Depletion of T lymphocytes is correlated with response to temozolomide in melanoma patients

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Therapeutic strategies to deplete lymphocytes, especially regulatory T cells, in cancer patients have been proposed to increase the benefits of (immuno)chemotherapy. In this study, we explored the influence of temozolomide (TMZ) on different T-cell populations and addressed if the depletion of CD4$^+$ T cells would be associated to the clinical benefits of TMZ. Patients were treated with TMZ (150 mg/m^2 daily, every two weeks on a 4-week schedule) until disease progression. Changes in T-lymphocyte subsets were characterized by flow cytometry. All patients enrolled in this study had histologically verified unresectable Stage IV melanoma. Objective responses were induced in 12.5% of the patients, while 42.5% of them obtained short-term disease stabilization. The median progression-free survival (PFS) of this patient cohort was 8.7 mo. Lymphopenia (< 0.7 × 10^9 cells/L, grade 2) developed in 71% of the patients after 3 treatment cycles (~100 d). The development of grade 2 lymphopenia after the 3rd cycle of therapy positively correlated with clinical outcome (p = 0.01), and was linked, though non-significantly, to prolonged median PFS (303 vs. 200 d). In addition, significant changes in CD8$^+$ T-cell subgroups were observed, notably a shift from naïve T cells toward more differentiated memory T cells. Finally, we demonstrated that specific CD8$^+$ T-cell responses against selected tumor associated antigens (TAAs) were enhanced by the administration of TMZ (p = 0.04), while virus-specific T-cell responses were stable. Thus, immunological monitoring in the course of TMZ treatment might become an important tool for clinical guidance in the future.

Introduction

The incidence of melanoma has steadily increased over the past 50 y in most fair-skinned populations in Europe, especially among people living in Nordic countries. Though the survival of such melanoma patients has reportedly improved in the last decades, the mortality-dependent mortality rate continues to rise in Europe.[1,2] The prognosis of patients affected by metastatic melanoma is poor, with a 5 year survival of approximately 10% and a median survival of 6–9 mo.[3]

Temozolomide (TMZ) is a cytotoxic alkylating agent used in the treatment of metastatic melanoma. TMZ is advantageous in that it can be administered orally and as it penetrates the blood-brain barrier. Moreover, TMZ has comparable efficacy to dacarbazine (DTIC).[4,5] As a standalone intervention against metastatic melanoma, TMZ is associated with an objective response rate of 4–20%.[6–9] In Denmark, TMZ is also used as a systemic therapy in selected metastatic melanoma patients.

Different dosing regimens of TMZ have been investigated, including (1) five consecutive days of treatment in a 28-d cycle (5/28-d schedule), (2) seven consecutive days of treatment alternating with 7 drug-free days (14/28-d schedule), (3) 21 consecutive days of treatment in a 28 d cycle (21/28-d schedule), and (4) a 6-week continuous, extended dosing followed by a 2-week stop, every 8 weeks (8-week schedule).[10] An unexpected high incidence of lymphopenia (60%) and occasional opportunistic infections have been retrospectively associated with the 8-week schedule back in 2004.[11] This tendency was confirmed prospectively by Rietschel et al. in 2008.[12] In our study, 150 mg/m^2 TMZ was given as a day 1–7 plus day 15–21 in a 28 d cycle (14/28-d schedule), which is the standard dosing for treatment of melanoma patients in Denmark.

Regulatory T cells (Tregs) are known to participate in the suppression of antitumor immune responses as mediated by multiple mechanisms.[13] Increased levels of Tregs in both the peripheral blood and the tumor microenvironment have been shown to negatively correlate with clinical outcome in patients affected by a variety of neoplasms. Thus, Tregs seem to be a major obstacle against the success of immunotherapy, and recent data suggest that Treg-eliminating strategies might constitute an important therapeutic tool.[14] Recently, Rosenberg et al. have demonstrated that the addition of lymphodepleting cytotoxic regimens to adoptive T-cell transfer lead to impressive clinical responses in melanoma patients.[15] TMZ-induced CD4+ lymphopenia might also be advantageous if the depletion mainly involve Tregs. This study was designed to address how 150 mg/m^2 TMZ given in a 14/28 d schedule affects the circulating levels of different T-cell subsets. We assessed changes in frequency and absolute counts of different T-cell subsets, i.e., CD3+ cells, CD4+ cells, Tregs, CD49d- Tregs, CD8+ cells and various CD8+ cell memory subsets including central memory (Tcm), CD45RA- effector memory (Tem), CD45RA+ effector memory (Temra) T cells, and of immunosuppressive myeloid-derived suppressor cells (MDSC), and evaluated if these changes were associated with clinical responses to TMZ treatment. Moreover, we detected CD8+ T-cell responses specific for viral antigens and melanoma-associated antigens in patients before and after TMZ therapy.

Results

Demographics. Forty patients with metastatic melanoma were enrolled in this prospective study. All patients received at least 3 cycles of TMZ and were evaluated for clinical responses using CT scans. Among these 40 patients, 6 patients were excluded from immunological assessments due to concomitant treatment with prednisone. The study population, including 3 patients with uveal melanoma, was constituted of 19 women and 21 men with a median age of 67 y (range 39–85 y). TMZ treatment represented the first line systemic regimen in 73% of the patients. Patients were not eligible for other first line systemic treatments due to age (54%), brain metastases (38%) or co-morbidity (8%). Thus, only 6 patients had formerly been treated with high dose interleukin-2/interferon α (IL-2/IFNα) (decrescendo regimen).[16,17] All patients had metastatic Stage IV disease, (M1a = 2, M1b = 9 and M1c = 29 patients), and the average number of completed TMZ cycles were 5.3 (range 3–13 cycles).

Clinical efficacy and overall survival time. The overall objective response rate in this study was 12.5%. Complete responses (CRs) were obtained in 2/40 patients and partial responses (PRs) in 3/40 patients. Disease stabilization (SD) for 3 mo was observed in 14/40 (35%) patients and SD for 6 mo in 3/40 (7.5%) patients. The rate of clinical benefit, defined as CR or PR or SD for at least 6 mo was 8/40 (20%), whereas disease progression (PD) at first evaluation was observed in 18/40 (45%) of the patients.

One patient manifesting a CR developed hemolytic anemia, severe lung infection and died shortly thereafter; whereas the other patient developing a CR has never evolved since, nowadays more than 18 mo. Three patients manifested PRs lasting from 2 to 5 mo and one of them had an almost CR in a brain lesion (**Fig. 1A**). All 3 patients undergoing SD for > 6 mo continue in the same status, nowadays for more than 16, 21 and 7 mo. Median progression-free survival (PFS) was calculated using the Kaplain Meier method[18] (**Fig. 1B**). PFS was defined as the time intervening between the first dose of TMZ and disease progression, death (for any cause) or—if neither of these events—data were censored at the date of last adequate disease assessment. Median PFS was 8.7 mo. The duration of treatment was defined as time intervening between the first and the last dose of TMZ. The median treatment duration was 3.9 mo (range 3.0–16.6 mo) (**Fig. 1C**).

Immunological assessments. Lymphocyte counts were assessed before treatment and after each TMZ cycle. Lymphopenia was graded according to the Common Toxicity Criteria for Adverse Events (CTCAE). Overall, lymphopenia (< 0.7×10^9 cells/L, CTCAE grade 2) developed in 71% of the patients after 3 TMZ cycles (~100 d). The lymphocyte reduction was highly significant p < 0.001 (**Fig. 2**).

In 15 patients, the mean frequencies (%) (**Fig. 3A–D**) and absolute counts (cells/μL) (**Fig. 4A–D**) of CD3+, CD8+, CD4+ and Tregs (defined as CD4+,CD25^{high}, CD127-) before and after the first and second cycles of TMZ were analyzed. After the first TMZ cycle, the frequencies and absolute numbers of CD3+ and CD8+ T cells were increased, whereas the frequencies and absolute counts of CD4+ T cells and Tregs decreased. After the second TMZ cycle, CD8+ cells kept increasing significantly and CD4+ cells kept decreasing significantly. The frequency of Tregs was non-significantly increased after the second TMZ cycle, whereas the absolute numbers of Tregs were lower after the second TMZ cycle than after the first one, though this decrease did not reach the threshold for statistical significance (p = 0.2).

Recently, a subpopulation of Tregs that do not express the surface marker CD49 was suggested to exert potent immunosuppressive effects, hence constituting "true Tregs."[19] Thus, to further characterize the Treg population we specifically quantified CD49d- Tregs, finding the percentage of CD49d- Tregs before TMZ treatment to be 3.9% (range 1.9–7.3%), and after TMZ treatment to be 3.6% (range 1.6–7.7%). Our data suggest that CD49d- Tregs undergo a small, non-significant decrease, in melanoma patients receiving TMZ (data not shown).

Another highly potent immunosuppressive cell population is constitute by myeloid-derived suppressor cells (MDSCs), which have been defined as HLA-DR-Lin-(CD3-CD19-CD56-CD33+CD11b+) cells. MDSCs can be further divided into two populations: monocytic MDSCs, which express CD14, and granulocytic MDSCs, which express CD15.[20] The percentage of monocytic and granulocytic MDSCs was not altered in our patient cohort by the administration of TMZ (data not shown).

T-cell alterations correlate to clinical response. Thirty-four patients were evaluable for the precise characterization of lymphopenia (i.e., they did not receive prednisone) as well as for clinical responses (by CT scans) after 3rd cycles of TMZ (**Fig. 5A**). We found a significant positive correlation between lymphocyte reduction and response to treatment (p = 0.01, Fisher exact test). After 3 cycles of TMZ, two patients subgroups, that
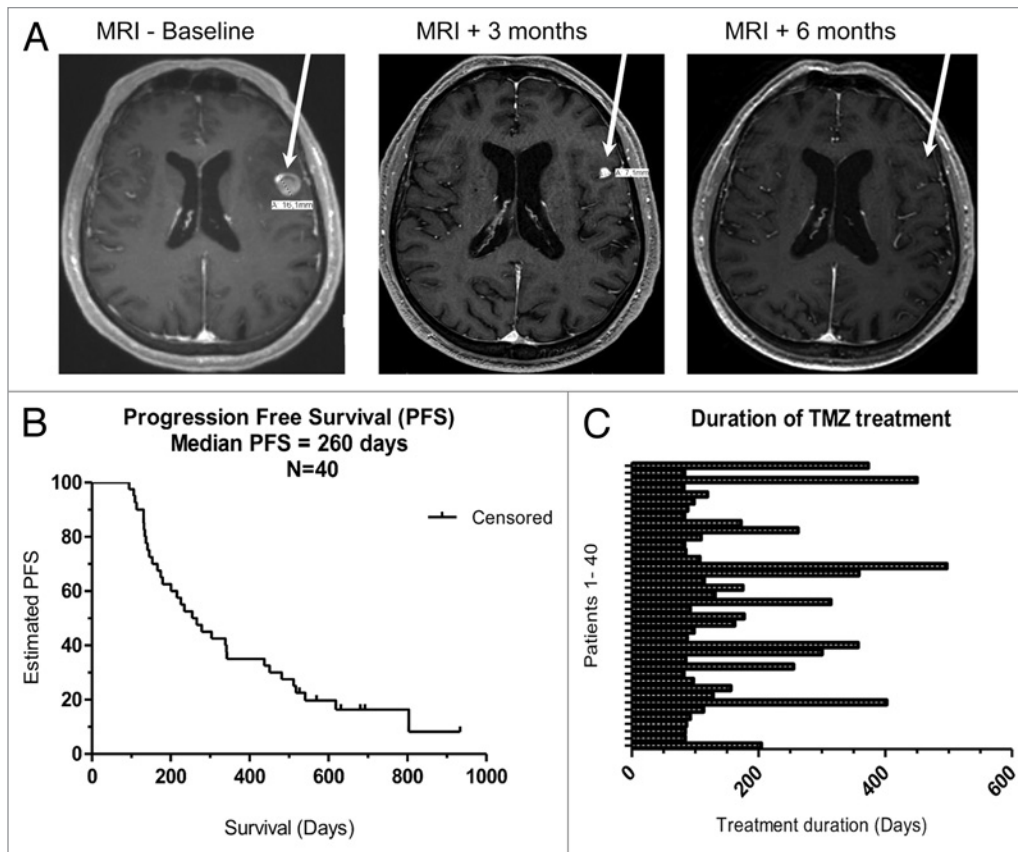
Figure 1. Clinical efficacy and overall survival of melanoma patients treated with temolozomide. (**A**) Magnetic resonance imaging (MRI) scans from a patient undergoing a partial response and demonstrating an almost complete response in a brain lesion after 6 mo of temolozomide (TMZ) treatment. (**B**) Kaplan-Meier plot of progression-free survival (PFS) for all patients included in this study (n = 40). Median PFS was 260 d. PFS was defined as the time intervening between the first dose of TMZ until disease progression or death from any cause. When no progression or death occurred, data were censored at the date of last adequate disease assessment. (**C**) Duration of TMZ treatment for all enrolled patients. The duration of treatment was defined as time intervening between the first and the last dose of TMZ. The mean treatment duration was 173 d (95% CI = 135.4–210.3), corresponding to ~5 cycles of TMZ treatment.

is, lymphocyte responders (defined as individuals with lymphocyte counts $\leq 0.7 \times 10^9$ cells/L; n = 23 patients) and lymphocyte non-responders (defined as subjects with lymphocyte counts $> 0.7 \times 10^9$ cells/L; n = 11 patients), showed a non-significantly (p = 0.2, Log-rank test) different median PFS (lymphocyte responders vs. non-responders 303 vs. 200 d (7 mo) (**Fig. 5B**).

To evaluate the impact of Tregs, we compared the Treg/CD4+ T-cell ratio in clinical responders (CR+PR+SD) and non-responders (PD). In responders the pre-treatment ratio was 0.02, after the first cycle 0.03, and after the second cycle 0.04, whereas the ratio of non-responders remained stable at 0.02 throughout TMZ cycles, suggesting perhaps that Tregs decrease in clinical responders. To characterize if clinical responders had a superior CD8+ T-cell profile than non-responders, the CD8+/CD4+ T-cell ratio was calculated during the first 2 cycles of TMZ. The CD8+/CD4+ T-cell ratio in clinical responders before treatment was 1.7, after the first cycle 2.9, and after the second cycle 5.22, whereas non-responders displayed a CD8+/CD4+ T-cell ratio before TMZ of 2.15, after the first cycle of 2.35 and after the second cycle of 3.16, indicating a comparatively stronger CD8+ T-cell profile among clinical responders (**Fig. 6A**). In addition,

we investigated the repertoire of distinct populations of CD8+ T lymphocytes, including naïve T cells as well as antigen-experienced Tcm, Tem and Temra, as described by Sallusto et al.[21] The frequencies of CD8+ naïve, Tcm, Tem, Temra T cells were measured in the course of TMZ treatment (n = 15). We found a significant decrease in the frequency of CD8+ naïve T cells (p = 0.05), and a significant increase in the frequency of the differentiated CD8+ T cells (i.e., CD8+ Tcm plus Tem plus Temra T cells; p = 0.02) after 2 cycles of TMZ (**Fig. 6B and C**).

We then analyzed CD8+ T-cell immune responses in melanoma patients before and after chemotherapy, focusing on a panel of 27 peptides that have recently been described as targets for melanoma infiltrating lymphocytes.[22,23] Peptides were selected from a library of previously described melanoma-associated antigens, including overexpressed, differentiation-associated and cancer-testis antigens presented on HLA-A1, HLA-A2, HLA-A3 and HLA-B7.[23] We also included a number of viral epitopes, to monitor the general status of the immune system. To screen the reactivity against these peptides using minimal amounts of patient material, we took advantage of the recently described technique for the combinatorial encoding of specific T-cells by fluorescent
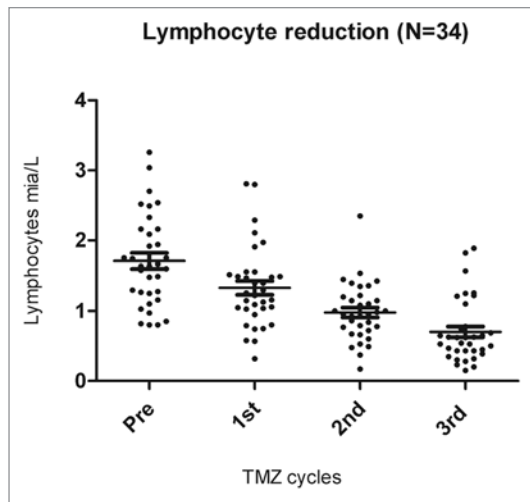
Figure 2. Lymphopenia induced by temolozomide in melanoma patients. Lymphocyte counts on freshly drawn blood samples (as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first, second and 3rd cycles of chemotherapy. **p < 0.01; ***p < 0.001 (paired Student's t-test).

MHC multimers[22,24] (**Fig. 7A and B**). In 7 patients, MHC multimer staining was performed to investigate if CD8+ T-cell responses against the selected peptides were influenced by TMZ. The majority of the patients analyzed (6/7) expressed HLA-A2 molecules, constituting the restriction element for most selected peptides (**Fig. 7A**). In 5/7 patients, we demonstrated significantly enhanced T-cell responses against different TAAs (p = 0.04), whereas in the remaining 2 patients no responses against TAAs were detected. In 5/7 patients, we also demonstrated preserved T-cell responses against selected viral epitopes after TMZ treatment (**Fig. 7B–D**). There was no correlation between the capability of patient material from these 7 individuals to recognize TAAs and clinical outcome.

Discussion

To improve the selection of patients that are likely to respond to therapy as well as to combine and develop new therapeutic approaches against melanoma, it is important to identify relevant biomarkers. In this sense, we estimated of value assessing T-cell subpopulations in the course of cytotoxic TMZ treatment. We have formerly shown that the levels of Tregs are lowered in patients receiving TMZ before entering an experimental dendritic cell-based vaccine protocol. In the present study, we have determined changes in the frequency and absolute abundance of different T-cell subsets in the peripheral blood of melanoma patients treated with TMZ (Danish standard doses) and compared these changes to clinical response.

Previously, it has been shown that TMZ given as a continuous dosing (8-week schedule) induces lymphopenia.[11,12] Rietschel et al. found that 75% of these patients develop lymphopenia after the 3rd cycle of TMZ, and that the incidence of lymphopenia increases with additional cycles. The same authors also reported that TMZ induces a steady decline of total CD3+ cells, mainly

as a result of CD4+ T-cell depletion, whereas the CD8+ T-cell population seems relatively less affected by TMZ.[12]

Here, we have demonstrated a statistically significant reduction of T lymphocytes in melanoma patients after 3 cycles of TMZ, which is in concordance with the hypothesis of the study. Moreover, we have shown that the development of ≥ Grade 2 lymphopenia after the 3rd TMZ cycle positively correlates with clinical response. Recently, we have shown a similar correlation between lymphopenia and clinical outcome in Stage IV melanoma patients receiving both TMZ and thalidomide.[25] In the present study, we have also shown that PFS is not correlated with treatment-dependent lymphopenia (< 0.7×10^9 cells/L).

It is generally accepted that Tregs influence oncogenesis and tumor progression, and that the host immune system plays a critical role in therapeutic responses. Treg-depleting strategies to achieve potent anticancer immune responses with therapeutic impact have been investigated by other groups. Targeting CD25 with denileukin has resulted in the partial depletion of circulating Tregs, resulting in an improved stimulation of tumor-specific T cells.[26] Rasku et al. have found that denileukin breaks peripheral tolerance and allow for the expansion of the effector T cells.[27] Golovina et al. have studied the clinical use of the anti-CD25 antibody daclizumab and demonstrated that Tregs are efficiently eliminated by this approach, suggesting that daclizumab might also be effective in modulating Tregs.[28] In our study, we focused on both the frequency and absolute abundance of Tregs and CD4+ T lymphocytes during TMZ treatment. We found decreasing levels of both CD4+ T cells and Tregs after the first cycle of therapy. However, after the second cycle the absolute counts of Tregs kept decreasing whereas their frequency had increased. This increase in frequency may be due to a global depletion of CD4+ T cells or to the re-expansion/re-trafficking of T cells from lymph nodes, as recently suggested by Rasku et al.[27] Surprisingly, even though a general depletion of CD4+ T cells and Tregs occurred, we found a Treg/CD4+ T-cell ratio favoring Tregs in clinical responders to TMZ therapy, which was not seen among non-responders.

Kleinewietfield et al. have shown how anti-CD49d antibodies can be an important tool in defining "true Tregs," as it allows for the removal of CD49d+CD127+ cells to effector cells from Tregs.[19] We have previously shown that Tregs with proven inhibitory function can be determined by CD49d expression on the cells surface.[29] In the present study, we performed a similar CD49d staining, which revealed only a marginal decline of this Treg population in the course of TMZ treatment.

Furthermore, we found increasing levels of CD8+ T cells. This suggests that the CD8+ T-cell population might be able to expand even though CD4+ cells are reduced. Importantly, we observed that the CD8+/CD4+ T-cell ratio was higher among clinical responders than in non-responders, suggesting that a better CD8+ T-cell profile might be advantageous for therapeutic responses. In this context, the depletion of T cells bearing a non-specific T-cell receptor (TCR) might generate space for development of a better-primed T-cell population bearing tumor-specific TCRs.[27]

To further investigate if a tumor-specific immune response was induced in the course of TMZ treatment, we analyzed the
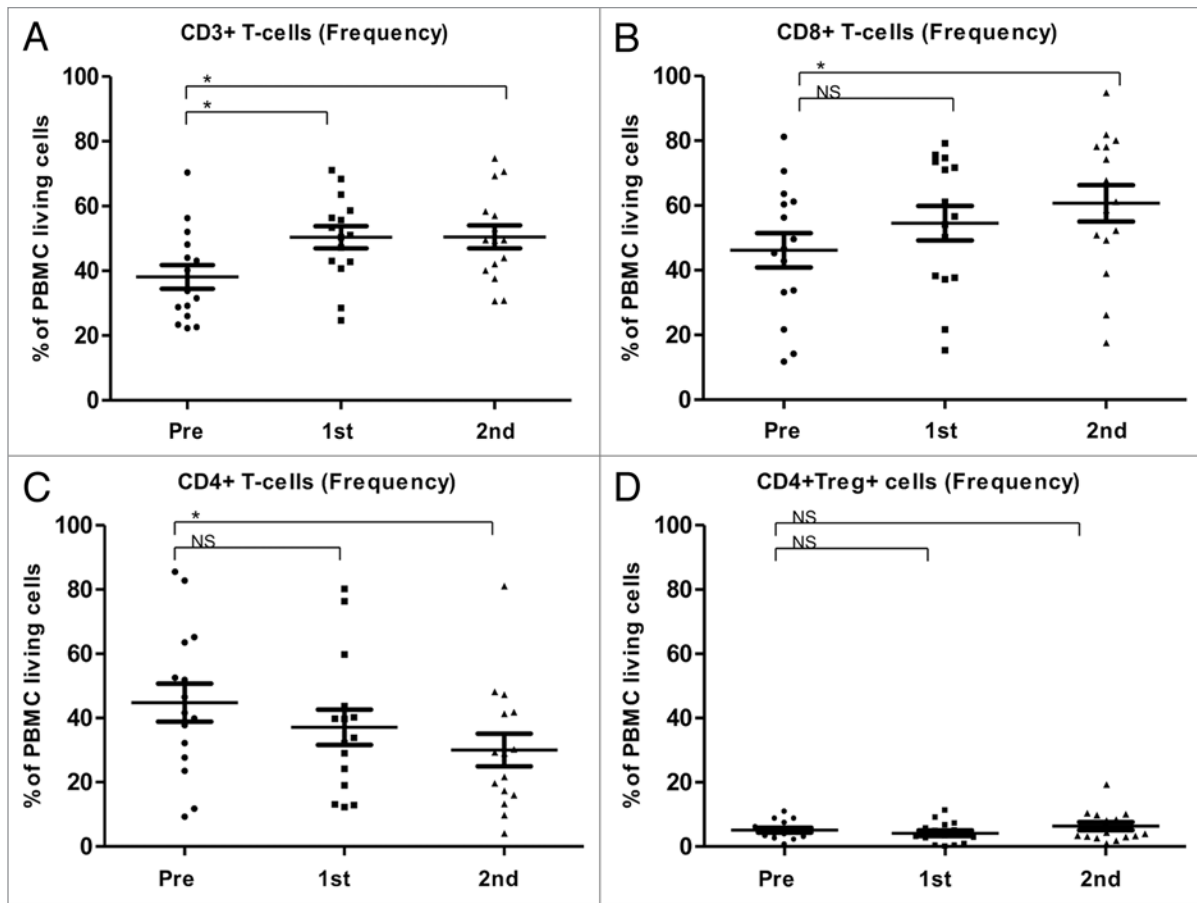
Figure 3. Alterations of the frequency T-cell subsets as induced by temolozomide in melanoma patients. (**A–D**) Frequency of T cell subpopulations on frozen/thawed peripheral blood mononuclear cells (PBMCs, as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first and second cycles of chemotherapy: *p < 0.05; NS = non-significant (paired Student's t-test).

capacity of patient material to recognize a library of TAAs-derived peptides that are known to be recognized by melanoma-infiltrating lymphocytes.[23,30] In 7 patients, we assessed TAA-specific T-cell populations by MHC multimer combinatorial encoding. We found that CD8+ T-cell responses against TAAs are significantly enhanced post TMZ, whereas virus-targeting CD8+ T-cell responses were stable, suggesting that the chemotherapy-induced killing of cancer cells might release TAAs that can be presented by antigen-presenting cells and hence elicit significant T-cell responses. As suggested by Galluzzi et al.,[31] classical cytotoxic agents may stimulate tumor-specific immune responses by inducing immunogenic cell death, a peculiar form of apoptosis involving the release of ATP and activating numerous immune effector mechanisms. This lends support to the idea of combining TMZ with anticancer vaccine strategies.

In general, we found that the absolute lymphocyte counts in our patients were rather low, probably due to their age (mean = 67 y). In line with this notion, Rech et al. found that absolute lymphocyte and CD4+ T-cell counts are low (< 200 cells/µL) in cancer patients treated with adjuvant chemotherapy or having metastatic disease as compared with healthy donors.[32] We found that the CD4+ T-cell population is more vulnerable to freezing/thawing procedures than its CD8+ counterpart, emphasizing the need

for using a marker of cell death when performing T-cell assays. Rasku et al. have also observed that the CD4+ T-cell population is more fragile than CD8+ T cells.[27]

We also investigated the repertoire of antigen-naïve CD8+ T cells as well as of distinct populations of antigen-experienced CD8+ T lymphocytes, including Tcm, Tem and Temra. We demonstrated a significant decrease of naïve CD8+ T cells along with a significant increase of differentiated CD8+ T cells (Temra plus Tcm plus Tem) after 2 cycles of TMZ, that is, a rise in the mature CD8+ compartment at the expenses of naïve CD8+ cells. It is well known that anti-melanoma CD8+ T cells are primed against cancer during oncogenesis and disease progression. Hence, an increase in these CD8+ cell populations might be critical for therapeutic responses. The naïve CD8+/differentiated CD8+ T-cell ratio was similarly reduced in the course of treatment in both clinical responders and non-responders. In our experience, the profile of CD8+ memory cells during TMZ treatment has not yet been investigated by others, and additional insights into memory cell subsets, including information on their antigen specificity, is needed to elucidate the importance of our data.

Another cell population that influence the therapeutic response of cancer patients, due to their potent immunosuppressive activity, is constituted by MDSCs. Solito et al. have demonstrated that
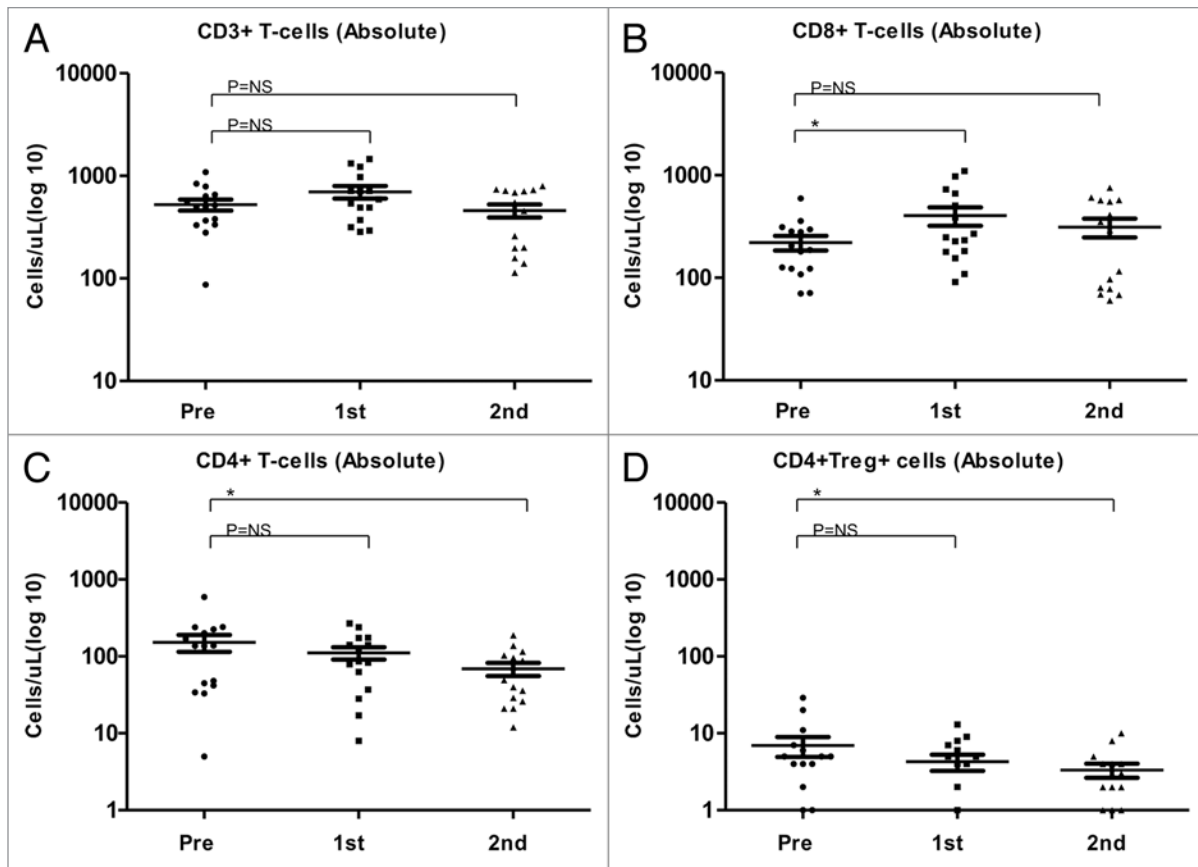
Figure 4. Alterations of the absolute abundance of T-cell subsets as induced by temolozomide in melanoma patients. (**A–D**) Absolute T-cell counts on frozen/thawed peripheral blood mononuclear cells (PBMCs, as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first and second cycles of chemotherapy. *p < 0.05; NS = non-significant (paired Student's t-test).

increased circulating levels of MDSCs correlate with worsened prognosis in patients affected by colorectal cancer,[20] though an unambiguous phenotype of MDSCs exerting immunosuppressive functions is intensively discussed. A recent review of murine and human MDSCs suggests that MDSCs mainly consist of a monocytic population characterized by the expression of CD14 but not CD15.[33] Finkelstein et al. have investigated the influence of MDSCs in renal cell carcinoma and melanoma patients treated with high-dose IL-2, finding increased levels of MDSCs after treatment even in patients with the most favorable clinical outcome.[34] In our study, we observed that the levels of monocytic and granulocytic MDSCs are unaffected in metastatic melanoma patients receiving TMZ.

The clinical efficacy of TMZ in our cohort (PFS = 8.7 mo and objective RR = 12.5%) was in accordance with previous studies in similar clinical settings.[12,35] One patient still manifests an ongoing CR, nowadays for more than 18 mo, and all 3 patients exhibiting SD for more than 6 mo have not yet progressed, nowadays exhibiting SD for more than 16, 21 and 7 mo. CRs in Stage IV melanoma patients treated with TMZ/DTIC do occur and are occasionally durable, though very infrequent.[11,12] Long-term survival after TMZ/DTIC treatment not always is induced by chemotherapy, but rather can results from host factors or from an indolent disease. Presumably, patients bearing indolent disease are

those who benefit the most from immunotherapy.[4] This suggests that TMZ treatment may be suitable for combination with other immunotherapies, e.g., melanoma antigen-specific vaccines. Kyte et al. have reported that the administration of TMZ together with hTERT-derived peptide vaccine resulted in one long-term survivor (> 5 y, PET-CT scan negative).[36] At our institution, we have set up a new clinical trial to investigate the combination of TMZ and a peptide vaccine in melanoma patients (NCT01543464).

TMZ has been shown to exert clinical efficacy in patients affected by aggressive tumors such as Stage IV melanoma. Here, we demonstrated that ≥ Grade 2 lymphodepletion after the 3rd TMZ cycle correlates with treatment response and that median PFS does not change significantly between lymphocyte responders and non-responders. In clinical responders, TMZ induced a significant increase in CD8+ T-cell memory subsets along with a drop in naïve T cells and a beneficial CD8+ T-cell profile. Furthermore, we proved that CD8+ T-cell responses specific for selected viral antigens and TAAs were preserved or even enhanced after TMZ treatment. Though these data need validation, our study indicates that the modulation of specific differentiated T-lymphocyte subsets could be important for the clinical efficacy of systemic chemotherapies. However, further studies are required to elucidate if CD4+ T-cell depletion in general provides clinical benefits to melanoma patients.
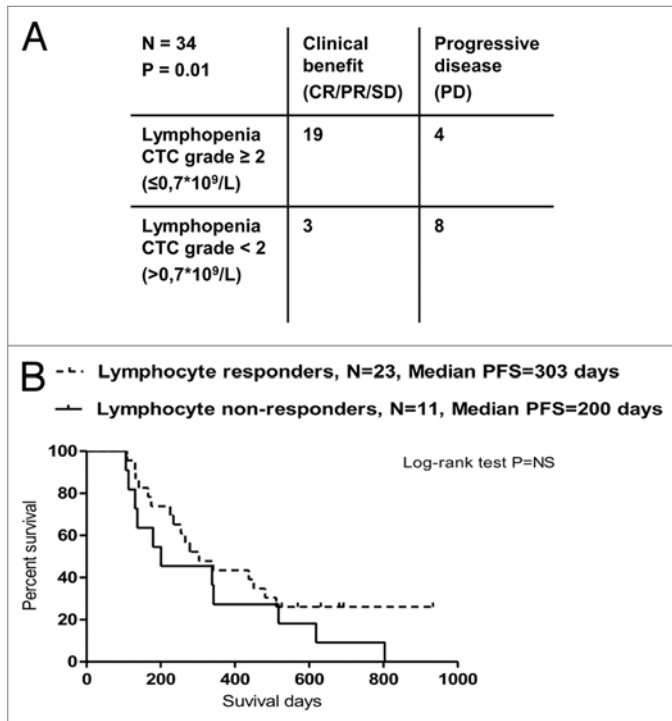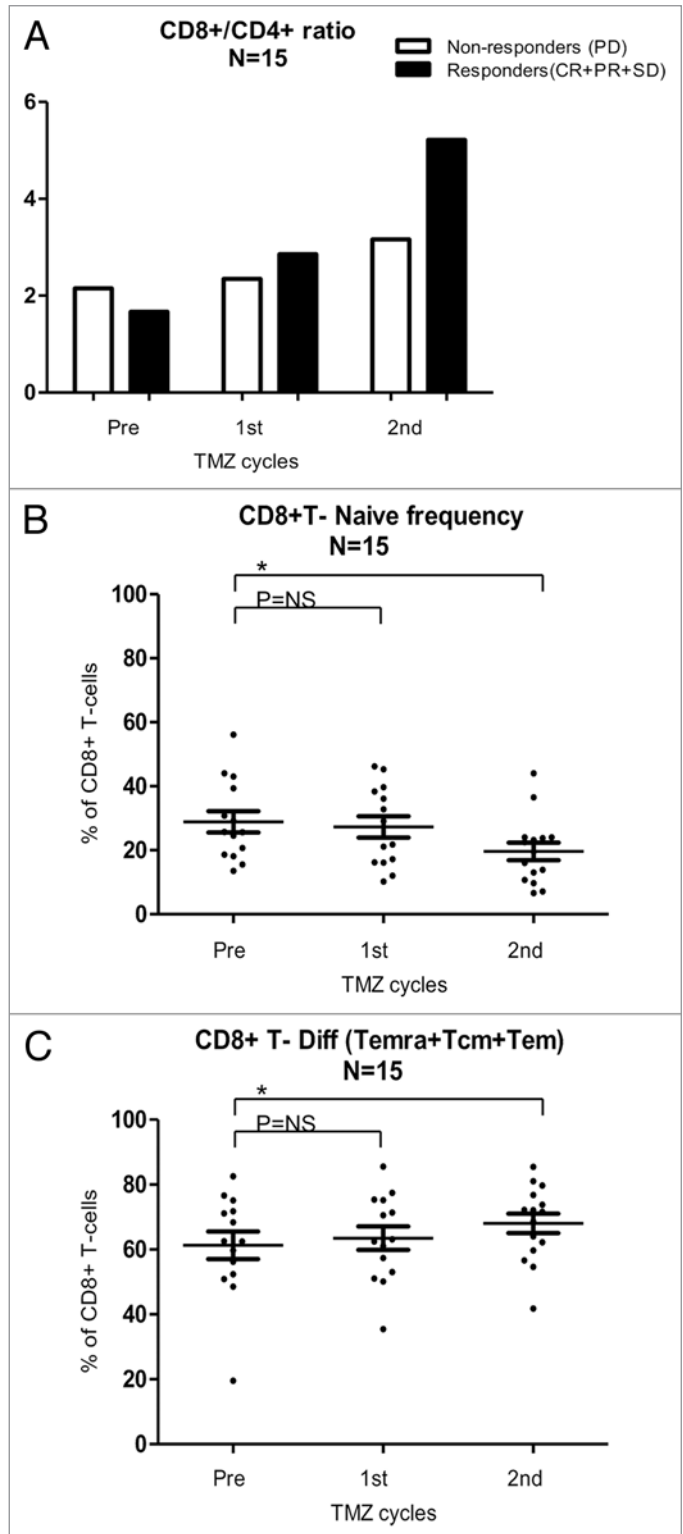
Figure 5. Correlation between temolozomide-induced lymphopenia and clinical parameters. (**A**) Correlation between the grade of lymphopenia (as determined on freshly drawned blood samples) and the response of n = 34 melanoma patients to 3 cycles of temolozomide (TMZ). p = 0.01 (Fischer's exact test). Lymphocyte counts were done directly on. (**B**) Progression-free survival (PFS) of lymphocyte responders (defined as individuals with lymphocyte counts ≤ 0.7 × 10⁹ cells/L, n = 23) and lymphocyte non-responders (defined as individuals with lymphocyte counts > 0.7 × 10⁹ cells/L, n = 11). The Kaplan-Meier estimate demonstrates a non-significantly (p = 0.2, Log-Rank test) prolongation in median PFS in lymphocyte responders (303 d) vs. lymphocyte non-responders (200 d).

Figure 6 (right). CD8^{+} T-cell subsets in melanoma patients responding or not to temolozomide. (**A**) CD8^{+}/CD4^{+} T-cell ratio (as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first and second cycles of chemotherapy to clinical responders, exhibiting either complete response (CR), either partial response (PR) or stable disease (SD), and clinical non-responders, exhibiting progressive disease (PD). (**B**) Frequency of naïve CD8^{+} T cells (as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first and second cycles of chemotherapy. *p < 0.05; NS = non-significant (paired Student's t-test). (**C**) Frequency of differentiated CD8^{+} T cell (Tcm plus Tem plus Terma) (as assessed by flow cytometry) before the administration of temolozomide (TMD) and after the first and second cycles of chemotherapy. *p = 0.02; NS = non-significant (paired Student's t-test).

Material and Methods

Patients and blood samples. Patients with Stage IV metastatic melanoma planned for TMZ therapy were eligible. All included patients fulfilled the following criteria: ≥ 18 y of age, histological confirmation of unresectable American Joint Committee on Cancer (AJCC) Stage IV melanoma with progressive disease according to RECIST v. 1.0, and a World Health Organisation (WHO) performance status of 0–2. Patients were treated daily with 150 mg/m² TMZ for 7 d every second week in 4-week cycles until unacceptable toxicity, progression of disease or patients wish. Peripheral blood samples of 30 mL were collected prospectively before the initiation of the treatment and after the first and second TMZ cycle. The protocol was approved by the Ethics
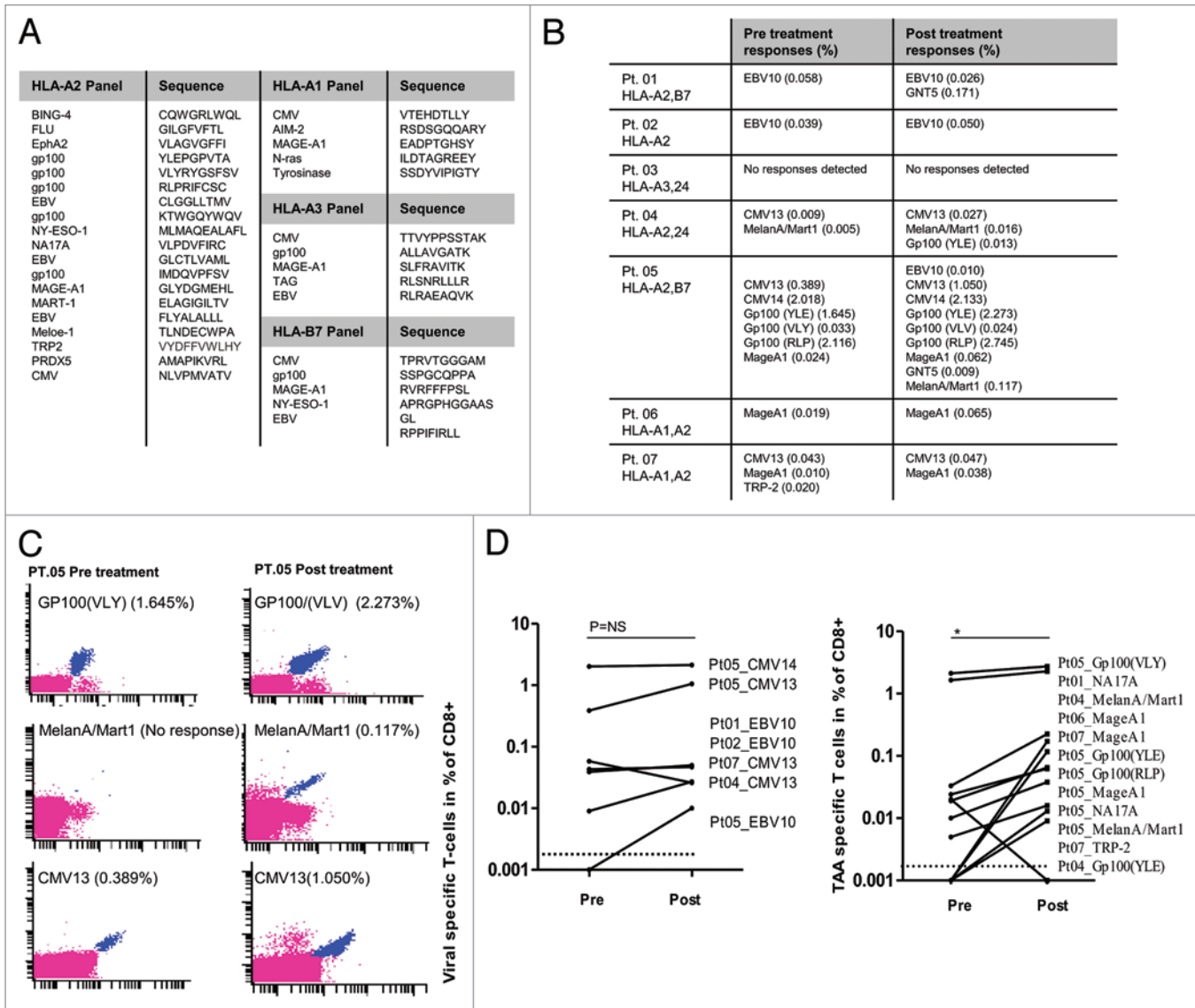
Figure 7. Tumor-associated antigen- and viral antigen-specific T-cell responses in melanoma patients receiving temolozomide. (**A**) Peptide panel used for screening T-cell responses in patients with tissue types HLA-A1, A2, A3 and B7. (**B**) Responses to viral and tumor-associated antigens evaluated before and after temolozomide (TMD) chemotherapy in n = 7 melanoma patients. (**C**) Examples of pre and post-chemotherapy T-cell responses in-patient 05, as detected by flow cytometry with MHC multimers. CD8+ T-cell responses were gated on living single CD8+ T cells (mean number of CD8+ cells for all experiments = 73,655; range = 8056–238,576). MHC multimer positive populations are depicted in blue, from the top recognizing (1) Gp100(VLY), labeled by PE and Q655, (2) MelanA/Mart1, labeled by Q585 and Q605 and (3) CMV13, labeled by Q605 and Pe-Cy7. (**D**) Viral antigen- and tumor antigen-specific responses pre- and post-TMZ chemotherapy. *p = 0.04; NS = non-significant (paired Student's t-test). The dotted line represents the detection limit = 0.002% CD8+ T cells.

Committee for The Capital Region of Denmark (H-A-2007–0124) and all patients gave written informed consent. The study was conducted in accordance with the Helsinki Declaration of 1975. Project blood samples were drawn simultaneously with routine blood samples during TMZ treatment. For multimer MHC combinatorial coding, tissue typing of 7 patients was performed by flow cytometry and PCR, screening patients for the expression of HLA-A1, 2, 3, 24 and B7. The resultant HLA expression pattern is provided in **Figure 7C**.

Cytofluorometric analyzes. *PBMCs.* PBMCs were obtained from the peripheral blood by gradient centrifugation according to the Lymphoprep technique. Isolated cells were frozen immediately with 10% DMSO and 90% humanised AB-serum and stored at -140°C.

CD4+/Treg assays. PBMCs were thawed and stained for cytofluorometric analyzis with a FACSCanto II cytometer (BD bioscience). Briefly, PBMCs were thawed and incubated with mouse serum (Invitrogen) followed by labeling with the following fluorochrome–conjugated antibodies or relevant isotype (surface antigens): AMCYAN-CD3 (BD bioscience), PerCP-CD4 (BD Bioscience), APC-CD25 (E-bioscience), FITC-CD27 (E-bioscience). PE-FOXP3 (E-bioscience) and isotype PE

rat IgG2a (E-bioscience) were used for intracellular staining. In this context, cells were fixed and permeabilized using a fixation/permeabilization kit (E-bioscience), according to manufactures instruction. The APC-Cy7 near IR (Invitrogen) fluorescent reactive dye was used as a dead-cell marker.

CD8+ naive, Tcm, Tem and Temra T-cell assays. For surface antigen staining the following antibodies were used: APC-CD3 (BD bioscience), FITC-CD4 (BD bioscience), PE-CD8 (BD bioscience), PECy7-CCR7 (BD PharMingen), HorizonV450-CD45RA (BD Bioscience) and the relevant isotype controls.

Treg CD4+CD49d− assays. For surface antigen staining the following antibodies were used: APC-Cy7-CD3 (BD bioscience), PerCP-CD4 (BD bioscience), Pacific blue-CD8 (DAKO), APC-CD25 (Ebioscience), FITC-CD127 (Ebioscience) and PE-CD49d (BD bioscience) and the relevant isotype controls.

Myeloid-derived suppressor cells (MDSC) assays. Cells were incubated with mouse serum (Invitrogen) to prevent unspecific labeling, and for surface antigen staining the following antibodies were used: PerCP-HLADR lineage (BD Bioscience), Pe-Cy7-CD3 lineage (BD PharMingen), Pe-Cy7-CD19 lineage (BD PharMingen) and Pe-Cy7-CD56 lineage (BD PharMingen), PE-CD33 (DAKO), APC-CD11b (BD PharMingen), APC-Cy7-CD14 (BD PharMingen) and Pacific blue-CD15 (Biolegend). For these assays, 200,000 lymphocytes were collected and gated on forward and side scatter profiles.

Combinatorial encoding with MHC multimers. Cells were thawed in Pulmozyme buffer and washed twice, then resuspended in FACS-PBS buffer and distributed into 96-well plates before centrifugation. A panel of 27 MHC multimers (each specificity having a specific color combination, 2 μL per specificity produced, as indicated below) were mixed with cells and incubated 10 min at 37°C, then the relevant fluorochrome-conjugated antibodies and dead-cell marker were added for further incubation, 30 min at 4°C. We used PerCP-CD8 (Invitrogen) and a dump channel (FITC), including: FITC-CD4 (BD Bioscience), FITC-CD14 (BD Bioscience), FITC-CD19 (BD Bioscience), FITC-CD40 (Serotec) and FITC-CD16 (BD Bioscience). Analyzes were recorded on an LSRII SORP cytometer and data were analyzed with FACSDiva Version 6.1.3 (BD Bioscience).

MHC multimer production. UV-sensitive ligands were synthesized, and recombinant human HLA-A1 and -A2 heavy chains as well as the human β₂-microglobulin light chain were produced in *Escherichia coli*, then refolded with the conditional ligand, and purified.[37] Specific peptide-MHC complexes were generated using the MHC peptide exchange technology. MHC multimers were produced by multimerization of the generated peptide-MHC complexes using eight different fluorescent-streptavidin (SA) conjugates, SA-PE, SA-APC and SA-PE-Cy7 (Biolegend), and Qdot 585, 605, 625, 655 and 705 (Invitrogen), as described. MHC multimers were frozen in 0.5% bovine serum albumin (BSA) and 16% glycerol. The final concentration of the MHC multimers was 100 μg/mL.[22,24]

Clinical assessment of response. Clinical response evaluation was performed by computed axial tomography of the chest and abdomen (CT scans) and evaluated by Response Evaluation Criteria In Solid Tumors (RECIST v. 1.0.). CT scans were performed before treatment start (baseline) and every 3rd month until disease progression or death. In patients with brain metastases, complementary magnetic resonance imaging (MRI scan) of the brain was performed within 4 weeks from the beginning of the treatment and every three treatment cycles thereafter.

Statistical procedures. All data were evaluated using the GraphPad Prism Software version 5. Individually serial samples of T-lymphocyte subsets were assessed using paired Student's t-test and results are given as means with 95% confidence intervals (CI). For the correlation of lymphocyte counts to treatment response, the Fischer exact test was used. Survival statistics for PFS were performed by the Kaplan-Meier method. Survival between lymphocyte responders and non-responders was compared by the Log-Rank test. In all analyzes, a two-sided p value < 0.05 was considered statistically significant.

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