Infectious Agents and Cancer

RESEARCH

Open Access

Tumor-infiltrating $CD4^+$ $CD25^+$ $FOXP3^+$ Treg is associated with plasma EBV DNA and disease progression in nasopharyngeal carcinoma

Enzi Feng[1†], Yaoyu Yang[1†], Jie Yang[1], Rongyi Hu[1], Ling Tian[1], Xinyu Yang[1], Meng Yang[1], Qianqian Qu[1], Yanxin Ren[1*] and Xiaojiang Li[1*]

Abstract

Background Regulatory T cells (Tregs) play a significant role in immune evasion within the tumor microenvironment (TME). Nasopharyngeal carcinoma (NPC) is strongly associated with Epstein-Barr virus (EBV) infection. Previous studies have shown that EBV can suppress immune activity. The relationship between plasma EBV DNA levels and Treg infiltration in NPC remains to be elucidated. Some studies have shown that FOXP3, a Treg marker, is a favorable prognostic factor in NPC. However, relying solely on FOXP3 for Treg identification may be unreliable due to its expression in other cell types. Therefore, this study investigated the impact of tumor-infiltrating Tregs identified by CD4, CD25, and FOXP3 triple markers in NPC and the relationship between these Tregs and EBV infection.

Methods In this study, 103 NPC patients were included. All tumor slides were stained using multi-immunofluorescence with CD4, CD25, and FOXP3. HALO software was used to analyze whole-slide images. The correlation between two factors was assessed using Spearman analysis. The prognostic value of factors was evaluated using Kaplan-Meier curves and Cox regression.

Results A significant positive correlation was observed between Treg infiltration in tumor tissues and plasma EBV DNA levels ($r=0.3428$, $p=0.02$). Higher Treg infiltration was significantly associated with poorer progression-free survival (PFS) ($p=0.03$) and was an independent risk factor for NPC progression ($p=0.045$). CD25 expression was positively correlated with plasma EBV DNA levels ($r=0.3229$, $p=0.03$). Furthermore, increased Treg infiltration was negatively correlated with peripheral $CD8^+$ T cells (r=-0.3556, $p=0.006$). The proportion of peripheral $CD8^+$ T cells in patients with advanced-stage NPC was significantly lower compared to those with early stage ($p=0.02$).

Conclusion This study identified tumor-infiltrating $CD4^+CD25^+FOXP3^+$ Tregs as an independent negative prognostic factor for NPC progression and found higher Treg infiltration positively associated with plasma EBV DNA levels.

Keywords Nasopharyngeal carcinoma, Regulatory T cell, Tumor-Infiltrating lymphocyte

†Enzi Feng and Yaoyu Yang are co-first authors.

*Correspondence:
Yanxin Ren
renyanxin8@163.com

Xiaojiang Li
xiaojiangli2024@163.com
[1]Department of Head and Neck Surgery, Third Affiliated Hospital of Kunming Medical University, No. 519 Kunzhou Street, Xishan District, Kunming 650118, China

Background

Nasopharyngeal carcinoma (NPC), arising from the nasopharyngeal epithelium, is one of the most common malignancies of the head and neck cancers. NPC exhibits a distinct geographical and ethnic distribution. According to the World Health Organization (WHO), 83.3% of new NPC cases in 2022 were reported in Asia, with China representing the largest proportion (50.9%) [1]. Research over the past several decades has demonstrated that NPC development is a complex, multifactorial process, primarily influenced by environment, genetics, and Epstein-Barr virus (EBV). NPC is classified histopathologically into four types: keratinizing squamous cell carcinoma (WHO type I), differentiated non-keratinizing squamous cell carcinoma (WHO type II), undifferentiated non-keratinizing squamous cell carcinoma (WHO type III), and basaloid squamous cell carcinoma [2]. Keratinizing squamous cell carcinoma is more common in regions with lower NPC prevalence, which accounts for one-third to one-half of NPC cases in Western countries. Conversely, in high-prevalence regions like China, over 95% of cases are non-keratinizing squamous cell carcinomas and are strongly associated with EBV infection [3, 4].

EBV is a human herpesvirus that is highly prevalent worldwide. EBV often causes asymptomatic infection during early childhood. However, delayed primary infection can manifest as infectious mononucleosis. Following primary infection, EBV can persist in the host for extended periods in a latent state. During latency, EBV expresses a subset of genes, many of which exhibit prooncogenic properties. Latent EBV proteins (EBNA1, LMP1, LMP2), EBV-encoded RNAs (EBERs) and BamHI A rightward transcripts (BARTs) RNA have been detected in infected nasopharyngeal epithelial cells. These viral products contribute to genomic instability and aberrant DNA methylation, promoting malignant transformation of nasopharyngeal epithelial cells [5]. Furthermore, EBNA1 has been implicated in promoting chemotaxis of regulatory T cells (Tregs) via upregulation of CXCL12 [6]. Tregs are a subset of immunosuppressive cells whose primary role is to prevent excessive immune responses that damage normal tissues. However, Tregs within the tumor microenvironment (TME) can facilitate tumor immune escape and suppress the anti-tumor activity of $CD8^+$ T cells. In many cancers, increased Treg infiltration is associated with poor prognosis. However, in NPC, greater Treg infiltration has been reported to be associated with improved prognosis [7, 8]. We notice that these studies relied solely on FOXP3 expression as Treg marker. FOXP3 expression alone is unreliable as a Treg marker because some activated effector T cells and certain cancer cells can express FOXP3, leading to potential misidentification of Tregs in tumor tissues. Consequently, the precise role of Tregs in NPC remains to be fully elucidated. Furthermore, quantification of EBV DNA in plasma serves as the most common clinical assessment of EBV viral load, and its association with the level of Treg infiltration in NPC remains unclear.

In this study, we analyzed Treg infiltration levels in NPC tissues from 103 patients using multi-immunofluorescence with more widely accepted Treg markers ($CD4^+CD25^+FOXP3^+$). We subsequently investigated the correlation between Treg infiltration and plasma EBV DNA levels, as well as the impact of tumor-infiltrating Tregs on the prognosis of NPC.

Methods

Patients

This study included 103 NPC patients at the Third Affiliated Hospital of Kunming Medical University between January 2017 and July 2021 who met the enrollment criteria. Detailed clinical data and tissue samples were collected for each patient. Clinical data included sex, age, tobacco and alcohol consumption, tumor stage, treatment received, and plasma EBV DNA copy number. Inclusion criteria were: (1) Pathologically confirmed diagnosis of NPC (2) No prior treatment, including radiotherapy, chemotherapy, or other cancer therapies (3) Availability of high-quality tissue samples for analysis (4) Absence of primary cancers in other organs or tissues (5) Completion of standard treatment protocols. Due to an updated EBV DNA testing method with increased sensitivity (from 5,000 copies/mL to 400 copies/mL) implemented in 2020, only the 44 most recent cases using new test were included in the analyses involving EBV DNA. Peripheral blood immune cell analysis ($CD8^+$ T cells; $CD4^+$ T cells; natural killer (NK) cells) was performed on 58 patients. Descriptive statistics of all cases was presented in Table 1. Tumor stage was determined according to the Chinese Society of Clinical Oncology (CSCO) staging system (2024 version). This study was approved by the Ethics Committee of the Third Affiliated Hospital of Kunming Medical University [KY201931].

Multi-immunofluorescence

All tissue slides were deparaffinized and rehydrated. Following heat epitope retrieval using citrate buffer, endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide solution in the dark. Hydrophobic barrier pen was applied to circle the tissue. Subsequently, non-specific binding sites were blocked with 3% bovine serum albumin (BSA) solution. The slides were incubated with the following primary antibodies diluted in antibody diluent overnight at 4 °C in a humidified chamber: CD4 (abcam, ab288724, 1:1000), CD25 (abcam, ab128955, 1:200), and FOXP3 (ServiceBio, GB11093, 1:400). Following washes with PBST, slides were

Table 1 Descriptive analysis of clinicopathological and molecular characteristics

Total patients	***n*** **= 103**	
Age	Median(range)	49(17–75)
Sex	Female	27(26.2%)
	Male	76(73.8%)
Smoker	Yes	43(41.7%)
	No	60(58.3%)
Alcohol	Yes	22(21.4%)
	No	81(78.6%)
Pathological grade	Undifferentiated carcinoma	59(57.3%)
	Differentiated carcinoma	44(42.7%)
cT	1	13(12.6%)
	2	20(19.4%)
	3	58(56.3%)
	4	12(11.7%)
cN	0	11(10.7%)
	1	27(26.2%)
	2	29(28.2%)
	3	36(35.0%)
cStage	I	12(11.7%)
	II	44(42.7%)
	III	42(40.8%)
	IV	5(4.9%)
Plasma EBV DNA[a] **(copies/mL)**	Median(Q1-Q3)	1221(407.8-2635.75)
Peripheral CD4$^+$cells%[b]	Median(Q1-Q3)	33.35(27.125–41.7)
Peripheral CD8$^+$cells%[b]	Median(Q1-Q3)	23(18.35–30.9)
Peripheral NK cells%[b]	Median(Q1-Q3)	17.85(13.5–27.9)
Tumor-infiltrating Treg% in CD4$^+$cells	Median(Q1-Q3)	1.65(0.45–3.67)
Treg Infiltration Score	Median(Q1-Q3) ($\times 10^{-4}$)	0.17 (0.03–0.52)
CD25 H Score	Median(Q1-Q3)	7.57(3.13–10.32)

a. All patients underwent EBV DNA test. However, because the EBV DNA testing method was updated in 2020, only the 44 most recent cases utilizing the new test were included in analyses involving EBV DNA

b. Peripheral blood immune cell analysis (including CD4$^+$ T cells, CD8$^+$ T cells, and NK cells) was performed on 58 patients

incubated with an HRP-conjugated secondary antibody (SeraCare, 5220 – 0336, 1:400) for 50 min at room temperature in the dark. Subsequently, TSA reagents (CY3 Tyramide, 488 Tyramide, and CY5 Tyramide, diluted in PBST with 0.003% H_2O_2) were applied and incubated for 20 min at room temperature. The epitope retrieval step was repeated prior to each staining cycle. This staining procedure was repeated for each marker. Finally, nuclei were stained with DAPI for 10 min at room temperature in the dark. Slides were then mounted using mounting medium. Coverslips were sealed with nail polish to prevent drying. Slides were observed under microscope and whole-slide images were acquired for analysis.

Image data analysis

HALO software (Indica Labs; Albuquerque, NM) was employed for the quantitative analysis of acquired images. Parameters were adjusted using the HALO Highplex FL analysis module. Following optimization of parameters, all images were analyzed using consistent thresholds. Co-localization analysis of CD4, CD25, and FOXP3 was conducted to quantify CD4$^+$CD25$^+$FOXP3$^+$ positive cells and determine their percentage within CD4$^+$ cells. Immune cells infiltration score was calculated as the number of positive cells per analyzed area (μm^2). CD25 expression was evaluated using a histochemistry score (H-Score), calculated as follows: H-Score = [1 × (weak positive cells%) + 2 × (moderate positive cells%) + 3 × (strong positive cells%)].

Survival analysis

Patients were stratified into two groups based on the median percentage of Tregs within CD4$^+$ T cell and the median CD25 H-Score. Progression-free survival (PFS) and overall survival (OS) were used as the primary endpoints. PFS was defined as the time interval between treatment initiation and disease progression, with the latter defined as either progression of the primary tumor or the development of new metastatic lesions. OS was calculated as the time interval from treatment initiation until death from any cause.

Statistical methods

Statistical analyses were performed using GraphPad Prism (version 9.0.0) and SPSS (version 27.0). All statistical tests were two-sided, and p-value less than 0.05 was considered statistically significant. Spearman analysis was used to assess correlations between variables. For group comparisons, one-way ANOVA test and Kruskal-Wallis test were employed, depending on whether the data met the assumptions of normality and homogeneity of variance. Dunn's test was used following a significant Kruskal-Wallis test. The log-rank test was used to compare Kaplan-Meier survival curves between groups. Univariate and multivariate analysis were using Cox proportional risk model to determine the influence of each variable.

Results

Tumor-infiltrating Treg positively correlated with plasma EBV DNA in NPC

In this study, CD4$^+$CD25$^+$FOXP3$^+$ cells were identified as Tregs, as illustrated in Fig. 1. Figure 2 showed weak, medium and strong staining of CD25. A positive correlation was observed between the proportion of Tregs

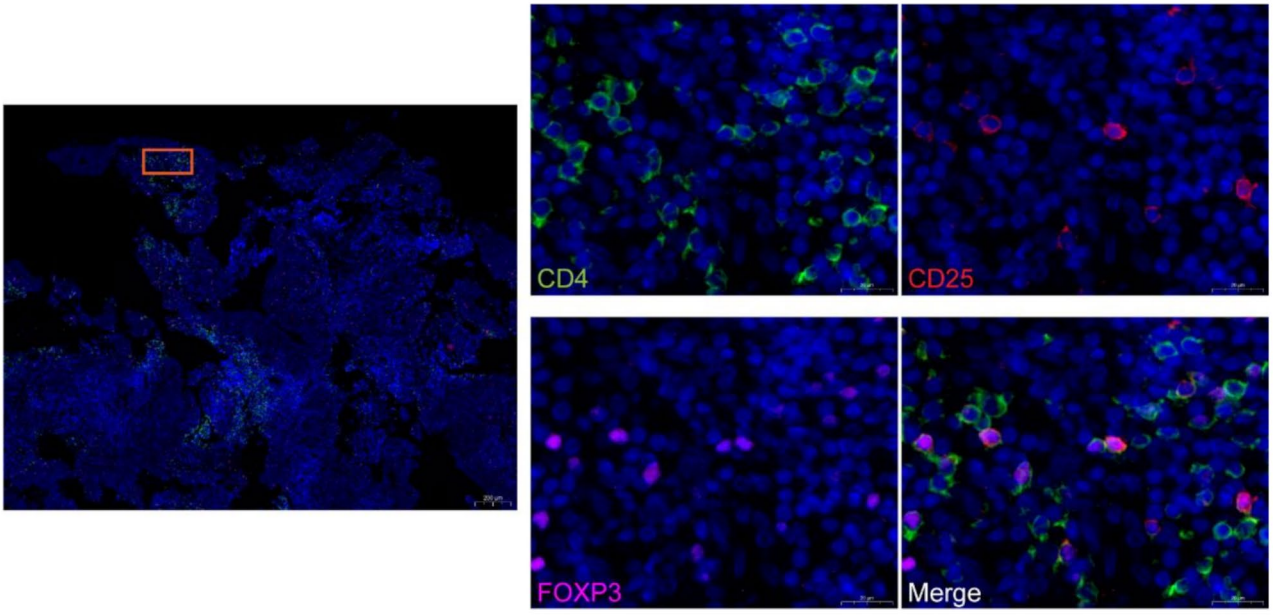

Fig. 1 The representative mIF image of NPC tissue (CD4: green; CD25: red; FOXP3: pink)

within the $CD4^+$ T cells in NPC tumor tissue and the corresponding copy number of EBV DNA in plasma. Specifically, an increase in EBV DNA was associated with an increase in the proportion of Tregs ($r = 0.3428$, $p = 0.02$) (Fig. 3A). A similar positive correlation was found between the Tregs infiltration score and plasma EBV DNA ($r = 0.31$, $p = 0.04$) (Fig. 3B). Given that CD25 expression is markedly elevated in activated Tregs, the correlation between CD25 expression and EBV DNA was also investigated. This analysis revealed a significant positive correlation between the CD25 H-Score and EBV DNA ($r = 0.3229$, $p = 0.03$) (Fig. 3C).

Tumor-infiltrating Treg is an independent risk factor for NPC progression

Survival analysis revealed that NPC patients with high levels of tumor-infiltrating Tregs exhibited a significantly greater PFS compared to those with low levels of tumor-infiltrating Tregs (HR = 2.268(1.107–4.645), $p = 0.03$) (Fig. 4A). NPC patients with high CD25 H Scores exhibited a shorter PFS, although this trend was not statistically significant (HR = 1.962(0.9577–4.019), $p = 0.07$) (Fig. 4B). Regarding OS, no statistically significant difference was observed (Treg%: HR = 1.051(0.2121–5.211), $p = 0.95$; CD25 H Score: HR = 2.102(0.4241–10.42), $p = 0.38$) (Fig. 4C-D). Subsequently, we conducted further analysis to assess the clinical significance of these two factors in the prognosis of NPC. Univariate analysis included the following variables: sex, age, tobacco and alcohol use, pathological grade, clinical stage, tumor-infiltrating Tregs and CD25 H Score. The results indicated that T3, T4, tumor-infiltrating Tregs and CD25 H Score were risk factors for NPC progression (Table 2). Multivariate analysis further revealed that T3, T4, and tumor-infiltrating Tregs were independent risk factors for PFS in NPC (Table 3). On the other hand, only stage IV was identified as a risk factor for OS in NPC (Table 4). We also analyzed the correlation between PFS and plasma EBV DNA copy number (Supplementary Fig. 1). Due to the small sample size ($n = 44$), no statistically significant difference was observed.

Correlation between tumor-infiltrating Tregs and peripheral immune cells

Furthermore, we investigated the correlation between tumor-infiltrating Tregs in NPC and $CD4^+$ T cells, $CD8^+$ T cells and NK cells in peripheral blood. A significant negative correlation was observed between peripheral $CD8^+$ T cells and tumor-infiltrating Tregs (r = -0.3556, $p = 0.006$). No significant correlations were identified between the other peripheral immune cells ($CD4^+$ T cells and NK cells) and tumor-infiltrating Tregs (Fig. 5A-C). Notably, NPC patients with stage III-IV exhibited significantly lower proportions of peripheral $CD8^+$ T cells compared to patients with stage I ($p = 0.02$). However, no statistically significant differences were observed in other peripheral immune cells and tumor-infiltrating Tregs across different stages (Fig. 5D-G).

Discussion

Plasma EBV DNA is a valuable tumor marker for NPC, exhibiting high sensitivity (96%) and specificity (93%) [9]. Evidence suggests that EBV in plasma and tumor tissue originate from the same viral clone. Plasma EBV DNA

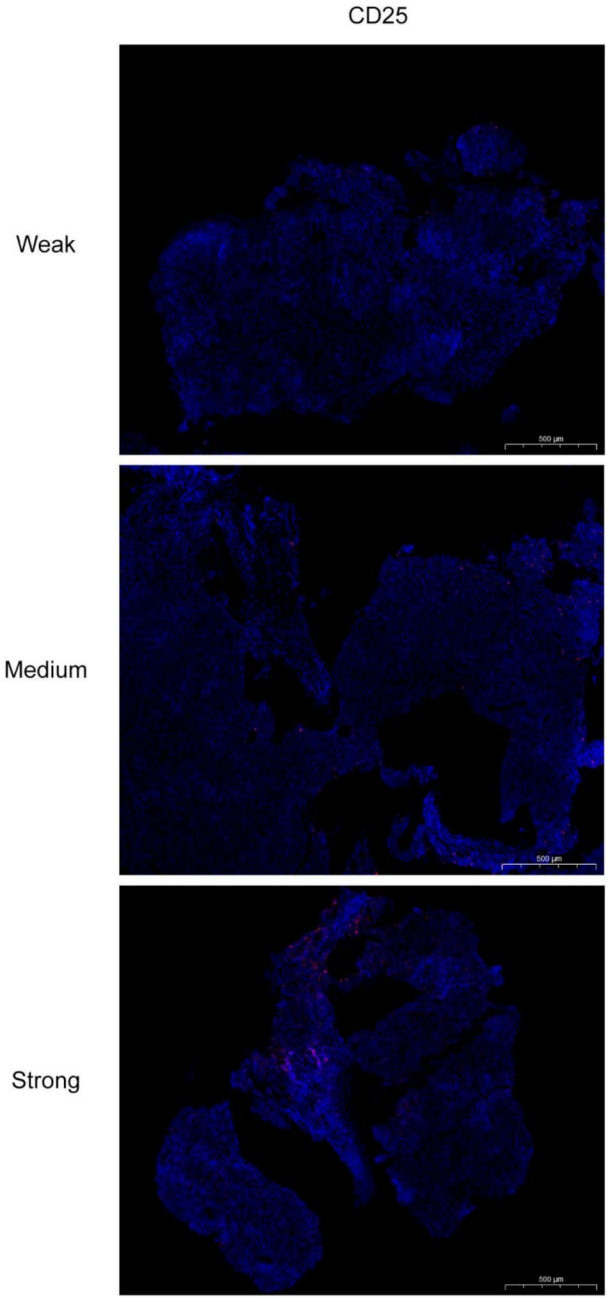

Fig. 2 The representative figure of weak, medium and strong staining of CD25

consists of small fragments derived from the disintegration and fragmentation of infected tumor cells (predominantly < 181 bp) [10, 11]. Therefore, plasma EBV DNA levels reflect both tumor burden and tumor EBV DNA load. Our study found a significant positive correlation between plasma EBV DNA and tumor-infiltrating Tregs in NPC, suggesting that high plasma EBV DNA is associated with a more immunosuppressive TME. This finding aligns with previous researches. K-M Lau et al. observed significant expansion of Tregs in the peripheral blood and the presence of Tregs in tumor tissues of EBV-positive NPC patients [12]. Moreover, Treg infiltration was found to be significantly higher in EBER-positive NPC tissues compared to EBER -negative tumour tissues [7]. Collectively, these findings suggest that EBV replication activity may be an indicative of the immunosuppressive status of NPC. In NPC, EBV nuclear antigen 1 (EBNA1) can stimulate the production of TGF-β1, promoting the differentiation of naive T cells into Tregs or enhancing the expression of chemokines like CCL20 and CXCL12, which facilitate Tregs migration [6, 13]. High Treg levels can impair the efficacy of immune effector cells, contributing to sustained tumor progression. Furthermore, the presence of Tregs within TME may contribute to the limited efficacy of targeted EBV tumor vaccines in patients with advanced NPC [14].

Survival analysis revealed that NPC patients with elevated tumor-infiltrating Treg levels demonstrated a higher propensity for disease progression. Moreover, Treg infiltration was identified as an independent risk factor for PFS. The impact of Treg infiltration on OS in NPC was also assessed. However, due to the very few death events of all cases, no statistically significant differences in OS were observed. Notably, our findings differ from those reported in previous studies. Stefan M Willems et al. found that, in NPC, higher tumour-infiltrating $FOXP3^+$ cell counts showed better OS [7]. Yi-Lan Zhang et al. observed a significant association between tumor-infiltrating Tregs and favorable OS and PFS in advanced NPC [8]. We believe that the discrepancy may stem from the use of different markers to identify Tregs. Previous studies rely solely on $FOXP3^+$ as Treg marker, which may lack sufficient specificity. Following stimulation of the human T cell receptor with CD3, non-suppressor effector T cells are observed to express FOXP3 [15]. Furthermore, FOXP3 expression has been reported in some tumor cells, including small-cell lung cancer, breast cancer, gastric cancer and colorectal cancer [16–19]. Our ongoing research indicates that FOXP3 is also expressed in EBV-positive NPC cells. Therefore, identifying the Treg population using $CD4^+CD25^+FOXP3^+$ as markers offers greater precision and clarity for elucidating its role in NPC development. Furthermore, a negative correlation was observed between tumor-infiltrating Tregs and peripheral $CD8^+$ T cell. Previous research has demonstrated that NPC patients with elevated circulating $CD3^+CD8^+$ T cell exhibit improved survival [20], providing indirect support for our finding that high infiltrating Tregs are associated with a poorer prognosis in NPC. On the other hand, our study analyzed complete tissue slides, enabling a more comprehensive assessment of Treg infiltration in NPC compared to studies that randomly select a few fields of view.

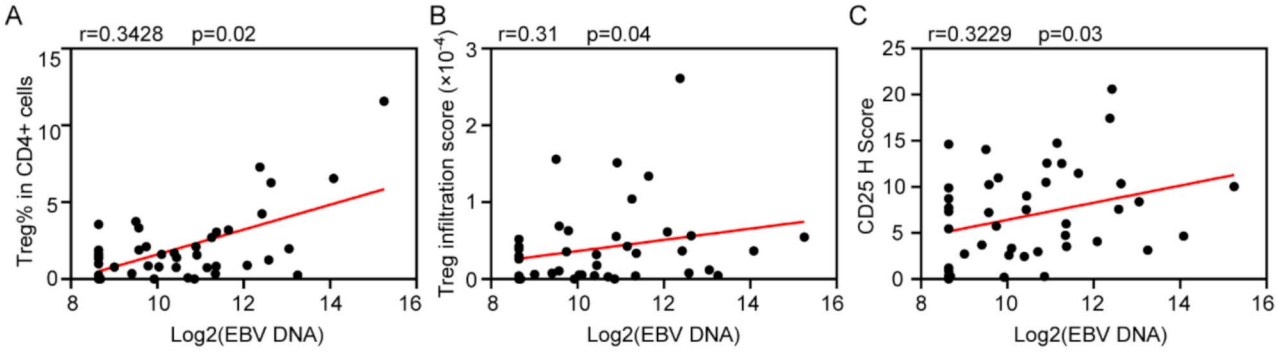

Fig. 3 The correlation between EBV DNA in plasma and Treg% (**A**), Treg infiltration score (**B**) and CD25 H Score (**C**) in NPC tissue

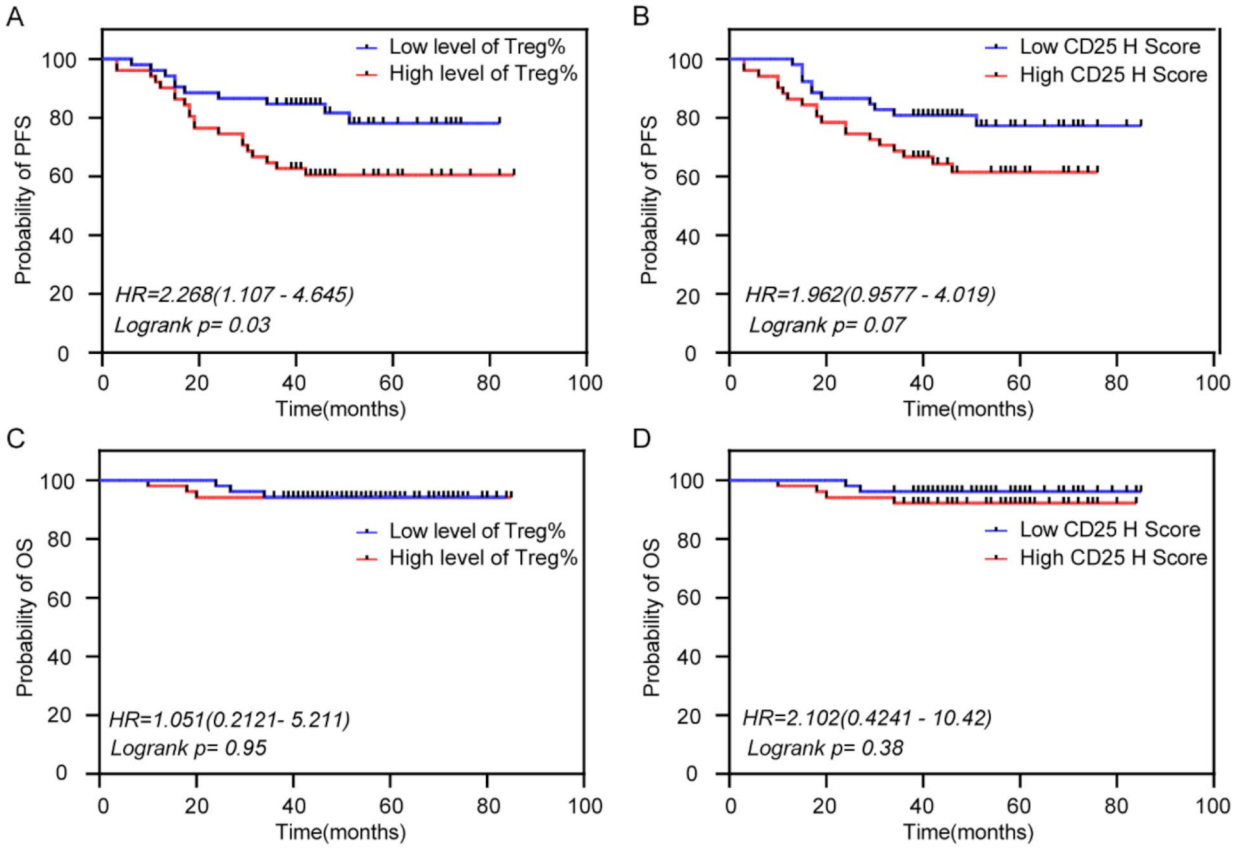

Fig. 4 Kaplan–Meier analysis of PFS and OS for high/low tumor-infiltrating Tregs groups (**A, C**) and high/low CD25 H Score groups (**B, D**)

CD25, the alpha chain of the interleukin-2 (IL-2) receptor, is highly expressed in Tregs. These cells inhibit the immune response by competitively binding IL-2 [21]. FOXP3 strongly inhibits IL-2 expression in Tregs, resulting in minimal IL-2 production. Consequently, Tregs depend on exogenous IL-2 for survival, leading to a depletion of IL-2 available for other immune cells. This hinders the differentiation of conventional T cells into effector T cell and its expansion, thus weakening the immune system's ability to eliminate harmful cells and contributing to tumor progression [22]. High CD25 expression in $CD4^+CD25^+FOXP3^+$ Treg has been reported as an independent risk factor for poor prognosis in colorectal cancer [23]. Our study also evaluated the predictive role of CD25 in NPC patients' survival. However, no statistically significant difference was observed in PFS between the high and low CD25 expression groups. The univariate analysis suggested that CD25 significantly influenced PFS, but this effect was not confirmed in the multivariate analysis. Our finding suggested that CD25

Table 2 Univariate survival analysis of clinical characteristics (PFS)

Statistic	HR (95%CI)	*p*
Sex		
Women vs. men	0.984(0.438–2.211)	0.97
Age	1.033(0.996–1.071)	0.08
Smoker		
Yes vs. no	0.677(0.317–1.446)	0.31
Alcohol		
Yes vs. no	0.723(0.277–1.888)	0.51
Pathological grade		
Undifferentiated carcinoma vs. differentiated carcinoma	1.430(0.699–2.926)	0.33
cT		
T3 vs. T1/2	3.580(1.049–12.218)	**0.04**
T4 vs. T1/2	17.132(4.675–62.774)	**< 0.001**
cN		
N2 vs. N0/1	1.136(0.423–3.050)	0.8
N3 vs. N0/1	2.022(0.872–4.687)	0.1
Treg% in $CD4^+$cells	1.817(1.050–3.143)	**0.03**
CD25 H Score	2.055(1.023–4.127)	**0.04**

Table 3 Multivariate survival analysis of clinical characteristics (PFS)

Statistic	HR (95%CI)	*p*
cT		
T3 vs. T1/2	3.570(1.045–12.193)	**0.04**
T4 vs. T1/2	18.569(4.917–70.121)	**< 0.001**
Treg% in $CD4^+$cells	1.990(1.016–3.895)	**0.045**
CD25 H Score	1.012(0.484–2.115)	0.97

Table 4 Univariate survival analysis of clinical characteristics (OS)

Statistic	HR (95%CI)	*p*
Sex		
Women vs. men	1.471(0.269–8.032)	0.66
Age	1.044(0.962–1.133)	0.3
Smoker		
Yes vs. no	0.269(0.031–2.304)	0.23
Alcohol		
Yes vs. no	0.034(0-134.656)	0.42
Pathological grade		
Undifferentiated carcinoma vs. differentiated carcinoma	0.682(0.125–3.725)	0.66
cStage		
III vs. I/II	0.434(0.045–4.174)	0.47
IV vs. I/II	8.866(1.469–53.507)	**0.02**
Treg% in $CD4^+$cells	2.203(0.686–7.077)	0.19
CD25 H Score	2.061(0.429–9.898)	0.37

may have potential predictive value in NPC and further investigation with a larger sample size is required to validate its significance as a prognostic factor.

Conclusions

This study utilized CD4, CD25, and FOXP3 triple markers to assess the degree of Treg infiltration within TME of NPC. Our analysis revealed a significant positive correlation between plasma EBV DNA and the tumor-infiltrating Tregs in NPC. Furthermore, patients exhibiting high Treg infiltration demonstrated a decrease in peripheral $CD8^+$ T cells and experienced inferior PFS. Whole-slide scanning was employed to enhance the accuracy of Treg infiltration quantification. However, due to limitations in specimen preservation and staining techniques, we were unable to achieve ideal staining results for earlier cases, and thus, only cases from the past five years were included, resulting in a relatively small sample size. Future studies with larger sample sizes are necessary to validate these findings. We will focus on elucidating the specific molecular mechanisms by which EBV modulates Treg infiltration, aiming to identify novel strategies for effective NPC immunotherapy.

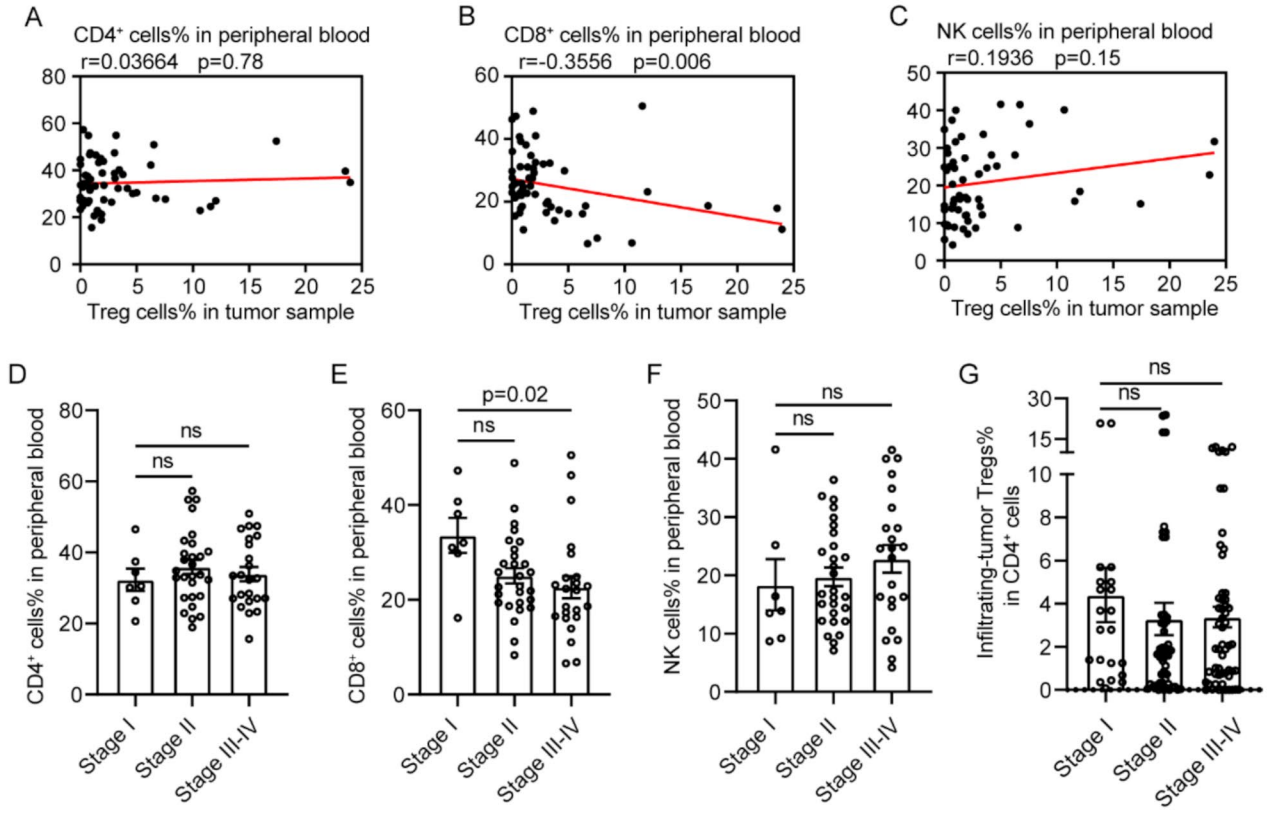

Fig. 5 The correlation between tumor-infiltrating Tregs and peripheral immune cells (**A-C**) and the change of immune cells across different clinical stage (**D-G**)

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13027-025-00660-4.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
X Li, Y Ren and E Feng contributed to the study conception and design. Data analysis and data visualization were performed by E Feng. Y Yang collected clinical data and followed all patients. J Yang helped slides preparation and staining. R Hu and L Tian collected all NPC samples. The first draft of the manuscript was written by E Feng. X Li and Y Ren reviewed the manuscript. X Yang, M Yang and Q Qu helped prepare the discussion.

Funding
This work was supported by the National Natural Science Foundation of China [Grant numbers 81960489, 82260485], Yunnan Ten Thousand People Plan"Young Top Talents"Project [Grant numbers YNWR-QNBJ-2019-056] and the Research Foundation of Education Bureau of Yunnan Province, China [Grant numbers 2023Y0658].

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Third Affiliated Hospital of Kunming Medical University [KY201931] and obtained consent from all patients for use of their tissue.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 7 December 2024 / Accepted: 29 April 2025
Published online: 09 May 2025

References

1. Freddie B, Mathieu L, Hyuna S, Jacques F, Rebecca LS, Isabelle S, Ahmedin J. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2024;74(3):229–63.
2. Zhi Yi S, Pui Yan S, Yu Yu L, Shiau-Chuen C. Epidemiology of nasopharyngeal carcinoma: current insights and future outlook. Cancer Metastasis Rev. 2024;43(3):919–39.
3. Hai-Yun W, Yih-Leong C, Ka-Fai T, Jacqueline SGH, Hai-Qiang M, Yan-Fen F, et al. A new prognostic histopathologic classification of nasopharyngeal carcinoma. Chin J Cancer. 2016;35:41.
4. Chen Y-P, Chan ATC, Le Q-T, Blanchard P, Sun Y, Ma J. Nasopharyngeal carcinoma. Lancet. 2019;394(10192):64–80.

5. Wong KCW, Hui EP, Lo K-W, Lam WKJ, Johnson D, Li L, et al. Nasopharyngeal carcinoma: an evolving paradigm. Nat Rev Clin Oncol. 2021;18(11):679–95.
6. Huo S, Luo Y, Deng R, Liu X, Wang J, Wang L, et al. EBV-EBNA1 constructs an immunosuppressive microenvironment for nasopharyngeal carcinoma by promoting the chemoattraction of Treg cells. J Immunother Cancer. 2020;8(2):e001588.
7. Ooft ML, van Ipenburg JA, Sanders ME, Kranendonk M, Hofland I, de Bree R, et al. Prognostic role of tumour-associated macrophages and regulatory T cells in EBV-positive and EBV-negative nasopharyngeal carcinoma. J Clin Pathol. 2018;71(3):267–74.
8. Zhang YL, Li J, Mo HY, Qiu F, Zheng LM, Qian CN, Zeng YX. Different subsets of tumor infiltrating lymphocytes correlate with NPC progression in different ways. Mol Cancer. 2010;9:4.
9. Allen KC, John C, Ann KSW, Benny K, Z CY, Jacky WK, Stephen L. Analysis of plasma Epstein-Barr virus DNA to screen for nasopharyngeal Cancer. N Engl J Med. 2017;377(6):513–22.
10. Allen KC, Jun C, Anthony Z, Kenny TCC, Sing-Fai IKL, Lisa L. Molecular characterization of Circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. Cancer Res. 2003;63(9):2028–32.
11. Allen KC, Anthony C, Sing-Fai TCC, Jesse L, Angela CSP, Joanna YMW. Investigation into the origin and tumoral mass correlation of plasma Epstein-Barr virus DNA in nasopharyngeal carcinoma. Clin Chem. 2005;51(11):2192–5.
12. Lau KM, Cheng SH, Lo KW, Lee SA, Woo JK, van Hasselt CA, et al. Increase in Circulating Foxp3 + CD4 + CD25(high) regulatory T cells in nasopharyngeal carcinoma patients. Br J Cancer. 2007;96(4):617–22.
13. Wang J, Luo Y, Bi P, Lu J, Wang F, Liu X, et al. Mechanisms of Epstein-Barr virus nuclear antigen 1 favor Tregs accumulation in nasopharyngeal carcinoma. Cancer Med. 2020;9(15):5598–608.
14. Hui E, Taylor G, Jia H, Ma B, Chan S, Ho R, et al. Phase I trial of Recombinant modified vaccinia Ankara encoding Epstein-Barr viral tumor antigens in nasopharyngeal carcinoma patients. Cancer Res. 2013;73(6):1676–88.
15. Gootjes C, Zwaginga JJ, Roep BO, Nikolic T. Defining human regulatory T cells beyond FOXP3: the need to combine phenotype with function. Cells. 2024;13(11):941.
16. Ziółkowska-Suchanek I, Żurawek M. FOXP3: A player of Immunogenetic architecture in lung Cancer. Genes. 2024;15(4):493.
17. Takenaka M, Seki N, Toh U, Hattori S, Kawahara A, Yamaguchi T, et al. FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. Mol Clin Oncol. 2013;1(4):625–32.
18. Ma G, Miao Q, Liu Y, Gao H, Lian J, Wang Y, et al. High FoxP3 expression in tumour cells predicts better survival in gastric cancer and its role in tumour microenvironment. Br J Cancer. 2014;110(6):1552–60.
19. Grimmig T, Kim M, Germer C, Gasser M, Waaga-Gasser A. The role of FOXP3 in disease progression in colorectal cancer patients. Oncoimmunology. 2013;2(6):e24521.
20. Zhu J, Fang R, Pan Z, Qian X. Circulating lymphocyte subsets are prognostic factors in patients with nasopharyngeal carcinoma. BMC Cancer. 2022;22(1):716.
21. Peng Y, Tao Y, Zhang Y, Wang J, Yang J, Wang Y. CD25: A potential tumor therapeutic target. Int J Cancer. 2022;152(7):1290–303.
22. Sakaguchi S, Mikami N, Wing J, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. Annu Rev Immunol. 2020;38:541–66.
23. Bergsland CH, Jeanmougin M, Moosavi SH, Svindland A, Bruun J, Nesbakken A, et al. Spatial analysis and CD25-expression identify regulatory T cells as predictors of a poor prognosis in colorectal cancer. Mod Pathol. 2022;35(9):1236–46.

Publisher's note