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Original Article

Characterization of the immune cell profile in metastatic nasopharyngeal carcinoma treated with chemotherapy and immune checkpoint inhibitors

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Abstract: Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV)-associated cancer, and immune checkpoint inhibitors (ICIs) have shown efficacy in its treatment. The combination of chemotherapy and ICIs represents a new trend in the standard care for metastatic NPC. In this study, we aim to clarify the immune cell profile and related prognostic factors in the ICI-based treatment of metastatic NPC. Programmed cell death ligand 1 (PD-L1) expression was measured in 81 metastatic tissue samples that had not received prior ICI treatment. The combined positive score (CPS) was positive in 58.0% of the samples, with a statistically significant correlation to median overall survival (OS) (CPS ≥ 1 vs. CPS < 1: 28 vs. 16 months, $P = 0.004$). For the combination treatment of metastatic NPC, 62 patients were enrolled in a retrospective analysis, yielding a median OS of 39.3 months. The objective response rate for this combination therapy was 71%, with a complete response rate of 45.2%. With a cutoff value of 4.8 for the pre-treated neutrophil-lymphocyte ratio (NLR) in peripheral blood (PB), the difference in median OS was statistically significant ($P = 0.021$). Thirty-seven patients received local treatment following the combination therapy of ICIs and chemotherapy, which provided additional survival benefits. Most hyper-responders exhibited a prolonged low NLR (< 3), a high total lymphocyte count, and an undetectable or stable EBV DNA load in PB during treatment. Peripheral blood mononuclear cells (PBMCs) from most patients receiving the combination treatment were rich in PD-1+CD8+ lymphocytes, which showed high expression of both IFN-γ and Granzyme B, demonstrating the ability to kill the EBV-positive NPC cell line and xenografts in vitro and in vivo. Responders also displayed increased levels of CD4+CD45RA-CCR7-CD28+CD57- cells (effector memory cell subset) in peripheral blood. These results indicate that in the context of combined chemotherapy and ICIs, high PD-L1 expression in pre-treated metastatic tumor tissue, a low NLR before treatment, a decrease in NLR after treatment, and local treatment can provide significant benefits for patients with metastatic NPC.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, immune checkpoint inhibitor, programmed death ligand 1, neutrophil-lymphocyte ratio, flow cytometry

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Introduction

Nasopharyngeal carcinoma (NPC) is prevalent in South Asia, including Taiwan [1]. A combination of host factors, environment, and *Epstein-Barr virus* (EBV) contribute to its pathogenesis [2]. Recently, rapid progress has been made in cancer immunotherapy, particularly in the use of immune checkpoint inhibitors (ICIs) including anti-cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), and PD-1 ligand (PD-L1) monoclonal antibodies (Mabs), for patient management [3]. Anti-PD-1/PD-L1 Mabs have shown a promising anti-tumor effect with a 20-31% overall response rate and acceptable side effects on multiply pre-treated recurrent or metastatic NPC in trials [4-7]. Furthermore, some Anti-PD-1/PD-L1 Mabs, including camrelizumab and toripalimab monotherapies or combinations with chemotherapy, have shown survival benefits in patients with recurrent/metastatic NPC in clinical trials [8, 9].

The main biomarkers used for the prediction of NPC response to ICI treatment are PD-L1 expression and the neutrophil-lymphocyte ratio (NLR) in the peripheral blood (PB) [4, 10]. NPC is unique in tumor immunology due to its high EBV expression rate in WHO types II and III, inflamed tumor microenvironment (TME) with dominant lymphocytes, low tumor mutation burden, and high PD-L1 expression [11-14]. Measurements of PB cells, including the neutrophil-lymphocyte ratio (NLR), absolute lymphocyte count, and platelet-lymphocyte ratio, have been shown to be practical biomarkers for the prediction of ICI treatment response [15, 16]. The NLR may reflect the balance between pro-tumoral inflammatory status and anti-tumoral immune response and serve as a prognostic factor, as a high NLR can lead to worse outcomes across several cancer types, including NPC [17-20]. Recent meta-analysis data reveal that elevated pretreatment NLR is associated with shorter overall survival and progression-free survival in NPC [18, 21].

Our current studies focus on the treatment of metastatic NPC patients and the search for immune cell profile and related prognostic factors. The use of chemotherapy combined with ICIs represents an updated trend in the standard care for metastatic NPC. Both the combined positive score (CPS) of PD-L1 expression in metastatic NPC tissue and NLR are strong prognostic predictors. Adding local treatment to the ICI-based regimen for these patients could potentially enhance long-term survival and improve quality of life. Additionally, our studies include immune cell profiling and functional analyses of ICI-based treatments in responders versus non-responders, which may provide a more comprehensive view of immune-related therapies.

Material and methods

Patients

Between March 2016 and June 2024, one hundred sixty patients with metastatic nasopharyngeal carcinoma were enrolled during the data collection period for this retrospective study at our institute.

The inclusion criteria were: (1) metastatic NPC, either de novo or recurrent; (2) histologically confirmed NPC at the primary or metastatic site; (3) treatment-naïve or having received ≥ 1 line of chemotherapy, with the chemotherapy regimen determined by the physician; (4) receipt of ≥ 1 course of immune checkpoint inhibitor (ICI) treatment (Pembrolizumab, Nivolumab, or Nivolumab + Ipilimumab); (5) eligibility for local therapy to the primary or metastatic site with either palliative or curative intent.

The exclusion criteria were: (1) chemotherapy alone; (2) ICI treatment alone; (3) receipt of targeted therapy, including tyrosine kinase inhibitors or monoclonal antibodies; (4) supportive care only.

We retrospectively reviewed the clinical data of 62 patients with metastatic nasopharyngeal carcinoma who received at least one cycle of immunotherapy combined with chemotherapy at Chang Gung Memorial Hospital, Linkou. This study was approved by the Institutional Review Board of CGMH (IRB No. 201801434A3D001).

Clinical and laboratory parameters

We collected the following parameters from the medical records of the patients: age at diagnosis of recurrent or metastatic nasopharyngeal carcinoma; gender; date of first exposure to immune checkpoint inhibitors; white blood cell count; differential white blood cell count; neu-

trophil-lymphocyte ratio (NLR); absolute lymphocyte count; and EBV DNA quantity before immunotherapy. In addition, we also recorded the lowest NLR parameters (total white blood cell count > 3000/*μL* and absolute neutrophil count > 2000/*μL)* during the follow-up period after immunotherapy to exclude the myelosuppression effect due to chemotherapy. In addition, data during infection, defined as concurrent use of antibiotics for infection status documented in medical records, were also excluded to avoid potential confounding effects. The NLR was determined by dividing the absolute neutrophil count by the absolute lymphocyte count. The timing of the pre-treatment hematological data collection was within one week before the first dose of a combination of ICIs and chemotherapy. Post-treatment was defined as the time with the lowest NLR within 3 months after the patient received a combination of ICI and chemotherapy. The cutoff value of NLR was determined by calculating the area under the curve using the receiver operating characteristic (ROC) curve method.

Treatment response was defined using the RECIST criteria with computed tomographic images every 3 months during the follow-up period; patients with complete response (CR) or partial response (PR) were classified as responders, while patients with stable or progressive disease were classed as non-responders. Hyper-responders were defined as patients who showed a survival of more than 24 months and received at least 7 cycles of ICI treatment with CR or PR. The overall survival time was calculated and defined as the length of time from the date of the 1st dose of a combination of chemotherapy and immunotherapy until the date of each patient's death or the last follow-up visit. The presence or lack of immune-related adverse events (irAEs) was reviewed in the patients' medical records.

Contents of treatments

The immune checkpoint inhibitors were administered as follows: Pembrolizumab 200 mg IVF every 3 weeks, Nivolumab 240 mg IVF every 2 weeks, and Ipilimumab 1 mg/kg combined with Nivolumab 3 mg/kg every 3 weeks. The combined chemotherapy regimens included TPF (Docetaxel 75 mg/m^2 on day 1, Cisplatin 75 mg/m^2 on day 1, and 5-Fluouracil 750 mg/m^2 for 24 hours on days 1-5 every 3 weeks); GP (Gemcitabine 1250 mg/m^2 on day 1 and day 8 and Cisplatin 70 mg/m^2 on day 1 every 3 weeks); Docetaxel (75 mg/m^2 on day 1 every 3 weeks); and PUL-B (Cisplatin 50 mg/m^2 on day 1, Bleomycin 8 mg/m^2 on day 1, oral tegafururacil 300 mg/m^2/day, and oral leucovorin 60 mg/day given on days 1-14 every 2 weeks). The ICIs and chemotherapies were combined on day 1 during each cycle. Local treatments, such as concurrent chemoradiotherapy (CCRT), radiotherapy alone, trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), or resection, were given 3-6 months later after the initiation of systemic therapy with the achievement of maximal optimal response. As best we know, the GP regimen is generally used as the first-line treatment for metastatic nasopharyngeal cancer in consideration of its promising efficacy and well-tolerated toxicity, although gemcitabine is not covered by Taiwan's National Health Insurance. Docetaxel-containing regimens, including TPF and Docetaxel alone, are alternative options of treatment. However, Docetaxel is not reimbursed in Taiwan, and its toxicity profiles, such as myelosuppression, neuropathy, and alopecia, may limit its opportunities for patient use. The PUL-B regimen is used mostly for patients who would find taxanes unsuitable or cannot afford gemcitabine/taxanes.

With the above concerns and rationales in mind, the chemotherapy regimens, ICIs, and local treatments were decided based on the clinical physicians' choices and patients' informed consent in this real-world study.

PBMC preparation

PBMCs were separated from the peripheral blood by using Ficoll Histopaque density gradient centrifugation following the manufacturer's directions. A total of 5 mL of PB was mixed gently with 5 mL of Ficoll Histopaque in a 15 mL centrifuge tube. The tube was centrifuged for 30 min at 100×g in 4°C and the PBMCs were removed for further study. PBMCs were amplified by IL-2 rapid expansion protocol [22]. Briefly, PBMCs were cultured with complete medium (CM) plus 6000 IU per mL of rhIL-2 in 24-well tissue culture plates. The CM consisted of RPMI 1640, 25 mmol/L HEPES pH 7.2, 100 U/mL penicillin, 100 μg/mL streptomycin, 2

mmol/L-glutamine, and 5.5 × 10^{-5} mol/L β-mercaptoethanol, supplemented with 10% human serum. Each round of PBMC culture was continued for 10-14 days and cryopreserved for further testing.

Cell growth assay (IC_{50})

The NPC-B13 cell line (EBV-positive) was maintained in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 2 mM L-glutamine (Gibco, Grand Island, NY, USA), and 100 U/mL penicillin-streptomycin (Gibco, Grand Island, NY, USA) [23]. A total of 10,000 NPC-B13 cells were plated in a 96-well culture plate for 3 days. Different amounts of amplified PBMCs were added to the plated NPC-B13 well for another 2 days. An MTT (3-{4,5-dimethylthiazol-2-yl}-2, 5-diphenyltetrazolium bromide) assay was used to detect IC_{50} [23].

Animal study

All experiments involving laboratory animals were conducted in accordance with the Guidelines for Animal Experiments of Chang Gung Memorial Hospital and were approved by the Animal Research Committee at Chang Gung Memorial Hospital (*IACUC No*. 2021080201). NPC PDX-B13 tumors were cut into shapes 3 mm in diameter and inserted subcutaneously into the flank areas of NOD/SCID mice (BioLASCO, Taiwan). When the tumors reached a size of 50-100 mm^3, 1 × 10^6 amplified PBMCs from patients after ICI treatment were injected into the mice via the tail vein twice a week [24]. A total of 4 doses of PBMCs were injected; the mice were sacrificed two weeks after the initial PBMC injection, and the tumors were characterized. A duplicated study was conducted for each patient.

Immunohistochemical (IHC) study of PD-L1 and CD8 expression

An IHC study was performed on 5 μm thick sections of FFPE tumor tissue and PD-L1 expression was assayed by using an IHC 22C3 pharmDx assay (Dako North America) [25]. The PD-L1 IHC staining of tumor samples was interpreted by two experienced pathologists and assessed using the tumor proportion score (TPS) and combined positive score (CPS) [26]. Anti-CD8 ab and clone C8/144B (DAKO) were used for an IHC study of PDX.

Flow cytometry

The enriched PBMCs were washed with RPMI-1640 supplemented with 10% FBS and once with FACS buffer (2% FBS in PBS). Subsequently, the samples were stained with antibodies in FACS buffer for 30 min at 4°C in the dark. After washing two times with FACS buffer, the samples were acquired using *BD FACSymphony* (BD Biosciences) and analyzed with FlowJo software [27]. The FCS files were compensated and gated in FlowJo before exporting channel values as CSV files. The CSV files were exported from live T cell (CD3+) gates. t-Distributed Stochastic Neighbor Embedding (tSNE), FlowSOM, and heatmap analyses were performed in R (version 4.3.2) using the default settings of the CATALYST package (version 1.24.0) [28]. The fluorochrome-conjugated antibodies used for staining included anti-CD57 (HNK-1, Biolegend), anti-KLRG1 (13F12F2, Invitrogen), anti-CCR6 (30-F11, Biolegend), anti-CCR8 (433H, BD bioscience), anti-CD69 (FN50, Biolegend), anti-SLC1A5 (KM4012, BD Biosciences), anti-CCR4 (1G1, BD Biosciences), anti-CD127 (HIL-7R-M21, BD Bioscience), anti-CD3 (UCHT1, BD Biosciences), anti-CD56 (NCAM16.2, BD Biosciences), anti-Foxp3 (259D/C7, BD Bioscience), anti-HLA-DR (G46-6, BD Bioscience), anti-VISTA (MIH65, BD Bioscience), anti-CD38 (HIT2, BD Bioscience), anti-CD4 (SK3, BD Bioscience), anti-ICOS (DX29, BD Bioscience), anti-CXCR3 (1C6/CXCR3, BD Bioscience), anti-TIM3 (7D3, BD Bioscience), anti-CXCR4 (12G5, BD Bioscience), anti-CD152 (BNI3, BD Bioscience), anti-CD45RA (HI100, BD Bioscience), anti-CD14 (MφP9, BD Bioscience), anti-CD19 (SJ-25C1, BD Bioscience), anti-CD8 (RPA-T8, BD Bioscience), anti-TCRγδ (11T2, BD Bioscience), anti-LAG3 (T47-530, BD Bioscience), anti-CXCR5 (J252D4, Biolegend), anti-CCR7 (2-L1-A, BD Bioscience), anti-TCF7 (S33-966, BD Bioscience), anti-TOX (TXRX10, Invitrogen), anti-Granzyme B (GB11, BD Bioscience), anti-CD161 (DX12, BD Bioscience), anti-CD25 (2A3, BD Bioscience), anti-PD1 (EH12.1, BD Bioscience), anti-TIGIT (A15153G, Biolegend), and anti-CD28 (CD28.2, Biolegend).

Statistics

Overall survival was estimated by using the Kaplan-Meier method, and the log-rank test was used to compare the survival difference between groups. Variables that were tested

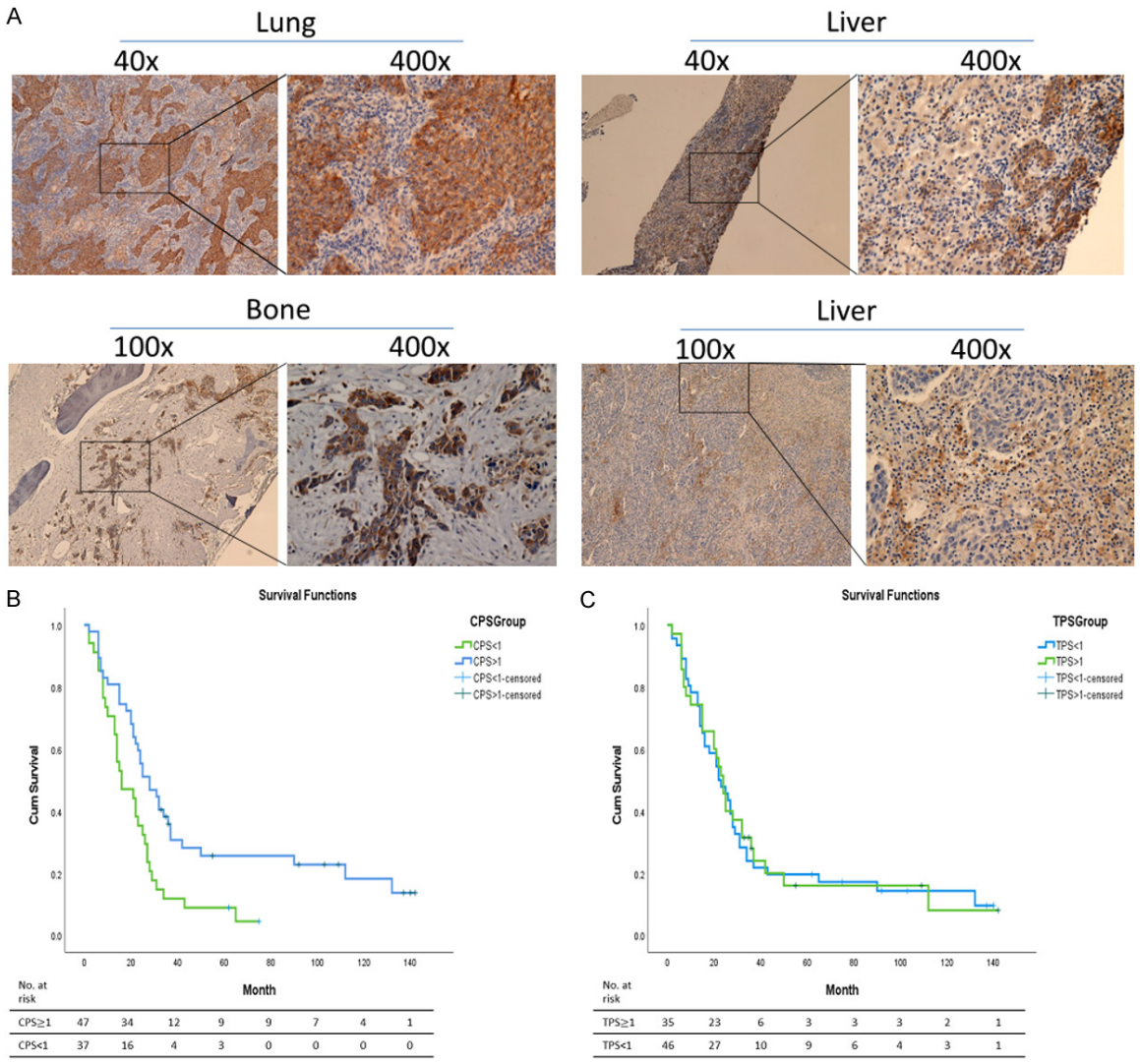

Figure 1. PD-L1 expression in metastatic NPC tissue. (A) PD-L1 expression in metastatic tissue identified with IHC staining. Kaplan-Meier curves of overall survival: (B) CPS ≥ 1 vs. CPS < 1, $P = 0.004$; (C) TPS ≥ 1% vs. TPS < 1%, $P = 0.929$.

with p-value < 0.05 in univariate analyses were selected into multivariate analyses. The hazard ratio and 95% confidence interval of OS in univariate and multivariate analyses were estimated by using the Cox regression method. Analyses were carried out by using SPSS version 27 (IBM Corp., Chicago, IL). All tests were two-sided, and statistical significance was defined as p-value < 0.05.

Results

We had check the metastatic tissue PD-L1 expression without ICI treatment from the tissue bank of CGMH, Linkou, during 2005-2019. Total 81 metastatic tissue were available including metastasis to lung 36 (44.4%), liver 19 (23.5%), bone 9 (11.1%), lymph node 7 (8.6%), bone marrow 6 (7.4%), and soft tissue 4 (4.9%). Using anti-PD-L1 antibody, 22C3 Dako, combine positive score (CPS) was positive in 58.0% (47/81) checked tissue and tumor proportional score (TPS) was positive in 43.2% (35/81) tissue respectively as shown in **Figure 1A**.

In an analysis of overall survival (OS) combined with PD-L1 expression in metastatic tissue, it was shown that median overall survival was significantly different between the CPS ≥ 1 group and CPS < 1 group, as shown in **Figure 1B** (28 vs. 16 months, $P = 0.004$). However, in terms of

TPS with a cutoff value of 1%, there was no statistical difference in median overall survival (24 vs. 22 months, $P = 0.929$) between the positive group (TPS ≥ 1%) and negative group (TPS < 1%), as shown in **Figure 1C**.

From 2015 onwards, ICIs became available in clinical practice, but are not covered by National Health Insurance in Taiwan. A consensus was reached regarding the generalized applicability of a combination of ICI and chemotherapy among our multi-disciplinary cancer team according to previous case-cohort experience, as presented in **Figure 2A**. For this retrospective analysis of ICI-related treatment, a total of 62 patients with metastatic NPC were enrolled: 26 with de novo metastasis and 36 with recurrent metastasis. All of them had received chemotherapy combined with ICI treatment, as shown in **Table 1**. Most (98.3%) patients showed NPC cells of WHO types II and III, and plasma EBV DNA was detected in 82.9% of enrolled patients. Fifty-five (88.7%) patients were undergoing first-line treatment when metastasis occurred, thirty-seven (59.7%) of whom received local treatment, including concurrent chemoradiotherapy, radiotherapy, radiofrequency ablation, trans-arterial chemoembolization, or the resection of metastatic sites, as shown in **Table 1**.

The median OS for all enrolled patients was 39.3 months. The objective response rate, as measured by the RECIST criteria for ICI-based regimens, was 71% (CR: 45.2%; PR: 25.8%) and the disease control rate was 85.5% (CR+PR+ stable disease). The median OS was not reached for the complete response group, whereas it was 31.1 months for the partial response group, 39.3 months for the stable disease group, and 5.9 months for the progressive disease group (P < 0.001), as shown in **Figure 2B**. Median **Table 1**. Main characteristics of patients with metastatic NPC receiving ICI-based treatment.

OS was not reached and 9.7 months, respectively, for responders and non-responders (P = 0.002) (**Figure 2C**). Using the area under the receiver operating characteristic (ROC) curve, the cutoff value for pre-treated NLR was determined to be 4.8. With this cutoff value, the difference in median OS between the two groups was statistically significant (P = 0.021), as shown in **Figure 2D**. Patients who received local treatment had better overall survival outcomes than patients without local treatment (P = 0.008) (**Figure 2E**).

There was a significant difference in pre-treatment NLR between responders and non-responders (P = 0.003). In responders, the median NLR decreased significantly after immunotherapy-based treatment from 3.09 to 1.53 (P = 0.002). We also found a lower median post-treatment NLR in responders than in non-responders (1.53 vs. 3.51, P < 0.001), as shown in **Figure 3A**. Regarding local treatment, only responders showed a statistically significant decrease in the NLR after local treatment (2.6 vs. 1.3, P = 0.045), as shown in **Figure 3B**.

The median post-treatment NLR of the seven patients defined as hyper-responders (survival of more than 24 months and receiving at least seven cycles of ICI treatment with CR or PR; the historical median OS for patients with metastatic NPC was 19 months in our institute [29]) was 1.1. Of our hyper-responders (**Figure 4**, patients A-G), most had a prolonged low NLR (< 5, even < 3), high total lymphocyte count (around 1000-3000 per microliter), and undetectable/stable EBV DNA load in the PB during treatment. The non-responder (patient I) in the combination treatment showed a high NLR fluctuation (> 5), low total lymphocyte count, and high EBV DNA load in the PB. Two responders (patients G and H) showed high NLR, low total lymphocyte count, and rapidly decreased or stable EBV DNA load that may have been caused by a strong effect of chemotherapy before the immunotherapy period began.

Based on the clinical and treatment-related characteristics, we performed univariate and multivariate analyses of overall survival to identify independent prognostic factors. **Table 2** shows that local treatment, chemotherapy regimens with gemcitabine and cis-platin, responder status, and pre-treatment NLR showed trends in better overall survival in our univariate analysis. In our multivariate analysis, we found that these characteristics were independent factors for overall survival with hazard ratios of 0.360 (95% CI: 0.130-0.996), 4.362 (95% CI: 1.431-13.293), 2.275 (95% CI: 0.784-6.607), and 4.310 (95% CI: 1.517-12.242), respectively. We observed that patients with metastatic NPC had a relatively low NLR, with most of the peripheral blood sampled showing an NLR of less than 3. After the immune prim-

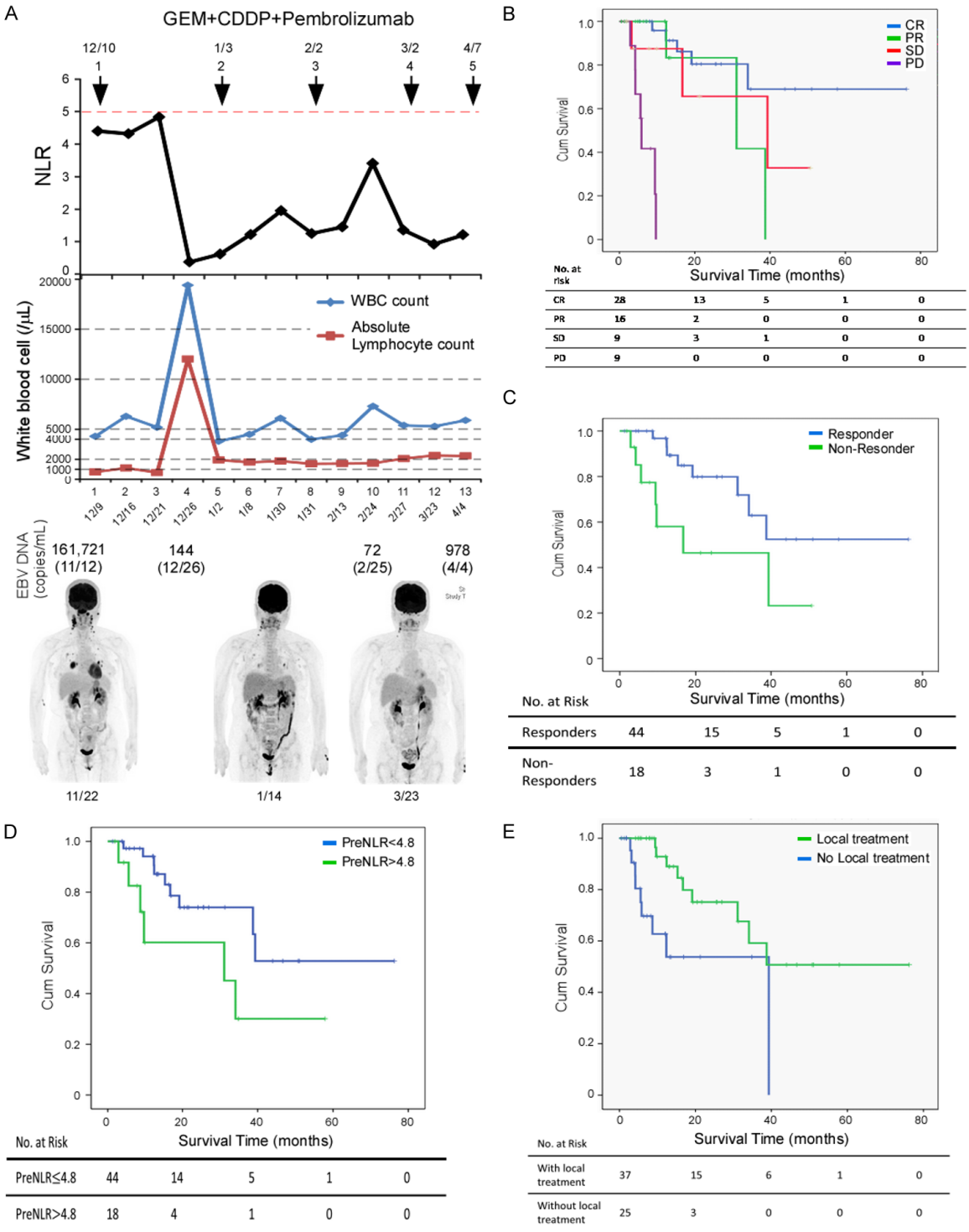

Figure 2. The clinical response and parameters of combined chemotherapy, ICIs, and local treatment in metastatic NPC. (A) A 48 y/o female patient was diagnosed as having NPC with lung metastasis. She was treated with combined chemotherapy and ICIs and a good partial response was achieved. Kaplan-Meier curves of overall survival: (B) treatment response, $P < 0.001$; (C) re-sponder vs. non-responder, $P = 0.002$; (D) pre-treatment NLR of cutoff value: 4.8, $P = 0.021$; and (E) local treatment, $P = 0.008$. Abbreviations: CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

ing process in the tumor immune cycle, the reactive CD8+ cytotoxic T lymphocyte may be detected in the PB of patients receiving ICI treatment [30].

Table 1. Main characteristics of patients with metastatic NPC receiving ICI-based treatment

Clinical parameters		All patients (N = 62)	
		N	%
Metastasis	*De novo*	26	41.9
	Recurrent	36	58.1
Median Age		53.5	
Gender	Male/Female	42/20	68.8/31.2
Primary tumor cell type	WHO type I	1	1.7
	WHO type II/III	61	98.3
Pre-treatment plasma EBV DNA	Not detectable	10	16.1
	Detectable	52	82.9
	(copies/ml) Ranging	0-233,517	
	Median	2436	
Metastatic sites	Lung	17	27.4
	Bone	12	19.4
	Liver	11	17.7
	Multiple	22	35.5
Chemotherapy when metastasis occurred before ICI treatment	0	55	88.7
	1	6	9.7
	≥ 2	1	1.6
ICI Treatment	Pembrolizumab	58	93.6
	Nivolumab	3	4.8
	Nivolumab + Ipilimumab	1	1.6
Chemotherapy Treatment*	TPF	6	9.7
	GP	42	67.8
	Docetaxel/Paclitaxel	10	16.1
	PUL-B	4	6.4
Treatment Cycles	1-6	43	69.3
	7-12	14	22.6
	≥ 13	5	8.1
Treatment Response	Complete Response	28	45.2
	Partial Response	16	25.8
	Stable Disease	9	14.5
	Progressive Disease	9	14.5
Local Treatment		37#	59.7
	CCRT	12	19.4
	Radiotherapy	17	27.4
	RFA/TACE	5	8.1
	Resection	2	3.2
irAE (all grades)		13§	21.0
	Hepatitis	3	4.7
	Adrenal Insufficiency	2	3.2
	Pneumonitis	3	4.8
	Dermatomyositis	1	1.6
	Diabetes mellitus Type I	1	1.6
	Neuropathy	1	1.6
	Hypophysitis	1	1.6
	Stevens-Johnson Syndrome	1	1.6
Grade 3/4 (patient no/total)		7/62	11.3

Abbreviations: CCRT: concurrent chemoradiotherapy; ICI: immune checkpoint inhibitor; irAE: immune-related adverse event; PDL1: programmed death ligand 1; RFA: radiofrequency ablation; TACE: transcatheter arterial chemoembolization. *Chemotherapy regimens: GP: gemcitabine + cisplatin, TPF: taxotere + cisplatin + 5FU, and PUL-B: cisplatin + UFUR (tegafur + uracil) + leucovorin + bleomycin. #Some individual patients received more than one type of local treatment. §Different types of irAEs may occur in one individual patient.

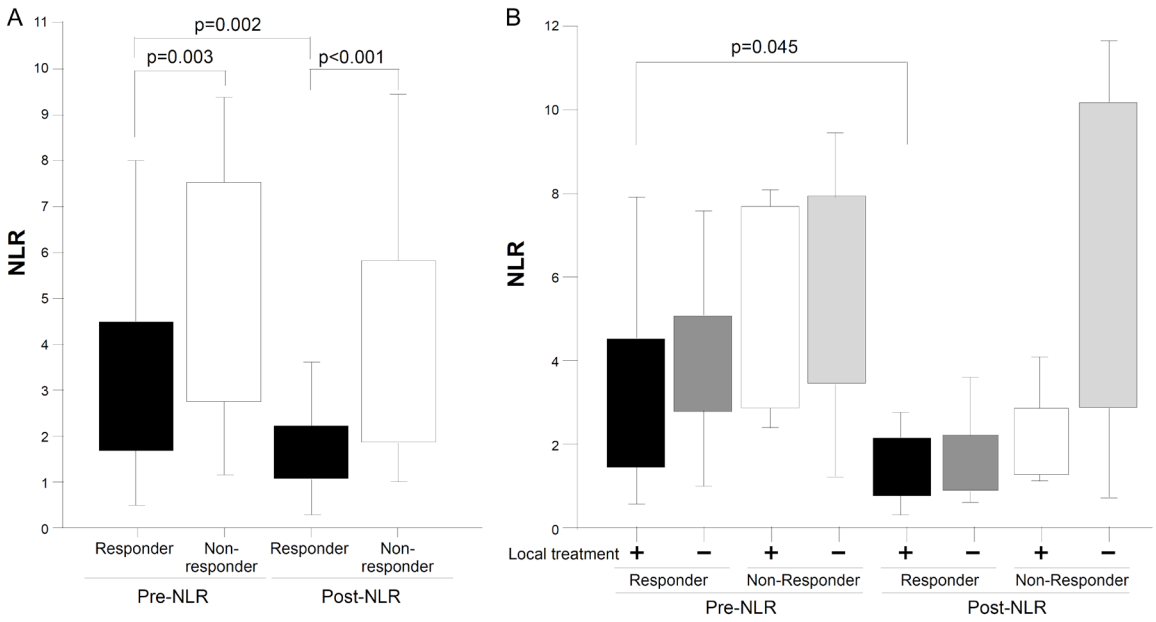

Figure 3. Changes in neutrophil-lymphocyte ratio (NLR): (A) among responders and non-responders; and (B) regarding response and local treatment status.

For ICI-based treatment, immune profile especially cytotoxic T lymphocyte (CTL) could be detected at peripheral blood. Using a rapid lymphocyte amplification protocol via IL-2 stimulation [22] for two weeks, the total PBMC count from most of our responders was amplified 1-10-fold and this amplitude was positively correlated with total lymphocyte count.

Through flow cytometry analysis (the gating strategies were shown in Figures S1 and S2), we found that these amplified PBMCs were lymphocyte-dominant and rich in CD8+ T cells, as shown in **Figure 5A** and **5B**. CD4 and CD8 T cells expressed the activation-induced marker ICOS and the inhibitory molecules TIM3, PD-L1, and PD-1 (**Figure 5B**). About 33.9% of the CD8+ T cells expressed both IFN-γ and Granzyme B (**Figure 5C**). Taken together, these data suggest that the expanded cells were activated by cytotoxic activity. We tested the cancer killing efficacy of highly amplified PBMC samples (≥ 10-fold) in vitro. As shown in **Figure 6A** and **6F**, the amplified lymphocytes could kill the cancer cell line in a ratio of 1:1 to 1:5 (seeding 10^4 cells/well could grow up to 2×10^4 cells/well in 3 days' culture; the IC_{50} for patient A was 1×10^4 and 2×10^3 for patient B).

We further tested the cancer-killing effect of these amplified PBMCs in vivo via an NPC patient-derived xenograft model. The amplified PBMCs inhibited xenograft growth efficiently, as shown in **Figure 6B-H**. Through IHC staining, we found human CD8+ cells infiltrating around the tested tumor, as shown in **Figure 6E**. These results demonstrated that the PBMCs from our patients with NPC treated with ICIs had a cancer-cell-killing function.

To identify cell populations that distinguished responders from non-responders, we used high-parameter flow cytometry to analyze T cell expression markers. PBMCs from 10 responders and 4 non-responders were examined with a 30-color flow cytometry panel, focusing on total T cells. The data were visualized using t-Distributed Stochastic Neighbor Embedding (t-SNE) and FlowSOM clustering, which group cells based on similarities in marker expression profiles (**Figure 7A**). Our analysis revealed a significant increase in clusters 6 and 9 among responders compared to non-responders (**Figure 7B**). A corresponding heatmap indicated higher expression levels of CD4 and CD28 and lower levels of CD45RA, CCR7, and CD57 in these clusters (**Figure 7C**), suggesting that they represent a CD4 effector memory phenotype. To further characterize this population, we manually gated for CD4+CD45RA-CCR7-CD28+CD57- cells. This gating strategy confirmed a higher prevalence of these cells in the

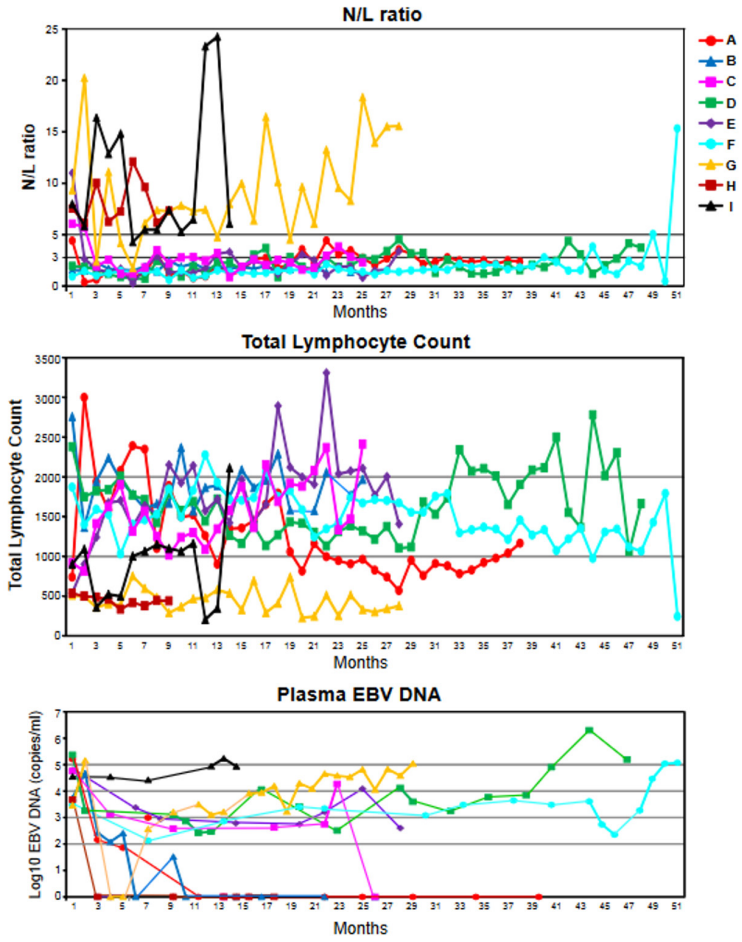

Figure 4. The peripheral blood NLR, total lymphocyte count, and plasma EBV DNA load in met-astatic NPC patients receiving ICI-based treatment. Patients A-G were hyper-responders (OS > 24 months, CR or PR, and ICI treatment > 6 cycles), patient H was a responder but with high NLR and low absolute lymphocyte count, and patient I was a non-responder.

responder group (**Figure 7D**), supporting the findings of our cluster analysis.

Discussion

At the time of metastatic tumor recurrence, previous local tumor biopsies may not represent the evolution process and characteristics of metachronous metastatic tumor cells. In our study, we examined metastatic tissues to reflect real-time PD-L1 expression, which is more suitable for evaluating real tumor characteristics compared to prior or synchronous primary biopsy specimen. Our data were sourced from pre-treatment metastatic tissue with high PD-L1 expression that correlated with longer survival. Whether this high PD-L1 expression in metastatic tissue could translate to good ICI treatment response requires further observation.

The prognostic value of the baseline NLR in different malignancy types treated with ICIs was investigated in many previous studies; a higher pre-treatment NLR was associated with significantly poorer overall survival and progression-free survival [17, 31]. However, these studies used different methods and patient populations, and there is still a lack of consensus regarding the cutoff values for pre-treatment NLR. As for NPC, the published studies demonstrated a similar trend, as a high pre-treatment NLR was a poor prognostic factor [18]. In our study, we found that a lower pre-treatment NLR with a cutoff value of 4.8 was a good prognostic factor for patients with metastatic NPC treated with ICIs and chemotherapy. In addition, changes in the NLR before and after intensity-modulated radiotherapy [32] or immunotherapy [33] was proposed as an independent predictive factor of survival. In our study, decreased NLRs after immunotherapy-based treatment were observed in both responders and non-responders, with a significant difference shown. A lower median post-treatment NLR was also found in responders with local treatment.

In a phase I clinical trial of recurrent/metastatic (R/M) NPC, a combination of chemotherapy and ICIs showed a high response rate (91%) compared to ICIs alone (31%), but with a high level of treatment-related side effects [6]; a high response rate (73%) of this combination was also shown in metanalysis data [34]. Traditional chemotherapy can kill cancer cells directly; induce myelosuppression status, reducing the “N” value in the NLR; and modulate immune function [35]. ICI treatment can augment immune CTL priming and cancer cell killing, which may maintain/increase the “L”

Table 2. Univariate and multivariate Cox regression analyses of overall survival

Characteristic	Univariate analyses			Multivariate analyses		
	HR	95% CI	p	HR	95% CI	p
Age (> 55.5/≤ 55.5)	0.387	0.150-0.999	0.050			
Gender (Male/Female)	0.970	0.363-2.594	0.951			
Metastatic Sites (Oligo/Multiple)	1.020	0.329-3.165	0.972			
Local Treatment (No/Yes)	0.297	0.116-0.763	0.012*	0.360	0.130-0.996	0.049*
Chemotherapy Regimens (GP/Non-GP)	4.385	1.537-12.513	0.006*	4.362	1.431-13.293	0.010*
Responder (Responder/Non-Responder)	3.923	1.539-10.000	0.004*	2.275	0.784-6.607	0.131
irAEs (No/Yes)	0.794	0.181-3.475	0.759			
preNLR (≤ 4.8/> 4.8)	2.888	1.125-7.415	0.028*	4.310	1.517-12.242	0.006*
Treatment Cycle (≤ 6/> 6)	0.410	0.143-1.179	0.098			
Decreased NLR (Yes/No)	2.070	0.755-5.676	0.157			

Abbreviations: irAEs: immune-related adverse events; NLR: neutrophil-lymphocyte ratio; GP: gemcitabine/cisplatin. *p<0.05.

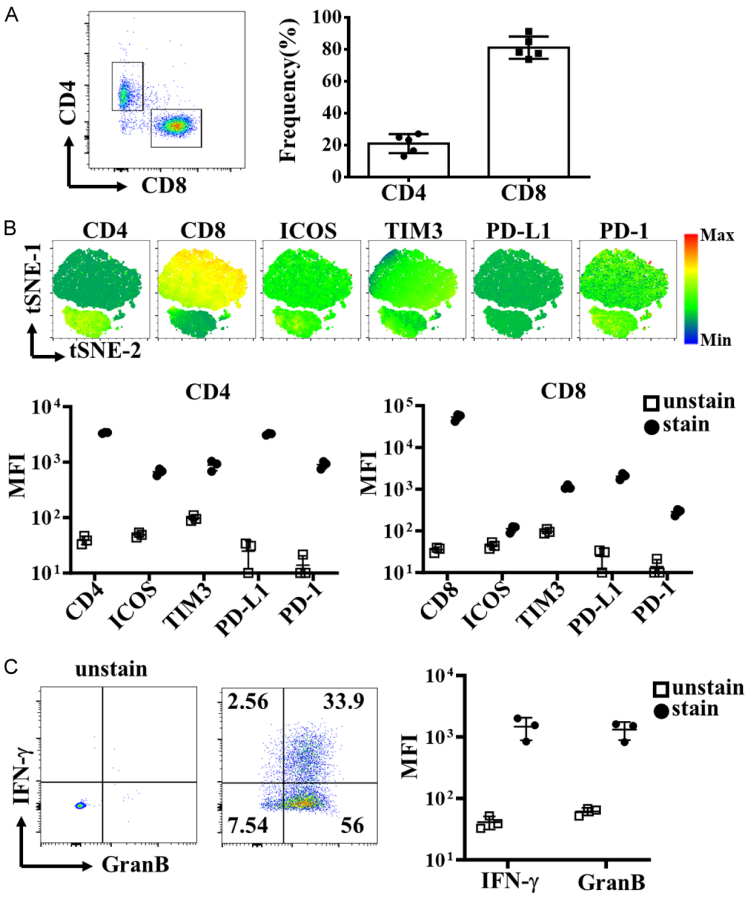

Figure 5. Flow cytometry analysis of amplified PBMCs. A. Lymphocytes were dominant and rich in CD8+ T cells. B. CD4 and CD8 T cells expressed activation-induced markers such as ICOS and the inhibitory markers TIM3, PD-L1, and PD-1. MFI: mean fluorescence intensity. C. In CD8+ T cells, about 33.9% expressed both IFN-γ and Granzyme B.

value in the NLR via a secondary increase in CTL function/proliferation. The presence of a lower NLR after a combination of chemotherapy and ICIs may provide a clinical clue towards better treatment response and survival.

CD8+ T lymphocytes have a pivotal role in anticancer immunity; high levels of tumor-infiltrating T lymphocytes are associated with good prognosis in cancer management, including in NPC [36]. A high-frequency early proliferation (Ki-67+) of PD-1+ CD8 T cells after ICI treatment is associated with better clinical outcomes in non-small-cell lung cancer and melanoma [37, 38]. In this study, after a combination treatment comprising chemotherapy, ICIs, and local treatment, a low NLR and high absolute lymphocyte count were shown and the PBMCs from the treatment responders was amplified by IL-2 with PD-1+ CD8+ dominance, the response to IFN-γ stimulation, and high Granzyme B (cytotoxic granules secreted by CTL) expression. These amplified PBMCs showed an anticancer capability against in vitro cell lines and inhibited PDX growth in an animal model in vivo. In comparison to non-responders, the responders showed increased levels of CD4+CD45RA-CCR7-CD28+CD57-

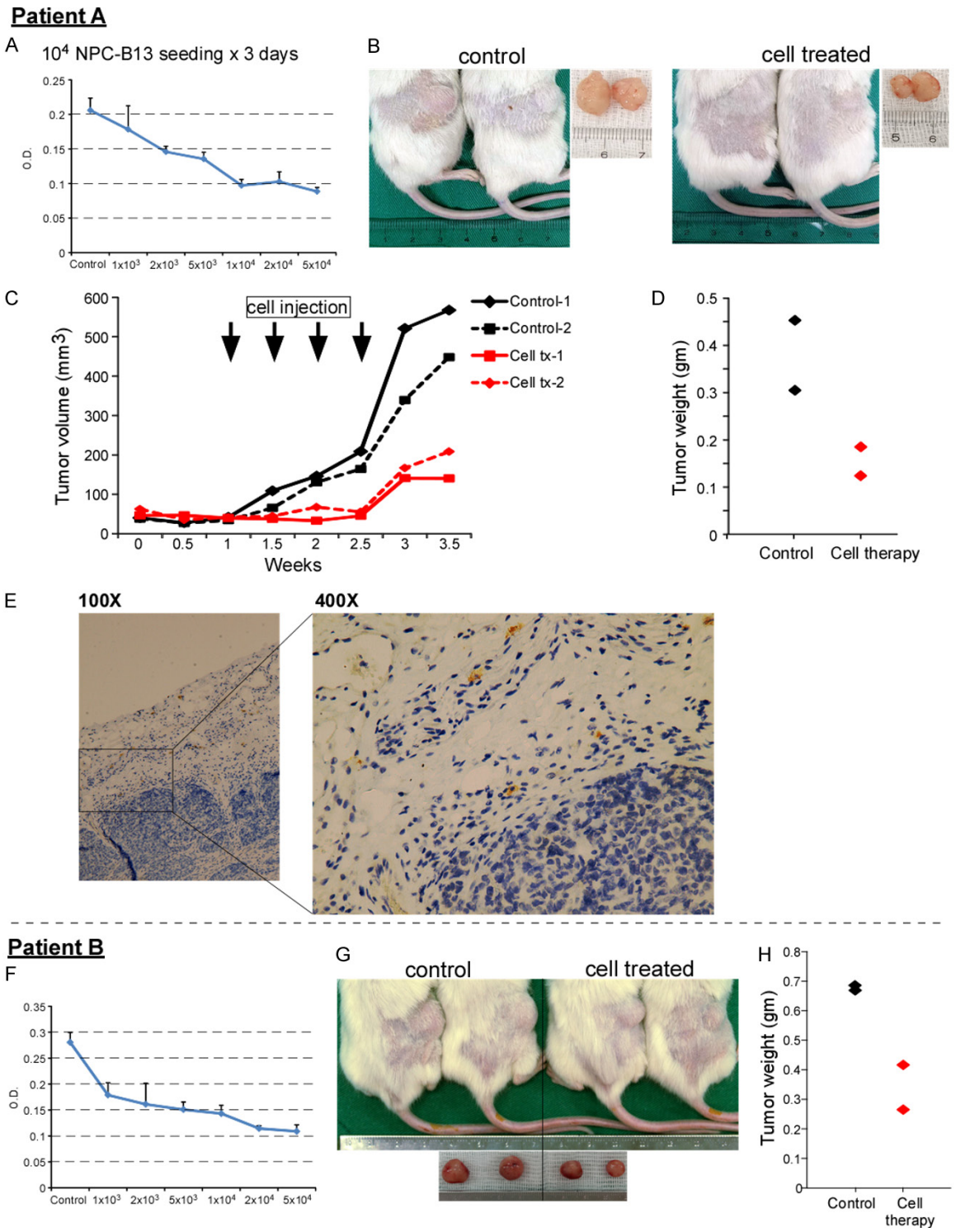

Figure 6. Ex vivo immune cell functional test. (A-E) Patient A: (A) In vitro functional assay of amplified PBMCs from Patient A, determining the IC_{50} of NPC-B13 cell line. In vivo functional test of amplified PBMCs from ICI-treated Patient A in NPC PDX-B13 model, showing (B) gross tumor, (C) tumor volume, (D) tumor weight, and (E) CD8+ lymphocyte infiltrated around the PDX tumor. (F-H) Patient B: (F) In vitro functional assay of amplified PBMCs from Patient B, determining the IC_{50} of NPC-B13 cell line. In vivo functional test of amplified PBMCs from ICI-treated Patient B in NPC PDX-B13 model, showing (G) gross tumor, (H) tumor weight.

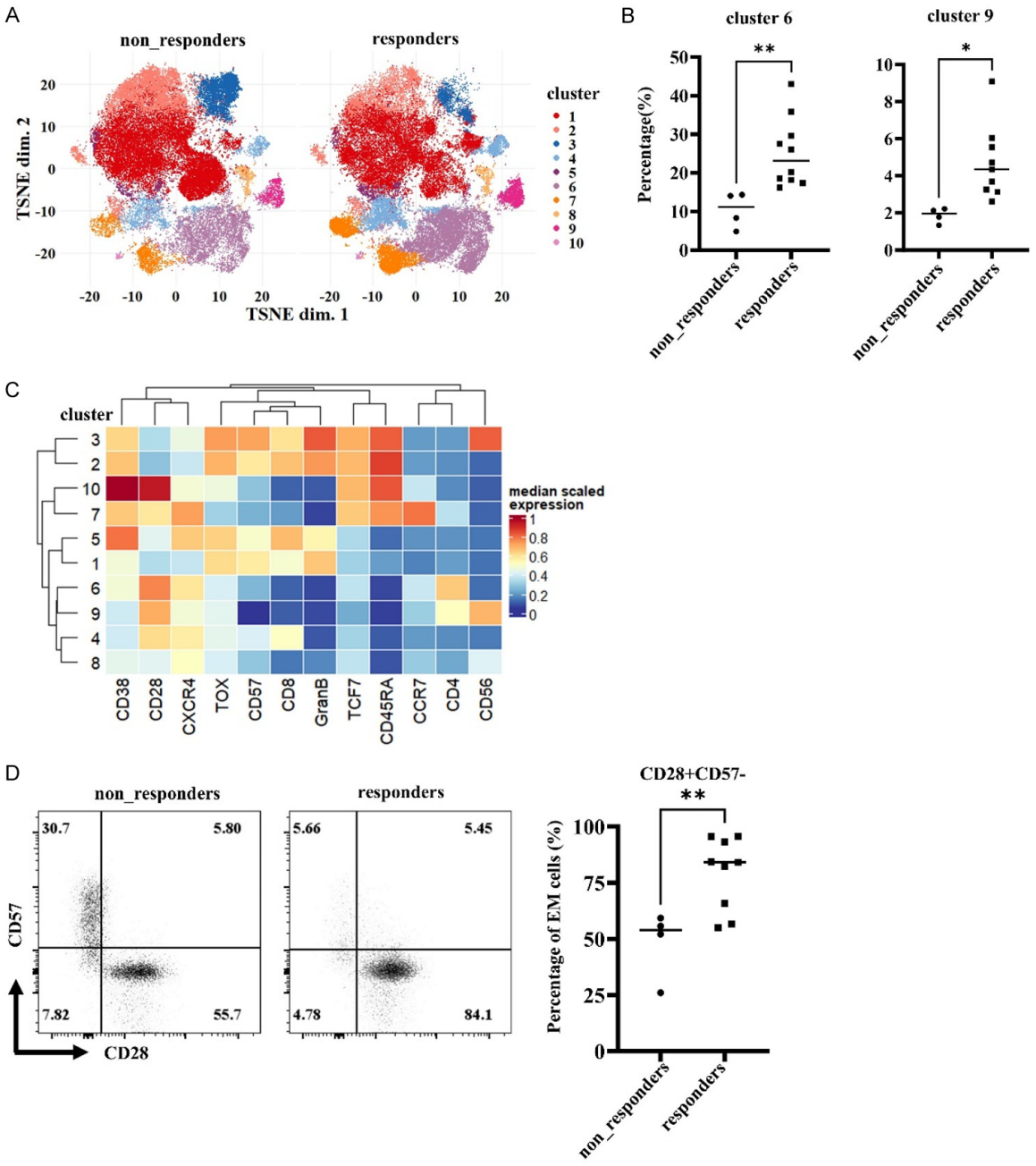

Figure 7. Increase in CD4+CD45RA-CCR7-CD28+CD57- cells in the responder group. A. t-SNE plots showing clustering of live T cells (CD3+) from non-responders and responders. B. The percentages of clusters 6 and 9 among T cells are displayed. C. Heatmap illustrating the expres-sion of selected markers within these clusters. D. Initial gating of T cells based on CD4+CD45RA-CCR7- expression; representative gates for CD57 and CD28 expression are shown in the left panel. The cumulative data representing the percentage of cells in the CD4+CD45RA-CCR7- effector memory (EM) cell subset is presented in the right panel.

cells (effector memory cell subset) in peripheral blood. These results may provide a good monitoring index for ICI treatment of NPC.

In combination with ICI treatment, local treatments, including radiotherapy, thermal ablation, and trans-arterial chemoembolization, may boost the abscopal anti-cancer effect via enhancing local tumor killing through tumor antigen release, allowing antigen-presenting cells to perform immune priming and activate cytotoxic T lymphocytes, and increasing tumor

killing at distant sites [39, 40]. In NPC, stereotactic body radiotherapy (SBRT) combined with PD-1 inhibitors benefited patients with PD-L1-positive recurrent/metastatic NPC, leading to stable tumor control [41]. Sequential local treatment for metastases after systemic palliative chemotherapy provided a survival benefit to metastatic NPC patients [28]. For responders to ICI treatment and chemotherapy, local treatment led to a consolidation of the responsive lesion sites. On the other hand, local control may be achieved through local treatment of stable or progressive lesions after a previous combination of ICIs and chemotherapy. In our study, we found that after a combined treatment of ICIs and chemotherapy, patients without local treatment showed a significantly decreased NLR, while responders with local treatment showed a trend of longer overall survival compared to the other groups of patients. In the current report, patients with metastatic NPC that received a combination of chemotherapy, ICIs, and local therapy showed prolonged low NLR, high absolute lymphocyte count, and undetectable/stable plasma EBV DNA load, which may have translated to a high response rate (70%), especially in terms of the CR rate (49%), and longer overall survival.

There were several limitations in our study. First of all, some incomplete data were identified in the follow-up period during a review of patient medical records. Second, an insufficient number of events and cases resulted in statistical insignificance. Third, there was a possibility of patient selection bias. In our study, ICIs were not reimbursed, and those patients who can afford ICIs may have better socioeconomic status and a greater awareness of the need for self-care and compliance, which may have had a positive in-fluence on response and survival outcomes. NPC is a chemotherapy-sensitive cancer associated with the EB virus. An objective response rate of 71.4% was reported by Zhou et al. when using a combination of ICIs and chemotherapy [42]. Chemotherapy provided a better response rate when combined with ICIs, which showed a durable response. Both components may be attributed to a longer survival benefit.

The prognostic value of baseline NLR in different malignancy types treated with ICIs was investigated in many previous studies and higher pre-treatment NLR was significantly associated with poorer overall survival and progression-free survival [17, 29]. However, those studies were diverse in different methods and patient populations, and there was still lack of consensus of cut-off values of pre-treatment NLR. As for NPC, published studies demonstrated a similar trend with high pre-treatment NLR was poor prognostic factor [18]. In our study, we found lower pre-treatment NLR with cut-off value of 4.8 was a good prognostic factor for patients with metastatic NPC treated with ICIs and chemotherapy. In addition, changes in NLR before and after intensity-modulated radiotherapy [30] or immunotherapy [31] was proposed as an independent predictive factor of survival. In our study, decreased NLR after immunotherapy-based treatment were observed in both responders and non-responders with significant difference. Lower median of post-treatment NLR was also found in responders with local treatment.

Combination of chemotherapy and ICI had been shown high response rate (91%) than ICI alone (31%) but with high treatment related side effect in one phase I clinical trial of recurrent/metastatic (R/M) NPC [6] and had high response rate (73%) of this combination in meta-analysis data [32]. Traditional chemotherapy may kill cancer cell directly, induce myelosuppression status which will reduce “N” value in NLR, and also modulate immune function [33]. ICIs treatment may augment immune priming and cancer cell killing by CTL which may maintain/increase “L” value in NLR via secondary increasing the CTL function/proliferation. Presence of lower NLR after combination of chemotherapy and ICIs may provide a clinical clue associated with better treatment response and survival.

CD8+ T lymphocyte had a pivotal role in anticancer immunity and high tumor infiltrating T lymphocyte had been shown to associate with good prognosis in cancer management including NPC [34]. High frequency of early proliferation (Ki-67+) of PD-1+ CD8 T cell after ICIs treatment had been reported to associate with better clinical outcome in non-small cell lung cancer and melanoma [35, 36]. In current study, after combination treatment including chemotherapy, ICIs, and local treatment, low NLR with high absolute lymphocyte count were found

and the PBMC from these treatment responders can be amplified by IL-2 with PD-1+CD8+ dominant, response to IFR-γ stimulation, and high in granzyme B (cytotoxic granules secreted by CTL) expression. These amplified PBMCs possess anti-cancer capability to kill cancer cell line in vitro and to inhibit PDX growth in animal model in vivo. In comparison to non-responders, the responders increased CD4+ CD45RA-CCR7-CD28+CD57- cells (effector memory cell subset) in peripheral blood. These results may provide good monitor index for ICIs treatment in NPC.

Conclusion

Our results demonstrated a strong correlation between pre-treatment NLR and changes in NLR during a combination of ICIs, chemotherapy, and local treatment. Further prospective randomized studies with longer follow-up periods and larger patient populations are needed to validate whether this strategy can lead to long-term survival in patients with metastatic NPC.

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Disclosure of conflict of interest

None.

Abbreviations

CCRT, concurrent chemoradiotherapy; EBV, Epstein-Barr virus; ICI, immune checkpoint inhibitor; NLR, neutrophil-lymphocyte ratio; PB, peripheral blood; PD-L1, programmed cell death ligand 1.

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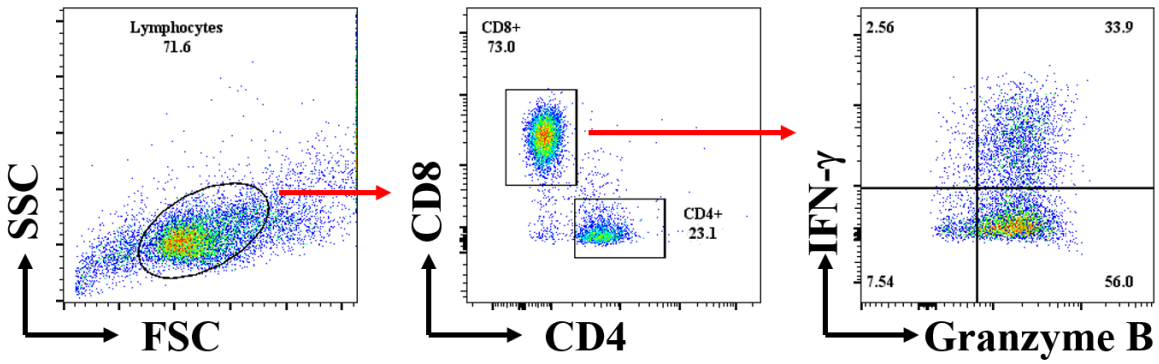

Figure S1. (Related to **Figure 5**): Gating strategy for IFN-g+ Granzyme B+ CD8 T cells.

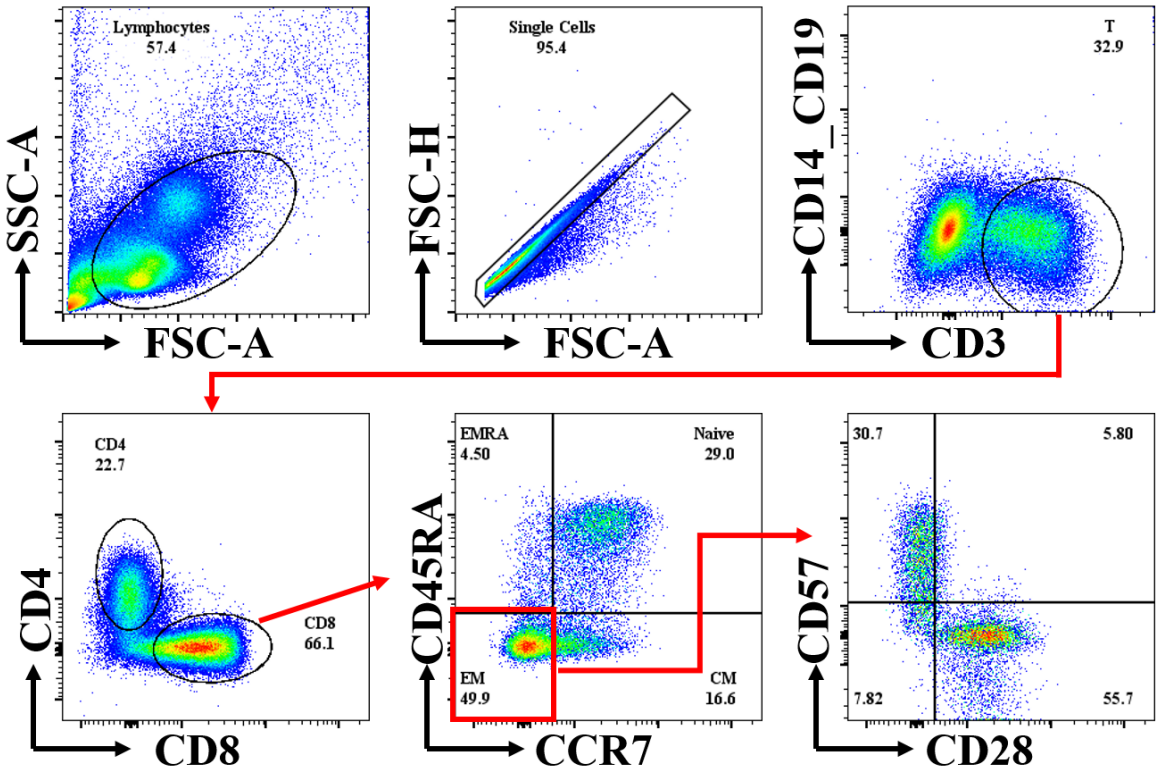

Figure S2. (Related to **Figure 7**): Gating strategy for CD4+CD45RA-CCR7-CD28+CD57- cells.