CHAPTER 6

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

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Int J Cancer 2015 Feb; 136: 85-94

Abstract

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

Introduction

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

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

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

Material and Methods

Patient material

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

Triple immunofluorescent confocal microscopy

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

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

Data analysis

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

Results

Patient characteristics

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

Table 1: Patient Characteristics

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

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

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

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

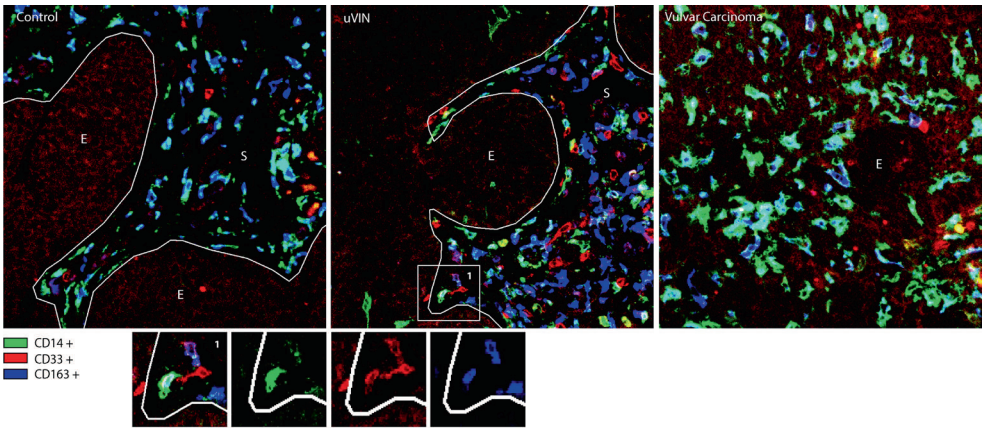

Figure 1: **Immunofluorescent staining of uVIN, vulvar carcinoma and control tissue with antibodies against CD14+ CD33+ and CD163+**

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

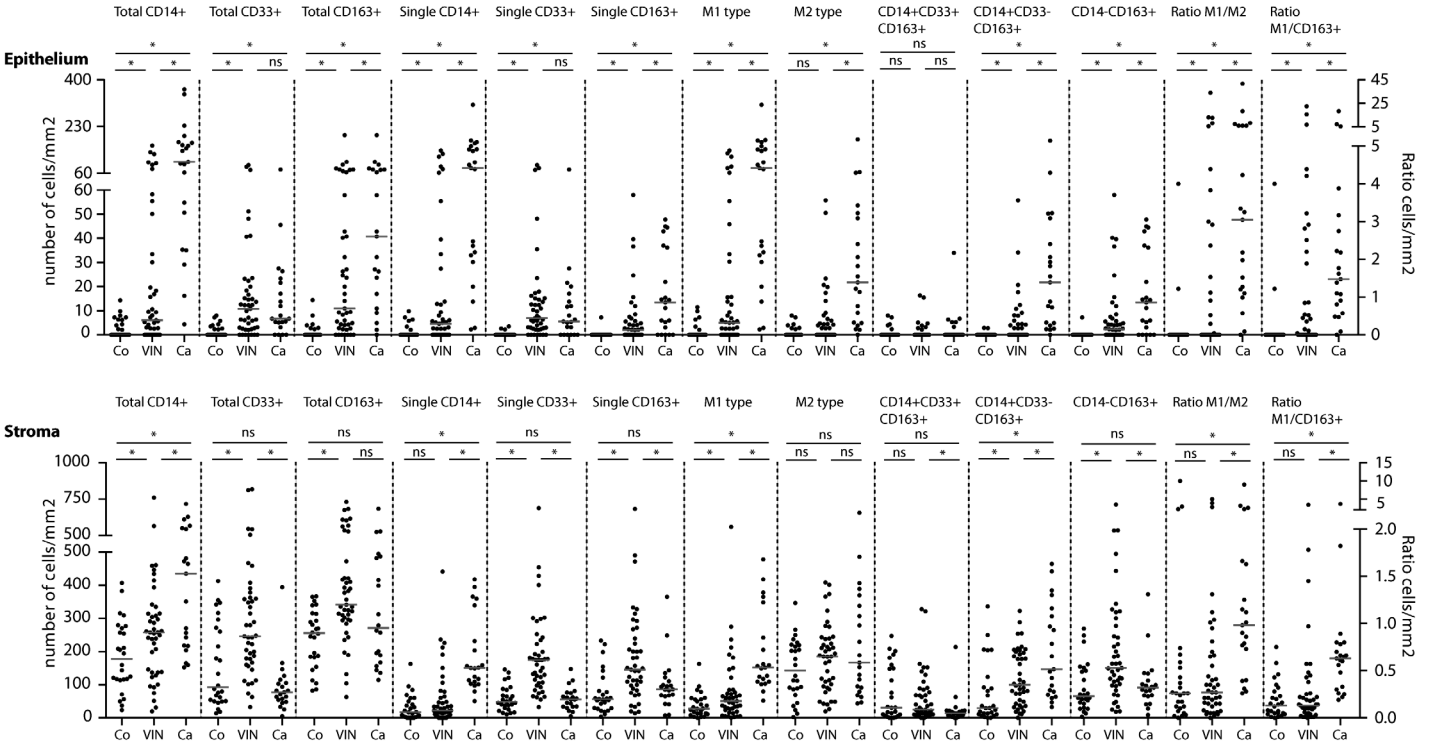

Figure 2: **Myeloid cell infiltrates during the progressive course of vulvar neoplasia and ratios of M1 monocytes in comparison to M2 monocytes and total CD163+ type 2 myeloid cells**

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

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

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

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

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

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

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

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

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

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

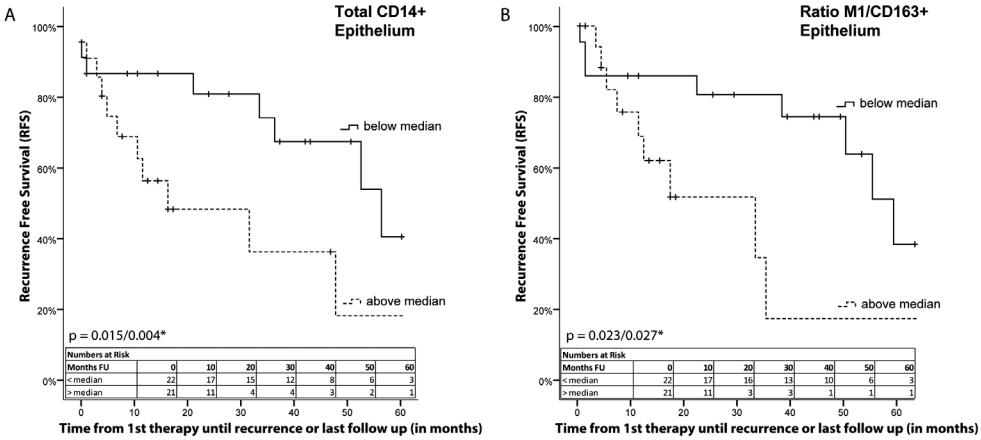

Figure 3: **Influence of intraepithelial CD14+ and ratio M1/CD163+ infiltrates on recurrence free survival**

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

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

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

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

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

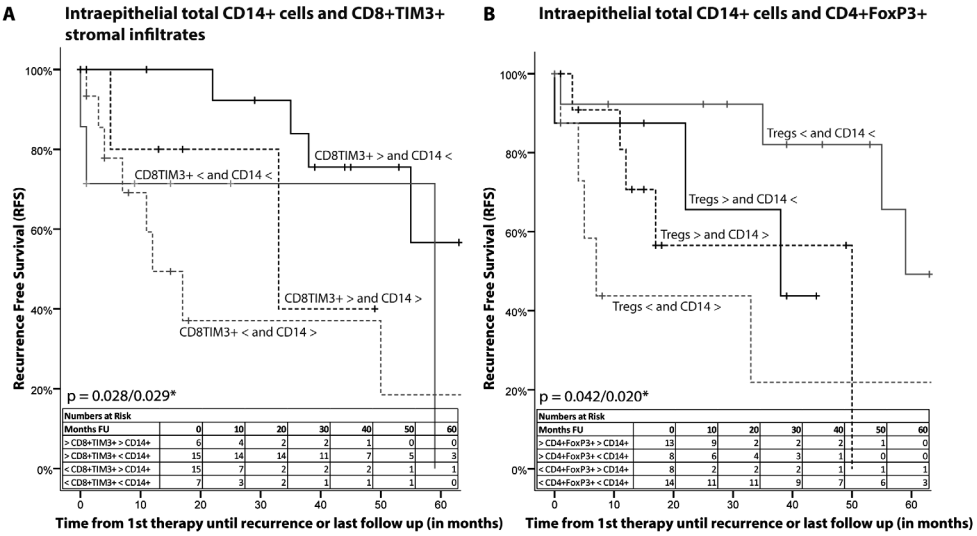

Figure 4: **Combinatorial analysis of intraepithelial CD14+ macrophages and stromal CD8+ TIM3+ T cells or intraepithelial regulatory T cells**

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

Discussion

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

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

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

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

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

Acknowledgements

We gratefully thank all the patients and healthy controls who have participated in this study (recruited from the HAGA teaching hospital, The Hague, The Netherlands, Dept. of Gynaecology: Dr. B.W.J. Hellebrekers and Dr. W. Kolkman, and from the Bronovo Hospital, The Hague, The Netherlands, Dept. of Plastic Surgery: Dr. T. Tan and Dept of Gynaecology: Dr. C.A.G. Holleboom and Dr. H.T.C. Nagel and from the Park Medical Centre Rotterdam, The Netherlands: Dr. T. Tan). Furthermore E.M. Osse and N. ter Haar for cutting of tissue sections and HPV typing, M.D. Trietsch for selection of the HPV positive vulvar carcinomas and F. Prins for advice in confocal microscopy all from the Department of Pathology, LUMC, Leiden, The Netherlands. The authors disclose no potential conflicts of interest.

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Supporting Information

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

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

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

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