Functional immunological parameters associated with long-term survival of patients with metastatic melanoma

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Manuscript 2: Weide B, **Zelba H**, Derhovanessian E, Pflugfelder A, Eigentler TK, Di Giacomo AM, *et al*. Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis. J Clin Oncol. 2012; 1835-41.

Manuscript 3: **Zelba H**, Weide B, Derhovanessian E, Pflugfelder A, Eigentler TK, *et al*. Phenotypic and Functional analysis of Melan-A and NY-ESO-1-reactive T cells and their influence on survival of late-stage melanoma patients *in preparation*

Contribution of author

Manuscript 1:

- Collection and assembly of immunological data
- Data analysis
- Final approval of manuscript

Manuscript 2:

- Conception and design
- Collection and assembly of immunological data
- Data analysis and interpretation
- Manuscript writing
- Final approval of manuscript

Manuscript 3:

- Conception and design
- Collection and assembly of immunological data
- Data analysis and interpretation
- Manuscript writing
- Final approval of manuscript

Introduction

Melanoma, the malignant tumour of the melanocytes, is one of the most rapidly increasing types of cancer worldwide (Ferlay 2007; Ferlay 2010). If detected at an early stage (stage I and II; see Figure 1.), surgical removal of visible tumours can be curative. In the case of progression and metastatic spread (stage III and IV), which occurs compared to other types of cancer rather quickly, melanoma becomes rapidly fatal.

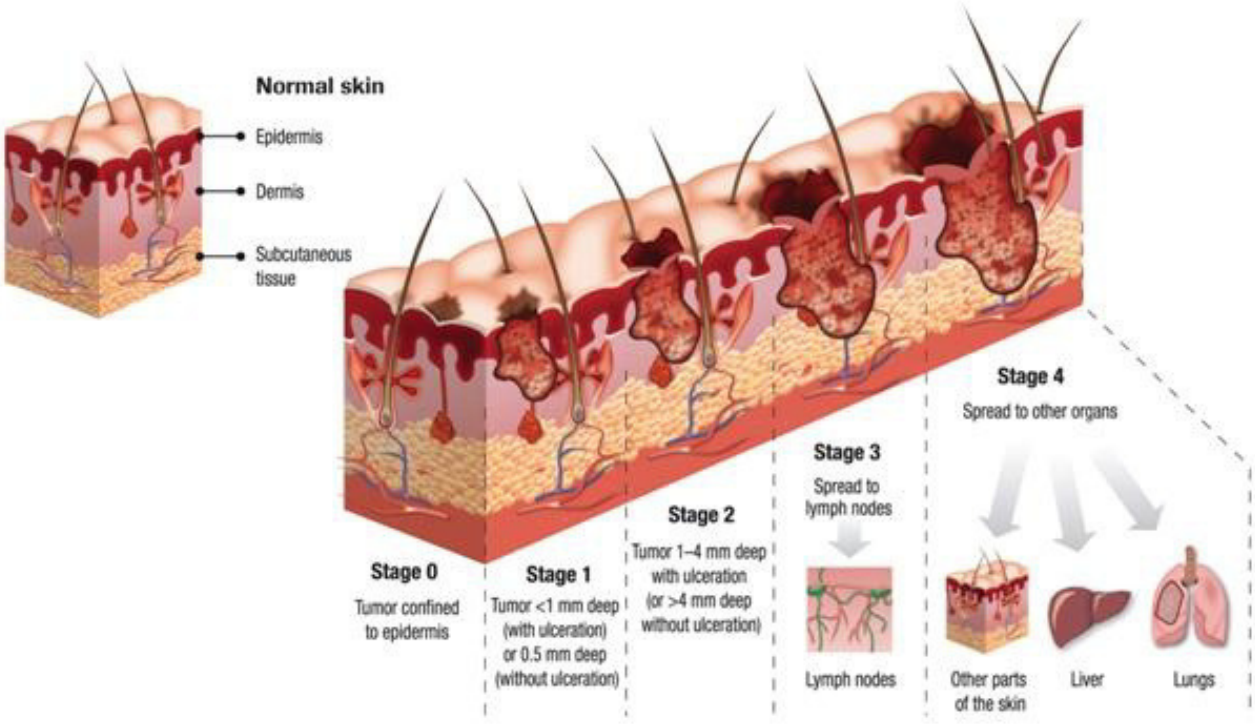

Figure 1: As soon as transformed melanocytes start to grow out of control, the first stage of melanoma has begun. If the tumour is more than 1 mm thick, the patient has stage II melanoma. In stage III melanoma, the thickness of the tumour is no longer relevant to the grading: the tumour has started to metastasise into local lymph nodes and vessels. Finally, as soon as the cancer has spread to other parts of the body (for example lung, liver and brain) stage IV is reached. Source: www.melanoma.org.nz

The median survival time of patients in advanced stages is 9 months and the 5 year survival rate is less than 20% (Neuman 2008). Conventional treatments like chemotherapy and radiotherapy remain the first choice, despite poor clinical efficacy resulting in a very poor prognosis (Mitchell 2004; Neuman 2008). Due to this modest success rate, other treatment modalities are urgently required, and immunotherapy is

increasingly being applied for melanoma management over the last decade (Zeiser 2012). Many immunotherapy trials targeting melanoma aim at two main goals: (i) generate or otherwise release from inhibition, a sufficient amount (generated either *in vivo* or *in vitro*) of activated anti-tumour **T cells specific for tumour-associated antigens (TAAs)** expressed by that individual patient´s tumour and furthermore (ii) these T cells must be in a position to circumvent tumours' **immune escape mechanisms**.**Tumour-associated antigens** are exclusively expressed by or over-expressed by transformed cells. Since the discovery of the first human TAA by van den Bruggen *et al.* (van der Bruggen 1991) much progress has been made in the search for possible TAAs, resulting in an ever-increasing list of tumour antigens, classified in different categories. Many of these antigens have been discovered on oncogenic melanocytes, making melanoma an appropriate model disorder for cancer immunotherapy.

A well-known antigen that is **overexpressed** in many tumours including melanoma is the anti-apoptotic protein **survivin**. This protein, 142 amino acids long, was first described in 1997 by Ambrosini and colleagues (Ambrosini 1997). The anti-apoptotic function of survivin seems to be achieved by a blockade of the activation of the two effector caspases 3 and 7 by a direct interaction (Tamm 1998) making cells expressing survivin unsusceptible for apoptosis. Recent findings suggest that survivin is involved in promoting metastatic spread (McKenzie 2012) which makes it a suitable target antigen to treat late-stage melanoma. **Differentiation antigens** are only expressed by tumour cells and normal cells from the same lineage. Most of the antigens of this type were identified in melanoma patients, where only malignant and healthy melanocytes expressed the antigen. Examples of such differentiation antigens are glycoprotein 100 (gp100) and melanocyte antigen (**Melan-A**) / melanoma antigen recognised by T cells (MART-1). Melan-A was discovered independently by two working groups in 1994 (Coulie 1994; Kawakami 1994). It is a 118-amino-acid-long protein with a single transmembrane domain which can only be found on melanocytes or on tumour cells which arise from them, making it an ideal target for melanoma immunotherapy (Romero 2002). The most targeted antigens for immunotherapy in general are the **cancer/testis antigens** (Caballero 2009). Aside from numerous cancer cells, they are exclusively expressed in immune-privileged tissue like testicular germ cells or placental trophoblasts. As germline cells lack MHC, therapies targeting cancer/testis antigens should only affect tumour cells and should avoid any autoimmune reactions. A prominent antigen of this category is melanoma-associated antigen 3 (**MAGEA3**), which was discovered on melanoma but is also expressed by many other tumours at the mRNA and protein level. MAGEA3, a member of the MAGEA family, is 314 amino acids long and was first described in 1994 (Gaugler 1994). Its function is not completely understood, but MAGEA proteins seem to interact with and block p53. Its level of expression is associated with prognosis, making MAGEA3 another appropriate target for cancer immunotherapy (Peled 2009). Another cancer/testis antigen that recently became the focus of

attention in cancer immunotherapy is New York esophageal squamous cell carcinoma 1 (**NY-ESO-1**), a 180-amino-acid-long protein of unknown function first described in 1997 (Chen 1997). In healthy individuals it is predominantly expressed in spermatogonia, the self-renewing stem cell population of germ cells in adult testis (Caballero 2009). It is expressed by many cancer types, including melanoma, where it is expressed in about 40% of patients. NY-ESO-1 is reported to be very immunogenic, hence, cellular responses in the peripheral blood and antibodies against NY-ESO-1 are frequently found, especially in late-stage melanoma patients. However, within the tumour, only a small number of cells express this antigen and the expression level can change with time. Its increasing relevance to immunotherapy was recently reviewed in detail by Cebon *et al.* (Cebon 2010).

Not all types of TAAs are presented by the thymic epithelial cells or are only presented to a small extent. For that reason, high avidity naïve T cells that are capable of recognising these antigens might not always be deleted by negative selection and should generally be present in the organism. The generation of antigen-specific T cells in an immunological relevant amount should therefore generally be possible.

(i) Tumor-specific T cells

The increase in the number of TAA-reactive cells can be approached by active vaccination by administration of antigen-loaded APCs or in other ways, and by adoptive immunotherapy using T-cell transfer. For active vaccination, DNA (Wolchok 2007) or RNA (Weide 2009) encoding the desired antigen, as well as peptides or the whole protein can be administered (Caballero 2009). The injected substance should be taken up by DCs, processed and presented to T cells in an immunogenic form. The same should happen if already-loaded DCs are injected into the patient (Lesterhuis 2008). Antigen-specific naïve or memory T cells must recognise their antigen and start clonal expansion and differentiation into effector T cells. These cells, as well as T cells administered by adoptive transfer (usually of autologous antigen-specific T cells first propagated *in vitro* and then reinfused into the patient (Rosenberg 2004)), should then attack those tumour cells that express the specific antigen(s).

However, most clinical trials this far have not taken into account whether or not targeting certain antigens rather than others is more likely to result in a measurable clinical benefit. To take this into consideration, Cheever *et al.* screened the literature in order to prioritise TAAs for immunotherapy. According to parameters like immunogenicity and therapeutic effect, well-known TAAs were listed according to which antigens should be preferentially targeted (Cheever 2009). But even if the most promising TAAs are targeted, a clinical response will only be noted if the patients' tumours actually express these TAAs. Some trials therefore analyse the expression

pattern of clinically removed tumours for each participating patient before therapy. But this approach has various disadvantages. It is very sophisticated, it cannot be applied to every patient but only to those with removable lesions and it is not known whether or not all metastases do constantly express the same antigens. One possibility to circumvent these drawbacks is the administration of a "**danger signal**" instead of a vaccination with a certain antigen. This signal, administered into the tumour, should attract immune cells to the site of injection and tumour degradation, enhance immune responses against antigens actually expressed by the tumour and lead to measureable tumour shrinkage optimally associated with prolonged survival. These clinical observations might also be associated with a detectable increase of antigen-specific T cells in the peripheral blood (as with those "Coley´s Toxins" successes). But not all melanoma patients, much less all cancer patients, can be treated with this method, as it requires visible tumours. Furthermore, none of these approaches consider that the tumour may still be able to inhibit the immune cells, even if they are highly antigen-specific and present in large numbers.

(ii) Tumour escape mechanisms

If we posit that the immune system can react to and control cancer ("immunosurveillance of cancer"), then most tumours must be definition have gained the ability to escape such immune surveillance (reviewed in detail by Campoli and Ferrone (Campoli 2008)). Multiple mechanisms contributing to tumour escape have been described over the years. In some tumours mutations within the cancer cells led to the downregulation of the major histocompatibility complex (MHC), resulting in a reduced or absent presentation of antigens.(Wang 2008) In other cases, the peptides derived from mutated proteins could not be embedded into the MHC molecule anymore, also resulting in an absence of antigen presentation. Production of inhibitory molecules, like cytokines Interleukin-10 (IL-10) or transforming growth factor β (TGF-β) which directly inhibit surrounding immune cells is also commonly observed (Derynck 2001; Dummer 1996). Tumours also stimulate immunosuppressive leukocyte populations like regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs) to focus their suppressive activity on those immune cells that should normally destroy the neoplastic cell, in the same way that these cells maintain tolerance to other self-tissues (Murdoch 2008; Teng 2011). Other mechanisms include upregulated expression of inhibitory receptors like Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) or Programmed cell death protein 1 (PD-1) by T cells as part of the normal immune control network usurped by tumours and leading to the inhibition of these antigen-specific T cells (Avogadri 2011; Flies 2011).

The circumvention of these latter escape mechanisms may be achievable by depleting the inhibitory cells (for example with agents like cyclophosphamide (Ghiringhelli 2007)), or by blockade of inhibitory molecules (for example with

neutralizing antibodies, here in a mouse model (Hagenbaugh 1997)) or the blockade of inhibitory receptors (for example with masking antibodies (Phan 2003)). Especially those recent clinical trials that targeted CTLA-4 and PD-1/PD-1 L have indeed yielded excellent clinical benefits, probably even cures in some patients (Hodi 2010; Topalian 2012). However, the fraction of patients responding favourably in any of these clinical trials has always been rather small, and many suffer serious side effects without receiving clinical benefit (Chmiel 2011; Minor 2009; Weber 2009). It is therefore of greatest interest to find independent biomarkers that predict a favourable outcome of cancer immunotherapy and provide mechanistic clues as to functional attributes required for maintaining long-term survival. Currently, only the plasma-level of Lactate dehydrogenase (LDH) is an accepted biomarker in many cancers, including melanoma (Balch 2001). Although other serum markers have been investigated for their prognostic power (Deichmann 1999; Tarhini 2009), none of them is widely accepted so far. Predictive markers are mostly absent thus far.

Aims of this thesis

Treatment modalities for late-stage cancer patients are very limited and, especially in melanoma, immunotherapy is currently under intense investigation. Nonetheless, clinical benefit has only be observed in rare cases. It is therefore of greatest interest to find (a) highly effective and safe therapies that can easily applied to a large proportion of melanoma patients; (b) those target antigens that lead to the best clinical results and (c) predictive markers for successful treatment. The selection of the best target-antigens and the treatment of only those patients bearing certain markers associated with a good course of disease might reduce the number of unsuccessful therapies within one trial to a minimum.

For this purpose, I first investigated the immunological impacts of intratumoural injected IL-2 for late-stage melanoma patients. Patients participating in this phase 2 trial received the cytokine as a danger signal in order to increase anti-tumour immune responses. I analysed whether or not the patients showed any systemic treatment-based increase of tumour-antigen-specific T cells in the peripheral blood. For that purpose, I assayed functional antigen-reactive T cells in the circulating blood using an intracellular cytokine staining (ICS) and flow cytometric analysis. In order to detect antigen responses in patients of any HLA type on the one hand and measure CD4 and CD8 responses at the same time on the other, I used overlapping peptide pools, representing the whole antigen for expansion and restimulation of specific T cells. The use of polychromatic flow cytometry, in contrast to commonly-used methods to detect antigen-reactive T cells like ELISPOT or tetramers, allowed me additionally to analyse multiple parameters of the phenotype and function of the specific T cell on a single cell basis.

Furthermore, I tried to identify biomarkers, which can independently predict the clinical outcome of an individual patient. Concerning melanoma, accumulating data suggested that the presence of certain antigen-reactive T cells within the tumour might be associated with a good clinical outcome, although this has never been observed for circulating cells from peripheral blood (Aarntzen 2010; Haanen 2006). I therefore sought T cells, specific for different melanoma-associated antigens, in the peripheral blood of stage IV melanoma patients. Patients with and without specific cells for each tested antigen, were clustered and analysed separately for survival. Additionally, I analysed the phenotype and function of each single antigen-specific T cell and investigated their possible impact on survival.

Results overview and discussion

Intratumoural treatment with Interleukin-2 (Manuscript 1)

51 stage III and IV melanoma patients were treated with IL-2, however, blood samples were unfortunately only available for one single patient with an exceptional good course of disease. I tested T-cell reactivity against four melanoma-associated antigens NY-ESO-1, Melan-A, MAGEA3 and survivin at the three different timepoints (before, during and after therapy).

The injection of IL-2 into the tumour was well tolerated and highly effective. The therapy led to a complete local response of all treated lesions in 69% of patients and resulted in a 2 year survival rate of 77% for stage III and 53% for stage IV patients. Interestingly, even after an intratumoural treatment, an increase of functional antigen-reactive T cells was observed in the peripheral blood, at least the one analysed stage III patient. This increase of antigen-reactive T cells was accompanied with an ongoing complete response, hinting at a possible systemic vaccination effect, which was also reported by others (Neville 2001). In another patient of this trial, vitiligo-like depigmentation as a possible sign of a specific immune response against differentiation antigens was observed. Furthermore, the possible systemic effect of a local application of IL-2 was also observed in preclinical trials, where the treatment led to the regression of untreated tumours and the generation of specific immunity (Van Es 2000).

Early studies of systemic IL-2 treatments with long-term follow-up already documented that immunotherapy can be extremely effective in a small minority even in advanced melanoma patients (Atkins 1999). Although the significance of the results presented in the Tübingen patient is limited and they do not allow the drawing of strict conclusions, these data are additional hints for the crucial role of antigen-reactive T cells, even for unspecific treatments like local IL-2 application. Although the results of this trial are very promising concerning complete responses and compatibility, it is still not an optimal treatment, as only visible lesions can be targeted. An improved approach to this trial, which was performed by the Department of Dermatology in Tübingen, used a fusion protein called L19-IL2, consisting of human IL-2 and an antibody fragment directed against the ED-B domain of fibronectin. This marker of angiogenesis is expressed in newly formed blood vessels, thus in cancer patients mainly at the tumour site. After being administered to the patient, the L19-IL2 will be distributed through the bloodstream and attach to newly-formed blood vessels, and should reveal the therapeutic effect of IL-2. This approach will theoretically target both visible and non-visible lesions in the patient.. The enrolment of the patients started in June 2010 and ended in October 2012. In total, 21 patients were recruited and we collected and cryopreserved PBMC samples from up to five different time points. It is planned to assay antigen-reactive T cells specific

for different melanoma-associated antigens at each time point. Furthermore we plan to investigate the role of certain immune cell types (naïve and memory T cells, dendritic cells, regulatory T cells, myeloid-derived suppressor cells etc.) and their potential influence on survival. Whether this approach will confirm the results from the first trial remains to be seen.

Functional Specific T Cells in Stage IV Melanoma (Manuscript 2)

In order to investigate the potential of antigen-specific T cells to predict the clinical outcome of end-stage melanoma patients, I assessed T cell responses against NY-ESO-1, Melan-A, MAGEA3 and survivin in 84 patients with unresectable distant metastases and available clinical follow-up after T-cell analysis. Circulating antigen-reactive T cells targeting TAAs were found to have indeed an important impact on survival. Univariate analysis showed that NY-ESO-1 ($p=0.001$) and Melan-A reactive T cells ($p=0.011$) both contributed to prognosis, and had an even stronger impact on survival than the M category ($p=0.024$) according to the American Joint Committee on Cancer (AJCC) staging system. Interestingly, therapies both prior and subsequent to blood draw did not have an impact on prolonged survival, clearly confirming the poor treatment options for late-stage melanoma patients. Patients with detectable MAGEA3-responsive T cells had a better course of disease than those without, but the observed survival differences were rather small and only just reached statistical significance ($p=0.042$). The presence of survivin-reactive T cells did not have an impact on survival ($p=0.374$). Finally, Multivariate Cox proportional hazards analysis revealed that only the presence of NY-ESO-1 ($p=0.001$) and Melan-A reactive T cells ($p<0.001$) were independently associated with a survival benefit. The median survival time (MST) for patients with NY-ESO-1 and/or Melan-A-reactive T cells was 21 months, compared to 6 months for patients that did not possess these cells.

A possible association between the presence of these cells and clinical benefit was also observed by others, albeit only in individual cases. It was reported that 6 of 8 melanoma patients with impressive clinical results after anti-CTLA-4 treatment possessed NY-ESO-1-specific T cells (Yuan 2008). Another patient that was also treated with anti-CTLA-4 antibody, showed a durable complete response in all metastases, accompanied by a massive increase of Melan-A-specific T cells (Klein 2009). Robbins *et al.* could reduce tumour mass in 5 of 11 melanoma patients by the adoptive transfer of genetically engineered T-cells, reactive to NY-ESO-1 (Robbins 2011).

Taken together, I confirmed the important role of NY-ESO-1 and Melan-A as target antigens of first choice for melanoma immunotherapy. The presence of these cells had an impact on survival superior to the AJCC M category and the applied therapy. Approaches that aim to generate or increase the amount of these specific cells should therefore be most probable to succeed. Furthermore, the absence of these cells might serve as a prognostic marker for stage IV melanoma.

Phenotype and Function of the antigen-specific T cell (Manuscript 3)

As shown in Manuscript 2, the presence of NY-ESO-1 and Melan-A reactive T cells has an independent impact on survival. As a next step, I wanted to analyse in detail the phenotype and function of each antigen-reactive T cell population. ICS and multi-colour flow cytometry revealed that both tested antigens were recognised predominantly by a certain subset, either CD4- or CD8-positive. When PBMCs were stimulated with overlapping peptides representing the whole NY-ESO-1 protein, it was mainly a CD4+ T-cell response that was triggered. 64% of all patients with NY-ESO-1-specific T cells showed a pure CD4 response, 8% a pure CD8 response and 28% both. On the other hand, Melan-A was predominantly recognized by CD8+ T cells. 59% of all patients with Melan-A-specific T cells showed a pure CD8 response, 14% a pure CD4 response and 27% both. Furthermore, responses against these antigens led to slightly different cytokine patterns that mainly affected IL-2 secretion, which was more often released by NY-ESO-1-specific T cells.

Interestingly, at least for Melan-A, both the phenotype and the function were associated with survival differences. Patients that possessed CD4+ Melan-A-reactive T cells showed a MST of 112 days, compared to 824 days for patients with CD8 T cell responses but without CD4 cell reactivity ($p=0.0040$). Concerning functional aspects of responses upon Melan-A stimulation, I observed that patients with specific T cells producing anti-inflammatory cytokines, had a markedly worse survival (MST: 83 days) compared to patients whose T cells produced only pro-inflammatory cytokines (MST: 1976 days; $p=0.0046$). Both findings might probably reflect the activation of Melan-A-specific regulatory T cells, whose increasing role in melanoma management was recently reviewed in detail by Jacobs *et al.* (Jacobs 2012). Interestingly, a positive impact on survival mediated only by CD8+ Melan-A specific T cells was also observed by Khammari *et al.*, where 7 of 14 melanoma patients treated with Melan-A-specific CTL clones, showed an objective response (complete and partial response or stable disease)(Khammari 2009).

Both findings might have strong impact on the success of upcoming immunotherapeutic trials. Approaches, that aim to increase the amount of a certain cell type, for example CD8+ NY-ESO-1-specific T cells, might be more likely to fail. Vaccination with predicted MHC class-I epitopes, in order to increase the number of CD8+ cytotoxic T cells might not lead to as promising results as targeting CD4+ T cells could for this target antigen. Furthermore, many monitoring projects are aimed at detecting NY-ESO-1-specific T cells with multimers bearing MHC-I restricted epitopes, especially after systemic treatment like chemotherapy or application of cytokines. However, the presence of CD8+ NY-ESO-1-specific T cells may not be particularly informative. This might be one of the reasons for the deficiency of data showing correlations between clinical outcome and immunological findings.

For Melan-A, targeting the CD4+ T cells might even lead to worse survival. Many clinical trials, especially using DNA, RNA or whole protein vaccination, lead to both Melan-A-specific CD4 and CD8 responses, which according to the results presented here, may be counterproductive. I further observed that the function, here the release of certain cytokines, also had a strong impact on survival, at least for Melan-A. Patients whose T cells produced anti-inflammatory cytokines (IL-10 and/or IL-4) upon Melan-stimulation, even if they also produced pro-inflammatory cytokines, showed a dramatically reduced survival. Vaccination trials which also induce anti-inflammatory responses are, based on our results, unlikely to succeed. Hence, adoptive T-cell transfer of CD8+ Melan-A-specific T cells, preselected for mediating a solely pro-inflammatory response might be the most promising immunotherapeutic approach for melanoma.

Additionally, I also observed that only about 70% of all patients (69% for NY-ESO-1 and 64% for Melan-A) responded upon antigen stimulation with the release of IFN-γ. Commonly applied methods to detect antigen-specific T cells, like ELISPOT, that are usually restricted to the detection of this one cytokine, might therefore miss a considerable proportion of specific cells.

Concluding remarks

Today, immunotherapy is a frequently used modality for treatment of advanced melanoma, although clinical benefits are only infrequently observed. Within this thesis I aimed at contributing data that might help to increase therapeutic success by finding promising treatment options, target antigens and predictive markers. In order to detect both CD4+ and CD8+ T cells, specific for various melanoma-associated antigens, I established a reliable method that can easily applied to patients of any HLA type. First applied in a study of local intratumoural application of IL-2 which demonstrated that this was a safe, easy applicable and most notably clinically successful method to treat melanoma, I observed that tumour-shrinkage was accompanied by an increase of peripheral antigen-specific T cells, at least in one patient. Although it can only be applied to patients with visible lesions, the obtained results are a rationale to further investigate and improve local IL-2-based therapies.

I could confirm the encouraging role of NY-ESO-1 and Melan-A as target antigens of first choice for immunotherapy in a large cohort of stage IV melanoma patients. Interestingly, I observed that each of the four investigated antigens was preferentially recognized by a certain cell type. Furthermore, I discovered that phenotype and function of Melan-A-specific T cells have a huge impact on survival. Both findings would have remained unobserved with other methods to detect antigen-specific T cells.

Most of these results were obtained from a homogeneous cohort of stage IV melanoma patients. Needless to say, the insights gained must be investigated in other melanoma patients, especially stage-III patients, as well. Late-stage melanoma patients have an extremely low life expectancy and the observed survival benefits for certain individuals might be even more significant in stage-III patients. Additionally, these data have to be investigated for other cancers expressing these two antigens. Especially NY-ESO-1, which is expressed by many other tumours (for example lung, breast and bladder carcinoma (Jungbluth 2001)), might be confirmed to be equally important. Furthermore, there are many other antigens that could play equally important roles for clinical benefit. Proteins that have characteristics (antigen category, level of expression, physicochemical properties etc.) similar to those of NY-ESO-1 and Melan-A might prove to be additional crucial target antigens in cancer immunotherapy.

Taken together, all the new insights obtained within this thesis might explain the lack of data that show correlations between clinical and immunological findings and should help to improve immunomonitoring and above all melanoma immunotherapy.

Manuscripts

High response rate after intratumoral treatment with interleukin-2: Results from a phase 2 study in 51 patients with metastasized melanoma.

Original Article

High Response Rate After Intratumoral Treatment With Interleukin-2

Results From a Phase 2 Study in 51 Patients With Metastasized Melanoma

Benjamin Weide, MD[1]; Evelyna Derhovanessian, PhD[2]; Annette Pflugfelder, MD[1]; Thomas K. Eigentler, MD[1]; Peter Radny, MD[1]; Henning Zelba[2]; Claudia Pföhler, MD[3]; Graham Pawelec, PhD[2]; and Claus Garbe, MD[1]

BACKGROUND: Systemic high-dose interleukin-2 (IL-2) achieved long-term survival in a subset of patients with advanced melanoma. The authors reported previously that intratumorally applied IL-2 induced complete local responses of all metastases in >60% of patients. The objectives of the current study were to confirm those results in a larger cohort and to identify patient or regimen characteristics associated with response. **METHODS:** Patients with melanoma who had a median of 12 injectable metastases received intratumoral IL-2 treatments 3 times weekly until they achieved clinical remission. The initial dose of 3 million international units was escalated, depending on the individual patient's tolerance. **RESULTS:** Forty-eight of 51 patients were evaluable. Only grade 1/2 toxicity was recorded. A complete response that lasted ≥6 months was documented in 70% of all injected metastases. A complete local response of all treated metastases was achieved in 33 patients (69%), including 11 patients who had between 20 and 100 metastases. Response rates were higher for patients who had stage III disease compared with patients who had stage IV disease. No objective responses of distant untreated metastases were observed. The 2-year survival rate was 77% for patients with stage IIIB/IIIC disease and 53% for patients with stage IV disease. Efficacy and survival did not differ between patients who had ≥20 lesions and patients who had <20 lesions. **CONCLUSIONS:** Intratumoral IL-2 treatment elicited complete local responses in a high percentage of patients. Further studies will be required to investigate the mode of action of this treatment and its impact on survival. ***Cancer* 2010;000:000–000.** © *2010 American Cancer Society.*

KEYWORDS: melanoma, in-transit metastases, interleukin-2, intratumoral treatment, in situ vaccination.

Metastatic melanoma has an unfavorable prognosis, and treatment options are limited.[1,2] In patients with stage III melanoma, the objective of treating locoregional metastases is the complete removal of any detectable tumor manifestation. In patients who have many in-transit metastases, surgery often is no longer feasible. In a subset of patients who have disease limited to 1 extremity, isolated limb perfusion can be applied but is associated with considerable toxicity.[3] In patients with stage IV melanoma and for patients who have unresectable metastases, palliative systemic treatments generally are preferred. However, surgical treatment is the first choice in stage IV if the patient can be rendered free of disease at all known metastatic sites; and, in 10% to 20% of patients with stage IV disease, this therapeutic option seems to be associated with a clear survival benefit.[4] Therefore, alternative direct treatment options are needed if disease is not completely resectable.

Interleukin-2 (IL-2) has been used in immunotherapy against cancer since the early 1980s, applied either as a single agent[5] or in combination with adoptively transferred, lymphokine-activated killer cells.[6] Another approach is the adoptive transfer of in vitro expanded, autologous tumor infiltrating lymphocytes[7] followed by high-dose IL-2.[8] A retrospective analysis of 8 trials that used systemic high-dose IL-2 in patients with melanoma demonstrated response rates of 16%, including 6% complete responses. Greater than 50% of the complete responders in those trials remained progression-free after 5 years.[9] These data led to US Food and Drug Administration approval of systemic high-dose IL-2 for the treatment of advanced melanoma in 1998.

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Intratumoral application of drugs is an appealing therapeutic concept, because high concentrations can be achieved within the tumor, which may be essential to attain the desired therapeutic effect. Furthermore, systemic concentrations of locally applied drugs are low in contrast to systemic treatments with the same agent, resulting in comparably low toxicity. Several cytokines have been used for intratumoral therapy with various results.[10-12] The first report that described the regression of melanoma metastases after intratumoral IL-2 treatment was published in 1994.[13] On the basis of those findings, we initiated a pilot study on 24 patients and observed complete responses of all treated metastases in >60%. In contrast to systemic, high-dose therapy, intratumoral application was associated with low toxicity.[14] The objectives of the current study were to confirm our previous results in a larger cohort and to identify relevant patient or regimen characteristics associated with response to treatment and with overall survival.

MATERIALS AND METHODS

Patients

The study protocol (National Clinical Trials [NCT] clinicaltrials.gov identifier NCT00204581) was approved by the local ethics committees, and patients were treated only after written informed consent was obtained. Inclusion criteria comprised age >18 years, histopathologically proven malignant melanoma, the presence of injectable dermal or subcutaneous metastases either in clinical stage III or clinical stage IV, and an expected survival >3 months. Excluded were pregnant or lactating women and patients with severe cardiac disease (New York Heart Association III and IV), alanine and aspartate aminotransferase levels >3 times above the upper limit of normal, creatinine >1.5 times above the upper limit of normal, and concomitant systemic therapy with steroids. Patients who required systemic chemotherapeutic treatment for metastatic disease and patients who had locoregional lymph node metastases were excluded. Previous systemic chemotherapy was allowed.

Study Design and Treatment

The trial had the design of an open, prospective phase 2 study. Patients were recruited at 2 study sites (Tuebingen and Homburg, Germany). For preparation of IL-2, 18 million international units (MIU) of recombinant human IL-2 (Proleukin; Novartis, New York, NY) were dissolved in 6 mL glucose 5% supplemented with 0.2% human serum albumin. IL-2 was injected intratumorally using 30-gauge needles for superficial injections and 27-gauge needles for deep injections, and single doses ranged between 0.3 MIU and 6 MIU, depending on the lesion size. One injection per lesion was applied for doses per lesion up to 3 MIU, and 2 injections were applied for doses >3 MIU per lesion. Treatment was initiated at 3 MIU IL-2 daily, and the dose was escalated by 1.5 MIU each treatment day up to the desired total dose according to the number of lesions and treatment guidelines (Table 1), provided that tolerability was maintained. The total daily dose was divided between all injectable lesions. For deep soft tissue metastases, sonography was used to guide injections. Up to 25 lesions were treated simultaneously; however, if more lesions were present, then they were treated alternately or subsequently. The treatment schedule was 3 times weekly on an outpatient basis. Treatment was terminated when clinical regression and/or necrosis of metastases was evident or if progression occurred that no longer was manageable by ongoing IL-2 injections. Adverse events were graded according to the Common Toxicity Criteria (version 3).

Table 1. Treatment Guidelines

Size of Individual Lesion, mm	Minimal Single Dose of IL-2 per Lesion, MIU	Volume Stock Solution, mL	Duration of Treatment, wk
<2	0.3	0.1	2
<5	0.6	0.2	2
<10	1.2	0.4	3
<20	3.0	1.0	4
≥20	6.0	2.0	4

IL-2 indicates interleukin-2; MIU, million international units.

Response Evaluation

Every treated metastasis was evaluated separately regarding clinical response between 4 weeks and 8 weeks after stopping IL-2 treatment and every 3 months thereafter. The following definitions were used: a complete response (CR) of a treated lesion was defined as the disappearance of any evidence of vital tumor and lack of tumor growth after stopping injections over a period of at least 6 months. A partial response (PR) was defined as a decrease ≥30% in the greatest dimension (longest diameter [LD]). Stable disease (SD) was defined as neither sufficient shrinkage to

qualify for a PR nor sufficient increase to qualify as progressive disease (PD). PD was defined as an increase $\geq$20% increase in the LD of the lesion. Subcutaneous metastases were evaluated by sonography. In nondistinctive cases (eg, if there was residual pigmentation), biopsies were taken for histopathologic confirmation of response.

Statistical Analysis

Statistical analyses were conducted using the SPSS 15.0 software package (SPSS Inc., Chicago, Ill). Follow-up was defined as the time from the first IL-2 injection to the date of last contact or death. For survival analyses, only deaths that were caused by melanoma were considered. The last survival update was done for all patients in April 2009. Kaplan-Meier analyses were performed to estimate overall survival. Differences in response rates were calculated by using a 2-tailed Fisher exact test.

Analysis of Specific Cellular Immune Responses

For 1 patient, the frequency of antimelanoma T cells was measured by flow cytometric analysis at different time points. Briefly, peripheral blood mononuclear cells (PBMCs) were incubated for 11 days in X-Vivo 15 (Lonza Verviers, Braine-l'Alleud, Belgium) with mixtures of overlapping peptides (PepMix; JPT Technologies, Berlin, Germany) that spanned the whole sequence of Melan-A, cancer-testis antigen (NY-ESO1), melanoma-associated antigen 3 (MAGE-A3), Survivin, and membrane protein M1/nucleocapsid protein from influenza virus, each at a concentration of 1 μg/mL. On Day 4, IL-2 was added (40 U/mL; Chiron Behring GmbH, Marburg, Germany). On Day 11, autologous PBMCs as stimulator cells were labeled with 5 μM carboxy fluorescein succinyl ester (CFSE) (Invitrogen, Karlsruhe, Germany) and incubated for 6 hours with each peptide mixture mentioned above. Cultured T cells were harvested and incubated at a 1:2 ratio with unpulsed or peptide-pulsed stimulator cells in the presence of 1 μL/mL Golgi-Plug (BD Biosciences, Mississauga, Ontario, Canada) for 12 hours. Thereafter, the cells were incubated with ethidium monoazide (EMA) (Invitrogen) followed by fixation and permeabilization with CytoFix/CytoPerm (BD Biosciences) and staining with cluster of differentiation (CD) 3 antibody (anti-CD3)-Pacific Orange (Caltag; Invitrogen), anti-CD4-peridinin chlorophyll protein complex, and anti-CD8-allophycocyanin-indocyanine 7 (BD Biosciences). Intracellular IFN-γ staining was done with a phycoerythrin/indocyanine 7-conjugated antibody (BD Biosciences) according to the manufacturer's instructions and was measured immediately using a BD LSR II flow cytometer (BD Biosciences). Flow cytometric data analysis was performed using FlowJo software (Tree Star, San Carlos, Calif) after the exclusion of CFSE-positive stimulator cells and EMA-positive dead cells. IFN-γ secretion was considered antigen-specific only when the frequency of IFN-positive T cells that responded to peptide-pulsed PBMCs was at least 2 times greater than the frequency of IFN secretion in response to the negative control (unpulsed PBMCs).

Table 2. Patient Characteristics

Characteristic	No. of Patients	%
Sex		
Men	21	44
Women	27	56
Stage		
Stage III	33	69
IIIB	17	
IIIC	16	
Stage IV	15	31
M1a	8	
M1b	3	
M1c	4	
Visceral metastases present	5	
Site of treated metastases		
Dermal only	28	58
Subcutaneous only	11	23
Combined	9	19
No. of treated metastases		
<20	33	69
≥20	15	31
Previous therapies		
Surgery in stage III/IV	28	58
Limb perfusion	2	4
Radiotherapy	2	4
Adjuvant interferon alpha	14	29
Systemic chemotherapy	11	23

RESULTS

Patients and Treatments

In total, 51 patients (44 in Tuebingen and 7 in Homburg) were enrolled between August 2003 and November 2007 and completed IL-2 treatment by December 2007. Two patients were excluded after they provided informed consent, because progressive visceral disease that was detected at baseline staging diagnostics required chemotherapy. For 1 patient, neither treatment nor follow-up data were available. The baseline data from the 48 remaining patients are summarized in Table 2. The median patient

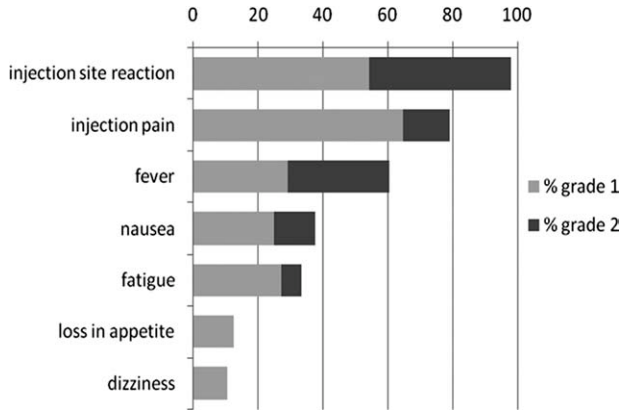

Figure 1. Frequent adverse events are illustrated in 48 patients with metastatic melanoma who received intertumoral interleukin-2.

age was 69 years (range, 37-88 years). Thirty-three patients were treated in stage III, and 15 patients were treated in stage IV. Among the patients with stage IV disease, 5 patients had lymph node/visceral metastases that were not accessible to local IL-2 treatment. Among the patients with stage III disease and the remaining 10 patients with stage IV disease, all metastases were accessible for injections. Most patients had received previous extensive therapy. Twenty-eight patients developed locoregional recurrences after undergoing surgery for metastatic disease. The last surgery occurred $\leq$8 weeks before the start of IL-2 therapy for 14 of 28 patients, and 22 of 28 patients underwent $\geq$2 previous surgeries. In 5 patients, limb perfusion or radiotherapy also was performed. Previous systemic treatments with interferon alpha (IFN-α) (11 patients), chemotherapy (8 patients), or both modalities subsequently (3 patients) had been applied before IL-2 treatment was initiated. The median duration of IL-2 treatment was 6 weeks (range, 1-32 weeks; some patients received several subsequent treatments for newly developing metastases), and the applied median total dose was 68.5 MIU IL-2 (range, 13.5-548.1 MIU). The median number of metastases treated per patient was 12. The highest daily dose was 16 MIU IL-2.

Toxicity

All 48 patients were included in the analysis of toxicity. The treatment generally was tolerated well, and only grade 1 and 2 toxicity was recorded. Intratumoral IL-2 therapy almost always caused an inflammatory injection site reaction (local swelling and erythema) followed by a selective necrosis of the tumor tissue that generally did not affect the surrounding normal tissue. Injection pain also was frequent but was manageable by the application of a local anesthetic cream and oral metamizole. The majority of patients experienced fever (58%) that could be controlled easily by acetaminophen. Fatigue (36%) and nausea (34%) usually were mild and of short duration. Frequent adverse events are presented in Figure 1. Adverse events that were observed in $<$10% of patients but that were at least possibly related to the treatment included stomach pain, myalgia, and headache (in 4 patients each); itching exanthema (in 3 patients); dry oral mucosa (in 2 patients); pruritus (in 2 patients); hair loss (in 1 patient); and diarrhea (in 1 patient). One patient presented with generalized urticaria, which was abated by antihistaminic treatment and did not recur after further IL-2 treatments under continued prophylactic medication. One patient observed worsening of a pre-existing atopic dermatitis, and 1 patient reported a single episode of mild cardiac arrythmia. One patient presented with vitiligo-like depigmentation around the treated metastases (Fig. 2).

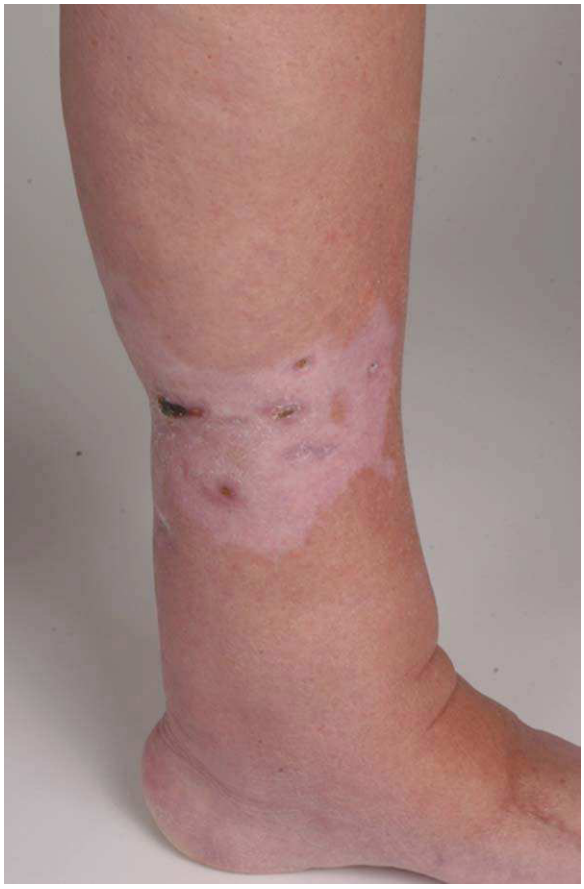

Figure 2. Vitiligo-like depigmentation is observed in an interleukin-2–treated area in a patient after treatment.

Table 3. Treatment Response

	No. of Metastases				Percentage				
	CR	PR	SD	PD	CR	PR	SD	PD	P^a
Response per metastases (n=894)	704	6	146	38	78.7	0.7	16.3	4.3	
Stage III metastases (n=509)	493	3	5	8	96.9	0.6	1	1.6	<.0001
Stage IV metastases (n=385)	211	3	141	30	54.8	0.8	36.6	7.8	
Visceral metastases not present (n=732)	677	4	35	16	92.5	0.5	4.8	2.2	<.0001
Visceral metastases present (n=162)	27	2	111	22	16.7	1.2	68.5	13.6	
Stage III dermal metastases (n=437)	428	3	4	2	97.9	0.7	0.9	0.5	.0034
Stage III subcutaneous metastases (n=72)	65	0	1	6	90.3	0	1.4	8.3	
Stage IV dermal metastases (n=353)	200	0	131	22	56.7	0	37.1	6.2	.0247
Stage IV subcutaneous metastases (n=32)	11	3	10	8	34.4	9.4	31.3	25	

CR indicates complete response; PR, partial response; DS, stable disease; PD, progressive disease.
[a] The calculation of differences between 2 groups of patients was based on the CR rate.

Clinical Responses to IL-2 Treatment

In total, 894 of 917 separately treated metastases (97.5%) could be evaluated for local tumor response (Table 3). The analysis revealed a 78.7% CR rate, a 0.7% PR rate, 16.3% stable metastases, and 4.3% progressive lesions. Differences regarding the rate of complete local responses of injected metastases were detected between stage III versus stage IV (96.9% vs 54.8%, respectively; $P < .0001$) and the absence or presence of visceral metastases (92.5% vs 16.5%, respectively; $P < .0001$). Furthermore, efficacy differed significantly between dermal versus subcutaneous injected lesions both for stage III disease (CR rate: 97.9% vs 90.3%, respectively; $P = .0034$) and stage IV disease (CR rate: 56.7% vs 34.4%, respectively; $P = .0247$). No objective responses of noninjected distant lesions were observed in 5 patients with stage IV disease who had visceral metastases. Figure 3 illustrates the clinical course of 3 patients who received intratumoral IL-2 treatment.

In 33 patients (69%) a complete local response of all treated metastases was achieved with a better outcome for patients who had stage III disease compared with patients who had stage IV disease (82% vs 40%; $P = .0067$). Of these, 32 patients were completely free of recognizable tumor after treatment. The percentage of patients with a complete local response of all treated metastases did not depend on the number of treated lesions (73% for patients who had $\geq$20 treated metastases vs 66% for patients who had <20 treated metastases; $P = .7458$).

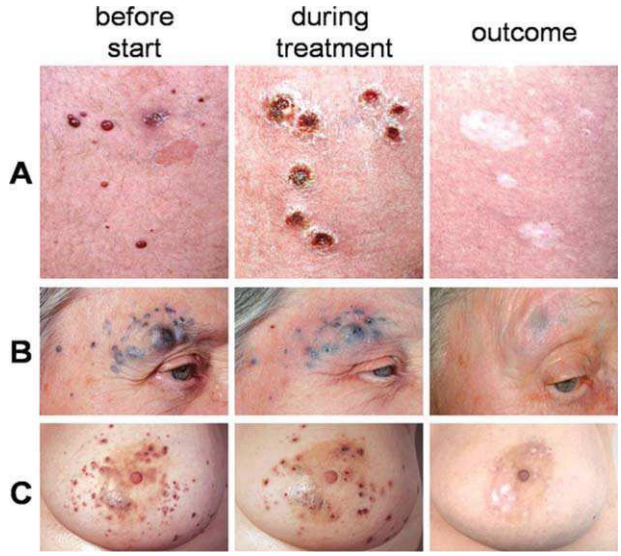

Figure 3. Clinical findings are shown before, during, and after intratumoral treatment with interleukin-2 (IL-2). (A) A man aged 59 years with stage IIIB melanoma had 17 lesions treated and has remained progression free since the start of IL-2 treatment in August 2003. Note the vitiligo-like depigmentation after therapy (right). (B) A woman aged 88 years with stage IIIB melanoma had 25 lesions treated and remained progression-free from the start of IL-2 treatment in December 2004 until her death in January 2009 (not melanoma-related). (C) A woman aged 47 years with stage IV melanoma (M1a) had 44 lesions treated and has remained progression free since June 2006.

Overall Survival

The median follow-up was 25 months from the start of IL-2 treatment (range, 4-68 months; median, 14 months for patients who died of their disease and 32.5 months for all others). Overall survival rates were promising for all patients (Fig. 4A). We did not record any deaths from melanoma later than 25 months after starting treatment, indicating a considerable chance for long-term survival in patients who survived the first 2 years. Patients who had stage III disease, as expected, had higher overall survival rates compared with patients who had stage IV disease (77% vs 53% after 2 years). It is noteworthy that overall survival for the subgroup of patients who had stage IV

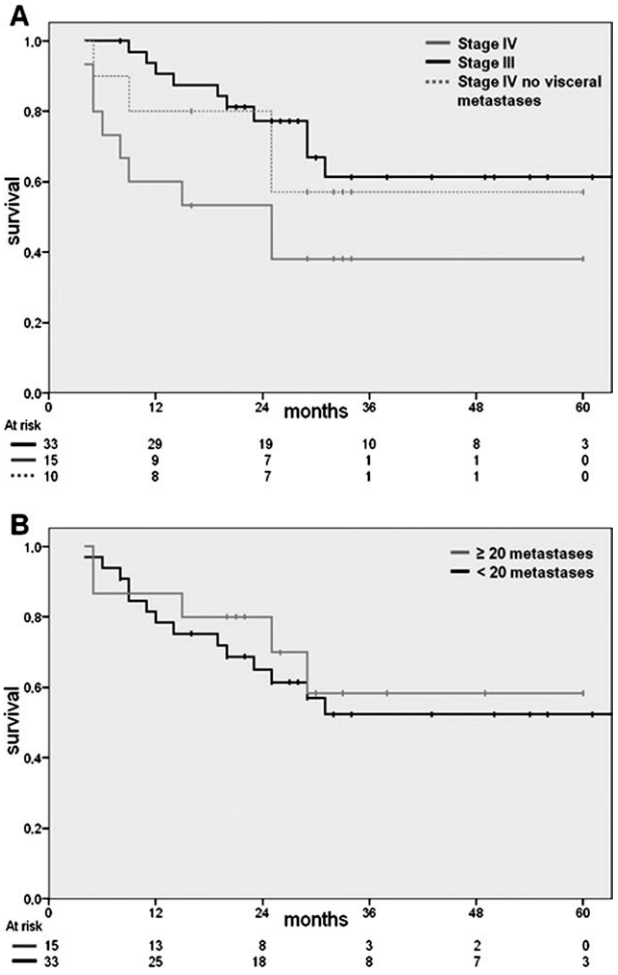

Figure 4. Overall survival was calculated from the start of interleukin-2 treatment. Survival probabilities calculated according to the Kaplan-Meier method are illustrated according to (A) disease stage and (B) the number of metastases at the start of treatment. Short vertical lines represent censored events.

disease without visceral metastases was comparable to that for patients who had stage III disease (Fig. 4A, dotted line). The long-term outcome did not depend on the number of treated metastases, because the overall survival for 15 patients who had $\geq$20 metastases was as good as that for the other 33 patients who had $<$20 metastases (Fig. 4B).

Specific Cellular Immune Responses

For 1 additional patient with stage III disease who was treated in 2008, peripheral blood samples were collected before IL-2 injections, 4 weeks after starting treatment, and 2 months after ending treatment. This woman had 19 dermal metastases, received 11 treatments (cumulative dose 55 MIU IL-2), and had an ongoing complete clinical and histopathologic response after therapy. There was a significant increase (3 times greater than background) in IFN-γ–positive, CD8-positive T cells that were specific for MAGE-A3, Melan-A, and NY-ESO-1 during treatment, and the increased frequency of MAGE-A3–specific, CD8-positive T cells remained present even 2 months after the end of treatment; whereas the frequency of influenza-specific, IFN-γ–positive T cells remained constant at all 3 time points (data not shown). Similarly, there was a marked increase (almost 2 times greater than background) in the frequency of IFN-γ–positive, CD4-positive T cells that were specific for MAGE-A3 during and after therapy, but the frequency of CD4-positive T cells that were specific for other antigens did not change significantly (Fig. 5).

DISCUSSION

Confirming the results from our pilot study in the current, larger trial, we again observed a complete local response of all treated metastases in 69% of patients (82% of patients with stage III disease and 40% of patients with stage IV disease). It is well established that systemic, high-dose IL-2 treatment is effective in a subset of patients with melanoma, and this subgroup appears to represent most patients who have soft tissue metastases. Phan et al reported a 53% response rate to systemic IL-2 treatment in a subgroup of 28 patients with subcutaneous and dermal metastases.[15] Thus, the observed efficacy in our current trial most likely was a result of the high intratumoral IL-2 concentration, which also has been described in animal models after intratumoral treatment.[16]

In contrast to systemic IL-2 treatment accompanied by severe toxicity, intratumoral IL-2 treatment is well tolerated. The good tolerability of this treatment obviously depends on the small amounts applied, with little systemic leakage, and on the application of treatment only 3 times weekly. This is reflected in the complete lack of any premature terminations of treatment because of toxicity in our study.

In the current study, we also tried to identify the patient characteristics that were associated with outcome. We observed that the presence of visceral metastases that could not be injected also was associated with a low local response rate of the injected metastases (CR, 16.7% of metastases; PR, 1.2% of metastases). This is an interesting observation and raises the issue of differences in the immune responsiveness of patients who have visceral metastases. These patients probably should be regarded as ineligible for this treatment. Efficacy also was greater for dermal metastases than for subcutaneous metastases in both stage III and

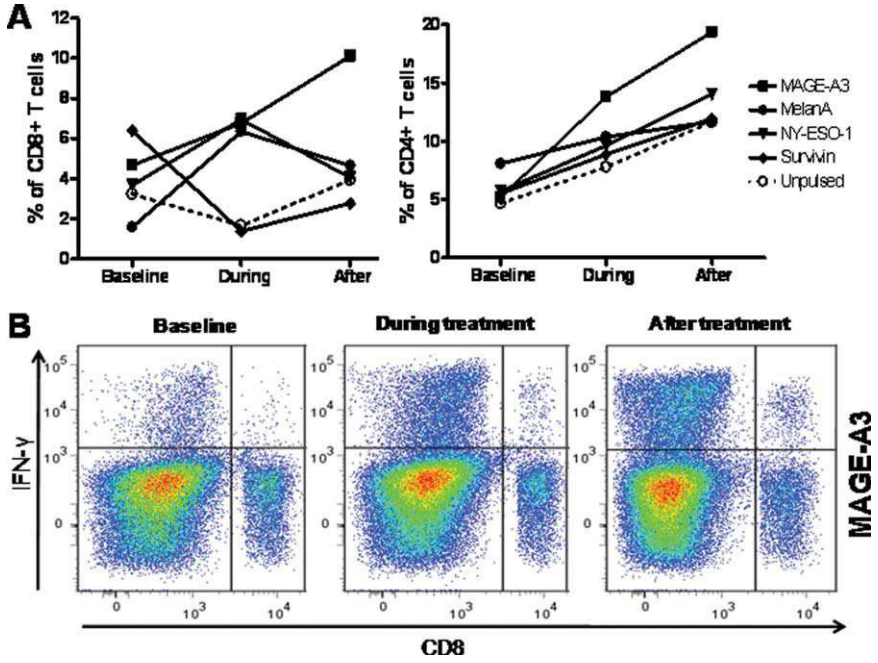

Figure 5. Specific immune responses are illustrated. Circulating interferon-γ (IFN-γ)-positive (IFN-γ+) T cells from 1 patient were analyzed before, during, and after therapy and were measured by flow cytometry. (A) The percentages of MAGE-A3, Melan-A, NY-ESO-1, and Survivin-specific CD8+ T cells (left) and CD4+ T cells (right) are illustrated. Cells were stimulated either with autologous peripheral blood mononuclear cells that were pulsed with corresponding peptides or with unpulsed cells as a negative control. (B) Corresponding dot plots of MAGE-A3–specific IFN-γ+ T cells are shown at baseline and before and after treatment.

stage IV disease. The greater size of subcutaneous lesions may help explain this finding, because we generally observed greater treatment efficacy in smaller metastases. Conversely, injections into subcutaneous metastases usually were applied after ultrasound-guided marking of the localization on the skin surface. This occasionally may have led to peritumoral injections instead of intratumoral injections, which are known to be less effective.[17] Another possible explanation may be that the dermis provides a better environment for inflammatory reactions than subcutaneous tissues.

The 2-year overall survival probability for patients who had stage IIIB/IIIC disease was 77% (stage IIIB, 94%; stage IIIC, 61%), and it was 53% for patients who had stage IV disease after starting local IL-2. It is noteworthy that the presence of a large number of metastases was not associated with a poorer long-term outcome than the presence of fewer metastases. In the current study, we treated 13 patients who had ≥20 metastases but no visceral lesions. Remarkably, 11 or those patients have remained free of any residual tumor after stopping IL-2 treatment despite the initially high number of lesions. Seven patients have remained free of any recurrence over the follow-up period, and only 3 patients have died from melanoma to date (data not shown). This subgroup of patients often is regarded as unresectable because of the high number of metastases. Therefore, systemic chemotherapy (with response rates <30% and without any proven benefit on overall survival) otherwise generally would be the only alternative treatment option for such patients.[2]

The exact mode of action of IL-2 is unknown. One hypothesis is that lymphokine-activated killer cells are induced by IL-2 and subsequently destroy the tumor by direct lysis.[6] Indeed, histopathologic evaluation of biopsies taken from IL-2–treated metastases revealed a dense intratumoral and peritumoral lymphocytic infiltrate. We observed that tumor cells were undergoing apoptosis and that the mononuclear infiltrate mainly consisted of CD8-positive, CD4-positive T cells, and (only to a minor extent) natural killer cells.[14] The favorable long-term outcomes that we observed suggest a possible beneficial systemic mode of action induced by the local treatment. Systemic responses after locally applied intratumoral IL-2 have been described to date in various animal models. Maas et al treated lymphoma-bearing mice and reported the regression of distant uninjected lesions after intratumoral treatment. The same IL-2 doses given systemically were far less effective.[18] Van Es et al studied a transplanted rabbit carcinoma model and demonstrated that

peritumoral IL-2 induced complete regressions of untreated contralateral tumors. A second challenge of the cured animals resulted in tumor rejection, suggesting the generation of specific immunity.[19] In accordance with these preclinical observations, we demonstrated vitiligo-like depigmentation in 1 patient as a sign for the induction of a specific immune response directed against differentiation antigens. In another patient, a marked increase in circulating antimelanoma T cells was observed, hinting at a possible systemic vaccination effect.[20] The presented trial was planned as a local treatment option for unresectable patients that did not take into account possible systemic modes of action. We only performed specific immune monitoring in 1 patient. Therefore, the significance of these data are limited, and additional patients need to be analyzed in subsequent trials. We are aware that the design of the current study does not allow us to reach any definite conclusions regarding long-term outcome, because the trial did not include a control group and may have been influenced by a positive selection bias. Moreover, a potential positive systemic effect of locally applied IL-2 is questionable because of the inability of the treatment to induce regression of distant metastases that were not treated directly in 5 of our patients. Nevertheless, our observations strongly support further pursuit of this therapeutic approach in a randomized trial that includes a control (surgery-alone) group.

In conclusion, intratumoral IL-2 treatment elicited complete local responses of all injected lesions in 69% of patients, especially in patients without visceral metastases. A high proportion of patients could be rendered completely free of injected metastases using this approach. A large number of metastases was not associated with a poorer long-term outcome. Therefore, intratumoral IL-2 may be regarded as a promising therapeutic option for a selected subgroup of patients with melanoma and also may be feasible for the treatment of other accessible solid tumor entities. Further studies will be required to investigate the mode of action and the impact on survival of intratumoral treatment with IL-2.

CONFLICT OF INTEREST DISCLOSURES

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Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis.

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Functional T Cells Targeting NY-ESO-1 or Melan-A Are Predictive for Survival of Patients With Distant Melanoma Metastasis

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B.W. and H.Z. contributed equally to this work.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose
To analyze the prognostic relevance of circulating T cells responding to NY-ESO-1, Melan-A, MAGE-3, and survivin in patients with melanoma with distant metastasis.

Patients and Methods
We examined 84 patients with follow-up after analysis (cohort A), 18 long-term survivors with an extraordinarily favorable course of disease before analysis (> 24 months survival after first occurrence of distant metastases; cohort B), and 14 healthy controls. Circulating antigen-reactive T cells were characterized by intracellular cytokine staining after in vitro stimulation.

Results
In cohort A patients, the presence of T cells responding to peptides from NY-ESO-1, Melan-A, or MAGE-3 and the M category according to the American Joint Committee on Cancer classification were significantly associated with survival. T cells responding to NY-ESO-1 and Melan-A (hazard ratios, 0.29 and 0.18, respectively) remained independent prognostic factors in Cox regression analysis and were superior to the M category in predicting outcome. Median survival of patients possessing T cells responding to NY-ESO-1, Melan-A, or both was 21 months, compared with 6 months for all others. NY-ESO-1–responsive T cells were detected in 70% of cohort A patients surviving > 18 months and in 50% of cohort B patients. Melan-A responses were found in 42% and 47% of patients in cohorts A and B, respectively. In contrast, the proportion was only 22% for NY-ESO-1 and 23% for Melan-A in those who died within 6 months.

Conclusion
The presence of circulating T cells responding to Melan-A or NY-ESO-1 had strong independent prognostic impact on survival in advanced melanoma. Our findings support the therapeutic relevance of Melan-A and NY-ESO-1 as targets for immunotherapy.

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INTRODUCTION

The prognosis of patients with melanoma with unresectable distant metastasis is poor, with a median survival of 9 months and 5-year survival of less than 20%.[1] Lactate dehydrogenase (LDH) is the only well-accepted serum biomarker in malignant melanoma. It has been part of the American Joint Committee on Cancer (AJCC) staging system since 2001.[2-4] Other serum markers of melanoma cell origin (eg, S100b, MIA) have been investigated, but none are as yet widely accepted.[5,6] Current peripheral blood biomarkers do not allow the identification of either patients with the worst prognosis or patients who may survive longer term.

In contrast to factors related to or derived from the melanoma cell itself, immune-related biomarkers have rarely been described. Long-term survival even in advanced disease was observed in a subset of patients after treatment with interleukin-2 (IL-2)[7] or ipilimumab.[8] Those agents are believed to act indirectly through the immune system. Immune system–based prognostic markers might therefore be useful in tumor-free patients to indicate the likelihood of long-term survival and may serve as predictive markers for response to immunotherapies.

Thus far, interferon alfa (IFN-α) –induced autoantibodies and pretherapeutic serum cytokine profiles have been described to predict outcome after treatment with IFN-α.[9,10] C-reactive protein, IL-6, and IL-10 concentrations in serum have likewise been reported to serve as prognostic markers, but their specificity seems limited.[11-14] The humoral

immune response against TA90 has been found to be prognostically relevant in patients receiving an allogeneic melanoma vaccine.[15,16] Whether the frequency of circulating regulatory T cells, natural killer cells, myeloid-derived suppressor cells, or others does have prognostic impact has only been analyzed in small cohorts of patients with melanoma, with equivocal results.[17-20]

Specific immunotherapy of solid tumors mainly aims to induce or increase the number of T cells directed against epitopes of tumor antigens; it has been under intense investigation. Although a high frequency of circulating antigen–specific T cells after vaccination has been reported in many trials, correlations with clinical outcome have been notoriously inconclusive.[21-23] On the other hand, a direct impact of these types of cells has been demonstrated by impressive clinical responses in up to 70% of patients with melanoma on adoptive transfer of tumor-infiltrating lymphocytes or T-cell receptor–transduced lymphocytes.[24,25] T cells specific for melanoma-associated antigens have also been detected in the peripheral blood of patients with melanoma without vaccination or other immunotherapies.[26,27] So far, only a single, tetramer-based study has analyzed the correlation of spontaneously occurring circulating specific T cells in patients with melanoma with survival, but it did not apply functional assays and failed to report a prognostic impact.[28]

The aim of the present study was to investigate the prognostic relevance of functional circulating T cells responding to the tumor-associated antigens NY-ESO-1, Melan-A, MAGE-3, and survivin on overall survival of patients with melanoma with distant metastasis.

PATIENTS AND METHODS

Patients

Cryopreserved peripheral blood mononuclear cells (PBMCs) were accessed from the Departments of Dermatology, University Medical Centers of Tübingen and Essen (Tübingen and Essen, Germany), and the Department of Medical Oncology and Immunotherapy, University Hospital of Siena, (Siena, Italy). Peripheral blood lymphocytes were obtained from the Nijmegen Centre for Molecular Life Sciences (Nijmegen, the Netherlands). Additional fresh blood samples were received from the Blood Bank and Department of Dermatology, University Medical Center of Tübingen. PBMCs were immediately isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved until use.

Available biobanked samples from patients with melanoma who fulfilled the following criteria were obtained from the clinical centers: cohort A, patients with unresectable distant metastases at the time of blood draw and available follow-up data after blood draw; cohort B, no radiologic or clinical evidence of disease at blood draw, ≥ 24 months since first diagnosis of distant metastasis, history of visceral metastasis other than lung or elevated LDH (long-term survivors); cohort C, no history of cancer. All patients provided written informed consent for biobanking. This study was approved by the ethics committee in Tübingen (approvals 147/2011BO2 and 432/2011BO2).

Detection of Antigen-Responsive T Cells

T-cell responses against NY-ESO-1, Melan-A, MAGE-3, and survivin were measured as described previously.[29] Briefly, cells were stimulated with protein-spanning overlapping peptides (1 μg/mL; PepMix; JPT Peptide Technologies, Berlin, Germany). After culture for 12 days, T cells were restimulated at a ratio of 1:2 with autologous, carboxyfluorescein succinimidyl ester (CFSE) –stained PBMCs (5 μmol/L CFSE; Invitrogen, Karlsruhe, Germany) either unpulsed (negative control) or presenting one of the antigens in the presence of Golgi-Plug (1 μL/mL; BD Biosciences, Toronto, Ontario, Canada). After 12 hours of coincubation, Fc receptors

Table 1. Patient Characteristics and Results of Survival Analysis Based on Kaplan-Meier Method

Factor	Patients No.	Patients %	Patients % Dead	1-Year Survival Rate* %	1-Year Survival Rate* 95% CI	*P*†
Age, years						.755
< 55	41	48.8	73.2	35.8	20.9 to 50.7	
≥ 55	43	51.2	81.4	50.1	35.0 to 65.2	
Sex						.715
Male	56	66.7	75.0	40.1	27.0 to 53.2	
Female	28	33.3	82.1	48.9	30.1 to 67.7	
M category						.024
M1a or b	22	26.5	68.2	58.7	37.9 to 79.5	
M1c	61	73.5	82.0	36.3	24.0 to 48.6	
NY-ESO-1–responsive T cells						< .001
Present	36	42.9	61.1	63.3	47.4 to 79.2	
Absent	48	57.1	89.6	27.5	14.6 to 40.4	
Melan-A–responsive T cells						.011
Present	22	31.9	63.6	59.1	38.5 to 79.7	
Absent	47	68.1	83.0	37.2	23.1 to 51.3	
Missing	15		80.0	37.5	12.0 to 63.0	
MAGE-3–responsive T cells						.042
Present	38	60.3	71.1	49.3	33.2 to 65.4	
Absent	25	39.7	92.0	28.0	10.4 to 45.6	
Missing	21		71.4	49.5	27.2 to 71.8	
Survivin-responsive T cells						.374
Present	8	15.1	62.5	58.3	21.8 to 94.8	
Absent	45	84.9	80.0	34.4	20.3 to 48.5	
Missing	31		77.4	51.6	34.0 to 69.2	
Prior systemic treatments						
Anti-CTLA4						.218
Yes	9	10.7	44.4	64.8	32.5 to 97.1	
No	75	89.3	81.3	40.2	28.8 to 51.6	
Other immunotherapy						.260
Yes	39	46.4	74.4	49.7	33.6 to 65.8	
No	45	53.6	80.0	37.6	23.5 to 51.7	
Monochemotherapy						.505
Yes	22	26.2	72.7	36.2	14.4 to 58.0	
No	62	73.8	79.0	45.1	32.8 to 57.4	
Polychemotherapy						.802
Yes	7	8.3	71.4	35.7	74.5 to 74.5	
No	77	91.7	77.9	43.5	32.3 to 54.7	
Biochemotherapy						.447
Yes	7	8.3	57.1	53.6	14.2 to 93.0	
No	77	91.7	79.2	42.0	30.8 to 53.2	
Subsequent systemic treatments						
Anti-CTLA4						.421
Yes	18	21.4	61.1	54.5	31.2 to 77.8	
No	66	78.6	81.8	39.9	27.9 to 51.9	
Other immunotherapy						.656
Yes	17	20.2	88.2	47.1	23.4 to 70.8	
No	67	79.8	74.6	42.0	30.0 to 54.0	
Monochemotherapy						.171
Yes	36	42.9	86.1	37.6	21.5 to 53.7	
No	48	57.1	70.8	47.0	32.7 to 61.3	
Polychemotherapy						.164
Yes	19	22.6	89.5	35.1	13.0 to 57.2	
No	65	77.4	73.8	45.4	33.2 to 57.6	
Biochemotherapy						.713
Yes	12	14.3	91.7	66.7	40.0 to 93.4	
No	72	85.7	75.0	39.0	27.4 to 50.6	

*Two- and 3-year survival rates provided in Appendix Table A1, online only.
†*P* values are results of log-rank tests, excluding patient cases with missing values.

were blocked with Gamunex (human immunoglobulin; Bayer, Leverkusen, Germany), and dead cells were labeled with ethidium monoazide (Invitrogen, Karlsruhe, Germany). Cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences) and stained with the following antibodies: CD3/Qdot655 and anti-CD4/Pacific Orange (Invitrogen); CD8/APC-H7, IL-4/APC, and IFN-γ/PE-Cy7 (BD Biosciences); TNF/PE (Miltenyi Biotec, Bergisch Gladbach, Germany); IL-10/Pacific Blue and IL-17/PerCP-Cy5.5 (eBioscience, San Diego, CA); and IL-2/Alexa700 (BioLegend, San Diego, CA). Samples were measured immediately using LSR II and FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). After removal of the duplicates using the forward-scatter area versus forward-scatter height plot, autologous stimulator cells were excluded by gating on the CFSE-negative cells. Next, CD4+ and CD8+ cells were gated within viable CD3+ lymphocytes and analyzed separately for the production of cytokines. For each cytokine, we evaluated the percentage of cytokine-producing cells among all gated T cells in sample one (restimulated with antigen-pulsed PBMCs) and sample two (restimulated with unpulsed PBMCs). For each cytokine, antigen-responsive T cells were defined as present if the stimulation index was ≥ 2 (sample one divided by sample two) and a clearly separate cytokine-producing population distinguishable from the nonproducing cells was present in sample one, as described previously.[30] The patient was defined as having antigen-responsive T cells for the analyzed cytokine if these criteria were met either for CD8+ or CD4+ T cells. The interpretation of fluorescence-activated cell sorting data and the response evaluation were performed according to established criteria for intracellular cytokine staining, as described previously.[29] All experiments were performed and analyzed centrally by one investigator (H.Z.). To ensure the quality of samples and the functional capacity of T cells, the cytokine response after stimulation by influenza matrix protein 1 and nucleocapsid protein (1 μg/mL; PepMix; JPT Peptide Technologies) was assessed as for the tumor-associated antigens. Only patients with detectable influenza-responsive T cells were further analyzed. Assay reproducibility was assessed in 61 additional independent experiments performed exclusively in patients participating in this study. The initial result (detection of antigen-responsive T cells, yes or no) was confirmed in 51 of these assays (84%).

Statistics

The presence of antigen-responsive T cells was analyzed separately for NY-ESO-1, MAGE-3, Melan-A, and survivin. Additional prognostic factors considered were age (dichotomized using the median of the distribution), sex, AJCC M category (M1a or b *v* M1c), and systemic treatments applied before and after blood draw. Therapies were aligned to the following categories: treatment with anti-CTLA4 antibody, immunotherapy other than anti-CTLA4 antibody, monochemotherapy, polychemotherapy, and biochemotherapy. Follow-up time was defined from the date when blood was drawn for T-cell analysis to the date of last follow-up or death. Disease-specific survival probabilities were calculated, and only deaths resulting from melanoma were considered, whereas deaths resulting from other causes were regarded as censored events. Estimates of cumulative survival probabilities according to the Kaplan-Meier method were described together with 95% CIs and compared using log-rank tests. Median survival times (MSTs) are presented. Multivariable Cox proportional hazards analyses were used to determine the independent effect of prognostic factors. All variables were considered in multivariable analysis. In the first model (model A), patients with missing values of significant factors were excluded. In the second model (model B), missing values were dummy coded to allow the inclusion of all patients. Both

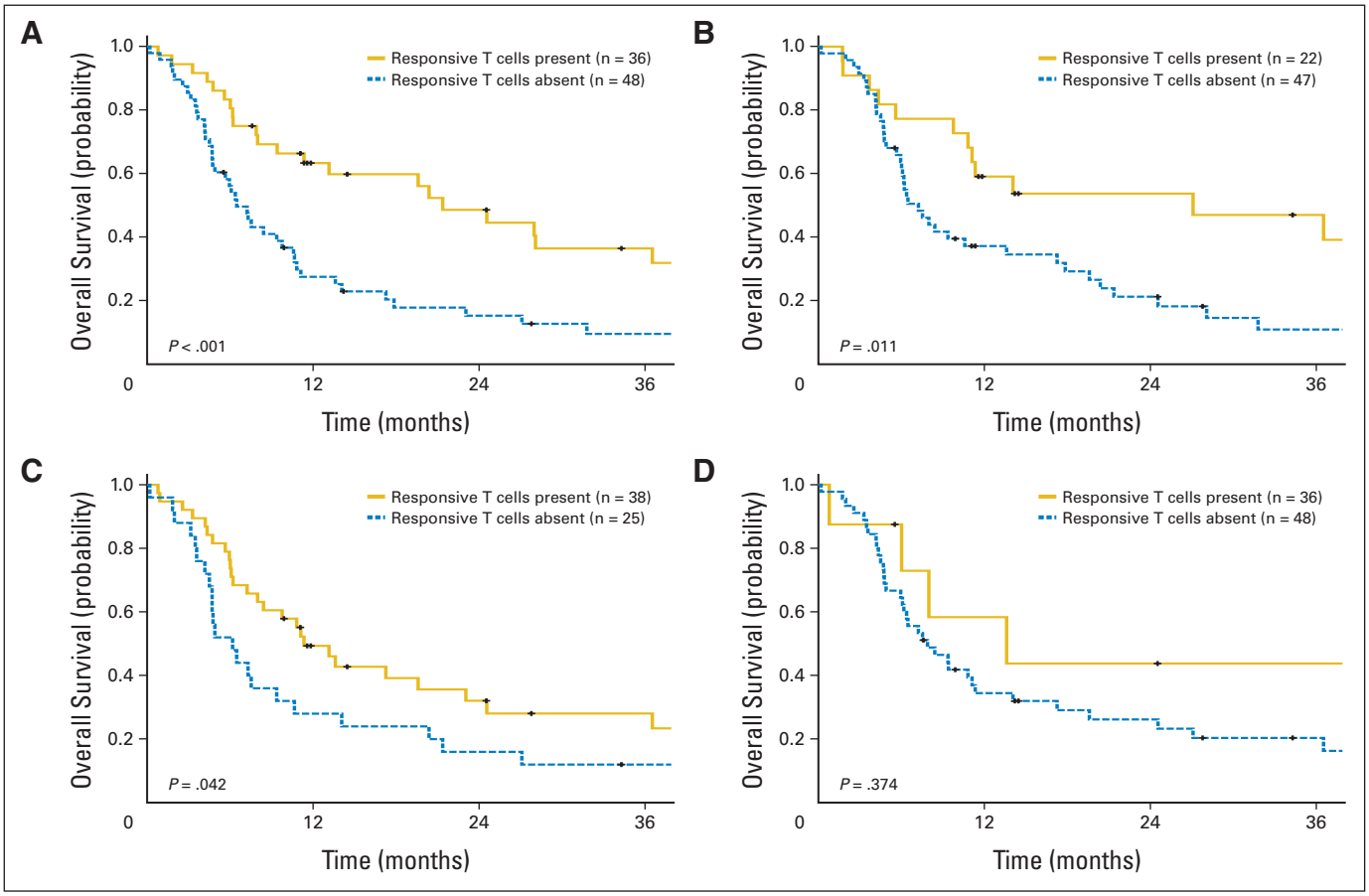

Fig 1. Survival of patients with unresectable stage IV disease according to the presence or absence of T cells responding to (A) NY-ESO-1, (B) Melan-A, (C) MAGE-3, and (D) survivin peptides.

models were established using backward and forward stepwise procedures. Remaining nonsignificant factors were assessed for potential confounding effects. Changes in the estimates of factors in a model by more than 5% were regarded as indicative for confounding. Results of the Cox model were described by means of hazard ratios (HRs) together with 95% CIs, and *P* values were based on the Wald test. Throughout the analysis, *P* values $<.05$ were considered statistically significant. All analyses were carried out using SPSS version 19 (SPSS, Chicago, IL).

RESULTS

Patients

PBMCs initially collected for biobanking purposes were requested by the participating clinical centers according to the inclusion criteria. One of 117 samples was excluded from further analysis because influenza-responsive functional T cells could not be detected. The other 116 patients were assigned to three cohorts: 84 patients with unresectable distant metastasis and clinical follow-up after T-cell analysis (cohort A), 18 tumor-free long-term survivors (cohort B), and 14 healthy controls (cohort C). In cohort A (67% male), the MST was 9.7 months. Median follow-up was 24 months for 19 patients who were alive at the last follow-up and 6.5 months for 65 patients who died; 73.5% of patients were assigned to the M category M1c. Median age was 55 years, with an interquartile range (IQR) of 45 to 67 years. The 18 tumor-free long-term survivors of cohort B (also 67% male) had a median age of 56.5 years (IQR, 48 to 68 years). Healthy controls had a median age of 45 years (IQR, 41 to 59 years), and 50% were men (cohort C). No patient had received NY-ESO-1 or Melan-A vaccines before blood draw for T-cell analysis.

T-Cell Analysis Correlates With Overall Survival

In univariate analysis of 84 cohort A patients, the presence of T cells responding to either NY-ESO-1, Melan-A, or MAGE-3 was associated with improved overall survival (Table 1; Fig 1; Appendix Table A1, online only). The most significant difference in survival for patients with responsive T cells versus those without was seen for NY-ESO-1 ($P < .001$), with Melan-A less significant at $P < .011$ and MAGE-3 at $P < .042$, whereas the presence or absence of T cells responsive to survivin peptides was not associated with survival in this analysis. The MST of patients possessing T cells responding to NY-ESO-1 and/or Melan-A was 21 months, compared with 6 months for all others (Fig 2A). The survival benefit was also evident when the analysis was limited to the detection of IFN-γ–producing T cells (Fig 2B) and when CD4+ and CD8+ T cells were analyzed separately (Appendix Fig A1, online only). The individual frequencies of IFN-γ–producing cells after stimulation with NY-ESO-1 and Melan-A for cohort A patients are listed in Appendix Table A2 (online only). As expected, the M category (Table 1) was also prognostic: the MST was 21 months for patients with soft tissue or lung metastases and normal LDH (M1a/b), whereas it was 8 months for those with other visceral metastases or elevated LDH (M1c; $P = .024$). Multivariable Cox proportional hazards analysis of 53 patients with complete data on all significant factors (model A) showed that the presence of T cells targeting NY-ESO-1 (HR, 0.29; $P = .001$) or Melan-A (HR, 0.18; $P < .001$) were independently associated with a survival benefit, whereas for the M category and T cells responding to MAGE-3, no additional significant prognostic impact was found (Table 2). Model B included patients with missing data. In agreement with model A, this analysis of 83 patients confirmed the prognostic impact of NY-ESO-1– and Melan-A–responsive T cells, whereas significant independent roles of the M category and MAGE-3 were likewise not observed (Table 3). A complete analysis of T-cell responses against all four antigens was performed in 44 patients. There was a strong correlation between the number of targeted antigens and survival. Patients with no response to

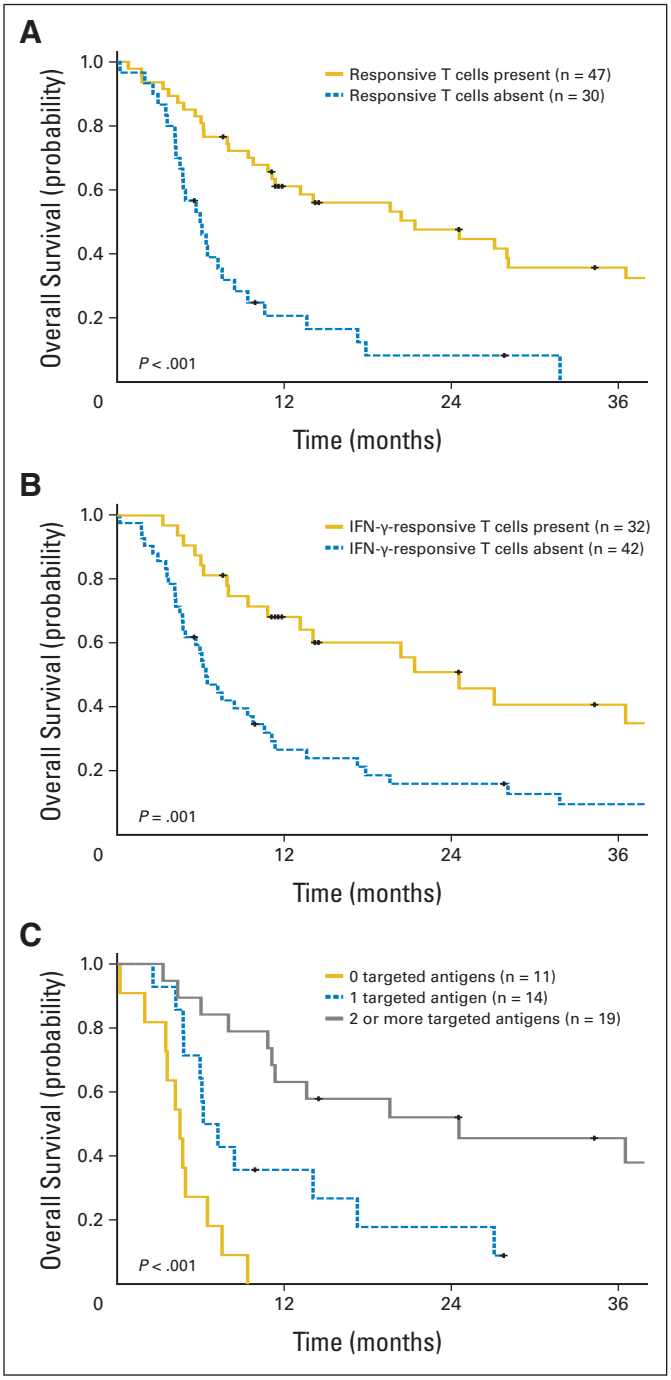

Fig 2. (A) Patients with circulating T cells responding to Melan-A and/or NY-ESO-1 by producing at least one of six cytokines have a median survival time of 21 months, compared with 6 months for those who are negative for both. (B) Impact of Melan-A– and/or NY-ESO-1–responding interferon-γ–positive T cells on survival. (C) Survival according to the number of targeted antigens per patient. Only patients with analysis of responses to all four antigens are considered.

Table 2. Final Model A of Multivariable Cox Proportional Hazards Analysis*

Factor	Patients No.	%	% Dead	HR	95% CI	P
M category						.943
M1a or b	18	34.0	77.8	0.97	0.48 to 2.00	
M1c	35	66.0	77.1	1		
NY-ESO-1–responsive T cells						**.001**
Present	23	43.4	60.9	**0.29**	**0.14 to 0.61**	
Absent	30	56.6	90.0	**1**		
Melan-A–responsive T cells						**< .001**
Present	16	31.9	56.3	**0.18**	**0.08 to 0.42**	
Absent	37	68.1	86.5	**1**		
MAGE-3–responsive T cells						.341
Present	33	62.3	69.7	0.73	0.39 to 1.39	
Absent	20	37.7	90.0	1		

NOTE. Bold font indicates statistical significance.
Abbreviation: HR, hazard ratio.
*Model A included 53 patient cases (those with missing values were excluded) and was adjusted for confounding effects of M category, presence of MAGE-3–responsive T cells and anti-CTLA4 treatment after T-cell analysis.

any antigen, a response to a single antigen, or responses to at least two of the four antigens had an MST of 4, 6, or 24 months, respectively (Fig 2C). No association between survival and the frequency of influenza-specific IFN-γ–producing CD4/CD8+ T cells was observed (data not shown).

Antigen-Responsive T Cells in Long-Term Survivors

T cells responding to NY-ESO-1 and Melan-A peptides were detectable in 50% and 47%, respectively, of long-term survivors in cohort B. Such T cells were also found in a high proportion of cohort A patients who survived > 18 months after analysis. In contrast, only 22% or 23% of patients who died within 6 months after T-cell analysis possessed detectable peripheral blood NY-ESO-1– or Melan-A–specific T cells, respectively. This was similar to the background level in healthy controls. MAGE-3–specific T cells were detectable in a high proportion of healthy controls (cohort C) but were equally frequent in patients of cohort A with the worst prognosis. Survivin-responsive T cells were rare and were found in only 14% of long-term survivors (Fig 3). Five recurrences but no deaths were observed among 18 cohort B patients after a median follow-up of 18 months. Interestingly, NY-ESO-1–responsive T cells were detected in only one (20%) of five patients who experienced relapse, in contrast to eight (62%) of 13 long-term survivors without recurrences thus far. Similarly, only one (25%) of four long-term survivors who experienced relapse versus seven (54%) of 13 long-term survivors who did not assessed for Melan-A had responsive T cells. Appendix Tables A3 and A4 (online only) list frequencies of antigen-responsive CD4+ and CD8+ T cells according to cytokine response to antigen stimulation.

Table 3. Final Model B of Multivariable Cox Proportional Hazards Analysis*

Factor	Patients No.	%	% Dead	HR	95% CI	P
M category						.064
M1a or b	22	26.5	68.2	0.56	0.31 to 1.04	
M1c	61	73.5	82.0	1		
NY-ESO-1–responsive T cells						**.002**
Present	35	42.2	62.9	**0.42**	**0.24 to 0.73**	
Absent	48	57.8	89.6	**1**		
Melan-A–responsive T cells						**.002**
Present	22	32.4	63.6	**0.36**	**0.19 to 0.69**	
Absent	46	67.6	84.8	**1**		
MAGE-3–responsive T cells						.103
Present	37	59.7	73.0	0.62	0.35 to 1.10	
Absent	25	40.3	92.0	1		

NOTE. Bold font indicates statistical significance
Abbreviation: HR, hazard ratio.
*Model B included 83 patient cases (one had missing information for M category and was excluded; those with missing values were assigned their own category per characteristic; missing value categories for Melan-A–specific T cells [n = 15] and MAGE-3 specific T cells [n = 21] were included) and was adjusted for confounding effects of M category and presence of MAGE-3–responsive T cells.

DISCUSSION

Functional circulating tumor antigen–reactive T cells were found to have an important impact on survival of patients with advanced melanoma in this study. Melan-A– and NY-ESO-1–reactive T cells contributed independently to prognosis and predicted survival better than the AJCC M category in patients with distant metastasis. Moreover, patients possessing T cells reactive to more than one antigen had better survival than those with T cells reactive to fewer. This finding further supports but does not prove the hypothesis that antigen-specific T cells play a causative role in the control of tumor cells. To our knowledge, this is the first report to associate spontaneously occurring functional T-cell responses to survival in patients with melanoma. The proportion of patients with an NY-ESO-1– or Melan-A–stimulated T-cell response was likewise high in the cohort of tumor-free long-term survivors, indicating a prognostic value independent of tumor burden. MAGE-3–reactive T cells were frequently found in healthy controls, and the proportion was lower in all analyzed melanoma

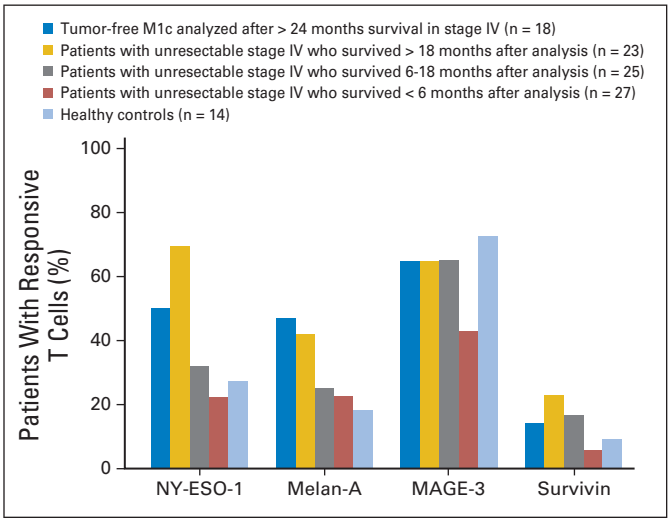

Fig 3. Proportion of patients with T cells responding to NY-ESO-1, Melan-A, MAGE-3, and survivin in long-term survivors, patients with unresectable stage IV disease with follow-up after T-cell assay, and healthy controls.

cohorts, suggesting a disease-related decrease of responsiveness to this antigen. The M category was strongly associated with prognosis according to univariate analysis but did not remain independently significant when tested in conjunction with NY-ESO-1– and Melan-A–responsive T cells in any model of the multivariate analysis. Nevertheless, a strong trend was found according to model B ($P = .064$), whereas model A negated any independent impact of the M category on survival. These differences might have been caused by the small sample size and need to be clarified in larger patient cohorts.

Biobanking efforts of different centers enabled us to include samples from patients for whom clinical follow-up was already available. Antigen-reactive T cells, either occurring spontaneously or after vaccination, have been investigated before, but their correlation with survival remains inconclusive, and most studies have not assessed any aspects of the actual functional integrity of the T cells.[21,22,26,28,31] Previous analyses have focused on different tumor-associated antigens and methods to analyze T cell status. Van Oijen et al[28] used tetramer staining to analyze spontaneously occurring T cells in 62 patients with distant metastasis. Specific T cells were detected in 68% of patients, but no correlation of any of these cells with survival was observed. It has been reported that the majority of tumor antigen–specific T cells in the blood of patients with melanoma are not able to lyse tumor cells or to produce any cytokines.[26] In contrast, here we analyzed functional response by measuring cytokine production on antigenic stimulation. To minimize the risk of underestimating the frequency of T cells in principle capable of responding to melanoma-associated antigens, we quantified production of six cytokines. Because of different and partially opposing functions of these cytokines in adaptive immunity, we also compared survival of cohort A patients based only on detection of IFN-γ–releasing T cells, which confirmed the strong impact of these functional T cells on survival. The frequency of tumor antigen–reactive memory T cells in vivo is too low to be detected directly ex vivo by intracellular cytokine staining. Therefore, we initially applied a limited 12-day in vitro stimulation to ensure expansion of memory and not naive T cells to a measurable extent. For stimulation, nested overlapping 15-mer peptides, spanning entire protein antigens, were used as stimuli, allowing us to include patients of any HLA type. Furthermore, considering the increasing appreciation of the importance of T helper responses for tumor rejection over the last few years,[32,33] it is noteworthy that both CD8 as well as CD4 responses are stimulated and analyzed simultaneously with this approach.[34]

The prognostic impact of spontaneously occurring memory T cells responsive to NY-ESO-1 and Melan-A was not restricted to patients with limited stage IV disease; 73.5% of cohort A patients presented with visceral metastases other than lung and/or elevated LDH. The analysis of their T-cell responses enabled us to identify patients with a chance of long-term survival, even among those in the M1c category.

Our work provides a rationale for refining vaccination and T-cell transfer strategies by targeting Melan-A and NY-ESO-1. Poor clinical responses in previous trials might be explained by tumor-induced escape mechanisms like downregulation of HLA class I or recruitment of regulatory T cells to metastatic sites. For upcoming specific immunotherapies, combinations with newly available agents targeting immune escape mechanisms should therefore be considered.

This analysis might also serve as a rationale for investigating the pretherapeutic detection of circulating T cells reactive to melanoma-associated antigens as a predictive marker for outcome after ipilimumab.[8] Although the mode of action of this agent is incompletely understood, it breaks tolerance by blocking CTLA-4 and amplifies preexistent memory immune responses, which might be detectable by our assay. Recent data implicate an association between outcome after ipilimumab treatment and the existence of post-treatment T-cell responses targeting NY-ESO-1.[35,36]

In conclusion, circulating functional T cells targeting melanoma-associated antigens in patients with melanoma with distant metastasis have strong prognostic impact. T cells responding to Melan-A peptides (HR, 0.18; $P < .001$) or NY-ESO-1 peptides (HR, 0.29; $P = .001$) were independently associated with a survival benefit and superior to the M category in predicting outcome according to Cox regression analysis. In addition, our findings provide a rationale for pursuing vaccination and T-cell transfer strategies targeting Melan-A and NY-ESO-1.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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Appendix

Table A1. Two- and 3-Year Survival According to Kaplan-Meier Method

Factor	No.	%	% Dead	2-Year Survival Rate		3-Year Survival Rate		*P**
				%	95% CI	%	95% CI	
Age, years								.755
< 55	41	48.8	73.2	29.5	14.8 to 44.2	21.1	6.8 to 35.4	
≥ 55	43	51.2	81.4	29.7	15.0 to 44.4	20.8	7.5 to 34.1	
Sex								.715
Male	56	66.7	75.0	27.0	14.1 to 39.9	20.2	7.7 to 32.7	
Female	28	33.3	82.1	33.8	16.0 to 51.6	22.6	6.7 to 38.5	
M category								.024
M1a or b	22	26.5	68.2	48.1	26.5 to 69.7	42.1	20.1 to 64.1	
M1c	61	73.5	82.0	20.9	9.7 to 32.1	11.6	2.4 to 20.8	
NY-ESO-1–responsive T cells								< .001
Present	36	42.9	61.1	48.6	31.0 to 66.2	36.4	18.6 to 54.2	
Absent	48	57.1	89.6	15.3	4.5 to 26.1	9.6	0.4 to 18.8	
Melan-A–responsive T cells								.011
Present	22	31.9	63.6	53.7	32.5 to 74.9	47.0	24.7 to 69.3	
Absent	47	68.1	83.0	21.3	8.8 to 33.8	10.9	0.3 to 21.5	
MAGE-3–responsive T cells								.042
Present	38	60.3	71.1	32.1	16.0 to 48.2	28.1	12.2 to 44.0	
Absent	25	39.7	92.0	16.0	1.7 to 30.3	12.0	0.0 to 24.7	
Survivin-responsive T cells								.374
Present	8	15.1	62.5	43.7	6.9 to 80.5	43.7	6.9 to 80.5	
Absent	45	84.9	80.0	26.2	12.7 to 39.7	20.4	7.7 to 33.1	
Prior systemic treatments								
Anti-CTLA-4								.218
Yes	9	10.7	44.4	32.4	0.0 to 80.2	32.4	0.0 to 80.2	
No	75	89.3	81.3	28.0	17.4 to 38.6	19.4	9.8 to 29.0	
Other immunotherapy								.260
Yes	39	46.4	74.4	34.5	18.6 to 50.4	31.0	15.3 to 46.7	
No	45	53.6	80.0	25.3	11.6 to 39.0	11.4	0.2 to 22.6	
Monochemotherapy								.505
Yes	22	26.2	72.7	27.1	4.8 to 49.4	18.1	0.0 to 38.9	
No	62	73.8	79.0	30.2	18.4 to 42.0	21.8	10.6 to 33.0	
Polychemotherapy								.802
Yes	7	8.3	71.4	0.0	0.0 to 0.0	0.0	0.0 to 0.0	
No	77	91.7	77.9	30.8	20.0 to 41.6	21.9	11.7 to 32.1	
Biochemotherapy								.447
Yes	7	8.3	57.1	53.6	14.2 to 93.0	26.8	0.0 to 68.7	
No	77	91.7	79.2	27.6	17.0 to 38.2	20.4	10.4 to 30.4	
Subsequent systemic treatments								
Anti-CTLA-4								.421
Yes	18	21.4	61.1	32.7	5.5 to 59.9	0.0	0.0 to 0.0	
No	66	78.6	81.8	28.1	16.9 to 39.3	21.1	10.7 to 31.5	
Other immunotherapy								.656
Yes	17	20.2	88.2	29.4	7.6 to 51.2	15.7	0.0 to 34.1	
No	67	79.8	74.6	29.9	17.9 to 41.9	23.0	11.6 to 34.4	
Monochemotherapy								.171
Yes	36	42.9	86.1	25.4	10.7 to 40.1	9.1	0.0 to 20.3	
No	48	57.1	70.8	32.4	17.9 to 46.9	29.5	15.2 to 43.8	
Polychemotherapy								.164
Yes	19	22.6	89.5	17.5	0.0 to 35.3	11.7	0.0 to 26.8	
No	65	77.4	73.8	33.3	21.1 to 45.5	24.4	12.6 to 36.2	
Biochemotherapy								.713
Yes	12	14.3	91.7	33.3	6.6 to 60.0	11.1	0.0 to 30.9	
No	72	85.7	75.0	29.7	18.3 to 41.1	23.6	12.6 to 34.6	

*P values are results of log-rank tests excluding patient cases with missing values.

Table A2. Frequencies of IFN-γ–Positive T Cells in 84 Cohort A Patients After Stimulation With NY-ESO-1 or Melan-A Peptides and in Unstimulated Controls

	NY-ESO-1 Stimulated (%)		Melan-A Stimulated (%)		Unstimulated Control (%)			NY-ESO-1 Stimulated (%)		Melan-A Stimulated (%)		Unstimulated Control (%)	
Patient No.	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	Patient No.	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
1	0.06	0.10	0.03	0.09	0.03	0.04	43	0.03	0.08	0.11	5.85	0.03	0.07
2	6.20	2.25	3.61	32.34	2.56	1.42	44	0.26	0.59	ND	ND	0.72	0.41
3	0.88	0.01	0.48	0.01	0.40	0.03	45	0.35	0.68	0.64	1.27	0.54	0.64
4	0.96	5.52	0.60	1.75	0.64	1.04	46	0.19	0.39	0.19	0.49	0.13	0.71
5	2.22	0.09	2.35	0.04	2.43	0.04	47	0.30	0.05	0.25	0.06	0.28	0.02
6	0.19	0.29	ND	ND	0.11	0.29	48	0.02	0.04	0.03	0.05	0.02	0.02
7	0.17	0.02	0.18	0.00	0.16	0.01	49	0.66	0.02	0.55	0.07	0.54	0.06
8	0.64	0.92	0.37	1.05	0.70	3.68	50	0.15	0.32	0.19	0.74	0.18	0.73
9	0.01	0.02	0.01	0.07	0.01	0.02	51	0.78	0.07	0.80	0.23	1.00	0.08
10	0.10	0.03	0.11	0.27	0.06	0.07	52	0.22	0.27	ND	ND	0.24	0.24
11	2.60	0.22	ND	ND	0.62	0.21	53	0.40	0.02	0.39	0.02	0.42	0.01
12	0.07	0.06	ND	ND	0.04	0.06	54	4.53	0.78	4.55	0.51	5.19	0.40
13	0.06	0.29	0.07	0.37	0.04	0.24	55	3.50	0.89	3.79	1.65	3.46	1.11
14	4.68	0.90	1.65	0.67	1.05	0.65	56	1.42	0.03	0.91	0.03	0.87	0.02
15	0.61	0.70	0.56	2.73	0.55	0.72	57	0.06	0.09	ND	ND	0.02	0.06
16	0.01	0.06	ND	ND	0.01	0.03	58	2.72	0.22	2.77	0.15	2.33	0.14
17	6.54	5.16	6.17	5.68	5.31	4.65	59	0.02	0.01	ND	ND	0.01	0.01
18	2.73	1.50	0.63	0.93	0.48	0.19	60	0.29	0.05	0.48	0.07	0.47	0.02
19	ND	ND	ND	ND	ND	ND	61	0.18	0.08	0.07	0.06	0.08	0.08
20	0.01	0.11	0.75	0.48	0.01	0.01	62	0.14	0.09	0.08	0.07	0.15	0.10
21	0.43	0.24	0.44	0.20	0.44	0.12	63	0.57	0.39	0.55	0.45	0.48	0.30
22	0.62	0.10	0.38	0.53	0.25	0.08	64	0.04	0.34	ND	ND	0.02	0.34
23	2.21	0.84	1.22	1.17	1.68	0.49	65	0.02	0.08	ND	ND	0.03	0.12
24	2.80	0.59	3.08	6.29	2.49	0.69	66	0.01	0.16	0.01	0.38	0.01	0.27
25	3.51	1.55	ND	ND	3.87	0.12	67	0.69	1.69	0.18	1.10	0.36	1.92
26	0.08	0.07	0.08	0.08	0.09	0.10	68	0.12	0.09	0.02	0.07	0.04	0.14
27	0.24	0.06	ND	ND	0.06	0.06	69	0.28	0.12	0.28	0.17	0.52	0.10
28	0.29	0.09	0.58	0.04	0.25	0.07	70	0.22	0.04	0.10	0.59	0.07	0.04
29	1.52	0.06	0.67	0.11	0.65	0.06	71	0.02	0.11	0.01	0.14	0.01	0.13
30	6.54	0.26	7.69	0.29	7.64	0.14	72	0.39	1.00	0.41	0.09	0.38	0.13
31	0.06	0.16	0.05	0.13	0.07	0.16	73	1.06	0.17	0.50	0.27	0.42	0.18
32	0.88	0.08	ND	ND	1.10	0.04	74	0.29	0.10	0.30	0.20	0.29	0.15
33	0.37	0.04	0.33	0.05	0.34	0.06	75	0.36	0.36	0.23	0.39	0.21	0.20
34	0.46	0.11	0.42	0.10	0.62	0.18	76	0.93	0.25	0.76	2.58	0.73	0.10
35	0.92	0.48	0.83	0.70	0.67	0.42	77	0.83	3.16	0.27	0.18	0.03	0.01
36	0.42	0.39	0.34	0.32	0.32	0.25	78	0.12	0.04	ND	ND	0.02	0.01
37	0.13	0.45	ND	ND	0.07	0.59	79	0.51	0.36	0.33	0.22	0.40	0.37
38	1.91	12.59	ND	ND	0.98	0.54	80	0.01	0.05	0.01	0.06	0.01	0.06
39	0.80	0.23	0.69	0.18	0.64	0.16	81	0.28	1.02	2.90	2.26	0.10	0.16
40	0.03	0.04	0.04	0.08	0.03	0.07	82	0.53	3.00	0.15	1.42	0.10	0.02
41	0.36	0.43	0.43	0.33	0.28	0.24	83	0.22	0.01	0.07	0.03	0.06	0.03
42	0.39	0.17	0.19	0.41	0.08	0.31	84	0.19	0.11	0.08	0.07	0.07	0.05

Abbreviations: IFN-γ, interferon-gamma; ND, not done.

Table A3. Frequencies of Antigen-Responsive CD4+ T Cells According to Six Cytokines and Five Antigens

Responsive T Cells Detectable	Mean Frequency ± Standard Deviation (%)					
	CD4+IFNγ+	CD4+IL-10+	CD4+IL-17+	CD4+IL-2+	CD4+IL-4+	CD4+TNF-α+
NY-ESO-1						
No						
Control	0.52 ± 1.10	0.06 ± 0.07	0.08 ± 0.33	0.18 ± 0.38	0.06 ± 0.18	1.48 ± 2.37
Stimulated	0.56 ± 1.04	0.07 ± 0.08	0.12 ± 0.38	0.28 ± 0.45	0.08 ± 0.16	2.10 ± 2.71
Yes						
Control	0.66 ± 1.23	0.14 ± 0.16	0.11 ± 0.15	0.17 ± 0.17	0.10 ± 0.14	2.22 ± 3.69
Stimulated	1.69 ± 3.14	0.13 ± 0.17	0.17 ± 0.30	0.46 ± 0.96	0.16 ± 0.23	4.12 ± 5.85
Melan-A						
No						
Control	0.61 ± 1.23	0.08 ± 0.09	0.13 ± 0.34	0.20 ± 0.21	0.08 ± 0.18	2.01 ± 2.43
Stimulated	0.67 ± 1.31	0.07 ± 0.08	0.15 ± 0.33	0.22 ± 0.23	0.11 ± 0.25	2.21 ± 2.69
Yes						
Control	0.51 ± 1.03	0.07 ± 0.07	0.07 ± 0.13	0.22 ± 0.49	0.09 ± 0.17	2.09 ± 3.12
Stimulated	1.31 ± 1.03	0.07 ± 0.07	0.08 ± 0.11	0.24 ± 0.49	0.24 ± 1.20	2.49 ± 3.04
MAGE-3						
No						
Control	0.60 ± 0.89	0.08 ± 0.12	0.09 ± 0.15	0.14 ± 0.13	0.05 ± 0.06	1.79 ± 2.37
Stimulated	0.56 ± 0.81	0.08 ± 0.10	0.09 ± 0.16	0.19 ± 0.21	0.05 ± 0.05	1.89 ± 2.77
Yes						
Control	0.62 ± 1.37	0.06 ± 0.07	0.13 ± 0.36	0.24 ± 0.40	0.10 ± 0.22	2.12 ± 2.89
Stimulated	1.56 ± 3.16	0.07 ± 0.07	0.21 ± 0.53	0.38 ± 0.56	0.21 ± 0.55	3.93 ± 5.90
Survivin						
No						
Control	0.66 ± 1.19	0.08 ± 0.11	0.13 ± 0.34	0.23 ± 0.37	0.07 ± 0.19	2.38 ± 3.07
Stimulated	0.71 ± 1.33	0.09 ± 0.12	0.15 ± 0.37	0.27 ± 0.41	0.10 ± 0.30	2.57 ± 3.16
Yes						
Control	0.77 ± 1.44	0.13 ± 0.18	0.12 ± 0.10	0.17 ± 0.16	0.12 ± 0.20	3.50 ± 4.67
Stimulated	0.95 ± 1.28	0.08 ± 0.08	0.12 ± 0.11	0.25 ± 0.20	0.26 ± 0.73	4.34 ± 4.25
Influenza						
Yes						
Control	0.86 ± 2.08	0.15 ± 0.29	0.10 ± 0.34	0.24 ± 0.44	0.07 ± 0.12	2.44 ± 3.42
Stimulated	8.87 ± 10.40	0.12 ± 0.21	0.21 ± 0.60	1.13 ± 1.64	0.71 ± 4.19	13.26 ± 12.72

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Table A4. Frequencies of Antigen-Responsive CD8+ T Cells According to Six Cytokines and Five Antigens

	Mean Frequency ± Standard Deviation (%)					
Responsive T Cells Detectable	CD8+IFN-γ+	CD8+IL-10+	CD8+IL-17+	CD8+IL-2+	CD8+IL-4+	CD8+TNF-α+
NY-ESO-1						
No						
Control	0.16 ± 0.32	0.09 ± 0.15	0.06 ± 0.11	0.08 ± 0.11	0.06 ± 0.10	0.79 ± 1.30
Stimulated	0.19 ± 0.28	0.10 ± 0.16	0.08 ± 0.08	0.12 ± 0.13	0.10 ± 0.13	0.93 ± 1.25
Yes						
Control	0.36 ± 0.86	0.12 ± 0.11	0.16 ± 0.24	0.11 ± 0.10	0.15 ± 0.20	0.99 ± 1.16
Stimulated	1.25 ± 2.96	0.12 ± 0.12	0.20 ± 0.25	0.13 ± 0.10	0.22 ± 0.30	2.28 ± 3.76
Melan-A						
No						
Control	0.33 ± 0.76	0.11 ± 0.16	0.11 ± 0.19	0.11 ± 0.09	0.09 ± 0.13	1.08 ± 1.30
Stimulated	0.35 ± 0.77	0.11 ± 0.21	0.20 ± 0.66	0.12 ± 0.11	0.20 ± 0.64	1.15 ± 1.52
Yes						
Control	0.14 ± 0.19	0.09 ± 0.09	0.10 ± 0.20	0.10 ± 0.12	0.13 ± 0.22	0.95 ± 1.33
Stimulated	0.96 ± 1.69	0.11 ± 0.11	0.14 ± 0.27	0.14 ± 0.17	0.63 ± 2.44	2.31 ± 3.12
MAGE-3						
No						
Control	0.35 ± 0.72	0.11 ± 0.16	0.11 ± 0.21	0.09 ± 0.11	0.11 ± 0.13	0.92 ± 1.54
Stimulated	0.31 ± 0.52	0.11 ± 0.21	0.07 ± 0.08	0.11 ± 0.16	0.10 ± 0.07	0.86 ± 1.52
Yes						
Control	0.21 ± 0.61	0.07 ± 0.09	0.10 ± 0.19	0.11 ± 0.11	0.11 ± 0.18	0.87 ± 0.98
Stimulated	2.09 ± 7.57	0.08 ± 0.09	0.17 ± 0.38	0.12 ± 0.14	0.27 ± 0.67	3.98 ± 11.78
Survivin						
No						
Control	0.19 ± 0.28	0.09 ± 0.12	0.10 ± 0.18	0.09 ± 0.11	0.09 ± 0.16	0.98 ± 1.28
Stimulated	0.24 ± 0.37	0.11 ± 0.19	0.16 ± 0.65	0.11 ± 0.15	0.15 ± 0.63	1.05 ± 1.48
Yes						
Control	0.51 ± 1.02	0.09 ± 0.10	0.22 ± 0.32	0.10 ± 0.07	0.17 ± 0.25	1.69 ± 1.63
Stimulated	0.28 ± 0.29	0.08 ± 0.05	0.09 ± 0.05	0.13 ± 0.10	0.32 ± 0.80	1.64 ± 1.96
Influenza						
Yes						
Control	0.38 ± 1.07	0.50 ± 1.29	0.13 ± 0.19	0.18 ± 0.39	0.14 ± 0.19	1.28 ± 2.52
Stimulated	12.98 ± 19.31	0.30 ± 0.61	0.17 ± 0.27	0.24 ± 0.30	0.55 ± 4.21	14.99 ± 20.11

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

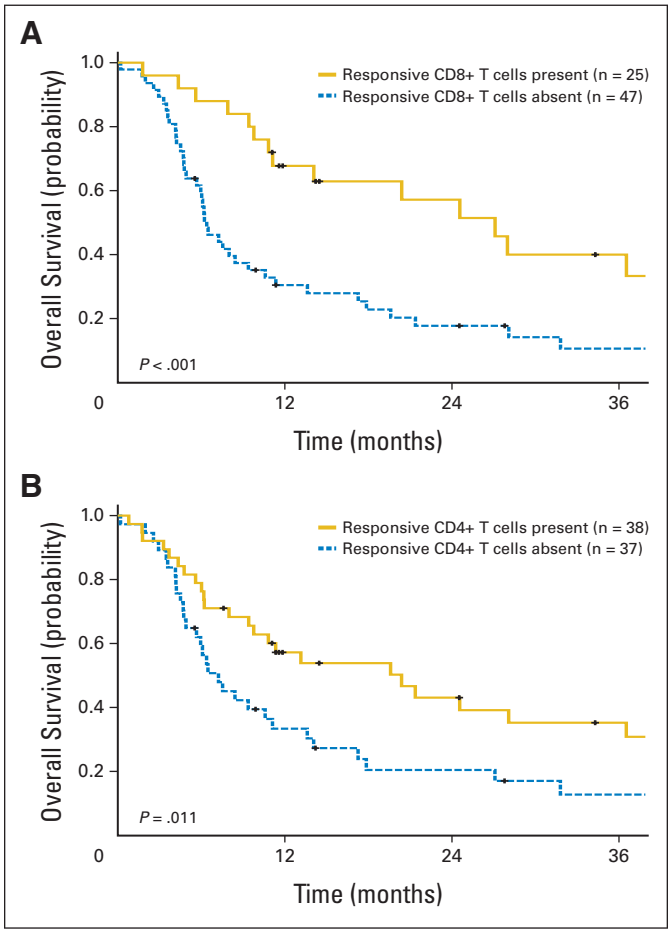

Fig A1. More favorable survival curves for patients with (A) CD8+ T cells and (B) CD4+ T cells responding to Melan-A or NY-ESO-1 when analyzed separately.

GLOSSARY TERMS

AJCC/UICC-TNM staging: The TNM Classification of Malignant Tumours (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue; N describes regional lymph nodes that are involved; M describes distant metastasis (spread of cancer from one body part to another). TNM is developed and maintained by the International Union Against Cancer (UICC) to achieve consensus on one globally recognized standard for classifying the extent of spread of cancer. The TNM classification is also used by the American Joint Committee on Cancer (AJCC). In 1987, the UICC and AJCC staging systems were unified into a single staging system. Prognosis of a patient is defined by the TNM classification.

Cox regression analysis: The Cox proportional hazards regression model is a statistical model for regression analysis of censored survival data. It examines the relationship of censored survival distribution to one or more covariates. It produces a baseline survival curve, covariate coefficient estimates with their standard errors, risk ratios, 95% CIs, and significance levels.

MAGE-3: Protein encoded by the MAGE A locus. *MAGE* genes are normally silent in normal tissues but expressed in several cancers.

Melan-A: A melanoma-related antigen. MART-1 is absent in all normal cells except for melanocytes and the retina. In addition, apart from melanomas, MART-1 has not been observed in any other cancers. Consequently, the MART-1 antigen is considered to have a melanoctye lineage.

NY-ESO-1: Also known as CTAG1B or cancer/testis antigen 1B, the gene codes for antigens recognized on neoplastically transformed T cells.

Survivin: IAPs suppress host cell death in response to viral infection. By binding to caspases, they directly inhibit apoptosis. Survivin and xIAP are members of this family, differing perhaps in binding to selective caspases. BIRC5: Tumor-associated protein belonging to the group of shared overexpressed antigens.

Phenotypic and Functional analysis of Melan-A and NY-ESO-1-reactive T cells and their influence on survival of late-stage melanoma patients.

Phenotypic and Functional analysis of Melan-A and NY-ESO-1-reactive T cells and their influence on survival of late-stage melanoma patients

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Abstract

Clinical trials of vaccines and immunomodulators are yielding impressive results, but still only in a minority of melanoma patients. Patient parameters associated with successful therapy are largely undefined, and whether targeting particular antigens can result in a better clinical outcome than others is not clear. Furthermore, predictive markers are mostly lacking. Initially, we observed in a cohort of late-stage melanoma patients that the presence of NY-ESO-1- and/or Melan-A-reactive T cells in the peripheral blood was significantly associated with prolonged survival. Here we report on the phenotypes and functions of these T cell antigen responses and their prognostic impact. We assayed functional antigen-reactive T cells with an intracellular cytokine staining protocol using multicolor flow cytometry to detect six cytokines simultaneously, allowing us to analyze the cell type (CD4+ or CD8+) and the released cytokines at the single-cell level. We observed that NY-ESO-1 was generally recognized more frequently by CD4+ than by CD8+ T cells under these conditions, both being associated with a positive effect on patient survival. In contrast, Melan-A mainly stimulated CD8+ T cells and additional or sole recognition by CD4+ T cells was associated with a poor clinical outcome. We also observed distinct cytokine profile differences between NY-ESO-1- and Melan-A-reactive T cells and between CD4 and CD8 responses. Pro-inflammatory responses seemed to be favourable for both antigens, but especially for Melan-A. Taken together, our data support NY-ESO-1 and Melan-A as target antigens of choice for melanoma immunotherapy. The finding that CD4+T cell recognition of Melan-A had a strong negative correlation with survival should be considered for upcoming trials attempting to stimulate both CD4- and CD8-mediated responses to this antigen.

Introduction

The annual increase of malignant melanoma lies between 3 and 7% per year and trends suggest that the incidence per year will even double within the next 10 to 20 years (1, 2). Once a patient has failed surgery, conventional treatments like chemo- and radiotherapy remain the first choice despite poor clinical efficacy resulting in a very poor prognosis (3, 4). Due to this modest success rate, other treatments are clearly necessary, and immunotherapy is increasingly being applied for melanoma management (in detail reviewed by Zeiser et al. (5)). Clinical approaches in melanoma immunotherapy commonly include attempts to generate either in vivo or in vitro a sufficient amount of activated anti-tumor T cells specific for tumor-associated antigens (TAAs) expressed by that individual patient´s tumor. Furthermore, these T cells must be in a position to circumvent tumors' immune escape mechanisms. There are many ways to address the above issues, but so far the results of melanoma immunotherapy have been generally rather disappointing. Nonetheless, several recent reports of immune treatments leading to impressive clinical responses are raising optimism in the field. In 2010, a randomized double blind placebo-controlled phase 3 trial of Ipilimumab, an anti-CTLA-4 antibody, resulted in a statistically significant survival benefit in stage IV advanced melanoma patients (6). These clinical benefits, including shrinkage or complete disappearance of large tumor masses, are often associated with the demonstrable presence of CD8+, antigen-specific T cells in peripheral blood (7). In a different approach, impressive clinical responses after adoptive transfer of TAA-specific T cells clearly reveal the importance of these cells for successful therapy (8). More recently, in a phase 1 study, different cancer patients were treated with an anti-PD-1 antibody resulted in objective responses (complete or partial responses) in about 20% of all treated patients (9). Furthermore, accumulating data suggest that even conventional anti-cancer treatments like chemotherapy or non-specific systemic immunotherapy (such as application of cytokines) depend for their long-term success at least partly on the generation of tumor-antigen-specific T-cells capable of maintaining anti-tumor immune responses (10, 11).

In this light, and given that only a fraction of patients responds well to each therapeutic modality, predictive markers for clinically efficient immunological treatments are required. Currently, only the amount of plasma Lactate dehydrogenase (LDH) is an established biomarker in malignant melanoma (12) and although several other serum markers have been investigated for their prognostic power (13,

14), none is widely accepted so far. It might be of great relevance to undertake functional testing of each patient´s anti-tumor repertoire prior to initiating therapy. Using PBMC and in vitro stimulation with tumor antigens, an integrated readout of the presence of, and nature of, potential anti-tumor activity may be valuable. In a previous study in a large cohort of stage IV melanoma patients, we showed that the presence in the peripheral blood of T cells responding to the cancer/testis antigen NY-ESO-1 or the well-studied differentiation antigen Melan-A had a strong independent prognostic impact on survival. Multivariate analyses showed that patients possessing T cells responding to NY-ESO-1 (NYE) or Melan-A (Melan) had a clear survival benefit compared to patients without these T-cells (15). Here, we analyzed in detail the importance of the nature of the T cell response, whether CD4+ and/or CD8+ T cell-mediated, and the balance of the production of 6 pro- and anti-inflammatory cytokines at the individual cell level.

Patients and Methods

Patients

47 patients with Melan-A and/or NY-ESO-responsive T cells, which were part of an already published report (15), were included here. Briefly, blood cells were collected from four different centers in Europe: Tübingen, Essen (both Germany), Nijmegen (Netherlands) and Siena (Italy). Only stage IV Melanoma patients with unresectable distant metastases at the time of blood draw and available survival follow up data were included. All patients had given their written informed consent for biobanking. The conduct of this study was approved by the Ethics Committee, Tübingen (approvals 147/2011BO2 and 432/2011BO2). All experiments were performed and analyzed centrally by one operator (H.Z.).

Intracellular cytokine assay

CD4 and CD8 T-cell responses against NYE and Melan were measured as described previously (15). Briefly, after thawing, cells were stimulated with protein-spanning overlapping peptides (1 µg/mL; PepMix™; JPT Peptide Technologies, Berlin, Germany), representing each of the two antigens. Additionally, cells were also stimulated with overlapping peptides representing the Influenza A-derived molecules Nucleocapsid protein and Matrix protein 1. These responses served as positive controls, because all patients had been exposed to influenza during their lives. After 12 days culture to amplify the memory cell response, antigen-reactive T-cells were restimulated in the presence of Golgi-Plug (1 µL/mL; BD Bioscience, Ontario, Canada) with autologous antigen-pulsed PBMCs (at a ratio of 1:2) that were previously stained with CFSE (5 µM; Invitrogen, Karlsruhe, Germany). Unpulsed PBMCs were used as a negative control. After 12 h restimulation, assay readout was intracellular staining for 6 different cytokines simultaneously allowing an analysis of the T cell phenotype and effector functions (pro- versus anti-inflammatory response) on a single-cell basis. The following antibodies were used: CD3 – Qdot655 (Invitrogen), anti-CD4 + Pacific Orange (Invitrogen), CD8 – APC-H7 (BD Bioscience), IFN-γ – PE-Cy7 (BD Bioscience), TNF-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), IL-2 – Alexa700 (BioLegend, San Diego, USA), IL-4 – APC (BD Bioscience), IL-10 – Pacific Blue (eBioscience, San Diego, USA) and IL-17 – PerCP-Cy5.5 (eBioscience). Flow Cytometry data were obtained using a 14-colour BD LSR II (BD Bioscience) together with the FACSDiva software (BD Bioscience).

Gating strategy

Data were analyzed using FlowJo software (Tree Star Inc, Ashland, USA). After removal of the doublets using the FSC-Area plotted versus the FSC-Height, restimulators were excluded by gating on the CFSE-negative cells. After that, CD4+ and CD8+ cells were gated within viable (EMA-negative), CD3+ lymphocytes. Subsequently, both the CD4+ and the CD8+ fraction were analyzed for the production of the 6 cytokines.

For the definition of a positive response, established criteria were used as previously described. A response was defined as positive if the percentage of cytokine-producing cells within all CD4+ or CD8+ T-cells of the sample restimulated with loaded APCs was at least twice that of controls incubated with unpulsed autologous APCs. Further, the dot plots of each positive response were again reviewed and only clearly separate cytokine-positive populations were finally defined as significant. Simultaneously measured responses against Influenza served as a positive control. Only patients that possessed a response against the Influenza-derived peptides were included in the analyses. Finally, the production of IL-4 and/or IL-10 was defined as anti-inflammatory response, whereas a detection of IFN-γ, TNF, IL-2 and/or IL-17 was classified as a pro-inflammatory response.

Statistics

The presence or absence of antigen-responsive T cells was analyzed separately for NYE, Melan and Influenza (Flu). CD4 and CD8 responses were analysed separately. If more than one cytokine could be detected, responses were defined as being polyfunctional (in comparison to monofunctional responses). Follow-up time was defined from the date when blood was drawn for T cell analysis to the date of last follow-up or death. Disease-specific survival probabilities have been calculated and only deaths due to melanoma have been considered, whereas deaths due to other causes were regarded as censored events. Estimates of cumulative survival probabilities according to Kaplan-Meier were described and compared using Log rank tests. Throughout the analysis, $P<0.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA).

Results

Patients

PBMCs that were initially collected for biobanking purposes were provided by the participating clinical centers. 47 patients with unresectable distant metastasis and clinical follow-up after blood draw could finally be included into the cohort. 22 patients had Melan-A and 36 patients had NY-ESO-1-reactive T cells. 11 patients had both. 64% of the patients were male and the median survival time of the whole cohort was 650 days. Median follow-up was 1286 days for 14 patients who were alive at the last follow-up and 338 days for 33 patients who died; 61.7% of patients were assigned to the M category M1c. Median age was 55 years, with an interquartile range (IQR) of 45 to 67 years. No patient had received NYE or Melan vaccines before blood draw for T-cell analysis.

22 patients had detectable Melan-A- and 36 NY-ESO-1-reactive T cells, including 11 patients who had both. Median survival time was 650 days for patients with NYE-reactive T cells (1-year survival rate 63.33%) and 679 days for patients responding to Melan-A (1-year survival rate 59.01%)

NY-ESO-1

Figure 1A shows the proportion of patients with CD4+ and/or CD8+ T cells responding to each antigen. For NY-ESO-1, 23 of 36 patients (64%) had only CD4+ NYE-reactive T cells, 3 of 36 patients (8%) had solely CD8+ T cells and 10 patients (28%) had both cell types responding to the antigen. When stimulated with NY-ESO-1, regarding both CD4 and CD8 driven responses, we mainly detected the pro-inflammatory cytokines IFN-γ (25 of 36 patients; 69%) and TNF (23 of 36 patients; 64%). IL-2 could be detected in 17 of 36 patients (47%) responding to NY-ESO-1, whereas IL-4 (7 patients; 19%), IL-17 (6 patients; 17%) and IL-10 (1 patient; 3%) were only infrequently released. In total, NY-ESO-1 stimulation resulted in the release of anti-inflammatory cytokines (IL-4 and IL-10) in 8 patients (22%) (Figure 2A).

Each antigen was preferentially detected by either CD4+ or CD8+ T cells (Figure 1). Therefore,, we next analyzed the CD4+ and CD8+ T cell responses separately for cytokine production (Figure 3A). Patients with NY-ESO-1-responsive CD4+ T cells produced mainly IFN-γ (21 of 36 patients; 58%), TNF (16 patients; 44%) and IL-2 (15 patients; 42%). IL-4 (7 patients; 19%) and IL-17 (6 patients; 17%) were only detected in a few patients. IL-10 could not be detected. Concerning the cytokine production patterns of CD8+ NY-ESO-1-reactive T cells we interestingly observed that TNF (10 of 36 patients; 28%) was the most frequently released cytokine, followed by IFN-γ (9 patients; 25%). IL-2 (2 patients; 6%), IL-4 (2 patients; 6%) and IL-10 (1 patient; 3%) were only occasionally detected. IL-17 was not detected.

We additionally investigated, whether the patients' antigen-specific T cells produced only one (single) or more cytokines (multiple cytokine response) in response to antigen (Figure 4). Concerning NY-ESO-1, we observed that stimulation with this antigen, led mainly to the release of multiple cytokines in the CD4 compartment (21 patients; 58%). Only 12 patients with CD4+ NY-ESO-1-reactive T cells responded upon stimulation with one single cytokine (33%). In the CD8 compartment, single cytokine responses were also only infrequently detected (4 patients; 11%). In 9 patients (25%), multiple cytokines were detected upon stimulation.

Finally, we stratified the patients according the presence or absence of the above-analyzed phentotypic or functional parameters, separately for the CD4 and CD8 compartment. A detailed overview of the univariate analyses is presented in Table 1A for NY-ESO-1 and 1B for Melan-A. Phenotypically, NY-ESO-1 was mainly recognized by CD4+ T cells. However, there was no correlation between the presence of CD4+ or CD8+ reactive T cells, or both, and survival. Any NY-ESO-1 reactivity was equally associated with better survival. Functionally, NY-ESO-1-reactive T cells were found to produce mainly pro-inflammatory cytokines upon antigen restimulation. We distinguished between "unopposed pro-inflammatory" (only release of IFN-γ, TNF, IL-2 and/or IL-17) and those

responses where anti-inflammatory cytokines (IL-4 and IL-10) were also present or only present (pure anti-inflammatory and mixed responses). Kaplan-Meier analyses revealed that neither pro- versus anti-inflammatory nor mono- versus polyfunctional responses had an impact for patients with T cells responding to NYE (for both CD4 and CD8 responses).

Melan-A

In contrast to NY-ESO-1 reactivity, most of the patients with measurable Melan-A responses, had only T cells restricted to the CD8 phenotype (13 of 22 patients, 59%). Only 3 of these 22 patients (14%) had solely CD4+ T cells responding to Melan-A, 6 patients showed both, CD4 and CD8 responses (27%) (Figure 1B).

Similar to NY-ESO-1, Melan-A-reactive T cells mainly released IFN-γ (14 of 22 patients; 64%) and TNF (11 patients; 50%). However, IL-2 was detected in only 2 patients (9%). IL-17 (3 patients; 14%), IL-4 (3 patients; 14%) and IL-10 (3 patients; 14%); were detected in equally low frequencies to NY-ESO-1 stimulated cells (Figure 2B). In total, anti-inflammatory cytokines could be detected in 27% of all patients with Melan-A-specific T cells (6 patients).

When we analyzed the CD4 compartment separately from the CD8 cells, we observed that IFN-γ was only detected in a few patients (2 of 22 patients; 9%). TNF (5 of 22 patients; 9%) and even IL-17 (3 patients; 14%) and IL-4 (3 patients; 14%) were detected more frequently. IL-2 and IL-10 could not be detected at all. CD8+ Melan-A-reactive T cells mainly produced IFN- γ (14 patients; 64%) and TNF (8 patients; 36%). IL-10 (3 patients; 14%), IL-2 (2 patients; 9%), IL-17 (1 patient; 5%) and IL-4 (1 patient; 5%) detection was rare (Figure 3B).

In contrast to all NY-ESO-1-driven responses, differences in single versus multiple cytokine responses were more balanced concerning Melan-A-reactivity. CD4+ Melan-A-reactive T cells released more frequently one single cytokine (5 patients; 23% versus 4 patients; 18% with multiple cytokine responses) whereas CD8+ Melan-A-reactive T cells released again mainly multiple cytokines (10 patients; 45% versus 9 patients; 41% with single cytokine responses) (Figure 4).

In contrast to the NY-ESO-1 data, Melan-A stimulated mainly CD8+ T-cells. Kaplan-Meier analysis revealed that neither a Melan-A response limited to CD8+ T cells (P=0.0951) or a mixed response of both CD4 and CD8 cells (P=0.8711) were associated with better survival, but the presence of responses limited to CD4+ cells were strongly associated with poor survival (Figure 4A; P=0.0040). These patients had a median survival time of 112 days, compared to 824 days for the remaining patients with Melan-reactive T cells (Hazard ratio = 36.67). In fact, there was a tendency for patients with CD4+ reactive cells to do worse even than those without any Melan-reactive cells.

In contrast, strikingly, the inflammatory nature of the CD4+ response after Melan stimulation had a significant impact on survival (Figure 4B; P = 0.0046). Patients whose CD4+ T cells responded to Melan stimulation with the production of anti-inflammatory cytokines had a MST of only 83 days. Patients that did not release IL-4 and/or IL-10 had a MST of 1976 days (Hazard ratio = 0.05). Mono- or polyfunctional responses did not have an impact for patients with T cells responding to Melan (CD4 and CD8).

Influenza

In all 47 patients, influenza antigens were recognized by both CD4+ and CD8+ T cells at the same time. Interestingly, IFN-γ was detected in every sample (100%). TNF (46 patients; 98%) and IL-2 (41 patients; 87%) responses were more frequent, IL-17 (22 patients; 47%), IL-4 (23 patients; 49%) and IL-10 (3 patients; 6%) only rarely detected.

Discussion

There are a number of immunotherapeutic approaches emerging that clearly show clinical efficacy, even in advanced melanoma patients. Early studies of interleukin 2 treatments with long-term follow-up documented that immunotherapy can be extremely effective in a small minority even of such patients (16). Active immunization protocols and treatment with immunomodulators such as anti-CTLA-4 and anti-PD-1 antibodies have also yielded excellent clinical benefits, possibly even cures, in some patients. However, the fraction of patients responding favorably to any of these approaches has always been rather small. Furthermore, all patients participating in these trials are at high risk for severe or even fatal side effects (17-19). Biomarkers, or in our case immune parameters which can predict a beneficial course of disease for late-stage melanoma patients, regardless of the nature of their previous or current therapies, are therefore urgently needed. We previously reported that the presence of circulating NY-ESO-1- and/or Melan-A-reactive T cells predicted survival better than the AJCC M category and independent of therapy (15). In contrast to commonly-applied approaches to detect antigen-specific T cells, such as ELISPOT or multimer stainings, we developed a functional assay enabling collation of data on type of cells responding and type of response generated, independent of the HLA type of the patient. Here, we have taken this analysis further by asking whether knowledge of the nature of the responding T cell (CD4+ or CD8+) and the nature of the response (unopposed pro-inflammatory, anti-inflammatory or mixed pro- and anti-inflammatory; mono- or polyfunctional) would facilitate a finer analysis of the immunological parameters, and/or might even predict long-term survival. Furthermore, our approach has some important advantages compared to established techniques. ELISPOT and other cytokine capture assays usually detect only one cytokine, routinely IFN- γ. Based on our data, only about 70% (69% for NY-ESO-1 and 64% for Melan-A) of patients with antigen-specific T cells would be detected if the assay readout was restricted to this cytokine alone. We would therefore recommend the additional detection of at least one more cytokine (TNF or IL-2) to cover a broader range of specific T cells. Multimer assays, on the other hand, are mainly performed using MHC class I epitopes and detect therefore only CD8+, antigen-specific T cells (including anergic T cells). However, our results suggest that every antigen is preferentially detected by a certain cell type and that amongst antigen-specific T cells, the CD8+ fraction can be rather small. We found that when patients´ PBMC are challenged with peptides representing the entire NY-ESO-1 molecule presented on a large number of different HLA alleles in multiple different patients, it is mainly a CD4+ T cell response that is triggered under these assay conditions. This may be important in the context of the many cancer immunotherapy trials exploiting NY-ESO-1 as a target antigen of choice in recent years. Approaches such as vaccination with predicted MHC class I epitopes aim to increase the number of CD8+ cytotoxic T cells which may not lead to as promising results as targeting CD4+ T cells could for the NY-ESO-1 antigen. Furthermore, monitoring projects using MHC-I-multimers, especially after systemic treatment like chemotherapy or application of cytokines, might be yield a distorted impression. CD8+ NY-ESO-1-reactive T cells may be underrepresented and unmeasured CD4+ responses (in our study 64% of all NY-ESO-1-specific T-cells) might be one of the reasons for the deficiency of data showing correlations between clinical outcome and immunological findings. For example, Jäger et al. found only 3 of 15 stage IV melanoma patients with detectable CD8+ NY-ESO-1-specific T cells, using tetramer technology (NY-ESO-1 $p_{157-165}$) (20). Valmori et al. were able to detect CD8+ NY-ESO-1-reactive T cells (using intracellular cytokine staining after in vitro stimulation) in only 6 of 11 vaccinated melanoma patients, whereas CD4+ responses could be detected in all but one patient (21).

Nevertheless, although stimulation with NY-ESO-1 led generally more often to CD4+ T cell responses, Kaplan-Meier analysis did not reveal an impact on individual patients' survival time based on whether they mediated a CD4+ and/or CD8+ T-cell response against this antigen. The possession of either CD4+ or CD8+ T cells, or both, able to recognize the NY-ESO-1 antigen, seems equally associated with better survival. Robbins et al. showed that the adoptive transfer of genetically engineered, CD4+ and CD8+ T-cells reactive to NY-ESO-1 led to a regression of tumor mass in 5 of 11 melanoma patients (22). Yuan et al. observed that 6 of 8 Ipilimumab-treated patients with evidence of clinical benefit (partial response, complete response, stable disease) showed CD4+ and CD8+ T cell

responses against 20-mer NY-ESO-1 overlapping peptides. Interestingly, none of these patients possessed a solely CD8+ response. Similar to our results, only 1 of 5 patients that progressed or died showed a response on NY-ESO-1 stimulation (23). Thus, the NY-ESO-1 antigen is a candidate target of choice for immunotherapy of malignant melanoma. Hunder et al. reported about durable clinical remissions, which were mediated by CD4+ T cell clones with specificity for NY-ESO-1, in an advanced melanoma patient (24).

In contrast to NY-ESO-1, we found that the T cells responding to Melan-A were mainly CD8+. Strikingly, the CD4 or CD8 nature of the patient´s responding T cells had a strong impact on survival time. When CD4+ T cells responding to Melan-A were present, the probability of long-term survival was low, perhaps reflecting preferential activation of CD4+ regulatory T cells, consistent with the findings of their IL-10 production. Alternatively, the reactivity of CD4+ T cells in the absence of CD8+ T-cells might imply a humoral immune response against Melan-A in these patients, which may be associated with disease progression. However, the former explanation seems most likely, as only 3/9 patients possessed Melan-A-reactive CD4+ T cells with a Th2-like cytokine profile, and because even when reactive CD8+ T cells were seen, the presence of reactive CD4+ T cells was still associated with poor survival. Klein et al. reported on a single advanced melanoma patient treated with Ipilimumab monotherapy. The patient showed increasing partial tumour remissions during therapy that finally resulted in a complete disappearance of all metastases. This was accompanied by a very large increase of CD8+ Melan-A-specific T cells (detected with tetramers; Melan-A_{26-35}). Interestingly, CD8+ NY-ESO-1-specific T cells could not be detected (25).

We also show here for the first time that the nature of the response observed in vitro, namely whether solely pro-inflammatory or also anti-inflammatory, had a great impact on the patients' course of disease, at least for Melan-A. The stratification of patients with Melan-A-reactive T cells according to whether these cells mediated an unopposed pro-inflammatory response (only IFN-g and/or TNF and/or IL-2 and/IL-17) or whether there were also anti-inflammatory mediators involved (IL-4 and/or IL-10) was informative in this regard. Patients with an unopposed pro-inflammatory response to Melan-A stimulation survived much longer than those with an anti-inflammatory response. Khammari et al. treated metastatic melanoma patients with Melan-A-Specific CTL clones (generated by in vitro stimulations with Melan-A peptide analog; ELAGIGILTV). Among 14 treated patients, six (43%) showed an objective response (complete and partial response), one patient had stable disease. It is reported that all selected clones were able to produce IL-2 upon stimulation with melanoma cell lines, but unfortunately, anti-inflammatory cytokines were not measured (26). This impact on survival could not be observed for patients with NYE-reactive T cells. However, consistent with our results, Ayyoub et al. observed that CD4+, NY-ESO-1-specific T cells (detected with MHC II/His-tag-peptide tetramers; DR52b/$ESO_{123-137}$) produce mainly IFN- γ, TNF and IL-2 and only minor proportions of IL-4 and IL-17. IL-10 could not be detected (27).

Both findings concerning Melan-A-reactivity may have important implications for the development of future immunotherapeutic protocols. Many clinical trials, especially using DNA, RNA or whole protein vaccination, lead to both Melan-A-specific CD4 and CD8 responses, which according to the results presented here, may be counterproductive. Additionally, the advantage of mediating a solely pro-inflammatory response may imply that vaccination is unlikely to succeed. Hence, adoptive T-cell transfer with previously-generated, Melan-A-specific CD8+ cells preselected for mediating a solely pro-inflammatory response might be the most promising way of exploiting immune responses against this particular target antigen. Concerning NY-ESO-1-reactivity, we confirmed the important role of both, CD4+ and CD8+ mediated responses for tumour management and strengthened the role of NY-ESO-1 as immunotherapeutic target. We believe that these types of analyses, even though they use peripheral blood and do not speak to what is actually happening inside the tumor, nonetheless reflect the latter events in an informative manner and at the very least allow patient stratification for selection of the most appropriate therapy.

Figures/Tables:

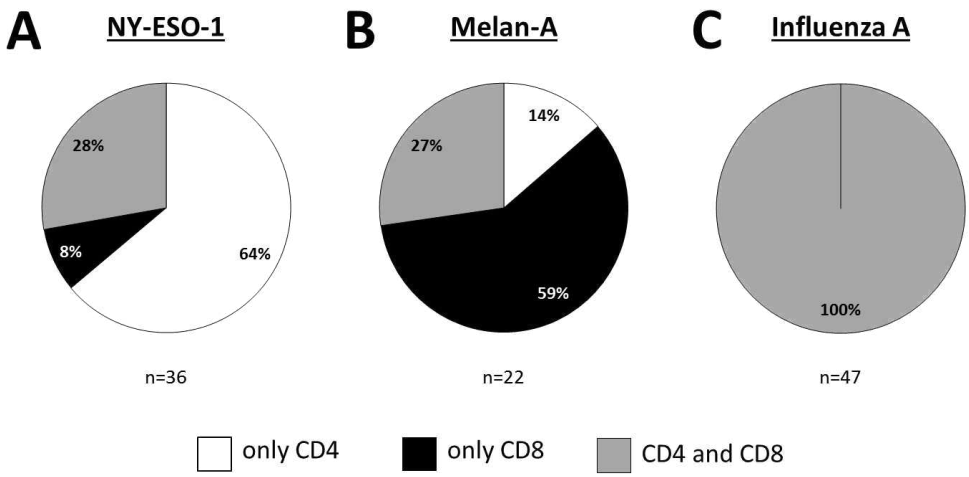

Figure 1:

Type of T cell (white = CD4+ T cell; black = CD8+ T cell) responding upon antigen stimulation. If the patient had both CD4+ and CD8+ antigen-specific T-cells, the response was defined as mixed (grey)

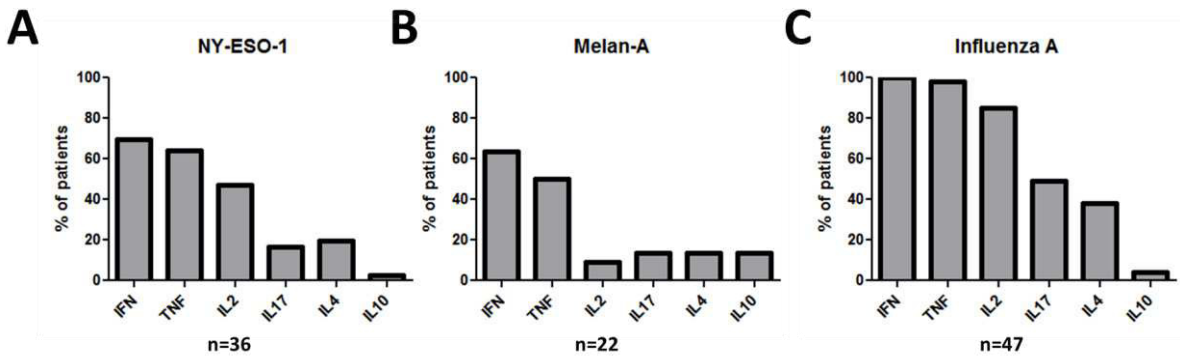

Figure 2:

Proportion of patients with T cells releasing different cytokines by all patients with detectable T cells responding to NY-ESO-1 (A), Melan-A (B) or Influenza (C)

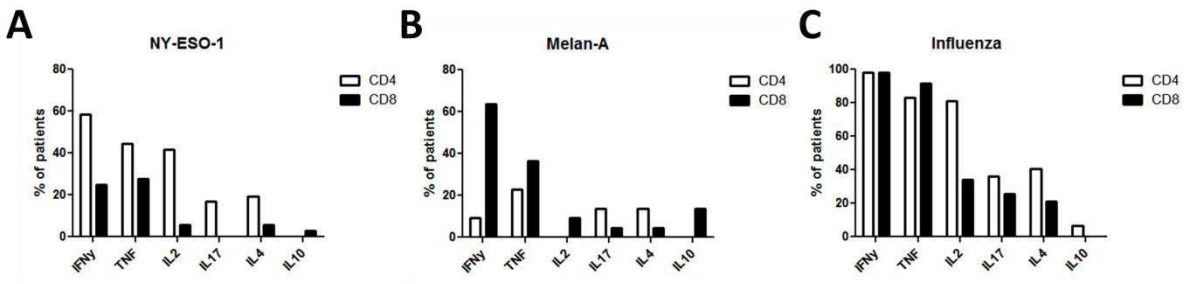

Figure 3:

Detected cytokines by antigen-reactive T cells after restimulation with corresponding antigens. Responses were stratified into CD4 (white) and CD8 (black) driven responses.

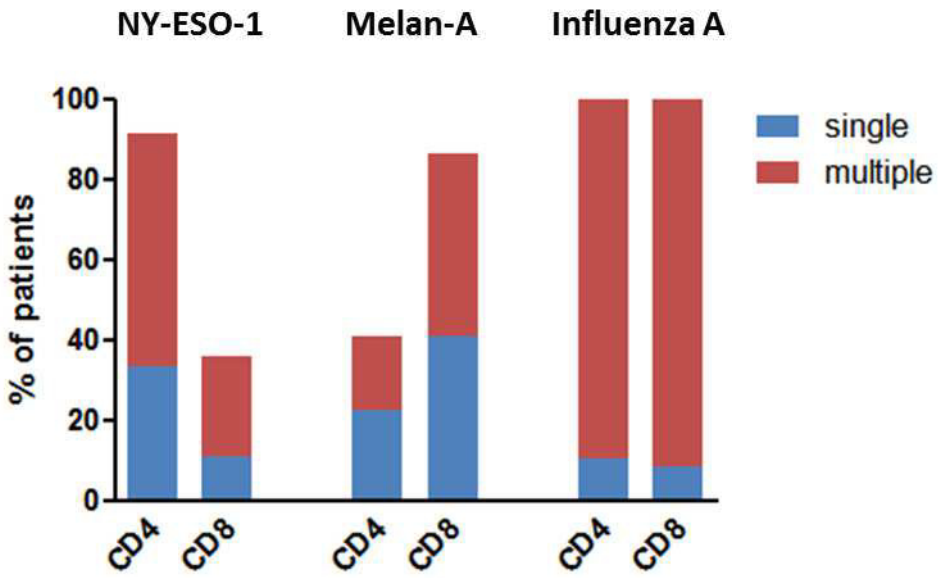

Figure 4:

Proportion of single (blue) and multiple (red) cytokine responses upon antigen stimulation in patients with NY-ESO-1 (left) and Melan-A (right) reactive T cells.

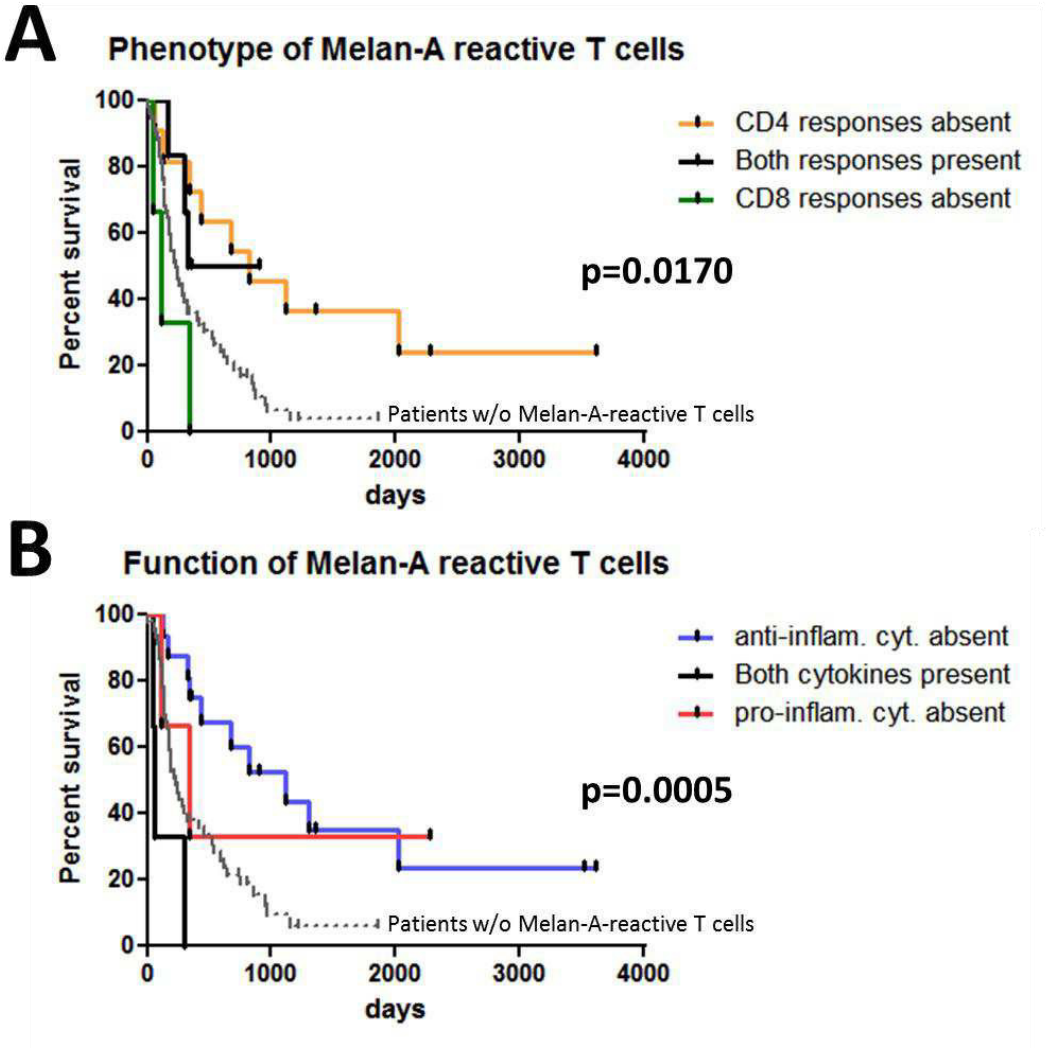

Figure 4:

Survival of stage IV melanoma patients with Melan-A-reactive T cells. Patients are grouped according to the absence CD4 (orange) or CD8 (green) responses or a mixed response (black) (A); or the absence of an anti- (blue) or pro-inflammatory (red) or combined (black) Melan-driven response respectively (B). Survival of patients without any detectable Melan-A-reactive T cells (Historical control) are plotted for comparison

Antigen	Factor	No.	% Dead	MST [days]	1-Year Survival Rate	P
NY-ESO-1	CD4 responses absent	3	100%	240	33,33%	
	CD8 responses absent	23	65%	650	60,29%	0,1444
	Both responses present	10	30%	n.d.	80,00%	
	Anti-inflammatory cytokines absent	28	64%	650	63,80%	
	Pro-inflammatory cytokines absent	0	-	-	-	0,7728
	Both cytokines present	8	63%	747	62,50%	
	Single cytokine response	8	63%	596	75,00%	0,8681
	Multiple cytokine response	28	64%	747	60,15%	
	Factor	**No.**	**% Dead**	**MST [days]**	**1-Year Survival Rate**	**P**
Antigen	CD4 responses absent	13	69%	1111	76,92%	
Melan-A	CD8 responses absent	3	100%	112	0,00%	**0,0170**
	Both responses present	6	50%	1968	50,00%	
	Anti-inflammatory cytokines absent	16	63%	1111	75,00%	
	Pro-inflammatory cytokines absent	3	67%	338	33,33%	**0,0005**
	Both cytokines present	3	100%	54	0,00%	
	Single cytokine response	9	78%	824	66,67%	0,8642
	Multiple cytokine response	13	62%	679	53,85%	

Table 1:

Results of survival analysis according to Kaplan-Meier. Patients with NY-ESO-1- (upper part) or Melan-A-reactive T cells (lower part) were grouped according to phenotypical and functional characteristics. P values are results of Log-rank (Mantel-Cox) Test

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Zusammenfassung

Das maligne Melanom, der bösartige Tumor der Melanozyten, ist einer der Krebsarten mit den weltweit am stärksten ansteigenden Neuerkrankungen. In frühen Stadien (Stadium I und II) stellt die chirurgische Entfernung des Tumors die wichtigste und erfolgreichste Therapieform dar. In fortgeschrittenen Stadien, welche beim Melanom verhältnismäßig früh erreicht werden, gelten die Chemo- und Strahlentherapie als wichtigste Behandlungsmethoden. Aufgrund der sehr geringen Heilungschancen sind diese Methoden jedoch eher als palliative Therapien anzusehen. Die geringe Aussicht auf Erfolg führte in den letzten Jahren vermehrt dazu, nach alternativen Therapieansätzen zu suchen und diese auch anzuwenden. Hierbei hat sich besonders die Krebsimmuntherapie als vielversprechend herausgestellt. Das Ziel solch einer Immuntherapie ist es, das Immunsystem des Patienten derart zu beeinflussen, dass sämtliche Krebszellen im Körper gezielt bekämpft und idealerweise beseitigt werden. Die Krebszellen sollen hierbei über Tumor-assoziierte Antigene (TAA), also Antigene die nur oder vermehrt auf Krebszellen vorkommen, erkannt werden. TAAs wurden zuerst und später besonders häufig auf Melanomzellen identifiziert. Die Antigene werden von unterschiedlichen Tumoren, unterschiedlich stark exprimiert und sind teilweise mit bestimmten klinischen Verläufen assoziiert. Die Entdeckung der TAAs auf Melanomzellen und der eindeutige Einfluss immunologischer Faktoren (Spontanremissionen, aggressive Verläufe bei Immunsupprimierten) führten dazu, dass die Immuntherapie schnell ein häufig eingesetztes Mittel zur Behandlung des malignen Melanoms wurde.
Unterschiedliche immuntherapeutische Ansätze führten teilweise zu beeindruckenden Erfolgen, allerdings nur in wenigen Patienten. Außerdem setzen sich alle Teilnehmer an klinischen Studien, schweren und teilweise lebensbedrohlichen Nebenwirkungen aus. Das Ziel dieser Dissertation war es deshalb zum einen die Sicherheit und zum anderen vor allem klinische und immunologische Wirksamkeit einer intratumoralen IL-2 Behandlungen zu untersuchen. Des Weiteren sollten Antigene identifiziert werden, die als Ziel einer spezifischen Immuntherapie zu besonders guten klinischen Ergebnissen führen. Schließlich sollte analysiert werden, ob sich die Präsenz bestimmter antigen-spezifischer T –Zellen als prädiktive Marker für einen guten Krankheitsverlauf eignen.
Ich konnte zeigen, dass die intratumorale Injektion des Zytokins IL-2 eine sichere und effektive Behandlungsmethode, zumindest für sichtbare Läsionen ist. Außerdem wurde in einem Patienten, simultan zu den Komplettremissionen, ein Anstieg von antigen-spezifischen T-Zellen beobachtet. Weitere Folgestudien sollen nun zeigen, ob dieser Anstieg einerseits auch in anderen Patienten beobachtet werden kann und andererseits mit einem guten klinischen Verlauf assoziiert ist.
Bezüglich Tumor-assoziierte Antigene konnte ich zeigen, dass T-Zellen die in der Lage sind die Antigene NY-ESO-1 und Melan-A zu erkennen, vermehrt in Patienten mit einem guten klinischen Verlauf detektiert werden können. Die Präsenz oder

Abwesenheit dieser Zellen erlaubte es außerdem, den klinischen Verlauf des einzelnen Patienten vorauszusagen, ungeachtet der angewendeten Therapie und der TNM Klassifizierung. Diese Fähigkeit konnte für die weiteren untersuchten Antigene nicht beobachtet werden. NY-ESO-1 und Melan-A scheinen sich deshalb hervorragend als Ziel einer Immuntherapie zu eignen.

Schließlich wurden der Phänotyp und die Funktion der einzelnen Antigen-spezifischen Zelle untersucht. Ich konnte zeigen dass jedes der getesteten TAAs hauptsächlich von einem bestimmten T-Zelltyp (CD4+ oder CD8+) erkannt wird und zu einer bestimmten T-Zell-Antwort, also der Freisetzung von bestimmten Zytokinen, führt. Hierbei war auffallend, dass bezüglich der Melan-A-Reaktivität, es für den Patienten essentiell war, welcher Zelltyp und welche Immunantwort involviert waren. Die Abwesenheit von CD8+ T Zellen und die Präsenz von anti-inflammatorischen Zytokinen war jeweils mit einem sehr schlechten klinischen Verlauf assoziiert. Immuntherapeutische Ansätze, die den falschen Zelltyp beeinflussen oder zu einer falschen Zytokinantwort führen, könnten vermutlich nicht zu klinischen Erfolgen führen. Ob die Immuntherapie des malignen Melanoms unter Berücksichtigung der Ergebnisse dieser Arbeit jedoch erfolgreicher wird, bleibt abzuwarten.