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ORIGINAL RESEARCH

Low CD4⁺/CD25⁺/CD127⁻ regulatory T cell- and high INF-γ levels are associated with improved survival of neuroblastoma patients treated with long-term infusion of ch14.18/CHO combined with interleukin-2

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ABSTRACT

Immunotherapy with the anti-GD_2 antibody (Ab) ch14.18/CHO in combination with interleukin 2 (IL-2) has improved survival of high-risk neuroblastoma (NB) patients. Here, we report immunotherapy-related effects on circulating NK cells, regulatory T cells (T_{regs}), granulocytes as well as on Ab-dependent cell-mediated cytotoxicity (ADCC) and cytokines IFN-γ, IL-6, IL-10, IL-18 and CCL2 and their association with progression-free survival (PFS).

In a closed single-center program, 53 patients received five cycles of 6×10^6 IU/m^2 subcutaneous IL-2 (d1-5; 8–12) combined with long-term infusion (LTI) of 100 mg/m^2 ch14.18/CHO (d8-18). Immune cells and cytokines were analyzed by flow cytometry and ADCC by calcein-AM-based cytotoxicity assay.

IL-2 administration increased cytotoxic NK cell-, eosinophil- and T_{reg} counts in cycle 1 (2.9-, 3.1- and 20.7-fold, respectively) followed by further increase in subsequent cycles, whereas neutrophil levels were elevated only after the ch14.18/CHO infusion (2.4-fold change). Serum concentrations of IFN-γ, IL-6, IL-10, IL-18 and CCL2 in cycle 1 were increased during the combinatorial therapy (peak levels of 3,656 ± 655 pg/ml, 162 ± 38 pg/ml, 20.91 ± 4.74 pg/ml, 1,584 ± 196 pg/ml and 2,159 ± 252 pg/ml, respectively). Surprisingly, we did not observe any correlation between NK-, eosinophil- or neutrophil levels and PFS. In contrast, patients with low T_{regs} showed significantly improved PFS compared to those who had high levels. T_{reg} counts negatively correlated with INF-γ serum concentrations and patients with high INF-γ and IL-18 had significantly improved survival compared to those with low levels.

In conclusion, LTI of ch14.18/CHO in combination with IL-2 resulted in T_{reg} induction that inversely correlated with IFN-γ levels and PFS.

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Introduction

Survival of patients with high-risk neuroblastoma (NB) is still poor despite multimodal therapy.[1] The introduction of anti-GD_2 monoclonal antibodies (mAbs) in combination with IL-2 and GM-CSF improved the event-free survival (EFS) of high-risk NB patients by about 20% compared to the standard treatment with isotretinoin,[2] but the majority of patients still experience a relapse of their disease. Therefore, further efforts to improve the treatment are needed.

Anti-GD_2 mAbs act through binding to the tumor-associated antigen GD_2 expressed by NB cells. This results in the attraction of immune cells such as cytotoxic natural killer (NK) cells and granulocytes (neutrophils, eosinophils), that can directly eradicate tumor cells via Ab-dependent cellular cytotoxicity (ADCC) known to be one of the main anti-tumor mechanisms of mAbs.[3–10] The level of ADCC depends on genetic and/or immune-modulating factors, such as polymorphisms in Fc-γ receptors (FCGR), and immune-stimulating cytokines. Indeed, high-risk NB patients with high-affinity polymorphisms of FCGR 2A and −3A that are expressed on neutrophils and cytotoxic NK cells, showed an increased ADCC and an improved EFS when treated with ch14.18/CHO in combination with IL-2 compared to those with low-affinity polymorphisms.[11]

The rationale to combine the immune-stimulating cytokine IL-2 with anti-GD_2 Ab was based on preclinical data showing an IL-2-dependent increase of Ab-mediated effector functions.[12] A prospective randomized phase 3 trial was conducted to assess the role of IL-2 in GD_2-directed immunotherapies.[13] Surprisingly, no improvement in progression-free survival (PFS) and overall survival (OS) was found in patients treated with IL-2 and ch14.18/CHO compared to patients treated with ch14.18/CHO alone.[13] One possible explanation for the observation that co-treatment with IL-2 is of no benefit may be the expression of the IL-2 receptor with high affinity to IL-2 on T_{regs} (CD25, CD122 and CD132) in contrast to NK cells expressing only the IL-2 receptor with intermediate affinity (CD122, CD132).[14,15] Besides the induction of T_{regs}, it has been shown that IL-2 pre-activated eosinophils resulting in increase of ADCC in -vitro.[9] Therefore, we also aimed to investigate effector cells of

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the myeloid lineage, such as neutrophils and eosinophils, in patients receiving ch14.18/CHO in combination with IL-2.

One further aspect to better understand the immune modulation induced by the co-treatment with IL-2 is the cytokine release. Besides exogenous IL-2, the interaction between mAb and effector cells (ADCC) itself induces a strong release of cytokines.[16,17] Therefore, we investigated cytokines marking lymphocyte activation and regulation (IFN-γ and IL-10) and cytokines activating and recruiting myeloid-derived suppressor cells (MDSCs; IL-6, IL-18 and CCL2).[18–22]

In summary, we evaluated circulating immune cells, in particular NK cells with a cytotoxic phenotype ($CD16^+$and $CD56^{dim}$)[23] and T_{regs} ($CD4^+$, $CD25^+$ and $CD127^-$), as well as the cytokines IFN-γ, IL-6, IL-18 and CCL2 and their impact on ADCC and PFS of 53 high-risk NB patients treated with a long-term infusion (LTI) of anti-GD_2 mAb ch14.18/CHO in combination with IL-2.

Results

Patient selection, treatment and analysis

Fifty-three patients with high-risk, primary refractory (≥2 lines of conventional treatment) NB in agreement to the INSS criteria[24,25] were enrolled in a closed single-center compassionate use program.[26] These patients received up to five cycles immunotherapy with each cycle consisting of 5d of 6 × 10^6 IU/m^2/d s.c. IL-2 alone (d1–5), followed by 10d continuous LTI of 10 mg/m^2/d ch14.18/CHO (d8-18) in combination with of 6 × 10^6 IU/m^2/d s.c. IL-2 (d8-12) and 160 mg/m^2/d oral isotretinoin (d19-32) (Figure 1).

We first determined immune cell counts and cytokine serum concentrations during the treatment with IL-2 and ch14.18/CHO. Then, we analyzed the effect of immune cells on d15 in cycle 1 (corresponding to d8 of Ab infusion) on ADCC levels, on PFS and on cytokine release.

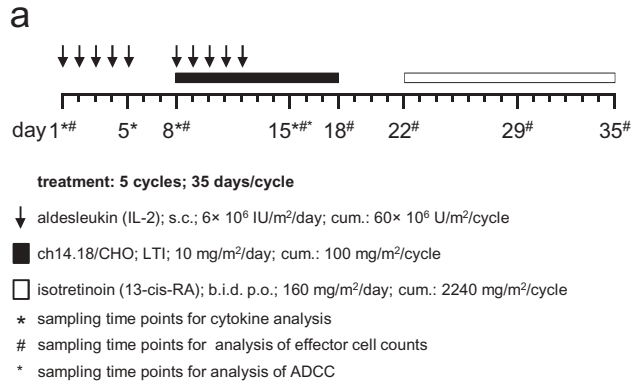

Figure 1. Schematic overview of treatment schedule and sampling time points. Fifty-three patients were enrolled in a closed single-center compassionate use program and received up to five cycles of anti-GD_2-based immunotherapy (35 d/cycle). In every cycle, patients were treated with 5d s.c. IL-2 (aldesleukin, horizontal black bar) given once a day (d1-5, 6 × 10^6 IU/m^2/day) followed by combined treatment of i.v. ch14.18/CHO administered by long-term infusion (d8-18, 10 mg/m^2/d; gray horizontal bar) and s.c. IL-2 (d8-12, 6 × 10^6IU/m^2/d). Starting on d22, treatment was continued with oral isotretinoin (white horizontal bar) given twice a day (b.i.d) (d22-35). Cumulative doses of IL-2, ch14.18/CHO and isotretinoin were 60 × 10^6 IU/m,^{2} 100 mg/m^2 and 2240 mg/m^2 per cycle, respectively. Serum samples for cytokine analysis were collected on d1, 5, 8, 10, 12 and 15 (*). For determination of effector cell counts, EDTA blood samples were collected on d 1, 8, 15, 18, 22, 29 and 35 (#). Sodium-heparin blood samples were collected on d1 and 15 for analysis of ADCC ($).

Impact of IL-2 and ch14.18/CHO treatment on immune cell populations

To show therapy-dependent effects on cytotoxic NK cells, $T_{regs,}$ neutrophils and eosinophils, the cell counts of these cell populations were determined in the blood of treated patients (sampling time points are described in Figure 1, Suppl. Table 1 for evaluable patients).

First, we observed a significant IL-2-dependent induction of NK cell expansion in every cycle after the first round of IL-2 (d8, prior to LTI of ch14.18/CHO combined with the second round of IL-2) compared to the baseline (2.9-, 3.0-, 3.2-, 3.5- and 3.4-fold increase, in cycle 1–5, respectively). Surprisingly, analysis of NK cells on d15 did not reveal any significant change of cell counts compared to d8 in all cycles, despite a second round of five IL-2 injections given in combination with ch14.18/CHO (Figure 2a). Importantly, the trough levels of NK cells with cytotoxic phenotype in subsequent cycles were constantly above the baseline (96 ± 12 cells/µl) (Figure 2a). Next, we observed a remarkable IL-2-dependent increase of T_{reg} counts on d8 in cycle 1 compared to the baseline (11.3- fold; 23 ± 4 cells/µl), followed by further increase in the subsequent cycles (20.7-, 22.9-, 26.0- and 23.4-fold increase on d8 compared to baseline, in cycle 2–5, respectively), indicating a sustained accumulation of these inhibitory cells during the entire treatment period (Figure 2b). Interestingly, the T_{reg} counts during the combined treatment of IL-2 and ch14.18/CHO (d15) remained at similar levels compared to d8 in the first cycle or even decreased in the following cycles (Figure 2b) suggesting a counteractive effect of ch14.18/CHO on further T_{regs} expansion after the second course of IL-2.

In contrast, neutrophil cell counts were reduced after the administration of five s.c. injections of IL-2 (d8) (0.8, 0.7, 0.7, 0.6, 0.8 fold change vs. baseline, for cycles 1–5, respectively) (Figure 2c and D). As expected, in cycle 1, 28% of the patients developed neutrophil levels under the threshold of 1000 cells/µl defined as neutropenia. However, at the end of ch14.18/CHO LTI (d18), a strong increase of neutrophil counts occurred (2.7-, 1.6-, 1.6-, 1.9- and 2.3 fold increase vs. baseline, for cycle 1–5, respectively), indicating a ch14.18/CHO-dependent effect on this cell population (Figure 2c and d). In contrast to neutrophils, we observed an increase of eosinophil counts following IL-2 on d8 (3.1-, 6.4-, 6.0-, 6.3- and 6.3 fold increase vs. baseline for cycle 1–5, respectively) that was further elevated during the combinatorial treatment in all cycles resulting on d15 in eosinophilia (threshold 350 cells/µl)[27,28] in 90% of the treated patients (9.5-, 6.2-, 6.9-, 6.2- and 9.1-fold increases vs. baseline for cycle 1–5, respectively) (Figure 2e). Trough levels on d1 in cycles 2–5 were about 3-fold increase compared to the baseline. Eosinophils reached peak levels at the end of the Ab-infusion which is 5 d after the last IL-2 injection (d18, 19.9-, 13.9-, 9.8-, 8.1- and 13.9-fold increases vs. baseline for cycle 1-5, respectively) suggesting effects of both IL-2 and ch14.18/CHO on eosinophil cell counts.

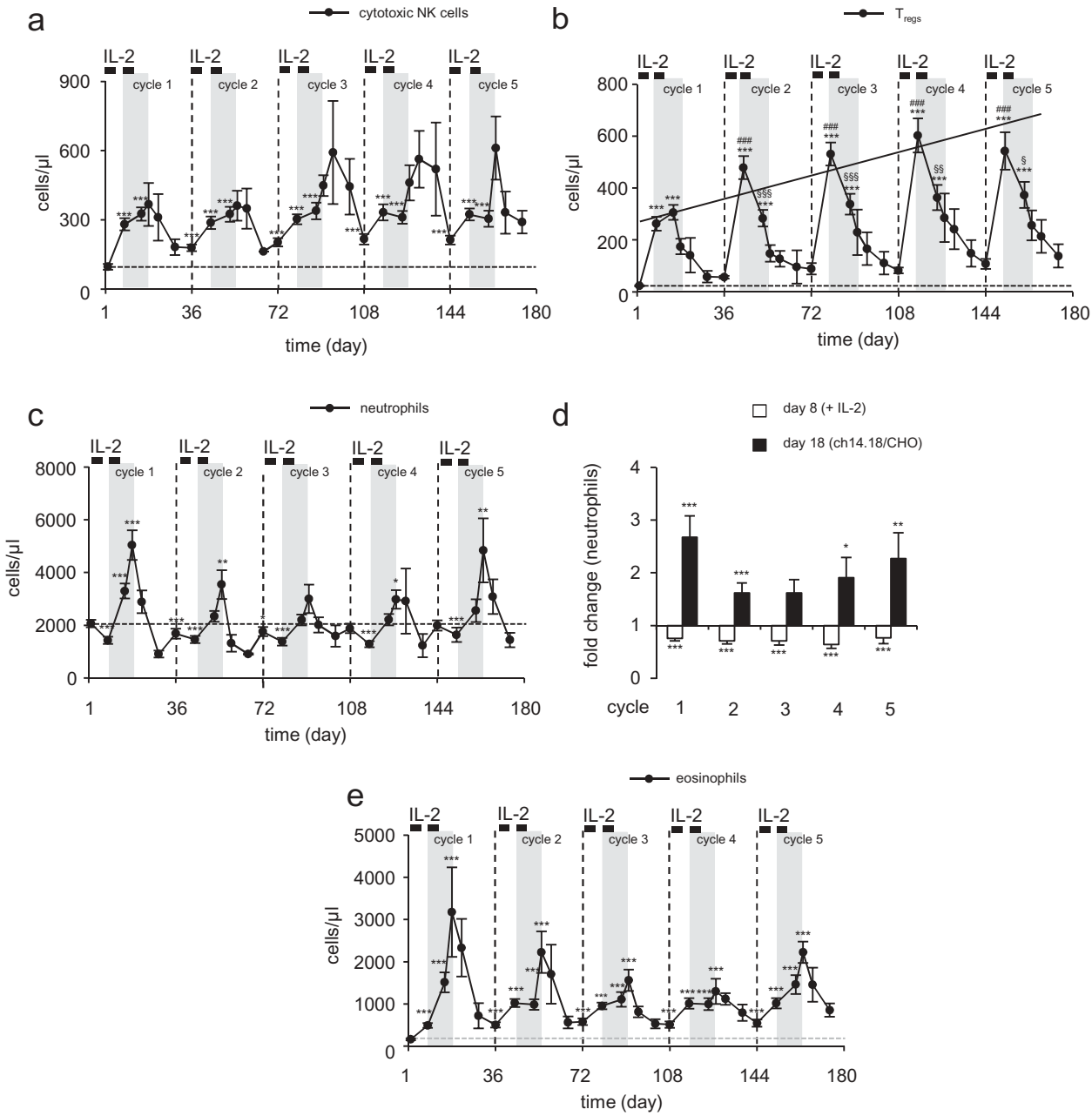

Figure 2. Effector cell counts during the entire treatment period. Cell counts of cytotoxic NK cells ($CD16^+/CD56^+$) (a), T_{regs} ($CD4^+/CD25^+/CD127^-$) (b) as well as neutrophils ($CD64^+$, black solid line, closed circles) and eosinophils (black solid line, open circles) (c) were evaluated on d1 (cycle 1, d1 is defined as baseline), d8 (after IL-2 treatment), d15 and d18 (during Ab infusion in combination with IL-2), d22 and d29 (after Ab infusion) in all five cycles (start of cycle 2–5 is indicated with vertical black dashed line) using flow cytometry as described in the "Methods" section. IL-2 administration (d1-5 and d8-12) is indicated as black horizontal lines and ch14.18/CHO treatment (d8-18) as gray vertical bars. Baseline values are indicated as dashed horizontal lines. Data are shown as mean ± SEM. (d) Fold change vs. baseline of neutrophil levels on d8 (white bars) and d18 (black bars) was analyzed in all cycle. Numbers of patients evaluable for this analysis are shown in suppl. Tab. 1. Statistical analysis was performed using Mann-Whitney-U-test. (a) ***P < .001 vs. baseline. (b) ***P < .001 vs. baseline. §§§P < .001 d15 vs. d8 of the respective cycle; §§P < .01 vs. d8, cycle 5; P < .001 vs. d8, cycle 1. (c and d) ***P < .001 vs. baseline; **P < vs. baseline. *P < .05 vs. baseline. (e) ***P < .001 vs. baseline.

In summary, we observed that IL-2 administration led to induction of NK cells with a cytotoxic phenotype, T_{reg} and eosinophils but reduced levels of neutrophils. The combined treatment with IL-2 and ch14.18/CHO did not further increase NK cells and T_{regs} but strongly elevated neutrophil and eosinophil levels suggesting a ch14.18/CHO-dependent effect on granulocytes (Figure 2c-e).

Impact of IL-2 and ch14.18/CHO treatment on cytokine serum concentrations

Since lymphocytes and MDSCs play an important role in immunotherapeutic efficacy, we analyzed whether cytokines are induced, which are typical for lymphocyte activation and regulation (IFN-γ and IL-10) and for MDSCs activation and recruitment[18-22] (IL-6, IL-18 and CCL2) at indicated time

points as shown in Figure 1. Following the first round of IL-2 treatment (d5), the serum concentrations of IFN-γ, IL-18 and IL-10 were significantly elevated (10.8-, 1.6- and 2.4-fold increase for IFN-γ, IL-18, and IL-10, respectively) compared to the baseline (Figure 3a, B and D). In contrast, IL-6 serum concentrations were only slightly increased (1.3-fold) on d5 compared to the baseline, but this difference was statistically not significant (Figure 3c). Notably, compared to IL-2 treatment alone (d5), the combined treatment with ch14.18/CHO and IL-2 (d8-12) resulted in a substantial increase of IFN-γ, IL-18, IL-6 and IL-10 serum concentrations on d12 (94.7-, 4.7-, 11.3- and 5.8 fold increase vs. baseline, respectively) (Figure 3a-d).

The kinetics of cytokine secretion over time was different between IFN-γ, IL-6, IL-10, and IL-18. Peak levels of IFN-γ, IL-6 and CCL2 were observed on d10 (221.0-, 17.4- and 2.8 fold increase vs. baseline, respectively) and significantly decreased on d12 compared to d10 (Figure 3a, c and e). In

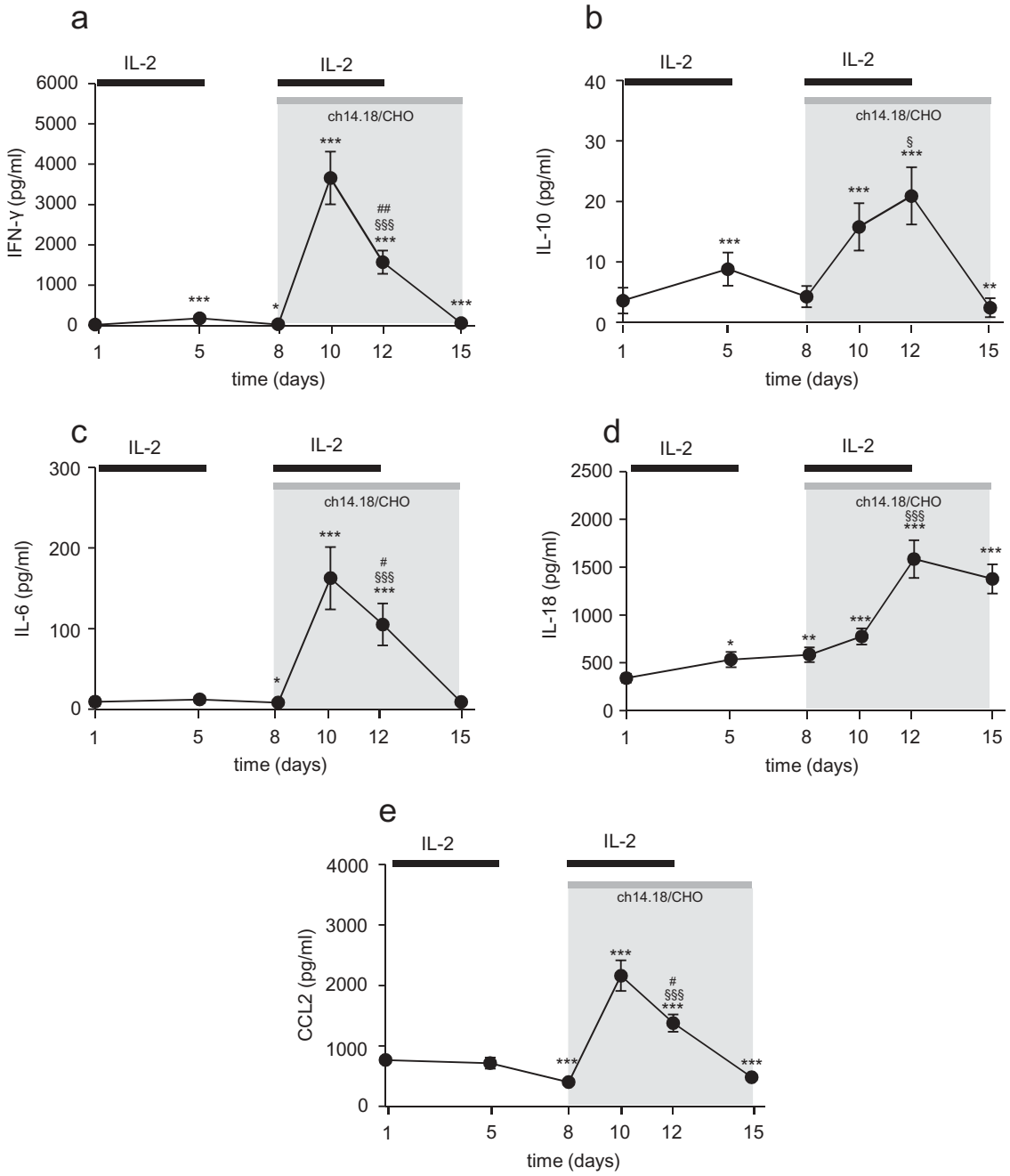

Figure 3. Cytokine serum concentrations. Serum levels of IFN-γ (a), IL-10 (b), IL-6 (c), IL-18 (d) and CCL2 (e) were analyzed in cycle 1 prior to the treatment start (d1, baseline), after 5 d of IL-2 administration (d5), prior to Ab infusion (d8) and during the Ab infusion in combination with IL-2 (d10, d12 and d15) using a bead-based immunoassay as described in the "Methods" section. Data are shown as mean values ± SEM of experiments performed in duplicates. Black horizontal bars indicate the IL-2 administration time period and gray areas that of the long-term infusion of ch14.18/CHO. Statistical differences between the means of serum concentrations were assessed using t-test or Mann-Whitney-U-test. (a) ***$P < .001$ vs. baseline; §§§$P < .001$ vs. d5; ##$P < .01$ vs. d10; (b) ***$P < .001$ vs. baseline, §$P < .05$ vs. d5, §§§$P < .001$ vs. d5; (c) ***$P < .001$ vs. baseline, *$P < .05$ vs. baseline, §§§$P < .001$ vs. d5, #$P < .05$ vs. d10; (d) ***$P < .001$ vs. baseline; (e) ***$P < .001$ vs. baseline, §§§$P < .001$ vs. d5, #$P < .05$ vs. d10.

contrast, IL-10 and IL-18 showed the highest serum concentrations on d12, suggesting a regulatory response to the combinatorial treatment (Figure 3a-d). On d15 (i.e. d8 of LTI of ch14.18/CHO) IFN-γ-, IL-6- and IL-10 cytokine serum concentrations dropped to the baseline level or even below (Figure 3a, c and d). In contrast, IL-18 serum concentrations on d15 remained at a significantly elevated level compared to the baseline (Figure 3a-d).

In summary, these results indicate, that the application of ch14.18/CHO in combination with IL-2 led to a substantial increase of IFN-γ, IL-18, IL-6, IL-10 and CCL2 compared to the treatment with IL-2 alone, underscoring the combined effect of both treatment components on the cytokines analyzed here.

Effect of immune cell counts on ADCC levels

Since NK cells, neutrophils and eosinophils mediate ADCC, we evaluated in cycle 1, whether their cell counts correlate with ADCC levels determined on d15 using a validated calcein-AM-based cytotoxicity assay.[29] We also investigated whether T_{regs} counts correlate with the ADCC, as they are known to inhibit NK cell functions.[30] For statistical analysis, patients were divided into high- and low responders using the median cell counts as a cutoff. The long-term infusion of ch14.18/CHO resulted in an ADCC of 21 ± 3% on d15 compared to baseline in all patients analyzed. Here, we observed that neither NK cell nor granulocyte counts correlate with the ADCC level in our pt cohort. Pts with high NK cell (>301 cells/μl) counts showed nearly the same ADCC levels compared to the pts with low cell counts (19 ± 5% vs. 21 ± 5%) (Figure 4). Moreover, we found in pts with high neutrophil and eosinophil counts (>2778 cells/μl and >1032 cells/μl) similar ADCC levels of 22 ± 5% and 21 ± 4% compared to those with low counts of 19 ± 3% and 19 ± 5%, respectively). Interestingly, we did not observe any correlation between ADCC levels and T_{reg} levels showing similar cellular cytotoxicity on d15 in cycle 1 in both patient cohorts (20 ± 5% and 21 ± 4% for high- and low responder cohorts, respectively; cutoff = median of 256 cells/μl) (Figure 4).

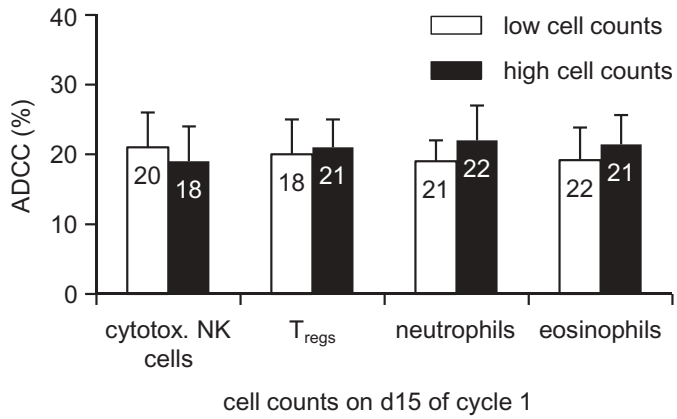

Figure 4. Impact of cytotoxic NK cell-, neutrophil-, eosinophil- and T_{reg} levels on ADCC. ADCC was evaluated using a validated calcein-AM-based assay as described in the "Methods" section. Patients were divided into two groups using the median effector cell count on d15 as a cutoff (low- and high cell counts). ADCC levels on d15 are shown for patients with low- (white bars) and high cytotoxic NK cell-, T_{regs}, neutrophil- and eosinophil counts determined on d15 (black bars). Data are shown as mean values ± SEM. The number of patients evaluable for ADCC analysis is shown within the bars. The differences in ADCC levels between the groups were statistically not significant ($P > .05$; Mann-Whitney-U-test).

Role of effector cell levels for PFS

Next, we addressed the question, whether NK- and neutrophil cell levels as well as T_{reg} counts on d15 in cycle 1 are linked to the differences in the outcome of the treated patients.

Patients with high T_{reg} levels (cutoff = median: 256 cells/μl) had a 4-y PFS of 14% (95% CI [0.00, 0.29]) compared to PFS of 38% (95% CI [0.17, 0.58]) in those with low counts ($P = .138$) (Figure 5b). Dividing the patient cohort in groups with very high- (>447 cells/μl), high- (>256 cells/μl), low- (>138 cells/μl) and very low cell counts (<138 cells/μl) revealed a 4-y PFS of 9% (95% CI [0, 0.26]), 20% (95% CI [0, 0.45]), 30% (95% CI [0.02, 0.58]) and 44% (95% CI [0.13, 0.74]), respectively. The differences between the groups with very low- and very high-T_{reg} counts were statistically significant (Figure 5c). In contrast, patients with either high NK cell- (cutoff = median: 301 cells/μl) or high granulocyte levels (cutoff = median: 2778 cells/μl and 1032 cells/μl for neutrophils and eosinophils, respectively) did not show any significant differences in PFS (Figure 5a, d and e). Taken together, we observed a significantly improved PFS in patients with low T_{reg} levels and did not observe any impact of other cell populations on PFS suggesting the major role of T_{regs} for the outcome to treatment with ch14.18/CHO in combination with IL-2.

Impact of cytokine serum concentration on PFS

We then analyzed whether the peak cytokine serum levels observed on d10 (IFN-γ, IL-6 and CCL2), d12 (IL-10 and IL-18) and for IL-18 additionally on d15 (plateau phase) correlate with PFS. In patients exhibiting elevated IFN-γ serum levels, we found a tendency toward an improved 4-y PFS of 37% (95% CI [0.16, 0.57]) compared to low levels of only 13% (95% CI [0, 0.27]) (d10; cutoff = median: 1921 pg/ml) ($P = .053$) (Figure 6a). Importantly, using the quartiles for splitting the cohort according to their IFN-γ levels in a very high- (>4080 pg/ml), high- (>1921 pg/ml), low- (>951 pg/ml) and very low group (<951 pg/ml), we observed a 4-y PFS of 46% (95% CI [0.17, 0.76]), 27% (95% CI [0.01, 0.54]), 18% (95% CI [0, 0.41]) and 8% (95% CI [0, 0.24]), respectively (Figure 6b). The differences between the very low- and very high groups were statistically significant (Figure 6b).

For IL-18, we observed a similar effect as for IFN-γ. Patients with high IL-18 levels (cutoff = median: 1409 pg/ml) on d12 showed a tendency toward a better survivalcompared to patients with low levels (data not shown; $P = .134$) and this changed to a significant difference on d15 (cutoff = median: >1139 pg/ml) (Figure 6e). Survival analysis of IL-6, IL-10 and CCL2 did not show any significant impact of these cytokines on PFS (Figure 6c, d and f) ($P = .801$, 0.603 and 0.728, respectively). In summary, we observed a significant association of high IFN-γ- and IL-18 levels with PFS.

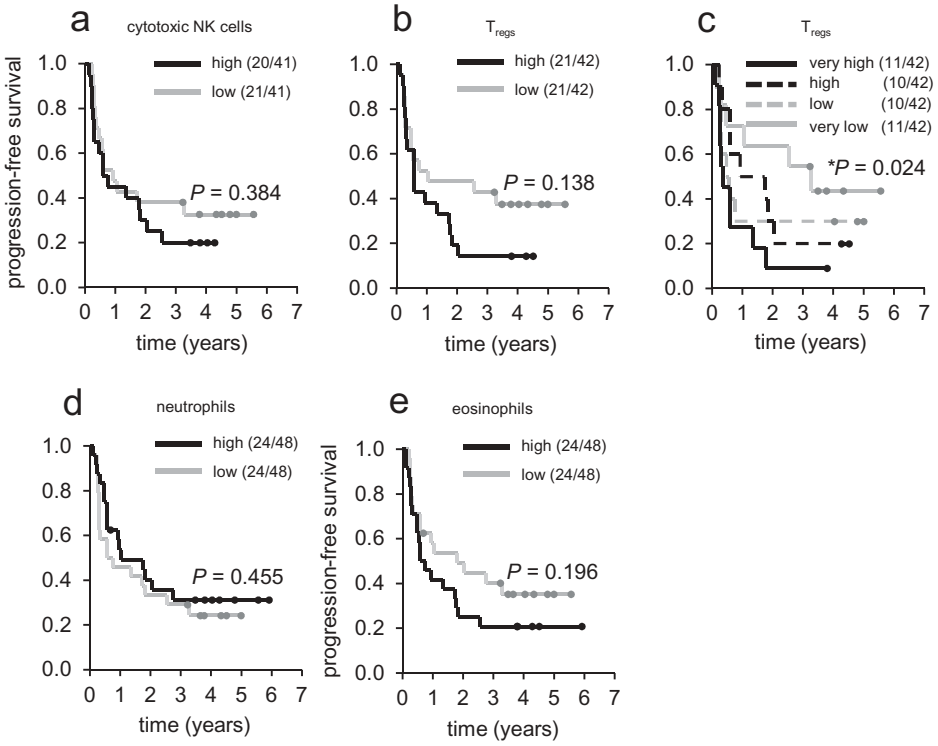

Figure 5. Impact of effector cell levels on PFS. The cohort of the treated patients was divided into two or four groups using median and quartile effector cell counts measured on d15 and then PFS probabilities were determined for each group. PFS probability of patients with high (black solid line) cytotoxic NK cell- (a; cutoff = median: 301 cells/µl), T_{reg} counts (b; cutoff = median: 256 cells/µl) neutrophil- (d; cutoff = median: 2778 cells/µl) and eosinophil counts (e; cutoff = median: 1032 cells/µl) compared to the patients with low cell counts of the respective cell population (gray solid line). (c) Comparison of PFS probabilities of patients with very high- (black solid line; <447 cells/ml), high- (black dashed line; >256 cells/µl), low- (gray dashed line; >138 cells/µl) and very low T_{reg} counts (<138 cells/µl) on d15 (gray solid line). Number of patients evaluable for the analysis is shown in brackets. Statistical analysis was performed using LogRank test.

Correlation between T_{reg} levels and cytokine serum concentrations

Our data indicate that systemic levels of IL-18 and IFN-γ as well as low T_{reg} counts are associated with an improved outcome following ch14.18/CHO therapy combined with IL-2. Therefore, we investigated whether there is any correlation between T_{reg} cell counts and cytokine serum concentrations that might explain the observed improvement of PFS in patients with low T_{reg} counts. For this, we analyzed the peak cytokine serum concentrations of IL-6, IL-10, IL-18, IFN-γ and CCL2 in patients with high- and low T_{reg} cell counts on d15 of cycle 1.

We found significantly higher IFN-γ peak serum concentrations on d10 in patients with low T_{reg} levels (4487 ± 981 pg/ml) compared to those who had high T_{reg} levels (1868 ± 369 pg/ml) (Figure 7a). Importantly, regression analysis showed a significant negative correlation between IFN-γ serum concentrations and T_{reg} counts, supporting our assumption that T_{reg} might adversely affect IFN-γ release or vice versa (Figure 7b).

Similarly, patients with low T_{reg} levels showed slightly increased IL-18 serum concentrations (1870 ± 378 pg/ml) compared to those with high T_{reg} levels (1285 ± 179 pg/ml) suggesting a similar correlation between T_{regs} and IL-18 as observed for IFN-γ. However, these differences were not significant (Figure 7a) (P = .361). One additional interesting observation was made for CCL2. In patients with low T_{reg} levels, CCL2 serum concentrations were significantly elevated compared to those with high levels (2603 ± 355 and 18642 ± 444 pg/ml) (Figure 7a).

Finally, we did not find any significant difference of IL-6 and IL-10 peak concentrations (d10 and d12, respectively) between patients with low- and high T_{reg} levels on d15 (Figure 7c and d).

Taken together, our data suggest an association between T_{regs} and IFN-γ-, IL-18- and CCL2 cytokine levels.

Discussion

Patients with high-risk NB benefit from anti-GD_2 Ab-based immunotherapies,[2] which included co-administration of cytokines, such as IL-2 and GM-CSF. Surprisingly, results of a randomized clinical trial evaluating IL-2 role did not show any clinical benefit of this cytokine in the context of the GD_2-directed immunotherapy with ch14.18/CHO.[13] Here, we provide a possible explanation for this observation demonstrating a strong IL-2-dependent T_{reg} induction and a correlation between high T_{reg} cell counts and poor treatment outcome of high-risk NB patients treated with IL-2 and ch14.18/CHO. Additionally, we found that T_{reg} levels inversely correlated with IFN-γ serum concentrations, suggesting T_{reg}-mediated inhibition of NK cells activity,[31] thus further confirming IL-2-dependent induction of a negative regulation of the immune response to the combinatorial immunotherapy. Thus, the treatment with IL-2 leads to a selective expansion of T_{regs}

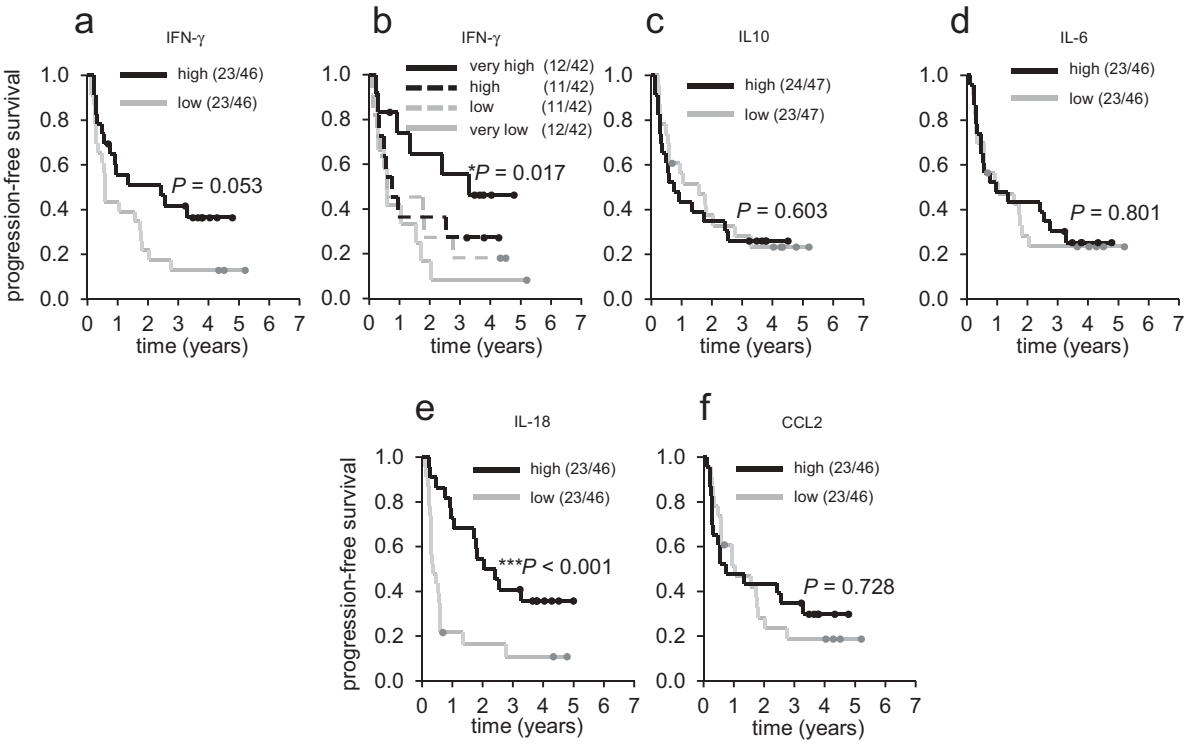

Figure 6. Correlation between cytokine levels and PFS. For evaluation of PFS probabilities, the cohort of treated patients was divided into equally sized groups according to the median or quartile cytokine serum concentration as a cutoff. For this analysis, the time point of the day was used, at which a maximum serum concentration was detected. PFS probability of patients with high serum concentrations (black solid line) of IFN-γ (a; d10, (cutoff = median of 1921 pg/ml), IL-10 (c; d12, cutoff = median of 8.8 pg/ml), IL-6 (d; d10, cutoff = median of 1265 pg/ml), IL-18 (e; cutoff = median of 1139 pg/ml) and CCL2 (f; d10, cutoff = median of 1590 pg/ml) were compared to patients with low serum concentrations on d15 (gray solid line). (b) Analysis of correlation between PFS and IFN-γ levels determined on d15 in patients with very high- (black solid line; >4080 pg/ml), high- (black dashed line; >1921 pg/ml), low- (gray dashed line; >951 pg/ml) and very low IFN-γ concentrations (gray solid line; <951 pg/ml). Number of patients evaluable is shown in brackets. Statistical analysis was performed using LogRank test. ***$P < .001$ vs. low IL-18; *$P < .5$ vs. very low IFN-γ.

rather than NK cells presumably limiting the anti-tumor effector functions of NK cells.[30] This contention is supported by studies in patients with melanoma and renal cancer treated with IL-2 who showed an increased frequency of T_{regs} secreting anti-inflammatory cytokines such as IL-10,[32–35] thus limiting the clinical efficacy of immunotherapies.

The rationale to combine the immune-stimulating cytokine IL-2 with anti-GD_2 Ab was based on the in vitro data showing increased ADCC when IL-2 was additionally given.[12] Based on these observations and on the fact that ADCC is one of the major mechanisms of action of Ab-based immunotherapies and NK cells mainly mediate ADCC,[3–7] the NK cell-stimulating cytokine IL-2 was included in the treatment protocol to increase ch14.18-mediated efficacy in patients.[2] Indeed, after the first 5 d of IL-2 administration, we found an about 3-fold increase of NK cell counts. Unexpectedly, the second course of IL-2 (in combination with ch14.18/CHO) did not lead to a further increase of NK cells, which could be related to the impact of T_{regs} that were strongly (about 11-fold) induced during the first IL-2 treatment course. This is supported by the biological role of T_{regs} to prevent excessive immune activation[36] and is in line with the previous reports that T_{regs} impair anti-tumor response through direct killing of NK cells via granzyme release.[37,38]

We also observed a strong release of inflammatory cytokines following this treatment.[17] Although IL-2 application alone resulted on d5 in a modest increase of IFN-γ, IL-6 and CCL2 serum levels, the combinatorial therapy of IL-2 with ch14.18/CHO largely increased the levels of these cytokines on d10 that was the second day of the Ab infusion. These results suggest that ch14.18/CHO rather than IL-2 mediates release of these cytokines. Interestingly, after the peak concentrations on d10, the IFN-γ-, IL-6- and CCL2 levels significantly dropped on d12 with further decrease to levels comparable to the baseline on d15 (Figure 3). This may be associated with the high T_{reg} levels observed during the combined treatment. This contention is supported by the strong increase of IL-10 levels on d12 which is 2 d after the cytokines IFN-γ, IL-6 and CCL2 achieved their peak levels (d10) and consistent with a cytokine-mediated anti-inflammatory response. Our data suggest a switch of the immune response from a pro-inflammatory to an anti-inflammatory direction during the combined treatment, thereby possibly limiting the efficacy of ch14.18/CHO-mediated immunotherapy. We also observed an inverse correlation between IFN-γ serum concentrations and T_{reg} levels that may be related to an inhibitory impact of T_{regs} on NK cells that was reported to be mediated by TGF-β.[30,31,39] In contrast to IFN-γ, IL-6, IL-10 and CCL2, which dropped already during the combined treatment, IL-18 serum concentrations remained at high levels (d12 and d15). Since IL-18 is mainly produced by myeloid cells, such as macrophages,[40] we suggest that ch14.18/CHO-dependent activation of macrophages results in strong release of IL-18 that has been

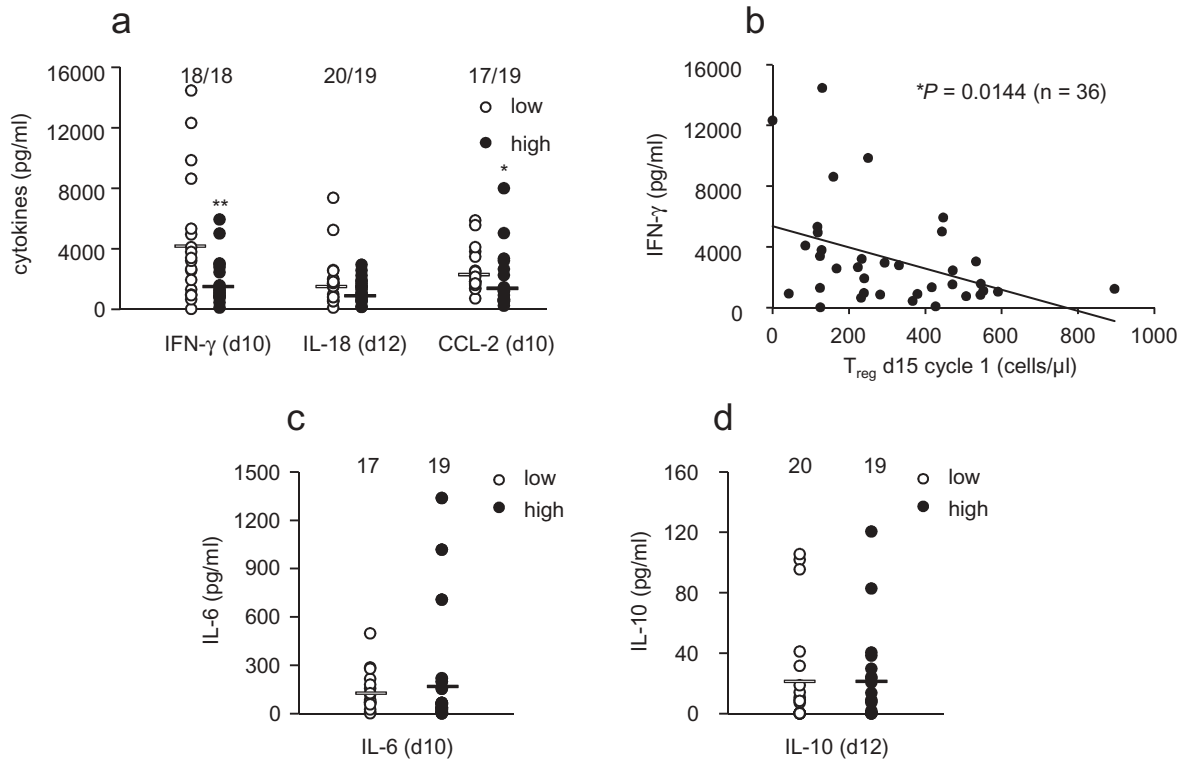

Figure 7. Correlation of T_{reg} counts and cytokine serum concentrations. The cohort of patients was divided into two groups using the median of T_{reg} counts determined on d15 as a cutoff. The time point of peak levels (pg/ml) for each cytokine was used for this analysis. Serum levels of IFN-γ (d10), IL-18 (d12), CCL2 (a; d10), IL-6 (c; d10) and IL-10 (d; d12) in patients with low- (open circle; <256 cells/ml) and high T_{reg} levels (closed circles; >256 cells/µl) determined on d15 were assessed with a bead-based immunoassay as described in the "Methods" section. The circles represent individual patients evaluable for the analysis. The total number of evaluable patients per group is indicated above the symbols representing the analyzed patients. (b) Scatter plot of IFN-γ serum concentrations on d10 (y-axis; pg/ml) vs. T_{reg} levels on d15 (x-axis, cells/µl) and regression analysis are shown for 36 evaluable patients. Statistical analysis was performed using t-test, Mann-Whitney -U-test (a, c and d) or linear regression analysis (b). (a) $^{**}P < .01$ vs. IFN-γ serum concentrations in patients with low T_{reg} levels on d15; $^{*}P < .05$ versus CCL2 serum concentrations in patients with low T_{reg} levels.

shown to stimulate neutrophils.[41] Similar to IL-18, neutrophil expansion was observed after the start and was further increased during the ch14.18/CHO LTI. A comparable effect was shown for eosinophil levels, which further increased at the end of the Ab treatment (approx. 20-fold vs. baseline). The expression of CD25 on eosinophils may provide a possible explanation for these effects.[42,43] Besides IL-2, GM-CSF is known as the second prominent cytokine included into the standard treatment protocols with anti-GD_2 Ab to stimulate granulocytes for ADCC improvement.[44] Since a strong expansion of granulocytes can be achieved by the ch14.18/CHO and IL-2 treatment regime, the question arises whether a co-treatment with GM-CSF is necessary particularly as GM-CSF is known to generate immune-suppressive MDCSs.[45]

Although we did not observe any correlation between the T_{reg} cell counts and ADCC levels, we found that the number of T_{regs} during the Ab treatment was associated with the outcome of the immunotherapy. Our observations are in line with other studies that report a negative effect of T_{reg} cell expansion on the outcome of immunotherapies despite the IL-2-dependent stimulation of NK cells.[32–35,46] Since it has been shown that inflammation facilitates the infiltration of T_{regs} into the tumor microenvironment and subsequently exerts immune inhibitory activity that negatively affects the efficacy of immunotherapies,[47] the analysis of the immune-modulatory effects of the immunotherapy on the tumor microenvironment would be of further interest. In order to address this question a systematic and sequential tumor re-biopsy strategy is needed, which is difficult to realize.

These studies and our observation of the inferior survival in patients with high T_{reg} counts could explain the results of the prospective randomized phase 3 trial in which PFS and OS of patients receiving ch14.18/CHO were nearly identical to that of patients treated with ch14.18/CHO in combination with IL-2,[13] and, importantly, to that of patients treated with ch14.18/SP2/0 in combination with IL-2 and GM-CSF.[2] These observations suggest that the cytokine co-treatment mediates both, pro- and anti-inflammatory effects and that in summary does not improve the efficacy of anti-GD_2 mAbs in currently used treatment regimens and underscore that the therapeutic mAb and not cytokines is the crucial component mediating the survival benefit for treated patients. Importantly, immunomodulatory effects caused by the single-agent treatment (anti-GD2 mAbs only) will be evaluated in ongoing randomized trials.

We also showed a positive impact of IL-18- and IFN-γ levels on PFS. Importantly, both cytokines were induced as a result of ch14.18/CHO and not IL-2 pre-treatment. Supporting our observations, IL-18- and IFN-γ were reported to mediate an anti-tumor response through activation of

cytotoxic NK cells and cytotoxic T lymphocytes that may largely contribute to the clinical benefit.[39,48–50]

Since co-treatment with IL-2 led to a strong induction of immune suppressive T_{regs}, the concomitant depletion of T_{regs} during an IL-2 treatment or replacing IL-2 by cytokines which do not stimulate immune suppressive cell populations may represent potential strategies for future.[51–53]

In summary, our study provides a mechanistic explanation for the absence of a beneficial role of IL-2 for immunotherapy with ch14.18/CHO as previously reported[13] due to its undesired expansion of immune regulatory T_{regs}.

Material and methods

Ethic statement

All procedures involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Treatment conducted under a compassionate use program and analysis protocols were approved by the ethical committee of the University Medicine Greifswald (ethical code: BB 179/15). Informed consent was obtained from all individual participants or their parents or legal guardians.

Patients

The patients were treated according to the protocol as previously described.[26] Briefly, 53 patients received up to five cycles immunotherapy with the anti-GD_2 Ab ch14.18/CHO in combination with IL-2 in a closed single-center program (APN311-303). They were first treated 5d with IL-2 (aldesleukin, s.c., d1–5, 6×10^6 IU/m^2/d) followed by a combined application of IL-2 (s.c., d8–12, 6×10^6 IU/m^2/d) with ch14.18/CHO (i.v., d8–18, 10 mg/m^2/d) given as an LTI and isotretinoin (p.o., d22–35) (Figure 1).

Sampling

For analysis of ADCC, sodium-heparin blood and serum were collected in every cycle prior to the start of the immunotherapy (d1) and on d15 (i.e. d8 of ch14.18/CHO infusion). To show effects of the treatment on relevant effector cell populations (NK cells, neutrophils, eosinophils and T_{regs}), EDTA-blood samples collected in every treatment cycle on d1, d8, d15, d18, d22 and d29 were used. For determination of cytokine serum concentrations (IFN-γ, IL18, IL-10, IL-6 and CCL2), serum samples were collected on d1 (baseline), during IL-2 treatment (d5 and d8) and during the combined treatment with IL-2 and ch14.18/CHO (d10, d12 and d15) and stored at −80°C until analysis.

Immunophenotyping

For analysis of Ab-dependent effects on effector cell counts, following effector populations were examined by flow cytometry: NK cells with a cytotoxic phenotype ($CD3^-/CD16^+/CD56^{dim}$),[23] neutrophil granulocytes ($CD64^+$), eosinophils (FSC-A vs. SSC-A) and T_{regs} ($CD4^+/CD25^+/CD127^-$). First, 3 ml EDTA-blood samples were incubated for 7 min with 3 ml erythrocyte lysis buffer (FACS Lysing Solution, BD Biosciences, 349202) in the dark followed by centrifugation for 5 min at RT, 300 × g. Cells were then washed once (5 min, 300 × g, RT) using wash buffer (1x PBS, 2% FCS, 0.1% NaN_3, pH 7.4). After supernatant was discarded, cells were stained for 20 min at +4°C in the dark with effector cell population-specific mAb (for NK cells: mouse anti-human CD16-PE (BD Biosciences, 555407), and mouse anti-human CD56-APC (BD Biosciences, 341027); for granulocytes: mouse anti-human CD45-PerCP (BD Biosciences, 347464) and mouse anti-human CD64-PE (Biozol, LS-C204449-100); for T_{regs}: mouse anti-human CD3-PerCP (BD Biosciences, 340663), mouse anti-human CD4-PerCP (BD Biosciences, 345770), mouse anti-human CD25-FITC (Merck, FCMAB189F) and mouse anti-human CD127-PE (BD Biosciences, 557938) in a total volume of 100 µl wash buffer. To exclude $CD64^+$ monocytes from the neutrophil population, monocytes were stained with CD14-FITC (DAKO, F0844). For each sample, 100,000 ungated events were estimated using FACS Calibur (BD Biosciences, Heidelberg, Germany). Data were analyzed with FlowJo V10 software (Ashland, OR, USA).

Analysis of ADCC

Patient-specific effector cells and serum were analyzed to evaluate the cellular ch14.18/CHO-dependent induction of tumor cell lysis. For this, a calcein-acetoxymethyl ester (AM)-based cytotoxicity assay was conducted as previously described.[29] Briefly, 5,000 LAN-1 NB cells were stained with 10 µmol/l calcein-AM (Merck, 17783-1MG) and coincubated with patient leucocytes (effector to target cell ratio: 40:1) and heat-inactivated patient serum to exclude complement-dependent cytotoxicity for 4 h. ADCC was evaluated by measuring the calcein release ((experimental release – spontaneous release)/(maximum release – spontaneous release)).

Evaluation of cytokine serum concentration

To evaluate serum concentrations of the cytokines IFN-γ, IL-6, IL-10, IL-18 and CCL2, a cytokine-specific bead-based immunoassay (Biolegend, 92919) was conducted using 25 µl serum samples according to the manufacturers' protocol (Biolegend, custom Legendplex™ panel). Statistical analysis was performed to analyze the differences between the baseline and all time points mentioned above.

Statistics

Differences between the groups were assessed using either the Mann-Whitney-U-test or Students *T*-test, if the assumption of normality was met. For statistical analysis, SigmaPlot software (Version 13.0, Jandel Scientific Software) was used. All data are presented as mean ± SEM (standard error of the mean). For survival analysis, we used the median cell counts and cytokine levels as a cutoff to define a high- or low patient cohort showing cell counts above and below the median, respectively. Additionally, for exploratory analysis, we divided our patient

cohort into four groups (denoted as very high-, high-, low- or very low phenotype) using quartile effector cell counts and cytokine levels. PFS probabilities were estimated using Kaplan-Meier analysis and compared using LogRank statistics. LogRank analysis was only performed, if the assumption of proportional hazard was met. Four-year PFS was shown as mean ± SEM in years and confidence interval (95% CI) was given in parenthesis. Correlation between two independent variables was assessed using linear regression. A P value of <0.05 was considered significant, <0.01 very significant and <0.001 highly significant.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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