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Bi 38-3 is a novel CD38/CD3 bispecific T-cell engager with low toxicities for the
treatment of multiple myeloma.

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Monoclonal antibodies (mAb) targeting CD38, such as daratumumab, have shown good therapeutic efficacy in Multiple Myeloma (MM), both alone (1) and in combination with normal standard of care regimens (2, 3). However, many patients eventually relapse because of resistance mechanisms, including FcγR dependent down regulation of CD38 on tumor cells as well as inhibition of complement dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and antibody dependent cellular phagocytosis (4). Bi-specific T-cell engaging (BiTE) antibodies belong to a new class of immunotherapeutic agents that can recognize, on the one hand, a specific antigen on the surface of the target cells (i.e. tumor antigen) and, on the other hand, the CD3ε chain on T lymphocytes (5). By activating T cells via the CD3 complex and recruiting them in proximity of target cells, BiTEs efficiently induce T-cell mediated cytotoxicity (6). In MM, bi-specific antibodies recognizing B-cell maturation antigen (BCMA) or FcRH5 (CD307) have been shown to eliminate tumor plasma cells in preclinical models (7-9). However, FcRH5 expression is not limited to tumor plasma cells and BCMA is abundantly secreted in MM patients (10, 11). These two features may therefore limit the specificity or efficiency of these cognate bi-specific antibodies in vivo. Recently, an anti-CD38 bispecific antibody, AMG424, has been shown to eliminate MM cells in preclinical models, but also to trigger “off tumor” T cell cytotoxicity on B, T and NK cells in vitro (12). Thus, development of efficient and safer bi-specific antibody could contribute to improve the treatment of MM.

We have developed a new BiTE (Bi38-3) that consists of two single-chain variable fragments derived from mouse hybridomas, producing anti-human CD38 and CD3ε (Online Supplementary Figure S1A and B). Purified Bi38-3 efficiently and specifically recognizes CD38 on MM cells and binds CD3ε expressing Jurkat T cells (Online Supplementary Figure S1C and D). To assess the function of Bi38-3 on the cytotoxic activity of T cells, we performed co-culture assays with effector T cells, isolated from peripheral blood mononuclear cells (PBMC) of healthy donors, on firefly luciferase expressing target KMS11 and MM1.S MM cell lines. We observed that T cells readily killed KMS11 cells (as measured
by luciferase activity) in a Bi38-3 dose dependent manner, with a half maximal effective concentration (EC\textsubscript{50}) around 5 ng/mL, the equivalent of 0.09 nM for this 55.6 Kd protein (Figure 1A). Bi38-3 mediated T cell cytotoxic activity was also observed in co-culture with MM1.S cells (Figure 1B). However, in this cell line, which expresses heterogeneous levels of CD38, the EC\textsubscript{50} was tenfold lower (0.5 ng/mL), indicating that Bi38-3 also triggered a strong T cell cytotoxicity in MM cells with weaker CD38 expression. In line with this, stimulation of donor T cells with Bi38-3 in the presence MM1.S cells led to robust proliferation, expression of activation markers CD25 and CD69, as well as production of Interferon-gamma (IFN\textgamma), Tumor necrosis factor-alpha (TNF\textalpha) and Interleukin-2 (IL-2) in a Bi38-3 dose dependent manner (Online Supplementary Figure S1E-H). The viability of MM1.S or KMS11 MM cells was not affected by co-culture with T cells or Bi38-3 alone (Figure 1A and B, right). In addition, Bi38-3 induced poor T cell-mediated killing of CD38 deficient MM1.S cells (MM1.S-KO), with around half of CD38-deficient MM1.S cells surviving the co-culture even at the highest dose of Bi38-3 (1µg/mL) (Figure 1C). These results indicate that, at lower doses, similar to those that are expected in patients, Bi38-3 directs efficient T-cell-cytotoxic activity specifically towards CD38 expressing MM cells. We next analyzed the potential of Bi38-3 to induce lysis of target (T) tumor plasma cells, isolated from 4 patients at diagnosis, by autologous effector (E) T cells. FACS analysis of overnight E:T co-cultures revealed that the numbers of viable CD138 positive MM cells were reduced in a Bi38-3 dose dependent manner, with the EC\textsubscript{50} ranging from 0.028 to 1.29 ng/mL, depending on the patient (Figure 1D and Online Supplementary Figure S2). Importantly, in the absence of T cells, Bi38-3 exhibited no toxicity against fresh tumor cells. Bi38-3-induced cytotoxicity of autologous T cells was further investigated on tumor plasma cells from 3 MM patients at relapse and demonstrated similar efficacy, with EC\textsubscript{50} ranging from 0.2 to 0.86 ng/mL (Figure 1E). These results indicate that, Bi38-3 triggered autologous T cell-mediated killing of tumor plasma cells from patients both at diagnosis and at relapse.
To investigate potential toxic effects of Bi38-3 on blood cells, PBMC from donors were treated with various concentrations of Bi38-3 for 24 hours and the mononuclear cell populations were individually analyzed by FACS (Online Supplementary Figure S3). We observed that the percentages of CD14 expressing monocytes included in the live gate were markedly reduced in a Bi38-3 dose dependent manner (Figure 2A). In contrast, the percentage of CD4 and CD8 T lymphocytes, that together represented around 60% of total PBMC cells, slightly increased in response to Bi38-3 probably due to the decrease in live CD14 positive cells. Similarly, the B (CD19+) and NK (CD56+) cell populations remained at similar levels (around 10% and 5% respectively), even at high concentrations of Bi38-3 (100ng/mL) (Figure 2A).

Next, we investigated whether expression of CD38 at the surface of blood cells was downregulated by Bi38-3. FACS analysis indicated that CD38 Mean Fluorescence Intensity (MFI) on T, B and NK cells remained similar in cultures containing increasing doses of Bi38-3 (Figure 2B). In line with this, CD38 expression was not dramatically reduced on CD14+ monocytes. Of note, this analysis could not be performed at high doses because these cells, which express higher levels of CD38, were sensitive to elevated concentrations of Bi38-3 (above 1ng/mL). To compare the activity of Bi38-3 on CD38 high MM versus CD38 intermediate cells, we performed co-culture assays with MM1.S, expressing high levels of CD38, freshly isolated B cells, expressing intermediate levels of CD38 (Figure 2C) and autologous T cells. Following an overnight culture, the percentages of viable CD20+ B cells and CD138+ MM1.S cells were analyzed by flow cytometry (Online Supplementary Figure S4A). We observed that the percentages of MM1.S cells dropped at Bi38-3 concentrations of 0.1ng/mL and this reduction was more dramatic at higher doses (Figure 2C). In contrast, compared to untreated conditions, the percentages of viable CD20+ B cells remained unchanged even at high concentrations of Bi38-3 (Figure 2C).

We developed a similar autologous tri-culture assay to investigate potential toxic effects of Bi38-3 on CD34+ bone marrow hematopoietic progenitors and on regulatory T cells (Treg), which both express low levels of CD38. While Bi38-3 readily induced MM cell killing at low concentrations ($10^{-2}$ ng/mL and above), we found that it triggered no significant T cell
mediated cytotoxicity on Foxp3+ Treg (Figure 2D). Similarly, there was no significant toxicity on CD34+ hematopoietic progenitors at concentrations below 10ng/mL and moderate toxicity (> 40% survival) at the highest concentrations (Figure 2E). Altogether, our results indicate that Bi38-3 does not impair the surface expression of CD38 and only triggers T cell mediated killing of cells expressing high levels of CD38 with no or limited toxicity against cells expressing intermediate levels of CD38, such as hematopoietic progenitors, B, T or NK cells.

The antitumor activity of Bi38-3 was further assessed in vivo using a human MM xenograft mouse model. MM1.S cells expressing luciferase (MM1.Sluc) were injected in the tail vein of immunodeficient (NSG) mice and luciferase levels measured by IVIS Imaging System every 3 or 4 days. Thirteen days after MM1.S injection, purified human T cells were transplanted I.V. with or without Bi38-3 (0.1 mg/kg). Treatments with Bi38-3 or vehicle were repeated daily for 9 days (Figure 3A). Seven days following tumor cell injection, all mice showed similar levels of Radiance (luciferase), indicating that MM cells had effectively engrafted in host animals prior to Bi38-3 treatment (Figure 3B). While control mice showed rapid tumor progression, all Bi38-3 treated animals displayed a marked reduction in tumor growth within the first 5 days of Bi38-3 treatment (Figure 3C). At the end of treatments, the level of luciferase expressing MM cells in Bi38-3 treated mice was only one tenth of the initial level and was on average thirty-fold lower than untreated controls (Figure 3C). These results show that Bi38-3 is able to efficiently control MM tumor progression in vivo.

We report here the development of Bi38-3, a new anti-CD38/CD3 BiTE, which triggers T cell-mediated lysis of CD38-positive MM cells in vitro, ex vivo and in vivo. Interestingly, Bi38-3 provokes no toxicity on B, T and NK cells in vitro and, therefore, is likely to have less "off tumor" effects than AMG424, a recently developed anti-CD38 bispecific antibody (12). Importantly, Bi38-3 efficiently triggers killing of MM cells from patients that are resistant to standard treatments. Furthermore, because it recognizes a specific epitope on CD38 and is devoid of the Fc region, Bi38-3 is expected to also be efficient in relapse patients following daratumumab therapy. Altogether, the data presented in this study identifies Bi38-3 as a
selective and efficient compound for the treatment of MM, that could be used both as a front-line agent or at relapse (alone or in combination with additional drugs), and which should be evaluated further in MM patients.

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References

FIGURE LEGENDS

Figure 1: Bi38-3 induces CD38-dependent T cell mediated MM cell lysis in vitro. KMS11-luc (A), MM1.S-luc (B) and CD38 deficient MM1.S-luc (MM1.S-KO-luc) (C) MM cell lines were co-cultured with T cells, isolated from peripheral blood samples from healthy donors, at an E:T cell ratio of 5:1 with increasing concentrations of Bi38-3 for 24 hours. Curves represent target cell lysis, monitored by luciferase activity and expressed as the percentage of the untreated condition (0% lysis). Data are the means of independent experiments with 4 (A), 9 (B) and 4 (C) different donors. Standard deviations (SD) are shown for each concentration. Histograms on the left show target cell lysis induced by Bi38-3 alone, donor T cells alone and T cells with Bi38-3 (10^1 ng/mL) on KMS11-luc (A), MM1.S-luc (B) and MM1.S-KO-luc (C). (D and E) Fresh tumor plasma cells were collected from buffy coat of bone marrow aspirates from myeloma patients, then CD138^+ cells were purified and co-cultured with autologous CD3^+ T cells isolated from PBMC at an E:T cell ratio of 5:1 for 24 hours. Cultures were analyzed by FACS to monitor the number of CD138^+ cells falling into the live gate. The average percentages of lysis of CD138^+ cells (relative to the untreated condition) in 4 different patients at diagnosis (D) and 3 different patients at relapse (E) are shown and the error bars indicate the SD. Histograms show the average effects of Bi38-3 alone, T cells alone and T cells with Bi38-3 (10^2 ng/mL) on tumor plasma cells from the same 4 patients at diagnosis (D) and 3 patients at relapse (E). Standard deviations are shown and P values were determined by two-sided Mann–Whitney U-test (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 2: Sensitivity of blood cells and bone marrow hematopoietic progenitors to Bi38-3. (A) PBMC from healthy donors (n=3) were cultured with medium (M) or various concentrations of Bi38-3 for 24 hours and the percentages of live T, myeloid, B and NK cells were determined by FACS analysis. Histograms show the average percentages of live CD4^+ T cells, CD8^+ T cells, CD14^+ monocytes, CD19^+ B cells and CD56^+ NK cells in Bi38-3 cultures compared to untreated controls in 3 independent donors and the error bars indicate the SD. (B) Mean
Fluorescence Intensity (MFI) were measured in MM.1S WT cells and in CD4, CD8, CD14, CD19 and CD56 expressing cell subsets based on FACS analysis of CD38 expression levels in PBMC cell populations cultured with various concentrations of Bi38-3 as in A. Histograms show average CD38 MFI of MM.1S cells and PBMC cells populations and error bars indicate the SD. ND: Not Determined due to lack of sufficient numbers of events. (C) Relative Bi38-3 mediated T cell lysis of B versus MM1.S cells. Purified paired B and T cells from healthy donors (n=5) were co-cultured with increasing concentrations of Bi38-3 for 24 hours in the presence of MM1.S cells. (D) Relative Bi38-3 mediated T cell lysis of Treg versus MM1.S cells. Purified T cells from healthy donors (n=3) were co-cultured with increasing concentrations of Bi38-3 for 24 hours in the presence of MM1.S cells. (E) Relative Bi38-3 mediated T cell lysis of CD34+ bone marrow hematopoietic progenitors versus MM1.S cells. Paired CD34+ hematopoietic progenitors and T cells purified from the bone marrow of healthy donors (hip surgery) (n=4) were co-cultured with increasing concentrations of Bi38-3 for 24 hours in the presence of MM1.S cells. In C, D and E, the numbers of live CD20+ (B cells), FoxP3+ (Treg cells), CD34+ (Hematopoietic progenitors) and CD138+ (MM1.S cells) were calculated by FACS using counting beads and expressed as a ratio to untreated controls, respectively. Histograms show the ratios of B, Treg, CD34+ hematopoietic progenitor and MM.1S cells for each Bi38-3 concentrations and error bars indicate the SD. The normality of the CD34+ populations was established with a Shapiro-Wilk normality test and P-values were determined by an unpaired Student’s t test (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 3: In Vivo Activity of Bi38-3 in the MM1.SLuc Xenograft mouse model. (A) Treatment schedule. Six to 12-week-old NOD/SCID/IL-2Rγnull mice were inoculated with 5.10^6 MM1.SLuc cells by tail vein injection (i.v) at day 0, followed, 13 days later, by infusion of 5.10^6 purified human T cells, isolated from healthy donors, with (or without) Bi38-3 at 0.1mg/kg (blue arrow). Treatment was initiated at day 13 when similar levels of luciferase expressing MM cells were detected in all mice. Tail veins injections of Bi38-3 (0.1 mg/Kg) or
PBS (for controls) were repeated daily for 9 days (black arrows). Bioluminescence was measured with the IVIS Imaging System at day 7, 11, 13, 15, 18 and 21 (or 22) after tumor injection (red arrows). (B) Serial bioluminescence imaging to assess myeloma progression/regression. Radiance was measured on the entire body of mice. Images on the left indicate bioluminescence at 7 days after inoculation with MM.1S myeloma cells and before the beginning of the treatment. Images on the right indicate 18 days after inoculation with MM.1S cells and 4 days after treatment with Bi38-3 (lower panel) or with vehicle (upper panel). The radiance color scale is represented on the right. (C) Longitudinal radiance levels of vehicle (blue lines) and Bi38-3 (red lines) treated mice. Red and blue curves represent, a group of 9 and 11 mice inoculated with MM.1SLuc and T cells and then treated with Bi38-3 (0.1 mg/Kg) and PBS, respectively. Black filled triangles indicate the first injection of Bi38-3 or PBS with T cells and white filled triangles indicate Bi-38 or PBS injections every day for 9 days. These experiments were performed from 2 independent donors. The normality of populations was established with the Shapiro-Wilk normality test, and P-values were calculated based on unpaired Student’s t test (* p < 0.05; ** p < 0.01; *** p < 0.001).
A. I.V. Injection of 5 $10^6$ MM1.S-luc

Day 0

I.V. Injection of 5.1$10^6$ T cells + Bi38-3 (0.1mg/Kg)

Day 7

Day 11

Day 13

Day 14

Day 15

Day 18

Day 21

Day 22

IVIS Acquisition

B. Day 7: Vehicle, Bi38-3

Day 18: Vehicle, Bi38-3

Luminescence

Radiance (p/sec/cm²/sr)

Color Scale

Min = 2.50e4
Max = 1.00e6

C. Graph showing luminescence over time for Vehicle and Bi38-3 treatments.
Legends for Supplementary figures

Supplementary figure S1: Structural and functional characterizations of Bi38-3. (A) Schematic of Bi38-3. The anti-human CD38 scFv and the anti-human CD3ε scFv domains are represented in red and blue respectively. Plain lines represent 15-amino-acid glycine-serine spacers. Myc-tag and His-tag are indicated by green and brown boxes respectively. (B) (Left) Western blot analysis of HEK293 cells transfected with Bi38-3 expression vector or an empty vector. Bi38-3 was detected with an anti-Myc antibody. β-actin was used as a loading control. (Right) Analysis of purified Bi38-3 by gel electrophoresis and Coomassie blue staining. (C) Binding of Bi38-3 to MM cell lines. (Left panel) FACS analysis of NCI-H929, KMS11, MM1.S and CD38-deficient MM1.S (MM1.S-KO) cells stained with PE-anti-CD38 (red) or isotype control (blue). (Right panel) Cells were stained with Bi38-3 (red) or left untreated (blue) and revealed with APC-anti-Fab′ antibody. The percentage of cells falling in the gate are indicated in the histogram. Representative of 3 independent experiments. (D) Binding of Bi38-3 to CD3+ Jurkat cells deficient or not for CD38 (KO). (First and second panels) FACS analysis of Jurkat and Jurkat-KO cells stained with, respectively, APC-anti-CD3/PE-anti-CD38 (red) or isotype control (blue). (Third panel) Cells were stained with Bi38-3 (red) or left untreated (blue) and revealed with APC-anti-Fab′ antibody. (E and F) Proliferation assessed by violet CellTrace dye dilution. Purified T cells, isolated from healthy donors (n=4), were stained with CellTrace violet dye and co-cultured with (or without) MM1.S target cells or CD38 deficient MM1.S (MM1.SKO) at an E:T ratio of 1:1 with fixed concentration of Bi38-3 (10¹ ng/mL) for 96 hours. T cell expansion index was calculated after a 96-hours culture in medium alone (M), CD3/CD28 beads (3/28) or Bi38-3 (10¹ ng/mL). Histograms show the average expansion indexes of T cells in 4 independent experiments with 4 different donors. Standard deviations are shown and P values were determined by two-sided Mann–Whitney U-test (* p < 0.05; ** p < 0.01; *** p < 0.001). (G) Activation markers CD25 and CD69 on CD4-positive and CD8-positive T cells was determined by FACS analysis. T cells were grown in medium (M) or stimulated with Bi38-3 at
$10^0$ or $10^2$ ng/mL, with (or without) MM1.S or CD38 deficient MM1.S (MM1.S-KO) at an E:T ratio of 5:1. Histograms show average percentages of CD25+ and CD69+ on CD4-positive and CD8 positive T cells and error bars indicate the SD. (H) Production of Interferon gamma (IFN$_\gamma$), Interleukin 2 (IL-2) and Tumor necrosis factor (TNF$_\alpha$) in MM1.S/T cell co-cultures at an E:T ratio of 5:1. Data represent the average of 4 independent experiments with 4 different donors. The normality of populations was established with the Shapiro-Wilk normality test and P-values were determined by the unpaired Student’s $t$ test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplementary Figure S2: Shown are the gating strategies to determine Bi38-3 mediated autologous T cell cytotoxicity on fresh tumor plasma cells used in Figure 1B. Purified bone marrow CD138$^+$ plasma cells from patients were co-cultured with autologous PBMC CD3$^+$ T cells at an E:T cell ratio of 5:1 for 24 hours. Cultures were analyzed by FACS to monitor the number of CD138$^+$ cells falling into the live gate as measured with counting beads. Culture conditions are indicated above each dot plot pair: FSC/SSC (top) and SSC/CD138 (bottom). Representative of 4 different patients at diagnosis and 3 at relapse.

Supplementary Figure S3: Shown are the gating strategies to determine Bi38-3 mediated autologous T cell cytotoxicity on PBMC cell subsets cells used in Figure 2A. PBMC from healthy donors (n=3) were cultured with various concentrations of Bi38-3 for 24 hours and the percentages of T, myeloid, B, NK cells were determined by FACS. Dot plots depict the percentages of live CD4$^+$ T cells, CD8$^+$ T cells, CD14$^+$ monocytes, CD19$^+$ B cells, and CD56$^+$ NK cells in the culture. Culture conditions are indicated on the left. The percentages of cells falling into the gates are shown is each dot plot. Representative of 3 independent experiments.
Supplementary Figure S4: (A) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and autologous B cells co-culture used in Figure 2C. Purified paired T and B cells from healthy donors (n=5) were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours. (B) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and FoxP3+ Treg cells co-culture used in Figure 2D. Purified T cells from donors were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours. (C) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and CD34+ bone marrow hematopoietic progenitors cells co-culture used in Figure 2E. Purified paired CD34+ hematopoietic progenitors and T cells from bone marrow samples from healthy donors (from hip surgery) (n=4) were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours.

Cultures were analyzed by FACS to monitor, respectively, the number of CD138+ cells (MM1.S), B cells (CD20+), Treg cells (FoxP3+) and hematopoietic progenitors (CD34+) falling into the live gate as measured with counting beads. Culture conditions are indicated above each dot plot pair: FSC/SSC (top) and CD20/CD138 (bottom A), FoxP3/CD138 (bottom B) or CD34/CD138 (bottom C). Data are representative of 5 (A), 3 (B) and 4 (C) independent donors.
SUPPLEMENTARY FIGURE 2
SUPPLEMENTARY FIGURE 3

Medium

PBMC 16.8

CD4

CD8

CD14

CD19

CD56

Bi38-3 10^{-1} ng/mL

CD4+ cells 41.1

CD8+ cells 17.3

CD14+ cells 15.5

CD19+ cells 5.6

CD56+ cells 0.78

Bi38-3 10^{-2} ng/mL

CD4+ cells 46.1

CD8+ cells 17.8

CD14+ cells 16.7

CD19+ cells 5.40

CD56+ cells 0.69

Bi38-3 10^{-3} ng/mL

CD4+ cells 47.2

CD8+ cells 18.9

CD14+ cells 2.24

CD19+ cells 5.88

CD56+ cells 0.37

PBMC 15.0

CD4+ cells 46.0

CD8+ cells 21.8

CD14+ cells 0.044

CD19+ cells 6.00

CD56+ cells 7.29