# APPROACHES TO POTENTIATE IMMUNOTHERAPY IN MULTIPLE MYELOMA

Laurens Emanuel Franssen

# APPROACHES TO POTENTIATE IMMUNOTHERAPY IN MULTIPLE MYELOMA

# BENADERINGEN OM IMMUNOTHERAPIE POTENTER TE MAKEN BIJ MULTIPEL MYELOOM

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Laurens Emanuel Franssen
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# Chapter 1

## GENERAL INTRODUCTION

## MULTIPLE MYELOMA AND IMMUNOTHERAPY

Multiple myeloma (MM) is a malignant disease, characterized by clonal proliferation of plasma cells in the bone marrow. It is a disease of the elderly, with a median age at diagnosis of 70 years and only five percent of patients below 40 years. MM accounts for 1% of all malignancies and approximately 10% of all hematological malignancies. The disease originates from a, generally asymptomatic, monoclonal gammopathy of undetermined significance (MGUS) to further evolve towards symptomatic MM, while undergoing sequential genetic and micro-environmental changes.[1] Clinical characteristics include osteolytic bone lesions, hypercalcemia, monoclonal protein depositions leading to renal failure, and progressive bone-marrow dysfunction with anemia and other cytopenias.[1,2] The treatment of MM has undergone significant changes and improvements over the last years. Next to the development of proteasome inhibitors (such as bortezomib, carfilzomib and ixazomib), especially the introduction of agents that have profound stimulatory effects on the immune system (such as thalidomide, lenalidomide and pomalidomide) and the development of novel immunotherapies has significantly improved the outcome of MM patients over the past decade.[3,4] However, even though median survival currently is 7-10 years, eventually all patients relapse. This indicates the need for potentiation of the currently available therapies by development of alternative treatment strategies and by gaining insight into resistance mechanisms to current treatments. This has been the main goal of studies described in this thesis. This introduction will first provide an outline of current immunotherapeutic strategies in MM.

## IMMUNOTHERAPY IN MM

Immunotherapy has evolved substantially over the past years, with a wide range of different modalities investigated. The general principle of cancer immunotherapy is to administer or activate immune cells, in order to gain a more targeted approach towards cancer, thereby reducing off target side effects. This approach is in contrast with traditional chemotherapeutic treatment modalities, which are generally non-specific cytotoxic therapies with substantial side effects. In MM, some immunotherapies are currently approved for the treatment of MM, while several other immunotherapeutic approaches are currently focus of active investigations.[5–7] Here, we will discuss a number of immunotherapeutic strategies currently applied for, or investigated in MM patients.

### Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (allo-SCT) can be considered one of the first immunotherapies applied in MM. In allo-SCT, stem cells of a donor are administered to a patient, who has been pretreated with radiation and/or chemotherapy. T cells of the donor, co-administered with the stem cells, can recognize and eliminate tumor cells resulting in the so called "graft-versus-tumor effect (GVT)". Unfortunately, donor T cells can also recognize normal tissue as foreign, thereby causing the detrimental graft-versus-host-disease (GVHD),

a major cause of treatment-related mortality (TRM). In an HLA-matched setting, the GVT effect is mainly mediated by the recognition of minor histocompatibility antigens (mHags) presented on malignant cells.[8] These transplantation antigens are peptides derived from the polymorphic regions of intracellular proteins. Upon binding to HLA molecules they are presented to the T cells of the donor. The polymorphism in the intracellular proteins between the donor and recipient is due to the evolutionary occurrence of, for instance, single nucleotide polymorphisms (SNPs) in the coding regions of the genome, creating peptides which differ in amino-acid sequence. Therefore mHags are foreign antigens for the donor and can therefore induce potent allo-immune T cell responses, even in the setting of HLA-matched transplantation (Figure 1).[9,10]

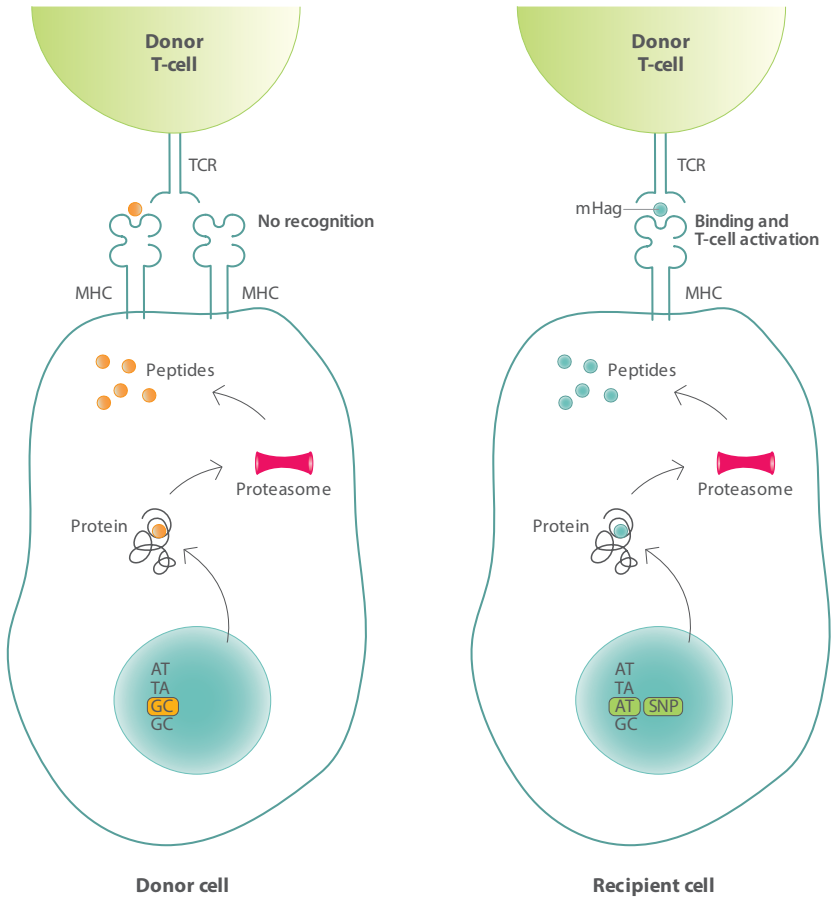


**Figure 1.** Schematic overview of mHag presentation on recipient cells leading to activation of donor T-cells. Genetic polymorphisms leading to differences in amino acids can give rise to differential presentation of mHags on cells of the recipient (right), whereas cells of the donor present no or a different antigen. *TCR:* T-cell receptor; *MHC:* major histocompatibility complex; *mHag:* minor histocompatibility antigen; *SNP:* single nucleotide polymorphism.







The tissue distribution of the mHags determines their contribution to either GVT and GVHD effects. mHags expressed exclusively on hematopoietic cells have a dominant role in the beneficial GVT effect, whereas mHags with a broad tissue expression cause both GVT and GVHD. In addition to the tissue distribution, the population frequency of the mHag is important. mHags with a very low or very high population frequency are not very relevant from an immunotherapeutic point of view, as chances of a mismatch between donor and recipient will be low. The chance for a mismatch is actually maximum with an mHag population frequency of 50%.[11,12]

While allo-transplantation can induce a powerful GVT effect, not present in an autologous transplantation (auto-SCT), in MM comparison of tandem auto-SCT versus allo-SCT in an upfront setting has shown conflicting results.[13–24] Some of these studies suggest that allo-SCT can overcome the unfavorable prognosis in patients with adverse cytogenetic aberrations.[14,25] However, the majority of these studies was performed before the introduction of proteasome inhibitors and immunomodulatory agents. This, together with a high rate of TRM of 10-30% has led to the disappearance of allo-SCT as upfront therapy. Patients with an early relapse (≤ 18 months) after auto-SCT have a poor prognosis, and guidelines recommend considering these patients to be treated with allo-SCT in the context of a clinical trial.[26–29] However, the outcome of allo-SCT in these patients is not well established. Key to improvement of allo-SCT results in MM patients is the development of less toxic conditioning regiments, improvement of (sustained) GVT responses and a reduction or better treatment of GVHD. Post-allo immunotherapy modalities are also subject of investigation, as will be discussed later in this thesis.

### Immunomodulatory agents

Although currently named 'immunomodulatory agents', the IMiDs were initially not designed as such. The initial focus was mainly on their direct anti-MM effects. At present, a lot of data on their immune-activating capacity has been published and it seems that their immune-modulating effects are at least as important as their direct anti-tumor effects.

Thalidomide was initially designed as a sedative, anti-emetic drug used in pregnancy. After the recognition of the teratogenic effects of thalidomide, the drug was withdrawn from the market in 1961. In 1990, the anti-myeloma effects of thalidomide were observed after administration of the drug to 5 patients with end-stage MM, which led to a rapid increase in clinical trials investigating thalidomide in MM.[30]

The second-generation IMiD was lenalidomide, which was approved in 2006 for relapsed/refractory myeloma in combination with dexamethasone. This combination showed a significantly better response rate compared to thalidomide-dexamethasone, and also showed activity in patients previously treated with thalidomide.[31,32] The third-generated IMiD, pomalidomide, was approved for treatment of relapsed/refractory MM patients who have received at least 2 prior lines of therapy including lenalidomide and bortezomib. Pomalidomide also has activity in lenalidomide-refractory patients, with overall response

rates of around 45% in these patients.[33] Immunomodulatory drugs (IMiDs) have multiple mechanisms of action including immune-stimulatory and anti-angiogenic properties, as well as direct anti-MM activity.[34] IMiDs bind to Cereblon, a substrate receptor for the ubiquitin E3-ligase complex $CRL4^{CRBN}$, which include damage-specific DNA binding protein (DDB1), cullin 4A (Cul4) and RING finger protein 1 (ROC1).[35–38] After binding, the specific substrate proteins IKZF1 (Ikaros) and IKZF3 (Aiolos) are recruited to the E3 ligase and targeted for ubiquitination and subsequent proteasomal degradation. The degradation of Ikaros and Aiolos is followed by downregulation of interferon regulatory factor 4 (IRF-4) and c-Myc leading to growth inhibition and apoptosis of MM cells (Figure 2).

Ikaros and Aiolos have also been shown to act as repressors of IL-2 transcription in $CD4^+$ and $CD8^+$ T cells.[36,39–42] The IMiD-induced, Cereblon-dependent degradation of Ikaros and Aiolos in immune cells thus increases IL-2 expression, while also enhancing the production of other cytokines (IFNγ, IL-4, IL-6, IL10, IL13 and GM-CSF).[41] This leads to activation of T cells and NK cells.[43,44] Lenalidomide in combination with dexamethasone is currently one of the standards of care for newly diagnosed MM patients who are not eligible for transplant.[45] In transplant eligible patients, bortezomib combined with lenalidomide-dexamethasone (VRD) or thalidomide-dexamethasone (VTD) are frequently used as induction regiments prior to autologous stem cell transplant. In the relapsed/refractory setting, IMiDs are used in several treatment combinations.[45] At this moment, several new IMiDs, such as CC220, are being investigated in phase 1 clinical trials in extensively pretreated MM patients.

**Monoclonal antibodies**

The substantial therapeutic effect of anti-CD20 monoclonal antibodies for the treatment of B cell lymphoma's indicates the potential of monoclonal antibodies in anti-cancer treatment.[46] In contrast to the genetic heterogeneity of MM, with sequential genetic changes while the disease is progressing, is the more homogeneous immune-phenotype. Furthermore, the different mode of action of immunotherapeutic treatment strategies makes cross resistance with direct anti-MM drugs less likely. Identification of proteins that are highly and stably expressed on MM cells during different disease stages might therefore be attractive to target with therapeutic monoclonal antibodies.

Two antibody targets, signaling lymphocytic activation molecule F7 (SLAMF7) and CD38, are of particular interest in MM. Elotuzumab, a humanized $IgG_1$ monoclonal antibody targets SLAMF7. SLAMF7 is highly expressed on plasma cells and NK cells, and to a lesser extent on a subset of activated T and B cells. Elotuzumab was shown to act primarily via antibody dependent cellular cytotoxicity (ADCC), but has no single agent activity in extensively pretreated MM.[47] However, when combined with lenalidomide and dexamethasone (Rd), objective response rates of 84% were achieved in advanced MM patients with 1-3 prior lines of therapy.[48,49] Preclinical studies showed that lenalidomide potentiates elotuzumab-mediated ADCC by improving NK cell activity.[50] In a randomized phase III trial, elotuzumab, lenalidomide and dexamethasone (Elo-Rd) was compared with Rd in relapsed and/or refractory (94% lenalidomide-naïve) MM patients. Overall response rates were 79% vs. 66%







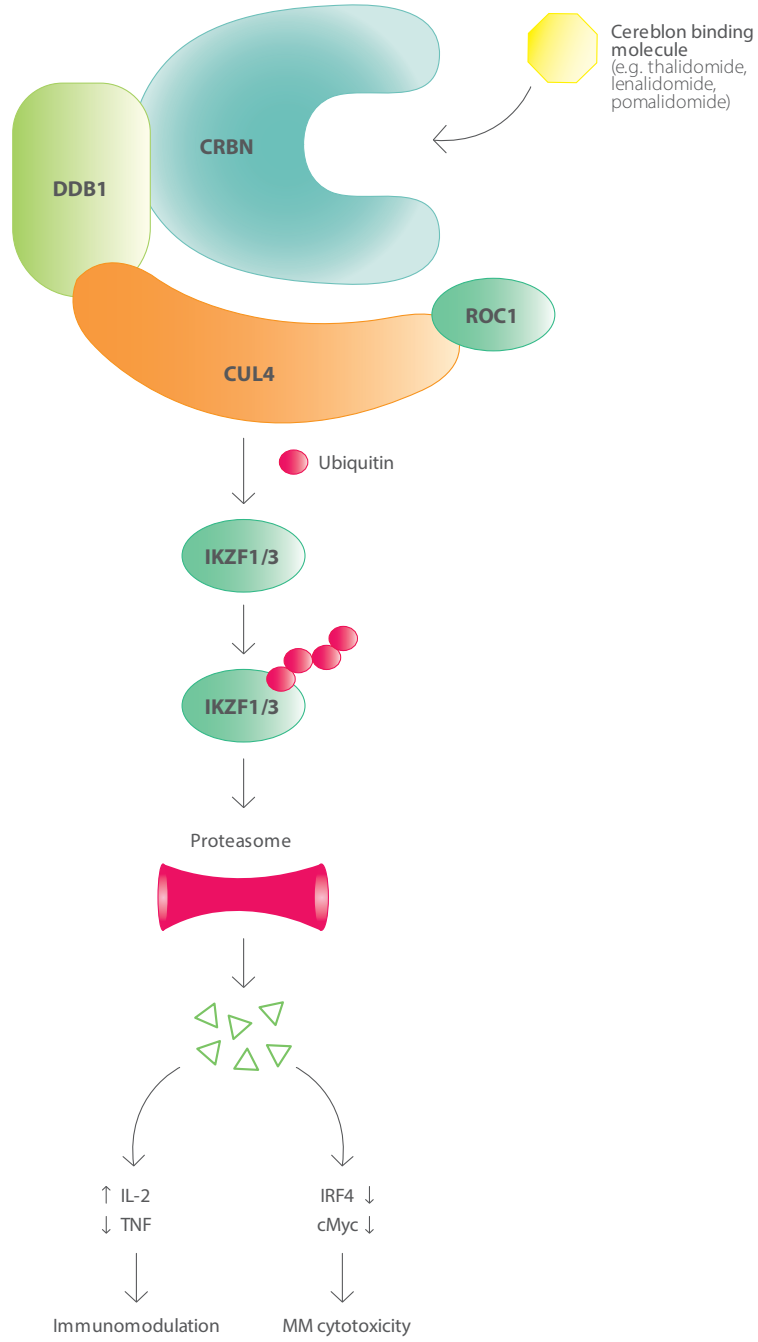


**Figure 2.** The ubiquitin E3-ligase complex $CRL4^{CRBN}$ causing ubiquitination of IKZF1 and IKZF3 leading to their proteasomal degradation and subsequent immunomodulatory and MM cytotoxic effects. *CRBN:* Cereblon; *DDB1:* damage-specific DNA binding protein; *CUL4:* cullin 4A; *ROC1:* RING finger protein 1; *IKZF:* Ikaros family zinc finger protein; *IL-2:* interleukin 2; *TNF:* tumor necrosis factor; *IRF4:* interferon regulatory factor 4; *MM:* multiple myeloma.

($P < 0.001$), with a median PFS of 19.4 months vs. 14.9 months respectively (hazard ratio 0.70, $P < 0.001$).[49] One, two and three-year OS rates for Elo-Rd were 91%, 73% and 60% respectively.[51] Most common grade 3-4 adverse events were neutropenia and thrombocytopenia. Infusion-related reactions occurred in 10% of patients. Elotuzumab has also been combined with bortezomib and dexamethasone in a randomized phase 2 trial, and compared with bortezomib-dexamethasone, resulting in superior PFS (hazard ratio, 0.72; median PFS 9.7 vs. 6.9 months).[52]

The second important antibody target in MM is CD38. CD38 is highly expressed on plasma cells, followed by NK cells and subpopulations of T and B cells. Furthermore, it is also expressed on myeloid cells, erythrocytes and platelets. Several CD38 targeting antibodies have been developed (daratumumab, isatuximab, and MOR202). These antibodies have multiple mechanisms of action, including complement-dependent cytotoxicity (CDC), ADCC and antibody-dependent cellular phagocytosis (ADCP).[52] Interestingly, daratumumab also reduces CD38+ regulatory cell populations as regulatory T cells, regulatory B cells and myeloid-derived suppressor cells, potentially leading to a better anti-tumor immune response.[52] Daratumumab has potent single-agent activity, as was shown by the GEN501 and Sirius trials published in 2015 and 2016.[53,54] Pooled analysis of these studies showed an overall response rate of 31.1%, a PFS of 4 months and an OS of 20.1 months in relapsed/refractory MM patients with a median of 5 prior lines of therapy.[55] Daratumumab was well tolerated. Infusion-related reactions (IRRs) were observed in 48% of patients and consisted of chills, nausea and respiratory conditions, but only 2.7% of patients had ≥ grade 3 IRRs.[55] Main hematological adverse events were anemia, thrombocytopenia and neutropenia. Most common non-hematological adverse events were fatigue, nausea and back pain. Based on the high activity of daratumumab in MM, other CD38-targeting antibodies were developed including MOR202 and isatuximab, which have similar single agent activity in advanced MM.[56,57] Similar to elotuzumab, combining daratumumab with lenalidomide increased NK cell mediated ADCC in preclinical studies, which led to several clinical trials combining anti-CD38 antibodies with an IMiD showing significantly improved outcome. In the POLLUX trial, lenalidomide-dexamethasone (Rd) was compared with daratumumab-Rd in patients with at least one prior therapy but not lenalidomide-refractory disease. PFS at 12 months was 60.1% for Rd versus 83.2% for dara-Rd. In addition, complete response rates and MRD negativity were significantly higher with dara-Rd.[58] Similarly, combining daratumumab with bortezomib and dexamethasone resulted in a significantly higher response rate, depth of response, and PFS in MM patients with at least one prior therapy, but not bortezomib-refractory disease.[59] Others investigated in a phase 1b study, the combination of pomalidomide-daratumumab and dexamethasone in relapsed/refractory MM patients (89% lenalidomide-refractory, 71% bortezomib-refractory and 30% carfilzomib-refractory). Overall response rates were 60% with median PFS and OS of 8.8 months and 17.5 months respectively, which is striking considering the study population.







**CAR T cells**

Successful clinical results have been obtained using CD19 targeting CAR T cells in B cell malignancies.[60,61] These results have led to the development of CAR T cells for other targets. In a chimeric antigen receptor (CAR), the antigen-recognition part of a monoclonal antibody is combined with a T cell receptor and costimulatory domains. The CAR gene can be introduced in T cells using viral transduction. This generates cells that can directly recognize (tumor) antigens, followed by an activation of the cytotoxic machinery of the T cell causing kill of the target cells.[59] The advantages of CAR T cells are the HLA independent mode of action (as compared to T cells), and a much more efficient kill compared to antibodies (ADCC). The expansion and activation of the CAR T cells may lead to the so-called cytokine-release syndrome. This is a potentially fatal clinical syndrome caused by a striking release of pro-inflammatory cytokines, and is characterized by fever, dyspnea, and hypotension and can lead to shock and multi-organ failure. However, the use of the anti-IL6 antibody tocilizumab and corticosteroids is effective in the majority of patients.

In MM, the use of anti B cell maturation antigen (BCMA) CAR T cells has recently been investigated.[62–65] BCMA, a member of the tumor necrosis factor superfamily, is expressed by some B cells, normal plasma cells and MM cells. In these studies, heavily pretreated, relapsed/refractory MM patients were treated with different doses of anti-BCMA CAR T cells after a short course of leukocyte-depleting chemotherapy. High response rates were observed, including some patients achieving a stringent CR. Cytokine-release syndrome was frequent, but well manageable.[62–65] Another study investigated CAR T cells targeting the kappa light chain in 7 MM patients.[66] No objective responses were observed, but treatment with the CAR T cells lead to stable disease ranging from 2-17 months, with no significant toxicities. Several preclinical studies have shown promising other MM antigens suitable for CAR T directed therapy, including CD38, CD44v6 and SLAMF7 which will probably be tested in clinical trials shortly.[67]

**Bispecific antibodies**

Despite the promising clinical results with the use of CAR T cells, particularly CD19 targeting CARs in B cell malignancies, their production requires the use of autologous cells and is considerably time-consuming. Bispecific antibodies (BsAbs) contain two antigen recognition domains. One of them designed to recognize (for example) CD3, which is expressed on almost all T cells, and the other targeting a tumor antigen. This technique enables bringing T cells in proximity to tumor cells, causing T cell proliferation and tumor cell lysis. Because it does not require autologous cells to manufacture, the BsAbs can potentially be generated in large quantities and stored until use, creating an 'of the shelf' product. Furthermore, the toxic side effects observed in CAR T cell therapy can theoretically be managed by halting infusion of the BsAb.[66] Bispecific T cell engagers (BiTEs) are a type of BsAbs consisting of two single-chain variable fragments of different antibodies. In line with studies on CAR T cells, successful clinical results have been obtained using the CD3/CD19 BiTE blinatumomab

targeting CD19 positive ALL,[68,69] relapsed/refractory DLBCL and other types of NHL.[70,71] In MM, thus far no clinical trials have been reported using BiTEs. In preclinical studies, several antigens have been investigated as targets. Among them B cell maturation antigen (BCMA), CD38, CD138, and Fc receptor like 5 (Fcrl5).[72,73] The mode of action of BiTEs requires well-functioning autologous T cells. However, as will be discussed in more detail below, MM is characterized by a defective immune-system, potentially limiting their effectiveness. Furthermore, regulatory T cells also express CD3 and can be activated upon the use of BiTEs further decreasing anti-tumor immune responses.[74]

**Immune-checkpoint inhibition**

The discovery that T cells have mechanisms to control their excess activity, and that tumor cells can efficiently exploit these mechanisms to evade immune T cells attack, caused a major shift forward in cancer immunotherapy, also in MM. Today, we know that activated T cells express several co-inhibitory molecules (immune checkpoint molecules) such as cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and programmed death-1 (PD-1). Binding of these receptors to their corresponding ligands (CD80/86 for CTLA-4 and PD-ligand-1/2 (PD-L1/PD-L2) for PD-1) on antigen presenting cells leads to a controlled inhibition of activated T cells, actually protecting us from immune-mediated diseases. However, soon after the discovery of these natural protection mechanisms, it appeared that tumor cells effectively exploited such feedback loops by upregulating the expression of these co-inhibitory receptors.[75] This has led to the development of antibodies blocking these co-inhibitory receptors (checkpoint inhibitors). Indeed, successful results have been obtained using checkpoint inhibitors in relapsed/refractory Hodgkin's lymphoma.[76,77] An increased expression of PD-L1 on malignant plasma cells has been observed in MM patients, together with PD-1 expression on T cells and NK cells.[78–81] Despite this, monotherapy with the PD-1 inhibitor nivolumab had no clinical activity.[82] However, the combination of pembrolizumab (an anti-PD-1 antibody), lenalidomide and dexamethasone had an overall response rate (ORR) of 44% in heavily pretreated, relapsed/refractory MM patients,[83] with and ORR increasing to 60% when lenalidomide is replaced by pomalidomide.[83]

**Dendritic cell vaccination**

The ultimate goal of dendritic cell (DC) vaccination strategies is the in vivo induction or stimulation of tumor specific T cell immunity against cancer cells in an antigen-dependent fashion, so that patients not only get rid of the tumor but also possess a long-term protection against an eventual relapse. DCs are professional antigen-presenting cells (APCs) capable of efficiently stimulating naïve T cells to build up an anti-tumor response.[84] DC-based immunotherapy has been studied in a wide range of malignancies, and has been shown to be safe.[85] In MM, different strategies have been studied, including autologous DCs pulsed with idiotype, fused with MM cells and loaded with myeloma-associated antigen mRNA (MAGE3, BCMA and survivin).[86–89] Although the use of idiotype pulsed DCs and DCs loaded with myeloma-associated antigen mRNA resulted in the induction of antigen specific T cells







in a subset of patients, clinical responses were disappointing. However, promising results have been observed using DC-MM fusion vaccines. In a phase II clinical study, the use of DC-MM fusion vaccines after autologous transplant was shown to induce anti-MM CD4+ and CD8+ T cells, with a conversion of partial response to complete response following vaccination in 24% of patients.[86]

Dendritic cells are also of interest following allogeneic transplantation, as it has been shown that the graft-versus-tumor effect depends on the presence of host DCs (capable of presenting host antigens).[90–92] However, allo-SCT results in rapid replacement of host DCs by donor DCs, which may hamper the alloreactivity of donor lymphocytes.[93] Therefore, the GVT effect can theoretically be boosted by administration of dendritic cells capable of presenting host antigens, as will be described in this thesis.

## IMMUNE-EVASION BY MM

Immunotherapy can induce potent clinical responses in MM, as is illustrated by long-term survival in a subset of patients after allo-SCT and the successful results of some of the immunotherapeutic approaches described above. However, like other malignancies, MM has several mechanisms to escape immune-mediated killing. Insight into these mechanisms might lead to potential interventions to improve immune therapy in MM. In the following section, a number of mechanisms of immune-evasion by MM cells are described.

**Immune-suppressor cells**

The two most studied immunosuppressive cell subsets that can be induced by MM cells are regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Regulatory T cells are a subset of T cells characterized by the expression CD4, CD25, and forkhead box P3 (FOXP3) and low expression of CD127 ($CD4^+CD25^+CD127^{low}FOXP3^+$). Tregs can be naturally occurring (generated in the thymus) or induced from effector T cells, and are biologically important for maintaining peripheral tolerance and limiting auto-immune disease. However, they can also suppress anti-cancer immune responses. Tregs exert their suppressive effect through several mechanisms. They secrete suppressive cytokines, such as TGF-β and IL-10, and have the capacity to kill B cells, NK cells and CTLs by secreting granzyme-B. In addition, they can suppress the function of dendritic cells by expression of CTLA-4 and lymphocyte activation gene 3 (LAG3) and induce expression of indoleamine 2,3-dioxygenase (IDO) by DCs.[94] Several reports show increased frequencies of Tregs in peripheral blood of MM patients.[95–99] Furthermore, MM cells were shown to induce Tregs from conventional T cells ex vivo.[100,101] However, others state that these Tregs are dysfunctional, or show similar levels of Tregs in MM patients compared to healthy donors.[102–105] These conflicting results can in part be explained by differences in phenotypes used, compartment of measurement (peripheral blood vs. bone marrow) and disease state. In line with the idea of MM-induced Treg expansion and active immune-suppression are two studies showing that lower Treg numbers in bone marrow and peripheral blood are associated with long-term survival in MM patients.[106,107]

Furthermore, recent reports show an increased CD38 expression on Tregs as compared to conventional T cells, whereby alleviation of Treg-induced immune suppression in MM can be achieved using CD38-targeting antibodies as daratumumab and isatuximab.[100,101,108]

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous, immature population of CD11b+CD33+HLA-DR– /low myeloid cells. Two main subtypes of MDSCs exist: polymorphonuclear (granulocytic) MDSCs, expressing CD15 and/or CD66b in addition to the phenotype mentioned above, and monocytic MDSCs expressing CD14. MDSCs exert their suppressive function through several distinct mechanisms. They deplete essential amino-acids like L-arginine and L-cysteine, and cause oxidative stress by production of reactive oxygen species and reactive nitrogen species, both inhibiting T cell function. Furthermore, they interfere with lymphocyte trafficking and viability, and induce regulatory T cells.[109]

MDSCs have been found at increased frequencies in peripheral blood and bone marrow of MM patients, compared to healthy donors.[110–114] In addition, MM cells were shown to induce MDSCs, and conversely, MDSCs contributed to disease progression in MM.[113] These results indicate an active immunosuppressive and disease promoting role of MDSCs in MM.

### Immune-suppressive cytokines

The MM microenvironment is characterized by production of several immunosuppressive cytokines. Expression of indoleamine 2,3-dioxygenase (IDO) in MM catalyzes the metabolization of the essential amino acid tryptophan, leading to immune suppression and recruitment of Tregs.[115] Increased production of soluble major histocompatibility complex class I-related chain A (sMICA) in MM leads to impaired T and NK cell function.[116] A key cytokine in pathogenesis and disease progression of MM is IL-6, produced by bone marrow stromal cells (BMSCs) and MM cells, which can inhibit NK cell function.[117] Furthermore, TGF-β production inhibits T, NK and dendritic cells.[118,119]

### Co-inhibitory molecules

As described above, T cells need co-stimulatory signals to become fully activated. After activation, the upregulation of several co-inhibitory molecules dampens the T cell activation. Tumor cells exploit this mechanism by upregulating co-inhibitory molecules and downregulating co-stimulatory molecules. A number of co-inhibitory molecules are described to be upregulated in MM. PD-L1 is expressed on antigen presenting cells and tumor cells and delivers an inhibitory signal to activated T cells through their ligand PD-1 on T cells. PD-L1 expression is increased on plasma cells of MM patients, can be induced by bone marrow stromal cells and can induce apoptosis and anergy of myeloma-specific T cells.[79,80,120] Besides PD-L1, MM cells express increased levels of carcinoembryonic antigen-related cell adhesion molecules (CEACAMs). CEACAM-6 has been shown to inhibit anti-MM T cell activity, which was completely abrogated by using anti-CEACAM-6 antibodies.[121] Furthermore, a higher expression of the transmembrane glycoprotein CD200 on MM cells at diagnosis correlates with decreased event-free survival after high dose therapy and autologous transplantation, possibly by inhibition of T cell activity after binding to its







ligand CD200R on T cells.[122] On the T cell side, increased expression of PD-1 and CTLA-4 was observed on T cells of MM patients in the bone marrow, indicating an exhausted phenotype which might be reversed by blocking these receptors.[81]

### Cell adhesion-mediated immune resistance

Bone marrow stromal cells (BMSCs) as well as vascular endothelial cells in MM patients can suppress cytotoxic T lymphocyte (CTL) and NK cell-mediated killing of MM cells by regulating anti- and pro-apoptotic pathways in the myeloma cells.[123,124] This immune-resistance was shown to be induced by cell-cell adhesion and decreased significantly when adhesion was abrogated. Upon cell-cell adhesion, investigators observed a downregulation in Fas and an upregulation in survivin and Mcl-1. Using the small molecule YM155, survivin and Mcl-1 could be suppressed, leading to an increased MM cell lysis by cytotoxic T lymphocytes.[123,124]

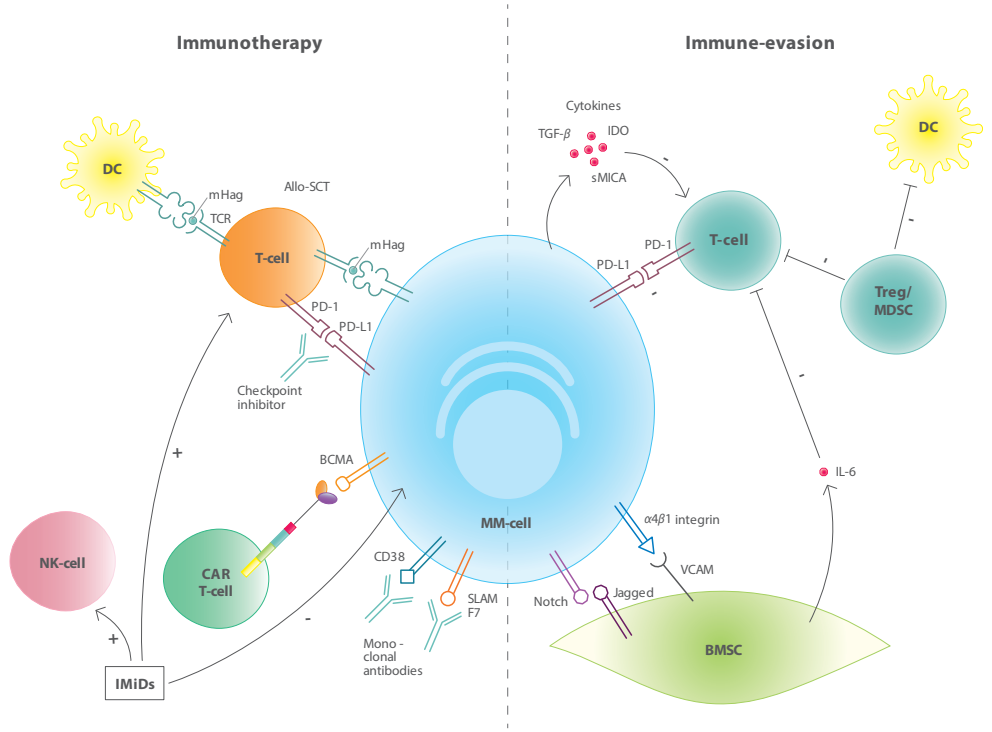


**Figure 3.** Schematic overview of immunotherapeutic strategies to target multiple myeloma (left panel) and immune-evasion strategies (right panel) in multiple myeloma described in this introduction. *DC:* dendritic cell; *mHag:* minor histocompatibility antigen; *Allo-SCT:* allogeneic stem cell transplantation; *TCR:* T-cell receptor; *PD-1:* programmed-death-1; *PD-L1:* programmed-death-ligand-1; *IMiDs:* immunomodulatory drugs; *BMSC:* bone marrow stromal cell; *Treg:* regulatory T-cell, *MDSC:* myeloid-derived suppressor cell.

## OUTLINE AND AIM OF THIS THESIS

The role of allo-SCT in MM is debated because of high treatment related mortality. However, the poor prognosis of high-risk patients, and the potential of allo-SCT to achieve long-term remission ask for optimization of transplantation schemes and better identification of patients who would benefit from allo-SCT. In **chapter 2** we retrospectively analyzed the outcome of one hundred and forty-seven patients with MM who received an allo-SCT in our institution. We aimed to identify patients with known high-risk disease who showed benefit from allo-SCT.

One of the strategies to consolidate or 'boost' the graft-versus-tumor effect after allo-SCT is DLI. Unfortunately, despite using DLI, the majority of patients eventually relapse. Therefore, in **chapter 3** we aimed to improve the efficacy of DLI, without increasing the risk of GVHD, by using mHag-based DC vaccinations administered in combination with a DLI.

In **chapter 4** we analyzed the impact of circulating regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) on the outcome of DLI in MM patients. MM cells can evade the immune system through induction of suppressor cells. This might contribute to a lack of sustained effect of DLI in many patients.

**Chapter 5** shows the results of a phase I/II clinical study in lenalidomide-refractory patients using a combination treatment consisting of lenalidomide, cyclophosphamide and prednisone (REP).

To gain more insight into resistance mechanisms to lenalidomide, we analyzed in **chapter 6** the protein expression of Cereblon and downstream targets in bone marrow-localized plasma cells of lenalidomide-refractory MM patients. Using a validated immunohistochemistry assay, we analyzed paired bone marrow samples at diagnosis and at the time of lenalidomide-refractory disease.

In **chapter 7** we further explored the hypothesis that despite a lenalidomide-refractory myeloma, lenalidomide could retain its immune-activating properties. We therefore analyzed peripheral blood immune cell subsets obtained during REP treatment by flow cytometry for expression of the Cereblon target proteins Ikaros and Aiolos. In addition, we studied the activation status and pro-inflammatory cytokine production of T cells in these patients during REP treatment.







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# Part 1

## ALLOGENEIC STEM CELL TRANSPLANTATION

# Chapter 2

## OUTCOME OF ALLOGENEIC TRANSPLANTATION IN NEWLY DIAGNOSED AND RELAPSED/REFRACTORY MULTIPLE MYELOMA: LONG-TERM FOLLOW-UP IN A SINGLE INSTITUTION

Laurens E. Franssen[1,2], Reinier A.P. Raymakers[1], Arjan Buijs[3], Marian F. Schmitz[1], Suzanne van Dorp[4], Tuna Mutis[2], Henk M. Lokhorst[1,2], Niels W.C.J. van de Donk[2]

[1] Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands. [2] Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands. [3] Department of Genetics, University Medical Center Utrecht, Utrecht, The Netherlands. [4] Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands.

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## ABSTRACT

Allogeneic stem cell transplantation (allo-SCT) has the potential to induce long-term remission in multiple myeloma (MM), but the role of allo-SCT in MM is controversial due to the high rate of treatment-related mortality (TRM). However, although proteasome inhibitors and immunomodulatory drugs have improved the outcome of patients with MM, high-risk patients still have a very poor prognosis. This indicates the need for new treatment strategies and identification of patients who might benefit from allo-SCT. We therefore analyzed the outcome of one hundred and forty-seven patients with MM who received an allo-SCT at our institution (58 in first line, 89 in relapsed/refractory setting) after a median follow-up of 88.8 months. For the first-line setting, median progression-free survival (PFS) and overall survival (OS) were remarkably good, with a CR rate of 48.3%, median PFS of 30.2 months, and 10-yr OS of 51%. We found no difference in outcome for patients with high-risk metaphase cytogenetics or FISH del(13q14), but efficacy in current standard high-risk patients could not be determined. The outcome in the relapsed/refractory setting was poor, especially in the subgroup of patients relapsing within 18 months after auto-SCT. Therefore, if applied at all in these patients, improvement of allo-SCT is needed, focusing on reduction of TRM and more effective immunotherapy.








## INTRODUCTION

Allo-stem cell transplantation (SCT) has the potential to induce long-term remissions due to the graft-versus-tumor effect.[1–6] However, the role of allo-SCT in multiple myeloma (MM) is debated. Comparison of tandem autologous transplantation (auto-SCT) versus upfront allo-SCT has shown conflicting results.[7–12] This, together with the high rate of treatment-related mortality and established effectiveness of novel drugs as induction and/or maintenance therapy, led to the disappearance of allo-SCT as upfront therapy for MM. Although the introduction of proteasome inhibitors and immunomodulatory drugs (IMiDs) has markedly improved the outcome of MM patients, patients with high-risk MM still have a very poor prognosis.[13–17] This indicates the urgent need for new treatment strategies for these patients.

Current guidelines recommend allo-SCT to be performed in the setting of clinical trials, with candidates being newly diagnosed patients with ultra-high-risk myeloma or patients with an early relapse after first line treatment including auto-SCT.[18–20] The effectiveness of allo-SCT in these patients is however not well established. In the current study, we present the results of allo-SCT for MM patients from a single institution. We will describe the outcome of patients transplanted upfront, including the impact of cytogenetic aberrations that were considered high risk in the treatment period of this cohort of patients. In addition, the outcome of patients transplanted in a relapsed/refractory setting is analyzed, specifically looking at patients with early progression after auto-SCT.

## METHODS

### Patients

Between April 2001 and January 2014, 147 MM patients underwent an allo-SCT in the University Medical Center Utrecht (UMCU), Utrecht, The Netherlands. One patient received two additional transplantations because of non-engraftment and another patient received one additional transplantation because of non-engraftment. Total follow up was until June 2014. During this period, standard practice in the UMC Utrecht was that all patients below 66 years of age with a suitable sibling donor were offered an allo-SCT as part of their first line therapy. The indication for allo-SCT in the relapsed setting was determined on an individual basis. Requirements included chemo-sensitive disease, a good performance status (WHO-2) and absence of severe organ abnormalities.

### Outcomes and definitions

Response to treatment and progression were determined according to the criteria formulated by the International Myeloma Working Group.[21] Overall survival (OS) was measured in months and defined from the date of allo-SCT to the date of death from any cause. Patients alive at their last follow-up were censored. Progression-free survival (PFS) was defined from the date of allo-SCT to the date of progression or death from any cause. Patients alive without progression at their last follow-up were censored. Non-relapse mortality (NRM) was

defined as death without previous occurrence of a relapse or progression. Relapse incidence (RI) was defined from the date of allo-SCT to the date of a relapse or progression. NRM and RI were considered competing events. Acute graft-versus-host-disease (GvHD) was defined as grade II-IV according to Seattle criteria.[22] Chronic GvHD was defined as limited and extensive according to Shulman et al.[23] starting from day 100 after allo-SCT. Death or progression/relapse without chronic GvHD were considered competing events. Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation were determined by quantitative PCR. Invasive aspergillosis was diagnosed if the patient fulfilled criteria for possible, probable, or proven aspergillosis.[24] Myeloablative conditioning (MAC) regimens included either busulfan and fludarabine or cyclophosphamide and TBI. Melphalan plus fludarabine and alemtuzumab, as was used in the HOVON 108 trial was considered semi-ablative. Regimens not meeting these criteria were considered to be non-myeloablative (reduced intensity) conditioning.

### Chromosomal and FISH analysis

Metaphase cytogenetics and analysis of deletion of chromosome 13 (del(13q14)) by FISH was done in 86 patients (58.5%). In three of these patients, only FISH analysis was performed. Any aberration found with metaphase cytogenetics (except hyperdiploidy) was considered as 'abnormal karyotype', as this has been described to be an adverse prognostic factor in MM.[25–28] Although del(13q14) is not an optimal prognostic marker for outcome, it was the only chromosomal aberration that was adequately analyzed by FISH in these patients. In addition, it is often associated with other adverse cytogenetic abnormalities (del(17p), t(14;16), t(14;20)), and as such it still is associated with adverse clinical outcome.[14] Cytogenetic analysis was performed at the time of diagnosis in all patients.

### High-risk patients

Two groups were defined as high-risk patients. The first being patients with high-risk cytogenetics. In our cohort we separately looked at an abnormal karyotype (excluding hyperdiploidy) and del(13q14) by FISH. The second being patients with early progression after auto-SCT (either within 12 or within 18 months).

### Statistical analysis

Survival curves were estimated using the Kaplan–Meier method, with group comparison by the log-rank test. Prognostic factors for PFS and OS were analyzed for statistical significance using the Cox proportional hazard model. Factors that showed a significance of $P \leq 0.1$ were included in a multivariate Cox regression model (backward stepwise regression, likelihood ratio test).

For competing-risk analyses, Cumulative Incidence Functions were estimated with group comparison using the Gray Test. NRM, RI and chronic GvHD were analyzed using Cumulative Incidence curves. For prognostic influence of chronic GvHD, analysis was restricted to patients surviving >100 days. Differences in continuous variables were determined using the Mann-Whitney-*U*-test or Kruskal-Wallis test. Differences in categorical variables were determined







with the Fisher's exact test for two by two tables and otherwise with the Pearson's $\chi 2$ test. A level of $P<0.05$ was considered significant. Analyses were performed using SPSS (IBM Statistics, version 20, IBM SPSS Inc., Armonk, NY, USA) and R (for Windows Ri386 3.1.0).

## RESULTS

### Patient and transplant characteristics

We included 147 MM patients. Median follow-up was 88.8 months. Fifty-eight allo-SCTs were performed as part of first-line treatment (39.5%) and 89 for relapsed or refractory MM (60.5%). Induction therapy prior to the allo-SCT included novel agents in 39.7% of the patients treated with allo-SCT in first line, and in 88.8% of the patients treated in the relapsed/refractory setting. Of the first-line patients, 57 (98.3%) received a tandem auto-allo-SCT according to the Seattle scheme.[29] For first-line patients, the remission status pre-allo-SCT was VGPR in 50% of patients, PR in 34.5% and less than PR in 15.5%. For the relapsed patients, the remission status pre-allo-SCT was CR in 12.4% of patients, VGPR in 36%, PR in 41.6% and less than PR in 10.1%. The majority of patients (93.2%) received peripheral blood stem cells. The conditioning regimen was myeloablative in only 3.4% of the transplantations. T-cell depletion was performed with anti-thymocyte globulin (ATG; *in vivo*) in case of an unrelated donor or HLA-mismatch in 53 transplantations (36.1%), or with alemtuzumab (*in vivo* as well as "in the bag") as part of the HOVON 108 trial in 30 transplantations (20.4%). In a subgroup of patients, cytogenetic analysis was performed (42 patients transplanted in first line (72.4%), 44 patients transplanted in the relapsed/refractory setting (49.4%)). No consolidation treatment was given after allo-SCT or after DLI, except for four patients receiving lenalidomide maintenance post-allo-SCT. For detailed characteristics, see Table 1.

### Response

Outcome after transplantation is shown in Table 2. Overall response rate (defined as a remission status of ≥PR after allo-SCT) was 87.9% in first line setting versus 87.6% in relapsed/refractory setting ($P=0.79$). Complete response rates after allo-SCT were higher in the first line setting compared to the relapse setting (48.3% versus 30.3%) ($P=0.06$).

### Survival

In the first line setting, median PFS was 30.2 months (95% CI: 21.4-39.0) and median OS was not reached (10-year OS was 51%). PFS and OS were significantly shorter in the relapsed/refractory setting. Median PFS was 8.0 months (95% CI: 6.4-9.7) and median OS was 28.7 months (95% CI: 16.4-41.0) ($P<0.0001$, Figure 1). To exclude worse survival due to multiple lines of relapse treatment before allo-SCT was given, we compared PFS and OS of patients treated with allo-SCT after first relapse/progression (n=58) with patients treated with allo-SCT after one or more lines of relapse treatment (n=31) which did not show any significant differences.

**Table 1.** Clinical characteristics

| Clinical characteristics | First line setting N= 58 | Relapse/refractory setting N= 89 |
|---|---|---|
| **Sex** | | |
| Male | 37 (63.8) | 63 (70.8) |
| Female | 21 (36.2) | 26 (29.2) |
| **Age (years)** | | |
| Mean | 53.30 | 55.64 |
| Range | 35-66 | 32-68 |
| **Line of therapy** | | |
| 1 | N.A. | n.a. |
| 2 | | 58 (65.2) |
| 3 | | 22 (24.7) |
| >3 | | 9 (10.1) |
| **Source** | | |
| PB | 56 (96.6) | 81 (91.0) |
| BM | 1 (1.7) | 7 (7.9) |
| Missing data | 1 (1.7) | 1 (1.1) |
| **Donor** | | |
| Sibling | 55 (94.8) | 32 (36.0) |
| MUD | 3 (5.2) | 57 (64.0) |
| **Sex mismatch** | | |
| Patient /donor M/F | 13 (22.4) | 21 (23.6) |
| **Myeloablation** | | |
| MA | 3 (5.2) | 2 (2.2) |
| NMA | 55 (94.8) | 58 (65.2) |
| Semi-ablative | 0 | 29 (32.6) |
| **T-cell depletion** | | |
| ATG/Alemtuzumab | 7 (12.1) | 76 (85.4) |
| None | 51 (87.9) | 13 (14.6) |
| **Type original M-protein** | | |
| IgA | 7 (12.1) | 21 (23.6) |
| IgG | 43 (74.1) | 52 (58.4) |
| IgM | 1 (1.7) | 1 (1.1) |
| IgD | 1 (1.7) | 0 |
| FLC only | 3 (5.1) | 13 (14.6) |
| Non-secretory | 3 (5.2) | 1 (1.1) |
| **Novel agents pre-allo** | | |
| Bortezomib based | 6 (10.3) | 6 (6.7) |
| Lenalidomide based | 0 | 4 (4.5) |
| Thalidomide based | 17 (29.3) | 19 (21.3) |
| Multiple types | 0 | 50 (56.2) |
| None | 35 (60.3) | 10 (11.2) |







**Table 1.** (continued)

| Clinical characteristics | First line setting N= 58 | Relapse/refractory setting N= 89 |
|---|---|---|
| **Time auto-SCT to allo-SCT (months)** | | |
| Mean | 4.08 | 32.79 |
| Range | 1.63-15.37 | 2.13-81.13 |
| **Relapse after auto-SCT*** | | |
| <12 months | N.A. | 17 (25.8) |
| <18 months | | 29 (43.9) |
| **Remission status pre-allo** | | |
| CR | 0 | 11 (12.4) |
| VGPR | 29 (50) | 32 (36.0) |
| PR | 20 (34.5) | 37 (41.6) |
| Less than PR | 9 (15.5) | 9 (10.1) |
| **Cytogenetic aberrations** | | |
| ***Metaphase cytogenetics (excluding hyperdiploidy)*** | | |
| Yes | 15 (25.9) | 13 (14.6) |
| No | 27 (46.6) | 29 (32.6) |
| Unknown | 16 (27.6) | 49 (53.9) |
| ***FISH del(13q)*** | | |
| Yes | 17 (40.5) | 18 (40.9) |
| No | 25 (59.5) | 26 (59.1) |
| Unknown | 16 (27.6) | 45 (50.6) |

* Only patients in relapse setting, n=89. Of those, we could determine time from auto-SCT to relapse/progression in 66 patients.

## NRM and relapse/progression incidence

In the first line setting, cumulative incidence of NRM at 10 years was 15.5% (95% CI: 7.6-26.0), compared to 18.8% (95% CI: 10.8-28.5) in the relapsed/refractory setting ($P$=0.72). Causes of NRM were infections in eight patients, heart failure of unknown origin in one, and GvHD in all others (n=41). Cumulative incidence of relapse or progression at 10 years was 53.3% (95% CI: 39.0-65.6) in the first line setting, compared to 75.1% (95% CI: 56.3-86.7) in the relapsed/refractory setting ($P$< 0.001, see Figure 2).

## GVHD

The incidence of acute GvHD (grade II-IV) in the first-line setting was 50.0%, compared to 30.3% in the relapse setting ($P$=0.024). Cumulative incidence of limited and extensive chronic GvHD at 10 years was higher in the first line setting compared to allo-SCT in the relapse setting (50% vs. 36.5%, $P$=0.133).

**Table 2.** Outcome after allo-SCT

| | Allo-SCT in first line N= 58 | Allo-SCT in relapse setting N= 89 |
|---|---|---|
| **Remission status after allo-SCT** | | |
| CR | 28 (48.3) | 27 (30.3) |
| VGPR | 18 (31.0) | 33 (37.1) |
| PR | 5 (8.6) | 18 (20.2) |
| Less than PR | 7 (12) | 9 (10.1) |
| Too early to evaluate | | 1 (1.1) |
| EBV reactivation | 8 (13.8) | 31 (34.8) |
| CMV reactivation | 10 (17.2) | 33 (37.1) |
| Aspergillus infection | 3 (5.2) | 12 (13.5) |
| Acute GVHD grade 2-4 | 29 (50) | 27 (30.3) |
| **Chronic GVHD** | | |
| Limited | 6 (10.3) | 12 (13.5) |
| Extensive | 23 (39.7) | 21 (23.6) |
| **Median PFS (months)** | | |
| Whole group (95% CI) | 30.20 (21.44-38.96) | 8.03 (6.38-9.68) |
| **Median PFS2 (months)** | | |
| Whole group (95% CI) | 61.63 (31.75-91.51) | 14.97 (11.60-18.34) |
| **Median OS (months)** | | |
| Whole group (95% CI) | NR (10-year survival of 51%) | 28.70 (16.39-41.01) |

CMV, cytomegalovirus; EBV, Epstein–Barr virus; OS, overall survival; PFS, progression-free survival; allo-SCT, allogeneic stem cell transplantation.

### High-risk cytogenetic abnormalities

In the first-line setting, cytogenetic aberrations defined by metaphase cytogenetics or FISH del(13q14) did not significantly influence PFS and OS in our cohort (Table 3).

In the relapsed/refractory setting, FISH del(13q14) was an unfavorable prognostic factor for OS, but not for PFS. Median OS was 13.4 months in patients with del(13q14) compared to 64.3 months in patients without this abnormality (P=0.007, HR 2.845, 95% CI: 1.330-6.082) (Table 4). Cytogenetic aberrations defined by metaphase cytogenetics did not influence survival in this group.

### Early relapse after auto-SCT

For 66 patients receiving allo-SCT for relapse/progression after previous treatment we were able to define the time from auto-SCT to relapse or progression (according to IMWG criteria). The other 23 patients did not receive auto-SCT (n=5), were primary refractory to auto-SCT (n=5), received tandem auto-allo-SCT for progression after previous non-high dose treatment (n=2), progressed without reaching the IMWG progression criteria (n=2) and for 9 patients the exact interval between auto-SCT and relapse/progression could not be calculated.







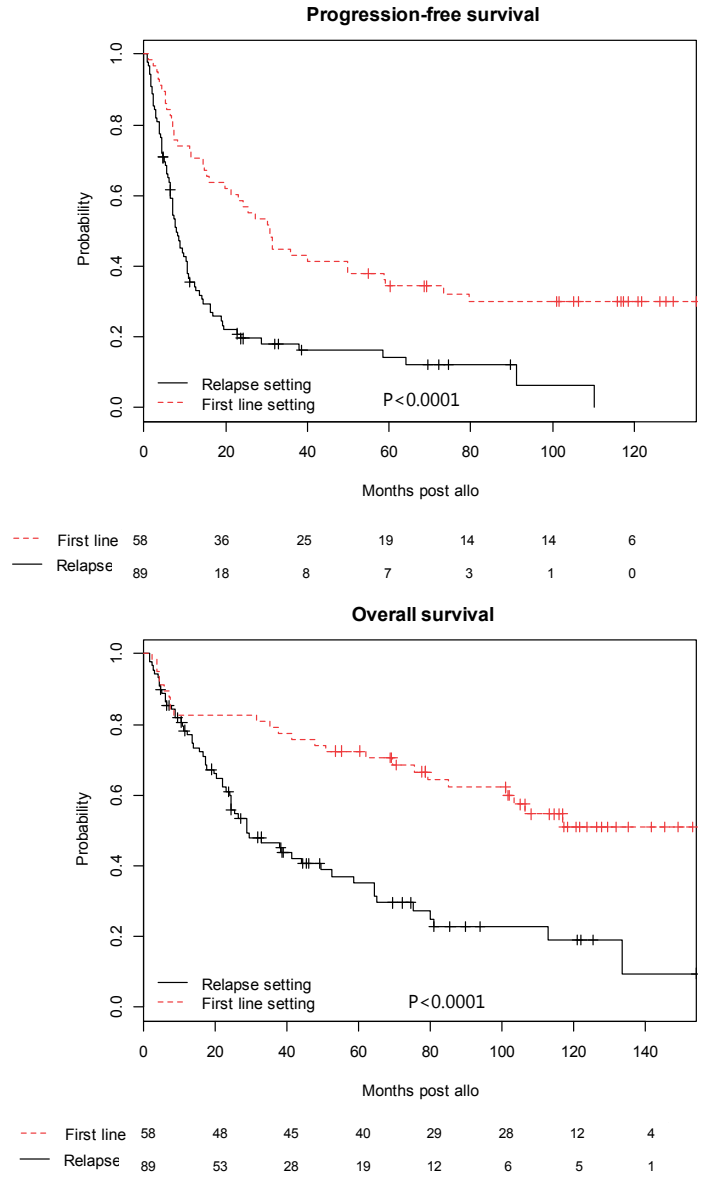


**Figure 1.** Progression-free survival and overall survival for patients transplanted upfront vs. patients transplanted in a relapsed/refractory setting. The log-rank test was used to test the statistical significance of the difference between the survival curves.

A relapse or progression within 18 months after auto-SCT significantly influenced both PFS and OS. Median PFS in patients relapsing within 18 months after auto-SCT (n=29) was 6.5 months (95% CI: 4.5-8.4) compared to 9.7 months (95% CI: 6.4-13.1) in patients relapsing after 18 months (n=37) (*P*=0.020). Median OS was 22.8 months (95% CI: 14.9-30.8) compared to 52.4 months (95% CI: 20.5-84.4) in patients relapsing within and after 18 months

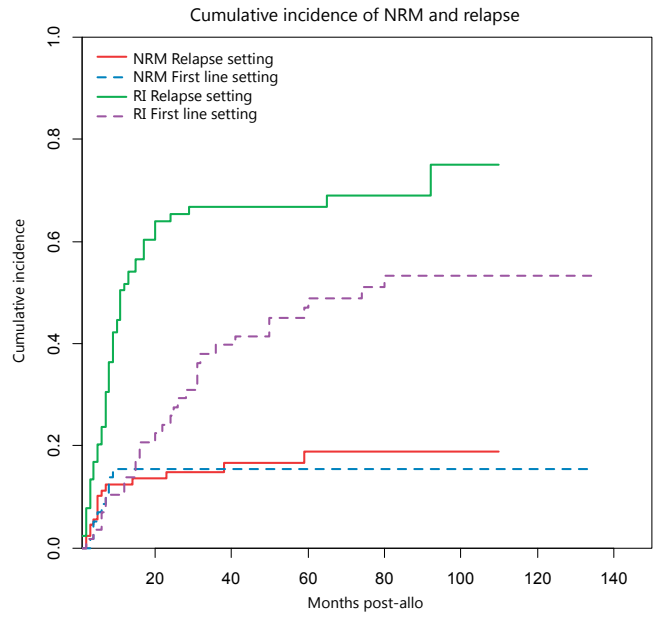


**Figure 2.** Cumulative incidence functions of non-relapse mortality (NRM) and relapse incidence (RI) in upfront vs. relapsed/refractory setting.

respectively (*P*=0.012)(see Figure 3). For patients relapsing within 12 months after auto-SCT (n=17), survival was not significantly different from patients relapsing within 18 months (median PFS 6.6 months and median OS 19.4 months, *P*=0.061 and *P*=0.068 respectively).

### Predictive factors for PFS and OS

Next to the defined "high risk myeloma" group described above, we also analyzed other possible predictive factors for PFS and OS.

For the upfront setting, univariate analysis for possible predictive factors for PFS and OS is depicted in Table 3. In the multivariate analysis, a remission status of ≥VGPR after allo-SCT was an independent predictor for longer PFS (HR 0.196, *P*<0.001) and OS (HR 0.259, *P*=0.001).

For the relapse/refractory setting, univariate analysis is depicted in Table 4. In multivariate analysis, independent predictive factors for PFS were age at allo-SCT (HR 1.059, *P*=0.012), ≥VGPR after allo-SCT (HR 0.395, *P*=0.006) and chronic GvHD (HR 0.371, *P*=0.002). For OS, FISH del(13q14) (HR 4.149, *P*=0.006), aspergillus infection (HR 7.336, *P*=0.003) and CR after allo-SCT (HR 0.184, *P*=0.010) were independent predictive factors.

### Relapse treatment and outcome

After allo-SCT, 90 patients (61.2%) had a relapse or progression of disease. Seventy patients were treated with novel agents post allo-SCT (15 bortezomib, 26 lenalidomide, 18 thalidomide, and 11 lenalidomide plus bortezomib). ORR (≥PR) to relapse treatment







**Table 3.** Univariate analysis of possible predictive factors for PFS and OS after allo-SCT in the upfront setting.

| | PFS; | | | OS; | | |
|---|---|---|---|---|---|---|
| | *P*-value | HR | 95% CI | *P*-value | HR | 95% CI |
| Sex (female) | 0.036 | 0.464 | 0.226-0.953 | 0.111 | 0.474 | 0.189-1.188 |
| Age | 0.256 | 1.023 | 0.984-1.064 | 0.110 | 1.042 | 0.991-1.096 |
| **Remission status pre-allo** | | | | | | |
| CR | N.A. | | | N.A. | | |
| CR and VGPR | 0.007 | 0.415 | 0.219-0.786 | 0.024 | 0.378 | 0.163-0.878 |
| Stem cell source (BM) | 0.461 | 2.126 | 0.286-15.818 | 0.146 | 4.571 | 0.590-35.419 |
| Donor type (Sib) | 0.947 | 0.953 | 0.229-3.956 | 0.331 | 0.487 | 0.114-2.078 |
| **Remission status after-allo** | | | | | | |
| CR | <0.001 | 0.217 | 0.109-0.432 | <0.001 | 0.160 | 0.059-0.435 |
| CR and VGPR | <0.001 | 0.196 | 0.093-0.414 | 0.001 | 0.259 | 0.115-0.582 |
| Aspergillus infection | 0.806 | 0.837 | 0.202-3.473 | 0.699 | 0.673 | 0.091-5.003 |
| EBV reactivation | 0.434 | 1.414 | 0.593-3.373 | 0.123 | 2.176 | 0.810-5.844 |
| CMV reactivation | 0.562 | 1.273 | 0.563-2.883 | 0.318 | 1.651 | 0.618-4.416 |
| Acute GVHD (grade 2-4) | 0.798 | 1.084 | 0.582-2.019 | 0.987 | 1.007 | 0.457-2.220 |
| Chronic GVHD (limited and extensive)* | 0.169 | 0.646 | 0.347-1.204 | 0.196 | 0.593 | 0.268-1.311 |
| Prior treatment with novel agents | 0.668 | 0.867 | 0.453-1.662 | 0.297 | 1.523 | 0.690-3.361 |
| Patient/donor (male/female) | 0.462 | 1.309 | 0.639-2.681 | 0.148 | 1.861 | 0.803-4.317 |
| T-cell depletion with ATG or alemtuzumab | 0.469 | 0.682 | 0.243-1.919 | 0.945 | 0.959 | 0.286-3.209 |
| Karyotyping abnormal (excluding hyperdiploidy) | 0.654 | 0.834 | 0.377-1.845 | 0.160 | 0.459 | 0.151-1.396 |
| FISH del(13q) | 0.351 | 1.425 | 0.677-2.997 | 0.798 | 0.883 | 0.342-2.282 |

CMV, cytomegalovirus; EBV, Epstein–Barr virus; OS, overall survival; PFS, progression-free survival; allo-SCT, allogeneic stem cell transplantation. * chronic GVHD was analyzed including the time-dependent variable >100 days.

was 51.4%, with a CR rate of 13.2%. Response rate was not significantly different between patients receiving allo-SCT as part of first line or relapse treatment. Median time from start of relapse treatment to subsequent progression or death (second PFS) was 8.1 months (95% CI: 6.6-9.7) and not significantly different between different types of novel agents. Median overall survival from the time of first relapse was 76.8 months (95% CI: 44.6-109.0) in newly diagnosed patients, compared to 22.1 months (95% CI: 10.3-33.9) in the relapsed/refractory setting (*P*=0.001). Thirty-seven of the 90 patients received a DLI, which was preceded by novel agents in 28 patients. Novel agents combined with DLI (n=28) resulted in an ORR (≥PR) of 60.7% and a CR rate of 10.7%. DLI without novel agents (n=9) resulted in an ORR of 77.8% and CR rate of 11.1%. For all patients receiving DLI as part of relapse treatment, median second PFS was 8.7 months (95% CI: 4.6-12.7).

Of the 90 patients receiving treatment for first progression after allo-SCT, 8 developed chronic GvHD, while 18 developed acute GvHD. There was no significant difference in GvHD occurrence between the different types of treatment, including DLI.

**Table 4.** Univariate analysis of possible predictive factors for PFS and OS after allo-SCT in the relapsed/refractory setting.

| | PFS; *P*-value | HR | 95% CI | OS; *P*-value | HR | 95% CI |
|---|---|---|---|---|---|---|
| Sex (female) | 0.301 | 0.749 | 0.434-1.294 | 0.583 | 1.187 | 0.644-2.187 |
| Age | 0.061 | 1.030 | 0.999-1.062 | 0.130 | 1.026 | 0.992-1.061 |
| **Relapse after auto-SCT** | | | | | | |
| <12 months | 0.061 | 1.743 | 0.976-3.116 | 0.068 | 1.796 | 0.957-3.372 |
| <18 months | 0.022 | 1.860 | 1.094-3.160 | 0.014 | 2.140 | 1.165-3.930 |
| **Remission status pre-allo** | | | | | | |
| CR | 0.543 | 0.796 | 0.382-1.660 | 0.240 | 0.576 | 0.230-1.445 |
| CR and VGPR | 0.224 | 1.336 | 0.838-2.130 | 0.312 | 1.302 | 0.781-2.172 |
| Stem cell source (BM) | 0.031 | 2.401 | 1.083-5.320 | 0.989 | 0.994 | 0.394-2.506 |
| Donor type (Sib) | 0.487 | 1.180 | 0.740-1.883 | 0.658 | 0.886 | 0.518-1.516 |
| Extent of prior therapy ($2^{nd}$ vs $3^{rd}$ vs $4^{th}$ line allo) | 0.156 | 1.240 | 0.922-1.668 | 0.057 | 1.369 | 0.991-1.892 |
| **Remission status after-allo** | | | | | | |
| CR | 0.001 | 0.400 | 0.234-0.683 | 0.018 | 0.464 | 0.246-0.875 |
| CR and VGPR | 0.005 | 0.495 | 0.302-0.812 | 0.203 | 0.704 | 0.410-1.209 |
| Aspergillus infection | 0.110 | 1.662 | 0.892-3.097 | <0.001 | 3.568 | 1.788-7.119 |
| EBV reactivation | 0.023 | 0.565 | 0.345-0.923 | 0.058 | 0.583 | 0.334-1.018 |
| CMV reactivation | 0.176 | 0.714 | 0.438-1.163 | 0.424 | 0.798 | 0.458-1.389 |
| Acute GVHD (grade 2-4) | 0.133 | 0.673 | 0.402-1.129 | 0.889 | 1.041 | 0.590-1.838 |
| Chronic GVHD (limited and extensive)* | 0.001 | 0.434 | 0.263-0.715 | 0.012 | 0.488 | 0.278-0.856 |
| Prior treatment with novel agents | 0.261 | 1.498 | 0.740-3.030 | 0.305 | 1.491 | 0.695-3.198 |
| Patient/donor (male/female) | 0.152 | 1.453 | 0.871-2.424 | 0.856 | 1.054 | 0.598-1.858 |
| T-cell depletion with ATG or alemtuzumab | 0.361 | 1.337 | 0.717-2.494 | 0.150 | 1.713 | 0.822-3.570 |
| Karyotyping abnormal (excluding hyperdiploidy) | 0.846 | 1.076 | 0.514-2.252 | 0.141 | 1.813 | 0.812-4.049 |
| FISH del(13q) | 0.528 | 1.237 | 0.638-2.397 | 0.007 | 2.845 | 1.330-6.082 |

ATG, antithymocyte globulin; CMV, cytomegalovirus; EBV, Epstein–Barr virus; OS, overall survival; PFS, progression-free survival; allo-SCT, allogeneic stem cell transplantation.

* chronic GVHD was analyzed including the time-dependent variable >100 d.

Of the 29 high-risk patients relapsing within 18 months after auto-SCT, 24 received treatment for their first progression after allo-SCT. Strikingly, the ORR to first relapse treatment in these patients was only 25% compared to 72% in patients relapsing after 18 months after auto-SCT (*P*=0.002). In addition, duration of response was significantly shorter in these patients (4.7 compared to 12.1 months, *P*=0.020). Median overall survival from the time of first relapse in these high-risk patients was 16.2 months (95% CI: 5.3-27.2), compared to 36.1 months (95% CI: 3.8-68.4) in patients relapsing after 18 months post auto-SCT (*P*=0.028). For patients with cytogenetic high-risk features (in this cohort defined as either an abnormal







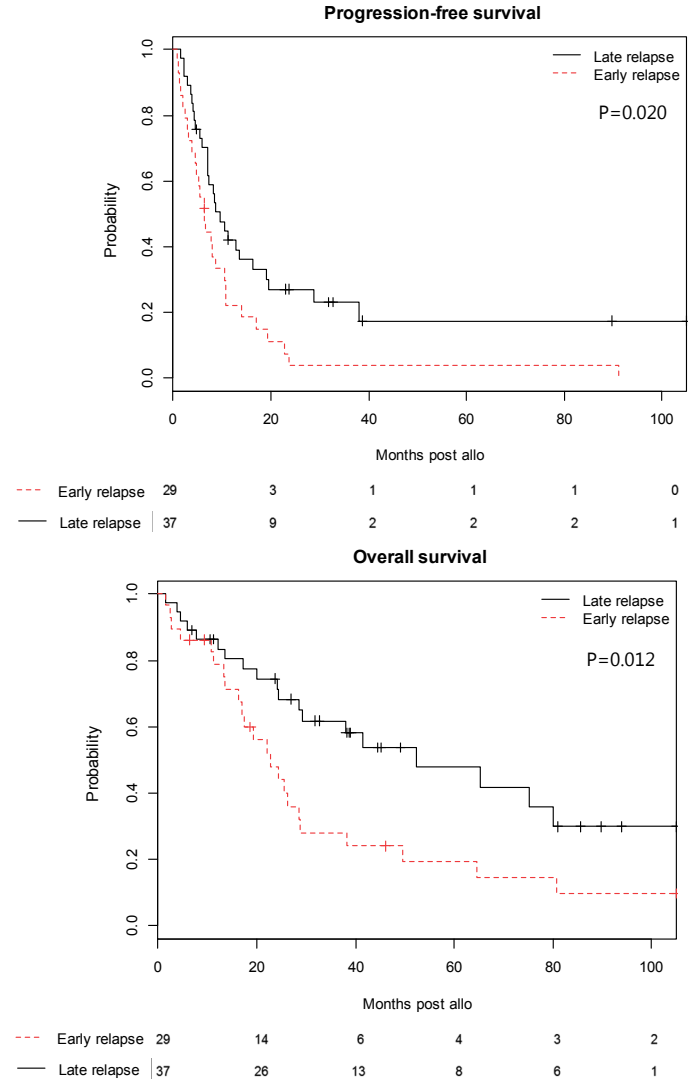


**Figure 3.** Progression-free survival and overall survival for patients transplanted for a relapse after auto-SCT within 18 months (early relapse) vs. patients transplanted for a relapse after auto-SCT beyond 18 months (late relapse). The log-rank test was used to test the statistical significance of the difference between the survival curves.

karyotype or FISH del(13q14)), response to and duration of first relapse treatment were not different compared to patients without these aberrations.

## DISCUSSION

In the present study, we describe our single center experience with allo-SCT in 147 MM patients. This is one of the largest single center reports on the outcome of allo-SCT in MM. Our goal was to describe the outcome of allo-SCT for newly diagnosed and relapsed/refractory MM, including the outcome in patients with abnormal metaphase cytogenetics or del(13q14) determined by FISH, and in the relapse setting in patients with an early relapse or progression after auto-SCT.[13–17,20] As almost all patients were treated with allo-RIC and peripheral blood stem cells (PBSC), we were not able to draw any conclusions on the use of PBSC vs. bone-marrow as stem cell source, or the difference of allo-MAC vs. allo-RIC.

The outcome of patients transplanted as part of first line therapy (being a tandem auto-allo approach in 98.3%) in our cohort is remarkably good, and compares favorably to results described in literature where "upfront" allo-SCT has mostly been compared to high dose chemotherapy and single auto-SCT or tandem auto-SCT in donor-versus-no-donor comparisons, with conflicting results.[8–12,30–34] We observed a high CR rate after allo-SCT of 48.3%. Median PFS was 30.2 months and median OS was not reached (10-year OS was 51% in our cohort). We also observed a plateau in the PFS and OS curves; however, it remains unclear whether these patients can be considered to be cured. Despite these encouraging survival outcomes, we also found a cumulative incidence of chronic graft-versus-host disease of 50% in this group. This probably has a major impact on quality of life.[35,36] Unfortunately, however, due to lack of collection of standardized quality of life data in this cohort, we were not able to report on quality of life in this analysis. Our subgroup analysis in patients with cytogenetic aberrations showed no difference in outcome compared to standard risk patients, suggesting that allo-SCT in the upfront setting might overcome the unfavorable prognosis of these cytogenetic aberrations.

Very few studies have described the outcome of high-risk cytogenetic patients after allo-SCT in an upfront setting. Long-term results of the EBMT-NMAM2000 study show an equal outcome for patients with and without del(13q14).[31] In addition, Kroger et al. found that allo-SCT overcomes the adverse prognosis of del(17p) and t(4;14), but it is unclear whether they included only upfront allo-SCT.[37] In a prospective study comparing patients with del(13q14), Knop et al. found a significantly increased PFS for the auto-allo arm, compared with double auto-SCT and also a significantly better OS in the allo-SCT arm for the subgroup with del(17p).[7] In contrast to these studies, the French group found no difference in outcome, in high risk MM patients also carrying del(13q14), when double auto-SCT was compared with tandem auto-allo.[33,34] However, the high dose ATG used in that protocol may have negatively influenced the outcome of the allogeneic transplantation.

Unfortunately, we were not able to obtain data on the presence of t(4;14), del(17p) and t(11;14) due to the retrospective character of this study and the fact that assessment of these cytogenetic aberrations was not routine practice at the time of transplant for most of these patients. Although del(13q) as determined by FISH is no longer seen as an independent risk factor because it often coincides with other adverse cytogenetic abnormalities, as such it still is associated with adverse clinical outcome.[14]







The discussion on whether allo-SCT should be considered an option for relapsed/refractory patients is still ongoing. A recent guideline by Giralt et al. describes that allo-SCT should be considered appropriate therapy for any patient with a relapse within 24 months after a primary auto-SCT.[20] We found a very poor outcome of allo-SCT in relapsed patients, with a short median PFS of 8.0 months and a median OS of 28.2 months. If we focus only on patients relapsing within 18 months after auto-SCT, median PFS was 6.5 months compared to 9.7 months in patients relapsing after 18 months. In addition, patients relapsing within 18 months after auto-SCT had inferior response rates and response duration to relapse treatment after allo-SCT, which translated in a worse median OS of 22.8 months compared to 52.4 months in patients relapsing within or after 18 months respectively. Although the overall survival of patients relapsing after 18 months is promising, the short PFS in this group suggests a poor graft-versus-myeloma effect and the extended OS probably reflects effective relapse treatment after allo-SCT with novel agents. For patients relapsing beyond 1.5-2 years after auto-SCT, we generally recommend novel agent-based therapy or, in case of transplant eligible patients, a second auto-SCT.[38–47]

In conclusion, although our results of allo-SCT as part of first line therapy are very encouraging, upfront allo-SCT is not considered an option for standard risk MM due to the high non-relapse mortality and currently available superior alternatives with novel agent-based combination therapies. For the subgroup of patients with ultra-high-risk myeloma (ISS 3 and high lactate dehydrogenase and del(17p) and/or t(4;14)) the chances of achieving a long-term remission are still very low.[16] In these patients, upfront allo-SCT in the setting of a clinical trial might be an option.

In the relapsed/refractory setting, outcome after allo-SCT is very poor, especially for patients with an early relapse after auto-SCT. If applied at all, new treatment options for allo-SCT are urgently needed. In this respect, the value of optimal induction, maintenance therapy and post allo-SCT immunotherapy should be explored, as well as strategies to lower NRM and acute and chronic GvHD.

## ACKNOWLEDGMENTS

None.

## CONFLICTS OF INTEREST

None.

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# Chapter 3

## A PHASE I/II MINOR HISTOCOMPATIBILITY ANTIGEN-LOADED DENDRITIC CELL VACCINATION TRIAL TO SAFELY IMPROVE THE EFFICACY OF DONOR LYMPHOCYTE INFUSIONS IN MYELOMA

L.E. Franssen*[1,2], M.W.H. Roeven*[3,4], W. Hobo[4], R. Doorn[5], R. Oostvogels[1,2], J.H.F. Falkenburg[6], N.W. van de Donk[2], M.G.D. Kester[6], H. Fredrix[4], K. Westinga[7], I. Slaper-Cortenbach[7], E. Spierings[5], M.J. Kersten[8], H. Dolstra[4], T. Mutis[2], N. Schaap[3] and H.M. Lokhorst[1,2]

* These authors contributed equally to this work

[1] Department of Hematology, University Medical Center Utrecht, The Netherlands. [2] Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands. [3] Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands. [4] Department of Laboratory Medicine, Laboratory of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands. [5] Laboratory of Translational Immunology, University Medical Center Utrecht, The Netherlands. [6] Department of Hematology, Leiden University Medical Center, The Netherlands. [7] Cell Therapy Facility, University Medical Center Utrecht, The Netherlands. [8] Department of Hematology, Amsterdam Medical Center, The Netherlands.

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## ABSTRACT

Allogeneic stem cell transplantation (allo-SCT) with or without donor lymphocyte infusions (DLI) is the only curative option for several hematological malignancies. Unfortunately, allo-SCT is often associated with graft-versus-host disease (GVHD), and patients often relapse. We therefore aim at improving the graft-versus-tumor effect, without increasing the risk of GVHD, by targeting hematopoietic lineage-restricted and tumor-associated minor histocompatibility antigens (mHags) using peptide-loaded dendritic cell (DC) vaccinations. In the present multicenter study, we report the feasibility, safety and efficacy of this concept. We treated nine multiple myeloma (MM) patients with persistent or relapsed disease after allo-SCT and a previous DLI, with donor monocyte-derived mHag-peptide loaded DC vaccinations combined with a second DLI. Vaccinations were well tolerated and no occurrence of GVHD was observed. In 5/9 patients we were able to show the induction of mHag specific $CD8^+$ T-cells in peripheral blood. Five out of nine patients, of which four developed mHag specific T-cells, showed stable disease (SD) for 3.5-10 months. This study shows that mHag based donor monocyte-derived DC vaccination combined with DLI is safe, feasible and capable of inducing objective mHag specific T-cell responses. Future research should focus on further improvement of the vaccination strategy, towards translating the observed T-cell responses into robust clinical responses.








## INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) with or without donor lymphocyte infusions (DLI) can induce durable remissions due to an allogeneic graft-versus-tumor (GVT) effect.[1] In an HLA-matched setting, this effect is mainly mediated by donor-derived T-cells directed against recipient's minor histocompatibility antigens (mHags) presented on malignant cells.[2] Unfortunately, allo-SCT is frequently associated with severe, sometimes life-threatening graft-versus-host-disease (GVHD). In addition, patients may relapse after achieving an initial remission, underscoring the need for novel strategies to improve the efficacy and specificity of allo-SCT and DLI. There is compelling evidence that the induction of GVT and GVHD crucially depends on the presence of professional antigen presenting cells (APCs), in particular dendritic cells (DCs), capable of presenting host antigens.[3,4] It has been postulated that accomplishing strong and specific GVT effects could be possible through vaccination of allotransplanted patients with DCs, capable of presenting tumor-associated host antigens to donor T cells. We and other investigators previously evaluated host DC vaccinations and tumor-associated antigen loaded DC vaccination after allo-SCT.[5–10] Thus far, these studies revealed that host- or donor-DC vaccination after allo-SCT, combined with DLI, is feasible, safe and can induce relevant tumor-associated immune responses. Encouraged by the initial studies, we evaluated in a multicenter phase I/II trial the feasibility, safety and clinical efficacy of treatment of DLI non-responders with a second equivalent dose of DLI combined with a DC vaccine, which was generated from donor monocytes and pulsed with peptides of hematopoietic-restricted mHags.

## METHODS

### Patients and definitions

The study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, ABR 39604, trial EudraCT number: 2012-002435-28) and conducted according to the Declaration of Helsinki after informed consent of patients and donors.

Candidates for the study were HLA-A2, -A24, -B7 and/or -B44 positive patients at an age of ≥ 18 years, who did not respond to DLI given for relapsed or residual disease after an HLA-matched allo-SCT. Included were patients who showed at least one mismatch with their donors for the hematopoietic-restricted mHags HA-1, HA-2, UTA2-1, LRH-1, ACC-1, ACC-2, PANE-1 and HB-1 in the GVT direction (i.e. patient positive, donor negative for the mHag).[11] Main exclusion criteria were WHO performance 3-4, rapidly progressive disease despite salvage treatment, concomitant use of immunosuppressive drugs and the presence of acute GVHD > grade 1 or extensive chronic GVHD. Maintenance therapy was allowed, but could not be started prospectively after DLI. Patients on maintenance therapy had to have stable disease (i.e. no ongoing response on maintenance) to allow assessment of the response to the study treatment. Included patients received an equivalently dosed subsequent DLI combined with DC vaccination. Patients received a total dose of 45-90 x $10^6$ DCs, administered in three vaccinations with 2-week intervals. In each vaccination, 2/3 of

the DCs were administered intravenously (i.v.) and 1/3 intradermal (i.d.) in the median site of the upper leg. The first DC administration was combined with the DLI. Response assessment was performed at week 14 and total follow-up was until week 20. Response criteria were based on the International Myeloma Working Group (IMWG) uniform response criteria.[12,13] Furthermore, for selected patients plasma cell chimerism was determined in freshly FACS purified bone marrow plasma cells using a previously reported method and used as an additional marker of disease activity.[14] Time to progression (TTP) was defined as time between the start of the first DC vaccination and the occurrence of progression or relapse. Toxicity was assessed using the latest available version of Common Toxicity Criteria (CTC) for Adverse Events (AE; version 4.0). The GVHD-assessment was done using IBMTR criteria, which classifies GVHD according to percentage of involved skin surface, level of serum bilirubin and amount of diarrhoea in ml per day in 4 classes.[15]

### Vaccine generation

The DC vaccine was generated using a 9-day culture protocol under good manufacturing practices (GMP) conditions from $CD14^+$ monocytes enriched from a leukapheresis product of the original stem cell donor. Isolation of $CD14^+$ monocytes was done using a CliniMACS© procedure. After enrichment, DCs were cultured in X-Vivo 15 medium supplemented with 2% Human Serum in the presence of GM-CSF (800IU/mL) and IL-4 (500IU/mL). After three days, fresh medium with GM-CSF and IL-4 was added, together with keyhole limpet hemocyanin (KLH) protein (20µg/mL). At day 7, the DCs were matured *ex vivo* using IL-6 (15ng/mL), IL-1β (5ng/mL), TNFα (600IU/mL) and PGE-2 (1µg/mL). At day 9, the mature DCs were then pulsed with 10µg/mL of the appropriate mHag peptide for 2.5-3 hours, after which the cells were washed. The peptide-loaded, mature DCs were then cryopreserved for quality control testing and release until administration. Cryopreservation had no effect on the antigen presenting capacity of the DCs.[10] Patients received a total dose of 45-90x$10^6$ peptide loaded DCs, administered in three vaccinations with 2-week intervals. A reference vial was thawed and used for quality evaluation determining sterility (endotoxin levels <1.6IU/mL; negative for anaerobe and aerobe bacteria, fungi and yeast), viability (>70% is 7AAD- on flow cytometry or trypan blue negative on light microscopy), purity (<30% expression of CD3 (BD Biosciences), CD14 (Beckman Coulter), CD66e (Sanquin), CD19 (Beckman Coulter) and CD56/16 (BD Biosciences)) and maturity (>70% of the CD11c+ (BD Biosciences) DCs express CD80, CD86, CD83 (all BD Biosciences) and HLA-DR (Biolegend)). Vaccines were manufactured locally at the two participating centers.

### Immune monitoring

Next to mHag-peptides, the DCs were also loaded with the MHC class II restricted protein KLH as an adjuvant to provide $CD4^+$ T-cell help for boosting cytotoxic $CD8^+$ T-cell responses, and to monitor immunological responses after vaccination. PBMC samples obtained from week 0 (baseline), week 1, 2, 4, 6, 8, 10, 14 and week 20 (end of follow-up) after vaccination







were incubated *in vitro* with 5µg/mL of KLH peptide for 5 days. After five days, cells were washed and proliferation was analyzed by staining with Cell Trace™ Violet Cell Proliferation Kit (ThermoFisher) and expressed as % of divided cells/total $CD3^+$ T-cells, as measured on a FACS Canto II (BD Biosciences). Activation was measured by staining the PBMCs for CD25, CD38 and HLA-DR, and depicted as the % of $CD3^+$ T-cells expressing CD25 or co-expressing CD38/HLA-DR (all antibodies from BD Biosciences). In addition, the presence of mHag-specific $CD8^+$ T-cells was determined on the obtained PBMC samples. mHag-specific $CD8^+$ T-cells were identified as viable (SYTOX blue negative, ThermoFisher), $CD45^+$ cells double positive for APC and PE peptide-MHC tetramers within the $CD3^+$ $CD8^+$ population.

## RESULTS

### Patient and vaccine characteristics

Between May 2014 and May 2016, nine MM patients were included in the University Medical Center Utrecht and the Radboud University Medical Center. Because of pre-defined stopping rules, inclusions were stopped after the 9$^{th}$ patient due to limited efficacy. Patients had a median age of 56 years (range 40-66) and were heavily pre-treated with a median number of previous treatment lines of 5 (range: 4-7) (Table 1). Patients were intended to receive a minimal dose of 45x$10^6$ and a maximal of 90x$10^6$ DCs. From all donors, we were able to generate sufficient mHag-loaded, pure and highly mature DCs (Table 2). The lowest number of DCs administered was 64x$10^6$. The vaccine was administered in three courses with 2-week intervals. In each vaccination, 2/3 of the DCs were administered intravenously (i.v.) and 1/3 intradermal (i.d.) in the median site of the upper leg. The first DC administration was combined with a DLI. None of the included patients had rapidly progressive disease requiring salvage treatment before DLI. Patient 9 mistakenly received a DLI dose of 40x$10^6$ instead of 5x$10^6$, which was reported as a protocol violation. This did not lead to any toxicity.

### Induction of peptide-specific T-cells

Analysis of collected PBMC samples showed a rapid induction of anti-KLH responses in all patients after the first vaccination, as shown by *in vitro* proliferation and activation of T-cells upon incubation with KLH (Figure 1A). However, in 7 out of 9 patients, these responses were lower after the second and third vaccination. Analyses of several T-cell subsets, including $CD4^+$, $CD8^+$ and regulatory T-cells did not reveal significant changes after vaccination (Supplementary data). Flow cytometric analyses of PBMCs with mHag peptide-MHC tetramers showed the induction of mHag-specific $CD8^+$ T-cells in 5/9 patients, with the highest frequency in patient 6 (Figure 1B, C). The percentage and time interval of occurrence varied between patients (Figure 1C).

### Clinical response

Response assessment was performed at week 14 and total follow-up was until week 20. No objective clinical responses (≥PR) were observed (Table 1). Median time-to-progression

DENDRITIC CELL VACCINATIONS IN MULTIPLE MYELOMA



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**Table 1.** Patient characteristics and outcome

| N | Sex | Age (y) | Treatment lines | M protein type | Response status before previous DLI | Dose 1st DLI 10E6 | Response status at inclusion | Maintenance | HLA- match | mHag mismatch | DLI dose 10E6 | Total DC dose 10E6 | Best response | TTP months | Last follow up | Minor specific T-cells | Anti-KLH response |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 56 | 6 | IgGκ | VGPR | 10 | VGPR | No | Sibling | LRH-1 | 10 | 75 | SD | 10 | PD | + | + |
| 2 | F | 48 | 4 | IgGκ | CR | 1,5 | CR | No | MUD 10/10 | UTA2-1 | 1,5 | 80 | PD | 2.2 | PD | - | + |
| 3 | F | 66 | 5 | κFLC | PD | 100 | PD | No | Sibling | UTA2-1 | 100 | 80,3 | PD | 2 | PD | + | + |
| 4 | M | 40 | 4 | IgGκ | VGPR | 10 | VGPR | No | MUD 9/10 | HA-1 + LRH-1 | 10 | 90 | SD | 7 | PD | + | + |
| 5 | M | 52 | 5 | IgGκ | PD | 1 | VGPR | No | Sibling | HA-1 | 1 | 76 | SD | 6 | PD | + | + |
| 6 | F | 44 | 7 | IgGκ | PD | 14 | VGPR | Cyclophosphamide/ Bortezomib | Sibling | UTA2-1 | 14 | 83,7 | SD | 7 | SD | + | + |
| 7 | M | 64 | 5 | IgGλ | PD | 10 | PD | No | Sibling | HA-1 + LRH-1 | 10 | 90 | PD | 1 | PD | - | + |
| 8 | M | 66 | 5 | IgGλ | PD | 10 | PD | No | Sibling | UTA2-1 | 10 | 77 | PD | 1.4 | PD | - | + |
| 9 | M | 66 | 5 | IgA | PD | 5 | PR | No | Sibling | UTA2-1 | 40 | 64 | SD | 3.5 | PD | - | + |

The total DC dose was divided into three doses with a 2-week interval, the first vaccination was combined with the DLI. *FLC* free light chains, *VGPR* very good partial response, *CR* complete response, *PD* progressive disease, *PR* partial response, *MUD* matched unrelated donor, *DLI* donor lymphocyte infusion, *TTP* time-to-progression.

(TTP) was 3.5 months (range: 1-10). Three patients were included with progressive disease (patient 3, 7 and 8). All of these patients showed ongoing progression after vaccination. Five patients remained in stable disease for a median of 7 months after vaccination (Pt 1, 4, 5, 6 and 9) (patient 6 still without signs of progression, although on maintenance treatment, patient 1,4,5 and 9 have all progressed). Patient 2, included with minimal residual disease (determined by chimerism analysis on purified plasma cells), received radiotherapy on a bone lesion 2.2 months after inclusion, and achieved a complete molecular remission (mCR) with 100% donor plasma cell chimerism shortly thereafter, and has remained in mCR for 23 months now.

### Toxicity

The vaccinations were well tolerated with only transient induration and erythema at the i.d. injection site and grade 2 fever after the second and third vaccination, lasting less than 24 hours, in all patients. There was no development of GVHD or any life-threatening toxicity. Five severe adverse events (SAE) were reported, four being possibly related to the vaccinations: one admission because of high fever and abdominal pain after the second vaccination, which resolved spontaneously, and three admissions with pneumonia. Other adverse events consisted of diarrhea causing hypophosphatemia, flu-like symptoms, liver enzyme elevation, herpes simplex infection and a basal cell carcinoma of the skin. All adverse events were CTC grade II or III.

## DISCUSSION

This study demonstrates the feasibility, safety and efficacy of mHag-peptide loaded donor DC vaccination combined with DLI in patients with persistent or relapsed disease after allo-SCT and previous DLI. To our knowledge, we are the first to describe the use of donor-derived DCs loaded with mHag peptides as vaccines in combination with DLI.

In the few vaccination studies performed in the allo-SCT setting, DCs were loaded with various antigens using different techniques. The source of DCs (autologous or allogeneic) also differed.[5–9] Thus far, all these studies have shown that the vaccinations were safe and well-tolerated. In none of the studies, except in one of our previous studies with autologous DCs[10], DC vaccination was combined with a second equivalently dosed DLI. We chose this strategy because T cells from the first DLI, as they did not induce any sustainable GVT effect, could have been anergized or dysfunctional, which is often the case in MM patients.[16–20] We did not increase the dose of the DLI with the idea that the effect of the first DLI could serve as a control for the effect of combinatorial DLI+DC vaccination. Several other decisions in the design of this study, such as the dose, administration intervals and administration route (i.v.+ i.d.) were based on our previous experience[10,21] with the intention to keep the variation between this and previous studies minimal. Nevertheless, it could well be that in a different design the outcome of DC vaccination in these patients could have been different. Therefore, the results of this study need to be interpreted within its specific design.

In agreement with our previous results, we observed a low toxicity from mHag peptide pulsed donor DC vaccinations, even after combination with a second DLI, demonstrating the safety of the approach. The observed immunological responses in this trial are also in line

**Table 2.** Generation and characteristics of the vaccine

| | | | | Total mHag loaded DCs | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| N | Purity CD14+ selection | Viability CD14+ | $10^E6$ | Sterility | Viability DCs | % non-DC | CD83 | CD80 | CD86 | HLA-DR | mHag |
| 1 | 99% | 97% | 197 | Sterile | 93% | 0,4% | 94% | 88% | 100% | 100% | LRH-1 |
| 2 | 100% | 97% | 210 | Sterile | 91% | 3,3% | 98% | 91% | 99% | 99% | UTA2-1 |
| 3 | 100% | 98% | 112,5 | Sterile | 78% | 4,8% | 97% | 97% | 99% | 99% | UTA2-1 |
| 4 | 100% | 95% | 140,5 | Sterile | 89% | 1,7% | 96% | 97% | 99% | 99% | HA-1 + LRH-1 |
| 5 | 88% | 96% | 65* | Sterile | 87% | 2,0% | 99% | 99% | 100% | 72% | HA-1 |
| 6 | 100% | 98% | 297 | Sterile | 100% | 0,4% | 99% | 95% | 100% | 100% | UTA2-1 |
| 7 | 87% | 97% | 130 | Sterile | 89% | 1,0% | 98% | 98% | 100% | 82% | HA-1 + LRH-1 |
| 8 | 90% | 97% | 155 | Sterile | 73% | 4,0% | 98% | 97% | 99% | 94% | UTA2-1 |
| 9 | 88% | 92% | 70 | Sterile | 83% | 3,0% | 76% | 98% | 100% | 70% | UTA2-1 |

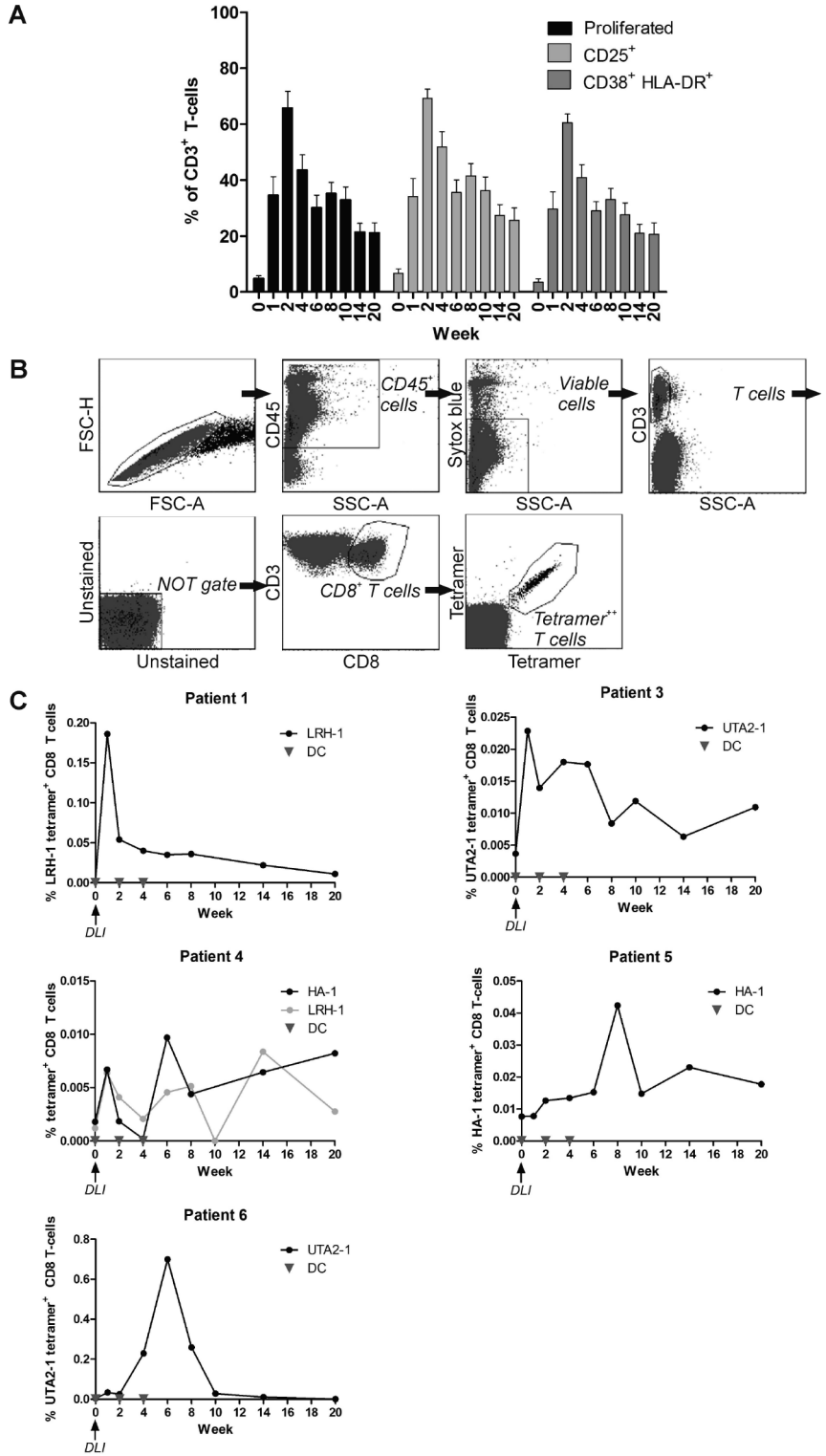
* This number is lower than the sum of separately counted thawed DC products upon administration (Table 1). This discrepancy is probably caused by a counting error before freezing.

**Figure 1.** mHag-loaded donor DC vaccinations induce anti-KLH responses and mHag-specific T cells in peripheral blood. (a) KLH-specific T-cell proliferation and activation are induced by mHag and KLH-loaded donor-derived DC vaccines. Vaccinations were administered at week 0, 2 and 4. DLI was administered at week 0. Shown are pooled data of nine patients, mean±s.e.m. (b) Gating strategy of mHag-reactive CD8 T cells. The presence of mHag-specific T cells was defined as the percentage of viable (SYTOX blue negative, ThermoFisher), $CD45^+$ cells double positive for APC and PE peptide-MHC tetramers within the $CD3^+$ $CD8^+$population. (c) Different patterns and variable levels of induced mHag-specific T cells after vaccination were observed. Vaccinations were administered at weeks 0, 2 and 4. DLI was administered at week 0. Results for five individual patients are shown.









with previous studies and provide evidence for the capacity of mHag-peptide loaded DCs to induce or boost tumor-reactive cytotoxic T-lymphocytes (CTLs). Nonetheless, in virtually all previous studies, not all patients developed CTLs. Although it is not well-understood why some patients do and some do not mount a CTL response, we can exclude DC related factors, because the quality of the DCs was more or less equal in all vaccine products.

Disease characteristics could also be of influence: We observed that in our study 3/4 of the patients who failed to induce CTLs (Pt 7,8 and 9) entered the study with a response status of PR or progressive disease, while such patients represented only 1/5 in the group who did develop CTLs upon vaccination. Although these differences did not reach statistical significance it may be, if possible, better not to include patients with a progressive disease in future trials.

Besides mHag-specific CTL induction, our main goal was to induce clinical responses. In this study, we observed that the developed CTL responses did not translate into robust clinical responses and even the induction of a considerably high frequency of mHag UTA2-1 specific CTLs in one patient (Pt 6) did not translate into a clear clinical response. Although not statistically significant, we noted that 4/5 patients (Pt 1,4,5 and 6) who developed mHag-reactive CTLs after vaccinations had a longer TTP than expected, while the TTP of the patients who did not develop mHag-specific CTLs was much shorter. These observations trigger the discussion why the clinical effects of our induced CTLs were not, or at best were hardly, visible. Among several possibilities, one could speculate on the limited persistence of the induced T cell responses. Are monocyte-derived DCs not capable of inducing stable CTLs? Or are the induced CTLs quickly inactivated?

Although these mechanisms need to be further elucidated in future trials, it seems highly plausible that the expansion or even the cytotoxic function of tumor specific CTLs could be actively suppressed by tumor (microenvironment)-related factors. In this respect, several well-described suppressive cytokines (e.g. TGF-β, IL-10), immune cells (e.g. regulatory T-cells, myeloid-derived suppressor cells) and immune checkpoint molecules, such as PD-L1, could play a role.[22,23] Regarding the latter, we have previously shown that the presence of immune-checkpoint molecules on DCs hampers sufficient priming and boosting of mHag-specific T-cells.[24,25] Importantly, siRNA-mediated silencing of PD-1 ligands on DCs increases the immunogenicity of the vaccine and thereby boosts the induction of mHag-specific T-cell responses.[26,27] A similar scenario is plausible when effector CTLs encounter PD-L1$^+$ tumor cells. Thus, as already suggested by others, the use of systemic immune-checkpoint inhibitors may improve the outcome of DC vaccination, but has to be used with caution in combination with DLI due to the potential risk of eliciting severe GVHD.[28,29]

In addition to these possible future perspectives, our current results point out that patient selection for vaccination strategies is crucial. Patients with a low tumor burden and stable disease are the best candidates for immune-therapy with DCs. But even in these patients, our focus should be on strategies targeting tumor-induced immune suppression, to overcome this important barrier to effective anti-tumor immune therapy.







In conclusion, our findings demonstrate that mHag based donor-derived DC vaccination is safe and well-tolerated when combined with DLI. In addition, this strategy is capable of inducing objective mHag specific T-cell responses. These findings warrant future investigations, focusing on improvement of the vaccination strategy, towards translating the observed T-cell responses into robust clinical responses.

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None.

## DISCLOSURES

None.

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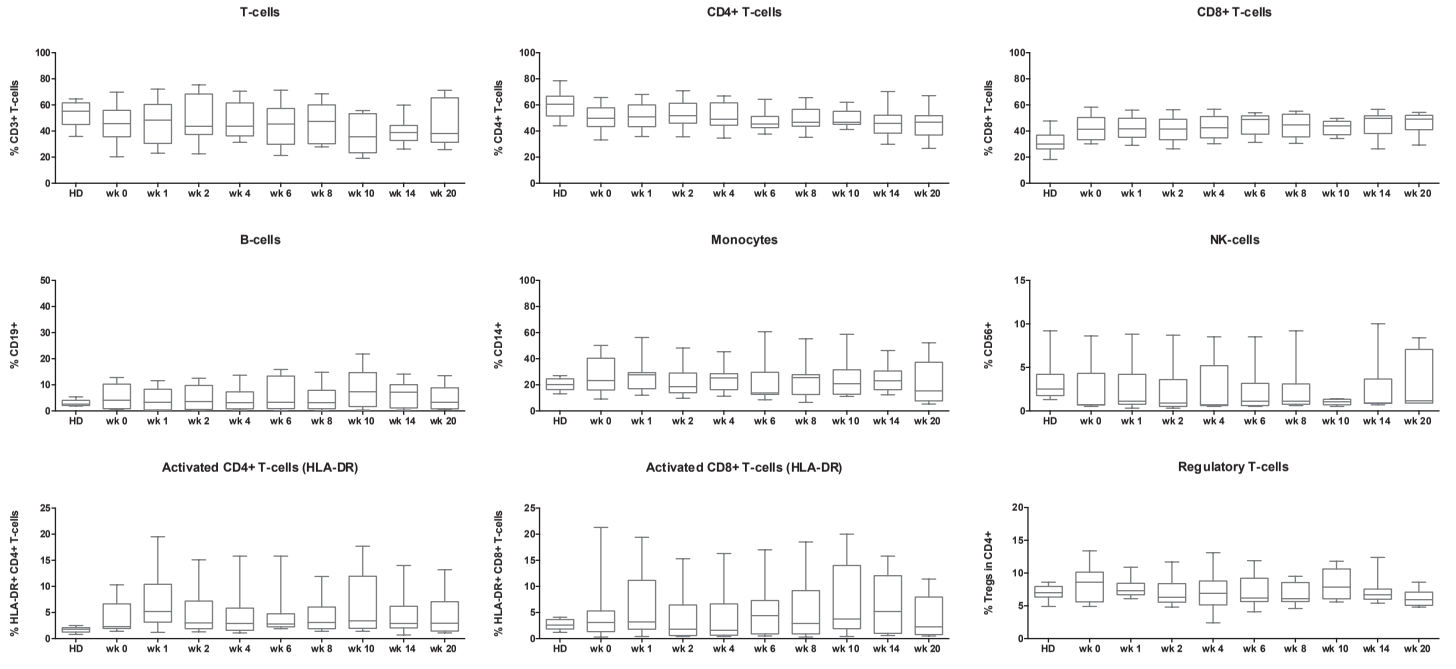


Peripheral blood mononuclear cells fluorescently labeled to stain for CD45 (lymphocytes), CD3 (T-cells), CD4, CD8, CD19 (B-cells), CD14 (monocytes) and CD56 (NK-cells). Activated T-cells were expressed as the percentage of CD4+ or CD8+ T-cells expressing HLA-DR. Regulatory T-cells were identified as CD3+/CD4+/CD25+/CD127-/dim and expressed as percentage of CD4+ T-cells. Dead cells were excluded using LIVE/DEAD fixable dead cell stain. PBMCs of nine healthy donors were also analyzed to compare with baseline levels in patients. Vaccinations were administered at week 0, week 2 and week 4. DLI was administered at week 0. The boxes represent first and third quartile with median value, and error bars represent the 5th- 95th percentile. HD (healthy donors), wk (week).

**Supplementary Figure 1.**

# Chapter 4

## THE IMPACT OF CIRCULATING SUPPRESSOR CELLS IN MULTIPLE MYELOMA PATIENTS ON CLINICAL OUTCOME OF DONOR LYMPHOCYTE INFUSIONS

Laurens E. Franssen[1,2], Niels W.C.J. van de Donk[2], Maarten E. Emmelot[2,3], Mieke W.H. Roeven[4], Nicolaas Schaap[4], Harry Dolstra[5], Willemijn Hobo[5], Henk M. Lokhorst[2] and Tuna Mutis[2,3].

[1] Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands. [2] Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands. [3] Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands. [4] Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands. [5] Department of Laboratory Medicine-Laboratory of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands.

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## ABSTRACT

Allogeneic stem cell transplantation (allo-SCT), followed by donor lymphocyte infusions (DLI) can establish long-term remissions in multiple myeloma (MM) patients. In many patients however, the immunotherapeutic graft-versus-tumor effect (GVT) is moderate and not sustained, implying immune suppression mediated, among other factors, by regulatory T-cells (Tregs) or myeloid derived suppressor cells (MDSCs). Towards a better understanding of, and eventually manipulating the immune regulatory mechanisms in transplanted MM patients, we retrospectively sought a correlation between DLI outcome and circulating $CD14^+$ MDSCs, $CD14^-$ MDSCs and Tregs in 53 MM patients prior to their first DLI. We found significantly elevated frequencies of highly suppressive $CD14^+$ MDSCs, $CD14^-$ MDSCs and Tregs in pre DLI samples of patients. Higher frequencies of Tregs, but not of MDSCs were significantly associated with non-responsiveness to DLI. Furthermore, a lower frequency of Tregs predicted the development of chronic Graft-versus-Host Disease, which, in turn, displayed a high-association with GVT. Elevated Treg frequencies prior to DLI were also associated with significantly shorter progression free- and overall survival. Hence, our data reinforce the idea of active suppression of anti-tumor responses by Tregs in MM patients and therefore suggest that targeting patient Tregs prior to DLI may improve outcome of DLI.








## INTRODUCTION

Multiple myeloma (MM) is characterized by malignant proliferation of clonal plasma cells in the bone marrow. Although the disease is still incurable, allogeneic stem cell transplantation (allo-SCT) followed by donor lymphocyte infusions (DLI) can induce durable responses by virtue of the graft-versus-tumor (GVT) effect in 30-50% of the patients. It is currently unknown why only a fraction of patients benefits from DLI. Furthermore, the success of the therapy is also hampered by the occurrence of graft-versus-host-disease (GVHD).[1–5] This underscores the notion that the improvement of this potentially curative immunotherapy for MM will be critically dependent on the identification and proper modulation of mechanisms that control GVT and GVHD.

Research over the past decades identified several immune cells that may regulate both GVT and GVHD. Among these are the extensively studied $CD4^+CD25^+FOXP3^+CD127^{-/low}$ regulatory T-cells (Tregs), and the more recently discovered myeloid-derived suppressor cells (MDSCs), a heterogeneous population of $CD11b^+CD33^+HLA\text{-}DR^{-/low}$ myeloid cells, with variable expression of CD14, CD15 and other markers.[6]

To date, numerous studies have demonstrated the protective role of Tregs on GVHD.[7–12] However, there is not a clear consensus yet on their impact on GVT. In some murine models, co-infusion of Tregs with bone-marrow transplantation does not impair GVT,[7,13] a poorly understood phenomenon, which was partly confirmed for human Tregs in a xenograft model.[13] However, in other murine models, Tregs not only regulate GVHD but also GVT after DLI.[14] These latter observations gained support from clinical studies, which showed increased Treg frequencies in chronic myeloid leukemia (CML) patients with relapse after allo-SCT[15] and an inverse correlation of Treg frequencies in DLI products with GVT.[16] Finally, the depletion of Tregs resulted in an improved GVT effect, strongly associated with the occurrence of GVHD[17], suggesting, despite the initial thoughts, a detrimental role of Tregs on GVT in the clinical setting.

Like Tregs, murine MDSCs were reported to expand post allo-SCT[18] and their co-infusion or removal from the graft was associated with alleviation or aggravation of GVHD, respectively.[19,20] A recent clinical study described an accumulation of $CD14^+$ MDSCs after allo-SCT, especially during acute GVHD of higher grade.[21] Although MDSCs are present in increased frequencies in MM patients, inhibit T-cell proliferation and promote MM cell growth in vitro[22–24], it is not yet known whether they can influence the outcome of cellular immunotherapy, such as DLI.

To gain more insight into the impact of both Tregs and MDSCs on the clinical outcome of DLI in MM patients, we retrospectively determined the suppressive capacity and frequencies of $CD4^+CD25^+CD127^{-/low}$ Tregs[25,26], and two types of MDSCs, namely the $CD14^+HLA\text{-}DR^{-/low}$ ($CD14^+$ MDSCs) and the $CD11b^+HLA\text{-}DR^{-/low}CD14^-CD33^+$ ($CD14^-$ MDSCs) in 53 MM patients after allo-SCT with persistent or progressive disease, prior to their first DLI, and correlated the findings with DLI outcome. We found significantly elevated frequencies of suppressive $CD14^+$ MDSCs, $CD14^-$ MDSCs and Tregs in pre DLI peripheral blood samples of patients,

compared to healthy controls. Higher frequencies of Tregs, but not of MDSCs were significantly associated with non-responsiveness to DLI, as well as reduced progression free- and overall survival.

## MATERIALS AND METHODS

### Patients and definitions

This retrospective study was conducted in accordance with the Declaration of Helsinki and performed with approval of the Institutional Medical Ethical Review Board. It included the analysis of peripheral blood samples from 53 MM patients who had received allo-SCT, prior to their first DLI, as well as 16 healthy controls. All patients had persistent or progressive disease after allo-SCT. According to local protocol, patients received a dose of $1.10^6$ T-cells/kg to $1.10^7$ T-cells/kg. Three patients received up to $1.10^8$ T-cells/kg. Response to therapy and progression were assessed according to the uniform criteria of the International Myeloma Working Group.[27,28] Response to DLI was defined as at least partial response (PR). Immunosuppressive drugs were stopped at least 1 month before DLI. Five patients received immunomodulatory drugs (lenalidomide 4, bortezomib 1) shortly before or after DLI. All analyses were performed with or without exclusion of these 5 patients with no significant variation in the results. Therefore, throughout the manuscript only the results of the whole cohort are presented.

Overall survival was measured in months and defined from the date of DLI to the date of death or last follow-up. Progression-free survival was defined as the time from DLI to date of progression or death from any cause or last follow-up. Acute and chronic GVHD were defined according to the Seattle criteria and according to Shulman *et al*, respectively, calculated from the time of DLI.[29,30]

### Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen. After thawing, cells were stained with CD11b-PE-Cy7, CD33-APC, CD14-FITC, CD15-PE and HLA-DR-APC-H7 monoclonal antibodies (MAbs) (BD Biosciences, San Jose, CA) to identify MDSC phenotypes. To identify Tregs, cells were stained with CD3-PE-Cy7, CD4-PerCP, CD25-FITC and CD127-PE MAbs (BD Biosciences). Dead cells were excluded using 7-AAD (BD Biosciences). Cells were analyzed on a FACSCanto II Analyzer (BD Biosciences). Frequencies shown are the percentage of suppressive cells within PBMCs (for MDSCs) or within the $CD4^+$ T-cells (for Tregs). The gating strategy for the identification of MDSCs and Tregs is depicted in figure 1.

### *In vitro* suppression assay

To determine the suppressive capacity of MDSCs and Tregs, we performed in vitro suppression assays for 3 patients and 4 healthy controls. $CD4^+CD25^+CD127^{-/low}$ Tregs, $CD11b^+CD14^-HLA\text{-}DR^{-/low}CD33^+$ MDSCs and $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were sorted with a FACSAria III





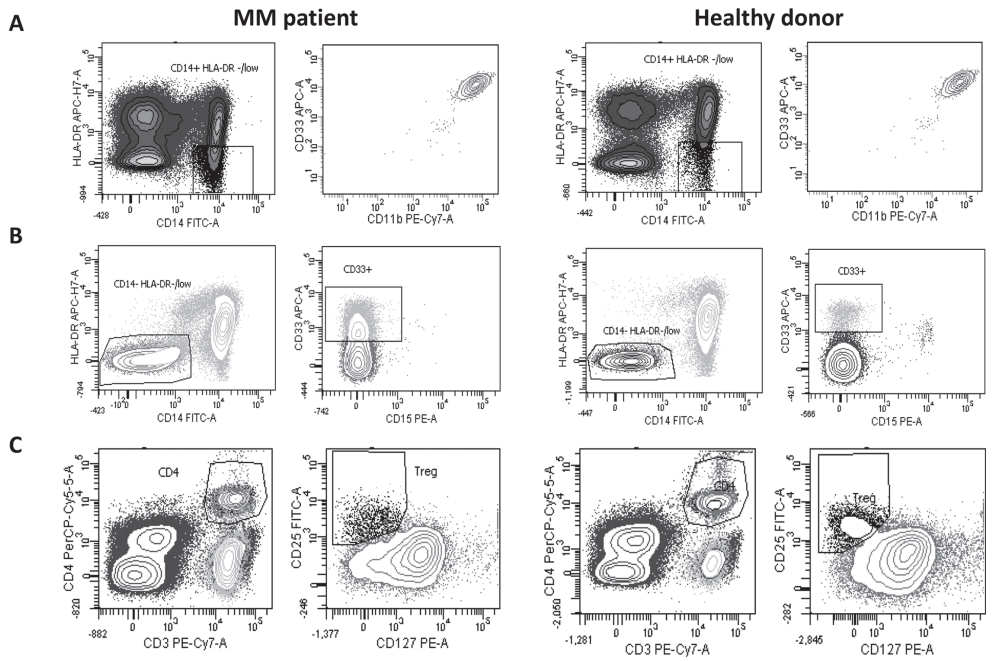



**Figure 1 A-C.** Representative multiparameter flow cytometry plots of MDSCs and Tregs. After excluding $7\text{-}AAD^+$ dead cells, gates were set around $CD14^+HLA\text{-}DR^{-/low}$ MDSCs (**a**), $CD14^-HLA\text{-}DR^{-/low}CD33^+$ MDSCs within the $CD11b^+$ population (**b**) and $CD4^+CD25^+127^{-/low}$ Tregs (**c**) as shown (left: patient; right: healthy donor).

(BD Biosciences) from PBMCs and cocultured with autologous PBMCs (at ratios of 1:1 and 1:0.5) in anti-CD3 (clone OKT3)-coated 96-well plates. Cells were cultured for 4 days in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Integro, Zaandam, the Netherlands) and standard antibiotics (1% penicillin/streptomycin). Proliferation was determined by measuring [$^3$H]-Thymidine incorporation.

### Statistical analysis

Prognostic factors for progression free- and overall survival were analyzed for statistical significance using the Cox proportional hazard regression model. Survival curves were designed using the Kaplan-Meier method, with group comparison by the log-rank test. The independent samples T-test (2-sided) or Mann-Whitney U test were used to determine differences in continuous variables, based on the distribution levels. Differences in categorical variables were determined with the Fisher's exact test for two by two tables and otherwise with the Pearson's $\chi 2$-test. A level of $P < 0.05$ was considered significant. For progression free- and overall survival, variables with $P \leq 0.05$ were entered into multivariate Cox regression analysis. Calculations were performed in SPSS version 20.0.0 (IBM SPSS, Inc.) and GraphPad Prism version 5.03 (GraphPad Software, Inc.).

# RESULTS

### Immune suppressive capacity of MDSCs and Tregs in MM patients prior to DLI

Because various phenotype markers are used to identify Tregs and different types of MDSCs in humans, we first evaluated the in vitro suppressive activity of the two MDSC subsets, which were identified as $CD14^{+}HLA\text{-}DR^{-/low}$ and $CD11b^{+}CD14^{-}HLA\text{-}DR^{-/low}CD33^{+}$ as well as of Tregs, which we defined as $CD4^{+}CD25^{+}CD127^{-/low}$ (Figure 1 A-C). Tregs and MDSCs were isolated from pre DLI samples of MM patients using FACS and cocultured with autologous PBMCs for 4 days in anti-CD3-coated wells at different effector:target ratios. All sorted cell types suppressed the T-cell proliferation induced by CD3 triggering (Figure 1 D). These results confirmed that $CD14^{+}$ MDSCs and $CD14^{-}$ MDSCs as well as Tregs, as defined by their surface markers in our study, represented functionally intact suppressor cell populations in the pre DLI PBMC samples of MM patients.

### Increased frequency of Tregs and MDSCs in MM patients pre DLI

After confirming the immune suppressor capacity of the Tregs and MDSC subsets, we determined the frequencies of these cell subsets in pre DLI samples of the whole cohort of 53 MM patients (blood withdrawal: mean 9.9 days before DLI, range 0 – 150 days) and compared this to healthy donors (n=13 and 16 for Tregs and MDSCs, respectively). No patients used immunosuppressants at the time of sampling and all patients were fully reconstituted. For detailed clinical characteristics of the patients see Table 1. We observed a significantly higher frequency of $CD14^{+}$ MDSCs (mean 4.45 vs. 0.73%, $P < 0.001$), $CD14^{-}$ MDSCs (mean 1.10 vs. 0.61%, $P < 0.01$) and Tregs (mean 11.82 vs. 5.59%, $P < 0.01$) in peripheral blood of MM patients compared to healthy donors (Figure 2). For 16 patients we could also measure the frequencies of these subsets 1-2 months after DLI. We found no significant difference in

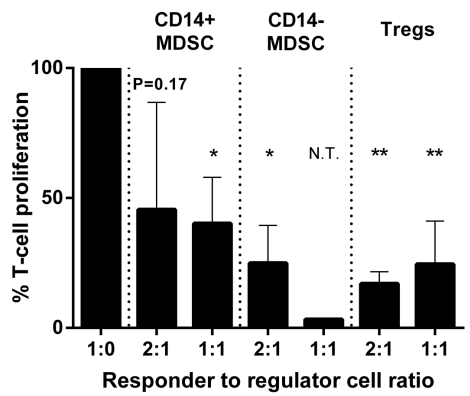



**Figure 1. D.** FACS-sorted patient suppressor cells cultured with autologous PBMCs for 4 days in anti-CD3-coated wells at indicated suppressor to responder ratios. Proliferation was in c.p.m. *$P$<0.05 and **$P$<0.01 in an unpaired $t$-test as compared with first column (no regulatory cells). Nt, statistical test was not possible as there were <2 samples. Mean±s.e.m.







frequencies before and after DLI (not shown). Remarkably, not all patients showed elevated levels of these suppressor cells. This suggested that, whatever the reason, the increase in Tregs and MDSCs in the peripheral blood of these extensively pretreated MM patients, months after allo-SCT, could affect DLI outcome.

### High frequency of Tregs is associated with a low response to DLI

To assess the possible impact of these circulating MDSCs and Tregs on DLI outcome, we first correlated their frequencies with response to DLI. We found a significantly higher level of Tregs (mean 13.73 vs. 8.67%, $P = 0.03$, table 2) in patients who did not respond to DLI, compared to responding patients. Levels of $CD14^{-}$ and $CD14^{+}$ MDSCs were not different between DLI-responders and non-responders (Figure 3, table 2). This indicated that high levels of Tregs, but not MDSCs, were significantly associated with non-response to DLI.

**Table 1.** Clinical characteristics.

| | **No. of patients** |
|---|---|
| **Age (years)** | |
| Median | 55 |
| Range | 36-68 |
| **Sex** | |
| Male | 40 |
| Female | 13 |
| **Conditioning** | |
| NMA | 45 |
| MA | 8 |
| **T-cell depletion in conditioning** | |
| ATG | 14 |
| Alemtuzumab | 13 |
| None | 26 |
| **Stem cell source** | |
| Sib | 30 |
| MUD | 23 |
| **Allo-SCT as part of first line treatment** | |
| First line | 21 |
| Not first line | 32 |
| **Time from Tx to DLI (months)** | |
| Median | 11.6 |
| Range | 3-107 |
| **DLI reason** | |
| Persistent disease | 17 |
| Progressive/relapse | 36 |

NMA = non-myeloablative; ATG = anti-thymocyte globulin; TBI = total body irradiation; Sib = sibling donor; MUD = matched unrelated donor; Tx = transplantation.

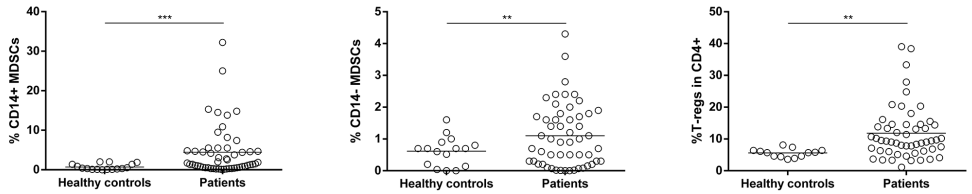



**Figure 2.** Increased frequency of Tregs and MDSCs in MM patients pre-DLI. Percentages of $CD14^+$ MDSCs (left) and $CD14^-$ MDSCs (middle) in viable PBMCs and Tregs (right) in $CD4^+$ cells of MM patients after allo-SCT, pre-DLI compared with healthy donors. *$P$<0.05, **$P$<0.01 and ***$P$<0.001.

**Table 2.** Predictive factors for response to DLI, univariate analysis.

| Characteristic | Total no. of patients | No. of patients with response ≥PR(%) | P |
|---|---|---|---|
| **Age** | | | |
| <55 | 24 | 11 (46) | 0.27 |
| ≥55 | 29 | 9 (31) | |
| **Sex** | | | |
| Male | 40 | 15 (38) | 0.95 |
| Female | 13 | 5 (38) | |
| **Acute GVHD after DLI** | | | |
| Grade 0-1 | 44 | 13 (30) | <0.01 |
| Grade 2-4 | 9 | 7 (78) | |
| **Chronic GVHD after DLI** | | | |
| Yes | 12 | 10 (83) | <0.01 |
| No | 41 | 10 (24) | |
| **Stem cell source** | | | |
| MUD | 23 | 9 (39) | 0.85 |
| Sib | 30 | 11 (37) | |
| **Reinduction chemotherapy** | | | |
| Yes | 37 | 12 (32) | 0.23 |
| No | 16 | 8 (50) | |
| **Response to reinduction** | | | |
| Yes | 21 | 8 (38) | 0.31 |
| No | 10 | 2 (20) | |
| **Disease status pre DLI** | | | |
| ≥VGPR | 19 | 7 (37) | 0.92 |
| PR, SD or progression | 34 | 13 (38) | |
| **Progressive disease pre DLI** | | | |
| Yes | 11 | 5 (45) | 0.55 |
| No | 42 | 15 (36) | |
| **DLI dose** | | | |
| ≥$1.10^7$/kg | 21 | 9 (43) | 0.60 |
| <$1.10^7$/kg | 31 | 11 (35) | |





**Table 2.** (continued)

| Characteristic | Total no. of patients | No. of patients with response ≥PR(%) | P |
|---|---|---|---|
| **Time between Tx and DLI (months)** | | | |
| <6 | 5 | 2 (40) | 0.62 |
| 6-12 | 23 | 7 (30) | |
| >12 | 25 | 11 (44) | |
| **Conditioning** | | | |
| NMA | 45 | 16 (36) | 0.44 |
| MA | 8 | 4 (50) | |
| **ATG/Alemtuzumab** | | | |
| Yes | 27 | 11 (55) | 0.78 |
| No | 26 | 9 (45) | |
| **Allo-SCT part of first line treatment** | | | |
| Yes | 21 | 8 (38) | 0.97 |
| No | 32 | 12 (38) | |
| **Characteristic** | **Mean (%)** | **SEM** | **P** |
| **$CD14^+$ MDSCs** | | | |
| Responders | 5.200 | 1.734 | 0.51 |
| Non-responders | 3.990 | 0.944 | |
| **$CD14^-$ MDSCs** | | | |
| Responders | 0.853 | 0.183 | 0.15 |
| Non-responders | 1.254 | 0.185 | |
| **Tregs** | | | |
| Responders | 8.665 | 0.857 | 0.03 |
| Non-responders | 13.733 | 1.710 | |

MUD = matched unrelated donor; Sib = sibling donor; Tx = transplantation; NMA = non-myeloablative; ATG = anti-thymocyte globulin. Percentages are quantitated as percentage of viable cells for MDSCs and of $CD4^+$ T-cells for Tregs.

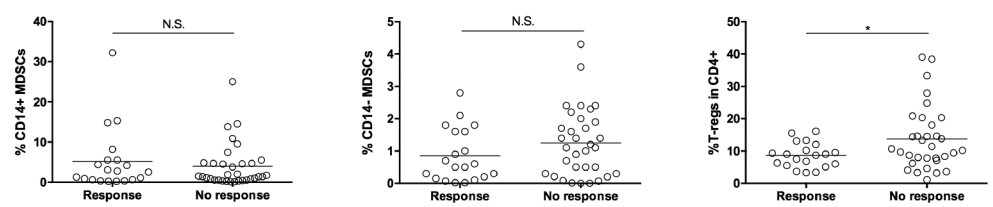



**Figure 3.** High frequencies of Tregs are associated with a low response to DLI. Percentages of $CD14^+$ MDSCs (left) and $CD14^-$ MDSCs (middle) in viable PBMCs and Tregs (right) in $CD4^+$ cells of MM patients after allo-SCT, pre-DLI. Responders to DLI (≥ PR) compared with non-responders. *$P$<0.05.

**Low Treg levels pre DLI are associated with the occurrence of chronic GVHD**

Among several other possible predictive factors analyzed for response to DLI, only acute and chronic GVHD showed a significant association with response (Table 2). Response to DLI was 78% in patients with aGVHD and 83% in patients with cGVHD, compared to only 30% of patients without aGVHD and 24% of patients without cGVHD ($P < 0.01$). Since suppressor cells may also influence GVHD, we also analyzed the correlation between the frequencies of Tregs, MDSCs and GVHD occurrence. Treg levels pre DLI were significantly higher in patients without cGVHD after DLI (mean 13.08 vs. 7.52%, $P = 0.03$, Figure 4). MDSCs did not show an association with cGVHD. Levels of suppressor cells prior to DLI showed no association with aGVHD. Other possible predictive factors for GVHD in this cohort were also tested (Supplementary Table 1), among these, only a higher dose of CD3+ cells was associated with increased chronic GVHD.

**The frequency of Tregs pre DLI is related to the OS and PFS in MM patients**

Finally, we assessed whether the levels of MDSC subsets and Tregs prior to DLI could predict progression free survival (PFS) and overall survival (OS). In univariate Cox analysis, higher frequencies of Tregs, but not of MDSCs prior to DLI were significantly associated with a reduced PFS ($P = 0.01$) and OS ($P = 0.001$) (Supplementary Table 2). In a Kaplan Meier analysis, patients with Treg levels in the highest quartile (>14.6% Tregs in $CD4^+$ T-cells) had a significantly decreased PFS (P<0.001; Figure 5A) and OS (P<0.01; Figure 5B) as compared to patients with lower levels. Median PFS was 4.2 months and the median OS was 25.5 months in the highest quartile versus a median PFS of 12.4 months and OS of 67 months in patients with lower levels of Tregs. After including other predictive factors for PFS and OS (see supplementary Table 1) in multivariate Cox regression analysis, we found a trend for decreased PFS in patients with high numbers of Tregs. Response to DLI and the remission status prior to DLI (≥VGPR vs. rest) were independent factors influencing PFS (Table 3;

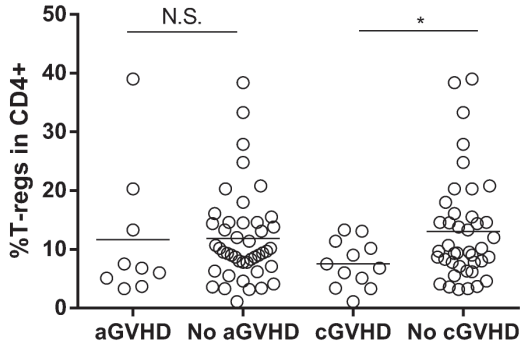


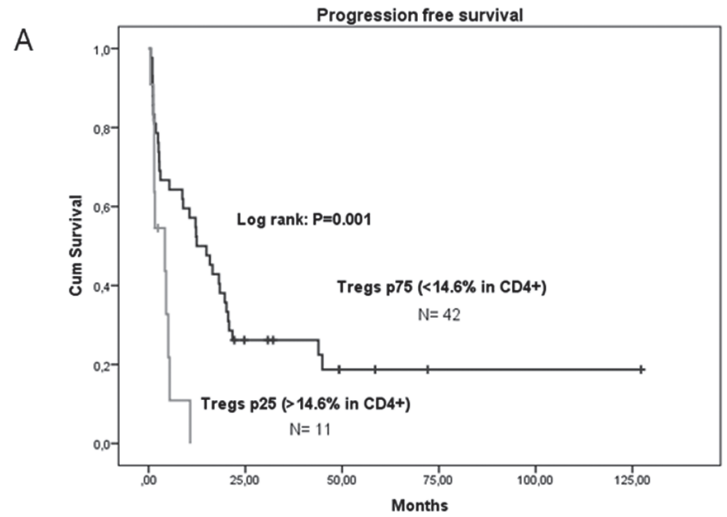

**Figure 4.** Low Treg levels pre-DLI are associated with the occurrence of chronic GVHD. Percentages of Tregs in $CD4^+$ cells in PBMCs of MM patients after allo-SCT, pre-DLI. Patients with GVHD after DLI compared with patients without GVHD after DLI. Acute and chronic GVHD were analyzed. *$P$<0.05.







$P = 0.004$ and $P = 0.003$ respectively). In the multivariate Cox regression analysis for OS, Treg levels ($P = 0.02$) and application of reinduction chemotherapy were independent predictors for OS (Table 3). In addition, for OS there was a trend towards significance for time from transplantation to DLI. These data show that in patients with persistent or progressive disease after allo-SCT, response to DLI is associated with a longer PFS. Furthermore, high Treg levels prior to DLI have a negative effect on OS, independent of response to DLI.



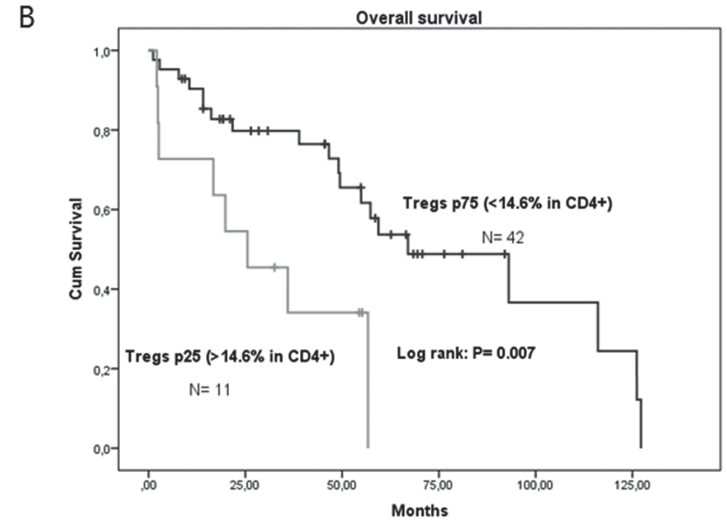




**Figure 5.** Kaplan–Meier survival curves: survival for patients with Treg levels in the highest quartile (>14.6% Tregs in $CD4^+$ T cells) compared with patients with lower levels (14.6% of $CD4^+$ cells). (a) PFS and (b) OS.

**Table 3.** Predictive factors for PFS and OS: multivariate analysis.

| | Factor | HR | 95% CI | P |
|---|---|---|---|---|
| PFS | Tregs pre DLI | 1.036 | 0.994 – 1.080 | 0.097 |
| | Response to DLI (≥PR) | 0.361 | 0.180 – 0.722 | 0.004 |
| | Remission status pre DLI (≥VGPR) | 0.327 | 0.157 – 0.679 | 0.003 |
| OS | Application of reinduction chemotherapy | 5.191 | 1.683 – 16.010 | 0.004 |
| | Tregs pre DLI | 1.067 | 1.010 – 1.127 | 0.021 |
| | Time Tx to DLI | 0.959 | 0.917 – 1.002 | 0.064 |

Factors with a $P \leq 0.05$ in univariate Cox analysis were included. Abbreviations: HR = hazard ratio; Tx = transplantation; CI = confidence interval; MUD = matched unrelated donor.

## DISCUSSION

In this study we show that circulating MDSCs and Tregs are found at increased frequencies in a subset of MM patients with persistent or progressive disease, months after allo-SCT. The increased levels of Tregs, but not of MDSCs, are associated with an impaired response to DLI, a lower incidence of cGVHD, as well as a decreased PFS and OS.

A major aim of this work was to gain insight into the possible modulation of therapeutic immune responses by MDSCs in MM patients. Despite being encountered at elevated levels in these patients, it is currently not well documented whether this suppressor cell subset can indeed modulate immune responses in MM. We also readdressed the impact of Tregs on the immunotherapeutic anti-tumor effect, as this is a frequently debated issue, with conflicting reports. We chose to address our questions in the DLI setting, because DLI is an important therapeutic modality for MM, and, as compared to the direct post allo-SCT setting, provides a better platform to evaluate the therapeutic impact of suppressor cells without being biased by GVHD prophylaxis, post-transplant homeostatic lymphocyte expansion and a post-transplant inflammatory state. Consistent with our goal, we correlated DLI outcome with the frequency of cell subsets present in patients prior to DLI. In this respect, our study differs from several others, which correlated the DLI/SCT outcome with suppressor cell levels present in the infused cell products.[10,11,16,31,32] Therefore, our findings point to a novel clinical strategy to improve the clinical outcome of DLI (see below).

With regard to MDSCs, we should note that due to the retrospective character of our study, we have not been able to analyze all previously described MDSC subsets. Especially the use of cryopreserved PBMCs, which are largely devoid of $CD15^+$ cells, forced us to discriminate the $CD33^+CD11b^+HLA\text{-}DR^{-/low}$ MDSCs only on the basis of their CD14 expression. Despite these limitations, consistent with recent reports[22,23] we found significantly elevated levels of CD14+ and CD14- MDSCs also in transplanted MM patients, prior to DLI. We also confirmed their functional competence (suppressor capacity) in these patients prior to DLI (Figure 1D). Thus it seems conceivable that the expansion of these suppressor cells is driven by persistent MM. However, since the possible causes of their elevated levels were







not a specific focus of our study, we did not further analyze this issue. Our focus was to analyze their clinical relevance. Despite earlier suggestions, however, we found no evidence for this: there was no association between MDSCs and DLI outcome. Yet, to be definitive, it is appropriate to study the impact of $CD15^+CD14^-$ granulocyte-like MDSCs as well, but in a prospective study, using freshly isolated PBMCs.

Regarding Tregs, contradictory results have been reported in various settings. While some, especially murine studies, found that Tregs do not hamper GVT responses[7,8,13], others demonstrated the opposite.[14–17] Some discrepancies between mouse and human studies may be related to species-specific aspects. We suspect however that the discrepancies could also be due to the fact that murine models do not take into account the heterogeneity seen in the human setting. Underscoring this, we have found a considerable level of heterogeneity in the Treg levels in pre DLI samples of MM patients. Remarkably, and confirming the results of several clinical studies[16,17], Treg levels in pre DLI samples were clearly associated with DLI responsiveness, which in turn was a good predictor of PFS. Importantly, we also found that high levels of Tregs were associated with impaired OS. It has previously been shown that Tregs modulate OS in newly diagnosed MM patients.[33] In that report, higher percentages of Tregs were also encountered in patients who died of infectious complications, suggesting that the modulation of OS by Tregs can be multifactorial. Also in our study, possible tumor growth promoting properties together with increased incidence of infectious complications could have been contributing to the decreased OS in patients who displayed higher levels of Tregs in the circulation, already before DLI.

Taken together, our findings showing that high pre DLI levels of Tregs are associated with DLI non-response and decreased OS, may have important clinical implications. First of all, our results suggest that GVHD prevention by Treg co-infusions in the DLI setting is not a favorable idea, due to the potential impairment of the GVT effect. Most importantly, our results suggest a careful monitoring of Treg levels prior to DLI, with the intention to apply a pre DLI Treg depletion strategy. Since the levels of Tregs in some patients are much higher than in healthy donors, we think that this could be a more effective strategy to improve DLI responses compared to Treg depletion from the DLI products. For example, the administration of a continuous, low dose of cyclophosphamide has been shown to selectively deplete Tregs and has also been combined with cellular immunotherapy.[34–37] Administration of continuous, low dose cyclophosphamide in those patients with elevated Treg levels compared to normal individuals prior to DLI could improve its antitumor effect. Furthermore, since low pre DLI levels of Tregs do not seem to increase aGVHD incidence in our cohort, it may be possible that this in vivo Treg depletion strategy may not induce the early morbidity and mortality associated with aGVHD.

In conclusion, our analyses point to, and warrant the clinical testing of a novel pre DLI Treg depletion strategy towards improvement of the DLI outcome.

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## DISCLOSURES

None.





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# SUPPLEMENTAL DATA

**Supplementary Table 1.** Predictive factors for GVHD, univariate analyses.

| Characteristic | Total no. of patients | No. of patients with aGVHD | P | No. of patients with cGVHD | P |
|---|---|---|---|---|---|
| **Age** | | | | | |
| <55 | 24 | 8 (33) | 0.01 | 6 (25) | 0.75 |
| ≥55 | 29 | 1 (3) | | 6 (21) | |
| **Male patient- female donor** | | | | | |
| Yes | 7 | 1 (14) | 1.00 | 4 (57) | 0.06 |
| No | 36 | 8 (22) | | 7 (19) | |
| **Stem cell source** | | | | | |
| MUD | 23 | 6 (26) | 0.15 | 4 (17) | 0.52 |
| Sib | 30 | 3 (10) | | 8 (27) | |
| **Reinduction chemotherapy** | | | | | |
| Yes | 37 | 7 (19) | 0.32 | 9 (24) | 0.32 |
| No | 16 | 2 (13) | | 3 (19) | |
| **DLI dose** | | | | | |
| ≥$1.10^7$/kg | 21 | 5 (24) | 0.46 | 8 (38) | 0.05 |
| <$1.10^7$/kg | 31 | 4 (13) | | 4 (13) | |
| **Conditioning** | | | | | |
| NMA | 45 | 9 (20) | 0.32 | 10 (22) | 1.00 |
| MA | 8 | 0 (0) | | 2 (25) | |
| **ATG/Alemtuzumab** | | | | | |
| Yes | 27 | 7 (26) | 0.14 | 6 (22) | 1.00 |
| No | 26 | 2 (8) | | 6 (23) | |
| **Allo-SCT part of first line treatment** | | | | | |
| Yes | 21 | 2 (10) | 0.29 | 5 (24) | |
| No | 32 | 7 (22) | | 7 (22) | 1.00 |
| **Characteristic** | **Mean (SEM)** | | **P** | **Mean (SEM)** | **P** |
| **$CD14^+$ MDSCs** | | | | | |
| GVHD | 4.289% (2.64) | | 0.94 | 5.542 (2.71) | 0.50 |
| No GVHD | 4.479% (0.92) | | | 4.127 (0.83) | |
| **$CD14^-$ MDSCs** | | | | | |
| GVHD | 1.124% (0.34) | | 0.94 | 1.153 (0.28) | 0.84 |
| No GVHD | 1.098% (0.15) | | | 1.088 (0.16) | |
| **Tregs** | | | | | |
| GVHD | 11.667% (3.86) | | 0.95 | 7.517 (1.15) | 0.04 |
| No GVHD | 11.852% (1.18) | | | 13.081 (1.40) | |
| **Time Tx to DLI (months)** | | | | | |
| GVHD | 15.074 (3.27) | | 0.56 | 16.733 (3.33) | 0.73 |
| No GVHD | 19.001 (2.93) | | | 18.802 (3.08) | |

MUD = matched unrelated donor; Sib = sibling donor; Tx = transplantation; NMA = non-myeloablative; ATG = anti-thymocyte globulin. Percentages are quantitated as percentage of viable PBMCs for MDSCs and of $CD4^+$ T-cells for Tregs.







**Supplementary Table 2.** Predictive factors for PFS and OS, univariate analysis.

| | PFS | | | OS | | |
|---|---|---|---|---|---|---|
| **Characteristic** | **HR** | **95% CI** | **P** | **HR** | **95% CI** | **P** |
| Age | 1.027 | 0.989 – 1.067 | 0.160 | 1.045 | 0.997 – 1.094 | 0.066 |
| aGVHD after DLI | 0.761 | 0.338 – 1.715 | 0.510 | 0.805 | 0.240 – 2.703 | 0.726 |
| cGVHD after DLI | 0.493 | 0.235 – 1.035 | 0.062 | 0.523 | 0.179 – 1.529 | 0.236 |
| Stem cell source (MUD) | 0.730 | 0.388 – 1.374 | 0.329 | 2.105 | 0.909 – 4.871 | 0.082 |
| Reinduction chemotherapy | 2.077 | 1.025 – 4.205 | 0.042 | 3.686 | 1.259 – 10.795 | 0.017 |
| Response to reinduction | 0.558 | 0.243 – 1.283 | 0.170 | 0.887 | 0.310 – 2.532 | 0.822 |
| Progression pre DLI | 1.435 | 0.687 – 3.000 | 0.337 | 0.655 | 0.240 – 1.787 | 0.409 |
| DLI dose ≥$1.10^7$ | 1.053 | 0.571 – 1.941 | 0.869 | 0.553 | 0.230 – 1.328 | 0.185 |
| Remission status pre DLI ≥VGPR | 0.420 | 0.213 – 0.828 | 0.012 | 0.786 | 0.341 – 1.810 | 0.571 |
| Response to DLI ≥PR | 0.399 | 0.208 – 0.765 | 0.006 | 0.467 | 0.197 – 1.111 | 0.085 |
| Response to DLI ≥VGPR | 0.245 | 0.118 – 0.510 | 0.001 | 0.360 | 0.136 – 0.953 | 0.040 |
| Time between Tx and DLI | 1.002 | 0.983 – 1.020 | 0.853 | 0.965 | 0.933 – 0.999 | 0.047 |
| $CD14^+$ MDSCs pre DLI | 1.015 | 0.969 – 1.064 | 0.530 | 1.003 | 0.928 – 1.085 | 0.937 |
| $CD14^-$ MDSCs pre DLI | 1.098 | 0.792 – 1.521 | 0.576 | 0.906 | 0.587 – 1.397 | 0.654 |
| Tregs pre DLI | 1.057 | 1.012 – 1.104 | 0.012 | 1.096 | 1.040 – 1.156 | 0.001 |

HR = hazard ratio; CI = confidence interval; Tx = transplantation; MUD = matched unrelated donor.

# Part 2

## IMMUNOMODULATORY ANTI-MM THERAPY

# Chapter 5

## PHASE 1/2 STUDY OF LENALIDOMIDE COMBINED WITH LOW-DOSE CYCLOPHOSPHAMIDE AND PREDNISONE IN LENALIDOMIDE-REFRACTORY MULTIPLE MYELOMA

Inger S. Nijhof[1,2], Laurens E. Franssen[1,2], Mark-David Levin[3], Gerard M.J. Bos[4], Annemiek Broijl[5], Saskia K. Klein[6], Harry R. Koene[7], Andries C. Bloem[8], Aart Beeker[9], Laura M. Faber[10], Ellen van der Spek[11], Paula F. Ypma[12], Reinier Raymakers[2], Dick-Johan van Spronsen[13], Peter E. Westerweel[3], Rimke Oostvogels[2], Jeroen van Velzen[8], Berris van Kessel[1], Tuna Mutis[1], Pieter Sonneveld[5], Sonja Zweegman[1], Henk M. Lokhorst[1], and Niels W.C.J. van de Donk[1]

[1] Hematology, VU University Medical Center, Amsterdam, The Netherlands. [2] Hematology, University Medical Center Utrecht Cancer Center, Utrecht, The Netherlands. [3] Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands. [4] Hematology, Maastricht University Medical Center, Maastricht, The Netherlands. [5] Hematology, Erasmus Medical Center, Rotterdam, The Netherlands. [6] Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands. [7] Hematology, St. Antonius Hospital, Nieuwegein, The Netherlands. [8] Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands. [9] Internal Medicine, Spaarne Hospital, Hoofddorp, The Netherlands. [10] Internal Medicine, Rode Kruis Hospital, Beverwijk, The Netherlands. [11] Internal Medicine, Rijnstate Hospital, Arnhem, The Netherlands. [12] Internal Medicine, HagaZiekenhuis, Den Haag, The Netherlands. [13] Hematology, Radboud University Medical Center, Nijmegen, The Netherlands.

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## ABSTRACT

The prognosis of multiple myeloma (MM) patients who become refractory to lenalidomide and bortezomib is very poor, indicating the need for new therapeutic strategies for these patients. Next to the development of new drugs, the strategy of combining agents with synergistic activity may also result in clinical benefit for patients with advanced myeloma. We have previously shown in a retrospective analysis that lenalidomide combined with continuous low-dose cyclophosphamide and prednisone (REP) had remarkable activity in heavily pretreated, lenalidomide-refractory MM patients. To evaluate this combination prospectively, we initiated a phase 1/2 study to determine the optimal dose and to assess its efficacy and safety in lenalidomide-refractory MM patients. The maximum tolerated dose (MTD) was defined as 25 mg lenalidomide (days 1-21/28 days), combined with continuous cyclophosphamide (50 mg/day) and prednisone (20 mg/day). At the MTD (*n*=67 patients), the overall response rate was 67%, and at least minimal response was achieved in 83% of the patients. Median progression-free survival and overall survival were 12.1 and 29.0 months, respectively. Similar results were achieved in the subset of patients with lenalidomide- and bortezomib-refractory disease as well as in patients with high-risk cytogenetic abnormalities, defined as t(4;14), t(14;16), del(17p), and/or ampl(1q) as assessed by FISH. Neutropenia (22%) and thrombocytopenia (22%) were the most common grade 3-4 hematologic adverse events. Infections (21%) were the most common grade 3-5 non-hematologic adverse events. In conclusion, the addition of continuous low-dose oral cyclophosphamide to lenalidomide and prednisone offers a new therapeutic perspective for multidrug refractory MM patients. This trial was registered at www.clinicaltrials.gov as #NCT01352338.








## INTRODUCTION

The introduction of immunomodulatory drugs (IMiDs), such as thalidomide and lenalidomide, and the proteasome inhibitor bortezomib, has considerably improved survival of multiple myeloma (MM) patients.[1;2] However, the prognosis of patients who become refractory to lenalidomide and bortezomib is very poor, with a median event-free survival of 5 months and overall survival (OS) of 9 months.[3] This clearly demonstrates that there is an urgent need for effective, well tolerated therapies for this category of patients.

In this respect, several new anti-myeloma agents have shown activity, including next generation IMiDs (pomalidomide) and proteasome inhibitors (carfilzomib), but also compounds with different mechanisms of action such as histone deacetylase inhibitors, kinesin spindle protein inhibitors, and monoclonal antibodies.[4] Next to the development of new drugs, the strategy of combining drugs with synergistic activity may also result in significant clinical benefit for patients with advanced myeloma. Importantly, several large randomized studies comparing lenalidomide-dexamethasone with or without a new agent (carfilzomib, ixazomib, elotuzumab, or daratumumab) have recently shown improved response rates and prolonged PFS in favour of the triplet regimens in MM patients who had received 1-3 prior treatments.[5-8] However, patients with lenalidomide-refractory disease were not eligible to participate in these clinical trials. In addition, patients with bortezomib-refractory disease were excluded from the studies evaluating the combination of lenalidomide-dexamethasone with ixazomib[7] or carfilzomib.[8]

Lenalidomide has multiple effects in MM, including direct anti-tumor activity, inhibition of adhesion of MM cells to stromal cells, and suppression of angiogenesis.[9] IMiDs also stimulate anti-tumor response of the immune system through promotion of T cell co-stimulation and increase in natural killer (NK) cell numbers and activation status.[10-12] Similarly, administration of cyclophosphamide, at a dose substantially lower than the maximum tolerated dose (MTD) (metronomic dosing),[13] has next to its direct anti-tumor activity several effects on the bone marrow microenvironment including immune stimulatory activity.[14-22] We hypothesized that the addition of low-dose metronomic oral cyclophosphamide to lenalidomide may be an attractive strategy for lenalidomide-refractory MM patients. Indeed, we previously showed in a small retrospective study that lenalidomide (Revlimid®) combined with continuous low-dose oral cyclophosphamide (Endoxan®) and prednisone (REP) has remarkable activity in heavily pretreated, lenalidomide-refractory MM patients.[23] To assess the optimal dose of this combination and to further evaluate the safety and efficacy of this combination, we initiated a prospective phase 1/2 study in lenalidomide-refractory MM patients.

Here we report the MTD, as well as safety and efficacy data from the phase 1/2 REPEAT-study.

## MATERIALS AND METHODS

### Study design

This study was a prospective, investigator-initiated, non-randomized, multicenter, open-label, phase 1 dose-finding trial (5 dose levels as indicated in Supplemental Table 1), followed by a phase 2 expansion at the recommended dose level (RDL) to evaluate the safety, tolerability, and efficacy of lenalidomide combined with continuous orally-dosed cyclophosphamide and prednisone (REP) in lenalidomide-refractory MM patients (REPEAT-study). This study enrolled a total of 82 patients (21 in phase 1 and 61 in phase 2) from August 2011 to November 2014. The dose escalation phase 1 study determined the MTD and RDL of lenalidomide combined with cyclophosphamide and prednisone. The MTD was the RDL for the patients treated in the phase 2 part of the REPEAT-study. This trial was conducted in 10 hospitals in the Netherlands. The REPEAT-study was approved by the institutional medical ethical committee in each participating center in accordance with the declaration of Helsinki. All participants provided written informed consent. The trial was registered at www.clinicaltrials.gov as #NCT01352338.

### Study objectives

The primary objective of the phase 1 study was to identify the MTD and RDL of lenalidomide in combination with cyclophosphamide and prednisone in patients with lenalidomide-refractory MM. The other primary objective of the study was to evaluate the overall response rate (ORR; ≥ partial response (PR)) of REP in patients treated at the MTD. Secondary objectives of the study were to evaluate the clinical benefit rate (≥ minimal response (MR)), to evaluate the safety of the combination, and to assess progression-free survival (PFS) and OS of patients treated at the MTD.

### Study population

Patients were eligible to participate in the study if they had lenalidomide-refractory MM following at least 1 prior therapy. Lenalidomide-refractory MM was defined as progressive disease during therapy, no response (less than PR) to prior lenalidomide-containing therapy, or within 60 days of discontinuation from lenalidomide-containing regimens, according to the International Myeloma Working Group criteria.[3] Patients were required to have measurable disease, defined by conventional criteria, as any of the following: (i) serum monoclonal protein ≥ 10 g/L, (ii) urine M-protein ≥200 mg/24 h, (iii) or serum immunoglobulin free light chain ≥ 100 mg/L and abnormal serum immunoglobulin kappa to lambda free light chain ratio. Furthermore, a WHO performance status of 0-3, a platelet count of ≥75 x $10^9$/L, an absolute neutrophil count (ANC) of ≥1.0 x $10^9$/L, and serum hepatic aminotransferases and bilirubin levels <3-fold the upper limit of normal were required. Patients were required to have an estimated creatinine clearance of ≥ 50 ml/min (Cockcroft-Gault calculation) in phase 1 and ≥ 30 ml/min in phase 2. Patients had to agree to use contraception in this







trial. Exclusion criteria included clinically relevant active comorbid medical or psychiatric conditions, or a history of malignancy within the last 5 years.

### Drug administration

All drugs were orally administered. Lenalidomide was used on days 1-21 of a 28-day cycle, and cyclophosphamide and prednisone were given continuously. REP therapy was given until progression of disease. All patients received thrombosis prophylaxis, consisting of daily aspirin (80 mg), or in patients with a prior history of venous thromboembolism (VTE), low-molecular-weight heparin. As infection prophylaxis, patients received cotrimoxazole (480 mg once daily). Patients in dose level 5 of the phase 1 study also received pegylated granulocyte colony-stimulating factor (PEG-G-CSF) on day 1 of each REP-cycle (supplemental Table 1).

### Dose Limiting Toxicity Assessment

See supplemental data.

### Dose modification

See supplemental data.

### Safety and efficacy assessments

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.03)[24]. Only adverse events of CTC grade 2 or higher were assessed during each cycle. Safety assessments were done throughout the study, from inclusion until 30 days after the administration of the last dose of any study drug. Treatment response was assessed at the end of each cycle according to the International Myeloma Working Group Uniform Response Criteria[25], with minimal response defined according to European Society for Blood and Marrow Transplantation criteria[26;27].

Response was also separately evaluated in patients with high-risk cytogenetic abnormalities as defined by the presence of t(4;14), t(14;16), del(17p) and/or ampl(1q) as determined by fluorescence in situ hybridization (FISH) on purified MM cells before start of REP-treatment. Similarly, response was also assessed in the subset of patients with bortezomib- and lenalidomide-double refractory MM.

### Statistics

The phase 2 part was designed to determine whether treatment with REP at the MTD warranted further investigation in clinical trials. In order to reject the null hypothesis (overall response rate (ORR): 15%) in favour of the alternative hypothesis (ORR: 30%) with power $1 - \beta = 0.80$ (2-sided significance level $\alpha = 0.05$), 53 eligible patients were required. However, in order to overcome dropout, 60 patients were included in the phase 2 part of the trial.

PFS was calculated from day 1 of treatment until progression or death, whichever came first. OS was measured from day 1 of treatment until death from any cause. Patients still alive

at the date of last contact were censored. PFS and OS were estimated using the Kaplan-Meier method. Differences between survival curves were tested for statistical significance using the 2-sided log-rank test. Predictive factors for response were determined with the Fisher's exact test in case of categorical variables and with the Mann-Whitney u-test for continuous variables. Univariate Cox regression was used to determine prognostic factors for OS and PFS. Unless otherwise specified, the analyses included either the 21 patients treated in phase 1, or the 67 patients treated at the MTD (dose level 4 phase 1 (n=6) and phase 2 (n=61)). All statistical analyses were performed using SPSS software (version 21.0) or Graphpad version 6.0. Data were monitored by an external contract research organization (Julius Clinical).

# RESULTS

## Patient characteristics

A total of 82 patients were enrolled in this phase 1/2 study. Twenty-one patients were enrolled in the phase 1 study, and 61 in the phase 2 study. Patient characteristics are shown in Table 1. The median age was 66 years (range, 41-82 years). Median number of prior therapies was 3 (range, 1-10 treatments). All 82 patients had lenalidomide-refractory disease: 66 of these patients (80%) had progressive disease during lenalidomide-based therapy, 11 patients (14%) had progression within 60 days after stopping lenalidomide-containing therapy, and 5 patients (6%) had no response to lenalidomide-containing therapy (see Table 1 for additional details on type of lenalidomide-containing therapy). Seventy-one patients (87%) were also exposed to bortezomib, including 54 (66%) with bortezomib-refractory disease. Autologous stem cell transplantation was previously applied in 50 patients (61%), and allogeneic stem cell transplantation in 8 (10%). Median time from diagnosis to study entry was 48 months (range, 5-169 months). FISH analysis on purified MM cells was performed before start of REP in 62 of 82 patients (76%); 32 of these 62 patients (52%) were classified as high-risk (presence of t(4;14), t(14;16), del(17p), and/or ampl(1q)).

## Maximum tolerated dose

We first evaluated the combination of lenalidomide, cyclophosphamide, and prednisone in the dose-finding, phase 1 part of the study, in which 21 lenalidomide-refractory patients were treated at 5 different dose levels (supplemental Table 1). The MTD was determined to be dose level 4 with 25 mg lenalidomide on days 1-21, combined with continuous cyclophosphamide (50 mg/day) and prednisone (20 mg/day). Details on the phase 1 part of the study are given in the supplemental data (including supplemental Tables 2 and 3).

## Safety of REP at the MTD

Sixty-one additional patients were subsequently treated at the MTD in the phase 2 part of the study to further assess the safety and activity profile of REP. In the safety analysis we also included the 6 patients treated at dose level 4 (MTD dose level) in the phase 1 part of the study (total of 67 patients).







**Table 1.** Patient characteristics.

| Characteristic | Phase 1 n=21 | Phase 2 n=61 | Total n=82 |
|---|---|---|---|
| Median age, years (range) | 69 (41-76) | 65 (43-82) | 66 (41-82) |
| **Sex, male, *n* (%)** | 16 (76%) | 42 (69%) | 58 (71%) |
| **Type of monoclonal heavy chain** | | | |
| IgG, *n* (%) | 11 (52%) | 32 (52%) | 43 (52%) |
| IgA, *n* (%) | 6 (29%) | 8 (13%) | 14 (17%) |
| IgD, *n* (%) | 0 (0%) | 1 (2%) | 1 (1%) |
| Light chain only, *n* (%) | 4 (19%) | 20 (33%) | 24 (29%) |
| **Type of light chain** | | | |
| Kappa, *n* (%) | 15 (71%) | 39 (64%) | 54 (66%) |
| Lambda, *n* (%) | 6 (29%) | 22 (36%) | 28 (34%) |
| **Median time from diagnosis till enrollment in months (range)** | 41 (18-96) | 51 (5-169) | 48 (5-169) |
| **Prior lines of therapy, median (range)** | 3 (2-10) | 3 (1-6) | 3 (1-10) |
| **Prior therapies** | | | |
| Lenalidomide | 21 (100%) | 67 (100%) | 82 (100%) |
| Bortezomib | 19 (90%) | 52 (85%) | 71 (87%) |
| Thalidomide | 16 (76%) | 36 (59%) | 52 (63%) |
| Melphalan | 21 (100%) | 58 (95%) | 79 (98%) |
| Cyclophosphamide | 10 (48%) | 37 (61%) | 47 (57%) |
| Prior autologous stem cell transplantation, *n* (%) | 13 (62%) | 37 (61%) | 50 (61%) |
| Prior allogeneic stem cell transplantation, *n* (%) | 3 (14%) | 5 (8%) | 8 (10%) |
| **Previous lenalidomide#** | | | |
| Refractory* | 21 (100%) | 61 (100%) | 82 (100%) |
| Progression while on lenalidomide-containing therapy[1] | 19 (90%) | 47 (77%) | 66 (80%) |
| No response during prior lenalidomide-based therapy[2] | 1 (5%) | 4 (7%) | 5 (6%) |
| Progressive disease within 60 days after stopping lenalidomide-based therapy[3] | 1 (5%) | 10 (16%) | 11 (14%) |
| **Lenalidomide and bortezomib double refractory*** | 16 (76%) | 38 (62%) | 54 (66%) |
| **International Staging System before start REP, *n* (%)** | | | |
| 1 | 7 (33%) | 15 (27%) | 22 (29%) |
| 2 | 9 (43%) | 25 (46%) | 34 (45%) |
| 3 | 5 (24%) | 15 (27%) | 20 (26%) |
| **WHO Performance Status, *n* (%)** | | | |
| 0 | 0 (0%) | 10 (17%) | 10 (12%) |
| 1 | 15 (71%) | 33 (56%) | 48 (60%) |
| 2 | 4 (19%) | 11 (19%) | 15 (19%) |
| 3 | 2 (10%) | 5 (8%) | 7 (9%) |
| **Beta 2-microglobulin median, nmol/L (range)** | 3.4 (1.7-10) | 3.4 (0.2-19.1) | 3.4 (0.2-19.1) |

**Table 1.** (continued)

| Characteristic | Phase 1 n=21 | Phase 2 n=61 | Total n=82 |
|---|---|---|---|
| **Laboratory values at baseline, median (range)** | | | |
| Absolute neutrophil count, x10$^9$/L | 3.2 (1.2-20.5) | 2.6 (1.1-7.9) | 2.6 (1.1-20.5) |
| Haemoglobin, mM | 6.6 (5.3-9.2) | 6.9 (4.5-9.1) | 6.9 (4.5-9.2) |
| Platelet count, x10$^9$/L | 183 (95-334) | 164 (50-369) | 167 (50-369) |
| Creatinine, µmol/L | 86 (58-117) | 86 (53-201) | 86 (53-201) |
| Calcium, mmol/L | 2.35 (2.15-2.64) | 2.31 (1.98-3.35) | 2.31 (1.98-3.35) |
| **Cytogenetic abnormalities** | | | |
| High-risk** | 10 (48%) | 22 (36%) | 32 (39) |
| Standard-risk | 10 (48%) | 20 (33%) | 30 (37%) |
| Not available | 1 (4%) | 19 (31%) | 20 (24) |

MM: multiple myeloma, *n*: number, FISH: fluorescence in situ hybridisation.

*Refractory disease is defined as progressive disease during therapy, no response (less than partial response), or progressive disease within 60 days of stopping treatment, according to the International Uniform Response Criteria for Multiple Myeloma.

**High-risk cytogenetic abnormalities were defined by the presence of t(4;14), t(14;16), del(17p) and/or ampl(1q) as determined by FISH analysis on purified MM cells before start of REP treatment. FISH analysis on purified MM cells was performed before start of REP in 62 of 82 patients.

#PFS for last lenalidomide-containing regimen was 11.2 months (median of 2 prior therapies). PFS was 11.1 months when last lenalidomide-containing regimen was lenalidomide-dexamethasone (median of 2 prior therapies).

[1]Fifty patients progressed while receiving lenalidomide (25 mg)-dexamethasone, 6 while receiving RVD (lenalidomide, bortezomib, and dexamethasone), and 10 while receiving lenalidomide maintenance therapy (10 mg).

[2]Three patients received lenalidomide (25 mg)-dexamethasone, one patient received 10 mg lenalidomide in MPR (melphalan, prednisone, lenalidomide), one patient received lenalidomide (10 mg) maintenance therapy.

[3]Ten patients received lenalidomide (25 mg)-dexamethasone, one patient received 10 mg lenalidomide in MPR.

All 67 patients could be evaluated for hematologic and non-hematologic adverse events, which were assessed from start of REP treatment until 1 month after stopping therapy. The most frequent adverse events in patients treated at the MTD were hematologic toxicities with grade 3 neutropenia in 13 patients (19%) and grade 4 in 2 patients (3%). These patients were successfully treated with granulocyte colony-stimulating factor in subsequent cycles without further interruptions of therapy. Grade 3 and 4 anemia occurred in 3 (4%) and 0 (0%) patients, respectively. Grade 3 and 4 thrombocytopenia occurred in 10 (15%) and 5 (7%) patients, respectively. These toxicities were well managed with dose delays or dose reductions. The most common non-hematologic toxicities were infections: 18% of the patients experienced a grade 3 infection during REP-treatment (mostly upper and lower respiratory tract infections), and 2 (3%) patients succumbed to pneumonia with septic shock. Cardiac disorders developed in 5 patients: 1 patient had grade 3 angina pectoris caused by anemia, which recovered completely after blood transfusion; 1 patient experienced palpitations caused by self-limiting unexplained ventricular arrhythmia; and 3 patients experienced heart failure (grade 3 in 2 patients and grade 4 in 1 patient). Two of these 3 patients with heart failure had a history of cardiac disease. Grade 3 venous







thromboembolism was reported in 3 patients: 2 patients with pulmonary embolism, despite low-molecular-weight heparin administered because of a history of previous pulmonary embolism, and 1 patient had a deep venous thrombosis, despite prophylactic therapy with aspirin. Treatment-emergent peripheral neuropathy was uncommon, with 4 patients experiencing grade 2 peripheral neuropathy. None of the patients developed a second primary malignancy (SPM). During the course of the study, toxicity led to at least one level of dose reduction for lenalidomide in 11 patients (16%), while there were no dose reductions for cyclophosphamide or prednisone. Eight patients (12%) discontinued therapy because of adverse events.

### Efficacy of REP at the MTD

Sixty-six of 67 patients treated at the MTD were evaluable for response; in 1 patient no response evaluation was performed during 2 courses of REP, after which treatment was stopped because of grade 3 fatigue, without signs of progression. Patients received a median

**Table 2.** Adverse events for patients treated at the MTD (dose level 4 of phase 1 and phase 2).

| | N=67 | | | | |
|---|---|---|---|---|---|
| **Events** | **Grade 2 N (%)** | **Grade 3 N (%)** | **Grade 4 N (%)** | **Grade 5 N (%)** | **Total N (%)** |
| **Hematologic** | | | | | |
| Neutropenia | 8 (12) | 13 (19) | 2 (3) | - | 23 (34) |
| Thrombocytopenia | 10 (15) | 10 (15) | 5 (7) | - | 25 (37) |
| Anemia | 4 (6) | 3 (4) | - | - | 7 (10) |
| **Non-hematologic** | | | | | |
| Thromboembolism | - | 3 (4) | - | - | 3 (4) |
| Constitutional | 21 (31) | 2 (3) | - | - | 23 (34) |
| Fatigue | 9 (13) | 1 (1) | - | - | 10 (15) |
| Muscle cramps | 12 (18) | 1 (1) | - | - | 13 (19) |
| Neurologic | | | | | |
| Sensory neuropathy | 4 (6) | - | - | - | 4 (6) |
| Dysesthesia | - | 1 (1) | - | - | 1 (1) |
| Infections | 25 (37) | 12 (18) | - | 2 (3) | 39 (58) |
| Upper respiratory | 12 (18) | 4 (7) | - | - | 16 (24) |
| Pneumonia | 2 (3) | 6 (9) | - | 2 (3) | 10 (15) |
| Gastro-intestinal | 5 (7) | 1 (1) | | | 6 (9) |
| Herpes zoster | 2 (3) | | - | - | 2 (3) |
| Other | 4 (7) | 1 (1) | - | - | 5 (8) |
| Cardiac disorders | - | 4 (7) | 1 (1) | - | 5 (7) |
| Congestive heart failure | - | 2 (3) | 1 (1) | - | 3 (4) |
| Arrhythmia | - | 1 (1) | - | - | 1 (1) |
| Angina pectoris | - | 1 (1) | - | - | 1 (1) |

of 9 REP-cycles (range 1-30+ cycles). The ORR (≥PR) was 67% (44 patients), including at least very good partial response (VGPR) in 15 patients (23%). Three patients achieved complete remission (CR) (5%), including 1 stringent CR (sCR). Eleven patients (16%) achieved an MR, translating to an overall 83% clinical benefit rate (≥MR). At least stable disease (SD) was achieved in 60 patients (91%) (Table 3). Median time to at least PR was 1.7 months (range, 0.5-22.8 months). Response to REP was better in 25%, similar in 44%, and inferior in 31% of patients, when compared with the preceding lenalidomide-containing regimen. Two of the 3 patients who achieved (s)CR during REP had also obtained CR in the preceding lenalidomide-containing regimen, whereas the other patient had a VGPR.



After a median follow-up of 24.5 months (range 1.1-33.9+), the median PFS was 12.1 months and the median OS was 29.0 months (Figure 1). Patients who reached ≥PR (median PFS: 15.6 months) or ≥VGPR (median PFS: not reached) had a significantly better PFS than those with responses less than PR (median PFS: 3.7 months). OS was also better in patients with PR (median OS: 29.0 months) or VGPR (median OS: 30.9 months) as compared to patients with less than PR (median OS: 11.9 months), but this did not reach statistical significance.

Median PFS for the preceding lenalidomide-containing regiment was 11.2 months (median of 2 prior therapies). Supplemental Table 4 shows the median PFS for patients treated with REP and with the preceding lenalidomide-containing regimen for several subgroups.

**Prognostic factors for response, PFS, and OS**

Forty-seven patients, treated at the MTD, were evaluated for cytogenetic abnormalities by FISH. Twenty-four of these patients (51%) had high-risk cytogenetic abnormalities. Response in these patients was similar to that observed in standard-risk patients (Table 3). Furthermore, PFS and OS did not differ between patients with high-risk and standard-risk as defined by FISH (median PFS: 12.1 vs 12.3 months, $P = 0.943$; median OS: 22.3 vs 29.0 months, $P = 0.982$

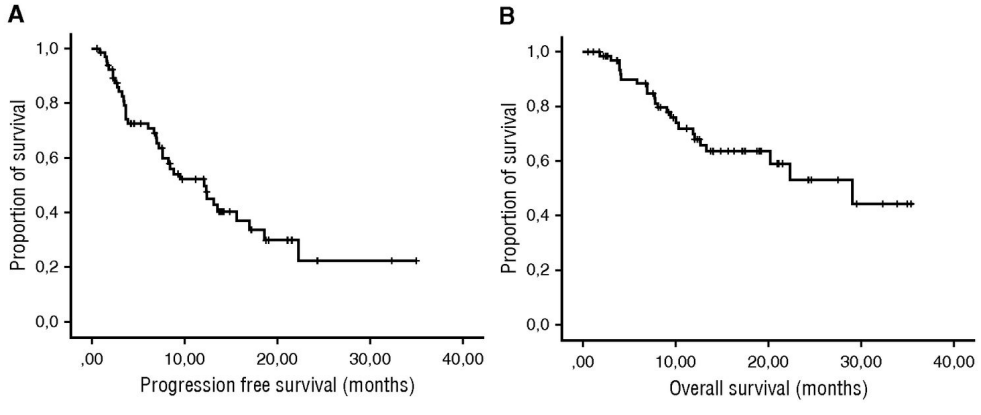


**Figure 1.** PFS and OS for patients treated at the MTD. (A) PFS and (B) OS of the 67 lenalidomide-refractory MM patients treated with REP at the MTD.





for high-risk and standard-risk patients respectively) (Figure 2A,B). Forty-two patients of the 67 patients treated at the MTD (64%), had disease refractory to both lenalidomide and bortezomib. Also in this subgroup response, PFS, and OS were not statistically different, as compared to patients who were not bortezomib refractory (Figure 2C,D). In addition, patients (n=46, 67%) who received REP directly after development of lenalidomide-refractory disease had similar response and survival, when compared to patients who received REP after one or more other lines of therapy (Table 3; Figure 2E,F).



WHO performance status before start of REP-treatment was the only variable, which was significantly associated with response (Table 4). Patients with WHO performance status of 2 or 3 had a significantly lower response rate than patients with performance status of 0 or 1. There were no differences in extent of dose-reduction of study medication between these 2 groups.

We also performed univariate Cox regression analysis to determine prognostic factors for PFS and OS. The only variable significantly associated with impaired PFS was an elevated pre-treatment β2-microglobulin level. High pretreatment LDH levels were significantly associated with reduced OS, while there was a trend towards impaired PFS. All other factors tested were not associated with PFS and OS (Table 4; Figure 2G,H).

## DISCUSSION

In this phase 1/2 trial, we evaluated the MTD, as well as the safety and efficacy of lenalidomide combined with low-dose cyclophosphamide and prednisone (REP) in heavily pretreated, lenalidomide-refractory MM patients (66% of the patients were also refractory to bortezomib). The MTD was determined to be 25 mg of lenalidomide on days 1-21 of a 28-day cycle, combined with continuous oral cyclophosphamide at a dose of 50 mg, and prednisone at a dose of 20 mg. The REP regimen was well tolerated and highly active with an ORR (≥PR) in 67% and a clinical benefit rate (≥MR) in 82%. The median PFS was 12.1 months and the median OS 29.0 months.

Hematologic toxicities in our study were acceptable and consistent with the observed toxicities in MM patients treated with lenalidomide-dexamethasone.[28;29] Similarly, when cyclophosphamide was added to pomalidomide and prednisone (PCP)[30] hematologic toxicity was comparable to the toxicity observed with pomalidomide-dexamethasone.[31;32] Altogether this suggests that low-dose cyclophosphamide does not significantly increase hematologic toxicity, when it is added to lenalidomide or pomalidomide. In contrast, melphalan has more profound myelosuppression, making this alkylating agent less attractive to use in combination with lenalidomide.[33;34] Non-hematologic toxicity of REP consisted mainly of infections. Discontinuations because of adverse events were uncommon, allowing patients to continue therapy until disease progression. No SPMs were observed in this study. Indeed, several other studies have demonstrated that lenalidomide combined with cyclophosphamide is associated with a markedly lower risk of SPM, when compared to lenalidomide plus melphalan.[35;36]

**Table 3.** Response of patients treated at the MTD (dose level 4 of phase 1 and phase 2).

| | **All patients (all len-refractory)** *n*=66 | **Len- and bor-refractory patients** *n*=42 | **Patients with high-risk cytogenetic abnormalities*** *n*=24 | **Patients treated with REP, directly following development of len-refractory disease (25 mg len or equivalent in case of renal insufficiency)** n=46 |
|---|---|---|---|---|
| sCR | 1.5% | 0% | 0% | 0% |
| CR | 3.0% | 2.4% | 0% | 0% |
| VGPR | 18.2% | 21.4% | 20.8% | 15.2% |
| PR | 44.0% | 36.1% | 45.9% | 50.0% |
| MR | 16.6% | 21.1% | 16.6% | 17.4% |
| SD | 7.6% | 9.5% | 4.2% | 10.9% |
| PD | 9.1% | 9.5% | 12,5% | 6.5% |
| ≥ VGPR | 22.7% | 23.8% | 20.8% | 15.2% |
| ≥ PR | 66.7% | 59.9% | 66.7% | 65.2% |
| ≥ MR | 83.3% | 81.0% | 83.3% | 82.6% |

Len, lenalidomide; bor, bortezomib; MM, multiple myeloma; FISH, fluorescence in situ hybridization; *n*, number; sCR, stringent complete response; CR, complete response; VGPR, very good partial response; PR, partial response; MR, minimal response; SD, stable disease; PD, progressive disease.*High-risk disease was defined by the presence of t(4;14), t(14;16), del(17p), and/or ampl(1q) as determined by FISH on purified MM cells before start of REP treatment.







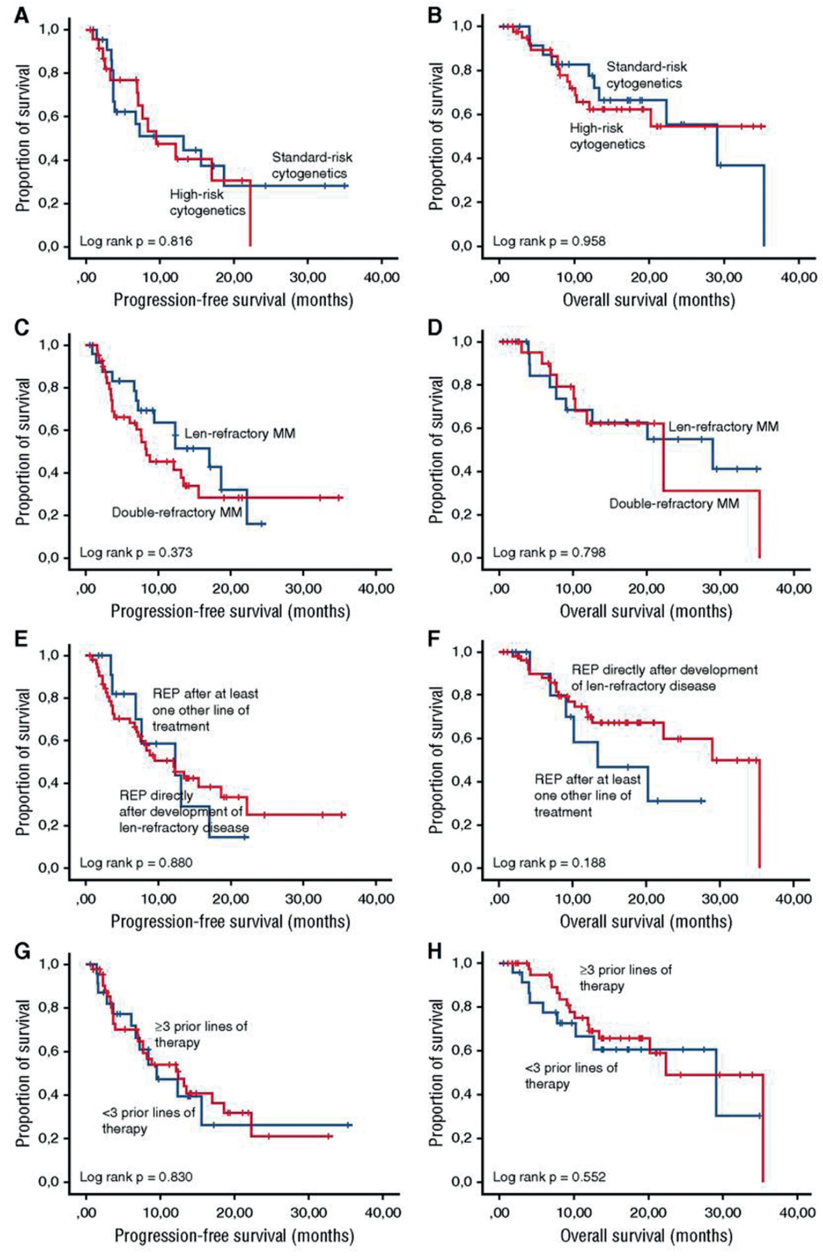


**Figure 2.** PFS and OS for patients treated at the MTD for different subgroups. (A) PFS and (B) OS for patients treated with REP at the MTD with high-risk disease (presence of t(4;14), t(14;16), del(17p), and/or ampl(1q) as determined by FISH) vs standard-risk disease. (C) PFS and (D) OS for patients treated with REP at the MTD with disease refractory to both lenalidomide and bortezomib (double-refractory MM) vs disease refractory to only lenalidomide. (E) PFS and (F) OS for patients treated with REP at the MTD with REP directly given after development of lenalidomide-refractory disease vs REP given after at least 1 other line of therapy after the development of lenalidomide-refractory disease. (G) PFS and (H) OS for patients treated with REP at the MTD with <3 vs ≥3 prior lines of therapy.

**Table 4.** Univariate analysis of possible predictive factors for PFS and OS.

| | ORR | PFS | | OS | |
|---|---|---|---|---|---|
| | P | HR (95% CI) | P | HR (95% CI) | |
| >65 years | .486 | .901 | 1.024 ( 0.545-1.993) | .810 | 1.105 (0.486-2.510) |
| Male | .705 | .315 | 1.415 (0.719-2.784) | .786 | 1.127 (0.476-2.669) |
| ≥ 3 lines of therapy | .144 | .830 | 1.079 (0.539-2.157) | .553 | 1.289 (0.557-2.981) |
| ≥ 4 lines of therapy | .855 | .651 | 0.859 (0.446-1.657) | .214 | 0.595 (0.262-1.351) |
| Creatinine clearance (≥50 ml/min) | .320 | .410 | 0.643 (0.225-1.837) | .418 | 2.296 (0.307-17.163) |
| Thrombocytes | .217 | .911 | 1.0 (0.995-1.005) | .209 | 1.004 (0.998-1.009) |
| Beta2-microgobulin | .697 | .050 | 1.122 (1.000-1.259) | .369 | 1.058 (0.935-1.197) |
| Albumin | .361 | .253 | 1.001 (0.999-1.003) | .147 | 1.002 (0.999-1.005) |
| LDH | .525 | .081 | 1.002 (1.000-1.004) | .001 | 1.004 (1.001-1.006) |
| High risk cytogenetics | .705 | .816 | 0.912 (0.420-1.981) | .958 | 0.974 (0.367-2.584) |
| WHO 0+1 vs 2+3 | .010 | .867 | 0.932 (0.405-2.141) | .238 | 0.567 (0.222-1.453) |
| Len-refractory vs len- and bor- refractory disease | .103 | .376 | 0.736 (0.374-1.450) | .798 | 0.896 (0.386-2.078) |
| Full-dose lenalidomide (25 mg or equivalent in case of renal insufficiency) before start REP-therapy | 1.00 | .497 | 1.299 (0.610-2.765) | .950 | 1.031 (0.402-2.643) |
| REP directly after development of len-refractory disease | .861 | .951 | 1.02 (0.532-1.957) | .785 | 1.122 (0.491-2.568) |

CI, confidence interval; HR, hazard ratio; LDH, lactate dehydrogenase.







We observed high activity of REP despite enrolling patients who were all lenalidomide-refractory and 66% also bortezomib-refractory. Although the importance of high-risk cytogenetic features in advanced relapsed/refractory MM has not been clearly defined, we observed similar response and survival in patients with high-risk cytogenetic abnormalities when compared to standard-risk patients. Outcome was also similar in patients with lenalidomide- and bortezomib, double-refractory, MM. Notably, we observed a median PFS of 14.3 months and median OS not yet reached in double-refractory MM patients, which compares favorably with historical controls of patients who were refractory to both IMiDs and bortezomib, who had a median event-free survival of 5 months and median OS of 9 months.[3] However, response was inferior in patients with WHO performance status score of 2 or 3, but this did not translate into reduced PFS or OS. In addition, in our study, elevated beta2-microglobulin was predictive for impaired PFS, while high LDH levels were associated with shorter OS. These subgroups involve relatively small numbers of patients and further analysis is needed to assess the impact of these variables on outcome with REP.

We previously showed that the two-drug combination of continuous low-dose cyclophosphamide and prednisone has also significant anti-MM activity in relapsed/refractory MM patients, who were not previously exposed to novel agents.[37] However, another study showed that low-dose cyclophosphamide (50 mg daily) combined with steroids has markedly lower activity in lenalidomide- and bortezomib-exposed patients (63% of these patients were double-refractory to bortezomib and IMiDs), with at least PR in 11.4% of these patients and a median PFS and OS of only 3.3 months and 10.0 months respectively.[38] This outcome is inferior to that observed with the REP regimen in double-refractory MM patients, and suggests clinical synergy between lenalidomide and low-dose cyclophosphamide.

The combination of lenalidomide, cyclophosphamide and steroid has also been studied in newly diagnosed MM.[35;39] Kumar et al demonstrated high efficacy of the 3-drug combination with weekly cyclophosphamide (≥PR: 85%).[39] However, lenalidomide combined with cyclophosphamide and prednisone was not superior to lenalidomide-dexamethasone in a phase 3 clinical trial with elderly newly diagnosed MM patients.[35] This may be explained in part by the low dose of lenalidomide (10 mg, days 1-21/28-day cycle) and cyclophosphamide (50 mg every other day), which were increased after protocol amendment.[35]

Other studies have also demonstrated a beneficial effect of addition of weekly cyclophosphamide to lenalidomide and corticosteroids in patients with relapsed/refractory lenalidomide-naive MM (≥PR: 65-94%).[40-42] Because of the high response and prolonged PFS reported in these studies, directly starting with the 3-drug regimen of lenalidomide, cyclophosphamide and corticosteroid may also be considered, as opposed to adding cyclophosphamide at the time of development of lenalidomide-refractory disease. Furthermore, a retrospective analysis showed high efficacy (≥PR: 68%) and good tolerability of lenalidomide, low-dose cyclophosphamide, and prednisone in relapsed/refractory MM patients who were previously exposed to lenalidomide-dexamethasone (39% lenalidomide refractory).[43] Similarly, it has recently been shown that addition of cyclophosphamide to pomalidomide and dexamethasone in lenalidomide-refractory MM increases the overall

response rate from 39% to 65% and median PFS from 4.4 to 9.5 months.[44] Larocca et al also showed that pomalidomide in combination with cyclophosphamide-prednisone is effective and well tolerated in lenalidomide- and bortezomib-refractory MM patients (≥PR: 50%; median PFS 8.6 months).[30] However, although our data suggest synergy between lenalidomide and cyclophosphamide, a formal comparison between REP and low-dose cyclophosphamide-prednisone alone would be needed to substantiate our findings.

Importantly, overall response rates with REP were higher and median PFS was longer, when compared to the outcome of next generation novel agents evaluated in lenalidomide-refractory MM. Treatment with pomalidomide plus dexamethasone results in at least PR in 31% of patients with a median PFS of 4.0 months (75% lenalidomide- and bortezomib-refractory MM patients).[32] Carfilzomib monotherapy induces at least PR in 19.1% of extensively pretreated patients with a median PFS of 3.7 months.[38] Daratumumab induces at least PR in 29-36% of patients with a median PFS of 3.7-5.6 months,[45;46] while elotuzumab[47] has no single agent activity in this setting. The outcome of the REP regimen also compares favorably to the results of carfilzomib- or pomalidomide-based combinations in relapsed/refractory MM patients (majority lenalidomide-refractory), such as pomalidomide-bortezomib-dexamethasone (≥PR: 85%, median PFS 10.7 months),[48] pomalidomide-carfilzomib-dexamethasone (≥PR: 50%, median PFS: 7.2 months),[49] and daratumumab-pomalidomide-dexamethasone (≥PR: 71%, PFS at 6 months: 66%).[50] Nevertheless, cross-trial comparisons must be interpreted with caution, since such a comparison might be biased by multiple factors as differences in trial sizes, patient populations, and study designs.

Other lenalidomide-based combinations were also evaluated in lenalidomide-refractory MM. Interestingly, there was synergy between lenalidomide and therapeutic antibodies such as pembrolizumab (anti-PD-1; ≥PR: 38%)[51] and isatuximab (anti-CD38; ≥PR: 48%)[52] in lenalidomide-refractory patients, suggesting that the immune system of these patients could still respond to the immunomodulatory effects of lenalidomide. Similarly, a retrospective study showed that the combination of lenalidomide, bortezomib and dexamethasone (RVD) may be effective in heavily pre-treated patients (50% lenalidomide-refractory) with an overall response rate of 47% and median PFS of 3.0 months.[53]

The efficacy of lenalidomide plus continuous low-dose oral cyclophosphamide in lenalidomide-refractory MM raises questions about mechanisms of action of this regimen. It is well known that metronomic low-dose cyclophosphamide has multiple effects including direct anti-tumor activity, anti-angiogenic effects,[18;19] modulation of the micro-environment,[21] and improvement of T and NK cell-mediated anti-tumor immune response via depletion of Tregs.[14-17;20;22] Also lenalidomide has pleiotropic effects on the tumor microenvironment, but via different pathways, which may explain the synergy between these drugs in lenalidomide-refractory patients.[9] It is currently unclear whether weekly higher-dose cyclophosphamide has the same effects on the bone marrow microenvironment and the same activity in patients, when compared to continuous low-dose cyclophosphamide. Additional studies are needed to resolve these questions.







Although, several of the new agents to treat lenalidomide- and bortezomib-refractory MM are now approved by the FDA and/or EMA, these therapies may not yet be available or reimbursed in many countries, while cyclophosphamide is widely available. In addition, REP is a fully oral three-drug combination, which is convenient for patients, but also likely associated with lower costs of patient care. Altogether this further highlights the importance of this effective salvage strategy for heavily pretreated relapsed/refractory MM patients.

In summary, REP, at the MTD of lenalidomide (25 mg, days 1-21/28 days), continuous low-dose cyclophosphamide (50 mg) and prednisone (20 mg), is a fully oral, well tolerated and active combination for patients with lenalidomide- and bortezomib-refractory MM. Therefore, the addition of continuous low-dose oral cyclophosphamide to lenalidomide and prednisone may offer new therapeutic perspectives for multidrug resistant MM patients.

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## DISCLOSURES

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# SUPPLEMENTAL DATA

## Dose Limiting Toxicity Assessment

In the phase 1 part of the trial three patients were assigned to each cohort via a 3+3 dose escalation scheme. As per protocol, if no dose-limiting toxicity (DLT) was observed in three evaluable patients during the first treatment cycle, the study proceeded to the next dose level. If one of three evaluable patients experienced a DLT, the cohort was expanded to six evaluable patients. If two or more of all six patients at that dose experienced a DLT, the MTD would be determined as one dose level below. Patients were evaluated for DLTs according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03. A DLT was defined as any of the following treatment-emergent toxicities that were attributable to at least 1 of the study drugs that occurred during cycle 1: grade 4 thrombocytopenia on more than one occasion; grade 4 neutropenia for more than 5 days; febrile neutropenia; grade 3 or higher non-hematologic toxicity except for inadequately treated nausea and vomiting; and death whatever the cause, except death due to MM. Five dose levels were tested as indicated in Supplemental Table 1.

## Dose modification

A new REP-cycle was allowed if ANC ≥ 1.0 x 109 /L and platelets ≥ 50 x 109 /L. A delay of 2 weeks was permitted without dose adjustments. Thereafter lenalidomide was dose-reduced first (from 25 to 15 mg/day, and subsequently to 10 and 5 mg/day). If a dose reduction was required and the patient used lenalidomide at a dose of 5 mg, then the dose of cyclophosphamide was reduced (from 100 to 50 mg/day, and thereafter to 50 mg every other day) and lenalidomide at a dose of 5 mg/day was continued. Growth factor support was given on day 1 of a new treatment cycle if the ANC dropped below 1.0 x 109 /L before the start of a new REP-cycle or if the ANC dropped below 0.5 x 109 /L during a REP-cycle. Grade 4 2 neutropenia and thrombocytopenia, as well as febrile neutropenia during

**Supplementary Table 1.** Dose levels and DLTs for each cohort of enrolled patients.

| | Cohort | Len (mg) | Cyclo (mg) | Pred (mg) | Peg G-CSF (day 1) | Patients *n* | DLT *n* | Type of DLT |
|---|---|---|---|---|---|---|---|---|
| Phase I | 1 | 10 | 100 | 20 | No | 3 | 0 | |
| | 2 | 15 | 50 | 20 | No | 3 | 0 | |
| | 3 | 15 | 100 | 20 | No | 3 | 0 | |
| | 4 | 25 | 50 | 20 | No | 6 | 1 | Grade 3* pneumonia |
| | 5 | 25 | 100 | 20 | yes | 6 | 2 | Grade 3* ACS |
| | | | | | | | | Grade 3* dyspnea |

Len: lenalidomide, Cyclo: cyclophosphamide, Pred: prednisone, PEG G-CSF: pegylated Granulocyte Colony Stimulating Factor, DLT: dose limiting toxicity, ACS: acute coronary syndrome, n: number. *According to CTCAE v4.03.

treatment, required immediate interruption of treatment and subsequent dose reduction at the start of the next cycle. However, in case of (febrile) neutropenia in a subject not receiving G-CSF therapy, growth factor support was to be initiated on day 1 of the next cycle without dose reductions. Non-hematologic adverse events were managed by supportive care or dose-reduction of the drug that was most likely associated with the adverse event.

### Dose-limiting toxicity of REP in the phase 1 part of the study

DLTs were not observed at dose levels 1, 2, and 3. At dose level 4, 1 of 6 patients experienced a grade 3 pneumonia. At dose level 5, 2 of 6 patients had a DLT, with 1 grade 3 acute coronary syndrome, and 1 grade 3 dyspnea. All these patients recovered completely

### Toxicity and efficacy of REP in the phase 1 part of the study

Adverse events of grade 2 or higher occurring in patients treated in the phase 1 part of the study are shown in Supplementary Table 2. All 21 patients were evaluable for response. The ORR (≥PR) was 67% (14 patients), including at least very good partial response (VGPR) in 7 patients (33%). No patients achieved CR. At least MR was achieved in 16 patients (76%) and at least stable disease (SD) in 18 patients (86%) (Supplementary Table 3). Maximum change in M-protein of the 21 patients treated in the dose-escalating phase 1 trial is depicted in a waterfall plot (Supplementary Figure 1). Although the number of patients in each group is limited, no significant differences in response rates were observed between patients receiving 50 or 100 mg/day of cyclophosphamide. Also response was similar between patients receiving lenalidomide at a dose of 25 mg/day or lower.







**Supplementary Table 2.** Adverse events during REP therapy in the phase 1 part of the study.

| | N=21 | | | | |
|---|---|---|---|---|---|
| **Events** | **Grade 2 N (%)** | **Grade 3 N (%)** | **Grade 4 N (%)** | **Grade 5 N (%)** | **Total N (%)** |
| **Hematologic** | | | | | |
| Neutropenia | 4 (19) | 6 (29) | - | - | 10 (48) |
| Thrombocytopenia | 3 (14) | 4 (19) | 1 (5) | - | 8 (38) |
| Anaemia | 3 (14) | 2 (10) | - | - | 5 (24) |
| **Non-hematologic** | | | | | |
| Thromboembolism | - | 1 (5) | - | - | 1 (5) |
| Constitutional | 4 (19) | 1 (5) | - | - | 5 (24) |
| Fatigue | 3 (14) | - | - | - | 3 (14) |
| Muscle cramps | 1 (5) | 1 (5) | - | - | 2 (10) |
| Neurologic | 2 (10) | 1 (5) | - | - | 3 (14) |
| Sensory neuropathy | 2 (10) | - | - | - | 2 (10) |
| Dysesthesia | - | 1 (5) | - | - | 1 (5) |
| Infections | 7 (33) | 7 (33) | - | 1 (5) | 15 (71) |
| Upper respiratory | 7 (33) | - | - | - | 7 (33) |
| Pneumonia | - | 5 (24) | - | 1 (5) | 6 (29) |
| Other | - | 2 (10) | - | - | 2 (10) |
| Cardiac disorders | - | 2 (10) | - | - | 2 (10) |
| Congestive heart failure | - | 1 (5) | - | - | 1 (5) |
| Acute coronary syndrome | - | 1 (5) | - | - | 1 (5) |

**Supplementary Table 3.** Response to REP therapy in the phase 1 part of the study.

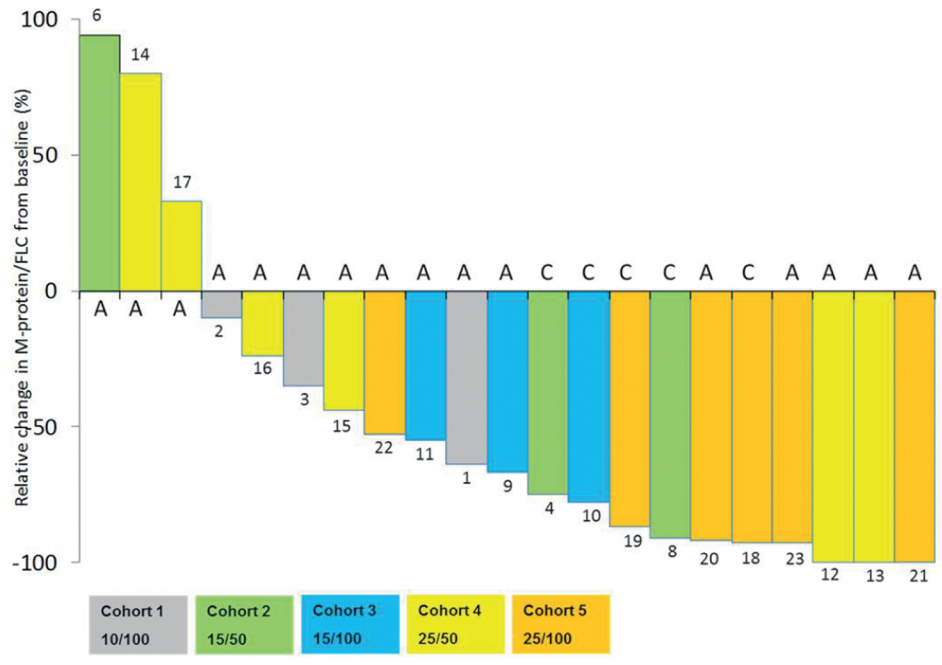
| | **All patients (all len-refractory), *n* =21** | **Len- and bor-refractory patients, *n*=16** | **Patients with high-risk cytogenetic abnormalities*, *n*=10** |
|---|---|---|---|
| VGPR | 33% | 31% | 44% |
| ≥ PR | 67% | 69% | 78% |
| ≥ MR | 76% | 75% | 89% |
| ≥ SD | 86% | 88% | 89% |
| PD | 14% | 12% | 11% |

Len, lenalidomide; bor, bortezomib; MM, multiple myeloma; FISH, fluorescence in situ hybridization; *n*, number; VGPR, very good partial response; PR, partial response; MR, minimal response; SD, stable disease; PD, progressive disease. *High-risk disease was defined by the presence of t(4;14), t(14;16), del(17p), and/or ampl(1q) as determined by FISH on purified MM cells before start of REP treatment.

**Supplementary Table 4.** Median PFS for patients treated with REP at the MTD and with their preceding lenalidomide-containing regimen.

| Characteristics at start REP treatment | Median PFS for preceding lenalidomide-containing regimen (months) | Median PFS for REP (months) |
|---|---|---|
| All patients | 11.2 | 12.1 |
| Progression while on len-containing therapy | 8.8 | 12.1 |
| No response during prior len-based therapy | NE | NE |
| Progressive disease within 60 days after stopping len-based therapy | 17.0 | 8.1 |
| Len- and bort-refractory | 9.8 | 14.3 |
| Len-refractory (not bort-refractory) | 12,2 | 17.0 |
| REP directly after development of len-refractory disease | 10.7 | 12.4 |
| REP after ≥1 other line of therapy after development of len-refractory disease | 15.2 | 12.1 |

Len, lenalidomide; bort, bortezomib; NE, not evaluable.




**Supplementary Figure 1.** Waterfall plot of maximum change in M-protein. Waterfall plot showing the maximum change in M-protein in 21 subjects treated in the phase 1 dose escalation trial, assessed from start of REP treatment until best response. Response is depicted as relative change in serum M-component (A), urine M-component (B) or serum free light chain (C), dependent on which biomarker was used to assess response. All 5 dose cohorts are indicated with different colors (the legend shows the dose of lenalidomide/cyclophosphamide for each dose cohort).

# Chapter 6

## CEREBLON LOSS AND UP-REGULATION OF C-MYC ARE ASSOCIATED WITH LENALIDOMIDE RESISTANCE IN MULTIPLE MYELOMA PATIENTS

Laurens E. Franssen[1], Inger S. Nijhof[1], Suzana Couto[2], Mark-David Levin[3], Gerard M.J. Bos[4], Annemiek Broijl[5], Saskia K. Klein[6], Yan Ren[2], Maria Wang[2], Harry R. Koene[7], Andries C. Bloem[8], Aart Beeker[9], Laura M. Faber[10], Ellen van der Spek[11], Reinier Raymakers[12], Roos J. Leguit[13], Pieter Sonneveld[5], Sonja Zweegman[1], Henk Lokhorst[1], Tuna Mutis[1], Anjan Thakurta[14], Xiaozhong Qian[14] and Niels W.C.J. van de Donk[1]

[1] Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands; [2] Department of Exploratory Toxicology, Celgene, San Diego, CA; [3] Department of Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands; [4] Department of Hematology, Maastricht University Medical Center, Maastricht, The Netherlands; [5] Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands; [6] Department of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands; [7] Department of Hematology, St. Antonius Hospital, Nieuwegein, The Netherlands; [8] Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [9] Department of Internal Medicine, Spaarne Hospital, Hoofddorp, The Netherlands; [10] Department of Internal Medicine, Rode Kruis Hospital, Beverwijk, The Netherlands; [11] Department of Internal Medicine, Rijnstate Hospital, Arnhem, The Netherlands; [12] Department of Hematology, University Medical Center Utrecht Cancer Center, Utrecht, The Netherlands; [13] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands; [14] Department of Translational Development, Celgene Corporation, Summit, NJ.

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Multiple myeloma (MM) patients who become refractory to anti-MM drugs have a very poor prognosis. Therefore, it is important to gain insight into the mechanisms of resistance to these drugs. Immunomodulatory drugs (IMiDs) have immune-stimulatory and anti-angiogenic properties, as well as direct anti-MM activity. Binding of IMiDs to Cereblon promotes ubiquitination and subsequent proteasomal degradation of the substrate proteins IKZF1 (Ikaros) and IKZF3 (Aiolos), which is followed by downregulation of interferon regulatory factor 4 (IRF-4) and c-Myc leading to growth inhibition and apoptosis of MM cells.[1,2] A low expression of Cereblon at baseline is associated with a decreased response to IMiD therapy.[3] Furthermore, in MM cell lines, acquired resistance to IMiDs is accompanied by a decrease in Cereblon expression.[4]

To gain insight into the expression levels of lenalidomide targets in lenalidomide-refractory patients, we analyzed the protein expression of Cereblon and downstream targets in BM-localized plasma cells using immunohistochemistry (Figure 1 and supplementary Figure 1). BM samples from 55 patients were obtained at the time of diagnosis and at the time of development of lenalidomide-refractory disease. Furthermore, BM biopsies taken at earlier time points during the disease course (after first line treatment (lenalidomide naïve), after second line treatment (lenalidomide naïve) and during lenalidomide treatment (responsive)) were analyzed.

The study population consisted of patients included in the REPEAT study,[5] a prospective, phase 1 dose-finding trial, followed by a phase 2 expansion at the recommended dose level to evaluate the safety, tolerability and efficacy of lenalidomide, low-dose oral cyclophosphamide and prednisone (REP) in lenalidomide-refractory MM patients. Patient characteristics are shown in Table 1 and details on the protocol are presented in the supplementary methods.

Sequential dual color immunohistochemistry (IHC) assays were performed as described in the supplemental data.[6] Briefly, the target markers Cereblon, Aiolos, Ikaros, IRF4, and c-Myc were evaluated in CD138-positive plasma cells in the BM to generate an H-score. H-scores range from 0 to 300 and take into account frequency and intensity of staining [H-score = (% at 1+) X 1 + (% at 2+) X 2 + (% at 3+) X 3]. For Cereblon, cytoplasmic and nuclear compartments were scored separately. The sum of cytoplasmic and nuclear H-Scores accounted for the total H-Score (0-600), which was generated for each sample.

There was marked heterogeneity in the intensity of Cereblon expression across patients. However, a significant reduction in Cereblon protein levels was observed, both nuclear and cytoplasmic, at the time patients developed lenalidomide-refractory disease as compared to time of diagnosis (Figure 2A). Seventy-seven percent of patients showed a reduction in overall Cereblon expression (combined H-score), with a median decrease in Cereblon in these patients of 53.1% (range: 6.6-99.2%). In 23% of patients, no decrease in Cereblon expression was observed (Figure 2A).

Levels of cytoplasmic and nuclear Cereblon H-scores were highly correlated, both at diagnosis as well as at the time of lenalidomide-refractory disease (Figure 2B). The expression levels of Ikaros, Aiolos and IRF4 did not change from diagnosis to development of lenalidomide-refractory disease, while there was a small, but significant, increase in

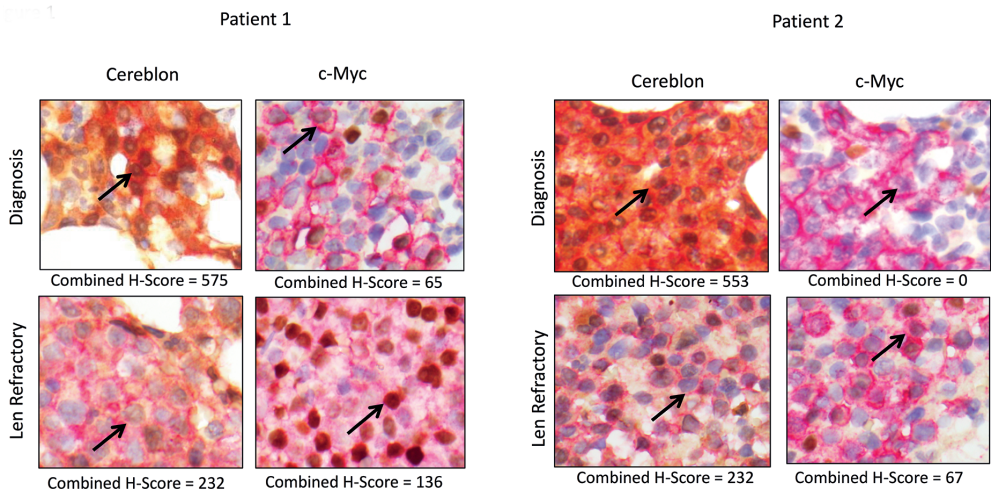



**Figure 1.** Immunohistochemical staining of Cereblon and c-Myc in CD138+ bone-marrow plasma cells of MM patients. Multiple myeloma BM core biopsies stained with dual CD138 (red membrane) and Cereblon (brown cytoplasm and nucleus) or c-Myc (brown nucleus). Shown are two representative patients at the time of diagnosis (upper panel) and lenalidomide-refractory disease (lower panel). Decreased Cereblon and increased c-Myc expression are noted upon development of lenalidomide resistance. Arrows point to tumor cells. H-scores range from 0 to 600 for Cereblon and from 0-300 for c-Myc. Abbreviations: *Len,* lenalidomide.

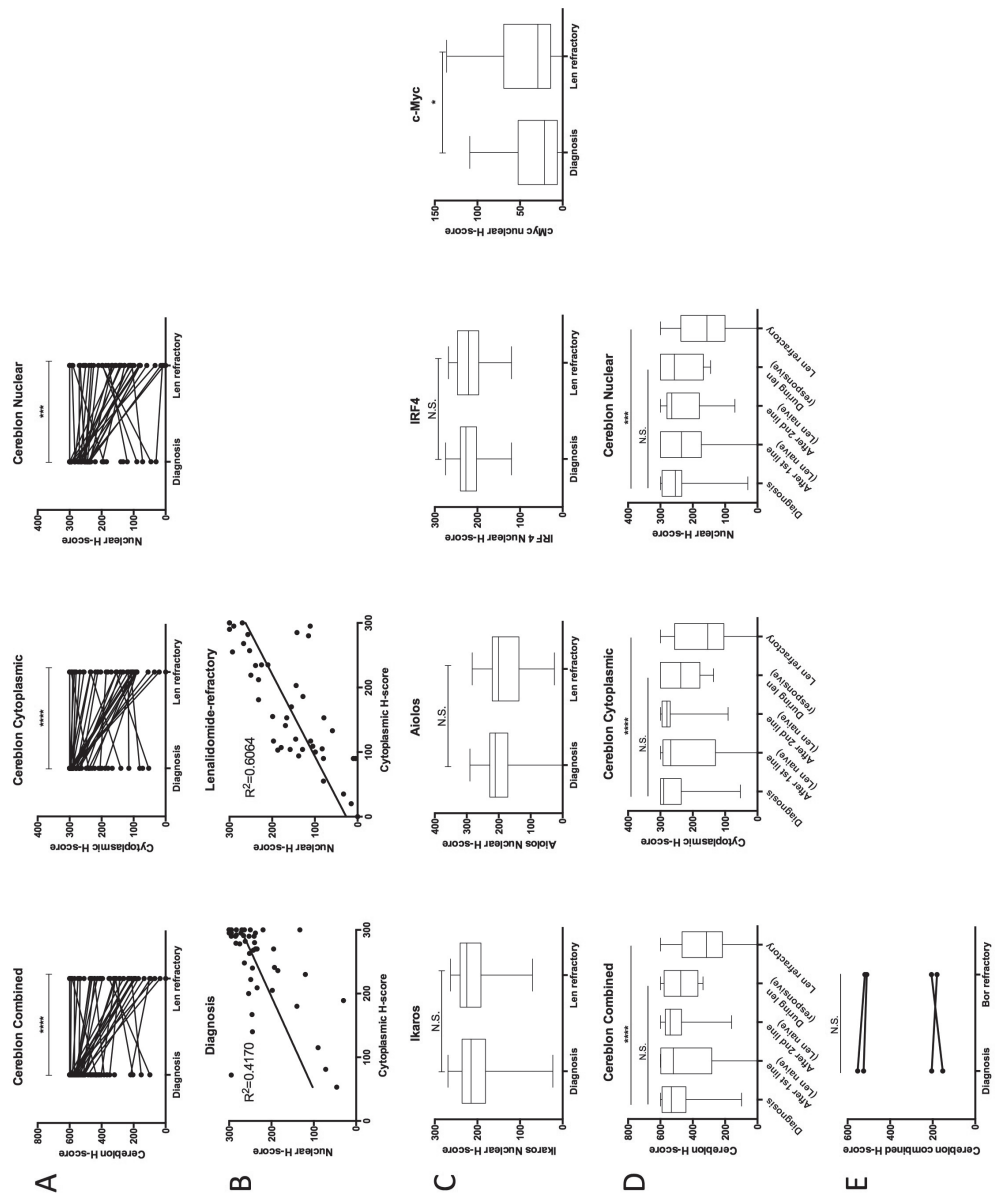
**Figure 2.** Longitudinal analysis of bone-marrow biopsies shows reduced Cereblon and increased c-Myc expression in MM cells from lenalidomide-refractory patients. Plasma cell protein expression levels of Cereblon and downstream targets were determined in 55 patients at the time of diagnosis and at the time of lenalidomide-refractory disease.(A) Cereblon expression (cytoplasmic, nuclear and combined expression [sum of the cytoplasmic and nuclear score]). (B) Correlations between nuclear and cytoplasmic H-scores at diagnosis (left panel) and at the time of lenalidomide-refractory disease (right panel). (C) Expression of Ikaros, Aiolos, IRF4, and c-Myc. (D) Cereblon expression was also analyzed after first line treatment (lenalidomide naive) (n=18), after second line treatment (lenalidomide naive) (n=11), and during lenalidomide treatment (lenalidomide sensitive myeloma, n=6). These additional time-points do not contain data from all patients, because of a limited number of available bone marrow biopsies. (E) Combined Cereblon H-score for 4 patients treated with bortezomib while lenalidomide-naïve, showing no change in Cereblon expression by bortezomib. Boxes represent first and third quartile with median value, and error bars represent minimum to maximum. Bars represent mean, and error bars represent the SEM. *P*-values were calculated by using Wilcoxon matched pairs signed rank test. Abbreviations: *Len,* lenalidomide; *Bor,* bortezomib; *N.S.,* not significant. ****, *P*<0.0001; ***, *P*<0.001; *, *P*<0.05.

c-Myc expression at the time of lenalidomide-resistant disease (Figure 2C). There was no correlation between c-Myc and Cereblon levels, or between change in c-Myc and Cereblon levels over time. To investigate the possibility that diminished Cereblon levels may also occur during other treatment lines before development of lenalidomide-refractory disease, we analyzed Cereblon expression of BM plasma cells in samples obtained at additional time









points during the patient's disease course (Figure 2D). There was no significant change in Cereblon expression at these other time points. Furthermore, we obtained paired samples from 4 patients both before start of bortezomib treatment, and at the time of development of bortezomib-refractory disease. The four patients were lenalidomide-naïve when these samples were taken. In contrast to lenalidomide treatment, bortezomib therapy did not alter Cereblon expression levels (Figure 2E). Altogether, these results indicate that the decrease in Cereblon protein levels is specifically associated with lenalidomide resistance.

There was no association between Cereblon expression at the time of inclusion in the REPEAT study and response (defined as PR or better), PFS or OS with REP treatment. The extent of change in Cereblon expression from diagnosis to inclusion in the REPEAT study was also not correlated with outcome. For c-Myc expression, no significant association with response or OS after REP treatment was observed. However, there was a trend for decreased PFS with high c-Myc expression levels at the time of inclusion in the REPEAT study (HR 1.011, 95% CI: 1.000-1.022, *P*= 0.053). Ikaros, Aiolos, and IRF4 were not associated with clinical outcome following REP. Furthermore, differential expression of Cereblon, c-Myc, Ikaros, Aiolos and IRF4 at the time of diagnosis did not affect OS. No correlative studies were done on other lines of treatment because of the small numbers of BM samples from the patients at the corresponding lines of treatment.

To our knowledge, this is the first study analyzing plasma cell expression levels of the Cereblon pathway proteins in paired samples at diagnosis and at the time of lenalidomide-refractory disease using a validated assay.[6] Our correlative analysis showed that development of lenalidomide-resistance is associated with reduced Cereblon protein levels in the majority of patients. Our observation that Cereblon expression levels did not show a significant change during other treatment lines indicates that the decrease in Cereblon is specifically associated with acquired lenalidomide resistance.

The underlying mechanisms of Cereblon downregulation remain to be determined. Epigenetic modifications of the Cereblon promotor region were previously described as a mechanism of Cereblon downregulation.[7] Other possible mechanisms may involve Cereblon gene mutations or chromosomal deletions although these mechanisms need to be addressed in future studies.

The median reduction in Cereblon of 53% can be considered modest. However, in preclinical studies, a similar degree of shRNA-mediated Cereblon depletion induced lenalidomide resistance in MM cell lines.[4,8] In fact, an absolute threshold of Cereblon protein expression for induction of lenalidomide resistance is currently unknown, especially not in patients. Therefore, we expect that the Cereblon reduction that we observed in the MM cells, contributes to the development of lenalidomide-refractory disease. However, Cereblon expression was not reduced in a subset of patients in our current analysis. Interestingly, 50% of these patients were primary refractory to lenalidomide-based treatment, versus only 13% in patients with a decrease in Cereblon expression (*P*=0.03), while there was no difference in baseline Cereblon expression between primary refractory patients and patients with an initial response to lenalidomide-based treatment. Other mechanisms of lenalidomide-







resistance are likely involved in these patients, such as Cereblon pathway mutations, Cereblon splice variants, or increased activity of the MEK/ERK pathway.[9,10] Given the limited availability of patient samples from our current study, no additional analysis of mRNA levels or analysis of Cereblon pathway mutations could be performed to further characterize these potential mechanisms.

The observed increase in c-Myc protein expression at the time of lenalidomide-refractory disease, as compared to levels at diagnosis, suggests that c-Myc increase may also contribute to development of lenalidomide resistance. Although c-Myc expression levels had not been studied in the setting of acquired lenalidomide resistance, c-Myc upregulation has been reported in the evolution from MGUS to MM.[11] Furthermore, c-Myc aberrations and/or overexpression are generally associated with adverse clinical features and poor survival.[11]

The lack of association between Cereblon levels and outcome of REP treatment indicates that the anti-myeloma effect of lenalidomide as part of the REP regimen may not be mediated via plasma cell Cereblon. Indeed, there is evidence that lenalidomide can still have immunomodulatory activity in the setting of lenalidomide-refractory disease,[12] which might be complementary to the previously described immune-activating effects of metronomically dosed cyclophosphamide.[13] Alternatively, modulation of residual Cereblon by lenalidomide may still be able to synergize with the other components of the REP regimen. In addition, downregulation of Cereblon may affect its recently described chaperon-like function that promotes maturation of CD147 and MCT-1 proteins, which regulate proliferation and survival of MM cells.[14]

In our analysis, the expression levels of Cereblon and its downstream substrates and effector proteins at diagnosis did not affect OS. We were not able to assess the effect of Cereblon expression at diagnosis on PFS or response after first-line treatment due to lack of clinical data. The association of Cereblon expression and survival in newly diagnosed MM patients has been assessed in several studies with conflicting results. When interpreting these studies, it is important to note that there is a lack of correlation between Cereblon mRNA and protein levels.[15] Furthermore, different techniques and scoring methods have been used for assessment of protein and mRNA expression in these studies.

In conclusion, we demonstrated in a longitudinal analysis of BM biopsy samples obtained from the REPEAT study, that a decrease in Cereblon protein levels is one of the characteristics of acquired resistance to lenalidomide. Other mechanisms of resistance, such as acquired Cereblon pathway mutations or defects in other components of the E3 ligase complex, may play a role in patients without reduction in Cereblon expression.

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## DISCLOSURES

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# SUPPLEMENTAL DATA

## Supplementary Methods

*Patients*

Patients were eligible to participate in the REPEAT study if they had lenalidomide-refractory disease following at least 1 prior therapy. Lenalidomide-refractory MM was defined as progressive disease during therapy, no response (less than partial response) to prior lenalidomide-containing therapy, or progression within 60 days of discontinuation from lenalidomide-containing regimens, according to the International Myeloma Working Group criteria.[1] The study was approved by the institutional medical ethical committee in each participating center in accordance with the declaration of Helsinki. All participants provided written informed consent. The trial was registered at www.clinicaltrials.gov as #NCT01352338.

*Immunohistochemistry*

Sequential dual color immunohistochemistry (IHC) assays were performed as previously described[2,3], except for the addition of Dako Protein Block (Catalog No. X0909) immediately before applying the CD138 antibody to reduce nonspecific background.

Primary antibodies used were: Cereblon, Celgene custom rabbit monoclonal, CRBN65, used at 1/2000; Aiolos, Celgene custom rabbit monoclonal, Clone 9B-9-7, used at 1/400; Ikaros, Celgene custom rabbit monoclonal, Clone 36-8-5, used at 1/12000; IRF4, mouse monoclonal, Dako, Catalog No. M7259, Clone MUM1P, used at 1/7500; c-Myc, rabbit monoclonal, Abcam, Catalog No. ab32072, Clone Y69, used at 1/200; CD138, mouse monoclonal, Dako, Catalog No. M7228, Clone MI15, used at 1/1200. Mouse monoclonal IgG1 (BD Bioscience; Catalog No 550878) and rabbit monoclonal IgG (Abcam, Catalog No. Ab172730) were used as isotype controls at the matched concentrations as the respective primary antibodies. All slides were counterstained with hematoxylin.

Evaluation of dual color IHC slides was performed by board-certified pathologists under the light microscope. The target markers Cereblon (cytoplasmic and nuclear), Aiolos (nuclear), Ikaros (nuclear), IRF4 (nuclear), and c-Myc (nuclear) were evaluated in at least 100 CD138 (membrane) positive plasma cells in the bone marrow to generate an H-score. H-scores range from 0 to 300 and take into account frequency and intensity of staining [H-score = (% at 1+) X 1 + (% at 2+) X 2 + (% at 3+) X 3]. For Cereblon, cytoplasmic and nuclear compartments were scored separately. The sum of cytoplasmic and nuclear H-Scores accounted for the total H-Score (0-600), which was generated for each sample. This dual color IHC assay has been validated previously, showing a high specificity for a wide range of Cereblon expression levels, a low coefficient of variation for cytoplasmic and nuclear H-scores of 5% and 2% respectively, and good inter-pathologist concordance with an average $R^2 = 0.73$.[2]







*Statistics*

Continuous variables were analyzed using Wilcoxon matched-pairs test or a Mann-Whitney U test. Differences in categorical variables were determined with the Fisher's exact test for two by two tables and otherwise with the Pearson's $\chi 2$ test. Results are expressed as 2-tailed *P* values. A level of $P < 0.05$ was considered significant. Progression-free survival (PFS) was calculated from inclusion in the REPEAT study until progression or death from any cause. Overall survival (OS) was measured from diagnosis and from inclusion in the REPEAT study until death from any cause. Prognostic factors for PFS and OS were analyzed for statistical significance using the Cox proportional hazard model. Calculations were performed in SPSS version 20.0.0 (IBM SPSS Inc., Armonk, NY, USA) and GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Correlations of various patient- and tumor-related factors with clinical response to REP treatment were only performed for patients treated at the RDL.



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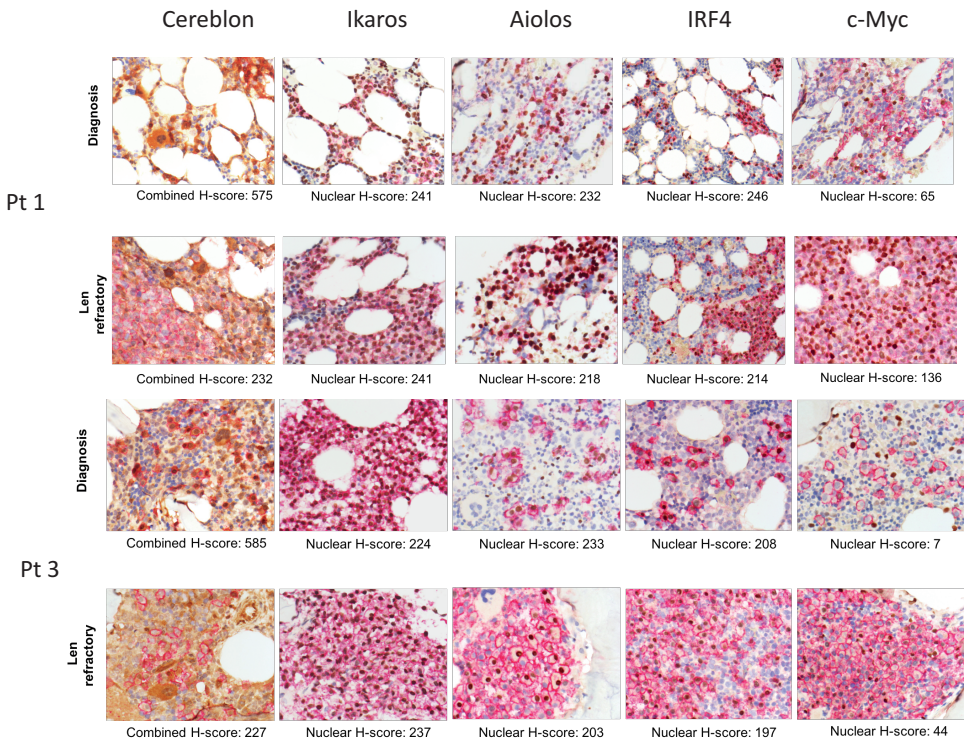
# SUPPLEMENTAL DATA




**Supplemental Figure 1.** Immunohistochemical staining of Cereblon, Ikaros, Aiolos, IRF4 and c-Myc in CD138+ bone-marrow plasma cells of MM patients. Multiple myeloma BM core biopsies stained with dual CD138 (red membrane) and Cereblon (brown nucleus and cytoplasm- 1st column), Ikaros (brown nucleus - 2nd column), Aiolos (brown nucleus -3rd column), IRF4 (brown nucleus - 4th column), or c-Myc (brown nucleus - 5th column). Shown are two representative patients at the time of diagnosis (upper panel) and lenalidomide-refractory disease (lower panel). Decreased Cereblon and increased c-Myc expression are noted upon development of lenalidomide resistance. Aiolos, Ikaros and IRF4 remain unchanged. H-scores range from 0 to 600 for Cereblon and from 0-300 for the remaining stains. Abbreviations: Len, lenalidomide.

# Chapter 7

## LENALIDOMIDE COMBINED WITH LOW-DOSE CYCLOPHOSPHAMIDE AND PREDNISONE MODULATES IKAROS AND AIOLOS IN LYMPHOCYTES, RESULTING IN IMMUNOSTIMULATORY EFFECTS IN LENALIDOMIDE-REFRACTORY MULTIPLE MYELOMA PATIENTS

Laurens E. Franssen,[1] Inger S. Nijhof,[1] Chad C. Bjorklund,[2]
Hsiling Chiu,[2] Ruud Doorn,[3] Jeroen van Velzen,[3]
Maarten Emmelot,[1] Berris van Kessel,[1] Mark-David Levin,[4]
Gerard M.J. Bos,[5] Annemiek Broijl,[6] Saskia K. Klein,[7]
Harry R. Koene,[8] Andries C. Bloem,[3] Aart Beeker,[9]
Laura M. Faber,[10] Ellen van der Spek,[11] Reinier Raymakers,[12]
Pieter Sonneveld,[6] Sonja Zweegman,[1] Henk M. Lokhorst,[1]
Anjan Thakurta,[2] Xiaozhong Qian,[2]
Tuna Mutis[1] and Niels W.C.J. van de Donk.[1]

[1]Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands; [2]Department of Translational Development, Celgene Corporation, Summit, NJ; [3]Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [4]Department of Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands; [5]Department of Hematology, Maastricht University Medical Center, Maastricht, The Netherlands; [6]Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands; [7]Department of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands; [8]Department of Hematology, St. Antonius Hospital, Nieuwegein, The Netherlands; [9]Department of Internal Medicine, Spaarne Hospital, Hoofddorp, The Netherlands; [10]Department of Internal Medicine, Rode Kruis Hospital, Beverwijk, The Netherlands; [11]Department of Internal Medicine, Rijnstate Hospital, Arnhem, The Netherlands; [12]Department of Hematology, University Medical Center Utrecht Cancer Center, Utrecht, The Netherlands.

*Oncotarget. Accepted*

## ABSTRACT

We recently showed that the outcome of multiple myeloma (MM) patients treated in the REPEAT study (evaluation of lenalidomide combined with low-dose cyclophosphamide and prednisone (REP) in lenalidomide-refractory MM) was markedly better than what has been described with cyclophosphamide-prednisone alone. The outcome with REP was not associated with plasma cell Cereblon expression levels, suggesting that the effect of REP treatment may involve mechanisms independent of plasma cell Cereblon-mediated direct anti-tumor activity. We therefore hypothesized that immunomodulatory effects contribute to the anti-MM activity of REP treatment, rather than plasma cell Cereblon-mediated effects. Consequently, we now characterized the effect of REP treatment on immune cell subsets in peripheral blood samples collected on day 1 and 14 of cycle 1, as well as on day 1 of cycle 2. We observed a significant mid-cycle decrease in the Cereblon substrate proteins Ikaros and Aiolos in diverse lymphocyte subsets, which was paralleled by an increase in T-cell activation. These effects were restored to baseline at day one of the second cycle, one week after lenalidomide interruption. *In vitro*, lenalidomide enhanced peripheral blood mononuclear cell-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant MM cells in a co-culture system. These results indicate that the Cereblon-mediated immunomodulatory properties of lenalidomide are maintained in lenalidomide-refractory MM patients and may contribute to immune-mediated killing of MM cells. Therefore, combining lenalidomide with other drugs can have potent effects through immunomodulation, even in patients considered to be lenalidomide-refractory.








## INTRODUCTION

Multiple myeloma (MM) is a malignant disease, characterized by clonal proliferation of plasma cells in the bone marrow. Clinical characteristics include osteolytic bone lesions, hypercalcemia, renal failure, and progressive bone-marrow dysfunction with anemia and other cytopenias. Significant advances have been made during the past decades in the treatment of MM, especially due to the development of immunomodulatory drugs (IMiDs) and proteasome inhibitors.[1,2] However, patients who become refractory to all available anti-MM agents have a very poor prognosis.[3,4] The mechanism of action of IMiDs has been shown to be dependent on Cereblon-mediated ubiquitination and subsequent proteasomal degradation of the substrate proteins Ikaros family zinc finger 1 (IKZF1) (Ikaros) and IKZF3 (Aiolos). This leads to downregulation of cMyc and Interferon regulatory factor 4 (IRF4) resulting in growth inhibition and apoptosis of MM cells.[5–11] In addition to these direct anti-tumor effects, Ikaros and Aiolos have been shown to act as repressors of IL-2 transcription. IMiDs induce Cereblon-dependent degradation of Ikaros and Aiolos in immune cells, which increases IL-2 expression, and also enhances the production of other cytokines (IFNγ, IL-4, IL-6, IL-10, IL-13 and GM-CSF), causing activation of both T- and NK-cells.[9,12-17] Resistance mechanisms to IMiDs however, are still poorly understood. A low expression of Cereblon has been correlated with IMiD resistance,[11,18–21] as are the presence or development of Cereblon pathway mutations, Cereblon splice variants, or increased activity of the MEK/ERK pathway.[22–24] We have recently reported in vivo evidence that plasma cell Cereblon downregulation is one of the characteristics of acquired lenalidomide resistance in patients who were subsequently treated in the REPEAT study.[25] In this study we showed remarkable activity of lenalidomide (Revlimid) combined with continuous low-dose oral cyclophosphamide (Endoxan) and prednisone (REP) in heavily pretreated, lenalidomide-refractory MM patients.[26] The outcome of REP treatment was better than what has been described with cyclophosphamide-prednisone alone, suggesting synergistic effects of the lenalidomide-cyclophosphamide combination.[27,28] Although several studies have shown a correlation between Cereblon expression in MM cells and clinical outcomes of IMiD based therapy,[19,20,29,30] the outcome with REP treatment was not associated with plasma cell Cereblon expression levels, suggesting that the effect of REP treatment may involve mechanisms independent of plasma cell Cereblon-mediated direct anti-tumor activity.[25] We therefore hypothesized that immunomodulatory effects contribute to the anti-MM activity of REP treatment, rather than plasma cell Cereblon-mediated effects. Consequently, we here analyzed the frequency and activity of lymphocyte subsets from patients treated in the REPEAT study, to characterize the effect of REP treatment on the immune system of lenalidomide-refractory MM patients.

# RESULTS

## Ikaros and Aiolos can still be modulated in lymphocytes from lenalidomide-refractory patients during REP treatment

Sixty-four lenalidomide-refractory MM patients were treated with REP in the phase 2 part of the REPEAT study (patient characteristics are shown in Table 1). Lenalidomide was administered on days 1 to 21 of a 28-day cycle, and cyclophosphamide and prednisone were given continuously. PBMCs obtained at the start of REP treatment, at day 14 and at day 1 of cycle 2 (before the administration of anti-MM agents) were analyzed for Ikaros and Aiolos expression using flow cytometry. The results revealed that two weeks of REP treatment caused a significant decrease in expression of Ikaros and Aiolos both in $CD4^+$ T-cells (median decrease: 63% and 46%, resp.) and $CD8^+$ T-cells (median decrease: 63% and 55%, resp.), NK-cells (median decrease: 59% and 57%, resp.), and B-cells (median decrease: 46% and 37%, resp.) (Figure 1A, B). There was a significant correlation between baseline Ikaros and Aiolos expression (Figure 1C; Pearson correlation: $CD3^+$ T-cells: $R^2$ 0.57, $P$<0.001; $CD4^+$ T-cells: $R^2$ 0.51, $P$<0.001; $CD8^+$ T-cells: $R^2$ 0.67, $P$<0.001; NK-cells: $R^2$ 0.59, $P$<0.001; B-cells: $R^2$ 0.50, $P$<0.001). Similarly, a significant correlation was observed between the extent of Ikaros and Aiolos downregulation (Figure 1D; Pearson correlation: $CD3^+$ T-cells: $R^2$ 0.90, $P$<0.001; $CD4^+$ T-cells: $R^2$ 0.88, $P$<0.001; $CD8^+$ T-cells: $R^2$ 0.91, $P$<0.001; NK-cells: $R^2$ 0.89, $P$<0.001; B-cells: $R^2$ 0.342, $P$<0.001) in all these immune cell subsets. Ikaros and Aiolos expression levels were restored to baseline levels at day 28, which was after one week without lenalidomide treatment. These results indicated that in these lenalidomide-refractory patients, Ikaros and Aiolos expression in lymphocytes can still be modulated by REP treatment. Nonetheless, the baseline expression of Ikaros and Aiolos levels or the extent of Aiolos/Ikaros reduction in these immune cells did not show a significant correlation with response, PFS or OS following REP treatment.

## REP treatment induces T-cell activation

Next, we investigated the impact of REP treatment in lenalidomide-refractory patients on the frequency of peripheral blood lymphocyte subsets and on T-cell activation status (HLA-DR expression) and cytokine production (IFNγ and IL-2). Frequencies of total $CD3^+$ T-cells, $CD4^+$ T-cells, and $CD19^+$ B-cells slightly decreased during the first 14 days, while NK-cells increased (Figure 2). These levels returned to baseline at day 1 of cycle 2. Levels of $CD8^+$ T-cells did not change. However, the observed changes were relatively small. There was a significant increase in the frequency of activated $CD4^+$ and $CD8^+$ T-cells mid-cycle, which decreased to baseline at the start of cycle 2 (Figure 3A). The production of IFNγ and IL-2 by $CD4^+$ and $CD8^+$ T-cells did not change significantly from baseline to mid-cycle. However, there was a significant reduction in IFNγ and IL-2 production at the start of cycle 2 compared to mid-cycle levels, which is after one week without lenalidomide treatment (Figure 3B, C). This indicates that in lenalidomide-refractory patients, Ikaros and Aiolos degradation in immune cells is associated with an increase in activated T-cells. Although there was no correlation between overall response (≥ partial response (PR)) and frequency of







**Table 1.** Patient characteristics.

| Characteristic | Total (n=64) |
|---|---|
| Median age, y (range) | 65 (43-82) |
| Sex, male, n (%) | 43 (67) |
| **Response, n (%)** | |
| ≥ PR | 43 (67) |
| ≥ VGPR | 15 (23) |
| **Type of monoclonal heavy chain, n (%)** | |
| IgG | 37 (57.8) |
| IgA | 8 (12.5) |
| IgD | 0 (0) |
| Light chain only | 19 (29.7) |
| **Type of light chain, n (%)** | |
| Kappa | 42 (65.6) |
| Lambda | 22 (34.4) |
| Median time from diagnosis until enrollment REPEAT study in months (range) | 51.5 (5.37-673) |
| Prior lines of therapy, median (range) | 3 (1-6) |
| **Prior therapies, n (%)** | |
| Lenalidomide | 64 (100) |
| Bortezomib | 53 (82.8) |
| Thalidomide | 38 (59.4) |
| Cyclophosphamide | 25 (39.1) |
| Autologous stem cell transplantation (HDM) | 35 (54.7) |
| Oral melphalan | 27 (42.2) |
| Allogeneic stem cell transplantation | 4 (6.3) |
| **Previous lenalidomide, n (%)** | |
| Refractory* | 64 (100) |
| Progression while on lenalidomide-based therapy** | 60 (93.8) |
| No response during prior lenalidomide-based therapy*** | 1 (1.6) |
| Progressive disease within 60 days after stopping lenalidomide-based therapy**** | 3 (4.7) |
| Primary lenalidomide refractory#, n (%) | 14 (21.9) |
| REP directly after development of lenalidomide-refractory disease, n (%) | 53 (82.8) |
| Lenalidomide and bortezomib double refractory*, n (%) | 38 (59.4) |
| **Cytogenetic abnormalities, n (%)** | |
| High risk## | 23 (35.9) |
| Standard risk | 19 (29.7) |
| Not available | 22 (34.4) |

Abbreviations: *HDM*, high-dose melphalan; *PR*, partial response; *VGPR*, very good partial response.

* Refractory disease is defined as progressive disease during therapy, no response (< PR), or progressive disease within 60 days of stopping treatment, according to the International Uniform Response Criteria for Multiple Myeloma.

** Forty-nine patients progressed while receiving lenalidomide (25mg)-dexamethasone, 2 while receiving lenalidomide, bortezomib and dexamethasone, 1 while receiving MPR (10mg lenalidomide), and 8 while receiving lenalidomide maintenance therapy (10mg).

*** One patient received lenalidomide (25mg)-dexamethasone.

**** Two patients received lenalidomide (25mg)-dexamethasone, and 1 patient received 10mg lenalidomide in MPR.

# Primary lenalidomide-refractory was defined as a best response on previous lenalidomide treatment of < PR.

##High-risk cytogenetic abnormalities were defined by the presence of t(4;14), t(14;16), del(17p), and/or ampl(1q) as determined by FISH analysis on purified MM cells before start of REP treatment.

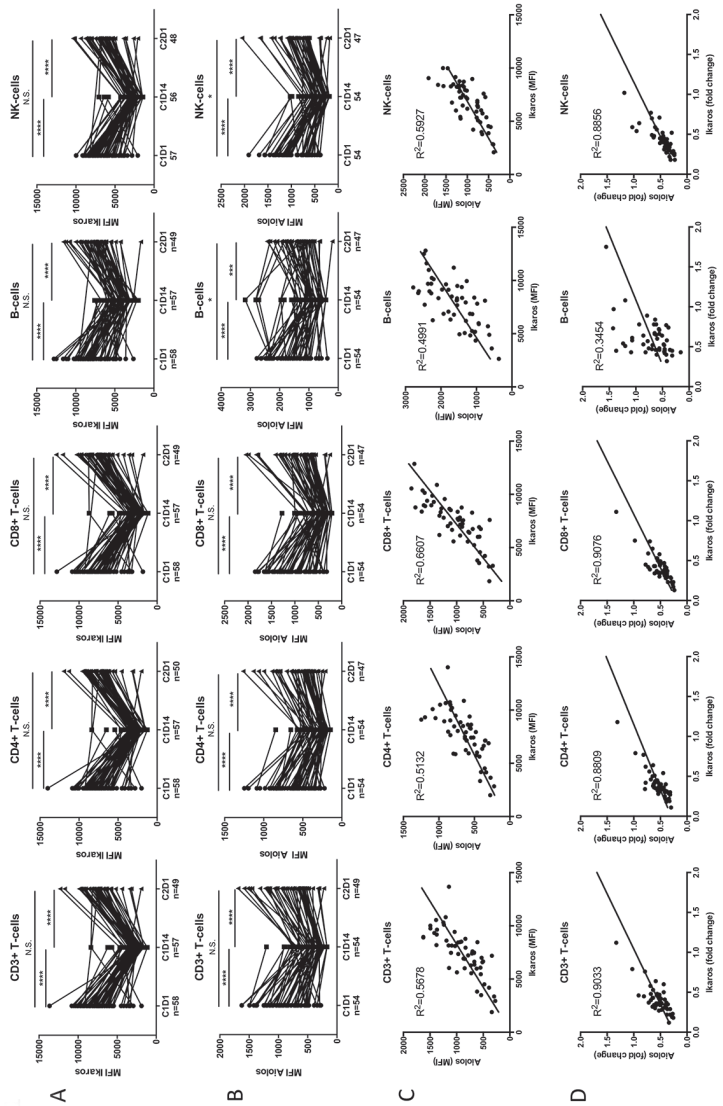



**Figure 1.** During REP treatment, Ikaros and Aiolos levels can be modulated in lymphocytes from lenalidomide-refractory patients. PBMCs obtained at the start of cycle 1 (C1D1), mid-cycle (C1D14) and start of cycle 2 (C2D1) were stained for CD3, CD4, CD8, CD19 and CD56 to identify the different lymphocyte subsets. Lymphocytes were then stained for intracellular Ikaros (A) and Aiolos (B) expression and analyzed by flow cytometry. (C) Correlation between baseline Ikaros and Aiolos expression levels in the different lymphocyte subsets. (D) Correlation between fold change from C1D1 to C1D14 in Ikaros and Aiolos expression in the different lymphocyte subsets. *P*-values were calculated using the Wilcoxon matched pairs, signed rank test. *** $P<0.0001$, **** $P<0.0001$, *N.S.* not significant.







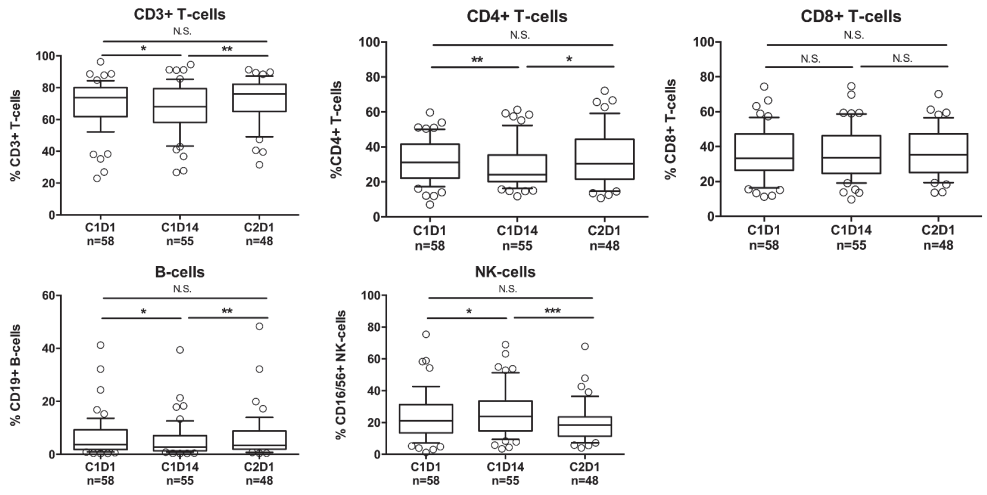



**Figure 2.** Changes in lymphocyte frequencies following REP treatment. Frequencies of T-cells, CD4⁺ T-cells, CD8⁺ T-cells, B-cells, and NK-cells before start of REP treatment (C1D1), mid-cycle (C1D14) and at the start of cycle 2 (C2D1). Boxes represent first and third quartile with median value, and error bars represent p10 to p90. *P*-values were calculated using the Wilcoxon matched pairs, signed rank test, or paired T-test, depending on the distribution. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, *N.S.* not significant.

activated T-cells or IFNγ/IL-2 production by T-cells, we observed that patients who achieved at least a very good partial response (VGPR) (n=13) had a significantly higher percentage of mid-cycle activated CD3⁺ T-cells (18.58% vs. 10.68%, $P=0.01$) and a significantly higher baseline (IFNγ⁺ CD3⁺ 13.11% vs. 8.14%, $P=0.02$) and mid-cycle IFNγ production by T-cells (IFNγ⁺ CD3⁺ 14.43% vs. 8.80%, $P=0.006$) when compared to patients with less than VGPR (n=45) (Figure 3D), suggesting the contribution of T-cell immune responses in achieving ≥VGPR.

### Regulatory T-cells and PD-1 expression on NK-cells increase during REP treatment

Several reports have shown that lenalidomide induces an increase in regulatory T-cells (Tregs), which may hamper the beneficial effect of an increased frequency of activated T-cells.[31–38] On the other hand, metronomic dosing of cyclophosphamide has been shown to deplete regulatory T-cells.[39–44] Therefore, we hypothesized that the addition of cyclophosphamide to lenalidomide treatment may prevent the lenalidomide-induced increase in Tregs. However, similar to earlier studies performed with lenalidomide alone, we observed a significant increase in Tregs during the first REP cycle (Figure 4A, left panel). Interestingly though, the increase in Tregs was more pronounced in non-responding patients (<PR) as compared to responding patients. Furthermore, non-responders showed an ongoing increase in Tregs, while responders showed equal frequencies of Tregs from mid-cycle to the start of cycle 2 (Figure 4A, right panel). These differences were not observed when response was defined as ≥VGPR. PD-1 expression on CD3⁺ T-cells and NK-cells was also analyzed. We observed no

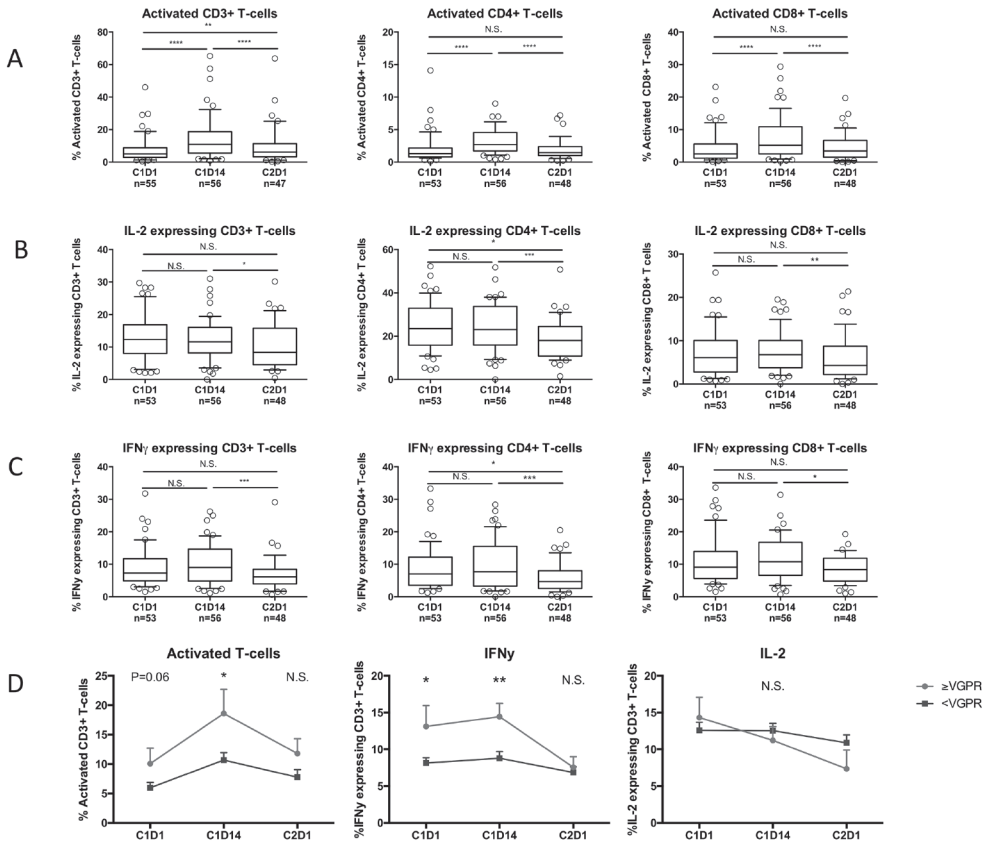



**Figure 3.** Effects of REP treatment on T-cell activation and cytokine production in lenalidomide-refractory patients. PBMCs were obtained before start of cycle 1 (C1D1), mid-cycle (C1D14) and before start of cycle 2 (C2D1). (A) Change in frequencies of activated (HLA-DR$^+$) T-cells during REP treatment. (B, C) Changes in expression of IL-2 (B) and IFNγ (C) in T-cells during REP treatment. Boxes represent first and third quartile with median value, and error bars represent p10 to p90. (D) Change in activated (left panel), IFNγ producing (middle panel) and IL-2 producing (right panel) T-cells in patients with a response ≥VGPR (red) versus <VGPR (blue) during REP treatment. Shown are mean +/- SEM. *P*-values were calculated using the Wilcoxon matched pairs, signed rank tests (A, B, C) and T-tests (D). * *P*<0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.0001, *N.S.* not significant.

changes in PD-1 expression on T-cells, and a modest increase in PD-1 expression on NK-cells (Figure 4B).

### Lenalidomide enhances PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant MM cells

To further investigate the immune-mediated effects of lenalidomide in lenalidomide-refractory MM, we used two lenalidomide-sensitive MM cell lines (MM1.S and L363) and





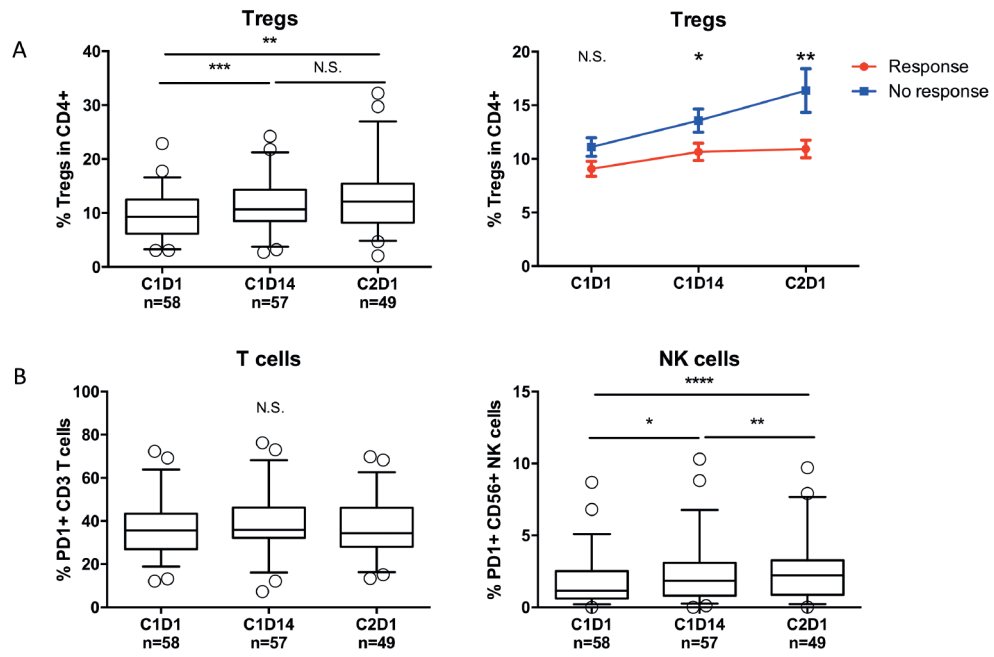



**Figure 4.** REP treatment causes an increase in regulatory T-cells and increased PD-1 expression on NK-cells. (A) Left panel: Frequencies of regulatory T-cells during REP treatment. Boxes represent first and third quartile with median value, and error bars represent p10 to p90. Right panel: Change in regulatory T-cell frequencies in responding patients (≥PR, red line) versus non-responding patients (<PR, blue line) during REP treatment. Shown are mean +/- SEM. (B) Frequencies of PD-1+ T-cells (left panel) and NK-cells (right panel) during REP treatment. Boxes represent first and third quartile with median value, and error bars represent p10 to p90. *P*-values were calculated using the Wilcoxon matched pairs, signed rank tests (A, left panel and B) and T-tests (A, right panel). * *P*<0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.0001, *N.S.* not significant.

generated lenalidomide-resistant progeny cell lines (MM1S/LR and L363/LR) as previously described (Figure 5A).[27] To eliminate direct effects of lenalidomide on the MM cells, PBMCs from healthy donors were pretreated for 72 hours with either vehicle control or lenalidomide in different concentrations, washed, and then co-cultured with the lenalidomide-sensitive and the lenalidomide-resistant MM cell lines for 4 hours. Lenalidomide was not cytotoxic to PBMCs (Supplementary Figure 2). Flow-cytometric analysis of MM cell viability after 4 hours revealed that lenalidomide enhanced the PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant cell lines (Figure 5B), with increased granzyme B release in the co-culture system (Figure 5C). Furthermore, lenalidomide treatment induced profound IL-2 secretion by PBMCs (Figure 5D). Altogether, these results indicate that lenalidomide induces an activation of the immune-system, capable of killing MM cells, independent of their lenalidomide-sensitivity.

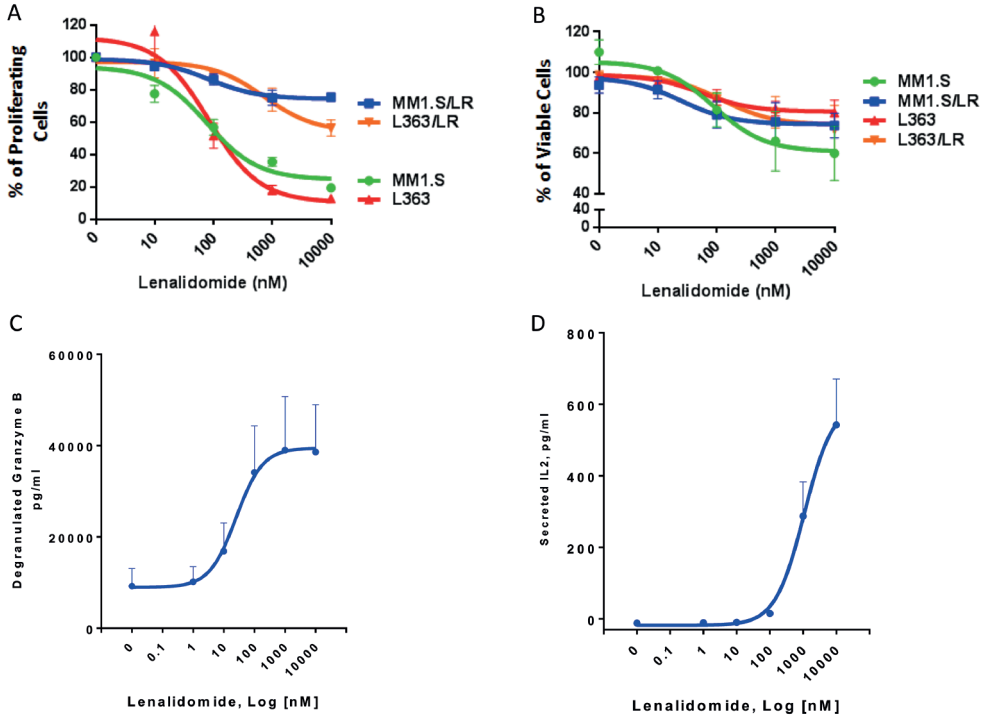



**Figure 5.** Lenalidomide enhances PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant MM cells. (A) $^{3}$H-thymidine incorporation in lenalidomide-sensitive MM1.S and L363 and their lenalidomide-resistant progeny cells (MM1.S/LR, L363/LR) following treatment with either vehicle control or lenalidomide (10-10000 nM). The results are presented as percent of the vehicle control. (B) The portion of viable MM cells as percent of the vehicle control in a co-culture assay with lenalidomide-pretreated PBMCs obtained from a healthy donor. CFSE-labeled lenalidomide-sensitive MM1.S and L363 and their lenalidomide-resistant progeny (MM1.S/LR, L363/LR) cells were cultured for 4 hours at 3:1 ratio with PBMCs, which were pretreated for 72 hours with muromonab-CD3 and lenalidomide (10-10000 nM). The viable MM cells were identified by Annexin-V/To-Pro-3 negative staining by flow cytometry. (C) ELISA measurement of granzyme B levels in the supernatant of the PBMC/MM cell co-culture as depicted in part (B). (D) Secreted IL-2 from PBMCs that were treated with lenalidomide (0-10000nM) for 72 hours. IL-2 levels were measured by ELISA from the supernatant of PBMCs that were subsequently used for the co-culture experiment as depicted in part (B). All figures shown are representatives of n = 3 experiments.

## DISCUSSION

In the present study, we show in peripheral blood samples collected from 64 lenalidomide-refractory MM patients that lenalidomide combined with low-dose cyclophosphamide and prednisone (REP) was capable of inducing degradation of Ikaros and Aiolos in T, NK and B-cells. This degradation was associated with an increase in NK-cells and activated T-cells. Due to limited availability of patient PBMC samples, we were not able to analyze the effect of REP treatment on NK-cell activation. Furthermore, we show that lenalidomide enhances







PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-refractory MM cells. Our results are consistent with a recent study, showing similar immune-activating effects of pomalidomide-dexamethasone in lenalidomide-refractory patients.[16,22] However, our observation that lenalidomide itself retains its immunomodulatory capacity despite the presence of lenalidomide-refractory MM has not been described before. Our observation that the immunomodulatory properties of lenalidomide are maintained despite a clinical lenalidomide-refractory status can have implications for other combination therapies. In fact, pre-clinical and clinical studies have already shown promising results combining lenalidomide and therapeutic antibodies such as isatuximab and daratumumab (anti-CD38) in lenalidomide-refractory MM patients.[46–49]

In our study, there was no significant increase in IL-2 and IFNγ production by T-cells at day 14 of cycle 1 compared to baseline levels of these cytokines. However, in vitro experiments indicate that lenalidomide-induced degradation of Ikaros and Aiolos already occurs after a 3-6 hour incubation,[14] and an increase in cytokine production can be measured ex vivo after 7 days.[16] In addition, our own *in vitro* data show a profound increase in IL-2 production by PBMCs after a 72-hour incubation with lenalidomide (Figure 5C). Therefore, it is possible that in our patient samples obtained at day 14 after start of REP treatment, the maximum effect has passed or the cytokines have already been consumed by the activated immune system. The observation that there was a significant reduction of IL-2 and IFNγ production in T-cells after one week without lenalidomide treatment (while cyclophosphamide and prednisone were given continuously), suggests a lenalidomide-mediated stimulation of cytokine production in these patients.

The decrease in activation status and cytokine production after one week of stopping lenalidomide may suggest that continuous lenalidomide administration, rather than intermittent dosing on days 1-21 of 28-day cycles, could be favorable. However, a previous report described the randomized comparison between continuous pomalidomide and 21/28 days pomalidomide administration, both combined with dexamethasone, in relapsed MM patients.[16] Both treatment arms showed an initial decrease in Ikaros expression in T- and NK-cells, but also in both arms this returned to baseline levels at day 28 (although less pronounced in the continuous treatment arm). This suggests that with continuous treatment, the effects on Ikaros and Aiolos are probably reversed after some time, which may induce an IMiD resistant phenotype of the immune system. Moreover, continuous treatment is probably associated with increased toxicity, when compared to intermittent dosing.

Despite the fact that all patients included in the REPEAT study were lenalidomide refractory and 66% were also bortezomib refractory, REP treatment showed a remarkable overall response rate (ORR) of 67% and a median PFS and OS of 12.1 and 29.0 months respectively.[26] This is markedly higher than what has been described with cyclophosphamide-prednisone treatment alone in relapsed/refractory MM, suggesting synergistic effects of the lenalidomide-cyclophosphamide combination.[27,28] While we could not exactly determine the nature of such a synergy, our results provide some insights for future studies. It seems unlikely that cyclophosphamide had a direct effect on Ikaros/Aiolos, because the levels of

Aiolos and Ikaros returned rapidly to baseline levels within one week without lenalidomide, but with continuous cyclophosphamide. Therefore, the combined effects should have more complex mechanisms. Continuous low-dose cyclophosphamide has been shown to mediate not only direct anti-tumor activity, but also improves anti-tumor immunity via depletion of regulatory T-cells.[39–44,50–52] When combined with lenalidomide, the depletion of Tregs may not be clearly visible, because it is known that lenalidomide causes an increase in the frequencies of regulatory T-cells,[31,41,53–56] probably as a compensatory mechanism for the activated T-cell response. Indeed, we observed only a relatively small increase in regulatory T-cell frequencies during REP treatment. Moreover, the increase in responding patients was less pronounced compared to non-responding patients and showed a stabilization between day 14 and day 28 in the first cycle of REP treatment. In addition, patients achieving at least VGPR had a higher percentage of mid-cycle activated T-cells and also higher baseline and mid-cycle IFNγ-producing T-cells when compared to patients with less than VGPR, also suggesting the contribution of an improved immune response in mediating the anti-MM activity of the REP regimen.

Importantly, due to the phase 2 design of the REPEAT study, it is not possible to define the relative importance of each individual drug to the observed immunomodulatory effects. It may also be possible that the combined effects of lenalidomide and cyclophosphamide are due to their known suppressive effects on angiogenesis and inhibition of MM cell adhesion to stromal cells, which have not been analyzed in this study.[51,57]

In conclusion, we show that in lenalidomide-refractory patients, Ikaros and Aiolos expression levels can be modulated in immune cells during REP treatment, which is paralleled by an increase in activated T-cells. In addition, lenalidomide enhances the PBMC-mediated killing of lenalidomide-refractory MM cells *in vitro*. These results indicate that combining lenalidomide with other immunotherapeutic drugs, such as monoclonal antibodies, can still have potent effects through immunomodulation, even in patients considered to be lenalidomide-refractory.

## MATERIALS AND METHODS

### Patients

The study population consisted of the patients in the phase 2 part of the REPEAT study that has been described in detail previously.[26] Briefly, the REPEAT study was a prospective, investigator-initiated, nonrandomized, multicenter, open-label, phase 1 dose-finding trial, followed by a phase 2 expansion at the recommended dose level (RDL) to evaluate the safety, tolerability, and efficacy of lenalidomide, low-dose oral cyclophosphamide and prednisone (REP) in lenalidomide-refractory MM patients. The maximum tolerated dose (MTD) in phase 1 was the RDL for the patients treated in the phase 2 part of the REPEAT study (25 mg lenalidomide (days 1-21/28 days), combined with continuous low-dose oral cyclophosphamide 50 mg/day and prednisone 20 mg/day). Patients were eligible to participate if they had lenalidomide-refractory disease following at least 1 prior therapy. Lenalidomide-refractory MM was defined as progressive disease during therapy,





no response (less than PR) to prior lenalidomide-containing therapy, or progression within 60 days of discontinuation from lenalidomide-containing regimens, according to the International Myeloma Working Group criteria.[3] REP therapy was given until disease progression. Response was defined according to the "International Myeloma Working Group Criteria for response and minimal residual disease assessment".[3] The study was approved by the institutional medical ethical committee in each participating center in accordance with the declaration of Helsinki. All participants provided written informed consent. The trial was registered at www.clinicaltrials.gov as #NCT01352338. In this analysis, only patients treated at the RDL were included (n=64). Patient characteristics are shown in Table 1.

### Immune-monitoring

Peripheral blood samples were collected at day 1 and 14 of cycle 1, as well as on day 1 of cycle 2 of REP treatment (before administration of the anti-MM agents). To identify the different immune cell subsets, nucleated cells of whole blood were stained with fluorochrome-conjugated antibodies after lysis of red blood cells (Lysing solution, BD Biosciences). The following antibodies were used: CD3-FITC, CD4-APC, CD8-PE and CD45-PerCP (T-cells), CD3-FITC, CD16/56-PE, CD45-PerCP and CD19-APC (NK-cells and B-cells), HLA-DR-PE, CD3-PerCP and CD8-APC (activated T-cells) (all antibodies from BD biosciences). Activated T-cells were defined as the percentage of T-cells expressing HLA-DR.

Flow cytometry was performed using a FACS Canto II or LSR Fortessa Analyzer (BD Biosciences) and data were analyzed using FACSDIVA v8.0.1 software (BD Biosciences). Additional analyses were performed with cryopreserved peripheral blood mononuclear cells (PBMCs), isolated by Ficoll density centrifugation. The LIVE/DEAD® Fixable Dead Cell Staining kit (Thermofisher Scientific) was used to determine the viability of the cells prior to the fixation and permeabilization and subsequent intracellular staining with antibodies against Ikaros, Aiolos, interferon-γ (IFNγ) and interleukin-2 (IL-2). The FOXP3/Transcription Factor Staining Buffer Set (eBioscience) was used for the fixation and intracellular staining according to the manufacturer's protocol. To measure intracellular Ikaros and Aiolos expression, PBMCs were labeled with appropriate surface markers to identify T, B and NK-cells and stained intracellularly for Ikaros and Aiolos using PE-conjugated antibodies (BD Pharmingen). Cytokine production (IL-2 and IFNγ) of T-cells was measured after stimulating PBMCs with CD3/CD28 Human T-activator Beads (Dynabeads®) in a 1:1 ratio for 5 hours at $37^0$ C in the presence of an inhibitor of intracellular protein transport (Brefeldin A, eBioscience). For the gating strategy used in the flow cytometry analysis to detect activated T-cells, T-cells producing IL-2 or IFNγ and regulatory T-cells, see Supplementary Figure 1A-C.

### Generation of lenalidomide-resistant cell lines

The human MM-derived lines MM1.S and L363 (ATCC, Manassas, VA, USA) were maintained and routinely tested for mycoplasma. Acquired lenalidomide-resistant cell lines were generated as previously described and cultured in the presence of 10 μM lenalidomide.[45]

Lenalidomide was removed from culture for a minimum of 5 days prior to any in vitro experiments described in the manuscript.

### PBMC co-culture with MM cells

PBMCs were isolated from buffy coats obtained from healthy donors via ficoll separation. Isolated PBMCs were treated with solvent control or lenalidomide for 1 h prior to stimulation with 3 mg/ml plate-bound anti-CD3 (OKT3; eBiosciences). After 72 hrs of culture, supernatants were collected for IL-2 ELISA (R & D Systems). The PBMCs were washed and subsequently co-cultured with carboxyfluorescein succinimidyl ester (CSFE, Invitrogen)-labeled MM cells at 3:1 ratio for 4 hours; supernatants were collected again at the end of the co-culture for Granzyme B release ELISA (Biolegend). The cells were then washed and stained with Annexin-V-PE (BD Biosciences) and To-Pro3-APC (Invitrogen) according to manufacturer's protocol. Live target cells were gated as $CFSE^+$, Annexin-$V^-$, and $Topro^-$ singlets by flow cytometry.



### IL-2 and Granzyme B ELISA

IL-2 and Granzyme B level in supernatant were determined using ELISA kit, following manufacturer's protocol from R&D and Biolegend, respectively.

### Statistics

Continuous variables were analyzed using Wilcoxon matched-pairs test or a (paired) T-test depending on the distribution levels. Differences in categorical variables were determined with the Fisher's exact test for two by two tables and otherwise with the Pearson's $\chi 2$ test. Results are expressed as 2-tailed *P* values. A level of $P < 0.05$ was considered significant. Calculations were performed in SPSS version 20.0.0 (IBM SPSS Inc., Armonk, NY, USA) and GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

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## DISCLOSURES

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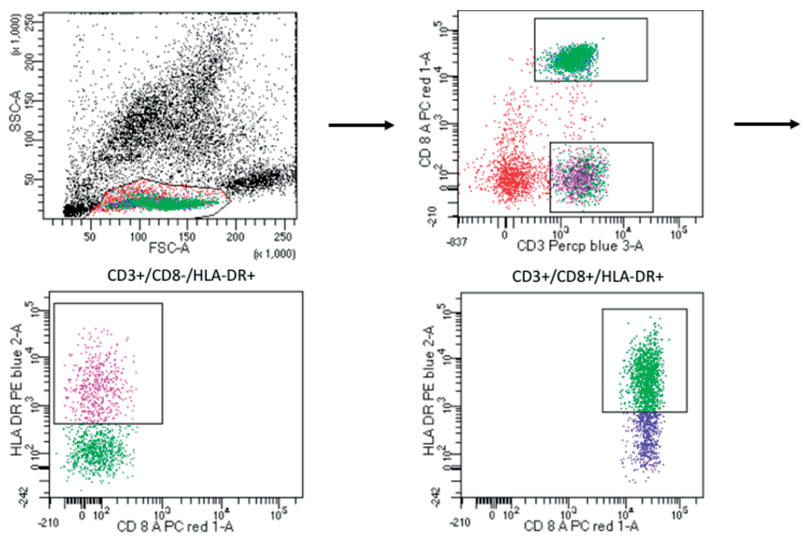
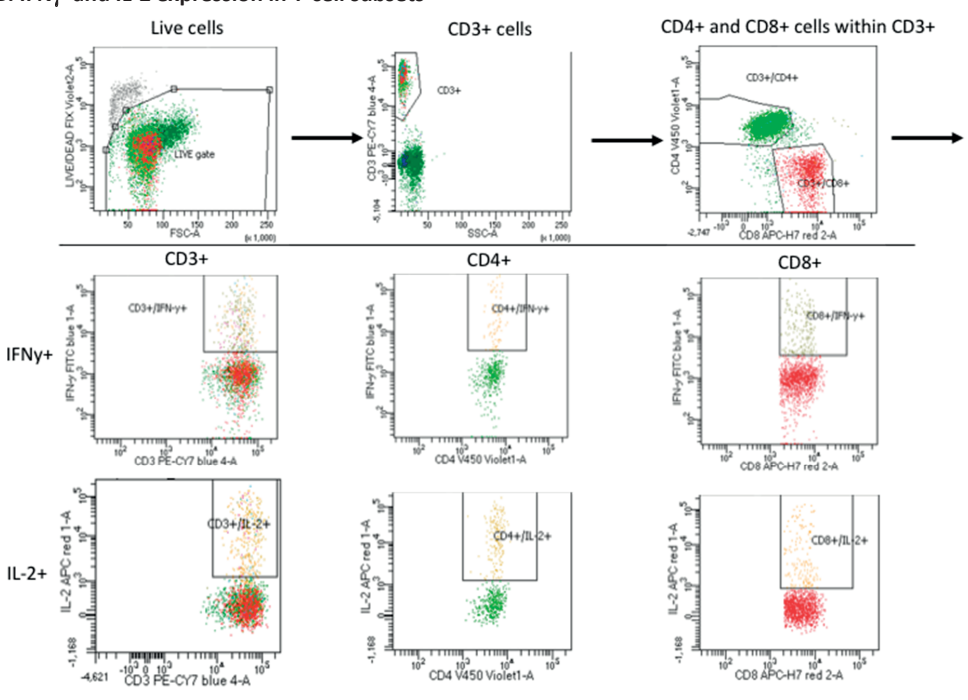
# SUPPLEMENTAL DATA

**A. Activated T-cells**




**B. IFNγ and IL-2 expression in T-cell subsets**



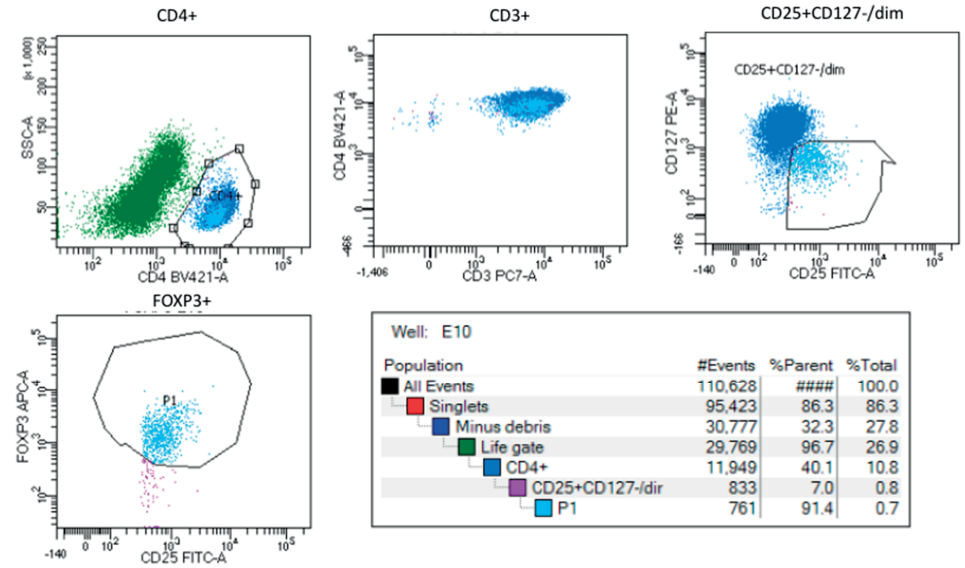

**Supplementary figure 1.** Representative flow cytometry plots showing the gating strategy to detect different lymphocyte subsets: **(A)** Activated T-cells, defined as the percentage of T-cells expressing HLA-DR. CD3+/CD8- cells were considered CD4+ T-cells. **(B)** IFNγ and IL-2 positive T-cells. Cytokine



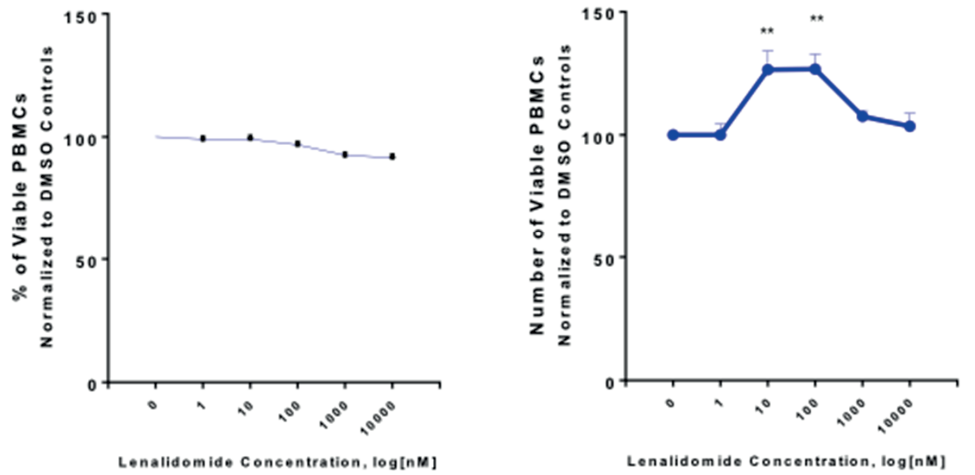




**C. Regulatory T-cells**




production (IL-2 and IFNγ) of T-cells was measured after stimulating PBMCs with CD3/CD28 Human T-activator Beads (Dynabeads®) in a 1:1 ratio for 5 hours at 37° C in the presence of an inhibitor of intracellular protein transport (Brefeldin A, eBioscience). **(C)** Regulatory T-cells were defined as CD3+/CD4+/CD25+/CD127-/dim. FOXP3 intracellular staining was used to confirm the FOXP3 positivity of our defined regulatory T-cells. The FOXP3/Transcription Factor Staining Buffer Set (eBioscience) was used for the fixation and intracellular staining according to the manufacturer's protocol. The LIVE/DEAD® Fixable Dead Cell Staining kit (Thermofisher Scientific) was used to determine the viability of the cells prior to the fixation and permeabilization and subsequent intracellular staining.






**Supplementary figure 2.** Effect of lenalidomide on viability of PBMCs. Following DMSO or lenalidomide treatment and anti-CD3 stimulation for 72 hours, and prior to co-culture, viability of PBMCs was determined by flow cytometry. After 2 washes with PBS, these PBMCs were stained with Annexin-V (BD Biosciences) and To-Pro3 (Invitrogen) according to manufacturer's protocol, and CountBright™ Absolute Counting Beads (ThermoFisher) were added for absolute cell count. Live PBMCs were gated as Annexin-V$^-$ and Topro$^-$ population, and enumerated by the counting beads according to manufacturer's protocol. **(A)** Percent viable PBMCs of total PBMCs was plotted (n=4). **(B)** Viable PBMCs were enumerated with Countbright ™ absolute counting beads according to manufacturer's protocol (n=4). *P*-values were calculated using one-way ANOVA, ** $P<0.01$.

# Part 3

## GENERAL DISCUSSION AND APPENDIX

# Chapter 8

## GENERAL DISCUSSION

The prognosis of multiple myeloma (MM) patients has significantly improved over the last decades. However, the disease is still incurable, and eventually all patients will relapse. Therefore, there is a continuing need for improvement of existing therapies and development of new, more effective treatments. Evidence of the immunogenicity of myeloma and potential of immunotherapy in MM comes from the observation that a subset of patients can experience long-term survival following allogeneic stem cell transplantation (allo-SCT), with or without donor lymphocyte infusions (DLI).[1] However, significant hurdles have to be overcome towards effective immunotherapy for MM. The presence of immunosuppressive cell subsets in the bone marrow microenvironment, production of immune-suppressive cytokines, expression of co-inhibitory molecules and cell adhesion-mediated immune resistance all hamper effective anti-MM immune responses.[2–15] In this thesis, we have studied two immunotherapy modalities for MM. First, we studied allo-SCT, because it has the potential to induce long-term disease remission, but its use in MM is hampered by high treatment related mortality and frequent relapses, indicating a need for improvement. Second, we studied MM patients who became resistant to immunomodulatory drugs (IMiDs), as these patients have a very poor prognosis and insight into the mechanisms of resistance and new combination therapies are needed in order to improve the outcome of these patients.



## ALLOGENEIC STEM CELL TRANSPLANTATION

In **chapter 2** we outlined our single-center experience with allo-SCT in 147 patients with MM with the primary aim to identify a subgroup of patients with known high-risk disease, who would benefit from allo-SCT. Therefore, we analyzed the outcome of patients transplanted as part of first line treatment, as well as patients in a relapsed/refractory setting. In addition, we studied two subgroups of high-risk patients: those with abnormal metaphase cytogenetics or del(13q14) determined by FISH, and in the relapsed setting patients with an early relapse or progression after autologous SCT (ASCT).[16–20] The outcome of patients transplanted as part of first-line therapy (being a tandem auto-allo approach in 98.3%) in our cohort was remarkably good and compared favorably to results described in the literature where 'upfront' allo-SCT has mostly been compared to high-dose chemotherapy and single auto-SCT or tandem auto-SCT in donor-vs.-no-donor comparisons, with conflicting results.[21–30] We observed a high complete response (CR) rate after allo-SCT of 48.3%. Median progression-free survival (PFS) was 30.2 months and median overall survival (OS) was not reached (10-yr. OS was 51% in our cohort). We also observed a plateau in the PFS and OS curves; however, it remains unclear whether these patients can be considered to be cured. Despite these encouraging survival outcomes, we also found a cumulative incidence of chronic graft-vs.-host disease (cGVHD) of 50% in this group. This probably has a major impact on quality of life.[31,32] In addition, cumulative incidence of non-relapse mortality (NRM) at 10-years in first line patients was 15.5%. Another important issue is the use of novel agents such as proteasome inhibitors and IMiDs. Fifty percent of our upfront patients were transplanted before 2005, when the use of novel agents was limited. Only 30% of our upfront

patients received thalidomide-based treatment, 10% bortezomib-based treatment and none received lenalidomide pre allo-SCT. This makes interpretation of these results difficult, given the extensive use of novel agents at the present time. The recent phase III EMN02/HO95 study compared single versus double autologous transplantation in newly diagnosed MM patients after bortezomib-cyclophosphamide-dexamethasone induction.[33] Three-year PFS was 73% versus 64% (P= 0.040) in the double versus single ASCT group, with a 3-year OS of 89% and 82% (*P*= 0.011) respectively. A randomized trial investigating lenalidomide-bortezomib-dexamethasone (RVD) alone or RVD followed by autologous transplantation in newly diagnosed MM patients, showed a median PFS of 50 months and a 4-year OS of 81% in the ASCT group.[34] A large retrospective study analyzing the outcome of novel agent based induction treatment followed by ASCT in newly diagnosed patients again confirmed the beneficial outcome of novel-agents based therapy with overall median PFS of 32 months and OS of 96.1 months.[35] Although a direct comparison with other trials is difficult, these results suggest that in the novel agent era, the outcome of newly diagnosed patients is better with a single or double ASCT than with upfront allo-SCT, without the treatment-related mortality (TRM) observed with allo-SCT.

Very few studies have described the outcome of patients with high-risk cytogenetic aberrations after allo-SCT in an upfront setting. Long-term results of the EBMT-NMAM2000 study show an equal outcome for patients with and without del(13q14).[27] In addition, Kroger et al. found that allo-SCT overcomes the adverse prognosis of del(17p) and t(4;14).[36] In a prospective study comparing patients with del(13q14), Knop et al. found a significantly increased PFS for the auto-allo arm, compared with double ASCT and also a significantly better OS in the allo-SCT arm for the subgroup with del(17p).[37] In contrast to these studies, the French group found no difference in outcome, in high-risk patients with MM (defined by high β2-microglobulin and presence of del(13q14)), when double ASCT was compared with tandem auto-allo.[29,30] However, the high-dose ATG used in that protocol may have negatively influenced the outcome of the allogeneic transplantation. Our subgroup analysis in patients with cytogenetic aberrations showed no difference in outcome compared with standard-risk patients, suggesting that allo-SCT in the upfront setting might overcome the unfavorable prognosis of these cytogenetic aberrations. Unfortunately, we were not able to obtain data on the presence of t(4;14), del(17p), and t(14;16) due to the retrospective character of our study and the fact that assessment of these cytogenetic aberrations was not routine practice at the time of transplant for most of these patients. It is important to keep in mind that in none of the trials described above (except some patients in the study by Kroger et al.[36]) patients received novel agents as induction treatment. This makes interpretation of these studies difficult in the current novel agent era. In the recent phase III EMN02/HO95 study described earlier, 26% of patients in de single ASCT and 21% of patients in the double ASCT group had high-risk cytogenetic features (defined as t(4;14) and/or t(14;16) and/or del(17p)).[33] Importantly, double ASCT overcame the adverse prognosis when looking at 3-year PFS (76% vs. 69% for standard risk patients, *P*=0.482) and OS. Therefore, we argue that







allo-SCT should not be considered an option for newly diagnosed MM patients, regardless of their cytogenetic risk.

The discussion on whether allo-SCT should be offered to relapsed/refractory patients is still ongoing. Recent guidelines describe that allo-SCT can be considered appropriate therapy for younger patients with a relapse within 18 months after a primary ASCT.[38] However, very few comparative studies have been published on the use of allo-SCT in MM relapsing after initial autologous transplant, all hampered by their retrospective nature.[39–41] Although these studies indicate a PFS and OS advantage in patients who are given an allo-SCT, high rates of NRM and GVHD incidence are observed. Furthermore, these results cannot be translated to the current treatment era with second- and third generation novel-agents available at relapse. We found a very poor outcome of allo-SCT in relapsed patients, with a short median PFS of 8.0 months and a median OS of 28.2 months. If we focus only on patients relapsing within 18 months after auto-SCT, median PFS was 6.5 months compared with 9.7 months in patients relapsing after 18 months. In addition, patients relapsing within 18 months after ASCT had inferior response rates and response duration to relapse treatment after allo-SCT, which translated in a worse median OS of 22.8 months compared with 52.4 months in patients relapsing within or after 18 months, respectively. Although the overall survival of patients relapsing after 18 months is promising, the short PFS in this group suggests a poor graft-vs.-myeloma effect and the extended OS probably reflects effective relapse treatment after allo-SCT with novel agents. Therefore, we conclude that if applied at all in relapsed myeloma, new treatment options for allo-SCT are urgently needed. In this respect, the value of optimal induction, maintenance therapy, and post-allo-SCT immunotherapy should be explored, as well as strategies to lower NRM and acute and chronic GvHD.

One of these post allo-SCT immunotherapy strategies was investigated in **chapter 3**. Here, we demonstrated the feasibility, safety and efficacy of minor histocompatibility antigen (mHag)-peptide-loaded donor dendritic cell (DC) vaccination combined with DLI in patients with persistent or relapsed disease after allo-SCT and previous DLI. DC vaccination was combined with a second equivalently dosed DLI, because T cells from the first DLI, as they did not induce any sustainable graft-versus-tumor (GVT) effect, could have been anergized or dysfunctional, which is often the case in MM patients.[11,42–45] In agreement with our previous results, we observed a low toxicity from mHag-peptide-pulsed donor DC vaccinations, even after combination with a second DLI, demonstrating the safety of the approach.[46] The observed immunological responses in this trial provide evidence for the capacity of mHag-peptide-loaded DCs to induce or boost tumor-reactive cytotoxic T lymphocytes (CTLs). However, the developed CTL responses did not translate into robust clinical responses and even the induction of a considerably high frequency of mHag UTA2-1-specific CTLs in one patient did not translate into a clear clinical response. The mechanisms behind this lack of clinical response should be evaluated in future studies, but as has been described in **chapter 4**, is seems highly plausible that the expansion or even the cytotoxic function of tumor-specific CTLs can be actively suppressed by tumor (microenvironment)-

related factors. In this respect, several well-described suppressive cytokines (for example, TGF-β and IL-10), immune cells (for example, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)) and immune-checkpoint molecules, such as PD-L1, could play a role.[47,48] Regarding the latter, we have previously shown that the presence of immune-checkpoint molecules on DCs hampers sufficient priming and boosting of mHag-specific T cells.[49,50] Importantly, siRNA-mediated silencing of PD-1 ligands on DCs increases the immunogenicity of the vaccine and thereby boosts the induction of mHag-specific T cell responses.[51,52] A similar scenario is plausible when effector CTLs encounter PD-L1+ tumor cells. Thus, as already suggested by others, the use of systemic immune-checkpoint inhibitors may improve the outcome of DC vaccination, but has to be used with caution in combination with DLI due to the potential risk of eliciting severe GvHD.[53,54] In addition to these possible future perspectives, our current results point out that patient selection for vaccination strategies is crucial. Patients with a low tumor burden and stable disease are the best candidates for immune therapy with DCs. But even in these patients, our focus should be on strategies targeting tumor-induced immune suppression, to overcome this important barrier to effective anti-tumor immune therapy.

In **chapter 4**, we further analyzed the role of immunosuppressive cell subsets in dampening the anti-tumor immune response post allo-SCT. We showed that circulating MDSCs and Tregs are found at increased frequencies in a subset of MM patients with persistent or progressive disease, months after allo-SCT. The increased levels of Tregs, but not of MDSCs, were associated with an impaired response to DLI, a lower incidence of cGVHD, as well as a decreased PFS and OS. We chose to address our questions in the DLI setting, because, as compared with the direct post-allo-SCT setting, this provides a better platform to evaluate the therapeutic impact of suppressor cells without being biased by GVHD prophylaxis, post-transplant homeostatic lymphocyte expansion and a post-transplant inflammatory state. In contrast with other studies, which correlated the DLI/SCT outcome with suppressor cell levels present in the infused cell products,[55–59] we correlated DLI outcome with the frequency of cell subsets present in patients before DLI. Consistent with previous reports,[5,60] we found significantly elevated levels of CD14+ and CD14– MDSCs also in transplanted MM patients, before DLI. We also confirmed their functional competence (suppressor capacity) in these patients before DLI. We found no evidence for an association between MDSCs and DLI outcome. However, due to the use of cryopreserved peripheral blood mononuclear cells (PBMCs), we were not able to study the CD15+CD14- granulocyte-like MDSC subset. Regarding Tregs, contradictory results have been reported in various settings. Although some, especially murine studies, found that Tregs do not hamper GVT responses,[61–63] others demonstrated the opposite.[57,64–66] Some discrepancies between mouse and human studies may be related to species-specific aspects. These discrepancies could also be because murine models do not take into account the heterogeneity seen in the human setting. Underscoring this, we have found a considerable level of heterogeneity in the Treg levels in pre-DLI samples of MM patients. Remarkably, and confirming the results of several clinical studies,[57,66] Treg







levels in pre-DLI samples were clearly associated with DLI responsiveness, which in turn was a good predictor of PFS. Importantly, we also found that high levels of Tregs were associated with impaired OS. It has previously been shown that Tregs modulate OS in newly diagnosed MM patients.[2] In that report, higher percentages of Tregs were also encountered in patients who died of infectious complications, suggesting that the modulation of OS by Tregs can be multifactorial. Our findings showing that high pre-DLI levels of Tregs are associated with DLI non-response and decreased OS may have important clinical implications. First, our results suggest that GVHD prevention by Treg coinfusions in the DLI setting is not a favorable idea, because of the potential impairment of the GVT effect. Second, our results suggest a careful monitoring of Treg levels before DLI, with the intention to apply a pre-DLI Treg depletion strategy. As the levels of Tregs in some patients are much higher than in healthy donors, we think that this could be a more effective strategy to improve DLI responses compared with Treg depletion from the DLI products. For example, the administration of a continuous, low dose of cyclophosphamide has been shown to selectively deplete Tregs and has also been combined with other cellular immunotherapies.[67–70] Importantly, as low pre-DLI levels of Tregs do not seem to increase acute GVHD (aGVHD) incidence in our cohort, it may be possible that this in vivo Treg depletion strategy may not induce the early morbidity and mortality associated with aGVHD.

In conclusion of the allo-SCT related studies described in this thesis, we show that although we obtained beneficial results with allo-SCT as part of first line treatment, allo-SCT should not be considered an option for first line treatment due to the high TRM and superior alternatives with novel agent-based therapy, regardless of cytogenetic risk. For the relapsed/refractory setting the data are less clear. At least in our institution the outcome after allo-SCT for patients with an early relapse after auto-SCT is very poor. If applied at all, strategies to lower NRM and GVHD incidence should be actively explored. In this respect, post-allo cyclophosphamide was described to lower GVHD incidence, while preserving immunity against infections and could serve as a platform for post-allo-SCT immunotherapy.[71–74] Furthermore, bortezomib as part of the reduced-intensity conditioning regimen, and given as maintenance treatment post allo-SCT was shown to be safe and might reduce GVHD occurrence and improve the GVT effect.[71,75–78]

The use of mHag-peptide-loaded donor DC vaccination combined with DLI in patients with persistent or relapsed disease after allo-SCT was shown feasible and safe. However, immune-responses were variable and clinical responses were lacking. This may be in part explained by the elevated frequencies of circulating immunosuppressive cell subsets as MDSCs and Tregs. In addition, it was previously shown that the presence of immune-checkpoint molecules on DCs hampers sufficient priming and boosting of mHag-specific T cells.[49,50] Both targeting of PD-L1 on DCs as well as targeting immunosuppressive subsets and cytokines might lead to better immune and clinical responses. Furthermore, the optimal type of DC subset (monocyte-derived, myeloid or plasmacytoid-DCs), as well as the optimal route of vaccination to induce anti-tumor responses are currently unknown.[79]

## IMMUNOMODULATORY DRUGS

In **chapter 5** we investigated in a phase 1/2 trial, the maximum tolerated dose as well as the safety and efficacy of the combination of lenalidomide, cyclophosphamide and prednisone (REP) in heavily pretreated, lenalidomide-refractory MM patients. The maximum tolerated dose was determined to be 25 mg of lenalidomide on days 1 to 21 of a 28-day cycle, combined with continuous oral cyclophosphamide at a dose of 50 mg, and prednisone at a dose of 20 mg. The REP regimen was well tolerated and highly active with an overall response rate (ORR) (≥ partial response (PR) of 67% and a clinical benefit rate (≥ minimal response) of 82%. The median PFS was 12.1 months, and the median OS was 29.0 months. Hematologic toxicities in our study were acceptable and consistent with the observed toxicities in MM patients treated with lenalidomide-dexamethasone.[80,81] Similarly, when cyclophosphamide was added to pomalidomide and prednisone,[82] hematologic toxicity was comparable to the toxicity observed with pomalidomide-dexamethasone.[83,84] Altogether, this suggests that low-dose cyclophosphamide does not significantly increase hematologic toxicity, when it is added to lenalidomide or pomalidomide. Nonhematologic toxicity of REP consisted mainly of infections. Discontinuations because of adverse events were uncommon, allowing patients to continue therapy until disease progression. No second primary malignancies were observed in this study. We observed high activity of REP despite enrolling patients who were all lenalidomide refractory and 66% who were also bortezomib refractory. Although the importance of high-risk cytogenetic features in advanced relapsed/refractory MM has not been clearly defined, we observed similar response and survival in patients with high-risk cytogenetic abnormalities when compared with standard-risk patients. Outcome was also similar in patients with lenalidomide and bortezomib, double-refractory, MM. Notably, we observed a median PFS of 14.3 months and median OS not yet reached in double-refractory MM patients, which compares favorably with historical controls of patients who were refractory to both IMiDs and bortezomib, who had a median event-free survival of 5 months and median OS of 9 months.[85]

We previously showed that the 2-drug combination of continuous low-dose cyclophosphamide and prednisone has also significant anti-MM activity in relapsed/refractory MM patients, who were not previously exposed to novel agents.[86] However, another study showed that low-dose cyclophosphamide (50 mg daily) combined with steroids has markedly lower activity in lenalidomide- and bortezomib-exposed patients (63% of these patients were double-refractory to bortezomib and IMiDs), with at least PR in 11.4% of these patients and a median PFS and OS of only 3.3 months and 10.0 months, respectively.[87] This outcome is inferior to that observed with the REP regimen in double-refractory MM patients and suggests clinical synergy between lenalidomide and low-dose cyclophosphamide-prednisone alone would be needed to formally demonstrate the synergy between lenalidomide and cyclophosphamide.







Importantly, ORRs with REP were higher and median PFS was longer, when compared with the outcome of next generation novel agents evaluated in lenalidomide-refractory MM. Treatment with pomalidomide plus dexamethasone results in at least PR in 31% of patients with a median PFS of 4.0 months (75% lenalidomide- and bortezomib-refractory MM patients).[83] Carfilzomib monotherapy induces at least PR in 19.1% of extensively pretreated patients with a median PFS of 3.7 months.[87] Daratumumab induces at least PR in 29% to 36% of patients with a median PFS of 3.7 to 5.6 months,[88,89] whereas elotuzumab[90] has no single-agent activity in this setting. The outcome of the REP regimen also compares favorably to the results of carfilzomib- or pomalidomide-based combinations in relapsed/refractory, mostly lenalidomide-refractory, MM patients, such as pomalidomide-bortezomib-dexamethasone (≥ PR: 85%, median PFS 10.7 months),[91] pomalidomide-carfilzomib-dexamethasone (≥ PR: 50%, median PFS: 7.2 months),[92] and daratumumab-pomalidomide-dexamethasone (≥ PR: 71%, PFS at 6 months: 66%).[93] Nevertheless, cross-trial comparisons must be interpreted with caution, because such a comparison might be biased by multiple factors as differences in trial sizes, patient populations, and study designs. Although several of the new agents to treat lenalidomide- and bortezomib-refractory MM are now approved by the US Food and Drug Administration and/or the European Medicines Agency, these therapies may not yet be available or reimbursed in many countries, whereas cyclophosphamide is widely available. In addition, REP is a fully oral 3-drug combination, which is convenient for patients but also likely associated with lower costs of patient care. Altogether, this highlights the importance of this effective salvage strategy for heavily pretreated relapsed/refractory MM patients.

In **chapter 6**, to gain insight into the expression levels of lenalidomide targets in lenalidomide-refractory patients, we analyzed the protein expression of Cereblon, Ikaros, Aiolos, IRF4 and c-Myc in BM-localized plasma cells using IHC. The patient population consisted of patients included in the phase 2 of the REPEAT study. Our correlative analysis showed that development of lenalidomide-resistance is associated with reduced Cereblon protein levels in the majority of patients. This Cereblon loss observed in clinical samples is consistent with results from previously conducted preclinical studies, which showed that lenalidomide resistant MM cell lines have reduced levels of Cereblon mRNA and protein.[94,95] Our observation that Cereblon expression levels did not show a significant change during other treatment lines indicates that the decrease in Cereblon is specifically associated with acquired lenalidomide resistance. In addition, we showed similar expression levels of Cereblon before and after treatment with bortezomib in lenalidomide-naïve patients, which is consistent with previous studies which showed that Cereblon expression is not correlated with response to bortezomib treatment.[94–98]

The underlying mechanisms of Cereblon downregulation remain to be determined. Epigenetic modifications of the Cereblon promotor region were previously described as a mechanism of Cereblon downregulation.[99,100] Other possible mechanisms may involve Cereblon gene mutations or chromosomal deletions although these mechanisms need to be addressed in future studies. Interestingly, Cereblon expression was not reduced in

a subset of patients in our current analysis. Other mechanisms of lenalidomide-resistance are likely involved in these patients without Cereblon downregulation, such as the presence or development of Cereblon pathway mutations, Cereblon splice variants, or increased activity of the MEK/ERK pathway.[101–103] Given the limited availability of patient samples from our current study, no additional analysis of mRNA levels or analysis of Cereblon pathway mutations could be performed to further characterize these potential mechanisms.

We found no association between Cereblon levels and overall response to REP treatment, which indicates that the anti-myeloma effect of lenalidomide as part of the REP regimen may not be mediated via plasma cell Cereblon. In this respect, there is evidence that lenalidomide can still have immunomodulatory activity in the setting of lenalidomide-refractory disease,[104] which might be complementary to the previously described immune-activating effects of metronomically dosed cyclophosphamide.[68] Alternatively, modulation of residual Cereblon by lenalidomide may still be able to synergize with the other components of the REP regimen.

In **chapter 7** we further investigated the hypothesis that despite the presence of lenalidomide resistance in the MM cells, the immune system might still be susceptible to lenalidomide-induced activation. To this extent, we analyzed immune cell subsets of patients treated in the REPEAT study, to characterize the effect of this treatment on the immune system *in vivo*. Peripheral blood samples were collected at day 1 and 14 of cycle 1, as well as on day 1 of cycle 2. We observed a significant mid-cycle decrease in the Cereblon substrate proteins Ikaros and Aiolos in diverse lymphocyte subsets, which was paralleled by an increase in T cell activation. These effects were reversed at day one of the second cycle, which was after one week without lenalidomide treatment. Our results are consistent with a recent study, showing similar immune-activating effects of pomalidomide-dexamethasone in lenalidomide-refractory patients.[101,105] However, our observation that lenalidomide itself retains its immunomodulatory capacity despite the presence of lenalidomide-refractory MM has not been described before. *In vitro*, lenalidomide enhanced PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant MM cells in a co-culture system. These results indicate that the Cereblon-mediated immunomodulatory properties of lenalidomide are maintained in lenalidomide-refractory myeloma patients and contribute to immune-mediated killing of MM cells. Therefore, combining lenalidomide with other drugs can still have potent effects, even in patients considered to be lenalidomide-refractory. In fact, pre-clinical and clinical studies have already shown promising results combining lenalidomide and therapeutic antibodies such as isatuximab and daratumumab (anti-CD38) in lenalidomide-refractory MM patients.[106–108] With regard to the REP combination, it seems unlikely that cyclophosphamide had a direct effect on Ikaros/Aiolos, because the levels of Aiolos and Ikaros returned rapidly to baseline levels within one week without lenalidomide, but with continuous cyclophosphamide. The beneficial effect of cyclophosphamide could also be related to its effect on Tregs, as cyclophosphamide has been described to improve anti-tumor immunity via depletion of Tregs.[67–69,109,110] When combined with lenalidomide, this effect may not be clearly visible, because it is known that lenalidomide causes an increase in the frequencies of Tregs,[111–115] probably as a compensatory mechanism for the activated T





cell response. In agreement with this idea, we observed only a small increase in regulatory T cell frequencies during REP treatment. Moreover, the increase in responding patients was less pronounced compared to non-responding patients and showed a stabilization between day 14 and day 28 in the first cycle of REP treatment. This idea should however be further evaluated in proper in vivo models. It may also be possible that the combined effects of lenalidomide and cyclophosphamide are due to their known suppressive effects on angiogenesis and inhibition of MM cell adhesion to stromal cells, which have not been analyzed in this study.[116,117]

In conclusion for part two, we show that Cereblon downregulation in MM cells is one of the characteristics of lenalidomide refractory disease. Several underlying mechanisms of Cereblon downregulation have been described.[99–103] It is interesting to speculate on the potential reversibility of some of these mechanisms, for example epigenetic modifications of the Cereblon promotor region.[100] In addition, other pathways involved in lenalidomide resistance may be targetable, such as increased activity of the MEK/ERK pathway.[102] Whether this would lead to re-inducing lenalidomide sensitivity in MM patients remains unknown. Interestingly, the immune system of lenalidomide-refractory MM patients is still susceptible to lenalidomide-induced activation, and lenalidomide enhanced PBMC-mediated killing of lenalidomide-sensitive and lenalidomide-resistant MM cells *in vitro*. Therefore, irrespective of the sensitivity of the MM cells to lenalidomide, its use in combination with other drugs can contribute to the anti-MM effect. One of these combinations, REP, was shown to be a well-tolerated and highly active combination for patients with lenalidomide- and bortezomib refractory MM.

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# Appendix



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# NEDERLANDSE SAMENVATTING

## Multipel myeloom

Multipel myeloom (MM) is een kwaadaardige ziekte die zich kenmerkt door ongecontroleerde deling van plasmacellen in het beenmerg. Het is een ziekte die vooral bij oudere patiënten voorkomt, met een mediane leeftijd bij diagnose van 70 jaar en slechts 5% van de patiënten onder de 40 jaar. Klinisch kenmerkt MM zich door bothaarden, te hoog calciumgehalte in het bloed, eiwitneerslagen in de nier wat aanleiding kan geven tot nierinsufficiëntie, en progressief beenmergfalen met bijbehorend bloedarmoede en een tekort aan andere bloedcellen zoals witte bloedcellen en bloedplaatjes. De behandeling van MM is de laatste decennia in een stroomversnelling gekomen met de ontwikkeling van vele nieuwe middelen. Desondanks is de ziekte nog steeds niet te genezen en is de mediane overleving na diagnose zo'n zeven tot tien jaar. Dit geeft aan dat er -ondanks de ontwikkeling van nieuwe behandelingen- een noodzaak is tot verdere verbetering van reeds bestaande therapieën en ontwikkeling van nieuwere, meer potente vormen van behandeling.

Immunotherapie heeft de laatste jaren een belangrijke rol gespeeld in de behandeling van kanker, zo ook bij MM: allogene stamceltransplantatie kan bij bepaalde patiënten voor een langdurige overleving zorgen, immuunmodulerende anti-MM middelen hebben de prognose van patiënten in sterke mate verbeterd, de ontwikkeling van monoclonale antilichaam therapie was een doorbraak voor patiënten die niet meer reageerden op verschillende lijnen van behandeling en de ontwikkeling van zogenoemde CAR-T cellen lijkt ook voor MM een belangrijke stap voorwaarts te zijn. Helaas is het MM in staat om effecten van het immuunsysteem, en dus ook immuuntherapie, te omzeilen of te onderdrukken. Zo kunnen MM cellen bepaalde celtypen aantrekken die op hun beurt het immuunsysteem kunnen onderdrukken (regulatoire T-cellen (Tregs) en myeloid-derived-suppressor cellen (MDSCs)), produceren ze stoffen die het immuunsysteem onderdrukken (cytokines als TGF-β en IL-6), zorgen ze voor eiwitten op hun celmembraan die na binding aan eiwitten op het immuunsysteem een anti-tumor reactie onderdrukken (PD-L1) en zorgt binding aan stromacellen in het beenmerg voor een verminderde gevoeligheid voor immuun-gemedieerde celdood van MM cellen. Al deze factoren compliceren het ontwikkelen van effectieve immuuntherapie. In dit proefschrift hebben we twee immuuntherapeutische behandelmodaliteiten onderzocht: allogene stamceltransplantatie en immuunmodulerende anti-MM middelen.

## Hematopoietische allogene stamceltransplantatie

Allogene stamceltransplantatie kan worden beschouwd als een van de oudste vormen van immuuntherapie bij MM. Bij een hematopoietische allogene stamceltransplantatie worden bloedvormende stamcellen van een donor toegediend aan de patiënt, die voorbehandeld is met bestraling en/of chemotherapie. De witte bloedcellen van de donor, die mee getransplanteerd kunnen worden, herkennen resterende tumorcellen in de patiënt als 'vreemd' en kunnen deze daardoor opruimen. Dit is het zogenoemde "graft-versus-tumor

effect" (GVT). Helaas herkennen witte bloedcellen van de donor niet alleen tumorweefsel, maar kan ook normaal weefsel voor 'vreemd' worden aangezien. De hierop volgende immuunreactie leidt tot vernietiging van dit gezonde weefsel: omgekeerde afstoting of "graft-versus-host ziekte" (GVH). Deze complicatie leidt tot aanzienlijke sterfte bij deze behandeling. De kans op omgekeerde afstoting wordt vooraf zo klein mogelijk gemaakt door patiënt en donor te "matchen" op basis van een weefseltypering (HLA-typering; HLA-matched). Echter, ondanks deze weefseltypering kunnen de weefsels toch van elkaar verschillen. Door bijvoorbeeld 'kleine' mutaties in coderende gebieden van het DNA kunnen eiwitten ontstaan die net iets verschillen in aminozuurvolgorde tussen patiënt en donor. Als deze eiwitten in de cel worden afgebroken tot kleine fragmenten (peptiden) en deze op het celoppervlak gepresenteerd worden ontstaan zogenoemde "minor antigenen". Als de witte bloedcellen van de donor deze minor antigenen vervolgens als vreemd herkennen kan een krachtige immuunreactie ontstaan. Als deze minor antigenen afkomstig zijn uit bloedcellen is deze immuunreactie gericht tegen de bloedcellen (waarbij in het geval van MM ook de kwaadaardige plasmacellen) en ontstaat het graft-versus-tumor effect. Als deze minor antigenen echter afkomstig zijn uit gezonde weefsels dan kan omgekeerde afstoting ontstaan.

Hoewel allogene stamceltransplantatie een krachtig GVT effect kan geven (welke niet aanwezig is bij een stamceltransplantatie met eigen stamcellen), zijn de resultaten bij MM in de eerste lijn van behandeling niet eenduidig. Sommige studies laten een voordeel zien van allogene stamceltransplantatie in vergelijking met een transplantatie van eigen stamcellen na behandeling met hoge dosis chemotherapie, maar andere vonden geen voordeel. De interpretatie van deze studies is lastig, omdat ze vrijwel allemaal verricht zijn voordat de nieuwe (veel effectievere) anti-MM middelen op de markt kwamen. Hier komt nog bij dat 10-30% van de patiënten komt te overlijden als gevolg van de allogene stamceltransplantatie. Dit heeft gemaakt dat het niet langer wordt gezien als 1e lijn behandeling bij MM. Patiënten die een recidief van hun MM hebben binnen 18 maanden na de hoge dosis chemotherapie en een stamceltransplantatie met eigen stamcellen, hebben een hele slechte prognose. Richtlijnen adviseren dan ook om een allogene stamceltransplantatie bij die patiënten te overwegen in studieverband. Echter, de resultaten van allogene transplantatie bij die patiëntengroep zijn niet goed beschreven.

Om de uitkomst van allogene stamceltransplantatie bij MM te verbeteren zijn een aantal zaken van belang: het ontwikkelen van minder toxische voorbereidende chemotherapeutica, het verbeteren van (aanhoudende) GVT effecten en reductie of betere behandeling van graft-versus-host ziekte.

In **hoofdstuk 2** beschrijven we een retrospectieve analyse van de uitkomsten van allogene stamceltransplantatie bij 147 MM patiënten. Ons doel was het identificeren van een hoog-risico patiëntengroep, welke mogelijk voordeel liet zien van behandeling met een allogene stamceltransplantatie. Patiënten die in 1e lijn werden behandeld hadden een opvallend goede uitkomst in vergelijking met eerdere studies. Echter, een groot deel van deze patiënten ontwikkelde een chronische omgekeerde afstoting. Tevens was de sterfte gerelateerd aan





de behandeling ruim 15%. Patiënten die pas getransplanteerd werden nadat ze een recidief kregen hadden een zeer slechte overleving. Belangrijk voor de interpretatie van de data is dat in onze patiëntengroep slechts een klein deel de nieuwere anti-MM middelen had gebruikt. Recente studies met deze nieuwe middelen gevolgd door een enkele of dubbele stamceltransplantatie met eigen stamcellen laten vergelijkbare, zo niet betere resultaten zien zonder de hoge sterfte gerelateerd aan behandeling. Daarom zijn wij van mening dat er in de eerste lijn behandeling van MM geen plaats is voor een allogene stamceltransplantatie. Aangezien patiënten met een vroeg recidief een zeer slechte overleving hebben (met of zonder allogene transplantatie) is er onzes inziens slechts ruimte voor allogene transplantatie in deze setting als dit minder toxisch en meer effectief gemaakt kan worden. Een van de manieren om een betere respons na allogene transplantatie te verkrijgen is het geven van afweercellen van de donor (donor lymfocyten infusie of DLI). Helaas laat de meerderheid van de MM patiënten na een DLI een recidief zien. In **hoofdstuk 3** hebben we geprobeerd om DLI effectiever te maken door patiënten te vaccineren met afweercellen van de donor (dendritische cellen) die "minor antigenen" specifiek voor de bloedcellen (en daarmee ook de kwaadaardige plasmacellen) van de patiënt kunnen presenteren aan de afweercellen in de DLI. De manier van vaccineren bleek veilig en haalbaar. Daarnaast vonden we bij vijf van de negen patiënten een inductie van minor-antigeen-specifieke donor afweercellen in het bloed. De klinische respons in alle patiënten was zeer beperkt, en uitte zich hooguit in stabiele ziekte, wat maakt dat verdere verbetering van deze methode noodzakelijk is. Zoals eerder beschreven zou het aantrekken van Tregs en MDSCs door de MM cellen het afweersysteem, en mogelijk ook de DLI, kunnen onderdrukken. In **hoofdstuk 4** hebben we gekeken naar de invloed van deze circulerende suppressor cellen (Tregs en MDSCs) op de respons op DLI bij MM patiënten. Hogere frequenties van Tregs, maar niet van MDSCs, waren significant geassocieerd met non-respons op DLI. Dit zou kunnen betekenen dat het verlagen van Tregs in MM-patiënten de respons op DLI kan verbeteren.

**Immuunmodulerende anti-MM middelen**

Hoewel ze nu collectief "immuunmodulerende middelen" genoemd worden zijn deze middelen initieel niet als immuunmodulerend ontworpen, maar lag de focus met name op hun anti-MM effecten. De laatste jaren werd echter meer en meer duidelijk dat de immuunstimulerende effecten van deze middelen wellicht minstens zo belangrijk zijn als de directe anti-tumor effecten.

Het bekendste immuunmodulerende middel is thalidomide, wat initieel ontwikkeld is als anti-misselijkheid middel tijdens zwangerschap. Nadat de schadelijke effecten van het middel op de foetus duidelijk werden is thalidomide van de markt gehaald. 29 jaar later werden de anti-MM effecten duidelijk, wat leidde tot een snelle toename in klinische studies met dit middel bij MM. De hierna ontwikkelde nieuwere immuunmodulerende middelen zijn lenalidomide en pomalidomide. Al deze middelen hebben een scala aan werkingsmechanismen, o.a. bestaande uit immuunactiverende-, anti-vaatnieuwvorming-, en directe anti-MM effecten. Het blijkt dat deze middelen binden aan het eiwit Cereblon,

wat samen met andere eiwitten (damage-specific DNA binding protein (DDB1), cullin 4A (Cul4) en RING finger protein 1 (ROC1)) een complex vormt (E3-ligase complex $CRL4^{CRBN}$). Na deze binding worden bepaalde eiwitten (Ikaros en Aiolos) naar het complex 'getrokken' en krijgen ze een specifieke markering die ervoor zorgt dat ze afgebroken kunnen worden. Het afbreken van de eiwitten Ikaros en Aiolos zorgt voor een verlaging van bepaalde factoren die van belang zijn voor de groei van MM cellen (IRF4 en cMyc), wat toxisch is voor de MM cellen. Daarnaast zorgt een verminderde aanwezigheid van Ikaros en Aiolos tot een verhoging van de productie van IL-2, een stof die zorgt voor activatie van bepaalde delen van het immuunsysteem. Het netto-effect van al deze middelen is dus grofweg een toxisch effect op de MM cellen en een activatie van het immuunsysteem.

MM patiënten die niet meer reageren op immuunmodulerende anti-MM middelen hebben een slechte prognose. Eerdere studies hebben goede effecten laten zien van continu gedoseerd lage-dosis cyclofosfamide gecombineerd met prednison bij MM patiënten. Onze hypothese was dat de immuunmodulerende effecten van lenalidomide nog aanwezig zouden kunnen zijn, ondanks het feit dat MM cellen niet meer op lenalidomide monotherapie reageren. Om te testen of deze immuunmodulerende eigenschappen synergie konden laten zien met de eerder beschreven combinatie van cyclofosfamide met prednison hebben we een klinische studie verricht. De resultaten hiervan beschreven we in **hoofdstuk 5**. In deze fase I/II studie werden patiënten die niet meer reageerden op lenalidomide (refractair) behandeld met de combinatie van lenalidomide, cyclofosfamide en prednison (REP). Deze behandeling liet een opvallend goede overall response zien van 67% met een mediane progressievrije- en overall overleving van 12,1 en 29,0 maanden respectievelijk. Dit is aanzienlijk hoger dan wat eerder beschreven werd met cyclofosfamide en prednison alleen en verhoudt zich ook gunstig ten opzichte van nieuwe combinatietherapieën bij patiënten die refractair zijn voor lenalidomide.

Om meer inzicht te krijgen in lenalidomide-refractair MM hebben we in **hoofdstuk 6** de eiwitexpressie van Cereblon en downstream eiwitten in de MM cellen bekeken met behulp van een gevalideerde immunohistochemische kleuring. We vonden dat een afname in Cereblon expressie in de MM cellen een van de karakteristieken van lenalidomide-refractair MM is. We vonden geen associatie tussen Cereblon expressie in de MM cellen en response op REP behandeling. Dit wijst dus op een werkingsmechanisme van REP dat niet afhankelijk is van Cereblon expressie in de plasmacel. Dit sluit aan bij onze eerdere hypothese dat de werking van lenalidomide als onderdeel van REP berust op de immuunmodulerende eigenschappen van lenalidomide, in plaats van de directe anti-MM effecten. Om dit verder te analyseren bekeken we in **hoofdstuk 7** verschillende immuuncellen in het bloed van patiënten die behandeld zijn in de REP studie. Hierin analyseerden we met behulp van flowcytometrie de expressie van de $CRL4^{CRBN}$ substraten Ikaros en Aiolos. We vonden dat Ikaros en Aiolos nog steeds afgebroken worden in de immuuncellen van deze patiënten tijdens REP behandeling. Dit ging gepaard met een toename van activatiestatus en immuunsysteem stimulerende cytokines van de T-cellen van de patiënten. Tevens vonden we in vitro dat lenalidomide witte bloedcellen stimuleert om MM cellen dood te maken.





Dat gold zowel voor MM cellen die gevoelig, als voor MM cellen die ongevoelig waren voor directe effecten van lenalidomide. Alles samengenomen bevestigt dit onze hypothese dat zelfs in patiënten waarbij het MM ongevoelig lijkt voor lenalidomide, het immuunsysteem nog wel gestimuleerd kan worden. Dit kan in combinatiebehandelingen zoals beschreven in **hoofdstuk 5** leiden tot een aanzienlijke verbetering van klinische respons.

# CURRICULUM VITAE

Laurens Franssen werd geboren op 21 september 1986 te Nijmegen. In 2004 behaalde hij zijn VWO diploma aan de Nijmeegse Scholengemeenschap Groenewoud. In datzelfde jaar begon hij met zijn studie geneeskunde aan de Radboud Universiteit Nijmegen. Voor de start van zijn coschappen verrichtte hij onder leiding van dr. B.A. van der Reijden en dr. R.A.P. Raymakers tijdens een wetenschappelijke stage onderzoek naar acute myeloide leukemie. In december 2010 behaalde hij zijn doctoraalexamen (cum laude) en in datzelfde jaar zijn artsexamen. In mei 2011 startte hij zijn opleiding tot internist in het Sint Antonius Ziekenhuis in Nieuwegein (opleider: dr. A.B.M. Geers). In 2013 onderbrak hij zijn opleiding om te starten met zijn promotieonderzoek in het UMC Utrecht onder begeleiding van Prof. dr. H.M. Lokhorst, Prof. dr. T. Mutis en dr. N.W.C.J. van de Donk. In 2014 verplaatste de onderzoeksgroep zich naar het VU Medisch Centrum in Amsterdam, waar hij zijn onderzoek voortzette. In januari 2016 hervatte hij zijn opleiding tot internist in het Sint Antonius Ziekenhuis (opleiders: dr. A.B.M. Geers en dr. P. Chr. de Jong), gevolgd door een jaar in het UMC Utrecht (opleiders: Prof. dr. H.A.H. Kaasjager en dr. J.J. Oosterheert). Sinds juli 2018 is hij hematoloog in opleiding in het VU Medisch Centrum in Amsterdam. Laurens is getrouwd met Myrthe Bink en woont in Utrecht.

# LIST OF PUBLICATIONS

**Franssen LE,** Nijhof IS, Bjorklund CC, Chiu H, Doorn R, van Velzen J, Emmelot M, van Kessel B, Levin M-D, Bos GMJ, Broijl A, Klein SK, Koene HR, Bloem AC, Beeker A, Faber LM, van der Spek E, Raymakers RAP, Sonneveld P, Zweegman S, Lokhorst HM, Thakurta A, Qian X, Mutis T, van de Donk NWCJ. Lenalidomide combined with low-dose cyclophosphamide and prednisone modulates Ikaros and Aiolos in lymphocytes, resulting in immunostimulatory effects in lenalidomide-refractory multiple myeloma patients. *Oncotarget: Accepted*

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