Potentially malignant oral disorders and oral cancer
- a study on immunosurveillance

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Cover illustration: Patient with a squamous cell carcinoma and a leukoplakia on the lateral border of the tongue (left). A histological section from a leukoplakia showing CD3 positive T cells (right).

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To my beloved boys, David, Julius and Elliot.

CONTENTS

ABSTRACT

The cancer immunosurveillance hypothesis postulates that the immune system can recognize cancer cell precursors and destroy those cells before a clinical manifestation occurs. During the last decades several groups have presented evidence of the influence and role of immune activation in oral squamous cell carcinoma (OSCC) patients; however, much less is known about the role of immune activation in potentially malignant oral disorders (PMOD). OSCC may be preceded by a PMOD. Two of the most common PMODs in the Western population are oral leukoplakia (LPL), defined as a predominantly white patch in the oral mucosa that cannot be characterized as any other definable lesion, and oral lichen planus (OLP) defined as a chronic inflammation in the oral mucosa manifested as bilateral white hyperkeratotic striations with or without erythema, ulceration, bullae or plaque.

The general aim of this thesis was to characterize the immune response in PMODs and oral cancer and to relate immune response to malignant transformation. Another aim was to address whether long-term immunosuppression in a large cohort of solid organ transplant (SOT) patients predisposes for cancer in the oral cavity and lip.

In papers I–III clinical data and biopsy specimens were analysed from patients with OLP and healthy oral mucosa (I), patients with LPL with and without dysplasia and OSCC (II) and those with LPL with dysplasia with (LPL-ca) or without (LPL-dys) malignant transformation (III). Immunohistochemistry was used to detect different cell types of interest, in particular, subtypes of dendritic Langerhans cells (LCs) and T cells. In paper IV a cohort of SOT patients were correlated with the Swedish Cancer Register for prevalence of oral and lip cancer and compared with the prevalence in the Swedish population. Overall 5-year survival in SOT patients with oral and lip cancer was compared to an age- and gender-matched control group with oral and lip cancer without previous SOT.

In paper I the results showed that OLP patients had a significantly higher number of dendritic Langerhans cells (LCs) in the epithelium and the connective tissue than in healthy control patients. Also, cells with dendritic morphology and expressing the maturation marker CD83 were found in clusters with lymphocytes in the connective tissue.

In paper II the results showed that both cytotoxic T cells and dendritic Langerhans cells were significantly increased in connective tissue in LPL with dysplasia compared to LPL without dysplasia, indicating an immune response to cells with cell dysplasia. In OSCC, the influx of T cells and LCs was increased almost a thousand-fold compared to LPL. Confocal laser scanning microscopy revealed a co-localization of LCs and T cells in LPL with dysplasia and OSCC, indicating possible immune activation

In paper III quantitative analyses showed that patients with LPL displaying cell dysplasia that transformed into OSCC had lower numbers of T cells than a group of patients with LPL with dysplasia that did not transform into OSCC during the observation period.

In paper IV the results showed a standardized incidence ratio (SIR) that was increased for both oral (SIR: 6.3) and lip cancer (SIR: 43.7) in SOT patients compared to non-SOT patients. Also, the overall 5-year survival was decreased for lip cancer in SOT patients compared to non-SOT lip cancer patients.

To conclude the findings in papers I, II and III, evidence of immunosurveillance in PMOD and OSCC are presented. After long-standing immunosuppression in patients with SOT there is an increased risk for both lip and oral cancer, and the overall survival for patients with lip cancer is also negatively affected.

The concept of immunosurveillance originally proposed by Dunn *et al.* in 2004 is well in line with the findings in this thesis of PMOD and oral cancer.

Keywords: immunosurveillance, potentially malignant oral disorders, oral cancer, solid organ transplantation, immunosuppression, T cells, Langerhans cells.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är en genetisk sjukdom som uppstår efter att ett antal förändringar i cellens viktiga reglerande gener har skett. Immunsystemets celler övervakar hela tiden vår kropp och angriper farliga mikroorganismer och celler som är infekterade eller celler som inte uppför sig normalt. När normala celler i vår kropp börjar få förändringar i sitt genetiska material (DNA) och utvecklas till cancerceller signalerar de till immunsystemets celler att det inte står rätt till och att dessa celler bör förintas. Vid många typer av cancer, inklusive munhålecancer, har man sett att immunsystemets förmåga att eliminera cancerceller påverkar prognosen för patienten. I munhålans slemhinna finns det potentiellt maligna sjukdomar som har en ökad risk att utvecklas till munhålecancer. De vanligaste potentiellt maligna orala sjukdomarna är leukoplakier och oral lichen planus. Kunskapen om närvaron av immunsystemets celler och hur dessa påverkar prognosen hos patienter med potentiellt maligna orala sjukdomar är idag i mångt och mycket okänt.

Huvudsyftet med den här avhandlingen har varit att karaktärisera immunsystemets celler i oral lichen planus, leukoplakier och munhålecancer. Vi har även velat undersöka om patienter som på grund av långvarig immundämpande medicinering efter organtransplantation löper större risk att utveckla cancer i munhåla och läpp samt om prognosen är sämre för dessa patienter än för patienter med cancer i munhåla och läpp utan långvarig immundämpande medicinering.

I första artikeln har vi undersökt om en subtyp av vita blodkroppar -Langerhans celler - i olika mognadsgrad, är fler i oral lichen planus jämfört med frisk oral slemhinna. I artikel II har vi undersökt om antalet Langerhans celler och T lymfocyter, en annan subtyp av vita blodkroppar, är färre i leukoplakier utan cellförändringar än i leukoplakier med cellförändringar och munhålecancer. I artikel III har vi jämfört antalet Langerhans celler och T lymfocyter i leukoplakier med cellförändringar där den ena gruppen sedan har utvecklat en munhålecancer. I artikel IV har vi tittat på patienter som har organtransplanterats mellan 1965 och 2010 och jämfört förekomsten av cancer i munhåla och läpp med den normala svenska populationen. Vi har även jämfört 5-årsöverlevnad hos patienter som har organtransplanterats och drabbats av cancer i munhåla och läpp jämfört med patienter som drabbats av cancer i munhåla och läpp utan någon tidigare organtransplantation.

Den första studien visade att antalet Langerhans celler i oral lichen planus är fler än i frisk slemhinna.

I den andra studien blev resultatet att i leukoplakier med cellförändringar och i munhålecancer finns det fler Langerhans celler och T lymfocyter än i leukoplakier utan cellförändringar.

I den tredje studien konstaterades att det fanns färre T lymfocyter i leukoplakier som har utvecklats till cancer än i de leukoplakier som inte har blivit cancer under uppföljningsperioden.

Patienter som har stått på långvarig immundämpande medicinering efter organtransplantation har 6 respektive 44 gånger så stor risk att utveckla cancer i munhåla respektive läpp. Patienter med immundämpande medicinering och läppcancer har även sämre 5-årsöverlevnad än patienter med läppcancer som inte organtransplanterats.

Resultaten i denna avhandling visar att det finns ett ökat antal immunceller i potentiellt maligna orala sjukdomar och oral cancer. Antalet T lymfocyter verkar även påverka om det ska ske en malign omvandling eller inte. Långvarig immundämpande medicinering ökar risken för att utveckla cancer i munhåla och läpp samt även försämra prognosen hos patienter med läppcancer.

LIST OF PAPERS

This thesis is based on the following original papers, which are referred to in the text by Roman numerals (I–IV):

I. **J Gustafson**, C Eklund, M Wallström, G Zellin, B Magnusson, B Hasséus.
Langerin-expressing and CD83-expressing cells in oral lichen planus lesions.
Acta Odontologica Scandinavica 2007 Jun; 65(3): 156–161.

II. **J Öhman**, B Magnusson, E Telemo, M Jontell, B Hasséus.
Langerhans cells and T cells sense cell dysplasia in oral leukoplakias and oral squamous cell carcinomas – evidence for immunosurveillance.
Scandinavian Journal of Immunology. 2012 Jul; 76(1): 39–48. doi: 10.1111/j.1365-3083.2012.02701.

III. **J Öhman,** R Mowjood, L Larsson, A Kovács, B Magnusson, G Kjeller, M Jontell, B Hasséus.
Presence of CD3-positive T cells in oral premalignant leukoplakia indicates prevention of cancer transformation.
Accepted for publication in Anticancer Research, vol. 35 (2015)

IV. **J Öhman**, H Rexius, L Mjörnstedt, H Gonzalez, E Holmberg, G Dellgren, B Hasséus.
Oral and lip cancer in solid organ transplant patients: a cohort study from a Swedish transplant centre.
Accepted for publication in Oral Oncol (2014), doi: http://dx.doi.org/10.1016/j.oraloncology.2014.11.007

ABBREVIATIONS

CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-Lymphocyte Associated protein 4 (CD152)
CLSM	Confocal Laser Scanning Microscopy
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
EBV	Epstein Barr Virus
ENT	Ear-, Nose- and Throat, otorhinolaryngology
FasL	Fas ligand (CD95L)
G-phase	Gap phases in mitosis
GVHD	Graft Versus Host Disease
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
ICD	International Classification of Diseases
IL	Interleukin
LC	Langerhans cell
LP	Lichen planus
LPL	Leukoplakia
LPL-dys	Leukoplakia with dysplasia but without malignant transformation
LPL-ca	Leukoplakia with dysplasia with malignant transformation
mAb	Monoclonal antibody
MDSC	Myelo-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
NKG2D-ligand	Natural Killer Group 2 member D-ligand
NSAID	Non-Steroidal Anti-inflammatory Drugs
OLP	Oral lichen planus
OSCC	Oral Squamous Cell Carcinoma
PD-L1	Programmed Death-Ligand 1 (CD274)
PMOD	Potentially Malignant Oral Disorder
PTLD	Post-Transplant Lymphoproliferative Disorder
PVL	Proliferative Verrucous Leukoplakia
SIR	Standard Incidence Ratio
SOT	Solid Organ Transplantation
TAA	Tumour Associated Antigen
TAM	Tumour Associated Macrophage
TCR	T Cell Receptor
TIL	Tumour Infiltrating Lymphocyte
TGF	Transforming Growth Factor
Th	T helper
TLS	Tertiary Lymphoid Structure
TNM	Tumour, Node, Metastasis. Classification system
TRAIL	Tumour necrosis factor-Related Apoptosis-Inducing Ligand
Treg	Regulatory T cell

1. INTRODUCTION

1.1 Background

Few diseases have greater impact on modern society than cancer. Unfortunately, the burden of cancer is increasing globally (1, 2). This evolvement may be associated with a steadily increasing global population, and by environmental factors and changes in lifestyle. In 2012, the World Health Organization (WHO) reported 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer in 2012, worldwide (3).

Oral cancer causes great morbidity and mortality for patients all over the world. Early detection is of great prognostic importance for patients with oral cancer. Even if improvements have been made in treatment modalities of oral cancer related to surgical technique, radiation and the use of directed therapy with monoclonal antibodies, the overall survival has not changed during the last decades. It has been reported that approximately 50% of the oral cancers have mucosal lesions in conjunction to the tumour, indicating that at least half of them are preceded by a potentially malignant oral disorder (PMOD). Leukoplakia (LPL) and oral lichen planus (OLP) are PMODs affecting the oral mucosa but there are no reliable risk factors at hand to predict which of these LPL and OLP lesions that will transform into a cancer. There is a great frustration both among health care providers and patients suffering from PMOD due to the lack of knowledge on how to treat and how to avoid the transformation into a cancer. There is a need for increased knowledge about biological mechanisms that are involved in cancer transformation in PMOD. This knowledge can eventually lead to better prognostic markers and to development of novel treatment strategies.

As the immune system plays an important role in protection of malignant diseases, the overall objective of this thesis is an attempt to increase our knowledge about the immune systems role in PMODs and oral cancer.

1.2 Hallmarks of cancer

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 tumour suppressor genes) leading to malignant transformation of normal cells (4, 5).

In 2000 Hanahan and Weinberg postulated the concept of 'the six hallmarks of cancer' in an effort to explain cancer biology. These theories describe important properties that potentially malignant cells need to acquire to favour carcinogenesis (6). The hallmarks are considered to be more or less universal for all cancers, regardless of organ or cell type. The properties listed by Hanahan and Weinberg attribute to the cancer cells dominant malfunctions of proteins that control cell

proliferation and differentiation. The cancer cells also acquire a loss of function in tumour suppressor proteins that normally govern induction of apoptosis, defects in DNA repair mechanisms and signalling that mediates cell cycle arrest. Genetic aberrations in genes that control angiogenesis, invasion and metabolism may also be affected and influence the capacity of the tumour to seed metastatic cells.

The importance of the surrounding tumour microenvironment has lately been highlighted in the process of tumorigenesis by several groups in both humans and animal models (reviewed in (7, 8)). The tumour mass is a complex network of cells consisting of cancer and stromal cells in a dynamic interaction. This new knowledge resulted in another publication from Hanahan and Weinberg in 2011, where four more hallmarks were suggested to be among the principal hallmarks of cancer (9) (fig. 1). Two of the next-generation hallmarks address the tumour cells' interaction with the tumour microenvironment, describing the tumour cells' ability to evade the antitumoral defence exerted by the peritumoral stroma and their ability to induce a more tumour-promoting inflammation to further favour the oncogenesis.

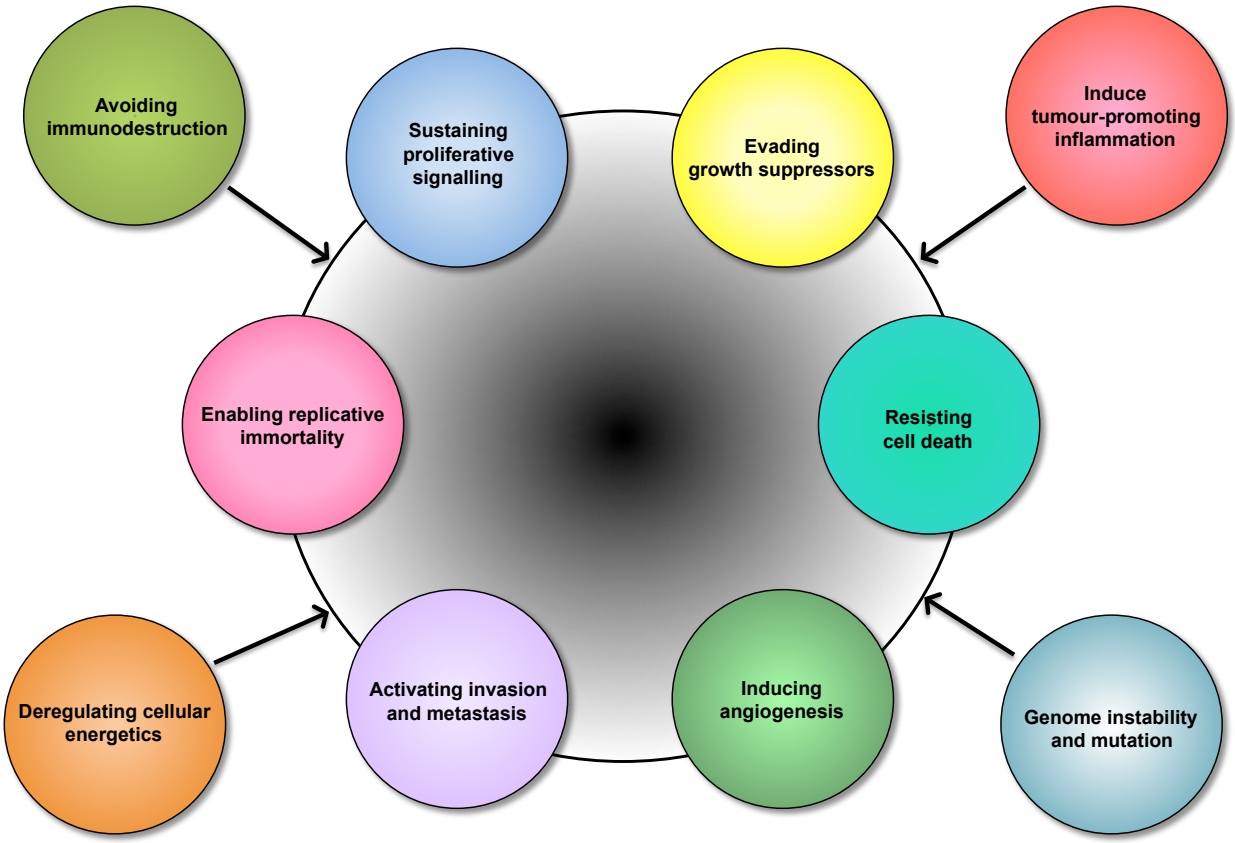


Figure 1. The hallmarks of cancer: the next generation. Modified from Hanahan and Weinberg in 2011 (9).

1.3 The immune system and cancer

Recently, the importance of the tumour-associated stroma has been highlighted by the scientific community (10, 11). Several studies have shown that infiltration of specific immune cells in the tumour microenvironment can impede the development of a cancer (reviewed in (8)). This could be looked upon as an extrinsic tumour suppressor mechanism when the intrinsic tumour suppressor mechanisms have failed.

1.3.1 Cancer immunosurveillance

Burnet and Thomas were first to describe immunosurveillance in the 1950s (12, 13), but lack of knowledge and experimental methods to investigate this field resulted in dormancy of research. Burnet and Thomas's theory fell into oblivion for more than 40 years. New evidence supporting this theory was presented at the end of the last century, suggesting that infiltration of immune cells and the immune response could be of importance regarding the protection of malignant transformation (14-16).

In the last 20 years a large number of reports have been published using cell culture, animal and human studies, recognizing that the immune system has an important role in preventing cancer (17). The mechanisms are studied foremost in various animal models where the experimental systems are well controlled. Experimental designs with genetically modified mice and adoptive transfer experiments in mice have shown that both tumour progression and regression can be modified by immunological mechanisms (7, 18). In the human setting convincing clinical evidence exists supporting the immunosurveillance hypothesis:

- Intra- and peritumoral immune responses predict patients' prognosis in a wide range of cancers (19-22).
- Systemic or remote immune response in serum and lymph nodes are seen in patients with cancer (23, 24).
- Pathologically and pharmacologically immunocompromised patients are at higher risk of several cancers, both virally and non-virally induced (25).
- Humans with inherited immunodeficiencies have an increased risk of developing cancers (26, 27).

The concept of immunosurveillance was suggested based on the findings, in animal models and in humans, as described above. Later on, a refined concept of immunosurveillance was suggested when it was recognized that the immune response not only protected the host but also edited the immunogenicity of tumours. The concept of immunoediting was then formulated by Dunn and Schreiber in 2002 (28).

1.3.2 Cancer immunoediting

Immunoediting can be divided into three parts: elimination, equilibrium and escape. This concept is an attempt to describe in a consecutive manner the interplay between the potentially malignant cells and the corresponding cells of the immune system (28).

Elimination

In the elimination phase, cells from both the innate and the adaptive immune systems are involved and participate in the elimination process (18). Early in carcinogenesis innate immune cells are alerted and recruited into the peritumoral stroma, forming a first line of defence (29). Granulocytes and macrophages contribute to antitumoral defence and secretion of proinflammatory cytokines (30). Natural killer (NK) cells continuously patrol and scavenge the tissue for cells with imbalances of activating and inhibitory molecules (31). An aberrant major histocompatibility complex (MHC) class I expression and signalling through killer cell immunoglobulin-like receptors (KIRs) results in elimination by cytotoxic mechanism or induction of apoptosis in cells out of line (32).

Dendritic cells (DCs) are potent antigen-presenting cells with a key role in evoking a T cell response (33). DCs also have a key role in initiating tumour-specific immune response and could be associated with prognosis of cancer (34, 35). DCs engulf, process and present tumour-associated antigens (TAAs) to naive or memory T cells, which causes T cell activation (36). When challenged with proinflammatory stimuli, DCs undergo a process of maturation characterized by upregulation of MHC class II and co-stimulatory molecules together with morphological changes that enhance migratory capacity (37).

DCs exist in different subsets, two of the main subsets being myeloid DCs (mDC) and plasmacytoid DCs (pDC). DCs direct immune responses against antigens towards either a cell-mediated or a humoral (antibody) response, depending on cell–cell interaction and cytokine production (38-40). TAAs can be recognized by cells of the immune system's adaptive arm by DCs presenting the antigens to T helper (Th) cells in context with MHC class II molecules, or cross-presentation together with MCH class I molecules (41).

Langerhans cells (LCs) are a subtype of mDCs that are localized in the epithelium of skin and mucosa. In those compartments, LCs are the key players in initiating adaptive immune response by engulfing and processing antigens from the epithelium of mucosal lining and skin, followed by migration to regional lymph nodes, where self or non-self antigen can be presented to naive or memory T cells. LCs are characterized by expression of CD1a, MHC class II and Langerin molecules. Intracellular Birbeck granules are LC-specific organelles (42).

pDCs are a subtype of DCs that are one of the main sources of IFN-γ but have a poor antigen-presenting capacity (43). Their presence in the peritumoral stroma is evident, but the clinical significance has not yet been clarified in cancer patients.

In parallel, T cell subsets have been further delineated and found to consist of at least five subpopulations: Th1, Th2, regulatory T cells (Tregs), cytotoxic T cells (CTLs), and finally, the recently discovered Th17. All subsets play important roles in mucosal immune response, including antitumoral responses. T cells represent approximately 10% of the total cells in a tumour mass (11).

Cytotoxic T cells execute the main antitumour defence mechanism of the adaptive immune cells. They have the ability to recognize and kill potentially malignant cells that present TAAs associated with major MHC class I on the surface with high specificity and sensitivity (44). This will result in an attack and killing via effector molecules such as perforin and granzyme. Tumour cells also get signals that induce apoptosis by interaction with FasL and TRAIL receptors expressed on DCs, NK cells or cytotoxic T cells (45, 46).

Th1 cells are the main orchestrator of antitumoral defence; they support cytotoxic T cells and NK cells by production of Il-2 and IFN-γ, and also enhance DCs' stimulatory capacity (47).

Th2 cells are mainly placed as a director of humoral response by activating B cells and a subsequent production of immunoglobulins. In cancer the role of B cells have so far not been extensively addressed. However, recent studies indicate an importance of B cells in tumour disease (48, 49).

Th17probably have a dual role in tumour-related inflammation. Th17 cells induce fibroblasts to produce proangiogenetic and protumoral factors; they also enhance the antitumoral defence by supporting cytotoxic T cells, NK cells and DCs (50).

In normal tissue homeostasis, Tregs are important for regulating the immune response by production of suppressive cytokines, primary TGF-β and IL-10, or by downregulating and limiting Th1 and cytotoxic T cell response by CTLA-4(51).
Tregs constitute only a minor population of T cells in healthy conditions, where they mediate peripheral tolerance and prevent autoimmune diseases from developing (52). It is important to limit an acute inflammation and not to overshoot the protective goal, thereby causing tissue damage and also suppressing any possible autoimmune reaction. However, in cancer these suppressive actions lead to a possibility for tumour cells to escape the antitumoral defence. In cancer patients increased frequency of Tregs in both the circulation and the tumoral tissue have been reported (53, 54), and their increased presence, are also related to a poorer outcome (55-57).

Myelo-derived suppressor cells (MDSCs) are a heterogeneous population of cells with immunosuppressive properties. MDSCs were first described in head and neck

cancer in 1995 as a (CD34+) immature cell with immunosuppressive functions (58). Their presence in the tumour microenvironment results in a direct immunosuppressive milieu with suppression of T cell response (59, 60).

Macrophages are scattered in the peritumoral stroma in many malignant tumours, where they are then referred to as tumour-associated macrophages (TAMs) (61). TAMS can be divided into two subsets with different modes of action; M1 and M2. M1 have antitumoral properties, while M2 is an immune regulatory and tumour-promoting phenotype (62). In the tumorigenesis process there seems to be a switch in polarization from M1 to M2 phenotype (63).

The elimination phase is a process that probably occurs all the time throughout life to prevent tumour disease from arising. If the immune cells do not successfully eradicate the cancer cells, they may be kept in an equilibrium stage.

Equilibrium

When immunosurveillance systems are not able to eradicate the tumour cells, the result may be tumour dormancy, where an equilibrium with defending cells occurs. The first line of defence capitulates and the adaptive branch of the immune system takes over and maintains a steady state between tumour cells and immune cells (64). Lymphocytes have the capacity to exert enough antitumoral effects to kill and limit tumour growth, and the tumour is thus kept under control.

More or less anecdotal examples have been reported of cancers in recipients of transplanted solid organs, where the tumour cells originated from the donors, who years earlier had had malignant tumours (65). This demonstrates that when the immune system is stunted, tumour cells that have been held in check in an immunocompetent donor are given free reign in an immunocompromised recipient, ending the equilibrium phase and beginning the escape phase.

Escape

The negative aspect of the antitumoral defence is that it favours less immunogenetic tumour cells to develop in accordance with the concept of immunoediting. There is a clonal evolution where the tumour cells gain new characteristics to avoid immune recognition and destruction. The antitumoral defence also shapes the tumour immunogenicity, enabling the selection for nonimmunogenic tumour variants (66). The tumour cells have multiple strategies to circumvent deletion, for example, induction of immune suppression, avoidance of recognition and lack of susceptibility (67). This may depend on an increased resistance to the cytotoxic effect of immune cells or the effector cells having lost their ability to annihilate tumour cells. The tumour cells gain properties that can suppress the antitumoral effects and recruit a more favourable milieu for metastasis and uncontrolled proliferation.
The inflammation-promoting arm of this response is counteracted by downregulating mechanisms, by Tregs, MDSCs, TAMs and tolerogenic DCs (51, 68).

Tumour cells themselves can also orchestrate the protumoral environment by producing for example, TGF-β and IL-10 as well as suppress the antitumoral activity by expression of PD-L1 and Fas ligand (45, 69-71). In a worst-case scenario downregulation of defence capacity occurs, resulting in impaired disease control. Thus, a delicate balance exists between an effective antitumoral response and a loss of defence capacity.

To decrease the ability for immune cells with antitumoral properties to enter the peritumoral tissue, homing receptors are downregulated and malformation of the vascular tree, hypoxia and interstitial pressure lead to a hostile microenvironment for infiltrating defence cells. To avoid recognition, tumour cells lose their antigen expression due to impaired processing or presentation of tumour-specific epitopes or downregulation of NKG2D ligand (72).

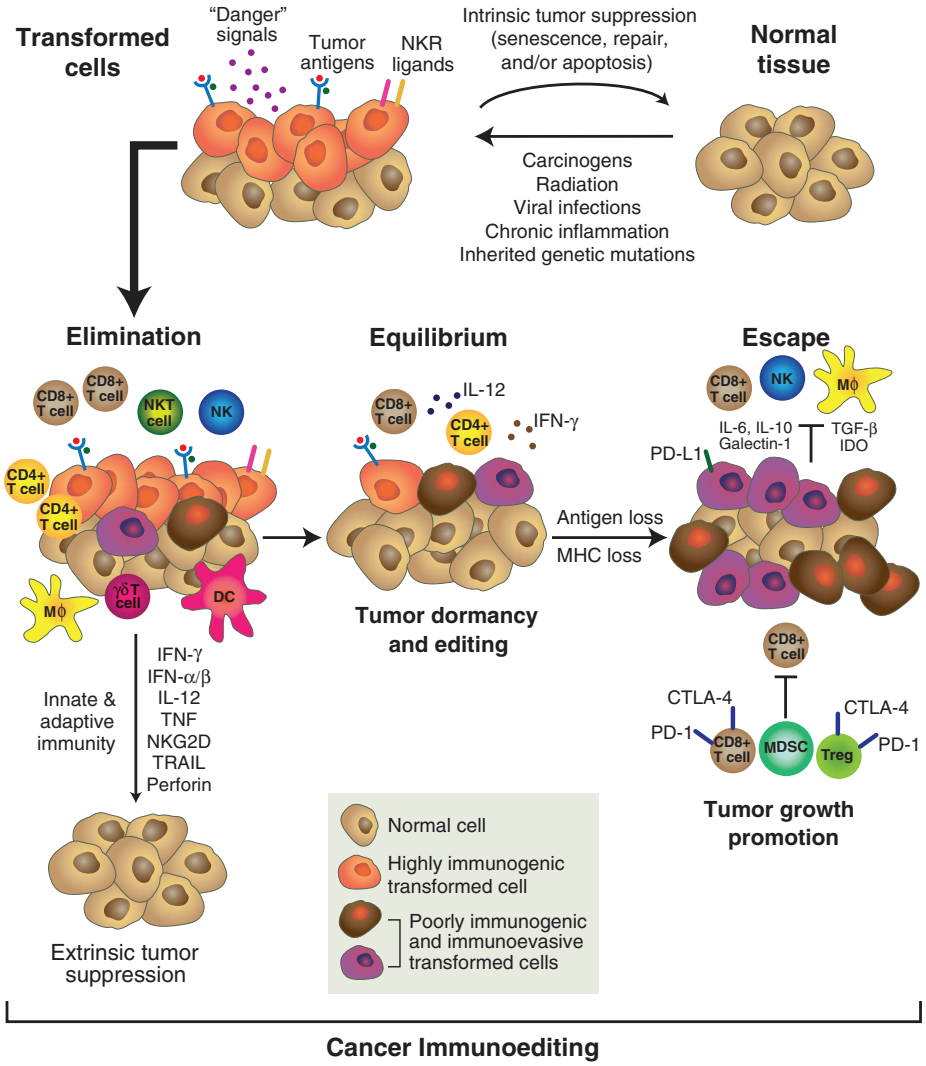


Figure 2. The cancer immunoediting concept: Elimination, Equilibrium and Escape. Cells involved in this process: CD4+ cells-T helper cells, CD3+ cells-cytotoxic T cells, Mϕ-macrophages, NK-natural killer cells, DC- dendritic cells, Treg-regulatory T cells, MDSC-myelo-derived suppressor cells, figure with permission from Schreiber R. Science 331, 1565 (2011) (73).

1.3.3 Chronic inflammation and cancer

The immune system has a dichotomous role and can both promote and prevent tumour development (74, 75). The cellular effects and mediators in inflammation can also create and sustain a favourable environment for tumours. In an inflammation there is a constant secretion of growth factors that enhance cell proliferation and angiogenesis but are also involved in tumorigenesis; a tumour resembles a wound that never heals (76). Common inflammatory mediators such as reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) cause DNA damage and degrade the connective tissue, facilitating migration of cells to exert inflammation. This microenvironment promotes growth stimulation, enhanced survival, enhanced invasion and increased angiogenesis (77). This phenomenon was eloquently explained by Balkwill et al.: 'If genetic damage is the match that lights the fire of cancer, some types of inflammation may provide the fuel that feeds the flames' (78).

Epidemiological evidence supports the link between chronic inflammation and cancer. De Martel et al. suggested that 16% of all cancers could be attributed to infections (79). Several chronic inflammatory disorders are associated with cancer development. Hepatitis B and C predispose for liver cancer, *Helicobacter pylori* infection predisposes for gastric cancer and Sjögren's syndrome is associated with an increased risk for lymphoma, while ulcerative colitis increases risk for colon carcinoma (80-83). In the oral cavity, oral lichen planus (OLP) is associated with an increased risk for oral cancer (84). This is believed to be attributable to a state of long-standing chronic inflammation (85).

When the chronic inflammation is reduced, the risk for cancer seems to decrease. The use of non-steroidal anti-inflammatory drugs (NSAID) has shown to decrease the risk of colon cancer (86, 87). Recently, postdiagnostic use of aspirin has been shown to reduce prostate cancer-specific mortality (88), which could be another piece of evidence for the important link between cancer and inflammation.

1.3.4 Immunosuppression and cancer

Several experimental models and clinical studies have highlighted the link between suppression of immunological pathways and cancer, including transgenic mouse models where deletion of important arms in the immune system make it possible to investigate the connection between immunological response and cancer development (17). In humans there are rare examples of hereditary genetic disorders resulting in severe immunodeficiencies such as Wiskott–Aldrich syndrome (89) and DiGeorge syndrome (27), where patients are more prone to develop cancers.

Under some circumstances immunosuppression occurs in humans as a result of pharmacological treatment or infection. Such conditions have made it possible to study the correlation between cancer and immunodeficiency in humans.

In the middle of the last century the procedures for transplantation of solid organs were developed. Over three decades ago the advancements in surgical sciences and discovery of rejection-preventing drugs led to the introduction of solid organ transplantation (SOT) into routine clinical care in centres with adequate resources.

A major breakthrough was the discovery that cyclosporin A, a substance retrieved from a fungus, could suppress the immune system by reducing the capacity of T cells to produce IL-2 (90). This cytokine works in auto- and paracrine loops and inhibits T cell proliferation. In a host-versus-graft rejection the cell-mediated immune response is a key player, and by dampening T cell activity, the rejection process can be kept under control (90). During the last decades new pharmacological agents, like mycophenolate mofetil, everolimus, tacrolimus and rapamycin, have been introduced (91). These new agents have the common goal to prevent the immune system from rejecting the transplanted organ.

There is an increased hazard of contracting malignant diseases in patients after solid organ transplantation (92-94). The overall risk for tumour diseases is increased at least two-fold after SOT compared to a normal population (95). This is believed to be a result of long-standing immunosuppression, which decreases immunosurveillance against tumours.

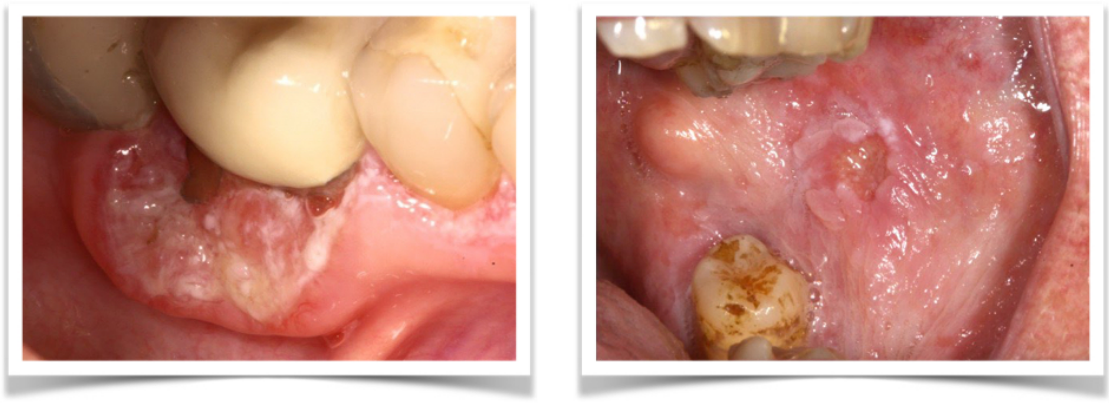
The improved clinical care of solid organ transplanted patients has resulted in better long-term survival, thereby increasing their risk of developing cancer over time (96). Three years after transplantation, malignancy is one of the major cause of death in SOT patients (96, 97).

However, there is a variation among types of cancer and types of transplanted organs. Non-melanoma skin cancer and post-transplant lymphoproliferative disorders (PTLD) are the tumour diseases that show the highest increase in prevalence in SOT patients (95, 98).

1.4 Oral cancer

Oral cancer is defined as cancer within the oral cavity (fig 3). The oral cavity is defined as the area from the anterior pharyngeal valves to the borderline between the mucosa and lip skin. The majority of oral cancers are squamous cell carcinomas (OSCC). Salivary gland tumours, bone tumours and malignant melanomas are more rarely seen in the oral cavity (99). Metastasis from primary tumours at other anatomical locations also occurs, with a predominance of breast, lung and prostate cancers (100). Oral cancer is more common in the later decades of life, but lately

early debut is more frequently observed (101). Irrespectively at what age the cancer occurs it causes great morbidity and mortality in patients.



A. B.

Figure 3. Squamous cell carcinoma in the gingival mucosa (A), squamous cell carcinoma in the buccal mucosa (B).

1.4.1 Incidence

There is a wide global variation in incidence of oral cancer, probably due to differences in tobacco and alcohol habits, environmental factors as well as socioeconomic status. In high-risk countries in Southeast Asia, such as Sri Lanka, India, Pakistan and Bangladesh, oral cancer is one of the most common cancers in men compared to northern and southern Europe, where the incidence is relatively low (99). The global estimated incidence for lip and oral cancer is approximately 300 000 cases per year, and the mortality rate is around 145 000 cases per year (3). The incidence of oral cancer is increasing in some parts of the world (102-104), while in other parts there seems to be a declining long-term incidence (105, 106). Lip and oral cancer combined occupies the 11th position on the worldwide cancer incidence list (3).

1.4.2 Aetiology

Smoking and smokeless tobacco is considered the main aetiological factor for OSCC (107, 108). Different types of smokeless tobacco such as betel quid (109), with or without concomitant smoking, result in an increased risk of OSCC (108, 110).

Whether alcohol as a sole aetiological factor participates in oral carcinogenesis is debated. Divergent results on the carcinogenic potential of alcohol have been presented (111-113). However, alcohol in combination with tobacco use seems to potentiate the risk for oral cancer (114, 115).

A correlation between HPV infection and oropharyngeal cancer has been revealed during the last two decades. The incidence of tonsillar and oropharyngeal cancers has increased in different parts of the world (106). The correlation between HPV and oral cancer is weaker than that of HPV and oropharyngeal cancer, but in a meta-analysis from 2011 Syrjänen et al. concluded that HPV significantly increases the risk for OSCC (116).

Confounding factors make it difficult to prove an association between oral cancer and socioeconomic status. However, in 2008 Conway et al. showed in a meta-analysis that there is an association between low socioeconomic status and oral cancer risk (117).

A subgroup of OSCC has been identified in younger individuals with absence of any identifiable 'traditional' risk factor such as longstanding use of tobacco or alcohol (118). This confirms our incomplete understanding of aetiological factors causing OSCC. However, the cancer related survival for the younger subgroup of OSCC does not seem to differ from that of older patients with OSCC (119, 120).

1.4.3 Treatment

During the last decades, the efforts in basic cancer research have resulted in a wide range of targeted therapies and personalized medicine strategies in different tumour diseases. Despite the advancement in these strategies for various cancers, the gold standard for treating OSCC is still surgical excision with tumour-free margins, with or without adjuvant radiotherapy or chemotherapy. Neck dissection with extirpation of regional lymph nodes is also standard therapy in efforts to prevent or treat metastasis. Functional and aesthetical consequences have a great impact on the patient's quality of life (121-123). A call for major improvement of cancer and reconstructive treatments, especially in younger patients with oral cancer, is of importance to reduce the adverse sequelae in a long-term perspective.

1.4.4 Prognosis

Early detection is the most significant factor in increased long-term survival for OSCC patients (124-126). Tumour stage, tumour grade and nodal stage are independent factors correlating with decreased overall survival of oral cancer patients (127). A Canadian follow-up study published in 2014 showed that after 2, 5, 15 and 25 years the overall survival rates were 65%, 49%, 23% and 12%, respectively, and the oral cancer-specific survival rates were 75%, 56%, 46% and 40%, respectively (128).

During recent decades the prognosis for OSCC patients does not seem to have changed to any large extent (129). There seems to be an increase in mortality among women in Europe whilst there is a small decrease in mortality in men (129).

In the Nordic population the overall survival after five years has not changed to any major extent in the last ten years for oral cancer patients, being just above 60% (101).

1.5 Lip cancer

Lip cancer, although also originating from squamous cell epithelium, differs from oral cancer in several aspects. Lip cancer has a great predominance in males compared to females (6:1) and occurs in the sixth or seventh decade of life. Lip cancer correlates with the cumulative lifetime exposure to UV light, and the majority of the cancers are located in the lower lip (130, 131). The highest incidence rates for cancers of the lip are reported in Caucasian populations in Canada and Australia (99).

The gold standard for treatment of lip cancer is surgery, although both external radiotherapy as well as brachytherapy is used with good results (132). Cancer of the lips has a more favourable prognosis than OSCC. Patients with lip cancer rarely contract lymph node metastasis, and the 5-year disease-specific survival rate is >85% (131, 133).

Lip cancers are sometimes preceded by actinic keratosis, which is classified as a potential malignant disorder, according to the WHO (134).

1.6 Immune response in oral cancer

In recent years several groups have presented evidence of the influence and role of immune activation in serum, saliva and sentinel lymph nodes as well as locally in the intra- and peritumoral tissues of OSCC patients (135-139). Both cell-mediated anti-tumoral responses and cytokine profiles have been shown to correlate with prognosis in a range of malignant diseases, including OSCC (140, 141).

Immunosuppressive pharmacological treatment significantly increases the risk of developing oral cancer (92, 142, 143). A large epidemiological study has also shown that pathological immunosuppression caused by the human immunodeficiency virus (HIV) increases oral cancer risk (144).

Tumour-infiltrating lymphocytes (TILs) are key players in the antitumoral response in OSCC (145). The intratumoral cytotoxic T cells are phenotypically inactive compared to the stromal cytotoxic T cells, probably as a consequence of the local immunosuppression which the tumour cells exert (146). Infiltration of both cytotoxic T cells and DCs in OSCC has shown to be an important positive prognostic factor for overall survival (139, 140, 147). These influxes also have an impact on tumour growth and the ability of tumour cells to cause metastasis (148). Al-Qathani et al. have reported that the number of regulatory T cells correlates with grade of

differentiation in OSCC (149), indicating the importance of the downregulating arm in immune response. The immune response in OSCC has been shown to be similar to that seen in other solid malignant tumours, as has the correlation with prognosis. An increased presence of DCs in the peritumoral stroma of OSCC is related to a better prognosis for the patients (147, 150).

Taken together, a complex immune network exists in OSCC as well as in other tumour forms. Interesting suggestions have been made for future perspectives and possible therapeutic approaches, against the background of this new knowledge of the microenvironment surrounding OSCC (151).

1.7 Potentially malignant oral disorders

Predicting how many OSCCs are preceded by a potentially malignant oral disorder (PMOD) is for obvious reasons hard. Petti and co-workers estimated that the annual oral cancer incidence rate due to leukoplakia (LPL) was between 6.2 and 29.1 per 100 000 (152). It has been reported that approximately 50% of OSCCs have a co-existing lesion (153, 154).

The WHO has defined a group of PMOD (134). Terminology and classification of oral mucosal diseases related to cancer risk have been under continuous debate in the scientific literature for decades. In 2005 a consensus conference of scientific experts agreed to define certain disorders as having a potential for malignant transformation: leukoplakia, erythroplakia, palatal lesions in reverse smokers, oral submucous fibrosis, actinic keratosis, oral lichen planus, discoid lupus erythematosus, dyskeratosis congenita, epidermolysis bullosa (134).

In a Western population LPL and OLP are the most prevalent PMODs, while erythroplakia has a prevalence below 0.1% (155). Tobacco-induced lesions such as oral submucous fibrosis and palatal lesions in reverse smokers are almost absent in the western countries, but are common in parts of Southeast Asia, where betel chewing and reversed smoking habits are more common. Actinic keratosis has a strong association with UV light exposure and age (156). Whether discoid lupus erythematosus possesses a malignant potential is unclear, but so far it is considered as a PMOD (157). Dyskeratosis congenita and epidermolysis bullosa are rare hereditary disorders, where oral lesions have been reported to evolve into OSCC (158, 159).

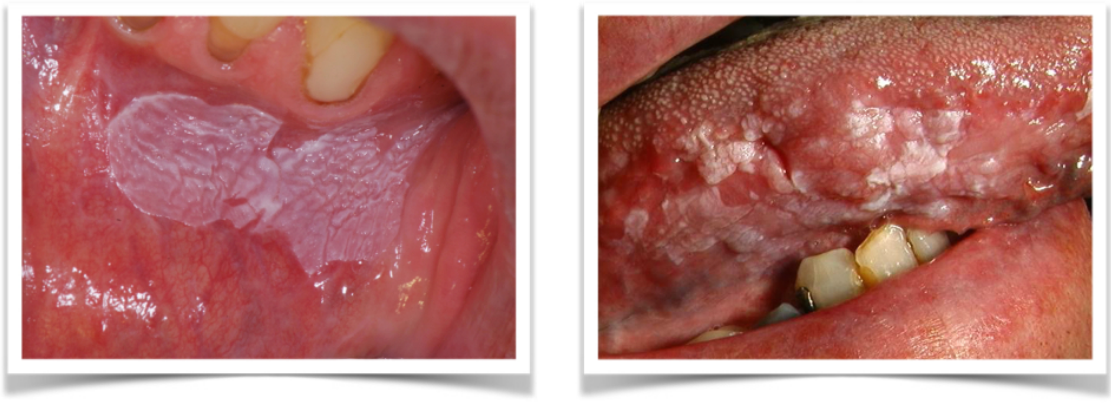
In the following chapter the main focus will be on LPL and OLP.

1.8 Leukoplakia

Leukoplakia (LPL) has been defined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion (160). In 2007

the WHO rephrased the definition as white plaques of questionable risk, (other) known diseases and disorders that carry no increased risk of cancers having been excluded (134). Thus the diagnosis is based on exclusion criteria rather than disease-specific characteristics. The definition has somewhat changed over the years from that of a premalignant lesion to that of a PMOD, to emphasize that the lesion does not always convert into cancer and that it is not only the lesion per se that has risk for malignant transformation. The cancer can also occur at a site distant from the LPL within the oral mucosa, and genetic aberrations can be detected in clinically healthy mucosa. This phenomenon is often referred to as 'field cancerization' (109, 161-163).

LPL is in most cases asymptomatic, and 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, and no other parameters are considered. Homogeneous LPLs are uniform white, flat, thin and well-demarcated lesions that can display a wrinkled surface (fig. 4A). Non-homogeneous LPLs are a more heterogeneous group, which can be further subdivided into different variants such as speckled, nodular and verrucous (fig. 4B). The non-homogeneous LPLs are most often diffuse in the demarcation to clinically healthy mucosa displaying a mix of erythematous and hyperkeratotic plaques. Verrucous LPL is characterized as a predominantly white lesion with a corrugated surface or projections of verrucous outgrowths. It can consist of one sole lesion or multiple lesions, the latter referred to as proliferative verrucous leukoplakia (PVL). This type of LPL cannot be distinguished clinically from a verrucous carcinoma.



A. B.

Figure 4. Homogeneous leukoplakia in the vestibular mucosa (A), non-homogeneus leukoplakia on the lateral border of the tongue

1.8.1 Prevalence

In contrast to cancer, PMODs are not registered in any national databases, making incidence figures hard to retrieve.

Prevalence data vary drastically, due to lack of strict inclusion criteria. Geographic regions, specific characteristics of the studied cohort and conditions under which the studies were conducted vary. Prevalence studies based on true idiopathic LPL are rare, and inclusion of subgroups such as tobacco-induced LPL, frictional keratosis, lichenoid contact reactions and other differential diagnoses influence study outcome.

Larger epidemiological studies have shown that the prevalence varies between 0.4% and 1,8% (153, 164-167). A meta-analysis of the prevalence showed that pooled estimation is around 2–3% worldwide (152). In a Swedish study conducted in 1987 with over 20 000 individuals examined, the prevalence of idiopathic LPL was 0.7%, while the prevalence of tobacco-associated lesions was estimated at approximately 3% (155).

LPLs are significantly more prevalent among males. A systematic review from Petti in 2003 reported that men have a three-fold increased risk of being affected compared to women (152).

1.8.2 Aetiology

The true aetiology of LPL is according to the definition unknown. However, genetic alterations in keratinocytes in epithelium are probably involved. Earlier studies have described gains and losses of chromosomal regions as well as loss of heterozygosity connected to malignant transformation (168-172).

However, associations have been suggested between some predisposing factors and LPL.

Tobacco and alcohol have been reported to be associated with LPL (173, 174).

Viral infections are of importance as co-factors in oncogenesis. An important causal relationship between LPL, OSCC and human papilloma virus (HPV) has been suggested (116).

Fungal infection occurs in LPL, rather as a consequence of the hyperkeratosis than as an aetiological factor. Wu et al. report that approximately 15% of LPLs are infected with *Candida albicans* (175). Fungal infection has been proposed as one aetiologic factor for malignant transformation. *C. albicans* isolated from LPL does produce acetaldehyde in carcinogenic levels, which may favour carcinogenesis (176).

1.8.3 Treatment

A wide range of suggested treatments for LPL have been proposed, but it is still a debate whether these modalities/interventions reduce the risk for malignant

transformation (177). Conventional surgery, laser surgery, photodynamic therapy, local and systemic medical treatment with both approved drugs and traditional medicine has been tested worldwide. No large scale randomized controlled clinical trials have been presented that evaluate the efficacy of surgery, and no evidence-based therapeutic recommendations have been issued for the above-mentioned treatments (178).

Excision, when possible, is considered to be the gold standard for managing LPL in the oral medicine, maxillofacial surgery and ENT community (179, 180). Lesions should be excised in toto. If excision is not feasible, multifocal incision biopsies ('mapping') to cover all possible reaction patterns are recommended. Several studies have reported that neither an active nor a passive approach in treating LPL could be found to affect the outcome (181-184). In contrast, others have reported that surgical intervention results in a risk reduction of malignant transformation (185, 186). Surgery is not seen as a curative treatment for LPL, since there still is a risk for recurrence after excision (187). However, to establish correct clinical and histopathological diagnoses, retrieval of tissue specimens is mandatory. It has been reported that there is a large risk of underestimation of the grade of dysplasia and OSCC when incisional biopsies are performed (188, 189). Holmstrup et al. showed that 8% of the cases with a primary histopathological diagnosis of no, slight, or moderate dysplasia contained OSCC when serial sectioning of tissue specimens was performed (189).

Since there is a lack of clear prognostic and diagnostic markers, the clinical evaluation of each lesion is fundamental. This makes the diagnostic process vulnerable, due to the inter- and intraobservational variability. Patients with LPL treated with excision surgery need to be controlled for recurrence in a prospective manner according to the risk.

1.8.4 Prognosis

A major risk factor after surgical treatment is recurrence of the excised lesion. Holmstrup et al. and Kuribayashi et al. have shown that approximately 15% of excised lesions recur (181, 187), while others have reported higher recurrence rates (184). Not surprisingly, inadequate resection margins seem to affect the recurrence rate (187).

The most severe risk of LPL is malignant transformation. It has been reported that approximately 7-17% of the LPL undergo malignant transformation (181, 184, 190-193). No reliable predicting factors foreseeing the outcome of LPL are presently at hand (194). Several studies have suggested potentially prognostic biomarkers to estimate the risk for malignant transformation (170, 172, 195, 196), but no conclusive evidence has been presented (169). Genetic aberrations such as DNA aneuploidy and loss of heterozygosity have been investigated and suggested as prognostic markers (171, 197). Despite more advanced and sophisticated techniques for genetic analyses, none of them are used routinely in clinical care.

Clinical and histopathological parameters are presently used to predict the outcome for the patient. The annual malignant transformation rate is estimated to be less than 1–3% in a Western population (184, 191, 198); therefore, regular checkups of patients at risk are important. Several clinical parameters, such as size and clinical classification, smoking habits and female gender, have been proposed as being associated with higher risk for malignant transformation (181, 183, 184, 191, 192). The degree of dysplasia also correlates with increased risk of malignant transformation (193, 197, 199); however, other researchers have not been able to confirm this (181, 184). The diagnostic and therapeutic challenges are that, despite all tools presently at hand, including molecular and genetic charting, no method with sufficient sensitivity and specificity is available. Creating a combined algorithm in which clinical, histopathological and genetic parameters are included is a challenge, but highly warranted in the clinical care of this patient group.

1.8.5 Immune response in leukoplakia

The immune response in LPL is not extensively studied. Previous groups have shown that an immune cell infiltrate was seen adjacent to the epithelium of LPL (200-202). Gannot et al showed that the influx of T helper, cytotoxic T cells and B cells were increased in LPL with moderate and severe dysplasia and OSCC compared to no and mild dysplasia (201).

1.9 Oral lichen planus

Lichenoid reaction is a group of disorders that present the same clinical and histopathological appearance; including oral lichen planus, lichenoid contact reaction, lichenoid drug eruptions and graft-versus-host disease (GVHD). It is not always possible to distinguish the disorders from each other without further investigating the patient's medical history or eliminating possible aetiological factors.

Lichen planus is an inflammatory disease of chronic nature, affecting skin and mucous membranes (203). The disease is thought to have an autoimmune cause, but no autoantigen, autoantibodies or autoreactive T cells have so far been identified (204, 205). In OLP the inflammatory pattern displays hallmarks of a T cell-mediated disease, with an influx of T cells creating a cellular infiltrate below the epithelial basement membrane (206). DCs are also present in the epithelium and in the inflammatory area (207).

OLP is subdivided according to the clinical appearance, displaying reticular, papular, plaque, erythematous and ulcerative presentations (fig.5). Reticular striations are considered to be pathognomonic for OLP (208).

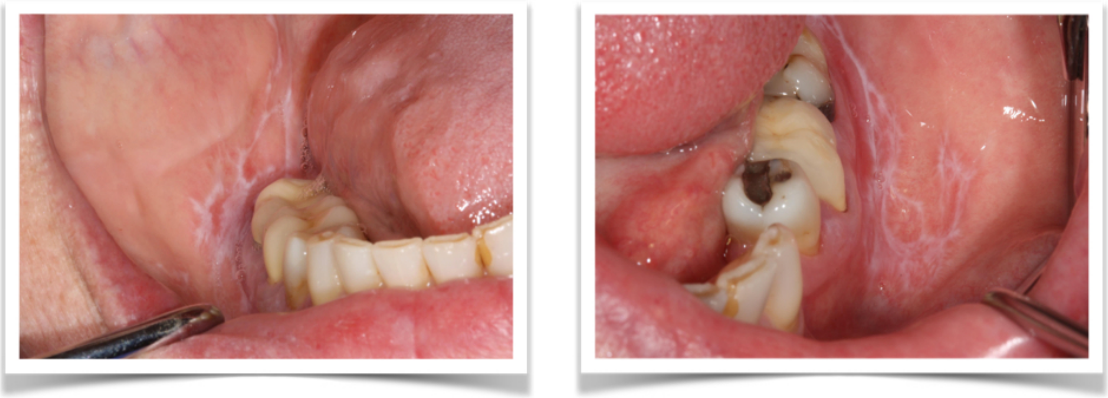


Figure 6. Bilateral reticular striations in the oral vestibular and buccal mucosa of an OLP patient.

The prevalence of OLP is thought to be around 0.5–2.5%, with a preponderance in women (155, 166, 209).

OLP has a premalignant potential, although the incidence for malignant transformation is lower than that of LPL. Studies of OLP patients show that approximately 0.5–1.5 % of the patients develop OSCC (84, 209-213). The state of inflammation persisting in OLP patients is in line with the current view of chronic inflammation as a risk factor in promoting cancer transformation (85, 214).

No causative cure for OLP is at hand, so treatment is symptomatic, and focus is on reducing inflammation, using local corticosteroids, or in severe cases, systemic immunosuppressive medication (215).

2. AIM

The general aim of this thesis was to characterize the immunological response in potentially malignant disorders and oral cancer and to relate the immunological response to prognosis regarding malignant transformation. We also wanted to address whether immunosuppression in a large cohort of patients predisposes for cancer in the oral cavity and lip.

2.1 Scientific questions

Paper I

- Do the presence and frequency of Langerhans cells, in relation to maturation stage, differ between healthy oral mucosa and oral lichen planus?

Paper II

- Do the presence and frequency of Langerhans cells and different subsets of T cells differ in leukoplakia with and without cell dysplasia, and from oral squamous cell carcinoma?
- Is there an interaction between T cells and Langerhans cells in patients with leukoplakia and oral squamous cell carcinoma?

Paper III

- Do the presence and frequency of T cells and Langerhans cells correlate with malignant transformation in LPL with cell dysplasia?

Paper IV

- Is there an increased risk of oral and lip cancer in a patient cohort treated with solid organ transplantation?
- Is the prognosis worse for patients with oral and lip cancer if they have undergone solid organ transplantation?

3. PATIENTS AND METHODS

Patients and methods used in this thesis are described in detail in the respective papers.

3.1 Patients

Paper I

Patients with a clinical diagnosis of oral lichen planus were included in this study. Following registration of anamnestic and clinical data, biopsies were performed to secure a histopathological diagnosis. Inclusion criteria were presence of a bilateral, lacelike network of slightly raised white striae with or without erythematous, ulcerative, bullous or plaque-like lesions, which are mandatory clinical features for OLP and a histopathological diagnosis of lichenoid reaction. Control specimens were obtained from patients with clinically healthy oral mucosa, undergoing surgery for removal of impacted wisdom teeth or installation of implants. Biopsies were collected in a consecutive, prospective manner, frozen within 30 min and stored at -80°C.

Papers II and III

Patients with clinical diagnoses of LPL (n = 23) or OSCC (n = 12) were included in study II. In paper III a total of 22 patient samples were collected; leukoplakia with malignant transformation (LPL-dys; n = 11) and leukoplakia without malignant transformation (LPL-ca; n = 11) within the follow-up period were included.

Data on clinical diagnoses were retrieved from patients' charts. Tissue specimens were collected from the archives of the Department of Oral Medicine and Pathology, Institute of Odontology, Sahlgrenska Academy, University of Gothenburg, Sweden.

Histopathological diagnoses were in paper II set by a senior pathologist, while two senior pathologists established diagnoses in paper III.

Presence of dysplasia is evaluated according to certain histological and cytological features (Table 1).

Table 1.

Architecture	Cytology
Irregular epithelial stratification	Abnormal variation in nuclear size
Loss of polarity of basal cells	Abnormal variation in nuclear shape
Basal cell hyperplasia	Abnormal variation in cell size
Drop-shaped rete ridges	Abnormal variation in cell shape
Increased number of mitotic figures	Increased nuclear size
Abnormally superficial mitoses	Atypical mitotic figures
Premature keratinization in single cells	Increased number and size of nucleoli
Keratin pearls within rete ridges	Hyperchromasia

Modified from Warnakulasuryia 2008 (216).

Grade of dysplasia in tissue specimens was recorded according to the WHO criteria (217). The grade of dysplasia is the architectural evaluation of the extent of the dysplastic features in the epithelium. Lesions with dysplastic features limited to the lower third are categorized as mild dysplasia. Moderate dysplasia is recognized when the dysplasia is extended into the lower two thirds of the epithelium, but if the cytological atypia is severe the lesion should be categorized as severe dysplasia.

For the lesion to be graded as severely dysplastic, the architectural and cytological atypia have to compose more than two thirds of the epithelium. When the cytological atypia is pronounced and extended into the whole epithelium without invading the connective tissue, it is categorized as cancer in situ (CIS), cancer within the epithelium.

Patients in paper III were in the LPL-dys group monitored for a median time period of 95 months (range, 21–159) without developing OSCC. Corresponding data for the LPL-ca group revealed a median period of 62 months (range, 7–192) before transformation into OSCC occurred.

Paper IV

The solid organ transplanted (SOT) patients analysed in this study were selected after a retrospective search in the register at the Transplant Institute at the Sahlgrenska University Hospital between 1 January 1965 and 31 December 2010 (n = 5755). Patients with more than one transplantation (n =1151) were excluded, due to a more complex medical situation, yielding 4604 patients for further analyses.

Also, patients with a history of oral and lip cancer before SOT and patients diagnosed within 30 days after SOT (n = 2) were excluded. Twelve patients with missing data were excluded from analysis. A cohort of 4590 SOT patients was then identified and subsequently analysed. The International Classification of Diseases (ICD) was used to identify anatomical sites of tumours. Diagnoses were identified by

ICD-7: oral cancer 141–144 (major salivary gland 142), tonsil 145 (data not shown), pharynx 146–148 (data not shown), and lip cancer 140.

A control group matched for sex and age was selected from the Cancer Registry, Region Västra Götaland, Sweden, and was used to compare the relative survival for SOT patients with that of non-SOT patients with oral and lip cancer. Oral cancer was diagnosed in 1849 non-SOT patients between 1975 and 2010, while 881 non-SOT patients were diagnosed with lip cancer during the same period.

3.2 Methods

3.2.1 Overview of markers for cell subset analyses

Dendritic cells

CD1a is a molecule belonging to the highly conserved group of CD1 transmembrane glycoproteins. The human CD1 family is structurally related to the major histocompatibility complex (MHC) proteins. It can form heterodimers with β2-microglobulin. It is exclusively expressed by Langerhans cells and thymocytes.

CD207 (Langerin) is a type II transmembrane protein. Its carbohydrate-recognition domain binds various monosaccharides, including fucose, mannose and N-acetylglucosamine in a calcium-dependent manner. CD207 is an endocytic receptor that functions as a potent inducer of Birbeck granules formation by the zipping and superimposition of cell membranes. The CD207 protein is expressed on immature Langerhans cells, cells that recently have reached the epithelial compartment and are not yet expressing the CD207 or cells migrating toward the connective tissue, presenting with a downregulation of the CD207 expression. Langerin expression on LCs is associated with antigen uptake, high endocytotic capacity, and low T cell stimulatory capacity.

CD83 is a member of the immunoglobulin superfamily and consists of a glycoprotein with a single extracellular Ig-like domain, a transmembrane region and a cytoplasmic domain. Human CD83, together with CD40, CD80, CD86 and human leucocyte antigen (HLA) A, B, C and DP, DG and DR class I and II, represents an important marker for the maturation of dendritic cells. CD83 molecules can also be expressed by B cells and T cells. CD83 is one of the well-known maturation markers for human DC, since it was first detected on human blood DC by Zhou and Tedder in 1995 (218). CD83 has been shown to be expressed on activated and mature human DC and is markedly upregulated together with co-stimulatory molecules CD80 and CD86 during DC maturation.

T cells

CD3 is a marker specific for the T cell lineage and a member of the immunoglobulin superfamily. CD3 expression is specific for all T cells, regardless of subtype. CD3 is a

complex of domains, part of the T cell receptor (TCR) complex. CD3 molecules generate activation signals to intracellular activation pathways when antigen binds to the peptide binding groove of the TCR.

CD4 is a glycoprotein and cell surface molecule expressed on T helper cells, which often are designated as CD4-positive T cells. CD4 is a member of the immunoglobulin superfamily. One of the four domains of CD4 interacts with MHC class II, and CD4 is an important co-receptor for the TCR to increase the response of MHC class II binding.
CD4+ T cells activate and stimulate the proliferation of cytotoxic T cells. They may also regulate B cell activity and promote the bactericidal activity of macrophages by producing IFN-γ. All subsets of T helper cells (Th1, Th2, Th17, Treg) express CD4, and it is not possible to differentiate between them by expression of CD4. CD4 molecules are also found on DCs and macrophages.

CD8 is a surface marker of cytotoxic/suppressor T cells and a member of immunoglobulin superfamily. CD8 is a transmembrane glycoprotein that consists of two domains. CD8 molecules also belong to the immunoglobulin superfamily. It can bind to MHC class I molecules and serve as a co-receptor for the T cell receptor. CD8 alpha domain can also be expressed on DCs and NK cells.

Cell cycle-associated proteins

Ki67 is a nuclear protein associated with proliferation of cells. This marker is present in all stages of cell division, G1, S and G2, except G0 phase. Ki-67 has been shown useful as a prognostic and predictive marker in various types of malignancies.

p53 is a tumour suppressor protein encoded by the TP53 gene. This protein is expressed and regulates the transition from G1 to S phase in cell cycle division. P53 is the key mediator of cell cycle arrest and induction of DNA repair or apoptosis as a response to cellular stress (ref). p53 is often referred to as the 'guardian of the genome'. Its prime function is to put cells with aberrant DNA into apoptosis and thereby prevent malignant transformation. P53 is mutated in more than 50% of all cancers.

3.2.2 Immunohistochemistry and confocal laser scanning microscopy

Paper I

The following monoclonal antibodies (mAbs) were used: anti-CD1a (NA1/34; Serotec Ltd, Oxford, UK), anti-CD83 (HB15A17.11; Serotec Ltd, Oxford, UK) and anti-Langerin (12D6; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK).

Sections from tonsils served as positive controls. Immunohistochemical stainings were done with isotype-matched irrelevant primary antibodies mouse

immunoglobulin G2a (IgG2a) Kappa (UPC10); Sigma, St. Louis, MO, USA) and mouse IgG2b (No. X0931; DAKO A/S, Glostrup, Denmark) as negative controls.

Paper II

The following mAbs were used: anti-CD1a (Clone 010; Dako A/S, Glostrup, Denmark), anti-CD207/Langerin (12D6; Abcam, Cambridge, UK), anti-CD3 (LN10; Novocastra, Newcastle, UK), anti-CD4 (1F6; Novocastra), anti-CD8 (4B11; Novocastra), Ki67 (MIB-1; Dako A/S). Sections from tonsils served as positive controls, while omission of primary antibodies served as negative controls.

Confocal laser scanning microscopy

The following primary mAbs were used: anti-CD1a (Clone 010; Dako A/S), anti-CD3 (SP7; Abcam) and anti-CD8 (SP16; Abcam).

As secondary mAB/fluorochromes Alexa Fluor 488 (Invitrogen, Eugene, OR, USA) and Alexa Fluor 647 (Invitrogen) were used. Sections were then mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Sections from tonsils served as positive controls, and omission of primary antibodies served as negative controls.

Paper III

The following mAbs were used: anti-CD3 (LN10; Novocastra), anti-CD1a (010; Dako A/S), anti-p53 (D07; DAKO A/S) and anti-Ki-67 (MIB-1; Dako A/S). Sections from tonsils served as positive controls, while omission of primary antibodies served as negative controls.

3.2.3 Quantification

In papers I, II and III quantitative analysis was performed on two sections per biopsy and two compartments per biopsy. The epithelium and the connective tissue were selected for quantitative analysis separately. Within the epithelium and connective tissue, positively stained nucleated cells were counted as positive.

In papers I and III counting of cells was done manually, while in paper II the sections were analysed with computer software BioPix iQ 2.0 (BioPix, Gothenburg, Sweden), calibrating the number of cells according to the stained area within a certain limit of hue, saturation, brightness and size of the positive cells.

The area was calculated with computer software Easy Image 3000 (Tekno Optik AB, Gothenburg, Sweden) in paper I, BioPix iQ 2.0 (BioPix, Gothenburg, Sweden) in paper II and CellSense (CellSense; Olympus, Hamburg, Germany) in paper III.

All cell counting was performed blinded by one observer, after calibration with two other observers.

3.2.4 Statistical analyses

Papers I and III

Analysis of differences between groups was performed using the Mann–Whitney U-test. A p-value < 0.05 was considered statistically significant.

Paper II

Analyses of differences between groups were carried out by the Kruskal–Wallis test, followed by the Mann–Whitney U-test as a post hoc test, utilizing the statistical software SPSS v17 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered as a significant difference.

Paper IV

Relative risk of cancer in SOT patients compared to the general population was expressed as standardized incidence ratio (SIR). A 95% confidence interval (CI) and a p-value <0.05 was considered statistically significant. Incidence rates in the Swedish population, by gender, 5-year age group and calendar year, were used to calculate the expected number of cancer cases among the SOT patients. A Poisson regression model was used to compare the relative survival between SOT patients and non-SOT patients with oral and lip cancer.

4. RESULTS

Do the presence and frequency of Langerhans cells, in relation to maturation stage, differ between healthy oral mucosa and oral lichen planus?

CD1a-, Langerin- and CD83-positive cells were detected in both epithelium and connective tissue in sections of healthy oral mucosa and OLP lesions, although in much higher numbers in epithelium than in connective tissue. Both epithelium and connective tissue of OLP lesions had a significantly higher number of CD1a-positive LCs ($p < 0.001$ and $p < 0.001$, respectively) and Langerin-positive cells ($p < 0.05$ and $p < 0.05$, respectively) per area unit. CD83-positive cells were significantly more abundant in the connective tissue in OLP compared to healthy mucosa ($p < 0.001$), but no significant difference could be detected in the epithelium ($p > 0.05$). CD1a-, Langerin- and CD83-positive cells were sometimes located in clusters of cells with lymphocyte morphology.

Do the presence and frequency of Langerhans cells and different subsets of T cells differ in leukoplakia with or without cell dysplasia, and from oral squamous cell carcinoma?

Regarding the CD1a-positive LCs in epithelium, there were no statistically significant differences between LPL with dysplasia, LPL without dysplasia and OSCC ($p = 0.238$; $p = 0.067$, respectively). In the connective tissue CD1a-positive LCs were found in higher numbers in LPL with dysplasia compared to LPL without dysplasia ($p = 0.007$).

The number of Langerin-positive LCs per area unit did not differ significantly either in epithelium or connective tissue between LPLs with or without dysplasia and OSCC ($p = 0.374$ and $p = 0.728$, respectively).

The number of CD3-positive T cells per area unit was significantly higher in connective tissue and in epithelium of LPLs with dysplasia compared to LPLs without dysplasia ($p = 0.002$ and $p = 0.008$, respectively). CD3-positive cells were significantly more abundant in OSCC in comparison with LPLs with dysplasia ($p = 0.0001$).

There were no significant differences in the number of CD4-positive cells per area unit between LPLs with or without dysplasia in either epithelium or connective tissue ($p = 0.636$ and $p = 0.783$, respectively). In contrast, OSCC showed significantly higher numbers of CD4-positive cells in epithelium in comparison with LPLs with dysplasia ($p = 0.0001$).

When comparing the epithelial compartments of LPLs without dysplasia with LPLs

with dysplasia, the number of CD8-positive T cells did not differ significantly (p = 0.076). Interestingly, there were significantly more CD8-positive cells per area unit in the connective tissue in LPLs with dysplasia than in LPLs without dysplasia (p = 0.042). OSCC tissue specimens contained significantly more CD8-positive cells than the epithelial compartment of LPL with dysplasia (p = 0.006).

Ki-67-positive cells were present in all three groups. There were no statistical differences between the groups of LPL with or without dysplasia, although there were differences in distribution and morphology of the Ki-67-positive cells. Not surprisingly, there were significantly more Ki-67-positive cells in OSCC.

Is there an interaction between T cells and Langerhans cells in patients with leukoplakia and oral squamous cell carcinoma?

With confocal laser scanning microscopy interaction were detected in a three-dimensional manner between CD1a-positive LCs and CD3- and CD8-positive T cells in all three groups. An increase in interaction, both in the epithelium and the connective tissue, could be seen in LPL with dysplasia and OSCC compared to LPL without dysplasia.

Do the presence and frequency of T cells and Langerhans cells correlate with malignant transformation in LPL with cell dysplasia?

The number of CD3-positive cells per area unit was significantly higher in the group of LPL without malignant transformation compared to the group with LPL with malignant transformation, in both the epithelium and the connective tissue (p = 0.016 and p = 0.016, respectively).

Regarding the number of CD1a-positive LCs in either the epithelium or connective tissue, no significant differences between the two groups were found (p = 0.630 and p = 0.248, respectively).

Both Ki-67 and p53 were present on cells in both epithelium and connective tissue in LPL-dys and LPL-ca, although in higher amounts in the epithelium than in the connective tissue, where they were rarely seen. No statistically significant difference was found when comparing the distribution of Ki-67-positive cells and p53-positive cells(p = 0.277 and p = 0.277, respectively) in epithelium between the two groups.

Is there an increased risk of oral and lip cancer in a patient cohort treated with solid organ transplantation?

Seventeen oral cancers and 34 lip cancers were registered in the cohort of 4590 single-transplanted patients. The expected number of oral cancers in the cohort was

0.7, and 17 patients with oral cancer were found, yielding a standard incidence ratio (SIR) of 6.3, with a confidence interval (CI) between 3.76 and 10.1. The expected number for lip cancer was 0.8, but 34 patients were identified with that diagnosis, yielding an SIR of 43.7 with a CI between 30.3 and 61.1.

Statistical comparisons show significantly increased risks for oral ($p < 0.02$) and lip cancer ($p < 0.001$) in solid organ transplantation (SOT) patients compared with what was expected to occur in the Swedish population.

Is the prognosis worse for patients with oral and lip cancer if they have undergone solid organ transplantation?

The five-year relative survival was used for comparison between SOT patients with oral and lip cancer and oral and lip cancer patients without any previous history of SOT. For oral cancer patients with SOT the five-year relative survival was 30.8% (CI 7.7–60.2%), and for patients with oral cancer without SOT it was 60.1% (CI 57.6–62.6%). Outcome comparison between the groups did not show a significant difference in relative survival ($p = 0.14$). The five-year relative survival for single SOT patients with lip cancer was 66.0% (CI 44.7–82.2%) and for lip cancer without SOT 94.2% (CI 91.1–96.9%), which results in a significantly reduced relative survival for SOT patients with lip cancer ($p < 0.001$) (fig. 6.)

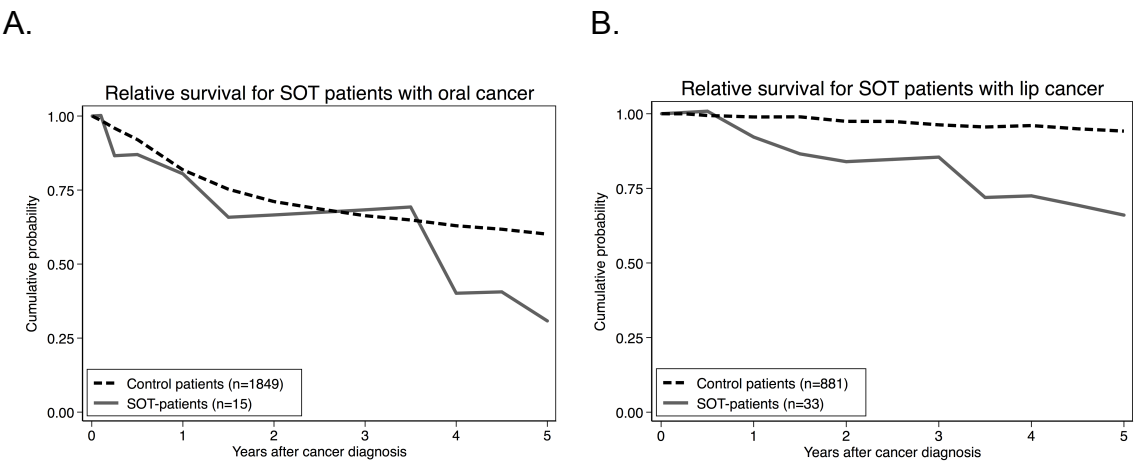


Figure 6. Relative survival for (A) SOT patients with oral cancer (solid line) and control patients with oral cancer (dotted line), and (B) for SOT patients with lip cancer (solid line) and control patients with lip cancer (dotted line).

5. GENERAL DISCUSSION

Tumour immunosurveillance pathways have been extensively studied in cancer, including oral cancer, for many years, but less knowledge exists on potentially malignant oral disorders (PMODs) like leukoplakia (LPL) and oral lichen planus (OLP). Immunosurveillance in PMODs probably is of importance in preventing cancer transformation. In the current thesis, an attempt has been made not only to characterize and relate the immune response in PMODs and oral cancer but also to study the effect of long-standing immunosuppression on cancer development in a large patient cohort with an immunodeficiency.

In paper I we addressed the presence of Langerhans cells at different stages of differentiation and maturation. Recognition of both foreign and non-self antigens is part of the normal function of the immune system. However, in autoimmune disorders there are malfunctions in the immune system recognizing self-antigens as non-self, which mounts a response to self-antigens (219).

Lichen planus (LP) affects skin and mucous membranes and clinically manifests as an inflammation, while at the cellular level a massive infiltration of T cells is seen (206, 220, 221). LP has been suggested to be an autoimmune disease, although no definite proofs have been provided (204, 205). T cell activation is dependent on professional antigen-presenting cells such as DCs, macrophages and B cells.

In the first study in this thesis LCs and T cells were identified in increased numbers in oral mucosa of patients with OLP compared with healthy patients (222). Another main observation in this study was that LCs were found clustering with subepithelial lymphocytes. Since the infiltration of T cells in OLP is dominated by T cells (206, 221), it is highly likely that subepithelial lymphocytes interspaced by LCs seen in the study are T cells. In addition, DCs in clusters expressed CD83 molecules, indicating a mature phenotype suitable for antigen presentation (223). The CD83 molecule is not specific for the dendritic cell lineage; it can also be expressed by macrophages and B cells under some pathological conditions (224, 225). Macrophages are present in OLP but are not the major antigen-presenting cell (226), and B cells are not present to any major extent in OLP (206, 227). Thus, a reasonable explanation is that clustering areas are sites of local autoantigen presentation of DCs to T cells.

In paper II the main observation was that the influx of CD8-positive T cells and CD1a-positive Langerhans cells were significantly increased in the connective tissue in LPL with dysplasia compared with LPL without dysplasia. CD3-positive T cells were more abundant in LPL with dysplasia than in LPL without dysplasia, both in connective tissue and epithelium (228). This finding gives support to the immunosurveillance theory, regarding T cells ability to sense dysplastic cells.

LCs seem to be recruited to the connective tissue in LPL with dysplasia. The number of CD1a-positive but not Langerin-positive LCs were significantly increased in the connective tissue of LPL with dysplasia in comparison with non-dysplastic LPL. The number of immature LCs does not seem to differ between LPL with or without dysplasia, and OSCC, indicating that recruitment of mature phenotypes of LCs is ongoing in dysplastic LPL. In actinic keratosis and cutaneous cell carcinoma a reduced number of immature LCs versus healthy skin has been observed, which supports the findings in paper II (229).

An interesting observation in this study was a shift in the ratio of CD4- and CD8-expressing T cells between dysplastic versus non-dysplastic lesions. A few groups have reported a similar shift in the ratio between CD4- and CD8-expressing T cells in presence of dysplasia(200, 230), but no other reports have so far observed this shift. A possible explanation is that with increasing dysplasia, CD8-positive cytotoxic T cells are recruited to the site of challenge and mount an antitumoral defence. A prerequisite may be that TAAs have been presented by DCs to T cells in an initial step. In the study a close interaction of CD1a-positive LCs and CD8-positive T cells in LPL with dysplasia and OSCC could be confirmed in confocal laser scanning microscopy analyses, which gives support to an ongoing interaction between LCs and T cells.

A huge variation in the count of CD8-positive T cells was seen both in LPL with dysplasia and OSCC. An explanation, in accordance with the concept of immunoediting (7), is that a difference in immunogenicity of the dysplastic and cancer cells is reflected by a variation in influx of CD8-positive T cells.

In patients with OSCC the tumours displayed a huge influx of CD3-positive T cells and CD4- and CD8-positive T cells. A thousand-fold increase in lymphocytes was seen in OSCC compared to epithelium of LPL. This finding could be of interest as an immunological diagnostic indicator of OSCC development in LPL. The influx of LCs and T cells varied greatly in the OSCC group. This has been shown in other reports (140, 147, 149) to be of prognostic value for the OSCC, although it has not been investigated in this thesis. This could indicate that the cancer itself is more or less prone to induce an immune response. In this study the regulatory subset of T cells was not assessed, but most probably differences are to be expected in both LPL and OSCC. Presence of Tregs signals a poor prognosis not only in OSCC but also in other malignant diseases (56, 57, 140, 149), and could therefore be of interest even in LPL as a prognostic marker.

To conclude the findings in paper II, evidence of an immunosurveillance activity in LPL and OSCC are presented. A reasonable scientific question arising from the results is whether LPL with dysplasia that transforms into OSCC presents a different immunosurveillance pattern from that of non-cancer-transforming LPL.

In paper III we wanted to differentiate the LPL group with dysplasia with regard to the clinical outcome of the patient.

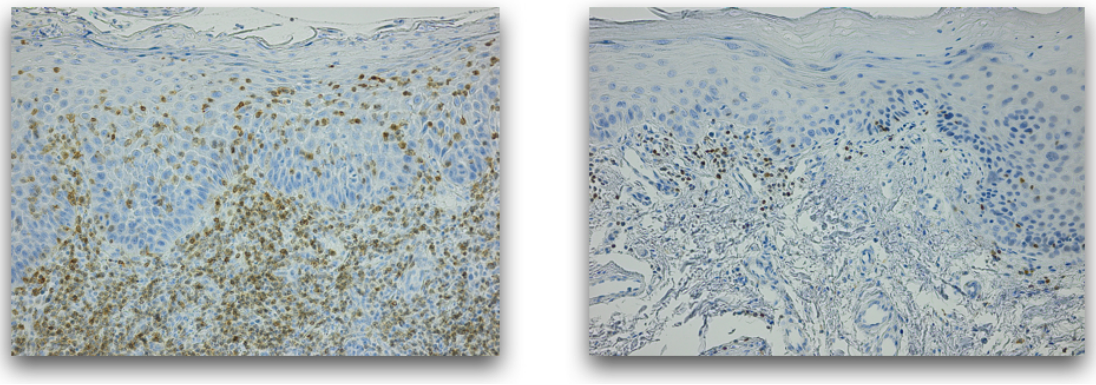
Cell dysplasia in LPL obviously causes an immune response, reflected in an influx of T cells. The migration of T cells to areas with epithelial cells showing an aberrant morphology can be interpreted as an immunosurveillance response that primarily aiming at eliminating cells that pose a risk for malignant transformation. Whether there is success in this process or failure decides the future. Either a state of equilibrium is achieved, in accordance with Dunn's immunoediting theory (7), or in a worst case scenario there is an escape of cells that have become cancer cells, and a tumour has formed. In a situation of cell dysplasia in patients with LPL it is reasonable to hypothesize that the influx of T cells in LPL with dysplasia seen in paper II also could influence whether LPLs will turn into cancer or not. In study III two groups of patients with LPL dysplasia were compared according to outcome during the follow-up period. The follow-up period can be questioned in the LPL group without malignant transformation (LPL-dys). Most LPL undergo malignant transformation with a mean of 2.5-7.5 years (181, 184, 191, 197). In our group of patients with malignant transformation (LPL-ca) we reported a median of 62 months with quite a wide range between 7 and 192 months. It is impossible to foresee how long one should have to follow up the patient to be certain that the lesion will not develop into cancer. The fact that it is impossible to know the actual time for onset of the lesion makes it hard estimate the time the patient has been living with the diagnosis before malignant transformation.

Outcome was defined as a registered cancer diagnosis or no cancer diagnosis during the observation period. The retrospective retrieval of clinical data together with histopathological enumeration of T cells revealed that the degree of T cell recruitment to epithelium and connective tissue in LPL with dysplasia that did not turn into OSCC was higher than in the LPL group that followed a path to malignant transformation. This result provides further evidence of the importance of immunosurveillance in LPL. Of course, in this small historical patient material it was not possible to control for tobacco habits and other factors of interest such as medical history. In the continued research, not only these factors will be controlled for but also assessment of T cell subsets, DCs subsets and other cells of the peritumoral stroma such as myelo-derived suppressor cells and tumour-associated macrophages with immunosuppressive functions.

Postulating that T cell activation and recruitment in response to cell dysplasia is dependent on DCs presenting TAAs to T cells, an increased number of LCs could be foreseen. However, no such statistically proven difference between cancer transforming and non-transforming LPL could be found in paper III. There are several possible explanations. A quite feasible explanation can be sought in the function of DCs. DCs are very potent inducers of naive T cell activation both in vivo and in vitro (231). The number of LCs found in the group of patients with cancer-transforming LPL may have been enough to induce a T cell response and subsequent influx into tissue areas in need of immunosurveillance. On the other hand, it may be that the LCs in the LPL-ca group had a defective antigen-presenting capacity, as seen

in several cancers (232-234), and in that way did not generate sufficient T cell response. Another explanation could be that the study only assessed one subset of DCs, namely LCs. In the connective tissue in mucosal membranes and skin several subsets of DCs reside. Apart from LCs, other cells with antigen-presenting capacity, such as monocyte-derived DCs, and macrophages are found (235). A possibility to be considered is that one of these subsets is a prime initiator or may counteract T cell response.

An important observation made in the study presented in paper III was that in some of the patients a clear recruitment of T cells into the dysplastic epithelium and into the corresponding connective tissue was seen (fig. 7A). This can be an indication of a higher grade of immunogenicity of the dysplastic cells, while other patients with marked dysplastic epithelium had only minor influx of T cells in epithelium or corresponding connective tissue (fig. 7B), indicating a more immunoedited phenotype. This is in accordance with the immunoediting theory (7).

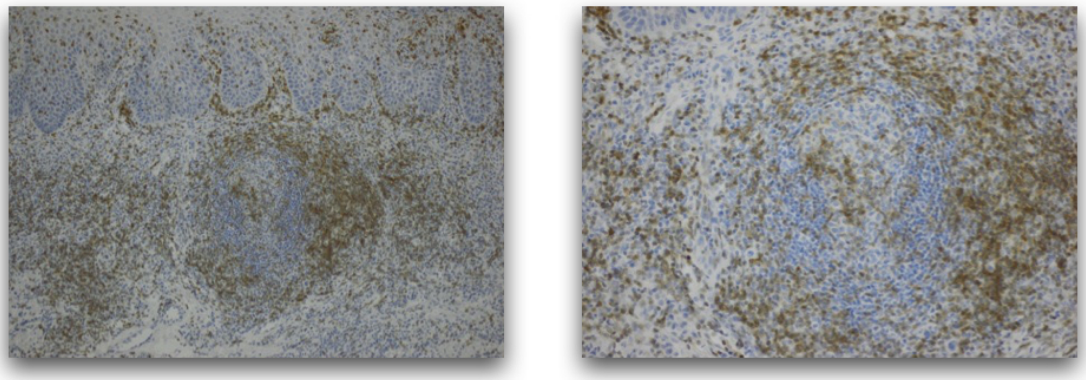


A. B.

Figure 7. LPL with dysplasia with major immune response (A), LPL with dysplasia with minor immune response (B) CD3 positive cells.

Another interesting observation was that clustering of LCs and lymphocytes could be seen in the connective tissue. The clustering areas were located in the connective tissue and clearly separated from epithelium (fig. 8). The clusters displayed similarities with what was observed in OLP lesions (222). Previously, tertiary lymphoid structures (TLSs) with structural and cellular similarities of secondary lymphoid organs exist in connection with various types of cancer (21, 236, 237). This may also be the case in PMODs such as LPL and OLP.

Presence of TLS seems to predict prognosis. Wirsing et al. reported that in oral cancer TLSs were found in one fourth of the cases (238). This group also presented data indicating that TLSs were found in larger amounts in small tumours. It could be speculated that in large tumours the immune system's defence has succumbed to the cancer cells and a state of escape prevails.



A. B.

Figure 8. Cluster formations resembling TLS, in connective tissue of LPL from paper III, magnification x100 (A) and magnification x200 (B) CD3-positive T cells stained brown.

Increase in cell proliferation inversely correlates with prognosis in patients with head and neck tumours (239). Ki67 is a marker of proliferation that has been used for decades in clinics and in science. Kovesi et al. and Vered et al. have reported that in LPL Ki67 expression correlates with degree of dysplasia and cancer transformation (240, 241). In contrast, a report from Torres-Rendon et al. could not confirm this correlation (242). Nor in the study presented in paper II comparing patients with LPL showing benign hyperkeratosis with LPL with dysplasia or in this study could a correlation be confirmed. Hence, a reasonable conclusion is that, although a valuable marker in established tumours, Ki67 expression is of less importance in PMODs like LPL.

P53 expression did not differ when comparing patients with cancer-transforming and non-transforming LPL patients in study III. This is in accordance with what Lee and co-workers have reported (243). A correlation between p53 expression and dysplasia has also been reported to exist (244). Nasser et al. concluded that Ki67 and p53 separately have a poor predictive value when used separately, but when used together and in combination with expression of $p16^{INK4a}$, the predictive value increases (245).

So far, most studies have used immunostaining of p53 as an enumeration tool. However, in cancer cells a mutant form of p53 occurs (246). The normal function of p53 is, in response to different types of stress, to become activated and govern several key biological activities in cell nuclei, such as DNA repair, cell cycle arrest and apoptosis. This normally functioning wild-type p53 is of outmost importance in guarding the genome (246). If mutant forms of p53 emerge as a result of mutations in the TP53 gene this process is sabotaged. The methodology used in paper III may not be sufficient to discriminate between wild-type and mutant p53. Thus, it could be speculated whether the p53-positive cells detected in the group of patients that contracted OSCC in fact had mutated p53, while patients in the non-cancer-

transforming group had wild-type, normally functioning p53 which protected from cancer development. Graveland and co-workers reported that they could see a correlation between LPL that did transform into OSCC and aberrations of the TP53 gene when sequencing the gene, and this correlation was not seen when the expression of p53 was detected with immunohistochemistry (196).

The findings in paper III regarding Ki67 and p53, reinforces the present knowledge that separately they are not useful markers of malignant transformation, but they may be so when combined.

The observations reported in this study indicate that immunosurveillance activity can be of importance for malignant transformation in LPL with cell dysplasia.

In papers I–III patients with no known immunodeficiencies were studied. Inherited or pharmacologically induced immunodeficiencies are known to predispose for cancer. Thus, a scientific question can be raised whether an impaired immunosurveillance against cancer cells give rise to an increase in oral cancer.

In the last paper of this thesis a large cohort of patients with longstanding immunosuppression were investigated.

The loss of immunosurveillance most certainly plays an important role in development of post-transplant malignancies. Clinical evidence that the immune system plays an important role regarding the progression and the prognosis of malignant tumours has been presented (19, 20, 140, 202).

Long-term downregulation of the immune system is a challenge in transplanted patients. Patients treated either by solid organ transplantation (SOT) or allogeneic hematopoietic stem cell transplantation are at an increased risk for cancer (95, 143). Multifactorial mechanisms probably interact in promoting oncogenesis. A primary cause in SOT patients, may be the long-lasting pharmacological immunosuppression resulting in deficient immunosurveillance over time (247). The hazard of cancer cells evading elimination by the immune system increases, and also opens pathways whereby viruses with oncogenic capability, like human papilloma virus (HPV) and Epstein–Barr virus (EBV) can cause infection-related tumours (248, 249). The immunodeficiency results in opening pathways for oncovirus-driven malignancies such as lymphomas related to EBV in transplanted patients, called post-transplant lymphoproliferative disorders (250). Oncoviruses that are normally present in humans transform normal cells into cancer cells continuously, but these cancer cells are eliminated in healthy, immunocompetent individuals in accordance with the concept of immunosurveillance (251). In a state of immunosuppression, virally tumour-transformed cells escape immunosurveillance, with cancer as a consequence.

Pharmacological immunosuppression per se may increase risk for cancer. The main immunosuppressive drugs used for decades, cyclosporin A and azathioprine, both have the capability to affect DNA repair mechanisms (252, 253); thus, a possible

oncogenic potential could be suspected.

The finding of a significantly increased prevalence for both lip and oral cancer in SOT patients compared to non-SOT patients, in the fourth paper are in accordance with those of previous groups (92, 95, 254, 255).

There was a clear switch in the ratio between the localizations of the investigated cancers. In a Nordic population lip cancer constitutes 27% of oral, salivary gland and lip cancers (101). There was a clearly skewed incidence of lip cancer in our SOT cohort, where this cancer constituted 66% of all oral, salivary gland and lip cancers. UV exposure of the skin reduces the antigen-presenting capacity of skin LCs (256). In addition, a subset of regulatory T cells expands after UV irradiation (257), which leads to an increased number of T regulatory cells and a decreased number of effector T cells in the skin (258). Thus, a shift in balance from effector T cells to Tregs occurs, which may cause a loss in immunosurveillance, promoting escape of cancer cells. Another important consequence of UV irradiation is that the number of LCs in skin is reduced (259). Hence, the skin loses scavenger LCs, which leads to reduced capacity of uptake both of endogenous and exogenous antigens.

Thus, the lip is subjected to a two-way hit from UV irradiation and pharmacological immunosuppression, while the oral cavity only suffers a one-way hit from immunosuppressive drugs. Thus, the lip is at higher risk for cancer than the oral cavity.

Several groups have reported an increased prevalence of LPL in the lips in SOT patients compared with a control group (254, 255). These LPL contained dysplastic features in a higher prevalence, than in LPL without any previous SOT, indicating that LPL in patients with long-term immunosuppression are of a more aggressive type.

The overall survival in SOT patients contracting cancer is an important issue to address. The results in paper IV show that SOT patients who contract lip cancer have a relative survival significantly less than that of a matched control group. This has not been reported before, but others have reported a decrease in survival for SOT patients with other malignancies (260-262), and this seems to correlate with lip cancer. Although we could not find a statistically significant difference in the overall survival between the oral cancer groups, there were a clear trend; it was probably due to lack of power that no statistical significance could be detected.

In this study, patient data regarding TNM classification were not retrievable on patients transplanted before 2008, as this classification was not recorded in the Swedish Cancer Register until 2008. TNM classification has been used for decades as a predictor for disease outcome and would have been of great interest to investigate. SOT patients undergo lifelong medical checkups, and it is reasonable to assume that especially lip, but also oral, cancers are diagnosed earlier in an SOT cohort than in the general population. Thus, if SOT and control patients were

matched for TNM stage the outcome would probably be even worse for the SOT patients.

In all likelihood a multifactorial scenario explains the reduced survival observed in SOT patients with cancer. A main reason most probably be attributed to long-standing immunosuppression, as several groups have pointed out (94, 260), but lifestyle factors before SOT can also be an explanation. Use of tobacco, alcohol and other drugs causes organ damage that can eventually lead to transplantation, but the physiological load caused by the aforementioned factors predisposes for cancer per se.

In this study the observation period began in 1965 and ended in 2010. During this period immunosuppressive regimens changed and new immunosuppressive drugs were introduced (91). How these time-dependent changes affect future disease panorama has to be addressed by future studies.

Another important factor is the changing panorama of infection-related cancers. There has been an increased incidence of HPV-related oropharyngeal cancer during the past twenty years, while incidence of oral cancer shows a more non-conclusive correlation with HPV (116). In this study the few observations of oropharyngeal cancers did not allow any conclusions.

In summary, SOT patients contract oral and lip cancer in increased numbers in comparison with a non-SOT control cohort. A common denominator for patients in the SOT group is long-standing pharmacological immunosuppression, which most like results in an impaired immunosurveillance against cancer cells.

Transplantation of solid organs has become an established treatment in end-stage diseases for many patients. Survival rates are increasing, and transplantation brings life back to many patients. However, tumour diseases pose a major threat to the overall survival in SOT patients. That is why it is important to rely on not only the power of immunosurveillance but also the power of close clinical surveillance of SOT patients.

6. CONCLUDING REMARKS

The concept of immunoediting originally proposed by Dunn et al., with phases of elimination, equilibrium and escape (7), is well in line with the findings in this thesis.

It can be speculated that leukoplakia represent a pattern of reaction in the oral mucosa, indicating that a state of equilibrium is reached where there is a counterattack from the immune system against potentially malignant cells. When the counterbalance is disrupted for various reasons, the state of equilibrium is lost and a malignant transformation occurs.

The new knowledge arising from this thesis needs to be brought into the clinical care of patients who have, or are at risk for, cancer. The goal for health care providers and researchers in this field is to minimize morbidity and decrease mortality of patients with PMODs and OSCC. It can be speculated that the immune response, both in PMODs and in OSCC, could in the future be used as a prognostic marker for malignant transformation and prognosis, and also used in immunologically based therapies.

Papers I–III consist of small samples, making it hard to draw strong conclusions, but considered as starting points, they confirm the hypothesis of immunosurveillance. Further large-scale prospective studies are needed to verify the results in this thesis. However, paper IV consists of a large cohort of patients with longstanding immunosuppression and where an increase in oral and lip cancer was detected, which lends support to the immunosurveillance hypothesis.

The oral cavity is a very important location for many reasons. The fact that it is easily accessible for inspection and sampling without any major risk to the patient or the need for advanced techniques makes it suitable for studying different cellular mechanisms. PMODs in the mucosa are easy to detect and clearly visible to the care provider. Thus, PMODs provide us with an excellent model for studying the malignant transformation process and the development of cancer.

Figure 9 is a concluding illustration of how the concept of Immunosurveillance could be interpreted in LPL.

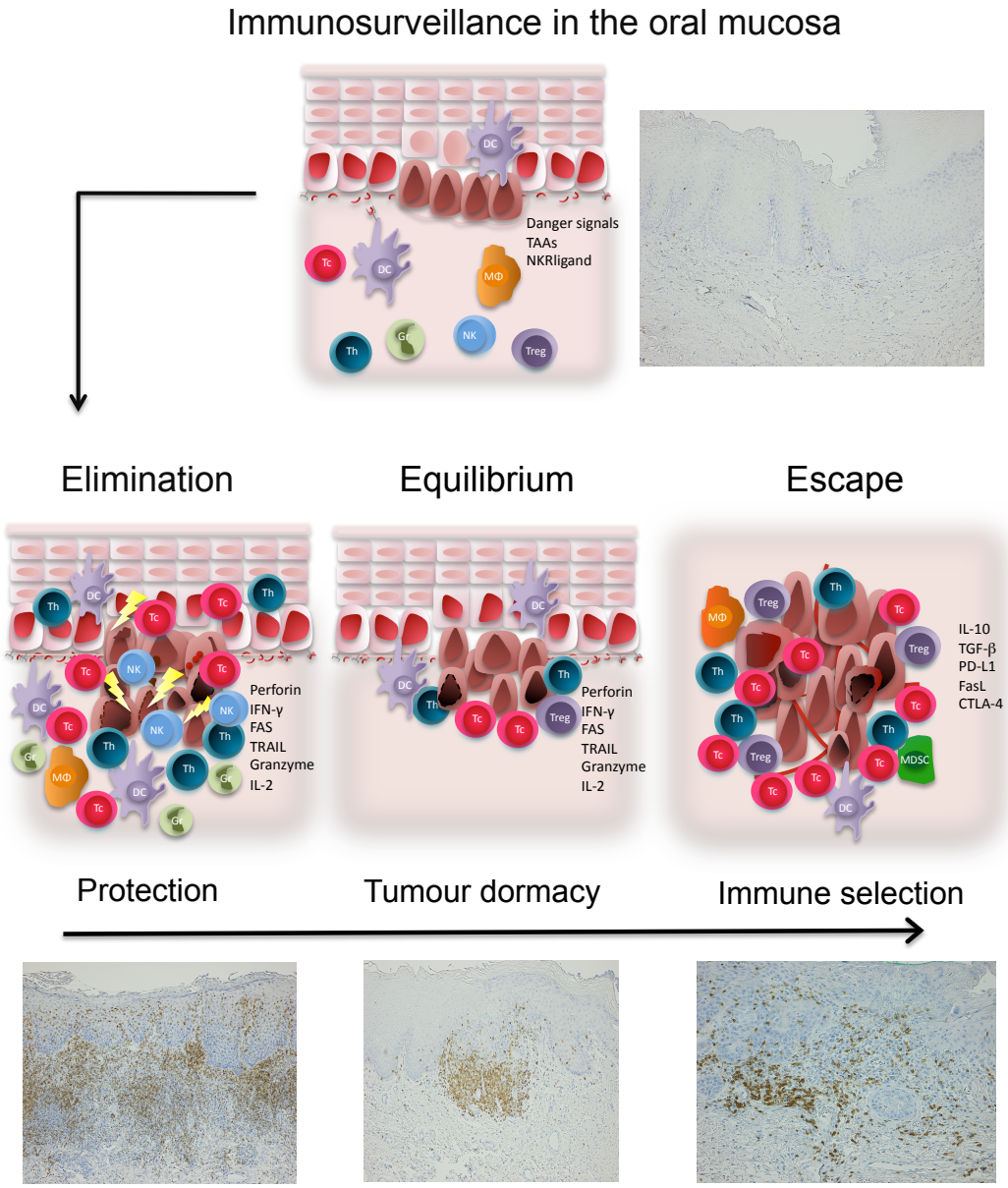


Figure 9. DC-dendritic cell, NK-natural killer cell, Th-T-helper cell, Treg- regulatory T cell, Tc-cytotoxic T cell, MDSC- Myeloderived suppressor cell, Gr-granulocyte

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