Functional T cells targeting tumor-associated antigens are predictive for recurrence-free survival of patients with radically operated non-small cell lung cancer

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ABSTRACT
In this prospective study, we examined postoperative follow-up and preoperative IFN-γ T cell responses against 14 non-small cell lung cancer (NSCLC)-associated antigens in the blood of 51 patients with NSCLC, 7 patients with benign pulmonary tumors, and 10 tumor-free patients by enzyme-linked immunospot assay. The phenotype and function of T cells specific for tumor-associated antigens (TAAs) in the blood or tumor tissue of 9 NSCLC patients were characterized in detail using TNF-α, IL-2, and IFN-γ cytokine capture assays. We found that circulating TAA-specific T cells were significantly enriched in NSCLC compared with tumor-free patients. The most frequently recognized TAAs were Aurora kinase A, HER2/neu, NY-ESO-1, and p53. TNF-α was the most abundant cytokine secreted by TAA-specific T cells in the blood as well as by in situ-activated tumor-infiltrating lymphocytes, most of which were effector memory cells. The absence of TAA-reactive T cells identified patients at higher risk of tumor recurrence, irrespective of tumor stage (OR = 8.76, 95% CI: 1.57–34.79, p = 0.008). We conclude that pre-existing TAA-reactive circulating T cells are a strong independent prognostic factor for recurrence-free survival. These data may help discriminating high-risk from low-risk patients, improving prognostication, and redirecting adjuvant therapy. Our findings suggest the therapeutic relevance of Aurora kinase A, HER2/neu, NY-ESO-1, and p53 as targets for immunotherapy. This study is registered on Clinicaltrials.gov with trial identification number: NCT02515760.

Introduction
Lung cancer is the most common cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all new lung cancer cases. For patients with early-stage NSCLC, complete resection is the treatment of choice, providing the highest probability of cure. However, despite complete surgical resection, a high proportion of patients experience tumor recurrence within 5 years. To reduce the risk of tumor recurrence, adjuvant platinum-based chemotherapy is recommended for completely resected stage II-IIIA NSCLC and high-risk stage IB, but this provides only a 5-year absolute survival benefit of 5%. Moreover, only about half of the respective NSCLC patients are eligible for adjuvant chemotherapy because of medical comorbidities or poor performance status, underlining the urgent need for new treatment options with fewer side effects.

Throughout their disease, many cancer patients develop endogenous T cell responses against their tumor cells. Upon appropriate restimulation and expansion, such tumor antigen-reactive T cells can be used therapeutically, suggesting that they could improve prognosis. In resected NSCLC, tumor-infiltrating lymphocytes (TILs) are correlated with a favorable prognosis, which is a hint that tumor-specific T cell responses have a protective role and provides a strong rationale for increasing the number of tumor-antigen specific T cells through tumor vaccination. To our knowledge, no studies have addressed the prognostic impact of endogenous tumor antigen-specific T-cell responses in NSCLC. Several studies, mainly in patients with advanced NSCLC, have shown a correlation between the specific immune response to a therapeutic tumor vaccination and increased survival or clinical response. This is in agreement with the results of a recent meta-analysis, which combined the data from all published tumor vaccine trials in NSCLC, showing significantly improved survival following vaccination. Critical to the successful use of antigen-specific immunotherapy is the choice of the tumor-associated antigen (TAA) to target, which is currently insufficiently defined. Moreover, data describing the functional characteristics of endogenous tumor-reactive T cells and their role in NSCLC are lacking. A pilot project at the National Cancer Institute revealed that immunogenicity, as determined by the presence of spontaneous T cell responses, is the most important criterion for target antigen selection.
This prospective study was conducted, first, to investigate circulating T cells in the peripheral blood of NSCLC patients responsive to a panel of NSCLC-associated TAAs, and second, to present a hierarchy of candidate antigens for antigen-specific cancer immunotherapy.

Results

TAA-reactive T cells are abundant in lung tumor patients

Eighty-six subjects were enrolled in this study between February 2014 and May 2015, of which were excluded post-operatively (see Suppl. Fig. 1). Ten tumor-free patients (median age: 57 years, range: 29–66), 7 patients with a benign tumor (median age: 46 years, range: 33–58), and 51 patients with NSCLC (median age: 64 years, range: 40–83) were included in enzyme-linked immunospot assay (ELISPOT) analysis. Although 60 NSCLC patients were originally selected for ELISPOT analysis, 9 patients were excluded from the analysis for technical reasons. Patient characteristics are summarized in Suppl. Tables 1–5. Suppl. Fig. 1 describes the exclusion criteria in detail.

We assessed endogenous T cell responses to a panel of synthetic peptides17–19 of 14 well-described tumor antigens (Fig. 1A and Suppl. Table 6) using an interferon (IFN)-γ ELISPOT assay as described previously.20 The data from one exemplary NSCLC patient are shown in Suppl. Fig. 2 and in Fig. 1A, demonstrating the presence of T cells reactive against several TAAs.

In total, we detected TAA-reactive T cells in 34 out of 51 NSCLC patients (67%), most of which showed simultaneous T cell reactivity against 2 to 3 different antigens (Fig. 1B). We also detected significant T cell reactivity against TAAs in 6 out of 10 (60%) tumor-free patients, though most tumor-free patients showed T cell reactivity against only one TAA (Fig. 1B), and in 5 out of 7 patients with non-malignant tumors (Suppl. Fig. 3). These results suggested that the generation of endogenous T cell responses against NSCLC-associated self-antigens is not exclusively related to malignant tumor disease.

Test wells were compared with negative control wells, and those having a fold change >2 with statistical significance were defined as showing a strong positive T cell response. In NSCLC patients, 32% of the tested samples showed strong T cell responses against individual TAAs, whereas in tumor-free patients, this was less than 13% (p < 0.001, 2-sided chi-square test). Moreover, in NSCLC patients, IFN-γ spot counts in TAA-containing wells were significantly higher overall than in negative-control wells (p < 0.001, Fig. 1C), whereas this was not the case in tumor-free patients. Likewise, NSCLC patients showed significantly higher frequencies of TAA-reactive T cells than tumor-free patients (Fig. 1C). In contrast, there were no statistically significant differences in responses to viral recall antigens between NSCLC patients and tumor-free patients (data not shown). Interestingly, in patients with benign tumors, T cell responses against TAAs were also significantly increased over both negative controls and tumor-free patients (p < 0.001 and p = 0.006 respectively) (Suppl. Fig. 4), but they did not differ significantly from the responses observed in NSCLC patients (Suppl. Figs. 5, 6). Thus, both benign and malignant lung tumors frequently induce endogenous T cell responses against NSCLC-associated antigens.

Regarding individual TAAs, T cell reactivity in NSCLC patients was high against p53 and NY-ESO-1 (both >25%), HER2/neu, and Aurora kinase A (both >30%) (Fig. 1D). We did not find significant T cell reactivity against NY-ESO-1 in 14 patients with non-malignant disease (8 tumor-free patients and 6 patients with benign tumor) (p = 0.033, 2-sided chi-square test), and rarely against Her2/neu (8%) (Fig. 1D and Suppl. Fig. 5). Statistical comparison of spot counts in the test and control wells revealed significantly increased T cell responses against heparanase, RHAMM, NY-ESO-1, and Aurora kinase A in NSCLC patients compared with tumor-free patients (Fig. 1E). TAA-reactive T cells were comparable in patients with benign tumors, with the exception of NY-ESO-1, which exerted stronger (though not significant) T cell responses in NSCLC patients (Suppl. Fig. 6).

Taken together, TAA-specific T cell responses were detected in 2-thirds of patients with NSCLC and were significantly increased compared with tumor-free individuals, but comparable to those in patients with benign tumors. P53, NY-ESO-1, Aurora kinase A, and HER2/neu were the most frequent targets of endogenous T cell responses in NSCLC patients. Thus, both benign and malignant lung tumors are associated with increased endogenous T cell responses against NSCLC-associated TAAs.

T cell subset composition in blood and tumors of NSCLC patients

To characterize tumor-reactive T cell subsets in the blood and tumor tissues, we analyzed 9 NSCLC patients in detail. CD4+ and CD8+ T cell subsets were identified by flow cytometry using established phenotypic marker panels21–24 as follows: T central memory (TCM): CD45RA−CD62L+, effector T cells (Teff): CD45RA+CD62L−, T effector memory (TEM): CD45RA−CD62L+, and naive T cells (TN): CD45RA+CD62L+. Recently, a small subset of antigen experienced stem-like memory T cells (TSCM) that share major phenotypic characteristics with TNS but differ from the latter in their capacity for early cytokine secretion after antigenic stimulation has been described.21 As our analysis did not allow phenotypic distinction between TN and TSCM, we designated this population as TN/SCM. The gating strategy used is shown for a representative NSCLC patient (Fig. 2A). To assess TILs, tumors were processed immediately after surgical resection. Memory T cells, particularly CD4+ and CD8+ TEM subpopulations, made up more than 80% of the T cell fraction in TILs, whereas peripheral blood-derived T cells (PBTCs) were predominantly of a TN/SCM phenotype. In contrast, very few TILs had a TN/SCM or effector T cell phenotype (Fig. 2B). Compared to PBTCs, the CD4+ / CD8+ ratio in TILs was significantly shifted toward CD8+ T cells (Fig. 2C). The effector-to-TN/SCM ratio was increased in TILs for both CD4+ and CD8+ T cell subsets, and furthermore, the mean TEM-to-TN/SCM ratio was 60-fold higher in TILs than in PBTCs (Fig. 2D). Thus, in contrast to the blood, TEMs represented the dominant T cell population in TILs, particularly in the CD8+ compartment.

TAA-reactive T cells in the blood predominantly secrete TNF-α

Next, we studied the contribution of the respective T cell subsets to the small subpopulation of tumor-reactive T cells
by combining flow-cytometric T cell phenotyping with cytokine capture assays that assess TAA-specific cytokine secretion by individual T cells. Previous observations in a murine infection model, in which different vaccines conferred varying degrees of protection, showed that T cell TNF-α, IFN-γ, and IL-2 secretions correlated best with immune protection. In contrast, in one colorectal cancer study, the prognostic significance of TILs was found to be associated with the TNF-α-secreting subpopulation. Therefore, we also analyzed the secretion of TNF-α and IL-2 in the T cell subpopulations.

Following stimulation with the TAAs p53, MUC-1, EGFR, and heparanase, compared with stimulation with the IgG negative control peptide, a strong and significantly increased level of TNF-α secretion was detected in both CD4+ and CD8+ PBTCs from cells of the 5 patients we tested (Fig. 3A). In contrast, TAA-specific stimulation resulted in only a small increase in IFN-γ secretion in CD4+ and CD8+ PBTCs, which did not reach statistical significance when considering the group of 5 tested patients as a whole (Fig. 3B). However, among these 5 patients, 2 had T cells that showed clear (>5-fold) increases in IFN-γ after TAA-challenge compared with unstimulated cells (Suppl. Fig. 7), demonstrating the presence of TAA-specific, IFN-γ-proficient T cells in their blood at frequencies (calculated to be 0.1–0.45% of the respective T cell subset) comparable to those measured by the IFN-γ ELISPOT assay, but much lower than those of TNF-α-secreting T cells. IL-2 secretion was not significantly increased overall in TAA-stimulated T cells (Fig. 3C), but there was a strong inter-individual variation within the group, with one patient having a strong TAA-specific IL-2 secretion response (Suppl. Fig. 8). In subsequent studies, we focused our phenotypic analysis of TAA-reactive PBTCs on the TNF-α-secreting subset, as only this subset could be compared among all patients.

Using gating on distinct T cell subsets, we determined their respective contribution to the TAA-reactive T cell population.

Figure 1. NSCLC patients have higher numbers of TAA-specific T cells than tumor-free patients. (A) Peripheral blood-derived dendritic cells (PBDCs) were pulsed with 14 polypeptides derived from TAAs or a peptide derived from human IgG as a peptide negative control. As positive controls, PBDCs + cytomegalovirus/adenovirus (CMV/AdV) and PBDCs + staphylococcal enterotoxin B (SEB) were used and compared with PBDCs alone and peripheral blood-derived T cells (PBTCs). The co-cultures were performed in triplicate. A patient was defined as responder against a TAA (indicated by the asterisks) when the triplicate IFN-γ spot counts for a specific peptide were significantly different compared with the IgG control peptide (p-value < 0.05 by t-test). Compared with the IgG control, this patient showed statistically significant responses against MUC-1, p53, Heparanase, EGFR, Survivin, RHAMM, CEA, NY-ESO-1, WT-1, and HER2/neu. (B) Response rate of PBTCs to NSCLC-associated antigens in 51 patients with NSCLC and 10 tumor-free patients categorized by the number of different TAAs recognized. (C) Fold increase in mean IFN-γ spot counts compared with mean IgG controls for all TAA-containing wells from NSCLC and tumor-free patients. p-Values were determined using a 2-tailed Mann–Whitney and Wilcoxon matched-pairs signed rank tests. (D) Response rate for each of the 14 TAA-specific peptides in NSCLC patients compared with tumor-free patients. Numerical data “X/Y” indicate the number of patients with responsive PBTCs for a given TAA (X) relative to the total number of patients tested for TAA responsiveness (Y). (E) Fold increase in mean TAA-specific IFN-γ spot counts relative to mean IgG IFN-γ spot counts. Data from all NSCLC (n = 51) and tumor-free (n = 10) responders and non-responders are presented. p-values were determined using a 2-tailed Mann–Whitney test.
The data for gated CD4+ and CD8+ TEMs from a representative NSCLC patient are shown in Fig. 3D, demonstrating the presence of high frequencies of TNF-α-secreting CD8+ and CD4+ cells within the TEM compartment, with TNF-α being specifically secreted in response to all tested TAAs, but not the control IgG peptide. The individual and cumulative data for all TNF-α-secreting CD4+ and CD8+ T cell subsets derived from 4 NSCLC patients are shown in Fig. 3E–G. Intracellular differences in the TAA responses were observed between the subsets, owing to the relative contribution of each T cell subset, particularly TCM, Teff, and TEM. Inter-individual differences in response patterns toward individual TAAs and in the overall strength of the T cell response were noted. A particularly strong TAA-specific TNF-α secretion response was found in patient 1 (Suppl. Table 4), who showed distinct TAA-specific TNF-α expression, particularly in CD8+ Teffs and TEMs (Fig. 3E; patient 1). This was the only patient in whom significant specific TAA-induced TNF-α expression was detected in CD4+ and CD8+ TCMs. The clinical characteristics of the 5 patients evaluated in this part of the study are summarized in Supplementary Table 4. Altogether, CD8+ PBTCs exhibited a slightly stronger cytokine response than CD4+ T cells. Cumulative data from the TNF-α capture assays for all tested patients suggest that within the different T cell subsets in the blood effector and effector memory PBTCs exert the strongest TAA-specific cytokine response. However, with regard to the very strong abundance of CD4+ T cells in the blood and among them the high frequencies of TN/SCM (Fig. 2B), CD4+ TN/SCM represented still a large source of TAA-specific TNF-α secretion (mean proportion: 37.9%; Fig. 3H). In contrast, CD8+ T cells, which made up only 23% of TNF-α-secreting PBTCs, displayed TAA-specific TNF-α secretion predominantly in the Teff and TEM compartments (mean proportion for CD8+ Teff: 10.2% and for CD8+ TEM: 13.0%; Fig. 3H).

**In situ-activated TEMs dominate type 1 effector cytokine secretion in TILs**

We studied the functional profile of TILs, as they are thought to be enriched in tumor-specific T cells and are affected by the tumor microenvironment.26 We processed resected tumor tissues immediately after resection by mechanical dissociation and subsequent cytokine capture analysis. Using this procedure, cytokine production is detected only in those TILs that already show secretory activity within the tumor before resection.20 We previously showed that TNF-α secretion by TILs is confined to the population of TAA-specific T cells.20 Thus, phenotyping of cytokine-secreting TILs provides information on the composition of tumor-reactive TIL subsets. A representative figure showing TNF-α production by intra-tumoral TEM TILs from one patient (Fig. 4A), clinical characteristics for all 6 patients (Suppl. Table 5), and cumulative data for all 6 patients and all cytokines tested (TNF-α, IFN-γ, and IL-2) are provided (Fig. 4B–D). Overall, the proportions of cytokine-secreting T cells were lower in TILs than in TAA-stimulated PBTCs. We detected a mean secretion of TNF-α in 0.69% (SD: 0.99), IFN-γ in 0.44% (SD: 0.38), and IL-2 in only 0.08% (SD: 0.11) of TILs, which is in agreement with the cytokine secretion profiles of TAA-reactive T cells in the blood. All TIL subsets showed similar
overall proportions of cytokine-secreting CD4$^+$ and CD8$^+$ T cells, suggesting that not only CD8$^+$ T cells, but also CD4$^+$ T cells, can be stimulated in the tumor tissue by direct interaction with tumor cells, e.g., through interactions with tumor-resident antigen-presenting cells. Nevertheless, taking into account the relative subset frequencies (Fig. 2B), cytokine secretion by in situ-activated TILs (which were highly dominated by TEM while only low numbers of TN/SCM...
were detectable in the tumor tissue) was almost entirely restricted to CD4\(^+\) and CD8\(^+\) TEMs (Fig. 4E). In summary, TILs showed TNF-\(\alpha\), IFN-\(\gamma\), and IL-2 expression predominantly in TEMs, demonstrating the presence of functionally competent TILs in NSCLC.

**Tumor-specific T cell response correlates with recurrence-free survival**

Immune reactivity signatures can predict outcomes in NSCLC.\(^{13,27}\) While it is conceivable that this effect is mediated by TAA-reactive T cells, the prognostic impact of the latter has not been directly addressed. Therefore, we followed the postoperative course of curatively resected patients, which were tested preoperatively for the presence of TAA-responsive PBTCs. Forty-one patients were eligible for recurrence-free survival (RFS) analysis (Suppl. Fig. 1). The median age was 66 years (range: 40–79), and the median follow-up time was 13 months (range: 3–19). Tumor recurrence occurred in 11 patients and the median RFS was 9 months (range: 3–15).

In univariable cox regression analysis, patients with responsive PBTCs exhibited statistically significantly improved RFS compared with those who were negative for any TAA (Fig. 5A and Table 1). Twenty-six patients were classified as responders to one or more of the 14 tested TAA-specific peptides. Three out of the 26 responders (11.5%) developed tumor recurrence (\(p = 0.004\), Suppl. Table 6). Tumor recurrence was statistically more frequent when TAA-reactive T cells were absent, and rare when T cells reactive against 2 or more different TAAs were present (Fig. 5B). The survival benefit was also evident for dichotomized IFN-\(\gamma\) spot counts at median, irrespective of whether a patient was classified as responder or non-responder (Fig. 5C and D). Survival analysis revealed comparable, statistically significant results, independent of the calculation mode for IFN-\(\gamma\) spot counts (fold increase to negative control in Fig. 5C and absolute increase Fig. 5D). Patients who experienced tumor recurrence had significantly lower preoperative IFN-\(\gamma\) spot counts than those who remained tumor-free during the observation period (HR, 4.59, 95% CI: 0.92–22.11, \(p = 0.031\)). Other factors influencing RFS were lymph node metastases, lymphangitic carcinomatosis, and being postoperative stage II and higher (Table 1). Twenty-six patients were classified as responders to one or more of the 14 tested TAA-specific peptides. Three out of the 26 responders (11.5%) developed tumor recurrence (\(p = 0.004\), Suppl. Table 6). Tumor recurrence was statistically more frequent when TAA-reactive T cells were absent, and rare when T cells reactive against 2 or more different TAAs were present (Fig. 5B). The survival benefit was also evident for dichotomized IFN-\(\gamma\) spot counts at median, irrespective of whether a patient was classified as responder or non-responder (Fig. 5C and D). Survival analysis revealed comparable, statistically significant results, independent of the calculation mode for IFN-\(\gamma\) spot counts (fold increase to negative control in Fig. 5C and absolute increase Fig. 5D). Patients who experienced tumor recurrence had significantly lower preoperative IFN-\(\gamma\) spot counts than those who remained tumor-free during the observation period (HR, 4.59, 95% CI: 0.92–22.11, \(p = 0.031\)). Other factors influencing RFS were lymph node metastases, lymphangitic carcinomatosis, and being postoperative stage II and higher (Table 1). After adjusting for variables within multivariable cox regression, i.e., taking into account the presence of lymph node metastases and lymphangitic carcinomatosis, patients with PBTCs responsive to TAA still had a 5-fold lower probability of developing tumor recurrence than those with no TAA-responsive PBTCs. This favorable effect remained statistically significant when modified multivariable models, e.g., with postoperative stage as an alternative variable, were applied (Table 1). Notably, age (Suppl. Table 7) and lymphangitic carcinomatosis (Suppl. Table 8) were significantly correlated with the presence of TAA-responsive PBTCs. Further, lymphangitic carcinomatosis was strongly correlated with the presence of lymph node metastases and being postoperative stage II and higher (Suppl. Table 9), and age (\(p = 0.047\), 2-sided \(t\)-test). Patients with NY-ESO-1-responsive PBTCs were statistically more frequent among those without tumor recurrence (\(p = 0.052\); Suppl. Table 6), and exhibited improved survival (HR, 4.17, 95% CI: 0.89–19.62; Fig. 5E). This was not the case for any other single TAA (Suppl. Fig. 9); however, it should be taken into account that the study group was too small to robustly assess the prognostic significance for all TAAs separately. Therefore, we constructed groups of TAAs and assessed a patient as responder if a significant responsiveness was detected against any of the TAAs in this group. Patients with a T cell response against p53, NY-ESO-1, Her-2, or Aurora kinase A had a significantly lower relapse rate than those without such T cell responses (Fig. 5F). T cell responsiveness against other TAA groups such as CEA, NY-ESO-1, WT-1, and MUC1 (\(p = 0.02\), Heparanase, MUC-1, EGFR, survivin, Mage A3, and RHAMM (\(p = 0.06\)), or CEA and NY-ESO-1 (\(p = 0.05\)) (Suppl. Fig. 10) was also associated with improved RFS. Notably, none of the patients with CEA/NY-ESO-1, CEA/NY-ESO-1/WT-1, and CEA/NY-ESO-1/WT-1/MUC1-1-responsive PBTCs developed tumor recurrence (Suppl. Table 6). Taken together, these data suggest that the T cell responses against all of these TAAs bear prognostic relevance. Thus, endogenous T cell responses against NSCLC-associated antigens correlate with lower risk of tumor recurrence in curatively resected NSCLC.

**Discussion**

This study revealed that the peripheral blood of NSCLC patients harbors responsive memory T cells against a broad panel of TAAs and that, despite the small patient cohort, preoperatively detected endogenous tumor antigen-reactive T cell responses correlate with a beneficial post-surgical outcome. In fact, the value of T cell responses for identifying patients with a high risk of tumor recurrence was close to that of tumor stage. T cell responses against TAAs were similarly detectable in patients with benign lung tumors, suggesting that the immune system may not respond toward malignant transformation in particular, but rather toward abundance and inflammatory infiltrates in TAA-reactive T cell subgroups in 4 NSCLC patients. (H) Contribution of CD4\(^+\) and CD8\(^+\) PBTC subsets to total TNF-\(\alpha\) expression demonstrating that the TN/SCM compartment, due to the over-representation of CD4\(^+\) Tcs and TN/SCM and the low numbers of TEM, represents a major source of TAA-reactive TNF-\(\alpha\) secretion in NSCLC patients (n = 4). TNF-\(\alpha\), tumor necrosis factor \(\alpha\); CEM, T central memory cells; TEM, T effector memory cells; Tc, effector T cells; TN/SCM, naive/stem-like memory T cells.
context of antigen release by dying tumor cells. This is in line with a previous study in non-malignant meningioma that provided evidence that a benign tumor is capable of inducing an antigen-specific immune response.28 We also observed TAA-reactive T cell responses in some of the tumor-free patients of the control group. We cannot rule out the possibility that in these patients the underlying disease might be associated with inflammation and has caused a T cell response against locally expressed self antigens, among them TAAs which, nevertheless, should be expressed to a lower level than in tumors and would also probably not reflect the broad bandwidth of TAA expression as simultaneously expressed by lung tumor cells. Indeed, both the frequencies of TAA-reactive T cells as well as the number of antigens simultaneously recognized was much lower in tumor-free patients than in tumor patients.

Within the framework of a prospective pilot trial, we tested for the presence of spontaneous T cell responses by focusing on antigen-stimulated IFN-γ production as reliable evidence of specific T cell activation.29 Antigen-responsive T cells are detected in several patients, irrespective of their particular HLA haplotype,30 which is an important advantage of the ELISPOT method used in this study. The findings in this study are basically in line with our previous observation in patients with colorectal cancer that improved prognosis is mediated by in situ activation of tumor antigen-specific T cells in the tumor tissue that are detectable only in patients who also show systemic tumor-specific T cell responses in the blood.30 These data support the concept of therapeutic vaccination in a situation of minimal residual disease; they also suggest that antigens that are recognized by endogenous tumor-reactive T cells are relevant targets for therapeutic vaccines. We found the highest response rates for the TAAs Aurora kinase A (32.6%), HER2/neu (32.5%), NY-ESO-1 (26.1%), and p53 (25.5%); in some cases, their frequencies reached those of responses against viral recall antigens. Regarding their individual impact on RFS, we found a statistical trend toward improved outcome in patients with a T cell response against NY-ESO-1, most likely due to the small cohort size, with only low numbers of patients showing a T cell response against a particular TAA. Nevertheless, NSCLC patients with T cell responses against one or more of the 4 most common TAAs had a clearly increased RFS compared with patients lacking T cell responses against any of these antigens. This demonstrates the prognostic relevance of these responses and might also point toward their suitability for therapeutic purposes, particularly, in the case of NY-ESO-1. However, patients showing T cell responses against other TAAs, e.g., against either one of heparanase, mucin-1, EGFR, survivin, Mage A3, or RHAMM, also exhibited at least a statistical trend toward improved RFS, suggesting that T cell responses against less-frequently recognized TAAs may also convey protection.
Although in the follow-up cohort we used IFN-γ as an indicator of TAA-specific type 1 T cell function, this does not necessarily imply that tumor-specific IFN-γ secretion plays a protective role in these patients. In the subgroup of patients analyzed for secretion of additional type 1 cytokines, we consistently detected strong TAA-specific secretion of TNF-α, whereas IFN-γ or IL-2 responses were less frequent. The question of whether TNF-α or IL-2 might be more, or less, predictive than IFN-γ with respect to clinical outcomes was not addressed and should be the subject of future studies. In the blood, considerable TAA-specific secretion of TNF-α was detected in all tested CD4⁺ and CD8⁺ T cell subsets. However, among CD4⁺ T cells, the T/N/SCM population contributed significantly to the total number of TNF-α-secreting T cells. Since naïve T cells cannot secrete type 1 effector cytokines in the context of a short-term cytokine capture assay, the cytokine secretion most likely arises from a small subset of TAA-specific TSCMs, which in this case would strongly contribute to systemic anti-tumor immunity. This issue warrants further investigation, as the role of TAA-specific TSCMs in cancer patients has not been addressed. Under conditions of minimal residual disease, TAA-reactive effector/memory T cells confer immune protection by controlling the outgrowth of occult micrometastases. As this requires their effector function, we sought evidence of intra-tumoral T cell activity in situ. IL-2, TNF-α, and IFN-γ have all been associated with therapeutic immune responses after vaccination. Increased numbers of TILs have been associated with better outcomes in NSCLC; however, their functional activity is unknown. Here, we detected TNF-α, IFN-γ, and even IL-2, secreting CD4⁺ and CD8⁺ T cells at low abundance in many, but not all, NSCLC TILs. However, inter-individual heterogeneity was considerable, likely reflecting differences in PBTC responses against endogenous TAA. Compared to blood, TILs were strongly enriched in TEMs; in both CD8⁺ and CD4⁺ TILs, cytokine expression was predominantly associated with this subset. Thus, at least a small proportion of NSCLC-infiltrating T cells are functional. In colorectal cancer, TNF-α secretion in situ is restricted to the population of tumor antigen-specific T

Figure 5. Preoperative T cell analysis correlates with recurrence-free survival (RFS). From the 51 patients assessed using ELISPOT analyses, 10 were excluded from survival analysis as outlined in Suppl. Fig. 1. (A) Survival according to the presence vs. absence of TAA-responsive peripheral blood-derived T cells (PBTCs). (B) Survival according to the number of TAA recognized by PBTCs per patient. (C–D) The level of IFN-γ spot counts correlates significantly with RFS. From all 51 patients, a median value for IFN-γ spot counts was calculated. Survival based on IFN-γ spot counts above vs. below the median was correlated with PBTCs responsiveness using 2 different modes of spot count calculation: either by division of TAA-specific spot counts by the corresponding IgG control counts (C) or by subtracting the IgG control counts from the corresponding TAA-specific spot counts (D). (E–F) Survival according to the presence or absence of PBTCs responsive to NY-ESO-1 (E) or to one of the TAA p53, NY-ESO-1, Her2/neu or Aurora kinase A (F). Survival curves are compared using the log-rank test.
and the respective frequencies of cytokine-secreting T cells were comparable to those in this NSCLC study. While we did not address TAA specificity in the TILs from NSCLC patients, our data do demonstrate the presence of functional TILs in NSCLC. It is tempting to speculate that treatment with immune checkpoint inhibitors\(^\text{34}\) aimed at activating tumor-reactive T cells in situ might further increase their numbers.\(^\text{35}\)Potentially, the efficacy of checkpoint inhibition therapy could be increased by antigen-specific immunotherapies, such as therapeutic vaccination, which would induce the formation of antigen-specific tumor-directed cytotoxic T cells and help overcome a possible lack of pre-existing tumor-specific T cells. Such combination might be particularly necessary since therapeutic vaccines alone have in the past failed to demonstrate clinical benefit suggesting that the increase of TAA reactive T cells alone may not be sufficient for effective cancer treatment. This is in accordance with more recent efforts to treat cancer with a multimodal approach incorporating surgery, immune checkpoint blockade, cytotoxic therapy, targeted therapy and therapeutic vaccination for optimal immune-mediated tumor control.\(^\text{36,37}\) Taken together, our data suggest that the TAAs, Aurora kinase A, HER2/neu, NY-ESO-1, and p53, all of which have been examined in clinical trials,\(^\text{38-41}\) are candidate target antigens for future immunotherapies. To our knowledge, this comprehensive analysis of spontaneous TAA-specific T cells in operable NSCLC is the first of its kind. The hierarchy of potential target antigens presented here may serve as guide through the plethora of TAAs suggested for cancer immunotherapy. Although this prospective study is limited by the relatively small sample size, the lack of a validation cohort, and the fact that detailed immunological assessment could not be conducted for all patients, the conclusions from this study raise the possibility that identifying patients with pre-existing TAA-reactive T cells in their blood could improve prognostication and help redirect adjuvant treatments including vaccinations and immune check point inhibition.

### Patients and methods

#### Patients

This prospective single institutional trial was conducted at the Department of Thoracic surgery at Thoraxklinik, Heidelberg University Hospital, Germany. The number of patients enrolled...
in the study and included in the analysis is outlined in the flow diagram in Suppl. Fig. 1.

**Patient samples**

Fresh peripheral blood samples were obtained preoperatively from 60 patients with lesions suspicious of being NSCLC, 7 patients with benign pulmonary lesions, and 10 tumor-free patients. Samples were subjected to Ficoll gradient centrifugation (Biochrom, Berlin, Germany), and cells in interphase were collected as described previously.\(^{11}\)

**Cell purification and culture**

Cell purification and culture for the IFN-γ ELISPOT and cytokine capture assays were performed as described previously.\(^{11,23}\) Briefly, non-adherent peripheral blood mononuclear cells (PBMCs) were cultured for 7 d in serum-free medium supplemented with 100 U/mL IL-2, plus 60 U/mL IL-4, and transferred into cytokine-free media for 12 h before T cells were purified using Dynabeads\textsuperscript{®} Untouched\textsuperscript{TM} Human T Cells Kit (Thermo-Fisher, Schwerte, Germany). For dendritic cell (DC) generation, plastic-adherent mononuclear cells were cultured for 7 d in serum-free medium supplemented with 50 ng/mL rhuGM-CSF (Essex Pharma, München, Germany) and 1,000 U/mL IL-4 (PromoCell, Heidelberg, Germany). DCs were enriched using anti-CD19, anti-CD3, and anti-CD56-coupled Dynabeads\textsuperscript{®} Pan Mouse IgG (Thermo-Fisher). For antigen presentation, DCs were pulsed overnight with 200 μg of test-antigens per 10\(^6\) cells/mL in cytokine-free X-VIVO 20 (Lonza, Köln, Germany).

**Antigens**

The Peptide Synthesis Facility of the German Cancer Research Center (DKFZ, Heidelberg, Germany) provided all polypeptides, which were designed to contain the known immunogenic HLA-A*0201 T cell epitope. The peptide sequences are shown in Suppl. Table 10.

**IFN-γ ELISPOT assay**

ELISPOT assays were performed as described previously\(^{11}\) with a few modifications. Briefly, antigen-pulsed DCs were incubated with autologous T cells (DC/T cell ratio = 1:5) for 40 h in ELISPOT plates. The number of IFN-γ spot-forming cells was quantified using a CTL Analyzer (Cellular Technology). As a negative control, DCs were loaded with human IgG (Endobulin, Baxter, Unterschleissheim, Germany), which was considered as nonspecific background. Staphylococcal enterotoxin B (SEB) and cytomegalovirus (CMV) were used as positive controls. Individuals were considered as responders if spot numbers in triplicate test wells significantly (2-sided Student's \(t\)-test with \(p < 0.05\) as responder criterion) exceeded those of control wells.

**Cytokine capture assay and analysis by flow cytometry**

Staining of PBMCs and TILs was conducted with 5–10 × 10\(^5\) cells. For PBMCs, T cells were cocultured with autologous DCs (5:1 ratio) pulsed with peptides, polyclonally stimulated with SEB as a positive control, or maintained in plain medium for 12–16 h as a negative control. For TILs, fresh tumor samples were cut into pieces in PBS containing Benzonase (Merck, 100 units/mL) and the cells were isolated by gentle mechanical disruption and then filtered using 100 μm cell strainers (BD Biosciences (BD) Falcon, 352360). Then, the cells were centrifuged at 300 g for 10 min. The pellet was resuspended in X-VIVO-20 medium (Lonza) and immediately stained and assessed for cell phenotype and cytokine expression. Non-specific binding was blocked by pre-incubation of the cells for 10 min on ice with hulgG (Kiovig, 1.7 mg/mL) followed by staining for secreted cytokines using the TNF-α and IFN-γ secretion assay detection kits (Miltenyi Biotec GmbH) as described previously.\(^{70}\) Dead cells were excluded by staining with a fixable yellow dead-cell stain (Life Technologies GmbH; L34959; 1:1,000), followed by T cell subset staining: anti-human CD4-PerCp-Cy5.5 (clone RPA T4, BD), CD8-V450 (RPA T8, BD), CD45RA-APC-H7 (clone H100, BD), and CD62L-PeCy7 (clone DREG56, eBio-science). Data were collected on a FACSAnity 2D (BD) with FACSDiva software. Data were analyzed using FlowJo 8.8.6. GraphPad Prism 7 was used for graphical representations and associated statistics.

**Surgery**

Patients underwent an open, or alternatively a video-assisted, thoracoscopic resection. Radical mediastinal and hilar lymphadenectomies were performed in cases of malignancy.\(^{42}\)

**Postoperative follow-up**

Postoperative follow-up consisted of chest X-rays every 3 months and a chest CT scan every 6 months. Tumor recurrence was confirmed by the institutional tumor board. The primary end point was RFS, calculated from the time of tumor resection. Patients with high-risk UICC stage IB, and stages II and higher, were considered for adjuvant chemotherapy.

**Statistical analyses**

The baseline characteristics of patients were reported as the summary measures of the empirical distributions. Experimental results were compared using paired and unpaired 2-tailed \(t\)-tests. To assess correlations, different statistical tests were used for group comparison. Survival curves were estimated by means of the Kaplan–Meier method. Median follow-up times were estimated with the reverse Kaplan–Meier method.\(^{13}\) The impact of specific T cell responses on RFS was analyzed separately for the 14 TAAs using several univariable cox models. Additional prognostic factors considered are depicted in Table 1. For multivariable analyses, we used the presence of any T cell response (T cell response present versus absent) as a factor. The proportional hazards cox regression model was used to investigate the influence of the variables on RFS. All reported tests and \(p\)-values are 2-sided. Owing to the high number of comparisons, the resulting \(p\)-values could not be adjusted for multiplicity and are therefore of a purely descriptive nature. All variables were assessed for potential confounding effects by
comparing the estimated regression parameters resulting from univariable and multivariable cox regression. Changes in the regression coefficients of more than 5% were regarded as indicative for confounding. The results of the cox model were summarized by reporting the estimated hazard ratios (HRs) together with the 95% confidence intervals (CIs); reported p-values were based on the Wald test. All analyses were performed with SPSS Statistics version 23 (IBM, Armonk, NY).

Study approval

All investigations were performed in accordance with principles embodied in the Declaration of Helsinki, and written informed consent was obtained from all participants. The ethics committee in Heidelberg approved this prospective study (approval number: S-515/2013), which has also been registered with ClinicalTrials.gov (trial registration ID: NCT02515760).

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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