Regulation and subversion of HPV16-specific immunity in cancer patients

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Chapter 7

Summary and general discussion

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1. Impact of tumor-infiltrating T cells.

Infiltration of tumors with CD8+ T cells is beneficial for patient survival in many types of tumors (1-6). In HPV-induced cervical cancer infiltration of CD8+ T cells is associated with an improved prognosis (Chapter 2) (7, 8), especially in patients who displayed a systemic HPV-specific immune response (Chapter 2). Consistent with earlier reports, we were able to show in a large cohort that these tumor infiltrating CD8+ T cells included T cells specific for the oncogenic HPV antigens E6 and E7 (Chapter 3) (9, 10). These CD8+ T cells can be negatively influenced by co-infiltration of CD4+ T cells as indicated by the ratio of CD8+/CD4+ T-cell ratio (Chapter 2). This indicates that these CD4+ T cells may have a suppressive phenotype. A recent study in a large cohort of cervical cancer patients revealed that the CD8/FOXP3 ratio was a favorable independent prognostic factor for patient survival (11). This implicates that the presence of regulatory T cells (Tregs) is indeed unfavorable and may suppress CD8+ T cells, these cytotoxic T cells potentially control tumor growth. Since the tumor infiltrating CD4 T cells comprised of HPV E6/E7-specific T cells (Chapter 3), they may very well include HPV-specific Tregs. To fully characterize the phenotype of these infiltrating T cells, it was necessary to analyze them on a functional level. In order to obtain enough T cells for functional analysis, expansion of the HPV-specific T cells in vitro was required.

2. Isolation and Culture of human Tregs

The most common method to obtain natural Tregs from the peripheral blood, is using flow cytometric isolation of CD4+ T-cells which highly express CD25 (12, 13). The numbers of these cells are generally enough to accommodate a few experiments. However, for in-depth analysis of the function and specificity of Tregs, larger numbers of cells and prolonged culture are required (14). Since several Treg subpopulations are characterised by their ability to produce IL-10 (reviewed in (15)), selection of T cells on the basis of specific IL-10 production by cytokine capture assays may prove to be a valuable tool to enrich for antigen specific CD4+ Tregs (Chapter 6) (16). The production of non-stimulatory factors (e.g. IL-10) by CD4+ Tregs precludes strong proliferation of the cells and as such it is difficult to expand Tregs in vitro. Different methods have been developed to circumvent these problems, which include antigen specific and non-specific expansion combined with different growth factors (17-19). T-cell receptor stimulation in combination with IL-2 can reverse the anergic state and can result in expansion of CD4+ Tregs (20, 21). IL-15, which efficiently promotes expansion of memory T cells (22, 23), enhances the expansion of Tregs as well (20, 24). IL-7 promotes survival of memory T cells (25) including CD4+ Tregs (26, 27). A recent study suggested that a combination of the common gamma chain cytokines IL-2, IL-4, IL-7 and IL-15, maintain the optimal regulatory function of human Tregs in a PI3K-dependent manner (27). Interestingly, the combination of rapamycin, an immunosuppressive drug used to prevent acute graft rejection in humans, and IL-2 promotes the expansion of Tregs while selectively depleting CD4+CD25- effector T cells both in vitro and in vivo (28). Similar to results from studies on tumor-specific effector T cells, the presence and type of Tregs in the peripheral blood may not necessarily reflect the Treg repertoire infiltrating the tumor (29-31). Therefore, studies on Tregs that contribute to the local immunosuppressive tumor environment require Tregs to be directy isolated from fresh tumor material and from tumor draining lymph nodes. Pioneers in the field were able to isolate and culture Tregs from tumor tissue and from tumor ascites using recombinant IL-2 (32-34). In our own studies the combination of T-cell growth factor (containing natural IL-2) in combination with IL-15 and IL-7 led to the successful isolation and expansion of tumor-infiltrating lymphocytes and tumor-draining lymph nodes-derived tumor-specific lymphocytes from a large cohort of patients with cervical cancer (Chapter 3).

3. HPV-specific Tregs isolated from cervical cancer patients suppress via antigen-dependent mechanisms

Using the isolation and expansion protocols as described above we were able to show in numerous cases cervical cancer derived T-cell cultures contained CD4+ Tregs. These could be cloned and studied extensively (>2 years) with respect to phenotype, antigen specificity and mechanism of suppression (Chapter 4.1). Both FOXP3+ and FOXP3- Treg clones were isolated (Figure 1). HPV-specific T-cell bulk cultures contained suppressive capacity in several but not all cases. Importantly, HPV-specific T-cell bulk cultures from healthy donor derived positive skin-test biopsies did not contain suppressive capacity. Suppressive capacity was also detected in a HPV-specific T-cell bulk culture isolated from premalignant lesion in one case but not in another (Chapter 5). This indicates that Tregs are already prominent in a proportion of the cervical pre-cancers. Moreover, Tregs were detected against both the E6 and E7 oncogenic proteins, indicating a broad specificity of the Tregs present in these patients (Chapter 4.1 and 5). In-depth analysis of HPV-specific Tregs on the clonal level revealed that they could suppress in an antigen-dependent manner (Chapter 4.1), which was also the case for a polyclonal T-cell culture isolated from HSIL lesion (Chapter 5). As HPV is not available in high enough quantities, we were unable to show that these Tregs were also capable to suppress upon stimulation by APC cross-presenting HPV antigens from infected cells. However, Treg clones specific for influenza M1 were able to suppress upon stimulation of APC infected with live influenza virus. This suggests that virus antigen-specific Tregs are capable of exerting their suppressive function upon stimulation when antigen is presented in natural physiological context (Chapter 6).

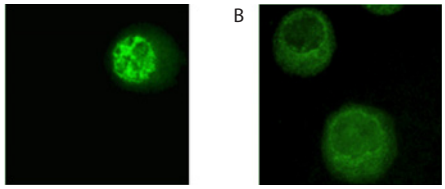

Figure 1. Staining of FOXP3 (PCH101, eBioscience) on cytospins of human papilloma virus-specific FOXP3+ (A) and FOXP3- (B) regulatory T-cell clones isolated from cervical cancer patients (Chapter 4.1). Clear intra-nuclear staining is visible in the FOXP3+ clone (A; C148.31), and not in the FOXP3- clone (B; C271.9)

4. Origin of CD4+ regulatory T cells

4.1 Thymus derived CD4+ Tregs

Literature on the origin of CD4+ Tregs focusses on two different developmental pathways. The first pathway is generation of natural CD4+CD25^{high} Tregs in the thymus and the second is generation of adaptive CD4+ Tregs in the periphery. Thymus derived CD4+ Tregs are generated as part of the natural differentiation of developing T cells in the thymus. Several antigens are selectively expressed on specialised tissue in the periphery. These tissue-restricted self antigens are also expressed in thymic epithelial cells under the control of the autoimmune regulator (AIRE) (35-38). AIRE+ medullary thymic epithelial cells (mTECs) play a central role in the generation of Tregs in the thymus by presentation of tissue restricted antigens either directly or indirectly (Figure 2A) (39, 40). Tumor antigens are in most cases aberrantly expressed self-antigens that are either also expressed in normal cells or are self-antigens

that are normally only expressed during embryogenesis (oncofetal antigens). Collectively these antigens are known as tumor associated antigens (TAA). Since both types of TAA are expressed in thymic epithelial cells as well, it is likely that at least part of the Tregs generated in the thymus is specific for these tumor associated antigens. In the case of HPV-specific Tregs this does not occur, as the E6 and E7 antigens are not expressed in the thymus. However, HPV-infected tumor cells overexpress different self-antigens as well which are expressed in the thymus, including hTERT and p16 (41-44). For this reason it is likely that Tregs specific for these antigens also infiltrate cervical tumors and contribute to the establishment of an immunosuppressive microenvironment in the tumor.

4.2 Induction of CD4+ Tregs in the periphery

In several mouse models it was shown that CD4+ T cells with suppressive capacity can also be generated from naïve precursors in the periphery (45-49). In these mouse studies, naïve CD4+ T cells were stimulated using high doses of antigen (oral tolerance protocols) or with a low antigen dose in the absence of co-stimulatory molecules. Furthermore, stimulation of naïve precursors in human peripheral blood mononuclear cells with an Epstein-Barr virus (EBV) encoded EBNA1 peptide resulted in the generation of CD4+ Tregs to EBV in vitro (50). Several studies investigated different methods to generate Tregs in vitro (19). Most of them focused on DCs, as DCs are the key players in the initiation of the immune response (51). These studies aimed at inducing CD4+ Tregs by stimulating naïve T cells with immature DCs (iDCs) (52). The generation of such Tregs was dependent on IL-10, which was likely to be produced by iDCs (53). Consistent with the idea that presentation of antigens by iDCs suppresses immunity, tumor derived vascular endothelial growth factor (VEGF) has been shown to inhibit maturation of DCs (54). Increased numbers of iDCs have been found in tumor patients as well, indicating that iDCs play a role in local immunosuppression, possibly through the induction of CD4+ Tregs (reviewed in (55)) (Figure 2CDG).

Next to iDCs a specialised tolerogenic DC (tDC) subset can also induce CD4+ Tregs (19, 56). These tDCs can be generated by compounds such as vitamin D3 and dexamethasone and by immunomodulatory cytokines including IL-10, TGFβ and IFNα (57-63). These tDCs may display similar or somewhat lower levels of costimulatory molecules such as CD40 and CD86 when compared to activated DCs (56, 64). However, tDCs also express other membrane molecules including ICOS-L, ILT3 and ILT4 which following cognate interaction activates tDCs to convert naïve CD4+ T-cells into Tregs (65, 66). In cancer patients, tDCs may be involved in the peripheral generation of Tregs, as DCs exposed to tumor cells acquire an immunosuppressive phenotype associated with the induction of Tregs (67-69) (Figure 2CDG).

The enzyme indoleamine 2,3-dioxygenase (IDO) when upregulated in plasmacytoid DCs, has also been shown to induce Tregs in vitro and activate mature Tregs in vivo (70-72). IDO is an enzyme which is involved in the tryptophan degradation pathway (73). The generation of Tregs is dependent both on tryptophan depletion and on the generation of its metabolites (71). IDO+ plasmacytoid DCs present in tumor-draining lymph nodes can directly activate lymph node resident

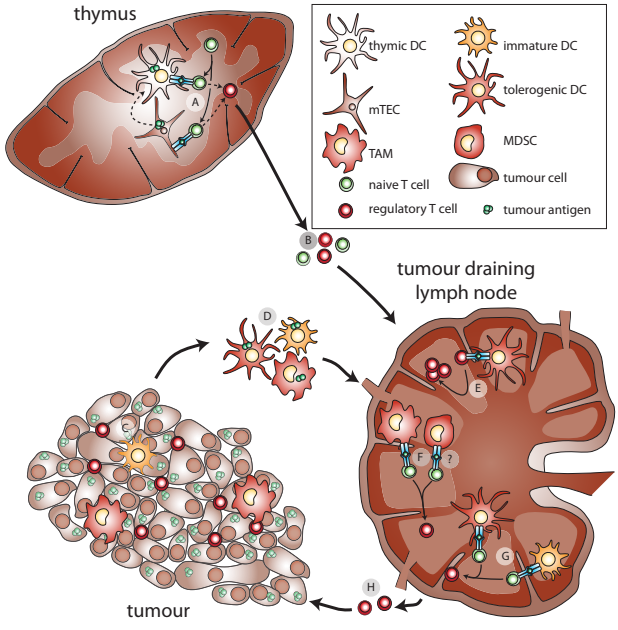

Figure 2. Proposed model of generation and migration of tumour-specific Tregs. (A) Tumour associated antigens are expressed by mTECs and presented directly and/or indirectly to naïve T cells, converting them to Tregs. (B) Both naïve T cells and thymus derived Treg migrate to the tumour draining lymph node. (C) Antigen derived from tumour cells is captured by immature DCs. (D) DCs remain immature or mature into a tolerogenic DC phenotype and migrate to the tumour draining lymph node along with tumor associated macrophages (TAMs). (E) Thymus derived tumor associated antigen-specific Tregs expand after stimulation by DC which present tumour antigen-derived epitopes. (F) Non professional APC, including TAMs and Myeloid derived suppressor cells (MDSC) may also induce Treg from naïve precursors. (G) Immature DC and/or tolerogenic DC present tumour antigen to naïve T cells, which are subsequently converted to Tregs. (H) Thymus derived and peripheral induced Treg migrate to the tumour, where they contribute to the immunosuppressive environment.

CD4+ Tregs. Thereby it prevents the activation of a proper antitumor response (72). Furthermore, IDO levels are elevated in different cancers which besides influencing Tregs, can also induce general T-cell anergy and apoptosis (reviewed in (74)).

Type 2 macrophages are abundant in many tumors (75). A recent study showed that Type 2 macrophages induce FOXP3+ Tregs from naïve precursors in a TGFβ dependent mechanism (76). The abundant presence of these cells may therefore be responsible of inducing Tregs in the tumor microenvironment (Figure 2CDF). However Tregs can also induce type 2 macrophages (77). This indicates that Tregs may also be partly responsible for the high numbers of type 2 macrophages in the tumor. These studies implicate an intimate relationship between type 2 macrophages and Tregs. This relationship is responsible for the generation and maintenance of an immunosuppressive milieu in the tumor microenvironment.

Myeloid Derived Suppressor Cells (MDSCs) are present in high quantities in the peripheral blood of cancer patients with different types of cancer (78). In mouse models these MDSCs have been shown to induce Tregs (79, 80) (Figure 2F). On the other hand MDSCs have been shown to expand Tregs and in a mouse model for B-cell lymphoma (81). However, other studies showed only limited or no effect of MDSCs on the expansion of Tregs (82, 83). These contradictory results may be partly explained by differences in models used in these studies. Therefore it is important to analyze the effect of MDSCs on Tregs in cancer patients to determine their interaction with Tregs in a clinically relevant setting.

Tregs are essential in limiting collateral damage as a result of exuberant immune responses against invading pathogens (84). Especially adaptive Tregs are important to limit immunopathology as was shown in a mouse model of allergic inflammation (85). Adaptive Tregs in humans include influenza-specific Tregs that are induced as a result of acute virus infection (Chapter 6), indicating that Tregs are part of the normal antiviral immune response. Therefore it is likely that Tregs specific for cancer antigens are already induced early during malignant transformation, but the balance between Tregs and effector T cells may have shift over time towards a more immunosuppressive milieu.

4.3 Origin of HPV-specific Tregs in cervical cancer

It is difficult to determine the origin and role of HPV-specific Tregs during disease progression in cervical cancer patients. It is highly likely that Tregs are induced as part of the normal immune response against HPV. Generally, HPV infections are cleared quite slowly (median of 6 months) (86), while acute viral infections such as influenza are cleared within weeks. Therefore, the immune system seems to be inefficient in clearing HPV infections. This may be caused by early interactions of the host with the virus at multiple levels. Firstly, Langerhans cells, which are the professional antigen presenting cells in initiating mucosal immune responses, are improperly activated upon encounter of L2-containing virus like particles (87). Secondly, HPV also interferes with the IFN pathway in infected keratinocytes, caused by the oncogenes E6 and E7 (Reviewed in (88)). This results in a more immunosuppressive microenvironment and may thereby promote the induction and expansion of HPV-specific Tregs. One or combinations of these interactions may result in enhanced induction of HPV-specific Tregs and as such induce a more immunosuppressive virus-specific immune response compared to acute viral infections. However, these observations do not explain why most people are able to clear persistent HPV infections, whereas a minority of the infected women are not able to cope with the virus and as a result develop cervical cancer. Both genetic and environmental factors have been implicated in HPV oncogenesis, however a clear picture is still missing (89).

Accumulating numbers of circulating Tregs (defined as CD4+CD25+) have been detected in the peripheral blood of HSIL patients as witness of an immunosuppressive milieu in these patients (90, 91). In line with these findings, substantial quantities of FOXP3+ Treg infiltrate have been detected in HSIL patients (92, 93), but no significant differences were observed between HSIL and LSIL (93). Moreover, HPV-specific Tregs were detected among cervical infiltrating lymphocytes in a patient with HSIL (Chapter 5). This is indicative of an immunosuppressive HPV-specific response in this patient. The immunosuppressive microenvironment in HSIL patients may subsequently favour the progression towards invasive carcinoma by evading immunosurveillance.

5 Mechanisms of CD4+ regulatory T-cell induced suppression

Several studies showed that Tregs have the capacity to suppress different members of adaptive immunity. Specifically, CD4+ Tregs have been shown to inhibit the proliferation and cytokine production of activated naïve CD4+ T cells as well as established T-helper 1 cells (Chapter 4.1 and Chapter 6) (32, 33, 52, 94-96). Moreover, a recent report showed that Tregs can induce a Treg phenotype in naïve T cells independent of APC in a TGFβ dependent manner (97). We and others reported that CD4+ Tregs were also able to prevent the activation of CD8+ T cells by preventing the expression of CD25 and by inhibiting IL-2 production by CD4+ T cells (Chapter 6) (98). Besides effects on T cells, Tregs were also shown to affect B cells. CD4+CD25^{high} Tregs were shown to directly interact with B cells resulting in an inhibition of antibody production, isotype switching and proliferation of B cells (99, 100). CD4+CD25^{high} Tregs are also able to suppress antigen-specific IFNγ production of γδ T cells (101).

Not only the members of the adaptive immune response are affected, but also cells from innate immunity. Thymus derived Tregs are capable to induce a suppressive phenotype in monocytes/macrophages (77). The interaction between Tregs and DCs may lead

to down regulation of CD80 and CD86, even in the presence of stimuli that normally would enhance cell surface expression of these molecules and increased IL-10 production by DC maturation (102, 103). Moreover, Tregs can activate the immunosuppressive tryptophan catabolism by the induction of IDO in DCs (104). The subset of TGFβ producing CD4+ Tregs was shown to inhibit the cytotoxicity, cytokine production and proliferation of natural killer cells both in vitro and in vivo through TGFβ dependent mechanisms (reviewed in (105)). Tumor infiltrating FOXP3+ Tregs have been indicated to downregulate VCAM and ICAM expression in large tumors, thereby hampering tumor-infiltration of CD8 T cells. Downregulation of the adhesion molecules could only be reversed by radiotherapy or prophylactic depletion of Tregs and not by therapeutic depletion of this T-cell subset (106).

Thymus derived Tregs function in a contact-dependent manner, as was shown in vitro by separating Tregs from responders by a semi-permeable membrane (94). A recent study showed that thymus derived Tregs in mice have contact with responder T cells trough gap-junctions (107). They further show that cAMP, known to be a potent inhibitor of proliferation and interleukin 2 synthesis in T cells, was present at high levels in thymus-derived Tregs and this was transferred to responder T cells through gap-junctions resulting in the suppression of the responder cells (107). However, we did not detect gap-junction formation for our Treg clones (unpublished data). Two membrane bound molecules (CD39 and CD73) have been shown to be expressed by Tregs and generate pericellular adenosine, which results in the suppression of effector T cells by ligation of the adenosine receptor 2A (Reviewed in (108)). We found that both molecules (CD39 and CD73) were expressed both by Treg as well as Thelper clones (unpublished data). Studies on adaptive Tregs show that the Tr1 subset of CD4+ Tregs act through the cytokines IL-10 and TGFβ (reviewed in (109)) (21, 110, 111), but we were unable to detect active TGFβ production by HPV and influenza-specific Tregs. Moreover, IL-10 produced by FOXP3+ Tregs is important in limiting inflammatory responses in vivo (112). Consistent with our results (unpublished data), others have reported that IL-10 production by natural Tregs is not involved in in vitro assays (113). Adaptive Tregs can also deploy a perforin and granzyme dependent pathway as a mechanism to control immune responses (114, 115). Such a mechanism may also be employed by thymic-derived Tregs (115). Both human and murine thymus derived Tregs were shown to secrete TNFRII, which inhibits the action of TNF-a and thereby limiting inflammation (116). Several molecules are able to counteract the suppressive function or expansion of certain subsets of Tregs. Leptin was shown to prevent proliferation of thymic-derived Tregs after engaging the leptin receptor ObR (117). IL-21 renders human CD4+ T cells resistant to the suppressive capacity of Tregs (118). Treatment with reserpine releases catecholamines from Tr1-type Tregs. This results in a decreased potential of these Tregs to produce IL-10 and TGFβ, as well as a decreased potential to inhibit proliferation of CD4+ T cells (119).

Furthermore, Tregs have been shown to express the toll-like receptors (TLR) 2, 5 and/or 8 (120-122). Ligation of TLR2 was shown to induce Treg proliferation and to cause temporal loss of suppressive phenotype (120), TLR8 ligation was shown to abrogate Treg mediated suppression (122), while ligation of TLR5 was shown to enhance the suppressive function of Tregs (121). A recent study showed that IL-10 producing CD4+ T cells induced in the presence of 1α,25-dihydroxyvitamin D3 express TLR9. Ligation of TLR9 on these cells inhibits the IFNγ and IL-10 production (123). In our hands, Treg clones displayed a heterogeneous TLR expression profile and no TLR was consistently expressed between clones. Moreover, almost all clones lacked TLR8 expression and TLR8 ligation by ssRNA40 did not result in decreased suppressive capacity of these clones (unpublished data). This indicates that TLR8 ligation cannot be used as general approach to directly abrogate Treg function.

Altogether, a variety of mechanisms for suppression utilized by Tregs have been described, but none of these mechanisms seem to be universal. Dominant mechanisms need to be identified to design potential clinical approaches that interfere with Treg function. On one hand, universal markers need to be identified, which may lead to more selective depletion strategies. So far, most studies focussed on natural Tregs versus naïve CD4 T cells, resulting in Treg associated markers, which are also present on activated T helper cells. More specific markers for subsets of Tregs (e.g. FOXP3+ vs FOXP3-) on the other hand will be greatly beneficial to determine which types of Tregs are present in tumors and cervical cancer in particular.

6 Impact of cancer immunotherapy on Tregs

Many of the known TAA and TSA are the prime components of therapeutic vaccines against cancer that are currently under development (reviewed in (124-126)). Tregs specific for several of these tumor antigens have been detected in cancer patients, including Tregs specific for the viral oncoproteins of HPV (Chapter 4.1 and 5) (32, 33, 127-129). Tumor-specific Tregs have also been detected in several tumor models in mice (130-132). Although therapeutic vaccines are designed to enhance CD4+ and CD8+ T-cell effector immunity, they may also activate pre-existing TAA/TSA-specific CD4+ Tregs present in the lymph nodes and tumors of cancer patients (Chapter 4.2) (128) (Figure 3). In mice, the boosting of Tregs after therapeutic vaccination was associated with subsequent failure of the anti-tumor immune response (131). Moreover, a recent study in vulvar intraepithelial neoplasia patients showed that patients who did not display a complete clinical response, mounted both HPV-specific effector T cells as well as HPV-specific Tregs following vaccination. In contrast, patients who displayed a complete clinical response mounted predominantly

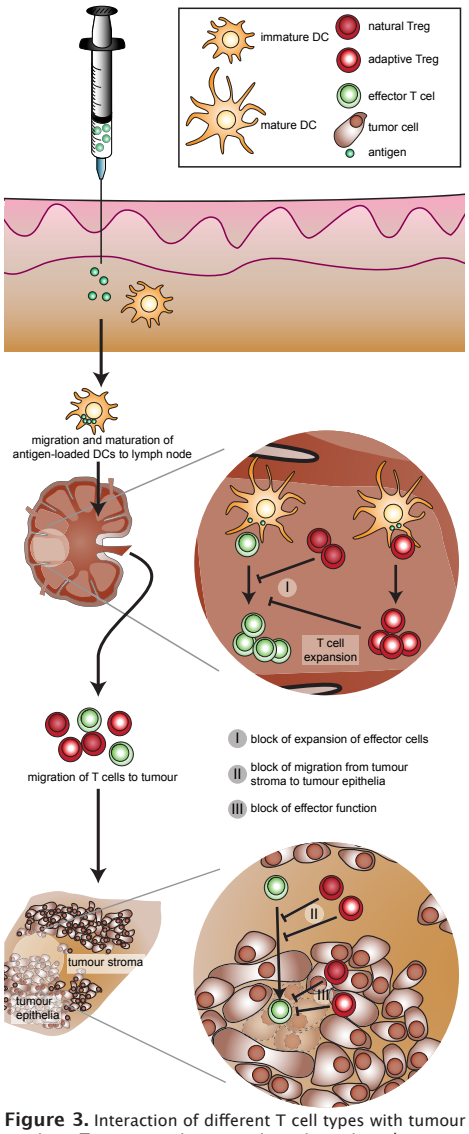

Figure 3. Interaction of different T cell types with tumour vaccines. Tumour vaccines contain antigens in order to activate both tumour-specific CD4+ T cells and CD8+ T cells. Injected antigens are taken up by local DCs and migrate into the draining lymph node. Here the DCs can stimulate both effector T cells and adaptive CD4+ Tregs. If self-antigens are injected also natural Tregs may get activated and expanded. In the draining lymph node the presence of natural Tregs as well as adaptive Tregs may limit the activation and expansion of effector T cells. Migrating effector T cells and Tregs may leave the blood vessels and enter the tumour stroma where the migration of effector T cells into the tumour cell nests may be dampened by co-migrating or resident Tregs. Furthermore, the function of effector T cells within the tumour cell nests may be downregulated by Tregs.

HPV16-specific T effector cells (Welters & Van der Burg, personal communication). These data indicate that those patients in which the current therapeutic approach is unsuccessful could benefit from an alternative therapy that includes the neutralization of Tregs (Figure 4).

7 Intervention strategies to bypass vaccination-induced Treg expansion

7.1 Depletion of Treg based on CD25 expression

In several mouse models, treatment with an anti-CD25 depleting antibody enhanced the anti-tumor immune response (reviewed in (133)). For translation to the clinic a hybrid molecule has been used (ONTAK). This molecule contains full-length IL-2 for binding to CD25 and the translocation and toxic domains of diphtheria toxin to induce apoptosis (134). In mice this molecule was able to deplete FOXP3+ Tregs in different compartments and was able to enhance vaccination-induced T-cell responses (135). In combination with vaccination, ONTAK is able to deplete Tregs and thereby boosting the tumor-specific immune response in renal cell carcinoma, CEA-positive and melanoma patients (136-138). In contrast, in one study ONTAK was unsuccessful in depleting Tregs in metastatic melanoma patients (139). Together, these studies show that ONTAK as supplementary therapy in vaccination trials may be promising, however caution is needed as this therapy is not always successful.

LMB-2 is another immunotoxin which targets CD25. LMB-2 is a hybrid molecule consisting of pseudomonas exotoxin A and the Fv chain of anti-CD25 (140). In a human study, LMB-2 was able to partially deplete Tregs, but no effect was seen on vaccine-induced responses (141). Since this is just one study showing in vivo depletion by LMB-2, further studies are required to investigate the efficacy of this molecule to deplete Tregs.

7.2 Depletion of Tregs based on cytotoxic chemotherapy

Low-dose cyclophosphamide, which is a cytotoxic alkylating compound, reduces both the number of Tregs as well as their function in mice (142). A recent study showed enhanced Treg depletion in the tumor when cyclophosphamide was used in combination with an agonistic anti-OX40 antibody. This regime induced hyperactivation and cell death in the Treg compartment (143). In animal models, low-dose cyclophosphamide was able to enhance vaccine-induced anti-tumor responses (144, 145). In humans, cyclophosphamide used as a single agent was shown to inhibit the Treg compartment, while the effector compartment was not negatively influenced (146, 147). Combinational therapy has not been studied in humans, but may prove to be an effective approach to enhance anti-tumor vaccination strategies.

7.3 CTLA-4 blockade to improve anti-tumor immunity

CTLA-4 is an inhibitory co-receptor that is expressed both on activated T cells and constitutively on thymus derived Tregs. In mouse models, it has been shown that combination therapy of CTLA-

4 blockade, especially together with CD25 depletion or GM-CSF secreting vaccine improves immunotherapy against established tumors (148-150). CTLA-4 blockade both on the effector population as well as on the Treg compartment is important in the enhancement of anti-tumor responses (151). Two monoclonal blocking antibodies (ipilimumab and tremelimumab) are currently being tested in clinical trials (152). Since these antibodies affect all T cells regardless of specificity, side effects of these antibodies include mild to severe autoimmunity (152, 153). Early promising clinical trials show enhanced anti-tumor T-cell responses upon treatment with anti-CTLA4 antibodies (154-156). Currently placebo-controlled phase II and III clinical trials are conducted to further investigate the potential of CTLA-4-blocking antibodies and treatment of the side-effects (152). These monoclonal antibodies may provide a window in which CTLA-4 blockade combined with antigen-specific immunotherapy may elicit an improved anti-cancer immune response that is able to eradicate tumors of cancer patients.

7.4 Blockade of PD-L1

Programmed Death 1 (PD1) is another inhibitory coreceptor which is expressed on activated T and B cells (including Tregs), APC and highly expressed on exhausted CD8+ T cells (157). The natural ligand PD-L1 (B7-H1) is expressed on APC and by several human tumors (158). In a variety of tumors, expression of PD-L1 is associated with poorer survival (159-166). Blockade of PD1 or PD-L1 improves anti tumor-responses in several mouse models (Reviewed in (157)). Unexpectedly, expression of cell-surface PD-L1 in cervical cancer patients was associated with improved survival (Karim et al, submitted). This phenomenon could be explained by incapacitation of infiltrating PD1+ Tregs through PD1:PD-L1 interactions. A humanized blocking antibody to PD-1 has been tested in a phase I trial in patients with hematological malignancies and was found to be well tolerated in these patients (167). Despite clinical benefit in a proportion of these patients, this antibody is likely to provoke adverse responses in cervical cancer patients as expression of PD-L1 is beneficial for patient survival. Alternatively, engagement of PD-1 in PDL-1 negative patients may suppress the Treg population, thereby enhancing the anti-tumor response.

7.5 Modulating antigen presenting cells

As described in paragraph 4.2 different subsets of APC have the capacity to induce Tregs. As these cell types are not affected using the strategies described above, depletion of Tregs does not exclude de novo induction of HPV-specific Tregs upon tumor-specific vaccination. Therefore, strategies to modulate these cells as well, may prove to be a valuable supplementary therapy to enhance tumor-specific immune responses.

Several approaches have been proposed to skew the phenotype of DCs in cancer patients from a tolerogenic into a pro-inflammatory phenotype (reviewed

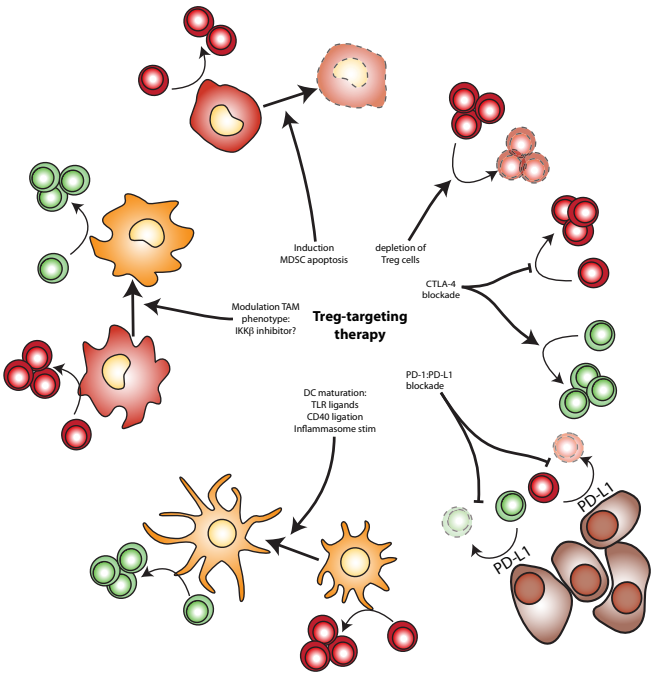

Figure 4. Strategies that could bypass vaccination-induced Treg expansion. Depletion of Treg before or during treatment with CD25-targeting compounds or with low dose cyclophosphamide decreases the initial numbers of Treg. Blockade of CTLA-4 signaling both dampens Treg as well as releasing the brakes on effector cells. Blockade of PD-1:PD-L1 interaction results in enhancement of effectotr responses, but also can enhance Treg function. Several agents can be used to skew the antigen presenting compartment to an immunogenic phenotype. These approaches include maturation of DC, modulation of macrophage phenotype and targeting myeloid suppressor cells (MDSC).

in (168)). These approaches include activation of DCs by anti-CD40 antibodies, TLR ligands, activation of the inflammasome and immunogenic cell death by chemotherapy and radiation therapy (169-171). These properly activated DCs may in turn shift the balance from a Treg dominated response into a CTL dominated response, which is able to mount a full-blown attack against the tumor.

Tumor associated macrophages promote the immunosuppressive microenvironment. Targeting these cells may therefore augment vaccination protocols. Two recent studies described that skewing of the phenotype towards a proinflammatory M1 phenotype by inhibition of IKKβ results in improved tumoricidal activity (172, 173). The M1 macrophages in turn may promote anti-tumor immune responses. Even though subversion of the phenotype of macrophages represents a promosing approach for anti-cancer therapy, agents are not yet available to promote M1 macrophage differentiation in the clinic.

Several agents are currently tested in preclinical models to inhibit expansion and function of MDSCs (Reviewed in (78)). These cells are implicated in the expansion of Tregs and are present in the peripheral blood of cancer patients in relatively high numbers. Therefore, depletion of MDSCs may result in abrogation of the immunosuppressive milieu, enabling effective vaccination without vigorous expansion of Tregs.

8 Final remarks

The local presence of HPV-specific Tregs provides a plausible explanation for the inability of the immune system of cervical cancer patients to cope with the tumor. Moreover, these HPV-specific Tregs are boosted upon vaccination with HPV16 systemic long peptides and correlate with clinical outcome. Therefore, elimination/ reduction of the Treg compartment either before or during vaccination, will likely shift the balance from a Treg dominated response to an effector T-cell dominated response. This will result in improved vaccination efficacy. Strategies that elicit potent anti-tumor immune responses may also lead to the induction of different escape mechanisms. These mechanisms could include antigen loss, loss of MHC-class I molecules and impaired antigen processing. Monitoring a substantial number of patients in who the therapy failed will give further insights in dominant escape variants that evolve in response to vaccination. These escape variants can subsequently be targeted in alternative approaches, such as vaccination against epitopes that are associated with impaired antigen processing (174).

Finally, the tumor microenvironment observed in cervical cancer patients has similar characteristics to other types of cancer. Knowledge gathered on inducing a potent anti-tumor immune therapy in cervical cancer patients may therefore be translated to other types of cancer as well.

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