

Review Article

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Cell intrinsic & extrinsic factors in cervical carcinogenesis

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Human papillomavirus (HPV) infection is a common sexually transmitted infection which a majority of infected women are able to clear by mounting an effective immune response. Individuals with a suboptimal immune response may be at increased risk of persistent HPV infection leading to sequelae of various grades of dysplasias and / or associated malignancy. Both cell intrinsic and extrinsic phenomena work in concert to bring about oncogenesis. Cell intrinsic factors for cervical carcinogenesis are: integration of the viral genome into the genome of the host's cell which correlates with the progression of low grade lesions into high grade ones, inactivation of tumor suppressor genes like p53 and pRB by HPV oncoproteins particularly E6 and E7, deregulation of cell cycle regulators, host DNA synthesis and apoptosis. Cell extrinsic elements include factors contributing towards immune tolerance; some incriminated in the multistep carcinogenesis of HPV induced cervical cancer are: immunoregulatory enzyme indoleamine 2,3-dioxygenase expressing antigen presenting cells, low numbers of invariant Natural Killer T cells, anergic cytotoxic T lymphocytes, regulatory T cells (Tregs), an immunoregulatory microenvironment comprising of increased IL10, TGF and reduced IL2; reduced intralésional ratios of effectors (CD4 and CD8) vs. Tregs; and different types of Tregs in the lesions of invasive squamous cell carcinoma. Notch signaling plays a crucial role in regulating T cell differentiation and activation including induction of Tregs. Increased expression of Notch receptor-Jagged 1 and number of Tregs were seen in invasive disease when compared to precancer in cervical cancer. Tregs impart their function either through cytokines or by cell to cell contact. Investigation of the consequences of interference of Notch signaling in terms of the dynamics of intratumoral Tregs in cervical cancer would be interesting.

Key words Carcinogenesis - cervical cancer - human papilloma virus - immune tolerance - immunoediting

Human papillomavirus (HPV) infection is a common sexually transmitted infection. Seventy per cent of infections clear within a year¹, and individuals with suboptimal immune responses may be at increased risk of persistent HPV infection and / or associated malignancy². Persistent HPV infections lead to sequelae of various grades of cervical dysplasias and also cervical cancer. HPV is thus considered to be the

major etiological factor for almost all cervical cancers, other anogenital cancers and a significant portion of oral cancers³. The major high risk genotypes associated with cervical cancer are HPV 16 and 18 and these two together are responsible for approximately 70 per cent of cervical cancers⁴.

HPV infection requires epidermal or mucosal epithelial cells that are proliferating (basal cells).

Following entry into the suprabasal layer, the viral genome replicates and in the upper layers of epidermis complete viral particles are released⁵. HPV infection thus results in enhanced proliferation of the infected cells and their lateral expansion. Most often, cervical cancer is marked by a premalignant phase of various grades of Cervical Intraepithelial Neoplasia (CIN I, II and III) which are characterized by a spectrum of histological abnormalities. On an average, it takes decades for cancer to arise. Cervical carcinogenesis thus is a multifactorial process and involves genetic, environmental, hormonal and immunological factors in addition to HPV².

HPV associated cervical carcinogenesis primarily affects the metaplastic squamous epithelium in the transformation zone (Fig.1) which is an irregular margin demarcating the squamous from the columnar epithelium. During metaplasia, foci of squamous cells are detectable amongst the endocervical glandular cells. Transgenic mouse models of HPV have suggested that metaplasia arises from the sub columnar reserve cells and estrogen along with the HPV oncogenes has a specific role in initiating cervical carcinogenesis from these cells^{5,6}. Reserve cells can differentiate into columnar or squamous cells and metaplasia arises as an alternative fate decision. Hence, subsequent to a collusion of cell autonomous and non-cell autonomous factors, transformation zone carcinogenesis is induced.

The three ‘E’s of cancer immunoediting

It was Rudolf Virchow in 1863, who first suggested a possible functional relationship between inflammatory

infiltrates and tumour growth. Nearly a century later, Burnet and Thomas postulated^{7,8} the existence of tumour immunosurveillance whereby lymphocytes were responsible for recognizing and eliminating continuously arising, precursors of cancer cells, before the disease becomes clinically apparent and hence act as an extrinsic tumour suppressor. Over the years this concept underwent some refinement and it is now recognized that both the innate and adaptive immune compartments participate in the process and serve not only to protect the host from tumour development but also to sculpt, or edit, the immunogenicity of tumours that may eventually form. Therefore, Schreiber and colleagues^{9,10} have proposed the use of a broader term “cancer immunoediting” comprising of three phases termed the “three Es” - Elimination, Equilibrium, and Escape (Fig. 2).

Six cell intrinsic (cell-autonomous) hallmarks of early oncogenesis are: cancer cells that characteristically provide their own growth signals, ignore growth-inhibitory signals, avoid cell death, replicate without limits, sustain angiogenesis, and invade tissues through basement membranes and capillary walls¹⁰. At the early stages of carcinogenesis, cell-intrinsic barriers to tumour development are paralleled by stimulation of an active antitumour immune response, whereas overt tumour development correlates with changes in the immunogenic properties of tumour cells. Hence as recently proposed by Schreiber and coworkers^{11,12}, defects or decreased efficiency in immunosurveillance could contribute to an increased incidence of malignancy

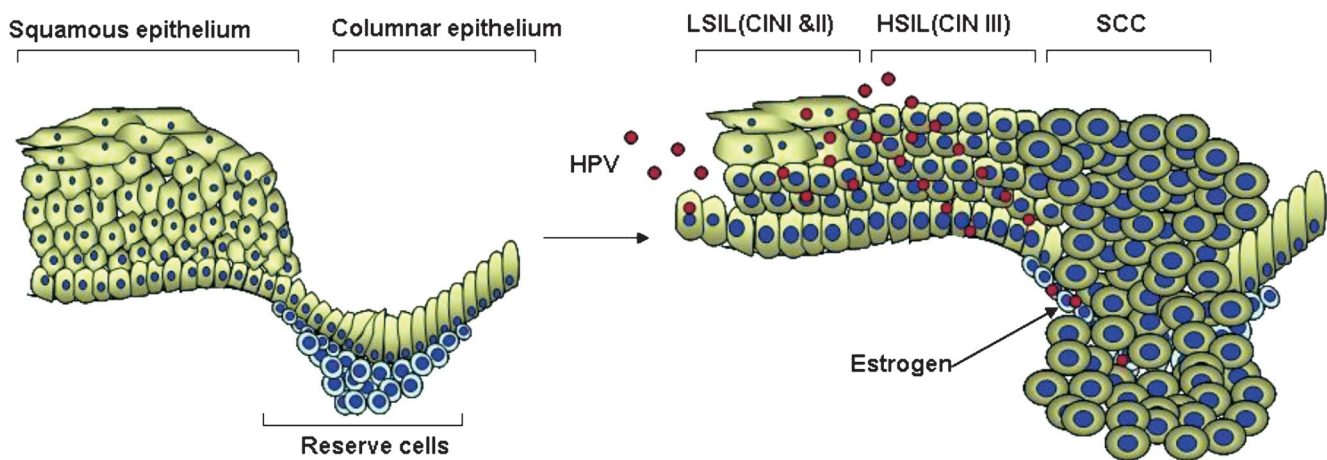


Fig. 1. HPV in cervical cancer progression. Human cervix is covered by squamous or columnar epithelium. The squamocolumnar junction has certain reserve cells that can differentiate into either of the cell fate. HPV infects the basal cells of squamous epithelium. Viral particles are released by the mature differentiated cells which can reinfect. With the expression of viral oncoproteins the lesions progress from Low grade Squamous Intraepithelial Lesion (LSIL) to High grade (HSIL). The presence of estrogen and high expression of viral proteins after the integration of the viral genome initiates squamous cell carcinoma (SCC) progression in the reserve cells of transition zone.

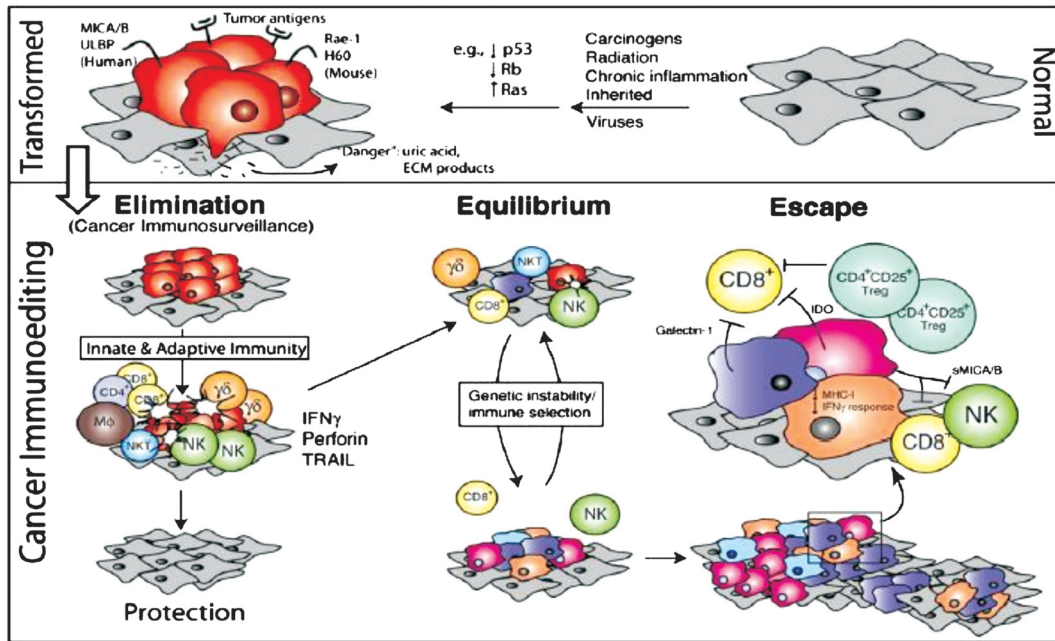


Fig. 2. The three Es of cancer immunoediting: host protective versus tumor sculpting actions of immunity. Following cellular transformation and the failure of intrinsic tumour suppressor mechanisms, a developing tumour is detected by the immune system and its ultimate fate is determined by whether or not it is eliminated by the host protective actions of immunity (*Elimination phase*), maintained in a dormant or equilibrium state (*Equilibrium phase*) or escapes the extrinsic tumour suppressor actions of immunity by either becoming non-immunogenic or through the elaboration of immunosuppressive molecules and cells (*Escape phase*). [Reprinted with permission from Elsevier (*Adv Immunol* 2006; 90 : 1-50)].

and might be the seventh hallmark of cancer which is mechanistically linked to the six established hallmarks¹³ (Fig. 3).

Cell intrinsic phenomena in HPV induced cervical carcinogenesis

HPV integration: HPV in the cervical cells could either be in an episomal state or an integrated state

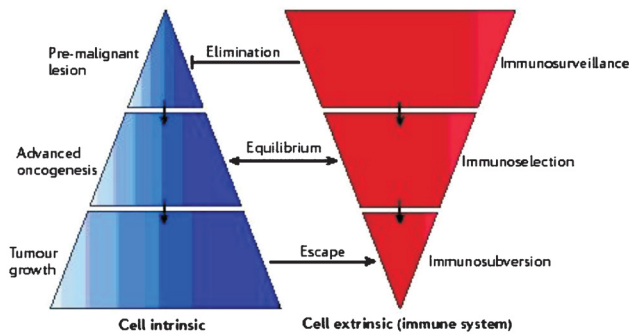


Fig. 3. Relationship between cell-intrinsic and cell-extrinsic aspects of tumour progression. This figure illustrates the central concept that multistep carcinogenesis results from crosstalk of cancer-cell-intrinsic factors and host immune system (cell-extrinsic) effects. [Reprinted with permission from Macmillan Publishers Ltd. (*Nature Rev Immunol* 2006; 6 : 715-27)].

or a mixed state that contains both forms of the virus. In the episomal state, viral gene expression is largely regulated by E2¹⁴, although limited expression of specific early genes (E5, E6 and E7) results in enhanced proliferation of infected cells. Viral integration into the host cell genome occurs downstream of E6 and E7, often in the E1 and E2 region and results in the loss of negative feedback control of oncogene expression (Fig. 4). Thus, integration of HPV DNA correlates with increased viral gene expression and cellular growth advantage, providing a selective advantage to cervical epithelial precursors of cervical carcinoma¹⁵. Integration of the HPV genome into host DNA usually correlates with the progression of low grade lesions to high grade ones. However, it has been shown that HPV oncogenes are necessary but not sufficient for cell immortalization and malignant phenotype^{5,6,16}.

HPV oncoproteins deregulate cell cycle regulators: The control of cell division in mammalian cells is brought about by the activity of cyclin dependant kinases (cdks) and their essential activating coenzymes, cyclins. The kinase activity of cdks is regulated by the abundance of their partner cyclins, phosphorylation and dephosphorylation events, and interaction with cdk

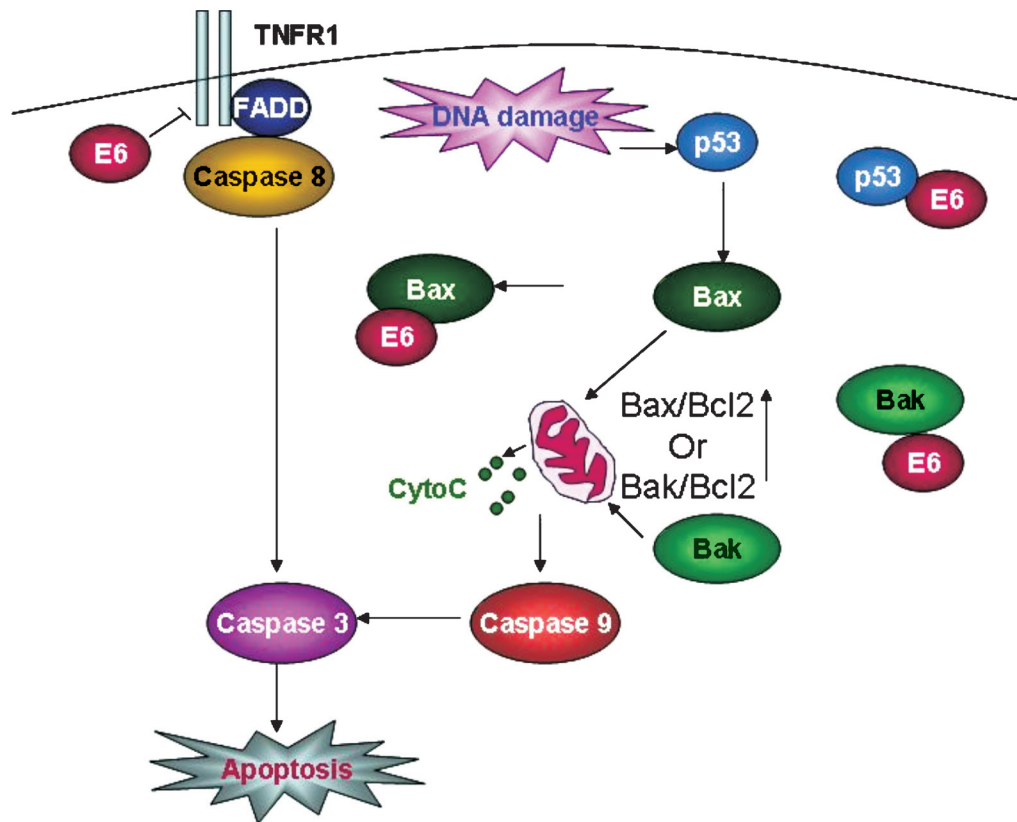


Fig. 4. HPV oncoprotein in cell cycle deregulation. The viral protein E2 represses E6 and E7 when expressed. The integration of viral genome into the host DNA disrupts E2 and hence functional E2 is not synthesized. E6 can bind to p53 and degrade the protein leading to the down regulation of cdk inhibitors, p21 and p27 and thus activates cell cycle progression through the activation of cyclin/cdk complexes. The active cyclin cdk complexes phosphorylate pRB and release E2F factor that is necessary for G/S transition. E7 can also induce cell cycle progression by binding and thereby inactivates pRB, p27 or p21.

inhibitory proteins (p15, p16, p21, p27 and p57). G1-S transition in normal cells requires phosphorylation of retinoblastoma protein, pRB by cdk2 thereby releasing E2F transcription factors which control various genes required for DNA synthesis and cell cycle control. For an optimal entrance into S-phase, the cell probably requires coordinated activation of both cyclin D and E- dependant kinases¹⁷ (Fig. 4).

The viral oncogene E6 is shown to bind to p53 and inactivate it by proteosomal degradation mediated by E6-AP¹⁸. This is thought to overcome the G1/S checkpoint by down regulation of cdk inhibitory proteins p21 and p27, which are downstream targets of p53. These cdk inhibitors can primarily regulate cyclin E/cdk2 pathway that is important for the phosphorylation of pRB. However, overexpression of p53 is noted in cervical cancer irrespective of high expression of HPV oncogenes¹⁹ suggesting that additional mechanisms are operating in the deregulation of this pathway. HPV oncoprotein E7 is shown to

associate with p21 and stabilize the complex abrogating the cdk inhibitory function of p21²⁰. Similarly, E7 can antagonize the ability of p27KIP1 to block cyclin E-associated kinase *in vitro*²¹. Apart from the inhibitory effects on cdk inhibitors, E7 can also directly regulate cyclin E expression. E7 binds to pRB and reduces the association between pRB and HDAC1, relieving their repressive effects on promoter of cyclin E²². E2F released from hyperphosphorylated pRB by the action of E7 may induce cyclin E transcription^{18,23}. Consistent with this, there is an increase in the protein levels of cyclin E in HPV infected lesions²⁴. But reports on the changes in the expression patterns of p21 and p27 in cervical carcinoma progression are contradictory²⁵.

E6 and E7 in apoptosis: Apoptosis is a genetically determined program, which leads to the induction of caspase activated deoxyribonucleases (Fig. 5). As a result, along with the high molecular weight DNA, viral DNA will also be cleaved that limits the spread of progeny

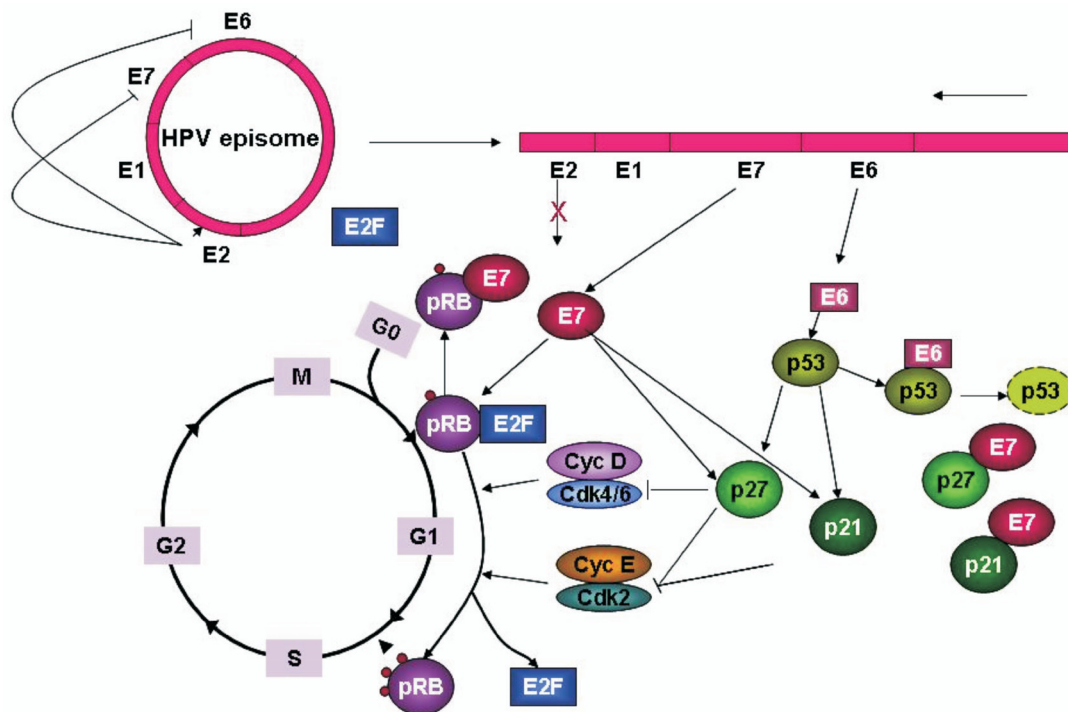


Fig. 5. HPV oncogenes block apoptosis. Apoptosis can be induced by activation of Caspase 3 by death receptor pathway or by DNA damage. Death receptor pathway activates caspase 3 through the activation of caspase 8. DNA damage induces p53 that activates Bax. Either the increase of Bax or Bak releases cytochrome C that activates caspase 9 which in turn can activate Caspase 3. E6 can block these processes at the receptor level or by degrading p53, Bak, or Bax.

viruses. Human pathogenic viruses have developed efficient strategies to modulate apoptotic responses upon infection²⁶. The most prominent function of E6 is the proteolytic cleavage of proapoptotic proteins such as, p53, Bak, Bax and c-Myc²⁷. Application of CD95 ligand rendered E7 immortalized cells to extensive apoptosis, while E6 and E6/E7 expressing keratinocytes were resistant²⁸. It has also been shown that the delivery of E6 can protect cells from TNF- mediated cell death in a p53 independent way. This resistance is attributed to the binding of E6 with TNF receptor (TNFR) and thereby the inability of TNFR1 intracellular death domain to interact with FADD²⁹. E7 controls the metabolic half life of pRB and thereby might block its anti-apoptotic role favoring apoptosis³⁰.

Cell extrinsic phenomena in HPV induced cervical carcinogenesis

As with all MALT (Mucosa Associated Lymphoid Tissue), the genital mucosa also has both inductor and effector arms of an immune response and HPV infection can interfere with the local immune vigilance mechanisms in both the arms². Though local and systemic immune vigilance determines latency in HPV infections and their evolution into high-grade

lesions²; cell mediated immune responses measured in the periphery are thought to be largely epiphenomenal, since the relevant immune response is local².

Immune infiltrates in the normal cervix: The normal uterine cervix is infiltrated by lymphocytes (CD4⁺, CD8⁺, plasma cells), dendritic cells (DCs) and macrophages either as individual cells or loose accumulation of cells akin to a lymphoid follicle^{31, 32}. There are also intraepithelial lymphocytes (IELs) in the ectocervix and the transformation zone (TZ) which are mostly CD8⁺ and less frequently CD4⁺^{31, 32}. The stroma on the other hand comprises submucosal lymphocytes predominantly of CD4⁺ type found just below the basement membrane³³ or more abundantly inside the LF in the TZ. NK cells are normally not found, but are present in infections. We and the others^{34, 35} have shown that the normal cervix is also under surveillance by a sparse number of natural regulatory T cells (nTregs) which are scattered in the stroma just below the basement membrane of the epithelium.

Immune infiltrates in cervicitis: The lesional infiltrate in cervicitis is rich in CD4⁺ and CD8⁺ phenotype cells but lack nTregs³⁵. In sharp contrast, a very small fraction

of cases of HPV positive cervicitis harbor a significant number of nTregs in the metaplastic squamous epithelium³⁵. Though such cases have not been followed up over time, we hypothesized that nTregs amidst the HPV infected epithelium may modulate effector T cell responses and may contribute to maintaining local tolerance - thus allowing the infection to persist. A similar phenomenon has been demonstrated during cutaneous infection with *Leishmania major*, where accumulation of nTregs at the site of infection leads to dampening of the response to the pathogen³⁶.

Immune infiltrates in cervical precancer: Regression of CIN 2/3 lesions is likely to be mediated by a local cell mediated immune (CMI) response which appears to be defective in high grade CIN lesions². Both the numbers of patrolling Langerhans cells (LCs) and their function are compromised: decreased secretion of TNF by keratinocytes and reduced expression of CD80 is thought to affect the antigen presentation capacity of LCs^{37,38}. Parallel to this change in LCs, there is increase in intralesional macrophages. Lymphoid follicles have been noticed to be more frequent in High grade Squamous Intraepithelial Lesion (HSIL) compared to normal cervix which may be indicative of increased immune activity locally³¹. The lymphocytic infiltrate comprises predominantly of CD4+ T cells in cervical stroma below the area of dysplasia and of CD8+ T cells within the dysplastic epithelium³⁹. However, the latter cells are thought to be anergic which might play a role in the persistence and progression of HPV induced lesions³⁹. The *in situ* pattern of distribution of nTregs has been shown to change from a predominantly intraepithelial distribution to that involving the stroma in the spectrum of HPV induced disease from infection to invasive cancer³⁵. This may be of relevance in the natural history of HPV infection and cervical cancer since Tregs may be exerting a “dominant” form of immunotolerance on many different cell types such as NK and CD8 cells⁴⁰. Literature on cytokine profile in precancerous lesions is varied^{35,41-45}. This variation could be attributed to different techniques used *e.g.*, RT PCR vs. IHC (immunohistochemistry), entire tumour specimens vs. micro dissected tissue specimen, HPV16+ vs. HPV18+ cases *etc.* Moreover, since there appears to be a dynamic immune equilibrium in precancerous lesions, the cytokine profiles observed could vary based on the type of the precancerous lesion (progressive, regressive or persistent) under study. Nevertheless a summary of cytokine profiles

in HSIL is: that there is increased expression of IL2R, IL4, TGF β and IL10 but decreased expression of IL2 and IFN γ reflecting an immunoregulatory milieu. Also, the immunoregulatory enzyme indoleamine 2, 3-dioxygenase (IDO) has been shown to be expressed in lesions of high grade CIN⁴⁵.

Immune infiltrates in invasive cervical cancer: Immune tolerance in cervical cancer is thought to be due to various reasons: tolerogenic DCs, invariant natural killer T cells (iNKT), $\gamma\delta$ cells, anergic cytotoxic T cells (CTLs), and Tregs, to name but a few. One of the reasons attributed to the relentless growth of cervical cancer in the presence of a good lymphocytic infiltrate is anergy of cytotoxic T cells⁴⁶. Low numbers of circulating iNKT cells have also been associated with poor prognosis⁴⁷. Moreover, antigen presenting cells at the invasive front in primary and metastatic lesions of cervical cancer and the cancer cells themselves have been found to be expressing IDO^{40,45}. Also, immature stromal macrophages within the lesions of high grade CIN are considered as pivotal in directing the differentiation of Tregs⁴⁵. We and the others^{34,35} have shown that the lesions of SCC are infiltrated with higher CD4+ and CD8+ cells when compared to CIN III, HPV positive cervicitis and normal cervix.

Natural Tregs have been observed to predominantly infiltrate tumour masses especially in the early phase of tumour progression⁴⁸ both in animal models and various human malignancies *viz.*, lung, breast, ovary, lymph nodes of human metastatic melanoma⁴⁹⁻⁵⁴ and in cervical cancer^{35,40,45,47} and play a major role in tumour immune evasion by strongly suppressing IL2 production and proliferation of antigen specific T cells⁴⁹. Also, Tregs isolated from SCCs specifically inhibited proliferation of naïve T cells to HPV 16 E6/E7 oncoproteins⁵⁵. Three different types of Tregs have been observed in equal proportions in the lesions of SCC: TGF β secreting Tregs, CD25- “inactive” n Tregs (? a reservoir of committed nTregs) and activated nTregs³⁵. CD25+ Tregs secreting TGF β has been demonstrated in lung cancer as well⁵³. High proportions of “inactive” nTregs within the tumour could pose a challenge for anti CD25 based therapy for eliminating nTregs. Although E6/E7 based vaccination is an attractive option for immunotherapy against cervical cancer, a serious concern is that such vaccination induces E6 / E7 specific Tregs simultaneous to the expansion of effectors⁵⁶. A similar phenomenon has been observed in EBNA1 peptide based vaccination against EBV associated malignancies⁵⁷. Low densities of peritumoral

infiltrates of CD3 and CD8 cells or lowered ratios of CD8/Tregs have been reported to be useful predictive markers of progressive disease in cervical and colorectal cancers^{34,58}. The latter may be relevant since the number of CD4+CD25+ Tregs is indexed to the number of IL2 producing CD4+ cells for the homeostatic control of different lymphocyte subsets⁵⁹.

An immunoregulatory microenvironment comprising of increased IL10 and TGFβ^{35,60} and reduced IL2 has been observed in invasive disease³⁵. Reports on the expression of IL2R and IFNγ have varied depending on the stage of SCC studied^{45,60,61}, since tumours are known to be heterogeneous in the later FIGO stages². One reason for this variation could be due to the differences in the methodology used in various studies – tumour infiltrating lymphocytes from patients with the same stage of SCC – were investigated differently^{62,63}. In addition, the possibility of IFNγ receptor negative mutants of the tumour getting sculpted as an immune escape mechanism should also be considered since such a phenomenon has been reported in other malignancies⁶⁴.

After the discovery of a new population of T cells *viz.*, the Th17 cells; the fact that one subset of Th17 cells also secrete IFNγ⁶⁵ and the reciprocal signaling between Tregs and Th17 cells⁶⁶, two aspects emerge: firstly that there may be a need to reinvestigate T cell infiltrates in SCC all over again. Secondly and more importantly, it would really be interesting to explore the consequences of converting Tregs into Th17 cells in SCC from a therapeutic angle.

The Notch signaling pathway plays a highly conserved role in regulating the cellular differentiation and proliferation events that characterize pattern formation in the embryo. Just as cells in the embryo respond to environmental signals, T-cells in the peripheral immune system also monitor their environment for antigens and respond accordingly by entering one of the several potential differentiation pathways. Cell to cell contact is a probable mechanism, through which Tregs impart their regulatory properties⁵⁰. Notch signaling appears to play a crucial role at multiple steps of T cell lineage development including the induction of Tregs⁶⁷. Experiments in mice have shown that over expression of Jagged 1 on dendritic cells induces antigen specific Tregs⁶¹. Concomitant ligation of Jagged1 by a Notch receptor modulates the consequences of these signals so that production of a Th1 or cytotoxic effector T cells is instead redirected

to the formation of Tregs. In addition, work from Sudhir Krishna's laboratory has shown that Jagged 1 is over expressed on the tumour cells of SCC⁶⁸. With this background information, it would be interesting to investigate whether interfering with notch signaling would reduce the number of Tregs in cervical cancer. Also, malignant cervical tumour cells are known to produce TGF⁴³, which in turn is known to induce Treg development⁶⁹, there is a possibility that this might be one of the mechanisms leading to increased Treg numbers in cervical cancer.

Hence, when immune cells and transformed cells localize to a common microenvironment, an assemblage of tumour eradicating and tumour promoting interactions take place; which though can coexist spatially; they might nevertheless be temporally distinct⁷⁰. This dynamic immune system – tumour cell interactions results in either destruction of the malignant cell by way of immunosurveillance or tumour outgrowth and can influence patient mortality. Immunologists in the field of HPV should define in detail the characteristics of those immune cells which are cast in a protagonist versus antagonist role in HPV mediated cervical cancer so as to potentially influence the process of immune mediated rejection of cervical tumours in the clinical setting.

Note: This manuscript is based on literature reviewed till May 2008.

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