STUDIES ON LOCAL APC AND HPV-SPECIFIC T CELLS AS PRELUDE TO THE IMMUNOTHERAPY OF HUMAN TUMORS

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CHAPTER 1

Introduction

1 INNATE AND ADAPTIVE IMMUNE CELLS

Our cellular immune system consists of an innate part that quickly responds to environmental cues and an adaptive component that is shaped and fine-tuned during immune responses and expands upon the presence of antigen. The first part is represented by antigen presenting cells (APC) that pick up antigen and signals from the environment and instruct T cells that belong to the second part; the adaptive compartment.

1.1 T cells

T cells express the T-cell receptor (TCR, CD3) that recognizes antigen in an MHC-dependent fashion. T cells develop in the bone marrow and by re-arrangement of the TCR genes, random specificities of the TCR are generated. Subsequently the cells are selected for recognition in the thymus. T cells that are able to recognize MHC and therefore express a functional receptor with a useful specificity get a survival signal but the cells that too strongly recognize self-antigens (and are potentially harmful for the body) are programmed to die by apoptosis. Alternatively, CD4+ T cells with weak affinity can differentiate into regulatory T cells (natural Tregs, expressing transcription factor FoxP3). After selection naïve CD4+ and CD8+ T cells enter the blood and cycle through the body between blood and lymph nodes (LN).

Upon their first encounter with antigen in the periphery, T cells develop in distinct functional phenotypes, depending on the second and third signal. Classically the CD8+ T cells were designated as the cytotoxic T cells (CTL), producing IFNγ, TNFα and granzymes to directly kill infected or transformed target cells. However, recently data emerged that also helper CD8+ T cells (TC2) and regulatory CD8+ T cells exist that suppress other T cells in infectious disease as well as in tumors [1,2].

CD4+ T cells have been named 'helper' cells in the past but are now known to comprise a number of distinct subsets. Their main characteristics are secreting cytokines and expressing CD40-L on their surface upon antigen recognition. Main functions of the CD40-CD40-L interaction are the class-switch in B cells and activation of APC. However keratinocytes also express CD40 and can interact with CD4+ T cells [3,4]. The CD4+ T cells are defined by their function and recently by the expression of transcription factors [5], although the latter is mostly defined in mice. Whether these factors are as strictly defining the subsets in humans too is currently under investigation. The 4 most studied CD4+ T cell subsets are shown in figure 1. Type one helper T cells (Th1) are mainly characterized by high T-bet expression and the secretion of pro-inflammatory IFNγ and -to a lesser extent- TNFα upon activation. Type 2 cells are characterized by the expression of gata-3 and production of IL-5 and IL-4. Their main function is activation and the induction of class-switching in B cells. Th17 cells were later discovered and the main characteristic of these cells is their production of IL-17 upon activation. Th17 cells maintain barrier function at mucosal sites but are also found to play a role in several autoimmune diseases and can be isolated from several human cancers (reviewed by [6]). The fourth distinct T cell subset are the regulatory T cells (Tregs), which are either directly derived from the thymus (natural Tregs) or are induced in the periphery (induced or adaptive Tregs). Natural Tregs express high CD25 and display constitutively the transcription factor FoxP3. Adaptive Tregs may or may not express FoxP3. They explore a number of mechanisms to suppress other T cells or APC. High expression of the IL-2 receptor (CD25) enables them to consume IL-2, depleting this source for other T cells. Also the secretion of suppressive cytokines like IL-10 and TGFβ directly

inhibits cells. Furthermore the expression of CD39 and CD73 generate adenosine mediated immune suppression and also direct killing of DC is described by granzyme A or B. Van der Burg *et al* showed that also cells that do not express FoxP3 and that produce IFNγ can suppress other T cells, underlining the heterogeneity and plasticity of T cells [7,8].

1.2 APC instruct T cells

T cells are instructed by APC that develop out of the myeloid lineage. CD14+ myeloid cells enter the tissue and develop into specialized antigen presenting cells (APC) the type of which depends on the local microenvironment [9]. The major myeloid populations that develop are Langerhans cells (LC) that reside in the epidermis of the skin, dendritic cells (DC) that reside in the dermis and in most tissues and are highly specialized in antigen presentation to T cells, and macrophages that also reside in all tissues and respond quickly to local inflammatory mediators.

DC and LC pick up antigen and environmental cues in the periphery and travel via the lymph towards lymph nodes (LN) where they can present the antigens and instruct T cells. Antigen presentation by non-activated APC results in T-cell tolerance. If APC are activated in the tissue by the recognition of pathogens or inflammatory cytokines they will initiate an adaptive T-cell response by activating T cells that recognize the presented antigen. These T cells will proliferate and travel to the periphery.

All APC express MHC class I and II on the cell surface. In these molecules small parts of inside the cell or environmental derived antigens are presented as peptides. MHC class I

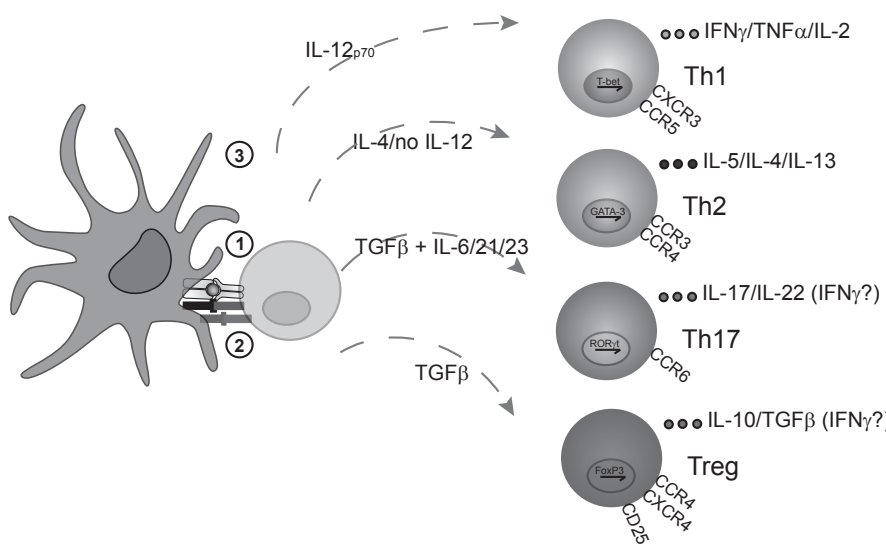

Figure 1. APC-T cell interaction and major CD4+ T cell subsets. APC present antigen to naïve T cells and provides 3 signals: 1) peptide presentation in the MHC molecule. 2) Co-stimulatory molecules interact with stimulating or inhibitory receptors of the T cells. Activated T cell expresses CD40-L (CD154) and hereby ligates CD40 which activates APC. 3) APC secretes cytokines that direct T-cell differentiation. 4 major T-cell subsets are shown on the right with the most important cytokines secreted by the APC that induce these subsets. Associated transcription factors that are expressed by these subsets are depicted in the cells as well as the associated chemokine receptors.

molecules present peptides derived from inside the cells. Antigen derived from outside the cell is presented as peptides in MHC class II molecules. Interestingly APC also can take up antigen from outside the cell and present this in MHC class I molecules, a process referred to as 'crosspresentation' [10]. In this way APC can take up antigen from infected or transformed cells and instruct T cells to kill these cells that express the same antigen in MHC class I.

Upon activation APC upregulate antigen presenting molecules thereby increasing the change that T cells recognize antigen presented by these cells. Along with this activating co-stimulatory molecules are expressed such as CD86, CD80, CD83, CD70 but also inhibiting receptors like PD-L1 and 2 or B7H4 which is referred to as 'signal 2'. These molecules ligate receptors expressed by T cells and provide activation or inhibitory signals to the T cells. On top of this the APC produce the third signal; cytokines. The type and level of cytokines informs the naïve T cell in what direction to differentiate. The concomitant 3 signals shape the T-cell response and therefore DC, LC and to a lesser extent macrophages are an important bridge between innate and adaptive immunity (Figure 1). For the induction of cytotoxic CD8+ T cells as well as type 1 IFNγ producing CD4+ T cells interleukin 12 (IL-12) is indispensable. The opposite cytokine is interleukin 10 (IL-10) which is associated with immune-shut down and suppression.

CD8 T cells recognize antigens that are presented in MHC class I molecules on cells. MHC class I is expressed by all nucleated cells. When a cell is virally infected or transformed like in cancer, abnormal antigens will be presented in MHC-class I molecules on the cell surface enabling CD8 T cells to recognize and kill this cell.

CD4+ T cells recognize antigens presented in MHC-class II molecules. MHC class II molecules are almost exclusively found on immune cells.

1.3 Macrophages

Besides becoming DC or LC myeloid cells can develop into macrophages. These innate cells are less specialized in antigen presentation but are present in most tissues and are involved in housekeeping and inflammatory responses in health and disease. Roughly two distinct differentiation states are recognized; the classically activated type 1 macrophage (M1) and the alternative differentiated type 2 macrophages (M2). M1 phenotype develops in response to bacterial products and IFNγ. They ingest and kill pathogen upon activation and are tumorocidal [11]. Furthermore, upon danger signal-mediated activation, they secrete cytokines that amplify the danger signal and attract more immune cells. Important features of M1 macrophages are the expression of iNOS, ROS and the production of the pro-inflammatory cytokine IL-12 for stimulation of NK and type 1 T cells. The opposite phenotype, M2 macrophages express high levels of scavenger receptors to take up cell debris and are associated with low production of pro-inflammatory cytokines and high production of IL-10, IL-1β, VEGF and matrix metalloproteinases (MMP). M2 macrophages play a role in chronic inflammation, parasite clearance and wound healing but they also polarize T cells to a Th2 phenotype and dampen immune responses. Notably, the terms M1 and M2 are a useful over-simplification of the type of macrophage that can be detected. Macrophages can adapt to a plethora of differentiation and activation states ranging between the M1 and M2 type.

1.4 APC activation by danger signals

There are a number of pattern recognition receptors known that allow APC to sense danger, including the Toll-like receptors (TLR) and Nod-like receptors (NLR). In humans there are 10 different TLR recognizing bacterial products (TLR 1,2,4,5,6) or viral products (TLR 3,7,8,9) and they can be expressed either on the cell surface (bacterial) or intracellular (viral) corresponding to where most likely a pathogen would be encountered. Not only pathogen products but also cell-derived products like HMGB-1 (TLR4) or heat shock proteins (HSP) can bind TLR. Ligands that act as agonists for these TLR and NLR are now explored as adjuvant in vaccines in order to induce an activated state of the APC. The choice of ligand is often based on *in vitro* APC activation assays or effects found in mouse models. However, expression differs between mice and man for some TLR. Furthermore, the fact that some of the TLR and NLR are expressed by other cells such as non-APC immune cells like keratinocytes and endothelial cells is generally not taken into account [12,13].

1.5 Immunology of tumors

In a recent update of the seminal paper of Hanahan & Weinberg on the hallmarks of cancer several features important for cancer progression were added including 2 immunological hallmarks; avoiding immune destruction and tumor promoting inflammation [14]. Tumors and tumor stroma can be infiltrated by high numbers of immune cells and the balance between these cells predicts disease outcome in several tumor types [15]. The current knowledge about key immunologic features in human tumors are listed below.

1.5.1 T cell infiltrate and immune escape in tumors

Mice lacking T-cell subsets are more likely to develop tumors indicating that the immune system operates as a significant barrier for tumor formation [16,17]. Immune deficient humans develop often tumors, albeit that the majority comprise virally-induced tumors. A growing number of studies show that in human tumors the infiltration of CD8+ T cells correlates with a certain level of tumor control resulting in better disease outcome for the patient [18]. For example, in breast carcinoma it was shown that high CD8+ T-cell infiltration or low CD4+ T-cell infiltration based on detection by immunohistochemistry could predict a better or worse disease outcome respectively [19]. In another large cohort of colorectal cancers the presence of CD3+CD8+ but also the number of CD8+CD4+ T cells was correlated with the absence of metastasis formation and better disease free survival [20]. On top of this, several studies show that the ratio between CD8+ and regulatory FoxP3+ cells predict outcome as for example is found for cervical cancer [21,22]. In contrast to the clear finding that the high infiltration of CD8 T cells contributes to or reflects a favorable patient outcome, the role of CD4 T cells is not as clear. This might be explained by the fact that not all studies dissect the type of CD4 T cell. The type of CD4+ T cells is of significance since in most studies a high number of regulatory T cells is associated with bad prognosis, however in colon carcinoma a protective role for regulatory T cells was reported [23].

Tumor cells exploit mechanisms to avoid recognition by CD8 T cells. MHC expression is down regulated as a result of loss of genes or defects in the antigen loading machinery [24]. Also expression of tumor antigens is lost during tumor development while intrinsic resistance to CTL is increased, processes that are thought to be a result of immunological pressure [25].

Another way for the tumor to avoid T-cell induced apoptosis is inhibiting the T cells directly. This can be executed during cell-cell contact by the expression of inhibitory co-receptors like programmed-death-ligand 1 (PD-L1) or B7-H4 molecules by the tumor cells that ligate with activation-receptors on the T cells and results in strong suppression of functions [26,27]. Secondly many immune suppressive factors can be produced by tumors that directly suppress T cells function. These include TGFβ, IDO, PGE2 and IL-10 [28,29]. Not only do these factors suppress local T-cell function, the APC in the microenvironment are also suppressed and therefore the induction of tumor-specific T-cell immunity is disturbed as well.

1.5.2 Tumor-promoting inflammation

Tumors require nutrients, oxygen and the ability to discharge metabolic waste and carbon dioxide. These needs are addressed by tumor associated neovascularization [14]. Macrophages are the perfect help in this process, especially the wound-healing subset since they are equipped to remodel tissue and produce VEGF. How tumors attract these myeloid cells has been excellently reviewed by Murdoch *et al* [30] .

Tumor cell and tumor-associated cell-produced CCL-2 attracts and shapes myeloid cells and interferes with osteoclasts in bone metastatic disease [31,32]. Blocking CCL-2 reduced the number of tumor-infiltrating macrophages in pre-clinical animal studies. Hypoxia Inducible Factor-1 (HIF-1) and Colony Stimulating Factor-1 (CSF-1/M-CSF) produced by tumor and stromal cells actively attract monocytes into tumor tissue. CSF-1 knockout mice displayed lower numbers of tumor-infiltrating macrophages and tumor progression was delayed [33]. Finally there are few reports on tumor-associated-cell produced CXCL-12 which in an autocrine loop fosters M2 development [34,35].

Many studies have been conducted to determine which factors induce macrophage differentiation upon arrival in the tumor. Cytokine levels in tumor fluids, in ascites or in blood have been measured but also gene expression analyses on tumor cells was performed. Interleukin-6 (IL-6) was often reported to be present in tumor fluids and was also found to be produced by tumor cell lines. IL-6 is a cytokine involved in many chronic inflammatory diseases such as rheumatoid arthritis [36]. A number of studies on the *in vitro* differentiation of M2 macrophages - when monocytes or immature DC are exposed to tumor cell supernatant derived from lung, ovarian or cervical cancer cell lines - showed that this differentiation partly depended on IL-6. In nearly all cases, IL-6 was shown to act in synergy with other factors [31,37-39]. Interestingly, IL-6 signaling results in STAT3 phosphorylation in both tumor and immune cells. Activation of transcription factor STAT3 suppresses the release of mediators necessary for immune activation [40].

CSF-1 (M-CSF) not only acts as an chemo attractant but can also differentiate monocytes into CD14+CD163+CD206high M2 macrophages in vitro [41]. A similar dual role has been suggested for VEGF which acts as an angiogenic factor when secreted by wound healing macrophages but which also can impair APC differentiation when secreted by tumor cells [42].

Prostaglandin E2 (PGE2), produced in chronic inflammatory environments by the COX enzymes, is another key-factor overexpressed in several human tumor-types [43]. Its role is well investigated in colorectal cancer where over expression of inflammatory induced COX-2 enzyme and production of PGE2 during smoldering inflammation eventually leads to oncogenesis. Blocking this pathway might prevent oncogenesis in the low-intestines [44]. Also

in 40-50 % of CxCa patients COX-2 is highly expressed and high intra-tumoral expression was linked to a bad prognosis [45-47]. Notably, a paracrine loop between high COX expression, induction of IL-6 and elevated levels of pSTAT3 has been suggested to exist in human tumor cell lines [48].

Human tumors are shown to harbor and shed high levels of Heat Shock Protein 27 (HSP27) (reviewed by [49]). High levels of HSP27 present in patient serum and breast carcinoma cells function as an endogenous danger signal augmenting cell activation by triggering TLR and scavenger receptors that directly affects APC differentiation and function in vitro [50,51].

Thus, inflammatory mediators attract myeloid cells to the tumor and actively interfere with APC differentiation towards tumor promoting M2 type macrophages. The type of macrophage that is found in tumor tissue is subject to local levels of many factors which can lead to the great variety in APC types present within a single tumor. Interestingly, most factors produced are also potently produced by macrophages themselves upon stimulation suggesting a feed forward loop.

1.5.3 Clinical impact of tumor associated macrophages

Studies on macrophage infiltration in human tumors often did not distinguish between macrophage differentiation (M1 or M2) but most studies suggest that a high number of tumor associated macrophages is beneficial for tumor growth and, therefore, associated with disease progression and poor prognoses (reviewed in [52]). However, a high number of infiltrating macrophages can correlate with a good prognosis. This discrepancy is found for instance in human papilloma virus (HPV) induced diseases. It was reported that macrophage infiltration correlated with disease progression of HPV-induced cervical intraepithelial neoplasia [53]. Yet, in cervical cancers the number of stromal macrophages positively correlated with the intratumoral expression of IL-12p40, which itself was associated with a favourable overall survival of patients [54]. In lung cancer, higher numbers of IL-10+ CD68+ macrophages correlated with worse survival of patients with late stage disease [55]. However, another study showed that high macrophage numbers may correlate with better survival. Importantly, in the latter study they focused on the phenotype of the macrophages revealing that improved survival was associated with a high M1/M2 ratio. The type of macrophage studied thus may explain the apparent discrepancy between the studies [56].

2. HUMAN PAPILLOMA VIRUS INDUCED TUMORS

2.1 HPV

The family of Human Papilloma Viruses (HPV) consists of over 100 DNA viruses that infect keratinocytes at the stratum basale of the epithelium and replicates inside the cell during the differentiation of these cells. Oncogenic HPV types like to infect mucosal sites at the junction of squamous and columnar epithelium and in the cervix are thought to enter through micro-lesions in the epithelium.

Human papilloma virus is a circular double stranded DNA virus, which encodes for 7 proteins, 3 regulatory proteins (E1, E2 and E5), 2 oncoproteins (E6 and E7) and 2 late proteins that form the viral capsid (L1, L2) [57]. All these proteins exert specific functions during the different stages of viral replication. Some proteins interfere with the differentiation and replication machinery

of the host cell to force the cell to maintain replication to ensure high virus production. As a consequence of activation of these 'oncoproteins' host cells keep dividing and lose their natural tumor suppressor functions. Certain viral types only cause warts (HPV 6 and 11) but HPV types of the alpha clade are more aggressive and may cause invasive cancer upon persistent viral infection.

In the episomal replicative phase of the virus, E2 protein suppresses E6 and E7 to ensure a high copy number of virus particles. During integration of the viral genome into the DNA of the host E2 often gets lost leading to deregulated expression of E6 and E7 which is a crucial step in the progression to cancer [58]. The E6 and E7 oncoproteins cause aberrant proliferation and inhibition of apoptosis which efficiently immortalizes cells. Importantly HPV oncogene E6 ubiquitinates P53 tumor suppressor protein and rapidly degrades it inducing high turnover of this protein and deregulating the cell cycle checkpoints upon cellular DNA damage. This high turnover may lead to increased expression of P53 in MHC and induction of P53-specific T cells and antibody responses.

2.2 Immunity in HPV-induced cervical cancer

HPV is the most prevalent sexual transmittable disease and almost all women and men get infected during adulthood. Usually the virus persists for a while at anogenital sites because

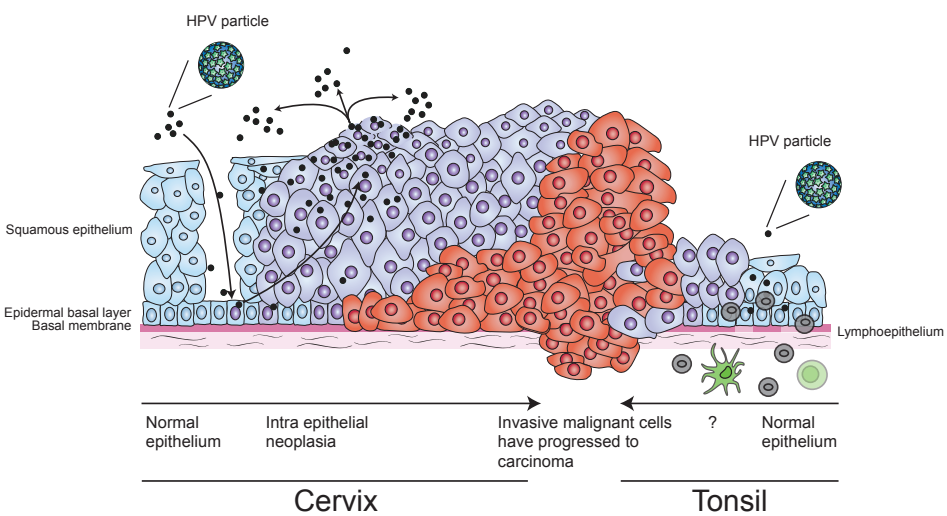

Figure 2. HPV mediated induction of cancer. LEFT: Normal squamous epithelium at the cervix consists of epithelial cells that differentiate during migration towards the surface (light blue cells). RIGHT: The tonsils and tonsillar crypts are covered with a layer of epithelial cells. This epithelium is referred to as lymphoepithelium because the basement membrane is often disrupted and the co-existence of epithelial and immune cells. Basel cells of the epithelial layer get infected by an oncogenic type of HPV. Early genes are expressed from episomal DNA during cell differentiation and movement towards the skin surface (blue cells). Upon integration of the viral episomes into the host genome, E2 is lost and E6 and E7 are continuously expressed (red cells). Overexpression results in carcinogenesis: cells disrupt the basal membrane and the lesion progresses to a malignant carcinoma. For the location of the cervix several stages of intra-epithelial neoplasia -before development into malignant disease- are described. For the location of the oropharynx it is not clear yet whether the same processes and disease stages evolve (depicted by a question mark).

it does not induce an overt inflammation. Microarray studies showed that HPV can actively suppress inflammatory responses in keratinocytes and thereby avoiding that the immune system gets alarmed [12]. Others showed that HPV manipulates LC residing in the epidermis and turns them into tolerogenic APC [59]. Although most women clear the virus, a minority is not able to do so and may develop viral induced neoplasia. HPV16 is the causative virus type in most cases followed by HPV 18, 31, 45 or sporadically other oncogenic types.

Though many studies have been performed the question why not all women clear the infection is not answered yet. Genetic risk factors like IL-10 polymorphisms or differences in interleukin-receptors are proposed. Clearance of the virus is achieved by activation of adaptive immune responses. In healthy subjects that do not show active HPV infection at the cervix functional circulating T cells are found in the peripheral blood mononuclear cells (PBMC). In the majority of these women proliferating T cells were found that recognize oncoproteins E2,E6 and E7 and produce IFNγ and IL-5 upon antigen encounter [60,61]. However failure of viral clearance was associated with a weak or complete lack of detectable HPV-specific T cells [62-64].

The progression of cervical intraepithelial neoplasia (CIN) is associated with the influx of immune cells. However in the progression towards CxCa local T cells lose their ability to produce IFNγ. Also macrophages are attracted to the site of disease and this is associated with a worse disease outcome. CXCL-12 and CCL-2 are important cytokines in attracting macrophages and regulatory T cells. Presumably these cells contribute to the wrong milieu by production of IL-10 and IDO [53,65]. Tumor cell produced PGE2 and TGFβ can directly suppress infiltrating immune cells [46,66]. In contrast to these suppressive parameters, the presence of type 1 T cells preludes a good prognosis. Infiltration of high numbers of granzyme B+ CD8+ T cells in pre-malignant CIN lesions is related to disease regression and in line with this is the finding that once CxCa has developed the composition of T-cell infiltrate is detrimental for the disease outcome [64]. Piersma et al showed the relation between a high CD8+/FoxP3 ratio and the absence of the, prognostic bad sign of LN metastasis. This finding was confirmed in another study that also investigated the antigen presentation machinery on tumors cells. Shut down of MHC-class I and the associated protein MICA further contributed to poor survival in these patients indicating that local T cells and their ability to recognize tumor cells is of great importance for disease outcome [21]. Another mechanism besides shut down of MHC-molecules on the surface of cancer cells is expression of receptors like Serpins to prevent the tumor cells from cytotoxic killing [25]. Interestingly although the inhibitory receptor PD-L1 is known to inhibit T cells, expression of this ligand by cervical tumors together with high numbers of Tregs correlated with a better prognosis [67].

Aforementioned parameters were all studied in the tumor microenvironment. The most important site for priming the T cells that are so important for survival happens in the tumor draining lymph node. Interesting work of Fattorossi *et al* showed that tumor draining lymph nodes with metastatic tumor cells contain more regulatory and Th2 type cells [68]. Tumor infiltrating T cells and LN derived T cells from patients with HPV16 or 18+ CxCa were shown to comprise HPV specific T cells in around 30% of TIL but all LN cultures [69]. However, from these same LN HPV specific regulatory T cells could be isolated that did not express high FoxP3, produced IFNg as well as IL-10 but clearly suppressed proliferation and cytokine production of stimulated T cells in an HPV-specific manner [7].

All these tumor specific T cells travel to the tumor via the blood. Whether the responses found in the blood are representative for what is happening in the tumor can be debated but in CxCa patients it is known that a detectable cellular adaptive immune response in the blood is often lacking in contrast to healthy women, as described previously.

2.3 Immunity in HPV-induced carcinoma of the head and neck region

In the 1970s the first reports emerged in northern Europe that HPV16 was found to be present in squamous cell carcinoma of the head and neck (HNSCC). The last decades steep increase in the incidence of HPV16+ HNSCC is reported worldwide and although the percentage differs per country it is reported that 30-50% of all oropharyngeal cancers harbor HPV16 (IPV conference 2011). HPV16 DNA is predominantly found to be involved in tumors that arise in the oropharynx and the base of the tongue and not in lower regions of the pharynx or the nasopharynx. Interestingly HPV type 16 is involved in >90% of HPV+ HNSCC whereas in CxCa also other types are involved [57]. In contrast to the known etiology of HNSCC (tobacco and alcohol use) that is often linked to mutated and over-expressed p53, patients with HPV16+ HNSCC present at younger age and often lack a history of smoking and drinking. Despite the fact that HPV+ patients present with more advanced stage of cancer they do much better in survival upon standard treatment compared to alcohol and tobacco-induced HNSCC [70]. Suggested reasons for this include better general health of these patients because of age, less morbity in general, less secondary malignancies by 'field cancerization' or the viral induced oncogenic pathway that makes the tumor more susceptible for radiotherapy.

Carcinomas of the head and neck region use the same mechanisms to hide for and suppress immune cells as described earlier. In HNSCC up to 88% of the tumors showed loss of HLA class I expression and especially in metastatic lesions HLA-class I expression was lost [71,72]. In at least 40% of HNSCC high expression of COX-2 is reported, and this related to the presence of metastasis, VEGF levels and poor survival [73,74]. In HNSCC the infiltration of s100+ infiltrating DC as well as T cells including CD4+ T cells, irrespective of their subtype, correlated to a better prognosis [75]. The majority of HPV+ HNSCC display an adaptive inflammatory response and infiltration of T cells as analyzed by gene array [76]. The group of Dalianis (based in Stockholm) reported higher number of CD8+ but also FoxP3+ cells in HPV16+ HNSCC compared to HPV negative (IPV conference 2011). This suggests that HPV-induced tumors are more immunogenic than non-virally induced HNSCC. Except for one study that reports circulating HPV16 E7-specific T cells and reports about the presence of circulating HPV specific antibodies, nothing is known HPV specific immunity in these patients.

3. TUMOR THERAPY – FOCUS ON REINFORCEMENT OF THE IMMUNE SYSTEM

3.1 Treatment of cervical and oropharyngeal cancer

Surgery, with the aim to remove all the malignant cells, is still the first choice of treatment for most cancers including CxCa and HNSCC. In cervical and oropharyngeal cancers, the diagnosis is based on a tissue biopsy. The immune-stimulating effects of biopsies are not clear but cases of spontaneous regression of CIN lesions after a biopsy exists and there is also a correlation

with detectable immune responses in the blood [62]. If tumors have invaded surrounding tissue or have metastasized, the patients are treated with chemotherapy, radiotherapy, monoclonal antibodies or combinations of these. Especially advanced HPV+ HNSCC is treated with radiotherapy combined with platinum based chemotherapy because surgical removal would be too disabling.

3.2 Platinum based chemotherapy

Worldwide, the therapy for advanced, persistent or recurrent CxCa primarily consists of cisplatin, carboplatin and combinations of platinum-based drugs with other types of chemotherapeutics [77]. In the Netherlands the current guideline is to treat patients with carboplatin, sometimes in combination with paclitaxel (www.oncoline.nl/cervixcarcinoom). Platinum-based chemotherapeutics act by binding to DNA. This results in the activation of various signal-transduction pathways, including those for DNA-damage recognition and repair, cell-cycle arrest and apoptosis. Compared to cisplatin, carboplatin forms DNA adducts more slowly and higher concentrations are required for similar efficacy, but the (nephro)toxicity is lower [78]. Unfortunately, many CxCa tumors are chemotherapy resistant and the response rate to cisplatin ranges from 20% to 30%, with an overall survival of 7 months [77]. Also for tumors of the oropharynx the first choice of chemotherapy is cisplatin.

Tumors of all origins can have or gain resistance to platinum based treatment. In search of predictors for response to chemotherapy a study was conducted by Ferrandina et al. in which they determined the expression of COX-2 enzymes - the inflammatory induced enzyme that produces PgE2- prior to adjuvant treatment with cisplatin based chemotherapy. The patients with high COX-2 expressing tumors did significantly worse in survival [45].

If cancer patients suffer from lymphopenia before the start of treatment they are less likely to respond to chemotherapy suggesting that immune cells are important for the response to chemotherapy [79]. Although the main goal of chemotherapy (and radiotherapy) is to kill the tumor cells, recent articles suggested that chemotherapy partly acts through the immune system. Several studies in mice showed synergy between platinum treatment or radiation with vaccination, indicating that these therapies make tumors more prone for immune attacks [80,81]. In patients with CxCa the composition of immune cells in the tumor draining lymph nodes was distorted by the treatment with cisplatin and radiation therapy. It was shown that a low dose of radiotherapy in combination with chemotherapy was crucial for the induction of a type 1 skewed T-cell response [82]. Remarkably, the higher dose of radiation resulted in loss of CD4+ T cells in the tumor draining lymph node. Since T cells are not susceptible for radiotherapy, probably the surrounding stroma was affected hampering CD4+ T cell outgrowth [82].

Recent work form the group of Zitvogel shows immunostimulatory effects by expression of calreticulin (CRT) and HMGB-1 and ATP release of tumor cells treated with oxaliplatin. These factors supported a better uptake of apoptotic cells (CRT) and activation of tumor draining APC by HMGB-1 mediated triggering of TLR4, thereby contributing to a shift in the local tumor milieu and the boosting of tumor-specific T-cell responses. Cisplatin induced HMBG-1 release in several cell lines but failed to elicit a strong immune response [83]. It must be said that this work until now only involves mouse models with prior treated, implanted tumors and therefore this pro-inflammatory effects of platinum-treated dying tumor cells have to be confirmed in other models and human tissue.

The above mentioned mechanisms can explain why the presence of immune infiltrate is a good marker to predict response to chemotherapy. However these correlations are mainly investigated for other classes of chemotherapeutics then platinum based chemotherapeutics [18,19,84,85].

3.3 Monoclonal antibody therapies

Over the past decade monoclonal antibodies (MAbs) have become an important treatment modality for several types of tumors. As in normal B-cell produced antibody responses, these MAbs consist of the 'fragment of antigen binding (Fab)' and the 'constant fragment (Fc)' of which the latter usually resembles the IgG family in therapeutic antibodies. In contrast to the subclasses of IgA and IgM that predominantly bind complement, IgG is suitable to bind to FcγReceptors of immune cells. With antibodies targeting specific tumor expressed molecules for example to B-cell antigen CD20 (Rituximab), growth factor receptors and prevention of signaling on tumor cells (EGFR, cetuximab, Her2, trastuzumab) or soluble factors (VEGF, bevacizumab) there is now a broad range of functional, clinically relevant antibodies. Although antibodies were first designed to directly block functional pathways on tumor cells, the increased response in patients expressing high affinity alleles for FcγR indicated that activation of immune cells contributed to the clinical effects [86,87].

Human immune cells express 3 Fc Receptors which is not the same in mice. FcγRI (CD64) is the high affinity receptor for IgG1 and IgG3 antibodies expressed on neutrophils, macrophages and eosinophils. FcγRII (CD32) is low affinity for IgG1 and IgG3 and expressed by the same cells as well as by platelets. The third receptor, FcγRIIIa (CD16) is expressed on natural killer (NK) cells and on macrophages. Although CD16 has less affinity then FcγRI, it is capable to induce antibody-dependent cell-mediated cytotoxicity. Mouse models and *in vitro* tests with human cells showed that NK cells, expressing FcγRIII can recognize and kill MAb opsonized cells, the phenomenon referred to as Antibody-Dependent Cellular Cytotoxicity (ADCC). Based on this, much effort has been put into optimizing the Fc region to better bind FcγRIII [88]. Figure 3 shows the several mechanisms of how MAbs are thought to mediate their effects.

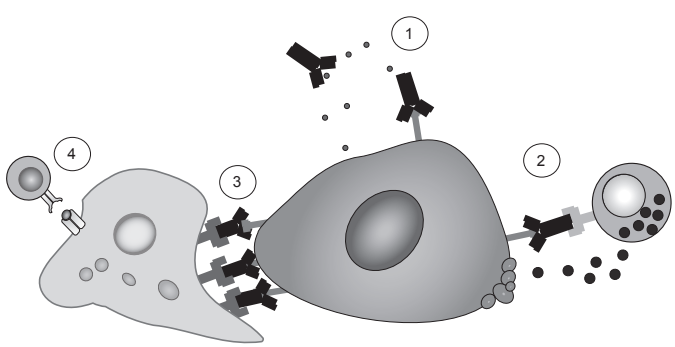

Figure 3. Antibody mediated anti-tumor mechanisms and immune cells. 1) MAbs bind on receptor on tumorcells and blocks autocrience loop of growth factor supply. MAb to soluble antigens do not interact with immune cells but deprive tumor cells from growth factors. 2) Tumor bound antibodies are recognized by FcgRIII (CD16) on NK cells and activated NK cells release granzymes and perforin to lyse tumor cells. 3) Antibody-opsonized tumor cells and tumor particles are engulfed by macrophages. 4) Macrophages and DC pick up antibody opsonized particles and cross present these antigens to CD8+ T cells.

MAb-based treatment are not included in standard regimes for CxCa. In oropharyngeal cancer the addition of Cetuximab to target epithelial growth factor receptor (EGFR) on tumors in combination with radiotherapy is currently tested for efficacy compared to chemo-radiation. Great improvement of disease free survival with the addition of the MAb bevacizumab which blocks soluble VEGF, was achieved in patients with colon cancer. Addition of Cetuximab to this treatment protocol did, unexpectedly, not further improve the survival of these patients [89,90].

OUTLINE OF THIS THESIS

In this thesis, several aspects and cellular players involved in the immune response to (HPV-induced) tumors were investigated.

In **chapter 2** a detailed analysis of HPV-specific immunity in a large group of patients with HPV-induced cervical cancer (CxCa) in relation to HLA-types and prognostic factors was performed. In 30% of the tested patients, circulating HPV specific T cells were found, most often in patients with deeply infiltrating tumors. In this group, patients with HPV specific proliferative immunity displayed better disease free survival.

It was shown earlier by Piersma *et al [69]* that only in 30% of TIL populations, HPV specific T cells were found. In **chapter 3** an in depth analysis of the breadth and type of HPV specific T-cell populations in tumor infiltrating T cell (TIL) cultures or LN cells from HPV 16 or 18 positive CxCa patients was performed. We found that if patients displayed a HPV-specific T cell response, this was surprisingly broad. Despite recognition, a number of cells did not produce type 1 cytokines and therefore we tested what TLR-agonist was able to support cytokine induction by these T cells.

In **chapter 4** we asked the question whether HPV-specific T cells play a role in HPV-induced HNSCC. We hypothesize that HPV-induced tumors are more immunogenic and stimulate strong T-cell reactivity to the viral oncoproteins in contrast to HPV-negative tumors. We set up a pilot study to investigate whether HPV is present in HNSCC in the Dutch patient population and at which anatomical site. Accordingly, blood and tumor infiltrating T cells were analyzed for the presence of functional HPV specific T cells.

The lack of T cell responses in CxCa patients and the 'poised' function of tumor-antigen specific T cells could be a lack of proper priming by DC. Therefore we investigated in **chapter 5A** the effects of CxCa produced soluble factors on the differentiation of antigen presenting cells (APC). Several tumor cell lines hampered DC differentiation or even skewed monocyte differentiation into tolerogenic tumor promoting M2 macrophages. We identified the factors responsible for this and investigated the outcome of the interaction of HPV specific T-cell clones with these macrophages.

Since patients with advanced or recurrent disease are treated with platinum based chemotherapy we investigated the immune-modulating effect of this therapy on tumor cells, tumor-modulated APC and subsequent interaction with T cells. This ongoing work is summarized in **chapter 5B**.

Therapeutic vaccination is being developed for treatment of chronic infections and cancer, and aims to generate protective T-cell immunity. Although some clinical successes have been reported, particularly in the field of cancer vaccination, there is still much to be gained in terms of efficacy [91,92]. Especially the adjuvant used and the route of administration of vaccines

are critical factors that determine the type and memory of the resulting T-cell response. Intradermal vaccination is an attractive method for diseases in the skin such as HPV induced tumors and melanomas since the induced T cells get skin-homing instructions. In chapter 5 we showed already that highly pure DC can become activated by the addition of several different TLR agonists *in vitro*. To assess the effect of these TLR agonists on the APC present in the dermis, a human skin-explant model was used to analyze the phenotype and function of the APC migrating out the skin upon TLR-injection. These results are described in **chapter 6**. Surprisingly, only few TLR-agonists turned out to induce activation of the migrating cells.

The current treatment of patients with advanced colorectal cancer consists of chemotherapy together with the MAb bevacizumab that blocks soluble VEGF. The addition of a second antibody that targets tumor expressed EGFR (cetuximab) to this treatment did not result in the expected disease free survival benefit in a large randomized phase III study (CAIROII). Analysis of gene polymorphisms in the FcγReceptors revealed that patients with the high affinity FCGR3A polymorphism did significantly worse upon addition of cetuximab to the standard treatment. As colon cancers are generally infiltrated with macrophages we tested the hypothesis that membrane bound antibodies could activate tumor promoting M2 macrophages and that this would happen more efficiently in patients with high affinity FCGRIIIA in **chapter 7**.

In **chapter 8** the work of this thesis is discussed in light of recent literature.

REFERENCES

1. Joosten SA, Ottenhoff TH: **Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination.** *Hum Immunol* 2008, **69:** 760-770.
2. Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM *et al.*: **CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer.** *Clin Cancer Res* 2007, **13:** 6947-6958.
3. Cao W, Cavacini LA, Tillman KC, Posner MR: **CD40 function in squamous cell cancer of the head and neck.** *Oral Oncol* 2005, **41:** 462-469.
4. Neppelberg E, Loro LL, Oijordsbakken G, Johannessen AC: **Altered CD40 and E-cadherin expression--putative role in oral lichen planus.** *J Oral Pathol Med* 2007, **36:** 153-160.
5. Murphy KM, Stockinger B: **Effector T cell plasticity: flexibility in the face of changing circumstances.** *Nat Immunol* 2010, **11:** 674-680.
6. Zou W, Restifo NP: **T(H)17 cells in tumour immunity and immunotherapy.** *Nat Rev Immunol* 2010, **10:** 248-256.
7. van der Burg SH, Piersma SJ, de Jong A, van der Hulst JM, Kwappenberg KM, van den HM *et al.*: **Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens.** *Proc Natl Acad Sci U S A* 2007, **104:** 12087-12092.
8. Vignali DA, Collison LW, Workman CJ: **How regulatory T cells work.** *Nat Rev Immunol* 2008, **8:** 523-532.
9. Steinman RM, Idoyaga J: **Features of the dendritic cell lineage.** *Immunol Rev* 2010, **234:** 5-17.
10. Yewdell JW, Norbury CC, Bennink JR: **Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines.** *Adv Immunol* 1999, **73:** 1-77.
11. Kaduka Y, Takeda K, Nakayama M, Kinoshita K, Yagita H, Okumura K: **TWEAK mediates anti-tumor effect of tumor-infiltrating macrophage.** *Biochem Biophys Res Commun* 2005, **331:** 384-390.
12. Karim R, Meyers C, Backendorf C, Ludigs K, Offringa R, van Ommen GJ *et al.*: **Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes.** *PLoS One* 2011, **6:** e17848.
13. Magrone T, Jirillo E: **The Impact of Bacterial Lipolysaccharides on the Endothelial System: Pathological Consequences and Therapeutic Countermeasures.** *Endocr Metab Immune Disord Drug Targets* 2011.

14. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation.** *Cell* 2011, **144:** 646-674.
15. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH: **Immune infiltration in human tumors: a prognostic factor that should not be ignored.** *Oncogene* 2010, **29:** 1093-1102.
16. Kim R, Emi M, Tanabe K: **Cancer immunoediting from immune surveillance to immune escape.** *Immunology* 2007, **121:** 1-14.
17. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ: **Immune-mediated dormancy: an equilibrium with cancer.** *J Leukoc Biol* 2008, **84:** 988-993.
18. Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G: **Prognostic and predictive impact of intra- and peritumoral immune infiltrates.** *Cancer Res* 2011, **71:** 5601-5605.
19. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF *et al.*: **Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy.** *Cancer Discovery* 2011.
20. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R *et al.*: **Effector memory T cells, early metastasis, and survival in colorectal cancer.** *N Engl J Med* 2005, **353:** 2654-2666.
21. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG *et al.*: **Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients?** *Clin Cancer Res* 2008, **14:** 2028-2035.
22. Piersma SJ, Jordanova ES, van Poelgeest MtIE, Kwappenberg KMC, van der Hulst JM, Drijfhout JW *et al.*: **High Number of Intraepithelial CD8+ Tumor-Infiltrating Lymphocytes Is Associated with the Absence of Lymph Node Metastases in Patients with Large Early-Stage Cervical Cancer.** *Cancer Res* 2007, **67:** 354-361.
23. Ladoire S, Martin F, Ghiringhelli F: **Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer.** *Cancer Immunol Immunother* 2011, **60:** 909-918.
24. Drake CG, Jaffee E, Pardoll DM: **Mechanisms of immune evasion by tumors.** *Adv Immunol* 2006, **90:** 51-81.
25. Kloth JN, Gorter A, Fleuren GJ, Oosting J, Uljee S, ter HN *et al.*: **Elevated expression of SerpinA1 and SerpinA3 in HLA-positive cervical carcinoma.** *J Pathol* 2008, **215:** 222-230.
26. Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P *et al.*: **B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma.** *J Exp Med* 2006, **203:** 871-881.
27. Zou W, Chen L: **Inhibitory B7-family molecules in the tumour microenvironment.** *Nat Rev Immunol* 2008, **8:** 467-477.
28. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P: **The polarization of immune cells in the tumour environment by TGFbeta.** *Nat Rev Immunol* 2010, **10:** 554-567.
29. Gottfried E, Kreutz M, Mackensen A: **Tumor-induced modulation of dendritic cell function.** *Cytokine & Growth Factor Reviews* 2008, **19:** 65-77.
30. Murdoch C, Giannoudis A, Lewis CE: **Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues.** *Blood* 2004, **104:** 2224-2234.
31. Roca H, Varsos ZS, Sud S, Craig MJ, Ying C, Pienta KJ: **CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization.** *J Biol Chem* 2009, **284:** 34342-34354.
32. Zhang J, Patel L, Pienta KJ: **CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis.** *Cytokine Growth Factor Rev* 2010, **21:** 41-48.
33. Bosco MC, Puppo M, Blengio F, Fraone T, Cappello P, Giovarelli M *et al.*: **Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration.** *Immunobiology* 2008, **213:** 733-749.
34. Rigo A, Gottardi M, Zamo A, Mauri P, Bonifacio M, Krampera M *et al.*: **Macrophages may promote cancer growth via a GM-CSF/HB-EGF paracrine loop that is enhanced by CXCL12.** *Mol Cancer* 2010, **9:** 273.
35. Sanchez-Martin L, Estecha A, Samaniego R, Sanchez-Ramon S, Vega MA, Sanchez-Mateos P: **The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression.** *Blood* 2011, **117:** 88-97.
36. Choy E: **Inhibiting interleukin-6 in rheumatoid arthritis.** *Curr Rheumatol Rep* 2008, **10:** 413-417.
37. Avila-Moreno F, Lopez-Gonzalez JS, Galindo-Rodriguez G, Prado-Garcia H, Bajana S, Sanchez-Torres C: **Lung squamous cell carcinoma and adenocarcinoma cell lines use different mediators to induce comparable phenotypic and functional changes in human monocyte-derived dendritic cells.** *Cancer Immunol Immunother* 2006, **55:** 598-611.
38. Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Leinster DA *et al.*: **Interleukin-6 as a Therapeutic Target in Human Ovarian Cancer.** *Clin Cancer Res* 2011, **17:** 6083-6096.

39. Duluc D, Delneste Y, Tan F, Moles MP, Grimaud L, Lenoir J *et al*.: **Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells.** *Blood* 2007, **110:** 4319-4330.
40. Yu H, Pardoll D, Jove R: **STATs in cancer inflammation and immunity: a leading role for STAT3.** *Nat Rev Cancer* 2009, **9:** 798-809.
41. Verreck FA, de Boer T, Langenberg DM, van der ZL, Ottenhoff TH: **Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation.** *J Leukoc Biol* 2006, **79:** 285-293.
42. Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S *et al*.: **Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells.** *Nat Med* 1996, **2:** 1096-1103.
43. Wang D, Dubois RN: **Eicosanoids and cancer.** *Nat Rev Cancer* 2010, **10:** 181-193.
44. Brown JR, Dubois RN: **COX-2: a molecular target for colorectal cancer prevention.** *J Clin Oncol* 2005, **23:** 2840-2855.
45. Ferrandina G, Lauriola L, Distefano MG, Zannoni GF, Gessi M, Legge F *et al*.: **Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients.** *J Clin Oncol* 2002, **20:** 973-981.
46. Herfs M, Herman L, Hubert P, Minner F, Arafa M, Roncarati P *et al*.: **High expression of PGE2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells.** *Cancer Immunol Immunother* 2009, **58:** 603-614.
47. Young JL, Jazaeri AA, Darus CJ, Modesitt SC: **Cyclooxygenase-2 in cervical neoplasia: a review.** *Gynecol Oncol* 2008, **109:** 140-145.
48. Dalwadi H, Krysan K, Heuze-Vourc'h N, Dohadwala M, Elashoff D, Sharma S *et al*.: **Cyclooxygenase-2-dependent activation of signal transducer and activator of transcription 3 by interleukin-6 in non-small cell lung cancer.** *Clin Cancer Res* 2005, **11:** 7674-7682.
49. Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C: **Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy.** *J Leukoc Biol* 2007, **81:** 15-27.
50. Banerjee S, Lin CF, Skinner KA, Schiffhauer LM, Peacock J, Hicks DG *et al*.: **Heat shock protein 27 differentiates tolerogenic macrophages that may support human breast cancer progression.** *Cancer Res* 2011, **71:** 318-327.
51. Laudanski K, De A, Miller-Graziano C: **Exogenous heat shock protein 27 uniquely blocks differentiation of monocytes to dendritic cells.** *Eur J Immunol* 2007, **37:** 2812-2824.
52. Bingle L, Brown NJ, Lewis CE: **The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies.** *J Pathol* 2002, **196:** 254-265.
53. Hammes LS, Tekmal RR, Naud P, Edelweiss MI, Kirma N, Valente PT *et al*.: **Macrophages, inflammation and risk of cervical intraepithelial neoplasia (CIN) progression--clinicopathological correlation.** *Gynecol Oncol* 2007, **105:** 157-165.
54. Zijlmans HJ, Fleuren GJ, Baelde HJ, Eilers PH, Kenter GG, Gorter A: **Role of tumor-derived proinflammatory cytokines GM-CSF, TNF-alpha, and IL-12 in the migration and differentiation of antigen-presenting cells in cervical carcinoma.** *Cancer* 2007, **109:** 556-565.
55. Zeni E, Mazzetti L, Miotto D, Lo CN, Maestrelli P, Querzoli P *et al*.: **Macrophage expression of interleukin-10 is a prognostic factor in nonsmall cell lung cancer.** *Eur Respir J* 2007, **30:** 627-632.
56. Ohri CM, Shikotra A, Green RH, Waller DA, Bradding P: **Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival.** *Eur Respir J* 2009, **33:** 118-126.
57. Chung CH, Gillison ML: **Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications.** *Clin Cancer Res* 2009, **15:** 6758-6762.
58. Moody CA, Laimins LA: **Human papillomavirus oncoproteins: pathways to transformation.** *Nat Rev Cancer* 2010, **10:** 550-560.
59. Fahey LM, Raff AB, Da Silva DM, Kast WM: **A major role for the minor capsid protein of human papillomavirus type 16 in immune escape.** *J Immunol* 2009, **183:** 6151-6156.
60. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A *et al*.: **Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects.** *Cancer Res* 2002, **62:** 472-479.
61. Welters MJ, de JA, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S *et al*.: **Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter.** *Cancer Res* 2003, **63:** 636-641.
62. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW *et al*.: **Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction**

of a dysfunctional HPV16-specific T-cell response. *Clin Cancer Res* 2008, **14:** 7188-7195.

63. Farhat S, Nakagawa M, Moscicki AB: **Cell-mediated immune responses to human papillomavirus 16 E6 and E7 antigens as measured by interferon gamma enzyme-linked immunospot in women with cleared or persistent human papillomavirus infection.** *Int J Gynecol Cancer* 2009, **19:** 508-512.
64. Woo YL, van den Hende M, Sterling JC, Coleman N, Crawford RA, Kwappenberg KM *et al.*: **A prospective study on the natural course of low-grade squamous intraepithelial lesions and the presence of HPV16 E2-, E6- and E7-specific T-cell responses.** *Int J Cancer* 2010, **126:** 133-141.
65. Kobayashi A, Weinberg V, Darragh T, Smith-McCune K: **Evolving immunosuppressive microenvironment during human cervical carcinogenesis.** *Mucosal Immunol* 2008, **1:** 412-420.
66. Kloth JN, Fleuren GJ, Oosting J, de Menezes RX, Eilers PHC, Kenter GG *et al.*: **Substantial changes in gene expression of Wnt, MAPK and TNF{alpha} pathways induced by TGF-{beta}1 in cervical cancer cell lines.** *Carcinogenesis* 2005, **26:** 1493-1502.
67. Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM *et al.*: **Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma.** *Clin Cancer Res* 2009, **15:** 6341-6347.
68. Fattorossi A, Battaglia A, Ferrandina G, Coronetta F, Legge F, Salutari V *et al.*: **Neoadjuvant therapy changes the lymphocyte composition of tumor-draining lymph nodes in cervical carcinoma.** *Cancer* 2004, **100:** 1418-1428.
69. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ *et al.*: **Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element.** *Int J Cancer* 2008, **122:** 486-494.
70. Psyrri A, Cohen E: **Oropharyngeal cancer: clinical implications of the HPV connection.** *Ann Oncol* 2011, **22:** 997-999.
71. Bandoh N, Ogino T, Katayama A, Takahara M, Katada A, Hayashi T *et al.*: **HLA class I antigen and transporter associated with antigen processing downregulation in metastatic lesions of head and neck squamous cell carcinoma as a marker of poor prognosis.** *Oncol Rep* 2010, **23:** 933-939.
72. Grandis JR, Falkner DM, Melhem MF, Gooding WE, Drenning SD, Morel PA: **Human leukocyte antigen class I allelic and haplotype loss in squamous cell carcinoma of the head and neck: clinical and immunogenetic consequences.** *Clin Cancer Res* 2000, **6:** 2794-2802.
73. Gallo O, Franchi A, Magnelli L, Sardi I, Vannacci A, Boddi V *et al.*: **Cyclooxygenase-2 pathway correlates with VEGF expression in head and neck cancer. Implications for tumor angiogenesis and metastasis.** *Neoplasia* 2001, **3:** 53-61.
74. Gallo O, Masini E, Bianchi B, Bruschini L, Paglierani M, Franchi A: **Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma.** *Hum Pathol* 2002, **33:** 708-714.
75. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NH *et al.*: **Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers.** *Clin Cancer Res* 2006, **12:** 465-472.
76. Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G *et al.*: **Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma.** *J Clin Oncol* 2010, **28:** 2881-2888.
77. Pectasides D, Kamposioras K, Papaxoinis G, Pectasides E: **Chemotherapy for recurrent cervical cancer.** *Cancer Treat Rev* 2008, **34:** 603-613.
78. Kelland L: **The resurgence of platinum-based cancer chemotherapy.** *Nat Rev Cancer* 2007, **7:** 573-584.
79. Ray-Coquard I, Cropet C, Van GM, Sebban C, Le CA, Judson I *et al.*: **Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas.** *Cancer Res* 2009, **69:** 5383-5391.
80. Spanos WC, Nowicki P, Lee DW, Hoover A, Hostager B, Gupta A *et al.*: **Immune response during therapy with cisplatin or radiation for human papillomavirus-related head and neck cancer.** *Arch Otolaryngol Head Neck Surg* 2009, **135:** 1137-1146.
81. Tseng CW, Hung CF, Alvarez RD, Trimble C, Huh WK, Kim D *et al.*: **Pretreatment with cisplatin enhances E7-specific CD8+ T-Cell-mediated antitumor immunity induced by DNA vaccination.** *Clin Cancer Res* 2008, **14:** 3185-3192.
82. Battaglia A, Buzzonetti A, Martinelli E, Fanelli M, Petrillo M, Ferrandina G *et al.*: **Selective changes in the immune profile of tumor-draining lymph nodes after different neoadjuvant chemoradiation regimens for locally advanced cervical cancer.** *Int J Radiat Oncol Biol Phys* 2010, **76:** 1546-1553.

83. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F *et al.*: **Immunogenic death of colon cancer cells treated with oxaliplatin.** *Oncogene* 2010, **29:** 482-491.
84. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M *et al.*: **Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer.** *J Clin Oncol* 2010, **28:** 105-113.
85. Zitvogel L, Kepp O, Kroemer G: **Immune parameters affecting the efficacy of chemotherapeutic regimens.** *Nat Rev Clin Oncol* 2011, **8:** 151-160.
86. Jiang XR, Song A, Bergelson S, Arroll T, Parekh B, May K *et al.*: **Advances in the assessment and control of the effector functions of therapeutic antibodies.** *Nat Rev Drug Discov* 2011, **10:** 101-111.
87. Nimmerjahn F, Ravetch JV: **Fcgamma receptors as regulators of immune responses.** *Nat Rev Immunol* 2008, **8:** 34-47.
88. Weiner LM, Surana R, Wang S: **Monoclonal antibodies: versatile platforms for cancer immunotherapy.** *Nat Rev Immunol* 2010, **10:** 317-327.
89. Pander J, Gelderblom H, Antonini NF, Tol J, van Krieken JH, van der Straaten T *et al.*: **Correlation of FCGR3A and EGFR germline polymorphisms with the efficacy of cetuximab in KRAS wild-type metastatic colorectal cancer.** *Eur J Cancer* 2010, **46:** 1829-1834.
90. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG *et al.*: **Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer.** *N Engl J Med* 2009, **360:** 563-572.
91. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP *et al.*: **Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia.** *N Engl J Med* 2009, **361:** 1838-1847.
92. Karbach J, Gnjatic S, Bender A, Neumann A, Weidmann E, Yuan J *et al.*: **Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival.** *Int J Cancer* 2010, **126:** 909-918.

CHAPTER 2

The detection of circulating Human Papillomavirus (HPV)-specific T cells is associated with improved survival of patients with deeply infiltrating tumors

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ABSTRACT

A detailed analyses of HPV-specific immunity was performed in a large group of patients with HPV-induced cervical cancer (CxCa) in relation to HLA-types and prognostic factors. Patients were HLA-typed and HPV16/18-specific T-cell immunity was assessed by proliferation assay and cytometric bead array using freshly isolated PBMC and by phenotypic analysis of HPV-specific T cells. The results were analyzed in relation to known disease-related HLA-types (DR7, DR13, DR15/DQ06), invasion-depth and size of tumor, lymph node (LN) status and disease free survival.

In total 119 HLA-typed patients with CxCa were analyzed. Patients expressing the HLA-DR13 haplotype were underrepresented as compared to the Dutch population ($p=0.014$), whereas HLA-DR7 was overrepresented in patients with HPV16+ CxCa ($p=0.006$). In 29 of 94 patients (31%) from whom blood could be tested, a proliferative response to HPV16/18 was detected, which was associated with increased numbers of HPV-specific CD4+CD25+ (activated) T cells ($p=0.03$) and HPV-specific CD4+CD25+FoxP3-positive T cells ($p=0.04$). The presence of both FoxP3-positive and negative HPV-specific CD4+CD25+ T cells was significantly correlated ($p=0.01$). Interestingly, the detection of HPV-specific proliferation was associated with invasion depth ($p=0.020$) but not with HLA type, tumor size nor LN status. Moreover, the detection of HPV-specific immunity was associated with an improved disease free survival ($p=0.04$) in patients with deeply infiltrating tumors.

In conclusion, HPV-specific proliferative T-cell response, comprising higher percentages of HPV-specific CD25+ and CD25+FoxP3-positive CD4+T cells, are more frequently detected in patients with deep infiltrating CxCa tumors and associated with an improved survival.

Novelty and impact of the study: Studies on the proliferative T-cell response against the two oncoproteins E6 and E7 of high-risk human papillomavirus type 16 and 18 have shown that the presence of a strong T-cell reaction was associated with protection against disease progression. This notion was sustained by our recent vaccine study in which such a response correlated with the regression of HPV16-induced high-grade lesions. This large prospective study reveals that especially the patients with deep stromal infiltrating cervical tumors display proliferative HPV-specific T-cell responses. Moreover, patients with deeply infiltrating tumors displaying such a HPV-specific proliferative response less often show recurrence of disease, suggesting that the HPV-specific T-cells have a protective effect. This implies that reinforcement of the HPV E6- and E7-specific T-cell response by vaccination may have a positive effect on disease free survival.

INTRODUCTION

Cervical cancer (CxCa) is the second most common cancer in women worldwide (1). It develops as a result of an uncontrolled, persistent infection with a high-risk type of human papillomavirus (HPV), in particular types HPV16 and HPV18 (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).

As the HPV proteins are foreign to the body one would expect the immune system to respond against these antigens when expressed in the cervical epithelium. Indeed, HPV16-specific Th1- and Th2-type CD4+ proliferative T-cell responses were frequently detected in PBMC cultures of healthy individuals (4-6) and both HPV16-specific CD4+ and CD8+ T cells are able to migrate upon antigenic challenge in exposed healthy individuals (7) showing that successful defense against HPV16 infection is commonly associated with the induction of a systemic effector T-cell response against these viral antigens. This notion is sustained by our most recent study showing that the full regression of HPV16-induced high-grade vulvar lesions is strongly associated with the strength of vaccine-induced HPV-specific immunity against these early antigens, as measured by proliferation and cytokine production (8).

A number of relatively small *in vitro* studies on the presence and function of circulating HPV16- or HPV18-specific T cells in patients with HPV16- or HPV18-induced cervical squamous intraepithelial lesion or cancer have suggested that the development of CxCa is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and cytotoxic T lymphocyte response and the induction of HPV-specific regulatory T cells (5;9-11). Furthermore, studies *in situ* suggested that CD8+ T cells may fail to migrate into the tumor cell nests and when tumors are infiltrated by CD8+ T cells it coincides with the infiltration by CD4+ T cells with a regulatory phenotype, as indicated by the expression of intra-nuclear FoxP3. (12) In addition, half of the tumor-infiltrating T cells express the programmed cell death receptor 1 as a sign of T-cell exhaustion (13). Moreover, the ratio between the tumor-infiltrating CD8+ T cells and co-infiltrating CD4+FoxP3-positive T cells is an independent prognostic factor for overall survival (14), indicating the key role of these different types of T cells in HPV-induced diseases such as cervical cancer.

The limited numbers as well as the response rate of patients analyzed in most of the studies so far precludes the assessment of the role or impact of HPV-specific immunity in

those patients with CxCa who did mount such a response. Therefore, we have performed a prospective study in which the HPV-specific proliferative immune response was measured before surgery of the primary tumor in a large group of patients with HPV16- or HPV18-induced CxCa. The presence or absence of an HPV-specific proliferative immune response – for which we previously showed that it correlated with protective immunity (5;8) – was analyzed with respect to several prognostic factors. These include the different HLA class II-alleles that have been suggested to be associated with protection or risk for HPV-related cervical disease (15-18) as it is believed that some HLA-class II molecules may be better or less well equipped to present the HPV protein-derived peptides to T cells. Furthermore, T-cell immunity in relation to tumor-size, depth of stromal invasion by the tumor, lymph node (LN) status – all known tumor characteristics associated with bad outcome (19) - as well as disease free survival was determined.

This prospective study reveals that a minority of the patients have circulating HPV-specific T cells that are able to proliferate when stimulated with cognate E6 or E7 antigen. HPV-specific proliferation is more often detected in patients with deep stromal infiltrating tumors and comprises both HPV-specific helper and CD4+CD25+FoxP3-positive T cells. Patients with deeply infiltrating tumors and an HPV-specific proliferative response present less often with recurrent disease.

MATERIAL AND METHODS

Patients

Women presenting with histologically proven CxCa at the Department of Gynaecology of the Leiden University Medical Center (LUMC) were enrolled in the CIRCLE study after signing informed consent. This CIRCLE study investigates cellular immunity against HPV in HPV-induced (pre)malignant lesions and was approved by the Medical Ethical Committee of the LUMC. Patient characteristics are described in Table 1. All patients underwent radical hysterectomy type III and pelvic lymph nodes (LN) were histologically evaluated for the presence of metastatic disease. Sixty percent of the patients received additional radiotherapy in the months after surgery. The subjects were tested for HPV status on DNA isolated from surgical resection specimens (20). Three years and 5-years follow up data were present for 86 % and 60% of the patients.

Blood samples were drawn at the day of and prior to surgery. PBMC were isolated by Ficoll density centrifugation. A proportion of these PBMC was tested directly ex-vivo in a proliferation assay and the remaining cells were cryopreserved in liquid nitrogen. DNA was isolated from granulocytes for the determination of the HLA class I and II type of the patient at the national reference laboratory for histocompatibility testing (LUMC, The Netherlands) by PCR using sequence-specific oligonucleotides and the IMGT/HLA database v2.24.0 (21).

Antigens and Lymphocyte stimulation test (LST)

Pools of overlapping 22-mer peptides spanning the entire HPV16 or HPV18 E6 and E7 proteins were used for the T-cell assays as described previously (6;22). Memory response mix (MRM), consisting of tetanus toxoid (0.75 limus flocculentius/mL; Netherlands Vaccine Institute), sonicated *Mycobacterium tuberculosis* (5 µg/mL; kind gift from Dr. P. Klatser, Royal Tropical Institute) and *Candida* (0.015%; HAL Allergenen Lab) was used as positive control (6;22).

Table 1 Patient group.

	N	%
No. Patients	119	
Mean age*	44,9	
years (range)	25-76	
FIGO stage		
≥ 1b	107	90
2a/b	12	10
HPV type		
HPV16+	77	65
HPV18+	27	23
other types'	7	6
NT or non detectable	8	7
LN metastasis'		
yes	35	29
no	81	68
unknown	3	3
Tumor size		
< 4cm	64	54
≥ 4 cm	44	37
unknown	11	9
Infiltration depth		
≥ 15mm	36	30
< 15 mm	76	64
unknown	7	6

*At time of intervention 'At time of surgery
'Others include HPV type 31(n=2), 33 (n=1) 39 (n=1), 45 (n=2), 69 (n=1)

The capacity of T cells to proliferate on stimulation with the antigen was determined by the lymphocyte stimulation test (LST) as described earlier (4). The average and SD of the 8 medium-only control wells were calculated and the cutoff was defined as this average plus 3 x SD. The stimulation index 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 of the 8 wells had to be above the cutoff value.

Phenotypical T-cell analysis by flow cytometry

PBMC were thawed and seeded in three wells of a 24-well plate (Costar) at a concentration of $1x10^6$ cells/ml in IMDM supplemented with 10% human AB serum (PAA laboratories). The complete set of overlapping 22-mer peptides of HPV16 E6 or E7 were added at final concentration of 5ug/ml. No peptide (medium only) served as negative control.

At the starting point and after 7 days of culture, cells were harvested, washed in cold PBS/5%BSA and stained for the surface markers CD4-APC, CD25-FITC, CD8-PerCP (BD Biosciences) and subjected to the intra-cellular staining protocol for FoxP3-PE (clone PCH101 Ebiosciences) or the isotype control according to the protocol of the manufacturer.

Two well validated clones, the intranuclear FoxP3- expressing clone C148.31 and the FoxP3 negative clone C271.9 (11;23) were used to discriminate between background staining of the cytosol (C271.9) and true high intra-nuclear staining (C148.31) of FoxP3 because we noticed that the official isotype control displayed less background staining than clone PCH101. The samples were measured by flow cytometry (FACS-CALIBUR, BD Biosiences) and evaluated using Cellquest software (BD Biosciences).

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-4 and IL-2 were determined. According to manufacturer's instructions the proposed detection limit was 20 pg/ml. However, for IFNγ the cut-off value

was set to 50 pg/ml. Positive antigen specific cytokine production was defined as a cytokine concentration above the cut-off value and at least two-fold above the concentration of the medium control (5).

Statistical analyses

The HLA types of CxCa patients were compared for dedicated alleles with a cohort of healthy blood donors derived from the area of Leiden as published by Schipper et al (24). For each of the tested alleles (DRB1*07, 13 or 15) the frequency present in CxCa patients was compared to the frequency in the control cohort by the 2-tailed Fischer's exact test. Note that no information was available about the linkage of DR and DQ alleles in this control group. The relationship between the presence or absence of an HPV-specific immune response and tumor size, LN status or infiltration depth was determined by the two-tailed Mann-Whitney test. The difference in the mean of CD4+CD25+ or CD4+CD25+Foxp3-expressing cells between patients with or without an HPV-specific proliferative response was determined by a two-tailed Mann-Whitney test. The association between the frequencies of both $CD4^{+}CD25^{+}$FoxP3-positive and $CD4^{+}CD25^{high}$Foxp3-negative T cells simultaneously present in PBMC of patients was tested by the Wilcoxon signed rank test. To determine the impact of the immune response on the survival of patients a logrank analysis using graphpad prism software version 4.02 was performed. Furthermore post-hoc analyses using the logrank test were performed to study the impact of immunity on disease free survival in subgroups of patients. Formal testing of these associations of tumor characteristics and the presence of HPV specific immunity was performed with Cox regression using SPSS version 17.0 software package for windows (SPSS inc. Chicago, USA). A p-value <0.05 was considered to be significant.

RESULTS

Patient population

One hundred and nineteen women with cervical cancer (CxCa; Figo stage 1b to 2b) enrolled in the Circle study onto April 2007 were studied. Patient characteristics are shown in table 1. The mean age of these 119 CxCa patients was 44.9 years (range 23.4 to 76.4 years). The majority (>95%) of the patients were from Dutch origin. PBMC used to determine the presence of HPV-specific T-cell immunity were isolated from blood samples drawn at the same day as but prior to surgery. Tumor tissue of all CxCa patients was analyzed for the presence of HPV-DNA. In 77 cases (65%) HPV16 and in 27 patients (23%) HPV18 was detected. Other virus types were only found at a low frequency and in 8 cases no HPV could be detected (Table 1). As >90% of all recurrences occur within 3 years after surgery (25) and follow-up for this period was available for almost all patients (86%), we analyzed the disease-free 3-years survival after surgery which was 81%, which is comparable to the percentage found previously (25). The group of patients for whom 5-years follow-up was available was too small (60%) to allow for a meaningful analysis.

The frequency of HLA alleles associated with risk or protection for CxCa

Three HLA class II alleles have consistently been reported to be related with either a risk (DRB1*07; DRB1*15/DQB1*0602) or with protection (DRB1*13) against cervical (pre-)malignant lesions (15;17;26). In order to confirm these respective associations in the current cohort – as this allows

Table 2. Expression of specific HLA-DR frequencies in CxCa patients.

	CxCa patients				
HLA[§]	**All n=119**	**HPV16+ n=77**	**HPV18+ n=27**	**Controls[˙]**	
DR* 07	30/89	25/52	3/24	459/1937	** 0,0059[˙]
	25%	**32%****	**11%**	**19%**	
DR* 13	21/98	17/60	4/23	669/1686	* 0,014[˙]
	18%*	**22%**	**15%**	**28%**	
DR* 15	35/84	25/52	6/21	414/1208	
	29%	**32%**	**22%**	**26%**	

[§]The frequency of HLA-DR types previously reported to be associated with disease (DR*07, DR*15) or protection (DR*13) were determined. Numbers indicate the number of patients positive/negative for this specific HLA-type within this group.
[˙]Controls are taken form a published Dutch cohort of healthy blood donors (Schipper et al. 1996) [˙] *P*-value, statistical analysis was performed with Fisher's exact test, two-sided.

a more meaningful analysis of a potential relationship between HPV-specific immunity and these disease-associated or protective alleles – the frequency of these described 'risk' or 'protective' alleles was determined in our study group and subsequently compared to the previously published Dutch control population that consists of more than 1622 healthy individuals (24).

Thirty of the 119 CxCa patients (25%) carried the DRB1*07 allele compared to 19.2% in the control group (p=0.13). When only the group of patients with an HPV16-induced tumor was analyzed the frequency of the DRB1*07 allele was significantly increased (p=0.006; Table 2). The DRB1*07 allele seems to be underrepresented in patients with HPV18-induced cancers but the group is too small to allow for a firm conclusion. The frequency of DRB1*15/DQB1*0602 is slightly but not significantly increased in our patient population (Table 2). Similar to previous observations DRB1*13 was found to be underrepresented in the total group of patients with CxCa as only 18% (21 patients) displayed this allele compared to 28.4% in the control population (p=0.014; Table 2).

A third of the patients with HPV-induced cancer mount HPV-specific immunity

In 94 patients with HPV16- or HPV18-induced cervical cancer we determined the presence of circulating HPV-specific T cells by a 7-day lymphocyte stimulation test (LST) (4;6). We have previously shown that this assay is geared towards the detection of predominantly CD4+ T-cell responses (5;6). Freshly isolated PBMC were stimulated with 4 different peptide pools of the E6 and 2 peptide pools of the E7 oncoproteins for HPV16 and HPV18. The immune response to the cognate HPV type was used to determine the response rate (Figure 1a). Only 29 out of 94 (31%) displayed a demonstrable HPV-specific proliferative T–cell response (Figure 1b; LST+). In 16 out of 23 responding HPV16+ patients, the T cells reacted against E6 and in 14 patients against E7. In 7 cases a response to the peptides of both oncoproteins was found. Six of the 18 HPV18+ patients responded to HPV18 peptides. Three responded only to E6 and 3 to the peptides of E7, including 2 patients who responded both to E6 and E7. Notably, this response rate is much lower as previously reported for healthy controls (5;6;22;27).

Supernatants, obtained at day 6 of these proliferation assays were analyzed for the presence of HPV-specific production of Th1 and Th2 cytokines by cytometric bead array. In only 8 of all 29 patients that showed a HPV-specific proliferative response (28%), this proliferation was

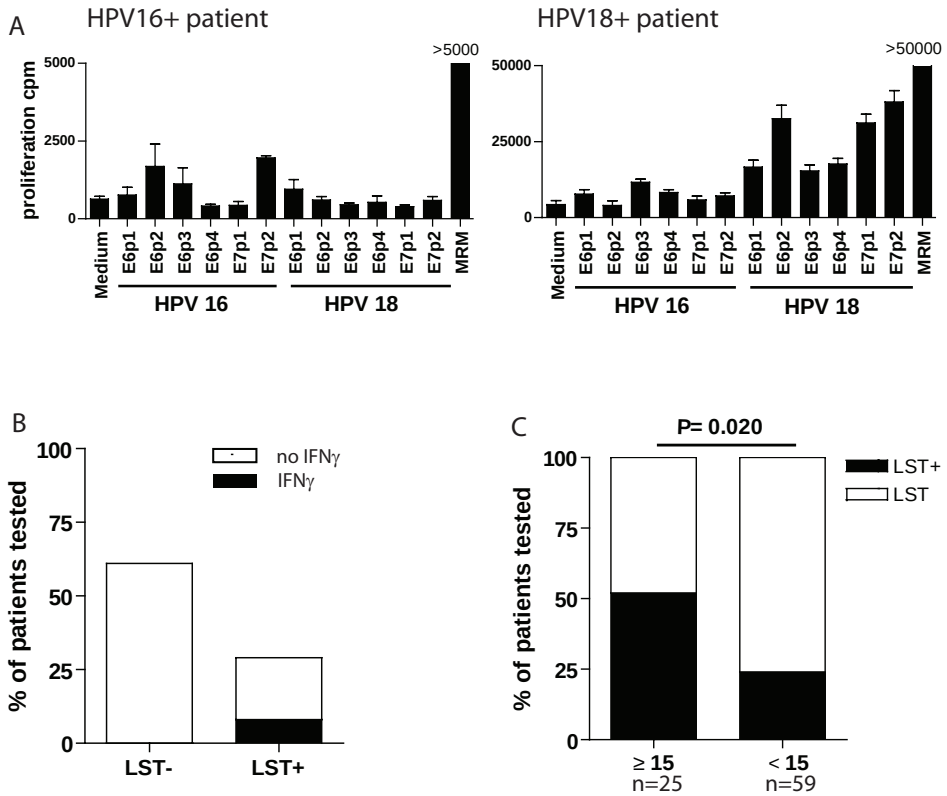

Figure 1 HPV-specific immunity in patients with cervical cancer. (A) PBMC obtained at the day of surgery were stimulated with HPV peptide pools representing either the HPV16 or HPV18 amino acid sequence of the two oncoprotein E6 and E7 (E6 pool 1-4, E7 pool 1-2) or memory response mix (MRM) and after 6 days the proliferation was measured using ^{3}H-thymidine incorporation. Shown is an example of the response detected in an HPV16-positive (left) or HPV18-positive (right) CxCa patient. (B) The percentage of patients (n=65) in whom no HPV-specific proliferative response (LST-) and patients (n=29) who did show an HPV-specific T-cell response (LST+) is depicted. In 8 of these 29 cultures proliferation was accompanied by IFNγ production. (C) The presence of an HPV-specific proliferative T-cell response is associated with deep stromal invasion by the tumor (p=0.02, two-tailed Mann-Whitney). Patients were divided into two groups on basis of the depth of infiltration (≤15mm or >15mm) and the percentage of patients within these groups displaying a systemic HPV-specific proliferative immune responses (LST+) is depicted.

accompanied by the production of detectable amounts of the Th1-effector cytokine IFNγ as depicted in Figure 1b and Table 3. In contrast, IFNγ is detected in two-thirds of the HPV-specific proliferative responses of healthy subjects (4;27). Furthermore, for 3 patients the specific production of IL-10 and in 4 cases TNFα was detected (Table 3). IL-2 was not detected in these cultures which highly likely can be contributed to it's consumption by the proliferating cells as this cytokine was not provided during the culture.

In each assay the positive control recall antigen mix MRM was taken along. This revealed that nearly all of the patients readily responded to bacterial antigens by proliferation and the production of IFNγ (data not shown).

Table 3. Overview of the responses in patients with HPV-specific cytokine production.

						cytokine production**	
patient	**HPV type†**	**Antigen‡**	**peptide pool§**	**SI***	**IFNγ**	**IL-10**	**TNFa**
A	16	E6	121-158	4,7	123	28	25
B	16	E7	51-98	4,6		21	
C	18	E7	1-98	6,7	121		
D	16	E7	51-98	6,4	259		
E	18	E6	41-92	7,6	2943		
		E7	1-62	7,3	2617	64	164
		E7	51-98	8,9	3150	47	67
F	18	E7	51-98	9,2	139		
G	18	E6	1-52	20	3400		56
		E6	41-92	9,5	195		
H	16	E7	51-98	5,8			65
I	16	E7	51-98	6,2	50		
J	16	E6	41-92	7,9	1244		

The 10 patients which in addition to proliferation also produced IFNg, TNF or IL-10 upon HPV-specific stimulation are depicted by a letter (A-J)
†HPV type as found in the tumor by PCR
‡indicates the antigen (E6 or E7) of the HPV type present in the tumor to which the patient responded
§the numbers indicate the first and the last amino-acid of the 22-mer peptide pool of the corresponding antigen to which the PBMC responded by proliferation
*SI=Stimulation index, the fold difference in proliferation when PBMC are stimulated with the indicated peptide pool when compared to PBMC stimulated with medium only
** Specific cytokine production (pg/ml) by PBMC stimulated with the indicated peptide as detected in the pooled supernatant of the 8 replicate test wells;
A specific response was defined as a peptide pool-induced production which was at least twice above background (medium control) and above the cut-off of 20 pg/ml (IL10 and TNFa) or 50 pg/ml (IFNγ)
Only the cultures with a positive cytokine response are shown

The detection of HPV-specific CD4+CD25+FoxP3-positive T cells coincides with that of HPV-specific CD4+CD25+ T cells

Previously we showed that CD4+CD25+Foxp3+ HPV-specific regulatory T cells can be isolated from tumors and lymph nodes of patients with CxCa (11) as well as can be detected in their blood as measured by the co-expression of the intra-nuclear transcription factor FoxP3 and the HPV-specific activation induced upregulation of CD25 ten days after antigen-specific stimulation (28). Although functional assays are the golden standard for the classification of Treg the intra-nuclear transcription factor FoxP3 is the best marker to date(29).Using this approach we determined the frequency of HPV-specific CD4+CD25+FoxP3-positive T cells in 41 patients with HPV16+ CxCa of whom enough PBMC were available. Two previously described HPV-specific regulatory T-cell clones (11;23) were used to set up a stringent gating strategy to ensure the enumeration of $CD25^{high}$ and $FoxP3^{high}$ cells only (Figure 2).

On average 3.4% of the peripheral CD4+ T cells expressed FoxP3 directly *ex-vivo* (Figure 2a), similar to observations in other types of cancer (30). Notably, non-stimulated PBMC cultured for 7 days comprised similar frequencies of FoxP3 expressing CD4+ T cells as PBMC stained *ex-vivo* (Figure 2a), demonstrating that our culture conditions do not induce non-specific FoxP3 expression.

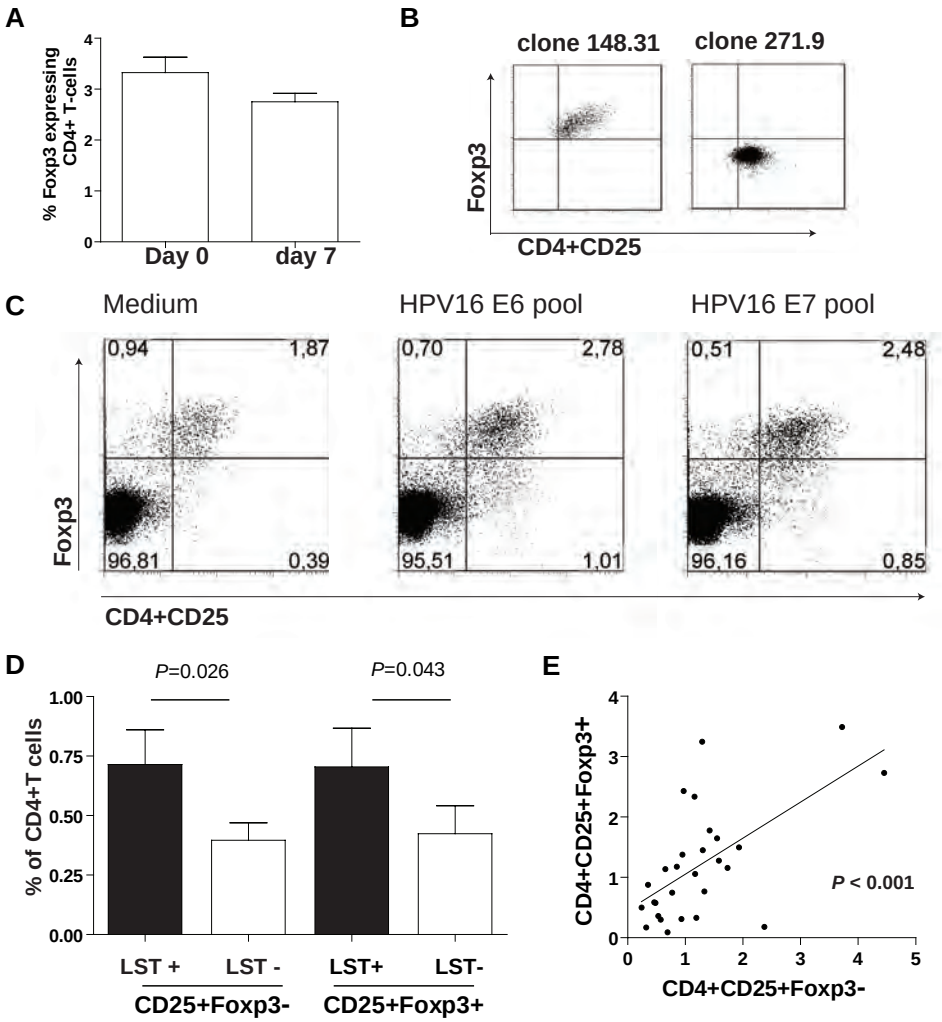

Figure 2 The detection of HPV-specific proliferative responses is associated with higher percentages of HPV-specific FoxP3-positive and-negative CD4+CD25+ T cells. PBMC of HPV16+ CxCa patients isolated before surgery were cultured for 7 days in the absence or presence of the cognate HPV peptides E6 or E7 and stained for the expression of CD25 and the transcription factor FoxP3. (A) On average 3% of the circulating CD4+ T-cells express FoxP3 directly ex-vivo (left bar) and this percentage is not altered when the T cells are cultured for 7 days in medium (right bar). (B) Two HPV-specific T-cell clones, either expressing intranuclear FoxP3 (148.31) or not (271.9) were used to optimize the gating strategy in the experiments. (C) Dot plot example of one HPV16+ patient showing CD4+CD25+ T-cells and CD4+CD25+FoxP3-positive T cells without stimulation (medium) or after stimulation with HPV16 E6 (middle) or E7 (right) as measured by flow cytometry. (D) Increased percentages of HPV-specific CD25+ (activated) and CD25+FoxP3-positive CD4+ T cells are found when patients display specific proliferation in the blood as compared to patients that do not show any T-cell response. The mean (±SEM) percentage of HPV E6- and E7-specific CD4+CD25+ or CD4+CD25+FoxP3-positive T cells is depicted for the group of patients lacking HPV-specific proliferation (LST-; n=27) or displaying HPV-specific T-cell proliferation (LST+; n=14). Means are calculated from stimulated cells after subtraction of PBMC in medium only. P-values were calculated using two-tailed Mann-Whitney. (E) In LST+ patients the detection of both types of HPV-specific CD4+ T-cells is correlated as analyzed by the Wilcoxon signed rank test.

Of the 41 patients tested, 14 displayed an HPV-specific proliferative response while the other 27 patients displayed no detectable proliferative response. Notably, CD4+CD25+FoxP3-positive T cells were already present in non-stimulated cell cultures (medium). The population of $CD25^{high}FoxP3^{high}$ T cells consisted on average of 0.83% (range 0.1-3.0) of total CD4+ T cells, whereas CD4+ T cells with a helper phenotype ($CD4+CD25^{high}$FoxP3-negative) were scarcely present in these control cultures (Figure 2). In the HPV peptides-stimulated cultures, however, both populations are present in the majority of the samples (Figure 2c). Significantly higher numbers of HPV-specific $CD4+CD25^{high}$FoxP3-negative and $CD4+CD25^{high}FoxP3^{high}$ T cells were detected in the HPV-stimulated PBMC cultures of the group of patients whom displayed an HPV-specific proliferative response (Figure 2d, p= 0.03 and p=0.04, respectively), when compared to the group of patients not able to mount an HPV-specific proliferative response. Moreover, the detection of HPV-specific T cells with either a $CD4+CD25^{high}$FoxP3-negative Th-phenotype or with a $CD4+CD25^{high}FoxP3^{high}$ T-cell phenotype was correlated in the patient group displaying HPV-specific proliferative responses ($p<0.001$; Figure 2e). No correlation between the presence of LN metastasis and the numbers of CD4+ $CD25^{high}$FoxP3-negative and $CD4+CD25^{high}FoxP3^{high}$ cells was found.

Notably, although recently CD8+FoxP3+ T cells were detected in the lymph nodes of early stage CxCa patients (31), we could not detect such a population in the PBMC of our CxCa patients (data not shown).

Deep tumor-infiltration of the surrounding tissue is associated with detectable HPV-specific immune responses

In order to assess whether relationships exist between the absence or presence of HPV-specific proliferative responses and the known disease related or protective HLA class II alleles the patients were divided according to immune status and presence or absence of a particular HLA allele (DRB1*07, DRB1*15 or DRB1*13). However, no relationship between the absence or presence of HPV-specific immunity and the presence of these alleles was found.

Known prognostic factors in cervical cancer are LN metastases (present or absent), the size of the tumor (< or ≥ 4cm) and the invasion depth of the tumor in the surrounding cervical tissue (< or ≥ 15mm) (19). These prognostic factors were entered into the Cox proportion hazard model, which revealed a correlation between tumor size and the presence of LN metastases ($p=0.003$) as well as with invasion depth of the tumor ($p=0.002$). However, depth of invasion was not correlated to LN metastasis in this cohort ($p=0.21$).

Subsequently, the presence of an HPV-specific immune response was also analyzed in relation to the LN status in 93 patients, tumor size (n=82) and invasion depth (n=84). Patients were grouped according to the presence or absence of an HPV-specific T-cell response. No differences were found between these groups with respect to tumor size at time of surgery.

In 48% of the patients with nodal metastases HPV-specific T cells were detected, whereas only 27% of the patients without metastasizing tumors displayed an HPV-specific T-cell response ($p= 0.06$; Fisher's exact two sided, data not shown). Interestingly, the group of patients with a deeply infiltrating tumor of at least 15 mm, significantly more often display a detectable T-cell response than patients with less invasive tumors (Figure 1c, $p=0.02$; Fisher's exact two-sided).

Patients with deep infiltrating tumors and HPV-specific immunity display improved survival

The great majority of recurrences occur within 3 years after surgery (25). When the disease-free 3-year survival curves of the CxCa patients are plotted on the basis of the aforementioned prognostic factors or the presence of HPV-specific immunity (Figure 3), the patients with large tumors (≥ 4 cm) are prone to have recurrent disease (Figure 3c, 37% vs 18%, p=0.02). In contrast to patients with less deep stromal invasion relatively more patients with a deeply infiltrating tumor (≥ 15mm) displayed a recurrence of disease within 3 years (Figure 3d 20% vs 35%; p=0.14). Survival was not different when all patients were grouped according to HPV-specific immune status (p=0.45; Figure 3a).

As we have found that HPV-specific immunity predominantly was detected in patients with deep infiltrating tumors, a subgroup analysis was performed in which the patients were divided according to the presence of an HPV-specific proliferative response and one of the prognostic parameters. These analyses suggested that the presence of HPV-specific proliferative T cells

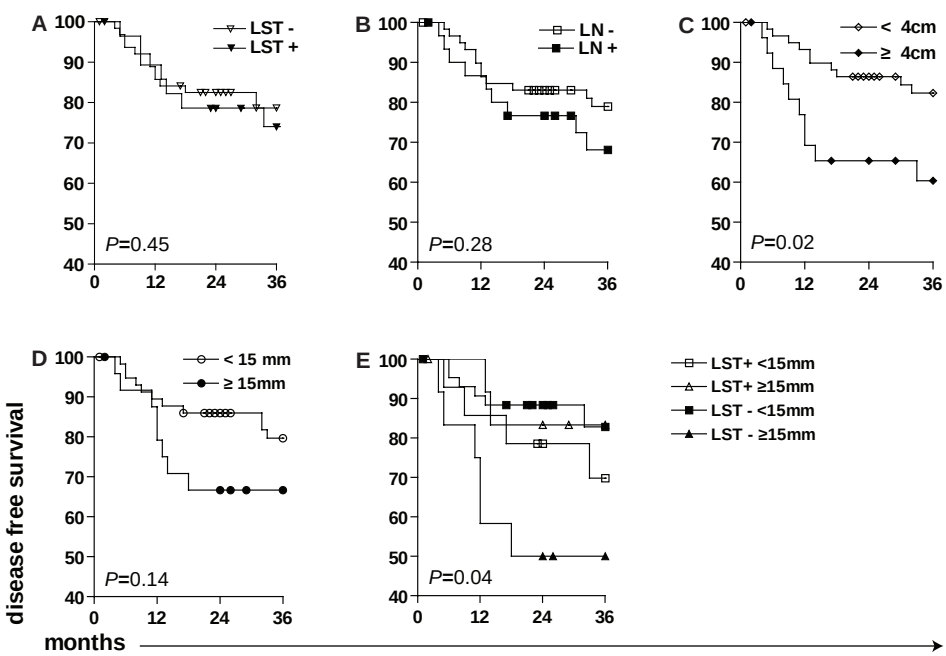

Figure 3. Disease free 3-year survival curves. Disease free survival for the patients is plotted when patients are divided on basis of (A) the presence of an detectable immune response by LST (LST-: open symbols n=64, LST+:closed symbol n=29 (B) the presence or absence of LN metastasis (LN-: open symbols n=60, LN+: closed symbols n=31), (C) a prognostic-defined relevant tumor size (< 4cm: open symbols n=60, ≥ 4cm: closed symbols n=27), or (D) a prognostic-defined relevant depth of stromal invasion (< 15mm: open symbols n=58, ≥ 15mm: closed symbols n=25). (E) Patients are grouped on the presence or absence of an immune response and the depth of infiltration. Notably, the group of patients with deeply infiltrating tumors is divided into 13 patients (open triangles) displaying an HPV-specific proliferative response and 12 patients lacking a detectable response (closed triangles). Symbols represent censored patients. P-values are determined by logrank analysis.

Table 4. Multivariate Cox regression analysis of immune response and invasion depth on risk of recurrence.

Immune response	Infiltration depth	n	HR	95% CI	*p*-value
LST -	< 15 mm*	44	1		
LST -	≥ 15 mm	12	4,33	1,45 - 12,96	0,01
LST +	< 15 mm	14	2,21	0,70 - 6,97	0,18
LST +	≥ 15 mm	13	1.178	0,24 - 5,68	0,84

HR, Hazard ratio; CI, confidence interval; * Reference category
LST- indicates no HPV-specific proliferation by lymphocyte stimulation test
LST+ indicates a HPV-specific proliferation by lymphocyte stimulation test

(LST+) is beneficial in patients with deep (≥ 15mm) infiltrating tumors as the percentage of patients with recurrence was much lower in this group than in those patients without a detectable HPV-specific proliferative response (Figure 3e 18% vs 50%; p=0.043, logrank test). Notably, all patients with invasion of ≥ 15 mm received radiotherapy as additional treatment. Analyses of HPV-specific immunity in relation to LN status or tumor size did not reveal any differences (not shown).

Multivariate analyses of these four groups by Cox proportion hazard analysis confirmed these observations (Table 4). While deep stromal invasion without a demonstrable immune response showed a highly increased disease recurrence risk (HR 4.33, 95% CI 1.45-12.96) as compared to having a less invasive tumor and no immune response, the presence of a detectable HPV-specific proliferative response was associated with a lower risk of recurrence in the patient group with deeply invading tumors (HR 1.18, 95% CI 0.24-5.68).

DISCUSSION

This is the largest prospective study of women with HPV-16 or -18-induced cervical cancer in which the HPV-specific immune response was analyzed in relation to known factors predicting disease prognosis. The PBMC of most (69%) of the 94 tested patients failed to proliferate when stimulated with cognate HPV E6 or E7 peptides *in vitro*. Furthermore only in a limited number of patients HPV-specific T cell responses were associated with the production of IFNγ. HPV-specific immunity - in patients who showed an HPV-specific proliferative response - consisted of circulating FoxP3-negative and –positive CD4+C25+ T cells. This large study confirms previous findings in small groups of patients (5;32-34). Moreover, it allows a more definitive conclusion with respect to the response rate and the type of the T-cell response to the tumor-specific HPV E6 and E7 antigens in patients with CxCa as well as scrutiny of the relationship between HPV-specific immunity and tumor characteristics or survival.

Interestingly, the presence of circulating HPV-specific proliferative T cells was associated with deep infiltration of the tumor in the surrounding normal tissue (p=0.02). Recently, we reported that surgery-mediated tissue destruction of lesions was strongly associated with the induction of HPV-specific immunity in patients with HPV16+ HSIL (10). Together these data suggest that the induction of HPV-specific immunity in patients with HPV-induced established cervical neoplasia is the result of local destruction of normal tissue and contact with antigen presenting cells therein, allowing the presentation of HPV antigen and the activation of naïve

or memory HPV-specific T cells. Possibly, this normally does not occur because of the tumor-induced lack of mobility and tolerogenic phenotype of DC within cervical tumors (35;36). The fact that tumor size is not related to the induction of HPV-specific immunity can be explained because generally tumors increase in size by growing into the lumen of the cervix.

Because the number of patients within our cohort was too low to perform an unselected HLA-association study, we chose to study the frequency of only three HLA class II alleles which have consistently been reported to be related with either a risk or with protection against cervical (pre-) malignant lesions. The combination of the two HLA class II alleles DRB1*13-DQB1*0603 was found to be protective in 9 out of 19 studies reviewed by Hildesheim (17). It is unclear which allele is important since DRB1*1301 and DQB1*0603 are in linkage disequilibrium. In our cohort DRB1*13 was also found to be underrepresented (p=0.014) when compared to a large group of Dutch controls (24). Furthermore, the allele DRB1*07 was found to be associated with an increased risk for the development of CIN lesions in Dutch patients (15). Here, we show that this allele is also present at a significantly higher frequency (p=0.006) in our group of patients with HPV16+ CxCa sustaining the observation that DRB1*07 is associated with a risk to develop HPV16-induced malignancies. The mechanism through which this may operate may relate to the absence or type of CD4+ T-cell response induced but we did not find a relationship between HPV-immunity and these HLA types in our cohort of cancer patients. The events -associated with protection or failure- possibly occur already at an early stage of the infection and as such, are no longer demonstrable in patients with cancer.

The size and design of our study, in which patients were followed up after surgery, allowed us to study the impact of HPV-specific immune responses in relation to disease-free survival at 3 years. We did not find a direct correlation between survival and the presence of circulating tumor-specific T cells. A similar study in patients with melanoma also failed to detect a direct correlation (37), albeit that there only the numbers and not the function of the tumor-specific T cells was studied. Here, the presence of HPV-specific T cells was determined by function (proliferation). Interestingly, subgroup analyses revealed that those patients with deeply infiltrating tumors – who in general display a low survival rate (67%, Figure 3d) – and circulating HPV-specific T cells able to proliferate when stimulated with E6 or E7 antigen, displayed a better 3-year disease free survival (82%) than patients lacking such an immune response (50%; p=0.043). Also, Cox proportion hazard analysis of these four subgroups showed a strong reduction of the risk of disease recurrence, in that the hazard ratio decreased from 4.3 to 1.2, when patients with deeply infiltrating tumors displayed an HPV-specific proliferative response (Table 4). Notably, both groups of patients comprised predominantly FIGO stage 1b tumors and all patients had received additional treatment with radiotherapy, excluding this as a variable. A recent study suggests that the presence of HPV-specific immunity reflects disease severity and is not associated with increased 3-year disease free survival in a small group of 32 patients (32).This is likely related to the stage of disease, as the group studied consisted mainly of patients with a more advanced stage of disease (FIGO 2b-3b), whereas we studied patients with an earlier stage (FIGO 1b-2b) of disease. We find an increase in proliferative responses in patients with LN metastasis when the subgroup analyses were performed on the basis of absence or presence of LN metastasis. It is described that T cells within tumor-draining metastasized LN might display a more suppressive phenotype suggesting that these suppressive T cells may suppress tumor-specific immunity (31). However, we did not observe differences in the phenotype of the PBMC of patients with LN metastatic

disease or without metastatic disease. Survival benefit of the presence of a HPV-specific response thus may be very specific for the group of patients with deeply infiltrating tumors.

The picture that emerges from these data is that during progression of tumors, as evidenced by the deeper infiltration of tumors in the normal surrounding tissue, HPV antigens can be taken up and presented by antigen presenting cells most likely in the less suppressive context of the adjacent tissue. This results in activation of HPV-specific T cells, defined by their capacity to either proliferate or suppress HPV-specific T cells. The latter is also supported by others who report that HPV-specific immunity coincides with enhanced levels of regulatory T cells (38;39). Also in melanoma the presence of circulating antigen-specific regulatory T cells has been reported (40).

A key effect of HPV-specific regulatory T cells is the suppression of proliferation and the production of cytokines by effector cells, including IFNγ (11). The low number of HPV-specific responses associated with the production of IFNγ suggests that such responses may have been suppressed. However, the detection of HPV-specific proliferation in 31% of the patients implies that if suppression occurs this is not always strong enough to suppress T cell function completely and HPV specific T cells can have a beneficial antitumor effect as the 3-year disease free survival of patients with deeply infiltrating tumors displaying an immune response is better than those without such a response.

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REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;**55(2)**:74-108.
2. Ho GYF, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural History of Cervicovaginal Papillomavirus Infection in Young Women. *N Engl J Med* 1998;**338(7)**:423-8.
3. zur Hausen H. Papillomavirus infections--a major cause of human cancers. *Biochim Biophys Acta* 1996;**1288(2)**:F55-F78.
4. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res* 2002;**62(2)**:472-9.
5. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, van der Burg SH. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. *Cancer Res* 2004;**64(15)**:5449-55.
6. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. *Cancer Res* 2003;**63(3)**:636-41.
7. van den Hende M, van Poelgeest MI, van der Hulst JM, de JJ, Drijfhout JW, Fleuren GJ, Valentijn AR, Wafelman AR, Slappendel GM, Melief CJ, Offringa R, van der Burg SH, et al. Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. *Int J Cancer* 2008;**123(1)**:146-52.

8. Kenter GG, Welters MJP, Valentijn AR, Lowik MJG, Berends-van der Meer D, Vloon APG, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, et al. Vaccination against HPV-16 Oncoproteins for Vulvar Intraepithelial Neoplasia. *N Engl J Med* 2009;**361(19)**:1838-47.
9. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Scheper RJ, Stacey SN, Duggan-Keen MF, Stern PL, Man S, Borysiewicz LK, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. *Int J Cancer* 2000;**88(1)**:92-8.
10. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW, Kenter GG, van der Burg SH. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. *Clin Cancer Res* 2008;**14(22)**:7188-95.
11. van der Burg SH, Piersma SJ, de JA, van der Hulst JM, Kwappenberg KM, van den HM, Welters MJ, van Rood JJ, Fleuren GJ, Melief CJ, Kenter GG, Offringa R. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. *Proc Natl Acad Sci U S A* 2007;**104(29)**:12087-92.
12. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW, Melief CJ, Kenter GG, Fleuren GJ, Offringa R, van der Burg SH. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 2007;**67(1)**:354-61.
13. Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, Melief CJ, van der Burg SH. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res* 2009;**15(20)**:6341-7.
14. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG, van der Burg SH, Fleuren GJ. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res* 2008;**14(7)**:2028-35.
15. Bontkes HJ, van DM, de Gruijl TD, Duggan-Keen MF, Walboomers JM, Stukart MJ, Verheijen RH, Helmerhorst TJ, Meijer CJ, Scheper RJ, Stevens FR, Dyer PA, et al. HPV 16 infection and progression of cervical intra-epithelial neoplasia: analysis of HLA polymorphism and HPV 16 E6 sequence variants. *Int J Cancer* 1998;**78(2)**:166-71.
16. Ghaderi M, Wallin KL, Wiklund F, Zake LN, Hallmans G, Lenner P, Dillner J, Sanjeevi CB. Risk of invasive cervical cancer associated with polymorphic HLA DR/DQ haplotypes. *Int J Cancer* 2002;**100(6)**:698-701.
17. Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. *Virus Research* 2002;**89(2)**:229-40.
18. Krul EJ, Schipper RF, Schreuder GM, Fleuren GJ, Kenter GG, Melief CJ. HLA and susceptibility to cervical neoplasia. *Hum Immunol* 1999;**60(4)**:337-42.
19. Singh N, Arif S. Histopathologic parameters of prognosis in cervical cancer--a review. *Int J Gynecol Cancer* 2004;**14(5)**:741-50.
20. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. *Am J Pathol* 1989;**135(4)**:703-9.
21. Verduyn W, Doxiadis II, Anholts J, Drabbels JJ, Naipal A, D'Amaro J, Persijn GG, Giphart MJ, Schreuder GM. Biotinylated DRB sequence-specific oligonucleotides. Comparison to serologic HLA-DR typing of organ donors in eurotransplant. *Hum Immunol* 1993;**37(1)**:59-67.
22. van der Burg SH, Ressing ME, Kwappenberg KM, de JA, Straathof K, de JJ, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. *Int J Cancer* 2001;**91(5)**:612-8.
23. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, Melief CJ, Hellebrekers BW, Fleuren GJ, Kenter GG, Offringa R, van der Burg SH. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. *Int J Cancer* 2008;**122(3)**:486-94.
24. Schipper RF, Schreuder GM, D'Amaro J, Oudshoorn M. HLA gene and haplotype frequencies in Dutch blood donors. *Tissue Antigens* 1996;**48(5)**:562-74.
25. Ayhan A, Celik H, Dursun P, Gultekin M, Yuce K. Prognostic and therapeutic importance of lymphadenectomy in gynecological cancers. *Eur J Gynaecol Oncol* 2004;**25(3)**:279-86.
26. Apple RJ, Erlich HA, Klitz W, Manos MM, Becker TM, Wheeler CM. HLA DR-DQ associations with cervical carcinoma show papillomavirus-type specificity. *Nat Genet* 1994;**6(2)**:157-62.

27. Welters MJ, van der LP, van den Eeden SJ, Kwappenberg KM, Drijfhout JW, Fleuren GJ, Kenter GG, Melief CJ, van der Burg SH, Offringa R. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. *Int J Cancer* 2006;**118(4)**:950-6.
28. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, Drijfhout JW, Valentijn AR, Wafelman AR, Oostendorp J, Fleuren GJ, Offringa R, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin Cancer Res* 2008;**14(1)**:178-87.
29. Piersma SJ, Welters MJ, van der Burg SH. Tumor-specific regulatory T cells in cancer patients. *Hum Immunol* 2008;**69(4-5)**:241-9.
30. Bignone PA, Banham AH. FOXP3+ regulatory T cells as biomarkers in human malignancies. *Expert Opinion on Biological Therapy* 2008;**8(12)**:1897-920.
31. Battaglia A, Buzzonetti A, Baranello C, Ferrandina G, Martinelli E, Fanfani F, Scambia G, Fattorossi A. Metastatic tumour cells favour the generation of a tolerogenic milieu in tumour draining lymph node in patients with early cervical cancer. *Cancer Immunol Immunother* 2009;**58(9)**:1363-73.
32. Delgado FG, Martinez E, Cespedes MA, Bravo MM, Navas MC, Combita Rojas AL. Increase of human papillomavirus-16 E7-specific T helper type 1 response in peripheral blood of cervical cancer patients after radiotherapy. *Immunology* 2009;**126(4)**:523-34.
33. Kaufmann AM, Stern PL, Rankin EM, Sommer H, Nuessler V, Schneider A, Adams M, Onon TS, Bauknecht T, Wagner U, Kroon K, Hickling J, et al. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. *Clin Cancer Res* 2002;**8(12)**:3676-85.
34. Visser J, van BD, Hoogeboom BN, Reesink N, Klip H, Schuuring E, Nijhuis E, Pawlita M, Bungener L, de Vries-Idema J, Nijman H, Miedema F, et al. Enhancement of human papilloma virus type 16 E7 specific T cell responses by local invasive procedures in patients with (pre) malignant cervical neoplasia. *Int J Cancer* 2006;**118(10)**:2529-37.
35. Herfs M, Herman L, Hubert P, Minner F, Arafa M, Roncarati P, Henrotin Y, Boniver J, Delvenne P. High expression of PGE2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells. *Cancer Immunology, Immunotherapy* 2009;**58(4)**:603-14.
36. Nakamura T, Shima T, Saeki A, Hidaka T, Nakashima A, Takikawa O, Saito S. Expression of indoleamine 2, 3-dioxygenase and the recruitment of Foxp3-expressing regulatory T cells in the development and progression of uterine cervical cancer. *Cancer Sci* 2007;**98(6)**:874-81.
37. van Oijen M, Bins A, Elias S, Sein J, Weder P, de Gast G., Mallo H, Gallee M, Van TH, Schumacher T, Haanen J. On the role of melanoma-specific CD8+ T-cell immunity in disease progression of advanced-stage melanoma patients. *Clin Cancer Res* 2004;**10(14)**:4754-60.
38. Molling JW, de Gruijl TD, Glim J, Moreno M, Rozendaal L, Meijer CJ, van den Eertwegh AJ, Scheper RJ, von Blomberg ME, Bontkes HJ. CD4(+)CD25hi regulatory T-cell frequency correlates with persistence of human papillomavirus type 16 and T helper cell responses in patients with cervical intraepithelial neoplasia. *Int J Cancer* 2007;**121(8)**:1749-55.
39. Visser J, Nijman HW, Hoogenboom BN, Jager P, van BD, Schuuring E, Abdulahad W, Miedema F, van der Zee AG, Daemen T. Frequencies and role of regulatory T cells in patients with (pre) malignant cervical neoplasia. *Clin Exp Immunol* 2007;**150(2)**:199-209.
40. Vence L, Palucka AK, Fay JW, Ito T, Liu YJ, Banchereau J, Ueno H. Circulating tumor antigen-specific regulatory T cells in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 2007;**104(52)**:20884-9.

CHAPTER 3

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

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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, 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 they are caused by the high-risk human papillomavirus (HPV) which encodes two 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 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 and 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 comprehensive analyses 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, 1b1/2) at the department of Gynecology of the Leiden University Medical Centre were enrolled in the CIRCLE study, which investigates cellular immunity against cervical lesions after providing informed consent. The study was approved by the Medical Ethical Committee. The subjects were tested for HPV status using HPV16 and HPV18 specific primers on DNA isolated from 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 with 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). At day 1, 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 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 1 hour at 37 ºC with collagenase (200 IU/ml, Sigma) and DNAse (50 μg/ml, Sigma), and 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 -20ºC 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 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 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 1μg/ml pool of E6 and E7 peptides, TCGF 10% and IL15. Toll like receptor (TLR) 4 ligand LPS 250 ng/ml (Sigma-Aldrich, USA), TLR3 ligand Poly(I:C) 12.5 μg/ml (InvivoGen, USA) and TLR1-2 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 these homeostatic cytokine-mediated expanded 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). All TIL reacted to PHA and proliferation was associated with large amounts of IFNγ and IL-10 (not shown).

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

				3 day Proliferation assay					Overnight activation analysis		
									Activated cells (%)§		
Patient	HPV type*	Origin	Days of culture††	Reactivity**	SI†	IFNγ‡	IL10‡	T-cell	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	>5000	315	CD4	0,8	63	0,9
								CD8	0	47	0,7
5	16	TIL	79	16E6	109	>5000	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	>5000	<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
** Reactivity indicates the HPVtype and protein to which the T-cell culture specifically reacted
† SI, Stimulation Index, average proliferation of test wells divided by the average profileration of medium control wells. SI>2 is positive
‡ Antigen-specific cytokine production in pg/ml is indicated when test value was above cut-off level and at least >2 times background production (4)
§ Percentage of total CD4 or CD8 T cell population expressing CD154 and/or CD137 after stimulation with indicated antigen corresponding to the HPV type present in the tumor. A response equal or more than 3x medium control was considered positive. || NT = Not tested

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, while HPV-specific CD8+ T cells predominantly expressed CD137, yet sometimes co-expressed CD154 (Figure 1a; Supplemental Figure 1). The percentage of HPV-specific T cells varied enormously between as little as 0.17% to as much as 66% (Table 1) of the

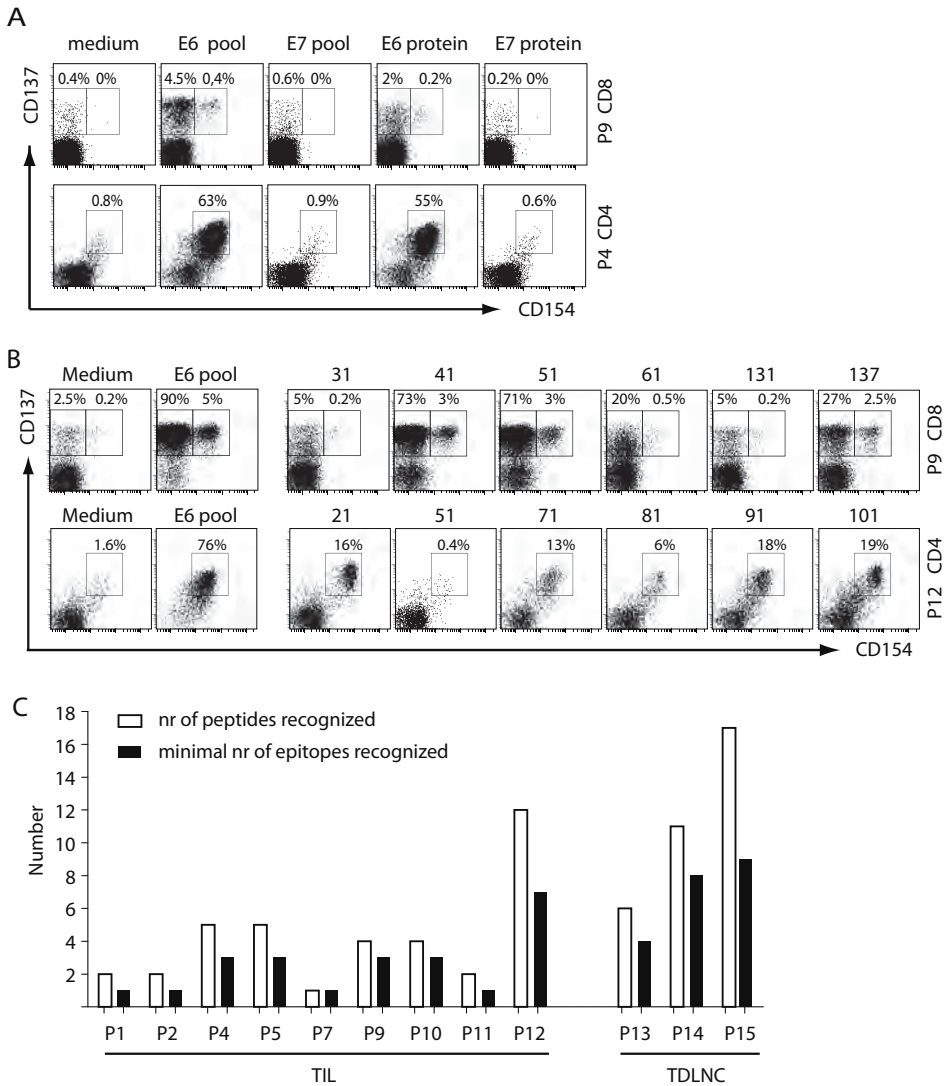


Figure 1. HPV-specific T cells in TIL and TDLNC. (A) An example of CD4 and CD8 HPV-specific responses measured by flow cytometry using the activation markers CD154 and CD137 in two TIL cultures tested before antigen-driven expansion *in vitro* (P4 and P9). (B) The breadth of the response was analyzed 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 22-mer peptides, which overlap by 12 amino acids, as one.

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

	Patient	Responding* T cell	Antigen	Peptides**	Number of TCRVß found per peptide** by FACS†			
					Dominant	Sub-dominant	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 TCR Vß analysis after antigen specific expansion by stimulation with E6 or E7 peptide pool

** The number indicates the first aminoacid 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 TCRVß consists of >10% of the activated T cells. A sub-dominant TCR Vß consists of between 3% and 10% of the activated T cells. A minor TCR Vß consists of < 3% of the activated T cells

‡ Additional TCRVβ's were found by PCR analysis for P7 and P15

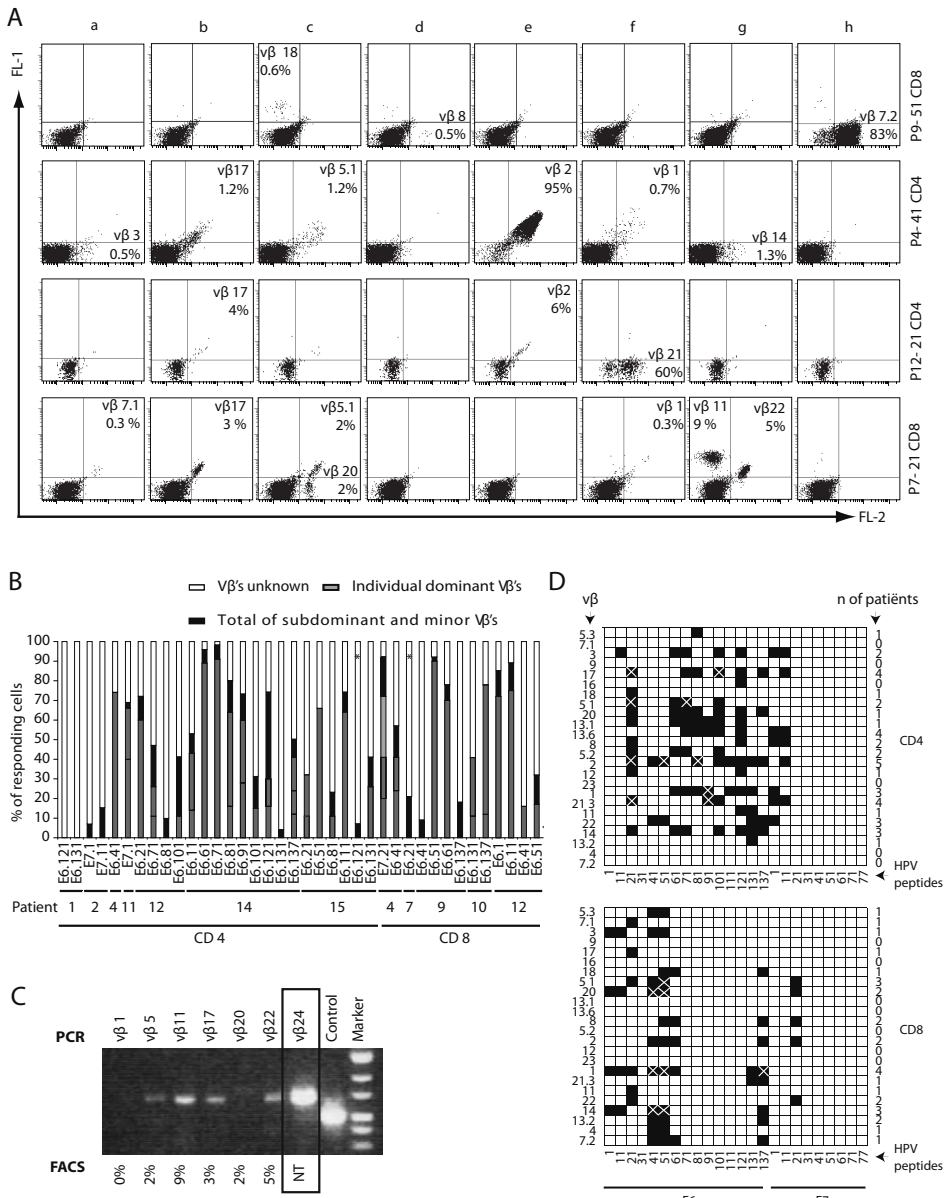
CD4+ or CD8+ T cells present in TIL and from 0.63% - 24% in TDLN. This was independent of the total numbers of T cells isolated, indicating a great variability in the contribution of HPV-specific T cells to the total local anti-tumor response between patients.

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 – based on antigen-specific expression of CD154 and/or CD137 - to single peptides in 12 patients (Figure 1b; Supplemental Table 1). The three TIL cultures tested negative before remained negative excluding priming *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 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 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 by 12 amino acids. The majority of the patients (8/12) recognized ≥3 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. Based on the patient's HLA-type 4 of the CD4- and 2 of the CD8-responses could involve a reaction against known HLA class I and II T-cell epitopes (not shown) (3;34;35). 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, within the population of specifically activated CD154+ and/or CD137+ T cells. Figure 2a shows examples of 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 2. The T-cell response to a single HPV epitope involves multiple dominant and subdominant TCRVβ families. (A) The TCRVβ families used by T cells responding to a single peptide were analysed within 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 TCRVβ was found.

Figure 2b). In a number of cases not all TCRVβ could be identified as the available antibodies cover approximately 70-80% of the full TCRVβ-repertoire (36). In one case (P7) where all cultured T cells 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 antibodies. 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 IFNγ and IL-2 is essential for an effective anti-tumor response and instrumental to functionally characterize antigen-specific T cells (37-42). Therefore, the *ex-vivo* enumerated HPV-specific CD154+ and/or CD137+ T cells were simultaneously analyzed for their production of these cytokines. When specifically gated on the population of CD4 or CD8 T cells that expressed CD154 and/or CD137 after antigen-specific stimulation, 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 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 TLR4 ligands can augment the tumor response (43-45). 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 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 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 within the CD154- and/or CD137-expressing HPV-specific T-cell population after this period (not shown).

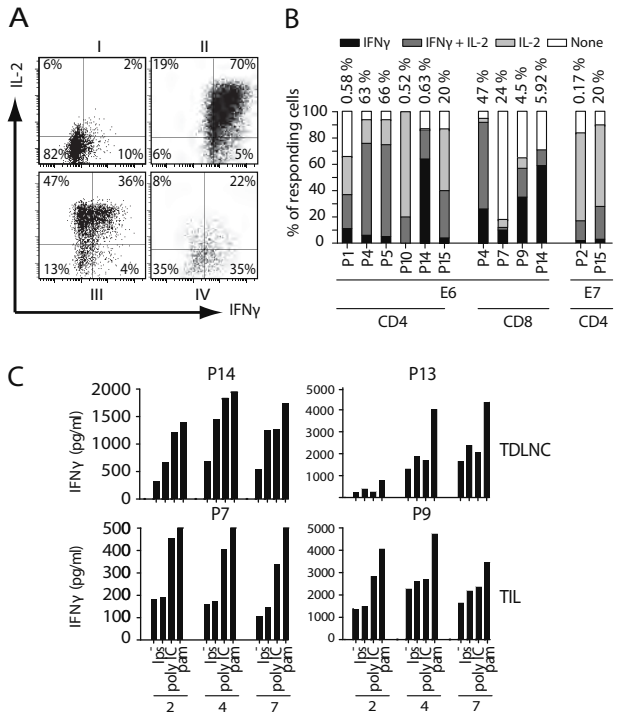


Figure 3. Functionality of HPV-specific TIL and TDLNC . (A) The specific activity to HPV16 or 18 E6 and E7 peptide pools by simultaneous analysis of CD154, CD137, IFNγ and IL-2 in homeostatic cytokine-mediated expanded TIL as well as directly *ex-vivo* in TDLNC. Four different cytokine profiles could be distinguished within the population of HPV antigen-induced CD154- and/or CD137-expressing T cells. 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.

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 in combination with the activation markers CD154 and CD137, 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't exclude that the quantification of HPV-specific T cells within 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, or T-cell responses to multiple different epitopes within a single peptide (Table 2, Figure 2). One could argue that our analysis 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 (36). 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 (46), and 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. The different dominant and subdominant HPV-specific T cells may 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 (47). 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. We deem it unlikely that this breadth is overestimated due to potential cross-reactivity of the T cells with non-related peptide-MHC complexes as even between the highly homologous sequences of HPV16 and HPV18 this has never been observed (3;13).

CD4+ and CD8+ T cells as well as the cytokines IFNγ and IL-2 play a key role in the protection against cancer (4;37-40) as well as in the control of chronic viral infections (41;42). 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 with respect to the production of these cytokines 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 (48). *In vitro* stimulation of these HPV-specific T cells with their cognate antigen resulted in an increase in the number of HPV-specific T cells (e.g. P9, compare Figure 1a and Figure 1b) as well as in an increased IFNγ production (data not shown). We recently reported that the majority of patients vaccinated with a HPV16 long peptide vaccine, displayed a 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 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), a pronounced increase of effector function is observed (Figure 3c). This suggests that local delivery of these innate immune-derived stimulating factors can stimulate a stronger anti-tumor response in human cancers similar to murine tumor models (43-45). Moreover, they may assist therapeutic vaccines in driving T-cell responses with increased function as shown for a melanoma peptide vaccine in combination with CpG (49). Such type of responses is highly required as they correlate with clinical efficacy in murine models (43) and in human trials (50). Interestingly, the widely used TLR4 ligand LPS did not overtly increase IFNγ production of TIL and TDLNC in our study.

In conclusion, 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.

REFERENCES

1. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol 1995 Apr 15;154(8):3961-8.
2. Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. Proc Natl Acad Sci U S A 2003 Jul 22;100(15):8862-7.
3. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, et al. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. Int J Cancer 2008 Feb 1;122(3):486-94.
4. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004 Aug 1;64(15):5449-55.
5. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, et al. Use of fluorogenic histocompatibility leukocyte antigen-A*0201/HPV 16 E7 peptide complexes to isolate rare human cytotoxic T-lymphocyte-recognizing endogenous human papillomavirus antigens. Cancer Res 2000 Jan 15;60(2):365-71.
6. Speiser DE, Baumgaertner P, Barbey C, Rubio-Godoy V, Moulin A, Corthesy P, et al. A novel approach to characterize clonality and differentiation of human melanoma-specific T cell responses: spontaneous priming and efficient boosting by vaccination. J Immunol 2006 Jul 15;177(2):1338-48.
7. Mandruzzato S, Rossi E, Bernardi F, Tosello V, Macino B, Basso G, et al. Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. J Immunol 2002 Oct 1;169(7):4017-24.
8. Sensi M, Traversari C, Radrizzani M, Salvi S, Maccalli C, Mortarini R, et al. Cytotoxic T-lymphocyte clones from different patients display limited T-cell-receptor variable-region gene usage in HLA-A2-restricted recognition of

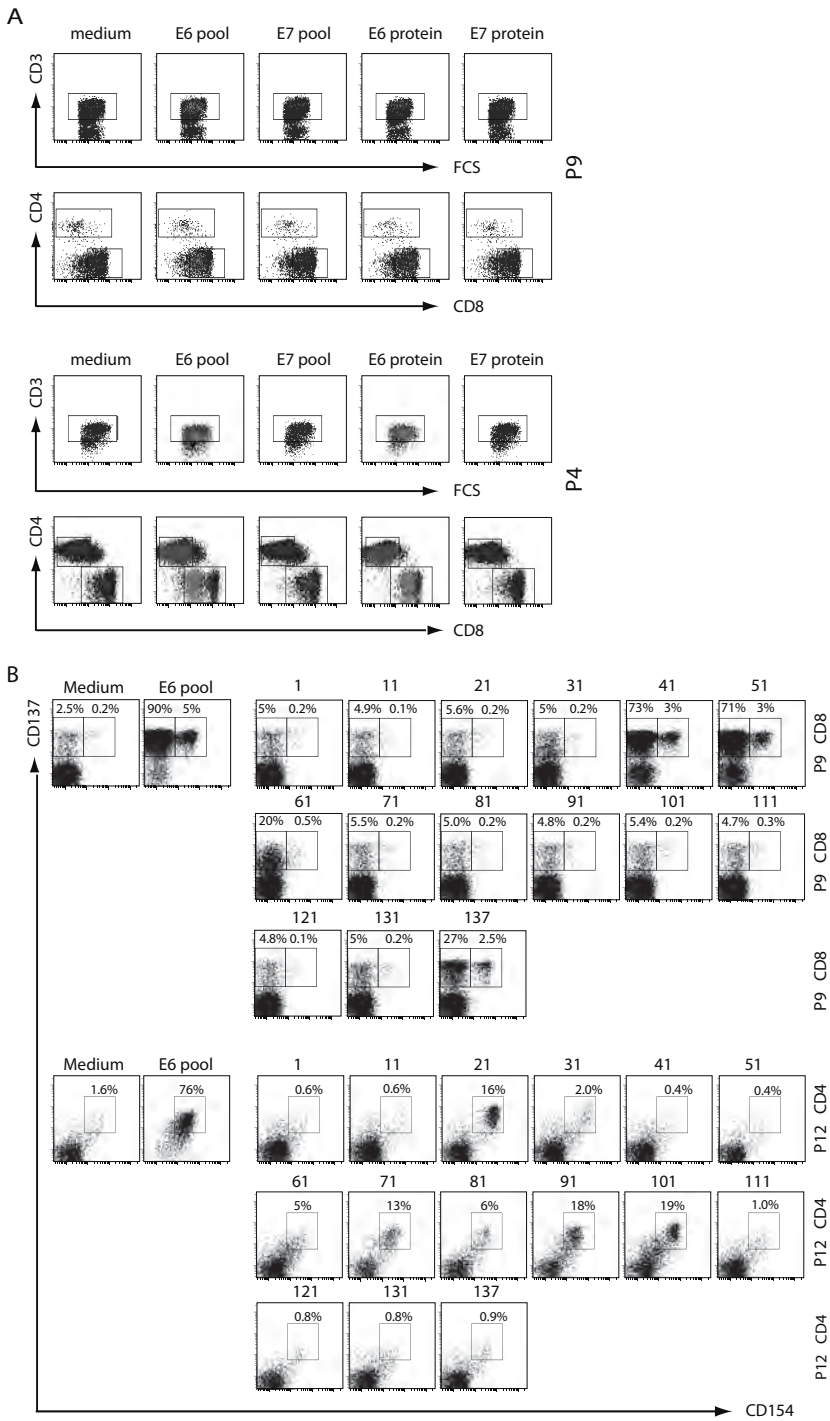
the melanoma antigen Melan-A/MART-1. Proc Natl Acad Sci U S A 1995 Jun 6;92(12):5674-8.

9. Lonchay C, van der BP, Connerotte T, Hanagiri T, Coulie P, Colau D, et al. Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. Proc Natl Acad Sci U S A 2004 Oct 5;101 Suppl 2:14631-8.
10. zur Hausen H. Papillomavirus infections--a major cause of human cancers. Biochim Biophys Acta 1996 Oct 9;1288(2):F55-F78.
11. Bouwes Bavinck JN, Berkhout RJ. HPV infections and immunosuppression. Clin Dermatol 1997 May;15(3):427-37.
12. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW, et al. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. Clin Cancer Res 2008 Nov 15;14(22):7188-95.
13. Welters MJ, van der LP, van den Eeden SJ, Kwappenberg KM, Drijfhout JW, Fleuren GJ, et al. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. Int J Cancer 2006 Feb 15;118(4):950-6.
14. Bontkes HJ, de Gruijl TD, Bijl A, Verheijen RH, Meijer CJ, Scheper RJ, et al. Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. J Gen Virol 1999 Sep;80 (Pt 9):2453-9.
15. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003 Feb 1;63(3):636-41.
16. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW, et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 2007 Jan 1;67(1):354-61.
17. Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res 1997 Jul 15;57(14):2943-50.
18. Hohn H, Pilch H, Gunzel S, Neukirch C, Freitag K, Necker A, et al. Human papillomavirus type 33 E7 peptides presented by HLA-DR*0402 to tumor-infiltrating T cells in cervical cancer. J Virol 2000 Jul;74(14):6632-6.
19. Yuan J, Gnjatic S, Li H, Powel S, Gallardo HF, Ritter E, et al. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. Proc Natl Acad Sci U S A 2008 Dec 23;105(51):20410-5.
20. Keilholz U. CTLA-4: negative regulator of the immune response and a target for cancer therapy. J Immunother 2008 Jun;31(5):431-9.
21. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother 2005 Apr;54(4):307-14.
22. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. Nat Rev Immunol 2004 Jan;4(1):46-54.
23. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res 2008 Jan 1;14(1):169-77.
24. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008 Jan 1;14(1):178-87.
25. Ferrara A, Nonn M, Sehr P, Schreckenberger C, Pawlita M, Durst M, et al. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. J Cancer Res Clin Oncol 2003 Sep;129(9):521-30.
26. Santin AD, Bellone S, Palmieri M, Zanolini A, Ravaggi A, Siegel ER, et al. Human papillomavirus type 16 and 18 E7-pulsed dendritic cell vaccination of stage IB or IIA cervical cancer patients: a phase I escalating-dose trial. J Virol 2008 Feb;82(4):1968-79.
27. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001 Mar 1;91(5):612-8.
28. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989 Oct;135(4):703-9.
29. van der Burg SH, Kwappenberg KM, Geluk A, van der KM, Pontesilli O, Hovenkamp E, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that

is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999 Jan 1;162(1):152-60.

30. Speetjens FM, Kuppen PJ, Welters MJ, Essahsah F, Voet van den Brink AM, Lantrua MG, et al. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin Cancer Res 2009 Feb 1;15(3):1086-95.
31. Heemskerk MH, Hoogeboom M, de Paus RA, Kester MG, van der Hoorn MA, Goulmy E, et al. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. Blood 2003 Nov 15;102(10):3530-40.
32. Wolfl M, Kuball J, Eyrich M, Schlegel PG, Greenberg PD. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. Cytometry A 2008 Nov;73(11):1043-9.
33. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 2005 Oct;11(10):1113-7.
34. Gallagher KM, Man S. Identification of HLA-DR1- and HLA-DR15-restricted human papillomavirus type 16 (HPV16) and HPV18 E6 epitopes recognized by CD4+ T cells from healthy young women. J Gen Virol 2007 May;88(Pt 5):1470-8.
35. Wang X, Moscicki AB, Tsang L, Brockman A, Nakagawa M. Memory T cells specific for novel human papillomavirus type 16 (HPV16) E6 epitopes in women whose HPV16 infection has become undetectable. Clin Vaccine Immunol 2008 Jun;15(6):937-45.
36. Bitmansour AD, Waldrop SL, Pitcher CJ, Khatamzas E, Kern F, Maino VC, et al. Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. J Immunol 2001 Aug 1;167(3):1151-63.
37. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001 Apr 26;410(6832):1107-11.
38. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993 Apr 9;73(1):5-8.
39. Melief CJ, Toes RE, Medema JP, van der Burg SH, Ossendorp F, Offringa R. Strategies for immunotherapy of cancer. Adv Immunol 2000;75:235-82.
40. Ostrand-Rosenberg S. CD4+ T lymphocytes: a critical component of antitumor immunity. Cancer Invest 2005;23(5):413-9.
41. Ciuffreda D, Comte D, Cavassini M, Giostra E, Buhler L, Perruchoud M, et al. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. Eur J Immunol 2008 Oct;38(10):2665-77.
42. Harari A, Vallelian F, Meylan PR, Pantaleo G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. J Immunol 2005 Jan 15;174(2):1037-45.
43. van Mierlo GJ, Boonman ZF, Dumortier HM, den Boer AT, Fransen MF, Nouta J, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol 2004 Dec 1;173(11):6753-9.
44. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BW. Targeting the effector site with IFN-alphabeta-inducing TLR ligands reactivates tumor-resident CD8 T cell responses to eradicate established solid tumors. J Immunol 2008 Feb 1;180(3):1535-44.
45. Asprodites N, Zheng L, Geng D, Velasco-Gonzalez C, Sanchez-Perez L, Davila E. Engagement of Toll-like receptor-2 on cytotoxic T-lymphocytes occurs in vivo and augments antitumor activity. FASEB J 2008 Oct;22(10):3628-37.
46. Jackson H, Dimopoulos N, Mifsud NA, Tai TY, Chen Q, Svobodova S, et al. Striking immunodominance hierarchy of naturally occurring CD8+ and CD4+ T cell responses to tumor antigen NY-ESO-1. J Immunol 2006 May 15;176(10):5908-17.
47. van der Burg SH, Piersma SJ, de JA, van der Hulst JM, Kwappenberg KM, van den HM, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 2007 Jul 17;104(29):12087-92.
48. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 2008 Mar 1;371(9614):771-83.
49. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 2005 Mar;115(3):739-46.
50. Kenter G, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Effective Treatment of Vulvar Intraepithelial Neoplasia by Vaccination against the Oncoproteins E6 and E7 of Human Papillomavirus Type 16. 2009.

SUPPLEMENTAL FIGURES



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 proteinactivated 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).

CHAPTER 4

Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer

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ABSTRACT

Squamous cell carcinomas of the head and neck (HNSCC), in particular those of the oropharynx, can be caused by human papilloma virus type 16 (HPV16). Whereas these HPV-induced oropharyngeal carcinomas may express the HPV16 E6 and E7 oncoproteins and are associated with better survival, the non-virally induced HNSCC are associated with over-expression of p53. In this study we assessed the presence of systemic and local T cells reactive against these oncoproteins in HNSCC.

An exploratory study on the presence, type and function of HPV16- and/or p53-specific T cells in the blood, tumor and/or metastatic lymph node as measured by several immune assays was performed in an unselected group of 50 patients with HNSCC. Tumor tissue was tested for HPV DNA and the overexpression of p53 protein.

Almost all HPV16+ tumors were located in the oropharynx. Circulating HPV16- and p53-specific T cells were found in 17/47 and 7/45 tested patients. T cells were isolated from tumor cultures and/or lymph nodes of 20 patients. HPV16-specific T cells were detected in 6 of 8 HPV+ tumors, but in none of the 12 HPV- tumors. Tumor-infiltrating p53-specific T cells were not detected. In depth analysis of the HPV16-specific T-cell response revealed that this response comprised a broad repertoire of CD4+ T-helper type 1 and 2 cells, CD4+ regulatory T cells and CD8+ T cells reactive to HPV16.

The local presence of HPV16-specific T-cell immunity in HPV16-induced HNSCC implicates a role in the antitumor response and support the development of immunotherapy for HNSCC.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer (1) and comprises cancers from the oral cavity, hypopharynx and oropharynx. Human papilloma virus type 16 (HPV16) induces HNSCC squamous cell carcinoma almost exclusively at the oropharynx, the incidence of which is rising worldwide (2). Patients with HPV-induced HNCSCC often present with advanced disease but their prognosis is better than HNCSCC associated with smoking and drinking (3-5). Notably, the majority but not all patients with an HPV+ tumor display a strong gene signature for adaptive immune response in their tumor (6) as well as strong tumor infiltration by T cells (4) suggesting that the presence of HPV may be related to an enhanced local tumor-specific T-cell response.

HPV16-induced cancer cells express two virally-derived oncoproteins E6 and E7 and because these oncoproteins are foreign to the body, they are expected to evoke an immune response. So far, there are only few reports on HPV16-specific T-cell immunity in HNCSCC. They describe elevated levels of circulating HPV16 E7-specific CD8+ T cells (7) as well as the presence of HPV16-specific IFNγ-producing T cells in *in vitro* cultures of PBMC from patients with HPV16+ HNSCC (8). In addition, circulating antibodies to HPV16 have been detected in HNCSCC patients with high viral load and it is suggested that the HPV-specific antibody status is related to clinical outcome (9, 10).

In non-viral induced, smoking and drinking-associated HNCSCC, p53 over-expression is one of the most common abnormalities identified. Mutation of p53 is correlated with poor prognosis. Mutations in p53 protein or in the p53 regulating pathway can cause overexpression of the protein. The expression of the HPV16 E6 oncoprotein, however, induces increased degradation of p53. Both mechanisms may result in an enhanced presentation of p53-derived peptides to T cells, the latter mechanism predominantly to CD8 T cells. P53-specific IgG antibodies have been found in patients with HNSCC and this was suggested to be linked with bad prognosis (11, 12). These p53-specific IgG responses indicate that p53- specific CD4+ T cells are also present in these patients. Indeed, the presence of both circulating and tumor-infiltrating p53-specific T cells has been reported (13, 14).

In HNSCC a number of cytokine-, antibody- and vaccine-based immunotherapeutic approaches have been tested or are currently underway (15-17). Recently, clinical success was achieved in the field of the immunotherapy of HPV16-induced (pre-)malignancies of the anogenital region, in particular with therapeutic vaccines (18-20). Similar vaccine approaches are tested for the treatment of p53-overexpressing cancers (21-23). There is a strong notion that the presence and type of pre-existing circulating and local tumor-specific immunity influences the clinical outcome as well as that of immunotherapeutic approaches (6, 18). Thus, in order to optimize immune resistance to HNSCC, a better understanding of the character and specificity of tumor-infiltrating-lymphocytes (TIL) in HNSCC is needed. Therefore, an explorative study was performed in which the blood of 50 patients and 20 successfully obtained T-cell cultures from their tumors and/or lymph nodes were studied for the presence, type and function of HPV- or p53-specific T cells. In addition the tumors were studied for the presence of HPV and p53-overexpression.

MATERIAL AND METHODS

Patients and material

Patients presenting with an epithelial lesion of the head and neck region were included after informed consent. After inclusion 50ml of blood and 2 (research and diagnostic) biopsies were taken in parallel with physical examination under sedation however for several patients a diagnostic biopsy had been taken 2-3 weeks earlier before the patients were referred to our hospital. None of the patients received treatment prior to study inclusion. Diagnosis was based on histopathological analysis of biopsies. PBMC were isolated by Ficoll-density centrifugation and obtained cells were directly tested in a lymphocyte stimulation test (LST). Remaining PBMC were preserved in liquid nitrogen until further use. In a number of cases, PBMC were transformed with EBV to obtain B-LCL lines.

Immunohistochemistry

Immunohistochemical analysis was performed on 3 µm paraffin sections, mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinized, rehydrated and treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. Antigen retrieval was performed (0.01 M citrate, pH 6.0) and sections were blocked with 1 % BSA. Subsequently, sections were stained for p53 (overnight, room temperature) using a 1:2000 dilution of anti-human p53 (Clone DO-7, Neomarkers, Fremont, CA) in PBS containing 1% BSA. Next the slides were incubated with Powervision-Poly/HRP (Immunogenic, Duiven, the Netherlands) and immune complexes were visualized with diaminobenzidine. Slides were evaluated by light-microscopy and scored for the presence, intensity and percentage of tumor cells expressing P53.

HPV typing

DNA was isolated from formalin-fixed, paraffin-embedded biopsy samples as previously described (24). The aqueous solution obtained from the DNA analysis was diluted 1:10 and 1:50. The presence of HPV in the diluted samples was determined by genotyping the samples that demonstrated 65 basepair PCR amplimers on a 3% agarose gel using an INNO-LiPA Genotyping Extra test (Innogenetics, Ghent, Belgium), according to the manufacturer's instruction. This assay allows the detection of the following HPV types: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69/71, 73, and 74. Hybridisation patterns were visually inspected and interpreted using a grid.

Antigens and lymphocyte stimulation test

Pools of overlapping 22-mer peptides spanning the entire HPV16 E6 and E7 proteins were used and pools of overlapping 30-mer peptides spanning the whole p53 protein as described previously (25, 26). The first and last amino acid within the sequence of the indicated antigen that is represented by the pooled peptides is indicated (*e.g.* E6 1-92). Memory response mix (MRM)was used as positive control (26, 27). The capacity of T cells to proliferate on recognition of the antigen was determined by LST as described earlier (27). The average of 8 tested wells divided by the average of the medium control plus 3 SD was calculated and is referred to as the stimulation index (SI).

Day 6 supernatant was analyzed for the presence of IFNγ and IL-10 by ELISA (Sanquin, the Netherlands). Cut-off value (IFNγ 50pg/ml; IL-10 20 pg/ml) and >2x the concentration of the medium control.

Isolation and culture of TIL

All T-cell cultures and tests were performed in IMDM supplemented with 10% human AB serum (Greiner Bio-one, Germany). Biopsies were incubated in IMDM+10% AB serum supplemented with Fungizone 25ug/ml, 1000 units pen/strep and 20ug/ml gentamycin (all Invitrogen) for 1 hour to decrease the bacterial load in the tissue. Subsequently, the biopsies were minced in to pieces of ~ $1mm^3$ and cultured in medium with 10% T-cell Growth Factor (Zeptometrix, Buffalo NY, USA) and 5 ng/ml IL-15 (Peprotech, Rocky Hill, NJ). During the first day 5 ng/ml IL-7 (Peprotech) was added. TIL were expanded for 2-3 weeks with only addition of medium and cytokines and analyzed for phenotype and function.

In 2 patients whit large lymph node metastases, a needle aspirate was taken and frozen in liquid nitrogen until further use.

Analysis of TIL specificity

Autologous PBMC were cultured for 2-3 days with GM-CSF and the obtained differentiated monocytes were loaded with peptide pools (5 ug/ml). When more then $1.5 x10^6$ T cells grew out also peptide pools of p53 were tested as well as separate smaller pools of HPV-peptides were used. Monocytes were washed and TIL were added (20.000-50.000) in fresh IMDM+10% AB serum. All tests were performed in triplicate and proliferation was determined by incorporation of 0.5 Ci/well [^{3}H]thymidine for the last 16 hr. Phytohaemagglutinin (PHA) (Remel, Germany) was taken along as a positive control for T-cell activation. 48 hr-supernatant was pooled and analyzed by Th1/Th2 cytometric bead array (CBA, BDbiosciences). Responses were considered positive when 3 times medium control and above cut-off value of 20 pg/ml. One TIL and LN culture were stimulated with E6 peptide pool in order to obtain enough HPV-specific T cells to measure the breadth of the response with respect to single peptide-antigens as described earlier (28). Data was recorded on a FacsCalibur or LSR-II (BD biosciences) and analyzed using Flowjo software (Treestar inc, USA).

Isolation of T-cell clones

T-cell clones from the TIL of patient 27 were isolated using limited dilution as described earlier (29). T-cell clones were tested for phenotype and TCRVβ usage by flow cytometry and specificity was tested by 48 hr proliferation assay on peptide or protein loaded B-LCL. Cytokine production was analyzed by ELISA and CBA.

Suppression assays

The capacity to suppress the proliferation of stimulated naive CD4+CD25+ cells by the T-cell clones of patient 27 was tested as previously described (30).

RESULTS

Circulating HPV16- and p53-specific T cells are present in patients with head and neck cancer

A total of 50 patients with HNSCC in a variety of regions were included in this study (Table 1). In 41 and 43 cases we were able to test the tumors tissue of the biopsy for the presence of HPV and the over-expression of p53 protein, respectively. The patients presenting with an HPV16+ tumor were slightly younger (average 60.4 years) compared to HPV16- and unknown oropharyngeal tumors (average 63.3 years) or all HPV- patients (average 65.8 years).

Of the 41 tested tumors, 12 contained DNA of HPV 16. It is known that HPV infection is often present in the oropharynx. Indeed, when we divided our cohort according to region 11 of the 12 HPV16+ tumors were located in the oropharynx region (52% of all oropharyngeal tumors) and one was present in the oral cavity (8% of oral cavity tumors). No HPV was detected in 10 tested tumors of the hypopharynx (Table 1). In 12 out of 43 tumors p53 was over-expressed, more often (6/10) in the tumors of the hypopharynx (Table 1).

PBMC were isolated and directly tested for the presence of specific T cells to peptides of the HPV 16 oncoproteins or p53 by LST (4 examples are shown in Fig. 1). In 17 of 47 tested patients a proliferative response to HPV16 was detected. Interestingly most responses to HPV were found in patients with an HPV+ tumor. There is a trend that the detection rate is higher in this group 7 out of 11 patients displayed a detectable response to HPV16 than in patients with a HPV- tumor (7 out of 28 patients; p= 0.061 Fisher's exact). Only in a minority of patients (5/17) this proliferative response was accompanied by detectable IFNγ production (patient 8, 9, 22, 28 and 52; range 77-440 pg/ml). The response of patient 8 was also accompanied by detectable IL-10 (28 pg/ml). Notably, in 4 of these 5 cases the patient's tumor was HPV16 negative.

Proliferative T cell responses to p53 were found in the blood of 7 out of 45 tested patients. In only one patient this response was associated with IFNγ-production (patient 7, 61 pg/ml). No correlation existed between p53 over-expression or the presence of HPV in the tumor and the detected p53-specific immune response in the blood. Only one of the patients with a p53 over-expressing tumor displayed p53-specific immunity (Table 1).

Thus, within an unselected group of patients treated with head and neck cancer 52% of patients with an oropharyngeal tumor presented with tumor-integrated HPV16 DNA. Furthermore, circulating HPV16-specific T cells are more often detected in this group.

The percentage of circulating and tumor-associated CD4+CD25+Foxp3+ T cells

For 12 patients of whom enough PBMC were available the number of regulatory T cells was determined. The mean percentage of CD25+Foxp3+ cells within the CD4+ T cell population in the blood was 2.7% (range 0.68-6.29, n=12) and no difference was found between patient groups or HPV status (data not shown). Interestingly a substantial number of CD4+ T cells present in the tumor cultures displayed Foxp3+, the transcription factor associated with regulatory function (average 9%; range 2-25 %, Fig 2a and not shown).

Table 1. Patients with squamous cell carcinoma of the Head and Neck region

					Tumor		Circulating T cells: HPV		Circulating T cells: P53		TIL[9]	
Region[1]	**ID**	**Stage[2]**	**M/F**	**age**	**HPV[3]**	**P53[4]**	**LST[5]**	**SI[6]**	**LST[7]**	**SI[8]**	**HPV**	**P53**
Oropharynx	17	2	M	62	NT	NT	-		-		-	
	18	2	M	62	NT	-	-		-		-	
	7	3	M	61	-	-	HPV16E6	6.5	P53-C	5,2		
	9	4B	M	65	-	-	HPV16E6	4,5	P53-C	9,3		-
	21	2	F	62	-	-	-		-		-	-
	34	3	M	66	-	-	-		-		-	-
	45	4B	M	56	-	NT	-		-			
	48	4A	M	81	-	-	-		-		-	
	52	3	M	66	-	-	HPV16E6	13.6	-		-	
	55	3	F	52	-	+	-		-		-	
	1	4A	M	65	HPV16	-	-		-			
	6	3	M	58	HPV16	-	HPV16E6+E7	12+13	P53-B	3,6	(LN)HPV16	
	26	4A	M	64	HPV16	-	HPV16E6	2.8	P53-C	2,5		
	27	1	F	49	HPV16	-	HPV16E6	6.7	-		HPV16	-
	28	3	F	65	HPV16	-	HPV16E7	4	-		HPV16	
	30	2	F	68	HPV16	-	HPV16E6	2.8	-		-	
	31	4A	F	63	HPV16	+++	-		-		-	-
	35	4A	M	47	HPV16	-	HPV16E6+E7	8+5	-		HPV16	
	46	4A	M	60	HPV16	-	-		NT		HPV16	
	50	4A	M	57	HPV16	-	HPV16E6	3.1	-			
	53	4A	M	54	HPV16	-	-		-		HPV16	
Oral cavity	2	2	F	75	HPV16	-	NT		NT			
	3	4A	M	67	NT	++	-		-			
	4	3	M	66	-	+++	-		-			
	8	4A	M	63	NT	NT	HPV16E6	7.6	-			
	12	2	F	77	-	-	HPV16E6	5.6	P53-B	5,8		

	13	4A	M	47	-	-	-		-		-	-
	15	1	M	61	NT	NT	HPV16E6	3	-		-	-
	23	2	F	87	-	-	-		-			
	25	1	F	63	-	-	-		-		-	-
	29	4A	M	75	NT	++	-		-			
	32	2	F	49	-	-	-		-		-	-
	36	2	F	76	-	-	-		-			
	39	0	F	45	-	-	-		-			
	42	2	F	79	-	-	-		-			
	43	4A	M	64	-	-	-		-			
	49	1	F	73	-	+	HPV16E6+E7	12+3.4	-			
Hypopharynx	5	3	M	64	-	+	HPV16E6+E7	27+24.6	P53-B	14		
	10	3	M	83	-	+++	-		-			
	11	4A	M	79	-	-	-		P53-B	10		
	14	2	M	55	-	++	-		-			
	16	2	M	65	-	+++	-		-			
	22	4A	M	74	-	+++	HPV16E7	3.1	-		-	-
	24	4B	M	73	-	-	NT		NT			
	33	3	F	76	NT	NT	-		NT			
	37	3	F	68	-	+++	-		-			
	38	4A	M	49	-	-	NT		NT			
	40	4B	F	60	NT	-	-		-			
	41	2	F	52	NT	NT	HPV16 E7	3.1	-			
	44	1	M	70	-	NT	-		-			

1) Location of primary tumor. 2) Stage of disease according to TNM clinical classification. 3) DNA of HPV16 present in tumor tissue; NT= Not Tested, - = no HPV16 found, HPV16= HPV16-DNA is found in tumortissue. 4) P53 overexpression in tumor tissue; NT=Not Tested, += overexpression in <25% of tumorcells, ++= overexpression in 26-75% of tumorcells, +++= overexpression in >75% of cells. 5) Lymphocyte stimulation test of PBMC; NT=Not Tested, - =no response to HPV, HPV16 = proliferation on peptides of the E6 or E7 protein. 6) Stimulation index of (5) 7) Lymphocyte stimulation test of PBMC NT=Not Tested, -=no response to P53, P53-A/B/C= proliferation on peptidepool A, B or C of the P53 protein. 8) Stimulation index of (7) 9) Tumor Infiltrating T-cells (TIL) tested are indicated by a "-" or by the protein that was recognised. (LN) is tested in tumor draining lymphenode.

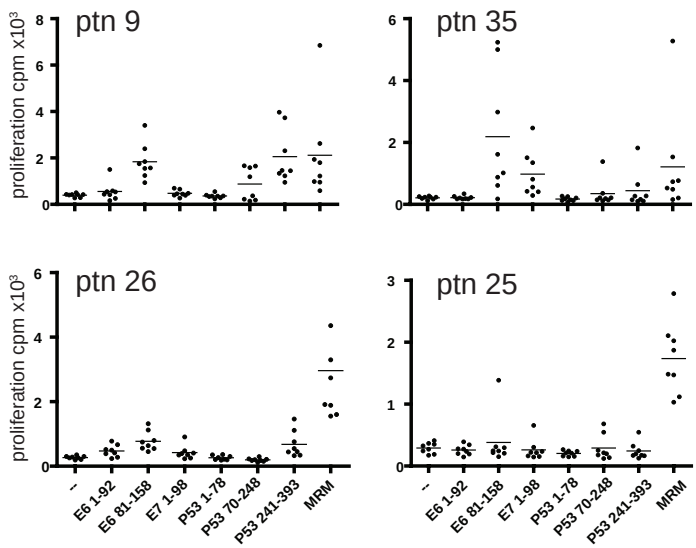

Figure 1. HPV- and p53-specific T cells circulate in the blood. Lymphocyte stimulation test of freshly isolated PBMC. Proliferation of T cells stimulated with pools of peptides indicated at the x-axis after 7 days of culture. The positive control is the memory response mix (MRM). Shown are the counts of 8 separate wells and the mean. Top: patient 9; positive response to peptide pools covering E6 aa81-158 and p53 aa241-393. Patient 35 responding to E6 aa81-158 and E7. Bottom: patient 26; weak response to E6 aa81-158. lower right: patient 25 no response.

HPV-specific T cells can be isolated from oropharyngeal tumors and draining lymph nodes

We were able to culture T cells from the tumor biopsies of 19 patients. In all cultures, both CD8+ and CD4+ T cells grew out (CD4/CD8 ratio median 2.45, average 5.1 and range 0.1-19) . The composition of T cells was not clearly influenced by the culture conditions (Fig. 2a). After a 3-week homeostatic expansion period (no exogenous antigen was added) the T cells were tested for their reactivity against autologous monocytes loaded with indicated pools of HPV16 E6 or E7 peptides (n=19) and when enough cells were available to monocytes pulsed with pools of p53 peptides (n=10). Non-specific stimulation with PHA revealed that the TIL from most tumors comprised a heterogeneous population of T cells that displayed a high capacity to proliferate and to produce the type 1 cytokines IFNγ and TNFα as well as the type 2 cytokines IL-5 and IL-4 (Fig. 2b and not shown).

While none of the TIL isolated from HPV negative tumors displayed a proliferative response to HPV (data not shown), we were able to detect HPV16-specific T-cell proliferation in 5 out of 7 patients with an HPV16+ tumor (Fig. 2b). TIL from patient 27 reacted against HPV16E6 by both proliferation and the production of IFNγ. TIL from patient 28 reacted with strikingly high proliferation and high production of IFNγ, TNFα and IL-5 against both HPV16 E6 and E7. The TIL from a third HPV16+ patient displayed a modest proliferative response to E6 but without the concomitant production of IFNγ (patient 35; Fig. 2b). In all these patients we had also detected an HPV16-specific response in the blood. The TIL of patient 46 displayed an IFNγ-associated HPV16 E7-specific proliferative response whereas the TIL of patient 53 displayed a potent IFNγ

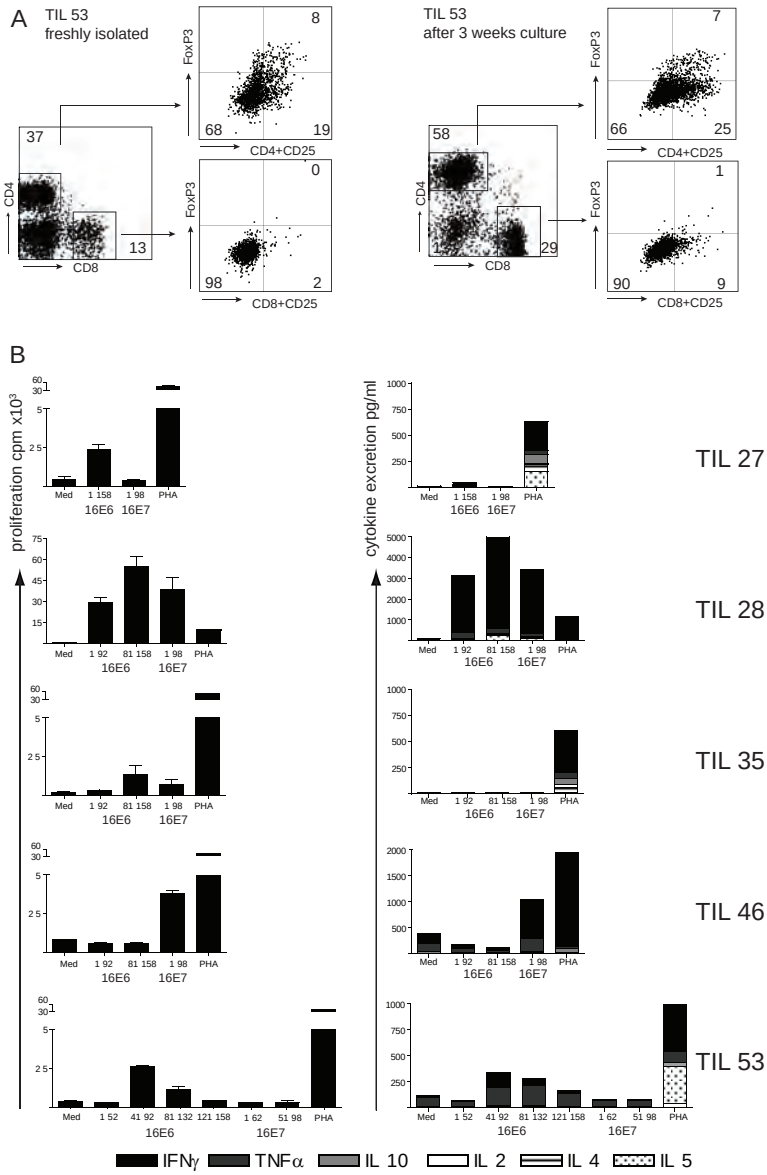

Figure 2. HPV-specific T cells are present in HPV+ tumors. A, Phenotypic analysis of freshly isolated T cells derived from tumor tissue of patient 53 (top panel) or after 3 weeks of culture (bottom panel) by flow cytometry. An increase in the percentage of CD4+ and CD8+ T cells (while their ratio remains relatively unchanged) during the 3 week culture period is observed. Within the CD4+ T cell population CD25+Foxp3+ regulatory cells are found. B, Proliferation assay of TIL of 5 different patients. Monocytes were loaded overnight with medium only (med), peptide pool of overlapping 22 mer peptides of the HPV16 E6 and E7 oncoproteins. As positive control for T-cell reactivity PHA stimulation was used (PHA). The number of conditions that could be tested depended on the number of TIL obtained. T cells were added and proliferation was measured at 48 hr by the incorporation of [^{3}H]-thymidine (top row). Mean of triplicate + SEM is shown. Supernatants isolated at 48 hrs of culture were analyzed by CBA (bottom row). All tumors were HPV16+.

and TNFα-associated HPV16 E6-specific T-cell response. In the latter two cases no response was detected in the blood suggesting that measurements of HPV16-specific immunity in PBMC may underestimate the total number of patients that have mounted an immune response. Of the 10 different TIL that could be tested for the presence of p53-specific T cells, none displayed p53-specific reactivity despite that 2 patients had tumors that over-expressed p53 (Patient 22 and 31; not shown).

In 2 patients large lymph node metastasis were palpable in the neck and a needle aspirate was taken (patient 6 and 35, both HPV16+). For patient 6, the cells were stimulated ones with pools of E6 or E7 peptides to obtain enough cells to test. These bulk cultures were tested by overnight activation and intracellular cytokine staining (28). As indicated by the up regulation of the activation markers CD137 and CD154, the lymph node cells comprised HPV16-specific T cells reactive to peptide pools of E6 and E7 and which were able to produce both IFNγ and IL-2 (Fig. 3b). The T cells were able to recognize their cognate antigen when naturally processed and

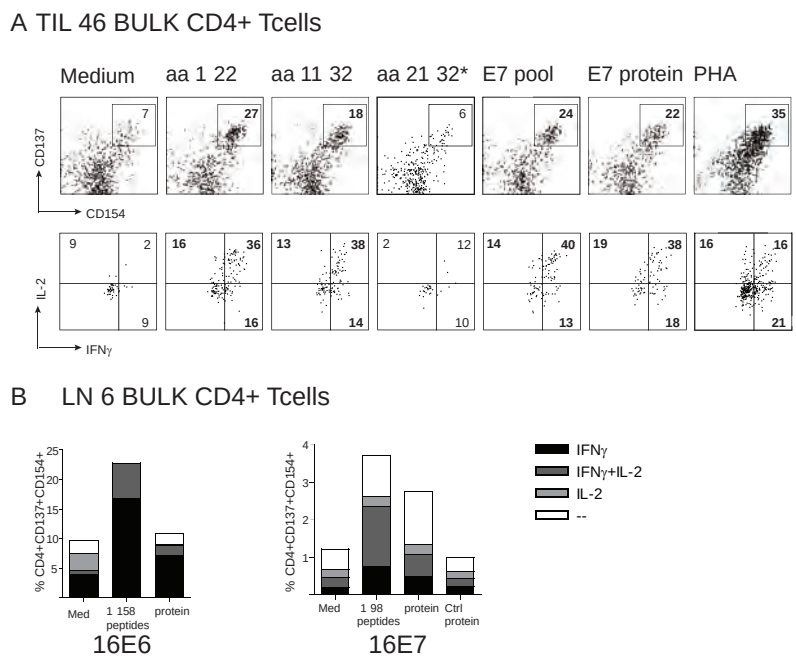

Figure 3. Specificity as well as IFNγ and IL-2 production of TIL by multiparameter flow cytometry. A, Analyses of the specificity and cytokine production of TIL of patient 46. T cells were stimulated with single peptides and analyzed for expression of activation markers CD137 and CD154 and the production of the cytokines IL-2 and IFNγ. Cells were gated on CD3+CD4+ expressing cells, the numbers represent the percentage of gated cells. Bottom row; IL-2 and IFNγ expression of CD154+,CD137+ cells gated in the plots of the top row. Numbers indicate percentage cells in that quadrant. * peptide 21-32 is representative for all other peptides covering E7 since no response was found in this group. B, Analysis of HPV-specific reactivity in LN cells of patient 6. T cells were cultured with B-LCL loaded with medium only (med) or peptide pools of E6 or E7, HPV16 E6 or E7 protein and HIV-RT protein (ctrl prot) measured by multi parameter flowcytometry as described in A. Shown is the percentage of CD4+ T cells expressing activation marker CD137 and CD154 in response to the indicated stimulus as well as the percentage of cells that produced the depicted cytokines within the activated cell population.

presented as E7 protein-pulsed APC were clearly recognized but not APC pulsed with control protein. Unfortunately in the LN of patient 35 we did not detect HPV-specific T cells although both the TIL and blood of this patient displayed HPV16-specific T-cell reactivity.

Thus in 6 out of 8 of patients with HPV-induced tumors of the oropharynx, we detected functionally active HPV-specific T cells in the tumor or lymph nodes.

The breadth of the HPV16-specific CD4+ and CD8+ T–cell response in TIL

To assess the type and specificity of the HPV16-specific T cells in the TIL, the tumor-derived T cells of patient 46 were stimulated overnight with autologous B-LCL pulsed either with a pool or each individual single 22-mer peptide of HPV16 E7 and analyzed by multiparameter flow cytometry. The CD4+ T cells within this TIL population responded to a sequence located in the first part of the HPV16 E7 protein by the expression of both CD154 and CD137 and the production of IL-2, IFNγ and IL-2, or IFNγ only (Fig. 3a). Not only B-LCL pulsed with the E7 22-mer peptides were recognized but also B-LCL loaded with whole protein confirming that these T cells recognized the naturally processed antigen. The population of CD4+ T cells predominantly produced IL-2 and IFNγ although some T cells only produced IL-2, IFNγ or neither cytokine upon antigen-specific stimulation.

As the number of TIL isolated from patient 27 was to low to do a direct analysis we decided to isolate T-cell clones from this TIL culture. This resulted in the isolation and expansion of 18 HPV16-E6 specific T-cell clones, 14 of these where CD4+ T cells and 4 CD8+ T-cell clones. Upon antigenic stimulation all T-cell clones proliferated and most T-cell clones produced high amounts of IFNγ. A limited number of clones produced IL-10 (Fig. 4a). Interestingly, in 2 clones IFNγ production was accompanied by high levels of IL-5 upon peptide or processed protein recognition (Fig. 4c and not shown). All CD4+ T cell clones tested responded to epitopes within the sequence covered by amino acids 41-72 of HPV16E6 and recognized naturally processed E6 protein. At least 3 distinct epitopes were recognized (examples see Fig. 4bc). Unfortunately, the exact CD8+ T-cell epitope recognized could not be determined since the *in vitro* life span of these cells was limited. Phenotypical analysis of surface markers and intra-cellular FoxP3 by flow cytometry on 2-3 weeks rested T-cell clones revealed that some CD4+ T cells expressed low levels of the transcription factor Foxp3 (Fig 5a, clone 4 and 245). Interestingly, 2 of the 3 HPV16-specific CD8 T-cell clones expressed high levels of CD25 and Foxp3 as well (Fig. 5a). The analysis of the T-cell receptorVβ (TCRVβ) usage of the T-cell clones revealed the presence of at least 5 different TCRVβ families. Since the T-cell clones expressing TCRVβ17 recognized different epitopes (Clones 4 and 142; E6 aa51-72 and Clone 245; E6 aa41-62) (Figures 4+5) and also the 2 T-cell clones expressing TCRVβ2 recognized different epitopes (Clone 49; E6 aa51-72 and Clone 251 E6 aa41-62 and 51-72) , we conclude that at least 6 distinct HPV16E6-specific CD4+ T cell populations were present in the tumor. It was reported for CD4+ T cells isolated from oropharyngeal tumors that they exert suppressive function via ectonucleotidases CD39 and CD73 (31). Interestingly, all isolated CD4+ T cells tested expressed the ectonucleotidases CD39 and CD73 on the surface whereas this is not the case for peripheral CD4+ T cells isolated from a healthy control (Fig 5b).

Based on the expression of Foxp3 by the isolated CD4+ T-cell clones, as well as on our experience with HPV16-specific T cells in cervical tumor, we tested the HPV16-specific CD4+ T cells for their capacity to suppress the proliferation and cytokine production of allogeneic naïve CD4+ T cells (Fig. 5c). This revealed that in this TIL population suppressive (clone 4) but

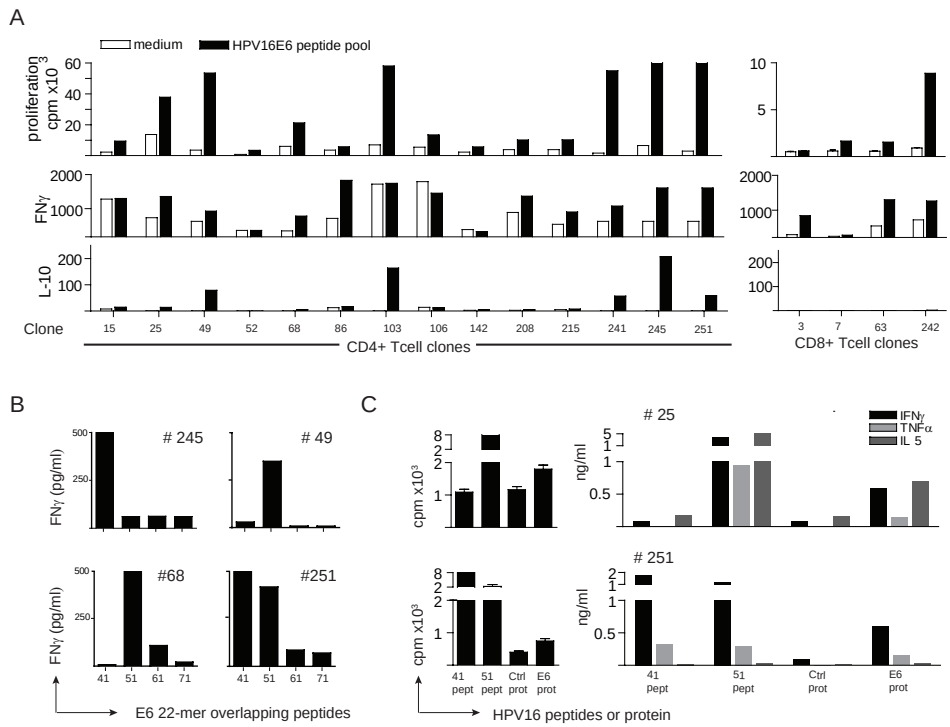

Figure 4. TIL isolated from patient 27 contains functional CD4+ and CD8+ HPV-specific T cells. Clones were isolated by limited dilution and tested for the recognition of HPV16E6 peptides loaded on autologous B-LCL (black) or B-LCL only (white). A, T cell function was tested by proliferation (top row), IFNγ production (middle) and IL-10 production (bottom) after 48 hrs. Clone number is depicted at the X-axis, 14 CD4+ and 4 CD8+ clones were isolated. B, all clones recognized a sequence between aa41 and aa82 of the E6 protein as shown by the IFNγ production upon stimulation with peptide loaded B-LCL. Shown are 4 typical examples of the recognition of aa41-62, aa51-72 or the overlapping peptides aa41-62 and aa51-72. C, Not only stimulation with peptide but also with naturally processed protein results in T-cell proliferation (left) and production of cytokines (right). Clone 25 produces IFNγ, TNFα as wells as high amounts of IL-5 whereas clone 251 mostly produced IFNγ . Two representative clones out of 6 are shown.

also helper T cells (clone 251) were present. Taken together we conclude that the local HPV-specific T-cell response comprises a mixed population of Th1, Th2 and regulatory T cells, as well as IFNγ producing CD8+ T cells.

DISCUSSION

In this explorative study of the tumor-specific immune response in 50 patients with HNSCC we found HPV16 DNA to be present in almost half of the patients presenting with a malignancy in the oropharynx but not in other regions. Circulating T cells reacting against HPV16 E6 or E7 peptides were detected in the blood, often in patients with an HPV16+ tumor, largely extending previous data on HLA-A2 restricted T cells in a small group of patients (7) Similar to what was

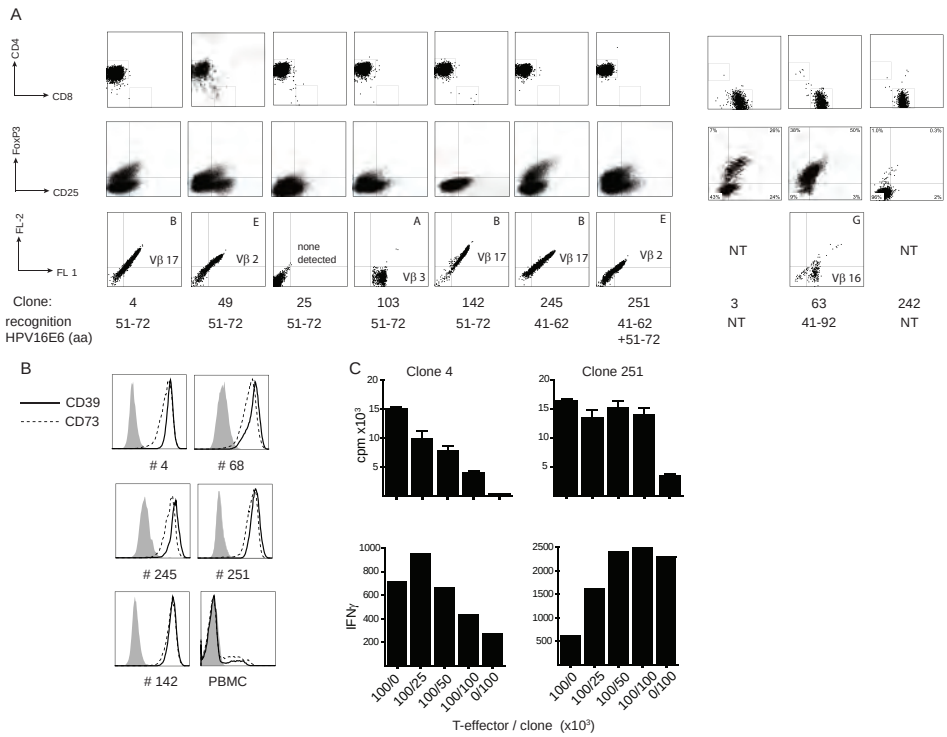

Figure 5. HPV specific helper and regulatory cells are present within the tumor. A, Flowcytometric analysis of resting clones by surface markers CD4 and CD8 (top row) and intracellular staining for the Treg markers CD25 and FoxP3 (second row). Gates are adjusted to unstained control. Bottom row shows the TCRVβ usage of the clones as determined with sets of antibodies. Shown is the antibody set that stained positive as depicted in the graphs and the concommitant TCRVβ is indicated. B. Ectonucleotidase expression was analyzed by flowcytometry for 5 clones and PBMC. All clones express high CD39 and CD73 receptor (grey fill is background fluorescence). C, The capacity of the T-cell clones to suppress the response of naive T cells was tested by the activation of CD4+CD25- T cells with a pool of mixed B-LCL anti-CD3 antibodies. Proliferation and IFNγ production is measured at 48 hr. The T-cell clones were added in increasing numbers (100/10, 100/50, 100/100). Example of suppressive clone 4 (left) and helper clone 251 (right). Proliferation test was performed in triplicate, mean with SEM.

observed in patients with cervical carcinoma the HPV16-specific T-cell proliferative responses in HNSCC were not/hardly ever accompanied by the production of IFNγ (26, 32). Although these responses in the blood clearly show that adaptive immune responses are induced upon exposure to the HPV viral oncoproteins, this reaction does not necessarily reflect an ongoing immune response against the tumor. This is exemplified by the detection of circulating HPV16-specific T cells in the blood of some patients with HPV-negative tumors, which in a number of cases displayed a similar cytokine profile as described for HPV16-specific reactivity in healthy females (27).

Here, we showed that HPV16-specific T cells are present in 5 out of 7 TIL populations and in 1 out of 2 tested tumor-draining lymph nodes from patients with HPV16+ tumors. Notably 2 out of these 6 patients displayed a local HPV16-specific T cell immunity without a concomitant

detectable response in the blood. This indicates that the peripheral T-cell compartment not always reflects the local anti-tumor response. Importantly our data indicate that HPV16-specific T cells are locally present in the majority of patients with HPV16-positive oropharyngeal tumors, this in sharp contrast to the only 32% of the patients with HPV16+ cervical cancers (29). We speculate that the location of the disease is likely to contribute to this as the oropharynx consists of lymphoid tissue and our studies on cervical tumor-draining lymph nodes suggest that they generally contain HPV-specific T cells (28, 29). In addition, the high load of bacterial and fungal co-infections present in the oropharynx of these patients may also act as stimulus for the local immune system.

So far, the data on TIL in HNSCC was limited to the quantification and phenotyping of tumor infiltrating immune cells by immunohistochemical studies and microarray (6, 33, 34). We now show that such infiltrating immune cells comprise functionally different T-cell subsets and importantly that they are reactive to the HPV16 oncoproteins. In TIL we find different subsets of HPV16-specific T cells (Th1, Th2, CTL and Tregs) with different cytokine profiles (IL-2, IFNγ, IL-5) comparable to what is found in anogenital HPV16 induced lesions (29, 35, 36). Together this advocates that local adaptive immune cells might play a role in the response to therapy and the altered survival of this patient group.

P53 specific CD8+ T cells have also been detected in HNSCC by means of tetramer staining, albeit that these cells were present at low frequencies (14). In this study 28 % of the tumors over-expressed p53 protein but we did not find evidence for p53-specific T cells in the 10 TIL tested. We have previously shown that it is possible to isolate p53-specific CD4+ T cells from the TIL of patients with ovarian cancer (23). Furthermore both p53-specific CD4+ and CD8+ T cells can be isolated from the vaccine site in our studies on p53 vaccination (22). Although the existence of HPV16-specific regulatory T cells is reported, there is no evidence for the existence of p53-specific regulatory T cells (22, 37). However other types of regulatory T cells may have suppressed the outgrowth of p53-specific T cells. We can not exclude that the high numbers of CD4+Foxp3+ T cells detected in the tumors may have played a role. In some patients circulating p53-specific T cells were detected but there was no obvious relation with either p53-overexpression or the potential HPV-mediated increased degradation of p53 as some tumors did not overexpress p53 and were HPV negative. Previously it has been argued that such p53-negative tumors may represent immune escape variants (13, 14), alternatively the tumors may harbour p53-mutations that do not result in overexpression.

We report that HNSCC are infiltrated by high numbers of CD4+CD25+Foxp3+ T cells and that the frequency of these cells exceeded the frequency in the blood. Furthermore, we isolated both HPV16-specific CD4+ and CD8+ T-cell clones that expressed high levels of Foxp3 under resting conditions. In HNSCC the ratio between CD8+ and regulatory T cells is associated with disease (5, 33). Similarly, in HPV16 induced genital malignancies the presence of FoxP3 positive as well as FoxP3 negative HPV16 specific regulatory T cells in tumor and LN is described (36, 38, 39) and a low CD8/Treg T-cell ratio is associated with worse outcome (40). While the regulatory function of the CD4+CD25+Foxp3+ T cells present in TILs needs to be confirmed, at least one of our isolated clones clearly exerted regulatory function. The expression of CD39 and CD73 has been found on CD4+ T cells as well as on the great majority of regulatory T cells in HNSCC, and has been implicated as a mechanism for T cell suppression (31). Analyses of these markers on the isolated T cell clones revealed that all CD4+ T-cell clones highly expressed CD39

and CD73 at the cell surface in resting state, but these included CD4 helper T cells that were not suppressive in a classical suppression assay. Notably, also influenza virus-specific CD4+ helper T cells and regulatory T cells that were isolated from PBMC cultures (41) express similar high levels of CD39 and CD73 (unpublished observations). Although the transcription factor Foxp3 is mainly reported in CD4+ regulatory T cells we found CD8+CD25+FoxP3^{high} T cells in TIL and in CD8+ T-cell clones derived from the tonsil region. Siegmund and colleagues describe this phenotype to be uniquely present in human tonsil tissue (42), however their presence was also demonstrated in human prostate cancer (43). Whether these T cells with tolerogenic phenotype are a reflection of suppressed local immunity or part of an ongoing adaptive immune response can not be concluded from this data.

Stimulation of local immunity might change the balance from a pro-tumor microenvironment to a hostile tumoricidal environment. Recently, different forms of immunotherapy – vaccination to enhance the HPV16-specific T cells response, imiquimod to enhance the local innate immune response, and combination hereof - have been successful in the treatment of HPV16-induced lesions of the vulva (19, 20, 44). Similar to HPV-induced lesions of the vulva, the HPV-induced HNSCC are relatively accessible and one could envisage that these strategies might be applicable to tumors of the oropharynx.

ACKNOWLEDGEMENTS

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REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Ramqvist T, Dalianis T. Oropharyngeal cancer epidemic and human papillomavirus. Emerg Infect Dis 2010;16:1671-7.
3. Dayyani F, Etzel CJ, Liu M, Ho CH, Lippman SM, Tsao AS. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). Head Neck Oncol 2010;2:15.
4. Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong A, et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 2009;74:553-61.
5. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. The Lancet Oncology 2010;11:781-9.
6. Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G, et al. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. J Clin Oncol 2010;28:2881-8.
7. Albers A, Abe K, Hunt J, Wang J, Lopez-Albaitero A, Schaefer C, et al. Antitumor Activity of Human Papillomavirus Type 16 E7-Specific T Cells against Virally Infected Squamous Cell Carcinoma of the Head and Neck. Cancer Res 2005;65:11146-55.
8. Hoffmann TK, Arsov C, Schirlau K, Bas M, Friebe-Hoffmann U, Klussmann JP, et al. T cells specific for HPV16 E7 epitopes in patients with squamous cell carcinoma of the oropharynx. Int J Cancer 2006;118:1984-91.
9. Smith EM, Pawlita M, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer. Int J Cancer 2010;127:111-7.
10. Kreimer AR, Clifford GM, Snijders PJ, Castellsague X, Meijer CJ, Pawlita M, et al. HPV16 semiquantitative viral load and serologic

biomarkers in oral and oropharyngeal squamous cell carcinomas. Int J Cancer 2005;115:329-32.

11. Chow V, Yuen AP, Lam KY, Ho WK, Wei WI. Prognostic significance of serum p53 protein and p53 antibody in patients with surgical treatment for head and neck squamous cell carcinoma. Head Neck 2001;23:286-91.
12. Bourhis J, Lubin R, Roche B, Koscielny S, Bosq J, Dubois I, et al. Analysis of p53 serum antibodies in patients with head and neck squamous cell carcinoma. J Natl Cancer Inst 1996;88:1228-33.
13. Hoffmann TK, Donnenberg AD, Finkelstein SD, Donnenberg VS, Friebe-Hoffmann U, Myers EN, et al. Frequencies of tetramer+ T cells specific for the wild-type sequence p53(264-272) peptide in the circulation of patients with head and neck cancer. Cancer Res 2002;62:3521-9.
14. Albers A, Ferris R, Kim G, Chikamatsu K, DeLeo A, Whiteside T. Immune responses to p53 in patients with cancer:enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites. Cancer Immunology, Immunotherapy 2005;54:1072-81.
15. Wolf GT, Fee WE, Jr., Dolan RW, Moyer JS, Kaplan MJ, Spring PM, et al. Novel neoadjuvant immunotherapy regimen safety and survival in head and neck squamous cell cancer. Head Neck 2011.
16. Timar J, Ladanyi A, Forster-Horvath C, Lukits J, Dome B, Remenar E, et al. Neoadjuvant immunotherapy of oral squamous cell carcinoma modulates intratumoral CD4/CD8 ratio and tumor microenvironment: a multicenter phase II clinical trial. J Clin Oncol 2005;23:3421-32.
17. Rapidis AD, Wolf GT. Immunotherapy of head and neck cancer: current and future considerations. J Oncol 2009;2009:346345.
18. van der Burg SH, Melief CJ. Therapeutic vaccination against human papilloma virus induced malignancies. Curr Opin Immunol 2011;23:252-7.
19. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 2009;361:1838-47.
20. Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL, et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. Br J Cancer 2010;102:1129-36.
21. Vermeij R, Leffers N, van der Burg SH, Melief CJ, Daemen T, Nijman HW. Immunological and Clinical Effects of Vaccines Targeting p53-Overexpressing Malignancies. J Biomed Biotechnol 2011;2011:702146.
22. Speetjens FM, Kuppen PJ, Welters MJ, Essahsah F, Voet van den Brink AM, Lantrua MG, et al. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin Cancer Res 2009;15:1086-95.
23. Leffers N, Lambeck AJ, Gooden MJ, Hoogeboom BN, Wolf R, Hamming IE, et al. Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial. Int J Cancer 2009;125:2104-13.
24. Krul EJ, van d, V, Schuuring E, Van Kanten RW, Peters AA, Fleuren GJ. Human papillomavirus in malignant cervical lesions in Surinam, a high-risk country, compared to the Netherlands, a low-risk country. Int J Gynecol Cancer 1999;9:206-11.
25. van der Burg SH, Menon AG, Redeker A, Franken KL, Drijfhout JW, Tollenaar RA, et al. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. Int J Cancer 2003;107:425-33.
26. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004;64:5449-55.
27. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002;62:472-9.
28. de Vos van Steenwijk PJ, Heusinkveld M, Ramwadhdoebe TH, Lowik MJ, van der Hulst JM, Goedemans R, et al. An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. Cancer Res 2010;70:2707-17.
29. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, et al. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. Int J Cancer 2008;122:486-94.
30. van der Burg SH, Piersma SJ, de Jong A, van der Hulst JM, Kwappenberg KM, van den HM, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 2007;104:12087-92.
31. Mandapathil M, Szczepanski MJ, Szajnik M, Ren J, Lenzner DE, Jackson EK, et al. Increased ectonucleotidase expression and activity in regulatory T cells of patients with head and neck cancer. Clin Cancer Res 2009;15:6348-57.

32. Heusinkveld M, Welters MJ, van Poelgeest MI, van der Hulst JM, Melief CJ, Fleuren GJ, et al. The detection of circulating Human Papillomavirus (HPV)-specific T cells is associated with improved survival of patients with deeply infiltrating tumors. Int J Cancer 2010.
33. Watanabe Y, Katou F, Ohtani H, Nakayama T, Yoshie O, Hashimoto K. Tumor-infiltrating lymphocytes, particularly the balance between CD8(+) T cells and CCR4(+) regulatory T cells, affect the survival of patients with oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:744-52.
34. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NH, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res 2006;12:465-72.
35. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008;14:178-87.
36. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc Natl Acad Sci U S A 2010;107:11895-9.
37. Bonertz A, Weitz J, Pietsch DH, Rahbari NN, Schlude C, Ge Y, et al. Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. J Clin Invest 2009;119:3311-21.
38. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW, et al. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. Clin Cancer Res 2008;14:7188-95.
39. van der Burg SH, Piersma SJ, de JA, van der Hulst JM, Kwappenberg KM, van den HM, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 2007;104:12087-92.
40. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? Clin Cancer Res 2008;14:2028-35.
41. Piersma SJ, van der Hulst JM, Kwappenberg KM, Goedemans R, van der Minne CE, van der Burg SH. Influenza matrix 1-specific human CD4+ FOXP3+ and FOXP3(-) regulatory T cells can be detected long after viral clearance. Eur J Immunol 2010;40:3064-74.
42. Siegmund K, R++ckert B, Ouaked N, B++rgler S, Speiser A, Akdis CA, et al. Unique Phenotype of Human Tonsillar and In Vitro-Induced FOXP3+CD8+ T Cells. The Journal of Immunology 2009;182:2124-30.
43. Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM, et al. CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer. Clin Cancer Res 2007;13:6947-58.
44. van Seters M, van Beurden M, ten Kate FJ, Beckmann I, Ewing PC, Eijkemans MJ, et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. N Engl J Med 2008;358:1465-73.

CHAPTER 5.1

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

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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 (TGFβ1), 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 integraded 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 mycoplasm 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^{0}C 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

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

			cytokine production (pg/ml)*			
Cell-line	**Passage***	**HPV§**	**IL-8**	**TGFβ-1****	**PgE2**	**IL-6**
CC-10B	P80	45	14000	0	0	10
CSCC-1	P22	16	1500	221	0	20
CC11 -	P32	67	425	398	403	3438
CaSKi	x	16	324	410	298	2335
CSCC-7	P9	16	9306	313	2781	263
CC-8	P41	45	1222	250	3662	2228
HeLa	x	18	4500	548	5389	12000

(*)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 TGF-β1 measured by ELISA after acidic treatment
x, HeLa and CaSKi not known

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 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).

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).

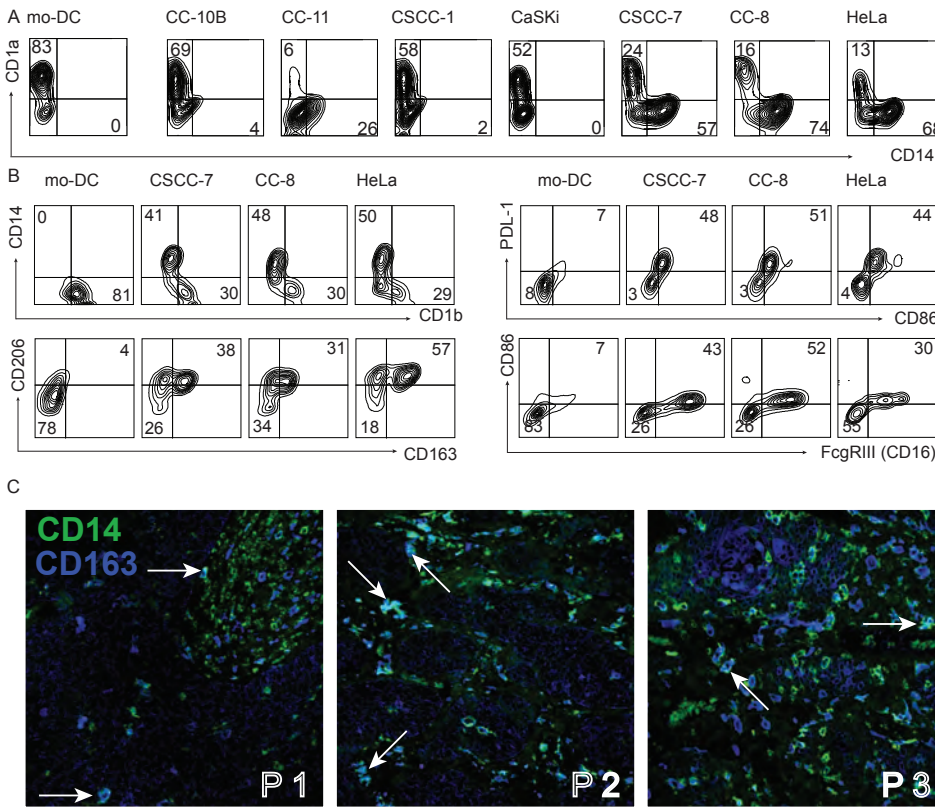

Figure 1 CxCa-secreted factors skew monocyte differentiation towards M2 macrophages. Monocytes were cultured for 6 days with GM-CSF and IL4 in culture medium with 20% tumor supernatant (TSN) of indicated CxCa cell lines or 20% control medium (mo-DC) and (A) analyzed for expression of CD14 and CD1a. TSN 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.

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.

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 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).

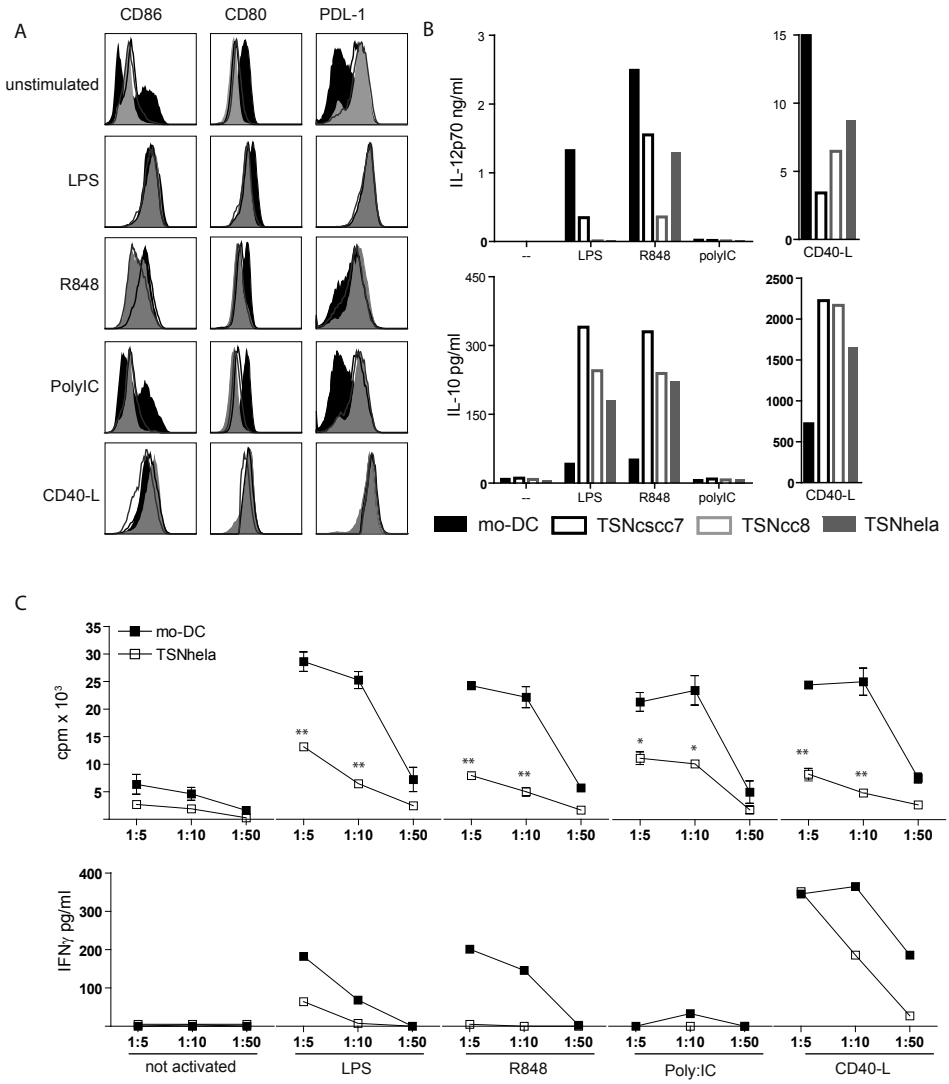

Figure 2 TSN-differentiated monocytes are phenotypically and functionally different from mo-DC. (A) Monocytes were differentiated in the presence of the indicated TSN and activated with single TLR-ligands or CD40-L cells for 48 hr. TSN-M2 cells express higher basal levels of CD86 and PDL-1. R848 or Poly-IC 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).

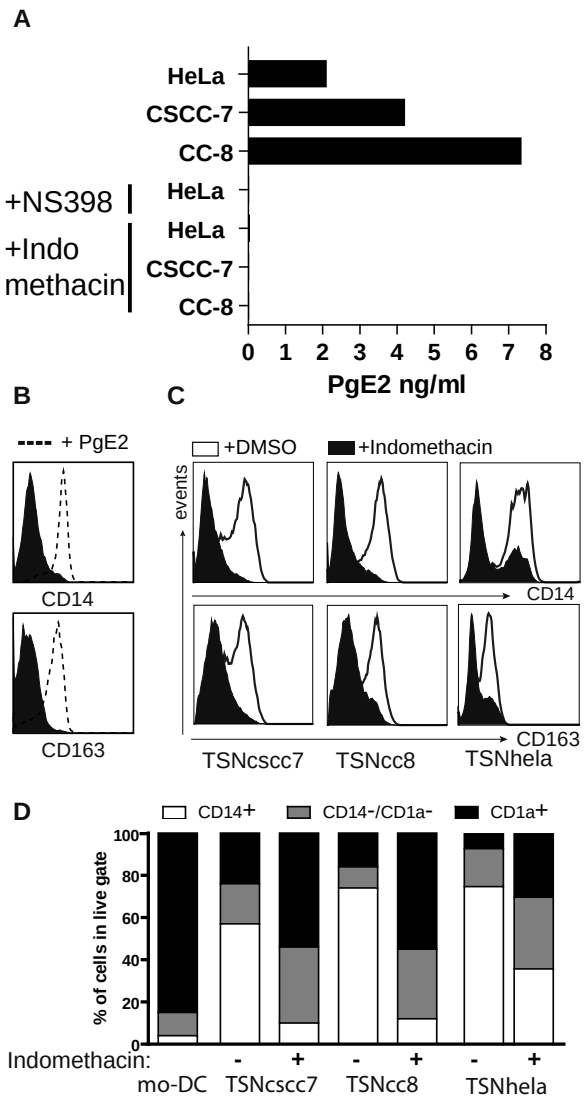

Figure 3 Cox-inhibition blocks PgE2 production and prevents M2-macrophage differentation. (A) Tumor cell lines were treated for 24 hr with DMSO (control) or Cox-inhibitor (NS398 or Indomethacin) followed by culturing in fresh medium without additives for 24 hrs. PgE2 levels were measured by ELISA. Inhibition 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).

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 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

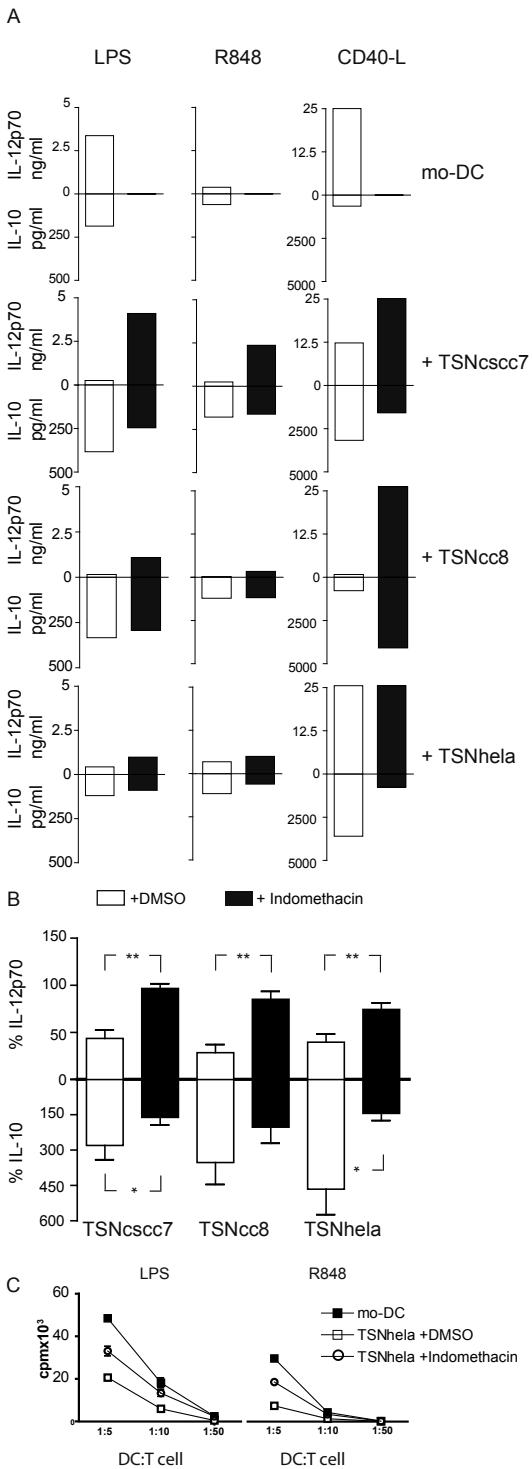

Figure 4 Restored cytokine production when PgE2 production by tumor cells is blocked. (A) The production of IL-12 and IL-10 by APC differentiated in the presence of TSN of Indomethacin-treated (black) tumor cells or TSN from non-treated tumor cells (white) upon 48 hr activation with LPS, R848 or CD40-L 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.

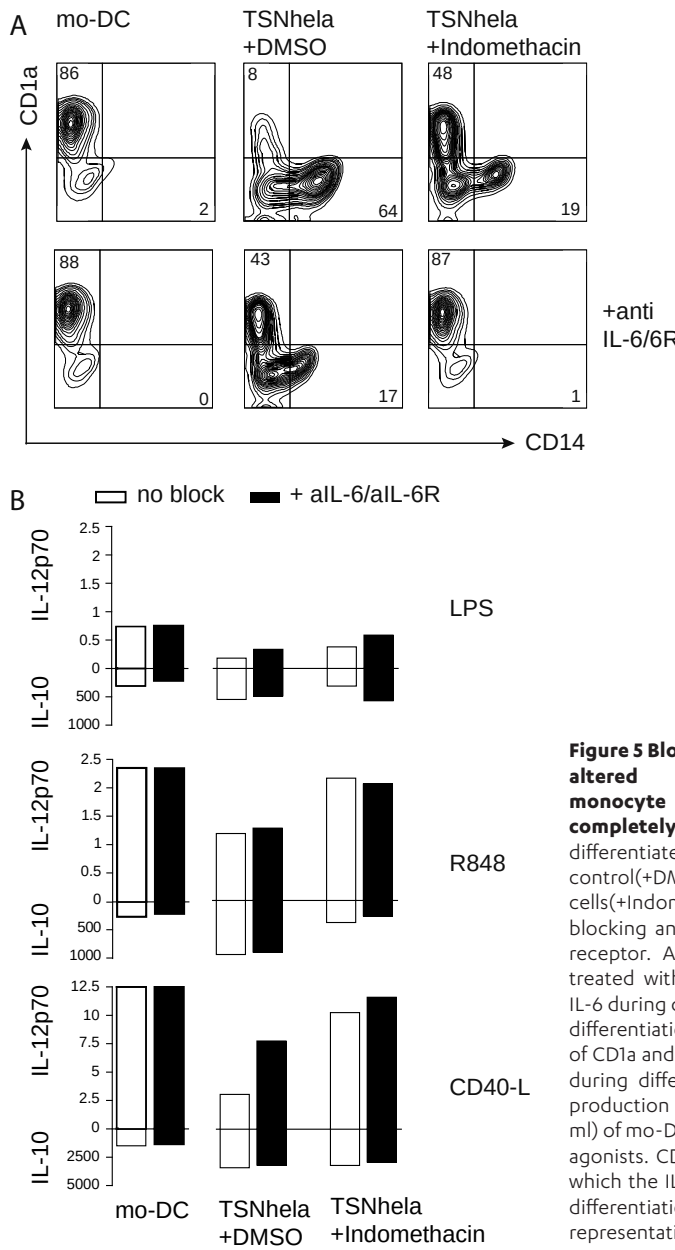

Figure 5 Blocking IL-6 and PgE2-induced altered differentiation restores monocyte to DC differentiation completely. (A) Monocytes were differentiated in the presence of control(+DMSO) or COX-inhibited HeLa cells(+Indomethacin) without or with blocking antibodies against IL-6 and IL-6 receptor. A combination 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.

supernatant of the co-cultures reflecting the activation of the T cells upon recognition of 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

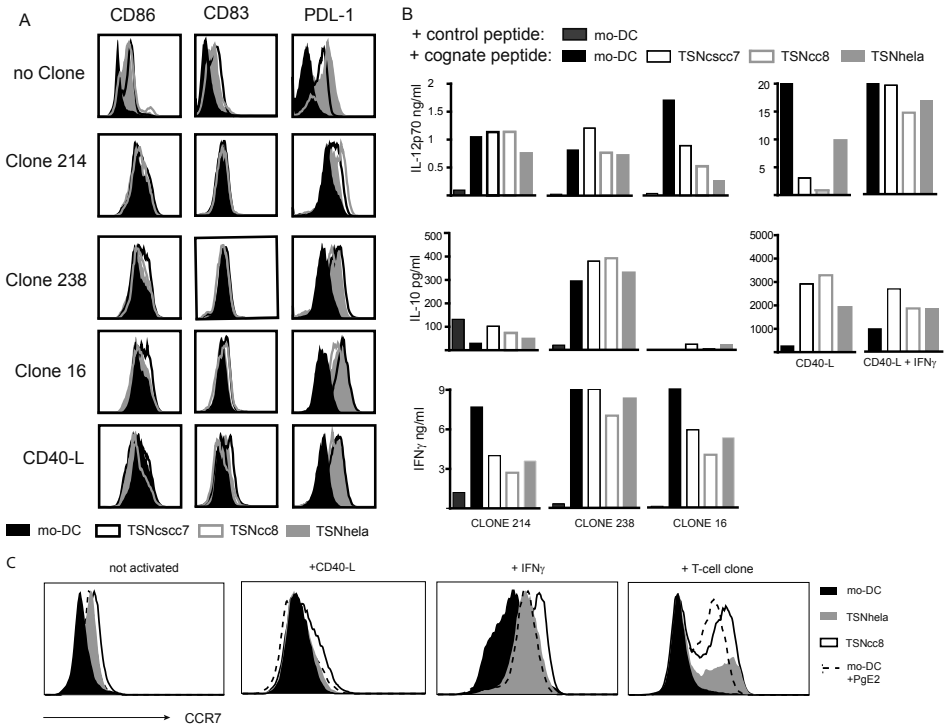

Figure 6 CD4+ T cells switch TSN-M2 to M1-like macrophages and activate them to produce IL-12p70. (A) Mo-DC or TSN-M2 were pulsed with the cognate Th1-peptide and co-cultured with HPV-specific CD4+ T-cell clones (2 DC:1 T cell) or CD40-L cells. T-cell interaction results in high expression of co-stimulatory 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.

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 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).

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REFERENCES

1. Ho, G. Y., R. Bierman, L. Beardsley, C. J. Chang, and R. D. Burk. 1998. Natural history of cervicovaginal papillomavirus infection in young women. *N. Engl. J. Med.* 338:423-428.
2. de Jong, A., M. I. van Poelgeest, J. M. van der Hulst, J. W. Drijfhout, G. J. Fleuren, C. J. Melief, G. Kenter, R. Offringa, and S. H. van der Burg. 2004. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. *Cancer Res.* 64:5449-5455.
3. de Vos van Steenwijk, PJ., S. J. Piersma, M. J. Welters, J. M. van der Hulst, G. Fleuren, B. W. Hellebrekers, G. G. Kenter, and S. H. van der Burg. 2008. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. *Clin. Cancer Res.* 14:7188-7195.
4. Heusinkveld, M., M. J. Welters, M. I. van Poelgeest, J. M. van der Hulst, C. J. Melief, G. J. Fleuren, G. G. Kenter, and S. H. van der Burg. 2010. The detection of circulating Human Papillomavirus (HPV)-specific T cells is associated with improved survival of patients with deeply infiltrating tumors. *Int. J. Cancer*.
5. Karim, R., E. S. Jordanova, S. J. Piersma, G. G. Kenter, L. Chen, J. M. Boer, C. J. Melief, and S. H. van der Burg. 2009. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin. Cancer Res.* 15:6341-6347.
6. Piersma, S. J., M. J. Welters, J. M. van der Hulst, J. N. Kloth, K. M. Kwappenberg, B. J. Trimbos, C. J. Melief, B. W. Hellebrekers, G. J. Fleuren, G. G. Kenter, R. Offringa, and S. H. van der Burg. 2008. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. *Int. J. Cancer* 122:486-494.
7. van der Burg, S. H., S. J. Piersma, A. de Jong, J. M. van der Hulst, K. M. Kwappenberg, H. M. van den, M. J. Welters, J. J. Van Rood, G. J. Fleuren, C. J. Melief, G. G. Kenter, and R. Offringa. 2007. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. *Proc. Natl. Acad. Sci. U. S. A* 104:12087-12092.
8. Jordanova, E. S., A. Gorter, O. Ayachi, F. Prins, L. G. Durrant, G. G. Kenter, S. H. van der Burg, and G. J. Fleuren. 2008. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin. Cancer Res.* 14:2028-2035.
9. Nedergaard, B. S., M. Ladekarl, J. R. Nyengaard, and K. Nielsen. 2008. A comparative study of the cellular immune response in patients with stage IB cervical squamous cell carcinoma. Low numbers of several immune cell subtypes are strongly associated with relapse of disease within 5 years. *Gynecologic Oncology* 108:106-111.
10. Piersma, S. J., E. S. Jordanova, M. t. I. E. van Poelgeest, K. M. C. Kwappenberg, J. M. van der Hulst, J. W. Drijfhout, C. J. M. Melief, G. G. Kenter, G. J. Fleuren, R. Offringa, and S. H. van der Burg. 2007. High Number of Intraepithelial CD8+ Tumor-Infiltrating Lymphocytes Is Associated with the Absence of Lymph Node Metastases in Patients with Large Early-Stage Cervical Cancer. *Cancer Res* 67:354-361.
11. Rabinovich, G. A., D. Gabrilovich, and E. M. Sotomayor. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.* 25:267-296.
12. Gottfried, E., M. Kreutz, and A. Mackensen. 2008. Tumor-induced modulation of dendritic cell function. *Cytokine & Growth Factor Reviews* 19:65-77.
13. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology* 23:549-555.
14. Whiteside, T. L. 2008. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 27:5904-5912.
15. Alcocer-Gonzalez, J. M., J. Berumen, R. Tamez-Guerra, V. Bermudez-Morales, O. Peralta-Zaragoza, R. Hernandez-Pando, J. Moreno, P. Gariglio, and V. Madrid-Marina. 2006. In vivo expression of immunosuppressive cytokines

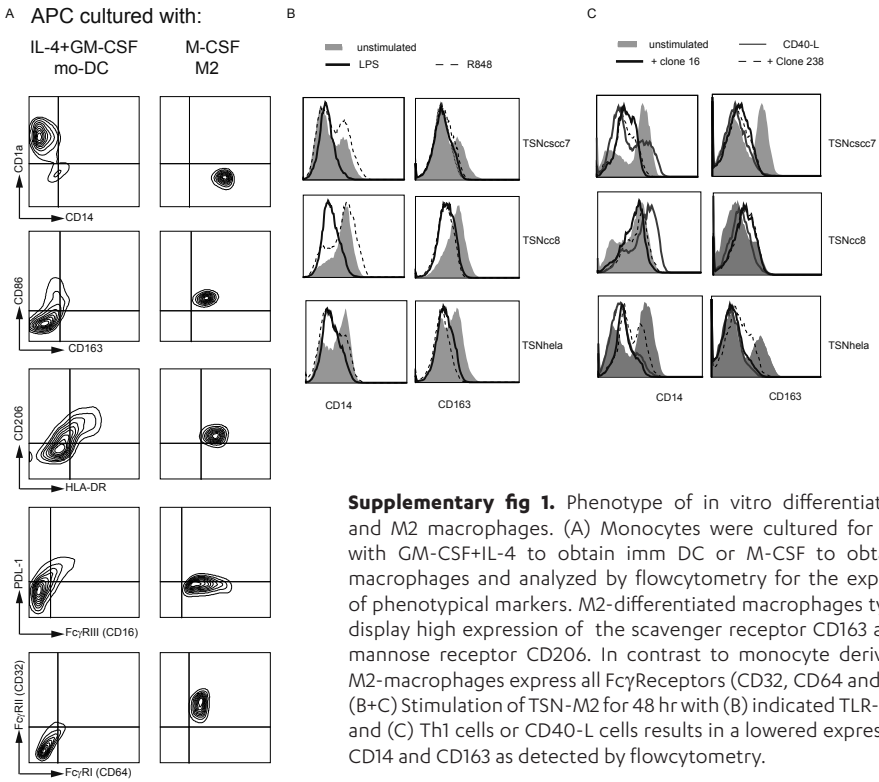
in human papillomavirus-transformed cervical cancer cells. *Viral Immunol*. 19:481-491.

16. Herfs, M., L. Herman, P. Hubert, F. Minner, M. Arafa, P. Roncarati, Y. Henrotin, J. Boniver, and P. Delvenne. 2009. High expression of PGE2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells. *Cancer Immunol. Immunother.* 58:603-614.
17. Kloth, J. N., G. J. Fleuren, J. Oosting, R. X. de Menezes, P. H. C. Eilers, G. G. Kenter, and A. Gorter. 2005. Substantial changes in gene expression of Wnt, MAPK and TNF{alpha} pathways induced by TGF-{beta}1 in cervical cancer cell lines. *Carcinogenesis* 26:1493-1502.
18. Kotowicz, B., M. Fuksiewicz, M. Kowalska, J. Jonska-Gmyrek, M. Bidzinski, and J. Kaminska. 2008. The value of tumor marker and cytokine analysis for the assessment of regional lymph node status in cervical cancer patients. *Int. J. Gynecol. Cancer* 18:1279-1284.
19. Srivani, R., and B. Nagarajan. 2003. A prognostic insight on in vivo expression of interleukin-6 in uterine cervical cancer. *Int. J. Gynecol. Cancer* 13:331-339.
20. Hennessy, E. J., A. E. Parker, and L. A. J. O'Neill. 2010. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* 9:293-307.
21. Tiersma, E. S., M. L. van der Lee, A. A. Peters, A. P. Visser, F. G. Jan, B. Garssen, K. M. van Leeuwen, S. le Cessie, and K. Goodkin. 2004. Psychosocial factors and the grade of cervical intra-epithelial neoplasia: a semi-prospective study. *Gynecol. Oncol*. 92:603-610.
22. van Dongen, M., N. D. Savage, E. S. Jordanova, I. H. Briaire-de Bruijn, K. V. Walburg, T. H. Ottenhoff, P. C. Hogendoorn, S. H. van der Burg, H. Gelderblom, and T. van Hall. 2010. Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. *Int. J. Cancer* 127:899-909.
23. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med*. 180:1263-1272.
24. Koopman, L. A., K. Szuhai, J. D. H. van Eendenburg, V. Bezrookove, G. G. Kenter, E. Schuuring, H. Tanke, and G. Jan Fleuren. 1999. Recurrent Integration of Human Papillomaviruses 16, 45, and 67 Near Translocation Breakpoints in New Cervical Cancer Cell Lines. *Cancer Res* 59:5615-5624.
25. Verreck, F. A., T. de Boer, D. M. Langenberg, Z. L. van der, and T. H. Ottenhoff. 2006. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *J. Leukoc. Biol*. 79:285-293.
26. Biswas, S. K., and A. Mantovani. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol*. 11:889-896.
27. Poschke, I., D. Mougiakakos, J. Hansson, G. V. Masucci, and R. Kiessling. 2010. Immature Immunosuppressive CD14+HLA-DRΓêÆ/low Cells in Melanoma Patients Are Stat3hi and Overexpress CD80, CD83, and DC-Sign. *Cancer Res* 70:4335-4345.
28. LeibundGut-Landmann, S., F. Osorio, G. D. Brown, and Reis e Sousa. 2008. Stimulation of dendritic cells via the dectin-1/Syk pathway allows priming of cytotoxic T-cell responses. *Blood* 112:4971-4980.
29. Schulz, E. G., L. Mariani, A. Radbruch, and T. H÷fer. 2009. Sequential Polarization and Imprinting of Type 1 T Helper Lymphocytes by Interferon-[gamma] and Interleukin-12. *Immunity* 30:673-683.
30. Flavell, R. A., S. Sanjabi, S. H. Wrzesinski, and P. Licona-Limon. 2010. The polarization of immune cells in the tumour environment by TGFbeta. *Nat. Rev. Immunol*. 10:554-567.
31. Kalinski, P., C. M. Hilkens, A. Snijders, F. G. Snijdewint, and M. L. Kapsenberg. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 159:28-35.
32. Chomarat, P., J. Banchereau, J. Davoust, and A. K. Palucka. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat. Immunol*. 1:510-514.
33. Menetrier-Caux, C., G. Montmain, M. C. Dieu, C. Bain, M. C. Favrot, C. Caux, and J. Y. Blay. 1998. Inhibition of the Differentiation of Dendritic Cells From CD34+ Progenitors by Tumor Cells: Role of Interleukin-6 and Macrophage Colony-Stimulating Factor. *Blood* 92:4778-4791.
34. Anastassiou, E. D., F. Paliogianni, J. P. Balow, H. Yamada, and D. T. Boumpas. 1992. Prostaglandin E2 and other cyclic AMP-elevating agents modulate IL-2 and IL-2R alpha gene expression at multiple levels. *J. Immunol*. 148:2845-2852.
35. Zijlmans, H. J., G. J. Fleuren, H. J. Baelde, P. H. Eilers, G. G. Kenter, and A. Gorter. 2007. Role of tumor-derived proinflammatory cytokines GM-CSF, TNF-alpha, and IL-12 in the migration and differentiation of antigen-presenting cells in cervical carcinoma. *Cancer* 109:556-565.
36. Kuang, D. M., Q. Zhao, C. Peng, J. Xu, J. P. Zhang, C. Wu, and L. Zheng. 2009. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune

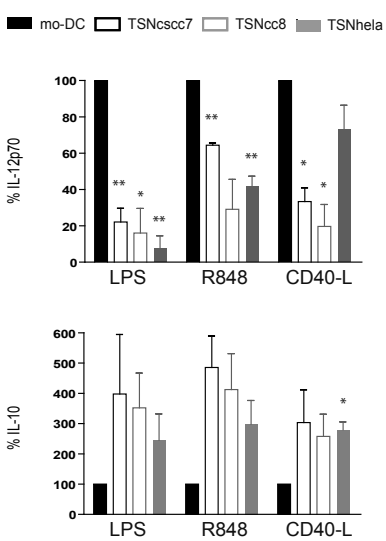
privilege and disease progression through PD-L1. *J. Exp. Med*. 206:1327-1337.

37. Hobo, W., F. Maas, N. Adisty, W. T. de, N. Schaap, d. van, V, and H. Dolstra. 2010. siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8+ T cells. *Blood* 116:4501-4511.
38. Dumitriu, I. E., D. R. Dunbar, S. E. Howie, T. Sethi, and C. D. Gregory. 2009. Human Dendritic Cells Produce TGF-{beta}1 under the Influence of Lung Carcinoma Cells and Prime the Differentiation of CD4+CD25+Foxp3+ Regulatory T Cells. *J Immunol* 182:2795-2807.
39. Hammes, L. S., R. R. Tekmal, P. Naud, M. I. Edelweiss, N. Kirma, P. T. Valente, K. J. SyrjΣnen, and J. S. Cunha-Filho. 2007. Macrophages, inflammation and risk of cervical intraepithelial neoplasia (CIN) progression--Clinicopathological correlation. *Gynecologic Oncology* 105:157-165.
40. Ratta, M., F. Fagnoni, A. Curti, R. Vescovini, P. Sansoni, B. Oliviero, M. Fogli, E. Ferri, G. R. la Cuna, S. Tura, M. Baccarani, and R. M. Lemoli. 2002. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* 100:230-237.
41. Roca, H., Z. S. Varsos, S. Sud, M. J. Craig, C. Ying, and K. J. Pienta. 2009. CCL2 and Interleukin-6 Promote Survival of Human CD11b+ Peripheral Blood Mononuclear Cells and Induce M2-type Macrophage Polarization. *Journal of Biological Chemistry* 284:34342-34354.
42. Sombroek, C. C., A. G. M. Stam, A. J. Masterson, S. M. Lougheed, M. J. A. G. Schakel, C. J. L. M. Meijer, H. M. Pinedo, A. J. M. van den Eertwegh, R. J. Scheper, and T. D. de Gruijl. 2002. Prostanoids Play a Major Role in the Primary Tumor-Induced Inhibition of Dendritic Cell Differentiation. *J Immunol* 168:4333-4343.
43. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.
44. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol*. 175:342-349.
45. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med*. 188:2357-2368.
46. Haabeth, O. A., K. B. Lorvik, C. Hammarstrom, I. M. Donaldson, G. Haraldsen, B. Bogen, and A. Corthay. 2011. Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer. *Nat. Commun*. 2:240.
47. Al Saleh, W., S. L. Giannini, N. Jacobs, M. Moutschen, J. Doyen, J. Boniver, and P. Delvenne. 1998. Correlation of T-helper secretory differentiation and types of antigen-presenting cells in squamous intraepithelial lesions of the uterine cervix. *J. Pathol*. 184:283-290.
48. de Vos van Steenwijk, PJ., M. Heusinkveld, T. H. Ramwadhdoebe, M. J. Lowik, J. M. van der Hulst, R. Goedemans, S. J. Piersma, G. G. Kenter, and S. H. van der Burg. 2010. An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. *Cancer Res*. 70:2707-2717.
49. Wu, W. K. K., J. J. Yiu Sung, C. W. Lee, J. Yu, and C. H. Cho. 2010. Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: An update on the molecular mechanisms. *Cancer Letters* 295:7-16.
50. Singh, J. A., S. Beg, and M. A. Lopez-Olivo. 2010. Tocilizumab for rheumatoid arthritis. *Cochrane. Database. Syst. Rev.* CD008331.
51. Welters, M. J., G. G. Kenter, de Vos van Steenwijk PJ, M. J. Lowik, Berends-van der Meer DM, F. Essahsah, L. F. Stynenbosch, A. P. Vloon, T. H. Ramwadhdoebe, S. J. Piersma, J. M. van der Hulst, A. R. Valentijn, L. M. Fathers, J. W. Drijfhout, K. L. Franken, J. Oostendorp, G. J. Fleuren, C. J. Melief, and S. H. van der Burg. 2010. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc. Natl. Acad. Sci. U. S. A* 107:11895-11899.
52. Kenter, G. G., M. J. Welters, A. R. Valentijn, M. J. Lowik, Berends-van der Meer DM, A. P. Vloon, F. Essahsah, L. M. Fathers, R. Offringa, J. W. Drijfhout, A. R. Wafelman, J. Oostendorp, G. J. Fleuren, S. H. van der Burg, and C. J. Melief. 2009. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med*. 361:1838-1847.

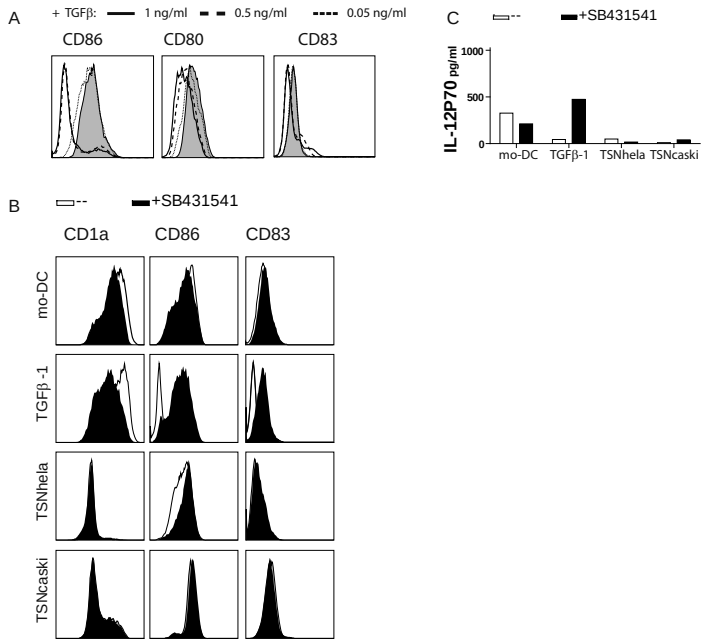
SUPPLEMENTARY FIGURES



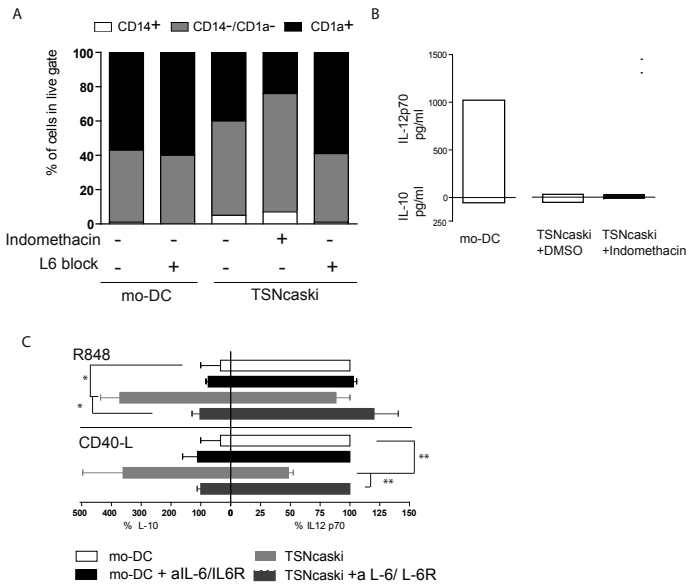
Supplementary fig 1. Phenotype of in vitro differentiated DC and M2 macrophages. (A) Monocytes were cultured for 6 days with GM-CSF+IL-4 to obtain imm DC or M-CSF to obtain M2 macrophages and analyzed by flowcytometry for the expression of phenotypical markers. M2-differentiated macrophages typically 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. 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 monocytes 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.



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. Flowcytometric 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 TGFb-1 (TGFb) 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 TGFb-treated cells to the same level as mo-DC, but does not restore IL-12 production by TSN-differentiated APC.



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 caski cells 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. Depicted is the percentage of all ells in live gate One epresentative of 3 experiments. (B) 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 compa e different donors the amount of cytokine produces is depicted as a percentage of the cytok ne when produced by mo DC a tivated 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 5.2

Modulation of tumor associated macrophages

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EM Dijkgraaf, V Jha, B Tummers, SH van der Burg**

Ongoing work

ABSTRACT

CxCa patients with recurrent disease are treated with cisplatin or carboplatin. These chemotherapeutics may not only affect tumor cells but also the local immune cells. Previously we showed that CxCa can skew monocyte differentiation toward type 2 macrophages (M2) by secretion of PGE2 and IL-6. Therefore we studied the effects of these drugs on soluble factor mediated effects of tumor cells lines on APC differentiation.

Tumor induced M2 can be skewed towards pro-inflammatory M1 by interaction with IFNγ+ CD4+ T cells. In CxCa-patients however, a considerable proportion of tumor antigen specific T cells display an non-Th1 phenotype. To study the activation of tumor supernatant skewed M2 by Th2 or Th17 cells we activated APC with CD40-L and several T-cell derived cytokines.

We show that only CD40-L+IFNγ can skew M2 cells into activated M1 cells. Furthermore while CD40-L stimulated M2 macrophages induced strong T cell proliferation, only when stimulation was in the presence of IFNγ, the responding T cells were polarized into Th1. Treatment tumorcells with cisplatin, and to a lesser extent carboplatin, resulted in increased production of PGE2 and IL-6, resulting in induction of more M2 cells. This is an unwanted immunological effect and could be prevented by treating the tumor cells with COX-inhibitors.

INTRODUCTION

Currently the therapy for advanced, persistent or recurrent cervical cancer (CxCa) consists of cisplatin or carboplatin treatment although many tumors are resistant and the response rate does not exceed 30% (1,2). One described predictor of poor response to treatment is high expression of COX-2 protein in the tumor (3). COX-2 , which is the inflammation induced rate limiting enzyme for the conversion of arachnoid acid into prostaglandin E2 (PGE2) is found in several human solid tumors and we recently showed that PGE2 skews local antigen presenting cells (APC) into type 2 macrophages (M2) (4). The presence of M2 macrophages in tumors is correlated to poor prognosis in several human cancers (5-7).

Platinum based therapeutics act by binding to DNA. This activates various signal transduction pathways including those for DNA repair, cell cycle arrest and apoptosis. As a result it may have immune-modulating side effects (8). In a mouse tumor model oxaliplatin caused immunogenic cell death, thereby activating local APC and enhancing anti-tumor T-cell responses. Cisplatin triggered the release of the TLR-4 stimulating protein HMGB-1 (9,10). In addition, several platinum based drugs down regulated surface molecules in tumor and human APC via regulation of STAT6 (11).

In contrast to TLR-agonists or CD40-L help, a signal of CD40-L+IFNγ supplied through interaction with Th1 CD4+ T cells can switch tumor cell-induced M2 macrophages (TSN-skewed APC) into activated M1 macrophages (4). Studies on murine macrophages confirmed macrophage plasticity by showing that exposure to a type 1 cytokine environment before activation directs the macrophages towards M1 macrophages, whereas exposure to IL-10 directed them to M2 (12,13). In line with this Dulluc *et al.* comprehensively showed that IFNγ can prevent and reverse M2 differentiation of human monocytes by ascites of ovarian cancer *in vitro* (14). Together this indicates that IFNγ is a strong polarizing cytokine for macrophages. However in several human tumors not only Th1 (IFNγ/TNFα) CD4+ T cells but also Th2 (IL-5/IL-4), Th17 (IL-17) and regulatory T cells (TGFβ, IL-10) play a role. Especially in cervical cancer draining lymph nodes considerable numbers of tumor-antigen specific T cells are found that do not express a clear type 1 cytokine profile.

Subsequently we questioned the impact of platinum based chemotherapy on the micro milieu of cervical tumors. We focused on APC differentiation influenced by chemotherapy treated tumor cell lines as well as the impact of cognate interaction with several types of T cells in our in vitro system geared to result in M2 macrophages. To study the potential role of such differently polarized CD4+ T-cell subsets in the activation of locally present APC we cultured mo-DC without or with supernatant of CxCa cell lines (TSN-skewed APC) with CD40-L expressing cells in the presence of several Th-cytokines to mimic interaction with the separate T cell subsets.

MATERIAL AND METHODS

Media and reagents

APCs and CxCa cell lines were cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% FCS (Greiner Bio-one, Germany), 2 mM L-glutamine (Cambrex, USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM β-mercaptoethanol (all Invitrogen), also referred to as complete or control medium. Adherent cell lines were harvested using trypsin/EDTA (Invitrogen).

The following factors were used to culture APCs: 500 U/mL IL-4 (Invitrogen), 800 U/mL GM-CSF (Immunotools, Germany), 5 ng/mL PGE2 (Sigma-Aldrich, Germany), 5 ng/ml IL-6 (immunotools) or 25 ng/ml M-CSF. The following was used to activate APCs. CD40-L expressing J558 mouse plasmacytoma cells ratio J558:APC 1:5 (The J558 cells were a kind gift of dr. E. de Jong, Amsterdam and dr. P. Lane, Birmingham); 10 µg/mL R848(CL097) 500 U/mL IFNγ (Bender Medsystems, Austria, this is approximately 1000 pg/ml), 2 ng/ml TNFα, 5 ng/ml IL-5, 2 ng/ml IL-17 (all immunotools). These concentrations were based on levels found in DC:T cell clone co-cultures or TIL cultures like described in chapter 6.

Chemotherapeutics and COX inhibitors

Cells were treated with 0.2-50 µg/mL cisplatin or 2-100 µg/mL carboplatin (Pharmachemie, NL) or the corresponding volume of PBS as well as with 25 µM indomethacin or the corresponding volume of DMSO.

Tumor cell line culture

The CxCa cell lines HeLa, CaSki, CSCC-1, CSCC-7, and CC-8 were typed and cultured as described earlier (4,15). Cell lines were cultured in 6- or 12- well plates (Corning, USA) for 24h, and treated with chemotherapeutics and/or COX inhibitors, as indicated. After 16h of treatment, cells were washed carefully and medium was refreshed. Supernatants were harvested after 24h and stored at -20ºC.

Only for RNA isolation cells were treated with chemotherapeutics for 18 hr, carefully washed and RNA was extracted. cDNA was prepared and gene expression was analyzed using pre-designed taqman probes of Applied Biosystems for GAPDH, IL-6, COX-1 (PTGS-1) and COX-2 (PTGS-2).

Transwell assay

Monocytes were cultured in a 24 well plate in complete medium containing IL-4 and GM-CSF and 0.5 or 2 µg/mL cisplatin, or PBS control. CC-8 cells (50,000 cells in 100 µL complete medium) were cultured in the upper compartment of a transwell 0.4 µm pore insert (Corning). To control wells, without transwell inserts, 100 µL control medium or tumor supernatant from untreated CC-8 or HeLa cells was added. After 3 days, complete medium with cytokines but without cisplatin was added. At day 6, cells were analyzed by flow cytometry.

Cell survival

To determine tumor cell line survival upon chemotherapeutics treatment, a MTT assay (Trevigen, USA) was performed according to the manufacturer's instructions. Cells were cultured as described above, in a 96 well flat bottom culture plate (Corning), but instead of harvesting the supernatant, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. Absorbance was determined at 570nm and corrected for absorbance at 655 nm. Cell survival was calculated as follows: ($OD_{570\text{-}655nm}$ for treated / $OD_{570\text{-}655nm}$ for untreated) x 100%. To determine APC survival upon chemotherapeutics treatment, cells were analyzed by flow cytometry (see below). Cell survival was calculated as follows: (% of cells in live gate for treated / % of cells in live gate for untreated) x 100%.

Flow cytometry

APC were phenotypically analized as described earlier (4).

Cytokine analysis

ELISA was used to quantify cytokine levels of IFNγ, IL-10 (Sanquin, NL) IL-12p70 (BD Biosciences) and chemokines CXCL-10 and CCL-22 (RndD systems), according to the manufacturer's instructions. Further analysis of inflammatory and T cell cytokines was performed by cytometric bead array (BD Biosicences). PGE2 levels were measured using the competitive PGE_2 immunoassay kit (R&D systems).

RESULTS

IFNγ is the only cytokine that skews M2 to activated M1

We found that in LN of CxCa patients tumor-antigen specific T cells produce IFNγ, TNFα, IL-5 or IL-17. Therefore we assessed whether these type of T cells also could activate TSN-skewed APC. Monocytes were cultured with IL-4 and GM-CSF in the presence of supernatant of HELA or CSCC-7 cells (TSN-skewed APC). Routinely this resulted in 30-40% cells expressing typical M2 markers (4). Addition of the cytokines IFNγ, TNFγα, IL-5 or IL-17 at quantities produced by patient derived T cell clones in antigen specific tests did not result in the production of IL-12, IL-10 or IL-23 by mo-DC or TSN-skewed APC. Only IFNγ induced the upregulation of costimulatory molecules CD86, CD80, CD83 and PD-L1 on APC(not shown).

To mimic T-cell help but to ensure no other receptors were involved we made use of the J558 cell line transfected with CD40-L (CD40-L). Upon stimulation with CD40-L the mo-DC and TSN-skewed APC expressed high co-stimulatory molecules and lost CD163 expression (not shown). Addition of cytokines did not induce any change in co-stimulatory molecule expression compared to CD40-L stimulation only. Upon CD40 stimulation, TSN-skewed APC produced lower levels of IL-12 but high IL-10 compared to mo-DC upon CD40-L stimulation. Only addition of the cytokine IFNγ to CD40-L stimulation resulted in increased IL-12 production indicating a switch from M2 into M1-like cultures (Figure 1a). No phenotypic or functional effects were observed upon TNFα, IL-5 or IL-17 addition to CD40-L. Also several combinations of cytokines were tested but no additive effect to IFNγ was seen (not shown).

APC secrete chemokines to attract other inflammatory cells. CXCL-10 is the ligand for $CXCR\text{-}3^+$ type 1 effector T cells whereas CCL-22 attracts CCR-4 expressing Th2 and regulatory T cells. CCL-22 was shown to be induced in human APC when activated in the presence of PGE2 (16). Unstimulated mo-DC or TSN-skewed APC did not produce any detectable CXCL-10 but did produce high levels of CCL-22 (mo-DC cultures 40 ng/ml or more). Interestingly, HELA-supernatant APC produced only half the quantities CCL-22 of mo-DC ($p < 0.043$ paired t-test). When APC were stimulated with IFNγ CXCL-10 production was increased almost 10-fold and this was the same for all APC cultures (fig 2b). No chemokine induction upon stimulation with TNFα, IL5 or IL-17 was seen.

CD40 mediated activation did not induce significant CXCL-10 release but induced even more production of CCL-22 in mo-DC as well as TSN-skewed APC. These results show that IFNγ is the only T-cell produced cytokine that can activate TSN-skewed APC and reverse the cells to pro-inflammatory cytokine production and attraction of type 1 T cells.

Activation of APC by Th1 signals determines type of T-cell induction

Previously, we showed that CD40ligation of mo-DC resulted in the production of high levels of IL-12, a known strong type 1 directing stimulus (4). To assess whether the type of T cell

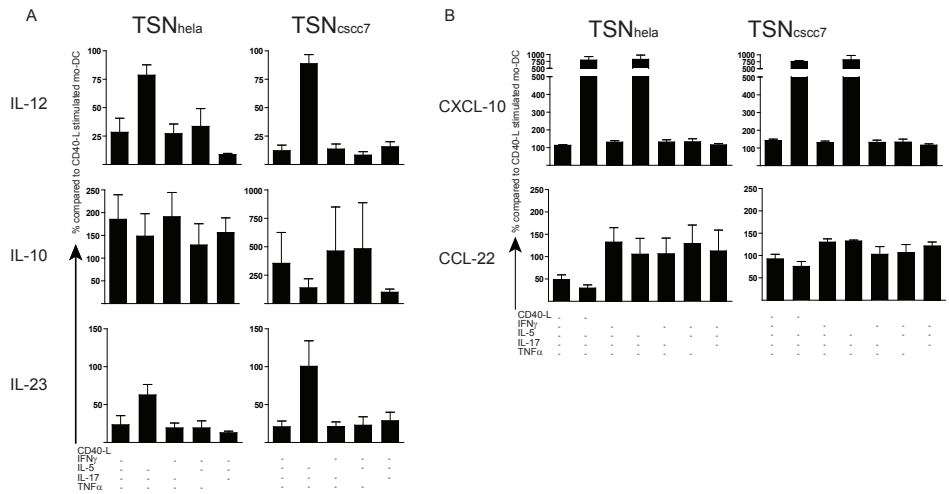

Figure 1. IFNγ activates tumor supernatant induced APC. Compared to mo-DC, supernatant induced cultures produce less IL-12 and more IL-10 upon CD40-L stimulation and only IFNγ can revert this balance. A) mo-DC or TSN-M2 (supernatant of HELA left, supernatant of CSCC-7 right) were activated with J558-CD40-L cells (ratio 5 DC:1 CD40-L cell) and recombinant cytokines (depicted at the x-axis). Supernatant was analyzed by ELISA after 48 hr. Mean cytokine production of CD40-L stimulated mo-DC: 24 ng/ml IL-12, 14 ng/ml IL-23 and 275 pg IL-10. Calculation: (activate TSN-M2 / Value mo-DC stimulated with CD40-L only)*100. Mean of 3 experiments + SEM. B) Chemokine production by cells described in A. Here also unstimulated and IFNγ only is shown. (In unactivated cultures no CXCL-10 was detected but CCL-22 was produced in all cultures.CCL-22 in mo-DC cultures >40 ng/ml) Mean of 3 experiments + SEM.

providing the activation signal may program the APC to subsequently instruct naïve T cells to adapt to a particular phenotype, we used a mixed lymphocyte reaction assay (MLR). Mo-DC or TSN-induced APC were activated for 48 hr with irradiated J558-CD40-L in the presence of recombinant cytokines. APC were extensively washed and cultured with allogeneic CD4+CD25- cells for 5 and 10 days. All APC types and activation signals induced comparable proliferation of the responder cells (fig 2. top left). However type 1 T-cell induction as reflected by the amounts of IFNγ and TNFα produced, was determined by the type of T cell providing the activation to the APC. Mo-DC activated with Th1 signals (CD40-L+IFNγ) clearly induced 4 fold higher type 1 cytokine production in the responder T cells. High IFNγ levels were also induced by TSN-induced M2 cultures stimulated with Th1 signals (figure 2, middle panels). Interestingly some type 2 cytokines were also detected in the cultures of T cells stimulated with CD40-L+IFNγ activated APC, but the production of these cytokines was more prominent in the T-cell cultures stimulated with TSN-skewed APC. Nine days after start of culture, the T cells were analyzed for expression of transcription factors. Figure 2 clearly shows that T cells display the highest T-bet expression following stimulation with mo-DC. Activation of APC in the presence of IFNγ resulted in higher T-bet expression in the responder T cells (figure 2 bottom left).

Different cytokines result in different macrophages in vitro

In vitro, macrophages can be obtained by culturing monocytes with only GM-CSF, M-CSF or as we reported previously with PgE2 and/or IL-6. Of note, the latter protocol induced

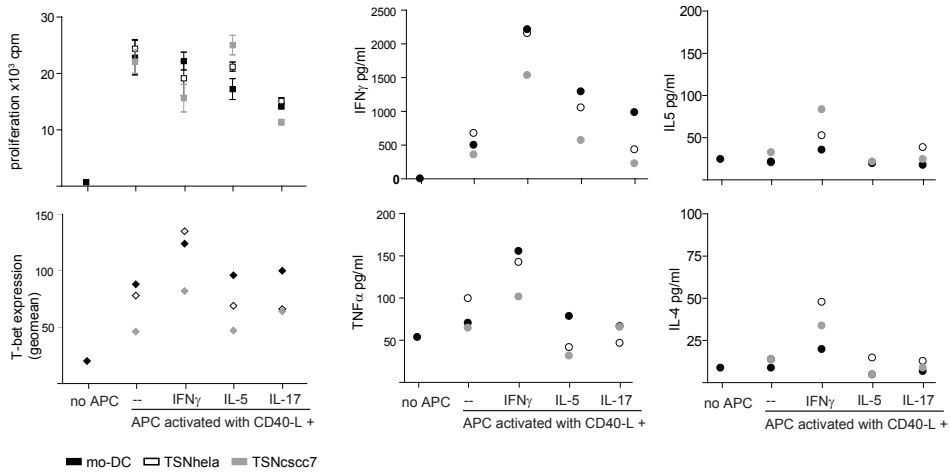

Figure 2. Th1 derived activation of APC instructs for type 1 T cell induction. Activation of APC in the presence of IFNγ results in higher T-bet expression and more type 1 cytokines. Allogeneic responder cells were cultured with mo-DC or TSN-skewed APC that were prior to co-culture activated with irradiated CD40-L only or in combination with cytokines (IFNγ, IL-5 or IL-17) as depicted at the x-axis. Top panel left; proliferation of T cell cultures at day 5 (initial ratio APC:T cell 1:5, triplo mean+SEM). Lower left panel: expression of the intra-cellular transcription factor T-bet at day 9 of T cell culture determined by intra-cellular staining and flowcytometry. Middle and right panels; cytokine levels in T-cell cultures at day 5 of co-culture. One experiment of 2 performed with IFNγ and IL-4 derived from MLR 17, IL-4 and TNFα from MLR 16.

macrophages even in the presence of the DC-skewing cytokines GM-CSF and IL-4. All cultures expressed macrophage marker CD14 and CD163 at day 6 with the latter being most prominently expressed by M-CSF induced M2 (data not shown). Since all these *in vitro* cultured macrophages display a similar phenotype, we tested whether they responded equally to T-cell interaction. Only the cytokine IFNγ upregulated co-stimulatory molecules on macrophages. CD40-L is a strong activator for mo-DC, less strong for GM-CSF or PgE2+IL-6-differentiated APC but hardly activated M-CSF-induced macrophages (Fig.3a). Compared to mo-DC, all macrophage subsets produced no to low levels of IL-12 upon activation with the indicated stimuli. In contrast IL-10 was produced by all cultures but PgE2+IL-6 derived macrophages produced extreme levels of IL-10 following activation via CD40 (fig 3b). Also here IFNγ was the only cytokine that clearly worked in synergy with CD40-L for activation of macrophages and the production of IL-12.

Platinum based chemotherapy of tumor lines increase M2 induction

Patients with recurrent or advanced disease are treated with cisplatin or carboplatin. It is reported that expression of the PgE2 converting enzyme COX-2 in tumors favors a poor response to this treatment (3,17). In view of recent literature indicating that chemotherapy may also influence the response of immune cells we assessed the impact of chemotherapeutics on tumor cells and the effect on APC differentiation. First monocytes that differentiate to DC as well as several tumor cell lines were treated with creasing doses of cisplatin or carboplatin. Figure 4a shows that all cell lines survive up to concentrations of 2 μg/ml for cisplatin and 20 μg/ml for carboplatin. In vitro treatment of APC revealed that these cells could tolerate the same

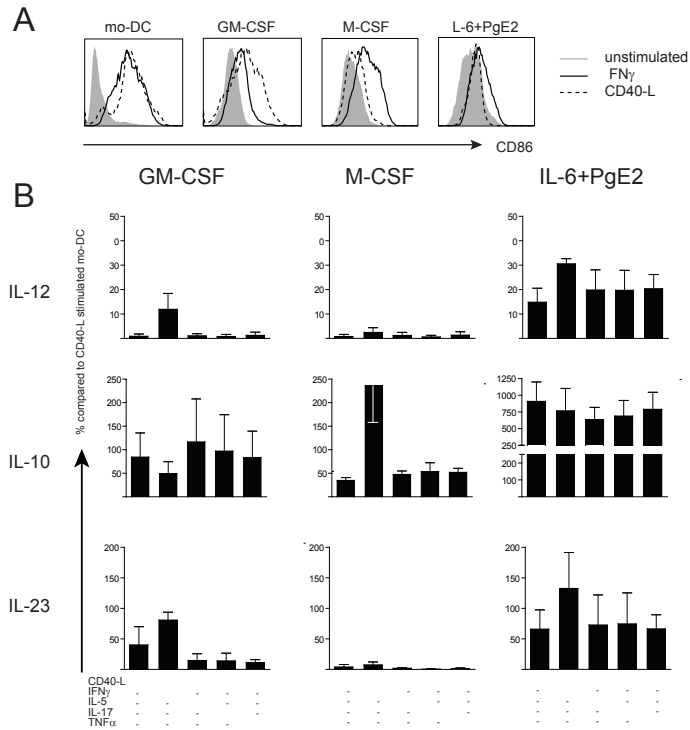

Figure 3. Different responses by cytokine induced macrophages. Macrophages were cultured with GM-CSF only, M-CSF only or to mimic tumorsupernatant IL-4/GM-CSF and 5 ng/ml IL-6+ 5 ng/ml PgE2 and compared to mo-DC. Cells were activated and analyzed after 48 hr for activation markers and cytokine production. A) CD86 expression unactivated or after activation, IFNγ only or CD40-L only. All other conditions as depicted in B did not induce CD86 expression. B) Depicted is the percentage of cytokine production compared to mo-DC in the same experiment stimulated with CD40-L only. Calculation: (Value TSN-M2 / Value mo-DC stimulated with CD40-L only)*100. Mean of 3 experiments + SEM.

doses (2 μg/ml cisplatin, 20 μg/ml carboplatin) with less than 10% dead cells but cell death was rapidly increasing at higher doses. No effect was seen on APC phenotype if chemotherapeutics were directly added during mo-DC or TSN-skewed APC differentiation.

Tumorcells were cultured and treated for 16 hr with cisplatin or carboplatin, carefully washed and cultured for another 24 hr. This supernatant was harvested and used in monocyte cultures. As shown earlier, TSN of line CC-8 and HELA skewed DC differentiation towards M2 macrophages. Strikingly, TSN from tumor cells treated with cisplatin induced a higher percentage of M2 macrophages. Carboplatin had the same effect on CC-8 cells but not on HELA cells (fig 4b). Remarkably, TSN of line CSCC-7 treated with cisplatin or carboplatin did not induce a higher percentage of M2 macrophages. The TSN of treated control lines CASKI and CSCC-1 did not show this effect (fig. 1c).

I*n vivo* tumor cells and immune cells are in the same microenvironment when treated with platinum. To better resemble the natural situation, monocytes and tumor cells were cultured together, using a transwell system in which chemotherapeutics were added at day

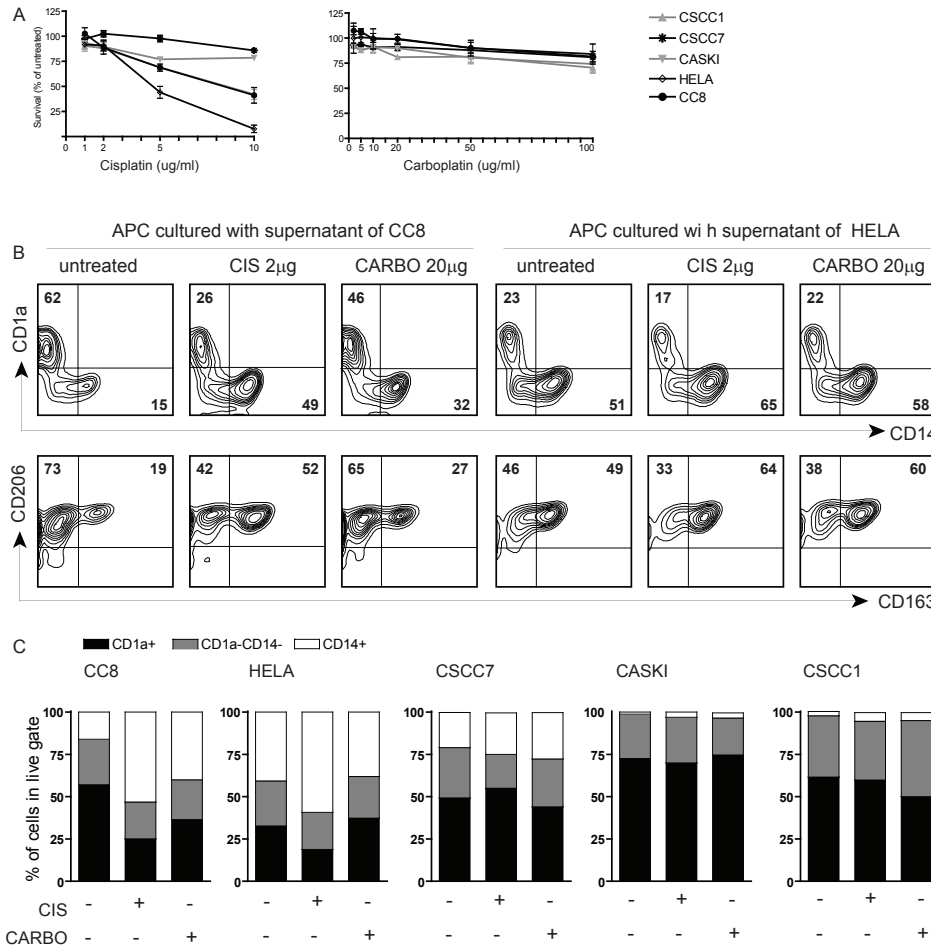

Figure 4. Platinum based chemotherapy treatment of CxCa Lines enhances M2 induction. Cxca cell lines that are treated with a dose of cisplatin or carboplatin that does not induce major cell death produce soluble factors that induce more type 2 macrophages. A) Survival of CxCa cell lines upon increasing doses of cisplatin (left) or carboplatin (right) measured in a MTT assay. Shown the mean of 3 experiments with SEM. Lines that induce M2 are shown in black. B) Monocytes were differentiated with IL-4 and GM-CSF in the presence of 20% tumor supernatant from CC-8 (left) or HELA(right) that were treated with 2 µg/mL cisplatin or 20 µg/mL carboplatin, or control medium (untreated). Differentiation was analyzed at day 5 by flow cytometry for expression of DC and M2 markers. Numbers in quadrants represent the percentage of cells within the live gate. Representatives of 5 independent experiments. C) $CD1a^+$, $CD1a^-CD14^-$ and $CD14^+$ subpopulations of cells within the live gate of APCs described in B. Mean of 3 (CC-8, HELA, CSCC7, CSCC1) or 2 (CASKI) experiments.

0 of culture. Again treatment with cisplatin resulted in an increased percentage of M2 cells when monocytes were cultured in the presence of cell line CC8 and cisplatin (fig.5a). However, chemotherapeutic treatment also resulted in a functional suppression of APC (fig. 5b). TSN induced APC produced less IL-12 and more IL-10 upon activation compared to mo-DC, but the production of both cytokines was lowered when tumor lines CC-8 and HELA were treated with

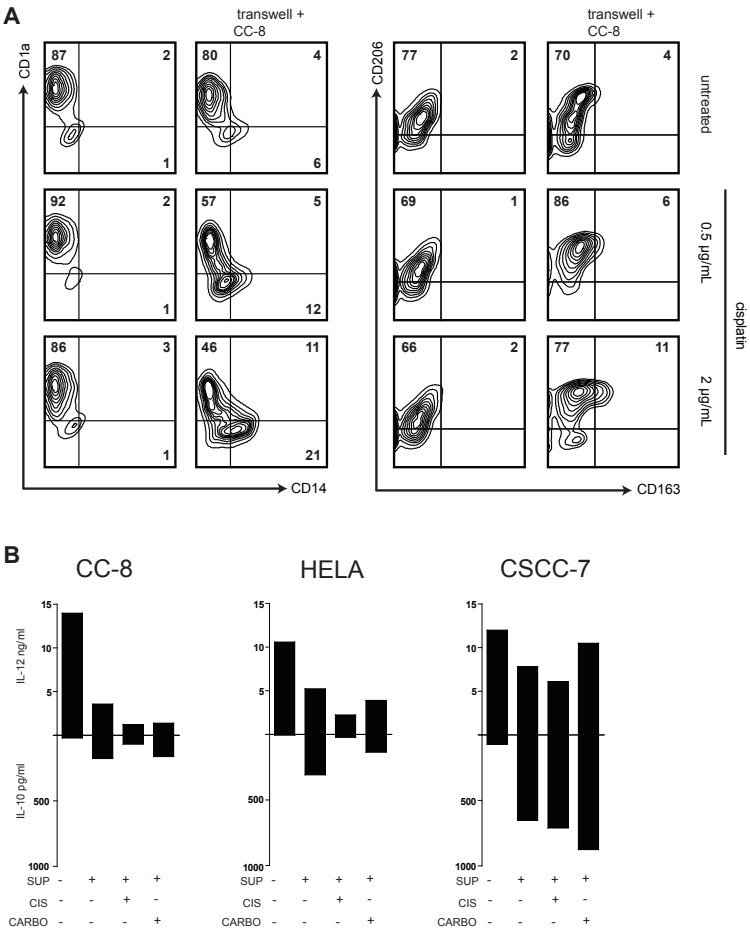

Figure 5. Platinum enhances macrophage induction and induce poor cytokine production. A) Monocytes were cultured with IL-4 and GM-CSF. In the upper chamber of a transwell tumor cells were added and in the lower chamber control (untreated) or cisplatin was added only at day 0 of culture. At day 3 fresh medium was added with IL-4 and GM-CSF. Cells were analyzed for phenotype at day 6. Numbers are percentages of cells in live-gate (livegate was similar for each condition). B) APC were cultured in the presence of supernatant of control or platinum treated tumor cells and stimulated with R848 for 48 hr. Cytokine production was measured by ELISA. All tumor lines were tested separately and this is one experiment of 2 of each.

chemotherapeutics. Line CSCC-7 did not induce more M2 macrophages after treatment and also the cytokine production upon activation by these APC was not affected (fig 5b, one of 2 experiments).

Chemotherapeutics enhance PgE2 and IL-6 production in tumor cells

M2 macrophage induction by CxCa cell lines depends on the levels of PGE2 and IL-6 (ref). Therefore we measured the amount of PGE2 and IL-6 produced by the cell lines upon platinum treatment. Cisplatin treatment increased the production of PGE2 2-7 fold by line CC-8 and HELA

but not by line CSCC-7. Carboplatin had no effect on PGE2 levels (fig 6a). Furthermore cisplatin and carboplatin increased the IL-6 production by CC-8 and HELA. Also IL-6 was induced in line CSCC-7, albeit at much lower levels compared to the other lines. The CASKI line, known to produce already high levels of IL-6 also responded to treatment with cisplatin by increasing the production of IL-6. Still this did not skew APC differentiation into M2 macrophages (fig 4c) (4). This indicates that both PGE2 and IL-6 in sufficient levels are needed to induce M2 differentiation.

As expected by the increase in cytokine levels, gene expression analysis revealed that IL-6 as well as COX-2 were induced upon platin treatment in both CC8 and CSCC7 cell lines with highest induction in CC8 cells. Furthermore levels of COX-1 mRNA were not increased by platinum treatment in both lines (fig 6b middle panel). Thus, cisplatin in a non-lethal dose,

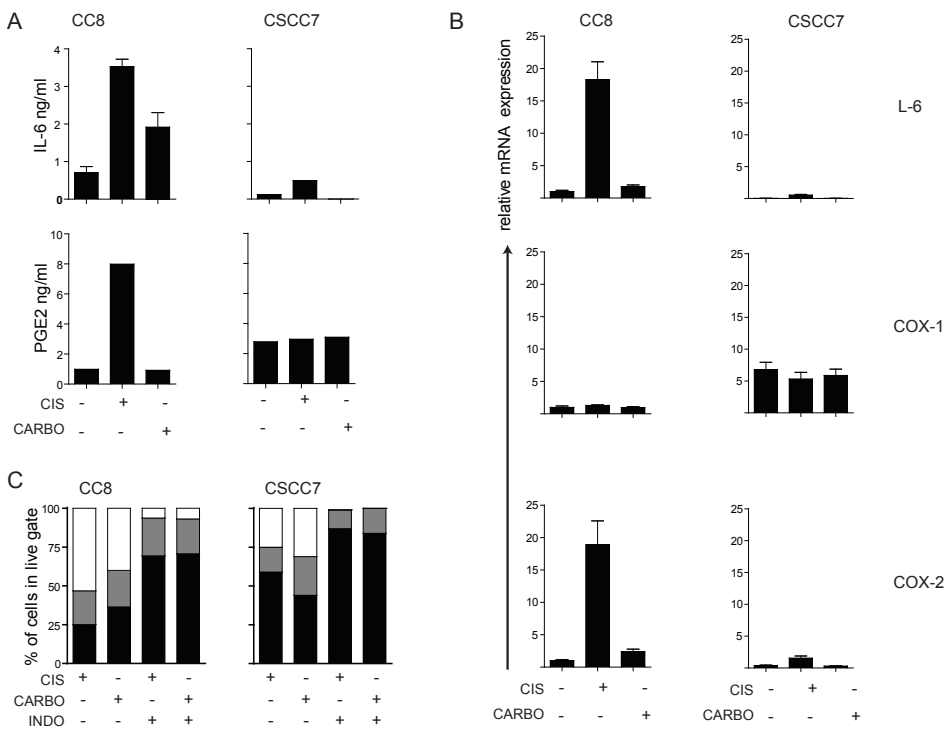

Figure 6. Induction of PgE2 and IL-6 by platinum based chemotherapy. Upon cisplatin production the levels of the M2-inducing factors PgE2 and IL-6 are increased in CxCa cell lines. A) Tumor cell lines were treated with cisplatin (2 ug/ml) or carboplatin (20 ug/ml) for 24 hours, carefully washed and cultured for another 24 hrs. This medium was analyzed for the production of IL-6 and PgE2 by ELISA. B) Tumor lines were treated as described in A, but after 18 hr total RNA was isolated and TaqMan gene expression assay was perfomed. Ct-values were corrected to GAPDH expression and depicted are relative mRNA levels compared to untreated CC-8 cells. Example of 1 cell culture, measured in triplo. C) Tumor Lines were treated with indomethacin (25 μM) and cis- or carboplatin for 24 hrs, washed and supernatant was used in APC cultures. APC cultured with supernatant were analyzed at day 5 and $CD1a^+$, $CD1a^-CD14^-$ and $CD14^+$ subpopulations of cells within the live gate are shown. M2 induction is abolished if cells are treated with COX enzyme inhibitor. Mean of 3 (CC-8) or one single experiment (CSCC7)

enhances the expression of COX-2 and IL-6 but not COX-1 and the production of PgE2 and IL-6 thereby inducing more M2 macrophages.

PgE2 is a critical factor in the *in vitro* skewing of monocytes to macrophages. If COX-enzymes are blocked with indomethacin in the tumorlines during chemo treatment, M2 induction is abolished (fig 6c). For line CC8 and HELA specific inhibition of COX-2 by NS398 resulted in abolished M2 induction, indicating that induced PGE2 levels upon platinum treatment are COX-2 mediated. For line CSCC-7 specific COX-2 inhibition was not yet tested.

DISCUSSION

Activation of mo-DC or TSN-skewed macrophages by CD40-L stimulation in the presence of helper T-cell produced cytokines all resulted in activation but no synergism of IL-5, IL-17 or TNFα with CD40-L was found for the induction of pro-inflammatory cytokines or chemokines. We showed that the presence of IFNγ clearly activates the TSN-skewed APC cultures to stimulate a type 1 inflammatory response reflected by the pre-dominant Th1 profile in subsequent stimulated T-cell cultures. In this model, we mimicked T-cell interaction with the use of CD40-L expressing cells that have a (artificially) high expression of this molecule. However when APC and T-cell crosstalk, more co-stimulatory molecules will be involved and therefore the activation is likely to be altered. T-cell receptor-peptide-MHC interaction results in the formation of immunological synapses –with locally high levels of cytokines- that is not mimicked in this system (18). The concentrations of cytokines used in our system were based on the amounts detected in patient derived HPV specific T-cell cultures. We did not find any response to IL-5, TNFα or IL-17 on mo-DC or TSN-skewed APC by upregulation of co-stimulatory molecules or IL12, IL23 or IL-10 production. Lack of IL-5 receptor may form one explanation as so far it was not reported to be present on unactivated myeloid cells (19,20). In contrast TNFα has been described in human monocytic cultures and mice DC to induce moderate activation (16,21) and high doses of IL-17 could activate human macrophages in vitro as well (22). Doses used in these studies outreached the amounts we detected in our in vitro T cell assays and used in our system.

PGE2 was described to induce CCL-22 production in human APC cultures if added during activation (16). Indeed in our cultures unactivated and activated APC produced high levels of CCL-22 but this was the same for mo-DC as well as TSN-skewed APC. Both HELA and CSCC7 supernatant contain PGE2 but addition of this supernatant induced CCL-22 production in the unactivated APC as high or lower then mo-DC cultured without PGE2.

Interestingly, IFNγ was the only stimulus that instructed mo-DC and TSN-skewed APC to produce the type 1 attracting chemokine CXCL-10. Thus, based on this limited study, the interaction of APC with antigen specific T cells may result in attraction of Th2/Treg cells which would contribute to shut down of the immune response. However when IFNγ is present, high levels of CXCL-10 are secreted which may attract more type 1 T cells.

In concordance with the IL-12/IL-10 cytokine balance produced by APC upon activation by CD40-L+IFNγ, induced naïve T cells become activated IFNγ-producing T cells even when APC were skewed into M2 by TSN.

Treatment of 2 out of 3 tumor cell lines that produce high PGE2 and IL-6 with a non-lethal dose of cisplatin resulted in an increased production of these inflammatory mediators and

subsequent M2 skewing in differentiating monocytes. Maximum doses in patients as measured in the blood for cisplatin or carboplatin are 5-6 ug/ml and 40-80 ug/ml respectively and assuming that levels in (poorly vascularized) tumor tissue is several times lower the used in vitro doses may be representative for the in vivo situation (23-25). Here we show that myeloid cells can survive the chemotherapeutic treatment but are affected by tumor released factors. The mRNA levels of COX-1 were higher expressed in line CSCC7 compared to line CC8 but were not influenced by treatment with cisplatin or carboplatin. In line CC8 the COX-2 and IL-6 mRNA levels clearly increased upon cisplatin treatment. This increase corresponds with the induction of M2 macrophages. The fact that supernatant of cisplatin treated CSCC7 did not result in more M2 induction correlates with the limited elevation of PGE2 and IL-6 upon treatment of this line. A possible explanation lies in the chemoresistence of this tumor cell line.

High COX-2 expression in CxCa identified the patients with a poor response to cisplatin and unfavorable prognosis (3). This effect was explained by enhanced apoptosis resistance of the tumor cells, but this was not reflected by our tested tumor cell lines. CCSC7 and CASKI cell lines were the most resistant to chemotherapy but did not express the highest levels of COX-2 and PGE2. Our data suggests an immunological explanation for the correlation of high COX-2 expression with poor response to treatment. Cisplatin increases the inflammatory mediators IL-6 and PGE2 in lines with high COX-2 expression, thereby promoting M2 macrophages and supporting tumor survival and growth. Interestingly these effects of cisplatin were only seen in the cell lines CC8 and HELA. Wang et al. showed that the chaperone protein PRDX-1 can join and stabilize the NF-kB complex at NF-kB promotor site of the COX-2 promotor region. This complex was shown to be selectively crosslinked to the DNA by cisplatin and therefore COX-2 gene expression is promoted (26).

Importantly, the effects of the supernatant on monocytes be reversed by inhibiting COX-enzymes in the tumor cells. The effects of IL-6 blocking is not yet addressed in this work.

Thus these results indicate that activation of tumor-skewed M2 by T cells without IFNγ results in IL-10 production and the induction of non-type 1 polarized T cells. Only CD40-L+IFNγ reverts TSN-skewed APC into Th1 inducing activated APC. Since spontaneous evoked T-cell responses often lack Th1 cytokines in patients with CxCa, therapeutic vaccination that can induce a strong type 1 T cell response is needed (4). This vaccine should not only aim for the induction of specific CTL but also strong IFNγ producing Th1 CD4+ T cells to activate the local macrophages and change the inflammatory balance in the tumor.

Concomitant treatment with cisplatin may have a devastating impact on local myeloid cells in tumors with high PGE2 and IL-6 production. Treatment of patients with COX-2 inhibiting drugs during chemotherapeutic treatment might prevent these unwanted immune related effects.

REFERENCES

1. Cadron, I., G. T. Van, F. Amant, K. Leunen, P. Neven, and I. Vergote. 2007. Chemotherapy for recurrent cervical cancer. *Gynecol. Oncol.* 107: S113-S118.
2. Kesic, V. 2006. Management of cervical cancer. *Eur. J. Surg. Oncol.* 32: 832-837.
3. Ferrandina, G., L. Lauriola, M. G. Distefano, G. F. Zannoni, M. Gessi, F. Legge, N. Maggiano, S. Mancuso, A. Capelli, G. Scambia, and F. O. Ranelletti. 2002. Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients. *J. Clin. Oncol.* 20: 973-981.
4. Heusinkveld, M., de Vos van Steenwijk PJ, R. Goedemans, T. H. Ramwadhdoebe, A. Gorter, M. J. Welters, H. T. van, and S. H. van der Burg. 2011. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J. Immunol.* 187: 1157-1165.
5. Jensen, T. O., H. Schmidt, H. J. Moller, M. Hoyer, M. B. Maniecki, P. Sjoegren, I. J. Christensen, and T. Steiniche. 2009. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J. Clin. Oncol.* 27: 3330-3337.
6. Kawamura, K., Y. Komohara, K. Takaishi, H. Katabuchi, and M. Takeya. 2009. Detection of M2 macrophages and colony-stimulating factor 1 expression in serous and mucinous ovarian epithelial tumors. *Pathol. Int.* 59: 300-305.
7. Shabo, I., O. Stal, H. Olsson, S. Dore, and J. Svanvik. 2008. Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival. *Int. J. Cancer* 123: 780-786.
8. Kelland, L. 2007. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* 7: 573-584.
9. Apetoh, L., F. Ghiringhelli, A. Tesniere, M. Obeid, C. Ortiz, A. Criollo, G. Mignot, M. C. Maiuri, E. Ullrich, P. Saulnier, H. Yang, S. Amigorena, B. Ryffel, F. J. Barrat, P. Saftig, F. Levi, R. Lidereau, C. Nogues, J. P. Mira, A. Chompret, V. Joulin, F. Clavel-Chapelon, J. Bourhis, F. Andre, S. Delaloge, T. Tursz, G. Kroemer, and L. Zitvogel. 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med.* 13: 1050-1059.
10. Tesniere, A., F. Schlemmer, V. Boige, O. Kepp, I. Martins, F. Ghiringhelli, L. Aymeric, M. Michaud, L. Apetoh, L. Barault, J. Mendiboure, J. P. Pignon, V. Jooste, P. van Endert, M. Ducreux, L. Zitvogel, F. Piard, and G. Kroemer. 2010. Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* 29: 482-491.
11. Lesterhuis, W. J., C. J. A. Punt, D. Eleveld-Trancikova, B. J. H. Jansen, G. Schreibelt, S. V. Hato, A. de Boer, C. M. L. van Herpen, J. H. Kaanders, J. H. J. M. van Krieken, G. J. Adema, C. G. Figdor, and I. J. M. de Vries. 2011. Platinum-drugs break STAT6-mediated suppression of immune responses against cancer. 115-125.
12. Stout, R. D., and J. Suttles. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J. Leukoc. Biol.* 76: 509-513.
13. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol.* 175: 342-349.
14. Duluc, D., Y. Delneste, F. Tan, M. P. Moles, L. Grimaud, J. Lenoir, L. Preisser, I. Anegon, L. Catala, N. Ifrah, P. Descamps, E. Gamelin, H. Gascan, M. Hebbar, and P. Jeannin. 2007. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood* 110: 4319-4330.
15. Koopman, L. A., K. Szuhai, J. D. H. van Eendenburg, V. Bezrookove, G. G. Kenter, E. Schuuring, H. Tanke, and G. Jan Fleuren. 1999. Recurrent Integration of Human Papillomaviruses 16, 45, and 67 Near Translocation Breakpoints in New Cervical Cancer Cell Lines. *Cancer Res* 59: 5615-5624.
16. Muthuswamy, R., J. Mueller-Berghaus, U. Haberkorn, T. A. Reinhart, D. Schadendorf, and P. Kalinski. 2010. PGE(2) transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. *Blood* 116: 1454-1459.
17. Nagai, N., X. Tian, K. Mukai, E. Hirata, T. Kusuda, Y. Shiroyama, K. Shigemasa, and K. Ohama. 2003. Overexpression of cyclooxygenase-2 protein and its relationship to apoptosis in cervical carcinoma treated with neoadjuvant chemotherapy. *Int. J. Mol. Med.* 12: 709-714.
18. Zou, W., and N. P. Restifo. 2010. T(H)17 cells in tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 10: 248-256.
19. Koike, M., and K. Takatsu. 1994. IL-5 and its receptor: which role do they play in the immune response? *Int. Arch. Allergy Immunol.* 104: 1-9.
20. Yamada, T., Q. Sun, K. Zeibecoglou, J. Bungre, J. North, A. B. Kay, A. F. Lopez, and D. S. Robinson. 1998. IL-3, IL-5, granulocyte-macrophage colony-stimulating factor receptor alpha-subunit, and common beta-subunit expression

by peripheral leukocytes and blood dendritic cells. *J. Allergy Clin. Immunol*. 101: 677-682.

21. Iwamoto, S., S. Iwai, K. Tsujiyama, C. Kurahashi, K. Takeshita, M. Naoe, A. Masunaga, Y. Ogawa, K. Oguchi, and A. Miyazaki. 2007. TNF-alpha drives human CD14+ monocytes to differentiate into CD70+ dendritic cells evoking Th1 and Th17 responses. *J. Immunol*. 179: 1449-1457.
22. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J. Immunol*. 160: 3513-3521.
23. Gaver, R. C., N. Colombo, M. D. Green, A. M. George, G. Deeb, A. D. Morris, R. M. Canetta, J. L. Speyer, R. H. Farmen, and F. M. Muggia. 1988. The disposition of carboplatin in ovarian cancer patients. *Cancer Chemother. Pharmacol*. 22: 263-270.
24. Gullo, J. J., C. L. Litterst, P. J. Maguire, B. I. Sikic, D. F. Hoth, and P. V. Woolley. 1980. Pharmacokinetics and protein binding of cis-dichlorodiammine platinum (II) administered as a one hour or as a twenty hour infusion. *Cancer Chemother. Pharmacol*. 5: 21-26.
25. Kroep, J. R., E. F. Smit, G. Giaccone, K. Van der Born, J. H. Beijnen, C. J. van Groeningen, W. J. Van der Vijgh, P. E. Postmus, H. M. Pinedo, and G. J. Peters. 2006. Pharmacology of the paclitaxel-cisplatin, gemcitabine-cisplatin, and paclitaxel-gemcitabine combinations in patients with advanced non-small cell lung cancer. *Cancer Chemother. Pharmacol*. 58: 509-516.
26. Wang, X., S. He, J. M. Sun, G. P. Delcuve, and J. R. Davie. 2010. Selective association of peroxiredoxin 1 with genomic DNA and COX-2 upstream promoter elements in estrogen receptor negative breast cancer cells. *Mol. Biol. Cell* 21: 2987-2995.

CHAPTER 6

Intradermal delivery of TLR agonists in a human explant skin model: preferential activation of migratory Dendritic Cells by Poly I:C and Peptidoglycans

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ABSTRACT

TLR agonists are attractive candidate adjuvants for therapeutic cancer vaccines as they can induce a balanced humoral and T-cell mediated immune response. With a dense network of dendritic cells (DCs) and draining lymphatics, the skin provides an ideal portal for vaccine delivery. Beside direct DC activation, TLR agonists may also induce DC activation through triggering the release of inflammatory mediators by accessory cells in the skin microenvironment. Therefore, a human skin explant model was used to explore the *in vivo* potential of intradermally delivered TLR agonists to stimulate Langerhans Cells (LCs) and dermal DCs (DDCs) in their natural complex tissue environment. The skin-emigrated DCs were phenotyped and analyzed for T-cell stimulatory capacity. We report that, of six tested TLR-agonists, the TLR-2 and -3 agonists PGN and Poly I:C were uniquely able to enhance the T-cell priming ability of skin-emigrated DCs, which in the case of PGN was accompanied by Th1/Th17 polarization. The enhanced priming capacity of Poly I:C stimulated DC was associated with a strong upregulation of appropriate costimulatory molecules, including CD70, while that of PGN-stimulated DC was associated with the release of a broad array of proinflammatory cytokines. Transcriptional profiling further supported the notion that the PGN- and Poly I:C-induced effects were mediated through binding to TLR2/NOD2 and TLR3/MDA5 respectively. These data warrant further exploration of PGN and Poly I:C, alone or in combination, as DC-targeted adjuvants for intradermal cancer vaccines.

INTRODUCTION

Vaccines aimed at the induction of neutralizing antibodies are widely used with great success to prevent microbial infections. Therapeutic vaccination is being developed for treatment of chronic infections and cancer, and aims to generate protective T-cell immunity. Although some clinical successes have been reported, particularly in the field of cancer vaccination, much is yet to be gained in terms of efficacy (1,2).

In vivo studies have shown that the vaccine delivery route determines the instruction of T cells by locoregional antigen-presenting cells (APCs), thereby affecting the homing capacity of primed T cells (reviewed by Kupper and Fuhlbrigge (3)). Langerhans cells (LCs) and dermal dendritic cells (DDCs) reside in the epidermis and dermis, respectively. Both subsets are well equipped to sense pathogenic threats in their microenvironment, to take up antigen and to migrate to the draining lymph nodes (LN) where they prime and program T cells. Hence, neoplastic diseases that present within the skin (*e.g.* melanoma and human papilloma virus (HPV)-induced tumors) may benefit most from intradermal vaccination, as in the LN the migrated LCs or DDCs will imprint the T cells to express a pattern of homing and chemokine receptors that facilitates migration to the skin. Immunization with poxviruses is a clear example of a remarkably successful induction of strong and long-lasting antibody and T-cell responses by vaccination via the skin (4). Also, intradermal vaccination with synthetic long peptides (SLPs) derived from HPV type 16 oncoproteins resulted in the induction of both $CD4^+$ and $CD8^+$ T cells specific for HPV that were able to migrate to the skin (5).

The choice of adjuvant will influence the type of immune response induced by therapeutic vaccines. More and more, it is realized that for effective anti-tumor immunity a balanced humoral and cell-mediated response with long-term T-cell memory is obligatory. Based on pre-clinical mouse models, human *in vitro* experiments and early clinical experience, compounds targeting toll like receptors (TLRs) are promising adjuvant candidates in this regard (6,7). TLRs are trans-membrane pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs). TLRs can be expressed at the cell membrane, to bind bacterial structural molecules such as peptidoglycans (PGN) and lipopolysaccharides (LPS) (TLR1,2,4,5,6), or intracellular in endosomal compartments, to bind viral or bacterial RNA or DNA (TLR 3,7,8,9). Subsequent signaling through MyD88 or TRIF leads to activation of the NF-κB and MAPK pathways resulting in APC activation and pro-inflammatory cytokine and chemokine release. Human epidermal LCs have been reported to express only virus-recognizing TLRs, to keep them from inadvertently being activated by commensal bacteria. In contrast DDCs express TLRs recognizing both bacteria and viruses (8). So far, the ability of TLR agonists to stimulate cutaneous DCs has mostly been tested *in vitro* (9). However, other skin-resident cells such as keratinocytes, melanocytes, endothelial cells and fibroblasts also express TLRs and can release cytokines that contribute to DC maturation and the induction and skewing of T cells (10,11). Moreover, alternative intracellular microbial PRRs, such as NOD-like receptors (NLR, NOD1 and NOD2) or RIG-I-like Helicases (RLH, MDA5 and RIG-I), can be expressed by DCs and accessory cells, and share ligands with TLRs (7). Therefore, a human skin explant model was used to explore the *in vivo* potential of TLR agonists to stimulate LCs and DDCs in their natural complex tissue environment (12). By intradermal delivery of TLR-ligands (TLR-Ls), intradermal vaccination with TLR-based adjuvants was thus mimicked *ex vivo*. The compounds tested included PAM_3CYSK_4.3HCl (PAM3CSK4, TLR1/2-L), PGN (TLR2-L/NOD2-L), LPS and its detoxified

active constituent monophosphoryl lipid A (MPLA, TLR4-L), Polyribosinic:polyribocytidic acid (Poly I:C, TLR3-L), and R848 (Resiquimod, TLR7/8-L), all of which are either used or considered as vaccine adjuvants (7). So-called crawl-out DCs, migrating from the skin explants, were analyzed for their expression of maturation and co-stimulatory molecules and their capacity to prime allogeneic T cells and induce Th1/2/17 responses. We report that Poly I:C and PGN are uniquely able to enhance the T-cell priming ability of skin-emigrated DCs. While for Poly I:C this appeared to be due to up-regulation of co-stimulatory molecules, PGN was a powerful inducer of pro-inflammatory cytokine release, thereby promoting Th1 induction. From these observations, we conclude that TLR2/NOD2 and TLR3 ligands are attractive candidate adjuvants for intradermally delivered therapeutic vaccines.

MATERIAL AND METHODS

Preparation and culture of skin explants. Healthy human skin was obtained after informed consent from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (Amsterdam, The Netherlands) or the Tergooi hospital (Hilversum, The Netherlands), following hospital guidelines, within 24h of surgery. Cytokines or TLR-Ls (endotoxin contaminations not exceeding 1 EU/mg, excepting MPLA and LPS) were dissolved in serum-free medium (IMDM) and injected intradermally into skin: GM-CSF (100 ng/biopsy, Berlex Laboratories Inc. Montville, NJ), IL-4 (10 ng/biopsy, R&D), PGN (10 μg/biopsy, InvivoGen), Poly I:C (20 μg/biopsy, InvivoGen), R848 (10 μg/biopsy, InvivoGen), LPS (250 ng/biopsy, Sigma Aldrich), MPLA (250 ng/biopsy, InvivoGen), PAM3CSK4 (10 μg/biopsy, InvivoGen). These amounts were based either on optimal maturational effects or on maximal sub-toxic doses found upon titration and were all found active on monocyte-derived DCs *in vitro* (see SFig.1). Of note, the TLR5 agonist flagellin was found to be too toxic in active concentration ranges (based on findings for monocyte-derived DCs) and was therefore not further included in these studies. Using a 500 μl microfine syringe (Becton Dickinson), 20 μl was injected into skin so that an urtica formed. A biopsy was immediately taken of the injection site with a 6 mm biopsy punch (Microtec) and floated in 1 well of a 48 well plate, which contained 1 ml IMDM supplemented with 5% Human Pooled Serum (HPS, Sanquin Blood Supply, Amsterdam, The Netherlands), 100 IU/ml sodium penicillin (Yamanouchi Pharma), 100 μg/ml streptomycin sulfate (Radiumfarma-Fisiopharma), and 2 mM L-glutamine (Invitrogen Life Technologies). Per experimental condition, 10-30 biopsies were taken. Biopsies and medium were placed in an incubator (37^{0}C, 5% CO_2) for 2 days, after which biopsies were discarded and migrated cells and conditioned media were harvested. Conditioned media from 48h-cultured full-thickness skin explants were analyzed for cytokine content with the inflammatory cytokine bead array (CBA) kit from BD Biosciences (San Jose, CA), and 1:2 diluted for CCL5 content with a DuoSet development system (R&D sytems, Minneapolis, MN), both according to the manufacturers' instructions.

Phenotypical analysis. Phenotypical analyses were performed by flow cytometry. Cells were washed in PBS supplemented with 1 % BSA and 0.02% NaN_3 and incubated for 30 min. at room temperature in the presence of appropriate dilutions of FITC or PE fluorochrome-conjugated specific mAbs to CD14, CD1a, B7-H1 (PD- L1), CD80, CD86, CD70, HLA-DR (BD, San Jose, CA), or CD83 (Beckman Coulter Immunotech), or corresponding isotype-matched control mAbs (BD, San Jose, CA). The cells were subsequently analyzed, using a FACSCalibur and Cellquest-Pro

FACS analysis software (Becton Dickinson, San Jose, CA). As described, DC were gated by their high Forward and Side Scatter properties (12).

Mixed Leukocyte Reaction (MLR). Migrated DCs were resuspended at $1x10^5$/ml in IMDM with 10% HPS, penicillin, streptomycin, and glutamine and 100 µl was plated in triplicate in a 96-well round bottom sterile culture plate. Peripheral blood lymphocytes (PBLs) were isolated from allogeneic healthy donors after plastic adherence to deplete monocytes and labelled with 3 µM CFSE (Invitrogen) for 10 minutes at 37°C. After washing, the labelled PBL were resuspended at $1x10^6$ /ml in IMDM with 10% HPS, penicillin, streptomycin, and glutamine and 100 µl was added to wells containing migrated DCs, and to 3 wells containing only medium. Control wells of unlabelled PBLs were also included. On days 3, 6, 8 and 10 cell culture samples were taken and analysed for proliferation by flow cytometry. On day 6, supernatants were collected and subsequently analyzed for cytokines secreted by the T cells using a Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Microarray analysis of TLR transcriptional profiles. Epidermal and dermal fractions were prepared from dermatome-sliced human skin (3mm) as described previously(13). After CD1a-guided MACS (Miltenyi, Bergisch Gladbach, Germany), RNA, isolated from LCs and DDCs from three different donors, was dissolved in TRIzol reagent (Invitrogen Life Technologies) and stored at –20°C. After chloroform extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol, lyophilized, and dissolved in 10 µl distilled water. Fragmentation, hybridization, and scanning of the Human Genome U133 Plus 2.0 arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA) and as described (13). The arrays were scanned with a GeneArray scanner (Affymetrix). Data analysis was performed with GeneSpring 7.1 software (Agilent Technologies). Gene expression levels of TLR, NOD2, RIG-I, or MDA5, present on the Human Genome U133 Plus 2.0 Arrays, are presented as mean signal values of triplicate DDC or LC samples (signal intensity range from 0- 22,000). Samples with an absent detection call in one to three of the replicate samples were set at 0 (i.e. no reliably detected expression).

Real-time quantitative RT-PCR. Cells were lysed and mRNA was specifically isolated by capture of poly(A)-RNA in streptavidin-coated tubes using an mRNA capture kit (Roche, USA). cDNA was synthesized using a reverse transcription system kit (Promega, USA) following the manufacturer's guidelines. cDNA was diluted 1:2 in nuclease-free water upon synthesis and stored at -20°C until analysis. Specific primers for human TLR1–10 were designed as described (14). Real-time PCR was performed using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems, USA) as previously described (15). Briefly, 4 µl of the Power SYBR Green master mix (Applied Biosystems) were mixed with 2 µl of a solution containing 5 nmol/µl of both oligonucleotides and 2 µl of a cDNA solution (1/100 of the cDNA synthesis product). The cycle threshold (Ct) value is defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value (fixed at 0.045 relative fluorescence units). GAPDH was selected as the endogenous reference gene from a set of 10 functionally unrelated housekeeping genes according to Garcia-Vallejo *et al*. (15). For each sample, the relative abundance of target mRNA was calculated from the obtained Ct values for both target and endogenous reference gene GAPDH by applying the following formula: relative mRNA expression =2[Ct(GAPDH) -Ct(target)].

Statistical analyses. DC subset frequencies, marker expression levels, cytokine release levels, and proliferated T cell fractions were compared between conditions using the 2-sided repeated measures one-way ANOVA test with post-hoc Tukey multiple comparison analysis. Prism 4.0 statistical software (GraphPad Software Inc., La Jolla, CA) was used. Differences were considered significant when $P<0.05$.

RESULTS

Effects of TLR-L on subset distribution among the skin-emigrated DC. We previously identified several DC subsets among DCs that had migrated from human skin explants, based on multicolor flow cytometry ((12) and Lindenberg, *submitted*). An overview of the phenotype of the four major discernable subsets is presented in Table I. Of note, LC and CD1a$^+$ DDC are mature subsets with a high T-cell stimulatory potential, whereas the CD1a$^+$CD14$^+$ and CD14$^+$ DDC subsets are immature cells with macrophage-like features and a poor capacity for T-cell stimulation (Table I, (12)). We previously reported that intradermal injection of GM-CSF and IL-4 prior to skin explant culture, led to predominant migration of the mature CD1a$^+$ subsets, while inhibiting migration of the CD14$^+$ immature subsets (12). Here we confirm these observations (Fig.1) and in all subsequent experiments GM-CSF+IL-4 was included as a positive control. TLR agonists were intradermally injected at subtoxic active doses (see Material and Methods) that induced phenotypic and functional activation in vitro of highly pure monocyte-derived DC (SFig.1). None of the intradermally delivered TLR-Ls significantly increased (or decreased) the absolute number of migrated DCs per explant at 48h after start of culture (range of mean absolute numbers: 1800-4000; data not shown). LPS injection into the dermis prior to explant culture significantly increased the frequency of migrating LC, while injection of either Poly I:C or LPS increased the proportion of migrated CD1a$^+$ DDC over that observed for medium controls (Fig.1). Of note, unlike GM-CSF+IL-4, none of the tested TLR-Ls was able to significantly down-regulate the frequencies of migrated immature CD14$^+$ DDC.

TLR-L effects on DC maturation, inflammatory cytokine release and T cell induction. Whereas intradermal delivery of most of the tested TLR-Ls had no effect on phenotypic maturation of the migrated cells, Poly I:C consistently upregulated the expression of CD80, CD83, CD86, and HLA-DR, as well as of the coinhibitory molecule PD-L1 (B7-H1) (Fig.2A). Similarly, LPS induced

Table I. DC subsets and their phenotype.

DC subset	Marker profile*
LC	CD1a^{hi}, Langerin$^+$, E-Cadherin$^+$, CD11c^{int}, CD1c$^+$, CD83$^{+,}$ CD80$^+$, PD-L1$^+$
CD1a+ DDC	DC-SIGN$^+$, CD11c^{hi}, CD1c$^+$, CD83$^+$, CD80$^+$, PD-L1$^+$
CD14+ DDC	DC-SIGN$^+$(30%)†, CD11c^{hi}, CD1c$^+$, CD163$^+$(15%)‡, CD83$^-$, CD80$^-$, PD-L1$^+$(20%)‡
CD1a+CD14+ DDC	DC-SIGN$^+$(20%)†, CD11c^{hi}, CD1c$^+$, CD163$^+$(35%)‡, CD83$^-$, CD80$^+$(50%), PD-L1$^+$(30%)‡

* Based on multicolor FACS analyses as described by de Gruijl *et al*. (12) and Lindenberg *et al*., *submitted,* marker expression was considered negative (-) when percentage positive cells did not exceed 10%; hi=high fluorescence intensity levels, int=intermediate fluorescence intensity levels.
†Up-regulated by intradermal GM-CSF and IL-4 injection prior to explant culture
‡Up-regulated by intradermal IL-10 injection prior to explant culture

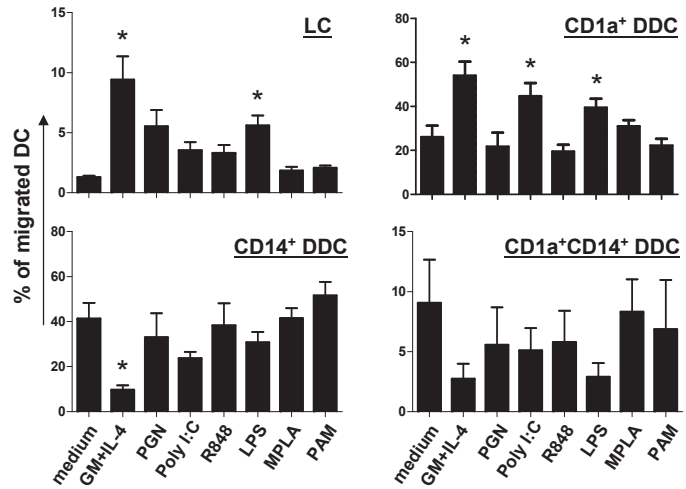

Figure 1. Subset distribution among skin emigrated DCs. Based on CD14 and C1a expression, four major subsets were discerned among skin-emigrated DCs (gated by high forward and side scatter properties) and their frequency determined for each of the test conditions. Means ± s.e.m. are shown based on 6 independent experiments. Asterisks denote significant differences vs. medium controls.

a significant upregulation of the percentages of DCs expressing CD83 and PD-L1, but the expression of CD80 and CD70 was enhanced by Poly I:C only (Fig.2B).

To assess the influence of the TLR-L on the local cytokine balance, explant-conditioned media were collected 48h after the start of culture, and tested for their inflammatory cytokine and chemokine content. As demonstrated in Fig.3, PGN by far proved to be a superior inducer of inflammatory cytokine release with high levels of IL-6, IL-8, and IL-1β, but also of the suppressive cytokine IL-10. IL-10 was produced upon administration of most TLR-Ls, with the notable exception of Poly I:C (Fig. 3). IL-12p70 was undetectable in the explant-conditioned media (not shown), while low but detectable levels of TNFα were induced by Poly I:C (Fig.3). In addition, variable but consistently elevated levels of the pro-inflammatory chemokine CCL5 were observed after intradermal delivery of Poly I:C (Fig.3).

Poly I:C and PGN were the only tested TLR-Ls that significantly enhanced the capacity of the skin-emigrated DC to induce the proliferation of allogeneic T cells (Fig.4A). Interestingly, DC migrated from GM-CSF+IL-4-injected skin explants, induced low but selective IL-4 release by the allogeneic T cells (Fig.4B, top panel) but this was not observed when DC were activated through their TLR. Whereas multiple TLR-Ls induced the release of IL-6, only PGN was able to induce the release of high levels of the Th1 cytokines IFNγ and TNFα (Fig.4B). In addition, PGN was uniquely able to induce the release of variable but consistently elevated levels of IL-17A by the primed T cells (Fig.4B, bottom panel).

PRR transcriptional profiles in LC, DDC and keratinocytes are conform the responsiveness to TLR-L. To assess PRR expression in the skin environment, transcriptional analysis of TLRs was performed in isolated LCs, CD1a$^+$ DDCs and keratinocytes from healthy human skin. As demonstrated by microarray data (Fig.5A), both LCs and DDCs generally expressed low levels of

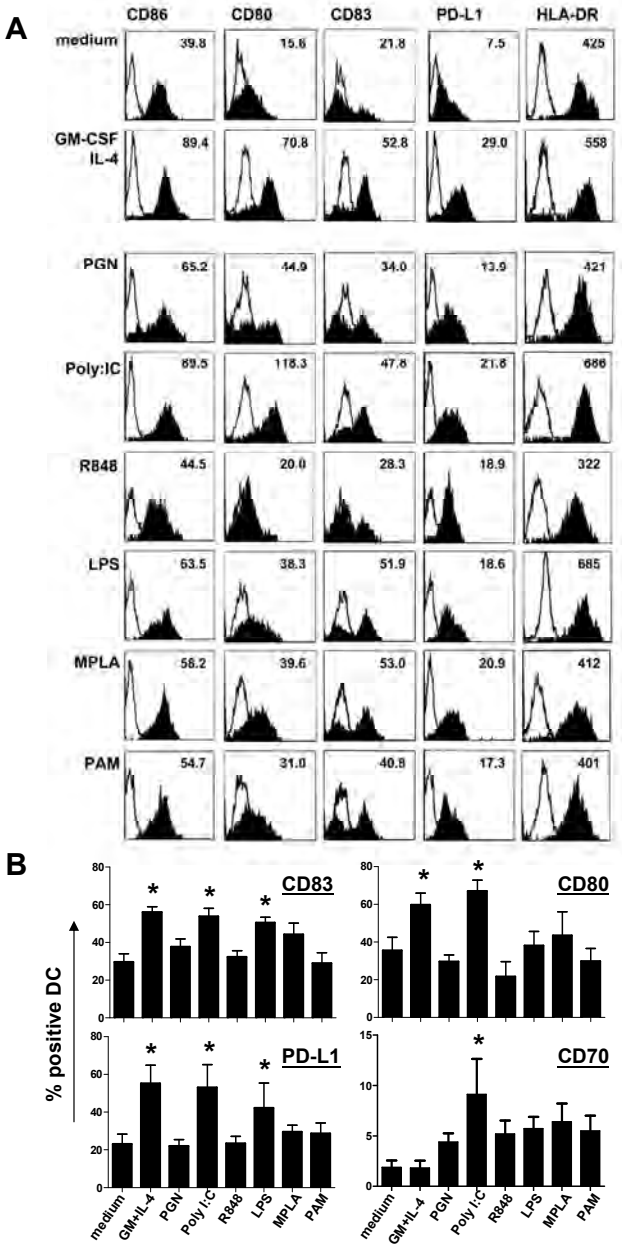

Figure 2. Expression of activation markers on skin-emigrated DCs. A) Expression of activation markers on the total population of skin-emigrated DCs at 48 hr after injection (and start of explant culture) of medium, GM-CSF+IL-4, or the denoted TLR agonists. Flowcytometric histograms of the expression of the indicated activation markers on all the cells within the live gate of one representative experiment. Fluorescence intensities are listed. Open histograms: IgG isotype controls; closed histograms: activation marker expression. B) Percentages of DCs that express the indicated activation markers as described in (A). Means ± s.e.m. of 3-6 experiments are shown. Asterisks denote significant differences vs. medium controls.

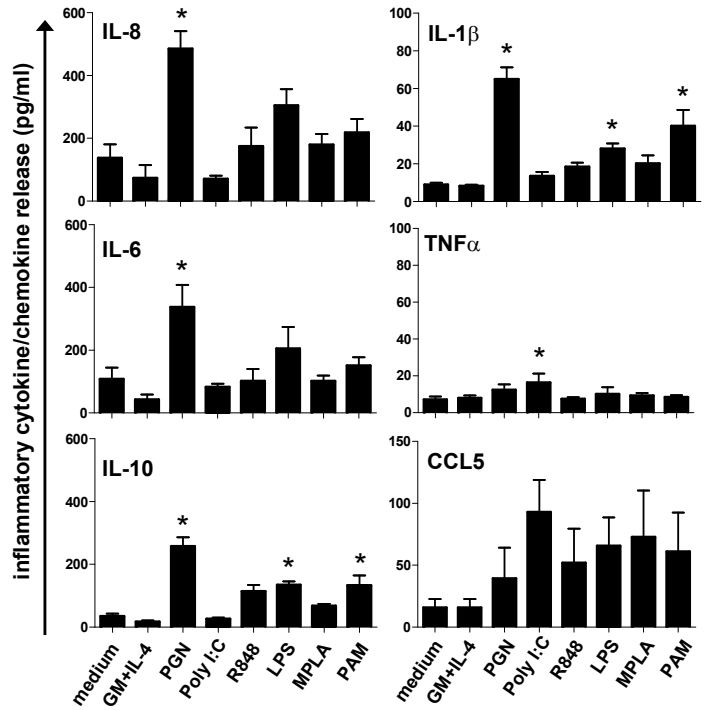

Figure 3. Inflammatory cytokine release from skin explants upon TLR-L injection. 48 hr after injection of TLR-Ls or controls and start of culture, skin explants were discarded and the medium was analyzed for the levels of the listed inflammatory cytokines by CBA or of CCL5 by ELISA. Means ± s.e.m. of 5 experiments are shown. Asterisks denote significant differences vs. medium controls.

TLR transcripts as compared to C-type Lectin Receptor (CLR) transcripts selectively expressed by LCs (Langerin, CD207) or by DDCs (Macrophage Mannose Receptor [MMR], CD206). Only TLR2 mRNA levels in DDC were relatively high. As PGN can also bind to NOD2 and Poly I:C to RIG-I/MDA5, expression of these receptors was also analysed. Relatively high expression levels were observed for both NOD2 and MDA5 in LCs as well as DDCs (Fig.5A). Epidermis-derived keratinocytes were tested by qRT-PCR for the expression of TLR transcripts. As shown in Fig.5B, resting keratinocytes expressed mostly TLR2, -3, and -5. Stimulation of keratinocytes with the TLR3-L Poly I:C led to up-regulation of TLR1-3, and -5 transcription, confirming functional expression of TLR3 on keratinocytes (Fig.5C). Thus, the observed DC activation by PGN and Poly I:C in the human explant cultures was in line with the detected expression of TLR2/NOD2 transcripts in DDCs and of MDA5 and TLR3 transcripts in LCs/DDCs and keratinocytes.

DISCUSSION

Immunization through the skin leads to the induction of T-cell mediated and humoral immunity (16). TLR agonists have been proposed as prime adjuvant candidates for therapeutic vaccines but most of our knowledge on TLR agonists and their effects on skin-mediated immunization

stems either from murine *in vivo* studies or from *in vitro* studies with isolated human primary LCs or DDCs, and more often from their monocyte-derived counterparts. However, vaccines and adjuvant deposited in the skin can interact with a complex mix of cells, amongst others DCs and keratinocytes. Therefore, we sought to assess the overall effect of these interactions in the context of intact human skin and intradermally injected a panel of TLR-Ls into human skin explants to study their effects on DC migration, activation, and subsequent T-cell priming in an organotypic culture model. Our studies point out Poly I:C and PGN as prime candidate adjuvants for clinical translation to skin-based cancer vaccines.

Of a panel of six tested TLR agonists, only the TLR3-L Poly I:C consistently induced phenotypic activation of the skin-emigrated DC population. This general lack of skin-derived DC activation upon TLR-L treatment is in striking contrast to our own observation for Monocyte-derived DCs (SFig.1). Strong activation by TLR-Ls was also reported for isolated monocyte-derived LCs (8,17). Of note, also overall migration rates of the skin APCs were not affected by intradermal injection of any of the tested TLR agonists. This general lack of reactivity in primary LCs and DDCs in the context of their tissue microenvironment may in part be explained by their low TLR transcript levels (Fig.5A), but may also be caused by specific local suppression. For instance, Jurkin *et al*. recently reported that selective overexpression of miR-146a in LCs interfered with TLR2-mediated signaling and caused their non-responsiveness to PGN (18). Alternatively, TLR triggering in DCs or skin-resident cells such as keratinocytes, melanocytes or fibroblasts, may have caused the release of suppressive IL-10 to

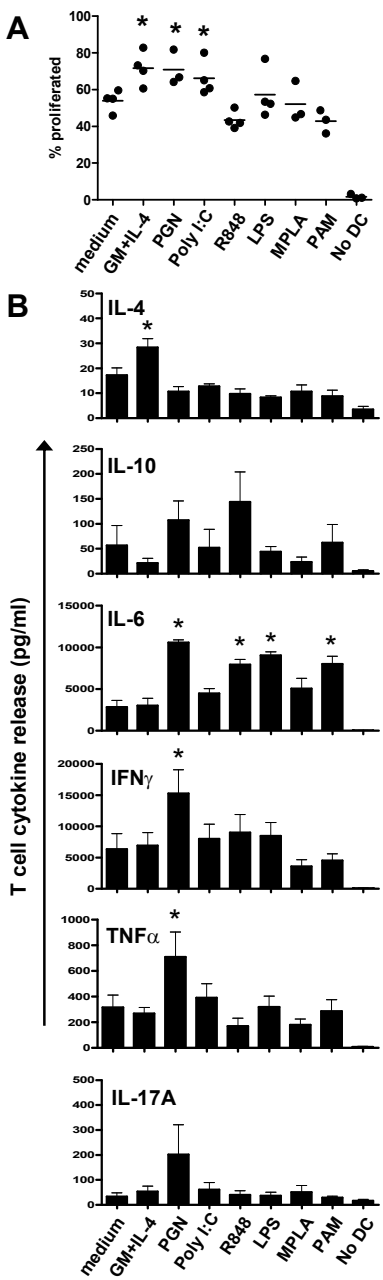

Figure 4. Allogeneic T cell priming and differentiation induction by skin-emigrated DCs. Emigrated DCs were washed and cultured with allogeneic CFSE-labeled PBLs for 6 days. The cells were analyzed by flow cytometry for proliferation by dilution of CFSE intensity (in % proliferated cells within the live lymphocyte gate). Means from 4 experiments are shown. B) Supernatants were collected from the mixed leukocyte cultures at day 6 and analyzed for Th cytokines by CBA. Means ± s.e.m. from 4 experiments are shown.

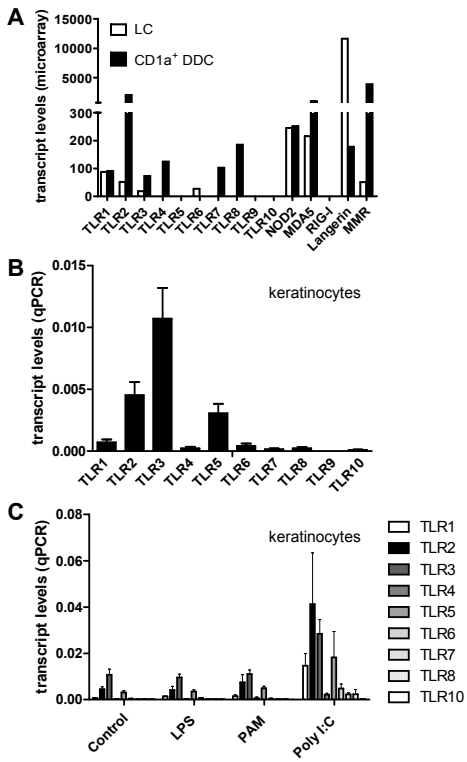

Figure 5. TLR mRNA expression in cell subsets of the skin. A) Freshly isolated LCs and CD1a$^+$ DDCs were analyzed by genome-wide transcriptional profiling. Shown are the transcript levels for the indicated Pattern recognition Receptor (PRR) and C-type Lectin receptor (CLR) genes (means from 3 separate donor samples; if one was negative the expression was set to 0 to ensure *bona fide* expression). Open bars: LC transcripts; closed bars: DDC transcripts. B) Skin-derived primary keratinocytes were profiled for TLR transcript expression by qRT-PCR. Transcript levels for TLR1-10 are indicated relative to GAPDH as a reference gene. C) Skin-derived keratinocytes were stimulated by the indicated TLR agonists (100ug) and again tested for TLR transcript expression as described under (B). Means ± s.e.m. are shown.

such an extent that it interfered with DC activation. In favor of the latter option, the only TLR agonist that did not induce elevated IL-10 release from the skin explants (i.e. Poly I:C, see Fig.3), was also the only agonist to consistently induce DC maturation. This elevated IL-10 release may also explain why intradermal injection of TLR-Ls, in contrast to GM-CSF+IL-4, did not reduce the migration frequency of CD14$^+$ DDCs (Fig.1B), as we previously described IL-10 to skew migratory DCs in skin from a mature to this immature CD14$^+$ phenotype (12,19).

Transcriptional profiling of cell types isolated from dissociated healthy human skin revealed TLR expression in LCs and CD1a$^+$ DDCs to be generally low, but other intracellular PRRs like MDA5 and NOD2 were also expressed. Keratinocytes expressed TLR2 and TLR3 and treatment of these cells with Poly I:C resulted in increased TLR expression, demonstrating functionality of the expressed TLR3. Roughly, our findings reflect the TLR expression profile previously described by van der Aar *et al.* for LCs and DDCs (8). Inconsistencies may be explained by differences in the employed methodologies or by differences in the isolated DC populations (notably CD1a$^+$ DDCs in our study versus all CD11c^{hi}HLA-DR$^+$ DDCs in their study). Also, the high TLR2 and -3 mRNA expression levels observed by us in isolated keratinocytes are in line with data from Karim *et al.* who reported high TLR2 and -3 expression as well as expression of RIG-I and MDA5 in foreskin, cervical, and vaginal keratinocytes (10). Importantly, these PRR expression profiles altogether support the observed stimulatory effects of PGN and Poly I:C on the skin-emigrated DCs, with PGN likely binding and activating through TLR2/NOD2 and Poly I:C through TLR3/MDA5 (7).

Intradermally delivered Poly I:C induced both DC maturation and an increased migration frequency of the mature CD1a$^+$ DDC subset, resulting in an overall significantly increased T-cell stimulatory ability of the skin-emigrated DCs in an MLR with allogeneic T cells. It is not clear if the effect of Poly I:C on DCs is solely caused by direct activation through TLR3/MDA5, as CD1a$^+$ DDCs express low levels of TLR3 but higher levels of MDA5, or indirectly. Both TLR3 and MDA5 are also functionally expressed by keratinocytes and as such may have triggered the release of DC-activating cytokines that subsequently can induce DC maturation (20). Interestingly, out of a panel of TLR-L based adjuvants subcutaneously injected with a DEC250-targeted HIV-gag protein vaccine in mice, Poly I:C was identified as the superior adjuvant (21). Its observed *in vivo* adjuvanticity, effecting efficient Th1 activation, depended on the release of type-1 IFN by stromal cells and DCs. Indeed, both keratinocytes and fibroblasts may be the source of Poly I:C-induced type-1 IFN (10, 21,22). Conceivably a similar mechanism may have been at work in our skin explant model. Recently, selective induction of the CD8$^+$ T cell activating co-stimulatory molecule CD70 was reported on LCs but not on DDCs in response to viral signals (23). Indeed, intradermal injection of Poly I:C (a synthetic viral PAMP mimic) resulted in a subset of skin-emigrated DCs *de novo* expressing CD70 (Fig.2B). Although Langerin double staining was not performed and their LC identity could thus not be unequivocally confirmed, the small percentages of CD70$^+$ cells correspond with the low frequencies of LCs among the skin-emigrated DCs (Fig.1B). As expected, CD70 was not highly up-regulated on Monocyte-derived DCs upon TLR triggering, in keeping with the reported exclusive up-regulation of CD70 on LCs (23).

PGN appeared to achieve a type-1 DC maturation through triggering the release of pro-inflammatory cytokines, either through TLR2/NOD2 binding in CD1a$^+$ DDCs or in keratinocytes (25).. Stimulation of T cells resulted in a Th1 polarization reflected by significantly elevated TNFα and IFNγ release, as well as in Th17 polarization, reflected by the release of IL-17 (Fig.4B). This confirms findings by others, showing Th1/Th17 induction by PGN through a TLR2/NOD2-dependent mechanism (24). Of note, the TLR2/TLR1 agonist PAM3CSK4 did not induce any DC maturation, in line with findings from a mouse study *in vivo* (21), thus confirming the likely involvement of the intracellular PRR NOD2 in these PGN-induced effects.

In addition, minor DC-activating effects were observed for LPS. Increased LC and CD1a$^+$ DDC migration frequencies and significantly enhanced CD83 and PD-L1 expression levels were observed. Although low TLR4 expression in LCs, DDCs and keratinocytes was observed, reported TLR4 expression in dermal fibroblasts (11) may have facilitated pro-inflammatory cytokine release (most notably IL-1β and IL-6, see also Fig.3), leading to the observed DC activation.

Remarkably, we observed no stimulatory effects for the TLR7/8 agonist R848 (Resiquimod), despite the fact that it emerged as an effective vaccine adjuvant –second only to Poly I:C from an *in vivo* study in humans (21). R848 was applied epicutaneously, like the closely related TLR7 agonist Imiquimod, which has previously been recognized as a possibly effective vaccine adjuvant (7). Conceivably, topical application more effectively addresses low-level TLR7/8 expressed in LCs (8). Moreover, topical application of Imiquimod has previously been described to lead to the recruitment of both myeloid and plasmacytoid DCs (26). Co-administration of systemic FLT3-L further mobilized DC precursors and enhanced the DC-stimulatory effects of Imiquimod (27). In line with these observations, we found R848 (like all the other TLR-Ls tested) to induce CCL5 release from the skin explants (although at highly variable levels, see Fig.3),

consistent with possible *in vivo* recruitment of DCs or their precursors from the blood. Clearly, the skin explant model employed in the current study is not suitable to address this issue.

In conclusion, Poly I:C and PGN, respectively binding TLR3/MDA5 and TLR2/NOD2, are attractive candidate adjuvants for intradermally delivered therapeutic vaccines targeting cutaneous DCs, and should be further explored as such. Based on *in vivo* findings from the Spörri and Reis e Sousa groups, direct APC activation by TLR agonists, rather than indirect activation through pro-inflammatory mediators released by accessory cells, ensures proper differentiation and long-term memory of Th1 and $CD8^+$ effector T-cells (28,29). In view of the relatively high expression levels of TLR2 and NOD2 in the mature and T-cell stimulatory $CD1a^+$ DDC subset and its proven ability to induce Th1 differentiation, inclusion of PGN in any TLR-targeted adjuvant formulation seems an attractive option in this regard. Indeed, as Poly I:C and PGN achieve their T-cell stimulatory effects through different mechanisms (Poly I:C through phenotypic DC activation and PGN through pro-inflammatory cytokine release and Th1/Th17 polarization), possible synergism through the combined administration of both deserves particular attention in future studies.

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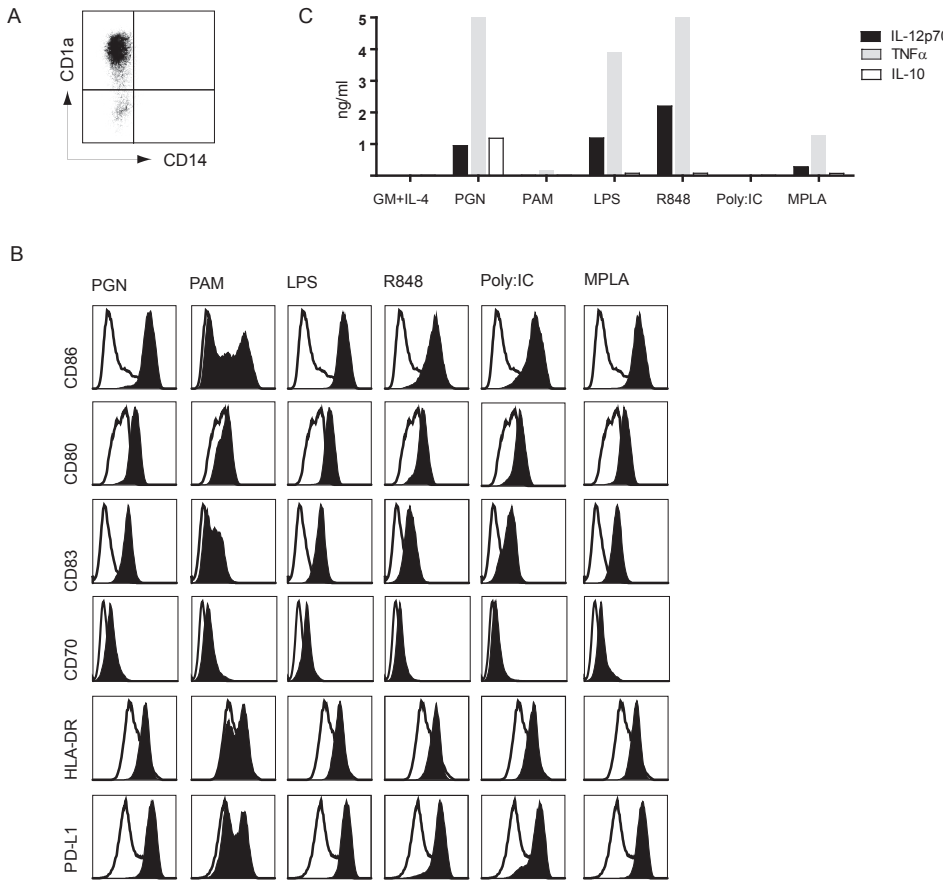
REFERENCES

1. Karbach, J., S. Gnjatic, A. Bender, A. Neumann, E. Weidmann, J. Yuan, C. A. Ferrara, E. Hoffmann, L. J. Old, N. K. Altorki, and E. Jager. 2010. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int. J. Cancer* 126:909-918.
2. Kenter, G. G., M. J. Welters, A. R. Valentijn, M. J. Lowik, Berends-van der Meer DM, A. P. Vloon, F. Essahsah, L. M. Fathers, R. Offringa, J. W. Drijfhout, A. R. Wafelman, J. Oostendorp, G. J. Fleuren, S. H. van der Burg, and C. J. Melief. 2009. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med.* 361:1838-1847.
3. Kupper, T. S., and R. C. Fuhlbrigge. 2004. Immune surveillance in the skin: mechanisms and clinical consequences. *Nat. Rev. Immunol.* 4:211-222.
4. Liu, L., Q. Zhong, T. Tian, K. Dubin, S. K. Athale, and T. S. Kupper. 2010. Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell-mediated immunity. *Nat. Med.* 16:224-227.
5. van den Hende, M., M. I. van Poelgeest, J. M. van der Hulst, J. J. de, J. W. Drijfhout, G. J. Fleuren, A. R. Valentijn, A. R. Wafelman, G. M. Slappendel, C. J. Melief, R. Offringa, S. H. van der Burg, and G. G. Kenter. 2008. Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. *Int. J. Cancer* 123:146-152.
6. Duthie, M. S., H. P. Windish, C. B. Fox, and S. G. Reed. 2011. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol. Rev.* 239:178-196.
7. Gnjatic, S., N. B. Sawhney, and N. Bhardwaj. 2010. Toll-like receptor agonists: are they good adjuvants? *Cancer J.* 16:382-391.
8. van der Aar, A. M., R. M. Sylva-Steenland, J. D. Bos, M. L. Kapsenberg, E. C. de Jong, and M. B. Teunissen. 2007. Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition. *J. Immunol.* 178:1986-1990.
9. Hari, A., T. L. Flach, Y. Shi, and P. R. Mydlarski. 2010. Toll-like receptors: role in dermatological disease. *Mediators. Inflamm.* 2010:437246.
10. Karim, R., C. Meyers, C. Backendorf, K. Ludigs, R. Offringa, G. J. van Ommen, C. J. Melief, S. H. van der Burg, and J. M. Boer. 2011. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. *PLoS. One.* 6:e17848.
11. Wang, J., K. Hori, J. Ding, Y. Huang, P. Kwan, A. Ladak, and E. E. Tredget. 2011. Toll-like

receptors expressed by dermal fibroblasts contribute to hypertrophic scarring. *J. Cell Physiol* 226:1265-1273.

12. de Gruijl, T. D., C. C. Sombroek, S. M. Lougheed, D. Oosterhoff, J. Buter, A. J. van den Eertwegh, R. J. Scheper, and H. M. Pinedo. 2006. A postmigrational switch among skin-derived dendritic cells to a macrophage-like phenotype is predetermined by the intracutaneous cytokine balance. *J. Immunol.* 176:7232-7242.
13. Santegoets, S. J., S. Gibbs, K. Kroeze, d. van, V, R. J. Scheper, C. A. Borrebaeck, T. D. de Gruijl, and M. Lindstedt. 2008. Transcriptional profiling of human skin-resident Langerhans cells and CD1a+ dermal dendritic cells: differential activation states suggest distinct functions. *J. Leukoc. Biol.* 84:143-151.
14. Chamorro, S., J. J. Garcia-Vallejo, W. W. Unger, R. J. Fernandes, S. C. Bruijns, S. Laban, B. O. Roep, B. A. 't Hart, and K. Y. van. 2009. TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program. *J. Immunol.* 183:2984-2994.
15. Garcia-Vallejo, J. J., H. B. Van Het, J. Robben, J. A. Van Wijk, D. Van, I, D. H. Joziasse, and D. W. Van. 2004. Approach for defining endogenous reference genes in gene expression experiments. *Anal. Biochem.* 329:293-299.
16. Romani, N., V. Flacher, C. H. Tripp, F. Sparber, S. Ebner, and P. Stoitzner. 2012. Targeting skin dendritic cells to improve intradermal vaccination. *Curr. Top. Microbiol. Immunol.* 351:113-138.
17. Peiser, M., J. Koeck, C. J. Kirschning, B. Wittig, and R. Wanner. 2008. Human Langerhans cells selectively activated via Toll-like receptor 2 agonists acquire migratory and CD4+T cell stimulatory capacity. *J. Leukoc. Biol.* 83:1118-1127.
18. Jurkin, J., Y. M. Schichl, R. Koeffel, T. Bauer, S. Richter, S. Konradi, B. Gesslbauer, and H. Strobl. 2010. miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. *J. Immunol.* 184:4955-4965.
19. Ouwehand, K., D. Oosterhoff, M. Breetveld, R. J. Scheper, T. D. de Gruijl, and S. Gibbs. 2011. Irritant-induced migration of Langerhans cells coincides with an IL-10-dependent switch to a macrophage-like phenotype. *J. Invest Dermatol.* 131:418-425.
20. Lai, Y., N. A. Di, T. Nakatsuji, A. Leichtle, Y. Yang, A. L. Cogen, Z. R. Wu, L. V. Hooper, R. R. Schmidt, A. S. von, K. A. Radek, C. M. Huang, A. F. Ryan, and R. L. Gallo. 2009. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat. Med.* 15:1377-1382.
21. Longhi, M. P., C. Trumpfheller, J. Idoyaga, M. Caskey, I. Matos, C. Kluger, A. M. Salazar, M. Colonna, and R. M. Steinman. 2009. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J. Exp. Med.* 206:1589-1602.
22. Farina, G. A., M. R. York, M. M. Di, C. A. Collins, S. Meller, B. Homey, I. R. Rifkin, A. Marshak-Rothstein, T. R. Radstake, and R. Lafyatis. 2010. Poly(I:C) drives type I IFN- and TGFbeta-mediated inflammation and dermal fibrosis simulating altered gene expression in systemic sclerosis. *J. Invest Dermatol.* 130:2583-2593.
23. van der Aar, A. M., G. R. de, M. Sanchez-Hernandez, E. W. Taanman, R. A. van Lier, M. B. Teunissen, E. C. de Jong, and M. L. Kapsenberg. 2011. Cutting edge: virus selectively primes human langerhans cells for CD70 expression promoting CD8+ T cell responses. *J. Immunol.* 187:3488-3492.
24. Volz, T., M. Nega, J. Buschmann, S. Kaesler, E. Guenova, A. Peschel, M. Rocken, F. Gotz, and T. Biedermann. 2010. Natural Staphylococcus aureus-derived peptidoglycan fragments activate NOD2 and act as potent costimulators of the innate immune system exclusively in the presence of TLR signals. *FASEB J.* 24:4089-4102.
25. Kobayashi, M., R. Yoshiki, J. Sakabe, K. Kabashima, M. Nakamura, and Y. Tokura. 2009. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br. J. Dermatol.* 160:297-304.
26. Adams, S., D. W. O'Neill, D. Nonaka, E. Hardin, L. Chiriboga, K. Siu, C. M. Cruz, A. Angiulli, F. Angiulli, E. Ritter, R. M. Holman, R. L. Shapiro, R. S. Berman, N. Berner, Y. Shao, O. Manches, L. Pan, R. R. Venhaus, E. W. Hoffman, A. Jungbluth, S. Gnjatic, L. Old, A. C. Pavlick, and N. Bhardwaj. 2008. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J. Immunol.* 181:776-784.
27. Shackleton, M., I. D. Davis, W. Hopkins, H. Jackson, N. Dimopoulos, T. Tai, Q. Chen, P. Parente, M. Jefford, K. A. Masterman, D. Caron, W. Chen, E. Maraskovsky, and J. Cebon. 2004. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. *Cancer Immun.* 4:9.
28. Kratky, W., Reis e Sousa, A. Oxenius, and R. Sporri. 2011. Direct activation of antigen-presenting cells is required for CD8+ T-cell priming and tumor vaccination. *Proc. Natl. Acad. Sci. U. S. A* 108:17414-17419.
29. Sporri, R., and Reis e Sousa. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat. Immunol.* 6:163-170.

SUPPLEMENTAL FIGURES



Supplementary figure 1. TLR-ligands activate human monocyte derived dendritic cells. A) CD14+ isolated monocytes (>95% pure) were differentiated with IL-4 and GM-CSF and analyzed at day 6 of culture by flowcytometry. B) mo-DC were stimulated with indicated TLR-agonists for 48 hr and analyzed by flowcytometry for expression of co stimulatory molecules Open histograms: immature/unactivated DC (GM+IL 4). Example of 2 5 different donors tested. C) Cytokine production of B analyzed by CBA (one representative experiment).

CHAPTER 7

Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab

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ABSTRACT

Purpose: In a recent randomized phase III clinical trial in metastatic colorectal cancer patients, the addition of the anti-epidermal growth factor receptor (EGFR) monoclonal antibody (MAb) cetuximab to bevacizumab and chemotherapy resulted in decreased progression-free survival, in particular for patients with the high-affinity FcγRIIIA.

Experimental Design: The presence of natural killer (NK) cells and type 2 (M2) macrophages in colorectal cancer was determined by immunohistochemistry using antibodies to lineage-specific markers, respectively, NKp46 and CD68 with CD163. Influence of tumor-bound cetuximab on M2 macrophages was performed *in vitro* with EGFR-expressing tumor cells and short-term differentiated monocytes from blood donors, who were typed for the FcγRIIIA polymorphism (CD16).

Results: ADCC by NK cells is generally proposed as one of the anti-tumor mechanisms of MAbs. We found that CD163-positive, M2 macrophages are much more abundant in colorectal carcinomas. *In vitro* analysis of M2 macrophages revealed high levels of Fc-gamma receptors (FcγRs) and PD-L1, and production of IL-10 and VEGF, but not IL-12. These anti-inflammatory and tumor-promoting mediators were released upon co-culture with EGFR-positive tumor cells loaded with low concentrations of cetuximab. Macrophage activation depended on EGFR expression on the tumor cells, FcγRs, target specificity of the MAb and mobility of antibody complexes. Cetuximab-induced macrophage responses were more pronounced for FCGR3A 158-valine (high affinity) carriers.

Conclusion: These results suggest that tumor-promoting M2 macrophages are activated by the therapeutic MAb cetuximab in the local tumor microenvironment and argue that this immune mechanism should be taken into account for the application of therapeutic antibodies.

Translational Relevance: In a recent clinical phase III study, the addition of the therapeutic MAb cetuximab was evaluated in colorectal carcinoma patients who received chemotherapy and bevacizumab. Surprisingly, patients with cetuximab inclusion in the protocol had a worse progression-free survival than those treated with standard treatment. This detrimental effect was more pronounced in patients with the high affinity Fc-binding receptor FcγRIIIA, implying a role for immune cells. In our consecutive study, we now show that macrophages with M2 differentiation profile are abundantly present in colorectal carcinomas, much more than ADCC-mediating natural killer cells. M2 macrophages are efficiently activated by low dose cetuximab, resulting in the release of immune suppressive and tumor-promoting mediators. Macrophages with the high affinity valine-encoding FcγRIIIA displayed an enhanced activation. We conclude that therapeutic monoclonal antibodies, such as cetuximab, can support tumor growth via tumor associated macrophages in the tumor microenvironment, in addition to their direct cytostatic activity.

INTRODUCTION

Monoclonal antibodies (MAbs) have become important agents for the treatment of many types of malignancies. Generally, their principal mechanism of action is blocking growth factor pathways that are essential for tumor growth and progression. So far, all clinically applied MAbs contain the Fc region of human IgG, which efficiently mediates activation through Fcγ receptors (FcγRs) on several types of immune cells. These IgG-binding receptors actually contribute to the clinical effect of MAbs, in addition to their direct inhibition on tumor growth (1). The role of immune activation is corroborated by several studies describing an association between the rs396991 polymorphism (*FCGR3A* 158Phe→Val) in the gene encoding FcγRIIIA (also known as CD16) and clinical outcome after treatment with the therapeutic MAbs rituximab (anti-CD20), trastuzumab (anti-HER2/*neu*) and cetuximab (anti-epidermal growth factor receptor, EGFR) (2-4). The high affinity valine allele has been associated with increased clinical response and survival in these studies, which is in line with *in vitro* studies indicating that antibody-dependent cellular cytotoxicity (ADCC) is more extensive for this allele (5).

Recently, the addition of cetuximab to bevacizumab plus chemotherapy resulted in decreased progression-free survival in a large clinical trial in metastatic colorectal cancer (CAIRO2 study) (6). To explain this unexpected result, we subsequently analyzed which gene polymorphisms were related to poor outcome in this cohort. This study revealed that patients expressing the high affinity allele for the FcγRIIIA (158Val) had a shorter progression-free time span, but only when cetuximab was added (7). Possibly, the detrimental effect of cetuximab is a consequence of activation of tumor promoting immune cells, rather than triggering tumoricidal ADCC by NK cells. FcγRs are also expressed by macrophages, which have been demonstrated in colorectal carcinomas (8, 9)Tumor-associated macrophages are characterized as M2-type cells and are known to possess anti-inflammatory, pro-angiogenic and tumor-promoting properties (10). Recent literature even implies an active involvement of FcγRs and myeloid cells in carcinoma development (11). We therefore hypothesized that therapeutic antibodies, such as cetuximab, might locally cross-link FcγRs on intratumoral M2-type macrophages by EGFR-binding on tumor cells, resulting in activation and release of tumor-promoting mediators.

These molecular interactions might explain the negative effect of the addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial.

MATERIALS AND METHODS

Immunohistochemical stainings

Formalin fixed and paraffin embedded tumors from ten untreated stage III colorectal cancer patients, of whom the majority was moderately differentiated, 1 well and 2 were poorly differentiated, were selected from the pathology archive of the Leiden University Medical Center. All patients showed recurrent disease at follow-up. Staining for NK cells and M2 macrophages was described before (12).

Cell-cultures

Colorectal adenocarcinoma cell lines LoVo and HCT-15 were kindly provided by Dr. van Wezel (Leiden University Medical Center, the Netherlands) and epidermoid skin cancer line A431 was obtained from ATCC (Manassas, VA, USA). M2-type macrophages and dendritic cells (DCs) were differentiated from purified $CD14^+$ monocytes (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated as previously described (12), using M-CSF (R&D, Minneapolis, USA) or GM-CSF (Invitrogen) with IL-4 (Invitrogen). Cells were activated by 250 ng/ml LPS (Sigma-Aldrich, St. Louis, USA) or tumor cells with MAbs cetuximab (Erbitux®, Merck, Darmstadt, Germany), rituximab or bevacizumab (Mabthera® and Avastin®, respectively; Roche, Welwyn Garden City, UK).

Experimental conditions

At day 6 of the monocyte differentiation cultures, tumor cell lines were plated in a density of 50.000 cells per well in 48-wells plates. After 2 h, 250 ng/ml LPS or MAbs were added together with M2 macrophages in a density of 100.000 cells per well. After 24 h, supernatants were collected and analyzed for IL-10 (Sanquin, Amsterdam, Netherlands), IL-8, VEGF (eBioscience, Vienna, Austria) and IL-12p70 (BD-Biosciences, Minneapolis, MN, USA) production. Macrophages were removed from the culture plates by scraping and stained with monoclonal antibodies (all purchased from BD-Biosciences, except for anti- PD1-L from eBioscience). Samples were recorded using a FACS Calibur with Cellquest software (BD-Biosciences). Data were analyzed with FlowJo software (Tree star, Ashland, USA). Macrophages were separated from tumor cell-lines by gating for HLA-DR.

Genotyping

Genomic DNA was isolated from monocytes with MagnaPure Compact (Roche, Almere, Netherlands) and genotyping for *FCGR3A* c.818A>C (C_25815666_10; rs396991) was performed as previously described (7).

RESULTS

Colon carcinomas are heavily infiltrated with type 2 macrophages, but not with NK cells.

To investigate immune cell infiltration of colorectal cancers, we stained ten tumors for the common macrophage marker CD68, and the scavenger receptor CD163, which is typically expressed by M2-type macrophages. All colorectal tumors were extensively infiltrated with this type of macrophages (figure 1a). By contrast, hardly any NK cells were observed using the NK-lineage specific receptor NKp46. We thus envisage that cetuximab treatment might impact on these infiltrating macrophages, and that local ADCC via NK cells plays a minor role.

The influence of cetuximab on macrophages was studied on freshly isolated monocytes that were differentiated *in vitro* into $CD1a^{-}CD14^{+}CD163^{+}$ macrophages (12). The expression of Fcγ receptors FcγRI (CD64), FcγRII (CD32) and FcγRIIIA (CD16) and release of cytokines after activation by the strong TLR stimulus LPS was examined (figure 1b, c). M2 macrophages strongly displayed all three Fc-binding receptors and produced high amounts of the anti-inflammatory IL-10, as well as IL-8 and the pro-angiogenic VEGF, but not the immunostimulatory IL-12. Control monocyte-derived DCs displayed an opposite profile, which is in line with their T-cell stimulating function. These data strongly suggested that M2-type macrophages could potentially be stimulated by MAbs to exert an anti-inflammatory and pro-angiogenic role in the tumor microenvironment.

Cetuximab induces activation of type 2 macrophages

M2 macrophages were then activated by cetuximab in the presence of tumor cells. Three tumor lines were used (A431, LoVo and HCT-15) and flow cytometry analysis showed that A431

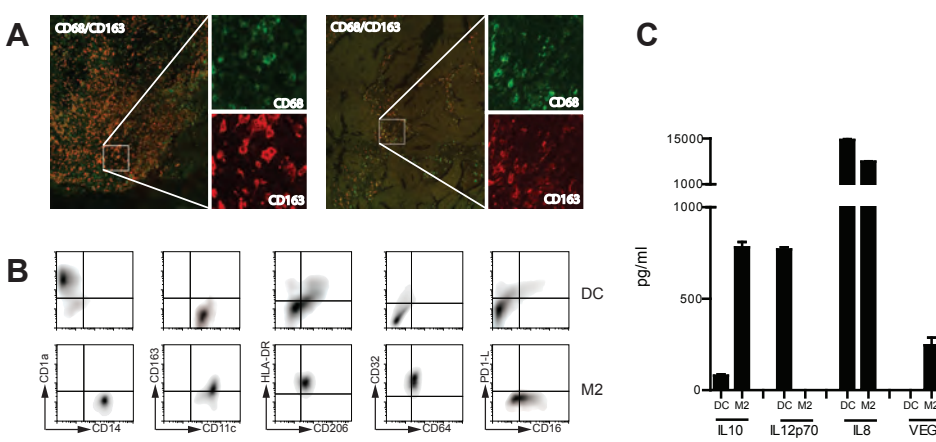

Figure 1. Detection and characterization of M2 macrophages in colorectal cancer. **A**, Two representative examples of two color immunofluorescent staining of stage III colorectal cancers with high (left panel) and low (right panel) infiltration of $CD68^{+}$ (green) macrophages expressing the typical type 2 marker $CD163^{+}$ (red). **B**, Flow cytometry analysis of M2 macrophages and monocyte-derived dendritic cells (mDC). Fcγ receptors are indicated by the CD nomenclature: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). **C**, Production of IL-10, IL-12p70, IL-8 and VEGF by mDCs and M2 macrophages after overnight stimulation with LPS.

highly expressed EGFR, whereas EGFR staining of LoVo and HCT-15 was much lower (figure 2a). Importantly, co-culture of macrophages with cetuximab-opsonized A431 tumor cells resulted in production of IL-10 and IL-8, whereas EGFR-low tumors LoVo and HCT-15 did not activate macrophages (figure 2b, suppl figure 1). Notably, the release of IL-8 upon cetuximab treatment exceeded that of the positive control LPS (suppl figure 1). The A431 tumor cells spontaneously produced VEGF, so this mediator could not be used in succeeding experiments to determine macrophage activation. To corroborate the notion that macrophage activation was the result of cross-linking Fcγ receptors, we incubated tumor cells with MAbs specific for the non-expressed CD20 (rituximab), or the soluble VEGF (bevacizumab), both containing the same IgG_1 isotype. This did not lead to IL-10 production (figure 2c), suggesting that only membrane-bound proteins as EGFR can efficiently trigger macrophages via FcγR. Furthermore, competition of Fc-binding by high concentrations of rituximab resulted in a dose-dependent decrease of IL-10 production (suppl figure 2). Interestingly, IL-10 was also not detected when cetuximab was coated on culture plates (figure 2c), suggesting that the molecular interaction of EGFR-cetuximab-FcγR required the flexibility of fluid membranes for proper cross-linking.

The cetuximab mediated activation of M2 macrophages was dose-dependent (figure 3) and concentrations as low as 10 ng/ml were sufficient to down-regulate cell surface levels of CD16 and to up-regulate the inhibitory molecule PD1-L (figure 3a) and to release IL-10 and IL-8 (figure 3b, suppl figure 1). These data showed that very low concentrations cetuximab induced the release of anti-inflammatory mediators from M2 macrophages through cross-linking of Fcγ receptors.

Effect of FCGRIIIA polymorphism

Addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial decreased the progression-free survival of metastatic colorectal cancer patients, especially for those with

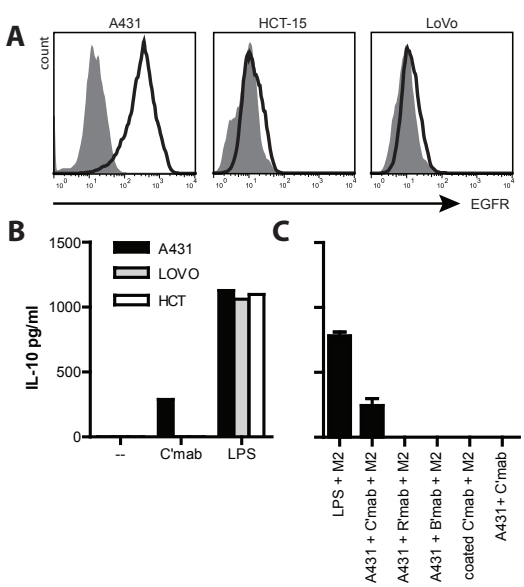

Figure 2. FcγR cross-linking by cetuximab activates M2 macrophages. **A**, Flow cytometry analysis of EGFR expression on tumor cell lines A431, HCT-15 and LoVo. Filled histograms represent isotype control antibody, solid lines indicate staining with anti-EGFR antibody. **B**, IL-10 production by M2 macrophages upon co-culture with tumor cells and 1 µg/ml cetuximab (c'mab). Addition of LPS (250 ng/ml) served as positive control. **C**, IL-10 production by macrophages depends on interaction of macrophages and cetuximab-pulsed A431 tumor cells. Plate-bound cetuximab (10 µg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibodies rituximab (r'mab, 1 µg/ml) or bevacizumab (b'mab, 1 µg/ml) could not replace cetuximab.

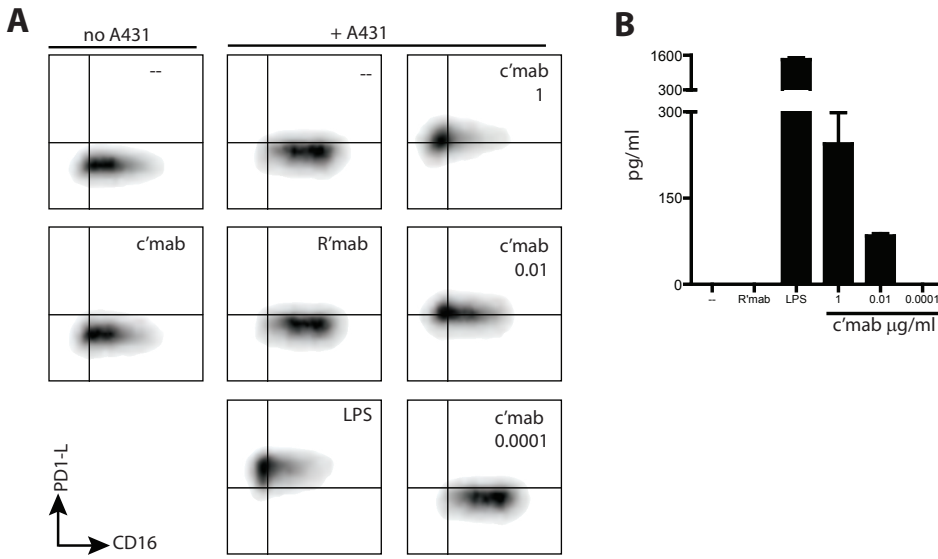

Figure 3. Degree of M2 macrophage activation depends on cetuximab concentration. **A**, Flow cytometry analysis of PD1-L up-regulation and CD16 down-regulation on M2 macrophages upon co-culture with EGFR-positive A431 tumor cells and different cetuximab (c'mab) concentrations. Rituximab (r'mab) served as negative control, LPS as positive control. **B**, Macrophage-derived IL-10 was measured in overnight supernatants.

high affinity FcγRIIIA genotype encoding the valine residue (6, 7). We examined the influence of this polymorphism on the degree of M2 macrophage activation by cetuximab using 22 healthy donors, consisting of 12 homozygous 158-Phe and ten 158-Val carriers (figure 4). Analysis of IL-10 release and CD16 down-regulation on M2 macrophages showed an apparent stronger activation of cells with the high affinity valine allele (figure 4). These differences did not reach statistical significance for cytokine release, most likely due to high variation within the groups and very high production (figure 4a and suppl figure 1, respectively). Notably, macrophage activation in this system is presumably also mediated by other Fcγ receptors, such as FcγRI, resulting in less pronounced differences between 158-Phe and 158-Val carriers. In conclusion, our data show that cetuximab can induce the release of anti-inflammatory mediators from M2 macrophages and that this effect might explain the negative clinical effect of this MAb in the recent CAIRO2 study.

DISCUSSION

Our data show that type 2 macrophages are abundantly present in colon carcinoma and are activated by cetuximab-opsonized tumor cells, resulting in anti-inflammatory and tumor promoting mediators, including IL-10 and VEGF. M2 macrophages are known to actively contribute to tumor growth via angiogenesis and immune suppression (10). Previous research on the immune mechanisms of therapeutic MAbs has focused on anti-tumor effects such as ADCC or phagocytosis. ADCC mediated by NK cells or PBMCs has been described for cetuximab

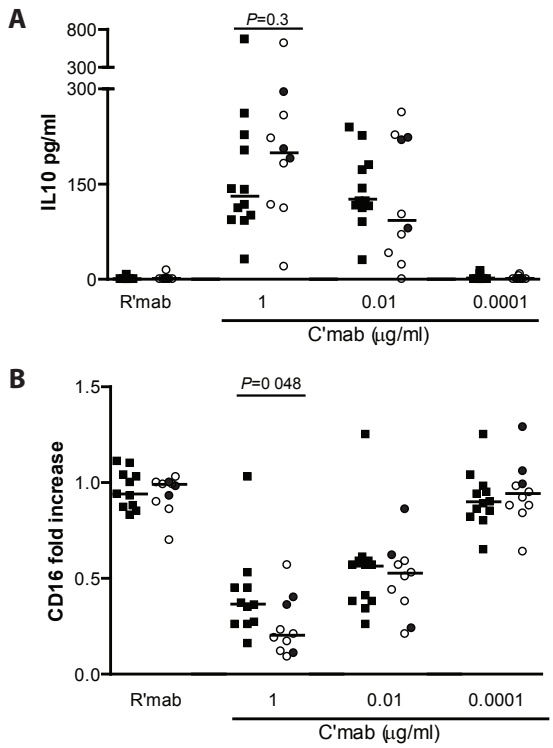

Figure 4. Activation difference of *FCGR3A* (CD16)-typed M2 macrophages. M2 macrophages of twelve homozygous 158-Phe (solid squares) and ten 158-Valine allele carriers (open circles are heterozygous and solid circles are homozygous) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. **A**, CD16 down-regulation and **B**, IL-10 release were measured. Lines represent median values and differences between the two groups at 1 µg/ml cetuximab were calculated by Mann Whitney test

(5, 13), however, staining for NK cells in colorectal carcinoma revealed that these cells are rare in colorectal cancers. Fcγ receptors are also expressed by macrophages and these cells were abundantly present in this tumor type (figure 1). Previous studies have shown that macrophages are present in all stages of colon tumors and that higher numbers of macrophages are found in more advanced stages of disease (8, 9). M2 macrophages are efficient in phagocytosis of rituximab-opsonized B cells (14), but we question the relevance of this FcγR-mediated process for solid tumors like colorectal carcinoma. Based on our findings, we rather suggest that activation of intratumoral M2 macrophages leads to release of tumor-promoting mediators.

The detrimental effect of cetuximab addition in the CAIRO2 trial was unanticipated (6), since the combination of cetuximab and anti-VEGF therapy appeared effective in mouse models (15-17) However, the Fcγ receptor-mediated effects by cetuximab could not be evaluated in these models, as the human Fc-region of cetuximab does not interact with the murine FcγRs. Future studies in mice expressing human FcγR might elucidate immune mechanisms of therapeutic MAbs and, importantly, better predict the outcome of combination studies. Our results indicate that the release of multiple anti-inflammatory and pro-angiogenic mediators by M2 macrophages could account for the decreased therapy efficacy for those patients that were treated with the combination of cetuximab, the anti-VEGF MAb bevacizumab and chemotherapy (6). The finding that M2 macrophages encoding the high affinity FcγRIIIA (valine-carriers) displayed a more pronounced activation (figure 4) corroborated our previous

observation that patients with this high affinity receptor had an even worse progression-free survival than those with 158-Phe homozygosity (7). Strikingly, removal of the high-affinity valine-carriers from the CAIRO2 cohort revealed that the addition of cetuximab did not lead to worse clinical outcome, compared with the trial arm of conventional therapy. On the contrary, the homozygous 158-Phe FcγRIIIA patients seemed to benefit from the addition of cetuximab. However, this analysis was performed on the *KRAS* wild type patients and groups sizes were too small to draw firm conclusions. Notably, bevacizumab binds soluble VEGF and therefore does not cross-link FcγRs and activate intratumoral macrophages (figure 2).

One intriguing question still remains: why does cetuximab mediate anti-tumor effects as a single agent (18, 19), whereas its addition to bevacizumab plus chemo-therapy leads to worse survival? Based on our findings, we speculate that cetuximab induces local release of pro-tumor mediators, amongst which VEGF, and thereby neutralizes the beneficial therapeutic effect of bevacizumab. Interestingly, a clinical study with cetuximab as monotherapy for metastatic colorectal cancers also revealed an increased progression-free survival for low-affinity carriers of FcγRIIIA, especially in combination with certain FCGR2A alleles (20). Combination with chemotherapy might tip the balance further towards macrophage activation by up-regulation of EGFR expression, as shown for fluoropyrimidines and irinotecan (21) and, importantly, also by recruiting immune-suppressive macrophages to the tumor site (22). These indirect immune effects might then overrule the direct growth-inhibiting effect of EGFR blockade.

In conclusion, therapeutic antibodies mediate a plethora of *in vivo* effector arms that reach beyond their on-target function or immediate complement-mediated cytotoxicity. These FcR-dependent mechanisms are diverse *in vivo* and several factors determine the outcome and employed effector arm, including the type of tumor (solid or circulating), type of immune infiltrate (macrophages or NK cells) and combinations with other therapeutics. We now propose an adverse mechanism by which therapeutic MAbs might promote tumor growth via activation of infiltrated macrophages, which are known for their pro-angiogenic and immune-suppressive functions. Clinical testing of engineered MAbs with Fc-regions with increased affinity to FcγRs should be performed very carefully (1, 23), because tumor-promoting effects by intratumoral M2 macrophages could lead to tumor promotion instead of tumor repression.

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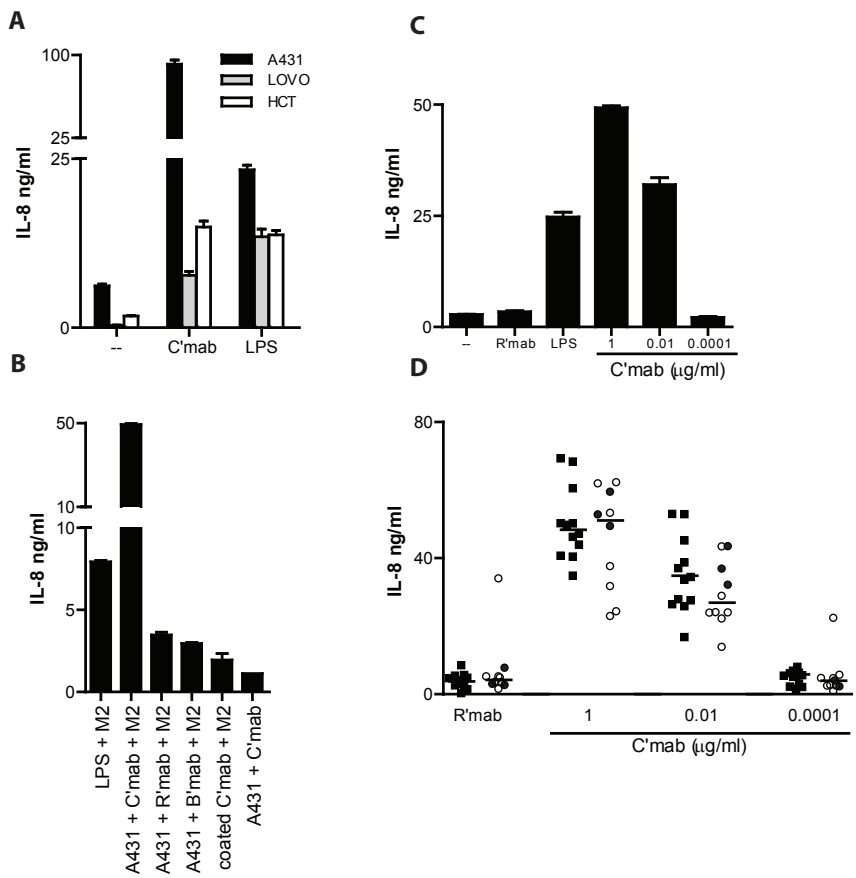
REFERENCES

1. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. Lancet 2009;373:1033-40.
2. Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, YchouM, BlanchardF, et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122-9.
3. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002;99:754-8.
4. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/*neu*-positive metastatic breast cancer. J Clin Oncol 2008;26:1789-96.
5. López-Albaitero A, Lee SC, Morgan S, Grandis JR, Gooding WE, Ferrone S, et al. Role of polymorphic Fc gamma receptor IIIa and EGFR expression level in cetuximab mediated, NK cell dependent in vitro cytotoxicity of head and neck squamous cell carcinoma cells. Cancer Immunol Immunother 2009;58:1853-64.
6. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 2009;360:563-72.
7. Pander J, Gelderblom H, Antonini NF, Tol J, van Krieken JH, van der Straaten T, et al. Correlation of FCGR3A and EGFR germline polymorphisms with the efficacy of cetuximab in KRAS wild-type metastatic colorectal cancer. Eur J Cancer 2010;46:1829-34.
8. Kang JC, Chen JS, Lee CH, Chang JJ, Shieh YS. Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. J Surg Oncol 2010;102:242-8.
9. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med 2007;5:62.
10. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010;11:889-96.
11. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcRγ activation regulates inflammation-associated squamous carcinogenesis. Cancer Cell 2010;17:121-34.
12. van Dongen M, Savage ND, Jordanova ES, Briaire-de Bruijn IH, Walburg KV, Ottenhoff TH, et al. Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. Int J Cancer 2010;127:899-909.
13. Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clin Cancer Res 2007;13:1552-61.
14. Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol 2009;182:4415-22.
15. Tonra JR, Deevi DS, Corcoran E, Li H, Wang S, Carrick FE, et al. Synergistic antitumor effects of combined epidermal growth factor receptor and vascular endothelial growth factor receptor-2 targeted therapy. Clin Cancer Res 2006;12:2197-207.
16. Shaheen RM, Ahmad SA, Liu W, Reinmuth N, Jung YD, Tseng WW, et al. Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. Br J Cancer 2001;85:584-9.
17. Jung YD, Mansfield PF, Akagi M, Takeda A, Liu W, Bucana CD, et al. Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer 2002;38:1133-40.
18. Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, Au HJ, et al. Cetuximab for the Treatment of Colorectal Cancer. New England Journal of Medicine 2007;357:2040-8.
19. Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009;360:1408-17.
20. Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol 2007;25:3712-8.
21. Correale P, Marra M, Remondo C, Migali C, Misso G, Arcuri FP, et al. Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-

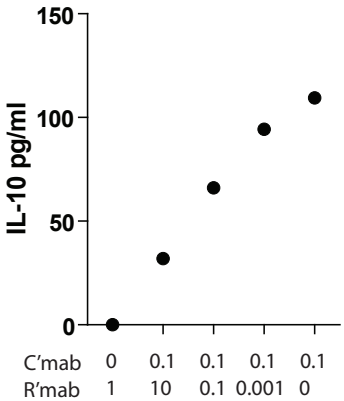
targeted antibody-dependent cell-mediated-cytotoxicity (ADCC). Eur J Cancer 2010;46:1703-11.

22. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. Cancer Discovery 2011.

23. Griggs J, Zinkewich-Peotti K. The state of the art: immune-mediated mechanisms of monoclonal antibodies in cancer therapy. Br J Cancer 2009;101:1807-12.



Supplemental figure 1. IL-8 production upon Fc-mediated M2 macrophage activation. **A**, IL-8 production after 24 h co-culture of M2 macrophages with cetuximab (c'mab, 1 μg/ml) opsonized A431 tumor cells (white bars), LoVo cells (light grey bars) and HCT-15 cells (dark grey bars). Addition of LPS (250 ng/ml) served as positive control. **B,** IL-8 production by macrophages depends on interaction of macrophages and cetuximab opsonized tumor cells. Plate-bound cetuximab (10 μg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibody rituximab (r'mab, 1 μg/ml) or bevacizumab (b'mab 1 μg/ml) could not replace cetuximab. **C,** Overnight IL-8 production by M2 macrophages is dependent on the concentration of cetuximab. **D,** Both the phenylalanine homozygote donors (solid squares, n = 12) and valine allele carriers (Phe/Val [open circles] and Val/Val [solid circles] donors combined; n = 10) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. IL-8 release was measured and lines represent median values.



Supplemental figure 2. Competition for Fc-binding by rituximab. A431 tumor cells were loaded without cetuximab (c'mab 0) or with a sub-optimal dose of 0.1 μg/ml cetuximab (c'mab 0.1). Rituximab (r'mab) was added in higher (10 μg/ml), equal (0.1 μg/ml) or lower concentration and M2 macorphages were added. IL10 release was measured after 24 hr by ELISA.

CHAPTER 8

General discussion

GENERAL DISCUSSION

The role of APC in the activation of T cells

In chapter 2 we analyzed the presence of HPV-specific proliferative T-cell responses in the blood of patients with HPV-induced cervical cancer. HPV-specific T cells were found only in a minority (31%) of the patients. T-cell responses were more often detected in patients presenting with a tumor that deeply invaded the surrounding tissue, which is on its own a bad prognostic sign. At that time we hypothesized that by the infiltration of healthy tissue, tumor antigens were delivered to APC not tolerized by the tumor milieu and, therefore, able to appropriately instruct T cells. However in view of the results presented in chapter 5, in which we showed that CxCa can induce macrophage differentiation, I would like to propose an alternative explanation. Macrophages contribute to tumor invasion by the production of metallo proteinases (MMP) which brake down collagen and facilitate infiltration by tumor cells in the surrounding tissue. Therefore, the deeply invading tumors described in chapter 2 could be the tumors that harbour high numbers of MMP-producing M1 or M2 macrophages. In chapter 2 we describe that half of the patients with deeply invading tumors have proliferating HPV specific T cells in the blood. After uptake of tumor cells or cell debris macrophages can present tumor-specific antigens to T cells and elicit T-cell responses. Analysis of the H&E staining of these deeply invading tumors showed that the patient group with T-cell proliferation in the blood had high immune cell-infiltrate in the tumor (Table 1). This suggest the presence of pro-inflammatory M1 macrophages, contributing to local invasion of the tumor but also inducing inflammation and T-cell responses.

In contrast, patients without HPV-specific proliferation in the blood predominantly lacked immune-cell infiltrate in the tumor. One can envisage that the patients without T-cell responses in the blood and low infiltrate density in the tumor (table 1) harbour M2 type macrophages in the tumor. These macrophages are not attracting or inducing T cells but promote tumor invasion and this correlates with the poor prognosis found for this group (chapter 2).

We showed that when HPV-specific T cells were present in the tumor, the T-cell reaction of a wide diversity and involved several CD4+ as well as CD8+ T-cell clones (chapter 3). *Ex-vivo,* these T cells recognized HPV but not all responding cells produced type 1 cytokines. Addition of TLR-agonist PAM3CSK4 and Poly I:C during antigen-specific stimulation of the T cells increased their production of IFNγ in primary cultures. To stimulate the T cells with antigen we made use of B-LCL as antigen presenting cell. Although B cells can present antigen, they are not as the specialized as DC and, therefore, these assays may not have been optimal for testing T-cell function. How important the type of APC is for the strength of activation in antigen-experienced cells is not clear, although it is known that the type of co stimulatory molecules present influence cytokine production [1]. Furthermore, we showed in chapter 5 that naïve responder T cells failed to produce high levels of IFNγ when stimulated with less well differentiated DC or macrophages – obtained when monocytes were cultured in the presence of tumor supernatant - as compared to T cell stimulated with fully differentiated DC. In addition, we observed that established HPV-specific T-cell clones produced less IL-2 upon activation with the above mentioned non-optimal APC (unpublished observations). Apparently the type of antigen presenting cell is important for the activation of naïve but also antigen experienced T cells. Finally, experiments on T-cell activation by peptide loaded monocytes revealed that increased antigen doses resulted in better cytokine production by T cells (*Singh*

et al, manuscript submitted). Hence, conclusions concerning the functionality of T cells (e.g. like we have called the T cells that did not produce cytokines 'poised' in chapter 3) should be drawn with great care.

TLR-agonists to boost inflammation?

Addition of TLR-agonists boosted the IFNγ production of the aforementioned cultures described in chapter 3 but not to the same extent in all patients. PAM3CSK4 and Poly I:C were the most promising adjuvant. In these cultures the exact cell type (APC, T cells and in lymph node tissue also B cells) that responded to the TLR-agonist was not dissected.

Several studies report that stimulation of TLR modulates T-cell responses after TCR triggering [2]. At the T-cell population level, TLR-stimulation enhanced IFNγ production by T cells but also abolished regulatory T-cell function [2]. So far, these studies were performed on unselected CD4+ T cell populations. In our study on functional TLR expression by T cells we made use of isolated HPV-specific T cell clones and conducted many *in vitro* experiments. Analysis of TLR expression by patient derived tumor-antigen specific CD4+ T-cell clones -including helper and regulatory T-cell clones- revealed that human T cells express a number of different TLRs at the mRNA level and that the TLR expression pattern differed per CD4+ T-cell clone, independent of their function. When these CD4+ T-cell clones were tested in various activation (proliferation, cytokine production) and suppression assays no consistent gain (proliferation/ IFNγ production) or loss in function was observed due to the presence of TLR-agonists. This suggests that TLR may not play a major role in direct stimulation of antigen experienced and fully polarized CD4+ T cells (unpublished observations).

The effect of several TLR-agonists on the activation of purified APC was investigated *in vitro* in chapter 5. Interestingly, the two agonists PAM3CSK4 and Poly I:C - showing the most promising activation of T-cell cultures in chapter 3 - were the least promising agonists in these assays (chapter 5a, figure 2 and not shown). Addition of TLR-agonists to mo-DC resulted in upregulation of co-stimulatory molecules at the cell surface, but Poly I:C as well as PAM3CSK4 failed to induce the production of IL-12. However, when Poly I:C was added to a less pure culture of mo-DC (derived by plastic adherence) splendid production of IL-12 was detected. These cultures are contaminated with T cells which may have provided additional signals via CD40-L or IFNγ [3,4] suggesting that Poly I:C in the presence of a second signal is a strong agonist (unpublished data).

This notion is sustained by our studies using skin-explant cultures as described in chapter 6. Poly I:C proves to be the strongest activator of dermal APC. Here too, other cell types are present that can provide synergistic signals to the local APC. Interestingly, R848 -the agonist of TLR7 and closely related to Imiquimod- did not induce any measurable activation or migration in the skin-explant model described in chapter 6. Keratinocytes, which functionally display TLR3 but not TLR7 may have supported the activation of APC by the production of IL-1, type I IFN and other factors ([5] and unpublished observations). Based on chapter 3 and 6, Poly I:C may form an attractive compound to apply locally on tumors or neoplastic lesions. Especially for HPV+ oropharyngeal tumors this could be a good alternative or adjuvant treatment. These tumors develop within a lymphoid structure and thus consist of lymphoid tissue and epithelial cells (e.g. keratinocytes). Since many HPV+ oropharyngeal tumors are infiltrated with tumor-antigen specific T cells, stimulation of local inflammation might tip the balance in favor of an anti-tumor response (chapter 4).

M2 and myeloid derived suppressor cells (MDSC)

Tumors attract myeloid antigen presenting cells and interfere with their differentiation. Gabrilovich *et al* showed in mouse models that tumor-induced myeloid cells suppressed surrounding T cells. These MDSC express GR1+ as well as CD11b, produce iNOS and arginase and are of pre-mature undifferentiated state [6]. MDSC are also found in humans, although the phenotype is less clear and there is a great variety between individuals and tumor types. Human MDSC express CD33, CD11b and IL4Rα, CD15, CD14 although the last 3 markers are not always reported [7]. In chapter 5 we showed that soluble factors produced by CxCa cell lines influence myeloid cell differentiation despite the presence of IL-4 and GM-CSF. Some cell lines only hampered DC differentiation but other cell lines clearly induced a macrophage phenotype. The APC differentiated in the presence of supernatant of CASKI cells expressed less CD1a and higher mannose receptor. Other markers like CD14 or IL-4Ra were not clearly expressed and, therefore, it was difficult to nail down the exact cell phenotype that was induced. The differentiation of these cells into DC was completely restored when IL-6 was blocked. Thus in our experiments IL-6 only hampered DC differentiation and induced higher IL-10 production but did not skew cell differentiation towards macrophages or MDSC.

Furthermore, we showed in chapter 5 that CxCa-produced PGE2 skewed mo-DC differentiation towards APC expressing CD14, CD163 and high levels of the mannose receptor. Based upon receptor expression profile and functional characteristics we called these cells type 2 macrophages. Similarly, Obermajer *et al*. recently published that the addition of PGE2 to mo-DC cultures induced suppressive cells which they called MDSC [8]. This is a clear example of the confusion and debate that is currently ongoing about the phenotype, function and role of myeloid subsets in human cancers. To complicate matters even further, we clearly found that activation with TLR-agonists or CD40-L of M2 macrophages resulted in phenotypic changes leading to loss of the typical M2 marker phenotype but exertion of their M2 function (chapter 5a supplementary. fig. 1). Thus the phenotype of APC is not always representing the function of the cell, even more because myeloid cells show great plasticity depending on the type of stimulus. Finally, tumors harbour distinct microenvironments that instruct locally present myeloid cells and results in heterogenic populations of myeloid cells within one tumor, both in mice and men [9-11]. These data plead for a less stringent separation of myeloid cell subsets. MDSC or macrophages are not different cell subsets but rather resemble a state of differentiation and activation of developing myeloid cells.

Chemotherapeutics induce immunological effects

Chapter 5b describes the preliminary data of studies in which tumor cell lines known to skew monocytes to M2 macrophages are treated with cisplatin or carboplatin. Treatment of these tumor lines with physiological but non-lethal doses of cisplatin resulted in an increased production of PGE2 and IL-6 by the tumor cells and augmented the skewing of monocytes to M2 macrophages. This illustrates that the quantity and the sum of factors released by the tumor cells bears impact on the final outcome. Possibly other factors are produced by the CxCa cell lines as well and contribute to the observed effects. The fact that COX-inhibition in tumor cells prevented the skewing to M2 macrophages illustrates that PGE2 is a key factor in this network.

If patients receive chemotherapy treatment it is likely that a peak dose emerges in the blood followed by a rapid decline. Ideally, all tumor cells die upon treatment but based on

the results of chapter 5b, one can envisage that if tumor cells are resistant and survive they might actually contribute to an increase in the number of local M2 macrophages and as such promote tumor growth. Ferrandina *et al*. performed a clinical study in patients with advanced CxCa and found that over-expression of COX-2 in the tumor correlated with a poor prognosis upon chemo radiation. This was explained by the fact that COX-2 expression in the tumor made the cells more resistant to apoptosis and thereby to chemotherapeutic treatment [12-15]. However the data of chapter 5b advocates an alternative explanation. An important predictor of response to therapy in several human tumor types is the number and type of T cells present in the tumor [16-21]. Maybe in cervical cancer these two known prognostic factors are related because the type and number of T cells infiltrating could reflect the type of macrophages that are present. High numbers of M2 macrophages correlate with more regulatory T cells infiltrate in human ovarian and GIST tumors [22,23]. Therefore the tumor expressing high regulatory T cell numbers is likely to be the COX+ tumor that induced the differentiation of M2 cells via elevated levels of PGE2 and IL-6 in its micro milieu. Besides the tumor promoting activity of the M2 macrophages, increased suppression of locally present T cells – either directly by the production of IL-10 by M2 macrophages or by the attraction of regulatory T cells - will occur and this is enhanced upon treatment with cisplatin (chapter 5b). This notion is sustained by a study in mice bearing lung tumors. Here, treatment with COX-inhibiting drugs resulted in marked T-cell infiltration in the tumor and delayed tumor outgrowth. The tumor infiltrating APC produced less IL-10 and more IL-12 upon treatment. This indicates that local PGE2 levels altered the APC type in the tumor with effects on T-cell infiltration [24]. Interestingly in lung cancer a poor response to cisplatin therapy is associated with activation of STAT3 protein. IL-6 signaling is known to activate STAT3, suggesting that cisplatin-mediated increase of factors like IL-6 – known to affect DC differentiation - might play a role in the poor response to cisplatin of these patients as well [25].

Unfortunately studies on the simultaneous presence of different T cells types in relation to the infiltrating APC on human tumors are scarce. This makes it difficult to find data corroborating above mentioned hypothesis. Studies in which these parameters are combined may reveal a relationship between these players, especially when analyzed before and after treatment. Currently, mouse tumor models are explored to further dissect the exact role of infiltrating APC and T cells upon in relation to treatment. A major drawback of such models is that only a few tumor lines are suitable to study this. As we have shown in chapter 5, pronounced differences between cell lines exist with respect to their capacity to influence immune cells. Mouse studies using tumor cell lines will poorly reflect the great variety in human tumors and thus their interplay with immune cells.

Notably, platinum based chemotherapeutics can have positive immune-related side effects. In a mouse model, Apetoh *et al*.showed that oxaliplatin induced immunogenic cell death via calreticulin exposure on tumor cells, thereby, stimulating the induction of an anti tumor T-cell response. Oxaliplatin and cisplatin induced release of HMGB-1 in dying tumor cells that activated APC via stimulation of TLR-4 [26,27].However, most of these experiments were done with only a few tumor-cell lines and intra-tumoral injection of chemotherapeutics or ex-vivo treatment of the tumor which does not resemble the clinical practice. This mechanism might be relevant in human cancer though since patients with breast cancer carrying a loss-of-function TLR-4 mutation relapsed more quickly after radiotherapy combined with (non-platin

based) chemotherapy [26], arguing for immune-dependent effects of chemotherapy. The data in chapter 5 shows that tumor induced macrophages respond to TLR-4 agonists, as reflected by the production of IL-10. Therefore, it is highly likely that chemotherapy-mediated release of HMGB-1 may not only activate DC but activate locally tumor-promoting macrophages as well. Activation of macrophages would add to the immune-suppressive tumor-promoting micro milieu in the tumor. Thus, platinum-based chemotherapy of solid tumors may both have positive and negative effects on the local anti-tumor immune response. The outcome is likely to depend on the constitution of the population of different APCs present within the tumor. Finally, most patients are treated with a combination of radiotherapy and chemotherapy but the combination of these two treatments is yet not addressed in the former and our own studies. Radiotherapy enhances antigen presentation by tumor cells, induction of tumor antigen specific antibodies and inflammation [28-30] and is, therefore, likely to contribute to the overall immune response as well.

Blocking COX-2 or IL-6 during treatment

Blocking the production of PGE2 with COX-inhibitors prevented the induction of tumor promoting macrophages *in vitro* (chapter 5) and, therefore, patients with COX overexpressing tumors may benefit from blocking these enzymes during therapy. A number of studies were performed in several solid cancers in which patients were treated with a COX-2 inhibiting drug during standard therapy. In a recent phase II study COX-2 inhibition favored the clinical outcome in patients with ovarian cancer [31]. Also a daily dose of celexocib (most clinically used COX-2 inhibitor) effectively prevented (pre-cancerous) colorectal polyp formation. Unfortunately, the latter trial was aborted because of serious adverse cardiovascular effects when patients were treated for several years [32]. Also in non-small-cell lung cancer the addition of celexocib during a shorter time period to chemotherapy was studied. Although no adverse cardiovascular side effects were reported also no survival benefit was found in two independent studies [33,34]. COX-inhibition in patients with neoplastic lesions was already advocated because the increased chemo-sensitivity of the tumor cells [13-15]. We provide with chapter 5b an immunological rationale for the addition of a COX-inhibitor to standard treatment. Also in combination with therapeutic vaccination, inhibition of COX-enzymes may contribute to a less hostile tumor microenvironment that is more vulnerable for the attack by T cells.

A second strategy to change the tumor microenvironment into an attractive place for pro-inflammatory macrophages and cytotoxic T cells is blocking IL-6. We found that cancer cell derived IL-6 skews DC differentiation towards APC that produce more IL-10 (chapter 4) as well as displayed elevated levels of activated STAT-3 (data not shown). Recently, a study was reported in which chemo resistant end-stage ovarian cancer patients were treated with an antibody to IL-6 (siltuximab). Stable disease was observed in some patients [35]. Concordantly, in a xeno-graft ovarian cancer mouse model blocking of IL-6 resulted in less macrophages infiltrating the tumor [35], indicating that tumor-rejection associated immune-effects can be achieved by blocking IL-6 signalling. In rheumatoid arthritis, a monoclonal antibody to IL-6Receptor is used with great clinical benefit by stopping inflammation upon treatment [36]. Treatment with blocking antibodies to IL-6 can break the autocrine supportive loop of IL-6 producing tumor cells as well as IL-6-mediated suppression of DC differentiation or induction of M2 macrophages. Therefore, addition of IL-6 blocking antibodies to standard treatment or therapeutic vaccination should be considered.

Monoclonal antibody can activate any type of local macrophage

In chapter 7 we describe that blocking growth receptors on tumors as anti-tumor therapy may have unwanted immunological side effects. We showed that tumor promoting M2 macrophages are activated by tumor bound cetuximab although this monoclonal antibody that targets EGFR on tumor cells was optimized to bind FcγReceptors on NK cells. For this, we used an in vitro culture system to differentiate monocytes into M2 macrophages by M-CSF. These macrophages responded to tumor bound antibodies with the production of the immune suppressive IL-10 and IL-8. Although M-CSF is widely used to differentiate human M2 macrophages in vitro, these might not resemble the tumor-supernatant induce M2 as described in chapter 5b. To study the differences, we analyzed M2 macrophage cultures derived by culture protocols with distinct cytokines (chapter 5b). Although these macrophages expressed similar phenotype, the response to stimuli was not equal. For example the cytokine production upon CD40-ligation was poor in the M-CSF cultures whereas the IL-6+PGE2 induced cultures (resembling CxCa cell line supernatant) produced high amounts of IL-10 upon this activation. Taken these together, the experiments described in chapter 7 might not resemble the real tumor associated macrophages as induced by CxCa cell lines (chapter 5b). However (unpublished) control experiments showed that TSN-M2 macrophages -as described in chapter 5- are also activated by tumor-bound cetuximab and not by control antibody, indicating that M-CSF derived M2 macrophages were appropriate cells for these proof-of-principle tests. Of note, if M1 macrophages, that also express FcReceptors, are present in the tumor they will get activated as well and engulfe or kill the cells upon binding cetuximab opsonized tumor cells. Therefore, the net outcome of a treatment may again be determined by the immune-cell repertoire that is present in each individual tumor. Based on the results with cetuximab, targeting the cell surface expressed IL6receptor by tocilizumab may also result in macrophage activation. Although it has been shown that this MAb did not activate DCs, no specific experiments with the MAb bound to IL-6R-expressing cells have been reported [37].

Repolarization of macrophages – a way to treat cancer?

The treatment of cancer may take advantage of therapies that interfere with M2 macrophages, if combined with standard or immunotherapeutic regimens. Therapeutic modalities may attack at several levels; the attraction, the differentiation or the activation of macrophages.

One therapeutic option is to interfere at the level of macrophage attraction and differentiation by abrogation of the PGE2 and the IL-6 and STAT-3 activation loop or other tumor produced cytokines [38,39]. This indirectly affects tumor growth and limits the induction of tolerogenic macrophages as described earlier. It might prove difficult to reach high enough antibody titers to block cytokine levels locally in the tumor, as they often are poorly vascularized at area's where macrophages tend to accumulate [40]. Care should be taken since as membrane bound antibodies may activate macrophages via cross linking of the FcγReceptors and thereby activate inflammatory cascades (chapter 7).

IFNγ in combination with the CD40-CD40L APC-activation signal effectively reprograms tumor-induced M2-like macrophages into activated IL-12 producing M1 cells (chapter 4). For this to occur in the tumor-microenvironment, it is essential that CD4+ Th1 cells are locally present. The capacity of tumor-specific Th1 cells to directly alter the tumor microenvironment has also been recognized in studies on tissue-infiltrating CD8+ T cells in mouse models. Th1 cells were essential for successful recruitment, local expansion and full effector function of large

numbers of CTL by modulation of the local environment [41,42], and this may have included the repolarization of macrophages. In order to obtain sufficient numbers of tumor-specific CD4+ Th1 cells one may make use of adoptive T-cell transfer protocols or apply strong vaccines [43-46]. These therapies should not only induce cytotoxic T cells but also type 1 polarized helper cells to help shifting the local tumor micromilieu from tumor-promoting and immune suppressive into anti-tumor inflammation.

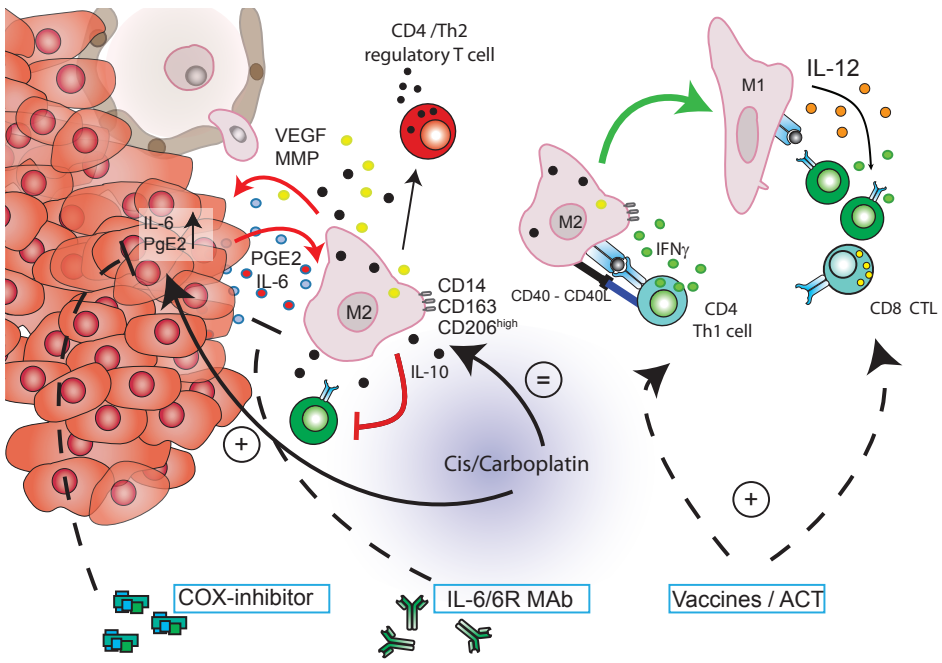


Figure 1. Modulation of tumor associated macrophages. Monocytes leaving the bloodstream and entering the tissue differentiate in APC but this is hampered by tumor produced soluble factors. In the proximity of a PGE2 and IL-6 producing tumor their differentiation is skewed towards M2 macrophages. These macrophages produce VEGF and MMP which support tumor growth but also high IL-10 and low IL-12 suppressing T-cell function and precluding type 1 T-cell induction. However if this M2 macrophage interacts with an IFNγ producing CD40-L expressing CD4+ T cell it can revert into a IL-12 producing macrophage capable of inducing type 1 T cells. Cisplatin or carboplatin treatment of a tumor that produce PGE2 and IL-6 (likely to be a COX-2 positive tumor) can induce higher levels of these factors and results in more M2 skewing. M2 induction by the tumor can be avoided by treating the patient with drugs that block COX-2 enzymes thereby preventing PGE2 production or blocking of the tumor produced IL-6 with MAb to IL-6 or IL-6Receptor. Induction of tumor specific IFNγ producing T cells by therapeutic vaccination or adoptive T cell transfer (ACT) can shift the balance in the tumor towards anti-tumor inflammation by T-cell mediated activation of the local APC. Therapeutic options are depicted in the blue boxes.

Immunogenic HPV16+ oropharyngeal tumors

Intriguingly, the incidence of HPV related cancers of the head and neck region are rapidly increasing. Another unresolved issue is how this virus can persist and cause cancer in an immunologic organ like the palatine tonsil of the Waldeyers ring. The palatine tonsils consist

of lymphoid follicles that are covered with squamous epithelium that form crypts [47,48]. The reticulated crypt epithelium, also called lymphoepithelium plays a key role in the initiation of immune responses in the palatine tonsils. Luminal antigens are taken up in the crypts and transported to sub epithelial spaces where they come in contact with lymphoid cells. Not only HPV virus can settle in these crypts and infect basal cells of the epithelium but also a high load of fungi, bacteria and other viruses pass by this tissue at daily basis [47].

In chapter 4 we describe that in 6 out of 8 HPV16+ oropharyngeal cancer patients HPV specific T cells were present in tumors or LN. Although the cohort is very small and the results need to be validated in a bigger cohort, these data suggest that local HPV-specific T cells are more often present or less suppressed compared to HPV16+ cervical cancer [49]. In two patients HPV-specific proliferating and IFNγ producing TIL could be readily detected in a direct ex-vivo analysis of a single cell suspension from a fresh biopsy (without the need for a homeostatic proliferation period). This indicates that a strongly activated tumor-antigen specific T-cell population is present within these tumors (data not shown). In the tonsil, local APC can take up tumor antigens but in parallel may get activated through stimulation of their pattern recognition receptors by normal bacterial and viral flora entering the body. This concomitant activation results in stronger signal 2 and 3 delivery of APC to T cells. We hypothesize that infection and inflammation caused by other pathogens may appropriately activate APC in this lymphoid structure to prime HPV16-specific T cells resulting in the strong T cell response found in these (few) patients. Furthermore I speculate that the type of pathogen co-infecting the tonsil at the moment of T-cell priming might be of importance for the T-cell response that is subsequently induced. This because we showed in chapter 5 and 6 that different TLR-agonists show unique activation patterns in APC resulting in different T cell stimulating abilities.

Lesions in the oral cavity known as leucoplakia progresses in 20% to cancer and studies on the presence of HPV in these lesions showed conflicting results. Although HPV was more often detected in lesions compared to healthy control tissue is was not exclusively detected in lesions. Furthermore the prevalence of HPV in lesions varied from 17%- 68% between studies (reviewed by [50,51]. Whether these lesions are caused by the virus or that the virus just better persists in ulcerated tissue and therefore is more often detected, is not known. Also pre-cancerous lesions of the Tonsil are not studied yet and this is the localization where HPV+ HNSCC develops. Thus whether oropharyngeal neoplasias develop in the same way as cervical neoplasias (progression of CINI, II, III to malignant carcinoma) remains an open question. Studies on the presence of pre-malignant lesions in combination with determination of the present flora in the tonsil and a detailed analysis of local T-cell infiltrate and specificity might elicit some answers to the previous mentioned discrepancies between HPV-induced CxCa and HPV induced oropharyngeal carcinoma.

We described that circulating HPV specific T cells in HPV+ CxCa (chapter 2) as well as HPV16+ oropharyngeal cancer (chapter 4) proliferate but often lack detectable cytokine production when stimulated *in vitro*. In contrast to the circulating T cells, HPV-specific tumor infiltrating T cells of most HPV+ oropharyngeal tumors produce type 1 cytokines upon peptide recognition (chapter 4). Also here, the design of the study and the APC used for the test might have influenced the results. However in both test monocytes have presented the peptides and in the patients with HPV-negative tumors 4 out of 7 responses were accompanied by IFNγ showing that monocytes can activate the T cells properly in these experiments.

Oropharyngeal tumor-derived TIL were able to respond to HPV oncoproteins with the production of type 1 cytokines when directly tested after isolation from a digested biopsy (n=2, data not shown) or short culture period (chapter 4). On the other hand we also showed that in addition to HPV16-specific IFNγ and TNFα producing Th1 cells also HPV16 specific IL-5 producing CD4+ Th2 cells, Treg and FoxP3 expressing CD8+ T cells could be isolated out of an oropharyngeal tumor. To understand why these patients, despite the presence of functional tumor-antigen specific T cells, can not fully control their growing HPV16+ tumors also the other tumor-infiltrating immune cells must be analyzed. For instance, one explanation could be that –similar to what is found in breast cancer [16] - CD4+ T cells promote pro-tumor inflammation by M2 macrophages in oropharyngeal tumors. Therefore, in depth analysis of the ex-vivo TIL population as described in chapter 3 for CxCa TIL should be performed including the analysis of Th2 and Th17 populations in this patients with oropharyngeal cancer. Furthermore, single cell suspensions of these tumors could be analyzed not only for lymphocytes but also for the presence and phenotype of the myeloid cells. Finally, analysis of T cells isolated from regressing lesions after therapy might shed light on the role of these cells in the better disease free survival that HPV+ HNSCC patients display.

For the treatment of HPV16+ high grade vulvar intra-epithelial neoplasia (VIN), promising results were obtained with a synthetic long peptide (SLP) vaccine that induced strong and broad Th1 type T cell immunity to E6 and E7 [52,53]. Although patients with HPV16+ HNSCC carcinoma respond surprisingly well to radiotherapy this heavy treatment is not without side effects. It is suggested that the immunogenic properties of these virally induced HPV16+ oropharyngeal tumors contribute to the good response to treatment ([54] further supported by the data of chapter 4). Therapeutic vaccination aiming to reinforce the T-cell response to the HPV16 oncoproteins, thus may form a promising treatment modality. Schiering *et al.* recently published that tumor specific CD4+ T cells that spontaneously had developed in mice, suppressed the induction of new T cells by vaccination [55]. In view of our data showing that HPV specific T cell responses are present in the majority of the circulating T-cell and TIL- populations of patients with HPV16+ oropharyngeal tumors (chapter 4), vaccination may predominantly lead to boosting of already existing HPV16-specific T-cell responses and increase the number of HPV16-specific Th2 cells and regulatory T cells [56]. Therefore a prospective study on the presence of HPV-oncoprotein specific immunity, the correlation with survival and what happens with these cells upon treatment in patients with HNSCC should be the next step.

REFERENCES

1. Schwartz RH: **Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy.** *Cell* 1992, **71:** 1065-1068.
2. Kabelitz D: **Expression and function of Toll-like receptors in T lymphocytes.** *Curr Opin Immunol* 2007, **19:** 39-45.
3. Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I *et al.*: **Activation of human dendritic cells through CD40 cross-linking.** *J Exp Med* 1994, **180:** 1263-1272.
4. Stout RD: **Macrophage activation by T cells: cognate and non-cognate signals.** *Curr Opin Immunol* 1993, **5:** 398-403.
5. Karim R, Meyers C, Backendorf C, Ludigs K, Offringa R, van Ommen GJ *et al.*: **Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes.** *PLoS One* 2011, **6:** e17848.
6. Youn JI, Gabrilovich DI: **The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity.** *Eur J Immunol* 2010, **40:** 2969-2975.
7. Ostrand-Rosenberg S: **Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity.** *Cancer Immunol Immunother* 2010, **59:** 1593-1600.
8. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P: **Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells towards stable myeloid-derived suppressor cells.** *Blood* 2011.
9. Kuang DM, Peng C, Zhao Q, Wu Y, Chen MS, Zheng L: **Activated monocytes in peritumoral stroma of hepatocellular carcinoma promote expansion of memory T helper 17 cells.** *Hepatology* 2010, **51:** 154-164.
10. Movahedi K, Laoui D, Gysemans C, Baeten M, Stange G, Van den Bossche J *et al.*: **Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes.** *Cancer Res* 2010, **70:** 5728-5739.
11. Pettersen JS, Fuentes-Duculan J, Suarez-Farinas M, Pierson KC, Pitts-Kiefer A, Fan L *et al.*: **Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated.** *J Invest Dermatol* 2011, **131:** 1322-1330.
12. Ferrandina G, Lauriola L, Distefano MG, Zannoni GF, Gessi M, Legge F *et al.*: **Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients.** *J Clin Oncol* 2002, **20:** 973-981.
13. Hida T, Kozaki K, Ito H, Miyaishi O, Tatematsu Y, Suzuki T *et al.*: **Significant growth inhibition of human lung cancer cells both in vitro and in vivo by the combined use of a selective cyclooxygenase 2 inhibitor, JTE-522, and conventional anticancer agents.** *Clin Cancer Res* 2002, **8:** 2443-2447.
14. Noordhuis MG, Eijsink JJ, Roossink F, de GP, Pras E, Schuuring E *et al.*: **Prognostic cell biological markers in cervical cancer patients primarily treated with (chemo)radiation: a systematic review.** *Int J Radiat Oncol Biol Phys* 2011, **79:** 325-334.
15. Souza RF, Shewmake K, Beer DG, Cryer B, Spechler SJ: **Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells.** *Cancer Res* 2000, **60:** 5767-5772.
16. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF *et al.*: **Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy.** *Cancer Discovery* 2011.
17. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M *et al.*: **Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer.** *J Clin Oncol* 2010, **28:** 105-113.
18. Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G: **Prognostic and predictive impact of intra- and peritumoral immune infiltrates.** *Cancer Res* 2011, **71:** 5601-5605.
19. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG *et al.*: **Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients?** *Clin Cancer Res* 2008, **14:** 2028-2035.
20. Piersma SJ, Jordanova ES, van Poelgeest MtIE, Kwappenberg KMC, van der Hulst JM, Drijfhout JW *et al.*: **High Number of Intraepithelial CD8+ Tumor-Infiltrating Lymphocytes Is Associated with the Absence of Lymph Node Metastases in Patients with Large Early-Stage Cervical Cancer.** *Cancer Res* 2007, **67:** 354-361.
21. Zitvogel L, Kepp O, Kroemer G: **Immune parameters affecting the efficacy of chemotherapeutic regimens.** *Nat Rev Clin Oncol* 2011, **8:** 151-160.
22. Kryczek I, Wei S, Zhu G, Myers L, Mottram P, Cheng P *et al.*: **Relationship between B7-H4, Regulatory T Cells, and Patient Outcome in**

Human Ovarian Carcinoma. *Cancer Res* 2007, **67:** 8900-8905.

23. van Dongen M, Savage ND, Jordanova ES, Briaire-de Bruijn IH, Walburg KV, Ottenhoff TH *et al.*: **Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors.** *Int J Cancer* 2010, **127:** 899-909.
24. Stolina M, Sharma S, Lin Y, Dohadwala M, Gardner B, Luo J *et al.*: **Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis.** *J Immunol* 2000, **164:** 361-370.
25. Yin Z, Zhang Y, Li Y, Lv T, Liu J, Wang X: **Prognostic significance of STAT3 expression and its correlation with chemoresistance of non-small cell lung cancer cells.** *Acta Histochem* 2011.
26. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A *et al.*: **Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy.** *Nat Med* 2007, **13:** 1050-1059.
27. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F *et al.*: **Immunogenic death of colon cancer cells treated with oxaliplatin.** *Oncogene* 2010, **29:** 482-491.
28. Demaria S, Bhardwaj N, McBride WH, Formenti SC: **Combining radiotherapy and immunotherapy: a revived partnership.** *Int J Radiat Oncol Biol Phys* 2005, **63:** 655-666.
29. Nesslinger NJ, Sahota RA, Stone B, Johnson K, Chima N, King C *et al.*: **Standard treatments induce antigen-specific immune responses in prostate cancer.** *Clin Cancer Res* 2007, **13:** 1493-1502.
30. Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK *et al.*: **Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy.** *J Exp Med* 2006, **203:** 1259-1271.
31. Legge F, Paglia A, D'Asta M, Fuoco G, Scambia G, Ferrandina G: **Phase II study of the combination carboplatin plus celecoxib in heavily pre-treated recurrent ovarian cancer patients.** *BMC Cancer* 2011, **11:** 214.
32. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K *et al.*: **Celecoxib for the prevention of sporadic colorectal adenomas.** *N Engl J Med* 2006, **355:** 873-884.
33. Groen HJ, Sietsma H, Vincent A, Hochstenbag MM, van Putten JW, van den Berg A *et al.*: **Randomized, Placebo-Controlled Phase III Study of Docetaxel Plus Carboplatin With Celecoxib and Cyclooxygenase-2 Expression As a Biomarker for Patients With Advanced Non-Small-Cell Lung Cancer: The NVALT-4 Study.** *J Clin Oncol* 2011, **29:** 4320-4326.
34. Koch A, Bergman B, Holmberg E, Sederholm C, Ek L, Kosieradzki J *et al.*: **Effect of celecoxib on survival in patients with advanced non-small cell lung cancer: a double blind randomised clinical phase III trial (CYCLUS study) by the Swedish Lung Cancer Study Group.** *Eur J Cancer* 2011, **47:** 1546-1555.
35. Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Leinster DA *et al.*: **Interleukin-6 as a Therapeutic Target in Human Ovarian Cancer.** *Clin Cancer Res* 2011, **17:** 6083-6096.
36. Choy E: **Inhibiting interleukin-6 in rheumatoid arthritis.** *Curr Rheumatol Rep* 2008, **10:** 413-417.
37. Betts BC, St Angelo ET, Kennedy M, Young JW: **Anti-IL6-receptor-alpha (tocilizumab) does not inhibit human monocyte-derived dendritic cell maturation or alloreactive T-cell responses.** *Blood* 2011, **118:** 5340-5343.
38. Dalwadi H, Krysan K, Heuze-Vourc'h N, Dohadwala M, Elashoff D, Sharma S *et al.*: **Cyclooxygenase-2-dependent activation of signal transducer and activator of transcription 3 by interleukin-6 in non-small cell lung cancer.** *Clin Cancer Res* 2005, **11:** 7674-7682.
39. Yu H, Pardoll D, Jove R: **STATs in cancer inflammation and immunity: a leading role for STAT3.** *Nat Rev Cancer* 2009, **9:** 798-809.
40. Murdoch C, Giannoudis A, Lewis CE: **Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues.** *Blood* 2004, **104:** 2224-2234.
41. Bos R, Sherman LA: **CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes.** *Cancer Res* 2010, **70:** 8368-8377.
42. Nakanishi Y, Lu B, Gerard C, Iwasaki A: **CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help.** *Nature* 2009, **462:** 510-513.
43. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R *et al.*: **Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1.** *N Engl J Med* 2008, **358:** 2698-2703.
44. Melief CJ, van der Burg SH: **Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines.** *Nat Rev Cancer* 2008, **8:** 351-360.
45. Verdegaal EM, Visser M, Ramwadhdoebe TH, van der Minne CE, van Steijn JA, Kapiteijn E *et al.*: **Successful treatment of metastatic melanoma by adoptive transfer of blood-derived polyclonal tumor-specific CD4+ and CD8+ T cells in combination with low-**

dose interferon-alpha. *Cancer Immunol Immunother* 2011, **60:** 953-963.

46. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F *et al.*: **Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses.** *Proc Natl Acad Sci U S A* 2010, **107:** 11895-11899.
47. Nave H, Gebert A, Pabst R: **Morphology and immunology of the human palatine tonsil.** *Anat Embryol (Berl)* 2001, **204:** 367-373.
48. Perry ME: **The specialised structure of crypt epithelium in the human palatine tonsil and its functional significance.** *J Anat* 1994, **185 (Pt 1):** 111-127.
49. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ *et al.*: **Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element.** *Int J Cancer* 2008, **122:** 486-494.
50. Syrjanen S, Lodi G, von B, I, Aliko A, Arduino P, Campisi G *et al.*: **Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review.** *Oral Dis* 2011, **17 Suppl 1:** 58-72.
51. Campisi G, Panzarella V, Giuliani M, Lajolo C, Di FO, Falaschini S *et al.*: **Human papillomavirus: its identity and controversial role in oral oncogenesis, premalignant and malignant lesions (review).** *Int J Oncol* 2007, **30:** 813-823.
52. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP *et al.*: **Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia.** *N Engl J Med* 2009, **361:** 1838-1847.
53. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM *et al.*: **Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine.** *Clin Cancer Res* 2008, **14:** 178-187.
54. Chung CH, Gillison ML: **Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications.** *Clin Cancer Res* 2009, **15:** 6758-6762.
55. Schiering C, Guarnerio J, Basso V, Muzio L, Mondino A: **Antigen-experienced CD4(+) T cells limit naive T-cell priming in response to therapeutic vaccination in vivo.** *Cancer Res* 2010, **70:** 6161-6170.
56. Welters MJ, de JA, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S *et al.*: **Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter.** *Cancer Res* 2003, **63:** 636-641.

ADDENDUM

Nederlandse Samenvatting
List of publications
Curriculum Vitae
Dankwoord

NEDERLANDSE SAMENVATTING

Ons afweersysteem bestaat uit twee samenwerkende delen: een aspecifiek deel dat snel kan reageren op signalen uit de omgeving en een specifiek deel dat unieke delen van eiwitten afkomstig van ziekteverwekkers (virus, bacterie etc.) of van afwijkende cellen (kanker) herkent en hier tegen geheugen op kan bouwen. Macrofagen behoren tot het eerstgenoemde aspecifieke deel. Dendritische Cellen (DC) vormen een brug tussen beide delen van het afweersysteem. Beiden zijn antigeen presenterende cellen (APC) wat inhoudt dat zij eiwitten van de ziekteverwekkers of kankercellen kunnen opeten en dan kleine stukjes hiervan (peptiden) in de context van zogenoemde MHC moleculen presenteren aan het specifieke deel van het afweersysteem, de T cellen. Macrofagen en DC hebben allerlei receptoren op hun celoppervlak die ervoor kunnen zorgen dat ze geactiveerd raken. Toll like receptoren zijn een voorbeeld hiervan; deze receptoren herkennen delen van bacterien en virussen. Het resultaat van herkenning via deze receptoren is dat de APC extra co-stimulatoire moleculen op het celoppervlak brengen en dat er bepaalde stoffen worden uitgescheiden zoals cytokines. T cellen die het MHC-peptide complex herkennen zullen door de aanwezige co-stimulatoire moleculen en cytokines geïnstrueerd worden om te gaan delen en een bepaalde functie uit te oefenen. In het geval van een ziekteverwekker zullen zij ten strijde trekken om de ziekteverwekker te vernietigen. Nadat de taak van de T cellen erop zit, blijft een kleine groep over, deze vormen een poel van reservekrachten die snel kunnen reageren mocht de ziekteverwekker opnieuw in het lichaam aanwezig zijn. De cellen van het afweercellen kunnen uitrijpen tot cellen met verschillende functies naar gelang het type activeringsignaal. Macrofagen rijpen uit in grofweg 2 types; type 1 pro-inflammatoire macrofagen stimuleren een ontsteking (inflammatie) en het specifieke deel van het afweersysteem. Type 2 macrofagen zijn betrokken bij wondheling, bloedvatvorming en de onderdrukking van T cel reacties. Ook T cellen ontwikkelen zich in verschillende typen. Type 1 cytotoxische (CD8+) en helper (CD4+) T cellen kenmerken zich onder andere door productie van IFNγ en hun vermogen om zieke of geïnfecteerde cellen aan te vallen en dood te maken. Wanneer CD4+ cellen (T helpers) een MHC-peptide complex op een APC herkennen zullen zij via CD40-CD40-L interacties een APC sterk activeren en hiermee helpen zij een specifieke immuunrespons op gang te houden. Daarnaast zijn er ook regulatoire T cellen die de ontwikkeling en functie van andere afweercellen kunnen onderdrukken.

Tumoren proberen een afweerreactie van T cellen te vermijden door middel van verschillende strategieën. Eén manier is om verschillende, voor de afweerreactie van belang zijnde, molekulen op het oppervlakte van de tumorcel aan te passen. Een tweede manier is factoren uitscheiden die T cellen onderdrukken. Ook gebruiken ze type 2 macrofagen om nieuwe bloedvaten te genereren en om T cel reacties te onderdrukken.

In verschillende diermodellen en in studies van menselijk materiaal is aangetoond dat een hoog aantal CD8 cellen en een laag aantal regulatoire T cellen in de tumor voorspellend is voor een betere overleving in patiënten met kanker. De aanwezigheid van een hoog aantal type 2 macrofagen in de tumor voorspelt een slechtere overleving. Dit duidt erop dat het afweersysteem een belangrijke rol speelt in controle van de tumor groei.

Het Humaan Papilloma Virus (HPV) is een DNA virus dat basale epitheel cellen infecteert. De tumorveroorzakende typen HPV (voornamelijk HPV 16 en 18) veroorzaken afwijkingen aan o.a. de baarmoederhals die uiteindelijk kunnen leiden tot baarmoederhalskanker. Nadat het virus een huidcel heeft geïnfecteerd komen virale eiwitten tot expressie in de cel.

Enkele virale eiwitten, genaamd E6 en E7, induceren ongecontroleerde celdeling en maligne transformatie en zijn verantwoordelijk voor het ontstaan en in stand houden van kanker. In een getransformeerde cel zijn dus altijd de eiwitten E6 en E7 aanwezig en omdat dit virale eiwitten zijn kan er een specifieke (T cel gecontroleerde) afweerreactie ontstaan. In dit proefschrift onderzoeken we T cellen die specifiek deze eiwitten herkennen.

Ruim de helft van de mensen die seksueel actief zijn wordt geïnfecteerd met een tumorverooorzakend type van HPV. Bijna alle geïnfecteerde vrouwen maken een afweerreactie en ruimen het virus weer op. Bij enkele mensen is het virus toch in staat om te blijven zitten en in de loop van de jaren kan dit tot kanker leiden. Waarom niet alle vrouwen het virus opruimen en enkelen toch een tumor ontwikkelen is nog steeds niet opgehelderd. Wel is er gevonden dat in voorstadia van een ontwikkelende tumor de T cellen een belangrijke rol spelen in het voorkomen van ernstige ziekte. In gezonde vrouwen worden sterke tegen HPV reagerende T cel reacties gevonden maar in vrouwen met een persisterende HPV infectie werden geen of zwakkere T cel reacties gemeten.

Nieuw is dat HPV (voornamelijk HPV16) steeds vaker wordt gevonden in tumoren van het hoofd-hals gebied, met name in de oropharynx (voornamelijk de amandelen). Hoewel er nog niet veel bekend is over de rol van het virus in deze tumoren, of de afweerreactie hiertegen, is het wel duidelijk dat patiënten met een tumor die HPV positief is veel beter reageren op (radio) therapie dan patiënten die een tumor in dit gebied hebben die niet veroorzaakt wordt door HPV.

De standaard behandeling voor tumoren is nog altijd chirurgie en het verwijderen van de tumor. Terugkerende baarmoederhalskanker wordt behandeld met platina houdende chemotherapie, zoals cisplatinum. Helaas reageert maar een klein deel van de patiënten goed op deze therapie (30%). Behandeling van tumoren met chemotherapie is gericht op destructie van de delende tumorcellen. Het is nog niet goed onderzocht wat de bijeffecten zijn van verschillende chemotherapeutica op de locale afweercellen. Een relatief nieuwe vorm van therapie voor kanker is de immunotherapie, het behandelen van kanker door (delen van) het afweersysteem te gebruiken. De eerste vorm van immunotherapie die in de kliniek als behandeling wodt gebruikt is monoklonale antilichamen. Dit zijn antistoffen die bijvoorbeeld een specifieke factor of receptor op tumorcellen binden en daarmee de groeifactoren van tumoren weg nemen. Een nieuwere vorm van immunotherapie is het gebruik van zogenoemde therapeutische vaccins. Deze vaccins hebben als doel de tegen de tumor gerichte CD4 en CD8 T cel reactie zodanig te versterken dat de tumorgroei onder controle komt.

In dit proefschrift zijn verschillende aspecten van de afweerreactie tegen (door HPV veroorzaakte) tumoren onderzocht. Door meer kennis van de locale afweerrespons te krijgen kunnen we immunotherapeutische strategieën gebaseerd op therapeutische vaccinatie beter ontwikkelen en toepassen.

In **hoofdstuk 2** hebben we de HPV-specifieke T cel reactie in het bloed van een grote groep baarmoederhalskanker patiënten prospectief geanalyseerd. Maar 31% van deze patiënten had circulerende HPV-specifieke T cellen. Een positieve T-cel reactie werd vaker gezien in de groep patiënten met diep infiltrerende tumoren. Hoewel diepe infiltratie van de tumor in het gezonde weefsel een slecht prognostisch teken is, bleek dat die patiënten die daarbij ook een circulerende HPV-specifieke T cel reactie hadden een betere 3-jaars overleving hadden dan de patiënten waarin zo'n reactie niet aangetoond kon worden.

Eerder is gepubliceerd dat in 30% van de HPV veroorzaakte baarmoederhals tumoren T cellen tegen dit virus in de tumor te vinden zijn. In **hoofdstuk 3** beschrijven wij een uitgebreide analyse van de breedte en het type respons van T cel populaties in de tumor of uit de tumor-drainerende lymfeklier. Als patiënten een HPV-specifieke reactie hadden dan was deze vaak verassend breed (meerdere eiwitdelen herkend, meerdere clonale T cel populaties). Een deel van de HPV-specifieke T cellen maakte niet de gewenste cytokines interferon-γ en IL-2. De toevoeging van APC-activerende signaalstoffen die aan de op APC aanwezige toll-like receptoren (TLR) binden resulteerde in hogere cytokine productie door de HPV-specifieke T cellen.

Niet alleen in de ano-genitale regio maar ook in de keel is HPV terug te vinden. **Hoofdstuk 4** beschrijft een eerste studie naar de HPV-specifieke T cellen in patiënten met kanker in de hoofd-hals regio. HPV16 DNA werd alleen gevonden in tumoren van de amandelen/oropharynx maar niet op andere locaties in dit gebied. We vonden in het bloed van verschillende patiënten HPV-specifieke T cellen terug, wat duidt op een eerdere infectie of een infectie die nog aanwezig is. In HPV16-positieve tumoren vonden we verrassend vaak HPV-specifieke T cellen. Dit zou een verklaring kunnen zijn waarom deze patiënten een grotere kans op overleving hebben op therapie; er is immers al een leger van afweercellen aanwezig die specifiek tumor antigeen herkent. Verder onderzoek naar de andere aanwezige afweercellen in deze tumoren moet uitwijzen waarom de tumor toch groeit ondanks sterke locale T cel reactie.

Het tekort aan HPV specifieke T cellen in patiënten met baarmoederhalskanker zou kunnen komen doordat ze een goede stimulatie door DC missen. In **hoofdstuk 5A** hebben we onderzocht of tumoren de uitrijping van monocyten naar DC kunnen beinvloeden. Meerdere in het laboratorium gekweekte tumorcellijnen bleken factoren te produceren die de ontwikkeling van DC verstoorde. Enkele tumorcellijnen dreven de uitrijping van monocyten in de richting van type 2 macrofagen. De type 2 macrofagen produceerden na stimulatie veel IL-10 en weinig IL-12 en waren daardoor slecht in staat een type 1 T cel reactie te induceren. De verantwoordelijke factoren voor de uitrijping van macrofagen waren de door tumorcellen uitgescheiden stoffen prostaglandine E2 (PGE2) en IL-6. Behandeling van de tumorcellen met COX-remmers (COX-enzymen vormen o.a. PGE2) resulteerde in restauratie van de ontwikkeling van DC. Interactie met interferon-γ producerende type 1 T cellen, via CD40- CD40-L interacties, is een sterk activerende stimulus voor dendritische cellen en macrofagen. In dit hoofdstuk laten we ook zien dat stimulatie van type 2 macrofagen via CD40 en de IFNγ receptor (via CD40-L positieve cellen en de toevoeging van IFNγ zoals zou worden geleverd door type 1 T-helper cellen) sterk genoeg is om deze te veranderen in geactiveerde type 1 macrofagen. Dit suggereert dat het remmen van COX-enzymen en IL-6 en daarnaast het verhogen van de aanwezigheid van type 1 T cellen een gunstig effect heeft op de lokale ontstekingsreactie in de tumor.

Als baarmoederhalskanker weer terug komt ondanks therapie worden patiënten vaak behandeld met platinum houdende chemotherapie. Het effect van chemotherapie op de lokale afweercellen is nog niet goed onderzocht. In **hoofdstuk 5B** onderzoeken we de immuun-modulerende effecten van cisplatinum op tumor cellen en de uitrijping van monocyten naar DC. Verrassend genoeg bleek dat de behandeling van tumorcellen met een fysiologische dosis cisplatinum de productie van PGE2 en IL-6 door tumorcellen verhoogt. Dit resulteerde in een toename van het percentage monocyten dat tot type 2 macrofagen was uitgerijpt. Deze macrofagen dragen niet bij aan de afweerreactie tegen tumoren maar produceren wel groeifactoren voor de tumor. Dit betekent dat een behandeling met cisplatinum van een

tumor waarvan niet alle cellen doodgaan maar wel M2 macrofagen kunnen induceren mogelijk averechts kan werken voor de patient. Dit mechanisme kan een alternatieve verklaring zijn voor een eerder beschreven observatie dat patiënten met een tumor die hoog COX-2 tot expressie brengt een slechtere respons op chemotherapie hebben. In dit hoofdstuk onderzoeken we ook of andere CD4 cellen die andere cytokinen dan interferon-γ produceren in staat zijn om type 2 macrofagen te veranderen naar een ander type macrofagen net zoals type 1 CD4+ T-helper cellen dat kunnen.

Er wordt momenteel veel geïnvesteerd in onderzoek naar therapeutische vaccinatie voor de behandeling van chronische infecties en kanker met het doel om beschermende T cel immuniteit te genereren. Het gebruikte adjuvant en de route van toedienen zijn belangrijk voor het type van de geïnduceerde T-cel respons. Intra-dermale injectie lijkt een goede methode om de afweer te stimuleren tegen ziekten die zich ontwikkelen in de huid, zoals melanoom en HPV geïnduceerde tumoren, omdat de geïnduceerde T cellen dan geïnstrueerd worden om naar de huid te migreren. In **hoofdstuk 6** bekijken we het effect van verschillende TLR stimulerende stoffen (agonisten) op de activering van uit de huid migrerende APC in een menselijk *ex-vivo* huid model. Het blijkt dat maar enkele TLR-agonisten activatie van in de huid aanwezige APC induceren in tegenstelling tot de *in-vitro* resultaten met zulke cellen zoals beschreven in de literatuur en dit kan implicaties hebben voor de keuze van adjuvant in vaccinatie strategien.

De behandeling van patiënten met uitgebreid colorectaal kanker bestaat momenteel uit chemotherapie samen met een monoklonaal antilichaam dat een tumorgroeifactor wegvangt (bevacizumab). Toevoeging aan dit regime van een tweede antilichaam dat de groeireceptor EGFR blokkeert op de tumor cellen (cetuximab), en daardoor verder de groei van deze cellen remt, resulteerde niet in de verwachte extra overleving (CAIROII studie). Verdere analyse liet zien dat patiënten met een bepaald polymorfisme in het gen voor FCGR3A (FcgRIIIa is een receptor die de staart van antilichamen bindt) het significant slechter deden als cetuximab was toegevoegd aan het regime. Dit polymorfisme geeft een hogere bindingsaffiniteit voor antilichamen aan deze receptor. Omdat macrofagen vaak aanwezig zijn in colorectale tumoren testen wij in **hoofdstuk 7** de hypothese dat aan tumorcel gebonden cetuximab de tolerogene, tumor geassocieerde type 2 macrofagen, die sterk FcgRIIIa positief zijn, activeert. Activering van deze tumorbevorderende macrofagen zou een verklaring kunnen zijn voor de slechtere overleving in de groep met cetuximab in de eerder beschreven studies. Onze *in-vitro* proeven laten zien dat inderdaad de type 2 macrofagen geactiveerd kunnen worden door dit antilichaam wanneer het gebonden is aan het oppervlak van tumor cellen en dit lijkt ook meer efficiënt te gebeuren in donoren met een receptor die een hogere bindingsaffiniteit voor antistoffen hebben. Dit heeft implicaties voor de toekomstige ontwikkeling van antilichamen die vaak extra geoptimaliseerd worden om aan de FcgRIIIa te binden. Dit omdat men de activatie van natural killercellen nastreeft, deze cellen werden echter nauwelijks in de colorectale tumoren aangetroffen.

LIST OF PUBLICATIONS

Intradermal delivery of TLR-ligands in a human explant skin model: preferential activation of migratory Dendritic Cells by Poly I:C and Peptidoglycans. D Oosterhoff*, M Heusinkveld*, S Lougheed, M Lindstedt, Y Van Kooyk, SH van der Burg, T de Gruijl
Submitted

Identification and manipulation of tumor associated macrophages in human cancers.
M Heusinkveld, SH van der Burg
Journal of translational medicine 2011 Dec 9:216

Systemic and local HPV-specific T-cell immunity in patients with head and neck cancer. M Heusinkveld, R Goedemans, RJP Briet, AJ Gelderblom, JWR Nortier, A Gorter, VTHBM Smit, APM Langeveld, JC Jansen, SH van der Burg
International Journal of Cancer 2011

Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab. J Pander*, M Heusinkveld*, T van der Straaten, E Jordanova, R Baak-Pablo, H Gelderblom, H Morreau, SH van der Burg, HJ Guchelaar, T van Hall
Clinical Cancer Research 17(17)5668

M2 macrophages induced by PgE2 and IL-6 by cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. M Heusinkveld, PJ de Vos-van Steenwijk, R Goedemans, THR Ramwhadoebe, A Gorter, MJ Welters, T van Hall, SH van der Burg
Journal of Immunology 2011 187:1157

An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. PJ de Vos-van Steenwijk, M Heusinkveld, THR Ramwhadoebe, M Lowik, JM van der Hulst, R Goedemans, SJ Piersma, GG Kenter, SH van der Burg
Cancer Research 2010 70(7):2707

The detection of circulating Human Papillomavirus (HPV)-specific Tcells is associated with improved survival of patients with deeply infiltrating tumors. M Heusinkveld*, MJ Welters*, MI van Poelgeest, JM van der Hulst, CJ Melief, GJ Fleuren, GG Kenter, SH van der Burg *International Journal of Cancer 2010 128:379*

CURRICULUM VITAE

Moniek werd geboren in Warnsveld in 1982. Tijdens haar middelbare schooltijd was ze elk weekend te vinden op het schip de Volharding waar ze veel heeft geleerd van boten, techniek en mensen. Na het behalen van het Atheneum aan het Baudartius College in Zutphen vertrok ze in 2000 naar Amsterdam voor het volgen van de beta-gamma propedeuse aan de UvA. Na het behalen van deze propedeuse -waarin ze koos voor wetenschapsdynamica en biologie- startte ze toch in het eerste jaar van Geneeskunde in het AMC. In het vierde jaar van deze studie deed zij 4 maanden onderzoek in het lab van Pawel Kalinski in Pittsburgh, USA. Dit onderzoek -naar de activatie van dendritische cellen voor kanker vaccinatie- resulteerde in de bewuste keus om immunologisch (lab) onderzoek te gaan doen na het behalen van het arts-diploma. Ze ronde haar co-schappen cumlaude af in 2007 en begon nog dat zelfde jaar in het lab van de klinische oncologie bij prof. Sjoerd van der Burg. Sinds december 2011 vervolgt ze haar weg met de opleiding tot medisch microbioloog aan de Vrije Universiteit in Amsterdam.

DANKWOORD

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