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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.
Expression of co-inhibitory receptors in the microenvironment in usual VIN

Introduction

Usual type vulvar intraepithelial neoplasia (uVIN) is caused by a persistent high risk HPV (hrHPV) infection, mainly type 16. The lifetime risk of an HPV infection is approximately 80% and around 40% of female adolescents become infected at least once with an hrHPV. HPV infections proceed asymptptomatically in 90% of cases if the immune system is capable to clear the infection within two years. Spontaneous regression and clearance of HPV is associated with systemic HPV-specific CD4+ and CD8+ T cell responses. uVIN lesions have a malignant potential in 10% of untreated and 3-4% of treated patients and recurrence rates are high after conventional treatments. Treatment for uVIN includes potential disfiguring interventions associated with psychosexual consequences. 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.

Although a few studies suggest that failure to respond to immunotherapy is related to a local immunosuppressive microenvironment, 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. Moreover, some uVIN lesions are infiltrated by high numbers of regulatory T cells (Tregs). 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.

Clinical response to immunotherapy in uVIN is associated with an increase in intralesional CD8+ T cells as well to low numbers of Tregs. 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 ). 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. The interaction between PD1 and PD-L1 impairs T cell function through downregulation of proinflammatory cytokine production or apoptosis. Monoclonal antibodies blocking the co-inhibitory molecules PD1 or PD-L1 resulted in an improved clinical outcome in cancer. 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 genotyipne 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$_2$O$_2$/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$_2$O$_2$ 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 nucliar 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 mm² 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 marker in our study cohort. 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.\textsuperscript{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>
<thead>
<tr>
<th>Immune cell types</th>
<th>Controls</th>
<th>uVIN</th>
<th>Carcinoma</th>
<th>P-value*</th>
<th>P-value*</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>uVIN vs. Controls</td>
<td>uVIN vs. Carcinoma</td>
<td>Controls vs. Carcinoma</td>
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<tr>
<td></td>
<td>N=26</td>
<td>N=43</td>
<td>N=21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+ (E)</td>
<td>209.92 (36-559)</td>
<td>185.99 (54-763)</td>
<td>240.99 (21-1222)</td>
<td>0.310</td>
<td>0.427</td>
<td>0.818</td>
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<td>CD3+ (S)</td>
<td>219.62 (34-976)</td>
<td>669.64 (28-2180)</td>
<td>981.37 (199-1928)</td>
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<td>0.307</td>
<td>0.000*</td>
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<td>CD4+ (E)</td>
<td>55.22 (3-406)</td>
<td>54.89 (3-245)</td>
<td>65.58 (7-293)</td>
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<td>0.563</td>
<td>0.774</td>
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<td>CD4+ (S)</td>
<td>149.26 (17-738)</td>
<td>362.62 (28-1305)</td>
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<td>0.983</td>
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<td>CD8+ (E)</td>
<td>131.34 (21-574)</td>
<td>107.93 (20-647)</td>
<td>127.90 (6-930)</td>
<td>0.373</td>
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<td>0.077</td>
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<td>CD4+FoxP3+ (E)</td>
<td>3.75 (0-27)</td>
<td>19.46 (0-152)</td>
<td>70.55 (4-610)</td>
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<td>CD4+FoxP3+ (S)</td>
<td>48.23 (11-208)</td>
<td>511.96 (49-2002)</td>
<td>872.59 (141-1500)</td>
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<td>0.000*</td>
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<td>Ratio CD8+/FoxP3+ (E)</td>
<td>9.67 (0.00-218.68)</td>
<td>4.13 (0.00-60.16)</td>
<td>1.52 (0.10-12.71)</td>
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<td>Ratio CD8+/FoxP3+ (S)</td>
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<td>0.55 (0.00-2.03)</td>
<td>0.58 (0.09-1.04)</td>
<td>0.000*</td>
<td>0.994</td>
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<td>Tbet (E)</td>
<td>99.64 (7.88-225.88)</td>
<td>55.31 (6.36-449.15)</td>
<td>70.93 (8.8-538.74)</td>
<td>0.116</td>
<td>0.679</td>
<td>0.843</td>
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<td>Tbet (S)</td>
<td>143.06 (9.61-361.57)</td>
<td>339.15 (24.02-2115.11)</td>
<td>303.32 (83.83-1028.28)</td>
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<td>0.679</td>
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<td>CD3+PD1+ (E)</td>
<td>191.08 (23-662.74)</td>
<td>96.18 (0.00-477.78)</td>
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<td>200.10 (70-18-539.40)</td>
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<td>71.32 (25.33-263.46)</td>
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<td>CD3+VNG2A+ (E)</td>
<td>17.43 (0.00-295.61)</td>
<td>14.23 (0.00-414.71)</td>
<td>11.93 (0.00-113.43)</td>
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<td>4.40 (0.00-161.85)</td>
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<td>CD3+T1M3+ (E)</td>
<td>5.28 (0.00-123.28)</td>
<td>45.49 (7.40-184.90)</td>
<td>51.62 (23.84-694.64)</td>
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<td>0.129</td>
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<td>CD3+T1M3+ (S)</td>
<td>22.65 (0.00-108.80)</td>
<td>122.91 (15.12-571.40)</td>
<td>191.01 (47.25-511.96)</td>
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<td>CD4+T1M3+ (E)</td>
<td>0.00 (0.00-70.88)</td>
<td>17.89 (0.00-69.54)</td>
<td>17.36 (4.92-167.28)</td>
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<td>CD4+T1M3+ (S)</td>
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<td>104.12 (10.81-462.48)</td>
<td>128.33 (8.8-538.74)</td>
<td>0.116</td>
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<td>0.843</td>
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<td>CD8+T1M3+ (E)</td>
<td>3.33 (0.00-64.48)</td>
<td>17.78 (0.00-165.93)</td>
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<td>CD8+T1M3+ (S)</td>
<td>4.51 (0.00-19.71)</td>
<td>22.86 (0.00-220.36)</td>
<td>67.38 (6-930)</td>
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<td>0.009*</td>
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<td>150.11 (21.36-743.36)</td>
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<td>1648.57 (753.08-5321.03)</td>
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<td>Galectin3 total (E)</td>
<td>95.26 (0.00-461.33)</td>
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<td>Galectin3 total (S)</td>
<td>693.63 (381.65-1083.60)</td>
<td>936.81 (246.89-3120.24)</td>
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<td>Galectin9 total (E)</td>
<td>76.57 (0.00-70.96)</td>
<td>98.21 (23.53-325.33)</td>
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<td>0.216</td>
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<td>Galectin9 total (S)</td>
<td>565.36 (221.28-1418.81)</td>
<td>790.14 (99.61-2319.27)</td>
<td>0.021*</td>
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<td>Galectin1/3/9 total (E)</td>
<td>1177.36 (618.00-2561.00)</td>
<td>1919.00 (922.23-5706.00)</td>
<td>0.000*</td>
<td>n.e.</td>
<td>n.e.</td>
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</tr>
</tbody>
</table>

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, * = 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/mm² 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/mm²) than control tissue (range 34-976 cells/mm²), 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 vulvar carcinoma. 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 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, 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 patients 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 and expression of the other galectins is more likely to be associated with neoplastic cell changes, 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+ (IFN-γ) 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 (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 (IFN-γ) 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.
Expression of co-inhibitory receptors in the microenvironment in usual VIN

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. 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. However, the expression of these receptors by activated T cells has been shown to primarily reflect T cell differentiation and activation. 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. 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. 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 while in vitro, Gal-9 exposed immature DCs become activated and produce IL-12. 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. 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. 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. 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. 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. 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.\textsuperscript{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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Expression of co-inhibitory receptors in the microenvironment in usual VIN

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Supporting Information


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