Tertiary lymphoid structure can be a potential therapeutic target for head and neck squamous cell carcinoma (Review)

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Received June 12, 2025; Accepted October 6, 2025

DOI: 10.3892/mco.2025.2916

Abstract. Tertiary lymphoid structure (TLS) is a kind of ectopic lymphoid tissues that form in chronic inflammation or tumor microenvironment (TME) and is structurally similar to secondary lymphoid organs such as lymph nodes, containing B cell follicles, T cell regions, and mature dendritic cells. It was reported that TLS is associated with improved prognosis and higher response rates to immunotherapy in a variety of tumors. However, the mechanism of TLS and its clinical significance in head and neck squamous cell carcinoma (HNSCC) remains unclear. In the present study, the latest advances of biological characteristics, research progress of TLS and its role in the TME were summarized. The relationship between the prognosis and response to immunotherapy of distinct cancers and the location, density and maturity of TLS were addressed. Predictive, therapeutic and prognostic values of TLS in HNSCC were also reviewed. In addition, the therapeutic value of TLS and the potential strategies for patients with HNSCC by regulating TLS were also discussed. Collectively, TLS can be regarded as an independent predictive biomarker for immunotherapy in HNSCC and appears to have a close correlation with the prognosis of HNSCC. Despite little acknowledge of mechanisms and few preclinical/clinical trials on TLS-targeted therapy, TLS-based therapy can provide a new potential therapeutic strategy.

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Key words: tertiary lymphoid structure, tumor microenvironment, head and neck squamous cell carcinoma, biomarker, immunotherapy

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1. Introduction

Tertiary lymphoid structure (TLS) refers to an assembly of immune cells with a definite organized structure that is acquired in non-lymphoid tissues out of the normal physiological conditions (1). It is a kind of ectopic lymphoid structure which is similar to secondary lymphoid organ (SLO). TLS mainly consists of B cells, T cells, dendritic cells (DCs), macrophages, fibroblast reticular cells, germinal centers (GCs) and high endothelial venules (2-4). TLS usually forms in the chronic inflammatory microenvironment derived of autoimmune diseases, chronic infections and tumors. TLS can be located in the stroma, intra-tumoral and peritumoral areas.

TLS initially forms in perivascular regions, which are rich in extracellular matrix, microvessels, lymphatic vessels and neurons (Fig. 1). These regions are relatively conserved in various organs of the organism, providing a unique niche for resident immune cells in the tissue. At first, TLS appears as small T/B cell aggregates, which then gradually expands and matures to form complex structures containing different B and T cell partitions (5,6). During the immature phase of TLS, antigen presentation and T cell-B cell interactions jointly drive lymphocyte activation and TLS maturation. Accompanied by this process, the previously resident fibroblasts undergo differentiation and switch to multiple phenotypes to facilitate the overall development of TLS (7). The core region of TLS is composed of $CD20^+$ B cells, which are closely surrounded by $CD3^+$ T cells, forming a lymphoid follicle structure similar to that in the SLO (8). Although the specific cellular composition of different TLS may vary, $CD4^+$ T follicular helper (Tfh) cells usually dominate the T cell area, and $CD8^+$ cytotoxic

T cells, $CD4^+$ T helper 1 (Th1) cells and regulatory T cells (Tregs) can also be observed (9). In addition to the B and T cell populations that constitute the major portion of TLS immune cells, TLS also contains a diverse subset of DCs, such as $CD21^+$ follicular DCs derived from the mesenchyme, which are primarily localized in the follicular region and are essential for TLS function (10). In addition, the follicles are interspersed with $CD68^+$ macrophages responsible for the clearance of apoptotic cells, a function similar to their role in SLO. According to the maturity of TLS, TLS can be at least divided into three different states: Lymphoid aggregate, immature TLS and mature TLS (Table I). Immature TLS could also be further divided into two subtypes: Conforming TLS and deviating TLS (11,12).

In the present review, an overview of the biological characteristics (such as components and classifications) was provided. The latest advances of the close correlation between the location, density and maturity of TLS and tumor prognosis and response to immunotherapy were reviewed. The predictive, therapeutic and prognostic values of TLS for head and neck squamous cell carcinoma (HNSCC) were also stated. Promising therapeutic strategies targeting TLS for HNSCC and potential methods to enhance the efficacy of current treatment were also summarized.

2. Predictive, therapeutic and prognostic values of TLS in distinct tumors

As an important site of tumor-specific immune response, TLS significantly affects the progression and outcome of tumors by enhancing local immune response (1,7,8). In the tumor microenvironment (TME), the formation and maintenance of TLS is the joint consequence of multiple immune cells and chemokines. B cells, T cells, DCs and other immune cells aggregate to form a complex cell network, thereby promoting the local reaction of immune cells and tumor-specific antigens (9). Since TLS has multiple potential roles in antitumor immunity, further study on its biological characteristics and application potential in tumor immunotherapy will provide new ideas and strategies for improving the efficacy of antitumor immunity. Previous studies revealed that continuous antigen presentation and recognition were observed within TLS. In addition, there is also a correlation between the formation of TLS and antigen-specific T cell responses. Presence of TLS and its higher degree of maturity are generally associated with an improved prognosis in malignancies and infection (13,14). In autoimmune diseases and organ transplantation, TLS often aggravates local immune reactivity. It has been reported that TLS is closely related to improved prognosis and higher response rate to the immunotherapy (15). TLS also exhibits predictive value for immune checkpoint blockade (ICB) treatment. Therefore, accurate assessment such as the location, density and maturity of TLS can provide great value for predicting the prognosis of patients and developing novel targeting therapies for malignancies.

The location of TLS shows significant influence on tumor prognosis. TLS is distributed both within the tumor and in the peritumoral regions. TLS in different locations shows certain differences in immune response and tumor prognosis. In most malignancies, stronger antitumor immune response and improved prognosis was found in patients with larger quantity of intra-tumoral TLS. By contrast, TLS that have an adverse effect on disease prognosis are more commonly found in the peritumoral region. There was a strong link between the intra-tumoral TLS formation and maintenance and improved tumor prognosis in oral cancers (16). It was reported that TLS within the tumor was correlated with more infiltrating B cells and $CD4^+$ T cells (17). TLS in the intra-tumoral areas was characterized with more memory B cells and macrophages. On contrary, TLS in the peritumoral areas tended to exhibit higher epithelial-mesenchymal transition activity and facilitated tumor progression and immune escape. TLS located in the intra-tumoral region was more likely correlated with lower TNM stage while TLS found in the peritumoral regions exhibited the opposite outcome (18,19). In a study on recurrent/metastatic HNSCC, patients with TLS which located close to tumor cells intended to have improved prognosis (15). Studies on breast cancer (20), melanoma (21), non-small cell lung cancer (NSCLC) (18) and colorectal cancer (22,23) have shown that intra-tumoral TLS was associated with an improved prognosis, while peritumoral TLS not. This indicated that the spatial distribution of immune cells in TLS had a great significance on tumor prognosis. Notably, TLS in the peritumoral regions was not always associated with poor prognosis (24,25). It was reported that TLS in the peritumoral regions had a close correlation with improved prognosis in hepatocellular carcinoma (HCC), while TLS in the intra-tumoral regions was associated with poorer prognosis and higher tumor invasiveness (26,27). The relationship between the spatial location of TLS and prognosis in different tumors shows significant heterogeneity. It remains unclear why TLS plays different roles in distinct tumors. It may be related to TLS maturity, cell composition in TLS, cytokines and immunogenicity. Because of this, it is crucial to analyze the impact of TLS on prognosis in combination with the specific type of tumor.

The density of TLS in the TME is closely related to the prognosis of tumor patients. More immune cells are contained in the TLS with higher density. Immune cells in TLS can recognize and fight tumor cells and enhance the immune response, thereby leading to improved survival benefits. TLS has been proved to be an independent prognostic marker, which could predict the response rate of immunotherapy in different tumors (22,28-33). Improved overall survival (OS) and lower recurrent rate were found in patients with high-density TLS (24,34,35). In a single-armed, phase II trial (ChiCTR2200066119) on advanced oral squamous cell carcinoma, neoadjuvant immunochemotherapy (treatment included PD-1 monoclonal antibody camrelizumab, nab-paclitaxel and cisplatin) was proved to significantly increase the density of TLS, along with bringing a promising response rate and prognosis (36). In a study on recurrent/metastatic HNSCC, the density of TLS was proved to predict the response to immunotherapy with 80% accuracy (37). Therefore, TLS density could be regarded as an independent predictive biomarker for patients with HNSCC which accepted ICB. In spite of this, there is no direct evidence to prove TLS to be an independent prognostic biomarker, and more preclinical studies and clinical trials should be carried out to link the density of TLS and prognosis of HNSCC.

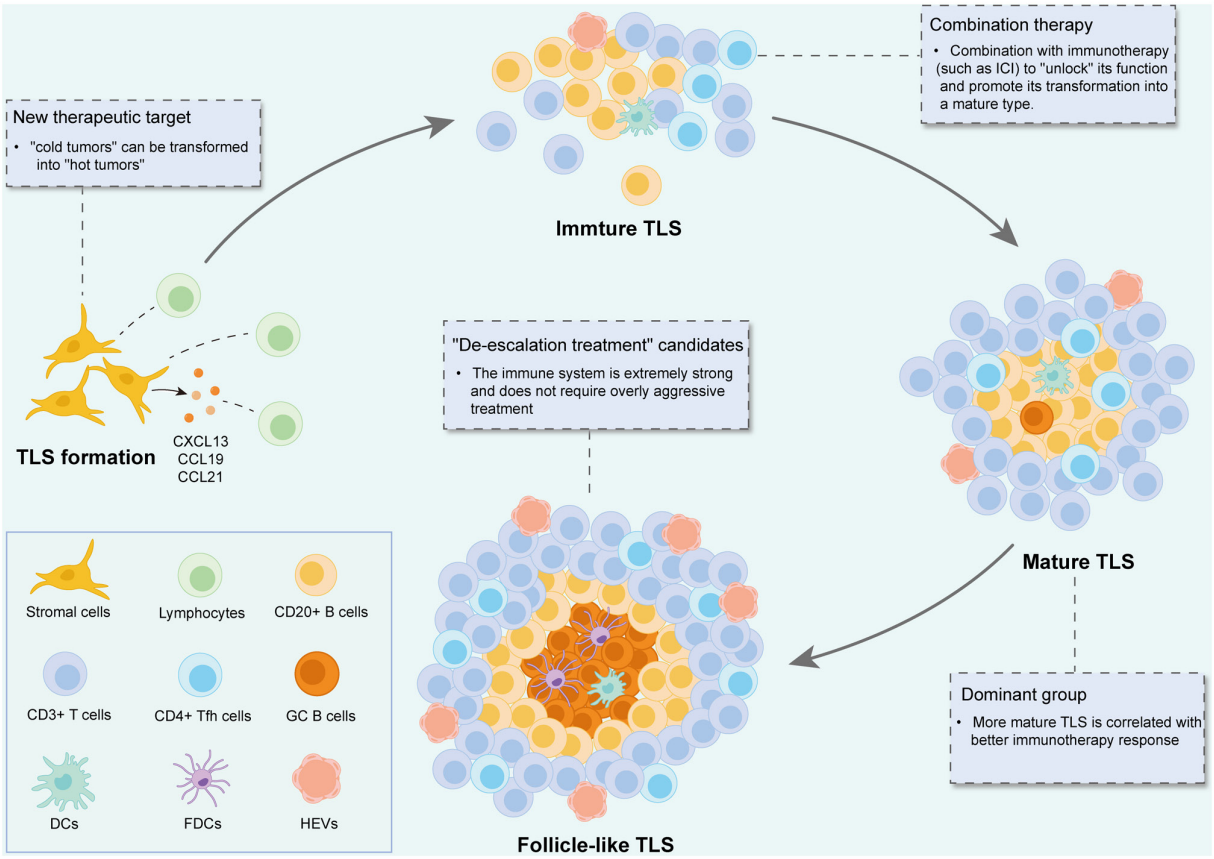

Figure 1. TLS formation, classification, and their respective clinical impacts across different cancers including head and neck squamous cell carcinoma. TLS, tertiary lymphoid structure.

The maturity of TLS may also play an important role in predicting the prognosis of tumor patients. Mature TLS is more likely to contain functional GCs which promote the production of high-affinity antibodies and induce immune responses against tumor antigens (13,14,38). By contrast, immature TLS may not be able to sufficiently activate immune cells, which in turn makes it difficult to recruit enough immune cells to eliminate the tumor cells. This may explain why immature TLS is associated with poorer prognosis. More mature TLS was proved to be related to improved prognosis of patients with HNSCC (39). In addition, higher GC activity was proved to have a correlation with improved OS of patients. This also illustrates that mature TLS could be regarded as a prognostic biomarker. In non-metastatic colorectal carcinoma, high density of TLS was proved to be correlated with high tumor mutation burden and low recurrent rate (22,40). A total of 3 different TLS was identified according to the maturity: early TLS, primary follicle-like TLS and secondary follicle-like TLS in laryngeal squamous cell carcinoma (LSCC) (41). In addition, follicle-like TLS could serve as an independent positive prognostic biomarker for LSCC, as more infiltrating immune cells and higher response rates were found in patients with follicle-like TLS. Studies on NSCLC (42) and pancreatic cancer (43) have also shown that the maturity of TLSs played an important role in influencing the immune response of patients. In a study on pancreatic ductal adenocarcinoma (PDAC), B cells within mature TLS were proved to exhibit an immunomodulatory efficacy and could activate the antigen presentation process (44). On the contrary, immature TLSs lack clear tissue structure, which does not significantly improve the prognosis of patients. Therefore, mature TLSs predict a stronger immune response and improved prognosis, thereby contributing to improve OS of patients.

3. TLS can be a biomarker and a potential therapeutic target for HNSCC

Since the composing proportions of diverse cells within TLS have been reported to vary in different types of tumors, the roles of TLS in distinct tumors can also be diverse. Different degrees of maturation and cell proportions of TLS have been demonstrated to determine the heterogeneity of TLS in distinct tumors (45). In a pan-cancer study, TLS was demonstrated to have a close relationship with gene mutations regarding tumor suppressor, immune regulation, DNA repair (46). Interestingly, increasing TLS was also proved to be linked to the Epstein-Barr virus infection in gastric cancer and human papillomavirus (HPV) infection in HNSCC (46-49). Patients with more TLS also had the characteristics of more T lymphocyte infiltration, higher TCR/BCR activation and more potent antigen presentation (50).

PD-L1 Combined Positive Score (CPS) is currently a clinically standard biomarker approved by the FDA for the selection of anti-PD-1/PD-L1-based immunotherapy (36). However, not all tumors with high PD-L1 expression rely

Table I. TLS formation, classification and their respective clinical impacts.

Descriptions			
TLS formation	Immature TLS	Mature TLS	Follicle-like TLS
Ectopic lymphoid tissue spontaneously formed by lymphocytes and stromal cells in chronic inflammatory tissues (such as tumors). 1. Trigger: Tumor antigen / DAMPs release, chronic inflammation. 2. Recruitment: Stromal cells secrete CXCL13/CCL19/ CCL21 to recruit lymphocytes. 3. Organization: Lymphocytes aggregate to form T/B cell partitions. 4. Maturation: With the assistance of Tfh cells, the GC is formed in the B-cell region.	Lymphocyte aggregates lacking GCs are the early stage of TLS formation. It is mainly a mixed aggregation of $CD3^+$ T cells and $CD20^+$ B cells, with no clear zoning. The main function is lymphocyte aggregation, which fails to effectively initiate high-affinity antibody responses and immune memory.	Structured TLS with clear T/B cell zoning and GCs, fully functional. The T-cell region ($CD3^+$) and B-cell region ($CD20^+$) are clearly defined. Within the B-cell region, there is a GC (composed of activated B cells with $CD23^+$ and $Ki67^+$ and $CD4^+$ $PD\text{-}1^+$ Tfh cells). The function is active: It can perform antigen presentation, T/B cell activation, clone screening and affinity maturation, and produce high-affinity antibodies.	A special mature TLS, whose structure is highly similar to the lymphoid follicles in secondary lymphoid organs with strong function. Structurally intact follicles, including dark/bright areas, follicular dendritic cell networks and high endothelial microveins. It can generate extremely powerful and long-lasting antitumor immune responses.
Clinical meanings			
New therapeutic target: By administering LT βR agonists, CXCL13, oncolytic viruses, etc. To induce TLS formation, 'cold tumors' can be transformed into 'hot tumors'.	Combination therapy is required: It needs to be used in combination with immunotherapy (such as immune checkpoint inhibitor) to 'unlock' its function and promote its transformation into a mature type.	Patients with more mature TLS are the advantageous population for immunotherapy: They can significantly benefit from immunotherapy alone.	May identify the potential 'de-escalation treatment' candidates: Due to their extremely strong immune system, they may not require overly aggressive treatment.

TLS, tertiary lymphoid structure; GC, germinal center.

on the PD-1/PD-L1 pathway for immune escape. This leads to a nonnegligible high rate of false positives for CPS. TP53 mutations impair DNA repair and lead to abnormal cell proliferation (51). Although TP53 mutation was recognized to be related to tumorigenesis of HNSCC, there is no evidence to identify TP53 as an independent predictive biomarker for HNSCC (52). EGFR overexpression facilitates tumor growth, invasion and therapy resistance. High EGFR expression was reported to be a negative prognostic factor in HNSCC. However, EGFR gene copy number and EGFR mutations just showed no prognostic value in HNSCC. EGFR appears to be unstable to be a predictive biomarker in HNSCC and need more acknowledge into its underlying mechanisms (53). The predictive value of tumor-infiltrating lymphocytes (TILs) may be compromised depending on the functional status. There may be a large number of exhausted T cells in the tumor, which are present but have lost their eliminating ability (37). Mature TLS comprises $PD\text{-}L1^+$ cells, $CD8^+$ T cells, $CD4^+$ Tfh cells, B cells and DCs. TLS integrates the information of PD-L1 and TILs and shows that there are effective interactions occurring among these components. This is more meaningful than measuring a single indicator alone. The existence of TLS

Table II. Clinical trials on TLS in HNSCC.

Trial name	Tumor	Purpose	Findings concerning TLS
NCT03906526	HNSCC	To evaluate the efficacy of αPD-1 immunotherapy combined with TLR8 agonist.	αPD-1 immunotherapy combined with TLR8 agonist increased TLS formation.
ChiCTR2200066119	OSCC	To evaluate the efficacy of camrelizumab combined with albumin-paclitaxel/cisplatin as preoperative neoadjuvant therapy for locally advanced OSCC.	Neoadjuvant immunochemotherapy significantly increased the density of TLS, along with bringing a promising response rate and prognosis.
NCT02519322	Melanoma	To evaluate the efficacy of nivolumab and relalimab combination therapy on patients with advanced melanoma.	Intra-tumoral TLS was associated with an improved prognosis, while peritumoral TLS not.

TLS, tertiary lymphoid structure; HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma.

suggests an active and ongoing antitumor immune response, rather than just the static presence of immune cells. The antibodies produced by B cells can form immune complexes, which further activate DCs and macrophages, amplifying the immune response (22).

Since TLS exhibits a great value for prediction and treatment for HNSCC (54-56), a widely recognized and standardized detecting and assessing procedure is in dire need. It was reported that TLS formation in the TME had a close correlation with the prognosis and response to immunotherapy in HNSCC (57-64). TLS in HNSCC was reported to be located in the peritumoral (tumor stroma) region and exhibit a positive correlation with the prognosis and response to immunotherapy for patients with HNSCC (65,66). TLS also exhibited a superior prognostic value compared with the current routine WHO grading and TNM staging system (62,67,68). A subtype of TLS was identified and proved to be related to higher immunotherapy response, which was mostly found in the HPV-positive HNSCC (69). However, the mechanism of how HPV infection regulates TLS stays unclear. TLS maturity was also demonstrated to be correlated with HPV infection. In an HPV negative HNSCC mouse model, enriched TLS was proved to improve the efficacy of αPD-1 immunotherapy and increase the infiltration of $CD8^+$T lymphocytes and DCs (70-72). Immature TLS in HNSCC was reported to be related to more infiltrating Tregs and induce resistance to PD-1/PD-L1 blockade immunotherapy (73,74). More TLS was also related to lower post-operative recurrence rate in HNSCC and HCC (75,76). Apart from being a predictive and prognostic biomarker for malignancies, TLS also exhibits a therapeutic value. Therefore, how to conduct precise regulation of TLS will be a promising therapeutic strategy for tumors in the near future. TLS is an important site for antitumor immune responses in the TME. Accordingly, it can also be a treatment alternation when TLS-targeting therapy combines with other antitumor treatment. A previous study on ovarian cancer indicated that TLS facilitate cytotoxic chemotherapy (77). In a study on PDAC, similar conclusion was also drawn that TLS improved the response to neoplastic cells (44). Lymphotoxin α (LTα) was found high expressed in the TLS regions, thus LTα was also applied to induce TLS formation in HNSCC throughout a CD8-dependent way (78). Different antitumor therapies can induce TLS formation, maturation and activation to improve the efficacy of antitumor therapies. Injection of CXCL 13 combined with CCL219 was proved to induce TLS formation within the orthotopic tumor (44). Activation of telomerase reverse transcriptase was also reported to have a correlation with TLS formation (79). In glioma, Toll-like receptor (TLR) agonists plus glioma antigens were used to successfully induce the TLS formation and improved immune cell infiltration (80). Low-dose STING agonist was also utilized to induce TLS formation and increase TILs in melanoma (81). Although there is no unified method to induce TLS, significant progress has been made in TLS induction through immunotherapy, targeted therapy, radiotherapy and chemotherapy. It has been reported that neoadjuvant ICB immunotherapy could activate TLS by inducing clonal expansion of B-cells (82,83). A triple therapy including IL-12, pembrolizumab (a PD-1 inhibitor) and cisplatin was demonstrated to elicit TLS formation and bring promising OS of HNSCC (84,85). A phase Ib clinical trial (NCT03906526) revealed that αPD-1 immunotherapy combined with TLR8 agonist significantly increased TLS formation and the infiltration of $CD8^+$ T cells and DCs (86, Table II). In the future, the induction of TLS may become a new target for enhancing tumor immune infiltration.

Although TLS formation in HNSCC has a close correlation with the prognosis and response to immunotherapy, how to directly induce TLS to develop into an antitumor phenotype stays an obstacle currently. The formation of TLS is a highly ordered process involving multiple cells (lymphocytes, stromal cells), cytokines (such as CXCL13, CCL19, CCL21 and LTαβ) and chemokines. Currently, there is no single 'universal drug' that can perfectly simulate this complex process. The use of a single agonist (such as LTβR agonists) may only induce structurally incomplete and functionally immature TLS (merely lymphocyte aggregation) but fail to form functional TLS with GCs. This would result in a significant reduction in therapeutic efficacy. In spite of this, combination with ICR, multiple induction and local delivery

system can be beneficial to TLS-based therapy. Therefore, TLS-targeted therapy depends on in-depth understanding of its complex biology and precise clinical manipulation capabilities.

4. Conclusion

The cellular component and biological characteristics of TLS were reviewed. The correlation and clinical meaning of the location, density and maturity of TLS in distinct tumors was stated. TLS with high density and maturity which was within the intra-tumoral regions exhibited a positive correlation with improved prognosis and higher response rates to immunotherapy in most malignancies. In several tumors (such as LSCC, NSCLC, breast cancer and melanoma), TLS was also regarded as an independent biomarker for prognosis or response to treatment. Predictive, prognostic and therapeutic values of TLS were also found in HNSCC. Studies and clinical trials have proved that TLS formation and activation significantly improved the efficacy of other treatments in HNSCC. Other therapeutic strategies such as neoadjuvant ICB were proved to successfully induce TLS in HNSCC. More studies are required to realize the precise regulation of TLS for HNSCC treatment in the future. To sum up, TLS could be a promising predictive, prognostic and therapeutic biomarker for HNSCC, and TLS-targeting therapeutic strategies could be a promising alternative treatment regime for patients with HNSCC in the future.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

JS approved the final list of included studies and revised the manuscript and performed the literature search and wrote the manuscript. XY performed the literature review. Data authentication is not applicable. Both authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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