"COMPARATIVE EVALUATION OF NATURAL KILLER CELL (CD57) EXPRESSION IN ORAL LEUKOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY"

Dissertation submitted to

Maharashtra University of Health Sciences, Nashik

in the Partial Fulfillment of Regulations

for the award of the Degree of

MDS

IN

ORAL PATHOLOGY AND MICROBIOLOGY

BRANCH VI

2019

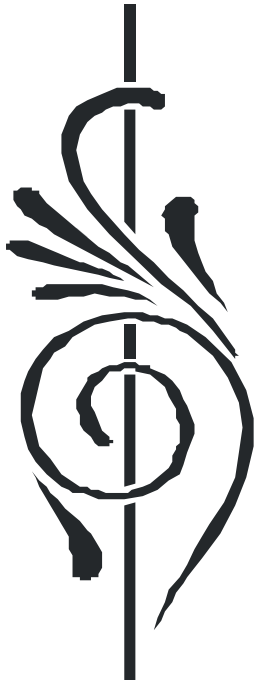
INDEX

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES

INTRODUCTION



INTRODUCTION

Hippocrates has rightly said:

"Natural forces within us are the true healers of disease"

"The difference between destruction of the immune system and stimulation of the immune system is the interpretation of a disease." - *Anonymous*.

Cancer is one of the most prevailing diseases in the World. Oral cancers rank from 6th to 8th as the most common cancers in the World[1] and account for about 30-40% of all malignancies in India.[2] Over 90% of oral cancers are squamous cell carcinomas.[3] Oral squamous cell carcinoma (OSCC) is a hyper-proliferative disorder.[4] Squamous cell carcinomas are heterogeneous cellular entities whose growth is dependent on reciprocal interactions between cells that are genetically altered and the

microenvironment in which they dwell. The microenvironment or more commonly referred as the tumor stroma is required for nutritional maintenance and removal of discarded products. It consists of connective tissue, blood vessels, innate and adaptive immune cells and so forth.[5] Throughout the World, extensive investigations have been undertaken during the past two decades, on the various aspects of oral cancer. Despite marked improvements in various therapeutic modalities, the rate of 5-year survival among oral cancer patients is still low.[6]

About 80% of oral cancers are preceded by potentially malignant oral disorders in India.[2] Leukoplakia is among the most common potentially malignant oral disorders.[2] Leukoplakia is a clinical term originally defined as a predominantly white lesion of the oral mucosa that cannot be characterized clinically or histopathologically as any other definable disease.[7,8] The prevalence of leukoplakia in India varies from 0.2% to 5.2%.[3] Malignant transformation rates in leukoplakia varied from 0.13% to 10% in various Indian populations.[9,10]

Immune system of an individual plays a key role in battling against carcinomas. When immunosuppression has been present for significant period of time, the likelihood of a malignant tumor appearing is enhanced.[11] Host immune reaction probably works for preventing malignant transformation and proliferation of the cells; however, if it is insufficient – or when it falls down – the tumor develops. Mononuclear immune inflammatory cell infiltration in the tumor stroma is thought to represent this immune reaction against tumor cells.[12] Stromal mononuclear immune inflammatory cell reaction is one of the typical features of the head and neck cancers, including oral location.[13]

The key role of human immune system is to confront cells undergoing carcinogenesis. Manipulation of the immune system against the tumor cell is multifactorial. The immune defence against tumor cell is mediated initially by the innate immune cells, i.e., Natural Killer (NK) cells, NKT cells, cytokines and complement proteins, and later by adaptive immune system (B and T cells). NK cells are one of the most important effector lymphocytes with an effective anti-tumor effect.[14,15]

NK cells are bone marrow derived large, granular lymphocytes comprising 10-20% of peripheral mononuclear cells and act as important immune effecter cells against tumors. NK cells are considered to be the first line of defence against tumor cells, cells showing dysplastic changes and virally infected cells. Immunosurveillance by NK cell is carried out by two mechanisms: cytokine release - interferon-γ and perforin dependent target cell elimination. The presence of NK cells is believed to have a good prognostic value for OSCC. Its role in leukoplakia is still under research.[15]

NK cell activation is determined by the balance between the stimulation of inhibitory and activating receptors. If the inhibitory receptor signalling is more prominent, NK cell activity will be inhibited; similarly, NK cell activation will result if the activating signal is dominant.[4]

CD57 was first identified on NK cells and was subsequently assigned the cluster of differentiation (CD) designation, CD57, at the Fourth International Workshop of Human Leukocyte Antigens in 1989.[17] $CD57^{+}$ NK cells are long-lived cells that have encountered pathogens and represent human "memory" NK cells.[14]

Progression from $CD56^{bright}$ to $CD56^{dim}$ $CD57^{-}$ to $CD56^{dim}$ $CD57^{+}$ reflects a maturation pathway for NK cells and rather than being a marker of anergy or immunosenescence, acquisition of CD57 represents a shift towards a higher cytotoxic capacity, greater responsiveness to signalling via CD16 and natural cytotoxicity receptors (NCRs) and decreased responsiveness to cytokines.[16] CD57 cluster expression is chosen for evaluation in this study because it represents the end stage of NK cell maturation. It is intriguing to understand that immature $CD57^{-}$ NK cells may contribute to autoimmune inflammation and tissue damage whereas more highly differentiated, cytotoxic, $CD57^{+}$ NK cells may fulfill an immunoregulatory role.[17,18]

The intent of this study was to characterize the immune response in Oral Leukoplakia and OSCC and to comparatively evaluate the specific expression of NK cells ($CD57^{+}$) in oral leukoplakia and different grades of OSCC.

After extensive research, it has been observed that this is the first study to evaluate and compare $CD57^{+}$ NK cell expression in Oral Leukoplakia and OSCC.

AIM AND OBJECTIVES

AIM AND OBJECTIVES

The present study will be an attempt to analyse the comparative expression of NK cells ($CD57^+$) in oral leukoplakia and in oral squamous cell carcinoma with the following aim:

AIM: To analyse the comparative evaluation of NK cells ($CD57^+$) in oral leukoplakia and oral squamous cell carcinoma.

The aim will be fulfilled with the help of the following objectives:

OBJECTIVES:

1. To evaluate expression of NK Cells ($CD57^+$) in oral leukoplakia.
2. To evaluate expression of NK Cells ($CD57^+$) in OSCC.
3. To compare the expression of NK Cells ($CD57^+$) in oral leukoplakia and OSCC.
4. To compare the expression of NK Cells ($CD57^+$) in different grades of OSCC.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cancer, the most dreaded disease, has always been in the discussion due to its extensive spread in the World. Today, oral cancer remains as the greatest problem facing oncologists in India. Statistically speaking, tumors of the oral cavity and oropharyngeal region rank first among males and third among females while constituting approximately 30% of all cancers. It is a matter of regret that the mortality rate from oral cancer is still high. This is often due to late diagnosis and lack of prognostic variables. It is unlikely that OSCCs arise directly from the normal epithelium. The epithelium first in all probability goes through a series of stages of initiation and promotion leading to precancerous lesions. These precancerous lesions further produce morphological changes in cells which result in clinically detectable lesions.[19]

The development of cancer is a multi-step process that starts with an accumulation of mutations, chromosomal rearrangement or amplification, or epigenetic changes in key genes (proto-oncogenes and tumor suppressor genes) leading to malignant transformation of normal cells.[19] In 2000, **Hanahan and Weinberg** postulated the concept of '**the six hallmarks of cancer**' in an effort to explain cancer biology.[20]

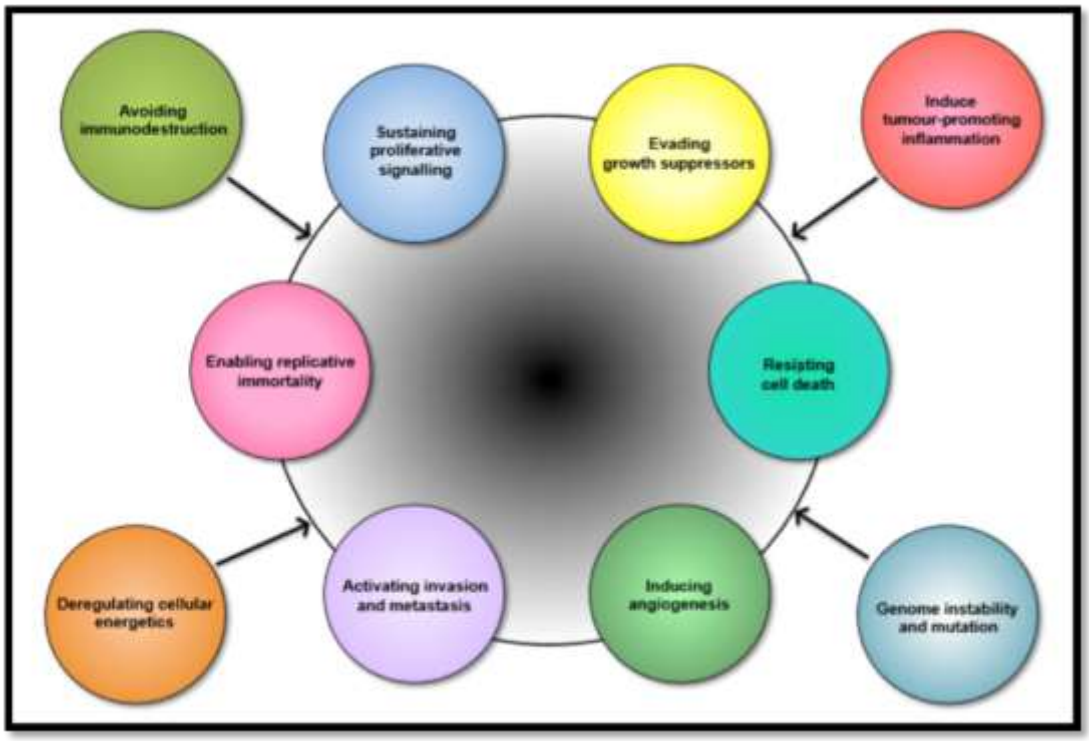
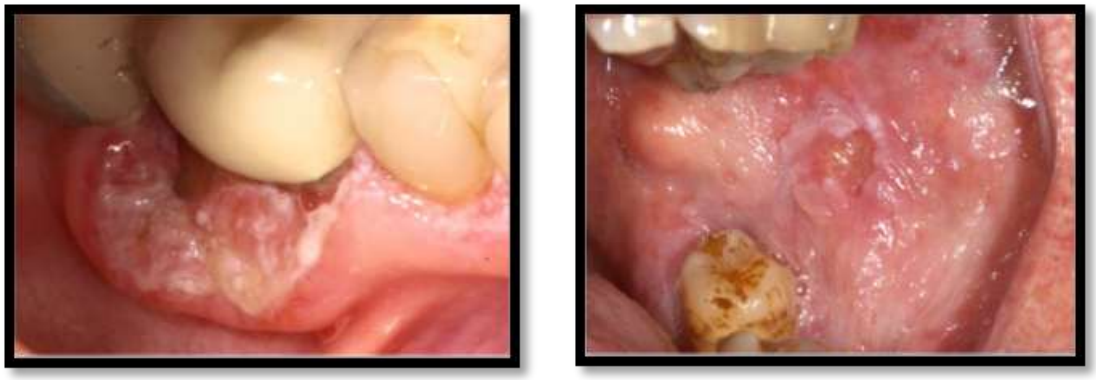


Fig. 1: Hallmarks of cancer.[20]

ORAL SQUAMOUS CELL CARCINOMA

Oral cancer is considered to be the sixth to eighth most common cancer around the World and has a great variability in incidence among countries.[21,22] OSCC accounts for about 90% of oral cancers affecting mostly adult males. Predominantly alcohol and tobacco users are affected between the sixth and seventh decades of life.[19] The most affected sites in decreasing order are the tongue, oropharynx, lip, floor of

mouth, gingiva (Fig. 2A), hard palate, and buccal mucosa (Fig. 2B).[23] Small lesions are often asymptomatic or may present with vague symptoms. On the other hand, locally advanced lesions usually present with pain, halitosis, difficulty in speech, deglutition and mastication.[24]

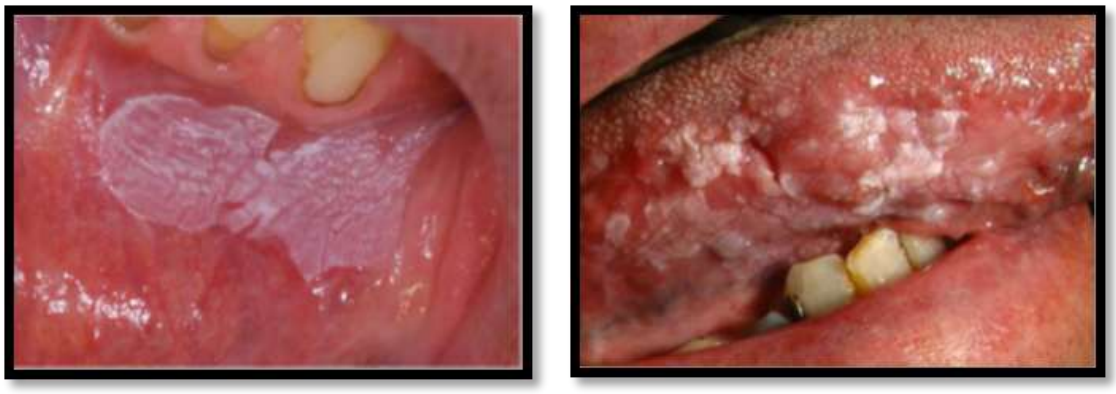


A B

Fig. 2: OSCC of the gingival mucosa (A) and buccal mucosa (B)[22]

LEUKOPLAKIA

About 80% of oral cancers are preceded by potentially malignant oral disorders in India.[2] Leukoplakia (LPL) is among the most common potentially malignant oral disorders.[2] The prevalence of leukoplakia in India varies from 0.2% to 5.2%.[3] Malignant transformation rates in leukoplakia varies from 0.13% to 10% in various Indian populations.[8] Leukoplakia in most cases is asymptomatic where the patient is not aware of its presence. Two major subgroups are described based on the clinical characteristics, i.e., homogenous and non-homogenous. This classification is based on the clinical appearance alone. Homogeneous LPLs are uniform white, flat, thin and well-demarcated lesions that can display a wrinkled surface (Fig. 3A) Non-homogeneous LPLs are a more heterogeneous group, which can be further subdivided into different variants such as speckled, nodular and verrucous (Fig. 3B).



A B

Fig. 3: Leukoplakia showing the homogenous variant (A) and Non-homogenous variant (B)[22]

Immune system of an individual plays a key role in battling against cancers and pre-cancers. The word 'immunity' comes from the Latin word '*immunis*' which means **free of** and was used in the milieu of being free of the burden of taxes or military conscription. The field of immunology is slightly more than 100 years old and **Louis Pasteur** is considered the '**father of immunology**'. Cellular immunology has a more recent history beginning in the late 1950's.[10]

THE IMMUNE SYSTEM IN POTENTIALLY MALIGNANT LESIONS AND CANCER

Recently, the importance of the tumor-associated stroma has been highlighted by the scientific society. Several studies have shown that infiltration of specific immune cells in the tumor microenvironment can impede the development of a

cancer. Infiltration of immune cells and immune response could be of importance regarding prevention of malignant transformation.[22]

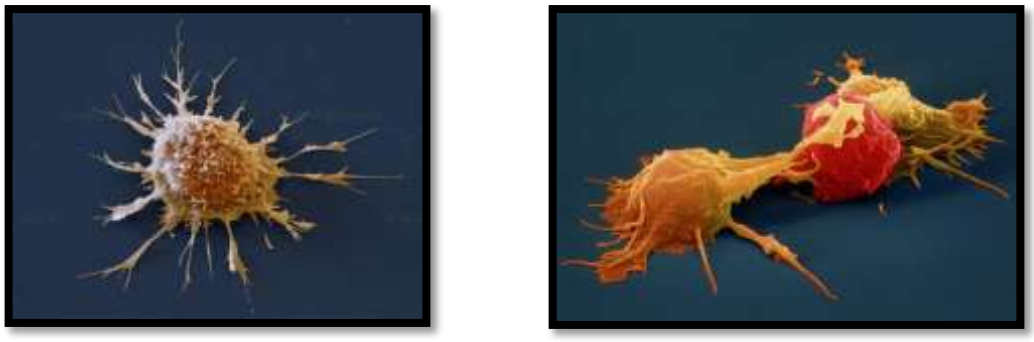
The host immune response to malignant tumor comprises the local response to tumor microenvironment as well as the systemic effects. The most frequent systemic alterations detected in solid tumor is neutrophilia and lymphopenia. Among the leukocytes associated with immunosuppression, T cells and **NK cells** are amongst the key factors that coordinate the host immune system to survey and eliminate cells with malignant transformation.[2] The presence of NK cells is believed to have a good prognostic value for OSCC.[4] Its role in leukoplakia is still under research.

NK CELLS

NK cells were described in the 1970s as **large granular lymphocytes** exhibiting "natural cytotoxicity" against several types of tumor cells.[25] NK cells display cytotoxic activity against a broad range of tumor cells as well as against cells infected with some but not all viruses. An extraordinary feature of these cells, which constitute 5% to 10% of lymphocytes in human peripheral blood, is their ability to recognize tumor or virus-infected cells despite lacking antigen-specific receptors.[26] NK cells are considered to be the first line of defence against tumor cells, cells showing dysplastic changes and virally infected cells.[4]

NK cells were initially identified by their morphology (Fig. 4 A & B). They were designated as 'large granular lymphocytes' based on them being slightly larger than resting T cells or B-cells and containing azurophilic granules. Later they were

found to contain proteases and cytolytic proteins (granzymes and perforin) responsible for their cytolytic function.[27]



A B

Fig. 4: NK cells (A and B)[28]

Origin and Localization of NK Cells

As with all hematopoietic cells, NK cells arise from hematopoietic stem cells originating in the bone marrow and further differentiate from the common lymphoid progenitor cells. NK cells are members of the innate lymphoid cell (ILC) family, which are distinct from B-cells and T cells in that they do not require recombination-activating genes for their development or generation of receptors to mediate their functions. Unlike T cells, NK cells do not require thymus for development. They can be found at lower frequencies in all lymphoid tissues, including bone marrow, lymph nodes and thymus as well as in the mucosal tissues, where they can be recruited during infections. NK cells recirculate through the bloodstream and their trafficking is regulated by the S1P5 (sphingosine-1-phosphate 5) receptor, which allows their egress from bone marrow and lymph nodes.[27]

NK Cell Receptors:[29]

Receptors present on the surface of NK cells provide the interface for interaction with other cells and thus are the basis for immunosurveillance. The receptors on NK cells is as follows:

A) Receptors belonging to the Immunoglobulin superfamily

1) Killer cell Ig like Receptors (KIR)

There are two types of KIRs; Activating and Inhibitory **(Table 1)**

TABLE I
KILLER CELL IG-LIKE RECEPTORS

KIR	Functionality	Protein domains	Ligand	Alias
2DL1	Inhibitory	2 Ig C2 (D1 + D2), TM, Cyp $_{2\ ITIM}$	HLA-C_{Lys80}	nkat1, cI-42, CD158A, p58.1
2DL2	Inhibitory	2 Ig C2 (D1 + D2), TM, Cyp $_{2\ ITIM}$	HLA-C_{Asn80}	nkat6, cI-43, CD158B1
2DL3	Inhibitory	2 Ig C2 (D1 + D2), TM, Cyp $_{2\ ITIM}$	HLA-C_{Asn80}	nkat2a, cI-6, CD158B1, p58
2DL4	Inhibitory + activating	2 Ig C2 (D0 + D2), TM_{Arg}, Cyp $_{1\ ITIM}$	HLA-G	KIR103AS, CD158D, p49
2DL5A	Inhibitory	2 Ig C2 (D0 + D2), TM, Cyp $_{2\ ITIM}$	?	CD158F
2DL5B	Inhibitory	2 Ig C2 (D0 + D2), TM, Cyp $_{2\ ITIM}$	?	KIR2DL5.2/3/4
2DS1	Activating	2 Ig C2 (D1 + D2), TM_{Lys}, Cyp_{short}	HLA-C_{Lys80}	EB6actl, CD158H
2DS2	Activating	2 Ig C2 (D1 + D2), TM_{Lys}, Cyp_{short}	HLA-C ?	nkat5, CD158J, cI-49
2DS3	Activating	2 Ig C2 (D1 + D2), TM_{Lys}, Cyp_{short}	HLA-C ?	nkat7
2DS4	Activating	2 Ig C2 (D1 + D2), TM_{Lys}, Cyp_{short}	HLA-C ?	nkat8, CD158I, cI-39
2DS5	Activating	2 Ig C2 (D1 + D2), TM_{Lys}, Cyp_{short}	HLA-C ?	nkat9, CD158G
3DL1	Inhibitory	3 Ig C2 (D0 + D1 + D2), TM, Cyp $_{2\ ITIM}$	HLA-B_{Bw4}	nkat3, NKB1, cl-2, cl-11, NKB1B, AMB11, CD158E1/2
3DL2	Inhibitory	3 Ig C2 (D0 + D1 + D2), TM, Cyp $_{2\ ITIM}$	HLA-A3, -A11	nkat4, cl-5, CD158K
3DL3	Inhibitory	3 Ig C2 (D0 + D1 + D2), TM, Cyp $_{2\ ITIM}$	?	KIRC1, KIR3DL7, KIR44, CD158Z
3DS1	Activating	3 Ig C2 (D0 + D1 + D2), TM_{Lys}, Cyp_{short}	HLA-B_{Bw4}	nkat10
2DP1	Pseudogene		–	KIRZ, KIRY, KIR15, KIR2DL6
3DP1	Pseudogene		–	KIRX, KIR48, CD158C, KIR2DS6

2) Leukocyte Immunoglobulin-Like Receptors (LILRs) **(Table 2)**

TABLE II
LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTORS

Gene	Functionality	Protein domains	Ligand	Alias	Predominant expression
LILRA1	Activating	4 Ig C2, TM_{Arg}, Cyp_{short}	HLA-B27	LIR-6, CD85i	Monocyte
LILRA2	Activating	4 Ig C2, TM_{Arg}, Cyp_{short}	HLA-interaction predicted	LIR-7, ILT1, CD85H	Monocyte, eosinophil, B-cell, NK-cell
LILRA3	Soluble	4 Ig C2	HLA-interaction predicted	LIR-4, HM43, ILT6, HM31, CD85e	Monocyte, B-cell, macrophage
LILRA4	Activating	4 Ig C2, TM_{Arg}, Cyp_{short}	?	ILT7, CD85g	Monocyte, dendritic cell
LILRA5	Activating	2 Ig C2, TM_{Arg}, Cyp_{short}	?	ILT11, LIR9, CD85f	Monocyte, neutrophil
LILRA6	Activating	4 Ig C2, TM_{Arg}, Cyp_{short}	?	ILT8, CD85b	
LILRB1	Inhibitory	4 Ig C2, TM, $Cyp_{4\ ITIM}$	HLA-A, B, C, E, F, G, UL18	LIR-1, ILT2, MIR-7, CD85j	Monocyte, dentritic cell, eosinophil, B cell, NK cell, T cell
LILRB2	Inhibitory	4 Ig C2, TM, $Cyp_{3\ ITIM}$	HLA-A, B, C, F, G, UL18	LIR-2, ILT4, MIR-10, CD85d	Monocyte, dentritic cell, eosinophil, B-cell, NK-cell
LILRB3	Inhibitory	4 Ig C2, TM, $Cyp_{4\ ITIM}$	?	LIR-3, HL9, ILT5, CD85a	Monocyte, dentritic cell, eosinophil, B cell
LILRB4	Inhibitory	2 Ig C2, TM, $Cyp_{3\ ITIM}$	?	LIR-5, ILT3, HM18, CD85k	Monocyte, mast cell, macrophage, NK cell, dentritic cell, B cell
LILRB5	Inhibitory	4 Ig C2, TM, $Cyp_{2\ ITIM}$	?	LIR-8, CD85c	NK cells
LILRP1	Pseudogene	4 Ig C2 exons	–		–
LILRP2	Pseudogene	4 Ig C2 exons	–		–

3) Low affinity receptor for the Fc fragment of Immunoglobulin-γ variant III B

Interacts with antibody aggregates and responsible for antibody-dependent cell-mediated cytotoxicity of NK cells.

4) Natural cytotoxicity receptors (NCR)

There are three types of NCRs – NCR I, NCR II and NCR III.

5) Other receptors

Other receptors of immunoglobulin superfamily are enlisted in the following table: **(Table 3)**

TABLE III
FURTHER RECEPTORS OF THE IMMUNOGLOBULIN SUPERFAMILY

Gene	Functionality	Protein domains	Ligand	Alias	Predominant expression
NCR1	Activating	2 Ig C2, TM_{Arg}, Cyp_{short}	?	NKp46	All NK cells, immature NK cells
NCR2	Activating	1 Ig V, TM_{Lys}, Cyp $_{1\ ITIM}$	?	NKp44, LY95	Activated NK cells, subset of γδT cells
NCR3	Activating	1 Ig V/C2, TM_{Arg}, Cyp_{short}	?	NKp30, 1C7	All NK cells, immature NK cells
FCGR3B	Activating	2 Ig, TM_{Arg}, Cyp	IgG	CD16b	NK cells
CD244	Activating	1 Ig V, 1 Ig C2, TM, Cyp $_{4\ ITSM}$	CD48	NKR2B4, NAIL, Nmrk, SLAMF4	All NK cells, subset of T cells, monocytes, mast cells, granulocytes
LAIR	Inhibitory	1 Ig C2, TM, Cyp $_{2\ ITIM}$	?	–	Most peripheral blood mononuclear leukocytes
CEACAM1	Inhibitory	1 Ig V, 3 Ig C2, TM, Cyp $_{2\ ITIM}$	CEACAM1, CEACAM5	BGP1, CD66A	Ubiquitous
SIGLEC7	Inhibitory	1 Ig V, 2 Ig C2, TM, Cyp $_{2\ ITIM}$	Sialic acid	p75/AIRM1, QA79	NK cells, monocytes, cytotoxic T cells
SIGLEC9	Inhibitory	1 Ig V, 2 Ig C2, TM, Cyp $_{1\ ITIM+1\ ITSM}$	Sialic acid	–	50% of NK cells, monocytes, neutrophils, bone marrow, placenta, spleen, and fetal liver

B) Receptors belonging to the C type lectin family (Table 4)

1) Subfamily A (KLRA)

2) Subfamily B (KLRB)

3) Subfamily C (KLR)

4) Subfamily D (KLRD)

5) Subfamily K (KLRK)

TABLE IV
NATURAL KILLER CELL RECEPTORS OF THE C-TYPE LECTIN FAMILY

Gene	Functionality	Domains	Known ligand	Alias	Predominant expression
KLRA1	Pseudogene	–	–	Ly49, LY49L	IL2-activated NK cells
KLRB1	Unclear	CRD, TM, Cyp	–	CD161, hNKRP1A, CLEC5B	Subset of NK cells, and T cells
KLRC1	Inhibitory	CRD, TM, Cyp $_{2\ ITIM}$	HLA-E	NKG2A, NKG2B, CD159A	Subset of NK cells, and T cells
KLRC2	Activating	CRD, TM $_{Lys}$, Cyp	HLA-E	NKG2C	Subset of NK cells, and T cells
KLRC3	Activating	CRD, TM $_{Lys}$, Cyp	HLA-E	NKG2E, NKG2H	Subset of NK cells
KLRC4	Pseudogene	$CRD_{truncated}$, TM $_{Lys}$, Cyp $_{ITIM}$	–	NKG2F	?
KLRD1	Partner in KLRC heterodimer	CRD, TM, Cyp	HLA-E	CD94	Subset of NK cells, and T cells
KLRG1	Inhibitory	CRD, TM, Cyp $_{ITIM}$	?	MAFA, 2F1, MAFAL	Subset of NK cells, activated T cells, mast cells and basophils
KLRK1	Activating	CRD, TM $_{Arg}$, Cyp	MICA, MICB, ULBPs, RAETs	NKG2D, KLR, D12S2489E	NK cells, $\alpha\beta$T cells, $\gamma\delta$T cells

C) Adaptor molecules of activating NK receptors (Table 5)

1) Hematopoietic cell signal transducer

2) TYRO protein Tyrosine Kinase Binding Protein

3) CD3Z Antigen, ζ polypeptide

4) High affinity receptor I for Fc fragments of IgE, γ-polypeptide

TABLE V
ADAPTOR MOLECULES OF ACTIVATING NK RECEPTORS

Adaptor	Official name	NK receptor	AS in TM	Alias
HCST	Hematopoietic cell signal transducer	KLRK1	Asp	DAP10, KAP10, DNAX, PIK3AP
TYROBP	TYRO protein tyrosine kinase–binding protein	KIR2DS1–5, KIR3DS1, KLRC2, -3, -4/KLRD1, Klrk1-s$_{mouse}$, NCR2	Asp	DAP12, PLOSL, KARAP
CD3ζ	CD3Z antigen, zeta polypeptide	NCR1, NCR3, CD16	Asp	CD3H, CD3Q
FcεR1γ	High-affinity Fc fragment of IgE receptor I gamma	*KIR2DL4*, LILRA1–6, NCR1, CD16	Asp	

Action of NK Cells

Immunosurveillance by NK cells is carried out by two mechanisms: cytokine release – IFN-γ and perforin dependent target cell elimination.[4]

Despite all the ways in which the NK cell recognizes target cell, secretory lysozyme exocytosis and perforin‑dependent target cell elimination are a requisite. This is divided into four stages:[15]

a. Activation of lytic immunologic synapse forms at the point of contact with the target cell thereby resulting in rearrangement of the actin cytoskeleton

b. Microtubules organizing centre of the NK cells and the secretory lysosomes are polarized towards the lytic synapse

c. Secretory lysosomes dock with the plasma membrane at the lytic synapse

d. Release of cytotoxic content

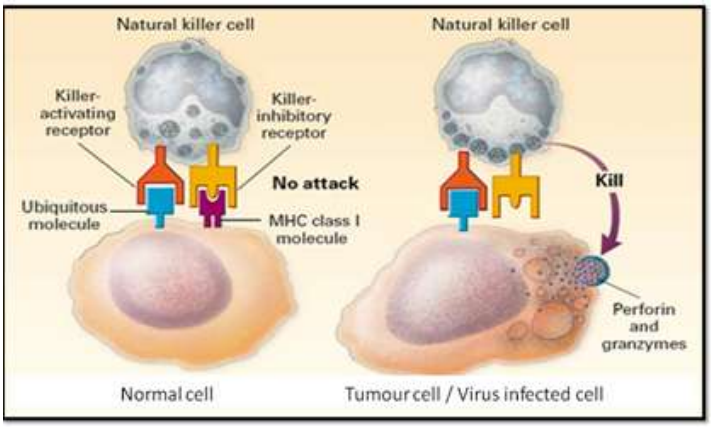


Fig. 5: Action of NK Cells[28]

NK Cell Activation:

Several general mechanisms exist for the activation of NK cells during an immune response. First, NK cells can be activated by cytokines alone without cognate recognition or interaction with a pathogen.[27]

In this case, epithelial cells, stromal cells, or myeloid cells that are stimulated by pathogens through their Toll-like receptors or intracellular sensors such as the nucleotide-binding oligomerization domain receptors secrete cytokines that can act directly on NK cells to induce their production of chemokines and cytokines.[27]

Thus, NK cells can serve as 'useful bystanders' responding to cytokines and secreting IFN-γ and other factors that can enhance the function of myeloid cells and

induce the expression of MHC Class II on antigen-presenting cells for interaction with CD4þ T cells.[27]

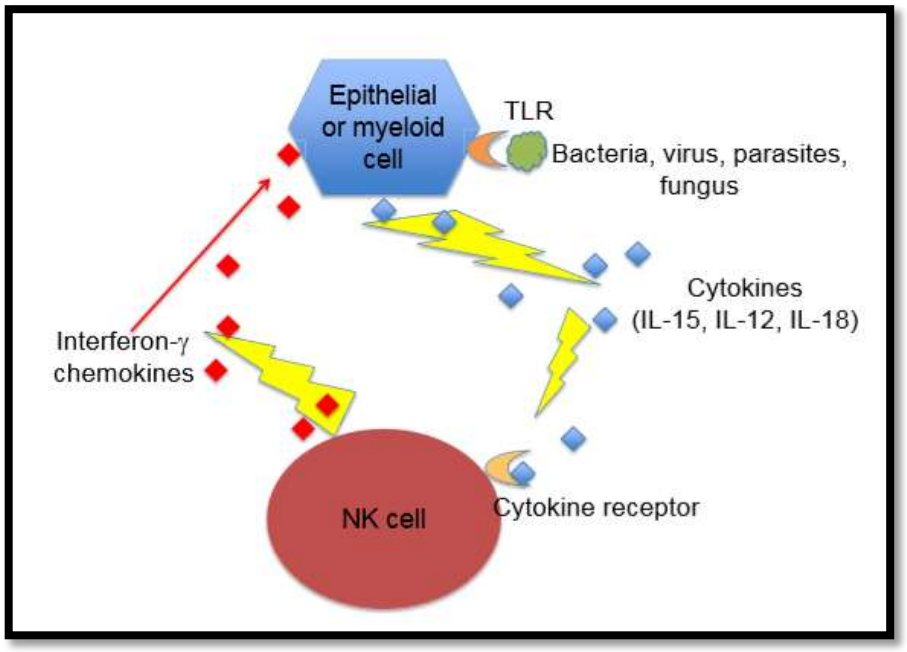


Fig 6. Activation of NK cells by cytokines during an immune response.[27]

In many cases, NK cells are activated by interaction with target cells that express 'stress-induced' self-ligands for their activating NK receptors. Ligands for the activating NKG2D receptor are frequently expressed on virus-infected cells and tumors, as well as hyperproliferating normal cells in the host, which can initiate cell-mediated cytotoxicity by NK cells resulting in the killing of these NKG2D ligand-bearing cells.[27]

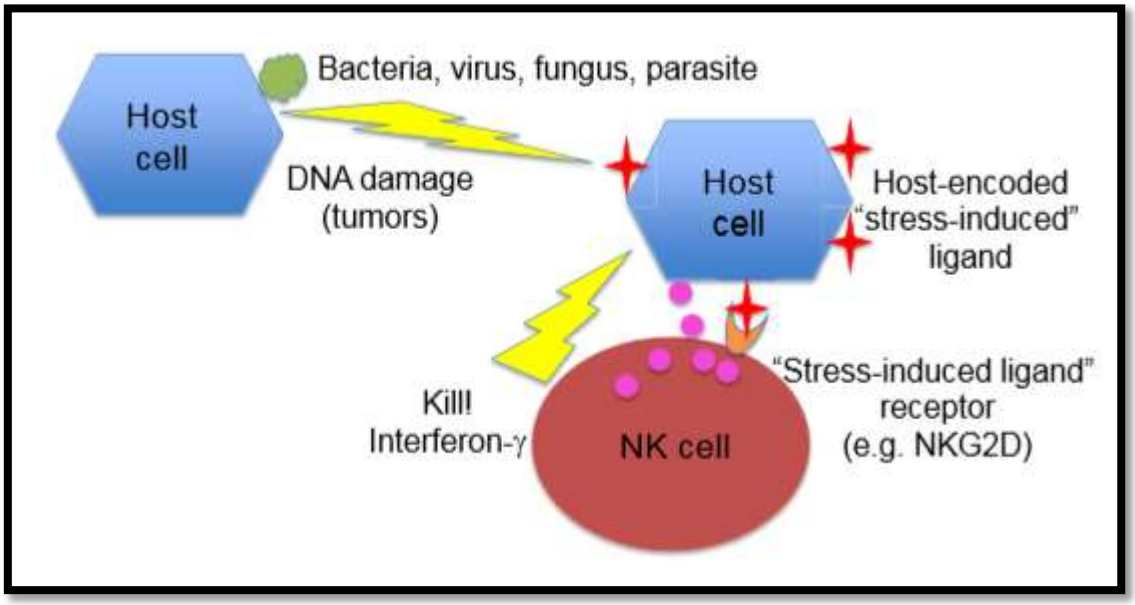


Fig 7. Activation of NK cells by recognition of 'stressed' cells expressing ligands for activating NK receptors such as NKG2D.[27]

In some cases, NK cells possess specific receptors that recognize pathogen-encoded ligands that can trigger killing of the pathogen-infected cells, secretion of cytokines, and proliferation of the responding NK cells. The best example of this latter mechanism is recognition of the m157 glycoprotein encoded by mouse cytomegalovirus (MCMV), which specifically binds to the activating Ly49H receptor. Activation of mouse NK cells through the Ly49H receptors can result in the generation of a self-renewing subset of MCMV-specific Ly49H þ NK cells that demonstrate immunological memory.[27]

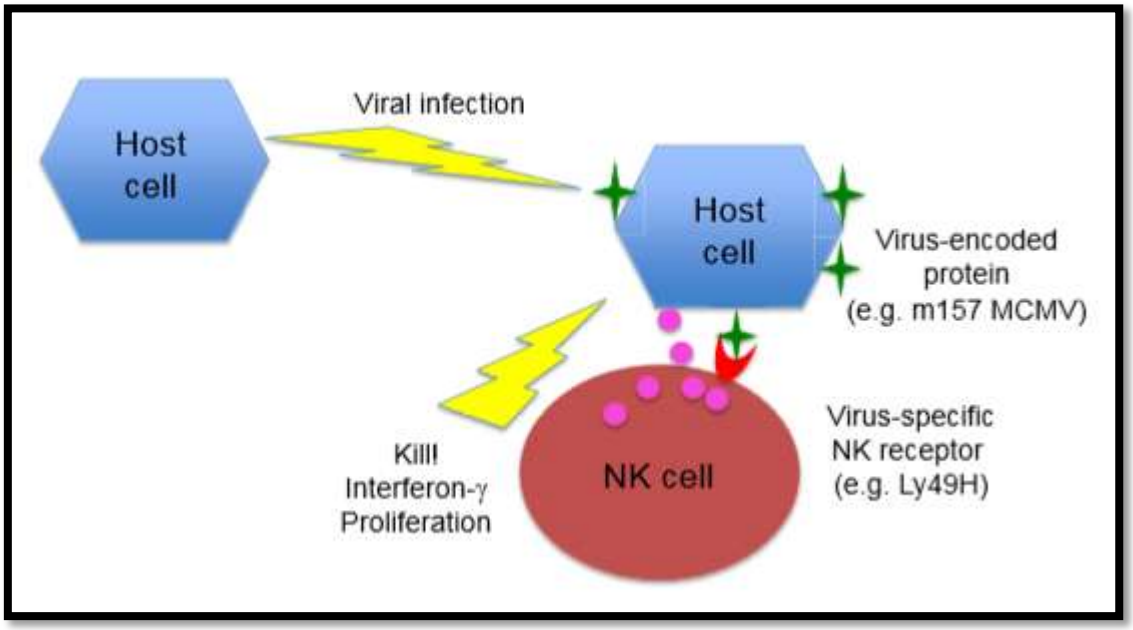


Fig 8. Activation of NK cells by recognition of specific pathogen-encoded ligands for activating NK receptors such as Ly49H.[27]

Another mechanism for an antigen-specific NK cell response is based on the ability of NK cells expressing the CD16-activating Fc receptor to kill IgG-coated target cells by a process referred to as antibody-dependent cellular cytotoxicity (ADCC). In this case, antigen specificity is conferred by the IgG, which allows for an exquisitely antigen-specific response by the NK cells.[27]

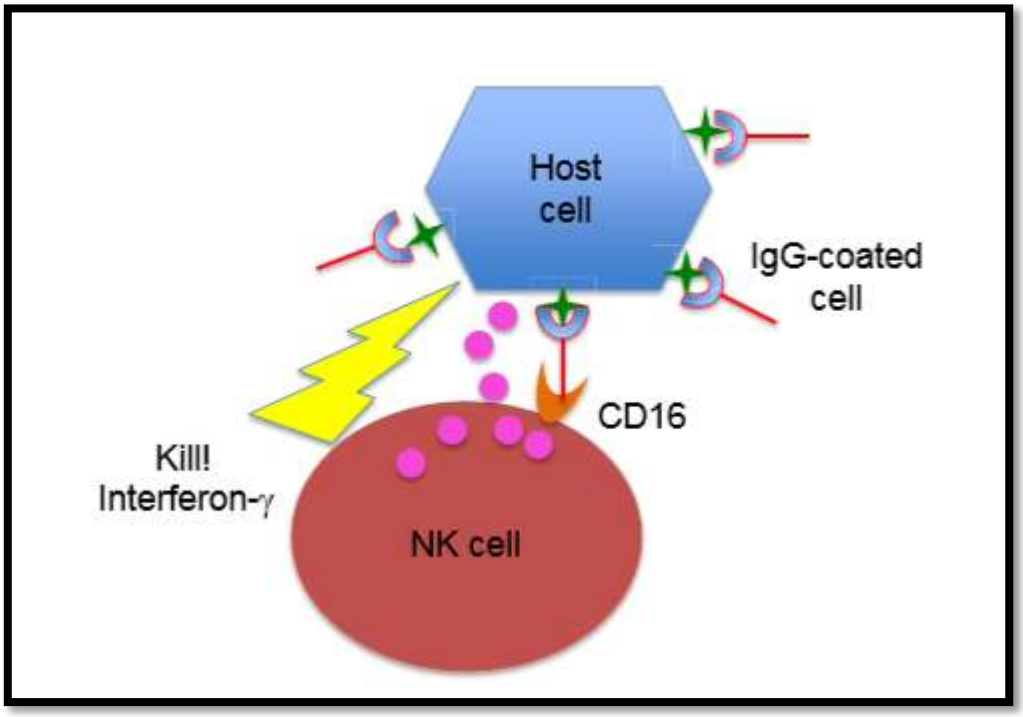


Fig 9. Activation of NK cells by recognition of IgG-coated cells that engage the activating Fc receptor CD16 on NK cells – a process ADCC.[27]

NK cells can be identified by CD56 and CD57 markers. Progression of NK cell sub-types from $CD56^{bright}$ to $CD56^{dim}CD57^{-}$ to $CD56^{dim}CD57^{+}$ reflects a maturation pathway for NK cells.[17] Surface molecule CD57, also known by the names HNK-1 or Leu-7, is a glycoprotein which is present in NK cells going through terminal differentiation, therefore many authors have been using CD57 as a marker for NK cells.[25] NK cells were initially identified by their ability to kill malignant cells and a large body of clinical and experimental evidence now supports their crucial role in cancer immunosurveillance. Reduced MHC Class I expression and *de novo* expression of stress related molecules (such as B7-H6, MICA, MICB, RAE-1, MULT1, and members of the ULBP family) in malignant cells alter the balance of inhibitory and activating signals for NK cells, leading to their activation. Elevated

frequencies of peripheral or tumor-associated $CD57^+$ NK cells are reported in carcinoma patients and – in sharp disparity to what has been seen for CD8+ T cells – have often been correlated to less severe disease and enhanced outcomes. This would be consistent with enhanced tumor surveillance/cytotoxicity of the mature, $CD57^+$ NK cell subset[17]

CD57 is a very valuable marker of NK cell maturation, identification of cells with potent cytotoxic potential along with decreased sensitivity to cytokines and reduced replicative potential. $CD57^+$ NK cells seem to be a stable sub-population, increasing with age and exposure to pathogens. Their presence is consistently associated with improved outcomes in cancer and autoimmune disease. Accurate understanding of the role of CD57 expression on NK cells necessitates a detailed dissection of the underlying biology of CD57, about which not much is known.

Immuno-surveillance in Oral Leukoplakia and OSCC

The cancer immunosurveillance hypothesis suggests that the immune system can recognize cancer cell precursors and destroy those cells before a clinical expression occurs. During the last decades several groups have presented evidence of the influence and the role of immunoactivation in OSCC patients. The failure of immunosurveillance has been reported to be an important factor in the development and progression of cancer. Tumor-specific antigens may be present both on the surface as well as in the interior of tumor cells. The ideas relating to the activities of the host immunity have evolved from the initial immunosurveillance concept which depicts the anti-tumorigenic effects of the immune system to the more lately proposed immunoediting concept. The notion of tumor immunoediting describes the dual action

of immune system in acting as tumor scavengers as well as tumor promoters. This has been described based on three stages of tumor immunoediting namely elimination, equilibrium and escape.[30] Balaram and Vasudevan[31] quantitated total lymphocytes, B-cells and the T-lymphocyte subsets, T_G cells (IgG Fc receptor bearing T cells) and T_M cells (IgM Fc receptor bearing T cells) and NK cells in the peripheral blood of oral cancer patients and normal subjects and found that the above mentioned cells are found in increased proportion in oral cancer patients. Specific antibodies to the neo-antigens may bind to the surface antigens leading to complement fixation and subsequent lysis of the tumor cells. Immunologically specific cytotoxic T cells may bind to surface antigens and destroy the tumor cells or inhibit their growth. NK cell activity was found to decrease with an increase in the tumor severity.[31] However, very little is known about immunoactivation in potentially malignant disorders.[22] NK cells being granular lymphocytes are summoned in the first line of action during a potential disease. $CD57^+$ NK cells are the most matured variant of NK cells and are the most cytotoxic. The role of immune activity in leukoplakia is still under research.

Mechanisms of Immunosuppression by Tumor Cells

Several mechanisms have been proposed for the functional inactivation of the tumor-associated lymphocytes. Tumor induced immunosuppression in mice has been associated with a decreased expression of the ζ-chain of the T cell receptor and the loss of mRNA for granzyme B. Nevertheless, caution should be exercised in continuing the results from mice cancer model to human disease model in terms of NK cell function because of an array of species specific differences. These dissimilarities could be due to the strain of mice used or the source from which purified NK cells were isolated in mice and humans; i.e. spleen vs. peripheral blood.[32]

They could be due to the inherent differences between human and mice NK cell function. Thus, mouse model "has been unimposing in producing informative and translatable models of human diseases" It is, however, a very expedient model which can be genetically manipulated and can give assets of knowledge if used in parallel with the human disease model thus confirming the observations obtained in human disease and not *vice versa*. Fascinatingly, most studies reported for tumor mediated NK cell immunosuppression were conducted using human NK cells. Therefore, decreased CD16 and its associated ζ chains were observed in tumor-infiltrating NK cells of patients with cancer. Tumor microenvironment may influence the function and phenotype of the NK cells. If there is a failure in expression of NK cell cytotoxicity, downregulation of NK cells results.[32]

Surface molecule CD57, also known as HNK-1 or Leu-7, is a glycoprotein present in NK cells going through terminal differentiation, therefore many authors have been employing CD57 as a marker for NK cells. CD57 protein has been used to probe the functional immune response in patients with cancer as well as other diseases with major involvement in the inflammatory component, such as in autoimmune diseases.[7] In tumor biology, CD57+ NK cell infiltration is associated to better prognosis in several types of cancer including OSCC.[19]

Karre K et al. (1986)[33] stated that the response of NK cells is determined by the balance of activating and inhibitory signals transmitted through these receptors when NK cells interact with the prospective target cells. In general, cells that have downregulated MHC Class I expression due to viral infection or transformation are more susceptible to NK cells attack due to lack of inhibitory signalling referred to as surveillance for 'missing self' by NK cells.

Pillai M et al. (1990)[34] studied interferon activation of latent NK cells and alteration in kinetics of target cell lysis with clinical implications for oral precancerous lesions. They concluded that reduced NK cell activity was observed in patients with oral leukoplakia and submucous fibrosis compared with normal control subjects. However, the number of target binding lymphocytes was found to be normal in these pre-cancers. Treatment of effector cells with interferon-α resulted in highly elevated active killer cell activity, even though no change was observed in target binding lymphocyte counts. This finding could imply that pre-cytotoxic cells which are activated by interferon exist in peripheral blood or that direct recruitment of a new cell population occurs. Additionally, altered target lysis kinetics was observed, with interferon-activated killer cells demonstrating a tremendous lytic activation that is completed so rapidly that a statistical kinetic analysis could not be precisely done. Because NK cell activity is an important effector system in immunosurveillance against tumors, its modulation with interferon may be an exciting clinical possibility in the control of malignant transformation of oral pre-cancers.

Pillai R et al. (1991)[35] studied immunological abnormalities in oral pre-cancers and concluded that the immune response plays a role in regulating the development and growth of oral pre-cancers. As a result, neoplastic transformation and proliferation could result from an impairment of the dynamic equilibrium between the inherent growth potential of the precancerous lesion and its ability to survive within the host. The host's immune response is a vital factor in the balance of this equilibrium and any defect in its composition or functioning can rapidly tilt the balance in favour of the precancer.

Vijaykumar T (1993)[6] studied immunological phenomena in human oral carcinoma in India and concluded that the primary role of the normally functioning immune system is to provide defence against malignant cells which are constantly arising throughout the body. Therefore, the failure of immune surveillance has been reported to be an important factor in the development of cancer. There is alteration both in humoral and cellular immune responses in oral cancer patients and the assessment of immunological status may be of use not only in the diagnosis and/or prognosis of oral cancer, but also for the effective management of the disease.

Kurosawas S et al. (1995)[36] showed that NK cells that infiltrated into the primary tumor site at an early stage of tumor development, were examined for their participation in the generation of anti-tumor cytotoxic T lymphocytes. NK cells, which were detected by anti-NK1.1 monoclonal antibody, increased in the peritoneal exudate cells (PEC) on days 3 and 7 after an intraperitoneal inoculation of syngeneic B16 melanoma cells. These tumor-infiltrating NK cells showed a high level of cytotoxic activity against NK-sensitive YAC-1 cells and an increased expression of IFN-γ mRNA and IL-2 mRNA. The *in vivo* depletion of NK cells with anti-NK1.1 monoclonal antibody, prior to intraperitoneal inoculation of B16 melanoma cells, resulted in an increased number of tumor cells in the PEC compared to NK cell non-depleted mice. Interestingly, the differences in tumor cell number between both groups were more prominent on days 7 and 14 than on day 3, which strongly suggested that early-infiltrating NK cells have a large influence on the subsequent anti-tumor response. *In vivo* diminution of NK cells preceding the immunization with melanoma cells abrogated the capability of the spleen cells to generate $CD8^+$ tumor-specific CTL after *in vitro* re-stimulation. This inability of generating anti-tumor CTL

was partially restored by additional intraperitoneal inoculation injections of recombinant IL-2 and/or IFN-γ simultaneously with the immunization of melanoma cells. The *in vitro* depletion of NK cells prior to the *in vitro* re-stimulation with melanoma cells partially impaired the anti-tumor CTL generation from the spleen cells of the immunized mice. Lastly, the *in vivo* depletion of NK cells before immunization with melanoma cells abolished the defensive immunity against melanoma cells at the re-challenge. On the whole, these results indicate that early-appearing tumor-infiltrating NK cells not only participate in the anti-tumor early defence by themselves, but also play a crucial role in the generation of anti-tumor CTL.

Lanier L (2000)[37] stated that NK cells preferentially recognize and kill cells that lack expression of MHC Class I. While inhibitory receptors expressing immunoreceptor tyrosine-based inhibition motifs prevent the NK cells from damaging tissues expressing normal levels of classical or nonclassical MHC Class I. The emerging concept is that NK cell function is determined by the integration of signals from several activating receptors that are regulated by the inhibitory receptors for MHC Class I, based on the density and array of ligands and class I molecules expressed by the antigen-presenting cell. Infection and inflammation are often accompanied by the release of local cytokines (e.g., IL-15, TNF-α, type I IFNs, chemokines). These cytokines may serve not only to set off NK cells, but also to upregulate ligands for adhesion or costimulatory receptors that are existent on NK cells. In this environment, rich in stimulatory cytokines and ligands for activating receptors, NK cells may grant a vital bridge between the innate and adaptive immune systems.

De Paula A and Gomez R (2001)[38] studied immunolocalization of p53, glutathione S-transferase π (GST-π) and CD57 antigen in oral leukoplakia using 70 biopsies streptavidin-biotin-peroxidase method to detect the antigen and concluded that the presence of CD57+ve lymphoid cells are indicative of immunosuppression. The severity of epithelial dysplasia and immunolabelling for GST-π are associated with local immune response alterations in oral leukoplakia.

Villegas F et al. (2002)[39] studied prognostic significance of tumor-infiltrating NK cells subset CD57 in patients with squamous cell lung cancer (SqCLC). Fifty patients with primary SqCLC were studied for the presence of tumor-infiltrating NK cells subset CD57 (TINK) after surgery. They found that together with TNM and age, the number of TINK observed at the time of surgery could also be used as a prognostic factor in SqCLC. They suggest that approaches to treatment must consider the introduction of drugs that enhance NK cell activities in these patients.

French A and Yokoyama W (2003)[40] suggested that the innate immune system, particularly NK cells, influence ensuing adaptive immune responses. By virtue of their capability to rapidly destroy abnormal cells and produce cytokines and chemokines, NK cells are placed for a key position in regulating autoimmune responses. The results abridged in this review demonstrate that NK cells are concerned with modulating responses to self antigens and that in some circumstances can directly or indirectly either suppress or augment autoimmunity. The associations found in humans and the pragmatic evidence from murine models suggest that additional research into the immunomodulatory role of NK cells in autoimmunity is justified and is likely to offer new insights into the pathogenesis of autoimmune disorders.

Reibel J (2003)[41] studied prognosis of oral pre-malignant lesions with significance of clinical, histopathological as well as molecular biological characteristics. He stated that the concept of a two-step process of cancer development in the oral mucosa that is the initial presence of a precursor subsequently developing into cancer has been well-established while oral leukoplakia is the most common precursor lesion. The substantiation that oral leukoplakias are pre-malignant is principally derived from follow-up studies showing that between less than 1 and 18% of oral pre-malignant lesions will develop into oral carcinomas. It has been proven that certain clinical sub-types of leukoplakia are at a higher risk for malignant transformation. The presence of epithelial dysplasia may even be more important in predicting malignant development than studying the clinical characteristics. There is a considerable necessity to improve the histologic assessment of epithelial dysplasia or, since epithelial dysplasia does not seem to be consistently associated with or even be a necessary prerequisite for malignant development, it may be essential to develop other methods for predicting the malignant potential of pre-malignant lesions. Molecular biological markers have been suggested to be valuable in the diagnostic and prognostic evaluation of leukoplakias. Markers of epithelial differentiation and in recent times, genomic markers could potentially be excellent candidates for improving the prognostic evaluation of precursors of oral cancer. These new markers could be taken into account complementary to conventional prognostic assessment.

Volz A and Radeloff B (2006)[29] in their article on natural killer cells discussed the characteristics, receptors and expression on NK cells. They suggested that NK cells can enter and defend a tissue almost as soon as it becomes infected since they do not require pathogen specific clonal expansion. NK cells need an activating

signal which is usually provided by the aberrant cell itself. Autoreactive NK cells usually do not form as NK cells check the target cell integrity with inhibitory receptors prior to elimination.

Gregoire C et al. (2007)[42] while studying the trafficking of NK cells found that NK cells recirculate through the bloodstream and their trafficking is regulated by the S1P5 receptor for sphingosine-1-phosphate, which allows their egress from bone marrow and lymph nodes.

Topham N and Hewitt E (2009)[43] studied NK cell cytotoxicity and molecular basis for NK cell secretory lysosome exocytosis with immunological consequences of defects in the exocytic machinery. They concluded that NK cells target and destroy abnormal cells, such as virally infected and tumorigenic cells. This eradication is mediated by cytotoxic molecules that are stored within secretory lysosomes, a specialized exocytic organelle that is found in NK cells. Target cell recognition stimulates the formation of a lytic immunological synapse between the NK cell and its target. The polarized exocytosis of secretory lysosomes is then stimulated and these organelles release their cytotoxic contents at the lytic synapse, specifically eradicating the target cell. The indispensable role that secretory lysosome exocytosis plays in the cytotoxic function of NK cells is highlighted by immune disorders that result due to the mutation of vital components of the exocytic machinery.

Lopez-vergès S et al. (2010)[18] stated that NK cells are innate immune lymphocytes which express a heterogeneous gamut of germ line-encoded receptors and endure a distinct pattern of maturation. CD57 is a marker of terminal differentiation on human CD8+ T cells while incredibly few new-born or foetal NK

cells express CD57. Nevertheless, the frequency of $CD57^+$ NK cells increases with age. They gauged that the transcriptional, phenotypic, and functional differences between $CD57^+$ and $CD57^-$ NK cells express a range of NK cell receptors, evocative of a more mature phenotype that proliferates less when stimulated with target cells or cytokines. In contrast, a higher frequency of $CD57^+$ NK cells produced interferon-γ and demonstrated more potent lytic activity when stimulated through the activating receptor CD16; however showed less response to stimulation by Interleukin (IL)-12 and IL-18. CD57 expression is induced on CD57 $CD56^{dim}$ NK cells after activation by IL-2. An amalgamation of a mature phenotype, a superior cytotoxic capacity, a higher sensitivity to stimulation via CD16 along with decreased receptiveness to cytokines and a decreased capability to proliferate suggest that $CD57^+$ NK cells are highly mature and may be terminally differentiated.

Jewett A and Tseng H (2011)[32] studied tumor induced inactivation of NK cell cytotoxic function and demonstrated that cytotoxic function of immune effectors is essentially suppressed in the tumor microenvironment by a numerous distinct effectors and their secreted factors. It has recently been shown that NK cells mediate noteworthy cytotoxicity against primary oral squamous carcinoma stem cells (OSCSC) compared to their more differentiated oral squamous carcinoma cells. In addition, human embryonic stem cells (ESCs), Mesenchymal Stem Cells (MSCs), dental pulp stem cells (DPSCs) and induced pluripotent stem cells (iPSCs) were all appreciably more vulnerable to NK cell-mediated cytotoxicity than their differentiated counterparts or progenitor cells. Total population of monocytes and those depleted of $CD16^+$ subsets were able to considerably prevent NK cell-mediated lysis of OSCSCs, MSCs and DPSCs. The results suggest that stem cells are major targets of the NK cell

cytotoxicity. The notion of split anergy in NK cells and its contribution to tissue repair and regeneration and in tumor resistance and progression was highlighted in this research paper.

Gayoso I et al. (2011)[44] studied immunosenescence of human NK cells and suggested that NK cells are a crucial component of innate immunity that are involved not only in the eradication of virus-infected or tumor cells but also in the regulation of the immune response by producing cytokines and chemokines which activate other cellular components of the immune system. NK cell subsets are differentially influenced by ageing. Whereas $CD56^{bright}$ cells are diminished in healthy old individuals, the $CD56^{dim}$ subset is expanded. The expression of CD57, a marker of highly differentiated NK cells, is increased in the senile. This supports the concept that a re-modelling process of NK cell subsets occurs as the individual ages with a steady decrease in more immature $CD56^{bright}$ NK cells and an increase in highly differentiated $CD56^{dim}$ $CD57^{+}$ NK cells. This NK cell redeployment can explain several phenotypic and functional changes in NK cells linked with healthy ageing such as decreased proliferation and the maintenance of CD16- dependent cytotoxicity.

Bryceson Y et al. (2011)[45] studied molecular mechanisms of NK cell activation and stated that with an assortment of activating and inhibitory receptors, NK cells can specifically eliminate infected and transformed cells. Target cell elimination is attained through directed release of lytic granules. Recognition of target cells also bring about production of chemokines and cytokines that can synchronize immune responses. After coming in contact with susceptible cells, an array of activating receptors can induce signals for adhesion. Engagement of the integrin leukocyte functional antigen-1 mediates firm adhesion, provides signals for granule

polarization and orchestrates the structure of an immunological synapse which facilitates efficient target cell elimination. Cytokine and chemokine release follow a diverse secretory pathway which requires phospholipase C-γ activation and store-operated Ca^{2+} entry. Recent studies of human NK cells have provided insights into a hierarchy of effector functions which result in graded responses by NK cell populations. Responses exhibit cellular heterogeneity and are influenced by environmental signals.

Ferlazzo G and Carrega P (2012)[46] studied NK cell distribution and trafficking in human tissues and stated that earlier studies have been often prejudiced by employing markers that were later on established to be either not sufficiently specific for NK cells or expressed only by subsets of NK cells. Presently, existing data confirmed that human NK cells populate blood, lymphoid organs, lung, liver, uterus (during pregnancy) and the gut. Numerous studies showed that NK cell homing appears to be subset-specific, as most secondary lymphoid organs and almost certainly several solid tissues are preferentially inhabited by $CD56^{bright}$ $CD16^{neg/dull}$ non-cytotoxic NK cells. Similar studies performed in the mouse model showed that lymph node and bone marrow are preferentially colonized by $CD11b^{dull}$ NK cells, while blood, spleen, and lung by $CD27^{dull}$ NK cells. Therefore, a significant topic to be addressed in the human system is the contribution of factors that control NK cell tissue homing and egress.

Raulet D et al. (2013)[47] studied regulation of NKG2D ligand activating receptor and stated that ligands for the activating NKG2D receptor are often expressed on virus-infected cells and tumors, as well as hyperproliferating normal

cells in the host. This can initiate cell-mediated cytotoxicity by NK cells ensuing the destruction of these NKG2D ligand-bearing cells.

Brahmbhatt B and Vora H (2014)[2] studied immune effector cells in leukoplakia and oral cancer and found that in comparison with healthy donors, decreased lymphocytes, naive helper cells, NKT subpopulations and increased effector cells were observed in leukoplakia patients. Similarly, decreased lymphocytes, NKT subpopulations and increased neutrophils, monocytes, helper and regulatory T cells were observed in OSCC patients as compared to healthy controls. Moreover, lymphocytes were decreased and regulatory T cells were increased during the progression of leukoplakia to OSCC. Additionally, concerning the clinicopathological parameters, cytotoxic cells were established to be diminished with increasing histological grade. Helper cells were found to be reduced in patients with tobacco and alcohol habit as well as with increasing tumor size. Further, in univariate survival analysis, increased incidence of relapse was observed in patients with low γδ and NKT cells. In multivariate survival analysis, low γδ T cells emerged as poor prognostic markers for disease-free endurance. High regulatory T cells materialize as poor prognostic markers for predicting overall survival. They concluded that altered systemic immune response was seen during malignant transformation and also found to be associated with patient's survival. Therefore, examination of circulating leukocyte and T cell subsets appears to be helpful for predicting patient's survival and to recognize immunosuppressed patients who may be benefited with immunotherapy.

Iida M et al. (2014)[16] studied increase of peripheral blood $CD57^{+}$T cells in patients with oral squamous cell carcinoma in 43 patients with OSCC by fluorescence-activated cell sorting analysis. They found that the proportion of $CD57^{+}$

T cells, including both CD8+ and CD4+ subsets, significantly increased with clinical stage, especially in parallel with tumor size. Increase in the population of $CD57^+$ T cells is a potent prognostic marker and may also influence the systemic immunity of patients with OSCC.

Bar E et al. (2014)[48] in their study – IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells – stated that NK cells provide shield by the release of IFN-γ, that supplements the function of myeloid cells and T cells. In case of fungal infections, NK cells can contribute to host defence by secreting granulocyte macrophage – colony stimulating factor (GM-CSF), which employs neutrophils that ingest the fungi in reaction to IL-17 formed by other cells in the mucosal tissues.

Gras Navarro A et al. (2015)[49] studied therapeutic potential and challenges of NK cells in the treatment of solid tumors and stated that NK cells are ILCs that possess incredible potential for effective immunotherapy for a large range of cancers. Due to the mode of NK cell killing, needing one-to-one target engagement and site-directed discharge of cytolytic granules, the therapeutic potential of NK cells has been expansively explored in haematological malignancies. However, their capacity to precisely kill antibody coated cells, cancer stem cells, and genotoxically altered cells, while maintaining forbearance to healthy cells makes them alluring therapeutic effectors for all cancer forms, including metastases. Due to their release of pro-inflammatory cytokines, NK cells may potently overturn the anti-inflammatory tumor microenvironment and boost adaptive immune responses by endorsing differentiation, activation, and recruitment of accessory immune cells to sites of malignancy. Nonetheless, integrated and coordinated mechanisms of subversion of NK cell

activity against the tumor and its microenvironment do exist. Although the perception of the receptor ligand interactions that regulate NK cell functionality has evolved outstandingly, the diversity of ligands and receptors is complex, as is their mechanistic foundations in regulating NK cell function. Exploiting NK cell subsets with best extrapolative KIR/HLA combinations might be valuable, since autologous NK cells formerly demonstrated restricted efficacy. For this to be effectual, tumor reduction prior to NK therapy is vital in order to give the NK cells a task they can manage. The cells could be injected into and around the resection cavity and administered as an adjuvant therapy after standard treatment that combines debulking surgery, concomitant Temozolomide chemotherapy and conformal ionizing radiation. If these results were exchangeable to humans, in a patient phase I trial, smaller dose escalations with maximally alloreactive subsets may be better endured, not least more reasonable with regards to *ex vivo* NK cell expansions.

Burga R et al. (2016)[50] studied improving efficacy of cancer immunotherapy by genetic modification of NK cells. They concluded that NK cells are members of the innate immune system which recognize target cells via activating and inhibitory signals that are received through cell receptors. Being derived from the lymphoid lineage, NK cells are able to produce cytokines and exert a cytotoxic effect on virally infected and malignant cells. It is their exclusive ability to lyse target cells rapidly without prior edification that provide NK cells a promising role as an effector cell for adoptive cell therapy. However, both viruses and tumors employ prevarication strategies to avoid attack by NK cells. This represents biological challenges that need to be harnessed to fully exploit the cytolytic potential of NK cells. Applying genetic modification, the function of NK cells can be enhanced to improve their homing,

cytolytic activity, *in vivo* persistence and safety. Preclinical studies have clearly demonstrated that such approaches are effective in improving NK cell function, homing as well as safety.

Taghavi N et al. (2016)[51] studied prognostic implication of CD57, CD16, and TGF-β expression in OSCC and concluded that CD57 expression and mode of invasion are independent prognostic factors of survival in OSCC patients.

Agarwal R et al. (2016)[4] carried out evaluation of NK cell (CD57) as a prognostic marker in OSCC by immunohistochemistry method. A total of 100 clinico-histopathologically diagnosed cases of OSCC of various grades were divided into two groups, i.e., Group I consisting of dead patients and Group II consisting of live patients and CD57 was detected in the tissues of these two groups by immunohistochemistry. They concluded that increase in the expression of CD57 in the tumor stroma of OSCC may serve up as a good prognostic marker for the patients.

Grossenbacher S et al. (2016)[52] demonstrated that activated NK cells may be exceptionally capable of targeting cancer stem cells (CSCs) of solid tissue cancers via stress ligand recognition. They assured that future studies will be needed to evaluate the immunological impact of NK cell killing of CSCs using immunocompetent models since this may aid in supplementing later T cell responses. It will also be decisive to optimize NK cell immunotherapy with regard to sustained *in vivo* effects as the highly activated NK cells are dependent on cytokines for their continued function and survival. Better targeting of CSCs by the NK cells, in all probability through monoclonal antibodies, may also increase efficacy. It is likely that NK cells require to be applied using a combination therapy approach as reduction of non-CSCs, which encompass the bulk of the tumor, needs to occur.

De Sousa Lopes M et al. (2017)[26] carried out evaluation of $CD57^+$ cells OSCC and their relationship with clinicopathological parameters. The results suggested that $CD57^+$ cells immune cells infiltration is a consistent finding in OSCC, regardless of clinicopathological features in these tumors.

Hofer E And Koehl U (2017)[53] stated that immunotherapies based on NK cells are amongst the most promising therapies under progress for the treatment of incurable forms of leukaemia and other types of cancer today. The importance of NK cells in controlling viral infections and cancer is supported among others by the findings that viruses and tumors use a massive amount of mechanisms to subvert and escape the NK cell system. Infections and malignant diseases can further lead to the shaping of NK cell populations with transformed reactivity. Counter methods of prospective therapeutic impact comprise the blocking of inhibitory communications between NK cell receptors and their cellular ligands, the augmentation of activating receptor signals and the infusion of large amount of *ex vivo* created and selected NK cells. Furthermore, the specific cross-linking of NK cells to their target cells using chimeric antigen receptors or therapeutic bi/tri-specific antibody reagents is an assuring line of attack. In this context, NK cells make a mark by their positive effects and safety established in most clinical trials conducted so far.

Dyck L and Lynch L (2018)[54] stated that NK cells are ILCs which are potent destroyer of malignant cells and they are particularly significant in limiting tumor metastasis. NK cells can diminish tumor growth by direct cytotoxicity through the release of cytolytic granules, by secretion of cytokines like IFN-γ, or indirectly by the orchestration of anti-tumor immunity. It is not only the secreted factors or the cellular and molecular composition of the tumor microenvironment, but also the architecture

of the tumor itself, that are key factors affecting metastasis formation. The findings that NKp46 ligation controls metastatic spread of tumor cells divulge a novel non-cytotoxic mechanism of NK cell anti-tumor control which could help to advance the development of potent NK cell immunotherapy strategies. It would be exciting to see whether modulation of NK cells *ex vivo*, such as enhancing NKp46 activity or IFN-γ production, could advance their anti-tumor activity upon adoptive transfer. Moreover, targeting the tumor architecture could symbolize a novel cancer therapy target. Overall, these findings emphasize the importance of activating receptors for potent NK cell-mediated anti-tumor immunity which could pave the approach for novel therapies.

Hu G and Wang S (2018)[55] stated that $CD57^+$ lymphocyte infiltration appreciably improved overall survival including 1-year, 3-year and 5-year survival, as well as disease-free survival in all types of solid tumors. In stratified analyses, $CD57^+$ lymphocyte infiltration was considerably associated with better overall survival in hepatocellular, oesophageal, head and neck carcinoma, non-small cell lung cancer. Five-year survival was associated with colorectal cancer, and three-year and five-year survival in gastric cancer. In addition, high density of intra-tumoral $CD57^+$ lymphocytes were radically correlated inversely with lymph node metastasis and TNM stage of solid tumor. In conclusion, they said that, $CD57^+$ lymphocyte infiltration lead to a good clinical outcome in solid tumors, suggesting that it is a useful biomarker for prognosis and adoptive immunotherapy based on these cells may be a hopeful choice for treatment.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present study titled "Comparative evaluation of NK cell (CD57) expression in Oral Leukoplakia and OSCC: an immunohistochemical study" was carried out in the department of oral pathology and microbiology.

The study was approved by the institutional ethics committee.

TYPE OF STUDY: Comparative, retrospective, analytical, observational study.

STUDY DURATION: Study was carried out from May 2017 to October 2018 over a period of 18 months.

MATERIALS:

The materials used for the study were as under:

1. **Paraffin embedded tissue sections** clinico-histopathologically diagnosed as Oral Leukoplakia and OSCC from archives.

2. **Olympus Trinocular Research Microscope** (Model – BX-51)

3. **Armamentarium and reagents for immunohistochemical detection of $CD57^+$ NK cells**

   - Glassware – Glass bottles, measuring jars, beakers, stirring rods
   - Optiplus Microscope Slides- BioGenex positively charged Glass slides (size 25 × 75 × 1.0 mm)
   - Hot plate
   - Water bath with thermometer
   - Glass cover slips
   - Analytical balance
   - pH meter
   - Incubator (56º- 60ºC)
   - Refrigerator (4ºC)
   - Staining Jars
   - Humidity chamber
   - Microtips and Micropipettes
   - E Z Antigen Retrieval system
   - Plastic vials for storage and making dilutions

- Primary monoclonal mouse antihuman antibody for CD57 and SS Polymer-HRP Detection Kit/DAB (Master Diagnostica)

The criteria for inclusion and exclusion for the paraffin embedded tissue sections was decided.

Inclusion Criteria:

- All the archived **paraffin blocks** of specimens that were **clinico-histopathologically diagnosed** as **Oral Leukoplakia** and **OSCC** from the department of oral pathology and microbiology.

Exclusion Criteria:

- Paraffin embedded biopsy specimens of **immuno-compromised** patients having oral leukoplakia or OSCC.*
- Paraffin embedded biopsy specimens of patients on **Immunosuppressant drug therapy** at the time of biopsy.*

***(based on the case-history records in the department)**

STUDY GROUPS:

The samples were divided into three groups:

Group I	**Normal oral mucosa (control group)**	**30 samples**
Group II	Clinico- Histopathologically proven samples of oral leukoplakia	30 samples
Group III	Clinico- Histopathologically proven samples of Oral Squamous Cell Carcinoma	30 samples

METHOD FOR IMMUNOHISTOCHEMICAL STAINING OF CD57^{+} NK CELLS

The immunohistochemical staining was carried out in the department of oral pathology and microbiology

REAGENT PREPARATION:

Prior to staining the following reagents were prepared.

a) Phosphate Buffered Saline (PBS) pH 7.4, 0.05 M.

For 1 Liter

- Sodium dihydrogen phosphate - 3.4gm.
- Disodium hydrogen phosphate- 12.0 gm.
- Sodium chloride - 8.5 gm.
- Distilled water - 1000 ml.

b) Preparation of Antigen Retrieval solution (pH -6)

For 500 ml.

Stock Solution 1

Citric Acid Buffer solution - 2.1 gm in 100 ml distilled water. (at 2 - 8^{o}C)

Stock Solution 2

Sodium citrate Buffer solution - 2.94 gm in 100 ml distilled water. (at 2 - 8^{o}C)

Working Antigen retrieval solution was made by mixing both the stock solutions

stock solution (1) - 9 ml of with

stock solution (2) - 41 ml.

Add distilled water to make up 500 ml,

pH of the solution obtained should be around 6.

PROCEDURE FOR IMMUNOHISTOCHEMICAL STAINING OF CD57^{+} NK CELLS:

- All the selected archived paraffin blocks of specimens of normal oral mucosa (from tissue removed during routine surgical procedures) and sections clinico-histopathologically diagnosed as Oral Leukoplakia and OSCC from the department of oral pathology and microbiology were processed, stained and evaluated for the expression of CD57 by immunohistochemical method. Following protocol was followed:

1. The slides were dehydrated by increasing grades of alcohol and brought to distilled water
2. Treated with hydrogen peroxide to eliminate endogenous peroxidase activity
3. Antigen retrieval with tri-sodium citrate for CD57 was done with primary antibody (monoclonal)
4. Sections were rinsed with PBS for five minutes

5. Blocking antibody was applied, incubated for 20 minutes at room temperature, residual fluid thrown off

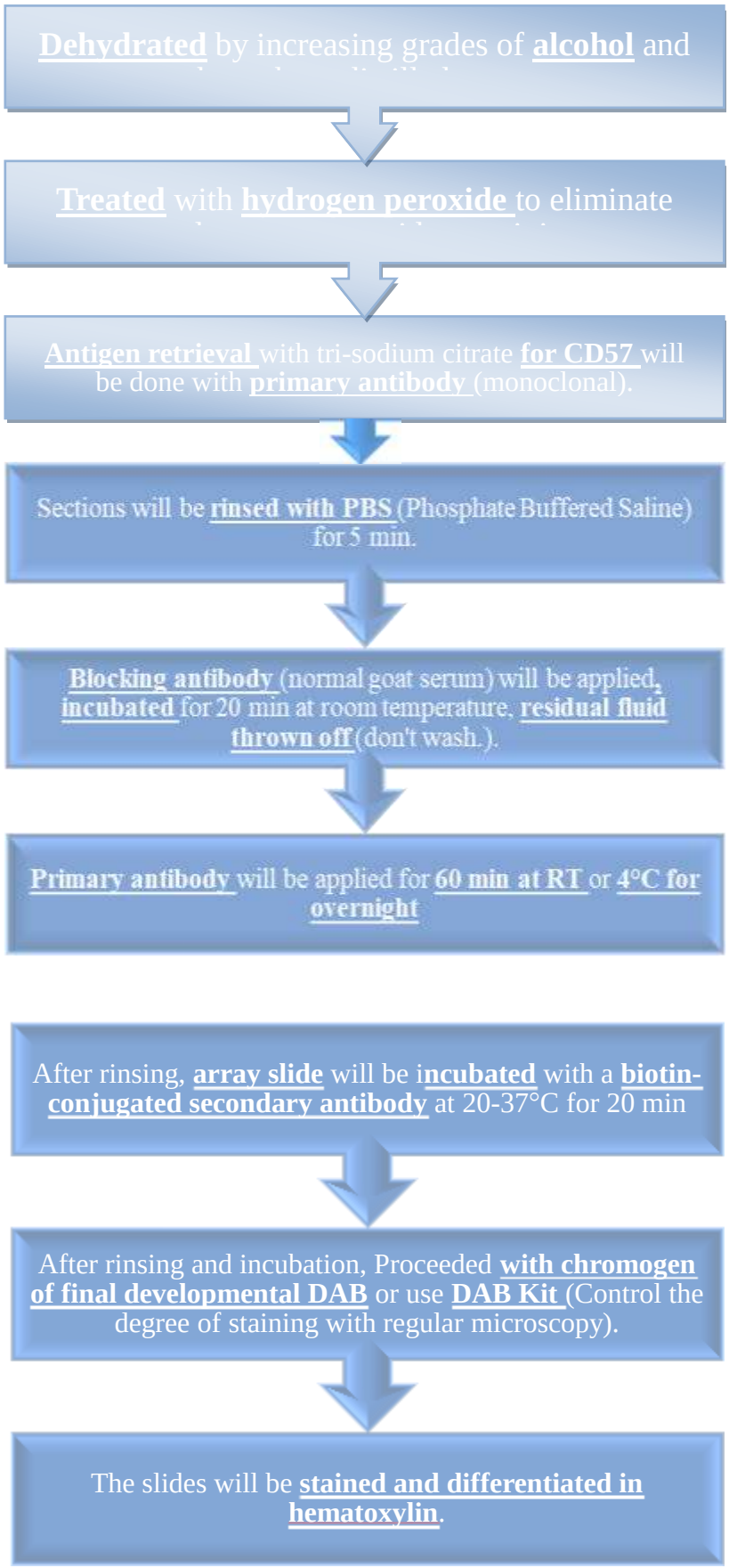
6. Primary antibody was applied for 60 minutes at room temperature

7. After rinsing, array slide was incubated with a biotin-conjugated secondary antibody at 20-37°C for 20 minutes

8. After rinsing and incubation, proceeded with chromogen of final developmental DAB or use DAB Kit (Control the degree of staining with regular microscopy)

9. The slides were stained and differentiated in hematoxylin

10. Positive and negative controls were examined for presence of staining

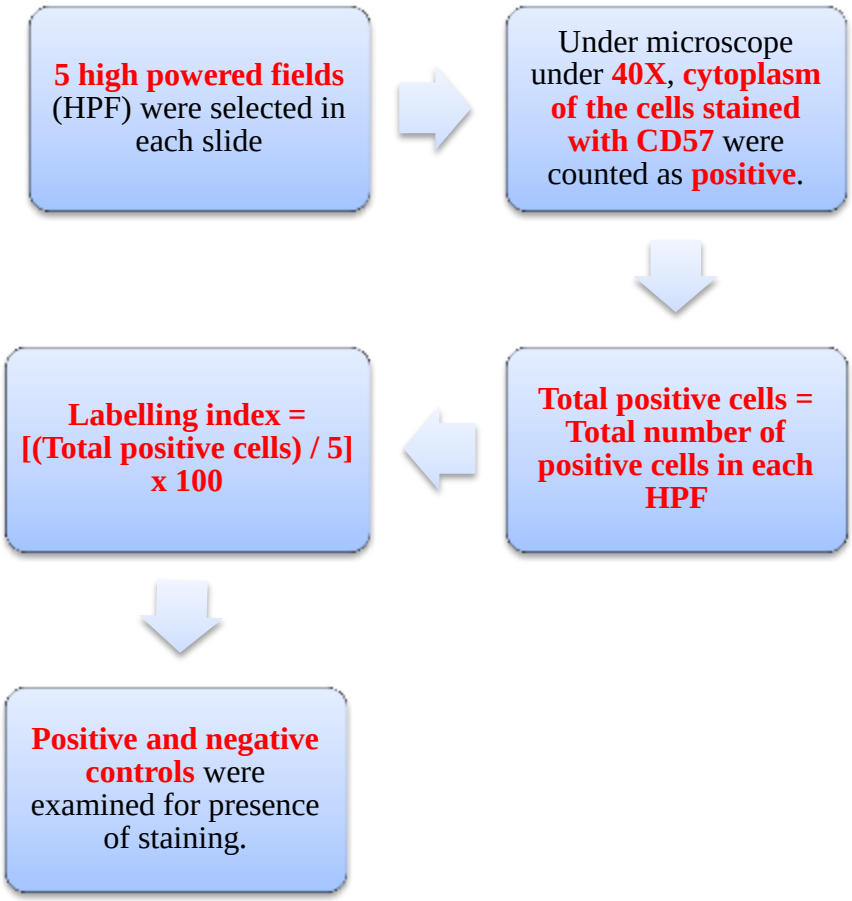

Dehydrated by increasing grades of alcohol and
Treated with hydrogen peroxide to eliminate
Antigen retrieval with tri-sodium citrate for CD57 will be done with primary antibody (monoclonal).
Sections will be rinsed with PBS (Phosphate Buffered Saline) for 5 min.
Blocking antibody (normal goat serum) will be applied, incubated for 20 min at room temperature, residual fluid thrown off (don't wash.).
Primary antibody will be applied for 60 min at RT or 4°C for overnight
After rinsing, array slide will be incubated with a biotin-conjugated secondary antibody at 20-37°C for 20 min
After rinsing and incubation, Proceeded with chromogen of final developmental DAB or use DAB Kit (Control the degree of staining with regular microscopy).
The slides will be stained and differentiated in hematoxylin.

CD57 LABELLING INDEX WAS EVALUATED

The method employed was as follows:

1. Five high powered fields (HPF) were selected in each slide
2. Under microscope under 40×, cytoplasm of the cells stained with CD57 were counted as positive
3. Total positive cells = Total number of positive cells in each HPF
4. Labelling index = [(Total positive cells) / 5] × 100

   [Cells were considered positive for CD57 in intracytoplasmic DAB staining (chromogen colour)]



- The results obtained were statistically analysed and compared.

CONTROL SLIDES

Positive Control:

According to the manufacturer's recommendation, known human liver samples and human tonsil samples showing good $CD57^+$ NK cell expression acted as positive control.

One Positive control was included for each immunohistochemical cohort.

This Positive control was observed for the presence of a coloured end product (DAB chromogen, brown coloured end product) at the site of target antigen.

The presence of brown coloured intracytoplasmic staining was interpreted as positive staining indicating proper performance of kit reagent.

Negative Control:

Studies show that normal human oral mucosa does not show the presence of $CD57^+$ NK cells.[4] The normal oral mucosa included in group I of the study acted as the negative control.

STATISTICAL METHODS EMPLOYED

The data obtained was compiled on a MS Office Excel Sheet (v 2010) and was subjected to statistical analysis using SPSS v 21.0, IBM (Statistical package for social sciences) software.

For all the statistical tests, $p<0.05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

- Chi-square test was used to assess gender distribution across oral lesions.

- One-way ANOVA was used to assess age distribution across oral lesions.
- One-way ANOVA was used to assess Distribution of CD 57^{+} NK cell expression as per oral lesions.

ETHICAL ISSUES INVOLVED - None

PLATE I

Fig 10. Positively charged microscope slides

Fig. 11 Glassware and pipettes

Fig 12a. Antigen retireval system

Fig 12b. Antigen retrieval system

PLATE II

Fig 13. CD57 Antibody kit with CD57 primary antibody and Super sensitive polymer-HRP detection system

Fig 14. Immunohistochemistry staining system

Fig 15. Image analyzer system - Used for Immunohistochemical analysis

OBSERVATIONS AND RESULTS

OBSERVATIONS AND RESULTS

The present comparative, retrospective, analytical, observational study consisted of a total of 90 samples. Out of these samples, 30 were clinico-histopathologically diagnosed as oral leukoplakia (OL) and 30 were OSCC. Among the 30 samples of OSCC, 15 samples were of well-differentiated OSCC and 15 samples of moderately-differentiated OSCC. Thirty samples of normal oral mucosa were also considered as a control group for comparative purpose.

STUDY GROUPS:

Group I - 30 samples of Normal oral mucosa (Control group)

Group II - 30 samples of OL

Group III - 30 Samples of OSCC

Each study group was evaluated for expression of $CD57^+$ NK cells stained irrespective of the intensity of staining in the connective tissue stroma of the stained sections. Labelling index for the $CD57^+$ NK cells was calculated in all the three groups.

The data was collected, tabulated and analysed by SPSS v21.0, IBM (Statistical package for social sciences) software and the statistical significance was tested at 5% level.

The observations of these studies are explained in detail in the following sections.

Chart 1: Pie diagram of distribution of lesion

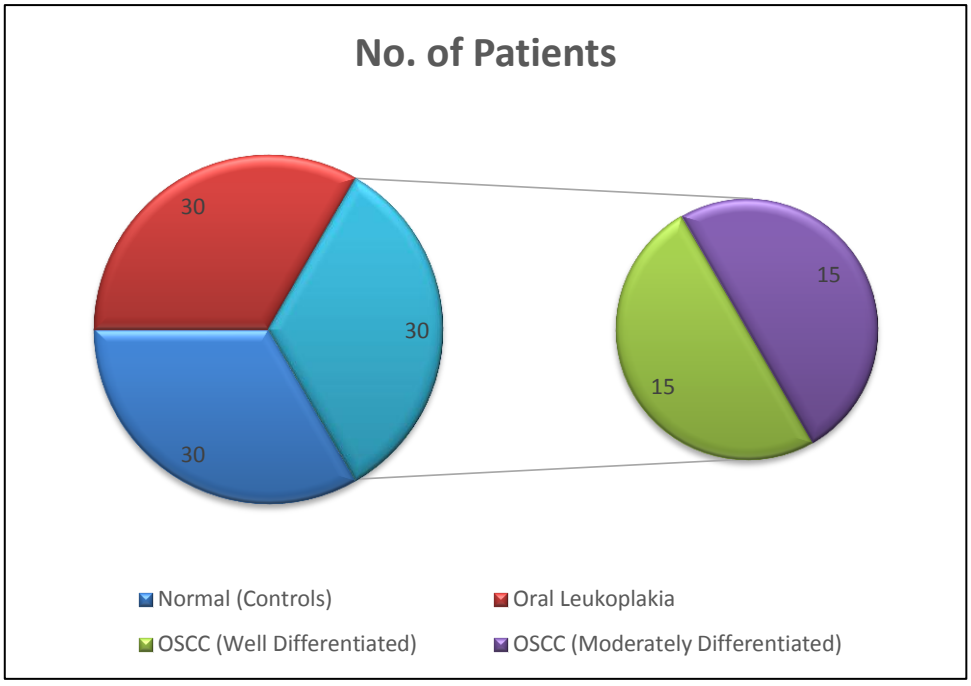


Table 6: Age distribution

Age Group (Years)	No. of Samples	% of Samples
≤ 30	8	8.9
31 – 40	13	14.4
41 – 50	23	25.6
51 – 60	22	24.5
61 – 70	21	23.3
> 70	3	3.3
Total	**90**	**100**

Comments (Table 6 and chart 2):

Out of 90 samples, 8 (8.9%) were ≤ 30 years, 13 (14.4%) were between 31 to 40 years, 23 (25.6%) were between 41 to 50 years, 22 (24.5%) were between 51 to 60 years, 21 (23.3%) were between 61 to 70 years and 3 (3.3%) were > 70 years of age.

Chart 2: Pie diagram of age distribution

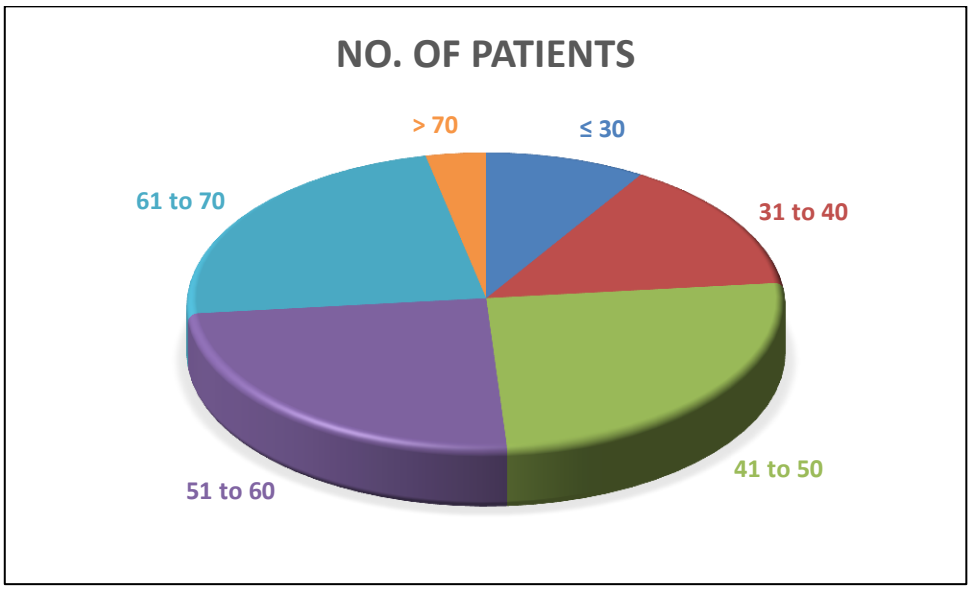


Table 7: Age distribution across the study groups

Disease	Mean Age (Years)	Standard Deviation (Years)	P Value
Normal oral mucosa (Control)	55.43	9.88	**0.059 (NS)**
OL	47.33	16.02	
OSCC	50.27	12.76	
Total	**51.01**	**13.41**	

Degree of freedom = 2,57; ANOVA (F) = 2.92716; P > 0.05, Not Significant (NS).

Comments (Table 7 and chart 3):

Mean age of the overall population was 51.01 years with standard deviation of 13.41 years. Mean age in normal oral mucosa group was 55.43 years with standard deviation of 9.88 years. OL had mean age of 47.33 years with standard deviation of 16.02 years and OSCC had mean age of 50.27 years with standard deviation of 12.76 years. This difference was statistically not significant with **P = 0.059 (>0.05).**

Result: Age and oral lesion are independent of each other.

Chart 3: Bar diagram of age distribution across the study groups

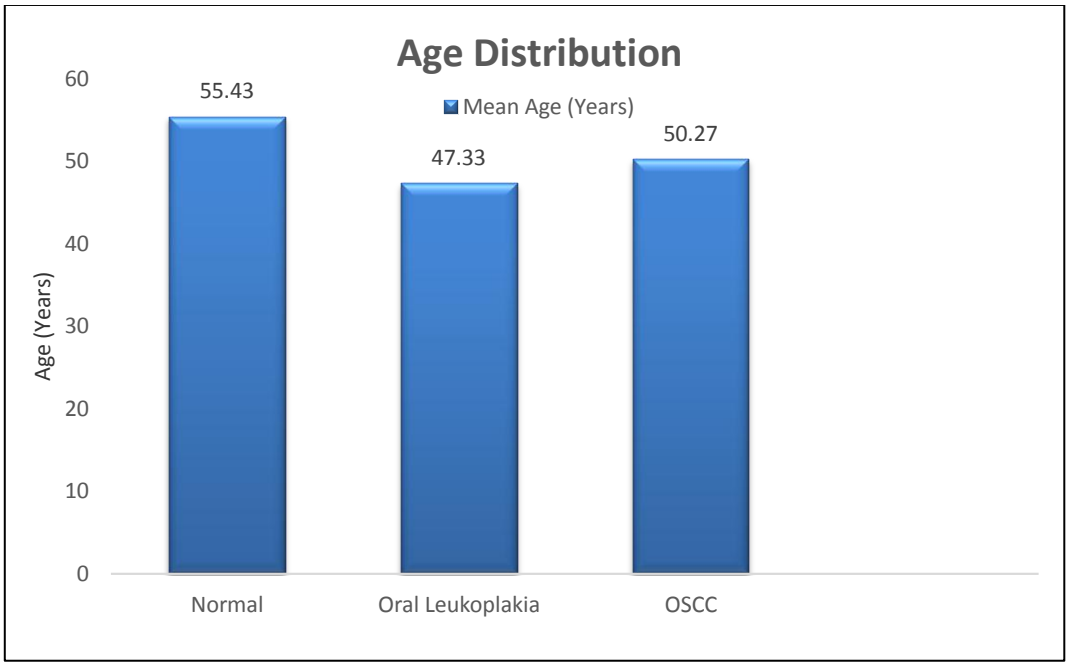


Table 8: Gender distribution

Gender	No. of Samples	% of Samples
Male	72	80
Female	18	20
Total	**90**	**100**

Comments (Table 8 and chart 4):

Out of 60 samples, 72 (80%) were males and only 18 (20%) were females

Chart 4: Pie diagram of gender distribution

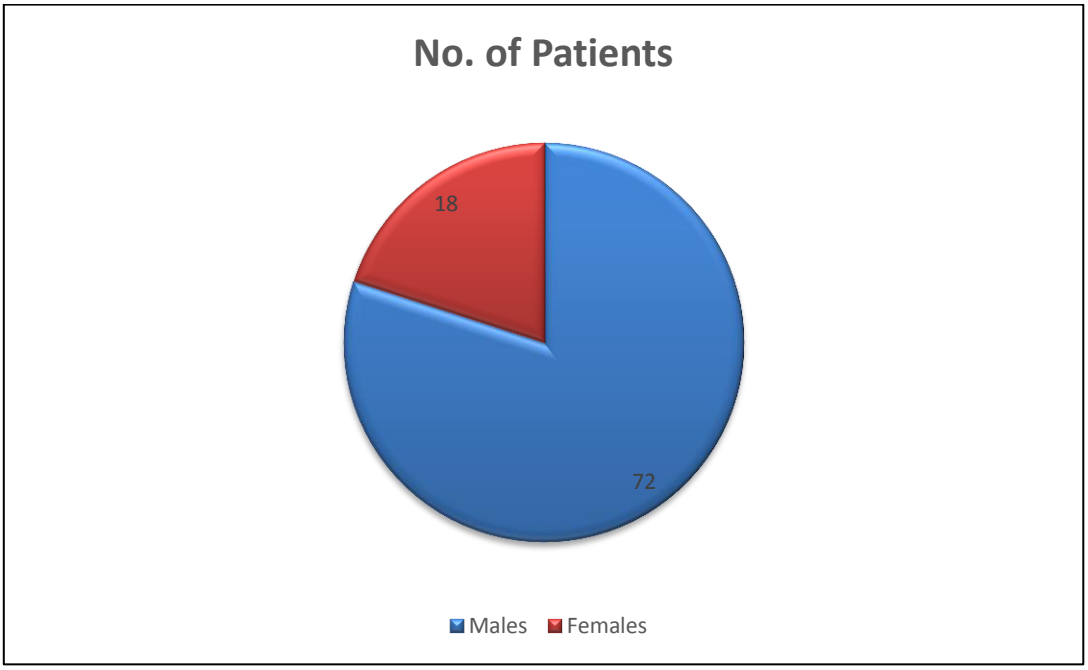


Table 9: Gender distribution across the study groups

Disease	**Normal Oral Mucosa (Control)**		**OL**		**OSCC**		**P Value**
Gender	**No. of Samples**	**% of Samples**	**No. of Samples**	**% of Samples**	**No. of Samples**	**% of Samples**	
Male	18	60.0	29	96.7	25	83.3	**0.0016 (S)**
Female	12	40.0	1	3.3	5	16.7	
Total	**30**	**100**	**30**	**100**	**30**	**100**	

Degree of freedom = 2, Chi-Square = 12.9167, P < 0.05, Significant (S).

Comments (Table 9 and chart 5):

Out of 30 samples with normal oral mucosa, 18 (60.0%) were males and 12 (40.0%) were females; out of 30 samples with OL, 29 (96.7%) were males and 1 (3.3%) was a female and out of 30 samples with OSCC, 25 (83.3%) were males and 5 (16.7%) were females. This difference is statistically significant with **P = 0.0016 (<0.05).** The statistical analysis was done using CHI-SQUARE TEST.

Chart 5: Bar diagram of gender distribution across the study groups

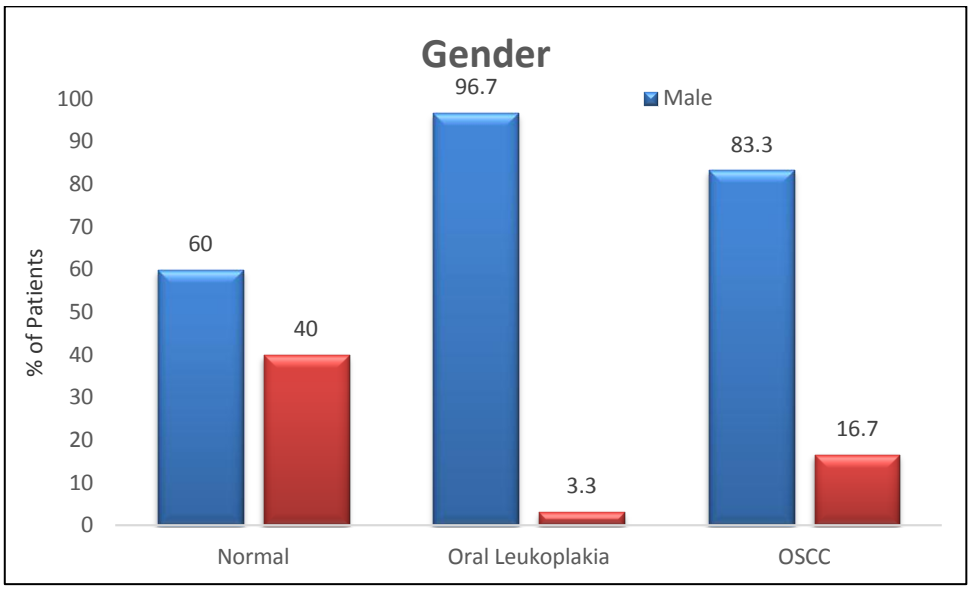


Table 10: Distribution of NK cell (CD 57) expression as per the study groups

Oral Lesion	Mean Labelling Index (/HPF)	Standard Deviation (/HPF)	P Value
Normal oral mucosa (Control)	00	--	**< 0.001**
Oral Leukoplakia	58.33	4.36	
Well-differentiated OSCC	30.80	1.91	
Moderately-differentiated OSCC	19.91	3.62	
Total	**41.84**	**17.46**	

Degree of freedom = 2,57; ANOVA (F) = 624.73; P < 0.05, Significant (S).

Comments (Table 10 and chart 6):

Overall, the expression of NK cells estimated as CD 57^+ cells, measured as mean labelling index per HPF was 41.84 / HPF with standard deviation 17.46 / HPF.

1) The $CD57^+$ NK cell expression in normal oral mucosa (control group) was negative.

2) In OL, the mean labelling index of $CD57^+$ NK cells was 58.33 / HPF with standard deviation of 4.36 / HPF.

3) In OSCC, well-differentiated OSCC had mean labelling index of 30.80 / HPF with standard deviation of 1.91 / HPF and moderately-differentiated OSCC had mean labelling index of 19.91 / HPF with standard deviation of 3.62 / HPF.

4) On comparing the expression of $CD57^+$ NK cells in OL and OSCC, the expression of $CD57^+$ NK cells was highest in oral leukoplakia which is seen to be markedly reduced on OSCC. **This difference was statistically significant with $P < 0.001$. Statistical analysis was done using ONE-WAY ANOVA.**

5) On comparing the expression of $CD57^+$ NK cells in different grades of OSCC, the expression of $CD57^+$ NK cells is lesser in moderately-differentiated OSCC as compared to well-differentiated OSCC. This signifies that the expression of $CD57^+$ NK cells goes on decreasing with increasing grade of OSCC. **The difference was statistically significant with $P < 0.001$. Statistical analysis was done using ONE-WAY ANOVA.**

Chart 6: Bar diagram as per NK cell expression as per the study groups

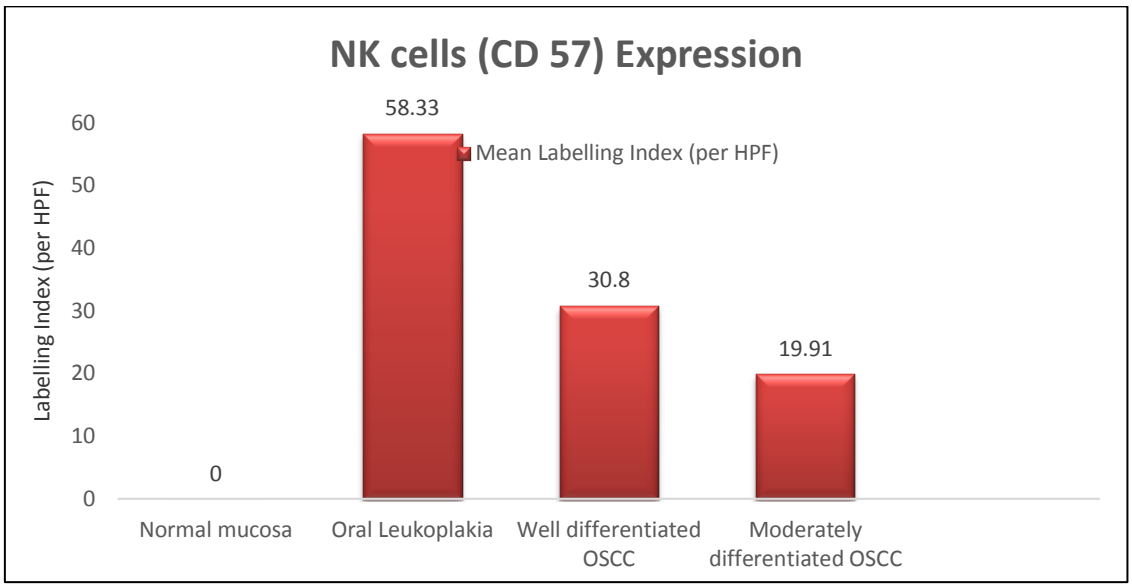


PLATE III

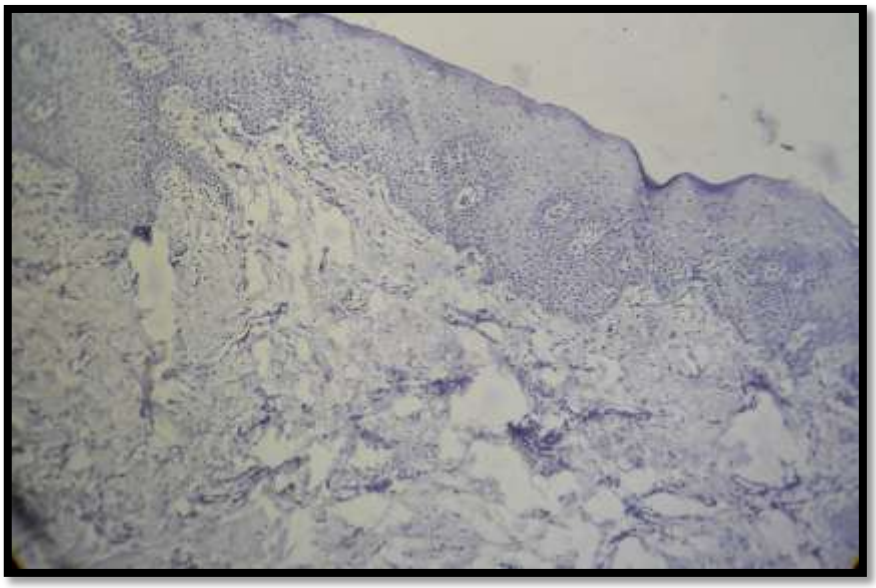


Fig 16. Negative IHC expression of CD57+ve NK cells in normal oral mucosa (10x) (Negative control)

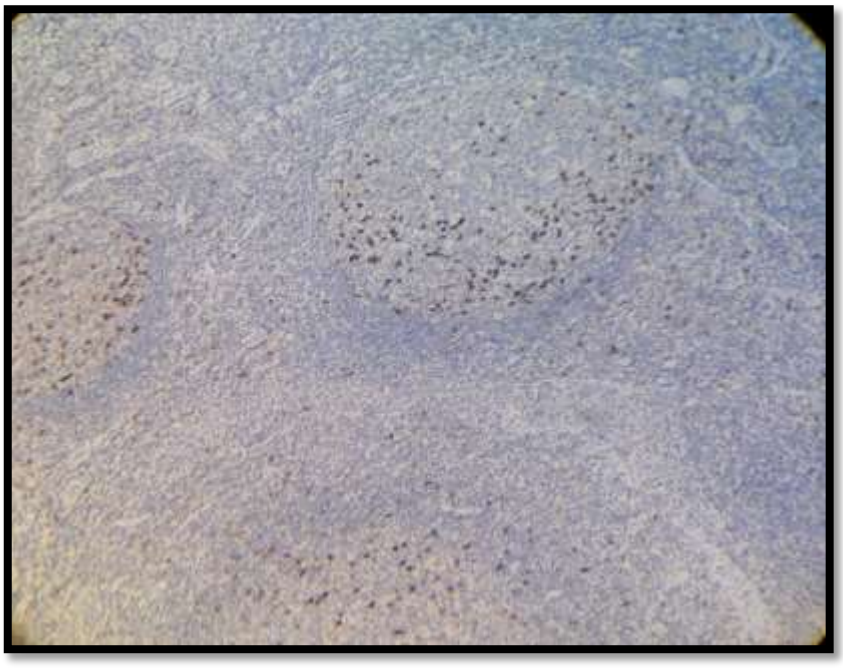


Fig 17. Positive IHC expression of CD57+ve NK cells in human tonsil (10x) (Positive control)

PLATE IV

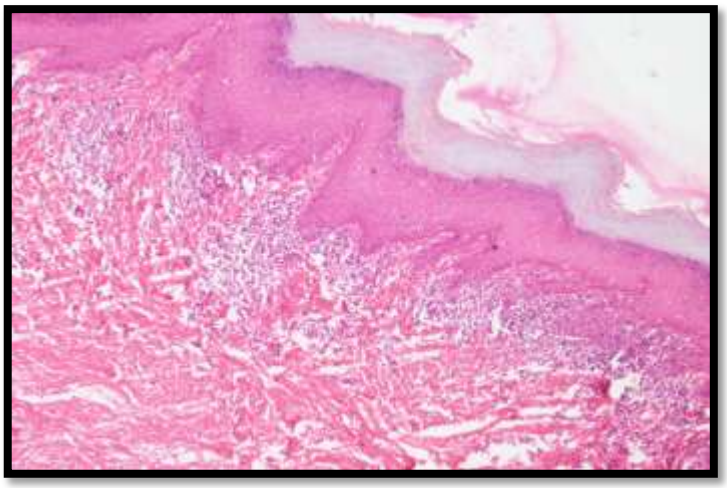


Fig 18. Oral leukoplakia H & E stained (10x)

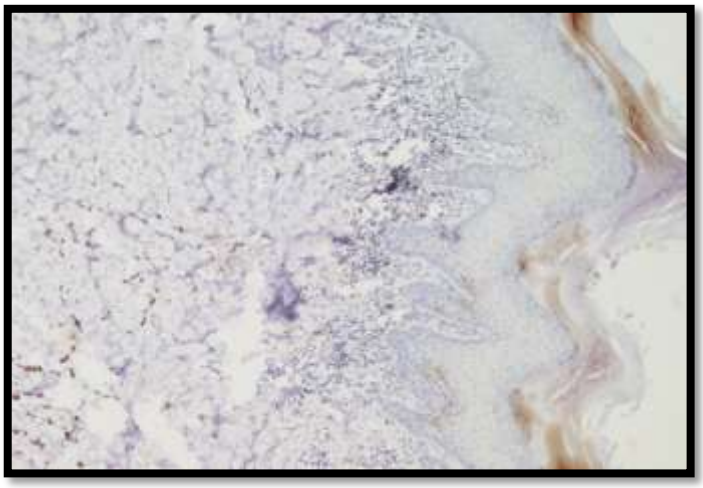


Fig 19 a. IHC expression of CD57+ve NK cells in oral leukoplakia (10x)

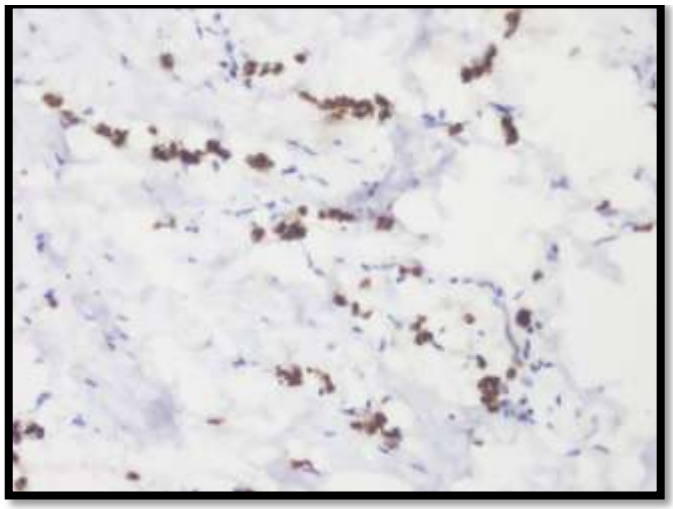


Fig 19b. IHC expression of CD57+ve NK cells in oral leukoplakia (40x)

PLATE V

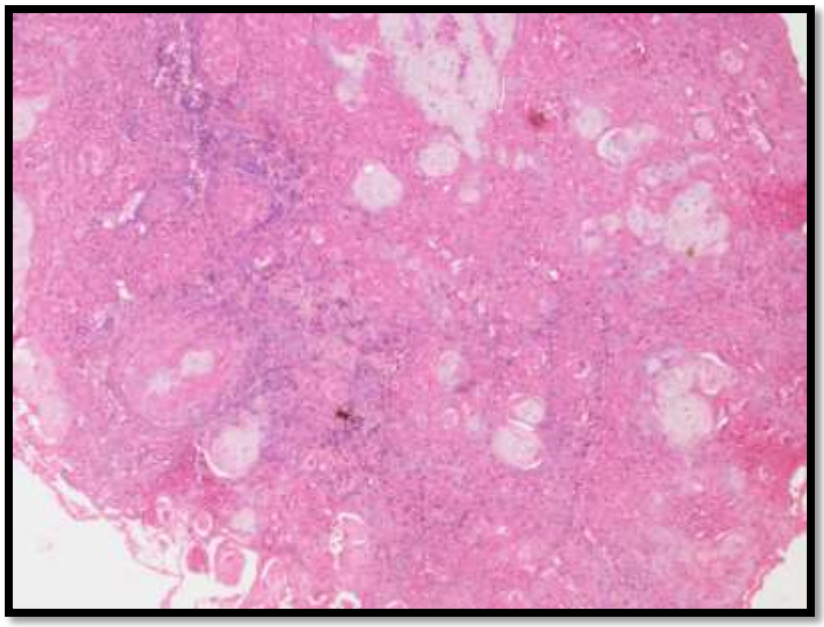


Fig 20. Well differentiated squamous cell carcinoma.
H & E stain (10x)

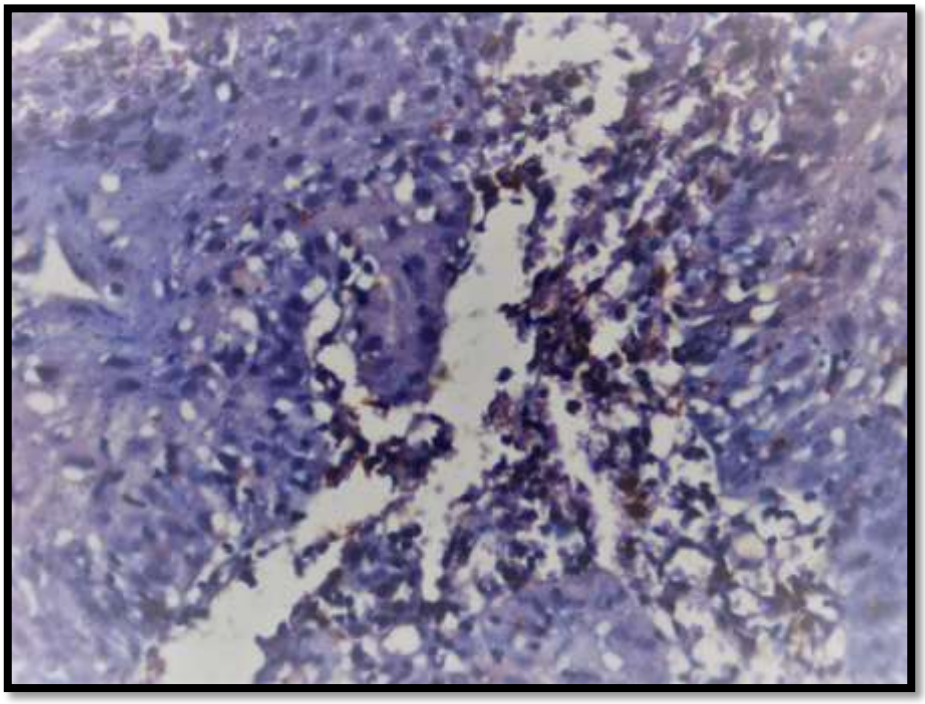


Fig 21. IHC expression of CD57+ve NK cells in well differentiated squamous cell carcinoma (40x)

PLATE VI

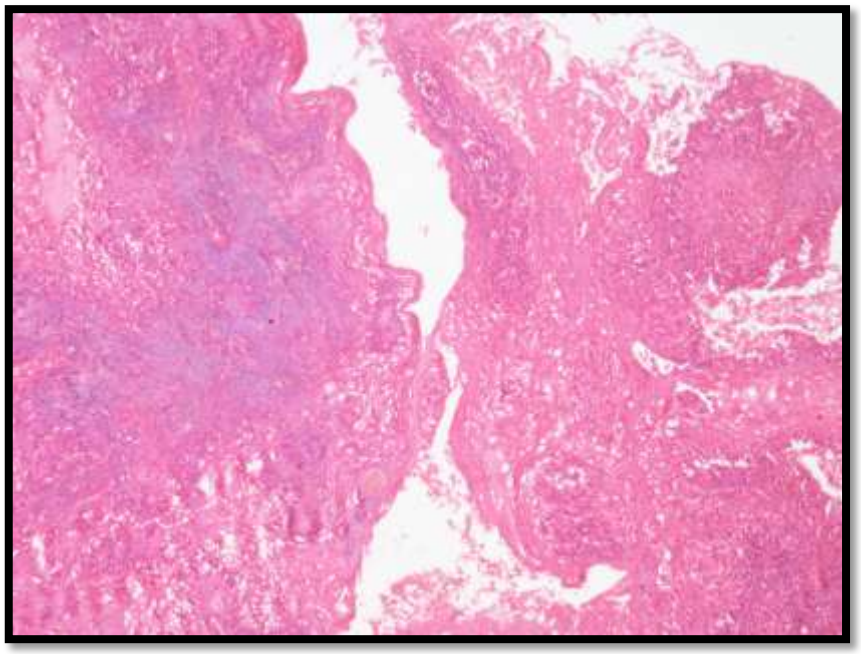


Fig 22. Moderately differentiated squamous cell carcinoma. H & E stain (10x)

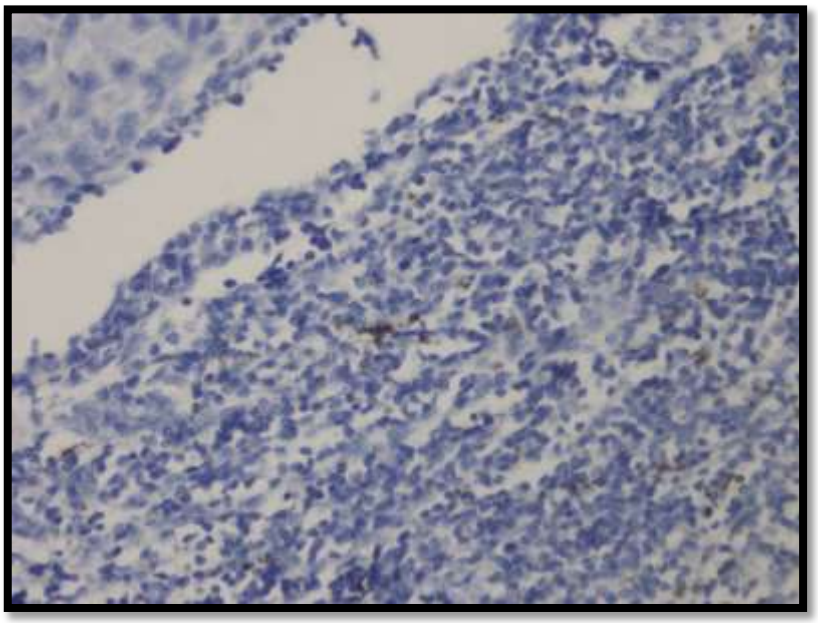


Fig 23. IHC expression of CD57+ve NK cells in moderately differentiated squamous cell carcinoma (40x)

DISCUSSION

DISCUSSION

Oral cancer is the sixth most common cancer reported globally having an annual incidence of over three lakh cases, 62% of which arise in developing countries. In India, oral cancer ranks among the top three types of cancers and accounts for 30% of all cancers in the country. The variation in incidence and pattern of the disease can be attributed to the combined effect of ageing as well as regional differences in the prevalence of disease specific risk factors. Cancer pertaining to the oral cavity is the 8^{th} most frequent cancer in the World among males and 14^{th} most among females. The main risk factors are tobacco use and alcohol.[56]

Over 95% of oral cancers are OSCCs. The development of OSCC is a multi-step and multifactorial process involving field carcinogenesis and intraepithelial clonal spread. The frequency of OSCCs in India remains one of the highest in the

World being 30-50% of the total global cancer incidence. In large part, it is attributed to exposure to carcinogens such as tobacco.[57] Notably, the great morbidity and mortality rates of this disease have not improved in decades.[58] Hence, early recognition is imperative in attempting to improve oral cancer survival rates, preserve function and enhance aesthetic and psychological outcomes.[59] OSCC is of significant public health importance in India. Firstly, it is diagnosed at a later stage resulting in low treatment outcomes and considerable cost to the patients who are typically of a low socio-economic status. Secondly, rural areas have an inadequate access to trained health care services. Thus, earlier detection of OSCC offers the best chance for long term survival and has the potential to improve the healthcare outcomes. Public health officials, private hospitals and academic medical centres within India have considered OSCC as a grave problem. Efforts to increase the body of literature on the knowledge, aetiology and regional distribution of the risk factors of this disease have begun to gain momentum. Oral cancer will remain a major health problem and efforts towards its early detection as well as prevention will reduce this burden.[56]

Multi-step theory of carcinogenesis describes that, oral cancer develops from potentially malignant mucosal lesions to invasive malignant changes which causes serial histological and clinical alterations. Common pre-malignant lesions present clinically as leukoplakia or erythroplakia. However, these lesions may have histologically diverse manifestations such as hyperkeratosis, dysplasia or even carcinoma.[60]

The term ‘potentially malignant disorders’ used by WHO is more widely accepted today as it conveys that not all lesions described under this term may

transform to cancer, rather, there is a family of morphological alterations among which some may have an increased potential for malignant transformation.[61]

Leukoplakias of the oral cavity are often encountered and have a well documented potential to develop into OSCC. A substantial part (15% to 20%) is reported to develop into carcinomas.[41] There have been wide variations in the incidence and prevalence of leukoplakia in India. A striking variation has been observed with 0.2% in Bihar and 4.9% in Andhra Pradesh. Gujrat has shown prevalence rate of 11.7%. The possibility of malignant transformation of leukoplakia depends on multiple factors. The frequency of dysplastic and malignant alterations in OL has ranged from 15.6% to 39.2% in several studies. In Indian studies, malignant transformation rates in leukoplakia varied from 0.13% to 10% in various Indian populations.[62,63]

During the last decades several studies have been carried out focusing on the importance of the immune system and prognosis of cancer.[64-66] The original description of the idea that the immune system could repress a potentially overwhelming frequency of carcinomas was envisioned by Paul Ehrlich in 1909. As the understanding of immunology expanded, Burnett and Thomas in 1957 proposed the immunosurveillance theory contemplating that the immune system was capable of destroying growing malignancies. The theory speculated that the lymphocytes acted as sentinels in recognizing and eliminating continuously arising, nascent transformed cell.[54]

Considerable evidence exists which suggests that the immune response plays a role in regulating the development and growth of oral pre-cancers. Investigation of the

inflammatory infiltrate into oral pre-cancers has yielded valuable information. The infiltrate has been shown to be mainly T cells though some NK (NK) and B-cells were present treatment of effector NK cells with interferon resulted in highly elevated killer cell activity. Biological response modifiers with the ability to stimulate NK cell activity, such as interferon, IL-2 and beta carotene could possibly modify the pathogenetic course of oral precancerous lesions.[35]

Progression in research with reference to the role of immune system in carcinogenesis led to the proposal of immunostimulation theory by Prehn (1970) who suggested that the immune system may actually promote the growth of tumors. Understanding the role of immune system in tumor rejection and tumor formation led to the modification of immunosurveillance concept into cancer immunoediting concept which describes the host protective and tumor sculpting actions of the immune system in preventing and shaping neoplastic disease. Cancer immunoediting encompasses three phases namely elimination, equilibrium and escape which encompasses the role of immune system in carcinogenesis.[30]

The concept of cancer immunoediting has resulted with phases of elimination, equilibrium and escape in a view of dynamic interaction between the immune system and dysplastic cells/tumor cells Thus, in an early phase of cell dysplasia or early tumor cell formation the immune system has the ability to eradicate cells with DNA damage that may result in, or already have resulted in, cancer. Immunoediting is probably of importance in the defence against dysplastic cells that may occur in leukoplakia and OSCC. The bulk of knowledge accumulated during the last decades has been almost exclusively gained in studies on established tumor s. Less is known about immune activation in pre-malignant disorders.[67] The primary role of the

normally functioning immune system is to provide defence against malignant cells. Therefore, the failure of immune surveillance has been reported to be an important factor in the development of cancer.[32]

Kiessling et al (1975)., coined the term 'NK cells to describe cells mediating this function, and it was soon appreciated that these cells were also capable of killing virus-infected cells, as well as allogeneic cells'. In the ensuing three decades, NK cells were shown to comprise a unique lineage of lymphocytes, distinct from T cells and B-cells, and their receptors used for tumor cell recognition, as well as the mechanisms employed to mediate cytotoxicity were identified. NK cells were initially identified by their morphology, being designated 'large granular lymphocytes' based on being slightly larger than resting T cells or B-cells and containing azurophilic granules, later found to contain the proteases and cytolytic proteins (granzymes and perforin) responsible for their cytolytic function.[27]

Effective immunosurveillance is imperative in the prevention of initiation and progression of carcinomas. Rapid progression of OL to oral carcinoma was observed after significant immunosuppression. Immunosuppression and tumor escape from immune recognition are thought to be the major factors that are responsible for the establishment and progression of cancer. However, the underlying physiological significance or the exact mechanisms by which immunosuppression occurs are still to be understood.[32]

A number of factors responsible for the suppression of NK cell cytotoxicity have been identified. Numerous mechanisms have been proposed for the functional inactivation of tumor-associated NK cells which take account of the over-expression

of Fas ligand , loss of mRNA for granzyme B as well as decreased CD16 and its associated zeta chain.[32]

The **mechanism of immune evasion** has been described in the following table[32]

Defect	**Mechanism**
Altered NK cell recognition	Decreased expression of NK receptor (NKG2D) ligands, increased MHC Class I expression, decreased expression of adhesion molecules, CD16 and zeta chai
Loss of NK cell function	Induction of anergy in NK cells. Activation of upstream transcription factors (NFκB) in differentiated tumors and tumor cell production of inhibitory factors (IL-10, IL-6, IL-1β, PGE2, GM-CSF, IL-8). Decreased IFN-γ secretion by the NK cells when co-cultured with increased NFκB function in tumors
Enhanced tumor cell survival/resistance to killing	Expression of anti-apoptotic molecules by tumor cells by means of activation of upstream transcription factors (c-Myc, AP-1, NFκB, STAT3)
Increased NK and T cell apoptosis	TNF-α induced apoptosis, expression of Fas ligand by tumor cells and membranous vesicles; Fas-mediated apoptosis of responding T cells, expression of DF3 and Muc1 in tumor cells

NK cells have two significant functions; one that relates to the elimination of stem cells that are either defective or disturbed or in general exceed the required number for the regeneration of damaged tissue. Therefore, they may select stem cells competent to achieve the highest ability to regenerate tissues.[32,43]

Alternatively, or in addition, NK cells may lyse other effectors in the connective tissue area in order to decrease inflammation as well as be conditioned to promote tissue regeneration. The subsequent important task for NK cells, therefore, is to support differentiation and promote tissue regeneration after altering their phenotype to cytokine secreting cells.[32]

This process not only would remove cells that are damaged and have flaws in the differentiation process, but it will also confirm the regeneration of tissues and the resolution of inflammation. Thus, any disturbance in the NK cell function may result in a chronic inflammatory process, causing continual tissue damage and recruitment of immune effectors to aid in tissue regeneration.[42] The inability of patient NK cells to contain CSCs due to the flooding of NK cells by proliferating CSCs and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer may progress and metastasize.[32]

Hence, there should be two definite strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells.[32.43] If this fails, downregulation of NK cells by the tumor takes place.

NK cells are frequently identified as CD3 - CD56þ lymphocytes, and further differentiated into immature CD3 CD16 $CD56^{bright}$þ and mature CD3- CD16þ- CD56þ

NK cells.[32] CD57 was first identified on cells with NK activity using the mouse monoclonal antibodies human NK-1 (HNK1) and Leu-7 and was subsequently assigned the CD designation, CD57, at the Fourth International Workshop of Human Leukocyte Antigens in 1989. HNK1/Leu-7/CD57 was initially believed to be uniquely expressed on NK cells – and was used to define this population – although it was soon apparent that CD57 was expressed only on a subset of functionally discrete NK cells. Progression from $CD56^{bright}$ to $CD56^{dim}CD57^{-}$ to $CD56^{dim}CD57^{+}$ reflects a maturation pathway for NK cells [35, 36] and rather than being a marker of anergy or immunosenescence, acquisition of CD57 represents a shift towards a higher cytotoxic capacity, greater responsiveness to signalling via CD16 and NCRs and decreased responsiveness to cytokines. The extent to which CD57 expression per se drives these changes in function, as opposed to being a marker for cells with altered expression of other characteristics of a mature NK cell, is not entirely apparent and may represent a fertile spot for further research. To add to this, a much better characterization is essential of the cell surface molecules that express the CD57 epitope, the mechanisms by which CD57 is induced on them and its functional consequences.[17]

Our study was an attempt to carry out a comparative evaluation of NK cell (CD57) expression in OL and OSCC by immunohistochemistry. For this study, total 90 paraffin blocks of oral lesions were chosen for the study. These were divided into three groups. Group I consisted of 30 paraffin blocks of normal oral mucosa taken as the control group. Group II consisted of 30 paraffin blocks clinico-histopathologically proven as OL. Group III consisted of 30 paraffin blocks clinico-histopathologically proven as OSCC. Group III was further divided into 15 paraffin blocks of well-differentiated squamous cell carcinoma and 15 paraffin blocks of moderately-

differentiated squamous cell carcinoma. The blocks were sequentially processed, stained and evaluated for the expression of $CD57^+$ NK cells by immunohistochemical method.

Mean age of the overall population was 51.01 years with standard deviation of 13.41 years. Mean age in normal oral mucosa group was **55.43 years** with standard deviation of 9.88 years. OL had mean age of **47.33 years** with standard deviation of 16.02 years and OSCC had mean age of **50.27 years** with standard deviation of 12.76 years.

According to the studies by R. Shenoi[68] (2012) and Vázquez-Álvarez R.[69] (2010), the highest occurrence of OSCC occurs in 50 to 60 years age group in OSCC and between 40 and 79 years in OL. Our results are consistent with the results in these studies.

Out of 30 patients with normal oral mucosa, 18 (60.0%) were males and 12 (40.0%) were females; out of 30 patients with OL, 29 (96.7%) were males and 1 (3.3%) was a female and out of 30 patients with OSCC, 25 (83.3%) were males and 5 (16.7%) were females. This signifies that males were affected more as compared to females.

In studies by R. Shenoi[65] (2012) and Sharma P[70] (2010), it has been mentioned that these diseases occur more commonly in males than in females as is evident in our results.

As per our results, **the expression of CD 57$^+$ NK cells in samples of normal oral mucosa (control) was negative.** Positive control was taken: Human tonsil and human liver tissue which stained positive for CD 57$^+$ NK cells.

On evaluation of CD 57$^+$ NK cells in OL, the mean labelling index of CD57$^+$ NK cells was 58.33 / HPF with standard deviation of 4.36 / HPF.

On evaluation of CD 57$^+$ NK cells in OSCC, well-differentiated OSCC had mean labelling index of 30.80 / HPF with standard deviation of 1.91 / HPF and moderately-differentiated OSCC had mean labelling index of 19.91 / HPF with standard deviation of 3.62 / HPF.

On comparing the expression of CD57$^+$ NK cell expression in OSCC and OL, expression of CD57$^+$ NK cells was highest in OL. The expression decreased in OSCC. This signified that there is increased expression of CD57$^+$ NK cells in OL as compared to OSCC.

After profound research, there has been one study found regarding the expression of CD57$^+$ NK cells in OL.

Pillai et al[35] (1991) studied immunological abnormalities in oral pre-cancers. They concluded that immune response plays a role in regulating the growth and development of oral pre-cancers. This signifies that the infiltration of immune cells is increased in oral precancer which correlates with our results.

According to **De Paula A and Gomez R**[38] (2001) the number of CD57$^+$ NK cells goes on increasing with the increase in epithelial dysplasia. However, in our

study the expression of $CD57^+$ NK cells was evaluated in OL irrespective of epithelial dysplasia. Expression of CD57 on NK cells is suggestive of highest maturity and low cellular activity. As there is lack of tumor cells in oral leukoplakia, the NK cell activity is less. Thus, more NK cells are seen in their matured state.[38] Moreover, lack of downregulation of NK cells due to absence of tumor cells in oral leukoplakia may be another reason for the increased expression of NK cells.[26]

. NK cells constitute an early defence system against foreign and autologous cells suffering from stress representing the first line of defence against tumors.[26] Immune cell exhaustion depicts the status of dysfunction of immune cells, usually under the settings of tumors.[71] At single cell levels, tumor-infiltrating NK cells produced decreased effector cytokines IFN-γ and GM-CSF in mouse models. NK cells in cancer patients showed diminished cytolytic activity, as evidenced by lower expression of cytolytic molecules, such as granzymes, perforin, FasL, and TRAIL.[71] It is well stated in literature, that $CD57^+$ NK cells represent the highest stage of NK cell maturity. Thus, these downregulated and exhausted NK cells in tumors fail to reach the maturation stage and therefore do not represent CD57 antigen. So, lesser and lesser $CD57^+$ NK cells are seen as the disease progresses from OL to OSCC. Thus, there is a decreased expression of $CD57^+$ NK cells in OSCC as compared to OL.

On comparing the expression of $CD57^+$ NK cells between different grades of OSCC, our study showed that the expression of $CD57^+$ NK cells was more in well-differentiated squamous cell carcinoma as compared to moderately-differentiated oral squamous cell carcinoma. This signifies that the expression of $CD57^+$ NK cells

decreased with increase in the grade of OSCC suggesting the downregulation of NK cells by the tumor cells.

In their study, **Agarwal et al**[4] stated that CD57 was expressed in OSCC in decreasing order from well-differentiated OSCC to poorly differentiated OSCC. The reason for this decrease is mostly due to the downregulation of NK cells as the tumor progresses.[71]

Our results showing decrease in CD 57^+ NK cells as the tumor progresses from well to moderately-differentiated OSCC, also correlate with a study by **Li et al**[72] who carried out a study on NK cell inhibition by gastric cancer cells and concluded that immunosuppressive barriers erected by tumor cells greatly hamper the anti-tumor activity of human NK cells thereby favouring tumor outgrowth and progression.

Lopes et al[26] (2017) carried out evaluation of CD 57^+ cells in immune cells of OSCC and their relationship with clinicopathological parametes. Their results showed that CD $57^=$ immune cells were a consistent finding in OSCC regardless of the clinicopathological features of these tumors.

Jamkar et al studied the NK cell activity in oral cancer patients and attempted to correlate the results with tumor size and metastasis in regional lymph nodes. NK cell activity was found to decrease with an increase in the tumor size. Increased involvement of regional lymph nodes however, was accompanied by an increase in NK cell activity.[32]

Very few studies have been carried out till date regarding the expression of $CD57^+$ NK cells in OL and OSCC. After profound research, it has been found that

ours is the first study which compares the expression of $CD57^+$ NK cells in OL and OSCC.

The expression of $CD57^+$ NK cells is associated with the prognosis of the disease. It has also been stated that more the number of $CD57^+$ NK cells, better is the prognosis.[4]

Ishigami et al.[13] carried out a similar study on gastric carcinoma cases and showed that a greater number of NK cell infiltrate in patients showed better prognosis than those showing less NK cell infiltrate.

Villegas et al.[39] also carried out similar study in lung squamous cell carcinoma concluding that patients with less NK cell infiltrate showed worse prognosis than those showing more NK cell infiltrate.

The association of decreased survival with decrease in CD57 expression could be due to[4]:

1. Cytotoxic T lymphocytic response is MHC Class I restricted as their activation is dependent on MCH Class I expression on target cells. However, there are various studies in the literature which have stated reduction or absence of MHC Class I molecule expression by neoplastic cells. Tumors with downregulated classical MHC Class I expression allows them to escape cytotoxic T^- lymphocytes immunosurveillance. Thus, NK cells play a major role as a cytotoxic cell for those tumor cells that have lost MHC Class I expression.

2. NK cells are also known to regulate haematopoiesis and antibody production by B-cells thereby increasing immunosurveillance.

3. Early-appearing, tumor-infiltrating NK cells play a crucial role in the generation of anti-tumor T lymphocytes. There is a possibility that NK cells have an influence on generation of anti-tumor cytotoxic T- lymphocytes through production of IFN- γ (Interferon gamma). This cytokine environment is important for the development of antigen- specific CD4+ and CD8+ T- cells. Furthermore, it is well documented that IFN- γ upregulates the expression of MHC Class I and MHC Class II molecule. Thus, the upregulation of MHC Class II on macrophages and MHC Class I on tumor cell allow T- cells to recognize tumor- specific antigen.

4. Evidence shows a connection between tumorigenesis, the DNA damage response and the immune response. DNA damaging agents or DNA lesions associated with tumorigenesis activate the DNA damage response in damaged cells. This response, as consequence, brings about upregulation of NKG2D ligand which stimulates the NK cells to attack the diseased cells.

5. NK cells express low affinity for immunoglobulin G receptor CD16 (activatory NKR), thus enabling them to recognize and kill target cells opsonized with antibodies by antibody-dependent cell- mediated cytotoxicity.

Recent advances regarding anti-tumor immune responses and cancer biology have shown a multifaceted dynamic interaction between the immune effectors and the tumor cells. The inability of patient's NK cells to contain CSCs due to the flooding of

NK cells by proliferating CSCs and conversion of NK cells to cytokine secreting cells may probably be one mechanism by which cancer may progress and metastasize. Therefore, there must be two distinct approaches by the NK cells to eliminate tumors, one that targets stem cells and the other that targets differentiated cells. Since CSCs were found to be resistant to certain chemotherapeutic drugs but sensitive to NK cell-mediated killing. Whereas, differentiated oral tumors were more resistant to NK cell-mediated killing but comparatively sensitive to chemotherapeutic drugs. Combination therapy should be taken into account for the elimination of both undifferentiated as well as differentiated tumors. In addition, since a huge majority of patient's NK cells have modified their phenotype to promote differentiation of the cells, they may not be effectual in eliminating the CSCs. Therefore, patients may benefit from repeated allogeneic NK cell transplantation at the site of the tumor for elimination of CSCs. In this view, depletion of immunosuppressive effectors in the tumor microenvironment, which condition the NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs should in theory provide a better strategy for triumphant targeting of tumors by the NK cells.[73]

LIMITATIONS:

1) Poorly differentiated OSCC were not included in the study.

2) A larger sample size would be helpful in confirming the results.

3) Follow-up of the patient can be done to evaluate the correlation between NK cell expression and prognosis.

FURTHER RESEARCH:

1) Research on genetic modification of NK cells by incorporation of γ-cytokine expressing NK cells or combination strategies could be carried out to unleash the full therapeutic potential of NK cells.

2) The incorporation of a safety switch—a suicide gene—in modified NK cells may be feasible. This may control any possible toxicity that may occur during immunotherapy interventions by incorporation of genetically modified NK cells.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

OSCC is a major global threat to public health with almost 3,00,000 new cases diagnosed worldwide on an annual basis. However, the morbidity and mortality rates of this disease have not improved since many decades. It has been reported that about 80% of oral cancers are preceded by potentially malignant oral disorders in India. Leukoplakia is among the most common potentially malignant oral disorders.

Immune system of an individual plays a key role in battling against carcinomas. When immunosuppression has been present for significant period of time, the likelihood of a malignant tumor appearing is enhanced. One of the effective immune mechanism against tumor cells is provided by NK cells. NK cells are considered to be the first line of defence against tumor cells, cells showing dysplastic changes and virally infected cells. CD57 was first identified on NK cells and was

subsequently assigned the CD designation, CD57, at the Fourth International Workshop of Human Leukocyte Antigens in 1989. CD57 cluster expression was chosen for evaluation in this study because it represents the end stage of NK cell maturation and represents the highest toxicity cluster of NK cells. The presence of NK cells is believed to have a good prognostic value for OSCC. Its role in leukoplakia is still under research.

In the present study, 30 samples with normal oral mucosa, 30 samples with OL and 30 samples with OSCC were considered out of which 15 belonged to well-differentiated squamous cell carcinoma and 15 belonged to moderately-differentiated squamous cell carcinoma with the aim of analysing the comparative expression of NK cells (CD57) in OL and in OSCC.

The paraffin blocks of all samples were processed and $CD57^+$ NK cells in the immunohistochemically stained slides were evaluated. A comparative analysis was done between the $CD57^+$ NK cells in OL and OSCC. In addition, the age and sex distribution across the diseases was also carried out.

On evaluating the age distribution across the study groups, we found that mean age in normal oral mucosa group was **55.43 years**, OL had mean age of **47.33 years** and OSCC had mean age of **50.27 years**. On evaluation of gender distribution of the disease, it was found that males were more profoundly affected than females.

On evaluation of $CD57^+$ NK (NK cells with highest maturation stage and cytotoxicity) cells in the study groups, it was found that the **normal oral mucosa stained negative for the presence of $CD57^+$ NK cells**. However, our study focused

on comparison of the expression of $CD57^+$ NK cells in OL and OSCC and reached a conclusion that the **expression of $CD57^+$ NK cells goes on decreasing as the severity of the disease increases**. The **expression of $CD57^+$ NK cells was highest in OL** whereas the **expression decreased in OSCC**. Two reasons may be attributed to this increased expression in OL: first, NK cells are less active in OL due to lack of tumor cells which explains most of them being in their matured state ($CD57^+$) signifying low activity and highest cytotoxicity; second, there is lack of downregulation of NK cells in OL due to the lack of tumor cells. In OSCC as well, the **$CD57^+$ NK cells decreased as the grade progressed from well-differentiated to moderately-differentiated OSCC**. This demonstrates the downregulation of NK cells by tumors and NK cell exhaustion.

Increased expression of $CD57^+$ NK cells may be associated with a better prognosis. This statement correlates with our results as well-differentiated OSCC has a better prognosis than moderately-differentiated OSCC.

Because of the inherent anti-tumor activity of NK cells, their therapeutic potential for the treatment of advanced malignancies is obvious to many in the adoptive cell therapy field. However, current knowledge of NK cell and tumor biology indicates that the use of such effector cell populations has limitations. Genetic modification of NK cells would unleash their complete therapeutic potential. Preclinical studies evaluating gene-modified NK cells reveal improved anti-tumor activity and persistence.

Hence, for further research, these results provide encouraging evidence that such strategies may have an impact clinically, and it is clear that γ-cytokine

expressing NK cells or combination strategies are expected to enter clinical trials in a near future. Moreover, it can be hypothesized that the incorporation of a safety switch, a suicide gene, in modified NK cells may be feasible and may serve to harness any possible toxicity that would appear during such immunotherapy interventions.

Consequently, we arrive again at the first line of the dissertation, a quote by Hippocrates, the father of modern medicine - ***Natural forces within us are the true healers of disease.***

REFERENCES

REFERENCES

1. Desai P. Clinical cancer research in India - present status and future prospects. Indian J Med Res 1983 Jul; 78: 8-28.

2. Brahmbhatt B, Vora H. Study of Immune Effector Cells in Leukoplakia and Oral Cancer. J Cancer Treat Res 2014; 2(2): 28–40.

3. Mehta F, Pindborg J, Gupta P, Daftary D. Epidemiologic and histologic study of oral cancer and leukoplakia among 50,915 villagers in India. Cancer 1969 Oct; 24(4): 832-849.

4. Agarwal R, Chaudhary M, Bohra S, Bajaj S. Evaluation of NK cell (CD57) as a prognostic marker in OSCC: An immunohistochemistry study. J Oral Maxillofac Pathol 2016; 20: 173-177.

5. Kufe D, Pollock R, Weichselbaum R, Bast R, Gansler T, Holland J, et al. Holland-Frei cancer medicine. 6th ed. Hamilton: BC Decker Inc; 2003.

6. Vijayakumar T, Shanavas K, Vasudevan D. Immunological Phenomena in Human Oral Carcinoma in India. Oral Oncol-Eur J Cancer 1993; 298(3): 181-185.

7. Axéll T, Pindborg J, Smith C, Van der Waal I. An International Collaborative Group on Oral White Lesions. Oral white lesions with special reference to precancerous and tobacco- related lesions: conclusions of an international symposium held in Uppsala, Sweden, May 18–21 1994. J Oral pathol med 1996 Feb; 25(2): 49-54.

8. Mehta F, Hamner I. Tobacco-Related Oral Mucosal Lesions and Conditions in India - A Guide for Dental Students, Dentists and Physicians, Bombay. Tata Institute of Fundamental Research. New Delhi: Jaypee Brothers; 1993.

9. Hathaway B, Ferris R, Gooding W, Whiteside T, Kuss I. Imbalance in Absolute Counts of T Lymphocyte Subsets in Patients with Head and Neck Cancer and Its Relation to Disease-1. Head and Neck Cancer 2005; 62: 161-172.

10. Rajendran R, Sivapathasundaram B. Shafer's Text-book of Oral pathology. 8th edition. New Delhi: Reed-Elsevier India Private Limited; 2016.

11. Stutman O. The immunological surveillance theory hypothesis. Basic and Clinical Tumor Immunology 1983; 1: 30-56.

12. Whiteside T, Letessier E, Hirabayashi H, Vitolo D, Bryant J, Barnes L, et al. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin-2 in patients with advanced squamous cell carcinoma of head and neck. Cancer Res 1993 Dec 1; 53(23): 5654–5662.

13. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, et al. Prognostic value of intra-tumoral NK cells in gastric carcinoma. Cancer 2000 Feb 1; 88(3): 577 - 83.

14. Miller J. Biology of NK cells in cancer and infection. Cancer Invest 2002 Jan 1; 20(3): 405–419.

15. Sun J, Beilke J, Lanier L. Adaptive immune features of natural killer cells. Nature 2009 Jan; 457(7229): 557–561.

16. Iida M, Takayama E, Naganawa K, Mitsudo K, Adachi M, Baba JI, et al. Increase of peripheral blood $CD57^{+}$ T-cells in patients with OSCC. Anticancer research 2014 Oct; 34(10): 5729-5734.

17. Nielsen C, White M, Goodier M, Riley E. Functional significance of CD57 expression on human NK cells and relevance to disease. Front Immunol 2013 Dec 9; 4: 1-8.

18. Lopez-vergès S, Milush J, Pandey S, York V, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human $CD56^{dim}$ $CD16^{+}$ NK cell subset. Blood 2010 Jan 1; 116(19): 3865–3874.

19. Marocchio L, Lima J, Sperandio F, Corrêa L, de Sousa S. Oral squamous cell carcinoma: an analysis of 1,564 cases showing advances in early detection. Journal of oral science 2010; 52(2): 267-73.

20. Hanahan D, Weinberg R. The hallmarks of cancer. Cell 2000 Jan 7; 100(1): 57-70.

21. Parkin D, Bray F, Ferlay J, Pisani P. Global cancer statistics 2002. Cancer J Clin 2005 Mar; 55(2): 74-108.

22. Ohman J. Potentially malignant oral disorders and oral cancer - a study on immunosurveillance. Sweden: Jenny Ohman; 2015.

23. Mc Dowell J. An overview of epidemiology and common risk factors for OSCC. Otolaryngol Clin North Am 2006 Apr 1; 39(2): 277-294.

24. Curado M, Hashibe M. Recent changes in the epidemiology of head and neck cancer. Curr Opin Oncol 2009 May 1; 21(3): 194-200.

25. Bryceson Y, Björkström N, Mjösberg J, Ljunggren H. NK Cells - The Autoimmune Diseases. 5th edition. New York: Rose & Mackay; 2014.

26. De Sousa Lopes M, De Silva Barros C, De Souto Medeiros M, De Costa Miguel M, De Souza L, Alves P, et al. Evaluation of CD57^{+} cells in oral squamous cells carcinoma and their relationship with clinicopathological parameters. J Oral Diag 2017; 2(1): 1-7.

27. Lanier L. NK Cells. Encyclopedia of Immunobiology, Volume 1. London: Elsevier Ltd; 2016.

28. LabRoots. (2018). "Supercharging" NK Cells to Kill Cancer. [online] Available at: https://www.labroots.com/ trending/immunology /3188/ supercharging-natural-killer-cells-kill-cancer [Accessed 29 Sep. 2018]

29. Volz A, Radeloff B. Detecting the unusual: NK cells. Progress in nucleic acid research and molecular biology 2006 Jan 1; 81: 473-541.

30. Gokul S, Sapna G. Immunosurveillance and Oral Cancer. Journal of Tumor 2014 May 18; 2(5): 136-141.

31. Balaram P, Vasudevan D. Quantitation of Fc receptor-bearing T-lymphocytes (TG and TM) in oral cancer. Cancer 1983 Nov 15; 52(10): 1837-1840.

32. Jewett A, Tseng H. Tumor induced inactivation of NK cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. Journal of Cancer 2011; 2: 443-449.

33. Karre K, Ljunggren H, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. Nature 1986 Feb; 319(6055): 675–678.

34. Pillai M, Balaram P, Kannan S, Sudha L, Nalinakumari K, Hareendran N, et al. Interferon activation of latent NK cells and alteration in kinetics of target cell lysis: clinical implications for oral precancerous lesions. OOOO 1990 Oct 1; 70(4): 458-61.

35. Pillai R, Balaram P, Reddiar K. Immunological abnormalities in oral precancers. Cancer letters 1991 Apr; 57(1):1-6.

36. Kurosawa S, Harada M, Matsuzaki G, Shinomiya Y, Terao H, Kobayashi N, et al. Early-appearing tumor-infiltrating NK cells play a crucial role in the generation of anti-tumor T lymphocytes. Immunology 1995 Jun; 85(2): 338-345.

37. Lanier L. Turning on NK cells. J exp med 2000 Apr 17; 191(8): 1259-1262.

38. De Paula A, Gomez R. Immunolocalization of p53, glutathione S-tranferase pi and CD57 antigens in oral leukoplakia. Anticancer Res 2001 Jan 1; 21: 379-385.

39. Villegas F, Coca S, Villarrubia V, Jiménez R, Chillón M, Jareño J, et al. Prognostic significance of tumor infiltrating NK cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002 Jan 1; 35(1): 23‑28.

40. French A, Yokoyama W. NK cells and autoimmunity. Arthritis Res Ther 2003 Feb; 6(1): 8-15.

41. Reibel J. Prognosis of oral pre-malignant lesions: significance of clinical, histopathological, and molecular biological characteristics. Crit Rev Oral Biol Med 2003 Jan; 14(1): 47-62.

42. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of NK cells. Immunol Rev 2007 Dec; 220(1): 169–182.

43. Topham N, Hewitt E. Natural killer cell cytotoxicity: how do they pull the trigger? Immunology 2009 Sep; 128(1): 7–15.

44. Gayoso I, Sanchez-Correa B, Campos C, Alonso C, Pera A, Casado J, et al. Immunosenescence of human natural killer cells. J innate immun 2011; 3(4): 337-43.

45. Bryceson Y, Chiang S, Darmanin S, Fauriat C, Schlums H, Theorell J, et al. Molecular mechanisms of natural killer cell activation. J innate immun 2011; 3(3): 216-226.

46. Ferlazzo G, Carrega P. Natural killer cell distribution and trafficking in human tissues. Front immunol 2012 Nov 29; 3: 347-355.

47. Raulet D, Gasser S, Gowen B, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. Annu Rev Immunol 2013 Mar 21; 31: 413–441.

48. Bär E, Whitney P, Moor K, e Sousa C, Leibundgut - Landmann S. IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. Immunity 2014 Jan; 40(1): 117–127.

49. Gras Navarro A, Björklund A, Chekenya M. Therapeutic potential and challenges of natural killer cells in treatment of solid tumors. Front immunol 2015 Apr 29; 6: 202-210.

50. Burga R, Nguyen T, Zulovich J, Madonna S, Ylisastigui L, Fernandes R, et al. Improving efficacy of cancer immunotherapy by genetic modification of natural killer cells. Cytotherapy 2016 Nov 30; 18(11): 1410-1421.

51. Taghavi N, Bagheri S, Akbarzadeh A. Prognostic implication of CD57, CD16, and TGF-b expression in OSCC. J Oral Pathol Med 2016 Jan; 45(1): 58–62.

52. Grossenbacher S, Canter R, Murphy W. Natural killer cell immunotherapy to target stem-like tumor cells. Journal for Immunotherapy of Cancer 2016 Dec; 4(1):19-26.

53. Hofer E, Koehl U. Natural killer cell-based cancer immunotherapies: From immune evasion to promising targeted cellular therapies. Front immunol 2017 Jul 12; 8: 745-750.

54. Dyck L, Lynch L. New Job for NK Cells: Architects of the Tumor Microenvironment. Immunity 2018 Jan 16; 48(1): 9-11.

55. Hu G, Wang S. Prognostic role of tumor-infiltrating CD57-positive lymphocytes in solid tumors: a meta-analysis. Oncotarget 2018 Jan 30; 9(8): 8111-8119.

56. Coelho K. Challenges of the oral cancer burden in India. J Cancer Epidemiol 2012; 1: 1-17.

57. Manzar W, Raghavan M, Aroor A, Keshavmurthy K. Evaluation of serum β2-microglobulin in oral cancer. Aust Dent J 1992 Feb; 37(1): 39-42.

58. Sudbo J. Novel Management of Oral Cancer: a paradigm of predictive oncology. Clin Med Res 2004 Nov 1; 2(4): 233-242.

59. Hyde N, Hopper C. Oral Cancer: the importance of early referral. The practitioner 1999; 243: 56-58.

60. Singh AP, Kumar N, Raju MS, Singh NN, Nagendra Reddy SG. Estimation of serum β2-microglobulin in potentially malignant disorders and squamous cell

carcinoma of the oral cavity: A clinicopathological study. Dent res J 2014 Jan; 11(1): 109-113.

61. Moore S, Pierce A, Wilson D. 'Oral cancer'—the terminology dilemma. Oral Dis 2000 May; 6(3): 191-3.

62. Singh M, Krishanappa R, Bagewadi A, Keluskar V. Efficacy of oral lycopene in the treatment of oral leukoplakia. Oral Oncol 2004 Jul 1; 40(6): 591-6.

63. Booth P. Tobacco-related oral mucosal lesions and conditions in India; a guide for dental students, dentists and physicians by FS Mehta, JE Hamner. Brit J Oral Max Surg 1995; 33(5): 336-345.

64. DeNardo D, Brennan D, Rexhepaj E, Ruffell B, Shiao S, Madden S, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov 2011 Apr 3; 1: 54-67.

65. Nordfors C, Grun N, Tertipis N, Ahrlund-Richter A, Haeggblom L, Sivars L, et al. CD8+ and CD4+ tumor infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. Eur J Cancer 2013 Jul 1; 49(11): 2522-2530.

66. Di Caro G, Bergomas F, Grizzi F, Doni A, Bianchi P, Malesci A, et al. Occurrence of tertiary lymphoid tissue is associated with T-cell infiltration and predicts better prognosis in early-stage colorectal cancers. Clin Cancer Res 2014 Feb 12; 20: 2147-2158.

67. Ohman J, Mowjood R, Larsson L, Kovacs A, Magnusson B, Kjeller G, et al. Presence of CD3-positive T-cells in oral premalignant leukoplakia indicates prevention of cancer transformation. Anticancer res 2015 Jan 1; 35(1): 311-317.

68. Shenoi R, Devrukhkar V, Sharma B, Sapre S, Chikhale A. Demographic and clinical profile of OSCC patients: A retrospective study. Indian J Cancer 2012 Jan 1; 49(1): 21-28.

69. Vázquez-Álvarez R, Fernández-González F, Gándara-Vila P, Reboiras-López D, García-García A, Gándara-Rey J. Correlation between clinical and pathologic diagnosis in oral leukoplakia in 54 patients. Med Oral Patol Oral Cir Bucal 2010 Nov 1; 15(6): 832-838.

70. Sharma P, Saxena S, Aggarwal P. Trends in the epidemiology of OSCC in Western UP: An institutional study. Indian J Dent Res 2010 Jul 1; 21(3): 316-319.

71. Bi J, Tian Z. NK cell exhaustion. Front immunol 2017 Jun 28; 8: 760-766.

72. Li T, Zhang Q, Jiang Y, Yu J, Hu Y, Mou T, et al. Gastric cancer cells inhibit natural killer cell proliferation and induce apoptosis via prostaglandin E2. Oncoimmunology 2016 Feb 1; 5(2): 1-9

73. Vijayakumar T, Shanavas K, Vasudevan D. Immunological Phenomena in Human Oral Carcinoma in India. Eur J Cancer 1993 Jan 1; 29(3): 181-185.

LIST OF ABBREVIATIONS

1.	NK cells	:	NK cells
2.	CD	:	Cluster of Differentiation
3.	KIRs	:	Killer cell Ig like receptors
4.	HNK	:	Human NK
5.	DL	:	Domain – Long
6.	D	:	Domain
7.	HLA	:	Human Leukocyte Antigen
8.	TM	:	Transmembrane
9.	Cyp	:	Cytochrome P
10.	NKAT	:	Nematode Kynurenine Amino Transferase
11.	ILT	:	Immunoglobulin-Like Receptors
12.	LILRs	:	Leukocyte immunoglobulin-like receptors
13.	HL	:	Human Leukocyte
14.	NCRs	:	Natural cytotoxicity trigerring receptors
15.	LIAR	:	Leukocyte associated immunoglobulin-like receptor
16.	FCGR3B	:	Fc fragment of IgG, low affinity 3B receptor

17.	ADCC	:	Antibody-dependent cellular cytotoxicity, (Also Antibody-dependent cell-mediated cytotoxicity)
18.	HPF	:	High powered fields
19.	ILC	:	Innate lymphoid cell
20.	KIR	:	Killer cell Ig like Receptors
21.	NCR	:	Natural cytotoxicity receptors
22.	NS	:	Not Significant
23.	OL	:	Oral leukoplakia
24.	OSCC	:	Oral squamous cell carcinoma
25.	OSCSC	:	Oral squamous carcinoma stem cells
26.	ESCs	:	Embryonic stem cells
27.	MSCs	:	Mesenchymal Stem Cells
28.	DPSCs	:	Dental pulp stem cells
29.	iPSCs	:	Pluripotent stem cells
30.	SqCLC	:	Squamous cell lung carcinoma
31.	TINK	:	Tumor-infiltrating NK cells
32.	PBS	:	Phosphate Buffered Saline

33.	PEC	:	Peritoneal exudate cells
34.	ANOVA	:	Analysis of Variance
35.	CTL	:	Cytotoxic T cell
36.	DAB	:	3,3’ Diaminobenzidine
37.	Ig	:	Immunoglobulin
38.	MCH	:	Mean Cell Hemoglobin
39.	MCMV	:	Murine Cytomegalovirus
40.	MHC	:	Major histocompatibility complex
41.	NKT	:	Natural killer T cell
42.	CSCs	:	Cancer Stem Cells
43.	TNM	:	Tumor, Nodes and Metastasis (Staging system)
44.	ULBP	:	Umbilical Cord Blood Plasma
45.	MIC	:	MHC Class I Polypeptide related sequence
46.	WHO	:	World health organization

GROUP 1: NORMAL ORAL MUCOSA

SR. NO.	AGE	GENDER	DIAGNOSIS	FIELD 1	FIELD 2	FIELD 3	FIELD 4	FIELD 5	LABELLING INDEX
1	33	M	Normal oral mucosa	0	0	0	0	0	0
2	43	M	Normal oral mucosa	0	0	0	0	0	0
3	67	F	Normal oral mucosa	0	0	0	0	0	0
4	54	M	Normal oral mucosa	0	0	0	0	0	0
5	65	F	Normal oral mucosa	0	0	0	0	0	0
6	46	M	Normal oral mucosa	0	0	0	0	0	0
7	65	F	Normal oral mucosa	0	0	0	0	0	0
8	58	M	Normal oral mucosa	0	0	0	0	0	0
9	55	F	Normal oral mucosa	0	0	0	0	0	0
10	56	M	Normal oral mucosa	0	0	0	0	0	0
11	48	F	Normal oral mucosa	0	0	0	0	0	0
12	50	F	Normal oral mucosa	0	0	0	0	0	0
13	47	F	Normal oral mucosa	0	0	0	0	0	0
14	64	F	Normal oral mucosa	0	0	0	0	0	0
15	68	M	Normal oral mucosa	0	0	0	0	0	0
16	59	M	Normal oral mucosa	0	0	0	0	0	0

17	60	M	Normal oral mucosa	0	0	0	0	0	0
18	51	F	Normal oral mucosa	0	0	0	0	0	0
19	57	M	Normal oral mucosa	0	0	0	0	0	0
20	36	M	Normal oral mucosa	0	0	0	0	0	0
21	43	F	Normal oral mucosa	0	0	0	0	0	0
22	66	M	Normal oral mucosa	0	0	0	0	0	0
23	70	M	Normal oral mucosa	0	0	0	0	0	0
24	68	M	Normal oral mucosa	0	0	0	0	0	0
25	65	F	Normal oral mucosa	0	0	0	0	0	0
26	58	M	Normal oral mucosa	0	0	0	0	0	0
27	51	M	Normal oral mucosa	0	0	0	0	0	0
28	49	F	Normal oral mucosa	0	0	0	0	0	0
29	64	M	Normal oral mucosa	0	0	0	0	0	0
30	47	M	Normal oral mucosa	0	0	0	0	0	0

GROUP: II – ORAL LEUKOPLAKIA

SR. NO.	AGE	GENDER	DIAGNOSIS	FIELD 1	FIELD 2	FIELD 3	FIELD 4	FIELD 5	LABELLING INDEX
1	47	M	Oral Leukoplakia	51	50	46	49	60	51.2
2	33	M	Oral Leukoplakia	60	64	68	70	61	64.6
3	30	M	Oral Leukoplakia	52	49	56	55	54	53.2
4	54	M	Oral Leukoplakia	48	56	50	51	55	52
5	21	M	Oral Leukoplakia	55	54	58	51	50	53.6
6	28	M	Oral Leukoplakia	66	65	64	60	58	62.6
7	18	M	Oral Leukoplakia	59	66	60	64	62	62.2
8	22	M	Oral Leukoplakia	58	64	54	50	60	57.2
9	38	M	Oral Leukoplakia	58	64	66	70	64	64.4
10	62	M	Oral Leukoplakia	60	64	59	50	48	56.2
11	62	M	Oral Leukoplakia	61	46	45	52	48	50.4
12	33	M	Oral Leukoplakia	64	59	60	54	68	61
13	28	M	Oral Leukoplakia	58	62	48	71	52	58.2
14	60	M	Oral Leukoplakia	76	62	62	45	49	58.8
15	48	M	Oral Leukoplakia	69	71	68	54	52	62.8
16	45	M	Oral Leukoplakia	65	70	49	54	50	57.6
17	37	M	Oral Leukoplakia	52	48	61	59	42	52.4
18	45	M	Oral Leukoplakia	60	58	56	52	64	58
19	58	M	Oral Leukoplakia	69	72	68	66	55	66

20	52	M	Oral Leukoplakia	68	60	58	54	56	59.2
21	70	M	Oral Leukoplakia	60	58	66	56	52	58.4
22	69	M	Oral Leukoplakia	58	60	59	61	52	58
23	63	M	Oral Leukoplakia	46	72	64	56	50	57.6
24	74	M	Oral Leukoplakia	56	64	52	70	68	62
25	45	M	Oral Leukoplakia	62	54	60	40	48	52.8
26	56	M	Oral Leukoplakia	54	56	59	60	56	57
27	50	M	Oral Leukoplakia	62	58	74	55	51	60
28	70	F	Oral Leukoplakia	64	62	74	61	58	63.8
29	62	M	Oral Leukoplakia	60	58	54	56	50	55.6
30	40	M	Oral Leukoplakia	66	65	47	78	60	63.2

GROUP: III – ORAL SQUAMOUS CELL CARCINOMA

SR. NO.	SLIDE NO.	AGE	GENDER	DIAGNOSIS	FIELD 1	FIELD 2	FIELD 3	FIELD 4	FIELD 5	LABELLING INDEX
1	4343/18	50	M	SCC-W	28	29	30	34	31	30.4
2	4279/17	42	M	SCC-M	26	24	20	18	16	20.8
3	4265/17	30	F	SCC-W	30	36	29	27	24	29.2
4	4033/17	50	F	SCC-M	21	25	28	18	16	21.6
5	4008/17	48	M	SCC-M	16	18	14	20	24	18.4
6	3045/17	32	M	SCC-W	28	34	32	30	29	30.6
7	2713/16	40	M	SCC-W	24	26	32	38	34	30.8
8	2407/16	60	M	SCC-W	30	35	28	26	31	30
9	2229/15	55	M	SCC-M	13	38	26	31	26	26.8
10	2128/15	35	F	SCC-M	20	10	14	12	19	15
11	2103/15	50	M	SCC-M	21	16	28	24	31	24
12	2031/15	35	M	SCC-M	28	18	20	24	16	21.2
13	2010/15	50	M	SCC-W	34	30	29	36	31	32
14	2008/15	65	M	SCC-W	32	31	28	26	30	29.4
15	1985/15	44	M	SCC-M	8	10	16	11	14	11.8
16	1954/15	58	F	SCC-M	18	24	14	28	10	18.8
17	1923/15	73	M	SCC-M	24	18	16	22	20	20
18	1911/15	54	M	SCC-W	28	30	29	31	28	29.2
19	1899/15	75	M	SCC-W	30	16	24	36	28	26.8

20	1896/15	58	M	SCC-W	38	30	29	35	27	31.8
21	1848/15	40	M	SCC-W	30	34	32	36	29	32.2
22	1833/15	63	M	SCC-W	34	36	38	29	25	32.4
23	1808/15	60	F	SCC-M	10	21	18	16	24	17.8
24	1807/15	62	M	SCC-W	38	40	31	29	36	34.8
25	1804/15	50	M	SCC-M	24	22	18	19	20	20.6
26	1800/15	36	M	SCC-W	34	32	38	29	30	32.6
27	1742/15	44	M	SCC-M	29	24	20	10	22	21
28	1738/15	54	M	SCC-W	29	34	26	24	36	29.8
29	1321/14	69	M	SCC-M	24	24	26	22	19	23
30	1207/14	26	M	SCC-M	20	15	18	10	26	17.8

SCC-M – Moderately-differentiated squamous cell carcinoma

SCC-W – Well-differentiated squamous cell carcinoma