When interpreting these findings, it should be kept in mind that a simple comparison for efficacy between different treatments is very difficult because the choice of treatment depends on several factors, for example uni- or multifocality of lesions and patient related factors.

Treatment with the immune response modifier imiquimod is more and more used since the publication of a large randomized controlled trial in patients with uVIN in 2008[24]. In this study, a reduction of >25% in lesion diameter was observed in 80% of the treated patients, with a complete disappearance of the lesions in 35% of the patients. HPV clearance occurred in 60% of patients[24]. Recurrences after imiquimod are established at approximately 20.5% vs. 53.5% after surgical therapies[35,36]. The small number of patients treated with imiquimod in our group, however show comparable results to excision regarding RFS in univariate analyses (*figure 1*). In this small cohort a complete response was achieved in one of six (16.7%) of patients primarily treated with imiquimod, the other patients with residual lesions were secondarily treated with laser therapy. Interestingly, clinical responses after imiquimod treatment in uVIN have been shown to be associated with normalization of immune cells in the lesions, and also with the presence of circulating HPV-specific T cells[11,24,35].

Other factors which have been associated with recurrence are smoking[30], HIV infection[37], use of immunosuppressive drugs[28] and p53 gene mutation[2,38]. We found no association between smoking and increased risk for recurrences in contrast to Wallbillich et al.[30] This may be related to the high number of smokers in our cohort, since only 8 patients never smoked or by longer median time of follow up in our group (21 months vs. 49 months). Other studies found no association of smoking and recurrences of uVIN either[28,29]. Smoking is however clearly associated with the development of uVIN and we feel that patients should be counseled to stop smoking during follow up visits since it is well known that smoking results in a decreased local immune response which makes it easier for the HPV virus to invade and persist[6,7,15].

In our cohort the number of progressive disease is 15.1% of which 60% are micro-invasive carcinomas (≤1mm), that are known to have excellent survival after wide local excision without lymph node dissection. Of the 11 patients with progression into invasive cancer,

4 were immune compromised. These patients are known to have an increased risk of multiple HPV induced anogenital lesions: over 80% of immune compromised uVIN patients were shown to have a history of CIN or developed CIN during follow-up in one study[8,39,40]. Importantly the immune compromised patients had an increased risk (27.3% vs. 12.9%) of progression into vulvar carcinoma and displayed a relatively faster (54 vs. 71.5 months) development of cancer compared to non immune compromised patients. A 50-fold increase in the risk to develop vulvar carcinoma in immune compromised patients was demonstrated before, and yearly cervical screening in combination with vulvar, vaginal and anal inspection is advised[16]. We could not detect an increased risk for recurrences; however, patient numbers were small.

Multicentric HPV-associated disease has been described in patients with cervical intraepithelial neoplasia (CIN) and uVIN[41,42]. Other studies have shown that 71% of uVIN patients had previous, concurrent or subsequent vaginal intraepithelial neoplasia (VAIN), (peri-)anal intraepithelial neoplasia ((P)AIN), CIN or cervical carcinoma[41,42]. This was confirmed in our study, in which 75% of patients were diagnosed previously or concurrent with cervical dysplasia or VAIN. Other studies showed that multicentric disease was associated with a higher risk of progression compared to isolated uVIN[43]. We could not confirm these data although 7 of 11 patients with progressive disease displayed multicentric HPV lesions. Multicentric disease may be a reflection of a higher susceptibility for HPV infections and not able to clear the infections which are able to induce transformation (e.g. HPV type 16)[40,42]. HPV testing of multicentric anogenital HPV induced neoplasias revealed an identical HPV infection in 46% of cases (most often HPV 16 in 69%, followed by HPV 33 in 13%). Most uVIN lesions are induced by HPV16 and HPV33, the types most often found in multicentric disease, explaining why these patients are at risk for HPV-induced disease affecting the cervix, vagina, and/or the anus. From these data it follows that patients with uVIN must be carefully screened for cervical dysplasia, in particular when they are immune compromised.

An important limitation of our study, and also from other studies on clinical characteristics in VIN patients, is that patient numbers are relatively small because of the rarity of the disease. This means that non-significant results, for example for the surgical margin status or type of treatment may relate to small patient numbers rather than being no clinical difference. Therefore, for the translation into clinical practice, larger prospective, eventually multicenter studies are needed in patients with uVIN.

In summary, our data indicate that multicentric disease is highly common in patients with uVIN. Recurrences after treatment are high and are only associated with multifocal lesions. No other clinical characteristics are correlated with a higher recurrence- or progression rate. Immune compromised patients show a trend towards a shorter progression free interval.

From this study and data from others it is clear that the immune system plays a crucial role in the development of and course of disease in patients with HPV-induced lesions. Future research should be aimed at a detailed analysis of the local immune environment in relation to the clinical outcomes of disease in patients with uVIN. Furthermore, the strengthening of the systemic and local immune immunity to HPV by (adjuvant) immunotherapies may assist in the improvement of the treatment for patients with uVIN and other HPV-induced disease.

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CHAPTER 4

ALTERATIONS IN CLASSICAL AND NON-CLASSICAL HLA EXPRESSION IN RECURRENT AND PROGRESSIVE HPV INDUCED VULVAR INTRAEPITHELIAL NEOPLASIA (VIN) AND IMPLICATIONS FOR IMMUNOTHERAPY

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Abstract

Immunotherapy of uVIN is promising however many patients still fail to show clinical responses, which could be explained by an immune escape through alterations in HLA expression. Therefore, we analysed a cohort of patients with a primary (n=43) and subsequent recurrent uVIN lesion (n=20), vaccine treated uVIN patients (n=12), patients with HPV-induced vulvar carcinoma (n=21) and healthy controls (n=26) for the expression of classical HLA-class I/II and non-classical HLA-E/-G and MICA. HLA-class I was downregulated in 70% of uVIN patients, including patients with a clinical response to immunotherapy. Downregulation of HLA-class I is probably reversible, as only 15% of the uVIN cases displayed loss of heterozygosity (LOH) and HLA-class I could be upregulated in uVIN keratinocyte cultures by IFNγ. HLA-class I downregulation is more frequently associated with LOH in vulvar carcinomas (25-55.5%). HLA-class II was found to be focally expressed in 65% of uVIN patients. Of the non-classical molecules, MICA was downregulated in 80% of uVIN whereas HLA-E and -G were expressed in a minority of cases. Their expression was more prominent in vulvar carcinoma. No differences were found between the alterations observed in paired primary and recurrent uVIN. Importantly downregulation of HLA-B/C in primary uVIN lesions was associated with the development of recurrences and progression to cancer. We conclude that downregulation of HLA is frequently observed in premalignant HPV-induced lesions, including clinical responders to immunotherapy, and is associated with worse clinical outcome. However, in the majority of cases downregulation may still be reversible.

Introduction

Usual vulvar intraepithelial neoplasia (uVIN) is a chronic premalignant skin condition, with an increasing incidence mainly in young women, which is caused by a persistent high risk human papilloma virus (HPV) infection in over 90% of cases.[1,2] uVIN causes complaints of severe and long-lasting pruritis, pain and sexual dysfunction and has a malignant potential of 3-4% in treated and of 9% of untreated patients. [1,3] Since conventional treatments for uVIN are characterised by high recurrence rates of 20-40% and psychosexual problems, there is a need for alternative therapies.[4-6] Failure of the immune system to induce a strong and effective immune response to HPV is known to cause viral persistence and development of these premalignant anogenital lesions.[7-9] The microenvironment of uVIN lesions is characterised by high numbers of infiltrating CD4+ T cells as well as regulatory T cells (Tregs) and low numbers of cytotoxic CD8+ T cells (CTLs) compared to controls.[10-12] Immunotherapy aims to overcome the inertia of the immune system and is therefore considered a possible effective treatment option for uVIN.[13-17] Immunotherapy by imiquimod (Aldara®,), photodynamic therapy (PDT) and/or therapeutic vaccination, in both standard and experimental settings, are nowadays widely used as an alternative to conventional treatments, and have shown promising results in uVIN.[13-17] Imiquimod is a topical immune response modulator which activates dendritic cells and induces proinflammatory cytokine expression and T cell activation.[14] PDT leads to tumor directed cell death and induces local inflammation which activates antigen presenting cells and induces effector T cells.[17] Therapeutic vaccines are designed to reinforce HPV specific CD4+ and CD8+ T cell responses, and particularly the protein peptide TA-CIN in combination with imiquimod and the HPV 16 synthetic long peptide (SLP) vaccine have met with clinical success.[13,15] Application of imiquimod, PDT and therapeutic vaccination or a combination of these therapies are all associated with an increase in intralesional CD4+ and CD8+ T cells. Clearance of HPV is associated with a normalization of immune cell infiltration.[12,15,16] Lack of efficacy of immunotherapy was shown to be associated with the presence and increase of Tregs.[11,15,16,18]

The majority of vulvar carcinomas and about 30% of VIN were shown to display down-regulation of human leukocyte antigen (HLA) class I in a single study.[12] This suggests that alterations in the expression of classical, and potentially also non-classical HLA-molecules may result in escape from a specific T cell response, as well as recurrence/progression of the lesions, or unresponsiveness to immunotherapy. Loss of HLA-class I was previously shown to be associated with non-responsiveness to PDT.[12] In analogy, in HPV-induced cervical cancer loss of HLA-A was associated with poor survival.[19] Alterations in HLA-class I expression are caused by a variety of mechanisms and these alterations may be reversible ('soft') or irreversible ('hard'), in case of molecular defects as loss of heterozygosity (LOH) or beta-2 microglobulin (β2M)/HLA-class I mutations.[20-23] Furthermore, downregulation of the non-

classical MHC class I chain related molecule A (MICA), which interacts with the co-stimulatory natural killer cell lectin-like receptor (NKG2)D receptor on natural killer (NK) cells and T cells[24], is observed in cervical cancer where it is associated with a decreased survival when analysed in the context of the CTL/Treg ratio and the expression of classical HLA molecules.[19] Elevated expression of HLA-class II and that of the non-classical molecules HLA-E and HLA-G has also been observed in HPV-induced cervical cancers.[25-29] HLA-E hampers the efficacy of NK and T cells by binding to the inhibitory CD94/NKG2A receptors expressed on tumor infiltrating lymphocytes (TILs). While the expression of HLA-E was associated with poor survival in ovarian cancer it's effect was limited in cervical squamous cell carcinoma and associated with better survival in cervical adenocarcinoma.[27,28] HLA-G inhibits the function of NK cells, T cells and antigen presenting cells (APCs) and induces Tregs and myeloid derived suppressor cells by direct binding to the inhibitory receptors immunoglobulin like transcripts ILT-2 and ILT-4 and the killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4).[30] The expression of HLA-G was preferentially expressed in cervical cancers with high number of TILs and may have caused T cell dysfunction.[29]

Based on these data in HPV-induced cervical cancer, as well as on the fact that HLA downregulation has also been found in vulvar carcinoma, we postulated that similar mechanisms may also play a role in premalignant HPV-induced vulvar neoplasia governing their progression to vulvar carcinoma and non-responsiveness to immunotherapy. Therefore, we analysed the expression of classical and non-classical HLA molecules in the lesions of patients with HPV-induced non-recurrent and recurrent uVIN, and vulvar carcinoma by immunohistochemistry and compared the results to healthy control tissue. Furthermore, the lesions of 12 patients, treated by HPV 16 SLP vaccination with or without clinical success, were analysed to determine the influence of HLA expression alteration on clinical responsiveness. Moreover, we studied whether HLA-class I downregulation could be regarded as 'hard' or 'soft' by LOH analysis and by comparing HLA expression on freshly isolated HPV-infected and non-infected keratinocytes after interferon (IFN)γ stimulation in vitro.

Material and Methods

Patient characteristics and material

All patients treated for uVIN in the LUMC between 1990 and July 2012 of whom archival formalin-fixed, paraffin embedded tissue of the first uVIN lesion was available, were selected and included in this study (N=43, age: mean 47.26 years; range 19-84). Of 20 patients recurrent uVIN lesions were also included. Recurrence free survival was determined as the interval between first therapy and diagnosis of recurrent disease or the last follow up

visit in case of no recurrent disease. Tissues from a cohort of HPV induced micro invasive- (<1mm infiltration) (N=8) and macro invasive vulvar carcinoma (>1mm infiltration) patients (N=13) were included as well to evaluate HLA expression in progressive vulvar neoplasia (age: mean 69.14 years; range 49-95). Samples from elective reductions of the labia minora, which were HPV negative (N=26), served as healthy controls (age: mean 32.96 years; range 16-54). All samples included in this study were typed for HPV by HPV 16 polymerase chain reaction (PCR) with a HPV 16 specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotyping *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV 16 negativity.[31,32] Histologic examination of all controls revealed no dysplasia or other abnormalities. Paraffin embedded uVIN biopsies of patients included in the HPV 16 SLP vaccination trial taken before vaccination were evaluated for tissue availability for HLA-class I immunofluorescent staining (N=12, of which 6 patients with and 6 patients without clinical response measured as lesion reduction).[13] The Leiden University Medical Ethic Committee approved this study on prospective collection of healthy control tissue and for keratinocyte isolation patients were enrolled in the Circle study which investigates cellular immunity against HPV induced neoplasia. Furthermore archival formalin-fixed paraffin embedded patients samples were handled according to the medical ethical guidelines described in the code of conduct for proper secondary use of human tissue of the Dutch federation of Biomedical Scientific Societies.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were stained according to standard protocols as described previously[27] with mouse monoclonal HLA-DR (anti HLA-DR clone TAL.1B5; DAKO (1:400)), mouse monoclonal HLA-DRDQDP (anti HLA-DRDQDPQ clone CD3/43; DAKO (1:2000)) which recognises all HLA class II molecules HLA-DR, -DQ and -DP, rabbit polyclonal MICA (anti MICA LS-B1377; Lifespan BioSciences (1:100)), mouse monoclonal HLA-E (anti HLA-E clone MEM-E/02; Serotec (1:200)) and mouse monoclonal HLA-G (anti HLA-G clone 4H84; Lifespan BioSciences (1:100)). Brown membrane and cytoplasm staining of epithelial cells was indicated positive for HLA expression. In case of no basal membrane staining and only cytoplasm staining this was scored as negative for HLA expression. Stromal and immune cells served as internal positive control and an extra section was stained without primary antibody as a negative control.

Immunofluorescence

Simultaneous detection of HLA-class I was performed by three color fluorescence staining according to standard procedure[27] using mouse monoclonal HCA-2 and HC-10 (anti HLA-A and anti HLA-B/C; kindly provided by Prof. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands (1:400 and 1:2500)) and rabbit polyclonal β2M (anti β2M; clone A-072;

DAKO (1:4000)). HLA-B/C antibody recognises all HLA-B and HLA-C molecules. Staining of lymphocytes was performed by use of rabbit polyclonal CD3 (anti CD3 clone ab828; Abcam (1:100)) and secondary antibodies were all goat anti mouse isotype specific antibodies with Alexa Fluorchromes; Alexa Fluor 488, 546, and 647 (Molecular Probes; 1:200) Five randomly selected representative images were captured using a confocal scanning microscope (LSM510, Zeiss) in a multitrack setting with a 25x/0.80 Plan-NEOFluar objective. Expression of the HLA molecules on the basal membrane was scored and stromal immune cells served as an internal positive control. Two additional sections were stained without primary or secondary antibody as a negative control.

Evaluation of HLA expression and lymphocyte infiltrate

HLA expression patterns were scored according to the scoring system by Ruiter et al.[33] Staining intensity of HLA at the basal membrane was scored as negative, weak, moderate or strong (0 to 3) (Supporting Information Fig.S1) and the percentage of positive cells was scored as: absent (<1%), sporadic (1-5%), local (6-25%), occasional (26-50%), majority (51-75%) or large majority (>75%). Final scores of both the intensity and the percentage were categorized into three groups: 0-1 (negative), 2-6 (weak/moderate) and 7-8 (strong). The slides were scored independently by two researchers (EvE and VB or EJ and MT) without prior knowledge of clinical or histopathological parameters. In case of discrepancies consensus was reached or a third researcher was consulted. Intraepithelial and stromal lymphocyte counts were represented as the number of cells per mm^2 for each slide (average of five 250x image slides) and were manually counted using the LSM 5 Image Examiner software.

LOH

DNA was extracted from micro dissected material (to obtain at least 70% of uVIN DNA) of 14 uVIN, 9 uVIN adjacent to micro invasive carcinoma, 13 HPV positive vulvar carcinoma cases and from the uVIN- and healthy control material from which primary keratinocytes were cultured (see below). DNA was extracted and analysed for LOH on chromosome 6p21, with a minimal input of 10ng DNA/PCR, by PCR amplification using the D6S273 and D6S265 microsatellite markers as previously described.[22] Definitions of thresholds for LOH >1.7, retention of heterozygosity (ROH) 0.76-1.3 and allelic imbalance ("grey area") 0.58-0.75 were used accordingly.[22]

Keratinocyte culture and IFNγ stimulation

Primary keratinocytes were isolated from biopsies or excisions from women undergoing surgery for uVIN, HPV induced vulvar carcinomas or elective reduction of the labia minora and cultured in E-medium in presence of irradiated 3T3J2 mouse fibroblasts and 5-10μM Rho-associated kinase (ROCK) inhibitor (Y-27632). The addition of ROCK inhibitor has proven to be a suitable method to indefinitely extend the life span of primary keratinocytes without

transduction of exogenous viral or cellular genes.[34,35] In case of successful culture the cells were adapted to keratinocyte serum-free medium (K-SFM; Medium 154 supplemented with HKGS kit, Invitrogen, Breda, The Netherlands) for one passage, after which they were stimulated with 0 or 100IU/ml IFNγ for 48 hours. HLA expression was analysed by flow cytometry. Details are given in Supplemental Information Materials and Methods. 3T3J2 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 2mM l-glutamine and 1% penicillin-streptomycin (complete DMEM medium) (Gibco-BRL, Invitrogen).

Statistical analysis

For data analysis the statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL) was used. Group comparisons of categorical data were performed by χ^2 test, and the Fishers exact test in case of small groups. The non-parametric Mann-Whitney U test was used for continuous variables. The paired McNemars categorical test or the paired Wilcoxon Signed Rank test for continuous variables were used to determine differences in primary and secondary lesions. The Spearman correlation coefficient was used to detect correlation in the non-parametric data and in case of normality Pearson correlation coefficient was used. The Shapiro-Wilk test was used to determine a normal distribution. The Bonferroni correction was applied for multiple testing revealing a *P*-value of <0.004 as significant. Both univariate and multivariate analysis, corrected for multifocality of uVIN, were performed by a Cox proportional hazard model for recurrence-free survival analysis (RFS) and progression free survival (PFS). Two sided *P*-values <0.05 were considered significant. GraphPad Prism 5.04 (Graphpad Software Inc., LA Jolla, CA, USA) was used to illustrate the data by graphs and figures.

Results

Patients

The clinical characteristics of the patient cohort are shown in Table 1. In 79% of uVIN patients HPV type 16 was detected, in the other uVIN samples HPV 33 (12%), HPV 73 (2%) or multiple high risk HPV infections (7%) were detected. First lesions were unifocal in 58.1% of cases and therapy consisted of excision in 51.2% (N=22), laser therapy in 30.2% (N=13), imiquimod in 7% (N=3) and a combination of laser and excision in 11.6% (N=5) of patients. Use of immunosuppressive medication was reported in 7 (16%) of patients and 80% of the patients were smokers.[36] The majority of patients with HPV-induced vulvar carcinomas had a low stage of disease according to the International Federation of Obstetrics and Gynaecologists (FIGO) and only 1 patient was diagnosed with a lymph node metastasis in the groins. None of these patients died in follow up and in 3 patients (14%) a recurrent vulvar carcinoma occurred after a median time of 7 months (range 6-46).

Table 1: Patient Characteristics

Characteristic	VIN patients (n=43)	Carcinoma patients (n=21)	HPV 16 SLP vaccination patients (n=12)	Controls (n=26)
Lesion histology				
High grade uVIN	43 (100%)	-	12 (100%)	-
Microinvasive carcinoma	-	8 (38.1%)	-	-
Macroinvasive carcinoma	-	13 (61.9%)	-	-
No dysplasia	-	-	-	26 (100%)
Age at diagnosis (years)				
Mean	47.3	69.1	38.2	33.0
SD	16.5	14.0	8.7	10.9
Range	19-84	49-95	29-60	16-54
Follow up time (months)				
Mean	85.2	96.5	24.1	n.a.
Median	50.0	8.5	23.7	
SD	85.0	76.5	2.1	
Range	0-307	2-242	20.5-28.1	
Lesion type				
Unifocal	25 (58.1%)	unknown	5 (41.7%)	n.a.
Multifocal	18 (41.9%)		7 (58.3%)	
Recurrent uVIN				
Yes	20 (46.5%)	9 (42.9%)	n.a.	n.a.
No	23 (53.5%)	12 (57.1%)		
Smoking status				
Yes	34 (79.1%)	9 (42.9%)	8 (66.7%)	-
No	6 (14%)	5 (23.8%)	-	-
Unknown	2 (4.7%)	7 (100%)	4 (33.3%)	26 (100%)
HPV type				
16	34 (79.1%)	14 (66.7%)	12 (100%)	-
33	5 (11.6%)	5 (23.8%)	-	-
16 + 33	1 (2.3%)	1 (4.8%)	-	-
Multiple hrHPV (e.g. 33,31,51,44)	2 (4.7%)	-	-	-
73	1 (2.3%)	-	-	-
18	-	1 (4.8%)	-	26 (100%)
Negative	-	-	-	-
Progression to carcinoma				
Yes	8 (18.6%)	n.a.	3 (25%)	n.a.
No	35 (81.4%)		8 (66.7%)	
Unknown	-		1 (8.3%)	
Immunosuppressive medication				
No	36 (83.7%)	20 (95.2%)	12 (100%)	26 (100%)
Yes (e.g. HIV, allograft recipient, autoimmune disease)	7 (16.3%)	1 (4.8%)	-	-

HLA expression in progressive course of disease

Expression of classical and non-classical HLA molecules at different stages of disease are depicted in Fig. 1 and Supporting Information Fig.S2. Partial downregulation of HLA-class I was found in 72% of uVIN lesions, in 88% of uVIN adjacent to micro invasive carcinoma, in 84% of vulvar carcinomas and in 8% of the controls. Total loss of HLA-A was detected in 9.3% of uVIN lesions and in 13% of uVIN lesions adjacent to microinvasive carcinoma. Total loss of HLA-B/C was found in 9% of uVIN lesions and in 38% uVIN lesions adjacent to micro invasive carcinoma whereas total loss of β2M was seen in 7% of uVIN, 13% of uVIN adjacent to micro invasive carcinoma and in 8.3% of vulvar carcinomas. Downregulation of β2M was more prominent in 83% - 100% of vulvar carcinoma compared with 58% of uVIN lesions (p=0.030). HLA-class II expression, determined by either HLA-DR or HLA-DRDQDP positivity, was focal in areas of the basal layer in 65% of uVIN, in 75% of micro invasive carcinomas, in 46% of the vulvar carcinomas and was absent in the controls. In 7% of uVIN lesions HLA-class II expression was limited to the expression of HLA-DQDP. The expression of the non-classical molecules HLA-E (14%) and HLA-G (16%) in uVIN lesions was low but increased with the progressive course of vulvar neoplasia. Both HLA-E and -G were expressed in 25% of micro invasive carcinoma and in 54% (p=0.041) and 46% (p=0.133), respectively of the macro invasive vulvar carcinomas. MICA was downregulated in approximately 80% of uVIN lesions, in a comparable number of the micro- and macro invasive vulvar carcinomas (100% and 92%, respectively) and in 7.7% of control tissues.

HLA expression in recurrent uVIN and recurrence free survival

We analysed 20 pairs of a primary uVIN lesions and the corresponding lesion that recurred after primary treatment. Analysis of the alterations in classical and non-classical HLA molecules revealed no overt differences between primary and subsequent recurrent lesions (Fig. 2). There was a clear correlation between the downregulation of HLA-B/C and the recurrences of uVIN lesions (p=0.029) as well as progression to carcinoma (p=0.016). Furthermore, our data suggest that the combination of HLA-class I and MICA downregulation was also associated with lesion recurrence (p=0.023) albeit that there was no difference in the risk of progression to cancer (Supporting Information Table S1). Expression of the other classical and non-classical HLA molecules was not predictive for recurrent uVIN lesions or progressive course of disease. Considering the time until recurrence or progression, the alterations in HLA expression had no influence on RFS neither on PFS (data not shown).

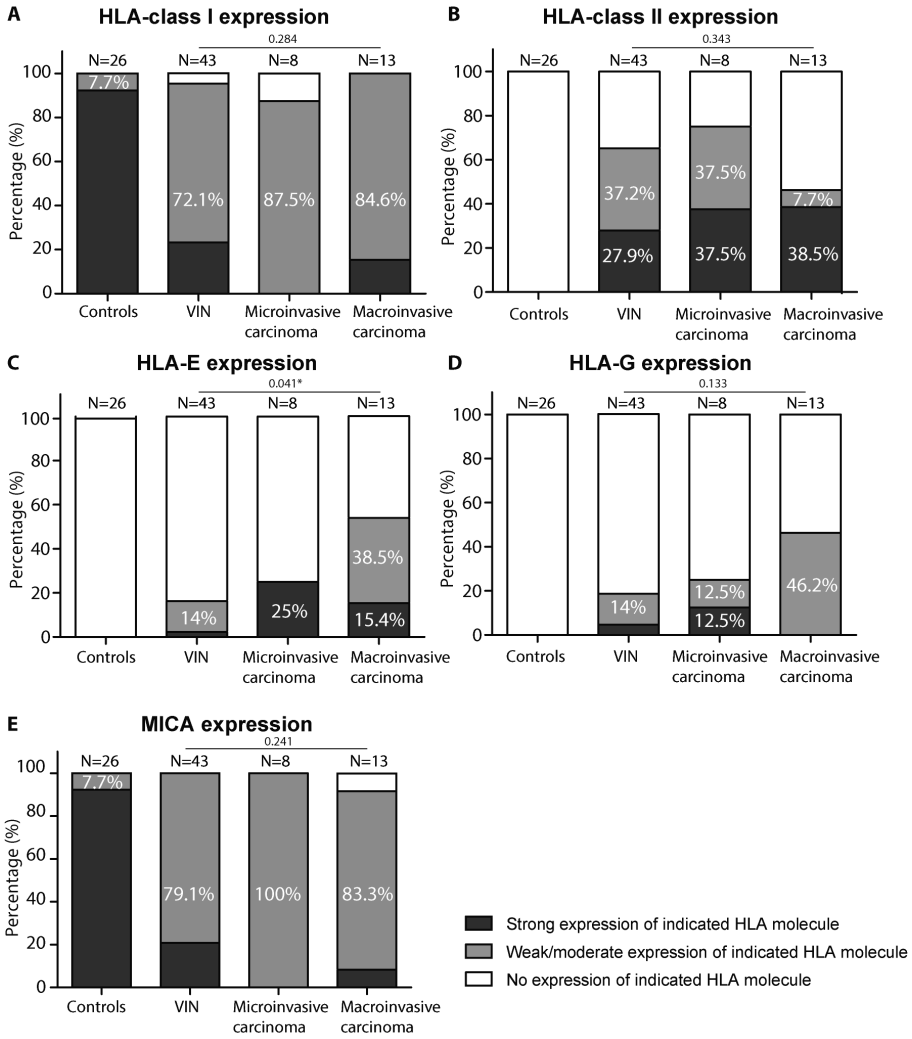

Figure 1: Classical HLA-class I, HLA-class II, HLA-E, -G and MICA expression in the progressive course of HPV induced vulvar neoplasia

Paraffin embedded tissues of healthy controls (n=26), high grade uVIN lesions (n=43), microinvasive- (n=8) and macroinvasive vulvar carcinomas (n=13) were stained by immunohistochemistry for the indicated HLA molecules. A: membranous expression of HLA class I was evaluated by use of antibodies to β2M, HLA-A (HCA-2 antibody) and HLA-B/C (HC-10 antibody). Weak HLA-class I expression was indicated in case of weak to moderate HLA-A and/or HLA-B/C membrane expression compared to stromal expression. No expression was indicated in case of total loss of β2M, HLA-A and HLA-B/C on the membrane of basal epithelial layer. B: expression of membranous HLA class II was evaluated by use of weak, moderate or strong membrane expression compared to stromal expression of HLA-DR or HLA-DRDQDP and healthy controls. Membranous expression of HLA-E (C), HLA-G (D), and MICA (E) was considered positive in case of moderate or strong expression. The expression of these molecules was considered downregulated in case of none or weak expression.
HLA expression is determined according to the scoring system of Ruiter et al.[33] (see example in supplemental figure 2)

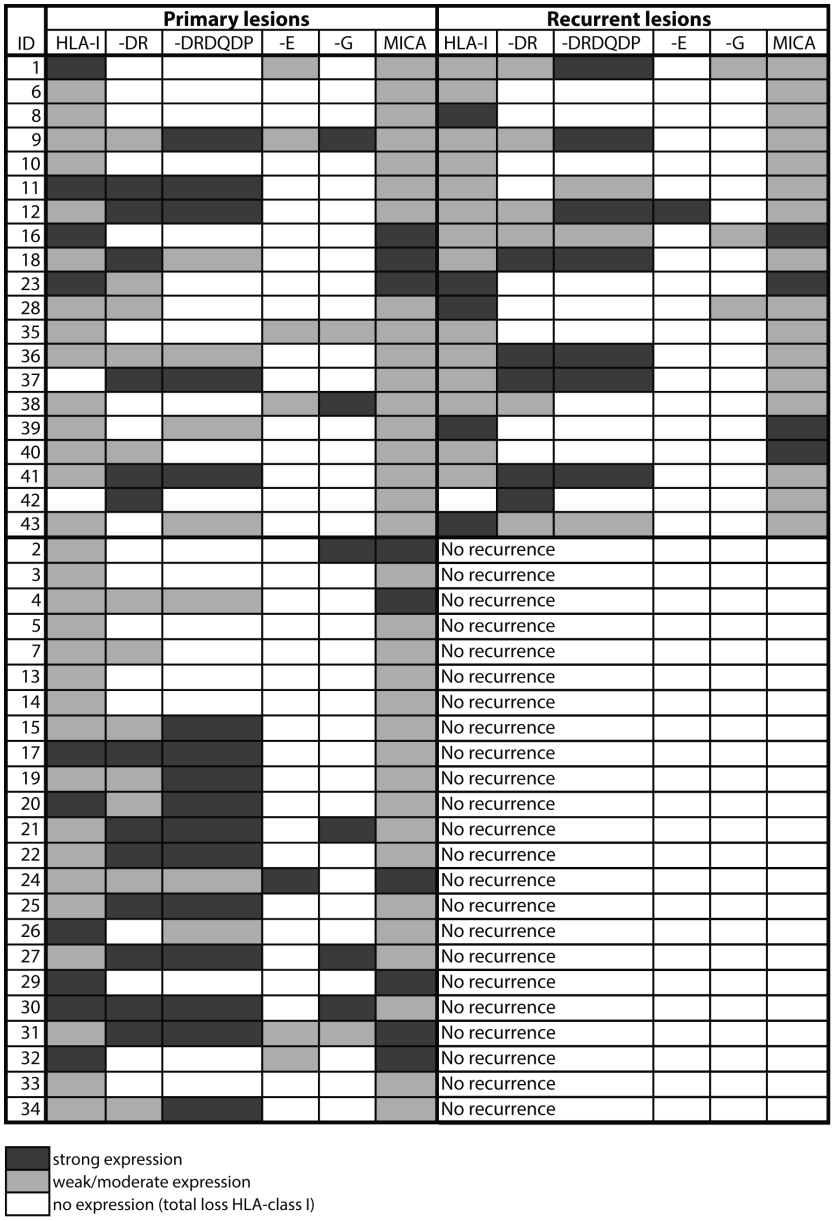

Figure 2: Classical and non-classical HLA expression in primary and recurrent uVIN lesions for each individual patient.

Primary lesions of high grade uVIN lesions (n=43) and concomitant secondary recurrent uVIN lesions (n=20) were analysed by immunohistochemistry for HLA-class I expression as described in figure 1. Depicted are the expression levels of all indicated molecules for each patient in the primary and the concomitant recurrent uVIN lesion showing that there are no distinct differences in alterations of classical and non-classical HLA molecules between the primary and recurrent lesions.

Hard and soft wired downregulation of HLA-class I in uVIN and vulvar carcinoma

In order to determine whether the observed downregulation of HLA by immunohistochemistry was 'hard'- or 'soft'-wired we selected 14 uVIN lesions based on tissue availability, 9 uVIN lesions adjacent to HPV positive micro invasive carcinomas and 12 HPV-positive macro invasive carcinomas for LOH evaluation (Supplemental Table 2). For 13 of the 14 uVIN lesions and for all carcinomas selected for LOH analysis, reliable results were obtained. Immunohistochemistry revealed downregulation of HLA-class I in 85% of these uVIN patients, in 100% of the micro invasive carcinomas and in 92% of the macro invasive carcinomas. The frequency of LOH was low (2 of 13; 15%) in the uVIN patients. In uVIN adjacent to micro invasive carcinoma LOH was found in 5 out of 9 cases (55.5%) and in 3 out of 12 (25%) of the macro invasive carcinomas LOH could be detected. In most cases the results of immunohistochemistry and LOH were concurrent. However, in 1 uVIN case LOH was found while immunohistochemistry revealed a strong expression of HLA-A, -B/C and β2M. The high percentage of partial HLA downregulation in uVIN lesions as detected by immunohistochemistry without concurrent detection of LOH suggests that the observed HLA-class I downregulation in uVIN is 'soft' wired and therefore, may be restored upon exposure to proinflammatory cytokines. To test this, we isolated keratinocytes from 3 healthy controls, 4 uVIN lesions and 2 vulvar carcinomas and analysed the expression of HLA-class I directly or after stimulation with IFNγ. The cultured keratinocytes of vulvar neoplasia patients displayed a lower expression of HLA-class I compared to controls (p=0.018; mean fluorescence 31.6 ± 19.1 vs. 66.1 ± 14.4) but stimulation with IFNγ resulted in an upregulation of HLA-class I expression in all samples (p=0.842; mean fluorescence index 143.7 ± 85.8 vs. 131.8 ± 25.8) (Fig. 3a). The expression level detected by in vitro analysis corresponded to the expression of HLA-class I obtained by immunohistochemistry on uVIN lesions of these patients (Fig. 4). In 2 cases, the cultured keratinocytes showed LOH and IFNγstimulation revealed upregulation of HLA-class I expression in both cases. This is probably explained by the upregulation of HLA-class I expression based on the one allele that was still present (Fig. 3a). As a positive control the IFNγ-mediated upregulation of HLA-DRDQDP expression was analysed. The expression of HLA-class II was upregulated in all cases and the expression levels did not differ (p=0.382; mean fluorescence 7.0 ± 4.4vs. 10.4 ± 4.7) (Fig. 3b).

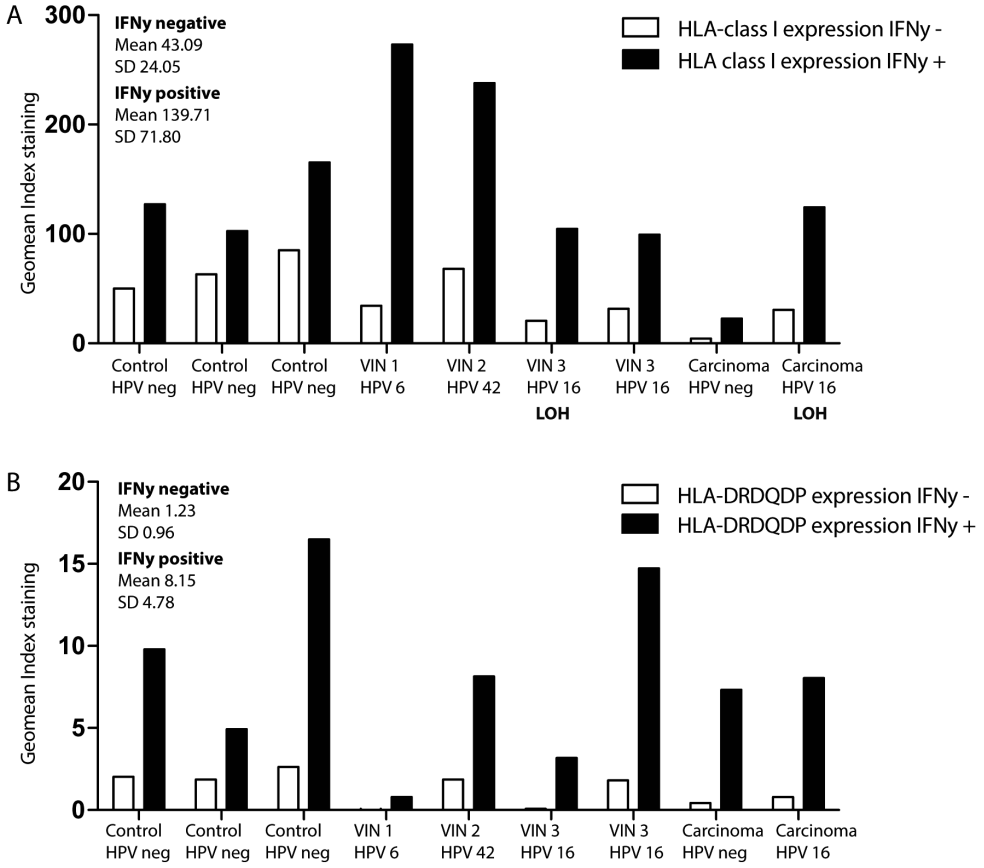

Figure 3: HLA-class I (A) and HLA-DRDQDP (B) expression on cultured patient-derived keratinocytes before and after 48h of IFNy stimulation.

Primary keratinocytes were isolated from biopsies or excisions of the vulva from women undergoing surgery for labia reduction, uVIN or HPV induced vulvar carcinomas. Keratinocytes were cultured in E-medium in presence of irradiated 3T3J2 mouse fibroblasts and 5-10µM Rho-associated kinase (ROCK) inhibitor (Y-27632). When cells grew out they were adapted to keratinocyte serum-free medium for one passage, after which they were stimulated with 0 or 100IU/ml IFNy for 48 hours. HLA expression was analysed by flow cytometry and the Geomean Index was used to depict the intensity of HLA-class I or - II staining. The cultured keratinocytes of vulvar neoplasia patients display lower expression of HLA-class I compared to controls whereas stimulation with IFNy results in an upregulation of HLA-class I expression in all samples. As a positive control the levels and IFNy mediated upregulation of HLA-DRDQDP expression was analysed.

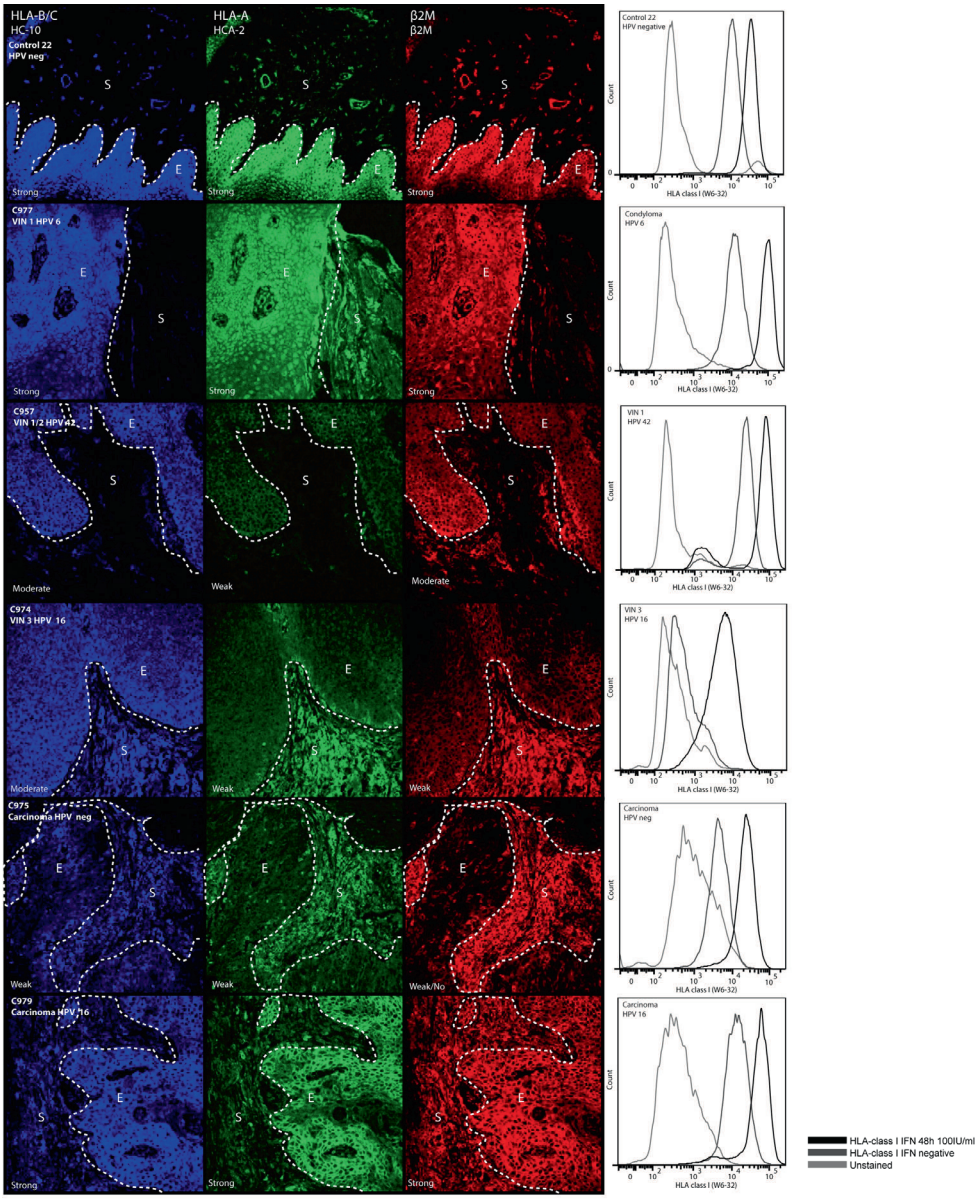

Figure 4: Immunofluorescent staining of HLA-class I in paraffin-embedded tissue and the effect of IFNy stimulation on HLA expression on corresponding isolated keratinocytes

From a number of patients with uVIN lesion we obtained fresh tissue for keratinocyte culture and paraffin embedded tissue of the same lesion used for histology. (left) Immunohistochemistry of HLA-B/C (blue), HLA-A (green) and β2M (red) staining of the paraffin embedded biopsy is shown. (right) Flow cytometry histograms representing HLA-class I expression (using the W6-32 antibody) of the keratinocytes from corresponding lesions with or without stimulation with 1000 IU/ml IFNy for 48 hours. Unstained is the background fluorescence of keratinocytes. The expression level (low, high) detected by in vitro analysis of keratinocyte cultures corresponded to the expression of HLA-class I determined by immunohistochemistry. E: Epithelium, S: Stroma

Lymphocyte infiltrates

Higher numbers of T cells, identified by the expression of CD3, were found in the stroma (p=<0.01) but not in the epithelium of uVIN and vulvar carcinoma lesions when compared to control tissues (Fig. 5). No correlations were found between CD3+ T cell infiltrates in stroma or epithelium and recurrent or progressive uVIN lesions (Supporting Information Table S1) neither was there an association with recurrence- or progression free survival (data not shown). Moreover the number of CD3+ cells did not correlate to the upregulation of HLA-class II, HLA-E or HLA-G or to the downregulation of HLA-class I, HLA-A, HLA-B/C or MICA.

HLA expression in uVIN biopsies of patients treated by HPV 16 SLP vaccination.

Evaluation of HLA expression in 12 uVIN biopsies taken before inclusion in a HPV 16 SLP vaccination trial[13] revealed that both clinical responders (n=6; ID 206, 207, 210, 216, 227, 229) and non-responders (n=6; ID 201, 202, 203, 212, 222, 228) showed partial downregulation of HLA-class I. Overall 75% of uVIN biopsies revealed partial downregulation. In 3 of the 6 non-responders a strong expression of HLA-class I could be observed whereas all 6 tested clinical responders revealed partial downregulation of HLA-class I. Of the clinical responders four patients displayed partial downregulation of HLA-A and –B/C whereas 2 patients displayed partial downregulation of HLA-A alone.

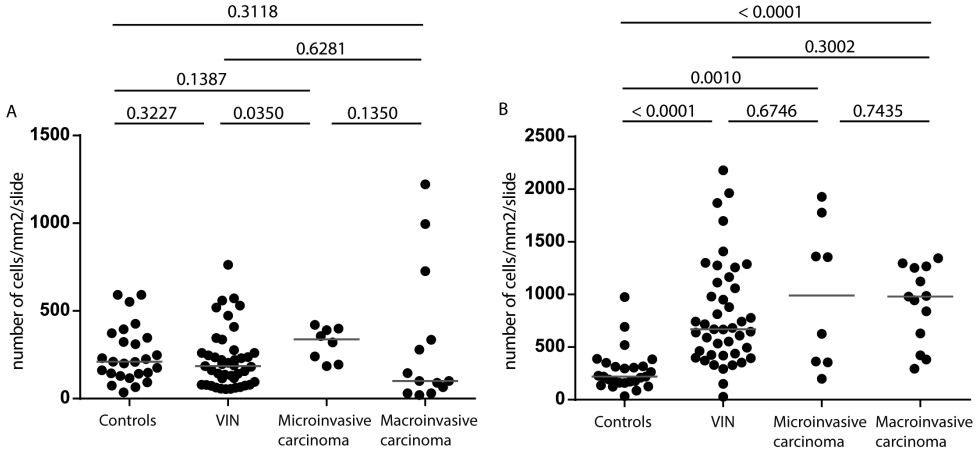

Figure 5: CD3 infiltrates in progressive course of HPV induced vulvar neoplasia

Paraffin embedded tissues of healthy controls (n=26), high grade uVIN lesions (n=43), microinvasive vulvar carcinomas (n=8) and macroinvasive vulvar carcinomas (n=13) were analysed for lymphocyte infiltration as identified by the expression of CD3 in both the epithelium (A) and stroma (B). An increased number of CD3+ T cells were found in the stroma (p=<0.01) but not in the epithelium of uVIN and vulvar carcinoma lesions when compared to healthy control tissues.

A: epithelial CD3+ infiltrates B: stromal CD3+ infiltrates

Discussion

This study shows that alterations in classical and non-classical HLA molecules is not limited to HPV-induced cancers but can already be observed at the premalignant stage of HPV-induced vulvar neoplasia. HLA-class I is partially downregulated in over 70% of uVIN lesions and in 80% of HPV-induced vulvar carcinomas. Loss of either one allele of HLA-A or HLA-B/C was found in only 9% of uVIN lesions and total HLA loss of both alleles in only 2 (5%) of uVIN lesions. These results do not completely correspond to the previously reported total loss in 19%, and partial downregulation of HLA-class I (allelic loss 9%) in uVIN lesions, probably due to the inclusion of HPV negative uVIN lesions in the previous study.[12] Importantly, our study revealed that both HLA-class I and MICA downregulation is an early event in the immune escape mechanism of HPV-induced premalignant uVIN lesions. HLA-B/C downregulation and possibly also the combined downregulation of HLA-class I and MICA are associated with the recurrence of uVIN after treatment. These data once again show the impact of the immune system in HPV-induced diseases as downregulation of HLA-class I and MICA are a well-documented events in both HPV-induced cervical intraepithelial neoplasia (CIN) and cervical cancer and both weak HLA-A and MICA expression are an independent prognostic factor for a decreased 5-year survival rate.[19,22,26,37] Alterations in the other HLA molecules (-class II, -E and -G) have no influence on recurrent or progressive course of uVIN lesions, and neither is the expression of both classical and non-classical HLA molecules altered from primary to recurrent uVIN lesions.

The high frequency of HLA-class I downregulation in uVIN may compromise the efficacy of immunotherapy because HPV peptides are insufficiently presented to T cells. However, in contrast to the vulvar carcinomas tested in this study, the majority of uVIN lesions display downregulation of HLA-class I expression that is not associated with LOH suggesting that HLA-class I downregulation is soft-wired and that expression can be restored upon changes in the microenvironment. Indeed, we were able to show that the lower expression of HLA-class I by HPV-infected keratinocytes freshly isolated from uVIN lesions could be increased by stimulation with IFNγ. Note that we only assessed general HLA-class I expression (by the W6-32 antibody) which may obscure subtle allele specific alterations. The reversible nature of the HLA-class I downregulation, however, may form an explanation for the lack of clinical impact of partial downregulation of HLA-class I in patients responding to HPV 16 SLP vaccination since for instance vaccine-induced HPV 16-specific type helper T cells infiltrating the lesion can provide IFNγ in the microenvironment.[13,18] The lack of overt differences in alterations of the expression of HLA between primary uVIN and the corresponding recurrent uVIN lesions suggests that the same possibility exists to restore HLA expression in recurrent uVIN lesions, however, maybe the recurrent lesions more often express LOH as we detected LOH in 55% of the uVIN adjacent to micro invasive carcinoma. The latter is in the same range as LOH in

CIN adjacent to carcinoma (75%) and in cervical cancer (50%).[22,23] We did not assess if there was a structural loss of β2M expression as total loss of HLA-class I expression was found in only 2 uVIN lesions. It will be important to test this as hard-wired HLA downregulation may directly affect the ability of lesions to regress. For instance, in melanoma it was the type of HLA-class I downregulation that determined the difference between regressing (soft-wired) and progressing (hard-wired) metastatic melanoma lesions.[38] Moreover in cervical cancer patients complete loss of HLA-class I was associated with a failure to induce systemic HPV-specific T cell responses as measured by CD4+ T cell responses.[25] This may indirectly cause a failure of HPV-specific T cell response induction since cancer cells with normal HLA-class I expression will be more efficiently destroyed by CTLs, resulting in an enhanced antigen uptake and presentation by HLA-class II on APCs that can stimulate the CD4+ T cells required for the maintenance and functions of CTLs.

The upregulation of HLA-class II is an early event in the carcinogenesis of vulvar neoplasia. HLA-class II is not expressed on non-dysplastic epithelium whereas in 65% of uVIN lesions and in 46-75% of vulvar carcinomas the expression is upregulated, corresponding to previous data on cervical cancer and 28% upregulation in uVIN (in a single study of only 7 cases).[25,26,39] The expression in uVIN is mainly focal (43.5% of cases <10% upregulation) and corresponds to high stromal infiltrates adjacent to the epithelium suggesting that upregulation is potentially related to cytokines in the local inflammatory environment. The effect of HLA-class II upregulation is unclear and may on one hand elicit activation of CD4+ T cells and enhance recruitment of CTLs but on the other hand it may as well hamper the immune response by induction or activation of Tregs.[24,40]

The expression of the non-classical molecules HLA-E and-G increases with the progressive course of HPV-induced vulvar neoplasia. These data are corresponding to cervical neoplasia where HLA-E expression gradually increases from CIN towards cancer and in cervical cancer was correlated to a large tumor size and lymph node metastasis.[27,28,41] HLA-E, expressed in 56-83% of cervical cancers and in 69% of cervical adenocarcinomas, is able to induce tumor tolerance by binding to its ligand CD94/NKG2A expressed on both NK and CTLs.[27,28,42] CD94/NKG2A expression can be induced by the cytokines interleukin (IL)-15 and tumor growth factor (TGF)-β present in the microenvironment of tumors.[43,44] Apart from its immune inhibitory function HLA-E can also bind to the immune stimulatory molecule NKG2C, which is expressed on the majority of NK cells and on some CTLs.[27] However the number of infiltrating NK cells in the microenvironment of both uVIN lesions and cervical cancer is low.[27]

HLA-G may induce immune evasion by inhibition of T cells, NK cells and APCs as well as by stimulating the production of Th2 cytokines.[45,46] Upregulation of HLA-G in tumor cells may be induced by IFNγ in the anti-tumor elimination phase, by epigenetic alterations of tumor cells, by hypoxia or immunosuppressive factors in the tumor environment such as IL-

10.[47] HLA-G expression was previously shown to be associated with progression of cervical lesions[29,41,48,49] and other types of cancer.[47] In addition, lack of HLA-G expression was an independent prognostic indicator of prolonged survival in patients with colorectal cancer.[50] These studies indicate that the increased expression of HLA-E and HLA-G in progressing lesions can contribute to escape from the immune system as well as form a barrier to successful immunotherapeutic approaches, although their rare expression in uVIN suggests that they are not able to mediate these effects during immunotherapy of uVIN.
In summary, alterations in classical HLA-class I and -II and non-classical MICA expression are already present in premalignant uVIN lesions and therefore of potential influence on immunotherapy in these lesions whereas expression of the non-classical HLA-E and -G are limited to the progressive course of disease. Remarkably, all evaluated uVIN patients who showed good clinical responses upon HPV SLP vaccination had partial HLA-class I downregulation indicating the potential reversibility of downregulation in premalignant uVIN upon proinflammatory cytokine response. These premalignant uVIN lesions may indeed be regarded as 'soft' lesions, considering the low percentage of LOH in uVIN and the reversible HLA-class I downregulation upon IFNγ stimulation in HPV infected keratinocytes.

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Supporting Information

Supplemental data available on: http://onlinelibrary.wiley.com.ezproxy.leidenuniv.nl:2048/doi/10.1002/ijc.28713/suppinfo

Supplemental Table 1: HLA expression and CD3 infiltrates associated with recurrence or progression of uVIN

Supplemental Table 2: LOH percentage in the progressive course of HPV induced vulvar neoplasia

Supplemental Figure 1: HLA expression intensity in immunohistochemistry staining according to Ruiter et al.[33]

Supplemental Figure 2: Expression of β2M, HLA-A, HLA-B/C and HLA-DR or total HLA class II (DRDQDP) in the progressive course of HPV induced vulvar neoplasia

CHAPTER 5

EXPRESSION OF COINHIBITORY RECEPTORS ON T CELLS IN THE MICROENVIRONMENT OF USUAL VULVAR INTRAEPITHELIAL NEOPLASIA IS RELATED TO PROINFLAMMATORY EFFECTOR T CELLS AND AN INCREASED RECURRENCE-FREE SURVIVAL

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Abstract

Human papillomavirus induced usual vulvar intraepithelial neoplasia (uVIN) are infiltrated by immune cells but apparently not cleared. A potential explanation for this is an impaired T cell effector function by an immunesuppressive milieu, co-infiltrating regulatory T cells or the expression of co-inhibitory molecules. Here, the role of these potential inhibitory mechanisms was evaluated by a detailed immunohistochemical analysis of T cell infiltration in the context of FoxP3, Tbet, IDO, PD1, TIM3, NKG2A and Galectins-1, -3 and -9. Paraffin-embedded tissues of primary uVIN lesions (n=43), recurrent uVIN lesions (n=20), vulvar carcinoma (n=21) and healthy vulvar tissue (n=26) were studied. We show that the vulva constitutes an area intensely surveyed by CD8+, CD4+, Tbet+ and regulatory T cell populations, parts of which express the examined co-inhibitory molecules. In uVIN especially, the number of regulatory T cells and TIM3+ T cells increased. The expression of the co-inhibitory markers TIM3 and NKG2A probably reflected a higher degree of T cell activation as a dense infiltration with stromal CD8+TIM3+ T cells and CD3+NKG2A+ T cells was related to the absence of recurrences and/or a prolonged recurrence free survival. A dense co-infiltrate with regulatory T cells was negatively associated with the time to recurrence, most dominantly when the stromal CD8+TIM3+ infiltration was limited. This notion was sustained in vulvar carcinoma's where the numbers of regulatory T cells progressively increased to outnumber co-infiltrating CD8+TIM3+ T cells and CD3+NKG2A+ T cells.

Introduction

Usual type vulvar intraepithelial neoplasia (uVIN) is caused by a persistent high risk HPV (hrHPV) infection, mainly type 16.[1] The lifetime risk of an HPV infection is approximately 80% and around 40% of female adolescents become infected at least once with an hrHPV.[2-4] HPV infections proceed asymptomatically in 90% of cases if the immune system is capable to clear the infection within two years.[2-4] Spontaneous regression and clearance of HPV is associated with systemic HPV-specific CD4+ and CD8+ T cell responses.[5,6] uVIN lesions have a malignant potential in 10% of untreated and 3-4% of treated patients and recurrence rates are high after conventional treatments.[7,8] Treatment for uVIN includes potential disfiguring interventions associated with psychosexual consequences.[7-9] Currently, conventional surgical treatments as local excision and laser therapy are increasingly replaced by immunotherapy, both in standardised (imiquimod) and experimental settings (photodynamic therapy (PDT) and therapeutic vaccination). Topical applied imiquimod induces T cell activation and proinflammatory cytokine release while therapeutic vaccines aim to reinforce HPV specific CD4+ and CD8+ T cell responses. Both immunotherapeutic approaches are promising and associated with clinical success however some patients are refractory to these therapies.[10-15] Although a few studies suggest that failure to respond to immunotherapy is related to a local immunosuppressive microenvironment[10,13-17], knowledge on the uVIN microenvironment is limited. The epidermis of uVIN is characterised by a decreased number of CD8+ T cells and increase of immature dendritic cells (DCs) and Langerhans cells (LCs) while the dermis underlying the lesion displays an influx of mature DCs, natural killer (NK) cells and both CD4+ and CD8+ T cells.[17,18] Moreover, some uVIN lesions are infiltrated by high numbers of regulatory T cells (Tregs).[10,14-17] Tregs may induce expression of Indoleamine 2,3-dioxygenase (IDO) by dendritic cells and IDO can induce an immunosuppressive microenvironment by suppression of effector T cell and NK cell function and enhance the function of Tregs.[19] Clinical response to immunotherapy in uVIN is associated with an increase in intralesional CD8+ T cells as well to low numbers of Tregs.[14-16,20]

The immune system prevents uncontrolled inflammation by expression of negative regulatory molecules, including Cytotoxic T-lymphocyte Antigen-4 (CTLA-4), T cell immunoglobulin mucin-3 (TIM3) and programmed cell death-1 (PD1), suppressing T cell function (reviewed in [21,22]). The ligands of PD1 are programmed death ligand-1 (PD-L1) and ligand-2 (PD-L2), which are induced upon exposure to inflammatory cytokines (*e.g.* IFNγ, IL-12, GM-CSF and IL-4) and can be expressed by resting B cells, T cells, macrophages, DCs and tumor epithelium.[23,24] The interaction between PD1 and PD-L1 impairs T cell function through downregulation of proinflammatory cytokine production or apoptosis.[23,24] Monoclonal antibodies blocking the co-inhibitory molecules PD1 or PD-L1 resulted in an improved clinical outcome in cancer.[25,26] Whereas T cells upregulate CTLA-4 and PD1 after activation, TIM3 is

only upregulated by differentiated T cells that produce IFNγ; CD4+ T helper-1 and CD8+ cytotoxic T cells.[22] CD4+ and CD8+ T cells expressing TIM3, especially in combination with PD1, are highly dysfunctional and produce less IFNγ, TNFα and IL-2.[27-29] TIM3 can suppress T cells following an interaction with Galectin-9.[30-32] Galectins (Gal) are a family of lectins expressed in a variety of solid tumors, and in particular Gal-1, Gal-3 and Gal-9 are known to play a pivotal role in tumor development.[33-35] Galectin-9 expression is upregulated by cells in response to proinflammatory cytokines in the microenvironment or upon activation via toll like receptors.[32,36] Gal-1 and -3 can suppress the local immune response via inhibition of CD8+ T cell responses, induction of tolerogenic antigen presenting cells (APCs), promotion of Tregs and stimulation of an IL-10 associated Th2 cytokine response (reviewed in [33-35,37]). Furthermore, activated T cells can express the CD94/NKG2A receptor which upon interaction with its ligand HLA-E inhibit T cell functionality.[38,39]

In order to assess the local immune suppressive microenvironment of uVIN we studied the infiltrating immune cells in the context of PD1, TIM3, NKG2A, Galectins 1, -3, and -9, IDO, and HLA-E in primary and recurrent uVIN lesions and their impact on clinical outcome. The results of this study highlight the pivotal role of uVIN-infiltrating activated T cells, reflected by the expression of TIM3 and NKG2A, in the protection against recurrent disease.

Material and Methods

Patient characteristics and material

Formalin-fixed, paraffin embedded tissue blocks from 43 patients treated for uVIN in the LUMC between 1990 and July 2012 were used. In case of recurrent disease (n= 20 patients), defined as diagnosis of uVIN after successful treatment without residual disease, the recurrent uVIN lesions were also included. In addition, tissues from patients with HPV-related micro invasive (≤1mm infiltration) (n=8) and macro invasive vulvar carcinoma (>1mm infiltration) patients (n=13) were included to evaluate TIM3 and NKG2A expression in relation to HLA-E expression in progressive vulvar neoplasia (mean 69.14 years; range 49-95).[40] Vulvar tissue from 26 healthy women undergoing labial reduction surgery served as healthy controls (mean 32.96 years; range 16-54). The Leiden University Medical Ethic Committee approved this study on prospective collection of healthy controls and use of archival FFPE blocks was according to Dutch Federation of Medical Research Association guidelines and histological analysis was performed by an experienced gynaecologic pathologist and classified according to the International Society for the Study of Vulvovaginal diseases (ISSVD) guidelines.[41] All samples included in this study were typed for HPV by HPV16 PCR with a HPV16 specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotypine *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV16 negativity (as described previously).[40]

All controls were HPV negative and histologic examination revealed no dysplasia and no other abnormalities.

Immunohistochemistry

For the analysis of Tbet, IDO, and NKp46 immunohistochemistry was used (Supplementary Fig. S1). Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-µm thick sections and were deparaffinised in xylene and dehydrated using graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/MeOH solution for 20 minutes. Antigen retrieval was achieved in boiling citrate (pH 6.0) buffer for 10 minutes for IDO and for Tbet and NKp46 EDTA buffer was used. Tissue sections were incubated with the primary antibody diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) at room temperature overnight in a humidified box. Subsequently the tissue sections were incubated at room temperature with BrightVision poly-HRP anti mouse, rabbit and rat IgG (ImmunoLogic BV, Duiven, the Netherlands) for 30 minutes. Slides were washed with PBS between incubation of antibodies for three times five minutes. The antigen-antibody reaction was visualized with 0.05M Tris-HCL buffer (pH 7.6) with 0.05% of 3,3'-diaminobenzidine and H_2O_2 for 10 minutes. Sections were counterstained with hematoxylin. The following primary antibodies were used: Tbet (anti-Tbet, rabbit, clone H210; Santa Cruz 1:400), IDO (anti-Indoleamine 2,3-dioxygenase, mouse-IgG3, clone 10.1; Millipore 1:400), and NKp46 (anti-NKp46, mouse IgG2b, clone 195314; R&D 1:400). Cells positive for Tbet and NKp46 displayed brown nuclair staining. Cytoplasmic staining of epithelial cells was indicated positive for IDO expression. Cervical cancer and tonsil were used as positive controls and a section stained without primary antibody served as negative control. Nuclear staining of Tbet was counted automatically by calculation of area and pixel value by Image J. The number of NKp46 cells was limited and therefore counted manually. IDO expression was scored by EE and EJ as expression intensity in stroma and cytoplasmic staining of the epithelium.

Immunofluorescence

Simultaneous detection of lymphocytes and co-inhibitory molecules was carried out by double or triple fluorescent staining and confocal microscopy (Fig. 1). In brief, sections were deparaffinised and antigen retrieval was performed in citrate buffer or EDTA as described above. Incubation with primary antibodies was overnight at room temperature and secondary antibodies were all isotype specific antibodies with Alexa Fluorchromes Alexa Fluor 488, 546, and 647 (Molecular Probes; 1:200 diluted in PBS/BSA 1%). Primary antibodies were used in the following combinations: *CD3+CD8+FoxP3+ staining*; CD3+ (anti-CD3+, rabbit, clone ab828; Abcam 1:100), CD8+ (anti-CD8+, mouse-IgG2b, clone 4B11; Novusbiologicals 1:200), FoxP3 (anti-FoxP3, mouse IgG1, clone 236A/E7; Abcam 1:200), *CD3+PD1+FoxP3+ staining*;

CD3+ (anti-CD3+, rabbit, clone ab828; Abcam 1:100), PD1 (anti-CD279, goat, clone AF1086; R&D 1:50), FoxP3 (anti-FoxP3, mouse-IgG1, clone 236A/E7; Abcam 1:200), *CD3+CD8+TIM3+ staining*; CD3+ (anti-CD3+, rabbit, clone ab828; Abcam 1:100), CD8+ (anti-CD8+, mouse-IgG2b, clone 4B11; Novusbiologicals 1:200), TIM3 (anti-HAVCR2, goat, clone AF2365; R&D systems 1:100), *CD3+NKG2A+ staining*; CD3+ (anti-CD3+, rabbit, clone ab828; Abcam 1:100), NKG2A (anti-NKG2A, goat, clone N19; Santa Cruz 1:50), *Galectin 1, -3, -9 staining*; Gal-1 (anti-Lgals1, rabbit, clone 25138; Abcam 1:1000), Gal-3 (anti-Lgals 3, rat, clone M3/38; Biolegend 1:50), Gal-9 (anti-Lgals 9, goat, clone AF2045; R&D 1:200). Five randomly selected representative images were captured using a confocal scanning microscope (LSM510, Zeiss) in a multitrack setting with a 25x/0.80 Plan-NEOFluar objective. Stromal and immune cells were used as internal positive control and two extra sections were stained without primary or secondary antibody as a negative control. Epithelium and stromal cells were manually counted using the LSM 5 Image Examiner software and represented as the number of cells per mm^2 for each slide (average of five 250x image slides).

Data analysis

The statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL) was used and the Shapiro-Wilk test was applied to determine a normal distribution. All variables were non-parametric and subsequently the Mann-Whitney U test was performed to compare continuous variables between patient groups. The paired Wilcoxon Signed Rank test was used to determine differences in paired primary and secondary recurrent lesions of the same patient. The Spearman correlation coefficient was used to detect correlation in the non-parametric data. Recurrence free survival (RFS) was determined as the interval between first therapy and diagnosis of recurrent disease or the last follow up visit. Patients were divided into groups based on the median of infiltrating cells and a univariate Log Rank (Kaplan Meier) analysis for recurrence-free survival was performed. Subsequently multivariate analysis, corrected for multifocality of uVIN lesion, was performed by a Cox proportional hazard model since multifocality was previously identified as prognostic markerin our study cohort.[42] Two sided p values <0.05 were considered statistical significant. GraphPad Prism 5.04 (Graphpad Software Inc, LA Jolla, CA, USA) was used to illustrate the data by graphs and figures.

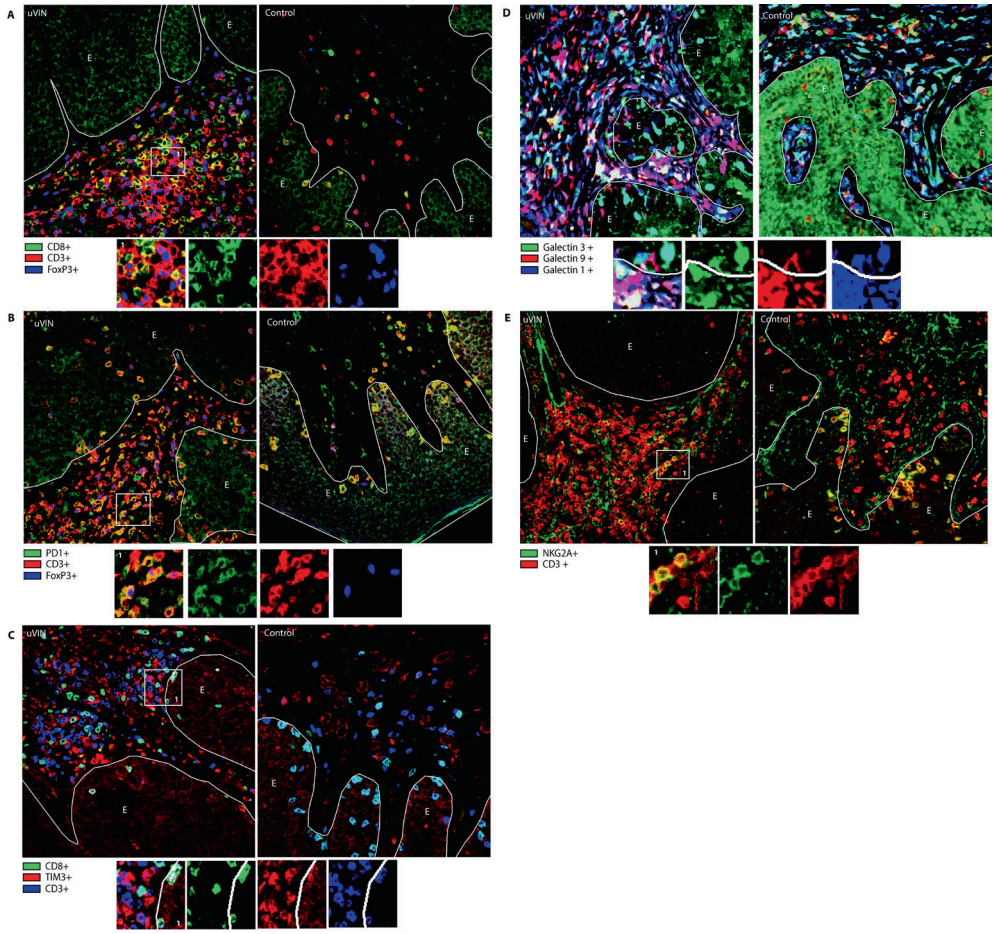

Figure 1: Examples of triple or double immunofluorescent confocal microscopy of T cell infiltrates and co-inhibitory molecules PD1, TIM3, NKG2A and immunosuppressive Galactins 1, -3 and -9

Paraffin-embedded tissue of healthy controls (n=26), primary uVIN lesions (n=43), recurrent uVIN lesions (n=20) and vulvar carcinoma (n=21) were analysed by triple or double fluorescent confocal microscopy with antibodies against: A: CD8+ (green), CD3+ (red) and FoxP3 (blue): double staining results in CD3+CD8+ (yellow), CD3+FoxP3+ (red with blue nuclei), B: PD1 (green), CD3+ (red), FoxP3+ (blue): double staining results in CD3+PD1+ (yellow), CD3+PD1+FoxP3+ (yellow with blue nuclei), CD3+FoxP3+ (red with blue nuclei), C: CD8+ (green), TIM3+ (red), CD3+ (blue): double staining results in CD3+TIM3+ (purple), CD3+CD8+TIM3+ (white), CD3+CD8+ (light blue), D: Galectin 3 (green), Galectin 9 (red), Galectin 1 (blue): double staining results in Galectin 1+3+ (light blue), Galectin 1+9+ (purple), Galectin 3+9+ (yellow), triple staining in Galectin 1+3+9+ (white), E: NKG2A+ (green), CD3+ (red): double staining results in CD3+NKG2A+ (yellow)

Results

Patients

The clinical characteristics of the patient cohort are shown in Supplementary Table S1. The clinical prognostic factors were previously described in detail.[40,42] HPV type 16 was detected in 79% of first uVIN lesions and 58.1% of uVIN lesions were unifocal. First therapy consisted of excision (51.2%), laser therapy (30.2%), imiquimod (7%) and a combination of laser and excision (11.6%). Use of immunosuppressive medication was reported in 7 patients which revealed no overt differences in immune infiltration as compared to the rest of the group of uVIN patients (data not shown). In 20 patients a recurrence occurred in a median time of 19.5 months (range 0-199). Patients with HPV-induced vulvar carcinomas had a low stage of disease according to the International Federation of Obstetrics and Gynaecologists (FIGO), except for one. None of these patients died during follow up and in 3 patients a recurrent vulvar carcinoma occurred after a median time of 7 months (range 6-46).

The microenvironment in uVIN is functionally different from healthy controls

Examples of the immunohistological stainings used to quantify the number of cells per square millimetre of vulvar tissue are shown in Fig. 1 and Supplementary Fig. S1. Substantial numbers of intraepithelial and stromal CD4+ and CD8+ T cells were found in healthy control tissue, uVIN and HPV related vulvar cancer (Table 1; Fig. 2), indicating that the vulvar skin is intensely surveyed by T cells. NK cells (NKp46+) were rare in both the dermis and epidermis of uVIN and controls (Supplementary Table S2).

Overall the intraepithelial CD3+ T cell infiltration (Table 1; Fig. 2) did not differ between healthy controls (range 36-592 cells/mm^2) and uVIN (range 54-763 cells/mm^2). In both cases, approximately 40% of these CD3+ cells were CD4+ and 60% were CD8+ (Fig.3). A closer look, combining T cell infiltration with the expression of functional markers, revealed that especially the number of CD4+FoxP3+ regulatory T cells (Table 1; Fig. 2) as well as that of intraepithelial CD3+, CD4+TIM3+, and CD8+ TIM3+ T cells was higher uVIN lesions than in healthy control tissue (Table 1; Fig.2; Fig.3). Moreover, the relative contribution of CD4+Foxp3+ T cells within the whole infiltrating T cell population increased (Fig.3). In contrast, the percentage of T cells expressing PD-1 or NKG2A was higher in controls than in uVIN lesions (Fig.3). No difference was found between unifocal and multifocal lesions (not shown).

Table 1: Comparison of infiltrating immune cell subsets in the microenvironment of healthy controls, uVIN and HPV induced vulvar carcinoma

Immune cell types	Controls Median (range) N=26	uVIN Median (range) N=43	Carcinoma Median (range) N=21	*P*-value* uVIN vs. Controls	*P*-value* uVIN vs. Carcinoma	*P*-value* Controls vs. Carcinoma
CD3+ (E)	209.92 (36-592)	185.99 (54-763)	240.99 (21-1222)	0.310	0.427	0.818
CD3+ (S)	219.62 (34-976)	669.64 (28-2180)	981.37 (199-1928)	**0.000***	0.307	**0.000***
CD4+ (E)	55.22 (3-406)	54.89 (3-245)	65.58 (7-293)	0.692	0.563	0.774
CD4+ (S)	149.26 (17-738)	362.62 (28-1305)	479.48 (131-1014)	**0.000***	0.983	**0.000***
CD8+ (E)	131.34 (21-574)	107.93 (20-647)	127.90 (6-930)	0.373	0.642	0.909
CD8+ (S)	69.88 (10-261)	294.28 (0-971)	522.72 (68-966)	**0.000***	0.077	**0.000***
CD4+ FoxP3+ (E)	3.75 (0-27)	19.46 (0-152)	70.55 (4-610)	**0.000***	**0.000***	**0.000***
CD4+ FoxP3+ (S)	48.23 (11-208)	511.96 (49-2002)	872.59 (141-1500)	**0.000***	**0.009***	**0.000***
Ratio CD8+/FoxP3 (E)	9.67 (0.00-218.68)	4.13 (0.00-60.16)	1.52 (0.10-12.71)	0.359	**0.005***	0.102
Ratio CD8+/FoxP3 (S)	1.64 (0.40-7.00)	0.55 (0.00-2.03)	0.58 (0.09-1.04)	**0.000***	0.994	**0.000***
Tbet (E)	99.64 (7.88-225.88)	55.31 (6.36-449.15)	70.93 (8.8-538.74)	0.116	0.679	0.843
Tbet (S)	143.06 (19.61-361.57)	339.15 (24.02-2115.11)	303.32 (83.83-1028.28)	**0.000***	0.679	**0.000***
CD3+PD1+ (E)	191.08 (23.07-662.74)	96.18 (0.00-477.78)	n.e.	**0.002***	n.e.	n.e.
CD3+PD1+ (S)	200.10 (70.18-539.40)	316.58 (40.08-1272.27)		**0.015***		
CD3+PD1+FoxP3+ (E)	3.65 (0.00-34.91)	8.08 (0.00-56.62)	n.e.	0.064	n.e.	n.e.
CD3+PD1+FoxP3+ (S)	71.32 (25.33-263.46)	163.35 (13.82-966.43)		**0.000***		
CD3+NKG2A+ (E)	17.43 (0.00-295.61)	14.23 (0.00-414.71)	11.93 (0.00-113.43)	0.346	0.683	0.178
CD3+NKG2A+ (S)	3.90 (0.00-31.14)	4.40 (0.00-161.85)	21.03 (0.00-94.24)	0.276	**0.004***	**0.001***
CD3+TIM3+ (E)	5.28 (0.00-123.28)	45.49 (7.40-184.90)	51.62 (23.84-694.64)	**0.000***	0.129	**0.000***
CD3+TIM3+ (S)	22.65 (0.00-108.80)	122.91 (15.12-571.40)	191.01 (47.25-511.96)	**0.000***	0.177	**0.000***
CD4+TIM3+ (E)	0.00 (0.00-70.88)	17.89 (0.00-69.54)	17.36 (4.92-167.28)	**0.000***	0.348	**0.000***
CD4+TIM3+ (S)	18.73 (0.00-95.09)	104.12 (10.81-462.48)	128.33 (16.17-379.30)	**0.000***	0.801	**0.000***
CD8+TIM3+ (E)	3.33 (0.00-64.48)	17.78 (0.00-165.93)	32.19 (8.77-527.37)	**0.000***	0.066	**0.000***
CD8+TIM3+ (S)	4.51 (0.00-19.71)	22.86 (0.00-220.36)	67.38 (6.93-163.33)	**0.000***	**0.009***	**0.000***
Galectin[a] 1 total (E)	119.39 (0.00-521.05)	150.11 (21.36-743.36)	n.e.	0.185	n.e.	n.e.
Galectin[a] 1 total (S)	863.87 (467.47-1953.02)	1648.57 (753.08-5321.03)		**0.000***		
Galectin[a] 3 total (E)	95.26 (0.00-461.33)	103.02 (6.92-553.27)	n.e.	0.552	n.e.	n.e.
Galectin[a] 3 total (S)	693.63 (381.65-1083.60)	936.81 (246.89-3120.24)		**0.000***		
Galectin[a] 9 total (E)	76.57 (0.00-370.96)	98.21 (23.53-325.33)	n.e.	0.216	n.e.	n.e.
Galectin[a] 9 total (S)	565.36 (221.28-1418.81)	790.14 (99.61-2319.27)		**0.021***		
Galectin[a] 1/3/9 total (E)	n.e.*	220.00 (51.61-766.00)	n.e.	n.e.	n.e.	n.e.
Galectin[a] 1/3/9 total (S)	1177.36 (618.00-2561.00)	1919.00 (922.23-5706.00)		**0.000***		

All evaluated immune cell subsets are described in Supplementary Table S2. *Significant *p*-values <0.05 by analysis with the non-parametric Mann-Whitney U test used to determine differences in myeloid cell type infiltrates between healthy controls, uVIN and vulvar carcinoma.
(E) = epithelium, (S) = stroma, [a] = galectin immune cell counts are presented, not the expression of keratinocytes
n.e. = not evaluable *due to strong epithelial keratinocyte expression of galectin-3 in healthy controls

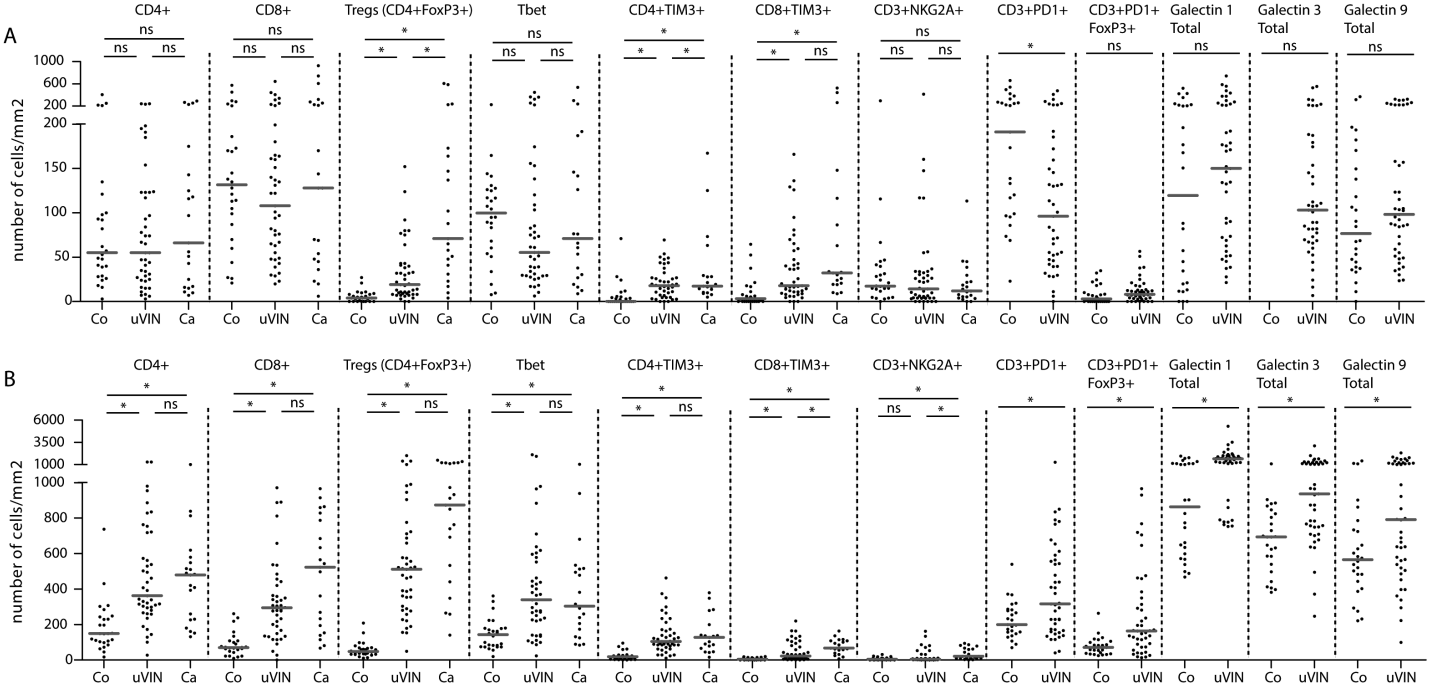

Figure 2: Intraepithelial and stromal T cell infiltrates in combination with expression of co-inhibitory molecules at different stages of disease

T cell infiltrates/mm^2 for each patient are depicted phenotypically characterised by immunohistochemical staining of paraffin-embedded tissue for CD4 (T helper cells), CD8 (cytotoxic T cells), CD4+FoxP3+ (regulatory T cells), Tbet (IFNγ) and by co-inhibitory markers PD1, TIM3 and NKG2A and total Galectin 1,-3 and -9 expression in healthy controls, uVIN and in vulvar carcinomas. * is significant (P<0.05) or non-significant (ns) by use of the non-parametric Mann-Whitney U test. A: intraepithelial infiltrates and B: stromal infiltrates.

In general, the stroma of uVIN was more densely infiltrated with CD3+ T cells (range 28-2180 cells/mm^2) than control tissue (range 34-976 cells/mm^2), albeit that the CD4+ (60-65%) and CD8+ (35-40%) composition was not different (Table 1, Fig.2; Fig.3). Analysis of T cell marker expression revealed that a much higher percentage of stromal uVIN-infiltrating CD4+ T cells expressed FoxP3 (80% vs 20% in healthy controls). In addition, Tbet was expressed in a higher number and percentage of cells (Table 1, Fig.2; Fig.3). The numbers and percentages of TIM3-positive CD3+, CD4+ and CD8+ T cells were much higher in uVIN lesions than controls (Table 1, Fig.2; Fig.3), however as indicated by the ratio's between TIM3+ and TIM3- T cells (<1), each lesion comprised more TIM3- T cells than TIM3+ T cells (Supplementary Table S2). Notably, the ratios between these cells were higher in uVIN lesions than in controls, indicating that uVIN lesions comprised both absolutely and relatively more infiltrating TIM3+ T cells than controls (Supplementary Table S2).

The number of stromal CD3+PD1+ T cells was higher in uVIN lesions than in controls but in both cases the CD3+PD1+ were outnumbered by the CD3+PD1- T cells as indicated by their ratios (Supplementary Table S2). Furthermore, the CD3+PD1- to CD3+PD1+ ratio was significantly higher for uVIN lesions than controls, suggesting that the uVIN lesions were infiltrated by higher numbers of CD3+PD1- T cells (Supplementary Table S2). Finally, stromal CD3+NKG2A+ T cells were scarce and did not differ (Table 1; Fig.2; Fig.3).

In 20 patients, the primary lesion as well as the recurrent lesion were analysed for the expression of inhibitory markers. Overall, there were no overt differences in the number of intraepithelial and stromal infiltrating immune cells between the primary and the recurrent lesions (Supplementary Table S3). In recurrent uVIN lesions a slightly higher number of intraepithelial CD3+PD1+FoxP3+ T cells and a lower number of stromal CD3+NKG2A+ T cells and a higher ratio of CD4+TIM3+/CD4+TIM3- T cells were seen reflecting a more immunotolerant microenvironment (Supplementary Table S3).

In summary, these data show that HPV-induced uVIN lesions display a T cell infiltrate characterised by higher absolute numbers of Foxp3+ Tregs and T cells expressing TIM3+.

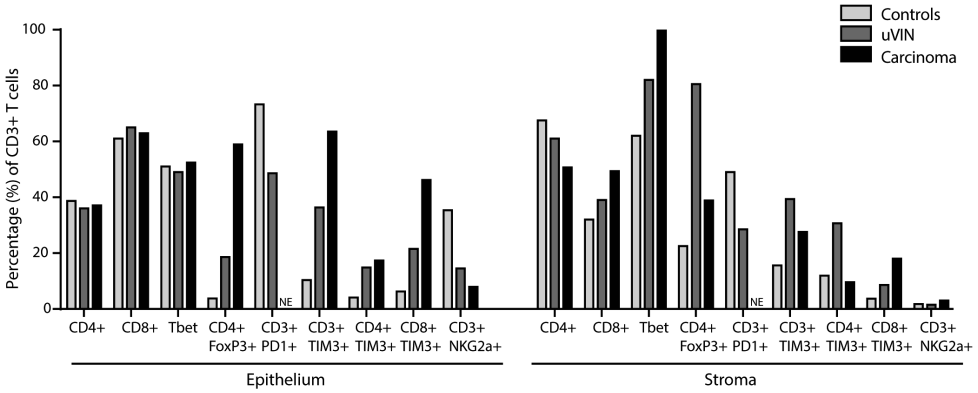

Figure 3: The relative distribution of T cell subsets within the infiltrating CD3+ T cell population

Bar graph representing the composition of the infiltrating T cells and the expression of co-inhibitory molecules FoxP3, PD1, TIM3 and NKG2A as a percentage (%) of the total amount of infiltrating CD3+ T cells, both in the epithelium and stroma of healthy controls, uVIN and vulvarcarcinoma. NE (not evaluated)

Higher numbers of stromal galectin-1 and/or - 9 expressing immune cells in uVIN.

As Gal-1, Gal-3 and Gal-9 are all involved in tumour development [33-35] their expression was also analyzed in uVIN lesions. Gal-9 expression by the uVIN epithelium itself was found in some uVIN patients but the expression was less intense compared to what was seen for keratinocytes in control tissue (Supplementary Table S2). Galectins can be expressed by virtually all immune cells [33-35], but based on the morphology of cells expressing Gal-9 in the microenvironment of uVIN and the number of infiltrating T cells (Fig.2), the majority of these cells were most likely myeloid cells. Whereas no differences were found in the intraepithelial immune cells expressing Gal-9, the total number of stromal Gal-9 expressing immune cells was increased in uVIN (Table 1 and Supplementary Table S2). Similarly, the number of stromal Gal-1 and Gal-3 positive immune cells was higher in uVIN than in controls (Table 1 and Supplementary Table S2). No difference was found in the number of intraepithelial Gal-1 expressing immune cells and due to the strong expression of Gal-3 by the keratinocytes in healthy controls, evaluation of Gal-3+ infiltrating immune cells was not possible (Supplementary Table S2). Gal-1 was not or weakly expressed by keratinocytes albeit the expression of Gal-1 by the HPV infected keratinocytes of recurrent uVIN lesions was increased (38.1%) compared to primary lesions (14.3%) (Supplementary Table S3). IDO was not expressed in epithelium of uVIN (Supplementary Table S2). Since PD-1 expression by T cells was higher in control tissue than in uVIN, we did not stain for its ligand PD-L1.

The presence of TIM3+and/or NKG2A+ T cells is associated with the absence of recurrences and a longer recurrence free survival

Twenty of the 43 uVIN patients developed recurrent lesions. A comparison of the data between the primary lesions of the non-recurrent and recurrent uVIN patients revealed that the primary lesions of non-recurrent uVIN lesions displayed a denser infiltration with intraepithelial CD3+TIM3+ T cells and Gal-9+ immune cells (Supplementary Table S4). Since Gal-9 is expressed upon exposure to proinflammatory mediators[36] and expression of the other galectins is more likely to be associated with neoplastic cell changes[33], the better outcome associated with intraepithelial Gal-9 expressing cells may in fact reflect the beneficial action of locally active proinflammatory T cells. Indeed, the number of intraepithelial Tbet+ (IFNγ) cells was positively associated with the total number of intraepithelial Gal-9+ cells (p= 0.028) as well as with the number of intraepithelial CD8+TIM3+ T cells (p=0.042) and in particular with a higher ratio of intraepithelial TIM3+ to TIM3- CD8+ T cells (p= 0.005) Altogether, this suggests a protective role for TIM3+ T cells against recurrence.

To sustain this notion we evaluated the time to recurrence of uVIN lesions in the context of the different immune infiltrates in uVIN by multivariate Cox analyses. The data were corrected for multifocality of uVIN lesions since this was a clinically prognostic factor for recurrence in this cohort[42] (Fig.4 and Supplementary Table S4). No differences in the time to recurrence were found when the intraepithelial populations of T cells were analysed (Supplementary Table S4). Analysis of the time to recurrence for cells in the stroma revealed that there were no direct associations with T cell functional type (CD4, CD8, Tbet, FoxP3), albeit that a low ratio of CD8+/Tregs in the stroma of uVIN, reflecting a relative high infiltrate with Tregs, is associated with a decreased RFS (Fig. 4a; p=0.016 HR 0.280). However, when the T cells were analysed in the context of their co-inhibitory marker expression, the presence of high numbers of absolute stromal CD3+TIM3+ or CD8+TIM3+ T cells (p=0.058 HR 0.377 and p=0.015 HR 0.282, respectively; Fig. 4b), or relative to their stromal CD8+TIM3-negative counterparts (p=0.015 HR 0.287; Fig. 4c), in the uVIN were associated with an improved RFS (Supplementary Table S4). Again, the infiltration of CD8+TIM3+ cells and that of Tbet (IFNγ) cells was associated with clinical benefit as a combined strong stromal infiltration with CD8+TIM3+ cells and Tbet+ cells was associated with a longer RFS (Supplementary Fig. S2a). Because of the association between proinflammatory cytokines and Gal-9 expression we also analysed the numbers of stromal TIM3+ cells in the context of Gal-9. Notably, a strong stromal infiltrate with CD3+TIM3+, and especially CD8+TIM3+ combined with high single expression of Gal-9 was associated with a much better recurrence free survival than when there were low numbers of these types of T cells and high single Gal-9 expression in a multivariate analysis (high vs low CD8+TIM3+: p=0.009 HR 53; Supplementary Fig. S2b and Supplementary Table S4).

Our data suggest that the expression of TIM3 by CD8+ T cells reflects a greater degree of T cell activation in uVIN. In order to understand if in a number of cases this is effectively counteracted by co-infiltrating Tregs we analysed their association with recurrence free survival by grouping the patients in 4 categories based on the median stromal CD8+TIM3+ and median stromal Treg number. This revealed no difference in RFS in epithelial compartment (Fig. 4d) and neither between patients displaying a low or high stromal infiltration with Tregs when the number of co-infiltrating stromal CD8+TIM3+ T cells was above the median (Fig. 4e). However, Tregs were indirectly associated with a worse RFS in case the numbers of stromal CD8+TIM3+ were lower than the median (Fig. 4e).

While, as expected based on our expression data, there was no association between T cell expressed PD1 and RFS, surprisingly a high ratio of CD3+NKG2A+ /CD3+NKG2A- was also associated with a prolonged RFS (*p*=0.001 HR 6.36) indicating that the presence of CD3+NKG2A+ T cells in stroma is favourable (Supplementary Fig. S2c and Supplementary Table S4).

Altogether these data suggest that patients with a better RFS display increased numbers of lesion infiltrating TIM3+ and/or NKG2A+ T cells as well as a more IFNγ-associated microenvironment.

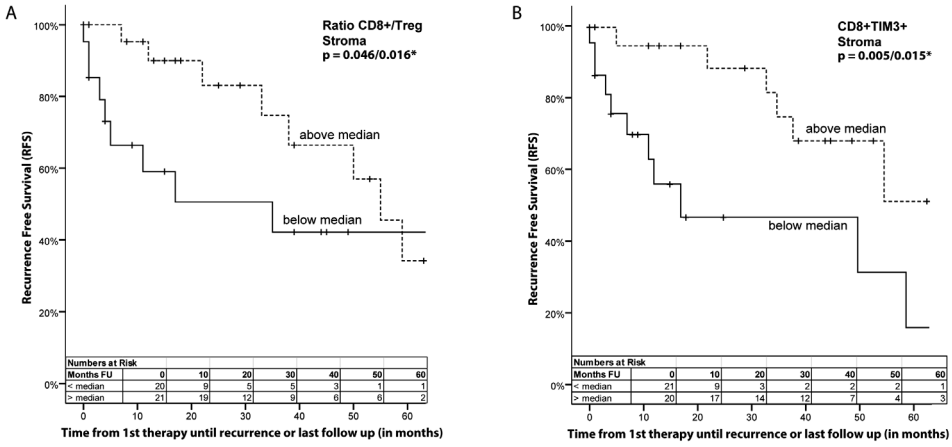

Figure 4 A+B

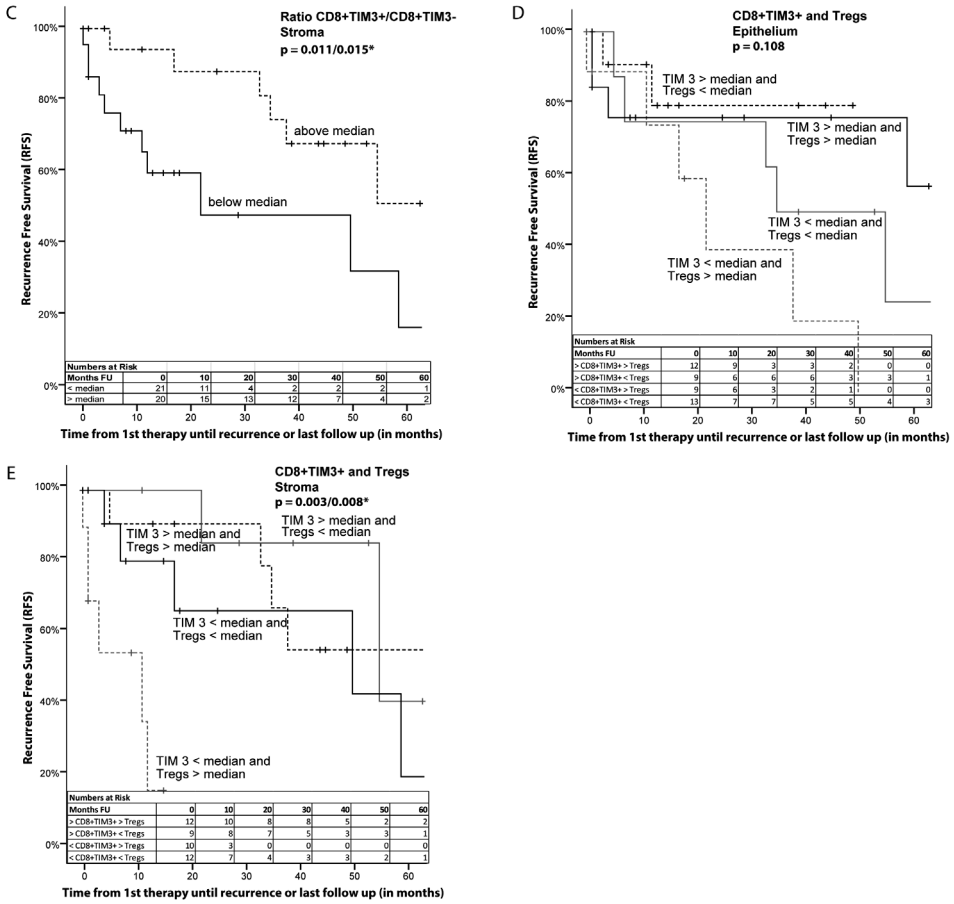

Figure 4 continued: The impact of TIM3 and NKG2A expressing T cells on recurrence free survival (RFS) in uVIN

Immune cell infiltrates in the microenvironment of uVIN expressing co-inhibitory molecules were grouped based on the median number of cells and analysed by Kaplan Meier (Log Rank) survival curves and *multivariate Cox analysis to determine the influence on the recurrence free survival of usual VIN lesions. Depicted are A: Ratio CD8+/Treg in stroma, B: CD8+TIM3+ in stroma, C: Ratio CD8+TIM3-/CD8+TIM3+ in stroma, D: Combination of CD8+TIM3+ and Tregs in epithelium and E: Combination of CD8+TIM3+ and Tregs cells in stroma.

T cells expressing Foxp3, TIM3, Tbet or NKG2a in HPV induced vulvar carcinomas

The number of stromal CD8+TIM3+ T cells, the combination of stromal CD8+ TIM3+ cells and T cells expressing Tbet, as well as the ratio's between stromal CD8+ T cells/Tregs and stromal CD3+NKG2A+/CD3+NKG2A- cells were all associated with protection against recurrence of uVIN. Therefore, we extended our study to determine the number of these T cells in a group of HPV-induced vulvar carcinomas (n=21). In comparison to uVIN lesions, these HPV induced vulvar carcinomas displayed a more dense intraepithelial and stromal CD4+, CD8+ and an

even stronger CD4+FoxP3+ T cells infiltration (Fig. 2;Fig.3; Supplementary Table S5). There was a strong reduction in the ratios of intraepithelial CD8+/Tregs, CD4/Tregs and Tbet+/Tregs. These ratios were also reduced in the vulva carcinomas stromal compartment albeit that the reductions were mild (Supplementary Table S5). Moreover the relative number of Tbet + effector T cells was decreased in stroma of vulvar carcinoma reflected by a higher ratio of CD8+/Tbet. Both the number of stromal NKG2A+ T cells and that of stromal CD8+TIM3+ T cells increased in vulva carcinomas in line with the increases seen for total CD3+ T cells and CD8+ T cells, but not as much as was seen for Tregs. While the relative number of stromal NKG2A+ to NKG2A- T cells showed a decrease in vulvar carcinoma, the relative numbers of stromal TIM3+ CD8+ T cells was enhanced (Supplementary Table S5). Interestingly, the number of NKG2A+ T cells was especially low in tumors expressing the NKG2A ligand, HLA-E (Supplementary Fig.S3).

Altogether, the progressive course of vulvar neoplasia is characterised by a more immune suppressed microenvironment where there is a marked increase in the number of regulatory T cells that readily outnumber the numbers of TIM3+ or NKG2A+ lymphocytes.

Discussion

Here we present a comprehensive study on the presence of T cells and expression of several immune inhibitory molecules in the microenvironment of primary and recurrent uVIN lesions, HPV-induced vulvar carcinoma, and in healthy controls. The results of our study clearly show that a stronger infiltration by T cells expressing the co-inhibitory markers TIM3 and NKG2A and relatively low numbers of regulatory T cells in primary uVIN lesions is associated with a longer recurrence free survival and the absence of recurrences. T cells expressing co-inhibitory markers are often regarded as exhausted T cells with impaired ability to become activated and exert their function.[21,22] It is beyond doubt that these T cells are vulnerable to suppression in case their ligands are expressed and that blocking of the receptor-ligand interactions (e.g. PD1-PD-L1) can stimulate tumor-specific T cell immunity.[25,26] However, the expression of these receptors by activated T cells has been shown to primarily reflect T cell differentiation and activation.[43-45] Based on their positive association with RFS, the increased expression of the co-inhibitory markers TIM3 and NKG2A by T cells in uVIN also is more likely to reflect a greater degree of T cell activation.

TIM3 is associated with exhaustion of T cells in several types of carcinoma and is expressed on terminally differentiated CD4+Th1+ [31], Th17 and CD8+ T cells.[36,46] In uVIN the numbers of both CD4+ and CD8+ T cells expressing TIM3 were increased when compared to healthy tissue. TIM3 expressed on CD4+ T cells did not bear any impact on clinical outcome, whereas CD8+ TIM3+ T cells were associated with the absence of recurrence and the

later development of recurrence in uVIN lesions. CD4+TIM3+ and TIM3 co-expressed on CD8+PD1+ T cells are associated with impaired T cell function resulting in less IFNγ, TNFα and IL-2 production in cancer.[27,29] In tumor bearing mice the administration of Gal-9 induced the apoptosis of CD4+ T cells while it increased the number of IFNγ producing CD8+TIM3+ T cells and CD86+TIM3+ DCs and prolonged their survival[47] while in vitro, Gal-9 exposed immature DCs become activated and produce IL-12.[35,47,48] Furthermore, treatment of PBMCs from healthy donors with Gal-9 induces apoptosis in 60% of T cells but the remaining cells expand and differentiate into central memory and IFNγ producing T cells.[49] Thus Gal-9 can mediate positive effects on CD8+TIM3+ T cells as long as they do not express PD1, and impair activated Th1 T cells.[31,49] In view of these data, the positive relation between IFNγ (Tbet), Gal-9 and TIM3 on CD4+ and CD8+ T cells in uVIN, in combination with a lower PD1 expression can explain the positive impact of (the presence of) CD8+ TIM3+ T cells on recurrences. Furthermore, it also helps to explain why the CD4+ TIM3+ T cells do not bear any impact.

It is likely that the T cells expressing the co-inhibitory markers in the presence of their ligands are functionally impaired and as such they will not be able to contribute in the antitumor response. This effect, however, may go unnoticed if their presence simultaneously indicates that overall there is much more effective stimulation of T cells within a highly active microenvironment. Then, the association between non-recurrence and RFS and the expression of these markers is highly likely a reflection of an effective local immune response, which one can envisage may even lead to regression when in addition such co-inhibitory molecules on the T cells are blocked.

Previous studies suggested that the stromal compartment of uVIN is the immunologically active area in uVIN.[16,17] Our data confirm that uVIN are intensely surveyed by the immune system as an abundant number of activated proinflammatory IFNγ-producing T cells, reflected by T cells expressing Tbet, TIM3 and/or NKG2A, and Gal-9 expressing immune cells were detected in the stroma. Moreover, our study reveals the importance of this stromal immunological activity by the association of such an activity with favourable clinical outcome. The stromal compartment in vulvar carcinomas show signs of immune suppression as reflected by the relative decrease in the number of stromal Tbet + effector T cells and a strong increase in the numbers of Tregs outnumbering stromal TIM3+ or NKG2A+ lymphocytes.

The epithelium in uVIN was considered to be in immunosuppressed state.[14-17,20] This fits with the data on myeloid cells and Tregs in our cohort of uVIN patients showing that a dense intraepithelial CD14+ cell infiltration was associated with high numbers of intraepithelial CD4+ Tregs but also with lower numbers of stromal CD8+TIM3+ T cells.[50] In vulvar carcinomas this was even more pronounced as evidenced by a strong reduction in the ratios of intraepithelial CD8+/Tregs, CD4/Tregs and Tbet+/Tregs. In general, the primary uVIN

immunological landscape is associated with the activation of a local IFNγ-associated T cell response. If the local response is less active (lower numbers of CD8+TIM3+ and NKG2A+ T cells; higher numbers of macrophages) recurrences are likely to appear more rapidly. Immunotherapy may strengthen the pre-existent activation of the local immune system and immunologically active primary lesions may be more likely to respond to immunotherapy, also because these lesions are characterised by lower numbers of regulatory T cells subsets, which have previously been shown to be associated with non-responsiveness to immunotherapy.[10,14,15] Along these lines, we expect that less immunologically active uVIN lesions, recurrent lesions and vulvar carcinoma may form a greater challenge for immunotherapy. We will now evaluate the expression of these potential biomarkers in the lesions of uVIN patients vaccinated with our HPV16-SLP vaccine combined with imiquimod applied at the vaccination site (manuscript in preparation), to explore their potential role in relation to the clinical response to vaccination.

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Supporting Information

Supplemental data available on: http://onlinelibrary.wiley.com.ezproxy.leidenuniv.nl:2048/doi/10.1002/ijc.29174/suppinfo

Supplementary Table S1: Patient characteristics

Supplementary Table S2: Complete overview of immune cell infiltration in healthy controls, the total uVIN group and subgroups of patients with recurrent and non-recurrent uVIN

Supplementary Table S3: Comparison of immune cell infiltration in primary and subsequent recurrent lesions

Supplementary Table S4: Overview of the associations between subsets of immune cells and recurrence free survival

Supplementary Table S5: Comparison of T cell infiltrates at different stages of vulvar disease

CHAPTER 6

INTRAEPITHELIAL MACROPHAGE INFILTRATION IS RELATED TO A HIGH NUMBER OF REGULATORY T CELLS AND PROMOTES A PROGRESSIVE COURSE OF HPV-INDUCED VULVAR NEOPLASIA

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Abstract

Human papilloma virus (HPV) induced usual type vulvar intraepithelial neoplasia (uVIN) is infiltrated by myeloid cells but the type and role of these cells is unclear. We used triple immunofluorescent confocal microscopy to locate, identify and quantify myeloid cells based on their staining pattern for CD14, CD33 and CD163 in a cohort of 43 primary and 20 recurrent uVIN lesions, 21 carcinomas and 26 normal vulvar tissues. The progressive course of uVIN is characterised by an increase in both intraepithelial and stromal mature M1 and M2 macrophages. While the M2 macrophages outnumber M1 macrophages in healthy controls and uVIN, they are matched in number by M1 macrophages in cancer. Importantly, uVIN patients with a dense intraepithelial infiltration with mature CD14+ macrophages (irrespective of M1 or M2 type) displayed approximately a six times higher risk to develop a recurrence and a high number of these cells constituted an independent prognostic factor for recurrence. In addition, a dense intraepithelial CD14+ cell infiltration was associated with high numbers of intraepithelial CD4+ Tregs and low numbers of stromal CD8+TIM3+ T cells. Patients with low numbers of intraepithelial CD14+ cells and high numbers of stromal CD8+TIM3+ cells showed the best recurrence free survival. These data clearly show the importance of the local immune response in HPV-induced vulvar neoplasia and may be of help in predicting the prognosis of patients or their response to immunotherapy.

Introduction

Usual vulvar intraepithelial neoplasia (uVIN) lesions are caused by a persistent high risk human papilloma virus (HPV) infection (mainly HPV 16) and are characterised by high recurrences, a low spontaneous regression rate of 1.5% and a malignant potential of 3-4% in treated patients.[1-3] Treatment of uVIN lesions is indispensable since 80% of patients suffer from symptoms as pruritis and pain.[2-4] Conventional treatment consistent of potential disfiguring surgical interventions is associated with psychosexual problems and is increasingly replaced by either standardised immunotherapy with imiquimod or immunotherapy in experimental setting by therapeutic vaccination or photodynamic therapy, with promising clinical successes.[2-8]

The incidence of hrHPV-induced dysplasia is increased in immunocompromised patients highlighting the essential role of the immune system in viral clearance.[9,10] Spontaneous regression of HPV is associated with systemic HPV specific CD4+ and CD8+ immune responses, however in most of the patients with uVIN these T cell responses are either weak or absent.[11,12] In addition, the local innate immune cell environment is known to influence innate and adaptive immune responses in tumors.[13,14] Monocytes are innate immune cells which can differentiate into dendritic cells (DCs) or macrophages in response to local factors.[13] DCs process and present antigens to T cells, stimulate cytotoxicity of NK cells and initiate the adaptive immune response.[15] Studies of the microenvironment in uVIN revealed that it is characterised by especially dermal immune activity as higher numbers of innate and adaptive immune cells are found in the stroma when compared to healthy control tissue.[16,17] In contrast, the epidermis shows reduced numbers of CD8+ T cells, CD1a+ mature Langerhans cells (LCs) and immature CD207+ LCs, while the number of intraepithelial macrophages is increased in uVIN.[16,17] Intralesional macrophages are derived from tissue immigrating monocytes and are generally categorised into tumor suppressive type 1 macrophages (M1) which produce IL-12 and TNFα and tumor promoting type 2 macrophages (M2) which are known to produce anti-inflammatory cytokines.[13,18,19] High numbers of tumor-associated macrophages (TAMs) induce tumor growth, progression and poor survival rates (reviewed in [13]). Interestingly, in HPV-induced cervical cancer the intraepithelial infiltration with high numbers of M1 macrophages is an independent prognostic factor for a favourable survival.[20] The type of myeloid cells infiltrate could influence the uVIN microenvironment and as such may also influence the outcome of immunotherapeutic approaches for HPV induced vulvar neoplasia.[5-8,21] Current knowledge of the character of uVIN infiltrating myeloid cells is limited. In this study we aimed to characterise intraepithelial and stromal (im)mature myeloid cells in uVIN lesions, HPV induced vulvar carcinoma and healthy vulvar tissue and determine their influence on the clinical course of disease. We analysed myeloid cell infiltrates in the microenvironment by triple fluorescent staining of CD14, CD33 and CD163 as previously

performed in cervical cancer.[20] CD14 is a marker for monocytes/macrophages and expressed as well on a subset of DCs[14,20,22,23], CD33 expression is lost along the differentiation pathway of myeloid cells and is expressed by immature monocytes, macrophages and myeloid DCs.[24] CD163 is a monocyte/macrophage specific marker that is mainly expressed on M2 macrophages as well as on immune suppressive DCs.[14,25-28] Our results demonstrate that under healthy conditions, intraepithelial myeloid cells are absent whereas in the stroma M2 macrophages dominate. The changes to vulvar dysplasia are characterised by an increase in both intraepithelial and stromal mature CD14+ M1 and M2 macrophages. The increase of intraepithelial mature CD14+ cells is an independent negative prognostic factor for recurrence free survival in uVIN.

Material and Methods

Patient material

Analysis of myeloid cell infiltrates in the microenvironment of vulvar neoplasia was performed on formalin-fixed, paraffin embedded tissue (FFPE) blocks from 43 first lesions of uVIN, 20 recurrent uVIN lesions, 21 HPV positive vulvar carcinomas and 26 HPV negative healthy controls who underwent labial reduction surgery. Selected uVIN patients were treated in the Leiden University Medical Center (LUMC) between 1996 and July 2012 and histological analysis was performed by an experienced gynaecologic pathologist and classified according to the International Society for the Study of Vulvovaginal Diseases (ISSVD) guidelines.[29] Patient selection and characteristics have been described previously.[30,31] The Leiden University Medical Ethic Committee approved the study on prospective collection of healthy controls and use of archival FFPE blocks was according to Dutch Federation of Medical Research Association guidelines. On all FFPE tissue from uVIN lesions HPV typing was performed by HPV16 PCR with a HPV16 specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotypine Extra line probe assay (Innogenetics, Ghent, Belgium) in case of HPV16 negativity.[32,33]

Triple immunofluorescent confocal microscopy

Simultaneous detection of monocytes was carried out by triple fluorescent staining and confocal microscopy as described previously.[20] In brief, sections were deparaffinezed and antigen retrieval was performed in pre-heated Tris-EDTA buffer pH 9.0. Primary antibodies: CD14 (anti-CD14, mouse IgG2a, clone 7; Novocastra 1:50), CD33 (anti-CD33, mouse IgG2b, clone PWS44; Novocastra (1:100) and CD163 (anti-CD163, mouse IgG1, clone 10D6; Novocastra 1:1600). Secondary antibodies were all isotype specific antibodies with Alexa Fluorchromes Alexa Fluor 488 (CD14-green), 546 (CD33-red), and 647 (CD163-blue) (Molecular Probes;

1:200). Five randomly selected representative images of immunofluorescent stained tissue sections were captured using a confocal scanning microscope (LSM510, Zeiss) in a multitrack setting with a 25x/0.80 Plan-NEOFluar objective. Cervical cancer tissue was used as a positive control and two extra sections were stained without primary or secondary antibody as a negative control. Epithelium and stromal cells were manually counted using the LSM 5 Image Examiner software and represented as the number of cells per mm^2 for each slide (average of five 250x images). By use of overlapping colors the following infiltrating myeloid cells were distinguished; CD14+CD33-CD163- (green), CD14-CD33+CD163- (red), CD14-CD33-CD163+ (blue), CD14+CD33+CD163- (yellow), CD14+CD33-CD163+ (light-blue), CD14-CD33+CD163+ (purple), CD14+CD33+CD163+ (white) (Fig. 1). These cells were prior to analysis categorized into total CD14+, CD33+ or CD163+ positives, M1 macrophages (CD14+CD33-CD163- and CD14+CD33+CD163-), M2 macrophages (CD14+CD33+CD163+ and CD14+CD33-CD163+) and non-macrophage M2-like cells (CD14-CD163+).

Data analysis

For data analysis the statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL) was used. Non-parametric Mann-Whitney test was used to compare continuous variables between patient groups and group comparisons of categorical data were performed by χ^2 test, and the Fishers exact test in case of small groups. The Shapiro-Wilk test was used to determine a normal distribution and revealed that all data on myeloid cell counts were non-parametric. The paired Wilcoxon Signed Rank test was used to compare primary and secondary lesions. The Spearman correlation coefficient was used to detect correlation in the non-parametric data. The Bonferroni correction was applied for multiple testing considering 12 variables (Supplementary Table S1), revealing a *P*-value of <0.004 as significant. Patients were divided into groups based on the median of infiltrating cells and both an univariate (Log Rank) and multivariate analysis corrected for multifocality of uVIN (Cox proportional hazard model) were performed for recurrence-free survival (RFS) analysis, since this was previously identified as prognostic marker in our study cohort[34]. Two sided *P*-values <0.05 were considered statistical significant. GraphPad Prism 5.04 (Graphpad Software Inc, LA Jolla, CA, USA) was used to illustrate the data by graphs and figures.

Results

Patient characteristics

The clinical characteristics of the patients and controls are shown in Table 1 and these were described previously.[30,31]

Table 1: Patient Characteristics

	uVIN patients (n=43)	Vulvar carcinoma patients (n=21)	Healthy controls* (n=26)
Lesion histology			
uVIN	43 (100%)	-	-
Microinvasive carcinoma	-	8 (38.1%)	-
Macroinvasive carcinoma	-	13 (61.9%)	-
No dysplasia	-	-	26 (100%)
Age at diagnosis (years)			n.a.
Mean	47.26	69.14	
Median	47.00	70.00	
SD	16.53	13.99	
Range	19-84	49-95	
Age at inclusion (years)			
Mean	47.72	62.90	32.96
Median	47.00	62.00	32.00
SD	16.55	13.30	10.91
Range	20-84	45-85	16-54
Follow up from 1st diagnosis (months)			n.a.
Mean	85.16	96.45	
Median	50	78.50	
SD	85.03	76.54	
Range	0-307	2-244	
Follow up from inclusion (months)			n.a.
Mean	79.35	59.60	
Median	46.00	56.50	
SD	85.95	40.15	
Range	0-307	1-133	
Lesion type		n.a.	n.a.
Unifocal	25 (58.1%)		
Multifocal	18 (41.9%)		
Recurrences after inclusion			n.a.
Yes	20 (46.5%)	3 (14.2%)	
No	23 (53.5%)	18 (85.8%)	
First treatment			
Excision	22 (51.2%)	15 (71.4%)	26 (100%)
Laser	13 (30.2%)	6 (28.6%)	-
Imiquimod	3 (7%)	-	-
Laser and Excision	5 (11.6%)	-	-
Smoking status			
Yes	34 (79.1%)	9 (42.9%)	-
No	6 (14%)	5 (23.8%)	-
Unknown	2 (4.7%)	7 (100%)	26 (100%)
HPV type			
16	34 (79.1%)	14 (66.7%)	-
33	5 (11.6%)	5 (23.8%)	-
16 + 33	1 (2.3%)	1 (4.8%)	-
Multiple hrHPV (e.g. 33,31,51,44)	2 (4.7%)	-	-
73	1 (2.3%)	-	-
18	-	1 (4.8%)	26 (100%)
Immunosuppressive medication			
No	36 (83.7%)	20 (95.2%)	26 (100%)
Yes (e.g. HIV, allograft recipient, autoimmune disease)	7 (16.3%)	1 (4.8%)	-
Carcinoma			n.a.
Before inclusion	-	-	
Diagnosed at inclusion	-	21 (100%)	
Progression after inclusion	8 (18.6%)	-	

* Healthy controls are HPV negative normal vulvar epithelium tissue sections obtained from labial reduction surgery.

The progressive course of vulvar neoplasia is characterised by an increase in mature CD14+ and CD14+CD163+ myeloid cells

Triple immunofluorescent confocal microscopy of CD14, CD33 and CD163 revealed several different combinations of myeloid cells staining patterns (Fig. 1). The stroma of HPV induced vulvar neoplasia is abundantly infiltrated with CD14+, CD163+ and CD14+CD163+ myeloid cells. Approximately four times more M2 macrophages compared to M1 macrophages are present in the stroma of uVIN and healthy controls. Vulvar carcinomas are characterised by increased numbers of CD14+ cells and especially the single positive CD14+ cells and M1 macrophages reach the level of the number of stromal M2 macrophages (reflected in the M1/M2 ratio; Fig. 2). The total number of CD33+ immature myeloid cells and CD163+ cells as well as the non-macrophage M2-like cells (CD14-CD163+) are increased in uVIN but in vulvar carcinoma they are back at the same level found in controls. The number of immature (CD33+) M2 macrophages in carcinoma is very low when compared to controls and uVIN lesions, suggesting only matured macrophages are present in the tumor microenvironment.

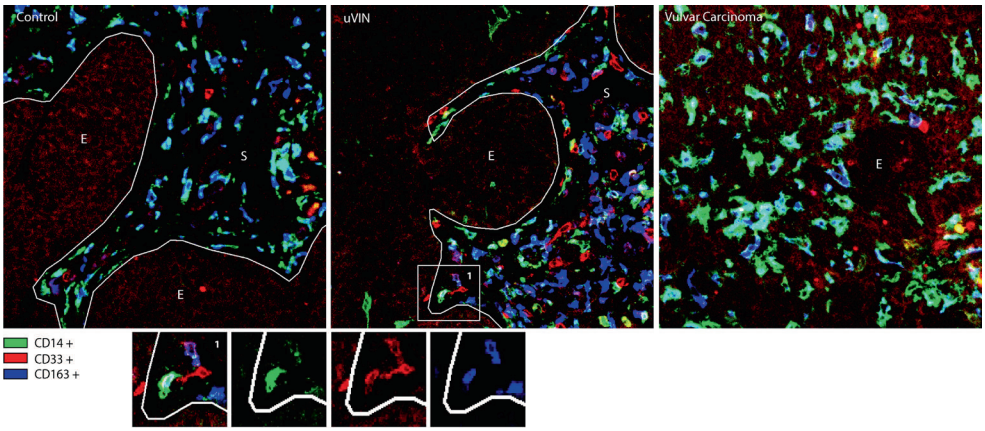

Figure 1: **Immunofluorescent staining of uVIN, vulvar carcinoma and control tissue with antibodies against CD14+ CD33+ and CD163+**

Epithelial (E) and stromal (S) infiltrates of myeloid cells in the progressive course of vulvar neoplasia were analysed with antibodies against CD14 (green), CD33 (red) and CD163 (blue). Representative examples of a healthy control, uVIN and vulvar carcinoma section are depicted. Of note: the vulvar carcinoma only tumorepithelium is depicted. In the enlarged section single positive cells of CD33 (red) and CD163 (blue) can be distinguished as well as a triple positive CD14+CD33+CD163+ (white) and a double positive CD33+CD163+ (purple) myeloid cell. In the picture of uVIN CD14+CD33+ (yellow) and CD14+CD163+ (light-blue) double positive cells can be seen.

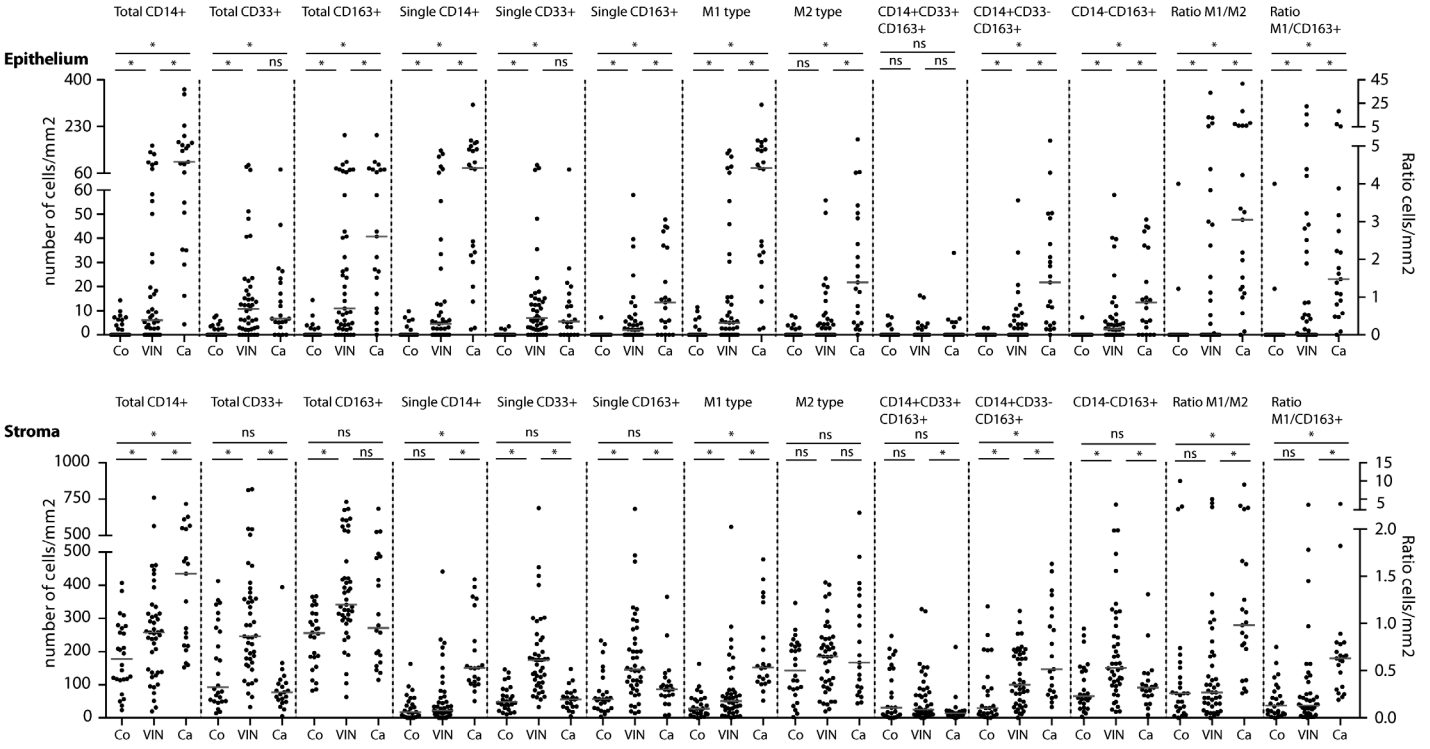

Figure 2: **Myeloid cell infiltrates during the progressive course of vulvar neoplasia and ratios of M1 monocytes in comparison to M2 monocytes and total CD163+ type 2 myeloid cells**

Infiltrating myeloid cells in the stroma and epithelium were characterised by expression of CD14, CD33 or CD163 and presented in relation to each other as cells/mm2 on the left vertical axis for healthy controls (Co), uVIN and vulvar carcinoma (Ca). These myeloid cells were subsequently categorised into M1 macrophages (CD14+CD33-CD163- and CD14+CD33+CD163-), M2 macrophages (CD14+CD33-CD163+ and CD14+CD33+CD163+) and non-macrophage M2 like myeloid cells (CD14-CD163+). Total CD163+ is a representation of all CD163+ cells (CD14+CD33-CD163+, CD14+CD33+CD163+, CD14-CD33-CD163+ and CD14-CD33+CD163+). Ratios of M1/M2 and M1/Total CD163+ myeloid cells were calculated and their depicted numbers are represented on the right vertical axis. Graphs represent their numbers or ratio's in the epithelium (A) and stroma (B) at different stages of disease.

In the epithelia of healthy tissue, myeloid cells were virtually absent (median 0.00 cells/mm2 for all types). However, these cells were clearly present in uVIN and vulvar carcinoma. Specifically a rise in the number of intraepithelial CD14+, single CD14+, CD163+, single CD163+ and CD14+(CD33-)CD163+ myeloid cells was found in uVIN and vulvar carcinoma (Table 2 and Fig. 2). In addition, a gradual increase in M1 macrophages, M2 macrophages and non-macrophage M2 like cells (CD14-CD163+) was observed from controls to uVIN to carcinoma. The increase in the numbers of the different intraepithelial myeloid cell subclasses (*e.g.* M1 and M2) was mutually correlated (Supplementary Table S1). The ratio between M1 and M2 macrophages in uVIN was still much lower than 1 indicating that the number of intraepithelial M2 macrophages still dominated.

A sharp increase in all the mentioned myeloid subsets marked the difference between uVIN and vulvar carcinoma. The great majority of CD14+ cells were CD163-negative indicative for a M1 type of infiltrating macrophages. Furthermore, these cells were mainly CD33-representing mature myeloid cells. This is also indicated by the CD33-/CD33+ cell ratio which is higher in vulvar carcinoma than in uVIN (data not shown p=0.002). Moreover, the ratio between intraepithelial M1 and M2 macrophages and the ratio between M1 macrophages and CD163+ cells was well above 1 indicating that in vulvar carcinoma the number of M1 macrophages was equal to or dominated M2 macrophages.

Overall, changes in the intraepithelial infiltration from healthy tissue to carcinoma reflected changes in the stromal compartment. Furthermore, uVIN and vulvar carcinoma display increased numbers in both stromal and epithelial myeloid cell populations when compared to healthy tissue. Whereas in uVIN the CD163+ (M2 and M2-like cells) cell populations still dominate, vulvar carcinoma is specifically characterised by yet an higher increase in myeloid cells infiltration and specifically an increased M1 infiltrate in comparable or higher numbers than the M2 population. A few patients presented with immunological disorders but they did not show overt difference in immune infiltration as compared to the rest of the group of uVIN patients (not shown).

Table 2: Myeloid cell infiltrates in the microenvironment of healthy controls, uVIN and HPV induced vulvar carcinoma

Myeloid cell types	Controls Median (range) N=26	uVIN Median (range) N=43	Carcinoma Median (range) N=21	*P*-value* uVIN vs. Controls	*P*-value* uVIN vs. Carcinoma	*P*-value* Controls vs. Carcinoma
CD14+ total (E)	0.00 (0.00-14.34)	6.03 (0.00-160.63)	101.01 (4.32-364.81)	**0.002***	**0.000***	**0.000***
CD14+ total (S)	177.18 (23.53-406.95)	257.67 (19.68-760.79)	435.74 (152.65-1134.99)	**0.036***	**0.008***	**0.000***
CD33+ total (E)	0.00 (0.00-7.96)	10.77 (0.00-90.13)	6.66 (0.00-73.87)	**0.000***	0.926	**0.000***
CD33+ total (S)	92.79 (13.82-412.93)	245.86 (32.13-818.66)	77.66 (4.60-394.28)	**0.001***	**0.000***	0.215
CD163+ total (E)	0.00 (0.00-14.42)	4.27 (0.00-108.49)	40.74 (0.00-199.02)	**0.000***	**0.000***	**0.000***
CD163+ total (S)	255.91 (82.55-366.76)	341.53 (62.35-731.87)	279.19 (114.67-684.19)	**0.000***	0.191	0.109
CD14+ single (E)	0.00 (0.00-9.82)	4.52 (0.00-143.19)	79.17 (2.26-309.56)	**0.001***	**0.000***	**0.000***
CD14+ single (S)	16.91 (0.00-162.69)	21.97 (0.00-442.04)	150.72 (50.28-418.28)	0.300	**0.000***	**0.000***
CD33+ single (E)	0.00 (0.00-3.45)	6.94 (0.00-90.13)	5.49 (0.00-73.87)	**0.000***	0.651	**0.000***
CD33+ single (S)	47.63 (10.61-145.80)	174.17 (32.13-687.30)	55.80 (4.60-147.11)	**0.000***	**0.000***	0.864
CD163+ single (E)	0.00 (0.00-7.21)	1.97 (0.00-57.98)	13.41 (0.00-47.76)	**0.000***	**0.003***	**0.000***
CD163+ single (S)	55.99 (3.84-233.01)	145.54 (13.88-681.90)	86.13 (7.58-365.46)	**0.000***	**0.005***	0.054
CD14+CD33+ (E)	0.00 (0.00-5.74)	0.00 (0.00-9.37)	0.00 (0.00-11.69)	0.130	0.227	0.854
CD14+CD33+ (S)	0.00 (0.00-55.16)	5.11 (0.00-141.83)	0.00 (0.00-83.40)	**0.014***	**0.044***	0.601
CD14+CD163+ (E)	0.00 (0.00-7.96)	0.00 (0.00-55.75)	21.74 (0.00-183.63)	0.078	**0.000***	**0.000***
CD14+CD163+ (S)	143.09 (2.14-346.94)	185.20 (19.68-408.68)	166.82 (43.16-656.37)	0.239	0.563	0.171
CD14+CD33+CD163+ (E)	0.00 (0.00-7.96)	0.00 (0.00-16.36)	0.00 (0.00-33.98)	0.935	0.268	0.411
CD14+CD33+CD163+ (S)	30.70 (0.00-247.18)	24.57 (0.00-328.31)	13.89 (0.00-214.13)	0.828	**0.021***	0.108
CD14+CD33-CD163+ (E)	0.00 (0.00-2.87)	0.00 (0.00-55.75)	21.74 (0.00-178.36)	**0.003***	**0.000***	**0.000***
CD14+CD33-CD163+ (S)	29.46 (0.00-336.41)	100.02 (0.00-322.95)	146.41 (32.25-464.94)	**0.006***	**0.035***	**0.000***
M1 type (E)[a]	0.00 (0.00-11.47)	4.97 (0.00-143.19)	79.17 (2.26-309.56)	**0.001***	**0.000***	**0.000***
M1 type (S) [a]	27.58 (0.00-162.69)	50.63 (0.00-559.27)	151.69 (51.97-478.62)	**0.047***	**0.000***	**0.000***
M2 type (E)[b]	0.00 (0.00-7.96)	0.00 (0.00-55.75)	21.74 (0.00-183.63)	0.067	**0.000***	**0.000***
M2 type (S) [b]	143.09 (2.14-346.94)	185.20 (19.68-408.68)	166.82 (43.16-656.37)	0.239	0.563	0.171
CD14-CD163+ total (E)[c]	0.00 (0.00-7.21)	2.37 (0.00-57.98)	13.41 (0.00-47.76)	**0.000***	**0.009***	**0.000***
CD14-CD163+ total (S) [c]	64.98 (3.84-269.45)	150.81 (18.41-712.19)	91.36 (8.62-373.11)	**0.001***	**0.003***	0.369
Ratio M1/M2 (E)	0.00 (0.00-4.00)	0.00 (0.00-33.87)	3.05 (0.00-41.63)	**0.008***	**0.000***	**0.000***
Ratio M1/M2 (S)	0.26 (0.00-10.00)	0.27 (0.00-4.99)	0.98 (0.26-9.00)	0.556	**0.000***	**0.000***
Ratio M1/CD163+ (E)	0.00 (0.00-4.00)	0.00 (0.00-22.58)	1.47 (0.00-18.44)	**0.001***	**0.001***	**0.000***
Ratio M1/CD163+ (S)	0.13 (0.00-0.75)	0.13 (0.00-3.41)	0.63 (0.19-3.65)	0.715	**0.000***	**0.000***

*Significant *p*-values <0.05 by analysis with the non-parametric Mann-Whitney U test used to determine differences in myeloid cell type infiltrates between healthy controls, uVIN and vulvar carcinoma.

[a] M1 = Single CD14+ and CD14+CD33+, [b] M2 = CD14+CD33+CD163+ and CD14+CD163+, [c] non-macrophage M2-like cells = CD14-CD163+ cells. Ratio M1/M2 = M1 type/ M2 type, Ratio M1/CD163 = M1 type/totalCD163+. (E) = epithelium, (S) = stroma

Intraepithelial CD14+ macrophage infiltration of uVIN is an independent prognostic factor for decreased recurrence free survival

The most prominent findings are the increase of CD14+ cells, CD163+ cells and CD14+CD163+ cells in the epithelium with a relative stronger increase of M1 cells over CD163+ cells at advanced stages of disease. To test if these myeloid cell populations contribute to the clinical course of uVIN we first analysed the myeloid cell infiltrates of the paired primary and recurrent lesions in a group of 20 patients. This analysis revealed no differences in the general myeloid cell infiltrates (Supplementary Table S2). Subsequently, we assessed if there were overt differences in the myeloid cell infiltration of the primary lesions when the patients with uVIN were divided into a group with or without recurrence. No gross differences were found between the two groups of patients based on the absolute numbers of infiltrating cells (Mann-Whitney U test) or based on the median cell count (χ^2 test) (Supplementary Table S3 for χ^2 analysis and data not shown of Mann-Whitney U test). Then we analysed the recurrence free survival of the patients in the context of these myeloid cells. This revealed that if the number of intraepithelial CD14+ macrophages (irrespective of M1 or M2 type) was high, patients more rapidly developed a recurrence. Moreover, the number of intraepithelial CD14+ macrophages formed an independent prognostic factor for a short recurrence free survival. Patients with a dense intraepithelial infiltration of CD14+ cells in the primary lesion had approximately a six times higher hazard ratio to rapidly develop a recurrence (HR 5.94; 95% CI 1.76-20.09). The influx of CD14+ M1 macrophages appeared to be the most important factor as a higher ratio of M1 cells over CD163+ cells was associated with a rapid development of an recurrence (Supplementary Table S3 and Fig. 3). Thus, while differences in the intraepithelial CD14+ cell infiltrate of the primary lesion is associated with a decreased recurrence free survival time and forms an independent prognostic factor, the immune infiltration of the primary and recurrent lesions within each individual patient with a recurrence do not differ.

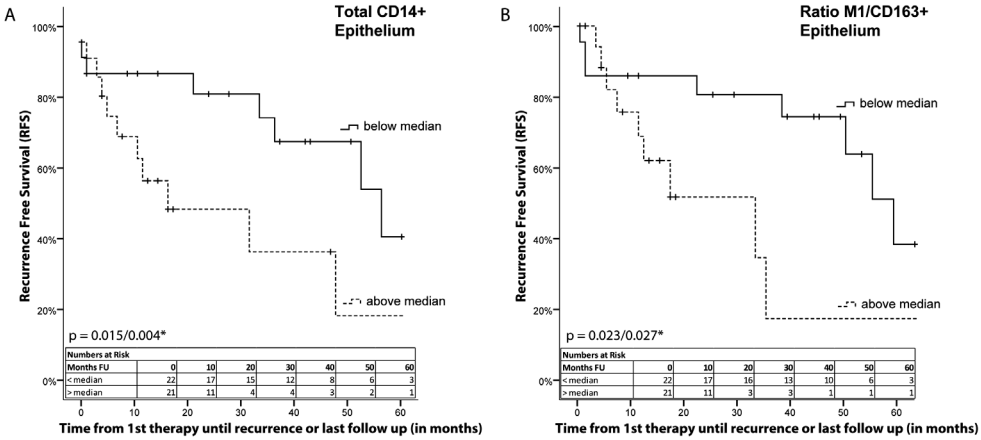

Figure 3: **Influence of intraepithelial CD14+ and ratio M1/CD163+ infiltrates on recurrence free survival**

Myeloid cell infiltrates in the epithelium and stroma of uVIN lesions were divided based on the median number of cells and analysed for their influence on the recurrence free survival (RFS) of the uVIN patients by univariate (Log Rank) and *multivariate Cox analysis. Depicted are Kaplan-Meir survival curves of RFS for; A: total intraepithelial CD14+ infiltrate, suggesting that low numbers are an independent favourable prognostic marker, B: the ratio of intraepithelial M1 monocytes and total CD163+ cells, indicating that relatively more numbers of intraepithelial M1 macrophages is associated with a decreased RFS.

Intraepithelial macrophage infiltration is correlated to a high number of regulatory T cells and low numbers of activated CD8+ stromal T cells

In parallel, we had analysed this patient cohort with respect to T cell infiltration and the expression of co-inhibitory molecules in the microenvironment of uVIN.[30] The outcomes of that study were then used to determine how changes in myeloid cell infiltration correlated with changes in uVIN-infiltrating T cells. Correlations of myeloid cell infiltrates to lymphocyte infiltrate and the expressed co-inhibitory markers are described in Supplementary Table S1. Regardless of the type of myeloid infiltrate, higher numbers of intraepithelial myeloid cells were correlated with the presence of intraepithelial regulatory T cells, represented by CD4+FoxP3+, CD3+PD1+FoxP3+ or CD4+TIM3+ T cells.[35] Moreover a high number of intraepithelial myeloid cells was strongly associated with lower numbers of stromal CD8+TIM3+ cells, a T cell population for which we previously showed to be associated with improved recurrence free survival.[30] The relation between stromal myeloid cells and different types of T cells was less clear.

In view of the independent prognostic effects of intraepithelial CD14+ myeloid cells (this study) and stromal CD8TIM3+ cells[30] their combined effects on recurrence free survival were examined. This revealed that patients with low numbers of intraepithelial CD14+ cells and high numbers of stromal CD8+TIM3+ cells have a much better recurrence free survival than patients with high number of intraepithelial CD14+ cells and low stromal CD8+TIM3+

T cells (Fig. 4a). Furthermore, since there was a direct association between the numbers of intraepithelial myeloid cells and that of intraepithelial Tregs, the latter of which we showed that they do not as a single entity impact RFS[30], we also analysed the RFS on basis of the combined intraepithelial CD14+ cell and Treg number in our patient cohort. This analysis revealed that patients with high numbers of intraepithelial CD14+ macrophages and intraepithelial Tregs more rapidly displayed recurrences (Fig. 4b).

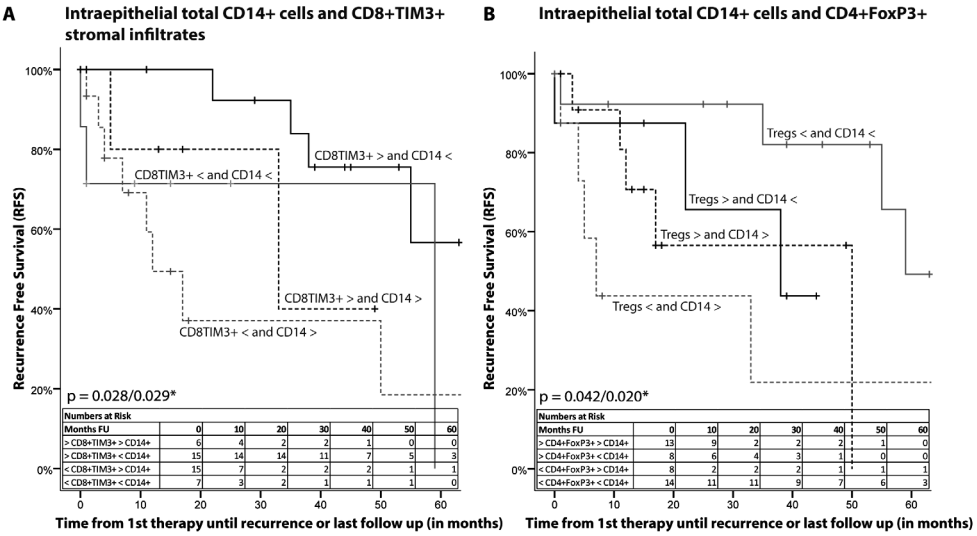

Figure 4: **Combinatorial analysis of intraepithelial CD14+ macrophages and stromal CD8+ TIM3+ T cells or intraepithelial regulatory T cells**

Based on the median number of innate and adaptive immune cells, combinatorial data of the importance of innate and adaptive immune infiltrates were analysed for their influence on recurrence free survival by univariate (Log Rank) and *multivariate Cox analysis. Kaplan-Meir survival curves of RFS are depicted for; A: total of intraepithelial CD14+ cells combined with stromal CD8+TIM3+ T cells, suggesting that uVIN lesions with low numbers of intraepithelial CD14+ cells and high numbers of stromal CD8+TIM3+ T cells have the best prognosis. B: low intraepithelial CD14+ cells are correlated with a low intraepithelial numbers of regulatory T cells, these patients have the best recurrence free survival.

Discussion

This is the first study on the presence and clinical impact of different myeloid cell populations in patients with HPV-induced non-recurrent and recurrent uVIN as well as HPV induced vulvar carcinoma. It demonstrates that under healthy conditions, intraepithelial myeloid cells are absent whereas in the normal stroma M2 macrophages dominate. The change from healthy vulvar skin to HPV-induced vulvar dysplasia is characterised by an increase

in both intraepithelial and stromal mature M1 and M2 macrophages. Vulvar carcinoma, however, is associated with a strong increase in especially mature CD14+ M1 macrophages. Whereas in the stroma of healthy controls and uVIN the M2 macrophages outnumber M1 macrophages, in vulvar carcinoma these M1 macrophages level up and generally dominate M2 macrophages. Importantly, the number of intraepithelial mature CD14+ cell infiltrate forms an independent negative prognostic factor for recurrence free survival in uVIN. Importantly, we detected no difference in the number and composition of the myeloid cell infiltrate between paired primary and secondary lesions of patients, suggesting that after treatment the patients may display a similar recurrence free period before a third lesion appears.

Recently, a study of the effects of imiquimod on the local immune signature also showed that the number of intraepithelial CD14+ cells was higher in uVIN when compared to healthy controls.[16] Although there was no significant difference between responding and non-responding patients, the non-responding group on average had the highest number of intraepithelial CD14+ cells suggesting that these non-responding patients immunologically were more similar to the recurrent uVIN and vulvar cancer patients. It might be perceived that the intraepithelial CD14+ or single CD14+ cell population, thus is unfavourable. However, this contradicts the finding that single CD14+ cells are related to a pro-inflammatory anti-tumor immune microenvironment[13,18,19], as well as previous findings that this intraepithelial CD14+ single cell population was related to an improved survival in patients with HPV-induced cervical cancer.[20] Other studies also showed a positive relationship between M1 infiltration and survival.[36-40] Notably, HPV induced vulvar carcinomas are known for their good overall prognosis and survival rate of approximately 80%.[41,42] In the current study, the increased presence of single CD14+ cells, representative for M1 macrophages, was related to a decreased RFS but so was the number of all intraepithelial CD14+ cells, comprising both M1 and M2 macrophages. Most notably, in uVIN the increase of one myeloid cell population coincided with the increase in all other myeloid populations (Supplementary Table S1) as well as with intraepithelial CD4+ regulatory T cells. Tregs are known to regulate monocyte differentiation and stimulate M2 macrophages.[13] We showed that in uVIN the population of (stromal and intraepithelial) M2 macrophages outnumber M1 macrophages by a factor 4 at least (M1/M2 ratio; Table 2), suggesting that in the epithelium of uVIN not the M1 macrophages but other immune suppressive immune cells prevail. This is in line with previous studies, the focus of which was mainly on T cell infiltrates and LCs/DCs in uVIN in relation to responses to immunotherapy.[5,7,8,16,17,21,43,44] From these studies it was concluded that the epithelium of uVIN lesions was suppressed as it lacked a strong CD8+ infiltration and displayed lower numbers of Langerhans cells.[16,17,21] In contrast, the dermis of uVIN lesions was thought to be the most active region as there was abundant infiltration of CD4+ and CD8+ T cells as well as mature DCs.[8,16,17] We recently showed that stromal CD8+

T cells expressing TIM3 reflected activated T cells, the presence of which was associated with local IFNγ production and an increased RFS.[30] Notably, in case of abundant Treg infiltration, the effect of CD8+ T cells on RFS in uVIN diminished.[30] In the current study, we found a clear inverse association between the number of these stromal CD8+TIM3+ T cells and intraepithelial macrophages. If the enhanced intraepithelial M1 and M2 macrophage infiltrate is considered to reflect, rather than cause, a process in uVIN associated with an unfavourable course of the disease, one can envisage that an active uVIN-resistant stromal immune response (e.g. more CD8+TIM3+ cells and less Tregs) prevents this process and thus the accumulation of intraepithelial macrophages.

Irrespective of their actual role in the prevention or stimulation of uVIN recurrences, an estimation of the number of intraepithelial macrophages may thus be of help in determining the prognosis of patients diagnosed with uVIN. Previously, we had identified multifocality of uVIN lesions, most often found in relation to larger lesions, as the only clinical prognostic factor for recurrence in this cohort.[34] A larger lesion size was found associated with a lower capacity to respond to therapeutic vaccination.[6] Potentially, intraepithelial myeloid cell infiltration may also influence the outcome of immunotherapeutic approaches since CD14+ and CD68+ cells were also higher in patients that failed to clear their VIN lesion after imiquimod treatment.[16] Hence, imiquimod or vaccination reinforced T cell responses[5-8,44] might be locally suppressed by the microenvironment associated with the dense intraepithelial CD14+ cell infiltration. Our combined analysis of the stromal infiltration with activated CD8+ T cells (CD8+TIM3+) and intraepithelial CD14+ cells suggests that lesions more rapidly occur when low CD8+ T cell infiltration is paralleled by a dense intraepithelial CD14+ infiltrate (Fig. 4). New studies on the innate and adaptive immune cell infiltrate of uVIN in pre-treatment samples should determine their relevance for the clinical responses to immunotherapy.

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Supporting Information

Supplemental data available on: http://onlinelibrary.wiley.com.ezproxy.leidenuniv.nl:2048/doi/10.1002/ijc.29173/suppinfo

Supplementary Table 1: Correlation analysis myeloid cells and lymphocyte infiltrates in the microenvironment of uVIN

Supplementary Table 2: Myeloid cell infiltrates in the microenvironment of paired primary and recurrent uVIN lesions of the same patient

Supplementary Table 3: Myeloid cell infiltrates in the microenvironment of recurrent and non-recurrent uVIN patients and their influence on recurrence free survival

CHAPTER 7

ANALYSIS OF MYELOID CELLS AND LYMPHOCYTES BY EXPRESSION OF CO-INHIBITORY MOLECULES AND PHOSPHORYLATION OF SIGNAL TRANSDUCTION ACTIVATORS OF TRANSCRIPTION (STAT) AFTER STIMULATION IN PERIPHERAL BLOOD OF PATIENTS WITH HIGH GRADE USUAL VULVAR INTRAEPITHELIAL NEOPLASIA

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Abstract

Different topical or systemic immunotherapies have shown partial clinical success in the treatment of human papillomavirus (HPV)-induced usual vulvar intraepithelial neoplasia (uVIN). The clinical response of patients with HPV16-induced uVIN to HPV type 16 synthetic long peptide (SLP) vaccination was associated with a stronger HPV16-specific T-cell response and a distinct peak in cytokine levels directly after the first vaccination. This suggested that differences in capacity to respond to HPV16-SLP vaccination are related to the immune status of patients before therapeutic intervention. Because cytokines and co-inhibitory molecules are important regulators of the immune response, circulating myeloid and lymphoid cells of uVIN patients were phenotyped and their phosphorylation of signal transduction activators of transcription (STAT) proteins (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) upon stimulation with different cytokines were analysed. A decreased number of circulating dendritic cells (DCs) and higher numbers of immature DCs and type 2 monocytes are related to recurrent disease. Increased frequencies of CD4+CD94+ and CD4+CD94+NKG2a+ T cells and lower numbers of CD8+TIM3+ T cells were related to the absence of recurrent disease. No differences were found in pSTAT response upon stimulation with IFNγ, IL-2, IL-4, IL-5, IL-6, IL-7 and IL-10. However, the CD14+CD33+ monocytes of uVIN patients responded differently when stimulated with IFNα and GM-CSF, specifically with respect to the response rate or levels of pSTAT5 and total pSTAT induction, suggesting that there are differences in the capacity of these immature immune cells to induce pro-inflammatory cytokine responses. Interestingly, the CD14+CD33+ cells of uVIN patients, who responded with a distinct peak in IFNγ upon HPV 16 SLP vaccination, displayed a stronger pSTAT1 response to IFNα stimulation. Altogether, our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients.

Introduction

Human papilloma virus (HPV) induced usual vulvar intraepithelial neoplasia (uVIN) is caused by a failure of the immune system to clear a persistent HPV infection and is related to the majority of uVIN lesions[1]. The incidence of uVIN is higher in immunocompromised patients, indicating the pivotal role of the immune system in viral clearance and lesion regression[2-5]. Indeed, the presence of HPV-specific T-cell reactivity is associated with protection against development and progression of HPV-induced neoplasia, but is lacking in most uVIN patients[6-9]. In addition, treatment of uVIN with immunotherapy aimed at the reinforcement of the immune response against HPV, shows promising clinical successes although the number of complete clinical responses varies up to 50%[10-13]. Furthermore, local immunity is associated with clinical outcome. The epithelium of uVIN lesions is characterised by lower numbers of infiltrating CD8+ T cells, a reduction of Langerhans cells (LCs) and an increase in CD14+ macrophages[14,15] and CD4+ regulatory T cells (Tregs)[12-16]. Importantly, a high number of intraepithelial macrophages are an independent prognostic factor for rapid recurrences[16] and high Treg numbers are related to non-responsiveness to immunotherapy[11-13]. Furthermore, a dense stromal infiltration by dendritic cells (DCs) and Tbet+, CD8+TIM3+ and CD3+NKG2a+ T cells as well as low numbers of Tregs are associated with the prevention of recurrences[14,15,17]. T-cell function has been related to expression of co-inhibitory molecules as CTLA-4, PD1, TIM3 and NKG2a after interaction with their ligands[18-21]. Monocytes and macrophages are important antigen presenting cells and mediate the innate immune responses by phagocytosis and activate and polarise the adaptive immune response depending upon cytokine production[22-24].

We recently showed that a high number of HPV16-positive uVIN patients clinically responded to treatment with a HPV16-SLP vaccine and that these clinical responses were strongly correlated with the strength of HPV16-specific proliferative T-cell responses characterised by high amounts of interferon gamma (IFNγ) and interleukin-5 (IL-5)[10,25]. The HPV-specific T-cell response in the clinical complete responders was characteristically accompanied by a distinct peak in cytokine levels three weeks after the first vaccination[10,25], suggesting that already before treatment a difference in the patients' capacity to respond to therapeutic vaccination may determine final outcome. Cytokines are well-known important immune regulators and the cytokine milieu plays an essential role in the functional differentiation of immune cells, including T cells and antigen presenting cells [22,26,27]. Binding of cytokines to their receptor triggers distinct signal transduction activators of transcription (STAT) pathways [28-30]. Phosphorylation of STAT (pSTAT) within cells modulates their activity and phosphorylation either leads to the activation of the protein, and triggers downstream activation of different regulatory pathways as NF-kB, MAP kinases and PI3K/Act[31] involved in differentiation, proliferation and apoptosis[32]. The JAK/STAT pathway is facing different challenges of the immune system from resisting infections and enforcement of barrier

function to maintenance of immune tolerance and protection against cancer (reviewed in[33]). Therefore, measurement of cytokine-induced pSTAT may form a method to analyse differences in responsiveness of the immune system between subjects. Due to the many different cell types involved in the immune response, the evaluation of changes in responsiveness requires quantification of phosphorylation events in individual cells by flow cytometry[29].

We hypothesized that differences in patients with respect to the response of immune cells to cytokines may offer an explanation for the observed difference in vaccine responses and associated clinical outcome. Because cytokines and co-inhibitory molecules are important regulators of the immune response we phenotyped circulating myeloid and lymphoid cells in uVIN patients and healthy controls as well as analysed the phosphorylation of STAT proteins (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) in these immune cells upon stimulation with different cytokines.

Our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients and that the measurement of circulating type 2 monocytes is related to recurrent disease, which is similar to our previous finding on the association between recurrences and type 2 macrophages in the local microenvironment.

Materials and Methods

Patient material

Patients presenting with histologically proven high-grade uVIN at the Leiden University Medical Center (LUMC) were enrolled in the Circle study, which is approved by the medical ethical committee of the LUMC and investigates cellular immunity against HPV-induced neoplasia. After meaningful signed informed consent was obtained, venous heparinized blood (60 mL) was collected prior to the therapeutic intervention and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation, cryopreserved at 10 million cells per vial and stored in the vapour phase of liquid nitrogen according to standard operating procedures (SOPs) in the laboratory of the Department of Clinical Oncology. Serum was collected in a clotting tube and frozen at -20°C until use.

Patient characteristics are summarized in Table 1. Fourteen patients with high-grade uVIN were included in this study (mean age at inclusion 51.1 year ± 49.5) of whom in 3 cases a micro-invasive carcinoma (<1mm invasion) was diagnosed after therapy. In 7 cases a recurrence of uVIN was diagnosed after Circle inclusion of which 2 patients were excluded from recurrence analysis because of a persistent uVIN lesion despite therapy. In the remaining 7 cases no recurrence occurred after Circle inclusion.

Table 1: Patient Characteristics

Characteristic	Healthy Controls (n=8)	uVIN patients (n=14)	HPV 16 SLP ISA101 vaccination patients (n=10)
Lesion histology			
High grade uVIN	-	11 (78.6%)	10 (100%)
Microinvasive carcinoma	-	3 (21.4%)	-
No dysplasia	8 (100%)	-	-
Age at diagnosis (years)	n.a.		
Mean		44.4	35.4
Median		42.0	36.0
SD		12.5	7.3
Range		30-71	25-47
Age at inclusion (years)			
Mean	46.4	51.1	41.3
Median	50.5	49.5	41.0
SD	13.1	9.8	7.4
Range	26-64	34-71	28-55
Follow up time from 1st diagnosis (in months)	n.a.		
Mean		129.5	84.4
Median		94.5	90.0
SD		98.4	49.1
Range		18-265	16-154
Time from first diagnosis until inclusion (in months)	n.a.		
Mean		77.2	70.0
Median		14.0	77.0
SD		100.4	47.9
Range		0-247	0-137
Follow up time from inclusion (in months)	n.a.		
Mean		51.5	15.0
Median		30.0	16.0
SD		45.2	4.1
Range		9-158	5-19
Recurrences before inclusion	n.a.		
Yes		7 (50%)	2 (20%)
No		7 (50%)	8 (80%)
Treatment type at inclusion	n.a.		
Excision		8 (57.1%)	-
Laser		5 (35.7%)	-
Laser and Excision		1 (7.1%)	-
HPV 16 SLP vaccination		-	10 (100%)
Recurrences after inclusion	n.a.		n.a.
Yes		6 (42.9%)	
No		8 (57.1%)	
Carcinoma	n.a.		
Before inclusion		2 (14.3%)	0
Diagnosed at inclusion		3 (21.4%)	0
Progression after inclusion		1 (7.1%)	2 (20%)*
HPV type	unknown		
16		9 (64.3%)	10 (100%)
33		4 (28.6%)	-
6 (low risk)		1 (7.1%)	-

The patient characteristics of the subsequent groups are given: the healthy controls, uVIN patients included in the Circle study and the uVIN patients who participated in the HPV 16 SLP ISA101 vaccination trial.

*Of the overall inclusions of the vaccination trial only 3 patients progressed, in this cohort however apparently a higher number was included.

Analyses were performed according to the occurrence of a recurrent uVIN lesion after Circle inclusion. The Circle patients were compared to a cohort of age-matched healthy controls (mean age at inclusion 46.4 year ± 13.1) with no known HPV medical history.
In order to analyse effects of STAT phosphorylation in patients who received therapeutic vaccination, an additional uVIN patient cohort was selected which included a cohort of 10 HPV16-positive uVIN patients who received 4 vaccinations with HPV16-SLP (ISA101) with or without application of imiquimod on the vaccination site. Pre-vaccination PBMC samples of 3 patients, who displayed a distinct peak of IFNγ production after the first vaccination and 5 patients without an IFNγ peak upon the first vaccination (determined by a median IFNγ production above 1000pg/ml in the cytokine bead array) were analysed[34,35].

HPV typing

HPV typing was performed on formalin-fixed, paraffin embedded tissue, resected during surgery or on a biopsy taken at enrolment in the vaccination study by HPV16 PCR with a HPV16-specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotyping *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV16 negativity[36,37]. Notably, at the same time the blood samples were collected.

Cell proliferation assay

The overall capacity of the T cells to proliferate upon mitogenic stimuli (i.e. phytohaemagglutinin (PHA), HA 16 Remel, Murex Biotech, Dartford, UK) was tested in a 3-days proliferation assay where the PBMC samples of uVIN patients and healthy controls were analysed against medium (Iscove's Modified Dulbecco Medium (IMDM, Life Technologies, Bleiswijk, the Netherlands) +10% Human AB serum (HAB, Greiner, Alphen a.d. Rijn, the Netherlands)) only (negative control) or after PHA stimulation (0.5 µg/mL), using a previously described method which was slightly modified[38]. The proliferation of PBMCs were measured in quadruplicate wells (50.000 cells/well) after 3 days of incubation by adding ^{3}H-thymidine for the last 16-18 hours of the incubation period after which its incorporation (expressed as counts per minute (cpm)) is measured. A cut-off was calculated by mean cpm of the 4 wells with unstimulated PBMCs (medium only) plus 3 times standard deviation (SD). The stimulation index (SI) was calculated by the ratio of the mean cpm of 4 wells with PHA stimulated PBMCs divided by the mean cpm of the 4 wells containing unstimulated PBMCs. A SI ≥3 plus mean cpm of the PHA stimulated wells above cut-off is considered a positive response.

Antigen presenting capacity of antigen presenting cells

The antigen-presenting capacity of antigen-presenting cells (APC) was tested in a mixed lymphocyte reaction (MLR). 1.5x10^{6} PBMCs of uVIN patients resuspended in 5 mL IMDM-

10%HAB were irradiated at 3000 rad to prevent proliferation of these cells. Thereafter, the cells were centrifuged, the supernatant removed and the cells resuspended in 1.5 mL IMDM-10%HAB. The irradiated patient's PBMCs were independently co-cultured with PBMCs obtained from two different healthy donors (differently from the healthy subjects) in quadruplicate wells (100.000 cells/well). Cells were incubated for 6 days at 37°C, 5% CO_2 in a humified incubator before supernatants (100 uL/well pooled) were harvested and stored at -20°C until cytokine level determination by the Th1/Th2 cytometric bead array kit (CBA, BD Biosciences, Breda, the Netherlands). Cells were pulsed with ^{3}H-thymidine for 16-18 hours and the proliferation was measured by ^{3}H-thymidine incorporation. The proliferative capacity of the two individual healthy donors co-cultured with the irradiated patients PBMCs is a measure for the APC quality of the patient. The SI was calculated by the ratio of the mean cpm of irradiated patient's PBMCs co-cultured with PBMCs of one healthy donor divided by the mean cpm of that particular healthy donor unstimulated PBMCs and a SI ≥3 is considered positive.

Phenotyping of PBMCs for macrophages, myeloid-derived suppressor cells and co-inhibitory molecule expression by flow cytometry analysis

The composition of the immune cells in the Circle and healthy control PBMC samples was determined by phenotyping for macrophages (macrophages set), myeloid-derived suppressor cells (MDSC set) and expression of co-inhibitory molecules on T cells (inhibitory T-cell set). Three sets of antibodies (10-11 antibodies per set) directed to specific surface markers for myeloid cells and T cells were used to stain these cells, which were thereafter analysed by multiparameter flow cytometry (LSR Fortessa, BD Biosciences). Thawed PBMCs were washed two times with Phosphate Buffered Saline (PBS)/0.5% Bovine Serum Albumin (BSA) and incubated on ice with PBS/0.5%BSA/10% Fetal Bovine Serum (FBS). Thereafter, PMBCs were stained for 30 minutes in the dark on ice with the 3 different antibody sets, washed two times with PBS/0.5%BSA and fixed with 1% paraformaldehyde (PA). Compensation beads were made for the different fluorchromes according to the protocol of BD Biosciences to optimize fluorescence compensation settings of the flow cytometer. Surface stainings were analysed using FACSDiva™ Software (BD Biosciences) after identification of the life gate in single cells.

Flow cytometry for analysis of macrophages

The following antibodies were used: anti-CD1a-FITC (Clone HI149, BD Biosciences), anti-CD3-Pacific Blue (PB;Clone UCHT1, DAKO), anti-CD11b-PE (Clone D12, BD Biosciences), anti-CD11c-Alexa Fluor (AF) 700 (Clone B-ly6, BD Biosciences), anti-CD14-PE-Cy7 (Clone M5E2, BD Biosciences), anti-CD16-PE-CF594 (Clone 3G8, BD Biosciences), anti-CD19-Brillian Violet (BV) 605 (Clone SJ25C1, BD Biosciences), anti-CD45-PerCP-Cy5.5 (Clone 2D1, BD Biosciences),

anti-CD163-APC (Clone 215927, R&D systems), anti-CD206-APC-Cy7 (Clone 15-2, Biolegend) and anti-HLA-DR-Horizon (H) V500 (Clone L234, BD Biosciences). In the analysis singlet cells and subsequently those in the life gate and CD45+ cell gate were plotted for CD3 and CD19. The CD45+CD3-CD19- were selected and further gated as HLA-DR+ cells. This population was divided upon differential expression of CD14 and CD11b, which reveals five subpopulations of myeloid cells in the CD45+HLA-DR+ population; activated monocytes (CD14Int+CD11b+ or CD14high+CD11b+), immature DCs/early differentiating monocytes (CD14-CD11b-), activated DCs or monocytes with loss of CD14 (CD14-CD11b+) and non-activated DCs or monocytes (CD14+CD11b-). Within these subcategories CD163, CD16, CD206 and CD11c were plotted for each of the populations to distinguish phenotypes (Supplementary Fig. S1). Differentiation markers used are CD163[39], CD11b (a monocyte activation marker[40]), CD11c (a marker of dendritic cells[41]), CD16 (macrophage Fcγ-Receptor III)[42] and CD206 (a mannose receptor)[42]. CD16 is expressed on IL-6 and IL-10 polarized CD14+ macrophages in co-expression of CD163+[43,44]. CD206 is an early marker for differentiation of monocytes into macrophages[45] and is expressed on IL-4 polarized macrophages where CD14 and CD163 are downregulated and CD200R upregulated[43,44]. Type 2 macrophages are mainly associated with Th2 responses and divided into four sub-categories; M2a macrophages (CD206+) induced by IL-4 and IL-13 associated with allergy and parasitic infections; M2b immunoregulatory macrophages (CD16+) induced by TLR activation and M2c (CD163+) regulatory macrophages induced by IL-10 and M2d macrophages[46-49]. Activation of macrophages may as well result in loss of CD14, CD163 and CD16 expression[42,50]. To compare these data between different patients the numbers of events were calculated as percentage of CD45+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry for analysis of MDSCs

Next to terminally differentiated myeloid cells such as macrophages and DCs, myeloid-derived suppressor cells (MDSCs) were stained and acquired, these immature myeloid cells counteract the anti-tumor immune response (reviewed in [22,51]). Two main MDSC phenotypes have been described: the monocytic (mMDSC) and polymorphonucleair MDSCs (granulocytic MDSC, gMDSC) [22,51], which were analysed using the following antibodies: anti-CD3-PB (Clone UCHT1, DAKO), anti-CD11b-FITC (Clone CBRM1/5, Biolegend), anti-CD14-AF700 (Clone M5E2, BD Biosciences), anti-CD15-PE-CF594 (Clone W6D3, BD Biosciences), anti-CD19-BV605 (Clone SJ25C1, BD Biosciences), anti-CD33-PE-Cy7 (Clone P67.6, BD Biosciences), anti-CD34-APC (Clone 581, BD Biosciences), anti-CD45-PerCP-Cy5.5 (Clone 2D1, BD Biosciences), anti-CD124-PE (Clone HiL4R-M57, BD Biosciences) and anti-HLA-DR-HV500 (Clone L234, BD Biosciences). In the MDSC antibody set CD45+ CD3- CD19-HLA-DR- singlet and viable cells were isolated and herein the CD14+ and CD15+ populations identified. Subsequently CD11b, CD124, CD33 and CD34 were plotted for both CD14+ and CD15+ cells (Supplementary Fig. S2).

We have evaluated these cell types upon CD45 and HLA-DR negative cells for expression of CD14+ and CD15- (mMDSC) and CD14- and CD15+ (gMDSC)[51]. Subsequently, hematopoietic progenitor markers CD33+ and CD34+ in combination with CD11b+ and CD124+ (IL-4Rα) were analysed (reviewed in[51]). Myeloid differentiation of the hematopoietic progenitor cells is characterised by loss of CD34 and expression of CD33[52]. The numbers of events were calculated as the percentage of the CD45+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry for analysis of co-inhibitory molecules on T cells

Expression of co-inhibitory receptors on T cells were analysed by the following antibodies: anti-CD3-PB (Clone UCHT1, DAKO), anti-CD4-PE-CF594 (Clone RPA-T4, BD Biosciences), anti-CD8-APC-Cy7 (Clone SK1, BD Biosciences), anti-TIM3-PE (Clone F38-2E2, Biolegend), anti-NKG2a-AF700 (Clone 131114, R&D Systems), anti-CTLA-4 (CD152)-PE-Cy5 (Clone BNI3, BD Biosciences), anti-CD94-FITC (Clone 131412, R&D Systems) and anti-PD1-BV605 (Clone EH12.2H7, Biolegend). After identification of the singlets and life gate the CD4+ and CD8+ cells were selected within the CD3+ cells. CD94, TIM3, NKG2a, CD152 and PD1 were plotted in these populations (Supplementary Fig. S3). The numbers of events were calculated as percentage of the CD3+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry analysis of STAT phosphorylation in PBMCs

Phosphorylation of signalling proteins within cells modulates their activity and leads to activation of the protein (MAP kinases and STAT transcription factors), whereas in some cases the phosphorylation leaves the proteins in an inactive state. To determine the activation state of PBMCs, intracellular phosphorylation of STAT1, -3, -5 and -6 were measured in CD3+, CD4+ and CD8+ lymphocytes and in CD14+CD33+ and CD14-CD33dim monocytes, after individual stimulation with eight different cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFNγ) or adjuvant immune modulators (IFNα and GM-CSF). PBMCs of uVIN Circle patients, healthy controls and pre-vaccination samples of HPV16-SLP treated uVIN patients were analysed using this phosphorylation cytometry assay. The protocol was adopted from G.P. Nolan (Stanford University, Stanford California)[29] and A. Cesano (Nodality, South San Francisco)[53]. Thawed PBMCs were resuspended in serum-free (SF) IMDM at a concentration of 1-1.5 x10^6/ml and incubated at 37°C for 1 hour to rest. Then, the PBMCs in SF medium were stimulated for 15 minutes at 37°C with the following cytokines and adjuvant immune modulators: IFNγ (20 ng/mL; Immunotools, Friesoythe, Germany), IL-10 (50 ng/mL; Immunotools), IL-2 (50 ng/mL; Aldusleukin 18x10^6IE, Novartis Pharma, Arnhem, the Netherlands), IL-5 (50 ng/mL; Immunotools), IL-6 (100 ng/mL; Immunotools), IL-4 (50 ng/mL; Invitrogen, Life Technologies), IL-7 (50 ng/mL; Peprotech, Huissen, the Netherlands),

IFNα (1000 IU/mL Roferon A, Roche, Woerden, the Netherlands), GM-CSF (4 ng/mL; Immunotools). These concentrations were first determined by titration assays (data not shown). After the stimulation the cells were immediately fixed in 1.5% paraformaldehyde (PA, pharmacy LUMC) for 10 minutes at room temperature (RT) and after centrifugation resuspended in 100 uL phosphate buffered saline (PBS, B. Braun, Melsungen, Germany)/0.5% bovine serum albumin (BSA; Sigma, St Louis, USA)/1.5% PA, and transferred into wells of a V-bottom 96-wells plate (Costar). After another wash step in 100 uL PBS/0.5% BSA the cell surface staining was performed for 30 minutes in the dark on ice with anti-CD3-PB (Clone UCHT, DAKO, Heverlee, Belgium), anti-CD4-HV500 (Clone RPA-T4, BD Biosciences), anti-CD8-APC-Cy7 (Clone SK1, BD Biosciences), anti-CD14-FITC (Clone M5E2, BD Biosciences) and anti-CD33-AF700 (Clone WM53, BD Biosciences). Then, the cells were washed two times with 100 uL PBS/0.5% BSA and permeabilized by adding 100 uL cold 100% Methanol (Sigma) for 10 minutes on ice. Thereafter, cells were washed again two times with 100 uL PBS/0.5% BSA and subsequently the intracellular staining was performed for 30 minutes in the dark at RT again with anti-CD33-AF700 (Clone WM53, BD Biosciences) and with anti-pSTAT1 (Clone 4a, BD Biosciences), anti-pSTAT3 (Clone 49-p-STAT3, BD Biosciences), anti-pSTAT5 (Clone 47, BD Biosciences), anti-pSTAT6 (Clone 18-p-STAT6, BD Biosciences) either coupled to fluorochromes PE or AF647. After this intracellular staining the cells were washed for two times with 100 uL PBS/0.5% BSA and fixated in 1% PA. Compensation beads were prepared for all the fluorochromes according to the protocol of BD Biosciences to properly set up the fluorescence compensation settings for the multicolour flow cytometric analyses by use of LSR Fortessa (BD Biosciences). Phosphorylation data were analyzed using FlowJo software (TreeStar Inc, Ashland, USA, version 7.6.5). The life gate was identified and subsequently CD3+ and CD3-negative populations were selected to distinguish between CD4+ and CD8+ T cells within the CD3+ population and single positive CD14 or CD33 or double positive (CD14+CD33+) myeloid cells within the CD3-negative population (Supplementary Fig. S4). The levels of pSTATs were analyzed as mean fluorescence intensity (MFI) and a fluorescence index (mean of stimulated sample divided by mean of unstimulated control sample) was calculated. A fluorescence index ≥ 2 was considered as a distinct upregulation of pSTAT after stimulation with the cytokine whereas an index between 1.5 and 2 was considered as a small up-regulation. Downregulation was defined as an index ≤ 0.5 or small downregulation when the index was between 0.5 – 0.7. Values between 0.7 and 1.5 were considered as no difference.

Statistical analysis

The non-parametric Mann-Whitney U test was used to compare continuous variables between patient groups and the $\chi 2$ test was used to compare categorical data with the statistical software package SPSS 20.0 (SPSS Inc., Chicago, USA). The Spearman correlation

coefficient was used to detect correlation in the non-parametric data. Patients were divided into groups based on the median of infiltrating cells and a univariate (Log Rank) analysis was performed for recurrence-free survival (RFS) analysis. Because of the small study population no multivariate analysis was performed. Two sided P values <0.05 were considered statistical significant. GraphPad Prism 5.04 (Graphpad Software Inc, LA Jolla, CA, USA) was used to illustrate the data by graphs and figures and for statistics in the total pSTAT induction.

Results

Antigen presenting capacity of APCs and T-cell proliferation of lymphocytes in uVIN patients and healthy donors

The antigen presenting capacity of blood-derived APCs from uVIN patients and healthy controls was tested by mixing them with lymphocytes of two unrelated healthy donors. In addition, the capacity of T cells to proliferate upon PHA stimulation was tested. No differences were observed between patients with uVIN and healthy controls (Supplementary Fig. S5), indicating that there are no overt general defects in the immune reactivity of patients with uVIN that may explain differences in response to immunotherapy.

Circulating myeloid cells and MDSCs in uVIN patients and healthy controls

An extensive panel of markers was used to distinguish monocytes/macrophages, dendritic cells and MDSC. The major populations of myeloid cells consisted of activated CD14+CD11b+ monocytes and account in total for approximately 15% of myeloid cells in both uVIN patients and healthy controls (Supplementary Table S1). The other three remaining populations represent minor categories of circulating myeloid cells and consist of immature DCs/early differentiating monocytes (CD14-CD11b-), activated DCs or monocytes with loss of CD14 (CD14-CD11b+)[42,50] and non-activated DCs or monocytes (CD14+CD11b-) accounting for <1% of CD45+HLA-DR+ cells.

Comparison of the different subpopulations between uVIN patients and healthy controls revealed no differences in the great proportion of activated CD14+ monocytes. Analysis of the smaller myeloid cell groups revealed that the frequency of circulating immature DCs/early differentiating monocytes (CD14-CD11b-, p=0.03) and type 2a and 2c monocytes (CD14-CD11b+CD206+CD16-, p=0.01 and CD14-CD11b+CD163+CD206-, p=0.02) were lower in uVIN patients than in healthy controls (Supplementary Table S1). The frequency of circulating MDSC populations was estimated below 0.05% and therefore not further analysed (Supplementary Table S2).

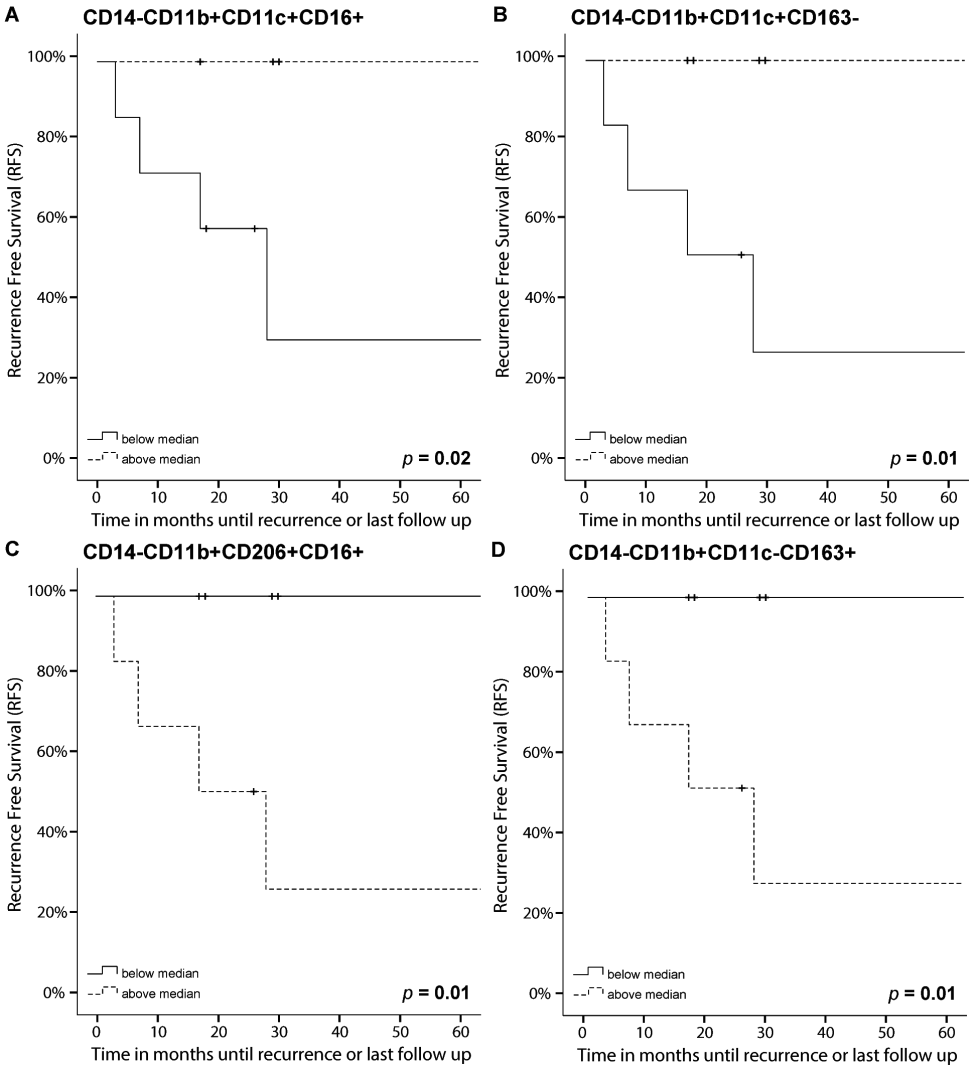


Figure 1: **Influence of systemic DCs and type 2 macrophages on recurrence free survival**

Differences in circulating myeloid cell types between recurrent and non-recurrent uVIN patients were divided based on the median percentage of cells and analysed by univariate Log Rank for their influence on recurrent free survival (RFS). Kaplan-Meir survival curves for activated DCs and type 2 macrophages on RFS are depicted. In A and B a high number of activated DCs (CD14-CD11b+CD11c+CD16+(A) and CD14-CD11b+CD11c+CD163-(B)) is related to an increased RFS and in C and D a high number of type 2a macrophages (CD14-CD11b+CD206+CD16+(C)) and type 2c macrophages (CD14-CD11b+CD11c-CD163+(D)) is related to a decreased RFS.

Previously we observed that the number of CD14+ intraepithelial myeloid cells had a prognostic value with respect to the recurrence and progression of uVIN lesions[16]. Therefore, we evaluated the frequency of circulating myeloid cells in this context. Recurrent uVIN patients have a lower, albeit not significant, percentage of circulating CD14High+CD11b+ activated monocytes compared to non-recurrent uVIN patients (9.5% vs 16.5%). Analysis of DCs by CD11c+ in this CD14High+CD11b+ monocyte population revealed that more DCs are present in non-recurrent uVIN patients, albeit not significant, probably due to the small group of patients analysed. Furthermore, patients with recurrent uVIN lesions displayed increased frequencies of type 2a and 2c monocytes/macrophages (CD14-CD11b+CD206+CD16+, p=0.01, CD14-CD11b+CD163+CD206-, p=0.01, and CD14-CD11b+CD11c-CD163+, p=0.01) and a lower number of activated DCs (CD14-CD11b+CD11c+CD16+, p=0.02 and CD14-CD11b+CD11c+CD163-, p=0.00). Moreover, a high number of activated DCs was associated with a prolonged recurrence free survival (Fig. 1a+b p=0.02/0.01) whereas a high frequency of type 2a and 2c monocytes/macrophages are associated with a decreased recurrence free survival (Fig. 1c+d p=0.01).

Expression of co-inhibitory molecules on PBMC from uVIN patients and healthy controls

In Table 2 the percentage of CD3+ lymphocytes of CD4+, CD8+ and expression of co-inhibitory molecules are represented. The majority of circulating lymphocytes is CD4+ (approximately 70%) and hardly express one of the tested co-inhibitory molecules on their surface, except for PD1 which was expressed in over 4% of CD4+ T cells and in approximately 3% of CD8+ T cells. TIM3 and NKG2a are expressed only by small proportions (<1%) of lymphocytes. Surface CTLA-4 (CD152) expression is scarce (< 0.05% of CD4+ and CD8+ T cells) and was therefore not analysed in detail. Thus, the differences in expression of the co-inhibitory molecules on T cells between healthy controls and uVIN patients are based on small cell populations. In the CD4+ T-cell populations the number of CD4+NKG2a+, either with or without co-expression of TIM3 and/or PD1, T cells is higher in uVIN patients(Supplementary Table S3). There were no differences in co-inhibitory molecule expression on CD8+ T cells observed between uVIN patients and controls (Supplementary Table S3).

Table 2: Expression of co-inhibitory molecules on CD3+ lymphoid cells in healthy controls and uVIN patients

Percentage of CD3+ T cells	PBMCs Healthy controls	PBMCs uVIN
CD4+	68.6%	69.7%
CD8+	23.2%	22.3%
PD1		
CD4+	4.1%	4.7%
CD8+	3.7%	2.7%
TIM3		
CD4+	0.21%	0.25%
CD8+	0.15%	0.13%
NKG2a		
CD4+	0.23%	0.43%
CD8+	0.38%	0.42%
CTLA4 (CD152)		
CD4+	0.04%	0.04%
CD8+	0.03%	0.03%
CD94		
CD4+	0.13%	0.15%
CD8+	1.17%	0.90%

In the microenvironment of the uVIN lesion, stromal expression of CD8+TIM3 and CD3+NKG2a+ T cells was related to an improved clinical outcome in uVIN patients[17]. Hence, we analysed the co-inhibitory molecule expression on lymphocytes in the context of recurrent disease. The main finding was that the frequency of CD4+ T cells expressing CD94+ was higher in patients who did not display a recurrent uVIN lesion. Moreover, a relatively higher number of circulating CD4+CD94+ T cells was associated with a longer recurrence free survival. This was not the case when patients were divided on basis of CD3+CD94+ and CD8+CD94+ T-cell frequencies (Fig. 2a+b+c). Expression of CD94 is related to the NKG2 family of receptors (e.g. NKG2a, -c, -d, -e and -h) by formation of heterodimers with either an activating or inhibitory function and are therefore always co-expressed[54,55]. Our study on the local immune infiltrate revealed that the expression of NKG2a was associated with an increased recurrence free survival (RFS). Therefore, we analysed RFS in the context of CD94+NKG2a+ T cells. The frequency of CD3+CD94+NKG2a+ and CD8+CD94+NKG2a+ T cells was not associated with differences in RFS while relatively higher numbers of CD4+CD94+NKG2a+ T cells were associated to no recurrences (Supplementary Table S3 + Fig. 2d-f). Because the CD3+CD94+NKG2a+/CD3+CD94+NKG2a- cell ratio in the uVIN microenvironment was associated with better clinical performance[17], we also analysed this ratio for cells in the circulation, but this lacked clinical significance (Supplementary Table S3 + Fig. 2g). The expression of TIM3 on circulating T cells was higher in patients with recurrent uVIN lesions albeit not directly related to RFS (Supplementary Table S3 and Fig. 2h-j). However, a relative higher number of circulating CD8+TIM3+ T cells was related to the recurrences and a worse RFS (Supplementary Table S3 + Fig. 2k).

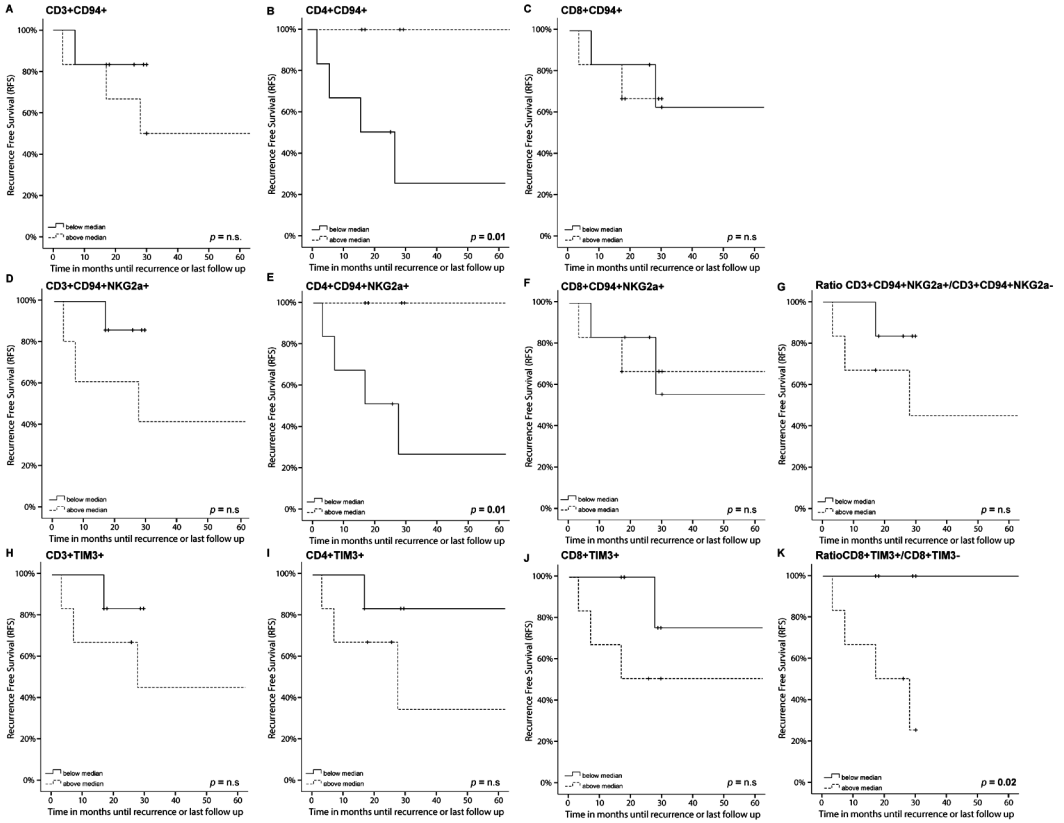
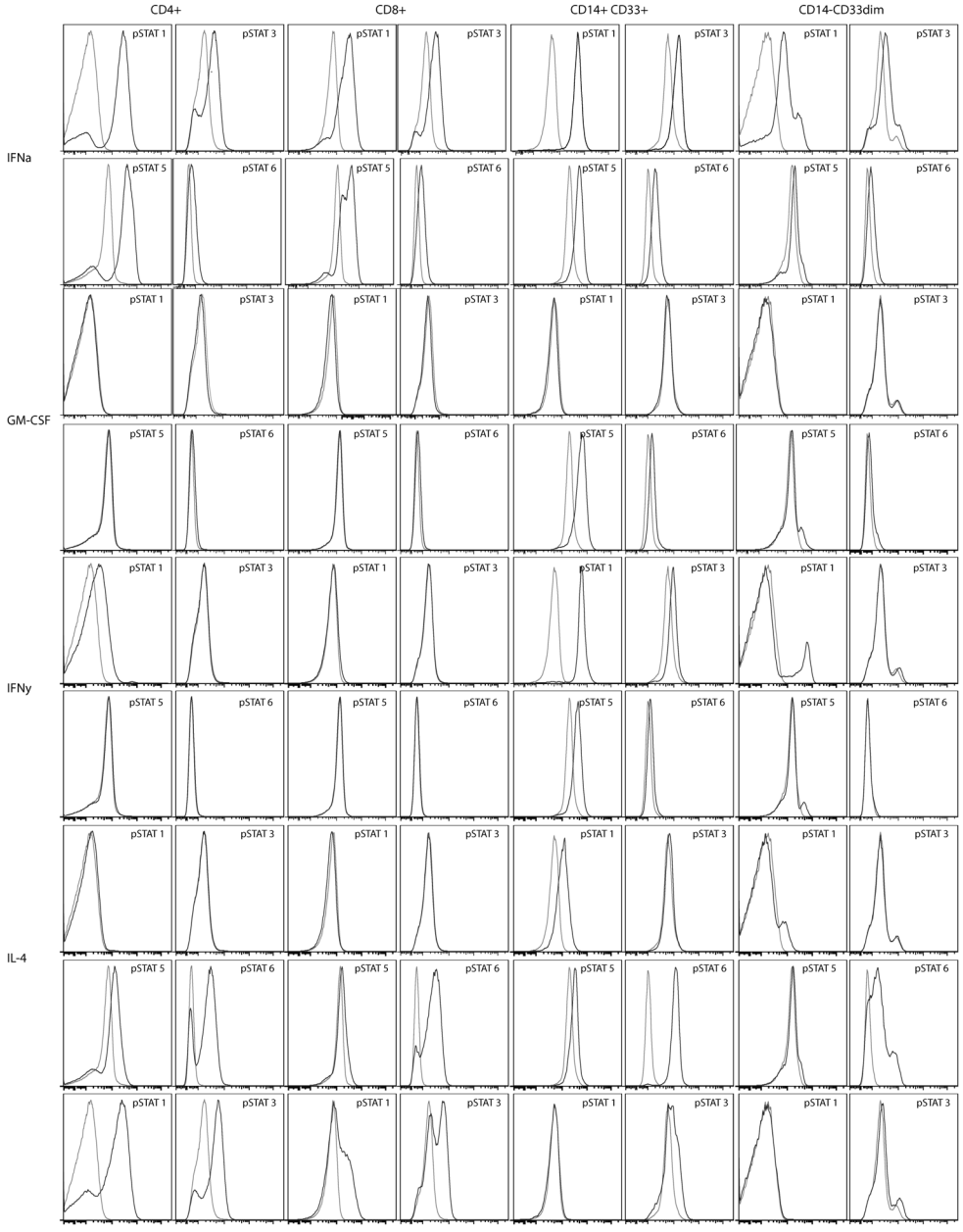


Figure 2: **Influence of CD94+, NKG2a+ and TIM3 expression on systemic lymphocytes on recurrence free survival**

Differences in circulating expression of co-inhibitory molecules on lymphocytes of recurrent and non-recurrent uVIN patients were divided based on the median percentage of cells and analysed by univariate Log Rank for their influence on recurrent free survival (RFS). Kaplan-Meir survival curves for expression of CD94+ on CD3(A), CD4(B) and CD8(C) as well as CD94+NKG2a+ on CD3(D), CD4(E) and CD8(F) and TIM3 on CD3(H), CD4(I), CD8(J) on RFS are depicted. Moreover in G and K, the ratios of CD3+CD94+NKG2a+/CD3+CD94- and CD8+TIM3+/CD8+TIM3- on RFS are depicted. In B and E a high number of CD4+CD94+ and CD4+CD94+NKG2a+ T cells are related to an increased RFS and in K a relative high number CD8+TIM3+ T cells is related to a decreased RFS.

No differences in STAT phosphorylation levels upon stimulation with cytokines

Since pathogens can reprogram intracellular immune-related signalling networks, potentially hindering immune activation, we set out to study the response of APCs and lymphocytes to various cytokines. In Figure 3 an example of STAT phosphorylation (pSTAT) upon cytokine stimulation in different immune cells is depicted.



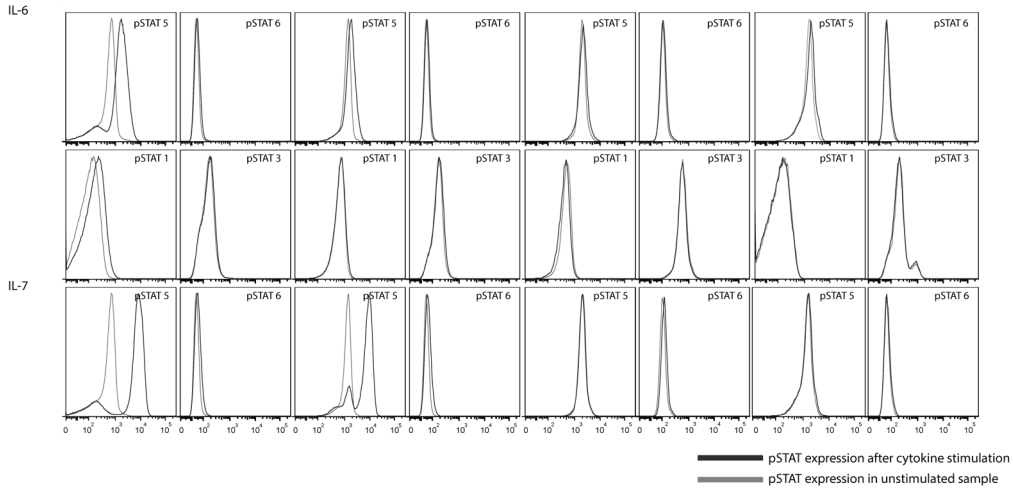


Figure 3: STAT phosphorylation upon stimulation with various cytokines or immune modulators in an example of one patient with uVIN

Intracellular phosphorylation of STAT1, -3, -5 and -6 were measured in CD3+, CD4+ and CD8+ lymphocytes and in CD14+CD33+ and CD14-CD33dim monocytes, after individual stimulation with eight different cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFNγ) or adjuvant immune modulators (IFNα and GM-CSF) to determine the activation state of PBMCs of uVIN patients and healthy controls. After identification of the lifegate in the CD3+ populations CD4+ and CD8+ T cells were distinguished whereas in the CD3- population either single or double CD14+ and CD33+ cells were identified. The levels of pSTATs were analyzed as mean fluorescence intensity (MFI) and a fluorescence index (mean of stimulated sample divided by mean of unstimulated control sample) was calculated. A fluorescence index ≥ 2 was considered as a distinct upregulation of pSTAT after stimulation with the cytokine whereas an index between 1.5 and 2 was considered as a small up-regulation. Downregulation was defined as an index ≤ 0.5 or small downregulation when the index was between 0.5 – 0.7. Values between 0.7 and 1.5 were considered as no difference. The differences in pSTAT induction in different immune cell populations upon cytokine stimulation are illustrated by use of FlowJo software (TreeStar Inc, Ashland, USA, version 7.6.5). The results for Il-2, IL-5 and IL-10 are depicted in Supplementary Fig. S7.

IFNγ is expected to induce a high increase in the levels of pSTAT1 in monocytes and somewhat lower levels in T cells[53]. Indeed, a strong pSTAT1 increase was uniformly detected in CD14+CD33+ monocytes and in 37.5% of CD4+ T cells in all uVIN patients and healthy controls. In addition, IFNγ induced increased levels of pSTAT5 in CD14+CD33+ monocytes in approximately half of patients and control subjects (Fig. 4 and Supplementary Fig. S7). IL-2 hardly showed an effect on STAT phosphorylation, except for the upregulation of pSTAT5 in 3 individuals (2 patients and a healthy donor). IL-4 is known to mediate its signals via pSTAT6 [56,57]. Indeed IL-4 stimulation resulted in a strong increase of pSTAT6 in both myeloid cells and T cells of uVIN patients as well as healthy controls (Fig. 4 and Supplementary Fig. S7). IL-5 is known for pSTAT5 induction[57], but this was not observed. IL-6 is both a pro-inflammatory as anti-inflammatory cytokine, which is well known for its activation of STAT3. Whereas IL-6 upregulated pSTAT3 in T cells, predominantly in the CD4+ T cell population in line with IL-6R

expression[58], it did not influence pSTAT3 levels in myeloid cells (Fig. 4 and Supplementary Fig. S7). Furthermore, IL-6 increased the levels of pSTAT1 and pSTAT5 levels in T cells, albeit that pSTAT5 upregulation was more infrequently observed. The upregulation of pSTAT1 was more pronounced in CD4+ than CD8+ T cells (Fig. 4 and Supplementary Fig. S7). IL-7 is known to induce pSTAT5[57]. Indeed CD4+ and CD8+ T cells but not myeloid cells expressed pSTAT5 (Fig. 4 and Supplementary Fig. S7). IL-10 is known to induce pSTAT3[57]. Approximately half of the uVIN patients and controls displayed increased levels of pSTAT3 in CD4+ and CD8+ T cells whereas in 53% of patients and 75% of controls pSTAT3 was increased in the CD14+CD33+ monocyte population upon IL-10 stimulation (Fig. 4 and Supplementary Fig. S7). Overall, we observed no strong differences in the activation or expression of pSTAT to cytokine stimulation in the immune cells between healthy controls and uVIN patients.

STAT phosphorylation upon stimulation with immune modulators that are used to enhance the efficacy of immunotherapy

IFNα and GM-CSF are used in clinical trials as immune modulators to boost and polarize the Th1/CTL response to therapeutic vaccination and in some cases as monotherapy[59-61]. IFNα is a well-known potent inducer of various pSTATs and it is known to have a close synergic function with IFNγ[53,62,63]. Indeed all types of tested immune cells responded with increased levels of pSTAT1. In T cells the levels of pSTAT5 increased, which was most pronounced for CD4+ T cells. Interestingly, pSTAT5 upregulation was more often observed in the CD8+ T-cell population of healthy controls than that of uVIN patients (88% controls vs 54% patients (*p*=n.s.)). Moreover, whereas the majority of healthy controls (75%) displayed increased pSTAT5 levels in their CD14+CD33+ monocytes, this was only the case in 37.5% of the patients (*p*=0.07). The levels of pSTAT5 expressed by healthy control-derived CD14+CD33+ monocytes were increased as well (*p*= 0.05; Supplementary Fig. S7). IFNα also stimulated the increase of pSTAT3 and pSTAT6, especially in the CD4+ T cells and the CD14+CD33+ monocytes. The immune modulator GM-CSF only induced an increase in pSTAT5 levels in CD14+CD33+ monocytes and in contrast to IFNα this was more often observed in CD14+CD33+ monocytes of uVIN patients (83%) than healthy controls (38%). Also the absolute levels of pSTAT5 were higher in these cells after stimulation with GM-CSF (p=0.018; Fig. 4, Fig. 5 and Supplementary Fig. S7). Comparison of the pSTAT responses to these two immune modulators between uVIN patients and healthy controls revealed differences only in the response of CD14+CD33+ monocytes. Healthy control-derived CD14+CD33+ monocytes displayed a stronger response on the basis of the overall number of pSTATs that were increased upon stimulation with IFNα (Fig. 6). In conclusion, comparison of the fold-increase of pSTATs showed that IFNα induced a stronger increase of pSTAT5 in CD14+CD33+ monocytes of healthy subjects while GM-CSF triggered a stronger upregulation of pSTAT5 in these particular monocytes of uVIN patients (Fig.5).

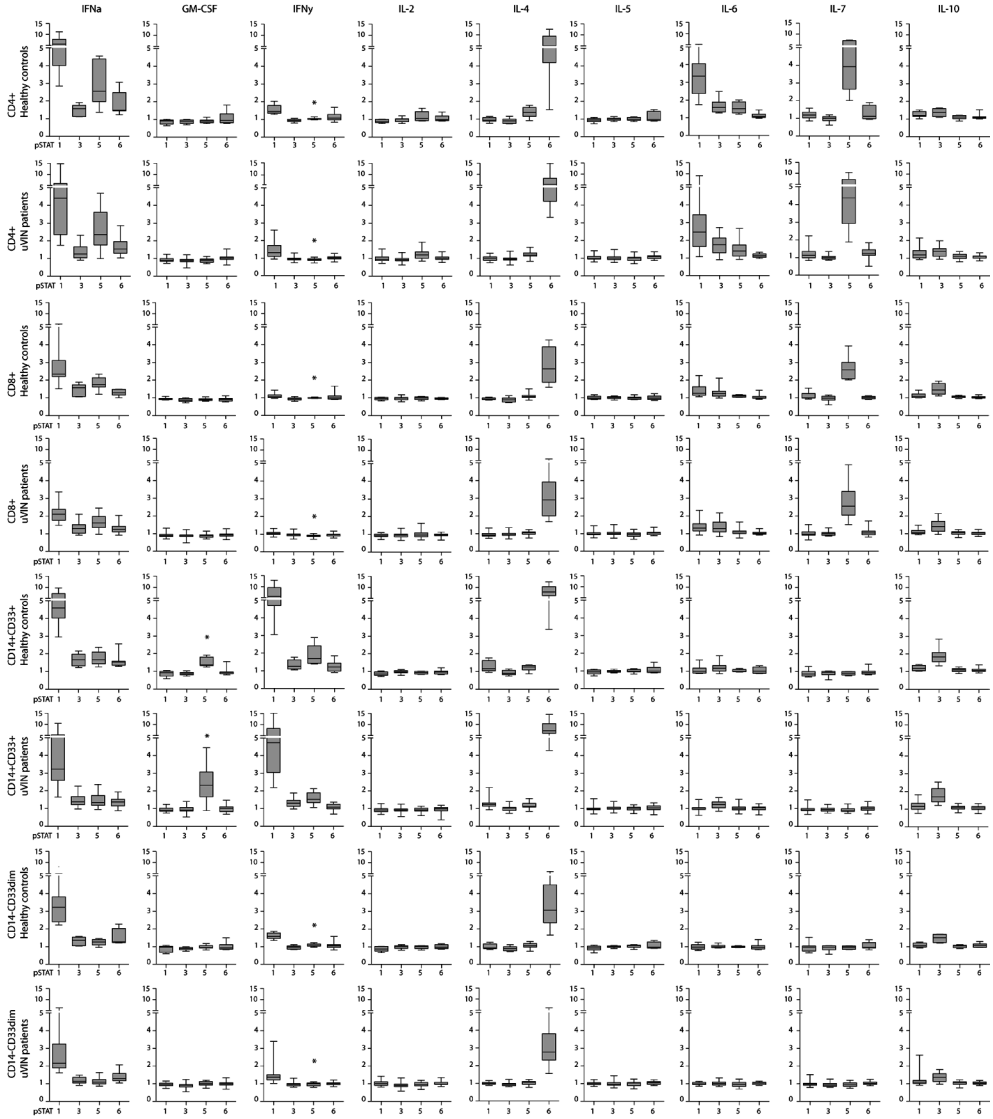


Figure 4 - van Esch et al.

Figure 4: **Boxplots of pSTAT induction upon stimulation with various cytokines or immune modulators in patients with uVIN and healthy donors**

The fold increase of different pSTATs upon cytokine stimulation in all immune cell populations are depicted for uVIN patients and healthy controls. Significant differences by comparison of absolute fold changes of pSTAT in uVIN patients and healthy donors by use of the non-parametric Mann-Whitney U test are indicated by *.

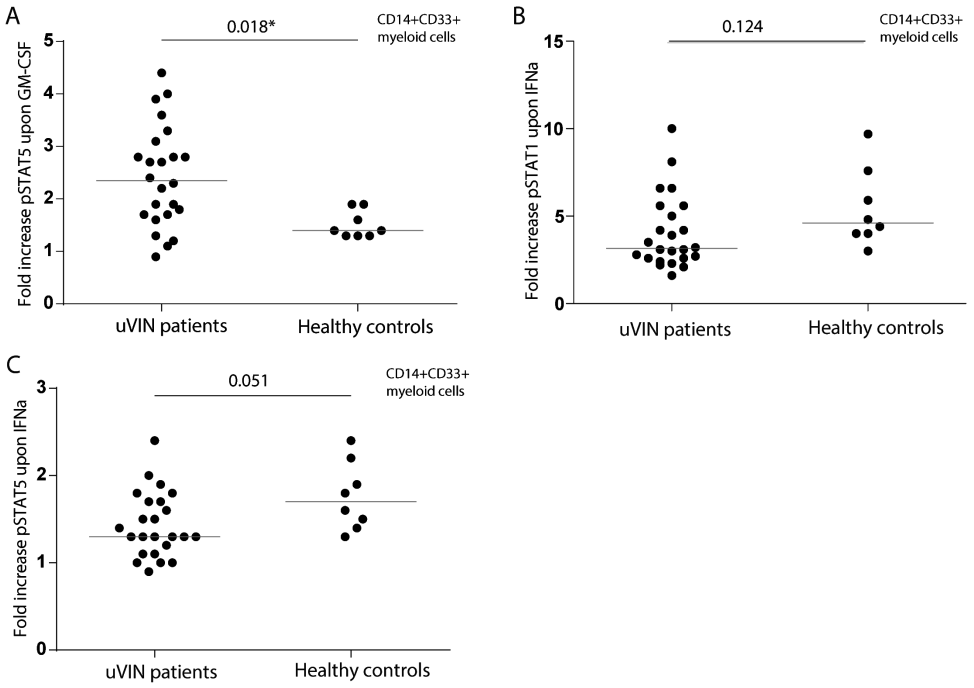


Figure 5: Phosphorylation of pSTAT5 after stimulation with GM-CSF and pSTAT1 and pSTAT5 upon IFNα in CD14+CD33+ monocytes in uVIN patients and healthy donors

The induction of pSTAT1 and pSTAT5 upon stimulation with immunomodulatory agents GM-CSF and IFNα. In figure A, a significant increase in pSTAT5 in CD14+CD33+ myeloid cells upon GM-CSF in comparison to the fold increase in healthy controls is depicted. In figure B the pSTAT1 and in figure C the pSTAT5 induction in CD14+CD33+ myeloid cells upon IFNα is depicted and albeit not significant in uVIN patients the fold induction is lower compared to healthy controls.

STAT phosphorylation of pre-treatment samples of patients treated with HPV-16 SLP ISA 101 vaccination

We then compared the STAT phosphorylation after stimulation with these immune modulators in pre-treatment samples of 3 uVIN patients which responded by a peak in T-cell associated IFNγ production upon the first vaccination with HPV16-SLP ISA101 to those (n=5) that did not[34]. The CD14+CD33+ cells of uVIN patients with a peak IFNγ response displayed a more pronounced increase in pSTAT1 induction upon IFNα stimulation than the other patients. Stimulation with GM-CSF did result in an overt pSTAT5 induction and there were no differences observed between the two patient groups (Fig. 7).

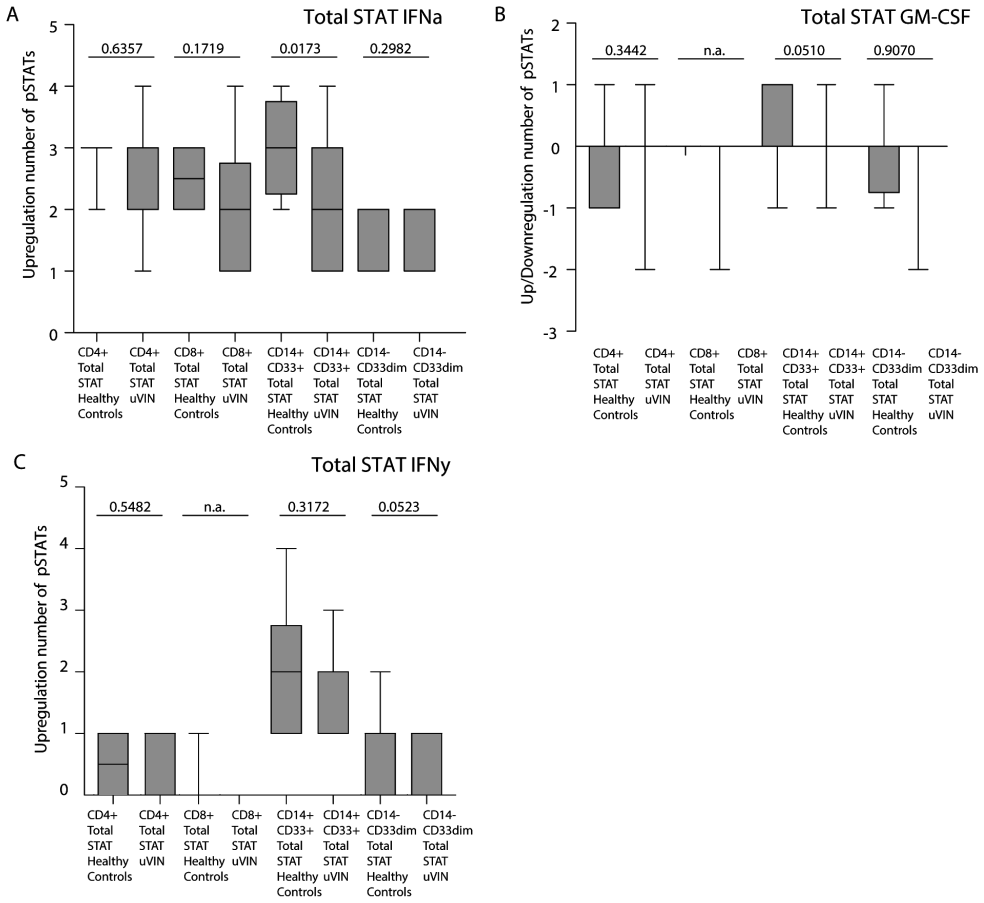


Figure 6: Total pSTAT upregulation in different immune cell types upon stimulation with IFNα, GM-CSF and IFNγ

Total pSTAT upregulation was calculated upon an upregulation or downregulation of one or more STATs upon stimulation with a specific cytokine. In case of upregulation +1 and in case of downregulation -1 was scored revealing a maximum score of 4 in case of pSTAT 1, 3, 5 and 6 upregulation and a minimum score of -4 for each patient. Subsequent analysis of the total pSTATs was performed by an unpaired T test in Graphpad Prism 5. In Figure A the total pSTAT induction upon IFNα is significantly higher in the CD14+CD33+ myeloid cell population in healthy controls. Differences in total pSTAT induction upon GM-CSF (Fig. B) and IFNγ (Fig. C) can be established albeit that IFNγ reveals a distinct upregulation of total pSTAT in CD14+CD33+ myeloid cells of both healthy controls as uVIN patients.

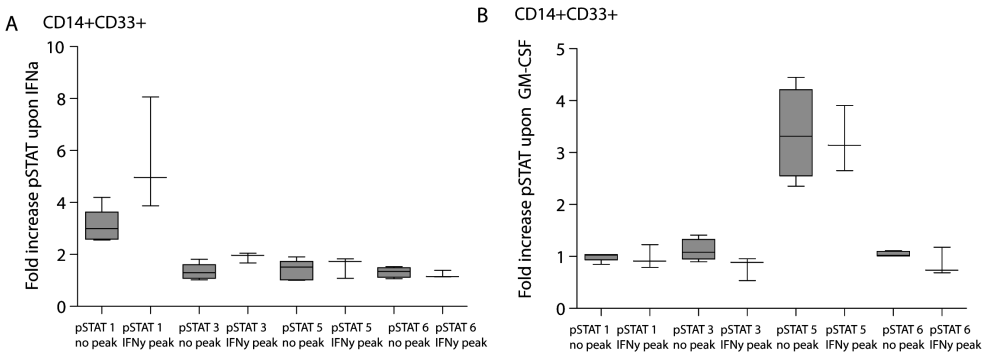


Figure 7: STAT phosphorylation upon stimulation with IFNα and GM-CSF in CD14+CD33+ myeloid cells of patients with and without an IFNγ peak response upon HPV 16 SLP ISA 101 vaccination

In patients where a distinct peak of IFNγ production upon the first vaccination with HPV 16 SLP ISA 101 is found, IFNα reveals a higher fold increase in pSTAT1 of CD14+CD33+ monocytes albeit not significant (A). Stimulation with GM-CS F does result in an overt increase in pSTAT5 in both peak responders and non-responders (B).

Discussion

In this study we analysed the type and function of circulating immune cells by an extensive phenotypic analysis and a novel technology that allows simultaneous measurements of changes in phosphorylation of protein levels of STATs after stimulation[29,53]. Our study revealed no differences in the potential of T cells to proliferate or the capacity of antigen presenting cells to stimulate T-cell responses between uVIN patients and healthy subjects. However, the frequencies of immature DCs or differentiating macrophages and activated monocytes/macrophages are lower in uVIN patients compared to healthy controls. Furthermore, patients with recurrent uVIN lesions display lower frequencies of DCs and higher frequencies of type 2 monocytes/macrophages than patients without recurrent disease. This alteration was also observed at the functional level since CD14+CD33+ monocytes/macrophages of uVIN patients displayed decreased levels of pSTAT5 upon IFNα stimulation but increased pSTAT5 levels when stimulated with GM-CSF when compared to healthy controls. Our data on the response of CD14+CD33+ cells from vaccinated patients to IFNα suggest that this may also be reflected in their capacity to respond to vaccination, but clearly more patients need to be analysed. Importantly, the relation between increased numbers of circulating type 2 monocytes/macrophages in the blood and a worse RFS is in concordance with our previous observation that M2 macrophages are abundantly infiltrating uVIN lesions and associated with recurrent disease as well[16].

A higher frequency of circulating CD4+CD94+(NKG2a+) T cells was found to be associated with the absence of recurrences. If cells express NKG2a than they can also be considered

positive for CD94 since CD94 forms a complex with the NKG2 family[54,55]. In our previous study on the expression of co-inhibitory molecules in the uVIN microenvironment we studied NKG2a expression in the context of CD3+ T cells and showed that its expression was associated with a better RFS[17]. Unfortunately, we did not distinguish between CD4+ and CD8+ T cells expressing NKG2a+ in that study. Our current data suggest that specifically the expression of NKG2a on CD4+ T cells is associated with better clinical outcome. New studies are required to analyse whether the NKG2a+ cells in the uVIN microenvironment are CD4+ or CD8+ and which of the two populations is associated with better RFS. It will be extremely important to know if the analysis of both the type 2 monocytes/macrophages and the CD4+CD94+NKG2A+ T cells in a blood sample could substitute for measurements of these cell types in the tissue as it may allow a relatively simple blood analysis to become a biomarker that is associated with local events and prognosis. Therefore, a new study is warranted in which both blood and tissue of the same patient population is studied to validate our current observations.

In contrast to the similarities between local and systemic NKG2a expression we did not observed such a relationship with respect to the presence of CD8+TIM3+ cells. While in the uVIN microenvironment the presence of CD8+TIM3+ cells correlated with pro-inflammatory T-cell responses, characterised by Tbet and Galectin-9 expression, and was found to be a favourable prognostic factor in recurrence free survival, the frequencies of circulating TIM3+ T-cell subpopulations were not directly associated. However, a relative higher number of circulating CD8+TIM3+ T cells over CD8+TIM3- T cells was found to be associated with worse clinical outcome. These opposite data suggest that either there is no reflection of the changes in the local environment by those measured in the circulation, or that the context in which CD8+TIM3+ cells are analysed is more important as it is expressed both during T-cell activation and exhaustion[64]. Future research should establish the exact role and function of these cells in uVIN patients.

Of interest for future immunotherapeutic studies are our data on the stimulation of immune cells with the immune modulators GM-CSF and IFNα. They are both involved in DC activation and used as adjuvants for different therapeutic vaccines aiming to enhance a Th1 polarized T-cell response[59-61]. The use of mainly IFNα has been proven beneficial to vaccine-induced T-cell responses, whereas GM-CSF resulted in different outcomes[60,61]. IFNα can be seen as a potent immune modulator revealing mainly pro-inflammatory associated STAT1 phosphorylation induction in both healthy controls, premalignant and cancer patients and it is known to have a close synergic effect with IFNγ[62,63]. A type I IFN response is essential for DC maturation and induction of CD4+ Th1 immunity[65]. The higher total induction of pSTAT in healthy subjects compared to uVIN patients may reflect the reduced potency of the precursor cells of patients' DCs and macrophages (CD14+CD33+) to respond to immune modulators. Of particular interest was our observation of opposite pSTAT5 responses in

CD14+CD33+ myeloid cells from uVIN patients and healthy controls when stimulated with IFNα or GM-CSF. Of note, IL-2 is known to induce pSTAT5 in DCs required for their functional maturation[66]. However, an IL-2 induced increase of pSTAT5 was not observed, potentially because the stimulation was for 15 minutes whereas in another study pSTAT5 was shown to be detectable in DCs only after 30 minutes of stimulation[66]. GM-CSF is known to generate granulocyte and macrophage populations from myeloid precursor cells and is of importance in survival, proliferation and differentiation of monocytes and macrophages by activation of JAK2 and STAT5 [24]. The CD14+CD33+ myeloid cells of uVIN patients responded to GM-CSF with higher levels of pSTAT5 but it is unclear how this may impact immunity. In patients with a cytomegalovirus (CMV) infection monocyte-derived DCs lack the ability to secrete IL-12 and to induce Th1 cell activation and this is associated with an impaired response of these monocytes to induce pSTAT5 upon GM-CSF stimulation[67], suggesting that GM-CSF induced pSTAT5 is required for a good immune response. Interestingly, leukaemia patients display an increased pSTAT5 response to GM-CSF stimulation when compared to healthy donors. Similar to what we observed in uVIN patients. This response in leukaemia patients was classified as hypersensitivity and correlated with high risk disease, higher peripheral leukocyte and monocyte count and altered signalling in the Ras pathway[68]. Signalling via STAT5 may result in pro-inflammatory responses but it also essential for the induction of peripheral tolerance[27]. pSTAT5 plays an essential role in Th2 differentiation since pSTAT5 expression results in IL-4 expression by cells lacking pSTAT6 and pSTAT5 induces IL-4 in T cells cultured under Th1 polarizing conditions. Moreover pSTAT5 is involved in regulatory FoxP3+ T-cell (Treg) development, induced by IL-2 and pSTAT5, by binding to the FoxP3 promotor[56]. The role of an altered pSTAT5 expression in myeloid cells of uVIN patients can thus be interpreted along two lines; a tolerance inducing role as well as a pro-inflammatory role when pSTAT5 is expressed in combination with pSTAT1. Interestingly, the increased expression of pSTAT1 upon stimulation with IFNα in uVIN patients with a peak IFNγ response upon vaccination fits this latter role of pSTAT5 well. Unfortunately, it remains difficult to unravel the activated interactive pathways *in vivo* that may explain the difference in the patients capacity to respond to immunotherapy by *in vitro* experiments of phosphorylation of STATs upon single cytokine stimulations due to heterodimerization of simultaneous STATs and crosstalk among the JAK-STAT pathways occur[32,57,62]. For example there is cross talk between type I and type II IFNs as pre-treatment with IFNγ sensitizes cells to IFNα and pre-treatment with IFNγ increases the level of pSTAT1 which can enhance the subsequent IFNγ response during macrophage activation[62,63]. Furthermore, IFNs inhibit IL-4 induced STAT6 expression in human monocytes and IL-10 can inhibit the activity of pro-inflammatory cytokines such as IFNα [62,69,70]. Potentially, combinations of cytokines to stimulate immune cells may better mimic the *in vivo* reaction upon secretion of multiple cytokines in response to infections or therapy.

In conclusion, our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients and that the measurement of circulating type 2 monocytes is related to recurrent disease. This relationship needs to be confirmed as it may serve as a blood biomarker that reflects the local microenvironment and is important for prognosis.

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The authors disclose no potential conflicts of interest.

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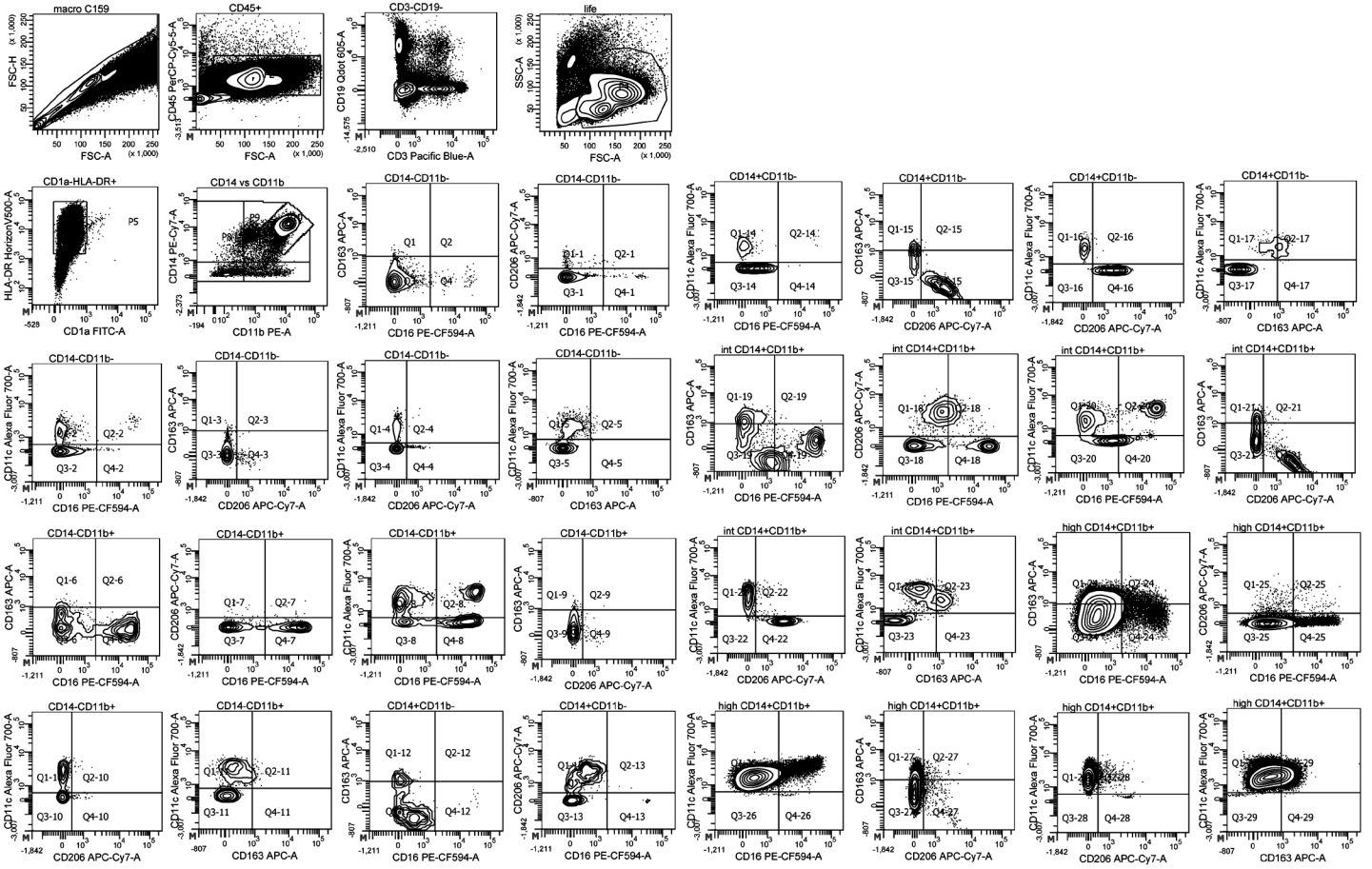
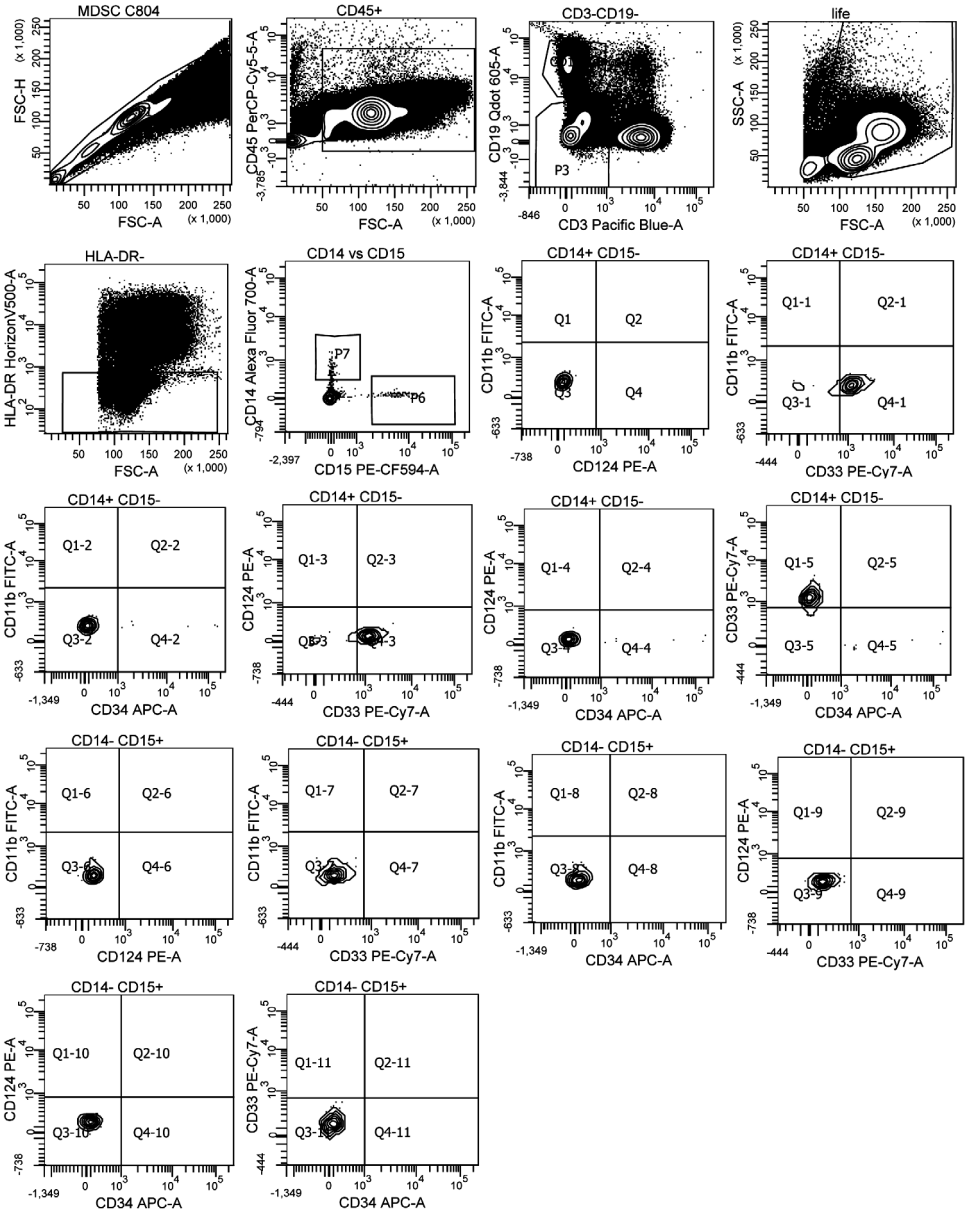


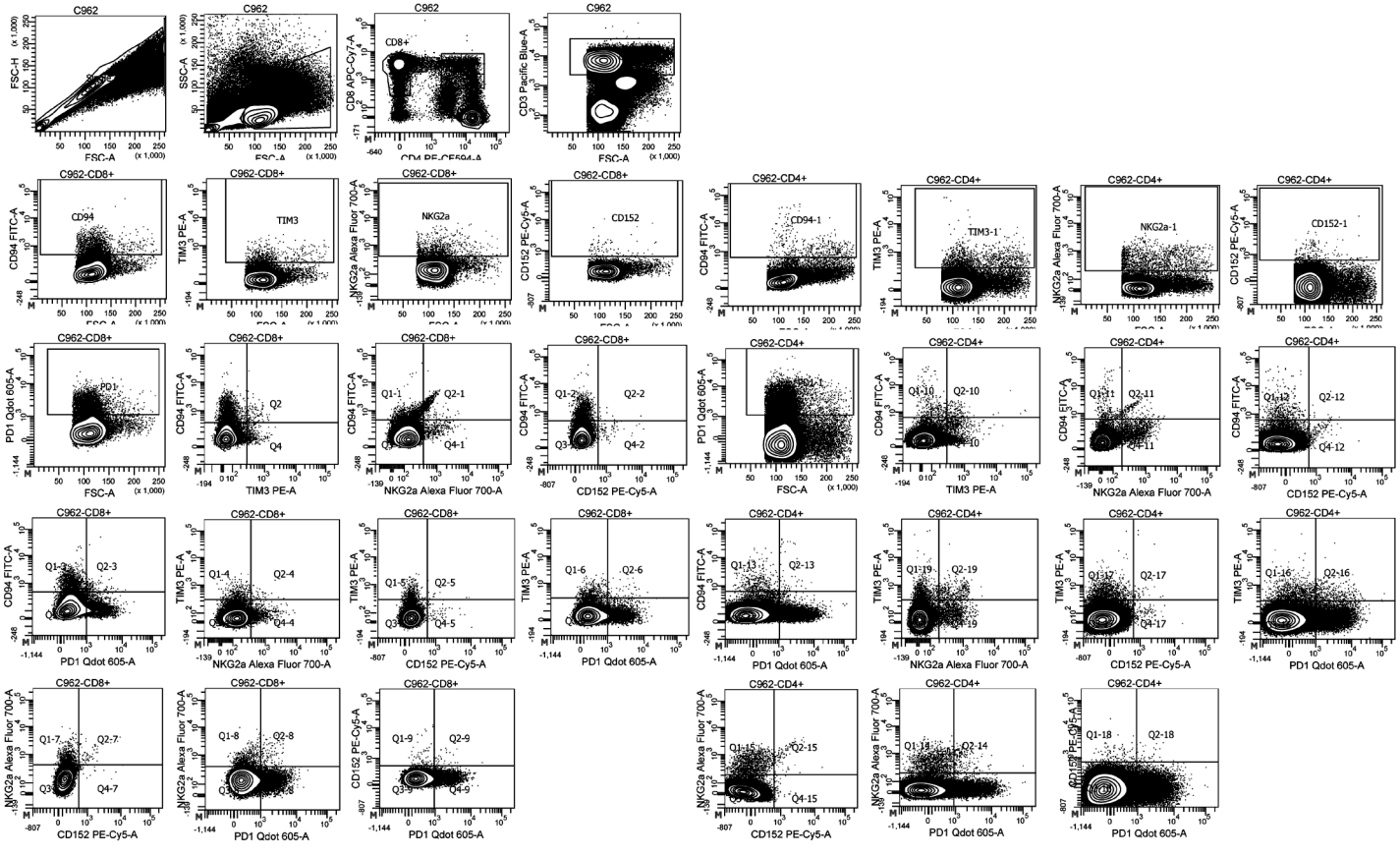
Supplementary Figure S1

Gating strategy example of monocytes/macrophages



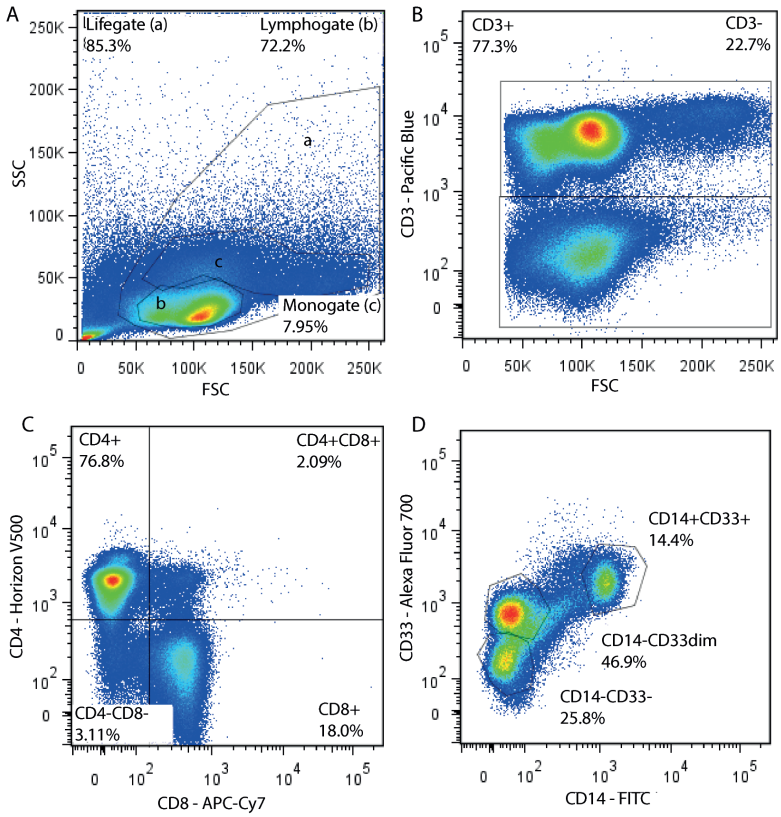
Supplementary Figure S2

Gating strategy example of myeloid derived suppressor cells



Supplementary Figure S3

Gating strategy example of co-inhibitionary molecules



Supplementary Figure S4

Gating strategy example of phosphorylation of STAT

PHA

Patient	SI	SI	SI	SI	SI
Buffy 9	689,0	867,5	495,3	443,6	655,4
Buffy 11	368,5	482,4	304,5	361,2	418,8
C883	539,3				
C889	455,4				
C977	201,6				
C988	423,3				
C612		237,1			
C887		400,9			
C296		491,3			
C333		170,1			
C80			1105,4		
C962			608,1		
C804			196,0		
C159				344,5	
C412				802,6	
C789				603,4	
C313				409,2	
HD ML					980,7
HD PV					245,4
HD SS					240,3
HD SM					768,3

HD = Healthy control
SI = Stimulation Index: calculated by the ratio of the mean of PHA stimulated PBMCs devided by the mean of the unstimulated PBMCs
SI >3 is considered positive (of mean plus 3 times the standarad deviation of unstimulated PBMCs)

MLR

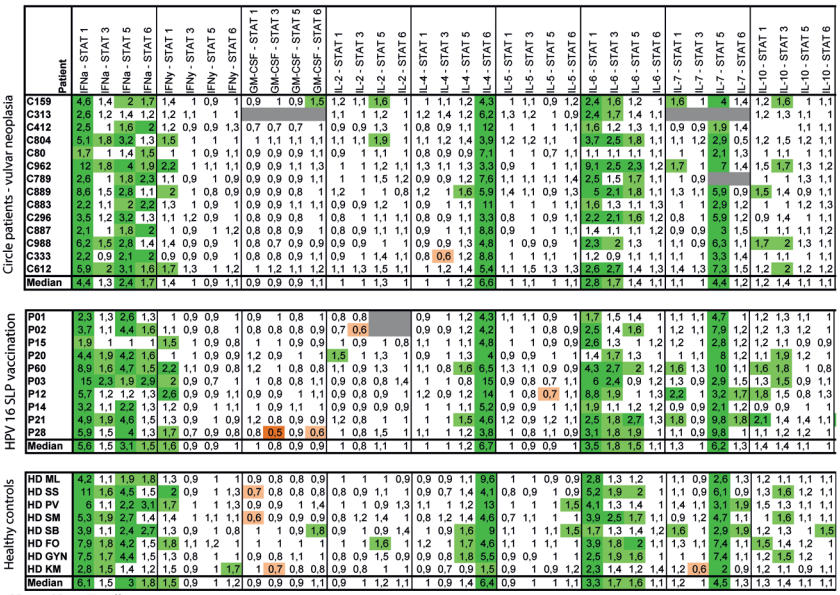
Patient	Buffy	SI	Circle	Buffy	SI	Median SI
C883	B9	44,2	C883	B11	48,4	46,3
C889	B9	33,9	C889	B11	6,6	20,2
C977	B9	49,3	C977	B11	15,6	32,5
C988	B9	8,4	C988	B11	14,3	11,3
C612	B9	23,1	C612	B11	23,9	23,5
C887	B9	23,5	C887	B11	10,2	16,9
C296	B9	65,2	C296	B11	24,8	45,0
C333	B9	3,5	C333	B11	45,2	24,4
C80	B9	2,4*	C80	B11	10,7	10,7
C962	B9	13,1	C962	B11	17,1	15,1
C804	B9	15,3	C804	B11	23,3	19,3
C159	B9	3,8	C159	B11	19,8	11,8
C412	B9	6,4	C412	B11	5,7	6,0
C789	B9	15,2	C789	B11	5,6	10,4
C313	B9	12,6	C313	B11	3,3	7,9
HD ML	B9	17,7	HD ML	B11	13,2	15,5
HD PV	B9	28,7	HD PV	B11	21,9	25,3
HD SS	B9	23,2	HD SS	B11	18,6	20,9
HD SM	B9	5,8	HD SM	B11	8,9	7,3

HD = Healthy control
* HLA typing B9 = HLA-DRB1 401 and C80 = HLA-DRB1 401
SI = Stimulation Index: calculated by the ratio of the mean of irradiated PBMCs co-cultured with healthy donor PBMCs divided by the mean of the healthy donor PBMCs alone
SI >3 is considered positve (of the mean plus 3 times the standard deviationof healthy donors alone)

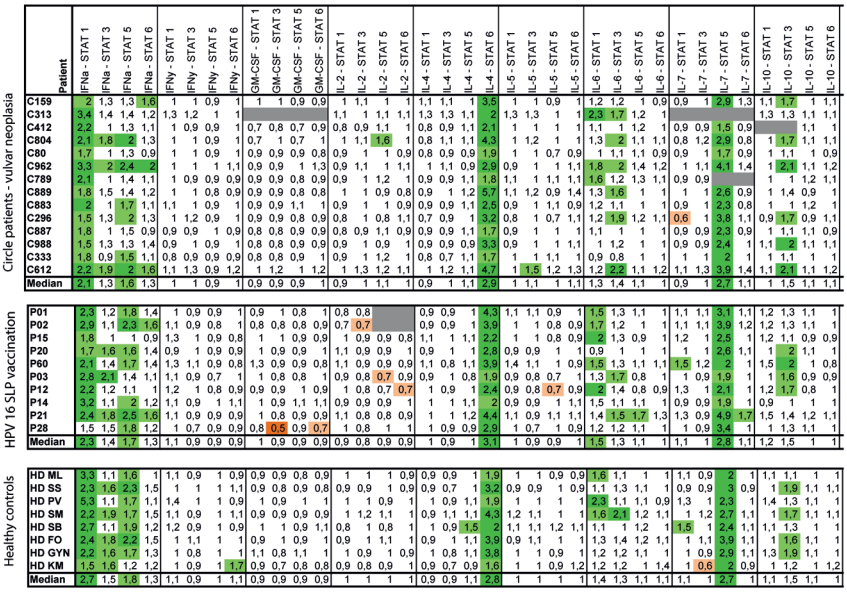
Supplementary Figure S5

Results of proliferation (PHA) and antigen presenting capacity (MLR) of PBMCs of uVIN patients. SI: stimulation index

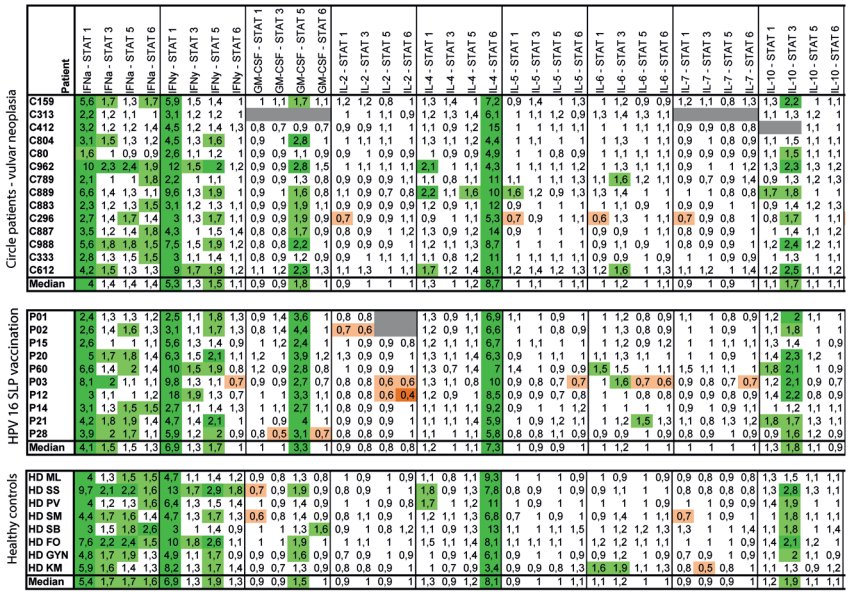
A: CD3+ CD4+ T cells



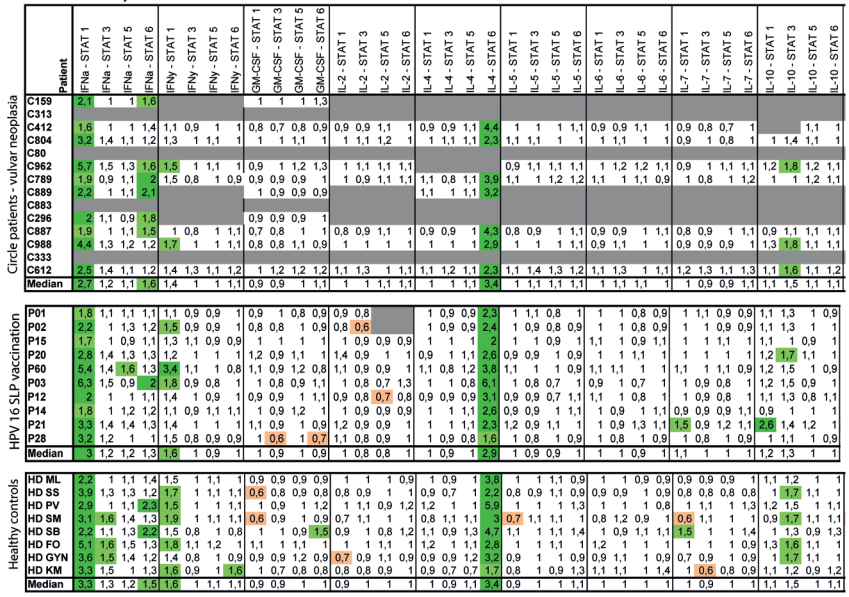
B: CD3+ CD8+ T cells



Supplementary Figure S6

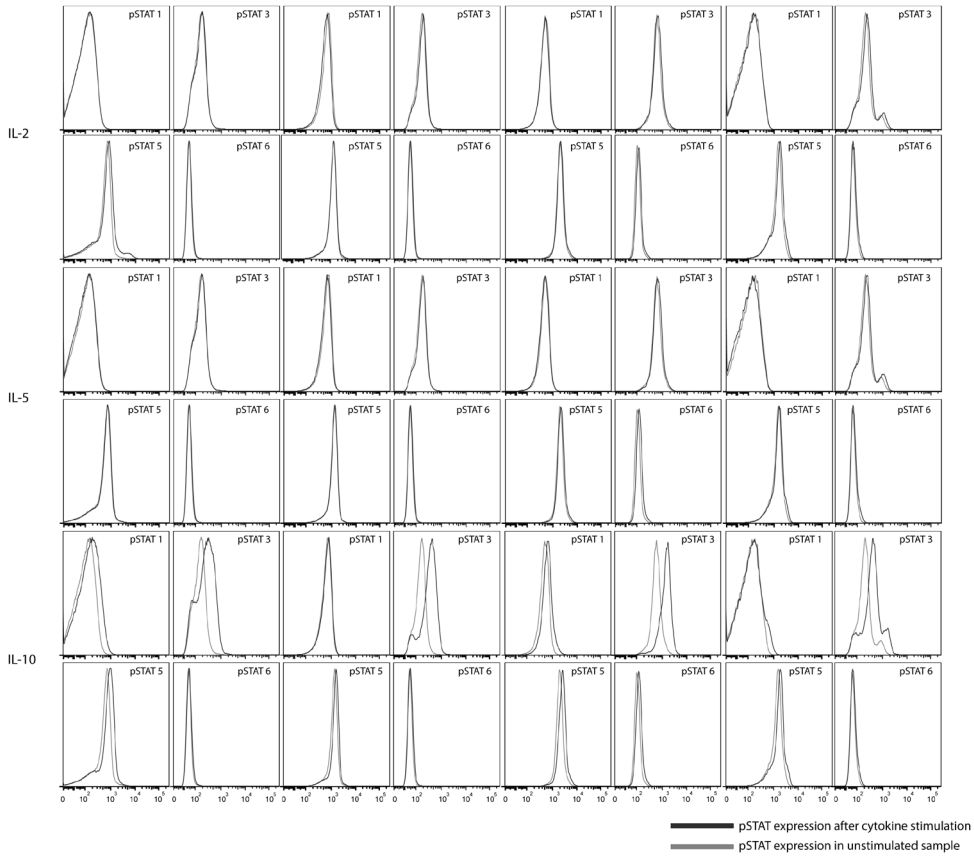


D: CD14- CD33dim myeloid cells



Supplementary Figure S6 - Continued

Overview of pSTAT upregulation in CD4+, CD8+, CD14+CD33+ and CD14-CD33dim immune cells upon stimulation with various cytokines or immune modulators in patients with uVIN and healthy controls



Supplementary Figure S7

Gating strategy example of phosphorylation of STAT for IL-2, IL-5 and IL-10

Supplementary Table S1 - Systemic expression of myeloid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.

*Non-parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant and in **bold** the p-values are marked where the cells numbers are >0.05%.

Myeloid cell type	Controls (median % of CD45+) N=8	uVIN (median % of CD45+) N=14	p =	Recurrent uVIN (median % of CD45+) N=5	Non Recurrent uVIN (median % of CD45+) N=7	p =	RFS
CD14- CD11b-	1.16 (0.58-2.41)	0.85 (0.30-1.72)	**0.029**	0.59 (0.30-1.17)	0.89 (0.42-1.72)	n.s.	0.295
CD163+ CD16-	0.03 (0.01-0.11)	0.004 (0.00-0.03)	0.005	0.005 (0.00-0.01)	0.003 (0.00-0.03)	n.s.	n.t.
CD163+ CD16+	0.0002 (0.00-0.00)	0.0001 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.000 (0.00-0.00)	n.s.	n.t.
CD163- CD16+	0.27 (0.04-1.51)	0.19 (0.02-1.02)	n.s.	0.05 (0.02-0.72)	0.19 (0.06-1.02)	n.s.	n.t.
CD206+ CD16-	0.007 (0.00-0.02)	0.01 (0.01-0.04)	0.042	0.01 (0.01-0.02)	0.02 (0.01-0.03)	n.s.	n.t.
CD206+ CD16+	0.001 (0.00-0.00)	0.003 (0.00-0.02)	n.s.	0.001 (0.00-0.002)	0.004 (0.001-0.01)	0.030	0.021
CD206- CD16+	0.27 (0.04-1.51)	0.19 (0.02-1.02)	n.s.	0.05 (0.02-0.71)	0.19 (0.06-1.02)	n.s.	n.t.
CD11c+ CD16-	0.33 (0.07-0.64)	0.21 (0.07-0.39)	n.s.	0.12 (0.10-0.20)	0.23 (0.07-0.31)	n.s.	n.t.
CD11c+ CD16+	0.27 (0.04-1.51)	0.78 (0.01-1.02)	n.s.	0.04 (0.01-0.71)	0.18 (0.05-1.02)	n.s.	n.t.
CD11c- CD16+	0.003 (0.00-0.03)	0.007 (0.00-0.02)	n.s.	0.003 (0.00-0.02)	0.007 (0.00-0.02)	n.s.	n.t.
CD163+ CD206-	0.03 (0.01-0.11)	0.005 (0.00-0.03)	0.006	0.006 (0.00-0.01)	0.004 (0.00-0.03)	n.s.	n.t.
CD163+ CD206+	0.0003 (0.00-0.00)	0.00 (0.00-0.00)	0.010	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.008 (0.00-0.02)	0.016 (0.01-0.06)	0.020	0.01 (0.0-0.03)	0.03 (0.01-0.03)	n.s.	n.t.
CD11c+ CD206-	0.64 (0.38-1.89)	0.37 (0.13-1.31)	n.s.	0.24 (0.13-0.91)	0.40 (0.17-1.31)	n.s.	n.t.
CD11c+ CD206+	0.004 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	0.001 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	n.t.
CD11c- CD206+	0.006 (0.00-0.01)	0.01 (0.00-0.05)	0.002	0.01 (0.00-0.02)	0.02 (0.01-0.03)	n.s.	n.t.
CD11c+ CD163-	0.60 (0.34-1.87)	0.34 (0.12-1.29)	n.s.	0.20 (0.12-0.89)	0.39 (0.16-1.29)	n.s.	n.t.
CD11c+ CD163+	0.03 (0.01-0.08)	0.005 (0.00-0.03)	0.004	0.006 (0.00-0.01)	0.005 (0.00-0.03)	n.s.	n.t.
CD11c- CD163+	0.0002 (0.00-0.03)	0.0002 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.	n.t.
CD14- CD11b+	0.61 (0.44-1.05)	0.42 (0.15-1.12)	n.s.	0.83 (0.15-1.12)	0.35 (0.24-0.90)	n.s.	0.255
CD163+ CD16-	0.006 (0.00-0.12)	0.005 (0.00-0.08)	n.s.	0.004 (0.00-0.01)	0.006 (0.00-0.02)	n.s.	n.t.
CD163+ CD16+	0.002 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	0.004 (0.00-0.01)	0.001 (0.00-0.01)	n.s.	n.t.
CD163- CD16+	0.26 (0.01-0.73)	0.20 (0.07-1.02)	n.s.	0.64 (0.07-1.02)	0.18 (0.13-0.78)	n.s.	n.t.
CD206+ CD16-	0.18 (0.00-0.42)	0.04 (0.00-0.19)	**0.005**	0.09 (0.06-0.18)	0.003 (0.00-0.19)	**0.048**	**0.011**
CD206+ CD16+	0.22 (0.00-0.66)	0.04 (0.00-1.00)	n.s.	0.34 (0.06-1.00)	0.006 (0.00-0.14)	**0.010**	**0.011**
CD206- CD16+	0.03 (0.00-0.24)	0.17 (0.01-0.77)	n.s.	0.09 (0.01-0.30)	0.17 (0.01-0.77)	n.s.	n.t.

CD11c+ CD16-	0.07 (0.02-0.33)	0.04 (0.00-0.24)	n.s.	0.005 (0.00-0.03)	0.07 (0.04-0.17)	0.003	0.011
CD11c+ CD16+	0.002 (0.00-0.23)	0.043 (0.00-0.77)	n.s.	0.002 (0.00-0.00)	0.15 (0.00-0.77)	**0.018**	**0.021**
CD11c- CD16+	0.008 (0.00-0.01)	0.01 (0.00-0.09)	n.s.	0.009 (0.00-0.01)	0.02 (0.00-0.05)	0.048	0.030
CD163+ CD206-	0.43 (0.12-0.90)	0.10 (0.00-1.08)	**0.016**	0.43 (0.12-1.08)	0.008 (0.00-0.33)	**0.010**	0.084
CD163+ CD206+	0.005 (0.00-0.01)	0.0007 (0.00-0.01)	0.001	0.003 (0.00-0.01)	0.000 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.003 (0.00-0.01)	0.009 (0.00-0.04)	0.003	0.006 (0.00-0.01)	0.01 (0.00-0.01)	n.s.	n.t.
CD11c+ CD206-	0.43 (0.15-0.87)	0.29 (0.10-1.07)	n.s.	0.40 (0.10-1.07)	0.25 (0.18-0.86)	n.s.	n.t.
CD11c+ CD206+	0.05 (0.01-0.14)	0.006 (0.00-0.04)	0.003	0.005 (0.00-0.03)	0.006 (0.00-0.04)	n.s.	n.t.
CD11c- CD206+	0.001 (0.00-0.06)	0.004 (0.00-0.03)	n.s.	0.00 (0.00-0.00)	0.005 (0.00-0.01)	0.018	0.218
CD11c+ CD163-	0.08 (0.02-0.43)	0.11 (0.00-0.84)	n.s.	0.005 (0.00-0.02)	0.22 (0.04-0.84)	**0.003**	**0.011**
CD11c+ CD163+	0.001 (0.00-0.13)	0.004 (0.00-0.08)	n.s.	0.00 (0.00-0.02)	0.008 (0.00-0.02)	n.s.	n.t.
CD11c- CD163+	0.25 (0.00-0.73)	0.03 (0.00-1.02)	n.s.	0.64 (0.07-1.02)	0.00 (0.00-0.16)	**0.010**	**0.011**
CD14+ CD11b-	0.07 (0.02-0.17)	0.08 (0.02-0.20)	n.s.	0.09 (0.02-0.17)	0.07 (0.03-0.20)	n.s.	0.663
CD163+ CD16-	0.003 (0.00-0.01)	0.0082 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD163+ CD16+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.048	n.t.
CD163- CD16+	0.02 (0.00-0.08)	0.018 (0.00-0.06)	n.s.	0.02 (0.00-0.02)	0.02 (0.01-0.06)	n.s.	n.t.
CD206+ CD16-	0.01 (0.00-0.15)	0.02 (0.01-0.12)	n.s.	0.03 (0.01-0.12)	0.02 (0.01-0.03)	n.s.	n.t.
CD206+ CD16+	0.003 (0.00-0.01)	0.003 (0.00-0.01)	n.s.	0.002 (0.00-0.00)	0.005 (0.00-0.01)	n.s.	n.t.
CD206- CD16+	0.009 (0.00-0.08)	0.012 (0.0-0.05)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.05)	n.s.	n.t.
CD11c+ CD16-	0.007 (0.00-0.03)	0.03 (0.00-0.11)	0.029	0.04 (0.00-0.05)	0.03 (0.01-0.11)	n.s.	n.t.
CD11c+ CD16+	0.02 (0.00-0.08)	0.02 (0.00-0.05)	n.s.	0.01 (0.00-0.02)	0.02 (0.00-0.05)	n.s.	n.t.
CD11c- CD16+	0.0004 (0.00-0.00)	0.001 (0.00-0.01)	0.042	0.000 (0.00-0.00)	0.003 (0.00-0.07)	0.030	0.142
CD163+ CD206-	0.002 (0.00-0.01)	0.006 (0.00-0.03)	n.s.	0.009 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD163+ CD206+	0.0002 (0.00-0.00)	0.0004 (0.00-0.00)	n.s.	0.001 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.02 (0.01-0.16)	0.03 (0.01-0.12	n.s.	0.03 (0.01-0.12)	0.03 (0.01-0.04)	n.s.	n.t.
CD11c+ CD206-	0.02 (0.01-0.08)	0.04 (0.00-0.16)	n.s.	0.04 (0.00-0.05)	0.04 (0.01-0.16)	n.s.	n.t.
CD11c+ CD206+	0.006 (0.00-0.01)	0.005 (0.00-0.01)	n.s.	0.002 (0.00-0.01)	0.006 (0.00-0.01)	n.s.	n.t.
CD11c- CD206+	0.01 (0.00-0.14)	0.02 (0.00-0.11)	n.s.	0.02 (0.01-0.11)	0.02 (0.00-0.03)	n.s.	n.t.
CD11c+ CD163-	0.02 (0.00-0.08)	0.036 (0.00-0.14)	n.s.	0.02 (0.00-0.04)	0.03 (0.02-0.14)	n.s.	n.t.
CD11c+ CD163+	0.002 (0.00-0.01)	0.007 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.02 (0.00-0.03)	n.s.	n.t.
CD11c- CD163+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	n.t.

Intermediate CD14+ CD11b+	0.54 (0.18-0.84)	0.61 (0.27-0.78)	n.s.	0.55 (0.27-0.78)	0.69 (0.40-0.78)	n.s.	0.829
CD163+ CD16-	0.04 (0.02-0.14)	0.035 (0.00-0.19)	n.s.	0.04 (0.00-0.19)	0.03 (0.02-0.04)	n.s.	n.t.
CD163+ CD16+	0.02 (0.00-0.08)	0.03 (0.00-0.08)	n.s.	0.04 (0.01-0.08)	0.03 (0.00-0.07)	n.s.	n.t.
CD163- CD16+	0.20 (0.00-0.50)	0.26 (0.09-0.58)	n.s.	0.30 (0.09-0.58)	0.21 (0.11-0.37)	n.s.	n.t.
CD206+ CD16-	0.09 (0.04-0.12)	0.07 (0.02-0.20)	n.s.	0.05 (0.02-0.08)	0.11 (0.04-0.20)	n.s.	n.t.
CD206+ CD16+	0.002 (0.00-0.01)	0.002 (0.00-0.04)	n.s.	0.002 (0.00-0.04)	0.002 (0.00-0.01)	n.s.	n.t.
CD206- CD16+	0.22 (0.00-0.58)	0.29 (0.11-0.62)	n.s.	0.32 (0.11-0.62)	0.21 (0.12-0.44)	n.s.	n.t.
CD11c+ CD16-	0.18 (0.05-0.26)	0.21 (0.10-0.36)	n.s.	0.15 (0.10-0.19)	0.29 (0.13-0.36)	n.s.	n.t.
CD11c+ CD16+	0.22 (0.00-0.55)	0.27 (0.10-0.58)	n.s.	0.29 (0.10-0.58)	0.21 (0.12-0.43)	n.s.	n.t.
CD11c- CD16+	0.004 (0.00-0.02)	0.008 (0.00-0.07)	n.s.	0.03 (0.00-0.07)	0.005 (0.00-0.02)	n.s.	n.t.
CD163+ CD206-	0.08 (0.04-0.12)	0.06 (0.02-0.21)	n.s.	0.06 (0.02-0.07)	0.10 (0.03-0.21)	n.s.	n.t.
CD163+ CD206+	0.008 (0.00-0.02)	0.004 (0.00-0.02)	n.s.	0.007 (0.00-0.02)	0.005 (0.00-0.02)	n.s.	n.t.
CD163- CD206+	0.09 (0.02 (0.164)	0.07 (0.03-0.26)	n.s.	0.08 (0.04-0.26)	0.06 (0.03-0.09)	n.s.	n.t.
CD11c+ CD206-	0.38 (0.13-0.68)	0.48 (0.17-0.68)	n.s.	0.42 (0.17-0.68)	0.55 (0.31-0.67)	n.s.	n.t.
CD11c+ CD206+	0.05 (0.01-0.11)	0.04 (0.01-0.10)	n.s.	0.03 (0.02-0.08)	0.04 (0.01-0.10)	n.s.	n.t.
CD11c- CD206+	0.04 (0.01-0.12)	0.03 (0.01-0.25)	n.s.	0.03 (0.01-0.25)	0.02 (0.01-0.04)	n.s.	n.t.
CD11c+ CD163-	0.31 (0.03-0.68)	0.40 (0.15-0.67)	n.s.	0.33 (0.15-0.67)	0.38 (0.26-0.56)	n.s.	n.t.
CD11c+ CD163+	0.10 (0.05-0.12)	0.07 (0.02-0.22)	n.s.	0.06 (0.03-0.08)	0.12 (0.04-0.22)	n.s.	n.t.
CD11c- CD163+	0.0001 (0.00-0.01)	0.001 (0.00-0.01)	n.s.	0.001 (0.00-0.00)	0.002 (0.00-0.01)	n.s.	n.t.
High CD14+ CD11b+	11.80 (2.33-18.46)	14.95 (3.96-24.65)	n.s.	9.45 (3.96-24.65)	16.52 (14.76-20.54)	n.s.	0.100
CD163+ CD16-	2.98 (1.15-5.17)	1.49 (0.14-6.76)	n.s.	0.89 (0.14-2.74)	2.05 (1.21-6.76)	n.s.	n.t.
CD163+ CD16+	0.08 (0.01-0.23)	0.09 (0.03-0.81)	n.s.	0.09 (0.03-0.59)	0.10 (0.03-0.29)	n.s.	n.t.
CD163- CD16+	0.35 (0.00-0.95)	0.62 (0.15-4.41)	n.s.	1.04 (0.15-4.41)	0.49 (0.44-0.94)	n.s.	n.t.
CD206+ CD16-	0.16 (0.13-0.33)	0.17 (0.00-0.66)	n.s.	0.09 (0.00-0.23)	0.20 (0.06-0.66)	n.s.	n.t.
CD206+ CD16+	0.04 (0.00-0.11)	0.04 (0.01-0.14)	n.s.	0.03 (0.02-0.06)	0.04 (0.01-0.14)	n.s.	n.t.
CD206- CD16+	0.35 (0.01-0.98)	0.75 (0.14-4.90	n.s.	1.35 (0.14-4.90)	0.66 (0.47-1.03)	n.s.	n.t.
CD11c+ CD16-	11.40 (2.29-17.34)	14.22 (2.55-22.46)	n.s.	8.41 (2.55-22.46)	15.5 (13.9-19.6)	n.s.	n.t.
CD11c+ CD16+	0.39 (0.01-1.10)	0.84 (0.18-4.98)	n.s.	1.39 (0.18-4.98)	0.65 (0.49-1.08)	n.s.	n.t.
CD11c- CD16+	0.0008 (0.00-0.00)	0.001 (0.00-0.01)	n.s.	0.003 (0.00-0.01)	0.000 (0.00-0.00)	n.s.	n.t.
CD163+ CD206-	2.70 (1.02-4.87)	1.30 (0.48-6.90)	n.s.	0.80 (0.48-2.78)	1.99 (1.16-6.90)	n.s.	n.t.
CD163+ CD206+	0.13 (0.04-0.16)	0.05 (0.00-0.36)	n.s.	0.04 (0.00-0.11)	0.04 (0.02-0.36)	n.s.	n.t.

CD163- CD206+	0.19 (0.04-0.44)	0.19 (0.02-0.51)	n.s.	0.18 (0.02-0.25)	0.21 (0.05-0.51)	n.s.	n.t.
CD11c+ CD206-	11.55 (2.15-17.89)	14.73 (3.60-24.45)	n.s.	9.11 (3.60-24.45)	16.23 (14.48-20.0)	n.s.	n.t.
CD11c+ CD206+	0.24 (0.16-0.55)	0.27 (0.02-0.89)	n.s.	0.19 (0.02-0.35)	0.28 (0.08-0.89)	n.s.	n.t.
CD11c- CD206+	0.007 (0.00-0.02)	0.001 (0.00-0.01)	0.000	0.003 (0.00-0.01)	0.001 (0.00-0.00)	n.s.	n.t.
CD11c+ CD163-	9.20 (1.13-15.50)	12.07 (3.27-23.60)	n.s.	7.83 (3.27-23.60)	14.46 (7.54-15.16)	n.s.	n.t.
CD11c+ CD163+	3.14 (1.16-5.49)	1.57 (0.66-7.20)	n.s.	1.02 (0.68-3.16)	2.16 (1.23-7.20)	n.s.	n.t.
CD11c- CD163+	0.001 (0.00-0.01)	0.002 (0.00-0.04)	n.s.	0.00 (0.00-0.00)	0.006 (0.00-0.04)	n.s.	n.t.

Supplementary Table S2: Systemic expression of myeloid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.
*Non parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant

Myeloid cell type	Controls (median % of CD45+ or CD45- cells) N=8	uVIN (median % of CD45+ or CD45- cells) N=14	p =*	Recurrent uVIN (median % of CD45+ or CD45- cells) N=5	Non Recurrent uVIN (median % of CD45+ or CD45- cells) N=7	p = *
CD14+	0.01 (0.00-0.08)	0.02 (0.00-0.30)	n.s.	0.002 (0.00-0.03)	0.02 (0.00-0.30)	n.s.
CD11b+ CD124-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD33+	0.01 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.001 (0.00-0.03)	0.007 (0.00-0.03)	n.s.
CD11b+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD34+	0.0004 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD33+	0.01 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.001 (0.00-0.03)	0.007 (0.00-0.30)	n.s.
CD124+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD34+	0.001 (0.00-0.00)	0.005 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33+ CD34-	0.010 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.002 (0.00-0.03)	0.007 (0.00-0.30)	n.s.
CD33+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33- CD34+	0.0004 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD15+	0.05 (0.00-0.27)	0.13 (0.01-1.21)	n.s.	0.16 (0.01-1.21)	0.10 (0.02-0.37)	n.s.
CD11b+ CD124-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	**0.030**
CD11b- CD33+	0.001 (0.00-0.01)	0.003 (0.00-0.05)	n.s.	0.002 (0.00-0.05)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD33+	0.00 (0.00-0.00)	0.003 (0.00-0.06)	n.s.	0.003 (0.00-0.06)	0.000 (0.00-0.00)	**0.030**
CD124+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33+ CD34-	0.001 (0.00-0.01)	0.0002 (0.00-0.05)	n.s.	0.002 (0.00-0.05)	0.000 (0.00-0.00)	**0.030**
CD33+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.

Supplementary Table S3: Systemic expression of co-inhibitory molecule expression on lymphoid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.
*Non-parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant and in **bold** the p-values are marked where the cells numbers are >0.05%.

Results Mann-Whitney U test	Controls (median % of CD3+ T cells) N=8	uVIN (median % of CD3+ T cells) N=14	p =	Recurrent uVIN (median % of CD3+ T cells) N=5	Non Recurrent uVIN (median % of CD3+ T cells) N=7	p =	RFS (Log Rank)
CD3+			x			x	
CD3+ CD94+	4.05 (1.78-5.57)	2.67 (1.05-5.02)	n.s.	3.15 (2.07-4.05)	2.33 (1.88-5.02)	n.s.	0.372
CD3+ TIM3+	0.77 (0.45-2.38)	0.86 (0.58-3.12)	n.s.	1.60 (0.58-1.73)	0.77 (0.58-3.12)	n.s.	0.249
CD3+ NKG2A+	1.93 (0.98-2.79)	1.98 (1.32-5.60)	n.s.	2.13 1.87-3.18)	1.97 (1.32-5.60)	n.s.	0.488
CD3+ CD152+	0.12 (0.11-0.16)	0.16 (0.08-0.27)	n.s.	0.20 (0.17-0.25)	0.11 (0.08-0.27)	**0.048**	0.082
CD3+ PD1+	10.01 (5.78-16.66)	8.70 (4.69-26.03)	n.s.	14.29 (4.69-26.03)	7.35 (4.83-21.215)	n.s.	0.750
CD3+ CD94+ TIM3-	3.39 (1.66-5.32)	2.14 (0.81-3.80)	n.s.	2.15 (1.11-3.80)	2.13 (1.32-3.13)	n.s.	n.t.
CD3+ CD94+ TIM3+	0.35 (0.06-1.50)	0.31 (0.15-2.17)	n.s.	0.98 (0.21-1.12)	0.29 (0.15-2.17)	n.s.	n.t.
CD3+ CD94- TIM3+	0.46 (0.22-0.97)	0.55 (0.31-1.07)	n.s.	0.67 (031-0.91)	0.51 (0.37-1.07)	n.s.	n.t.
CD3+ CD94+ NKG2A-	2.73 (1.22-3.57)	1.50 (0.56-3.28)	n.s.	1.51 (0.88-3.28)	1.49 (1.17-2.43)	n.s.	n.t.
CD3+ CD94+ NKG2A+	1.38 (0.47-2.13)	0.97 (0.53-3.41)	n.s.	1.23 (0.80-1.71)	0.80 (0.55-3.41)	n.s.	0.142
CD3+ CD94- NKG2A+	0.65 (0.44-0.85)	1.05 (0.55-2.21)	**0.002**	1.10 (0.55-1.96)	0.99 (0.76-2.21)	n.s.	n.t.
CD3+ CD94+ CD152-	4.00 (1.76-5.51)	2.61 (0.99-4.94)	n.s.	3.04 (1.99-3.99)	2.32 (1.82-4.94)	n.s.	n.t.
CD3+ CD94+ CD152+	0.07 (0.03-0.10)	0.07 (0.03-0.14)	n.s.	0.10 (0.09-0.14)	0.05 (0.03-0.11)	**0.018**	n.t.
CD3+ CD94- CD152+	0.05 (0.04-0.07)	0.07 (0.05-0.20)	n.s.	0.08 (0.07-0.12)	0.05 (0.05-0.20)	0.048	n.t.
CD3+ CD94+ PD1-	2.90 (1.38-4.84)	1.70 (0.81-4.18)	n.s.	1.84 (1.25-2.55)	1.67 (0.32-4.18)	n.s.	n.t.
CD3+ CD94+ PD1+	0.84 (0.10-1.50)	0.77 (0.20-1.34)	n.s.	1.30 (0.76-1.34)	0.64 (0.20-1.17)	n.s.	n.t.
CD3+ CD94- PD1+	9.48 (5.50-15.78)	8.55 (4.06-25.06)	n.s.	13.28 (4.06-25.06)	7.42 (4.39-20.59)	n.s.	n.t.
CD3+ TIM3+ NKG2A-	0.43 (0.20-1.00)	0.35 (0.27-0.80)	n.s.	0.53 (0.27-0.80)	0.33 (0.29-0.36)	n.s.	n.t.
CD3+ TIM3+ NKG2A+	0.30 (0.20-1.28)	0.44 (0.15-2.83)	n.s.	0.77 (0.15-1.24)	0.44 (0.29-2.83)	n.s.	n.t.
CD3+ TIM3- NKG2A+	1.33 (0.76-2.46)	1.58 (0.92-2.81)	n.s.	1.69 (1.10-2.36)	1.60 (0.92-2.81)	n.s.	n.t.
CD3+ TIM3+ CD152-	0.75 (0.42-2.34)	0.83 (0.48-3.04)	n.s.	1.53 (0.52-1.65)	0.73 (0.48-3.04)	n.s.	n.t.
CD3+ TIM3+ CD152+	0.03 (0.02-0.06)	0.05 (0.03-0.14)	0.010	0.06 (0.03-0.09)	0.03 (0.03-0.14)	n.s.	n.t.
CD3+ TIM3- CD152+	0.08 (0.07-0.10)	0.09 (0.05-0.17)	n.s.	0.13 (0.09-0.17)	0.06 (0.05-0.11)	**0.005**	n.t.
CD3+ TIM3+ PD1-	0.50 (0.31-1.45)	0.67 (0.21-2.64)	n.s.	0.84 (0.31-0.92)	0.38 (0.21-2.64)	n.s.	n.t.

CD3+ TIM3+ PD1+	0.30 (0.05-0.95)	0.34 (0.17-0.88)	n.s.	0.70 (0.20-0.88)	0.38 (0.17-0.52)	n.s.	n.t.
CD3+ TIM3- PD1+	9.86 (5.79-16.60)	8.76 (4.54-25.54)	n.s.	13.72 (4.64-25.54)	7.47 (4.54-21.42)	n.s.	n.t.
CD3+ NKG2A+ CD152-	1.82 (0.86-2.67)	1.84 (1.09-5.42)	n.s.	1.99 (1.72-2.98)	1.86 (1.09-5.42)	n.s.	n.t.
CD3+ NKG2A+ CD152+	0.10 (0.06-0.13)	0.13 (0.05-0.19)	n.s.	0.17 (0.14-0.19)	0.08 (0.05-0.18)	**0.030**	n.t.
CD3+ NKG2A- CD152+	0.02 (0.01-0.04)	0.03 (0.02-0.09)	n.s.	0.03 (0.03-0.07)	0.02 (0.02-0.09)	0.048	n.t.
CD3+ NKG2A+ PD1-	1.30 (0.65-2.22)	1.17 (0.93-4.82)	n.s.	1.25 (1.13-1.53)	0.99 (0.93-4.82)	n.s.	n.t.
CD3+ NKG2A+ PD1+	0.58 (0.16-1.11)	0.78 (0.31-1.60)	n.s.	0.87 (0.70-1.60)	0.77 (0.31-1.11)	n.s.	n.t.
CD3+ NKG2A- PD1+	9.74 (5.72-16.54)	8.45 (4.06-25.03)	n.s.	13.31 (4.22-25.03)	7.05 (4.06-20.13)	n.s.	n.t.
CD3+ CD152+ PD1-	0.05 (0.04-0.06)	0.07 (0.02-0.13)	n.s.	0.07 (0.07-0.09)	0.05 (0.02-0.13)	n.s.	n.t.
CD3+ CD152+ PD1+	0.07 (0.04-0.09)	0.08 (0.05-0.16)	n.s.	0.10 (0.09-0.16)	0.07 (0.05-0.13)	**0.018**	n.t.
CD3+ CD152- PD1+	10.37 (6.00-17.20)	9.06 (4.84-26.51)	n.s.	14.63 (4.84-26.51)	7.76 (5.11-22.10)	n.s.	n.t.
Ratio CD3+NKG2a+/ CD3+NKG2a-	0.02 (0.01-0.03)	0.02 (0.01-0.06)	n.s.	0.02 (0.02-0.03)	0.02 (0.01-0.06)	n.s.	n.t.
Ratio CD3+CD94+NK-G2a+/CD3+CD94+NK-G2a-	0.49 (0.31-0.77)	0.64 (0.24-1.93)	n.s.	0.88 (0.24-1.37)	0.45 (0.37-1.93)	n.s.	n.t.
Ratio CD3+PD1-/ CD3+PD1+	8.99 (5.00-16.30)	10.78 (2.84-20.34)	n.s.	6.00 (2.84-20.34)	12.59 (3.73-19.71)	n.s.	n.t.
Ratio CD3+TIM3+/ CD3+TIM3-	0.008 (0.005-0.024)	0.009 (0.006-0.032)	n.s.	0.016 (0.006-0.018)	0.008 (0.006-0.32)	n.s.	n.t.
CD4+	68.59 (59.79-79.45)	69.74 (55.46-81.11)	n.s.	75.76 (55.46-81.11)	67.74 (59.26-79.89)	n.s.	0.181
CD4+ CD94+	0.13 (0.08-0.27)	0.15 (0.05-0.43)	n.s.	0.07 (0.05-0.13)	0.30 (0.07-0.43)	**0.010**	**0.011**
CD4+ TIM3+	0.20 (0.08-0.43)	0.27 (0.13-0.61)	n.s.	0.34 (0.13-0.61)	0.24 (0.13-0.35)	n.s.	0.100
CD4+ NKG2A+	0.22 (0.09-0.33)	0.44 (0.18-0.82)	**0.005**	0.65 (0.24-0.82)	0.31 (0.18-0.57	n.s.	0.884
CD4+ CD152+	0.04 (0.02-0.07)	0.05 (0.03-0.08)	n.s.	0.05 (0.04-0.08)	0.04 (0.03-0.08)	0.048	0.017
CD4+ PD1+	4.14 (3.10-6.55)	4.42 (2.02-10.50)	n.s.	5.24 (2.02-8.16)	3.64 (2.43-10.50)	n.s.	0.750
CD4+ CD94+ TIM3-	0.11 (0.06-0.22)	0.10 (0.04-0.36)	n.s.	0.05 (0.04-0.10)	0.18 (0.05-0.36)	**0.030**	n.t.
CD4+ CD94+ TIM3+	0.03 (0.01-0.07)	0.05 (0.01-0.22)	n.s.	0.01 (0.01-0.04)	0.09 (0.02-0.13)	0.010	n.t.
CD4+ CD94- TIM3+	0.16 (0.06-0.41)	0.17 (0.08-0.57)	n.s.	0.29 (0.12-0.57)	0.11 (0.08-0.33)	n.s.	n.t.
CD4+ CD94+ NKG2A-	0.06 (0.04-0.21)	0.09 (0.03-0.35)	n.s.	0.05 (0.03-0.09)	0.14 (0.05-0.35)	**0.018**	n.t.
CD4+ CD94+ NKG2A+	0.06 (0.03-0.13)	0.06 (0.02-0.28)	n.s.	0.04 (0.02-0.05)	0.13 (0.03-0.19)	**0.018**	**0.011**
CD4+ CD94- NKG2A+	0.16 (0.06-0.28)	0.32 (0.13-0.77)	**0.006**	0.61 (0.22-0.77)	0.20 (0.13-0.55)	**0.048**	n.t.
CD4+ CD94+ CD152-	0.12 (0.08-0.26)	0.14 (0.05-0.42)	n.s.	0.07 (0.05-0.14)	0.30 (0.07-0.42)	**0.018**	n.t.
CD4+ CD94+ CD152+	0.003 (0.00-0.01)	0.004 (0.00-0.02)	n.s.	0.003 (0.00-0.00)	0.008 (0.00-0.02)	0.003	n.t.
CD4+ CD94- CD152+	0.02 (0.01-0.03)	0.03 (0.01-0.06)	n.s.	0.04 (0.03-0.06)	0.01 (0.01-0.04)	0.018	n.t.
CD4+ CD94+ PD1-	0.11 (0.07-0.22)	0.12 (0.04-0.41)	n.s.	0.06 (0.04-0.10)	0.15 (0.05-0.41)	**0.018**	n.t.

CD4+ CD94+ PD1+	0.02 (0.01-0.06)	0.03 (0.01-0.19)	n.s.	0.02 (0.01-0.04)	0.03 (0.02-0.19)	0.048	n.t.
CD4+ CD94- PD1+	4.60 (3.49-7.27)	4.94 (2.32-11.38)	n.s.	5.59 (2.32-9.10)	4.20 (2.80-11.38)	n.s.	n.t.
CD4+ TIM3+ NKG2A-	0.18 (0.07-0.28)	0.35 (0.14-0.62)	**0.020**	0.55 (0.22-0.62)	0.18 (0.14-0.50)	**0.048**	n.t.
CD4+ TIM3+ NKG2A+	0.03 (0.01-0.06)	0.08 (0.02-0.20)	0.010	0.09 (0.02-0.20)	0.07 (0.04-0.17)	n.s.	n.t.
CD4+ TIM3- NKG2A+	4.63 (3.54-7.25)	4.96 (2.31-11.47)	n.s.	5.52 (2.31-8.99)	4.26 (2.83-11.47)	n.s.	n.t.
CD4+ TIM3+ CD152-	0.20 (0.08-0.31)	0.41 (0.15-0.78)	n.s.	0.61 (0.21-0.78)	0.30 (0.15-0.54)	n.s.	n.t.
CD4+ TIM3+ CD152+	0.02 (0.01-0.02)	0.02 (0.01-0.03)	0.035	0.02 (0.02-0.03)	0.02 (0.01-0.03)	n.s.	n.t.
CD4+ TIM3- CD152+	0.008 (0.01-0.03)	0.01 (0.01-0.04)	0.035	0.01 (0.01-0.03)	0.008 (0.01-0.04)	n.s.	n.t.
CD4+ TIM3+ PD1-	0.16 (0.06-0.31)	0.20 (0.08-0.42)	n.s.	0.24 (0.08-0.42)	0.11 (0.10-0.30)	n.s.	n.t.
CD4+ TIM3+ PD1+	0.04 (0.01-0.11)	0.07 (0.03-0.18)	n.s.	0.08 (0.05-0.18)	0.06 (0.03-0.13)	n.s.	n.t.
CD4+ TIM3- PD1+	4.53 (3.46-7.09)	4.86 (2.24-11.35)	n.s.	5.48 (2.24-8.95)	4.19 (2.78-11.35)	n.s.	n.t.
CD4+ NKG2A+ CD152-	0.20 (0.08-0.42)	0.25 (0.12-0.58)	n.s.	0.32 (0.12-0.58)	0.20 (0.13-0.33)	n.s.	n.t.
CD4+ NKG2A+ CD152+	0.004 (0.00-0.01)	0.008 (0.00-0.05)	0.004	0.01 (0.00-0.02)	0.007 (0.00-0.05)	n.s.	n.t.
CD4+ NKG2A- CD152+	0.02 (0.01-0.03)	0.02 (0.01-0.05)	n.s.	0.03 (0.02-0.05)	0.02 (0.01-0.02)	0.010	n.t.
CD4+ NKG2A+ PD1-	0.02 (0.01-0.03)	0.03 (0.01-0.04)	n.s.	0.03 (0.02-0.04)	0.02 (0.01-0.03)	0.048	n.t.
CD4+ NKG2A+ PD1+	0.005 (0.00-0.01)	0.009 (0.00-0.03)	0.005	0.01 (0.01-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD4+ NKG2A- PD1+	4.75 (3.62-7.44)	5.13 (2.39-11.81)	n.s.	5.67 (2.39-9.31)	4.43 (2.98-11.81)	n.s.	n.t.
CD4+ CD152+ PD1-	0.16 (0.07-0.32)	0.12 (0.07-0.31)	n.s.	0.20 (0.07-0.31)	0.10 (0.07-0.18)	n.s.	n.t.
CD4+ CD152+ PD1+	0.04 (0.01-0.10)	0.09 (0.01-0.43)	0.029	0.07 (0.01-0.43)	0.11 (0.06-0.21)	n.s.	n.t.
CD4+ CD152- PD1+	0.16 (0.08-0.27)	0.32 (0.13-0.67)	**0.008**	0.41 (0.23-0.67)	0.23 (0.13-0.37)	n.s.	n.t.
Ratio CD4+TIM3+/ CD4+TIM3-	0.003 (0.00-0.01)	0.004 (0.00-0.014	n.s.	0.005 (0.00-0.01)	0.004 (0.00-0.00)	n.s.	n.t.
CD8+	23.18 (17.20-31.36)	22.33 (12.28-35.92)	n.s.	20.10 (14.36-35.92)	26.33 (12.28-27.93)	n.s.	0.460
CD8+ CD94+	1.17 (0.76-2.73)	0.91 (0.46-1.78)	n.s.	0.83 (0.47-1.78)	0.92 (0.76-1.69)	n.s.	0.829
CD8+ TIM3+	0.15 (0.06-0.26)	0.13 (0.02-0.67)	n.s.	0.16 (0.13-0.67)	0.11 (0.02-0.15)	**0.010**	0.100
CD8+ NKG2A+	0.38 (0.13-0.96)	0.38 (0.13-0.90)	n.s.	0.67 (0.22-0.90)	0.33 (0.13-0.71)	n.s.	0.625
CD8+ CD152+	0.03 (0.02-0.05)	0.03 (0.01-0.16)	n.s.	0.06 (0.02-0.09)	0.03 (0.01-0.16)	n.s.	0.807
CD8+ PD1+	3.70 (1.09-7.15)	2.71 (0.84-17.88)	n.s.	2.46 (1.07-17.88)	2.72 (0.84-8.19)	n.s.	0.663
CD8+ CD94+ TIM3-	1.33 (0.77-2.75)	0.94 (0.45-2.83)	n.s.	0.95 (0.66-2.83)	0.94 (0.75-1.67)	n.s.	n.t.
CD8+ CD94+ TIM3+	0.01 (0.01-0.03)	0.01 (0.00-0.08)	n.s.	0.03 (0.01-0.07)	0.007 (0.00-0.02)	0.005	n.t.
CD8+ CD94- TIM3+	0.11 (0.03-0.16)	0.08 (0.01-0.40)	n.s.	0.09 (0.07-0.40)	0.08 (0.01-0.10)	n.s.	n.t.
CD8+ CD94+ NKG2A-	0.77 (0.59-1.90)	0.70 (0.31-1.53)	n.s.	0.51 (0.31-1.53)	0.78 (0.55-1.37)	n.s.	n.t.

CD8+ CD94+ NKG2A+	0.24 (0.06-0.92)	0.18 (0.07-0.72)	n.s.	0.17 (0.11-0.72)	0.18 (0.07-0.55)	n.s.	0.684
CD8+ CD94- NKG2A+	0.15 (0.08-0.22)	0.16 (0.08-0.67)	n.s.	0.22 (0.10-0.67)	0.16 (0.08-0.25)	n.s.	n.t.
CD8+ CD94+ CD152-	1.17 (0.79-2.77)	0.91 (0.44-1.73)	n.s.	0.82 (0.46-1.73)	0.91 (0.75-1.68)	n.s.	n.t.
CD8+ CD94+ CD152+	0.005 (0.00-0.01)	0.008 (0.00-0.02)	n.s.	0.008 (0.00-0.02)	0.008 (0.00-0.01)	n.s.	n.t.
CD8+ CD94- CD152+	0.02 (0.01-0.06)	0.03 (0.01-0.16)	n.s.	0.04 (0.02-0.08)	0.02 (0.01-0.16)	n.s.	n.t.
CD8+ CD94+ PD1-	1.05 (0.67-2.59)	0.76 (0.38-1.28)	n.s.	0.78 (0.38-1.28)	0.75 (0.67-1.04)	n.s.	n.t.
CD8+ CD94+ PD1+	0.20 (0.04-0.36)	0.21 (0.02-0.65)	n.s.	0.21 (0.04-0.48)	0.21 (0.02-0.65)	n.s.	n.t.
CD8+ CD94- PD1+	3.84 (1.04-7.21)	2.83 (0.98-18.34)	n.s.	2.63 (1.12-18.34)	2.87 (0.98-8.61)	n.s.	n.t.
CD8+ TIM3+ NKG2A-	0.10 (0.04-0.18)	0.09 (0.01-0.45)	n.s.	0.11 (0.08-0.45)	0.007 (0.01-0.11)	**0.048**	n.t.
CD8+ TIM3+ NKG2A+	0.006 (0.01-0.02)	0.009 (0.00-0.05)	n.s.	0.02 (0.01-0.05)	0.006 (0.00-0.01)	0.018	n.t.
CD8+ TIM3- NKG2A+	0.40 (0.14-1.09)	0.39 (0.14-0.89)	n.s.	0.69 (0.22-0.89)	0.34 (0.14-0.75)	n.s.	n.t.
CD8+ TIM3+ CD152-	0.10 (0.04-0.20)	0.09 (0.02-0.50)	n.s.	0.11 (0.09-0.50)	0.07 (0.02-0.11)	**0.010**	n.t.
CD8+ TIM3+ CD152+	0.002 (0.00-0.00)	0.004 (0.00-0.02)	n.s.	0.004 (0.00-0.01)	0.002 (0.00-0.02)	n.s.	n.t.
CD8+ TIM3- CD152+	0.30 (0.01-0.05)	0.03 (0.01-0.15)	n.s.	0.05 (0.02-0.08)	0.02 (0.01-0.15)	n.s.	n.t.
CD8+ TIM3+ PD1-	0.10 (0.03-0.17)	0.07 (0.02-0.42)	n.s.	0.10 (0.07-0.42)	0.06 (0.02-0.10)	**0.030**	n.t.
CD8+ TIM3+ PD1+	0.01 (0.01-0.04)	0.02 (0.00-0.06)	n.s.	0.03 (0.01-0.06)	0.01 (0.00-0.04)	n.s.	n.t.
CD8+ TIM3- PD1+	4.03 (1.22-7.56)	2.96 (0.97-18.55)	n.s.	2.67 (1.15-18.55)	3.01 (0.97-9.01)	n.s.	n.t.
CD8+ NKG2A+ CD152-	0.38 (0.12-1.04)	0.31 (0.13-0.87)	n.s.	0.61 (0.20-0.87)	0.30 (0.13-0.70)	n.s.	n.t.
CD8+ NKG2A+ CD152+	0.02 (0.01-0.03)	0.02 (0.01-0.14)	n.s.	0.05 (0.02-0.08)	0.02 (0.01-0.14)	n.s.	n.t.
CD8+ NKG2A- CD152+	0.007 (0.00-0.03)	0.009 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD8+ NKG2A+ PD1-	0.26 (0.11-0.92)	0.27 (0.10-0.49)	n.s.	0.30 (0.18-0.49)	0.25 (0.10-0.42)	n.s.	n.t.
CD8+ NKG2A+ PD1+	0.11 (0.02-0.18)	0.11 (0.02-0.52)	n.s.	0.21 (0.03-0.52)	0.07 (0.02-0.32)	n.s.	n.t.
CD8+ NKG2A- PD1+	3.96 (1.22-7.40)	2.91 (0.95-18.03)	n.s.	2.63 (1.13-18.03)	2.95 (0.95-8.81)	n.s.	n.t.
CD8+ CD152+ PD1-	0.02 (0.01-0.04)	0.02 (0.00-0.14)	n.s.	0.02 (0.01-0.06)	0.01 (0.00-0.14)	n.s.	n.t.
CD8+ CD152+ PD1+	0.01 (0.00-0.02)	0.01 (0.00-0.04)	n.s.	0.03 (0.01-0.04)	0.01 (0.00-0.03)	n.s.	n.t.
CD8+ CD152- PD1+	4.07 (1.24-7.65)	2.98 (0.98-18.67)	n.s.	2.72 (1.17-18.67)	3.02 (0.98-9.11)	n.s.	n.t.
Ratio CD8+TIM3+/ CD8+TIM3-	0.007 (0.002-0.11)	0.005 (0.001-0.35)	n.s.	0.009 (0.00-0.03)	0.04 (0.00-0.01)	**0.030**	**0.018**
Ratio CD8+CD94+NK-G2a+/CD8+CD94+NK-G2a-	0.33 (0.04-0.56)	0.26 (0.10-1.39)	n.s.	0.42 (0.16-1.39)	0.23 (0.10-0.51)	n.s.	n.t.

CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

A diagnosis with usual vulvar intraepithelial neoplasia (uVIN), caused by a persistent infection with the human papilloma virus (HPV), has major impact on the patient since this chronic skin disease is associated with severe long-lasting complaints as pruritus, high recurrence rates, a malignant potency, psychosexual implications and frequent outdoor patient clinic follow up visits. The incidence of uVIN is increasing, mainly in young women and there is a need for alternative therapies. The immune response clearly plays a role in protection against this disease and different immunotherapies are being developed to treat the persistent HPV infection and related anogenital neoplasia. In contrast to patients with cervical intraepithelial neoplasia (CIN) for whom the conventional loop electrosurgical procedure (LEEP) therapy is a relatively simple and uncomplicated therapy, the conventional therapies for uVIN are associated with potential mutilation and high recurrence rates. Therefore uVIN patients form an exquisite patient group to evaluate the potency of immunotherapy in HPV-induced premalignant disease. In the past decade clinical successes have been achieved in the treatment of uVIN by different immunotherapeutic approaches, albeit that still a notable number of patients does not respond to these therapies. Despite the fact that uVIN is the first HPV-induced disease successfully treated by immunotherapy, the knowledge of the immune response in uVIN is relatively limited when compared to the well-studied CIN and cervical cancer lesions. Through the studies in this thesis we gained more knowledge on the local and systemic immune responses. This may help to understand the non-responsiveness to immunotherapy of some patients which can be used to optimize these therapies and to foster individualised (immune) therapies.

Local immune cell infiltrates in the microenvironment of uVIN lesions

The importance of the immune infiltration in protection and regression of uVIN lesions is indicated by the normalization of immune cell counts when the lesion is resolved[1-4]. The epithelium of the uVIN lesion has been characterised as immunosuppressive reflected by a lower number of CD8+ T cells, the presence of immature DCs and LCs whereas the stroma is the immune active compartment with higher numbers of mature DCs, NK and T cells[1,5,6]. Regulatory T cells (Tregs), which are known to suppress induction of pro-inflammatory Th1 cells required to subsequently attract effector CD8+ T cells to the lesion, abundantly infiltrate uVIN lesions and are consistently associated with non-responsiveness to immunotherapy [1-3,7-11]. Intralesional CD8+ T cells are indispensable as illustrated by the decrease in the number of CD8+ T cells in the progression of uVIN as well as in an increase of mainly CD8+ lymphocytes in clinical responders to immunotherapy[1-4,12]. We confirmed the abundant expression of CD4+ and CD8+ lymphocytes and regulatory T cells mainly in the stroma of uVIN lesions. Moreover a high stromal CD8+/Treg ratio was related to a prolonged recurrence free survival rate in uVIN lesions highlighting the importance of intralesional effector T cells. In the progressive course of the disease towards HPV-positive vulvar

carcinomas the number of regulatory T cells increase and outnumber the CD8+ T cells, as reflected by a lower CD8+/Treg ratio *(Chapter 5)*.

Alterations in HLA expression in uVIN patients

Alterations in HLA expression (*e.g.* HLA class I expression) in uVIN might allow these premalignant lesions to escape immune surveillance by specific CD8+ T cells because of insufficient antigen presentation to T cells. In a previous study downregulation of HLA class I was reported in 30% of uVIN lesions and in the majority of vulvar carcinomas. HLA class I downregulation was associated with non-responsiveness to photodynamic therapy (PDT)[2]. In *Chapter 4* we show that alterations in HLA expression are already present in HPV-induced uVIN. Over 70% of uVIN lesions and 80% of vulvar carcinomas display (partial) downregulation of HLA class I. Total loss of HLA-A and/or HLA-B/C, however, is scarce and found only in <10% of uVIN lesions although total loss of HLA B/C increased from 9% in uVIN to 38% in uVIN adjacent to micro invasive carcinoma. Downregulation of HLA B/C was related to the recurrences and progression of uVIN lesions. The (partial) HLA-class I downregulation seems reversible in uVIN since IFNγ stimulation of uVIN keratinocytes in vitro resulted in the upregulation of HLA class I. Moreover, only 15% of uVIN cases showed a genetically caused downregulation of HLA class I, through loss of heterozygosity (LOH). The reversibility of HLA class I downregulation is a potential explanation for the fact that HLA class I downregulation showed no clinical impact on the results of our HPV16 SLP vaccination trial, where all clinical responders showed partial downregulation of HLA class I and non-responders could still fully express HLA class I. Potentially, vaccine induced IFNγ-producing HPV16-specific CD4+ T cells that infiltrate the lesion mediate the upregulation of HLA class I in uVIN lesion without genetic cause of HLA downregulation. In vulvar carcinomas however LOH was more often associated with HLA class I downregulation (25-55.5%) suggesting that more advanced stages of HPV induced neoplasia are increasingly difficult to treat by immunotherapy. MICA, which serves as a stimulatory molecule for CD8+ T cells and NK cells through interaction with NKG2d, was downregulated in 80% of uVIN and carcinomas. A combination of HLA-class I downregulation and MICA was associated with recurrent disease. The alterations of the classical HLA molecules and MICA seem an early event in HPV induced neoplasia which may allow lesions to develop. Expression of the non-classical HLA molecules -E and -G was associated with the progressive course of vulvar neoplasia, and found in approximately 50% of carcinoma cases. Negative feedback through NKG2a, suppressing activated T cells and NK cells, may add to the difficulty to treat carcinoma.

Intralesional infiltration of myeloid cells and characterisation of lymphocytes

The observed intralesional lymphocytic and myeloid cell infiltrates in uVIN by several independent study groups[1-6,13], are apparently not able to clear the uVIN lesions and HPV.

This may be caused by an impaired function of those infiltrating immune cells as result of an immunosuppressive microenvironment. In *Chapter 5* we presented a detailed analysis of several immune inhibitory molecules in the uVIN lesions and observed the expression of co-inhibitory molecules PD1, TIM3 and NKG2a on a proportion of infiltrating lymphocytes. The negative regulatory molecules as CTLA-4, TIM3 and PD1 are expressed to suppress T-cell function and prevent uncontrolled inflammation of the immune system[14,15]. TIM3 is upregulated in IFNγ producing CD4+ and CD8+ differentiated T cells whereas CTLA-4, PD1 and NKG2a can be upregulated on T cells after activation[15-17]. In our study, the expression of TIM3 was correlated with high numbers of infiltrating Tbet positive T cells as well as with higher numbers of immune cells expressing its ligand Galectin-9, which is known to be upregulated in response to pro-inflammatory cytokines (e.g. IFNγ) or upon activation via TLRs[18,19]. Upon Gal-9 TIM3 interactions, T-cell function can be suppressed[18,20,21], but Gal-9 TIM3 interactions in CD8+ T cell may enhance their function if these cells do not co-express PD1[21,22]. Interestingly the expression of the markers TIM3 and NKG2a on lymphocytes in uVIN lesions seemed a reflection of T-cell activation rather than inhibition since relatively higher numbers of CD8+TIM3+ and CD3+NKG2a+ T cells in the stroma of uVIN lesions were related to a prolonged recurrence free survival. However, when co-infiltrating Tregs outnumber these CD8+TIM3+ T cells the recurrence free survival is decreased. This is also observed in vulvar carcinomas. Unfortunately, we were not able to evaluate CTLA-4 by immunohistochemistry due to aspecific staining of different antibodies in paraffin embedded tissue but analysis of PD1 showed a number of activated PD1+ T cells in uVIN lesions, the presence of which did not seem to have clinical impact. Interestingly, the number of PD1+ and NKG2a+ T cells was higher in the epithelium of control tissue compared to uVIN tissue suggesting that immune cells in HPV infected tissue are less activated. Although scarcely present, stromal NKG2a expression on CD3+ T cells was associated with an improved clinical outcome in uVIN lesions. Potentially, because its ligand HLA-E was almost not expressed in uVIN lesions[23]. The expression of NKG2a is thus a potential reflection of an adequate local pro-inflammatory T-cell response in uVIN lesions. This notion would fit with the observation that in HLA-E expressing vulvar carcinomas the number of stromal CD3+NKG2a+ T cells was remarkably lower.

The progressive course of HPV induced vulvar neoplasia is characterised by an increase in both epithelial and stromal Tregs as well as intraepithelial and stromal matured M1 and M2 macrophages (*Chapter 6*). In uVIN lesions M2 macrophages outnumber M1 macrophages whereas the numbers of M1 level up in vulvar carcinoma. In case of a dense number of intraepithelial CD14+ macrophages (irrespective of type M1 or M2) the risk of a recurrence is markedly enhanced and this is an independent prognostic factor for recurrent disease. The presence of these CD14+ macrophages is associated with an increase in intraepithelial Tregs and with low numbers of stromal CD8+TIM3+ T cells. This indicates a shift towards

an immunosuppressed microenvironment since the combination of these parameters was associated with rapid recurrences *(Chapter 6)*. Interestingly, patients not responding to imiquimod display an increased average (not-significant) number of CD14+ and CD68+ cells suggesting that macrophage infiltration in uVIN lesions may have impact on clinical responses to immunotherapy[1]. Based on our data we expect that the patients with higher numbers of CD14+ and CD68+ macrophages will also display a stronger infiltration with regulatory T cells whereas the clinical responders to imiquimod therapy probably will show the presence of pro-inflammatory infiltrating T cells.

Systemic immunity in patients with uVIN lesions

Systemic cellular HPV specific T-cell responses characterized by relatively robust proliferative IFNγ- and IL-5-producing CD4+ T-cell responses against early viral proteins E2, E6 and E7 are associated with better control of HPV16 infections[24,25]. In uVIN patients the systemic HPV 16-specific IFNγ-associated type 1 T-cell responses against E2, E6 and/or E7 are either weak or non-detectable in up to 50% of the patients[26-28]. Perhaps the patients with detectable systemic HPV-specific T-cell responses are also the patients where a pro-inflammatory effector T-cell response is detected in the microenvironment. The presence of such circulating HPV-specific T-cell responses is unfortunately not associated with spontaneous lesion clearance but is associated with a better clinical response to imiquimod or PDT[26,29]. Therapeutic vaccination studies demonstrated the importance of a strong and broad systemic HPV specific pro-inflammatory immune response to resolve uVIN[3,13,30,31]. Interestingly, the capacity of patients to respond to therapeutic vaccination differs extensively. Some of the patients display relatively weak vaccine-induced responses of limited breadth associated with no clinical response, whereas in others the vaccine-induced T-cell response was strong and broad and associated with lesion regression. These data suggested that the patients' capacity to respond to the vaccination varies and potentially this depends on their immune status. In two hypothesis generating studies we explored the phenotypic (co-inhibitory molecule expression) and functional (cytokine stimulated STAT phosphorylation) analysis of peripheral circulating lymphocytes as well as the type and number of myeloid cells (macrophages, DCs and MDSCs) in uVIN patients in comparison to that of healthy controls.

Phenotypic analysis of circulating lymphocytes and myeloid cells

The expression of the inhibitory markers on peripheral lymphocytes was limited to a small percentage (<1%) of all CD3+ lymphocytes apart for PD1, which is expressed in approximately 3-5% of CD4+ or CD8+ T cells in both uVIN patients and healthy controls. No overt differences were observed although in uVIN patients the proportion of CD4+PD1+ and CD4+TIM3+ T cells was slightly increased compared to healthy controls. Interestingly, there was a higher frequency of CD4+CD94+ T cells in non-recurrent uVIN patients when

compared to patients with a recurrence. In addition, a higher frequency of these cells was associated with a prolonged recurrence free survival (*Chapter 7*). Interestingly, high numbers of CD3+NKG2a+ T cells in the microenvironment were also associated with a favourable clinical outcome*(Chapter 5)*. Importantly, if a cell is NKG2a+ one can consider it positive for CD94+ as well because NKG2a forms a complex with CD94 and cells should be positive for CD94 when they express NKG2a[17]. While we observed that the frequency of circulating CD4+CD94+ and CD4+CD94+NKG2a+ T cells were related to non-recurrent uVIN and a prolonged recurrence free survival, this was not the case for CD8+ T cells. In our analysis of lesion infiltrating lymphocytes we were not able to distinguish between CD4+NKG2a+ or CD8+NKG2a+ T cells in the microenvironment but this is an important goal for the future analysis since a potential direct correlation between systemic and local immunity in relation to clinical outcome would provide a valuable biomarker. Unlike NKG2a+ expression, the frequency of local and circulating CD8+TIM3+ T cells was associated with opposite clinical outcomes. Circulating CD8+TIM3+ T cells are related to recurrences and a decreased recurrence free survival period *(Chapter 7)*. The relation between CD8+TIM3+ cells and their opposite relations with clinical outcome based on the origin of the biological sample they are measured in requires further investigation.

Phenotyping of circulating myeloid cells revealed that the frequencies of CD14+CD11b+ monocytes are comparable in uVIN patients and controls and account for the largest population of myeloid cells. The percentage of CD14highCD11b+ monocytes was lower in recurrent uVIN lesions than in non-recurrent lesions (9.5% vs 16.5%) albeit that this percentual difference was not significant, probably due to the small group of patients analysed. Minor groups are represented by populations of <1% and are formed by CD14+IntCD11b+ mature macrophages/DCs, non-activated CD14+CD11b- monocytes and CD14-CD11b+ myeloid cells which may be activated DCs or monocytes with loss of CD14[32,33]. A comparison of the myeloid cell populations revealed that patients with recurrences displayed lower frequencies of circulating immature DCs/early differentiating monocytes and activated mature DCs whereas the proportion of circulating type 2 monocytes/macrophages was increased. This is in accordance with the observation that an increased number of lesion-infiltrating M2 was associated with worse outcome. Patients with relatively higher frequencies of DCs and lower frequencies of circulating type 2 monocytes/macrophages show a s favourable clinical outcome and prolonged recurrence free survival (*Chapter 7)*.

Notably, DCs are indispensable in antigen presentation and subsequent regulation of tumor-specific immune responses[34] and their activation is impaired by immunosuppressive tumor associated myeloid cells as macrophages[35]. The immature or non-activated DCs which we observed more frequently in PBMCs of recurrent uVIN patients are also frequently observed in tumors and they do not contribute to anti-tumor immune responses[34]. The higher number of type 2 monocytes/macrophages in PBMCs of recurrent uVIN patients, which were also

found to be increased within the lesion (*Chapter 6*) are probably involved in the impairment of DC.

Phosphorylation of signal transduction activators of transcription (STAT) in uVIN patients and healthy controls

We analysed the activation of different signal transduction routes in cytokine stimulated immune cells as a measure of their immune responsiveness (*Chapter 7*). We observed no differences in the activation rate or expression levels of pSTAT between uVIN patients and healthy controls for most of the cytokines tested. The only observations that we made were that healthy donor derived CD8+ T cells more often upregulated pSTAT5 upon IFNα stimulation when compared to uVIN patients. In an earlier study HPV-specific T cells from patients with recurrent respiratory papillomatosis display a reduced IFNγ and IL-2 secretion as well as lower STAT5 phosphorylation when compared to healthy controls suggesting that the HPV-specific T-cells were anergic. Their function could be restored by IL-2 implying that interventions restoring pro-inflammatory cytokine responses could improve clinical outcome and reverse T-cell anergy[36]. In addition, we observed that the CD14+CD33 monocytes of uVIN patients displayed lower levels of pSTAT5 upon IFNα stimulation whereas upon stimulation with GM-CSF higher levels of pSTAT5 were induced. Potentially, the cytokine signalling in these precursor antigen presenting cells has been altered in uVIN patients.

The data obtained with the immune modulators GM-CSF and IFNα, both involved in DC activation, are of interest for future immunotherapeutic studies. They are both used as adjuvants to different therapeutic vaccines in order to enhance a Th1 polarized T-cell response[37-39] but mainly the use of IFNα has been proven beneficial to vaccine induced T-cell responses[38,39]. The use of GM-CSF to enhance T-cell immunity has met with mixed outcomes[38,39]. Our data suggested that IFNα can indeed be regarded as a potent immune stimulator. It synergizes with IFNγ and may be a promising immune modulator during immunotherapy of uVIN patients. IFNα therapy has already shown promising enhancement of immune responses and potentially is related to clinical outcomes[37,38,40-42]. In a small number of vaccinated uVIN patients, with a peak of IFNγ upon the first HPV-16 SLP ISA101 vaccination, pSTAT1 expression was increased upon stimulation with IFNα as well although it is difficult to unravel the activated interactive pathways *in vivo* that may explain the difference in the patients' capacity to respond to immunotherapy.

In conclusion, the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients and our phenotypical analysis of circulating immune cells revealed two potential biomarkers associated with a better clinical outcome. The first is the frequency of CD4+CD94+ cells whereas the second is formed by the frequency of certain myeloid cell subsets, in particular DCs and type 2 monocytes/macrophages.

Future prospects for the immunotherapy of premalignant uVIN lesions

The prerequisites for successful immunotherapy of uVIN consist of adequate T-cell priming by APCs, a balance towards effector instead of regulatory T cells, an increase of intralesional effector T cells, prevention of immune exhaustion and creating a pro-inflammatory microenvironment in which the activated HPV-specific effector T cells can exert their function in order to resolve the infection and lesion[43,44]. Different immunotherapies may act on parts of this. For instance, the topical immune modifier imiquimod enhances the migration of effector T cells in the lesion but apparently lacks the capacity to induce systemic T-cell activation to HPV antigens[1,7,26]. Therapeutic HPV vaccination results in adequate T-cell priming and pro-inflammatory T-cell responses and is in two studies related to clinical responses (reviewed in [45]) of which the TA-HPV results in an increase in intralesional influx of T cells in responders if combined with imiquimod[3] whereas this remains unknown for the HPV-16 SLP vaccine[30,31]. We recently submitted the results of a vaccination study with HPV16 SLP ISA101, in which patients were randomised for vaccination with or without imiquimod application on the vaccination sites[46]. This trial confirmed the clinical efficacy of the vaccine as well as the relation of clinical responses to the strength of the vaccine induced pro-inflammatory HPV specific T-cell response. PDT may also result in the priming of lesion-specific T cells[47], but is not likely to be as effective as vaccines. Since the vaccine-induced immune responses in the two HPV16 SLP trials are quite strong, one can envisage that meaningful improvements of clinical efficacy are not likely to come from further improvements of the vaccine itself but need to come from manipulation of the microenvironment where the T cells need to execute their function[30,46].

We have shown that the premalignant uVIN lesions are actually immune supportive compared to progressed vulvar lesions. This is reflected by reversibility of HLA-class I downregulation, the infiltration with relative high numbers of activated CD8+ effector T cells and IFNγ-producing (Tbet+) T cells as well as relatively low numbers of regulatory T cells and intraepithelial CD14+ monocytes, all of which are related to time to recurrence and progression of the disease. We hypothesize that for immunotherapy one may best focus on this group of patients as, with such a supportive immunological profile of which it is likely that they will be responsive. For instance, the lesions of patients within this group show no impaired migration of T cells or local immune suppression which may counteract a therapeutic vaccine-induced or boosted T-cell response.

The non-responders to current immunotherapies are likely to be among the group of patients with lesions that are characterised by loss of HLA expression and show a strong infiltration with Treg and CD14+ macrophages. These lesions are more alike to HPV induced vulvar cancers. Here one can expect that immunotherapy requires a strategy including methods to overcome the different aspects of immunological failure which may come from the field of cancer immunotherapy. Potentially, a combination of therapeutic vaccination with

imiquimod on the lesion can be used. Imiquimod is a topical immune modifier which acts through activation of innate immune cells by binding to TLR7 and 8 on DCs, which induces activation of NF-kB and subsequent secretion of multiple pro-inflammatory cytokines and activation of DCs, resulting in an influx of immune cells in the vulvar lesions and in increased antigen presentation because of LC migration to the draining lymph nodes[1,3,4,7,48,49]. Recently, it was also shown to upregulate the local expression of CXCL9 and CXCL10, chemokines involved in recruitment of CD4+ and CD8+ T cells[50]. Thus imiquimod may be used to change the local microenvironment cytokine milieu resulting in M1 polarization of the macrophages and a better attraction of CD8+ T cells to a number that outbalances Tregs. Treatment does not result in expansion of HPV-specific T cells[26] but the combination is very likely to be successful as pre-existing HPV-specific immunity is related to better clinical responses upon imiquimod therapy[26] and data from our clinical vaccination trials show that vaccinated patients who were initially not responding to vaccination, but are subsequently treated with imiquimod, generally display a complete and durable lesion regression[30,46]. Furthermore, the combination of vaccination and local imiquimod resulted in increased lesion-infiltrating immune cells and disease control in an animal model [50] and in uVIN patients[3]. A similar observation was made when imiquimod was combined with PDT[3,4]. In contrast to the combination of PDT and imiquimod, the numbers of intralesional T cells did not return to levels before imiquimod application when it was combined with vaccination suggesting that the vaccine-induced HPV 16 specific T-cell response resulted in increased T-cell infiltration and subsequent higher numbers of clinical responders[1-4,31].

Of note, our associations between immune cell infiltrates and clinical outcome are based on a cohort of uVIN patients treated with conventional therapies. It will be of utmost importance to estimate and validate these associations in a second patient cohort consisting of patients treated with immunotherapies such as imiquimod and therapeutic vaccines, in particular with HPV16 SLP vaccination.

As already established the combination of local imiquimod and systemic immunotherapy to increase both intralesional and circulating immune responses promoted the clinical success rate[3].

Furthermore depletion of Tregs may be of additional value to enhance cytotoxic T-cell mediated responses since we showed the importance of Tregs in the recurrence and progression of uVIN and the association of local Treg infiltration in non-responsiveness to immunotherapy. Cyclophosphamide is a well-known Treg depleting agent and anti-CD25, anti-CTLA-4 and anti-GITR monoclonal antibodies have been used as well[51-54]. Anti-CTLA4 has been shown to deplete tumor infiltration regulatory T cells, which express high levels of CTLA4, via an Fc dependent mechanism[55-59]. In a murine model of HPV tumor bearing mice a single dose of cyclophosphamide prior to therapeutic HPV-16 DNA vaccination resulted in an increase in anti-tumor responses related to a reduction in infiltration of regulatory T cells

and increased number of HPV specific CD8+ T cells[52]. Moreover in patients with genital warts administration of cyclophosphamide reduces the number of regulatory T cells and improves the microenvironment resulting in prevention of recurrence in patients with large genital warts after laser therapy[60]. In ovarian cancer patients pre-treated with cyclophosphamide before p53 SLP vaccination the systemic number of Tregs nor their function was altered but induced higher IFNγ specific T cells compared to p53 SLP vaccination although the influence on the local regulatory T cell infiltrates was not established[61].

The targets for monoclonal antibodies on either blocking of co-stimulatory or co-inhibitionary pathways on effector T cells, monocytes/APCs or regulatory T cells to release the brake on T-cell proliferation and activation are extensive (reviewed in [14,62-64]). Our current data do not support the use of such antibodies to improve immunity, however, more specific phenotyping of the cells expressing these inhibitory molecules as well as their ligands are needed before firm conclusions on this topic can be made.

Another target that should be considered to optimize immunotherapy of uVIN may be the depletion or re-programming of macrophages as they were found to be associated with recurrent disease and increased numbers of regulatory T cell infiltrates in uVIN lesions. In our data the absence of intraepithelial macrophages, irrespective of type 1 or type 2 macrophages, is favourable. Notably, the CD14+CD163-negative cells that are thought to be M1 macrophages may also reflect a population of CD14+CD11c+PDL1+ regulatory DCs which have been correlated to Tregs in metastatic lymph nodes of cervical cancer patients[65]. This still needs to be studied in uVIN. If so, depletion of macrophages might be the first choice. On the other hand, we found that M2 macrophages strongly outnumber the M1 macrophages in uVIN lesions which may have masked a potential positive influence of M1 macrophages. In tumors, M1 macrophages were an independent prognostic factor for better clinical outcome[32,66]. Furthermore, our research group showed that a population of inflammatory macrophages was required for vaccine induced regression of tumors[67]. In case M1 macrophages are as essential in uVIN lesions, therapies resulting in a switch from M2 to M1 macrophages are required. As production of PGE2 and IL-6 is known to induce M2 macrophages and hamper DC differentiation[68,69], blocking with anti-IL-6 (tocilizumab) or COX-inhibition which blocks production of PGE2 (celexocib) can induce re-polarization of macrophages and may improve clinical outcome[68-72]. Moreover blockade of colony-stimulating factor 1 receptor (CSF1 inhibitors) results in improved anti-tumor T-cell responses by decreasing the number of tumor associated macrophages (TAM) but also reprogram remaining TAMs to support antigen presentation and include T-cell activation revealing reduced local immune suppression and IFN**γ** responses[73-75]. Re-differentiation of macrophages can as well be induced in response to IFNγ in combination with CD40-CD40L[68] since upon CD40 ligation DCs mature and produce pro-inflammatory cytokines and upregulate co-stimulatory molecules to induce effector T cells[76,77]. Other repolarisation

options rely in triggering of TLRs[78,79]. By triggering of TLR3 by poly I:C in mice tumor supporting macrophages were converted into tumor suppressing M1 macrophages rapidly producing inflammatory cytokines[78]. Furthermore blocking of IL-10 by antibodies in combination with the TLR9 ligand CpG resulted in a shift from M2 to M1 infiltrating macrophages [79].

Recently antiviral therapy by cidofovir 1%, which may induce apoptosis of the HPV infected cells[80], showed comparable results to imiquimod making this a feasible and active alternative in treatment of uVIN lesions[81]. It will be important to establish if the clinical effect of this compound relies on the immune system. If so, the effects of this compound may be improved by combination with one or more immunotherapeutic agents.

Last but not least if pre-existing immune infiltrates can function as biomarkers in the individual patient to predict the patients' responsiveness to the suggested therapy we could prevent unnecessary side effects of immunotherapy as well as delay in effective therapy and prevent potential progression in this period. In the ideal situation combination of local immunotherapy, as imiquimod or cidofovir, to induce local inflammation and effector T-cell homing, combined with therapeutic vaccination to induce a strong and proliferative systemic HPV T-cell response would be combined with an additional immune modulating therapy depending on the immune infiltrates profile present in the local environment of the patients' uVIN lesion.

Final conclusion

All steps achieved in the last decade regarding the knowledge of immune infiltrating cells in (pre)malignant lesions as well as steps taken in immunotherapeutic approaches, makes that we now know that these high grade HPV uVIN lesion can undergo an immune-driven regression and we are challenged to further improve the promising established immunotherapies. Individualisation of patients therapy based on the immune infiltrates prior to therapy requiring for example depletion of immune suppressive macrophages or Tregs or enhancement of the pre-existent pro-inflammatory environment should be a goal to keep in mind in order to minimize the side effects of therapies and to improve the number of responding patients.

The recent introduction of prophylactic HPV vaccination to prevent HPV related (pre) malignancies is expected to lower the incidence and impact of HPV related disease as uVIN in the future[82]. However it will take a long time until the prophylactic vaccination will actually decrease the burden of HPV-induced (pre)malignancies over the general population especially since the coverage of HPV vaccination is lower as expected[83]. These prophylactic vaccines are not able to treat already infected HPV women[84]. Therefore, new strategies to effectively treat HPV-induced (pre)malignancies as uVIN are still needed.

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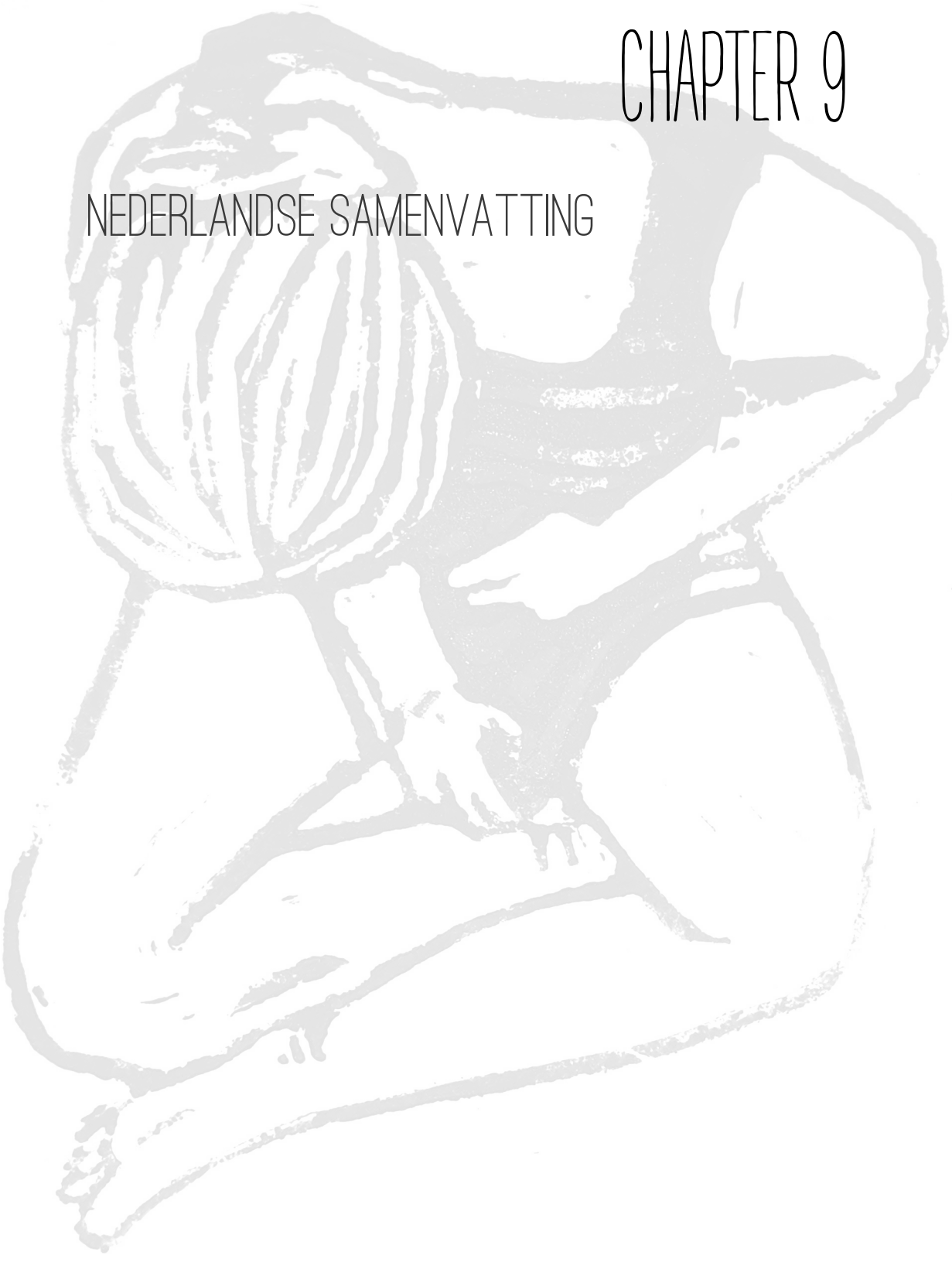
CHAPTER 9

NEDERLANDSE SAMENVATTING



Vulvaire intra-epitheliale neoplasie (VIN) is een relatief weinig voorkomende afwijking aan de schaamlippen met vaak een langdurig beloop en recidiverend karakter. VIN is een voorstadium van schaamlipkanker. Er zijn twee typen van VIN te onderscheiden; differentiated VIN (dVIN) en usual VIN (uVIN). uVIN wordt veroorzaakt door een voortdurende infectie met het humaan papilloma virus (HPV) bij patiënten van wie het afweersysteem er niet slaagt om de infectie op te ruimen. Door de stijging in het aantal HPV infecties is er ook sprake van een toename van uVIN in met name jonge vrouwen. De kans op het spontaan verdwijnen van uVIN afwijkingen is met 1.5% laag. De kans dat uVIN zich tot schaamlipkanker ontwikkeld is 10% als er geen behandeling plaats vindt en als de uVIN afwijking wel wordt behandeld dan verminderd de kans naar 3-4%. uVIN geeft bij de meeste vrouwen klachten als jeuk, pijn en/of branderigheid maar ook psychoseksuele klachten. Indien de uVIN afwijking geen klachten geeft en er geen verdenking is dat er kankervorming plaatsvindt, kan er gekozen worden voor een afwachtend beleid met regelmatige poliklinische controles. Een behandeling van uVIN is in de meerderheid van de patiënten echter wel nodig, omdat ze klachten ervaren. Standaard chirurgische behandelingen voor uVIN zijn het chirurgisch verwijderen van de afwijking of lasertherapie. Na deze standaard behandelingen komt de afwijking in 20-40% van de gevallen weer terug. Daarnaast kan een chirurgische behandeling leiden tot misvorming van de schaamlippen, wat vaak samen gaat met psychoseksuele klachten. Dit is de reden dat er momenteel behandelingen worden ontwikkeld die het afweersysteem activeren om zo het HPV virus en de daardoor veroorzaakte uVIN afwijking te bestrijden.

Sinds de jaren 70 is het bekend dat HPV baarmoederhalskanker veroorzaakt en in 1982 toonde onderzoek aan dat HPV ook VIN afwijkingen veroorzaakt. HPV is een seksueel overdraagbaar virus waarvan er meer dan 100 soorten bekend zijn. Deze zijn onderverdeeld in zogenoemde laag risico virussen die bijvoorbeeld wratten kunnen veroorzaken en in hoog risico virussen, zoals HPV 16 en 18, die baarmoederhalskanker en ook uVIN kunnen veroorzaken. Ongeveer 80% van de mensen wordt in zijn leven besmet met één of meerdere HPV infecties. In de meeste gevallen zal het afweersysteem deze infecties binnen 1-2 jaar onder controle krijgen zonder dat er klachten ontstaan. Indien het afweersysteem niet in staat is om dit virus op te ruimen, wat in ongeveer 10% van de mensen het geval is, dan is er sprake van een chronische HPV infectie die afwijkingen in de slijmvliezen van zowel het genitale gebied (baarmoederhals, schaamlippen, vagina en anus) als in de hoofd-hals regio kan veroorzaken (amandelen). Risicofactoren voor het krijgen van een voortdurende HPV infectie zijn het aantal seksuele partners, roken en gebruik van orale anticonceptie. Sinds een aantal jaar is er een preventief vaccin beschikbaar tegen de meest vóórkomende typen hoog risico HPV 16 en 18, die in 2010 is opgenomen in het rijksvaccinatieprogramma voor meisjes. Het doel van deze vaccinatie is om een HPV 16 en 18 infectie te voorkomen en daarmee het aantal HPV-veroorzaakte afwijkingen te verlagen. Helaas is het percentage

jonge meisjes dat gevaccineerd in Nederland lager dan gehoopt (variërend per regio van 31% tot 61%). Doordat het een lange tijd duurt voordat een afwijking zich door HPV ontwikkelt, zal het ook lang duren voordat men het beschermende effect van het preventieve vaccin ziet. Daarom is behandeling en opsporing van HPV veroorzaakte afwijkingen nog steeds van groot van belang.

Het afweersysteem is een zeer complex geheel met het doel de mens te beschermen tegen indringers van buiten en afwijkingen binnen het lichaam. Het kan globaal worden onderverdeeld in het aangeboren afweersysteem en het verworven afweersysteem. Het aangeboren afweersysteem kan direct, snel en altijd met dezelfde kracht reageren op indringende ziekteverwekkers in de omgeving. Cellen die onderdeel uitmaken van het aangeboren afweersysteem zijn onder andere: natural killer (NK) cellen en antigeen presenterende cellen (APC) zoals macrofagen en dendritische cellen (DCs). APC's zijn in staat zijn om het verworven afweersysteem activeren.
Het verworven afweersysteem is heel specifiek en kan tegen delen die uniek zijn voor ziekteverwekkers zoals virussen, bacteriën of kanker cellen reageren. Het duurt langer voordat een verworven afweerreactie op volle sterkte is, maar de reactie is zeer krachtig en specifiek gericht tegen de afzonderlijke ziekteverwekkers. Bovendien zorgt deze afweerreactie ervoor dat er een geheugen opgebouwd kan worden tegen deze ziekteverwekkers. Dit geheugen zorgt ervoor dat wanneer men dezelfde ziekteverwekker nog een keer tegenkomt, men er dan niet meer ziek van wordt. In de verworven afweerreactie spelen vooral de witte bloedcellen (lymfocyten) een belangrijke rol.
Subpopulaties van lymfocyten zijn onder andere B- en T-cellen. B-cellen maken antistoffen die de buitenkant van virussen kunnen herkennen en op die manier kunnen voorkomen dat het virus het lichaam kan infecteren. De B-cellen zijn belangrijk voor de werking van het preventieve HPV vaccin. T-cellen zijn van groot belang in de afweer tegen onder andere virale infecties zoals HPV wanneer de infectie al heeft plaatsgevonden. Er zijn verschillende soorten T-cellen te onderscheiden: de T-helper cellen (CD4+), cytotoxische T-cellen (CD8+) en ook regulatoire T-cellen (Treg). De CD4+ T-helper cellen activeren B cellen, ondersteunen de werking van cytotoxische CD8+ T-cellen en kunnen het gewenste type afweerreactie stimuleren. Globaal kunnen er twee T-helper afweerreacties onderscheiden worden die het afweersysteem sturen: een type 1 en een type 2 afweerreactie. De type 1 reactie resulteert in het opruimen van geïnfecteerde cellen door stimulatie van macrofagen en cytotoxische T cellen en kenmerkt zich door de productie van signaalstoffen (cytokines) zoals IFNγ, TNFα en IL-2. De type 2 reactie resulteert activatie van B cellen en is een chronische ontstekingsreactie die zich kenmerkt door cytokines zoals IL-4, IL-5 en IL-10. Cytotoxische T-cellen kunnen geïnfecteerde cellen en tumorcellen opruimen en zijn van groot belang in een effectieve afweerreactie. Regulatoire T-cellen remmen de afweerreactie omdat ze in

staat zijn om de activatie van andere afweercellen te onderdrukken. Dit is belangrijk om bijvoorbeeld allergie of autoimmuunziekten te voorkomen, maar ook om te zorgen dat er geen lichaamsschade optreed bij een te sterke afweerreactie.

Het is bekend dat in het geval van voortdurende HPV infecties en het ontstaan van uVIN zowel de aangeboren als verworven afweerreactie tegen HPV onderdrukt of niet geactiveerd is. Het merendeel van de macrofagen in uVIN zijn gericht op het dempen van de afweerreactie. Er zijn minder T-cellen gericht tegen HPV terwijl diegene die er zijn minder actief zijn. Bovendien worden ze in de uVIN vaak ook vergezeld door grotere aantallen van regulatoire T-cellen.
Aangezien we weten dat het falen van het afweersysteem om HPV te controleren in een klein deel van de patiënten tot het ontwikkelen van uVIN leidt, is er de afgelopen jaren veel aandacht uitgegaan naar immunotherapie als behandelmethode voor uVIN. Immunotherapie heeft als doel om het afweersysteem zodanig te activeren dat de HPV infectie en de afwijking ten gevolge van HPV worden opgeruimd.
Er kunnen twee soorten immunotherapie worden onderscheiden. Lokale immunotherapie grijpt direct aan op de afwijking zelf en beoogt de activering van het afweersysteem in de uVIN zelf. Een voorbeeld hiervan is het gebruik van imiquimod crème (Aldara®). Systemische immunotherapie zorgt voor activatie van het afweersysteem in het hele lichaam. Therapeutische HPV vaccinatie is hiervan een voorbeeld. Het vaccin stimuleert de lymfocyten van het verworven afweersysteem om tegen delen van het HPV te reageren. Deze lymfocyten circuleren door het lichaam om HPV infecties/afwijkingen op te sporen en daarna te vernietigen. Therapeutische vaccins bestaan in verscheidene vormen die heden ten dage in onderzoekverband worden getest. Eén type vaccin, het HPV 16 synthetic long peptide (HPV16 SLP) vaccin, ontwikkeld in het Leids Universitair Medisch Centrum (LUMC), geeft een krachtige stimulus aan het verworven afweersysteem wat samen gaat met het (deels) verdwijnen van uVIN in een groot aantal patiënten. Zowel de lokale als systemische immunotherapie van uVIN heeft geresulteerd in veel belovende resultaten in meer dan de helft van alle behandelde patiënten. Een aanzienlijk deel van de patiënten reageert helaas (nog) niet op deze therapieën.

Dit proefschrift richt zich op het lokale en systemische afweersysteem in uVIN patiënten. Het doel is om meer inzicht te krijgen in de rol en het falen van het afweersysteem in uVIN en hoe dit de reactie op immunotherapie kan beïnvloeden. Hiertoe hebben we bij een geselecteerde groep uVIN patiënten uit het LUMC, de patiënt karakteristieken, de lokale en de systemische afweerreactie in kaart gebracht.

In **hoofdstuk 2** van dit proefschrift wordt een gedetailleerd overzicht van de eigenschappen van de aangeboren en verworven afweerreactie tegen HPV, en bij uVIN in het bijzonder, gegeven. Daarnaast beschrijven we verschillende vormen van immunotherapie voor uVIN en wordt er een overzicht gegeven van mogelijke oorzaken van het falen van immunotherapie in uVIN. Dit review vormt de basis voor de verschillende studies in dit proefschrift.

In **hoofdstuk 3** beschrijven we de patiënt karakteristieken van onze onderzoeksgroep van uVIN patiënten. Hieruit blijkt dat in onze onderzochte populatie alleen het op meerdere plekken aanwezig zijn van uVIN afwijkingen een voorspeller is voor het snel terugkomen van uVIN nadat deze eerder door een chirurgische behandeling was verwijderd. Eerder beschreven voorspellende patiënt karakteristieken als roken en het nog aanwezig zijn van uVIN in de randen van het operatiegebied waren in deze groep niet van belang voor het verdere beloop van de ziekte. Het multifocaal aanwezig zijn van uVIN als ongunstige voorspeller hebben we mee laten wegen in alle volgende onderzoeken over het belang van afweercellen in het verdere beloop van de uVIN afwijking.

Veranderingen in de lokale afweerreactie in uVIN

uVIN wordt gekenmerkt door een ruime hoeveelheid aan verschillende typen afweercellen die zich in de afwijking bevinden. Alhoewel deze afweercellen aanwezig zijn in de uVIN afwijking blijken ze toch niet in staat te zijn om de HPV infectie op te ruimen en de uVIN afwijking te laten verdwijnen. De functie van deze cellen is dus verminderd of niet van het juiste type benodigd voor het opruimen van de uVIN cellen. In een effectieve afweerreactie tegen uVIN is de hoeveelheid en verhouding van cytotoxische CD8+T-cellen (die de geïnfecteerde cellen op kunnen ruimen) en regulatoire T-cellen (die de afweerreactie onderdrukken) van groot belang. Het recidiveren van uVIN, het ontstaan van schaamlipkanker en het niet reageren op immunotherapie wordt namelijk gekenmerkt door minder cytotoxische T-cellen en meer regulatoire T-cellen (**hoofdstuk 5**).

De effectiviteit van T-cellen, maar ook NK cellen,wordt beïnvloedt door de aan- of afwezigheid van het zogenoemde humaan leukocyten antigeen (HLA) molecuul op afweercellen. Dit molecuul toont aan de buitenkant van de geïnfecteerde cel een klein stukje van het virus waardoor de cel herkent kan worden door T-cellen. In **hoofdstuk 4** hebben we daarom de aanwezigheid van verschillende HLA moleculen bestudeerd. HLA klasse I, dat nodig is voor de herkenning van geïnfecteerde cellen door cytotoxische T-cellen, is verminderd aanwezig bij de meerderheid van uVIN afwijkingen. Dit verlies aan HLA klasse I lijkt echter geen probleem te zijn in de effectiviteit van immunotherapie.. Het verlies aan HLA klasse I in uVIN was meestal niet het gevolg van een genetische irreversibele verandering. In reactie op IFNy (een cytokine kenmerkend voor een type 1 verworven afweerreactie) werd de expressie

HLA klasse I op huidcellen van uVIN weer gestimuleerd. In uVIN patiënten die behandeld werden met het therapeutische HPV-16 SLP vaccin zagen we bovendien dat patiënten die niet reageerden op het vaccin wel HLA klasse I tot expressie te brachten, terwijl patiënten die wel reageerden soms minder HLA klasse I hadden. In schaamlipkanker lijkt dit anders te zijn. Minder HLA klasse I is hier wel vaak het gevolg van een genetische verandering. De niet klassieke HLA moleculen -E en -G, die een remmende werking op T en NK cellen hebben, worden niet tot expressie gebracht in uVIN maar wel in ongeveer de helft van de patiënten met schaamlipkanker. De combinatie van HLA-E en -G expressie met irreversibele genetische veranderingen in HLA klasse I, maakt immunotherapie in schaamlipkanker mogelijk moeilijker dan in uVIN.

De functie en het type van de aanwezige afweercellen in uVIN kunnen beïnvloed zijn. Ze kunnen in een uitgeputte of niet functionele staat verkeren waardoor het afweersysteem de afwijking niet voldoende aanvalt. In **hoofdstuk 5** hebben we daartoe naast het vaststellen van de hoeveelheid en verhouding van de verschillende T cellen ook de aanwezigheid van zogenoemde co-inhibitie moleculen op T-cellen onderzocht. In **hoofdstuk 6** hebben we tevens de hoeveelheid en het type macrofagen in uVIN onderzocht waarna we beide datasets aan elkaar gekoppeld hebben. Een effectieve afweerreactie in uVIN kenmerkt zich door geactiveerde IFNy producerende T-cellen en de relatieve afwezigheid van macrofagen en regulatoire T-cellen. Deze combinatie van afweercellen in de uVIN afwijking is geassocieerd met het (lang) uitblijven van recidieven.
In het lokale afweersysteem van uVIN is het dus van belang dat immunotherapie een goede activatie van het afweersysteem bewerkstelligd die zich kenmerkt door IFNy productie. Een gunstige samenstelling van afweercellen voorafgaand aan immunotherapie zal het waarschijnlijk makkelijker maken om de uVIN afwijking te laten verdwijnen. De afweercellen zijn immers al aanwezig, ze hoeven niet nog naar de afwijking toe te komen en hebben geen verminderde functie. Een boost van de al aanwezige afweerrespons kan dan genoeg zijn, terwijl immunotherapie in patiënten met een onderdrukte, niet effectieve afweerrespons, lastiger zal zijn. Deze patiënten zullen baat hebben bij een bredere aanpak om zowel de functie als het type aanwezige afweercellen te beïnvloeden.

Veranderingen in de systemische afweerreactie in uVIN
Systemische HPV specifieke afweer is van belang voor het verdwijnen van HPV afwijkingen. In het bloed van uVIN patiënten is deze afweer meestal zwak of niet aanwezig. Het opwekken van deze HPV specifieke afweerrespons na therapeutische HPV 16 SLP vaccinatie leidt tot het verdwijnen van de uVIN afwijking. Niet alle patiënten blijken in staat tot het opwekken van sterke HPV specifieke afweer reactie. Dit duidt erop dat er verschillen bestaan in de mogelijkheid van het afweersysteem om te reageren op de therapeutische vaccinatie.

In **hoofdstuk 7** worden, net als eerder voor het lokale afweersysteem, verschillen in type en functie van afweercellen aanwezig in het bloed onderzocht. De functie van T-cellen om signaalstoffen uit te scheiden en van macrofagen om afweercellen te activeren, is gelijk in gezonde controles. Co-inhibitie moleculen op T-cellen zijn nauwelijks aanwezig. Echter net als bij het lokale afweersysteem, zijn IFNγ-producerende T-cellen van belang. In patiënten met recidiverende uVIN afwijkingen komen minder van deze actieve T-cellen voor. Naast deze actieve T-cellen zijn er in patiënten met recidiverende uVIN afwijkingen minder dendritische cellen en meer type 2 (afweer remmende) monocyten. In uVIN patiënten met recidieven lijkt er sprake van een verminderde mogelijkheid tot activatie van de HPV specifieke afweer door een tolerant, minder effectief afweersysteem.
De mogelijkheid tot activatie van afweercellen in reactie op signaalstoffen (cytokines) en het type afweerreactie hierop hebben we onderzocht met behulp van het meten van phosforylatie van signal transduction activators of transcription (STAT). STAT phosforylatie in afweercellen is na stimulatie met verschillende cytokines gelijk in uVIN patiënten en gezonde controles. Stimulatie met afweer stimulerende stoffen IFNα en GM-CSF resulteerde wel in verschillen. In uVIN patiënten lijken de voorloper cellen van antigeen presenterende cellen minder in staat om IFNy geassocieerde responsen op te wekken. In de uVIN patiënten die behandeld werden met therapeutische HPV 16 SLP vaccinatie bleken de patiënten waar het afweersysteem goed reageerde op vaccinatie, ook beter in staat om IFNy geassocieerde responsen op te wekken na stimulatie met IFNα. IFNα kan gezien worden als een sterke afweer modulator die vooral IFNy geassocieerde responsen opwekt. IFNα zou mogelijk aanvullend kunnen zijn op bestaande immunotherapie om hun effecten te versterken.
Bovenstaande resultaten suggereren dat de systemische myeloide cellen (voorlopers van) dendritische cellen en macrofagen) in patiënten met uVIN zowel in type als functie veranderd zijn. Het recidiveren van uVIN is gerelateerd aan type 2 monocyten in het bloed is en de aanwezigheid van macrofagen in de uVIN afwijking. Nader onderzoek is nodig om te kijken of het meten van deze type 2 monocyten kan dienen als bloedmarker. Het meten van deze type 2 monocyten is dan mogelijk een reflectie van het lokale afweersysteem en kan van belang zijn voor de prognose van de patiënt.

Discussie en toekomstperspectieven

In **hoofdstuk 8** worden de bevindingen van dit proefschrift samengevat en worden mogelijke combinaties van immunotherapieën en toekomstige onderzoeksmogelijkheden uitgelicht. Een succesvolle (combinatie van) immunotherapie resulteert zowel lokaal als systemisch in: adequate T-cel activatie, een toename van effector T-cellen, weinig regulatoire T-cellen en het creëren van een IFNy geassocieerde afweerreactie. In deze omstandigheden kan het afweersysteem zijn functie optimaal uitoefenen en de HPV infectie en de afwijking opruimen.

Lokale imiquimod therapie is in staat om het afweersysteem in de uVIN afwijking gunstig te beïnvloeden. Imiquimod resulteert in meer CD8+ cytotoxische T-cellen, minder regulatoire T-cellen, minder macrofagen en minder immature dendritische cellen. Het (deels) verdwijnen van uVIN na imiquimod is niet gerelateerd aan de van te voren aanwezige afweercellen in de afwijking. Dit betekend dat imiquimod een krachtige activator is van het lokale afweersysteem. Imiquimod heeft geen effect op de algehele HPV specifieke afweerreactie in het bloed. Systemische immunotherapie, met name HPV 16 SLP vaccinatie, resulteert wel in sterke systemische HPV specifieke T-cel responsen die geassocieerd zijn met het (deels) verdwijnen van uVIN. In de uVIN afwijking heeft systemische immunotherapie daarentegen weer weinig invloed op het lokale afweersysteem. Het verdwijnen van de uVIN afwijking na systemische immunotherapie is geassocieerd met de van te voren aanwezige afweer cellen. De combinatie van lokale en systemische immunotherapie combineert mogelijk het beste uit twee werelden: activatie van zowel het lokale als het algehele HPV specifieke afweersysteem. Een studie waar patiënten therapeutisch gevaccineerd werden na lokale behandeling met imiquimod liet inderdaad zien dat bij meer vrouwen de uVIN afwijking (deels) verdween.
De verbetering in uitkomst lijkt met name te zitten in het veranderen van het lokale afweer milieu waar de T-cellen hun functie moeten uitoefenen. Er treden al sterke systemische reacties op na therapeutische HPV vaccinatie, ook zonder het aanbrengen van imiquimod.
Patiënten met een bij voorbaat gunstig afweer milieu, zoals aangetoond in dit proefschrift, met reversibele HLA klasse I expressie, veel CD8+ cytotoxische T-cellen, weinig regulatoire T-cellen en weinig tot geen macrofagen, zullen naar alle waarschijnlijkheid goed reageren op immunotherapie. uVIN patiënten die geen gunstige uitgangssituatie hebben voorafgaand aan immunotherapie, zijn mogelijk meer gelijk aan HPV geïnduceerde schaamlipkankers. Bij deze groep patiënten zullen de verschillende aspecten van het falen van het afweer systeem aangepakt moeten worden om wel resultaat te boeken. Naast een combinatie van therapeutische vaccinatie met lokaal imiquimod kan ook gedacht worden aan vermindering van regulatoire T-cellen of macrofagen.
De karakteristieken van afweercellen, aanwezig in uVIN voorafgaand aan immunotherapie, kunnen mogelijk dienen als biomarkers in de individuele patiënt. Deze biomarkers kunnen dan idealiter een reactie op therapie voorspellen. Op deze manier zorg je er voor dat onnodige bijwerkingen van immunotherapie en de eventuele ontwikkeling van schaamlipkanker in de periode van immunotherapie kunnen worden voorkomen.
De kennis van de afweercellen die een rol spelen in uVIN en de progressie die geboekt is in het veld van immunotherapie voor uVIN, maakt dat we nu weten dat deze uVIN afwijkingen in staat zijn om te verdwijnen bij de juiste activatie van het afweersysteem. Deze kennis vormt een basis om in de toekomst verder te gaan met verbetering van immunotherapieën en geeft wellicht de mogelijkheid om therapieën te individualiseren voor de patiënten op basis van de aanwezige afweercellen.

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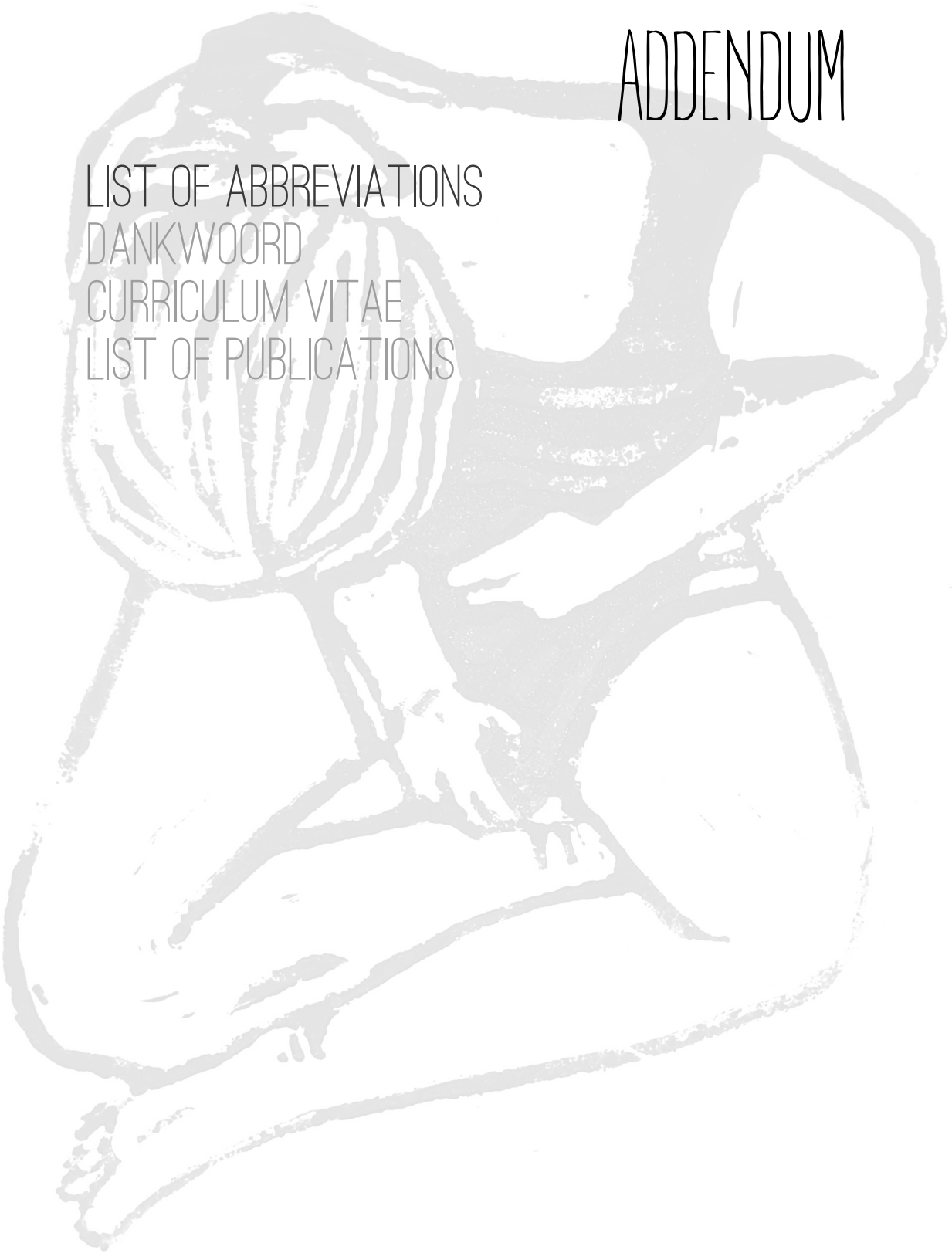

List of Abbreviations

AIN	Anal intraepithelial neoplasia
APC	Antigen presenting cell
β2M	Beta-2 microglobulin
BSA	Bovine Serum Albumin
CBA	Cytometric bead array
CD	Cluster of differentiation (cellular surface receptors that identify cell types and stage of differentiation)
CIN	Cervical intraepithelial neoplasia
COX	Cyclooxygenase
CTL	CD8+ cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4 (CD152)
DAB	Diaminobenzidine
DC	Dendritic cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded tissue
FIGO	International Federation of Gynecology and Obstetrics
FSC	Forward scatter
Gal	Galectin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HPV	Human papillomavirus
hrHPV	High-risk human papillomavirus
lrHPV	Low-risk human papillomavirus
HRP	Horse radish peroxidase
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
ISSVD	International Society for the Study of Vulvovaginal Disease
KC	Keratinocytes
KIR	Killer-immunoglobulin-like receptors
LC	Langerhans cell
LEEP	Loop electrosurgical procedure

LOH	Loss of heterozygosity
LUMC	Leiden University Medical Center
M1	Type 1 macrophages
M2	Type 2 macrophages
MDSC	Myeloid-derived suppressor cell
MICA	MHC class I chain-related molecule A
MHC	Major-histocompatability class
MLR	Mixed lymphocyte reaction
NF-kB	Nuclear factor-kappa B
NK	Natural killer
NKG2	Natural killer receptor family
NLR	Nucleotide-binding oligomerization domain-like receptors
PAIN	Peri-anal intraepithelial neoplasia
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFS	Progression free survival
PHA	Phytohemagglutinine
PD1	Programmed cell death 1
PDL1	Programmed cell death ligand 1
PDT	Photodynamic therapy
PGE2	Prostaglandin E2
PRR	Pathogen recognition receptor
RFS	Recurrence free survival
RLR	RIG-1 like receptors
SCC	Squamous cell carcinoma
SSC	Sideward scatter
SI	Stimulation index
SF	Serum-free IMDM medium
SLP	Synthetic long peptide
STAT	Signal transduction activators of transcription
pSTAT	Phosphorylation of signal transduction activators of transcription
TCR	T-cell receptor
Th	CD4+ T helper
TIL	Tumor infiltrating lymphocyte
TIM3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor

TNF	Tumor necrosis factor
Treg	Regulatory T cell
VAIN	Vaginal intraepithelial neoplasia
VIN	Vulvar intraepithelial neoplasia
dVIN	Differentiated type vulvar intraepithelial neoplasia
uVIN	Usual type vulvar intraepithelial neoplasia

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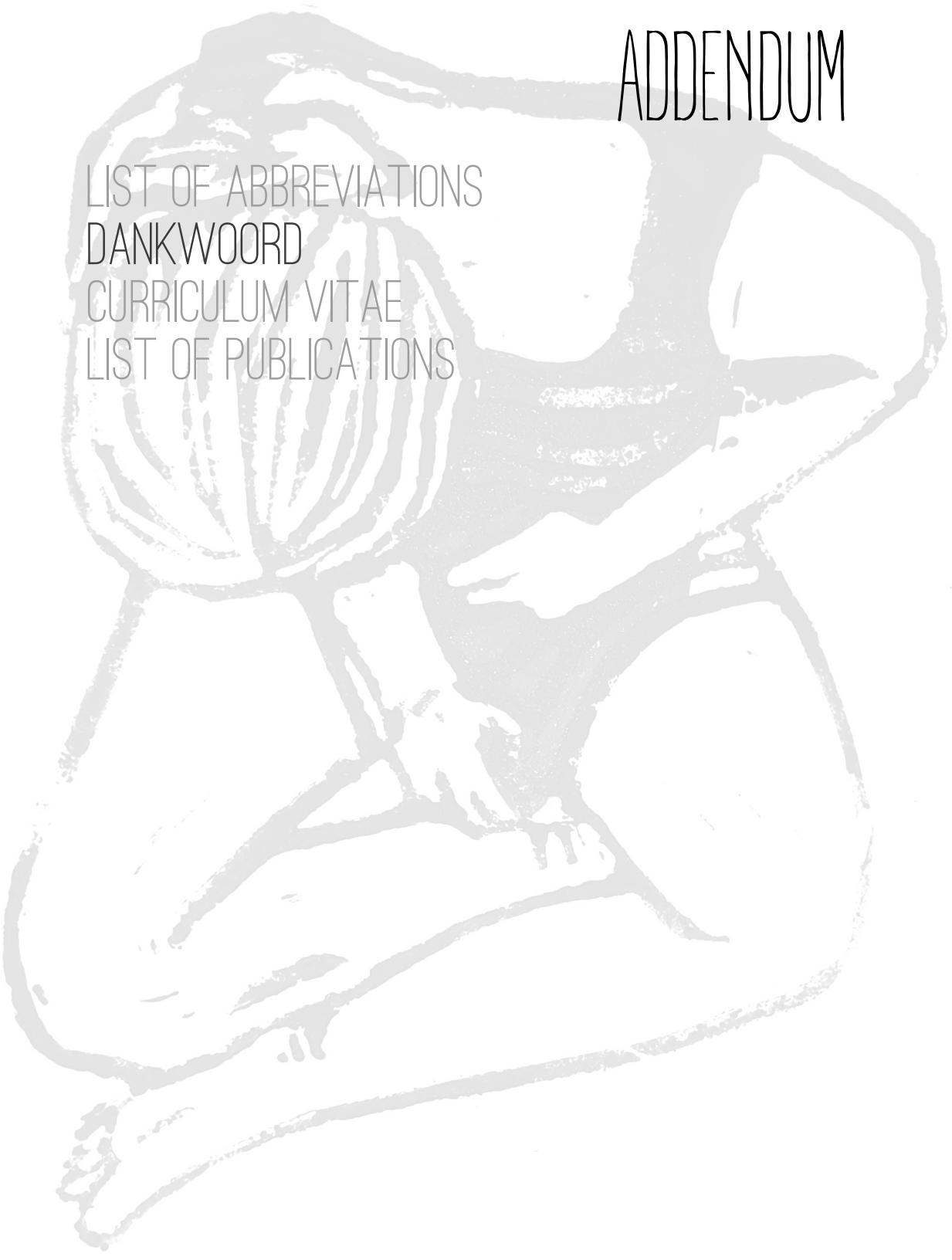

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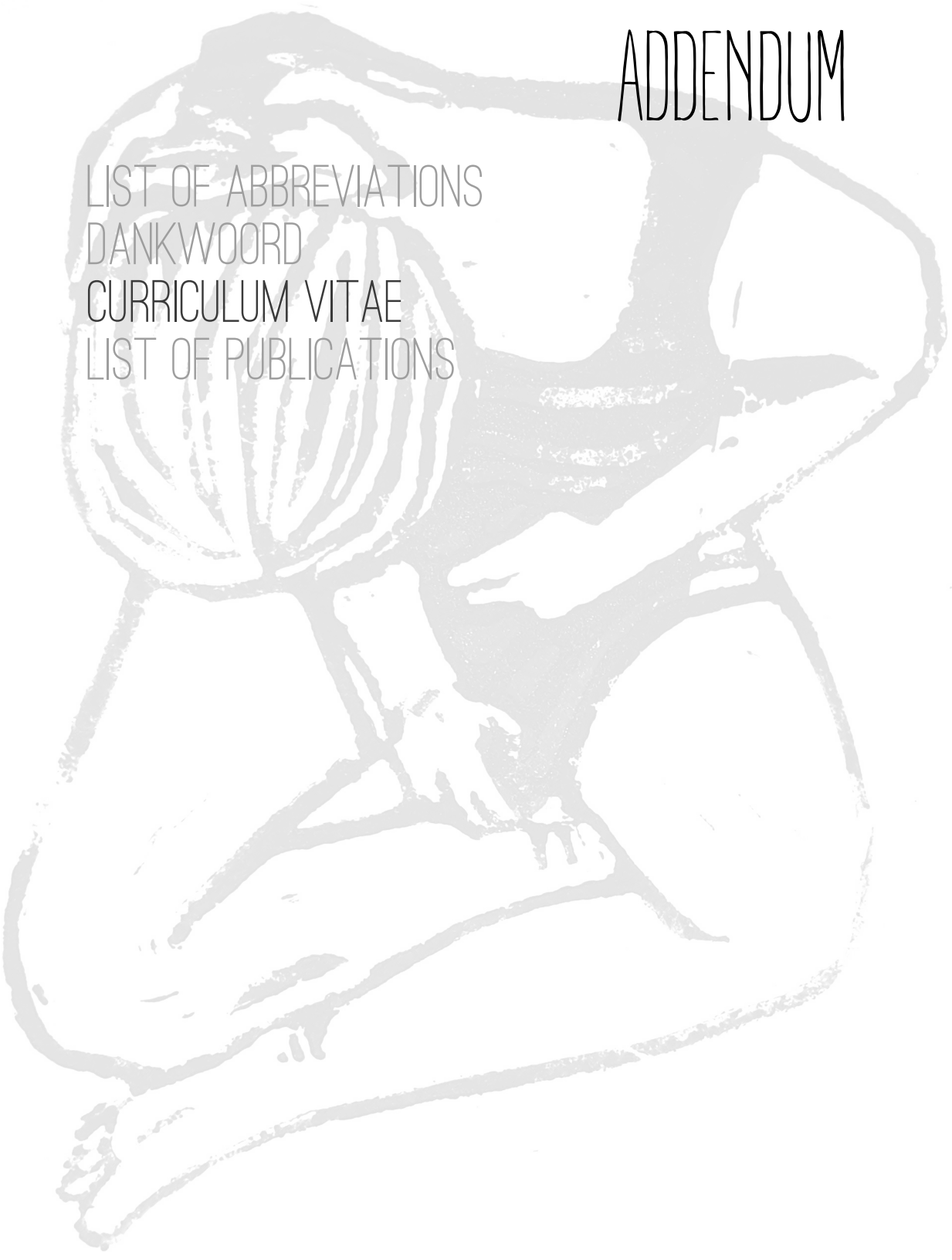

Curriculum Vitae

Edith van Esch was born on the 5th of July 1984 in Tilburg. In 2002 she graduated from secondary school at the Theresia Lyceum (Atheneum) in Tilburg and started medical school at the Leiden University. During her study she went to Indonesia for an (research) internship via the Female Cancer Foundation where her enthusiasm for Obstetrics and Gynaecology was born. In 2007, she followed an Italian language course in Rome for a period five months, after which she continued her medical study with her internships. In 2009 she attained her medical degree and started working as a physician (ANIOS) in Obstetrics and Gynaecology at the Bronovo Hospital, The Hague. In February 2011 she was given the opportunity to start her research which resulted in this thesis at the Departments of Gynaecology, Clinical Oncology and Pathology at the Leiden University Medical Center (LUMC), Leiden, which she initially combined with her work as a physician at the Bronovo Hospital. In October 2011 she was selected for an 'AGIKO' (MD clinical research trainee) grant and the PhD project was continued full-time. The studies in this thesis on clinical implications of immune cell infiltration in vulvar intraepithelial neoplasia were performed at the three departments in the LUMC under supervision of Prof. S.H. van der Burg (Dept. of Clinical Oncology), Dr. M.I.E. van Poelgeest and Prof. J.B.M.Z. Trimbos (Dept. of Gynaecology) and Prof. G.J. Fleuren (Dept. of Pathology). In April 2014 she started her residency in Obstetrics and Gynaecology at the Groene Hart Hospital, Gouda (Dr. J.C.M. van Huisseling and Dr. C.A.H. Janssen) and in September 2015 she continued her residency training at the Department of Obstetrics and Gynaecology at the LUMC, Leiden (Prof. Dr. J.M.M. van Lith). In 2002 she met Joris Hendriks in Leiden with whom she lives together in The Hague with their two daughters Fenne (2013) and Annelot (2015).

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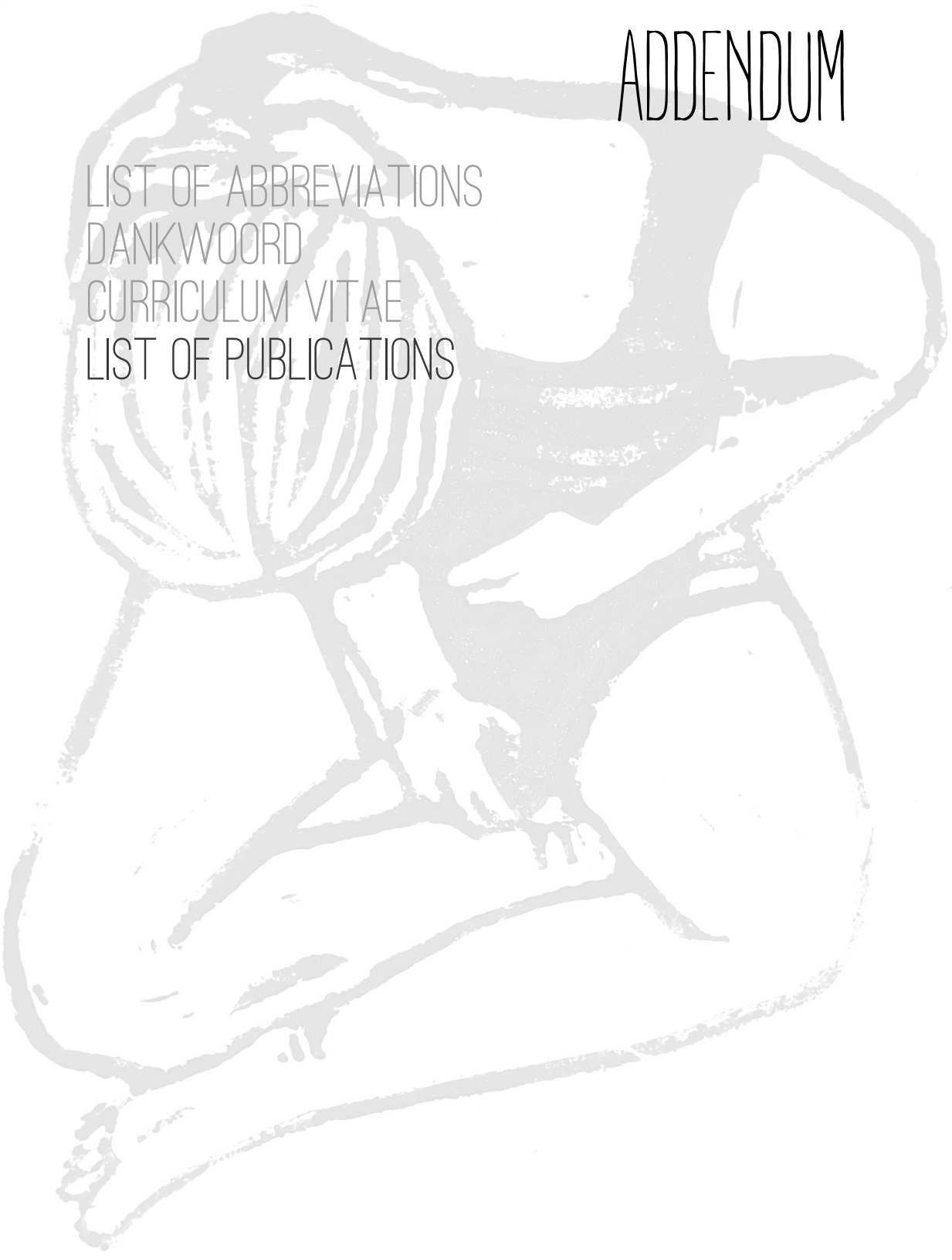

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