Novel Immunochemotherapy Approaches Towards Improved Targeting of Multiple Myeloma

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Novel Immunochemotherapy Approaches Towards Improved Targeting of Multiple Myeloma

Nieuwe immunochemotherapie benadering voor
verbeterde aanpak van Multiple Myeloma

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

1

General introduction

Introduction to Multiple Myeloma

Multiple myeloma (MM) is a haematological cancer originating from antibody producing plasma cells. Clinical symptoms may include bone pain, infections, renal failure and fatigue due to anemia. The disease is often mistakenly called a bone disease as the cancer usually resides in the bone marrow and invades the bone tissue, resulting in multiple bone lesions, accidental and sudden break of bones due to the skeletal destruction(1;2). MM is typically a disease of middle aged and elderly people. Only 5% of patients are under 40 years. The median age for MM is 59 (range 26-84) years(3). Nonetheless, MM has an important worldwide clinical impact: it accounts for 10% of all haematological disorders and 1% of all cancers. Diagnosis is based on 10% or more clonal plasma cells in the bone marrow examination or a biopsy-proven plasmacytoma plus evidence of end-organ damage felt to be related to the underlying plasma cell disorder(4). The pathophysiology of the disease is complex and involves tight crosstalk with the supportive cells in the bone marrow and with the extracellular matrix. Through these interactions malignant MM cells gain survival advantage, invade surrounding tissues and may develop therapy resistance. Despite several achievements in treatment modalities in the past years, MM is still considered an incurable disease. Overall survival however for younger patients has improved in the last decade and is now 5-6 years for patients with standard risk disease. The survival for high risk patients like those with a 17p deletion, t(4;14), t(14;16), t(14;20), a karyotypic deletion 13 or hypodiploidy is still poor(4). Also for the elderly patient the prognosis has improved however less impressive and the overall survival is now median 3-4 years. Improvement of MM therapy is a major task for investigators in the translational research field. This general introduction is intended to provide a general overview about the current status of standard and novel treatment modalities for multiple myeloma and will outline the work presented in this thesis, which is aimed at designing novel approaches by combining chemotherapy with immunotherapy.

Treatment of Multiple Myeloma

Over the last 2 decades important changes in the treatment of myeloma patients have occurred. First, intensive therapy followed by autologous stem cell transplantation was initiated for the younger myeloma patient. Subsequently novel anti-myeloma agents like thalidomide, bortezomib and lenalidomide were introduced initially for treatment of relapsed and refractory disease. Nowadays, these novel agents are part of front line therapies frequently in combination with classical anti-myeloma agents like melphalan, cyclophosphamide and doxorubicin(5;6). A newer therapy which is not yet used as front line therapy, but is an strong and upcoming field is immune therapy, for example antibody treatment(7-12). Below a brief explanation and summary is given on the action of the separate chemotherapeutic agents and their impact in currently used clinical therapies.

Melphalan

Melphalan is one of the older chemotherapeutic agents still being used in the clinic. It is a nitrogen mustard alkylating agent, which generally mediate their cytotoxic effects by adding an alkyl group (C_nH_{2n+1}) to DNA. Intravenous High Dose Melphalan (HDM 200mg/m^2) followed by autologous stem cell transplantation is now routinely applied in younger patients(7;13;14). For elderly patients orally melphalan in lower dosages is usually combined with prednison . For over 40 years the combination of melphalan and prednisone (MP) has been used as front line therapy for elderly patients. The efficacy of this regimen has improved by combining it with the novel agents thalidomide (MP-T), bortezomib (MP-V) or lenalidomide (MP-R). All these combinations have been or are being tested in randomized phase III trials.

Thalidomide

Thalidomide is a racemic agent, containing both left- and right-handed isomers in equal amounts. The (R) is effective against morning sickness but the (S) is teratogenic, because it binds and inactivates the protein cereblon, which is important in limb formation. Thalidomide however is highly beneficial in MM treatment. It appears to inhibit the disease progression by several mechanisms, such as inhibition of angiogenesis, inhibition of the production of interleukin-6 (IL-6), activation of apoptotic pathways through caspase 8-mediated cell death and by induction of c-jun terminal kinase-dependent release of cytochrome-c and Smac into the cytosol of cells. It also activate T cells to produce IL-2, thereby increasing the amount and function of natural killer cells (NK cells)(15). The drug is generally used in combination. Randomized studies comparing the combination of MP-T with the standard MP revealed significant benefits of adding thalidomide in the MM regiments(13).

Lenalidomide

Lenalidomide (Revlimid®)(LEN), a second-generation immunomodulatory drug (iMiD), is a structural analogue of thalidomide with a similar but more potent biologic. It has reduced teratogenic properties(16;17). LEN has multiple mechanisms of action; it induces tumor cell apoptosis directly and indirectly by inhibition of bone marrow stromal cell support, it has anti-angiogenic and anti-osteoclastogenic effects, and it has immunomodulatory activity(18). LEN similar to thalidomide enhances survival and immunostimulatory effects on T-cell and NK cell responses. Especially the NK cell stimulatory properties suggest that LEN may be highly effective in combination with therapeutic antibodies, which induce antibody-dependent cell-mediated cytotoxicity (ADCC)(19;20). In a several studies LEN alone or in combination with other drugs such as dexamethasone offers a significantly higher overall response with markedly higher proportion of patients achieving a complete response (CR) and a longer median time to progression(21-25).

Bortezomib

Bortezomib (Velacade®) is a potent proteosome inhibitor by specific blocking the catalytic site of the 26S proteasome(26). Proteosome inhibition in MM cells has an important impact on activation of NF-κB, a transcription factor that appears to be critical for the growth and resistance to apoptosis (NF-κB)(27). By inhibiting the degradation of Inactive KB, bortezomib inhibits the translocation of NF-kB into the nucleus, thus blocking the activation of its downstream targets(27;28). Proteasome inhibition by bortezomib also decreases the adherence of MM cells to bone marrow stromal cells, which is a critical component of the microenvironment necessary for sustaining MM cell growth(29;30). The beneficial activity of bortezomib against MM has been demonstrated in several nonrandomized and randomized trials(26;31-33).

Resistance to chemotherapy

Resistance of cancers to chemo and immune therapy is a major complication in the field of cancer treatment. Genetic alterations, micro-environment, and several other factors contribute to resistance in treatment(33-37). Gene expression profile on the basis of a molecular characterization model has identified 7 genetic subsets of MM, which include the HY group (hyperdiploid), the MF group (maf overexpression), the MS group (fibroblast growth factor receptor 3 (FGFR3) overexpression), the PR group (proliferative), ME (myeloid), CD1 and CD2 overexpression of cyclin dependent kinase 1 (CDK1) and CDK2. These genetic subsets of MM likely have real implications on prognosis, response to and durability of therapy. Therefore these genetic subsets are increasingly being used in the era of novel targeted agents to shape the decisions being made about approaches to therapy in the induction therapy setting, as well as in the relapsed and refractory disease setting(36;38;39). Of course new research, novel drugs and new drug combinations are being tested to overcome resistance of cancer(40-42).

Stem cell transplantation

Autologous stem cell transplantation

Intensive chemotherapy followed by autologous stem cell transplantation is now standard of care for the younger myeloma patient. Several phase III trials have shown improved progression free and overall survival as compared to standard chemotherapy(43). Currently the efficacy of more effective induction regimens before and maintenance therapy following autologous SCT are explored in large phase III trials to further improve response progression free and overall survival(24;44-48).

Allogeneic stem cell transplantation

While autologous transplantation is applied as a salvage therapy in combination with high dose chemotherapy, allogeneic transplantation is given as a form of immunotherapy to treat the disease. Although it is probably the only strategy with a potential curative potential, the

role of allogeneic Stem cell transplantation in myeloma is disputed because it's application is hampered by severe morbidity and a high treatment related mortality(45;48;49). Due to the severe, and sometimes fatal side effects allogeneic SCT is not recommended for patients with standard risk features and are only explored in high risk patients included in prospective trials. The potential curative therapeutic effect, generally known as 'graft vs myeloma' is largely mediated by cytotoxic donor T cells present in the graft. Donor T cells, which are generally directed at alloantigens of the recipient, are capable of localizing and eliminating the residual MM cells. Therefore, if allogeneic stem cell transplantation fail to induce long term remissions in the first instance or in case of relapse, treatment is usually followed by infuson of donor lymphocytes (DLI)(45;50-52).

Challenges for Immune Therapy

Although MM patients can benefit from allogeneic transplantation, followed by DLI(44;48;52), relapse rates are also high, illustrating the capability of MM cells to escape immunity. Thus there is an urgent need for more effective strategies. On the other hand, an important challenge of allotransplant strategy is to reduce the high morbidity and mortality rates due to GvHD. Since the beneficial effect of allo transplantation and DLI is frequently associated with GvHD, innovative strategies are necessary to separate the beneficial effects from the side effects. For instance novel IMIDs such as lenalidomide have enhanced the efficacy of allo transplantation(53-55), though major problems, including GvHD occur if treatment is given at a non appropriate time(56). Towards efficient and specific immune therapies for MM it seems not only important to identify relevant target antigens of MM cells but also their escape mechanisms from immune system: Regarding the latter, it has been demonstrated that MM cells can produce several cytokines including, IL-10, IL-6 and TGF-β that can hinder the immune response(57-59). MM cells also express surface molecules such as PD-L1 that can down regulate immune responses(60). Tackling these immunosuppressive mechanisms is a challenge. Also it is relevant to understand the role of microenvironment on the outcome of immune therapy since, as illustrated in figure 1, the interaction between MM cells with the cells of bone marrow microenvironment may trigger many mechanisms at various cellular levels; not only to induce drug resistance but also to establish an immune suppressive microenvironment. Modulating this immune suppressive environment may require the combination of immune therapy with carefully selected chemotherapies.

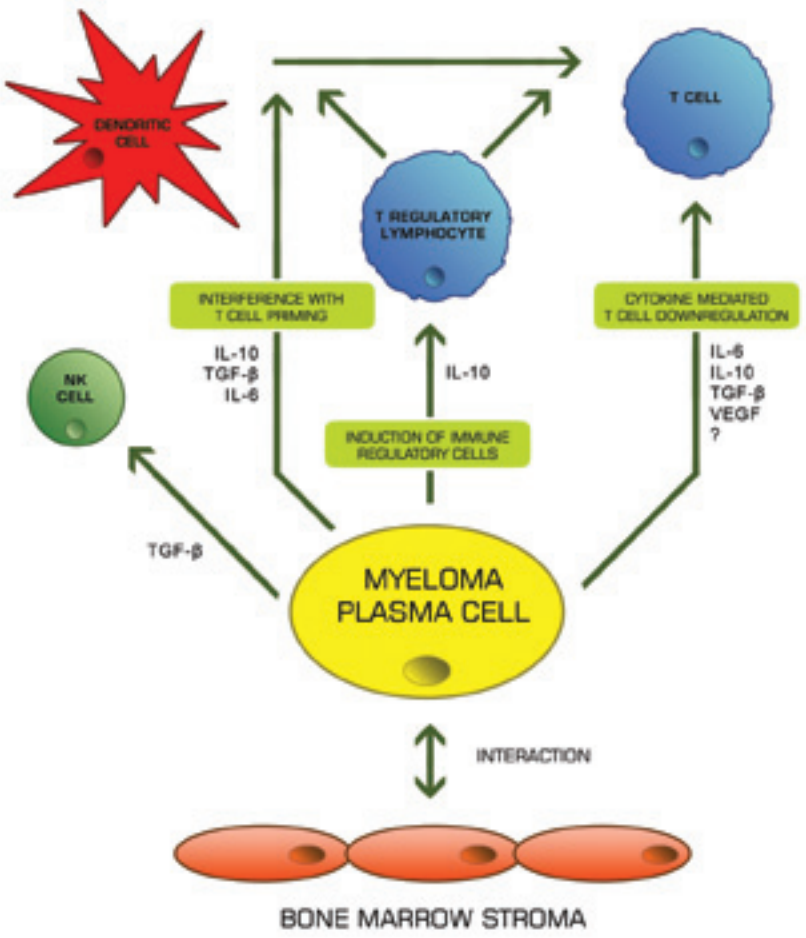

Figure 1: Regulation of immune functions by Multiple Myeloma. Adapted From Br J Haematol. 2007

Antibody mediated therapy

Another increasingly interesting therapy in MM and other cancers is monoclonal antibody therapy. The success of monoclonal antibody therapy is exemplified by rituximab(61), a chimeric antibody directed against CD20, which has been very successful in the treatment of a number of B cell malignancies in the last decade(61). However, rituximab has hardly any effect in MM(62) as in the far majority of cases the malignant plasma cells do not express CD20. Therefore in the MM field, a number of different antibodies against tumor/lineage specific surface molecules, growth factors/ activation molecules or against adhesion molecules are in development(63). For instance a candidate antibody is anti-CD40 (Dacetuzumab; SGN-40), a normally expressed molecule on B cells, dendritic cells, several types of epithelial

cells, and endothelial cells. But CD40 has also a relatively high expression on the majority of MM cells and plays an important roles in MM cell proliferation, migration and adhesion to bone marrow stroma. Therefore targeting CD40-CD40L interactions is thought to be relevant in MM. Antibodies targeting growth factors and receptors are a chimeric neutralizing mAb against IL-6 (siltuximab; CNTO 328)(64) and a humanized mAb against VEGF (bevacizumab ; Avastin).

The primary rationale behind targeting adhesion molecules with antibodies is to inhibit the cell adhesion mediated drug resistance (CAM-DR) by disrupting the interaction between MM and stromal cells. For instance antibody Elotuzumab (HuLuc63)(65;66) against the MM cell specific adhesion molecule CS1 indeed inhibits MM cell adhesion and blocks the stimulatory effects of BM stromal cells on myeloma cell growth and survival. In addition, Elotuzumab has been shown to kill MM cells through antibody dependent cellular cytotoxicity (ADCC). Syndecan-1(CD138)(67), a plasma cell specific molecule, functions as a receptor for the extracellular matrix molecules collagen and fibronectin. Treatment of MM cells with the murine CD138 antibody B-B4 linked to the potent antimicrotubule agent DM1 (B-B4-DM1) results in MM cell death, even in the presence of bone marrow stromal cells, indicating that this immunoconjugate overcomes cell adhesion-mediated drug resistance (CAM-DR).

In addition to the latter, antibodies improving the effector cell functions are being evaluated in MM. Important ones are a fully human anti-KIR antibody (1-7F9 or IPH2101) directed against the inhibitory KIR2DL1/2/3 receptors(68) and CT-011 a humanized anti-PD-1 antibody which appear to enhance NK cell function against MM cells through increased NK cell trafficking, improved immune complex formation between myeloma cells and NK cells, and increased NK cell cytotoxicity(69).

Thus there are several interesting antibodies being studied in vitro as well as in vivo mouse models and in phase I/II trials in clinic. Finding novel targets and understanding the mechanisms involving MM can clearly benefit this increasingly larger and upcoming field. Antibodies can also work synergistically with the newly developing IMiDS which have an enormous boosting impact on CD4, CD8 and NK cells. Also combination of antibodies may be able to negate chemo resistance and perhaps can help lower doses of chemotherapeutic agents without compromising disease free survival and overall survival.

Scope of the thesis

After decades of clinical and experimental experience, the medical and scientific community increasingly appreciate that effective targeting of MM inevitably requires innovative approaches and combination therapies. With this respect, it also seems now very timely to fully understand and exploit the potential power of the immune system to battle MM. The immune system alone, but certainly in combination with a new generation of immunomodulatory chemotherapeutics may be the key to achieve long lasting complete remissions in MM patients. At the same time, tumor immunologists and hemato-oncologists

increasingly become aware of the fact that several tumors, including MM are extremely capable of evading immune attack by several mechanisms. Improvement of cellular immune therapy seems impossible without understanding and modulating these immune evasion mechanisms. On the other hand, alternative immune therapy approaches, such as targeted antibody therapy may offer new perspectives, as have been already shown by the success of CD20 targeted immune therapy.

Thus, towards the improvement of MM immune therapy, this thesis focuses on two important aspects: one is the possible improvement of cellular immunotherapy by a) combination with immune modulatory drugs and b) by understanding the mechanisms of immune evasion by MM. The other important aspect is investigating the antibody mediated therapy alone or combined with novel MM drugs as a new modality for MM treatment. To this end, Chapter 2 describes the clinical and immunological effects of the immunomodulatory drug lenalidomide alone or together with a classical MM drug dexamethasone after a well known cellular immunotherapy approach, the allogenic stem cell transpantation. This chapter demonstrates that the clinical administration of LEN alone or together with DEX in allo transplanted patients, significantly increases the response rates. The treatment is curiously associated with the rise of $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells. The risk of GvHD was however still present but seemed limited when LEN was combined with DEX. Chapter 3 investigates the cytokine mediated immune escape as a mechanism for immune escape by MM cells. This chapter demonstrates the important role of BM stromal cells in the establishment/ improvement of this mode of immune escape. Most MM cells, if not all, produce high levels of anti-proliferative cytokines to inhibit T cell proliferation when cultured together with BM stromal cells. Identification, modulation of these cytokines or their pathways, or modulation of stroma-MM interactions may improve the outcome of cellular immune therapy.

Chapter 4 introduces a novel therapeutic human antibody, daratumumab(DARA) directed against a highly expressed molecule on MM cells, the CD38 molecule. This CD38 specific antibody is selected to effectively kill MM tumor cells by antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), but also by effectively inhibiting the ADP-ribosyl cyclase activity of CD38. The in vitro and in vivo results presented in this chapter indicate the potential of this antibody to effectively eliminate MM.
The study presented in Chapter 5 demonstrates that DARA can be effectively combined and synergizes with lenalidomide, which is known to activate NK cells, a major effector cell of ADCC induced by DARA. This chapter also introduces a novel assay to measure MM cell lysis in the BM-MNCs without the need to isolate the malignant cells from their microenvironment which supports their growth.

Chapter 6 signifies the combination of DARA with newly emerging novel chemotherapy combinations of lenalidomide, bortezomib, melphalan, dexamethasone and prednisone. In all cases addition of DARA to drug cocktails significantly enhances the MM cell lysis. DARA synergizes with LEN/Bort therapy even in patients who are known to be resistant to these drugs, indicating that targeted antibody therapy of MM may have important contributions in

the future MM therapy even for patients .MM patients who relapse while under chemotherapy.

Finally the potential impact of the findings in this thesis is discussed in chapter 7 with a specific focus on DARA as the most promising modality, which can be readily tested in the clinical trials.

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2

Lenalidomide alone or in combination with Dexamethasone is highly effective in patients with relapsed Multiple Myeloma following allogeneic stem cell transplantation and increases the frequency of $CD4^+Foxp3^+$ T cells

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Lenalidomide has demonstrated significant clinical activity in patients with newly diagnosed and relapsed multiple myeloma (MM).(1) Its actions are believed to be mediated by apoptosis, anti-angiogeneic and anti-proliferative activity, inhibition of cytokine production and stimulation of T and Natural Killer (NK) cell immunity and cytotoxicity.(2) Especially these immuno-stimulatory properties suggest that lenalidomide can be very effective following allogeneic stem cell transplantation (allo-SCT) or donor lymphocyte infusions (DLI) by enhancing the donor T cell or NK cell mediated Graft-versus-Myeloma (GvM) effect.

To explore this, we have given lenalidomide to 16 MM patients who relapsed after allo-SCT. To benefit optimal from the immuno-stimulatory effects, dexamethasone was not routinely given. In addition, we analysed peripheral blood collected from 8 patients before, during and after the first cycle of treatment to evaluate the influence of lenalidomide on the cellular immune responses.

Sixteen MM patients (11 male, 5 female; median age 58.5 years (range 43-67), who relapsed after an allo-SCT started lenalidomide treatment between September 2005 and December 2007 (Table 1). Three patients started within 3 months after the allo-SCT, 15 patients had received several other treatments for multiple relapses after allo-SCT including thalidomide (given to 14 patients; 10 became refractory) and bortezomib (given to 11 patients; 7 became refractory)(Table 1).

Table 1: Patients characteristics and clinical outcome

Table 1 Patients characteristics and clinical outcome

Patient no.	Age/sex	Refractory	Dex	Resp Dex	Resp +Dex	GvHD	Toxicity (CTC grade)	PFS (days)	OS (days)
1[a]	62/F	Yes	No	CR	NA	No	No	427	427
2[a]	57/F	No	No	CR	NA	Acute grade II	Grade 3 L-penia	193	225
3	64/M	No	No	CR	NA	Acute grade II chronic k	No	516	516
4[a]	56/F	Yes	No	VGPR	NA	Acute grade IV	No	363	682[b]
5	64/M	No	No	PR	NA	No	Grade 4 pulmonary embolism Grade 1 L-penia	400	400
6	62/F	Yes	No	PR	NA	BOS improved	Grade 2 pneumonia Grade 1 L-penia Grade 1 T-penia	230	406[b]
7[a]	58/M	No	No	PR	NA	Acute grade III	No	133	168[b]
8[a]	63/M	Yes	No	MR	NA	Acute grade III	No	37	37[b]
9[a]	44/M	Yes	Yes	MR	PR	No	Grade 1 fatigue Grade 3 diarrhea	319	432
10[a]	43/M	No	Yes	MR	PR	No	Grade 2 T-penia Grade 4 L-penia	352	352
11	53/M	No	Yes	MR	PR	No	Grade 3 T-penia Grade 2 anemia	158	250
12	66/M	No	Yes	MR	VGPR	No	Grade 1 L-penia Grade 3 muscular pain	283	551
13[a]	59/M	Yes	Yes	SD	SD	No	Grade 2 L-penia	93	197
14	67/F	Yes	Yes[c]	NA	CR	No	Grade 2 L-penia Grade 1 fatigue	400	400
15	51/M	No	Yes[c]	NA	CR	No	Grade 3 deep venous thrombosis	338	338
16	49/M	Yes	Yes[c]	NA	PR	BOS stable	Grade 1 fatigue	391	391

Abbreviations: BOS, bronchiolitis obliterans syndrome; CTC, common toxicity criteria; CR, complete response; Dex, dexamethasone added to treatment; GvHD, graft-versus-host disease; L-penia, leukopenia; MR, minimal response; NA, not applicable; OS, overall survival; PFS, progression-free survival; PR, partial response; Resp, response; SD, stable disease; T-penia, thrombocytopenia; VGPR, very good partial response.
[a]Blood sampling in the first cycle.
[b]Patients who died.
[c]Dexamethasone at the starting of the treatment.

Eight patients switched to lenalidomide as relapse treatment due to peripheral polyneuropathy. Two patients had been treated with lenalidomide before allo-SCT (Table 1) and reached a partial remission (PR) after 1 and 3 cycles, respectively and then proceeded to allo-SCT. Eleven patients had received DLI(s), 7 of them started with lenalidomide within 3 months after their DLI due to progressive disease. All patients except 3 (Table 1, patients 2, 10 and 13,) had experienced episodes of Graft versus Host Disease (GvHD). At start of therapy, 3 patients had symptoms of chronic GvHD.. One patient had an acute GvHD grade 2 of the skin. Other GvHD episodes were in remission. Lenalidomide (provided by Celgene International Sarl, Switzerland) was administered for 21 days at 25 mg/day, followed by 1 week rest for a maximum of 6 courses. Lenalidomide was combined with pulsed Dexamethasone (40 mg/day, days 1-4 and 15-18) after 2 cycles in 5 patients, and from the start in 3 patients (Table 1, patients 9-16).

Response and progression were assessed according to the European Group for Blood and Marrow Transplantation (EMBT) criteria with addition of very good partial relapse (VGPR), indicating a response greater than 90%.(3) Response rates are based on the disease measurements within 3 weeks before starting treatment. GvHD was graded according to the Glucksberg criteria.(4) Heparinized blood samples were collected from 8 patients before, during (between days 7 and 14) and after the first cycle only of treatment. Just prior to the lenalidomide only patient nr 1 had received thalidomide, another immunomodulatory drug. All patients provided written informed consent for the use of their blood for immunomonitoring purposes.

Peripheral blood mononuclear cells (PBMC) were isolated and cryopreserved in liquid nitrogen until use. FITC, Per-CP, PE or APC conjugated antibodies against CD_3, CD_4, CD_8, CD_{25}, CD_{56}, $CD_{40}L$ (Becton Dickinson, San Jose, CA), $V\alpha_{24}$, $V\beta_{11}$ (Immunotech, Marseille, France), were used in 3- or 4-color flow cytometry to determine the frequency of different cell subsets in PBMC. $FOXP_3^+$ cells were detected using an intracellular (i.c.) human $FOXP_3$ staining kit (eBioscience, San Diego, CA). Prior to i.c, $FOXP_3$ staining, cells were surface labelled with fluorescent antibodies against CD_4 and CD_{25}. IFN-γ-secreting cells were detected by a cytokine secretion capture assay (Miltenyi Biotec) after stimulation of PBMC with anti-CD_3/anti-CD_{28} beads (Dynalbeads, Invitrogen, Carlsbad, CA) for 24h. Cells were additionally stained with fluorescent antibodies against CD_4 and CD_{14} to determine the phenotype of IFN-γ secreting cells. Data were analyzed by FACScalibur (Beckton Dickinson) flowcytometer and by CellQuest software. Friedman test was used to determine the statistical significance of the relative differences in the frequencies of different T cell subsets before during and after treatment. P values < 0.05 were considered significant. A total of 13 patients started their treatment with lenalidomide only (Table 1, patients 1-13). Six of these patients (patients 1-6) responded including 3 with a CR.. One patient responded with a MR only, he died shortly after start treatment. Patients 9 to 13, received a combination of lenalidomide and dexamethasone after the second lenalidomide cycle because of insufficient response after 2 cycles. Four of these 5 patients responded to the combination therapy. In 3

patients (pts 14-16) lenalidomide was combined with dexamethasone from the start due to a very rapidly progressing disease. All of these patients responded..

Maximal response to lenalidomide was observed after a median of 2 cycles (range 1-5). After a median follow up of 396 days (range 37-682), the median progression free survival was 328 days (range 37 – 516) and the median overall survival was 395 days (range 37-682). Two patients (7 and 8) died from infections associated with GvHD, patient 4 and 6 died from progressive disease.

In summary, 46% (6/13) of the patients responded to lenalidomide alone, and most poor responders benefited from the combination with dexamethasone, resulting in an overall response rate of 87.5%. This high response rate is remarkable for relapsed or refractory MM patients and may be due to the direct anti-tumor effects of these drugs. However, the clinical responses observed with lenalidomide alone are probably also accountable to its immunostimulatory effects. Supporting this idea, 5 out of 13 patients starting with lenalidomide alone developed acute GvHD between 2 -13 days after start of treatment. The occurrence of acute GvHD was a good predictive factor for clinical response: 4 out of these 5 GvHD patients developed a good response (the 5th GvHD patient lived too short to determine the anti-tumor response). Only 3 patients responded without developing GvHD. Interestingly, all 5 GvHD patients had received an allo-SCT or DLI shortly before (5 months or earlier) lenalidomide treatment, whereas the 3 responders without GvHD had received the allo-SCT or DLI at least 1,5 years before. Patients who started lenalidomide with dexamethasone did not develop GvHD, regardless of the interval time.

To evaluate the immunomodulatory effects, blood samples were taken from 8 patients during the first treatment cycle. None of them used dexamethasone at the time of blood sampling. In the peripheral blood, low frequencies of dendritic cells and NKT cells were detected and seemed stable (data not shown). Alterations in the frequencies of $CD3^-CD56^+$NK cells were not consistent (figure 1A). However, the frequencies of $CD4^+$ and $CD8^+$ T cells declined during treatment and recovered thereafter ($p<0.05$) (figure 1B, C). In contrast, the frequencies of $CD4^+FOXP3^+$ cells showed a significant increase in 7 of 8 patients during lenalidomide administration and later declined ($p<0.001$) (figure 1D, E). $FOXP3$ is considered a specific marker for regulatory T cells (Tregs). However, activated effector T cells may also show temporary expression of $FOXP3$. (5) To evaluate this we determined the frequencies of IFN-γ secreting $CD4^+$ T cells and sought a correlation between $FOXP3^+$ and IFN-γ+ T cells (Figure 1F). Unlike $FOXP3^+$ cells, the frequencies of IFN-γ^+ T cells remained stable or tended to decline during treatment. In fact, the $FOXP3^+$ T cells and the IFN-γ^+ T cells seemed to show an inverse relation, as suggested by the significant increase in the $FOXP3^+$ to IFN-γ^+ T cell ratios during treatment ($p=0.008$) (Figure 1G). Such a significant relation was not found between $FOXP3^+$ cells and NK cells(figure 1H). Taken together, these results most probably reflect the increase of Tregs and their downregulatory effects on (IFN-γ secreting) $CD4^+$ and $CD8^+$ T cells.

Tregs have been associated with disease progression in MM patients and may also downregulate GvHD after allo-SCT.(6, 7) However, in our small cohort the impact of the increase in Tregs to the clinical outcome is not clear. Probably due to small number of analysed patients, we did not find any association between $FOXP_3$+ T cells, GvHD or clinical responses. The development of severe acute GvHD, despite high levels of circulating $FOXP_3$+ cells in a number of patients indicate that Tregs may not always be capable of neutralizing the immuno-stimulatory effects of lenalidomide. On the other hand, the fact that GvHD did not develop in other patients, and that the symptoms of chronic GvHD improved in 2 patients, could be interpreted as an effective compensation of the immuno-stimulatory effects of lenalidomide by Tregs. Nonetheless, since several other explanations are also possible, larger studies are necessary to unravel the precise mechanism and consequences of the increase of $FOXP_3^+$ T cells during lenalidomide treatment.

In conclusion, our study show for the first time that lenalidomide therapy in relapsed MM patients after allo-SCT is very effective with a response rate of 87,5%. When given as monotherapy within months after allo-SCT or DLI it can elicit a very rapid immune response causing severe acute GvHD. This supports the *in vitro* data on the immuno-stimulatory capacity of lenalidomide. The exact role of the novel observation of elevated $FOXP_3^+$ T cells in these patients needs further investigation.

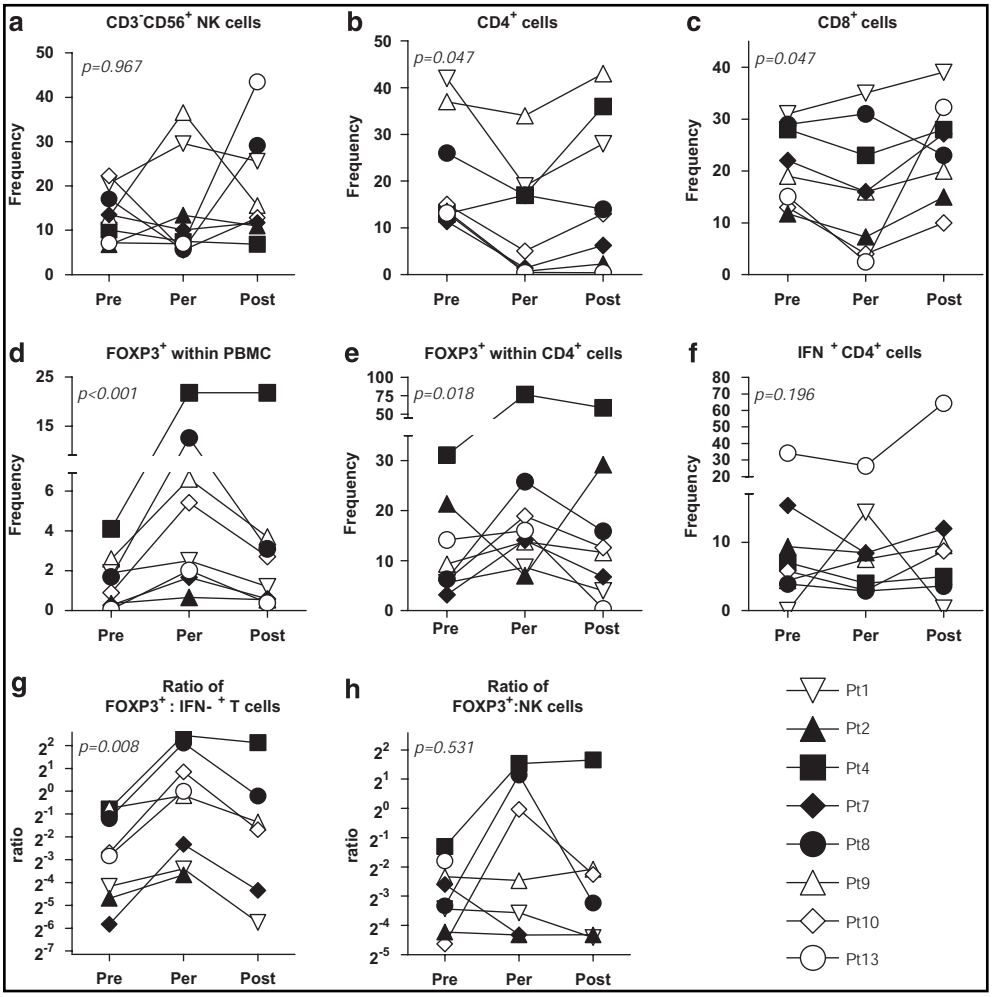

Figure 1. Immunomonitoring of lenalidomide treatment Mononuclear cells obtained from blood of patients treated with lenalidomide, before (pre) during (per) or after (post) treatment, were analyzed by FACS. Graphs A to F show frequency of different cellular subtypes. Graphs G and H show ratio of FOXP3+ cells compared to either INF-y producing T-cells or NK cells.Patients with filled symbols (pts 2, 4, 7, 8) experienced acute GvHD grade II-IV (see also Table 1). P values were calculated using the Friedman test.

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3

The impact of bone marrow stroma on T cell downregulation by Multiple Myeloma cells

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Submitted

Abstract

Despite the proven capacity of T cells to recognize and eliminate Multiple Myeloma (MM) cells, cellular immunotherapy frequently fails to induce complete remissions in MM patients, indicating the in vivo ability of MM cells to evade cellular immune attack. To gain insight into the extent and possible mechanisms of this immune evasion, we investigated the T cell inhibitory capacities of several MM cell lines (n=12) and primary MM cells (n=5). Only four MM cell lines inhibited the CD_3/CD_{28} triggered T cell proliferation. However, most MM cell lines and primary MM cells gained significant suppressive activity after co-culture with bone marrow stromal cells. The profound suppressive activity of MM lines RPMI8226, UM_3 and XG 1 was mainly mediated by a novel soluble factor with a molecular mass of 18-25 kDA, did not involve regulatory T cells and was directed at both unprimed or primed CD_4^+ and CD_8^+ T cells. Remarkably, the inhibitory factor secreted by RPMI8226 affected only the proliferation by inhibiting CD_{25} upregulation, without altering IL-2,IFN-γ IL-4 or IL-10 secretion or cytotoxic activity. Finally, this antiproliferative activity was partially but significantly reconstituted by lenalidomide and bortezomib, suggesting that overcoming the immune evasion mechanisms of MM cells, which seem to be mainly controlled by stroma may be possible by inhibiting stroma-MM cell interactions, neutralizing antiproliferative cytokines or by the use of carefully selected and tested immune modulatory chemotherapeutic agents.

Introduction

Multiple myeloma (MM), the malignant disorder of antibody producing plasma cells, accounts for approximately 2 % of all cancer mortality worldwide[1]. Despite significant progress in understanding the biology of the disease and the development of new anti-myeloma agents, MM remains incurable due to the development of drug resistance[2]. Clinical and experimental studies, on the other hand, indicate the possibility to combat MM via cellular immune therapy[3;4]. This is perhaps best illustrated by the induction of long term remissions, after allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusions (DLI) in a subset of patients[5-7]. In support to these clinical studies, we and others have shown that T cells, isolated from the peripheral blood of successfully transplanted MM patients, can effectively kill MM cells *in vitro*[8-12]. In addition, using a humanized murine model, we have demonstrated the possibility to eradicate bone-marrow residing MM cell tumors by adoptive transfer of donor-derived $CD4^+$ Cytotoxic T cells[13].

Despite this proven capacity of T cells to recognize and eliminate MM cells, clinical response rates of MM patients to allo-SCT, DLI, and newly developed vaccination strategies are moderate[14-17]. Most patients eventually progress to relapse, suggesting the ability of MM cells to escape from T cell mediated immune responses. Indeed, a number of earlier studies reported quantitative and qualitative defects in the cellular immune responses of MM patients. MM cells were suggested to suppress immunity by the expression of high levels of Fas ligand[18;19] or by the expression of co-inhibitory molecules such as PD1-L[20;21] and CD200[22]. Furthermore, a number of studies suggested the production of transforming growth factor β (TGF-β) as an effective mode of immune escape[23-26]. It is however not well known how these receptor- and cytokine-mediated immune inhibition mechanisms are acquired, to what extent they are universally operational, and whether they can be modulated in a strait forward way to improve immunotherapy. Since development of more effective immune t herapy strategies can benefit from insight into these issues, we here investigated the ability of several human MM cell lines and purified primary MM cells to downregulate T cell responses. In particular, to assess the role of bone marrow stromal cells in the immune escape, we executed several assays in the absence and presence of bone marrow stromal cells. We demonstrate that only a minority of MM cell lines inhibit proliferative T cell responses by themselves. However most of them gain significant T cell inhibitory capacity after co-culture with bone marrow stromal cells. An in depth analyses indicated that a number of MM cell lines mediate this T cell inhibition predominantly by secreting an anti-proliferative cytokine with a molecular mass of 18-25 KD. Finally we collected evidence that the T cell inhibition could be partially reconstituted by the novel anti-MM drugs lenalidomide and bortezomib. In the light of these results, we discuss the potential benefits of immunochemotherapy, targeting stroma-MM cell interactions and the importance of the identification of MM-secreted anti-proliferative cytokines to improve the outcome of cellular immune therapy in MM.

Material and Methods

Myeloma Cell lines

MM cell lines, RPMI8226, U266, OPM-1, OPM-2, L363, UM3, UM-6, UM8, UM-9, UM11, XG-1 and MDN, were maintained as previously described[27;28].

Primary Myeloma Cells. $CD138^+$ primary MM cells were isolated from BM-MNCs of MM patients by immunomagnetic cell purification as described elsewhere[29]. All experiments with patient material are performed after written informed consent and after approval of the institutional medical ethical committee.

Bone marrow derived Stromal cells

The bone marrow derived stromal cell line HS-5 was cultured in DMEM medium containing 10% FBS. The cells were used in the experiments after culturing for no more than 5 passages.

Peripheral blood Mononuclear cells (PBMC)

Peripheral blood was obtained from healthy volunteers after written informed consent. PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation and cryopreserved in liquid nitrogen until use as effector cells in ^{3}H-Thymidine or CFSE-based proliferation assays[30;31]. All procedures involving material from healthy donors were approved by the institutional medical ethical committee.

Primary responder cells for proliferation assays. Where indicated, $CD4^+CD25^-$ T cells were purified from PBMCs by immunomagnetic cell sorting according to the protocols of the manufacturer (Miltenyi Biotech)[32]

Cloned responder T cells. $CD4^+$ T cell clones specific for Tetanus Toxoid were generated, characterized, cryopreserved and maintained as described elsewhere[33].

Concentrated cell-free supernatants of MM cells

For a number of experiments, secreted proteins of MM cells were concentrated from the culture supernatants using two-compartment bioreactor flasks (Celline classic 1000 (DC-90005); Sartorius), in which the compartments are separated with a 10kDA filter. For these assays, MM cells were cultured in X-vivo 15 medium at an initial seeding density of 1×10^7 cells/flask as recommended by the manufacturer. The concentrated supernatant from >10kD chamber was collected after 3 days of culture. The supernatants were either used directly in proliferation experiments or were stored at 4°C for maximum two weeks before use.

FPLC fractionation of MM supernatants

To estimate the molecular size of the inhibitory cytokine, MM supernatants were fractionated by AKTA-FPLC system (GE Healthcare) using a HiLoad 26/60 Superdex 200 size-exclusion column, running at 1ml/minute. 4 ml samples were collected and used in proliferation assays.

3H-Thymidine- and CFSE-dilution based T cell proliferation assays.
Standard 72-hour ^{3}H-Thymidine incorporation-based proliferation assays were carried out by stimulation of responder cells (PBMC's or T cells; 2 x 10^{4}cells/well) with immunostimulatory beads coated with anti-CD3/CD28 (immunostimulatory beads) at a 10 beads to 1 responder cell ratio in U bottomed 96 well plates in a total volume of 200 µl/well. Culture medium consisted of RPMI-I640 +10% human serum and antibiotics. During the last 16 hours, 1 µCi of ^{3}H Thymidine was added in the wells. Cells were harvested on glass-fibre filters and ^{3}H-Thymidine incorporation in the cellular DNA was measured by liquid scintillation.

Proliferation assays based on dilution of fluorescent dye CFSE (Carboxyfluorescein succinimidyl ester) were carried out by labeling responder cells (20000c/well) with CFSE following the instructions of the manufacturer (Invitrogen). Responder cells were then stimulated with immunostimulatory beads using the same conditions for ^{3}H-Thymidine assays. After 3-5 days, cells were collected, counterstained for CD_3, CD_4 and CD8 using appropriate fluorescent conjugated antibodies (Becton Dickinson). CFSE dilution in each T cell subset was determined by three color flow cytometry on a FACS Calibur device (Becton Dickinson). FACS data were analyzed using the Cell Quest Pro software. The inhibitory cytokines specifically affected the proliferation of T cells after two divisions. Therefore T cell suppression after two divisions was calculated by putting appropriate gates on the cells divided more than two times during the assay period.

To assay the influence of bone marrow stromal cells on the T cell inhibitory capacity of MM cells, freshly cultured stromal cell line, HS-5, was plated at 4 x 10^{3} cells/well in 96-well flat bottom plates. After overnight incubation, MM cells were added at 10^{4} cells/well, together with CFSE labeled PBMC (10^{4} cells/well) and beads coated with immunostimulatory beads. The experiments were terminated on day 3 or 4 to measure the CFSE dilution in T cells.
To assay the contribution of known inhibitory cytokines, proliferation experiments were carried out by adding neutralizing/blocking antibodies against anti-IL10 (50ng/ml), anti-IL10R (0,3µg/ml), anti-TGFβ (10µg/ml) or VEGF (20µg/ml) during the whole assay period.

T cell Proliferation assays using transwell inserts. To determine the contribution of soluble factors to the T-cell inhibitory capacity of MM cells, CFSE labeled T cells (2 x 10^{4}) cells were stimulated with anti-CD3/CD28 beads in the transwell inserts (0.4 µm pore) of 96-well plates (Costar), while MM cells alone or together with HS-5 cells were cultured in the main well.

Cytokine production by T cells. Prior to termination of some proliferation assays, 50 µl of cell free supernatants were collected to measure T cell derived cytokines IL-2,IFN-γ IL-4 and IL-10 as described elsewhere[33].

Luciferase Based compartment specific Cytotoxicity assay. Luciferase transduced MM cell line UM-9 was co-cultured with a relevant cytotoxic T cell clone (3AB11[34]) at various effector target ratios in 96 well flat bottomed opaque plates in the absence of presence of 50% cell free supernatant of RPMI8226. After overnight incubation, the bioluminescence signals from the surviving MM cells were measured as described elswhere[34].

Statistics. Differences between treatment groups were tested for significance in students' tests with 2-tailed 95% confidence intervals. P-values below 0.05 were considered significant.

Results

The impact of Bone Marrow Stroma on the inhibition of T cell proliferation by Multiple Myeloma cells.

To evaluate the T cell inhibitory capacities of MM cells, we first stimulated PBMC from healthy individuals with immunostimulatory beads in the presence or absence of four different MM cell lines. The MM cell line RPMI8226 significantly inhibited T cell proliferation in a dose-dependent manner (figure 1a). However, other cell lines were either non-inhibitory or even improved (i.e.U266) T cell proliferation (figure 1a).

Separation of the MM cell line RPMI8226 from the responder T cells in a transwell assay system only slightly influenced its inhibitory activity, indicating that the inhibitory effect was predominantly mediated by a soluble factor (figure 1c). Using this transwell system, we next addressed whether bone marrow stromal cells, which are known to support the growth of MM cells in their natural microenvironment, would influence the T cell inhibitory capacity of MM cells. Thus, MM cells alone or together with the bone marrow stromal cell line HS-5 were cultured in the bottom chamber of transwell system while the PBMC was stimulated in the upper chamber (figure 1c). The presence of HS-5 cells increased the T cell inhibitory capacity of RPMI8226. Even more remarkably, the UM11 cell line, which did not inhibit T cell proliferation alone, displayed substantial T cell inhibitory activity in the presence of HS-5 cells (figure 1c). This enhanced/induced T cell inhibitory activity was not due to production of inhibitory cytokines by HS-5 cells since a) HS-5 cells alone did not inhibit T cell proliferation and b) the T cell inhibition was completely abrogated, even in the presence of HS-5 cells, when MM cells (RPMI8662) were fixated (figure 1c). Taken together, these results indicated that similar to RPMI8226, the UM11 cell line could secrete T cell inhibitory factors, but only after interaction with bone marrow stromal cells. Therefore, we evaluated the T cell inhibitory capacity of a larger panel of MM cell lines (n=11) in the transwell system in the presence and absence of stromal cells (figure 1d). Only four MM cell lines displayed substantial inhibition (>30%) of T cell responses in the absence of stromal cells. In contrast, 9 of 11 MM cell lines displayed substantial T cell suppressive activity in the presence of HS-5 cells, attributing an important role to bone marrow stromal cells in the initiation/facilitation of T cell inhibitory mechanisms.

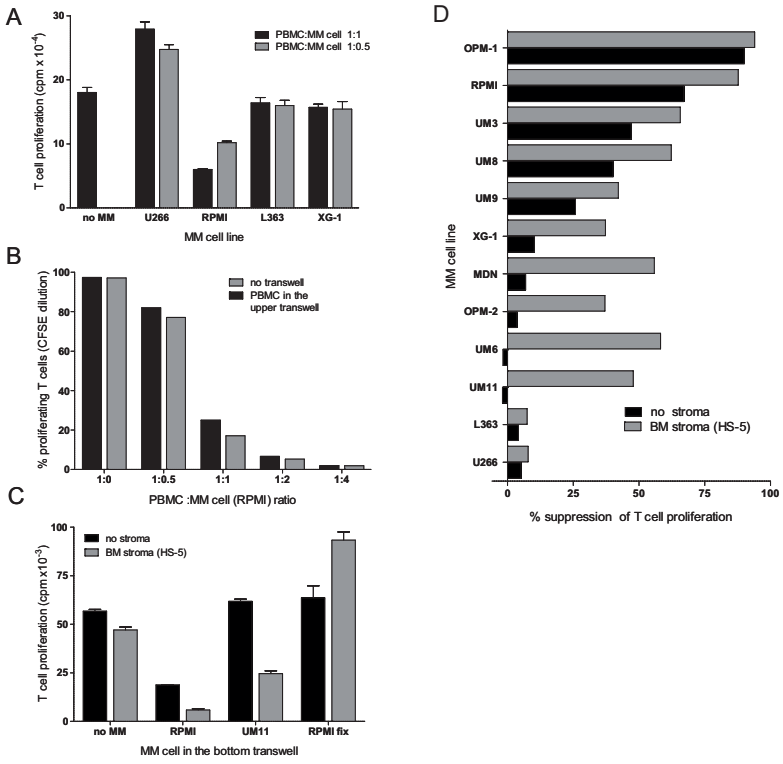

Figure 1: BM Stroma-triggered, cytokine mediated inhibition of T cell proliferation by Multiple Myeloma cell lines. (A). Inhibition of T cell proliferation by the MM cell line RPMI8226 in a dose dependent manner. Responder PBMC is stimulated with anti-CD3/CD28 beads in the presence or absence of MM cells at the indicated PBMC: MM cell ratios. 3H Thymidine incorporation was determined as indicated in material and methods. Error bars represent the SEM of triplicate cultures. Data is representative for 7 independent assays. (B) Dose dependent inhibition of T cell proliferation by RPMI8226 in a transwell culture system. CFSE labeled responder PBMC were incubated in the transwell insets; MM cells were added at the indicated responder: MM ratios in the main chamber. The % proliferating cells was determined by enumerating the CFSE labeled CD3+ T cells that have divided >2 divisions as detected by the typical CFSE dilution on FACS. As control the responder cells and MM cells were co-cultured in the main chamber. (C, D) The inhibition of T cell proliferation by MM cell lines in the presence of absence of bone marrow stromal cells. Responder cells were incubated in the transwell insets; MM cells alone or together with BM stromal cell line HS-5 (1:1 ratio) were incubated in the bottom chamber. T cell proliferation was measures by 3H-Thymidine incorporation. In (C) The MM cell line RPMI8266 was also used after fixation with 1% parafomaldehyde for 30 minutes at 37°C (RPMI fix). In D, the suppression of T cell proliferation was calculated using the formula: [proliferation in the presence of MM (or MM + stromal cells) – (without MM (or stromal) cells]/ proliferation without MM (or stromal) cells x 100%.

Subsequently, we used in similar settings >90% pure, $CD138^+$ primary MM (pMM) cells, which were directly cryopreserved after isolation from the bone marrow of MM patients. The pMM cells displayed little or no T cell inhibition in the absence of stromal cells (figure 2a). However, their T cell inhibitory capacities were upregulated upon co-culture with HS-5 cells (figure 2a). For one MM cell (pat#5) we could also demonstrate that the T cell inhibition was mediated by a soluble factor since this MM cell inhibited T cell responses also in the transwell system (figure 2b).

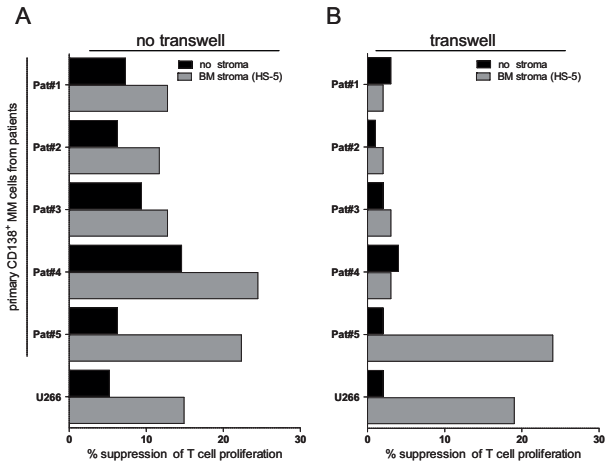

Figure 2: BM Stroma-triggered, cytokine mediated inhibition of T cell proliferation by primary Multiple Myeloma cells. CFSE based-proliferation assays (see legend figure 1) were executed in the presence or absence of >80% purified CD138+ MM cells isolated from MM patients. Stromal cell line HS-5 was added in the assay as indicated. The MM cell line U266 was used as a control.

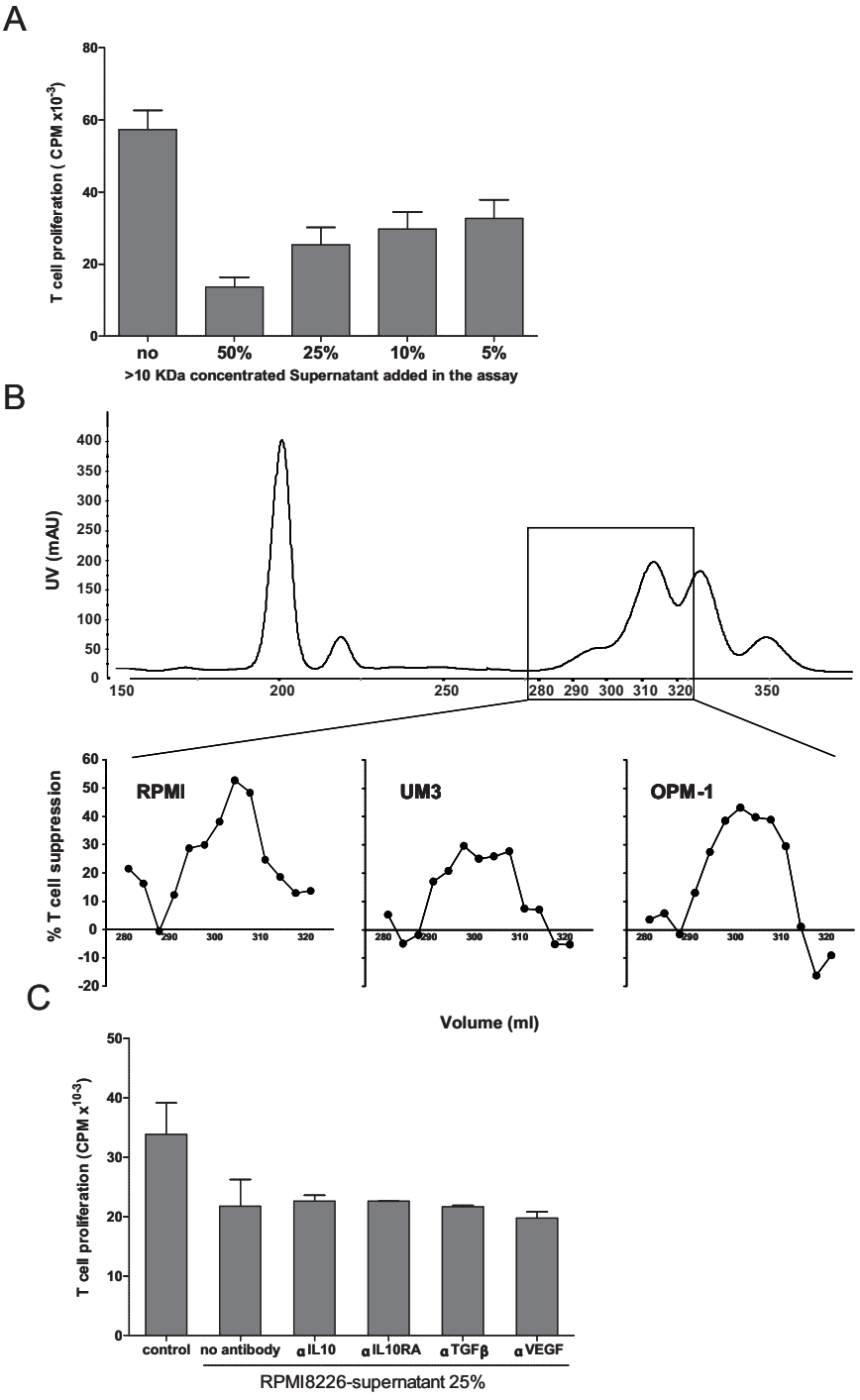
A
T cell proliferation (CPM x10-3)
80
60
40
20
0
no
50%
25%
10%
5%
>10 KDa concentrated Supernatant added in the assay
B
UV (mAU)
400
350
300
250
200
150
100
50
0
150
200
250
280 290 300 310 320
350
% T cell suppression
60
50
40
30
20
10
0
-10
-20
RPMI
UM3
OPM-1
280
290
300
310
320
Volume (ml)
C
T cell proliferation (CPM x10-3)
50
40
30
20
10
0
control
no antibody
αIL10
αIL10RA
αTGFβ
αVEGF
RPMI8226-supernatant 25%

Figure 3. Culture supernatants of MM cell lines contain a novel inhibitory cytokine.
(A) Dose dependent T cell inhibitory capacity of cell free supernatant of MM cell line RPMI8226. Culture supernatants of MM cells were concentrated by culturing MM cells in two chambered flasks divided with a10KDa filter. These were added in T cell proliferation assays at the indicated dilutions. The error bars represent the SEM of 8 independent assays. The supernatant of MM cell lines UM3 and OPM-1 revealed similar results (not shown) (B) FPLC fractionation of the cell free supernatants of RPMI, UM3 and OPM1 revealed the similar pattern of protein elution depicted in the upper panel. All fractions were tested for T cell inhibitory capacity in a similar fashion shown in (A). The plots in the lower panel indicates the level of T cell suppression using the FPLC fractions of supernatants from RPMI8266, UM3 and OPM-1 at the depicted region. No suppressive activity was detected in other fractions. (C) T cell inhibition by supernatants (added at 25%) of RPMI8226 is not restored by neutralizing/blocking antibodies against known inhibitory cytokines. T cell proliferation is measured by CFSE dilution, antibodies are added in the assay as described in material and methods.

The nature and the properties of inhibitory factor produced by MM cells

After establishing the role of BM stromal cells in upregulating the T cell inhibitory cytokines by MM cells, we questioned the nature and working mechanisms of the inhibitory factor secreted by T cell inhibitory MM cell lines RPMI8226, OPM-1 and UM-3. We first established that supernatants of these cell lines concentrated over a 10 kDa barrier could readily inhibit the T cell responses in a dose dependent manner (figure 3a). Subsequently, in an attempt to identify the inhibitory factor, the supernatants were fractionated by FPLC using a size exclusion column. Remarkably, the T cell inhibitory activity of all three MM cell lines eluted exactly at the same time, corresponding to a range of 18-25 KD molecular weight (figure 3b) and suggested the secretion of a common cytokine. A strait forward identification of the inhibitory factor was however not possible since mass-spectrometric analyses indicated that the fractions contained >50 cytokines, which could not be separated by an ion exchange strategy (data not shown). Nonetheless, it seemed that the inhibitory cytokine was a novel one, since neutralizing or blocking the receptors of a number of well known T cell inhibitory cytokines (IL-10, TGF-β and VEGF, IL-9, IFN-α) did not influence the inhibitory activity (figure 3c).

T cell inhibition by RPMI 8226 does not involve regulatory T cells and affects only the proliferation.

To date several T cell inhibitory factors have been described to act via the induction or activation of regulatory T cells (Tregs). Therefore, we explored the involvement of Tregs in T cell inhibition mediated by MM cell line RPMI8226. Stimulation of PBMC in the presence of RPMI8226 for 4 or 5 days did not increase the percentage of $CD4^+FOXP3^+$ cells, even in the presence of increasing doses of IL-2 (figure 4a and b). Furthermore, RPMI8226 was readily able to abrogate the proliferation of >95% pure $CD4^+CD25^-$ or $CD8^+CD25^-$ primary non regulatory T cells (figure 4c) as well as of Th1-like cloned T cells specific for Tetanus Toxoid (figure 4d).

Taken together these results excluded the possibility that the T cell inhibition by RPMI8226 required Tregs, or involved conversion of non-Tregs into Tregs. Furthermore, in an extended functional analysis, we observed that RPMI8226 or its supernatants inhibited only T cell proliferation and CD25 upregulation (figure 5a) but not the secretion of IL-2, IFN-γ, IL4 or IL-10 (figure 5b). In compartment specific cytotoxicity assays, supernatants of RPMI8266 also showed no inhibitory effect on the cytotoxic activity of a $CD8^+$ T cell clone towards its specific target cell (figure 5c). Thus, the inhibitory cytokine produced by RPMI8226 solely inhibited CD25 upregulation and subsequent expansion of T cells without influencing their effector/helper functions.

Anti MM agents Lenalidomide and Bortezomib partially reverse the inhibitory effect of MM cell line RPMI8226.

Finally, we tested whether the T cell inhibitory effect of RPMI8226 could be abrogated by novel anti MM agents Lenalidomide (LEN) and Botezomib (BORT). In an assay system where RPMI8226 cells were cultured in the presence of HS-5 cells, the chemotherapeutic agents were either directly added in the assay (figure 6 upper panel) or used to pretreat MM cells before the assays (figure 6 lower panel) at carefully selected doses by which no MM cell kill occurred. T cell suppression induced by RPMI8226 was partially but significantly reversed both by LEN en BORT, when the agents were added in the assay. There was however no effect of LEN when it was used to pretreat MM cells, suggesting that LEN probably acted by decreasing the susceptibility of T cells for immune regulation, while the effect of BORT was directly on the MM cells.

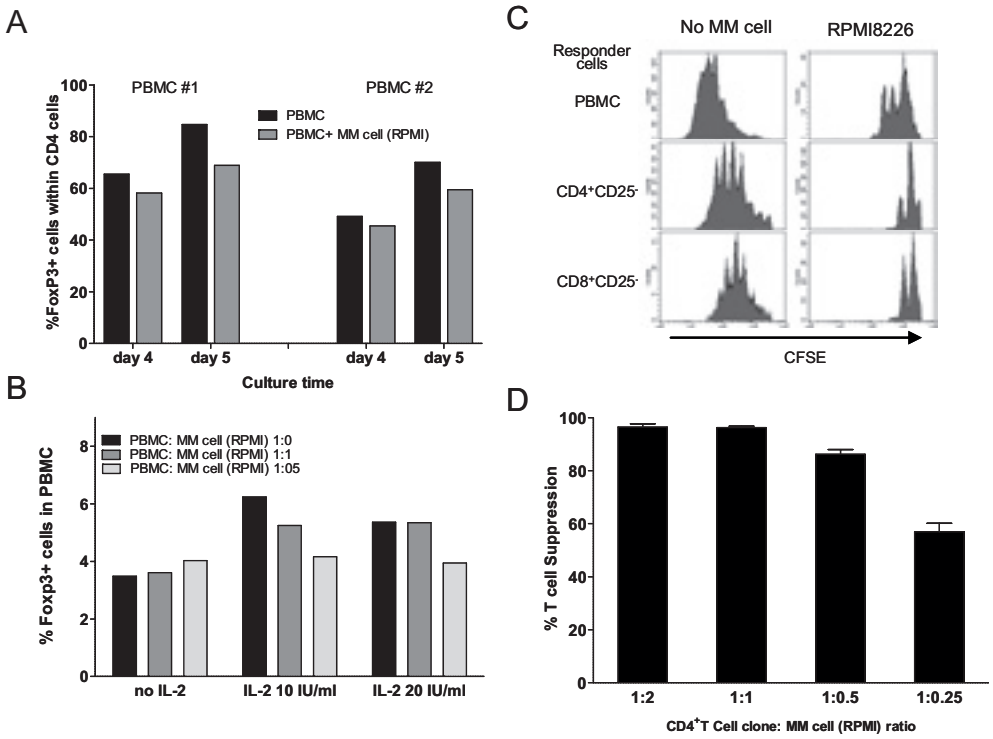

Figure 4. T cell inhibition by RPMI does not involve regulatory T cells. The percentage of Foxp3+ T cells within CD4+ cells or within whole PBMC during culture with immunostimulatory beads in the presence of RPMI8226 cells was determined by intracellular FOXP3 staining and surface counterstaining with CD4+ (A,B). In B indicated concentrations of IL-2 was added in the assay. Addition of IL-2 did not reverse the T cell inhibition by RPMI8226 (data not shown). (C) Suppression of T cells in PBMC as well as MACS purified (purity >90%) CD4+CD25- and CD8+CD25- T cells by soluble factors produced by RPMI8226. CFSE labeled T cells were stimulated in the transwell insets while RPMI8226 was cultured in the main well as described in the material and methods. The plots depict the CFSE dilution. (D) Inhibition of a Th-1 like CD4+ T cell clone specific for tetanus toxoid by RPMI8226. The T cell clone was stimulated with immunostimulatory beads in the presence or absence of RPMI8226 as indicated. T cell proliferation was measured by 3H-Thymidine incorporation. T cell suppression was calculated relative to the proliferation of the wells where no RPMI8226 was added. Error bars represent the SEM of triplicate cultures.

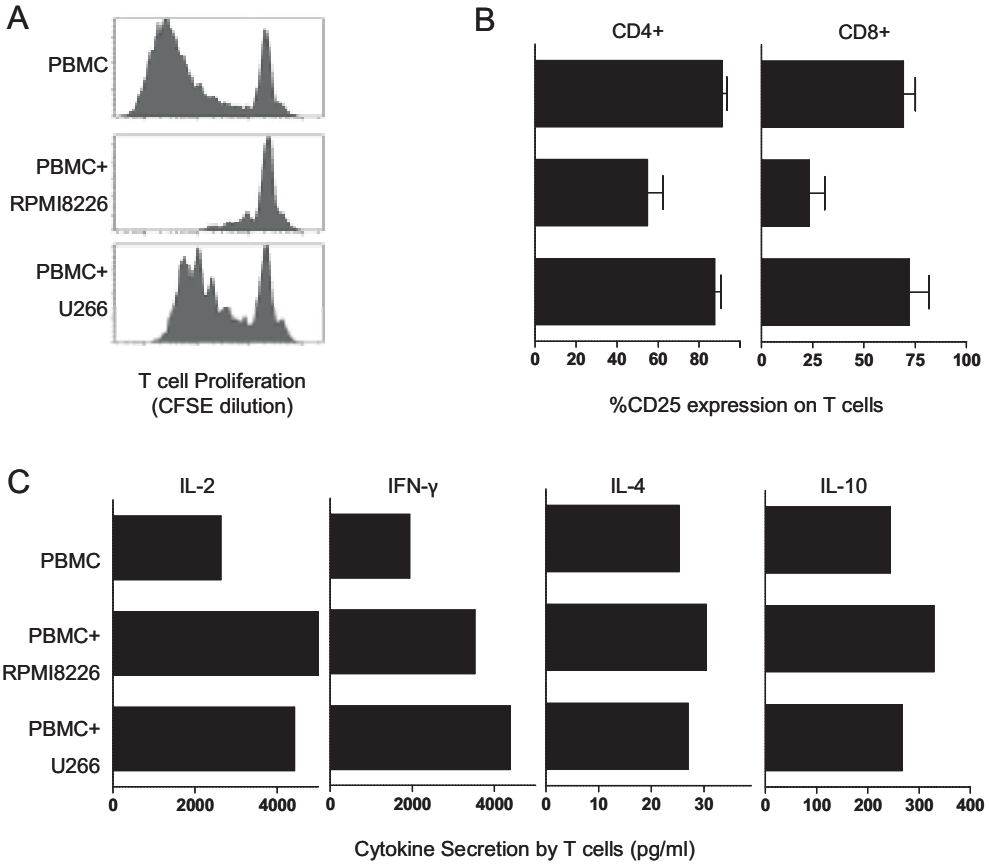
A
PBMC
PBMC+
RPMI8226
PBMC+
U266
T cell Proliferation
(CFSE dilution)
B
CD4+
CD8+
0 20 40 60 80
0 25 50 75 100
%CD25 expression on T cells
C
IL-2
IFN-γ
IL-4
IL-10
PBMC
PBMC+
RPMI8226
PBMC+
U266
0 2000 4000
0 2000 4000
0 10 20 30
0 100 200 300 400
Cytokine Secretion by T cells (pg/ml)
D
no Supp
Supp RPMI8226
% specific lysis
0 20 40 60 80 100
0:1 0.06:1 0.25:1 0.5:1 1:1
E:T ratio

Figure 5: Inhibitory cytokines produced by RPMI8226 only affect T cell proliferation and CD25 upregulation. (A,B) T cell proliferation in PBMC by CFSE dilution assay as measured in a transwell assay system as described in the material methods. In B, T cells were counterstained for CD4, CD8 and CD25 with fluorescent conjugated appropriate antibodies to determine the expression levels of CD5 within CD4 and CD8 subsets. The percentage of T cells expressing the CD25 is depicted. Error bars represent the SEM of triplicate cultures. (C) Cytokine production during T cell inhibition by RPMI. The cytokines were measured in the culture supernatants after stimulatin of PBMC with immunostimulatory beads for 3 days in the absence or presence of MM cells. Similar results were obtained in three independent assays. (D) Culture supernatants (25%) of RPMI8226 was added in a bioluminsescence-based cytotoxy assay, in which the cytotoxic activity of a CD4+ T cell clone was measured against the luciferase transduced target cell (UM9). Bioluminsecence signals from surviving target cells were determined after 24 hours and the % specific lysis was calculated relative to the wells in which no T cells were added.

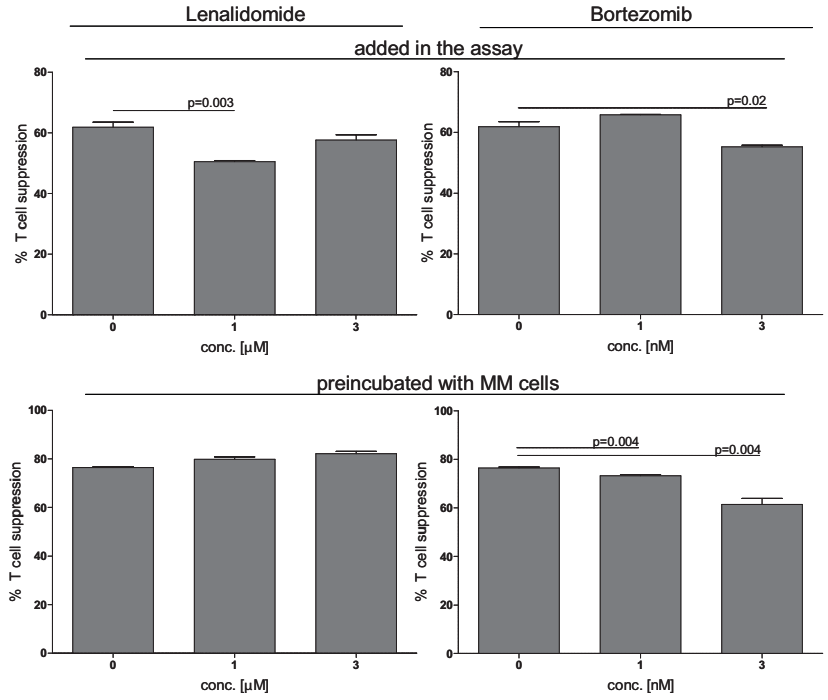

Figure 6: Chemotherapy partially reverses T cell inhibiton by RPMI8226.
(A) CFSE labeled PBMC from healthy individuals were stimulated with immunostimulatory beads for 3days in the presence of RPMI8226 and the BM stromal cell line HS-5 with or without addition of the indicated doses of lenalidomide and bortezomib (A). In B the MM cells were pretreated with LEN or BORT for 24 hours prior to assays. Assays were executed after extensive washing of the agents. Percentage T cell suppression was calculated relative to the proliferation in the wells stimulated in the absence of MM cells and stromal cells. The differences between groups was tested for statistical significance using two tailed, unpaired t tests.

Discussion

Understanding the extent and mechanisms by which multiple myeloma cells evade the immune system is a vital aspect to improve cellular immune therapies against MM and other cancers. To date several possible mechanisms, such as antigen loss, downregulation of HLA expression or costimulatory molecules, upregulation of co-inhibitory molecules, activation of regulatory T cells, induction of T cell apoptosis have been proposed. Several inhibitory cytokines such ad TGF-β or VEGF, which directly or indirectly suppresses activation of T cells were also implicated as immune escape mechanisms (reviewed in [35-38]). However, a common, readily controllable mode of immune evasion mechanism is yet to be demonstrated. In a series of experiments we now observed the potential important role of bone marrow stromal cells, which are the most important accessory cells of MM microenvironment, in the development of T cell inhibitory properties of MM cells. Stromal cells enabled most, if not all MM cell lines to secrete anti-proliferative cytokines. Bone marrow stromal cells also appeared to induce a suppressive phenotype in primary MM cells which were directly isolated from patients. Thus stromal cells appear to provide signals for MM cells to hamper the development of an effective anti myeloma response. Our findings are in agreement with the current notion that cells of the bone marrow environment, in particular the stromal cells, play key roles in MM cell growth and in the induction of the drug resistance by continuous cross talk with MM cells via surface receptors, growth factors, cytokines and other bioactive molecules[39]. Our results extend this notion by demonstrating another important outcome of MM-stroma interactions, the induction of immune escape mechanisms.

Also in other tumor models, such as in melanoma, the importance of tumor-stroma interactions in the immune evasion is increasingly being appreciated. For instance, it has recently been shown that mouse and human melanomas expressing the chemokine CCL21 can reorganize tumor stroma into a lymphoid-like reticular stromal network, associated with an immunotolerant cytokine milieu and the attraction of regulatory leukocyte subsets[40]. Nonetheless, in this and in several reports until now, the role of the microenvironment to reinforce the tumor with immunosuppressive properties has generally been uninvestigated. Thus, our results now, by revealing a new dimension in the immune escape phenomenon, provides another possible mechanism how the microenvironment of MM and other tumors could be converted into an immunotolerant milieu.

We are also highly intrigued by the fact that BM stromal cells induced/enhanced the T cell inhibitory capacity in almost all MM cells, even in many of the fast growing, apparently "stroma independent" MM cell lines, which are therefore generally considered to represent most malignant and aggressive forms of MM. Our results however reveal that many of these MM cell lines are still dependent on stroma to develop T cell inhibitory capacities. Thus stroma-tumor interactions may be a prerequisite to develop immune evasion mechanisms, even required by highly malignant, aggressive types of MM. Thus, careful disturbance of stroma-myeloma interactions may represent an attractive and common strategy to eliminate

immune escape mechanisms in a wide range of MM patients even in late stages of the disease. It should be noted that, currently not many possibilities are available for specific disturbance of stroma-MM interactions, without disturbing the interactons between T cells and MM cells. Therefore, an alternative strategy could be the identification and neutralization of the anti-proliferative cytokine secreted by MM cells. Our results suggest that several MM cell lines secrete the same inhibitory cytokine which is different than TGF-beta, IL-10 or VEGF. Howewer, it appears that advanced multi-dimensional separation techniques are necessary for its identification since since the crude size exclusion fractionation,followed by mass-spectrometry indicated that the fractions contained > 50 candidate cytokines.

Our results suggest however that that another and apparently more convenient way to downregulate the T cell inhibitory action of MM cell may be the use of carefully selected novel agents. Indeed we observed that the T cell inhibition by RPMI 8226 was partially significantly reverted by BORT and LEN. While BORT seemed indeed to work on MM cells, the effect was LEN was not directly on MM cells. Thus in our experimental setting BORT seemed to be a better choice to combine with immunotherapy. Obviously, the slight reversal of T cell inhibition by BORT and LEN in our assays is not impressive; it may be argued that these results may have no biological relevance. However, we need to emphasize that, in our assays we deliberately used BORT and LEN at quite low doses,by which no MM cell death was observed. We did this because at higher doses of the agents, strong but biased "reversal of T cell inhibition" occurred due to massive death of MM cells in the assay. Thus our results, due to technical reasons, may be underestimating the real impact of these novel agents. Our future studies will therefore focus on the evaluation of the outcome of the combination immunochemotherapy in a suitable in vivo model, such as our recently developed graft vs myeloma model in the immune deficient Rag2-/-γc-/- mice.

In conclusion, our results now provide evidence on the importance of bone marrow stroma to induce/facilitate the T cell inhibitory properties of MM cells and open new areas of investigation toward improvement of cellular immune therapy. Major future challenges are designing strategies for specific modulation of stroma-MM interactions, the identification and neutralisation of anti-proliferative cytokines secreted by MM cells, and investigation of the *in vivo* significance of modulating T cell inhibitory properties of MM cells by immunochemotherapy.

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4

Daratumumab, a Novel Therapeutic Human CD38 Monoclonal Antibody, Induces Killing of Multiple Myeloma and Other Hematological Tumors

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Abstract

CD38, a type II transmembrane glycoprotein highly expressed in hematological malignancies including multiple myeloma (MM), represents a promising target for mAb-based immunotherapy. In this study, we describe the cytotoxic mechanisms of action of daratumumab, a novel, high-affinity, therapeutic human mAb against a unique CD38 epitope. Daratumumab induced potent Ab-dependent cellular cytotoxicity in CD38-expressing lymphoma- and MM-derived cell lines as well as in patient MM cells, both with autologous and allogeneic effector cells. Daratumumab stood out from other CD38 mAbs in its strong ability to induce complement-dependent cytotoxicity in patient MM cells. Importantly, daratumumab-induced Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity were not affected by the presence of bone marrow stromal cells, indicating that daratumumab can effectively kill MM tumor cells in a tumor-preserving bone marrow microenvironment. In vivo, daratumumab was highly active and interrupted xenograft tumor growth at low dosing. Collectively, our results show the versatility of daratumumab to effectively kill CD38-expressing tumor cells, including patient MM cells, via diverse cytotoxic mechanisms. These findings support clinical development of daratumumab for the treatment of CD38-positive MM tumors.

CD38, a type II transmembrane glycoprotein highly expressed in hematological malignancies including multiple myeloma (MM), represents a promising target for mAb-based immunotherapy. In this study, we describe the cytotoxic mechanisms of action of daratumumab, a novel, high-affinity, therapeutic human mAb against a unique CD38 epitope. Daratumumab induced potent Ab-dependent cellular cytotoxicity in CD38-expressing lymphoma- and MM-derived cell lines as well as in patient MM cells, both with autologous and allogeneic effector cells. Daratumumab stood out from other CD38 mAbs in its strong ability to induce complement-dependent cytotoxicity in patient MM cells. Importantly, daratumumab-induced Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity were not affected by the presence of bone marrow stromal cells, indicating that daratumumab can effectively kill MM tumor cells in a tumor-preserving bone marrow microenvironment. In vivo, daratumumab was highly active and interrupted xenograft tumor rowth at low dosing. Collectively, our results show the versatility of daratumumab to effectively kill CD38-expressing tumor cells, including patient MM cells, via diverse cytotoxic mechanisms. These findings support clinical development of daratumumab for the treatment of CD38-positive MM tumors.

Multiple myeloma (MM) is a malignant disorder of the B cell lineage, characterized by neoplastic monoclonal expansion of plasma cells in the bone marrow resulting in destruction of adjacent bone tissue. MM remains an incurable disease (median overall survival, 4.4–7.1 y) despite the recent availability of new agents that, especially when used in combination regimens, have dramatically improved initial response rates (1–3). Therefore, new approaches that induce long-term tumor regression and improve disease outcome are needed and urgently sought for.

Targeted immunotherapy with mAbs has become critical for the successful treatment of many forms of cancer. This is exemplified by rituximab, a chimeric CD20 Ab, which has revolutionized the treatment of several B cell malignancies such as follicular lymphoma (4, 5). B cells, however, lose CD20 expression upon terminal differentiation into plasma cells, and rituximab consequently has conveyed very limited benefit to the treatment of MM (6–8).

In cancer immunotherapy, effective therapeutic Abs are typically thought to require both cytostatic and cytotoxic abilities (9). This can be optimally achieved if Ab binding to the target directly interrupts signaling and, in addition, activates potent cytotoxic immune effector functions such as Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Thus, cell surface-expressed molecules that 1) play an important role in cell growth or survival and that 2) are highly overexpressed on malignant cells represent attractive targets. Both of these requirements are in place for CD38 (10–12).

CD38 is a 46-kDa type II transmembrane glycoprotein with a short 20-aa N-terminal cytoplasmic tail and a long 256-aa extracellular domain (13). Functions ascribed to CD38 include receptor-mediated adhesion and signaling events, as well as important bifunctional ectoenzymatic activities that contribute to intracellular calcium mobilization (10). Under

normal conditions, CD38 is expressed at relatively low levels on lymphoid and myeloid cells and in some tissues of nonhematopoietic origin (14). The relatively high expression of CD38 on all malignant cells inMM(12) in combination with its role in cell signaling suggest CD38 as a potential therapeutic Ab target for the treatment of MM.

In the past, several Abs to human CD38 have been generated that induce killing of neoplastic B cell lines (15–17). Two CD38 mAbs are currently in clinical development: a humanized mAb (SAR650984) (18) and a human mAb (daratumumab; current work). Daratumumab represents an Ab with unique cytotoxic activities. Significantly, daratumum was found to be able to effectively kill MM tumor cells that were freshly isolated from patients by ADCC and CDC. Additionally, daratumumab was active at low concentrations in a SCID mouse xenograft tumor model. Daratumumab is currently in a phase I/II safety and dose finding study for the treatment of MM.

Materials and Methods

Cells

NIH 3T3 cells were transfected to express human CD38 (NIH-3T3-CD38) and used for immunizations. Briefly, NIH 3T3 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were transfected with pCIpuroCD38 vector (provided by Prof. M. Glennie, Tenovus Research Laboratory, Southampton General Hospital, Southampton, U.K.) using lipofectamine (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. By using limiting dilution, a high-expressing cell line was selected. Human CD38 transfected CHO cells (CHO-CD38) were provided by Prof. M. Glennie. Daudi cells were obtained from the American Type Culture Collection (LGC Standards, Teddington, U.K.).

Daudi-luc cells and UM-9-luc cells were generated as described previously (19–21). Ramos cells were provided by Prof. J. Golab (Department of Immunology, Center of Biostructure Research, The Medical University of Warsaw, Warsaw, Poland). Cells were cultured in RPMI 1640 medium supplemented with 1% (v/v) sodium pyruvate (BioWhittaker, Walkersville, MD), 1% (v/v) L-glutamine, 1% (v/v) penicillin and streptomycin, and 10% (v/v) cosmic calf serum (HyClone, Logan, UT).

All CD138-expressing MM cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). They were provided by sources previously described (22–24), the American Type Culture Collection (Manassas, VA), or by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Primary CD138-positive MM cells from patients were obtained after Institutional Review Board-approved (Dana-Farber Cancer Institute) informed consent using positive selection with CD138 microbeads (Miltenyi Biotec, Auburn, CA). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Residual CD138-negative bone marrow mononuclear cells were cultured in RPMI

1640/10% FCS for 3–6 wk to generate bone marrow stromal cells (BMSCs), as previously described (23).

In other experiments, mononuclear cells from bone marrow samples of MM patients were obtained from both untreated and relapsed patients, after informed consent and after approval by the Medical Ethical Committee (University Medical Center, Utrecht, The Netherlands). Relapsed patients had been treated extensively with a median of three treatment regimens that included induction therapy with anthracyclines followed by autologous stem cell transplantation, thalidomide, and bortezomib. In these experiments, negative selection was performed to obtain MM tumor cells using Abs directed against CD33 mAb (BD Pharmingen), CD11b mAb (BD Pharmingen), CD32 mAb, CD45RA mAb, CD45 mAb, anti-glycophorin A mAb, CD3 mAb, CD14 mAb (all from Dianova), and magnetic beads coated with human anti-mouse IgG (Invitrogen). After this negative selection procedure, the percentage of tumor cells varied between 75 and 92%, as determined by assessing the fraction of CD138-positive cells in flow cytometry.

Construction of hemagglutinin-CD38 expression vector pEE13.4HACD38

The extracellular domain of human CD38 was amplified from plasmid pCIpuroCD38 (obtained from Prof. M. Glennie). By PCR, sequences encoding an efficient signal peptide, an N-terminal hemagglutinin (HA)- tag, suitable restriction sites and an ideal Kozak sequence (GCCGCCACC) for optimal expression were introduced. The resulting PCR fragment was cloned into expression vector pEE13.4 (Lonza Biologics, Slough, U.K.) and after confirmation of the complete coding sequence, this construct was named pEE13.4HACD38.

Transient expression in HEK-293F cells

Freestyle 293F (HEK-293F) cells were obtained from Invitrogen and transfected with pEE13.4HACD38 and with the construct carrying the mutation S274F, according to the manufacturer's protocol using 293fectin (Invitrogen), producing recombinant HA-CD38 (extracellular domain of human CD38 with an N-terminal HA-tag). Culture supernatants of transfected cells were used in ELISA for CD38 mAb binding studies.

Purification of HA-CD38

HA-CD38 was purified from the supernatant using a CD38-Ab column, by coupling Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) to CD38 mAb AT13/5. For immunization, HA-CD38 was coupled to mariculture keyhole limpet hemocyanin (KLH) using the Imject Immogen EDC conjugation kit (Pierce/Thermo Fisher Scientific; Perbio Science, Etten- Leur, The Netherlands) according to the manufacturer's instructions.

Ab generation

CD38 Abs were generated by immunization of HuMAb-mice (Medarex, Milpitas, CA) with purified HA-CD38 recombinant protein alone or alternating with NIH-3T3-CD38

cells. After isolation of mouse splenocytes and lymph node cells and fusion, the resulting hybridomas were tested for binding to CHO-CD38 cells. All HuMAbs described in this article were of the IgG1, κ subclass. From this panel of 42 Abs, daratumumab was selected on basis of its unique ability to induce CDC of Daudi cells. In all experiments, anti-KLH, a human IgG1 Ab directed at KLH, was used as negative control (25).

Flow cytometry

Experiments were performed on ice. CHO-CD38 and Daudi cells were incubated with serial dilutions of CD38 mAb or isotype control. After washing and incubation with FITC-conjugated rabbit anti-human IgG (Dako, Heverlee, Belgium), cell-associated fluorescence was determined using an FACSCalibur with CellQuest Pro software (BD Biosciences, Erembodegem-Aals, Belgium).

MM tumor cells were incubated with mouse anti-human CD138 Ab (Dako) and serial dilutions of FITC-conjugated CD38 mAbs. After washing and incubation with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), cell-associated fluorescence was again assessed by flow cytometry, and analysis was performed after gating for CD138 expression.

ADCC

Lysis of plasma cell leukemia cells and MM tumor cells by ADCC was measured in a [^{51}Cr]-release assay as described (26). In experiments with plasma cell leukemia cells, an E:T ratio of 40:1 was used. In experiments with MM tumor cells, an E:T ratio of 100:1 was used. In some cases,MM cell lines and CD138-purified patientMM cells were subjected to calcein acetoxymethyl ester (calcein-AM) release ADCC assays as described elsewhere (23). The percentage of specific lysis was determined in the similar formula as in [^{51}Cr]-release ADCC assay.

Complement-dependent cytotoxicity

Lysis of Daudi cells and MM cells via CDC was measured by use of flow cytometry (FACSCalibur) after measurement of the percentage of propidium iodide (PI)-positive cells, as described previously (27, 28). Pooled human serum (10%) was used as source of complement. MM tumor cells were first stained for CD138 and goat anti-mouse IgG FITC as conjugate (Jackson ImmunoResearch Laboratories). MM cell lysis was determined after calculating the viable cells within the CD138-positive cell population. For data shown in Table I, GraphPad software was used to generate doseresponse curves, and top curve values were used to determine maximal lysis, which was reached between 0.3 and 1.2 μg/ml.

In a separate set of experiments, MMcells were subjected to calcein-AM release CDC assays in which a similar protocol was used as in the calcein-AM release ADCC assay. MMcells were incubated with Ab in the presence of 10% pooled human complement serum.

Epitope mapping

For the Pepscan approach, epitope mapping was performed essentially as described by Teeling et al. (28). In short, overlapping 20-mer linear and 15-mer looped peptides were synthesized covering 138 aa at the C terminus of human CD38. Furthermore, based on the sequence at the C terminus, single-looped peptides of different size were made covering region KNIYRPDKFLQCVKNPEDSSCTSEI, region CVHNLQPEKVQTLEAWVIHGG, and region CLESIISKRNIQFSAKNIYRC. Additionally, extra sets were designed to reconstruct double-looped regions that were composed of SKRNIQFSCKNIYR and EKVQTLEAWVIHGG. Native cysteines were replaced by alanines. Peptides were screened in an ELISA assay using credit card format mini-Pepscan cards.

For site-directed mutagenesis, mutations in the extracellular regions of human CD38 were introduced using the QuikChange XL site-directed mutagenesis kit, according to the manufacturer's instructions (Stratagene Europe, Amsterdam, The Netherlands). Mutagenesis was checked by sequencing (AGOWA, Berlin, Germany). Culture supernatants of transfected HEK-293F cells were used in ELISA. In this assay, anti-HA Ab (Sigma-Aldrich) was used as coating to capture HA-tagged CD38 proteins. Bound CD38 Abs were detected with HRP-conjugated goat anti-human IgG Abs (Jackson ImmunoResearch Laboratories, Newmarket, U.K.).

Mouse tumor xenograft models

Female, 8- to 10-wk-old C.B-17 SCID mice (C.B-17/IcrCrl-scid-BR), purchased from Charles River Laboratories (Maastricht, The Netherlands), or female 9- to 18-wk-old $RAG2^{-/-}\gamma c^{-/-}$ mice (29) were housed in filter-top cages in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands). Water and food were provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee. Mice were checked three times a week for signs of discomfort and for general appearance.

For the Daudi tumor xenograft model, experiments were performed essentially as decribed by Bleeker et al. (19). Briefly, 2.5 x 10^6 Daudi-luc cells/mouse were injected i.v. Mice were treated with a single dose of CD38 mAb or isotype control Ab HuMab-KLH. Two different treatment settings (early and late treatment) were used. In the early treatment setting varying doses of Ab (0.01–100 μg/mouse i.p.; as described in the text) were administered within 1 h after tumor cell inoculation. In the late treatment setting Ab (10 μg/mouse i.p.) was administered 14 d after administration of cells. At weekly intervals mice were injected with synthetic D-luciferin (acid form; Biothema, Haninge, Sweden) and luminescence was measured. For imaging, mice were anesthetized by i.p. injection of a mixture of ketamine, xylazine, and atropine before synthetic D-luciferin was given i.p. (2.5 mg in 200 μl 10 mM Tris base/mouse). Mice were then placed in a light tight box and after 3 min imaging was started using a VersArray 1300B liquid nitrogen cooled charge-coupled device detector (Roper Scientific, Vianen, The Netherlands). Photons emitted from the luciferase system were counted over an exposure period of 5 min. Under illumination, black-and-white images were

made for anatomical reference. MetaVue software (Universal Imaging/Molecular Devices, Downingtown, PA) was used for data collection and image analysis.

For the mouse UM9 tumor xenograft model, experiments were performed essentially as decribed by Rozemuller et al. (20). Briefly, 20 x 10^6 UM9-luc cells were injected i.v. $RAG2^{-/-}\gamma c^{-/-}$ mice were treated with a single dose of 50 mg daratumumab or isotype control Ab 3 wk after tumor injection. Bioluminescent imaging using synthetic D-luciferin was as described for the mouse Daudi tumor xenograft model.

Statistical analysis

Statistical analysis was performed by Student *t* test or by repeated measures one-way ANOVA followed by a Tukey post hoc test. In vivo differences were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. Significance of the dose escalation experiment was tested with a normal linear mixed effect model. Treatments were considered significant when $p < 0.05$.

Results

Four competition groups

Human Ig transgenic mice were immunized with recombinant CD38 protein and CD38-transfected NIH 3T3 cells (NIH 3T3- CD38) until CD38-specific serum titer development. After fusion of spleen and lymph node cells with SP2/0 myeloma cells, hybridomas secreting CD38 Abs were cloned by limiting dilution. A panel of 42 human mAbs that specifically bound to CD38- transfected CHO cells (CHO-CD38) was identified. By competition binding experiments on CHO-CD38 cells, we were able to divide the mAbs into four competition groups (data not shown). Fig. 1A and 1B show binding of exemplary Abs from each competition group (designated competition groups 1–4) to CHO-CD38 cells and Daudi cells. Whereas good to excellent binding to CHOCD38 and Daudi cells was found with Abs from competition groups 2, 3, and 4 (HM-003, daratumumab, and HM-029, respectively), somewhat weaker binding, particularly for Daudi cells, was found with the Ab from competition group 1 (HM-037). In a separate experiment, we demonstrated that daratumumab and HM-003 also bound to fresh MM tumor cells (Fig. 1C).

Induction of CDC

Tumor cell killing by CDC is considered an important mechanism of action for therapeutic Abs. The ability to induce CDC was tested in a first set of experiments with Daudi cells. After incubation of Daudi target cells with Abs and serum followed by staining with PI, killing was monitored by flow cytometry. Interestingly, only a single Ab in our panel of 42 human mAbs was able to induce CDC of Daudi cells. Fig. 1D shows that daratumumab from competition group 3 induced efficient complement-dependent lysis, with an EC_{50} of 0.16 μg/ml and achieving a maximal lysis of 56%, in contrast to prototypic Abs from the other

competition groups. Fig. 1E shows that daratumumab was also unique among competition group 3 mAbs in its ability to induce CDC. Our data show that no complement-mediated cell lysis of Daudi cells was observed with any of the other mAbs from this group.

Next, we investigated whether daratumumab would also kill freshly isolated MM cells via CDC. To this end, mononuclear cells containing MM cells were obtained from bone marrow of 13 previously untreated or relapsed MM patients. After incubating these cells with normal human serum in the presence of Ab, complementmediated lysis was determined. Daratumumab induced a concentration-dependent lysis of MM cells in 11 patients. The maximal lysis achieved for MM cells from each patient is shown in Table I (range, 10–90%; median, 51%). HM-003 was used as a control and did not induce CDC in any of the MM cell samples tested (data not shown).

Because the bone marrow microenvironment protects MM cells against cell death, we also studied whether daratumumab is able to effectively mediate CDC against MM cells in the presence of BMSCs. Importantly, daratumumab mediated effective CDC against XG-1 MM cells under these conditions (Fig. 1F), suggesting that daratumumab may also induce CDC in the bone marrow microenvironment.

Collectively, our results show that daratumumab is effective in killing MM cells via CDC.

Epitope mapping

The CDC experiments showed that the ability to induce complement-mediated killing is a unique characteristic of competition group 3 Ab daratumumab. To investigate whether this was linked to the epitope recognized, we first mapped daratumumab using a constrained peptide approach (Pepscan), which located its epitope to two β-strand–containing amino acids 233–246 and 267–280 of CD38 (Fig. 2A). We next investigated whether fine epitope heterogeneity existed between competition group 3 mAbs using a site-directed mutagenesis approach, in which we made use of the observation that epitope group 3 mAbs did not bind to CD38 from cynomolgus monkey (Macaca fascicularis) (data not shown). Mutant molecules incorporating all five amino acid differences between human and monkey CD38 located in the area shown in Fig. 2A were generated. Daratumumab, HM-025, HM-028, and HM-034 bound strongly to wild-type CD38 (Fig. 2B). Interestingly, replacing serine at position 274 for phenylalanine (S274F) completely abolished daratumumab binding, whereas binding of the other competition group 3 Abs was not affected (EC_{50} values: HM-025, 15 ng/ml; HM-028, 15 ng/ml; HM-034, 17 μg/ml) (Fig. 2C). Daratumumab thus seems to bind a unique fine epitope on the CD38 molecule.

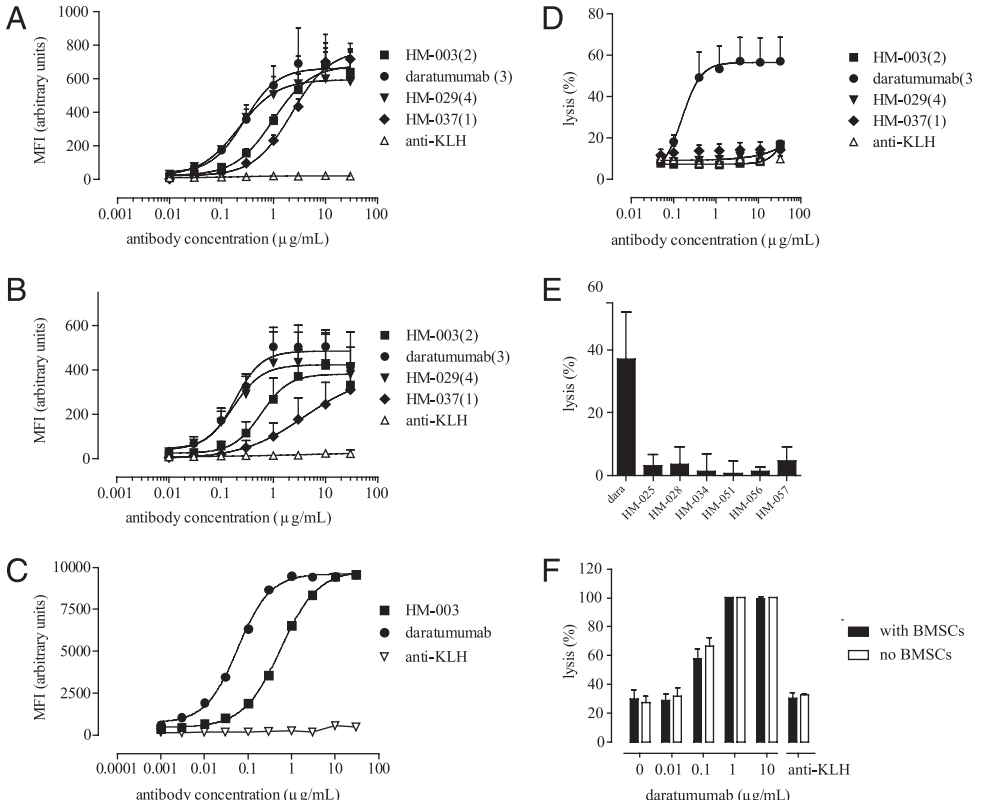

FIGURE 1. Binding and CDC of CD38 Abs. A, Binding of CD38 Abs from each competition group (numbers in parentheses) to CD38-transfected CHO cells. B, Binding to Daudi cells. Cells were incubated with various concentrations of Ab, and Ab binding was detected with an FITC-conjugated polyclonal secondary Ab. An anti-KLH mAb was used as an isotype control. Designated competition groups are given in parentheses. n, HM-003 (n = 4); d, daratumumab (n = 3); ▼, HM-029 (n = 4); ♦, HM-037 (n = 2); 4, isotype control anti-KLH (n = 3). Each data point shows the mean 6 SD obtained from independent experiments. C, Binding of daratumumab and HM-003 to fresh MM tumor cells. MM tumor cells were incubated with mouse anti-human

CD138 mAb and serial dilutions of daratumumab or HM-003. D, Daudi cells were incubated with CD38 Abs from each competition group in the presence of 10% pooled human serum from healthy donors as source of complement. Data show mean lysis and SD. Lysis was detected by measurement of the percentage of PI-positive cells by flow cytometry. Designated epitope groups are given in brackets. E, CDC of Daudi cells after incubation with different

Abs from competition group 3 in the presence of 10% pooled human serum from healthy donors as source of complement. Data show mean and SD after correction for lysis induced by anti-KLH. F, MM target cells were cultured in the absence (open bars) or presence (filled bars) of BMSCs in CDC assays. XG-1 cells were used as target cells. Anti-KLH (10 mg/ml) was used as negative control Ab. Data represent means of triplicates and SD.

Induction of ADCC against MM cells

Ab-dependent killing via FcR-bearing effector cells is also considered an important mechanism of action for therapeutic Abs (30, 31). We assessed daratumumab-mediated ADCC against Daudi cells and a panel of drug-sensitive and drug-resistant MM cell lines in the presence of PBMCs enriched for NK cells. ADCC was measured by use of a calcein-AM release assay. Daratumumab induced a dose-dependent lysis of Daudi cells (EC_{50}, ~0.01 μg/ml) and CD38-expressing MM cell lines (n = 14) but not of CD38-negative U266 MM cells. Data in Table II show percentage lysis measured at a concentration of 0.1 mg/ml, a concentration at which all Abs had achieved maximal lysis (median, 39%; range, 6–65%). Specificity was confirmed in control experiments in which CD38-transfected CHO cells were efficiently killed by daratumumab-mediated ADCC (specific lysis, 30% at 0.1 μg/ml) but not parental, nontransfected, CHO cells (4% at 0.1 μg/ml) (data not shown).

Next, we investigated the ability of daratumumab to induce killing of MM cells via ADCC obtained from four MM patients (patient status: one untreated, two relapsed, one refractory). The mononuclear cell fraction was enriched for tumor cells (range, 38– 92%). Effector cells from eight different healthy donors were used (A–H). Data in Fig. 3A show that daratumumab (1.0 μg/ml) could induce specific killing of patient MM cells in most experiments, but killing varied dependent on the patient and effector cells used (median, 21.6%; range, 3–46%).

We also investigated whether daratumumab would induce lysis of patient MM cells in the presence of patient-derived PBMC effector cells in an autologous setting. Importantly, daratumumab induced significant ADCC against autologous CD138-positive myeloma cells obtained from nine MM patients (Fig. 3B), as compared with the KLH isotype control Ab ($p = 0.016$, paired t test).

In an additional experiment, daratumumab was added to freshly isolated tumor cells obtained from a patient with a CD38/CD138- positive, chemotherapy-refractory, plasma cell leukemia. Effector cells from three healthy donors were used. Daratumumab (EC_{50}, 0.05 mg/ml; maximum lysis, 27%) and to a lesser extent HM-003 (EC_{50}, 0.08 μg/ml; maximum lysis, 17%) induced dose-dependent lysis of these leukemic plasma cells by ADCC (Fig. 3C).

Finally, we studied whether daratumumab is able to effectively mediate ADCC against MM cells in the presence of BMSCs. Daratumumab-mediated ADCC, of either dexamethasone-sensitive MM1S or dexamethasone-resistant MM1R cells, showed a similar dose response in the absence or presence of BMSCs (shown for MM1R cells in Figs. 3D). Note that the apparent decrease in killing in the presence of BMSCs appears to be due to a generalized effect of these cells on MM survival. These observations suggest that daratumumab may also induce ADCC in the bone marrow microenvironment.

As a control experiment, we incubated PBMCs from healthy donors with daratumumab in the presence or absence of active complement. No daratumumab-mediated lysis of primary human B and T cells, activated T cells, NK cells, and monocytes was observed (data not shown). This suggests that daratumumab selectively kills MM tumor cells.

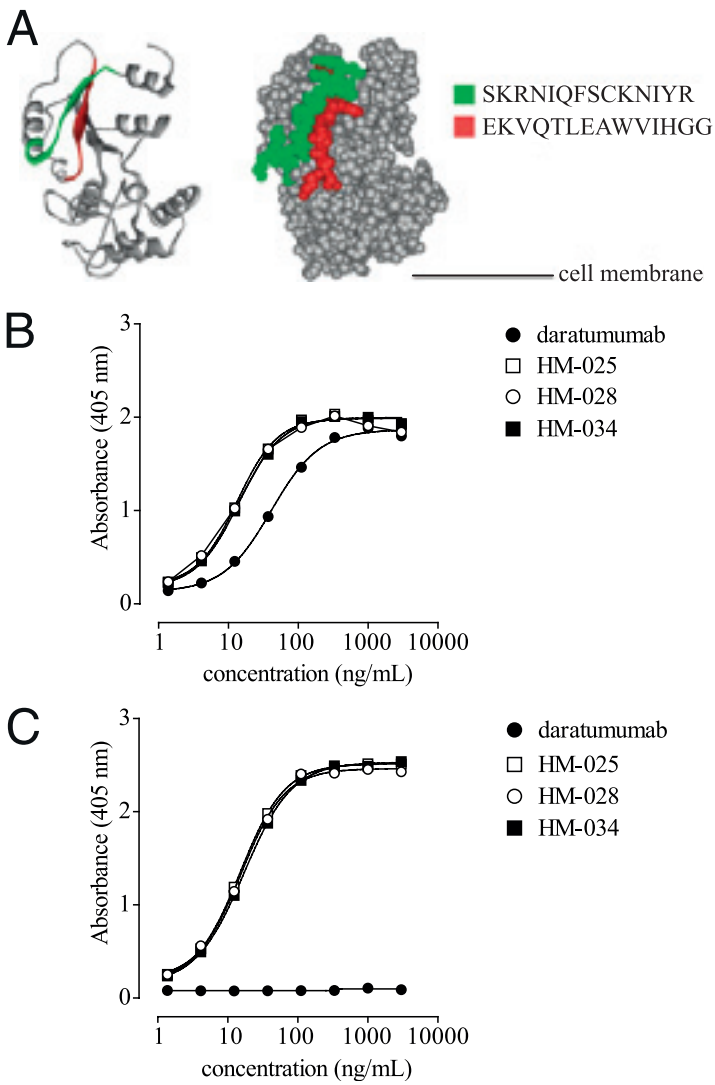

FIGURE 2. CDC induction by CD38 Abs is related to a specific epitope. A, The epitope recognized by daratumumab was mapped by use of a constrained peptide approach. The epitope was localized to two b-strands depicted in green and red in two representations of the crystal structure of the extracellular domain of CD38, and it was visualized by use of visual molecular dynamics molecular modeling software (51). B, Competition group 3 Ab binding to wild-type CD38. C, Ab binding to S274F mutant CD38. Binding of the CD38 mAbs to HA-CD38 was monitored by measuring absorbance at 405 nm in ELISA. ● Daratumumab; □, HM-025; ○, HM- 028; ■, HM-034. Figure shows data from one experiment that has been repeated with similar results.

Table I. Percentage maximum complement-mediated lysis induced by daratumumab in fresh MM cells obtained from tumor patients.

Patient	Clinical Status	Lysis (%)
1	Relapse	90
2	Relapse	12[a]
3	Relapse	88
4	Untreated	63
5	Relapse	81
6	Relapse	36[a]
7	Relapse	22
8	Untreated	68
9	Relapse	18
10	Relapse	53
11	Relapse	10[a]
12	Relapse	49
13	Relapse	84[a]

Data show lysis at 1 μg/ml.
[a]Top value could not accurately be determined by GraphPad software (see Materials and Methods)

Table II. Daratumumab induces ADCC in CD38-expressing MM cell lines

Cell Line	Daratumumab (% Lysis)
Daudi	61.5
MM1R	43.2
MM1S	47.5
28BM	23.8
28PE	6.1
12BM	15.4
12PE	39.1
RPMI 8226	54.2
LR5	39.2
DOX40	39.3
L363	52.1
H929	65.2
INA6	18.0
LP-1	54.6
UM-9	27.2
U266	0

Data show maximal killing at 0.1 μg/ml obtained in a representative experiment. The experiment was repeated two to three times, depending on the cell line tested.

Daratumumab shows potent anti-tumor activity in mouse xenograft tumor models

Daratumumab was able to induce potent cytotoxic effects in vitro. To examine whether these characteristics translated into anti-tumor efficacy in vivo, we evaluated daratumumab in two mouse xenograft tumor models, based on Daudi lymphoma and UM-9 multiple myeloma cells stably expressing the luciferase gene (19, 20).

CD38-expressing Daudi-luc cells were injected i.v. into the tail vein of SCID mice. In initial experiments, we assessed whether daratumumab could prevent tumor outgrowth of Daudi-luc cells by administering Abs and cells on the same day (early treatment). Data in Fig. 4A show that tumor growth was observed from day 14 and onward in the mice receiving control treatment. In contrast, mice receiving daratumumab at a dose of 5 mg/kg were fully protected against tumor development, and no growth of tumor cells could be detected. On day 28, a significant difference was apparent between the control and daratumumab-treated mice ($p > 0.001$; Fig. 4A). In an additional experiment the minimal effective dose was assessed for this early treatment setting. Mice were treated with daratumumab at different dose levels, ranging from 0.01 (0.5 μg/kg) to 10 μg per mouse (0.5 mg/kg). Inhibition of tumor growth at all tested dose levels was significantly different from the control group from day 21 onward ($p > 0.001$; Fig. 4B). At 1 μg per mouse (peak serum levels of ~500 ng/ml), we observed a similar effect as with the saturating dose, but with lower doses the antitumor effect decreased (although group size is not sufficient to show significance with two-way ANOVA). The remaining anti-tumor activity of daratumumab at the 0.01 mg dose (peak serum level of ~5 ng/ml) demonstrates that the Ab is still active at subsaturating concentrations. The common half-maximal effect dose was estimated to be 0.0013 μg (95% C.I. 0.00036–0.0043 μg), which corresponds to 0.065 μg/kg body weight (95% C.I. 0.018–2.15 μg/kg). This experiment demonstrated the significant anti-tumor potential of daratumumab.

To evaluate daratumumab in a late treatment setting (Fig. 4C), Ab was injected i.p. at a dose of 0.5 mg/kg 14 d after challenge with Daudi-luc cells. Daratumumab effectively inhibited tumor growth compared with control-treated animals in which tumor grew rapidly and therefore required euthanasia on day 35. On day 28 and day 35, tumor size in daratumumab-treated animals was significantly different from the control ($p > 0.01$, two-way ANOVA).

Finally, daratumumab was tested in aMMmouse tumor model in a late treatment setting with UM9 cells (Fig. 4D). Administration of daratumumab 3 wk after tumor cell inoculation resulted in significant inhibition of tumor cell growth as compared with control treatment ($p > 0.05$ at day 35; $p > 0.001$ at day 42, twoway ANOVA).

Collectively, our data show that daratumumab is highly efficacious in two different in vivo tumor models, in both early and late treatment settings.

Discussion

A panel of 42 novel human CD38 mAbs was generated using the proven technology of human Ab transgenic mice (32, 33). In this panel of Abs, the Ab daratumumab stood out. It was shown to bind to a unique region on the CD38 molecule, which facilitated killing of cell lines derived from various hematological tumors by ADCC, CDC, and apoptosis. Interestingly, daratumumab also potently induced killing of a large panel of patient MM tumor cells via ADCC and CDC. Our xenograft data suggest that this translates into a CD38 mAb with high anti-tumor activity in vivo.

Daratumumab was shown to induce CDC in a range of tumor cell samples from MM patients. Several studies have stressed the importance of CDC for therapeutic efficacy of Abs. For example, alemtuzumab (CD52 mAb) has been found to kill B cell tumor cells mainly via CDC (34), and the in vivo efficacy of rituximab (a chimeric CD20 Ab) is largely blocked by depletion of complement (35). In certain CLL patients, infusion of rituximab led to consumption of .90% of the patients' hemolytic complement activities for periods of days to weeks (36). Moreover, the efficient killing of CLL cells by the human CD20 Ab ofatumumab is correlated with potent CDC (27, 28, 37). In contrast to rituximab, however, only minimal amounts of complement were needed for ofatumumab to induce CDC (38). Data suggest that the positioning of ofatumumab after binding to its target may be ideal for complement activation and generation of the membrane attack complex (38). The same may hold true for daratumumab. In fact, induction of CDC was a very specific feature of daratumumab, as no other CD38 Ab from our panel was able to kill tumor cells in the presence of complement. Daratumumab was initially clustered in competition group 3 with a number of other CD38 Abs that all compete for binding. Interestingly, fine epitope mapping using constrained peptides and sitedirected mutagenesis revealed that daratumumab binds to a unique fine epitope on CD38 distinct from the epitope recognized by the other competition group 3 Abs. Presumably, the binding of daratumumab to this unique CD38 epitope clusters and positions the Ab Fc in a way that facilitates more optimal binding and/or activation of complement proteins.

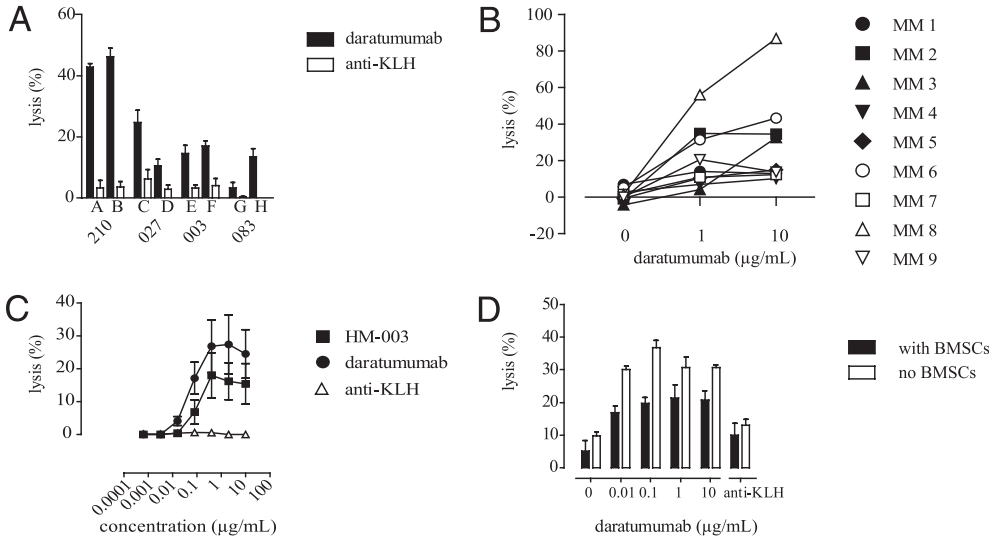

FIGURE 3. Daratumumab induces ADCC against MM cells. A, Percentage of maximum lysis of daratumumab-induced ADCC of tumor cells isolated from the bone marrow of four MM patient (patients are depicted by numbers). Cytotoxicity was determined by [51Cr] release. PBMCs were used as effector cells. For each patient sample, an experiment was performed with effector cells from two healthy donors (A–H). Filled bar, daratumumab; open bar, anti-KLH. B, Daratumumab induced dose-dependent ADCC against autologous patient MM cells (MM 1–9) as measured by calcein-AM release assays. CD138-purified myeloma cells from MM patients were used. C, A [51Cr]-release assay was performed using mononuclear cells from three healthy donors as effector cells. PBMCs of a patient with plasma cell leukemia were used as target cells. Data show mean 6 SEM of three experiments. n, Lysis induced by HM-003; d, lysis induced by daratumumab; 4, lysis induced by anti-KLH, the isotype control Ab. D, MM target cell line MM1R was cultured in the absence (open bars) or presence (filled bars) of BMSCs in calcein-AM release ADCC assays. NK cells from one healthy donor were used as effector cells in the ADCC assay. Anti-KLH (10 mg/ml) was used as negative control Ab.

In MM patients, the complement system may be compromised due to decreased levels of components of the classical and alternative complement pathways (39–41). In an in vitro setting we could demonstrate that daratumumab is able to induce maximal complement-mediated lysis of MM cells in medium containing only 10% human serum. Furthermore, daratumumab-induced lysis could be restored in C1q-depleted serum by the addition of low amounts of C1q (representative of ~7% of normal C1q serum levels, data not shown). This suggests that daratumumab is still effective under complement-limiting conditions occurring in MM patients. Nevertheless, it will be important to monitor these aspects in the clinic.

In addition to CDC, daratumumab induced ADCC of many different tumor cell lines

with varying CD38 expression, patient MM tumor cells, and plasma cell leukemia cells. In line with this, it has been described that chimeric and humanized CD38 Abs were also able to induce ADCC in Burkitt lymphoma cell lines (15, 16). ADCC through IgG FcγR-bearing immune effector cells is thought to be an important mechanism of action by which therapeutic Abs exert their effect in vivo: in mice lacking expression of activating FcγR, cancer therapeutic Abs lose their effect on tumor growth (30), and in cancer patients FcγR polymorphisms directly impact therapeutic responses to Abs (31, 42, 43).

These data are in line with those of Stevenson et al. (15). They demonstrated that blood samples from myeloma patients who had undergone a variety of chemotherapeutic schedules were still able to mediate ADCC, resulting in lysis values that were not different from those obtained with effector cells from healthy controls (15). In fact, lytic activity of one of the major cell types involved in ADCC induction, NK cells, in peripheral blood of MM patients was comparable to normal control values and even increased in the bone marrow (44). Thus, engagement of immune effector cells in MM patients, and also in the bone marrow where tumor cells reside, is expected to occur. Our observations that daratumumab was capable of inducing ADCC against patient MM cells mediated by autologous PBMC effector cells (Fig. 3B) and that it effectively triggered myeloma cell lysis via ADCC as well as CDC in the presence of BMSCs are indeed significant. These results suggest that daratumumab should also be active in the patients' bone marrow microenvironment, which usually supports MM growth, survival, and the development of drug resistance.

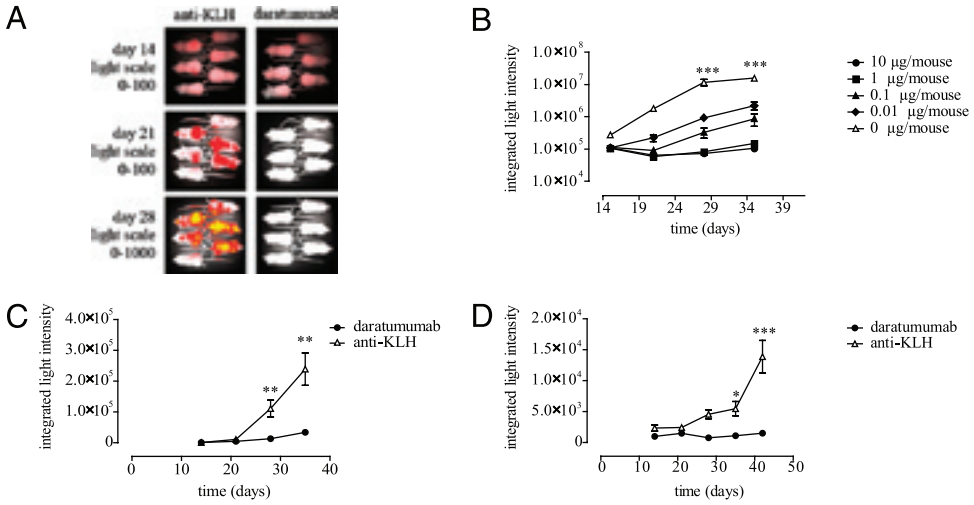

FIGURE 4. Daratumumab inhibits outgrowth of CD38-expressing tumor cells in two different SCID xenograft models. A–C, SCID mice were inoculated with luciferase-expressing Daudi cells. A, Immediate treatment with daratumumab (100 mg/mouse). Control animals were euthanized on day 28 to avoid discomfort per protocol. Light emission from the back side of individual mice was collected at different time points. B, Immediate treatment (0.01–10 mg/mouse). Control mice were euthanized on day 35. Light emission collected from the back sides of mice was integrated over the total body areas of individual mice and plotted in time as measure of tumor mass development. A linear mixed effect model was used to analyze for significance. A significant difference was found between the control group (0 mg) versus all other groups, ***p , 0.001. C, Treatment was started on day 14 (10 mg/mouse). Mice were euthanized on day 35. Light emission collected from the back sides of mice was integrated over the total body areas of individual mice and plotted in time as measure of tumor mass development. D, RAG22/2gc2/2 mice were injected with luciferase-expressing UM-9 cells. Mice were treated with 50 mg daratumumab 3wk after cell inoculation. Mice were euthanized on day 42. Data represent mean and SEM (six to eight mice per group). For data shown in C and D, a repeated-measures two-way ANOVA followed by a Bonferroni post hoc test on transformed data were used for statistical analysis. *p , 0.05; **p , 0.01; ***p , 0.001.

Recently, Benson et al. (45) showed that upregulated expression of the program death receptor-1 on NK cells of MM patients downmodulates the NK cell-mediated killing of MM cells via interaction with program death ligand-1. Although we show that daratumumab is able to induce ADCC of MM primary tumor cells in an autologous setting (Fig. 3B), we cannot exclude that interference with the program death receptor-1/program death ligand-1 axis, such as by downregulation of program death ligand-1 by lenolidomide treatment, could further enhance killing efficacy (23, 45–47).

The in vitro cytotoxic effects of daratumumab prompted us to investigate its activity in vivo. In an early treatment setting, daratumumab at a low dose of 0.5 µg/kg almost completely

inhibited Daudi cell tumor growth. Our analysis estimates a half-maximal effective dose of 0.065 μg/kg body weight (95% C.I. 0.018–2.15 μg/kg). Importantly, in the late treatment settings (14 or 21 d after tumor establishment), a single dose of 0.5 or 2.5 mg/kg daratumumab suppressed tumor growth of Daudi cells or UM-9 cells, respectively, for at least 5 wk.

We observed unusually high efficacy of daratumumab in our Daudi tumor xenograft mouse model (half-maximal effective dose of 0.065 μg/kg). Although in this experiment treatment was performed at a low tumor load, this represents a dose at which the number of CD38 molecules expressed on the injected Daudi cells largely exceeds the number of administered daratumumab molecules. This indicates that daratumumab can already induce antitumor effects when only a small fraction of the CD38 molecules is occupied.

We should take into account that dosing requirements may be quite different in patients. Thus, pharmacokinetics can be affected by binding of daratumumab to CD38 molecules expressed on tumor cells and normal cells. Additionally, soluble CD38 molecules present in sera and ascites from myeloma patients (48) may affect Ab activity and clearance, although Stevenson et al. (15) did not observe binding of CD38 Abs to soluble CD38 in sera from multiple myeloma patients. The effect of high tumor load on pharmacokinetics has, for example, been demonstrated for CD20 Abs in patients with B cell tumors, where, after the first dose, plasma concentrations were much lower than expected. However, upon repeated dosing, CD20 Ab clearance decreases and effective serum Ab concentrations are obtained (49, 50). Therefore, we think that also for daratumumab the possible impact of cellular or soluble CD38 expression will be manageable in clinical applications.

In conclusion, we have generated a human IgG1 CD38 mAb, daratumumab, that binds the CD38 molecule at a unique epitope and is effective in killing tumor cells via multiple mechanisms of action, including CDC and ADCC. Importantly, daratumumab induced substantial autologous ADCC against patient MM cells and cell killing was still observed in the presence of BMSCs. Finally, daratumumab demonstrated a potent ability to disrupt MM and B cell tumor growth in two in vivo xenograft models. These data indicate that daratumumab is a therapeutic Ab with high potential for the treatment of CD38-positive MM tumors.

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5

Towards effective immunotherapy of myeloma: enhanced elimination of myeloma cells by combination of lenalidomide with the human CD38 monoclonal antibody daratumumab

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Abstract

Background
We evaluated the potential benefits of combining the immune modulatory drug lenalidomide (LEN) with daratumumab (DARA) in our efforts to develop novel effective treatment regimens for multiple myeloma (MM). DARA is a novel human CD38 monoclonal antibody, which induces killing of $CD38^+$ cells via complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated-cytotoxicity (ADCC) and apoptosis.

Design and method
Using a CD38+ MM cell line, purified MM cells and full BM-MNCs of MM patients containing 2-50% malignant plasma cells in FACS based cytotoxicity, we ascertained the effect of lenalidomide combined with the antibody daratumumab.

Results
We demonstrate that DARA-induced ADCC of purified primary MM cells, as well as of a CD38+ MM cell line UM-9, was significantly augmented by pre-treatment of PBMC effector cells with LEN. Importantly, the availability of a novel assay allowed us to investigate and demonstrate the synergy between LEN and DARA-induced ADCC directly in whole bone marrow MNC, thus without isolating MM cells from their natural environment.

Conclusions
Finally, the significant upregulation of DARA-induced ADCC in PBMC derived from three MM patients while under LEN treatment underscored the notion that powerful and complementary effects may be achieved by combining LEN and DARA in clinical MM management.

Introduction

Over the past years, experimental and clinical studies have indicated that the use of chemotherapeutic agents with strong immune stimulatory properties can offer promising perspectives for multiple myeloma (MM) patients (1;2). Perhaps the best example of such an agent is lenalidomide (LEN)(3-5), a structural analogue of thalidomide (THAL) with a similar but more potent immunomodulatory activities (6-8). Since it's approval by the FDA, LEN demonstrated impressive results in patients with newly-diagnosed and relapsed MM (9;10) and also improved Graft vs. MM effects after allogeneic stem cell transplantation(11). In several initial studies, LEN has been frequently used alone or in combination with other chemotherapeutical agents(12-15). Nonetheless, such strategies are possibly not exploiting the full immunomodulatory capacities of LEN. Especially its NK cell stimulatory properties suggest that LEN could be highly effective in combination with therapeutic antibodies capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC)(16-18). Supporting this idea, a number of earlier studies showed that both THAL and LEN can enhance rituximab-mediated ADCC(19-21). Furthermore, MM cell lysis was significantly improved when LEN was combined with a humanized CD40 antibody(22;23). Indeed, promising results are being reported from a number of recently started clinical trials combining LEN with rituximab or CD40 antibodies for the treatment of chronic lymphoid leukaemia, lymphoma and MM (24-26).

A highly interesting target for antibody therapy in MM is the CD38 molecule, a 46 kDa type II transmembrane glycoprotein with a short N-terminal cytoplasmic tail (20 amino acids) and a long extracellular domain (256 amino acids)(27;28). CD38 is expressed at low or moderate levels on various haematopoietic cells and in some solid tissues; but its extremely bright and uniform expression on all MM cells suggests CD38 as an optimal therapeutic target for antibody therapy (29;30). Recently we have developed a new human CD38 antibody, daratumumab (DARA) and we have shown that it induces tumor cell killing via anti-Fc-mediated effector functions, e.g. complement dependent cytotoxicity (CDC), (NK) cell-mediated ADCC and apoptosis upon secondary cross-linking. Therefore, we now investigated the possibility that combining LEN with DARA would significantly enhance MM tumor cell killing. In a series of experiments using a CD38+ MM cell line, purified MM cells and full BM-MNCs of MM patients containing 2-50% malignant plasma cells, we demonstrate that LEN significantly improves DARA-dependent lysis of MM cells, mainly by activating the effector cells of ADCC. Furthermore, PBMCs isolated from patients during or just after LEN treatment show increased capacity to mediate DARA-dependent ADCC against MM cells, underscoring the potential clinical benefits that can be obtained by combination of DARA with LEN in the clinical setting.

Design and Methods

Primary MM cells &. MM cell lines

Primary $CD138^+$ MM cells were isolated from bone marrow of MM patients after written informed consent using anti CD138 (Becton Dickinson) coated Rabbit-anti-Mouse microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. Isolated primary MM cells were immediately used in experiments after determining the expression of CD138 and CD38. The CD38+ MM cell line UM9 has been generated and were maintained as previously described (31;32).

PBMC from healthy donors and MM patients: All procedures involving material from healthy donors and MM patients were approved by the institutional medical ethical committee. Peripheral blood was obtained from healthy volunteers and from MM patients after written informed consent. PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation(33). Freshly isolated PBMC from healthy individuals were used either immediately or after culturing with LEN (Cellgene, 3µmol/L) for 3 days as effector cells in ADCC assays. PBMC from MM patients were frozen prior to use as effector cells in ADCC assays.

Bone marrow mononuclear cells (BM-MNC) from MM patients: All procedures involving BM material were approved by the institutional medical ethical committee. MNC from BM were isolated by Ficoll-Hypaque density-gradient centrifugation(34) and contained 2-50% malignant plasma cells. Freshly isolated BM MNCs from patients were treated immediately with LEN (3µmol/L) and/or DARA (Genmab, 10 µg/ml) for 48 hours and used in FACS based BM ADCC assays.

Immunophenotyping: PBMC, MM cells and BM-MNC were labeled with the indicated Fluorescein conjugated antibodies. The cells were washed and analyzed by Flow cytometry using FACScalibur device (Becton Dickinson). Data was analyzed by using Cellquest Pro data acquisition software.

Antibody-dependent cell-mediated cytotoxicity (ADCC against purified MM cells. ADCC was measured by standard 4 hour chromium-51 (^{51}Cr) release assays using 2500 cells/well (^{51}Cr labeled) and a 100 fold of effector cells in the presence or absence of antibodies (0.01-1 µg/ml). Percentage specific lysis is calculated as follows: % specific lysis = (Mean experimental release – mean spontaneous release) / (Mean maximum release – mean spontaneous release) x100.

FACS-based ADCC and CDC assays in BM-MNC: These assays are executed as described previously (35) with slight modifications: Briefly, BM-MNC were isolated by Ficoll-Hypaque

density-gradient from full BM aspirates and incubated with or without 10 μg/ml antibody and 3 μM LEN. After 24 to 48 hours BM-MNCs were stained with CD138 PE mAbs (BD) for ADCC. For CDC, BM-MNCs were treated with LEN for 24 hours and 10 μg/ml antibody and complement were added 45 minutes prior to FACS measurements, in which absolute numbers of cells were determined by using flow cytometric count beads as a constant. The percentage MM cell lysis was determined relative to controls with the following formula: % lysis cells = (1- (absolute number of surviving cells in treated wells / absolute number of surviving cells in control wells) x 100%. To determine the overall lysis percentages the wells treated with control antibody was used as control values.

Statistics. Differences between treatment groups were tested for significance in repeated measures ANOVA, using Tukey's post-hoc multiple comparison tests and in paired students' tests with 2-tailed 95% confidence intervals. P-values below 0.05 were considered significant. We tested whether the effect of DARA and LEN is proportional (the product of the effects of the individual treatments) or results in an extra effect (Figure 3b). To this end, the ratios between cell counts with DARA relative to medium and the combination of LEN+DARA relative to LEN were calculated and the log-transformed ratios were fitted by a mixed model, with fixed effect LEN (yes, no) and a random effect for patient sample.

Results

Lenalidomide significantly improves daratumumab-dependent antibody-dependent cell-mediated-cytotoxicity by activating effector cells.

Since ADCC via NK cells is an important mechanism of action for DARA, and LEN is known to increase the activity of NK cells, we first investigated the possible improvement of DARA-dependent ADCC by LEN. Hereto, we first used standard four-hour ADCC assays, in which PBMC from different healthy individuals (n=10), untreated or treated with LEN for 72 hours, were used as effector cells against the MM cell line UM-9 (n=5) or against primary MM cells (n=6) freshly isolated from the BM of patients (Figure 1). Untreated PBMC from five different healthy individuals mediated significant DARA-induced ADCC against UM9 cells (Figure 1A). A representative dose-response curve is depicted in figure 1B. ADCC against UM-9 was significantly enhanced by pretreatment of PBMC with LEN ($p<0.001$). Likewise, LEN pretreatment of PBMC resulted in significant enhancement of ADCC against primary MM cells; $p<0.05$) (figure 1C), indicating that LEN can improve the effects of DARA through activation of effector cells mediating ADCC.

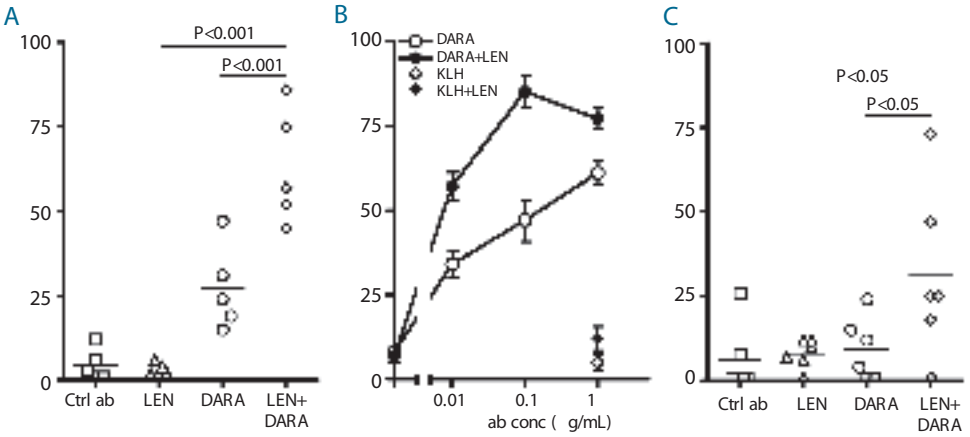

Figure 1: Enhancement of DARA induced ADCC by preincubation of effector PBMC with LEN. PBMC from healthy individuals were incubated with LEN (3 μM) for 72 hours prior to ADCC assays in which UM9 (A, B) and purified MM cells (C) were used after addition of DARA (0.1 μg/ml) or a control antibody against KLH (1μg/ml). A representative DARA dose-response curve is depicted in (B). P values are calculated by the Tukey's post-hoc analysis of a repeated measures ANOVA.

Next to its immunomodulatory properties, LEN can directly induce apoptosis in MM cells. LEN was also shown to sensitize MM cells to CD40–induced cell death. We therefore also evaluated whether LEN could sensitize MM cells for DARA-induced ADCC. To test this,

we treated primary MM cells or the UM9 cells with LEN for 24 hours prior to ADCC assays in which PBMC from healthy donors were used as effector cells. Pretreatment of MM cells did not change the expression of CD38. Also the expression of MHC class I and LFA-1, surface molecules that can influence NK cell mediated lysis remained unchanged (data not shown). Pretreatment of MM cells with LEN did not result in significant improvement of ADCC (Figure 2) indicating that LEN does not sensitize MM cells for DARA-mediated ADCC.

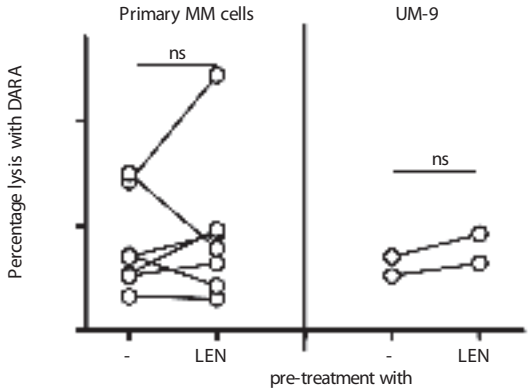

Figure 2: Absence of sensitization of MM cells by LEN for DARA-induced ADCC. Primary MM cells (N=7, left) or UM9 (n=2, right) where pretreated for 24 hours with LEN (3μM). A standard ADCC was performed with DARA (0,1 μg/ml) and PBMC from healthy donors. Percentage lysis is calculated as indicated in material and methods. P values are calculated by a paired t test.

Lenalidomide improves daratumumab-induced antibody-dependent cell-mediated-cytotoxicity of multiple myeloma cells by whole bone marrow mononuclear cells.

To evaluate the outcome of the combination therapy in a more physiological manner, we conducted a new series of ADCC assays without isolating MM cells from their natural environment. Thus, BM-MNC of MM patients containing stromal cells and 2-50% malignant plasma cells (n=14) were treated with LEN, DARA or with the combination for 48 hours. The survival of MM cells in the BM was then determined by enumeration of CD138+ cells by FACS staining. In these assays, 0-5% MM cell lysis was observed in the presence of the control anti KLH antibody. As illustrated in figure 3A, LEN alone induced poor to moderate levels of MM cell lysis. In contrast DARA induced substantial MM cell lysis, which was significantly increased when it was combined with LEN. Interestingly, a mixed model analysis revealed that LEN and DARA acted in a synergistic fashion to generate 20% extra effect as compared to expected additive effects of the agents (figure 3B).

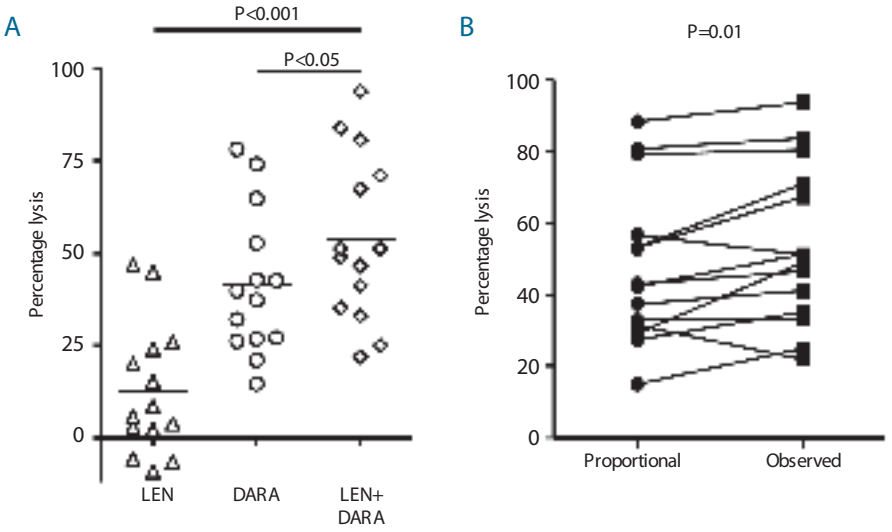

Figure 3: Improvement of DARA-induced ADCC by LEN in BM-MNC of MM patients. (A) BM-MNC of MM patients (n=14) were incubated for 48h hours with LEN (3 μM) and DARA (0.1 μg/ml). Surviving MM cells were enumerated by FACS analysis of CD138+ cells. Percentage lysis of MM cells in LEN, DARA and LEN+ DARA treated were calculated using the MM survival of wells treated with the control antibody. P values are calculated by the Tukey's post-hoc analysis of repeated measures ANOVA. (B) The observed effect (% lysis) of the combination treatment was compared to the expected proportional effect of the combined treatments. Mixed model analysis (see material and methods) supports the conclusion that the combination treatment is synergistic.

Lenalidomide does not interfere with daratumumab-induced complement-dependent cytotoxicity

DARA has documented strong CDC effects on primary MM cells (submitted). Here, we determined the impact of LEN on DARA-mediated CDC in our whole BM-MNC system. We treated freshly isolated BM-MNC of MM patients (n=16) with LEN for 24 hours, added DARA in the last 45 minutes in the absence or presence of active complement (heat inactivated or inactivated human serum) and determined the lysis of MM cells by FACS (figure 4). LEN alone induced little or no lysis of MM cells in the absence or in the presence of complement. DARA mediated moderate lysis of MM cells in the absence of complement, but as expected, the lysis was significantly upregulated by the addition of complement. The combination of LEN and DARA did not further improve cell killing via CDC as compared to killing with DARA alone (figure 4).

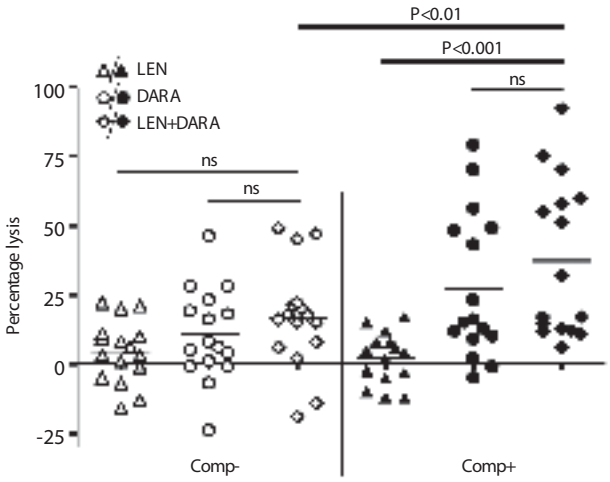

Figure 4: MM cell lysis in the BM of MM patients by the combination of DARA+LEN in the absence or presence of complement. BM-MNCs from MM patients (n=16) were subsequently incubated for 24h hours with LEN and for 45 minutes with or without complement in the absence or presence of DARA. P values are calculated by the Tukey's post-hoc analysis of a one way repeated measures ANOVA.

PBMC from lenalidomide treated patients show significant antibody-dependent cell-mediated-cytotoxicity in the presence of daratumumab.

Since LEN appeared to improve the DARA-mediated effects primarily through activation of effector cells, we questioned whether the effector cells of MM patients that received LEN treatment would also show increased ADCC when treated with DARA ex vivo. To this end, we used PBMCs from MM patients who were under treatment with LEN as effector cells to mediate ADCC against UM-9 cells. The effector PBMCs were isolated from MM patients before, during and one week after their first treatment cycle with LEN and were stored in liquid N_2 until use. As depicted in figure 5, PBMCs isolated from patients during or just after LEN treatment showed a significantly increased capacity to mediate DARA-dependent ADCC against UM-9 cells. Thus, also in this ex vivo clinical setting we show that DARA-mediated MM tumor cell killing is significantly augmented by stimulating effector cells.

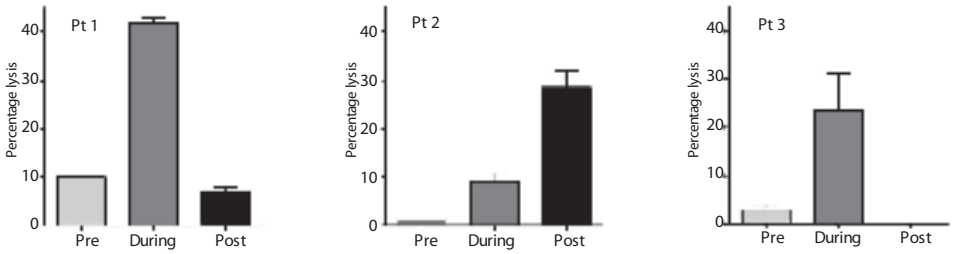

Figure 5: Significant DARA-dependent ADCC in PBMC form patients treated with LEN. Frozen PBMC obtained from patients 0-7 days before (Pre), during (During) or 0-1 days after (Post) the first cycle of LEN treatment was tested in ADCC assays after addition of DARA and control antibody KLH. Control antibody against KLH did not induce any ADCC (not shown) Error bars represent the SEM of the duplicate conditions. P values are calculated using a student's t test. Each graph signifies 1 patient.

Discussion

The survival of MM myeloma patients has improved over the last decade as a result of the introduction of novel immunomodulatory agents such as LEN and bortezomib with remarkable efficacy in relapsed and refractory myeloma(36). Nonetheless, MM still remains an incurable disease and almost all patients will eventually relapse. In an attempt to develop an effective therapy for MM we here evaluated the potential benefits of combining LEN with a novel human antibody DARA, which targets the CD_{38} molecule highly expressed on MM cells. In a series of experiments using purified MM cells, MM cell lines, full BM-MNC suspensions derived from MM patients and PBMC isolated from LEN treated patients, we show that the combination of LEN and DARA significantly increases the lysis of MM cells as compared to either of the single agents.

Obviously, the increased lysis of MM cells by the combination of LEN and DARA is to a certain extent due to separate actions of these agents. For instance DARA is known to induce its effects against MM predominantly via CDC and ADCC, while LEN induces MM cell apoptosis via a mechanism involving increase of p21WAF-1 expression and cell arrest in Go-G1 phase(37-39). Obviously, targeting MM cells via various mechanisms is a primary goal of combination strategies to diminish the chance of developing resistance. A combination therapy becomes however more powerful if the agents work synergistically to improve each others' actions. From this point of view, LEN-DARA combination appears a highly interesting choice since our results demonstrate that LEN significantly synergises with DARA to improve the MM cell lysis. Our assays demonstrate that the most important mechanism of this synergism is the ability of LEN to activate the effector cells of ADCC since LEN appeared not to interfere with CDC and improved ADCC predominantly after pre-incubation of PBMCs but not of MM cells.

While our standard ADCC or CDC assays using purified MM cells or MM cell lines

were suitable and sufficient to reveal the basic lytic mechanisms of the LEN and DARA combination, we also aimed to assess the influence of the micro environment on such therapy, in this study. This is of particular interest, because the interaction of MM cells with BM stromal cells is crucial for their survival (40-42) and may even induce a certain level of resistance against immune mediated lysis mechanisms. Therefore, in this study we used a new assay to measure ADCC and CDC assays without isolating MM cells from their natural environment. Thus we incubated LEN en DARA directly with MNCs isolated from full BM, which contains not only MM cells but also stromal cells. Setting up the assays in this way, provided not only a more physiologic platform but also allowed us to measure ADCC without being forced to determine the effector to target cell ratios arbitrarily. Also the results of these assays clearly demonstrated a synergistic improvement of DARA mediated ADCC by LEN, thus strongly substantiating the idea that significant anti-MM effects can be expected from DARA-LEN combination even in the presence of stromal cells that support MM cell growth. Interestingly, in these "full BM assays" we observed 20% extra effect of LEN-DARA combination above the expected additive effects of separate agents, which could translate into a substantial improvement in the clinical setting.

We argue that a final and strong indication for the clinical relevance of the DARA-LEN combination therapy is the significant induction/augmentation of DARA-dependent ADCC in PBMC isolated from three MM patients during or just after oral treatment with LEN. The results obtained from these patients are remarkable and strongly suggest that oral administration of LEN also stimulates the effector cells of MM patients to enhance DARA-dependent lysis of MM cells. Taken together, our results demonstrate the potential benefits of combining LEN with the novel CD38 antibody DARA in MM treatment.

Currently a growing number of studies are addressing the efficacy of therapies based on combination of lenalidomide with antibodies that can successfully target tumor cells. While in several $CD20^+$ B cell malignancies, such as lymphoma or CLL the rutiximab-LEN combination is tested, in the MM setting promising antibodies are considered to target CD40, CS-1 and FGFR3, surface molecules highly expressed on MM cells. Our results now indicate that DARA, which effectively targets CD38 and capable of inducing ADCC as well as CDC is a highly interesting additional candidate to combine with LEN to exploit its full immunomodulating power. We believe that our results merit further evaluation of the LEN-DARA combination in clinical trials, and a phase I/II clinical trial based on dose-escalation principle will be the choice of strategy to evaluate the feasibility, safety and efficacy of this novel approach in MM management.

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The therapeutic human CD38 antibody daratumumab improves the anti-myeloma effect of newly emerging multi-drug therapies

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In press

Letter to the Editor,

Multiple Myeloma (MM) is an incurable malignancy of antibody producing clonal plasma cells. The mean life expectancy of patients has remained at less than 3 years during the past few decades. The introduction of a new generation drug regimens including an immunomodulatory agent such as lenalidomide (LEN) or a proteasome inhibitor such as bortezomib (BORT) used alone or in combination with classical anti-MM drugs melphalan (MEL), dexamethasone (DEX) or prednisone (PRED) has significantly improved the overall survival of MM patients[1,2]. All treatment strategies, including multidrug regimens, however are eventually hampered by the development of drug resistance3. Targeted immunotherapy, based on human antibodies against relevant tumor antigens has shown to be a feasible and highly promising approach in haematological malignancies which can be effectively combined with chemotherapy to further increase the potency of anti-tumor effects. For instance in several B cell malignancies, clinically approved human antibodies against CD20 are now being successfully combined with fludarabine, cyclophosphamide or LEN[4,5]. Building on this knowledge and to achieve a similar goal in the MM setting, we recently generated daratumumab (DARA), a human CD38 antibody with broad-spectrum killing activity[6]. We have shown that DARA mediates strong lysis of MM cells via CDC (Complement dependent cytotoxicity) as well as ADCC (antibody dependent cellular cytotoxicity), although the potency of autologous ADCC was donor-dependent. In our initial work to combine DARA with novel chemotherapeutics, we have demonstrated that DARA mediated cellular lysis of MM cells is significantly improved by LEN, mainly due to the potent capacity of LEN to activate the effector cells of ADCC[7]. Current clinical practice however shows that the future of successful MM treatment lies in the use of drug combination regimens. It appears essential to identify regimens in which individual components synergize to obtain the greatest achievable effects. Therefore, we now explored the potential clinical benefit of combining targeted DARA therapy with newly emerging multi-drug chemotherapy regimens. To this end, we employed a recently developed ex vivo flow cytometry-based assay platform[7], which enables us to enumerate and subsequently deduce the drug/antibody mediated lysis of primary $CD138^+$ MM cells directly in bone marrow samples from MM patients. The assays are performed with bone marrow mononuclear cells (BM-MNC), thus without the need for separating malignant cells from autologous effector cells and tumor-supporting accessory cells, such as stromal cells. With this ex vivo assay system, we first addressed the benefits of combining DARA with both LEN and BORT, since not only LEN but also BORT may enhance the therapeutic efficacy of DARA by sensitizing tumor cells for antibody mediated lysis. In a series of experiments, we incubated BM-MNC from 16 MM patients, containing 2-20 % malignant plasma cells, either with medium alone or with combinations of LEN, BORT and DARA at carefully selected individual concentrations inducing half-maximal lysis of MM cells. An antibody against an irrelevant antigen (Keyhole Limpet Hemocyanin (KLH)) was used as an isotype control. After 48 hours, we harvested the cells, labeled them with a monoclonal CD138 antibody and

enumerated the surviving CD138$^+$ MM cells using single-platform flow cytometry, to assess the percentage of MM cell lysis in each sample (Figure 1A) relative to that obtained with the control antibody KLH, which induced negligible MM cell lysis (data not shown). LEN and BORT alone or in combination caused low to moderate lysis of MM cells (mean lysis 10%, 18% and 25 %, respectively). Addition of DARA significantly increased the MM cell lysis by more than 2-fold in all combinations (p=0.001). The highest MM cell lysis was observed with the triple LEN-BORT-DARA combination. Notably, combination with DARA seemed to improve MM cell lysis especially in the samples that poorly responded to LEN and BORT (MM cell lysis < 30%; Fig 1A). To evaluate this, we analyzed the data of LEN/BORT high and low responders separately (Fig. 1B, C). A significant improvement of MM cell lysis by DARA was observed in the LEN/BORT low responder subset particularly (Fig. 1C) and the effect was synergistic. This group included cells from five patients who had been treated in the clinic with LEN and/or BORT without success (Fig. 1D).

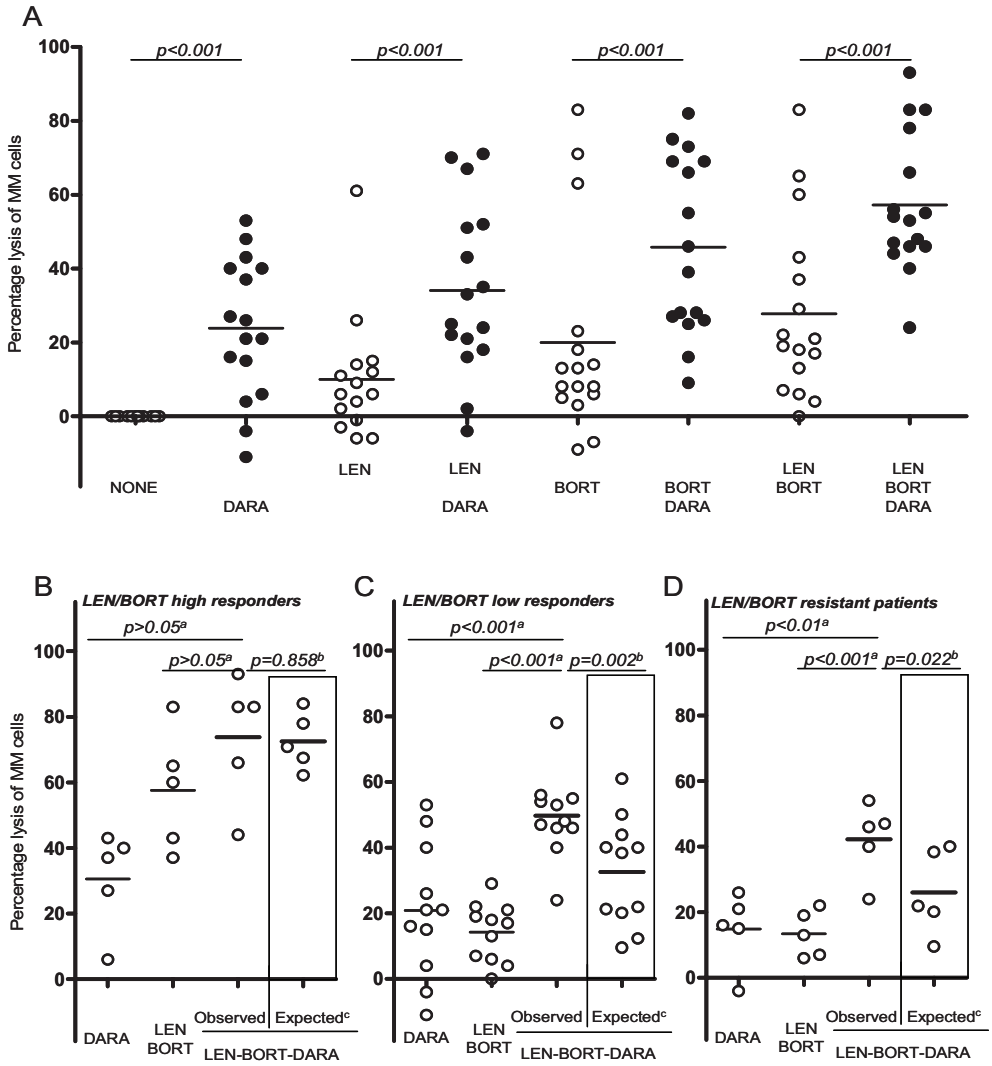
A
p<0.001
p<0.001
p<0.001
p<0.001
Percentage lysis of MM cells
100
80
60
40
20
0
NONE
DARA
LEN
LEN
DARA
BORT
BORT
DARA
LEN
BORT
LEN
BORT
DARA
B
LEN/BORT high responders
p>0.05a
p>0.05a
p=0.858b
C
LEN/BORT low responders
p<0.001a
p<0.001a
p=0.002b
D
LEN/BORT resistant patients
p<0.01a
p<0.001a
p=0.022b
Observed
Expectedc
DARA
LEN
BORT
LEN-BORT-DARA

Figure 1: Addition of DARA to BORT-LEN significantly increases the MM cell lysis in BM-MNC of MM patients, particularly in LEN/BORT low responder or refractory patients. (A) BM-MNC from MM patients (n=16), containing 2-20% CD138+ MM cells as detected by flow cytometry were incubated with LEN (3μM), BORT (3nM) and DARA (10 μg/ml) alone or in combination for 48 hours in RPMI+ 10%FBS in 96 well u bottom plates in fully humidified incubators at 37°C, 5% CO2-air mixture . Surviving MM cells were enumerated by the single platform FACS analysis of CD138+ cells in the presence of Tru-Count® beads. Percentage lysis of MM cells in LEN, DARA and LEN+DARA treated conditions were calculated using the MM survival of wells treated with the control KLH antibody alone. Differences between indicated groups were tested for significance in repeated measures ANOVA, using Bonferonni's post-hoc multiple comparison tests with 2-tailed 95% confidence intervals. In B-D, data is analysed for low LEN/BORT responders, high LEN/BORT responders and LEN/BORT refractory patients respectively. aP values were calculated using a repeated measures ANOVA. bP values were calculated by a paired t test. c Expected values were calculated to test the null hypothesis that there is no synergism between DARA and LEN/BORT using the following formula: % expected lysis = 100- %survival after DARA* %survival after LEN/BORT.

Interestingly, the synergy between DARA and LEN/BORT treatment was also apparent for cells from the 5 LEN/BORT resistant patients (Fig. 1D, as illustrated by the fact that observed levels of MM cell lysis with DARA/LEN/BORT treatment were significantly higher than the expected levels of MM cell lysis, calculated on the assumption that there was no treatment interaction). Although we have only been able to evaluate a small number of samples from resistant patients to date, this remarkable synergy suggests the maintenance of anti-tumorigenic properties of LEN and BORT, despite the development of drug-resistance. Taken together these results indicate the potential clinical benefits of combining DARA with these two novel anti-MM agents and warrant further investigation even in patients who are low responders or have become resistant to the latter drugs.

After showing the potential benefits of combining DARA with LEN and BORT, our further investigation focused on two recently introduced and so far the most successful first-line combination therapies based on these two novel agents, namely the triple combination of LEN, BORT, DEX (RVD) and MEL, PRED, BORT (MPV). To assess the impact of combining DARA with these combination chemotherapies, we prepared cocktails of these agents, by mixing them at concentrations causing +/- 30% of the maximal lytic effect on various MM cell lines (data not shown). We then incubated BM-MNC of MM patients with serial dilutions of these cocktails alone or in the presence of DARA and assessed MM cell lysis. As expected, cocktails of RDV (Fig 2A) as well as MPV (Fig 2B) induced dose-dependent lysis of MM cells. Addition of DARA to both RDV and MPV significantly increased the treatment efficacy by almost doubling the lysis levels especially at lower doses of the cocktails.

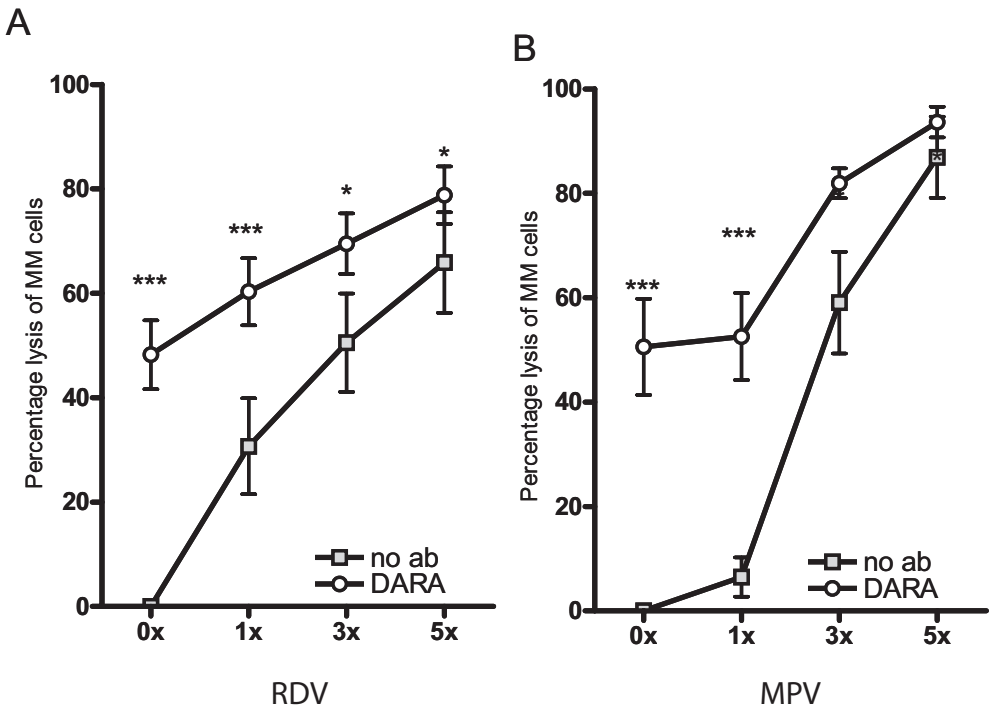

Figure 2: DARA increases response against MM in triple chemotherapy treatments BM-MNC form patients (n=7) were incubated with increasing dilutions of a cocktail of (A) LEN, BORT and DEX (1x dilution contains: 1μM LEN, 1nM BORT and 1μM DEX) or with a cocktail of (B) MEL, PRED, BORT (1x dilution contains 1μM MEL, 1μM PRED and 1nM BORT) in the presence or absence of DARA (10 μg/ml) for 48 hours. Surviving MM cells were enumerated by FACS analysis of CD138+ cells. P values were calculated by a paired t test. * <0.01p<0.05, ** p= <0.001 p<0.01 *** =p<0.001

These results illustrate that targeted immunotherapy of MM by DARA holds a significant potential to improve the clinical outcome of currently available novel combination therapies. Recent studies have indicated that combination of multiple drugs are superior over single or double agent combinations[2]. Addition of new drugs to the available regimens can mediate their clinical benefit due to the induction of a higher rate of initial complete responses, which in turn improves the relapse free and overall survival[8]. Contingent on the premise that the combined agents have non-overlapping and synergistic mechanisms of actions, immediate and effective targeting of tumors with multiple agents appears a successful strategy to improve the clinical outcome of MM therapy. Indeed, such a strategy is in full agreement with the emerging concept that the genetic signature of MM, and consequently, the individual patient's susceptibility to a specific agent will be highly heterogeneous and this eventually may lead to drug resistance. Nevertheless, the complete response rate of the best chemotherapeutic combinations is currently less than 50 %, and all current combination

therapies eventually induce drug resistance[2;9]. In this respect, DARA, with its immediate and effective cell mediated cytotoxic effects against MM cells, and the observed remarkable synergy with LEN/BORT even in LEN/BORT refractory patients, may potentially improve the achievement of first CR in MM when combined with these agents either alone or in multidrug chemotherapy regimens. In conclusion, our study, in which we demonstrate the potential benefits of combining DARA mediated targeted therapy with newly emerging chemotherapy options, warrants the evaluation of this approach in MM in clinical phase I/II trials.

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7

General discussion

A modified version of this chapter is submitted for publication.
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Preface

Multiple myeloma (MM), the malignant disorder of antibody producing plasma cells, is characterized by the accumulation of malignant cells in the bone marrow (BM), development of lytic bone lesions, inhibition of normal blood cell production, immunodeficiency, and renal failure. The disease is the second frequent haematological cancer and accounts for almost 10% of the haematological cancers. While the overall survival rates were less than one year before 1960's(1-3), the introduction of Melphalan, an alkylating agent, significantly improved survival rates(4). This trend is continued in the late nineties after the discovery that thalidomide, a teratogenic but an immunomodulatory agent (IMID), is beneficial in MM treatment(5). Currently, with the application of allogeneic transplantation(6) autologous stem cell transplantation combined with high dose chemotherapy(7) and with the discovery of several other effective agents such as the proteasome inhibitor bortezomib(8) and the thalidomide analogue lenalidomide(LEN)(9), the life expectancy of MM patients is significantly improved, with a median overall survival of 4.4-7.1 years(3). However, the majority of MM patients ultimately develop drug resistance from all combinations of chemotherapy, underscoring the continuing need for new and more effective treatment strategies. While on one hand, second generation of (IMiDs) such as Pomalidomide(10) and new proteasome inhibitors like Carfilzomib(11) are entering clinical trials to tackle drug resistance. Immunotherapy, particularly targeted immunotherapy by antibodies rapidly gains impact, opening new possibilities for new immunochemotherapeutical combination therapies.

The focus of this thesis was two fold: concerning the possible future treatment methods of MM, we searched for possibilities to improve cellular immune therapy and natural killer cells (NK)/antibody mediated therapy by combining with chemotherapy.

Cellular immune therapy

LEN in allogeneic stem cell transplantation

In chapter 2 we report on a preclinical trial in which refractory or relapsed patients received LEN alone or in combination with dexamethasone after allogeneic stem cell transplantation(12). On a clinical level we observed that 46% (6/13) of the patients responded to LEN alone, and most poor responders benefited from the combination with dexamethasone, resulting in an overall response rate of 87.5%. Also 5 out of 13 patients starting with LEN alone developed acute GvHD between 2 -13 days after start of treatment. A detailed analysis of patient blood samples showed us that the frequencies of $CD4^+FOXP3^+$ cells significantly increased in 7 of 8 patients during LEN administration. Since the patient group was limited we could not further investigate the correlation between increased $CD4^+FOXP3^+$ cells and GvHD. What we did however observe and was later also reported by Kneppers et al.(13) that administration of LEN within a early time frame after allogenic stem cell transplantation caused severe GvHD in patients. An explanation could be that the potency of an allogenic transplantation

in combination with the strong immuno modulatory effects of an IMID such as LEN induces an uncontrolled alloreactivity, which can not be controlled by elevated levels of regulatory T cells. The observed elevation of $CD_4^+FOXP_3^+$ cells may be thus a natural attempt of the body to control the vigorous immune response caused by LEN. Further investigation is necessary whether the graft vs MM effects of allogenic transplantation can be potentiated by combination of other drugs , such as bortezomib, which is known to reduce GvHD in murine models when applied properly.

The impact of BM stroma on T cell downregulation by MM cells

In chapter 3 we investigate the potential role of BM stromal cells on MM. In a series of experiments we observed the potential important role of BM stromal cells in the development of T cell inhibitory properties of MM cells. Stromal cells enabled most, if not all MM cell lines to secrete anti-proliferative cytokines. Stroma cells also appeared to induce a suppressive phenotype in primary MM cells directly isolated from patients. Thus stromal cells appear to provide signals for MM cells to hamper the development of an effective anti MM response. The production of T cell inhibitory cytokines upon stroma-MM interactions may be a major barrier towards the success of immune therapies. Therefore, future *in vitro* and *in vivo* investigation of strategies that can modulate MM-stroma interactions as well as identification and neutralizing of anti-proliferative cytokines seem relevant towards improvement of cellular immunotherapy including allogeneic transplantation, DLI, adoptive immunotherapy and therapeutic DC vaccination studies. Not only blocking the interaction between stroma and MM or neutralizing T cell inhibitory cytokines produced by MM cells, but also blocking the T cell inhibitory molecules present on MM cells may be also relevant. With this respect, it is worth to mention the PD1. This molecule, known as a co-inhibitory molecule is associated with downregulatin of T cells immune responses. In several cancer models, recent studies indicate that interruption of PD1/PD-L1 interactions leads to improved antitumor T-cell responses and tumor control(14;15). mAbs developed against both PD1 and PD-L1/B7H1 are being evaluated in phase I/II clinical trials in patients with a variety of cancers(16). It may be therefore highly relevant to evaluate whether PD1 expression on MM cells is also under control of MM-stroma interactions.

NK/antibody mediated therapy

Anti-CD38 antibody daratumumab

In chapters 4, 5 and 6 we discuss the in vitro efficacy of a novel CD38 monoclonal fully human antibody, daratumumab (DARA) and the combination of this antibody with clinically used chemotherapy treatments.

The success of monoclonal antibody therapy is typically illustrated by Rituximab, a chimeric antibody directed against the B cell specific molecule CD20(17). Since its first clinical application, Rituximab has been very successful in the treatment of a number of B cell

malignancies including non-Hodgin lymphoma, follicular lymphoma, chronic lymphocytic. The CD38 is a 46 kDa type II transmembrane glycoprotein (18), which is involved in receptor-mediated adhesion and signaling events by binding its ligand CD31. CD38 also contributes importantly to intracellular calcium mobilization with its important ectoenzymatic activity (19).

Previously, a number of chimeric antibodies have been generated against human CD38(20;21). Although these antibodies mediates significant lysis of malignant B cell lines, like many other chimeric antibodies, they did not make to the clinical development due to rapid clearance from the body by the human immune system. In chapter 4 we report of the fully humanized DARA, generated using the proven technology (22) of human antibody transgenic mice. DARA recognizes a unique fine epitope on CD38. It was selected as the best candidate from a panel of 42 novel CD38 mAb's due to its superior capacities to lyse $CD38^{+}$ malignant cells through ADCC, CDC and induction of direct apoptosis (figure 1) (23). In extensive in vitro analyses we have first demonstrated that DARA induces strong CDC in a range of primary tumor cell samples from MM patients. In fact, induction of CDC is a very specific feature of DARA, as no other CD38 antibody from our panel mediated its effects via CDC. Furthermore the strong lysis through CDC is probably an essential feature, since several other therapeutic antibodies such as alemtuzumab(24), Rituximab(17) and Ofatumumab(25) exert strong tumor cell lysis predominantly via CDC. In addition to CDC, ADCC through IgG Fc receptor (FcγR)-bearing immune effector cells such as NK cells and monocytes, is another important mechanism by which therapeutic antibodies exert their effect. With this respect DARA showed also superior capacities: it induced ADCC against many different tumor cell lines with varying CD38 expression, patient MM cells and plasma cell leukemia cells. It is important to note that DARA dependent ADCC occurs even when the effector cells are derived from MM patients and in even in the presence of BM stromal cells, which are known to support MM cell survival and mediate drug resistance(26).

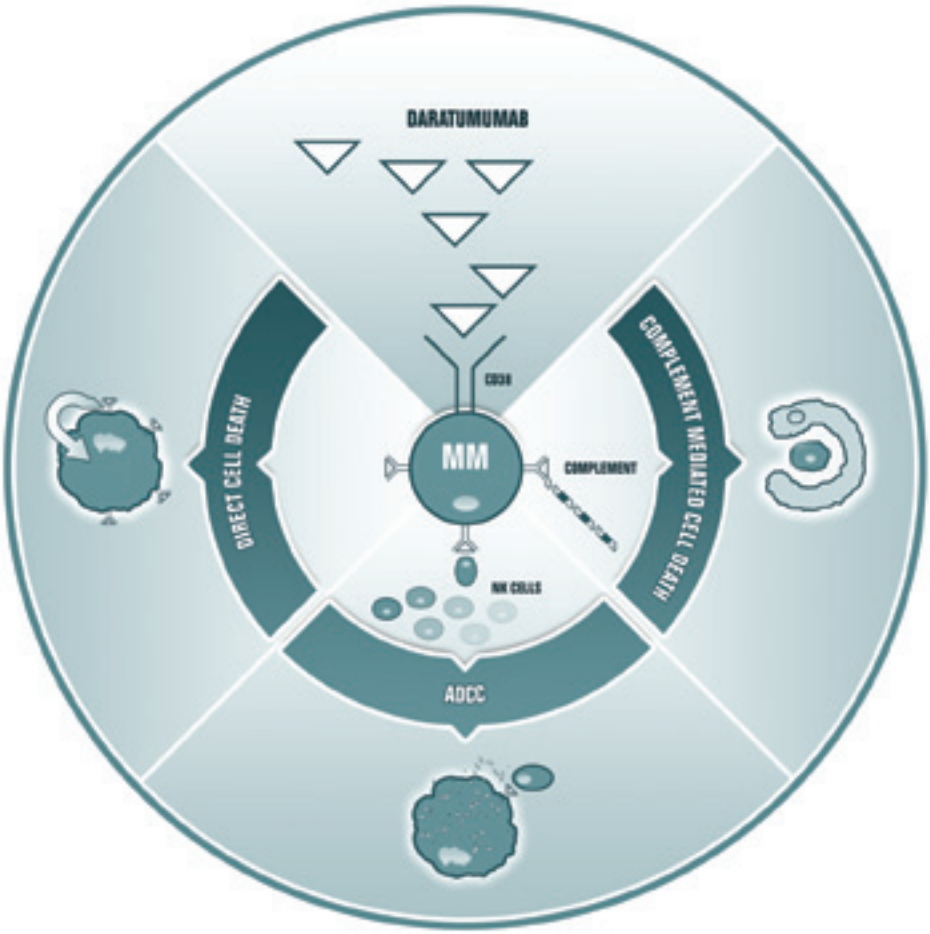

Figure 1: The 3 major effects of daratumumab, complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxiciy (ADCC) and direct cell death

Finally, in two different xenograft models we showed the potent capacity of DARA to inhibit the growth of $CD38+$ tumor cells such as Daudi and the MM cell line UM-9. All together, our preclinical analyses strongly indicated the potential value of DARA as a therapeutic Ab for the treatment of $CD38^{+}$ malignant diseases such as MM .

Combining DARA with novel anti-MM agents

An important part of our aim towards the clinical application was to investigate whether DARA could be incorporated into the new therapeutic regimes that are implemented in

clinic. Currently, several treatment regimens utilize LEN, and the potent proteasome inhibitor bortezomib (BORT) alone or in a combination with classical anti-MM drugs melphalan (MEL) and steroids like dexamethasone (DEX) or prednisone (PRED)(27;28). Our initial interest was to evaluate the combinatorial effects of DARA and LEN, because LEN, with its significant stimulatory activities on NK cells, could potentiate the ADCC mediated by DARA. In deed, a number of in vitro studies and recent clinical trials had already reported that LEN enhanced rituximab-mediated ADCC(29;30); MM cell lysis was also significantly improved when LEN was combined with a humanized CD40 antibody(31). In chapter 5 we showed by using a CD38+ MM cell line (UM-9) and purified MM cells from patients, we have clearly demonstrated that the combination of LEN significantly increases the DARA mediated ADCC of MM cells in a synergistic way(32). LEN appeared to improve ADCC primarily by activating the effector cells, not by sensitizing MM cells. Importantly, the synergism between LEN and DARA was also observed in novel assays, in which we investigated ADCC directly in whole BM MNC of MM patients, thus using the patient's autologous effector cells and without the need for isolating MM cells from their natural environment. The beneficial effects of combining DARA with LEN therapy was finally suggested by the significant upregulation of DARA-induced ADCC in PBMC derived from three MM patients who were treated with LEN. Thus in vitro assays so far suggest that LEN-DARA combination may be a highly interesting choice to evaluate in clinical trials.

Current clinical practice indicates that combination of multiple drugs are superior over single or double agent treatments(33;34). Addition of new drugs to the available regimens appear to mediate their clinical benefit due to the induction of a higher rate of initial complete responses, which in turn improves the relapse free and overall survival(3). Therefore, we extended our preclinical evaluation by combining targeted DARA therapy with newly emerging multi-drug chemotherapy regimens.

In chapter 6 (submitted for publication) we show, by measuring the lysis of MM cells ex vivo in the BM MNC of MM patients, that addition of DARA significantly increases the MM cell lysis by more than 2-fold in BM-MNC samples treated with LEN, BORT alone or with a combination of LEN and BORT. The highest MM cell lysis levels is achieved using the triple LEN-BORT-DARA combination. Remarkably, DARA could improve MM cell lysis by LEN and BORT in a synergistic way, especially in the BM samples that poorly responded to LEN and BORT. Notably, this low responder group included samples from five patients who had became refractory to clinical LEN and/or BORT treatment, indicating the maintenance of immunomodulatory properties of LEN/BORT, despite the development of drug-resistance in these LEN/BORT refractory patients. Thus, although our data are limited at the moment to only five patients, it is tempting to speculate that combining DARA with these two novel anti-MM agents might be beneficial even in patients who are low responders or have become resistant to LEN and/or BORT. Extended studies and clinical trials will reveal the relevance of this speculations. Finally we extended our preclinical evaluation to

two recently introduced and so far the most successful first-line combination therapies based on these two novel agents, namely the triple combination of LEN, BORT, DEX (RVD) and MEL, PRED, BORT (MPV). In both combinations, addition of DARA significantly increased the lysis of MM cells in our in vitro assays, illustrating that targeted immunotherapy of MM by DARA bears a significant potential to improve the clinical outcome of currently available novel combination therapies. In conclusion our preclinical experience with the human CD_{38} antibody DARA reveals that it shares the strong capacity to induce ADCC and CDC and direct apoptosis with several successful therapeutic antibodies. DARA may be highly potential in the treatment of MM patients alone or in combination of newly emerging multi-drug chemotherapeutical regimens. Stimulated by these findings we have already initiated a clinical phase I trial in which, the feasibility and optimal safe dosage of DARA is being evaluated. Several other important aspects for clinical application such as the duration of application, anti-tumor efficacy and the optimal dose in combination with other chemotherapeutics need to be evaluated in further clinical trials. Since our results indicate that DARA synergizes with LEN/BORT even in patients refractory to the chemotherapeutic agents, a specific target group of DARA treatment may be the patients who have developed drug resistance. Thus DARA may find an optimal application area not only to induce long term complete remissions in combination with other anti-MM drugs but also to overcome drug resistance towards further improvement of the life expectancy of MM patients.

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8

Nederlandse samenvatting

Dankwoord

Curriculum vitae

Nederlandse samenvatting

In Nederland overlijdt een groot gedeelte van mensen aan kanker. Kanker kan uit elke cel in je lichaam ontstaan, doordat tijdens de deling van cellen kleine mutaties ontstaan. Sommige mutaties zijn niet schadelijk, andere mutaties zorgen ervoor dat de gemuteerde cel meteen apoptose ondergaat (een vorm van celdood). Er kan echter een mutatie ontstaan die de groei en levensduur van een cel beïnvloed. In zo'n geval kan er kanker ontstaan. Zo ook uit je eigen afweercellen. De kankervorm die in deze thesis besproken wordt ontstaat uit gerijpte B cellen en wordt multiple myeloma (MM) ofwel de ziekte van Kahler genoemd, naar Otto Kahler, die de ziekte als eerste beschreef. Normaal gesproken wordt een B cel geactiveerd wanneer T cellen in je lichaam een micro organisme detecteren, deze sturen dan signalen, genaamd cytokinen, naar B cellen die dan rijpen en antilichamen gaan produceren om het micro organisme aan te vallen. In het geval van MM zijn deze cellen zomaar gerijpt en produceren ze niet relevante antilichamen. De ziekte komt voor in je botten en zorgt voor botafbraak en de overmatige productie van antilichamen overbelasten je nieren. Een patiënt met de diagnose MM heeft een gemiddelde levensduur van 5 jaar na bepaling van de ziekte, zelfs met de nieuwste therapieën. In deze thesis wordt zowel gekeken naar de verbetering van huidige therapieën met nieuwe chemotherapie en antilichaam therapie als ook naar de rol die het beenmerg micro milieu heeft op de immuun verstoring van MM.

In hoofdstuk 2 hebben we gekeken naar de mogelijkheid om lenalidomide (LEN) (Revlimid) aan patiënten te geven na allogene transplantatie. LEN is een IMiD, een chemotherapie die het immuun systeem stimuleert en MM ook gelijk doodt. Er is gekeken naar de klinische uitkomst van behandeling van patiënten met deze therapie, waaruit een indicatie kwam dat de therapie een goede overal response gaf. Echter veel patiënten ontwikkelden graft versus host disease (GvHD) en 1 patient overleed aan de gevolgen. Ook werd er bloed afgenomen van de patiënten. Hieruit kwam voort dat patiënten een verhoogd aantal $CD_4^+CD_{25}^+$ T cellen, ook wel regulatoire T cellen (Tregs) genoemd, in hun bloed hadden na de therapie. De ontstane hypothese was dat imuun simulerende LEN in samenwerking met de krachtige immuun response van de allogene transplantatie GvHD veroorzaakte en dat de stijging van Tregs een biologische response was van het immuun systeem om deze krachtige immuun response te temperen.

In hoofdstuk 3 is de rol van stroma op MM bekeken. In het verleden is gebleken dat desondanks het afweer MM kan doden complete remissie niet plaatsvindt, dit zou indiceren dat MM in staat is in vivo om het immuun systeem te beïnvloeden. Uit gedane experimenten bleek dat sommige MM cellijnen in staat waren om de proliferatie van cellen te remmen. Dit effect werd nog duidelijker door beenmerg stroma toe te voegen waarna de meeste MM cellijnen en MM cellen direct uit patiënten gehaald een inhiberend karakter kregen. Uit onderzoek bleek dat een tot nu toe onbekend cytokine werd uitgescheiden dat alleen de proliferatie van de cellen beïnvloedde. Zowel de cytotoxische alsmede de mogelijkheid om cytokinen die proliferatie ondersteunen te produceren, werd niet

beïnvloed. Onderzoek wees uit dat dit cytokine tussen de 18 en 25 kD is. Verder onderzoek naar dit cytokine moet nog plaatsvinden, maar het vinden van deze onbekende cytokine en het mogelijk onschadelijk maken van dit is een stap naar de verbetering van immuun therapie.

In hoofdstuk 4, 5 en 6 bespreken we de productie van een nieuw volledig gehumaniseerd antilichaam, daratumumab en de combinatie van dit nieuwe antilichaam in huidig gebruikte klinische therapieën. Daratumumab (DARA) is een antilichaam gericht tegen CD38, een 46 kDa type II transmembraan glycoproteine, dat betrokken is bij receptor-gemedieerde adhesie en signalering door binding met zijn ligand CD31. Er waren eerder antilichamen tegen CD38 gecreëerd, deze hadden echter nog een muis gedeelte en werden daardoor snel opgeruimd door het afweersysteem van de patiënt. DARA is volledig humaan gemaakt en zou daardoor mogelijk dit ongewenste effect niet hebben.
Uit testen van verschillende anti-CD38 antilichamen bleek DARA het beste was in directe cytotoxische dood en zowel in antilichaam gemedieerde dood (ADCC) en complement gemedieerde dood (CDC). DARA bleek ook effectief de groei van tumorcellen te inhiberen in 2 verschillende muismodellen, waarvan een met MM cellen en een met een B cel lymfoma.

Combinatie van DARA met LEN een immuun stimulerende chemotherapie bleek uiterst effectief zoals aangetoond in hoofdstuk 5. LEN heeft de eigenschap om NK cellen te stimuleren, deze NK cellen zijn een essentieel onderdeel van ADCC. In een model waarin we niet alleen MM cellijnen maar ook MM cellen die we uit patiënt beenmerg hebben verkregen en dus in het beenmerg stroma milieu verkeren bleek dat de combinatie van deze 2 middelen niet alleen zorgde voor een verwachte versterkte MM cel dood, maar een synergistisch versterkte celdood. Deze resultaten spraken een goede indicatie in vitro dat het gebruik in vivo een goede mogelijkheid voor behandeling zou zijn.

In Hoofdstuk 6 breiden wij de combinatie van mogelijke therapieën waarbij DARA een rol zou kunnen spelen uit. Als eerste werd de combinatie van LEN met bortezomib (BORT) onderzocht. BORT is een proteosome inhiberende chemotherapie en wordt in combinatie met LEN in de kliniek gebruikt als therapie. De combinatie LEN-BORT-DARA werkte beter dan de therapieën los of duo therapieën. Ook bleek dat patiëntenmateriaal van patiënten die eerder BORT, LEN of beide hadden gehad en in vitro slecht reageerden op deze chemotherapie door toevoeging van DARA significant beter op de drievoudige therapie wat indiceert dat er een mogelijkheid bestaat dat DARA chemoresistentie in een bepaalde mate kan verhelpen.
Ook werd gekeken naar de toegevoegde waarde van DARA aan drievoudige chemotherapie combinaties van LEN, BORT, dexamethasone (DEX)(RVD) en meplhalan (MEL), prednison (PRED), BORT (MPV). Ook hieruit bleek dat DARA een toegevoegde waarde had bij combinatie met deze chemotherapie. Deze in vitro onderzoeken bieden een hoopvol perspectief voor toekomstig gebruik in de kliniek.

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Curriculum vitae

Michael Steve van der Veer werd geboren op 18 augustus 1981 te Den Helder. In 1998 behaalde hij zijn HAVO diploma aan scholengemeenschap Schravenlant, te Schiedam. Dat zelfde jaar begon hij aan de Hoger laboratorium opleiding, HLO, aan de Hogeschool Rotterdam. Na een succesvolle stage op de afdeling tumor immunologie in het LUMC in 2001-2002 werd hem hier na afronding van zijn HBO, in 2002, een baan aangeboden. Gedurende 2 jaar werkte hij als research analist onder begeleiding van Dr. Thorbald van Hall. In 2004 begon hij met de opleiding biomedische wetenschappen aan de Vrije Universiteit, te Amsterdam. Na een stage in het LUMC bij de groep van Prof. Dr. Tom Ottenhof in 2005 en een stage in La Jolla Insitute for Allergy and Immunology in San Diego onder begeleiding van Dr. Stephen Schoenberger en Dr. Monika Wolkers in 2005, behaalde hij in 2006 zijn bull. In 2007 begon hij als AIO bij de afdeling klinische chemie en haematologie, te Utrecht, onder begeleiding van Dr. Tuna Mutis en Prof. Dr. Henk Lokhorst. De vruchtvolle resultaten van dat onderzoek staan beschreven in dit proefschrift. In december van 2011 begint het onderzoek als post-doc in het VUMC Cancer Center op de afdeling angiogenese onder begeleiding van Prof. Dr. Arjan Griffioen naar novel targets als drug tegen angiogenese in kanker.