Progressive natural killer cell dysfunction associated with alterations in subset proportions and receptor expression in soft-tissue sarcoma patients

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List of abbreviations

7-AAD	7-Aminoactinomycin
CLL	Chronic lymphatic leukemia
GIST	Gastrointestinal stromal tumor
HLA	Human leukocyte antigen
HD	Healthy donor
Hsp70	Heat shock protein 70
IL-2	Interleukin-2
KIR	Killer cell immunoglobulin-like receptor
MCA	3-methylcholanthrene
MHC	Major histocompatibility complex
NK cell	Natural killer cell
NKG2D	Natural-killer group 2, member D receptor
PBMC	Peripheral blood mononuclear cells
RCC	Renal cell carcinoma
STS	Soft-tissue sarcoma
TKD	14 amino acid derivative of Heat shock protein 70
TKI	tyrosine-kinase inhibitor

Keywords

Natural killer cells, NK cell dysfunction, NK cell subsets, soft-tissue sarcoma, cancer progression, chemotherapy, immunotherapy

Abstract

Immunotherapy is currently investigated as treatment option in many types of cancer. So far, results from clinical trials have demonstrated that significant benefit from

immunomodulatory therapies is restricted to patients with select histologies. To broaden the potential use of these therapies, a deeper understanding for mechanisms of immunosuppression in patients with cancer is needed. Soft-tissue sarcoma (STS) presents a medical challenge with significant mortality even after multimodal treatment. We investigated function and immunophenotype of peripheral Natural killer (NK) cells from chemotherapy-naïve STS patients (1^{st} line) and STS patients with progression or relapse after previous chemotherapeutic treatment (2^{nd} line). We found NK cells from peripheral blood of both STS patient cohorts to be dysfunctional, being unable to lyse K562 target cells while NK cells from renal cell cancer (RCC) patients did not display attenuated lytic activity. *Ex vivo* stimulation of NK cells from STS patients with interleukin-2 plus TKD restored cytotoxic function. Furthermore, altered NK cell subset composition with reduced proportions of $CD56^{dim}$ cells could be demonstrated, increasing from 1^{st} to 2^{nd} line patients. 2^{nd} line patients additionally displayed significantly reduced expression of receptors (NKG2D), mediators (CD3ζ), and effectors (perforin) of NK cell activation. In these patients we also detected fewer NK cells with CD57 expression, a marker for terminally differentiated cytotoxic NK cells. Our results elucidate mechanisms of NK cell dysfunction in STS patients with advanced disease. Markers like NKG2D, CD3ζ and perforin are candidates to characterize NK cells with effective anti-tumor function for immunotherapeutic interventions.

Introduction

Natural killer (NK) cells are lymphocytes with the ability to lyse virus-infected and neoplastic cells.[1] To distinguish infected or transformed cells from healthy tissues, NK cells utilize a multitude of receptors to establish a complex balance of activating and inhibitory signals.[2] Stressed or transformed cells express antigens that are usually not found on healthy tissues. Activating NK cell receptors like NKG2D bind these antigens and subsequently shift the balance into the activating direction.[3] In contrast, killer cell immunoglobulin-like receptors (KIRs) bind human leukocyte antigen (HLA) molecules, expressed on all healthy cells, and mediate inhibitory signals.[4] With their ability to recognize and lyse transformed cells, NK cells can play an important role in the immunosurveillance of cancer, and are currently investigated for use in immunotherapy.[5] Subjects with reduced NK cell-specific cytotoxicity have been shown to have a higher incidence of malignant diseases in a prospective longitudinal study[6], and familial cancer has been associated with reduced NK cell cytotoxicity.[7] Moreover, patients with newly diagnosed cancer commonly display alterations of the cytotoxic capacity of NK cells.[8-11] The underlying mechanisms of NK cell dysfunction are still incompletely understood, but downregulation of activating NK cell receptors can often be found on NK cells of patients suffering from neoplastic disease.[12-14] So far, it has not been possible to differentiate if the cancer itself causes these alterations, or if the neoplastic disease is the result of an impaired NK cell cytotoxicity. In established human tumors, NK cell infiltrates often are non-existent, or NK cells isolated from tumor tissue show signs of NK cell dysfunction.[15,16] Numerous reports have shown that insufficient recognition of tumors by NK cells, e.g. by disrupting the function of activating NK cell receptors, enables tumor growth and dissemination.[17] Reestablishing sufficient lytic activity of NK cells might be a promising therapeutic approach for patients suffering from cancer.

Soft-tissue sarcomas (STS) are a group of heterogeneous and rare malignant tumors that arise in any of the mesodermal tissues of the body. Currently, more than 50 different histologic subtypes of STS have been described. Standard of care for high-risk patients (with large tumors and deep location) is a multidisciplinary approach including surgery with sufficient resection margins and – when possible – radiation.[18] Chemotherapy can be applied in a neoadjuvant or adjuvant setting for locally advanced disease as part of a multimodal therapy, and is the mainstay of therapy for patients with metastatic disease.[19-21] Despite comprehensive efforts to identify new agents that are efficient in the treatment of STS patients[22], however, relapse of disease even after wide resection of the tumor is common. Ultimately, approximately 40% of the patients still die from STS[23], illustrating the urgent medical need for new therapeutic options. Mouse models have demonstrated that NK cells are involved in the elimination of 3-methylcholanthrene (MCA)-induced STS.[24,25] Augmentation of NK cell cytotoxicity might therefore be beneficial for STS patients. However, NK cell function of patients with STS has not been characterized yet.

Here, we report a significant decrease in NK cell-specific cytotoxicity for PBMCs of chemotherapy-naïve STS patients, referred to as 1st line patients and STS patients with progression or relapse after previous chemotherapeutic treatment, referred to as 2nd line patients. To characterize the underlying mechanisms of NK cell dysfunction, we analyzed NK cell subsets and the expression of activating NK cell receptors important for the initiation of cytotoxicity. We found significant alterations in NK cell subset proportions and the expression of activating receptors and differentiation markers of 2nd line STS patients. Apparently, the observed deviations were specific for STS patients since NK cells of patients with renal cell carcinoma (RCC) did not show these alterations and displayed features similar to NK cells from healthy donors (HD). Incubation with interleukin-2 plus TKD (a 14 amino acid derivative of heat

shock protein 70) *ex vivo* was able to reverse NK cell dysfunction and might increase the efficacy of immunotherapeutic regimens in STS patients.

Results

Cytotoxicity of peripheral NK cells is impaired in STS patients but can be restored by *ex vivo* stimulation with IL-2

NK cells can recognize and kill tumor cells, and impaired NK cell cytotoxicity may be a means of immune escape. We evaluated the NK cell-specific cytotoxicity of PBMCs from patients with STS and RCC by chromium release assay using K562 cells as NK cell-specific targets.[26] We observed a significantly lower NK cell-specific cytotoxic capacity for PBMCs from STS patients (Fig. 1A), reduced to approximately 1/5 of the activity of PBMCs of HD, whereas PBMCs from RCC patients showed NK cell-specific lysis of target cells comparable with PBMCs of HD.

Our cohort of STS patients consisted of two different subgroups. The first subgroup encompassed patients who had been diagnosed with STS within weeks before inclusion in our analyses (n=13). All patients in this group had locally advanced disease but no documented metastases. None of them had received any tumor-specific medication (chemotherapy) before blood withdrawal. Based on these criteria, patients in this STS subgroup were termed 1st line patients (Table 1).

The second subgroup, termed 2nd line patients (n=11), included STS patients who had received chemotherapy before inclusion in our analyses. All had progressive disease after cytostatic treatment, whereby progression did not necessarily occur during chemotherapeutic treatment, but might have also emerged after the completion of (e.g. adjuvant) chemotherapeutic treatment. Thus, time intervals since last cytostatic drug treatment varied between patients. Two thirds of the 2nd line

patients had metastatic disease at the time of blood withdrawal, and one third had local progression without documented metastases (Table 1).

A comparison of the cytotoxicity of PBMCs of 1st and 2nd line STS patients revealed that 2nd line patients had almost undetectable lytic activity (median 0.6%), whereas PBMCs of 1st line patients displayed a significantly higher NK cell-specific cytotoxicity (median 7.5%, Fig. 1B), which, however, still was significantly below the NK cell-specific cytotoxic capacity of PBMCs of HD and RCC (p=.001 and .03, respectively; data not shown).

For 2nd line patients, median time interval since last chemotherapeutic treatment was 5 months, with a minimum of 4 weeks. There was neither a significant correlation between the time since last cytotoxic treatment and NK cell-specific cytotoxicity (Pearson's *r*=.31; p=.34, data not shown) for 2nd line patients, nor was the NK cell-specific cytotoxicity significantly different between patients who had their blood withdrawn within 5 months of last chemotherapy (median time interval to last treatment) and patients with longer time intervals to last chemotherapeutic treatment (Suppl. Fig. 1A). 2nd line patients with metastatic disease had a lower NK cell-specific cytotoxicity than 2nd line patients with non-metastatic, e.g. locally relapsed or progressive disease (Suppl. Fig. 1B). However, the lytic activity of PBMCs from non-metastatic 2nd line patients still was lower than that of PBMCs of (all non-metastatic) 1st line patients (Suppl. Fig. 1C).

Interleukin-2 (IL-2) is a strong activator of NK cell cytotoxicity. TKD can further augment the stimulatory capacity of IL-2.[27] We evaluated whether the suppressed NK cell function of STS patients could be restored by stimulation with IL-2/TKD. This experiment employed select samples obtained from STS patients and HD analyzed above. Levels of NK cell-specific cytotoxicity from unstimulated PBMCs of these samples were representative for the respective group. We found that cultivation of

PBMCs with IL-2/TKD significantly increased NK cell-specific cytotoxicity in all three groups, with PBMCs of 2nd line STS patients reaching lytic activities comparable to activated PBMCs of HD (median 47.5% and 51.0%, respectively). PBMCs of 1st line STS patients also responded to IL-2/TKD stimulation, but the increase in NK cell-specific cytotoxicity remained below that of IL-2/TKD-stimulated PBMCs of HD (median 33.0%, Fig. 1C, left panel). When cytotoxicity after stimulation was normalized to values of the uncultured samples, the relative increase upon IL-2/TKD stimulation was 2-, 5- and 23-fold for PBMCs from HD, 1st line and 2nd line STS patients, respectively. Thus, 2nd line patients showed a significantly higher increase compared with both HD and 1st line STS patients (Fig. 1C, right panel), and stimulation with IL-2/TKD could reinstate cytotoxic capacity in PBMCs from patients with STS, even with severely suppressed cytotoxicity in 2nd line patients.

NK cell frequencies and NK cell subset distributions are altered in STS patients

Polychromatic flow cytometry was performed to identify mechanisms that might explain the observed impairment in NK cell-mediated cytotoxicity.

NK cells were identified as $CD3^-CD56^+$ cells among live single PBMCs (Suppl. Fig. 2A), and the two main NK cell subsets were distinguished based on the intensity of their CD56 expression (Fig. 2A and Suppl. Fig. 2B). As commonly observed for PBMCs of HD, the frequency of NK cells ($CD3^-CD56^+$) in our HD collective ranged from 4.6% to 32.0% (mean 13.7%) with 13.0% belonging to the $CD56^{dim}$ subset and 0.7% being $CD56^{bright}$. Regarding the RCC samples, the overall frequency and the subset distribution was not significantly different to the HD samples. However, in PBMCs of STS patients, the NK cell frequency was lower compared with HD and RCC reaching significance in 2nd line PBMCs. Notably, it was the $CD56^{dim}$ NK cell subset that was significantly reduced in 2nd line STS patients (mean 7.1% and 2.1% in 1st and 2nd line STS patients, respectively, versus 13.0% in HD and 10.1% in RCC

patients) while the fraction of $CD56^{bright}$ NK cells was comparable between all patient and donor groups. The $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets were further subgrouped with respect to their expression level of CD16, distinguishing 3 groups, $CD16^{+}$, $CD16^{low}$ and $CD16^{-}$ (Fig. 2B/C and Suppl. Fig. 2B). Among the $CD56^{dim}$ NK cells of HD, $CD16^{+}$ cells were the most frequent subset while $CD16^{-}$ were sparse. A comparison of the CD16 distribution in $CD56^{dim}$ NK cells of HD, 1st line and 2nd line STS patients revealed that the fraction of $CD16^{+}$ cells within the $CD56^{dim}$ NK cells was lower in STS patients than in HD with a progressive decrease from 1st line to 2nd line STS patients (median of 82.9% in HD, 80.8% in 1st line and 31.6% in 2nd line STS) (Fig. 2B). In accordance with the loss of $CD16^{+}$ NK cells in the $CD56^{dim}$ subset there was a gradual gain in the $CD16^{low}$ (median 15.3% in HD, 17.5% in 1st line and 65.2% in 2nd line STS) and $CD16^{-}$ (median 3.0% in HD, 3.0% in 1st line and 4.6% in 2nd line STS) populations of $CD56^{dim}$ NK cells. Within the $CD56^{bright}$ NK cells, no significant differences were observed between 1st and 2nd line STS patients and HD (Fig. 2C).

Since the frequency of NK cells was reduced in PBMCs of STS patients, the diminished NK cell-mediated cytotoxicity, as observed, might be caused by reduced numbers of these cells. However, differences between HD and STS patients remained after normalizing the cytotoxicity values to the frequency of NK cells or $CD56^{dim}$ NK cells among PBMCs, indicating that other mechanisms limit the NK cell cytotoxicity in STS (Suppl. Fig. 3A/B).

Percentages of NKG2D- and CD3ζ-expressing NK cells are reduced in 2nd line STS patients

As the reduced NK cell-specific lytic capacity of PBMCs from 1st line patients cannot be fully explained by reduced proportions of NK cells within PBMCs, we analyzed the expression of NKG2D and NKp46, two of the main activating NK cell receptors that

trigger NK cell cytotoxicity.[28,29] Additionally, expression of CD3ζ, a signaling adaptor protein for NKp46, and the expression of the inhibitory receptor CD94 were assessed.

NKG2D is frequently downregulated on peripheral NK cells of cancer patients.[13,30] This downregulation correlates with decreased cytotoxic activity.[31] Percentages of $NKG2D^+$ NK cells were similar for 1st line STS patients, RCC patients and HD (range 41-86%). In contrast, significantly lower percentages were observed for 2nd line STS patients (median 37%, range 28-69%) (Fig. 3 A/B, left panels).

NKp46 has been found to show reduced expression in patients with cancer, e.g. melanoma and acute myeloid leukemia.[14,32] However, we did not observe significant differences in the frequency of $NKp46^+$ NK cells in all analyzed patient cohorts (Fig. 3 A/B, middle panels).

CD3ζ-deficiency is often seen in NK cells of cancer patients and is associated with impaired cytotoxicity.[33] Proportions of $CD3\zeta^+$ NK cells were comparable for 1st line STS patients, RCC patients and HD (median 99% for all groups), however, the frequency of $CD3\zeta^+$ NK cells was significantly reduced in 2nd line STS patients (median 84%) (Fig. 3 A/B, right panels).

CD94, when dimerized with NKG2A, is an inhibitory NK cell receptor.[34] No significant differences in the percentage of $CD94^+$ NK cells were seen between patient groups and HD (data not shown).

Percentages of perforin-positive NK cells and NK cells expressing CD57 are reduced in 2nd line STS patients

Cytotoxic activity of NK cells depends on their ability to secrete lytic effector proteins, i.e. perforin and granzyme B.[35] Intracellular staining of PBMCs showed that percentages of $perforin^+$ NK cells were not different between 1st line STS patients, RCC patients and HD. In contrast, 2nd line STS patients had significantly lower

proportions of perforin^{+} NK cells (median 48%) compared with HD (97%). (Fig. 4 A/B, left panels). For granzyme B, patient and donor groups showed comparable proportions of positive cells, and NK cells from RCC patients showed a trend towards higher percentages of granzyme B^{+} cells compared to HD (median 83% vs. 63%; Fig. 4 A/B, middle panels).

CD57 is a marker for terminally differentiated, cytotoxic NK cells.[36] CD57^{+} NK cells contain high amounts of granzyme B and perforin.[37] High frequencies of CD57^{+} tumor-infiltrating NK cells have been associated with improved outcomes for different kinds of cancer.[38] Frequencies of CD57^{+} NK cells were comparable between HD (median 48%), 1st line STS (41%) and RCC patients (62%). On the other hand, in line with observed lower frequencies of CD56^{dim} and perforin^{+} NK cells, 2nd line STS patients showed a significantly reduced percentage of CD57^{+} NK cells (median 5%) compared with NK cells of RCC patients (27%) and HD (26%) (Fig. 4 A/B, right panels).

Discussion

NK cells and CD8^{+} T lymphocytes can act as major players in antitumor responses, and inhibition of their function has been associated with tumor immune escape.[39,40] Here, we examined the NK cell-specific cytotoxicity of PBMCs of patients with STS and RCC in comparison with healthy controls, and aimed to identify differences between patient groups and the underlying mechanisms of NK cell activation and inhibition. RCC patients were chosen as a second reference group due to the documented prognostic importance of tumor-infiltrating NK cell for this histology.[41-44]

We observed that the cytotoxic function of NK cells from STS patients was profoundly impaired, while, notably, NK cells of RCC patients exhibited a cytotoxic ability similar to HD. To our knowledge, this is the first description of functional deficits of NK cells

from patients with STS. While suppressed NK cell activity has been demonstrated for a variety of epidermal and mesenchymal neoplasias[8,10,11,30,45,46], functional impairment of NK cells from peripheral blood does not seem to be a universal phenomenon in cancer patients, as NK cell-mediated cytotoxicity was normal in patients with pancreatic cancer and RCC.[47,48] Interestingly, patients with gastrointestinal stromal tumors (GIST), a STS sub-entity with distinct clinical and pathophysiological features separating it from other STS, have also been shown to display unaltered NK cell-specific cytotoxicity compared with HD.[49]

We attempted to gain insight into the mechanisms that might cause the NK cells' functional deficit, as this might reveal means of intervention that could help prevent the development of dysfunction or restore activity. Surgery has been described to cause NK cell dysfunction[50], but these effects were of limited duration, with cytotoxic capacity returning to pre-surgery values after approximately 30 days.[47] In our cohort of STS patients, blood samples were taken at least four weeks after surgery. Therefore, post-surgical effects seem unlikely as explanation of impaired NK cell-specific cytotoxicity. Additionally, there was no difference between patients who had undergone tumor resection and patients who had been diagnosed by tumor biopsy without surgery (data not shown). Age can also be excluded as an influential factor[51] since RCC patients, who were older (median age 65 years) than STS patients (median 44 and 34 years for 1st and 2nd line STS patients, respectively), showed even higher levels of NK cell cytotoxicity in comparison with STS patients.

Administration of cytostatic agents needs to be considered as one factor with impact on NK cell-specific cytotoxicity, in particular since the analyzed STS cases include samples from patients who had or had not received chemotherapy before blood withdrawal. Reports on the effects of cytostatic treatment on NK cell-specific cytotoxicity *in vivo* are scarce, and results vary depending on the treated cancer and

the used cytostatic agents, documenting inhibition as well as augmentation of NK cell-specific cytotoxicity.[52-56] *In vitro* assays show distinct effects of different chemotherapeutic agents on NK cell cytotoxicity. Doxorubicin and epirubicine, anthracyclines frequently used in the treatment of STS patients, are among those agents that have been shown to only marginally influence NK cell lytic activity.[57] However, these results have to be interpreted with caution, as *in vitro* assays cannot consider drug metabolism and interactions relevant for NK cell activation. Moreover, detectable effects are restricted to short-term outcomes and thus cannot reveal effects that are associated with or depend on NK cell proliferation.

In our study, all 2nd line patients had been treated with anthracyclines and usually ifosfamide, but not with any tyrosine-kinase inhibitors (TKI), and were included following relapse or progression of disease. The NK cells of the 2nd line patient cohort showed strikingly poor cytotoxicity with levels significantly below those of chemotherapy-naïve 1st line STS patients. This might suggest negative effects of systemic chemotherapy on the function of peripheral NK cells. However, when 2nd line STS patients were divided into two groups according to the median time interval between last chemotherapeutic treatment and blood withdrawal (5 months), patients who had not received cytotoxic medication for years did not show higher NK cell-specific cytotoxicity compared with patients who had received their last chemotherapy within five months before blood withdrawal. Interestingly, one of the 2nd line patients (red triangle in Suppl. Fig. 1A) had a NK cell-specific lytic activity that was comparable to 1st line STS patients. This patient had very late relapse of clear cell sarcoma almost 18 years after initial treatment. The observed preserved NK cell cytotoxicity supports the assumption that this late reoccurrence might rather be a secondary *de novo* transformation than an actual relapse of the original disease.

Since NK cell dysfunction still persists in 2nd line STS patients with very long chemotherapy-free periods, the detrimental effects of chemotherapy on NK cell activity would have had to be very long-lasting if they were to explain the observed NK cell dysfunction. However, such very long-lasting effects seem unlikely considering that NK cell numbers quickly recover after chemotherapy[58] and cytotoxicity is restored quickly even after maximally invasive procedures like allogeneic transplantation.[59]

Therefore, in addition – or alternatively – to chemotherapy, disease burden and dissemination might explain the difference between 1st line and 2nd line STS patients. For different neoplasias decrease in NK cell cytotoxicity has been described to be stage-dependent. For patients with lymphoma, melanoma and head and neck cancer, a more advanced, e.g. metastatic/disseminated disease was associated with lower NK cell lytic activity compared to patients with localized, early-stage disease.[9,10,60] Of the 11 2nd line STS patients in our study, 7 had metastatic disease, while none of the patients in the 1st line cohort had evidence of metastasis. The differences in NK cell cytotoxicity between 1st and 2nd line patients might therefore be associated with the more advanced disease status of the 2nd line patients. Accordingly, subgroup analyses revealed that PBMCs of 2nd line patients with metastatic disease had lower NK cell-mediated cytotoxicity than those of 2nd line patients without metastasis. However, in addition to disease status, and contradictory to the observation that NK cell dysfunction did not recover in a time-dependent manner after chemotherapy, chemotherapeutic treatment possibly had an impact on the cytotoxic capacity of NK cells, since NK cell cytotoxicity of non-metastatic 2nd line STS patients was significantly lower compared with the cytotoxicity of PBMCs of (all non-metastatic) 1st line patients. We observed no obvious correlation between NK cell-specific cytotoxicity and tumor volume, and 1st line STS patients with resected

tumors (hence absent/minimal tumor burden) did not show significantly higher NK cell cytotoxicity than patients who had not received surgery (data not shown). Taken together, disease dissemination can be seen as an important factor that influences NK cell cytotoxicity in STS patients. However, other factors like chemotherapeutic treatment also seem to affect the cytotoxic capacity of NK cells. Yet, these interpretations have to be taken with caution due to the low number of patients available for the analyses.

Finally, we tested a scenario that patients with low NK cell-specific cytotoxicity might have a higher probability of relapse after 1st line therapy. This would enrich the group of 2nd line STS patients with individuals showing low NK cell cytotoxicity. However, in our cohort, patients with progressive disease or relapse after 1st line therapy had comparable NK cell-specific cytotoxicity with patients that did not experience relapse (data not shown). While this refutes such consideration, caution is indicated, as only 13 patients were assessed. Prospective serial measurements of NK cell function over the course of disease and therapy are warranted in order to substantiate the rejection of this hypothesis. Interestingly, new small molecule agents used for targeted therapy, e.g. BRAF inhibitors or TKIs like imatinib have been shown to positively affect NK cell proliferation[61] and to increase NK cell cytotoxicity.[49] Furthermore, imatinib therapy leads to increased tumor infiltration of immune cells, including NK cells, in GIST.[15] As TKIs like pazopanib become important in the treatment of non-GIST STS, it will be exciting to see how these relatively new agents influence NK cell-specific cytotoxicity of STS patients, and if the effects of TKIs are different from those of “classic” cytostatic agents. The possible promotion of tumor infiltration with NK cells by these agents will be of special interest, as STS have been shown to display only minor infiltration by NK cells.[15]

To define the cellular basis of the poor NK cell function, we analyzed the NK cell population by flow cytometry. We observed drastic changes in the composition of peripheral NK cell populations of patients that intensified from 1st to 2nd line STS patients. Alterations included reduced NK cell frequencies and altered distributions of NK cell subsets. Notably, mainly CD56^{dim} NK cell subsets were affected. As this subset is considered the main cytotoxic subset of NK cells[62], the pronounced reduction of this NK subset could be an explanation for the poor cytotoxicity of PBMCs of STS patients. Yet, other factors still contribute since NK cell cytotoxicity values of PBMCs of STS patients remained below those of HD and RCC patients after normalization to the percentage of NK cells. One contributing factor could be the reduction of CD16 expression on CD56^{dim} NK cells, as CD16 in synergy with other activating receptors like NKG2D or NKp46 is required to activate cytotoxicity.[63]

PBMCs of 1st line STS also exhibited a notable reduction of CD56^{dim} NK cell frequencies compared with HD and RCC. Values of CD56^{dim}CD16^{+} subgroup proportions of 1st line STS patients were between those of HD and 2nd line patients. As 1st line STS patients did not receive systemic cytostatic therapy, these changes may be disease-associated. Notably, not all cancer types seem to exert effects on peripheral NK cells as NK cells from RCC patients did not differ from those of HD. Disturbances in NK cell subsets seem to follow disease progression, as they worsened from 1st line to 2nd line STS patients. Besides increasing tumor burden, a contribution of chemotherapy to the development of these alterations cannot be excluded. However, such long lasting effects are to date unprecedented as discussed before.

The drastic alterations of NK cell subset composition in PBMCs of 2nd line STS patients compared to HD and RCC patients provided a plausible explanation for the impaired NK cell reactivity. Yet, alterations in 1st line STS patients were moderate

406 and did not reach statistical significance, thus they cannot convincingly explain the
407 poor cytotoxicity of NK cells of 1st line STS patients. Subsequent analyses included
408 expression of activating NK receptors (NKG2D, NKp46) as well as inhibitory CD94
409 receptor or signaling adaptor molecule CD3ζ and cytotoxic proteins (perforin,
410 granzyme B), all of which contribute to the level of NK cell activity and are frequently
411 downregulated in cancer patient NK cells.[14,45] Again, our observations failed to
412 provide an explanation for the deficient NK cell activity of 1st line STS patients as
413 none of the markers showed any difference between 1st line STS patients and HD or
414 RCC patients.
415 On the other hand, NK cells of 2nd line STS patients showed deficits in several of
416 these markers, including reduced proportions of NK cells expressing NKG2D, CD3ζ,
417 perforin and CD57. Interestingly, percentages of NK cells expressing granzyme B,
418 NKp46 or CD94 were not significantly altered.
419 CD57 is a marker for terminally differentiated and cytotoxic NK cells.[36,37] The reduced
420 percentage of $CD57^+$ NK cells in 2nd line STS patients might hint at a relative
421 predominance of immature NK cells in the peripheral blood of these patients, and
422 higher proportions of immature NK cells might be seen as an effect of recent
423 cytostatic treatment, as chemotherapy temporarily suppresses bone marrow function.
424 Arguing against this interpretation, however, is the observation that the expression of
425 NKp46, which is only found on NK cells at later maturation stages[1], was comparable
426 between HD, RCC, and sarcoma patients in our study.
427 The observation that deficits in marker expression of NK cells were only seen in 2nd
428 line STS might indicate that chemotherapy negatively affects the quality of NK cells.
429 Yet, as discussed above, all 2nd line patients had month-long treatment-free periods
430 before blood withdrawal. Moreover, published results do not suggest that strong
431 effects of cytostatic treatment are to be expected. For example, neoadjuvant

chemotherapy had no effect on NKG2D expression on NK cells of breast cancer patients[54], and doxorubicin did not alter NKG2D expression in a murine system *in vivo*.[64] For NKp46, chemotherapy increased expression in melanoma patients.[65] Influence of chemotherapeutic treatment on CD3ζ expression in NK cells has not been addressed yet. In T cells, treatment of CLL patients with fludarabin lead to an decrease in the percentage of $CD3\zeta^+$ cells.[66] Taken together, there is no convincing evidence that the observed changes in NK cell marker expression might be linked to previous cytostatic treatment. Thus, disease-specific effects, e.g. mediated by cytokine release or receptor shedding[3,67,68], might be an explanation for the observed changes.

In summary, we could demonstrate profound alterations of NK cell characteristics in the peripheral blood of STS patients, including reduction in NK cell frequency and deviations in subset distribution, as well as changes in the expression of activating receptors, signaling and cytotoxic molecules, and differentiation markers. Interestingly, none of these changes were seen in NK cells of RCC patients. 1st and 2nd line STS patient cohorts differed regarding the degree of alterations, which was considerably more pronounced in 2nd line patients. The observed changes in subset composition and expression of molecules involved in the activation of NK cells suffice to explain the lack of cytotoxicity of NK cells in 2nd line STS patients. However, attempts to elucidate the reduced NK cell cytotoxicity of 1st line STS patients remain unsatisfactory, presently, although reduced NK cell frequency, particularly in the cytotoxic subset of $CD56^{dim}CD16^+$ NK cells were discernable. Further analyses to better characterize the NK cells of 1st line STS patients are warranted.

Our study provides important information for potential immunotherapeutic approaches in patients with STS. STS negatively affects the NK cell population concerning frequency but also, and most importantly, concerning cytotoxic function.

This is apparently specific for soft-tissue sarcoma, as NK cells of RCC patients were not affected. Yet, despite severe suppression, NK cells of STS patients can recover activity when stimulated with IL-2/TKD reaching levels of cytotoxicity comparable with those of similarly activated PBMCs of healthy donors. This indicates that STS patients may benefit from immunotherapeutic approaches tailored to activate NK cells. We did not perform flow cytometry analyses of the activated cells, so the reversion of alterations observed in 2nd line STS patients after stimulation still need to be addressed. This knowledge might, however, be of interest for future immunotherapeutic interventions for STS patients, as protocols using *ex vivo* expanded and activated NK cells as adoptive immunotherapy have provided disappointing outcomes for different tumor entities.[69-73] To improve the outcome of these therapies, NK cell markers and effector molecules that describe functional NK cells have to be defined. Receptors and effector molecules we observed to be aberrantly expressed might be suitable as objectives to optimize NK cells used for immunotherapeutic approaches in STS patients.

Patients, materials and methods

Patients and healthy donors

Blood samples were taken from patients and donors after they gave written informed consent. The study was approved by the Institutional Review Board on Medical Ethics, and the Declaration of Helsinki was observed.

STS patients were enrolled between 2008 and 2014 (Klinikum Großhadern Medical Center, Munich, Germany and Schön Klinik Starnberger See, Berg, Germany). Of the 24 STS patients, 13 (median age 44 years, range 31-75 years) had not received previous chemotherapy (1st line patients), whereas 11 patients (median age 34 years, range 24-77 years) had been treated with anthracycline-based chemotherapy before enrollment (2nd line patients). Patients with GIST were excluded. All 1st line patients had macroscopic tumor burden or had had tumor resection within 4-16 weeks before inclusion. All 2nd line patients showed progression or relapse of disease at the time of enrollment, whereby progression did not necessarily occur during previous chemotherapeutic treatment, but could also emerge after the end of (e.g. adjuvant) chemotherapeutic treatment. Time intervals since last cytostatic drug treatment therefore varied between patients. Detailed patients' characteristics are shown in Table 1. Blood samples of 1st line patients were taken after definite diagnosis (by biopsy or resection) 4-8 weeks after the diagnostic procedure (surgery in 8 of 11 patients), but always before the initiation of cytostatic treatment. For 2nd line patients, blood samples were taken directly before the initiation of the non-1st line chemotherapy. The time interval between last cytostatic treatment and blood withdrawal was always 4 weeks or longer. Longitudinal analyses of single individuals were not possible, as no patient was recruited consecutively in both the 1st and the 2nd line patient group.

All RCC patients (n=11, median age 65 years, range 42-80 years) had not received prior systemic treatment. Blood was taken before nephrectomy. Detailed patients' characteristics are shown in Table 2.

32 healthy donors (HD) were recruited at HelmholtzZentrum Munich. Initially, HD were stratified into two age groups (24-59 years and 60-68 years, respectively) to match them to younger STS and older RCC patient groups in our cohort. Since we did not observe significant differences in all analyzed data between the two HD groups (data not shown), experiments were pooled for graphical presentation.

Isolation of PBMC and stimulation of NK cell cytotoxicity

PBMCs were isolated from venous blood using density gradient separation with Pancoll (PAN-Biotech, no. P04-60500). Cells from the interphase were either cryo-preserved or used immediately.

Where indicated, PBMCs were cultured in RPMI 1640 (Sigma-Aldrich, no. R0883), supplemented with 10% fetal calf serum, L-glutamine (both from PAN-Biotech, no. P30-1302 and P04-80100, respectively) and penicillin/streptomycin (Gibco/Thermo Fisher Scientific, no. 15140-122) and NK cells were stimulated with interleukin-2 (IL-2, 1000 IU/ml, Gibco/ Thermo Fisher Scientific, no. PHC0021) and TKD (14 amino acid derivative of Hsp70, 2 µg/ml, gift from G. Multhoff, Munich)[27] for 96h at 37°C and 5 % CO_2.

Chromium release assay

The MHC class I-deficient cell line K562 (ATCC no. CCL-243) was cultured in RPMI 1640 (Sigma-Aldrich, no. R0883), supplemented with 10% fetal calf serum, L-glutamine, amino acids (BME amino acids solution, all from PAN-Biotech, no. P30-1302, P04-80100 and P08-2000, respectively) and penicillin/streptomycin (Gibco/ Thermo Fisher Scientific, no. 15140-122). NK cytotoxicity was determined in a standard 4h chromium release assay.[74] Briefly, 3×10^5 K562 cells were incubated

with 3.7 × 10^6 Bq $Na_2{}^{51}CrO_4$ (Hartmann Analytic, no. Cr-RA-8) for 90 min and then washed twice. 1.2 × 10^5 PBMC in 100µl RPMI 1640 were added as triplicates to a 96-well U bottom plate. 1:1 dilutions of the PBMC were performed to create serial dilutions. 3 × 10^3 K562 in 100 µl medium were added to achieve 40:1, 20:1, 10:1 and 5:1 effector to target ratios. After 4h the supernatants were collected in solid scintillator coated microplates (LumaPlate, Perkin Elmer, no. 6006633). Activity of the dried plates was measured in a gamma counter. To determine the spontaneous release, supernatants from wells with K562 targets without added effector PBMC were collected. Maximum release was assessed from wells with K562 cells collected directly after incubation with $Na_2{}^{51}CrO_4$. The lytic activity was calculated as

(experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

PBMCs from all RCC patients were cryo-conserved and used directly after thawing, with no cytokines added to the medium. PBMCs of HD or STS patients were either cryo-conserved and used directly after thawing with no cytokines added to the medium, or were used freshly within 48h after isolation. IL-2 was added only for stimulation assays. All assays included PBMCs from HD as positive control. Spontaneous release was always < 20% of maximum release.

Multiparameter flow cytometry

Cells were stained and analyzed as previously described.[75] Antibodies are listed in Suppl. Table 1. Dead cells were excluded with propidium iodide (0.4 µg/ml, Invitrogen, no. P3566) or 7-AAD (10 µg/ml, Sigma, no. A9400). NK cells were gated as $CD3^-CD56^+$ cells within live, single leukocytes (selected based on forward scatter/side scatter characteristics). The gating strategy is exemplified in Suppl. Fig. 2A. The percentage of marker-positive cells within the gated $CD3^-CD56^+$ NK cells was determined using an internal population negative for the analyzed marker or

isotype controls as reference, respectively. Identification of NK cell subsets is exemplified in Suppl. Fig. 2B. PBMCs of STS and RCC patients were always analyzed in parallel with samples from HD. As 1st and 2nd line STS patients were enrolled at different time points, they were analyzed in independent experiments. Moreover, HD and RCC patients employed as controls were different for 1st and 2nd line STS patient analyses. Therefore, results of HD and RCC measurements were not pooled, but displayed in separate graphs. Age matching of HD samples was later omitted since initial analyses did not show significant differences in the analyzed markers between younger and older HD (see above).

Statistical analyses

Comparisons of multiple subgroups (e.g. expression levels of NK cell antigens) were performed by non-parametrical Kruskal-Wallis test with Dunn's post hoc tests or 2-way ANOVA with Bonferroni's post hoc test (for paired values, e.g. cytotoxicity before and after stimulation). For comparisons of two subgroups (e.g. percentages of leukocyte subgroups of patients and HD), non-parametrical Mann-Whitney U tests were performed. Correlation analyses were done with Pearson's correlation and linear regression tests. Graphpad Prism (Version 6, Graphpad software) was used for all statistical analyses.

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Figure legends

Fig. 1 Peripheral NK cells of patients with STS are less cytotoxic than NK cells of RCC patients and HD, but regain cytotoxicity after incubation with IL-2/TKD. NK-specific cytotoxicity against radiolabeled K562 target cells was assessed by 4h ^{51}Cr release assay. Displayed are the specific lysis values of PBMC/K562 at a ratio of 20:1. **(A)** NK-specific cytotoxicity of HD (n=32), STS (n=24) and RCC patients (n=4). **(B)** NK-specific cytotoxicity of STS patients without previous chemotherapy (1st line, n=13) compared with STS patients with prior chemotherapy (2nd line, n=11). **(C)** Left panel: NK-specific cytotoxicity of HD (n=10) and STS patients (1st line n=6, 2nd line n=6), assessed before and after 96h of incubation in medium containing interleukin-2 (IL-2) and 14-mer heat shock protein 70 (hsp70) peptide TKD. Only paired samples are shown. Right panel: Normalized data of left panel; relative increase in cytotoxicity for HD, 1st and 2nd line STS patients after 96h of incubation with IL-2 and TKD. For A, B and C (left panel), box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample. For C (right panel), mean values with standard errors are shown. For statistical analyses, Kruskal-Wallis tests with Dunn's post hoc tests (A), Mann-Whitney-U test (B) and 2-way ANOVA with Bonferroni's post hoc test (C) were used.

Fig. 2 Percentage of CD56^{dim} NK cells among PBMCs is reduced and CD16 expression on CD56^{dim} NK cells is diminished in 2nd line STS patients. (A) Relative distributions of NK cells and NK cell subsets (CD56^{dim} and CD56^{bright}) among PBMCs were assessed by polychromatic flow cytometry. Mean values with standard errors of indicated PBMC subtype cells among live, single, small lymphocytes (FSC/SSC) are shown. Asterisks indicate p values resulting from comparisons of total

NK cells ($CD3^-CD56^+$ cells) and the $CD56^{dim}$ NK cell subset. Comparison of the percentages of $CD56^{bright}$ NK cells revealed no statistically significant difference. **(B, C)** show the frequencies of $CD16^+$, $CD16^{low}$ and $CD16^-$ cells among $CD56^{dim}$ and $CD56^{bright}$ NK cells, respectively. Relative distributions are depicted as percentages of the respective NK cell subset, defined as $CD3^-CD56^{bright}$ or $CD3^-CD56^{dim}$ cells among live, single, small PBMCs (FSC/SSC), as assessed by polychromatic flow cytometry. Box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample, and comparisons of 1^{st} line STS (n=8), 2^{nd} line STS (n=5), RCC patients (n=11) with HD (n=21) are depicted. For statistical analysis, Kruskal-Wallis test with Dunn's post hoc test was used. * p< .05, *** p< .001.

Fig. 3 2^{nd} line STS patients show reduced percentages of cells expressing the activating NK cell receptor NKG2D and the CD3ζ signaling adaptor protein in peripheral NK cells. (A) Expression of NKG2D (left panel), NKp46 (middle panel) and CD3ζ (right panel) of HD (n=4), 1^{st} line STS patients (n=6) and RCC patients (n=4) analyzed by polychromatic flow cytometry of uncultured PBMCs. **(B)** Marker expression in HD (n=13), 2^{nd} line STS patients (n=5) and RCC patients (n=7). **(A-B)** Percentages of marker-positive cells among NK cells ($CD3^-CD56^+$ cells within live, single, small (FSC/SSC) PBMCs) are depicted. Box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample. For statistical analyses, Kruskal-Wallis test with Dunn's post hoc tests was used.

Fig. 4 2^{nd} line STS patients show reduced percentages of perforin$^+$ and $CD57^+$ cells in peripheral NK cells. (A) Expression of perforin (left panel),

granzyme B (middle panel) and CD57 (right panel) of HD (n=4), 1st line STS patients (n=6) and RCC patients (n=4) analyzed by polychromatic flow cytometry of uncultured PBMCs. **(B)** Marker expression in HD (n=13), 2nd line STS patients (n=5) and RCC patients (n=7). **(A-B)** Percentages of marker-positive cells among NK cells ($CD3^-CD56^+$ cells within live, single, small (FSC/SSC) PBMCs) are depicted. Box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample. For statistical analyses, Kruskal-Wallis test with Dunn's post hoc tests was used.

Tables

Table 1 Characteristics of STS patients. Median age of 1st-line patients was 44 years, of 2nd-line patients was 34 years. All 2nd-line patients had been treated with anthracyclines before. Months since initial diagnosis indicates the time interval to blood sample withdrawal. TNM stage indicates tumor stage, assessed by CT/MRI imaging, at time of blood withdrawal, with resection status in brackets. Months since last cytostatic treatment indicates the time interval from last application of chemotherapeutic agents to blood sample withdrawal.

Patient No.	Sex	Age (years)	Histology	Months since diagnosis	TNM stage	Previous cytostatic treatments (n)	Months since last cytostatic treatment
1	f	46	Synovial sarcoma	3	m.d. (R2)	0	n/a
2	m	62	Mesenchymal chondrosarcoma	1	T2bN0M0 (-)	0	n/a
3	m	31	Spindle cell sarcoma	4	T2bN0M0 (R1)	0	n/a
4	m	75	Myxofibrosarcoma	1	T2bN0M0 (-)	0	n/a
5	m	50	Synovial sarcoma	2	T1bN0M0 (R1)	0	n/a
6	f	44	Leiomyosarcoma	3	T2bN0M0 (Rx)	0	n/a
7	f	38	Epitheloid fibrosarcoma	1	m.d. (-)	0	n/a
8	f	39	Malignant solitary fibrous tumor	2	T2bN0M0 (-)	0	n/a
9	f	62	Leiomyosarcoma	1	T1bN0M0 (-)	0	n/a
10	m	31	Malignant peripheral nerve sheath tumor	3	T2bN0Mx (R1)	0	n/a
11	f	32	Synovial sarcoma	1	T1bN0M0 (R0)	0	n/a
12	f	39	Synovial sarcoma	2	T2bN0M0 (R0)	0	n/a
13	m	50	Malignant peripheral nerve sheath tumor	2	T2bN0M0 (R1)	0	n/a
14	f	34	Malignant peripheral nerve sheath tumor	11	T2bNxM1 (-)	2	1
15	m	35	Alveolar soft part sarcoma	23	T2bNxM1 (-)	3	3
16	m	24	Desmoplastic small round cell tumor	16	T2bN1M1 (-)	1	3
17	f	57	Leiomyosarcoma	34	TxNxM1 (-)	2	5
18	f	31	Malignant peripheral nerve sheath tumor	68	T2bN0M0 (-)	2	56
19	f	25	Clear cell sarcoma	219	T2bN0M0 (Rx)	1	212
20	f	30	Chordoma	85	T2bN0M0 (-)	1	2
21	f	66	Rhabdomyosarcoma	54	TxN0M1 (-)	2	15
22	m	24	Epitheloid sarcoma	5	T2bNxM1 (-)	1	1
23	f	77	Leiomyosarcoma	40	TxNxM1 (-)	1	6
24	m	71	Liposarcoma	45	T2bN0M0 (-)	1	32

Abbreviations: n: number of previous chemotherapeutic treatments. n/a: not applicable. m.d.: missing data. f: female. m: male. R0: complete resection of the tumor, with no microscopic evidence of tumor infiltration of resection margins. R1: complete macroscopic resection of the tumor, with microscopic evidence of tumor infiltration of resection margins. R2: incomplete resection of the tumor, with macroscopic tumor burden remaining in situ. Rx: resection with unknown resection margins. (-) No tumor resection performed.

Table 2 Characteristics of RCC patients. Median age of all patients was 65 years. TNM stage indicates tumor stage, assessed by CT/MRI imaging and pathologic diagnosis, at time of blood withdrawal. None of the patients had received systemic treatment or had tumor-related surgery before blood was withdrawn. Patients 1-4 were assessed as comparative group for cytotoxicity assays and flow cytometry assays for 1st-line STS patients, patients 5-11 were assessed as comparative group for flow cytometry assays for 2nd-line STS patients.

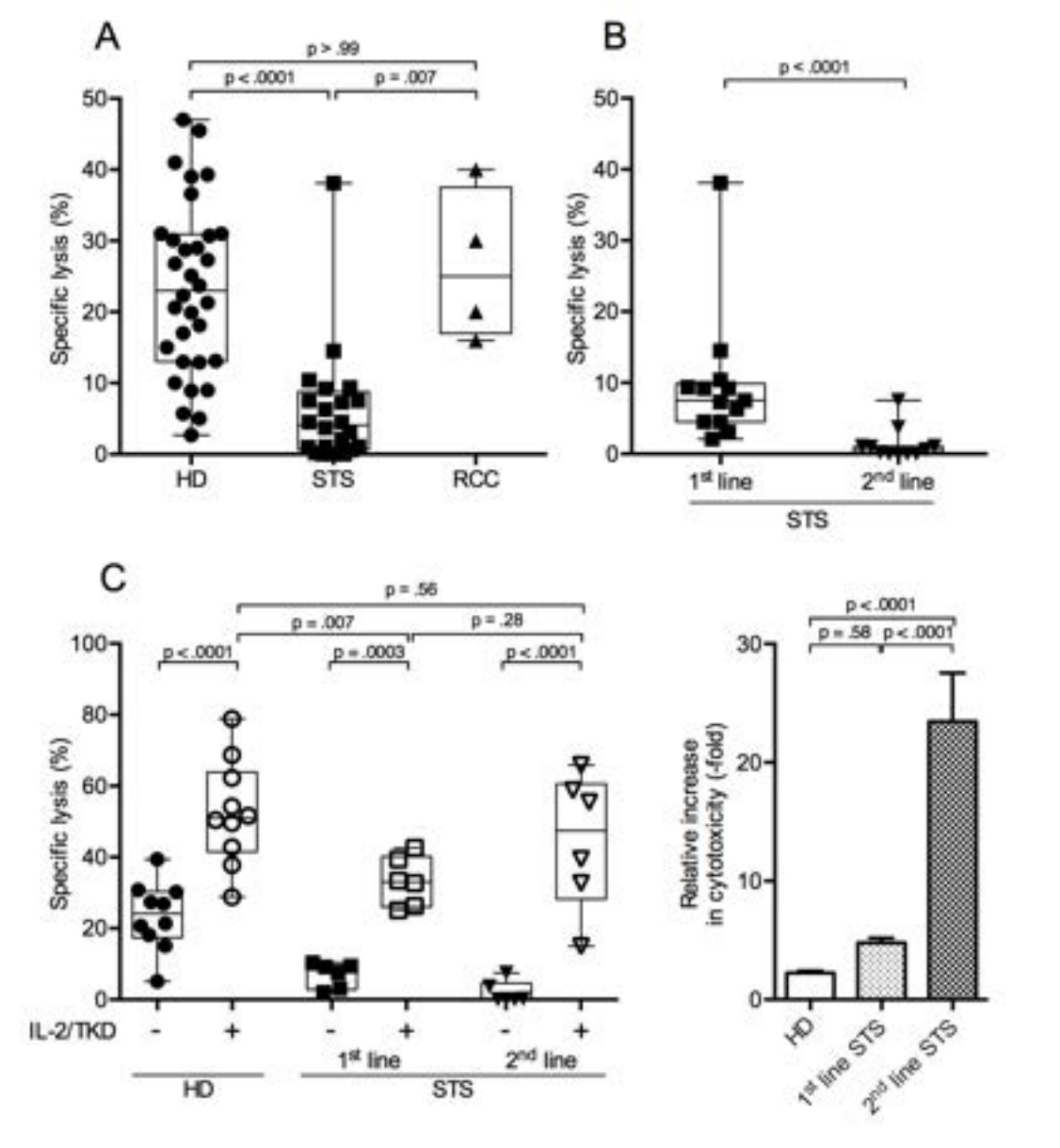
Patient No.	Sex	Age (years)	Histology	TNM stage
1	m	68	Clear cell renal cell carcinoma	T3bN0M0
2	f	80	Clear cell renal cell carcinoma	T3bN2M0
3	f	76	Clear cell renal cell carcinoma	T3bN0M0
4	m	47	Clear cell renal cell carcinoma	T1bN0M0
5	f	61	Sarcomatoid renal cell carcinoma	TxNxM1
6	f	80	Clear cell renal cell carcinoma	T3bN0M1
7	m	61	Clear cell renal cell carcinoma	m.d.
8	m	42	Adrenocortical carcinoma	T3N0M0
9	m	64	Clear cell renal cell carcinoma	TxNxM1
10	m	65	Clear cell, sarcomatoid renal cell carcinoma	T3aN0M0
11	f	70	Papillary renal cell carcinoma	T1N1Mx

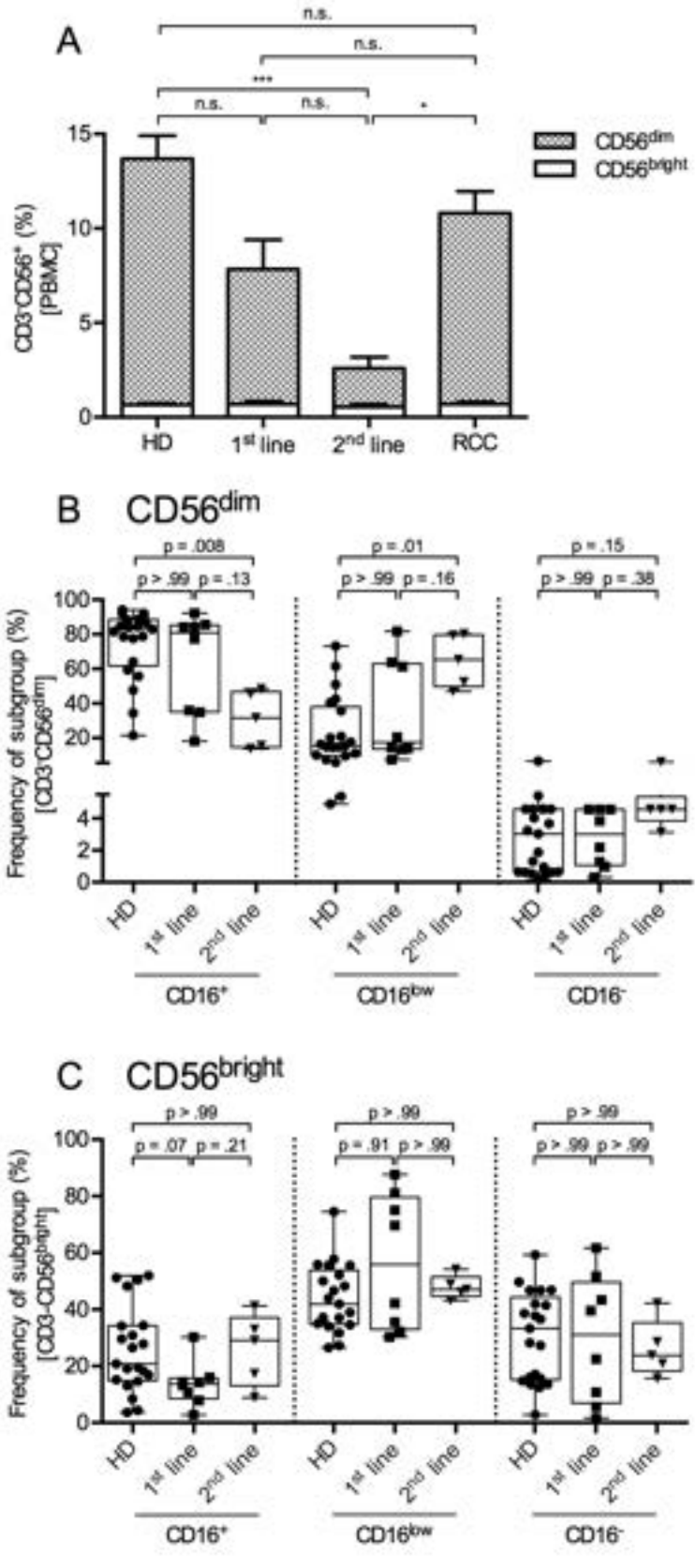
Abbreviations: m.d.: missing data. f: female. m: male.

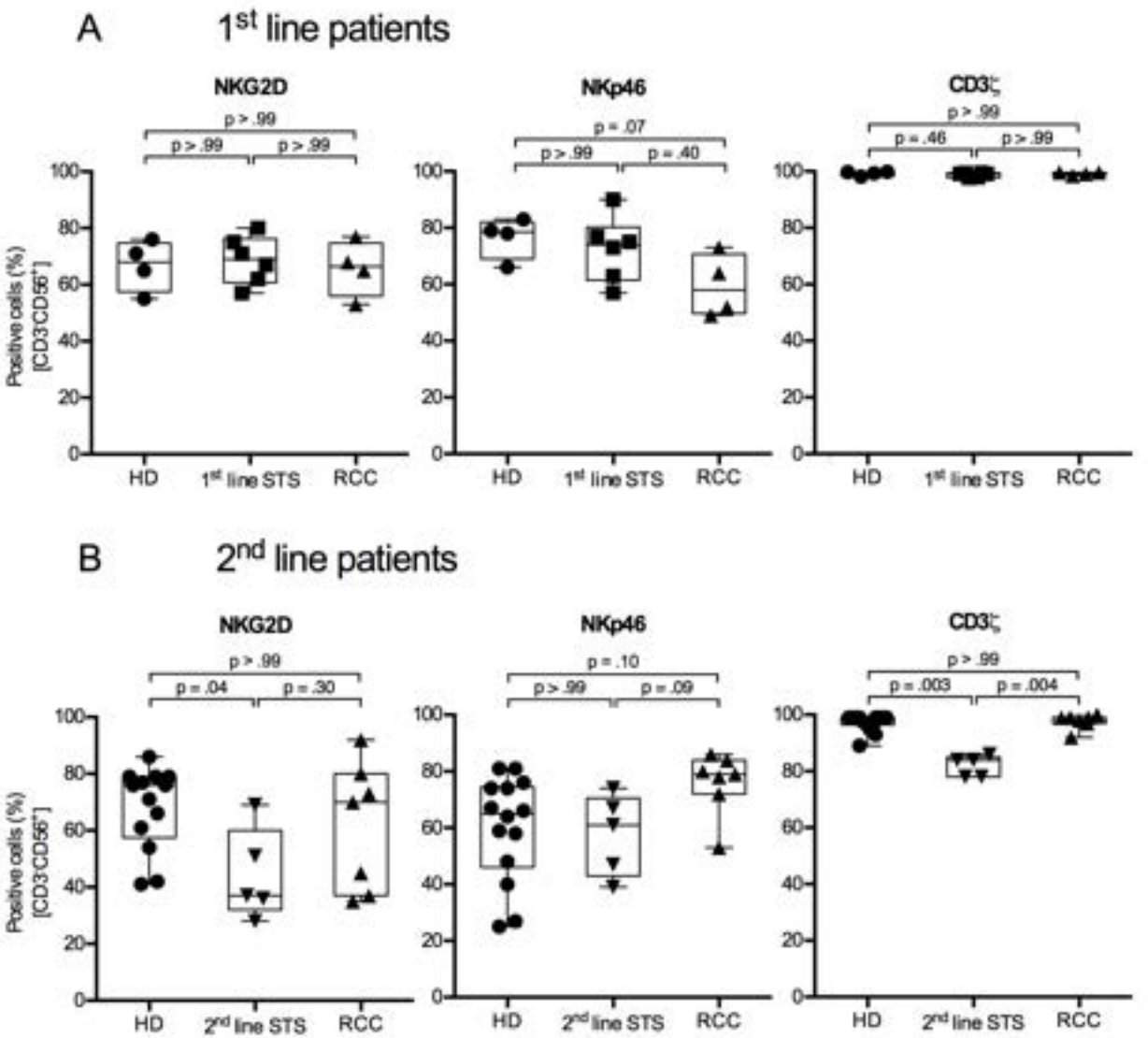
Supplemental Table 1 Antibodies used for flow cytometry.

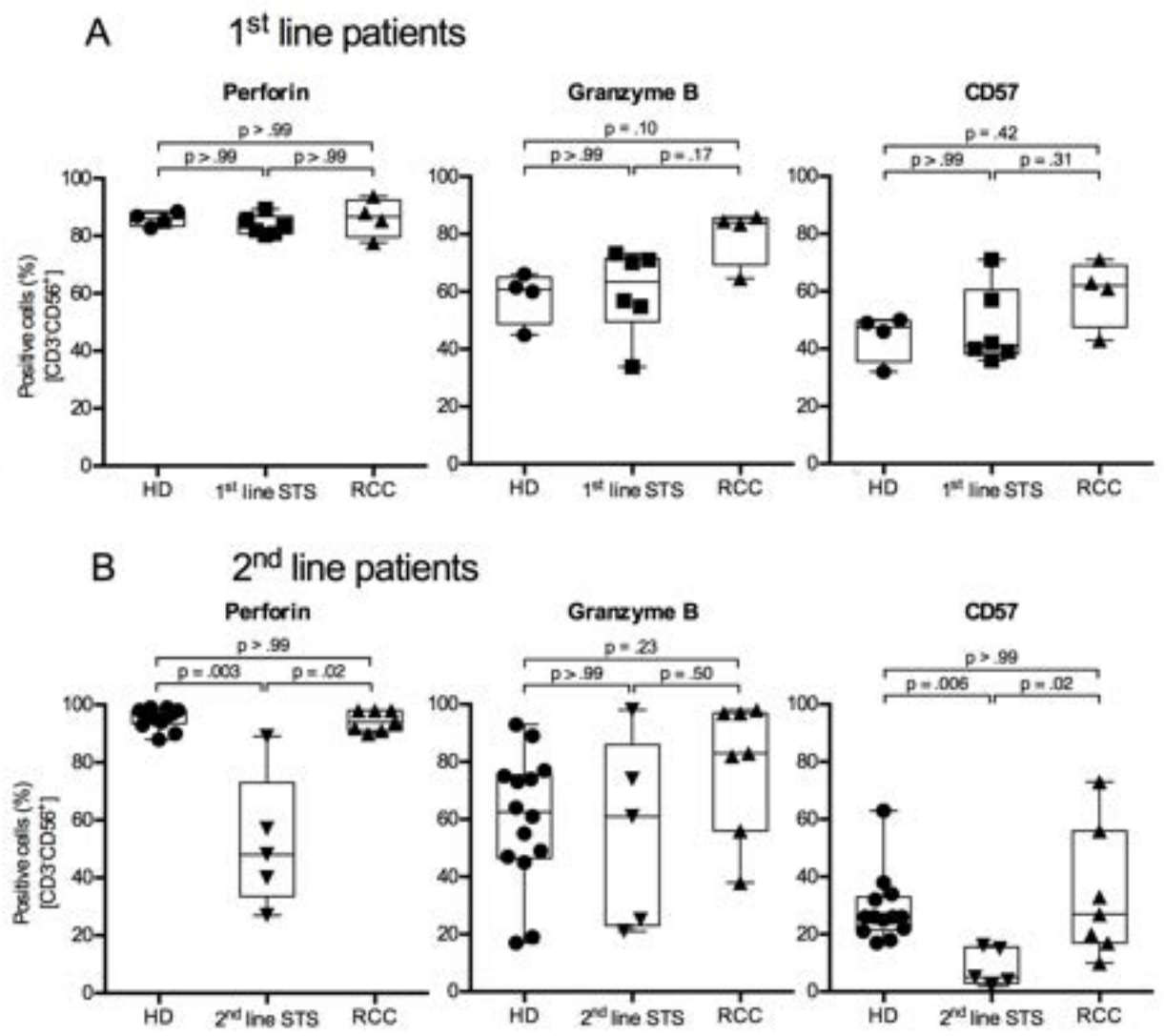
Antibody	Fluorochrome	Clone	Species and Isotype	Manufacturer	No.
CD3	Pacific blue	UCHT1	Mouse IgG1	Biolegend	300431
CD8	V500	RPA-T8	Mouse IgG1	BD Biosciences	560774
CD14	APC-Alexa Fluor 750	TüK4	Mouse IgG2a	Thermo Fisher Scientific	MHCD1427
CD16	A700	3G8	Mouse IgG1	Thermo Fisher Scientific	MHCD1629
CD19	APC-Alexa Fluor 780	HIB19	Mouse IgG1	eBioscience	27-0199
CD56	APC	N901	Mouse IgG1	Beckman Coulter	IM2474
CD56	PE	B159	Mouse IgG	BD Biosciences	555516
CD57	FITC	TB01	Mouse IgM	eBioscience	11-0577-42
CD94	FITC	HP-3D9	Mouse IgG1	eBioscience	555888
CD247/CD3zeta	FITC	G3	Mouse IgG2a	AbD Serotech	MCA1297
NKp46	PE	9E2	Mouse IgG1	BD Biosciences	557991
NKG2D	APC	91D11	Mouse IgG1	BD Biosciences	558071
Granzyme B	PE	GB11	Mouse IgG1	BD Biosciences	561142
Perforin	APC	dG9	Mouse IgG2b	eBioscience	17-9994

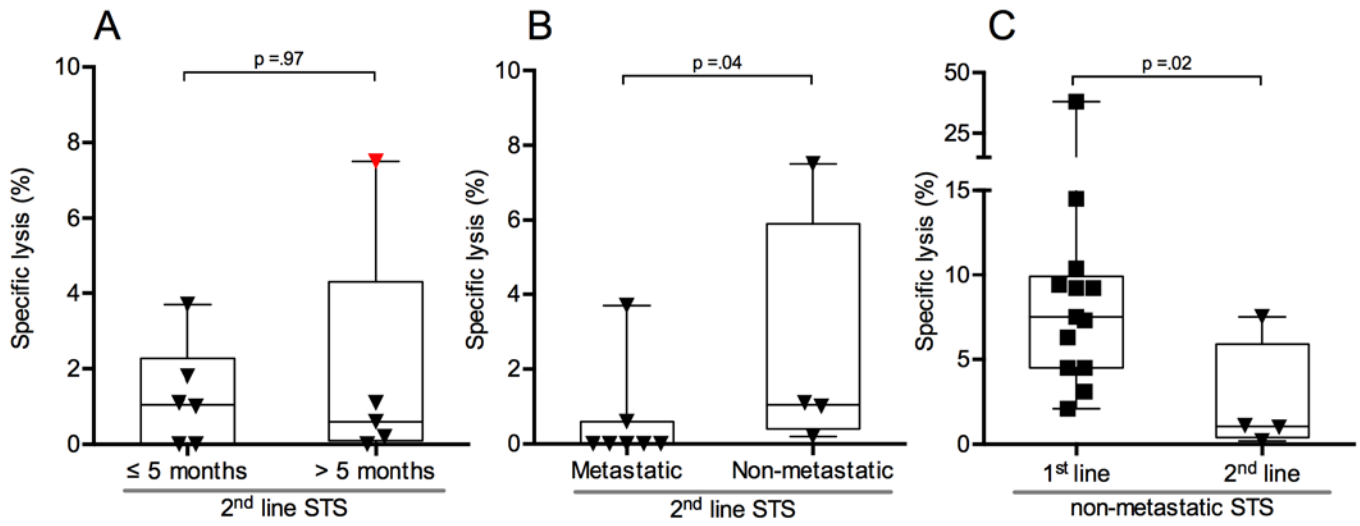
Abbreviations: APC = allophycocyanine, FITC = fluorescein isothiocyanate, PE = phycoerythrin


A
p > .99
p < .0001
p = .007
Specific lysis (%)
HD
STS
RCC
B
p < .0001
Specific lysis (%)
1st line
2nd line
STS
C
p = .56
p = .007
p = .28
p < .0001
p = .0003
p < .0001
Specific lysis (%)
IL-2/TKD
HD
1st line
2nd line
STS
p < .0001
p = .58
p < .0001
Relative increase in cytotoxicity (-fold)
HD
1st line STS
2nd line STS

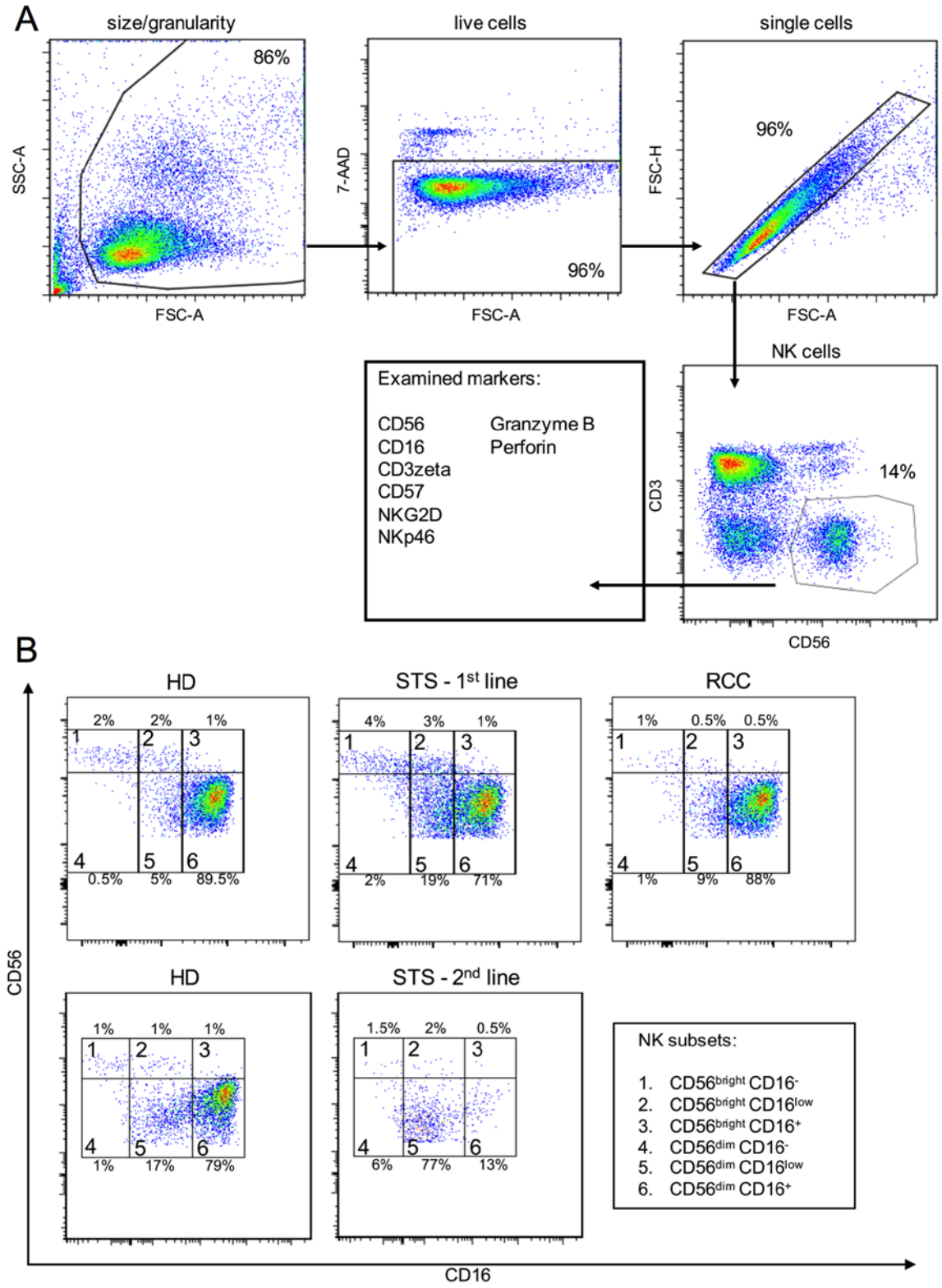

A
n.s.
n.s.
***
n.s.
n.s.
*
CD56dim
CD56bright
CD3-CD56+ (%) [PBMC]
15
10
5
0
HD
1st line
2nd line
RCC
B CD56dim
p = .008
p > .99 p = .13
p = .01
p > .99 p = .16
p = .15
p > .99 p = .38
Frequency of subgroup (%) [CD3-CD56dim]
100
80
60
40
20
4
2
0
HD
1st line
2nd line
CD16+
CD16bw
CD16-
C CD56bright
p > .99
p = .07 p = .21
p > .99
p = .91 p > .99
p > .99
p > .99 p > .99
Frequency of subgroup (%) [CD3-CD56bright]
100
80
60
40
20
0
HD
1st line
2nd line
CD16+
CD16bw
CD16-


A
1st line patients
NKG2D
NKp46
CD3ζ
p > .99
p > .99
p > .99
p = .07
p > .99
p = .40
p > .99
p = .46
p > .99
Positive cells (%) [CD3-CD56+]
100
80
60
40
20
0
HD
1st line STS
RCC
B
2nd line patients
NKG2D
NKp46
CD3ζ
p > .99
p = .04
p = .30
p = .10
p > .99
p = .09
p > .99
p = .003
p = .004
Positive cells (%) [CD3-CD56+]
HD
2nd line STS
RCC

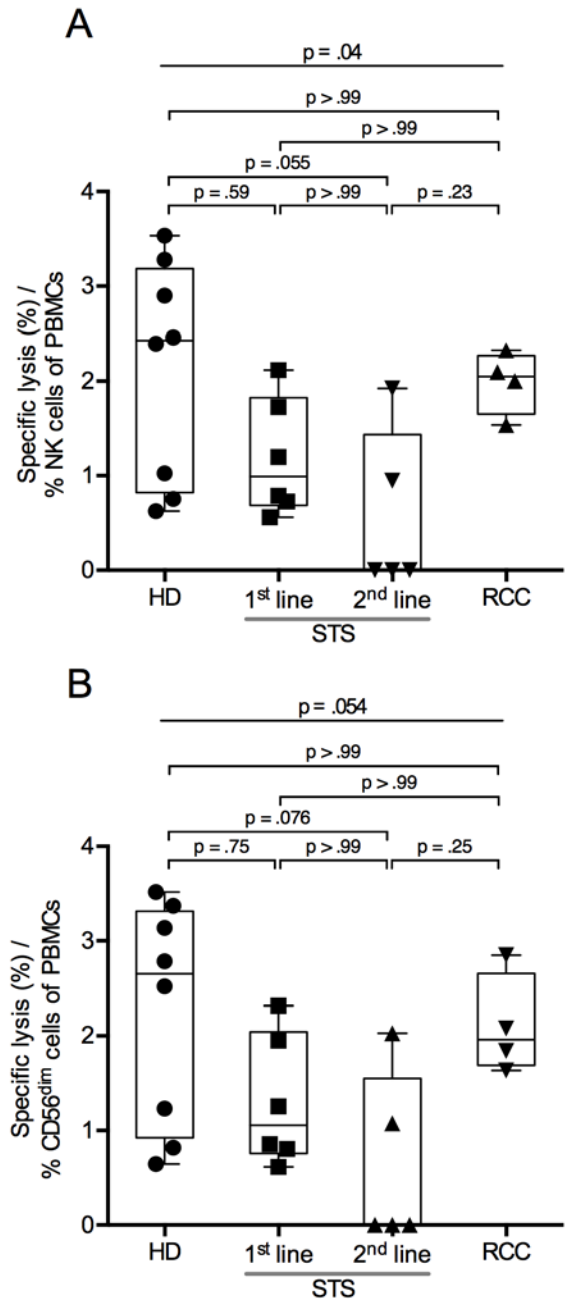

A
1st line patients
Perforin
Granzyme B
CD57
p > .99
p > .99
p > .99
p = .10
p > .99
p = .17
p = .42
p > .99
p = .31
Positive cells (%) [CD3-CD56+]
HD
1st line STS
RCC
B
2nd line patients
Perforin
Granzyme B
CD57
p > .99
p = .003
p = .02
p = .23
p > .99
p = .50
p > .99
p = .006
p = .02
Positive cells (%) [CD3-CD56+]
HD
2nd line STS
RCC



Suppl. Fig. 1 NK cell-specific cytotoxicity of PBMCs of 2nd line STS patients is independent of the time interval since last chemotherapy, but decreases with disease progression. (A) NK-specific cytotoxicity of PBMCs of 2nd line STS patients with a time interval of ≤ 5 months (n=6) and > 5 months (n=5) since last chemotherapeutic treatment. The red triangle marks the NK cell-specific lysis of a patient with very late relapse of clear cell sarcoma almost 18 years after initial treatment (see discussion). **(B)** NK-specific cytotoxicity of PBMCs of 2nd line STS patients with metastatic (n=7) and non-metastatic (n=4) disease at time of blood withdrawal. **(C)** NK-specific cytotoxicity of PBMCs of non-metastatic 1st line STS patients (n=13) and non-metastatic 2nd line STS patients (n=4). **(A-C)** Cytotoxicity was assessed against radiolabeled K562 target cells using the 4h ^{51}Cr release assay. PBMC/K562 ratio was 20:1 for all experiments. Box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample. For statistical analysis, Mann-Whitney-U test was used.



Suppl. Fig. 2 **(A)** NK cell identification by gating $CD3^{-}CD56^{+}$ cells among live single PBMCs. **(B)** NK cell subset identification exemplified by representative dot plots of HD, 1st, 2nd line STS and RCC patients.



Suppl. Fig. 1 NK-specific cytotoxicity, normalized to the percentage of NK cells among PBMCs, differs significantly between HD and STS patients. (A) NK-specific cytotoxicity of PBMCs of healthy donors (HD, n=8), 1st line STS patients (n=6), 2nd line STS patients (n=5) and RCC patients (n=4) against radiolabeled K562 target cells was assessed by 4h ^{51}Cr release assay and normalized to the percentage of $CD3^{-}CD56^{+}$ NK cells among PBMCs (as assessed by polychromatic flow cytometry). **(B)** NK-specific cytotoxicity of PBMCs of healthy donors (HD, n=8),

1st line STS patients (n=6), 2nd line STS patients (n=5) and RCC patients (n=4) against radiolabeled K562 target cells was assessed by 4h ^{51}Cr release assay and normalized to the percentage of $CD3^-CD56^{dim}$ NK cells among PBMCs (as assessed by polychromatic flow cytometry). **(A, B)** PBMC/K562 ratio was 20:1 for all experiments. Box plots represent the median, .75 and .25 percentiles, with whiskers showing minimum and maximum values. Each symbol corresponds to one sample. For statistical analysis, Kruskal-Wallis with Dunn's post hoc test was used. The uppermost p value represents the result of the Kruskal-Wallis test, whereas the p values below (with bracketed lines) represent results of the post hoc tests.