# Chapter V:

## Study of NK cell polarization towards a decidual phenotype in the Tumor Microenvironment.

## 5.1 INTRODUCTION:

Reproduction in any living organism is considered to be a predominant trait,necessary for the continuity of the species. Therefore, all the biological functions and mechanisms which are involved in the process of reproduction are known to be powerful drivers of the cell cycle. In humans, the interface driving the maternal and the fetal dynamics allows various dynamic chances to allow the allogenic fetus to develop in spite of being marked as foreign by the maternal immune system(1).During the first trimester of pregnancy, almost 40% of all the cells in the decidua are the immune cells which are mainly responsible for the protection of the fetus from pathogens. In addition, these immune cells also provide tolerance to the embryo in early decidua.

The Natural Killer cells (NK), which are part of the Group 1 innate lymphoid cells (ILC), are responsible for the protection of the host from viral infections and diseases like cancer. Interestingly, approximately70% of the immune cells in decidua are NK cells, however, the nature of the decidual NK cells (dNK), changes from its original cytotoxic nature to a more of “nurturing” cell which produces the required cytokines for the protection and development of the fetus.(1, 2). Theoretically, dNK cells are considered as a type of NK that shows a CD56 bright CD16-/KIR + phenotype and are characterized by the expression of CD9 marker which is member of tetraspanin family. CD9 is known to be associated with integrin adhesion receptors which regulates cell invasion and migration and are also found to be present in the exosomes. At present, dNK1, dNK2, and dNK3 are the three types of dNK cells that have been identified by sequencing of human maternal and fetal interface and all of them expressing CD9 (3). One of the most important function of dNK cells is the production of large amounts of IFN-γandproangiogenic cytokines which are very crucial factors for the formation of spiral arteries and vascularization of the decidua.(4-6). In addition, dNK cells plays a critical role in maintenance of the immune balance in the decidua and as an immune suppressant whenever required. Data from various investigators have suggested that dNK cells plays a pivotal role in maintenance of immune tolerance in the decidua by controlling local inflammation. Therefore, in order to provide a microenvironment that supports a healthy pregnancy,dNK cells are found to lose the original killer phenotype of NK cells(1).

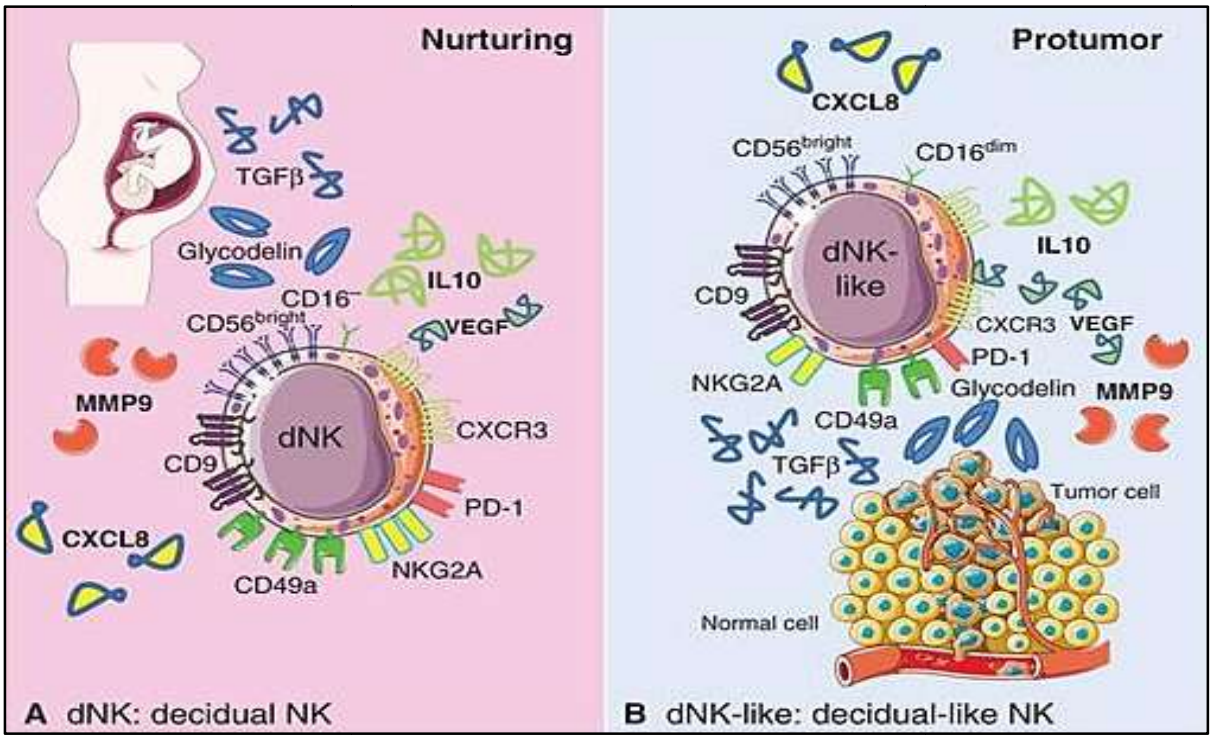



Figure 21: (**A)**CD56brightCD16−CD9+, CD49a+ dNK cells nurturing in the reproductive system, helping embryo implant and fetal development. dNK cells produce proangiogenic factors(**B)** CD56brightCD16−, CD9+, and CD49a+ dNK-like cells, displaying protumoractivities, and nurturing in cancer.

It is well established in various preclinical as well clinical studies that most of the tumors are the target of immune clearance by the NK cells and also in many instances may carry distinct advantages over T cells(7). However, it has also been found in many studies that various tumors have also developed strategies that helps them to evade the NK cell mediated immune cytotoxicity which includes disruption of the interactions of the NK cells receptors and ligands. In addition, the more recent strategy that has been in focus is the polarization of the NK cells into a decidual cell type phenotype in the tumor microenvironment(8).Several recent reports have suggested that NK cells with CD56brightCD16−/lowphenotype in tumor microenvironment expresses CD9 and shows a decidual-like phenotype that mimics a program that is typical for embryo development and fetal growth(8, 9). Additionally, the deployment of proangiogenic and non-cytotoxic NK cells in the tumor microenvironment might occur through several mechanisms that includes: (i) Reprogramming of NK cells according to the TME, (ii) NK cells decidualization by migrating poorly cytotoxic peripheralCD56brightCD16− NK cells in

the TME, and (iii) Survival and proliferation of non-cytotoxic proangiogenic NK cells (9, 10).

TGFβ, a common and vital component of the TME is found to be present in the decidua involving in promotion of the dNK phenotype. It has been observed in various studies that upon treatment with TGFβ, the cytolytic CD56dimCD16+ NK cells polarizes towards aCD56bright phenotype which shows proangiogenic, non-cytotoxic and an immuno-suppressed characteristic(9). Further, it was found that these subpopulations of NK cells were unable to provide immunity against tumor growth (10). It is noteworthy that although the presence of CD56brightCD16−/low cells in the tumor microenvironment and its consequences have been the prime focus of investigation in recent years, less emphasis have been given to the role of the decidual marker CD9 in the tumor infiltrated NK cells. There are few evidences that the characteristics of the CD56brightCD16−/low NK cells in normal tissue differs vastly from those found in the TME (9-11).Recently, studies have reported the expression of CD9 marker along with the presence of CD56brightCD16−/low NK cells inbreast cancer,melanoma, glioblastoma,lung andcolorectal cancers(11-14). Therefore, it can be hypothesized that the mechanisms that are involved in the recruitment and functioning of CD56brightCD16−/lowNK cells in the decidual environment is mimicked in the TME in patients, and thus have a potential to be a valuable marker in several types of cancer. In the present study, we initiated a preliminary study for the hypothesisthat NK cells in the tumor microenvironment may become polarized toward adecidual phenotypethereby aiding cancer growth instead of killing cancercells.

## 5.2. Materials and methods:

### 5.2.1.Study site, study design, and participants

The study site, study design and study participants are the same as described in section 3.2.1. Single-cell suspension culture from the different parts of HNSCC tumor tissue was standardized. Five samples were collected immediately after the surgery and processed for a single cell suspension culture in the laboratory. The isolated single cells were further checked for NK cell phenotype activation using specific markers (CD56, CD16 and CD9) by flow cytometric analysis.

### 5.2.2. Preparation of cells from whole blood

The process of determination of NK cell phenotypes by the flow cytometer (BD Biosciences, India) was first standardized in whole blood of the healthy individuals and gating was determined for the NK phenotypes. The protocol used for the determination of NK phenotype using whole blood is briefly summarized below-

Collection of 100µL of whole blood

↓

Incubated with fluorochrome-conjugated (BD, USA, CD3 PerCP-Cy5.5, CD16 PE+CD56 APC, and CD9 FITC) for 15 minutes at room temperature

↓

RBC lysis using 2mL FACS lysing solution (BD Biosciences, United States)

↓

Centrifugation at 1500 rpm for 5 mins

↓

Cell pellet obtained washed with 2mL of PBS

↓

Resuspended in 500 µL of PBS

↓

Flow cytometric analysis for NK Phenotype in BD accuri C6 plus flow cytometer (BD, Biosciences, United States

↓

At least 10,000 cells from each sample were analysed in the P1 gate.

### 5.2.3Single-cell suspension culture of tumor tissue

To determine the NK cell phenotypes in tissue, flow cytometric analysis was performed using monocyte specific markers– CD56, CD16 and CD9. For that, single cells were isolated from the freshly obtained post operated tumor tissues using both the enzymatic method as well as explant culture method. The protocol used for the single-cell isolation is briefly summarized below-

Tissue samples collected in RPMI media (RPMI+antibiotic+antimycotic solution)

↓

Washed with PBS three times and then washed with incomplete media

↓

Cut the tissue into small pieces using a surgical blade

↓

Incubated with the enzyme cocktail (elastase+ hyaluronidase + collagenase + trypsin) for 30-45 minutes at 37°C temperature with continuous stirring and checked for cell separation

↓

The supernatant was collected, the enzyme inhibitor was added and then centrifuged at 600g for 5 minutes at 4°C to obtain the pellet

↓

Pellet was washed with chilled PBS and re-suspended in culture medium and cells with media were strained in a cell strainer

↓

Cells were checked for cell viability under the microscope and Muse™ Cell Analyzer (Millipore, Burlington, USA)

↓

100µL of the cells were taken and incubated with CD56 and CD16 antibodies (BD Biosciences, India) as per manufacture's instruction

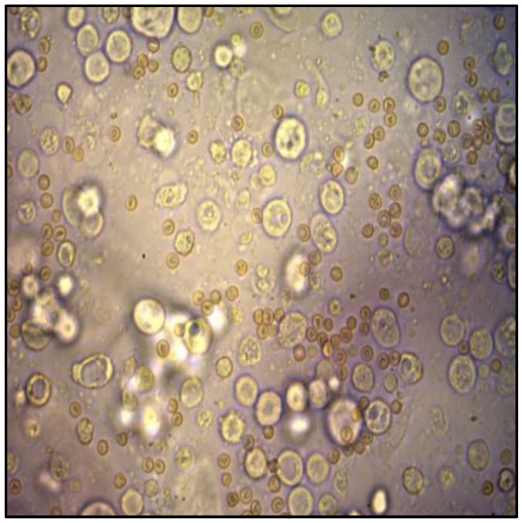
↓

Washed with 2ml of PBS and resuspended in 500 µL of PBS for flow cytometric analysis in BD accuri C6 plus flow cytometer (BD, Biosciences, United States)

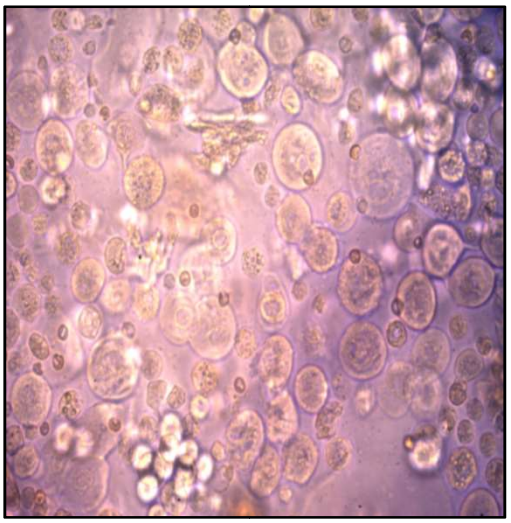
**5.3 Results:**

**5.3.1. Single-cell suspension culture**

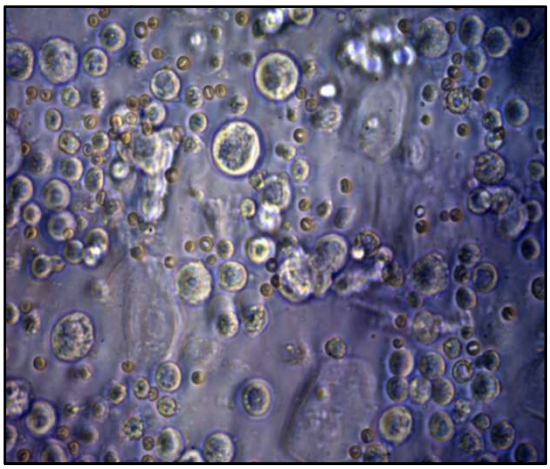
NK cell phenotypes were determined in patient blood and single cells isolated from the tumor tissues. Single-cell suspension culture was standardized, and cells were isolated by enzymatic treatment method as well as explant culture as presented. Cell viability was determined in Muse™ Cell Analyzer (Millipore, Burlington, USA). Isolated cells were viable with 99-100% viability.



(A)

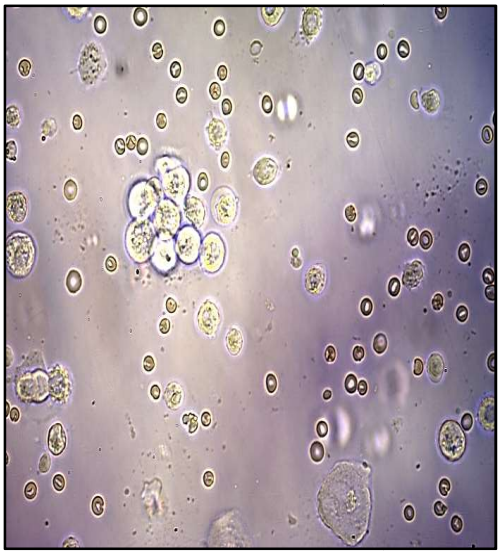


(B)

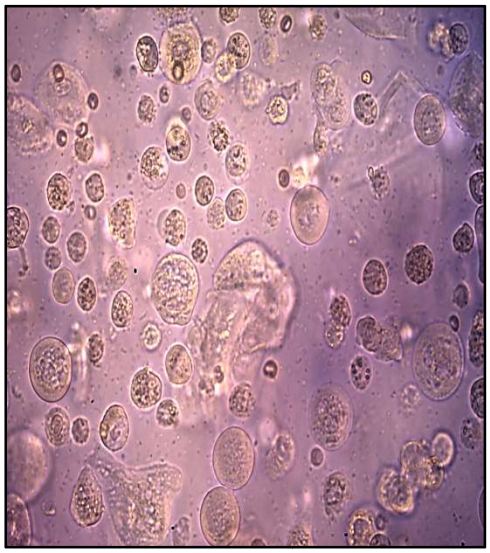


(C)

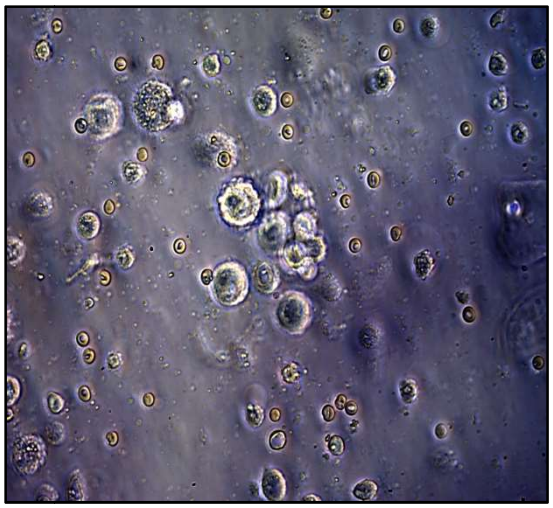
Figure 22: Single cell suspension culture of post operated tumor tissue by enzymatic method - Cells in RPMI and DMEM media obtained from explants method (400X)RPMI media (A), DMEM media (B) and Phase contrast image (C)



(A)



(B)



(C)

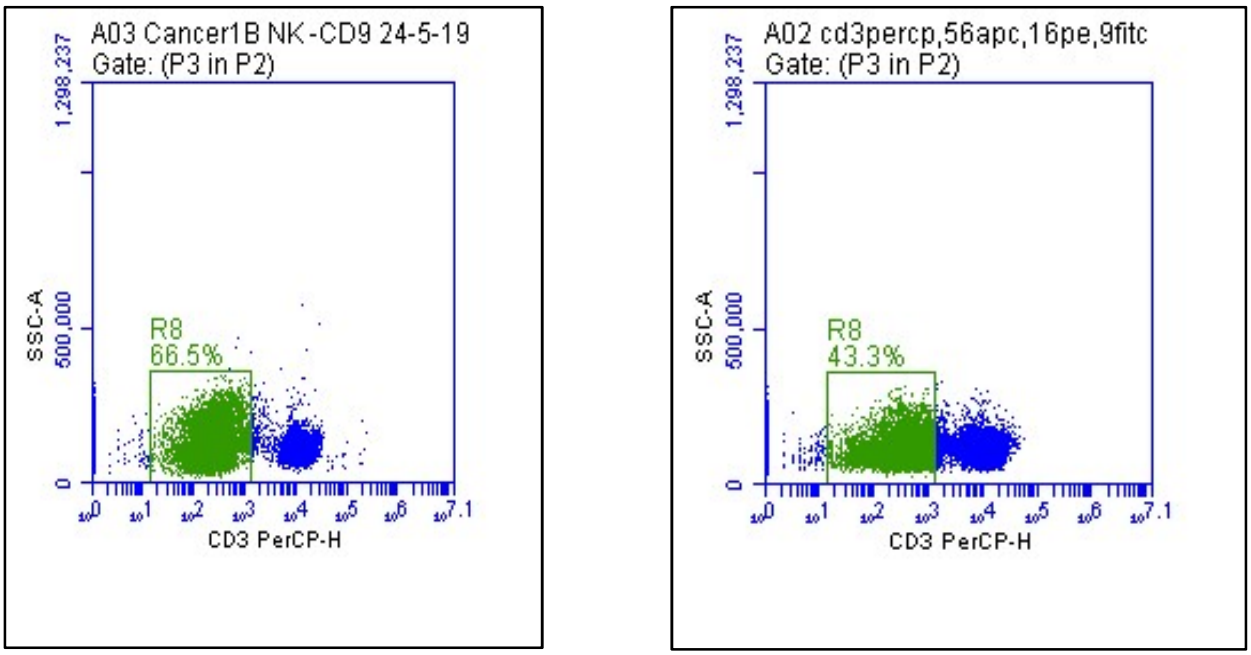
Figure 23: Single cell suspension culture of post operated tumor tissue by explant culture - Cells in RPMI and DMEM media obtained from explants method (400X) RPMI media (A), DMEM media (B) and Phase contrast image (C)

### 5.3.2 Tumor resident NK cells did not show decidual NK cell phenotype:

CD16+CD56dim cells comprised 60.8% of the NK cell population in the tumor tissue and 57.3 % in patients' blood, suggesting the predominance of mature NK cells in tumor tissue and peripheral circulation. An intermediate stage of matured NK cells i: e CD16+CD56+ was also seen that comprised 35.4 % and 20.8% of the cells in blood and tissue extracts respectively. CD9+ decidual NK cells were seen at 0.03%-0.05% of the total NK cells in both blood and tumor tissue negating the assumption that the non-cytolytic characteristic of tumor NK cells was due to the recruitment/ predominance of decidual NK cells in the tumor microenvironment.

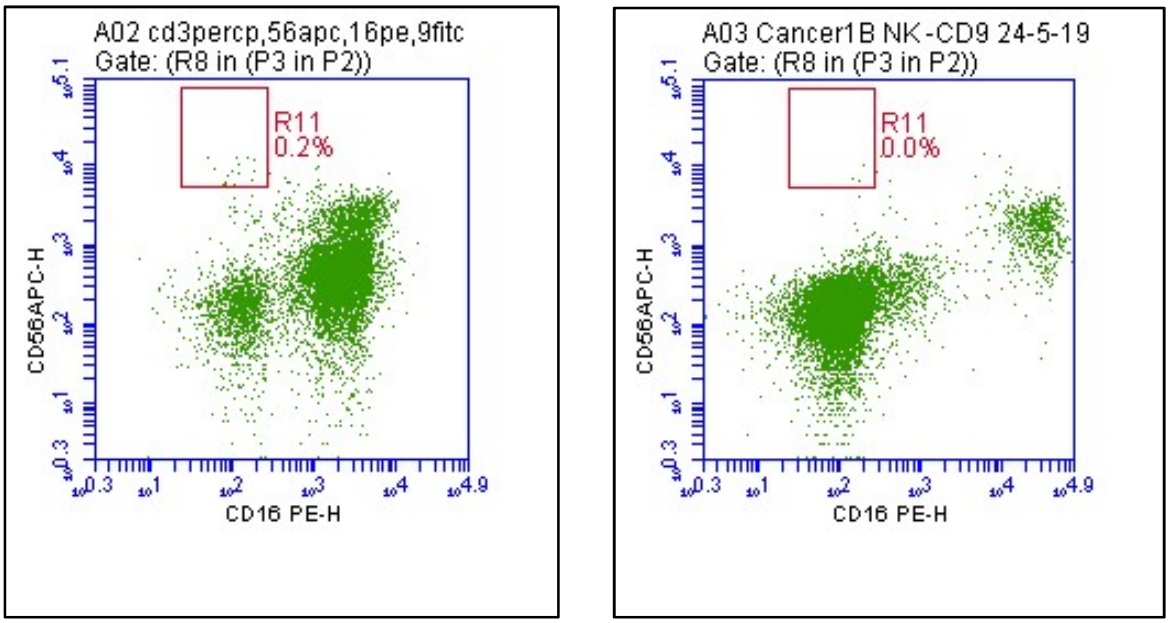
| | Blood | Tumor Tissue |
|---|---|---|
| **CD16+ CD56$^{dim}$ (Mature)** | 57.3% | 60.8% |
| **CD16+CD56+ (Intermediate)** | 35.4 % | 20.8% |
| **CD16-CD56$^{Bright}$ (Immature)** | 0.57% | 0.4% |

**Table 11:** Study of NK cells maturation status in HNSCC patients' blood and tumor tissue.




(A) (B)

Fig 24: Exclusion of B-cells and NK cells from CD3+ T cells in (A) Controls (B) HNSCC Patient




(A) (B)

Fig 25: Graphical representation of CD56 vs CD16 Plot in (A) Controls (B) HNSCC patient

**5.4: Discussion:**

Natural killer (NK) cells are the primary innate immune cells that mediate anti-viral and anti-tumor responses and are regarded as promising candidate for NK-based immunotherapy(15). Predominantly, most of the NK cells are CD56dimCD16+ cytotoxic cells and only about 5% of the circulating NK cells are found to be of CD56brightCD16 phenotype. NK cells primarily have a regulatory activity towards the cells of both innate and adaptive immune system and upon activation, produces large amount of cytokines like IFN-g and TNF-a(16-19). In cancer, NK cells have the ability to counter and control both tumor growth and metastasis (16-20); however, several investigators have reported that the cytotoxic activity of the NK cells are sometimes compromised in some patients with cancer (5, 21, 22).In addition, it has been found that normal cells especially innate immune cells from the tumor microenvironment display proangiogenic characteristics and the invasive and migratory ability of those cells allows them to infiltrate the tumor and contribute in tumor malignancy by becoming towards a pro-tumorigenic phenotype(23-25). In that context, one of the most important immune cells that have shown a dual faced

characteristics in the Natural Killer cells which can change to builders from killers and can act as an ally to the cancer (26). Interestingly, the particular subpopulation of NK cells that have been characterized in the tumor-microenvironment in some cancers is the same NK cell subpopulation that has been defined within the decidua during the first trimester of pregnancy. That subpopulation is termed as uterine or decidual NK (dNK) cells(6, 27-29).

Decidual NK cells are characterized by a CD56brightCD16 phenotype as well as by the presence of specific cell surface markers like CD9 and are comprised of almost 30-5-% of all the lymphoid cells in that particular tissue.

Data from various researchers have shown that numerous mechanisms are employed by the tumor microenvironment to evade the antitumor responses from the immune cells and possess the ability to polarize and destabilize the innate immune cells that includes the NK cells, macrophages and dendritic cells or the cancer associated fibroblasts(30, 31).One of the most commoncytokine that has been observed in various cancers is TGF-β. Multiple investigators have reported the TGF-β mediated down-regulation of NK cell functions and activating receptors resulting in invasion, proliferation, angiogenesis and immunosuppression (32).

From our study also, we found that the expressions of the key pro-inflammatory cytokines having a role in NK cell activation – TNF-α (2.45-fold) along with IL-18 (1.46-fold) and anti-inflammatory cytokine– TGF-β (2.47-fold) was found higher in HNSCC patients. Also, the transcript expression of IFN-γ, a key cytokine of NK cells and a master regulator of immune responses along with IL-12 was found to be downregulated in patients. The above observations suggested an immunosuppressive microenvironment in HNSCC patients favoring the proliferation of suppressive immune cells. In addition, expression of VEGF was seen in patients from all tumor stages whereas, Ki67 expression was higher in stage III-IV patients whereas. Previous studies from our lab have also reported thetumor resident NK cells to have lower cytolytic ability(33).We reasoned that the lower cytolysis of the NK cells may be related to either the maturation status of the NK cells or their immune suppression in the tumor microenvironment (TMO). However, the preliminary data from the present study negates the possible recruitment of immature NK cells during cancer and

suggested that although the NK cells are matured, there is diminished cytotoxic activity due to the immunosuppressive tumor microenvironment in HNSCC patients.

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