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The role of novel immune suppressing cytokine-IL-35 in head and neck cancer

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Introduction

One in two people will be diagnosed with cancer in their lifetime.[1] Head and neck cancer is the sixth most common malignancy worldwide, with nearly 600,000 new cases and 300,000 deaths per year.[2] Head and neck cancer includes cancers of the oral cavity, pharynx and larynx and 90% of these are squamous cell carcinomas of the head and neck (HNSCC).[3] HNSCCs have a common origin in the squamous mucosa of the epithelial linings of the upper aerodigestive tract.[4,5] Most patients express with locally advanced stage diseases (III to IVb) and tumour progression of locoregional failure and distant metastases is common.[6,7] Due to this, the 5-year estimated survival rate is only 40-50%.[5] Research development into the mechanisms employed by HNSCCs is essential to improve patient prognosis. Smoking tobacco and drinking alcohol, as well as maintaining poor oral hygiene, represent key lifestyle risk factors for development of malignancies in the head and neck area.[8] Smoking and drinking are often done simultaneously and this exhibits a negative and synergistic effect on development risk.[9] Inherited genetic disorders, such as Fanconi anaemia, can also predispose a patient to HNSCC,[10] along with specific genetic mutations, most commonly *TP53* inactivation.[11] Some viral infections are also associated HNSCC risk factors. Epstein-Barr virus infection is a longstanding risk factor for nasopharyngeal carcinomas,[12] and human papillomavirus (HPV) is strongly associated with both oropharyngeal squamous cell carcinoma[4] and HNSCC carcinogenesis.[13] Interestingly, survival rates for HPV-positive HNSCC patients are higher than those with HPV-negative tumours.[14] This may be due to tumour intrinsic factors, as genetic pathogenesis can increase drug sensitivity and decrease proliferation rates, or host intrinsic factors, as patients are likely to be healthier than those suffering from a drinking or smoking-induced disease.[4,5] HPV-negative HNSCC patient outcomes have seen little improvement in the last 30 years and this is partly due to a fundamental lack of knowledge in the molecular mechanisms of their pathogenesis.[15] HNSCCs are regularly treated with aggressive multimodality therapy including surgery, chemotherapy, radiotherapy, recent CAR-T cell immune therapy and immune check point therapy or a concurrent combination of these.[16] The chimeric immunoglobulin G monoclonal antibody cetuximab targets the epithelial growth factor receptor (EGFR) and is recommended as a chemotherapeutic agent for locally advanced HNSCC.[17] However, cetuximab has a large side effect profile with over 80% of patients suffering from skin reactions and is very expensive.[17] A large number of patients also experience a reduction in swallowing or speech function.[18] Coupled with poor tumour responses, cetuximab has therefore only had limited anti-HNSCC therapy success.[19] Alternatively or as well as cetuximab, cisplatin is used in combination with fluorouracil to also combat HNSCC, but this has a predominantly palliative benefit.[19] HPV-negative HNSCCs have a poorer response than HPV-positive HNSCCs to both chemotherapy and radiotherapy and have been reported as radiation-resistant.[13] Due to the ineffective nature of current anti-HNSCC therapy, new research, especially in the less responsive HPV-negative disease, is essential for production of novel therapies.

Macrophages are heterogeneous cell populations present in all living tissues that have an essential role in host innate immunity.[20] They differentiate from circulating monocyte precursor cells[21] and can be split into 2 types: M1 macrophages are pro-inflammatory and are 'classically' activated M2 macrophages are anti-inflammatory and 'alternatively' activated.[20] These two macrophage types both have the potential to phagocytose pathogens but work in unison to balance pro and anti-inflammatory host responses.[22] M1 macrophages are activated in an inflammatory environment to produce pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα), whereas M2 macrophages are activated to produce anti-inflammatory cytokines such as TGF-β1, IL-10 and are thus involved in wound healing and tissue repair.[20] Phenotype switching between the two macrophage types can occur in order to better respond to a particular pathogen, and this is dependent on the presence of cytokines in the immediate environment and demonstrates the high plasticity of macrophages.[20,23]

Tumour cell survival is dependent on avoiding host immune detection, known as immune tolerance. One mechanism, by which immune tolerance is induced, is through tumour-macrophage bidirectional communication and thus formation of a microenvironment that favours tumour proliferation and immune system avoidance.[18] Tumour-associated macrophages (TAMs) are macrophage cells found in close proximity to tumour cells, and they have been shown to have pro-tumour effects.[24] These are commonly M2 macrophages and aid cancer in evading the immune system by promoting angiogenesis within cancer cell masses and aiding the release of primary tumour cells for metastasis.[25] The release of anti-inflammatory cytokines by TAMs initiates a vicious immunity cycle as the microenvironment becomes heavily anti-inflammatory. This can initiate M1 to M2 phenotype switching in other surrounding macrophages, causing immunosuppression and further increasing anti-inflammatory cytokine production.

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The role of a disintegrin and metalloprotease (ADAM) and tumour necrosis factor α (TNFα)

A disintegrin and metalloproteases (ADAMs) are a family of proteases involved in the proteolysis of cell surface proteins.[26] They are related to the matrix metalloproteinase family.[27] Two specific ADAMs with very similar structures,[28] ADAM10 and ADAM17, are recognised as important in the pathophysiology of HNSCC.[29-31] ADAM17 is also known as TNFα-converting enzyme due to its ability to shed pro-TNFα from the cell surface of molecules to release soluble TNFα.[32] ADAM10 has also been documented to shed pro-TNFα.[33] Inhibition of ADAM10 and ADAM17 has been proposed as a potential therapeutic target for HNSCC[30,31] due to cetuximab-resistant HNSCCs exhibiting higher ADAM10 and ADAM17 levels.[31]

hTNFα is a pro-inflammatory cytokine with the ability to induce apoptosis, necrosis or necroptosis in target cells. Apoptosis is induced via the activation of TNF receptor 1 and subsequent caspase-mediated signalling cascades.[34] However, HNSCCs have largely developed resistance to the effects of hTNF.[35] This may be due to upregulation of nuclear factor κB and thus increased expression of apoptosis-inhibiting proteins.[36] HNSCCs have also been shown to produce hTNFα and this has been suggested as beneficial towards tumour cell survival.[37] Also, through stimulating the production of vascular endothelium growth factor, hTNFα promotes angiogenesis in the tumour cell microenvironment.[8] hTNFα has been shown to exert both pro-tumour and anti-tumour effects. Further research into the role of hTNF in the tumour-macrophage interaction is needed to elucidate its role in tumour carcinogenesis and immune tolerance.

The role of interleukin-35

One method utilised by tumour cells for immune tolerance is the production of IL-35, an anti-inflammatory and immunosuppressive cytokine.[38] However, the mechanism by which IL-35 does this is not yet fully understood. IL-35 is a cytokine of the IL-12 family and is composed in a heterodimer formation, meaning it is made up of two independent macromolecular protein subunits: Epstein-Barr virus-induced gene 3 (*Ebi3* which encodes IL-27) and interleukin-12 alpha (*Il12a* which encodes IL-12α/p35) (Figure 1).[38,39] The IL-35 receptor (IL-35R) is made up of two corresponding subunits: glycoprotein 130 and interleukin-12 receptor β2.[39] These receptor subunits may be arranged either in homodimeric or heterodimeric forms, with the heterodimeric formation resulting in the strongest immune suppression on activation.[40]

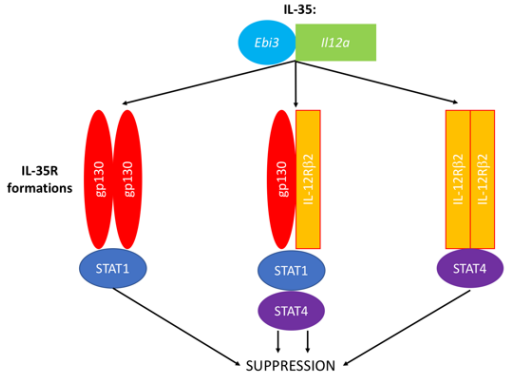

Figure 1 The interleukin-35 (IL-35) heterodimer and corresponding IL-35 receptor (IL-35R) homodimeric and heterodimeric formations. IL-35 binding to the heterodimeric IL-35R exhibits maximal suppression through activation of both single transducer and activator of transcription (STAT) 1 and 4. *Ebi3,* Epstein-Barr virus-induced gene 3 *Il12a,* interleukin-12α gp130, glycoprotein 130 IL-12Rβ2, interleukin-12 receptor β2.

IL-35 isn't exclusively produced by tumour cells. T regulatory (T_{reg}) cells ($CD4^+$ T cells in human and mouse) within the immune system of the body express IL-35 to maintain self-tolerance and prevent autoimmunity, as well as provide anti-inflammatory effects.[38] This release of IL-35 is carefully regulated to prevent suppression of the natural immune response to pathogens. IL-35 expression has been demonstrated in many different cancer cell types,[39,41]and has even been suggested as a potential prognostic indicator for hepatocellular carcinoma.[42] HNSCCs specifically have been shown to express IL-35.[43,44] These tumour cells hijack the immunosuppressive potential of IL-35 to avoid host immune detection and thus promote tumour cell survival.

IL-35 has been found to be a suppressor of both the immune and inflammatory system.[39] It has been shown to suppress the activity of pro-inflammatory T helper (T_H) cells 1 and 17, as well as causing the downregulation of pro-inflammatory cytokines such as IL-17.[39,43,45] Further, IL-35 promotes upregulation of anti-inflammatory cytokines, such as IL-10, and can expand T_{reg} cells, thus promoting T_{reg}-mediated suppression of the immune response.[43,45] IL-35 can also convert cytotoxic T cells into T_{reg} cells[46] and induce alternative macrophage phenotype switching from M1 to the anti-inflammatory M2 form.[47] These events work synergistically to suppress the immune system.

Contrastingly, in response to TNFα and interferon-δ stimulation, intrinsic IL-35 over-expression has been shown to inhibit cancer cell growth in vitro.[39] This occurred through serum starvation-induced apoptosis via downregulation of cyclin D1 and survivin expression,[39] as cyclin D1 is factored in the G1/S cell cycle transition[48] and survivin is an apoptosis suppressing protein.[49] Further, hyper-expression of intrinsic IL-35 was shown in the same study to increase tumour apoptosis via both the extrinsic pathway, through upregulation of the *Fas* gene, and the intrinsic pathway, through downregulation of the B-cell lymphoma 2 (*Bcl-2*) gene.[39] This demonstrates that both upregulating intrinsic IL-35 expression and downregulating extrinsic IL-35 expression are potential targets in the fight against cancer.

Long *et al.*, 2016, linked IL-35 to anti-tumour activity in hepatocellular carcinoma, as lower IL-35 concentrations at the site of the tumour were recorded at more advanced stages of the disease.[50] They confirmed that over-expression of IL-35 in these cells may cause upregulation of HLA-ABC (human leukocyte antigen-ABC) and cluster of differentiation 95 (CD95).[50] HLA-ABC is a gene complex that encodes MHC molecules,[51] so upregulation makes the cancer more easily detected by the immune system.[50] The contrasting results compared to previous literature may be due to the IL-35R having homodimeric or heterodimeric formations, with differing cell signalling cascades resulting in each case.[50] This highlights the importance of the IL-35R as well as IL-35 itself in immune tolerance mechanisms.

The mechanisms through which IL-35 exerts its effects are still not fully understood. IL-35 has been shown to be immunosuppressive[38] as well as demonstrating anti-tumour activity.[39,50] Tumour-derived IL-35 poses a clinical problem to medicine as it enables tumour cells to develop immune tolerance. Greater understanding of the specific mechanisms by which IL-35 induces immune tolerance therefore has clinical relevance as novel therapeutic drug targets to treat tumours could be discovered.

The target cell lines

Heterogeneity within different HNSCCs, such as the disease anatomical site and drainage routes through veins and lymph nodes, results in different pharmacological therapy mechanisms with each

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disease.[4] Therefore, HNSCCs should not be considered as a single disease type and we thus investigated four HNSCC cell lines: FADU, H357, C1 and VB6. The HaCat and MG-63 cell lines were investigated as positive and negative controls respectively. FADU was isolated from a human hypopharyngeal squamous cell carcinoma[52] and has been shown to express both IL-35 subunits.[53] This epithelial cell line is HPV-negative[54] and is characterised by overexpression of the EGFR.[55] H357, C1 and VB6 are HPV-negative[56] progressive cell lines linked to one another, dependent on the presence of the αvβ6 integrin.[57] This integrin has been highlighted as important in HNSCC.[58] H357 is a polygonal cell line and was established from a human oral squamous cell carcinoma of the tongue.[57,59] H357 is negative for the αv and β6 integrin subunits.[60] αv transfection of H357 produced the V3 cell line,[61] which was infected with a retrovirus containing β6 cDNA to yield the αvβ6 positive VB6 cell line.[58,60] C1 is a null transfectant control cell line of VB6, so is αv positive and β6 negative.[58,60] H357, C1 and VB6 have all been shown to express both IL-35 subunits.[53] The MG-63 cell line was isolated from a human osteosarcoma and is fibroblastic.[62] The HaCat cell line was established as an immortalised (>140 passages) form of human adult skin keratinocytes, and occurred through spontaneous transformation.[63,64] HaCat cells are epithelial and differentiate normally, so are not tumourigenic.[63] The THP-1 monocyte cell line was isolated from human acute myeloid leukaemia and was used to represent human macrophages in vitro.

The HNSCC cell lines C1, H357 and VB6, as well as the human keratinocyte cell line HaCat, induced significant hTNFα production by THP-1 cells in co-culture in vitro. The HNSCC cell line FADU and human osteosarcoma cell line MG-63 failed to induce hTNFα production. H357, VB6 and HaCat CM induced significant hTNFα production by THP-1 cells in co-culture, suggesting that soluble factors released by these cell lines are responsible for THP-1-mediated hTNFα production. THP-1 cells treated with IL-35 were subject to a significant reduction in hTNFα production when co-cultured with VB6. hTNFα is an integral cytokine in the tumour-macrophage relationship between HNSCC cell lines and the host immune system. The role of IL-35 in tumour immune tolerance and as a therapeutic drug target is an exciting future prospect for further research.

Acknowledgments

None.

Conflicts of Interest

None.

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