

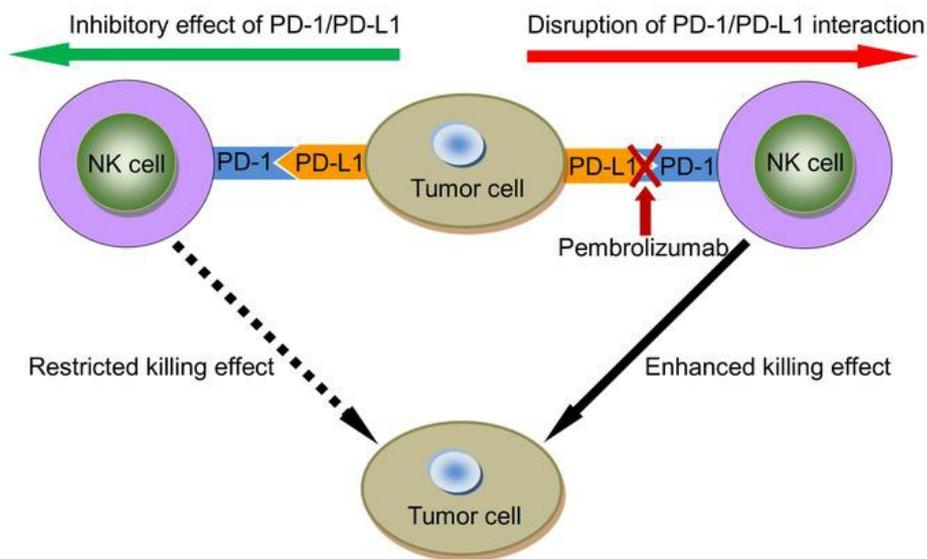
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Pembrolizumab plus allogeneic NK cells in advanced non-small cell lung cancer patients

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Conflict of interest

The authors have declared that they have no conflicts of interest.

Abstract

Background: The anti-programmed cell death 1 (PD-1) antibody pembrolizumab is clinically active against non-small cell lung cancer (NSCLC). In addition to T-cells, human natural killer (NK) cells, reported to have the potential to prolong the survival of advanced NSCLC patients, also express PD-1. This study aimed to investigate the safety and efficacy of pembrolizumab plus allogeneic NK cells in patients with previously treated advanced NSCLC.

Methods: In total, 109 enrolled patients with a programmed death ligand 1 (PD-L1) tumor proportion score (TPS) $\geq 1\%$ were randomly allocated to group A (55 patients, pembrolizumab plus NK cells) and group B (54 patients, pembrolizumab alone). The patients received intravenous pembrolizumab (10 mg/kg) once every 3 weeks and continued treatment until the occurrence of tumor progression or unacceptable toxicity. The patients in group A continuously received two cycles of NK cell therapy as one course of treatment.

Results: In our study, Group A patients had better survival than group B patients (median overall survival [OS]: 15.5 months vs. 13.3 months; median progression-free survival [PFS]: 6.5 months vs. 4.3 months, $P < 0.05$). In group A patients with a TPS $\geq 50\%$, the median OS and PFS were significantly prolonged. Moreover, the group A patients treated with multiple courses of NK cell infusion had better OS (18.5 months) than those who received a single course of NK cell infusion (13.5 months).

Conclusions: Pembrolizumab plus NK cell therapy yielded improved survival benefits in patients with previously treated PD-L1-positive advanced NSCLC.

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Introduction

In the past few years, the incidence of lung cancer has increased at a rate of 7.5%, making it the most common malignancy and a serious danger to human health (1, 2). Approximately 80% of lung malignancies are non-small cell lung cancer (NSCLC), which has a 5-year survival rate of only 15% (3). Platinum-based chemotherapy has been the standard-of-care first-line treatment for advanced NSCLC (2, 4). However, the toxicity and side effects of chemotherapy influence the health and quality of life of patients, and treatment efficacy is limited and appears to have plateaued in the last decade (5, 6). Although treatment for NSCLC has improved with the development of targeted drugs for patients with the corresponding mutations (7, 8), only a small proportion of tumors harbor these mutations, and most tumors become resistant to targeted treatment (9).

Manipulating the immune system for therapeutic benefit in NSCLC patients has been studied for decades (10, 11). Immunotherapy targeting the programmed cell death 1 (PD-1)/ PD-1 ligand 1(PD-L1) inhibitory axis has produced spectacular results in the treatment of a wide variety of tumors (10, 12-15). With the recent development of immune checkpoint inhibitor therapy that blocks the PD-1/PD-L1 pathway, pembrolizumab monotherapy has replaced chemotherapy as the first-line treatment for NSCLC patients (2, 16), and pembrolizumab plus platinum-based chemotherapy is administered to patients with nonsquamous histology, regardless of their PD-L1 expression status (17).

Natural killer (NK) cells are innate lymphocytes with cytotoxic activity against cancer cells mediated by the release of cytokines and chemokines (18). NK cells participate in immune responses against solid and hematopoietic cancers owing to their capacity to recognize characteristic molecular patterns of stressed cells. Unlike T cells, NK cells are able to recognize cancer cells without requiring neoantigen or self-antigen overexpression, and loss of major histocompatibility complex (MHC) expression increases the susceptibility of tumor cells to NK cell-mediated death (19). The killer cell immunoglobulin-like receptors (KIRs) are expressed on the

surface of NK cells, and different KIRs recognize and bind different human leukocyte antigen (HLA) ligands (20). It is advisable to select KIR and its ligand, HLA class I molecules from mismatched alloreactive blood donors (21). In our previous studies, allogeneic NK cells achieved a certain inhibitory effect on tumor growth (19, 22). Furthermore, evidence of PD-1 expression on human NK cells has recently emerged in several cancer types (23, 24).

Based on the promising results of those studies, we conducted a randomized clinical trial with the aim of investigating the safety and efficacy of pembrolizumab in combination with NK cells as second- or third-line treatment for advanced NSCLC patients.

Results

Enrolled patients. Between July 2016 and November 2017, 379 patients were screened for enrollment, of which 249 (65.7%) were assessed for PD-L1 expression. Of the 186 (74.7%) PD-L1-positive patients, 78 (31.3%) had PD-L1 expression on at least 50% of their tumor cells. The PD-L1-positive patients were screened for the eligibility criteria, and 109 (58.6%) were finally enrolled in the study and assigned randomly to group A (55) or group B (54) (Figure 1A). Baseline characteristics were balanced between the two groups (Table 1). The majority of the patients enrolled in the randomized trial were current or former smokers, had tumors with nonsquamous histology, and had previously received first-line systemic treatment. Only a few patients had tumors with an epidermal growth factor receptor (EGFR)-sensitizing mutation or anaplastic lymphoma kinase (*ALK*) gene translocation. Baseline characteristics were also similar among the group A patients who received NK cell infusion (Table 1).

Safety evaluation. Treatment was well tolerated throughout the trial. Our previous studies confirmed that NK cell infusion had no serious side effects (19, 22), so the adverse events should be attributed to pembrolizumab. The most common adverse events during the trial and the proportions of treatment-related adverse events by grade are shown in Figure 2. There was no significant difference in the incidence of adverse events between the two groups ($P>0.05$). All

adverse events were below grade 4, with grade 2 events composing the majority. All symptoms were relieved after symptomatic treatment. No pembrolizumab-related grade 4 adverse events were observed in the patients in this study. Two patients (1.8%) discontinued pembrolizumab treatment.

Immune parameters. Immune parameters were evaluated, and we found that there was no significant difference between group A and group B before treatment ($P>0.05$) (Figure 3). After combination treatment, the accumulation of lymphocytes, especially NK cells, significantly increased in group A (Figure 3A). A representative flow cytometry result from a group A patient is shown in Supplementary Figure 6. Before treatment, the absolute numbers of total T cells, CD8⁺ T cells, CD4⁺ T cells, and NK cells per microliter were 811.4, 420.1, 315.0, and 66.1, respectively. After combination therapy, the absolute numbers of the same subpopulations of lymphocytes per microliter increased to 1115.7, 569.2, 444.5, and 125.6, respectively. The percentages of total and subtypes of T cells and NK cells are shown in Supplementary Table 5. Notably, NK cells increased from $8.76 \pm 4.06\%$ of the total population to $20.67 \pm 5.31\%$ after combination treatment. Interestingly, the levels of Th1 cytokines, including interleukin (IL)-2, tumor necrosis factor (TNF)- β , and interferon (IFN)- γ , increased significantly in group A after treatment (Figure 3B).

Tumor markers. There were no significant differences in the levels of carcinoembryonic antigen (CEA), cytokeratin 19 fragment antigen 21-1 (Cyfra21-1), or carbohydrate antigen (CA)125 between the two groups before treatment, although the levels were above the normal range ($P>0.05$). Importantly, the levels of CEA, Cyfra21-1, and CA125 in group A were significantly decreased after combination treatment, despite some efficacy for pembrolizumab treatment being found in group B (Figure 3C).

Circulating tumor cells (CTCs). There was no significant difference in the median number of CTCs between group A and group B before treatment ($P>0.05$) (Figure 3D). In comparison with the pembrolizumab treatment regimen in group B, the combined treatment regimen in group A showed a more significant effect on the reduction of CTCs in 7.5 mL of blood (13.0 ± 5.3 before treatment

to 10.8 ± 3.1 after treatment; $P < 0.05$). A representative flow cytometry result from a group A patient is shown in Supplementary Figure 7.

Tumor response. The objective response rate (ORR) was assessed by radiological review and judged according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines. The ORR in the total population was 27.5%, and the ORR in group A (36.4%) was superior to that in group B (18.5%) (Table 2). All responses were partial. Representative computed tomography (CT) images for two patients who received combination therapy are displayed in Supplementary Figure 8. The tumor maximum transverse diameters (MTDs) of all patients are shown in Figure 4. Although there was no significant difference in the median tumor MTD between the two groups before treatment ($P > 0.05$), it decreased significantly after combination treatment with pembrolizumab plus NK cells ($*P < 0.05$).

Overall survival (OS). At the time of data cutoff, 86 patients had died, including 38 of 55 (69.1%) patients in group A and 48 of 54 (88.9%) patients in group B. Among the patients with a PD-L1 tumor proportion score (TPS) $\geq 50\%$, 39 died, including 17 of 23 (73.9%) in group A and 22 of 24 (91.7%) in group B. The median OS of the group A patients was 15.5 months (Figure 5A), which was longer than that of the group B patients ($P < 0.05$). The benefit of combination therapy received by the patients in group A was consistently observed in all subgroups that were analyzed (Figure 5B). In the patients with a PD-L1 TPS $\geq 50\%$, the median OS of patients in group A was 17.0 months, which was longer than that of patients in group B ($P < 0.05$) (Figure 5C). Furthermore, the median OS was significantly longer in patients who received multiple courses of NK cell infusion than in those administered only a single course of NK cell infusion ($P < 0.05$) (Figure 5D).

Progression-free survival (PFS). Of the 109 enrolled patients, 94 died or had disease progression, i.e., 43 patients from group A and 51 patients from group B died or developed disease aggravation. There were 47 patients with a PD-L1 TPS $\geq 50\%$, of whom 43 had experienced disease progression, including 20 in group A and 23 in group B. The median PFS in group A was 6.5

months (Figure 6A), which was superior to that in group B ($P<0.05$). The benefit of combination therapy received by group A patients was consistently observed in all subgroups that were analyzed (Figure 6B). The median PFS in group A patients with a PD-L1 TPS $\geq 50\%$ was 7.0 months, which was longer than that of the corresponding group B patients ($P<0.05$) (Figure 6C). However, we failed to detect a difference in the median PFS between patients who received a single course of NK cell infusion and those who received multiple courses of NK cell infusion ($P>0.05$) (Figure 6D).

Discussion

Outcomes are poor for patients with previously treated advanced or metastatic NSCLC, and systemic chemotherapy provides only modest benefits (4). PD-L1 is an immune checkpoint protein expressed on tumor cells and tumor-infiltrating immune cells that downregulates the antitumor function of T cells through binding to PD-1 (25). Clinical studies of anti-PD-1 antibodies, such as nivolumab and pembrolizumab, have established the therapeutic value of targeting the PD-1/PD-L1 pathway (26, 27). In our study, pembrolizumab was well tolerated with a good safety profile, which is consistent with the results of a previous study by Herbst et al. (14).

PD-1 was reported to be expressed in activated T cells, B cells, NK cells, and NKT cells and play an important role in the regulation of the threshold, strength, duration, and properties of antigen-specific immunological responses (28). In the current study, we found that PD-1 was expressed at a higher level in the peripheral NK and T lymphocytes of patients than in those of healthy donors, which explains the phenomenon that high expression of inhibitory molecules leads to dysfunction and apoptosis of lymphocytes, thus favoring tumor growth and metastasis. The high PD-1 positivity in cancer patients was thought to be due to prolonged stimulation by tumor-derived ligands. After blocking the interaction between PD-1 and PD-L1 with pembrolizumab, the phenomenon induced by high PD-1 expression was reversed, and the secretion of IFN- γ was increased, which enhanced immune function. Furthermore, PD-1 was expressed at a lower level in patients who received combination therapy, indicating that the combination therapy was more

beneficial than pembrolizumab alone, which was explained by the functional recovery of activated NK cells. Kamata et al. found that PD-1 expression was elevated in freshly isolated circulating iNKT cells from peripheral blood mononuclear cells (PBMCs) of NSCLC patients and blockade of PD-L1 augmented Th1 cytokine production in iNKT cells, resulting in cytotoxicity mediated by NK cells (29).

It is commonly believed that T cells are the only important mediator of the antitumor response unleashed by PD-1 blockade. However, this point of view is challenged by at least two observations: (a) PD-1 blockade is still effective in some human tumors with low expression of HLA-I molecules (30), and (b) a strong clinical response to PD-1 blockade is observed in Hodgkin's lymphomas that do not express HLA-I molecules (31). Unlike T cells, NK cells respond to MHC-deficient tumors (32) through activation by ligands whose expression is usually increased upon oncogenic stress (33). Based on these premises, we hypothesized that PD-1 blockade might activate NK cell responses.

In this study, human high-activity NK (HANK) cells with activated and cytotoxic status were derived from appropriate allogeneic donors. After infusion of the expanded NK cells, the number of NK cells in patients was significantly increased, but the patients treated with pembrolizumab alone failed to show any significant effect on the number of NK cells in the blood, indicating that the increase of NK cells in patients was caused by NK cells of donor origin. A previous study by Bouchlaka, et al. reported that using nonradioactive isotope fluorine-19 (^{19}F) to label and track NK cells in preclinical models by magnetic resonance imaging (MRI) (34). However, in view of the potential influence on the therapeutic effect and clinical safety, we didn't test the presence of NK cells of donor origin with this method. In addition, our result also demonstrated that the expanded NK cells had a stronger cytotoxicity, which was in line with a previous study performed by Kamiya and colleagues (35). Furthermore, we found that combining pembrolizumab with allogeneic NK cell therapy significantly enhanced immune functions, especially cellular immunity, which could be explained by the increased proportion of NK cells in the blood (36). In line with this result,

combination therapy also significantly reduced the levels of CTCs and tumor markers. It's well established that reduction of CTCs reflects the fact that NK cells prevent metastasis and alleviate the residual tumor load by targeting CTCs in the blood (37).

Garon et al. assessed the efficacy and safety of PD-1 inhibition with pembrolizumab and found that pembrolizumab had an acceptable side-effect profile and showed antitumor activity in patients with advanced NSCLC (27). Our study demonstrated that, in comparison with pembrolizumab alone, combined therapy of pembrolizumab plus NK cells had a higher ORR, indicating that the combination therapy was superior to pembrolizumab monotherapy. Intriguingly, the combination therapy met the prespecified criteria for improving OS and PFS in all patients, especially those with a TPS $\geq 50\%$. A survival benefit associated with the combined therapy was also observed in all subgroups. In addition, the patients treated with NK cells had better OS than those never treated with NK cell infusion, and the improvement in OS was associated with the NK cell treatment course, which was consistent with the results of previous reports (22, 25). Notably, less than 10% of the patients enrolled in our trial harbored sensitizing mutations, and treatment with tyrosine kinase inhibitors (TKIs) failed and these patients displayed tumor progression. The PFS and OS of patients with sensitizing mutations were 3.1 months and 9.7 months, respectively, which were much lower than those in the rest of the cohort. However, further studies with a larger patient sample are needed to confirm these results.

This exciting phenomenon confirmed that pembrolizumab plus NK cells exhibited an enhanced effect *in vivo*, suggesting that PD-1/PD-L1 blockade elicits an antitumor response by NK cells. This is consistent with the results of previous studies showing that PD-L1 blockade enhances the antitumor efficacy of NK cells (38, 39). Based on our results and other reports, it is reasonable to propose that, in addition to T cells, NK cells participate in the clinical benefit of anti-PD-1/PD-L1 antibody therapy by directly killing tumor cells and/or recruiting T cells (39). NK cells exhibit cytotoxicity against various tumors, but they fail to eliminate tumors completely *in vivo*, because the high expression of inhibitory molecules (i.e., PD-1) leads to dysfunction and apoptosis of NK

cells (29). In addition, several studies have reported that PD-L1 expression in cancer cells resulted in reducing NK cell responses and PD-1/PD-L1 blockade stimulates NK cell responses in vivo (40, 41), which supports our finding that the combination of NK cells with a checkpoint inhibitor improved the antitumor effect of NK cells.

Checkpoint inhibitors have revolutionized cancer treatment. Recently, it was reported that PD-1 was expressed on human NK cells in several cancer types, including Hodgkin's lymphoma (23, 24). There are a few recent mechanistic in vivo studies examining whether and how PD-1 inhibits NK cell responses to tumors and whether PD-1/PD-L1 blockade stimulates NK cell responses. Hsu et al. revealed that PD-1/PD-L1 blockade relieved the inhibition of T cells and NK cells by cancer cells, and combination therapy with antibodies against PD-1 or PD-L1 enhanced the antitumor effects of NK cells (39). Oyer et al. found that combinatorial application of PM21-NK cells and anti-PD-L1 treatment improved NK cell function and significantly extended survival in an animal model of aggressive disseminated peritoneal ovarian cancer (38). Benavente et al. reported that the combination of PD-1 antibody and EGFR blockade in head and neck cancer patients with high PD-L1 expression improved their clinical response by reversing NK cell dysfunction (41). In addition, Kevin C et al. found that the NK/dendritic cell (DC) axis defined responsive tumor microenvironments in melanoma, which revealed that NK cells, through the production of *FLT3LG* in the tumor, controlled the levels of stimulatory DCs (SDCs) and further improved the responsiveness to anti-PD-1 immunotherapy (42). Dong et al. showed that the binding of anti-PD-L1 monoclonal antibody to PD-L1⁺ NK cells induced a strong antitumor activity both in vitro and in vivo, so PD-L1⁺ NK cells might be another important immune effector for checkpoint inhibitor-based cancer immunotherapy (40). Andre et al. reported that the immune checkpoint NKG2A/HLA-E was used by cancer cells to evade attack by the immune system, and administration of monalizumab, a humanized antibody against NKG2A, enhanced NK cell activity against various tumor cells and rescued CD8⁺ T cell function (43). These various mechanistic studies indicated that immune checkpoint blockade stimulated NK cell responses.

In conclusion, this is the first clinical trial to investigate the safety and efficacy of the combination of pembrolizumab and NK cell infusion. Pembrolizumab plus NK cells yielded an improved survival benefit in patients with previously treated, PD-L1-positive, advanced NSCLC. Our results suggest that there are opportunities for therapy with an anti-PD-1 or anti-PD-L1 antibody combined with NK cells to enhance antitumor effects (44), which provides a novel strategy for the treatment of previously treated, PD-L1-positive advanced NSCLC patients.

Methods

Eligibility criteria. Patients with stage IIIB or stage IV NSCLC, according to the American Joint Committee on Cancer (AJCC) criteria, with documented malignant pleural effusion proven by histology or cytology were enrolled in our study. Other enrollment criteria included the following: measurable disease according to the RECIST guidelines, age ≥ 18 years, progression after chemotherapy or appropriate TKI treatment for those patients with an EGFR-sensitizing mutation or *ALK* rearrangement, a life expectancy ≥ 3 months, and a PD-L1 TPS $\geq 1\%$. The most important exclusion criteria were proven brain metastasis, a history of level 3 hypertension, severe coronary disease, myelosuppression, autoimmune disease, pneumonitis, chronic viral disease, pregnancy, or previous treatment with a therapeutic antibody against CTLA4, PD-L1, or PD-1 or PD-L1/PD-1 pathway-targeting agents.

Pembrolizumab monotherapy. Patients received intravenous pembrolizumab (10 mg/kg) on day 1 of a 21-day cycle. Pembrolizumab treatment was continued as long as the patients received clinical benefit according to investigator assessment (absence of unacceptable toxicity or symptomatic deterioration attributed to disease progression after an integrated assessment of radiographic data, biopsy results, and clinical status) and the patients consented to continuation.

HANK cell preparation. A human high-activity NK (HANK) cell in vitro preparation kit (Hank Bioengineering Co., Ltd, Shenzhen, China) was used to prepare NK cells with high quantity, purity, and activity from PBMCs. The detailed method for HANK cell expansion and activation is

described in the Supplementary data.

HANK cell cytotoxicity assay. NK cell viability was assessed by Chromium-51 (⁵¹Cr) release assay as described in the Supplementary data.

Detection of HANK cell-related surface receptors. The receptors on the cell surface were analyzed with FACS before expansion and 12 days after expansion as detailed in the Supplementary data.

Donor selection. The donors were selected based on genotyping mismatch between the KIR of allogeneic donors and the HLA class I molecules of patients. The detailed method for KIR detection and HLA class I molecule genotyping is shown in the Supplementary data.

Treatment procedures. Using a random number table, patients were divided into group A, which received pembrolizumab plus NK cell therapy, and group B, which was treated with pembrolizumab alone. The treatment schedule is summarized in Figure 1B. Patients in group A received pembrolizumab plus one to three courses of allogeneic NK cells; one NK cell treatment course was designed to contain two cycles in a 28-day period, i.e., infusion on days 12, 13, and 14 for the first cycle and days 26, 27, and 28 for the second cycle. The patients in group B received pembrolizumab therapy regularly. Peripheral blood was drawn from a donor for NK cell amplification. Administration of pembrolizumab was once every three weeks, and continued until disease progression or unacceptable toxicity occurred. Tumor assessment was performed by CT imaging until disease progression.

Safety monitoring. Safety assessments included regular interviews with patients and monitoring of hematological and blood chemistry parameters. Adverse events and complications during treatment were closely observed and recorded. All adverse effects were assessed and reported according to the National Cancer Institute Common Terminology Criteria for Adverse Events (v4.0).

Detection of PD-1. Peripheral blood (6 mL) was obtained from healthy donors and patients before treatment and 90 days after treatment for the detection of PD-1. Details can be found in the Supplementary data.

Detection of immune function. Peripheral blood (2 mL) was obtained from patients before treatment and 90 days after treatment for the detection of immune function by flow cytometry with a FACSCanto™ II (BD Biosciences, San Jose, CA, USA). The multitest 6-color TBNK reagent (#337166, BD Biosciences) with Trucount tubes were used to detect the absolute numbers of CD3⁺CD4⁺ cells, CD3⁺CD8⁺ cells, total CD3⁺ cells, CD3⁻CD19⁺ cells, and CD3⁻CD16⁺CD56⁺ cells. A human Th₁/Th₂ cytokine kit II (#551809, BD Biosciences) for a cytometric bead array was used to detect the expression of IL-2, IL-4, IL-6, IL-10, TNF-β, and IFN-γ. The tests were carried out according to the manufacturer's protocols. Patients with parameters within the reference range defined by the manufacturer were considered to have normal immune function. Patients with one or more parameters with a below-normal value were considered to have immune dysfunction.

Analysis of CTCs. Peripheral blood (7.5 mL) was obtained from patients before treatment and 90 days after treatment for the detection of CTCs. The samples were stored at room temperature and processed within 6 h of collection. Mononuclear cells were separated from other blood components using a human PBMC separation liquid (#LDS1075CB, Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China). The cells were centrifuged at 1,800 ×g for 20 min at 4°C and washed twice with sterile Hank's balanced salt solution (#24020117, Life Technologies, Carlsbad, CA, USA). The isolated cells were enriched by magnetic CD326 (epithelial cell adhesion molecule) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, a phycoerythrin (PE)-labeled antibody against CD45 (#5B1, 10 μL), fluorescein isothiocyanate (FITC)-labeled antibodies against cytokeratins 8, 18, and 19 (#REA885, 10 μL), and an allophycocyanin (APC)-labeled antibody against CD326 (#HEA-125, 10 μL) (Miltenyi Biotec) were added to the enriched cells, followed by incubation in the dark for 12 min at room temperature. The cell pellets were resuspended in 500 μL of PBS, and the samples were analyzed by a FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with a CD45⁻/CK⁺/CD326⁺ gating strategy. The absolute number of CD45⁻CK⁺ CD326⁺ cells was used to quantitate the CTC levels.

Tumor marker detection. The serum concentrations of CEA, Cyfra21-1, and CA125 were

evaluated by a chemiluminescent immunoassay before treatment and 90 days after treatment.

Tumor response. The tumor responses of the enrolled patients were assessed by CT in accordance with RECIST v1.1. Tumor imaging was scheduled for weeks 6 and 12 and was then performed every 9 weeks through week 48 and every 12 weeks thereafter. According to the degree of change in the largest transverse diameter, the therapeutic effect was classified as one of the following: complete response (CR): arterial enhancement imaging of all target lesions disappeared; partial response (PR): the total reduction in the diameters of the target lesions was more than 30%; stable disease: tumor regression failed to reach the PR definition or tumor progression failed to reach the progressive disease definition; or progressive disease: total progression of the tumor with an increase in the diameter of more than 20%. The curative effect had to be maintained for more than 4 weeks, and CR+PR represents the ORR.

PFS and OS. PFS was defined as the interval between treatment initiation and local relapse, distant metastasis, or death, whichever occurred first. OS was calculated as the interval from treatment initiation to death. All patients were consistently monitored after treatment by our manual and intelligent follow-up system.

Statistical analysis. The primary endpoints were OS and PFS. Safety, immune parameters, CTCs, tumor markers, ORR, and other indexes were the secondary endpoints. The results are presented as the mean \pm standard deviation or median \pm range. All statistical tests comparing the treatment groups were two-sided Student's *t* test, and $P < 0.05$ was considered statistically significant. To evaluate whether the combined treatment had a more significant effect on the reduction in CTCs, the analysis of covariance (ANCOVA) method was used. Kaplan-Meier analysis was used to estimate survival and draw survival curves. We used the stratified log-rank test to assess differences in PFS and OS based on treatment. We used stratified Cox proportional hazard models to calculate hazard ratios (HRs) and associated 95% confidence intervals (CIs). SPSS v 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses.

Study approval. This clinical trial was approved by the Ethics Committee of Guangzhou Fuda Cancer Hospital, Jinan University (Guangzhou, China). Informed consent was obtained from all 109 patients in accordance with the Declaration of Helsinki.

Author contributions

ML contributed to data acquisition, data interpretation, statistical analysis and drafting of the manuscript. HHL, SZL and JBC contributed to data acquisition and statistical analysis. AHL, LZN and YJ contributed to the study design and critical revision of the manuscript. All the authors have final approval of the submitted manuscript and reached agreement to be accountable for all aspects of the work.

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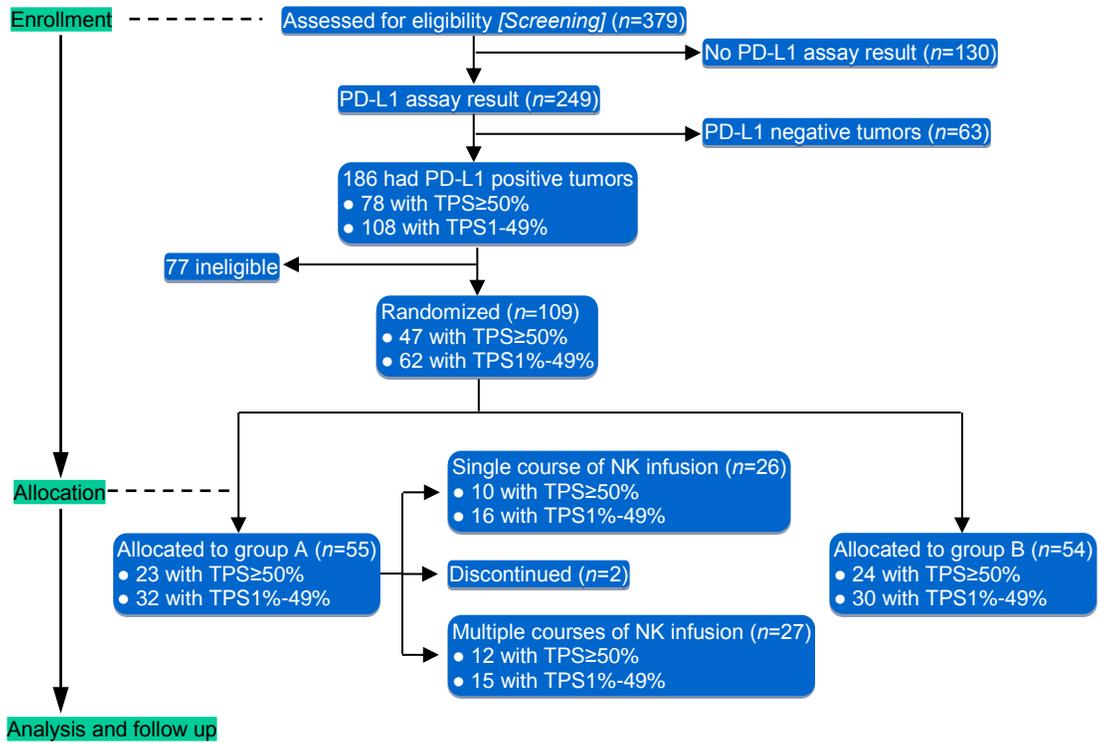
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A



B

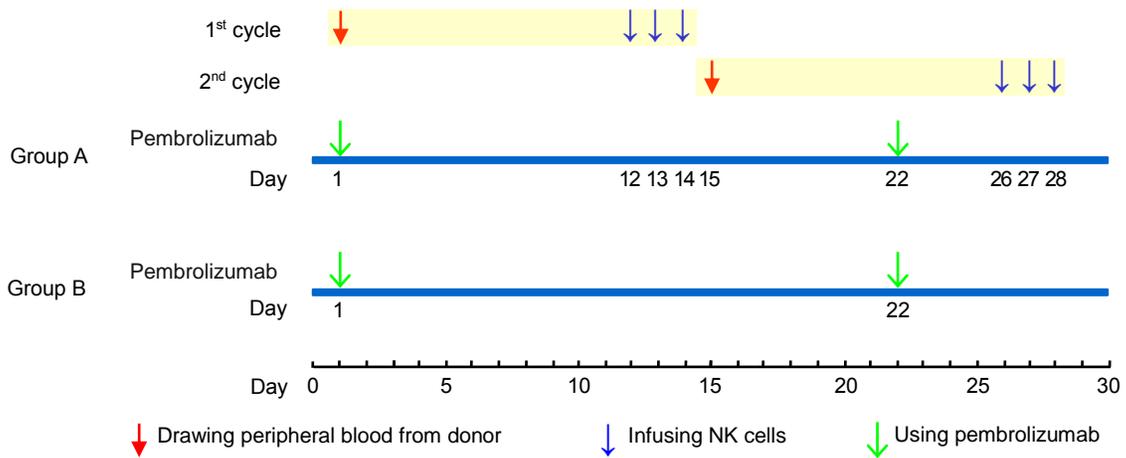


Figure 1. The clinical trial profile and treatment schedule. (A) The clinical trial profile. In total, 379 patients were selected for enrollment, of whom 249 (65.7%) were assessed for PD-L1 expression. For the 186 (74.7%) PD-L1-positive patients, 78 (31.3%) had PD-L1 expression on at least 50% of their tumor cells. The PD-L1-positive patients were screened for the eligibility criteria, and 109 (58.6%) were enrolled in the study and assigned randomly to group A or B. (B) Clinical treatment schedule. Patients in group A received pembrolizumab plus one to three courses of allogeneic NK cells; one NK cell treatment course was designed to contain two cycles, totaling six NK cell infusions in 28 days, i.e., days 12, 13, and 14 for the first cycle and days 26, 27, and 28 for the second cycle. Patients in group B received regular pembrolizumab therapy with intravenous injection of pembrolizumab (10 mg/kg) on day 1 of a 21-day cycle, and the treatment was continued until disease progression or unacceptable toxicity occurred. $n = 109$.

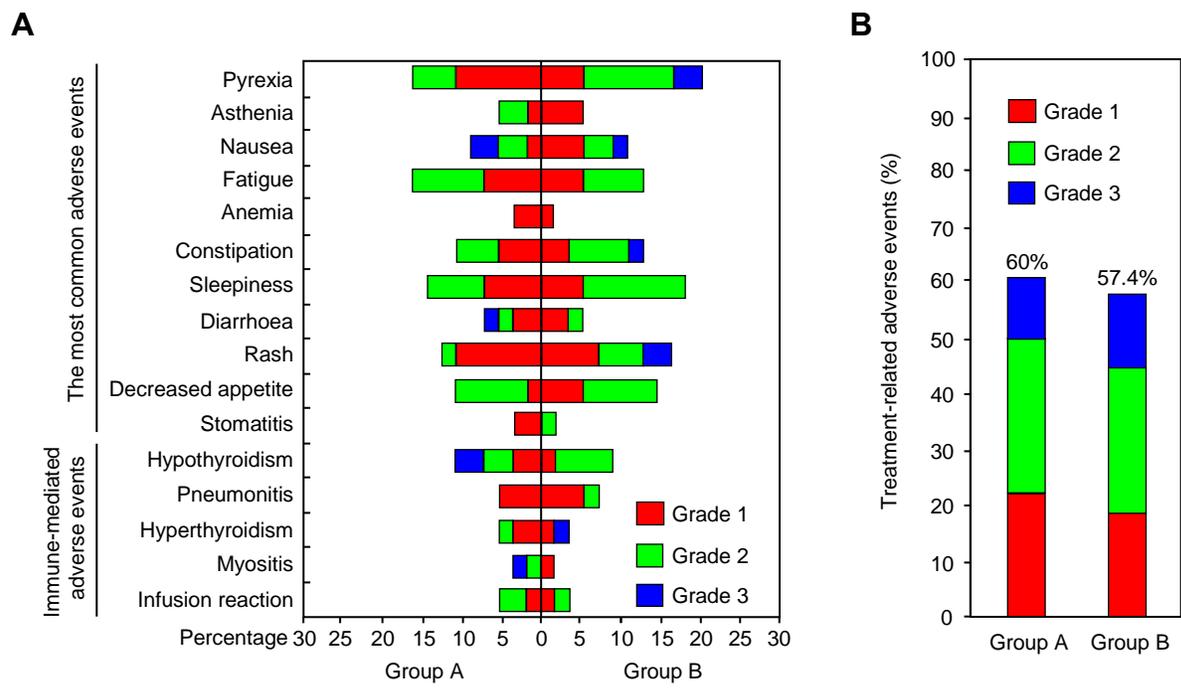


Figure 2. All-cause adverse events in the safety population. (A) All-cause adverse events with a difference of no less than 5% between the study groups. (B) Proportions of patients with treatment-related adverse events presented by grade. There was no significant difference between the two groups. $n = 109$. $P > 0.05$. Chi-square test.

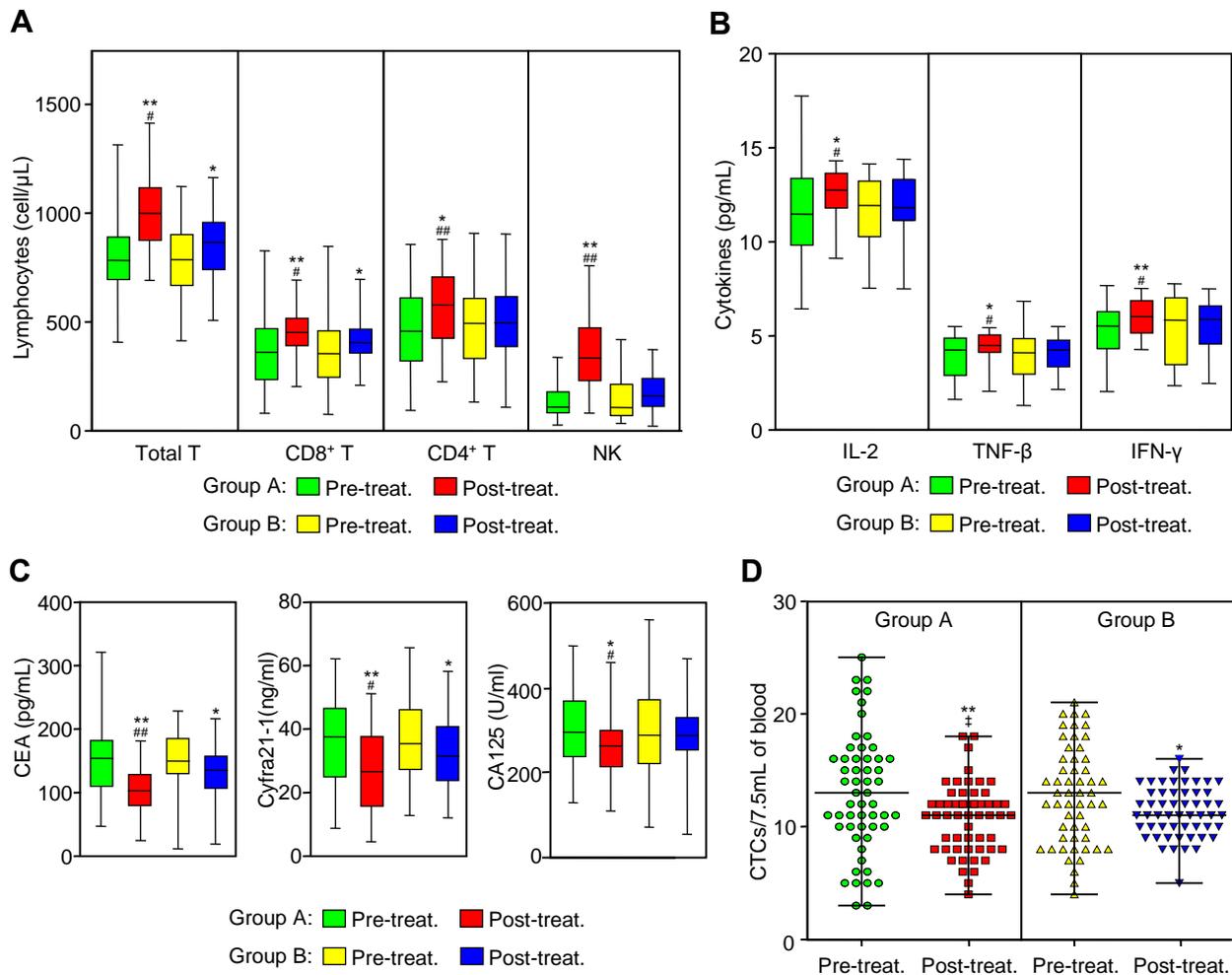


Figure 3. Evaluation of immune parameters, tumor markers, and CTCs before treatment and 90 days after treatment. (A) Flow cytometry was performed with the 6-color TBNK reagent to detect lymphocytes in the blood. $n = 109$. Data are presented as the median \pm SD. Two-sided Student's t test. Comparison within groups: * $P < 0.05$, ** $P < 0.01$; comparison between groups: # $P < 0.05$, ## $P < 0.01$. Pre-treat.: before treatment; Post-treat.: 90 days after treatment. (B) Flow cytometry was performed with the Cytometric Bead Array Human Th1/Th2 Cytokine Kit II to detect cytokines in the blood. (C) The levels of tumor markers including CEA, Cyfra21-1, and CA125 were quantitated by a chemiluminescent immunoassay. (D) The number of CD45-CK⁺CD326⁺ cells (CTCs) was acquired with a FACSCanto™ II. ANCOVA was used to analyze the effect of combined treatment on the reduction in CTCs in 7.5 mL of blood compared to pembrolizumab alone. $n = 109$. † $P < 0.05$.

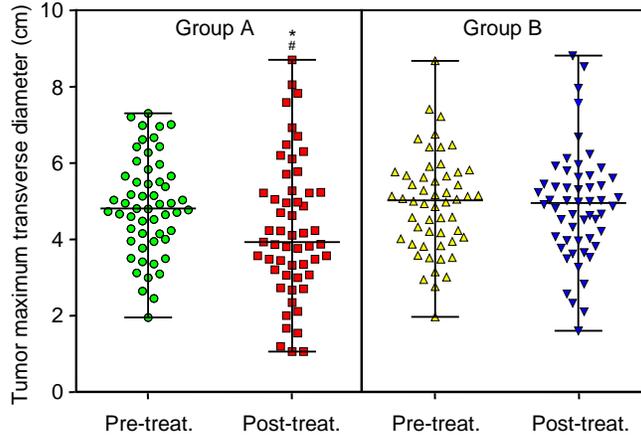


Figure 4. The effect of combination therapy on the tumor maximum transverse diameter of NSCLC patients. Data are presented as the median \pm SD. * $P < 0.05$, compared with before treatment. # $P < 0.05$, compared with group B patients. Pre-treat.: before treatment; Post-treat.: 90 days after treatment. $n = 109$. Two-sided Student's t test.

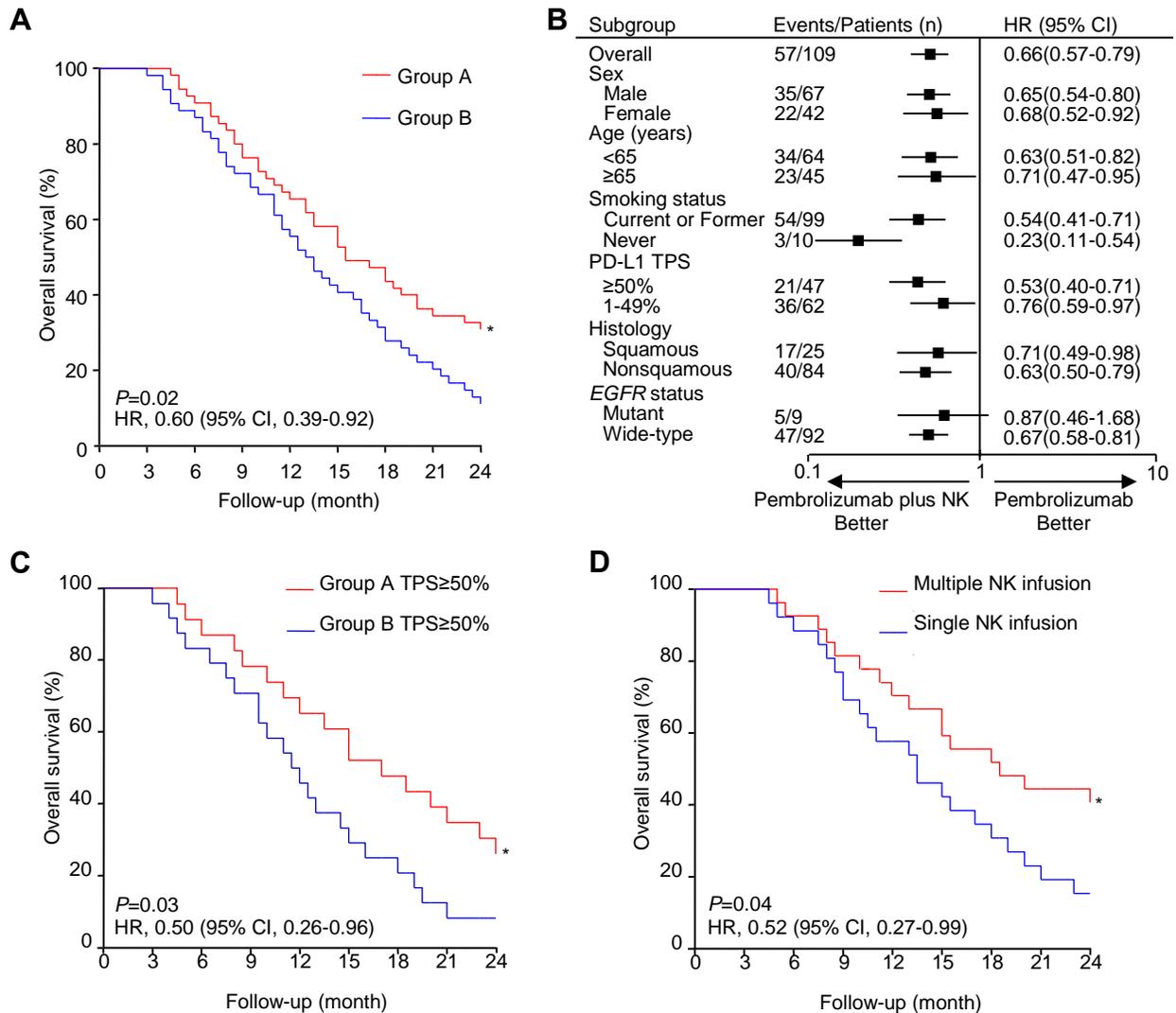


Figure 5. OS analysis. (A) Kaplan-Meier analysis was used to estimate the OS of the total population. $n = 109$. $*P < 0.05$. (B) Multivariate Cox regression analysis of OS for key subgroups. $n = 109$. (C) Kaplan-Meier analysis was used to estimate the OS of patients with a TPS $\geq 50\%$. $n = 47$. $*P < 0.05$. (D) Kaplan-Meier analysis was used to estimate the OS of group A patients who received a single course of NK cell infusion or multiple courses of NK cell infusions. $n = 53$. $*P < 0.05$.

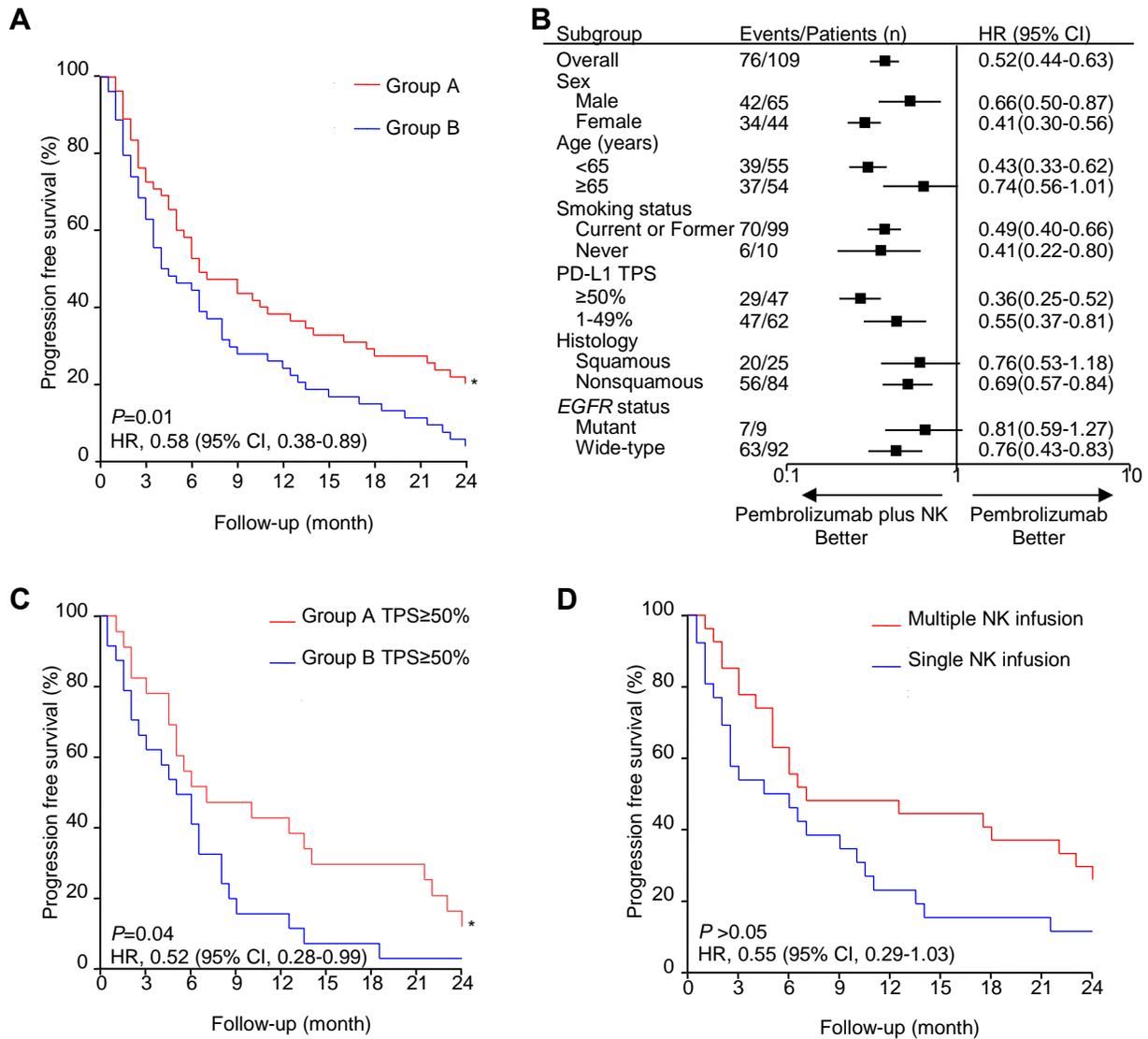


Figure 6. PFS analysis. (A) Kaplan-Meier analysis was used to estimate the PFS of the total population. $n = 109$. $*P < 0.05$. (B) Multivariate Cox regression analysis of PFS for key subgroups. $n = 109$. (C) Kaplan-Meier analysis was used to estimate the PFS of patients with a TPS $\geq 50\%$. $n = 47$. $*P < 0.05$. (D) Kaplan-Meier analysis was used to estimate the PFS of group A patients who received a single course NK cell infusion or multiple courses of NK cell infusions. $n = 53$. $P > 0.05$.

Table 1 Demographic and disease characteristics of the patients at baseline

	All patients		Patients in group A	
	Group A (n=55)	Group B (n=54)	Multiple NK (n=27)	Single NK (n=26)
Age(years)	62.0(56.0-69.0)	64.0(58.0-70.0)	60.0(54.0-68)	63.0(56.0-69.0)
Male sex	34(61%)	33(62%)	16(60%)	15(58%)
Smoking status				
Current or Former	45(82%)	44(81%)	22(81%)	20(78%)
Never	10(18%)	10(19%)	5(19%)	6(22%)
Histology				
Squamous	13(24%)	12(22%)	6(21%)	5(19%)
Non-squamous	42(76%)	42(77%)	21(79%)	21(81%)
PD-L1 TPS				
≥50%	23(41%)	24(44%)	12(44%)	10(40%)
1-49%	32(59%)	30(56%)	15(56%)	16(60%)
EGFR status				
Wild-type	47(85%)	45(83%)	23(86%)	22(84%)
Mutant	4(8%)	5(9%)	2(6%)	2(9%)
Unknown	4(7%)	4(8%)	2(8%)	2(7%)
ALK translocation				
No	48(88%)	49(90%)	23(87%)	22(86%)
Yes	1(1%)	1(1%)	0(1%)	0(1%)
Unknown	6(11%)	4(9%)	4(12%)	4(13%)
Previous therapy				
Adjuvant	3(5%)	2(4%)	0(1%)	1(2%)
Neoadjuvant	1(2%)	1(1%)	1(2%)	0(1%)

TPS- tumor proportion score.

Table 2 Tumor response

Response	Total population		Group A		Group B	
	N	Rate, %	N	Rate, %	N	Rate, %
CR	0	0	0	0	0	0
PR	30	27.5	20	36.4	10	18.5*
SD	59	54.1	30	54.5	29	53.7
PD	20	18.3	5	9.1	15	27.8*
ORR	30	27.5	20	36.4	10	18.5*

*: Significant difference ($P<0.05$).