Clinical Protocol

Intraperitoneal Infusion of *ex vivo*-cultured Allogeneic NK Cells in Recurrent Ovarian Carcinoma Patients (a phase I study)

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form (General Assessment and Registration form) is the application form that is required for submission to the accredited Ethics Committee (ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
AR	**Adverse Reaction**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials GCP Good Clinical Practice**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
METC	**Medical research ethics committee (MREC); in Dutch: Medisch Ethische Toetsing Commissie**
(S)AE	**Serious Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

LIST OF SPECIFIC ABBREVIATIONS

AML	**acute myeloid leukemia**
BM	**bone marrow**
CR	**complete remission**
EOC	**Epithelial ovarian carcinoma**
G-CSF	**granulocyte-colony stimulating factor**
GM-CSF	**granulocyte macrophage-colony stimulating factor**
GMP	**good manufacturing practice**
GVHD	**graft-versus-host disease**
GVT	**graft-versus-tumor**
Hi-Cy/Flu	**high-dose cyclophosphamide and fludarabine**
HLA	**human leukocyte antigen**
hr	**hour**
IL	**interleukin**
IFN	**interferon**
IP	**Intraperitoneal**
IV	**Intravenous**
KIR	**killer immunoglobuline-like receptor**
LAK	**lymphokine activated killer**
MHC	**major histocompatibility complex**
NK	**natural killer cells**
OC	**ovarian carcinoma**
PB	**peripheral blood**
PBMC	**peripheral blood mononuclear cells**
PFMC	**Peritoneal fluid mononuclear cells**
RT-PCR	**reverse transcriptase-polymerase chain reaction**
Q-PCR	**quantitative polymerase chain reaction**
SCF	**stem cell factor**
SCT	**stem cell transplantation**
SR1	**Stemregenin-1**
TNF	**tumor necrosis factor**
TRAIL	**TNF-related apoptosis-inducing ligand**
TPO	**thrombopoietin**
UCB	**umbilical cord blood**

SUMMARY

Rationale: Recurrent ovarian carcinoma is incurable, but control of disease and prolonged survival are possible in some patients. The estimated 5-year survival is 46% for all stages of ovarian cancer, and only 28% for metastasized disease. Notably, almost 70% of women with ovarian cancer present with Stage III or IV disease, for which the rate of recurrence is 60-70%.[1] As most women with relapsed or metastatic cancer will die of progressive disease, there is an urgent need for novel therapeutic strategies. This study investigates an innovative treatment for recurrent ovarian cancer exploiting *ex vivo*-generated allogeneic natural killer (NK) cells with or without preceding non-myeloablative conditioning chemotherapy.

Objective: The primary aim of our study is to evaluate safety and toxicity of intraperitoneal infusion of *ex vivo*-expanded NK cells from CD34+ umbilical cord blood (UCB) progenitor cells with and without a preceding non-myeloablative immunosuppressive conditioning regimen in patients suffering from recurrent ovarian, fallopian tube or primary peritoneal cancer. Secondary objectives are to compare the *in vivo* lifespan, expansion and biological activity of intraperitoneal infused NK cell products with and without preparative chemotherapy, and effects on disease.

Study design: This study is a phase I safety and feasibility study in a series of 12 patients who are suffering from recurrent ovarian, fallopian tube or primary peritoneal cancer. Prior to NK cell infusion, a laparoscopy is performed to place a catheter in the peritoneal cavity. The first cohort of three patients will receive an intraperitoneal infusion of between 1.5x 10^9 and 3x10^9 allogeneic UCB-NK cells generated *ex vivo* from CD34+ hematopoietic progenitor cells obtained from an allogeneic UCB unit without a preparative regimen. In the second group of three patients the same UCB-NK cell dosage will be given with a preparative regimen of four days non-myeloablative immunosuppressive conditioning regimen with cyclophosphamide and fludarabine (CyFlu). If no severe toxicity is seen in these 6 patients, an extension cohort of 6 patients will be included to answer the secondary objective.

Study population: Patients suffering from their second recurrence of ovarian, fallopian tube or primary peritoneal cancer, detected by an significant rise in serum level of CA-125 on two successive time points.

Intervention: Allogeneic UCB-NK cell products will be transfused into the peritoneal cavity of patients (single dose between 1.5 x10^9 and 3x10^9 UCB-NK cells) with or without a preparative immunosuppressive conditioning consisting of cyclophosphamide (900 mg/m^2/day) and fludarabine (30 mg/m^2/day) on days -6, -5, -4, -3, in order to prevent direct rejection and facilitate engraftment. To reach the whole abdominal cavity with the NK cell infusion, a peritoneal catheter will be placed during laparoscopy. Monitoring will be done for toxicity, biological parameters and remission status.

Main study parameters/endpoints: Primary endpoints are safety and toxicity. Secondary endpoints are *in vivo* detection and expansion of the transfused UCB-NK cells, detection of biological NK cell activity and effect on CA-125 levels.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Based on literature and preliminary results of other groups, we don't expect severe toxicity of the UCB-NK cell product, nor from the intraperitoneal infusion. The Cy/Flu treatment dosage is the same as in the study we performed with UCB-NK cells in elderly AML patients, the PLMA25 study and lower than in multiple myeloma patients, where no severe toxicity is seen.

For IP treatment an IP catheter is placed during laparoscopy, this catheter remains in place for 28 days after UCB-NK cell infusion. Every laparoscopy brings a risk of infection, bleeding and thrombosis. A laparoscopy after primary surgery holds an increased risk of organ injury. To prevent this, an open laparoscopy will be performed. For follow-up peripheral blood will be collected at 7 time points, and peritoneal fluid will be collected through the IP catheter at maximal 5 time points.

This study investigates the safety and feasibility of a promising new cellular therapy in a group of patients with a poor prognosis. If UCB-NK cell therapy is safe and shows expansion without Cy/Flu pre-treatment, the next step could be UCB-NK cell infusion after regular second line chemotherapy.

1. INTRODUCTION AND RATIONALE

1.1. Treatment of recurrent ovarian carcinoma

Recurrent ovarian carcinoma (OC) is an incurable and lethal disease, but control of the disease and prolonged survival are possible in some patients. The estimated 5-year survival is 46% for all stages of ovarian cancer, and only 28% for advanced stage disease. Notably, almost 70% of women with ovarian cancer present with Stage III or IV disease, for which the rate of recurrence is 60-70%[1]. As most women with relapsed or metastatic cancer will die of progressive disease, there is an urgent need for novel therapeutic strategies. Current therapy consists of 6 cycles of palliative chemotherapy, which are not always completed due to side effects. A new drug in the palliative treatment, Bevacuzimab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), which is used in combination with gemcitabine and carboplatin chemotherapy, shows an improvement in progression free survival, but not in overall survival[2]. It is known that treatment of recurrent ovarian carcinoma based solely on elevation of the tumor marker CA-125 does not improve quality of life over treatment starting after first symptoms of recurrence. The time between elevation of CA-125 and first symptoms has a mean of 4.8 months[3].

This study investigates an innovative immunotherapeutic treatment for advanced ovarian cancer that uses natural killer (NK) cells in the time between elevation of CA-125 and the occurrence of symptoms. NK cells are part of the innate immune system and are unique in the capacity to react to tumor cells without prior immunisation and use several killing mechanisms[4]. These mechanisms are similar to CD8+ cytotoxic T cells and include release of cytotoxic granules containing perforin and granzymes[5,6], TRAIL-dependent cytotoxicity and activation of Fas-mediated apoptosis[6]. Tumor cell recognition by NK cells is controlled by germline-encoded NK cell stimulatory and inhibitory receptors[7-9]. The discovery of these receptors emerged from early observations showing that NK cell cytotoxicity was triggered by tumor cells lacking self MHC class I molecules. These characteristics, referred to as 'missing-self' recognition, led to the identification of inhibitory killer immunoglobulin-like receptors (KIR) and C-type lectin receptors of the CD94-NKG2A complex. Inhibitory KIR recognize groups of HLA-A, -B and -C alleles, and CD94-NKG2A recognizes HLA-E[10,11]. Each NK cell expresses a different combination of inhibitory and stimulatory receptors such that at least one inhibitory KIR specific for a self MHC class I allele is expressed. NK cells expressing a KIR for a particular HLA molecule will not be inhibited by tumor cells that lack this molecule, resulting in tumor cell killing. In the setting of allogeneic adoptive NK cell therapy this 'KIR-ligand' mismatch has shown to contribute to the anti-tumor effect. To explore NK cell adoptive therapy we developed unique GMP-compliant culture protocols for the generation of large numbers of highly functional NK cells from umbilical cord blood (UCB)-derived CD34+

hematopoietic progenitor cells[12,13,26.] Previously, we have demonstrated that intravenous (IV) infusion of a first generation allogeneic UCB-NK cell product up to a dose of $30x10^6$/kg after lymphodepleting chemotherapy is feasible, safe and well tolerated in older acute myeloid leukemia (AML) patients[26].

Recently, we have demonstrated that an improved second generation UCB-NK cell product can efficiently attack OC spheroids as well as OC tumors in a preclinical mouse model after intraperitoneal (IP) infusion[13]. In addition, we found that the number of CD56+ NK cells in ascites of ovarian carcinoma patients is related to survival (unpublished data). In a cohort of 21 patients a significantly better survival was seen in patients with more CD56+ NK cells. However, we observed a completely different distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cells in ascites from OC patients. While in blood this is normally 90% vs 10%, we found in the patient's peritoneal fluid a distribution of 55% vs 45%, respectively. Furthermore, on our *ex vivo*-generated UCB-NK cells far more activating receptors are available in comparison to the ascites-derived NK cells. These findings indicate that UCB-NK cells are much better armed against ovarian carcinoma than the available NK cells in the abdominal cavity of patients, and support the rationale of IP infusion of highly activated UCB-NK cells for targeting OC tumor deposits in recurrent OC patients.

1.2. NK cell-based immunotherapy against cancer

The information we have nowadays on NK cell therapy originates mainly from the field of haematological cancers. Important insights into NK cell function in cancer eradication originates from the allogeneic stem cell transplantation (SCT) settings. Ruggeri and colleagues clearly demonstrated that NK cell alloreactivity following haplo-identical SCT can control relapse of AML without causing graft-versus-host disease (GVHD)[14,15]. This stimulated further exploitation of allogeneic NK cell infusions in the non-transplant setting. Here, it has been observed that adoptive NK cell therapy can exert anti-AML activity without inducing GVHD and is clinically well-tolerated[16,32-35]. Allogeneic NK cell therapy is performed following cyclophosphamide and fludarabine (Cy/Flu) based lymphodepleting chemotherapy, thereby preventing immediate rejection of transferred allogeneic NK cells. Moreover, it eliminates tolerogenic immune cells and promotes cytokine availability for *in vivo* NK cell persistence and expansion[16,19,26]. In 2005, Miller and collaborators demonstrated that allogeneic intravenous NK cell infusions up to $2x10^7$ NK cells/kg body weight were well tolerated[16]. In this study a heterogeneous group of 43 patients with advanced cancer status (melanoma, renal cell carcinoma, AML) received NK cell infusions together with IL-2. Toxicity was limited to constitutional symptoms including low-grade fever, chills and myalgias mostly due to low-dose IL-2 injections post-NK cell infusion. Furthermore, transferred NK cells can persist and expand *in vivo* (>1% engraftment at day 7 and beyond). Most importantly, five

out of 19 patients with advanced AML achieved complete remission (CR) and in six out of 16 solid tumor patients stable disease was achieved. In 2014, updated studies at the University of Minnesota showed that poor-prognosis AML patients with relapsed/refractory disease achieved hematologic CR in 53% (8/15) of cases after infusion of purified peripheral blood NK cells in combination with prior IL-2-diphtheria toxin fusion protein (IL-2DT) treatment to deplete Tregs and post-NK IL2 administration[34]. Interestingly, this increased CR rate allowed several patients to become eligible for potentially curative allo-SCT. In line with these results, we and others demonstrated that infusion of purified NK cells[32,33] as well as UCB-NK cells[26] is feasible in older AML patients as post-CR consolidation strategy.

Next to haematological malignancies, other clinical studies have explored NK cell-based adoptive immunotherapy in solid tumor patients. In 2008 Arai et al. published results of a clinical trial using the NK-92 cell line in melanoma and renal cell cancer patients, showing stable disease in 4 out of 10 RCC patients and partial response in 1 melanoma patient[17]. Furthermore, Brehm et al. have shown in 2011 CR in neuroblastoma patients after allogeneic IL-2-stimulated NK cell infusion[18]. Altogether these results suggest that allogeneic NK cell adoptive immunotherapy can also inhibit tumor growth in solid tumors. Regarding NK cell therapy against ovarian carcinoma the first trial, exploiting IL-2 activated peripheral blood (PB)-derived NK cells was conducted in 2010. This trial showed that adoptive transfer of haplo-identical NK cells intravenously after lymphodepleting chemotherapy is associated with transient donor chimerism in 15 out of 20 patients[19]. Currently there is a first trial going on in the USA (NCT02118285), investigating intraperitoneal NK cell therapy in combination with an immune modulating agent (see appendix 6).

1.3. Intraperitoneal infusion of NK cells

Since NK cells can target human tumors, several strategies for therapeutic use of NK cells are under investigation. From biodistribution studies we know that a major part of the NK cells accumulates in the liver and lungs after IV infusion[20,21]. In ovarian carcinoma cases the disease is mostly confined to the peritoneal cavity till the last stage. Therefore, intraperitoneal infusion of NK cells, represents an attractive alternative approach to enhance anti-tumor activity at the tumor site itself in ovarian cancer patients. In mouse studies, we have demonstrated that UCB-NK cells can persist *in vivo* and that NK cell therapy slows down tumor progression and enhances survival. This effect was more pronounced in mice treated with IP UCB-NK cells[13]. In line with our data, the group of Geller et al has shown similar results with a different OC cell line and different NK cell products[22].

In the regular primary chemotherapeutic treatment of ovarian carcinoma, intraperitoneal treatment shows better OS and PFS[23]. For this treatment an intraperitoneal catheter is placed during laparotomy or laparoscopy. Our hospital is one of the expert centres in the

Netherlands were intraperitoneal chemotherapy is frequently administered. Miller et al are currently performing a phase 1 toxicity study in ovarian carcinoma patients, exploring the toxicity of intraperitoneal NK cell therapy in combination with an immune modulating agent. Their first results show that IP NK cell infusion is safe, has no NK specific side effects and the transferred NK cells robustly expand in the peritoneal cavity (see appendix 6)

1.5. *Ex vivo* generation of NK cells from CD34+ hematopoietic progenitor cells

In order to exploit NK cell adoptive immunotherapy for cancer treatment, we have developed a novel stroma cell-free culture protocol based on the combination of several selected cytokines for the *ex vivo* generation of NK cells from CD34+ hematopoietic progenitor cells enriched from thawed UCB units. The developed NK cell generation procedure consists of two steps. The first step involves the expansion of NK progenitors from CD34+ UCB cells in 14 days of culture. The second step consists of the differentiation of the expanded progenitor cells into the NK cell lineage, which requires an additional 3-week culture period. Pre-clinical testing has been performed using CD34+ cells obtained from UCB after full-term delivery or bone marrow/mobilized PB from transplant donors after informed consent. CD34+ cells are enriched by anti-CD34 immunomagnetic beads selection. After checking purity with flow cytometry, CD34+ cells with an enrichment of >70% cells are used to initiate cell expansion. For this, CD34+ cells are cultured for 14 days in a basal medium supplemented with 10% human serum, a specific combination of cytokines including SCF, Flt3L, IL-7 and TPO and the aryl hydrocarbon receptor (AHR) antagonist Stemregenin-1 (SR1)[13,25]. Overall, we observe a stable and reproducible expansion rate between 50- and 150-fold during the first 14 days of culture from UCB-derived CD34+ cells[12,13,24,25]. Subsequently, expanded progenitor cells are differentiated into NK cells using differentiation medium consisting of basal medium supplemented with 2% human serum, SCF, Flt3L, IL-7 and cytokines like IL-2, IL-12 and IL-15, which are essential for the differentiation of NK cells[13,24,25]. Extensive phenotypic analysis revealed reproducible outgrowth of CD56+ NK cells within the first two weeks of differentiation, from approximately 10% after one week until 60-75% after two weeks. In the last week of the differentiation step, we observed robust expansion of CD56+ cells resulting in a homogeneous final cell product of CD56+CD3- cells with a purity of >85%[13].

Subsequently, we performed upscaling studies using CD34+ cells enriched from thawed UCB units by the CliniMACS cell separator (Miltenyi Biotec). The average yield and purity of these enrichments were $2.2x10^6$ (range $0.9\text{-}6.7x10^6$; n=9) and 71% (range 52-92 %; n=9) CD34+ cells (see for details IMPD). Similar CD34+ cell enrichment results were obtained in our recently completed phase I study with the Heparin/IL-15/IL-2 based first generation UCB-NK cell product[26]. In our improved second generation UCB-NK cell culture protocol, enriched

CD34+ UCB cells ($2x10^5$/ml) were subsequently cultured for 10 days in VueLife™ culture bags (CellGenix) with basal medium plus 10% pooled human serum (Sanquin Bloodbank), SCF, Flt3L, IL-7 and TPO and SR1. Further differentiation and expansion using the combination IL-15 and IL-12 resulted in the generation of UCB-NK cell products with a purity of 92 ± 3% (range 89-94%; n=3; see for details IMPD). The remaining cells represented mature myeloid cells expressing CD14, CD15 and/or CD33. Importantly, the final NK cell products contained <0.01% CD3+ T cells and <0.01% CD19+ B cells. The mean expansion rate in the VueLife™ culture bags was >1,000 fold, resulting in the generation of ~$2x10^9$ CD56+CD3- NK cells. These upscaling data show that the developed two-step expansion and differentiation protocol reproducibly generates CD56+CD3- NK cell products from UCB applicable for adoptive immunotherapy. Based on these validation studies, we expect to generate between 2-$5x10^9$ NK cells from 2-$5x10^6$ UCB-derived CD34+ cells within maximal 5 weeks of culture. In this clinical phase I study, we will infuse a single-dose between $1.5x10^9$ and $3x10^9$ UCB-NK cells intraperitoneally in combination with IL-2 support.

2. OBJECTIVES

2.1. Primary Objective:

The study is designed as a phase I toxicity study in a series of 12 patients suffering from their second recurrence of ovarian, fallopian tube or primary peritoneal cancer, detected by an elevated serum level of CA-125 on two successive time points with 28 days in between reaching a level of more than 35 U/ml, to evaluate:

- feasibility, safety and toxicity of intraperitoneal CD34+ UCB progenitor-derived allogeneic NK cell infusions with a fixed dose of between $1.5x10^9$ to $3x10^9$ *ex vivo*-expanded UCB-NK cells with and without an immunosuppressive conditioning therapy.

2.2. Secondary Objectives:

- evaluation of the *in vivo* lifespan and expansion of UCB-NK cells following intraperitoneal infusion with and without immunosuppressive conditioning therapy.
- exploration of the biological and clinical activity of UCB-NK cell infusion in study participants.

3. STUDY DESIGN

This is a prospective phase I toxicity study with the aim to treat a total of 12 patients suffering from a second recurrence of ovarian, fallopian tube and primary peritoneal cancer, with *ex vivo*-expanded allogeneic UCB-NK cells following a non-myeloablative immunosuppressive preparative regimen. In the first two cohorts of three patients, the toxicity of intraperitoneally

infused UCB-NK cells with and without Cy/Flu pre-treatment will be studied. See appendix 7 for a flow chart. Currently used adoptive transfer protocols involve pre-conditioning of patients with lymphodepleting Cy/Flu chemotherapy. This approach allows proliferation of NK cells through depletion of host lymphocytes. However, it is currently not known whether pre-conditioning is necessary in the setting of intraperitoneal delivery in OC patients. Therefore a key objective is to compare UCB-NK cell survival and expansion with and without preconditioning. This question will be addressed in the extension cohorts of three patients without and three patients with a preparative non-myeloablative immunosuppressive chemotherapy regimen.

- Patients with a second recurrence of ovarian, fallopian tube and primary peritoneal cancer, detected by a raise in serum CA-125 level of 2 times nadir on two following time points with 28 days in between, reaching a level of more than 35 U/ml. After informed consent, these patients will undergo a laparoscopy to place the intraperitoneal catheter and to score the abdominal cavity for adhesions. If possible a small biopsy will be taken. The catheter will only be placed if 5 out of 9 compartments of the abdominal cavity are accessible.
- These patients will be typed for HLA class I alleles. Eligible ovarian carcinoma patients are those for whom a suitable allogeneic UCB unit displaying the highest HLA match for HLA-A and HLA-B at antigen level can be found. For this purpose, we will use UCB units within the Cord Blood Bank Nijmegen that meet the criteria of cell numbers for this protocol. Immediately after allocation, available UCB units will be screened for selecting an appropriate donor for *ex vivo* NK cell expansion.
- Five weeks prior to NK infusion (day -35), the suitable allogeneic UCB unit will be thawed and CD34+ cells will be enriched by using a CliniMACS cell separator after binding with CD34 coupled to immunomagnetic particles (Miltenyi Biotec). Enriched CD34+ UCB cells will be used for *ex vivo* differentiation and expansion of CD56+ NK cells products according to the validated SR1/IL-15/IL-12 culture procedure. Cell isolation, enrichment and culture procedures will be performed under GMP conditions in a clean room and using established SOPs according to JACIE, NETCORD FACT and GMP guidelines (see for details IMPD).
- The trial design is depicted in Appendix 7. First, three patients will be treated in Cohort 1 without Cy/Flu conditioning. If no grade 3 toxicity (specified in paragraph 7.1) of the investigational treatment is observed we will continue with Cohort 2 with Cy/Flu regimen. If 1 of 3 patients in Cohort 1 has ≥ grade 3 toxicity of the investigational treatment we first extend this cohort with 3 patients to a total of 6 patients (i.e. Cohort 3). If none of the additional patients in Cohort 3 has ≥ grade 3 toxicity we will then proceed with Cohort 2 (including Cy/Flu). Finally, if <2 of 6

patients in Cohort 3 and <2 of 3 patients in Cohort 2 has ≥ grade 3 toxicity of the investigational treatment we extend the Cy/Flu cohort with 3 patients to a total of 6 patients (i.e. Cohort 4).

- For cohort 1 and 3: Patients will be treated with UCB-NK cells without intravenous non-myeloablative immune suppression, with IL-2 support.
- For cohort 2 and 4: Patients will be treated with UCB-NK cells after intravenous non-myeloablative immune suppression, with IL-2 support similar as for the first cohort.
    - Six days before infusion of the *ex vivo*-generated UCB-NK cell product, patients receive intravenous non-myeloablative immunosuppression consisting of cyclophosphamide (900 mg/m^2/day) and fludarabine (30 mg/m^2/day) on days -6, -5, -4, -3. This Cy/Flu regimen will be administered in an inpatient hospitalized setting.
    - Six days before infusion of the *ex vivo*-generated UCB-NK cell product, with the start of non-myeloablative immune suppression patients receive opportunistic infection prophylaxis consisting of ciprofloxacin (2 dd 500 mg until recovery of neutropenia), valaciclovir and co-trimoxazol (1 dd 480 mg) in combination with folic acid (1 dd 5 mg) until 6 months after infusion of UCB-NK cells. On day 8 patients receive a single dose of pegfilgrastim (6 mg s.c.) to shorten neutropenia.
- All patients receive an IP infusion of UCB-NK cells on day 0. NK cells will be infused with a single dose of between 1.5 x10^9 and 3x10^9 cells dissolved in 500 ml of infusion medium (i.e. 0.9%NaCl plus 5% HSA; see IMPD). Prior to infusion, the patient will receive premedication consisting of acetaminophen 500 mg orally and clemastine 2 mg intravenously. Patients will be evaluated including physical examination, toxicity scores and standard blood tests. To examine the response to treatment, we will collect peripheral blood from patients (pre-study, day 7, 14, 28 and 56 after NK cell infusion) and peritoneal fluid aspirates (at time of catheter placement, 7, 14, 21 and 28 days after NK cell infusion).
- All patients receive intraperitoneal IL-2 at a dose of 6x10^6 units/dose, 3 times a week with a total of 6 dosages starting at day 0.
- We will not continue with the extension cohort of six patients (i.e. Cohort 3 and 4), which consist of three patients without preconditioning and three patients with preconditioning, if we don't reach an NK cell percentage in the abdominal fluid of more than 5% in the cohort of 3 patients who received the standard preconditioning chemotherapy regimen combined with intraperitoneal UCB-NK cell therapy and IL-2 support.
- Furthermore we will use the following stopping criteria with regard to toxicity in the

different study cohorts (see Appendix 7):

- Cohort 1 without Cy/Flu regimen: If 1 of 3 patients has grade 4 or if 2 of 3 patients have grade 3 toxicity (specified in paragraph 7.1) related to the investigational treatment we will stop the study. If 1 of 3 patients has ≥ grade 3 toxicity of the investigational treatment we extend this cohort with 3 patients to a total of 6 patients (i.e. Cohort 3). If 0 of 3 patients has ≥ grade 3 toxicity of the investigational treatment we first continue with Cohort 2 with Cy/Flu regimen.
- Cohort 3 without Cy/Flu regimen: If 2 of 6 patients (in Cohort 1+3) have ≥ grade 3 toxicity related to the investigational treatment we will stop the study.
- Cohort 2 with Cy/Flu regimen: If 1 of 3 patients has grade 4 or if 2 of 3 patients have grade 3 toxicity (specified in paragraph 7.1) related to the investigational treatment we will stop this Cy/Flu arm of the study. If 1 of 3 patients has ≥ grade 3 toxicity of the investigational treatment we extend this cohort with 3 patients to a total of 6 patients (i.e. Cohort 4).

4. STUDY POPULATION

4.1. Population

Patients diagnosed with their second recurrence of epithelial ovarian cancer, fallopian tube, or primary peritoneal cancer, without gastrointestinal symptoms. Recurrence will be diagnosed by an ascending serum level of CA-125 on two successive time points with 28 days in between, reaching a value of more than 2 times nadir and above 35 U/ml. Patients must have a life expectancy of >6 months and are only enrolled after prior written informed consent. Patients should be able to undergo a laparoscopy. If at laparoscopy there is exposure of less than 5 out of nine compartments of the peritoneal cavity, patient will be excluded. Furthermore, patients on immunosuppressive treatment, with active infections that require specific therapy and patients with renal, pulmonary or myocardial impairment following chemotherapy treatment will be excluded. We expect to have at least 20 patients a year that meet these criteria. The study is completed at day 56, further treatment will be guided by patients own physician. If there is a need to start regular palliative treatment, investigational treatment will be stopped and patient will be excluded from participation.

4.2. Patient inclusion criteria

- Patients suffering from their second recurrence of ovarian, fallopian tube or primary peritoneal cancer, with an elevated serum level of CA-125 on two successive time

points with 28 days in between, reaching a value of more than 2 times nadir and above 35 U/ml without gastrointestinal symptoms.

- Able to undergo laparoscopic IP port placement and IP treatment administration
- Adequate organ function
- Age 18 years or older
- Age under 76 years.
- Karnofsky performance status >70% (see appendix 2)
- Life expectancy > 6 months
- All anti-cancer treatment should be stopped at least 28 days prior to administration of investigational treatment.
- Grade 2 or higher toxicity of previous ant-cancer treatment should be resolved except neuropathy
- Written informed consent
- Availability of a partially HLA-matched UCB unit

4.3. Patient exclusion criteria

- Patients on immunosuppressive drugs
- Patients with active infections (viral, bacterial or fungal) that requires specific therapy. Acute anti-infectious therapy must have been completed within 14 days prior to study treatment
- Laparoscopic adhesion score >4 out of 9.
- Severe cardiovascular disease (arrhythmias requiring chronic treatment, congestive heart failure or symptomatic ischemic heart disease (appendix 4)
- Severe pulmonary dysfunction (CTCAE III-IV) (appendix 4)
- Severe renal dysfunction (MDRD<50) (appendix 4)
- Severe hepatic dysfunction (serum bilirubin or transaminases > 3 times normal level) (appendix 4)
- Severe neurological or psychiatric disease

5. TREATMENT OF SUBJECTS

5.1. Investigational product/treatment

This study is a phase I toxicity trial, using *ex vivo*-generated NK cells from CD34+ UCB cells from allogeneic partially HLA-matched donors. The *ex vivo*-generated allogeneic UCB-NK cells will be infused intraperitoneally into patients suffering from recurrent ovarian, fallopian

tube or peritoneal cancer with or without Cy/Flu conditioning. This immunosuppressive conditioning regimen is often applied with intravenous NK cell infusion to prevent direct rejection but might not be necessary if NK cells are administered IP. The investigational CD56+CD3- UCB-NK cell products will be ≥70% pure and almost devoid of CD3+ T cells (*i.e.* $<1x10^5$ cells/kg body weight), thereby minimizing donor T cell-mediated GVHD. NK cell therapy is combined with IL-2 cytokine support, at a dose of $6x10^6$ units/dose, 3 times a week with a total of 6 dosages. Study participants will undergo clinical and immunological evaluation (see appendix 1).

5.2. Description and justification of route of administration and dosage

Laparoscopy

To be able to infuse the UCB-NK cells in the abdominal cavity, a single lumen 9.6fr attachable silicone catheter (Bard Medical) is placed per laparoscopy. If there are too many adhesions to reach 5 out of 9 compartments of the abdominal cavity, no catheter is placed.

Pre-treatment

All patients will receive an intraperitoneal infusion of between $1.5x10^9$ and $3x10^9$ allogeneic NK cells generated *ex vivo* from CD34+ cells obtained from an allogeneic UCB unit. This dosage is based on results of published trials with PB-enriched NK cells and on our UCB-NK trial in elderly AML patients[26]. Miller et al are currently executing a trial on peritoneal infusion of enriched PB-NK cells with IL-2 support in ovarian carcinoma patients. In the first two patients significant survival and expansion has been observed. No adverse NK cell related toxicity is observed in these first two patients (see appendix 6). IP infusion might be a valuable stategy, since the immunologic environment in the abdomen is completely different from the situation in blood. The hypothesis is that our UCB-NK cells can survive and expand in the abdominal cavity without preparative lymphodepleting regimen. However, lymphodepleting chemotherapy is currently the standard clinical practice for adoptive cellular therapy in cancer patients. In the first cohort, toxicity of UCB-NK cell therapy with IL-2 support will be monitored. The second cohort of 3 patients in this study will receive a non-myeloablative immunosuppressive preparative regimen of cyclophosphamide (900 mg/m^2/day) and fludarabine (30 mg/m^2/day) on days -6, -5, -4, -3. Ample experience with this preparative conditioning regimen has been obtained in the transplant setting with patients suffering from multiple myeloma and high-grade Non-Hodgkin's lymphoma. In addition, we recently published the results of our trial with first generation UCB-NK cell products in elderly AML patients where no severe toxicity is seen[26]. Currently this is the standard preparation for cellular adoptive therapy. To be able to draw conclusion on the survival and expansion of UCB-NK cells in the abdominal cavity with and without myeloablative preconditioning, an

extension cohort of 3 patients per group will be performed to have 6 patients per group, see statistical calculation in 10.2.

UCB-NK cells

In eligible patients, UCB-NK cell products will be administered to the patient IP at a dose of minimum $1.5x10^9$ to a maximum $3x10^9$ UCB-NK cells, in the first cohort UCB-NK cells will be infused three days after a Cy/Flu regimen. The UCB-NK cell dosage is based on our PLMA25 study in which we show that following Cy/Flu conditioning UCB-NK cell products could be administered to AML patients up to $3x10^7$/kg body weight i.e. $3x10^9$ cells in total for a patient of 100kg, without induction of GVHD or severe toxicity. Furthermore, we want to investigate whether UCB-NK cells are safe to administer intraperitoneally. Chemotherapy is administered IP in standard ovarian carcinoma therapy, for better targeting of the intraperitoneal disease. Miller et al are currently executing an intraperitoneal NK cell trial and has not observed any toxicity of the PB-NK cells up to a dose of $3x10^9$ cells. Intraperitoneal infusion may be a valuable strategy, because it gives NK cells the opportunity to come directly in contact with the tumor cells, without first being trapped in the liver or lungs. Since the immunologic environment in the abdominal cavity is different from the environment in blood and lymphoid organs, the hypothesis is that the UCB-NK cells may survive and expand in the abdominal cavity without the preparative regimen. Based on the route of administration we don't expect severe toxicity.

IL-2

IL-2 (proleukin) will be administered IP to prolong NK cell survival and expansion. Infusion of IL-2 is also intraperitoneal to be sure that the IL-2 is in the same compartment as the UCB-NK cells. In the study of Miller et al the same dosage and frequency is applied, without severe side effects.

5.3. Preparation and labelling of Investigational Medicinal Product

5.3.1. Availability of cryopreserved UCB units for experimental cell therapy

UCB units within the Cord Blood Bank Nijmegen that meet the criteria of cell numbers for this protocol, will be used for enrichment of CD34+ hematopoietic stem cells and subsequent generation of allogeneic UCB-NK cell products. At present, 350 UCB units are available in our Cord Blood Bank for the selection of an allogeneic partially HLA matched NK cell donor. Furthermore, the continuation of banking activities of the Cord Blood Bank guarantees that sufficient numbers of UCB units will be available for this trial.

5.3.2. CD34+ isolation from thawed UCB units

CD34+ UCB cells will be enriched according to JACIE standards of the Stem Cell Laboratory (SOPs MCTS039 and MCTS021) performed in the clean room facility of Laboratory of Hematology. UCB units stored in liquid nitrogen will be thawed at 37^{o}C and resuspended in CliniMACS buffer containing 5% HSA, 3.5 mM $MgCl_2$ and 100 U/ml Pulmozyme (clinical grade DNAse). All media are clinical-grade and allowed to be used for this purpose. After 30 minutes of incubation, UCB cells will be washed and CD34+ cells will be enriched using a CliniMACS cell separator after binding with CD34 coupled to immunomagnetic particles according to standard procedures (Miltenyi Biotec, Bergish Gladbach, Germany).

5.3.3. *Ex vivo* generation of NK cells from CD34+ UCB cells

UCB-NK cell products will be generated from CD34+ UCB cells according to the established protocol SOP's (see for details IMPD). In brief enriched CD34+ UCB cells will be cultured in VueLife^{TM} culture bags (CellGenix) in NK MACS medium Miltenyi Biotec**)** containing 10% virus-free pooled human serum (Sanquin), 5 μM SR1 and GMP-grade recombinant SCF (25 IU/ml), Flt3L (25 IU/ml), IL-7 (2000 IU/ml), TPO (500 IU/ml), (cytokines are from Miltenyi Biotec). At day 9/10, TPO will be replaced with IL-15 (1000 IU/ml). And at day 14/15, Flt3L will be replaced with low-dose IL-12 (0.25 IU/ml). From day 14/15 onward, expanded NK progenitor cells will be differentiated into NK cells in NK MACS medium, 2% virus-free pooled human serum, SCF (25 IU/ml), IL-7 (2000 IU/ml), IL-15 (1000 IU/ml), IL-12 (0.25 IU/ml) and 5 μM SR1. From day 24/25/26, no SR1 will be added anymore to the cultures. Cell cultures will be maintained in humidified atmosphere at 37°C with 5% CO_2. The final UCB-NK cell product will be washed and resuspended in infusion medium (0.9% sodium chloride containing 5% HSA). Cell culturing will be performed according to GMP standards in the M850 cleanroom facility of the Department of Pharmacy equipped with all necessary devices such as centrifuges, CO_2 incubators, microscope and automated cell counters.

5.3.3. Release criteria UCB-NK cell product

Ex vivo generated UCB-NK cell products will be tested for the following release criteria (see for details IMPD):

- Sterility: negative for bacterial, and fungal contamination. Test result will be reported to clinic 2 weeks after infusion.
- Endotoxin: <0.7 EU/ml. Test result will be reported to clinic 2 weeks after infusion.
- Phenotype: > 30% positivity for NKG2A, NKp30, NKp44, NKp46, NKG2D and DNAM-1 on CD45+CD56+CD3- cells as determined by flow cytometry.
- Purity: ≥ 70% CD56+CD3- NK cells as determined by flow cytometry.
- T cell contamination: < 1x10^5 CD3+ T cells/kg body weight of the patient

- B cell contamination: < $3x10^5$ CD19+ B cells/kg body weight of the patient
- Viability: ≥ 70% of CD56+CD3- cells as determined by 7-AAD exclusion.

Results of QC testing will be evaluated by an immunologist/expert QC of the Laboratory of Hematology, Radboudumc. All QC results as well as in-process and monitoring results during production will be documented in the release dossier. The product will be released by one of the QPs when the UCB-NK cell product meets all release criteria. The results from all UCB-NK cell end-product control tests, except for the sterility and endotoxin content of the end product, are available prior to release of the UCB-NK cell end-product. Final certification and release of the SR1-expanded UCB-NK cell product is performed by the QP after assessment of the results of the final tests (sterility and endotoxin, environmental monitoring) of the end product. A procedure is in place that describes the measures that are taken in case an out of specification test result is obtained after conditional release of the product. In short, the contaminant micro-organism will be identified and an antibiogram will be established. Time limits for reporting results of sterility testing and antibiograms are laid down in the Quality Agreement between Bactimm and the Hospital Pharmacy. The QC pharmacist will report deviant and out of specification results to the QP who informs the head of production, PI and physician of the patient.

5.4. Potential risks/toxicity

Laparoscopy

Diagnostic laparoscopy is an invasive technique under general anesthesia with a low complication rate, and is a routinely performed technique by gynecologists. In 1997 a prospective multicenter study in the Netherlands was published on complications of laparoscopic surgery. The rate of complications was 2.7 for 1000 diagnostic procedures and 4.5 per 1000 for tubal ligation[27]. Emergency laparotomy was required in 1.6 ‰. Chi and colleagues reported their complication rate in patients who underwent laparoscopy in a gynecological oncology service[28]. Laparoscopies were done for diagnosis, second look after treatment and therapeutically, like hysterectomies and lymphadenectomies. In the whole group the overall complication rate was 9%, the major complication rate 2.5% and mortality rate was 0.2%. Older age, malignancy, previous radiation therapy, and previous abdominal surgery were risk factors for complications and conversions. In the diagnostic group there were 10% grade 1-2 complications, 5% grade 3-5 complications, and 10% conversion rate to laparotomy. In 1999 Eltabbakh et al published comparable results in 204 patients with gynecological oncological conditions and laparoscopic operative procedures as hysterectomy and lymph node dissection[29]. The significant complication rate was 5% and the conversion

rate to laparotomy was 10%, however, only operative procedures and no diagnostic procedures were included in this analysis. Most complications occur at entering the abdominal cavity, either with the first or second trocar. That is why we will perform an open laparoscopy. The Radboudumc has ample experience with intraperitoneal catheter placement and is one of four Dutch centers for intraperitoneal chemotherapy.

Pre-treatment

Possible toxicities include constitutional symptoms such as fever, chills, myalgias, malaise and allergic reactions. Cy/Flu conditioning can induce acute toxicity related to the agents themselves and additionally pancytopenia requiring transfusions and increasing the probability of (severe) infections. Patients will be hospitalized during Cy/Flu conditioning and UCB-NK cell infusion. In the PLMA25 trial no infections were seen related to the Cy/Flu conditioning regimen.[26]

Cyclophosphamide was used in treatment of ovarian carcinoma till the use has slowly been phased out of standard clinical practice following the publication of studies showing the inferiority of platinum and cyclophosphamide compared with platinum and paclitaxel. Cyclophosphamide in this setting may have a cytoreductive effect.

UCB-NK cell therapy

Toxicity that might be expected from UCB-NK cell infusion includes abdominal pain, tumor lysis syndrome and GVHD (see appendix 3). During conditioning and after infusion, patients will be observed under supervision of experienced hematologists, oncologists and gynecologists during hospitalization. All patients will be seen under supervision of an experienced gynecologists or hematologist in the outpatient clinic twice weekly until recovery of the bone marrow. So appropriate measures can be taken in case of any unforeseen reaction to the cells takes place. Any life-threatening or other grade-4 toxicity (CTCAE toxicity criteria appendix 4) has to be reported immediately to the study coordinator. In case of GVHD is induced patients will be treated with immunosuppressive drugs (prednisolon, methylprednisolon, cyclosporine A) according the international guidelines for treatment of GVHD.

In February 2009 tumor lysis syndrome was added as a possible risk associated with NK cell infusion after a patient enrolled in a preceding study died approximately 24 hours after receiving NK cells. It appears her cause of death was consistent with tumor lysis syndrome. In response to this event the following preventative measures were added: 1) all patients (except those with known allergy) are to receive allopurinol 300 mg every day beginning before chemotherapy and continuing until day +14; 2) lytes, LDH, uric acid, and phosphorus will be monitored until day +14, and 3) hydration will be administered per institutional guidelines; however any elevation of daily metabolic monitoring would be treated with more aggressive hydration, but with being attentive to fluid overload.

IL-2

Toxicities associated with IV IL-2 are as follows: flu-like syndrome (fever, chills, tiredness, headache, muscle and joint pain), low blood pressure, nausea, diarrhea, weakness, fluid retention and weight gain, hypothyroidism, abnormal liver function test. By infusing the IL-2 IP we expect to have a lower peak level in the blood and less side effects. In the study of Miller et al the same regimen was administered without severe side effects.

In a study in women with platinum resistant or refractory ovarian cancer the IP administration of single agent IL-2 weekly at dose higher than used in this study was generally well tolerated. Grade 1 and 2 constitutional symptoms (flu-like symptoms, GI and neurological) were most common, but controlled with medication and not requiring dose reduction.[30]

Risk Classification Risk classification	Light damage	Moderate damage	Serious damage
Small chance	Negligible risk	Negligible risk	Moderate risk
Moderate chance	Negligible risk	Moderate risk	High risk
High chance	Moderate risk	High risk	High risk

Based on the risk classification table of the NFU, the overall risk is intermediate. With a high chance on minimal toxicities by the chemotherapy and IL-2 and a low chance on intermediate toxicity by the cellular product.

5.5. Concomitant therapy

During chemotherapy and leukopenia all patients will be treated with preventive antibiotics and antiviral therapy according the local guidelines for severe immune compromised patients. No other concomitant therapy will be given during UCB-NK cell infusion.

6. STUDY PROCEDURES

The visit schedule and checklist with required clinical evaluations, laboratory tests and follow-up are depicted in appendix 1.

6.1. Before start of treatment

Patient eligibility criteria should be assessed before laparoscopic placement of the catheter. In the same session a biopsy will be taken if there are visible tumor deposits and the abdominal cavity will be scored for adhesions with the laparoscopic adhesions score. If less than 5 compartments of the abdomen are accessible, the patient is excluded. The start of *ex vivo* generation of NK cells from allogeneic UCB units will be after laparoscopy. Screening

assessments should be performed prior to start of the ex vivo UCB-NK cell generation procedure. The following tests will be performed:

- History, physical examination including performance status, weight, pulse, blood pressure, and body temperature. Blood sampling for CRP, Hb, Ht, CBC, differential, platelets, serum sodium, potassium, calcium, phosphorus, creatinine, bilirubin, albumin, total protein, alkaline phosphatase, gGT, ASAT, ALAT, LDH, urea and CA-125.
- Heparinized blood (10 ml) will be obtained for EBV and CMV PCR analysis.
- Heparinized blood (30 ml) and blood for serum (3 ml) will be obtained for immunological studies.
- Ultrasound, CT, abdominal washing.

One week prior to admission in the clinic repeat necessary blood test as described above.

6.2. During treatment

Prior to infusion and during evaluation of the treatment the following tests will be performed (see also appendix 1):

- History, physical examination including vital signs and performance status, toxicity assessment, complete blood count and biochemistries.
- Heparinized blood (30 ml) and blood (3 ml) for serum will be obtained for immunological studies.
- EDTA blood (10 ml) will be obtained for chimerism analysis.
- Peritoneal fluid will be aspirated through the IP catheter on day 7, 14, 21 and 28, after the last washing/aspiration the catheter will be removed.

6.3. After the end of treatment (Follow-up)

After completing the study treatment (day 56), further treatment will be guided by patients own physician.

7. CRITERIA OF EVALUATION

7.1. Main study parameter/endpoint

The primary endpoint of this study is to evaluate safety and toxicity of an escalating dose of intraperitoneal infused *ex vivo*-generated UCB-NK cells with IL-2, with and without Cy/Flu conditioning. UCB-NK cells will be infused at a dose of between $1.5x10^9$ and $3x10^9$ NK cells. A total of 12 patients will be included in this study.

Because there is no evidence of the necessity of Cy/Flu pretreatment on the survival and expansion of intraperitoneally administered NK cell therapy, our primary endpoint is also to investigate these parameters. Without Cy/Flu conditioning, the toxicity might be different from our previous study conducted in older AML patients with intravenous UCB-NK cell therapy[26].

We don't expect severe toxicity of the UCB-NK cell infusion, based on preliminary results of the IP PB-NK cell trial in ovarian carcinoma patients of Miller et al. thus far.

Toxicity of the immunosuppressive conditioning regimen and UCB-NK cell infusions will be separately evaluated. Acute toxicity caused by the Cy/Flu conditioning is generally low and has proven to be safe in patients, although the immunosuppressive state of the patient may cause severe infections caused by the transient pancytopenia induced by this regimen. All patients will be evaluated intensively for toxicity caused by the conditioning regimen using the CTCAE toxicity criteria (appendix 4) and GVHD (appendix 3). If the toxicity of the conditioning regimen in this population of patients exceeds the common accepted toxicity for this kind of treatment the study will be stopped. If a patient dies due to the conditioning regimen itself, the study will also be stopped immediately.

Toxicity caused by laparoscopic catheter placement could include damage to intra-abdominal organs or infection of the port-a-cath (PAC). This toxicity will also be scored by the CTCAE criteria. In addition there are expected side effects of the placement of the PAC (for example, post operative pain), which will not be scored as toxicity of the investigational treatment.

Toxicity caused by the UCB-NK cell infusions may predominantly consist of GVHD. In the study reported by Miller et al. no significant GVHD was observed and a dose limiting toxicity of IV PB-NK cells was not reached[16]. In our PLMA25 study no GVHD was observed after IV UCB-NK cell infusion in patients receiving up to $3x10^9$ NK cells[26]. From intraperitoneal infusion we don't expect severe toxicity. In 1987 Stewart et al conducted a study exploring the anti ovarian cancer effect of intraperitoneal LAK cells[31]. Patients experienced abdominal pain and nausea but no severe toxicities. Miller et al currently conducting a phase 1 study combining IP PB-NK cell therapy with IL-2 and an IDO inhibitor. The first 2 patients in that protocol did not experience any toxicity of the NK cell infusion (see appendix 6). Grade 3 or greater related non-hematologic toxicity other than constitutional symptoms and fatigue, and not caused by the immunosuppressive pre-treatment will be scored as toxicity caused by the NK cell infusions (CTCAE toxicity criteria, acute GVHD >grade 1, or any form of chronic GVHD appendix 3 and 4).

Toxicity will be monitored till 56 days (8 weeks) after UCB-NK cell infusion. Only if no severe toxicity is observed in the first patient, we will continue with the second. In case 1 patient will experience toxicity, analysis will be done for the cause of toxicity. After discussion with the researcher, the cohort can be increased with 3 patients. If the first 2 patients of group 1 have no severe toxicity, more patients will be included in an overlapping setting.

7.2. Secondary study parameters/endpoints

Determination of the *in vivo* lifespan, expansion and cytolytic activity of the infused UCB-NK cells following Cy/Flu conditioning is a secondary endpoint. Therefore, we will determine the percentage and absolute number of donor-derived NK cells in peripheral blood and in peritoneal fluid after infusion and the amount of tumor cells in peritoneal fluid. In parallel, we will determine IL-15 plasma and peritoneal fluid levels, which will be correlated with absolute lymphocyte count and *in vivo* NK cell persistence and expansion. Finally, we will assess cytolytic activity of donor-derived NK cells using functional assays.

Determination of the effect of NK cell infusion on measurable disease is another secondary endpoint. Presence of residual disease will be evaluated by monitoring of the specific tumor marker CA-125.

If in cohort two with Cy/Flu pretreatment no more than 5% donor-derived NK cells are found in the abdominal fluid, we will not continue with the extension cohort with and without Cy-Flu pre-treatment, to prevent patients from unnecessary exposure to cellular therapy and chemotherapy.

7.3. Response criteria

The primary endpoint of this study is evaluation of the toxicity, and secondary endpoints are NK cell survival, NK cell expansion, NK cell cytolytic activity and progression of disease with and without preceding immune suppressive chemotherapy. Patients will serve as their own controls. Pre- and post-infusion values will be generated and statistical analysis by the Mann-Whitney U-test will be applied to these paired data. Linear regression will be used to study relationship between the absolute cell numbers, functional activity and the IL-15 concentrations. CA-125 levels will be monitored.

7.3.1. Toxicity

Toxicity is described in section 5.4 and 7.1 and will be measured using the CTCAE toxicity criteria (see appendix 4). Possible toxicities include constitutional symptoms such as fever, chills, myalgias, malaise and allergic reactions. Cy/Flu conditioning can induce acute toxicity related to the agents themselves and additionally pancytopenia requiring transfusions and increasing the probability of (severe) infections. Intraperitoneal UCB-NK cell infusion may result in abdominal pain or GVHD, which will be scored and treated if indicated according to standard guidelines (Glucksberg and Shulman criteria, see appendix 3).

7.3.2. Immunologic response

To study the immunologic response, we will determine the percentage and absolute number of donor-derived NK cells in peripheral blood (PB) and peritoneal fluid (PF) after infusion. These analyses will be done by flow cytometry and DNA chimerism analysis. In parallel, we will determine the donor NK cell engraftment and IL-15 plasma level in blood, which will be compared to PFNK cell survival and expansion. Finally, we will assess cytolytic activity of donor-derived NK cells using functional assays.

To assess these biological parameters, PB samples will be taken on day 0 (pre-study sample) and day 1, day 3, day 7, day 14, day 21, day 28 and day 56 after IP-NK cell infusion. Blood samples will be immediately monitored for phenotypical expressions, and PBMC will be isolated by density gradient centrifugation, stored in liquid nitrogen and used for monitoring of NK cell responses. Plasma samples will be collected at each time point and stored at -20^{o}C until use for the determination of cytokines that drive NK cell expansion. PF aspirates will be obtained at time of catheter placement (pre-study sample), day 7, day 14, day 21 and day 28 after NK cell infusion. First, we will attempt to obtain a PF sample of 50 ml via the catheter. In case less than 50 ml is collected, peritoneal washing will be performed. For this, a volume of maximum 2 times 250 ml pre-warmed 0.9% sodium chloride plus 5% HSA will be infused via the catheter into the abdomen. After infusion, the patient will be asked to change position at 5-minute intervals to ensure adequate intra-abdominal distribution. At least 50 ml of infusate will then be retrieved through the catheter. If after this no infusate can be retrieved, the process will be terminated. The IP-wash samples will be immediately monitored for phenotypical expressions, and remaining cells will be used for DNA analysis and cryopreserved for monitoring of NK cell responses. The following immunologic tests will be conducted on blood and IP-wash samples:

a. Phenotypic analysis of NK cells: To study the effect of treatment on the number of leukocyte subsets, immunophenotyping will be performed directly on PB samples and PF samples after collection following treatment. The standard whole blood phenotype assay will quantify the proportion of the following lymphocyte subsets: NK cell subsets (CD3-CD56^{bright} and CD3-CD56^{dim}), NKT cells (CD56+CD3+), T cell subsets (CD3+CD4+ and CD3+CD8+ T cells in combination with CCR7, CD45RA, CD27, CD25, CD127, FoxP3) and B cells (CD19+). Absolute numbers of circulating lymphocyte subsets will be determined by single-platform flow cytometry analyses using counting beads. A positive expansion rate of the infused NK cells requires the presence of ≥5% donor-derived NK cells within the circulating and/or peritoneal cell fraction after infusion. In addition, a multi-color panel will be used to determine the frequency and phenotype leukocyte subsets in the IP-wash samples. Next to the above mentioned lymphocyte subsets, we will determine the proportion of macrophages and residual CD45- tumor cells. Furthermore, in both blood and IP-wash samples, the CD56+CD3- NK cell fraction will be analyzed for the expression of KIRs (CD158a, CD158b,

CD158e), c-type lectin receptors (CD94/NKG2A, NKG2C, NKG2D), NCR (NKp30, NKp44, NKp46), activation markers (DNAM-1, CD69, CD16), cytotoxic mediators (Perforin, TRAIL), cytokine receptors (CD25, CD127, CD122) and homing receptors (CXCR3, CD62L).

b. Enumeration of donor-derived NK cells: To assess *in vivo* survival, persistence and expansion of the infused NK cells, we will perform standard chimerism analysis on the obtained PB and IP-wash samples. This will be performed when all samples of the patient are collected. Donor chimerism will be determined by real-time quantitative PCR using single nucleotide polymorphisms (SNPs) discriminative between the UCB donor and recipient. For this, DNA will be extracted from blood and IP-wash samples (Qiagen DNA blood mini kit) and used to determine the percentage recipient and donor cells by real-time quantitative SNP-PCR. Patient and donor DNA collected before infusion will be used to screen for discriminative SNP in a panel of polymorphic genes that is able to discriminate >95% donor/recipient pairs in both directions. Alternatively, informative HLA alleles specific for the donor can be used. Donor and recipient-specific signals will be normalized for the amount of input DNA by quantitative PCR for the albumin gene. A positive expansion rate of the infused NK cells requires the presence of ≥5% donor-derived NK cells within the circulating and/or peritoneal cell fraction after infusion.

c. Detection of cytokines in plasma: Pre- and post-infusion plasma samples will be serially diluted and cytokine concentrations will be determined by a commercial ELISA or LUMINEX assay. In cohort 2 and 4, plasma and PF collected before and after Cy/Flu conditioning will be tested for the increase of endogenous cytokine levels (IL-15, IL-7). In addition, we will determine the levels of inflammatory cytokines (IFN-γ, TNF-α, IL-6). This monitoring measurement will be performed when all samples of all 12 patients have been collected. Cytokine levels will be correlated with absolute lymphocyte and NK cell counts as well as NK cell chimerism.

d. Functional activity of donor-derived NK cells: To enumerate the number of NK cells reactive against the patient ovarian carcinoma cells, we will analyze CD107a degranulation and intracellular IFN-γ using flow cytometry. For this, PBMC and PFMC samples will be cocultured with different ovarian carcinoma cell lines (*e.g.* SKOV-3, OVCAR, IGROV1), patientderived ovarian carcinoma cells and/or K562 cells (positive control). After overnight incubation, the percentage CD107a+ and/or IFN-g+ cells within the CD56+CD3- NK cell population will be determined by flow cytometry. In parallel, we will determine the amount of IFN-γ production by the stimulated NK cells using ELISA. These monitoring measurement will be performed when all samples of all 12 patients are collected. A positive cytotoxic response requires significant increase in functional activity of the CD56+CD3- NK cell fraction against patient-derived ovarian carcinoma cells and/or cell lines.

e. Phenotypic analysis and enumeration of tumor cells present in peritoneal fluid: To study the effect of UCB-NK cell infusion on the frequency and phenotype (activating and inhibitory ligands) of tumor cells in the peritoneal fluid, immune phenotyping will be performed on the PF samples before and after treatment. This analysis will be performed directly on PF samples after collection.

7.3.3. Response on residual disease in treated patients

Determination of the effect of NK cell infusion on measurable residual disease is another secondary endpoint. Clinical effects will be investigated by determining CA-125 serum levels.

8. WITHDRAWAL OF INDIVIDUAL SUBJECTS

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The clinical investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.1. Specific criteria for withdrawal

Treatment is discontinued when any of the following occurs:

- Patient refusal or non compliance
- Unacceptable toxicity or life-threatening events
- Adverse event(s) that, in the judgment of the Investigator, may cause severe or permanent harm or which rule out continuation of the study
- Withdrawal of consent.
- Progression of symptoms of disease needing palliative chemotherapy.

8.2. Replacement of individual subjects after withdrawal

We will define the patients as included from the moment they have started the treatment; either Cy/Flu or UCB-NK cell infusion. In total we will include 12 patients. If patients drop out before starting treatment, we will include a new patient.

8.3. Follow-up of subjects withdrawn from treatment

At treatment discontinuation, subjects will undergo safety assessments as per the schedule in appendix 1. All subjects who discontinued treatment will continue to be followed by their attending physician at standard intervals until progression or death.

8.4. Premature termination of the study

Serious, life threatening adverse events or grade 4 toxicity which can be possibly related to the UCB-NK cell infusion protocol will be a reason to terminate the study. If in the first cohort with Cy/Flu pretreatment no more than 5% donor derived-NK cells are found in the abdominal fluid, we won't continue with the extensioncohort with and without Cy/Flu pretreatment, to prevent patients from unnecessary exposure to cellular therapy and chemotherapy.

9. SAFETY REPORTING

9.1. Section 10 WMO event

In accordance to section 10, subsection 1 of the WMO, the investigator will inform the subjects and the reviewing CCMO if any event occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the CCMO, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

9.2. Adverse and serious adverse events

Adverse events are defined as any undesirable experience occurring to a subject during a clinical trial, whether or not considered related to the investigational drug or procedure. All adverse events reported spontaneously by the subject or observed by the investigator or his staff, occurring from the start of the investigational treatment, as defined in paragraph 8.2 until 180 days after infusion of NK cells will be recorded in the CRF.

Adverse Events have to be reported on the Adverse Events CRF. Adverse Events will be scored according to the NCI Common Terminology Criteria for Adverse Events, version 4.0 Pre-existing relevant conditions will be collected on the medical history CRF, i.e. active (symptomatic) diseases of CTCAE grade > 2 diseases under treatment, chronic diseases and long term effects of past events as present at the time of baseline assessment. As CTCAE grade 3 toxicities are scored all treatment emergent non-hematological grade 3 toxicity lasting more than 48 hours except for transient constitutional symptoms and diarrhea not requiring systemic therapy.

The following events are in this study not considered as AE:

- A pre-existing condition that does not increase in severity; the pre-existing condition should be reported on the baseline concomitant diseases CRF

- AE's of CTCAE grade 1 and 2
- Hematological toxicities (except cytopenias: these are reported in the CRF), alopecia, nausea and vomiting
- Abnormal laboratory values that have been recorded as being not clinically significant by the investigator in the source documents
- Progression of the disease under study; complaints and complications as a result of disease progression remain reportable Adverse Events
- AE's expected due to chemotherapy

A serious adverse event is any untoward medical occurrence or effect that at any dose;

- results in death;
- is life threatening (at the time of the event);
- requires hospitalization or prolongation of existing inpatients' hospitalization;
- results in persistent or significant disability or incapacity;
- is a new event of the trial likely to affect the safety of the subjects, such as an unexpected outcome of an adverse reaction, lack of efficacy of an IMP used for the treatment of a life threatening disease, major safety finding from a newly completed animal study, etc.

SAEs will be reported through the web portal ToetsingOnline by the investigator to the CCMO that approved the protocol, according to the requirements of and timelines indicated by the CCMO. SAEs occurring from the start with immunosuppressive conditioning until 180 days after infusion of NK cells will be reported to the CCMO. SAE that occur outside of the SAE detection period (after the 180-days period), will be reported if it is considered to have a reasonable possibility to be related to the investigational product or study participation.

The following situations are in this study not considered to be SAEs:

- Elective hospitalization for pre-existing conditions that have not been exacerbated by trial treatment
- Hospitalization for protocol therapy administration. Hospitalization for a complication of therapy administration will be reported as a Serious Adverse Event.
- A hospitalization which was planned before the patient consented for study participation and where admission did not take longer than anticipated
- Hospitalization for blood or blood product transfusion
- Medical or surgical procedure (e.g. endoscopy, appendectomy); the condition that leads to the procedure is an (S)AE
- Hospitalization or prolonged hospitalization for technical, practical or social reasons, in absence of adverse events.

- Anticipated day-to-day fluctuations of pre-existing disease(s) or condition(s) present or detected at the start of the study that do not worsen.
- Adverse events which are expected side effects of immunosuppressive/cytoreductive conditioning. If side effects are more severe than expected they will be reported.
- Clinical events related to the primary cancer progression are not to be reported as SAEs, even if they meet any of the seriousness criteria from the standard SAE definition, unless the event is more severe than expected and therefore the investigator considers that their clinical significance deserves reporting.

9.3. Suspected unexpected serious adverse reactions (SUSAR)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered. All adverse events judged by either the investigator or the sponsors as having a reasonable suspected causal relationship to an IMP qualify as adverse reaction. Unexpected adverse reactions are adverse reactions, of which the nature, or severity, is not consistent with the applicable product information (*e.g.* Investigator's Brochure for an unapproved IMP or Summary of Product Characteristics (SPC) for an authorised medicinal product).

The investigator will report expedited all SUSARs to the Medicines Evaluation Board (CBG), the Ministry of VWS and CCMO. The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

9.4. Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

9.5. Annual safety report

In addition to the expedited reporting of SUSARs, the investigator will submit, once a year throughout the clinical trial, a safety report to the CCMO, competent authority, Medicine Evaluation Board.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;

- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

The annual safety report will be combined with the annual progress report.

9.6 Data safety management board

In addition to the expedited reporting of events and reactions above, a data safety management board will be installed. This board will check all data, qualifications, events and reactions after day 7 and day 28 of the first, third and sixth patient of both cohorts, and once in the end of the study. The DSMB will consist of 2 persons, Prof dr H. Nijman professor in gynaecological oncology UMC Groningen and dr. D.J. van Spronsen, haematologist/ oncologist Radboudumc. The DSMB will also be involved in the evaluation of the toxicity criteria for stopping or continuation of the patient cohorts according to the study design scheme in Appendix 7.

10. STATISTICAL ANALYSIS

10.1. Sample size calculation

Six patients will be included (cohort 1 and 2) to evaluate the primary study objective, which is safety and toxicity of intraperitoneal infusion of *ex vivo* generated UCB-NK cells with and without Cy/Flu conditioning. Grade 3 or greater related non-hematologic toxicity (CTCAE toxicity criteria, other than constitutional symptoms and fatigue), acute GVHD > grade 1, or any form of chronic GVHD (appendix 3 and 4), will be scored as toxicity. An extension cohort of 6 patients (cohort 3 and 4) will be included if no toxicity is seen in the first 6 patients, to be able to draw conclusions on the secondary objectives.

10.2. Descriptive statistics

This is a phase I study design with the primary aim of evaluating the safety and toxicity of IP infusion of ex vivo-generated UCB-NK cells in two different conditions with and without Cy/Flu pre-conditioning. A total of 3 patients will be included into each treatment arm in order to appropriately determine UCB-NK cell treatment-related toxicity. To answer the other key question whether Cy/Flu pre-conditioning is required for successful UCB-NK cell engraftment and expansion in the peritoneal cavity, we will perform statistical analyses on the immunomonitoring results. The proportion of patients with NK cell expansion as well as the number of NK cells in the peritoneum and blood will be statistically compared between cohort 1 and 3 (without Cy/Flu) and cohort 2 and 4 (with Cy/Flu) using the Mann-Whitney U-test.

Using the statistical program R, we calculated that a group size of 6 per cohort is sufficient to answer if pre-conditioning is required or not. We expect that pre-conditioning results in a frequency of 50±20% UCB-NK cells IP at day 7 and/or 14 determined by flow cytometry and DNA chimerism analyses. When >3-fold lower NK cell expansion is seen without pre-conditioning, i.e. 15±20%, then this is statistically less efficient with significance level of 0.05 and a power of 0.80. So after the classic three by three toxicity study, we continue with the extension cohort of three patients per group. If no statistical difference is observed in the total 6 patients per group, we will define that pre-conditioning is not required for IP UCB-NK cell engraftment and expansion in OC patients.

In case <5% donor-derived NK cells within the peritoneal CD56+CD3- cell fraction will be detected with pre-conditioning in cohort 2, this is not successful and the trial will be stopped, which means that the extension cohort will not be exposed to an ineffective experimental treatment and chemotherapy. The secondary goal of clinical (CA-125) response will have a descriptive nature. Data management will be conducted in Castor (Castor EDC, CIWIT B.V. Amsterdam) in collaboration with our trial bureau.

11. ETHICAL CONSIDERATIONS

11.1. Patient protection

The responsible investigator will ensure that this study is conducted in agreement with the Declaration of Helsinki.

The design of this study follows current views of the European Medicine Agency (EMEA) for Advanced Therapy Medicinal Products (ATMP) in general and for Advanced Cellular Therapeutics (ACT) in particular.

The protocol has been written, and the study will be conducted according to the guidelines for Good Clinical Practice and Good Manufacturing Practice issued by the European Union.

11.2. Informed consent

All patients will be informed about the aims of the study, the possible adverse events, the procedures and possible hazards to which he/she will be exposed, and the mechanism of treatment allocation. They will be informed as to the strict confidentiality of their patient data, but that authorized individuals other than the treating physicians may review their medical records for trial purposes.

It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the protocol whenever he/she wants. This will not prejudice the patient's subsequent care. Documented informed consent must be obtained for all patients

included in the study before they are registered at the Data Center, Department of Hematology, Radboud University Medical Centre Nijmegen. For European Union member states, the informed consent procedure must conform to the ICH guidelines on Good Clinical Practice. This implies that “the written informed consent form should be signed and personally dated by both the physician and the patient”. The patient will receive a copy of the informed consent form.

Blood, peritoneal fluid and serum samples collected in this study will be used to study immunologic and disease responses induced by UCB-NK cell infusion. Remaining material will be stored in the Biobank Hematology, part of the Radboud Biobank for a maximum of 15 years and potentially used for future translational research on immunology and ovarian carcinoma. Translational research is required for the development and evaluation of treatment for ovarian carcinoma. These studies will be performed under strict confidentiality of patient data. Samples can only be used after permission of the local CMO. Documented informed consent must be obtained for the collection of patient material for translational research.

12. COMPENSATION FOR INJURY

The RadboudUMC has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

13. ADMINISTRATIVE ASPECTS

13.1. Registration

The patient will be registered (after checking inclusion and exclusion criteria) at the trialbureau hematologie/oncologie, Radboud University Medical Centre Nijmegen. Registration is possible either by phone call 024-3614794 or by E-mail: trialbureauhemat-onco@radboudumc.nl

13.2. Forms and procedures for collecting data

All information is gathered by the co-investigator, Department of Hematology, Radboud University Medical Centre Nijmegen and registered in case report forms. A safety monitoring board will be installed, composed of 2 independent physicians with knowledge of the field of research. For this purpose a standardized monitor form will be used (see appendix 5).

Monitoring frequency is planned as follows:
- an initiation visit
- First visit 7 days after NK cell infusion of first andthird patient of cohort 1 and 2.
- Second visit 28 days after NK cell infusion of first and third patient of cohort 1 and 2.
    - Third visit after day 28 of patient (nr 9)
- a close out visit after completion of last patient.

At initiation visit monitoring includes the following:
- completeness of the Study Master File
- check procedures: availability of SOP

See attachment for details.

For 6 patients (50% of total included patients) monitoring will take place as follows. The data safety monitoring board will verify that all case report forms are complete, accurate, consistent and reliable. This reliability will be verified by means of a check with the available source documents. In addition, drug accountability will be verified.
For all patients electronic CRF's will be checked for completeness and monitoring will take place of all grade 3-4 AE, all SAE, informed consent, eligibility criteria and primary endpoint.

13.3. Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the CCMO once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

13.4. End of study report

The investigator will notify the CCMO [and the Ministry of VWS] of the end of the study within a period of 8 weeks [90 days]. The end of the study is defined as the last patient's last visit.
In case the study is ended prematurely, the investigator will notify the CCMO [and the Ministry of VWS within 15 days], including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the CCMO [and the Ministry of VWS].

14 STRUCTURED RISK ANALYSIS

14.1 Potential issues of concern

a. Level of knowledge about mechanism of action

The mechanism of action of NK cells is that NK cell can kill cancer cells without prior sensitization, and that NK cells don't attack healthy cells. Toxicity that might be expected from UCB-NK cell infusion includes abdominal pain, tumor lysis syndrome and GVHD (see appendix 3 and). The preparative chemotherapy regimen can induce fever, chills, myalgias, malaise and allergic reactions and additionally pancytopenia requiring transfusions, increasing the probability of (severe) infections. Toxicities associated with IV IL-2 are as follows: flu-like syndrome (fever, chills, tiredness, headache, muscle and joint pain), low blood pressure, nausea, diarrhea, weakness, fluid retention and weight gain, hypothyroidism, abnormal liver function test.

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

The UCB-NK cell dosage is based on our PLMA25 study in which we show that following Cy/Flu conditioning UCB-NK cell products could be administered to AML patients up to $3x10^7$/kg body weight i.e. $3x10^9$ cells in total for a patient of 100kg, without induction of GVHD or severe toxicity. Furthermore, we want to investigate whether UCB-NK cells are safe to administer intraperitoneally. Chemotherapy is administered IP in standard ovarian carcinoma therapy, for better targeting of the intraperitoneal disease. Miller et al are currently executing an intraperitoneal NK cell trial and has not observed any toxicity of the PB-NK cells up to a dose of $3x10^9$ cells with preparative Cy/Flu chemotherapy.

In a study in women with platinum resistant or refractory ovarian cancer the IP administration of single agent IL-2 weekly at dose higher than used in this study was generally well tolerated. Grade 1 and 2 constitutional symptoms (flu-like symptoms, GI and neurological) were most common, but controlled with medication and not requiring dose reduction.

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?

We have demonstrated that an improved second generation UCB-NK cell product can efficiently attack OC spheroids as well as OC tumors in a preclinical mouse model after intraperitoneal (IP) infusion. To be able to see the effect of UCB-NK cells on ovarian carcinoma in a human immune system in the abdominal cavity, it is

necessary to perform this study. We did our best to perform all pre-clinical studies possible.

d. Predictability of effect

Because of our PLMA study and the study of Miller in a comparable patient category, we have confidence that we won't see unexpected toxicity, and we hope to see some effect. All safety measures and monitoring is available for unexpected toxicity. Effect will be monitored by CA-125 levels, we know that this a relatively aspecific marker, which is elevated by all kinds of peritoneal irritation.

e. Can effects be managed?

During conditioning and after infusion, patients will be observed under supervision of experienced hematologists, oncologists and gynecologists during hospitalization. All patients will be seen under supervision of an experienced gynecologists or hematologist in the outpatient clinic twice weekly until recovery of the bone marrow. So appropriate measures can be taken in case of any unforeseen reaction to the cells takes place.

14.2 Synthesis

In this phase 1 toxicity study we expect to see toxicity related to the CyFlu treatment and the IL2 injection. From the NK cell infusions we don't expect severe toxicity and hope to see some effect. Hopefully we can give the NK cell infusion without preparative chemotherapy, because than the patrn af side effects could be very mild. We think this study is valuable, because we can possibly introduce a new therapy for a group where no curative treatment is available.

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APPENDIX 1

Follow up Study	Pre-study	Day -7	day 0	day 1	day 2	day 5	day 7	day 14	day 28	day 56	month 3 & 6
Informed consent	X										
History and Physical		X	X	X	X	X	X	X	X	X	X
Performance status (a)	X									X	X
Toxicity assessment (b)		X	X	X	X	X	X	X	X	X	X
CBC,Diff.,Platelets 3 ml (c)		X	X				X	X	X	X	X
Biochemistries 6 ml (d)		X	X				X	X	X	X	X
EBV and CMV PCR (e)		X							X		
HLA and KIR typing (f)	X										
Immunophenotyping 5 ml (g)			X				X	X	X	X	
Blood draw for plasma 3 ml (h)		X	X				X	X	X	X	
Immunologic testing 30 ml (i)		X	X				X	X	X	X	
Chimerism analysis 10 ml (j)	X						X	X	X	X	
Peritoneal fluid aspiration (k)			X				X	X	X		
CT scan	X										X

Flow sheet with Clinical, Laboratory and Immunologic Evaluations.

a. See appendix 3.
b. See appendix 6.
c. CBC, Diff, Platelets.
d. Serum creat, urea, Na, K, bili, AF, gGT, ASAT, ALAT, LDH, Ca, P, total protein, albumin. CA125 day 0, 7, 28 and 3&6months.
e. Serological tests for EBV and CMV (pre-study) followed by PCR-EBV and CMV.
f. 10 ml blood in ACD solution A for HLA and KIR typing.
g. 5 ml heparinized blood for immunophenotyping.
h. 3 ml blood for plasma to be stored at -20^{0}C.
i. 25 ml heparinized blood for isolation of PBMC to be stored in liquid nitrogen for cellular immunological studies.
j. 10 ml EDTA blood for DNA isolation for chimerism analysis.
k. Peritoneal fluid aspiration for isolation of PFMCs for cellular immunological studies and for collection of supernatant for cytokine analysis.

APPENDIX 2

KARNOFSKY PERFORMANCE - WHO PERFORMANCE STATUS

	Karnofsky	WHO
Normal; no complaints; no evidence of disease.	100%	
Able to carry on normal activity; minor signs or symptoms of disease	90%	0
Normal activity with effort; some signs or symptoms of disease.	80%	1
Cares for self. Unable to carry on normal activity or to do active work.	70%	
Requires occasional assistance but is able to care for most of his needs.	60%	2
Requires considerable assistance and frequent medical care	50%	
Disabled; requires special care and assistance.	40%	
Severely disabled; hospitalization is indicated although death is not imminent.	30%	3
Very sick; hospitalization necessary; active supportive treatment necessary.	20%	4
Moribund; fatal processes progressing rapidly.	10%	
Death		5

APPENDIX 3

CLINICAL CLASSIFICATION AND GRADING OF ACUTE AND CHRONIC GVHD

A. CLINICAL CLASSIFICATION OF ACUTE GVHD (GLUCKSBERG)

	Skin	**Liver**	**Gastrointestinal**
0	No rash	Bilirubin < 2 mg / dl (< 34 ↑mol/L)	Diarrhea < 500 ml/day
1	Maculopapular rash on < 25% of body surface	Bilirubin 2-3 mg/dl (34-50 ↑mol/L)	Diarrhea 500-1000 ml/day
2	Maculopapular rash on 25-50% of body surface	Bilirubin > 3-6 mg/dl (51-102 ↑mol/L)	Diarrhea 1000-1500 ml/day
3	Generalized erythroderma	Bilirubin > 6-15 mg/dl (103-255 ↑mol/L)	Diarrhea > 1500 ml/day
4	Generalized erythro- derma with formation of bullea and desquamation	Bilirubin > 15 mg/dl (> 225 ↑mol/L)	Severe abdominal pain with or without ileus

B. GRADING OF ACUTE GVHD

Overall grade	**Stage**		
	Skin	**Liver**	**Gut**
I (mild)	1 or 2	0	0
II (moderate)	1-3	1	1
III (severe)	2 or 3	2 or 3	2 or 3
IV (life-threatening)	2-4	2-4	2-4

C. CLINICAL CLASSIFICATION OF CHRONIC GVHD (SHULMAN)

Limited chronic GVHD Either or both:	**Extensive chronic GVHD** Either:
1. Localized skin involvement	1. Generalized skin involvement: or
2. Hepatic dysfunction due to chronic GVHD	2. Localized skin involvement and/or hepatic dysfunction due to chronic GVHD, plus:
	a. Liver histology showing chronic aggressive hepatitis, bridging necrosis or cirrhosis; or
	b. Involvement of eye: Schirmer's test with less than 5 mm wetting; or
	c. Involvement of minor salivary glands or oral mucosa demonstrated on labial biopsy; or
	d. Involvement of any other target organ.

APPENDIX 4

Common Terminology Criteria for Adverse Events

The grading of toxicity and adverse events will be done using the most recent version of the NCI Common Terminology Criteria for Adverse Events, CTCAE version 4. A complete document may be downloaded from http://ctep.cancer.gov/reporting/ctc.html.

APPENDIX 5

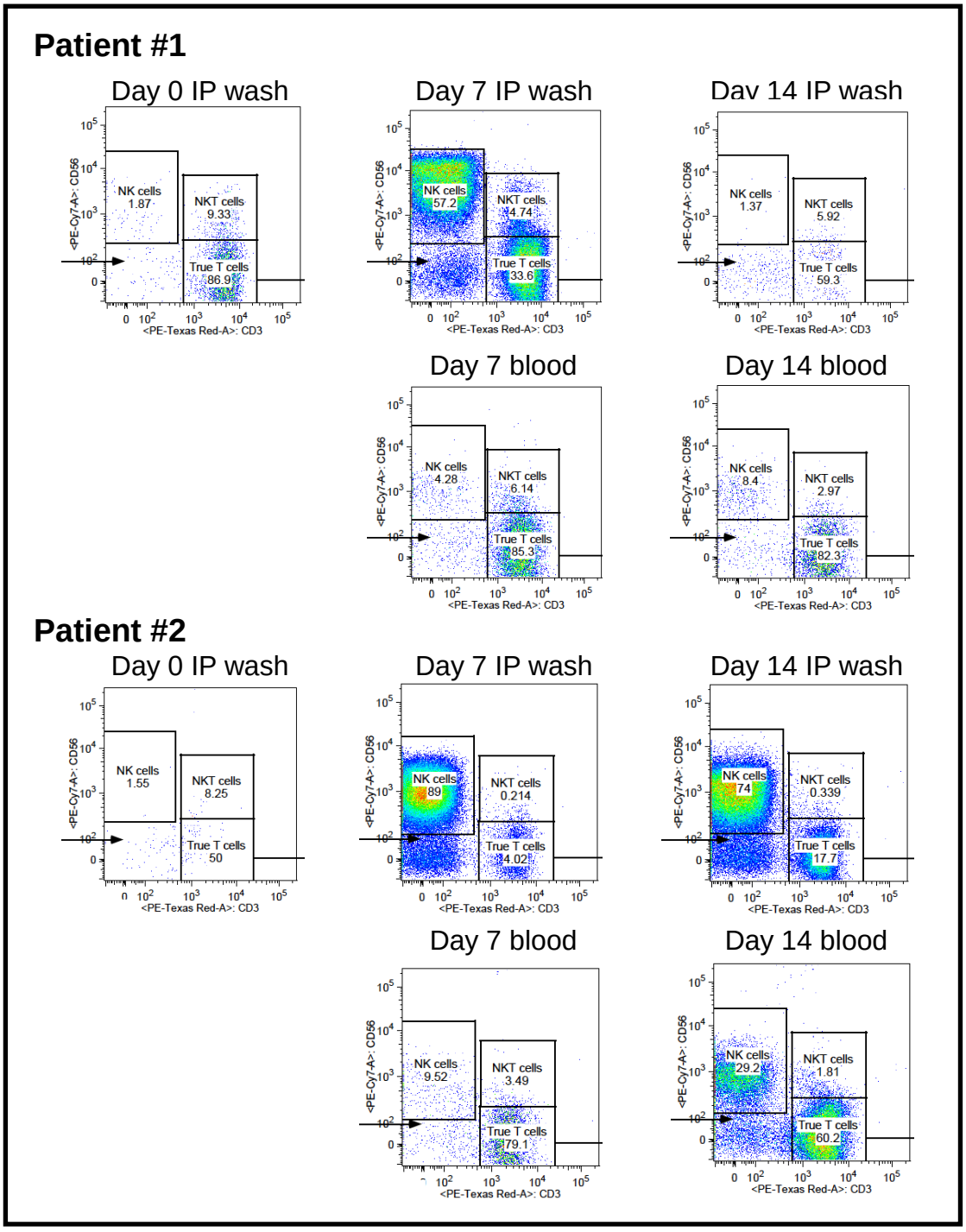
STUDY MONITOR FORM (completed at study initiation visit)

	Comments	**Monitor Paragraph**
Is the signed protocol, including IB and CMO approval available at study centre		
Does the study master file contain all essential documents including a list of all members of the study staff, with their signatures		
Are all CV's of study staff members available at study centre		
Are CRFs available at study centre		
Are normal values for lab tests available at study centre		
Has the project been properly introduced at CRCN, department of clinical chemistry and clinical pharmacy?		

Subject number:

Standard checks	**Comments**	**Monitor Paragraph**
Volunteer complies with inclusion/exclusion criteria		
Source document contains copy of informed consent, all lab results, and completed evaluation form		
CRF is filled in completely		
Monitoring of all grade 3-4 AE and all SAE		
Primary endpoint		

APPENDIX 6



Flow cytometry analysis of allogeneic peripheral blood enriched NK cells following IP infusion in recurrent OC patients. Data are from ongoing clinical trial (NCT02118285) at the Masonic Cancer Center, and obtained from our collaborators Drs. Melissa Geller, Sarah Cooley and Jeffrey Miller. FACS plots from blood and peritoneal washings from patient #1 and #2 before (day 0), and day7 and 14 after NK cell infusion. A clear NK cell expansion was noted in the peritoneum of patient #1 (57% at day 7) and patient #2 (89% and 74% at day 7 and 14, respectively). The percentage donor DNA chimerism was 36% and 4% for patient #1, and 24% and 31% for patient #2 at day 7 and 14, respectively. The percentage DNA chimerism in blood was <2% for both patients.

Appendix 7 Flow chart trial design