Beyond the Horizon

New treatment strategies for relapsed/refractory multiple myeloma

Inger S. Nijhof

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New treatment strategies for relapsed/refractory multiple myeloma

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Beyond the Horizon

New treatment strategies for relapsed/refractory multiple myeloma

De horizon voorbij
Nieuwe behandelstrategieën voor het recidief en therapieresistent multiple myeloom
(met een samenvatting in het Nederlands)

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“Twenty years from now you will be more disappointed by the things you didn’t do than by the ones you did do. So throw off the bowlines. Sail away from the save harbor. Catch the trade winds in your sails. Explore. Dream. Discover.”

-Mark Twain-

Voor de patient

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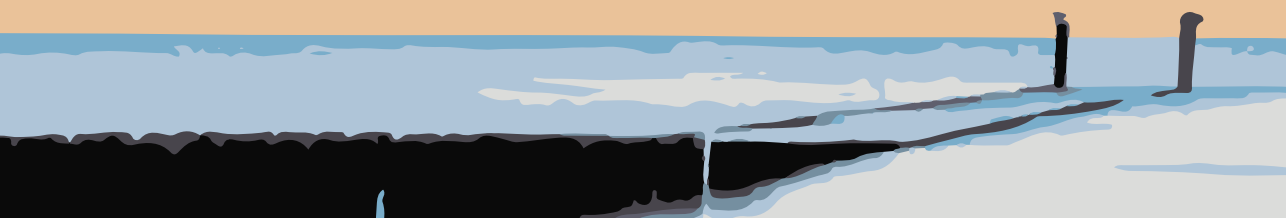
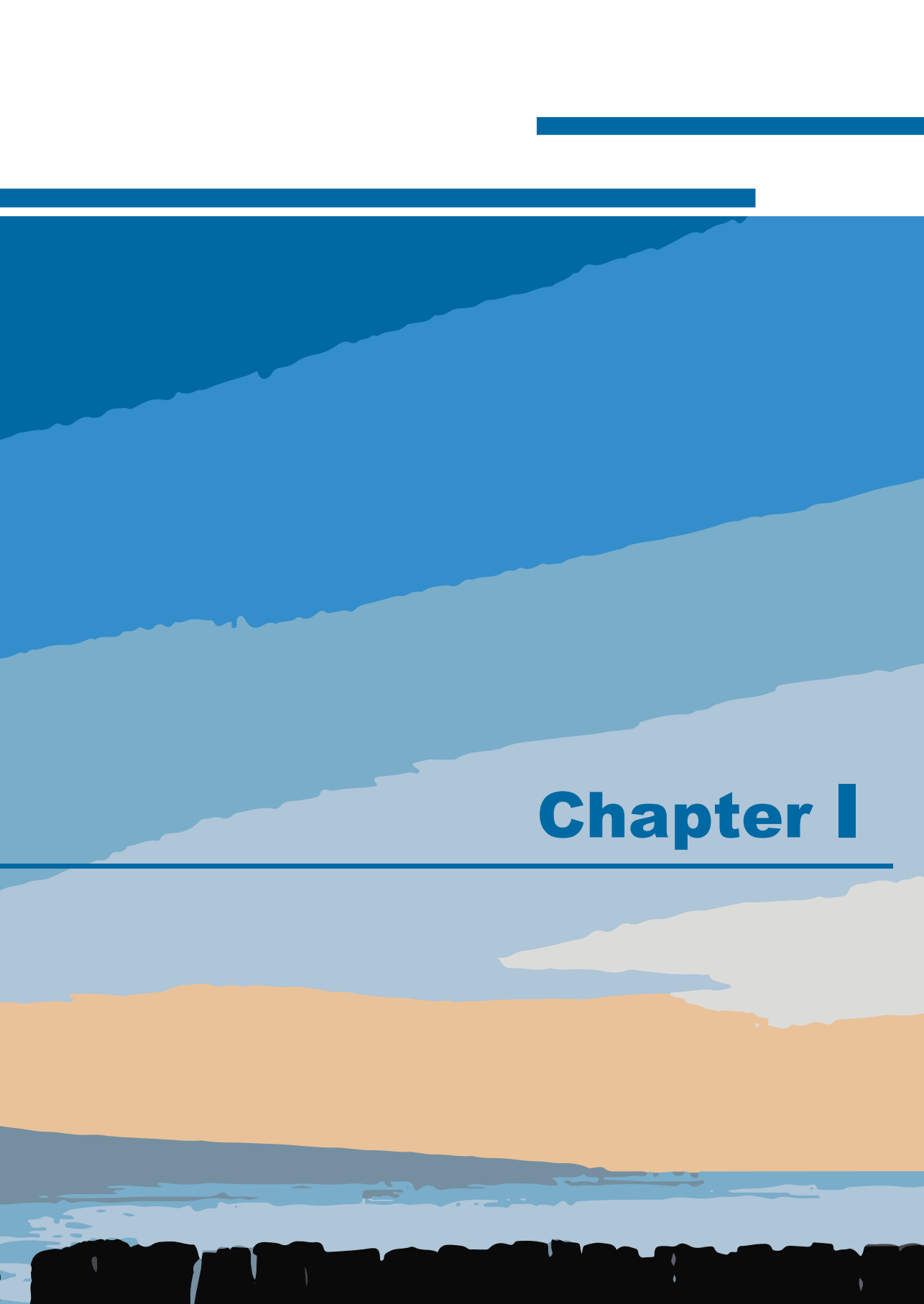
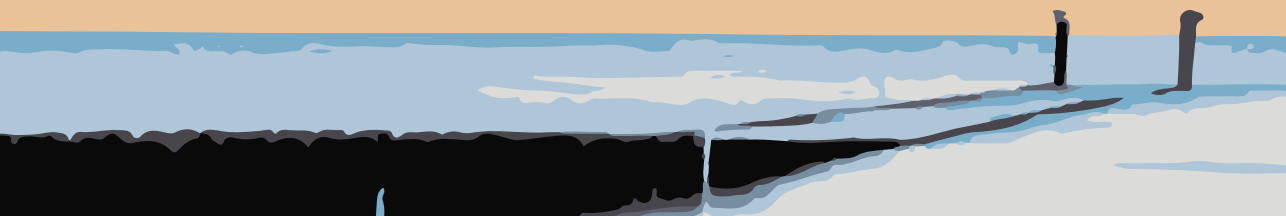



Chapter I

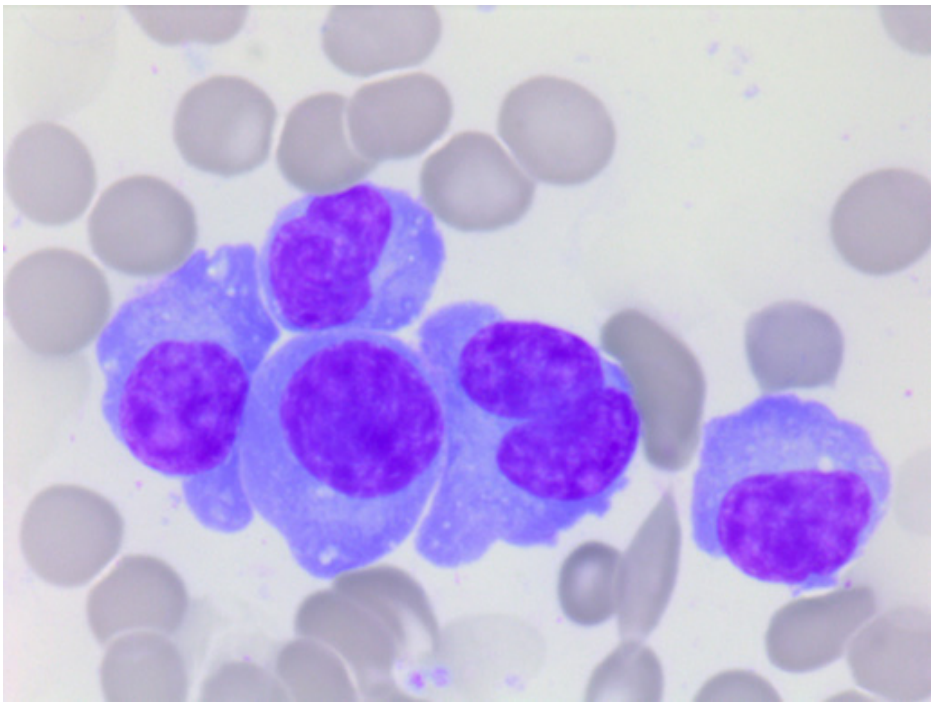
General introduction
rationale and outline of the thesis



Multiple myeloma

Multiple myeloma is characterized by the accumulation of monoclonal plasma cells in the bone marrow (Figure 1) and/or extramedullary sites.[1] The neoplastic cells are long-lived cells with a low proliferation index, are characterized by strong bone marrow dependence, and are typically accompanied by the secretion of monoclonal immunoglobulins that are detectable in the serum or urine (M-protein). In about 20% of the myeloma patients only free light chains (FLC) are produced which can also be secreted in the urine as Bence Jones proteins. The amount of the M-protein/FLC in the serum/urine is a reliable measure of tumor burden and can therefore be used as an excellent tumor marker.[2,3] 2% of the patients have non-secretory myeloma, characterized by the absence of detectable M-protein or FLC in serum and urine. The tumor cells and the M-protein are responsible for a number of organ dysfunctions and symptoms, including hypercalcemia, renal failure, anemia, bone disease (these typical manifestations of organ damage caused by multiple myeloma are named CRAB-criteria), but also increased susceptibility to infections, and sometimes clotting abnormalities or manifestations of hyperviscosity.

FIGURE 1



Smear of a bone marrow aspiration of a multiple myeloma patient.

Recently the criteria for multiple myeloma were redefined by the International Myeloma Working Group (IMWG).[4] These revised criteria are depicted in Table 1. Importantly, recent studies have shown that asymptomatic patients with a bone marrow plasma cell percentage ≥60%, a free light-chain ratio ≥100, provided the involved FLC level is 100 mg/L or higher, or >1 focal lesion on MRI (myeloma-defining events) have a 80% risk of developing symptomatic MM within 2 years. These patients are now considered to have MM requiring therapy, similar to patients with symptomatic disease (in Table 1 these criteria are depicted as biomarkers of malignancy).

TABLE 1

Revised International Myeloma Working Group diagnostic criteria for multiple myeloma[5]

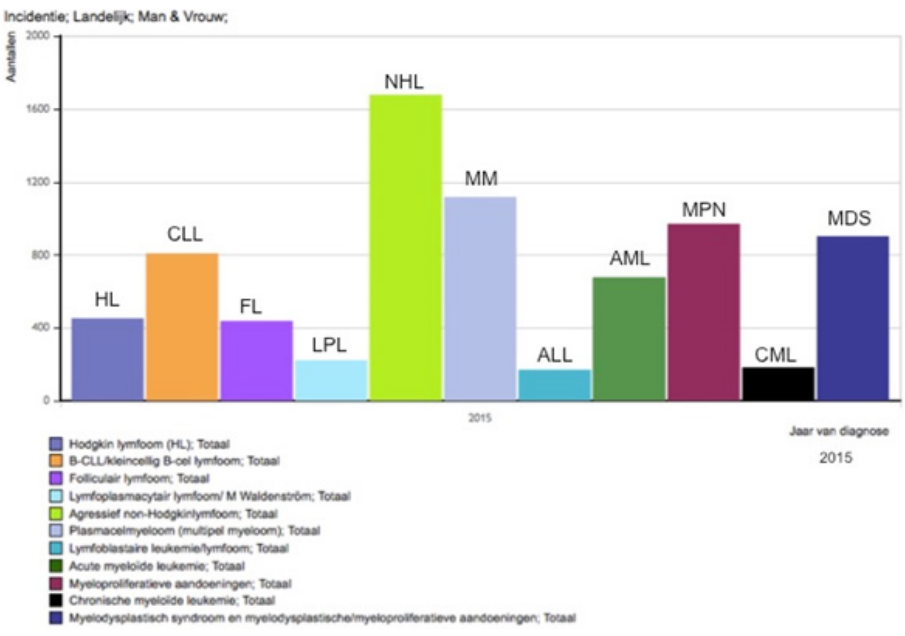
Definition of multiple myeloma
Clonal bone marrow plasma cells ≥ 10% or biopsy-proven bony or extramedullary plasmacytoma and one or more of the following myeloma defining events:
• Evidence of end organ damage (CRAB-criteria) that can be attributed to the underlying plasma cell disorder, specifically:
- Hypercalcemia: serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or > 2.75 mmol/L (11 mg/dL)
- Renal insufficiency: creatinine clearane <40 ml/min or serum creatinine >177 mol/L (>2 mg/dL)
- Anemia; hemoglobin value of >20 g/L (>1.25 mmol/L) below the lower limit of normal, or a hemoglobin value <100 g/L (<6.20 mmol/L)
- Bone lesions: one or more osteolytic lesions on skeletal radiography, CT or PET-CT
• Or any one or more of the following biomarkers of malignancy:
- Clonal bone marrow plasma cell percentage ≥60%
- Involved:uninvolved serum free light chain ratio ≥100 (the involved free light chain must be ≥100 mg/L)
- >1 focal lesions on MRI studies (each focal lesion must be 5 mm or more in size)

Epidemiology

Multiple myeloma accounts for 1.4% of all cancers worldwide and more than 10% of all malignant hematologic diseases, representing the second most prevalent hematologic malignancy with an age-adjusted incidence of six per 100 000 per year

in the USA and Europe.[6,7] In the Netherlands 1100 new MM patients are diagnosed each year (Figure 2). The median age of diagnosis is 69 years, with three-quarters of patients being diagnosed above the age of 55 years.

FIGURE 2



Incidence of hematologic malignancies in the Netherlands in 2015 (source: IKNL.nl)

Biology

Monoclonal gammopathy of undetermined significance (MGUS) is one of the most common premalignant disorders. IgG and IgA MGUS are precursor conditions of multiple myeloma (MM), whereas light-chain MGUS is a precursor condition of light-chain MM. Smoldering MM (SMM) is a precursor condition with higher tumor burden and higher risk of progression to symptomatic MM compared to MGUS.[8] All myeloma cases are preceded by MGUS and/or SMM.[9,10] A fraction of the individuals with MGUS or SMM will evolve to symptomatic MM, but most of the MGUS cases will remain asymptomatic. Why some MGUSs will remain totally asymptomatic for decades whereas others will evolve to overt MM is currently unknown. The main hypothesis is the occurrence of “malignant” genetic events in evolving patients as depicted in Figure 3.

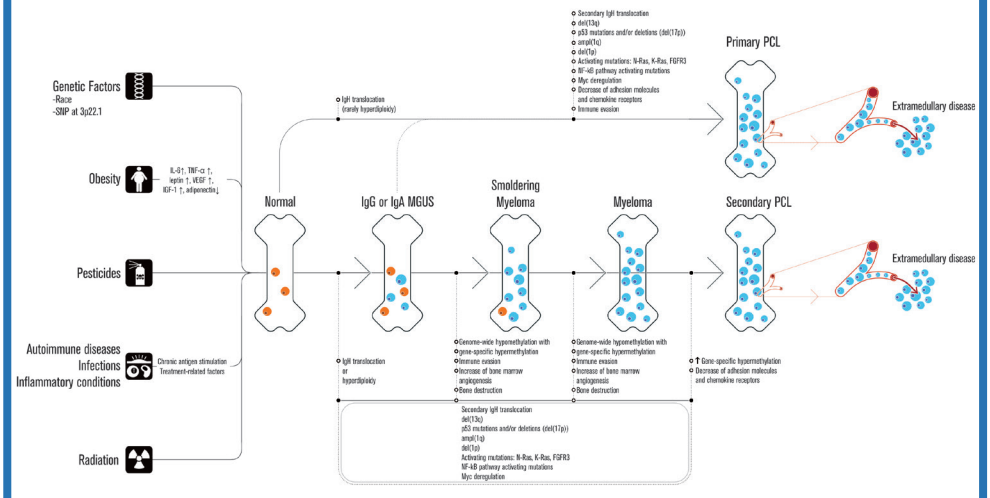
Figure 3 shows a model for the mechanisms that contribute to the development and progression of MGUS to MM. Obesity, exposure to pesticides, radiation exposure, and personal history of autoimmune diseases, inflammatory

conditions and infections have been described to be associated with an increased risk of MGUS. In addition, there seems to be a genetic predisposition to MGUS.[8] Primary immunoglobulin heavy chain (IgH) translocations with 5 recurrent chromosomal partners (4p16, 6p21, 11q13, 16q23, 20q11) and hyperdiploidy are early events and are associated with the initiation of limited clonal plasma cell proliferation in non-IgM MGUS.

Acquisition of secondary chromosomal abnormalities (such as deletions of (parts of) chromosomes or secondary chromosomal translocations) and mutations involving individual genes results in the stepwise progression from MGUS to newly diagnosed symptomatic MM, and finally aggressive forms of MM such as secondary plasmacel leukemia (sPCL) or extramedullary MM. During this process there is a progressive replacement of normal/polyclonal plasma cells (orange) by clonal plasma cells (blue).

Progression of the plasma cell disorder is also accompanied by altered interactions of the tumor cells with various components of their micro-environment such as osteoclasts, endothelial cells, and cells of the immune system. Recent evidence suggests the presence of intraclonal heterogeneity in MGUS, adding a further level of genetic complexity to the initiation and progression of myeloma.[8]

FIGURE 3



Development and progression of MGUS to MM (source: vd Donk et al, Haematologica, 2014)[8]

Role of the microenvironment

Multiple myeloma cells are protected by their bone marrow microenvironment by cell–cell and cell–matrix interactions, growth factors and cytokines, that provide optimal conditions for myeloma cell proliferation and survival. Furthermore, the bone marrow microenvironment contributes to therapy resistance by inducing anti-apoptotic mechanisms and facilitating the escape to the antitumor immune system.[11–13] Cellular components of the microenvironment include bone marrow stromal cells, osteoblasts, endothelial cells, and cells of the innate and adaptive immune system, including regulatory T cells and myeloid-derived suppressor cells. Crosstalk between multiple myeloma and its microenvironment is bidirectional, e.g. in the case of myeloid-derived suppressor cells, which on the one hand induce the growth of multiple myeloma cells by suppressing immune effector cells, but on the other hand are increased in number by multiple myeloma cells.[7,14]

Clonal evolution and competition in multiple myeloma

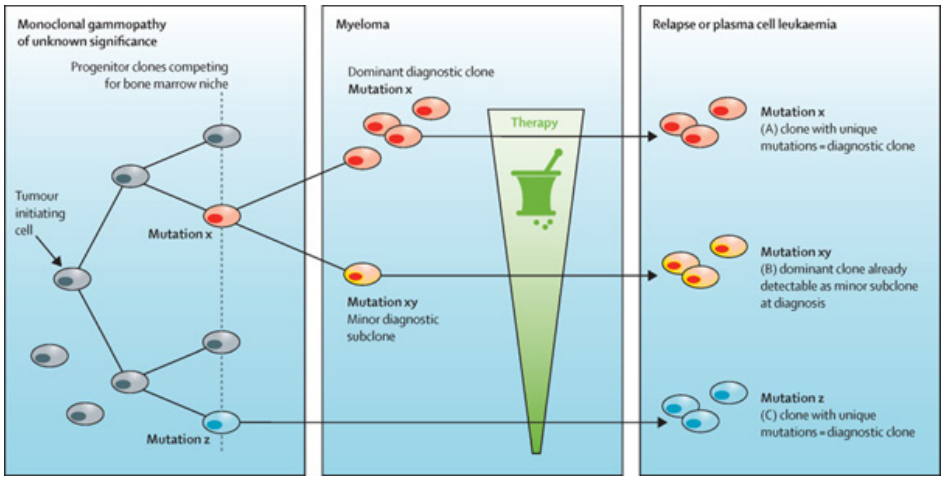
MM is a clonal malignancy characterized by the secretion of a monoclonal Ig or monoclonal free light chains. However, recently, detailed analyses at the molecular level have revealed a more complex situation. In 2012, 3 publications reported a certain degree of heterogeneity within the tumor clone, by comparing DNA abnormalities present at diagnosis and at relapse.[15–17] Based on SNP array or high-throughput sequencing, all 3 reports demonstrated that the clone observed at relapse could be slightly different from the one observed at diagnosis. In some cases, the relapse clone presented more chromosomal/genetic changes, but on the same background, representing a classical linear clonal evolution. However, in other patients, the relapse clone presented pieces of DNA, which were absent at the time of diagnosis, arguing for the existence of minor subclones, emerging during time and/or therapy and responsible for relapse.[18] Figure 4 depicted the clonal composition of MM during disease course and therapy.

The contribution of this clonal heterogeneity to disease progression and resistance to therapy is increasingly being recognized in cancer. In acute lymphoblastic leukemia, for example, it has been reported that tumors follow 1 out of 4 evolutionary pathways: no change over time, linear evolution, evolution from ancestral clones, and genetically distinct relapses supporting a variable branching architecture of tumor evolution.[19–21]

Keats et al. found that approximately one-third of patients with MM have stable genomes, particularly those with low-risk hyperdiploid disease, potentially explaining their more favorable clinical outcomes. In another one-third of patients, genome changes over time were evident that are best explained by the existence of clonal heterogeneity at diagnosis. In the final one-third of patients, a pattern consistent with linear evolution was the dominant characteristic. The last 2 groups included the high-risk patients, suggesting high-risk tumors are less stable and more prone to change with time.[16] These findings might have therapeutic implications.

For instance, to offer, especially in the high-risk and relapsed and refractory setting, combination therapies which confer anti-myeloma effects through different and complementary mechanisms, presumably targeting all coexisting disease subclones, will probably be particularly important, while sequential single-agent therapy might lead to preferentially eradicating the more indolent clone, making room for the more aggressive ones to expand. Another consideration might be that currently retreatment of a patient with a regimen on which they have previously progressed is avoided because of the assumption of continued drug resistance.
However, with intervening therapy a sensitive subclone may have re-emerged, and retreatment might be effective again. Nevertheless, the waves of different multiple myeloma clones evolving during the natural course of disease and the shifts in dominant and subdominant clones emerging during therapy and relapse are still chiefly an unraveled field and these findings require additional exploration before they may lead to personalized treatment care in MM.[7]

FIGURE 4



Clonal composition of MM during disease progression and therapy. During the progression from MGUS to MM, tumor initiating cells can give rise to subclones that are predominantly detected in diagnostic samples and harbor unique mutations, x, which might also be detectable later on in relapsed disease. Especially in high-risk disease, minor subclones that might have been hardly detectable at the time of initial diagnosis might acquire additional driving mutations, xy, during therapy, which later dominate the clonal composition at relapse. Subclones derived from tumor-initiating cells not detectable at the time of initial diagnosis might awake from dormancy at a later point in time and evolve as the dominating clone at relapse with a different founding mutation, z. Whereas clones with unique non-linear mutations x or z are supposed to be more susceptible to salvage therapy, subclones driven by newly acquired mutations (xy) are likely to be resistant to conventional therapies. (Source: Röllig C et al. The Lancet, 2015.)[7]

Prognostic factors

The course of MM is highly variable and depends on a variety of prognostic factors, including unfavorable high-risk cytogenetic abnormalities (del17p, t(4;14), add 1q, t(14;16)), a high-risk gene expression profile, high serum levels of lactodehydrogenase (LDH), a high International Staging System (ISS) stage (defined by the level of serum albumin and β2-microglobulin), plasma cell leukemia, or rapid progression or relapse during or after treatment.[22,23] Over the last decade, MM management has undergone profound changes thanks to advances in our understanding of the biology of the disease and improvements in treatment and supportive care approaches. The survival of MM patients has significantly improved due to the application of autologous stem cell transplantation (ASCT) in younger patients and the introduction of novel drugs such as the immunomodulatory agents (IMiDs) thalidomide and lenalidomide and the proteasome inhibitor (PI) bortezomib.[24,25] With these advances in treatment modalities the median overall survival has increased from 3 years to 6 years in the past two decades.[6] However, considerable heterogeneity exists in the survival outcomes among patients diagnosed with MM. Recently, the IMWG proposed a combined ISS-genetic prognostic system as the new standard to define high-risk, normal-risk and low-risk disease.[26] This risk stratification includes serum albumin and beta-2 microglobulin for ISS staging, and FISH for t(4;14), deletion 17p13 and 1q21 gain. Using these markers, a high-risk group of patients can be defined by ISS stage II/III and the presence of either t(4;14) or 17p13.[27] This high-risk group covers approximately 20% of MM patients and these patients have a described median OS of only 2 years. At the same time, a low-risk group can be defined by age less than 55 years, ISS stage I or II and normal results for the three FISH markers.[28] Also this group of MM patients covers approximately 20% of patients and have a described median overall survival of >10 years. The standard-risk MM patients are all other MM patients and concerns about 60% of MM patients. These patients have a median OS of approximately 7 years.[26]

Newly diagnosed multiple myeloma

Current treatment strategies for the newly diagnosed (ND) MM patient consists of novel-agent-based induction followed by up-front high dose therapy and ASCT in medically fit patients.[29–31] Novel agents have been incorporated as induction regimens with the objective of increasing the response prior to ASCT, and as consolidation or maintenance treatment to increase the depth of response and prolong the duration of response. Thalidomide or lenalidomide maintenance have been shown to increase progression-free survival[32–34] and, as recently been shown in a large meta-analysis, also benefits overall survival.[35]
During recent years melphalan-prednisone-bortezomib or melphalan-prednisone-thalidomide were the standards of care for transplant-ineligible NDMM patients.
Recently, both the US and European regulatory authorities expanded their approvals

of lenalidomide to include NDMM in combination with dexamethasone in the United States and in Europe, based on the results of the MM-020/FIRST-trial.[36,37] Recent update analyses of this study showed a superior efficacy, PFS and OS of continuous lenalidomide-dexamethasone over a fixed 18 cycles of lenalidomide-dexamethasone at the expense of a modest increase in toxicity. [38]

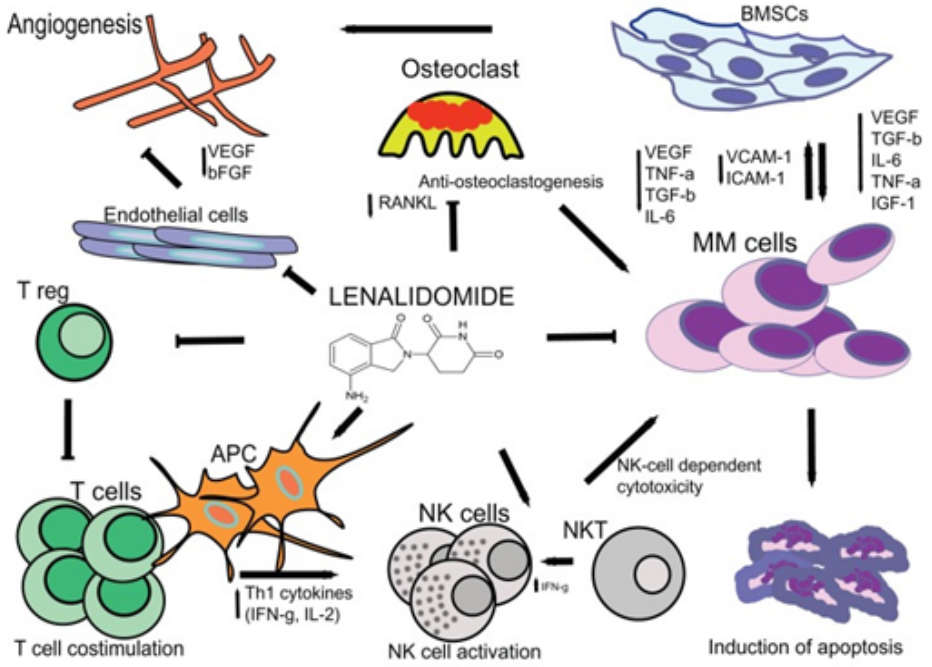
Older age and comorbidities can negatively affect patient outcomes. An analysis of 869 patients with newly diagnosed MM from three clinical trials divided elderly patients with MM in three groups (fit, intermediate fitness, and frail) on the basis of age,comorbidities, and cognitive and physical statuses that were predictive of risk of mortality and toxicity.[39] Results suggested that patient fitness should be considered when making treatment decisions, because this factor may affect the optimal balance between efficacy and safety.[39]

As stated above, the introduction of the novel agents lenalidomide and bortezomib has substantially improved survival for MM patients. Both IMiDs and proteasome inhibitors differ from conventional therapies by their modes of action. Importantly, next to direct anti-tumor effects, they show potent immunomodulatory effects, which contributes significantly to their clinical benefits, and are also important for rational combination strategies.

Mechanisms of action of lenalidomide

Lenalidomide is the more potent and less toxic amino-substituted derivative of the immunomodulatory drug (IMiD) thalidomide. IMiDs have direct anti-proliferative and cytotoxic effects on the myeloma tumor cell, as well as anti-angiogenic activity and immunomodulatory effects (Figure 5).[40] IMiDs directly kill MM cells by the induction of cell cycle arrest and caspase-dependent apoptosis.[41,42] In addition, they impair MM cell survival and proliferation through interference with the protective properties of bone marrow stromal cells, including the downregulation of adhesion molecules such as VICAM-1 and ICAM-143 and inhibition of the production of cytokines like IL-6 and TNF-α.44 Furthermore, IMiDs inhibit angiogenesis by downregulation of vascular endothelial growth factor and β−fibroblast growth factor[45,46] and impair osteoclastogenesis by reducing RANKL levels.[47] IMiDs also have immunomodulatory effects including stimulation of T cell proliferation, increased production of IL-2 and IFNγ[48] and enhancement of cytotoxic T lymphocyte, natural killer T, and natural killer effector cell activity against MM cells.[49,50] IMiDs have been described to decrease the development of regulatory T cells in MM.[51] However, others have described that lenalidomide alone or combined with dexamethasone may increases the frequency of regulatory T cells (Tregs) in newly diagnosed and in relapsed/refractory MM patients.[52–58] Finally, lenalidomide has been described to inhibit myeloid cell-mediated inflammatory function by decreasing the secretion of IL-6, TNFα, and IL-10.[59]

FIGURE 5



The proposed mechanisms of action of lenalidomide in multiple myeloma include immune modulation, interference with tumor microenvironment interactions, and direct antitumor effects. Immunomodulation by lenalidomide includes T cell co-stimulation, suppression of Tregs, increased production of Th1 cytokines, and activation of NK and NKT cells. Lenalidomide mediates disruption of myeloma cell-microenvironment interactions via several mechanisms including antiangiogenesis, anti-inflammatory effects, antiosteoclastogenic properties, modulation of cytokine production, and downregulation of adhesion molecules. IMiDs also exert direct effects on myeloma cells via cell cycle arrest and induction of apoptosis.[40]
Abbreviations: T reg, regulatory T cell; BMSCs, bone marrow stromal cells; APC, antigen-presenting cell; NK cells, natural killer cells; NKT cells, natural killer T cells; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; RANKL, receptor activator of nuclear factor kappa-B ligand; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; IGF-1, insulin-like growth factor 1; TNF-α, tumor necrosis factor α; IL-6, interleukin-6.
(source: van de Donk et al. Cancer Manag. Res. 2012).[40]

Mechanisms of action of bortezomib

Bortezomib belongs to the group of proteasome inhibitors and works by targeting specific cell receptors, proteins and signaling pathways. Proteasomes are 26S ATP-dependent protein complexes within the ubiquitin-proteasome pathway. They are present in all cells and are responsible for processing the majority of intracellular proteins.[60] Cancer cells generally have higher levels of proteasome activity when compared with normal cells and are therefore more sensitive to

proteasome inhibition,[60] leading to disruption of cellular tumor growth and survival. By inhibiting the proteasome, bortezomib acts through multiple mechanisms to suppress tumor survival pathways and to arrest tumor growth, tumor spread, and angiogenesis.[61] Bortezomib directly induces apoptosis of tumor cells, inhibits the activation of NF-κB in cells and in the tumor microenvironment, reduces adherence of myeloma cells to bone marrow stromal cells, blocks production and intracellular signaling of IL-6 in myeloma cells, stops the production and expression of proangiogenic mediators, and overcomes defects in apoptotic regulators, such as Bcl-2 overexpression and alterations in tumor suppressor p53.[61,62]

In addition, proteasome inhibition with bortezomib has been shown to induce the endoplasmic reticulum stress response,associated with disruption of the unfolded protein response, an important aspect of the mechanism of action of proteasome inhibition in MM because of the high production of immunoglobulins by MM cells.[63]

TABLE 2

International Myeloma Working Group relapse criteria for multiple myeloma

Definition of relapsed multiple myeloma

Recurrence of the disease after prior response, defined on:

- Objective laboratory criteria:
  - ≥25% increase of the serum M-protein (the absolute increase must be ≥5 g/L)
  - or urine monoclonal protein (the absolute increase must be ≥200 mg/24 h)
  - or ≥25% difference between involved and uninvolved serum free light chains (the absolute increase must be ≥100 mg/L) from its nadir, respectively,
- Or objective radiologic criteria:
  - the definite development of new lytic bone lesions or soft tissue plasmacytomas
  - or definite increase in the size of existing bone lesions or soft tissue plasmacytomas increase, defined as a ≥50% increase and at least increase of ≥1 cm as measured serially by the sum of the products of the cross-diameters of the measurable lesion
- Or the development of hypercalcemia that can be attributed solely to the plasma cell proliferative disorder.
- In patients with non-secretory disease, relapse is defined as an increase of the bone marrow plasma cell percentage (the absolute rise in % must be ≥10%).

Definition of relapsed/refractory multiple myeloma

With the advances in treatment modalities due to the introduction of IMiDs and bortezomib in the treatment of multiple myeloma patients, the median overall survival has substantially increased from 3 years to 6 years in the past two decades.[6] Consequently, these 'novel' agents form nowadays the backbone of therapy in multiple myeloma. Unfortunately, also these novel anti-myeloma strategies are eventually hampered by the development of drug resistance. As a consequence the far majority of myeloma patients relapse and may develop refractory disease. The IMWG published definitions of relapsed multiple myeloma, as well as refractory myeloma, and also treatment indications in 2006, 2009 en 2011.[64–66] Relapsed multiple myeloma is regarded as a recurrence of the disease after prior response, and has been defined based on objective laboratory and radiologic criteria as depicted in Table 2.

Refractory to prior treatment indicates progressive disease on last prior treatment, best response of stable disease to last prior treatment, or progressive disease within 60 days. There are 2 categories of refractory myeloma: "relapsed-and-refractory myeloma" and "primary refractory myeloma". Relapsed and refractory myeloma is defined as relapse in patients who must have achieved at least minimal response (MR), which either becomes non-responsive while on salvage therapy or progresses within 60 days of last treatment.[64,67] Primary refractory myeloma is defined as disease that is non-responsive; patients who have never achieved a MR or better with any therapy.[67]

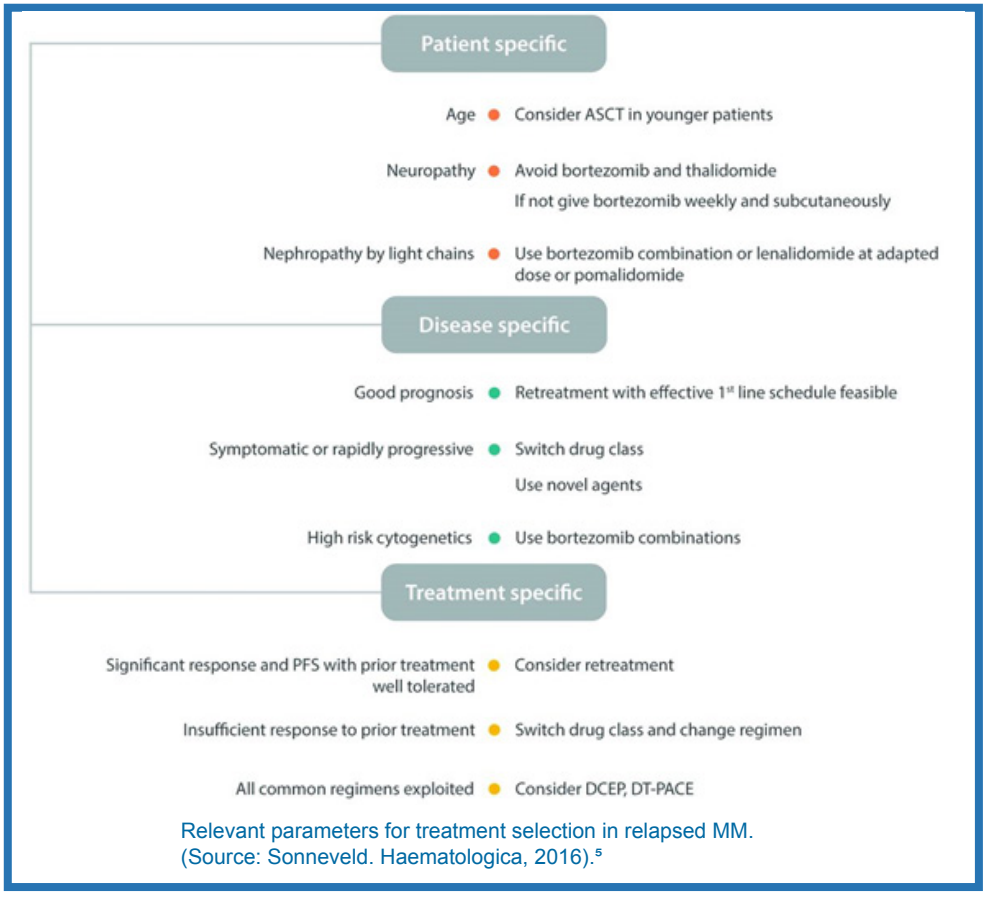
Treatment indications for relapsed and/or refractory multiple myeloma

In general, an indication for relapse treatment has been defined as either the appearance or reappearance of one or more CRAB criteria or a rapid and consistent significant biochemical relapse, defined as doubling of the M-component in 2 consecutive measurements separated by less than or equal to 2 months; or an increase in the absolute levels of serum M protein by more than or equal to 10 g/L, or urine M protein increase by more than or equal to 500 mg/24 hours, or increase of the involved FLC level by more than or equal to 200 mg/L (plus an abnormal FLC ratio) in 2 consecutive measurements separated by less than or equal to 2 months.[66] In the presence of high-risk factors, such as aggressive disease at diagnosis, a short treatment-free interval with a suboptimal response to the previous treatment line, imminent risk for organ dysfunction such as previous light chain-induced renal impairment, aggressive bone lesions or unfavorable cytogenetics as t(4;14) or del17p, treatment should be initiated at the stage of biochemical relapse before serious symptomatic disease develops.

Treatment options at first relapse

When selecting second-line therapy, it is important to consider prior response to previous treatment. Also previous tolerability is an important consideration; factors like myelosuppression, neuropathy and thrombosis may influence the choice and dosing of therapy. To this respect, additional challenges arise towards the end stage of MM when many patients with cytopenia due either to extensive bone marrow involvement or exhausted hematopoiesis due to prior therapies may be prevalent. Furthermore, treatment choices are also influenced by age, frailty status and the expectations of the patients and their families.[5] Figure 6 shows the relevant parameters for treatment selection in relapsed MM.

FIGURE 6



Relevant parameters for treatment selection in relapsed MM. (Source: Sonneveld. Haematologica, 2016).[5]

Frontline treatment strategies, as well as treatment options for relapsed MM patients, is a fast-moving field as rapid and exciting developments are currently ongoing. Therefore, following suggestions for relapse treatment are expected to change quickly in the upcoming years.

At the time of relapsed MM when second-line treatment is necessary, it is currently adviced that patients not previously treated with a novel agent, should be treated with a bortezomib-based, an IMiD-based or combination of both-regimen. Patients previously treated with an IMiD-based regimen should preferably be treated with a bortezomib-containing regimen at relapse and of course the other way around; patients initially treated with a bortezomib-containing, should preferably be treated with an IMiD-containing regimen as second-line salvage treatment. In general, doublet or triplet regimens are preferred above single agents for inducing optimal effect. In addition, retreatment with an agent previously used is considered feasible, provided there was no acquired refractory status and prior treatment with the regimen produced a clinically meaningful response of adequate duration with acceptable toxicity. In general, the minimal depth of response should be partial response, while the minimal duration of response should be at least 6 months.[5] When transplant-eligible MM patients had sufficient benefit from upfront therapy with ASCT (>18-24 months remission period) or have not been treated with high dose therapy and ASCT at frontline, then second-line induction therapy followed by high dose therapy and ASCT might be a valuable salvage option. Also allogeneic stem cell transplantation may be still considered for young patients with high-risk disease at the time of relapse, but preferably in the context of a clinical trial. Despite the significant improvement in survival of MM patients over the last two decades, myeloma is stil considered an incurable disease. Virtually all anti-myeloma strategies are eventually hampered by the development of drug resistance and as a consequence the far majority of myeloma patients relapse and develop refractory disease. In addition, second and later remissions tend to show shorter durations because of more aggressive tumor behavior at each relapse due to the selection of resistant clones and the development of refractory disease.[17] The prognosis of patients with multiple myeloma who become refractory to the 'novel agents' bortezomib and lenalidomide is very poor with an event-free survival and overall survival of only 5 and 9 months, respectively.[68] This clearly demonstrates that there is an urgent need for additional active agents and treatment strategies with distinct mechanisms of action, especially for the relapsed and refractory MM patients.

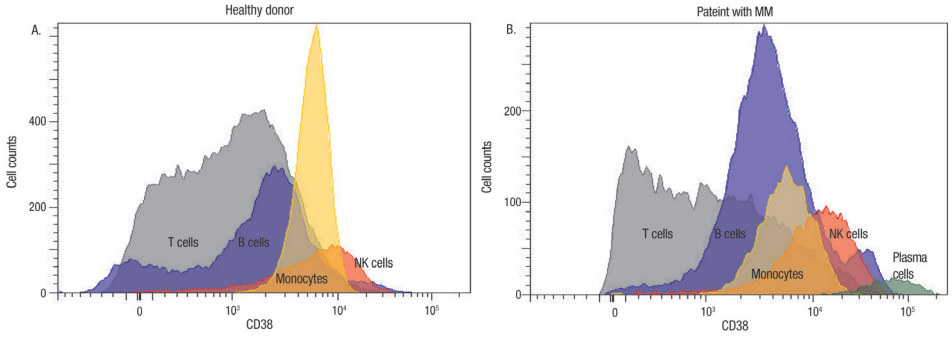
Treatment options for lenalidomide- and/or bortezomib-refractory myeloma

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 mTOR inhibitors.[69] Furthermore, the success of anti-CD20 mAbs in the treatment of non-Hodgkin lymphomas and chronic lymphocytic leukemia, illustrated that antibody-mediated immunotherapy can also represent a powerful therapeutic option for hematologic malignancies and stimulated the investigation of various antibody therapies for multiple myeloma.[70] In this respect CD38 might be an interesting target in MM while CD38 is highly and ubiquitously expressed on virtually all MM cells.

CD38

CD38 is a multifunctional cell surface protein that has receptor as well as enzyme functions.[71] Early functional studies showed that CD38 regulates adhesion events between circulating lymphocytes and endothelial cells by binding to CD31 (also known as PECAM-1).[72] The CD38-CD31 interaction not only plays a role in the binding and migration of leukocytes to endothelial cells[73], but also triggers activation and proliferation of human leukocytes and is involved in the differentiation of B cells.[72;74] Next to its receptor function, CD38 has bifunctional ecto-enzymatic activity.[71,75] Via the NAD^+-pathway CD38 regulates Ca^{2+} mobilization from the cytosol, whereby activating signaling pathways that control various biological processes, such as lymphocyte proliferation.[76] Interestingly, recent studies suggest a role of CD38-dependant adenosine production in immune suppression mediated by NK cells[77] and involvement of CD38 in immune modulation mediated by myeloid-derived suppressor cells.[78] The CD38 gene is located on chromosome 4 (4p15) and comprises seven introns and eight exons that encode the CD38 protein. Gene transcription is regulated at multiple levels; the promotor region is controlled by methylation and this promotor region has potential binding sites for various immunological transcription factors, including those for T cell-specific transcription factor alpha (TCF-1α), nuclear factor-IL-6 (NF-IL-6), and interferon responsive factor-1 (IRF-1).[79] Another level of transcriptional control is positioned within intron 1, which contains responsive elements for retinoic acid.[80,81] Finally the transcription factor E2A mediates CD38 gene transcription in response to environmental signals, such as interleukin-2 and TLR-9 ligands.[82] The CD38 protein is generally expressed at low levels on various hematological cells, while plasma cells express particularly high levels of CD38 (Figure 7).

FIGURE 7



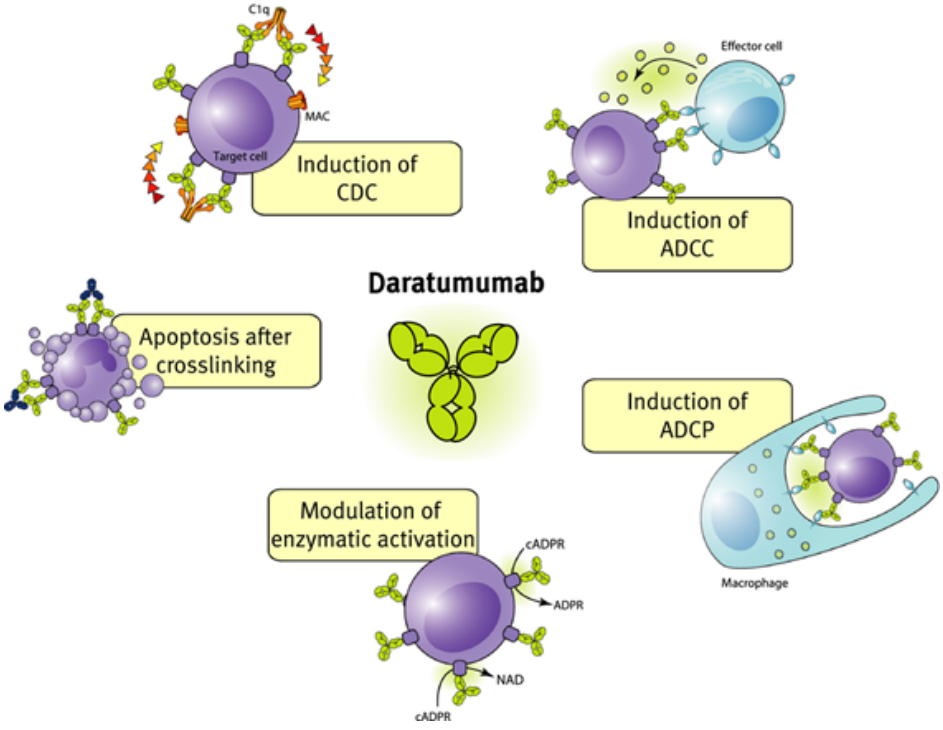
Histograms show expression of CD38 in T cells, B cells, monocytes, NK cells, in a healthy donor (A) and in a patient with multiple myeloma (B). CD38 expression in plasma cells from the myeloma patient is also shown. (Images created by Berris van Kessel, scientific analyst in the research projects described in this thesis)

Expression of CD38 in hematopoietic cells depends on the differentiation and activation status of the cell. Approximately 80% of NK cells and monocytes express CD38 at low levels, as do various other hematological cell types, including lymph node germinal center lymphoblasts, intrafollicular cells, dendritic cells,[83] erythrocytes[84] and platelets.[85]
Although CD38 is widely expressed on cells of hematopoietic origin, including committed stem cells, the hematopoietic compartment remained unaltered in CD38 knockout mice,[71,86] suggesting that CD38 is not critical to hematopoiesis or lymphopoiesis in mice.[71] With regard to non-hematopoietic tissues, CD38 is expressed at low levels in some specific cell types, such as Purkinje cells and neurofibrillary tangles in the brain, epithelial cells in the prostate, β-cells in the pancreas, osteoclasts in the bone, retinal cells in the eye, and sarcolemma of smooth and striated muscle.[87]
CD38 is a highly interesting target for antibody therapy in MM and related disorders, as virtually all MM cells express high levels of CD38 on their cell surface.[88] However CD38 expression has also been reported in CLL,[89] Waldenström's macroglobulinemia,[90] primary systemic amyloidosis,[91] mantle cell lymphoma,[92] acute lymphoblastic leukemia,[93] acute myeloid leukemia,[94] NK cell leukemia,[95] NK/T-cell lymphoma,[96] and plasma cell leukemia.[97]
Therefore, next to MM, also in other hematopoietic or solid malignancies expressing CD38, CD38 might be an interesting potential therapeutic target, particularly when combined with most suitable combination strategies with synergistic activities. With the upcoming experience in the field of MM with CD38-antibodies, the exploration within other hematologic and even solid malignancies might be an interesting field of research in the upcoming years.

Daratumumab

Daratumumab (also referred to as HuMax-CD38 and IgG1-005) is an immunoglobulin G1 kappa (IgG1κ) human monoclonal antibody (mAb) that specifically binds a unique epitope present on the CD38 molecule.
Daratumumab showed strong anti-tumor activity in tumor models *in vitro* and *in vivo* and engages multiple mechanisms of action (figure 8). The antibody has a unique binding profile and effectively triggers Fc-dependent effector functions, including complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP), while Fc cross-linking induces apoptosis of daratumumab-opsonized cells. Furthermore, daratumumab modulates the enzymatic activities of CD38, blunting the cyclase activity, and enhancing the hydrolase activity. While all of these actions are involved in the anti-tumor activity mediated by daratumumab, the exact contribution of each mechanism to the observed clinical activity needs to be elucidated. Based on the preclinical activity of daratumumab, a phase 1/2 study was initiated in MM patients with relapsed/refractory disease (GEN501 study).[98] In the first-in-human dose-escalation part of the study, the maximum-tolerated dose was not reached with dose

FIGURE 8



Mechanisms of action of daratumumab (Source: van de Donk et al. Immunol Rev. 2016).[71]

levels up to 24 mg/kg. In the phase 2 part of the study, patients with a median of 4 prior lines of therapy (majority refractory to lenalidomide and bortezomib) were treated with daratumumab at a dose of 8 mg/kg or 16 mg/kg. Daratumumab was well tolerated. The most frequent adverse events were infusion-related reactions, which occurred in 71% of patients. The majority of these reactions included grade 1 and 2, and were characterized by rhinitis, cough, headache, pyrexia, and dyspnea. The most infusion-related reactions occurred during the first daratumumab infusion and only few patients (<10%) had infusion-related reactions with more than one infusion[98]. The overall response rate (at least PR) was 36% in the 16 mg/kg cohort and 10% in the 8 mg/kg group. 2 of 42 patients treated with 16 mg/kg daratumumab achieved complete response. The median PFS in the 8 mg/kg and 16 mg/kg groups were 2.4 and 5.6 months, respectively. The 12-month survival was 77% for both groups.[98]The SIRIUS (MMY2002) study, in which 106 MM patients with a median of 5 prior lines of therapy (95% of these patients were refractory to lenalidomide and bortezomib), received daratumumab monotherapy at a dose of 16 mg/kg, and confirmed the results from GEN501 study by demonstrating single agent activity of

daratumumab with a favorable toxicity profile.[99] At least PR was achieved in 29% of patients with stringent complete response (CR) in 3%. The median duration of response was 7.4 months. The median progression-free survival was 3.7 months and 1-year overall survival was 65%. Notably, subgroup analysis showed that in the group of patients who were refractory to lenalidomide, pomalidomide, bortezomib, and carfilzomib, PR or better was achieved in 21% of these patients. Also in this study, infusion-related reactions were observed in 43% of the patients (predominantly grade 1 and 2), and could be managed with interruption of the infusion or extra corticosteroids and anti-histamines.
As ADCC is an important mechanism of action of daratumumab,[100] it might be valuable to enhance NK cell function to optimize mAb-therapy with daratumumab.

KIRs and IPH2102

NK cells appear to play an important role in the immune response to multiple myeloma. NK cells work mainly via antigen-independent cytotoxicity and lyse tumor targets directly via the perforin/granzyme pathway. They also express Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which directly trigger death receptor pathways inducing tumor apoptosis.[101,102]
Unlike T and B lymphocytes, NK cells do not rearrange genes encoding for specific antigen receptors; rather, their recognition of targets is regulated through a balance of activating and inhibitory signals.[103] Even in the presence of an activating ligand, inhibitory ligands can initiate overriding signals that culminate in a net suppression of NK cell function. NK cells recognize major histocompatibility class (MHC) I and class I-like molecules through surface expression of killer immunoglobulin-like receptors (KIRs).[104] MHC class I molecules on tumor cells ligate NK cell-inhibitory KIR, suppressing NK cell function.
Multiple myeloma cells utilize NK cell-specific immune-evasive strategies, including the upregulation of the surface expression of HLA class I molecules, which serve as inhibitory KIR ligands. The upregulation of these KIR ligands prevents NK cell recognition and lysis, making this receptor-ligand axis a provocative target for NK-cell mediated therapeutics.[105] IPH2102 (formerly 1-7F9 and IPH2101) is a hinge-stabilized, human IgG4 monoclonal antibody that blocks the interaction of the three main inhibitory KIR receptors (KIR2DL-1, -2, -3) with their ligands, the human leukocyte antigen-C (HLA-C) molecules and thereby promotes NK cell recognition and lysis of tumor cells.
The predecessor of IPH2102, IPH2101 (a wild-type IgG4 version of the antibody), was shown to increase *in vitro* NK-cell cytotoxicity against MM cells, but not against normal healthy cells.[106,107] Furthermore clinical trials conducted with IPH2101 in patients with relapsed/refractory MM and smoldering myeloma revealed that the clinical use of IPH2101 is safe and tolerable at doses that achieve full inhibitory KIR saturation, with disease stabilization as the best observed response to IPH2101.[108,109]

Combination strategies

Next to the development of new drugs, also the strategy of combining drugs with synergistic activity may offer new therapeutic perspectives for multi-drug refractory MM patients. For instance, bortezomib and lenalidomide have different, but at the same time overlapping mechanisms of action, that include activation of apoptosis mediated by caspase-9 and/or caspase-8, and inhibition of nuclear factor-kB signaling. Additionally, lenalidomide sensitizes MM cells to both bortezomib and dexamethasone,[110] whereas the anti-MM activity of dexamethasone is enhanced by both bortezomib and lenalidomide,[111] suggesting that combination therapy may enhance clinical activity. Indeed, the combination lenalidomide, bortezomib and dexamethasone (RVD) is an active and well-tolerated regimen in patients previously treated with lenalidomide, bortezomib, or thalidomide with an overall response rate of 64%, a median progression-free survival (PFS) of 9.5 months and median OS of 26 months.[112-115]

Also other combination strategies may work synergistic in relapsed and refractory multiple myeloma patients. We have previously shown in a retrospective study that lenalidomide combined with continuous low-dose cyclophosphamide and prednisone (REP) had remarkable activity in heavily pretreated, lenalidomide-refractory MM patients.[116] However the optimal dose of the combination has not been established previously. Lenalidomide has multiple well-described antitumor effects in MM, including direct anti-proliferative and cytotoxic effects on the myeloma tumor cell, as well as anti-angiogenic activity and immunomodulatory effects through promotion of T cell co-stimulation and increase in natural killer (NK) cell numbers and activation status. Similarly, administration of cyclophosphamide, at a dose substantially lower than the maximum tolerated dose (MTD) (metronomic dosing), has next to its direct anti-tumor activity several other effects including anti-angiogenic effects, modulation of the micro-environment, and improvement of T and NK cell-mediated anti-tumor immune response via depletion of Tregs. Furthermore continuous low-dose therapy has the potential to maintain the suppression of residual disease, prolong the time to subsequent relapse and extent OS rates.

Aims and outline of the thesis

Altogether, in this thesis studies were conducted with the aim to create new treatment strategies for MM patients refractory to IMiDs and PIs who have a very poor prognosis to date. We performed:

1. **Translational research**
   - > to improve daratumumab-mediated cell lysis to provide new combination strategies with higher anti-myeloma activity
   - > to gain insight into the host- and tumor-related factors in multiple myeloma patients treated with daratumumab that might contribute to response and innate or acquired resistance with the aim to establish predictive factors for response and/or resistance to daratumumab and to develop new rationally designed combination therapies with daratumumab
2. A phase 1/2 prospective **clinical trial** to determine the maximal tolerated dose as well as tolerability and efficacy of Revlimid®, Endoxan® and prednisone (REP) in lenalidomide-refractory multiple myeloma patients.

Chapter 2 explored the possibility of improving daratumumab-mediated cell-mediated cytotoxicity by blocking natural killer cell inhibitory receptors with the human monoclonal anti-KIR antibody IPH2102, next to activation of natural killer cells with the immune modulatory drug lenalidomide.

Chapter 3 addressed the potential value of daratumumab alone or in combination with lenalidomide or bortezomib in the treatment of lenalidomide- and bortezomib-refractory patients. We explored the underlying mechanism of the synergistic effect of the combination of daratumumab and lenalidomide in the setting of lenalidomide-refractory disease.

Chapter 4 studied the effect of CD38 expression levels on the efficacy of daratumumab to induce MM cell lysis via ADCC and CDC. We also examined other potential determinants of daratumumab sensitivity including the extent of previous therapy and the frequency of effector cells. In addition, we explored the effect of all-trans retinoic acid (ATRA) on daratumumab-mediated ADCC and CDC.

Chapter 5 focuses on the predictive value of CD38 expression on MM cells in daratumumab-treated patients, as well as the contribution of the expression levels of complement-inhibitory proteins on MM cells for the development of response and resistance. We also looked at the effect of ATRA on daratumumab-mediated ADCC and CDC at the time of progressive disease.

Chapter 6 explores the impact of daratumumab on CD38+ immune-suppressive populations, T-cell proliferation and activation. Furthermore T-cell receptor (TCR) clonality was evaluated.

Chapter 7 shows the results of the REPEAT-study. The REPEAT-study is a prospective, investigator-initiated, non-randomized, 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 combined with continuous orally low-dosed cyclophosphamide and prednisone (REP) in lenalidomide-refractory MM patients.

Chapter 8 summarizes the main findings of this thesis and discusses the implications for patient care and future research.

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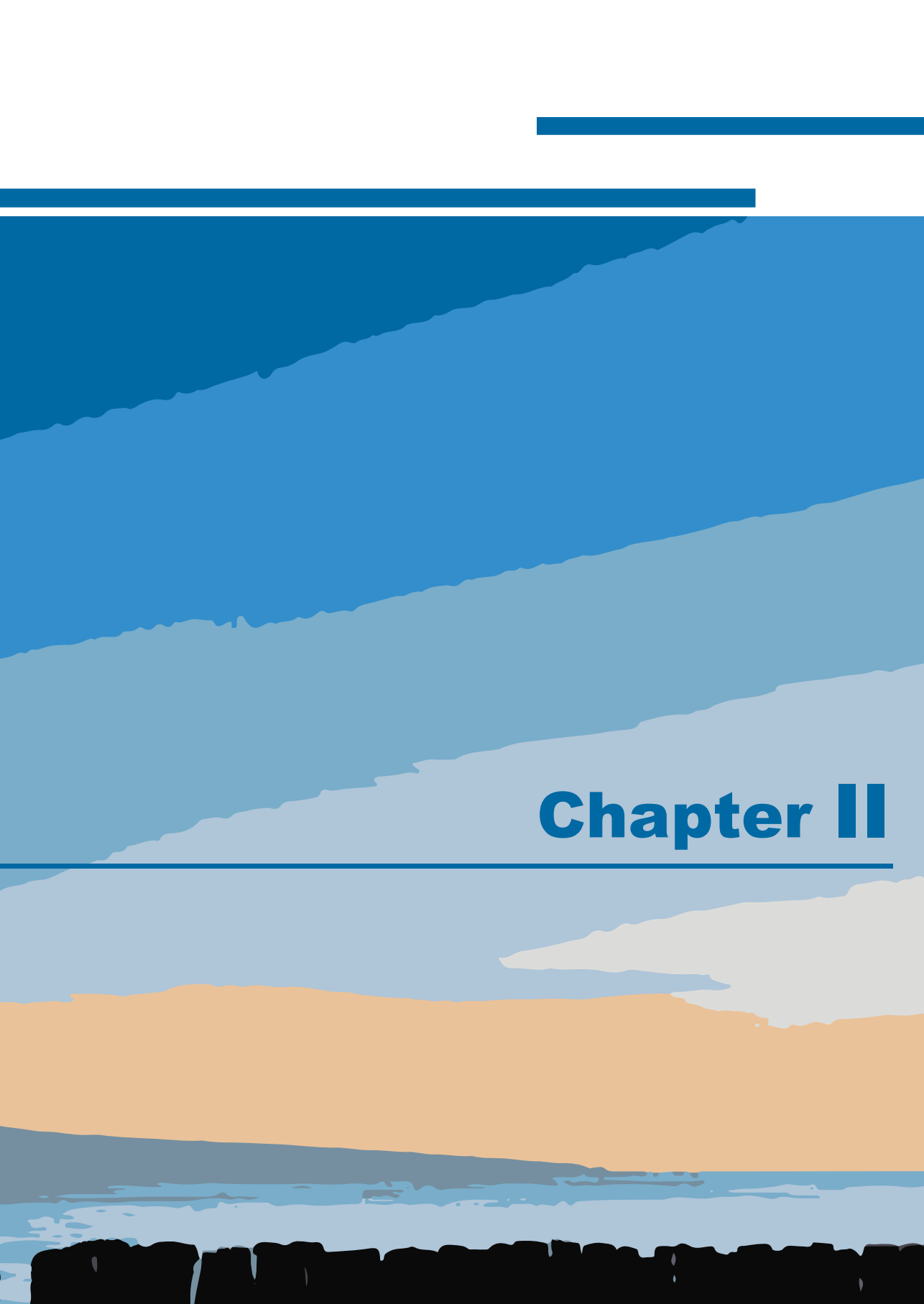
Chapter II

Daratumumab-mediated lysis of primary multiple myeloma cells is enhanced in combination with the human anti-KIR antibody IPH2102 and lenalidomide

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Abstract

Despite recent treatment improvements, multiple myeloma remains an incurable disease. Since antibody dependent cell-mediated cytotoxicity is an important effector mechanism of daratumumab, we explored the possibility of improving daratumumab-mediated cell-mediated cytotoxicity by blocking natural killer cell inhibitory receptors with the human monoclonal anti-KIR antibody IPH2102, next to activation of natural killer cells with the immune modulatory drug lenalidomide. In four hour antibody dependent cell-mediated cytotoxicity assays, IPH2102 did not induce lysis of multiple myeloma cell lines, but it did significantly augment daratumumab-induced myeloma cell lysis. Also in an *ex vivo* setting, IPH2102 synergistically improved daratumumab-dependent lysis of primary myeloma cells in bone marrow mononuclear cells (n=21), especially in patients carrying the FcγRIIIa-158F allele or the FcγRIIa-131R allele, who bind IgG1 with lower affinity than patients carrying the FcγRIIIa-158V allele or the FcγRIIa-131H allele. Finally, a further synergistically improved myeloma cell lysis with the daratumumab-IPH2102 combination was observed by adding lenalidomide, which suggests that more effective treatment strategies can be designed for multiple myeloma by combining daratumumab with agents that independently modulate natural killer cell function.

Introduction

Multiple myeloma (MM), the progressive malignancy of clonal plasma cells is the 2nd most common hematologic neoplasia[1] and accounts for 1.4 % of all cancers and for 1.8 % of all cancer mortality worldwide.[2] Despite encouraging improvements in the survival of MM myeloma patients over the last decade, the disease remains incurable, even by combination therapies with effective novel pharmacological agents.[2-5]

A novel appealing alternative to these treatments is the targeting of MM with therapeutic antibodies, as already standard-of-care in several other hematological malignancies. Therefore, we generated the CD38-specific human monoclonal antibody, daratumumab (DARA), which induces MM cell death via various mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[6] Based on these preclinical data, DARA is currently being evaluated in patients with relapsed/refractory MM, with encouraging results.[7]

In previous studies, we demonstrated that DARA-mediated ADCC can be significantly improved by lenalidomide (LEN), mainly due to the potent capacity of LEN to activate NK cells.[8,9] Based on these observations, we hypothesized that the efficacy of DARA-induced, NK cell-mediated ADCC may be further enhanced via modulation of NK cell regulatory signals transmitted via the inhibitory and activating NK receptors (KIRs; killer-cell immunoglobulin-like receptors).[10,11] Since the signals transmitted by inhibitory KIRs may prevent NK cell-mediated ADCC, even in the presence of an activating receptor-ligand interaction,[12] we set out to test the possibility to improve DARA efficacy by blocking inhibitory KIRs.

IPH2102 (formerly 1-7F9 and IPH2101) is a hinge-stabilized, human IgG4 monoclonal antibody that blocks the interaction of the three main inhibitory KIR receptors (KIR2DL-1, -2, -3) with their ligands, the human leukocyte antigen-C (HLA-C) molecules. The predecessor of IPH2102, IPH2101 (a wild-type IgG4 version of the antibody), was shown to increase in vitro NK-cell cytotoxicity against MM cells, but not against normal healthy cells.[13,14] Clinical trials conducted with IPH2101 in patients with relapsed/refractory MM and smoldering myeloma revealed that the clinical use of IPH2101 is safe and tolerable at doses that achieve full inhibitory KIR saturation, with disease stabilization as the best observed response to IPH2101.[15,16] This suggested that this antibody likely requires inclusion in a combination regimen such as with a potent ADCC-inducing antibody and/or with NK cell activating agents like LEN. Hence, in a series of *ex vivo* assays we explored the potential benefits of combining DARA with IPH2102 and LEN. We demonstrate that DARA-induced killing of primary MM cells increases synergistically when combined with these NK-enhancing agents.

Methods

Bone marrow mononuclear cells (BM-MNC) from MM patients

All patients' samples were collected and stored under institutional review board-approved protocols. All procedures involving bone marrow material were in

accordance with the declaration of Helsinki and approved by the local medical ethical committee. MNC from the BM were isolated by Ficoll-Hypaque density-gradient centrifugation and contained 2-35% MM cells as detected by flow cytometry. Freshly isolated BM-MNC from patients were immediately used in experiments (see below). Patient characteristics of the tested BM-MNC are summarized in table 1.

Tabel 1

Baseline characteristics of the tested MM patients' BM-MNC aspirates

Parameter	MM patients, (n=21)
Median age, years (range)	64 (37-78)
Sex, male, n (%)	9 (43%)
Type of monoclonal heavy chain	
IgG, n (%)	12 (57%)
IgA, n (%)	4 (19%)
Light chain only, n (%)	5 (24%)
Type of light chain	
Kappa, n (%)	9 (43%)
Lambda, n (%)	12 (57%)
Prior lines of therapy, median (range)	2 (0-10)
No prior treatment, n, (%)	9 (43%)
Prior LEN treatment, n (%)	8 (38%)
LEN refractory status*, n (%)	6 (29%)
Prior bortezomib treatment, n (%)	6 (29%)
Bortezomib refractory status*, n (%)	4 (19%)

*LEN-and/or bortezomib-refractory disease is defined as progressive diseave on therapy, no response (less than partial response) to therapy, or progressive disease within 60 days of stopping a LEN-and/or bortezomib-containing regimen, according to the International Uniform Response Criteria for Multiple Myeloma[5]

MM Cell lines

The CD38 positive, luciferase (LUC)-transduced MM cell lines UM9 and RPMI8226 were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, California, USA), supplemented with 10% fetal bovine serum (FBS; Integro BV, Salisbury, North Carolina, USA) and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin; both Invitrogen Life Technologies) as previously described.[17]

Peripheral blood mononuclear cells (PBMC) from healthy donors

All procedures involving material from healthy donors were approved by the local institutional medical ethical committee. Peripheral blood from healthy volunteers was obtained after written informed consent. PBMC were isolated by Ficoll-Hy paque density-gradient centrifugation to use as effector cells in ADCC assays.

Immunophenotyping by flow cytometry

Cell surface expression of various receptors was determined by FACS analysis using fluorochrome-conjugated monoclonal antibodies (mAb). PE-conjugated IPH2102 was produced by Innate Pharma. All other mAbs used for phenotyping were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Flow cytometry was performed using a FACS-Calibur device (BD Biosciences, Franklin Lakes, New Jersey, USA); data were analyzed using CellQuest software.

Test antibodies

The anti-KIR mAb IPH2102 (formerly 1-7F9/IPH2101) is a hinge-stabilized human IgG4 antibody, anti-KIR2DL1/L2/L3/S1/S2, produced in a CHO cell line and was generated by Innate Pharma (Marseille, France). Polyclonal human IgG4 (Sigma-Aldrich, St. Louis, Missouri, USA) was used as an isotype control for IPH2102. DARA was provided by Genmab (Utrecht, The Netherlands). Human IgG1-b12 against an innocuous antigen (HIV-1 gp120), donated by Genmab, was used as an isotype control for DARA.

Bioluminescence imaging-based ADCC assay using LUC-transduced MM cell lines

LUC-transduced MM cell lines UM9 and RPMI8226 were co-cultured with effector cells (freshly isolated PBMCs from healthy donors) at an effector to target ratio of 25:1 in white opaque 96-well flat bottom plates (Costar) in the presence of solvent control, or IgG1-b12 control antibody (1.0 µg/mL), or DARA (0.0001, 0.001, 0.01, 0.1 and 1.0 µg/mL) and/or IgG4 control antibody (10.0 µg/mL), or IPH2102 (10.0 µg/mL) for four hours. The survival of LUC+-MM cells was then determined by bioluminescence imaging (BLI), 10 minutes after addition of the substrate luciferin (125 µg/mL; Promega, Leiden, The Netherlands). Lysis of MM cells was determined using the following formula: % lysis = 1 - (mean BLI counts in treated wells / mean BLI counts in control wells) × 100%.

Flow cytometry-based ADCC assays using BM-MNC aspirates from MM patients

BM-MNCs derived from 21 MM patients, containing 2-57% CD138+ tumor cells, but also autologous effector cells, were used in flow cytometry-based ADCC assays as previously described.[8,9] BM-MNC were suspended in RPMI + 10% FBS and incubated with serial concentrations of DARA, IPH2102, control antibodies and LEN (Celgene, Utrecht, The Netherlands) alone or in combination in 96 wells U bottom plates. BM-MNC were incubated in fully humidified 5% CO2-air mixture at 37°C for 48 h. Sample viability at incubation was more than 98%, as assessed by using ToPro-3 (Invitrogen Life Technologies). Surviving MM cells after 48 h were enumerated by single platform flow cytometric analysis of CD138+ cells in the presence of Flow-Count Fluorospheres (Beckman Coulter) and ToPro-3 to determine absolute numbers of viable MM cells. The percentage of daratumumab-mediated ADCC was then calculated using the following formula: % lysis cells = 1 - (absolute number of surviving CD138+ cells in treated wells / absolute number of surviving CD138+ cells in control wells) x 100%. Where indicated, the DARA and IPH2102-specific lysis values were calculated by using the counts of surviving MM cells in IgG1-b12 and IgG4 treated wells, respectively.

Fc gamma receptor polymorphism genotyping

After isolating the patients' genomic DNA from PBMC, the typing for FcγRIIIa (CD16) polymorphisms (158V/F) on immune effector cells, including NK cells and macrophages, and FcγRIIa (CD32) polymorphisms (131H/R) on immune effector cells, including monocytes, was performed as described by Koene et al.[18] and Jiang et al.,[19] respectively.

Statistics

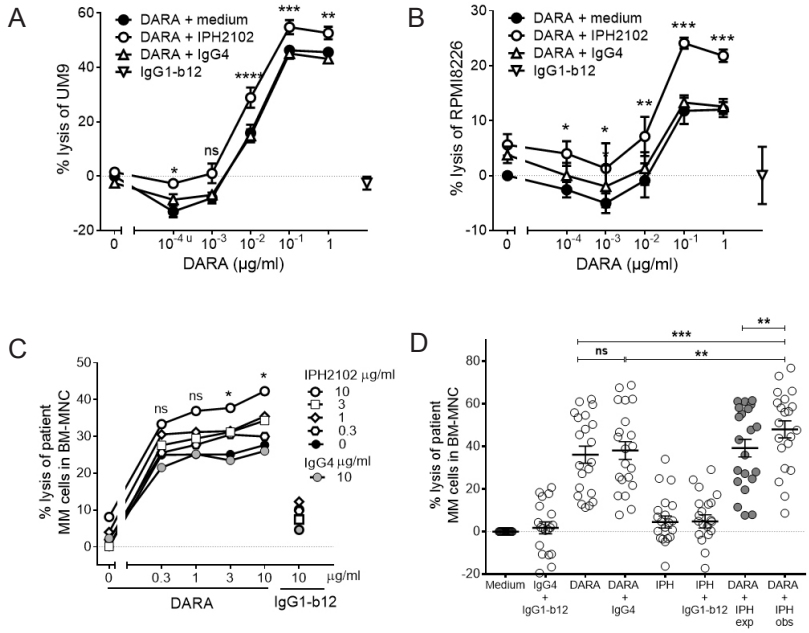
Differences between indicated groups were analyzed for significance in two-tailed paired Student's t tests analyses using Prism software (Graphpad Software Inc. version 5). P-values below 0.05 were considered significant.
Where indicated, expected lysis values from combinatorial treatments were calculated using the formula: % expected lysis = (% lysis by Agent 1 + % lysis by Agent 2) – % lysis by Agent 1 x % lysis by Agent 2. This formula assumes that there is only an additive effect between the combined agents. Paired t-tests were then used to test the statistical difference between the observed and expected values. The additive interaction was rejected, and synergy was concluded, if the observed values were significantly higher than the expected values. In triple combinations, the combination of the first two agents was considered as Agent 1.

Results

IPH2102 increases DARA-mediated ADCC of MM cells

We first investigated the potential of combining DARA with IPH2102 in standard four-hour ADCC assays using MM cell lines UM9 and RPMI8226 as target cells and PBMC from different healthy donors (n=4) as effector cells. An irrelevant polyclonal IgG4 antibody and an irrelevant IgG1 antibody (IgG1-b12) were used as isotype controls. As depicted in Figure 1A and B, DARA, but not IPH2102, induced ADCC of MM cells in a dose-dependent manner. Interestingly, however, DARA-dependent ADCC was significantly augmented in the presence of IPH2102 ($p<0.05$), with a somewhat larger effect in the cell line RPMI8226 which is relatively resistant to DARA. These results suggested that blockade of KIR receptors can improve NK cell-mediated lysis by DARA. To confirm these results in a more physiological setting, we employed our previously described *ex vivo* flow cytometry-based cytotoxicity assays, in which we measure the survival of primary CD138+ MM cells in patients' BM-MNC, without separating malignant cells from their microenvironment and autologous effector cells.[8] In this setting, incubation of 10 BM-MNC in serial dilution (0-10 µg/mL) of DARA and IPH2102 in a checkerboard fashion, confirmed the dose-dependent induction of MM cell lysis by DARA. Again, IPH2102 induced little or no lysis alone, even at a concentration of 10 µg/mL, which has been shown to saturate all KIR receptors.[14,15] However, 10 µg/mL of IPH2102 combined with DARA (>3 µg/mL), significantly enhanced DARA-mediated killing ($p<0.05$, Figure 1C). Hence, in an extended analysis, in which we assessed BM-MNC from 21 patients, we tested both antibodies only at a saturating concentration of 10 µg/mL (Figure 1D). Again,

FIGURE 1

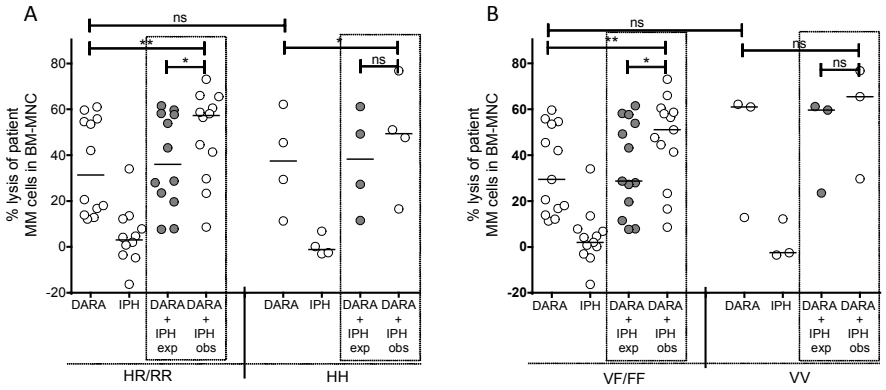


Dose dependent enhancement of DARA-mediated ADCC by IPH2102
(A,B) Four hour ADCC assays were performed using PBMC from four healthy donors as effector cells. The MM cell lines UM9 (A) and RPMI8226 (B) were used as target cells at an effector to target ratio of 25:1. Data show mean lysis and SEM of four experiments. DARA was used at indicated concentrations; IPH2102 was used at 10 µg/mL. IgG4 (10 µg/mL) was used as isotype control for IPH2102. IgG1-b12 was used at 1 µg/mL as isotype control for DARA. P-values were calculated by a paired t-test comparing DARA + IPH2102 vs. DARA + IgG4; ns means non significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Negative lysis levels indicate MM cell growth. (C) BM-MNC of 10 MM patients were incubated with DARA and/or IPH2102 and/or controls at serial concentrations (0-10 µg/mL) for 48h. IgG4 was used as isotype control for IPH2102, IgG1-b12 was used as isotype control for DARA. Surviving CD138+ MM cells were enumerated by flow cytometry. Percentage lysis of MM cells were calculated as indicated in methods. The mean lysis values are calculated and depicted. P-values were calculated by a paired t-test comparing DARA + IPH2102 vs. DARA + IgG4; ns means non significant, * p<0.05. (D) BM-MNC of 21 MM patients were incubated with DARA and/or IPH2102 and/or control antibodies at 10 µg/mL for 48h. Data show mean lysis and SEM. The observed (obs) lysis levels of MM cells with DARA-IPH2102 were compared to the expected lysis levels (exp), which were calculated with the assumption that the combinatorial effect is achieved by additive effects as indicated in methods. The statistical differences between the observed and expected levels were tested by a paired t-test; ns means non significant, * p<0.05, ** p<0.01, *** p<0.001. Every circle represents an individual BM-MNC patient sample. The black bar indicates the group mean ± SEM.

IPH2102 alone was not able to induce MM cell lysis, but it significantly improved the DARA-dependent ADCC in these primary patient samples. A mixed model analysis indicated the synergistic action of DARA and IPH2102 to generate an average of 10% extra tumor cell lysis as the observed amount of tumor cell lysis

significantly exceeded the expected levels, which were calculated with the assumption that the combinatorial effect was achieved only by additive effects (Figure 1D).

FIGURE 2

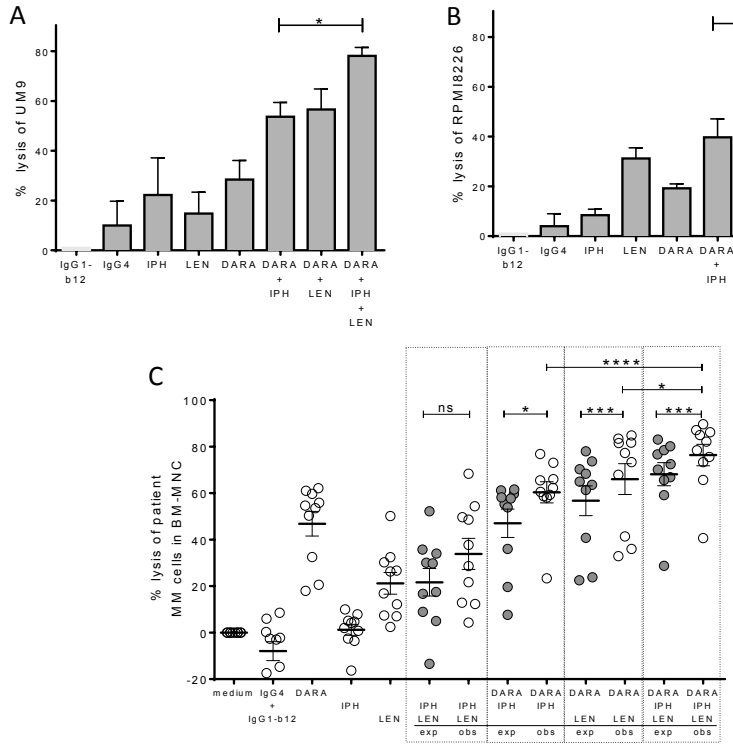


Impact of FcγR polymorphisms on the DARA-IPH2102 combination effect
Genomic DNA available from PBMC from 16 patients tested in Figure 1D were typed for (A) FcγRIIa 131 H/R and (B) FcγRIIIa 158 V/F polymorphisms on effector cells.
The data obtained in figure 1D were then grouped according to the respective polymorphisms. Every circle represents an individual patient sample. The black bars indicate the group median value. The observed (obs) lysis levels of MM cells with DARA-IPH2102 were compared to the expected lysis levels (exp), which were calculated with the assumption that the combinatorial effect is achieved by additive effects as indicated in methods.
P-values were calculated by a paired t-test; ns means non significant, * $p<0.05$, ** $p<0.01$.

Impact of FcγR polymorphisms on the DARA-IPH2102 combination effect

Although the MM cell lysis was significantly increased by DARA-IPH2102 combination, the observed heterogeneity in the results prompted us to analyze the impact of the FcγRIIIa 158 V-F and FcγRIIa 131 H-R polymorphisms on the outcome. FcγRIIIa (CD16) is expressed on immune effector cells, including NK cells. FcγRIIa (CD32) is expressed on immune effector cells, including monocytes.
The homozygous FcγRIIa His 131 and FcγRIIIa Val 158 have a higher affinity for IgG1.[20] FcγR polymorphisms influence the efficacy of ADCC[21] and might also influence effectiveness of monoclonal antibody-based immunotherapy.[22] Genomic DNA material was available from 16 of the tested MM patients and used for typing the FcγRIIa and FcγRIIIa polymorphism. We did not observe a significant association between Fc-receptor polymorphisms and DARA-mediated MM cell lysis in our cohort. However, the synergism between DARA and IPH2102 appeared to occur only in patients carrying a FcγRIIIa-158F allele (V/F and FF) and in patients carrying a FcγRIIa-131R allele (H/R and R/R) (Figure 2A,B). This suggests that the synergistic effects between IPH2102 and DARA are most pronounced in patients carrying an FcγRIIIa or FcγRIIa polymorphism with lower affinity to DARA.

FIGURE 3



Improvement of DARA-IPH2102 combinatorial effects by LEN
Four hour ADCC assays were performed using PBMC from healthy donors as effector cells and the MM cell lines UM9 (A) and RPMI8226 (B) as target cells. DARA, IPH2102 and LEN were used at 0.01 µg/mL, 10 µg/mL and 3 µM, respectively. P-values are calculated using a paired student t test; ns means non significant, * p<0.05. Data shown represent mean ± SEM of 4 experiments (C) BM-MNC of 10 MM patients were incubated with DARA (10 µg/mL), LEN (3 µM), IPH2102 (10 µg/mL), combination treatments or control antibodies for 48h. The observed (obs) lysis levels of MM cells with DARA-IPH2102-LEN were compared to the expected lysis levels (exp), which were calculated with the assumption that the combinatorial effect is achieved by additive effects as indicated in methods. The black bars indicate the group mean value ± SEM. P-values are calculated using a paired student t test; ns means non significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

IPH2102 further augments DARA-mediated killing of MM in the presence of LEN

After demonstrating the improvement of DARA-mediated MM cell lysis by IPH2102, we questioned whether adding LEN to this combination could further improve MM cell lysis. In our previous studies, LEN improved DARA-dependent MM cell lysis mainly via the activation of NK cells[8,9,23] and this effect was best visible after 48h incubation of effector cells with LEN. Hence in 4 hour ADCC assays, using MM cell lines UM9 and RPMI8226 as target cells and PBMC from different healthy donors (n=4) as effector cells, we not only added LEN in the assay, but also pretreated the effector PBMC for 48h with LEN to determine the effect of LEN. As expected LEN

increased the DARA-dependent MM cell lysis in UM9 and RPMI8226 cells, but also significantly upregulated MM cell lysis in combination with DARA-IPH2102 treatment (Figure 3A,B). To confirm this effect on primary MM cells, we used our 48 h flow cytometry-based MM cell lysis assays and incubated the BM-MNC of 10 MM patients with DARA, IPH2102, control antibodies or LEN, alone or in combinations (Figure 3C). In these assays, DARA, LEN and IPH2102 induced a median MM cell lysis of 54%, 22% and 3%, respectively. Combining DARA with IPH2102 and LEN further increased tumor cell lysis compared to DARA plus IPH2012 alone ($p<0.05$). With a median lysis level of 81% the triple combination significantly exceeded the levels observed for DARA+LEN (75%) or DARA+IPH2102 (61%). The MM cell lysis observed for the triple combination were found to be synergistic, since the observed values significantly exceeded the calculated expected values presuming additive effects. Taken together, these data suggest superiority of the DARA, IPH2102 and LEN triple combination compared to respective double combinations of these agents.

Discussion

In this study we explored the possibility to further improve DARA-mediated killing of MM cells by combining it with the anti-KIR antibody IPH2102 and the immunomodulatory drug LEN. The rationale behind this exploration is that a further augmentation of the activity of DARA might lead to stronger *in vivo* responses in order to increase the chances for long term sustained remissions. To develop this concept, we previously tested the combination of DARA with LEN and demonstrated that its potent activating effects on NK cells, can significantly improve DARA-dependent ADCC of MM cells.[8] In this regard, adding IPH2102 to this combination was a logical next step because this novel monoclonal antibody, was shown to increase NK cell lysis of malignant cells by blocking the three main inhibitory KIR receptors (KIR2DL1/2/3) on NK cells.[14] It therefore could, conceivably further potentiate DARA-dependent ADCC of MM cells independent of LEN. In a series of experiments, we discovered that IPH2102 alone, in contrast to earlier studies (see below), did not induce significant lysis of MM cells. However, it did augment DARA-dependent ADCC, and this combinatorial anti-MM effect was further improved by LEN. Our analyses strongly suggest that the enhancement of DARA-dependent ADCC by IPH2012, in the absence or presence of LEN, is established through synergistic action of the agents since the observed combinatorial tumor cell lysis levels significantly exceeded expected levels, which were calculated with the assumption that the effects were merely additive. A synergistic effect is consistent with the assumed mechanisms of action, because IPH2102 and LEN most probably increase NK cell cytotoxicity via non-overlapping, independent mechanisms. While IPH2102 blocks KIR-inhibitory signaling of NK cells,[14] LEN stimulates the proliferation of NK cells and activates them to increase the production of IFN-γ, TNFα and granzyme B.[8,23-26] As mentioned, in our experiments IPH2102 alone induced little or no MM cell lysis (Figure 1-3). This is apparently inconsistent with previous reports, in which IPH2101 was found to induce NK cell cytotoxicity against lymphoma, acute myeloid leukemia (AML) cells and MM cells.[14,27] While this latter study used IPH2101 at a higher

(30 µg/mL) concentration than we did (10 µg/mL), this is probably not the reason for the observed apparent discrepancy, since earlier tests have demonstrated that KIRs are fully saturated above 10 µg/mL IPH2102.[14] On the other hand, it needs to be noted that the study in the AML setting was performed using purified and IL-2 activated NK-cells from HLA-C-matched healthy donors, which were furthermore used at higher NK: tumor ratios (10:1) to induce visible and significant tumor cell lysis above background levels. By contrast, we executed our experiments mainly in patient-derived BM-MNC, thus without isolating the patient's own MM cells or NK cells from their natural environment. Consequently, in these experiments we readily achieve a median NK cell: MM cell ratio of 1:1 (data not shown), which generally does not induce significant MM cell lysis without addition of ADCC-inducing antibodies, like DARA. Another apparent discrepancy, which might be related to the differences in the experimental approach, is the absence of IPH2102-mediated MM cell lysis when combined with LEN in our setting, while previously IPH-LEN combinations were shown to improve MM cell lysis in both AML and MM settings.[13] In addition to the aforementioned experimental differences, our approach applied a lower dose of LEN (3 µM vs. 10 µM in other studies) since in a clinical setting it may be difficult to obtain LEN plasma levels above 3 µM without inducing adverse toxicities.[28] Our results, combining DARA with IPH2102, are fully compatible with the recent findings that anti-KIR antibodies augment the efficacy of ADCC-inducing antibodies like rituximab.[27] Accordingly, our results advocate that the best strategy to exploit the beneficial effects of IPH2102 may be in combination with ADCC-inducing antibodies. Furthermore, as we also show here, the ADCC-enhancing effects of IPH2102 with DARA can be further potentiated by addition of LEN. In this light, it is interesting that significant synergistic effects between DARA and IPH2102 were especially observed in bone marrow samples from MM patients carrying alleles for the low affinity variants for the activating receptors for ADCC, FcγRIIa or FcγRIIIa (Figure 2). In summary, this preclinical evaluation suggest that a further increase in the potency of MM treatment strategies can be designed by combining DARA with the immune modulatory agent LEN as well as with the KIR-blocking antibody IPH2102, whereby synergistic elimination of MM tumors by NK cells may be achieved. These preclinical data, together with the preliminary results of the first clinical studies with DARA monotherapy and IPH2101 monotherapy, support the use of a combination of DARA with LEN and IPH2102 in a clinical trial.

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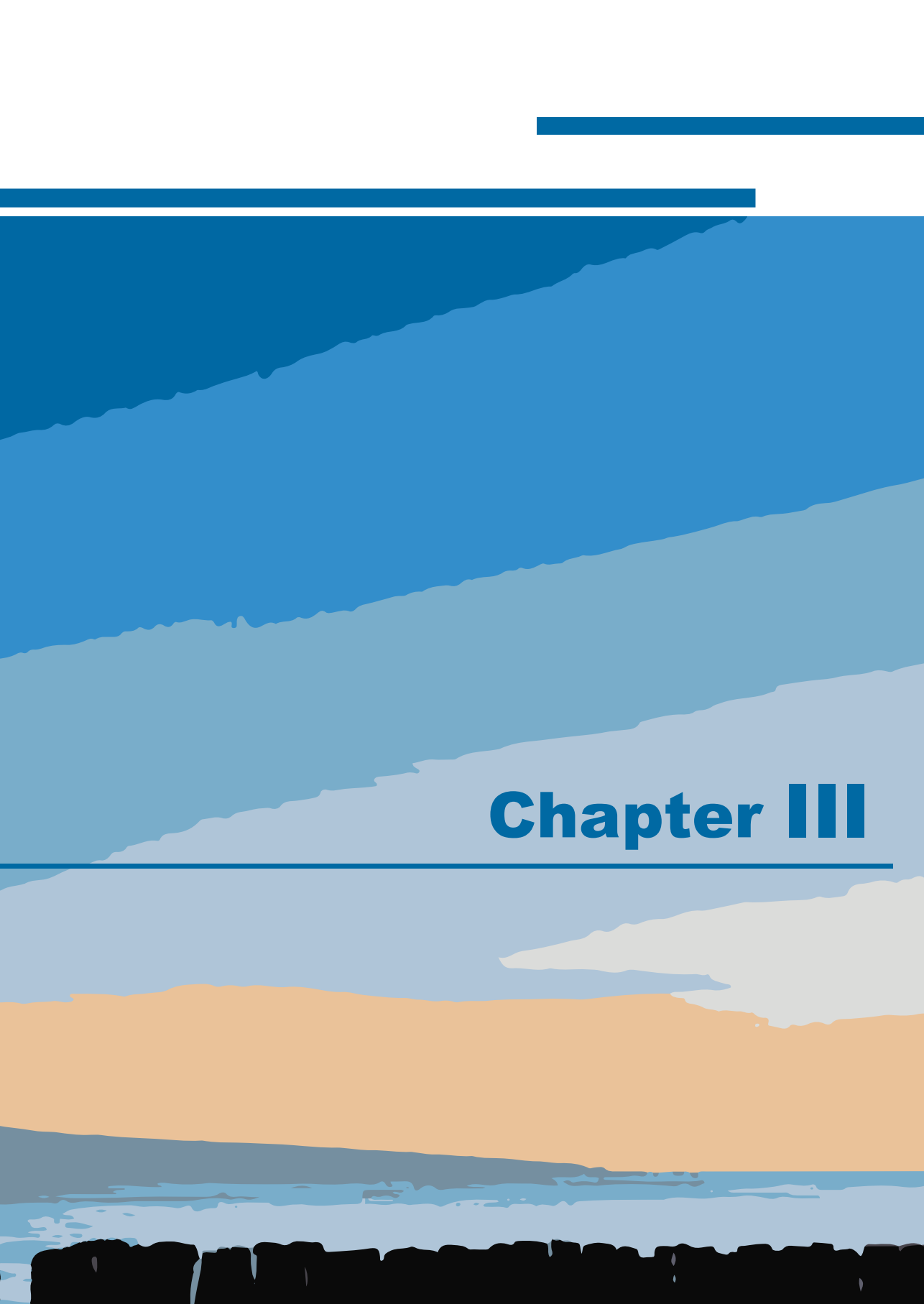
Chapter III

Preclinical evidence for the therapeutic potental of CD38-targeted immuno-chemotherapy in multiple myeloma patients refractory to lenalidomide and bortezomib

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Abstract

Novel therapeutic agents have significantly improved the survival of multiple myeloma (MM) patients. Nonetheless, the prognosis of MM patients who become refractory to the novel agents lenalidomide (LEN) and bortezomib (BOR) is very poor, indicating the urgent need for new therapeutic options for these patients. The human CD38 monoclonal antibody daratumumab (DARA) is being evaluated as a novel therapy for MM. Prompted with the encouraging results of ongoing clinical phase I/II trials, we now addressed the potential value of DARA alone or in combination with LEN or BOR for the treatment of LEN-and BOR-refractory patients. In *ex vivo* assays, mainly evaluating antibody-dependent cellular cytotoxicity (ADCC), DARA induced significant lysis of LEN/BOR-resistant MM cell lines and of primary MM cells in the bone marrow mononuclear cells derived from LEN- and/or BOR-refractory patients. In these assays LEN but not BOR, synergistically enhanced DARA-mediated MM lysis through activation of natural killer (NK) cells. Finally, in an *in vivo* xenograft model, only the combination of DARA with LEN effectively reduced the tumorigenic growth of primary MM cells from a LEN- and BOR-refractory patient, providing the first preclinical evidence for the benefit of DARA plus LEN combination for LEN- and BOR-refractory patients.

Translational Relevance

The prognosis of multiple myeloma (MM) patients who become refractory to the novel anti-myeloma agents lenalidomide and bortezomib is very poor, indicating the need for new therapeutic strategies for these patients. The human CD38 monoclonal antibody daratumumab is currently being evaluated in phase I/II clinical trials as a novel therapy for MM. Here, we demonstrate the *in vitro* as well as *in vivo* susceptibility of malignant cells, derived from lenalidomide- and bortezomib-refractory MM patients, to daratumumab. Thus, this novel CD38 antibody may offer new therapeutic perspectives for multi-drug refractory MM patients. Furthermore, we discovered that lenalidomide, although no longer effective as a therapeutic drug in these patients, can still synergistically potentiate the anti-myeloma effects of daratumumab through the activation of natural killer cells. Therefore, the therapeutic perspectives of multi-drug refractory patients may be improved by combination therapy with daratumumab and lenalidomide.

Introduction

Multiple Myeloma (MM), the malignant disease of antibody producing plasma cells, is still considered incurable. Over the past decade, the survival of MM patients has significantly improved, mainly due to the introduction of the novel immunomodulatory agent lenalidomide (LEN), the potent proteasome inhibitor bortezomib (BOR), and the application of high-dose conventional therapy with autologous stem cell rescue.[1] Nonetheless, virtually all anti-myeloma strategies are eventually hampered by the development of drug-resistance. The prognosis of MM patients who become
refractory to BOR and LEN is very poor with an event-free survival and overall survival of only 5 and 9 months, respectively.[2] Therefore, especially for this specific group of (multi-)refractory MM patients, new approaches that induce long-term tumor regression are urgently needed. In this respect, several new antimyeloma agents hold promise, including next generation IMiDs (pomalidomide) and proteasome inhibitors (carfilzomib).[3]
On the other hand, the success of anti-CD20 monoclonal antibodies (mAbs) in the treatment of non-Hodgkin's lymphomas and chronic lymphocytic leukemia, illustrated that antibody-mediated immunotherapy can also represent a powerful therapeutic option for hematological malignancies and stimulated the investigation of antibody therapies for MM.[4] Daratumumab (DARA) is a human IgG1 kappa
monoclonal antibody that targets CD38, a type II transmembrane glycoprotein, which is highly and uniformly expressed in MM.[5] Previously we have demonstrated that DARA induces effective MM cell death mainly via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[5] We have also shown in *ex vivo* assays that the anti-MM efficacy of DARA can be potentiated by immunomodulatory agents such as LEN or BOR, alone or in combination with other antimyeloma agents.[6,7] *In vivo*, DARA controlled or even eliminated MM cell
line-derived tumors growing in the bone marrow of RAG2-/-γc-/- mice.[8] In a more advanced *in vivo* model, DARA also mediated significant anti-tumor activity against tumors derived from patients' MM cells, growing in a humanized bone microenvironment.[8] With these properties, DARA monotherapy represents a promising
therapeutic strategy for the treatment of MM. DARA is currently evaluated in phase I/II studies.[9, 10] Encouraging preliminary results of these studies prompted us to evaluate the potential value of DARA in LEN- and BOR-refractory MM patients, who have a very poor prognosis at present. We hypothesized that, despite the drug resistance of the malignancy, the immune system of these patients could still respond to the previously described immunomodulatory effects of LEN[11,14] and BOR.[15,16] Therefore we now evaluated DARA alone or in combination with LEN or BOR as a potential therapy for LEN- and BOR-refractory patients.
In *in vivo* and *ex vivo* assays we show the susceptibility of LEN- and BOR-refractory MM cells to DARA treatment. Confirming our hypothesis, the combination of DARA with LEN or BOR significantly enhanced lysis in LEN- and/or BOR- refractory patients. While the combination of DARA with BOR resulted in additive effects, LEN synergized with DARA through the activation of effector cells, especially NK cells. Finally, in an *in vivo* xenograft model, the combination of DARA with LEN, in the

presence of human NK cells, effectively delayed the tumorigenic growth of primary MM cells from a LEN- and BOR-refractory patient, underscoring the potential beneficial anti-MM effects of the DARA plus LEN combination for LEN- and BOR-refractory patients. These results suggest that DARA combined with LEN or BOR may be an effective therapeutic combination in LEN- and BOR-refractory patients.

Tabel 1

Baseline characteristics of the tested BM-MNC of 11 MM patients

Parameter	MM patients n=11
Median age, years (range)	64 (42-75)
Sex, male, n (%)	7 (64%)
Type of monoclonal heavy chain	
IgG, n (%)	5 (46%)
IgA, n (%)	2 (18%)
Light chain only, n (%)	4 (36%)
Type of light chain	
Kappa, n (%)	7 (64%)
Lambda, n (%)	4 (34%)
Previous therapy	
Prior lines of therapy, median (range)	4 (2-6)
Prior stem cell transplantation	8 (73%)
autologous	8 (73%)
allogeneic	0 (0%)
Prior LEN treatment, n (%)	11 (100%)
LEN refractory status*, n (%)	11 (100%)
Prior bortezomib treatment, n (%)	10 (91%)
Bortezomib refractory status*, n (%)	8 (73%)

Abbreviations: BOR, bortezomib; LEN, lenalidomide.
*LEN- and/or bortezomib-refractory disease is defined as progressive disease on therapy, no response (less than partial response) to therapy, or progressive disease within 60 days of stopping a LEN- and/or bortezomib-containing regimen, according to the International Uniform Response Criteria for Multiple Myeloma.[2]

Material and Methods

Patients:

All patient materials were collected after written informed consent as approved by the institutional medical ethical committee. All samples originated from LEN- and BOR-refractory patients, who were extensively treated with a median of 4 preceding regimens that included, but were not limited to, induction therapy with anthracyclines followed by autologous stem cell transplantation, thalidomide, LEN

and BOR. LEN- and BOR-refractory disease is defined as progressive disease on LEN- and BOR-therapy, no response (less than partial response) to LEN- and BOR-therapy, or progressive disease within 60 days of stopping a LEN- and BOR-containing regimen, according to the International Uniform Response Criteria for Multiple Myeloma.[2] Characteristics of the tested MM patients are summarized in Table 1.

Bone marrow and peripheral blood mononuclear cells

All samples from patients and healthy individuals were collected and stored using protocols/procedures approved by the institutional medical ethical committee in accordance with the declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) from healthy donors and MM patients and bone marrow mononuclear cells (BM-MNC) from MM patient BM aspirates were isolated by Ficoll-Hypaque density-gradient centrifugation. Freshly isolated PBMC were used after culturing with and without LEN (3 μM; Celgene, Utrecht, The Netherlands) for 48 h as effector cells in classical 4 hour flow cytometry-based cytotoxicity assays, in which MM cell lines were used as target cells.

Bioluminescence imaging (BLI)-based ADCC assays using luciferase (LUC)-transduced MM cell lines

LUC-transduced (LEN/BOR-resistant) MM cells were co-cultured with effector cells (freshly isolated PBMC or MACS-enriched PBMC fractions; see below) at the indicated effector to target ratios in the wells of white opaque 96 well plates (Costar, Washington D.C., USA) in the presence of a previously determined optimal concentration of DARA (0.1 μg/ml) for four hours. The survival of LUC+-MM cells was then determined by BLI, 10 minutes after addition of the substrate luciferin (125 μg/ml; Promega, Leiden, The Netherlands). The percentage MM lysis was determined using the following formula: % lysis = 1- (mean BLI signal in the presence of effector cells and daratumumab / mean BLI signal in the presence of effector cells and control antibody) x100%. In some assays PBMC were enriched for T cells and monocytes using immunomagnetic MACS beads coated with CD3 and CD14, respectively, following the instructions of the manufacturer. The purity of both T and monocyte fractions were >90%. The negative fractions of CD3-enriched PBMC, which were highly enriched for NK cells (>90 %), were also used as effector cells in the in vitro and in vivo assays.

Flow cytometry-based *ex vivo* cell lysis assays in BM-MNC

Freshly isolated BM-MNC, containing 2-35% malignant plasma cells as determined by flow cytometry, were immediately used in *ex vivo* experiments. The BM-MNC, containing the malignant plasma cells, as well as the patient's own effector cells, were incubated with DARA (10 μg/mL; Genmab), LEN (3 μM; Celgene) and BOR (3 nM; Millennium Pharmaceuticals, Cambridge, Massachusetts, USA) alone or in combination in RPMI + 10% fetal bovine serum in 96 wells round bottom plates in fully humified incubators at 37°C, 5% CO2-air mixture for 48 h. The survival of primary CD138+ MM cells was determined by flow cytometry as previously described.[7] Sample viability at incubation was more than 90%, as assessed using ToPro-3 (Invitrogen Life Technologies, Carlsbad, California, USA). Percentage lysis of MM cells was deduced using the following formula: % lysis cells = 1 - (counts of surviving CD138+ cells in treated wells / counts of number of surviving CD138+ cells in control wells) × 100%.

Mesenchymal stromal cells (MSC)

The mononuclear cell fraction from healthy donor BM aspirates was obtained after Ficoll-Hypaque centrifugation and seeded in culture flasks in α-MEM (Gibco, Paisley, UK), supplemented with 5% human platelet lysate, penicillin and streptomycin (100 units/mL and 100 µg/mL, respectively; both Invitrogen Life Technologies), and 10 IU/ml heparin (Leo Pharma, Breda, The Netherlands). Expanded MSC were re-plated at a confluence of approximately 90%, passaged once, and collected and stored at -196°C for later use.

MM Cell lines and culture

The CD38+ MM cell lines UM9, L363-CD38 cl2.2, LME-1 and RPMI8226 were cultured in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% fetal bovine serum (FBS; Integro BV, Salisbury, North Carolina, USA) and antibiotics (100 units/mL penicillin, 100 µg/ml streptomycin; both Invitrogen Life Technologies) as previously described.[7]

Antibodies and reagents

Daratumumab was provided by Genmab. IgG1-b12, a human mAb against an innocuous antigen (HIV-1 gp120), was used as an isotype control as previously described.[7]

In vivo treatment of primary MM tumors growing in humanized bone marrow niches in immunodeficient mice

In vivo experiments were conducted in a recently described human MM model in the RAG2-/-γc-/- mice[8] after approval of the local Ethical Committee for Animal Experimentation and in compliance with the Dutch Animal Experimentation Act.

Briefly, to enable the engraftment of primary MM cells, a humanized microenvironment was generated in mice by subcutaneous (s.c.) implantation of ceramic scaffolds that were seeded with human MSC (2×10^5 cells/scaffold) and *in vitro* cultured for 7 days in osteogenic medium, containing ascorbic acid and dexamethasone.

Eight weeks after implantation, mice received a sublethal irradiation dose (3 Gy, 200 kV, 4 mA) and luciferase-gene-marked primary MM cells were injected directly into the scaffolds (1×10^6 cells/scaffold). Luciferase transduction of primary MM cells was carried out using the lentiviral construct pRRL-cPPT-CMV-Luc2-IRES-GFP-PRE-SIN as described previously.[8,17] Tumor growth was monitored by BLI using a charge-coupled device camera, controlled by Photo Vision software and analyzed with M3Vision software (Photon Imager; Biospace Laboratory, Paris, France). When tumors became clearly detectable, mice were distributed over the following treatment groups: 1) control, 2) T-cell depleted PBMC (PBMC-T), 3) PBMC-T plus LEN, 4) PBMC-T plus DARA, and 5) PBMC-T plus LEN plus DARA. LEN (1 mg/kg) was given in a 5 days on 2 days off schedule for 2 weeks (days 49–53 and 56-60) and both DARA (8 mg/kg) and PBMC-T (8×10^6 cells/mouse) were given on day 49 and 56. PBMC-T were prepared by Ficoll-Hypaque density-gradient centrifugation of buffy coats, and subsequent depletion of T cells by CD3-beads using the EasySep™-technology (STEMCELL Technologies, Vancoucer, Britsch Colombia, Canada).

Immunophenotyping by flow cytometry

Cell surface expression of all tested antigens was determined by flow cytometry using FITC-, PE-, PerCP-, or APC-conjugated monoclonal antibodies (all from BD Biosciences, Franklin Lakes, New Jersey, USA). Flow cytometry was carried out using a FACS-Calibur device (BD Biosciences);
the data were analyzed using Cell Quest software.

Statistics

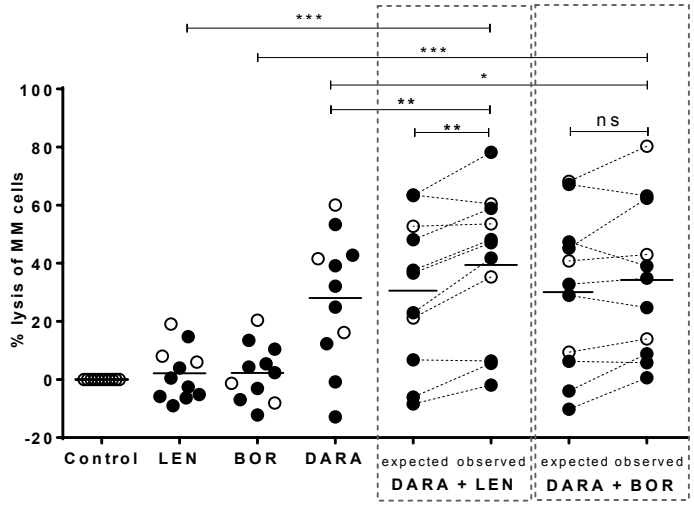
Differences between groups were analyzed for significance in two-tailed paired Student's t tests using Prism software (Graphpad Software Inc. version 5).
P-values below 0.05 were considered significant. In case of combinatorial treatments of DARA and LEN or BOR, the expected lysis values were calculated to test the null hypothesis that there is only an additive effect between DARA and the drug (LEN or BOR), using the following formula: % expected lysis = (% lysis with DARA + % lysis with drug) – % lysis with DARA × % lysis with drug.[7,18] The null hypothesis of "additive effects" was rejected, if the observed values were significantly higher ($p<0.05$) than the expected values.

Results

LEN but not BOR, improves DARA-mediated ADCC of MM cells from LEN- and BOR- refractory patients

We first questioned whether the MM cells of LEN- and/or BOR-refractory patients would respond to DARA alone and whether improvement of MM cell lysis is possible when combining DARA with LEN or BOR in LEN- and/ or BOR- refractory MM patients. To address these questions we used our previously developed *ex vivo* flow cytometry-based cytotoxicity-assay.[7] BM-MNC from 11 LEN-refractory patients, of whom 8 were also BOR-refractory, were incubated with DARA, LEN and BOR alone or in combination to measure the lysis of CD138+ MM cells (Figure 1). LEN and BOR mono treatment induced virtually no MM cell lysis in refractory patients, consistent with the LEN- and/or BOR-refractory clinical status of these patients. In contrast, DARA induced significant levels of MM cell lysis in the BM-MNC from refractory MM patients (mean 29.7%, 95% CI 13.5 – 45.9%). Nonetheless, there was a large degree of heterogeneity in the lysis induced in LEN- and BOR-refractory patients, with less than 30% of MM cell lysis in 5/11 patients. Remarkably however, the MM cell lysis of 29.7% with DARA alone was significantly improved to 39.4% (95% CI 22.1 – 56.7%; $p=0.0041$) upon combination of DARA with LEN, although all tested patients were refractory to LEN. Such an effect was not observed with BOR. Assuming drug combinations would be additive, we calculated the expected lysis values,[7,18] as described in material and methods, and compared them with the observed results. The DARA plus LEN combination appeared synergistic, as illustrated by the significant difference between the expected versus observed results, while the DARA plus BOR combination was indeed additive.

FIGURE 1



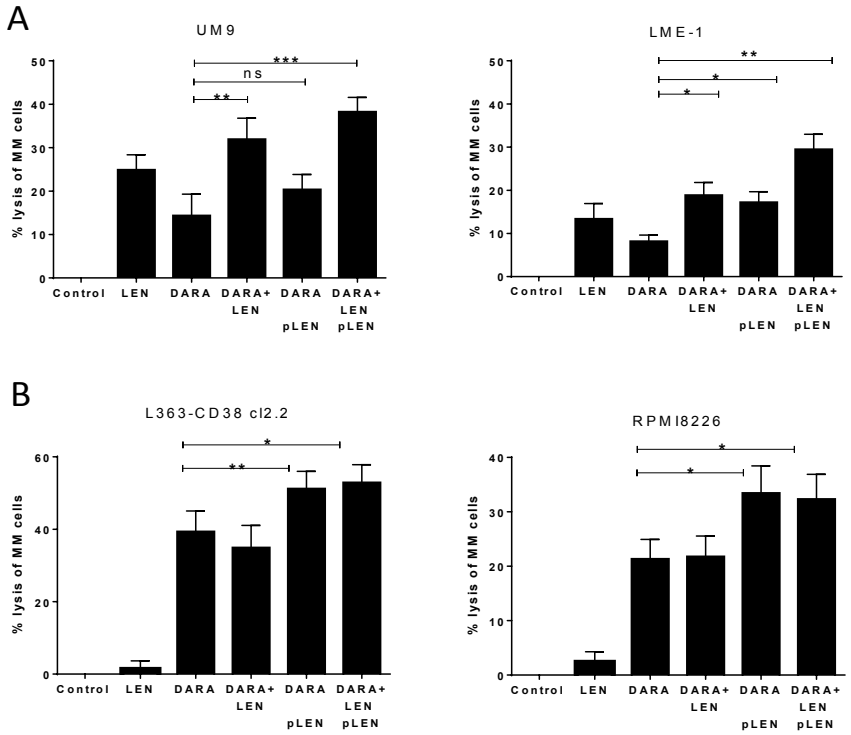
LEN, but not BOR, significantly improves DARA-mediated lysis of primary MM cells from LEN- and BOR-refractory patients

BM-MNC of 11 LEN-refractory MM patients, of whom 8 patients were also BOR-refractory (the black circles represent the LEN/BOR double-refractory patients) were incubated with control antibody (control) or with DARA (both at a concentration of 10 µg/mL), alone or in combination with LEN (3 µM) or BOR (3 nM) for 48h. Surviving MM cells were enumerated by FACS-analysis of CD138+ cells. Percentage lysis of MM cells was calculated as indicated in material and methods. DARA and LEN acted synergistically, as illustrated by significant differences between the expected vs. observed results, while the combination of BOR and DARA shows an additive effect, as illustrated by the non-significant differences between the expected vs. observed results as indicated in material an methods. P-values were calculated by a paired t test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns not significant.

NK activation by LEN contributes to LEN and DARA synergism in LEN- and BOR-refractory patient MM cells

To investigate effector cell contributions to the mechanism of synergy between LEN and DARA, we performed standard 4-hour cytotoxicity assays, in which PBMC from random healthy donors (n=5) were used as effector cells against the LEN-resistant MM cell lines L363-CD38 cl2.2 and RPMI8226, and the LEN-sensitive cell lines UM9 and LME-1. As expected, LEN alone induced MM cell lysis and also enhanced the DARA-dependent lysis in the LEN-sensitive cell lines (Figure 2A). In the LEN-resistant cell lines DARA, but not LEN, induced significant MM cell lysis (Figure 2B). DARA-mediated lysis of MM cells was not improved when MM cells were pre-incubated with LEN in LEN-resistant cell lines (Figure 2B). Interestingly, when we pretreated the PBMC (effector cells; Plen) with LEN for 48h, the DARA-mediated MM cell lysis was significantly enhanced, not only in LEN-

FIGURE 2

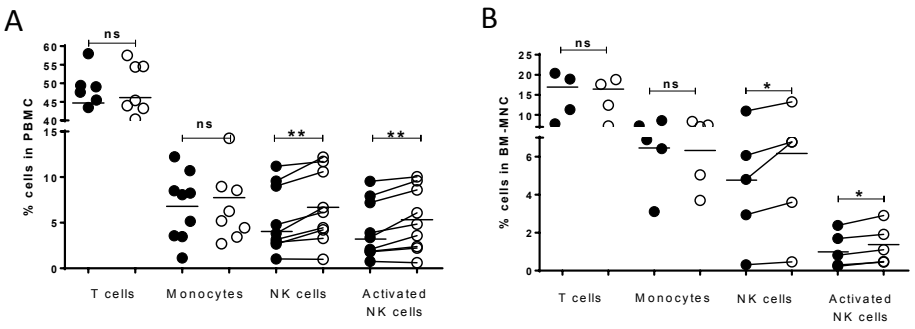


Potent capacity of LEN to activate ADCC effector cells as explanation for the LEN and DARA synergy in LEN-resistant MM cells

(A, B) Results of standard 4h ADCC performed using PBMC from healthy donors as effector cells. Target cells were the LEN-sensitive MM cell lines UM9 and LME1 in (A), and the LEN-resistant cell lines L363-CD38 cl2.2 and RPMI8226 in (B). Data show mean and SEM of at least five experiments. Control antibody (control) or DARA was added in the assay at a concentration of 0.1 µg/mL. LEN was either added in the assay (LEN) and/or pre-incubated with effector PBMC for 48h (pLEN) at a concentration of 3 µM. P-values between the indicated groups were calculated using a paired student's t test; * p<0.05, ** p<0.01, *** p<0.001; ns not significant.

sensitive, but also in LEN-resistant cell lines (Figure 2A,B). Based on these results, the synergy between LEN and DARA appeared to be due to the action of LEN on the effector cells present in PBMC and not via direct effects on the tumor cells. To identify the effector cell population activated by LEN we determined the frequencies of various cell subsets in the PBMC of 9 healthy donors, and in BM-MNC of 5 LEN- and BOR-refractory MM patients. LEN-treatment had no effect on the frequencies of T cells or monocytes, but significantly increased the frequency of CD3-CD56+ NK cells and the activated fraction of CD3-CD56+CD16+ NK cells in healthy donor PBMC (Figure 3A) and in LEN- and BOR- refractory MM patient BM-MNC (Figure 3B). To functionally validate these results, we pretreated PBMC from healthy donors and a heavily pretreated LEN- and BOR-refractory MM patient with LEN or solvent control

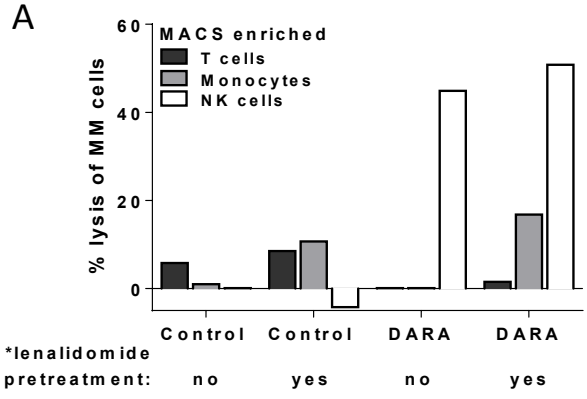
FIGURE 3



Effect of LEN on NK cells in healthy PBMC and BM-MNC derived from LEN- and BOR-refractory patients

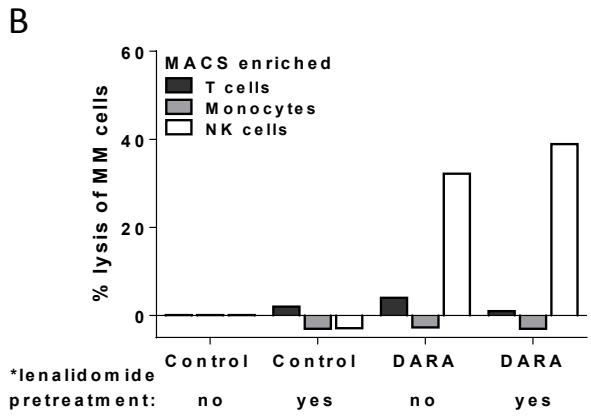
Effect of 48h LEN pre-incubation on immune cell subsets in PBMC of healthy donors (A) and on BM-MNC of LEN- and BOR-refractory MM patient' full bone marrow aspirates (B). Results represent percentages of cell subsets in untreated (black circles) and LEN-treated (white circles) samples, determined by FACS-analysis. T cells, monocytes and NK cells were identified as CD3+, CD14+ and CD3-CD56+ cells respectively. Activated NK cells were identified as CD3–CD56+CD16+ cells.[32,33] The statistical differences between untreated and treated groups were calculated using a paired student's t test; * $p<0.05$, ** $p<0.01$; ns not significant. For (activated) NK cells the paired samples are depicted with a connection-line, for the clarity.

FIGURE 4



and used their T cell-, NK cell- or monocyte-enriched fractions as effector cells in a 4-hour ADCC-assay (Figure 4A and B). Regardless of the source of the sample, the MM cell lysis was mainly mediated by CD3-/CD56+-enriched NK cell fraction in these assays. LEN pre-incubation of PBMC improved the NK cell-mediated MM cell lysis, but did not induce or enhance T cell- or monocyte-mediated MM cell lysis, confirming that the synergism between LEN and DARA is mainly due to the activation of NK cells.

FIGURE 4

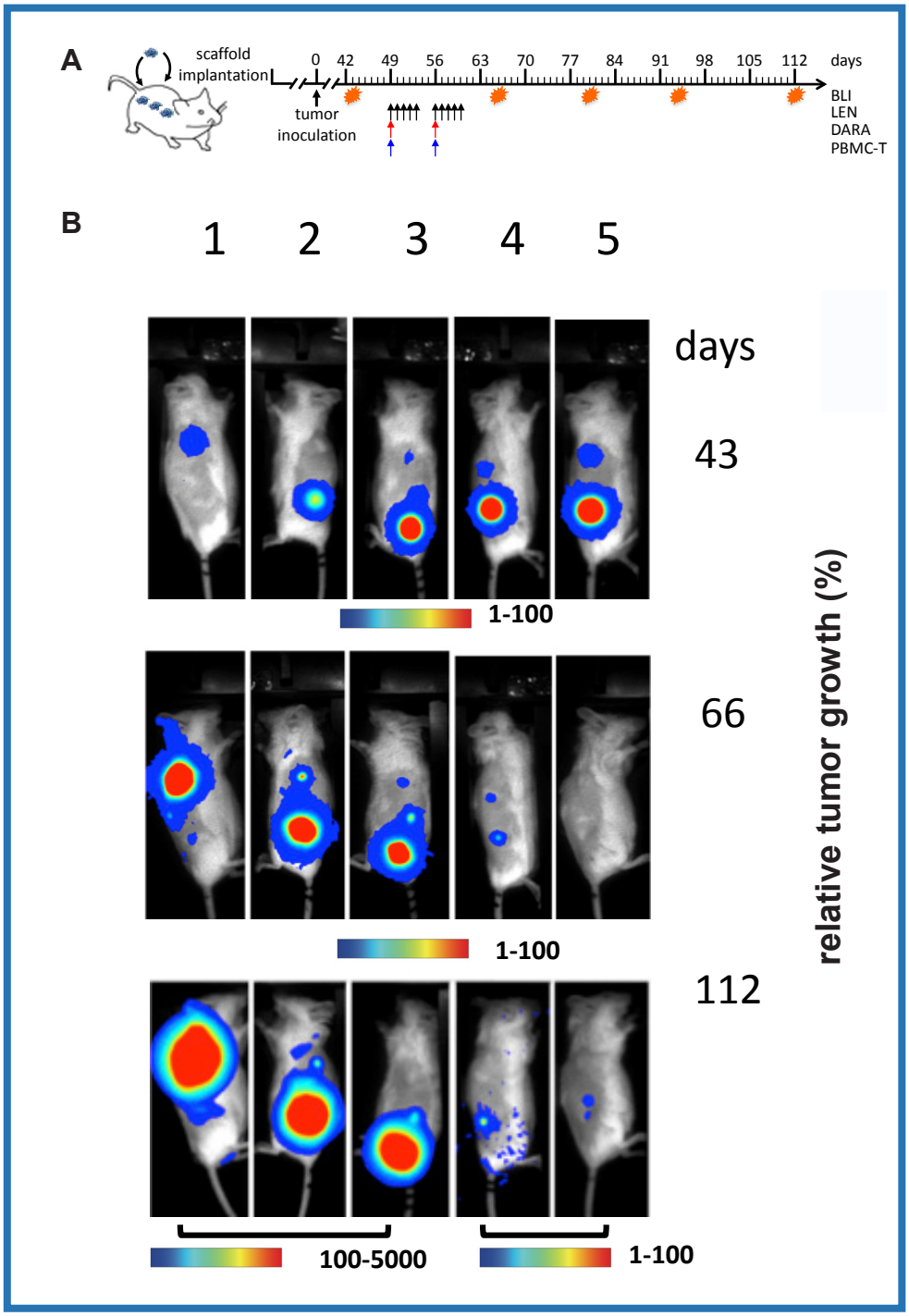


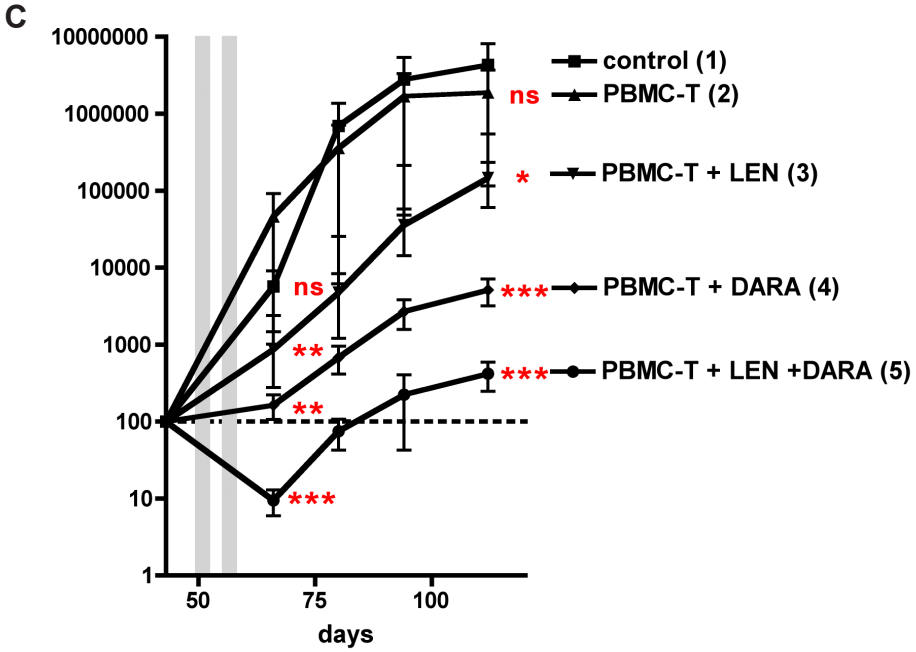
NK cells as mediators of DARA-dependent ADCC
PBMC were pretreated with LEN for 48h at a concentration of 3 μM were indicated (*). Then MACS-enrichment for monocytes, T cells and NK cells was performed as described in material and methods. MACS-enriched monocytes, T cells and NK cells from the PBMC of a healthy donor (A) and the PBMC of a heavily pretreated LEN- and BOR-refractory MM patient (B) were tested in 4 hour ADCC assays as effector cells. RPMI8226 MM cell line was used as target cells. We show a epresentative example of three performed experiments. See for further details the legend of Figure 2 and material and methods.

LEN improves responses to DARA in humanized mice engrafted with MM cells derived from a LEN- and BOR-refractory patient

To evaluate the added value of LEN to DARA-mediated treatment for LEN- and BOR-refractory patients *in vivo*, we used our recently developed model in which primary MM cells from patients can be grown in humanized bone scaffolds implanted in RAG2-/-γc-/- mice.[8] We inoculated the humanized scaffolds with luciferase-marked primary MM cells, derived from a LEN- and BOR-refractory patient. After confirmation of tumor growth by BLI, mice were treated with DARA and LEN alone or in combination (Figure 5A). To optimally evaluate effects of LEN and DARA, we also co-injected the mice with NK cell-enriched (T cell-depleted) PBMC of a healthy donor in combination with DARA and/or LEN as RAG2-/-γc-/- mice are devoid of NK cells. As expected, MM tumors from the LEN- and BOR-refractory patient

FIGURE 5


A
scaffold implantation
0 42 49 56 63 70 77 84 91 98 105 112 days
tumor inoculation
BLI
LEN
DARA
PBMC-T
B
1 2 3 4 5
days
43
66
112
1-100
1-100
100-5000
1-100
relative tumor growth (%)



LEN improves response to DARA in LEN- and BOR-refractory MM patient-derived tumor cells in an in vivo mouse model

(A) Humanized bone marrow environment scaffolds implanted in Rag2-/-γc-/- mice were inoculated with luciferase-marked primary MM cells, derived from a LEN- and BOR-refractory patient. Mice were monitored biweekly by BLI. When visible tumor growth was observed, mice were treated with T cell-depleted PBMC (PBMC-T), combined with LEN, or DARA, or the respective combination, or vehicle as a control at the indicated time points. (B) Images of representative mice per treatment group: 1) control, 2) PBMC-T, 3) PBMC-T plus LEN, 4) PBMC-T plus DARA, and 5) PBMC-T plus LEN plus DARA. Shown are BLI images before (day 43; top panels), 2 weeks after the initiation of treatment (day 66; middle panels) and at the end of the experiment (day 112; lower panels).

(C) Quantification of the therapeutic intervention. BLI results are expressed as relative tumor growth with the intensity at day 43 (before start of treatment) set to 100% (indicated by dashed line). Lines represent mean BLI signal of at least 12 individual tumors followed in time (control n=12, PBMC-T n=16, PBMC-T plus LEN n=17, PBMC-T plus DARA n=13, PBMC-T plus LEN plus DARA n=15) ± SEM. The treatment cycles are indicated by the grey bars. The statistical differences between groups directly after treatment and at the end of the experiment are calculated using a Mann-Whitney test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns not significant.

showed tumor growth after treatment with LEN alone (Figure 5B and C).

Confirming *in vitro* results, treatment with DARA alone suppressed the tumor growth significantly. However, only the combination of DARA plus LEN was able to reduce the tumor volume (Figure 5B and C) indicating synergistic effects. Notably, the tumor growth was suppressed/delayed for 6 weeks, while the controls showed a 4-log increase in tumor growth. These results provided the first preclinical *in vivo* evidence that clinically beneficial, and sustained anti-MM effects can be achieved in LEN- and BOR-refractory MM patients with DARA therapy in combination with LEN.

Discussion

Despite significant advances in the management of MM over the past decades, the development of drug resistance remains a significant obstacle to the long-term survival of MM patients. In the present study, using well established *in vivo* and *ex vivo* models, we now show that DARA, especially when combined with LEN, can be effective as a targeted treatment for LEN- and BOR-refractory patients. Specifically, we demonstrate that the MM cells of these patients can be killed by DARA-dependent ADCC and these anti-MM effects can still be potentiated by LEN in a synergistic fashion not only *in vitro* but also in an optimized *in vivo* model.

We have previously shown that DARA induces effective lysis of MM cells not only via ADCC, but also via CDC, via apoptosis upon cross-linking with anti-immunoglobulin G antibody and via anti-enzymatic activity.[5] In our current *in vitro* assays, however, we mainly focused on assays that measure (NK cell-mediated) ADCC. From this point of view, our results may underestimate the whole potential therapeutic benefits that can be achieved by combining DARA with BOR or with LEN. Nonetheless, we think to have measured one of the most relevant parameters, because the previously described immunomodulatory effects of LEN and BOR are especially beneficial for ADCC. For instance, LEN has been shown to increase the NK cell numbers and activation status of these cells.[11,13] LEN has further been shown to upregulate FcγRIIIa (CD16) expression,[13] and to inhibit T-regulatory cell function.[12] BOR on the other hand, has been described to downregulate expression of inhibitory NK cell ligands[16] and to upregulate CD95 and TRAILR2 on MM tumor cells,[15,19] whereby enhancing the susceptibility of myeloma cells to NK cell mediated killing *in vitro*.The potential benefit of targeting MM tumor cells by specific mAbs alone or in combination with other anti-MM agents has also been demonstrated by other recent experimental and clinical studies.[4, 20–26] For instance, BOR was described to synergistically enhance the lysis of ADCC mediated by the CS1 mAb elotuzumab *in vitro*.[27] Perhaps due to the limitations of our experimental setting, we observed only additive effects of BOR combined with DARA. Some caution is therefore needed to interpret these results. Nonetheless, the apparent discrepancy between the aforementioned elotuzumab-study and ours may also be due to the fact that a significant enhancement of elotuzumab-mediated ADCC was only observed at doses of BOR ≥ 5 nM[27], while we have specifically chosen a dose of 3 nM for BOR, and 3 μM for LEN, because these dose levels are clinically achievable in humans without causing severe side effects and comparable with concentrations found in the serum of patients treated with therapeutic doses of BOR and LEN.[28,29] Furthermore, it is possible that, depending on the condition, BOR mitigates ADCC. For instance, BOR was also described to disrupt TRAIL-expression on MM cells and to downregulate the activating NK-receptor NKp46, resulting in diminished ADCC-activity mediated by NK cells.[30,31]. In contrast to the DARA-BOR combination, the combination of DARA with LEN, dosed at clinically achievable and tolerable doses, showed synergistic effects in LEN- and BOR-refractory patients in our *ex vivo* BM assays. We found that the synergism between DARA and LEN was mainly mediated through the activation of NK cells. These results confirmed our hypothesis that in the BM-MNC of these multi-refractory patients, despite heavily pretreated with several

lines of anti-myeloma therapy, the NK cells were still able to respond to the well described immunomodulatory effects of LEN. We confirmed the synergistic anti-MM effects of the DARA plus LEN combination *in vivo* in a setting where primary MM cells from a LEN- and BOR-refractory patient were growing in humanized niches in RAG2-/-γc-/- mice. In fact, in this model we have previously shown that DARA monotherapy can mediate significant antitumor activity against tumors growing from patient-derived primary MM cells.[8] However, to study the synergy between DARA and LEN, we needed to optimize the model, since the RAG2-/-γc-/- mice lack NK cells, which according to our *in vitro* results, appeared necessary to establish the synergistic effect (Figure 4A,B). Therefore, we co-infused NK cell-enriched, T cell-depleted PBMC together with LEN and DARA in the mice and showed indeed a significant improvement of DARA-mediated anti-MM effect with LEN.

Although in this *in vivo* setting the PBMC (NK cells) are not autologous to the MM tumor, these results indicate that an optimal preclinical testing of ADCC-inducing mAbs in RAG2-/-γc-/- and NOD/scid/γc-/- (NSG)-based xenotransplant models will highly benefit from such an NK cell repletion approach. While NK cell repletion can be performed by several ways, our NK cell enrichment method, via T cell depletion, can become a standard procedure because it is convenient, effective, and will not induce T cell-mediated xenogeneic graft-versus-host disease. It needs to be noted that for optimal preclinical testing of mAbs the *in vivo* models may also need further adjustments. For instance, NSG-based models may also need complement injections, because these heavily immunodeficient mice not only lack NK cells but also complement, which often is required for optimal tumor lysis by mAbs.

Collectively, we provide evidence that the combination of LEN with DARA increases the lysis of MM cells in a synergistic fashion in LEN- and BOR-refractory MM patients. To date this multi-refractory MM patient group has a very poor prognosis. Our findings warrant the testing of this combination approach in clinical trials for these multi-refractory MM patients.

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Chapter IV

Upregulation of CD38 Expression on Multiple Myeloma Cells by All-Trans Retinoic Acid Improves the Efficacy of Daratumumab

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Abstract

Daratumumab is an anti-CD38 monoclonal antibody with lytic activity against multiple myeloma (MM) cells, including ADCC (antibody-dependent cellular cytotoxicity) and CDC (complement-dependent cytotoxicity). Due to a marked heterogeneity of response to daratumumab therapy in MM, we investigated determinants of the sensitivity of MM cells towards daratumumab-mediated ADCC and CDC. In bone marrow samples from 144 MM patients, we observed no difference in daratumumab-mediated lysis between newly diagnosed or relapsed/refractory patients. However, we discovered, next to an expected effect of effector (NK cells/monocytes) to target (MM cells) ratio on ADCC, a significant association between CD38 expression and daratumumab-mediated ADCC (127 patients) as well as CDC (56 patients). Similarly, experiments with isogenic MM cell lines expressing different levels of CD38, revealed that the level of CD38 expression is an important determinant of daratumumab-mediated ADCC and CDC. Importantly, all-trans retinoic acid (ATRA) increased CD38 expression levels, but also reduced expression of the complement-inhibitory proteins CD55 and CD59 in both cell lines and primary MM samples. This resulted in a significant enhancement of the activity of daratumumab *in vitro*, but also in a humanized MM mouse model. Our results provide the preclinical rationale for further evaluation of daratumumab combined with ATRA in MM patients.

Introduction

The introduction of autologous stem cell transplantation as well as novel agents such as bortezomib and the immunomodulatory drugs (IMiDs) thalidomide and lenalidomide, has significantly improved long-term outcome of MM patients[1]. However, the increased survival is less evident in patients who present with high-risk disease. Also patients with lenalidomide and bortezomib-refractory MM have a very poor outcome with a median overall survival of only 9 months[2].

Altogether, this clearly demonstrates that there is a need for new treatment approaches especially for these categories of patients. In this respect, several new antimyeloma agents hold promise, including next generation IMiDs (pomalidomide) and proteasome inhibitors (carfilzomib), but also compounds with different mechanisms of action [3, 4]. One of the most promising novel agents is the human IgG1 anti-CD38 monoclonal antibody daratumumab[5, 6].

CD38 is highly and uniformly expressed on all MM cells[7]. CD38 is a type II transmembrane glycoprotein with ectoenzymatic activity involved in the catabolism of extracellular nucleotides[7, 8]. Other functions ascribed to CD38 include receptor-mediated adhesion by interacting with CD31 or hyaluronic acid, regulation of migration, and signaling events[7-10].

Daratumumab induces killing of MM cells mainly via the activation of potent cytotoxic immune effector functions, including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC)[11]. Another mechanism of action is induction of apoptosis upon secondary crosslinking[12]. Anti-myeloma activity has also been demonstrated in mouse xenograft models[11], and more importantly, in a humanized mouse model[13, 14].

Daratumumab is currently being evaluated as a single agent in two clinical studies in heavily pretreated relapsed/refractory MM patients. Preliminary results show that daratumumab monotherapy is well tolerated and that in the 16 mg/kg cohort at least a partial response can be achieved in 35% of the patients including CR in 10%[15]. Based on preclinical evidence showing potential benefit of combining daratumumab with lenalidomide[16-18], another phase 1/2 study is evaluating the combination of daratumumab plus lenalidomide and dexamethasone in relapsed/refractory MM. Preliminary data show a manageable toxicity profile and high efficacy of this three-drug regimen[19, 20]. Several phase 3 trials with daratumumab are underway in the both relapse and front-line settings. Treatment with daratumumab is clinically effective, but there is a marked heterogeneity of response with a fraction of MM patients that does not respond to daratumumab as a single agent.

It is currently unknown which mechanisms underlie the differential therapeutic efficacy of daratumumab. Because daratumumab is being evaluated as a single agent and in combination with various regimens in the clinical trial setting[6], it is important to better understand host- and tumor-related factors that predict response. An improved understanding of mechanisms that contribute to innate or acquired resistance may result in the rational design of new daratumumab-based combinations with higher antimyeloma activity. Here, we studied the effect of CD38 expression levels on the efficacy of daratumumab to induce MM cell lysis via

ADCC and CDC. We also examined other potential determinants of daratumumab sensitivity including extent of previous therapy and the frequency of effector cells. Finally, we show that all-trans retinoic acid (ATRA) improves daratumumab-mediated ADCC and CDC against MM cells by upregulation of CD38 expression. Furthermore, ATRA reduces the expression of the complement-inhibitors CD55 and CD59 on MM cells, thereby further enhancing daratumumab-mediated CDC.

Material and Methods

Antibodies and reagents

Daratumumab was provided by Janssen Pharmaceuticals. Human IgG1-b12 (Genmab), a human mAb against an innocuous antigen (HIV-1 gp120),was used as an isotype control as described previously[16,21]. All-trans retinoic acid (ATRA) was purchased from Sigma-Aldrich.

Bone marrow mononuclear cells (BM-MNCs), peripheral blood mononuclear cells (PBMCs), MM cell lines, and immunophenotyping by flow cytometry

Details are given in the supplemental methods.

Generation of MM cell lines with higher CD38 expression

The luciferase (LUC)-transduced MM cell lines UM9 and L363, were transduced with the human CD38 gene to obtain CD38 expression levels comparable to primary myeloma cells. For this, the amphotropic Phoenix packaging cell line (Phoenix Ampho) was transfected, using calcium phosphate precipitation, with the pQCXIN vector in which the gene encoding human CD38 was inserted. These cell lines are referred to as UM9-CD38 and L363-CD38.

Bioluminescence imaging (BLI)-based ADCC assay using LUC-transduced MM cell lines

LUC-transduced MM cell lines were co-cultured with effector cells (freshly isolated PBMCs from healthy donors) at an effector to target ratio of 25:1 in white opaque 96-well flat bottom plates (Costar) in the presence of solvent control, IgG1-b12 control antibody, or daratumumab for four hours. The survival of LUC+-MM cells was then determined by BLI, 10 minutes after addition of the substrate luciferin (125 µg/mL; Promega). Lysis of MM cells was determined using the following formula: % lysis = 1- (mean BLI signal in the presence of effector cells and daratumumab / mean BLI signal in the presence of effector cells and control antibody) x100%.

BLI-based CDC assays using LUC-transduced MM cell lines

Solvent control, IgG1-b12 control antibody, or daratumumab were added to MM cell lines in medium supplemented with pooled unheated (native) human serum or pooled heat-inactivated (56 ^{o}C for 30 minutes) human serum (10%; Sanquin). After a 1-hour incubation at 37 ^{o}C, MM cell survival was determined by BLI, 10 minutes after addition of luciferin (125 µg/ml), and the lysis of cells was calculated using the following formula: % lysis = 1 - (mean BLI signal in the

presence of native human serum / mean BLI signal in the presence of heat-inactivated serum) x 100%. In separate experiments we showed that daratumumab was also able to trigger CDC using patients' serum (Supplemental Figure 1).

Flow cytometry-based *ex vivo* ADCC and CDC assays in BM-MNC

BM-MNCs derived from 144 MM patients, containing 2-57% CD138+ tumor cells, but also autologous effector cells, were used in ADCC and CDC assays. ADCC results were previously reported for 21 of these patients[17, 22]. Sample viability at incubation was more than 98%, as assessed by using ToPro-3 (Invitrogen/Molecular Probes). For ADCC assays, BM-MNCs were incubated in RPMI + 10% fetal bovine serum with control antibody or daratumumab (0.01–10 μg/mL) in 96-well flat-bottom plates for 48 hours. For CDC assays, BM-MNCs were treated with daratumumab (0.3–10 μg/mL) and 10% pooled human serum or autologous patients' serum as a source of complement for 1 hour prior to flow cytometric analysis. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow cytometry as previously described[16, 21]. In both assays, surviving MM cells were enumerated by single platform flow cytometric analysis of CD138+ cells in the presence of Flow-Count Fluorospheres (Beckman Coulter) and ToPro-3 to determine absolute numbers of viable MM cells. The percentage of daratumumab-mediated ADCC was then calculated using the following formula: % lysis cells = 1 - (absolute number of surviving CD138+ cells in the presence of daratumumab / absolute number of surviving CD138+ cells in the presence of control antibody) x 100%. Complement-dependent lysis was calculated using the following formula: % lysis = 1 - (absolute number of surviving CD138+ cells in the presence of native human serum / absolute number of surviving CD138+ cells in the presence of heat-inactivated serum) x 100%.

In vivo efficacy of the combination of ATRA and daratumumab against MM tumors growing in a humanized microenvironment.

Hybrid scaffolds consisting of three 2-3 mm biphasic calcium phosphate particles were coated *in vitro* with human mesenchymal stromal cells (MSCs; $2x10^5$ cells/scaffold). After a week of *in vitro* culture in a osteogenic medium, humanized scaffolds were implanted subcutaneously into RAG2-/- γc-/- mice, as described previously[13, 23]. Eight weeks after implantation, mice received a sublethal irradiation dose (3 Gy, 200 kV, 4 mA) and XG1 cells were injected directly into the scaffold (1x106 cells/scaffold). Three weeks after inoculation, when there was visible tumor growth in the scaffolds by BLI, different groups of mice were treated with 1) vehicle, 2) ATRA plus T-cell depleted PBMC as effector cells (PBMC-T), 3) daratumumab plus PBMC-T, and 4) daratumumab plus ATRA plus PBMC-T. Daratumumab (8 mg/kg) was given intraperitoneally on days 23, 30, and 37; PBMC-T ($8×10^6$ cells/mouse) were given intravenously on days 24, 31, and 38; and ATRA (10 mg/kg) was given via intraperitoneal injection on days 21-24, 28-31, and 35-38. PBMC-T were prepared by Ficoll-Hypaque density-gradient centrifugation of buffy coats, and subsequent depletion of T cells by CD3-beads using the EasySep™-technology (STEMCELL Technologies). Tumor growth was monitored by weekly BLI

measurements as described previously[13]. All animal experiments were conducted after acquiring permission from the local ethical committee for animal experimentation and were in compliance with the Dutch Animal Experimentation Act.

Table 1

Patient characteristics (n=144)

Parameter	NDMM patients n=45	RRMM patients n=99
Median age, years (range)	65 (38-82)	64 (38-83)
Sex, male, n (%)	21 (47%)	59 (60%)
Type of monoclonal heavy chain		
IgG, n (%)	31 (69%)	46 (47%)
IgA, n (%)	8 (18%)	24 (24%)
IgM, n (%)	0 (0%)	1 (1%)
IgD, n (%)	0 (0%)	1 (1%)
Light chain only, n (%)	6 (13%)	27 (27%)
Type of light chain		
Kappa, n (%)	25 (56%)	62 (63%)
Lambda, n (%)	20 (44%)	37 (37%)
Previous therapy		
Prior lines of therapy, median (range)	-	3 (1-10)
Prior stem cell transplantation, n (%)	-	56 (57%)
Autologous, n (%)	-	55 (56%)
Allogeneic, n (%)	-	12 (12%)
Prior lenalidomide treatment, n (%)	-	56 (57%)
Lenalidomide refractory status*, n (%)	-	49 (50%)
Prior bortezomib treatment, n (%)	-	54 (55%)
Bortezomib refractory status*, n (%)	-	44 (44%)
Prior lenalidomide & bortezomib treatment, n (%)	-	47 (47%)
Lenalidomide & bortezomib refractory status*, n (%)	-	41 (41%)

NDMM: newly diagnosed multiple myeloma; RRMM: relapsed/refractory multiple myeloma; n: number.*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.

Statistics

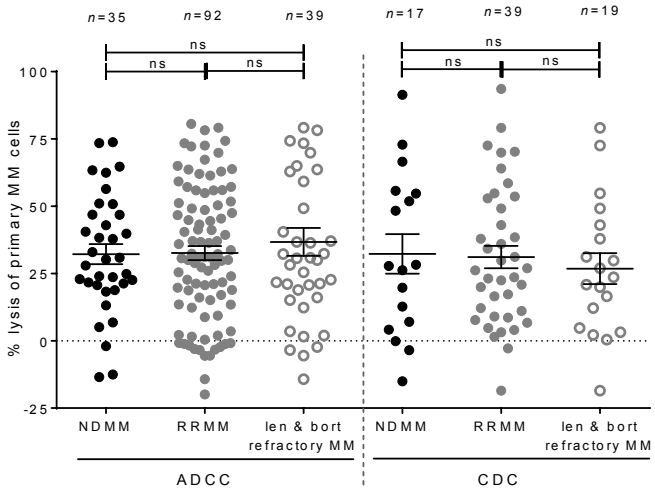
Comparisons between variables were performed using two-tailed (paired) Student's t-test. The statistical differences between the different treatment groups in the mice experiments were calculated using a Mann-Whitney test. P-values below 0.05 were considered significant.

Results

Daratumumab-mediated lysis is not affected by extent of prior treatment

During their treatment course MM cells become increasingly resistant towards anti-myeloma agents. To evaluate whether clinical resistance to bortezomib and/or lenalidomide also results in resistance to daratumumab, we analyzed daratumumab-mediated killing of tumor cells from different groups of MM patients. The characteristics of these 144 patients are shown in Table 1. ADCC and CDC assays with 10 µg/mL daratumumab were performed in 127 and 56 patients, respectively. Sufficient BM-MNCs for both assays were obtained from 39 patients. In these experiments, BM-MNCs, containing tumor cells as well as autologous

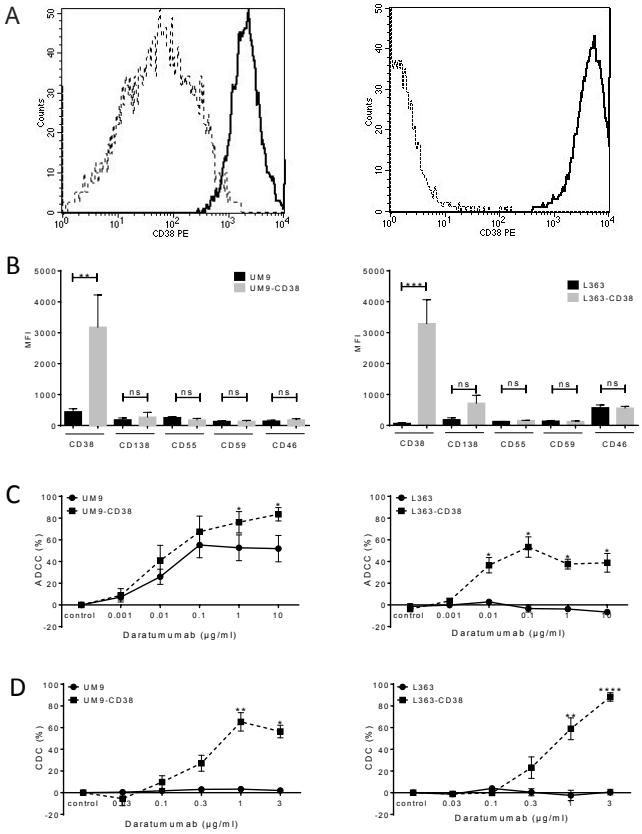
FIGURE 1



No difference in daratumumab-mediated ADCC and CDC against MM cells from newly diagnosed or relapsed/refractory patients BM-MNCs from 144 patients with newly diagnosed MM (NDMM) as well as relapsed/refractory MM (RRMM), including patients with lenalidomide (len) and bortezomib (bort)-refractory disease, were used in ADCC- and CDC-assays with 10 µg/mL daratumumab. ADCC- en CDC-assays were performed as described in materials and methods. Bars represent means ± standard error of the mean (SEM). P-values between the indicated groups were calculated using a student's t test; ns not significant.

effector cells, were treated with daratumumab, after which survival of MM cells was determined by enumeration of viable CD138+ cells by flow cytometric analysis. Daratumumab-mediated ADCC against the MM cells was variable and ranged from -19.9% (negative values indicate MM cell growth) to 80.6%

FIGURE 2



CD38 expression levels correlate with the susceptibility towards daratumumab-mediated ADCC and CDC of MM cell lines.

(A) The histograms represent cell surface expression of CD38 on the L363 and UM9 cell lines, as well as on CD38-transduced UM9 (UM9-CD38) and L363 (L363-CD38) cells. (B) Comparison of the expression levels of CD38, CD138, CD55, CD59 and CD46 between UM9 and UM9-CD38 cell lines, as well as L363 and L363-CD38 cells. P-values between the indicated groups were calculated using a student's t test. (C) Results of 4-hour ADCC assays and (D) 1-hour CDC assays. UM9, UM9-CD38, L363, and L363-CD38 MM cell lines were incubated with IgG1-b12 control antibody or with daratumumab in the presence of PBMCs derived from healthy donors as effector cells in ADCC assays, or with medium supplemented with pooled human serum (10%) as a source of complement or heat-inactivated human serum as control in CDC assays. Survival was determined by BLI. The percentage lysis in ADCC and CDC assays was calculated as outlined in the materials and methods section. Depicted are the mean and SEM of at least three independent experiments. P-values between the indicated groups were calculated using a student's t test; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant.

(median 30.8%). Also complement-dependent lysis of primary MM cells was very heterogeneous and ranged from -18.5% to 93.6% (median 27.4%). Importantly, there were no significant differences in ADCC or CDC between patients with newly diagnosed disease or relapsed/refractory MM (Figure 1). Also in the subgroup of heavily pretreated, lenalidomide- and bortezomib-refractory MM patients the efficacy of daratumumab was similar to that observed in the newly diagnosed patients or in relapsed patients with less previous therapies. These data suggest that resistance to classic antimyeloma agents such as steroids, anthracyclines, and alkylators, as well as novel agents (IMiDs and proteasome inhibitors), is not associated with decreased sensitivity to daratumumab-mediated ADCC and CDC *in vitro.*

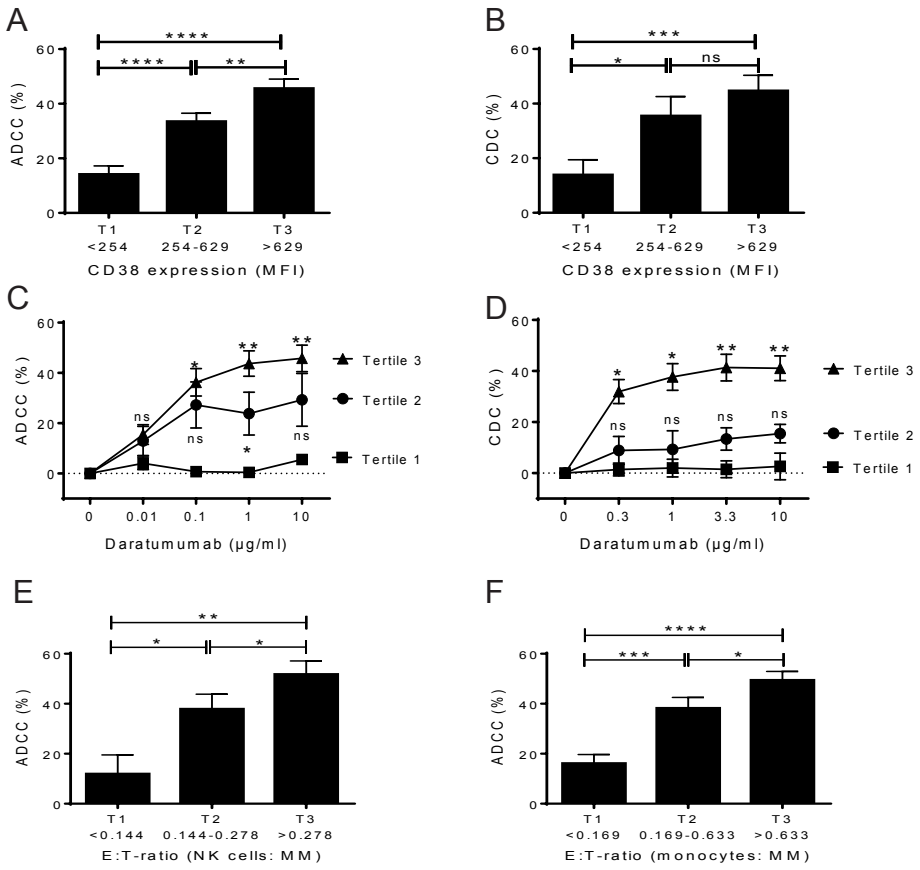
Effect of CD38 expression on ADCC and CDC in MM cell lines

Since previous therapy does not explain the variability in daratumumab-mediated ADCC and CDC, we examined other factors that may affect the susceptibility of tumor cells towards daratumumab. We hypothesized that cell surface expression of CD38 on MM cells is associated with the extent of daratumumab-mediated ADCC and CDC. We first tested this hypothesis in a controlled experimental setting in which CD38 expression level was the only variable. To this end, we generated clones of the MM cell lines UM9 (UM9-CD38) and L363 (L363-CD38) with higher levels of CD38 expression (Figure 2A). The expression of CD138 and the complement-regulatory proteins (CD46, CD55, and CD59) was similar between the non-transduced parental cell lines and the clones (Figure 2B). Importantly, in ADCC as well as CDC assays daratumumab-mediated lysis of CD38 transgenic clones was significantly better compared with the non-transduced parental cell lines (Figure 2C and 2D).

Effect of CD38 expression on ADCC and CDC in primary MM cells

The impact of CD38 expression levels on daratumumab-mediated killing was further examined by using patients' samples. As expected, all MM cells expressed CD38 antigen in these patients' samples (n=144), but there was a marked heterogeneity in intensity of CD38 expression with median fluorescence intensity (MFI) ranging from 19.99 to 2642 (median 401.3). No significant difference in CD38 expression was observed between newly diagnosed and relapsed/refractory patients. Next, we divided the patients into tertiles according to CD38 expression on their MM cells. As illustrated in Figure 3A, ADCC against primary MM cells (n=127 patients' samples) mediated by 10 μg/mL daratumumab was only 14.2% in the lowest tertile of CD38 expression, while it was significantly higher in the mid-tertile (33.5%) and the highest tertile (45.6%). We also evaluated the association between CDC induced by 10 μg/mL daratumumab and CD38 expression in 56 patients. In the lowest tertile of CD38 expression, daratumumab-mediated CDC was significantly worse (13.9%), when compared to the mid-tertile (35.5%) and highest-tertile (44.7%) (Figure 3B). In 25 patients, we harvested enough BM-MNCs to evaluate different concentrations of daratumumab in ADCC and CDC assays. The dose-response curve of patients in the lowest tertile according to CD38 expression clearly reflects the lower efficacy of daratumumab against MM cells with low CD38 expression, when

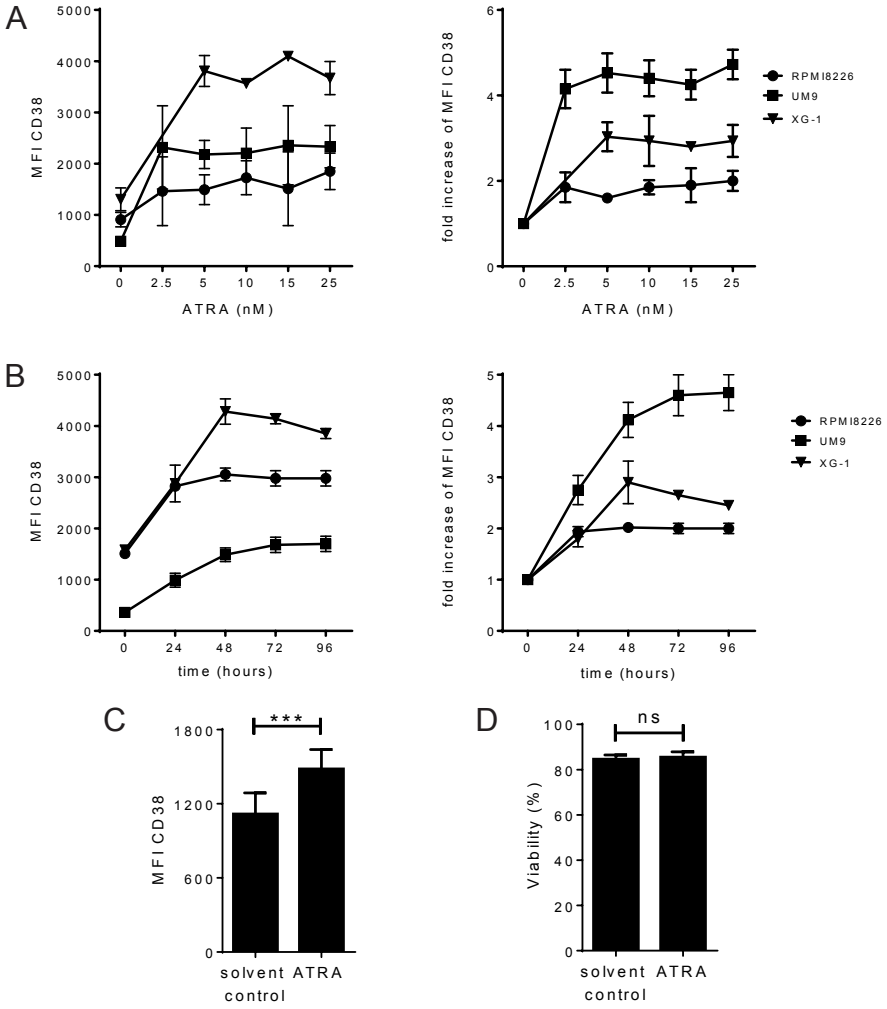
FIGURE 3



CD38 expression levels are associated with extent of daratumumab-mediated ADCC and CDC against primary MM cells.

(A) Means ± SEM of ADCC mediated by 10 µg/mL daratumumab at 48 hours according to tertiles of CD38 expression levels on primary MM cells from 127 patients, as determined by flow cytometry. Tertiles were computed in the whole group of 144 patients (MFI CD38: <254, 254-629, >629; T, tertile). (B) Means ± SEM of CDC mediated by 10 µg/mL daratumumab at 1 hour according to tertiles of CD38 expression levels on primary MM cells from 56 patients, as determined by flow cytometry (T, tertile). ADCC- and CDC-assays were performed as described in materials and methods. (C) Dose-response curves for ADCC according to tertiles of CD38 expression on primary MM cells (n=25 patients). ADCC in tertiles 2 and 3 was compared with ADCC in tertile 1. (D) Dose-response curves for CDC according to tertiles of CD38 expression on primary MM cells (n=25 patients). CDC in tertiles 2 and 3 was compared with CDC in tertile 1. (E) Mean estimates ± SEM of ADCC mediated by 10 µg/mL daratumumab at 48 hours according to tertiles of E:T-ratio with CD3-/CD56+ NK cells as effector cells and CD38+/CD138+ MM cells as target cells (NK cell:MMcell ratio: <0.144, 0.144-0.278, >0.278; n=25 patients; T, tertile) or (F) with CD14+ monocytes as effector cells and CD38+/CD138+ MM cells as target cells (monocyte: MM cell ratio: <0.169, 0.169-0.633, >0.633; n=85 patients; T, tertile). P-values between the indicated groups were calculated using a student's t test; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant.

FIGURE 4



ATRA increases CD38 expression on MM cell lines and primary MM cells.
(A) RPMI8226, UM9, and XG1 MM cell lines were incubated with solvent control or with ATRA ranging from 2.5 – 25 nM for 48 hours or (B) incubated with 10 nM ATRA for 24, 48, 72 or 96 hours, after which cells were harvested to analyze CD38 expression levels by flow cytometry. Left panels show median fluorescence intensity (MFI) of CD38, and right panels show fold increase compared to solvent control. There was no significant difference in cell viability of ATRA- or solvent control-treated cells. (C) BM-MNCs from 26 MM patients were incubated with solvent control or with 10 nM ATRA for 48 hours. Cells were then harvested to determine CD38 expression levels by flow cytometry using a FACS-Calibur device (Becton Dickinson). The data were analyzed using CellQuest software. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using a paired student's t test; *** P<0.001. (D) Viability of primary MM cells treated with solvent control or 10 nM ATRA. Viability of primary CD138+ MM cells in the BM-MNCs was determined by flow cytometry using ToPro-3 staining. P-values between the indicated groups were calculated using a paired student's t test; ns, not significant.

compared to MM cells with higher CD38 levels (Figure 3C and D). Altogether, this indicates that the differential susceptibility of the MM cells towards daratumumab-mediated ADCC and CDC can be partly explained by the level of CD38 expression.

Impact of the ratio between effector and target cells on daratumumab-mediated ADCC

We have previously demonstrated that ADCC against MM cell lines is mainly mediated by NK cells[17, 18]. Since the patients' samples contained variable amounts of NK and MM cells, we also analyzed whether part of the heterogeneity in ADCC could be explained by naturally occurring differences in the effector:target ratio (E:T ratio) in the BM-MNCs. The frequency of total CD3-CD56+ NK cells in the BM-MNCs from 25 MM patients ranged from 1.1 to 8.9% (median 3.8%). There was also a great variation in the frequency of MM cells in these samples (range 2-57%, median 17.7%). Daratumumab-mediated ADCC against MM cells was significantly inferior (12.0%) in the lowest tertile according to total NK cell:MM cell ratio, while it was 37.9% and 51.8% in the mid-tertile and highest tertile, respectively (Figure 3E). Similar results were obtained when we considered the activated fraction of NK cells (defined as CD3-CD56+CD16+) as effector cells (data not shown). In addition, a high monocyte:MM cell ratio was associated with improved ADCC (Figure 3F).

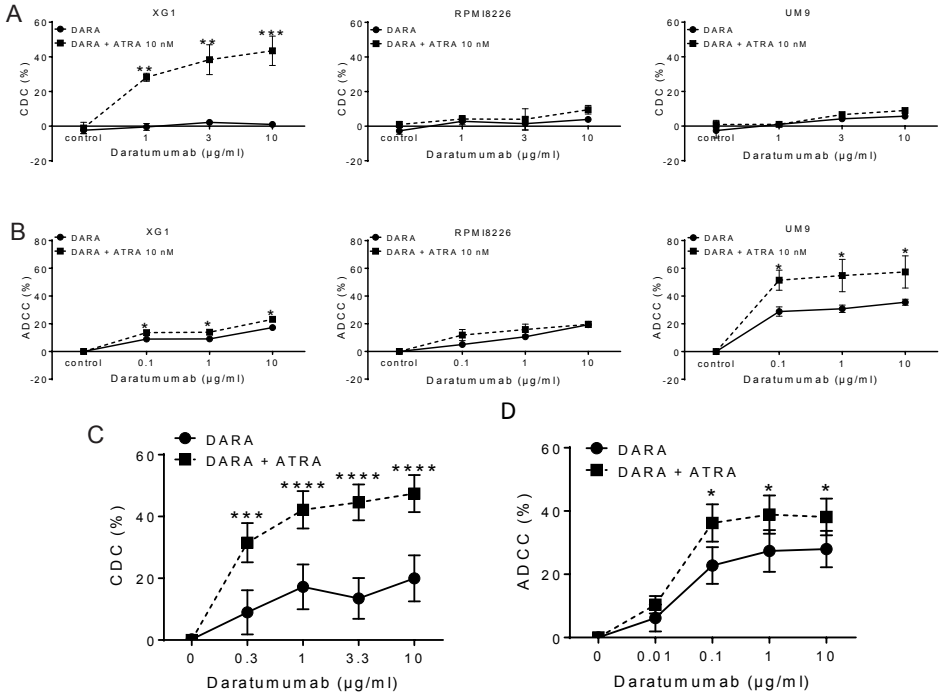
ATRA increases CD38 expression

Our experiments demonstrate that CD38 expression on MM cells is an important determinant of susceptibility to both daratumumab-mediated ADCC and CDC.
Therefore, we hypothesized that an increase in CD38 expression may enhance the efficacy of daratumumab. Because interaction of ATRA with nuclear retinoic acid receptors results in altered expression of target genes including induction of CD38 expression[8,24] we evaluated the effect of ATRA on CD38 expression on MM cells, and the impact of the combination of ATRA and daratumumab on MM cell lysis. Here we show that as little as 10 nM ATRA was sufficient to induce a 1.9–4.4-fold increase in CD38 expression on the MM cell lines RPMI8226, UM9, and XG1. Higher doses of ATRA did not further enhance CD38 expression (Figure 4A). Maximum enhancement of CD38 expression occurred at 48 hours (Figure 4B).
Similarly, *ex vivo* ATRA exposure (10 nM, 48 hours) of BM-MNCs from 26 MM patients resulted in induction of CD38 expression on MM cells (mean increase: 32.7%; P=0.0001) (Figure 4C). There was also a significant upregulation of CD138 expression levels (mean increase: 86.9%; P<0.0001), which is characteristic of MM cell differentiation. In contrast, no significant changes in the expression of other plasma cell antigens, such as HLA A/B/C or CD56, were observed in response to ATRA. Viability of primary MM cells was similar between ATRA treated cells and solvent-control treated cells (87.1 vs 84.6%; Figure 4D).

ATRA enhances daratumumab-mediated ADCC and CDC against MM cells

We first evaluated the effect of ATRA-mediated upregulation of CD38 on ADCC and CDC by using MM cell lines. Cells were treated with 10 nM ATRA or solvent control for 48 hours, followed by incubation with or without daratumumab in the presence of human serum as complement source for CDC (Figure 5A), or in the

FIGURE 5



ATRA enhances daratumumab-mediated CDC and ADCC against MM cell lines and primary MM cells.

(A) Results of 1-hour CDC assays and (B) 4-hour ADCC assays. XG1, RPMI8226, and UM9 cells were pretreated with solvent control or with 10 nM ATRA for 48 hours. This was followed by treatment with IgG1-b12 control antibody or daratumumab, in medium supplemented with pooled human serum (10%) as a source of complement or heat-inactivated human serum in the CDC assays, or in the presence of PBMCs derived from healthy donors as effector cells in the ADCC assays. Daratumumab-mediated lysis was determined by BLI. The results are the mean and SEM of at least three independent experiments. P-values between the indicated groups were calculated using a paired student's t test. (C) Pooled results of 1-hour CDC and (D) 48-hour ADCC assays, using BM-MNCs of 16 and 11 MM patients, respectively. BM-MNCs were pretreated for 48 hours with solvent control or 10 nM ATRA, followed by incubation with IgG1-b12 control antibody or daratumumab. Pooled human serum (10%) was used as a source of complement. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow-cytometry. Percentage lysis of MM cells was calculated as indicated in materials and methods. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using a paired student's t test; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant.

presence of PBMCs as effector cells in ADCC assays (Figure 5B). Pretreatment of MM cell lines with ATRA alone induced no or only a minimal increase in MM cell death, but significantly enhanced daratumumab-mediated CDC in XG1 cells, and ADCC in XG1 and UM9 cells, compared with solvent control. In RPMI8226 cells there was no significant improvement in daratumumab-mediated ADCC and CDC, which may be explained, in part, by the significantly smaller relative increase in CD38

expression levels in this cell line when compared to the other cell lines (Figure 4A). We subsequently used MM cells derived from patients, including those with lenalidomide- and bortezomib-refractory disease, to further explore the combination of ATRA and daratumumab. ATRA alone for 48 or 96 hours did not significantly affect MM cell viability when compared to solvent control (non-viable cells at 48 hours: 13% vs 15% and at 96 hours 12% vs 15% for ATRA and solvent control, respectively). However, also in primary MM cells, pretreatment with ATRA for 48 hours resulted in a significant increase in their susceptibility to daratumumab-mediated CDC in 13 out of 16 patients and ADCC in 8 out of 11 patients. Pooled results from these patients show that ATRA improved CDC mediated by 10 µg/mL daratumumab from 16.1 % to 43.9 % ($P < 0.0001$), and ADCC from 25.1 % to 39.5 % ($P=0.0315$) (Figure 5C and 5D, respectively). ADCC and CDC data, as well as CD38 levels with or without ATRA treatment from individual patients' samples are shown in Supplemental Figures 2 and 3A, respectively. Importantly, ATRA augmented daratumumab-mediated CDC and ADCC against MM cells with low, intermediate or high levels of CD38 expression, as well as against MM cells derived from patients with double-refractory disease. Furthermore, ATRA improved the efficacy of daratumumab in MM cells which were resistant to daratumumab as a single agent in CDC and/or ADCC assays (Supplemental Figure 2). Similar improvement in CDC was seen when autologous patients' serum was used (see Supplemental Figure 4). Altogether, this suggests that ATRA is an attractive strategy to enhance CD38 expression and to improve daratumumab activity in MM.

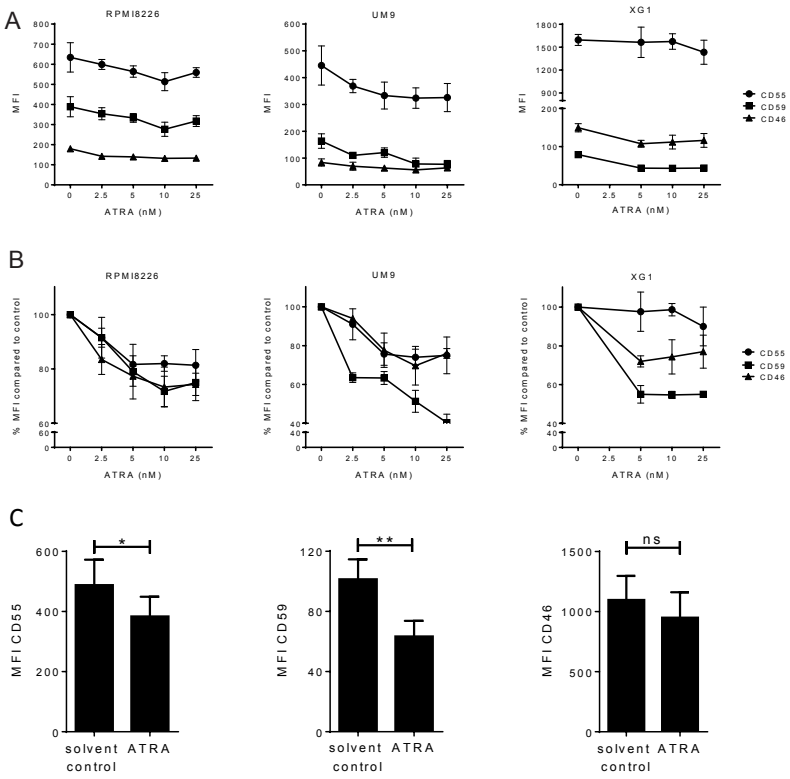
ATRA reduces CD55 and CD59 expression on MM cells

Since ATRA improved CDC to a higher extent than ADCC, we evaluated the effect of ATRA on the complement-inhibitory proteins, CD46, CD55, and CD59, which confer protection against several therapeutic antibodies[25]. Interestingly, ATRA also reduced expression levels of CD55, CD59, and CD46 in a dose-dependent way in MM cell lines (Figure 6A and B). Similarly, in primary MM cells, ATRA (10 nM, 48 hours) reduced CD55 (mean reduction: 21.3%; $P=0.019$) and CD59 expression levels (mean reduction: 37.5%; $P=0.0047$), without significantly affecting CD46 levels (Figure 6C). There was a significant correlation between ATRA-induced increase in CD38 expression and downregulation of CD59 ($r=-0.503$; $P=0.0047$), but not with reduction of CD55 expression. The CD46, CD55 and CD59 expression levels with or without ATRA treatment of the 16 patients' samples tested in the CDC assays are depicted in Supplemental Figure 3B. We also analyzed the effect of ATRA on effector cells. Preincubation of PBMCs with ATRA prior to ADCC assays did not result in enhancement of daratumumab-mediated ADCC against MM cell lines (Supplemental Figure 5).

ATRA enhances the anti-MM activity of daratumumab in a humanized mouse model

Finally, we evaluated whether ATRA enhanced the anti-MM effect of daratumumab in a recently developed *in vivo* model[13,23] in immunodeficient RAG2-/-γc-/- mice, in which the LUC-transduced MM cells develop into aggressive tumors in a humanized bone

FIGURE 6

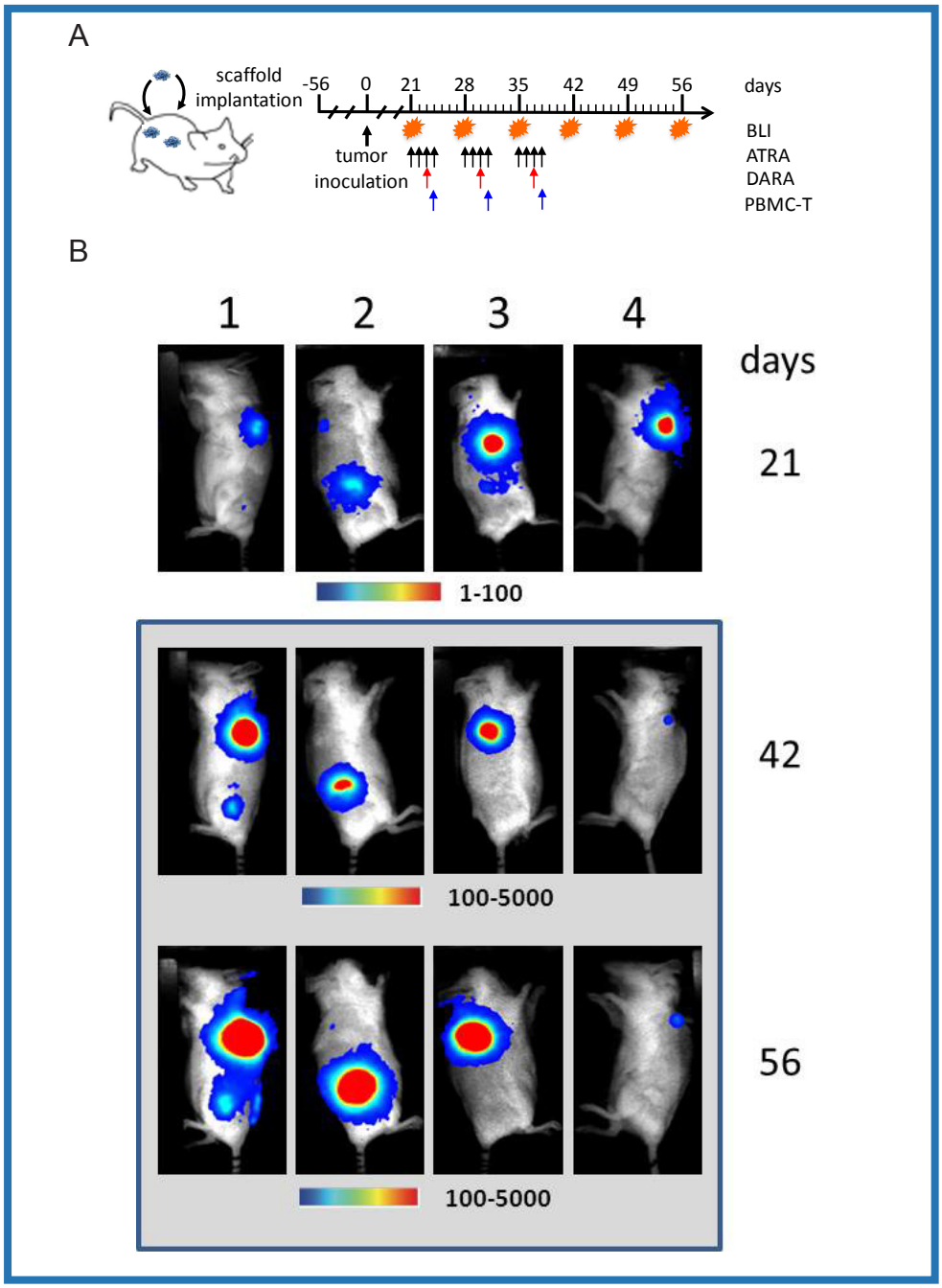


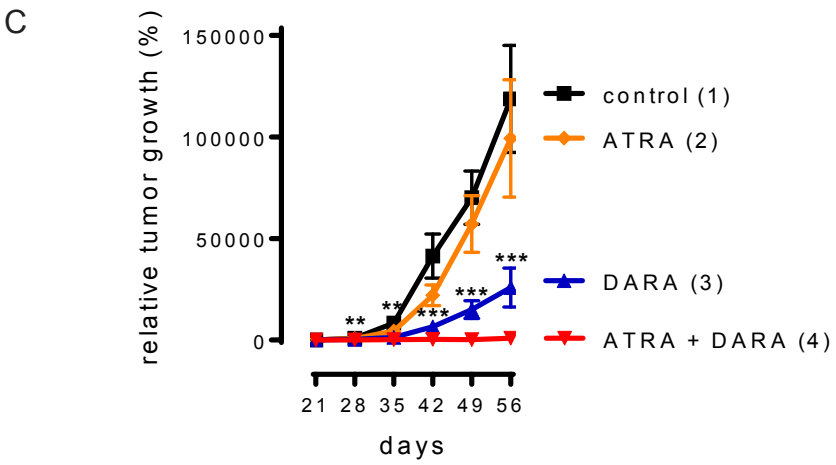
ATRA reduces complement-inhibitory proteins on MM cell lines and primary MM cells. (A) RPMI8226, UM9, and XG1 MM cell lines were incubated with solvent control or with ATRA ranging from 2.5 – 25 nM for 48 hours. Cells were then harvested to determine CD55, CD59, and CD46 expression levels by flow cytometry using a FACS-Calibur device. Upper panels (A) show median fluorescence intensity (MFI) of these complement-inhibitory proteins, and lower panels (B) show their expression levels normalized to solvent control. (C) BM-MNCs from 26 MM patients were incubated with solvent control or with 10 nM ATRA for 48 hours. Cells were then harvested to determine CD46, CD55, and CD59 expression levels by flow cytometry using a FACS-Calibur device (Becton Dickinson). The data were analyzed using CellQuest software. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using a paired student's t test; *P<0.05, ** P<0.01, ns not significant.

marrow microenvironment generated by subcutaneous implantation of MSC-coated ceramic scaffolds. Three weeks after inoculation of XG1 cells in the humanized scaffolds when there was visible tumor growth, we treated the mice with either vehicle, daratumumab, ATRA, or with the combination of daratumumab plus ATRA (Figure 7A). To optimally evaluate the effects of daratumumab and ATRA, we also co-injected the mice with NK cell-enriched (T cell-depleted) PBMCs of a healthy donor in combination with daratumumab and/or ATRA as RAG2-/- γ c-/- mice are devoid of NK cells. To follow the outgrowth of the tumor, BLI was performed weekly for 5 weeks. As shown in Figure 7B and C, daratumumab markedly slowed tumor progression,

whereas ATRA as single agent had no effect. Furthermore, also in this humanized mouse model ATRA significantly enhanced the anti-MM effect of daratumumab (Figure 7C).

FIGURE 7





ATRA improves response to daratumumab in a humanized MM mouse model. (A) Rag2-/-γc-/- mice carrying MSC-coated scaffolds were inoculated with LUC-transduced XG1 cells. Mice were treated with control, ATRA plus T-cell depleted PBMCs as effector cells (PBMC-T), daratumumab plus PBMC-T, or daratumumab plus ATRA plus PBMC-T, at the indicated time points. Mice were monitored weekly by BLI. (B) Images of representative mice per treatment group: 1) control, 2) ATRA plus PBMC-T, 3) daratumumab plus PBMC-T, and 4) daratumumab plus ATRA plus PBMC-T. Shown are BLI images before (day 21; top panels), 3 weeks after the initiation of treatment (day 42; middle panels) and at the end of the experiment (day 56; lower panels). (C) Analysis of tumor load per treatment group with 4 mice per group and each mouse with 4 scaffolds. The statistical differences between mice treated with daratumumab and mice treated with daratumumab plus ATRA were calculated using the Mann-Whitney U-test. * P<0.05, ** P<0.01, *** P<0.001; ns not significant.

Discussion

Immunotherapy with daratumumab is clinically effective, but there is a significant variability in quality of response among patients[15]. Mechanisms that influence daratumumab efficacy will most likely be multifactorial and include both host- and tumor-related factors. In this study, we evaluated daratumumab-mediated CDC and ADCC against primary MM cells, in relation to CD38 expression on the tumor cells, frequency of effector cells in the bone marrow, and extent of previous treatment of the patient. To this end, we used mononuclear cells isolated from BM derived from MM patients, which contain not only MM cells, but also autologous effector cells and stromal cells. The current study shows that there is a significant positive association between CD38 expression levels on MM cells from patients and the efficacy of daratumumab to induce cell death by ADCC as well as CDC. The importance of CD38 expression was further strengthened by the observation that an enforced increase in CD38 expression levels on UM9 and L363 cells resulted in an increase in daratumumab-mediated CDC and ADCC. This indicates

that CD38 may be useful as a biomarker in daratumumab-based therapies. Several other studies have also demonstrated that efficacy of monoclonal antibodies is partly dependent on expression levels of their target. For example, preclinical studies show that rituximab-mediated killing of chronic lymphocytic leukemia and lymphoma cells is dependent on CD20 expression[26-29], and also clinical studies show an inferior outcome in patients with weak CD20 expression, treated with rituximab-based immunochemotherapy[30, 31]. Furthermore, ofatumumab-mediated CDC effects on lymphoma and CLL cells are largely dependent on CD20 expression levels[28]. The association between the levels of the target antigen and clinical outcome has also been demonstrated for alemtuzumab[32] and trastuzumab[33, 34]. The variability in daratumumab-mediated ADCC and CDC was not solely explained by the differential expression of CD38. For this reason, we also evaluated CD38-independent factors influencing ADCC and/or CDC, including effector cell frequencies and extent of previous therapy. Importantly, the efficacy of daratumumab in terms of ADCC and CDC did not differ between newly diagnosed and heavily pretreated lenalidomide and bortezomib double-refractory patients. Similarly, we have previously shown in a humanized mouse model that daratumumab is highly effective in killing MM cells derived from double-refractory patients[17, 18]. This suggests that mechanisms of resistance towards prior therapies, such as IMiDs and proteasome inhibitors, do not affect the susceptibility of MM cells to daratumumab-mediated ADCC and CDC. These findings are in agreement with the high efficacy of daratumumab as single agent in heavily pretreated relapsed/refractory MM patients[15]. In addition, we found that the ratios between NK cells, activated NK cells, or monocytes, to MM cells are positively associated with susceptibility to ADCC. It is possible that the activity of these effector cells may be affected by preceding or concomitant therapy such as steroids and IMiDs[16]. In addition, inhibitory signals transmitted to NK cells by MM cells, may result in NK cell dysfunction[35].

Modulation of determinants of daratumumab sensitivity with novel therapeutic approaches may lead to more effective daratumumab-based regimens with increased quality of response and improvement in survival. Because, CD38 levels may influence the efficacy of both daratumumab-mediated CDC and ADCC against MM cells, we hypothesized that upregulation of CD38 expression levels could increase MM cell kill and thereby enhance the response rate of the antibody. Indeed, we demonstrated that ATRA increased expression levels of CD38 on MM cells. This resulted in enhanced ADCC and CDC in both cell lines and patient samples, including those with low CD38 expression or complete resistance to daratumumab-mediated CDC and/or ADCC. In addition, the anti-MM activity of daratumumab was also significantly enhanced by ATRA in our recently developed humanized mouse model. Interestingly, the improvement in CDC was more pronounced than the enhancement of ADCC, suggesting that ATRA also modulates CD38-independent determinants of CDC but not of ADCC. Indeed, we did not observe enhancement of ADCC by preincubating effector cells with ATRA, but we did find a significant downregulation of the complement-inhibitory proteins CD55 and CD59 with ATRA. It is possible that ATRA has additional CD38-independent activities that result in improvement of daratumumab-mediated lysis of MM cells. Importantly, the enhancing effect of ATRA was evident at a dose of 10 nM, which is a clinically

achievable and safe concentration of ATRA. Retinoic acids can influence gene expression and protein production in different ways[36]. Previous studies have demonstrated that the retinoic acid receptor (RAR) plays an important role in the induction of CD38 by ATRA[8, 24, 37]. Indeed, the CD38 gene contains a retinoic acid-responsive element in the first intron[38]. However, also nonclassical retinoic acid signaling, independent of the conventional RAR pathway, has been demonstrated in CD38 upregulation[37]. This includes ATRA-induced CD38 induction via response elements in the 5'-flanking region, which is mediated by protein kinase Cδ[37]. Also phosphatidylinositol 3-kinase is involved in ATRA-induced upregulation of CD38 on human hematopoietic cells[39]. Similar RAR-dependent and -independent mechanism may be involved in ATRA-mediated suppression of CD55 and CD59.

In conclusion, this study has identified multiple factors that influence the extent of MM cell lysis mediated by daratumumab. These factors may serve as biomarkers to predict response in daratumumab-based regimens. A better understanding of mechanisms underlying variability in sensitivity to daratumumab-mediated killing may also lead to novel strategies to enhance the effectiveness of daratumumab therapy. Here, we provide the rationale for clinical evaluation of ATRA and daratumumab in MM patients.

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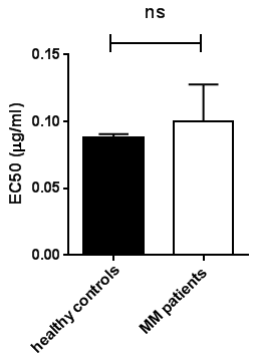
Supplemental methods

Bone marrow and peripheral blood mononuclear cells

All samples from patients and healthy individuals were collected and stored after obtaining written informed consent, using protocols/procedures approved by the institutional medical ethical committee in accordance with the declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) from healthy donors and MM patients, and bone marrow mononuclear cells (BM-MNCs) from MM patient BM aspirates were isolated by Ficoll-Hypaque density-gradient centrifugation. BM-MNCs that contained 2-57% CD138+ MM cells as detected by flow cytometry, were used in flow-cytometry-based ADCC and CDC assays. Freshly isolated PBMCs from healthy individuals were used as effector cells in bioluminescence imaging-based ADCC assays, in which MM cell lines were used as target cells.

Supplementary
FIGURE 1



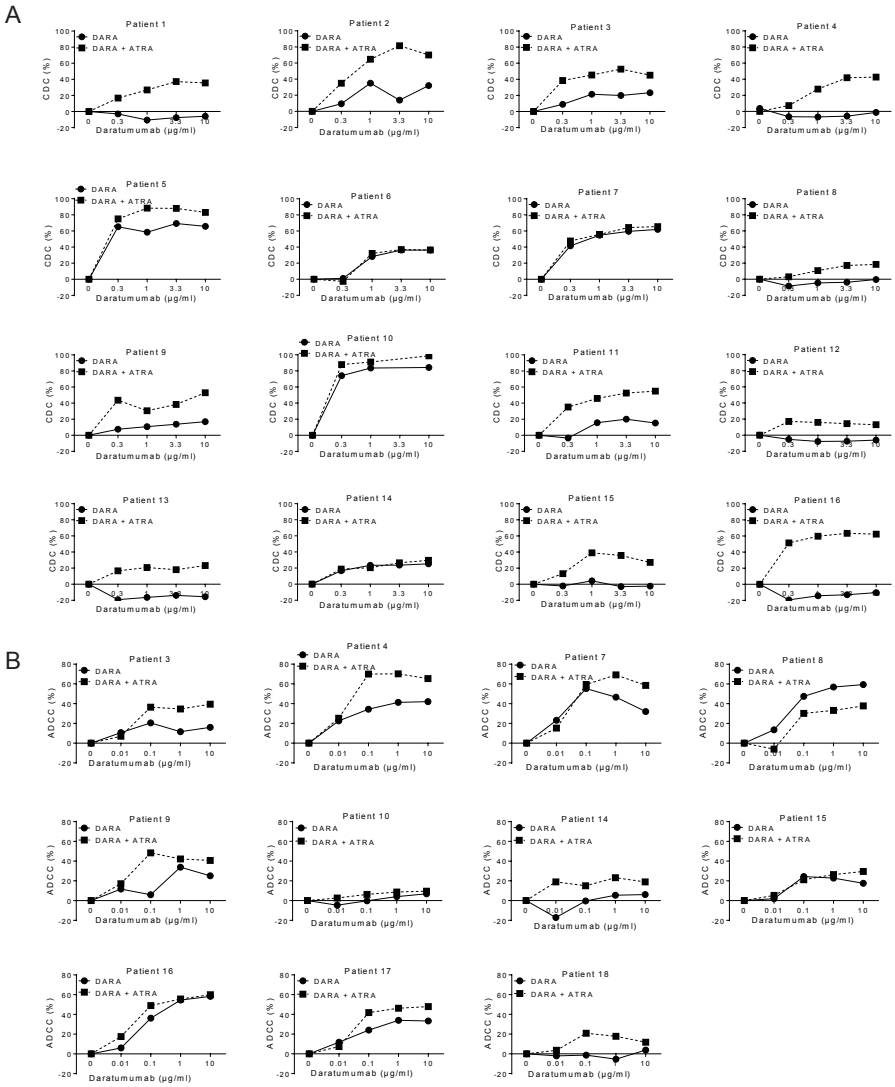
Daratumumab-mediated CDC against Daudi tumor cells evaluated with serum obtained from healthy controls and MM patients.
Daudi cells were incubated with IgG1-b12 control antibody or daratumumab (0.00390-1 µg/ml) with medium supplemented with serum (10%), derived from 7 healthy controls or 4 MM patients, as a source of complement. Survival was determined after a two-hour incubation; for each serum sample tested maximum CDC was >95%. The EC50 was calculated for each healthy control serum or patient serum tested. We defined EC50 as the concentration of daratumumab that gives half-maximal response. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using an unpaired student's t test; ns not significant.

MM Cell lines and culture

The luciferase (LUC)-transduced multiple myeloma cell lines UM9, UM9-CD38, L363, L363-CD38, XG1, and RPMI8226 were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Lonza) and antibiotics (100 units/mL penicillin, 100 µg/ml streptomycin; both Life Technologies).

Supplementary

FIGURE 2



Daratumumab-mediated CDC and ADCC against primary MM cells is improved by ATRA

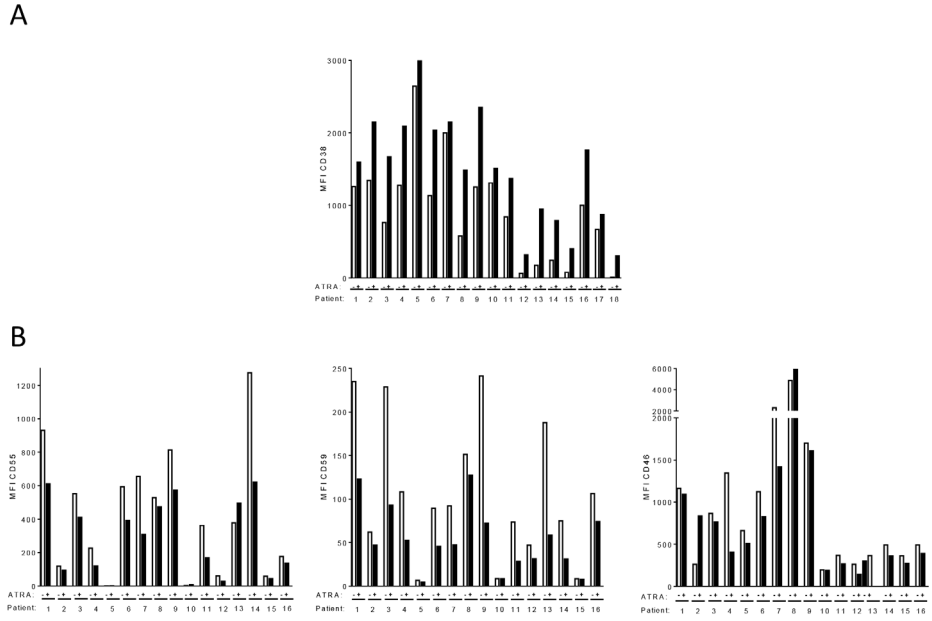
(A) Results of 1-hour CDC and (B) 48-hour ADCC assays, using BM-MNCs of 16 and 11 MM patients, respectively. BM-MNCs were pretreated for 48 hours with solvent control or 10 nM ATRA, followed by incubation with IgG1-b12 control antibody or daratumumab. Pooled human serum (10%) was used as a source of complement. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow-cytometry. Percentage lysis of MM cells was calculated as indicated in materials and methods. Except for patient 6 who had newly diagnosed MM, all patients had relapsed/refractory MM, including 11 with lenalidomide- and bortezomib double refractory disease (patients 1, 2, 3, 4, 5, 9, 10, 13, 14, 16, and 18).

Immunophenotyping by flow cytometry

Expression of several cell surface proteins was determined by flow cytometric analysis using FITC-, PE-, Per-CP-, or APC-conjugated monoclonal antibodies. Anti-CD38, anti-CD138, and anti-CD56 were purchased from Beckman Coulter; anti-CD3, anti-CD16, anti-CD55, anti-CD59 from BD Biosciences; and anti-CD46 from Biolegend. Flow cytometry was done using a FACS-Calibur device (Becton Dickinson); the data were analyzed using the CellQuest software.

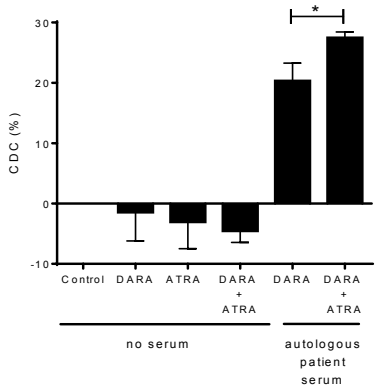
Supplementary

FIGURE 3



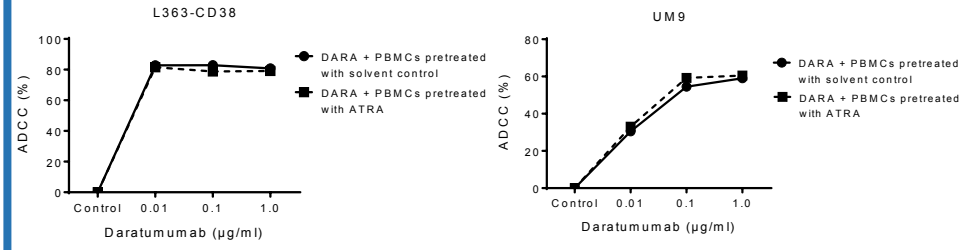
Effect of ATRA on expression levels of CD38, CD46, CD55, and CD59.
(A) Expression levels of CD38 on primary MM cells incubated with solvent control or with 10 nM ATRA of the patients tested in the ADCC and CDC assays in Supplementary Figure 2.
(B) Expression levels of CD46, CD55 and CD59 on primary MM cells incubated with solvent control or with 10 nM ATRA of the 16 patients tested in the CDC assays in Supplementary Figure 2.

Supplementary
FIGURE 4



Daratumumab-mediated CDC against primary MM cells is improved by ATRA in the presence of autologous serum.
Results of 1-hour CDC assay using BM-MNCs obtained from a MM patient. BM-MNCs were pretreated for 48 hours with solvent control or 10 nM ATRA, followed by incubation with IgG1-b12 control antibody or daratumumab. Autologous serum (10%) was used as a source of complement. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow-cytometry. Percentage lysis of MM cells was calculated as indicated in materials and methods. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using a paired student's t test; * P<0.05.

Supplementary
FIGURE 5



ATRA does not enhance the activity of effector cells in ADCC assays.
L363-CD38 and UM9 MM cell lines were treated with IgG1-b12 control antibody or daratumumab (0.01-1.0 µg/mL) for 4 hours in the presence of either solvent-control or 10 nM ATRA-pretreated PBMCs (for 48 hours) derived from healthy donors as effector cells. Daratumumab-mediated ADCC was determined by BLI. Data show mean and SEM of at least three experiments. Of note, the MM cells in these ADCC assays were not exposed to ATRA.

Chapter V

CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma

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Blood. 2016; 128(7): 959-970

Abstract

The anti-CD38 monoclonal antibody daratumumab is well tolerated and has high single agent activity in heavily pretreated relapsed and refractory MM. However, not all patients respond and many patients eventually develop progressive disease to daratumumab monotherapy. We therefore examined whether pretreatment expression levels of CD38 and complement-inhibitory proteins (CIPs) are associated with response and whether changes in expression of these proteins contribute to development of resistance. In a cohort of 102 patients treated with daratumumab monotherapy (16 mg/kg), we found that pretreatment levels of CD38 expression on MM cells were significantly higher in patients who achieved at least partial response (PR), when compared to patients who achieved less than PR. However, cell surface expression of the CIPs, CD46, CD55 and CD59, was not associated with clinical response. In addition, CD38 expression was reduced in both BM-localized and circulating MM cells, following the first daratumumab infusion. CD38 expression levels on MM cells increased again following daratumumab discontinuation. In contrast, CD55 and CD59 levels were significantly increased on MM cells only at the time of progression. All-trans retinoic acid increased CD38 levels and decreased CD55 and CD59 expression on MM cells from patients who developed daratumumab resistance, to approximately pretreatment values. This resulted in significant enhancement of daratumumab-mediated complement-dependent cytotoxicity. Together, this data demonstrates an important role for CD38 and CIP expression levels in daratumumab sensitivity, and suggests that therapeutic combinations that alter CD38 and CIP expression levels should be investigated in the treatment of MM.
The trials were registered at www.clinicaltrials.gov as NCT00574288 (GEN501) and NCT01985126 (Sirius).

Introduction

In the last decade survival of MM patients has markedly improved.[1] However, patients with disease refractory to IMiDs and proteasome inhibitors have a median overall survival of only 9 months,[2] underscoring the need for additional active agents with novel mechanisms of action.[3,4] Antibodies against target antigens expressed on MM cells are an important new class of agents, and preliminary results are very promising in this group of patients.[5]

The anti-CD38 monoclonal antibody, daratumumab, is well tolerated and has high single agent activity. In the 16 mg/kg cohort of the GEN501 study, at least a partial response was achieved in 36% of the patients including CR in 5%.[6] Similar efficacy was observed in the Sirius study.[7] Based on these results, the FDA recently approved daratumumab for the treatment of MM patients who have received at least 3 prior lines of therapy including a proteasome inhibitor and an IMiD or who are double-refractory to a proteasome inhibitor and an IMiD. The mechanisms implicated in daratumumab-mediated killing of tumor cells include a direct apoptotic effect[8] and, more important, activation of potent cytotoxic immune effector functions, including, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC).[9–13] In fact daratumumab was selected for further development because of its high efficacy to kill tumor cells via CDC.[11] Moreover, myeloid-derived suppressor cells, regulatory B-cells, and a subset of regulatory T cells also express CD38 and are susceptible to daratumumab-mediated lysis.[14] It has also been shown that daratumumab modulates the enzymatic activity of CD38, which potentially leads to a reduction in immunosuppressive adenosine levels.[15,16] This shift away from an immunosuppressive environment is hypothesized to result in an improved host-anti-tumor immune response.[14]

Despite the well-established clinical efficacy of daratumumab, not all of the heavily pretreated patients respond to single agent daratumumab, and the majority of patients who initially respond eventually progress. This indicates the need for new insights into mechanisms of resistance. We have previously shown that expression of CD38 on MM cells correlates with daratumumab-mediated ADCC and CDC *in vitro*.[17] However, the predictive value of CD38 expression on MM cells for achieving clinical benefit from daratumumab is currently unknown. In addition, not all the variability in daratumumab-mediated killing *in vitro* could be explained by differences in CD38 expression. To further understand CD38-independent tumor-related mechanisms influencing daratumumab sensitivity, we investigated expression of complement-inhibitory proteins on MM cells.

Host cells are protected against accidental complement attack by fluid-phase regulators as well as by expression of membrane-associated complement-inhibitory proteins, such as CD46 and the glycosyl-phospatidylinositol-anchored proteins, CD55 and CD59.[18,19] Overexpression of these complement-inhibitory proteins in cancer plays a role in tumor immune evasion and resistance against therapeutic antibodies.[20–28] Here, we demonstrate that daratumumab-treated patients, who achieve at least PR have higher CD38 baseline levels, when compared to patients with less than PR. No differences in pretreatment expression

levels of the complement-inhibitory proteins were observed between both groups of patients. Furthermore, we show that development of resistance towards daratumumab was associated with upregulation of CD55 and CD59 on the MM cells. In addition, reduced surface expression of CD38 on non-depleted MM cells may also confer protection against daratumumab. Finally, we demonstrate that all-trans retinoic acid (ATRA) increases CD38 and reduces CD55 and CD59 expression on daratumumab-resistant MM cells, thereby improving CDC against MM cells.

Material and Methods

Patients and protocols

Data on expression levels of CD38 and complement-inhibitory proteins on bone marrow (BM)-localized MM cells were derived from patients with relapsed or refractory multiple myeloma treated with 16 mg/kg daratumumab monotherapy and who were enrolled in two clinical studies (NCT00574288 [GEN501] and NCT01985126 [Sirius]) that have been described in detail elsewhere.[7,29]

Briefly, in the GEN501 study, patients had MM requiring systemic therapy and relapsed from or refractory to at least 2 prior therapies.[29] In the Sirius study, patients had received at least 3 prior lines of therapy including a PI and an IMiD, or were refractory to both classes of drugs.[7] In both studies patients had age ≥18 years; life expectancy ≥3 months; Eastern Cooperative Oncology Group performance status of ≤2; and measurable disease. Exclusion criteria included other malignancies; uncontrolled infections; cardiovascular and respiratory conditions; or meningeal involvement of MM. Before start of daratumumab therapy, BM aspirates were obtained from 102 patients treated in the GEN501 and Sirius studies. In addition, in a subset of 21 patients, treated in the GEN501 study, BM aspirates were also obtained approximately 14 weeks after initiation of treatment, and at the time of progression. In this group of patients, peripheral blood was also obtained before start, at multiple time points during treatment, and after administration of the last daratumumab infusion. Study site ethics committees or institutional review boards approved the protocols, which were conducted according to the principles of the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice. All patients gave written informed consent.

Antibodies and reagents

Daratumumab was provided by Janssen Pharmaceuticals. Human IgG1-b12 (Genmab), a human mAb against an innocuous antigen (HIV-1 gp120), was used as an isotype control as described previously.[30,31] All-trans retinoic acid (ATRA) was purchased from Sigma-Aldrich.

Flow cytometric analysis of bone marrow and blood samples from patients treated with daratumumab monotherapy

CD38, CD46, CD55, and CD59 expression levels were determined on both BM-localized and circulating MM cells by using flow cytometric analysis. Additional methods are presented in the Supplemental Data.

Results

CD38 expression levels on pretreatment MM cells are associated with clinical response to daratumumab

We have previously shown that there is a significant positive association between CD38 expression levels on MM cells from patients and the efficacy of daratumumab to induce cell death by ADCC as well as CDC.[17] We hypothesized that cell surface expression of CD38 on MM cells may predict response to daratumumab monotherapy. We therefore analyzed CD38 expression on pretreatment BM-localized MM cells from patients, who were subsequently treated with 16 mg/kg daratumumab as single agent in the phase 2 part of the GEN501 study[29] or in the Sirius study.[7] Pretreatment BM samples for this analysis were available for 102 out of a total of 148 MM patients treated with 16 mg/kg daratumumab. The clinical characteristics of these heavily pretreated patients are shown in Table 1.

Table 1

Patient characteristics

Parameter	MM patients n=102
Median age, years (range)	64 (32-84)
Sex, male, n (%)	57 (56%)
M-protein type	
IgG, n (%)	51 (50%)
IgA, n (%)	21 (21%)
IgM, n (%)	0 (0%)
IgD, n (%)	3 (3%)
Biclonal, n (%)	2 (2%)
Light chain only, n (%)	25 (25%)
Previous therapy	
≤ 3 lines of therapy	24 (24%)
> 3 lines of therapy	78 (76%)
Prior therapy to which disease was refractory* before start of daratumumab	
lenalidomide refractory, n (%)	86 (84%)
bortezomib refractory, n (%)	85 (83%)
lenalidomide and bortezomib refractory, n (%)	78 (76%)
pomalidomide refractory, n (%)	54 (53%)
carfilzomib refractory, n (%)	36 (35%)

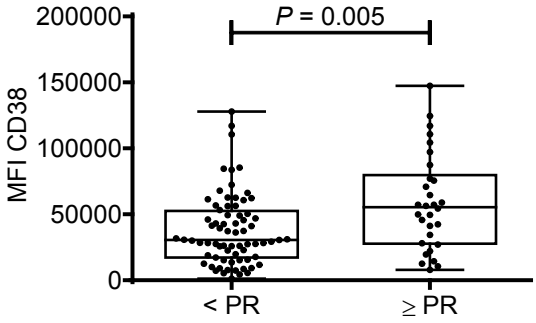
Abbreviations: n, number.

*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.

As expected, all MM cells expressed CD38 antigen in these patients' samples, but there was a marked heterogeneity in the intensity of CD38 expression. In this group

of 102 patients at least PR was achieved in 30 patients (29%). The MM patients who achieved at least PR had significantly higher baseline CD38 expression levels on their tumor cells, when compared to patients who achieved less than PR (median MFI CD38: 55424 versus 30659; P=0.005; Figure 1).

FIGURE 1



CD38 expression levels on primary MM cells before start of therapy are significantly higher in responders versus non-responders in GEN501 and Sirius studies.
CD38 expression levels were analyzed by flow cytometry in 102 BM samples obtained from relapsed/refractory MM patients before start of treatment with daratumumab monotherapy at a dose of 16 mg/kg in the GEN501 and Sirius studies. HuMax-003 FITC was used as CD38 antibody. Shown are median, 25th-75th percentile (box), and minimum and maximum value (whiskers). CD38 expression levels on MM cells were compared between responders (defined as partial response or better) and non-responders. P-values between the indicated groups were calculated using the Mann-Whitney U test. Every dot represents a patient. PR: partial response; N: number.

Accordingly, response to daratumumab was markedly higher in the highest tertile of CD38 expression (≥PR: 48.5%), compared to the mid-tertile (22.2%), or lowest tertile (18.2%). Importantly, CD38 levels on MM cells were similar in patients with or without double-refractory (lenalidomide and bortezomib-refractory), triple-refractory (lenalidomide, bortezomib, and either pomalidomide or carfilzomib-refractory), or quadruple refractory disease (lenalidomide, pomalidomide, bortezomib, and carfilzomib-refractory). Also age, sex, tumor load, creatinine clearance, lenalidomide treatment before daratumumab, LDH levels, beta2-microglobulin levels, and ISS stage did not affect CD38 expression. Soluble CD38, which may also bind daratumumab, was evaluated in 110 out of the 148 patients, and detected in only 2 cases. Both of them achieved PR.

Expression levels of complement-inhibitory proteins on pretreatment MM cells are not associated with daratumumab-mediated CDC or clinical response to daratumumab

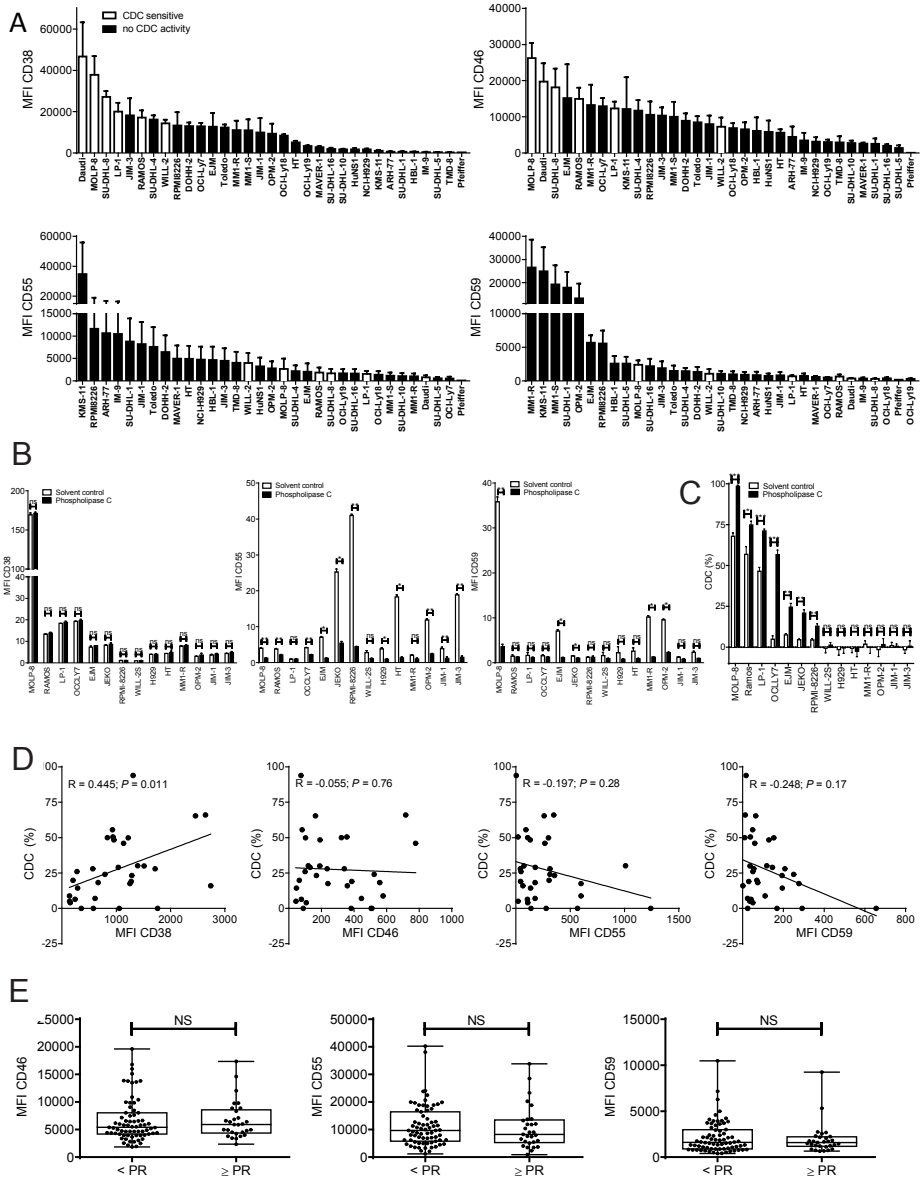
Given the overlap in CD38 expression levels between responding and non-responding patients, we concluded that CD38 levels alone do not explain the whole variability in response to daratumumab therapy. Since daratumumab has high CDC

activity,[11] which explains the consumption of complement proteins C2 and C4 (and to a lesser extent C3, but not C1q) after the first daratumumab infusion (Supplementary Figure 1), we hypothesized that cell surface expression of the complement-inhibitory proteins CD46, CD55, and CD59 on MM cells could be associated with the extent of daratumumab-mediated CDC as well as response to therapy. Therefore, we evaluated the impact of complement-inhibitory proteins on daratumumab's ability to kill tumor cells by using cell lines, primary MM cells, and also pretreatment samples from patients treated in the GEN501 and Sirius studies.

We first analyzed 14 MM and 19 non-Hodgkin's lymphoma cell lines in CDC assays. Twenty-seven cell lines were completely resistant and 6 cell lines were sensitive to daratumumab-mediated CDC (Figure 2A). The sensitive cell lines had higher CD38 (P=0.0019) and lower CD59 (P=0.0006) expression levels, when compared to the resistant ones. In addition, there was a trend towards higher levels of CD55 expression in CDC-resistant cell lines, while elevated CD46 levels were not associated with daratumumab resistance. The role of CD55 and CD59 as possible determinants of susceptibility towards daratumumab, was further investigated by pretreating cells with phospholipase-C, which removed the GPI-anchored CD55 and CD59 molecules from the cell surface but had no effect on CD38 expression (Figure 2B).

Pretreatment with phospholipase-C rendered 7 out of 14 cell lines more susceptible to complement-mediated lysis (Figure 2C). The cell lines without improved CDC after pretreatment with phospholipase-C had significantly lower CD38 expression, when compared to the 7 cell lines which showed enhanced CDC (P=0.02). Next we examined the impact of complement-inhibitory proteins on daratumumab-mediated CDC by using BM samples from 32 daratumumab-naive MM patients. Twenty-one of these 32 patients were subsequently treated with daratumumab monotherapy in the GEN501 study. The characteristics of these patients are shown in Supplementary Table 1. Daratumumab (10 μg/mL)-mediated CDC against MM cells was very heterogeneous and ranged from -15.5% (negative values indicate MM cell growth) to 93.9% (median 22.5%). There was a positive correlation between susceptibility of primary MM cells to CDC and the level of CD38 (R=0.45; P=0.011), which is in agreement with our previous analysis in a different cohort of patients.[17] However, expression levels of CD46, CD55, and CD59 were not correlated with CDC (Figure 2D). We also analyzed complement inhibitor expression on pretreatment MM cells from the 102 patients with available baseline BM samples, who subsequently received daratumumab treatment in the GEN501 and Sirius studies (Figure 2E). There was no significant difference in baseline expression levels of CD46, CD55, and CD59 between patients who achieved at least PR, when compared to patients with less than PR. We also tested whether the combined biologic effects of CD38 and complement inhibitors were associated with response by calculating the ratios of CD38/CD46, CD38/CD55, and CD38/CD59. However, there was no improvement in the strength of the association with response when compared to CD38 expression levels alone (data not shown). Therefore, to gain a more detailed insight on the role of these molecules during treatment, we next performed an in depth longitudinal analysis of CD38 and complement-inhibitory proteins in a subset of 21 GEN501 patients.

FIGURE 2



Expression of complement-inhibitory proteins and susceptibility to daratumumab-mediated CDC.

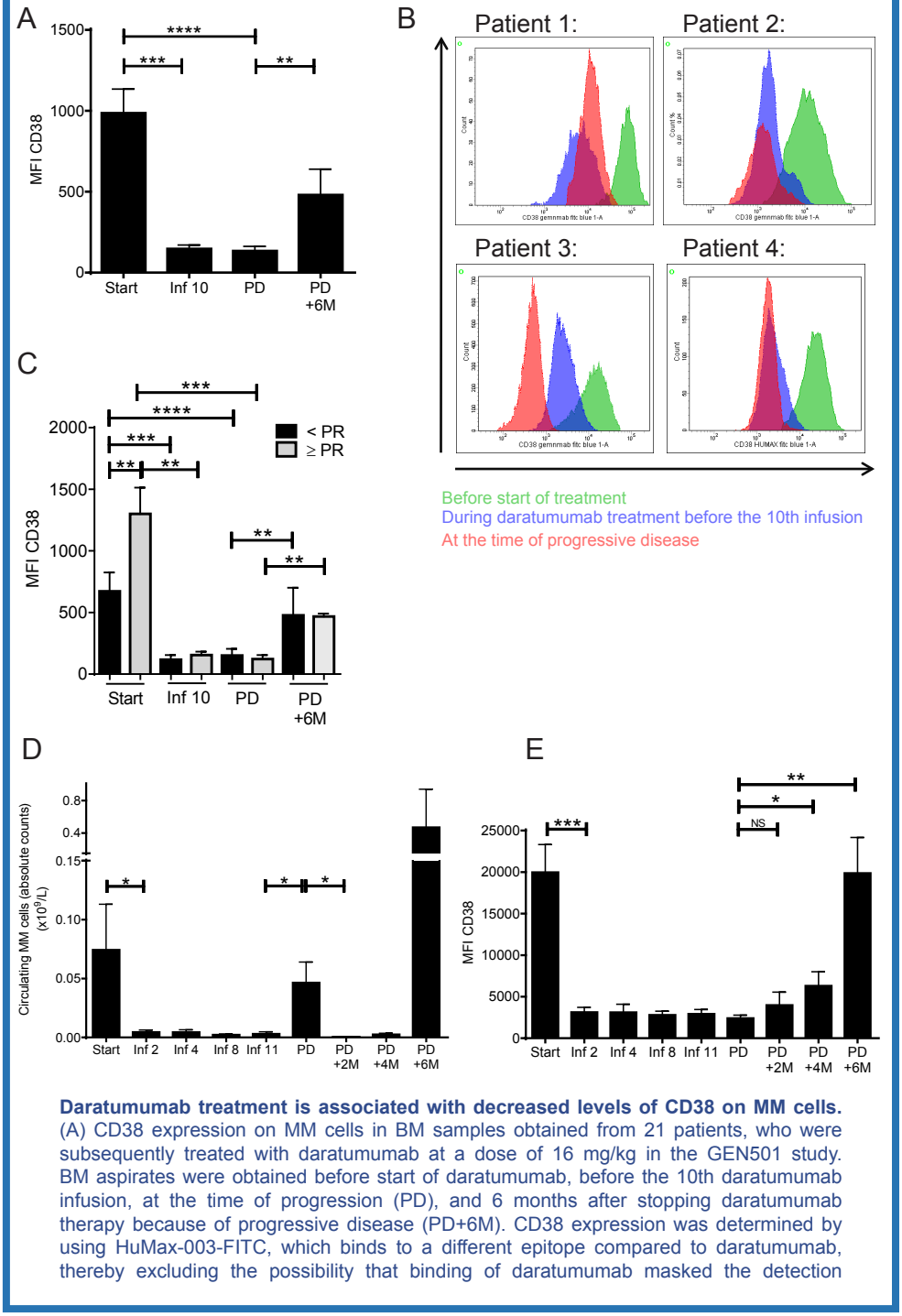
(A) Susceptibility of 33 MM and lymphoma cell lines towards daratumumab-mediated CDC according to expression levels of CD38, CD46, CD55, and CD59 as determined by flow cytometry. Daratumumab induced CDC in 6 out of 33 cell lines (white bars), whereas the others were completely resistant (black bars). Shown are mean +/- s.e.m. of 6 experiments. (B) Expression levels of CD38, CD55, and CD59 on 14 MM and lymphoma cell lines, after

incubation with solvent control (white bar) or phospholipase-C (black bar) for 30 minutes. Shown are mean +/- s.e.m. P-values between the indicated groups were calculated using paired student t-tests. (C) CDC assays were performed with the same 14 cell lines as shown in Figure 2B, which were pretreated with phospholipase-C (black bars) or solvent control (white bars) for 30 minutes. CDC-assays were performed with 10 µg/mL daratumumab or IgG1-b12 control antibody as described in Materials and Methods. Shown are mean +/- s.e.m. of 3 experiments. P-values between the indicated groups were calculated using a paired Student's t-test; *P<0.05, ** P<0.01, *** P<0.001, ns not significant. (D) Positive correlation between CD38 expression levels on primary MM cells and CDC mediated by 10 µg/mL daratumumab at 1 hour (n=32 MM patients). No significant correlation between CD55, CD59, and CD46 expression levels on primary MM cells and CDC mediated by 10 µg/mL daratumumab at 1 hour (n=32 MM patients). Expression levels were determined by flow cytometry. CDC-assays were performed as described in Materials and Methods.
Correlations between variables were analyzed using the Spearman's rank correlation coefficient. (E) CD46, CD55 and CD59 expression levels were analyzed by flow cytometry in 102 BM samples obtained from relapsed/refractory MM patients before start of treatment with daratumumab monotherapy at a dose of 16 mg/kg in the GEN501 and Sirius studies. Shown are median, 25th-75th percentile (box), and minimum and maximum value (whiskers). CD46, CD55 and CD59 expression levels on MM cells were compared between responders (defined as partial response or better) and non-responders. P-values between the indicated groups were calculated using the Mann-Whitney U test. Every dot represents a patient. PR: partial response; N: number.

Reduced CD38 expression on MM cells during daratumumab treatment

Since CD38 expression on MM cells is an important determinant of susceptibility towards daratumumab,[17] we hypothesized that residual daratumumab-resistant MM cells may have decreased levels of this protein. To this end, we analyzed CD38 expression on BM-localized MM cells in a subset of GEN501 patients (n=21) before start of treatment, 14 weeks after the initiation of daratumumab treatment, and also at the time of progression during daratumumab therapy. We used an anti-CD38 monoclonal antibody, HuMax-003-FITC, that binds to a different epitope compared to daratumumab. This excluded the possibility that binding of daratumumab masked the detection of CD38. Interestingly, 14 weeks after the first daratumumab infusion the MM cells had significantly lower CD38 expression levels, compared to baseline values (median MFI CD38: 866.0 versus 124.2, P= 0.0001) (Figure 3A and B).
Similarly, at the time of progression MM cells had low CD38 expression levels (median MFI CD38: 85.1). The reduced expression of CD38 is a transient phenomenon since approximately 6 months after the last daratumumab infusion BM-localized MM cells regained CD38 expression (Figure 3A). There was no difference between patients who did or did not achieve PR or better and decrease in CD38 expression during daratumumab treatment (Figure 3C).To gain further insight into the kinetics of CD38 reduction, we analyzed CD38 expression on circulating MM cells in the same GEN501 subgroup. Peripheral blood clonal plasma cells could be detected before start and during daratumumab therapy in 11 out of 21 patients (52%). Circulating MM cells were rapidly cleared by daratumumab (Figure 3D). Already after the first daratumumab infusion the

FIGURE 3



Daratumumab treatment is associated with decreased levels of CD38 on MM cells. (A) CD38 expression on MM cells in BM samples obtained from 21 patients, who were subsequently treated with daratumumab at a dose of 16 mg/kg in the GEN501 study. BM aspirates were obtained before start of daratumumab, before the 10th daratumumab infusion, at the time of progression (PD), and 6 months after stopping daratumumab therapy because of progressive disease (PD+6M). CD38 expression was determined by using HuMax-003-FITC, which binds to a different epitope compared to daratumumab, thereby excluding the possibility that binding of daratumumab masked the detection

of CD38. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant. (B) Flow cytometry histogram overlays depicting cell surface expression of CD38 on MM cells from 4 representative patients treated with daratumumab in the GEN501 trial at different time points: before start of treatment (green histogram), during daratumumab treatment before the 10th infusion (blue histogram), and at the time of progressive disease (red histogram). HuMax-003 FITC was used as CD38 antibody.
(C) Longitudinal data representation of CD38 expression levels on MM cells from the 21 patients presented in panel A, according to the response achieved to daratumumab monotherapy (partial response (PR) or better (grey bars) versus less than partial response (black bars)). P-values between the indicated groups were calculated using a Student's t-test; ** P<0.01, *** P<0.001, **** P<0.0001. (D) Longitudinal data representation of absolute circulating MM cell counts over time in peripheral blood. Circulating MM cells were observed in 11 out of 21 patients tested. Peripheral blood was obtained before start of treatment with daratumumab, during treatment with daratumumab, at the time of progression (PD), as well as 2, 4 and 6 months after development of progressive disease (PD). Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05. (E) CD38 expression on circulating MM cells before start of daratumumab treatment, during daratumumab treatment, at the time of progression (PD), as well as 2, 4 and 6 months after the development of progressive disease (PD)(n=11 patients). CD38 expression was determined by using HuMax-003-FITC. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05, ** P<0.01, *** P<0.001, ns not significant.T, tertile) or (F) with CD14+ monocytes as effector cells and CD38+/CD138+ MM cells as target cells (monocyte: MM cell ratio: <0.169, 0.169-0.633, >0.633; n=85 patients; T, tertile). P-values between the indicated groups were calculated using a student's t test; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant.

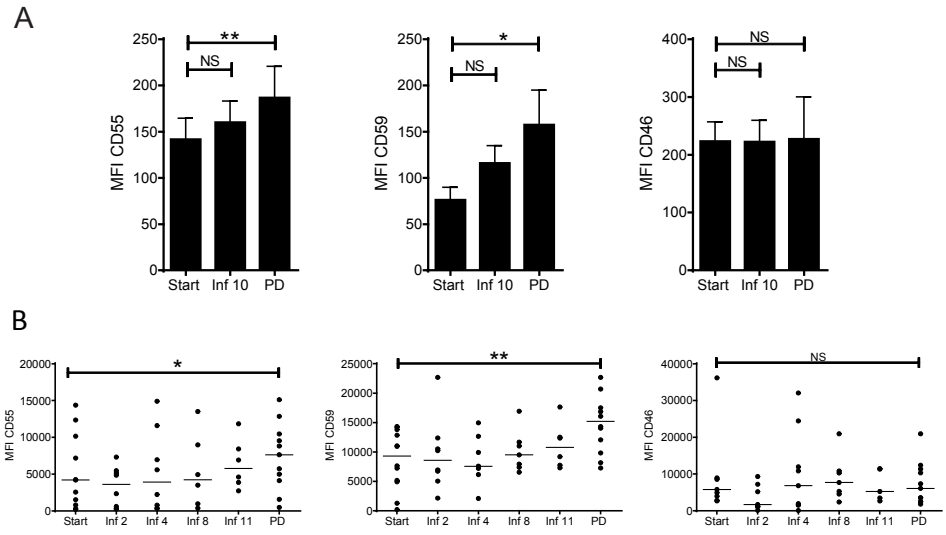
non-depleted circulating MM cells had significantly lower CD38 expression levels, when compared to baseline (P= 0.0006; Figure 3E). Examination of peripheral blood samples, taken at different time points after disease progression, showed that CD38 expression gradually increased to baseline levels after approximately 6 months post-daratumumab treatment. Also on the circulating MM cells, there was no difference in the reduction of CD38 levels during daratumumab treatment between patients who achieved PR and those with less than PR (Supplementary Figure 2).

Increased CD55 and CD59 levels on MM cells at the time of progression during daratumumab therapy

To further analyze mechanisms of resistance to daratumumab, we analyzed expression of the complement-inhibitory proteins on BM-localized MM cells in the GEN501 subgroup (n=21). There was no change in expression of the complement-inhibitory proteins when patients were still responding to daratumumab or had stable disease. However, there was a significant increase in CD55 and CD59 expression levels on BM-localized MM cells at the time of progression, when compared to levels before start or during daratumumab treatment (median MFI CD55: 109.0 vs 167.0, P = 0.01; median MFI CD59: 50.3 vs 98.2, P = 0.018 for samples taken at baseline and at the time of progression, respectively; Figure 4A). Importantly, CD46 protein levels did not increase at the time of progression.
Similarly, on circulating MM cells, CD55 and CD59 levels did not change until patients developed progressive disease (increase in median MFI CD55 of 81.5%, P=0.031; increase in median MFI CD59 of 63.3%, P=0.0049,

for samples taken at baseline and at the time of progression). CD46 expression on circulating MM cells remained unchanged (Figure 4B).

FIGURE 4

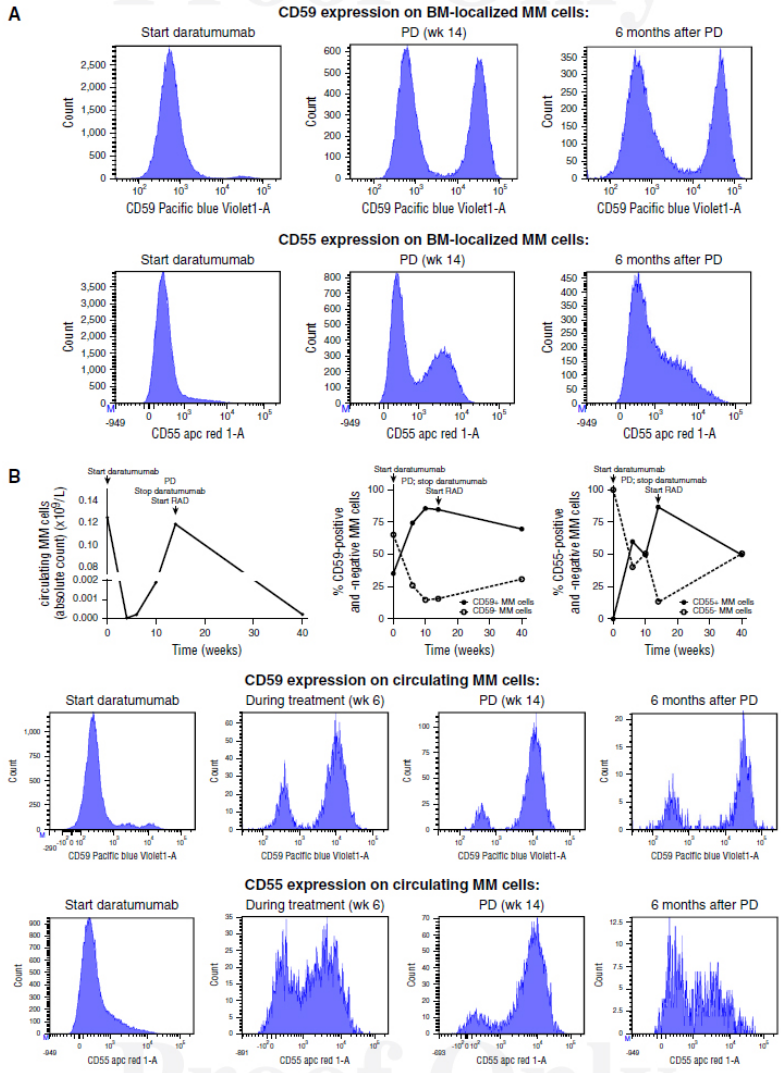


Expression levels of CD55 and CD59 on MM cells increase at the time of progression. (A) CD55, CD59 and CD46 expression levels on MM cells from BM samples obtained from 21 MM patients, who were subsequently treated with daratumumab at a dose of 16 mg/kg in the GEN501 study. BM samples were obtained before start of daratumumab, before the 10th daratumumab infusion, and at the time of progression (PD). Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05, ** P<0.01, ns not significant. (B) CD55, CD59 and CD46 expression levels on circulating MM cells before start of daratumumab treatment, during daratumumab treatment, and at the time of progression (PD) (n=11 patients). Expression levels of the complement-inhibitory proteins were analyzed by flow cytometry. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05, ** P<0.01, ns not significant.

Daratumumab treatment selects for MM clones with high expression levels of CD55 and CD59

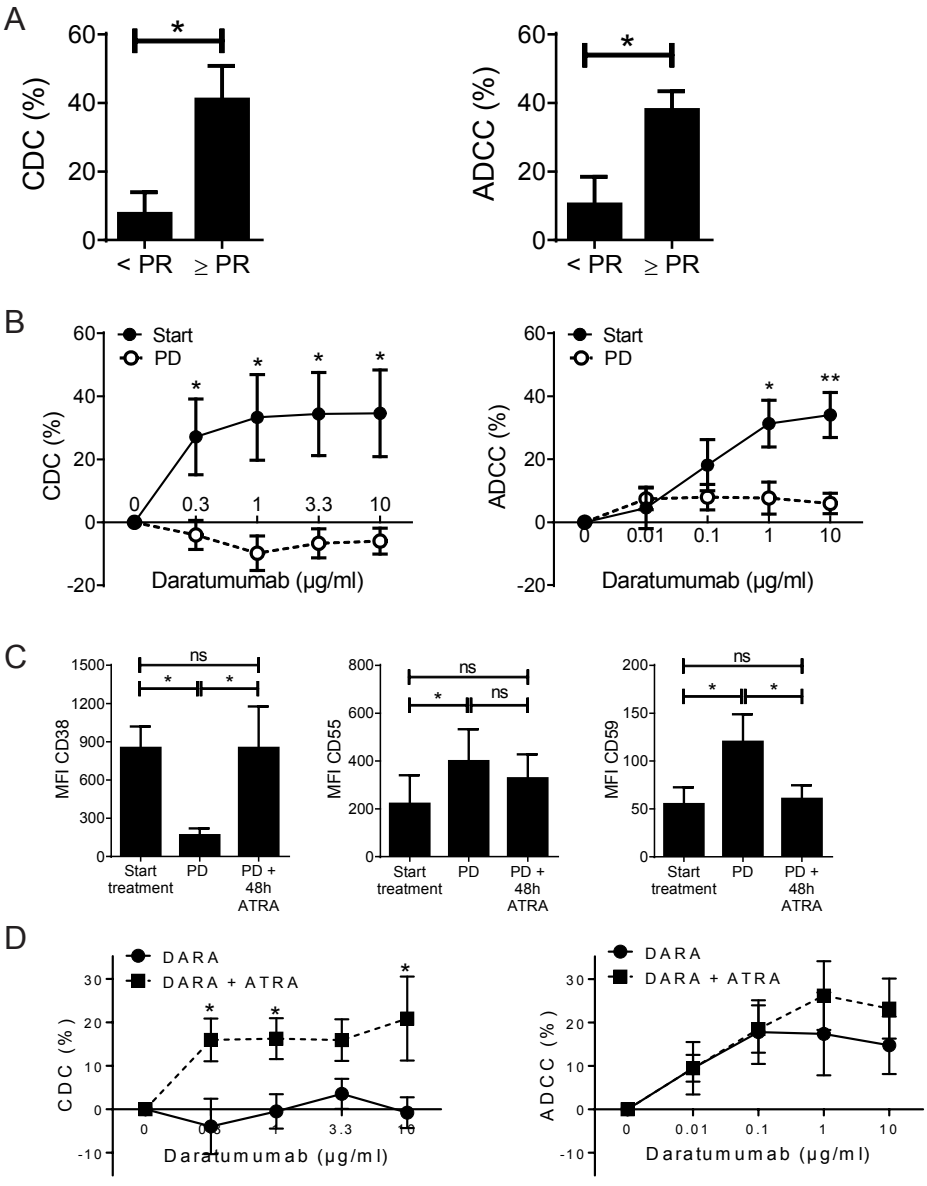
There is increasing evidence of existence of competing subclones in MM.[32–36] We therefore investigated the presence of phenotypic subclones based on CD38 and complement-inhibitory protein expression levels. In the majority of patients, one phenotypic population of MM cells was detected based on expression of CD38 and complement inhibitors. However, in two out of 21 patients we detected two coexisting populations of MM cells based on differential expression of CD55 and/or CD59. To investigate the effect of daratumumab on the relative frequencies of these different subpopulations, we analyzed several blood and/or BM samples from these patients before the start of treatment, during daratumumab treatment, at the time of

FIGURE 5



Selection of populations of MM cells with high expression of complement-inhibitory proteins during therapy with daratumumab.
Two out of 21 GEN501 patients had co-existing populations of MM cells, which differed in CD55 and/or CD59 expression. (A) BM-localized MM cells from patient 5 differed in expression levels of CD55 (absent or strongly positive) and CD59 (absent or strongly positive). Bone marrow samples were obtained before start of daratumumab therapy, at the time of progressive disease (PD), and 6 months after the last daratumumab infusion. Flow cytometry histograms for these samples are shown. (B) Similarly, circulating MM cells from patient 5 also differed in expression levels of CD55 (absent or strongly positive) and CD59 (absent or strongly positive). Serial blood samples were obtained during daratumumab monotherapy and during the treatment given after development of progressive disease (PD). Longitudinal data representation of absolute circulating MM cell counts over time, and of the frequency of the different subpopulations based on complement-inhibitory protein expression. Representative histograms are also shown for this patient.
Abbreviations: RAD, lenalidomide, adriamycin, and dexamethasone.

FIGURE 6



ATRA reverses resistance to daratumumab-mediated CDC by increasing CD38 expression and reducing expression levels of complement-inhibitory proteins on MM cells. (A) *Ex vivo* CDC and ADCC assays were performed with BM aspirates from 15 patients, who were subsequently treated in the GEN501 study. One-h CDC and 48-h ADCC assays were performed as described in Materials and Methods. Shown are means ± s.e.m of CDC and ADCC mediated by 10 µg/ml daratumumab according to response achieved

in the GEN501 study. P-values between the indicated groups were calculated using a Student's t-test; * P<0.05. (B) Serial BM aspirates were obtained from 5 patients both before start of daratumumab and at the time of progression during daratumumab. One-h CDC and 48-h ADCC assays were performed as described in Materials and Methods with pretreatment samples and samples obtained at the time of progression. We expect that at the time of progression MM cells have already daratumumab, given to the patient via intravenous infusion, bound to their cell surface; therefore these graphs show the effect of freshly added daratumumab. To evaluate the effect of possible prebound daratumumab in CDC assays, we analyzed the effect of the control antibody IgG1-b12 (no CDC activity). IgG1-b12 in the presence of heat-inactivated serum or native human serum did not induce CDC against MM cells obtained at the time of progression (lysis: 0.81% and -1.14%). This indicates that prebound daratumumab does also not induce CDC at the time of progression. Dose-response curves for ADCC and CDC were constructed according to treatment status. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05, ** P<0.01. (C) BM-MNCs were obtained from 8 patients before the first daratumumab infusion and at the time of progression (PD). BM-MNCs obtained at the time of progression were subsequently incubated with solvent control or with 10 nM ATRA for 48 hours. Cells were then collected to determine CD38, CD55, and CD59 expression levels by flow cytometry. HuMax-003-FITC was used to detect CD38 expression. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05, ns not significant. (D) Pooled results of 1-h CDC and 48-h ADCC assays, using BM-MNCs of 6 patients. BM-MNCs were pretreated for 48 h with solvent control or 10 nM ATRA, followed by incubation with IgG1-b12 control antibody or daratumumab. Pooled human serum (10%) was used as a source of complement. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow cytometry. Percentage lysis of MM cells was calculated as indicated in Materials and Methods. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a paired Student's t-test; * P<0.05.

progression, and also during additional follow-up. In both patients, we observed a change in subclone phenotypes with rapid selection of daratumumab-resistant clones with high expression of complement-inhibitors (Figure 5 [patient 5] and Supplementary Figure 3 [patient 6]). CD38 expression levels were similar in the different MM cell populations from patient 5. However, baseline CD38 expression levels were lower in CD59-positive cells from patient 6 when compared to the CD59-negative cells, which may have contributed to the selection of the CD59-positive subpopulation.

ATRA reverses resistance to daratumumab by increasing CD38 expression levels

From 15 of the previously described 21 GEN501 patients, we harvested sufficient BM-MNCs with which we performed *ex vivo* CDC and ADCC assays.
Daratumumab-mediated CDC and ADCC in these pretreatment samples was associated with clinical response to daratumumab therapy (Figure 6A). In serial samples (n= 5), there was a marked reduction in daratumumab-mediated CDC and ADCC against MM cells from aspirates taken at the time of progression, when compared to paired pretreatment samples (CDC with 10 µg/mL daratumumab: 34.6 to -6.0%, P= 0.02; ADCC with 10 µg/mL daratumumab: 34.1 to 5.9%, P= 0.007) (Figure 6B). Thus, the results from these *ex vivo* experiments are consistent with

the daratumumab-resistant phenotype of the MM cells in the patients at the time of progression. Importantly, daratumumab that is possibly bound to MM cells after initiation of daratumumab treatment had no CDC activity, since the IgG1-b12 control antibody in the presence of native human serum did not induce lysis of MM cells (lysis: -1.14%).

We have previously shown that ATRA increases CD38 expression levels and reduces CD55 and CD59 levels on MM cells. This resulted in enhanced ADCC and CDC in both MM cell lines and patient samples.[17] However, these MM cells were not previously exposed to daratumumab. Since our data suggest that reduced CD38 expression and increased CD55 and CD59 levels contribute to acquired resistance to daratumumab, we hypothesized that ATRA may also be of value at the time of progression. We first tested whether ATRA could restore CD38 expression and reduce CD55 and CD59 levels on daratumumab-resistant MM cells. To this end, we incubated BM-MNCs, obtained from 8 MM patients with progressive disease during daratumumab therapy, with ATRA. This resulted in a significant upregulation of CD38 expression, but also in a reduction of CD55 and CD59 levels on the MM cells, almost to pretreatment values (Figure 6C).

Next, we treated BM-MNCs obtained from patients with progressive disease during daratumumab therapy with solvent control or ATRA for 48 h, followed by incubation with or without daratumumab. ATRA alone for 48 or 96 h did not significantly affect MM cell viability, when compared to solvent control. However, pretreatment with ATRA for 48 h improved daratumumab-mediated CDC in 4 out of 6 patients and ADCC in 2 out of 6 patients. Pooled results show that ATRA pretreatment improved daratumumab (10 µg/ml)-mediated CDC from -0.8 to 20.9% (P= 0.031) and ADCC from 14.8 to 23.2% (P=0.31) (Figure 6D).

Discussion

Treatment with single agent daratumumab is clinically effective in relapsed and refractory MM, although there is a fraction of these heavily pretreated MM patients that does not respond to daratumumab. Furthermore, the majority of responding patients will develop resistance over time. Mechanisms that influence daratumumab efficacy are probably multifactorial including both host- and tumor-related factors. This study combined prospective clinical data with correlative measurements on blood and BM specimens to evaluate the impact of several tumor-related factors on response and development of resistance to daratumumab monotherapy.

The current study shows that CD38 expression on MM cells is associated with response to daratumumab therapy. Several other clinical studies have also shown that efficacy of monoclonal antibodies including rituximab,[37,38] alemtuzumab[39], and trastuzumab[40,41] is partly dependent on target antigen expression. Importantly, it is unlikely that CD38 is a general prognostic factor in MM. Firstly, CD38 gene expression levels were not associated with response in the Hovon-65/GMMG-HD4 study, in which patients did not receive daratumumab (data not shown). Moreover, we found that CD38 expression was not affected by prior therapies or by markers of aggressive disease. Therefore, our data indicate that CD38 is a predictor of

response to daratumumab. However, the variability in clinical outcome following daratumumab treatment cannot solely be explained by differential expression of CD38, which precludes its use as a definitive predictive biomarker of response to daratumumab in clinical practice. It is likely that other tumor-related factors such as genetic abnormalities and activation status of signaling pathways, as well as differences in the composition of the bone marrow microenvironment including frequency of immune effector and suppressor cells, also contribute to the variability in response to daratumumab. Furthermore, extent of target saturation is an important determinant of response, with 16 mg/kg daratumumab as the lowest tested dose with pharmacokinetics that are consistent with target saturation[29].

Since daratumumab has potent CDC activity, we also evaluated expression levels of the complement inhibitory proteins CD46, CD55, and CD59. Although, reduction of CD55 and CD59 expression improved daratumumab-mediated CDC in cell lines with substantial CD38 expression, expression levels of complement-inhibitory proteins were not associated with response in MM patients treated with daratumumab monotherapy.

However, analysis of serial blood and BM samples revealed that CD55 and CD59 levels were increased on MM cells at the time of progression, when compared to baseline values. Importantly, *ex vivo* experiments showed that at the time of progression MM cells were resistant to daratumumab-mediated killing. Altogether, this suggests that CD55 and CD59 protein levels increase during the acquisition of a resistant phenotype. Similarly, CD55 and CD59 expression levels are not correlated with susceptibility to rituximab-mediated CDC and do not predict clinical outcome in lymphoma and CLL patients treated with rituximab.[25,42–44] However, there is an increase in CD55 and CD59 levels on CLL cells that were not cleared from blood by rituximab therapy.[43] Interestingly, there is also a positive correlation between naturally occurring anti-MUC1 antibody levels and expression of the complement-inhibitory proteins CD46, CD55, and CD59 in patients with bladder cancer.[28]

Altogether, this indicates that complement-inhibitory proteins may be a broad resistance mechanism for monoclonal antibodies that function through CDC.

Furthermore, we show that daratumumab treatment resulted in a rapid reduction of CD38 levels on both BM-localized and circulating MM cells. There are several possible explanations for the reduction of CD38 levels on MM cells. First, in responding patients, daratumumab may select for tumor cells with lower CD38 expression, while preferentially eliminating MM cells with high CD38 levels. In addition, downregulation of CD38 may be an active process to evade daratumumab-mediated killing. Furthermore, recent *in vitro* studies suggest that binding of daratumumab to CD38 may cause redistribution of CD38 molecules, formation of distinct polar aggregates, and subsequent release of tumor microvesicles.[16] Finally, trogocytosis of CD38-daratumumab complexes by Fcγ receptor-expressing effector cells and direct internalization may also play a role in loss of CD38.[45] Downregulation of CD38 on MM cells is a transient event, since approximately 6 months after the last daratumumab infusion CD38 expression increases again. This may be explained by persisting circulating daratumumab during this period resulting in continuous selective pressure. Daratumumab concentrations in serum were not determined after administration of the last

infusion. However, interference of daratumumab in the indirect antiglobulin test, as a result of binding to CD38-positive donor erythrocytes, persisted 2-6 months after the last daratumumab infusion,[46] indicating that daratumumab remains present in serum for up to 6 months. Similarly, measurable circulating rituximab can persist for up to 6 months after treatment.[47] Importantly, since CD38 levels return to baseline values approximately 6 months after the last daratumumab infusion, retreatment with daratumumab may be effective and warrants further investigation. Similarly, recent studies demonstrated substantial and rapid reduction of CD20 in CLL[48–51] and lymphoma[52] patients treated with rituximab or ofatumumab[53], which has been linked to development of acquired resistance.[48,51–54] Although the rapid loss of CD38 may allow MM cells to escape from daratumumab-mediated killing, CD38 reduction was observed in patients with both <PR and ≥PR, including those with sustained clinical response, which raises the possibility that the continuous pressure to maintain MM cells in a CD38-/low state offers a clinical benefit in the treatment of CD38-positive malignancies. Physiological ligands for CD38 are CD31 present on BM stromal cells and endothelial cells, as well as hyaluronic acid, which is an extracellular matrix component. It has recently been demonstrated that overexpression of CD38 on MM cells results in increased adherence to BM stromal cells probably via CD38-CD31 interactions[55]. Reduced expression of CD38 may therefore lead to loss of cell-cell and cell-matrix contacts, which may contribute to reduced MM cell growth and survival. In addition, CD38 also functions as an ectoenzyme and in this role it has been implicated in immune suppression through production of adenosine in the BM microenvironment.[56,57] Daratumumab-mediated reduction of CD38 on MM cells may therefore contribute to an improved host-anti-tumor immune response.[14]

By using immunophenotypic analysis, we demonstrated in two patients the presence of unique subpopulations with different expression levels of complement inhibitors, whose relative frequencies changed during daratumumab therapy and also after stopping this treatment. This dynamic picture of back and forth competition between phenotypic subclones during and after daratumumab is similar to what has been previously demonstrated with the use of fluorescence in situ hybridization, array comparative genomic hybridization, and whole-exome/genome sequencing.[32–35] Our data support the importance of intraclonal heterogeneity in MM with multiple clones having a different clinical behaviour and differential sensitivity to treatment.[36]

Modulation of determinants of daratumumab sensitivity with novel therapeutic approaches may lead to more effective daratumumab-based regimens with increased quality of response and improved survival. As CD38 levels are significantly reduced following daratumumab treatment and CD38 expression determines susceptibility to daratumumab, we hypothesized that upregulation of CD38 expression levels would lead to resensitization of daratumumab-resistant MM cells. We have previously shown that ATRA increases CD38 expression on daratumumab-naive MM cells.[17] In this study, we demonstrated that ATRA restored expression of CD38, but also reduced CD55 and CD59 levels to close to pre-infusion levels, on daratumumab-resistant MM cells from patients with progressive disease. This resulted in significant enhancement of CDC and a modest improvement in ADCC. This differential effect can be explained by the fact that ATRA-mediated reductions of CD55 and CD59 will improve CDC, but not ADCC.

Furthermore, the moderate enhancement of ADCC may also be related to low frequencies of NK cells at the time of progression when compared to baseline values (manuscript in preparation), without ATRA affecting NK cell levels or their activity.[17]
In conclusion, this study demonstrates that CD38 expression is associated with response to daratumumab monotherapy. We also showed that the development of daratumumab resistance may occur by acquisition of a new drug-induced phenotype with higher CD55 and CD59 expression levels, or as a result of the emergence of a pre-existing subpopulation that is already relatively resistant to daratumumab prior to initiation of therapy. We also provide the rationale for retreatment with daratumumab after sufficient time to allow CD38 expression levels to return to baseline on remaining MM cells, or by adding ATRA to daratumumab regimens. These hypotheses should be explored in upcoming clinical studies with daratumumab.

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Supplemental Methods

Flow cytometric analysis of bone marrow and blood samples from patients treated with daratumumab monotherapy

BM-localized MM cells were identified and analyzed for CD38 expression levels by staining 1x10^6 cells with CD38 V450, CD138 PercP-Cy5.5, CD56 Pe-Cy7 (all Becton Dickinson), HuMax-003 FITC (this antibody binds to an epitope distinct from the epitope bound by daratumumab; Genmab/Janssen Pharmaceuticals; HuMax-003 FITC was used for analysis of CD38 expression in sequential samples obtained before start of daratumumab, during treatment with daratumumab, and after the last infusion of daratumumab), CD45 Pacific Orange (Invitrogen), CD19 APC-A750 (Beckman Coulter, Immunotech) combined with cytoplasmic staining for immunoglobulin light chains using monoclonal anti-kappa APC (Becton Dickinson) and polyclonal anti-lambda PE (DAKO). No significant difference in CD38 expression was noted between patients treated in GEN501 when compared to Sirius. In addition, MM cells were analysed for CD46, CD55 and CD59 expression by staining 1x10^6 cells with HuMax-003 FITC (Genmab/Janssen R&D), CD138 PercP-Cy5.5, CD56 Pe-Cy7 (Becton Dickinson), CD46 PE (Biolegend), CD59 Pacific blue (Exbio), CD55 Alexa 647 (Exbio), CD19 APC-A750 (Beckman Coulter, Immunotech) and CD45 Pacific orange (Invitrogen). Red blood cells were lysed (BD Pharm Lyse, Becton Dickinson) directly before analysis by flow cytometry. These samples were analyzed within 48 hours from the time the bone marrow aspirate was collected. Expression levels of CD38 and the complement inhibitors were stable during the first 48 hours after bone marrow sampling.

Quantitation of circulating MM cells and analysis of their CD38, CD46, CD55 and CD59 expression levels, was performed by the incubation of 35 x10^6 cells with sufficient amounts of antibodies in the combinations described above for 30 minutes at room temperature in the dark. Cells were then washed with PBS, suspended in 2 ml of PBS/human serum albumin and directly analyzed by flow cytometry. MM cells were quantitated by using the calculated ratio of MM cells and B cells in the sample and the established absolute number of B cells in the same blood sample using the single platform analysis described above.

Flow cytometry was performed using a 3-laser Canto II flow cytometer (Becton Dickinson, Mountain view, CA). Fluorescent labeled beads (CS&T beads, Becton Dickinson) were used to monitor the performance of the flow cytometer and verify optical path and stream flow. This procedure enables controlled standardized results and allows the determination of long-term drifts and incidental changes within the flow cytometer. No changes were observed which could affect the results.

Compensation beads were used to determine spectral overlap, compensation was automatically calculated using Diva software.

MM and lymphoma cell lines and culture

MM cell lines (MOLP-8, LP-1, JIM-3, RPMI-8226, EJM, MM1.S, MM1.R, JIM-1, OPM-2, ARH-77, IM-9, HuNS1, NCI-H929, and KMS-11) and lymphoma cell lines (Daudi, SU-DHL-1, SU-DHL-4, SU-DHL-5, SU-DHL-8, SU-DHL-10 SU-DHL-16, Ramos, WILL-2, DOHH-2, OCI-LY7, OCI-LY18, OCI-LY19, Toledo, HT, MAVER-1,

HBL-1, TMD-8, and Pfeiffer) were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Lonza) and antibiotics (100 units/mL penicillin, 100 µg/ml streptomycin; both Life Technologies) as previously described[55].

CDC assay with MM and lymphoma cell lines

Solvent control, IgG1-b12 control antibody, or daratumumab were added to MM cell lines in medium supplemented with pooled unheated (native) human serum or pooled heat-inactivated (56 ^{o}C for 30 minutes) human serum (10%; Sanquin). After a 1-hour incubation at 37 ^{o}C, cell viability was assessed with CellTiter-Glo assay (Promega), and the lysis of cells was calculated using the following formula: % lysis = 1 - (mean signal in the presence of native human serum / mean signal in the presence of heat-inactivated serum) x 100%.

Bone marrow mononuclear cells

BM mononuclear cells (BM-MNCs) from MM patient BM aspirates were isolated by Ficoll-Hypaque density-gradient centrifugation. BM-MNCs that contained 2-57% CD138+ MM cells as detected by flow cytometry, were used in flow-cytometry-based ADCC and CDC assays.

Flow cytometry-based *ex vivo* ADCC and CDC assays in BM-MNCs

BM-MNCs derived from 32 MM patients, containing 2-57% CD138+ tumor cells, but also autologous effector cells, were used in ADCC and CDC assays. Twenty-one of these 32 patients were subsequently treated with daratumumab monotherapy in the GEN501 study. Sample viability at incubation was more than 98%, as assessed by using ToPro-3 (Invitrogen/Molecular Probes). For ADCC assays, BM-MNCs were incubated in RPMI + 10% fetal bovine serum with control antibody or daratumumab (0.01–10 µg/mL) in 96-well flat-bottom plates for 48 hours.

For CDC assays, BM-MNCs were treated with daratumumab (0.3–10 µg/mL) and 10% pooled human serum as a source of complement for 1 hour prior to flow cytometric analysis. The survival of primary CD138+ MM cells in the BM-MNCs was determined by flow cytometry as previously described[56;57]. In both assays, surviving MM cells were enumerated by single platform flow cytometric analysis of CD138+ cells in the presence of Flow-Count Fluorospheres (Beckman Coulter) and ToPro-3 to determine absolute numbers of viable MM cells. The percentage of daratumumab-mediated ADCC was then calculated using the following formula: % lysis cells = 1 - (absolute number of surviving CD138+ cells in the presence of daratumumab / absolute number of surviving CD138+ cells in the presence of control antibody) x 100%. Complement-dependent lysis was calculated using the following formula: % lysis = 1 - (absolute number of surviving CD138+ cells in the presence of native human serum / absolute number of surviving CD138+ cells in the presence of heat-inactivated serum) x 100%.

Phospholipase-C treatment

Cells were treated with phospholipase-C (6 U/ml) for 30 minutes at 37 ^{o}C. After three wash steps, CD38, CD55 and CD59 expression levels were determined by flow cytometry, and daratumumab-mediated CDC was determined by using the CellTiter-Glo luminescent cell viability assay (Promega).

Immunophenotyping by flow cytometry of in vitro experiments

Expression of several cell surface proteins on cell lines was determined by flow cytometric analysis using FITC-, PE-, Per-CP-, or APC-conjugated monoclonal antibodies. Anti-CD38, anti-CD138, and anti-CD56 were purchased from Beckman Coulter; anti-CD3, anti-CD16, anti-CD55, anti-CD59 from BD Biosciences; and anti-CD46 from Biolegend. Flow cytometry was done using a FACS-Calibur device (Becton Dickinson); the data were analyzed using the CellQuest software.

Complement measurements

Complement assays were performed at baseline and at regular time points after initiation of daratumumab therapy in the MMY2002 study at the National Jewish Laboratory (Denver, CO). C2 and C1q levels were measured in serum by radial immunodiffusion (RID) using polyclonal anti-C2 or C1q. Specimens were pipetted into duplicate wells cut into the agarose, placed in a covered humidified box in the cold, and allowed to diffuse into the gel to form immunoprecipitation rings around the application well. The gels were washed to remove unprecipitated proteins, dried and stained. The area of the precipitin ring (minus the area of the well) was proportional to the amount of antigen that was present in the test specimen. The outside diameter of the ring of precipitated antigen (C2 or C1q) was measured and recorded.
Then the inner ring (hole) diameter was measured and recorded. From these two measurements the area of precipitation was calculated using A =π2. The total area of the precipitin is given by (Aprecipitin) = (Aouter ring) - (Ainner ring).
Linear regression was used to determine the parameters of the best-fit line, with the values for Aprecipitin used for the dependent variable and the known concentration of the standard specimens as the independent variable. The value of each individual specimen was calculated from its Aprecipitin value. The results were reported as percent of the standard, defined as 100%. C3 and C4 levels were measured by endpoint nephelometry. The specimen to be tested was mixed with a fixed concentration of excess polyclonal antibodies to the analyte of interest. This leads to the formation of large antigen-antibody immune complexes. A diode laser light source that emits at 670 nm was passed through the cuvette containing the reaction mixture. These immune complexes scatter light and the amount of the scattered light was proportional to the amount of complex formed and was detected by the photodiode. For each specimen to be assayed, a scatter reading was taken at the beginning of the antibody/antigen reaction (blank or 1st reading), followed by a second scatter reading at a fixed time. The analyte concentration was calculated using the difference between these two readings.

Statistics

Comparisons between variables were performed using two-tailed (paired) Student's t-test, or Mann-Whitney U test or Wilcoxon matched-pairs signed-rank test in case the data do not follow a normal distribution. Correlations between variables were made using the Spearman's rank correlation coefficient. P-values below 0.05 were considered significant.

Supplementary

Table 1

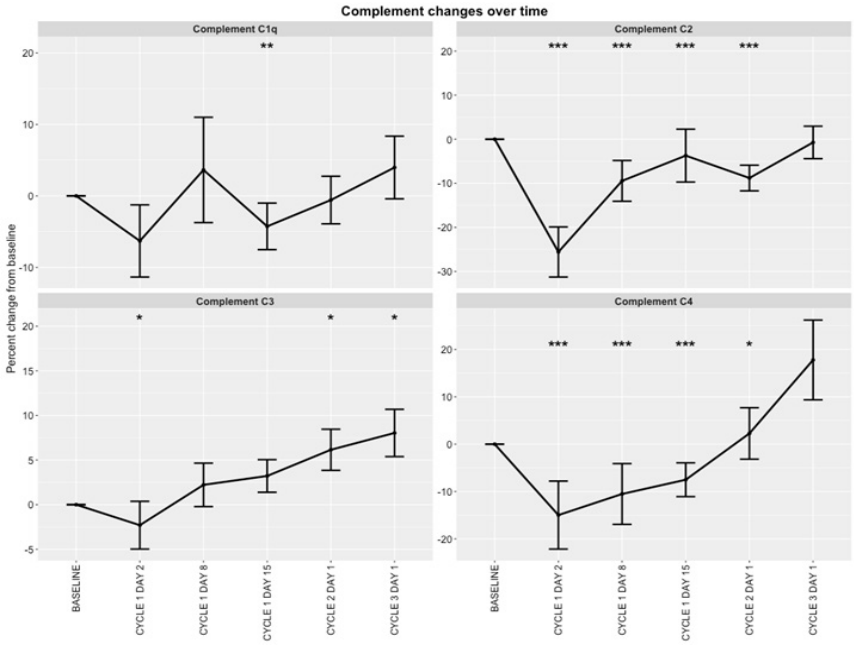
Patient characteristics

Parameter	MM patients n=32
Median age, years (range)	63 (43-75)
Sex, male, n (%)	22 (71%)
M-protein type	
IgG, n (%)	9 (28%)
IgA, n (%)	8 (26%)
IgM, n (%)	0 (0%)
IgD, n (%)	2 (6%)
Light chain only, n (%)	13 (42%)
Previous therapy	
Newly diagnosed MM, n (%)	2 (6%)
Relapsed/refractory MM, n (%)	30 (94%)
≤ 3 lines of therapy	16 (53%)
> 3 lines of therapy	14 (47%)
Prior therapy to which disease was refractory* before start of daratumumab	
lenalidomide refractory, n (%)	24 (80%)
bortezomib refractory, n (%)	21 (70%)
lenalidomide and bortezomib refractory, n (%)	19 (63%)
pomalidomide refractory, n (%)	3 (10%)
carfilzomib refractory, n (%)	1 (3%)

Abbreviations: n, number.

*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.

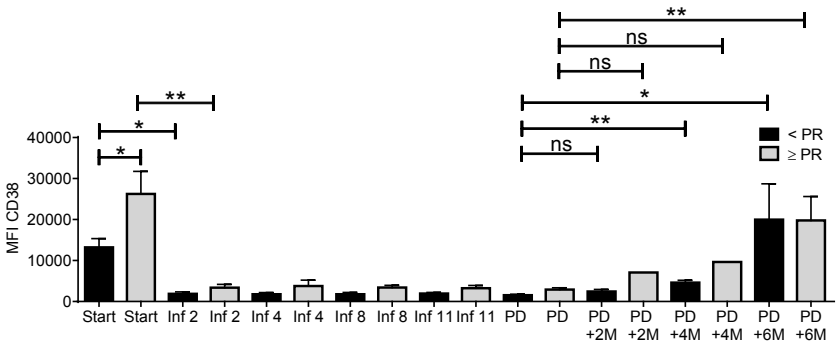
Supplementary
FIGURE 1



Supplementary Figure 1. Changes in complement factor levels during treatment with daratumumab.
Levels of the complement factors C1q, C2, C3, and C4 in peripheral blood were analyzed in samples obtained before the first daratumumab infusion and at regular timepoints during daratumumab treatment. Daratumumab was administered once weekly during cycles 1 and 2 (on days 1, 8, 15, and 22 of a 28-day cycle). Shown is the percent change from baseline in complement factors. Data are presented as mean ± s.e.m. Asterisks denote significant changes from baseline as calculated with ANOVA; * P<0.05, ** P<0.01, *** P< 0.001.

Supplementary

FIGURE 2



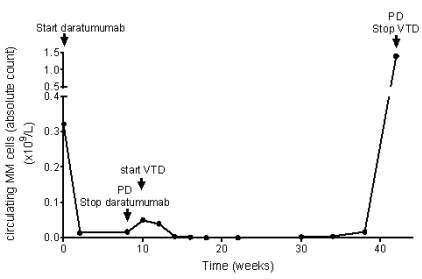
CD38 expression levels decrease on circulating MM cells irrespective of response to daratumumab therapy.

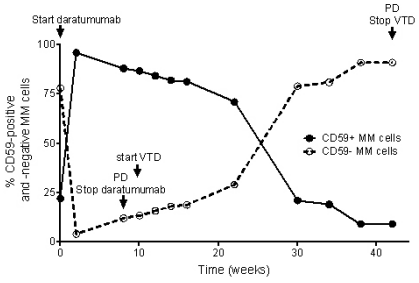
Longitudinal data representation of CD38 expression on circulating MM cells according to best response achieved in the GEN501 study (PR or better versus less than PR).

Peripheral blood was obtained from 11 patients during treatment with daratumumab, at the time of progression (PD), as well as 2, 4, and 6 months after development of progressive disease (PD). CD38 expression was determined by using HuMax-003-FITC, which binds to a different epitope compared to daratumumab, thereby excluding the possibility that binding of daratumumab masked the detection of CD38. Data are presented as mean ± s.e.m. P-values between the indicated groups were calculated using a Student's t-test; * P<0.05, ** P<0.01, ns not significant; PR, partial response.

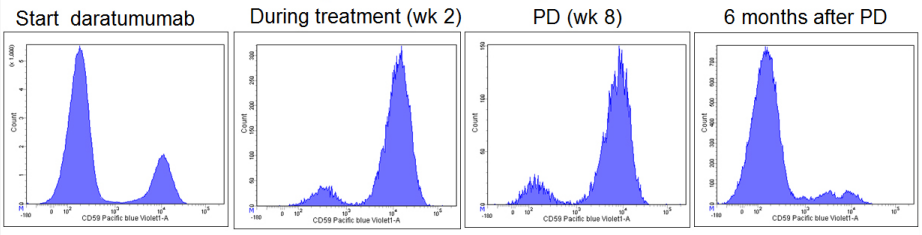
Supplementary

FIGURE 3

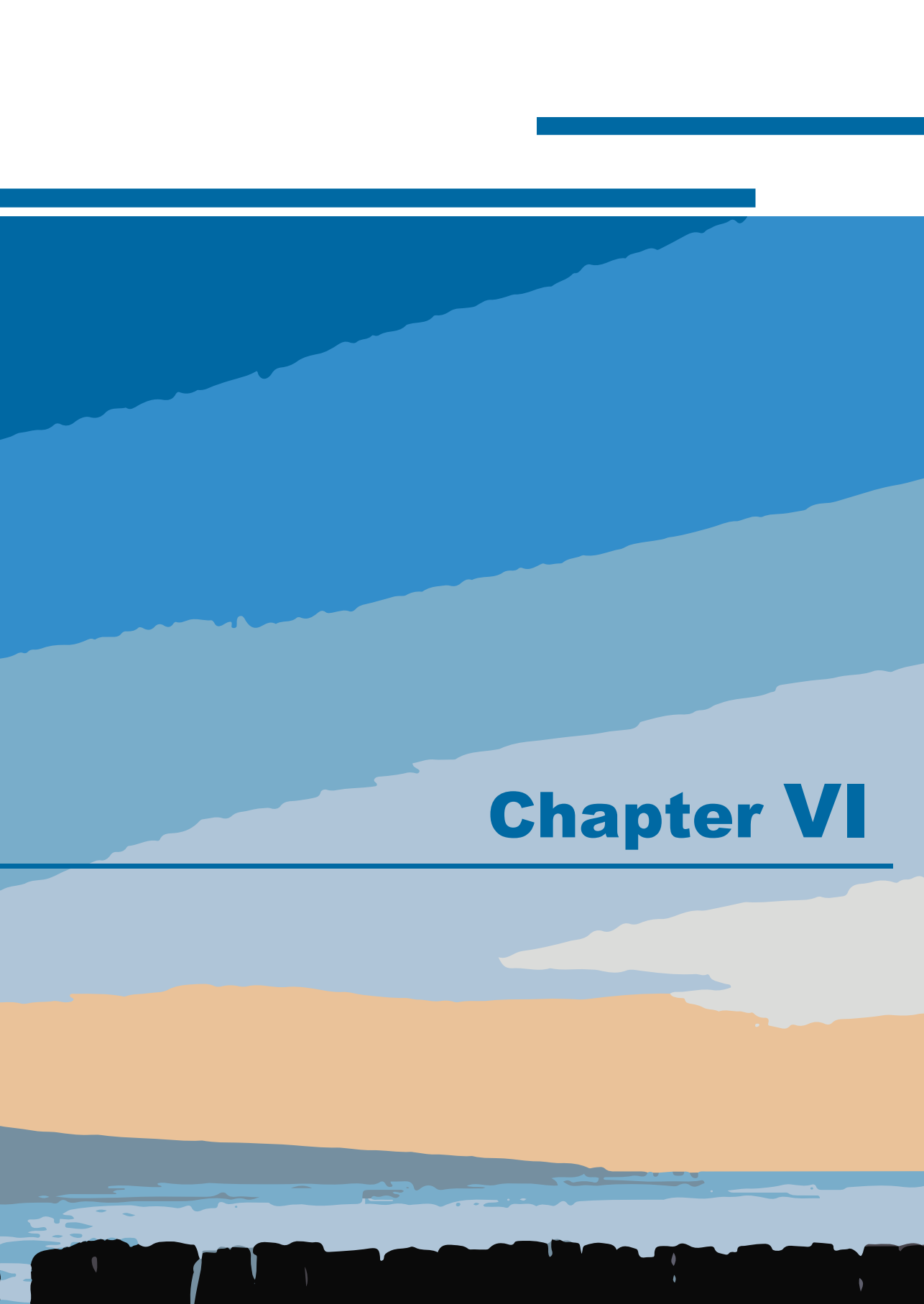




CD59 expression on circulating MM cells:



Selection of MM cells with high CD59 expression during therapy with daratumumab. Patient 6 had two coexisting populations of MM cells based on differential expression of CD59. Serial blood samples were obtained from this patient during daratumumab monotherapy and during the treatment given after development of progressive disease (PD). Longitudinal data representation of absolute MM cell counts over time, and of the frequency of the different subpopulations based on CD59 expression. Representative histograms are also shown for patient 6. Abbreviations: VTD, bortezomib, thalidomide, and dexamethasone.


Chapter VI

Daratumumab Depletes CD38+ Immune-regulatory Cells, Promotes T-cell Expansion, and Skews T-cell Repertoire in Multiple Myeloma

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Abstract

Daratumumab targets CD38-expressing myeloma cells through a variety of immune-mediated mechanisms (complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent cellular phagocytosis) and direct apoptosis with cross-linking. These mechanisms may also target non-plasma cells that express CD38, which prompted evaluation of daratumumab's effects on CD38-positive immune subpopulations.

Peripheral blood (PB) and bone marrow (BM) from patients with relapsed/refractory myeloma from two daratumumab monotherapy studies were analyzed before and during therapy and at relapse. Regulatory B cells (Bregs) and myeloid-derived suppressor cells (MDSCs), previously shown to express CD38, were evaluated for immuno-suppressive activity and daratumumab sensitivity in the myeloma setting. A novel subpopulation of regulatory T cells (Tregs) expressing CD38 was identified. These Tregs were more immunosuppressive *in vitro* than CD38-negative Tregs and were reduced in daratumumab-treated patients. In parallel, daratumumab induced robust increases in helper and cytotoxic T-cell absolute counts. In PB and BM, daratumumab induced significant increases in CD8+:CD4+ and CD8+:Treg ratios, and increased memory T cells while decreasing naïve T cells. The majority of patients demonstrated these broad T-cell changes, although patients with a partial response or better showed greater maximum effector and helper T cell increases, elevated antiviral and alloreactive functional responses, and significantly greater increases in T-cell clonality as measured by T-cell receptor (TCR) sequencing. Increased TCR clonality positively correlated with increased CD8+ PB T-cell counts.

Depletion of CD38+ immune suppressive cells, which is associated with an increase in T-helper cells, cytotoxic T-cells, T-cell functional response, and TCR clonality, represent possible additional mechanisms of action for daratumumab and deserve further exploration.

Introduction

Proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) have improved outcomes in patients with multiple myeloma (MM).[1–3]
Despite these advances, prognosis for patients with relapsed MM remains poor, particularly for those who have relapsed after PI and IMiD treatment.[4] New therapies with novel mechanisms of action are needed for resistant patient populations.
Myeloma is associated with immune dysfunction,[5] including immune evasion through expression of immune checkpoint ligands on plasma cells,[6] elevated adenosine receptor and adenosine activity,[7,8] and immune suppression through myeloid-derived suppressor cells (MDSCs) and regulatory T cell (Treg) activity.[9–11] CD38 is ubiquitously expressed on MM cells,[12,13] but is also present on other immune cells, including MDSCs and regulatory B cells (Bregs).[14,15] These CD38-positive (CD38+) immune-suppressive cell populations are associated with decreased immune function and disease progression. Thus, the role of CD38 in myeloma and immune cell biology may be important for the treatment of disease.
Daratumumab is a human immunoglobulin G1 (IgG1) monoclonal antibody that targets CD38, inducing tumor cell death through multiple mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity, and antibody-dependent cellular phagocytosis (ADCP).[16,17]
Daratumumab has shown promising anti-myeloma activity in two clinical studies (GEN501 and SIRIUS) in patients with relapsed and refractory MM, resulting in remarkable response rates that include stringent complete responses (sCRs) and prolonged clinical responses in heavily pretreated patients.[18,19] Based on these data, daratumumab was approved by the US Food and Drug Administration for patients with multiple myeloma who have received at least three prior lines of therapy, including a PI and an IMiD agent or who are double refractory to a PI and an IMiD.[20]
The observation that CD38 is expressed on various immune cells prompted an evaluation of the potential immunomodulatory effects of daratumumab monotherapy in patients with relapsed or relapsed refractory MM. The impact of daratumumab on CD38+ immune-suppressive populations, T-cell proliferation and activation, and T-cell receptor (TCR) clonality was evaluated.

Methods

Clinical Study Design

Immune profiling and assessments of functional activity were performed in samples from patients with relapsed or refractory MM treated with 16-mg/kg daratumumab monotherapy and who were enrolled in two concurrent clinical studies (ClinicalTrials.gov Identifiers: NCT00574288 [GEN501] and NCT01985126 [SIRIUS]) that have been described in detail elsewhere.[18,19]
Briefly, in the GEN501 study, a phase 1/2 dose escalation and expansion study, patients had documented MM and had relapsed from or were refractory to at least

two prior therapies.[18] In SIRIUS, a phase 2 study, patients had received more than three prior therapies, including a PI or an IMiD, or were refractory to both classes of agents.[19] Patients enrolled in these studies also received low to intermediate doses of corticosteroids to manage infusion-related reactions before and after daratumumab dosing.

Best overall clinical responses were determined using the International Myeloma Working Group consensus recommendation for MM treatment response criteria.[21] Patients were grouped into responders (i.e., patients with best overall responses of partial response [PR], very good PR [VGPR], complete response [CR], or stringent CR) and nonresponders (ie, patients with a best response of minimal response, stable disease, or progressive disease).

The investigators and sponsors were responsible for the study design and statistical analysis plan. The investigators and their research teams collected the data.

Janssen Research and Development and Genmab compiled the data for summation and analysis and confirmed the accuracy of the data. All the investigators had full access to the data and analyses and were not restricted by confidentiality agreements. Ethics committee or institutional review boards at each study site approved the study protocols and the statistical analysis plans. The studies were conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. All the patients provided written informed consent.

Blood cell collection

Peripheral blood and bone marrow samples were collected in heparinized tubes at baseline, immediately prior to the first infusion, and at specified time points during treatment. The majority of samples were evaluated using real-time flow cytometry, as they arrived at a central laboratory, [24–48] hours after collection.

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood, isolated by density-gradient centrifugation, and stored frozen until analysis. For the T-cell activation and clonality assays, pre- and post-treatment samples were analyzed at the same time, using frozen PBMC samples.

Immune cell phenotyping and quantification

Blood and bone marrow samples were stained with the following multifluorochrome antibody panels; cell lineage panel: PerCPCy5.5α-CD19 (cloneHIB19; Becton Dickinson [BD]), APCα-CD24 (SN3; eBioscience), PC7α-CD3 (UCHT-1; Beckman Coulter), V500α-CD16 (3G8; BD), and PEα-CD56 (MY; BD); regulatory T cell (Treg) panel: APCα-CD25 (2A3; BD), PEα-CD127 (HIL-7R-M21; BD), APC-H7α-HLA-DR (G46-6; BD), and PerCPα-CD4 (L200; BD); naive/memory T-cell panel: APC-H7α-CD4 (RPA-T4; BD), PerCP-Cy5.5α-CD8 (RPA-T4 BD), PEα-CD62L (SK11; BD), and APCα-CD45RA (HI100; BD). In addition, all samples were stained with FITCα-CD38 (HuMax-003; Genmab/Janssen Research and Development), an antibody that binds to an epitope distinct from the epitope bound by daratumumab.[22] Blood samples were lysed with FACSTM lysing solution (BD), and FIX and PERM® cell permeabilization reagent (Invitrogen) was used for bone marrow aspirates.

Analysis was performed using FACS Canto II flow cytometers, and data were analyzed using FACSDiva software. Absolute cell counts were calculated for peripheral blood samples, and immune cell subpopulations were represented as a percentage of total lymphocytes for bone marrow samples.

MDSC phenotyping and daratumumab-mediated ADCC lysis

PBMCs from three normal healthy donors were co-cultured with myeloma tumor cell lines (RPMI8226, U266, H929) for 6 days, and evaluated for the production of granulocytic-like MDSCs (G-MDSCs; CD11b+CD14–HLA–DR–CD15+CD33+), as previously described.[23] G-MDSCs were not present in normal healthy PBMCs; however, following co-culture with all three myeloma cells lines, G-MDSCs were present as 5%-25% of the total PBMC population (data not shown). Gating strategy for flow cytometric evaluation of G-MDSC included CD11b+ as the first gate, followed by CD14– and HLA–DR– gating, and then followed by CD15+ and CD33+ gating. G-MDSCs were cell sorted and evaluated for CD38 expression levels and sensitivity to daratumumab-mediated ADCC.[16] To evaluate the effect of daratumumab on ADCC/CDC of MDSCs, serum containing complement or an isotype control was added to ADCC assays.

Naive and memory cell analysis

Heparinized peripheral blood samples were obtained from patients prior to each infusion of daratumumab. PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation and stored in a cryopreservation medium (RPMI supplemented with 10% human serum and 10% dimethyl sulfoxide) in liquid nitrogen.
For FACS analysis, PBMCs were thawed, and 2×106 cells/panel were resuspended in phosphate-buffered saline with 0.05% sodium azide and 0.1% HAS.

T-cell clonality

DNA from frozen patient PBMCs was assessed for TCR rearrangements using an ImmunoseqTM assay (Adaptive Biotechnologies),[24] and analysis was performed using prequalified multiplex polymerase chain reaction (PCR) assays (TR2015CRO-V-019), which were composed of forward and reverse primers that directly targeted the family of variable (V) genes (forward primers) and joining (J) genes (reverse primers). Each V and J gene primer acted as priming pairs to amplify somatically recombined TCRs, and each primer contained a specific universal DNA sequence. Following the initial PCR amplification, each amplicon was amplified a second time with forward and reverse primers containing the universal sequence and adaptor sequence needed for DNA sequencing by Illumina.

T-cell responses to viral and alloantigens

Patient PBMCs were seeded on 96 well plates (2×105 cells/well) and stimulated for 5 days with a cocktail of 23 major histocompatibility complex class I–restricted viral peptides from human cytomegalovirus, Epstein-Barr virus, and influenza virus (2 µg/mL; CEF peptide pool; PANATecs®) or an equivalent number of 25-Gy–irradiated allogeneic PBMCs from healthy donors. Unstimulated PBMCs and PBMCs stimulated with anti-CD3/CD28–coated beads served as negative and positive

controls, respectively. On Day 5, interferon γ (IFN-γ) from cell-free supernatant was measured by sandwich enzyme-linked immunosorbent assay (ELISA; Human IFN gamma ELISA Ready-SET-Go; eBioscience) and served as a surrogate marker for T-cell activation.

Carboxyfluorescein succinimidyl ester dilution assay

PBMCs from healthy donors were labeled with PerCP-Cy5.5α-CD3 (SK7; BD), KOα-CD45 (J33; Beckman Coulter), V450α CD4 (SK3; BD), PEα-CD25 (M-A251, BD), PE Cy7α-CD127 (HIL-7R-M21; BD), and APCα-CD38 (HB-7; BD) and sorted by FACS Aria (BD). Sorted effector cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; eBioscience) and stimulated with anti-CD3/CD28–coated beads in the presence or absence of CD38+ or CD38− Tregs (1:1 Treg to effector cell ratio) in RPMI plus 10% fetal calf serum. After 72 hours, flow cytometry was performed and the percent dilution of CFSE was used as a surrogate for T-cell proliferation.

Statistical Analyses

The patient population was defined as all patients who had an evaluable response and received more than one dose of daratumumab, from whom more than one on treatment sample had been collected. All data analyses and output generation were performed using the programing language R, with all reproducible code contained in an R package. Modeling of the populations of absolute T-cell counts in peripheral blood was presented on a log scale as a function of the relative visit only using linear mixed-effect models[25,26] with a random intercept and slope. In a separate analysis using mixed modeling, associations between time and response were assessed as well. Baseline measurements were mapped relative to Day 1. The normality of the data was evaluated using a Shapiro-Wilk test for two-group comparisons.[27] If the data were not normally distributed, all two-group comparisons were evaluated using rank tests and a t test, following a Box-Cox transformation. In all cases, the results from the t test and rank tests agreed; thus, only the P values of the rank tests were reported. Differences between the sums of absolute expansion in the T-cell clone repertoire, and the maximum expansion of a single clone between responders and nonresponders were evaluated using a Wilcoxon rank-sum test.[28] Paired Wilcoxon signed-rank tests were used to evaluate CD8+:CD4+ cell ratio changes over time, differences between T-cell clonality at baseline compared with on-treatment, and CD8+-naive T cells prior to the eighth infusion compared with samples at time of relapse, and to compare CD8+ effector memory T cells at baseline and at relapse.[28] For the analysis of CD8+:CD4+ cell ratios, only data from patients with observations at all three (for peripheral blood) or two (for bone marrow) time points were included. The timing of bone marrow aspiration/biopsy varied by a few days from patient to patient and, for this reason, all on-treatment visits were grouped into a single “on-treatment” time point. If a patient had multiple on-treatment bone marrow measurements, the last on-treatment data point was used. Experimental data were aligned by mapping the relative study day to fixed week windows (Supplementary Appendix).

The percent changes of HLA-DR T cells, CD8-naive cells, and CD8 effector memory

cells were modeled in function over time, as categorical variables, using linear mixed-effect models with a random intercept and slope, with or without response variables.[25,26]

Results

Clinical Findings

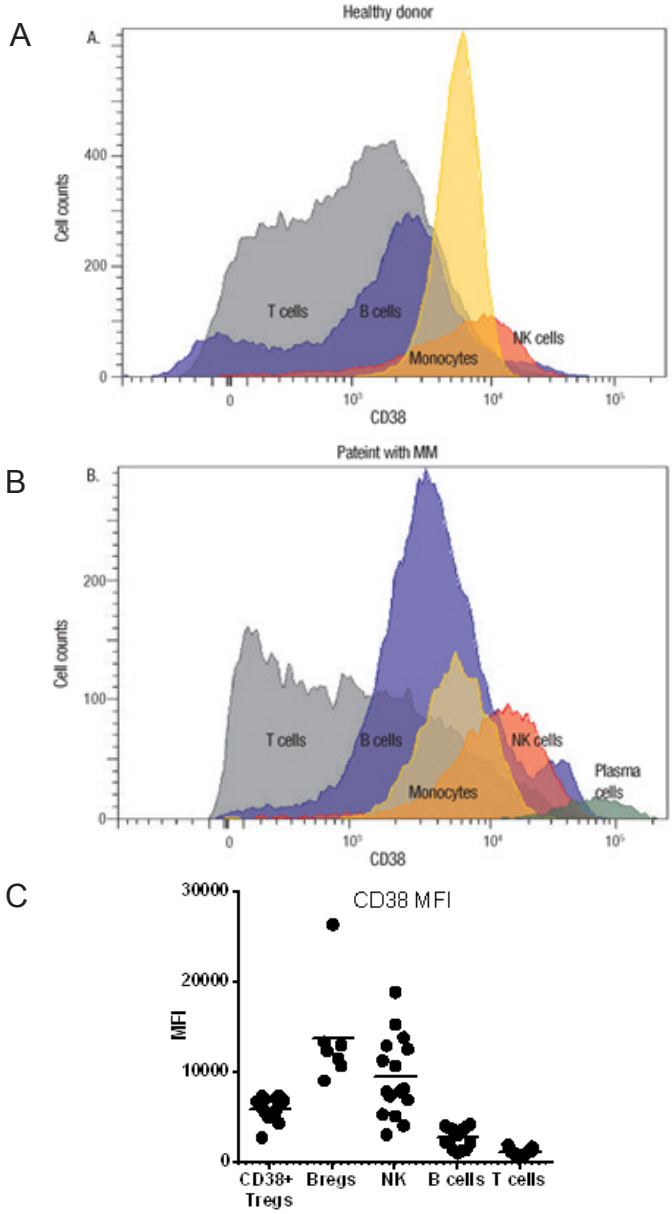
To assess the effects of daratumumab on CD38+ immune subpopulations, response evaluable peripheral blood and bone marrow samples from a total of 148 patients with myeloma who received daratumumab in the GEN501 (n = 42) and SIRIUS (n = 106) studies were analysed by flow cytometry, functional assays, and TCR sequencing. The median (range) age of the patients and time since diagnosis were 64 (31-84) years and 5.12 (0.77-23.77) years, respectively. Fifty-three percent of patients were men. The population was heavily pretreated and 76% of patients had received more than three prior lines of therapy, 91% were refractory to their last line of treatment, and 86% were refractory to both a PI and an IMiD. Additional patient characteristics for this combined data set are summarized in Table S1 in the Supplementary Appendix. The overall response rates were 36% in the GEN501 study[18] and 29% in the SIRIUS study.[19]

Effect of Daratumumab on Regulatory Cells Expressing CD38

We confirmed the previously reported observation that CD38 was highly expressed on plasma cells of patients with myeloma,[12] but was also present on natural killer (NK) cells, monocytes, B cells, and T cells in PBMCs of healthy donors (Figure 1A) and patients with myeloma (Figure 1B). A comparison of the mean fluorescent intensity (MFI) of CD38 across these cellular populations, showed that after plasma cells, NK cells expressed the highest levels of CD38, followed by subpopulations of B and T cells (Figure 1C). Given recent literature that indicated regulatory and suppressive immune populations like MDSCs and Bregs can express CD38,[14,15] we evaluated these immune cells for their response to daratumumab treatment. Since MDSCs were not readily detectable in frozen PBMC samples,[29] G-MDSCs (CD11b+CD14–HLA–DR–CD15+CD33+) were generated *in vitro* using a previously described co-culture model.[23] These G-MDSCs expressed elevated CD38 and were highly sensitive to daratumumab-mediated ADCC/CDC compared to an isotype control (Figure 2A & B). Bregs (CD19+CD24+CD38+) were measured in daratumumab-treated patients (n = 16; Figure 2C). These Bregs produced IL-10 (Figure 2D), were reduced following the first dose of daratumumab (P = 0.0018 at Week 1 compared with baseline), and remained low while patients were on treatment (Figure 2E).Given the high levels of CD38 expression in these immune suppressive populations, we evaluated regulatory T cells for expression of CD38 and their sensitivity to daratumumab. A novel subpopulation (10% ± 10%) of peripheral Tregs (CD4+CD25+CD127dim) expressed high levels of CD38 prior to activation (Figure 3A). These CD38+ Tregs were highly sensitive to daratumumab treatment and exhibited a significant and almost immediate decline following the first dose of daratumumab (n = 17 patients; P = 8.88×10−16 at Week 1 versus baseline).

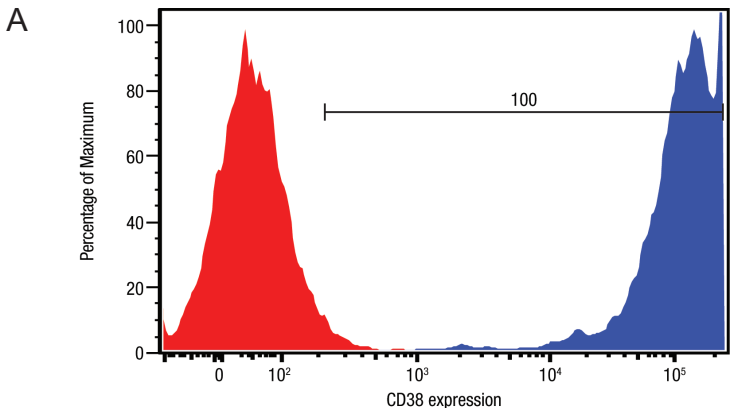
These CD38+ Tregs remained depleted throughout daratumumab treatment

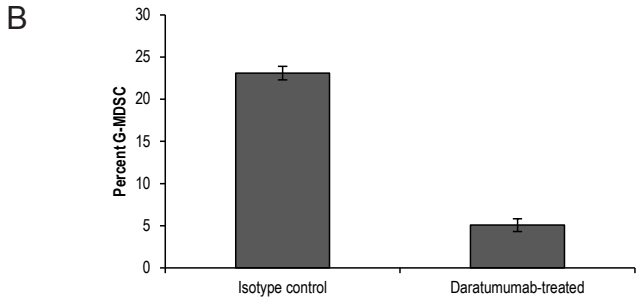
FIGURE 1



CD38 Expression in Various Cell Populations.
Histograms show expression of CD38 in T cells, B cells, monocytes, NK cells, in a healthy donor (A) and in a patient with multiple myeloma (B). CD38 expression in plasma cells from the myeloma patient is also shown. Subpopulations of NK cells, regulatory T cells (Tregs), regulatory B cells (Bregs), and MDSCs from relapsed and refractory myeloma patients expressed higher levels of CD38 compared to CD8+ and CD4+ T cells (C). NK, natural killer; MFI, mean fluorescent intensity; Tregs, regulatory T cells; Bregs, regulatory B cells.

FIGURE 2 A/B/C


A
Percentage of Maximum
100
80
60
40
20
0
100
0
10^2
10^3
10^4
10^5
CD38 expression


B
Percent G-MDSC
30
25
20
15
10
5
0
Isotype control
Daratumumab-treated

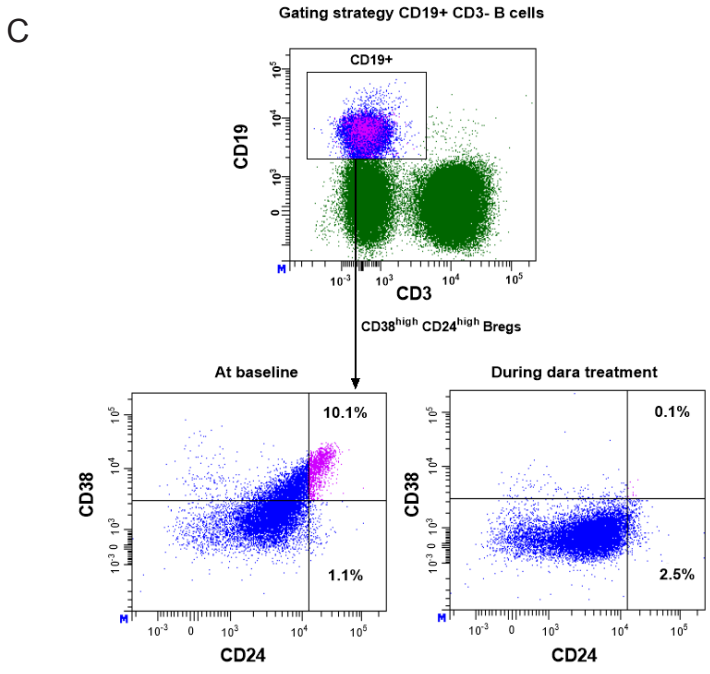
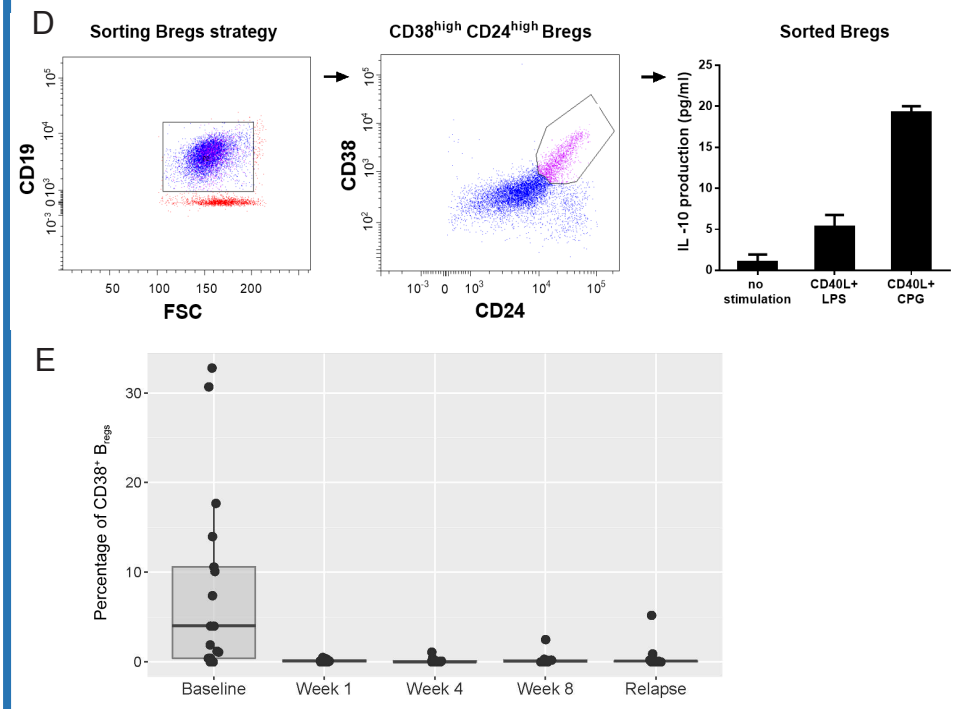

C
Gating strategy CD19+ CD3- B cells
CD19+
CD19
CD3
$CD38^{high}$ $CD24^{high}$ Bregs
At baseline
During dara treatment
10.1%
1.1%
0.1%
2.5%
CD38
CD24

FIGURE 2 D/E



Immunosuppressive Cells Express CD38 and are Sensitive to Daratumumab. Granulocytic-like MDSC (CD11b+CD14–HLA–DR–CD15+CD33+) were generated from six day co-cultures with healthy donor PBMC and myeloma cell lines as previously described. G-MDSC were sorted and evaluated for CD38 expression (red represents the isotype control and blue indicates the CD38+ staining; A) and sensitivity to isotype or daratumumab-mediated ADCC/CDC (B). Regulatory B cells (Bregs) express CD38 (C) and, when stimulated, produced IL-10 (D); CD38+ Bregs decreased and remained depleted following daratumumab treatment (E).

(P = 8.88×10-16, 1.11×10−15, and 1.50×10−11 at Weeks 1, 4, and 8, respectively, versus baseline; Figure 3B). In these studies, CD38 evaluation was performed using HuMax-003. It was confirmed that daratumumab did not interfere with the binding of HuMax-003 to CD38 that was expressed on total PMBCs, lymphocytes, monocytes, NK cells or a CD38+ MM cell line. (Figure S1 in the Supplementary Appendix).
To assess the possible biological relevance of CD38 expression in Tregs, we measured the suppressive capacity of CD38+ Tregs versus CD38− Tregs on autologous CD3+ T cells. In a series of experiments performed with samples from multiple healthy donors, CD38+ Tregs suppressed T-cell proliferation more robustly than CD38− Tregs or negative controls (Figure 3C).

Daratumumab induces helper and cytotoxic T-cell expansion

Given the reduction in CD38+ Tregs, Bregs, and MDSCs, we evaluated other

FIGURE 3[A/B]

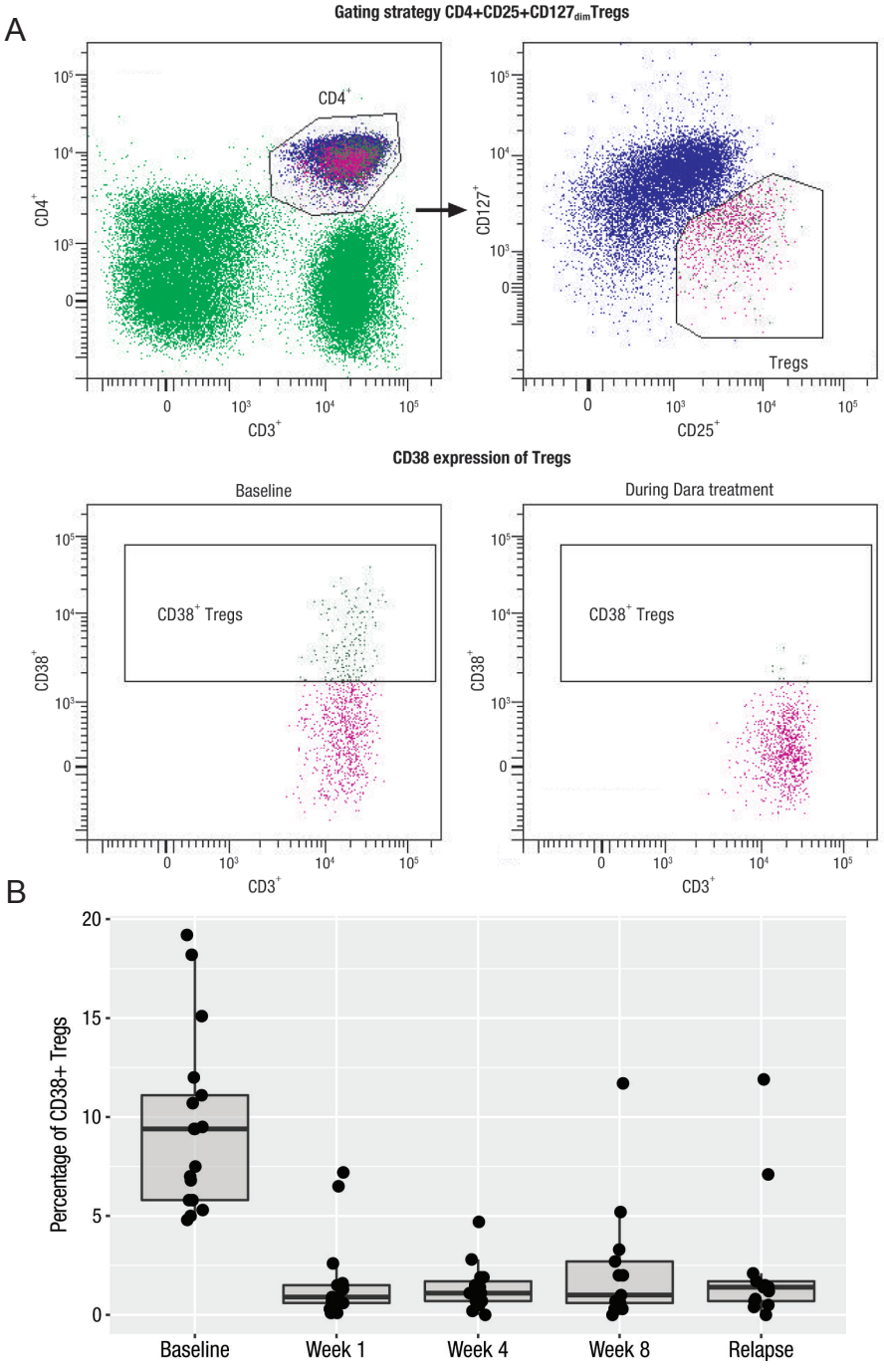
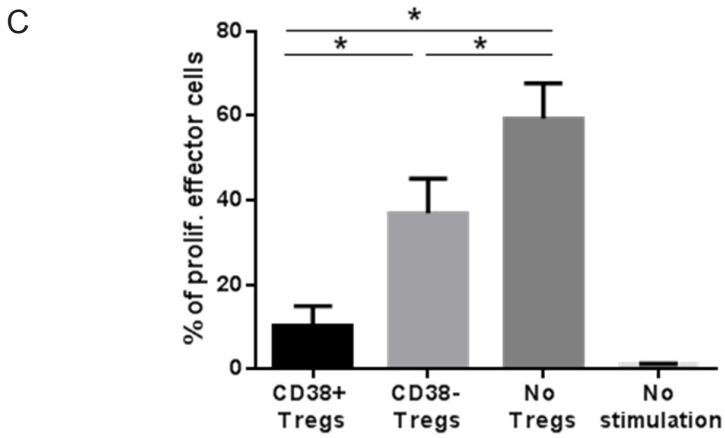

A
Gating strategy CD4+CD25+CD127dimTregs
CD4+
CD3+
CD127+
CD25+
Tregs
CD38 expression of Tregs
Baseline
During Dara treatment
CD38+ Tregs
CD38+ Tregs
CD38+
CD3+
B
Percentage of CD38+ Tregs
20
15
10
5
0
Baseline
Week 1
Week 4
Week 8
Relapse

FIGURE 3[C]



In a representative experiment, the gating strategy of Treg cells is shown in (A, upper panels); 18.2% of Tregs expressing CD38 were depleted after the first daratumumab infusion (A, lower panels). CD38+ Tregs were reduced and remained low during Weeks 1, 4, and 8 of daratumumab therapy (B). In experiments performed on samples from multiple healthy donors, cell proliferation was assessed through the dilution of carboxyfluorescein succinimidyl ester (CFSE) dye due to cell division; strong inhibition of cell division was observed in the presence of CD38+ Tregs, in contrast to the partial inhibition of proliferation observed in the presence of CD38− Tregs or negative controls (C). Error bars represent standard error. Asterisks denote significant changes.

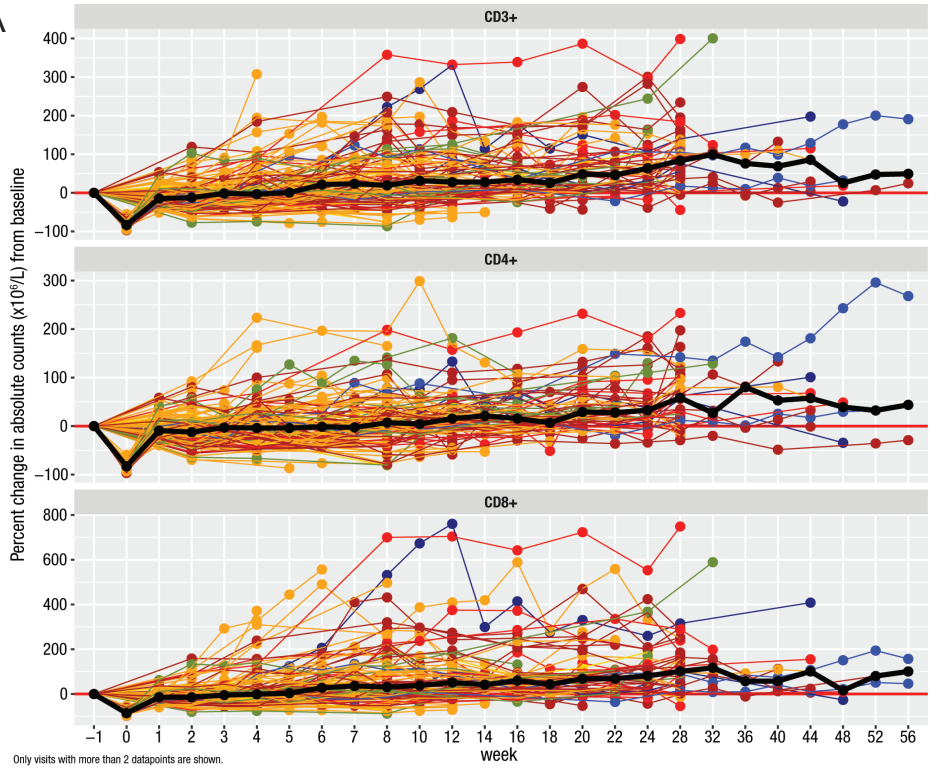
lymphocyte populations. NK cells expressed the highest level of CD38 and were reduced with daratumumab treatment (manuscript in preparation). B cells (CD19+) were maintained with daratumumab treatment, and did not change significantly (data not shown). Interestingly, absolute counts for peripheral total CD3+ T cells, as well as CD4+ and CD8+ T-cell subsets, increased significantly with daratumumab 16-mg/mg treatment (CD3+: 0.16 [95% confidence interval (CI) = 0.14-0.19]; CD4+: 0.12 [95% CI = 0.1-0.14]; CD8+: 0.21 [95% CI = 0.17-0.25] on log scale per 100 days) equivalent to an average increase of approximately 44%, 32%, and 62%, respectively, per 100 days (Figure 4A). Similar T-cell increases were observed in bone marrow, with median maximal (range) increases from baseline in CD3+, CD4+, and CD8+ T-cell percentages of lymphocytes of 19.95% (−40.40% to 121.60%), 5.66% (−60.70% to 125.50%), and 26.99% (−10.89% to 187.90%), respectively (Figure 4B).

Ratios of CD8+:CD4+ and CD8+:Tregs were evaluated at baseline and at Weeks 8 and 16 of treatment in the peripheral blood (Figure 5A) and at baseline and on treatment (Week 12 ±1 cycle) in bone marrow (Figure 5B). The CD8+:CD4+ and CD8+:Treg ratios increased significantly over time in peripheral blood and bone marrow with daratumumab treatment; no significant differences in CD8+:CD4+ or CD8+:Treg ratio were observed between responders and nonresponders.

In peripheral blood, the median (range) ratio of CD8+:CD4+ T cells (n = 58)

FIGURE 4

A



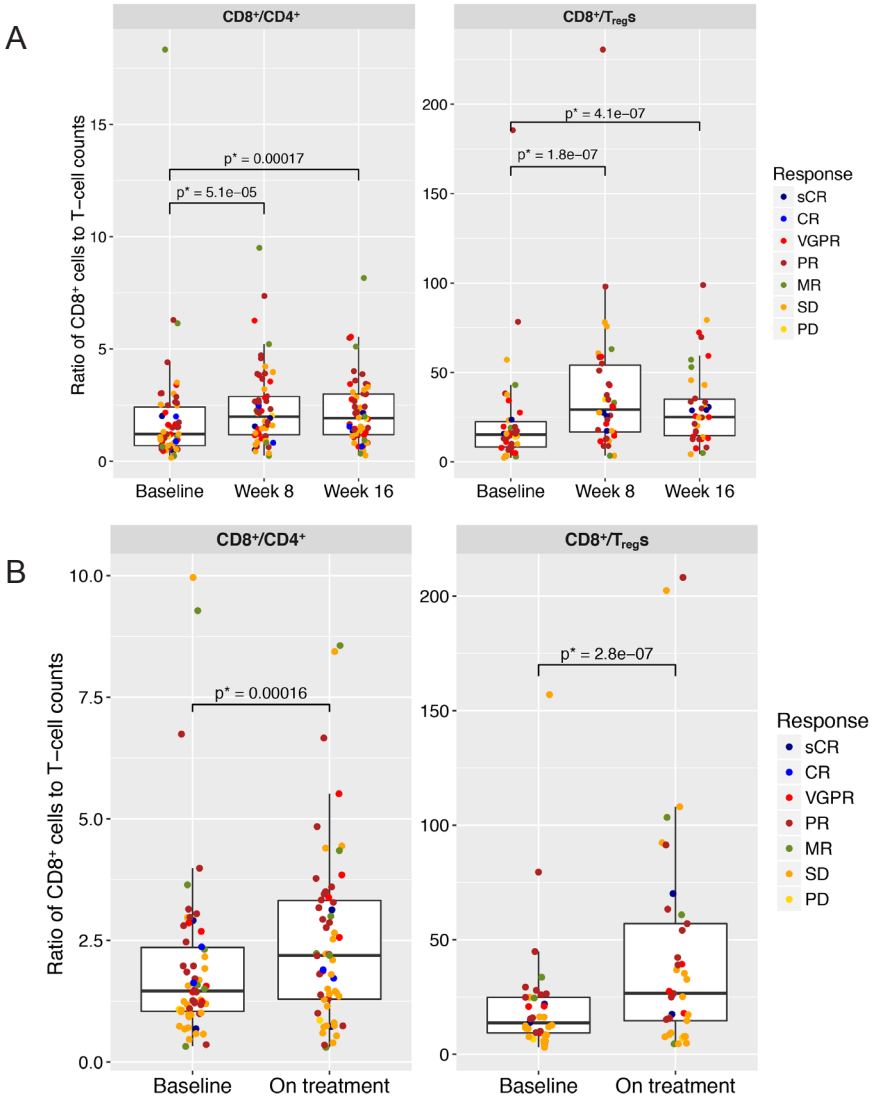
B

	CD45+CD3+ (*)	CD45+CD3+CD4+ (*)	CD45+CD3+CD8+(*)
Min	-40.4	-60.7	-10.89
1st Q	12.13	-8.666	14.58
Median	19.95	5.66	26.99
Mean	29.28	13.42	39.09
3rd Q	47.65	25.34	53.71
Max	121.6	125.5	187.9

* Percent changes to baseline at on-treatment visit of T cells as a percentage of lymphocytes of 58 subjects.

Effect of Daratumumab Treatment on Helper and Cytotoxic T Cell Counts.
Longitudinal data representation of the percent change from baseline of absolute CD3+, CD4+, and CD8+ T-cell counts over time in peripheral blood (A); lines represent connected data points of individual patients that are colored by best overall clinical response; Blue represents sCR, orange VGPR, brown PR, yellow SD, green MR. The black bold line shows the overall median percent change over time. Only visits with more than two data points are shown. Median (range) percent change from baseline in bone marrow T cells (as a percentage of lymphocytes) of daratumumab-treated patient is shown in (B).

FIGURE 5



Ratio of CD8+ to CD4+ or Tregs Over Time.
Median ratios of CD8+:CD4+ (A, left panel; n = 58) and CD8+:Tregs (A, right panel; n = 38) increased at Weeks 8 and 16 compared to baseline in the peripheral blood. Only patients with baseline, Week 8, and Week 16 measurements were included. Similarly, in bone marrow, median ratios of CD8+:CD4+ (B, left panel) and CD8+:Tregs (B, right panel) increased on treatment (Week 12 ±1 cycle) compared to baseline. No significant differences were observed between responders and nonresponders. CR, complete response; PR, partial response; sCR, stringent CR; SD, stable disease; VGPR, very good PR.calculated as indicated in materials and methods. Data are presented as mean ± SEM. P-values between the indicated groups were calculated using a paired student's t test; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns not significant.

significantly increased at Weeks 8 and 16: 1.99 (0.26-9.51; P = 5.1×10–5) and 1.92 (0.26-8.17; P = 0.00017), respectively, versus 1.21 (0.16-18.35) at baseline. Similar changes were observed for CD8+:Tregs ratio (n = 38) at Week 8, 29.21 (3.5-230.67; P = 1.7857×10–7) and Week 16, 25.05 (4.33-98.91; P = 4.1245×10–7), versus 15.23 (2.33-185.5) at baseline. In the bone marrow, the median (range) CD8+:CD4+ ratio (n = 58) increased from 1.46 (0.33-9.97) at baseline to 2.19 (0.30-8.56; P = 0.00016) at Week 12 (±1 cycle). Similarly, CD8+:Treg ratios increased in the bone marrow (n = 37) from 13.72 (3.06-157) at baseline to 26.57 (4.51-208.25; P = 2.7758×10–7) at Week 12 (±1 cycle). In peripheral blood, a decrease in absolute Tregs counts (including CD38+ and CD38– populations) occurred with daratumumab treatment (Figure S2A in the Supplementary Appendix), suggesting that the observed reduction in CD38+ Tregs in response to daratumumab (Figure S2B in the Supplementary Appendix) was due to an overall reduction in Tregs, not a downregulation in CD38 expression. CD8+:Treg ratios were significantly higher at Week 8 in patients who showed a response to daratumumab (P = 0.00955; Figure S2C in the Supplementary Appendix).

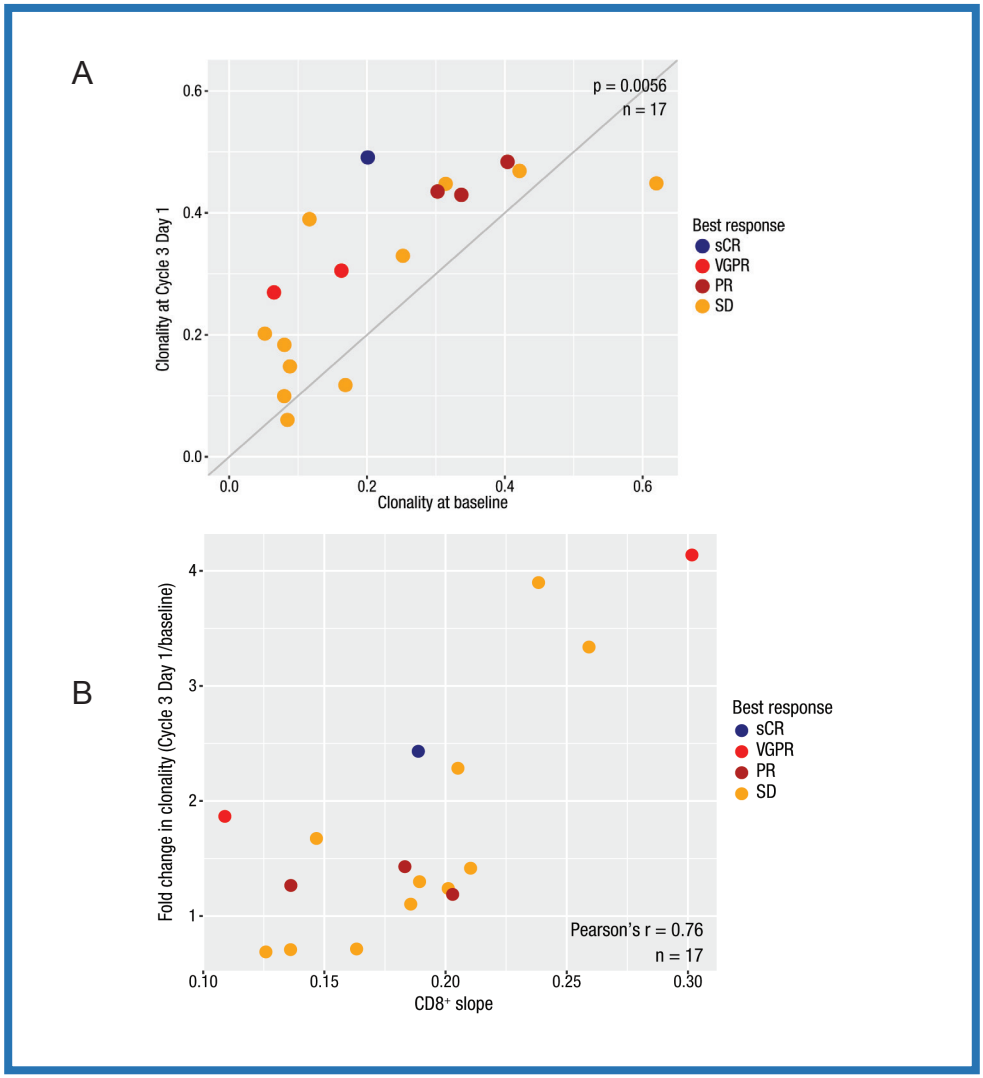
The median maximum percent increases in total CD3+, CD4+, and CD8+ T-cell counts were higher in patients who responded to treatment (n = 45) compared with those who did not respond (n = 93; Figure S3A in the Supplementary Appendix). The median (range) percent increases from baseline of total CD3+, CD4+, and CD8+ T cells in patients who responded to daratumumab compared with those who did not were 86.76% (−16.1% to 398.71%) compared with 35.44% (−67.11% to 400.47%; P = 0.00012); 56.16% (−21.05% to 296.00%) compared with 26.97% (−68% to 298.89%; P = 0.00031), and 111.99% (−7.07% to 760.51%) compared with 42.5% (−66.22% to 589.12%; P = 0.00018), respectively (Figure S3A and Table S2 in the Supplementary Appendix). However, statistical significance is confounded by interactions between time and clinical response because patients who responded to daratumumab were observed for longer than those who did not respond (Figure S3B in the Supplementary Appendix).

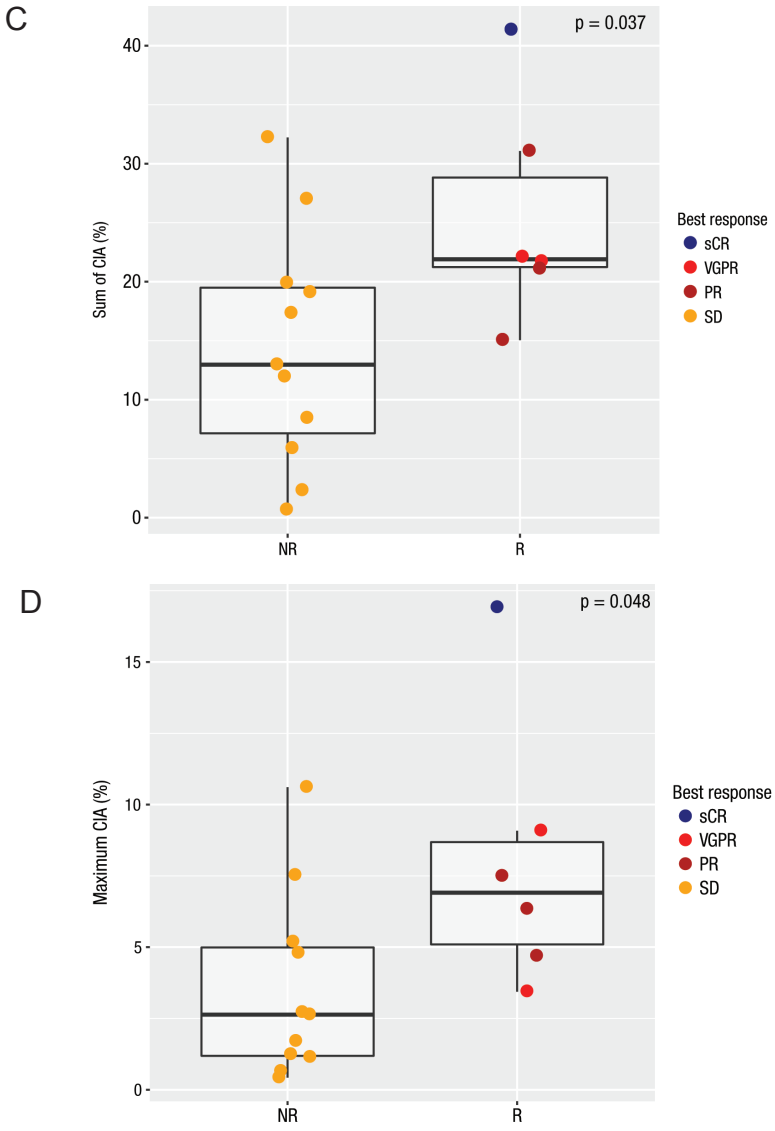
In a more detailed analysis of a subset of 17 patients enrolled in the GEN501 study, levels of peripheral HLA-DR+ CD8+ T cells were significantly increased during daratumumab treatment compared with baseline (Figure S4A in the Supplementary Appendix; P = 1.25×10–5 at Week 8) and declined when patients relapsed. This was accompanied by a decrease in the quantity of naive CD8+ T cells (Figure S4B in the Supplementary Appendix; P = 1.82×10–4) and a concomitant increase in the quantity of CD8+ effector memory T cells (Figure S4C in the Supplementary Appendix; P = 4.88×10–2). These expanding effector memory T cells expressed low levels of CD38 (Figure S4D). At relapse, the quantity of naive CD8+ T cells was similar compared with those at Week 8, while CD8+ effector memory T cells returned to baseline levels (Figure S4B and S4C in the Supplementary Appendix; P = 0.3 and 1, respectively). In addition, a significantly greater decrease in CD8+-naive T cells was apparent in patients who responded to treatment (P = 0.046 at Week 8).

To assess daratumumab's effects on T-cell activation and functionality, IFN-γ production from peripheral T cells in response to viral and alloantigens was measured in daratumumab-treated patients (n = 7) with a range of clinical outcomes (Figure S5A in the Supplementary Appendix). Patients with a PR or better demonstrated significant increases in IFN-γ secretion in response to viral and

alloantigens following daratumumab treatment, compared with baseline, for at least one time point during treatment, suggesting that T-cell function is not impaired by low CD38 expression. This increase appeared to be more marked in patients who responded to daratumumab than those who did not. Consistent with these results, virus-reactive T cells demonstrated an increase in proliferative capacity during daratumumab treatment (Figure S5B in the Supplementary Appendix).

FIGURE 6





Effect of Daratumumab Treatment on T-Cell Clonality.
Patients with pre- and posttreatment samples were evaluated (n = 17). TCR clonality increased during treatment with daratumumab (A) and was correlated with increases in CD8+ T cells (B). The sum of absolute change in abundance (CIA) in responders and nonresponders is shown for each expanded T-cell clone (C) and maximum CIA of a single T-cell clone (D). CR, complete response; PR, partial response; sCR, stringent CR; SD, stable disease; VGPR, very good PR; NR, nonresponder; R, responder.

Daratumumab skews the T-cell repertoire and increases TCR clonality

To investigate whether the observed T-cell expansion was clonal in nature, TCR clonality was assessed in a subset of patients (n = 17) through PCR/next-generation sequencing. TCR clonality increased significantly during daratumumab treatment in the majority of patients measured (14/17 = 82%; P = 0.0056; Figure 6A).

Interestingly, the TCR clonality change observed upon treatment was positively correlated with the increase in CD8+ T cells (Pearson's r = 0.76; Figure 6B).

In addition, patients who responded to daratumumab had significantly greater increases in the sum of change in abundance (CIA) for each expanded T-cell clone in the TCR repertoire (P = 0.037; Figure 6C), as well as greater maximum CIA of individual clones (P = 0.048; Figure 6D) compared with patients without a clinical response.

Discussion

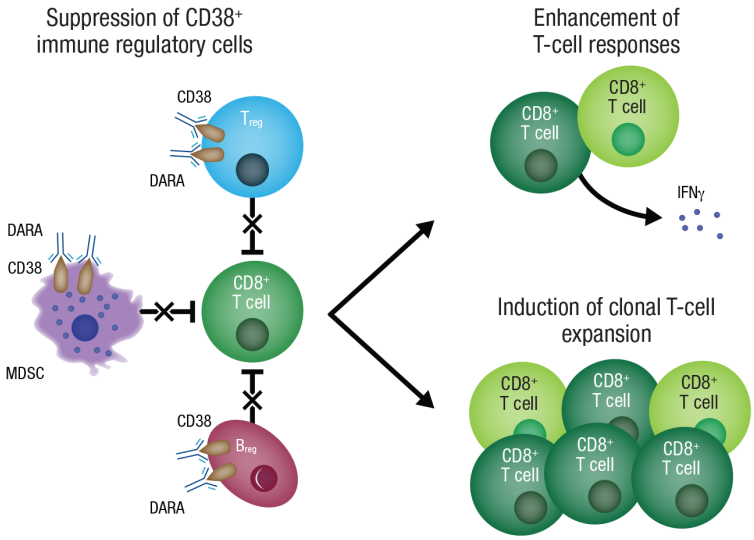
Daratumumab, the first monoclonal antibody approved for use in myeloma, has demonstrated promising activity as a monotherapy in patients with relapsed and refractory myeloma. Overall response rates of 36% and 29% in heavily treated patients administered 16-mg/kg daratumumab monotherapy included rapid responses that deepened over time to include VGPRs, CRs, and sCRs.[18,19] Responses were durable; among patients receiving 16 mg/kg monotherapy, the median durations of response were 6.9 and 7.4 months.[18,19]

This study describes previously unknown immunomodulatory effects of daratumumab through reduction of CD38+ immune suppressive cellular populations and concomitant induction of helper and cytotoxic T-cell expansion, production of IFN-γ in response to viral peptides, and increased TCR clonality, indicating an improved adaptive immune response. The extent of direct anti-tumor responses arising as a result of this adaptive response remains to be determined along with their contribution to clinical responses.

Concurrent with recent literature,[14,15] this study demonstrates that, in the myeloma setting, MDSCs and Bregs express CD38 and, in addition, were susceptible to daratumumab treatment. These cells are known to be present in the tumor microenvironment and contribute to tumor growth, immune evasion, angiogenesis, metastasis, and production of suppressive cytokines.[30] In addition to these CD38+ suppressive cellular subsets, a novel subpopulation of regulatory T cells (CD4+CD25+CD127dim) was identified that also expressed high levels of CD38 and demonstrated superior autologous T-cell suppressive capacities. These cells were also sensitive to daratumumab and were significantly reduced in patients receiving treatment. Daratumumab-mediated elimination of these CD38+ immune-regulatory cells may reduce local immune suppression within the myeloma microenvironment and allow positive immune effector cells to expand and contribute to antitumor response. Indeed, significant increases in broad T cells populations, including both CD4+ and CD8+, were observed in both peripheral blood and within the tumor (ie, the bone marrow). As would be expected for this heavily pretreated population there was broad inter-patient variability in the response to daratumumab. Specific CD8+

subpopulations were altered with daratumumab therapy, including significant decreases in naive T cells and concomitant significant increases in effector memory CD8+ T cells, indicating a shift in effector T cells towards an antigenic experienced phenotype that retained immunological memory and may be reactive against tumor antigens. Ratios of CD8+:CD4+ and CD8+:Tregs also increased significantly with treatment, demonstrating a shift in positive versus negative immune regulators.
To evaluate whether expanded CD4+ and CD8+ T cells were clonal in nature, the

FIGURE 7



Multifactorial Mechanism of Action of Daratumumab.
Daratumumab induces antimyeloma effects via multiple mechanisms of action. Based on the present study, an additional mechanism of action is proposed in which treatment with daratumumab eliminates a population of highly immune suppressive CD38+ regulatory T cells (Tregs) and T-cell modulating myeloid-derived suppressor cells (MDSCs) and regulatory B cells (Bregs) and thus stimulates T-cell effector functions. DARA, daratumumab; MDSC, myeloid-derived suppressor cells; Treg, regulatory T cells; Breg, regulatory B cells.

T-cell repertoire was examined in a subset of patients. T-cell clonality significantly increased with daratumumab treatment, even in patients who had a best response of SD or who progressed. Therefore, increased T-cell clonality cannot be due simply to reduction in tumor burden. However, the skew in T-cell clonality was greater in patients with a good clinical response, and was correlated with the increase in CD8+ T cells, suggesting the observed T-cell expansion with daratumumab treatment was antigen-driven. This is remarkable in this patient population, which was heavily pretreated (median of 5 prior lines of therapy) and not expected to be able to mount a strong antitumor immune response.
In addition to increased TCR clonality, patients with a response to daratumumab

demonstrated increased T-cell responses to preexisting viral- and alloantigens, suggesting the rescue of the immune system from an immunosuppressive state. Future clinical studies will evaluate the anti-myeloma effects of expanded T cells following daratumumab treatment.

Treatment with daratumumab caused a reduction in immune suppressive MDSCs and regulatory T and B cells, although not all MDSC subtypes were evaluated due to limitations in the clinical samples collected. Ongoing clinical studies are focused on appropriate sample collection and further evaluation of daratumumab's effects on additional subtypes of regulatory cellular populations. The reductions in MDSCs, Tregs and Bregs described here were concomitant with an expansion of CD4+ T-helper cells and CD8+ cytotoxic T-cells. T-cell clonality and functional anti-viral responses as measured by IFNγ production also increased with daratumumab treatment. These observations indicate that T cells continued to function properly, despite low CD38 expression, and suggest that increased T-cell response may be due to depletion of regulatory cells. Further, these changes in T-cell expansion, activity, and clonality were more pronounced in patients who responded to daratumumab compared with those who did not. Relapse from daratumumab therapy was associated with reversal of many of these changes.

This suggests an additional, previously uncharacterized mechanism of action of daratumumab through immunomodulation that may contribute to clinical responses and its efficacy in such a heavily treated patient population (Figure 7).[19] Whether these immune effects result in targeted anti-myeloma T-cell reactivity or represent a more generalized T-cell response will need to be addressed in future studies.

Recently, antibodies that promote antitumor immune responses, rather than targeting the cancer directly, have demonstrated efficacy in a range of settings. Antibodies inhibiting CTLA 4 and PD-1 promote T-cell expansion and enhance T-cell activation, respectively, resulting in prolonged survival and delayed disease recurrence in patients with advanced solid tumors and hematologic malignancies such as Hodgkin lymphoma.[31–35] By enhancing anticancer immunity, these immunomodulatory antibodies may not only induce clinical responses, but also prevent disease recurrence.[36] Interestingly, these agents have not demonstrated efficacy as monotherapies in MM, suggesting that enhancing immune function and at the same time targeting malignant cells may be required to maintain long-term clinical responses in MM.

In conclusion, these data suggest a previously unknown, multidimensional, immunomodulatory role for daratumumab that may contribute to deeper clinical responses and enhanced survival. Future studies will be needed to determine whether these expanded T cells are active against tumor-specific targets, and what role these immune modulatory effects play in overall clinical response. In addition, these data suggest a broader immune-regulatory role of CD38 that may be applicable in other malignancies.

Acknowledgements

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Supplementary appendix

Additional Methods

Interval mapping of study days

Daratumumab dose frequency changed from every week to every 2 weeks, and eventually every 4 weeks, with more frequent sample collection in earlier cycles compared with later cycles. Relative study days of the two studies were therefore mapped and aligned to time intervals as follows:

Time Interval	Time Interval (Study Day)	Target Time Point
Baseline = Week −1	Day ≤ 1 before 1st infusion	0
Week 0	Day =1	1
Week 1	2 ≤ Day ≤ 11	8
Week 2	12 ≤ Day ≤ 18	15
Week 3	19 ≤ Day ≤ 25	22
Week 4	26 ≤ Day ≤ 32	29
Week 5	33 ≤ Day ≤ 39	36
Week 6	40 ≤ Day ≤ 46	43
Week 7	47 ≤ Day ≤ 53	50
Week 8	54 ≤ Day ≤ 63	57
Week 10	64 ≤ Day ≤ 77	71
Week 12	78 ≤ Day ≤ 91	85
Week 14	92 ≤ Day ≤ 105	99
Week 16	106 ≤ Day ≤ 119	113
Week 18	120 ≤ Day ≤ 133	127
Week 20	134 ≤ Day ≤ 147	141
Week 22	148 ≤ Day ≤ 161	155
Week 24	162 ≤ Day ≤ 182	169
Week 28	183 ≤ Day ≤ 210	197
Week 32	211 ≤ Day ≤ 238	225
Week 36	239 ≤ Day ≤ 266	253
…	…	…
Week X	X*7 – 13 ≤ Day ≤ X*7 + 14	X*7 + 1

Supplementary

Table 1

Baseline Demographics and Clinical Characteristics of Patients Receiving 16 mg/kg Daratumumab

Characteristic	N = 148
Median (range) age	64 (31-84)
18-< 65 years, n (%)	80 (54)
65-< 75 years, n (%)	52 (35)
≥75 years, n (%)	16 (11)
Sex, n (%)	
Male	79 (53)
Female	69 (47)
Race, n (%)	
White	116 (78)
Black or African American	16 (11)
Asian	4 (3)
Other	2 (1)
Not reported	10 (7)
Prior lines of therapy, n (%)	
≤3, n (%)	35 (24)
>3, n (%)	113 (76)
Median (range) time from diagnosis, months	5.12 (0.77-23.77)
Refractory to, n (%)	
Last line of prior therapy	135 (91)
Both PI and IMiD	128 (86)
PI only	6 (4)
IMiD only	5 (3)
Neither PI nor IMiD	9 (6)
PI + IMiD + ALKY	100 (68)
BORT	125 (84)
CARF	58 (39)
LEN	124 (84)
POM	82 (55)
THAL	41 (28)
ALKY	107 (72)
BORT + LEN	114 (77)
CARF + POM	44 (30)
BORT + LEN + CARF	49 (33)
BORT + LEN + POM	70 (47)
BORT + LEN + CARF + POM	38 (26)
BORT + LEN + CARF + POM + THAL	15 (10)
Baseline type of myeloma, n (%)	
IgG	73 (49)
IgA	26 (18)
IgD	4 (3)
IgM	1 (1)
Biclonal	3 (2)
Kappa	22 (15)
Lambda	17 (11)

Abbreviations: n, number.

*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.

Supplementary

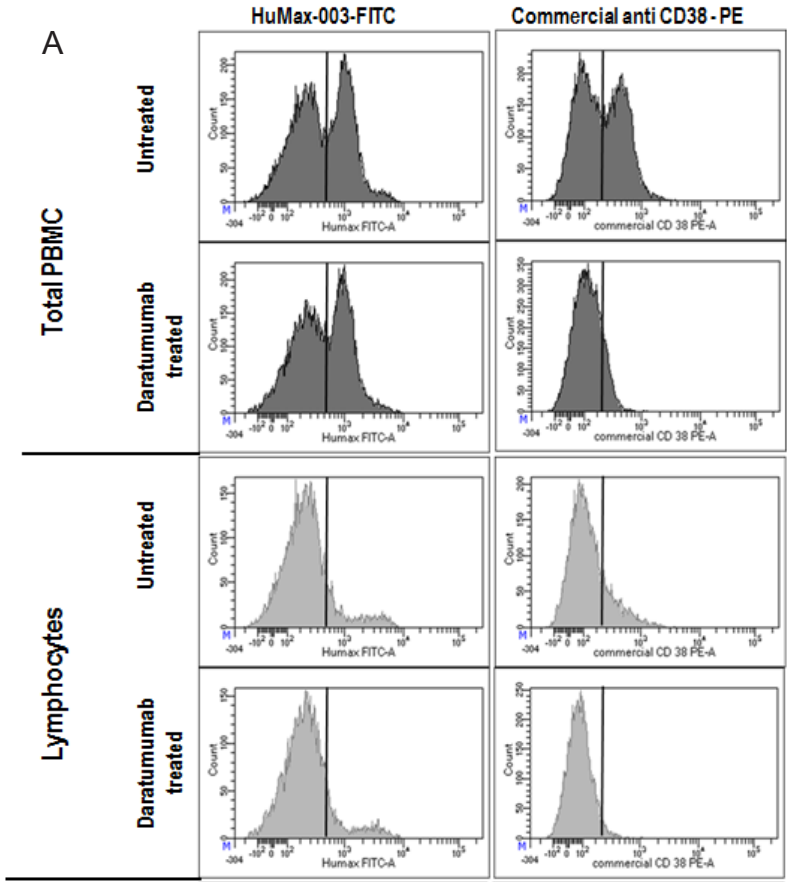
Table 2

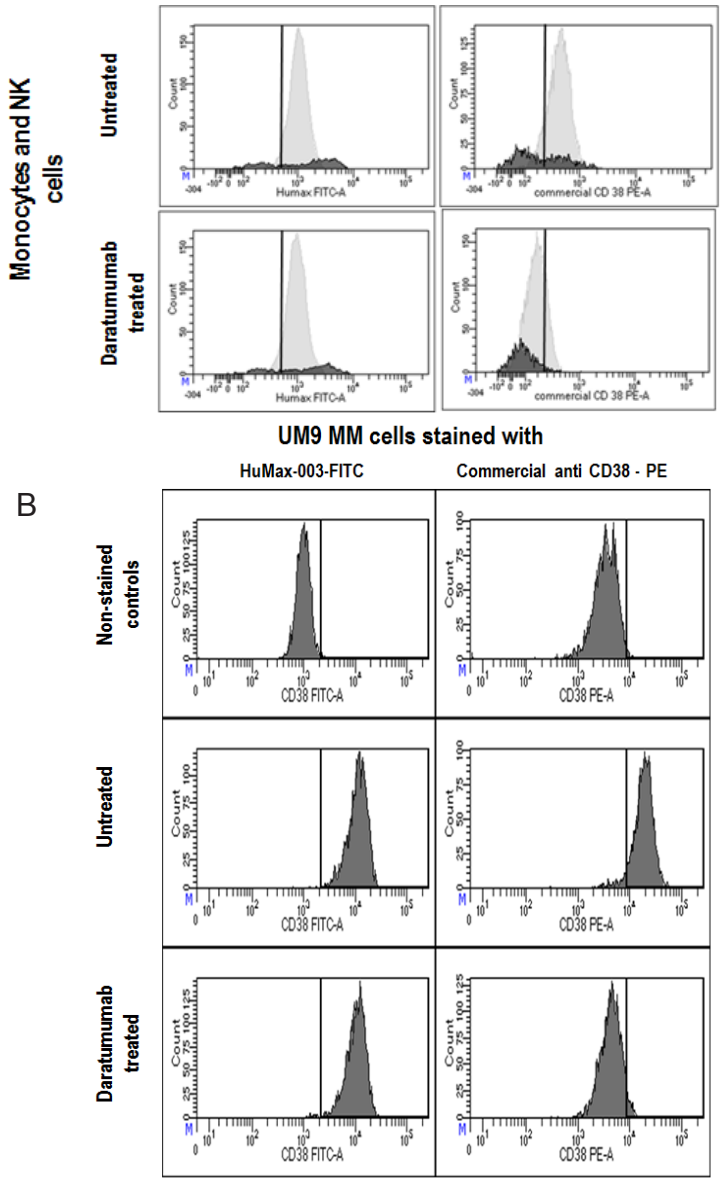
Summary Statistics of Maximum Percent Change from Baseline in Absolute CD3+, CD4+, and CD8+ T-cell Counts in Peripheral Blood of Responders and Nonresponders.

	CD45+CD3+		CD45+CD3+CD4+		CD45+CD3+CD8+	
	Responders	Non-responders	Responders	Non-responders	Responders	Non-responders
n	45	93	45	93	45	93
Median	86.76	35.44	56.16	26.97	111.99	42.5
Range	−16.1 to 398.7	−67.1 to 400.5	−21.1 to 296	−68 to 298.9	−7.1 to 760.5	−66.2 to 589.1
P value		0.00012		0.00031		0.00018

Supplementary

Figure 1

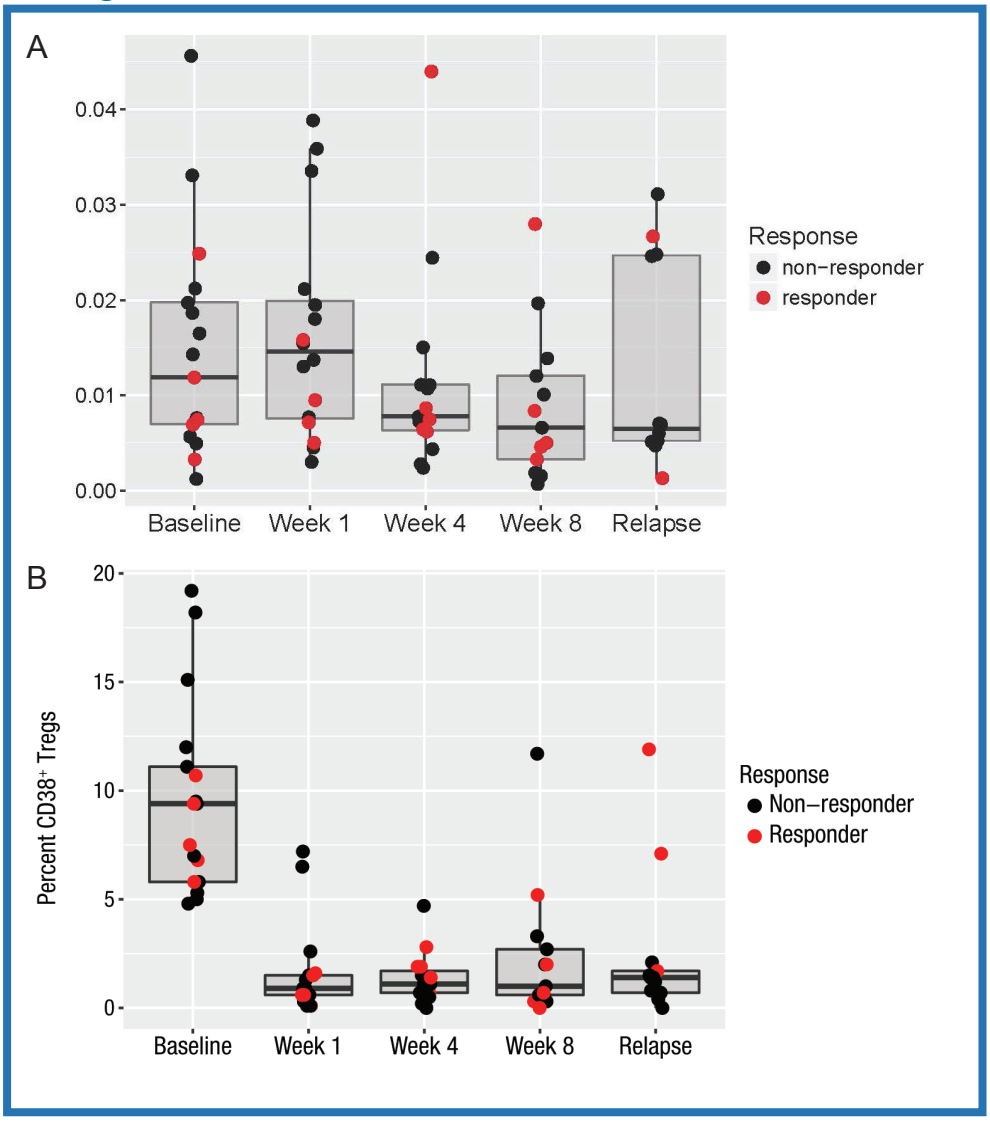


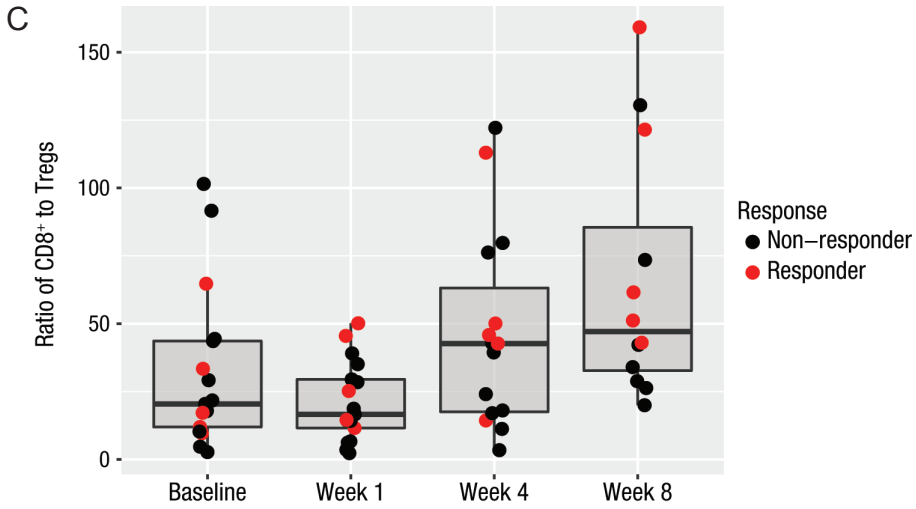


Daratumumab does not inhibit staining with HuMax-003. Normal human PBMC (A) and CD38+ UM-9 MM cells (B) were either left untreated or pretreated with daratumumab (1 µg/mL) for 20-30 minutes before staining with either HuMax-003-FITC or a commercial anti-CD38-PE antibody (BD, clone HB-7) for 20-30 minutes at 4˚C. The cells were then washed and analyzed on a FACS-CANTO II flow cytometer. The results were analyzed with FACS Diva software. In (A) staining of total PBMC, lymphocytes, monocytes (gray) and NK cells (black) are indicated separately. Note that pre-incubation of the cells with daratumumab did not affect staining with HuMax-003-FITC compared with the complete abrogation of staining with the commercial antibody.

Supplementary

Figure 2



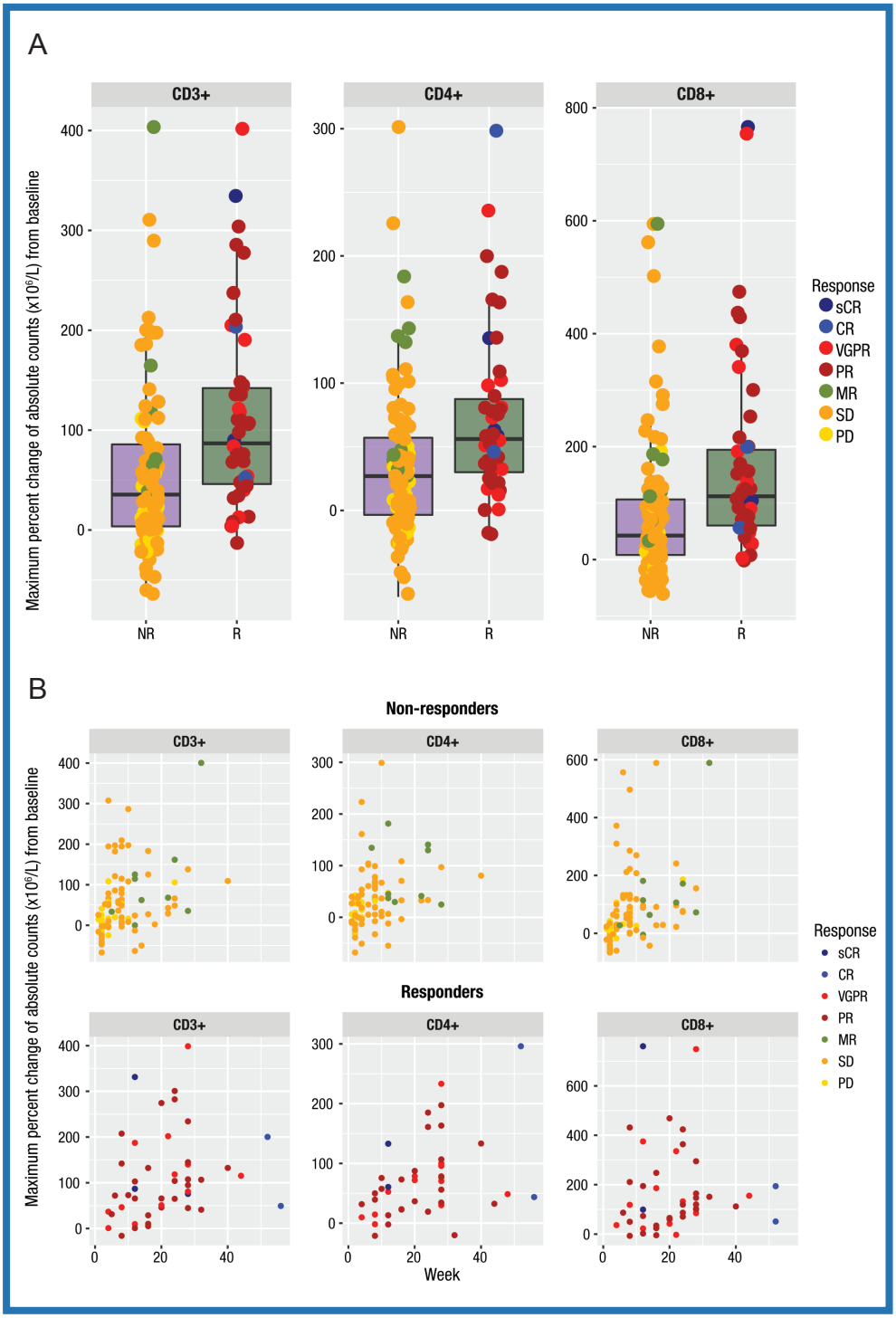


Linear Mixed Modeling of Regulatory T cell (Treg) Populations in Responders versus Non-responders.

Absolute Treg counts (A), percent changes in CD38+ Tregs (B), and ratio of CD8+ T cells to Tregs (C) in peripheral blood prior to and during daratumumab treatment (Weeks 1, 4, and 8) and at relapse are shown, by response. Red dots represent responders and black dots represent nonresponders

Supplementary

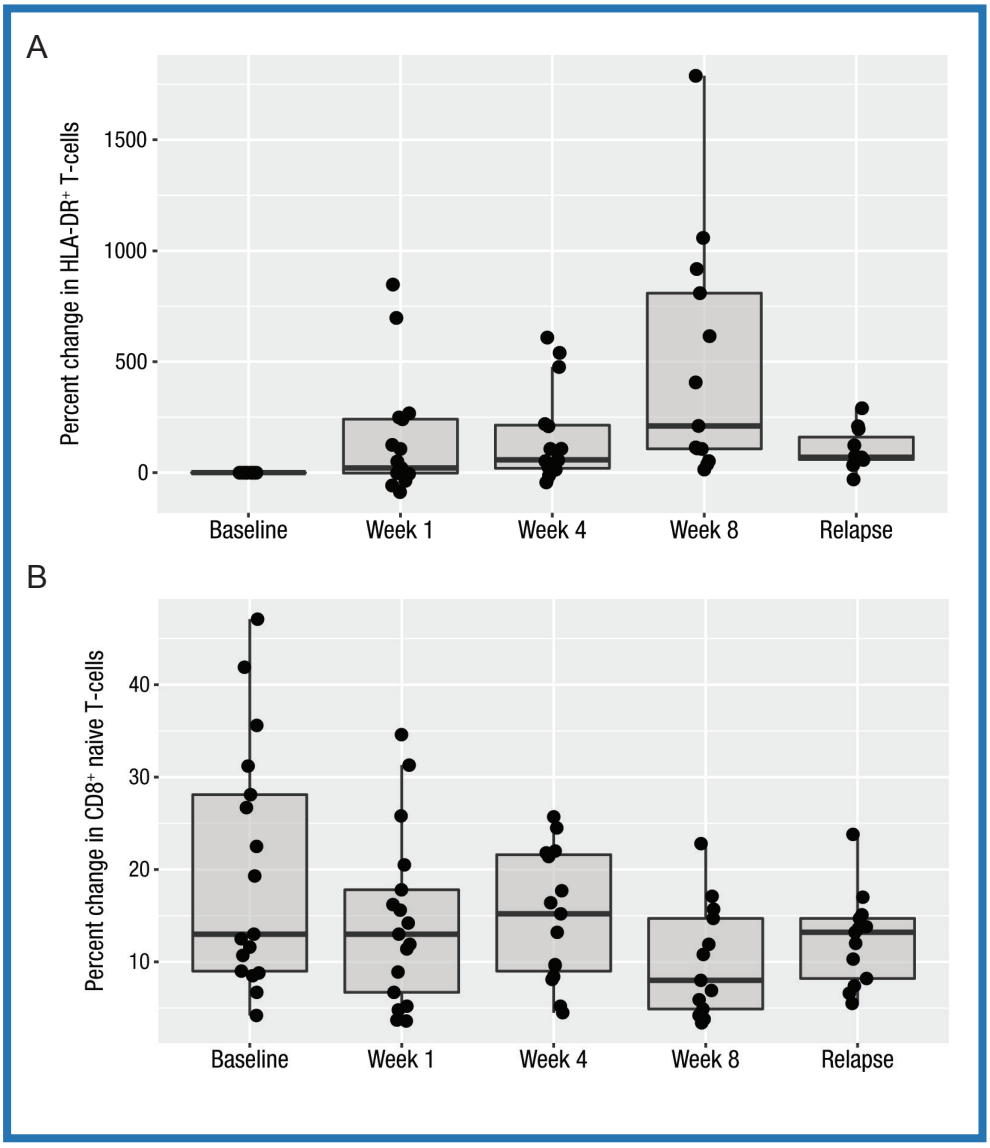
Figure 3

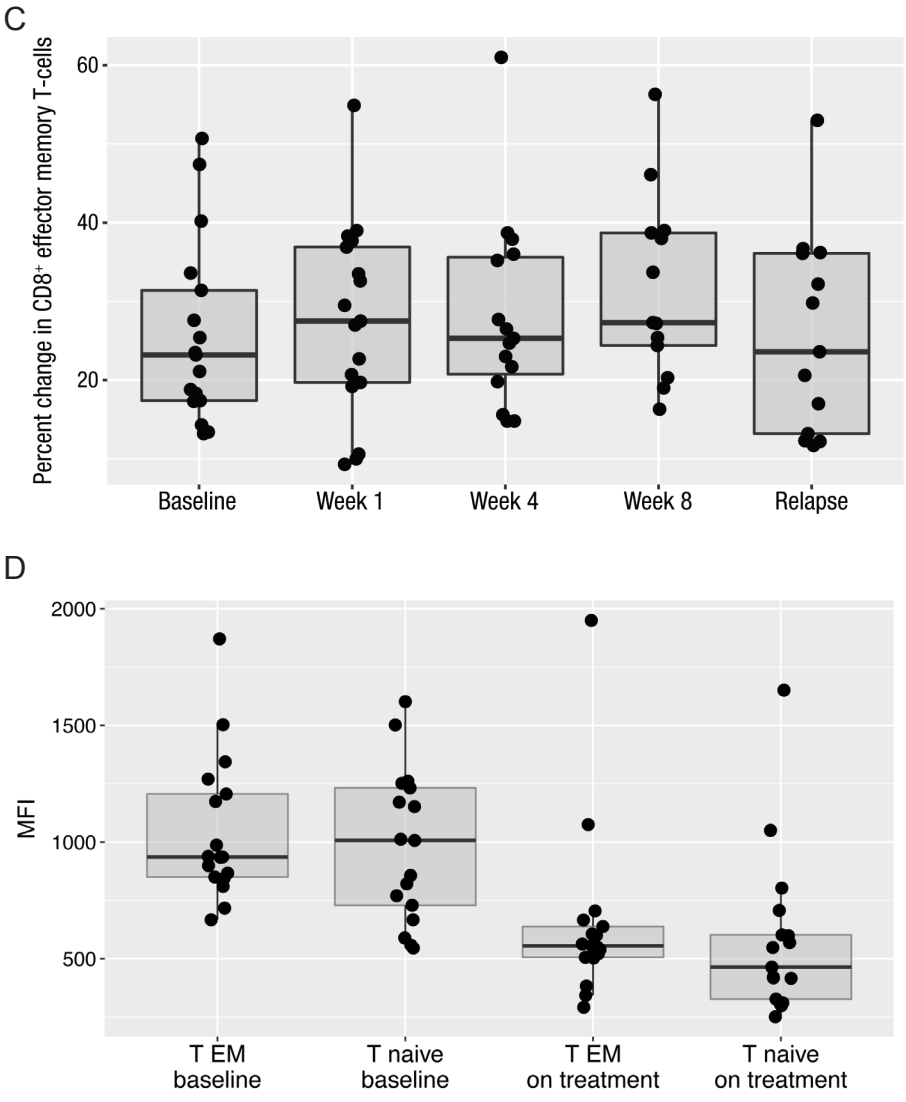


Maximum Percent On-treatment Increases From Baseline in T Cells.
Maximum percent change from baseline in absolute total CD3+, CD4+, and CD8+ T-cell counts of daratumumab-treated patients by response in peripheral blood (A). Maximum percent change in absolute counts from baseline as a function of the week at which this maximum increase was observed, per patient (B). CR, complete response; MR, minimal response; PD, progressive disease; PR, partial response; sCR, stringent CR; SD, stable disease; VGPR, very good PR; NR, non-responder; R, responder.

Supplementary

Figure 4

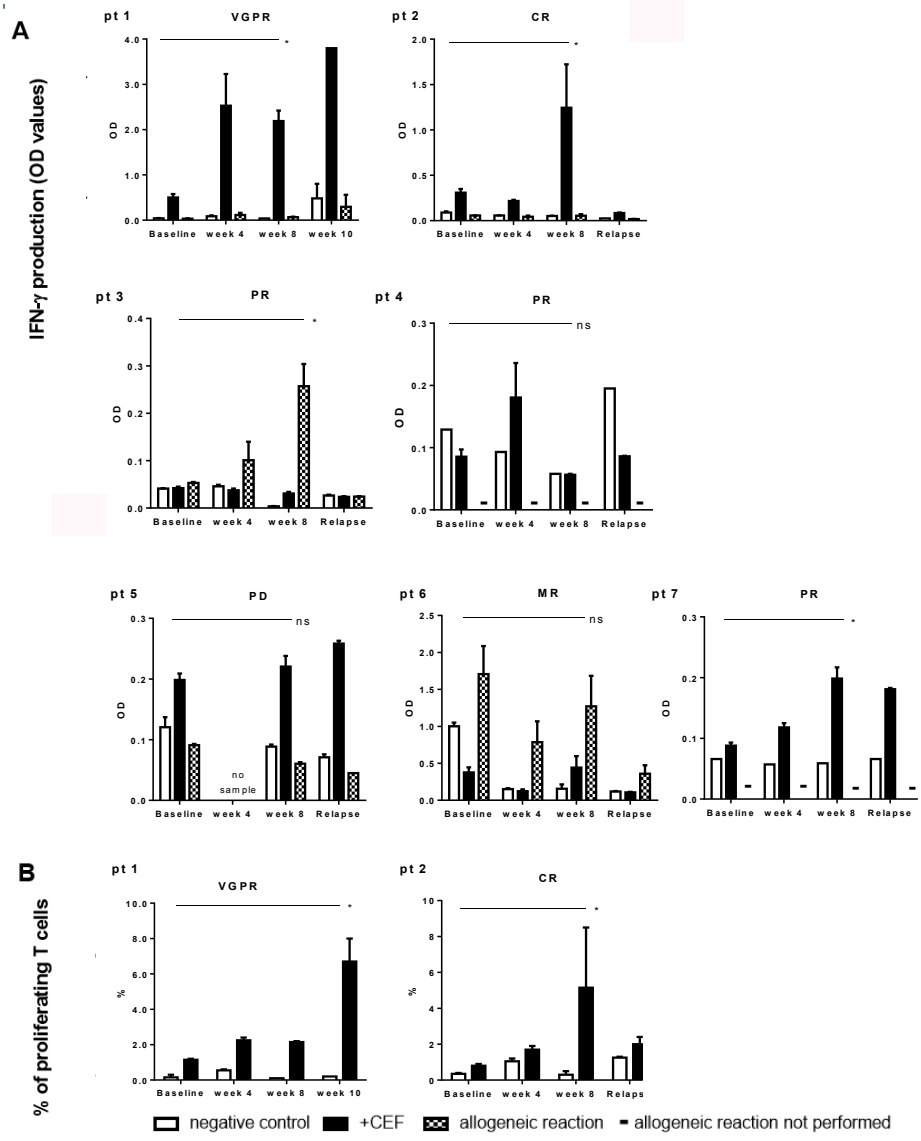




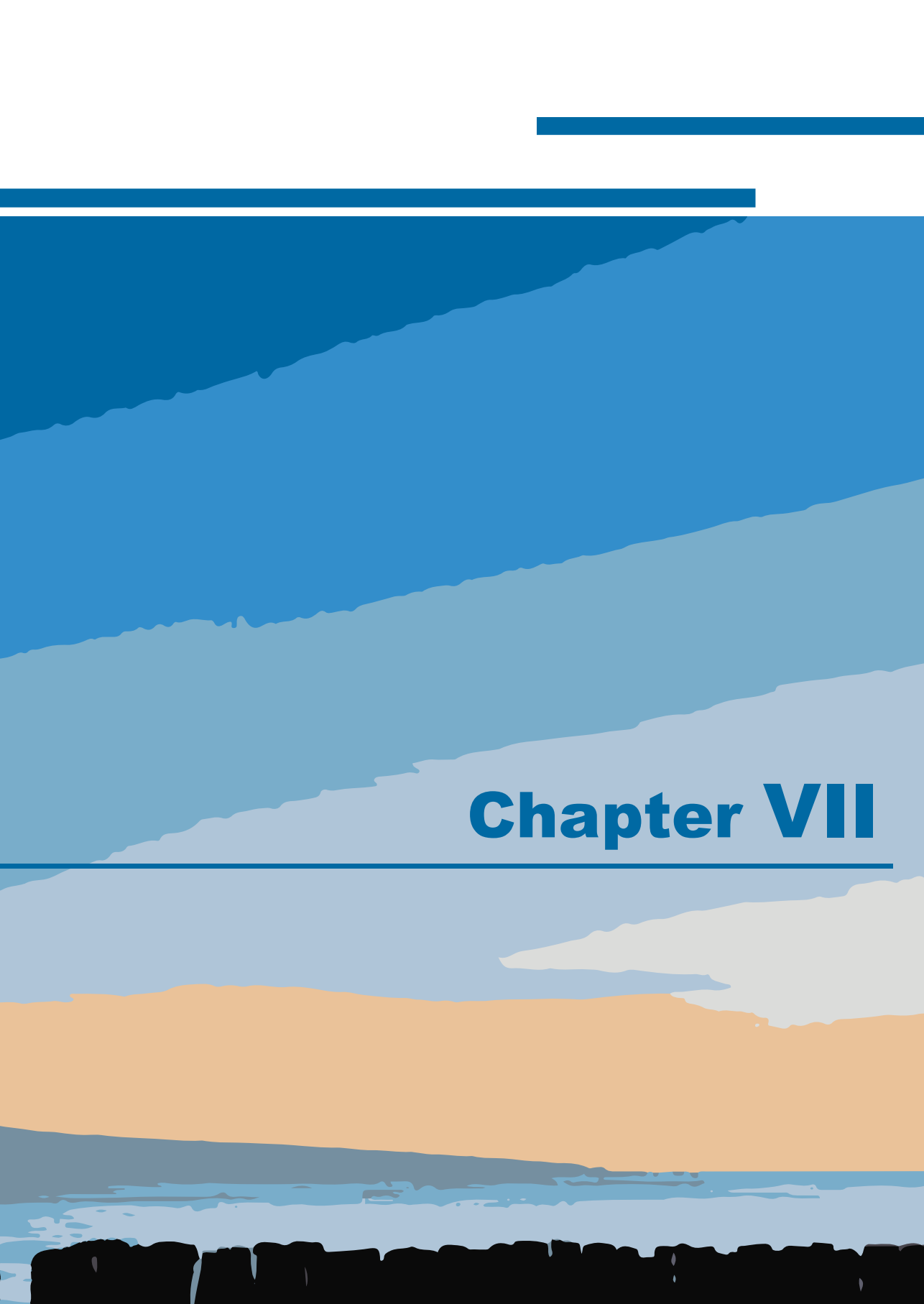
Changes in CD8+ T-Cell Phenotype During Daratumumab Treatment.
Percent increase in HLA-DR+ CD8+ T cells (A), percent increase in naive CD8+ (CD3+CD8+CD45RA+CD62L+) cells (B), and percent increase in effector memory CD8+ T cells (CD3+CD8+CD45RA-CD62L−) (C) in peripheral blood prior to and during daratumumab treatment (Weeks 1, 4, and 8) and at relapse are shown. CD38 expression in expanded effector memory cells and naïve cells at baseline and on treatment (D).

Supplementary

Figure 5



Increase in T-cell Response in Responders.
Antiviral T-cell response 5 days after stimulation, as measured through cytomegalovirus-, Epstein Barr virus–, and influenza virus (CEF)-specific interferon γ (INF-γ) production in peripheral-blood mononuclear cells (PBMCs) from daratumumab-treated patients, is shown (A). Virus-reactive T cells showed increased proliferative capacity during daratumumab treatment (B). Error bars represent standard error of the mean of duplicate cultures. Asterisks denote significant changes. Best clinical response per Independent Review Committee is shown. OD, optical density; MR, minimal response, CR, complete response; PR, partial response; VGPR, very good PR; PD, progressive disease; ns, not significant.


Chapter VII

Phase 1/2 study of lenalidomide in combination with cyclophosphamide and prednisone (REP) in patients with lenalidomide-refractory multiple myeloma (REPEAT-study)

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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 PFS and OS 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.

Lenalidomide has multiple effects in MM, including direct anti-tumor activity, inhibition of adhesion of MM cells to stromal cells, and suppression of angiogenesis.[5] 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.[6–8] Similarly, administration of cyclophosphamide, at a dose substantially lower than the maximum tolerated dose (MTD) (metronomic dosing),[9] has next to its direct anti-tumor activity several other effects including anti-angiogenic effects,[10,11] modulation of the micro-environment,[12] and improvement of T and NK cell-mediated anti-tumor immune response via depletion of Tregs.[13–18]

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.[19] 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, 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)), 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 109/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 cotrimoxazol (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 (Table 1).

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[20].

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 Table 1.

Table 1

Dose levels and DLTs for each cohort of enrolled patients

	Cohort	Len (mg)	Cyclo (mg)	Pred (mg)	Peg G-CFS (day 1)	Patients n	DLT n	Type of DLT
Phase 1	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[20]

Dose modification

A new REP-cycle was allowed if ANC ≥ 1.0 x 10^9/L and platelets ≥ 50 x 10^9/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 10^9/L before the start of a new REP-cycle or if the ANC dropped below 0.5 x 10^9/L during a REP-cycle. Grade 4 neutropenia and thrombocytopenia, as well as febrile neutropenia during 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.

Safety and efficacy assessments

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.03)[20]. 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[21], with minimal response defined according to European Society for Blood and Marrow Transplantation criteria[22,23].

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 - β = 0.80 (2-sided significance level α = 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 2. 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 (Table 2). 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 (Table 1).
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. Therefore, 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).

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 1.
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 2). 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.

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

TABLE 2

Patient characteristics phase 1/2 REPEAT-study

Characteristic	Phase 1	Phase 2	Total
	n=21	n=61	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)

Laboratory values at baseline, median (range)			
Absolute neutrophil count, x109/L	3.2 (1.2-20.5)	2.6 (1.1-7.9)	2.6 (1.1-20.5)
Hemoglobin, mM	6.6 (5.3-9.2)	6.9 (4.5-9.1)	6.9 (4.5-9.2)
Platelet count, x109/L	183 (95-334)	164 (50-369)	167 (50-369)
Type 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

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).
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 G-CSF 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;

TABLE 3

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

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)
Arythmia	-	1 (1)	-	-	1 (1)
Angina pectoris	-	1 (1)	-	-	1 (1)

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. 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.

TABLE 4

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 hybridisation; 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.

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 of 9 REP-cycles (range 1-30+ cycles). The ORR (≥PR) was 67% (44 patients), including at least VGPR in 15 patients (23%). Three patients achieved complete remission (CR) (5%), including 1 stringent CR (sCR). Eleven patients (16%) achieved a MR, translating to an overall 83% clinical benefit rate (≥MR). At least SD was achieved in 60 patients (91%) (Table 4). Median time to at least PR was 1.7 months (range, 0.5-22.8 months). 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 1A en B). 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.

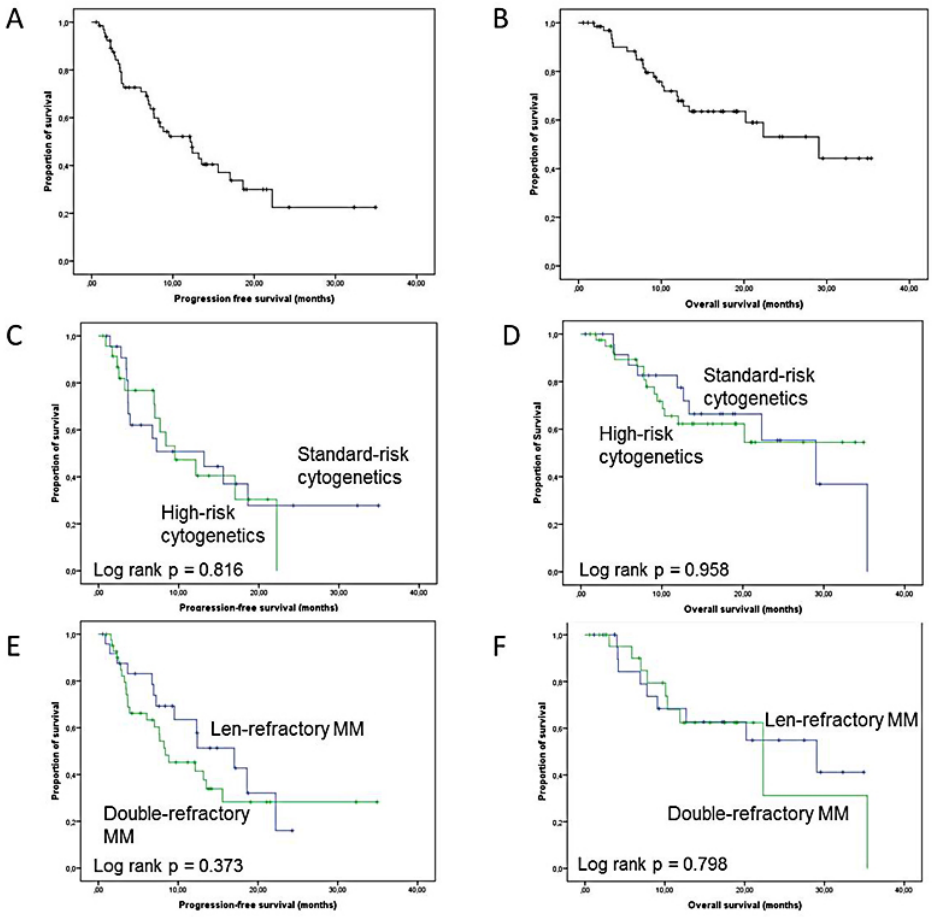
TABLE 5

Univariate analysis of possible predictive factors for PFS and OS

	ORR	PFS		OS	
	P-value	P-value	HR (95% CI)	P-value	HR (95% CI)
>65 years	0.486	0.901	1.024 (0.545-1.993)	0.81	1.105 (0.486-2.510)
Male	0.705	0.315	1.415 (0.719-2.784)	0.786	1.127 (0.476-2.669)
≥ 3 lines of therapy	0.144	0.830	1.079 (0.539-2.157)	0.553	1.289 (0.557-2.981)
≥ 4 lines of therapy	0.855	0.651	0.859 (0.446-1.657)	0.214	0.595 (0.262-1.351)
Creatinine clearance (≥50 ml/min)	0.320	0.410	0.643 (0.225-1.837)	0.418	2.296 (0.307-17.163)
Thrombocytes	0.217	0.911	1.0 (0.995-1.005)	0.209	1.004 (0.998-1.009)
Beta2-microgobulin	0.697	0.050	1.122 (1.000-1.259)	0.369	1.058 (0.935-1.197)
Albumin	0.361	0.253	1.001 (0.999-1.003)	0.147	1.002 (0.999-1.005)
LDH	0.525	0.081	1.002 (1.000-1.004)	0.001	1.004 (1.001-1.006)
High risk cytogenetics	0.705	0.816	0.912 (0.420-1.981)	0.958	0.974 (0.367-2.584)
WHO 0+1 vs 2+3	0.010	0.867	0.932 (0.405-2.141)	0.238	0.567 (0.222-1.453)
Len-refractory vs len- and bor-refractory disease	0.103	0.376	0.736 (0.374-1.450)	0.798	0.896 (0.386-2.078)
Full-dose lenalidomide (25 mg or equivalent in case of renal insufficiency) before start REP-therapy	1.00	0.497	1.299 (0.610-2.765)	0.950	1.031 (0.402-2.643)
REP directly after development of len-refractory disease	0.861	0.951	1.02 (0.532-1.957)	0.785	1.122 (0.491-2.568)

ORR, overall response rate; PFS, progression-free survival; OS, overall survival

FIGURE 1



Progression-free survival and overall survival.
(A) Progression-free survival (PFS) and (B) overall survival (OS) of the 67 lenalidomide-refractory MM patients treated with REP at the MTD. (C) PFS and (D) 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) versus standard-risk disease. (E) PFS and (F) OS for patients treated with REP at the MTD with disease refractory to both lenalidomide and bortezomib (double-refractory MM) versus disease refractory to only lenalidomide.

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 4). 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 for high-risk and standard-risk patients respectively) (Figure 1C and D).
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 1E and F). 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 (Tables 4 and 5). WHO performance status before start of REP-treatment was the only variable, which was significantly associated with response (Table 5). 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 beta2-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 5).

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[24,25]. Similarly, when cyclophosphamide was added to pomalidomide and prednisone (PCP)[26] hematologic toxicity was comparable to the toxicity observed with pomalidomide-dexamethasone[27,28]. 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[29,30]. Non-hematologic toxicity of REP consisted mainly of infections. Discontinuations because of adverse events were uncommon, allowing patients to continue therapy until disease progression.We observed high

activity of REP despite enrolling patients who were all lenalidomide-refractory and 66% also bortezomib-refractory. Response and survival were similar in patients with high-risk cytogenetic abnormalities or 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.[31]

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 18% of these patients and a median PFS of only 3.3 months.[32] 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. Other studies have also demonstrated a beneficial effect of addition of cyclophosphamide to lenalidomide and corticosteroids in patients with newly diagnosed MM[33] or relapsed/refractory lenalidomide-naive MM (≥PR: 65-94%).[34-36]

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.[37] 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).[26]

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.

The outcome of the REP regimen compares favorably to the results of next generation novel agents evaluated in lenalidomide-refractory patients. 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).[28] Carfilzomib monotherapy induces at least PR in 19.1% of extensively pretreated patients with a median PFS of 3.7 months.[32] Daratumumab induces at least PR in 29-36% of patients with a median PFS of 3.7-5.6 months,[38,39] while elotuzumab[40] has no single agent activity in this setting. 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. 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 favor of the triplet regimens in MM patients who had received 1-3 prior treatments.[41–44] 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[43] or carfilzomib.[44]Although, several of the new agents to treat lenalidomide- and bortezomib-refractory MM are now approved by the FDA a nd/or EMA, these therapies may not yet be available or reimbursed in many countries. 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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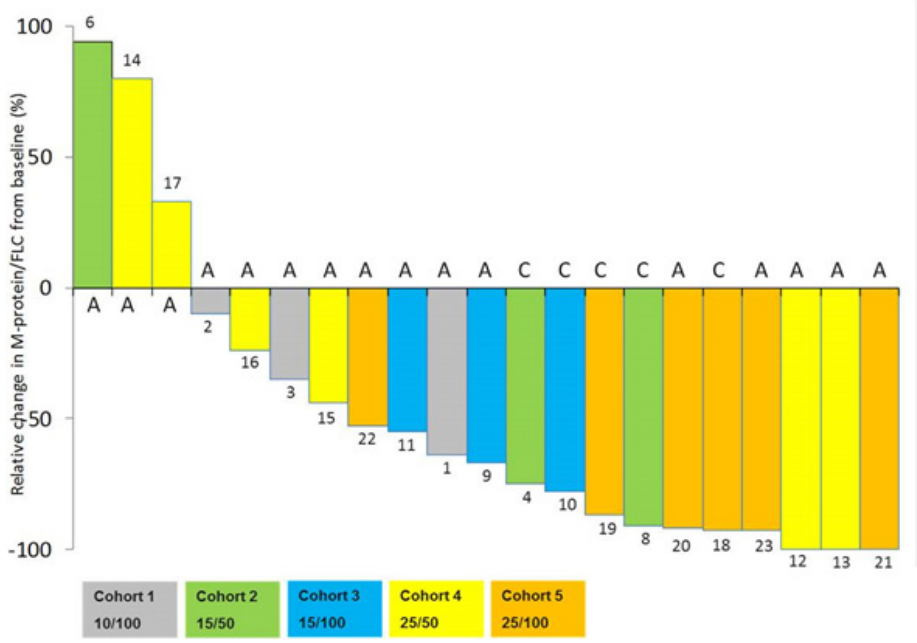
Supplementary appendix

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).

Supplementary

TABLE 1

Adverse events during REP-therapy in the phase 1 part of the study

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)
Anemia	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)

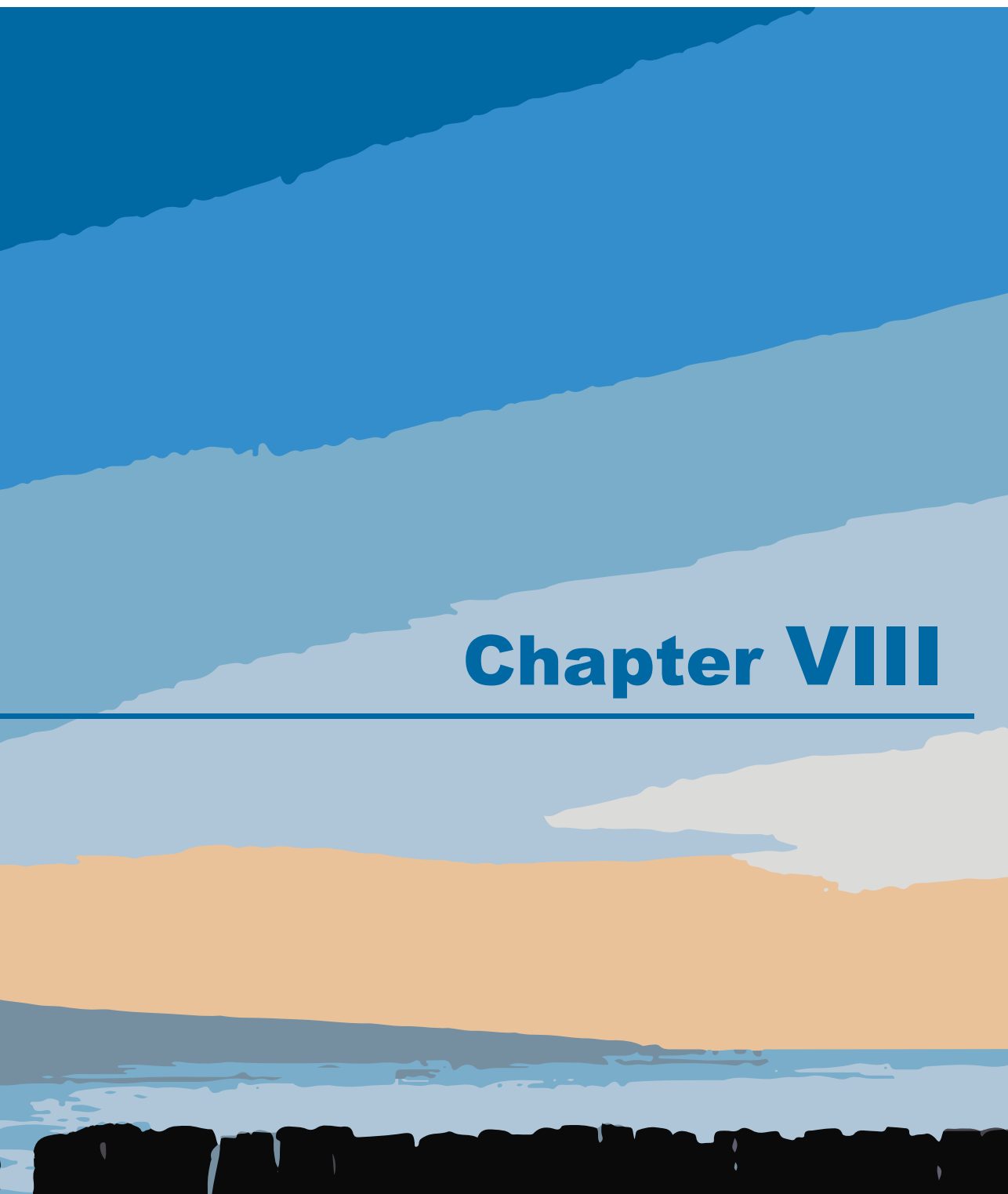
Supplementary

TABLE 2

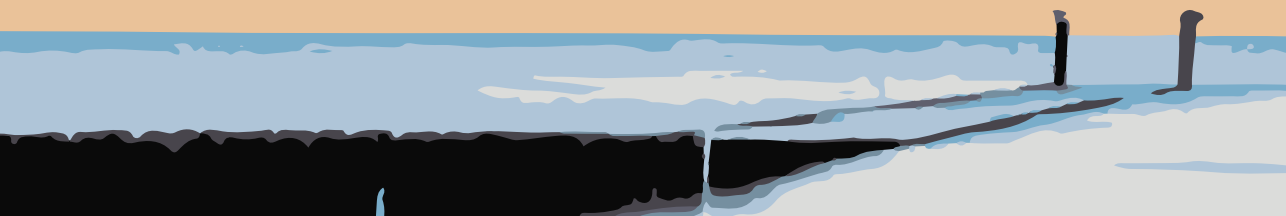
Response to REP-therapy in the phase 1 part of the study

	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 hybridisation; 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.


Chapter VIII

General discussion



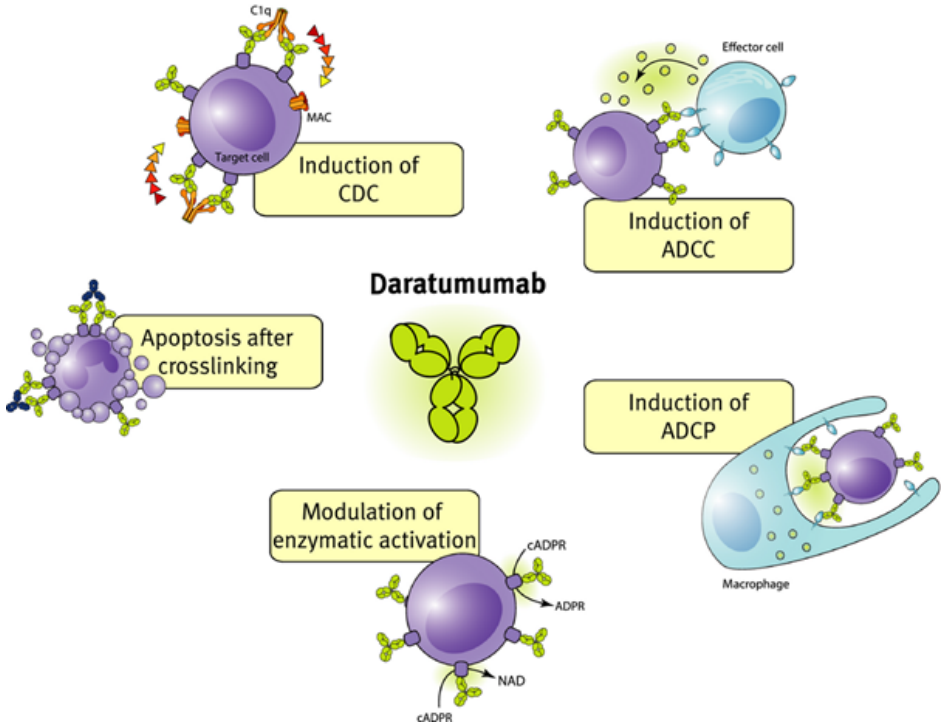
General discussion

Multiple myeloma (MM) is a neoplasm of the B-cell lineage that is characterized by the proliferation of plasma cells in the bone marrow. This typically results in anemia and increased bone resorption by osteoclasts, frequently leading to bone fractures or hypercalcemia. MM is further characterized by the production of excess immunoglobulin (M-protein), or fragments thereof (kappa or lambda free light chains). Large amounts of free light chains may result in damage to tubular cells in the kidney, leading to acute renal failure.[1] In MM, the introduction of the proteasome inhibitor (PI) bortezomib and the immunomodulatory agents (IMiDs) thalidomide and lenalidomide, have significantly improved prospects of MM patients.[2] However, the far majority of patients still relapse even when intensive therapy is combined with IMiDs and PIs.[3] Patients who are refractory to these novel agents have a very poor prognosis with a median event-free survival and a median overall survival (OS) of only 5 and 9 months respectively.[2,4] This clearly demonstrates that there is an urgent need for additional active agents with distinct mechanisms of action, especially for the relapsed and refractory MM patients.

Immunotherapy, aimed at engaging the immune system in the fight against malignant cells, is an attractive concept in cancer treatment. A number of different strategies can be distinguished, such as monoclonal antibodies (mAbs), chimeric antigen receptor (CAR) T cells, checkpoint inhibition, and vaccination. mAbs are already a widely used treatment modality in hematology, with rituximab being the most prominent example. The agent was approved for the treatment of lymphoma in 1997 by the US Food and Drug administration (FDA) and in 1998 by the European Medicines Agency (EMA),[5] and is now considered a standard therapy in lymphoma that has had a major impact on survival.[6]
Until recently, no appropriate mAbs were available for MM. However, with various candidates FDA- and EMA-approved now and others in late-stage clinical development, mAbs are an emerging class of agents for the treatment of MM.[7] Among them daratumumab, an immunoglobulin G1 kappa (IgG1κ) human monoclonal antibody, that specifically binds to an unique epitope present on the CD38 molecule. CD38 is a multifunctional cell surface protein that possesses receptor as well as enzyme functions and might be an interesting target for antibody therapy in MM, since virtually all MM cells express high levels of CD38 on their cell surface.[8] However, CD38 is also expressed, although with lower expression rates, on lymphoid and myeloid cells as well as on several non-hematopoietic tissues.[9] Daratumumab was originally developed by Genmab.[10] The antibody was selected from a panel of 42 different CD38-specific mAbs that were generated with the HuMAb technology using human Ab transgenic mice.[11] Daratumumab was selected because it stood out in its exceptional ability to initiate complement-dependent cytotoxicity (CDC) in CD38-overexpressing cell lines as well as in patient-derived MM cells.[10] In addition to CDC, the binding of daratumumab to CD38 on the surface of tumor cells has also been shown to induce antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), tumor cell apoptosis, and modulation of the enzymatic

activity of CD38 (Fig. 1).[1;10;12]
Prior preclinical studies, conducted by us and others, have shown promising results for daratumumab in the treatment of MM and has led to the conduction of two clinical studies with daratumumab as a single agent. These first clinical studies showed good tolerability and marked activity in heavily pretreated relapsed and refractory

FIGURE 1



Mechanisms of action of daratumumab (source: van de Donk et al. Immunol Rev. 2016).[1]

MM patients.[13,14] With these emerging results, the FDA approved daratumumab (Darzalex) monotherapy for MM patients who have received three or more prior lines of therapy, including a PI and an IMiD, or who are double refractory to a PI and an IMiD in November 2015. Also the EMA granted a priority review and approved daratumumab recently in May 2016. However, although treatment with daratumumab has shown remarkable clinically efficacy in heavily pretreated multidrug-refractory MM patients, there is still a marked heterogeneity of response with a fraction of MM patients not responding to daratumumab as a single agent. Furthermore, during treatment responding patients develop eventually progressive disease again. It is currently unknown which mechanisms underlie this differential therapeutic efficacy or development of refractory disease to daratumumab. In addition, combi-

nations of therapeutic agents are often superior in terms of depth of response and survival, when compared to treatment with a single agent, while combination therapies simultaneously target multiple pathologic pathways and might prevent escape and resistance mechanisms of tumor cells.
This also applies for combinations of pharmacological agents with therapeutic monoclonal antibodies (mAbs), which often result in superior effects compared to monotherapy. Indeed, the combination of mAbs targeting cell surface antigens on tumor cells with conventional chemotherapy or novel agents is already standard-of-care in several other hematologic malignancies such as non-Hodgkin's lymphoma and CLL as well as in solid tumors including breast cancer and colon carcinoma.[15]
Taken together, translational research is very important to modulate possible determinants of sensitivity of the tumor cell toward CD38-targeting antibodies with novel therapeutic approaches that consequently may lead to more effective regimens with presumable higher anti-myeloma activity with the ultimate aim of reaching increased quality of response and improvement in survival with the best possible quality of life.

Next to the introduction of (combinatorial) mAb-therapy in MM, also the evolution of other new treatment strategies is important to further improve outcome of refractory MM patients. In the last part of this thesis we present a clinical trial in which we combined continuous low-dose cyclophosphamide and prednisone with lenalidomide to initiate presumed synergistic activity in heavily pretreated lenalidomide-refractory MM patients. Also this strategy may offer new therapeutic perspectives for multi-drug refractory MM patients.

Altogether, the aim of this thesis is to create new treatment strategies for MM patients refractory to IMiDs and PIs who have a very poor prognosis to date. Studies were conducted on the following topics:

1. **Translational research**
   a. to improve daratumumab-mediated cell lysis to provide new combination strategies with higher anti-myeloma activity
   b. to gain insight into the host- and tumor-related factors in MM patients treated with daratumumab that might contribute to response and innate or acquired resistance with the aim to establish predictive factors for response and/or resistance to daratumumab and to develop new rationally designed combination therapies with daratumumab
2. A phase 1/2 prospective **clinical trial** to determine the maximal tolerated dose as well as tolerability and efficacy of Revlimid®, Endoxan® and prednisone (REP) in lenalidomide-refractory MM patients.

Translational studies

Combination strategies that simultaneously target multiple pathways might prevent tumor escape and development of resistance whereby presumably further improving efficacy of daratumumab. Since ADCC is an important effector mechanism of daratumumab, we explored the possibility of improving daratumumab-mediated cell-mediated cytotoxicity by blocking natural killer (NK) cell inhibitory receptors with the human monoclonal anti-KIR antibody IPH2102, next to activation of NK cells with the immune modulatory drug lenalidomide (**chapter 2**).
IPH2102 improves NK cell activity by blocking the three main killer cell immunoglobulin-like receptors (KIR2DL1/2/3) present on the cell surface of NK cells with their ligands, the human leukocyte antigen-C (HLA-C) molecules, present on tumor cells. Since the signals transmitted by inhibitory KIRs may prevent NK cell-mediated ADCC, even in the presence of an activating receptor-ligand interaction,[16] we set out to test the possibility to improve daratumumab-mediated efficacy by blocking these inhibitory KIRs. Lenalidomide, on the other hand, has been described to increase NK cell numbers and activation status.[17,18]
In 4 hour ADCC assays, IPH2102 did not induce lysis of MM cell lines, but it did significantly augment daratumumab-induced myeloma cell lysis. To confirm these results in a more physiological setting, we employed *ex vivo* flow cytometry-based cytotoxicity assays, in which we measured the survival of primary CD138+ MM cells in patients' bone marrow mononuclear cells (BM-MNCs), without separating malignant cells from their microenvironment and autologous effector cells. Also in this *ex vivo* setting, IPH2102 synergistically improved daratumumab-dependent lysis of primary myeloma cells in BM-MNCs (n=21), especially in patients carrying the FcγRIIIa-158F allele or the FcγRIIa-131R allele, who bind IgG1 with lower affinity than patients carrying the FcγRIIIa-158V allele or the FcγRIIa-131H allele.
Finally, adding lenalidomide to the daratumumab-IPH2102 combination further improved daratumumab-mediated lysis synergistically. These results suggest that more effective treatment strategies can be designed by combining daratumumab with agents that independently modulate NK cell function. These preclinical data, together with the results of the first clinical studies with daratumumab monotherapy and IPH2101 monotherapy, support the use of a combination of daratumumab with lenalidomide and IPH2102 in a clinical trial.
In **chapter 3** we demonstrated the susceptibility of malignant plasma cells, derived from lenalidomide- and bortezomib-refractory MM patients, to daratumumab. We showed *in vitro* and *ex vivo* that daratumumab may offer new therapeutic perspectives, also in lenalidomide and bortezomib-refractory MM patients, as has indeed recently been confirmed in the GEN501 and Sirius daratumumab monotherapy clinical studies. Furthermore, we hypothesized that, despite drug resistance of the malignancy, the immune system of these patients could still respond to the previously described immune-modulatory effects of lenalidomide[17–19] and bortezomib.[20,21] Confirming our hypothesis, the combination of daratumumab with lenalidomide or bortezomib significantly enhanced lysis, also in lenalidomide- and/or bortezomib- refractory patients. While the combination of daratumumab with bortezomib resulted in additive effects, lenalidomide synergized with daratumumab

through the activation of effector cells, especially NK cells. We showed that, although no longer effective as a therapeutic drug in these patients and patients being heavily pretreated, lenalidomide can still synergistically potentiate the anti-myeloma effects of daratumumab through the activation of NK cells. Finally, in an *in vivo* xenograft model, the combination of daratumumab with lenalidomide, in the presence of human NK cells, effectively delayed the tumorigenic growth of primary MM cells from a lenalidomide- and bortezomib-refractory patient, underscoring the potential beneficial anti-MM effects of the daratumumab plus lenalidomide combination for lenalidomide- and bortezomib-refractory MM patients. Advances in the daratumumab field are impressively fast. Our preclinical evidence showing that lenalidomide enhances daratumumab-mediated anti-MM activity formed the rationale for an ongoing phase 1/2 study of daratumumab and lenalidomide plus dexamethasone (GEN503) in relapsed/refractory myeloma. Preliminary results showed high response rates (an overall response rate of 83% with at least VGPR in 63% of patients treated with ≥4 cycles in part 2) that improved over time.[22] Based on these results, a multicenter randomized controlled phase 3 study was started comparing lenalidomide–dexamethasone with or without daratumumab in the relapsed/refractory setting (POLLUX study). A late-breaking abstract presented at last EHA showed encouraging activity and little additional toxicity, other than infusion-related reactions.[23]

In the perspective of other developments for relapsed and refractory MM patients, table 1 (and 2) summarizes other phase 3 studies testing combination strategies with lenalidomide (and bortezomib respectively). These tables may not be used for direct cross-trial comparison, since such a comparison might be biased by multiple factors as differences in trial sizes, patient populations, and study designs. Rather these tables are meant to give insights in the different tested combination strategies in the relapsed and refractory MM patient setting to date. Importantly, all summarized

TABLE 1

Clinical phase 3 combination studies with lenalidomide in the RRMM setting (1-3 prior lines, in lenalidomide-naive or lenalidomide-sensitive MM patients)

Study	N	Median lines of prior treatment	ORR (%)	Treatment	Median PFS (m)	PFS HR (95% CI)	Median OS (m)	OS HR (95% CI)
Pollux[23]	569	1 (1-11)	93 vs 76	DRd vs RD	NE vs 18.4	0.37 (0.27-0.52)	NE vs NE	0.64 (0.40-1.01)
Aspire[24]	792	2 (1-3)	87 vs 67	KRd vs Rd	26.3 vs 17.6	0.69 (0.57-0.83) p=0.0001	NE vs NE	0.79 (0.63-0.99) p=0.04
Eloquent-2[25;26]	646	2 (1-4)	79 vs 66	ERd vs Rd	19.4 vs 14.9	0.70 (0.57-0.85) P<0.001	43.7 vs 39.6	0.77 (0.61-0.97) p=0.257
Tourmaline-MM1[27]	722	2 (1-3)	78 vs 72	NRd vs Rd	20.6 vs 14.7	0.74 (0.59-0.94) p=0.012	NE vs NE	NE

clinical studies in Table 1 excluded patients with lenalidomide-refractory MM, while in Table 2 patients with bortezomib-refractory MM were excluded.
Also, based on ours and others provided preclinical data, the combination of daratumumab with bortezomib is evaluated in the relapsed/refractory setting in the ongoing, randomized controlled phase 3 CASTOR study, which compares bortezomib–dexamethasone with or without daratumumab in MM patients with at least one prior line of therapy (enrollment was completed September 2015).

TABLE 2

Clinical phase 3 combination studies with bortezomib in the RRMM setting (1-3 prior lines, in bortezomib-naive or bortezomib-sensitive MM patients)

Study	N	Median lines of prior treat-ment	ORR (%)	Treat-ment	Median PFS (m)	PFS HR (95% CI)	Median OS (m)	OS HR (95% CI)
Castor[28]	498	2 (1-10)	83 vs 63	DVd vs Vd	NE vs 7.2	0.39 (0.28-0.53) p<0.0001	NE vs NE	0.77 (0.47-1.26)
Endeavor[29]	929	2 (1-2)	77 vs 63	Kd vs Vd	18.7 vs 9.4	0.53 (0.44-0.65) p=0.0001	NE vs 24.3	0.79 (0.58-1.08) p=0.13
Panorama-1[30]	768	1 (1-3)	61 vs 55	PVd vs Vd	12.0 vs 8.1	0.63 (0.52-0.76) P<0.001	43.7 vs 30.4	0.77 (0.69-1.10) p=0.26

Table 2 summarizes the preliminary results from the Castor study, as presented at ASCO and EHA this year, as well as other combination phase 3 studies with bortezomib in the RRMM setting. In **Chapter 4** we investigated determinants of the sensitivity of MM cells towards daratumumab-mediated ADCC and CDC *in vitro*. In bone marrow samples from 144 MM patients, we observed no difference in daratumumab-mediated lysis between newly diagnosed or relapsed/refractory MM patients. These data suggest that resistance to classic anti-myeloma agents, such as steroids, anthracyclines, and alkylators, as well as novel agents (IMiDs and PIs), is not associated with decreased sensitivity to daratumumab-mediated ADCC and CDC. Furthermore, we showed, next to a clear association between effector (NK cells/monocytes) to target (MM cells) ratio and ADCC, a significant association between CD38 expression and the extent of daratumumab-mediated ADCC (127 patients) as well as CDC (56 patients). No significant difference in median CD38 expression on MM cells was observed between newly diagnosed and relapsed/refractory MM patients. In addition, experiments with isogenic MM cell lines expressing different levels of CD38, revealed that the level of CD38 expression is an important determinant of daratumumab-mediated ADCC and CDC.
Interaction of all-trans retinoic acid (ATRA) with nuclear retinoic acid receptors has been described to result in induction of CD38 expression. We confirmed an

upregulation of CD38 expression in MM by ATRA in our *in vitro* and *ex vivo* setting, which significantly improved daratumumab-mediated ADCC and CDC against MM cells. Importantly, ATRA augmented daratumumab-mediated CDC and ADCC against MM cells with low, intermediate or high levels of CD38 expression, as well as against MM cells derived from patients with double-refractory disease. Furthermore, ATRA improved the efficacy of daratumumab in MM cells, which were resistant to daratumumab as a single agent in CDC and/or ADCC assays.

Interestingly, the improvement in CDC was more pronounced than the enhancement of ADCC in both cell lines as primary MM samples, suggesting that ATRA also modulates CD38-independent determinants further benefiting CDC or compromising ADCC. We did not observe a decrease in the extent of ADCC by preincubating effector cells with ATRA, but we did find a significant downregulation of the complement-inhibitory proteins CD55 and CD59 induced by ATRA. It is possible that ATRA has additional CD38-independent activities that result in improvement of daratumumab-mediated lysis of MM cells. Finally, ATRA also significantly enhanced activity of daratumumab in a humanized MM mouse model.

Altogether, this study has identified multiple factors that influence the extent of MM cell lysis mediated by daratumumab. These factors may serve as biomarkers to predict response in daratumumab-based regimens. A better understanding of mechanisms underlying variability in sensitivity to daratumumab-mediated killing may also lead to novel strategies to enhance the effectiveness of daratumumab therapy. This study provided the rationale for clinical evaluation of ATRA and daratumumab in MM patients.

Daratumumab has been evaluated as a single agent in two clinical studies in heavily pretreated relapsed/refractory MM patients to date. These studies have shown that daratumumab monotherapy is well tolerated and that in the 16mg/kg cohort at least a partial response can be achieved in 35% of the patients including CR in 10%.[13,14] However, despite the well-established clinical efficacy of daratumumab, not all patients respond to single agent daratumumab, and the majority of patients who initially respond eventually progress. This indicates the need for new insights into mechanisms of response and resistance. Since CD38 expression is associated with the extent of daratumumab-mediated ADCC and CDC *in vitro* we hypothesized that cell surface expression of CD38 on MM cells might be one of the important factors predicting for response to daratumumab monotherapy in the clinical setting.

In **chapter 5** we demonstrated that daratumumab-treated patients who achieve at least partial response (PR) have higher CD38 baseline levels, when compared to patients with responses less than PR. Accordingly, response to daratumumab (at least PR) was markedly higher in the highest tertile of CD38 expression (48.5%), compared to the mid-tertile (22.2%), or lowest tertile (18.2%) of CD38 expression on MM cells in the pretreatment bone marrow samples of patients treated with daratumumab monotherapy. Importantly, CD38 gene expression levels were not associated with response in the Hovon-65/GMMG-HD4 study, in which patients did not receive daratumumab (data not shown). Moreover, we found that CD38 expression was not affected by prior therapies or by markers of aggressive disease, suggesting that CD38 expression is a marker for response to daratumumab rather than being a prognostic factor in general. However, given the considerable overlap

between responding and non-responding patients concerning CD38 expression, clinical outcome following daratumumab treatment cannot solely be explained by differential expression of CD38, which precludes its use as a definitive predictive biomarker of response to daratumumab in clinical practice. Therefore, CD38 expression is more a relative indicator of the likelihood of response, rather than a binary predictor of response. To further understand CD38-independent tumor-related mechanisms influencing daratumumab sensitivity, we investigated expression levels of complement-inhibitory proteins (CIPs) on MM cells. Overexpression of these CIPs in cancer is known to play a role in tumor immune evasion and resistance against therapeutic antibodies.[31–39]

Although reduction of CD55 and CD59 expression with phospholipase-C improved daratumumab-mediated CDC in cell lines with substantial CD38 expression, baseline expression levels of CIPs on MM cells were not associated with response in ptients treated with daratumumab monotherapy. However, at the time of development of resistance towards daratumumab we showed a significant upregulation of CD55 and CD59 on the MM cells. Also, *ex vivo* experiments showed that at the time of progression MM cells were resistant to daratumumab-mediated killing. Altogether, this suggests that CD55 and CD59 protein levels increase during the acquisition of a resistant phenotype. Next to the above described factors, it is likely that also other tumor-related factors such as genetic abnormalities and activation status of signaling pathways, as well as differences in the composition of the bone marrow microenvironment including frequency of immune effector and suppressor cells, might contribute to the variability in response to daratumumab.

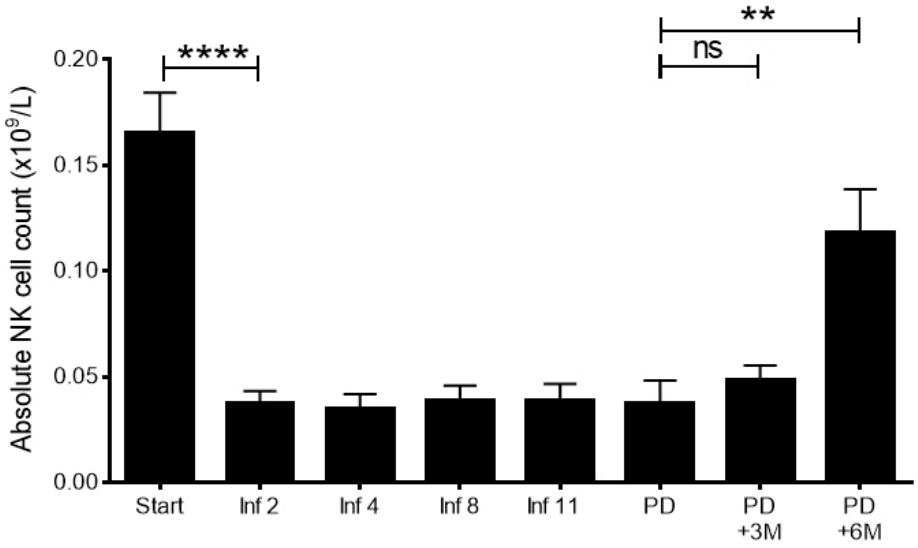
In two out of 21 patients we detected two coexisting populations of MM cells based on differential expression of CD55 and/or CD59. In both patients, we observed a change in subclone phenotypes with rapid selection of daratumumab-resistant clones with high expression of complement-inhibitors. These data support the importance of intraclonal heterogeneity in MM with multiple clones having a different clinical behavior and differential sensitivity to treatment.

In addition to the significant enhancement of CIPs at the time of progressive disease, we noticed a clear reduction in CD38 expression on non-depleted MM cells during treatment, which may also contribute in conferring protection against daratumumab. The reduced expression of CD38 is a transient phenomenon since approximately 6 months after the last daratumumab infusion BM-localized MM cells regained CD38 expression again. There was no difference between patients who did or did not achieve PR or better and extent of decrease in CD38 expression during daratumumab treatment. Importantly, to measure CD38 expression in our experiments, we used an anti-CD38 monoclonal antibody, HuMax-003-FITC, which binds to a different epitope compared to daratumumab.

This excluded the possibility that binding of daratumumab masked the detection of CD38. There might be several possible explanations for the reduction of CD38 levels on MM cells. First, in responding patients, daratumumab may select for tumor cells with lower CD38 expression, while preferentially eliminating MM cells with high CD38 levels. In addition, downregulation of CD38 may be an active process to evade daratumumab-mediated killing. Recent *in vitro* studies suggest that binding of

daratumumab to CD38 may cause redistribution of CD38 molecules, formation of distinct polar aggregates, and subsequent release of tumor microvesicles.[40] Finally, trogocytosis of CD38-daratumumab complexes by Fcγ receptor-expressing effector cells and direct internalization may also play a role in loss of CD38.[41]
Probably multiple of these mechanisms act together in the reduction of CD38 expression. Another important finding is the rapid decrease in absolute numbers of total and activated NK cells in whole blood and BM samples during daratumumab treatment (Figure 2). This reduction in NK cell numbers might be due to

FIGURE 2



NK cell numbers at baseline, during daratumumab treatment, at the time of progressive disease and post treatment

relatively high levels of CD38 expression on NK cells, which make them presumably more susceptible to daratumumab-mediated ADCC and CDC. Alternatively, NK cell numbers may decrease due to consummation during ADCC. Post-treatment NK cell numbers recovered to baseline values again as shown in Figure 2.
While baseline levels of NK cells (total and activated) between responders and non-responders were similar, a trend towards higher overall response rate was associated with greater reduction in NK cells following daratumumab treatment, suggesting that depletion of NK cells did not interfere with the clinical activity of daratumumab. No correlations were observed between reductions in the number of NK cells and onset of grade ≥3 adverse events (AEs), infections of any grade, grade ≥3 infections, or herpes zoster.[42] Spontaneous recovery of NK cell numbers, as well as of the restore of CD38 expression levels to baseline levels on MM cells post-treatment takes approximately 6 months after stopping daratumumab. We think that

this might be related to persisting circulating daratumumab during this period of time, resulting in continuous selective pressure. This is consistent with the fact that interference of daratumumab with the indirect Coombs test may persist up to 6 months (which is caused by binding of daratumumab to CD38-positive red blood cells). Importantly, since CD38 levels return to baseline values approximately 6 months after the last daratumumab infusion, retreatment with daratumumab may be effective and warrants further investigation.

Although the rapid loss of CD38 may allow MM cells to escape from daratumumab-mediated killing, CD38 reduction was observed in patients with both <PR and ≥PR, including those with sustained clinical response, which raises the possibility that the continuous pressure to maintain MM cells in a $CD38^{-/low}$ state might offer a clinical benefit in the treatment of CD38-positive malignancies. Physiological ligands for CD38 are CD31 present on BM stromal cells and endothelial cells, as well as hyaluronic acid, which is an extracellular matrix component. It has recently been demonstrated that overexpression of CD38 on MM cells results in increased adherence to BM stromal cells probably via CD38-CD31 interactions[43]. Reduced expression of CD38 may therefore lead to loss of cell-cell and cell-matrix contacts, which may contribute to reduced MM cell growth and survival. In addition, CD38 also functions as an ectoenzyme and in this role it has been implicated in immune suppression through production of adenosine in the BM microenvironment.[44,45] Daratumumab-mediated reduction of CD38 on MM cells may therefore contribute to an improved host-anti-tumor immune response.[46]

Another remarkable finding are the overall survival rates of MM patients treated with daratumumab monotherapy; even the patients with minimal response and stable disease show remarkable long OS rates with a median of 17.5 months, while the median PFS is only 3.7 months for this particular group of patients, suggesting that daratumumab might induce changes in drug resistance, for example by intervening with protective effects of the micro-environment or stimulating an antitumor immune effect or intervening with more aggressive subclones, whereby more sensitive or more indolent clone may re-emerge.

In the framework of mechanisms of action of daratumumab, it is possible that CDC, ADCC, direct effects and ADCP might be crucial for initial inducement of response; the significant association between CD38 and CDC/ADCC, NK cells/monocyte to MM cell ratio associated with ADCC, extent of CDC/ADCC associated with response, and baseline levels of CD38 associated with response, as well as the consumption of complement proteins C2 and C4 (and to a lesser extent C3, but not C1q), as the lowering of NK cells numbers (which might be due to consumption too) after the first daratumumab infusion, may advocate this hypothesis. During treatment these effector mechanisms of action might be less sufficient due to lower levels of CD38. However, during treatment, the $CD38^{-/low}$ state induced mechanisms of action might become important in containing and further deepening response by modulating the immune system. In **chapter 5** we demonstrated that ATRA is able to restore expression of CD38 and to reduce CD55 and CD59 levels on daratumumab-resistant MM cells coming from patients with progressive disease under daratumumab treatment. This resulted in significant enhancement of CDC and a modest improvement in ADCC. This differential effect can be explained by the fact that ATRA-mediated

reductions of CD55 and CD59 will improve CDC, but not ADCC. Furthermore, the moderate enhancement of ADCC may also be related to low frequencies of NK cells at the time of progression when compared to baseline values as described above, without ATRA affecting NK cell levels or their activity. Altogether, in **chapter 5** our data demonstrated an important role for CD38 and CIP expression levels in daratumumab sensitivity, which suggests that therapeutic combinations that alter CD38 and CIP expression levels should be investigated in the treatment of MM. Following these results, excitingly, a phase 1/2 clinical trial, conducted by us and others, combining ATRA with daratumumab at the time of progression to daratumumab monotherapy has start recruitment very recently.Since other tumors treated with mAb therapy show an increase in CD55 and CD59 levels on residual tumor cells as well,[47–39] this indicates that CIPs may be a broad resistance mechanism for mAbs that function through CDC. Therefore it might be worth to combine ATRA with other non-CD38 mAbs with the hypothesis to lower CIP-expression whereby enhancing antibody-mediated CDC. The same holds true for the previous described combination strategy with daratumumab and the IMiD lenalidomide; all antibodies working via ADCC might benefit from combination strategies with lenalidomide because of the described increase in NK cell numbers and activity benefiting ADCC, even though there are no expected direct effects with the respective tumor itself. Possibly the combination of both lenalidomide and ATRA with mAb therapy might be even more interesting. However, clinical studies should be performed to show safety and efficacy of such triple combination strategies.

MM is associated with immune dysfunction,[48] including immune evasion through expression of immune checkpoint ligands on plasma cells,[49] elevated adenosine receptor and adenosine activity,[50,51] and immune suppression through myeloid-derived suppressor cells (MDSCs) and regulatory T cell (T_{reg}) activity.[52–54] Daratumumab targets CD38-expressing myeloma cells through a variety of previously described immune-mediated mechanisms as CDC, ADCC, ADCP, and direct apoptosis with cross-linking. As described before, CD38 is ubiquitously expressed on MM cells,[55,56] but is also present on other immune cells. In **chapter 6** we explored daratumumab's effects on immune cell subsets and the following immunomodulatory effects during daratumumabtreatment.Given recent literature that regulatory and suppressive immune populations like MDSCs and regulatory B cells (Bregs) can express CD38,[57,58] we first evaluated these immune cells for their response to daratumumab treatment. Longitudinal analyses revealed that, in the myeloma setting, MDSCs and Bregs express relatively high levels of CD38 and that these cells were susceptible to daratumumab treatment. In addition to these $CD38^+$ suppressive cellular subsets, a novel subpopulation of Tregs ($CD4^+CD25^+CD127^{dim}$) was identified that also expressed high levels of CD38 and demonstrated superior autologous T-cell suppressive capacities. These cells were also sensitive to daratumumab and were significantly reduced in patients receiving treatment.

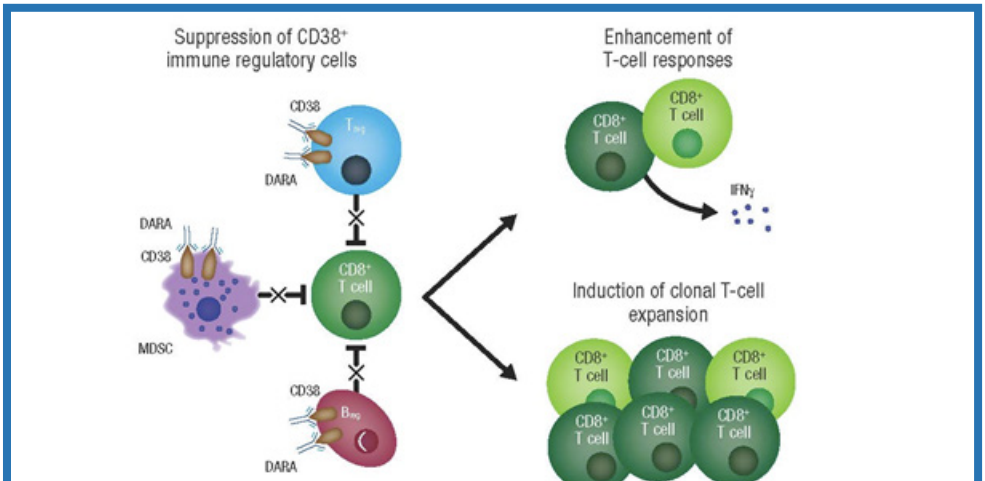
Daratumumab-mediated elimination of these $CD38^+$ immune-regulatory cells may reduce local immune suppression within the myeloma microenvironment and allow positive immune effector cells to expand and contribute to antitumor response.

Indeed, we observed significant increases in broad T cells populations, including both $CD4^+$ and $CD8^+$, in both peripheral blood and in the BM during

daratumumab treatment. Specific CD8+ subpopulations were altered with daratumumab therapy, including significant decreases in naive T cells and concomitant significant increases in effector memory $CD8^+$ T cells, indicating a shift in effector T cells towards an antigenic experienced phenotype that retained immunological memory and may be reactive against tumor antigens.

Ratios of $CD8^+$:$CD4^+$ and $CD8^+$:T_{reg}s also increased significantly with treatment, demonstrating a shift in positive versus negative immune regulators. Subsequently we assessed daratumumab's effects on T-cell activation and functionality. Patients demonstrated significant increases in IFN-γ secretion in response to viral and alloantigens following daratumumab treatment compared with baseline, for at least one time point during treatment, suggesting that T-cell function is not impaired by low CD38 expression. This increase appeared to be more marked in patients who responded to daratumumab with a PR or better than those who did not. Consistent with these results, virus-reactive T cells demonstrated an increase in proliferative capacity during daratumumab treatment. Interestingly, TCR clonality increased significantly during daratumumab treatment in the majority of patients measured and the TCR clonality change observed upon treatment was positively correlated with the increase in $CD8^+$ T cells. In addition, patients who responded to daratumumab had significantly greater increases in the sum of change in abundance (CIA) for each expanded T-cell clone in the TCR repertoire as well as greater maximum CIA of individual clones compared with patients without a clinical response. Altogether **chapter 6** describes previously unknown immunomodulatory effects of daratumumab through reduction of $CD38^+$ immune suppressive cellular populations and concomitant induction of helper and cytotoxic T-cell expansion, production of IFN-γ in response to viral peptides, and increased TCR clonality, indicating an improved adaptive immune response, as depicted in Figure 3. The extent of direct anti-tumor responses arising as a result of this adaptive response remains to be determined along with their contribution to clinical responses.

FIGURE 3



Immunomodulatory effects of daratumumab

TABLE 3

Clinical studies with daratumumab

————+	Phase	Disease	Setting	Treatment	Status
NCT00574288 (GEN501)	1/2	MM	Relapsed/ refractory	Daratumumab as single agent	Ongoing, not recruiting
NCT01985126 (SIRIUS; MMY2002)	2	MM	Relapsed/ refractory	Daratumumab as single agent	Ongoing, not recruiting
NCT01615029 (GEN503)	1/2	MM	Relapsed/ refractory	Daratumumab combined with lenalidomide–dexamethasone	Ongoing, not recruiting
NCT02076009 (POLLUX)	3	MM	Relapsed/ refractory	Lenalidomidedexamethasone versus lenalidomide–dexame-thasone plus daratumumab	Ongoing, not recruiting
NCT02136134 (CASTOR)	3	MM	Relapsed/ refractory	Bortezomib- dexamethasone versus bortezomib–dexamethasone plus daratumumab	Ongoing, not recruiting
NCT02519452	1	MM	Relapsed/ refractory	Daratumumab combined with rHuPH20 in subcutaneous formulation	Recruiting
NCT01998971	1b	MM	Relapsed/ refrac-tory and newly diagnosed	Daratumumab combined with pomalidomide–dexamethasone (RR), carfilzomib–dexamethasone (RR), bortezomib–dexamethasone (ND), bortezomib–thalidomide–dexamethasone (ND), bortezomib–melphalan–pred-nisone (ND) or carfilzomib–lenalidomide–dexamethasone (ND)	Recruiting
NCT02751255	1/2	MM	Relapsed/ refractory	Daratumumab combined with ATRA	Recruiting
NCT02807454 (FUSIONMM-03)	1/2	MM	Relapsed/ refractory	Daratumumab combined with durvalumab versus daratumumab combined with durvalumab, pomalidomide and dexamethasone	Not yet open
NCT02252172 (MAIA)	3	MM	Newly diagnosed, non-transplant eligible	Lenalidomide–dexamethaso-ne versus lenalidomide–dexamethasone plus daratumumab	Recruiting

NCT02195479 (ALCYONE)	3	MM	Newly diagnosed, non-transplant eligible	Bortezomib–melphalan–prednisone versus bortezomib–melphalan–prednisone with daratumumab	Recruiting
NCT02541383 (CASSIOPEIA)	3	MM	Newly diagnosed, transplant eligible	Randomization 1: bortezomib–thalidomide–dexamethasone induction therapy – high-dose melphalan plus autologous stem cell rescue – bortezomib–thalidomide–dexamethasone consolidation versus bortezomib–thalidomide–dexamethasone with daratumumab induction therapy – high-dose melphalan plus autologous stem cell rescue – bortezomib–thalidomide–dexamethasone with daratumumab consolidation Randomization 2: daratumumab as single agent in maintenance versus observation only	Recruiting
NCT02316106 (CENTAURUS)	2	Smoldering MM	Not previously treated	Daratumumab as single agent	Recruiting
NCT02816476 (AMYDARA)	2	AL-amyloidosis	Relapsed/refractory	Daratumumab as single agent	Not yet open
NCT02413489 (CARINA)	2	CD38-positive mantle cell lymphoma, diffuse large B-cell lymphma, or follicular lymphoma	Relapsed/refractory	Daratumumab as single agent	Recruiting

Clinical studies

Above described preclinical and translational studies performed by us, as well as studies conducted by others, provided a strong rationale for several clinical (combinatorial) studies with daratumumab in MM patients with relapsed and refractory as well as with newly diagnosed disease. Current and upcoming daratumumab trials are summarized in Table 3.

Next to above described clinical studies in table 3 with rationally designed combination strategies to further improve efficacy of daratumumab, also combination strategies with conventional therapeutics and 'novel' agents with supposed synergy might be a valuable salvage strategy for multi-refractory MM patients.

As described before, lenalidomide has multiple effects in MM, including direct anti-tumor activity, inhibition of adhesion of MM cells to stromal cells, and suppression of angiogenesis.[59] 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.[60–62] In addition, we have previously shown that, despite extensive pretreatment and lenalidomide-refractory myeloma, the immune system of lenalidomide-refractory MM patients is still able to respond to the immunomodulatory effects of lenalidomide.[63]

Similarly, administration of cyclophosphamide, at a dose substantially lower than the maximum tolerated dose (MTD) (metronomic dosing),[64] has, in contrary to high pulse-dosed cyclophosphamide, several immunomodulatory effects including anti-angiogenic effects,[65;66] modulation of the micro-environment,[67] and improvement of T and NK cell-mediated anti-tumor immune response via depletion of Tregs.[68–73]

We hypothesized that the addition of metronomically dosed cyclophosphamide to lenalidomide may be an attractive strategy for lenalidomide-refractory MM patients, via improvement of the patient anti-tumor immune response. Indeed, we have shown 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.[74]

To evaluate this combination prospectively, we describe in **chapter 7** a phase 1/2 study to determine the optimal dose and to assess its efficacy and safety in lenalidomide-refractory MM patients. The 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 PFS and OS 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.

The outcome of the REP regimen compares favorably to the results of next generation novel agents evaluated in lenalidomide-refractory patients. 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, REP-therapy was very tolerable; hematologic toxicities in our study were acceptable and consistent with the observed toxicities in MM patients treated with lenalidomide-dexamethasone[75,76]. 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. Discontinuations because of adverse events were uncommon, allowing patients to continue therapy until disease progression.

Although, the FDA and/or EMA approved several new well-appreciated agents in

relapsed/refractory MM as daratumumab, elotuzumab and carfilzomib to date, it may take some time before these therapies may be widely available in the clinic or reimbursed in many countries. REP, however, consists of medications generally available in the (outpatient) clinic. In addition, REP is a fully oral three-drug combination, which is convenient for patients, and likely associated with lower costs of patient care. Altogether this further highlights the importance of REP as effective salvage strategy for heavily pretreated relapsed and multidrug-refractory MM patients.

Other developments in the RRMM field

Next to above described combination strategies, also new generation novel agents are currently being evaluated in clinical trials, including second-generation IMiDs, such as pomalidomide, and second-generation proteasome inhibitors, such as carfilzomib, MLN9708 and marizomib. Furthermore, drugs belonging to other classes, such as histone deacetylase inhibitors, Akt inhibitors, mTOR inhibitors and several monoclonal antibodies, next to daratumumab, as elotuzumab (directed against SLAMF7/CS1) and tabalumab (directed against a B cell-activating factor) hold promise for improving the outcome of MM patients with lenalidomide and bortezomib refractory disease. Importantly, also other immunotherapeutic strategies, next to mAb therapy, such as CAR T cells, (combination therapy with) checkpoint inhibition and tumor vaccinations studies are promising in the treatment field of MM. These new treatment strategies together are expected to change the therapeutic scenario for multidrug-refractory MM patient who have a very poor prognosis to date.

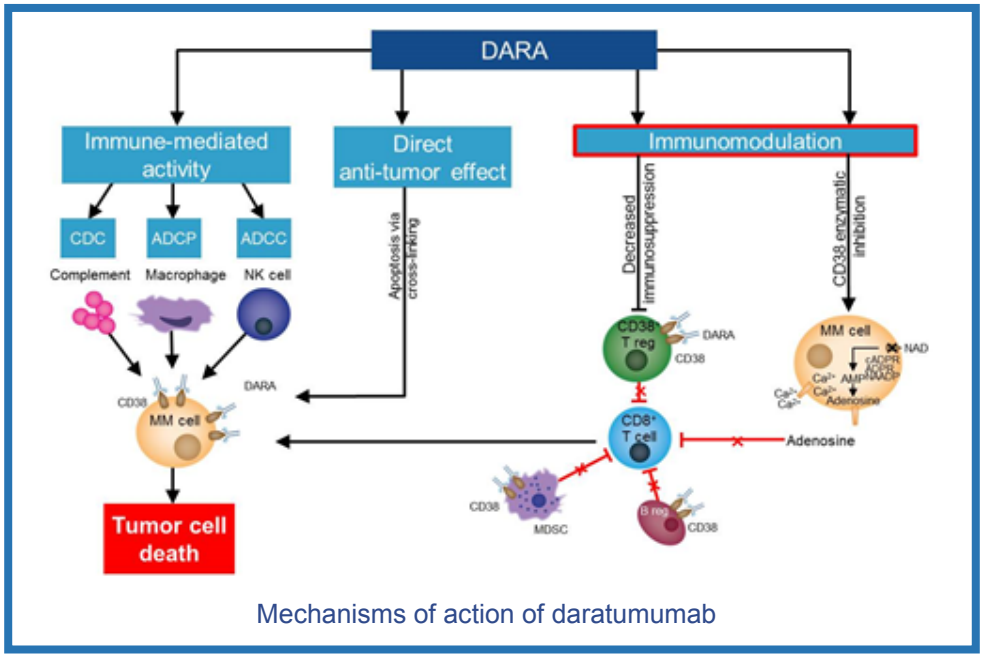
Conclusions and further perspectives

Recent advances with novel classes of drugs, including proteasome inhibitors and immunomodulatory agents, have significantly improved the prospects of MM patients. However, the vast majority of patients will eventually develop disease that is refractory to all available agents, which confers a very poor prognosis.[2;4] This thesis aimed to broaden the horizon especially for this category of multidrug-refractory MM patients.

A substantial part of the research described in this thesis involves daratumumab, a CD38-specific mAb that showed strong anti-tumor activity and engages multiple mechanisms of action, as depicted in Figure 4. While all of these actions are involved in the anti-tumor activity mediated by daratumumab, the exact contribution of each mechanism to the observed clinical activity needs to be further elucidated.

Several factors might have impact on the expected clinical outcome of daratumumab therapy, including host-related factors, such as frequency and activity of effector cells which may be affected by various prior anti-MM therapies, and tumor-related factors such as target antigen expression levels and expression of complement-inhibitory

FIGURE 4



Mechanisms of action of daratumumab

proteins. Also genetic variations in certain genes including FcγR polymorphisms[77] and KIRs may influence response to mAb-based therapy. Other factors of interest might be expression of inhibitory receptors such as PD-L1, which inhibits PD-1-positive NK and T cells, or CD47, which impairs phagocytosis, and may impact clinical outcome.

Daratumumab has shown anti-myeloma activity as a single agent, but enhancement of the anti-tumor efficacy has been preclinically demonstrated in combination with several agents. For example combinations with proteasome inhibitors and especially immunomodulatory agents and daratumumab has shown promising results in preclinical studies and excitingly also in recently presented clinical data (as presented in Table 1 and 2). Also ATRA combined with daratumumab might be a promising combination strategy, since ATRA enhances expression of the target antigen CD38 and lowers CIP-expression on MM cells, whereby significantly enhancing daratumumab-mediated efficacy in the preclinical setting and *in vivo* studies. A clinical phase 1/2 study with the combination of ATRA and daratumumab has started recruitment this year. Also triple combinations with daratumumab, ATRA and lenalidomide for example, or the combination with a checkpoint inhibitor may be interesting. However, the optimal combination strategies as well as the most appropriate clinical setting (e.g., smoldering, induction, maintenance, relapse) needs to be further elucidated.

In addition to MM, CD38 expression is observed in various other hematological malignancies, such as NHL and CLL, suggesting that CD38 mAbs and combination strategies with daratumumab, may be active in these indications as well. Since CD38 expression is in general lower on these malignancies than on MM cells, also

here, the combination with ATRA might be very interesting. In addition, following on the success of both rituximab and trastuzumab with regard to the development of subcutaneous delivery of mAbs in oncology, subcutaneous delivery of daratumumab in combination with recombinant human hyaluronidase is currently under investigation in a phase 1 clinical trial. A subcutaneous product will offer significant expected advantages over intravenous delivery in terms of convenience and the potential side effect profile. Finally, also combinations strategies with already well-established conventional regimens and novel agents might be valuable salvage strategies for multidrug-refractory MM patients, as we have shown for REP-therapy in lenalidomide-refractory MM patients. Importantly, REP is a well-tolerated and all oral regimen. Tolerability and convenience of therapy are of course very important factors in the decision for further treatment, especially in this heavily pretreated MM patient group. Translational research to unclear the underlying mechanisms of the supposed synergy between continuous low-dose cyclophosphamide and lenalidomide in the refractory setting is ongoing. These findings may subsequently lead to new rationally designed combination strategies with REP in the near future with the aim to further improve responses and outcome.
In conclusion, CD38 is an emerging therapeutic target for the treatment of hematological malignancies, in particular MM. The CD38 mAb daratumumab has shown encouraging activity as single agent in heavily pretreated MM patients.
Daratumumab has differentiating mechanisms of action and a limited toxicity profile, which allows favorable combination therapies with existing as well as emerging therapies. It is expected that the introduction of CD38 mAbs as a novel treatment option will significantly contribute to improved outcomes of myeloma patients.
Furthermore, daratumumab has therapeutic potential in indications beyond MM, in particular in other CD38-positive hematological malignancies. Recent, exciting data indicate that CD38-targeted inhibition of myeloid-derived suppressor cells could mediate anti-tumor activity through the release of T-cell suppression. In addition to hematological tumors, this immune stimulatory potential suggests a role for CD38 antibodies in the treatment of solid tumors. Also the combination with ATRA and/or lenalidomide might be interesting in this setting. Similarly mAbs working via CDC might benefit from combination strategies with ATRA, while mAbs working via ADCC might benefit from lenalidomide while its effect is mainly via the immune system as shown in the lenalidomide-refractory MM setting. However, further studies addressing this are warranted. Finally, the addition of continuous low-dose oral cyclophosphamide to lenalidomide and prednisone may offer a valuable salvage therapy for multi-refractory MM patients. The excellent response rate as well as the prolonged PFS, OS suggest true synergy between lenalidomide and cyclophosphamide, even in the lenalidomide-refractory setting. Translational studies to further investigate these presumed synergistic effects are ongoing and may yield new insights and new proposed combination strategies with REP in the near future to further improve prognosis for multidrug-refractory MM patients. Altogether our translational and clinical efforts has led to already available new treatment options as well as several new clinical trials with rational designed combination strategies with presumed synergy in lenalidomide- and bortezomib-refractory MM patients. Hopefully this may lead to new perspectives on the horizon for these multidrug-refractory MM patients.

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Nederlandse Samenvatting

Introductie

Multipel myeloom, ook wel de ziekte van Kahler genoemd, is een vorm van beenmergkanker gekarakteriseerd door ongecontroleerde woekering van monoklonale plasmacellen in het beenmerg. Het is de op één na meest voorkomende hematologische maligniteit in de Westerse wereld. Per jaar wordt in Nederland bij ongeveer 1100 mensen de diagnose multipel myeloom gesteld. In totaal zijn er in Nederland circa 4000 patiënten met deze ziekte. Het gaat vooral om mensen boven de 60 (80%) en er zijn iets meer mannen dan vrouwen met het multipel myeloom.
Plasmacellen behoren tot de witte bloedcellen, en zijn verantwoordelijk voor een deel van de afweer tegen bacteriën en virussen door de productie van antistoffen. Infiltratie van de maligne plasmacellen in het beenmerg kan leiden tot destructie van de architectuur van het beenmerg en disfunctioneren van de normale hematopoëse. Productie van paraproteïne door de myeloomcel kan leiden tot orgaandisfunctie. Dit kan zich uiten in hypercalciëmie, nierinsufficiëntie, anemie, lytische bothaarden, en verhoogde vatbaarheid voor infecties, de zogenaamde CRAB-criteria.
De overleving van patiënten met het multipel myeloom is de afgelopen 2 decennia verbeterd, met name door toepassing van autologe stamceltransplantatie bij de jongere patiënten en door de introductie van nieuwe geneesmiddelen, zoals de immuunmodulerende drugs (IMiD's, zoals thalidomide en lenalidomide) en de proteosoominhibitor bortezomib. Deze middelen zijn effectief bij zowel nieuw gediagnosticeerde myeloompatiënten, als ook in de recidiefsetting. IMiD's en proteosoomremmers hebben naast direct anti-myeloomactiviteit ook verscheidene indirecte anti-tumor effecten, zoals immuunmodulatie en remming van de angiogenesis.
Het merendeel van de myeloompatiënten reageert aanvankelijk goed op anti-myeloom therapie, maar uiteindelijk overlijdt de meerderheid van de patiënten aan multi-drug resistente ziekte. Met name lenalidomide- en bortezomib-refractaire patiënten hebben een zeer slechte prognose.
Een recente analyse van lenalidomide- en bortezomib-resistente patiënten toonde een mediane overleving van slechts 9 maanden. Er is geen standaardbehandeling voor deze groep patiënten. Onderzoek naar nieuwe therapeutische mogelijkheden voor deze groep patiënten is daarom erg belangrijk.

Een belangrijke recente toevoeging aan het arsenaal van nieuwe therapeutische mogelijkheden is de ontwikkeling van de monoklonale antistof daratumumab, gericht tegen CD38. CD38 komt sterk tot expressie op multipel myeloom-cellen, maar ook op normale plasmacellen en andere cellen zoals NK-cellen, monocyten, onrijpe en geactiveerde B-cellen en geactiveerde T-cellen. Daratumumab doodt myeloom tumorcellen via verschillende mechanismen, waaronder complement-afhankelijke cytotoxiciteit (CDC), antistof-afhankelijke celgemedieerde cytotoxiciteit (ADCC) en antistofafhankelijke-cellulaire fagocytose (ADCP). In 2 recente studies met daratumumab-monotherapie (GEN501- en Sirius-studie) werd bij uitgebreid voorbehandelde patiënten met multipel myeloom minstens een partiële respons behaald bij 30% van de patiënten. Er werden zelfs complete responsen behaald, wat bij deze groep patiënten uitzonderlijk is. In het fase 2-deel van de GEN501-studie

resulteerde behandeling met 16 mg/kg daratumumab in een mediane progressievrije overleving van 5,6 maanden en een algehele overleving na 1 jaar van 77%. In de Sirius-studie was de mediane responsduur 7,4 maanden, de mediane progressievrije overleving 3,7 maanden en de algehele overleving na 1 jaar 65%. Op basis van deze 2 studies is daratumumab als monotherapie inmiddels door de FDA in de Verenigde Staten en door de EMA in Europa goedgekeurd voor de behandeling van patiënten met recidief multipel myeloom die minstens 3 voorgaande behandelingen hebben gehad, waaronder therapie met lenalidomide en bortezomib, of indien er sprake is van dubbel-refractaire ziekte voor lenalidomide en bortezomib. Daratumumab werd in deze studies goed verdragen en geeft niet de toxiciteit zoals die bij de huidige beschikbare middelen nogal eens wordt gezien, zoals beenmergsuppressie of neuropathie. Helaas ontwikkelt ook bij daratumumab-therapie het grootste deel van de patiënten een recidief van het multipel myeloom. Het is belangrijk om te weten te komen welke factoren van belang zijn voor respons op daratumumab en welke factoren bijdragen aan de ontwikkeling van resistentie tegen daratumumab. Zo zou je rationele keuzes kunnen maken of je de therapie aan zou moeten bieden aan de betreffende patiënt. Daarnaast kan een beter begrip van mechanismen, die van belang zijn voor respons en resistentie van myeloom tumorcellen, bijdragen aan de ontwikkeling van nieuwe specifieke en effectieve combinatietherapieën voor de behandeling van het multipel myeloom, die de werking van daratumumab naar verwachting effectiever zal maken. Andere mogelijkheden om het behandelarsenaal van de multi-drug refractaire multipel myeloom patiënt uit te breiden, met als doel de levensverwachting en de kwaliteit van leven te verbeteren, kunnen ook combinaties van bestaande middelen zijn, die naar verwachting zodanig synergistisch werken dat ze effectief zijn bij multipel myeloompatiënten die refractair zijn voor lenalidomide en bortezomib.

Samenvatting van de hoofdstukken

Hoofdstuk 1 geeft een introductie over het multipel myeloom. Ook wordt hierin de rationale en inhoudsopgave van het proefschrift beschreven. In **Hoofdstuk 2** beschrijven we de *in vitro* en *ex vivo* resultaten van het verrichte translationele onderzoek gericht op het versterken van het werkings-mechanisme ADCC van daratumumab. ADCC vindt met name plaats via natural killer (NK) cellen. NK cellen bezitten echter receptoren die de werking van NK-cellen kunnen remmen (killer cel immunoglobulin (KIR-) receptoren genoemd), wanneer deze receptoren zich binden aan hun ligand (HLA klasse I moleculen). Multipel myeloomcellen brengen dit HLA klasse I ligand hoog tot expressie, waardoor naar verwachting de NK-cellen niet optimaal gestimuleerd kunnen worden tot ADCC door daratumumab. Om de binding tussen deze KIR-receptoren op de NK-cellen en hun ligand op de myeloom tumorcel te blokkeren, om zo dit remmende effect op NK-cellen teniet te doen en naar verwachting de daratumumab-geïnduceerde ADCC te verbeteren, hebben we daratumumab in het laboratorium gecombineerd met het humane monoklonale anti-KIR antilichaam IPH2102. Inderdaad was de mate van ADCC significant hoger bij de combinatie van daratumumab met IPH2102 in zowel

de *in vitro* uitgevoerde experimenten met myeloom cellijnen en gezonde donor NK-cellen, als in de *ex vivo* experimenten met patiëntenbeenmerg, waarin de myeloomcellen en de NK-cellen van de patiënt zelf in het experiment getest werden. Wanneer we lenalidomide aan deze combinatie toevoegden, waarvan reeds bekend is in de literatuur dat het in staat is het aantal en de activatiestatus van NK-cellen te verhogen bij myeloompatiënten, werd de ADCC verder significant verhoogd. Zowel de combinatie daratumumab en IPH2102, als de toevoeging van lenalidomide had een synergistisch effect. Deze laboratoriumresultaten suggereren dat daratumumab effectiever zijn werk zou kunnen doen wanneer gecombineerd met NK-cel stimulerende therapieën. In **hoofdstuk 3** hebben we zowel *in vitro*, *ex vivo*, en *in vivo* middels een muizen experiment laten zien dat daratumumab ook werkzaam is bij lenalidomide- en bortezomib-refractaire myeloompatiënten, ondanks de uitgebreid voorbehandelde status van deze patiënten. Verder hebben we laten zien dat de combinatie bortezomib en daratumumab additief werkt, maar dat de combinatie lenalidomide en daratumumab synergistisch samenwerken, zelfs in de setting van het bortezomib- en lenalidomide-refractair multipel myeloom. We toonden aan dat bij lenalidomide-refractaire ziekte de direct toxische effecten van lenalidomide op de myeloom tumorcel weliswaar niet meer aanwezig zijn, maar dat het immuunstimulerende effect, waaronder NK-cel stimulatie, nog wel mogelijk is in deze setting.

De bovenbeschreven preklinische studies hebben geleid tot nieuwe klinische studies zoals de fase 3 Castor en Pollux studies in de setting van het recidief multipel myeloom, waarin bortezomib-dexamethason al dan niet gecombineerd met daratumumab en lenalidomide-dexamethason al dan niet gecombineerd met daratumumab gerandomiseerd werden getest. Beide studies lieten veelbelovende resultaten zien. In de Castor-studie behaalden 83% van de patiënten die behandeld werden met de combinatie daratumumab-bortezomib-dexamethason een partiële respons of beter versus 63% van de patiënten die behandeld werden met bortezomib-dexamethason. In de Pollux-studie behaalde zelfs 93% van de patiënten behandeld met daratumumab-lenalidomide-dexamethason een partiële respons of beter versus 76% van de patiënten behandeld met lenalidomide-dexamethason. Voor betrouwbare data ten aanzien van progressievrije overleving en mediane overleving is de follow-up nu nog te kort.

In hoofdstuk 4 hebben we onderzocht welke factoren van belang zijn voor daratumumab-geïnduceerde ADCC en CDC. In beenmergsamples van 144 myeloom-patiënten hebben we *ex vivo* laten zien dat er geen significant verschil bestaat in daratumumab-geïnduceerde kill tussen nieuw gediagnosticeerde myeloom patiënten versus recidief patiënten versus gerecidiveerde multi-refractaire myeloom patiënten. Dit suggereert dat de opgedane weerstand van de myeloom tumorcel door eerdere behandelingen, zoals door eerdere therapie met steroïden, anthracyclines, alkyleerders, IMiD's en proteosoomremmers, geen effect heeft op de gevoeligheid voor daratumumab-gemedieerde myeloom celdood. Factoren van belang voor daratumumab-geïnduceerde ADCC was de ratio NK-cellen en

myeloomcellen in het beenmerg, de ratio monocyten en myeloomcellen in het beenmerg en de mate van CD38 expressie op de myeloomcel. De mate van CD38 expressie was ook geassocieerd met de mate van daratumumab-geïnduceerde CDC. De mediane CD38 expressie was niet verschillend tussen nieuw gediagnosticeerde myeloom patiënten versus recidief patiënten versus gerecidiveerde multi-refractaire myeloom patiënten. We hebben in **hoofdstuk 4** ook laten zien dat all-trans retinoic acid (ATRA) *in vitro* en *ex vivo* de CD38 expressie op de myeloomcel omhoog brengt en dat de combinatie ATRA en daratumumab de daratumumab-gemedieerde ADCC en CDC daarmee significant verhoogd. Opvallend was echter dat in deze experimenten de CDC in een grotere mate werd verbeterd dan de ADCC. Bij nader onderzoek bleek ATRA, naast CD38 verhoging op de myeloomcel, tevens een significante verlaging van CD55 en CD59 (beschermende eiwitten tegen CDC) te veroorzaken, waardoor de tumorcel minder bescherming heeft tegen CDC en beter kan worden gedood door daratumumab-gemedieerde CDC wanneer gecombineerd met ATRA. Ook in *in vivo* muizen experimenten zagen we dat de combinatie daratumumab met ATRA superieur was boven daratumumab monotherapie.

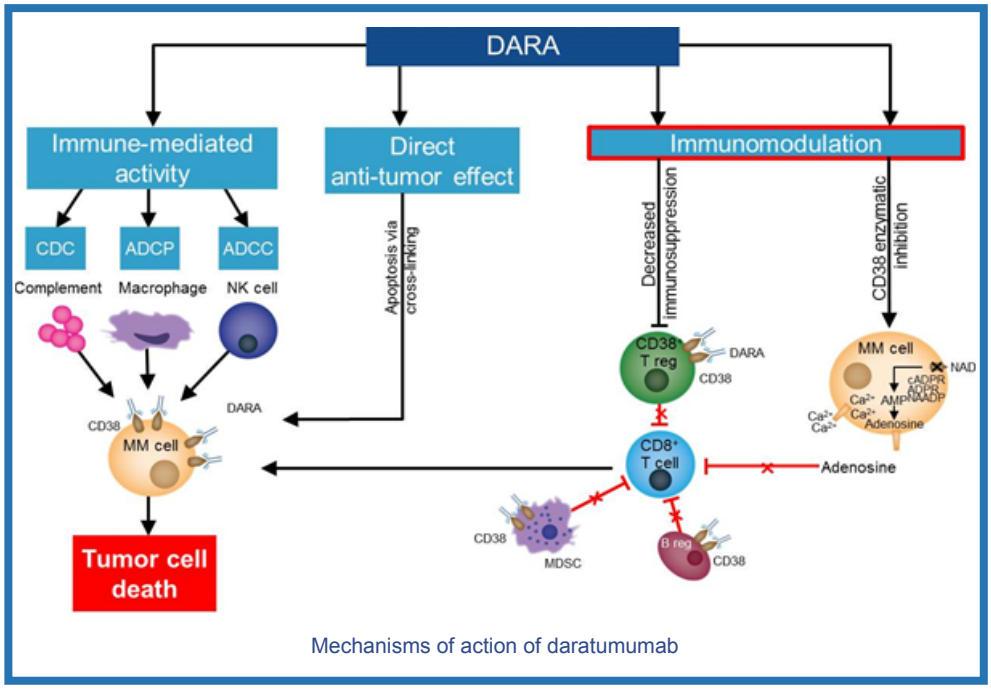
In **Hoofdstuk 5** hebben we het bloed en beenmerg getest van de myeloom patiënten behandeld met daratumumab monotherapie in de GEN501- en Sirius-studie. We hebben gekeken naar factoren belangrijk voor respons en (de ontwikkeling van) resistentie tegen daratumumab. De mate van expressie van CD38 op de myeloomcel voor start behandeling is een belangrijke factor voor respons; zo hebben patiënten met een partiële respons of hoger op daratumumab-behandeling als groep een significant hogere CD38 expressie op de myeloomcel voorafgaande aan start behandeling dan patiënten die minder dan een partiële respons hebben behaald op daratumumab therapie. Omgekeerd hebben we de CD38 expressie op de myeloomcel voor start behandeling met daratumumab ingedeeld in tertielen en is het aantal responders (partiële respons of beter) significant beter in de hoogste CD38 expressie tertiel (48.5%), ten opzichte van de middelste (22.2%) of laagste tertiel (18.2%). De mate van CD38 expressie op de myeloomcellen van de patiënten werd niet beïnvloed door voorgaande therapieën, danwel door markers passende bij agressieve ziekte. Verder was in de HOVON65 studie, een 1e-lijnstudie met multipel myeloom patiënten waarin geen behandeling met daratumumab, de CD38 genexpressie niet geassocieerd met respons.
Dit suggereert dat CD38 inderdaad een marker is voor de kans op respons op daratumumab, en niet een prognostische marker voor het multipel myeloom in het algemeen. Echter, gezien de overlap in CD38 expressie op de myeloomcel voor start behandeling tussen responders en non-responders, is het niet de enige factor van belang voor respons en is de mate van CD38 expressie daarmee niet te gebruiken als een selectieve predictieve marker voor respons om te bepalen welke patiënten je wel of niet de behandeling met daratumumab aan zou moeten bieden. De mate van expressie van CD55 en CD59 (complement-remmende eiwitten op de celmembraan) op de myeloomcel voor start daratumumab had geen voorspellende waarde voor respons. Daarentegen was de expressie van CD55 en CD59 op de myeloomcel wel significant verhoogd ten tijde van het ontstaan van refractaire ziekte

tov start behandeling, terwijl er geen significante verhoging van de expressie van deze eiwitten plaatsvind tijdens behandeling, wanneer de patiënt nog responsief is op behandeling. Mogelijk spelen deze complement-remmende eiwitten dus een rol bij het refractair worden van de myeloomcel tegen daratumumab. Andere factoren die mogelijk een rol zouden kunnen spelen bij de mate van respons en het ontstaan van resistentie zijn genetische factoren en activatiestatus van signaling pathways, of veranderingen in het beenmerg micromilieu tijdens behandeling, zoals ook de hoeveelheid en activatiestatus van effector cellen en immuunsuppressieve cellen. Een andere belangrijke bevinding van deze studie is de significante daling van CD38-expressie op de myeloomcel tijdens behandeling met daratumumab. Dit zien we bij alle patiënten en treedt onafhankelijk van de mate en duur van respons op. Deze significante daling zien we al na 1 gift daratumumab optreden en blijft aanwezig gedurende de behandeling tot +/- 6 maanden na staken daratumumab-therapie, waarna herstel van CD38-expressie richting de uitgangssituatie plaatsvindt. Deze gemeten CD38-daling is een reële daling, gezien de CD38-antistof die we gebruiken voor het vervolgen van de CD38 expressie tijdens daratumumab behandeling aan een ander epitoop op het CD38 molecuul bindt dan dat daratumumab zich bindt. Er zijn meerdere mogelijke verklaringen voor deze CD38-daling tijdens daratumumab therapie; het zou het gevolg kunnen zijn van selectie, doordat de cellen met hoge CD38 expressie geattaqueerd worden door daratumumab en de cellen met de laagste expressie overblijven. Maar het zou ook een actief proces van de myeloom tumorcel kunnen zijn om te ontsnappen aan daratumumab-gemedieerde kill.
Recente *in vitro* studies laten zien dat ten gevolge van daratumumab therapie er redistributie van de CD38 moleculen plaatsvindt met dientengevolge clustering van CD38 op de celwand, waardoor de CD38 in microvesicles loslaat van de myeloomcelwand. Ook trogocytose (het 'afknabbelen' van CD38-moleculen van de celwand door effectorcellen als monocyten) of internalisatie (het naar binnen verplaatsen van de CD38-moleculen naar het binnenste van de cel ipv CD38 op de celwand houden) zouden een rol kunnen spelen. Mogelijk spelen meerdere mechanismen hier een rol. Tijdens deze studie zagen we ook een daling van het absolute aantal NK-cellen tijdens daratumumab-behandeling optreden, zowel in bloed als beenmerg.
Deze daling zou het gevolg van verbruik van NK cellen tijdens ADCC kunnen zijn. Een alternatieve verklaring is dat NK-cellen door hun relatieve hoge CD38 expressie geattaqueerd worden tijdens daratumumab-therapie. Het absolute aantal NK-cellen herstelt naar uitgangswaarde circa 6 maanden na staken van therapie met daratumumab. Deze 6 maanden na de laatste gift daratumumab komt overeen met de periode dat daratumumab nog aanwezig lijkt in het bloed, gezien de coombstest ook tot ongeveer 6 maanden na laatste gift daratumumab positief blijft, wat veroorzaakt wordt door binding van daratumumab aan CD38 op de rode bloedcel.
Tot slot hebben in **hoofdstuk 5** laten zien dat ATRA ook ten tijde van progressieve ziekte tijdens daratumumab behandeling in staat is om de CD38-expressie op de myeloomcel te verhogen richting uitgangssituatie en de CD55 en CD59 expressie te verlagen richting uitgangssituatie, wat resulteerde in een significante verbetering van met name de CDC. ADCC was in deze fase van behandeling niet goed te

herstellen, wat verklaard zou kunnen worden door het lage aantal NK-cellen tijdens behandeling met daratumumab. Bovenbeschreven laboratoriumstudies hebben de rationale gevormd voor een nieuwe klinische fase 1/2 studie met daratumumab en ATRA, welke recent geopend is voor inclusie. In deze studie wordt bij uitvoerig voorbehandelde patiënten daratumumab gecombineerd met ATRA, indien de respons op daratumumab-monotherapie beperkt is of als er progressie is tijdens daratumumab monotherapie.

Het multipel myeloom is een ziekte geassocieerd met immuundisfunctie ten gevolge van effecten van de myeloomcel en zijn interacties met het beschermende micromilieu, zoals ontsnappen van de myeloom tumorcel aan het immuunsysteem door het tot expressie brengen van het immuun checkpoint ligand PDL1 op myeloom cellen, waardoor de activatie van het immuunsysteem wordt geremd, en door de verhoogde aanwezigheid van immunosuppressieve cellen bij patiënten met het multipel myeloom, zoals regulatoire T-cellen (Tregs), regulatoire B-cellen (Bregs), en monocyt-afkomstige suppressieve cellen (MDSC's). In **hoofdstuk 6** hebben we ons gefocust op het effect van daratumumab op immuuncellen en de effecten op het immuunsysteem die ten gevolge hiervan zouden kunnen optreden. Immunosuppressieve cellen, zoals Tregs, Bregs, en MDSC's brengen CD38 tot expressie. *In vitro* onderzoek met het bloed en beenmerg van patiënten behandeld met daratumumab monotherapie in de GEN501- en Sirius-studie laat zien dat het aantal Bregs, Tregs en MDSC's daalt tijdens behandeling met daratumumab. We zagen dat met name de Tregs die CD38 hoog tot expressie brengen, en

FIGURE 1



Mechanisms of action of daratumumab

dientengevolge het meest worden weggevangen tijdens therapie met daratumumab, het meest immunosuppressief blijken te zijn in laboratoriumtesten. Ten gevolge van deze absolute daling van CD38+ immunosuppressieve cellen, vindt toename en activatie van cytotoxische T-cellen en T-helpercellen plaats, neemt de productie van interferon-Ɣ (IFN-Ɣ) toe en zagen we ook een verhoogde T-celreceptor klonaliteit, wat zou kunnen wijzen op een adaptieve anti-tumor immuunrespons ten gevolge van daratumumab. Dit is een nieuw beschreven werkingsmechanisme van daratumumab. De mate en specificiteit van deze anti-myeloom immuunrespons, alsmede de bijdrage aan klinische respons is momenteel onderwerp van verder onderzoek. De werkingsmechanismen van daratumumab, zoals nu bekend, staan weergegeven in figuur 1.

Naast de rationale combinatiestrategieën met daratumumab, die oa uit voorgaand translationeel onderzoek zijn voortgekomen en die de daratumumab-behandeling naar verwachting effectiever zullen maken, kunnen ook combinaties tussen conventionele therapieën en/of met novel agents dusdanig synergistisch werken, dat ook een dergelijke combinatiestrategie een welkome aanvulling kan zijn op het arsenaal van therapeutische mogelijkheden voor de multi-drug resistente myeloompatiënt.

In **hoofdstuk 7** beschrijven we de resultaten van de REPEAT-studie, een multicentrum, prospectieve, niet-gerandomiseerde, open-label fase 1/2-studie.
Patiënten met een recidief MM refractair voor lenalidomide kwamen voor deze studie in aanmerking en werden in deze studie behandeld met opnieuw lenalidomide (Revlimid®), met hieraan toegevoegd laag gedoseerd cyclofosfamide (endoxan®) en prednison (het REP-regimen). Deze combinatietherapie wordt gegeven tot aan progressie. Cyclofosfamide en prednison worden continu gegeven. Lenalidomide wordt 21 dagen per 4 weken ingenomen. Alle middelen zijn oraal gedoseerd.
We hebben in ons studieprotocol gekozen voor het toevoegen van een continu lage orale dosis cyclofosfamide (‘metronomic dosing’) in verband met de beschreven anti-angiogene en immunomodulatoire effecten die hiervan uitgaan. De rationale voor de combinatie van lenalidomide, cyclofosfamide en prednison wordt gevormd door de verwachte synergie tussen lenalidomide en metronoom gedoseerd cyclofosfamide, gezien de beschreven immunologische effecten van deze middelen die naar verwachting complementair zijn en synergistisch zouden kunnen werken. We hebben eerder in laboratoriumstudies reeds laten zien dat patiënten die refractair geworden zijn voor lenalidomide weliswaar geen direct toxische effecten op de myeloom tumorcel meer laten zien, maar dat de immunomodulatoire effecten van lenalidomide in deze setting behouden blijven.
Vandaar dat deze combinatie, ondanks de setting van lenalidomide-refractair myeloom, hypothetisch toch heel mooi samen zou kunnen werken. Ook hebben we in een kleine retrospectieve analyse laten zien dat de volledig orale combinatie van lenalidomide, cyclofosfamide en prednison haalbaar en effectief is bij uitgebreid voorbehandelde lenalidomide-refractaire multipel myeloompatiënten.
De optimale dosis van lenalidomide met continu cyclofosfamide en prednison was echter nog niet bekend. Dit heeft geleid tot de huidige fase I-studie, om de maximaal

tolereerbare dosis (MTD) van lenalidomide en cyclofosfamide vast te stellen om met deze dosis een fase II-studie te verrichten om de effectiviteit en toxiciteit te kunnen beoordelen; de REPEAT-studie.
De MTD werd in de fase I studie bepaald middels een 3+3 dosis escalatie design, waarbij de dosis lenalidomide per dosis level geleidelijk werd opgehoogd (van 10 naar 15 naar 25mg) en werd getest met dagelijks 50 of 100mg cyclofosfamide oraal in de verschillende dosis niveaus. De MTD bleek te zijn: 25mg lenalidomide (dag 1-21/28 dagen) met dagelijks 50mg cyclofosfamide en 20mg prednison (beiden dag 1-28). Na bereiken van een stabiele respons werd de dosis prednison naar 10mg/dag verlaagd. In het fase I gedeelte van de studie werden 21 patiënten behandeld, waarvan 6 patiënten in het dosisniveau van de uiteindelijke MTD. Tromboseprofylaxe werd gegeven middels ascal of profylactisch fraxiparine (indien trombose in de voorgeschiedenis), antibiotica-profylaxe werd gegeven met cotrimoxazol. Tevens werd maagbescherming gegeven en osteoporoseprofylaxe volgens lokale richtlijnen. In het fase 2 gedeelte van de studie werden 61 patiënten behandeld. 67 patiënten werden in totaal in deze studie behandeld in de MTD-dosering.
Het percentage responders (partiële respons of beter) in deze uitgebreid voorbehandelde lenalidomide-refractaire myeloom patiënten (waarvan ook 66% bortezomib-refractair) was 67%. 83% van de patiënten behaalden een minimale respons of beter. De mediane progressievrije overleving en mediane overleving was respectievelijk 12.1 en 29.0 maanden. De REP-therapie werd goed verdragen; het percentage patiënten met hematologische toxiciteit kwam overeen met de toxiciteit die we kennen van de patiënten die we behandelen met de combinatie lenalidomide-dexamethason. Neutropenie (22%) en trombopenie (22%) waren de meest voorkomende hematologische bijwerkingen. Infecties (21%) waren de meest voorkomende niet-hematologische bijwerkingen. Er waren weinig patiënten die moesten stoppen met REP-therapie vanwege bijwerkingen; de meeste patiënten verdroegen de therapie dusdanig goed dat ze de behandeling konden voortzetten tot ontstaan van progressieve ziekte. Wat belangrijke voordelen zijn van deze behandeling voor de patiënt, is dat de middelen allemaal oraal kunnen worden genomen, wat prettig is voor de patiënt en ook relatief weinig ziekenhuisbezoek met zich meeneemt. Er is inmiddels ruime ervaring met deze orale middelen. Deze medicijnen zijn in vrijwel alle landen geregistreerd en goedgekeurd, wat de combinatietherapie toegankelijk maakt voor de patiënt en relatief betaalbaar maakt. We zien REP dan ook als een effectieve en belangrijke aanvulling aan het arsenaal van behandelmogelijkheden voor de multi-drug refractaire myeloom patiënt.

Analyses op het bloed en beenmerg van de patiënten, verzameld voor start en tijdens behandeling met REP, zijn gaande en zullen naar verwachting meer inzicht geven in de onderliggende verklaringen voor de vermeende synergie tussen lenalidomide en metronoom gedoseerd cyclofosfamide bij de lenalidomide-refractaire multipel myeloom patiënten in deze studie.

Conclusie

In mijn proefschrift hebben we translationeel onderzoek verricht naar factoren die belangrijk zijn voor respons op daratumumab en naar factoren belangrijk in het ontstaan van refractaire ziekte op de CD38 antistof daratumumab. Dit heeft richting gegeven aan nieuwe rationele combinaties van therapie met daratumumab in deze multidrug-refractaire patiëntengroep om de werking van daratumumab en daarmee de prognose van deze patiëntengroep te verbeteren. Deze opgedane kennis leidde tot de initiatie van nieuwe klinische studies om deze combinatiestrategieën verder te evalueren. Daarnaast hebben we aangetoond dat daratumumab een T-celrespons lijkt te induceren, die mogelijk ook bijdraagt aan het anti-myeloomeffect van daratumumab. Tot slot hebben we een klinische studie verricht in lenalidomide-refractaire myeloom patiënten waarin we hebben aangetoond dat de combinatie van lenalidomide-cyclofosfamide-prednison (het REP-regimen) goed verdragen wordt en erg effectief kan zijn in deze uitgebreid voorbehandelde groep multi-drug resistente patiënten.
Tezamen heeft dit onderzoek geleid tot nieuwe behandelstrategieën voor deze uitgebreid voorbehandelde, multi-drug refractaire multipel myeloom patiënten. Deze nieuwe behandelstrategieën zullen hopelijk in de nabije toekomst de horizon van de multidrug-refractaire myeloom patiënt verbreden door de overleving en kwaliteit van leven van deze patiëntengroep te verbeteren.

Curriculum Vitae

Inger Nijhof werd, met haar tweelingzusje Sanne, op 21 juni 1982 geboren in Hoorn. Samen met haar jongere zusje Linde groeiden ze op in Ooltgensplaat. In 2000 behaalde zij haar VWO-diploma aan het Montessori Lyceum te Rotterdam.
Aansluitend studeerde zij geneeskunde aan de medische Universiteit te Utrecht. Tijdens haar studie liep zij onderzoeksstage bij de immunotherapie op de afdeling dermatologie aan de medische faculteit van de Universiteit Utrecht. Tussen haar 5e en 6e jaar geneeskunde is zij samen met haar studiegenootje Manon 4 maanden naar Rome gegaan om daar Italiaans en kunstgeschiedenis te studeren. In haar laatste jaar geneeskunde liep zij haar semi-arts stage (oudste co-schap) bij de interne geneeskunde, afdeling nefrologie, te UMC Utrecht. Na afronding van de studie begin 2007 werkte zij gedurende 3 jaar met veel plezier als arts-assistent in opleiding tot internist op de afdeling interne geneeskunde van het Diakonessenhuis te Utrecht. Haar opleiders waren dr. Willem Hustinx en later dr. Alex Muller. Begin 2010 vervolgde zij haar opleiding tot internist in het UMC Utrecht onder supervisie van achtereenvolgens prof. dr. Elsken van der Wall, prof. dr. Douwe Biesma, prof. dr. Margriet Schneider en prof. dr. Karin Kaasjager. In 2011 onderbrak zij haar opleiding om promotieonderzoek te verrichten op de afdelingen hematologie en translationele immunologie in het UMC Utrecht en later VUmc, onder leiding van prof. dr. Henk Lokhorst, dr. Niels van de Donk en dr. Tuna Mutis, wat geresulteerd heeft in dit proefschrift. Januari 2015 heeft zij de opleiding tot internist weer opgepakt en is zij begonnen met de specialisatie hematologie in het UMC Utrecht (opleider, dr. Reinier Raymakers).
Zij zal eind 2016 haar opleiding hematologie vervolgen in het Antonius ziekenhuis Nieuwegein onder supervisie van dr. Harry Koene, waarna zij in januari 2017 zal starten in het VUmc onder supervisie van prof. dr. Sonja Zweegman.
Inger is getrouwd met Ron Huisman; zij wonen in Utrecht met hun 2 zoontjes Thijs (2012) en Sebas (2014).

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Dankwoord

Dankwoord

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