Sepantronium bromide (YM155) improves daratumumab-mediated cellular lysis of multiple myeloma cells by abrogation of bone marrow stromal cell-induced resistance

Targeted immunotherapy with the human anti-CD38 antibody daratumumab has recently emerged as a promising strategy for the treatment of multidrug resistant patients with multiple myeloma (MM). In experimental *in vitro* and *in vivo* settings, and in recently completed clinical trials, daratumumab achieves highly promising response rates.[1-3] Despite these encouraging results, there is a marked heterogeneity in the response of patients to daratumumab as a single agent,[1-3] suggesting that the clinical efficacy of daratumumab may still be improved by exploring and effectively tackling the mechanisms behind this heterogeneity. We have recently demonstrated that the CD38 expression levels on MM cells are an important parameter for daratumumab sensitivity and proposed combining daratumumab with CD38 up-regulating agent all trans retionoic acid (ATRA).[4] Nonetheless, poor responses to daratumumab, observed even in the pres-

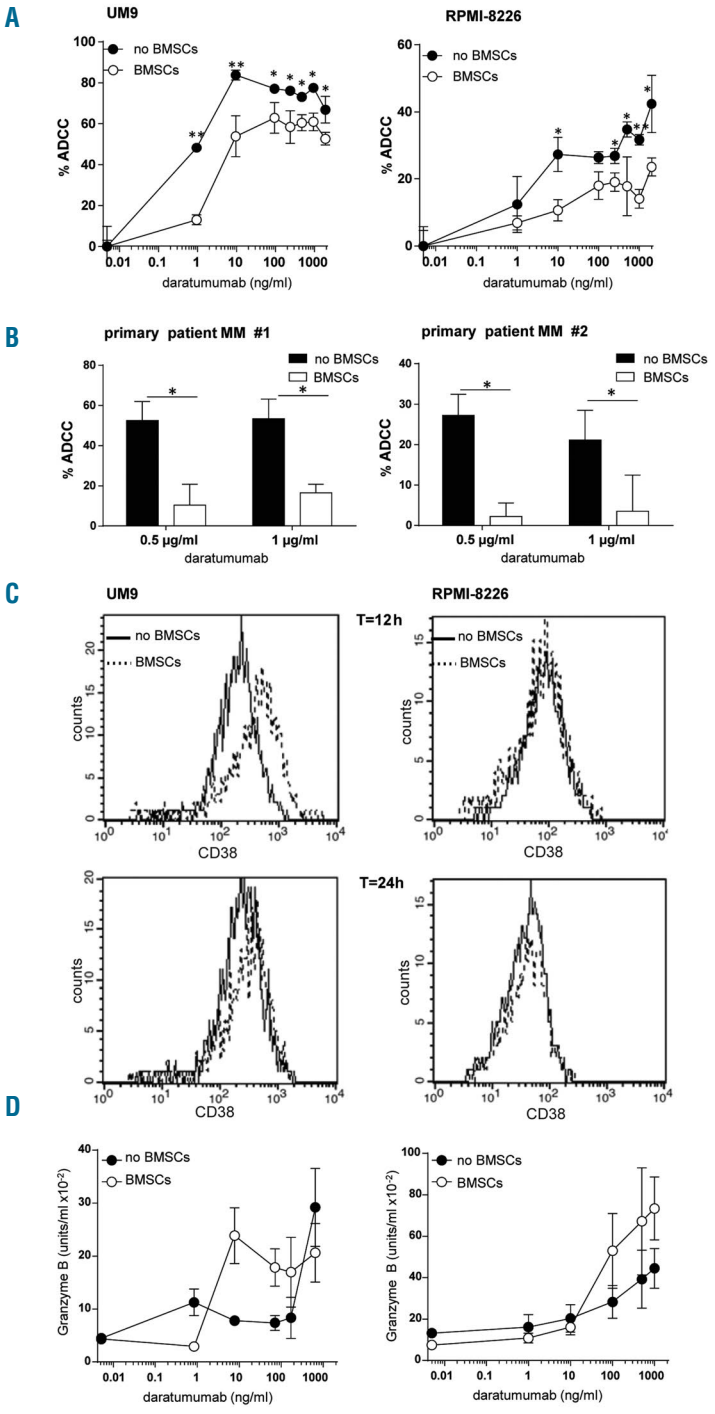

Figure 1. Bone marrow stromal cells (BMSC) protect multiple myeloma (MM) cells against daratumumab-induced antibody-dependent cellular cytotoxicity (ADCC) without CD38 modulation or immune suppression. (A) In compartment-specific cellular cytotoxicity assays,[6] luciferase-transduced CD38$^+$ MM cell lines UM9 and RPMI8226 were cultured in presence or absence of healthy donor-derived BMSC for 16 h prior to incubation with serial concentrations of daratumumab and healthy donor (HD)-derived peripheral blood mononuclear cells (PBMC) at a PBMC:MM cell ratio of 40:1. All cellular material was collected using protocols and procedures approved by the institutional medical ethical committee in accordance with the Declaration of Helsinki. MM cell viability was determined after 4 h by bioluminescence imaging (BLI). MM cell lysis was calculated relative to the viability without daratumumab. Error bars indicate the standard error of mean (SEM) of triplicate measurements. Differences between cultures with or without BMSCs were tested with an unpaired *t*-test; *P<0.05. Data are representative of 3 independent experiments. (B) In earlier developed assays,[4] BM-MNCs of 2 MM patients, from whom BMSCs were also available, were cultured in presence or absence of autologous BMSC for 16 h and then treated with daratumumab at indicated concentrations. Since bone marrow mononuclear cells (BM-MNC) already contain natural killer (NK) cells as effector cells, no additional effector cells were added. The topro-3 negative viable CD138$^+$ MM cells in the cultures were counted after 24 h by flow cytometry using Tru-Count beads, as described earlier.[4] Daratumumab-dependent lysis of MM cells was calculated relative to the cultures without daratumumab incubation. Error bars indicate the SEM of triplicate measurements. Differences between cultures with or without BMSCs were tested with an unpaired *t*-test; *P<0.05. (C) UM9 and RPMI8226 cells cultured in presence or absence of HD-BMSCs were measured for CD38 expression by flow cytometry at indicated time points. (D) The cell-free supernatants of the experiments in (A) were analyzed for the presence of GzB by ELISA. Error bars indicate the SEM of measurement in triplicate.

ence of elevated CD38 expression,[1,4] point to additional tumor escape mechanisms, possibly operating in the tumor microenvironment. Such mechanisms may interfere with the main tumor cell lysis mechanisms of daratumumab, including complement-mediated cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular cytotoxicity (ADCC).[4] With respect to ADCC, a potential microenvironment-related resistance mechanism might be the well-documented cell adhesion-mediated apoptosis resistance of MM cells,[5] because, like in the case of several pharmacological agents, ADCC mediated by natural killer (NK) cells eliminates MM cells *via* the induction of apoptosis. Increasing the likelihood of this happening, we

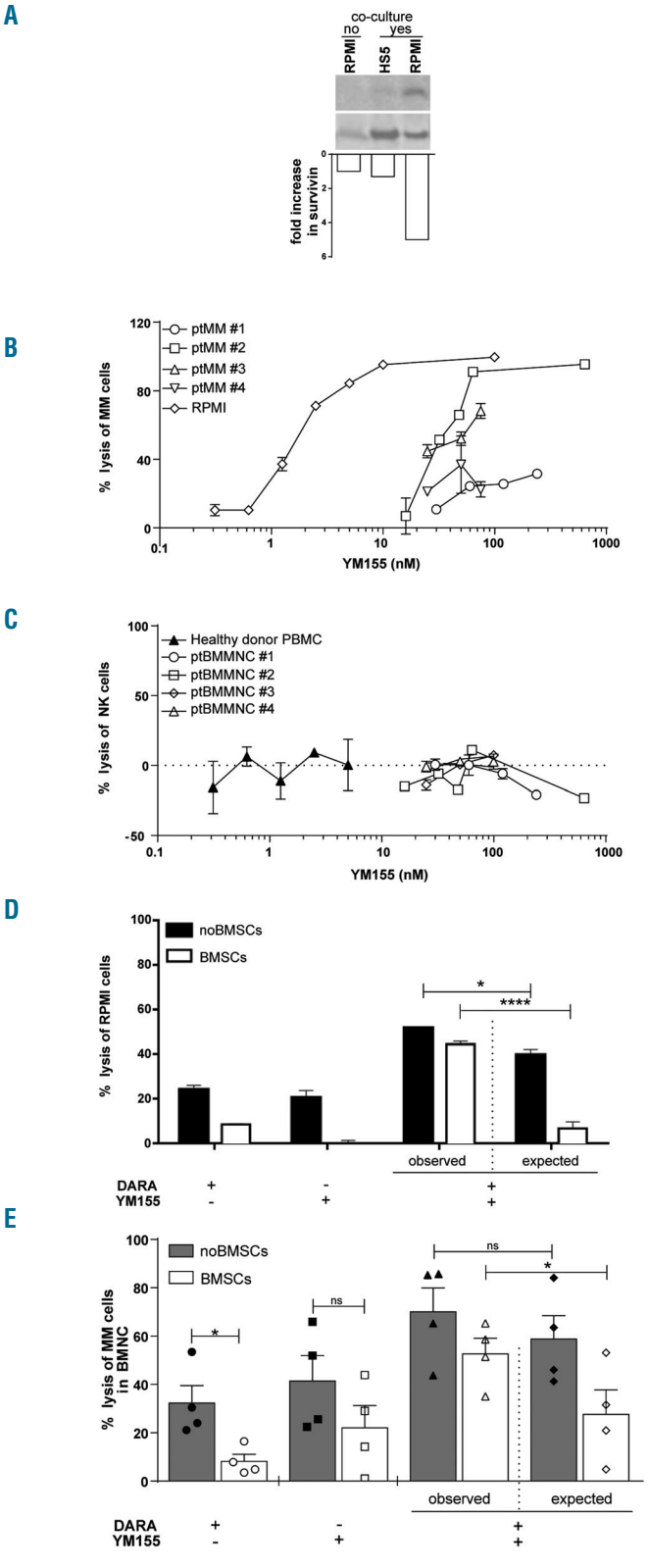

Figure 2. YM155, a small molecule survivin suppressant, synergizes with daratumumab to improve the lysis of multiple myeloma (MM) cell lines and primary patient MM cells in the presence and absence of stroma. (A) Upregulation of survivin expression in RPMI-8226 cell line after incubation with stromal cells. Standard western blots were carried out as described earlier[6] using the lysates of the indicated cells incubated alone, or separated by mechanical pipetting force from each other after 24 h of co-culture. The separated populations were 89% pure. The survivin and control β-actin bands were quantified using J-image software. Relative survivin expression was then plotted after correcting for the control β-actin. (B and C) The lytic activity of YM155 on MM cells (B) or natural killer (NK) cells (C) present in healthy donor (HD)-derived peripheral blood mononuclear cells (PBMC) and patient bone marrow mononuclear cells (BM-MNC). The results are obtained after incubating the cells with the indicated concentrations of YM155 for 24 h. The absolute numbers of topro-3 negative viable (B) CD138$^+$ MM cells or (C) CD3- CD56$^+$ NK cells was determined by flow cytometry using Tru-Count beads and the percent lysis was calculated using the untreated samples as negative control. (D) Luciferase-transduced RPMI8226 cells were cultured in presence or absence of healthy donor-derived bone marrow stromal cells (BMSC). Daratumumab (1 ng/mL) and YM155 [1 nM; concentration was determined as inducing sub-maximal lysis as shown in B], were added in all wells, HD-PBMC were added at a PBMC:MM ratio of 40:1 as a source of effector cells of antibody-dependent cellular cytotoxicity (ADCC). Survival of RPMI8226 was determined after 4 h by bioluminescence imaging (BLI). % lysis was calculated relative to the survival of RPMI8226 cells that received PBMC only. In case of combinatorial treatments of daratumumab and YM155, the expected lysis values were calculated to test the null hypothesis that there is only an additive effect between daratumumab and YM155, using the following formula: % expected lysis = (% lysis with daratumumab +% lysis with Ym155) −% lysis with daratumumab ×% lysis with YM155. The null hypothesis of "additive effects" was rejected if the observed values were significantly higher (P<0.05) than the expected values. These were determined in an unpaired t-test; ***P<0.005, *P<0.05. (E) Bone marrow mononuclear cells (BMNC) containing CD138$^+$ MM cells from 4 MM patients (45%, 5.5%, 10.2% 21.6% for Patients #1-4, respectively) were cultured in presence or absence of autologous MM-BMSCs. Daratumumab (1 ng/mL) and titration-dependent concentrations of YM155 (and 48, 120, 50 and 25 NM for Patients #1-4, respectively, determined as sub-maximal concentrations; Figure 1B) were added. Since BM-MNCs contained sufficient NK cells (7.9, 7.9, 10.3 and 9.5%, respectively), no additional effector cells were added. After 24 h, the viable CD138$^+$ MM cells were enumerated in each condition *via* flow cytometry. % lysis was calculated relative to the survival of MM cells in BM-MNCs which were cultured at the same conditions but did not receive any treatment.

have recently demonstrated that accessory bone marrow (BM) cells protect MM cells from lysis by cytotoxic T cells (CTLs), mainly through the upregulation of the anti-apoptotic molecule survivin.[6] CTLs and NK cells possess similar mechanisms of target cell lysis. Therefore, we explored the potential impact of BM stroma-MM cell interactions on the ADCC response to daratumumab. We started testing the efficacy of daratumumab-mediated ADCC against two CD38^{+} MM cell lines, UM9 and RPMI8226 in the absence *versus* presence of healthy donor-derived bone marrow stromal cells (BMSCs) and healthy donor-derived peripheral blood mononuclear cells (PBMCs) as effector cells. In the absence of BMSCs, daratumumab induced a dose-dependent ADCC against both MM cell lines. Remarkably, however, both cell lines were significantly less sensitive to ADCC in the presence of BMSCs (Figure 1A). We then explored whether BMSCs can also protect primary MM cells from daratumumab-dependent ADCC, applying flow cytometry-based ADCC assays, in which the lysis of primary CD138^{+} MM cells is determined in whole bone marrow-mononuclear cells (BM-MNCs) without separation of MM cells from autologous NK cells already present in the BM-MNCs.[4] Also here, daratumumab-mediated ADCC against primary MM cells was substantially inhibited after the addition of autologous BMSCs in the cultures (Figure 1B). Taken together, these results confirmed that stromal cells of tumor microenvironment could protect MM cells from ADCC.

In order to gain insight into the mechanisms of BMSC-mediated escape from ADCC, we first evaluated whether the interaction with BMSCs negatively influenced the membrane expression of CD38 on MM cells. Co-culture of MM cells with BMSCs, however, did not down-regu-

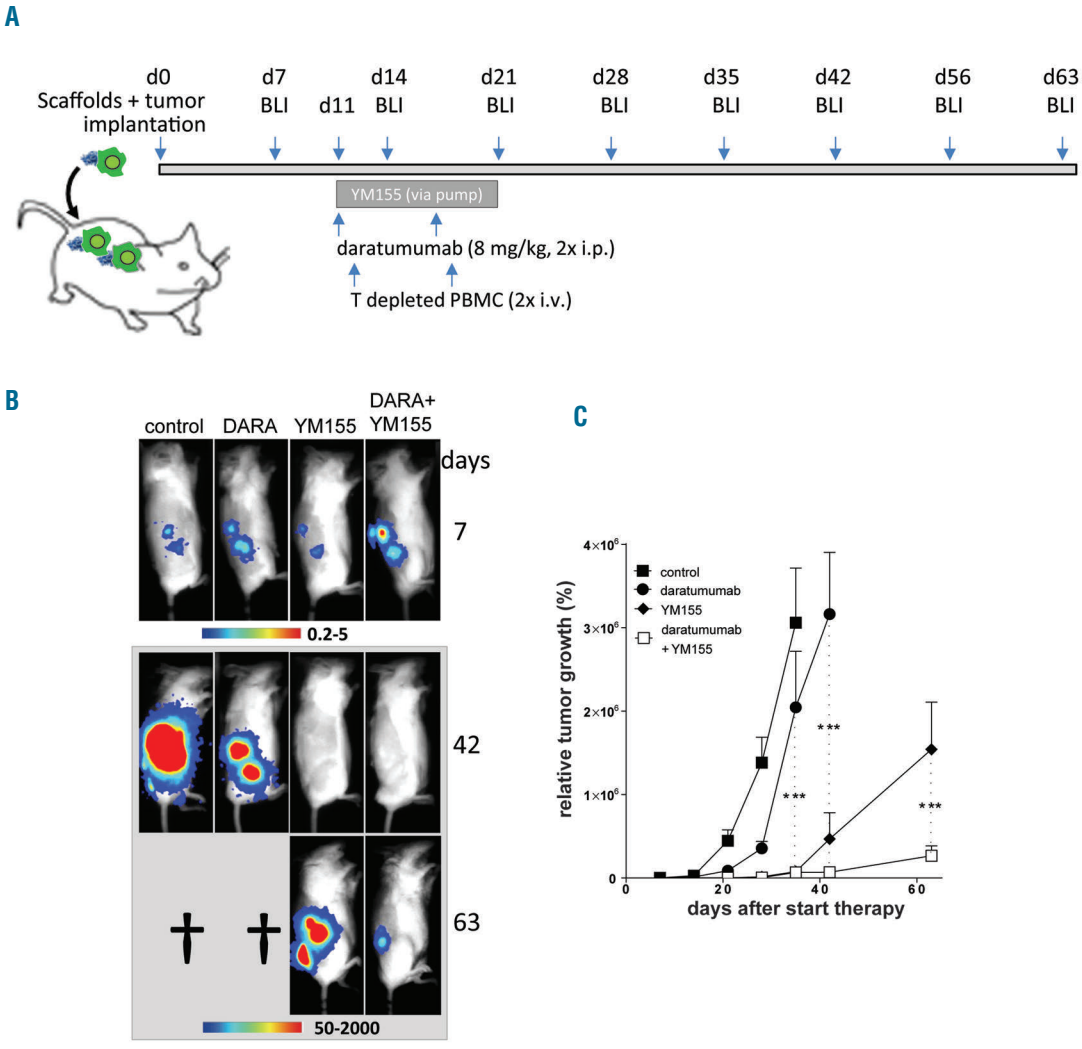

Figure 3. ***In vivo*** **antitumor effect of daratumumab and YM155 combination therapy.** (A) Schematic overview of the assay. Hybrid scaffolds *in vitro* coated with human mesenchymal stromal cells (MSC) and loaded with luciferase-transduced multiple myeloma (MM) cell line UM9 were implanted subcutaneously at the back of RAG2$^{-/-}\gamma c^{-/-}$ mice (4 scaffolds per mice). Ten days after implantation, growing tumors were visualized and quantified by bioluminescence imaging (BLI), as described elsewhere.[8] Different groups of mice (n=4) were then treated with: i) vehicle (control); ii) YM155; iii) daratumumab; iv) daratumumab plus YM155. YM155 or its vehicle (phosphate buffer solution) was delivered with subcutaneous pumps (Alzet 1007D) at a rate of 1 mg/kg/d YM155 for ten days. Each mouse, including those in the control group, received T-cell-depleted HD-PBMC (5x10^{6} cells) as a source of human natural killer (NK) cells to induce antibody-dependent cellular cytotoxicity ADCC. Mice were monitored weekly by BLI. (B) Images of representative mice per treatment group: control, daratumumab, YM155 or daratumumab plus YM155. (C) Analysis of tumor load per treatment group. Results are expressed as mean tumor load in each scaffold. Error bars represent the standard error of mean (SEM). Statistical differences between mice treated with daratumumab and mice treated with daratumumab plus YM155 were calculated using the Mann-Whitney U-test. *P<0.05, **P<0.01, ***P<0.001; ns: not significant.

late their CD38 expression (Figure 1C). We then questioned whether the escape from ADCC could be due to the suppression of NK cells, as BMSCs are known to produce several immunosuppressive factors, such as indolamine 2,3-dioxygenase (IDO), or prostaglandin E2 (PGE2), that can down-regulate NK-cell cytotoxic machinery.[7] To evaluate this, we measured the daratumumab-mediated granzyme B (GzB) secretion from effector cells in the supernatants of ADCC assays (Figure 1A). There was no downregulation of GzB levels in the presence of BMSCs (Figure 1D). Therefore, we also ruled out the possibility that the BMSC-mediated escape from ADCC was due to suppression of NK cells.

After excluding CD38 expression-dependent and immune suppression-mediated escape mechanisms, we questioned whether a survivin-mediated apoptosis resistance mechanism, which we have recently described for CTLs,[6] could also explain the BMSC-mediated protection of MM cells from ADCC. Therefore, we tested the susceptibility of MM cells toward daratumumab-dependent ADCC not only in the absence *versus* presence of BMSCs but also in the absence *versus* presence of the small molecule YM155 that effectively suppresses survivin expression in tumor cells, but also modulates other anti-apoptotic molecules like MCL.[1,8,9] Prior to these assays, we confirmed that survivin is up-regulated in MM cells upon interaction with stromal cells (Figure 2A), and, importantly, we carefully determined the dose range of YM155 that induced a sub-maximal level of MM cell lysis (Figure 2B), but was completely non-toxic for NK cells (Figure 2C). At these dose ranges, YM155 was also non-toxic to BMSCs, as we have documented previously.[6] As expected, daratumumab-mediated ADCC of the MM cell line RPMI-8226 was inhibited by BMSCs (Figure 2D). YM155 alone induced 26% and no lysis in the absence *versus* presence of BMSCs, respectively (Figure 2D). When combined with daratumumab, it significantly and synergistically enhanced the daratumumab-mediated ADCC both in the absence and in the presence of BMSCs, and largely abrogated the protective effects of BMSCs (Figure 2D). Also for primary MM cells (n=4), the daratumumab-mediated ADCC of MM cells was significantly inhibited by the addition of autologous BMSCs and markedly increased, in a synergistic fashion, by the addition of YM155, especially in the presence of BMSCs (Figure 2E). These results confirmed that BMSCs did indeed protect MM cells from daratumumab-mediated ADCC and suggested that combination of daratumumab with pro-apoptotic small molecules, like YM155, could achieve significantly better clinical results. To test this, we used a preclinical xenograft model in RAG2$^{-/-}\gamma c^{-/-}$ mice in which MM tumors are grown in a humanized BM-like niche created by subcutaneous implantation of ceramic scaffolds coated with human BMSCs.[10] In this model, we loaded the BMSC-seeded scaffolds with luciferase transduced UM9 MM cells *in vitro* and then incubated these tumor-loaded scaffolds in the backs of the mice. Ten days later, when a clear BLI signal was detected from growing tumors, we treated different groups of mice (n=4) with either: i) vehicle control (PBS); ii) daratumumab; iii) YM155; iv) daratumumab plus YM155. YM155 or PBS was delivered with subcutaneous pumps at a rate of 1 mg/kg/day YM155 for ten days (Figure 3A). Each mouse, including the control group, received T-cell-depleted healthy donor PBMCs (5x10^6 cells) as a source of human effector cells to induce ADCC. Daratumumab showed an objective but marginal effect on tumor growth (Figure 3B and C). The anti-MM effect was more pronounced with YM155, which delayed the *in vivo* tumor growth significantly better than daratumumab. Although at day 35 there was no visible difference between YM155 and the YM155-DARA combination, the tumor growth curves indicated that the tumor was more effectively controlled by the combination. Indeed, while tumors in the YM155 group showed rapid expansion from day 35 onwards, the tumor growth was considerably slower in the group treated with combination of daratumumab and YM155 (Figure 3B and C). On day 63, at the end of the experiment, all mice in the daratumumab group were sacrificed due to extremely large tumors; tumors in the YM155 group also reached large dimensions, but in the daratumumab + YM155 group only a small mass was visible, showing significantly improved anti-MM effects compared to YM155. These findings supported the *in vitro* data and suggested that a clinical benefit could, indeed, be expected from the combination of daratumumab with YM155.

The results of this study are consistent with large series of earlier reports, documenting that MM cells optimally exploit their natural niche in the BM to develop resistance against various pro-apoptotic therapies.[5,11,12] Until recently, microenvironment-induced apoptosis resistance was almost exclusively studied for pharmacological agents.[9] Now our current findings, our recent demonstration that microenvironment protects MM cells from CTLs,[6] and reports from others indicating that BMSCs can protect lymphoma cells from rituximab-induced apoptosis,[13] together provide increasing evidence that microenvironment-mediated apoptosis resistance is also an important tumor escape mechanism from immune therapies. Our current and recent results[6] indicate survivin as a possible mediator of this immune escape mechanism and are consistent with the observations that the efficacy of rituximab can also be enhanced by YM155.[14] Nonetheless, these results do not necessarily rule out the involvement of other anti-apoptotic molecules, such as the BCL-2 family of molecules, which are also critical in the balance between pro- and anti-apoptotic signals.[15] In this regard, it needs to be noted that YM155 not only suppresses survivin, but also down-regulates MCL-1,[9] which possibly potentiates the pro-apoptotic effect. We observed that addition of YM155 to daratumumab improved ADCC also in the absence of BMSCs, suggesting that YM155-daratumumab combination could be beneficial even if MM cells are not in direct contact with BMSCs, such as in plasma cell leukemia.

These results taken together show the potential benefits of combining daratumumab with YM155 in MM, which may increase clinical efficacy over daratumumab as a single agent. Theoretically, the combination of daratumumab with YM155 or another survivin inhibitor could also be beneficial for other CD38$^+$ hematologic tumors, such as acute myeloid leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma, which not only express CD38 but also high levels of survivin.[16]

Sanne J. de Haart,[1] Lisa Holthof,[2] Willy A. Noort,[2] Monique C. Minnema,[3] Maarten E. Emmelot,[3] Tineke Aarts-Riemens,[1] Parul Doshi,[4] Kate Sasser,[4] Huipin Yuan,[5] Joost de Bruijn,[5] Anton C.M. Martens,[2] Niels W.C.J van de Donk,[2] Henk M. Lokhorst,[2] Richard W.J. Groen,[2] and Tuna Mutis[1,2]

[1]Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, the Netherlands; [2]Department of Hematology, VU University Medical Center, Amsterdam, the Netherlands; [3]Department of Hematology, University Medical Center Utrecht, the Netherlands; [4]Janssen Research & Development, Spring House, PA,

USA; and [5]Xpand Biotechnology BV, Bilthoven, the Netherlands

Correspondence: t.mutis@vumc.nl
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