Progression of Lung Cancer Is Associated with Increased Dysfunction of T Cells Defined by Coexpression of Multiple Inhibitory Receptors

Daniela S. Thommen1,2, Jens Schreiner2, Philipp Müller2, Petra Herzig2, Andreas Roller3, Anton Belousov5, Pablo Umana4, Pavel Pisa5, Christian Klein6, Marina Bacac4, Ozana S. Fischer5, Wolfgang Moersig5, Spasenija Savic Prince6, Victor Levitsky4, Vaios Karanikas4, Didier Lardinois5, and Alfred Zippelius1,2

Abstract

Dysfunctional T cells present in malignant lesions are characterized by a sustained and highly diverse expression of inhibitory receptors, also referred to as immune checkpoints. Yet, their relative functional significance in different cancer types remains incompletely understood. In this study, we provide a comprehensive characterization of the diversity and expression patterns of inhibitory receptors on tumor-infiltrating T cells from patients with non–small cell lung cancer. In spite of the large heterogeneity observed in the amount of PD-1, Tim-3, CTLA-4, LAG-3, and BTLA expressed on intratumoral CD8+ T cells from 32 patients, a clear correlation was established between increased expression of these inhibitory coreceptors and progression of the disease. Notably, the latter was accompanied by a progressively impaired capacity of T cells to respond to polyclonal activation. Coexpression of several inhibitory receptors was gradually acquired, with early PD-1 and late LAG-3/BTLA expression. PD-1 blockade was able to restore T-cell function only in a subset of patients. A high percentage of PD-1hi T cells was correlated with poor restoration of T-cell function upon PD-1 blockade. Of note, PD-1hi expression marked a particularly dysfunctional T-cell subset characterized by coexpression of multiple inhibitory receptors and thus may assist in identifying patients likely to respond to inhibitory receptor–specific antibodies. Overall, these data may provide a framework for future personalized T-cell–based therapies aiming at restoration of tumor-infiltrating lymphocyte effector functions.

Introduction

T-cell dysfunction has been recognized as an important target in cancer immunotherapy highlighted by the successful introduction of antibodies targeting inhibitory receptors on lymphocytes, such as programmed death-1 (PD-1), into the clinical setting. However, we lack a clear picture of the mechanisms governing T-cell responsiveness in human cancer. In this study, we investigated the expression of multiple inhibitory receptors on tumor-infiltrating CD8+ T cells from non–small cell lung cancer (NSCLC) patients. Further, we characterized the impact of cumulative receptor expression on T-cell function and tumor progression.

In contrast with circulating tumor antigen–specific T cells, which have full functional competence (1, 2), T cells with the same antigen specificity residing in the tumor are functionally tolerant with severely impaired inflammatory and cytotoxic functions (3–5). The functional impairment comprising T-cell exhaustion was originally described in the context of murine chronic lymphocytic choriomeningitis virus (LCMV) infection (6, 7), but has been observed also in human chronic infections (8). During persistent antigen stimulation, T cells undergo loss of effector function, typically in a hierarchical manner: with early defects in IL2 production, proliferation, and cytotoxicity, followed by the loss of TNFα, IFNγ, and β-chemokine production at later stages (7). Tumor-specific T cells residing in the tumor are exposed to high antigen load and consequent chronic antigen exposure, which may present the persistent stimulus that leads to repeated activation and finally dysfunction of the T cells. As the latter occurs during a dynamic adaptation process (9), tumor infiltrating lymphocytes (TIL) represent a heterogeneous cell population with distinct individual states of dysfunction.

A deeper understanding of T-cell dysfunction in chronic infection arose from global gene expression profiling studies, which showed that T-cell exhaustion was a unique state of T-cell differentiation (10, 11). Within this process, T cells overexpress inhibitory receptors (commonly referred to as “immune checkpoints”), such as PD-1, T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3), cytotoxic T-lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), or the B- and T-cell lymphocyte attenuator (BTLA and CD272; refs. 10, 12, 13), and display major alterations as compared with fully functional...
effector or memory T cells. During tumor development, T cells progress through functional stages with expression of multiple inhibitory receptors. Of note, the coexpression of PD-1 with Tim-3 and BTLA has been shown to correlate with T-cell dysfunction in melanoma and murine acute myelogenous leukemia. However, despite these data, the distinctive expression patterns of inhibitory receptors in different tumors, including NSCLC, are largely undefined.

Blockade of immune checkpoints by targeting inhibitory receptors on T cells is a promising therapeutic strategy in human cancer. This approach has been pioneered with the antibody ipilimumab, which blocks CTLA-4 and has demonstrated survival benefits in patients with melanoma. The PD-1–blocking antibody nivolumab improved overall survival in lung adenocarcinoma compared with standard second-line chemotherapy with docetaxel. However, to date, only a minority of patients has achieved a long-term benefit from immunotherapies. A deeper understanding of the pathogenetic mechanisms and functional relevance of these inhibitory receptors is necessary to develop more effective therapeutic strategies for the efficient targeting of these receptors in oncology.

Our analysis of expressed inhibitory receptors on tumor-infiltrating CD8 T cells from NSCLC patients revealed a diverse expression with complex patterns of coexpression. Cumulative expression of PD-1, Tim-3, CTLA-4, and BTLA on CD8 T cells was correlated with nodal status and tumor stage. T-cell activation and effector function were impaired with increasing expression of inhibitory receptors and, notably, tumor progression. PD-1 blockade could restore effector function, though only in a subset of patients. These patients were characterized by higher numbers of T cells expressing intermediate amounts of PD-1 only. In contrast, T cells from patients with predominance of PD-1hi expressing T-cell subsets showed only marginal effects upon PD-1 blockade in vitro. These results indicate that T-cell dysfunction in human NSCLC is largely determined by cumulative expression of multiple inhibitory receptors. In our experimental setting, the level of PD-1 expression predicted the response to PD-1–blocking antibodies. These findings offer essential insights into the functional state of intratumoral T cells in patients with NSCLC and provide a rational basis for optimizing T-cell-based therapies, thereby unleashing the full armamentarium of T-cell effector functions.

**Materials and Methods**

**Patients and tumor sample processing**

Fresh tumor tissues and malignant effusions were collected from 32 patients with NSCLC undergoing surgery or puncture between January 2013 and August 2014 at the University Hospital Basel, Switzerland. Detailed patient characteristics are provided in Supplementary Table S1. The analyzed samples comprised 25 solid tumor–derived single-cell suspensions and 7 malignant effusions from patients with NSCLC. The mean age of the patients was 68.8 ± 10.1 years. The study was approved by the local Ethical Review Board (Ethikkommission Nordwestschweiz), and all patients consented in writing to the analysis of their tumor samples. Staging was based upon the 7th edition of the AJCC/UICC tumor–node–metastasis (TNM) staging system. An experienced lung pathologist carried out the histologic subtype analysis.

The solid tumor lesions were mechanically dissociated and digested using accutase (PAA), collagenase IV (Worthington), hyaluronidase (Sigma), and DNase type IV (Sigma), directly after excision. Single-cell suspensions were prepared. The cellular fraction of malignant effusions was isolated by density gradient centrifugation using Histopaque-1119 (Sigma). All samples were stored in liquid nitrogen until further usage.

**Antibodies and flow cytometry**

Anti–CD45-PerCP-Cy5.5 (clone 2D1), anti–CD8-PE-Cy7 (SK3), anti–CD8-FTC (SK1), anti–BTLA-Biotin (MH26), anti–CTLA-4-PE (14D3), and anti–LAG-3-APC (3DS223H) were purchased from eBioscience; anti–CD25-BV605 (2A3), anti–granzyme B-PE (GB11), anti–PD-1-BV605 (EH121), Steptavidin-BV711 were purchased from BD Bioscience; and anti–CD45RA-BV421 (HI100), anti–CCR7-AlexaFluor647 (G043H7), and anti–Tim-3-BV421 (F38-2E2) were purchased from Biolegend.

Cryopreserved tumor digests were thawed, washed, suspended in PBS, and blocked with Fc receptor-blocking agent (eBioscience). Dead cells were stained with live/dead Fixable Blue (Invitrogen). Cells were washed, resuspended in FACS buffer (PBS + 2mmol/L EDTA + 0.1% Na-Acid + 2% FCS), and antibodies for surface staining were added. Corresponding isotype antibodies were used as a control (data not shown). After washing, a secondary antibody was added where indicated. For CTLA-4 and granzyme B staining, cells were washed twice, fixed using IC Fixation Buffer (eBioscience), permeabilized using 1× permeabilization Buffer (eBioscience), and stained with the respective antibody. Cells that were not fixed/permeabilized were incubated in 200 µl Fixation buffer (eBioscience), washed, and resuspended in FACS buffer. Stained cells were analyzed on a BD LSR Fortessa Cell analyzer (BD Bioscience). Data were collected using the BD FACS Diva Software version 7 and further analyzed with FlowJo v10.6.0 (Tree Star Inc.) and GraphPad Prism v6.0e (GraphPad Software Inc.).

All tumor samples were comprehensively characterized by multiparameter flow cytometry. Viable cells were identified by lack of live/dead blue staining. Viable CD45/CD3 double-positive cells were gated for CD8 T cells and then divided into subpopulations based on the expression of CD45RA, CCR7, PD-1, Tim-3, CTLA-4, and BTLA. Flow cytometric data for distinct parameters were quantified either as percentage of positive cells or as mean fluorescence intensity (MFI).

Flow cytometry heat mapping was performed using an Excel-based conditional formatting program. Briefly, a 3-color scale was set with 0% as the lower threshold and 100% as the upper threshold. Color coding was then defined in 10% steps reflecting the breadth of inhibitory receptor expression.

**Enzyme-linked immunosorbent assay**

Supernatants from cultured tumor digests were collected and analyzed by ELISA. The human IL2, IFNγ, and TNFα ELISA kits were all obtained from BD Bioscience, and ELISAs were performed according to the manufacturer’s instructions. Briefly, ELISA plates were coated with 100 µl capture antibody overnight at 4°C. The next day, the plates were washed, blocked with Assay diluent for 1 hour, and washed again. Samples were incubated for 2 hours. Afterwards, Working Detector (Detection Ab + SAV-HRP) was added, incubated for 1 hour, extensively washed again, and mixed with Substrate solution. After addition of Stop Solution, the plates...
were analyzed at 450 nm with λ correction 570 nm. Cytokine secretion was normalized to the amount of 1 × 10^6 CD3^+ T cells in the culture for all samples.

**Polyclonal stimulation using anti-CD3/CD28 antibodies**
A 96-well flat-bottom plate was precoated with 0.5 μg/ml anti-CD3ε (OKT3; Biologend) for 2 hours at 37°C. Afterwards, the antibody solution was removed and the plate washed extensively. Frozen tumor suspensions were thawed, washed, and cultured at 3 × 10^5 cells/200 μl/well in complete medium [DMEM + sodium pyruvate (1 mM) + MEM nonessential AA (1×)]. CXCL12 (50 ng/ml) + 10% human Serum was added to each well. Cells were incubated for 24 hours, and the supernatants were collected, washed, and analyzed by flow cytometry.

**Restoration of T-cell function by PD-1 blockade**
Tumor digests were stimulated by agonistic anti-CD3 and anti-CD28 antibodies, as described above, in the presence or absence of 10 μg/ml anti-PD-1 antibody (clone MDX-5C4, provided by Roche Glycart) per well and incubated for 24 hours. After 24 hours, supernatants were collected, washed, and analyzed by flow cytometry for expression of activation markers, e.g., CD25 and T-cell effector functions, e.g., granzyme B and IFNγ. Supernatants were collected for IL2, IFNγ, and TNFα ELISA.

**Statistical analysis**
Values are reported as mean ± SD as indicated. Comparison between groups (P) was calculated using one-way ANOVA or Wilcoxon rank sum tests. In order to investigate the impact of multiple inhibitory receptors in CD8^+ T cells, an inhibitory receptor score was defined as the sum of the quintiles of the corresponding distributions of the markers in the present cohort. For every sample, depending on the relative expression of the particular marker, the marker got a discrete value ranging from 0 to 3: 0 for relative expression from the first quartile of the corresponding distribution of marker values in the cohort. By applying this procedure, for each sample, an inhibitory receptor score in the range of 0 ≤ inhibitory receptor ≤ 15 was obtained. To determine the percent inhibition of the total population of CD8^+ T cells, the score was normalized by 15.

**Results**

**Immunophenotyping of tumor-infiltrating T-cell subsets in NSCLC**
We investigated the expression of inhibitory receptors and differentiation markers on TIL subsets from 32 patients with NSCLC. Detailed patients' characteristics are provided in Supplementary Table S1. Analyzed samples comprised 25 tumor lesion-derived single-cell suspensions and 7 malignant effusions from patients with adenocarcinoma (n = 16), squamous cell carcinoma (n = 8), pleomorphic carcinoma (n = 1), and mixed histologies (n = 4). Three tumors were NSCLC not otherwise specified (NOS). Patients receiving systemic treatment or irradiation for their tumors before surgery or resection were excluded from the study. CD3^+CD8^+ T cells were characterized in all malignancies using multiparameter flow cytometry. Flow cytometry panels of one representative patient and one healthy donor for CD8 and PD-1, Tim-3, CTLA-4, LAG-3, and BTLA, respectively, with the respective isotype controls, are shown in Fig. 1A. Analyzed cell subsets were heat mapped based on the percentage of expression, with the use of an Excel conditional formatting program (ref. 23; Fig. 1B). In the vast majority of tumors, we observed a predominance of CCR7^−CD45RA^low effector memory CD8^+ T cells (mean, 71.1%; SD, ± 21.4%), whereas CCR7^−CD45RA^high naïve CD8^+ T cells were virtually absent (7.8% ± 16.3%; Fig. 1B and C). In peripheral CD8^+ T cells from healthy donors, CCR7^−CD45RA^low effector memory CD8^+ T cells were much less abundant (35.6% ± 15.5%; Fig. 1C). The main inhibitory receptor expressed on tumor-infiltrating CD8^+ T cells was PD-1. Expression of PD-1 varied substantially between patients (43.5% ± 21.5%; Fig. 1B and D). Similarly, though expressed at lower levels, we documented expression of Tim-3 (23.3% ± 16.3%), CTLA-4 (25.3% ± 16.6%), LAG-3 (11.7% ± 14.0%), and BTLA (1.42% ± 1.77%; Fig. 1B and D). In contrast, peripheral CD8^+ T cells from healthy donors showed almost no expression of PD-1 (2.8% ± 1.8), Tim-3 (1.0 ± 0.6), CTLA-4 (0.4 ± 0.3), and LAG-3 (0.1 ± 0.03; Fig. 1D), with the exception of BTLA (25.1 ± 6.0), which is expressed mainly on naïve T cells (20).

**Single and cumulative expression of inhibitory receptors increases with tumor progression**
T-cell dysfunction is considered a dynamic process in which the quality of the T-cell response gradually adapts during chronic diseases, such as viral infections and cancer (9). We therefore assessed whether the expression of inhibitory receptors and T-cell function is associated with tumor progression. Tumor-infiltrating CD8^+ T cells from tumor digests of 22 NSCLC patients with distinct tumor stages for whom all clinical and histopathologic information are available were analyzed for the expression of PD-1, Tim-3, CTLA-4, LAG-3, and BTLA. We found that the expression of most inhibitory receptors increased during tumor progression, although only the increased expression of PD-1 and Tim-3 in advanced tumor stages reached statistical significance in this rather small patient cohort (Fig. 2). No clear correlation was observed for the expression of CTLA-4, which may indicate that this receptor is differentially regulated. BTLA was generally expressed at a low percentage of cells, and only a small increase was found in advanced tumor stages (Fig. 2).

As the coexpression of inhibitory receptors was associated with greater T-cell exhaustion both in chronic viral infections and cancer (3, 17, 24), we investigated the impact of multiple inhibitory receptors on T-cell functionality by defining an inhibitory receptor score which reflects the cumulative expression of these receptors. To this end, the percentage of expression of PD-1, Tim-3, CTLA-4, LAG-3, and BTLA was analyzed in all NSCLC samples. The inhibitory receptor score was defined as the sum of inhibitory receptor expression (see Materials and Methods and Supplementary Fig. S1 for a more detailed description). We found...
a statistically significant increase in the cumulative expression of inhibitory receptors, as reflected by the inhibitory receptor score, in patients with nodal-positive cancers and advanced tumor stages, whereas primary tumor size did not significantly correlate with the inhibitory receptor score (Fig. 3). These data suggest a gradual and continuous upregulation of inhibitory receptors during tumor progression, which are most likely involved in T-cell exhaustion in NSCLC.
Inhibitory receptors are gradually expressed on tumor-infiltrating T cells

To explore the role of simultaneous expression of distinct inhibitory receptors on single T cells, we analyzed the concomitant expression of these receptors in CD8+ T cells (Fig. 4) relative to the expression of any of the five analyzed receptors. Expression is shown as a heat map displaying the percentage of expression of inhibitory receptors for the individual patients (Fig. 4A) and as radar plots, which analyze tumor-infiltrating CD8+ T cells with expression of the indicated inhibitory receptor for the expression of the additional inhibitory receptors (Fig. 4B). We found that CD8+PD-1+ T cells expressed on average the lowest percentages of other inhibitory receptors, whereas CD8+BTLA+ T cells expressed high amounts of the four other inhibitory receptors, suggesting that BTLA marks a particularly exhausted T-cell subset (Fig. 4A and B). We observed an increase in the number of coexpressed inhibitory receptors from CD8+Tim-3+ T cells over CD8+CTLA-4+ T cells to CD8+LAG-3+ T cells (Fig. 4). These findings indicate a gradual acquisition of inhibitory receptors with PD-1 as a broadly expressed, early marker, whereas BTLA is upregulated rather late during T-cell exhaustion.

Cumulative expression of inhibitory receptors defines T-cell dysfunction

During T-cell exhaustion in chronic infection and cancer, T-cell effector functions, such as proliferation, cytokine production, and cytotoxicity, are continuously impaired (8). We utilized polyclonal T-cell receptor stimulation with anti-CD3 and anti-CD28 antibodies to assess the impact of inhibitory receptor expression on T-cell dysfunction. We applied a suboptimal dose of anti-CD3/CD28, as potent stimulatory signals may mask some of the functional deficits of the T cells (25). Peripheral blood mononuclear cells (PBMC) from healthy donors were used as a control (Supplementary Fig. S2). The effect of anti-CD3/CD28 stimulation on T-cell activation, as exemplified by CD25 expression, and on T-cell effector function, as analyzed by IFNγ, TNFα, and IL2 production; as well as granzyme B expression, as determined by flow cytometry (Fig. 5A) and ELISA (Fig. 5B), varied substantially between patients. Of note, IL2 secretion was
generally low, which is compatible with reduced production during early T-cell exhaustion (7) and thus suggests functional T-cell impairment in the vast majority of patients. Importantly, we observed different stages of T-cell dysfunction, varying from patients with largely preserved T-cell function (i.e., upregulation of CD25 and granzyme B expression, as well as induction of IL2, IFNγ, and TNFα production) to patients with completely abrogated T-cell function (lack of induction of CD25 and granzyme B expression and no cytokine production).

In a next step, we investigated whether the pattern and the number of receptors simultaneously expressed by CD8 T cells was correlated with impaired T-cell function. To this end, induction of CD25 and granzyme B expression as well as cytokine production upon polyclonal stimulation was correlated to the inhibitory receptor score, as defined above. Tumor-infiltrating CD8 T cells with a high inhibitory receptor score (i.e., T cells coexpressing multiple inhibitory receptors) showed only a marginal response upon polyclonal stimulation, compatible with a highly dysfunctional state, whereas T cells with a low inhibitory receptor score could be strongly activated using polyclonal stimulation (Fig. 5C and D).

Rescue of T-cell function by PD-1–blocking antibodies depends on the amount of PD-1 expression

Owing to the widespread expression of PD-1 as observed in our analysis (Fig. 1D) and the recent therapeutic successes of PD-1/PD-L1–blocking antibodies (26, 27), we hypothesized whether PD-1 blockade might restore T-cell function. Utilizing the anti–PD-1 antibody nivolumab (MDX-5176), T-cell effector functions, such as production of IL2, IFNγ, and TNFα, could be restored in TILs from some NSCLC patients, whereas in other patients, only a marginal recovery of T-cell functions could be achieved (Fig. 6A).

As observed in a chronic murine LCMV infection model (28), we identified PD-1hi and PD-1int subsets in tumor-infiltrating CD8+ T cells from NSCLC patients (Fig. 6B). The increase in cytokine production upon exposure to anti-CD3/CD28 stimulation in combination with the PD-1–blocking antibody was compared with the percentage of PD-1hi CD8+ T cells in the PD-1–positive population of each patient. Of note, the increase in cytokine expression upon PD-1 blockade inversely correlated with the percentage of PD-1hi T cells, indicating that patients expressing larger numbers of PD-1hi T cells respond poorly to PD-1 blockade alone (Fig. 6C). As T-cell dysfunction correlates with the expression of multiple inhibitory receptors (i.e., patients with a high inhibitory receptor score) and response to a PD-1–directed therapy correlates with the amount of PD-1 on CD8+ T cells, we further analyzed the expression of Tim3, CTLA-4, LAG-3, and BTLA in PD-1hi and PD-1int CD8+ T cells. Remarkably, PD-1hi T cells expressed significantly more additional receptors compared with PD-1int subsets (Fig. 7). Thus, PD-1hi and PD-1int may identify two distinct T-cell populations where PD-1hi T cells may exhibit a more exhausted phenotype, which cannot be recovered by PD-1 blockade alone.

Discussion

Although T-cell exhaustion can unambiguously impair effective antitumor immunity, a clear picture of tumor-induced dysfunction is currently lacking. A comprehensive characterization of the distinct functional traits of tumor-infiltrating T cells is of great clinical relevance, as personalized strategies are likely required in a disease-specific fashion to reverse their dysfunctional state and facilitate antitumor immunity.
We performed a comprehensive phenotypical and functional analysis of tumor-infiltrating CD8⁺ T cells from patients with NSCLC. We found that these cells mainly possess an effector memory phenotype (CCR7⁻/CD45RAlo) and show large heterogeneity in expression of inhibitory receptors, such as PD-1, Tim-3, CTLA-4, LAG-3, and BTLA. Nevertheless, the number of receptors expressed on TILs from late-stage tumors was clearly increased, which reflects progressive T-cell dysfunction during tumor development. Effector functions of TILs were impaired in the vast majority of patients, which correlated with the expression of inhibitory receptors. To recover T-cell function in a clinically relevant setting, we combined polyclonal T-cell stimulation with antibody-mediated inhibition of PD-1. The effect of PD-1 blockade on T-cell functionality varied between TILs from different patients, but could be predicted by assessing the percentage of CD8⁺ T cells that highly expressed PD-1.

Tumors foster the activation of a plethora of immunosuppressive mechanisms that may act in concert to counteract effective antitumor immune responses. Though inhibitory T-cell receptors may be transiently expressed on functional effector T cells, their sustained expression is a hallmark of progressive exhaustion (10, 29). T cells bearing inhibitory receptors represent a heterogeneous population with distinct individual states of dysfunction, which have been originally described in the context of chronic viral infections (6, 7).

Figure 4.
Coexpression of inhibitory receptors by TILs from NSCLC patients. Tumor-infiltrating CD8⁺ T cells expressing the indicated receptor are analyzed for the expression of the additional inhibitory receptors. The results are shown as a heat map (A) or as a radar plot (B). The latter indicates the mean expression (black line) and SD (gray area) of the four other receptors.
if these receptors are functionally overlapping and to what degree coexpression of inhibitory receptors correlates with and affects T-cell function. Li and colleagues have reported that PD-1⁺ and Tim-3⁺ T cells are two distinct T-cell populations in patients with hepatocellular carcinoma (HCC), suggesting two separate pathways that lead to the upregulation of these inhibitory receptors within the HCC microenvironment (31). One may speculate that these are either redundant pathways, which result in T-cell dysfunction, or are differently regulated within the tumor environment by nonoverlapping signaling pathways.

Along this line, distinct cellular functions have been described for individual receptors, such as CTLA-4 (32), PD-1 (33), or BTLA (34). Here, we could demonstrate that the functionality of TILs can be correlated with and is largely affected by the number and amounts of inhibitory receptors. Of note, even T cells expressing few inhibitory receptors showed some degree of impaired functionality, as the secretion of IL2 was impaired in the vast majority of patients. Overall, the activation and effector function of CD8⁺ T cells was inversely correlated with the cumulative expression of inhibitory receptors, indicating a direct contribution of different

**Figure 5.** Cumulative expression of inhibitory receptors defines T-cell dysfunction. Whole tumor digests were stimulated for 24 hours with agonistic anti-CD3 and anti-CD28 or left untreated (control). The expression of CD25 and Granzyme B in CD8⁺ T cells as well as IL2, IFNγ, and TNFα secretion was determined by flow cytometry (A) or ELISA (B), respectively. The increase of these parameters was correlated to the cumulative expression of the inhibitory receptors PD-1, Tim-3, CTLA-4, LAG-3, and BTLA as reflected by the inhibitory receptor (iR) score (C + D). The P values were calculated using the one-way ANOVA test.
inhibitory pathways to T-cell dysfunction in NSCLC. However, a deeper understanding of the functional relevance of the distinct inhibitory receptors is clearly warranted to develop novel and more effective therapeutic strategies for the efficient targeting of these receptors for cancer immunotherapy.

In many human cancers, the accumulation of TILs expressing inhibitory receptors, such as PD-1, is associated with tumor progression. Intratumoral infiltration of PD-1-positive lymphocytes predicted advanced tumor stages and reduced overall survival in renal cell carcinoma (35), soft tissue sarcomas (36), and follicular lymphoma (37). Our findings for NSCLC are in line with reports from chronic viral infections, such as HIV in which upregulation of CTLA-4 correlates with disease progression (38).

Our analysis of five inhibitory receptors on tumor-infiltrating CD8 T cells showed a clear increase of the single and cumulative expression of these inhibitory receptors in tumor tissues from NSCLC patients presenting with advanced tumor stages, in particular lymph node–positive disease. Larger patient cohorts with long-term follow-up are necessary to fully address this question.

Expression of CTLA-4 differed from the other four receptors with the highest percentage of positive cells at early stages, suggesting a distinct role of CTLA-4 in regulating T-cell immunity (39). Coexpression analysis of additional inhibitory receptors on single cells, relative to the expression of one given receptor, showed a gradual expression, with early and late upregulation of PD-1 and BTLA, respectively. This observation may reflect a dynamic process of T-cell development; further experimental work will define the evolution and spatiotemporal hierarchy of expression of these receptors on dysfunctional intratumoral T cells. It needs to be explored if their increased expression is induced specifically in advanced tumors or may simply result from a longer in vivo "residence" of later-stage tumors, thus allowing more time to induce T-cell exhaustion. Nevertheless, our findings underscore the clinical relevance of inhibitory receptor expression during NSCLC tumor progression, associated with progressive failure of immune control during tumor growth (40, 41).

Blockade of inhibitory receptors, such as PD-1, is attracting increasing attention as a viable therapeutic option in different cancer types, including NSCLC. Though objective and deep responses are achieved in patients with pretreated and advanced cancers, only a minority of patients experience durable responses (27, 42). Reliable markers that allow prediction of initial and subsequent responses upon treatment with immune checkpoint-blocking antibodies are needed. We document here two populations of CD8+ tumor-infiltrating T cells characterized by different PD-1 expression (PD-1hi and PD-1int subsets). The number of PD-1hi T cells did not correlate with the percentage of PD-1 expression. Instead, we observed that the effect of PD-1 blockade correlated with the amount of PD-1 expression, with minimal effects on responsiveness of TILs with high proportions of PD-1hi subpopulations. These findings are in line with experiments in a murine chronic LCMV infection model where the subset of PD-1hi DbGP33-specific CD8+ T cells could be restored upon PD-1 blockade. In contrast, the PD-1hi subset appeared more "exhausted" and responded poorly to PD-1 blockade (28). Thus, the amount of PD-1 expression may represent a novel marker to define distinct T-cell subsets in human cancers and may serve as a predictor of responses to treatment with PD-1-blocking antibodies.
Thorough characterization of these subsets as predictive markers in cancer immunotherapy is needed. Our findings raise the question of how and whether T-cell effector functions in TILs are affected by the strength of PD-1 signaling. Upon adoptive transfer of T cells into a tolerizing mouse model, PD-1hi CD8\(^{+}\) T cells exhibited an impaired proliferative and inflammatory capacity (43). In another system, persistent antigen stimulation during LCMV-clone 13 infection leads to an accumulation of PD-1hi Eomes\(^{+}\) CD8\(^{+}\) T cells, which express more additional inhibitory receptors compared with their PD-1int counterparts (44).

We here confirm these findings in human cancer and extend this observation by showing that TILs with excessive PD-1hi cells are nonresponsive to PD-1 blockade, supporting the nonredundant function of inhibitory receptors. Whether PD-1int and PD-1hi T cells truly represent two different subsets, which differ in their generation and developmental program, is not clear. Future work needs to address representation of antigen-specific tumor-reactive T cells in these distinct PD-1-expressing subsets. The recovery of effector function in exhausted T cells and control of viral infection by simultaneous blockade of different inhibitory receptors, such as dual blockade of the PD-1 and LAG-3 pathways, is greater than that achieved by blockade of one pathway alone. Therefore, these combinatorial approaches may be crucial for the functional rescue of PD-1hi T cells.

In summary, we describe herein the cumulative expression of inhibitory receptors as a hallmark of dysfunctional T cells and tumor progression in NSCLC. Inhibitory receptors were gradually expressed, with PD-1 as the predominant receptor. PD-1 amounts defined two distinct CD8\(^{+}\) T-cell subsets with different patterns of coexpressed inhibitory receptors and responses to PD-1 blocking antibodies. Great progress has been achieved in dissecting the spatiotemporal dynamics of T-cell responses and defining predictive models in patients receiving therapies that block the PD-1/PD-L1 axis (45). However, major efforts are still necessary to standardize methods and to evaluate and validate eventual clinical usefulness (46). Thus, although the amount of PD-1 expression might be helpful in identifying patients who may benefit from therapeutic PD-1 blockade, tailoring therapeutic antibody combinations for each patient according to the individual tumor-immune profile may be beneficial. We suggest that the assessment of TILs as performed in our study may provide additional insights when combined with immunohistological analysis of TILs and ligands of inhibitory receptors. Our approach, also translated to other types of cancer and embedded into prospective clinical trials with checkpoint inhibitors, could increase the therapeutic efficacy and pave the way for more successful, personalized cancer immunotherapies.

**Disclosure of Potential Conflicts of Interest**

V. Karanikas has ownership interest (including patents) for Roche. A. Zippelius has received speakers bureau honoraria from Bristol-Myers Squibb, MSD, and Roche. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: D.S. Thommen, P. Müller, P. Umana, P. Pisa, C. Klein, M. Bacac, V. Levitsky, V. Karanikas, A. Zippelius Development of methodology: D.S. Thommen, J. Schreiner, A. Roller, A. Belousov, V. Karanikas, A. Zippelius Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.S. Thommen, J. Schreiner, P. Herzig, S.S. Prince, D. Lardinois, A. Zippelius
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.S. Thommen, J. Schreiner, P. Müller, A. Roller, A. Belousov, P. Pisa, V. Karanikas, D. Lardinois, A. Zippelius

Writing, review, and/or revision of the manuscript: D.S. Thommen, P. Müller, A. Roller, A. Belousov, P. Umana, P. Pisa, C. Klein, M. Bacac, W. Moersig, S.S. Prince, V. Levitsky, V. Karanikas, D. Lardinois, A. Zippelius

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.S. Thommen, J. Schreiner, C. Klein, O.S. Fischer, A. Zippelius

Study supervision: D.S. Thommen, P. Müller, P. Umana, M. Bacac, A. Zippelius

Other (conducted experiments): P. Herring

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