DISCUSSION-Part I

Immune suppression is an early event in tumorigenesis that continues during progression to metastatic disease. The number, phenotype and functional status of Leukocytes can be altered by interaction with tumor cells at tumor site and also in circulation via immunosuppressive factors released by tumor cells. Further, high Neutrophil and Monocyte count and low Lymphocyte count are most frequent alteration detected in peripheral blood of patients with solid tumors. Moreover, presence of defective Lymphocytes, specifically T cells, in blood and tumor site is one of the causes for immune dysfunction. Human T cells are phenotypically and functionally heterogeneous. The identification and enumeration of T cell subsets in peripheral circulation and in tissue of patients with Leukoplakia and Oral Squamous Cell Carcinoma (OSCC) is primary requirement for understanding their role in the host immune responses. Further, the identification and characterization of these cells may be useful for immunomonitoring and clinical trial based on T cell immunotherapy. Many studies including few Indian studies till date have evaluated T cells in patients with Head and Neck Cancer (Kuss et al, 2004; Hathaway et al, 2005; Schaefer et al, 2005; Manchanda et al, 2006; Lau et al, 2007; Strauss et al, 2007; Bose et al, 2008; Millrud et al, 2012, Gaur et al, 2012). However, very few studies have examined T cell subsets in both premalignant and malignant oral lesions (Pilai et al, 1987; Gannot et al, 2002; Zancope et al, 2010; Lee et al, 2010; Ohman et al, 2012). In present study, we analysed T cell subsets in both premalignant and malignant lesions of oral cavity.

Further, Tobacco and alcohol are major risk factors for Head and Neck cancer. Persons with habit of tobacco consumption in form of betel quid chewing or smoking
and alcohol consumption have a higher risk for development of premalignant as well as malignant lesion of oral cavity. In studies exploring immune dysfunction in Head and Neck cancer, more than 90% of patients have habit of chewing betel quid (BQ), smoking cigarettes and alcohol consumption (Kuss et al, 2004; Manchanda et al, 2006; Zancope et al, 2010; Lee et al, 2010). Moreover, some study groups also analysed alteration of systemic immune response in relation with different habit in Oral Cancer patients (Manchanda et al, 2006; Lee et al, 2010). In our study, 91% patients with OSCC had habit of tobacco, among them 66% were chewers, 13% were smokers and 12% had both tobacco chewing and smoking habit, while 2% patients were alcohol users. Further, among Hyperplasia patients, 53% were tobacco chewers and 47% were smokers, while among Dysplasia patients, 73% were chewers and 27% were smokers.

In the first part of this study, we evaluated the mean percentage of Leukocyte and T cell subsets in the peripheral circulation of healthy controls, Leukoplakia and OSCC patients. Further, mean percentage of Leukocyte and T cell subsets were compared between these groups and then correlated with clinicopathological parameters and disease status.

With respect to Leukocyte subsets, in comparison with healthy controls, significantly decreased Lymphocytes with increased Neutrophils to Lymphocytes ratio was observed in Leukoplakia patients with similar mean percentage of Neutrophils and Monocytes.

Further, in comparison with healthy controls, significant decrease in mean percentages of Lymphocytes, increase in mean percentage of Neutrophils and Monocytes, and enhanced Neutrophils to Lymphocytes ratio was observed in OSCC patients.
In comparison with Leukoplakia, significantly decreased mean percentage of Lymphocytes was noted in OSCC patients with similar mean percentage of Neutrophils, Monocytes and Neutrophils to Lymphocytes ratio.

The increase in Neutrophils and Monocytes and decrease in Lymphocytes reflect induction of inflammatory response in OSCC patients. Accordingly, high Neutrophils to Lymphocytes ratio in OSCC and Leukoplakia patients indicate ongoing systemic inflammation in these patients as explained by Zahorec et al (2001).

Further, depletion of Lymphocytes in Leukoplakia and OSCC patients in present study indicates deprived cellular immune response created by tumor during carcinogenesis. This observation is in accordance to the experimental animal model findings, which demonstrated that Lymphocytes deficient mice had increased mean percentage of tumor development (Shankaran et al, 2001). Further, studies on primary immunodeficient patients demonstrated lymphopenia showed increased risk for developing Head and Neck Squamous Cell Carcinoma in these patients (Penn et al, 1981; Kaplan et al, 1985).

Further, with respect to T cell subsets, Leukoplakia patients showed increased mean percentage of Effector cells (CD8+CD45RA−) and Memory T cells (CD4+CD45RO+) and decreased mean percentage of Naive T cells (CD4+CD45RA+), CD161+CD56+ and CD161−CD56+ NK T subpopulations as compared to healthy controls. However, mean percentage of total T cells, αβ and γδ T cells, Cytotoxic T cells, Naive T cells (CD8+CD45RA+), Helper T cells, Regulatory T cells, CD161+CD56− NK T subpopulation and Helper to Cytotoxic T cells were found similar in these groups.
Further, in present study, OSCC patients had significantly increased mean percentage of Helper T cells with increased Helper to Cytotoxic T cell ratio, increased mean percentage of Regulatory T cells and decreased mean percentage of CD161+CD56+ NK T subpopulation as compared to healthy controls. A trend of decreased mean percentage of CD161+CD56+ NK T subpopulation was also observed in OSCC patients as compared to healthy controls. However, mean percentage of total T cells, αβ and γδ T cells, Cytotoxic T cells, Naive (CD8+CD45RA+) and Effector T cells (CD8+CD45RA−), Naive (CD4+CD45RA+) and Memory T cells (CD4+CD45RO+), CD161+CD56− NK T subpopulation were found similar in these groups.

Moreover, OSCC patients showed significantly decreased mean percentage of Effector T cells (CD8+CD45RA−) and increased mean percentage of Naive T cells (CD4+CD45RA+) and Regulatory T cells as compared to Leukoplakia patients. However, mean percentage of total T cells, αβ and γδ T cells, Cytotoxic T cells, Naive T cells (CD8+CD45RA+), Helper T cells, Regulatory T cells, CD161+CD56− NK T subpopulation and Helper to Cytotoxic T cells were found similar in these groups.

Similar to our findings, Charazinska et al (2008) demonstrated decreased percentage of Naive T cells and increased percentage of Memory T cells in patients with Oral lichen planus. Moreover, study by Johannisson et al (1995) showed that transition of Naive (CD45RA+CD45RO−) to Memory (CD45RA−CD45RO+) phenotype is accompanied by proliferation and activation of T cells, thus decreased mean percentage of Naive T cells (CD4+CD45RA+), increased mean percentage of Effector (CD8+CD45RA−) and Memory T cells (CD4+CD45RO+) in Leukoplakia patients as compared to healthy controls.
present study indicate rapid turnover of Naive T cells and activation of systemic immune response in these patients.

Further, the relationship of NK T cells with Leukoplakia condition is not clear as no study has evaluated its role in Leukoplakia patients till date, yet, the study from Molling et al (2007) demonstrated low level of circulating NK T cells in Head and Neck Cancer patients than healthy controls, which were not restored even after treatment, suggesting that low number of circulating NK T cells may precede the development of cancer and represent a risk factor for the development of malignancies. In view of this observation, low mean percentage of NK T cells in blood of Leukoplakia patients in our study might reflect the defective innate immune response. However, some reports have also demonstrated association of reduced CD161⁺CD3⁺ cells with inflammatory diseases (Guebre-Xabier et al, 2000; Li et al, 2005). Therefore, the reduction of NK T subpopulations in Leukoplakia patients in present study can also be linked with inflammatory condition of patients. Moreover, in accordance with our study, Lee et al (2010) did not find significant difference in Helper and Cytotoxic T cells between Leukoplakia and healthy controls. Also, no study has evaluated T cell receptor expression and Regulatory T cells in Leukoplakia patients.

In present study, the mean percentage of Helper T cells included combined value of Th1 and Th2 type of Helper T cells. The Th1 type of cells are characterized by the production of interferon (IFN)-γ, interleukin (IL) 2, IL12 and IL18, whereas Th2 type of cells are characterized by the production of IL4, IL6, IL10 and IL13. Also, the increased percentage of Regulatory T cells in peripheral circulation is known to be linked with the pro inflammatory cytokines such as IL10 (Strauss et al, 2007; Alhamarneh et al, 2010).
In our study, the increased mean percentage of Helper T cells along with enhanced Helper to Cytotoxic ratio and increased Regulatory T cells in OSCC as compared to healthy controls may indicate the activation of Th2 phenotype of Helper T cells in OSCC patients. However, this result needs to be confirmed by expression of activation markers along with pro inflammatory cytokines on Helper T cells. Furthermore, increased mean percentage of Regulatory T cells in peripheral circulation in OSCC patients in present study is in accordance with other published reports of Head and Neck cancer (Strauss et al, 2007; Bose et al, 2008; Boucek et al, 2010; Gasparoto et al, 2010; Alhamarneh et al, 2010), which may be linked with the down regulation of immune response leading to immunosuppression in OSCC patients. Moreover, the increase in Regulatory T cells not only down regulates other immune cells but also interferes with the antigen presentation of dendritic cells (Zou et al, 2006).

Further, low mean percentages of NK T subpopulations were observed in OSCC patients as compared to healthy controls in our study. In accordance with our findings low number of NK T cells in circulation of Head and Neck cancer patients was demonstrated by some study groups (Molling et al, 2007; Bose et al, 2008; Singh et al, 2013). This alteration may result from NK T cell death, impaired NK T cell proliferation, or an accumulation of NK T cells in the tumor tissue (Molling et al, 2005 and 2007). Moreover, Tahir et al (2001) demonstrated that NKT cells in cancer patients are functionally impaired and produce less IFNγ.

Further, in present study, similar mean percentage of Cytotoxic and total T cells was found in OSCC patients as compared to healthy controls. In accordance with our findings, few study groups have observed similar percentage of Cytotoxic T cells (Kim et
al, 2004; Chikamatsu et al, 2007; Boucek et al, 2010) and total T cells (Kuss et al, 2005; Boucek et al, 2010) in Head and Neck Cancer patients as compared to healthy controls. However, in contrast to our findings, some study groups (Hoffman et al, 2002; Manchanda et al, 2006; Noguchi et al, 2014) showed significantly decreased percentage of total T cells, αβ and γδ T cells in cancer patients indicate down regulation of immune response in these patients. However, Bas et al (2006) showed higher percentage of γδ T cells in Head and Neck Cancer patients as compared to healthy controls. Study by Roden et al (2008) showed increased number of circulating γδ T cells were associated with its activation, hence, high percentage of γδ T cells in study of Bas et al indicate activation of γδ T cells in their study, which was not observed in our study.

Also, some study groups have observed shift from Naive to Memory phenotype of T cells in Head and Neck Cancer patients as compared to healthy controls (Boucek et al, 2010; Turksma et al, 2013; Noguchi et al, 2014). This finding was not observed in the present study which might suggest defective T cell activation in OSCC patients. Moreover, OSCC patients showed significantly decreased mean percentage of Effector cells (CD8⁺CD45RA⁻), increased mean percentage of Naive T cells (CD4⁺CD45RA⁺) and Regulatory T cells as compared to Leukoplakia patients, which also suggest lack of proliferation and T cell activation during carcinogenesis, which in turn results in suppression of systemic immune response.

Further, mean percentage of total T cells, αβ and γδ T cells, Cytotoxic T cells, Helper T cells and NK T subpopulations was comparable between Leukoplakia and OSCC patients. Similar to our findings, Lee et al (2010) also observed no change in Cytotoxic
and Helper T cells between Leukoplakia and OSCC patients. However, they have not analysed the other T cell subsets.

In relation to clinicopathological parameters, regarding Leukocyte subsets, high mean percentage of Neutrophils was observed in patients with buccal mucosa patients as compared to other anatomical sites. Also, high mean percentage of monocytes was found in patients whose mucosal margins were involved by tumor as compared to patients without margin involvement suggesting increased systemic inflammation in patients with buccal mucosa cancer and margin involvement. Further, significant correlation was not found for Neutrophils and Monocytes with other clinicopathological parameters such as age, gender, habit, TMN staging, histological grade, lymphatic permeation, vascular permeation and neural invasion. Moreover, Lymphocytes did not show significant correlation with any of the clinicopathological parameters.

Regarding T cell subsets, significantly reduced mean percentage of total T cells was found in males than females, which is in agreement with study of Saxena et al (2004), who analysed circulating T cell subsets in Indian population and observed low mean percentage of CD3^+ T cells in Indian males than females. Further, significantly low mean percentage of total T cells was found in patients without vascular permeation as compared to patients with vascular permeation. Moreover, a trend of low total T cells was found in patients with habit of tobacco and alcohol as compared to patients without habit. In accordance with these findings, Manchanda et al (2006) also observed tobacco related depletion of CD3^+ cells in oral squamous cell carcinoma. However, in present study, total T cells did not show significant correlation with age, anatomic site, TNM staging, histological grade, lymphatic and vascular permeation, neural invasion and
mucosal margin involvement. Similar to our findings, some study groups have not observed significant correlation of circulating T cells with TNM stage (Kuss et al, 2005; Lee et al 2010). Further, Yan et al (2010) examined effect of age on different circulating T cell subsets and observed similar mean percentage of total T cells with increasing age, which is also in accordance with our findings. Regarding αβ T cells, a trend of low mean percentage of αβ T cells was seen in males than females, which may be associated with low total T cells in male patients as examined αβ T cell subsets were examined on CD3^+ cells. Low mean percentage of αβ T cells was observed in patients with T3 tumor size as compared to other tumor size. Further, αβ T cells did not show significant correlation with age, site, habit, nodal status, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement in present study. Further, in contrast to our findings, Yan et al (2010) showed decrease in αβ T cells with age. Moreover, γδ T cells did not show significant correlation with any clinicopathological parameters in the present study.

Regarding Cytotoxic T cells, a significantly low mean percentage of Cytotoxic T cells was seen in histological grade II and III tumors as compared to grade I tumor, which could be due to apoptosis of circulating Cytotoxic T cells in poorly differentiated tumors as these cells are more sensitive to apoptosis as demonstrated by Hoffman et al (2002). In contrast to our findings, Boucek et al (2010) observed increased mean percentage of circulating Cytotoxic T cells with increasing histological grade. However, Cytotoxic T cells did not show significant correlation with age, gender, habit, anatomic site, TNM staging, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement. In contrast to our findings Yan et al (2010) observed decline in
Cytotoxic T cells with age. Further, Uppal et al (2003) observed high percentage of Cytotoxic T cells in Indian males than females, which was not observed in this study. Further, regarding Cytotoxic T cell subsets, a significantly decreased mean percentage of Naive (CD8⁺CD45RA⁺) T cells and increased mean percentage of Effector T cells (CD8⁺CD45RA⁻) was observed in patients with lip lesion as compared to other anatomic sites suggesting Naive to Memory shift in patients with lip lesion. Patients with Squamous Cell Carcinoma of lip usually have different biological behavior from the oral cavity because of the associated etiology and high infiltrate of inflammatory cells (Massano et al, 2006; Batista et al, 2009). Moreover, Zancope et al (2010) demonstrated increased tumor infiltrating CD8⁺ cells in lip squamous cell carcinoma than oral cavity. In view of these findings, shift of Naive to Effector cells in Squamous Cell Carcinoma of lip in our study suggests the effective immune response in lip as compared to other anatomical sites. Further, Cytotoxic T cell subsets did not show significant correlation with age, gender, habit, TNM staging, histological grade, lymphatic permeation, vascular permeation, neural invasion, mucosal margin involvement.

Regarding Helper T cells, significantly decreased mean percentage of Helper T cells and trend of low total T cells was found in patients with habit of tobacco and alcohol as compared to patients without habit. These findings are in accordance with the findings of Manchanda et al (2006), who observed tobacco related depletion of CD4⁺ cells in Oral Squamous Cell Carcinoma. Moreover, Mortaz et al (2009) showed that cigarette smoke suppress the proliferation of Helper T cells via unknown mechanism. Further, patients with T₂, T₃ and T₄ tumor size had significantly low mean percentage of Helper T cells.
cells as compared to T\textsubscript{1} tumor size in present study. This observation is different from the findings of Mirllud et al (2012), who observed increased Helper T cells in patients with advanced tumor status of Head and Neck Cancer. However, they carried of study in 20 patients only and none of the patient in their study had <2 cm tumor size. Moreover, in our study, a trend of increased mean percentage of Helper cells was found in N\textsubscript{1} and N\textsubscript{2} patients than N\textsubscript{0} patients, whereas, Helper T cells did not show significant correlation with age, gender, anatomical site, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement. Similar to our study, the percentage of Helper T cells did not change with age in study of Yan et al (2010). However, Uppal et al (2003) observed low percentage of Helper T cells in Indian males than females, which was not observed in this study. This discrepancy might be because of less number of female patients in our study.

Regarding Helper T cell subsets, significantly decreased mean percentage of Naive (CD4\textsuperscript{+}CD45RA\textsuperscript{+}) cells was observed in patients with > 45 years of age as compared to patients with ≤45 years of age. Similar to our observation, Yan et al (2010) observed the significant decline in Naive T (CD3\textsuperscript{+}CD45RA\textsuperscript{+}). Further, a trend of reduced Naive (CD4\textsuperscript{+}CD45RA\textsuperscript{+}) and increased Memory T cells (CD4\textsuperscript{+}CD45RO\textsuperscript{+}) was found in N\textsubscript{1} and N\textsubscript{2} patients than N\textsubscript{0} patients indicating shift from Naive to Memory phenotype in node positive patients. This suggests an attempt of Helper T cells to resist tumor load in node positive patients. Similar to this observation Milrrud et al (2012) observed high mean percentage of activated T cells in N\textsubscript{1} and N\textsubscript{2} nodal status. Moreover, decreased mean percentage of Naive T cells (CD4\textsuperscript{+}CD45RA\textsuperscript{+}) was observed in patients with margin involved by tumor as compared to patient without margin involvement. Further, Helper T
cell subsets did not show significant correlation with gender, habit, anatomical site, tumor size, disease stage, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement in present study.

Regarding Regulatory T cells, a trend of high mean percentage of Regulatory cells was observed in patients with lip lesion as compared to other anatomic sites. However, Regulatory T cells did not show significant correlation with age, gender, habit, TNM staging, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement in present study. Similar to our observation, Boucek et al (2010) did not observe significant correlation with clinicopathological parameters. Moreover, Yan et al (2010) also did not observed significant difference in Regulatory T cells mean percentage with age.

Regarding NK T cell subpopulations, male patients showed low mean percentage of CD161\(^+\)CD56\(^-\) NK T subpopulation than female patients, which may be associated with low total T cells in male patients as CD161\(^+\)CD56\(^-\) NK T subpopulation was examined on CD3\(^+\) cells. Further, a trend of increased mean percentage of CD161\(^-\)CD56\(^+\) NK T cell subpopulation was found in patients without habit than patients with habit. Also, a trend of increased mean percentage of CD161\(^+\)CD56\(^+\) NK T cell subpopulation was observed in smokers than tobacco chewers, chewers and smokers and alcohol users. However, NK T cells did not show significant correlation with age, anatomical site, TNM staging, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement in present study.

To assess the prognostic significance in univariate survival analysis, Leukocytes and T cell subsets were correlated with clinical outcome of patients. With respect to Leukocyte
subset, a trend of high incidence of disease relapse with reduced disease free survival (DFS) was seen in patients with high mean percentage of Neutrophils and elevated Neutrophils to Lymphocytes ratio. Neutrophilia is considered as independent prognostic factor, associated with reduced survival in various human cancers (Schmidt et al, 2005; Fogar et al, 2006; Donskov et al 2006). Moreover, increased pre treatment Neutrophils to Lymphocyte ratio is found to be associated with reduced survival in colorectal and ovarian cancer (Halazun et al, 2008; Cho et al, 2009).

With respect to T cell subsets, a trend of high incidence of death with reduced overall survival (OS) was observed in patients with low mean percentage of αβ T cells as compared to its counterpart. This observation indicates down regulation of immune status in OSCC patients and furthermore, immune status in such patients may be restored with adaptive T cell immunotherapy (Noguchi et al, 2014). Moreover, significantly high incidence of disease relapse with reduced DFS was observed in patients with low mean percentage of γδ T cells than high mean percentage. In contrast to our findings, Bas et al (2006) observed increased mean percentage of γδ T cells in recurrent Head and Neck Squamous cell carcinoma. However, activated γδ T cells are known to release Interferon γ and Tumor Necrotic Factor (TNF) α and kill cancer cell like Natural killer cells (Gao et al 2003; Urban et al, 2010). Further, these cells are found to be involved in combating infectious diseases. Study by Roden et al (2008) has revealed that increase in circulating γδ T has been associated with activation of immune system either by infection (50% case) or autoimmune disease (25% case). In view of this observation high incidence of relapse in low γδ T subgroup in present study may indicate γδ T cells as incompetent and lead to immune suppression. Moreover,
activated autologous γδ T cells can also be used as immunotherapy for recurrent non-small cell lung cancer patients (Nakajima et al, 2010). This suggests that decreased mean percentage of γδ T cells may be used to identify patients for autologous γδ T cells immunotherapy.

Further, CD161^+CD56^- NK T subpopulation showed significant association with disease status; reduction of this subpopulation was correlated with poor clinical outcome of patients, which is in accordance with the report of Molling et al (2005) suggesting low level of NK T cells predicts poor outcome of Head and Neck Cancer patients. Moreover, pre-treatment measurement of NK T cells may be useful for selecting patients for glycolipid α-galactosylceramide (α-GalCer)/DC therapy which enhances immune system by activating NK T cells (Yamasaki et al, 2011).

Further, other T cell subsets such as total T cells, Cytotoxic T cells, Helper T cells, Naive T cells, Effector T cells and Regulatory T cells did not show significant correlation with disease status in univariate survival analysis.

In multivariate survival analysis of peripheral blood Leukocytes and T cell subsets along with clinicopathological parameters, presence of vascular permeation entered at step 1, followed by decreased γδ T cells at step 2 and mucosal margin involvement at step 3 as independent prognostic factors for predicting reduced DFS and increased circulating Regulatory T cells was found as independent prognostic factor for predicting reduced OS.

The prognostic significance of Regulatory T cells in Head and Neck cancer is controversial. According to Loose et al (2008) a higher density of CD4^+CD25^+ Regulatory T cells is linked to good prognosis in Head and Neck Cancer, while some
studies (Strauss et al, 2007; Boucek et al, 2010) showed that the increase is linked to worse prognosis in HNSCC patients. Similar to our findings, Alhamarneh et al (2010) have not observed significant correlation of circulating Regulatory T cells in univariate survival analysis, however, multivariate survival analysis have not been carried out by this study group. Moreover, presence of increased Regulatory T cells has been associated with high death hazard and reduced survival in ovarian carcinoma (Curiel et al, 2004) which supports our finding that increased Regulatory T cells were associated with poor OS in multivariate survival analysis. Moreover, prevalence of Regulatory T cells helps to identify high risk patients, who are benefited with selective elimination of Regulatory T cells by targeting therapy like denileukin diftitox (Danull et al, 2005).

In conclusion, marked systemic inflammation was observed in Leukoplakia and OSCC patients. Moreover, significantly deprived innate and humoral immune response was observed in OSCC patients, which was mainly mediated through Regulatory T cells by down regulation of other immune cells including NK T cells. Further, factors like age and habit of tobacco and alcohol affects the circulating Naive and Helper T cells. Moreover, increasing tumor size down regulates the function of Helper cells. Altered Neutrophils to Lymphocytes ratio along with low γδ T cells, NK T cell in circulation predicts poor disease free survival and increased Regulatory T cells predicts poor overall survival of OSCC patients. Such patients might be benefited with treatment of immune enhancers and adoptive T cells and dendritic cells therapy. Leukoplakia patients showed effective systemic immune response by activated T cells. However, low NK T cells in circulation is linked with inflammatory condition of Leukoplakia. Further, altered systemic immune response including noticeable lymphopenia, defective T cells proliferation and activation
was seen during malignant transformation. Thus, investigation of circulating Leukocyte and T cell subsets seems to be useful predictors of survival and have been proposed as independent prognostic factors. Also, these parameters could be useful as stratification factors to identify patients with a poor prognosis prior to surgery and in clinical trials for immunotherapy.
DISCUSSION-Part II

In the second part of this study, we evaluated the number of tumor infiltrating T cell subsets including Cytotoxic T cells, Helper T cells and Regulatory T cells in Oral Squamous Cell Carcinoma (OSCC) patients. Further, tumor infiltrating T cell subsets of OSCC patients were correlated with clinicopathological parameters and disease status as well as peripheral T cell subsets. Moreover, Cytotoxic and Regulatory T cells have been evaluated in 23 Hyperplasia and 20 Dysplasia patients and correlated with OSCC patients to understand change in immune response during the progression of oral squamous cell carcinoma. Lymphocytes migrate from peripheral blood to tumor site through angiogenesis and therefore, Microvessel Density (MVD) was evaluated and compared between Hyperplasia, Dysplasia and OSCC patients. Also, MVD was correlated with clinicopathological parameters, circulating and tumor infiltrating Cytotoxic T cells, Helper T cells and Regulatory T cells.

Tumor infiltrating T cells was found to be distributed within different tumor compartment i.e. tumor nest, tumor stroma and tumor margin. Moreover, the density of tumor infiltrating T cells was found to be heterogeneous within tumor area; in some patients strong T cells infiltration was uniformly observed in all three tumor compartments, while in other patients major T cells infiltration was observed in tumor stroma and in tumor margin. The maximum number of T cells was observed in tumor stroma followed by tumor margin and tumor nest. Further, in tumor nest, tumor stroma and tumor margin the number of Cytotoxic T cells was found to be higher as compared to Helper and
Regulatory T cells. Moreover, the ratio of Regulatory to Cytotoxic and Helper T cells was found to be elevated in tumor nest as compared to tumor stroma and tumor margin.

The solid tumors are found to be infiltrated by cells of innate and adaptive immunity. Among immune cells infiltration, lymphocytes are found to be located in specific areas of tumor, as observed in study of Dieu-Nosjean et al (2008). They analysed lymphocytes distribution and observed that T cells are mainly distributed in tumor core that is in contact with tumor, in invasive margin and in adjacent lymphoid structure. Further, NK cells are distributed in stroma and B cells are distributed in invasive margin and in adjacent lymphoid structure which is similar to secondary lymphoid follicle structure that contains Naive and Memory T cells.

Further, the maximum infiltration of T cells in tumor stroma in present study might indicate stromal immune response by macrophage and dendritic cells which are abundantly present in tumor stroma (Fridman et al, 2013) and act as antigen presenting cells which stimulates T cells accumulation in tumor stroma. Moreover, tumor cells produce many chemokines which may be varying in different tumor compartment and therefore, variable densities of tumor infiltrating T cells were observed within different tumor compartment (Fridman et al, 2013).

Further, the distribution of T cells also varies between tumor types; some study groups show that subsets of T cells are present in the core, in stroma and in the invasive margin of the tumor in Colorectal Cancer (Deschoolmeester et al, 2010; Dahhlin et al, 2011) and in Head and Neck Cancers (Balermpas et al, 2014), while T cells are found only in the invasive margin in liver metastases of Colon Cancer (Halama et al, 2011).
Moreover, the variable density and location of T cells between tumors in different individuals with same type of Head and Neck Cancer have been observed by some study groups (Katou et al, 2007; Balermpas et al, 2014).

Further, the variation in T cell distribution and increased Regulatory to Cytotoxic and Helper T Cell ratio in present study indicated suppression of tumor infiltrating lymphocytes in tumor nest, which might affect clinical outcome.

Further, Cytotoxic T cells in tumor nest when correlated with clinicopathological parameters, a trend of low number of Cytotoxic T cells in tumor nest was seen in patients with vascular permeation as compared to patients without vascular permeation. However, Cytotoxic T cells in tumor nest did not show significant correlation with age, gender, anatomical site, TNM stage, histological grade, lymphatic permeation, neural invasion and mucosal margin involvement.

Regarding Cytotoxic T cells in tumor stroma, low number of Cytotoxic T cells in tumor stroma was observed in patients with ≤ 45 years of age and in males as compared to their respective counter parts. Further, with respect to disease stage, significantly low number of Cytotoxic T cells in tumor stroma was found in stage III disease as compared to stage I, stage II and stage IV disease. Similar to our observation, some study groups (Reichert et al, 2002; Distel et al, 2009; Cho et al, 2011) observed low number of tumor infiltrating CD8⁺ cells in advanced stage of Head and Neck Carcinoma. The depletion in Cytotoxic T cells in advanced stage patients indicated apoptosis of tumor infiltrating CD8⁺ cells by PD1/PDL1 pathway which has been reported in carcinoma of lung, tongue, and renal cells (Konishi et al, 2004; Katou et al, 2007; Thompson et al, 2007).
Also, tumor infiltrating Cytotoxic T cells are found to be disabled to accomplish perforin mediated cytolytic activity due to less production of Granzyme B (Deschoolmeester et al, 2010). Also, a trend of low number of Cytotoxic T cells in tumor stroma was seen in patients whose mucosal margin were involved by tumor as compared to patients without margin involvement. However, Cytotoxic T cells in tumor stroma did not show significant correlation with anatomical site, habit, histological grade, lymphatic permeation, vascular permeation, neural invasion.

Similarly, low number of Cytotoxic T cells in tumor margin was observed in patients with ≤ 45 years of age and in males as compared to their respective counter parts. Further, low number of Cytotoxic T cells in tumor margin was seen in patients with habit of tobacco and alcohol as compared to patients without habit. Surprisingly, a trend of low number of Cytotoxic T cells in tumor margin was observed in patients without lymphatic permeation as compared to patients with lymphatic permeation. However, Cytotoxic T cells in tumor margin did not show significant correlation with anatomical site, TNM stage, histological grade, vascular permeation, neural invasion and mucosal margin involvement. In contrast to our observation, Cho et al (2011) found significant correlation of peritumoral CD8⁺ cells with tumor size, lymph node metastasis in OSCC patients. Further, similar to our observation, another study by Cho et al (2003) did not observe significant correlation of Cytotoxic T cells with grade, tumor size, nodal status and margin in Esophagus Carcinoma.

Further, regarding Helper T cells in tumor nest, a trend of low number of Helper T cells in tumor nest was seen in patients with vascular permeation as compared to patients
without vascular permeation. CD4$^+$ T cells are essential for induction of antigen specific response of CD8$^+$ cells. Furthermore, cooperative role of CD4 and CD8 T cells is well established (Marzo et al, 1999; Huang et al, 2002; Cho et al, 2003). Reduction of Helper T cells and Cytotoxic T cells in tumor nest of patients with vascular permeation in present study suggest impaired function of these cells during progression of disease. However, Helper T cells in tumor nest did not show significant correlation with age, gender, anatomical site, TNM stage, histological grade, lymphatic permeation, neural invasion and mucosal margin involvement.

Regarding Helper T cells in tumor stroma and tumor margin, low number of Helper T cells in tumor stroma as well as in tumor margin was seen in patients with neural invasion as compared to patients without neural invasion which also suggested defective Helper T cells function during disease progression. However, Helper T cells in tumor stroma and in tumor margin did not show significant correlation with age, gender, TNM staging, histological grade, lymphatic permeation and mucosal margin involvement. Similar to our findings, two study groups (Cho et al, 2003; Badoual et al, 2006) did not observe significant correlation of tumor infiltrating CD4$^+$ cells with clinicopathological variables such as tumor site, tumor size, nodal status, histological grade and surgical margin.

Regarding Regulatory T cells in tumor nest, a trend of high number of Regulatory T cells in tumor nest was observed in histological grade III tumors as compared to grade I and II tumors indicating immunosuppressive role of Regulatory T cells in disease aggressiveness. Similar to our observation, Al-Qahtani et al (2011) observed that
moderately and poorly differentiated OSSC had high expression of FOXP3$^+$ cells than well differentiated tumors. Also, Ghebeh et al (2008) observed high number of FOXP3$^+$ cells in poorly differentiated breast tumors. However, in our study low number of Regulatory T cells in tumor stoma as well as in tumor margin was seen in histological grade III tumors than grade I and II tumors. This observation indicates prominent immunosuppression in tumor nest than tumor stroma and tumor margin.

Further, no significant difference in tumor infiltrating Regulatory T cells was observed with age, gender, habit, anatomic site, TNM staging, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement. In accordance with our observation, Al-Qahtani et al (2011) did not observe significant correlation of tumor infiltrating FOXP3$^+$ cells with age, gender and habit. However, they did not correlate with other pathological parameters.

Further, regarding Regulatory to Cytotoxic T cell ratio, significantly increased ratio was observed at tumor margin of males than females. However, Regulatory to Cytotoxic T cell ratio showed no significant correlation with age, anatomic site, habit, TNM staging, histological grade, lymphatic permeation, vascular permeation, neural invasion and mucosal margin involvement. Further, Regulatory to Helper T cells ratio was not found to alter when correlated with any of clinicopathological parameters.

To assess the prognostic relevance of tumor infiltrating T cells in univariate survival analysis, T cell subsets were correlated with clinical outcome of patients. With respect to DFS, a trend of high incidence of disease relapse along with reduced DFS was seen in patients with high number of Regulatory T cells than low number of Regulatory cells in
tumor nest. Similarly, a trend of high incidence of disease relapse along with reduced DFS was seen in patients with increased Regulatory to Cytotoxic T cell ratio as compared to decreased ratio in tumor nest. Also, a trend of high incidence of disease relapse along with reduced DFS was seen in patients with increased Regulatory to Cytotoxic T cell ratio than the decreased ratio in tumor stroma.

Further, with respect to OS, a trend of high incidence of death along with reduced OS was seen in patients with high number of Regulatory T cells than low number of Regulatory cells in tumor nest. Similarly, a trend of high incidence of death with reduced OS was seen in patients with increased Regulatory to Cytotoxic T cell ratio than the decreased ratio in tumor nest. Moreover, a trend of high incidence of death with reduced OS was observed in patients with low number of Helper T cells as compared to the high number in tumor stroma. However, tumor infiltrating Cytotoxic T cells was not found to be correlated with clinical outcome in present study.

The association of tumor infiltrating T lymphocytes and survival benefits of cancer patients is well known. However, it is important to characterise different types of T lymphocytes because they all have different functions in the tumour microenvironment, thus each may influence patient’s survival differently. In the present study, increased Regulatory T cells in tumor nest and tumor stroma were found to be associated with worse clinical outcome, whereas Regulatory T cells in tumor margin did not influence disease recurrence. Like circulating Regulatory T cells, tumor infiltrating Regulatory T cells also showed contradictory finding regarding their prognostic value in OSCC. Some study groups described tumor infiltrating Regulatory T cells as poor prognosticator of
Head and Neck Cancer (Strauss et al, 2007; Watanabe et al, 2010), whereas others found them as a better prognosticator for locoregional control (Badoual et al, 2006; Zhang et al, 2010). However, few study groups (Distel et al, 2009; Balermpas et al, 2014) found no correlation of tumor infiltrating Regulatory T cells with survival.

Further, in present study, low number of Helper T cells in stroma was found to be associated with poor OS which was also observed by other study groups (Cho et al, 2003; Badoual et al, 2006). Tumor specific Helper T cells can mediate an anti-tumor effect via various mechanisms including enhancing the immune environment by cytokine secretion like IFN γ and IL2 which stimulate the recruitment of Cytotoxic T cells and Dendritic cells (Knutson et al, 2005). Moreover, Pages et al (2009) have demonstrated that most of CD4+ T cell subsets form cluster with mature Dendritic cells. Thus, low number of Helper T cells in tumor microenvironment may cause defective stimulation of Cytotoxic T cells and Dendritic cells, which might be linked with poor OS of patients. However, two study groups did not find association of tumor infiltrating Helper T cells with clinical outcome of Head and Neck Cancer patients (Distel et al, 2009; Balermpas et al, 2014).

Unexpectedly, in present study tumor infiltrating Cytotoxic T cells did not show correlation with patients’ survival, which is contradictory to the findings of majority of study groups (Nedergaard et al, 2007; Distel et al, 2009; Pretscher et al, 2009; Denkert et al, 2010; Dahlin et al, 2011; Balermpas et al, 2014) who have shown high tumor infiltrating Cytotoxic T cells as a better prognosticator in Head and Neck Cancer. However, similar to our findings, some study groups have not observed correlation of
tumor infiltrating CD8\(^+\) cells with survival of Head and Neck Cancer (Guo et al, 1987; Badoual et al, 2006).

Further, in the present study, in multivariate survival analysis that includes tumor infiltrating T cells along with clinicopathological parameters, increased tumor size (a known prognosticator) emerged as independent prognostic factor at step 1, which is in accordance with findings of two study groups (Cho et al, 2003; Badoual et al, 2006) followed by presence of vascular permeation at step 2 and increased Regulatory to Cytotoxic T cell ratio in tumor stroma at step 3 for predicting poor DFS. In contrast to our findings, Badoual et al (2006) found high tumor infiltrating CD4\(^+\)FOXP3\(^+\) T cells as better predictor for DFS in multivariate survival analysis. Further, tumor size entered at step 1, followed by low number of Helper T cells in tumor nest at step 2 for predicting OS, which is similar to findings of Badoual et al (2006), who observed tumor stage and activated tumor infiltrating Helper T cells as predictor for better OS.

Further in patients with Leukoplakia, Cytotoxic and Regulatory T cell subsets were found to be distributed in epithelial and lamina propria layer in Hyperplasia and Dysplasia patients. The maximum number of T cells was found to be present in lamina propria as compared to proliferative or dysplastic epithelium.

Comparing Hyperplasia and Dysplasia patients, a trend of increased number of Cytotoxic as well as Regulatory T cells were observed in epithelial layer of Dysplasia patients as compared to Hyperplasia patients.
Further, comparing Dysplasia and OSCC patients, significantly increased number of Cytotoxic T cells and Regulatory T cells in tumor nest and tumor stroma were seen as compared to epithelial and lamina propria layer of Dysplasia patients.

It is well known that infiltration of inflammatory immune cells in tissue have role in inhibition of tumor development and progression. In an earlier study, Gannot et al (2002) examined the mononuclear cell infiltration in oral epithelial lesions and correlated them with various stages of epithelium transformation such as hyperkeratosis, mild, moderate and severe dysplasia and squamous cell carcinoma. They have observed that severe pathological changes such as dysplasia or SCC are accompanied by higher level of immune cell infiltrates as compared to hyperkeratosis. Moreover, steps towards malignancy had a distinct lymphocyte profile. As the lesion became malignant, the total amount of infiltrating cells increased and the population of the infiltrating cells changed from non-specialized infiltrating cells to T and B lymphocytes. In line with their findings, in the present study, increased infiltration of Cytotoxic and Regulatory T cells in epithelial layer was observed as the epithelium changed from hyperplasia to dysplasia to squamous cell carcinoma. Moreover, this change is gradual as lesion become malignant. Similarly, other two study groups (Zancope et al, 2010; Ohman et al, 2012) have found increased number of CD8$^+$ T cells in OSCC as compared to Dysplasia. However, these study groups have not analysed Regulatory T cells in their study.

Another study group, Yip et al (2008), observed increased number of FOXP3$^+$ cells in nasopharyngeal carcinoma as compared to non malignant nasopharyngeal tissue, which was also observed in the present study. However, in contrast to our observation,
this study group observed low number of CD8$^+$ T cells in nasopharyngeal carcinoma as compared to non malignant nasopharyngeal tissue.

Further, in our study, survival analysis in Dysplasia patients was analysed and in relation to clinical outcome of Dysplasia patients, Cytotoxic and Regulatory T cells individually did not affect the disease status of patients, but their ratio affected the clinical outcome of these patients. In the present study, increased ratio of Regulatory to Cytotoxic T cells in Dysplasia patients was found to be associated with increased probability of developing squamous cell carcinoma indicating that immune suppression is linked with malignant transformation progress. Furthermore, a study of Von Boehmer et al (2005) has revealed that inflamed tissue facilitates specific suppression that is initiated by antigenic stimulation and local recruitment of Regulatory T cells together with cytotoxic lymphocytes. Thus, our results support the hypothesis that Regulatory T cells can localise and expand together with Cytotoxic T cells.

Lymphocyte migration is a key event in immune response. They migrate from peripheral blood to tissue through angiogenesis and therefore, in the present study, Microvessel density (MVD) was evaluated in patients with Hyperplasia, Dysplasia and OSCC. MVD is most commonly used method for identification of angiogenesis. Several studies have examined MVD in different cancer including Oral Cancer by Immunohistochemistry using the markers against endothelial cells such as CD34, CD31 and Factor VIII antigen. In the present study, MVD was evaluated using CD34 which is known to be present in all types of blood vessels and lymphatic vessels. Different study groups found conflicting results regarding MVD in premalignant and malignant lesions of Head and Neck Cancer. Tae et al (2000) did not observed significant difference in MVD from
adjacent normal epithelium to premalignant lesion to cancerous lesion, while Astekar et al (2012) observed increased MVD from normal epithelium to dysplasia to OSCC. However, they did not analyse MVD in Hyperplasia patients. In present study, the significant increase in number of MVD was found during the progression of Dysplasia to OSCC indicating increased angiogenesis which provides nutrition for initial establishment of tumor growth. However, it also indicated high immune cell infiltration at tumor site. Further, no significant difference in number of MVD was found from epithelial transformation of Hyperplasia to Dysplasia. These discrepancies in results due to variations in immunohistochemical protocols, for example, level of section in tissue block (superficial or deep) and the tissue of hot spot selection may have contributed to variations in the results.

Further, in relation with clinicopathological parameters, we did not observe significant correlation of MVD with clinicopathological parameters such as age, gender, habit, TNM staging, vascular permeation, neural invasion and mucosal margin involvement. Similar to our observation, Tae et al (2000) also did not observe significant correlation of MVD with clinicopathological parameters. However, in the present study, a trend of high MVD was observed in stage I disease as compared to stage II, III and IV and in patients without lymphatic permeation as compared to patients with lymphatic permeation which was unexpected. These discrepancies could be due to use of pan epithelial marker CD34; which stained both blood and lymphatic vessels. Further, high endothelial venules (HEV), specialized blood vessels for lymphocytes recruitment, have been recently observed in human solid tumor which facilitate tumor destruction by high levels
of lymphocyte infiltration into tumor and also correlate with better survival (Martinet et al, 2008 and 2011).

Assuming that CD34 stains blood vessels which include HEVs, we further correlated circulating and tumor infiltrating T cell subsets with MVD. In relation with circulating T cell subsets, significantly increased incidence of high MVD was observed in patients with low circulating Regulatory T cells as compared to high circulating Regulatory T cells indicating the migration of Regulatory T cells from blood to tumor site. However, MVD did not show significant correlation with circulating total T cells, Cytotoxic and Helper T cells.

Moreover, in correlation with tumor infiltrating T cell subsets, a trend of increased incidence of high MVD was observed in patients with high Helper T cells as compared to low Helper T cells in tumor nest. However, MVD did not show significant correlation with tumor infiltrating Cytotoxic and Regulatory T cells.

Further, in multivariate survival analysis of peripheral blood Leukocyte, T cell subsets, tumor infiltrating Lymphocytes along with clinicopathological parameters, an increased ratio of Regulatory to Cytotoxic T cell subsets in tumor stroma emerged as independent prognosticator at step 1, presence of vascular permeation at step 2, increased percentage of Regulatory T cell in blood at step 3, and reduced percentage of CD161⁺CD56⁺ NK T subpopulation in blood at step 4 for predicting reduced DFS. Further, low number of Helper T cells in tumor stroma was found to be an independent prognostic factor for predicting poor OS.
Thus, our data emphasize on quantitative analysis of T cell subsets in peripheral blood and tumor tissue of OSSC patients which is important to identify immunosuppressed patients, who are at high risk for recurrence. These patients can be benefited with immunotherapy to enhance their immune response.

Early experimental model showed that nonspecific depletion of CD4$^+$ T cells induced efficient antitumor immune response (Fu et al, 2000) and targeting specifically Regulatory T cells by CD25 monoclonal antibody induced the development of CD8$^+$ Effector cells and NK cells (Shimizu et al, 1999). Immune response to malignant tumor was found to be weak and ineffective and therefore, sole depletion of Regulatory T cells might not always result in tumor regression. Thus, depletion of Regulatory T cells together with other immune-stimulatory interventions like CTL4 blockage (Sutmuller et al, 2001), DC based vaccination and transfer of activated T cells might be more beneficial (Prasad et al, 2005). In rat colon cancer model, administration of cyclophosphamide depleted Regulatory T cells and delayed the outgrowth of tumor (Ghiringhelli et al, 2004).

Further, Dunull et al (2005) carried out first clinical trial for selective elimination of Regulatory T cells by Denileukin diftitox (diphtheria toxin conjugated with IL-2) in cancer patients. Moreover, other novel approaches were also examined which manipulates Regulatory T cells such as depletion of Regulatory T cells, blocking their migration to tumor by CCL22 specific antibody and reducing their differentiation and signaling by blocking FOXP3 signals (Zou et al, 2006).
In conclusion, tumor infiltrating lymphocytes, especially Regulatory T cells are important clinical and prognostic indicator in OSCC. The prevalence of Regulatory T cells in different tumor compartment indicates strong immune suppression within tumor microenvironment. Moreover, the number of tumor infiltrating Regulatory T cells along with their ratio to other T cell subsets specifically Cytotoxic T cells helps to identify a subgroup of OSSC patients with high probability of disease recurrence and shorter DFS. Further, our data indicates infiltration of T lymphocytes increased in step wise manner from Hyperplasia to Dysplasia to Carcinoma. Moreover, the predominance of Regulatory T cells in Dysplasia is significantly associated with disease progression indicating that the host immune surveillance is prominent in the premalignant lesion, whereas it decreases during carcinogenesis. Hence, analysis of circulating and tumor infiltrating T cell subsets helps to identify immunosuppressed patients who are at high risk for relapse. Such patients may potentially benefit from the implementation of immunotherapy along with conventional treatments to stimulate T cell immune response.