Immune-based therapies in ovarian cancer

Dijkgraaf, E.M.

Citation

Dijkgraaf, E. M. (2017, June 13). *Immune-based therapies in ovarian cancer*. Retrieved from https://hdl.handle.net/1887/49549

Version: Not Applicable (or Unknown)

License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden

Downloaded from: https://hdl.handle.net/1887/49549

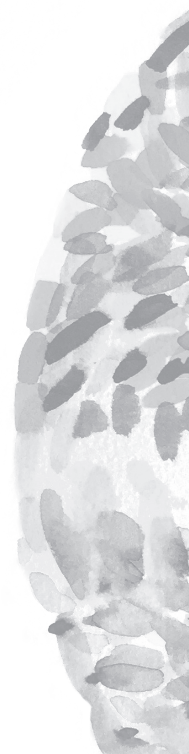
Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/49549 holds various files of this Leiden University dissertation.

Author: Dijkgraaf, E.M.
Title: Immune-based therapies in ovarian cancer
Issue Date: 2017-06-13

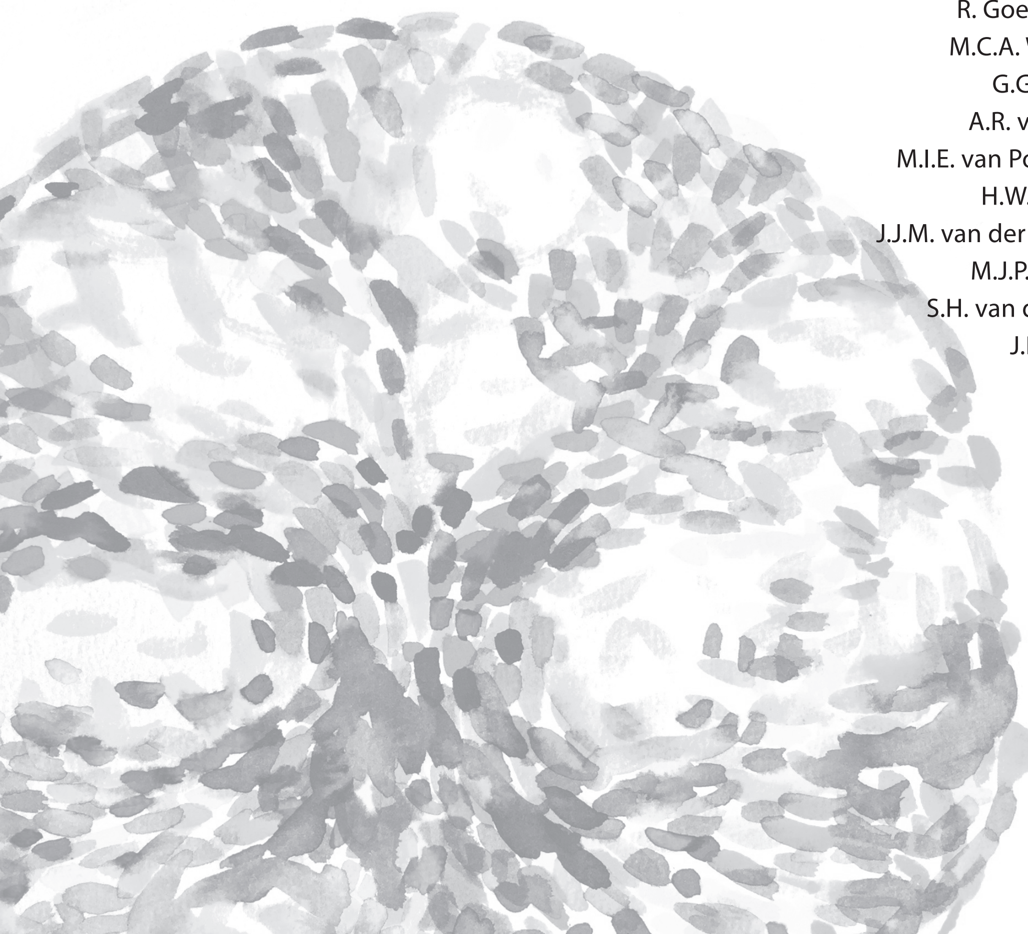


CHAPTER 5

A phase I trial combining carboplatin/doxorubicin with tocilizumab, an anti-IL-6R monoclonal antibody, and interferon-α 2b in patients with recurrent epithelial ovarian cancer

Ann Oncol. 2015 Oct; 26(10): 2141-9

E.M. Dijkgraaf
S.J.A.M. Santegoets
A.K.L. Reyners
R. Goedemans
M.C.A. Wouters
G.G. Kenter
A.R. van Erkel
M.I.E. van Poelgeest
H.W. Nijman
J.J.M. van der Hoeven
M.J.P. Welters
S.H. van der Burg
J.R. Kroep



ABSTRACT

Background: The immune system is important in epithelial ovarian cancer (EOC). Interleukin-6 is associated with chemoresistance and an immune-suppressive tumor microenvironment. We investigated whether a combination of chemotherapeutics, blockade of IL-6 receptor (IL-6R; tocilizumab) and immune enhancer interferon-α (Pegintron) is feasible, safe and able to enhance immunity in patients with recurrent EOC.

Patients and methods: In this dose-escalation study, patients received tocilizumab 1, 2, 4, or 8mg/kg i.v., q4 weeks during the first three cycles of carboplatin (AUC5) plus doxorubicin (pelgylated liposomal doxorubicin (PLD) 30mg/m^2 or doxorubicin 50mg/m^2 i.v., day 1, q4 weeks, for six cycles). At the highest tocilizumab dose (8mg/kg), Pegintron (1µg/kg s.c.) was added. Peripheral blood mononuclear cells were collected for immunomonitoring at baseline, after three and six cycles. Dose-limiting toxicity (DLT), CA-125, and radiologic response were evaluated.

Results: In the 23 patients enrolled, no DLT was established. The most frequent grade 3/4 adverse events (CTCAE v4·03) were neutropenia (23%), febrile neutropenia (19%) and ileus (19%). No treatment-related deaths occurred. Using CT evaluation, eleven of 21 evaluable patients responded, six had stable disease and three progressive disease. Patients receiving highest-dose tocilizumab showed a functional blockade of IL-6R with increased levels of serum IL-6 (p=0·02) and soluble IL-6R (p=0·008). Consequently, immune cells displayed decreased levels of pSTAT3, myeloid cells produced more IL-12 and IL-1β while T-cells were more activated and secreted higher amounts of effector cytokines IFN-γ and TNF-α. An increase in sIL-6R was potentially associated with a survival benefit (p=0·03).

Conclusions: Functional IL-6R blocking is feasible and safe in EOC patients treated with carboplatin/(pegylated liposomal)doxorubicin, using 8mg/kg tocilizumab. This combination is recommended for phase 2 evaluation based on immune parameters.

INTRODUCTION

EOC has the highest mortality rate of female cancers responsible for a five-year survival of only 35%, requiring more effective systemic therapies. The immune system plays a prognostic role in ovarian cancer (1-3) and accumulating evidence suggests that immunotherapy in combination with chemotherapy holds potential for improved clinical outcomes in EOC (4, 5). Increased levels of IL-6 have been associated with resistance to chemotherapy, including ovarian cancer (3, 6, 7). Importantly, many of the available chemotherapeutics not only act via direct killing of tumors cells but also operate by eliciting, unmasking or reactivating tumor-specific immune responses in the tumor microenvironment (8). Because of its immunemodulating capacity, IL-6 associated resistance to chemotherapy may occur via intrinsic mechanisms but extrinsic immune-related mechanisms will also play a role. IL-6 can alter the tumor microenvironment causing the differentiation of tumor-infiltrating monocytes towards immunosupressive, M2-like macrophages (9) and attraction of FoxP3-positive regulatory T-cells (T-regs) (10). Tocilizumab, a humanized monoclonal IgG1 antibody against IL-6 receptor (IL-6R), is effectively used to treat IL-6-driven autoimmune diseases (11-13) and the macrophage activation syndrome (14).

Carboplatin/pegylated-liposomal-doxorubicin (PLD) therapy has a good therapeutic index in platinum-sensitive EOC and supports activation of tumor-specific T-cell responses (8). However, carboplatin can enhance IL-6 production by tumor cells (9). We hypothesized that suppression of IL-6 signaling by blocking the IL-6R may abolish (platinum) drug resistance and amplify host immunity. Additionally, interferon-α was added monthly as an adjunct to tocilizumab to improve the induction of tumor-specific CD8+ T-cells (15, 16). We present data of the first, phase I dose-finding study of tocilizumab combined with chemotherapy and interferon-α 2b in patients with advanced EOC. Safety, feasibility, the effect of chemo-immunotherapy on the immune system, and its relation with clinical outcome was studied.

MATERIALS AND METHODS

Study design. This is a multicenter, phase 1, dose-escalation study (standard "3+3-design") to evaluate safety and feasibility of tocilizumab in combination with carboplatin/(pegylated liposomal) doxorubicin and interferon-α 2b (Pegintron) in patients with recurrent EOC. The secondary objective was to determine the effect of this chemo-immunotherapy on the immune system and to study the relation between immune parameters and clinical outcome. This study was approved by the Medical Ethics Committee Leiden and registered (NCT01637532).

Patients and treatment scheme. Women with progressive or relapsed EOC who did not receive chemotherapy within three months (in- and exclusion criteria: see **Supplementary**

Materials & Methods) were treated with carboplatin (AUC5; Carbosin, Pharmachemie, The Netherlands) plus pegylated liposomal doxorubicin (PLD; Caelyx® 30mg/m^2, Janssen-Cilag, The Netherlands) or doxorubicin (Adriamycin 50mg/m^2 i.v., Pharmachemie), day 1, q4 weeks for six cycles. The first three patients served as a control for immunomonitoring, thereafter patients (three per cohort) received tocilizumab (RoActemra, purchased from Roche, The Netherlands) 1, 2, 4, or 8mg/kg i.v., day 1, q4 weeks during the first three cycles of chemotherapy. The last cohort (*n*=6) received Pegintron (1µg/kg s.c., Schering-Plough, The Netherlands) day 1, q4 weeks in addition to tocilizumab at optimal dose during first three cycles chemotherapy (**Supplementary Figure 1**). Dose limiting toxicities are described in **Supplementary Material & Methods.**

Initially, patients were treated with a standard combination of carboplatin and PLD. After inclusion of seven patients, PLD was worldwide no longer available for clinical use due to production problems. It was decided to continue this study with non-pegylated doxorubicin.

Clinical Monitoring. All AEs, reported spontaneously by the patient or observed by the treating physician, were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events v4·03 (NCI CTCAE v4·03) and their relationship to the study drugs was recorded. Serum CA-125 levels and computerized tomography (CT-scan) performed before treatment, after three cycles and after the last cycle of chemotherapy, evaluated according to *Response Evaluation Criteria In Solid Tumors (*RECIST) criteria 1.1 (17) were used to determine tumor response.

Immunomonitoring. The authors support the minimal information about T cell assays (MIATA) effort and therefore describe the immunomonitoring according to this guideline (18). Detailed descriptions of all tests are given in **Supplementary Materials and Methods.**

Statistical analysis. (Non)parametric tests (Wilcoxon signed-rank test and paired t-test for paired samples, Mann-Whitney and unpaired t-test for unpaired samples) were performed as appropriate. All statistical tests were performed at the 0·05 significance level, confidence intervals were 95% 2-sided intervals. Survival was tested using Kaplan-Meier method, and statistical significance of the survival distribution was analyzed by log-rank testing. Statistical analyses were performed using SPSS for Windows version 20·0 (IBM, USA) and GraphPad Prism 6·02 (San Diego, USA). Because this is a dose-finding, hypothesizing generating study, the data is not corrected for multiple comparisons.

RESULTS

Patient characteristics

In total, 23 patients, two of whom failed screening, were enrolled from 2011-2013 at the LUMC and UMCG in the Netherlands. **Table 1** shows baseline characteristics of the patients. The time from last chemotherapy to inclusion was 30·5 ± 13·8 weeks (mean ± SD). Four

Table 1. Patient characteristics and outcome

		ID	Age	Histology	Time (weeks)		Current treatment	Treatment			Clinical respons		Survival (weeks)	
					from diagnosis	from last chemotherapy		Doxorubicin	*	Cycles	Radiology results	CA125		
								DR (cycle)		completed			PFS	OS
	CONTROL	**P01**	55	serous	51	23	Second line	75% (4)	*	6	SD	PD	33	37
		P02	53	serous	45	15	Second line	100%	*	6	PR	PR	33	107
		P03	62	serous	130	25	Third line	75% (5)	*	6	PR	PR	27	54
Tocilizumab	1mg/mL	**P04**	50	endometrioid	133	40	Third line	75% (2)	*	6	CR	PR	48	71
		P05	54	serous	60	31	Second line	75% (2)	*	6	PR	PR	42	51
		P07	69	serous	52	42	Second line	75% (5)	*	6	PR	CR	53	104
Tocilizumab	2mg/mL	**P08**	56	serous	47	34	Second line	100%	*	6	CR	CR	38	79
		P10	78	serous	56	33	Second line	100%		6	PR	nm	111	**ongoing**
		P11	46	serous	59	29	Second line	75% (5)		6	PD	CR	30	52
Tocilizumab	4mg/mL	**P12**	60	serous	132	34	Third line	100%		6	SD	PR	35	**ongoing**
		P13	71	clear cell	308	12	Seventh line	75% (2)¥		**3**	PD	PD	12	16
		P14	62	clear cell	163	64	Fourth line	75% (2)		**3**	SD	SD	15	20
Tocilizumab	8mg/mL	**P15**	53	serous	95	16	Third line	75% (3)		6	SD	PR	26	37
		P16	61	clear cell	57	35	Second line	75% (2)		**3**	SD	PD	16	28
		P17	65	serous	123	19	Fourth line	75% (2)		6	SD	SD	33	49
Tocilizumab	8mg/mL + IFN-α	**P18**	59	serous	79	65	Second line	75% (2); 60% (4)		6	CR	CR	76	**ongoing**
		P19	70	serous	51	24	Third line	100%		**1**	na	na	na	20
		P20	65	serous	56	28	Second line	60% (4)		6	PR	CR	56	**ongoing**
		P21	57	serous	104	25	Second line	100%		6	PR	CR	43	**ongoing**
		P22	74	endometrioid	112	19	Third line	75% (1)		**2**	PD	PD	19	24
		P23	46	endometrioid	123	27	Second line	100%		6	PR	CR	58	**ongoing**

* received pegylated liposomal doxorubicin; ¥ P13 did not receive carboplatin during third cycle; PFS: progression free survival (date from inclusion of study will progression of disease); OS: overall survival
(date from inclusion untill death); SD: stable disease; PD: progressive disease; PR: partial response; CR: complete response; na: not available; nm: not measurable

patients discontinued treatment due to progression of disease and one patient due to toxicity (P19; febrile neutropenia).

Safety

No DLT or treatment related death occurred. **Table 2** summarizes the toxicity.

Severe adverse events. Main grade 3/4 AEs were febrile neutropenia for < 7 days (19%), ileus (19%) and nausea/vomiting (10%). Four patients experienced an ileus; three of them (P01, P04, P13) had already a (partial) obstruction due to tumor mass at baseline. P13 again developed an ileus after 3 cycles of chemotherapy due to tumor progression. SAEs are summarized in **Table 2**. Haematological toxicity is shown in Figure 1. Neutrophil count decreased in all dosage groups. In the group combining 8mg/kg tocilizumab and Pegintron four out of the overall five patients experienced $\geq$ grade 3 neutropenia (p=0·01).

Adverse events The majority of side effects were grade 1–2. The most common treatment-related events were fatigue (76%), nausea/vomiting (71%) and anorexia (38%). The AEs occurring in more than 15% of patients are listed in **Table 2**. Potential tocilizumab-induced effects on total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were not observed.

Reduction of chemotherapy dose. Dose modification of chemotherapy based on toxicity occurred in all treatment groups (**Table 1**). Dose adjustment was performed after a median of three cycles. Seven patients (33%) received all cycles of chemotherapy at 100% dose level.

Clinical efficacy

A complete response (CR) was seen in three patients, partial response (PR) in eight patients, stable disease (SD) in six patients and progressive disease (PD) in three patients by CT-scan. Based on CA-125 levels, seven CR, six PR, two SD and four PD were observed. At the end of registration (October 2014), the median overall survival was 54 weeks with six patients (29%) still being alive (**Table 1**).

Immunological efficacy

Changes in plasma signature. To determine the known effects of tocilizumab (11), we measured IL-6, sIL-6R, TNF-α and CRP at baseline, after three and six cycles of therapy. Levels of IL-6 and sIL-6R increased in the group of patients receiving tocilizumab at 8mg/kg (p=0·02 and p=0·008 respectively) and normalized afterwards, but did not change in patients receiving no or a low dose of tocilizumab (**Figure 2**). Since the plasma signature revealed a clear disparity in responses of patients treated with a low dose (1 or 2mg/kg) and a high dose (8mg/kg) of tocilizumab, we categorized our data in three groups: control, low (tocilizumab 1 or 2mg/kg) and high (tocilizumab 8mg/kg with or without Pegintron). Since materials to analyze the response of patients treated with 4mg/kg were restricted to one patient only, these data were omitted. In supplementary figures, all separate patient groups are shown.

Table 2. Toxicity

	Control	Tocilizumab 1mg/kg	Tocilizumab 2mg/kg	Tocilizumab 4mg/kg	Tocilizumab 8mg/kg	Tocilizumab 8mg/kg + IFN-α
	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=6)
Serious Adverse Events						
Febrile neutropenia	1		1			2
Ileus	1	1		1	1	
Nausea/Vomiting					2	
Thromboembolic event			1			
Neuropatic pain						1
Perforation sigmoid						1
Diarrhea				1		
Dyspnoe					1	
Haematological Adverse Event Grade 3/4						
WBC count						1
Neutrophil count		1				4
Platelet count		1				
Blood Chemistry Adverse Events Grade 3/4						
AST	1					
ALT	1					1
AF	1			1		
Adverse Events in > 15% of patients						
Fatigue	3	2	2	2	3	4
Nausea/Vomiting	2	2	2	3	3	3
Anorexia	1	1		3	1	2
Infection	1		1	1		2
Constipation	1	1	2	1		
Diarrhea		1	1			2
Abdominal pain	1		1	1	1	
Dysgeusia			1	1	1	1

Febrile neutropenia occurred twice in the 8mg/kg plus Peg-Intron group(P12 and P14), once in the control group (P01) and in one patient receiving 2mg/kg tocilizumab (P11). Four patients experienced an ileus; three of them (P01, P04, P13) had already a (partial) obstruction due to tumor-mass at baseline. P13 again developed an ileus after 3 cycles of chemotherapy due to tumor progression. P15 and P16 were admitted to the hospital because of nausea and vomiting caused by progressive ascites (P15) and not taking the pre-described anti-emetics (P16), respectively. After ascites drainage respectively intake of appropriate anti-emetics both patients quickly recovered. A pulmonary embolism was diagnosed in P11; she had a history of multiple embolisms for which she already used low molecular heparin. The therapy was switched to fenprocoumon with good results. The observed dyspnoea (P17) was due to pleuritis carcinomatosa just after the first cycle of treatment which was already present at baseline. A sigmoid perforation occurred after the second cycle of therapy, due to tumor invasion, which led to treatment discontinuation of P22. One patient (P18) experienced neuropatic pain of the upper leg, not otherwise specified. Patient P13 suffered from diarrhea which led to dehydration.

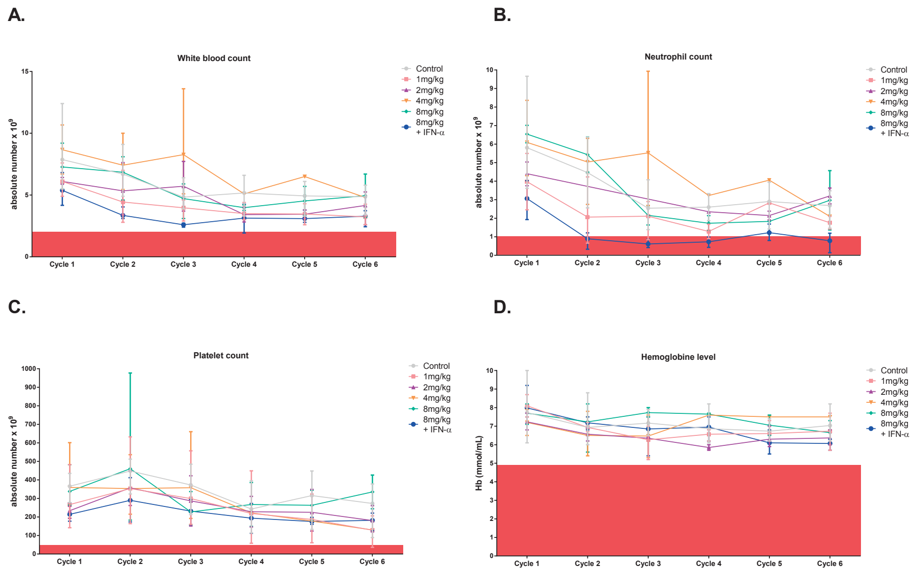


Figure 1.

Figure 1. Haematological adverse events.
All treatment groups are shown: control (carboplatin/doxorubicin), 1mg/kg (carboplatin/doxorubicin and 1mg/kg tocilizumab), 2mg/kg (carboplatin/doxorubicin and 2mg/kg tocilizumab), 4mg/kg (carboplatin/doxorubicin and 4mg/kg tocilizumab), 8mg/kg (carboplatin/doxorubicin and 8mg/kg tocilizumab), and 8mg/kg + Pegintron (carboplatin/doxorubicin and 8mg/kg tocilizumab and Pagintron). Mean levels with range are depicted. Red box indicates AE grade ≥ 3. A) White blood count B) Neutrophil count C) Platelet count D) Hemoglobin levels

Levels of CRP completely normalized after three therapy cycles in patients treated with high-dose tocilizumab (**Figure 2C**). TNF-α decreased in all patients after three and six cycles compared to baseline (p=0·02 and p=0·05, respectively; **Figure 2D**). No specific changes due to Pegintron were observed (**Supplementary Figure 5).**

Phenotypic changes in immune cells. In all patients, we observed a decline in B-cells (CD3-CD19+ cells), explicitly in tocilizumab-treated patients after three cycles and in all patients after six therapy cycles (**Supplementary Figures 6-7**). No changes in the frequencies of total CD3+, CD4+ and CD8+ T-cells, myeloid cells (CD45+CD3-CD19- cells), and T-regs (CD25+CD127-FoxP3+ cells) occurred. In-depth analysis of the T-cell (CD4+, CD8+, T-regs) and myeloid-cell lineages (myeloid derived suppressor cells (MDSC), DC and macrophages) showed no changes during treatment, except for the M1 macrophages, which increased after three and six cycles of therapy in all patients irrespective of tocilizumab or Pegintron (**Supplementary Figure 8).**

Functional changes in immune cells. Expression of IL-6R was low on CD8+ cells and high on B-cells, CD4+ T-cells and myeloid cells. Treatment with high-dose tocilizumab resulted in an increased expression of IL-6R on different subsets of macrophages while the expression

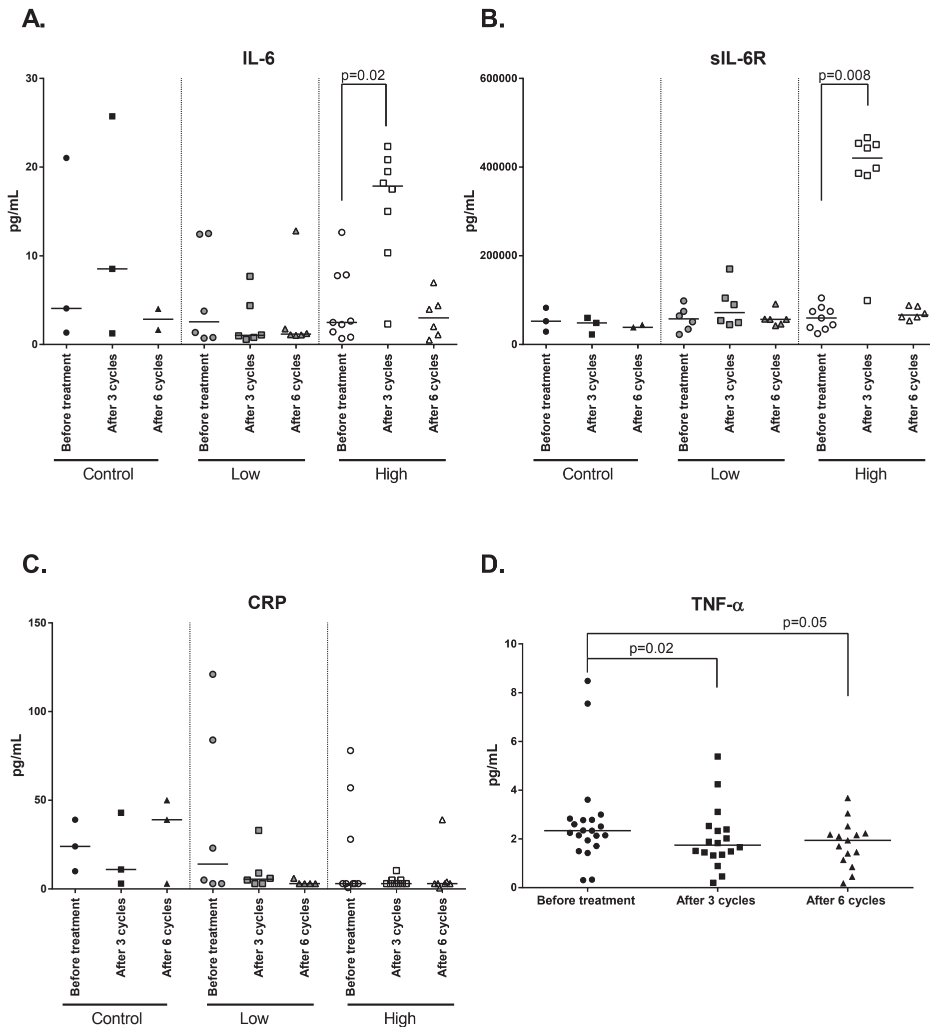


Figure 2. Changes in plasma signature upon treatment.

Patients are divided in three groups, unless otherwise stated; control, low dose tocilizumab (1 and 2mg/kg) and high-dose tocilizumab (8mg/kg). Median levels are represented by a bar. A) IL-6 is significantly increased upon treatment with high-dose tocilizumab (*p*=0·02). This is restored after 6 cycles. B) sIL-6R is significantly increased upon treatment with high-dose tocilizumab (*p*=0·008). This is restored after 6 cycles. C) CRP is completely normalized in patients with high-dose tocilizumab, while in other groups there are elevated levels detectable. D) TNF-α (all patients) is decreased in all patients after 3 (*p*=0·02) and 6 cycles (*p*=0·05), suggesting an effect of chemotherapy, not tocilizumab.

of IL-6R on B-cells and T-cells remained unaltered (**Figure 3A**). A tocilizumab-associated decrease in phosphorylated STAT3 (pSTAT3) was observed in the myeloid cells and CD4+ T-cells at all doses, while CD8+ T-cells displayed down-regulation only after treatment with

the highest dose of tocilizumab (**Figure 3B**), consistent with levels of IL-6R displayed by these cells. The levels of pSTAT1 and pSTAT5 followed a similar pattern while pSTAT6 seemed to be increased in CD4+ T-cells (**Supplementary Figure 9**).

All T-cell subsets displayed an increased expression of Ki67 but not CTLA-4 or CD45RA. While Ki67 expression by CD4+ and T-regs were not related to tocilizumab (**Supplementary Figure 10**), its expression by CD8+ T-cells was only detected in the high dose tocilizumab group, resulting in an increase in the CD8+Ki67+ (activated T-cell) to T-reg ratio (**Supplementary Figure 10**).

Mitogenic stimulation of T-cells revealed no change in their proliferative capacity, but the levels of secreted IFN-γ were increased in both the low- and high-dose tocilizumab group (p=0·03). This increase was only retained in patients receiving high-dose tocilizumab (**Figure 3C**). Furthermore, an increase in secreted TNF-α was observed in the high-dose tocilizumab group (p=0·02). The capacity of APC to produce IL-12, IL-1β, and IL-6 following stimulation with CD40L was increased and retained in patients receiving high-dose tocilizumab (**Figure 3D**).

Survival analysis. To explore a relation between the effects of tocilizumab on the IL-6 axis and clinical outcome, we first constructed survival curves on basis of pretreatment IL-6-levels, revealing that a relatively higher level of IL-6 at baseline was associated with a worse prognosis (p<0·0001; **Supplementary Figure 11**), despite treatment. When patients were grouped according to the presence of a good functional response to tocilizumab after three cycles, as defined by the treatment related increase in sIL-6R (defined by >20·000pg/ml sIL-6R levels upon treatment), a potential survival benefit was observed in patients with such a strong response, in essence patients treated with high-dose tocilizumab (p=0·03; **Supplementary Figure 11**).

DISCUSSION

This is the first study focusing on blocking of the IL-6R in cancer. It demonstrates that, in combination with chemotherapy, blocking of the IL-6R has an acceptable safety profile and a possible immunological benefit in patients with advanced EOC.

No unexpected toxicity was observed. The main ≥ grade 3 AE was neutropenia, probably caused by chemotherapy but it has also been reported for tocilizumab (19), as well as Pegintron (20). Unfortunately, PLD had to be substituted for doxorubicin, of which the working mechanism is comparable. PLD has a favourable safety profile, therefore, there is no underestimation of the toxicity when doxorubicin is used. All AEs were manageable. Future well-powered studies should investigate whether possible increased toxicity of this new treatment strategy compared to chemotherapy alone can be compensated by better outcome.

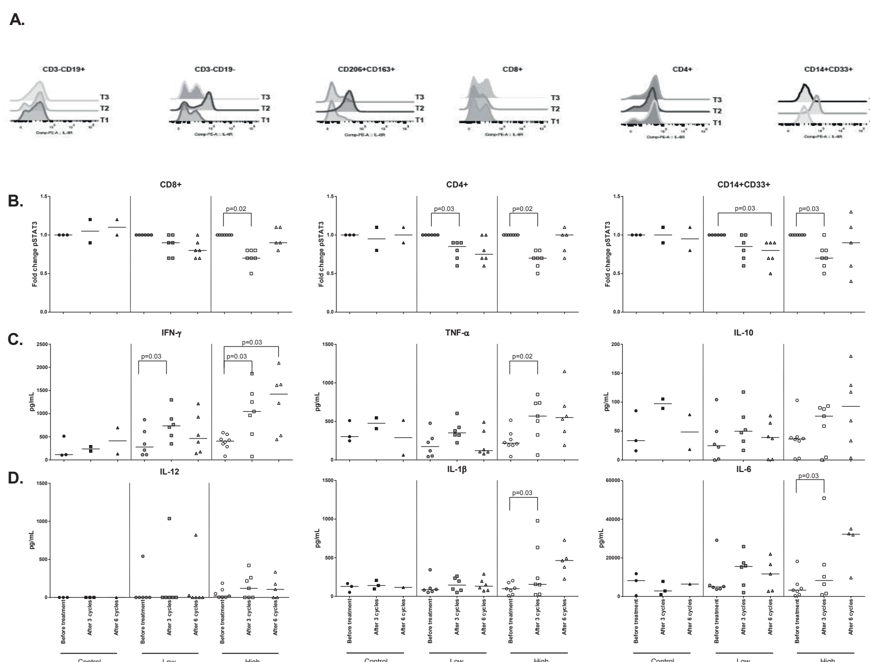


Figure 3. Functional changes in immune cells upon treatment.
Patients are divided in three groups; control, low-dose tocilizumab (1 and 2mg/kg) and high-dose tocilizumab (8mg/kg). Patients receiving Pegintron are depicted in red. The bar represents the median. A) Flow cytometry example of IL-6R expression on different cell subsets; B-cells (CD3-CD19+), myeloid cells (CD3-CD19-), M2-like macrophages (CD3-CD19-HLA-DR$_{high}$CD1a-CD11b$_{int}$/+CD14$_{int}$/+CD206+CD163+), CD8+ T cells, CD4+ T-cells and MDSCs (CD3-CD19-HLA-DR$_{low}$CD15-CD14+CD33+CD11b+). B) Fold change of pSTAT3. Levels of pSTAT3 at baseline were divided by pSTAT3 levels after treatment. pSTAT3 expression decreases upon treatment with high-dose tocilizumab. This effect could not be ascribed specifically to Pegintron. C) Upon treatment with high dose tocilizumab, T cells produce more cytokines (IFN-γ, TNF-α and IL-10) upon mitogenic stimulation. No beneficial effect of the addition of Pegintron is observed. D) Upon stimulation with CD40L, APCs of patients receiving high-dose tocilizumab produce more IL-12, IL-1β and IL-6. There are no explicit changes visible in patients additionally receiving Pegintron.

We demonstrated that treatment with 8mg/kg tocilizumab resulted in increased levels of IL-6 and sIL-6R and non-detectable levels of CRP one month after the last of three infusions with tocilizumab. The absence of CRP suggests the presence of more than 1ug/ml of unbound tocilizumab indicating complete functional blockade of IL-6 activity *in vivo* (11). However, one patient receiving 8mg/kg tocilizumab, did not show these effects when blood was taken after treatment but this patient stopped treatment after two cycles due to progression of disease. The dose dependent change in the serum levels of IL-6, sIL-6R and CRP thus reflects functional blocking of IL-6R by tocilizumab for at least a month after the last administration in the 8mg/kg group.

Consequently, a number of tocilizumab-associated immunological effects could be detected, most of them at the functional level. There was a decline in B-cells potentially due to chemotherapy and/or tocilizumab (21), and no overt changes in the number or phenotype of T-cells or myeloid cells were found, comparable to previous studies with tocilizumab in patients with autoimmunity (12, 22). Additionally, there was an effect on the phosphorylation of STATs, including pSTAT3 by both myeloid cells and the different populations of T-cells. The ablation of pSTAT3 is known to result in an increased production of Th1 cytokines and to alleviate immune suppression (23). Indeed, an increased production of the tumor-immunity promoting cytokines IL-12 and IL-1β (24, 25) by myeloid cells in the high-dose tocilizumab group was observed. Within this group, an increase in the activation status of especially CD8+ T-cells was detected, in accordance with a previous study (26), and the production of anti-tumor associated effector cytokines IFN-γ and TNF-α by T-cells. As the addition of Pegintron enhanced neutropenia but displayed no additive effects on changes in the immune profile, its use should probably be omitted.

The effective blocking of IL-6 signaling during the first three cycles of chemotherapy resulted in a series of functional immunological changes that could be considered as a change towards an immune response less associated with suppression. The sIL-6R level acted as a potential biomarker for these changes. Interestingly, patients with a strong increase in sIL-6R display a longer median overall survival, suggesting a possible survival benefit. However, this may be confounded by the antibody dose effect, but not by tumor cell type, as we excluded the patients with clear cell carcinoma known to have a bad prognosis, in the final analyses. Whereas the immunological changes and clinical outcome observed fit into the model of better tumor control through revamping of immunity, tocilizumab may also have acted directly on tumor growth by blocking IL-6 signaling in the tumor cells as we have previously demonstrated (9).

Our current clinical trial was not powered to demonstrate clinical efficacy but based on the observed immunological changes still present one month after infusion, monthly injections of 8mg/kg of tocilizumab combined with the current standard chemotherapy is recommended for phase 2 testing.

ACKNOWLEDGEMENT

We are greatly indebted to all patients participating in our study. Furthermore, we thank Prof.dr. V.T.H.B.M. Smit (Department of Pathology, LUMC) for his assistance during the clinical trial.

REFERENCES

1. Gooden M, Lampen M, Jordanova ES, et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8(+) T lymphocytes. Proc Natl Acad Sci U S A 2011; 108:10656-10661
2. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348:203-213
3. Reinartz S, Schumann T, Finkernagel F, et al. Mixed-polarization phenotype of ascites-associated macrophages in human ovarian carcinoma: correlation of CD163 expression, cytokine levels and early relapse. Int J Cancer 2014; 134:32-42
4. Lavoue V, Thedrez A, Leveque J, et al. Immunity of human epithelial ovarian carcinoma: the paradigm of immune suppression in cancer. J Transl Med 2013; 11:147
5. Charbonneau B, Goode EL, Kalli KR, et al. The immune system in the pathogenesis of ovarian cancer. Crit Rev Immunol 2013; 33:137-164
6. Coward J, Kulbe H, Chakravarty P, et al. Interleukin-6 as a Therapeutic Target in Human Ovarian Cancer. Clin Cancer Res 2011; 17:6083-6096
7. Plante M, Rubin SC, Wong GY, et al. Interleukin-6 level in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer. Cancer 1994; 73:1882-1888
8. Zitvogel L, Apetoh L, Ghiringhelli F, et al. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8:59-738.
9. Dijkgraaf EM, Heusinkveld M, Tummers B, et al. Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. Cancer Res 2013; 73:2480-2492
10. Preston CC, Maurer MJ, Oberg AL, et al. The ratios of CD8+ T cells to CD4+CD25+ FOXP3+ and F. PLoS One 2013; 8:e80063
11. Nishimoto N, Terao K, Mima T, et al. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. Blood 2008;112:3959-3964
12. Shirota Y, Yarboro C, Fischer R, et al. Impact of anti-interleukin-6 receptor blockade on circulating T and B cell subsets in patients with systemic lupus erythematosus. Ann Rheum Dis 2013; 72:118-128
13. Illei GG, Shirota Y, Yarboro CH, et al. Tocilizumab in systemic lupus erythematosus: data on safety, preliminary efficacy, and impact on circulating plasma cells from an open-label phase I dosage-escalation study. Arthritis Rheum 2010; 62:542-552
14. Grupp SA, Kalos M, Barrett D, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 2013; 368:1509-1518
15. Zeestraten EC, Speetjens FM, Welters MJ, et al. Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. Int J Cancer 2013; 132:1581-1591
16. Bukowski R, Ernstoff MS, Gore ME, et al. Pegylated interferon alfa-2b treatment for patients with solid tumors: a phase I/II study. J Clin Oncol 2002; 20:3841-3849
17. Rustin GJ, Vergote I, Eisenhauer E, et al. Definitions for response and progression in ovarian cancer clinical trials incorporating RECIST 1.1 and CA 125 agreed by the Gynecological Cancer Intergroup (GCIG). Int J Gynecol Cancer 2011; 21:419-423
18. Janetzki S, Britten CM, Kalos M, et al. "MIATA"-minimal information about T cell assays. Immunity 2009; 31:527-528

19. Smolen JS, Beaulieu A, Rubbert-Roth A, et al. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. Lancet 2008; 371:987-997
20. Koskinas J, Zacharakis G, Sidiropoulos J, et al. Granulocyte colony stimulating factor in HCV genotype-1 patients who develop Peg-IFN-alpha2b related severe neutropenia: a preliminary report on treatment, safety and efficacy. J Med Virol 2009; 81:848-852
21. Roll P, Muhammad K, Schumann M, et al. In vivo effects of the anti-interleukin-6 receptor inhibitor tocilizumab on the B cell compartment. Arthritis Rheum 2011; 63:1255-1264
22. Carbone G, Wilson A, Diehl SA, et al. Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid arthritis. Int J Biol Sci 2013; 9:279-288
23. Lee H, Pal SK, Reckamp K, et al. STAT3: a target to enhance antitumor immune response. Curr Top Microbiol Immunol 2011; 344:41-59
24. Dinarello CA Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009; 27:519-550
25. Tugues S, Burkhard SH, Ohs I, et al. New insights into IL-12-mediated tumor suppression. Cell Death Differ, 2014
26. Maccio A, Lai P, Santona MC, et al. High serum levels of soluble IL-2 receptor, cytokines, and C reactive protein correlate with impairment of T cell response in patients with advanced epithelial ovarian cancer. Gynecol Oncol 1998; 69:248-252

SUPPLEMENTARY MATERIALS & METHODS

Patients and treatment scheme. Patients (at the Leiden University Medical Center (LUMC) and University Medical Center Groningen (UMCG)) were eligible for inclusion in the study if they were at least 18 years of age, had progressive or relapsed EOC $\geq$ 3 months after previous standard therapy with platinum and measurable disease (RECIST 1.1 or elevated cancer antigen-125 (CA125) > 2 times the upper limit (ULN) of normal within 3 months). Patients also had to have a WHO 0-2 status, life expectation > 3 months, and adequate bone marrow, liver and renal function with a calculated creatinine clearance ≥ 50 mL/min. Patients were excluded when they had a previous malignancy in the past 5 years (except a previous basal cell carcinoma of the skin or pre-invasive carcinoma of the cervix), serious other diseases, known hypersensitivity reaction to any of the components of the treatment, pregnancy or lactating or medical or psychological condition which in the opinion of the investigator would not permit the patient to complete the study or meaningful sign informed consent.
Cell samples. PBMCs were isolated using Ficoll density gradient centrifugation washed with Phosphate Buffered Saline (PBS), resuspended in cold Fetal Calf Serum (FCS; PAA Laboratories, Pasching, Austria) and cooled on ice for 15 minutes. After dropwise addition in a 1:1 ratio of freezing medium (80% FCS and 20% DMSO (Sigma Aldrich)), PBMCs were cryopreserved at 10 million per ml per cryovial and stored in equal aliquots in the vapor phase of liquid nitrogen until use. The handling, cryopreservation and storage of the PBMCs were done according to the standard operation procedures (SOPs) of the department of Clinical Oncology at the LUMC by trained personnel.
Analyses of lymphocyte proliferation assay. Four peptide pools of Influenza Matrix protein 1 containing 4 of the in total 16 sequential overlapping (with 14 amino acids) 30-mer peptides was used. As a positive control, memory recall mix (MRM), a mixture of tetanus toxoid (150 limus flocculentius/ml; RIVM, Bilthoven, the Netherlands), Tuberculin PPD (1mg/ml; Statens serum institute, Copenhagen, Denmark) and Candidida Albicans (30mg/ml; HAL Allergenen Lab, Haarlem, the Netherlands) were used. PBMCs were incubated at 37°C, 5% CO_2 in a humidified incubator in 4-replicate wells of a 96-wells plate in medium (IMDM, Lonza) with 10% autologous serum in the presence of the indicated antigens and purified phytoheamagglutinin (PHA – Murex Biotech, Dartford, United Kingdom). On day three, 50 µl supernatant was harvested and frozen for cytokine analysis whereafter the cells were pulsed overnight with ^{3}H Thymidine (Perkin Elmer – Groningen, The Netherlands).
Evaluation of the antigen presenting capacity within PBMC population. Patient PBMCs as well as PBMCs obtained from buffy coats of two healthy donors were thawed. 1·5 million cells of patients PBMCs were irradiated in 5 mL at 3000 rad. 100·000 of these cells were incubated with the same amount of healthy donor cells in four-fold in a 96-wells plate for 6 days at 37°C, 5% CO_2 in a humidified incubator. Patients PBMCs as well as donor PBMCs

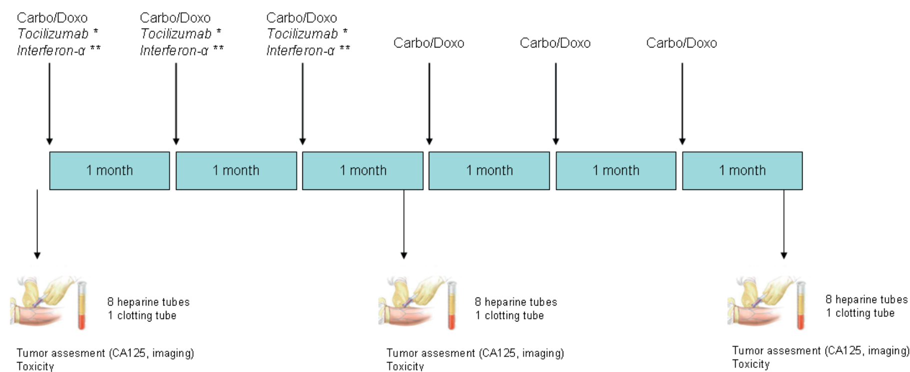
were incubated alone as a control. At day 6, 50 µl supernatant was harvested and frozen for cytokine analysis whereafter the cells were pulsed overnight with ^{3}H Thymidine.

Immunophenotyping by flow cytometry. In short, a million cells were spin down and afterwards, 1mL lysisbuffer was added for 1 minute. After addition of 9 mL IMDM + 10% fetal cow serum (FCS), cells were centrifuged and staining was performed in PBS / 0·5% BSA. Fc-receptor was blocked 10 minutes on ice by adding 50 µl PBS / 0·5% BSA / 10% FCS. Cells were incubated for 30 minutes with a mixture of the following antibodies: CD1a (FITC, clone HI149 - BD, Breda, The Netherlands), CD3 (Pacific Blue, clone UCHT1 - DAKO, Heverlee, Belgium or V450, clone UCHT1 - BD), CD4 (Horizon V500, clone RPA-T4 - BD), CD8 (APC-Cy7, clone SK1 - BD), CD11b (PE, clone D12 - BD or Alexa Fluor (AF) 488, clone CBRM1/5 - Biolegend, Uithoorn, the Netherlands), CD11c (AF700, clone B-ly6 - BD), CD14 (FITC, clone M5E2 - BD or PE-Cy7, clone M5E2 - BD or AF700, clone M5E2 - BD), CD15 (PE CF594, clone W6D3 - BD), CD16 (PE CF594, clone 3G8 - BD), CD19 (Brilliant Voilet (BV) 605, clone SJ25C1 - BD), CD33 (AF700, clone WM53 - BD or PE-Cy7, cloneP67·7 - BD), CD34 APC, clone 581- BD), CD45 (PerCP-Cy5·5, clone 2D1 - BD), CD 56 (APC-Cy7 - Biolegend), CD124 (IL-4R; PE, clone HiL4R-M57 - BD), CD126 (IL-6R; PE, clone M5 - BD), CD163 (APC, clone 215927 - R&D, Systems, Minneapolis, MN), CD206 (Mannose Receptor; APC-Cy7, clone 15-2 - Biolegend), LIVE-DEAD ® Fixable yellow dead cell stain kit (Q-dot585 - Life technologies, Oregon, USA), HLA-DR (V500, clone L243 - BD), pSTAT1 (PE, clone py701 - BD), pSTAT3 (AF647, clone 49 - BD), pSTAT5 (PE, clone pY694 - BD), pSTAT6 (AF648, clone 18 - BD). The data were acquired on a the Fortessa (BD) and analysed with DIVA software version 6.2 and FlowJo version 7.0.

For the detection of T-regs 1 million PBMC were used per condition. Cell surface antibody staining of PBMC was performed in PBS / 0·5% / BSA / 0·02% sodium-azide (PBA) buffer for 30 minutes at 4ºC. Intracytoplasmic/intranuclear staining was conducted with the BD Pharmingen Transcription Factor Buffer set (BD) according to manufacturers' protocol. The antibodies used are: CD3 (V500, clone UCHT1 - BD), CD4 (AF700, clone RPA-T4 - BD), CD25 (PE-CY7, clone 2A3 - BD), CD127 (BV650, clone HIL-7R-M21 - BD), CD45RA (APC-H7, clone HI100 - BD), CD8 (PerCPCy5·5, clone SK1 - BD), FoxP3 (PE-CF594, clone 256D/C7 - BD), CTLA-4 (BV421, clone BNI3 - BD), Ki67 (FITC, clone 20Raj1 - eBiosciences, Vienna, Austria), Helios (APC, clone 22F6 - Biolegend) and LIVE-DEAD ® Fixable yellow dead cell stain kit (Q-dot585). The data were acquired on a the Fortessa (BD) and analysed with DIVA software version 6.2

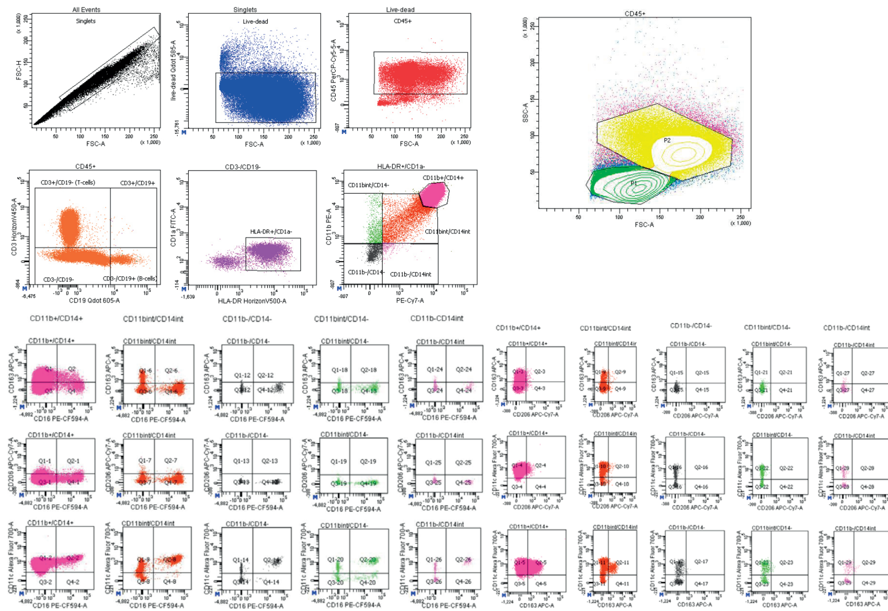
Laboratory environment. The immunomonitoring assays were performed in the laboratory of the department of Clinical Oncology (LUMC) that operates under research conditions, externally and internally audited with respect to immunomonitoring, following SOPs, with pre-established definitions of positive responses and using trained staff. This laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (CIP; of which SHvdB and MW are steering committee members; http://www.cimt.eu/workgroups/cip/) to validate its SOPs as well as many of the proficiency panels of the USA-based Cancer Immunotherapy Consortium (CIC of the Cancer Research Institute).

SUPPLEMENTARY DATA

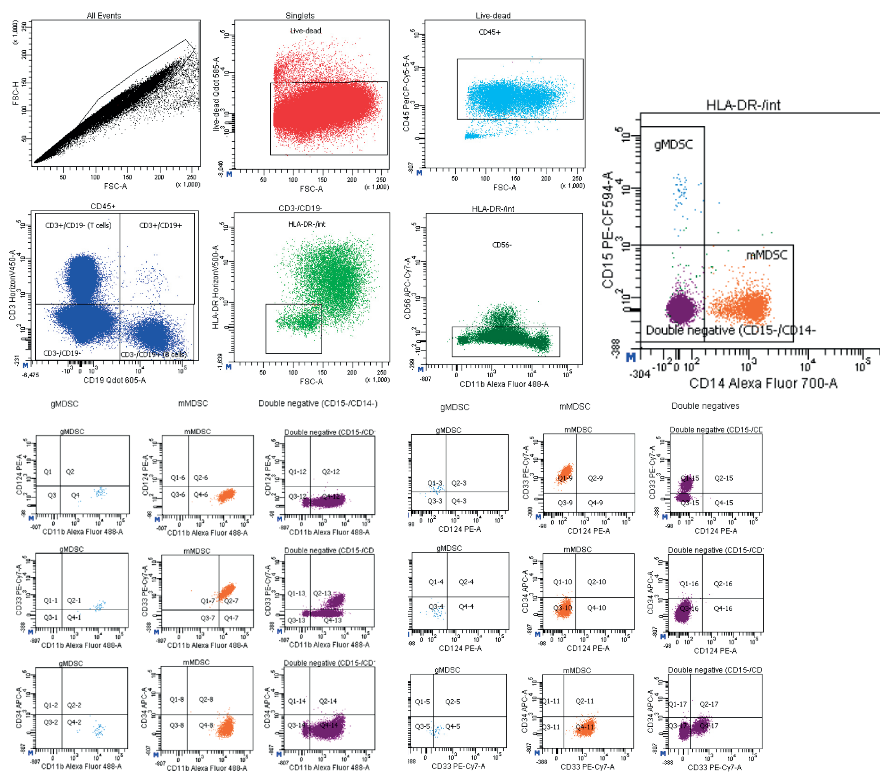


Supplementary Figure 1. Study scheme

Patients were treated in a 3+3 design: first three patients served as a control for immunomonitoring and received chemotherapy (carboplatin/(pegylated lisposomal) doxorubicin) only every months with a total of six cycles. * Depending on study groups, patients were treated in a dose escalation scheme with 1, 2 4 or 8 mg/kg tocilizumab during the first three cycles of chemotherapy. ** Only last cohort of 6 patients received Pegintron in addition to treatment with chemotherapy and tocilizumab during the first three cycles.

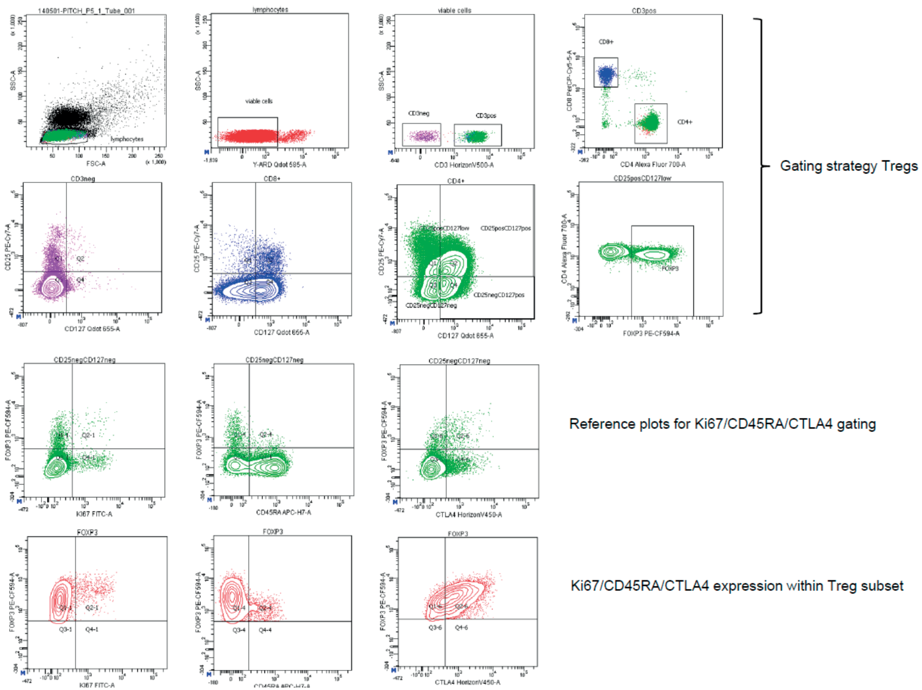


Supplementary Figure 2. Gating strategy macrophages
Singlets were gated based on FCS-A / FCS-H properties, after which the dead cells were excluded through gating on Yellow-Fluorescent Reactive Dye (Y-ARD)-negative cells. Leukocytes were gated on FSC-A / CD45. Subsequently, T-cells (CD3+CD19-), B-cells (CD3-CD19+) and myeloid cells (CD3-CD19-) were gated on differential expression of CD3 and CD19. Myeloid cells were further divided into HLA-DRpos / CD1aneg cells. Different macrophage subsets were split defined on CD14 and CD11b staining, resulting in 5 subcategories: CD11b+CD14+, CD11bintCD14int, CD11b-CD14-, CD11bintCD14- and CD11b-CD14int. These subsets were then further subdivided based on CD163, CD16, CD206 and CD11c.



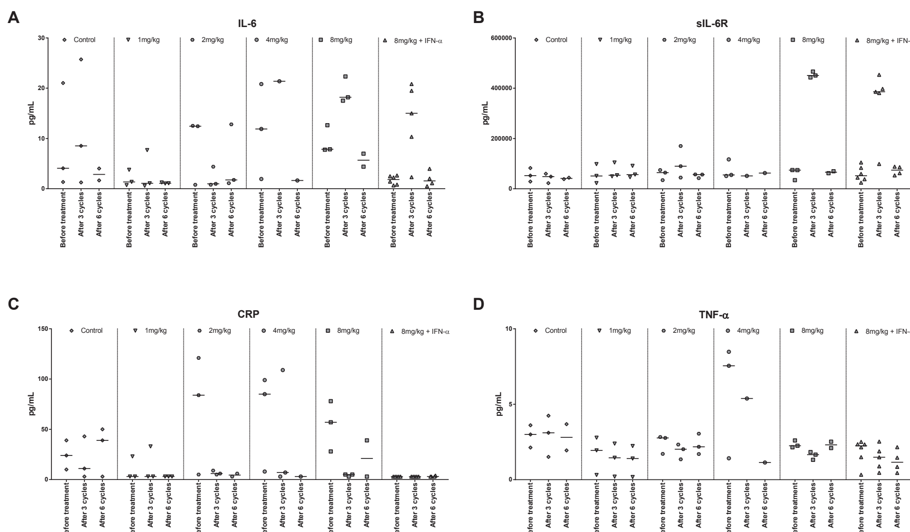
Supplementary Figure 3. Gating strategy MDSCs

Singlets were gated based on FCS-A / FCS-H properties, after which the dead cells were excluded through gating on Yellow-Fluorescent Reactive Dye (Y-ARD)-negative cells. Leukocytes were gated on FSC-A / CD45. Subsequently, T-cells (CD3+CD19-), B-cells (CD3-CD19+) and myeloid cells (CD3-CD19-) were gated on differential expression of CD3 and CD19. Myeloid cells were further gated on HLA-DRneg cells and CD56neg cells. Then, three subsets were recognized: gMDSC (CD15posCD14neg), mMDSC (CD15negCD14pos) and double negatives (CD15negCD14pos). Cells were further subdivided based on CD124, CD11b, CD33 and CD34.

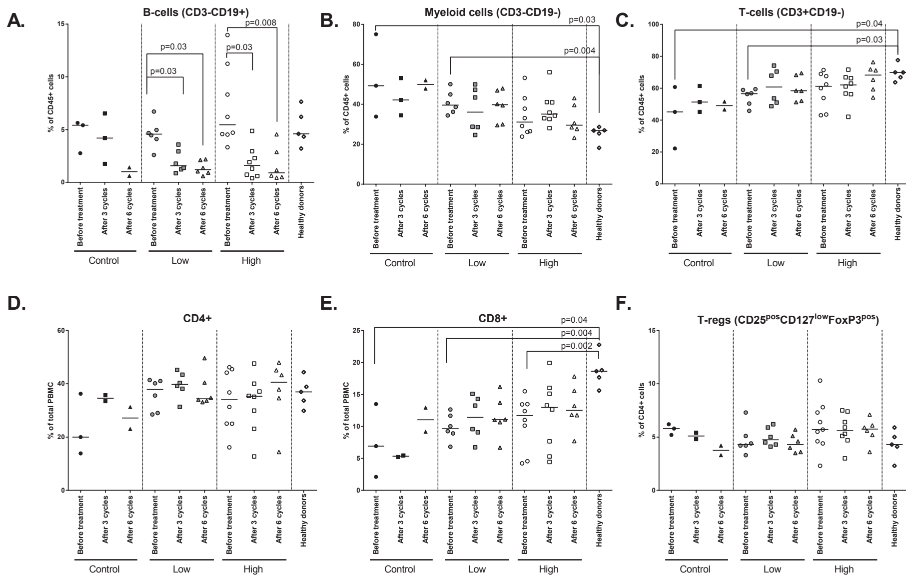


Supplementary Figure 4. Gating strategy T-regs

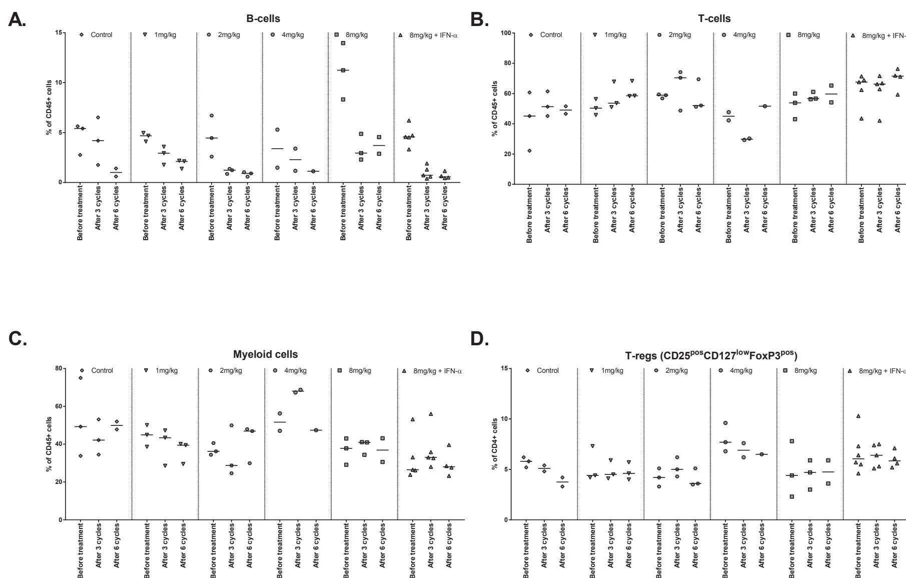
Lymphocytes were gated based on FSC-SSC properties, after which the dead cells were excluded through gating on Yellow-Fluorescent Reactive Dye (Y-ARD)-negative cells. Subsequently, CD3neg and CD3pos populations, as well as the CD4pos and CD8pos populations within the CD3pos cells were identified. Next, gates for CD25 and CD127 were set on CD3neg and CD8pos T cells respectively, and this gate was subsequently applied to the CD4pos T cells. FoxP3 was gated on CD25posCD127neg T cells. Gates for Ki67, CD45RA and CTLA4 were set on CD25negCD127neg T cells and applied to the CD25posCD127negFoxP3pos Tregs.



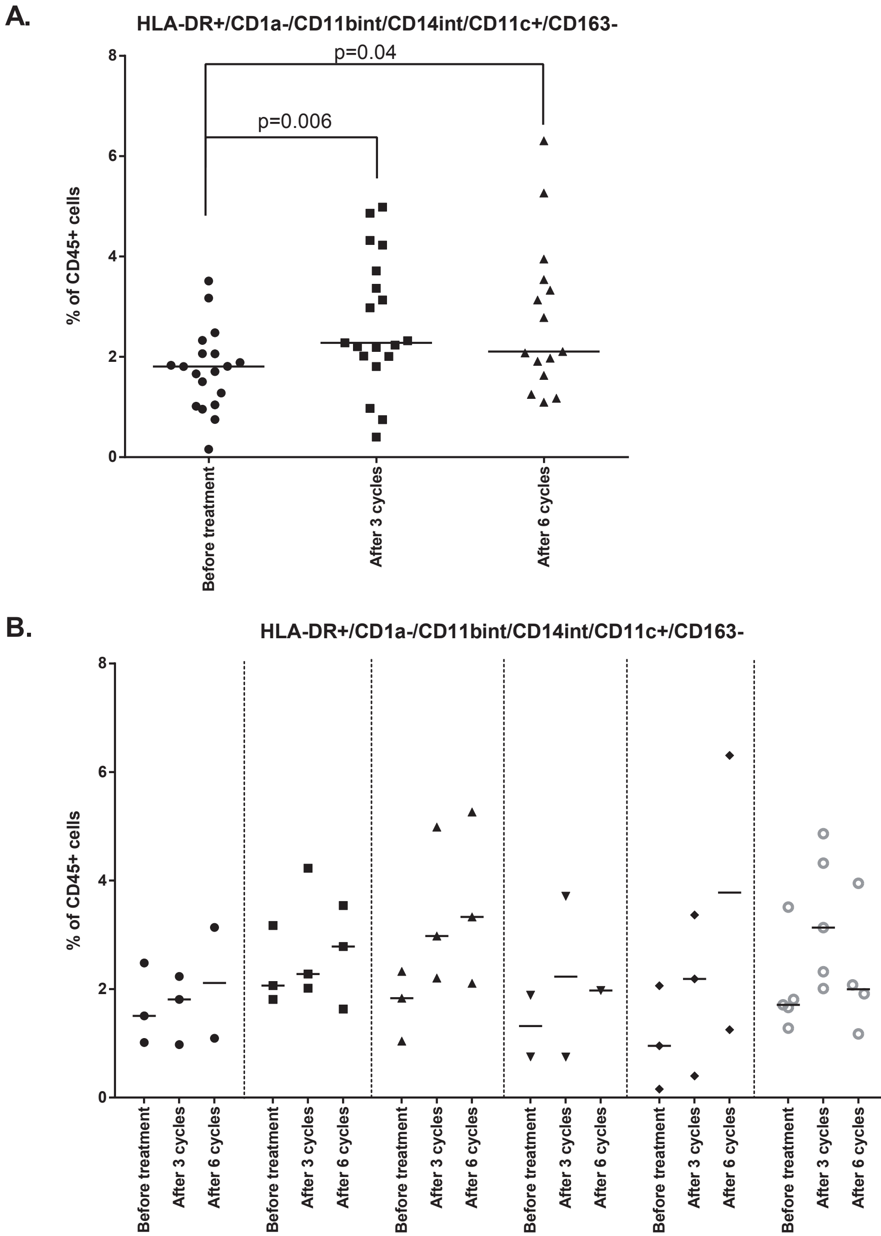
Supplementary Figure 5. Changes in plasma signature upon treatment divided per group.
A) IL-6 B) sIL-6R C) CRP D) TNF-α (all patients)



Supplementary Figure 6. Changes in immunological cell subsets upon treatment.
A) B-cells B) T-cells C) Myeloid cells D) T-regs (CD25+FoxP3+)

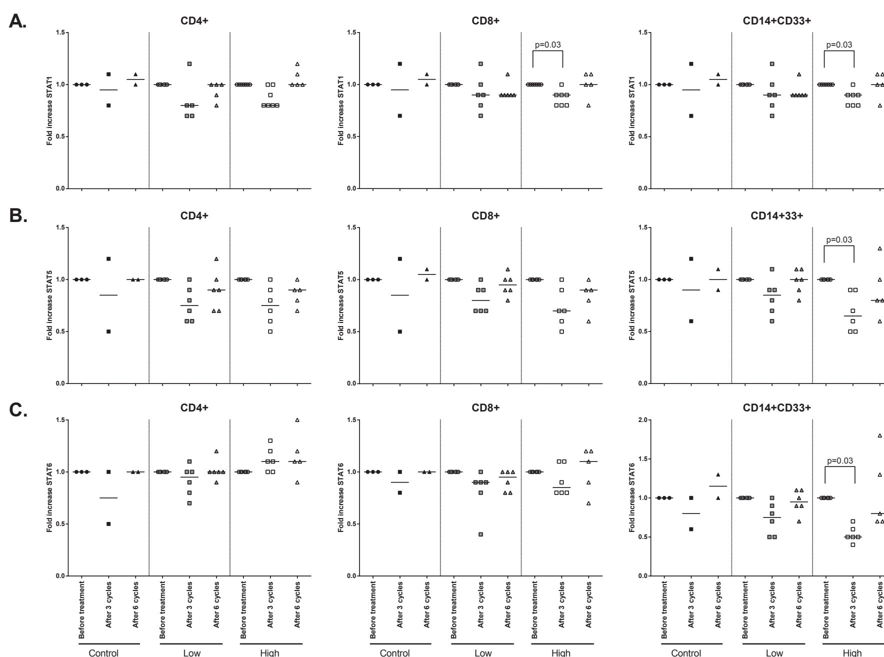


Supplementary Figure 7. Changes in immunological cell subsets upon treatment divided per group.
A) B-cells B) T-cells C) Myeloid cells D) T-regs (CD25+FoxP3+)

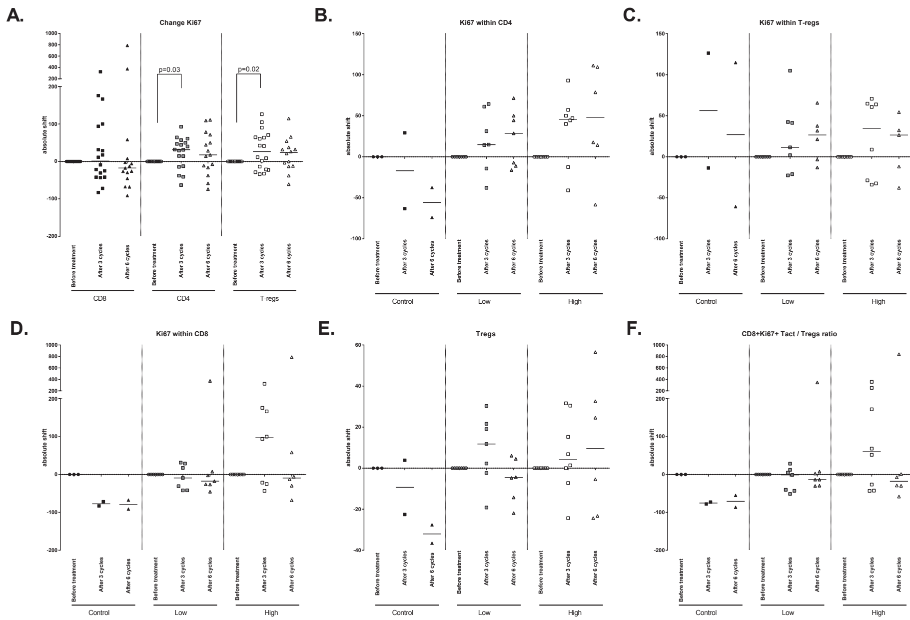


Supplementary Figure 8. Phenotypical changes upon treatment.

A) M1 macrophages (CD3-CD19-HLA-DR+/CD1a-/CD11bint/CD14int/CD11c+CD163-) are increased upon treatment – all patients B) M1 macrophages (CD3-CD19-HLA-DR+/CD1a-/CD11bint/CD14int/CD11c+CD163-) are increased upon treatment – divided per treatment group

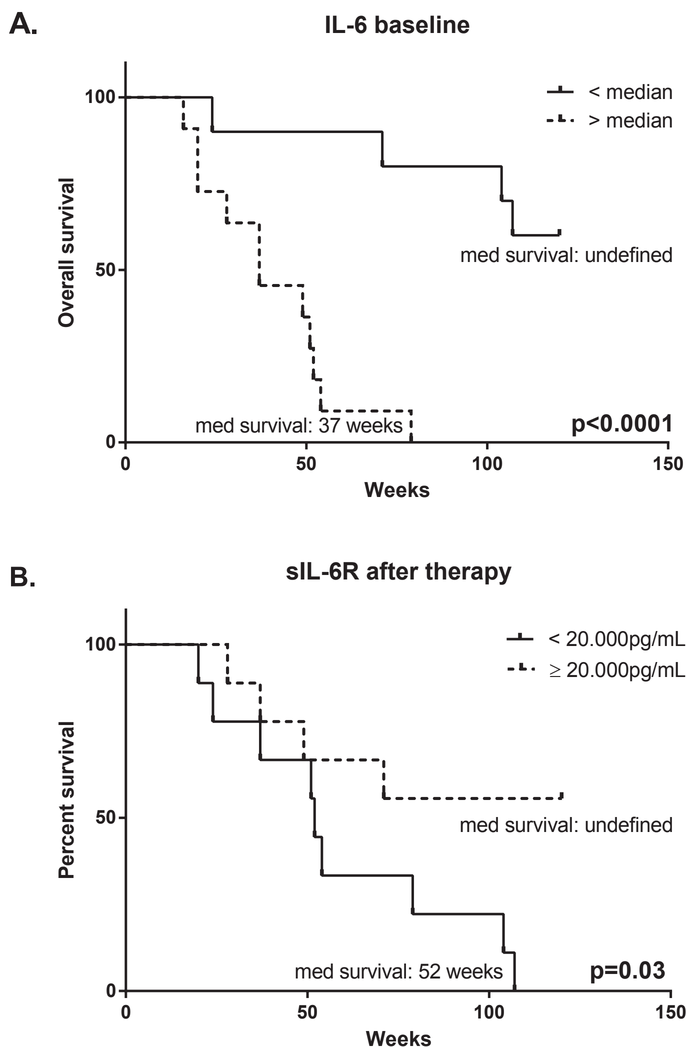


Supplementary Figure 9. Changes in levels of phosphorylated STAT1, 5 and 6 upon treatment.
Fold increase is measured by dividing the pSTAT-levels before treatment by the pSTAT-levels after 3 and after 6 cycles. A) pSTAT1 levels B) pSTAT5 levels C) pSTAT6 levels



Supplementary Figure 10. Activation status of different T cell subsets upon treatment

A) Change in Ki67 in CD4+ and CD8+ T cells as well as Tregs B) Ki67 expression within CD4+ cells depicted per treatment group (control, low tocilizumab, high tocilizumab) C) Ki67 expression within Tregs depicted per treatment group (control, low tocilizumab, high tocilizumab) D) Ki67 expression within CD8+ cells depicted per treatment group (control, low tocilizumab, high tocilizumab) E) Absolute shift of Tregs depicted per treatment group (control, low tocilizumab, high tocilizumab) F) Ratio of activated CD8+ cells (CD8+ki67+) and activated Tregs



Supplementary Figure 11. Survival benefit for patients treated with high-dose tocilizumab. A) Kaplan Meier-curve of IL-6 levels at baseline (p<0·0001) B) Kaplan-Meier curve indicating that patients with effective levels of sIL-6R show an increased survival benefit (p=0·03)