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

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Abstract

Purpose: Topical application of the immune response modifier imiquimod is an alternative approach for the treatment of human papillomavirus (HPV)–positive vulvar intraepithelial neoplasia (VIN) and aims at the immunologic eradication of HPV-infected cells. We have charted HPV16-specific immunity in 29 patients with high-grade VIN and examined its role in the clinical effect of imiquimod treatment.

Experimental Design: The magnitude and cytokine polarization of the HPV16 E2-, E6-, and E7-specific $CD4^+$ T-cell response was charted in 20 of 29 patients by proliferation and cytokine bead array. The relation between HPV16-specific type 1 T-cell immunity and imiquimod treatment was examined in a group of 17 of 29 patients.

Results: HPV16-specific proliferative responses were found in 11 of the 20 patients. In eight of these patients, T-cell reactivity was associated with IFNγ production. Fifteen of the women treated with imiquimod were HPV16$^+$, of whom eight displayed HPV16 E2- and E6-specific T-cell immunity before treatment. Imiquimod neither enhanced nor induced such immunity in any of the subjects. Objective clinical responses (complete remission or >75% regression) were observed in 11 of the 15 patients. Of these 11 responders, eight patients displayed HPV16-specific type 1 $CD4^+$ T-cell immunity, whereas three lacked reactivity. Notably, the four patients without an objective clinical response also lacked HPV16-specific type 1 T-cell immunity.

Conclusions: HPV16-specific IFNγ-associated $CD4^+$ T-cell immunity, although not essential for imiquimod-induced regression of VIN lesions, may increase the likelihood of a strong clinical response ($P = 0.03$).

Genital infections with high-risk human papillomaviruses (HPV) are very common (1–3). Fortunately, the majority of infected subjects clear the infection (4, 5). A persistent infection with a high-risk HPV, mostly HPV16, can lead to neoplasia of the anogenital tract, of which cervical intraepithelial neoplasia and cervical carcinoma are the most well known (6, 7). HPV16 infection may also cause a chronic skin disorder of the vulva known as vulvar intraepithelial neoplasia (VIN; refs. 8–10). In contrast to cervical intraepithelial neoplasia, which in general is effectively treated by eradication of the area involved, VIN is a chronic disease with high relapse rates after standard treatments (11–13).

Imiquimod therapy has been put forward as an alternative approach for the treatment of VIN. This immune response modifier acts through Toll-like receptor seven of the innate immune system resulting in the secretion of a multitude of proinflammatory cytokines. There is recent evidence that imiquimod also possesses direct proapoptotic activity against tumor cells (14–16). Topical application preserves the anatomy and function of the vulva, whereas surgical excision or ablation of affected skin may be extensive and disfiguring and can carry considerable psychosexual morbidity. Clinical success rates differ and are estimated on 30% to 87% (17–21).

The HPV16 early antigens E2, E6, and E7 are among the first of proteins that are expressed in HPV-infected epithelia. Our previous studies on HPV-specific T-cell immunity against these early antigens showed that type 1 (IFNγ) T-cell memory against the early antigens can be detected in the majority of healthy sexually active individuals but is weak or absent in

patients with HPV16-induced cervical neoplasia (22–24). In combination with earlier reports that point at a role for CD4+ T cells in the protection against progressive HPV infection (reviewed in ref. 25), our data argue that the CD4+ type 1 T-cell response against the early antigens of HPV16 plays an important role in the protection against progressive HPV16-induced disease.

To examine the role of HPV16-specific CD4+ T-cell immunity in the success or failure of treatment with imiquimod, we have done a detailed analysis with respect to the magnitude and cytokine polarization of the HPV16-specific CD4+ T-cell response in patients with high-grade VIN. Furthermore, HPV16-specific type 1 immunity was analyzed before, during, and after topical treatment with imiquimod. Our data indicate that chronic exposure of the immune system to the HPV16 viral proteins results in the induction of type 1 T-cell immunity in about half of the patients. Importantly, the presence of these type 1 T-cell responses is likely to be associated with a more favorable clinical response to imiquimod treatment.

Materials and Methods

Patients. Twenty-nine women with high-grade VIN (age range, 24-73 years; median age, 47 years) were recruited from the departments of gynecology of the Academic Medical Center and Leiden and Erasmus University Medical Center, The Netherlands. On the average, these patients had been diagnosed with VIN3 5.4 years before enrollment in the study (range, 6 months to 15 years). Eighteen women had undergone previous treatments for VIN3 [surgical excision, laser therapy, or imiquimod treatment (patients 20, 21, 24, 27)] before study entry.

Seventeen of these 29 subjects (age, 29-60 years; median, 43 years) were experimentally treated with a 5% imiquimod cream. The patients were asked to apply the cream to the affected areas on the vulva twice weekly overnight for a maximum period of 16 weeks. To analyze the effect of imiquimod treatment on the HPV16-specific immune response, we collected serial blood and serum samples before the start of imiquimod treatment ($T = 0$), after 8 weeks of treatment ($T = 8$), and at the end of treatment ($T = 16$). Vulvar lesions were assessed by direct measurement and photographic records at entry and after 8 and 16 weeks of treatment. Clinical responses were defined as a complete response; a partial response type 1, as defined by a reduction in lesion diameter from 76% to 99%; a partial response type 2, as defined by a reduction in lesion diameter from 26% to 75%; or no clinical response.

From 20 of 29 women peripheral blood mononuclear cells (PBMC) were isolated and directly used to analyze HPV16-specific proliferative T-cell reactivity. Of these 20 women, eight patients had also participated in the imiquimod study. In six cases blood was taken 3 months (patient 1), 4 months (patient 10), 10 months (patient 5) to over 1 year (patients 12, 13 and 15) after the end of the imiquimod study, in the other two cases (patients 2 and 4) blood was taken within 4 weeks after the start of treatment. Serum was collected to study the presence of virus-like particle L1 (VLP)–specific antibodies.

All subjects were typed for HPV by GP5+/6+ PCR followed by reverse line blot analysis as described previously (26). The study design was approved by the Medical Ethical Committees and all women gave written informed consent.

Antigens. A set of peptides spanning the whole HPV16 E2, E6, and E7 protein were used for the T-cell proliferation assays. The E2 peptides consisted of twenty-two 30-mer peptides with a 15-amino-acid overlap and the COOH-terminal peptide with a length of 35 amino acids. For the T-cell proliferation assays, the E2 peptides, 32-mer peptides of the E6 protein, and the 35-mer peptides of the E7 protein with an overlap of 14 amino acids were used in pools of two peptides per pool. For the IFNγ enzyme-linked immunospot (ELISPOT) assays, the peptides used

spanned the HPV 16 E2, E6, and E7 protein and consisted of the most immunogenic regions of the E2 30-mer peptides (22) and 15 E6 and nine E7 overlapping 22-mer peptides. The peptides were synthesized and dissolved as described previously (27). The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (e.g., E2$_{1-45}$, residues 1-30 and 16-45). Memory response mix, consisting of a mixture of tetanus toxoid (0.75 *Limus flocculentius*/mL final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 μg/mL; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands), and *Candida albicans* (0.005%, HAL Allergenen Lab., Haarlem, The Netherlands), was used as a positive control.

Short-term T-cell proliferation assay. Freshly isolated PBMCs were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, four pools of E6 32-mer peptides, and two pools of E7 35-mer peptides (each pool consisted of two overlapping peptides). PBMCs were seeded at a density of 1.5×10^5 cells per well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 125 μL of Iscove's medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% autologous serum. HPV16 E2-, E6-, and E7-derived peptides were added at a concentration of 10 μg/mL/peptide. Medium alone was taken along as a negative control, and memory response mix (dilution, 1:50) served as a positive control. For each peptide pool, eight parallel microcultures were incubated. Fifty microliters of supernatant from the microcultures were taken at day 6 after incubation and stored at $-20°C$ until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [^{3}H]-thymidine incorporation. Cultures were scored positive when the proliferation of ≥75% of the test wells exceeded the mean proliferation + 3× SD of the control wells containing medium only, and the stimulation index, defined as the mean of all test wells divided by the mean of the control wells, was ≥3 (22).

Analysis of cytokines associated with HPV16-specific proliferative responses. The detection of cytokines in the supernatants of the short-term proliferation assays was done using the cytometric bead array (Becton Dickinson, Erebodegem-Aalst, Belgium). This technique allows the simultaneous detection of six different Th1 and Th2 cytokines IFNγ, tumor necrosis factor α, interleukin 2 (IL-2), IL-4, IL-5, and IL-10. The cytometric bead array was done according to the manufacturer's instructions. Cutoff values were based on the standard curves of the different cytokines (50 pg/mL for IFNγ and 10 pg/mL for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and >2× the concentration of the medium control (23, 28).

Analysis of HPV16-specific T-cell reactivity by IFNγ enzyme-linked immunospot. The number of IFNγ producing HPV-specific T cells, present in the peripheral blood of the 17 patients treated with imiquimod, was quantified using ELISPOT that was done as described previously (29, 30). Briefly, PBMC were thawed, washed, and seeded at a density of 2×10^6 cells per well of a 24-well plate (Costar) in 1 mL of Iscove's modified Dulbecco's medium (Bio Whittaker) enriched with 10% human AB serum, in the presence or absence of indicated HPV 16 E2, E6, and E7 peptide pools. Peptides were used in pools of four to five peptides at a concentration of 5 μg/mL/peptide. The peptides, as indicated by their first and last amino acid in the protein, were used in the following pools: *E2-I*: 1-30, 16-45, 31-60, 46-75; *E2-II*: 61-90, 76-105, 91-120, 106-135; *E2-III*: 121-150, 136-165, 151-180, 166-195; *E2-IV*: 271-300, 286-315, 301-330, 316-345, 331-365; *E6-I*: 1-22, 11-32, 21-42, 31-52; *E6-II*: 41-62, 51-72, 61-82, 71-92; *E6-III*: 81-102, 91-112, 101-122, 111-132; *E6-IV*: 111-132, 121-142, 131-152, 137-158; *E7-I*: 1-22, 11-32, 21-42, 31-52; *E7-II*: 41-62, 51-72, 61-82, 71-92, 77-98. Following 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100 μL Iscove's modified Dulbecco's medium enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ-catching antibody (Mabtech AB, Nacha,

Sweden). Further antibody incubations and development of the ELISPOT was done according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (Biosys, Frankfurt, Germany). Specific spots were calculated by subtracting the mean number of spots + 2× SD of the medium control from the mean number of spots in experimental wells provided that the mean number of spots of the medium control wells were either <10 or >10 with a SD <20% of the mean. Antigen-specific T-cell frequencies were considered to be increased when specific T-cell frequencies were ≥1 in 10,000 and at least ≥2× background (30). The background number of spots was 2.6 ± 2.2 (mean ± SD), with one exception (patient 23, 51 ± 10 spots).

HPV16 virus-like particle ELISA. For the detection of HPV16-specific antibodies in serum we used an ELISA method previously described by Kirnbauer et al. (31). Each serum sample was tested for reactivity against HPV16 VLPs (baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus capsids, the latter disrupted by treatment with 0.1 mol/L carbonate buffer to serve as a negative control. Both VLP and bovine papillomavirus were kindly provided by Prof. Dr. J. Dillner (LUNDS University, Sweden). The patients were tested for both HPV16-specific IgG and IgA. A set of sera of healthy children (*n* = 8; mean age, 7.3 years; range, 4.3-14.1 years) was tested to determine background reactivity. For HPV16 L1-VLP IgG type responses a cutoff absorbance value of 0.230 was used (mean *A* = 0.060; range, −0.056 to 0.150; mean + 2× SD = 0.230). For IgA type responses a cutoff of *A* = 0.215 was used (mean *A* = 0.189; range, 0.171-0.205).

Statistical analysis. Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was done using Fisher's exact test. Fisher's Exact test (two tailed) was used to analyze HPV-specific immunity to clinical response upon treatment with imiquimod.

Statistical analyzes were done using Graphpad Instat Software (version 3.0).

Results

HPV16-specific cellular and humoral responses in patients with high grade vulvar intraepithelial neoplasia. VIN forms a unique aspect of HPV-induced disease because patients are frequently treated, but the infection often persists. HPV-16 is found most often. To gain a more profound insight in the CD4$^+$ T-cell response against HPV16 in VIN, we charted the magnitude, specificity, and functionality of HPV16 E2, E6, and E7-specific proliferative T-cell responses in a group of 20 women with HPV16-associated high-grade VIN.

PBMC isolated from VIN patients were stimulated with peptides derived from HPV16 proteins E2, E6, and E7 as well as with a mix of common recall antigens (memory response mix), in a short-term proliferation assay. We have previously shown that this assay is geared towards the detection of CD4$^+$ T-cell responses (23). HPV16-specific proliferative T-cell responses against E2 and/or E6 were detected in 10 of 20 patients (Table 1). E7-specific responses were detected in 5 of 20 subjects. Analysis of the supernatants of these T-cell cultures for the presence of type 1 and type 2 cytokines revealed the secretion of the Th1 cytokine IFNγ in 8 of 20 patients. In some of the patients, the production of tumor necrosis factorα, IL-5,

Table 1. HPV16-specific proliferative T-cell responses in VIN

	E2												E6				E7		MRM
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	1	2	
P1			5.5															5.2	61
P2	7.5	6.9																	62
P3				29						40							36		256
P4																			110
P5		12																	112
P6																			7.5
P7				4.6															190
P8																			15
P9																			281
P10																			69
P11																		3.0	8.8
P12		7.6											7.0					11	49
P13	6.1	7.8				3.4	5.3		3.1					5.7		8.5		6.9	37
P14																			9.8
P15				5.4	13										9.2				30
P16																			12
P17				6.0															203
P18																			64
P19																			12
P20		8.4											5.6	5.4	4.8				25

NOTE: Freshly isolated PBMCs from 20 patients with high-grade HPV16-associated VIN were tested in short-term proliferation assays using a complete set of HPV16 E2-, E6-, and E7-derived peptide pools. Responses were scored positive when the proliferation (cpm) of ≥6 of eight test wells exceeded the mean proliferation ± 3× SD of the control (medium only) wells, and the mean stimulation index of all test wells over control wells was ≥3. Memory response mix, consisting of a mixture of recall antigens, was used as a positive control. The stimulation indices of responses scored positive are indicated.
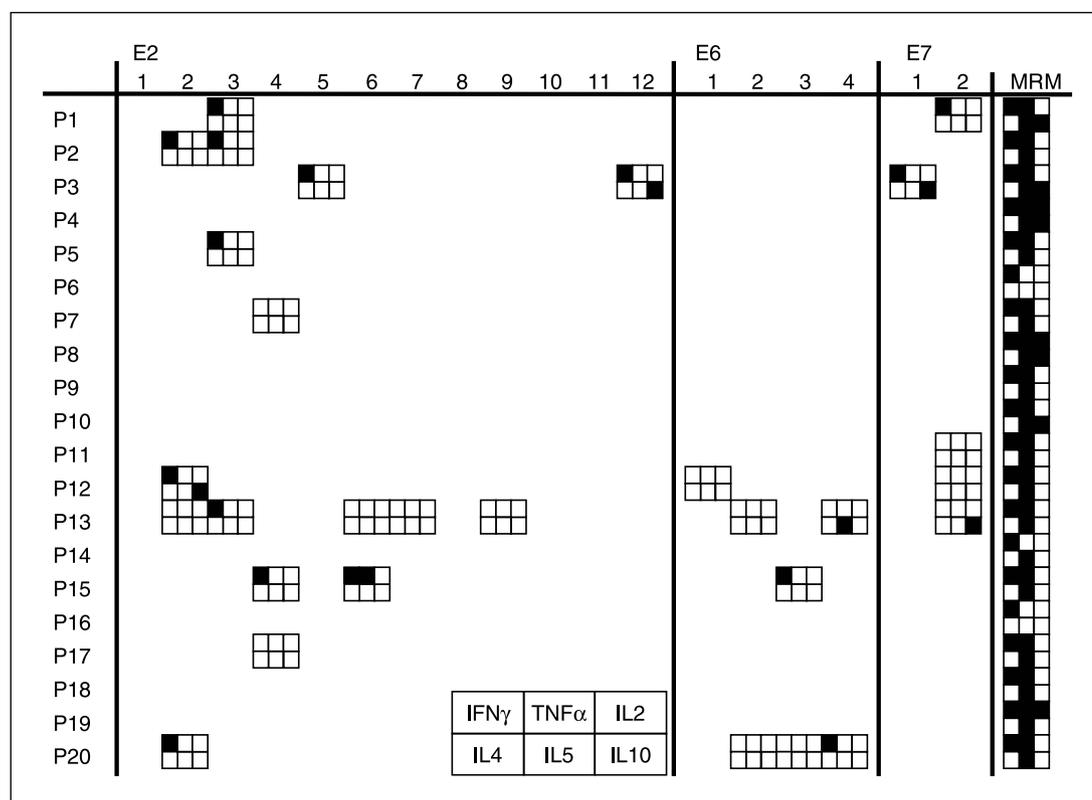Abbreviation: MRM, memory response mix.

Fig. 1. Supernatants of the positive proliferative responses indicated in Table 1 were analyzed for the presence of IFNγ, tumor necrosis factor-α, IL-2, IL-4, IL-5, and IL-10 by cytometric bead array. The indicated layout is used for the six measured cytokines. Antigen-specific cytokine production (■). Cutoff values were based on the standard curves of the different cytokines (50 pg/mL for IFNγ and 10 pg/mL for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and ›2× the concentration of the medium control.

and IL-10 was occasionally detected (Fig. 1). Although the overall frequency of proliferative responses is similar when compared with that previously found for cervical cancer patients, the number of patients with IFNγ-associated HPV-specific T-cell responses in these VIN patients was higher (8 of 20 versus 4 of 17, respectively; ref. 23).

In addition to T-cell immunity, the humoral response to HPV16 was measured in 28 VIN patients by ELISA using HPV16 L1-VLP as antigen. Overall, HPV16 L1-VLP IgG and IgA antibodies were detected in 25 of 28 (89%) and 13 of 28 (46%) subjects, respectively (Table 2). Based on the absorbance values, the HPV16 L1-VLP-specific IgG response exceeded that of IgA (Table 2). In general, HPV16-specific IgA responses were detected when patients displayed relatively high levels of HPV16-specific IgG. If IgG absorbance values were ≥0.5, 11 of 19 (58%) of the samples contained HPV16 L1-specific IgA, whereas at IgG levels of <0.5 only two of nine samples were IgA seropositive.

In conclusion, HPV16 L1-specific humoral immunity was detected in the great majority of patients, whereas HPV16 E2-, E6-, and/or E7-specific IFNγ-associated type 1 T-cell reactivity was detected in about half of the patients tested.

HPV16-specific immunity is associated with a more favorable clinical response on immunomodulatory treatment with imiquimod. Our analysis of HPV16-specific proliferation indicates that a high number of the proliferative T-cell responses is associated with IFNγ production. To examine the role of these HPV16-specific type 1 T-cell responses in the success or failure of treatment with the immunomodulator imiquimod, we studied this immune response in a group of patients with high-grade HPV16+ VIN. PBMC were isolated before ($T = 0$), during ($T = 8$), and after ($T = 16$) treatment and stored in liquid nitrogen. HPV-specific T-cell reactivity against HPV16 peptides E2, E6, and E7

was analyzed by IFNγ ELISPOT. This is a sensitive method for the analysis of antigen-specific type 1 T-cell reactivity on frozen material (32, 33). Three of these patients had been treated with imiquimod in the year before inclusion in our study (Table 3; patients 21, 24, and 27). Of these 17 patients, 15 were HPV16 positive. Preexisting IFNγ-associated T-cell responses ($T = 0$) were detected in 8 of 15 patients by IFNγ ELISPOT. In 5 of 15 patients, HPV16-specific T-cell reactivity against E2 was detected, whereas 4 of 15 patients displayed a response against E6 (Table 3). None of these patients showed preexisting T-cell responses against HPV16 E7. In two cases the $T = 0$ sample was not available and the reaction in PBMC from $T = 8$ are shown (Table 3; patients 1 and 22).

Despite that for some patients one of the two follow-up samples was not available (patients 5, 13, 27, and 28), it was clear that we could not detect a direct influence of imiquimod

Table 2. Distribution of absolute absorbance values among IgG- and IgA-seropositive samples

Immunoglobulin type ($n_{positive}$/28)	$\Delta A_{415\ nm}$	
	Mean (SD)	**Median (range)**
IgG seropositive ($n = 25$)	1.10 (0.61)	1.22 (0-1.93)
IgA seropositive ($n = 13$)	0.34 (0.26)	0.31 (0-0.88)

NOTE: HPV16 L1 IgG and IgA antibodies detected in the sera of 28 VIN3 patients. Serum antibody responses were measured by VLP-ELISA. Depicted are the absolute absorbance values at 415 nm. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

Table 3. HPV16-specific T-cell responses in patients treated with imiquimod

Patient*	HPV type	Clinical response[†]	VLP[‡]	T	E2 peptides				E6 peptides				E7 peptides		MRM
					1[§]-75	61-135	121-195	271-365	1-52	41-92	81-132	111-158	1-52	41-98	
1	16	No	++	8	—	—	—	—	—	—	—	—	—	—	179
2	16	PR1	+	0	**52**	**54**	**15**	**31**	—	—	*3*	—	*9*	—	295
4	16	CR	++	0	—	—	—	—	—	*1*	—	—	—	—	124
5	16	CR	++	0	—	**32**	*9*	*3*	—	—	—	—	—	—	32
10	16	PR1	—	0	—	*4*	—	—	—	—	**84**	—	*5*	—	54
12	16	PR1	++	0	**15**	—	—	—	—	—	—	—	—	—	32
13	16	CR	++	0	—[‖]	—	—	—	—	—	**104**	—	—	—	105
15	16	PR1	+	0	—	—	—	—	—	—	—	—	—	—	179
21	Negative	PR1	++	0	—	—	—	—	—	—	—	—	—	—	364
22	16	PR2	++	8	*8*	—	—	*4*	—	—	—	—	—	—	25
23	16	PR1	++	0	—	*1*	**59**	*5*	—	**74**	—	—	*21*[¶]	*11*	52
24	16	No	++	0	—	—	—	—	—	—	—	—	—	—	172
25	Negative	CR	+	0	—	—	—	*4*	—	—	—	—	—	—	444
26	16	CR	++	0	—	—	—	—	—	—	—	—	—	—	21
27	16	No	++	0	—	*4*	—	—	—	—	—	—	—	—	70
28	16	CR	++	0	*6*	—	**45**	**18**	—	*7*	*3*	*2*	*3*	—	20
29	16	CR	—	0	—	*2*	*7*	—	—	**40**	—	—	—	*2*	157

Abbreviations: CR, complete response; PR, partial response; MRM, memory response mix.

*PBMC from 17 VIN3 patients were tested for type 1 T-cell reactivity against HPV16 peptides. PBMC were stimulated with different pools of HPV16 E2, E6, and E7 peptides and tested for antigen-specific IFNγ production by ELISPOT.

† Clinical responses were defined as no clinical response; a partial response type 1, as defined by a reduction in lesion diameter from 76% to 99%; a partial response type 2, as defined by a reduction in lesion diameter from 26% to 75%; and a complete response.

‡ Sera of the patients were tested for the presence of HPV16 L1-VLP specific IgG antibodies. Indicated is the presence (+) or absence (−) of antibodies.

§The first and last amino acid in the indicated protein of the peptide pool used are indicated.

‖Per patient, T-cell responses on T = 0 are shown. In case of a missing T = 0 sample, data from T = 8 are shown. Specific responses were calculated by subtracting the mean number of spots ± 2× SD of the medium control from the mean number of spots of experimental wells. The number of specific spots per 100,000 PBMCs are given. Responses were considered positive if peptide pool specific T-cell frequencies were ≥10 in 100,000 PBMCs. These values are indicated in bold. Values below this threshold are shown in italics. (−), no specific response to E6 or L1. MRM was used as a positive control.

¶Responses considered negative because values did not exceed ≥2× the medium control.

on the numbers of HPV-specific T cells. In none of the patients was a clear-cut increase of HPV16-specific T cells detected upon imiquimod treatment (Fig. 2A-B). In some cases, patients had already been treated with a course of imiquimod before this study, but even this repeated treatment did not result in an increase of HPV 16 specific T cells (Table 3; patients 21 and 24). In addition, the HPV16 VLP-specific IgG and IgA response did not overly change when patients were treated with imiquimod (Fig. 3).

Thirteen of the 17 women treated (76%) displayed an overt clinical response upon treatment with imiquimod as indicated by 76% to 100% reduction in the size of their lesion (complete response or partial response 1; Table 3; Fig. 2C-D). Three patients showed no reduction in size of the affected area of vulvar disease and one woman showed only minimal improvement upon treatment.

Importantly, when the group of HPV16+ patients (n = 15) was divided in patients either with or without an HPV-specific Th1 immune response, all eight patients with an HPV-specific immune response displayed a complete or near complete clinical response (complete response or partial response 1) upon imiquimod treatment (Table 3). In contrast, patients without an HPV-specific immune response were less likely to show such a clinical improvement (P = 0.03, two-sided Fisher's exact test).

Taken together, chronic viral antigen exposure can induce type 1 CD4+ T-cell immunity against the HPV16 early antigens E2, E6, or E7 in patients with VIN3. The presence of these HPV16-specific Th1 cells as detected by IFNγ ELISPOT, although not essential for imiquimod-induced regression of VIN lesions, does increase the likelihood of a strong clinical response. The presence of L1-specific humoral reactivity was not correlated with imiquimod-induced regressions.

Discussion

We have analyzed the HPV16 E2-, E6-, and E7-specific CD4+ T-cell responses in a group of 29 patients with high-grade VIN, 17 of whom were treated with the immunomodulator imiquimod. HPV16-specific type1 (IFNγ) CD4+ T-cell proliferative immunity is present in about half of patients with VIN3 (8 of 20). Virus-specific CD4+ Th1-type T cells have emerged as an essential component in the immune response to chronic viral infection, fulfilling a multifactorial role, including the activation of antigen-presenting cell maturation for efficient CD8+ priming, the release of cytokines important in CD8+ T-cell proliferation and differentiation, and in the recruitment of other effector cells such as eosinophils and macrophages. Indeed, a substantial number of patients with VIN3 were reported to display high frequencies of
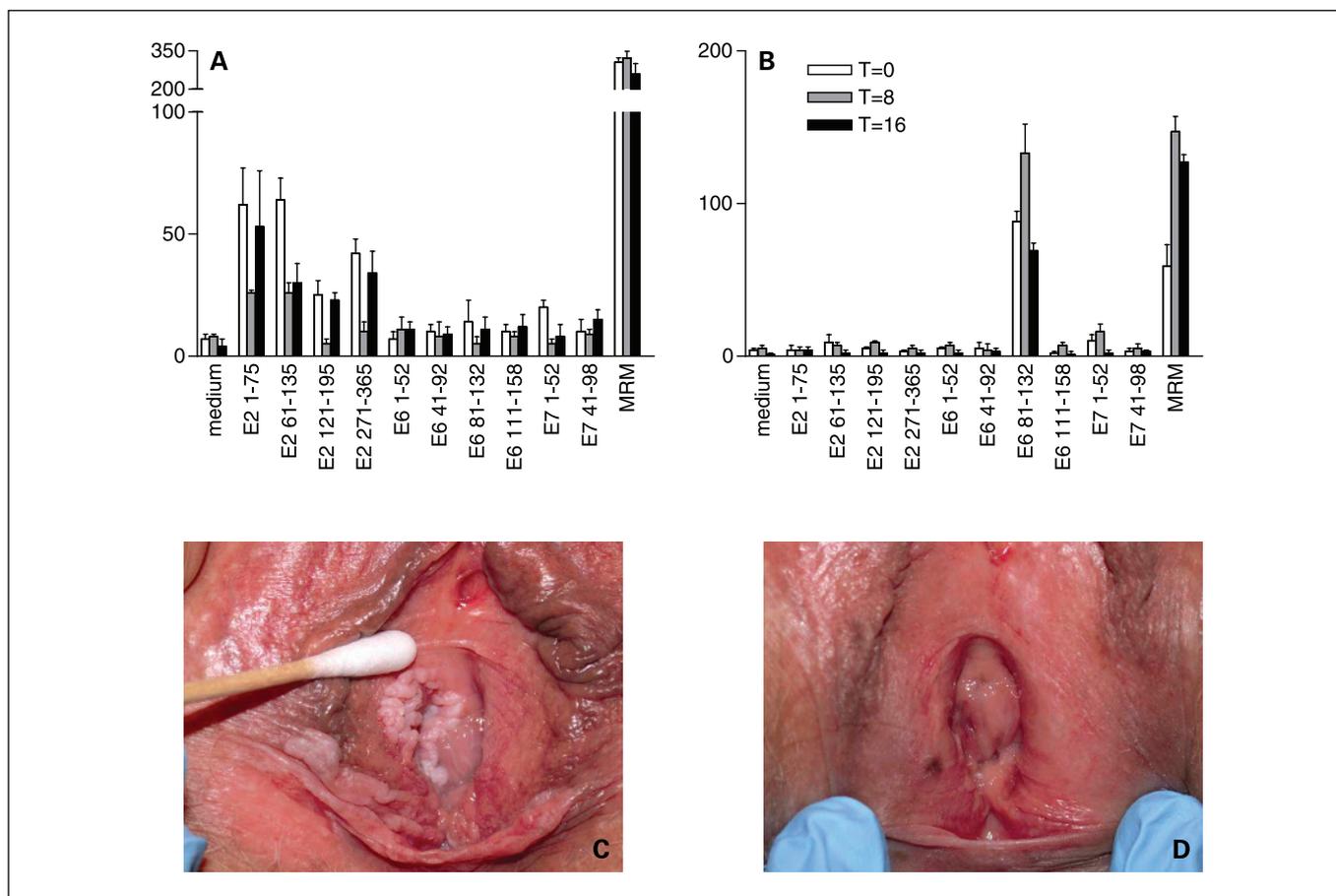
Fig. 2. *A* and *B,* HPV16-specific IFN-γ-producing T-cell responses in two representative patients with high-grade VIN (patient 2, *left* and patient 10, *right*). T-cell responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). Local application of 5% imiquimod containing cream does not result in enhanced systemic HPV16-specific T-cell responses. Note that the magnitude of the T-cell responses varies slightly over the different time points. The mean number of spots and SE induced by the medium control or the peptides present in the E2, E6, and E7 pools per 100,000 PBMCs are depicted. As positive control, the memory recall mix (*MRM*) was used. *C* and *D,* patients with preexisting HPV16-specific T-helper type 1 responses show objective clinical responses after imiquimod treatment. A typical example is shown. *C,* biopsy-proven VIN3 lesion of patient 5 before imiquimod treatment. *D,* the same vulvar area of patient 5 after 16 weeks of treatment.

HPV16-specific CD8u T cells (34–36). In contrast, only in a few occasions HPV16-specific CD8+ T-cell reactivity was detected in patients with cervical intraepithelial neoplasia 3 and cervical carcinoma (37–40). However, these latter types of patients display an impaired HPV16-specific CD4+ T-cell response (23).

Topical application of imiquimod neither enhanced the preexistent HPV16-specific CD4+ T-cell responses nor resulted in the induction of such responses in any of the other subjects. Todd et al. made a similar observation with respect to HPV16-specific CD8+ T cells (36). Notably, we found that a preexisting HPV-specific type 1 T-cell response was associated with a more favorable clinical outcome upon topical imiquimod treatment of VIN3. This indicates that a combination therapy, in which the HPV16-specific T-cell response is induced or boosted by vaccination and the affected skin is treated with imiquimod, may increase the number of patients that benefit from treatment.

Compared with normal vulvar skin, a number of VIN lesions display increased infiltration of CD4+ and CD8+ T cells (41–43). The clinical consequences of the infiltration of immune cells in these VIN lesions are poorly understood, but the immunologic make-up of the vulvar microenvironment may
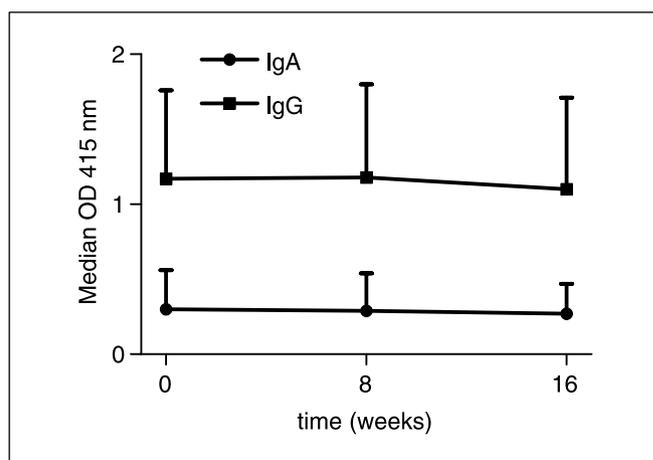

Fig. 3. IgG and IgA reactivity to HPV16 VLPs over time in 17 VIN3 patients treated with imiquimod. At least two serum specimens were tested in every patient. Serologic responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). The absorbance (*OD*) values are depicted as median ± SD of positive responses. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

determine the clinical outcome (43). The local cytokine microenvironment in high-grade cervical neoplasia is associated with a decreased expression of the proinflammatory Th1 cytokines, tumor necrosis factor-α and IFNγ (44–46). It is conceivable that similar to cervical intraepithelial neoplasia, the vulvar microenvironment also lacks proinflammatory cytokines. Imiquimod is known to directly stimulate Langerhans cells and macrophages (17, 47), of which the latter are increased in VIN lesions (42). Furthermore, it stimulates natural killer cells and T-helper type 1 cells via indirect mechanisms (17, 47). Upon stimulation, the antigen-presenting cells release proinflammatory cytokines, predominantly IFN-α, tumor necrosis factor-α, and IL-12 (14, 15, 17, 47). This may restore an inducive environment in which the innate effector cells, macrophages, and natural killer cells, as well as activated HPV16-specific T cells may act in concert to form an effective immune response. The requirement for these additional signals to activate T cells is sustained by recent observations in animal models. In the HPV16 E7-transgenic skin transplantation model, Matsumoto et al. (48) showed that despite the presence of large numbers of E7-specific memory T cells, E7+ skin transplants were not rejected, except when these E7-specific memory T cells were activated through vaccination. This suggested that the presence of the HPV16 E7 antigen itself is not sufficient to evoke a strong skin-destroying immune response but that additional activating signals were required.

Similarly, Van Mierlo et al. (49) showed that adenovirus-specific CD8+ T cells developed in the draining lymph nodes of mice bearing adenovirus-positive tumors, indicating that tumor-antigen was detected by T cells of the immune system. The tumor was rejected only when strong proinflammatory signals were provided. Likewise, HPV16-induced VIN3 lesions may fail to endow the immune system with strong inflammatory signals and exogenously provided signals will be required to provide a state of inflammation. These signals can be delivered by imiquimod, electrocoagulation (50), or by vaccines (32, 33, 43).

Currently, it is not clear whether immune activation causes the HPV16-specific IFNγ-producing CD4+ T cells to migrate into the HPV-infected tissue or whether these T cells should simply provide help to activate effector cells in the draining lymph nodes. Therefore, we are currently examining both local and systemic immune response in patients with high-grade VIN.

Acknowledgments

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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

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