



Th9 cytokines curb cervical cancer progression and immune evasion

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ABSTRACT

Cervical cancer is one of the most common cancers among women in developing countries. Persistent infection with high-risk human papillomavirus (HPV) is the major determinant for the development of cervical cancer. Role of newly discovered T helper 9 (Th9) cells in cervical cancer pathogenesis is yet unfolded. In this study, we observed a huge infiltration of PU.1⁺ cells and overrepresentation of IL-9R in tissue biopsy specimens of CIN patients in cervical cancer cases. Treatment with Th9 signatory cytokines, IL-9 and IL-21, suppressed proliferation, enhanced apoptosis and stimulated the expression of MHC I and e-cadherin on HeLa cell lines. Th9 thus seems enhance antitumor immune response through T cell cytotoxicity and play crucial role in a controlling malignant cell transformation. Therefore, this study helps in firmer understanding of relevance of Th9 in cervical cancer immunity.

1. Introduction

Cancer of uterine cervix (CaCx) is one of the most common cancer among women worldwide, with an estimated 485,000 new cases and 236,000 death in 2013 with more than 80% cases occurring in developing countries [1]. In India, cervical cancer is the leading gynecological cancer that shares about one fourth of the global cervical cancer burden [1].

Epidemiologically, it has been well established that persistent infection with high-risk human papillomavirus (HPV) is the major risk factor for the development of cervical cancer. Over 111 genotypes of HPV have been described till date but about 30 of them are associated with anogenital cancer. Though HPV types 16 and 18 are considered to be the most prevalent high-risk types worldwide, HPV 16 is exclusively high in India. Host immune response is considered to be a key determinant in cervical carcinogenesis after persistent infection with oncogenic, high risk HPV.

Lower rate of clearance of HPV in HPV-associated lesions of immune-deficient as compared to immune-competent patients clearly prove the critical role of Cell Mediated Immune (CMI) in HPV disease outcome [2]. Therefore, whether HPV infection will clear out or persist, rely heavily on type of T cell subset that make up the tumor micro-environment. Infiltration of those T cell subsets which inhibit tumor growth discourage cancer development and vice versa. Th9 is a newly discovered T cell subset that has been reported to impede tumor growth

in multiple cancers [3–5]. The ETS family transcription factor PU.1 is mandatory for Th9 development [6]. IL-9 and IL-21 are its signatory cytokines which seems to be instrumental in preventing and suppressing the development of cancers [4,7–9]. Recently Purwar et al. demonstrated that Th9 cells mediate robust tumor immunity against solid tumors through IL-9 production [10]. However, their role in cervical cancer immunity is yet to be unfolded.

2. Materials and Methods

2.1. Tissue specimen

A total of 82 biopsy tissue samples were obtained from the Department of Obstetrics and Gynecology at Safdarjang Hospital and LokNayakJaiprakash hospital, New Delhi. 28 cervical squamous cell carcinomas, 30 precancer and 24 from normal controls (UV-prolapse tissues, non-neoplastic). The clinico-pathological parameters of patients were collected as described by Bahnassy et al. 2006 [11]. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification [12]. Histo-pathological grades and clinical staging were evaluated according to standard criteria by two pathologists independently. Tissue samples were divided into two parts: one part was sent to histopathological diagnosis and other half was stored in -70°C for molecular investigations.

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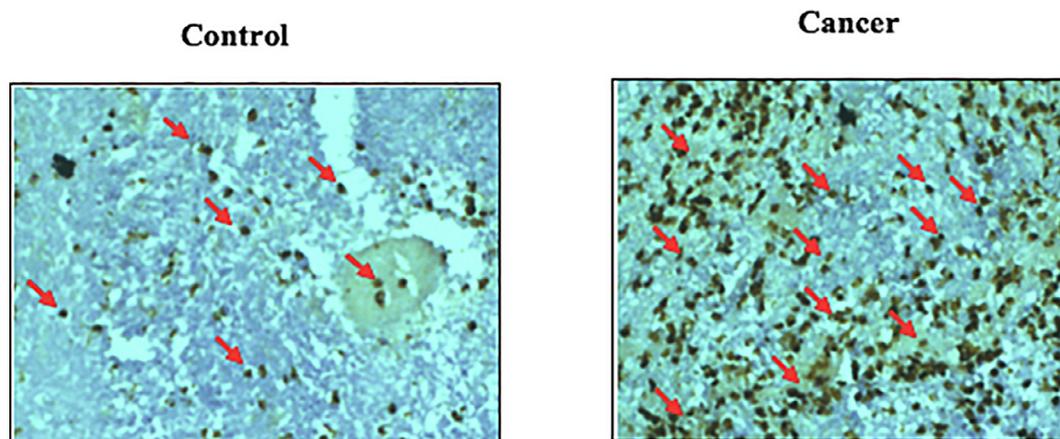


Fig. 1. Immunohistochemical localization of PU.1⁺ cells in tissue sections obtained from control and cervical cancer patients. The tissues were stained by DAB and counterstained with hematoxylin, as described in Section “Materials and Methods”. The presence of PU.1 transcription factor is stained brown and hematoxylin in blue. Right column: dense PU.1⁺ cell infiltration in representative tissue specimens from patients with advanced stages of cervical cancer. Left column: meager PU.1⁺ cell staining in representative control samples. The experiment was performed using tissues from 36 subjects. Original magnification 40 ×.

2.2. DNA extraction and HPV detection

Genomic DNA was extracted from cervical cancer tissue biopsy as well as control samples by standard proteinase K digestion followed by phenol/chloroform/isopropanol treatment. HPV diagnosis was performed by polymerase chain reaction (PCR) amplification using consensus primers MY09 and MY11 [13,14].

2.3. Immunohistochemistry

Immunohistochemical analyses were performed as described elsewhere [15]. Briefly, sections were cut from formalin fixed, paraffin wax embedded biopsy samples and mounted on glass slides, dewaxed and endogenous peroxidase activity was blocked. Antigen retrieval was performed and incubation with serum was done to block nonspecific binding. Slides were then incubated overnight with primary antibody diluted with 0.5% BSA, followed by fluorescently labeled secondary antibodies (Santa Cruz Biotechnology).

2.4. Cell culture

The HeLa, SiHa cells (NCCS, Pune, India) were cultured in DMEM (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 2 mmol L-glutamine (Gibco-BRL) and 50 mg/mL penicillin/streptomycin (Gibco-BRL). Cells were maintained in a humidified atmosphere of 5% CO₂ and 95% room air. Sub-cultivation of all cell lines was performed using 0.25% trypsin and 5 mmol ethylenediaminetetraacetic acid (EDTA) (Gibco-BRL). All experiments were performed during the logarithmic growth phase 15–20 h prior to the experiments.

2.5. Antibodies and reagents

Purified or polyclonal antibodies against human HLA-A,B,C (#311402), STAT3 (AP0365), PSTAT3 (y705#BS4181), PU.1 (R212#BS2649), CD4 (A429#BS1617), IL-9 (bs7697), IL9R (L514#bs1669), Ki67 (V3242#BS1454), FROM BIOWORLD (Recombinant IL-9 (Human, carrier free, #594402), recombinant IL-21 (Human, carrier free, #571202) from Bio legend.

2.6. Staining, and flow cytometric analysis

Cultured cells were harvested at treatment. Cell numbers were adjusted to 1 × 10⁷ cells/ml, and cell suspensions of 100 μL were

incubated with Fc receptor-blocking antibodies anti-CD16/32 (BD Biosciences). Subsequently, cells were stained with fluorescently labeled antibodies, as depicted. Cells were acquired on a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using Flowing software.

2.7. Statistical analysis

We used the Student *t* test for statistical analysis. Differences were considered statistically significant at P values less than 0.05.

3. Results

3.1. Prevalence of human papillomavirus infection

Persistence infection with high-risk HPV is one of the major causes for the development and advancement of cervical cancer. So, the collected samples were first screened for HPV infection. HPV infection was found to be in 80% (24/30) in precancerous lesions, 93% (26/28) in cervical cancer cases where as in controls, HPV prevalence was noticed only 8% (2/24). The analysis showed the significantly higher prevalence of HPV infection in pre-cancerous lesions ($p = 3.7 \times 10^{-25}$) and cancer cases ($p = 7.9 \times 10^{-36}$) as well in comparison to controls (supplementary table). We selected only HPV infected cases i.e. 26 cervical squamous cell carcinomas and 24 precancer (9 CIN1, 05 CIN2 and 10 CIN3) for further analysis. All the controls ($n = 22$) analyzed in the study were HPV negative.

3.2. Increased numbers of PU.1⁺ Th9 cells in the intra-tumoral tissue

The tumor-infiltrating immune cells play a vital role in deciding the kinetics of tumor growth, either via direct contact or cytokine-related signaling pathways. The type of T cell infiltration in tumor is associated with tumor immune response depending upon their biological function. We investigated whether there is any intratumoral recruitment of Th9 cells during cervical cancer development. We analyzed both normal and advanced cervical cancer tumor tissue. The PU.1⁺ Th9 cell density in the intratumoral tissues was much higher than in the normal tissue 9.6 (range, 2–21) vs. 1.8 (range, 0–11; $P < 0.0001$) (Fig. 1).

3.3. Increase in IL-9R

IL-9 binds to a complex composed of the specific IL-9R and γ_c , a subunit shared with IL-2, IL-4, IL-7 and IL-15 [16]. The cytokine

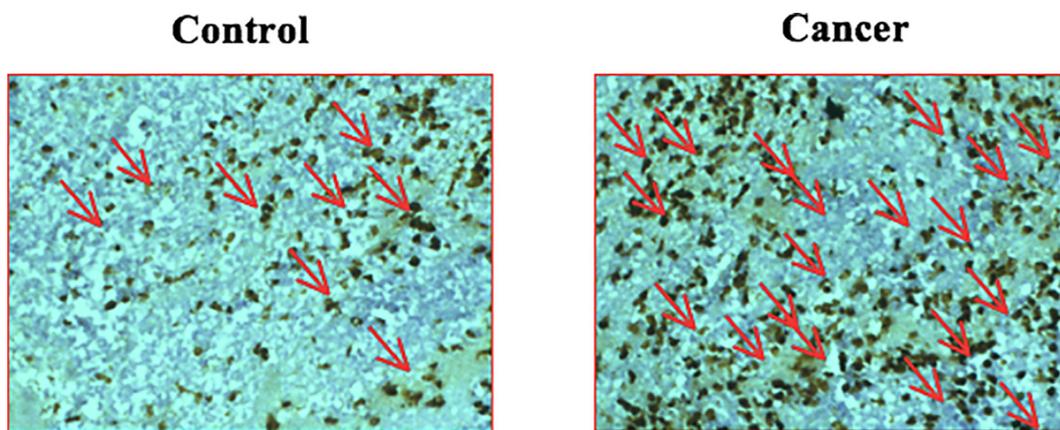


Fig. 2. Immunohistochemical localization of IL-9R⁺ cells in tissue sections obtained from control and cervical cancer patients. The tissues were stained by DAB and counterstained with hematoxylin, as described in Section “Materials and Methods. The expression of IL-9 receptor is stained brown and hematoxylin in blue. Right column: dense IL-9R⁺ cell in representative tissue specimens from patients with advanced stages of cervical cancer. Left column: meager IL-9R⁺ cell staining in representative control samples. The experiment was performed using tissues from 36 subjects. Original magnification 40 \times .

receptor expression levels are the limiting factor for signal transduction and cytokine mediated regulation. It has been previously shown that the level of expression of the hIL-9R is correlated with the intensity of STAT signaling and the ability of hIL-9 to inhibit cell growth [17]. The IL-9 signaling is mediated via IL-9 receptors that lead to activation of STAT1, STAT3, and STAT5 [18]. Here, we observed increased expression of IL9R⁺ and PU.1⁺ cells (as discussed above) in advanced stages of cervical cancer 13.8 (range, 3–32) vs. 5.6 (range, 2–17; $P < 0.0001$) as compared to control. Exogenous rIL-9 and rIL-21 increased expression of IL-9 receptor on cervical cells. Hence, we observed that there was not only enhanced recruitment of Th9 cells in intra-tumoral environment but also overexpression of IL-9R. The enhanced expression of IL-9R may be mandatory for IL-9 to mediate its aggravated anti-tumor effect (Fig. 2).

3.4. Change in morphology

A visual inspection of changes in the morphology of the cervical cancer cells was done 24 and 48 h after exogenous rIL-9 and rIL-21 treatment by phase contrast microscope. Cell shrinkage started at around 22 h in ~30% of cells, losing their elongated form. By 48 h, 50% of the cells showed cytoplasmic condensation in comparison with untreated cells at concentration of 10 ng/ml of cytokine. Cervical cells showed rounded shape leading to their selective detachment and a subsequent reduction in adherent cultured cell number (Fig. 3).

3.5. Exogenous rIL-9 and rIL-21 inhibited proliferation and promoted apoptosis of cervical cells

Optimal expression of both IL-9 and IL-9 receptor (IL-9R) is mandatory for tumor inhibition mediated via IL-9 signaling. Administration of rIL-9 has been reported to have protective effect against tumor progression. Adoptive transfer of Th9 cell have displayed the strongest reduction in melanoma tumor growth and induced melanoma cell apoptosis *in vitro*, among the different effector T-cell subsets, which was mediated partially through IL-9 signaling [10]. Th9 has been reported to induce immunosuppressive effect and hamper tumor progression [19,20]. Enough evidences have accumulated that demonstrate the anti-tumor response of both Th9/IL-9 [10,21].

These results support the critical and decisive role played by IL-9 and Th9 cells in tumor immunity. To assess the role of exogenous rIL-9 and rIL-21 in cervical cell growth, we examined the Ki 67 staining of both treated and untreated cells. Ki-67 antigen is highly expressed in the nuclear region of proliferating cells. We observed a considerable reduction in proliferation of cells after 48 h treatment of exogenous rIL-

9 and rIL-21 as compared to untreated cells. The results show that the cytokine induced significant accumulation of cells in G0/G1 phases and reduced the number of proliferating cells by approximately 25% (Fig. 4).

3.6. Exogenous rIL9 and rIL 21 upregulated e cadherin expression

E-cadherin plays a major role in controlling malignant cell transformation, tumor development and progression either by inducing apoptosis, inhibiting proliferation and growth of cells [22,23]. There are several lines of evidence that reflect the potency of e-cadherin expression in regulating cell proliferation and apoptosis in various types of cancers like esophageal, pulmonary, squamous head and neck, pancreatic and cervical [22,24]. Their loss is associated with increased invasiveness and metastasis of tumors [25], resulting in it being referred to as the “suppressor of invasion” gene. Here, we demonstrated an increase in e cadherin expression in cervical cells in response to exogenous rIL-9 and rIL-21 by 17.5% (Fig. 5). Hence, IL-9 and IL-21 seem to play critical role in baring the masked shielded tumor cells to host immune surveillance and help in controlling tumor growth.

E-cadherin is a well-known tumor suppressor protein, and the loss of its expression in tumor cells, in association with the epithelial–mesenchymal transition (EMT), occurs frequently during tumor progression and metastasis [26–29].

3.7. Exogenous rIL9 and rIL21 increased MHC I expression

The immune system has developed multiple ways to counteract immune evasion mechanism of tumor cells. Up-regulating suppressed MHC-1 expression, inhibiting tumor progression either by down-regulating its proliferation or inducing apoptosis are crucial ways to negate their evasion (Th)1 cells curb cancer development via mechanisms commonly involving their production of interferons and cytotoxins [30].

Countering the immune escape mechanisms of tumor cells, preventing them from shielding away from immune surveillance and making them easily accessible to T cell cytotoxicity are the functional features of secreted cytokines that embed antitumor effect. Antigen presentation of tumor associated antigens by MHC-I allows recognition and destruction of tumor cells by CD8 + T cells which distinguish between normal and cancerous cells by monitoring intensity of MHC-I on cell surface [31,32]. Here, we demonstrated an increase in major histocompatibility complex class I (MHC-I) expression in cervical cells in response to exogenous rIL-9 and rIL-21 by approximately 26%. This will expose the otherwise hiding tumor cells to immune check as

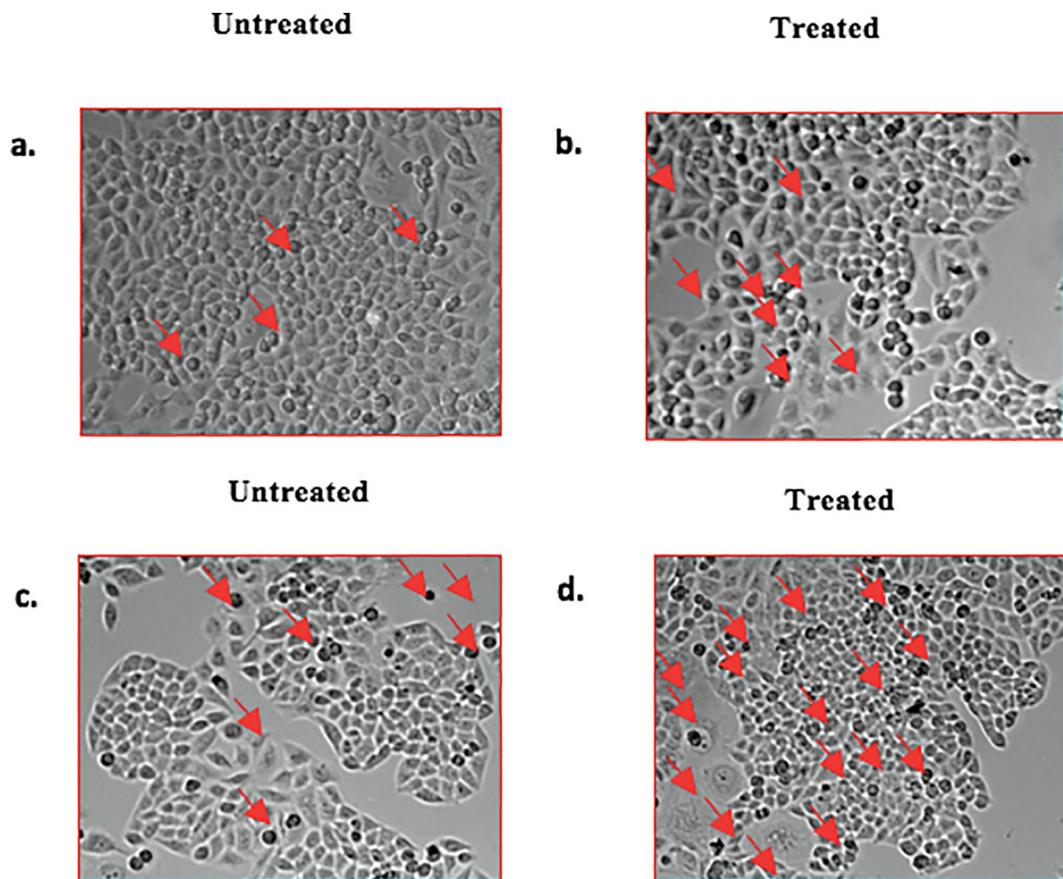


Fig. 3. Morphological observations of HeLa cells under light microscopy. a. Untreated HeLa cells, 24 h. b. HeLa cells treated with 10 ng/ml of IL-9 and IL-21, showing rounded apoptotic bodies, 24 h. c. Untreated HeLa cells, 48 h, showing few apoptotic bodies. d. HeLa cells treated with 10 ng/ml of IL-9 and IL-21, showing high frequency of rounded apoptotic bodies and cytoplasmic shrinkage as compared to untreated HeLa cells.

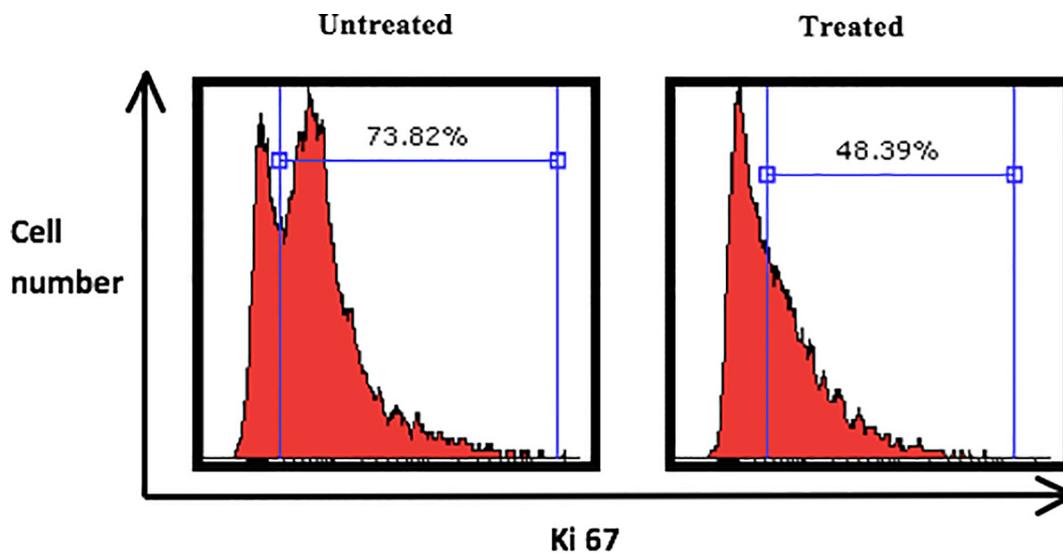


Fig. 4. Effects of IL9 and IL21 on proliferation of treated HeLa cells. Cells incubated with or without IL9 and IL21 (10 ng/ml) for 48 h and analyzed through FACS for Ki67staining. Biopsy cells from untreated and treated cells were stained with antibodies against Ki 67. Expression of Ki 67 on biopsy cells was analyzed.

downregulating MHC I expression is a key strategy in tumor immune evasiveness [33]. Here, we demonstrated an increase in major histocompatibility complex class I (MHC-I) expression in cervical cells in response to exogenous rIL-9 and rIL-21. Hence, IL-9 and IL-21 seem to play critical role in baring the masked shielded tumor cells to host immune surveillance and help in controlling tumor growth (Fig. 6).

4. Discussion

Our study has revealed that there is increase in infiltration of Th9 cells to tumor site in cancerous cases as compared to control. Th9 is a newly discovered T cell subset that is characterized by ETS family transcription factor PU.1 that is mandatory for it’s development [6]. Numerous studies have shown that Th9 impede tumor growth in

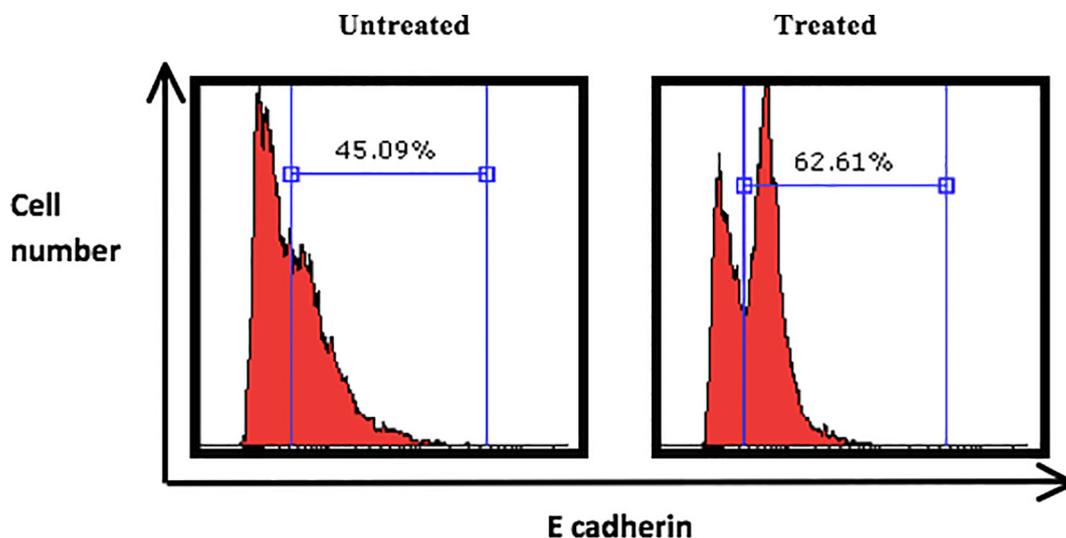


Fig. 5. Effects of IL9 and IL21 on proliferation of treated HeLa cells. Cells incubated with or without IL9 and IL21 (10 ng/ml) for 48 h and analyzed through FACS for e cadherin staining. Biopsy cells from untreated and treated cells were stained with antibodies against e cadherin. Expression of e cadherin on biopsy cells was analyzed.

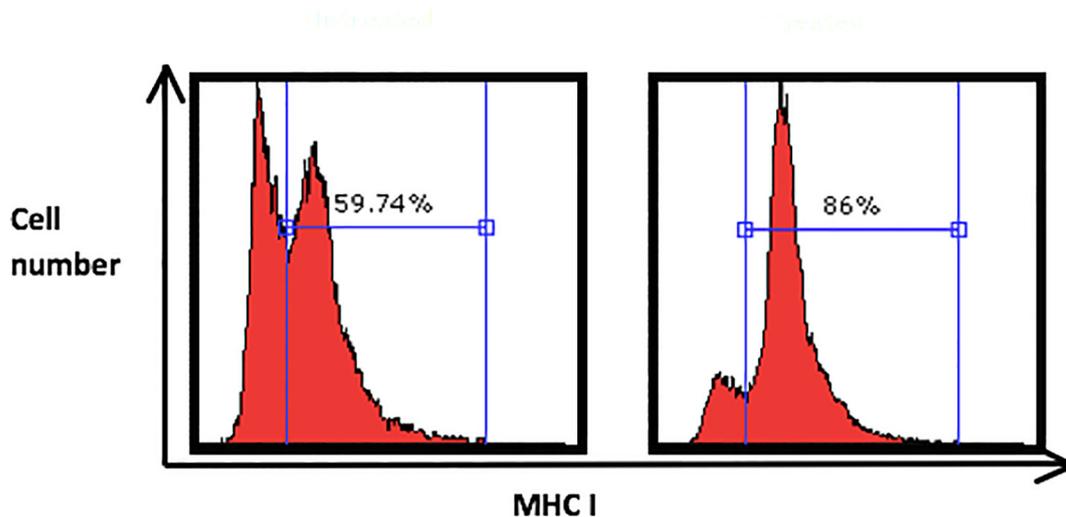


Fig. 6. Effects of IL9 and IL21 on proliferation of treated HeLa cells. Cells incubated with or without IL9 and IL21 (10 ng/ml) for 48 h and analyzed through FACS for MHC I staining. Biopsy cells from untreated and treated cells were stained with antibodies against Ki 67. Expression of MHC I on biopsy cells was analyzed.

multiple cancers [3–5]. We found that $PU.1^+$ are recruited immensely to the tumor site. This infiltration was also associated with an increase of MHC-I expression on cervical cells.

The antitumor effect of IL-9 has been reported to be due to upregulation of immunosuppression mediated by Treg cells and mast cells [19,20]. To investigate the potential role of Th9 in cervical cancer pathogenesis, we detected the effects of its signature cytokines (rIL-9 and rIL-21) on cervical cell apoptosis and proliferation. We found that treatment with exogenous rIL-9 and rIL-21, increased the apoptosis of cervical cells and hampered its proliferation. In addition to this, exogenous treatment of cervical cells with these cytokines changed the morphology of the cervical cancer cells where cell shrinkage, cytoplasmic condensation, rounding of cells and loss of adherence to surface was clearly evident. Hence, Th9 signature cytokines dampens the viability, proliferation and growth of HPV cervical cell lines. Consistent with this notion, previous reports have shown that IL-9 and IL-21 are Th9 signatory cytokines that are instrumental in preventing and suppressing the development of multiple cancers [10,34–36].

For an effective immunotherapy it is essential that the markers which expose the tumors to immune surveillance should increase in

expression, MHC-I is one such critical marker which has been observed to be otherwise downregulated in tumors to escape CTLs [37,38]. The capacity of Th9 to exhibit immune suppressive activity can further be explained by observed upregulation of e cadherin and MHC I expression in HPV cervical cell lines when treated with exogenous rIL-9 and rIL-21. Hence, these cells were able check the evasion mechanism tumor cells were using to escape immune surveillance. Thus, our study suggest that Th9 contribute to antitumor immune response with the help of multiple mechanisms.

In summary, we found that IL-9 receptor is markedly overexpressed in cervical cancer cells to mediate antitumor immune response of rIL-9 and rIL-21 that are secreted by $PU.1^+$ Th9 cells. These cytokines restrict tumor proliferation, enhance apoptosis and induce expression of MHC I and e-cadherin expression on cervical cells so that they express tumor antigens and don't escape immune check. This effect of Th9 seems to be independent of STAT3 signaling. Hence the intratumoral recruitment of $PU.1^+$ cells, IL9 and IL9 receptor overexpression may be involved in regressing cervical cancer progression and may be beneficial in immune check as has been reported elsewhere [39,40]. Therefore, this study helps us to get a better understanding about the role newly discovered

Th9 cells in cervical cancer and its use as a potentially therapeutic target for cervical cancer patients in the future.

5. Limitation

As the whole study was performed in Human Papillomavirus infected samples/cell lines, so we could not dissect the role of HPV in current study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Author contributions

S.R.C. designed the study, searched the literature, performed the experiments, analysed data, interpreted the results, and wrote the draft manuscript. P.S. Collected samples, interpreted the results and corrected the manuscript. U.S. and K.B. performed the experiments. K.C. designed the study, contributed reagents/material/analysis tools, interpreted the results, and corrected the manuscript. M.B. designed the study, provided the reagents/material/analysis tools and corrected the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.humimm.2019.09.009>.

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