Clinical implications of immunecell infiltration in vulvar intraepithelial neoplasia

Esch, Edith Margarethe Gertrude van

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.

References

1. Terlou A, van Seters M, Kleinjan A, Heijmans-Antonissen C, Santegoets LA, Beckmann I et al. Imiquimod-induced clearance of HPV is associated with normalization of immune cell counts in usual type vulvar intraepithelial neoplasia. Int J Cancer 2010; 127(12):2831-2840.
2. Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, Stern PL, Moore JV, Corbitt G et al. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res 2001; 61(1):192-196.
3. Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. Br J Cancer 2010; 102(7):1129-1136.
4. Winters U, Daayana S, Lear JT, Tomlinson AE, Elkord E, Stern PL et al. Clinical and immunologic results of a phase II trial of sequential imiquimod and photodynamic therapy for vulval intraepithelial neoplasia. Clin Cancer Res 2008; 14(16):5292-5299.
5. van Seters M, Beckmann I, Heijmans-Antonissen C, van BM, Ewing PC, Zijlstra FJ et al. Disturbed patterns of immunocompetent cells in usual-type vulvar intraepithelial neoplasia. Cancer Res 2008; 68(16):6617-6622.
6. Santegoets LA, van Seters M, Heijmans-Antonissen C, Kleinjan A, van BM, Ewing PC et al. Reduced local immunity in HPV-related VIN: expression of chemokines and involvement of immunocompetent cells. Int J Cancer 2008; 123(3):616-622.
7. van Seters M, van Beurden M, ten Kate FJ, Beckmann I, Ewing PC, Eijkemans MJ et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. N Engl J Med 2008; 358(14):1465-1473.
8. Winters U, Daayana S, Lear JT, Tomlinson AE, Elkord E, Stern PL et al. Clinical and immunologic results of a phase II trial of sequential imiquimod and photodynamic therapy for vulval intraepithelial neoplasia. Clin Cancer Res 2008; 14(16):5292-5299.
9. Oldenhove G, de HM, Urbain-Vansanten G, Urbain J, Maliszewski C, Leo O et al. CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. J Exp Med 2003; 198(2):259-266.
10. Nakanishi Y, Lu B, Gerard C, Iwasaki A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. Nature 2009; 462(7272):510-513.
11. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer Res 2010; 70(21):8368-8377.
12. Gul N, Ganesan R, Luesley DM. Characterizing T-cell response in low-grade and high-grade vulval intraepithelial neoplasia, study of CD3, CD4 and CD8 expressions. Gynecol Oncol 2004; 94(1):48-53.
13. Davidson EJ, Boswell CM, Sehr P, Pawlita M, Tomlinson AE, McVey RJ et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63(18):6032-6041.
14. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013; 13(4):227-242.
15. Anderson AC. Tim-3, a negative regulator of anti-tumor immunity. Curr Opin Immunol 2012; 24(2):213-216.
16. Sheu BC, Chiou SH, Lin HH, Chow SN, Huang SC, Ho HN et al. Up-regulation of inhibitory natural killer receptors CD94/NKG2A with suppressed intracellular perforin expression of tumor-infiltrating CD8+ T lymphocytes in human cervical carcinoma. Cancer Res 2005; 65(7):2921-2929.
17. Gooden M, Lampen M, Jordanova ES, Leffers N, Trimbos JB, van der Burg SH et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8 T lymphocytes. Proc Natl Acad Sci U S A 2011; 108(26):10656-10661.
18. Rodriguez-Manzanet R, DeKruyff R, Kuchroo VK, Umetsu DT. The costimulatory role of TIM molecules. Immunol Rev 2009; 229(1):259-270.
19. Gieseke F, Kruchen A, Tzaribachev N, Bentzien F, Dominici M, Muller I. Proinflammatory stimuli induce galectin-9 in human mesenchymal stromal cells to suppress T-cell proliferation. Eur J Immunol 2013; 43(10):2741-2749.

20. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 2002; 415(6871):536-541.
21. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 2005; 6(12):1245-1252.
22. Gooden MJ, Wiersma VR, Samplonius DF, Gerssen J, van Ginkel RJ, Nijman HW et al. Galectin-9 activates and expands human T-helper 1 cells. PLoS One 2013; 8(5):e65616.
23. van Esch EM, Tummers B, Baartmans V, Osse EM, Ter HN, Trietsch MD et al. Alterations in classical and nonclassical HLA expression in recurrent and progressive HPV-induced usual vulvar intraepithelial neoplasia and implications for immunotherapy. Int J Cancer 2014; 135(4):830-842.
24. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62(2):472-479.
25. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63(3):636-641.
26. van Poelgeest MI, van Seters M, van Beurden M, Kwappenberg KM, Heijmans-Antonissen C, Drijfhout JW et al. Detection of human papillomavirus (HPV) 16-specific CD4+ T-cell immunity in patients with persistent HPV16-induced vulvar intraepithelial neoplasia in relation to clinical impact of imiquimod treatment. Clin Cancer Res 2005; 11(14):5273-5280.
27. Bourgault Villada I, Moyal BM, Berville S, Bafounta ML, Longvert C, Premel V et al. Human papillomavirus 16-specific T cell responses in classic HPV-related vulvar intra-epithelial neoplasia. Determination of strongly immunogenic regions from E6 and E7 proteins. Clin Exp Immunol 2010; 159(1):45-56.
28. Baldwin PJ, van der Burg SH, Boswell CM, Offringa R, Hickling JK, Dobson J et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9(14):5205-5213.
29. Bourgault Villada I, Moyal BM, Ziol M, Chaboissier A, Barget N, Berville S et al. Spontaneous regression of grade 3 vulvar intraepithelial neoplasia associated with human papillomavirus-16-specific CD4(+) and CD8(+) T-cell responses. Cancer Res 2004; 64(23):8761-8766.
30. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 2009; 361(19):1838-1847.
31. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc Natl Acad Sci U S A 2010; 107(26):11895-11899.
32. Heusinkveld M, van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med 2011; 9:216.
33. Pander J, Heusinkveld M, van der Straaten T, Jordanova ES, Baak-Pablo R, Gelderblom H et al. Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab. Clin Cancer Res 2011; 17(17):5668-5673.
34. Melief CJ. Cancer immunotherapy by dendritic cells. Immunity 2008; 29(3):372-383.
35. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 2012; 12(4):253-268.
36. James EA, DeVoti JA, Rosenthal DW, Hatam LJ, Steinberg BM, Abramson AL et al. Papillomavirus-specific CD4+ T cells exhibit reduced STAT-5 signaling and altered cytokine profiles in patients with recurrent respiratory papillomatosis. J Immunol 2011; 186(11):6633-6640.
37. Talebian YM, Keene KR, Hiemstra PS, van der Burg SH. Recent progress in peptide vaccination in cancer with a focus on non-small-cell lung cancer. Expert Rev Vaccines 2014; 13(1):87-116.
38. Rizza P, Moretti F, Belardelli F. Recent advances on the immunomodulatory effects of IFN-alpha: implications for cancer immunotherapy and autoimmunity. Autoimmunity 2010; 43(3):204-209.
39. Clive KS, Tyler JA, Clifton GT, Holmes JP, Mittendorf EA, Ponniah S et al. Use of GM-CSF as an adjuvant with cancer vaccines: beneficial or detrimental? Expert Rev Vaccines 2010; 9(5):519-525.

40. Zeestraten EC, Speetjens FM, Welters MJ, Saadatmand S, Stynenbosch LF, Jongen R et al. Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. Int J Cancer 2013; 132(7):1581-1591.
41. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol 2003; 3(11):900-911.
42. Hu X, Herrero C, Li WP, Antoniv TT, Falck-Pedersen E, Koch AE et al. Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. Nat Immunol 2002; 3(9):859-866.
43. van der Burg SH, Melief CJ. Therapeutic vaccination against human papilloma virus induced malignancies. Curr Opin Immunol 2011; 23(2):252-257.
44. van der Burg SH, Arens R, Melief CJ. Immunotherapy for persistent viral infections and associated disease. Trends Immunol 2011; 32(3):97-103.
45. Stern PL, van der Burg SH, Hampson IN, Broker TR, Fiander A, Lacey CJ et al. Therapy of human papillomavirus-related disease. Vaccine 2012; 30 Suppl 5:F71-F82.
46. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends- van der Meer TM, et al. Therapeutic Vaccination in Vulvar/Vaginal Intraepithelial Neoplasia: a Randomized Controlled Study with Imiquimod as Adjuvant. Submitted 2015.
47. Daayana S, Winters U, Stern PL, Kitchener HC. Clinical and immunological response to photodynamic therapy in the treatment of vulval intraepithelial neoplasia. Photochem Photobiol Sci 2011; 10(5):802-809.
48. Schon MP, Schon M. Immune modulation and apoptosis induction: two sides of the antitumoral activity of imiquimod. Apoptosis 2004; 9(3):291-298.
49. Schiller M, Metze D, Luger TA, Grabbe S, Gunzer M. Immune response modifiers--mode of action. Exp Dermatol 2006; 15(5):331-341.
50. Soong RS, Song L, Trieu J, Knoff J, He L, Tsai YC et al. Toll-like receptor agonist imiquimod facilitates antigen-specific CD8+ T-cell accumulation in the genital tract leading to tumor control through IFNgamma. Clin Cancer Res 2014; 20(21):5456-5467.
51. Brode S, Cooke A. Immune-potentiating effects of the chemotherapeutic drug cyclophosphamide. Crit Rev Immunol 2008; 28(2):109-126.
52. Peng S, Lyford-Pike S, Akpeng B, Wu A, Hung CF, Hannaman D et al. Low-dose cyclophosphamide administered as daily or single dose enhances the antitumor effects of a therapeutic HPV vaccine. Cancer Immunol Immunother 2013; 62(1):171-182.
53. Menard C, Ghiringhelli F, Roux S, Chaput N, Mateus C, Grohmann U et al. Ctla-4 blockade confers lymphocyte resistance to regulatory T-cells in advanced melanoma: surrogate marker of efficacy of tremelimumab? Clin Cancer Res 2008; 14(16):5242-5249.
54. Leao IC, Ganesan P, Armstrong TD, Jaffee EM. Effective depletion of regulatory T cells allows the recruitment of mesothelin-specific CD8 T cells to the antitumor immune response against a mesothelin-expressing mouse pancreatic adenocarcinoma. Clin Transl Sci 2008; 1(3):228-239.
55. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000; 192(2):303-310.
56. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med 2009; 206(8):1717-1725.
57. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med 2013; 210(9):1695-1710.
58. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA et al. Activating Fc gamma receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med 2013; 210(9):1685-1693.
59. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. Cancer Immunol Res 2013; 1(1):32-42.
60. Cao Y, Zhao J, Yang Z, Cai Z, Zhang B, Zhou Y et al. CD4+FOXP3+ regulatory T cell depletion by low-dose cyclophosphamide prevents recurrence in patients with large condylomata acuminata after laser therapy. Clin Immunol 2010; 136(1):21-29.

61. Vermeij R, Leffers N, Hoogeboom BN, Hamming IL, Wolf R, Reyners AK et al. Potentiation of a p53-SLP vaccine by cyclophosphamide in ovarian cancer, a single arm phase II study. Int J Cancer 2011.
62. Yao S, Zhu Y, Chen L. Advances in targeting cell surface signalling molecules for immune modulation. Nat Rev Drug Discov 2013; 12(2):130-146.
63. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012; 12(4):252-264.
64. Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. Clin Exp Immunol 2009; 157(1):9-19.
65. Heeren AM, Koster BD, Samuels S, Ferns DM, Chondronasiou D, Kenter GG et al. High and Interrelated Rates of PD-L1+CD14+ Antigen-Presenting Cells and Regulatory T Cells Mark the Microenvironment of Metastatic Lymph Nodes from Patients with Cervical Cancer. Cancer Immunol Res 2015; 3(1):48-58.
66. de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, Doorduijn EM, van Ham JJ, Gorter A et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. Int J Cancer 2013.
67. van der Sluis TC, Sluijter M, van Duikeren S, West BL, Melief CJ, Arens R et al. Therapeutic peptide vaccine-induced CD8 T cells strongly modulate intratumoral macrophages required for tumor regressions. Submitted 2015
68. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, Ramwadhdoebe TH, Gorter A, Welters MJ et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. J Immunol 2011; 187(3):1157-1165.
69. Dijkgraaf EM, Heusinkveld M, Tummers B, Vogelpoel LT, Goedemans R, Jha V et al. Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. Cancer Res 2013; 73(8):2480-2492.
70. Legge F, Paglia A, D'Asta M, Fuoco G, Scambia G, Ferrandina G. Phase II study of the combination carboplatin plus celecoxib in heavily pre-treated recurrent ovarian cancer patients. BMC Cancer 2011; 11:214.
71. Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Leinster DA et al. Interleukin-6 as a therapeutic target in human ovarian cancer. Clin Cancer Res 2011; 17(18):6083-6096.
72. Dijkgraaf EM, Welters MJ, Nortier JW, van der Burg SH, Kroep JR. Interleukin-6/interleukin-6 receptor pathway as a new therapy target in epithelial ovarian cancer. Curr Pharm Des 2012; 18(25):3816-3827.
73. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. Cancer Res 2014; 74(18):5057-5069.
74. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L et al. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. Cancer Res 2014; 74(1):153-161.
75. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. Cancer Res 2013; 73(3):1128-1141.
76. Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. Semin Immunol 2009; 21(5):265-272.
77. Tummers B, Goedemans R, Jha V, Meyers C, Melief CJ, van der Burg SH et al. CD40-mediated amplification of local immunity by epithelial cells is impaired by HPV. J Invest Dermatol 2014; 134(12):2918-2927.
78. Shime H, Matsumoto M, Oshiumi H, Tanaka S, Nakane A, Iwakura Y et al. Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors. Proc Natl Acad Sci U S A 2012; 109(6):2066-2071.
79. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res 2005; 65(8):3437-3446.
80. Preti M, Scurry J, Marchitelli CE, Micheletti L. Vulvar intraepithelial neoplasia. Best Pract Res Clin Obstet Gynaecol 2014.

81. Tristram A, Hurt CN, Madden T, Powell N, Man S, Hibbitts S et al. Activity, safety, and feasibility of cidofovir and imiquimod for treatment of vulval intraepithelial neoplasia (RTVIN): a multicentre, open-label, randomised, phase 2 trial. Lancet Oncol 2014.
82. Hampl M, Sarajuuri H, Wentzensen N, Bender HG, Kueppers V. Effect of human papillomavirus vaccines on vulvar, vaginal, and anal intraepithelial lesions and vulvar cancer. Obstet Gynecol 2006; 108(6):1361-1368.
83. Rondy M, van Lier A, van de Kassteele J, Rust L, de MH. Determinants for HPV vaccine uptake in the Netherlands: A multilevel study. Vaccine 2010; 28(9):2070-2075.
84. Hildesheim A, Herrero R, Wacholder S, Rodriguez AC, Solomon D, Bratti MC et al. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. JAMA 2007; 298(7):743-753.