**T-helper and T-regulatory cells modulation in head and neck squamous cell carcinoma**

Daniele Maggioni, Lorenzo Pignataro, and Werner Garavello

Department of Surgery and Translational Medicine, University of Milan-Bicocca Monza, Italy; Department of Otorhinolaryngology, Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy; Department of Otorhinolaryngology, School of Medicine and Surgery, University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy

**ABSTRACT**

Head and neck squamous cell carcinoma (HNSCC) is one of the most diffused cancer types, characterized by a high reoccurrence rate, mainly due to the inability of current therapeutic approaches to completely eradicate cancer cells. HNSCC patients often have defective immune functions, thus allowing cancer immune escape and cancer spreading. Particularly important in driving immune escape during HNSCC progression are T-helper and T-regulatory cells. New insights into their mechanisms of action might support the development of effective and long-lasting immunotherapy.

**Abbreviations:** AE, adverse effect; DCR, disease control rate; five-FU, 5 fluorouracil; MTD, maximum tolerated dose; ORR, overall response rate; OS, overall survival; PFS, progressive-free survival; RT, radio therapy

**INTRODUCTION**

Cancer is still a global health problem, as the number of deaths due to cancer is growing rapidly due to population aging and increased cancer risk behavior, especially in low-developed countries.

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy and the most common neoplasm arising in the upper aerodigestive tract. It is the sixth most diffused cancer worldwide, with an estimated incidence of almost 50,000 new cases per year in United States only.

Despite therapeutic improvements that have brought 5 y survival rate over 60% in Western Countries, such as United States, HNSCC treatment is characterized by high failure rates. The currently applied treatments in fact often fail in completely eradicating the primary tumor, thus allowing local or distant reoccurrence within short time.

**HNSCC and immune escape**

In recent years, considerable attention has been focused on the involvement of immune system in the development of a long-lasting anticancer immunity and consequently on how cancer cells may escape such a surveillance. Multiple studies have indicated that HNSCC is associated with immune suppression and this could explain the high reoccurrence rate of this type of cancer.

Malignant HNSCC cells may escape immune surveillance by different strategies: they can avoid immune recognition or they may affect immune system efficiency. For instance, recognition of tumor cell is hampered by the significant decrease of antigen-presenting cells and their effectiveness. In addition, tumor cells may avoid immune surveillance by downregulating histocompatibility molecules, such as HLA class I gene, required to proper antigen presentation. Besides that, many cancer cells release cytokines and chemotactic factors in an unregulated way that might result in an ineffective and more tumor favorable immune response.

Several pieces of evidence have demonstrated that immune functions are usually compromised or at least defective in HNSCC patients. Some reports have shown a decrease of tumor-infiltrating lymphocytes (TIL) in HNSCC, but the clinical outcome seems to depend more on the type of TIL rather than on their total number. For instance, cytotoxic CD8+ T cells are significantly decreased in HNSCC patients if compared with healthy controls, probably due to an increase apoptotic rate among this cell population in HNSCC. On the top of that T cells isolated from HNSCC patients are significantly less responsive to in vitro stimulation than those isolated from healthy controls.

**T-helper cells and HNSCC development**

T-helper (Th) lymphocytes, characterized by CD4+ glycoprotein expression, are key players in directing the immune responses. They include many cell subtypes, classified as Th1, Th2 or Th17, and their differentiation relies on acquisition of differential cytokine production. Th1 cells are characterized by the production of INFγ and IL-2. Th2 cells instead mainly release IL-4, IL-6 and IL-10. Finally, Th17 cells are characterized by the production and release of IL-17, a pleiotropic inflammatory cytokine.
As in many other cancer types, a progressive increase in Th2 response has been described in HNSCC, nevertheless, the shift toward Th2 cells seems incomplete. Indeed, a concomitant increase of both Th1 and Th2 cells with a minimal variation of Th1 over Th2 cells ratio has been often reported in HNSCC.

Similarly, a shift toward Th2-related cytokines, with an increase of IL-4, IL-10 and TGFβ, and a subsequent decrease of the most important Th1 mediator, INFγ, is often observed in HNSCC. The increase of IL-10, known as one of the strongest immune suppressive factors, is correlated with tumor staging, nodal involvement and to a worst prognostic factor. Moreover, IL-10 levels markedly decrease after chemotherapeutic treatment, thus suggesting that cancer cells are the main source of this cytokine in HNSCC. These results demonstrate that HNSCC induces a powerful change in Th1 and Th2 cytokines, producing an environment that is no more effective in promoting a proper cell-mediated antitumor response. Nevertheless, the shift toward Th2 response is characterized by an incomplete decline of Th1 cytokines, as the level of Th1-associated cytokine IL-2 remains high during HNSCC progression, while the levels of IL-12 and IL-22, two important mediators of Th1 differentiation and function, are not modulated during HNSCC progression, as they are the same in HNSCC patients and healthy controls.

HNSCC development is accompanied by a significant increase of both circulating and tumor-infiltrating Th17 cells with a concomitant decrease of Th1/Th17 ratio. Furthermore, enhanced levels of Th17 cells strongly correlated with metastasis occurrence. Th17 cells proliferation is probably fostered by the high level of IL-6 and IL-23 released by cancer cells. These data are sustained by studies in a murine model of 4-nitroquinoline-1-oxide induced oral carcinogenesis, where the progression from premalignant lesion to oral squamous cell carcinoma (OSCC) was characterized by a progressive increase of Th17 cells and IL-17. How Th17 cells increase may be a favorable factor in tumor progression is still a matter of debate; nevertheless, one convincing hypothesis is that Th17 cells might have a significant role in promoting intra-tumor angiogenesis.

IL-17 steadily increases during HNSCC progression. Indeed, although HNSCC patients have lower IL-17 plasma levels than patients harbouring premalignant lesions, their levels are higher than those in healthy controls. Above reported data demonstrate that IL-17 increases during HNSCC progression, although a slight decrease is often observed in the latest phases, probably due to the high levels of TGFβ, which may inhibit Th17 differentiation while promoting regulatory T (Treg) cells differentiation.

On the other hand, Punt and coworkers did not find any significant variation in T cells frequency between OSCC and healthy controls. Their results showed that Th17 cells infiltrate more easily HPV+ than HPV− tumors. Interestingly, they found high levels of IL-17+ but non-Th17 cells in HPV+ tumors, and this is correlated with a worst prognostic value. The authors concluded that Th17 cells are associated with better prognosis, while elevated levels of IL-17− cells, whose only 6% are Th17 cells, are related to a poor prognosis. These data also suggest that many previously reported studies could be biased by the high levels of IL-17 releasing non-Th17 cells, such as macrophage and granulocytes, that might have been counted as Th17 cells.

The mechanisms of HNSCC immune escape may be briefly resumed as follows: in premalignant lesions, there are high levels of inflammatory cytokines, such as IL-2, IL-6 and IL-17, released mainly by IL-17+ Th17 cells, whose proliferation is sustained by the elevated levels of IL-23. However, immune response may fail to counteract tumor growth with a subsequent decrease of many pro-inflammatory cytokines and with a switch toward Th2 cytokines, probably driven by mediators, i.e., TGFβ, released by cancer cells themselves. Therefore, in the latest phases of HNSCC progression, the elevated levels of TGFβ seem to unbalance the ratio between Th17 and Treg cells, promoting Treg differentiation with a consequent increase of anti-inflammatory cytokine IL-10 (Fig. 1). As a result, the original antitumor response is converted into a more tumor favorable response.

All available studies regarding Th cells and Th-related cytokines modulation in HNSCC are summarized in Tables 1 and 2, respectively.

### Regulatory T cells in HNSCC development

Treg cells represent a minor heterogenic subset of CD4+ Th lymphocytes, accounting for less than 5% of them in peripheral blood. Treg are committed to regulate immune response to prevent an excessive immune reactivity and their activity is mainly toward other immune cells such as effector T cells. These cells are often involved in cancer; indeed, unfortunately, the mechanisms that prevent autoimmunity are the same that limit the immune system to recognize tumor cells since the majority of tumor-associated antigens are self-antigens or only minimal modified self-antigens harboring genetic modifications.

A definitive Treg marker has not been discovered yet. Recent approaches have used multiple markers to delineate Tregs, including the IL-2 receptor CD25, the α chain of IL-7 receptor CD127, the cytokotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and forkhead/winged-helix transcription factor box P3 (FOXP3). The levels of expression and the intracellular localization of such markers are important factors for Treg identification. For instance, high expression level of CD25 is a peculiar sign of activated Treg. Conversely, CD127 expression on Treg inversely correlates to Foxp3 expression; therefore, low CD127 expression level is considered a reliable marker of activated Treg. Thus, a general now accepted panel for Treg identification is CTLA-4+, CD25high, CD127low and nuclear expression of Foxp3. Nevertheless, Foxp3 expression has been reported also in HNSCC cells; thus, it is hard to surety state if studies that evaluated Treg infiltrate analyzing Foxp3 expression have identify proper Treg and not Foxp3+ cancer cells.

Elevated levels of Treg have been found in several cancer types, including lung, breast and pancreatic cancer. Multiple pieces of evidence show a general increase of both circulating and infiltrating Treg during HNSCC development. Furthermore, Treg levels increase accordingly with tumor staging and they are particularly elevated in patients with active disease. A murine model of oral carcinogenesis demonstrated a cancer cells promoted skew toward Treg, probably due to the
decreased levels of IL-23, also observed in the late phase of HNSCC development in humans. Th17 and Treg cells share a common lineage and a high plasticity degree, even though their mediator effects are opposing. Thus, a shift in cytokine milieu from the Th17 sustaining pro-inflammatory IL-23 toward TGFβ may easily result in a significant skew toward Treg as premalignant lesions progress to oral cancer.

**Treg significance in HNSCC prognosis**

An agreement regarding the prognostic value of Treg in HNSCC has yet to be found. Several data strongly sustained a negative prognostic index associated to high levels of Tregs, but other studies described a completely different situation, with high numbers of Treg associated to an improved loco-regional control and better overall survival. These apparent contradictions might be explained, in part by the difficulties to properly identify Treg cells and in part by the tissue dependent modulation of Treg levels; indeed, tumors arising in the rich lymphoid tissue oropharynx have higher level of infiltrating Treg than cancer types arising in larynx. Furthermore, synchronous multifocal cancers, the cancers arising at the same time in different oral cavity area, have more elevated numbers of Treg. This aspect sustains a model where Treg increase is a promoting factor rather than a consequence of cancer development.

**HPV infection is an important etiologic factor for oropharyngeal SCC. HPV+ HNSCC have a significantly better**

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**Figure 1.** Cytokines modulation during HNSCC progression. HNSCC development is characterized by the progressive decline of Th1 related cytokine INFγ. Conversely, IL-23 levels increase steadily, probably as the result of an inflammatory response against cancer. Nevertheless, as HNSCC progresses, a significant rise of TGFβ and a concomitant decrease of IL-23 levels promote Treg differentiation with a consequent decline of Th17 cells. As a result, in the latest phase of HNSCC progression, anti-inflammatory and anti-immune cytokines such as IL-4 and IL-10 prevail.

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**Table 1.** Studies evaluating T-helper cells modulation in HNSCC.

| Specimens | Methods | Main findings | Reference |
|-----------|---------|---------------|-----------|
| 45 HNSCC, stage I–IV | Flow cytofluorimetric analysis of PBMC | Decrease of CD11+ DC, increase of Th1 and Th2 cells, no variation in Th1/Th2 ratio | 21 |
| 42 HNSCC stage I–IV | Flow cytofluorimetric analysis of PBMC | Decrease of total CD4+ T cells, increase of Th1 and Th2 cells (upon PHA stimulation), no variation in Th1/Th2 cells | 61 |
| 10 HNSCC stage III–IV | Flow cytofluorimetric analysis of PBMC | Reduced count of total white cells, reduction of CD4+ and CD8+ T cells | 9 |
| 12 OSCC | Flow cytofluorimetric analysis of PBMC | No variation in CD4+ and CD8+ T cells | 51 |
| 25 HNSCC, stage not specified | Flow cytofluorimetric analysis of PBMC and TIL | Increase amount of circulating and infiltrating TH17 cells, decrease of circulating Th1 cells, decrease of Th1/Th17 cells ratio | 29 |
| 25 HNSCC, stage not specified | Flow cytofluorimetric analysis of peripheral blood and tumor infiltrating CD4+ T-helper cells | Decrease of circulating and infiltrated CD161+ Th17 cells, decrease of CD161+ Th17 cells in metastatic HNSCC | 19 |
| 67 HNSCC, stage I–IV | Flow cytofluorimetric analysis of peripheral blood and tumor infiltrating Th17 | Increased levels of Th17 cells, correlating to tumor stage | 30 |
| 54 HNSCC, stage I–IV | Flow cytofluorimetric analysis of PBMC | Increase amount of CD8+ T cells in HPV+ tumors | 15 |
| 42 Laryngeal SCC, stage I–IV | Flow cytofluorimetric analysis of PBMC | No variation in CD4+ and CD8+ T cells, Th1 cells decrease | 20 |
| 30 OSCC, stage I–IV | Flow cytofluorimetric analysis of peripheral blood and tumor infiltrating T lymphocytes | Decrease of circulating and infiltrating CD4+ T cells | 13 |
| 162 OSCC, stage I–IV, HPV+ vs. HPV– tumors | Immunofluorescence staining for CD3 and IL-17 in TIL | Higher levels of CD3+ T cells and Th17 cells in HPV+ tumors | 33 |
prognosis than HPV− tumors.47 The role of immune cells in this aspect has been investigated by Punt and colleagues in a set of oropharyngeal SCC. They found a higher infiltration of Foxp3+ Treg cells both in stroma and in the epithelium of HPV+ tumors, which significantly correlated with improved survival.13 These data are in agreement with the findings from Mandal and coworkers, reporting high levels of both CD8+ T cells and Treg and in a large set of HPV+ HNSCC.46 Interestingly, HPV+ tumors had almost twice ratio of total T cells over Treg cells if compared with HPV− tumors, thus suggesting that the better prognosis of HPV+ tumors might not be directly related to Treg infiltration but rather to the higher number of immune cells infiltrated. Consistently, Mandal and coworkers emphasized that CD8+/Treg cells ratio was higher in patients having a relative low immune infiltrate if compared with patients with high levels of T cells infiltration.46 This suggests that immune-high HNSCC are subjected to a higher degree of immunoregulation and only the relationship between the different players of immune response could give significant information about prognosis and potential immunotherapy effectiveness.

**Mechanism of Treg accumulation in HNSCC**

The mechanisms underlying the potential Treg driven pro-tumor effect remain elusive. Four independent studies have studied the immunolocalized Treg cells in HNSCC and they all highlighted a marked presence of cells in the tumor microenvironment.20,37,39,42 Two studies prevalently found Tregs at the tumor margins,39,42 thus sustaining the hypothesis that these cells might oversee the interactions between SCC cells and effector immune cells at the border between tumor and normal tissue. The accumulation of Treg in tumor might be the result of CD4+CD25+ T-cells recruitment from the periphery or of the activation of resting naive CD4+ T cells directly into the tumor tissue. The proportion of Foxp3+ cells is significantly more elevated in TIL than in peripheral blood mononuclear cells (PBMC).43 Moreover, immune-staining data show high Treg frequency in the peripheral area of the tumor, sometimes in the stroma, but never in the tumor nest,11 in such a way supporting the hypothesis that these cells are recruited from peripheral circulation into the tumor by chemotactic factors released by cancer cells. Consistently with this hypothesis the increased expression of Monocyte Chemotactic Protein-1 (MCP-1), C-C motif Chemokine Ligand 22 (CCL22), C-C chemokine receptors type 4 and 7 (CCR4 and CCR7), that are associated to a migratory phenotype, has been observed on HNSCC isolated Treg and in HNSCC tissue.10,11,35,48 In addition, ligands for CCR4 and CCR7 were more expressed in HNSCC if compared with the adjacent healthy tissue.19 In partial disagreement with the above-reported data, Sun and coworkers found high level of CCR7+ cells in the peripheral blood of HNSCC, but CCR7 expression was elevated in all T lymphocytes, irrespective of the subsets.48 Only the increased expression of CCR4 is a peculiar characteristic of Treg isolated from HNSCC patients. CCR4 expression is a marker of Treg activation as CCR4+ Tregs are readily suppressive in vitro, while CCR4− Treg needs further activation before becoming suppressive.49 The fundamental role of CCR4 in driving Treg migration toward tumor tissue has been further confirmed by experiments in mice, where blocking of MCP-1/CCR4 signaling came out in a lower frequency of Treg infiltrating the tumor and a significant inhibition of tumor growth.48

Tumor-isolated Treg have a more immunosuppressive phenotype, with elevated expression of ectonucleotidase CD39, TGFβ and CTLA-4. These cells are also more effective in inhibiting T cells proliferation in vitro than those isolated from peripheral blood, hence sustaining the activator effect of tumor microenvironment.43 Importantly, a murine model unveiled that peripheral CD4+CD25+FoxP3+ T cells convert extra-thymically into FoxP3+ Treg cells under conditions found in tumor environment.50 Therefore, it is likely that peripheral

| Specimens                          | Methods                                     | Main findings                                                                 | References |
|------------------------------------|---------------------------------------------|-------------------------------------------------------------------------------|------------|
| 101 HNSCC, stage I−IV              | ELISA analysis of serum cytokine levels     | Increased levels of IL-1b, IL-2, IL-4, IL-6, IL-10. Decreased levels of INFγ    | 28         |
| 58 HNSCC, stage I−IV               | ELISA analysis of serum cytokine levels     | Increased IL-6, IL-10 levels, positive correlation to stage. Increased levels of IL-12, negative correlation to stage | 25         |
| 55 pre-surgical HNSCC              | ELISA analysis of serum cytokine levels     | Increase levels of IL-12, decreased level of IL-10                            | 26         |
| 10 HNSCC stage III−IV              | ELISA analysis on culture supernatant of HNSCC PBMC | Increase levels of IL-4 and IL-10                                             | 9          |
| 25 HNSCC, stage not specified      | Flow cytometric analysis on cancer cell suspension and TIL | High amount of IL-1b, IL-23 and IL-6 positive cells in tumor tissue           | 29         |
| 150 HNSCC, stage I−IV              | ELISA analysis on serum of HNSCC patients pre- vs. post-treatment | IL-10 detected in more HNSCC patients than in healthy controls, no differences between pre-treatment or post-treatment | 27         |
| 17 larynx SCC, 6 larynx dysplasia  | Flow cytofluorimetric analysis on serum     | Increased levels of IL-6, IL-8, IL-10 in both SCC and dysplasia                | 22         |
| Pre-malignant oral lesion and OSCC, stage II−IV | Flow cytofluorimetric analysis of cytokine in plasma, tissue lysate and supernatants of dissociated tissue | Increased levels of IL-2, IL-4, IL-17 and INFγ in premalignant lesions       | 32         |
| 78 OSCC, mainly stage III−IV       | ELISA analysis of serum cytokine levels     | Increase levels of IL-2, IL-4, IL-10, INFγ and TGFβ                           | 23         |
| 34 premalignant oral lesion, 61 HNSCC, stage II−IV | Cytometric array analysis of plasma and tissue lysate | Increased levels of IL-6 and IL-17 in premalignant lesion and in HNSCC        | 31         |
Treg are recruited into the tumor tissue where they are induced toward a highly immunosuppressive phenotype by tumor-associated factors, such as TGFβ.

On the other hand, some reports have also shown the increased frequency of activated Treg in peripheral blood of HNSCC patients. Moreover, activated CD4+CD25+Foxp3+ T cells in peripheral blood of HNSCC patients are significantly more effective in inhibiting effector T cells than those from healthy controls. Therefore, it is conceivable that cancer-released cytokines might affect Treg cells development outside the tumor tissue, although at a lesser degree than in tumor stroma.

**Mechanisms of Treg-induced immunosuppression in HNSCC**

Treg-mediated immunosuppression may occur by cell-to-cell contact or by cytokines secretion.

Many pieces of evidence sustain a soluble factor mediated suppressive action of Treg. A significant increase of IL-10 and TGFβ levels has been reported in tumor, probably promoted by Tregs since Treg isolated from OSCC significantly induced IL-10 and TGFβ release from co-cultured allogenic PBMCs.

Nevertheless, Strauss and colleagues demonstrated that, at least for circulating Treg, immune suppression is mainly due to direct cell-to-cell interaction. In fact, co-incubation of peripheral blood isolated Treg with autologous T cells separated by permeable transwell insert resulted in no suppression, thus indicating that soluble factors diffusion is not enough to mediate immune suppression and that it rather relies on direct cell-to-cell contact.

The ectonucleotidases CD39 and CD73 and the Immune-Checkpoint Receptors (ICRs) have been proposed as key molecules for Treg immunosuppression. ICRs include CTLA-4, the programmed cell death-1 (PD-1), expressed on activated lymphocytes and glucocorticoid-induced tumor necrosis factor receptor (GITR), a T-cell stimulatory molecule.

CTLA-4 prevents the activation of the co-stimulatory molecule CD28 on T cells, hence significantly reducing T-cell proliferation. CTLA-4 blockade inhibits immunosuppressive activity of Treg.

Anti-CD39 blocking antibodies almost completely abrogate Treg immunosuppressive function while restoring effector T cells activity. Jie et al. evaluated the expression of ICRs on Treg. They observed a higher percentage of CTLA-4 and PD-1-positive Treg cells in TIL compared with circulating paired. Similarly, CD39 and CD73 expression levels were higher in TIL than in peripheral blood. In particular, the majority of tumor-infiltrated Treg co-expressed both CTLA-4 and CD39, suggesting that these two molecules are fundamental in mediating immunosuppression in the tumor microenvironment.

Another molecule potentially involved in cell-to-cell mediated immune suppression is β-galactoside binding protein (β-GGBP), β-GGBP is overexpressed in both OSCC cells and activated Treg cells and its blockade significantly attenuate the inhibitory effects of Treg cells. The surface bound form of β-GGBP mediates T-cell suppression by interacting with glycoproteins on T-cell surface and by inducing growth arrest and apoptosis on activated T cells. β-GGBP plays a double role since it promotes cancer cell proliferation and at the same time it impairs T-cell effectiveness by promoting IL-10 and IL-35 release. Intriguingly, using a specific β-GGBP inhibitor, thiogalactoside, a decrease of IL-10 and IL-35 Tregs and an impairment of cancer cells growth were observed.

The data reported above, although far from definitely depicting the Treg way of action in HNSCC, suggest that cytokine mediators released by cancer cells activate and recruit circulating Treg. As a result, Treg migrate into the tumor tissue, where they are further activated by the cytokine milieu found in tumor stroma. Finally, Treg suppress the activity of cytotoxic and effector cells via both cell-to-cell contact and humoral mechanisms, thanks to the expression of CTLA-4, β-GGBP and CD39 and the release of immune modulating factors such as IL-10, IL-35 and TGFβ.

**Immune therapy: A new promising strategy for HNSCC treatment**

Immune therapy has recently gained consideration and it is now considered the new frontier in anticancer strategies. The aim of immune therapy is to modulate immune response, shifting the balance toward an anti-tumor response, to achieve long-lasting tumor suppression.

Current immunotherapies adopt different strategies to sustain immune response by adoptive T-cell transfer, dendritic cells vaccine or by infusion with adjuvant cytokines, such as IL-1β, IL-2, INFγ and TGFα. Recent approaches tried to modulate immune response by directly targeting molecules involved in the interactions between cancer and the immune system. In particular, several experimental pieces of evidence have demonstrated that Treg eradication by chemical drugs or specific antibodies is a promising strategy to induce a significant anticancer immune response. Low doses of anti-proliferative drugs such as Indomethacin or Cyclophosphamide and human monoclonal antibodies against CTLA-4, PD-1 and GITR have been used to selectively target Treg. Nevertheless, these approaches are often hampered by the occurrence of autimmune diseases; therefore, it is necessary to refine these strategies allowing to selectively target Treg inside tumor tissue, without depleting them systemically.

Some of the above-mentioned therapies have proved promising in preclinical studies and they are now in clinical trials; indeed, a recent query in the ClinicalTrials.gov database for “HNSCC and immunotherapy” revealed 54 ongoing studies, the most of them being phase I and II.

One of the most promising approaches to prevent suppression of anticancer immunity is the blockade of immune checkpoint receptors (ICRs) by blocking antibodies. Thus, new monoclonal antibodies against PD-1 and CTLA-4, such as Pembrolizumab, Nivolumab, Durvalumab, Tremelimumab and Ipilimumab, have been developed and they are now in clinical trials. Ongoing trials for antibodies targeting ICR are summarized in Table 3.

Pembrolizumab is a humanized antibody targeting PD-1 receptor; it has been approved for melanoma treatment by Food and Drug Administration (FDA) and is in clinical trials...
| Trial NCT   | Phase     | No. of patients | Status              | Eligibility                                      | Treatment                                      | Primary end point                                                                 |
|------------|-----------|----------------|---------------------|--------------------------------------------------|------------------------------------------------|------------------------------------------------------------------------------------|
| NCT02475213 | Phase I   | 75             | Recruiting          | Refractory cancers, including HNSCC              | Pembrolizumab + Enoblituzumab                  | AE, plasma levels, antibodies, tumor volume                                         |
| NCT02626000 | Phase I   | 40             | Recruiting          | Recurrent or metastatic HNSCC                    | Pembrolizumab + Talimogene                     | AE, dose-limiting toxicity, ORR, OS                                                 |
| NCT02819752 | Phase I   | 36             | Not yet recruiting  | Advanced HNSCC                                    | Pembrolizumab + cisplatin + RT                 | AE                                                                                  |
| NCT02586207 | Phase I   | 39             | Recruiting          | HNSCC                                            | Pembrolizumab + cisplatin + RT                 | Acute toxicity, expression of peripheral immune-inflammatory markers, distant metastasis, OS, |
| NCT02775812 | Phase I   | 56             | Recruiting          | Stage III–IV HNSCC                               | Pembrolizumab + cisplatin + RT                 |                                                                                   |
| NCT02718820 | Phase I/II | 22             | Recruiting          | Recurrent or metastatic HNSCC                    | Pembrolizumab + docetaxel                      | ORR, AE, OS                                                                         |
| NCT02452424 | Phase I/II | 400            | Recruiting          | Melanoma + HNSCC                                 | Pembrolizumab + PLX3397                        | ORR                                                                                |
| NCT02521870 | Phase I/II | 156            | Recruiting          | Recurrent or metastatic HNSCC                    | Pembrolizumab + SD-101                         | dose limiting toxicity, PFS, OS                                                    |
| NCT02289209 | Phase II  | 48             | Recruiting          | Inoperable or second primary HNSCC               | Pembrolizumab + RT                             | Acute AE, PFS                                                                       |
| NCT02609503 | Phase II  | 29             | Recruiting          | Adenocarcinoma                                    | Pembrolizumab + RT                             |                                                                                   |
| NCT02454179 | Phase II  | 74             | Active not recruiting | Advanced HNSCC                                   | Pembrolizumab + Acalabrutinib                  | ORR                                                                                |
| NCT02255097 | Phase II  | 172            | Active not recruiting | Recurrent or metastatic HNSCC                    | Pembrolizumab + cetuximab + cisplatin          | ORR, AE, OS                                                                         |
| NCT02707588 | Phase II  | 114            | Not yet recruiting  | Advanced HNSCC                                    | Pembrolizumab + cetuximab + RT                 | ORR, reduction of PD-L1 expression, AE, OS                                           |
| NCT02892201 | Phase II  | 24             | Recruiting          | HNSCC                                            | Pembrolizumab                                  |                                                                                   |
| NCT02841748 | Phase II  | 100            | Not yet recruiting  | Potential recurrent HNSCC                        | Pembrolizumab                                  | OS, PFS                                                                            |
| NCT02296684 | Phase II  | 46             | Recruiting          | Surgically resectable HNSCC                      | Pembrolizumab + RT                             | AE, locoregional control                                                           |
| NCT02252042 | Phase III | 466            | Active not recruiting | Recurrent or metastatic HNSCC                    | Pembrolizumab vs. methotrexite, docetaxel, cetuximab | PFS, OS                                                                           |
| NCT02358031 | Phase III | 825            | Active not recruiting | Recurrent or metastatic HNSCC                    | Pembrolizumab vs. Pembrolizumab + cisplatin or 5-FU or cetuximab | PD-L1 expression, PFS, OS                                                        |
| NCT03040999 | Phase III | 780            | Not yet recruiting  | Advanced HNSCC                                    | Pembrolizumab vs. cisplatin + RT               | AE, OS                                                                             |
| NCT02827838 | Phase III | 20             | Recruiting          | Oral cavity and oropharynx cancer                | Durvalumab                                     | AE                                                                                  |
| NCT02626741 | Phase I   | 69             | Active not recruiting | HNSCC                                            | Durvalumab + Tremelimumab ± paclitaxel, carboplatin, 5-FU | AE, ORR                                                                            |
| NCT02658214 | Phase I   | 60             | Active not recruiting | Advanced solid tumors including HNSCC            | Durvalumab + Tremelimumab ± paclitaxel, carboplatin, 5-FU | AE                                                                                  |
| NCT02264678 | Phase I   | 114            | Recruiting          | Solid tumors including HNSCC                     | Durvalumab + AZD6738                           | AE, ORR, PFS                                                                       |
| NCT01936612 | Phase I   | 264            | Recruiting          | Solid tumors including HNSCC                     | Durvalumab or Tremelimumab                     | MTD, AE, ORR, PFS, OS                                                              |
| NCT02499328 | Phase III | 147            | Recruiting          | Advanced and metastatic HNSCC                    | Durvalumab ± AZD9150 or AZD9069                | MTD, AE, ORR                                                                       |
| NCT03019003 | Phase I/II | 59             | Not yet recruiting  | Recurrent or metastatic HNSCC                    | Durvalumab + Tremelimumab, azacitidine          | ORR, PFS, OS                                                                        |
| NCT02207530 | Phase II  | 112            | Active not recruiting | Recurrent or metastatic PD-L1 positive HNSCC     | Durvalumab                                     | ORR                                                                                |
| NCT02319044 | Phase II  | 543            | Active not recruiting | Recurrent or metastatic PD-L1 positive HNSCC     | Durvalumab ± Tremelimumab                      | ORR, PFS, DCR, OS                                                                  |
| NCT02369874 | Phase III | 720            | Recruiting          | Recurrent or metastatic PD-L1 positive HNSCC     | Durvalumab ± Tremelimumab vs. standard chemotherapy | ORR, PFS, DCR, OS                                                                     |
| NCT02551159 | Phase III | 760            | Recruiting          | Recurrent or metastatic HNSCC                    | Durvalumab ± Tremelimumab vs. standard chemotherapy (cisplatin, 5-FU, cetuximab) | ORR, PFS, OS                                                                       |
| NCT02471846 | Phase I   | 276            | Recruiting          | Advanced solid tumors including HNSCC            | Atelizumab or GDC-0919                         | Dose-limiting toxicity, AE                                                        |
| NCT02764593 | Phase I   | 120            | Recruiting          | High risk, advanced HNSCC                       | Nivolumab + cetuximab + cisplatin + RT         | Dose-limiting toxicity, AE                                                      |
| NCT Number     | Phase | ID | Status     | Tumor Type                  | Treatment                                                                 | Endpoints                                      |
|---------------|-------|----|------------|-----------------------------|---------------------------------------------------------------------------|-----------------------------------------------|
| NCT02903914   | I     | 236| Recruiting | Solid tumors including HNSCC | Nivolumab ± CB-1158, Nivolumab + ABBV-927                                 | MTD, ORR, PFS                                  |
| NCT02988960   | I     | 180| Recruiting | Solid tumors including HNSCC | Nivolumab ± motolimod vs. cetuximab + motolimod                           | Immune biomarkers: FcγR genotype, NK activation, tumor infiltration and serum cytokines, T-cell activation |
| NCT02124850   | I     | 24 | Recruiting | HNSCC                       | Nivolumab ± motolimod vs. cetuximab + motolimod                           |                                               |
| NCT02834247   | I     | 120| Recruiting | Solid tumors including HNSCC | Nivolumab + TAK-659, Nivolumab + enadenotucirev                          | MTD, AE, ORR, PFS, OS                        |
| NCT02636036   | I     | 30 | Recruiting | Metastatic epithelia tumors including HNSCC | Nivolumab + varilimumab                                                  | Dose-limiting toxicity, ORR, OS               |
| NCT0235918    | I/II  | 205| Recruiting | Refractory cancers, including HNSCC | Nivolumab + varilimumab                                                  |                                               |
| NCT03003637   | I/II  | 32 | Not yet recruiting | Advanced or recurrent HNSCC | Nivolumab + ipilimumab                                                   | Toxicity, tumor response, Best overall response |
| NCT02684253   | II    | 40 | Recruiting | metastatic HNSCC            | Nivolumab + RT                                                            | ORR, PFS, OS                                  |
| NCT02823574   | II    | 315| Recruiting | Recurrent or metastatic HNSCC | Nivolumab + ipilimumab                                                   | ORR, PFS, OS                                  |
| NCT02105636   | III   | 506| Active not recruiting, with results | Recurrent or metastatic HNSCC | Nivolumab vs. cetuximab or methotrexate or docetaxel                      | ORR, PFS, OS                                  |
| NCT02741570   | III   | 490| Recruiting | Recurrent or metastatic HNSCC | Nivolumab ± ipilimumab vs. cisplatin + cetuximab                           | ORR, PFS, OS, PD-L1 expression                |
for HNSCC therapy. Although all the studies are ongoing, preliminary results confirmed that Pembrolizumab is better tolerated than traditional chemotherapy and it has achieved encouraging results as 25% of the patients responded to treatment.\textsuperscript{60} Three phase III studies have been recently set up to evaluate Pembrolizumab efficacy in increasing overall survival in metastatic HNSCC patients in comparison with 5-fluorouracil or cetuximab plus a platinum-based drug therapy (NCT03040999, NCT02252042 and NCT02358031).

Nivolumab is another humanized antibody targeting PD-1, approved by FDA for the treatment of melanoma and renal carcinoma. Interestingly, a phase III study aiming to unveil whether Nivolumab would improve overall survival in comparison to cetuximab, carboplatin or methotrexate in patients with recurrent or metastatic head and neck carcinoma completed the results analysis. Nivolumab showed a significant superior performance of the understudied item (NCT02105636).

Durvalumab instead targets the ligand of PD-1, PD-L1, and it has been recently approved for use in bladder cancer by FDA. CTLA-4 is another hotspot of immune therapy in HNSCC; indeed, there are several clinical trials for antibodies targeting this receptor. Ipilimumab is a monoclonal human antibody that targets CTLA-4; it was approved by FDA in 2011 for the treatment of advanced melanoma and it is now being evaluated for HNSCC. In particular, a large phase III trials has been recently set up to analyze its efficacy in combination with Nivolumab in patients with recurrent or metastatic HNSCC (NCT02741570).

Safety and tolerability of Tremelimumab, a human monoclonal antibody against CTLA-4, is being evaluated in eight studies, in combination therapy with Durvalumab and standards of care such as cisplatin, carboplatin, azacitidine, 5-fluorouracil (please see Table 3 for references). Two phase III trials are now recruiting participants to determine the efficacy and safety of Tremelimumab plus Durvalumab combination therapy versus standards of care in HNSCC.

Conclusion

The above-reported data strongly confirmed the role of immune system in regulating HNSCC development. In particular, Treg cells play a pivotal role in modulating immune system response in a tumor favorable way. New insights coming from preclinical studies are mandatory since they will further unveil the mechanisms underlying this process, thus allowing the optimization of new strategies to let the immune system re-gain control over tumor development.

Given the extremely complex relations between immune system and cancer cells, it is necessary to deeply analyze the immune scenario characterizing each patient and each tumor. The role of Th and Treg cells in cancer is context dependent and it should always be examined in relation to other effector T cells.

The fragile balance between the different Th subtypes and effector T cells seems the true determinant of the immune response; hence, the success of any potential therapy aiming to restore cancer immune surveillance would rely on a slight modulation of such a balance. Successful therapy should target a precise molecular target in a tailored fit treatment, where the ability to hit a chosen mechanism on a wanted cell subtype would be more important than the quantity of the modulation achieved.

In this context, immunotherapy is a very promising strategy for the treatment of HNSCC, as sustained by preliminary data from clinical trials. Intriguingly, immunotherapy would permit to obtain long-lasting effects and a better disease control over time, thus preventing re-occurrence and metastasization. It is likely that a combination of traditional standards of care and immunotherapy would be particularly effective. Nevertheless, the preliminary results obtained so far need to be confirmed in enlarged phase III and IV trials. Moreover, the optimization of knowledge about Treg cell interactions with both cancer cells and other Th cells will surely provide better tool to selectively target cancer driven immune suppression, thus avoiding undesirable non-selective unbalance of immune regulation that could come out in autoimmunity.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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