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Author: Vos van Steenwijk, P.J. de
Title: Immunology and immunotherapy of high grade cervical lesions and cancer
Issue Date: 2015-05-19

IMMUNOLOGY AND IMMUNOTHERAPY OF HIGH GRADE CERVICAL LESIONS AND CANCER

P.J. de Vos van Steenwijk

Immunology and Immunotherapy of high grade cervical lesions and cancer
Thesis, Leiden University, the Netherlands
Peggy Jacqueline de Vos van Steenwijk

ISBN: 978-94-6169-662-5

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Cover: Lotte van Lieshout
Layout and Printing: Optima Grafische Communicatie (www.ogc.nl)

This PHD project was partly funded by Zon MW

The publication of this thesis was financially supported by:
BD Sciences, Greiner Bio-One B.V., Memidis Pharma, BMA BV (Mosos), ISA Pharmaceuticals, Bronovo Research Fund, SEO adviesbureau Huizing, the department of Clinical Oncology LUMC à Graag Bronovo Research Fund

IMMUNOLOGY AND IMMUNOTHERAPY OF HIGH GRADE CERVICAL LESIONS AND CANCER

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden.
op gezag van de Rector Magnificus prof . mr. C.J.J.M. Stolker
volgens besluit van het College voor Promoties

te verdediging op
dinsdag 19 mei 2015 klokke 16.15 uur

door

Peggy Jacqueline de Vos van Steenwijk
Geboren te Stamford, USA
in 1978

PROMOTIECOMMISSIE

Promotores:	Prof.dr. S.H. van der Burg
	Prof.dr. G.G. Kenter
Co-promotores:	Dr M.I.E van Poelgeest
	Dr E.S. Jordanova
Overige leden:	Prof. Dr. H.W. Nijman (Universitair Medisch Centrum Groningen)
	Prof. Dr. T.H.M. Ottenhoff
	Prof. Dr. T.D. de Gruijl (VU medisch Centrum)

For Penny

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Chapter 1

General Introduction

Immunology and Immunotherapy of Cervical cancer

CERVICAL CANCER

Cervical cancer is preceded by pre-malignant dysplastic changes in the epithelium known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). Affected cells comprising of more than one-third of the epithelium are called CIN 2/ 3 or high-grade SIL (HSIL). HSIL is caused by persistent infection with the human papillomavirus (HPV), a DNA virus infecting the basal cells of the cervical epithelium (1-3). There are over 100 types of HPV, which can be divided into low-risk (non-oncogenic) and high-risk (oncogenic) HPV (4). HPV is the most common sexually transmitted virus (5) with a lifetime risk of infection of 80% (6-8). In the majority of cases infection is controlled after approximately 2 years, but persistence of infection occurs in about 10% of the women (6- 7). These women are at risk of developing CIN, but other areas can be infected as well (vagina, anus, vulva, penis and/ or head and neck) with risk of progression to invasive squamous(adeno)carcinoma (5-7, 9, 10). The HPV genome importantly encodes the two oncoproteins, the early antigens 6 (E6) and 7 (E7), which are expressed in HSIL and tumour cells which are required for the onset and maintenance of the malignant transformation (4).

IMMUNOLOGY

The immune systems defense against HPV-induced cervical lesions.

Both the innate and adaptive immune system play an important role in the protection against HPV. Keratinocytes, the first line of defense express among other pathogen receptors, Toll-like receptor (TLR)-9, which can recognize HPV DNA. Activation of TLR9 should lead to activation of NF-kB, which results in the up regulation of proinflammatory cytokines (eg GM-CSF, IL-1b, TNF-a, IL-10, Il-12) and chemokine's, inducing the migration and the activation of antigen-presenting cells (APC's) e.g. Langerhans cells (LC's), Dendritic cells (DC's) and macrophages. Activation of the adaptive immune system requires viral antigen to be cross-presented by activated APC's (11). Cues in the microenvironment will evoke APC to differentiate and migrate to the local lymphoid organs in order to present antigens to locally present naive T cells. Depending on different co-stimulatory or -inhibitory molecules and cytokine production (e.g., IL-12 or IL-10), a T-cell response will be induced which may comprise various CD8+ cytotoxic T cells (CTLs), CD4+ helper T cells (Th cells) and/or CD25+FoxP3+ regulatory T cells (Tregs) (11). As HPV proteins are foreign to the body they should be able to trigger a strong immune reaction when presented in the cervical epithelium.

In the circulation of healthy individuals HPV-16 specific Th1, Th2 cells and CTLs are detected against a broad array of epitopes of the viral early (E2, E6, E7) and late (L1) antigens.

These cells are able to leave the circulation and migrate to areas where the antigen is present (12-19). The importance of the adaptive immune system is shown by the high incidence of HPV infections, HSIL and cervical cancer in immune suppressed individuals (20) and at the time of spontaneous regression of HPV-infected genital warts, lesions are infiltrated with CTL, CD4+ T cells and macrophages (21). A broad HPV specific T-cell response, consisting of Th1, Th2 and CTLs, seems desired for viral control, yet in patients with HPV induced disease this response is often not present. It deems logical that restoration of this profile is one of the aims of immunotherapeutic strategies.

Escape from the immune system

Failure of immune system to control infection is reflected by the fact that only one third of cervical cancer patients display a detectable systemic HPV-specific response against E6 and E7, and when it is present, the response is generally not associated with the production of IFN-γ and consists of mostly Th2 cells, non-polarized T cells or Tregs (14, 22-26). In the course of HPV infection to cervical cancer, various factors seem to play an important role. Pathogen recognition receptor (PPR) signaling in keratinocytes is suppressed, human leukocyte antigen (HLA) expression is down regulated, immunosuppressive cytokines are produced, Tregs are induced while Th cells and CTLs may be rendered dysfunctional via the expression of co-inhibitory molecules. Furthermore, APC's are hampered in their function.

- Viral recognition receptors

Keratinocytes are the first line of defense against invading pathogens. Viruses are recognized via several PRR. The viral DNA of HPV can be recognized by cells expressing TLR9. TLR9 is expressed in differentiating keratinocytes, but not in the undifferentiated cells of the basal layer which is the port d'entree of HPV. During infection with high risk HPV, TLR9 expression in the epithelium is not affected (2, 27), but its downstream signaling, as well as that of several other PRR which might recognize parts of HPV, is suppressed by the upregulation of the cellular protein ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) that dampens the production of interferon's, cytokines and chemokine's needed for the attraction and full blown activation of the adaptive immune system (2, 28). This renders the virus less visible, allowing HPV to persist.

- HLA expression

Essential elements for successful recognition of infected (or transformed) cells by the immune system is the presentation of viral peptides by HLA class I and II molecules. Downregulation of HLA class I on HPV transformed cells, possibly resulting in escape from CTL attack, is observed in cervical neoplasia (29, 30) and in patients with cervical cancer where the loss of HLA-A expression is associated with worse survival (31). HLA class II expression is found on most tumour cells, but it is not clear whether this leads to recognition by Th

cells, which are needed for the promotion of CTL recruitment and cytolytic function (32). In colorectal carcinoma HLA class II expression is a favorable prognostic marker (33) but its role in cervical cancer is less clear. Other non-classical HLA types have been described to play a role in the persistence of HPV induced lesions. HLA-G is found on cervical tumour cells (34-35) and might inhibit the function of NK cells (not frequently found in HPV induced lesions) and T cells through the binding of inhibitory receptors. The expression of HLA-G is associated with progression of pre-malignant to malignant lesions (34, 36). HLA-E is another non-classical HLA type which is over expressed in HPV induced lesions. HLA-E can inhibit the function CTL's expressing the CD94/NKG2A receptor which are frequently found in HPV induced lesions (37). A third non-classical HLA molecule is the MHC class I chain related molecule A (MICA) which interacts with the stimulating NKG2D receptor on CTL's and NK cells and enhances the effector function of these cell types (31, 38). MICA is expressed on normal epithelium but is weak or absent in about 60% of cervical tumours and is associated with worse survival when analysed in combination with a low ratio of CTL/ Tregs (31).

- *Tumour expressed inhibitory molecules*

T cell infiltration and function can be hindered through tumour expressed molecules. Versican is expressed in cervical tumours and thought to prevent the homing of T cells into the tumour. High expression was associated with low numbers of tumour infiltrating T cells (TIL's) in cervical tumours (39). Furthermore, co-inhibitory molecules expressed by chronically activated CD4+ and CD8+ T cells can interact with their ligands, when locally expressed. Examples are Cytotoxic T-Lymphocyte Antigen 4 CTLA-4, program death 1 (PD-1) and T cell immunoglobulin mucin-3 (TIM-3) expressed on T cells (40-42). The interaction between PD-1 receptor on T cells and program death ligand 1 (PD-L1[B7-H1]) and/or PD-L2 (B7-DC) results in the induction of apoptosis, anergy or exhaustion of effector T cells (43-45). Many PD-1 positive T cells are found infiltrating cervical cancer, yet its ligand PD-L1 is found less frequently on the cancer cells (45, 46). Interaction of TIM-3 and its ligand Galectin-9 results in a decreased Th1 and CTL immunity by inducing apoptosis of Th1 cells as well as by inhibiting the function of CTLs and Th1 cells (40, 42, 47). Increase in Galectin-9, has been associated with malignant differentiation in cervical cancer (48).

- *Antigen presenting cells*

HPV has been reported to also regulate the function and migration of APCs present in the epithelia, thereby hampering the activation of the adaptive immune response. The L2 protein of HPV is able to suppress the maturation of LC's causing inadequate antigen presentation to T cells (3, 49). Not only the function, but also the number of LCs seems reduced in HPV-infected epithelia (50-52). Furthermore, the absence of pro inflammatory signals in HPV-infected epithelia can result in inappropriately activated APCs (53). The

expression of HLA class II on tolerogenic APC's in the tumour could have pro-tumoural effects by rendering Th cells anergic through the lack of stimulation via co stimulatory molecules (eg CD40) and/or by the production of inhibitory cytokines, or by the induction and activation of Tregs (54). Tumour associated macrophages (TAM), are a heterogeneous myeloid cell population originating from monocytes in the circulation, which are able to undergo specific differentiation depending on the different stimulatory signals within the tumour microenvironment. Macrophages display the flexibility to continuously adapt to such cues in the local microenvironment allowing them to differentiate into cells that phenotypically and functionally fit between the two extreme polarization states known: M1-like pro-inflammatory, tumoricidal macrophages (M1) and M2-like anti-inflammatory, tumor-promoting macrophages (M2). Both are frequently found infiltrating tumours, but opposed to M1, M2 have a poor antigen-presenting capacity, preventing T-cells to be properly activated, furthermore they can enhance angiogenesis and metastasis (55) and have been associated with worse survival in various tumour types (56).

- Regulatory T cells and cytokines

In addition to CTL and Th cells the tumour milieu may also comprise Treg. Tregs display a high expression of CD25 and express the forkhead box protein 3 (FOXp3). Tumour infiltrating FOXp3+ T cells have been studied extensively and have been correlated with a poorer prognosis in various cancer types (57-64). Tregs found in tumours can inhibit the proliferation and function of Th cells and CTL's by preventing the expression of Il-2 receptor or inhibiting Il-2 production (24). The ratio of tumour-infiltrating CD4+, CD8+ and tumour-infiltrating Tregs is strongly correlated to survival in patients with cervical cancer (24, 31, 65, 66) and HPV specific Tregs have been found infiltrating cervical cancer (24, 25). Tregs further suppress the antitumor response by the induction of suppressive M2 macrophages, the up regulation of Il-10, the induction of tolerogenic indoleamine 2,3-dioxygenase (IDO)-positive APCs and the production of TGFβ (24). Il-10 and TGFβ can also be produced by tumour cells. Together, tumour cells, TAM and IDO+ APC may form a strongly immunosuppressive milieu. IDO, which is an immunosuppressive molecule, was found to be highly expressed in HSIL and cervical cancer on myeloid cells in the tumour stroma and the expression of IDO by tumour cells was correlated with an unfavourable survival in patients with cervical cancer (67-69). TGFβ is highly expressed in SIL and cervical cancer (70, 71), and prevents the infiltration of T cells into tumours, inhibiting their activation and inducing Tregs (72).

IMMUNOTHERAPY OF CANCER

With all evidence pointing in the direction that failure of the immune system leads to the development of cervical cancer, restoration of an effective anti-tumour immunity seems the logical way forward. Various modalities have been developed with limited success, affecting often a minority of patients with progressive disease. As discussed above a multitude of mechanisms can be responsible for the tumours escape from the hosts' immune system. Therefore successful immunotherapy probably lies in multiple therapeutic strategies aiming at the enhancement of immune-mediated tumour destruction as well as simultaneously counteracting the tumour-induced immune suppression. Three main modalities have been developed to achieve this goal, but it is likely that this arsenal will increase tremendously in the coming years.

Therapeutic vaccination

Various therapeutic vaccines aiming at inducing and/or restoring the tumour-specific T-cell response have been investigated (73). Vaccines based on recombinant viral vectors, recombinant proteins, DNA, antigen-pulsed DC's and peptides have been developed. For cervical cancer two promising vaccination strategies have been developed. One is a recombinant HPV16 E6E7L2 fusion protein (tissue antigen-cervical intraepithelial neoplasia, TA-CIN) (74) showing clinical responses when the vaccine was combined with Imiquimod treatment of the lesion. The other is a synthetic long overlapping peptide vaccine of HPV16 E6 and E7 (HPV16 SLP) which showed a good immunogenicity in cervical cancer patients (75, 76) and a clinical response in patients with VIN (77). This clinical response was associated with the induction of a strong HPV-specific CD4+ T cell and CD8+ T cell response. Furthermore, unresponsive patients showed an increase in the numbers of HPV16-specific Tregs infiltrating the lesion.

Antibodies and cytokines

Nonspecific immune stimulation with the use of monoclonal antibodies or recombinant cytokines can activate the immune system directly or counteract tumour induced immune suppression. Blocking or inhibiting immune inhibitory pathways are widely being investigated in cancer immunotherapy. CTLA4 blockade with Ipilimumab results in a broad enhancement of the immune system by T-effector stimulation and depletion of Tregs as reviewed by Blank et al (78). Blocking of CTLA-4 has been found to mediate a significant mean survival benefit in patients with advanced melanoma and an impressive long-term survival rate of a subgroup of patients (18% surviving longer than 2 years) (79). PD-1 is another immune checkpoint molecule, expressed by activated effector or Tregs within tumours (45, 80, 81), while its ligand, PD-L1, is expressed by tumour cells of many different tumour types and on macrophages and DC's (44, 45). Interaction results in the inhibition

of T-cell function. Blockade of this pathway (Nivolumab) has been demonstrated to have success in pre-clinical studies (82). Phase 1 trials are showing promising response rates with Anti-PD-L1 and Anti-PD-1 antibodies with less toxicity that CTLA-4 (83, 84). Combining Ipilimumab and Nivolumab showed objective responses in more that 40-50% of melanoma patients (85). The stimulation of costimulatory receptors on the other hand is also a promising potential strategy. OX40 (CD134) and 4-1BB (CD137) are co stimulatory receptors of the TNF family that are expressed on activated CD4 and CD8 T-cells. Agonistic antibodies to these receptors have powerful capabilities to activate CD8+ T cells to produce IFN-γ (86, 87). Various recombinant cytokine have found their way in to clinical trials with promising results. Examples are GM-CSF, inducing the proliferation of DC's and macrophages, Il-2 inducing T-cell activation and proliferation (88), Il-12 creating IFNγ producing T-cells (89) and type I Interferons which are known to have direct anti-viral properties, induce the differentiation of Th cells, generate CTL's and prolong the survival of all T cells (90). Other potential candidate cytokines are Il-7, Il-15 and Il-21 able to stimulate T cells or chemokines (e.g. CXCL9 and CXCL10) to attract T cells to the tumour.

ADOPTIVE CELL THERAPY

In adoptive cell therapy (ACT), lymphocytes are ex-vivo isolated from peripheral blood, tumour-draining lymph nodes or the tumour of cancer patients. Following an in vitro expansion the (partially) tumour-specific lymphocyte population is re-infused into the patient. This strategy may offer advantages over in vivo vaccination as the T cells are activated and expanded outside the cancer-induced immunosuppressive milieu, potentially allowing them to acquire more potent anti-tumoural properties. Moreover, in vivo lymphocyte depletion prior to infusion may also deplete pre-existing Tregs. In melanoma patients ACT has led to significant and durable remissions as reviewed in Phan *et al* (91).

OUTLINE OF THIS THESIS

This thesis firstly investigates the natural immune response against HPV in patients with (pre-) cancerous lesions of the cervix with an emphasis on the tumour microenvironment, by studying the local HPV-specific T-cell responses in HSIL and cervical tumours and draining lymph nodes, the presence of myeloid cells in cervical tumours, their interaction with the microenvironment and the effect on survival. The last two chapters describe two clinical trials in which patients with pre-cancerous lesions of the cervix are vaccinated with an HPV16 E6/E7 synthetic overlapping long-peptide vaccine (HPV16-SLP).

In the face of developing therapeutic vaccination strategies, it is of vital importance to gain a better understanding of the local tumour environment and the pre-existing local anti-tumour response. In **Chapter 2** we investigated the systemic and local HPV16 T cell response in patients with HSIL. Only a minority of the patients with HPV16+ HSIL have an IFNγ associated HPV16 specific T cell response. Proliferative responses were more often found, especially in patients with persisting infections after previous surgical treatment. Moreover, we showed that these premalignant lesions could be infiltrated with HPV-specific Tregs. The results of this chapter formed a good basis for the design and interpretation of immunotherapeutic vaccine approaches as treatment modality for HPV-induced in pre-cancerous lesions.

Previous work showed that HPV specific T cells can be found infiltrating the tumour and its draining lymph nodes. We wanted to know the potential of these cells to aid tumour immunity and, therefore, studied the properties of these T cells. In **Chapter 3** we comprehensively analysed the spontaneous tumour-specific immune response in patients with cervical cancer by dissecting local HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells. We describe a large polyclonal repertoire of HPV-specific T cells present in the tumour and lymph nodes of cervical cancer patients, whereby we distinguished four different cytokine signatures based on the production of IFNγ and Il-2. This work shows TIL's can be isolated and cultured for use in ACT.

The focus of **Chapter 4** was to further improve our knowledge of the local microenvironment by investigating the tumour infiltrating myeloid cells. The different types of myeloid cells, their clinical impact and their co-operation with T cells in the cervical cancer microenvironment was analysed. A strong intraepithelial infiltration with CD14+CD33-CD163- myeloid cells associated with a large influx of intraepithelial T lymphocytes, improved disease-specific survival and formed an independent prognostic factor for survival. This study provided a profound insight on the role of myeloid cells in the microenvironment, how they can work side by side with T cells to control tumours and forms a major addition to the current discussion about the impact of tumour-infiltrating myeloid cells in human cancers.

The influence of human cervical cancer cells on myeloid cell (monocyte) differentiation is presented in **Chapter 5**. It shows that the majority of cancer cells either hamper monocyte to DC differentiation, or skew their differentiation towards M2-like macrophages. Blocking studies revealed that M2-differentiation was caused by tumour-produced PgE2 and IL-6. Furthermore, upon cognate interaction with Th1 cells these tumour-induced M2-macrophages could be switched back to activated M1-like macrophages. These data show

the plasticity of tumour-induced tolerogenic APC and suggest that increased numbers of tumour-infiltrating Th1 cells may stimulate a tumour-rejecting environment by switching M2-macrophages to classical pro-inflammatory tumouricidal M1 macrophages. Vaccination aimed at the induction of HPV specific Th1 cells could be beneficial.

The aim of **Chapter 6** was to investigate the capacity of the HPV16-SLP vaccine to stimulate the HPV16-specific T-cell response and to enhance the infiltration of HPV16-specific type 1 T-cells into the lesions of patients with HPV16+ HSIL and HPV clearance. This was a placebo controlled randomized phase II study in patients with HPV16 positive HSIL. Vaccination of HSIL patients resulted in increased Th1 HPV16-specific T-cell immunity.

Chapter 7 investigates the capacity of a low dose of the HPV16-SLP vaccine to induce an HPV16-specific T-cell response in patients with low grade abnormalities of the cervix, to determine the long term memory response after vaccination and finally to evaluate the need and potency of a booster vaccination after one year. We concluded that two low dose injections of HPV16-SLP can induce a strong and stable HPV16-specific Th1 T-cell response that lasts at least for 1 year. The booster injection resulted in increased Th2 responses.

In **Chapter 8** the contribution of our results to the current knowledge of the immune response to HPV induced lesions and cancer is discussed. In addition, the present and future of immunotherapeutic strategies in cervical cancer according to recent international literature is reviewed.

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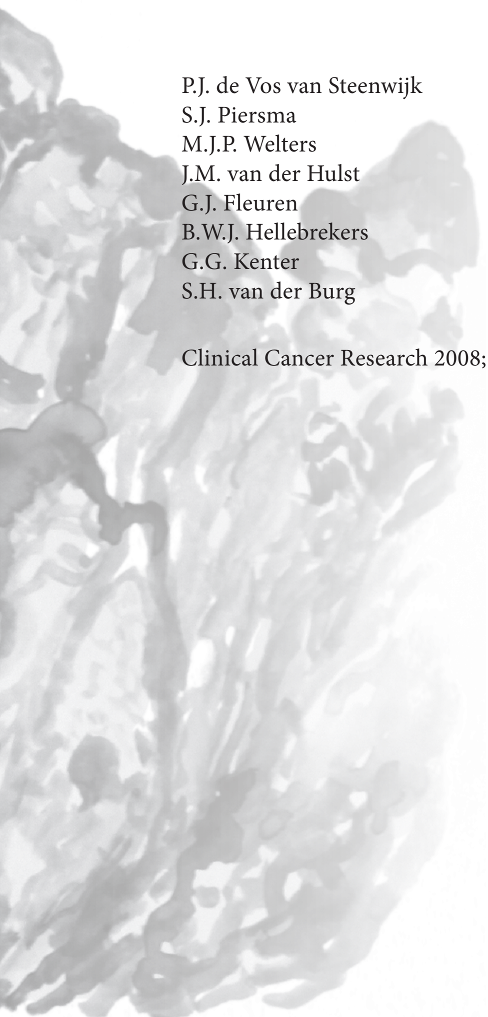
Chapter 2

Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16 specific T-cell response



P.J. de Vos van Steenwijk
S.J. Piersma
M.J.P. Welters
J.M. van der Hulst
G.J. Fleuren
B.W.J. Hellebrekers
G.G. Kenter
S.H. van der Burg

Clinical Cancer Research 2008;14(22)

ABSTRACT

Purpose: To characterize HPV16 E6- and E7-specific T-cell immunity in patients with high-grade squamous intraepithelial lesions (HSIL).

Experimental design: PBMC isolated from 38 patients with HPV16+ HSIL were used to determine the magnitude, breadth and polarization of HPV16-specific T-cell responses by proliferation assays and cytokine assays. Furthermore, HSIL-infiltrating T-cells isolated from 7 cases were analyzed for the presence of HPV16 E6 and/or E7-specific T-cells, phenotyped and tested for the specific production of IFNγ and IL-10 as well as for their capacity to suppress immune responses.

Results: HPV16-specific T-cell responses were absent in the circulation of the majority (~60%) of patients who visit the clinic for treatment of an HPV16+ HSIL lesion. Notably, HPV16-specific T-cell reactivity was predominantly detected in patients returning to the clinic for repetitive treatment of a persistent or recurrent HPV16+ HSIL lesion after initial destructive treatment. The majority (>70%) of these HPV16-specific T-cell responses did not secrete pro-inflammatory cytokines, indicating that most of the subjects, although in principle able to mount an HPV16-specific immune response, fail to develop protective cellular immunity. This notion is sustained by our observation that only 3 HSIL-infiltrating T-cell cultures contained HPV16-specific T-cells, one of which clearly consisted of HPV16 E7-specific regulatory T-cells.

Conclusions: The presence of HPV16-specific T-cells with a non Th1/Th2 cytokine and even suppressive signature in patients with HSIL may affect the outcome of vaccine approaches aiming at reinforcing HPV-specific immunity to attack HPV-induced lesions.

INTRODUCTION

Cervical cancer is preceded by well defined stages of changes in the epithelium known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL), which are caused by persistent infection with human papillomavirus (HPV). Changes that affect more than one-third of the epithelium are diagnosed as CIN 2 or 3 or high-grade SIL (HSIL). These HSIL have a high chance of progressing to cancer if left untreated (1). Most HSIL are associated with the presence of a high-risk HPV type, in particular HPV type 16 (2). The HPV genome encodes two oncoproteins, E6 and E7, which are constitutively expressed in high-grade cervical lesions and cancer since they are required for the onset and maintenance of the malignant cellular phenotype (3).

The key role of the adaptive cellular immune system in the protection against HPV-induced lesions is indicated by the high incidence of persistent HPV-infections and subsequent HPV-related malignancies in immunosuppressed individuals (4) as well as by the fact that only a fraction of infected subjects develop progressing epithelial lesions or cancer (5). Since HPV proteins are foreign to the body, one would expect the immune system to mount a response against these antigens when expressed in the cervical epithelium. Indeed, HPV16 E6, E7 and E2-specific Th1- and Th2-type CD4+ T-cell responses were frequently detected in PBMC cultures of healthy individuals (6-8) and both HPV16-specific CD4+ and CD8+ T cells are able to migrate upon antigenic challenge {van den Hende, 2008 176 /id} showing that successful defense against HPV16 infection is commonly associated with the installment of a systemic effector T-cell response against these viral antigens.

The local microenvironment in an HSIL is associated with an increase in IL-10 production and a decrease in proinflammatory cytokines (10-13), which poses a harsh milieu for the immune system and is likely to affect both the systemic and local immune response. Indeed, patients with HSIL show evidence of non-specific suppression of type 1 T-helper cell cytokine production (11;14) but how this affects the priming and the character of an HPV-specific immune response is only slowly elucidated. So far, the presence of HPV-specific immunity has either been studied in a number of really small cohorts of patients with HSIL (7;15-21), or in somewhat larger cohorts that focused on a highly restricted set of antigenic peptides (22), and/or studies using only one single immunological parameter (23-26) to decide whether a response is present. Unfortunately, huge differences between these studies in design and outcomes complicate the development of a unifying picture on how HPV-specific immunity and HSIL co-evolve.

So-called therapeutic vaccines are being developed for the treatment of individuals who contracted a high-risk type of HPV and were unable to control the viral infection - as dem-

onstrated by the presence of an HPV-induced (pre-)malignant lesion (27-31). A number of therapeutic vaccines have been tested in patients with cervical and non-cervical high-grade genital lesions, but with modest success (32-38). Since these therapeutic vaccines aim at reinstating an effective T-cell response against HPV16 E6 and/or E7 it will become very important not only to know the presence of pre-existing HPV16-specific T-cell immunity in patients with HSIL but also to understand the functionality, as this may bear impact on vaccine efficacy (39;40).

Here we have studied the presence and function of spontaneously induced HPV16-specific T cells in a large group of patients with HPV16+ HSIL. HPV16-specific proliferative T-cell responses were detected in less than half of the patients and the majority of the responses were not associated with the production of IFNγ or other pro-inflammatory cytokines. Notably, even HPV16-specific regulatory T-cells could be isolated from HSIL tissue. The induction of these HPV-specific T-cell responses most likely is the product of surgical treatment with recurrence or persistence of disease.

MATERIALS AND METHODS

Patient inclusion and sample collection

Patients visiting the colposcopy clinic at the department of Gynaecology of the Leiden University Medical Centre or the Haga Teaching Hospital in The Hague were recruited in the CIRCLE study, which investigates cellular immunity against HPV16- positive cervical lesions. The study design was approved by the Medical Ethical Committees of the Leiden University Medical Center and the Haga teaching hospital. Patients were eligible for the current study if they had a histologically proven cervical intraepithelial neoplasia at the time of diagnostic colposcopy or loop electrical excision procedure (LEEP). Notably, patients with chronic HSIL were more motivated to participate in this study hence this group pf patients is somewhat overrepresented in our cohort. Informed consent was obtained from all patients. Seventy ml of blood was drawn on the day prior to LEEP. Serum was obtained and peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood samples by Ficoll (Sigma) density centrifugation for the analysis of HPV-specific T-cell reactivity. In a number of cases tissue from the lesion was obtained for research purposes.

HPV typing

Patients with a HSIL were typed for HPV on paraffin-embedded sections of biopsies using 3 general HPV primer sets (CPI/II [1], MY 9/11[2], GP 5+/6+ [3]) followed by sequencing. Sequencing results were analysed by the NCBI BLAST programme. As controls beta-globine PCR and a blank sample were included (41-43).

Antigens

A set of 22 amino acid long peptides, overlapping by 12 amino acids, and indicated by the first and last amino acid in the sequence of the E6 and E7 protein of HPV16 (e.g. E6.1-22 and the last peptides E6.137-158 and E7.77-98), were used for the screening of T-cell responses. The peptides were mixed into four pools of E6 peptides and two pools of E7 peptides (i.e. E6.1 - E6.4, E7.1 and E7.2). These pools consisted of four 22-mer peptides. Notably, peptide pools E6.3 and E6.4 both contained peptide E6.111-132, whereas peptide pool E7.2 harbored the last five peptides of HPV16 E7. The peptides were synthesized and dissolved as described previously (44). Memory response mix (MRM) consisted of tetanus toxoid (0.75 Limus Flocculentius per ml; Netherlands Vaccine Institute, Bilthoven, The Netherlands), sonicated Mycobacterium tuberculosis (5 μg/ml; kind gift from Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and Candida (0.015%, HAL Allergenen Lab, Haarlem, The Netherlands). The response to MRM was used as positive control in the assays (8) to confirm the capacity of the antigen presenting cells that are present in PBMC to process and present antigens to memory T-cells.

Proliferative capacity of HPV16-specific T-cells by lymphocyte stimulation test (LST)

The capacity of T-cells to proliferate upon stimulation with the antigen was determined by short-time proliferation assay as described earlier (6;8;45). Briefly, freshly isolated PBMC (1.5 x 10^5) were seeded into 8-replicate wells of a 96-well U-bottom plate (Costar, Cambridge, MA) to which the indicated peptide pools were added at a final concentration of 10 μg/ml. Medium without antigen served as background control and MRM was taken along as a positive control. The test was conducted in IMDM (BioWhittaker, Verviers, Belgium) containing 10% autologous serum. On day 6, supernatant was harvested for cytokine analysis and subsequently the cells were pulsed with 0.5 μCi [3H]Thymidine (Pelkin Elmer, Boston, USA) per well and incubated for an additional 18 hours. Then, the cells were harvested onto filters (Wallac, Turku, Finland) using the Micro-cell Skatron harvester (Skatron Instruments AS, Lier, Norway) and counted on the 1205 Betaplate counter (Wallac, Turku, Finland). The average and standard deviation of the 8 medium only control wells were calculated and the cut-off was defined as this average plus 3xSD. The stimulation index (SI) was calculated as the average of tested 8 wells divided by the average of the medium control 8 wells. A positive proliferative response was defined as a stimulation index of at least 3 and the counts of at least 6 out of the 8-wells must be above the cut-off value (7).

Cytokine analysis

The supernatants isolated on day 6 of the proliferation assay were subjected to a Th1/Th2 inflammation cytokine bead array (CBA) kit (BD Biosciences, Erembodegem, Belgium). In this array the levels of IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2 were determined. According

to firm prescription the proposed detection limit was 20 pg/ml. However, for IFNγ the cut-off value was set to 100 pg/ml because the standard curve showed linearity starting at a concentration of 100 pg/ml. Positive antigen specific cytokine production was defined as a cytokine concentration above the cut-off value and >2x the concentration of the medium control (7).

Culture of CIN Infiltrating Lymphocytes

CIN Infiltrating Lymphocytes (CIL's) were isolated and cultured as described previously (46). Briefly, CIL cultures were expanded using a mix of irradiated autologous Epstein-Barr virus transformed B cell lines (B-LCL) and 5 ug/ml cognate peptide in IMDM, supplemented with 10% human AB serum (PAA laboratories, Pasching, Austria), 10% T-Cell Growth Factor (TCGF, Zeptometrix, Buffalo NY, USA) and 5 ng/ml recombinant human IL-15 (Peprotech, Rocky Hill NJ, USA).

Analysis of T-cell specificity

T-cell cultures (25,000-50,000 cells/well) were stimulated with autologous monocytes or irradiated autologous B-LCL's pulsed with their cognate peptide (ID2 HPV16 E7.71-92; ID23 HPV16 E7.51-72; 5 μg/ml) (47) and protein (10 μg/ml) in triplicate wells in a 3-day proliferation assay. After 48 hours supernatant was harvested and stored at -20°C for cytokine analysis. Antigen-specific IFN-γ and IL-10 production was measured by ELISA as described earlier (19).

Detection of CD4+ CD25+Foxp3+ T-cells

HPV16-specific CIL lines were stained 3 weeks after their last antigen-specific activation *in vitro* first for surface markers CD25 (anti-CD25 FITC; clone M-A251, BD Pharmingen), and CD4 (anti-CD4-APC; clone RPA-T4, BD Pharmingen) before the cells were fixed and permeabilized. Blocking was performed with 2% normal rat serum followed by the addition of anti-human Foxp3 (PCH101, eBiosciences, San Diego, CA) antibody or rat isotype IgG2a control. Then the cells were washed and analyzed by flowcytometry. As a positive control a previously isolated HPV16-specific CD4+CD25+Foxp3+ regulatory T-cell clone (C148.31) and as negative control an HPV16-specific CD4+CD25+Foxp3- T-cell clone (C271.9) (47) were used. The fluorescence intensity of these two control clones was used to set the gates for the other samples in which the CD25+Foxp3+ expression of the stimulated polyclonal T-cell populations were analyzed.

HPV16-specific T-cell Suppression Assay

T-cell suppression assay's were performed as described previously (47). Briefly, the CIL lines were co-cultured with allogenic CD4+CD25- responder cells in the presence of 1 μg/ml agonistic anti-CD3 (OKT-3; Ortho Biotech, Bridgewater, NJ) and APC mixture of 5

different B-LCL cell lines. Suppression of the responder cells was analyzed on proliferation and IFNγ production as described previously (47). HPV16 antigen-dependent suppression was measured using a flowcytometry based proliferation assay. Responder cells were labeled with CFSE and co-cultured with PKH -26 labeled CIL lines at a 1:1 ratio. The responder cells were stimulated with a pool of 5 allogenic B-LCLs, the HPV-specific CIL lines were stimulated with 5 ug/ml cognate peptide and autologous B-LCL in the presence of IL-2 (300 IU/ml). After 4 days of culture, the allo-specific proliferation of responder T-cells was analyzed by flowcytometry. HPV-specific CIL lines were treated with 50 µg/ml mitomycin C (Kyowa, Hakko, Japan) for 1 h followed by irradiation (2,000 rad) to prevent proliferation but not effector function.

Statistical analyses

In order to evaluate the effect of a previous treatment for high-grade CIN and persistence of the lesion afterwards on HPV-specific immunity, the patients were divided into two groups. The group of patients with a persistent lesion after treatment consisted of patients who had already undergone a surgical treatment for a HSIL after which the lesion persisted at least for 8 months (range 8-72 months) as indicated by the detection of Pap3a or higher in follow-up smears, or a HSIL at follow-up colposcopy for which these patients all had to undergo a second surgical treatment at the time that blood was drawn for the detection of HPV-specific immunity. Patients without a persistent infection where defined as patients who had no prior treatment before the drawing of blood for the immunological assay. These groups were then subdivided into patients who did show an HPV-specific immune response or in whom no specific immune response was detected and analyzed by a two-sided Fisher's exact test.

RESULTS

Patients and HPV distribution

During a period of 5 years a total of 74 patients with a HSIL were included in this study, 16 patients who were diagnosed with a CIN2 and 58 with a CIN3 (Table 1). The median age was 38 years with a range of 24-68 years. HPV typing revealed that 60 patients (81%) were HPV 16 positive and 2 (3%) were HPV 18 positive. In 11 patients (15%) another HPV type was found (HPV 45, 33, 31) and 1 patient was HPV negative (Table 1).

Patients with HSIL fail to induce a strong HPV16 E6- and E7-specific T-cell response

From 38 HSIL patients (Table 2) freshly isolated PBMC were stimulated with peptides derived from HPV16 proteins E6 and E7 as well as with a mix of common recall antigens

Table 1. HPV distribution in HSIL patients

Patients included	*N* = 74
Median age (range)	38 (24-68)
Histology CIN	
CIN 2	16
CIN 3	58
HPV typing (%)	
HPV16	60 (81)
HPV18	2 (3)
HPV45	3 (4)
HPV33	1 (1)
HPV31	7 (10)
HPV⁻	1 (1)

Table 2. Summary of patient characteristics and HPV16-specific immunity

ID	Age	Histology	Treatment at time of analysis	Previous treatment	LST*	CIL (antigen) †	Follow-up	
							6 mo	12 mo
1	28	CIN 2	LEEP		-		Pap 1	Pap 1
2	36	CIN 2	LEEP		-	CIL (E7)	Pap 1	
3	45	CIN 2	LEEP		-		CIN 3	
4	33	CIN 2	LEEP		-		Pap 1	Pap 1
5	40	CIN 2	Conization		-		Pap 2	Pap 2
6	61	CIN 2	LEEP		-			
7	41	CIN 2	LEEP		-		Pap 3a	Pap 2
8	29	CIN 2	LEEP		+		Pap 1	Pap 1
9	35	CIN 2	no treatment		+		Pap 3a	CIN 3
10	43	CIN 2	Conization	LEEP 2×	+		Pap 2	Pap 2
11	35	CIN 3	LEEP		-		Pap 1	Pap 1
12	30	CIN 3	LEEP		-		Pap 1	Pap 2
13	31	CIN 3	Conization	LEEP	+		Pap 1	
14	33	CIN 3	LEEP	LEEP	+		Pap 1	Pap 1
15	31	CIN 3	LEEP		-		Pap 1	Pap 1
16	35	CIN 3	LEEP		-		Pap 1	Pap 1
17	29	CIN 3	Conization		-		Pap 3b	CIN 3
18	61	CIN 3	LEEP		-	CIL	Pap 2	Pap 1
19	27	CIN 3	LEEP		-		Pap 1	
20	43	CIN 3	LEEP		-			Pap 1
21	35	CIN 3	LEEP		-	CIL (E7)		Pap 1
22	25	CIN 3	LEEP		-		Pap 3a	Pap 3a
23	26	CIN 3	LEEP		-	CIL (E7)	Pap 1	Pap 1
24	44	CIN 3	LEEP	LEEP	-		Pap 1	Pap 1
25	35	CIN 3	LEEP		-		Pap 1	Pap 3b
26	41	CIN 3	LEEP		-		Pap 1	
27	51	CIN 3	LEEP		-		Pap 1	
28	41	CIN 3	LEEP	LEEP	+		Pap 3a	
29	42	CIN 3	LEEP		-			
30	34	CIN 3	LEEP	LEEP	+		Pap 3a	Pap 3a
31	52	CIN 3	Conization	LEEP	+		Pap 3a	Pap 1
32	36	CIN 3	Conization	LEEP 2×	+		Pap 2	Pap 2
33	27	CIN 3	LEEP		+	CIL	Pap 1	Pap 1
34	46	CIN 3	LEEP		+	CIL	Pap 1	
35	47	CIN 3	Conization	LEEP	+	CIL	Pap 1	Pap 1
36	45	CIN 3	LEEP		+		Pap 3b	CIN 3
37	34	CIN 3	LEEP		+		Pap 3a	Pap 1
38	39	CIN 3	No treatment	LEEP	+		Pap 1	Pap 1

*Lymphocyte stimulation test; PBMC of patients failed to respond (-) or did proliferate (+) on stimulation with HPV16 E6 and/or E7 peptide pools.
† From this patient, a biopsy from the HSIL lesion was cultured. When the infiltrating T cells were found to react to either HPV16 E6 or E7 in a 3-d proliferation assay, the antigen recognized (E6 or E7) is specified in parentheses. Diagnosis at 6 and 12 mo follow-up by Pap smear or by histology is indicated, when available.

(MRM) in a short-term proliferation assay (Figure 1A). We have previously shown that this assay is geared towards the detection of CD4+ T-cell responses (7;8;14;22). HPV16-specific T-cell responses were detected in 15 (39%) of the 38 patients (Table 3). In 6 cases proliferation was detected against E6, in 3 cases to E7 and in 6 cases to both E6 and E7 (Table 3). 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 6/15 (40%) patients with a pro-

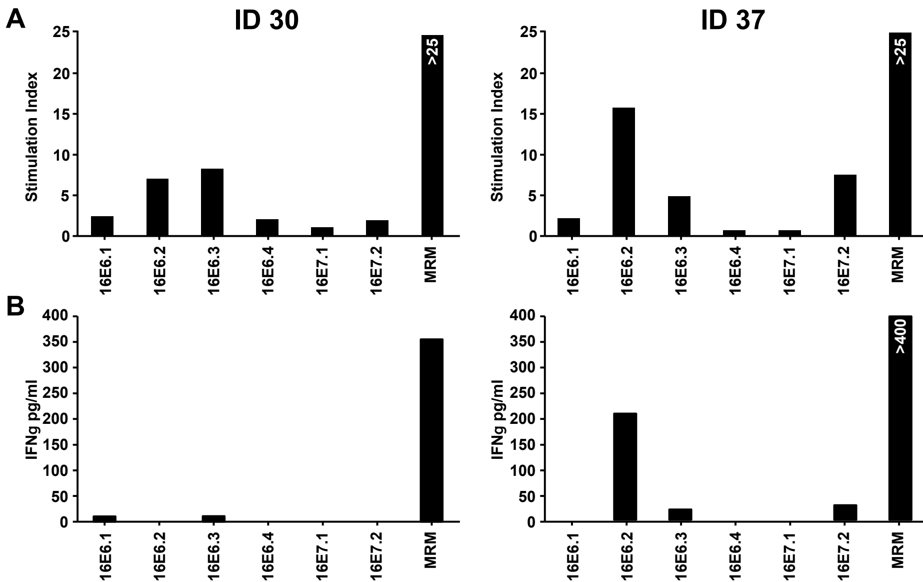


Figure 1. Short-term proliferation assay (LST) and associated IFNg production. (A) Representative examples of the HPV16-specific proliferation following the stimulation of PBMC from two HSIL patients (ID30, ID37). Cultures were tested in a 7 day lymphocyte proliferation assay upon stimulation with HPV16 E6 and E7 peptide pools. ID 30 tested positive for HPV16 peptide pool E6.2 and E6.3; ID 37 was positive for HPV16 peptide pools E6.2, E6.3 and E7.2. (B) Analysis of cytokine production by Cytokine Bead Array showed that only the culture from ID 37 stimulated with peptide pool E6.2 was associated with the production of IFNg, whereas in ID 30 the HPV16-specific T cells did not produce this typical Th1 cytokine.

liferative T-cell response (Figure 1B & Table 3). Occasionally, low levels of TNFα and IL-5 were produced by the HPV16-specific responding cells. Of all the proliferative responses measured (n=31) in these patients, only 9 (<30%) were associated with the production of a pro-inflammatory cytokine (Table 3).

HPV specific T-cell responses are correlated with the persistence/recurrence of HSIL

The majority of HSIL lesions are precancers that are destined to persist (1) and for that reason are surgically removed. As a small percentage of HPV16-positive HSIL may spontaneously regress after a biopsy (48) we hypothesized that the combination of HPV antigens and an invasive (surgical) treatment may deliver sufficient antigenic stimulation and danger signals to activate an HPV16-specific T-cell response. Because a number of our patients (Table 2) had already been surgically treated in the near past for HSIL, the patients were divided into two groups and analyzed with respect to the absence and presence of HPV16-specific immunity. Group 1 (n=28) consisted of those patients who were treated for the first time for an HSIL at the time of testing for HPV-specific immunity, whereas group

Table 3. Summary of HPV16-specific T-cell responses in patients with HSIL

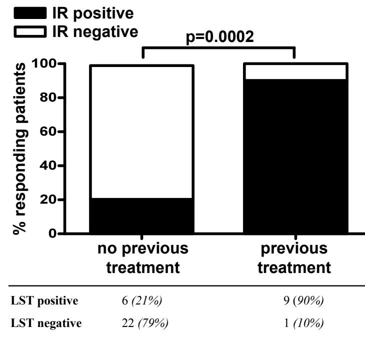
ID	Specificity*	SI †	IFN-γ ‡	Tumor necrosis factor-α ‡	IL-5 ‡
8	E6.4	4	<100	<20	<20
9	E6.2	11	299	<20	<20
10	E6.3	4	167	<20	<20
13	E6.2	4	<100	<20	<20
	E6.3	4	<100	<20	<20
	E7.2	3	<100	<20	<20
14	E6.3	6	<100	<20	<20
	E6.4	10	<100	29.2	<20
	E7.2	3	<100	<20	<20
28	E7.2	6	<100	<20	<20
30	E6.2	7	<100	<20	<20
	E6.3	8	<100	<20	<20
31	E6.2	7	<100	<20	<20
	E6.3	3	<100	<20	<20
	E7.2	6	<100	<20	<20
32	E7.2	13	<100	<20	<20
33	E6.1	5	<100	<20	<20
	E6.2	10	473	<20	31.2
	E6.3	6	<100	<20	<20
	E6.4	10	<100	<20	27.5
	E7.2	8	112	<20	<20
34	E6.1	3	<100	<20	<20
	E6.2	6	<100	<20	<20
	E6.3	11	<100	<20	<20
35	E7.2	7	447	<20	<20
36	E6.2	5	<100	<20	<20
	E7.2	5	<100	<20	<20
37	E6.2	16	210	<20	<20
	E6.3	5	<100	<20	<20
	E7.2	7	<100	<20	<20
38	E6.2	8	781	<20	60

*The peptide pool to which PBMC of the indicated patient proliferated.
† The magnitude of the response indicated by the stimulation index (SI) is shown.
‡ The amount of cytokine produced in the cultures with HPV16-specific PBMC is depicted in pg/mL. Production of IL-4, IL-10, and IL-2 was undetectable in the cultures. Cutoff values are 100 pg/mL for IFN-γ and 20 pg/mL for tumor necrosis factor-α and IL-5. Positive antigen-specific cytokine production was defined as a cytokine concentration above the cutoff value and more than two times the concentration of the medium control (7).

2 (n=10) consisted of patients with HSIL that persisted/recurred after a first destructive treatment and whom were treated again at the time that their HPV-specific immune status was assessed. The latter group displayed a significantly higher response rate to HPV16. The percentage of positive systemic responses in the group with persistent/ recurrent lesions (group 2) was 90% versus 21% in group 1 (p=0.0002, Figure 2). All together our results suggest that a systemic immune response, most often of a non Th1/ Th2 type, develops against HPV16 E6 and/or E7 and is often induced if the lesion persists or recurrs after initial surgical treatment of HSIL.

HPV16 E7-specific regulatory T cells infiltrate cervical HSIL lesions

From seven patients we were able to receive a small piece of cervical tissue for research purposes in order to characterize HPV16-specific T cells present in the HSIL lesion. As part of a larger study we showed that 3 of the 7 of these cervical infiltrating lymphocyte (CIL) cultures contained T-cells which recognized HPV16 E7 (46). From two of these CIL cultures (ID 2 and ID23) sufficient numbers of T cells were obtained for a more in-depth analysis. Upon stimulation with their cognate peptide both CIL cultures produced IFNg



	no previous treatment	previous treatment
LST positive	6 *(21%)*	9 *(90%)*
LST negative	22 *(79%)*	1 *(10%)*

Figure 2 . HPV16-specific immune responses are more frequently observed in patients with previous treatment of HSIL, than in patients treated for the first time. Of the 28 patients scheduled with a diagnosed HSIL for the first time, only 6 responded (21%) to HPV16 E6 and E7 peptide pools in a short-term lymphocyte stimulation test (LST) whereas a significant proportion of patients with persistence or recurrence of the lesion after initial treatment (90%; p=0.0002) displayed an HPV16-specific immune response (IR).

(Figure 3a). In addition, the culture of ID2 produced low levels of IL-10 and a small population of the CD4+ CIL population co-expressed CD25 and FoxP3, as measured by flowcytometry (Figure 3ab). As we had observed that the presence of HPV16-specific T cells with such a phenotype in cervical carcinoma represented regulatory T cells with the capacity to suppress immune responses (47) we assessed the suppressive capacity of the HPV-specific

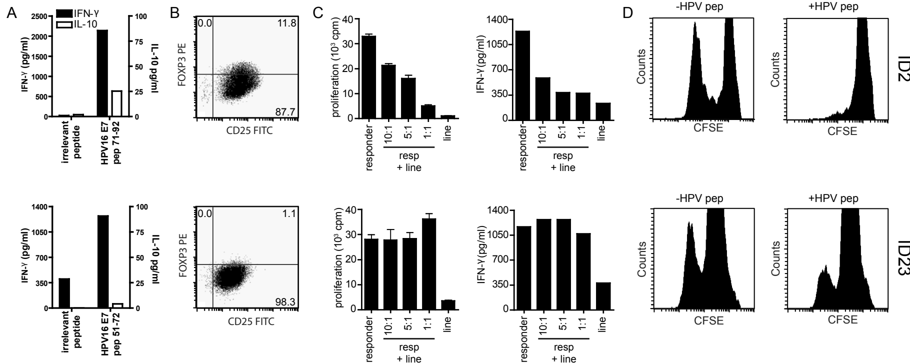


Figure 3. HPV16-induced HSIL lesions can be infiltrated by HPV16-specific regulatory T cells. Cervical infiltrating lymphocyte (CIL) cultures of ID2 and ID23 contained T-cells which recognized HPV16 E7 (46), and were analyzed for (A) their capacity to produce IFNγ and IL10 upon stimulation with cognate antigen, (B) the co-expression of the with regulatory T-cell associated markers CD25 and Foxp3. (C) Their capacity to suppress CD4+CD25- responder T-cells, cultured with the HPV-specific CIL lines at indicated different ratios, was measured by proliferation (left) and IFNγ production (right). T-cells of ID2 suppressed both proliferation and INFγ production. (D) The antigen-dependency of the HPV16 E7-specific regulatory T cells of ID 2 to exert their suppressive function was shown in a CFSE-based proliferation assay (47). Left and right panels depict responder cell proliferation in the absence or presence of their cognate peptide respectively.

CIL cultures in a classical suppression assay (Figure 3c). The CIL culture of patient ID2 was able to suppress both proliferation and IFNg production of CD4+CD25- responder T cells, whereas the CIL culture of patient ID23 did not contain such suppressive capacity. In order to prove that the HPV16-specific T cells were responsible for this suppressive effect, a CFSE-based suppression assay was performed in which the suppressive action of the CIL culture in the presence and absence of HPV16 peptide was tested (47). As expected, the CIL culture of patient ID2 almost completely suppressed the proliferation of the responder cells (85%) dependent on stimulation with HPV peptide, whereas the CIL culture of patient ID23 did not show substantial suppression (32%) when stimulated with its cognate HPV peptide (Figure 3d). These data demonstrate that HPV-specific regulatory T cells not only are present in patients with cervical carcinoma, but can already develop earlier during the malignant transformation of a persistently HPV16 infected cervix.

DISCUSSION

In this study we show that a systemic proliferative T-cell response against HPV16 is often absent in the majority of patients who visit the clinic for treatment of an HPV16+ HSIL lesion. In a number of cases HPV16-specific T-cell reactivity can be detected in the form of proliferative responses which are not associated with HPV16-specific secretion of the pro-inflammatory Th1 or Th2 signature cytokines. Importantly, HPV16-specific T-cell reactivity is predominantly detected in patients returning to the clinic for repetitive treatment of an HPV16+ HSIL lesion because of persistence or recurrence of the lesion after initial destructive treatment. This was not the case in patients visiting the clinic for a first treatment of their HPV16 HSIL lesion (Figure 2). The observation that these responses lack a clear pro-inflammatory signature indicates that this type of immune activation should not be regarded as beneficial, but rather as a reflection of the fact that most of the subjects, although in principal able to mount an HPV16-specific T-cell response, fail to develop a cellular immune response that is associated with protection against HPV-induced lesions (7;18;22;25). This notion is sustained by our data showing for the first time that already at this pre-malignant phase a population of regulatory T cells, which specifically recognize HPV16 E7 antigen, arises and infiltrates the HPV16+ HSIL lesion (Figure 3).

In a previous study, in which the immune response to HPV16 E6 and E7 was studied in patients with cervical carcinoma, we were able to detect an HPV16-specific proliferative response in about half of all patients, but only in 1 of the 8 tested subjects with an HPV16+ HSIL (7). Similar observations regarding the low frequency of responders were made in small cohort studies performed by others (18;22). The reason why fewer patients with HSIL mounted an immune response to E6 and E7 in comparison to cervical cancer patients at that time remained unclear. Our current study of a large group of 38 HPV16+ HSIL patients

confirms that the majority of HSIL patients fail to mount an HPV16 E6/E7-specific T-cell response. The absence of an HPV16-specific immune response in patients with HSIL and the presence of such responses in about half of the patients with cancer previously led us to hypothesize that the long-term presence of the HPV16 E6 and E7 antigens in a developing tumor may eventually trigger the induction of a CD4+ T-cell response (7). Here, we identified a larger group of HPV16+ HSIL patients which was able to mount an HPV16 E6/E7-specific immune response. Importantly, most patients within this group suffered from a persistent/recurrent HSIL lesion and had already been treated by destructive treatment before this immunological analysis was performed. This suggests that the HPV16 E6/E7-specific immune response detectable in the circulation of cervical cancer patients has developed as part of long term exposure to the HPV16 antigens in the persistent/recurrent HSIL in combination with the danger signals delivered by the previous invasive treatment. This notion is sustained by our observation that the cytokine profile of the HPV16-specific immune response in persistent/recurrent HSIL patients is similar to that of what we observed in cervical cancer patients (7) as well as the fact that the majority of established high-grade CIN lesions will evolve towards cervical carcinoma when left untreated (1). As such, the group of patients with recurrent/persistent HSIL may reflect our 'missing link' with respect to the absence of HPV16 E6/E7-specific immune responses in first time diagnosed HSIL patients and the presence of these responses in patients with cervical cancer. As yet, it is unclear whether either the previously given destructive treatment, the persistence of the lesion, and as such HPV antigens, or the combination of both is responsible for the activation of the HPV16-specific proliferative response. Notably, a previous study in HSIL patients showed that activation of HPV16 E7-specific immunity shortly after a local invasive procedure occurred only in 2 out of 18 patients (26). In that study immunity was measured by IFNγ ELISPOT while we showed that most of the HPV16-specific proliferative responses detected in our group of patients were not associated with the production of IFNγ (Figure 1, Table 3). The former study, thus, may have underestimated the response rate after local invasive procedures. Interestingly, a history of a long period (15-51 months) of persistent HPV16 infection is also associated with the activation of HPV16 E7-specific immunity, albeit that these responses are weak considering the fact that an indirect measurement consisting of a highly sensitive cellular bio-assay for IL-2 production was needed to detect these responses (23). Taken together, these studies build a strong case supporting the idea that local danger signals and long-term exposure to sufficient amounts of antigen are key in the development of the weak and dysfunctional immune responses observed in HSIL patients.

The number of circulating, regulatory T cells as defined by CD4+CD25^{high} T cells (49) or CD4+CD25^{high} CTLA4+ T cells (50), is increased in patients with HSIL when compared to healthy controls. In addition, immunohistochemical analysis of HSIL lesions not only revealed that HSIL lesions represent immunosuppressive environments (13) but also that

immune cells possessing a suppressive phenotype – as defined by CD25+TGFβ+ and CD4+TGFβ+ immune cells – may infiltrate such HSIL lesions (12). However, the specificity of these circulating and HSIL-infiltrating regulatory T-cells was never determined. Previously, we showed that the CD4+ subset of T cells infiltrating cervical carcinomas consisted of HPV16-specific regulatory T cells able to suppress proliferation and cytokine production of responder cells (47). Similar functional analyses of the T-cell populations infiltrating HSIL not only showed that indeed fully functional regulatory T cells can infiltrate pre-malignant cervical lesions but also that these regulatory T-cells exerted their action upon recognition of their cognate HPV16-specific antigen (Figure 3). Interestingly, a higher number of circulating CD4+CD25^{high} CTLA4+ regulatory T cells coincides with the presence of HPV16-specific T cells in the blood of patients with HSIL (50) indicating that such responses may co-evolve. Recently, we have developed an assay to measure the percentage of HPV16-specific regulatory T-cells in the circulation of patients with cervical cancer (27). Similar analyses in patients with HSIL may reveal to which extent the detected HPV16-specific responses lacking a clear cut pro-inflammatory signature (Figure 2 and Table 3) may actually represent HPV16-specific regulatory T cells. The lack of PBMC precluded such an analysis in the current group of patients but this question will be addressed in a new study.

The detection of HPV16-specific T cells with a non Th1/Th2 cytokine and even immune suppressive signature in patients with HSIL bears implications to immunotherapeutic vaccine approaches aiming at reinforcing HPV-specific immunity to attack HPV-induced lesions. Recently, we showed that such a vaccine also activates/boosts an unwanted pre-existing HPV16-specific T-cell repertoire in cervical cancer patients (27) suggesting that strategies to overrule or eliminate the responses of these subsets of T-cells in cancer patients should be considered for immunotherapeutic strategies against HPV-induced cervical lesions.

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Chapter 3

An unexpectedly large polyclonal repertoire of HPV-specific T cells are poised for action in patients with cervical cancer

Peggy J. de Vos van Steenwijk
Moniek Heusinkveld
Tamara H. Ramwadhdoebe
Margriet J. Löwik
Jeanette M. van der Hulst
Renske Goedemans
Sytse J. Piersma
Gemma G. Kenter
Sjoerd H. van der Burg

Cancer research 2010; 70(7), 2707-17

Translational Relevance
In the face of developing therapeutic vaccination strategies, it is of vital importance to gain a better understanding of the local tumor environment and the preexisting local anti-tumor response. In this study we have comprehensively analyzed the spontaneous tumor-specific immune response in patients with cervical cancer by dissecting local HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells, showing a large polyclonal repertoire of poised non-functional HPV-specific T cells. Addition of immuno-activating compounds could be key in increasing the effector function of these T cells with beneficial effects for therapeutic strategies.

ABSTRACT

The diversity and extent of the local tumor-specific T-cell response in a given individual is largely unknown. We have performed an in-depth study of the local T-cell repertoire in a selected group of cervical cancer patients, by systematic analyses of the proportion, breadth and polarization of HPV E6/E7-specific T cells within the total population of tumor-infiltrating lymphocytes (TIL) and tumor-draining lymph node cells (TDLNC). Isolated T cells were stimulated with sets of overlapping E6 and E7 peptides and analyzed by multiparameter flow cytometry with respect to activation, cytokine production and T-cell receptor Vβ (TCRVβ) usage. HPV-specific CD4+ and CD8+ T-cell responses were detected in TIL and TDLNC and their relative contribution varied between <1% to 66% of all T cells. In general, these HPV-specific responses were surprisingly broad, aimed at multiple E6 and E7 epitopes and involved multiple dominant and subdominant TCRVβ's per single peptide-epitope. In most patients only few IFNγ-producing T cells were found and the amount of IFNγ produced was low suggesting that these are poised T cells, rendered functionally inactive within the tumor environment. Importantly, stimulation of the TIL and TDLNC with cognate antigen in the presence of commonly used Toll like receptor-ligands, significantly enhanced the effector T-cell function. In conclusion, our study suggests that within a given patient with HPV-specific immunity many different tumor-specific CD4+ and CD8+ T cells are locally present and poised for action. This vast existing local T-cell population is awaiting proper stimulation and can be exploited for the immunotherapy of cancer.

INTRODUCTION

Our current knowledge on the diversity and extent of tumor-specific T-cell immunity is largely based on pooled T-cell response data of many different subjects (1-4), the T-cell response to a specific epitope (5;6) and/or studies of tumor-specific T-cell clones (7-9). However, very little is known about how extensively one individual's immune system can simultaneously respond to one or more tumor antigens. Cervical carcinoma offers an excellent opportunity to answer this question as these tumors are caused by the high-risk human papillomavirus (HPV) which encodes the two clearly defined tumor-specific viral antigens E6 and E7 that are constitutively expressed in each cancer cell (10). Notably, cervical cancer arises more frequently in immunocompromised individuals (11), illustrating the role of T cells in this type of cancer.

Low levels of circulating HPV E6- and E7-specific T cells in patients with cervical cancer or premalignant lesions (4;12-15) indicate that these oncoproteins activate an anti-tumor response. Indeed, cervical tumors are infiltrated by lymphocytes (16) and both CD8+ and CD4+ T cells isolated from such tumors are able to recognize the E6 and E7 tumor antigens (17;18). Furthermore, we showed that 43% of the isolated tumor-infiltrating lymphocyte (TIL) and tumor-draining lymph node cell (TDLNC) cultures from a large cohort of HPV16 or HPV18 positive patients contained T cells specific only for the E6- and/or E7-peptides corresponding to the HPV type present in the tumor (3). When the tumor was negative for HPV16 or 18, the TIL did not react to these HPV16 or HPV18 peptides (3). While these studies clearly indicate that HPV-specific T cells can infiltrate HPV-induced cervical cancer in a substantial number of patients, they do not allow a full comprehension of the contribution and role of these HPV-specific TIL and TDLNC to the total tumor-specific immune response. The size of the HPV-specific T-cell pool in the TIL population, the polarization of these T cells as well as the breadth of this local HPV-specific response within a given individual with an HPV-specific response is unknown. It is important to gain such insights because of several therapeutic strategies under development (19-21). These include vaccines to enhance E6- and/or E7-specific T-cell reactivity (22-27) the results of which could be influenced by the presence of a preexisting HPV-specific local immune response.

Therefore, we performed a more in-depth study of the populations of TIL and TDLNC of patients for which we previously showed that they comprised HPV16- or HPV18-specific T cells (3) by a comprehensive analysis of the HPV-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T cells.. Our study revealed that many different HPV-specific T cells are present, but need proper stimulation to become full effector cells.

METHODS

Subjects

Women presenting with histologically proven cervical neoplasia (FIGO 1a2, 1b1 or 1b2) at the department of Gynecology of the Leiden University Medical Centre were enrolled in the CIRCLE study, which investigates cellular immunity against HPV16- positive cervical lesions after providing informed consent. The study design was approved by the Medical Ethical Committee. The subjects were tested for HPV status using HPV16 and HPV18 specific primers on DNA isolated from surgical resection specimens (28).

Antigens

A set of 22-mer peptides overlapping by 12 residues spanning both HPV16 and HPV18 E6 and E7 protein were synthesized and dissolved as described earlier (13;27) and used for T cell stimulation assays.

Isolation and culture of T cells

Cervical tumor biopsies were obtained from patients scheduled for radical hysterectomy as described previously (3). Briefly, fresh cervical tissue was minced and cultured in IMDM, supplemented with 10% human AB serum (PAA laboratories, Pasching, Austria), 10% T Cell Growth Factor (TCGF, Zeptometrix, Buffalo NY, USA) and 5 ng/ml IL-15 (Peprotech, Rocky Hill NJ, USA). On the first day 5 ng/ml IL-7 (Peprotech) was added to cultures to drive homeostatic expansion of T cells. This does not alter the CD4/ CD8 T-cell composition of the TIL(3), but it allows the acquisition of sufficient numbers of T cells for immunological assays. After 2-3 weeks of T-cell expansion (mean $18x10^{6}$ cells, range 4 - $40x10^{6}$) the T cells were harvested and stored in liquid nitrogen.

TDLN derived from the pelvic region contained tumor cells, indicative of metastatic cancer. The TDLN were cut into pieces and incubated for one hour at 37 °C in the presence of collagenase (200 IU/ml, Sigma) and DNAse (50 μg/ml, Sigma), then put through a cell strainer (BD, Erebodemgem, Belgium) to obtain single cells. TDLNC were not expanded but directly stored in liquid nitrogen.

Analysis of T-cell specificity by proliferation assay

T cells (25,000-50,000/well) were stimulated with autologous monocytes or irradiated autologous Epstein-Barr virus transformed B cell lines (B-LCL's) pulsed with HPV16 or 18 E6 and E7 peptides (5 μg/ml) in triplicate wells in a 3-day proliferation assay. After 48 hours supernatant was harvested and stored at -20°C for cytokine analysis. During the last 16 hours of culture 0.5 μCi/well [3H]thymidine was added to measure proliferation (27) The stimulation index (SI) was calculated as the average of test wells divided by the average of the medium control wells. An SI >2 was considered a positive response. Antigen-specific IFNγ

and IL-10 production was measured by ELISA (29). Antigen-specific cytokine production was defined by a cytokine concentration above the cut-off value (IFNγ 100pg/ml; IL-10 20 pg/ml) and >2x the concentration of the medium control (4).

Analysis of T-cell specificity by multiparameter flow cytometry

T cells were examined directly *ex-vivo* (TDLNC) or after homeostatic expansion (TIL) to quantify the number of HPV-specific T cells. B-LCL were pulsed with 5 ug/ml HPV-16 or 18 E6 and E7 peptide pools. TIL's or TDLNC were thawed, rested in IMDM (BioWhittaker, Verviers, Belgium) containing 10% Fetal Calf Serum (FCS, PAA laboratories, Pasching, Austria) for 5 hours and seeded into a 96-wells round bottom plate at 200,000 cells per well and 40,000 antigen-pulsed B-LCL were added. After one hour Brefeldin A (10ug/ml) was added to the culture and left overnight. Cells were stained with antibodies to CD154-PECy5, CD137-APC, CD3-Pacific Blue, CD4-PECy7, CD8-APCcy7, IFNγ-FITC and IL-2-PE (all from BD Pharmingen, the Netherlands)(30).

In addition, TDLNC and TIL were stimulated with a mix of irradiated autologous B-LCL and 5 ug/ml HPV16 or HPV18 E6 or E7 peptide pools and irradiated allogeneic PBMC pool, in order to obtain enough HPV-specific T cells to measure the breadth of the response with respect to single peptide-antigens and for TCRVβ usage analyses (8 sets of antibodies). After a 3 week rest period these cells were tested for their specificity by overnight incubation with each single peptide. Responses were considered positive when the percentage of HPV stimulated CD154 and/or CD137 positive cells was at least three times the medium control.

Analysis of the breadth of the HPV-specific T-cell response

In vitro expanded T cells were stimulated with the indicated single peptides of HPV-16 or 18 E6 and E7 (5 ug/ml). Per peptide 500.000 cells were analyzed by multiparameter flow cytometry as described above. One day later this analysis was repeated for those peptides found positive, but then the antibodies to the cytokines were replaced by antibodies to different TCRVβ (Beckman Coulter, Immunotech, France). A TCRVβ was considered dominant (>10%), subdominant (3-10%) or minor (<3%) on basis of the percentage of HPV-specific cells using the same TCRVβ.

RT-PCR

Expanded bulk cultures were enriched for CD8+ T cells by negative selection using CD4+ isolation dynal beads (Invitrogen, the Netherlands). After RNA was isolated with the RNAeasy mini isolation kit (Qiagen, the Netherlands), cDNA was synthesized using the iScript cDNA Synthese kit (Biorad). Vβ PCR was performed on amplicons as previously described (31). Primers were kindly provided by dr M.H. Heemskerk.

In vitro stimulation with peptides and TLR ligands

TDLNC were thawed, rested for 5 hours and stimulated with 1ug/ml pool of E6 and E7 peptides, TCGF 10% and IL15. Toll like receptor (TLR) 4 ligand LPS 250 ng/ml (Sigma-Aldrich, USA), TLR 3 ligand Poly(I:C) 12.5 μg/ml (InvivoGen, USA) and TLR 1-2 ligand Pam3CSK4 20 μg/ml (InvivoGen, USA) were added at the start of culture where indicated. To stimulate TIL, monocytes were pulsed with 1 ug/ml of E6 and E7 peptide pool and the indicated TLR ligands. After 5 hours monocytes were washed and TIL were added. Supernatant was taken every 2 days and analyzed by human Th1/Th2 cytometric bead array (BD Pharmingen, USA). Cells were left to rest for 2-3 weeks before analysis of the percentage of activated and cytokine producing cells by flow cytometry.

RESULTS

Quantification of HPV-specific T cells in tumor and lymph nodes

TDLNC and homeostatic cytokine-mediated expanded TIL isolated from a selected group of 16 cervical carcinoma patients, comprising 10 patients of whom it was known that their TIL (8 patients) or TDLNC (2 patients) contained T cells that specifically reacted to the peptides of the HPV type present in the tumor and 3 patients in whom we previously failed to detect HPV-specific immunity (3), and 3 patients with unknown reactivity.

The presence of HPV-specific T cells within the TIL cultures was analyzed by their capacity to proliferate upon stimulation with HPV E6 and E7 peptides (Table 1). As expected proliferating HPV-specific T-cells were found in 8/12 tumors tested. All HPV-specific T-cell cultures produced IFNγ, yet the amount of production varied greatly (103 - >5000 pg/ml), irrespective of the proliferative capacity of the cells. In addition, 4/8 positive cultures produced IL-10 (45 – 836 pg/ml). The TIL all reacted to PHA and proliferation was associated with large amounts of IFNγ and IL-10 (not shown).

To type and enumerate HPV-specific T cells within the TIL or TDLNC populations, the percentage of CD4+ and CD8+ T cells specifically expressing the activation markers CD137 and/or CD154 when stimulated with E6 and E7 peptides or proteins was analyzed. CD137 is known as an activation marker for CD8+ T cells and CD154 for CD4+ T cells (32;33). The TIL cultures that were negative in the proliferation assay were taken along as control. In 7/10 TIL cultures tested and 2/4 TDLNC, HPV specific activated T cells were detected and comprised CD4+ or CD8+ T cells or both (Table 1). In two cases not enough TIL were available to perform this analysis. In one TIL culture – in which we previously failed to detect HPV-specific T cells by proliferation - an HPV-specific CD8+ T-cell response was detected. The percentage of HPV-specific T cells generally corresponded with the strength of proliferation. HPV-specific CD4+ T cells expressed both CD154 and CD137 (Figure 1a), while HPV-specific CD8+ T cells predominantly expressed CD137, yet sometimes co-

Table 1. Analysis of TIL and TDLNC before antigen specific expansion *in vitro*

Patient	HPV type*	Origin	Days of culture†	Proliferation assay (3 d)				T cell	Overnight activation analysis		
									Activated cells (%)‡		
				Reactivity§	SI‖	IFNγ¶	IL-10¶		Medium	E6	E7
1	16	TIL	9	16E6	2.9	103	<20	CD4	0.16	0.58	0.26
2	18	TIL	13	18E7	5.4	763	45	CD4	0.04	0.03	0.17
3	16	TIL	15	—				—	—	—	—
4	16	TIL	26	16E6	104	>5,000	315	CD4	0.8	63	0.9
								CD8	0	47	0.7
5	16	TIL	79	16E6	109	>5,000	836	CD4	3.1	66	2.7
6	18	TIL	25	—				—	—	—	—
7	16	TIL	12	—				CD8	2.9	24	3.4
8	16	TIL	29	—				—	—	—	—
9	16	TIL	17	16E6	2.6	110	<20	CD8	0.4	4.5	0.6
10	16	TIL	27	16E6	4.8	>5,000	<20	CD4	0.13	0.52	0.14
11	18	TIL	30	18E7	8.9	244	66	NT**			
12	16	TIL	68	16E6	4	276	<20	NT			
13	18	LN	0	NT				—	—	—	—
14	16	LN	0	NT				CD4	0.12	0.63	0.16
								CD8	0.23	5.92	0.11
15	16	LN	0	NT				CD4	0.51	24	20
16	16	LN	0	NT				—	—	—	—

*HPV type found in the tumor by PCR.
†Days of cytokine-mediated homeostatic expansion before immune assay.
‡Percentage of total CD4 or CD8 T-cell population expressing CD154 and/or CD137 after stimulation with the indicated antigen corresponding to the HPV type present in the tumor. A response equal or more than three times the medium control was considered positive.
§Reactivity indicates the HPV type and protein to which the T-cell culture specifically reacted.
‖SI, stimulation index, average proliferation of test wells divided by the average proliferation of medium control wells. SI > 2 is positive.
¶Antigen-specific cytokine production in pg/mL is indicated when test value was above cutoff level and at least more than two times the background production (4).
**NT, not tested.

expressed CD154 (Figure 1a). The percentage of HPV-specific T cells varied enormously between as little as 0.17% to as much as 66% (Table 1) of the CD4+ or CD8+ T cells present in TIL and from 0.63% - 24% in TDLN. This was independent of the total numbers of T cells isolated, indicating a great variability in the contribution of HPV-specific T cells to the total local anti-tumor response between patients.

The local HPV-specific response consists of a broad T-cell repertoire

To study the breadth of the HPV-specific TIL/TDLNC-repertoire the isolated cells were stimulated with pools of E6 or E7 peptides as otherwise there would not be enough cells to study the response to single peptides. This allowed the analysis of the breadth of the HPV-specific T-cell response to single peptides in 12 patients (Figure 1b; Supplemental Table 1). The three TIL cultures tested negative before remained negative excluding that the responses detected are primed *in vitro*. In most of the patients the HPV-specific CD4+ T-cell response was highly diverse as in 5/10 patients CD4+ T cells responded to 5 or more different peptides and in another 4 patients the CD4+ T cells recognized 2 different peptides (Supplemental Table 1). HPV-specific CD8+ T-cell reactivity was detected in 6 of the 12

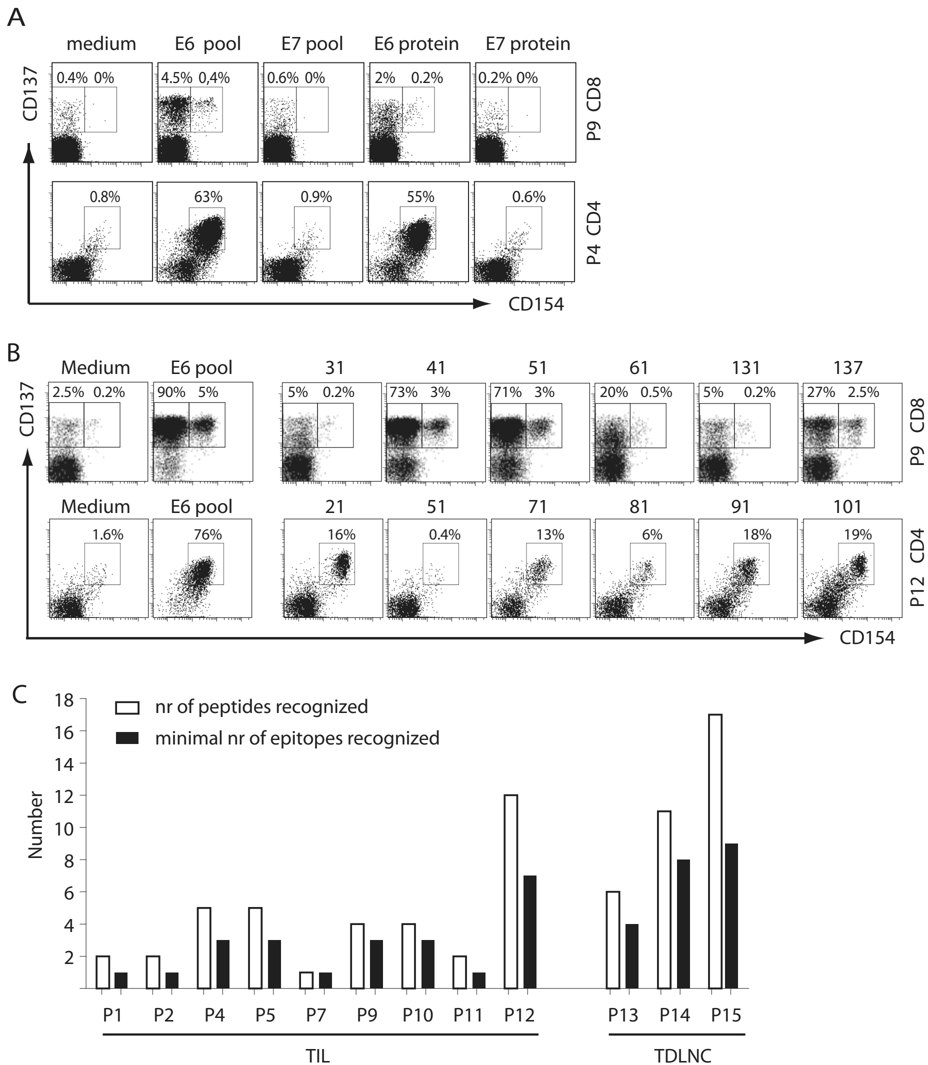

Figure 1. HPV-specific T cells in TIL and TDLNC.
(A) An example of a CD4 and a CD8 HPV-specific response measured by flow cytometry using the activation markers CD154 and CD137 in two TIL cultures tested before antigen-driven expansion *in vitro* (P4 and P9). (B) The breadth of the response was analyzed after antigen-driven expansion. TIL and TDLNC cultures were stimulated with single peptides and analyzed for the expression of CD154 and CD137. P9 displayed a CD8 response against four different single peptides (notably 41, 51, 61 and 137). P12 displayed a CD4 response against six different single peptides (notably 21, 61, 71, 81, 91 and 101). (C) Summary of the number of different peptides recognized by each single culture (white bars) after antigen-driven expansion. The minimal number of epitopes recognized (black bars) was estimated by counting the response to two overlapping peptides as one, because the peptides used are 22-mers, overlapping in 12 amino acids.

tested patients, five of whom displayed CD8+ T-cell reactivity to 2-6 different peptides (Figure 1b; Supplemental Table 1).

The minimal number of T-cell epitopes recognized per patient was estimated by counting the response to two overlapping peptides as one because they overlapped in 12 amino acids. The majority of the patients (8/12) recognized 3 or more different T-cell epitopes (Figure 1c). The TDLNC populations reacted against 4-9 different epitopes (Figure 1c). Thus the tumor-induced HPV-specific T-cell repertoire is directed against multiple T-cell epitopes.

As each individual T-cell epitope can be recognized by different T-cell clones, we studied the number of TCRVβ families involved in the recognition of each epitope by using a commercially available TCRVβ-specific antibody kit. The different T-cell clones were operationally defined as the cohort of activated HPV single peptide-specific CD4+ or CD8+ T cells expressing the same TCRVβ-chain. Figure 2a shows a number of examples on the contribution of several T-cell receptor families reactive to one single peptide. Often one or two dominant TCRVβ's were found (Table 2 and Figure 2b), as well as several sub-dominant and minor TCRVβ's. For example, the HPV-specific CD4+ T-cell response of patient P14 reacted to 9 different peptides and - on the basis of the different TCRVβ's present in the population of activated T cells - this involved the activation of at least 43 different T-cell clones (Table 2 and Figure 2b). In a number of cases not all TCRVβ could be indentified as the available antibodies cover approximately 70-80% of the full TCRVβ-repertoire (34). In one case (P7) of whom all the cells in the cultured T-cell population responded exclusively to one peptide - but for which only 22% of the HPV-specific T cells the TCRVβ was accounted for by antibodies (Figure 2a, fourth row and Table 2)- a semi-quantitative RT-PCR was applied revealing the presence of TCRVβ24 (Figure 2c). In another case (P15) only 30% of the TCRVβ were accounted for by flow cytometry. Here, three additional TCRVβ 6C, 6D and 15 (not shown) were detected by RT-PCR. No skewing to a certain TCRVβ within this patient group was found, nor was there any skewing of certain TCRVβ families to individual peptides observed (Figure 2d). Thus the HPV-specific T-cell repertoire consists of a polyclonal T-cell population able to respond to many different CD4 and CD8 T-cell epitopes.

HPV-specific TIL generally lack type 1 polyfunctional T cells

The production of type 1 cytokines (IFNγ, IL-2) is essential for an effective anti-tumor response and instrumental to functionally characterize antigen-specific T cells (35-40). Therefore, the *ex-vivo* enumerated HPV-specific CD154+ and/or CD137+ T cells were simultaneously analyzed for their production of IFNγ and IL-2. Four distinct cytokine profiles were found (Figure 3ab). Profile 1 was found in one patient (P7). Despite the presence of a high percentage of HPV E6-specific CD8+ T cells (24%), only about 4% produced either one of the cytokines. The second profile comprised HPV-specific T cells of which the majority produced both IFNγ and IL-2 (*e.g.* P4 and P5), HPV-specific T cells in the third profile mainly produced IL-2 (*e.g.* P1, P2, P10 and P15), whereas they produced mainly IFNγ in

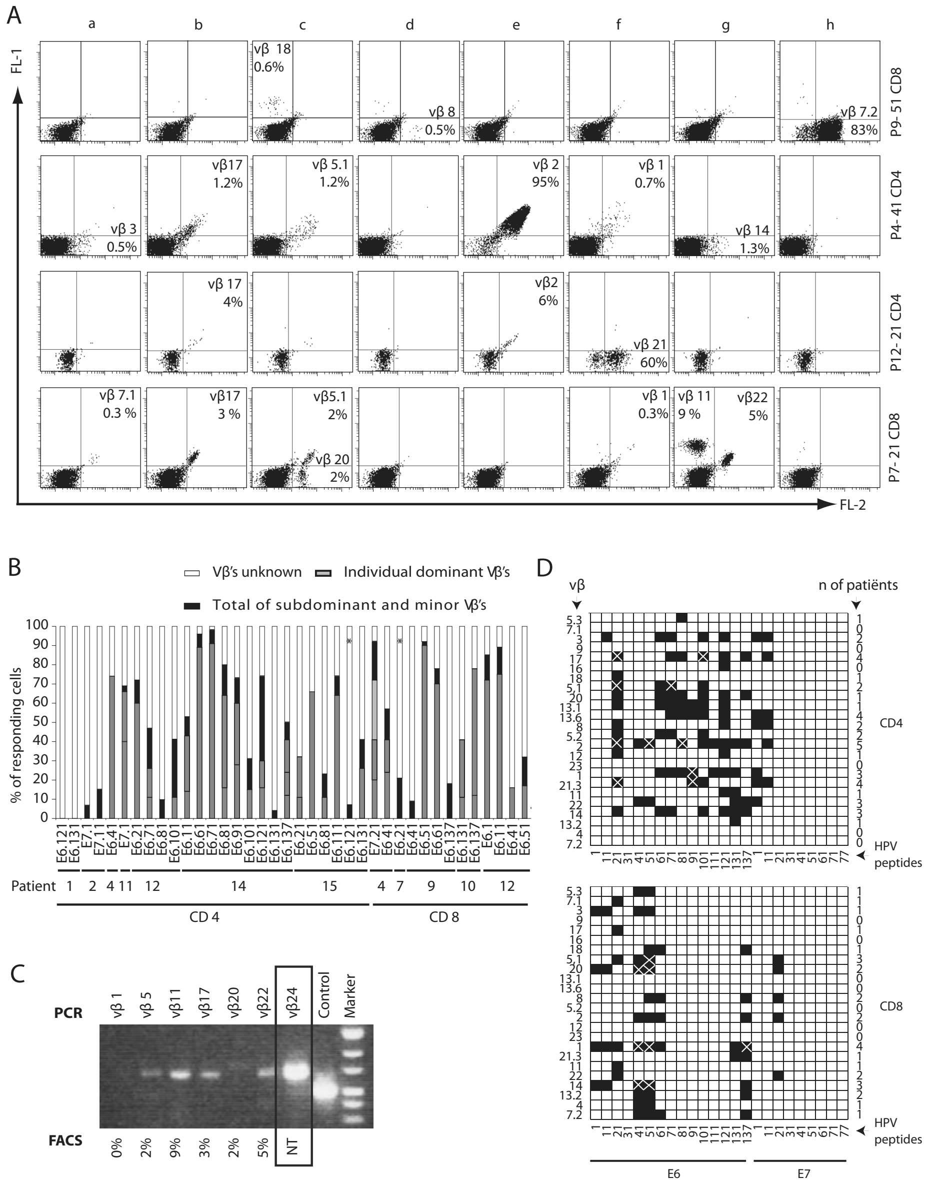

Figure 2. The T-cell response to a single HPV epitope involves the use of multiple dominant and subdominant TCRVβ families.

(A) The TCRVβ families used by T cells responding to a single peptide were analysed in the T-cell population with high expression of activation marker(s) after peptide stimulation. Depicted is the response against one peptide for four different patients (P4, P7, P9 and P12). The letters (a-h) indicate the eight different antibody pools, each consisting of three differently labeled antibodies (FITC (FL1), PE (FL2) or FITC-PE combined) specific for three different TCRVβ, plotted in separate quadrants. The involved TCRVβ and the percentage of responding cells are indicated in the plots.

(B) The relative contribution of dominant and subdominant TCRVβ families to the overall HPV-specific T-cell response is depicted. The grey stacked bars indicate the percentage of HPV-specific T cells comprising a dominant TCRVβ(s) and the black stacked bars indicate the accumulated percentage of all the sub-dominant and minor TCRVβ families (individual percentages for each family not shown) of all T cells responding to one peptide-epitope. The white bars indicate the percentage of HPV-specific T cells for which we could not identify the TCRVβ families involved using the TCRVβ kit. Patient numbers and 22-mer peptides recognized are indicated by the protein name and the first amino acid in the sequence of this protein. The * identifies the two cultures for which an additional TCRVβ analysis was done by RT-PCR allowing the detection of other TCRVβ's not present in the TCRVβ kit. (C) RT-PCR for TCRVβ in TIL of patient P7, revealing the presence of TCRVβ24. The percentage of each TCRVβ that was detected by flow cytometry is indicated on the left.
(D) A fingerprint overview of all TCRVβ's detected upon response to a single peptide-epitope for the patients investigated. The black squares indicate that the TCRVβ (indicated at the left) was involved in response to that particular peptide (indicted at bottom). A white cross means that this TCRVβ was found in two independent patients responding to that peptide, the right column indicates in how many patients this particular TCRVβ was found.

profile four (P14 and P9). These data indicate that while most of the HPV-specific TIL can produce either one of the type 1 cytokines, there are only few patients (2/9) in whom the majority of their HPV-specific TIL simultaneously produce IFNγ and IL-2 (Profile 2; Figure 3ab).

Activation of HPV-specific T cells in the presence of TLR ligands increases the type 1 cytokine effector response

Our results showed that in many cases the HPV-specific T-cell response is not associated with strong production of IFNγ (Table 1 and Figure 3ab). In mouse models, the local injection of TLR2, TLR3 or TLR 4 ligands can augment the tumor response (41-43). To mimic the local delivery of antigen and TLR ligand, homeostatic expanded TIL from 2 patients were stimulated with HPV antigen-pulsed TLR-activated autologous monocytes whereas TDLNC from 2 other patients, which already contained APC, were activated with their cognate HPV-antigens in the absence or presence of TLR-agonist directly *ex-vivo.* Cytokine analyses revealed a faster and higher production of IFNγ during the first 7 days after activation in all four TIL and TDLNC cultures tested when PAM3CSK4 (TLR2) was added and in 3 out of 4 of the cultures with poly(I:C) (TLR3) (Figure 3c). The use of the TLR4 agonist LPS boosted the IFNγ-response in one patient (P14), but with somewhat slower kinetics. Notably, PAM3CSK4 (TLR2) also increased the production of the Th2 cytokine IL-5 in 3 out of 4 cultures (not shown). Analysis of the constitution of the responding cell population after 14 days, allowing the activated T cells to come to rest which is needed to decrease background staining for the activation markers and cytokines, revealed no overt differences in the number of activated cells or the percentage of IFNγ, IL-2 or double- producing T cells after this period (not shown).

Table 2. Different TCRVβ families found within the HPV-specific T cell population

	Responding*			Peptides†	Number of TCRVβ found per peptide by FACS‡			
	Patient	T cell	Antigen		Dominant	Subdominant	Minor	Total
TIL	2	CD4	E7	1	0	0	4	4
				11	0	2	4	6
	4	CD4	E6	41	1	0	0	1
				51	1	0	0	1
		CD8	E6	41	2	1	3	6
				51	2	1	3	6
		CD8	E7	21	3	2	0	5
	7	CD8	E6	21	0§	3	4	7§
	9	CD8	E6	41	0	1	1	2
				51	1	0	3	4
				61	1	1	3	5
				137	0	2	5	7
	10	CD8	E6	131	2	0	0	2
				137	2	0	0	2
		CD4	E7	1	2	0	0	2
	11	CD4	E6	21	1	2	0	3
				71	2	0	1	3
				81	0	1	1	2
				101	1	3	0	4
	12	CD8	E6	1	1	0	3	4
				11	1	0	3	4
				41	1	1	4	6
				51	1	3	2	6
LN	14	CD4	E6	11	2	0	8	10
				61	1	1	5	7
				71	1	1	5	7
				81	2	2	0	4
				91	2	2	0	4
				101	1	3	3	7
				121	2	6	2	10
				131	0	0	2	2
				137	3	2	0	5
	15	CD4	E6	21	2§	0	0	2§
				51	1	0	0	1
				81	1	2	1	4
				111	1	1	0	2
				121	0	2	0	2
				131	1	1	3	5

*Depicted are all patients of whom enough T cells were available for TCRVβ analysis after antigen-specific expansion by stimulation with E6 or E7 peptide pool.

†The number indicates the first amino acid of the 22-mer peptide of the antigen that the culture specifically responds to by the expression of the activation markers CD154 and/or CD137.

‡A dominant TCRVβ consists of >10% of the activated T cells. A subdominant TCRVβ consists of between 3% and 10% of the activated T cells. A minor TCRVβ consists of <3% of the activated T cells.

§Additional TCRVβs were found by PCR analysis for P7 and P15.

DISCUSSION

We have comprehensively analyzed the spontaneous tumor-specific immune response in patients with cervical cancer by dissecting local HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells. The expression of the two known tumor antigens E6 and E7 in all cervical cancer cells and the use of overlapping peptide arrays for immunomonitoring in combination with the activation markers CD154 and CD137,

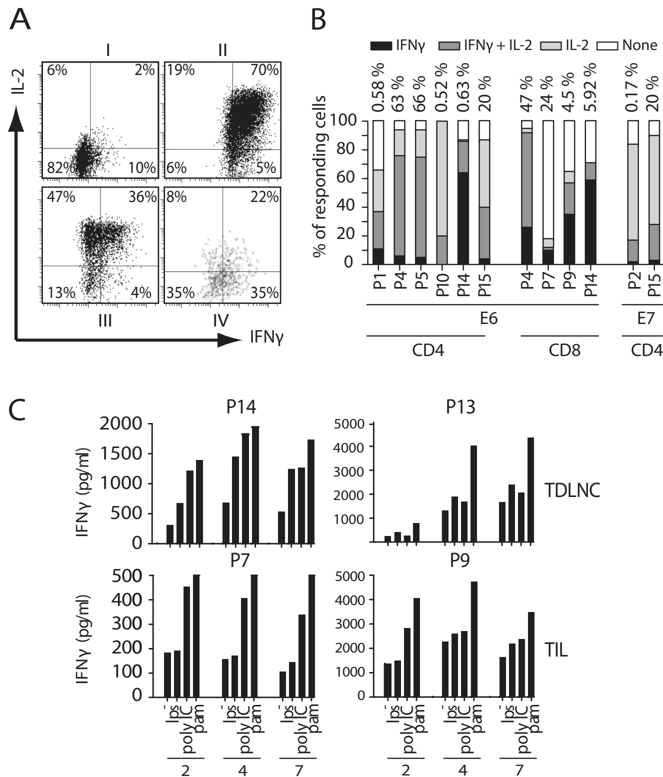

Figure 3. Functionality of HPV-specific TIL and TDLNC .
(A) The specific activity to HPV16 or 18 E6 and E7 peptide pools by simultaneous analysis of CD154, CD137, IFNγ and IL-2 in homeostatic cytokine-mediated expanded TIL as well as directly *ex-vivo* in TDLNC. Four different cytokine profiles could be distinguished. Profile I: HPV-specific T-cells with few T-cells producing cytokines (P7). Prolife II: Predominant production of IFNγ and IL-2 (P4). Profile III: predominant production of IL-2 (P15). Profile IV: mainly IFNγ producing HPV-specific T cells (P9). (B) Overview of the cytokine production. The percentage of activated HPV-specific T-cells is indicated on top of the bars. The stacked bars indicate the percentage and type of cytokines (black: IFNγ, grey: IL-2, hatched: IFNγ+IL-2 and white: no IFNγ or IL-2 (none)) produced within the activated HPV-specific T-cell population. (C) The homeostatic expanded TIL of patients 7 & 9 as well as the TDLNC of patients 13& 14 were stimulated with HPV16/18 E6 or E7 peptide pools and TLR-agonist when indicated (medium control indicates peptide stimulation without addition of TLR-agonist). After 2, 4 and 7 days supernatant was harvested and analyzed for cytokine production by cytokine bead array.

offered the advantage to study the complete cervical cancer-specific local T-cell repertoire - independently of the knowledge of defined T-cell epitopes and not restricted to particular HLA-types – in a quantitative manner. We used a selected panel of HPV16- and HPV18-typed cervical cancer patients for whom we previously showed that their TIL comprised HPV type-specific T cells, indicating that the current set of data applies to about 40-50% of all patients with an HPV16- or HPV18-positive cervical carcinoma (3). Our data show that

while HPV-specific T-cell responses can be detected within the tumors and tumor draining lymph nodes of this group of patients with cervical cancer their relative contribution to the overall local anti-tumor response varied enormously, ranging from <1% to 66% (Table 1). While we can not exclude that the quantification of HPV-specific T cells among the total population of TIL is accurate as it likely to be biased due to the isolation procedure, the results obtained in the ex-vivo measurement of HPV-specific T cells among TDLNC still sustains this notion. Strikingly the HPV-specific response of most patients tested was broad as it targeted multiple peptide-epitopes within the E6 and E7 tumor-specific antigens and the T-cell response to each and every peptide-epitope involved multiple dominant and/ or subdominant TCRVβ families (Table 2, Figure 2). One could argue that our analyzes concerning the breadth of the response is biased through the expansion of TIL by either homeostatic cytokines or peptide stimulation, as these rounds of expansion may not equally amplify all possible responding cells and less well proliferating HPV-specific T-cell clones may even become extinct. Yet in view of the broad responses observed already this would only mean that in reality the response is even broader and even now is still underestimated.

The broad and hierarchical responses closely resemble the published pattern of CD4+ and CD8+ T-cell responses to genetically stable viruses, such as CMV (34). This brings forward the question whether the HPV-specific T-cell responses observed in these cancer patients reflect a characteristic antiviral response or an anti-tumor response. As shown previously, patients with HPV-induced pre-malignant disease either fail to mount HPV-specific immunity (4;12) or induce a non-beneficial HPV-specific T-cell response during progression of disease (4;12). Therefore, we deem it more likely that the T-cell responses studied here reflect a typical tumor-specific T-cell response. Indeed, a similar hierarchy of the spontaneous T-cell response was observed in a study of two patients responding to NY-ESO-1 (44), as well as in the HLA-A*0201-restricted Melan-A/MART-1_{26-35}-specific CD8 T-cell response (6). The presence of single peptide-specific dominant and subdominant (based on TCRVβ-chain expression) T cells within the tumor tissue implies that subdominant TIL participate in the immune surveillance of tumors and not simply act as a reservoir. It is likely that the different dominant and subdominant HPV-specific T cells have different functions. This is illustrated by the isolation of both HPV-specific T-helper and T-regulatory cells from the same tumor in a group of cervical patients studied previously (45). Overall, the local HPV-specific immune response consists of a polyclonal T-cell population able to respond to many different CD4 and CD8 T-cell epitopes.

CD4+ and CD8+ T cells as well as the cytokines IFNγ and IL-2 play a key role in the protection against cancer (4;35-38) as well as in the control of chronic viral infections (39;40). In most of the *ex-vivo* tested TIL and TDLNC the population of IFNγ and IL-2 producing T cells or IFNγ-producing T cells among the total population of HPV-specific T cells as well as the amount of IFNγ produced was low (Table 1 & Figure 3b), suggesting that most of the HPV-specific TIL and TDLNC are rendered functionally tolerant within the

tumor environment and implying that the local tumor-specific immune response in cervical cancer patients does not differ from others, such as melanoma (46). *In vitro* stimulation of these HPV-specific T cells with their cognate antigen resulted in an increase in the total population of HPV-specific T cells (*e.g.* P9 and P12, compare Figure 1a and Figure 1b) as well as in an increased IFNγ production (P9; compare Table 1 & Figure 3c). We recently reported that the majority of patients vaccinated with a HPV16 E6 and E7 long peptide vaccine, showed a clearly detectable and broad vaccine-induced HPV-specific immune response as detected by IFNγ-ELISPOT (23;24). Most likely this vaccine taps the broad available T-cell repertoire we identified in this study and either primes (in the group of patients without HPV-specific reactivity) or boosts their number. Interestingly, when TIL and lymph node derived T cells are *ex-vivo* stimulated with cognate antigen in the presence of TLR ligands, such as PAM3CSK4 or poly (I:C), an even more pronounced increase of effector function is observed (Figure 3c). This suggests that local delivery of these innate immune-derived stimulating factors can overcome functional tolerance in human cancers as was also observed in murine tumor models (41-43). Moreover, they are likely to assist therapeutic vaccines in driving T-cell responses with increased function as shown previously for a melanoma peptide vaccine in combination with CpG (47). Such type of responses is highly required as they do not only correlate with clinical efficacy in murine models (41), but also in human trials (48). Interestingly, the widely used TLR4 ligand LPS did not overtly increase IFNγ production of TIL and TDLNC in our study.

In conclusion, the TIL or TDNLC isolated from 40-50% of patients with an HPV16 or HPV18-induced cervical tumor contain HPV type-specific T cells (3). Our study of the local T-cell repertoire within this group of patients suggests that within the tumor environment or tumor-draining lymph node of a given patient many different tumor-specific CD4+ and CD8+ T cells are poised for action but are awaiting proper stimulation.

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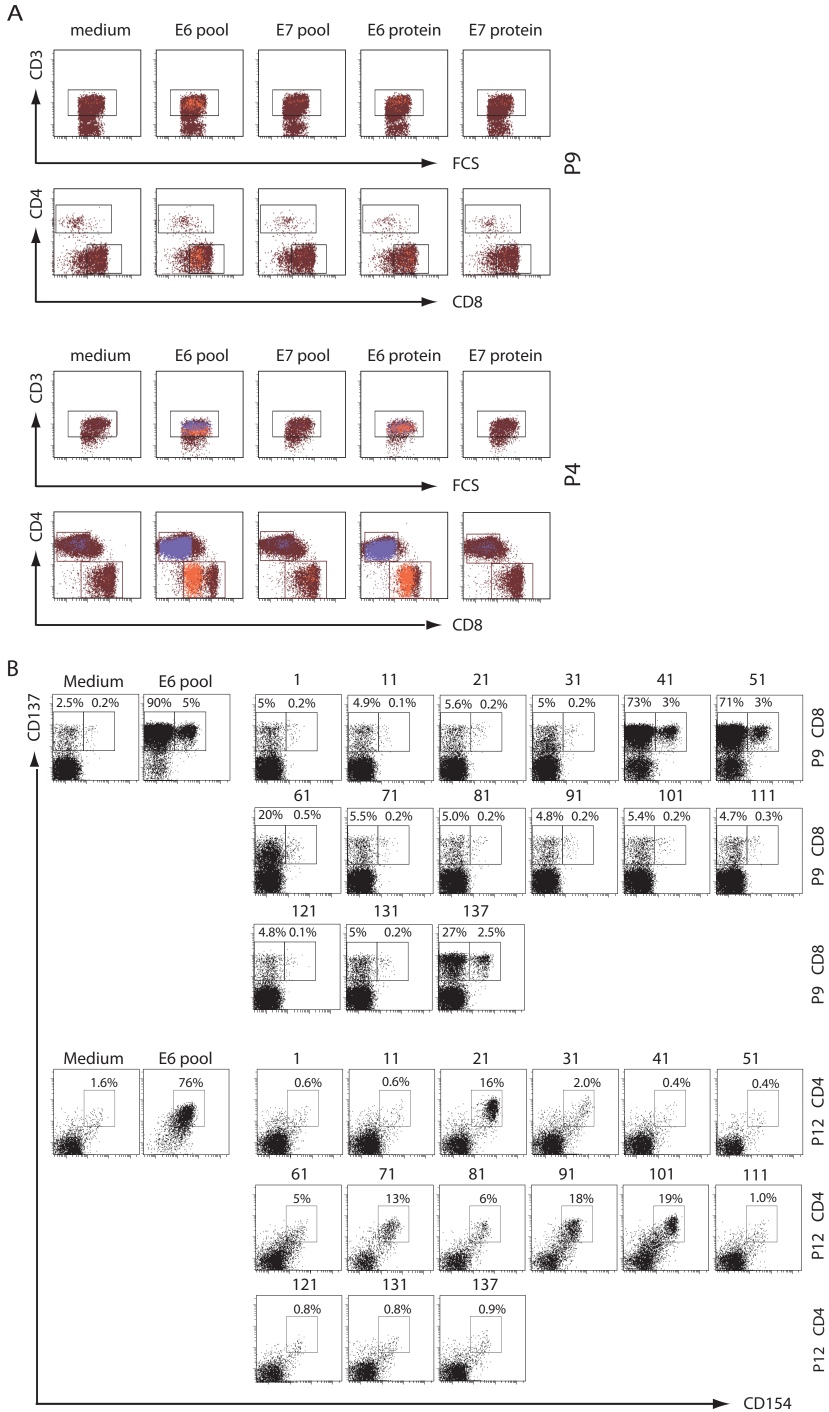
A
medium
E6 pool
E7 pool
E6 protein
E7 protein
CD3
FCS
P9
CD4
CD8
P4
B
Medium
E6 pool
CD137
CD154
2.5% 0.2%
90% 5%
1
11
21
31
41
51
5% 0.2%
4.9% 0.1%
5.6% 0.2%
5% 0.2%
73% 3%
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20% 0.5%
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19%
1.0%
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0.9%
P12 CD4

Supplemental figure 1.

(A) Activated cells (CD154+ and/or CD137+) within CD3+ T cell population and within the CD3+ T-cell gated population expressing either CD4 or CD8. In these plots the homeostatic expanded TIL from two patients (P9 and P4) after stimulation with medium only, E6 or E7 peptide pools and E6 or E7 protein is shown. The peptide or protein-activated CD154+ and/or CD137+ T cells as shown in Figure 1 where back-gated and indicated by a red color (activated CD8 T cells) or a blue color (activated CD4 T cells). Down regulation of CD8 is seen in the CD8+ T-cell population stimulated with the peptide pool and to a lesser extent in CD8 T cells stimulated with protein and this is not observed for CD4 on activated CD4 T cells.

(B) An overview of all the single peptides used for analysis of the breadth of response after antigen-driven expansion for P9 and P12 (as partly shown in figure 1). Shown here are all the positive and negative responses as measured by the expression of CD154 and CD137. P9 displayed a CD8 response against four different single peptides (notably 41, 51, 61 and 137). P12 displayed a CD4 response against six different single peptides (notably 21, 61, 71, 81, 91 and 101).

Supplemental table 1. Breadth of the HPV specific response detected in TIL and TDLNC

	Responding*				Activated
	Patient	**T-cell**	**Antigen**	**Peptides****	**cells (%)†**
TIL	**1**	CD4	E6	medium	2
			E6	121	22
			E6	131	23
	2	CD4	E7	medium	2
			E7	1	10
			E7	11	10
	4	CD4	E6	medium	4
			E6	41	89
			E6	51	65
		CD8	E6	medium	0,6
			E6	41	73
			E6	51	60
		CD8	E7	medium	2
			E7	21	15
	5	CD4	E6	medium	13
			E6	31	51
			E6	51	60
			E6	61	64
			E6	71	56
			E6	81	60
	7	CD8	E6	medium	12
			E6	21	47
	9	CD8	E6	medium	3
			E6	41	73
			E6	51	71
			E6	61	20
			E6	137	27
	10	CD4	E6	medium	9
			E6	1	33
		CD8	E6	medium	0.4
			E6	1	18
			E6	131	13
			E6	137	14
	11	CD4	E7	medium	5
			E7	1	92
			E7	11	67
	12	CD4	E6	medium	2
			E6	21	16
			E6	61	5
			E6	71	14
			E6	81	6
			E6	91	18
			E6	101	19
		CD8	E6	medium	1
			E6	1	47
			E6	11	42
			E6	41	37
			E6	51	27
			E6	61	2
			E6	71	3

	Responding				Activated
	Patient	**T-cell**	**Antigen**	**Peptides****	**cells (%)†**
LN	**13**	CD4	E6	medium	0.1
			E6	1	1
			E6	41	1
			E6	51	1
			E6	71	1
			E6	121	1
			E6	131	1
	14	CD4	E6	medium	0.1
			E6	11	15
			E6	61	78
			E6	71	78
			E6	81	2
			E6	91	3
			E6	101	3
			E6	121	9
			E6	131	6
			E6	137	2
		CD8	E6	medium	4
			E6	21	29
			E6	137	58
	15	CD4	E6	medium	1
			E6	1	14
			E6	21	2
			E6	41	6
			E6	51	6
			E6	71	2
			E6	81	4
			E6	111	5
			E6	121	56
			E6	131	38
			E6	137	20
		CD4	E7	medium	1
			E7	1	39
			E7	11	37
			E7	41	4
			E7	51	5
			E7	61	3
			E7	71	14
			E7	77	4

* Depicted are all patients of whom T cells were tested after antigen specific expansion with E6 or E7 peptide pools

** HPV 16 or 18 (corresponding to the HPV type present in the tumor) E6 and E7 22-amino acid long overlapping peptides were used to test specificity
Medium is activity present in cultures when no E6 or E7 22-mer peptide is added
The number indicates the first aminoacid of the peptide the culture reponded to or no peptide (medium)

† The percentage of HPV-specific T cells that responded by upregulation of activation markers CD154 and/or CD137 to the peptide depicted in the column on the left. A response is considered positive when the percentage is more than 3 times medium control

Chapter 4

Tumor-infiltrating CD14 positive myeloid cells and CD8 positive T cells prolong survival in patients with cervical carcinoma

PJ de Vos van Steenwijk
TH Ramwadhdoebe
R Goedemans
EM Doorduijn
JJ van Ham
A Gorter
T van Hall
ML Kuijjer
MIE van Poelgeest
SH van der Burg
ES Jordanova

International Journal of Cancer 2013; 133, 2884–2894

Brief description: This study on the different types of myeloid cells, their clinical impact and their co-operation with T cells offers a profound insight on the role of myeloid cells in the microenvironment and how they can work side by side to control tumors. As such, our data are a major addition to the current discussion about the impact of tumor-infiltrating myeloid cells in human cancers and offer potential new strategies to improve survival.

ABSTRACT

One of the hallmarks of cancer is the influx of myeloid cells. In this study we investigated the constitution of tumor-infiltrating myeloid cells and their relationship to other tumor-infiltrating immune cells, tumor-characteristics and the disease-specific survival of patients with cervical cancer. Triple color immunofluorescence confocal microscopy was used to locate, identify and quantify macrophages (CD14), their maturation status (CD33) and their polarization (CD163) in a cohort of 86 patients with cervical carcinoma. Quantification of the numbers of myeloid cells revealed that a strong intraepithelial infiltration of CD14+ cells, and more specifically the population of CD14+CD33-CD163- matured M1 macrophages, is associated with a large influx of intraepithelial T lymphocytes (p=0.008), improved disease-specific survival (p=0.007) and forms an independent prognostic factor for survival (p=0.033). The intraepithelial CD8+ T-cell and Treg ratio also forms an independent prognostic factor (p=0.010) and combination of these two factors reveals a further increased benefit in survival for patients whose tumor display a dense infiltration with intraepithelial matured M1 macrophages and a high CD8 T-cell/Treg ratio, indicating that both populations of immune cells simultaneously improve survival. Subsequently we made a heatmap including all known immune parameters for these patients, whereby we were able to identify different immune signatures in cervical cancer. These results indicate that reinforcement and activation of the intratumoral M1 macrophages may form an attractive immunotherapeutic option in cervical cancer.

INTRODUCTION

Cervical cancer (CxCa) is caused by high risk human papilloma virus (HPV) (1). Studies on HPV-specific T-cell response in patients with premalignant disease suggest that spontaneous regression occurs when circulating HPV early antigen-specific CD4+ and CD8+ T-cells are present and when the lesions are infiltrated with effector T-cells that outnumber regulatory T-cells. Moreover, the presence of circulating HPV-specific CD4+ T-cells is associated with T-cell infiltration in the lesion and favorable clinical outcome in high-grade squamous intraepithelial lesion (HSIL) after treatment (2;3). The development of CxCa is associated with a weak systemic and local immune response to HPV, reflected by low numbers of tumor-infiltrating T-cells comprising CD8+ cytotoxic T-cells, CD4+ T-helper cells and regulatory T-cells (4-6). The T-cells present often lack cytotoxicity (7) and/or express co-inhibitory molecules such as programmed cell death protein 1 (PD-1), CD94 and NKG2a (8;9). Tumors also down regulate Human Leukocyte Antigen (HLA) class I and MHC class I chain-related molecule A (MICA) and up regulate HLA-E and PD-L1 to further restrain the CD8+ T-cell response (2;8-10). The presence of circulating HPV-specific T-cells associates with better survival and high numbers of T-cells correlate with the absence of metastases or a relapse (2;6;11;12). Importantly, the ratio between tumor-infiltrating CD8+ and Foxp3+ T-cells was found to be the first immune-associated independent prognostic factor in CxCa (2).

Tumors mediate systemic and local effects, altering the accumulation and differentiation of myeloid cells and redirecting their function to sustain tumor outgrowth. Three groups of terminally differentiated myeloid cells are essential for innate and adaptive immunity – macrophages, dendritic cells (DC) and granulocytes – and monocytes are the major precursor of the first two populations in humans. Macrophages are a heterogeneous population of tissue-resident monocytes, which display different functions depending on the microenvironment. Roughly two types are recognized; the classically activated type 1 macrophages (M1) which are tumoricidal and produce IL-12 and the alternative activated type 2 macrophages (M2) which sabotage immunity by producing IL-10, prostaglandin E2 (PgE2), TGFβ and CCL22 (13-15). Extensive literature demonstrates that high numbers of tumor associated macrophages (TAMs) facilitate tumor growth, disease progression and poor prognosis (reviewed in (16)). The development from premalignant cervical lesions towards carcinoma is associated with high numbers of infiltrating CD68+ macrophages (17;18). However, in CxCa TAM were never associated with clinical parameters (12;19;20) or clinical outcome. It is conceivable that differences in macrophage subsets exist between patients, as some CxCa produce PgE2 and IL-6 (21;22), shown to be important for M2 macrophages differentiation (23). Furthermore, there are vast differences between patients concerning density and type of tumor-infiltrating T-cells (2;6), which may also effect the type of macrophages present (24;25).

This study uses a unique cohort of CxCa patients, for which many immune parameters are known, to investigate the presence of infiltrating myeloid cells and their relationship to other tumor-infiltrating immune cells, tumor characteristics and patient survival. We used fluorescence confocal microscopy to quantify macrophages (CD14), their maturation status (CD33) and their polarization (M2; CD163). CD14 is a specific monocyte/macrophage marker, although it can also be found on subsets of dendritic cells (26). CD33 is expressed on non-terminally differentiated myeloid cells (27) and CD163 is linked to macrophage anti-inflammatory functions (26;28;29). We demonstrate that the density of tumor-epithelium infiltrating CD14+CD163- cells is an independent prognostic factor for prolonged disease-specific survival. Furthermore, unsupervised clustering of patients based on 40 known immune markers suggested that a tumor microenvironment that allows the accumulation of high numbers of CD14+CD33-CD163- myeloid cells forms a prerequisite for tumor-infiltrating CD8+ T-cells to exert their antitumor effect.

MATERIALS AND METHODS

Patient Material

Formalin-fixed, paraffin-embedded tissue blocks from 86 CxCa patients undergoing a radical hysterectomy type III with pelvic lymphadenectomy (1985-2000) were retrieved from the Pathology Department (Leiden University Medical Center, Leiden, the Netherlands). Patients had not received radio- or chemotherapy before surgery. A trained pathologist reviewed all H&E-slides. All material was used according to Dutch Federation of Medical Research Associations guidelines.

Immunohistochemistry

Characterization of tumor-infiltrating myeloid cells (TIM) was carried out with triple immunofluorescent staining and confocal microscopy. Anti-CD33 (1:50, mouse-IgG2b, clone PWS44, LeicaMicrosystems, Rijswijk, the Netherlands), anti-CD14 (1:100, mouse-IgG2a, clone 7, Abcam, Cambridge, UK) and anti-CD163 (1:400, mouse-IgG1, Clone 10D6, LeicaMicrosystems) mixture was applied after EDTA (pH 9) pretreatment. CD68 (Mouse-IgG2a, Clone 514H12, Serotec, Düsseldorf, Germany)/CD163 double-staining was also performed. The following fluorescently-labeled antibodies were used: goat-anti-mouse IgG2b-AlexaFluor546, goat-anti-mouse IgG2a-AlexaFluor488 and goat-anti-mouse IgG1-AlexaFluor647 (MolecularProbes, Bleiswijk, the Netherlands). Images were captured with a confocal laser-scanning-microscope (Zeiss LSM 510, Germany) in a multitrack setting. Per slide, five randomly selected images were captured. Negative control slides, omitting the primary antibody, were included. Tumorcell-nests and stroma were measured using the

Zeiss LSMImageExaminer and myeloid subsets were manually counted and presented as number/mm^2.

In vitro differentiation assay of M2 macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (29). CD14+ monocytes were isolated using MACS cell-separation (Miltenyi Biotec). Monocytes were cultured with 25 ng/ml M-CSF (R&D systems, United Kingdom) to induce M2 macrophages (23). On day 0 and 8, one sample was taken for FACS analysis and another was paraffin-embedded and stained with CD14 AF700 (clone M5E2, BD Biosciences, the Netherlands), CD33 PEcy7 (clone P67.6, BD Biosciences, the Netherlands) and CD163 APC (clone 215927, R&D systems, United Kingdom) .

Statistical Analysis

Patients were divided into groups based on the median of the infiltrating cells. Cumulative disease-specific survival rate was calculated by the Kaplan-Meier method and analyzed by the log-rank test (Statistical Package for the Social Sciences software package 17 (SPSS)). A Cox regression analysis was used for the univariate and multivariate survival analyses of lymph node status, tumor size, tumor infiltration depth, vasoinvasion and parametria involvement as well as the different types of tumor-infiltrating immune cells. A p-value of <0.05 was considered statistically significant. The two-sided χ^2 and the Fisher's exact tests were used to associate TIMs, clinical variables, tumor-infiltrating lymphocytes (TILs) and expression of other tumor ligands. The Bonferroni correction was applied for multiple analyses. The Pearson's correlation was used within the myeloid cells group.

For the creation of a heatmap, immune cell counts were divided into quartiles, except the CD3+CD8+CD57+ cells which were displayed in two groups due to low positive cell number. Tumor ligand expression was divided into two or three groups: positive or negative (HLA-E (9), HLA-G (unpublished), HLA-class II (2;10;30), MICA (2), PD-L1 (8), Serpin A1/A3 (31)) or negative, weak, strong (Chemokine (C-X-C motif) ligand (CXCL)12 , C-X-C chemokine receptor (CXCR)4 (32), Indoleamine2,3-dioxygenase (IDO) (unpublished), CXCR7 (32), HLA-class I (2;10;30) and epidermal growth factor receptor (EGFR) (33)). The function 'heatmap' of the 'stats' package in R (Development Core Team, a language and environment for statistical computing, reference index version 2.14.0. 2005 Foundation for Statistical Computing, Vienna, Austria) was used. Standard settings were used: euclidean distance and complete-linkage clustering (34). Data on the different lymphocitic sub-populations was previously generated (2;6). Tumor expression of the various ligands was measured by the Ruiter-system (35). Changes from the lowest to the highest quartile are reflected by a darker color, white blocks are missing data.

RESULTS

The CxCa microenvironment comprises a large variety of myeloid cells

Patient characteristics are displayed in table 1. To evaluate the infiltration of TIMs within the tumor-epithelium and stroma 86 tumors were analyzed for CD14, CD33 and CD163. Vast differences in the number and type of myeloid cells was found between tumors (Figure 1 and Table 2). In general, the stroma was more densely infiltrated with TIMs. The most common TIMs within the tumor-epithelium were CD14+CD33-CD163-, CD14+CD33+CD163+ and CD14-CD33-CD163+. These were also abundantly found in the stroma (Table 2). To confirm that the CD14+ cells were macrophages a staining was performed with the macrophage specific marker CD68 as well as CD163 in 10 CxCa. About 70% of the CD68+ cells expressed CD163 (M2 macrophages) (Supplementary Figure S1a). Additionally, in vitro M-CSF-mediated differentiation of monocyte to M2 macrophages showed that these cells clearly expressed CD14 and high levels of CD163 (Supplementary Figure S1b). All together, we showed that tumors are infiltrated with variable M2 (CD14+CD163+) and M1 (CD14+CD163-) macrophage numbers. In both our analyses using CD68 or CD14 as a macrophage marker we observed CD163+ cells that were CD68- (Supplementary Figure S1a) or CD14-, suggesting the presence of non-macrophage CD163+ myeloid cells, also observed by others (29).

Table 1. Patient characteristics

	N (%)
No. of patients	86
Average age (years)	49.6
Ethnicity	
Caucasian	74 (86)
Suriname	5 (6)
Unknown	7 (8)
FIGO[1] stage	
1b1	37 (43)
1b2	28 (33)
2	21 (24)
HPV type	
16	45 (52)
18	19 (22)
Other	13 (15)
Negative	9 (11)
Histopathology	
Squamous	53 (61)
Adeno (squamous)	33 (38)
Lymph node metastasis	
Negative	60 (70)
Positive	25 (29)
Unknown	1 (1)
Tumor size	
<4 cm	44 (51)
≥4 cm	36 (42)
Unknown	6 (7)
Infiltration depth	
<15 mm	43 (50)
≥15 mm	42 (49)
Unknown	1 (1)
Vasoinvasion	
No	37 (43)
Yes	44 (51)
Unknown	5 (6)
Parametrial involvement	
No	76 (88)
Yes	10 (12)
Mean follow-up time (months)	49.3
5-year survival rate	65 (76)

[1]International Federation of Obstetricians and Gynecologists.

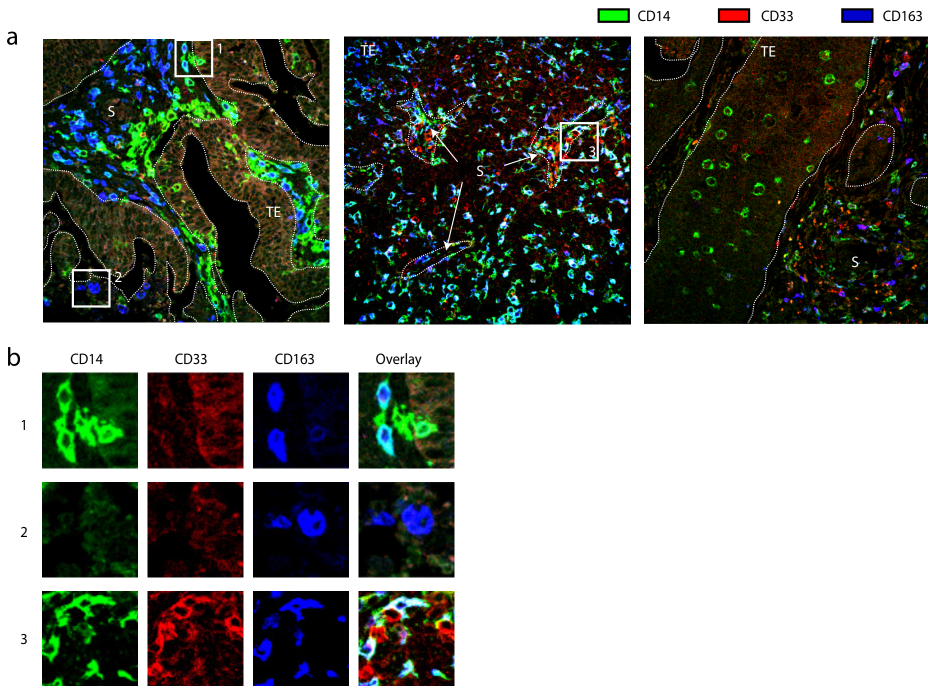

Figure 1. Immunofluoresence staining of cervical carcinoma with antibodies against CD14 (green), CD33 (red) and CD163 (blue). (a) The tumor-epithelium (TE) and tumor stroma (S) are marked. The left panel depicts an example of an adenocarcinoma with dense infiltration of the stroma by CD14+CD33-CD163- (green), CD14+CD33-CD163+ (turquoise) and CD14-CD33-CD163+ (blue) cells. In the middle, a squamouscarcinoma with dense infiltration of the tumor-epithelium by various myeloid cells is shown. In the right panel is an example of CD14+CD33-CD163- cells (green) infiltrating the tumor-epithelium. (b) An example of CD14+CD33-CD163- (green) and CD14+CD33-CD163+ cells (turquoise; number 1) staining; CD14-CD33-CD163+ cells (blue; number 2); various myeloid marker combinations found in close proximity (number 3).

Table 2. Cellular distribution in tumor epithelium and stroma

Cell type[1]	Epithelium, median[2] (interquartile range)	Stroma, median[2] (interquartile range)
CD14+CD33−CD163−	12 (4–28)	40 (20–69)
CD14+CD33+CD163−	4 (0–16)	13 (2–38)
CD14+CD33−CD163+	9 (2–21)	39 (14–84)
CD14+CD33+CD163+	10 (1–49)	59 (14–198)
CD14−CD33+CD163−	3 (0–15)	19 (7–42)
CD14−CD33+CD163+	8 (1–26)	31 (7–72)
CD14−CD33−CD163+	10 (2–26)	52 (15–113)

[1]The identification of different myeloid cell subsets on the basis of the expression of CD14, CD33 and CD163.
[2]Number of cells per mm^2.

CHAPTER 4

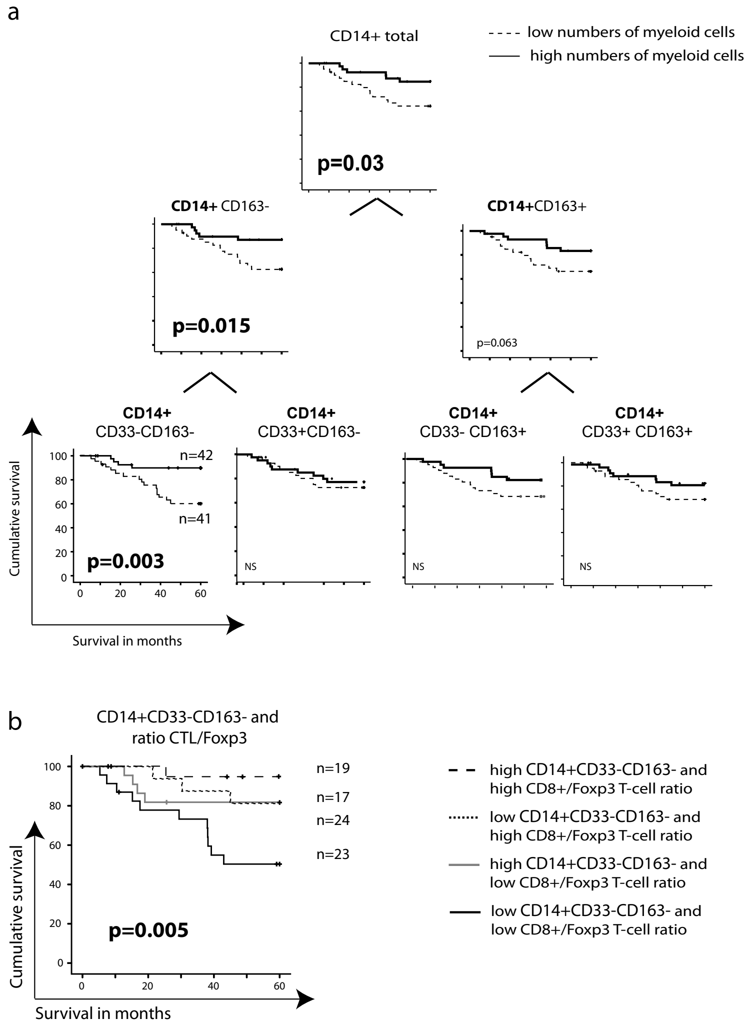

Figure 2. Myeloid cell populations were divided into two groups based on the median number of intraepithelial myeloid cells. (a) Kaplan Meijer curves of the total CD14+ cells showed a significant survival benefit for patients with more than median CD14+ tumor-infiltrating cells. When these cells were divided adding the expression of CD163, the survival benefit was seen in the group of patients with more than median CD14+CD163- infiltrating cells, yet a trend was still seen for the CD14+CD163+ myeloid cells. The main sub-population of the CD14+CD163- cells was CD33- (75%), which was again significantly correlated to a prolonged survival. (b) The ratio of CD8+ and Foxp3+ T-cells infiltrating the tumor-epithelium was previously identified as an independent factor of disease-specific survival in cervical carcinoma patients. The Kaplan Meijer of this ratio combined with the CD14+CD33-CD163- myeloid cells, showed a greatly increased 5-year disease-specific survival for patients with high numbers of both cell types and the poorest disease-specific survival in patients with low numbers of both cell types.

Intraepithelial M1 macrophages are associated with a better disease-specific survival

While on average CxCa is well infiltrated by the three major subsets of myeloid cells, a clear difference exists between individual patients as indicated by the interquartile ranges provided for each cell population (Table 2). To assess the prognostic impact of these myeloid populations, the 5-year Kaplan-Meier estimate and log-rank test were used. Patients were divided into two groups based on the median number of myeloid cell subset per mm^2 in the tumor-epithelium or in stroma.

Firstly the patients were analyzed according to the total myeloid cell population expressing CD14+, CD33+ or CD163+. Patients with tumors displaying high numbers of intraepithelial CD14+ myeloid cells, showed a better 5-year disease-specific survival (p=0.03; Figure 2). No survival benefit was seen based on the number of intraepithelial CD33 or CD163 expressing cells (Supplementary Figure S2). Further division of the CD14+ myeloid cell population based on CD163 expression revealed that patients with high numbers of intraepithelial CD14+CD163- cells (M1 macrophages) displayed a survival benefit (p=0.015). Unexpectedly a trend towards a better survival was seen in the patients with high numbers of intraepithelial M2 macrophages (p=0.063). Pearson's correlation test showed that the numbers of CD14+CD163- and CD14+CD163+ cells were strongly related in both tumor-epithelium (p=0.002) and stroma (p=0.008), suggesting that the observed trend between M2 macrophages and disease-specific survival could be a confounder. The non-macrophage CD14-CD163+ myeloid cells were not correlated to any of the other groups of myeloid cells in epithelium or stroma (0,086 and 0,083 respectively).

Further subdivision based on CD33 expression revealed that especially the number of intraepithelial CD14+CD33-CD163- cells (mature M1 macrophages), which made up 75% of all cells in the CD14+CD163- group (Table 2), was associated with improved disease-specific survival (p=0.003; Figure 2). Notably, the trend between intraepithelial M2 macrophages and disease-specific survival was lost. No associations with survival were found for cells present in stroma.

M1 macrophages and T-cells act together to improve disease-specific survival

Previously we have shown that CxCa can be abundantly infiltrated by CD8+ T-cells, but also by Foxp3+ T-cells (2;6). Therefore, the relationship between the infiltration of the different types of TIMs and the other previously enumerated tumor-infiltrating immune cells in this group of patients was studied. The presence of high numbers of intraepithelial CD14+CD163- cells (predominantly CD14+CD33-CD163- cells), correlated with a high influx of intraepithelial T lymphocytes (p=0.008; Table 3). There was no correlation with any specific lymphocyte subset. In contrast, the intraepithelial population of non-macrophage CD163+ myeloid cells, irrespectively of CD33 expression, was positively correlated to the

Table 3. Correlations to other tumoral and environmental parameters

Intraepithelial myeloid subgroups[1]	CD3+CD8− Foxp3−	CD3+CD8+ Foxp3−	CD3+CD8− Foxp3+	Ratio CD4/ Foxp3	Ratio CTL/ Foxp3	Immune infiltrate
CD14+CD163−	0.382	0.183	0.15	0.32	0.925	**0.008 (0.048)**[2]
CD14+CD33−CD163−	**0.037**	0.24	0.26	0.51	0.82	**0.012**
CD14+CD33+CD163−	0.33	**0.036**	0.69	0.38	0.97	**0.038**
CD14+ CD163+	**0.017**	0.089	0.859	0.65	0.877	**0.03**
CD14+CD33−CD163+	0.27	0.35	0.42	0.32	0.32	0.19
CD14+CD33+CD163+	0.98	0.87	0.59	0.062	0.59	0.27
CD14−CD163+	0.083	0.28	**0.006 (0.036)**[2]	**0.021**	0.062	0.519
CD14−CD33+CD163+	0.49	0.05	0.15	0.059	**0.02**	0.99
CD14−CD33−CD163+	**0.03**	0.12	0.15	0.9	0.87	0.51

	HLA class 1	HLA class 2	MICA	IDO	PDL1
CD14+CD163−	0.887	0.928	0.475	0.586	0.22
CD14+CD33−CD163−	0.43	0.62	0.52	0.27	0.078
CD14+CD33+CD163−	0.22	0.64	0.33	0.52	0.62
CD14+ CD163+	0.194	0.594	0.18	0.726	0.734
CD14+CD33−CD163+	**0.032**	0.81	0.38	0.15	0.96
CD14+CD33+CD163+	**0.027**	0.12	0.47	0.57	0.26
CD14−CD163+	0.78	0.093	0.478	0.44	0.071
CD14−CD33+CD163+	0.2	0.88	0.26	0.73	0.087
CD14−CD33−CD163+	**0.02**	**0.005 (0.025)**[2]	0.81	0.32	0.22

	Negative lymph nodes	Tumor size <4 cm	Infiltration depth <15 mm	No vasoinvasion	No parametrial involvement
CD14+CD163−	0.081	0.632	0.504	0.744	0.165
CD14+CD33−CD163−	**0.009 (0.045)**[2]	0.73	0.58	0.12	**0.043**
CD14+CD33+CD163−	0.7	0.7	0.91	0.48	0.93
CD14+ CD163+	**0.005 (0.03)**[2]	0.685	0.513	0.133	0.143
CD14+CD33−CD163+	0.86	0.27	0.82	0.74	0.54
CD14+CD33+CD163+	**0.007 (0.035)**[2]	0.84	0.38	**0.019**	**0.039**
CD14−CD163+	0.519	0.77	0.513	0.745	0.191
CD14−CD33+CD163+	0.758	0.543	0.827	0.22	0.38
CD14−CD33−CD163+	0.86	0.68	0.82	0.57	0.52

[1]For the assessment of relationships between histopathological parameters and myeloid subpopulations, the latter were divided based on the 50th percentile.
[2]The values in bold indicate the p-values that are considered to be significant, the values between the brackets indicate the *p*-value after Bonferroni correction, which are still considered significant.

number of intraepithelial CD3+CD8-Foxp3+ Tregs (p=0.006; Table 3). No correlations were found between stromal TIMs and intraepithelial immune cells (data not shown).

We have previously shown that it is not a single population of TIL that is associated with survival, but specifically the ratio between the tumor-infiltrating CD8+ and Foxp3+ T-cells (2). We therefore assessed the influence of the intra-epithelial CD14+CD33-CD163- myeloid population and the intra-epithelial CD8+/Foxp3+ T-cell ratio by dividing the patients into 4 groups based on the median number of both cell types.. The best disease-specific survival was seen in the group of patients with high numbers of intraepithelial CD14+CD33-CD163- myeloid cells and a high CD8+/Foxp3 T-cell ratio (n=19, p=0.005; Figure 2b). Conversely, patients with low numbers of these myeloid cells and a low CD8+/Foxp3 T-cell ratio displayed the worst survival (n=23). Interestingly, the groups with only high numbers of CD14+CD33-CD163- myeloid cells or a high CD8+/Foxp3+ T-cell ratio,

did equally well (Figure 2b), suggesting that M1 macrophages and CD8+ T-cells have an additive anti-tumor effect.

We also assessed the association between TIM and expression of HLA-class I/II, MICA (2;30), IDO (36;37) and PD-L1 (8) by the tumor cells, factors which may also play a role in the local immune response to tumors, all of which have been studied previously in this group of tumors. While there was no association with the macrophage populations, the intraepithelial sub-population of non-macrophage CD14-CD33-CD163+ myeloid cells correlated to strong HLA-class II expression (p=0.005; Table 3).

M1 macrophages form an independent prognostic factor for disease-specific survival

The clinical pathological factors known to influence CxCa prognosis are lymph node status, tumor size, tumor infiltration depth, vasoinvasion and parametria involvement (38-41). To further analyze the correlation between myeloid cells and prognosis, we investigated their correlation with these clinical pathological factors. Intraepithelial CD14+CD33-CD163- myeloid cells (p=0.006), as well as CD14+CD163+ myeloid cells (p=0.005) and specifically the CD14+CD33+CD163+ subpopulation (p=0.007), were associated with lack of metastasis (Table 3). The intraepithelial population of non-macrophage CD14-CD163+ myeloid cells was not correlated to any clinical pathological factors, again indicating that these cells form an unrelated group of tumor-infiltrating myeloid cells.

Because of the relationships found between M1 macrophages, lymph node status and T-cell infiltration, we assessed their prognostic impact in a multivariate analysis. Firstly, stratification of all patients based on the known histopathological risk factors showed that lymph node metastasis (p<0.001), tumor size (p<0.001), infiltration depth (p=0.011) and parametrial involvement (p<0.001) were all associated to a worse disease-specific survival by Cox regression analysis. A multivariate Cox regression analysis of these clinical parameters identified only lymph node status (p=0.004) and tumor size (p<0.001) as independent predictors of disease-specific survival.

Secondly, a Cox regression analysis of the tumor-infiltrating immune cells identified a significant correlation between survival and the total CD14 population (p=0.038), CD14+CD163- cells (p=0.016) and CD14+CD33-CD163- cells (p= 0.007). Finally, the independent clinical pathological risk factors, the number of myeloid cells, as well as the CD8+/ Foxp3+ T-cell ratio were analyzed in a multivariate Cox regression analysis. We confirmed that lymph node status (p<0.001), tumor size (p=0.001) and the CD8+/Foxp3+ intraepithelial T-cell ratio (p=0.015) were all independent survival factors. Furthermore we found that dense M1 macrophages (CD14+CD163-) tumor-epithelial infiltration is an independent survival factor (p=0.011). Analysis with the matured M1 macrophages (CD14+CD33-CD163-) did not reach significance (p=0.053).

Unsupervised clustering based on immune parameters identifies different immune microenvironments associated with survival

This cohort of 86 patients has been extensively studied for the total lymphocyte infiltration, as well as the various lymphocytes sub-populations CD3+CD8-Foxp3-, CD3+CD8-Foxp3+, CD3+CD8+Foxp3-, CD3+CD8+CD57+, CD3-CD8+CD57+, CD3+CD8-CD57+ and CD3-CD8-CD57+ (2;6). Furthermore, this group has been studied for the expression of CXCR7, CXCL12, CXCR4 (32), EGFR (33), MICA (2), HLA-E (9), HLA-G (unpublished), PD-L1 (8) IDO (unpublished), Serpin A1/A3 (31) as well as HLA-class I/II (2;10). To gain a better insight in the immunologic make-up of CxCa, a heatmap of all parameters including the ratio between intraepithelial CD8+ and Foxp3+ T-cells (2) and the intraepithelial myeloid cell populations identified in this study, was constructed. Unsupervised clustering divided the patients into two major groups (Group I and II) that are both subdivided into two subgroups (Ia and Ib, IIa and IIb) and then further into nine smaller subgroups (Figure 3a).

A clear difference between group I and II was the much denser matured M1 macrophage (CD14+CD33-CD163-) infiltration in group II, which co-clustered with different types of immature (CD33+) myeloid cells. Within group I, the tumors of group Ia generally expressed both HLA-class I/II, displayed a strong infiltration of all types of lymphocytes, as well as strong CD33-CD163+ myeloid cells infiltration, irrespective of CD14 expression. Group Ib distinguishes itself from group Ia by a lower lymphocytic infiltrate as well as loss or weak expression of HLA-class I/II in a substantial number of patients. The tumors in group IIa have less intraepithelial T-cell infiltrate and less infiltration of the above mentioned CD33-CD163+ (CD14+ or CD14-) myeloid cells, which are all abundantly present in group IIb. Kaplan-Meier survival curves and log-rank analysis for these groups of patients revealed a significantly better survival for group II ($p=0.045$; Figure 3b), yet no differences were found between subgroups (Ia versus Ib or IIa versus IIb; Supplementary Figure S2b).

Subsequently, survival of the nine small subgroups was analyzed. The clustering of these groups seemed to be based mainly on 4 parameters: HLA-class I/II expression, number of CD8+ T-cells, CD8+/Foxp3+ T-cell ratio and matured M1 macrophages number, indicated by the red boxes in Figure 3a. Subgroup 1 includes tumors with low matured M1 macrophages infiltration and a low CD8+/ Foxp3 T-cell ratio (despite good HLA expression and moderate numbers of CD8+ T-cells). Patients in this group had the worse survival (Figure 3c). Subgroup 2 reflects tumors strongly infiltrated with CD8+ T-cells with a beneficial CD8+/Foxp3+ T-cells ratio, but lacking matured M1 macrophages. This group displayed better survival (Figure 3c). Group 1b (subgroups 3-5) included tumors with low HLA expression, few CD8+ T-cells, a low CD8+/Foxp3+ T-cell ratio and few matured M1 macrophages. The survival in these groups was generally poor (Figure 3c). Group IIb was divided into the small subgroups 7, 8 and 9. They were all abundantly infiltrated with CD14+CD33-CD163- myeloid cells. The main difference was a lower CD8+/Foxp3 intraepithelial T-cell ratio in subgroup 9 and the strongest intraepithelial CD8+ T-cell infiltration in subgroup

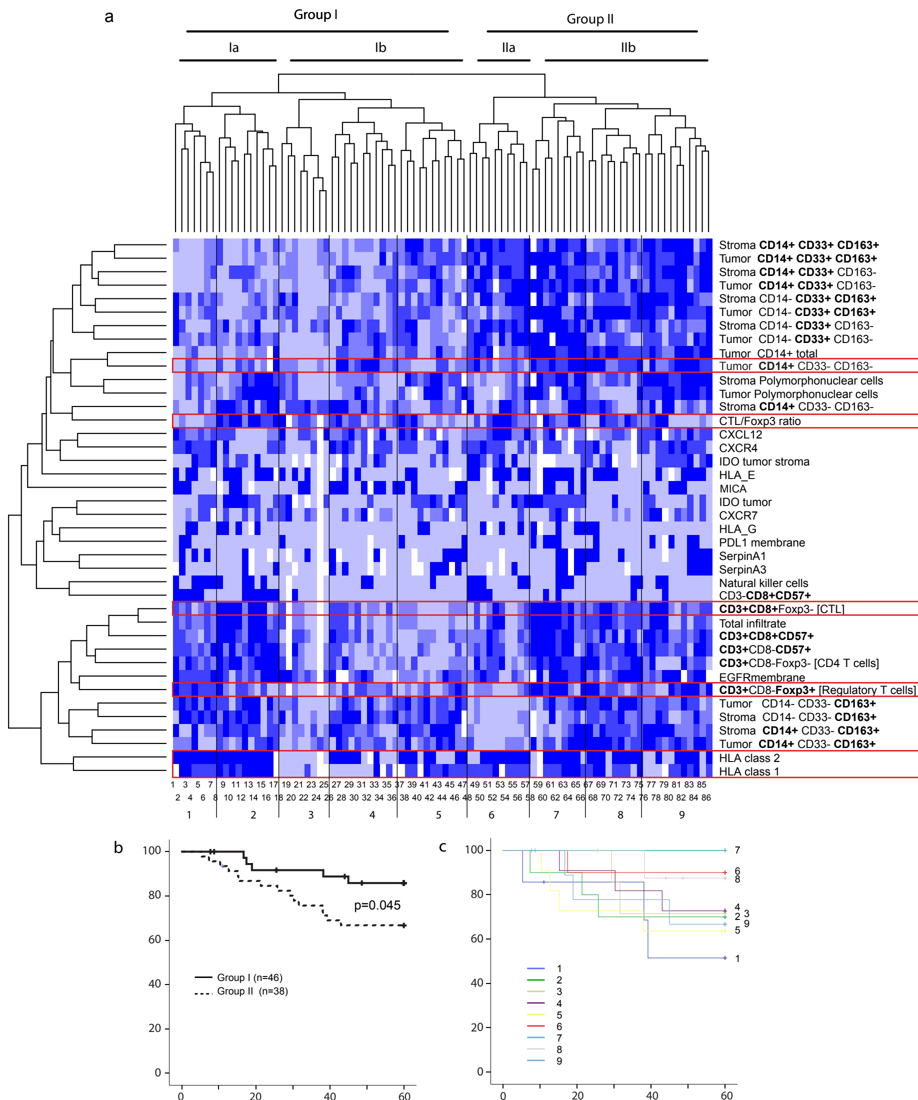

Figure 3. A heatmap was created based on all immunological parameters determined in this patient cohort, followed by unsupervised clustering. The changes from the lowest to the highest quartile are reflected by a darker color, the white boxes are missing data. Along the Y-axis are the 86 patients, with all the immunological parameters indicated to the right. Each column is the unique fingerprint of one patient. The brackets to the left and along the top indicated the unsupervised clustering. Patients are clustered into two groups (Group I and II) based mainly on the CD33+ expressing cells, which co-cluster together with the CD14+CD33-CD163- cells. Both groups can be subdivided into two major groups (Ia, Ib, IIa and IIb) and then further into nine subgroups (indicated along the bottom). The red boxes indicate the parameters which seem important in determining the sub-group clustering. (b) Survival analysis by Kaplan Meijer method and log-rank test of the two major groups, shows a significant disease-specific survival benefit for Group II versus Group I (p<0.05). (c) Disease-specific survival analysis by Kaplan Meijer method of all the subgroups 1-9.

7. Analysis of these subgroups revealed that subgroup 9 had the worst survival (similar to patients in subgroups 1-5), whereas patients in subgroup 7 had a 100% 5 year survival (Figure 3c). Subgroup 6 showed a similar survival to patients in subgroup 8, the main difference being the lower numbers of CD8+ T-cells, although a good CD8+/Foxp3 T-cell ratio was maintained.

In conclusion, the unsupervised clustering of patients based on several immune parameters confirmed our earlier observations that M1 macrophages, CD8+ T-cells and Foxp3+ Tregs are tightly associated with the disease-specific survival of CxCa patients.

DISCUSSION

Here we investigated the constitution of tumor-infiltrating myeloid cells in relationship to other tumor-infiltrating immune cells, tumor-characteristics and the disease-specific survival of CxCa patients. Quantification of myeloid cell populations based on the expression of CD14, CD33 and CD163, revealed that a strong intraepithelial infiltration of CD14+ cells, and specifically CD14+CD33-CD163- matured M1 macrophages, is associated with significantly improved disease-specific survival and is an independent prognostic factor as determined by multivariate analysis. The other independent prognostic immune-related factor in CxCa found so far is the intraepithelial CTL/Treg ratio (Figure 2c). Combination of these two factors revealed a substantial increase in survival in the group of patients with tumors displaying dense intraepithelial matured M1 macrophage infiltrate and a high CD8+/Foxp3+ T-cell ratio. Furthermore, immune profiling by unsupervised clustering of 40 different immune parameters revealed a fingerprint that was clearly associated with improved disease-specific survival. The main determinants were the presence of matured M1 macrophages (clustering together with various immature CD33+ myeloid cells) and a high CD8+/Foxp3+ T-cell ratio, both independent prognostic factors. Subgroup analyses provided new insights in the type of immune responses key in the protection against progressive CxCa. We observed that all groups of patients with few M1 macrophages and with low numbers of tumor-infiltrating Tregs (subgroups 2-5), displayed a similar intermediate percentage of disease-specific survival independent of the level of CD8+ T-cell infiltration. This implies that the tumor-infiltrating T-cells are less likely to exert a proper antitumor effect within a tumor microenvironment that does not allow the accumulation of high numbers of M1 macrophages. Indeed in subgroups 6-8, reflecting patients who do very well, the tumors were infiltrated with relatively high numbers of M1 macrophages and displayed a high CD8/Treg ratio (Figure 3).

Previous studies on the number of macrophages in CxCa did not reveal any relationship with clinical parameters (12;19;20), most likely because differences in function were not taken into account. Here, we quantified the number of immature and mature M1 and M2

macrophages. A few recent studies analyzing M2-type macrophages indicated that a dense M2 macrophage infiltration is associated with poor survival in different carcinomas (43-46). In our study, we did not observe this association, finding instead that the number of tumor-infiltrating M1 macrophages was positively associated with survival. Similar observations correlating M1 macrophages to better survival were made in non-small lung cancer (42;43) and breast carcinoma (44).

Studies on colorectal carcinoma also demonstrated that CD14+ TAM were associated with a favorable prognosis and that these CD14+ cells expressed CD40 and often CD80/CD86 (45;46), which can be considered markers of matured antigen presenting cells. In our study, the strongest correlation was found between survival and fully matured (CD33 negative) M1 macrophages, suggesting that the accumulation of matured M1 macrophages reflects a tumor-rejecting microenvironment. The group of M1 macrophages comprised about 25% of cells that expressed CD33+, however, these cells did not provide any disease-specific survival advantage, possibly, because these cells are still not fully activated. Alternatively these CD33+ cells may reflect myeloid derived suppressor cells if they also display a low expression of HLA-DR (14), but this was not tested in our study.

Apart from M2 macrophages a group of myeloid cells was found in vivo that was CD163+ but CD14-. The numbers of these cells did not correlate with the other two myeloid subgroups, yet correlated to intraepithelial Foxp3+ lymphocytes. Based on our *in vitro* data, showing CD14 expression on monocytes and macrophages, as well as the presence of CD68-CD163+ cells in vivo, we conclude that these non-macrophage CD163+ myeloid cells are likely to reflect immunosuppressive DC's or DC-derived macrophages (29;47).

Our results are important for the development of new strategies to combat cancer. We show that tumor-infiltrating matured M1 macrophages are associated with better survival, irrespective of CD8+ T-cells infiltration. Therapies to block macrophage infiltration – for instance by blocking chemokines or their receptors - thus are not warranted *per definition*. Furthermore, our data suggest that the density of tumor-infiltrating M2 macrophages does not have an impact on overall survival. Therefore, selective inhibition of M2 macrophages without stimulation of M1 macrophages may also prove to be unsuccessful. Therapies should rather aim at reprogramming the abundantly present M2 macrophages towards an M1 phenotype. This can be achieved via multiple pathways (14), including blocking of IL-6 and cyclooxygenase-2 (COX2), both of which are associated with poor survival in CxCa and are known to induce M2 macrophages (21-23), or by using taxoids (48). Activation of the tumoricidal function of M1 macrophages can be achieved through ligation of CD40. In pancreatic ductal adenocarcinoma combining gemcitabine and agonistic CD40-antibody induced tumor regression due to the activation of tumoricidal macrophages and subsequent depletion of tumor stroma (49). Here, the myeloid derived suppressor cells (MDSC) were probably effectively removed by the use of gemcitabine (50). Notably, CD4+ type 1 helper T-cells are well equipped to reprogram CD14+CD163+ macrophages towards activated M1

macrophages via CD40-CD40L interaction and the production of Interferon (IFN)γ (23). Our observations that patients whose tumors are infiltrated with a dense T-cell population comprising relatively low numbers of Foxp3 regulatory T-cells and a dense population of mature CD14+CD163- myeloid cells, have the best clinical performance, suggest that these two populations collaborate to resist tumor cells. These results should be validated by prospective trials. Current immunotherapy trials aim at reinforcing the tumor-specific T-cell response to CxCa, but our data argue that these therapies are most likely to have success if the tumors are infiltrated with matured M1 macrophages. Therefore, pre-selection of patients based on dense CD14+ cells infiltration may help to improve success rates. However, therapy combining the induction of tumor-specific IFNγ-producing CD4+ T-helper cell and cytotoxic CD8+ T-cell responses, the attraction of M1 macrophages or reprogramming of resident myeloid cells, may be beneficial to a larger group of patients.

Acknowledgements

We would like to thank E.M. Osse (Department of Pathology) for embedding the cells in paraffin and Prof GJ Fleuren (Department of Pathology) for examining the slides of all the tumors. This study was financially supported by The Netherlands Organization for Health Research and Development (ZonMW 92003425). The authors disclose no potential conflicts of interest

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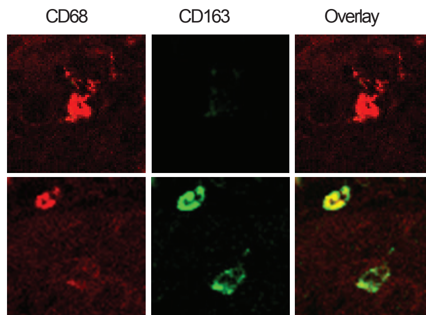
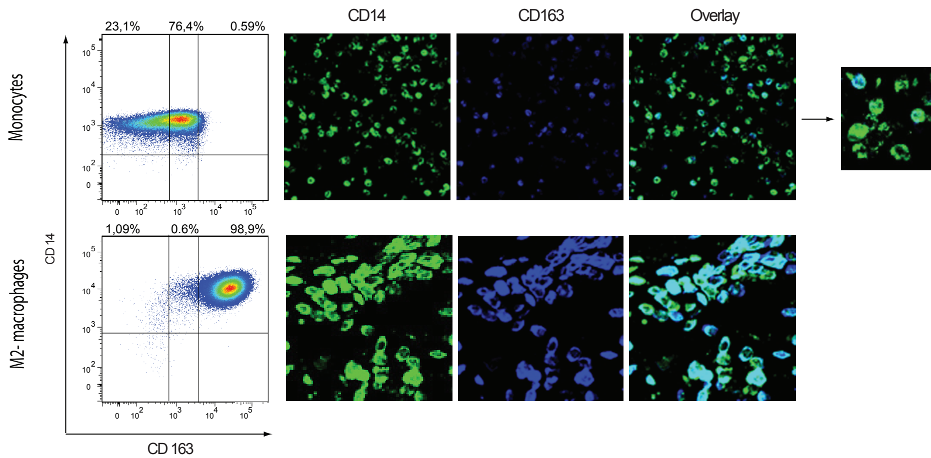
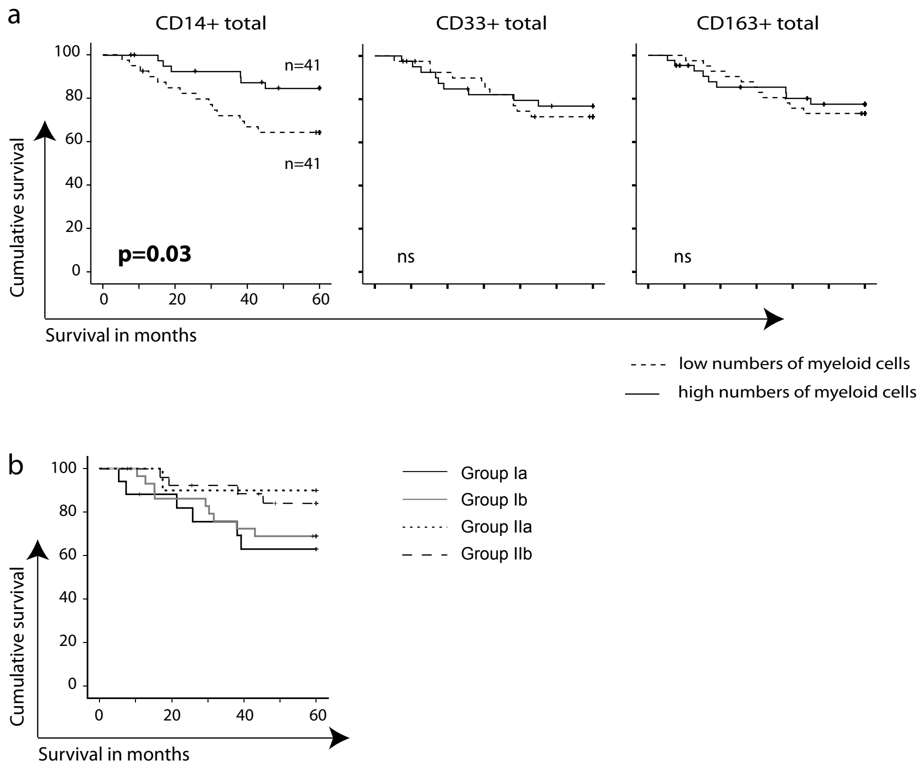


Supporting information 1. Immunohistochemical staining for CD68 and CD163 showing CD68+CD163- single staining, double staining CD68+CD163+ and CD68-CD163+ single staining.



Supporting information 2. 76% of freshly isolated monocytes show intermediate expression of CD163, while cultured M2 macrophages express up to 1000x more. Immunohistofluorescence staining of monocytes shows mainly CD14+CD163- (green) cells, whole most of the M2 cells were positive for both markers (CD14+CD163+, turquoise).



Supporting information 3. (a) Kaplan Meijer curves of intraepithelial myeloid cell populations based on the total CD14+, CD33+ and CD163+ cells shows a significant survival benefit for the total CD14+ cells. (b) Survival curves of the different subgroups of patients (Ia, Ib, IIa and IIb) created in the heatmap revealed no significant differences.

Chapter 5

M2 macrophages induced by PgE2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells

Moniek Heusinkveld
Peggy J. de Vos van Steenwijk
Renske Goedemans
Tamara H. Ramwadhdoebe
Arko Gorter
Marij J.P. Welters
Thorbald van Hall
Sjoerd H. van der Burg

Journal of Immunology 2011;187(3),1157-65

ABSTRACT

Monocytes attracted by tumor-induced chronic inflammation differentiate to antigen-presenting cells (APC), the type of which depends on cues in the local tumor milieu. Here, we studied the influence of human cervical cancer cells on monocyte differentiation and showed that the majority of cancer cells either hampered monocyte to DC differentiation or skewed their differentiation towards M2-like macrophages. Blocking studies revealed that M2-differentiation was caused by tumor-produced PgE2 and IL-6. TGFβ, IL-10, VEGF or M-CSF did not play a role. Notably, these $CD14^+CD163^+$ M2-macrophages were also detected *in situ.* Activation of cancer cell-induced M2-like macrophages by several TLR-agonists revealed that when compared to DC these M2-macrophages displayed a tolerogenic phenotype reflected by a lower expression of co-stimulatory molecules, an altered balance in IL-12p70 and IL-10 production and a poor capacity to stimulate T-cell proliferation and IFNγ production. Interestingly, upon cognate interaction with Th1 cells these tumor-induced M2-macrophages could be switched to activated M1-like macrophages that expressed high levels of co-stimulatory molecules, produced high amounts of IL-12 and low amounts of IL-10, as well as acquired the lymphoid homing marker CCR7. The effects of the interaction between M2-macrophages and Th1 cells could partially be mimicked by activation of these APC via CD40 in the presence of IFNγ. Our data on the presence, induction and plasticity of tumor-induced tolerogenic APC in cervical cancer suggest that tumor-infiltrated Th1 cells can stimulate a tumor-rejecting environment by switching M2-macrophages to classical pro-inflammatory M1 macrophages.

INTRODUCTION

Cervical cancer (CxCa) is induced by human papilloma virus (HPV) (1). In many cases, the development of CxCa is associated with a weak systemic and local immune response to HPV, reflected by low numbers of tumor-infiltrating T cells that comprise functionally impaired T-helper cells and regulatory T cells (2-7). When the tumor-specific immune response is stronger and more in favor of a Th1/CTL response, this is associated with an improved prognosis (4,8-10).

Tumors foster a tolerant microenvironment by the activation of a plethora of immunosuppressive mechanisms, including the modulation of antigen presenting cells (APC) that otherwise may stimulate adaptive immunity against cancer (11). Monocytes are attracted by the chronic inflammation caused by tumors and differentiate into a variety of tumor-associated macrophage (M2) and dendritic cell (DC) subtypes depending on local mediators (12-14). Factors secreted by tumor cells that have been implicated in the prevention or modulation of DC differentiation and/or function are vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF), transforming growth factor (TGFβ), IL-10, IL-6 and prostanoids(*e.g.* PgE2) (12). Cervical cancers are known also to secrete immunomodulatory compounds but their effect on APC is yet unknown (15-19).

Therefore, we studied the effect of cervical cancer cells on monocyte differentiation and function. We found that DC differentiation was hampered or even skewed towards the tolerogenic M2 macrophages by tumor-derived PgE2 and IL-6. Subsequently, we assessed the effects of APC activation by several different Toll like receptor (TLR)-agonists, which are currently used or tested for the treatment of cancer in human beings (20), CD40 stimulation or cognate interactions with Th1 cells. Notably, the interaction with Th1 cells resulted in a switch to activated M1-like macrophages expressing high levels of co-stimulatory molecules and producing high amounts of IL-12p70. Our data suggests that a highly immune-stimulatory local microenvironment might be achieved by utilizing COX-inhibitors and IL-6 blocking antibodies to prevent M2-differentiation and vaccine-mediated stimulation of Th1 cells to switch M2 macrophages to tumor-rejecting M1 macrophages.

MATERIAL AND METHODS

Immunofluorescent staining of tumor tissue

Ten patients with cervical cancer with FIGO stage I and II underwent radical hysterectomy (type III) in our hospital. Patients had not received radiotherapy or chemotherapy before surgery. Tumors were HPV typed by PCR and sequencing, as described previously (21). The use of clinical material was approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

Staining was performed on 4 μm tissue-sections of formalin-treated and paraffin embedded tumor material. Immunostaining was performed with a monoclonal antibody mix of anti-CD14 (clone 7, Abcam USA) and anti-CD163 (clone 10D6, Novocastra United Kingdom) and after incubation overnight stained with fluorescent antibody conjugates (IgG2a-Alexa Fluor 488 and IgG1- alexa Fluor 647, Invitrogen USA)(22). Control staining with only secondary antibodies were included to ensure specificity. Images were captured at 25 X magnification with a confocal laser scanning microscope (Zeiss LSM 510, Germany) in a multitrack setting.

Media and reagents

APC and tumor cell-lines were grown in RPMI (Invitrogen) supplemented with 10% Fetal Calf Serum (Greiner bio-one Germany), penicillin/streptomycin (Invitrogen) and L-glutamine (Cambrex USA). Adherent cell-lines were treated with trypsin/EDTA 1x (PAA, Austria). T cell clones were grown in IMDM (Lonza Switserland) supplemented with 10% FCS (PAA), P/S and L-glutamine. The following factors and final concentrations were used to generate APC: 500 U/ml IL-4, 800 u/ml GM-CSF (Gibco, USA), ,10-100 ng/ml M-CSF (R&D systems USA), 50-1000 pg/ml TGFβ1 (BD biosciences USA), 1-50 ng/ml prostaglandin E2 (Sigma-Aldrich Germany). TLR ligands used for activation: 25 μg/ml Poly(I:C), 10 μg/ml R848/CL097 (all from Invivogen, USA) and 0.25 μg/ml LPS (Sigma-Aldrich). Optimal concentrations were used based on maximal cytokine release in mo-DC. To mimic T-cell interaction APCs were stimulated with irradiated CD40-L expressing mouse fibroblasts (23).

Blockade of TGFβ signaling was achieved with 1μM SB431542 hydrate (Sigma-Aldrich) after optimization of the dose. IL-6 was blocked by adding 2.5 ug/ml antibody to IL-6 receptor (clone B-R6) and 2.5 ug/ml antibody to IL-6 (B-E8) (Abcam, USA) to the culture.

Supernatant of Cervical Cancer cell-lines

To confirm the origin of the established lines HeLa and CaSKi, lines were tested for the presence of integrated HPV 16 or 18 DNA using the INNO-LiPa HPV Genotyping procedure (Innogenetics). CSCC-1, CSCC-7, CC-8 , CC-10B and CC-11- were typed and cultured as described earlier (24). Stock ampoules were thawed and cultured for 10 passages and tested for the presence of mycoplasma monthly.

Cell-lines were grown in flasks at 80-90% confluence, harvested with trypsin/EDTA. 100.000 cells were plated in 2 ml/well of 6-well culture plate and cultured for 5 days. Supernatant was stored at -20 degrees. In case cultures were treated with COX-inhibitors, 250.000 cells in 2 ml were plated in 6-well plates in the presence of 25μM Indomethacin or 5 μM NS-398 (Cayman Chemical, USA) dissolved in DMSO or as a control only with the corresponding concentration of DMSO. Medium was replaced after 24 hr and then harvested after 24 hours of culture.

DC culture

PBMC were obtained from buffy coats of healthy donors. CD14+ monocytes (>95% purity) were isolated using the MACS cell separation (Miltenyi Biotec Germany) and stored in liquid nitrogen until further use. Monocytes were thawed and cultured in 48- or 24-well plates in a density of 0.25 or 0.5 million cells/well respectively in the presence of IL-4 and GM-CSF (mo-DC). After 2 days fresh medium with cytokines was added. At day 5-6 the cells were analyzed for differentiation by flow cytometry and activated in the culture medium or harvested, washed and activated in fresh medium. Tumor supernatant (TSN)-APC are cultured as described above but 20% supernatant of tumor cell-lines or medium was added. Titrations showed that 20% supernatant gave the best reproducible results between donors. DC were activated at day 6 and after 48 hr the supernatant was harvested and stored at -20^0 C for cytokine analysis and cells were stained for flow cytometric analysis.

Mixed Lymphocyte Reaction

Naïve CD4 cells were isolated from PBMC by CD25+ cell depletion using MACS and subsequently isolation of CD4+ cells with the DynalBead system (Invitrogen) to a purity of > 99%. These CD4+CD25- cells were plated in a 96-well plate at 50.000 cells per well. Matured DCs were added at different doses up to 10.000 cells/well in triplicate. T-cell proliferation was measured after 5 days by [3H]thymidine incorporation (0.5 uCi/well). Supernatant was taken at several time points and stored at -20^0C for cytokine analysis.

CD4+ T cell helper clones

HPV specific CD4+ T cell clones were obtained by limited dilution of LN cells of a patient with a HPV16+ cervical tumor. Clones were stimulated every 2 weeks with B-LCL loaded with the cognate HPV peptide, feeder-cells, TCGF and IL-15. Clones were used for DC activation after 2.5 weeks resting period.

Clone 214 recognized HPV16E6 aa61-82, clone 238 recognized HPV16 aa61-82 and clone 16 recognized HPV16E6 aa 11-32 and all clones produced IFNγ and IL-2 but only clone 238 produced IL-10 upon antigen-specific activation. HLA-class II matched APC were loaded with an irrelevant or the cognate peptide for CD4+ Th1 clones and co-cultured at different DC:T-cell ratio's in medium containing 20% TSN. After 48 hours supernatant was analyzed and APC were phenotyped.

Flow cytometry

Mouse monoclonal antibodies to human CD80, CD86, HLA-DR, CD206, CD1c(FITC) and CD83, CD86, CD14, CD16, CD163 (PE) and CD14, HLA-DR (PERCP) or CD11c, CD1a, CD4 (APC) (all from BD-biosciences) and CD163 (R&D systems) and PD-L1 (Ebioscience) were used. Cells were recorded (20.000/live gate) using a BD-FACS calibur with Cellquest software (BD-biosciences) and analyzed by Flowjo software (Tree star, inc. USA).

Cytokine analysis

IL-12p40 and IL-12p70 were analyzed using ELISA kits from BD-biosciences or by inflammatory CBA (BD-biosciences). Il-10 and IFNγ was measured with ELISA (Sanquin, the Netherland). To evaluate the cytokines present in supernatant of tumor cells IL-6, IL-8 and IL-10 were determined by CBA, M-CSF by Bioplex (BioRad). PgE2 was measured with the prostaglandin E2 parameter assay kit (R&D systems), TGFβ-1 with the human TGFβ1 ELISA from Ebioscience. Samples were tested with and without acidic treatment to determine active and latent TGFβ1 in the cultures.

RESULTS

DC differentiation is altered by cervical carcinoma cells

To explore the effects of soluble factors secreted by CxCa on the differentiation of monocytes, in vitro cultures were set up to analyze the direct effect of tumor supernatant (TSN) derived from five early passage CxCa cell lines (Table 1) (17,24) and the two well known cell lines CaSKi and HeLa. GM-CSF and IL-4 differentiated monocyte-derived DC (mo-DC) are defined as antigen presenting cells that lack the expression of CD14 but display the lineage marker CD11c, HLA-class I and II and CD1a. Healthy donor-derived monocytes were differentiated in the presence of 20% TSN of the 7 tumor cell cultures. The presence of TSN had a striking effect on their differentiation as shown by evaluation of surface marker expression typical for monocytes, DC, MDSC and macrophages. Mo-DC cultures typically contain >80% CD1a+ cells but when monocytes were differentiated in the presence of TSN from CC11-, CSCC1 or CaSKi this percentage dropped, reflecting poor DC-differentiation (Fig. 1a). TSN of the cell lines CCSC-7, CC-8 and HeLa did not only hamper CD1a expression but skewed the differentiation of monocytes towards the macrophage lineage as reflected

Table I Immuno-suppressive factors produced by CxCa cell-lines

			Cytokine Production (pg/ml)[c]			
Cell Line	Passage[a]	HPV[b]	IL-8	TGF-β1[d]	PGE_2	IL-6
CC-10B	P80	45	14,000	0	0	10
CSCC-1	P22	16	1,500	221	0	20
CC-11	P32	67	425	398	403	3,438
CaSKi	x	16	324	410	298	2,335
CSCC-7	P9	16	9,306	313	2,781	263
CC-8	P41	45	1,222	250	3,662	2,228
HeLa	x	18	4,500	548	5,389	12,000

(‡)number of passages since origin - HeLa and CaSKi not known
(§)integraded DNA found to be present in the tumor (24)
(*)100.000 cells/2ml cultured for 5 days in RPMI/10%FCS
(**)latent form of TGFβ-1 measured by ELISA after acidic treatment

by the high expression of CD14. Further evaluation of these CD14+ cells revealed that they expressed CD163 and CD206 as well. Notably these TSN induced cells expressed all human Fcγ-receptors (CD16, CD32 and CD64) as well as PD-L1 and HLA-class II, while CD1b and CD1c were absent (Fig.1b and not shown). This profile is highly similar to that of *in vitro* M-CSF-induced M2-macrophages and distinct from monocyte derived DC (Supplemental Fig. 1) (13,25). TSN of cell line CC-10B did not overtly alter the differentiation of monocytes to DC, indicating that CxCa supernatant does not *per se* result in phenotypical changes. Analysis of CD33, CD11b and CD124 expression revealed no evidence for skewing of monocytes to MDSC (data not shown).

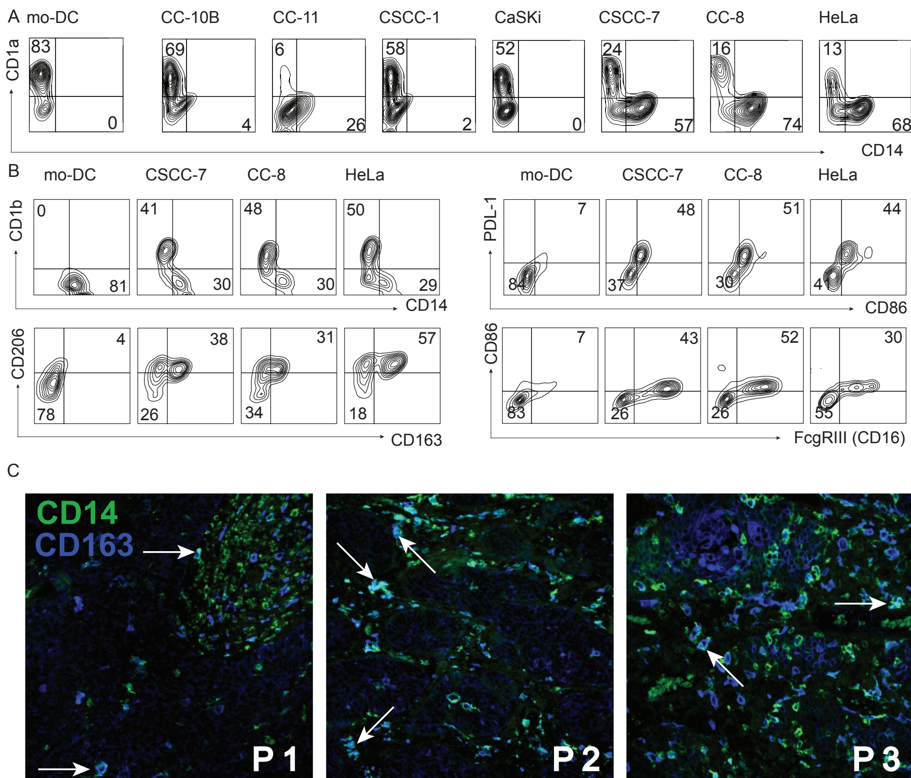


Figure 1. CxCa-secreted factors skew monocyte differentiation towards M2 macrophages.
Monocytes were cultured for 6 days with GM-CSF and IL4 in culture medium with 20% tumor supernatant (TSN) of indicated CxCa cell lines or 20% control medium (mo-DC) and (A) analyzed for expression of CD14 and CD1a. TSN reduced CD1a expression and in 3 cases induced CD14+ expression. (B) The cultures of monocytes differentiated with CD14+ cell-inducing TSN were analyzed for the expression of typical M2-macrophage markers. Numbers in quadrants represent percentage of cells within live gate. One representative of 5. (C) *In situ* immunofluorescence stainings of cervical tumor tissue of 3 out of 10 different patients for CD14 (green) and CD163 (blue) and measured by confocal microscopy (25 x magnification). CD14+CD163+ display as turquoise, white arrows indicate M2 macrophages.

To verify that these different cell types reside in the tumors of patients, paraffin embedded tissue sections of 10 patients with FIGO stage I or II CxCa were stained for macrophages ($CD14^+$) and M2-macrophages ($CD14^+CD163^+$) (26,27). Figure 1c shows the presence of CD14 single positive cells (macrophages), CD163 single positive cells but also CD14+CD163+ M2-polarized macrophages.

Functional impairment of APC by TSN

Next we assessed the capacity of these tumor-modulated APC to respond to 5 different TLR agonists or CD40-L expressing fibroblast cells (CD40-L) to mimic T-cell interaction. Since the supernatant of CSCC-7, CC-8 and HeLa induced strikingly induced these M2-macrophages (TSN-M2), which are known to foster immune tolerance, we focused on these cell lines for further evaluation and compared the results to those obtained with normal differentiated mo-DC.

Stimulation of mo-DC with LPS or R848 for 48 hours resulted in a strong increase in the expression of CD86, CD80, CD83, HLA-DR and PD-L1 (Fig. 2a and not shown). PolyI:C was the least potent TLR-agonist. TSN-M2 expressed higher basal levels of CD86, HLA-DR and PD-L1 but stimulation with R848 or PolyI:C did not raise their expression level to that of mo-DC (Fig. 2a and not shown).

For the induction and polarization of cytotoxic T cells and Th1 cells the secretion of IL-12p70 by APC is essential (28,29). Mo-DC produced the biologically active IL-12p70 upon stimulation with LPS, R848, or CD40-L cells. The amounts varied per donor of whom the mo-DC were prepared (Fig. 2b, Supplemental Fig. 2). Relatively to stimulated mo-DC, the TSN-M2 almost completely lacked the ability to produce IL-12p70 when stimulated with TLR agonists. The strong activation signal induced by CD40-L cells allowed TSN-M2 to produce IL-12p70, albeit at significantly lower concentrations than CD40-stimulated mo-DC. In contrast, the production of the cytokine IL-10 – which varied greatly between donors -was at least 2-fold increased when TSN-M2 were stimulated with TLR agonist or with CD40-L cells in 2 out of 3 experiments (Fig. 2b, Supplemental Fig. 2). The alterations in APC function were imprinted during the differentiation of the monocytes since activation of TSN-M2 cells in fresh medium without additives gave similar results (data not shown).

Subsequently, the capacity of TSN-M2 to induce proliferation and cytokine production of T cells was compared to mo-DC. Graded doses of APC were co-cultured with a fixed number of allogeneic CD4+CD25- T cells. Clearly, the activated TSN-M2 displayed a lower capacity to induce T-cell proliferation and/or concomitant IFNγ release (Fig. 2c). The percentage of CD25+Foxp3+ T cells, which can be increased upon stimulation with immature APC (30), was not clearly altered after 10 days of culture with TSN-M2 (data not shown). These results indicated that TSN-skewed APC were both phenotypically and functionally shifted towards that of M2 macrophages and that TLR mediated activation of TSN-M2 reinforced their tolerogenic profile.

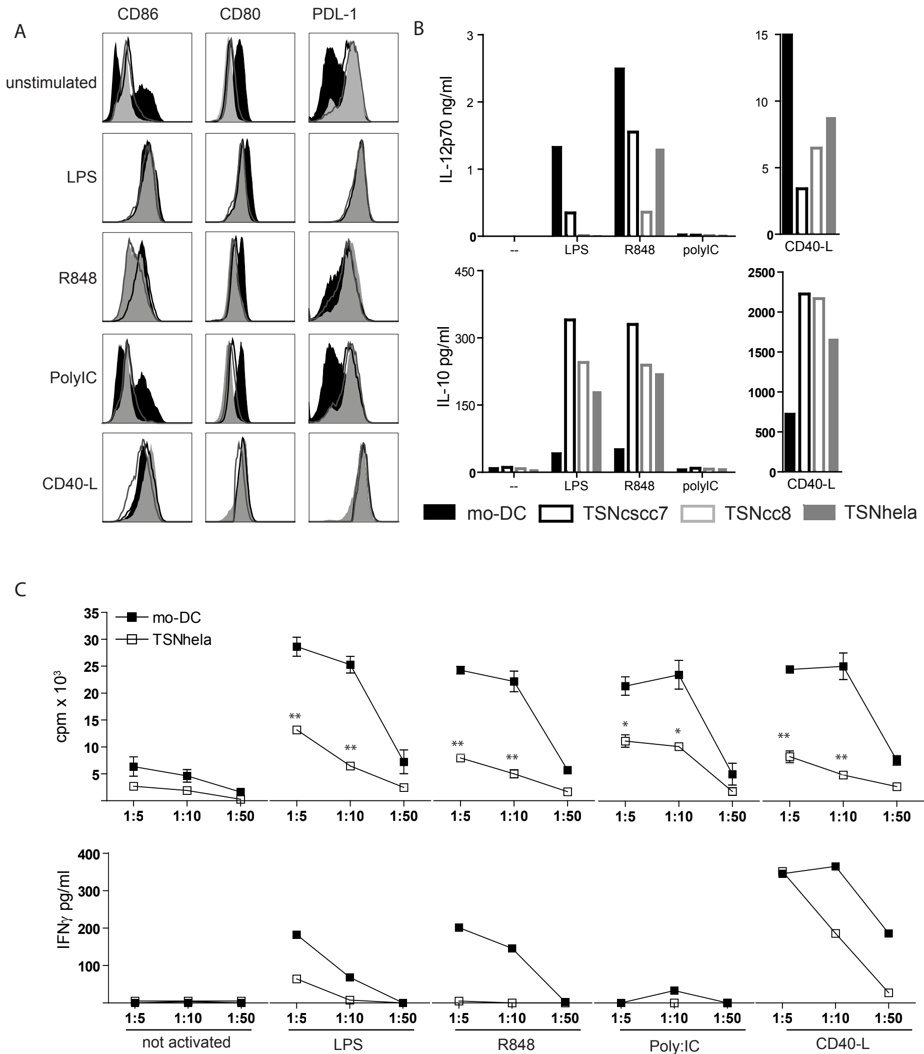


Figure 2. TSN-differentiated monocytes are phenotypically and functionally different from mo-DC. (A) Monocytes were differentiated in the presence of the indicated TSN and activated with single TLR-ligands or CD40-L cells for 48 hr. TSN-M2 cells express higher basal levels of CD86 and PDL-1. R848 or Poly-IC can not induce equal expression of CD80 and CD86 in all APC types. (B) Supernatants of the cultures described in (A) were tested for the presence of IL-12p70 and IL-10 revealing that TSN-M2 produce more IL-10 and less IL12 than mo-DC. (C) CD4+CD25- allogenic responder T cells were cultured with activated mo-DC or TSN-M2 (obtained with TSN from HeLa cells) at indicated ratio's (DC:T cell). Top row shows the higher proliferation of T cells when stimulated with upon activated mo-DC at day 5 as measured by [3H]-thymidine uptake. Test performed in triplicate, shown is mean with SEM. Means were compared by unpaired t-test, $*p < 0.05$, $**p<0.005$. Bottom row shows the IFNγ production within these cultures measured in the supernatant isolated at day 4 by ELISA. One representative of 3 experiments (A-C).

Mediators of altered APC differentiation

Numerous mediators may cause the altered differentiation of monocytes to DC, including TGFβ, PgE2, IL-6, IL-8, IL-10 and M-CSF (12). TSN of CxCa cell cultures were analyzed for these compounds. Latent TGFβ was produced by almost all cell lines, except for CC-10B. Three cell-lines produced high amounts of PgE2, and significant amounts of IL-6 were

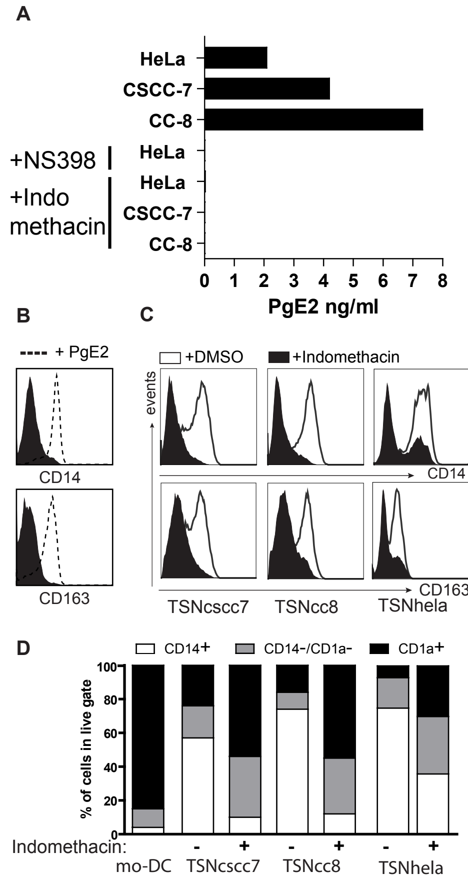


Figure 3. Cox-inhibition blocks PgE2 production and prevents M2-macrophage differentation.
(A) Tumor cell lines were treated for 24 hr with DMSO (control) or Cox-inhibitor (NS398 or Indomethacin) followed by culturing in fresh medium without additives for 24 hrs. PgE2 levels were measured by ELISA. Inhibition of COX totally abrogates PgE2 production. P<0.005 for all comparisons of PgE2 production by cells treated or not with indicated COX-inhibitor in 2 separate experiments (B) Culturing mo-DC (black fill) in the presence of 10 ng/ml PgE2 (dotted line) induce CD14+CD163+ expressing APC. (C) Flowcytometric analysis of CD14 and CD163-associated M2-macrophage marker expression on monocytes differentiated in the presence of TSN of Indomethacin-treated (black) tumor cells or TSN from non-treated tumor cells (white). (D) Comparison of CD1a and CD14 expressing populations following the differentiation of monocytes in the presence of TSN of Indomethacin-treated or non-treated tumor cells shows that TSN of COX-inhibitor treated cell lines induce less CD14+ cells and more CD1a+ DCs. Representative of 3 experiments (B-D).

produced by 5 cell lines. IL-8 was present in all TSN, of which CC-10B produced the highest levels (Table 1). Since the tumor supernatant of CC10B did not affect monocyte to DC differentiation, IL-8 was not further evaluated. IL-10 and M-CSF were not detected in the tumor supernatants.

The three likely candidates, TGFβ, PgE2 and IL-6 were further evaluated. The addition of TGFβ during differentiation of monocytes to DC did not induce the expression of CD14 but resulted in higher expression of CD1a. Likewise, blocking of the TGFβ pathway in TSN-APC cultures did not restore the phenotype to that of mo-DC, indicating that TGFβ was not responsible for the observed effects of TSN (Supplemental Fig. 3).

Skewing of APC to a macrophage phenotype can occur at concentration of > 2 pg/ml PgE2 (31). In fact, mo-DC differentiated in the presence of 1-50 ng/ml PgE2 resulted in CD14+ macrophages that are polarized to CD163+ M2-like macrophages (Fig 3b). To test if PgE2 was the M2-inducing factor in the TSN, the tumor cells were treated with specific COX-enzyme inhibitors. After treatment, the tumor cells were washed and incubated with fresh medium to obtain COX-blocked tumor supernatant. This procedure was chosen to avoid interaction of the inhibitor with COX in APC. Indeed, PgE2 production was totally abrogated by inhibition of COX 1 and 2 using Indomethacin or COX-2 by NS-398 (Fig. 3a).

Depletion of PgE2 in TSN by preventing its production revealed a striking effect on the DC-differentiation of monocytes. The expression of CD14 and CD163 was completely reversed (Fig. 3c) but the phenotypic differentiation towards DC was only partly restored as indicated by the percentage of $CD14^{-}CD1a^{+}$ APCs that was still lower than observed in mo-DC cultures (Fig. 3d). Furthermore, the capacity to produce IL-12p70 upon activation was restored while that of IL-10 was lowered (Fig. 4ab). The most pronounced effect of COX-inhibition was shown for CSCC-7 as the resulting APC from this COX-blocked tumor supernatant were completely comparable to mo-DC. The effects of COX-inhibition in lines CC-8 and HeLa on the function of TSN-altered APC was predominantly shown in CD40-activated APC. The functional restoration was reflected also by partial up regulation of their T-cell stimulatory capacity (Fig. 4c). As a control, TSN of Indomethacin treated CaSKi cells - which hardly produce PgE2 - was tested and neither clear differences in the hampered differentiation of the APC nor in LPS-induced IL-12 production were observed (Supplemental Fig. 4ab).

Since restoration of the phenotype and function of APC induced by PgE2 producing cell lines treated with Indomethacin was not complete and the cell lines CC-8 and HeLa produced significant amounts of IL-6, we explored the possibility that IL-6 mediated also an effect. Mo-DC were cultured with or without 20% TSN of the non-treated or the COX-inhibitor treated HeLa cell line in the presence of monoclonal antibodies to IL-6 and IL-6-receptor (32,33). The differentiation of mo-DC, nor the production of cytokines was altered by the presence of these antibodies (Fig. 5). However, blocking of IL-6 showed a profound effect on the phenotype of TSN-M2 in that the cultures contained a higher

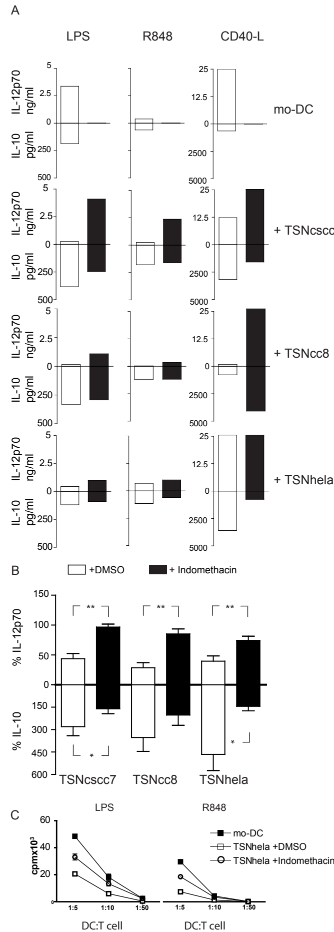


Figure 4. Restored cytokine production when PgE2 production by tumor cells is blocked.
(A) The production of IL-12 and IL-10 by APC differentiated in the presence of TSN of Indomethacin-treated (black) tumor cells or TSN from non-treated tumor cells (white) upon 48 hr activation with LPS, R848 or CD40-L as measured by ELISA. Top row shows the cytokine production of untreated mo-DC. One representative experiment of 3. The block of PgE2 production by tumor cells alters the balance in IL-12 and IL-10 towards that observed in the corresponding mo-DC.
(B) Comparison of the cytokine production by APC differentiated in the presence of TSN from non-treated tumor cells (white) to Indomethacin-treated (black) tumor cells that where activated for 48 hours (combined data of LPS, R848 and CD40-L stimulated cells; n=3 experiments). IL-12, all p<0.003. IL-10, TSNcscc7 p=0.015; TSNcc8 p=ns; TSNhela p=0.026.
(C) Mo-DC or TSN-M2 obtained with TSN of untreated (+DMSO) or Indomethacin-treated HeLa cells (+Indomethacin) were activated with LPS or R848 for 48 hours and used to stimulate allogeneic responder cells. APC differentiated in TSN of Indomethacin-treated cells induce better T-cell proliferation at day 5 of culture as measured by [3H]-thymidine incorporation. Test performed in triplicate, shown is mean with SEM. Means were compared by unpaired t-test. Comparison of non versus indomethacin treated HeLa cells (1:5 ; 1:10) for LPS p=0.009; p =0.024, and for R848 p=0.0003; p =ns. Comparison of mo-DC versus indomethacin treated HeLa cells (1:5 ; 1:10) for LPS p=0.004; p =ns, and for R848 p =0.009; p =ns. One representative out of 2 experiments.

percentage of CD14-CD1a+ APC. IL-6 blocking acted synergistically with the inhibition of COX since the combined treatment resulted in a complete phenotypical restoration of TSN-M2 to mo-DC (Fig. 5a). Blocking of IL-6 during the culture resulted in TNS-altered APC that after CD40-activation produced more IL-12p70 although this was not significant ($p=0.079$, n=3 experiments). There were no significant alteration in cytokine production ($p>0.05$, n=3 experiments) when the APC were activated by the TLR agonists (fig 5b). No major synergistic effect of IL-6 blocking on IL-12p70 and IL-10 production was found when COX-inhibited TSN was used. Since the supernatant of CaSKi cells, which hampered DC differentiation and function (Supplemental Fig. 4), also contained high levels of IL-6 we blocked this cytokine during the differentiation of monocytes to DC with TSNcaski. Blocking of IL-6 restored both CD1a expression and the balance between IL-12p70 and IL-10 to what is found for mo-DC (Supplemental Fig. 4c). Together, these data showed that PgE2 predominantly influenced the expression of the macrophage markers, whereas IL-6 altered CD1a expression. While both pGE2 and IL-6 affect the balance between IL-12p70 and IL-10, PgE2 had a more dominant negative effect. Blocking of these two mediators prevents M2-skewing and restores normal monocyte to DC differentiation.

CD4+ Th1 T cells can switch tumor-induced M2 to activated M1 macrophages

Initially, we had used CD40-L cells to mimic the interaction between T cells and TSN-M2. Since CD40-activation was the best stimulus to induce IL-12p70 production by APC we investigated the phenotypical and functional changes in TSN-M2 following cognate interactions with CD4+ T cells. Co-cultures of TSN-M2 and Th1 cells were performed in the same TSN-containing culture medium since tumor secreted factors may directly suppress T-cell function (34). Three different CD4+ Th1 clones were clearly able to fully activate mo-DC and TSN-M2 in an antigen dependent manner (Fig. 6). Notably, Th1-mediated activation of TSN-M2 resulted in a number of changes that suggested a shift from M2-like to M1-like macrophages. The levels of the co-stimulatory molecules readily increased to the same level as mo-DC (Fig. 6a). In addition, the expression levels of PD-L1 increased. The strong activation was also reflected by the high amounts of IL-12p70 produced reaching IL-12 levels similar to that of mo-DC, as there is no significant difference ($p>0.05$, n=3 T-cell clones) between mo-DC or TSN-M2 (Fig. 6b and not shown) and much higher than previously observed after TLR- or CD40-activation (compare Fig. 2b and 6b). Strikingly, the production of IL-10 remained low and around the same level as the corresponding mo-DC cultures ($p>0.05$ for TSNcscc7 and TSNcc8, $p=0.03$ for TSNhela; n=3 T-cell clones). After the interaction with Th1 cells, the typical M2-like macrophage markers CD206 and CD163 were lost. This was also observed when TSN-M2 were stimulated with LPS, R848 and CD40-L cells indicating that this is a reflection of APC activation (Supplemental Fig. 1bc). Furthermore, high amounts of T-cell produced IFNγ, TNFα and IL-2 were detected in the supernatant of the co-cultures reflecting the activation of the T cells upon recognition of

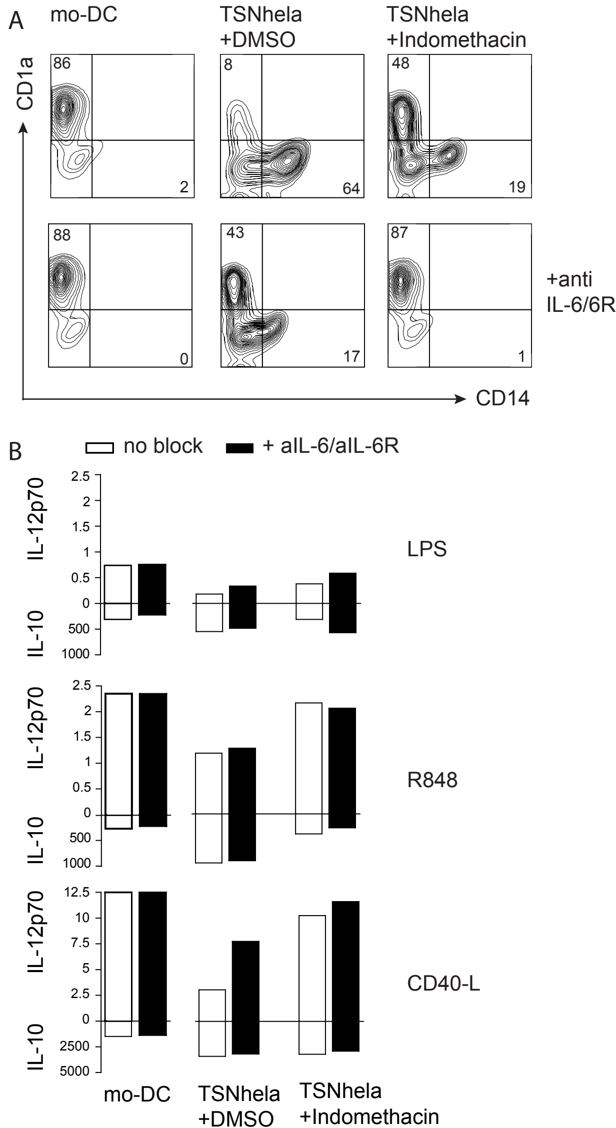


Figure 5. Blocking IL-6 and PgE2-induced altered differentiation restores monocyte to DC differentiation completely.

(A) Monocytes were differentiated in the presence of control(+DMSO) or COX-inhibited HeLa cells(+Indomethacin) without or with blocking antibodies against IL-6 and IL-6 receptor. A combination of tumor cells treated with Indomethacin and blocking IL-6 during differentiation results in full DC differentiation reflected by high expression of CD1a and lack of CD14. (B) Blocking IL-6 during differentiation did not affect the production of IL-12 (ng/ml) and IL-10 (pg/ml) of mo-DC upon activation by indicated agonists. CD40-activated TSN-M2 cells of which the IL-6 in TSN was blocked during differentiation, produced more IL-12. One representative experiment out of 3.

their cognate peptide (Fig. 6b and not shown). Apart from IL-12p70 and IFNγ for which it is clear that they are only produced by the APC or T cells, respectively, IL-10 may be produced by both cell types and this can not be distinguished by ELISA. It is likely that the IL-10 detected in co-culture with clone 238 is produced by the T cell clone as mo-DC stimulated with the other 2 clones do not produce IL-10. Our previous experiments indicated that ligation of CD40 could not switch M2 to M1 macrophages, therefore, we analyzed if one of the T-cell produced cytokines synergized with CD40-L cell-mediated activation to switch

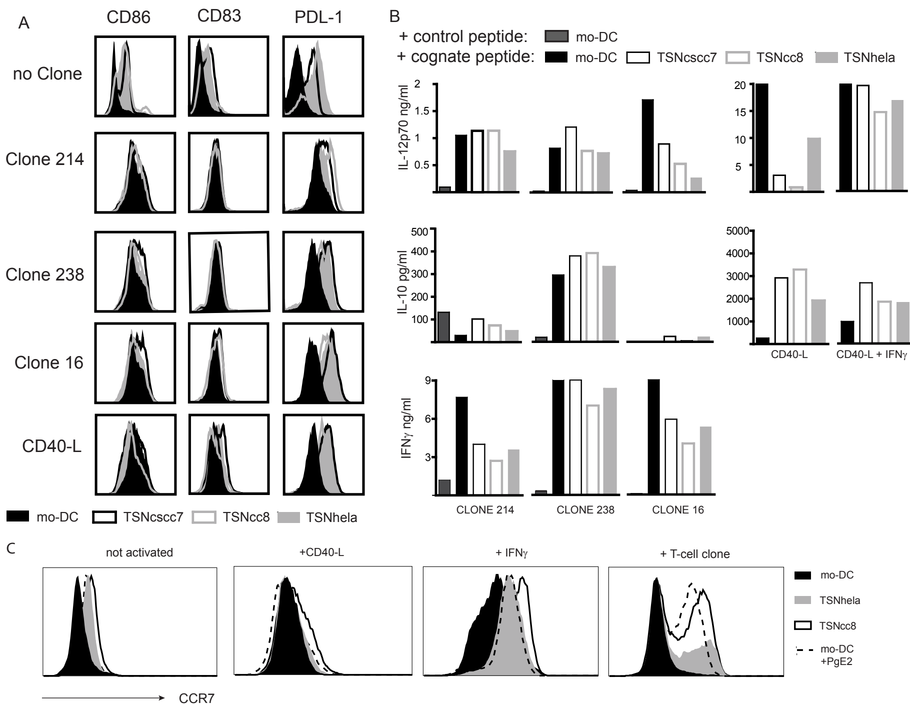


Figure 6. CD4+ T cells switch TSN-M2 to M1-like macrophages and activate them to produce IL-12p70. (A) Mo-DC or TSN-M2 were pulsed with the cognate Th1-peptide and co-cultured with HPV-specific CD4+ T-cell clones (2 DC:1 T cell) or CD40-L cells. T-cell interaction results in high expression of co-stimulatory molecules and PDL-1. One representative experiment out of 3. (B) Top row: Cognate interaction of Th1 cells with TSN-M2 results in high IL-12 production. Stimulation with CD40-L cells in combination with 500 pg/ml IFNγ induces IL-12p70 production in TSN-M2 cultures to the level of mo-DC. Middle row: Cognate interaction with Th1 cells induced the production of IL-10 in TSN-M2 cultures at similar low levels detected for mo-DC. The addition of IFNγ to CD40-stimulation results in similar production of IL-10 as after CD40-L cell stimulation. Bottom row: The Th1 clones produce IFNγ when stimulated with peptide-pulsed mo-DC and TSN-M2. Shown are the results of the experiment in which all conditions and 3 clones were combined. Similar results were obtained in experiments where individual clones and TSN-M2 combinations were tested (C) APC cultured in the presence of M2-inducing TSN or PgE2 were activated with CD4+ T-cell clone 16, CD40-L expressing fibroblasts or IFNγ and analyzed for the expression of CCR7 at 48 hr after activation. Cognate Th1 cell interaction induces high CCR-7 on TSN-M2 and this can be mimicked by activating DC with IFNγ. The level of CCR7 is enhanced when PgE2 is present during the differentiation of monocytes. One representative experiment out of 3.

M2 to M1-like macrophages. TSN-M2 were activated with CD40-L and IFNγ or TNFα. This revealed that the combination of CD40-L cells with IFNγ but not CD40-L cells or IFNγ alone resulted in high levels of IL-12 not only in mo-DC but also in TSN-M2 cultures (Fig. 6b right and not shown). This capacity of Th1 cells or CD40-L cells + IFNγ to switch M2-like macrophages to M1-like macrophages could be reproduced in co-cultures with M-CSF-induced M2 macrophages (not shown).

TSN-M2 expressed higher levels of CCR7 than mo-DC following their cognate interaction with CD4+ Th1 cells (Fig. 6c). As we had already found that IFNγ synergized with CD40-activation to switch M2- to M1-like macrophages we tested the hypothesis that IFNγ or TNFα secreted by the T-cell clone either alone or in combination with PgE2 present in TSN was responsible for the high levels of CCR7. Indeed, incubation with IFNγ but not TNFα induced the expression of CCR7 on mo-DC and a very high expression on TSN–M2. Furthermore, pretreatment of mo-DC with PgE2 during the differentiation phase resulted in similar high expression of CCR7 as found on TSN-M2 (Fig. 6c).

Thus, cognate interaction with IFNγ-producing T cells can switch the tumor-promoting M2-like polarized macrophages to activated classical M1-like macrophages that express high levels of co-stimulatory molecules, produce high amounts of IL-12 and gain the expression of the lymphoid homing receptor CCR-7.

DISCUSSION

Our analysis on the effect of tumor-secreted factors from 7 different cervical cancer cell lines on the differentiation of monocytes to dendritic cells and their functional capacity revealed that these cancer cells can be sorted into two major categories; -1- cancer cells that hamper DC differentiation and function and -2- cancer cells that induce M2-like macrophages. These two categories comprised similar HPV types ruling out that the effects seen were HPV type specific. Tumor secreted PgE2 and /or IL-6 were clearly responsible for these effects while no role was found for TGFβ, IL-8, IL-10 or M-CSF. In vivo, such APC are present at different levels of differentiation in stroma and epithelial compartments of HPV-induced cervical cancer and these include next to immature DC, mature DC, macrophages and type II macrophages (Fig. 1)(10,35).

Category 2 cancer cells stimulated the differentiation of $CD14^+$, $CD16^+$, $CD206^+$, $CD163^+$ M2-like macrophages. Consequently, these TSN-M2 displayed an altered cytokine profile and a poor capacity to stimulate T cells when compared to mo-DC. Careful evaluation of the expression of co-stimulatory molecules, cytokine production and T-cell stimulatory capacity of these TSN-M2 showed that stimulation with a number of clinical applicable TLR-agonists or CD40-L cells could not provoke the same phenotypical and functional activity as found for mo-DC. Interestingly, unstimulated TSN-M2 cells expressed PD-L1

at higher levels than mo-DC. While stimulation of mo-DC and TSN-M2 resulted in an increased expression of PD-L1, the expression on TSN-M2 remained higher on TSN-M2, suggesting that TSN-M2 display an altered co-stimulatory/inhibitory molecule ratio on the cell surface as compared to mo-DC. High levels of PDL-1 expression on monocytes have been shown to effectively suppress tumor-specific T cell immunity and to contribute to the growth of human hepatocellular carcinoma cells in vivo(36). Furthermore, knockdown of PD-L1 in activated DC has been shown to increase the IFNγ and IL-2 production of reacting T cells (37). We are currently investigating the role of PD-L1 expression level with respect to the lack of responsiveness of naive T cells in our experiments. Notably, comparison of the two different agonists R848 and LPS to stimulate TSN-M2 revealed clear differences in their effects on co-stimulatory molecule expression and cytokine production, indicating that previous results reported with the TLR4 agonist LPS – most often used to stimulate tumor-induced DC in vitro (16,38)– can not be translated to other TLR agonists. Earlier studies identified macrophages (CD68+) within the CxCa microenvironment and showed that an increase in macrophages is inversely correlated with survival (39). The presence of macrophages correlated with a high production of IL-6 by tumor cells, the latter of which was associated with poor survival (19). IL-6 was shown in vitro to hamper the DC differentiation and allogeneic T-cell stimulatory capacity and could even switch monocyte differentiation from DC to macrophages (32,33,40-42). Others showed that the over-expression of COX-enzymes in HPV-induced lesions is associated with a loss in CD1a+ cells and PgE2 was suggested to mediate this effect (16). Under our experimental conditions IL-6 alone was able to hinder DC-differentiation and function but PgE2 was responsible for the conversion of monocytes to M2 macrophages. Differentiation of monocytes to M2 macrophages could be prevented by inhibition of the production of PgE2 in tumor cells and blocking IL-6 during the differentiation period of the monocyte.

Importantly, when fully polarized M2 macrophages present antigen to Th1 cells within the context of a M2-polarizing milieu – as represented by the M2-inducing tumor supernatant – this interaction not only results in re-polarization of M2- to M1-macrophages but it also activated these M1 macrophages to express high levels of co-stimulatory molecules, to produce IL-12 and to express the lymph node homing marker CCR7. The switch from M2- to M1-macrophages is in concordance with the plasticity of macrophages to change their functional phenotype from classically activated macrophages to wound-healing or regulatory macrophages and *vice ver*se (43,44). This switch could be reproduced by stimulating TSN-M2 with CD40-L cells and IFNγ. While activation via CD40 was enough to induce changes in the typical M2 markers, IFNγ provided the necessary signals for the macrophages to produce IL-12 without additional IL-10 production. Mouse models have elegantly demonstrated the importance role of the Th1-macrophage axis in anti-tumor immunity. Tumor-resident macrophages were shown to process and present tumor antigen to Th1 cells which in return activated these macrophages – through the local release of

IFNγ - to become tumoricidal and to induce a CD4+ T-cell dependent tumor protection (45,46). Our data suggest that alteration of the suppressive tumor microenvironment by tumor-infiltrating Th1 cells - which change the tolerogenic M2-macrophage phenotype to that of activated M1-macrophages- could be one of the underlying mechanisms of this tumor protection system.

The local presence of IFNγ-producing T cells responding to antigen presented by these altered APC may restore proper tumor-rejecting immune function, but such T cells are often absent in tumors. (47,48). COX-inhibiting drugs are widely used in the clinic for treatment of auto-immune diseases and trials are now ongoing, with the aim to determine the effect of low dose NSAID on tumor prevention by disrupting the COX-2 meditated oncogenic pathways (49). Furthermore, monoclonal antibodies to IL-6 receptor are already in clinical use for the treatment of autoimmune diseases (50). It can be envisaged that a combination therapy consisting of COX-inhibition, IL-6 blocking and the induction of a strong Th1 T cell response by currently available vaccines may form the next generation of immunotherapy for the treatment of cervical cancer(51,52).

ACKNOWLEDGEMENTS

We like to thank Jaap van Eendenburg for the isolation and culture of fresh CxCa cell-lines. Also we like to thank Nigel Savage for determining the presence of M-CSF in our supernatants, Bart Everts for his helpful suggestions and Michelle Osse for confirming the origin of the cell lines by analyzing the integrated HPV types.

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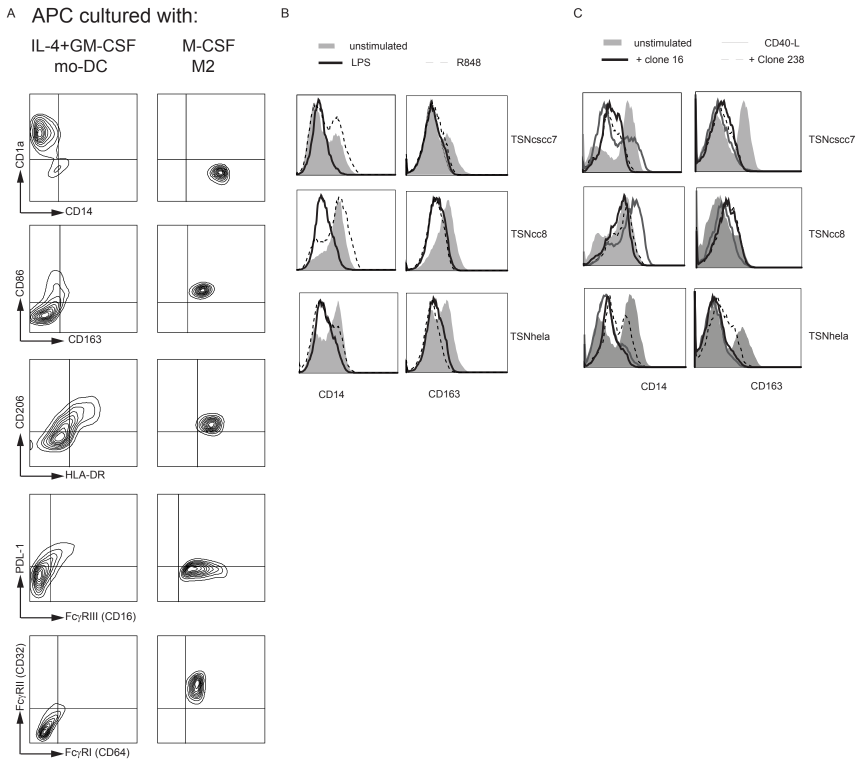
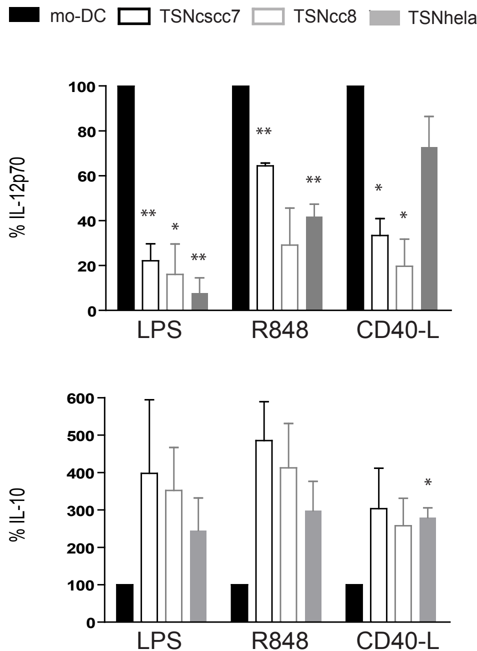


Supplementary fig 1. Phenotype of in vitro differentiated DC and M2 macrophages.
(A) Monocytes were cultured for 6 days with GM-CSF+IL-4 to obtain imm DC or M-CSF to obtain M2 macrophages and analyzed by flowcytometry for the expression of phenotypical markers. M2-differentiated macrophages typically display high expression of the scavenger receptor CD163 and the mannose receptor CD206. In contrast to monocyte derived DC M2-macrophages express all FcγReceptors (CD32, CD64 and CD16). (B+C) Stimulation of TSN-M2 for 48 hr with (B) indicated TLR-ligands and (C) Th1 cells or CD40-L cells results in a lowered expression of CD14 and CD163 as detected by flowcytometry.



Supplementary fig 2 Function of DC is hampered by TSN.
Compared to mo-DC, TSN-M2 cultures produce less IL-12 and more IL-10 upon depicted stimulus. We observed large variation in the amounts of cytokines produced by each donor to the different TLR-ligands, especially for IL-10. To compare 3 different donors with respect to the amounts of cytokine produced; the relative production of cytokines of each condition within one experiment was calculated. The amount of cytokines produced by mo-DC to the indicated stimulus was set to 100%. Three experiments combined, mean with SEM. For each stimulus means were compared to mo-DC by paired t test. * indicates p<0.05, ** indicates p<0.005.

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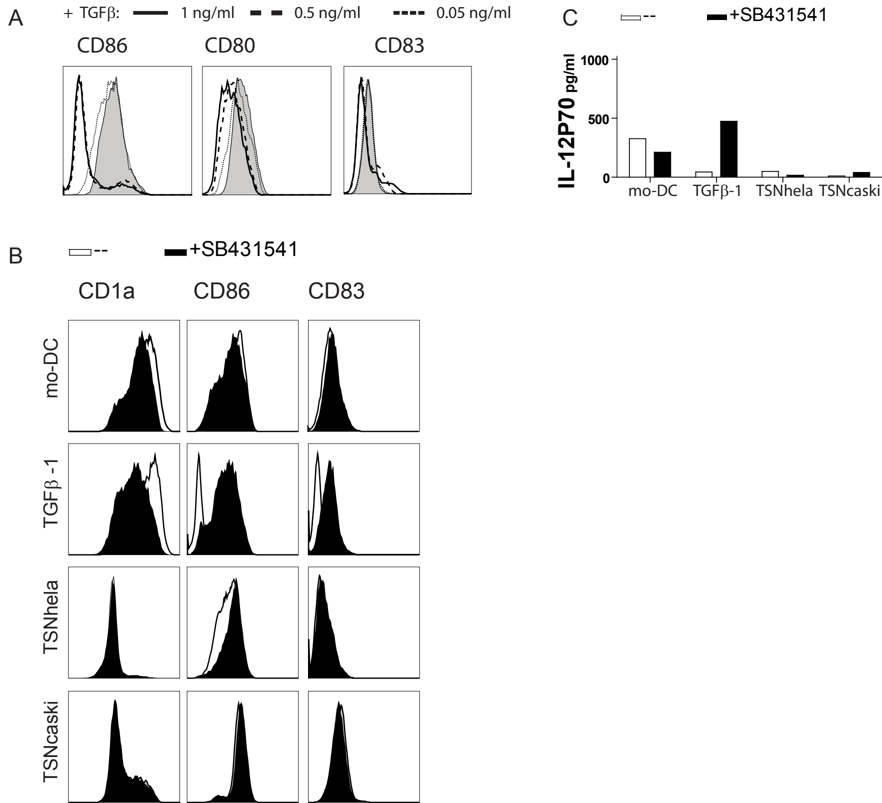
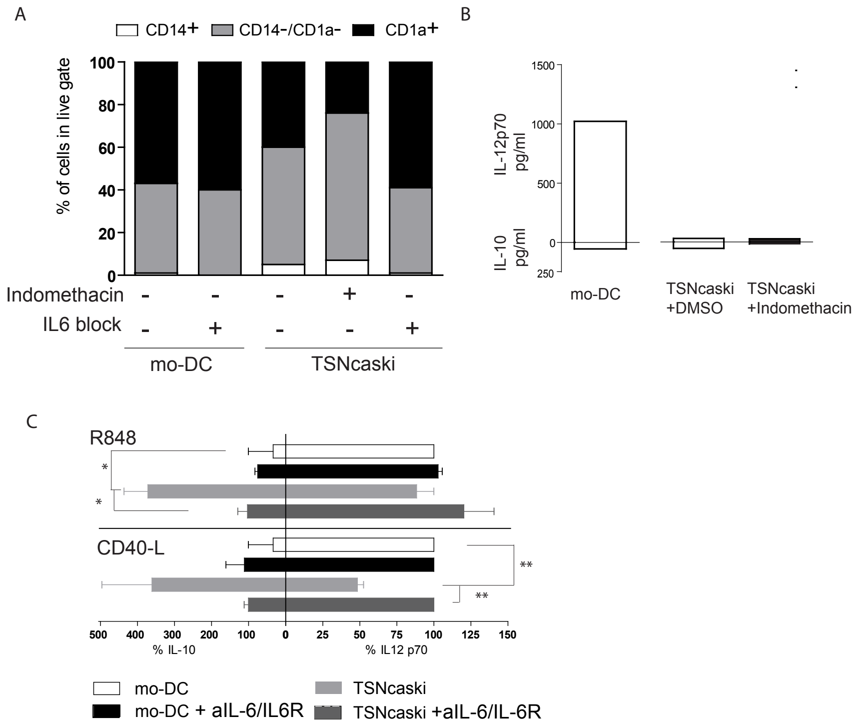


Supplementary fig 3 Blocking of the TGFβ pathway in TSN differentiated APC.
(A) Mo-DC were cultured with increasing concentration of TGFβ and activated for 48 hrs with LPS. Flow-cytometric analysis show that high levels of TGFβ make APC unresponsive to LPS as reflected by a failure to upregulate the expression of co-stimulatory molecules. One representative experiment of 3. (B) Mo-DC or APC differentiated in the presence of 500 pg/ml TGFβ-1 (TGFβ) or 20% TSN (obtained from HeLa or CaSKi) were treated without (white) or with the ALK-inhibiting compound SB431542 (black) during culture and activated with LPS for 48 hr. Although the effect of TGFβ on the unresponsiveness to LPS is completely abrogated by compound SB431541, no effect of this compound is found on the expression of CD1a, CD86 and CD83 by APC in TSN cultures. (C) The production of IL12p70 upon 48 h LPS stimulation of control (white) or compound SB431542 treated (black) cultures. The compound restores the IL12p70-production by TGFβ-treated cells to the same level as mo-DC, but does not restore IL-12 production by TSN-differentiated APC.

CHAPTER 5



Supplementary fig 4 Blocking IL-6 in the TSN of CaSKi cells abrogates the suppressive effect on DC differentiation and function.

(A) A lower percentage of monocytes differentiated in the presence of 20% supernatant of caskicells expresses CD1a after 6 days of culture, reflecting poor DC differentiation. Addition of IL-6 and IL-6Receptor blocking antibodies to the culture does not influence normal monocyte to DC differentiation but increases the percentage of CD1a expressing cells in cultures with TSNcaski. Treating the CaSKi-cells with COX-inhibitor indomethacin does not affect the effect on monocyte differentiation. Depicted is the percentage of all cells in live gate. One representative of 2 experiments. (B) Monocytes were differentiated in the presence of control (+DMSO) or COX-inhibited CasKi supernatant and activated with LPS. Cytokine production was analyzed after 48 hr. (C) APC differentiated in the presence of TSNcaski produce more IL-10 and less IL-12 upon activation by TLR-ligands or CD40-L when compared to fully differentiated mo-DC. Blocking of IL-6 in the supernatant during differentiation of monocytes restores the capacity of the resulting cells to produce IL-12 while reducing the production of IL-10. To compare different donors, the amount of cytokine produces is depicted as a percentage of the cytokine when produced by mo-DC activated by the same stimulus without blocking of IL-6. Three experiments combined. Means were compared by the two-tailed unpaired t-test using Graphpad software. * indicates $p<0.05$, ** indicates $p<0.005$.

Chapter 6

The long-term immune response after HPV16 peptide vaccination in women with low-grade pre-malignant disorders of the uterine cervix: a placebo-controlled phase II study

Peggy J. de Vos van Steenwijk
Mariette I. E. van Poelgeest
Tamara H. Ramwadhdoebe
Margriet J. G. Löwik
Dorien M. A. Berends-van der Meer
Caroline E. van der Minne
Nikki M. Loof
Linda F. M. Stynenbosch
Lorraine M. Fathers
A. Rob P. M. Valentijn
Jaap Oostendorp
Elisabeth M. Osse
Gert Jan Fleuren
Linda Nooij
Marjolein J. Kagie
Bart W. J. Hellebrekers
Cornelis J. M. Melief
Marij J. P. Welters
Sjoerd H. van der Burg
Gemma G. Kenter

Cancer Immunol Immunother 2014 ;63(2):147-60

ABSTRACT

The capacity of a low-dose HPV16 synthetic long-peptide vaccine (HPV16-SLP) to induce an HPV16-specific T-cell response as well as to establish long-term immunologic memory in patients with low-grade abnormali-ties of the cervix was determined in a placebo-controlled, double-blinded phase II study. In addition, the effect of a booster vaccination after 1 year was evaluated. Patients received either the HPV16-SLP or a placebo at the start of the study. After 1 year, the vaccinated patients were again randomized to receive the HPV16-SLP or a placebo. Patients were followed for 2 years. HPV16-specific T-cell responses were determined in pre- and post-vaccination blood samples by ELISPOT, proliferation assay and cytokine assays. We show that the HPV16-specific T-cell responses detected after vaccination are clearly due to vaccination and that reactivity was maintained for at least 2 years. Interestingly, a booster vaccination after 1 year especially augmented the HPV16-specific Th2 response. Furthermore, pre-existing immunity to HPV16 was associated with a stronger response to vaccination and with more side effects, reflected by flu-like symptoms. We conclude that two low-dose injections of HPV16-SLP can induce a strong and stable HPV16-specific T-cell response that lasts for at least 1 year. If booster vaccination is required, then polarizing adjuvant should be added to maintain the Th1 focus of the vaccine-induced T-cell response.

INTRODUCTION

The development of (pre)cancers of the anogenital tract is associated with persisting human papillomavirus (HPV) infections [1]. The risk of progression of squamous intraepithelial lesions (SIL) is related to the severity of dysplasia. Up to 40 % of low-grade cervical squamous intraepithelial lesions (LSIL) will not spontaneously regress [2]. Small lesions can easily be treated by loop electrosurgical excision procedure (LEEP), yet LEEP of larger lesions can leave positive margins causing lesion recurrence requiring repeated surgery [3]. For the group of patients with a child wish, this can pose a problem due to distortion of the cervix and pre-term delivery [4].

Vaccines have been developed to prevent persistent infection with HPV but these prophylactic vaccines are not effective in patients already infected with HPV16 or HPV18 [5]. Virus-specific, interferon-γ (IFNγ)-producing CD4+ T helper (Th) cells and CD8+ cytotoxic T lymphocytes (CTL) are essential components in controlling chronic viral infections [6, 7].

Healthy donors display relatively robust proliferative T-cell responses against the viral early proteins E2, E6 and E7, characterized by Th cells that produce IFNγ and IL-5 [8–10]. In addition, the majority of subjects who clear HPV16 display HPV16 E6-specific CTL responses [11, 12]. These findings suggest that successful defense against HPV16 infection is associated with a systemic HPV-specific T-cell response. Therapeutic vaccination can be clinically effective in patients with histologically confirmed HPV16+ vulvar epithelial neoplasia grade 3 (VIN3). Complete regression of lesions was seen after vaccination with a protein vaccine [13] or an HPV16 E6/E7 synthetic long-peptide vaccine (HPV16-SLP) [14]. Clinical success correlated with the induction of strong and broad HPV16-specific Th responses and HPV16-specific CD8+ T-cell activity [13–15]. In patients with high-grade cervical squamous intraepithelial lesions (HSIL), immunization with 300 μg per peptide of the HPV16-SLP vaccine-induced robust immune responses [16].

In addition to women with high-grade lesions, also women with persistent low-grade lesions may be treated by therapeutic vaccination. As low-grade cervical lesions are not considered a severe disorder, we decided to immunize such individuals with the lowest dose (50 μg/peptide) previously shown to be immunogenic in patients with cervical cancer [17]. To this end, patients with LSIL or persistent mild cytological cervical abnormalities received either placebo or were vaccinated twice. The group of vaccinated patients was then randomized to receive a placebo or a booster vaccination after 1 year. All patients were followed for 2 years and their HPV-specific immune response was tested at several time points during the study. The aim of this phase II study was threefold. (1) To study the capacity of a low-dose vaccine

to induce HPV16-specific T-cell responses in patients with LSIL or persistent mild cytological cervical abnormalities, (2) to evaluate the long-term memory response after vaccination and (3) to study the effect of revaccination after 1 year on the HPV16-specific T-cell response.

PATIENTS AND METHODS

Patients

In this placebo-controlled, double-blinded study, 50 patients with histological evidence of LSIL or persistent mild cytological cervical abnormalities were included from the out-patient departments of the Leiden University Medical Center (Leiden, the Netherlands), the Haga Teaching Hospital (the Hague, the Netherlands) and the Medical Centrum Haaglanden (the Hague, the Netherlands). Patients were included between May 2007 until March 2010 after oral and written informed consent. Eligibility required pre-treatment laboratory findings of leukocytes >3 × 10^9/L, lymphocytes >1 × 10^9/L, thrombocytes >100 × 10^9/L and hematocrit >30 % and no radiotherapy, chemotherapy or other potentially immunosuppressive therapy administered within 4 weeks prior to the immunotherapy. The study was approved by the Dutch Central Committee on Human Research (CCMO, https://toetsingo nline.ccmo.nl/ccmo_search.nsf/dossier number NL14057 000 06) and the medical Ethical Committee of the Leiden University Medical Center and the Haga teaching Hospital. Monitoring for adverse events and injection-site reactions, clinical assessments and laboratory tests were performed as described previously [17]. Data were gathered on previous HPV-related disease [PHD, defined as surgical or topical treatment of SIL of the cervix or vulvar intraepithelial neoplasia (VIN)], atopic constitution and smoking habits. Adverse events were classified according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. The flu-like syndrome was defined as having two or more of the following complaints: fever, chills, head-ache, malaise, fatigue, myalgia, nausea, anorexia, vomiting or diarrhea after vaccination. In most patients, the symptoms subsided within 72 h, any symptoms persisting longer than 72 h or starting 72 h after vaccination were scored separately.

Vaccine and vaccination scheme

The HPV16-synthetic long-peptide vaccine (HPV16-SLP) used in this study consists of two separate drug products, together representing the entire sequence of the E6 and E7 oncoproteins of HPV16. The clinical grade peptides (9 E6 and 4 E7 peptides of 25–35 amino acids long with an over-lap of 10–14 amino acids) were synthesized, vialed and formulated at the GMP facility of the department of Clinical Pharmacy and Toxicology of the LUMC as described previously [14–18]. Peptides were dissolved in dimethyl sulfoxide (DMSO) and admixed with 20 mM phosphate buffer (pH 7.5) and Montanide ISA-51 (final volume ratio 20/30/50, respectively), and patients received the vaccine at a dose of 50 μg/peptide. This dose has previously

been shown to induce HPV16-specific immunity in end-stage cervical cancer patients [17]. Patients were assigned to one of the two treatment groups (block size 5). Randomization was blinded for patients and the immunomonitoring laboratory. Four out of five patients, in total 40, were randomized to receive two sequential HPV16-SLP vaccinations at a 3-week interval (50 μg/peptide), *T* = 0 week and *T* = 3 weeks (Group 1, Fig. 1). They received a mix of nine synthetic long HPV16 E6 peptides in the left arm or thigh and four synthetic long HPV16 E7 peptides in the right arm or thigh. Ten (one out of five) patients were randomized to receive phosphate-buffered saline (PBS) in both arms or thighs in the same regime (Group 2). After 1 year (*T* = 1 year), a second randomization took place. Half the patients from group 1 were randomized to receive a booster vaccination of 50 μg peptide of the HPV16-SLP (group 1A) and the other half was randomized to receive PBS (group 1B). Patients in group 2 received PBS throughout the study. All vaccinations were performed in the LUMC. Patients stayed at the ward for 1–2 h after vaccination during which any experienced local and/or systemic adverse events were recorded. Patients recorded any adverse events experienced in the weeks between and after vaccinations in a diary or were asked to report any adverse events. Venous blood (70 ml) for immune monitoring was drawn at five time points; before (*T* = 0 week), 7 weeks (*T* = 7 weeks) and 1 year (*T* = 1 year) after first vaccination, 3 weeks after booster vaccination (*T* = 1 year + 3 weeks) and finally 2 years after first vaccination (*T* = 2 years; Fig. 1). An extra Pap smear was taken before vaccination and at 1 and 2 years after vaccination for histology and HPV typing.

HPV-specific T-cell immunity monitoring

In acknowledgment of the minimal information about T-cell assays (MIATAproject.org) detailed information about the sample, the assay, the data acquisition, the data analysis and the laboratory environment is provided [19, 20]. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (prepared by LUMC pharmacy) gradient centrifugation within 4 h after blood was drawn (70 mL in 9 mL heparine tubes, kept at room temperature). The median PBMC numbers obtained were 56 million and ~10 mil-lion cells were used directly in the lymphocyte stimulation test (LST) to test for HPV16 specificity. The proliferative response accompanying cytokines were measured by the cytometric bead array (CBA). The remaining cells were cryopreserved (~5–10 million/vial) in 90 % fetal bovine serum and 10 % DMSO in 1 mL cryovials (Greiner) using a controlled freezing apparatus and immediately stored in the vapor phase of the liquid nitrogen vessel until used (median pre-sample 24 months, post-samples 5 months). Thawed PBMCs were subjected to the IFN-γ-Elispot and geared to determine Th type 1 (Th1) responses. The median cell recovery post-thaw was 73.6 % with a median viability of 76.8 %. Cell counts and viability was obtained using trypan blue (0.4 %, Sigma) staining and counting using the hemocytometer. In this set of complementary T-cell immunomonitoring assays (LST and IFN-γ-Elispot), six pools of 22 amino acid long peptides overlapping by 12 amino acids were used. All tests

have previously been described, and positive responses have been pre-defined [21]. For all T-cell assays, a vaccine-induced response was defined as at least a threefold increase in the response after vaccination when compared with the results before vaccination ($T = 0$ week). Similarly, a booster vaccination-enhanced response was defined as an at least threefold increase in the immune response after the booster vaccination compared with the HPV-specific immune response before booster vaccination. The T-cell assays were performed in the lab-oratory of the Department of Clinical Oncology (LUMC, Leiden) that operates under exploratory research conditions following standard operating procedure (SOPs) and using trained staff. This laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (http://www.cimt.eu/workgroups/cip/) as well as in IFNγ-Elispot panels of the Cancer Immunotherapy Consortium (http://www.cancerresearch.org/cic), which both aim to harmonize the assays used for T-cell monitoring and the reporting thereof. For each different type of immune assay, the strength of the immune response was defined as the median-specific spot count (ELISPOT), stimulation index (LST) or amount of cytokine production (CBA) obtained for all six different peptide pools of all patients in one group. Raw data was stored for verification. Comparisons of the strength of the different types of immune responses at different time points within one group of patients were made by the non-parametric Wilcoxon matched-pairs signed rank test and between groups by the nonparametric Mann–Whitney test using GraphPad InStat Software. For the comparison of the immune responses and patients characteristics, patients were divided into two groups based on the presence or absence of HPV-specific immune response and calculated using the Statistical Package for the Social Sciences (SPSS) software package 17 . All reported *P* values are two-sided and have not been adjusted for multiple comparisons. $P \leq 0.05$ was considered significant.

HPV testing

DNA was isolated from cervical smears or formalin-fixed, paraffin-embedded biopsy samples as previously described [22]. Betaglobin polymerase chain reaction (PCR) was performed using primers RS40 and RS42 to determine whether the isolated DNA was suitable for amplification. The DNA was subjected to a short PCR fragment assay using the SPF_{10} primer set, according to the manufacturer's instructions (Innogenetics, Ghent, Belgium). Each experiment was performed with separate positive and several negative controls. The presence of HPV was established using a microtiter plate–based hybridization assay, and SPF_{10}-PCR products from HPV DNA-positive cases were directly genotyped using a reverse hybridization line probe assay (Inno-LiPa HPV Genotyping Extra; Innogenetics). With this assay, 28 individual HPV genotypes can be identified simultaneously: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74 and 82.

RESULTS

Patients and vaccinations

Between May 2007 and March 2010, 99 patients were screened of whom 51 patients were accrued for the study (Fig. 1). The average age was 40 years. 47 % of the patients had a medical history of PHD (Table 1). The majority (73 %) of the patients was infected by at least one high-risk HPV-type (Table 1) and 33 % were infected with HPV16. Patients were diagnosed with a LSIL at inclusion or had signs of persistent HPV infection (persistent mild cytological cervical abnormalities). The 51 patients were assigned to one of the two treatment groups at the start of the study (Table 1; Fig. 1). Group 1 was assigned to receive the HPV16-SLP twice at $T = 0$ week and at $T = 3$ weeks ($n = 41$) and Group 2 was to receive PBS-placebo ($n = 10$). The study was temporarily stopped in 2009 and 2010 due to serious adverse events in one of the other HPV16-SLP clinical trials. Figure 1 shows the drop-outs at the different time points. One year after first vaccination, half of group 1 should have been re-vaccinated with the HPV16-SLP (group 1A) and the other half should have received the PBS-placebo (group 1B; Fig. 1). As vaccination with PBS is not considered immunogenic, patients in group 1A who did not receive a booster vaccination at $T = 1$ year were grouped in group 1C and analyzed together with group 1B at $T = 2$ years.

Adverse events

In total, 40 patients received one or more vaccinations with the HPV16-SLP. Of these, five patients discontinued the study pre-maturely because of adverse events four because of local adverse events, and one patient (1001) with a history of systemic lupus erythematosus who developed an acute exacerbation of cutaneous lupus erythematosus (LE) 3 days after the first vaccination. Adverse effects did not exceed grade 2 (Table 2). The most frequent systemic adverse event after vaccination was the flu-like syndrome (FLS; 26 %). Fifty-four percent of the patients experienced a second flare of systemic and/or local side effects several days (5–21 days) after the initial reaction had subsided. In the placebo group, no side effects exceeded grade 1 (Table 2). After booster vaccination with the HPV16-SLP, the flu-like syndrome (23 %) was the most frequently experienced adverse event, but it did not exceed grade 1 (Group 1A). Almost all patients in group 1 had grade 2 or 3 injection-site reactions in the weeks following vaccination with swellings beyond 8 cm (Table 2) accompanied by redness, pain and/or itching. Patients were evaluated for remaining local adverse events at 1 and 2 years after vaccination. A painless swelling was still palpable in 41 % of the patients after 1 year and in 48 % after 2 years (56 % in group 1A and 41 % in group 1B). Three patients developed an ulcer at the site of injection. The first developed the ulcer (<2 cm) 6 months after first vaccination. Wound culture revealed a secondary infection with *Staphylococcus aureus*. After 2 years, there was scarring at the site of injection. Two other ulcers developed within the second year of the study. Both were sterile ulcers, showing a granulomatous in-

Table 1 Patient characteristics and vaccination scheme

ID	Age	PHD[a]	Allergic[b] constitution	Smoking[c]		Cytology/Histology			HPV[d]			Nr of Given injections	Reason for stopping	$T=0$ SLP/ Placebo[e]	$T=1$ year SLP/ Placebo[e]	Immuno-monitoring 2 years[f]
				Yes/no	years	$T=0$ week	$T=1$ year	$T=2$ years	$T=0$ week	$T=1$ year	$T=2$ years					
1001	34	+	UK	–	15	Dysplasia/ Pap3a	*Stopped (SLE)*	*Stopped (SLE)*	82	*Stopped (SLE)*	*Stopped (SLE)*	*1*	*SLE*	1	2	–
1002												*0*		*1*	*1*	–
1003	54	+	UK	–	1.5	CIN2 (LEEP)/ Pap3a	Pap2	*NT*	81	54.74	*NT*	3		1	2	1b
1004	32	+	UK	–	0.6	CIN 2–3 (LEEP)/ Pap3a	Pap 2	*LTF*	16	44.53	*LTF*	3		2	2	–
1005	66	+	UK	–	1.5	Pap3a (no dysplasia)/ Pap1	Pap1	Pap 1	Neg	Neg	74	3		1	1	1a
1006	45	–	UK	–	0	Pap2, Pap2	Pap 1	Pap2	Neg	Neg	Neg	3		1	2	1b
1007	33	+	UK	+	19	CIN1	Pap3a (LEEP CIN1)	Pap2	31, 33, 44, (52), (54)	31, 44, 53, (54)	Neg	3		1	2	1b
1008	36	+	+	+	20	Pap3a, Pap3a	Pap2	Pap3a	31	31, 44, (33), (52), (54)	31, 33, 44, (52), (54)	3		1	1	1a
1009	27	+	+	+	17	CIN1	Pap1	Pap 1	70	16, 53, 70	70	3		1	2	1b
1010	50	–	–	+	60	Pap3a (no dysplasia)	Pap1	Pap2	52	Neg	Neg	3		2	2	2
1011	47	–	UK	+	1.5	Pap2, Pap2	Pap1	Pap1	31.44	31, 44, 35, (52), (54)	52	3		2	2	2
1012	32	+	UK	+	1.5	Pap2, Pap1, VIN, Pap3a	Pap3a	Pap 2	31.53	31	31, 33, 44	3		1	1	1a
1013	51	–	UK	–	10	Pap2, Pap2	Pap1	Pap1	Neg	Neg	Neg	3		1	1	1a
1014	41	+	UK	+	18	Pap3a, Pap3a	Pap2	Pap3b	16	16	16	3		1	2	1b
1015	42	–	UK	–	9	Pap2, Pap3a	Pap2	Pap3a	(52), 53	53	53.43	3		1	1	1a
1016	51	–	–	+	10	CIN1	Pap3b (LEEP CIN2)	*Pap1*	58	16, 74	*LSE*	*2*	*LSE*	1	1	–
1017	28	+	UK	+	2	CIN1	Pap1	Pap1	39	Neg	*NT*	3		2	2	2
1018	42	+	UK	+	18	CIN1	Pap1	Pap1	16.51	45.51	45.51	3		1	2	1b
1019	37	–	–	–	0	Pap3a, Pap3a	Pap1	Pap1	16	16	16	3		1	2	1b
1020	32	+	–	+	8.5	CIN1	Pap1	Pap1	16	16	16	3		1	2	1b
1021	39	–	–	–	12	Pap2, Pap3a	Pap1	Pap1	Neg	Neg	Ntb	3		1	2	1b

Table 1 continued

ID	Age	PHD[a]	Allergic[b] constitution	Smoking[c]		Cytology/Histology			HPV[d]			Nr of Given injections	Reason for stopping	$T = 0$ SLP/ Placebo[e]	$T = 1$ year SLP/ Placebo[e]	Immuno-monitoring 2 years[f]
				Yes/no	years	$T = 0$ week	$T = 1$ year	$T = 2$ years	$T = 0$ week	$T = 1$ year	$T = 2$ years					
1022	21	–	+	–	5	Pap3a	Pap3a (LEEP CIN2)	Pap1	16, 53	16	54	3		1	2	1b
1023	34	–	–	+	7.5	Pap2, Pap2	Pap1	Pap1	51	Neg	Neg	3		2	2	2
1024	46	–	+	+	30	CIN1	Pap2	Pap2	56	56, 59	56	3		1	2	1b
1025	43	–	–	–	0	Pap2, Pap2	Pap1	Pap1	Neg	Neg	Neg	3		1	2	1b
1026	42	–	+	–	0	Pap3a, Pap2	Pap2	Pap1	16, 51, 66	51	Neg	3		1	1	1a
1027	36	–	+	–	17	Pap2, Pap2	Pap1	Pap1	39, 52, 58	31.39	58,31/54	3		1	2	1b
1028	51	–	–	–	6	Pap1, Pap1	Pap1	Pap1	Neg	66	Neg	3		1	1	1a
1029	26	–	+	+	7.5	CIN1	Pap2	*LSE*	31	31.56	*LSE*	*2*	*LSE*	1	1	1c
1030	46	–	–	+	22	CIN1	Pap1	Pap1	35, 53, 54	35, 53, 54	52	3		2	2	2
1031	26	–	–	+	1	Pap3a, Pap3a	Pap1	Pap1	18.35	16, 53, 6	53	3		2	2	2
1032	32	–	+	–	0	CIN1	Pap2	Pap1	×	×	×	*2*	*Pregnancy*	1	1	1c
1033	45	–	+	–	0	CIN1	Pap1	Pap1	68, (39)	68	18	3		1	1	1a
1034	46	–	+	–	0	CIN1	Pap2 (LEEP CIN2)	Pap2	66.82	NT	33	3		1	2	1b
1035	49	–	–	+	11	Pap1, Pap3a (no dysplasia)	Pap3a	Pap1	Neg	Neg	Ntb	3		1	1	1a
1036	38	+	+	–	0	CIN2-VAIN-Pap3a	Pap1	Pap2	16	Neg	Neg	3		1	1	1a
1037	28	+	–	+	8.5	Pap2, Pap1	Pap1	Pap1	16	16	Neg	3		1	1	1a
1038	38	+	–	+	1	Pap2, Pap3a	Pap1	Pap1	56, (74)	Neg	*NT*	3		1	2	1b
1039												*0*		*2*	*2*	–
1041	49	+	–	–	0	Pap2, Pap3a	Pap1	Pap1	Neg	Neg	Neg	3		1	2	1b
1042	32	–	+	–	12	Pap3a, Pap3a	Pap1	Pap1	52	52	52	3		1	1	1a
1043	36	+	–	–	6	Pap3a, Pap3a	Pap2	*Study stop*	16	16	*Study stop*	*2*	*Study stop*	2	2	–
1044	42	+	–	–	0	CIN1	Pap3a	*Hysterectomy*	45	Neg	*Hysterectomy*	*2*	*Study stop*	1	2	1c
1045	39	+	–	–	0	Pap2, Pap3a	Pap2	*Pap1*	45	Neg	*LTF*	*2*	*Study stop*	1	1	–
1046	47	–	–	–	0	Pap2, Pap2	Pap3a	Pap3a	56	56	56, 66	*2*	*Study stop*	1	2	1c
1047	46	+	–	–	7	CIN1	Pap1	Pap1	16, 58	*Study stop*	*Study stop*	*1*	*Study stop*	2	2	–
1048	28	–	UK	+	2.5	CIN1	*LTF*	*LTF*	16	*LTF*	*LTF*	*1*	*LTF*	1	2	–

Table 1 continued

ID	Age	PHD[a]	Allergic[b] constitution	Smoking[c]		Cytology/Histology			HPV[d]			Nr of Given injections	Reason for stopping	$T = 0$ SLP/ Placebo[e]	$T = 1$ year SLP/ Placebo[e]	Immuno-monitoring 2 years[f]
				Yes/no	years	$T = 0$ week	$T = 1$ year	$T = 2$ years	$T = 0$ week	$T = 1$ year	$T = 2$ years					
1049	57	+	+	+	14	Pap2, Pap1	Pap1	Pap1	Neg	*LSE*	*LSE*	*1*	*LSE*	1	1	–
1050	41	+	–	+	25	Pap3a, Pap2	Pap1	Pap1	35	58	*LSE*	2	*LSE*	1	1	1c
1051	49	–	+	+	17	CIN1	*Study stop*	Pap1	31	31, 33, 44	*Study stop*	2	*Study stop*	1	2	–

Six patient did not strictly fit the inclusion criteria, yet had extensive histories of abnormal cervical lesions or clear indication of HPV infection

The italics indicate patients who did not receive all three vaccinations or missing samples

UK unknown, *SLE* systemic lupus erymatosus, *LSE* local side effects, *LTF* lost to follow-up

[a] PHD previous HPV-related disease

[b] Allergic constitution: Atopy (atopic syndrome) is a syndrome characterized by a tendency to be "hyperallergic." A person presents with one or more of the following: eczema (atopic dermatitis), allergic rhinitis (hayfever), allergic conjunctivitis or allergic asthma

[c] Smoking at the time of inclusion. 20 cigarettes/day/year is one smoking year

[d] HPV testing was done by Inno-LiPA that detects high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82), a number of low-risk HPV genotypes (6, 11, 40, 43, 44, 54, 70) and some additional types (69, 71, 74). HPV X indicates that the HPV type was not found by Inno-LiPA. Neg indicates no HPV

[e] At $T = 0$, patients were randomized to receive two vaccination with HPV16 SLP (1) or a placebo (2). At $T = 1$ year, patients in group 1 were again randomized to receive the HPV16-SLP or placebo

[f] For the immunomonitoring at 2 years patients who had received 3× HPV16-SLP were group 1a; 2× HPV16-SLP and 1× placebo group 1b; 2× HPV16-SLP vaccination followed by nothing at 1 year were allotted to group 1c and analyzed together with group 1b. Patients who had received 2 or 3× placebo were allotted to group 2

fection as seen in foreign body reactions. Ulcers took a longtime to resolve with periods of healing followed by renewed sterile drainage. In one patient, we were able to do a skin test that revealed a type IV allergic reaction to the montanide.

HPV-specific memory T-cell responses are detected at 1 year after vaccination

Blood samples were drawn before vaccination (T = 0 week), at 7 weeks (T = 7 weeks) and 1 year (T = 1 year) after the first vaccination for immunomonitoring (Fig. 2). Pre-vaccination, 35 % of the patients in group 1 displayed an HPV16-specific T-cell response against a median of two peptide pools (out of 6) as detected by IFNγ-Elispot. At T = 7 weeks and T = 1 year, this response was significantly boosted and 97 % of the patients reacted against a median of five peptide pools (P value <0.0001; Fig. 2a). In the placebo group, no HPV16-specific responses were found at T = 0 week or T = 7 weeks by IFNγ-Elispot. At T = 1 year, two patients in group 2 had developed a response (against 1 and 4 peptide pools). The proliferation assay (LST) revealed an HPV16-specific T-cell response in 49 % of the patients in group 1 against a median of two peptide pools at T = 0. After vaccination, all patients (100 %) displayed a significantly increased proliferative response against a median of five peptide pools at T = 7 weeks and T = 1 year ($P < 0.0001$; Fig. 2a). HPV16-specific cytokine production was also boosted by vaccination ($P < 0.0001$; Fig. 2a), albeit

Table 2 Safety and toxicity

	ID	Systemic toxicity[a]				Local toxicity[b]						
		1st		2nd		Booster		$T = 7$ weeks	$T = 1$ year	Booster	$T = 2$ years	
		Grade 1	Grade 2	Grade 1	Grade2	Grade 1	Grade2				Swelling[c]	Pigmentation
Group 1a	1035	3, 4	–	4	1	1	–	3	0	2	0	0
	1037	–	–	–	1	13	–	2	0	1	1	0
	1040	–	–	3	1, 2	1	–	2	1	2	Ulcer	1
	1042	11	–	–	1, 2	–	–	2	0	1	0	0
	1015	4	–	–	3	3	–	1	0	1	0	0
	1012	–	1	–	–	–	–	2	1	1	1	1
	1008	–	3	2	–	3, 7	–	2	1	1	1	1
	1005	–	–	–	–	4	–	2	0	1	LTF	
	1013	–	–	14	–	–	–	2	0	1	0	0
	1026	–	–	–	–	–	–	2	1	0	0	0
	1028	–	–	–	–	–	–	2	1	1	1	0
	1033	–	–	1	–	1, 5	–	2	0	1	0	0
	1036	–	–	–	–	–	–	3	0	1	1	0
	1016	7	–	–	1			2	2		Ulcer	
	1029	9	–	1	–			1	Ulcer			
	1032	9	–	1	–			3	1		1	1
	1045	3	–	3	–			2	0			
	1050	–	–	–	–			2	1		2	1
	1049	13	–					2	1			
Group 1b	1003	–	–	–	–	–	–	2	0		LTF	LTF
	1006	–	–	–	1, 5	1	–	3	1	0	1	0
	1007	–	–	6		–	–	3	0	0	0	0
	1009	–	–	3	–	–	–	2	0	0	0	0
	1014	–	–	–	–	–	–	2	0	0	0	0
	1018	1	–	1	6, 8	–	–	2	0	0	1	0
	1019	4	–	4	–	–	–	3	0	0	0	1
	1020	9	–	–	1	–	–	2	1	0	1	1
	1021	–	–	–	–	–	–	1	0	0	0	0
	1022	3	–	–	–	–	–	2	1	0	UK	UK
	1024	1, 9	–	9	–	–	–	2	1	0	1	0
	1025	–	–	9, 11	–	–	–	1	0	0	1	1
	1027	1, 5, 3	–	1	5, 3	4	–	2	0	0	0	0
	1034	3, 4	–	4	1	–	–	2	0	1	0	0
	1038	1	–	–	–	–	–	2	1	0	1	1
	1041	1	–	–	–	–	–	1	1	0	0	0
	1044	–	–	–	–			1	1		0	0
	1046	–	–	–	–			3	0		0	0
	1051	3	–	1	2, 8			3			1	1
	1001	Exacerbation SLE					UK					
	1048	LTF	LTF					UK				
Group 2	1004	–	–	–	–	–	–	1	0	1	LTF	LTF
	1010	–	–	–	–	–	–	0	0	0	0	0
	1011	–	–	–	–	–	–	0	0	0	0	0
	1017	–	–	–	–	–	–	0	0	0	0	0
	1023	3	–	–	–	–	–	0	0	0	0	0
	1030	–	–	–	–	–	–	0	0	0	0	0
	1031	4	–	–	–	–	–	0	0	0	0	0
	1043	10	–	10	–			0	0			
	1047	–	–					0				

Up to ID 1014, no diaries were handed out to patient

LTF lost to follow-up, *UK* unknown

[a] 1 Flu-like syndrome; 2 rash; 3 fatigue; 4 headache; 5 depression; 6 pruritis; 7 hotflushes; 8 hand and foot syndrome; 9 myalgia; 10 anorexia; 11 throat ache; 12 constipation; 13 nausea and 14 other

[b] The swelling shortly after up to 3 weeks after vaccination are noted here. If more than one vaccinations sit reacted, the biggest was recorded. Grade 0: no swelling, grade 1: <4 cm; grade: 2 ≥4 cm; grade 3: ≥8 cm

[c] Swelling <4 cm

that the levels of cytokines were low in most patients rendering the median level under the cutoff value for all cytokines except IL-5 (Fig. 2a, dashed lines). Representative data for the responses measured by these assays are shown for one patient in Supplemental figure 1. In order to define which of the peptide pools were responsible for the HPV16-SLP-induced response in group 1 patients, the response was analyzed with respect to the individual peptide pools (Fig. 2b). This showed an HPV16-specific T-cell response that was detected against all peptide pools as measured by both IFNγ-Elispot and LST, with peptide pool E6.Two being the most immunogenic, and peptide pools E6.1 and E7.2 the least. There was no association between pre-existing HPV16-specific T-cell responses and various patient characteristics: i.e., HPV16 status at $T = 0$ ($n = 11$), PHD, allergic constitution and smoking (data not shown). There was no difference in response between patients with or without an active HPV16 infection in response to the HPV16-SLP.

The occurrence of a flu-like syndrome is correlated to the strength of the HPV16-specific T-cell response after vaccination

One of the major adverse events seen was the occurrence of the flu-like syndrome in group 1. The Mann–Whitney test was used to determine the association between the FLS and the HPV16-specific response. Patients who had a FLS displayed a significantly higher HPV16-specific T-cell response by all tests after vaccination at $T = 7$ weeks than patients with no FLS ($P < 0.0001$; Fig. 3a). Interestingly, patients with FLS also displayed a stronger pre-existing proliferative response associated with the production of cytokines to HPV16. This observation was not made using the IFNγ-ELISPOT assay, probably because of the shorter assay length. The assay time of the proliferation test and associated cytokine production allows low-magnitude T-cell response to expand before measurement. The same correlations were found with the immune response to MRM, suggesting a correlation between the occurrence of FLS, a stronger response to vaccination and the overall immune status of patients (supplemental figure 2).

Re-vaccination at 1 year boosts the immune response

One year after first vaccination, 13 patients from group 1 were re-vaccinated with the HPV16-SLP (group 1A) and 16 received the PBS-placebo (group 1B; Fig. 1). Five patients did not receive booster vaccination and were grouped into group 1C and analyzed together with group 1B at $T = 2$ years (Fig. 1; Table 1, supplemental Figure 3). A significant effect of the booster vaccination was seen on the HPV16-specific responses as measured by IFNγ-Elispot and IL-5 production in the patients in group 1A (Fig. 3b). No significant increase was seen after booster vaccination on HPV16-specific proliferative responses and the associated produced cytokines IFNγ, TNFα and IL-10 compared with patients in group 1B + 1C (data not shown). Interestingly, analysis of the HPV16-specific cytokine responses revealed that patients who received a booster vaccination at $T = 1$ year maintained a Th1

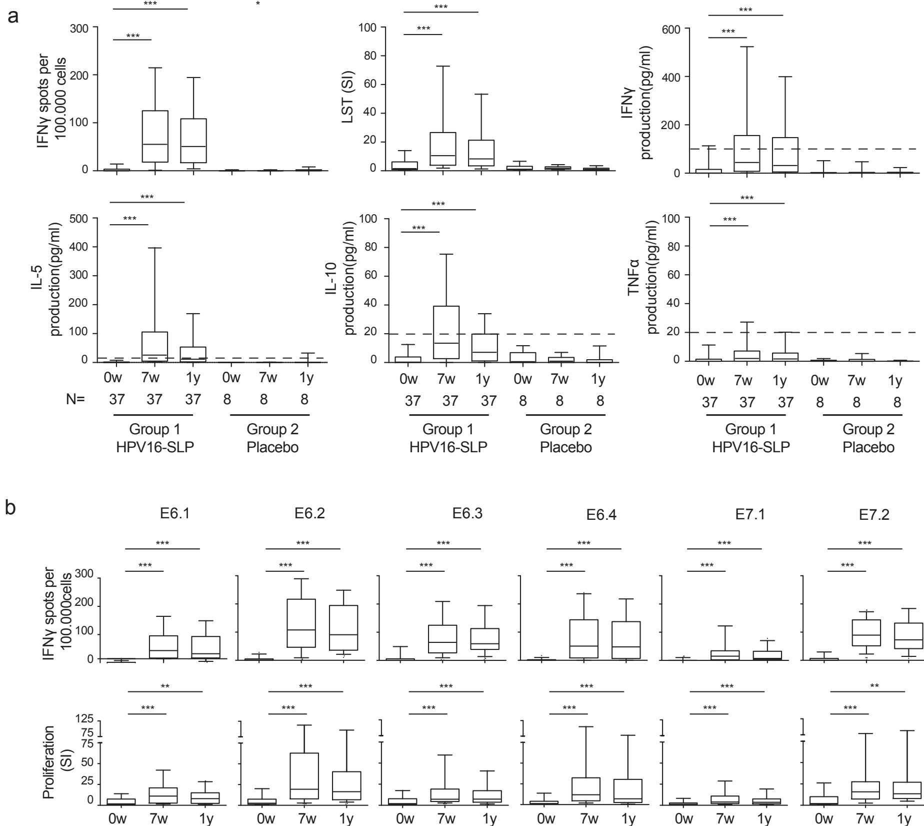


Figure 2. Strong vaccine-induced T-cell immunity was seen after two vaccinations with HPV16-SLP vaccine at 50μg/peptide. (a) HPV16-specific T-cell reactivity was determined using PBMCs before (T=0w), 7 weeks (T=7w) and 1 year (T=1y) after vaccination as determined by IFNγ-Elispot, lymphocyte stimulation test (LST) and cytometric bead array (CBA). The median (line), interquartile range (boxes) and 10-90% range (bars) of the HPV16-specific T-cell responses are shown for patients in group 1 (HPV16-SLP; n=37) and group 2 (PBS-placebo; n=8). The Wilcoxon matched-pairs signed rank test shows a significant increase after vaccination of HPV16-specific responses in all tests at T=7w and T=1y in group 1 (* 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001). In group 2 no differences in responses were seen except between T=0 and T=1y in the IFNγ-Elispot. (b) Analysis of the individual peptide pools in PBMCs from patients of group 1 shows broad responses with the greatest immunogenicity against E6.2, E6.4 and E7.2 and hardly any responses to E6.1.

response, but also started to develop a Th2 response, indicated by the increased production of IL-5. This did not occur in patients who did not receive a booster vaccination at 1 year. Patients in group 2 did not show any significant increase in any of the tests for the duration of the study (supplemental Figure 4).

Clinical and virological follow-up

Clinical and virological responses were not endpoints of this study but all patients were followed according to standard clinical practice. HPV typing was performed at three time points. At T = 1 year in group 1, 51 % (19/37) had regressed to a Pap1, 43 % (16/37) had a Pap2/3a (of whom three patients had a LEEP performed after diagnosing 2× a CIN2 and 1× a CIN1) and 3 % (1/37) had progressed to a Pap3b after two vaccinations (which after LEEP excision turned out to be a CIN2) (Table 1). In group 2, 78 % (7/9) of the patients had returned to a Pap1 and 22 % (2/9) still had a Pap2 at T = 1 year. At 2 years follow-up, 69 % (9/13) had a Pap1 in group 1A and 30 % (4/13) a Pap2/3a. In group 1B and 1C, 63 % (15/24) had a Pap1 at T = 2 years, 21 % (5/24) a Pap2/3a and 4 % (1/24) a Pap3b. Four per-cent (1/24) had undergone a hysterectomy (myoma) and 8 % (2/24) was not tested. At T = 0 year, nine patients in group 1 tested HPV16 positive. In the patients that were followed up for 1–2 years, the clearance rate at T = 1 year was 3/8 and T = 2 years 5/8. In group 2, two patients tested positive at T = 0, the clearance rate was 1/2 and 1/1 at T = 1 year and T = 2 years, respectively. For comparison, six patients in group 1 tested HPV31 positive at T = 0 and the clearance rate was 0/6 and 2/4 at T = 1 year and T = 2 years. In group 2, one patient tested positive for HPV31 at T = 0, the clearance rate was 0/1 and 1/1 at T = 1 year and T = 2 years, respectively.

DISCUSSION

In this randomized trial, patients received either a placebo or two vaccinations of a therapeutic HPV16-SLP vaccine with or without a booster vaccination after 1 year. The differences in the HPV16-specific T-cell responses detected between the patients in the vaccine and placebo groups clearly showed that the HPV16-SLP vaccine is responsible for a strong HPV16-specific T-cell response after vaccination. Furthermore, the study showed that the most immunogenic parts of the vaccine are E6 peptide pool two (amino acid 41–92), pool four (amino acid 111–158) and E7 pool two (amino acid 41–98). These regions are similar to those that are recognized by the spontaneously induced HPV16-specific T-cell response found in healthy volunteers [9, 23]. An important factor for the clinical efficacy of a therapeutic vaccine is its capacity to induce a CD4-mediated Th1 cell response [24]. The first two vaccinations augmented the HPV16-specific Th1 response and this response is still detected after 1 year, albeit that the strength of the response is somewhat lower. The group of patients receiving a booster vaccination, not only showed a threefold increase in the number of HPV16-specific IFNγ-producing T cells as detected by IFNγ-Elispot but also an increase in the HPV16-specific production of IL-5 (compare groups 1A *vs* 1B/1C). While the combination of HPV16-specific production of both IFNγ and IL-5 is commonly found in the spontaneous T-cell response to HPV infection in healthy volunteers and also in

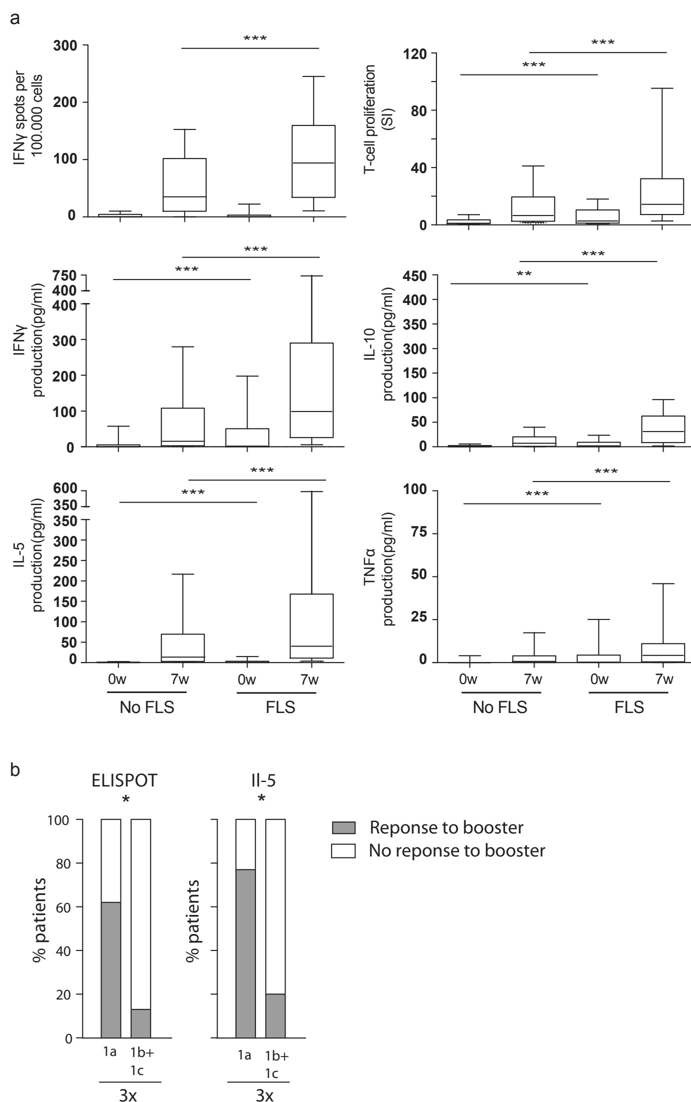


Figure 3. (a) Stronger HPV16-specific T-cell responses were seen in patients that had the Flu-like syndrome (FLS) after vaccination compared to patients with no FLS in patients of group 1. The median (line), interquartile range (boxes) and 10-90% range (bars) of the HPV16-specific T-cell response by IFNγ-Elispot, lymphocyte stimulation test (LST) and cytometric bead array (CBA) are shown for both groups. Patients with FLS had significantly stronger responses after vaccination by all tests (* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$). This difference was already seen before vaccination in the LST and CBA. (b) A booster vaccination enhanced response was defined as a threefold increase in the immune response after the booster vaccination compared to the HPV-specific immune response before booster vaccination. Booster vaccination significantly increased the response in the IFNγ-Elispot assay and IL-5 production.

the vaccine-induced response of patients clinically responding to HPV16-SLP vaccination [15, 17, 18], the specific rise in a Th2 type cytokine indicates an undesirable polarization toward a Th2 response. The addition of an adjuvant with the capacity to skew toward a Th1 response, therefore, seems warranted. Recently, IFNα has been used as adjuvant in a clinical trial in which colorectal cancer patients were injected with a p53-SLP vaccine. This trial showed that the addition of IFNα enhanced the frequency of IFNγ-producing p53-specific T-cells in vaccinated patients [25], and thus may also be used as adjuvant in HPV16-SLP vaccines.

A second concern about this vaccination scheme in patients with pre-cancerous lesions is the adverse events observed during vaccination, in particular the delayed local reactions at the vaccination sites occurring several weeks to months after vaccination. In our other studies using the HPV16-SLP and Montanide ISA51, though the adverse events did not exceed grade 2, the local injection-site reactions could be severe with large swelling formation with itching, redness and pain [14, 16, 17]. Therefore, in this study, a low-dose vaccine (50 μg/peptide) was administered with Montanide ISA51 as adjuvant. Montanide ISA 51 is not a component of any approved human vaccine, but has been used in many previous trials of candidate HIV, malaria and cancer vaccines and has been shown to cause severe injection-site reactions with occasional sterile abscess formation [26–29]. For patients with low-grade cervical disease, the short- and long-term local adverse events of the HPV16-SLP vaccine are difficult to accept [16]. To be successful in this patient group, the formulation of the HPV16-SLP should be changed in such a way that it remains effective, yet with reduction in the adverse events. A potential strategy to reach this goal is intradermal vaccination [30] or to use SLP formulations were an adjuvant is directly coupled to the peptide allowing Montanide to be omitted. Recently, Pam_3Cys-conjugated SLP were reported to be highly immunogenic and to display low toxicity at the low doses that are still effective in pre-clinical trials [31].

An interesting finding in this trial was the association between an overall higher active immune system - as based on the higher response to recall antigens - the appearance of FLS and a stronger response to vaccination. Apparently, such a profile associates with a stronger response to vaccination with more adverse events. We did not find this in our previous HPV16-SLP vaccination trials in patients with malignant disease, suggesting that this phenomenon specifically becomes apparent in patients with low-grade disease with a more alert immune system.

In conclusion, vaccination with 50 μg/peptide of HPV16-SLP induces a broad and strong immune response in patients with low-grade pre-malignant disorders of the uterine cervix. This response remains at a steady state high level for at least 2 years. A booster vaccination

after 1 year specifically increases Th2 responses. Future use of such a vaccination scheme thus may require better adjuvant to steer the immune response toward the desirable Th1 response. Notably, for this group of patients with pre-malignant lesions of the cervix, the often long lasting local adverse events make vaccination with HPV16-SLP as currently formulated inappropriate. The use of other adjuvant, peptide formulations or injection routes may overcome these problems.

ACKNOWLEDGMENTS

This study was financially supported by The Netherlands Organization for Health Research and Development (ZonMW 92003425).

Conflict of interest This study has been conducted by the Leiden University Medical Center (LUMC), which holds a patent on the use of synthetic long peptides as vaccine (US 7.202.034). Cornelis J.M. Melief and Sjoerd H. van der Burg are named as inventors on this patent. The LUMC does not share the financial benefit from this patent with its employees. Cornelis J.M. Melief has been employed part-time (75 %) since January 20, 2008, by ISA Pharmaceuticals, which exploits this long-peptide vaccine patent, and has been granted options on ISA Pharmaceuticals stock. All other authors declare that they have no conflict of interest.

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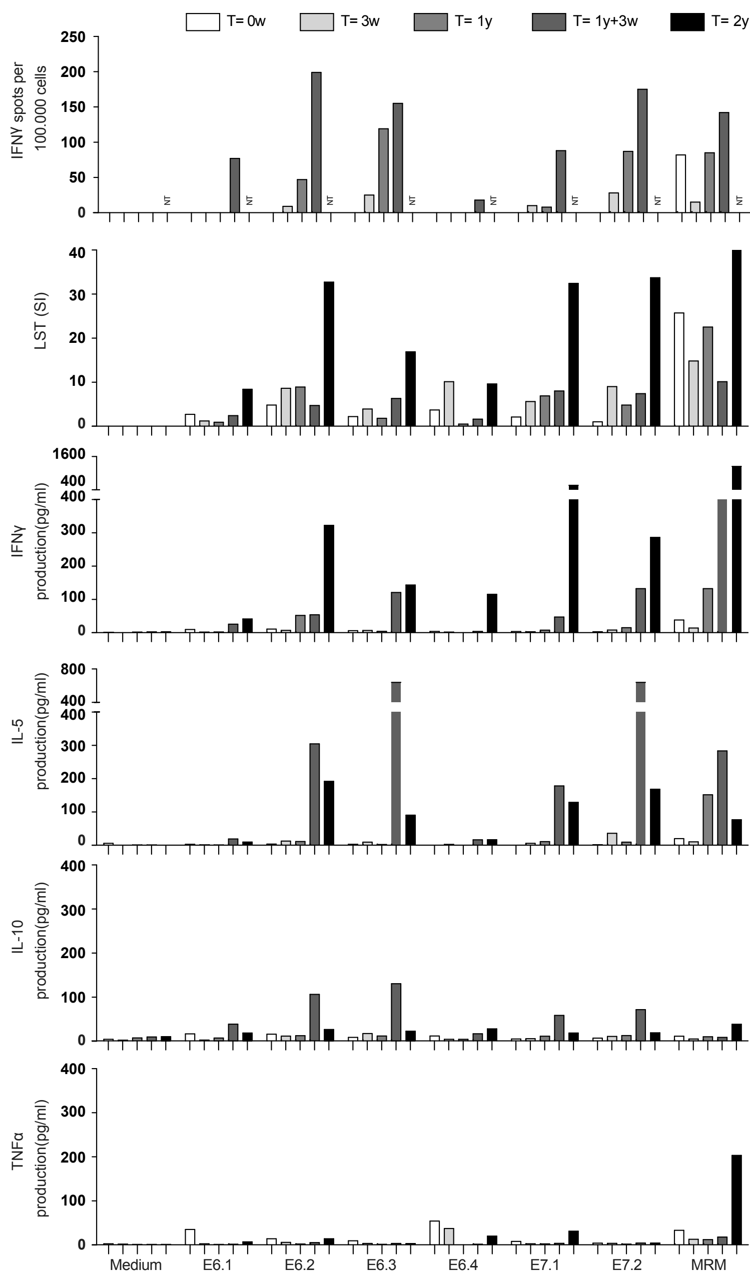
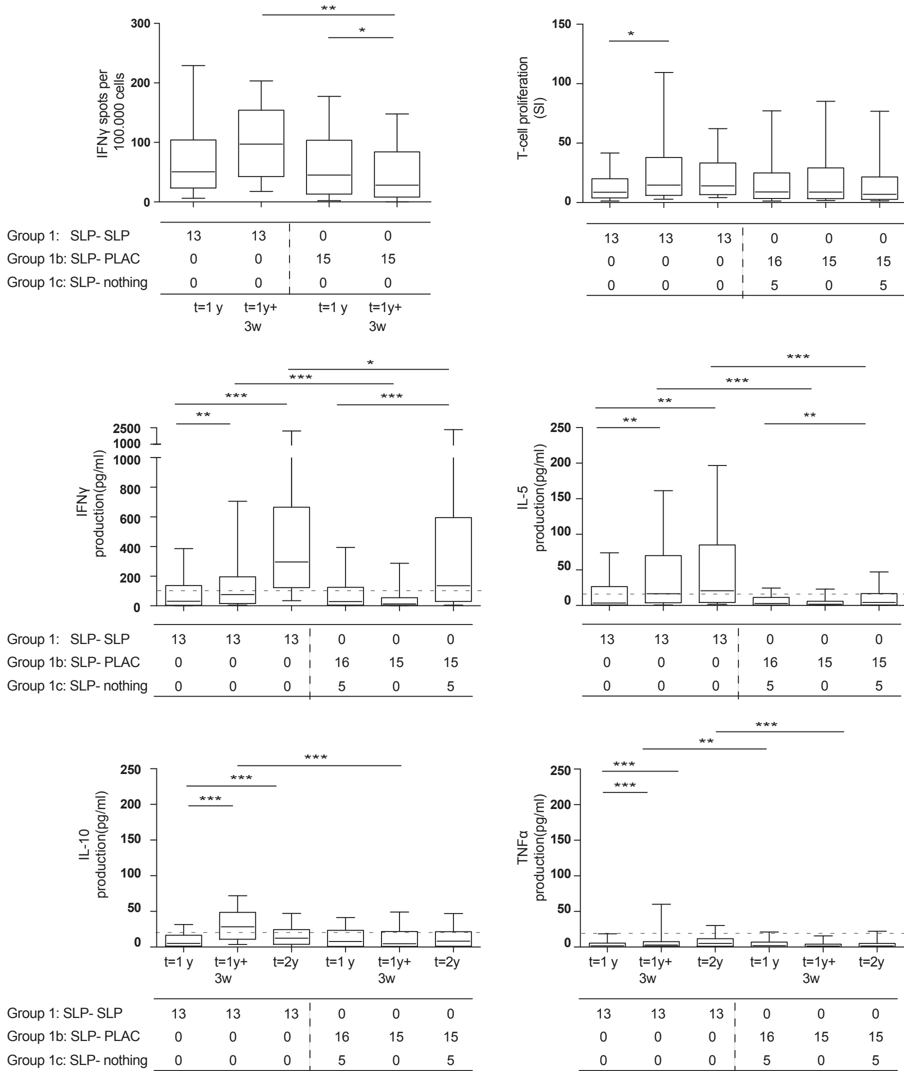
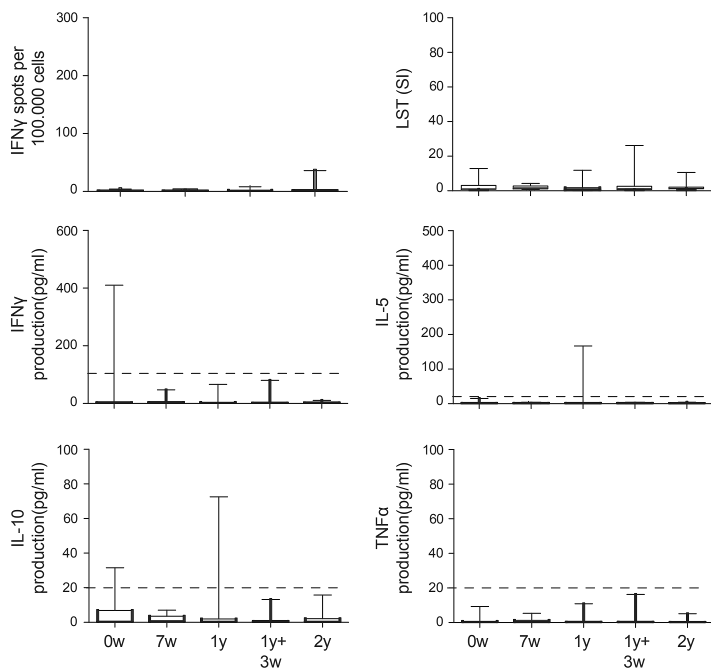


Supplemental Figure 1. Representative immunomonitoring data for patient 1026 is shown. Responses are measured by IFNγ- Elispot, proliferation assay (LST) and cytokine production (IFNγ, IL-5, IL-10 and TNFa). This patient was allotted to group 1a, receiving the HPV16-SLP twice at T=0w and T=3w and again at T=1y. A response is measured in the Elispot, LST and Il-5 after vaccination. After booster vaccination (T=1y+3w) a further augmentation (3x pre-booster vaccination) is seen in the Elispot, IFNγ, Il-5 and Il-10 assays.



Supplemental Figure 2. Box plots of the response to booster vaccination showing the median (line), interquartile range (boxes) and 10-90% range (bars) during the second year. Responses are shown for time-points T=1y, T=1y+3w and T=2y (except for the IFNγ-Elispot) for group 1A (who received the HPV16-SLP at T=0, T=3w and T=1y), Group 1B (who received the HPV16-SLP at T=0, T=3w and PBS at T=1y) and Group 1C (who received the HPV16-SLP at T=0, T=3w and nothing at T=1y). The results of Group 1B and 1C are depicted combined as both patient groups did not receive the HPV16-SLP vaccine. HPV16-specific T-cell responses are shown when measured by IFNγ Elispot, proliferation assay (LST) and proliferation associated production of IFNγ, IL-5, IL-10 and TNFα. In groups 1B and 1C a certain degree of fluctuation was seen, although significant increases in cytokine production were observed more often in group 1A after booster vaccination with HPV16-SLP.



Supplemental figure 3. The HPV16-specific T-cell response for patients in group 2, who received placebo at all time-points are depicted. No significant changes were observed in the immunological tests conducted: IFNγ-Elispot, proliferation assay (LST) or cytometric bead array (CBA) for the indicated cytokines.

Chapter 7

A placebo controlled randomized HPV16 synthetic long peptide vaccination study in women with high-grade cervical squamous intraepithelial lesions

Peggy J. de Vos van Steenwijk
Tamara H. Ramwadhdoebe
Margriet J.G. Löwik
Caroline E. van der Minne
Dorien M.A. Berends-van der Meer
Lorraine M. Fathers
A. Rob P.M. Valentijn
Jaap Oostendorp
Gert Jan Fleuren
Bart W.J. Hellebrekers
Marij J.P. Welters
Mariette I. van Poelgeest
Cornelis J.M. Melief
Gemma G. Kenter
Sjoerd H. van der Burg

Cancer Immunolgy Immunotherapy 2012; 61(9), 1485-92

ABSTRACT

The aim of this study was to investigate the capacity of an HPV16 E6/E7 synthetic overlapping long-peptide vaccine to stimulate the HPV16-specific T-cell response, to enhance the infiltration of HPV16-specific type 1 T-cells into the lesions of patients with HPV16+ high-grade cervical squamous intraepithelial lesion (HSIL) and HPV clearance. This was a placebo controlled randomized phase II study in patients with HPV16 positive HSIL. HPV16-specific T-cell responses were determined pre- and post vaccination by ELISPOT, proliferation assay and cytokine assays in PBMC and HSIL-infiltrating lymphocytes and delayed-type hypersensitivity skin tests. Motivational problems of this patient group to postpone treatment of their premalignant lesions affected the inclusion rates and caused the study to stop prematurely. Of the accrued patients 4 received a placebo and 5 received 1-2 vaccinations. Side-effects mainly were flu-like symptoms and injection site reactions. A strong HPV-specific IFNγ-associated T-cell response was detected by ELISPOT in all vaccinated patients. The outcome of the skin tests correlated well with the ELISPOT analysis. The cytokine profile associated with HPV16-specific proliferation varied from robust type 1 to dominant type 2 responses. No conclusions could be drawn on vaccine-enhanced T-cell infiltration of the lesion and there was no HPV clearance at the time of LEEP excision. Thus, vaccination of HSIL patients results in increased HPV16-specific T-cell immunity. Further development of this type of treatment relies on the ability to motivate patients and in the reduction of the side effects.

INTRODUCTION

Persisting Human Papillomavirus (HPV) infections, in particular HPV type 16 are associated with the development of (pre)cancers of the anogenital tract. The risk of progression of squamous intraepithelial lesions (SIL) of the cervix is related to the severity of dysplasia [1, 2]. Small lesions are easily treated by loop electrosurgical excision procedure (LEEP), while larger lesions often require repeated surgery for recurrences [3]. Virus-specific, interferon-γ–producing CD4+ T cells and CD8+ cytotoxic T lymphocytes are essential components in controlling chronic viral infections [4, 5]. The majority of subjects who clear HPV16 display HPV16 E6-specific CD8+ cytotoxic T-lymphocyte (CTL) responses [6, 7], and relatively robust proliferative T-cell responses against early viral proteins E2, E6 and E7, characterized by CD4+ T cells that produce Interferon-γ (IFNγ) and IL-5 [8-10]. Such IFNγ-associated T-cell responses are weak or absent in most patients with HSIL [7, 10-13].

Recently, two studies reported that therapeutic vaccination with an HPV16 E6/E7 protein or synthetic long-peptide vaccine (HPV16-SLP) resulted in the complete regression of HPV16-induced high grade lesions of the vulva [14, 15]. Clinical success correlated with the induction of strong and broad HPV16-specific T-cell responses [14-16]. Non-responders had bigger lesions [15] and increased numbers of HPV-specific regulatory T cells [14, 16]. The aim of this study was to investigate the capacity of the HPV16-SLP vaccine to stimulate the HPV16-specific T-cell response in patients with HPV16+ high grade lesions of the cervix.

MATERIALS AND METHODS

Patients and Vaccination

This was a placebo controlled randomized blinded phase II study aiming to include 34 patients, 17 in each arm. The aim of this study was to investigate the capacity of an HPV16 E6/E7 synthetic overlapping long-peptide vaccine to stimulate the HPV16-specific T-cell response, to enhance the infiltration of HPV16-specific type 1 T-cells into the lesions of patients with HPV16+ high-grade cervical squamous intraepithelial lesion (HSIL) and HPV clearance.

Patients with histologically proven HPV16+ HSIL were included after oral and written informed consent and randomized into two groups. Eligibility required pre-treatment laboratory findings of leukocytes > $3x10^9$/L, lymphocytes > $1x10^9$/L, thrombocytes > $100x10^9$/L and haematocrit > 30% and no radiotherapy, chemotherapy or other potentially immunosuppressive therapy administered within 4 weeks prior to the immunotherapy. Patients consented to HPV testing and to having an extra biopsy taken for culture of HSIL infiltrating lymphocytes at colposcopy (Figure 1a). HPV typing was done on paraffin embedded

sections of biopsies or smears as published previously [17-19]. Patients either received the vaccine at a dose of 300µg per peptide twice with a 3 week interval or a placebo, Phosphate Buffered Saline (PBS). Blood was drawn at week 0, 7 and 9-11. Both at screening and LEEP excision an extra biopsy was taken for the culture of HSIL infiltrating lymphocytes. A delayed type hypersensitivity (DTH) skin test was performed 2-4 weeks after LEEP excision. The study was approved by the Dutch Central Committee on Human Research (CCMO, https://toetsingonline.ccmo.nl/ccmo_search.nsf/ dossier number NL14015.000.06) and the medical ethical committee of the Leiden University Medical Centre and the Haga teaching Hospital. Monitoring for adverse events was performed as described previously [20] and adverse events were classified according to the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE). The vaccine consisted of a mix of 13 overlapping 25-35mer peptides representing the entire sequence of the E6 and E7 proteins of HPV16 (HPV16-SLP) dissolved in dimethylsulfoxide (DMSO) and admixed with 20 mM phosphate buffer (pH 7.5) and Montanide ISA-51. The vaccine was produced at the GMP facility of the Leiden University Medical Center (LUMC) [15, 16, 20, 21].

T-cell assays, data acquisition, analysis and interpretation

The peripheral blood mononuclear cells (PBMC) were tested for HPV16-specificity by a set of complementary T-cell immune monitoring assays including: IFN-γ-ELISPOT, lymphocyte proliferation assay (LST) and cytokine bead array (CBA), using pools of 22 amino acid long peptides, overlapping by 12 amino acids. All tests have previously been described and positive responses have been defined [22]. For all T-cell assays, a vaccine-induced response was defined as at least a 3-fold increase in the response after vaccination when compared to the results before vaccination. A semi-quantative analysis of local changes in immune infiltrate was done on haemotoxyline-eosine stained sections before and after vaccination. HSIL-infiltrating lymphocytes were isolated, cultured and tested for HPV16-specific proliferation and cytokine production as described previously [16].

The T-cell assays were performed in the laboratory of the department of Clinical Oncology (LUMC, Leiden) that operates under research conditions, following standard operating procedure (SOPs) and using trained staff. This laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (http://www.cimt.eu/workgroups/cip/), as well as in IFNγ ELISPOT panels of the Cancer Immunotherapy Consortium, which aim is to harmonize the reporting and assays used for T-cell monitoring [23-25].

Delayed-Type Hypersensitivity Skin tests

Delayed-Type Hypersensitivity reactions can be used as a sensitive and simple method for in vivo measurement of HPV-specific cellular immune responses and were used as previously described [26].

RESULTS

Vaccinations

A total of 47 patients visiting the out-patient department of two hospitals in the Netherlands gave informed consent to screening for this study between June 2007 and December 2009. Due to the anxiety of patients with a HSIL to postpone the LEEP procedure, accrual proved an obstacle. Of the 27 eligible patients only 10 consented, and one patient (placebo group) never showed up for vaccination. Within the vaccine group two patients (3006 and 3003) did not receive the second vaccination due to side effects and one (3010) due to a study stop (Figure 1b)

Safety and toxicity

Placebo patients did not display adverse reactions. As expected on the basis of our previous trials [15,20,21], all 5 patients in the vaccination group displayed adverse reactions not exceeding grade 2 according to CTCAE, and included injection site reactions with itching, redness, swelling and pain. All patients experienced swellings of more than 8 cm which lasted for several days. Systemic reactions consisted of a headache (80% of the patients); diarrhea, fatigue and/or dizziness (40% of the patients) and nausea, chills, myalgia, rash, fever, uticaria, edema of the limbs or an allergic reaction needing antihistamines (20% of the patients). Two patients (3002 and 3007) experienced stronger side effects after the second vaccination. In 4 cases there was a renewed reaction to the vaccine 5 to 14 days after vaccination consisting of increased injection site reactions with or without systemic reactions. The skin test caused mainly itching at the site of the test.

Spontaneous and vaccine-induced HPV16 E6- and E7-specific T-cell immunity

Systemic HPV16-specific T-cell reactivity from all three time points was simultaneously determined by IFNγ-ELISPOT (Figures 1b and 2a, Online resource 1). Only two of the nine patients (3006 and 3007) showed a weak pre-existing HPV16-specific immunity, one against E6 and one against E7 (10 and 11 spots per 100,000 PBMC). All vaccinated patients showed strong responses to 2-5 of the peptide pools (5/5 patients against E6 peptide pools and 4/5 against E7.2) 7 weeks after first vaccination, with reactivity up to 255 HPV-specific IFNγ producing cells per 100,000 cells (Figure 1b). Of the patients receiving placebo, 2 subjects (3004 and 3005) showed a weak IFNγ-associated HPV-specific response to one peptide pool (13 and 10 spots per 100,000 cells, against E7.2 and E6.1 respectively).

None of the patients tested, displayed an HPV16-specific proliferative response at the start of the study. Three of the four patients receiving a placebo (3001, 3004 and 3009) remained unresponsive to HPV16 E6 and E7 throughout the duration of the study (Figure 1c, Online resource 2). One patient (3005), received a placebo, yet developed a broad proliferative response after colposcopy with biopsy (week 7), which subsided after LEEP excision (week

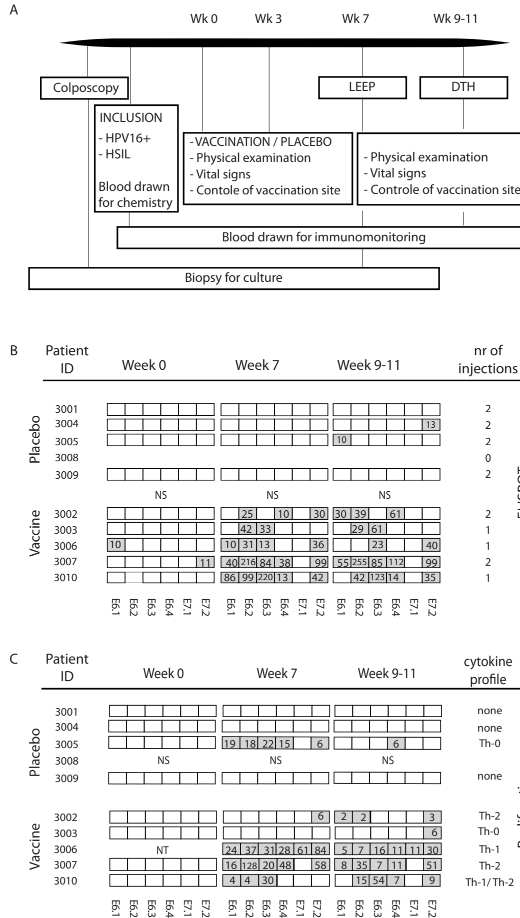


Figure 1. Schematic representation of the placebo-controlled randomized trial and summary of immunological results. (a) Patients were recruited at colposcopy visit. After informed consent, HPV testing was performed by PCR and an extra biopsy was taken for the culture of HSIL infiltrating lymphocytes. Patients with histological proven HPV16+ HSIL then consented to the vaccination study at which time blood was drawn for chemistry and base-line immunomonitoring. Patients in arm 1 received the vaccine at a dose of 300μg per peptide twice with a 3-week interval; patients in arm 2 received a placebo (PBS). Seven weeks after the first vaccination a LEEP excision was performed at which time an extra biopsy of the HSIL was taken and blood drawn for immunomonitoring. DTH skin test was performed 2-4 weeks after surgery at which time blood was drawn to measure the effect of the LEEP excision on the systemic immune response. (b) Immunological results of all the patients using PBMC from three different time-points. Week 0 (pre-vaccination), week 7 (post-vaccination) and week 9-11 (after LEEP excision). Systemic HPV16-specific T-cell reactivity against six peptide pools (4 E6 and 2 E7 peptide pools) was determined by IFNγ-ELISPOT. The boxes in grey show the number of HPV-specific IFNγ producing T-cells per 100,000 cells. (c) HPV specificity determined by the proliferation assay (LST). The grey boxes indicate the (stimulation index) SI of the HPV-specific proliferative responses. The culture at week 0 of patient 3006 was not tested due to technical problems. To the right the overall cytokine profile based on the outcome of tested supernatants of the LST by cytokine bead array (CBA) is indicated. A Th-0 response indicates weak cytokine production inconclusive for a Th-1 or Th-2 response. Patient 3008 was randomised, but never showed up for vaccination (NS= Not Started).

9-11). All 5 vaccinated patients developed an HPV16-specific proliferative response after vaccination. Patients 3006 and 3007 developed the broadest responses to 5-6 peptide pools and the other three patients responded to 1-4 pools (4/5 against E6 peptide pools and 5/5 against E7 peptide pools) at week 9-11. (Figure 1b and 2b, Online resource 2).

The supernatant of the proliferation assays was tested for the presence of HPV16-specific produced cytokines IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2. Before vaccination, no HPV16-specific cytokine production was found (Online resource 3 and figure 2c). At the time of LEEP treatment HPV16-specific IFNγ production – ranging between 146-1582 pg/mL - was found in 3 of the five vaccinated patients (3006, 3007 and 3010). Only in one patient (3006) did we find a robust T-helper type 1 response (Online resource 3). Two patients displayed a Th-2 response (3002 and 3007) with the production of IL-5 and IL-10. One patient (3003) had a weak polarisation (Th-0) with very low amounts of IL-5 production and one patient (3010) had a weak mixed Th-1 and Th-2 response producing little amounts of IFNγ, TNFα, IL-5 and IL-10 (Online resource 3). Patient 3005 who was not vaccinated had a Th-0 response with very low amounts of IFNγ against one pool and IL-5 against another, despite a broad proliferative response. This is typical for HPV16-specific immunity in patients invasively treated for a persistent or recurrent lesion [13]. IL-4 or IL-2 are most likely consumed by T cells during the culture.

Systemic immunity to recall antigens

In order to test the general immune status of the patients, the capacity of their T cells to proliferate and produce cytokines when stimulated with a mix of recall antigens (MRM) was tested. All patients, except 3002, displayed a proliferative response to MRM at all time points. MRM-specific IFNγ production was detected in the culture supernatants of patients 3001, 3003, 3005, 3006 and 3009 and in patient 3010 by IFNγ-ELISPOT. Patient 3004 failed to produce MRM-specific cytokines. Patient 3002 and 3007 produced IL-10 (33 pg/mL) and IL-5 (53 pg/mL), respectively.

Local changes in HSIL biopsies

All patients were diagnosed with HSIL before vaccination. One patient (3001) in the placebo group had two biopsies (punch and LEEP) taken before the trial because of a discrepancy between the PAP smear (Pap4) and the first biopsy (no dysplasia, the second showed a CIN2). In the LEEP specimen after vaccination no dysplasia was found. In none of the other patients was a change in the histological and viral disease status found at the time of LEEP. A semi-quantative analysis of local changes in immune infiltrate on haemotoxyline-eosine stained sections revealed a change from a scattered pattern to a dense immune infiltrate in 3 (3002, 3003, 3010) of the 5 vaccinated patients. A similar change was observed in one (3004) of the three patients in the placebo group.

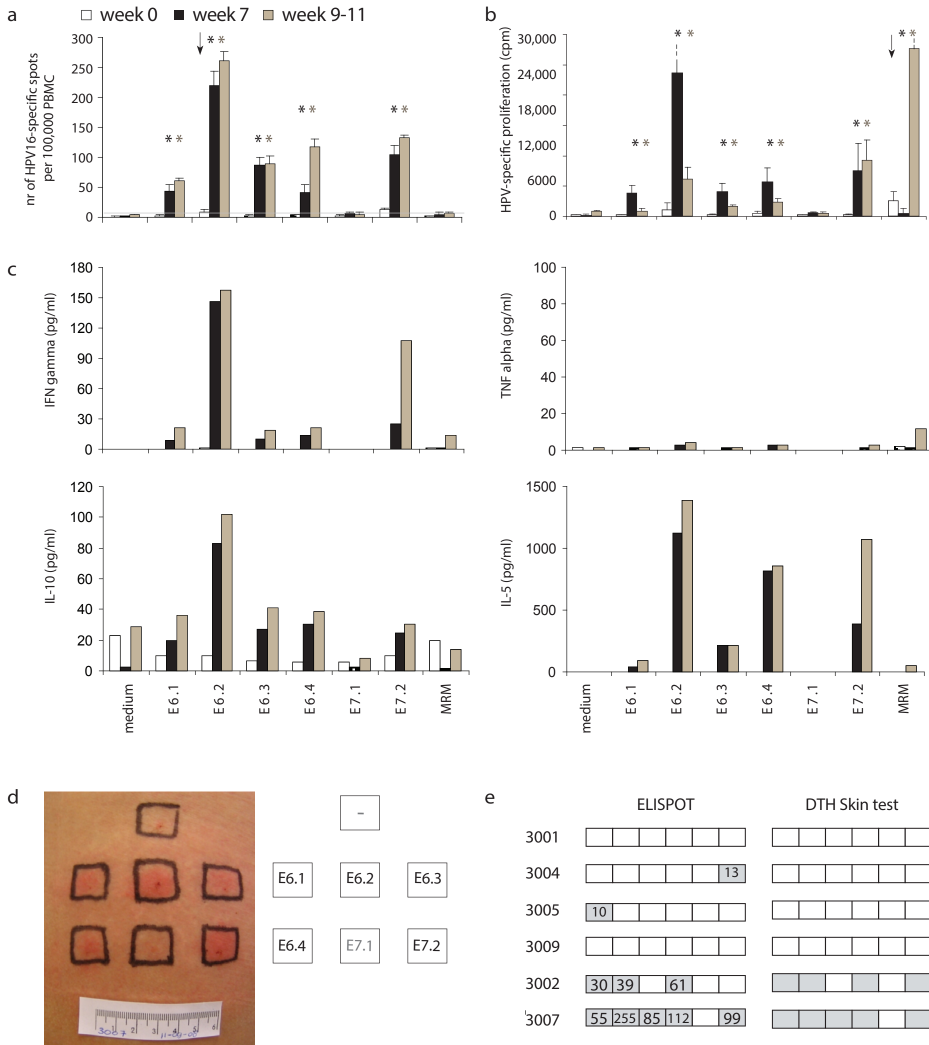


Figure 2. Example of the results from immunomonitoring. The results of patient 3007 who received two vaccinations and of whom blood was tested at week 0 (pre-vaccination; white), at week 7 (post-vaccination; black) and at week 9-11 (after LEEP excision; grey). The arrows (▼) indicate a pre-existing response and the stars (*) indicate a positive reaction during the course of the study. (a) Results of the IFNγ-ELISPOT assay. (b) Results of the proliferation assay showed no pre-excising HPV-specific reactivity. (c) Cytokine bead array (CBA) was used to test the HPV16-specific production of the indicated cytokines measured in the culture supernatants of the proliferation assay. (d) DTH results showing clear redness and swelling of sites injected with E6.1, E6.2, E6.3, E6.4 and E7.2. (e) Overview of the IFNγ-ELISPOT results compared to the DTH skin test results.

We received biopsies from all patients before vaccination for T-cell culture. After vaccination we received biopsies from 3 placebo treated patients and 3 vaccinated patients. In three of the 9 biopsies taken at the start of the study, enough T cells could be isolated to

test for the presence of HPV16-specific T cells. Only in the culture of patient 3010 was a proliferative response detected against monocytes pulsed with the combined peptide pools E6.1 and 2 as well as against monocytes pulsed with protein. After vaccination we received 6 biopsies for culture (3 from vaccinated patients and 3 from placebo treated patients). Only two cultures, both from vaccinated patients (patient 3006 and 3003) had enough T-cells to be tested. Neither showed evidence of HPV16-specificity. We did not receive a biopsy after vaccination from patient 3010 (who tested positive pre-vaccination).

Responses to an HPV16 peptide-based skin test

Skin tests, based on DTH reactions against HPV16 peptides, can be used for in vivo measurement of HPV-specific cellular immunity [26]. Patients receiving placebo showed no skin reactions. Patients 3003, 3006 and 3010 who stopped after one vaccination did not receive the skin test. Patients 3002 and 3007 showed strong DTH reactivity after two vaccinations matching the results of the IFNγ-ELISPOT assay (Figure 2de).

DISCUSSION

Therapeutic HPV vaccination is a promising strategy for HPV-induced pre-cancerous lesions and cancer as shown for patients with high grade VIN lesions by us and others [14, 15]. The aim of this study was to examine the systemic and local HPV16-specific T-cell responses after HPV16-SLP vaccination in patients with HPV16-induced HSIL. We were able to identify enough patients within 18 months, yet we experienced problems in accrual, due to patient anxiety at having to postpone standard surgical treatment. The study was extended in time, however, the accrual stayed extremely low and it was decided to stop the study prematurely. These problems have been described before in other attempts to test potential vaccines in patients with HSIL [27, 28]. Overall, the inclusion rate in this study was 19%. This was quite unexpected as the inclusion rate in our previous trials in which this vaccine was tested in patients with cancer or VIN was well over 60% [15, 20]. In contrast to patients with VIN3 - for whom treatment is mutilating, disfiguring and of which the effects are mostly transient as recurrences are high [15, 20, 29] - this is not the case for patients with HSIL as they have no symptoms of their lesion and can be treated relatively easily by surgery. The side effects including amongst others swellings of 8cm of the injection site and flu-like symptoms, were expected on the basis of our earlier trials [15, 20, 21]. However, though they did not bear much impact on the study in patients with VIN3, it did cause a high drop out of patients in this trial. This clearly shows that strong disparities in the side effects and benefit between the standard of care and new therapies may outweigh the potential benefits of newly tested therapeutic modalities and affect clinical testing.

This was the first placebo controlled trial with this HPV16 SLP vaccine. Although the numbers were small it allowed us to show that the standard care, which includes a biopsy, can induce a broad and strong HPV16-specific response. However, this response was neither associated with the production of IFNγ nor with a positive skin test. In contrast to the placebo group, all vaccinated subjects displayed a strong vaccine-induced IFNγ-associated T–cell response as measured by *ex-vivo* IFNγ-ELISPOT. This placebo-controlled trial thus sustains our notion to use the IFNγ-ELISPOT assay to determine vaccine-induced HPV16-specific T-cell reactions. The skin test assay may be an alternative as the pattern of skin reactions found in the 2 vaccinated and 4 placebo treated patients tested matched well the results of the IFNγ-ELISPOT assay, confirming our previous observations that they have quite similar detection rates [26]. Notably, IFNγ-ELISPOT reactivity correlated with clinical responsiveness in our previous study in patients with vulvar lesions [15]. Only 1 vaccinated patient (3006) showed a robust Th-1 profile at week 7 after receiving only one vaccination.

An earlier randomized blinded placebo-controlled study with E6 and E7 protein in ISCOMATRIX in HSIL patients reported stronger HPV16-specific T-cell responses in immunized subjects than in placebo recipients. No clinical effects were observed [27]. In addition, a recent report on the use of an encapsulated plasmid DNA vaccine revealed that about half of the patients mounted a transient HPV-specific CD8 T-cell response [30]. Furthermore, HSIL patients vaccinated with a MVA viral vector expressing HPV16 E6 and E7 as well as IL-2 displayed some clinical efficacy at six months but the correlation with vaccine-induced T-cell reactivity was not assessed [28].

Overall, our placebo-controlled study shows that the HPV16-SLP vaccine is capable of increasing the numbers of circulating IFNγ-producing HPV16-specific T cells in patients with HSIL. These responses can be reliably detected using a DTH skin test. Importantly, motivational problems and the local and systemic side-effects of the HPV16-SLP vaccine in HSIL patients must be taken into account when considering further studies in patients with pre-malignant lesions for whom an effective treatment is available. Future efforts should be focused on the development of a well tolerated formulation, capable of inducing strong immune responses in patients with premalignant HPV-induced disease.

ACKNOWLEDGMENTS

Financial support: This study was financially supported by grants from the Dutch Cancer Society (KWF 2006-3679) and The Netherlands Organization for Health Research and Development (ZonMW 92003425)

Conflict of interest statement: This study has been conducted by the Leiden University Medical Center (LUMC), which holds a patent on the use of synthetic long peptides as vaccine (US 7.202.034). C.J.M.M. and S.H.v.d.B. are named as inventors on this patent. The

LUMC does not share the financial benefit from this patent with its employees. C.J. M. M. has been employed part-time (75%) since January 20, 2008, by ISA Pharmaceuticals, which exploits this long-peptide vaccine patent, and has been granted options on ISA Pharmaceuticals stock. All other authors declare that they have no conflict of interest

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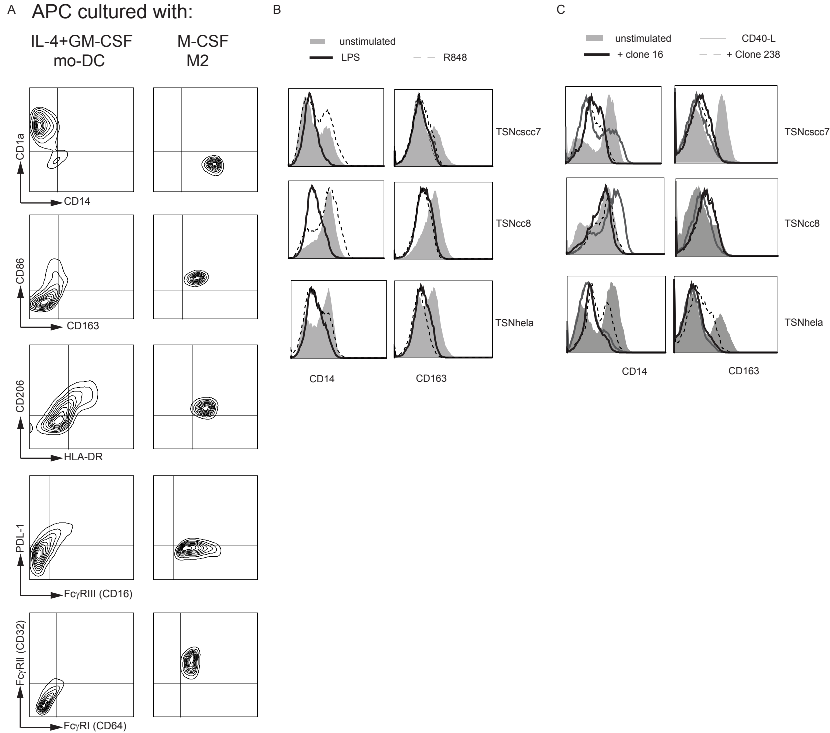
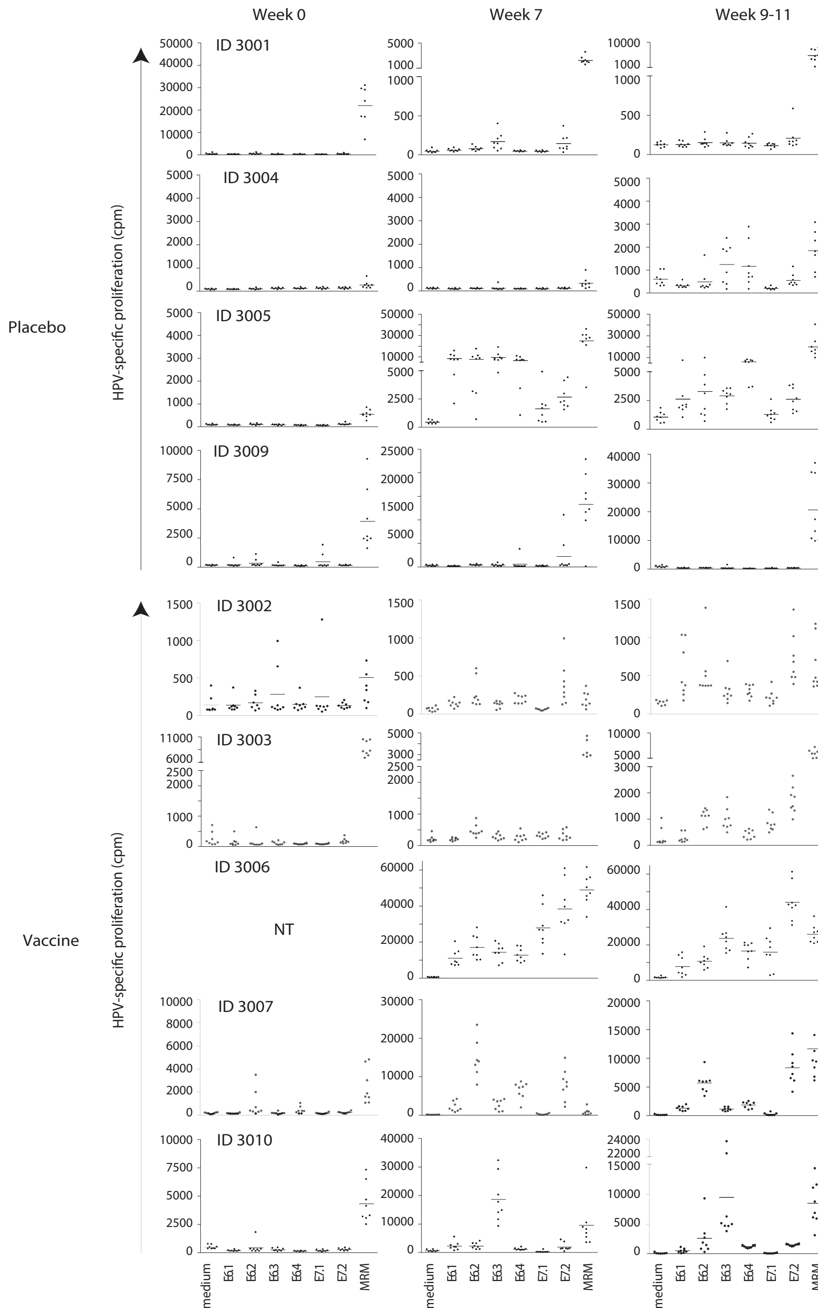
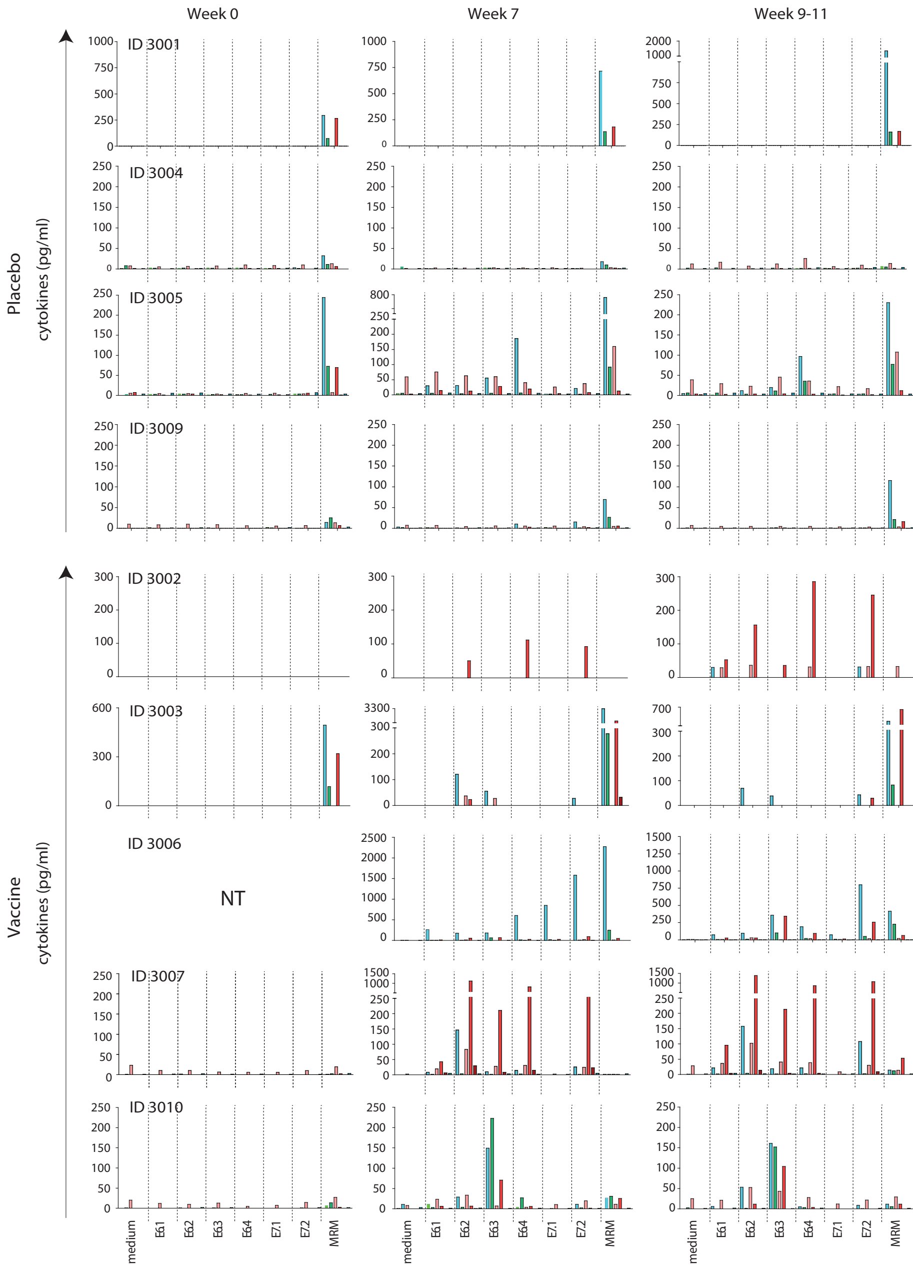


Supplementary fig 1. Phenotype of in vitro differentiated DC and M2 macrophages.
(A) Monocytes were cultured for 6 days with GM-CSF+IL-4 to obtain imm DC or M-CSF to obtain M2 macrophages and analyzed by flowcytometry for the expression of phenotypical markers. M2-differentiated macrophages typically display high expression of the scavenger receptor CD163 and the mannose receptor CD206. In contrast to monocyte derived DC M2-macrophages express all FcγReceptors (CD32, CD64 and CD16). (B+C) Stimulation of TSN-M2 for 48 hr with (B) indicated TLR-ligands and (C) Th1 cells or CD40-L cells results in a lowered expression of CD14 and CD163 as detected by flowcytometry.



Online Resouce 2. Immunomonitoring by proliferation assay (LST). The top four patients received a placebo and the bottom five patients received the vaccine. The proliferative responses were measured after 7 days of culture by [3H]-thymidine incorporation for the last 24 hours and are displayed as count per minute (CPM). The results are shown for week 0 (pre-vaccination), week 7 (post-vaccination) and week 9-11 (after LEEP excision). The data of patient 3006 at week 0 were non evaluable due to a technical problem



Online Resource 3. Immunomonitoring by cytokine bead array (CBA). Supernatants of the 7-day proliferative cultures (LST) were harvested at day 6 and subjected to CBA to measure the production of IFNγ (light blue), TNFα (dark green), IL-10 (pink), IL-5 (red), IL-4 (brown) IL-2 (dark blue). The results are shown for the three time points. The upper 4 patients received placebo the lower 5 received the vaccine. No results are available for patient 3006 at week 0.

Discussion

Future perspectives of immunotherapy in cervical cancer

Conventional therapies like surgery, radiotherapy and chemotherapy, are of essential clinical value in the combat against cervical cancer. Yet, recurrent and metastatic disease are still often incurable due to limitations of toxicity and loss of efficacy of treatment. Based on new insights, novel immune-based therapies are gaining a undisputed place in the treatment of many cancers. Understanding the mechanisms underlying the generation of tumour immunity is vital if immunotherapeutic strategies are to join ranks in standard cancer therapy. This thesis contributed to the ongoing research into the role of the immune system during the development of cervical cancer and the search for effective and innovative immunotherapeutic strategies to combat it.

Previous studies in healthy individuals show that systemic HPV16 specific T-cell responses are frequently present in the healthy population (+-80%) (1, 2) indicating that a successful defence against HPV16 infection is commonly associated with the presence of a systemic effector T-cell response against these viral antigens. Our studies revealed that the HPV-specific T cell response is different at multiple levels in patients with HPV induced disease (systemically and locally), varying from no response, or a dysfunctional response, to an anti-inflammatory response in the presence of Tregs. In patients with cervical cancer, HPV16 specific systemic immune responses are detected in only about half of all patients (3) and are predominantly not associated with the production of pro-inflammatory cytokines. We found that this immune failure against HPV starts earlier and is already present at the time of pre-cancerous disease (HSIL) (chapter 2). We found an HPV 16 specific proliferative T-cell response against HPV16 in a minority of patients, and similar to cervical cancer patients, these T-cell responses mostly lacked a pro-inflammatory signature.

Tumours can mediate systemic and local effects in their effort to escape the immune system. Circulating HPV-specific lymphocytes have to home to the lesion and overcome the often hostile anti-inflammatory tumour microenvironment. Previous *in situ* immunohistochemical studies suggest that CD8+ T cells fail to migrate into the tumour cell nests and when they did this it usually coincided with the infiltration of regulatory CD4+ T cells (4). Yet immunohistochemistry cannot define the tumour specificity of these infiltrating lymphocytes. TIL cultures suggest that only 50-60% of the HPV16 or 18-positive cervical carcinoma, and 4 out of 7 HSIL, contain no HPV specific T cells in the tumour or its draining lymph nodes ((5) chapter 2). When HPV specific T cells were found they comprised not only HPV-specific effector cells but also HPV-specific regulatory T cells ((6); chapter 2). In the face of developing therapeutic vaccination strategies, it is important to gain a better and more complete understanding of the local tumour environment and the preexisting local anti-tumour response. This led to our in depth analysis of the spontaneous local tumour-specific immune response whereby we dissected the HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells (chapter 3). We discovered that large polyclonal

repertoires of HPV-specific T cells can be present in tumours. At that time, we called these T-cells poised and non-functional yet in retrospect many tumours do contain HPV-specific T cells that seem well polarized and are able to produce IFNγ and IL-2 when stimulated *ex vivo* with their cognate antigen. This again shows the diversity of local HPV-specific responses varying from no HPV-specific T-cells, to regulatory T-cells, to dysfunctional or poised T-cells. Overcoming this immune failure is the key to successful immunotherapy and we found that the effector function of infiltrating T-cells could be enhanced when cultured in the presence of TLR ligands, such as PAM3CSK4 or poly(I:C) (chapter 3). The use of immune activating compounds, like TLR ligands, could have beneficial effects for therapeutic strategies. Imiquimod, which is used in the clinic for the treatment of genital warts and VIN, is an immune response modifier which triggers the TLR7 receptor. In patients with VIN the majority that responded to treatment had a pre-existing systemic HPV specific T-cells response (7). Although the local HPV specific T cell response was not investigated it deems plausible that this pre-existing HPV-specific T-cell response was activated in response to treatment.

It is clear that the tumour microenvironment is a precarious balance between tumour cells, infiltrating immune cells and the cytokines they produce. One of the hallmarks of cancer is the influx of myeloid cells which can be found infiltrating tumours in great numbers. In chapter 4 we aimed to improve our current understanding of the local microenvironment in cervical carcinomas. We focused on the constitution of tumour-infiltrating myeloid cells (TIM) and their relationship to other tumour-infiltrating immune cells, tumour characteristics and the disease-specific survival of patients with cervical cancer. Extensive literature demonstrates that high numbers of TAM facilitate tumour growth, disease progression and poor prognosis in various cancer types as reviewed by Heusinkveld et al (8). However, in CxCa, TAMs were never associated with clinical parameters or clinical outcome (9-11). Quantification of myeloid cell populations revealed a large variety between patients in type and amount of TIM, varying from no myeloid cells, to an abundance of mature or immature M1 and M2 macrophages. Analysis showed that a strong intraepithelial infiltration of matured M1 macrophages (CD14+CD33−CD163−), is associated with significantly improved disease-specific survival and is an independent prognostic factor as determined by multivariate analysis. Moreover, analysis with the CTL/Foxp3 ratio revealed a substantial increase in survival in the group of patients with tumours displaying dense intraepithelial matured M1 macrophage infiltrate and a high CD8+/Foxp3+ T-cell ratio. This work shows the importance of taking the whole tumour microenvironment into account and offers a profound insight on the important role of myeloid cells in the microenvironment and how they can work side by side with T cells to control tumours. Extensive studies in colorectal cancer have accentuated the importance of the number, function and location of infiltrating immune cells in the tumour microenvironment, leading to the development an immune score with a strong correlation to survival (12-14). Indeed in colorectal tumors B and T

cell infiltration can be linked to the production of IL-15, which in turn can be traced back to chromosomal changes in the tumor, underling the fact that multiple mechanisms can be involved in the creation of the tumour microenvironment. We succeeded in finding immunological fingerprints for cervical cancer by performing unsupervised clustering using 40 different immune parameters of the tumour microenvironment. The main determinants for a better survival were the presence of matured M1 macrophages and a high CD8+/Foxp3+ T-cell ratio, both independent prognostic factors. We found that the tumour-infiltrating T-cells are less able to exert a proper antitumour effect within a tumour microenvironment that does not allow the accumulation of high numbers of M1 macrophages. Patients with a better survival often have tumours that are infiltrated with relatively high numbers of M1 macrophages and displayed a high CD8/Treg ratio.

In view of our findings, selective inhibition of M2 macrophages together with the stimulation of M1 macrophages would seem a possible beneficial therapy in cervical cancer. In order to explore this possibility we analysed the effect of tumour cells on myeloid cell differentiation in order to explore the possibilities of reprogramming the abundantly present M2 macrophages toward an M1 phenotype. Our vitro analysis in chapter 5 shows that cancer cells can hamper DC differentiation and function and can induce M2-like macrophages by the production of IL-6 and PGE2. Blocking these two cytokines during the differentiation period of the monocyte prevented the differentiation of monocytes to M2 macrophages. Furthermore, fully polarized M2 macrophages could switch to M1 macrophages when interacting with Th1 cells. A combination therapy consisting of COX inhibition, IL-6 blocking, and the induction of a strong Th1 T cell response could be a promising form of immunotherapy for the treatment of CxCa. COX-inhibiting drugs and mAbs to IL-6 receptor are used in the clinic for treatment of autoimmune diseases and further exploration of their use in the treatment of cancer is underway in our group. HPV-specific Th1 T-cell responses can be elicited by therapeutic vaccination.

Vaccination is a powerful method to induce humoral and cellular adaptive immune responses. The treatment of cancer strongly depends on the activation of antigen-specific CD4 and CD8 T cells with the ultimate aim of destroying the tumour cells. The capacity of the immune system to combat cancer is shown by the approval of a cell-based vaccine for the treatment of prostate cancer (Provenge) and an aspecific T-cell stimulating therapeutic antibody for the treatment of melanoma (Ipilimumab). Synthetic peptide vaccines were initially developed in order to elicit tumour-specific CTL responses and consisted of short peptides. These early vaccine were able to elicit CTL responses able to protect against tumour challenge in preclinical studies, yet in time after an initial expansion phase could result in functional deletion of the antigen-specific T cells, leading to enhanced tumour outgrowth (15). The discovery that long peptides prevented tolerance induction, induced CD4 helper responses and increased the diversity of the anti-tumour response, which could help reduce selective tumour antigen-loss during treatment (16), led to the development of

an HPV16 overlapping long synthetic peptide vaccine consisting of the whole length of the oncogenic proteins E6 and E7 emulsified in Montanide (17). Montanide ISA-51 is a water in oil emulsion that is frequently used with peptide vaccines as adjuvants. It's main function is a depot formation which inhibits immediate systemic bio-distribution of the peptides (which can lead to the cytokine release syndrome) and improves uptake by APCs. The group previously reported that vaccination with HPV16-SLP (25–35 amino acids) was highly immunogenic in end-stage cervical cancer patients (18, 19) and could result in complete and durable regression of human papilloma virus-induced premalignant lesions of the vulva by induction of a strong and broad multifunctional CD4 and CD8 T-cell reaction (20, 21).

Our first placebo-controlled study was in a group of patients with HSIL (CIN3 trial). Vaccination in patients with precancerous lesions has a distinct advantage as the development of cancer can be prevented. The study was set up to evaluate the local response after vaccination, but we ran into motivational problems in this patient group for whom there is a treatment available. Furthermore the patients experienced considerable systemic (flu-like symptoms) and local side effects (redness, swelling, itching and pain) which caused drop-out leading to premature closure of the study. The side effects had been expected as they were seen in our previous trials, yet in this group they seemed more severe and were less well accepted in the light of the fact that patients with a HSIL experience no symptoms of their lesions and have an available therapy. However, this was the first placebo-controlled trial with the HPV16-SLP vaccine and although the numbers were small, it allowed us to show that vaccination compared to the standard care, which includes a biopsy, can induce a broad and strong HPV16-specific response associated with the production of IFNγ, as measured by ex vivo IFNγ-ELISPOT and it showed that the responses are detectable by a skin test.

Our second randomized controlled trial included patients with low grade lesions of the cervix (CIN1 trial). The purpose of this trial was to study the long term memory response to the vaccine. In addition, as in this trial a lower vaccine dose was used it also allowed us to evaluate if this would lead to less side-effects without loss of immunogenicity. The differences in the HPV16-specific T-cell responses detected between the patients in the vaccine and placebo groups clearly showed that the lower dose HPV16-SLP vaccine is responsible for a strong HPV16-specific T-cell response after vaccination. This response, still detectable after one year, was boosted by re-vaccination. However a rise in Th2 type cytokines were seen, possibly warranting the addition of a polarizing adjuvants. Though less severe than in the HSIL group, the side-effects found is some patients were still difficult to accept. Especially the delayed local reactions at the vaccination sites, which included sterile abscesses, occurring several weeks to months after vaccination are cause for concern. Montanide ISA 51 is not a component of any approved human vaccine, but has been used in many previous trials. Recently more reports have been published outing concern about severe injection-site reactions with occasional sterile abscess formation (22-24) These side-effects

may be acceptable in the treatment of patients with (recurrent) cancer, yet to patients with pre-clinical lesions they are unacceptable. Based on these data, new clinical trials have been set up to test the immunogenicity of the HPV16-SLP when injected intradermally without montanide, and a trial in which the vaccine peptides are conjugated to a TLR2 ligand and injected intradermally.

So what is the way forward for immunotherapy in cervical cancer? Our and other studies on the mechanisms underlying the generation of anti-tumour responses and the immune evasion by tumours have underscored that multiple mechanisms restrain the host's immune system to rise to the challenge of combating the tumour. On the other hand favourable immune profiles have been highlighted that need boosting in order to keep the balance in favour of tumour eradication. Therapy should combine various synergistic approaches, and old and new therapies should be used side by side in order to enhance vaccination efficacy and counteract tumour suppression. In order to boost the T cell response to CxCa one should put further effort in the combination with better adjuvants, delivered separately or conjugated to a vaccine. Continued research in pre-clinical and clinical settings within our group is investigating the possibilities of using HPV16 SLP vaccination in combination with other adjuvants such as IFNα (25) and Imiquimod (TLR 7), or a TLR2 ligand conjugated to the HPV16-SLP (unpublished data). Based on a study where healthy individuals showed an HPV 16 specific T-cell response after placement of a skin test, intradermal injection or delivery by tattoeage are being further investigated as a possibility of avoiding the use of Montanide. Preliminary data within our group show that tocilizumab, used in the treatment of rheumatoid arthritis, can functionally block the IL-6 receptor in patients with ovarian cancer. As cervical carcinoma, similar to ovarian cancer, produces IL-6 which is linked to a worse survival (26, 27) further research to its use in cervical cancer patients is warranted. Current studies within our group show alterations in the number and phenotype of circulating and local myeloid cells in both an animal model for CxCa and in patients with CxCa when compared to controls. The current standard therapy (carboplatin + paclitaxel) normalizes the myeloid cell population as well as synergizes with therapeutic vaccination (Welters & van der Sluis & van Meir, in preparation) Alternatively, one could enhance the number of tumour-specific T cells via adoptive cell transfer of ex-vivo cultured tumour-infiltrating lymphocytes as we showed in chapter 3. Current studies show it is feasible and a phase 1 clinical trial is being discussed. Other possibilities lie in the blocking of inhibitory receptors. In cervical carcinoma PD-1 is brought to expression in about half of the infiltrating CD8 T cells (28, 29), suggesting that the blocking of PD-1 or its ligand PD-1L could have therapeutic benefits. Agonistic antibodies to co-stimulatory receptors can also be considered. We showed that CD40 can stimulate a shift from M2 to M1 in the presence of IFNγ. Combining a monoclonal antibody to CD40 with a vaccine or other standard treatments *e.g.* surgery, radiation or chemotherapy are being investigated in clinical trials in patients with melanoma, haematological malignancies, pancreatic and prostate cancer as

reviewed by Khong et al (30) and VonderHeide et al (31) and should be further investigated in cervical cancer.

Our ongoing research has led to new insights into the role of the immune system in HPV induced disease and to various immunotherapeutic options which are being tested in pre-clinical and clinical trials. A future of possibilities lies ahead, all new immunotherapeutic strategies and combinations of therapies need extensive and accurate exploration as to dose optimisation, interaction, timing of delivery and feasibility. We should proceed with optimism, yet with great care.

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ENGLISH SUMMARY

Cervical Cancer

Cervical cancer is preceded by pre-malignant dysplastic changes in the epithelium known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). Affected cells comprising of more than one-third of the epithelium are called CIN 2/ 3 or high-grade SIL (HSIL). HSIL is caused by persistent infection with the human papillomavirus (HPV), a DNA virus infecting the basal cells of the cervical epithelium. There are over 100 types of HPV, which can be divided into low-risk (non-oncogenic) and high-risk (oncogenic) HPV. HPV is the most common sexually transmitted virus with a lifetime risk of infection of 80%. In the majority of cases infection is controlled after approximately 2 years, but persistence of infection occurs in about 10% of the women. These women are at risk of developing CIN, but other areas can be infected as well (vagina, anus, vulva, penis and/or head and neck) with risk of progression to invasive squamous(adeno)carcinoma. The HPV genome importantly encodes the two oncoproteins, the early antigens 6 (E6) and 7 (E7), which are expressed in HSIL and tumour cells which are required for the onset and maintenance of the malignant transformation.

Immunology

Keratinocytes are the first line of defense against invading pathogens. They express among other pathogen receptors, Toll-like receptor (TLR)-9, which can recognize HPV DNA. Activation of TLR9 should lead to activation of NF-kB, which results in the up regulation of proinflammatory cytokines (e.g. GM-CSF, IL-1b, TNF-a, IL-10, Il-12) and chemokine's, inducing the migration and the activation of antigen-presenting cells (APC's) e.g. Langerhans cells (LC's), Dendritic cells (DC's) and macrophages. Cues in the microenvironment will evoke APC to differentiate and migrate to the local lymphoid organs in order to present antigens to locally present naive T cells. Depending on different co-stimulatory or -inhibitory molecules and cytokine production (e.g., IL-12 or IL-10), a T-cell response will be induced which may comprise various CD8+ cytotoxic T cells (CTLs), CD4+ helper T cells (Th cells) and/or CD25+FoxP3+ regulatory T cells (Tregs). As HPV proteins are foreign to the body they should be able to trigger a strong immune reaction when presented in the cervical epithelium.

In the circulation of healthy individuals HPV-16 specific Th1, Th2 cells and CTLs are often detected against HPV. The importance of the adaptive immune system is shown by the high incidence of HPV infections, HSIL and cervical cancer in immune suppressed individuals. A broad HPV specific T-cell response, consisting of Th1, Th2 and CTLs, seems desired for viral control, yet in patients with HPV induced disease this response is often not present. It deems logical that restoration of this profile is one of the aims of immunotherapeutic strate-

gies. Failure of immune system to control infection is reflected by the fact that only one third of cervical cancer patients display a detectable systemic HPV-specific response against HPV, and when it is present, the response is generally not associated with the production of IFN-γ and consists of mostly Th2 cells, non-polarized T cells or Tregs. In the course of HPV infection to cervical cancer, various factors seem to play an important role. Pathogen recognition receptor (PPR) signaling in keratinocytes is suppressed, human leukocyte antigen (HLA) expression (important for the recognitions of cancer cells by lymphocytes) is down regulated, immunosuppressive cytokines are produced, Tregs are induced, while Th cells and CTLs may be rendered dysfunctional via the expression of co-inhibitory molecules. Furthermore, APC's are hampered in their function.

Immunotherapy of cancer

With all evidence pointing in the direction that failure of the immune system leads to the development of cervical cancer, restoration of an effective anti-tumour immunity seems the logical way forward. Various modalities have been developed with limited success, affecting often a minority of patients with progressive disease. As discussed above a multitude of mechanisms can be responsible for the tumours escape from the hosts' immune system. Therefore successful immunotherapy probably lies in multiple therapeutic strategies aiming at the enhancement of immune-mediated tumour destruction as well as simultaneously counteracting the tumour-induced immune suppression. Three main modalities have been developed to achieve this goal, therapeutic vaccination, antibody or cytokine therapy and adoptive cell transfer, but it is likely that this arsenal will increase tremendously in the coming years.

This Thesis

In the face of developing therapeutic vaccination strategies, it is of vital importance to gain a better understanding of the local tumour environment and the pre-existing local anti-tumour response. This thesis firstly investigates the natural immune response against HPV in patients with (pre-) cancerous lesions of the cervix with an emphasis on the tumour microenvironment. The last two chapters describe two clinical trials in which patients with pre-cancerous lesions of the cervix are vaccinated with an HPV16 E6/E7 synthetic overlapping long-peptide vaccine (HPV16-SLP).

In Chapter 2 we show that failure of the immune system is already present in a pre-cancerous phase. Only a minority of the patients with HPV16+ HSIL have an IFNγ associated HPV16 specific T cell response. Proliferative responses were found more often, especially in patients with persisting infections after previous surgical treatment. Moreover, we showed that these premalignant lesions could be infiltrated with HPV-specific Tregs. The results of this chap-

ter formed a good basis for the design and interpretation of immunotherapeutic vaccine approaches as treatment modality for HPV-induced in CIN.

In Chapter 3 we comprehensively analyzed the spontaneous tumour-specific immune response in patients with cervical cancer. We describe a large repertoire of HPV-specific T cells present in the tumour and lymph nodes of cervical cancer patients, whereby we distinguished four different cytokine signatures. This work shows lymphocytes can be isolated and cultured from tumour tissue for use in adoptive cell transfer.

A tumour is surrounded by stroma which is made up of, among others, fibroblasts, blood and lymph vessels and various immune cells. The tumour cells, immune cells and the cytokines they produce form a precarious balance, which is called the tumour microenvironment. The focus of Chapter 4 was to further improve our knowledge of the local microenvironment. We show an important role for myeloid cells, whereby, a strong intraepithelial infiltration with CD14+CD33-CD163- myeloid cells is associated with a large influx of intraepithelial T lymphocytes and an improved disease-specific survival. Furthermore we identified various immune fingerprints by analyzing 40 different immune parameters in the tumour microenvironment which are associated with the survival of patients. Chapter 5 further investigates the influence of human cervical cancer cells on myeloid cell (monocyte) differentiation. It shows that cancer cells can either hamper monocyte to DC differentiation, or skew their differentiation towards M2-like (immune suppressive) macrophages. Blocking studies revealed that M2-differentiation is caused by tumour-produced PgE2 and IL-6. Furthermore, upon CD40 activation or interaction with Th1 cells, these tumour-induced M2-macrophages could be switched back to activated M1-like macrophages. Blocking of Il-6 or PGE2 (through COX inhibitors) together with vaccination aimed at the induction of HPV specific Th1 cells could be beneficial in changing the tumour microenvironment towards an pro-inflammatory tumour rejecting profile.

The last two chapters describe two clinical studies testing the HPV16 synthetic long peptide vaccine (SLP) in patients with CIN. Chapter 6 describes a placebo controlled randomized phase II study in patients with HPV16 positive high grade CIN (CINII/III). The aim of the study was to investigate the capacity of the HPV16-SLP vaccine to stimulate the HPV16-specific T-cell response and to enhance the infiltration of HPV16-specific type 1 T-cells into the lesions and HPV clearance. Vaccination of HSIL patients resulted in increased Th1 HPV16-specific T-cell immunity, yet encountered problems of accrual and unacceptable local side-effects. Chapter 7 investigates the capacity of a low dose of HPV16-SLP vaccine to induce an HPV16-specific T-cell response in patients with low grade abnormalities of the cervix, to determine the long term memory response after vaccination and to evaluate the need and potency of a booster vaccination after one year. We concluded that two low

dose injections of HPV16-SLP can induce a strong and stable HPV16-specific Th1 T-cell response that lasts at least for 1 year. The booster injection resulted in increased Th2 responses. In order to further develop the HPV16-SLP in this group of patients it is essential to reduce the side-effects.

The future of immunotherapy in cervical cancer

So what is the way forward for immunotherapy in cervical cancer? Our and other studies on the mechanisms underlying the generation of anti-tumour responses and the immune evasion by tumours have underscored that multiple mechanisms restrain the host's immune system to rise to the challenge of combating the tumour. On the other hand favorable immune profiles have been highlighted that need boosting in order to keep the balance in favor of tumour eradication. Therapy should combine various synergistic approaches, and old and new therapies should be used side by side in order to enhance vaccination efficacy and counteract tumour suppression. Continued research in pre-clinical and clinical settings within our group is investigating the possibilities of using HPV16 SLP vaccination in combination with other adjuvants such as IFNα and Imiquimod, or a TLR2 ligand conjugated to the HPV16-SLP. Furthermore intradermal injection or delivery by tattooing are being further investigated as a possibility of avoiding the use of Montanide. Preliminary data within our group show that Tocilizumab, can functionally block the IL-6 receptor in patients with ovarian cancer. IL-6 is produced in cervical cancer affecting myeloid cell populations, further research to its use in cervical cancer patients is warranted. Preliminary results within the group shows that the current standard therapy (carboplatin + paclitaxel) for cervical cancer, normalizes the myeloid cell population as well as synergizes with therapeutic vaccination. Alternatively, one could enhance the number of tumour-specific T cells via adoptive cell transfer of ex-vivo cultured tumour-infiltrating lymphocytes as we showed in chapter 3. Current studies show it is feasible and a phase 1 clinical trial is being discussed. Other possibilities lie in the blocking of inhibitory receptors. In cervical carcinoma PD-1 is brought to expression in about half of the infiltrating CD8 T cells, suggesting that the blocking of PD-1 or its ligand PD-1L could have therapeutic benefits. Agonistic antibodies to co-stimulatory receptors can also be considered. We showed that CD40 can stimulate a shift from M2 to M1 in the presence of IFN-γ. Combining a monoclonal antibody to CD40 with a vaccine or other standard treatments *e.g.* surgery, radiation or chemotherapy should be further investigated in cervical cancer.

Our ongoing research has led to new insights into the role of the immune system in HPV induced disease and to various immunotherapeutic options which are being tested in preclinical and clinical trials. A future of possibilities lies ahead, all new immunotherapeutic strategies and combinations of therapies need extensive and accurate exploration as to dose optimization, interaction, timing of delivery and feasibility.

NEDERLANDSE SAMENVATTING

Immunologie en Immunotherapie van premaligne afwijkingen en kanker van de baarmoederhals

Baarmoederhalskanker

Baarmoederhalskanker (het cervixcarcinoom) wordt veroorzaakt door een virus, het humaan papillomavirus (HPV). Er zijn meer dan 100 verschillende soorten HPV ingedeeld in laag-risico (niet-oncogene) virussen en hoog-risico (oncogene) virussen. Voordat er sprake is van een maligniteit, kan er jaren lang sprake zijn van premaligne veranderingen in het epitheel van de baarmoedermond, beter bekend als Cervicale Intra-epitheliale Neoplasie (CIN). HPV is de meest voorkomende seksueel overdraagbare aandoening, ongeveer 80% van alle vrouwen zal ooit geïnfecteerd raken. De meerderheid van de infecties zal binnen twee jaar geklaard worden, echter in 10% van de gevallen zal het virus persisteren, met kans op het ontstaan van CIN. Het HPV brengt twee oncogene eiwitten tot expressie (E6 en E7). Deze eiwitten, die aanwezig zijn in alle hooggradige CIN (CIN II/III) en kankercellen, zorgen voor een maligne transformatie van de cellen, maar kunnen ook als markers op de cellen gebruikt worden om de kwaadaardige cellen te herkennen tussen de omliggende gezonde cellen.

Immunologie

Het immuunsysteem beschermt het lichaam tegen virussen, bacteriën en parasieten, maar ruimt ook afvalstoffen en zieke cellen (zoals kankercellen) op. Keratinocyten (epitheel cellen) zijn de eerstelijns verdediging tegen indringers doordat ze receptoren tot expressie brengen (Toll-like receptoren, TLR) die pathogenen, zoals HPV, kunnen herkennen. Als ze in aanraking komen met een pathogeen wordt de TLR geactiveerd, wat een cascade in de cel teweegbrengt, met als doel de productie van allerlei afweer stimulerende cytokinen (b.v. GM-CSF, IL-1b, TNF-a, IL-10, IL-12) en chemokinen die zorgen voor het aantrekken van antigeen-presenterende cellen (APC's) o.a. Langerhans cellen (LC), Dendritische cellen (DC's) en Macrofagen. De APC's reageren op signalen in de omgeving en zullen daardoor op een bepaalde manier differentiëren en migreren naar de lymfeklieren, om daar de antigenen te presenteren aan naïeve T-cellen (lymfocyten). Afhankelijk van co-stimulatoire of inhibitoire moleculen (bv IL-12 vs. IL-10) zal een T-cel reactie geïnduceerd worden bestaande uit CD8+ cytotoxische T-cellen (CTLs), CD4+ helper T-cellen (Th cellen) en/ of CD25+FoxP3+ regulatoire T-cellen.

Het belang van een goede T-cel respons in de verdediging tegen HPV, wordt evident bij immuun gecomprimeerde personen, waar een hoge incidentie van CIN II/III en baarmoederhalskanker wordt gevonden. In de circulatie van gezonde individuen wordt vaak een breed HPV specifiek Th1, Th2 en CTL immuun respons gevonden wat gepaard gaat

met de productie van pro-inflammatoire cytokinen (bv Interferon (IFN-)γ). Een breed HPV-specifiek respons lijkt essentieel om het virus onder controle te krijgen. Het falen van het immuunsysteem om HPV onder controle te krijgen wordt gezien in patiënten met baarmoederhalskanker, waarbij maar in een derde van de patiënten een systemische HPV-specifieke T-cel respons tegen de HPV E6 en E7 wordt gevonden. Een minderheid van die responsen gaat gepaard met de productie van IFN-γ en word er voornamelijk Th2 cellen, niet-gepolariseerde cellen en regulatoire T cellen gevonden.

In de loop van een HPV infectie zijn er meerdere mechanismen die een rol lijken te spelen in het ontwijken van het immuunsysteem. Zo wordt in keratinocyten de cascade na activatie van TLR onderdrukt. Het HLA expressie (humaan leukocytenantigen, belangrijk voor de herkenning van kanker cellen door, en de activatie van lymfocyten) wordt onderdrukt en er worden immunosuppressieve cytokinen geproduceerd. Regulatoire T-cellen worden geactiveerd terwijl Th cellen en CTLs dysfunctioneel worden gemaakt door de expressie van co-inhibitoire moleculen. Tot slot worden de APC's ondermijnd in hun functie.

Immunotherapie bij kanker

Het falen van het immuunsysteem kan dus leiden tot het ontstaan van kanker. Logischerwijs is het herstellen van een goed immuun profiel één van de belangrijke doelen van immunotherapeutische strategieën. Verschillende therapieën zijn in de loop van de jaren ontwikkeld, vaak met gelimiteerd succes, waarbij maar een kleine gedeelte van de patiënten baat lijkt te hebben. Dit zou dit kunnen liggen aan het feit dat er meerdere mechanismen betrokken bij het ontwijken van de tumor aan het immuunsysteem (tumor escape). Een succes ligt dan waarschijnlijk ook in een gecombineerde aanpak waarbij meerdere therapeutische strategieën worden gecombineerd. Enerzijds moet het immuun-gemedieerd tumorvernietiging bevorderd worden en anderzijds moet het tumor-gemedieerde immuun suppressie uitgeschakeld worden. Er zijn grofweg drie immunotherapeutische strategieën: therapeutisch vaccineren, antilichaam- of cytokinetherapie en adoptieve T cel therapie. De verwachting is dat het aantal strategieën in de komende jaren sterk zal toenemen.

Dit proefschrift

In de zoektocht naar effectieve en innovatieve immunotherapeutische behandelingen van baarmoederhalskanker, is het essentieel om meer te weten over de bestaande anti-tumorale immuniteit in het bloed en in de tumor zelf. Dit proefschrift draagt bij aan lopend onderzoek naar de rol van het immuunsysteem in het ontstaan van baarmoederhalskanker met nadruk op het lokale tumor micromilieu en een verdere ontwikkeling van een therapeutisch vaccin.

In Hoofdstuk 2 laten we zien dat het falen van het immuunsysteem reeds in de premaligne fase begint. Slechts een kleine minderheid van de patiënten met HPV16 positieve CIN II/

III laesies, heeft een IFN-γ geassocieerde immuunrespons tegen HPV16. Dysfunctionele proliferatieve responsen worden vaker gevonden bij patiënten met persisterende infectie na chirurgische behandeling. Ook hebben we HPV16 specifieke regulatoire T-cellen kunnen kweken uit CINII/III laesies. Dit onderzoek laat zien dat er ruimte is voor immunotherapie bij premaligne afwijkingen van de baarmoedermonden en het heeft de basis gelegd voor het ontwikkelen van een therapeutisch vaccin voor patiënten met CIN.

Hoofdstuk 3 beschrijft een uitgebreide onderzoek naar de lokale HPV16 E6 en E7 specifieke T-cellen, gekweekt uit baarmoederhalstumoren en de drainerende lymfeklieren. Grote repertoires aan HPV specifieke T-cellen kunnen aanwezig zijn die ingedeeld kunnen worden in verschillende cytokine profielen. De cellen zijn goed te geïsoleerd en te kweken en zouden gebruikt kunnen worden in adoptieve T-cel therapie voor patiënten met baarmoederhalskanker.

Een tumor is omgeven door een stroma opgebouwd uit o.a. fibroblasten, bloed- en lymfevaten en immuun cellen. Er bestaat een precaire balans tussen tumor cellen, infiltrerende immuun cellen en de cytokinen die geproduceerd worden. Dit wordt het tumor micromilieu genoemd. In hoofdstuk 4 brengen wij het tumor micromilieu verder in kaart bij patiënten met baarmoederhalskanker. Wij laten een belangrijke rol zien voor myloide cellen. Intraepitheliale CD14+CD33-CD163- myloide cellen worden geassocieerd met een sterkte intraepitheliale toename van T cellen en een verbeterde overleving van de patiënten. Tevens brengen we 40 bekende immuun parameters van het tumor micromilieu in kaart, waarbij we verschillende immuun profielen vinden, die geassocieerd zijn met de overleving van patiënten. In hoofdstuk 5 gaan we dieper in op het tumor micromilieu en laten we zien hoe baarmoederhalskanker cellen de myeoide cellen kunnen beïnvloeden door de differentiatie van monocyten tot DC te verstoren tot anti-inflammatoire macrofagen (M2). Wij identificeren twee cytokinen, geproduceerd door de kankercellen, die hiervoor verantwoordelijk zijn (PgE2 en Il-6). Wij zijn vervolgens op zoek gegaan naar manieren om dit proces weer terug te draaien en laten zien dat dit bereikt kan worden door het blokkeren van PgE2 en IL-6, maar ook door CD40 activatie door interactie met geactiveerde T cellen. Bij patiënten met baarmoederhalskanker zou het blokkeren van Il-6 en/of PgE2 (door COX-inhibitoren) samen met de inductie van sterk geactiveerde T cellen (door bv. vaccinatie) kunnen leiden tot een veranderde pro-inflammatoir tumor micromilieuIn de laatste twee hoofdstukken beschrijven we twee klinische studies, waarin een HPV16 synthetisch lange peptide (SLP) vaccin in een Montanide adjuvant, getest wordt bij patiënten met CIN. Hoofdstuk 6 beschrijft een placebo gecontroleerde fase II studie waarin het HPV16 SLP wordt getest bij patiënten met een CIN II/III. Het doel van de studie was de immuniciteit van het vaccin in deze patiëntengroep, de capaciteit van het vaccin om T cellen te laten migreren naar de laesie en de klaring van het virus. Vaccinatie van patiënten met CIN II/III liet een sterke

toename in Th1 HPV16 specifieke immuniteit zien, echter stuitte het onderzoek op inclusie problemen en onacceptabele bijwerkingen ter plekke van de vaccinatie plek. Hoofdstuk 7 onderzoekt de capaciteit van een lage dosering van het HPV16 SLP om een HPV16 specifieke response te initiëren in patiënten met een afwijkend uitstrijkje of CIN I. Ook wilden we de lange termijn respons en de noodzaak tot een herhaling van het vaccin na 1 jaar te bekijken. Aan de hand dit onderzoek kunnen we concluderen dat twee lage doseringen van het HPV16 SLP een sterke immuunrespons kan induceren en dat deze respons langer dan een jaar intact blijft. Het revaccineren na 1 jaar initieerde voornamelijk een Th2 respons. Om het HPV16 SLP vaccin verder te ontwikkelen in deze patiënten groep is een reductie in de lokale bijwerkingen essentieel. Lopend onderzoek zal moeten uitwijzen of het intradermaal injecteren van het HPV16 SLP, eventueel geconjugeerd aan TLR2 zonder Montanide, de bijwerkingen kan verminderen zonder verlies van effectiviteit.

De toekomst van immunotherapie bij baarmoederhalskanker?

Ons onderzoek benadrukt dat er meerdere mechanismen zijn die voorkomen dat het immuun systeem erin slaagt een tumor te bestrijden. Wij laten echter ook zien dat er gunstige immuun profielen bestaan die versterkt of nagebootst kunnen worden om het balans weer in het voordeel van tumor destructie te drijven. Het antwoord tot succes ligt waarschijnlijk in het gebruik van meerdere synergistische therapieën en het combineren van oude en nieuwe therapieën, om de effectiviteit te vergroten en immuun suppressieve werking van de tumor tegen te gaan. Preklinische en klinische studies binnen onze onderzoeksgroep richten zich op het optimaliseren van het HPV16 SLP vaccin en het ontwikkelen van nieuwe therapieën. Om de bijwerkingen van het vaccin te verminderen en de effectiviteit te vergroten, wordt het gebruik van andere adjuvanten bekeken (bv IFNα, Imiquimod, TLR2) en andere methodes van vaccineren (bv tatoeage) ontwikkelt. Een onderzoek in patiënten met eierstokkanker laat zien dat Tocilizumab de productie van Il-6 kan onderdrukken. Gezien het effect van Il-6 op myeloide cellen, beschreven in hoofdstuk 5, moet dit verder onderzocht worden in patiënten met baarmoederhalskanker. De standaard chemotherapie tegen baarmoederhalskanker is Carboplatin en Taxol. Recente resultaten van onze onderzoeksgroep laten zien dat behandeling met Carboplatin en Taxol de myeloide populaties in patiënten normaliseert en dat het een synergistische werking heeft op het HPV16-SLP vaccin. Een ander alternatief is het versterken van het aantal tumor specifieke T cellen door middel van adoptieve T cel therapie, zoals beschreven in hoofdstuk 3. Een fase 1 studie wordt momenteel opgezet. Stimulatie van CD40 in het bijzijn van IFN-γ kan een shift van M2 naar M1 cellen teweegbrengen, zoals beschreven in hoofdstuk 5. Een monoclonale antilichaam tegen CD40, gecombineerd met een vaccin of standaard therapieën zoals chirurgie, chemotherapie of radiotherapie, moet verder onderzocht worden in patiënten met baarmoederhalskanker. Een andere mogelijkheid is het blokkeren van inhiberende receptoren bijvoorbeeld PD-1. PD-1 is een immuun inhiberende receptor dat wordt gevonden op de helft van de tumor

infiltrerende CD8+ T cellen in baarmoederhalskanker. Het blokkeren van PD-1, of zijn ligand PD-1L, zou therapeutische consequenties kunnen hebben voor patiënten met baarmoederhalskanker.

Ons onderzoek geeft nieuw inzicht in de rol van het immuun systeem in HPV geïnduceerde ziekten en in verschillende immunotherapeutische opties die verder worden uitgewerkt in lopende studies. De toekomst van de immunotherapie staat voor ons open. We dienen alle nieuwe behandelingen, en combinaties van behandelingen, nauwkeurig te testen op optimale dosering, interactie, timing en haalbaarheid.

LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
APC	Antigen-presenting cells
CD	cluster of differentiation
CIN	Cervical intraepithelial neoplasia
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CxCa	Cervical cancer
CXCL	chemokine (C-X-C motif) ligand
DC	Dendritic cells
E	Early antigen
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HPV	Human papillomavirus
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
L1	Late antigen
LC	Langerhanscells
M1	Macrophage type 1
M2	Macrophage type 2
mAbs	Monoclonal antibodies
MICA	MHC class I chain related molecule A
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKG2D	natural-killer group 2, member D
PD-1	Program death 1
PD-L	Program death ligand
PPR	Pathogen recognition receptor
SIL	Squamous intraepithelial lesions
SLP	Synthetic long-peptide
TAM	Tumour associated macrophages
TGF	Transforming growth factor
Th	T helper
TIL	Tumour infiltrating T cells
TIM	Tumour infiltrating myeloid cell
TIM-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T cells
UCHL1	Ubiquitin carboxyl-terminal hydrolase L1

AUTHOR AFFILIATIONS

From the Department of Clinical Oncology, LUMC
Burg van der S. H.
Doorduijn E.M.
Goedemans R
Ham van J.J
Hall van T.
Heusinkveld M.
Hulst van der J.M.
Loof N. M.
Minne van der C. E.
Piersma S.J.
Ramwadhdoebe T. H.
Stynenbosch L. F. M.
Welters M. J. P.

From the Department of Gynecology, LUMC
Berends-van der Meer D. M. A.
Kenter G. G. (*Present Address:* Center of Gynecologic Oncology Amsterdam)
Löwik M. J. G.
Poelgeest van M. I. E.
Vos van Steenwijk de P.J.

From the Department of Pathology, LUMC
Fleuren G. J.
Gorter A.
Jordanova E.S.
Kuijjer M.L.
Osse E. M.

From the Department of Clinical Pharmacology and Toxicology, LUMC
Fathers L. M.
Oostendorp J.
Valentijn A. R. P. M.

From the Department of Immunohaematology and Blood Transfusion
Melief C. J. M.

From the Department of Obstetrics and Gynecology, Haga Teaching Hospital, The Hague
Hellebrekers B. W. J.

From the Department of Obstetrics and Gynecology, Centrum Haaglanden, the Hague
Kagie M. J.
Nooij L.

ISA Pharmaceuticals, Leiden
Melief C. J. M.

ACKNOWLEDGEMENTS

A lot of people have contributed to this thesis to whom I owe many thanks. Clinical trials and translational research cannot be performed without optimal cooperation between departments and people. Building bridges between the laboratory and clinical practice is essential, challenging and inspiring.

With special thanks to:
Sjoerd van der Burg, for your fast mind, action and scientific wit. Your matter of fact way of thinking, your focus and dedication have taught me a lot. It is because of you I started this thesis and because of you I finished it.

Gemma Kenter, for creating the terms and conditions that have brought me where I am.

Mariëtte van Poelgeest, your passion and dedication to your patients, your precise way of working and perseverance are true examples to me.

Katja Jordanova, master of so many traits. Nothing is impossible for you, where there is a will there is a way.

D5 Clinical Oncology. A top team working together towards a communal goal for patients with cancer. The past years I have had the pleasure of being part of that team. I have learned so much from you and, for somebody who had to learn how to hold a pipette, there was a lot to learn. So many of you have contributed to the work in this thesis and all of you have contributed to a great time. It always feels a bit like coming home on the D5.

Tamara Ramwadhdoebe, side by side we shared years of facsing, culturing, pulsing, ficolling, freezing, planning, Elispotting and LST-ing. Followed by even more time working out the results again and again..... and yes again! This thesis would not be without your help.

Margriet Löwik, Dorien Berends-van der Meer and Anneke Martins, without people like you there would be no clinical trial. Your commitment to the patients is fantastic.

Department of Pathology, for the invaluable contribution that *in vivo* work can bring and for all your help.

Ellen Hiemstra and Sabrina van den Tillaart for being great colleagues, for sharing laughter, frustration, scientific highs and lows. Edith van Esch, with whom I can share this crazy passion for immunotherapy. We have been through a lot together, hopefully we will continue

to do so in the future. All other colleagues of the Jay-seven and further, for all the scientific 'kroketten' lunches, meetings at Den Haspel and other 'conferences'.

My mother, how to thank someone who has given so much. You are the haven to which the ship can always return.

Egbert for giving me space to follow my heart, for trust, for humour, for family and most of all for love. As Kahlil Gibran wrote: the oak tree and the cypress grow not in each other's shadow.

And finally with thanks to Jaap, Martijn and Tygo without whom this book would probably have been finished a lot sooner.

CURRICULUM VITAE

Peggy Jacqueline de Vos van Steenwijk was born in Stamford USA on the 6th of March 1978. When she was one year old she moved with her family to Arnhem in the Netherlands. In 1982 the family went to live in France where Peggy attended the British School of Paris for four years after which she moved to Brussels, Belgium where she first attended the Prinses Juliana Dutch School and later the European School of Brussels, where she obtained the European Baccalaureate in 1996. Peggy chose to return to the Netherlands to study Medicine at the Erasmus University in Rotterdam where she completed her theoretical medical studies and then spent five months undertaking research at the Department of Surgery. Subsequently she spent six months working pro bono in a hospital in Tamil Nadu and travelling through India before returning to Rotterdam to complete her medical degree finishing with a final internship at the Queen Elisabeth Central Hospital in Blantyre, Malawi, Africa.

Peggy started work as an intern at the Bronovo Hospital in The Hague and left after one year to undertake research in Paris. She spent one and a half years at the Department of Clinical Immunology at the Institut Curie in Paris on a Mayer Rothschild Grant. Here she became interested in tumor immunology and started working on HPV induced pre-cancerous lesions and cervical cancer. Upon her return to the Netherlands in 2006 she began her training in Gynaecology at the Bronovo Hospital and the Leiden University Medical Center (LUMC). After she was awarded a ZonMW AGIKO grant, she committed to a 3 year PhD course at the Clinical Oncology and Gynaecology departments at the LUMC. Since then her work in clinical Gynaecology and PhD research have alternated each other.

Peggy lives in the Hague, is married to Egbert Huizing and they have three beautiful sons together Jaap, Martijn and Tygo.

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