Improvement of daratumumab- or elotuzumab-mediated NK cell activity by the bi-specific 4-1BB agonist, DARPin α-FAPx4–1BB: A preclinical study in multiple myeloma

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ABSTRACT

Multiple myeloma (MM) progression is closely dependent on cells in the bone marrow (BM) microenvironment, including fibroblasts (FBs) and immune cells. In their BM niche, MM cells adhere to FBs sustaining immune evasion, drug resistance and the undetectable endurance of tumor cells known as minimal residual disease (MRD). Here, we describe the novel bi-specific designed ankyrin repeat protein (DARPin) α-FAPx4–1BB (MP0310) with FAP-dependent 4–1BB agonistic activity. The α-FAPx4–1BB DARPin simultaneously binds to FAP and 4–1BB overexpressed by activated FBs and immune cells, respectively. Although flow cytometry analysis showed that T and NK cells from MM patients were not activated and did not express 4–1BB, stimulation with daratumumab or elotuzumab, monoclonal antibodies (mAbs) currently used for the treatment of MM, significantly upregulated 4–1BB both *in vitro* and in MM patients following mAb-based therapy. The mAb-induced 4–1BB overexpression allowed the engagement of α-FAPx4–1BB that acted as a bridge between FAP+FBs and 4–1BB+NK cells. Therefore, α-FAPx4–1BB enhanced both the adhesion of daratumumab-treated NK cells on FBs as well as their activation by improving release of CD107a and perforin, hence MM cell killing via antibody-mediated cell cytotoxicity (ADCC). Interestingly, α-FAPx4–1BB significantly potentiated daratumumab-mediated ADCC in the presence of FBs, suggesting that it may overcome the BM FBs' immunosuppressive effect. Overall, we speculate that treatment with α-FAPx4–1BB may represent a valuable strategy to improve mAb-induced NK cell activity fostering MRD negativity in MM patients through the eradication of latent MRD cells.

1. Introduction

Multiple myeloma (MM) is a haematological disease characterized by the expansion of tumor plasma cells (MM cells) in the bone marrow (BM) [1]. It is usually preceded by premalignant phases of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM) with 5 % and 50 % risk of progressing into MM within 5 years, respectively [2]. The BM microenvironment (e.g. fibroblasts [FBs], endothelial cells, osteoblasts and immune cells) fosters MM progression by creating a protective niche that sustains tumor cell growth, angiogenesis, apoptosis resistance and immune deregulation [3]. Specifically, immune dysfunction correlates with disease progression and poor prognosis [4–6]. Deregulation of immune cells involves quantitative, phenotypic and functional defects in adaptive immune cells, *i.e.* T

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and B cells, and innate immune cells, including natural killer (NK) cells [4–6]. During cancer immunoediting, MM cells acquire the ability to evade innate immune cells by preventing NK cell recognition and function [7]. Otherwise, NK cells from MM patients are exhausted and have impaired cytotoxic activity that correlate to poor prognosis and a worse disease-free survival [8–10].

Among BM stromal cells, FBs sustain immune dysfunction shaping an immunosuppressive microenvironment *via* the release of TGF-β [11]. We demonstrated that MM FBs are the main component of the supportive BM niche enabling tumor onset, progression, drug resistance, and angiogenesis [12]. In BM biopsies from MM patients, activated FBs were in close contact with MM cells allowing pharmacological resistance and immune evasion that entail tumor cell survival [12,13]. The persistence of surviving MM cells, known as minimal residual disease (MRD), correlates to poor patient prognosis and relapse. Therefore, MRD negativity is currently considered the main goal of anti-myeloma therapy.

Based on the pivotal role of the immune system, the newest therapeutic approaches (e.g. immunomodulatory drugs [IMiDs], checkpoint inhibitors, CAR-T cells, monoclonal antibodies [mAbs]) target the immune microenvironment in order to enhance the anti-tumor response [14] and promote MRD negativity. Nevertheless, the endurance of undetectable surviving MM cells in the BM still impairs the efficacy of anti-myeloma strategies [15–17].

Among the new therapeutic approaches, co-stimulation with 4–1BB agonists has received significant interest with its potential to enhance the efficacy of cancer immunotherapy [18]. Currently, over 40 second generation 4–1BB agonists are in development, mostly multi-specific molecules with tumor-targeted activity to avoid toxicity associated with systemic activation [19]. Most of their activity is considered to be driven by an increase in activation of NK and CD8 T cells, as they are the major cell types expressing 4–1BB [20]. Based on a platform of Designed Ankyrin Repeat Proteins (DARPins) [21,22], a fibroblast activation protein (FAP)-targeted bi-specific 4–1BB agonist DARPin, α-FAPx4–1BB (MP0310), has been designed to have tumor-localized activity [23]. The α-FAPx4–1BB simultaneously binds to 4–1BB and FAP, expressed on cancer-associated fibroblasts, triggering activation of immune cells exclusively in the tumor.

As MM cells adhere to FBs in the BM niche, here we speculate that α-FAPx4–1BB may direct 4–1BB^{+} NK cells towards FAP[+] FBs enhancing the recognition and killing of hidden MM cells. We demonstrated that mAbs currently used for the treatment of MM patients, namely the anti-CD38 daratumumab and the anti-SLAMF7 elotuzumab induce NK cell activation and 4–1BB expression *in vitro* and *in vivo* in MM patients. Interestingly, α-FAPx4–1BB significantly improves the anti-myeloma activity of mAb-treated NK cells suggesting its potential therapeutic use in combination strategies with daratumumab and/or elotuzumab.

2. Materials and methods

2.1. Patients

Fifty-five patients (32 M/23 F, age 38–85, median 61.5 years old) fulfilling the International Myeloma Working Group diagnostic criteria for MM were studied. The study was approved by the Ethics Committee of the Azienda Ospedaliero-Universitaria Policlinico of Bari (0083036/8/10/2019 - Study number: 6053), and all patients provided informed consent according to the Declaration of Helsinki.

2.2. Cell cultures

CHO-K1 (CCL-61) and HT-1080 (CCL-121) human fibrosarcoma cell lines as well as the CD38-positive MM1R and the CD38-negative U266 MM cell lines were obtained from ATCC (Manassas, Virginia, U.S.A). HT1080 hu4–1BB-NFκB-luc reporter cells were generated by stable transfection with cDNA, coding for full length human 4–1BB (OriGene Technologies, RC200664) and the pNiFty3-N-Lucia reporter gene

(Invivogen; pnf3-lc2). CHO-huFAP cells overexpressing human FAP were generated by stable transfection of CHO-K1 cells with a plasmid containing the ORF of human FAP sub-cloned at Molecular Partners AG, Zurich-Schlieren, Switzerland, from cDNA (OriGene Technologies, #RG204692) without GFP fusion. All the cell lines were cultured according to the supplier's instructions and used until the tenth passage.

Primary BM cells from MM patients were obtained from heparinized BM aspirates by centrifugation on a Ficoll gradient (Ficoll-HyPaque, Pharmacia Biotech, Uppsala, Sweden). After centrifugation, BM mononuclear cells (BMMCs) were seeded on Petri dishes and cultured overnight to obtain the non-adherent BM lymphocytes (BMLs) population. Primary NK cells were purified from BMLs by incubation with immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in RPMI-1640 containing 10 % fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MN, USA) and 1 % antibiotic/antimicotic (Euroclone, Milan, Italy). MM FBs were purified from BM adherent cells through D7-FIB-conjugated (anti-FBs) microbeads (Miltenyi) and cultured in DMEM medium containing 20 % FBS and 1 % antibiotic/antimicotic. BMLs and NK cells were freshly purified and used for *in vitro* studies, while primary MM FBs were used until the fifth passage.

2.3. Cell co-cultures, stimulation with mAbs and α-FAPx4-1BB treatment

BMLs or NK cells were co-cultured with the MM cell line MM1R or U266 (effector:target [E:T] cell ratio 10:1 or 1:1, respectively) and treated or not with daratumumab (1 µg/ml) or elotuzumab (10 µg/ml) for 2 hours. Daratumumab-treated/untreated BMLs or NK cells were co-cultured with MM cell lines (E:T cell ratio 10:1 or 1:1, respectively) in the presence/absence of MM FBs and treated or not with α-FAPx4–1BB (0.5 nM) for 4 hours. After treatment, BMLs or NK cells were analyzed by flow cytometry for 4–1BB, perforin and CD107a expression, and used for functional assays.

2.4. 4–1BB reporter cell assay

NFκB-downstream signaling after 4–1BB activation was measured by co-culturing 4–1BB agonists with 4×10^4 HT1080 hu4–1BB-NFκB-luc reporter cells in the presence of 4×10^4 CHO-huFAP cells or FAP-negative wild-type CHO cells in 96-well plates (Greiner Bio-One, #655083) at 37 °C, 5 % CO$_2$. After 20 hours, the supernatant was collected, mixed with QUANTI-Luc reagent (Labforce; #rep-qlc1), and luciferase activity was immediately measured using a Tecan M1000 microplate reader (Tecan). EC50 values were determined by fitting the data with a four-parameter logistical fit model using Graphpad Prism software (version 7.02).

2.5. Activation of human PBMCs for 4–1BB binding assay

Human PBMCs were isolated from the buffy coats of healthy donors obtained from Zurich Blood bank, Switzerland. U-bottom 96-well plates were coated with anti-human CD3 at 1 ug/ml (OKT-3, eBioscience) and incubated for 90 minutes at 37°C or overnight at 4°C. PBS was used in unstimulated control wells. To stimulate 4–1BB expression, isolated PBMCs were added to the pre-coated plates and cultured for 24 hours in RPMI media containing 10 % FCS and 1 % Pen/Strep.

2.6. Cell binding assay

To quantify cell binding, CHO-huFAP cells or activated human PBMCs were incubated with α-FAPx4–1BB or controls in U-bottom 96-well plates (Corning, Falcon #353910). His-tagged versions of the DARPins were used for cell binding experiments in order to detect binding with anti-Penta-His Alexa Fluor 647 secondary antibody (Qiagen). For assays with activated PBMCs, anti-CD4 FITC (eBiosciences, No. 11–0049–42) and/or anti-CD8 PE (BD, No. 555367) antibodies were additionally added to allow gating of T cell populations, following

exclusion of dead cells by Fixable Live Dead Aqua (Thermo Fisher, #L34957). Cells were washed and fixed with CellFix buffer (BD, #40181) for 15 minutes at room temperature then resuspenspended in FACS buffer. Samples were acquired using the AttuneNxT flow cytometer (Thermo Fisher) and analyzed using FlowJo software (version 10.0.3).

2.7. X-ray crystallography

For crystallization, the complex of the untagged extracellular domain of human 4–1BB (aa 25–162) together with the untagged anti-4–1BB DARPin domain was produced to a final concentration of 34.1 mg/ml in purification buffer (10 mM HEPES/NaOH pH 7.0 and 150 mM NaCl). Briefly, DARPin production has been conducted as described previously, using an N-terminal 6xHis-TEV sequence [24]. Analog, 4–1BB-(aa 25–162)-TEV-8xHis was expressed in Hi5 insect cells at Proteros Biostructures GmbH, Munich, Germany and non-tagged material were generated via a His-Trap using Ni-NTA, followed by TEV-digestion, a negative His-Trap and finally a SEC in purification buffer. Purified DARPin and 4–1BB target were mixed at a 1:1.2 ratio, excess of DARPin was removed from the complex via SEC in purification buffer. By applying standard crystallization screens, crystals of the DARPin-4–1BB complex were obtained with sitting-drop technique at 20° using 0.1 M citric acid pH 5.0 / 1.6 M $(NH_4)_2SO_4$ buffer at a 1:1 protein-buffer ratio. Data collection was performed with 25 % (v/v) glycerol as cryo-protectant at the SWISS LIGHT SOURCE (SLS, Villigen, Switzerland) using cryogenic conditions. The diffraction data were processed using STARANISO with anisotropic resolution cutoffs at 3.26 Å, 2.81 Å and 3.37, and the programs autoPROC [25], XDS [26] and AIMLESS of the CCP4 program suite [27]. The phases were obtained by Molecular Replacement using the solved DARPin structure (PDB 6mi2) as search model. The model building and refinement was performed with COOT [28] and REFMAC5 (CCP4 program suite, applying NCS restraints). Water molecules were added with COOT in the peaks of the F_o-F_c map contoured at 3.0 followed by refinement with REFMAC5 and use of the validation tool of COOT and visual inspection. Eight DARPin-4–1BB complexes were located in the asymmetric unit. The electron density of complex allowed to resolve residues 11–136 of the DARPin and residues 25–161 of the human 4–1BB. One short loop in 4–1BB domain of chain J (residue 41–42) is not fully defined by electron density and have thus not been included in the model. Since the interface of the eight complexes displayed a very similar structure (RMSD: 0.54 Å, calculated on the Cα pairs of the DARPin domain and the 4–1BB receptor, aa 27–160), the complex A:B was used as representative. All figures were generated with PyMol (Version 2.0 Schrödinger, LLC). Information regarding the data collection parameters and refinement statistics are indicated in Table S1. Data related to the biological structure shown in this study are deposited in Worldwide Protein Data Bank (wwPDB).

2.8. Flow cytometry

Expression of CD56, CD16, CD45, CD3, 4–1BB, perforin, and CD107a was evaluated by using the mAbs (Becton Dickinson-BD, San Jose, CA, USA) listed in Table S2. For perforin and CD107a detection, samples were treated with Protein Transport Inhibitor (containing Brefeldin A; Becton Dickinson-BD), fixed and permeabilized with cytofix/cytoperm (Becton Dickinson-BD) according to manufacturer's instructions.

Samples were acquired by flow cytometry (FACScanto II) and analyzed using FACS Diva software (Becton Dickinson-BD).

2.9. Adhesion assay

Daratumumab-stimulated or unstimulated NK cells were labeled with Calcein AM (Dojindo Laboratories, Munich, Germany) (5 μM) for 20 minutes. Cells were seeded at 4×10^4 NK cells/well in MM FB-coated 96-well plates and treated with different concentrations (0–5 nM) of α-FAPx4–1BB for 4 hours. After removal of non-adherent cells, the fluorescence intensity was read at 495 nm on a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer Inc., Shelton, Connecticut, U.S. Massachusetts). Data were normalized to untreated NK cells.

### 2.10. Antibody-Dependent Cell Cytotoxicity (ADCC)

MM cell lines MM1R or U266 were labeled with Calcein AM (5 μM) for 20 minutes. Daratumumab-stimulated or unstimulated BMLs were added to cell co-cultures in an E:T cell ratio 10:1 and were treated/untreated with α-FAPx4–1BB (5 nM) for 4 hours in a MM FB-coated/uncoated 12-well plate (final volume= 1 ml). We set up these experimental conditions to prevent effector and target cell interactions. Maximum release was assessed following treatment of the co-culture with Triton100x at a final concentration of 2.5x. After a 4 hour incubation, 100 μL supernatant was loaded on to a 96-well plate and Calcein AM release was measured at 495 nm using a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer Inc.). Calculation of specific cell lysis (%) was performed using the following equation:

$$\frac{\text{mean of calcein AM release in experimental condition} - \text{mean of spontaneous calcein AM release}}{\text{mean of calcein AM maximal release} - \text{mean of spontaneous calcein AM release}} \times 100$$

The results are shown as percentage of specific cell lysis.

3. Results

3.1. The α-FAPx4-1BB bi-specific DARPin drug candidate MP0310 is designed to induce tumor-targeted 4-1BB agonism only in the presence of FAP

Based on the original concept from Muller et al. [29], the α-FAPx4–1BB DARPin drug candidate (MP0310) was designed to simultaneously bind human 4–1BB and FAP to avoid systemic 4–1BB activation through FcR cross-linking due to absence of an Fc domain as well as prevent liver inflammation induced by 4–1BB antibodies [30]. The α-FAPx4–1BB molecule consists of a chain of 5 covalently linked DARPin domains: an N-terminal anti-HSA (human serum albumin) domain for half-life extension [31], an anti-human FAP domain for tumor localization and clustering, and two identical anti-human 4–1BB domains for immune activation (Fig. 1A). Flow cytometry analysis demonstrated binding of α-FAPx4–1BB to 4–1BB expressed on activated primary CD8+ T cells from healthy human donors in a dose-dependent manner. No binding of α-FAPx4–1BB to non-activated 4–1BB negative primary human T cells was observed (Fig. 1B). Binding to cellular FAP was confirmed using CHO-K1 cells transfected to express human FAP (Fig. 1C). Binding to 4–1BB in the presence of FAP induced activation of 4–1BB signaling in human 4–1BB NF-κB-reporter cells when co-cultured in the presence of CHO cells expressing FAP (Fig. 1D). To select the optimal format for α-FAPx4–1BB, constructs with different valencies of 4–1BB domains were tested. DARPin constructs including one FAP binding domain and one (α-FX), two (α-FXX) or three (α-FXXX) 4–1BB
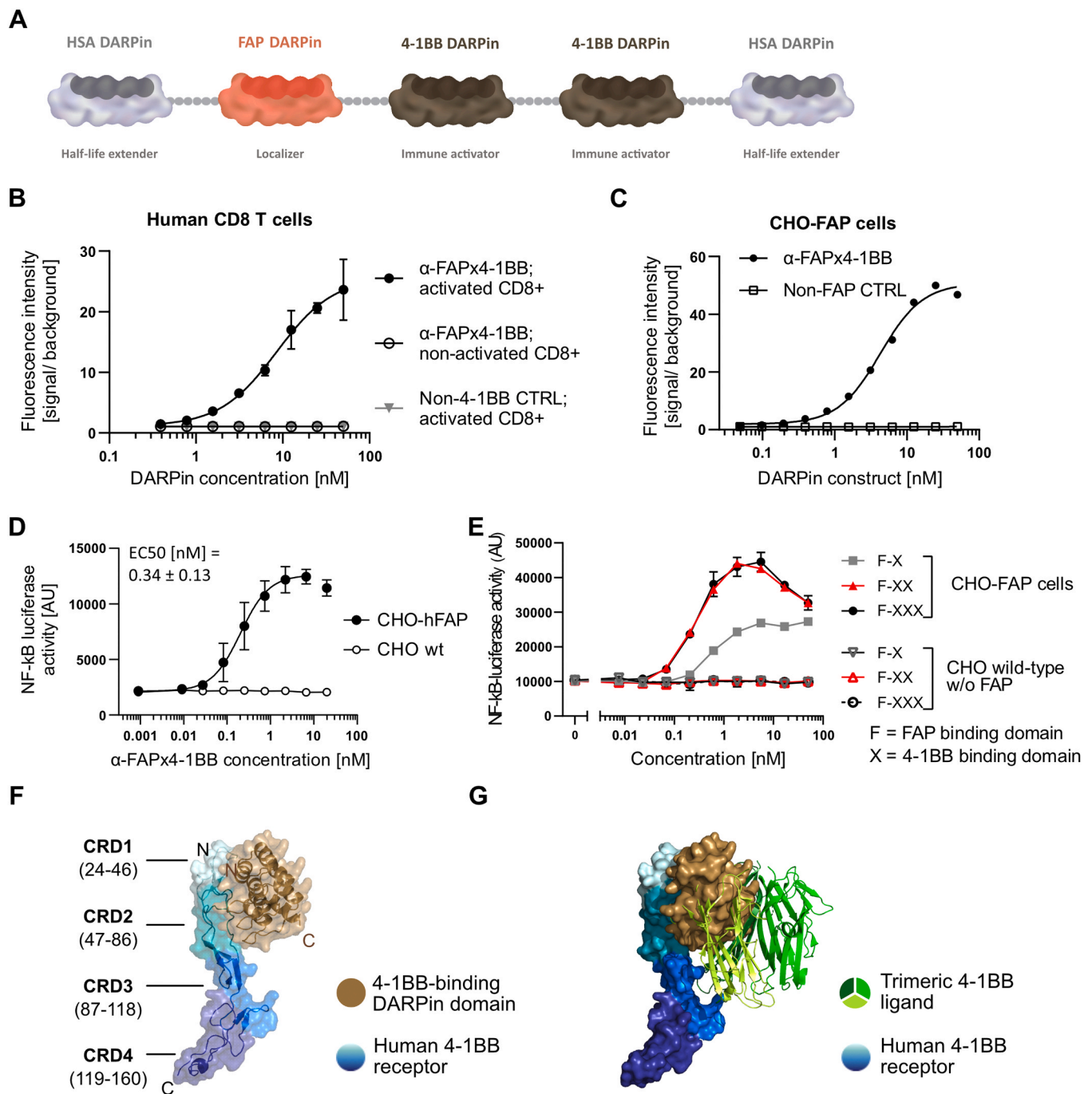
Fig. 1. Design of α-FAPx4–1BB (MP0310), a bi-specific DARPin 4–1BB agonist for tumor-localized immune activation. (A) Schematic representation of the α-FAPx4–1BB DARPin drug candidate consisting of an anti-human FAP, two identical human 4–1BB-binding domains as well as two flanking HSA-binding domains. (B) Binding of α-FAPx4–1BB or control DARPin to 4–1BB on anti-CD3 activated primary human CD8$^+$ T cells was assessed by flow cytometry. Non-activated T cells were used as an additional control. Data are shown as fluorescence signal (95th percentile) over background (n = technical duplicates). (C) Binding of α-FAPx4–1BB to human FAP was assessed by flow cytometry using huFAP-transduced CHO-K1 cells. Data are shown as fluorescence signal over background ratio of the median fluorescence intensity of the Alexa Fluor 647 labelled anti-penta-His detection antibody (n= technical duplicates). (D, E) HT1080 hu4–1BB-NFκB-luc reporter cells were co-cultured in the presence of huFAP-expressing CHO-K1 cells as well as FAP-negative wildtype CHO-K1 cells and α-FAPx4–1BB (D) as well as variants of α-FAPx4–1BB containing 1, 2, or three 4–1BB binding domains (E). Luciferase activity after 20 hours shown as arbitrary units (AU) of released light (n = technical duplicates). EC50 values are indicated as mean (SD). (F) Crystal structure of the 4–1BB-binding domain of α-FAPx4–1BB (sand) binding to the CRD-1 and CRD-2 of the extracellular domain of the human 4–1BB receptor (different colors of blue). (G) 4–1BB-binding domain of α-FAPx4–1BB competes with trimeric 4–1BB ligand (4–1BBL, shown in green) for binding to 4–1BB. Trimeric 4–1BBL from PDB ID 6mgp [32] was added by superimposing on structural well conserved 4–1BB receptor residues 1–105.

domains were compared for their functional activity using the 4–1BB *in vitro* reporter assay in the presence of FAP-expressing cells (Fig. 1E). Two α-4–1BB domains (α-FXX), compared to one (α-FX), increased the biological activity *in vitro;* inclusion of a third domain (α-FXXX) did not further improve the activity. The format with two 4–1BB DARPin domains (α-FXX) was therefore selected to generate α-FAPx4–1BB. In all cases, the 4–1BB agonistic activity was completely dependent on clustering by binding to FAP since no signal was observed in the absence of FAP-positive cells.

Structure analyses using X-ray crystallography revealed that the 4–1BB domain of α-FAPx4–1BB binds to cysteine rich domain (CRD) 1 (aa 24–46) and CRD-2 (aa 47–86) of the 4–1BB receptor (Fig. 1F), overlapping with the ligand binding site (Fig. 1G) by superimposing the 4–1BB/4–1BBL complex (PDB ID 6mgp) [32]. Thus, in contrast to the 4–1BB agonist urelumab, which binds the 4–1BB CRD1 away from the ligand binding site, 4–1BB binding DARPin domains compete with 4–1BBL for binding to 4–1BB. This is in line with ELISA competition experiments (data not shown). Accordingly, an α-FAPx4–1BB molecule including two binding domains to 4–1BB, is not expected to cluster 4–1BB/4–1BBL hexamers. This is in line with the absence of functional activity of α-FAPx4–1BB without presence of FAP-positive cells in the NF-κB-activation assay. Together, these data indicate that α-FAPx4–1BB is a 4–1BB agonist with strictly FAP-dependent tumor-targeted activity.

3.2. α-FAPx4–1BB drug candidate in MM settings: therapeutic mAbs daratumumab and elotuzumab induce 4–1BB expression on NK cells

We demonstrated that FBs from MM patients have high expression of FAP [12]. Accordingly, based on the FAP-dependent activity of α-FAPx4–1BB, we aimed to verify the potential use of α-FAPx4–1BB as a therapeutic candidate for the treatment of MM patients. Flow cytometry analysis confirmed that FBs from MM patients at different clinical stages (i.e. 1st diagnosis and relapse) strongly expressed FAP (Fig. S1), suggesting that α-FAPx4–1BB may be able to engage FAP$^+$ FBs through 4–1BB binding.

As 4–1BB is a costimulatory antigen expressed on activated T and NK cells [18,19], we investigated its expression on NK cells (Fig. 2A,B) and T cells (Fig. S2A) from MM patients by flow cytometry and. As illustrated in Fig. 2B, NK cells showed a very low or absent expression of 4–1BB (range 0–1.3 %), indicating that BM NK cells were not activated and thus unable to kill tumor cells. Similar results were obtained by analyzing 4–1BB expression on CD8$^+$ T cells (Fig. S2B).

Based on literature data showing the ability of mAbs to stimulate NK cells [33], we wondered whether the *in vitro* treatment of NK cells with daratumumab and elotuzumab may induce 4–1BB expression. BM lymphocytes were incubated with daratumumab or elotuzumab for 2 hours, and then analyzed by flow cytometry. Analysis showed that *in vitro* treatment significantly increased the percentage of 4–1BB$^+$ NK cells compared to untreated cells (Fig. 2C), implying that both mAbs activate *in vitro* NK cells. No effect was observed on CD8$^+$ T cells (Fig. S2C).

To verify whether mAbs activate NK cells *in vivo*, 4–1BB expression was investigated on BM NK cells from MM patients treated with mAb-based regimens or with the proteasome inhibitor, carfilzomib. As shown in Fig. 2D, treatment of MM patients with daratumumab or elotuzumab significantly induced 4–1BB expression on BM NK cells compared to carfilzomib, corroborating the ability of these mAbs to stimulate NK cells.

### 3.3. α-FAPx4-1BB induces adhesion of daratumumab-treated NK cells on FBs

Since α-FAPx4–1BB simultaneously binds 4–1BB and FAP, we investigated whether it may affect the adhesion of mAb-stimulated NK cells to FBs. To this purpose, daratumumab-treated/untreated NK cells, labelled with Calcein AM, were seeded on FB-coated wells in the presence of increasing doses (0–5 nM) of α-FAPx4–1BB. As illustrated in
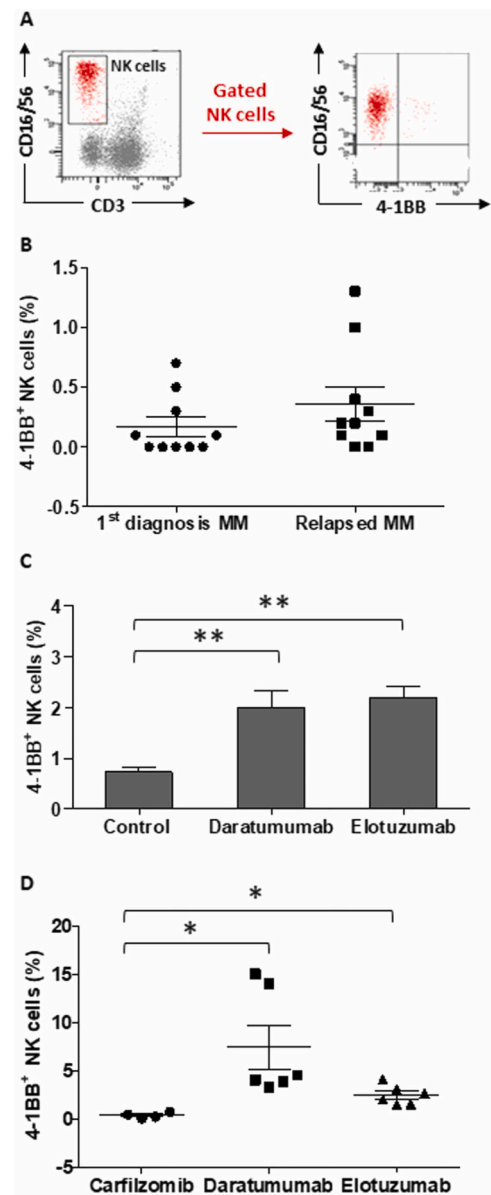
Fig. 2. Expression of 4–1BB on BM NK cells. (A) Representative dot plot analysis of 4–1BB expression on gated CD3$^-$CD16$^+$CD56$^+$ NK cells in BMMCs. (B) Flow cytometry analysis of 4–1BB expression on gated BM NK cells from patients with 1st diagnosis (n=10) or relapsed (n=10) MM. (C) 4–1BB expression on BM NK cells (n=10) co-cultured with MM cells and untreated (control) or treated with daratumumab (1 µg/ml) or elotuzumab (10 µg/ml) for 2 hours. Data are expressed as mean ± S.D. (D) 4–1BB expression on gated BM NK cells from MM patients treated with carfilzomib (n=4), daratumumab (n=6) or elotuzumab (n=6). Data are expressed as mean ± S.D. Note that mAbs treatment increased 4–1BB expression on NK cells *in vitro* and *in vivo*.* $p < 0.05$, ** $p < 0.005$.

Fig. 3A, α-FAPx4–1BB (0.5 nM) significantly increased the adhesion of daratumumab-treated NK cells to MM FBs (Fig. 3A) by up to 80 %. Higher doses of α-FAPx4–1BB (1 nM and 5 nM) lowered NK cell adhesion suggesting a bell-shaped activity of α-FAPx4–1BB. In contrast, no effect was observed in daratumumab-untreated NK cells (Fig. 3B), implying that daratumumab-dependent 4–1BB upregulation is required for α-FAPx4–1BB engagement. Similar results were obtained using elotuzumab-treated NK cells (Fig. S3).

Overall, these data suggest that α-FAPx4–1BB acts as a bridge between activated NK cells and MM FBs, fostering the adhesion of mAb-treated NK cells on MM FBs.
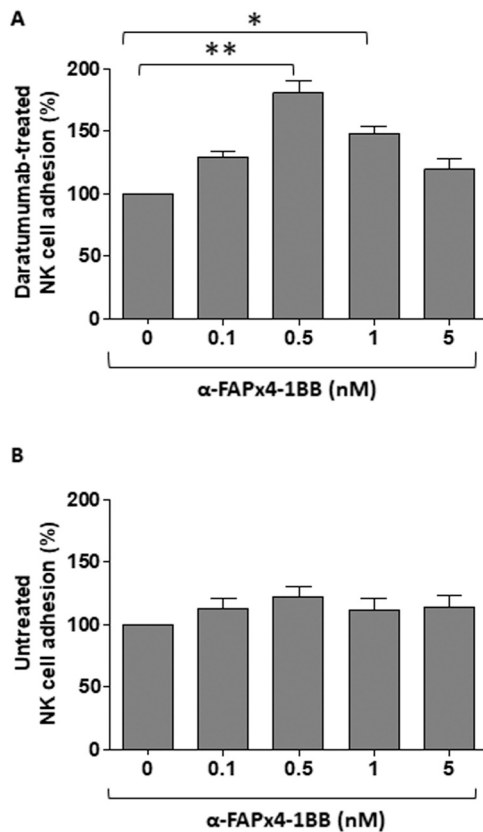
Fig. 3. Adhesion of BM NK cells on MM FBs. NK cells (n=5) were treated with daratumumab (1 µg/ml) for 2 hours (A) or untreated (B), labelled with Calcein AM, seeded on FB-coated plates and treated with increasing doses (0–5 nM) of the α-FAPx4–1BB molecule. Note that α-FAPx4–1BB increases the adhesion of daratumumab pre-treated NK cells on MM FBs. Data are expressed as mean ± S. D and normalized *vs* untreated cells (α-FAPx4–1BB [0 nM]). * $p < 0.05$, ** $p < 0.005$.

3.4. In vitro effect of α-FAPx4-1BB on NK cell activity

As the concurrent engagement of both FAP and 4–1BB results in 4–1BB clustering and activation (Fig. 1C,D), we evaluated the effect of α-FAPx4–1BB on NK cell activity by analyzing the expression of CD107a, a cell degranulation marker, and perforin, a pore forming cytolytic protein, on daratumumab-treated NK cells co-cultured with MM cells and FBs. Daratumumab *plus* α-FAPx4–1BB co-treatment triggered a higher expression of CD107a (Fig. 4A) and perforin (Fig. 4B) compared to control NK cells and to daratumumab-treated NK cells, suggesting the ability of α-FAPx4–1BB to improve daratumumab-mediated anti-myeloma effect.

Finally, we investigated the effect of α-FAPx4–1BB on ADCC by using a Calcein AM assay. In order to promote NK cell activity, the ADCC assay is usually performed in 96-round bottom well plates [34]. By contrast, we performed the ADCC assay using flat bottom 12-well plates to better mimic the BM milieu and to hamper MM cells and NK cell engagement. Furthermore, experiments were performed using either the CD38-positive MM1R cell line or the CD38-negative U266 cell line as target cells.

As shown in Fig. 4C, analysis of Calcein AM release, as an index of MM1R cell lysis, showed that the anti-myeloma effect of daratumumab was reduced in the presence of MM FBs because of their immunosuppressive effect. In contrast, the simultaneous treatment of daratumumab and α-FAPx4–1BB improved the MM cell killing in the presence of FBs, overcoming their immunosuppressive activity. Noteworthy, no effect was observed in the absence of FBs (Fig. 4C) proving that the
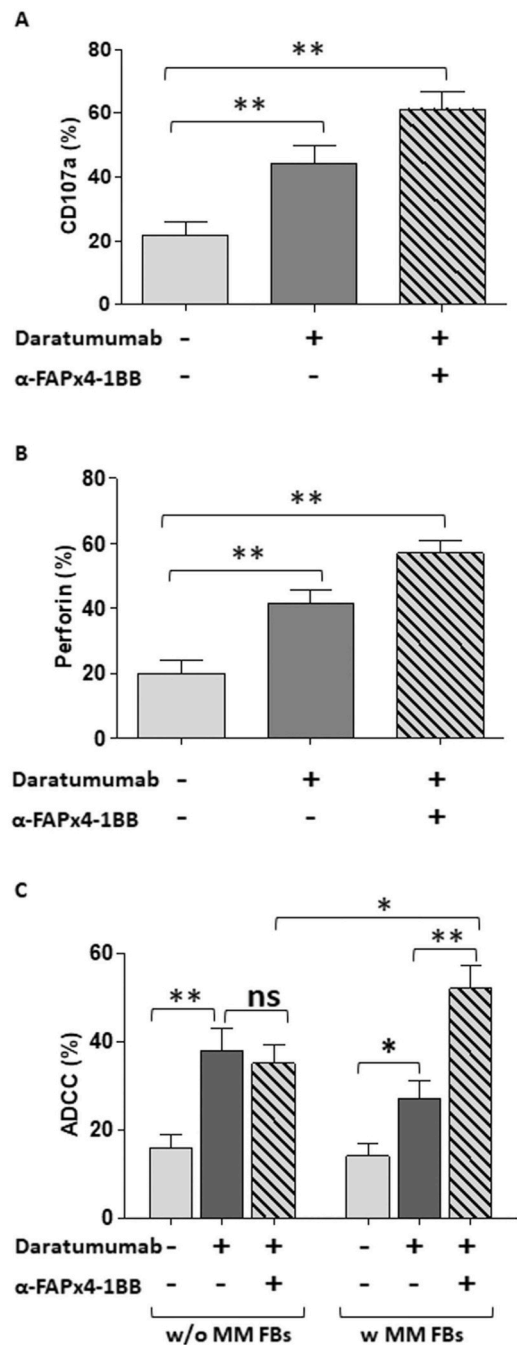
Fig. 4. *In vitro* effect of α-FAPx4–1BB on daratumumab-activated NK cells. (A-B) BMLs and MM cells were co-cultured in 12-well plates coated with MM FBs with/without daratumumab (1 µg/ml) and with/without α-FAPx4–1BB (0.5 nM) for 16 hours. Bar graphs show the percentage of CD107a (A) and perforin (B) expression on NK cells (n=6). (C) Analysis of daratumumab-mediated ADCC against the CD38-positive cell line (n=6). BMLs and MM cells were co-cultured in 12-well plates coated with/without MM FBs, with/without daratumumab (1 µg/ml) and with/without α-FAPx4–1BB (0.5 nM) for 4 hours. *In vitro* effect of α-FAPx4–1BB on daratumumab-mediated ADCC against the CD38-positive MM1R MM cells. Data are expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.005$.

simultaneous engagement of both FAP and 4–1BB is required for α-FAPx4–1BB activity.

The ADCC assay performed in the same experimental conditions using the CD38-negative U266 MM cell line as target control, displayed no significant increase in cell lysis (Fig. S4).

4. Discussion

Activation of BM immune microenvironment has been identified as a key factor in reducing MRD, hence, potentially improving overall survival of MM patients [35]. Some novel anti-myeloma agents including IMiDs, mAbs and proteasome inhibitors stimulate the immune system, triggering the anti-tumor response through the activation of T and NK cells and the suppression of Tregs [36]. Bispecific antibodies have also gained great interest for their ability to induce the re-direction, engagement and activation of effector cells. To date, several bispecific mAbs (targeting BCMA/CD3, CD38/CD3, GPRC5D/CD3 antigens) are undergoing preclinical and clinical investigation for MM treatment [37, 38].

Herein, we investigated the effects of a bi-specific α-FAPx4–1BB DARPin (MP0310) that was developed to simultaneously bind 4–1BB and FAP expressed on activated T and NK cells and on FBs, respectively. *In vitro* studies showed that the 4–1BB agonistic activity was strictly dependent on the FAP engagement, advocating a tumor-localized activity that may overcome the peripheral toxicities of 4–1BB agonistic molecules. Moreover, the absence of the Fc domain prevents the systemic 4–1BB activation, thereby avoiding liver inflammation that may occur with conventional bi-specific mAbs [39].

In the BM milieu, MM cells adhere to FAP$^+$ FBs that sustain MM cell survival, immune evasion and MRD [12,13]. Hence, we supposed that α-FAPx4–1BB could drive NK cells closer to MM FBs, enhancing the recognition and killing of MM cells resident in the BM niche. Nevertheless, NK and T cells from MM patients do not express 4–1BB, indicating their poor activation and inability to recognize tumor plasma cells. These results were in line with literature data showing that a permissive immune milieu contributes to immunological escape and disease progression [40,41].

Recently, mAb-based therapies have gained increasing traction in cancer immunotherapy for their ability to eliminate tumor cells through the activation of the immune system [15]. For instance, the interaction of Fc-receptor (CD16) with mAb-targeted tumor cells induced 4–1BB expression on NK cells [33,42]. Accordingly, treatment with daratumumab or elotuzumab significantly upregulated 4–1BB expression in MM patients following mAb-based regimens as well as in NK cell:MM cell co-cultures. The inducible 4–1BB upregulation involves 4–1BB synthesis triggered by mAbs treatment after Fc/Fc-Receptors interactions [data not shown; 42]. These results are in line with literature data showing that stimulation of cytolitic and/or T cells with PMA, ionomycin or viruses induces 4–1BB mRNA transcription and protein synthesis as consequence of protein kinase C activation and of intracellular Ca^{2+} increase [43,44].

Because of the mAb-induced 4–1BB overexpression, α-FAPx4–1BB may act as a bridge between FBs and NK cells through the simultaneous engagement of 4–1BB and FAP. Indeed, α-FAPx4–1BB increased the adhesion of daratumumab- and elotuzumab-treated NK cells on MM FBs. As consequence of 4–1BB and FAP engagement, α-FAPx4–1BB enhances MM cell killing *via* NK cell degranulation and daratumumab-mediated ADCC. Dose-response analysis revealed a bell-shaped activity of α-FAPx4–1BB with an optimum working concentration at 0.5 nM that decreased at higher doses. Several bi-specific antibodies have a similar response due to target saturation that leads to inefficient cross-linking and a decrease of cell cytotoxicity [45,46]. Noteworthy, ADCC was performed in unusual experimental conditions [34] (i.e. increased cell culture media) to mimic the BM milieu and disadvantage effector and target cell recognition. These data support our hypothesis that α-FAPx4–1BB may be a drug candidate able to drive NK cells closer to tumor cells.

MM FBs have an immunosuppressive role due to the release of cytokines and growth factors, including TGF-β, CCL-2, IL-6, IL-4, and IL-8 that promote M2 polarization, and other chemokines (e.g. CXCL-9, −10, −12) that increase peripheral Th2, Th17 and regulatory T-cells [20,41, 47]. All these features contribute to the creation of a permissive niche further sustaining the immunological and therapy escape, ultimately leading to daratumumab resistance [41]. Accordingly, we observed a reduction of the anti-myeloma activity of daratumumab in the presence of MM FBs. Interestingly, α-FAPx4–1BB improved the NK-mediated ADCC despite the presence of FBs, overcoming their protective and immunosuppressive effect. The analysis of cell apoptosis showed that α-FAPx4–1BB did not have any effect on MM FBs (data not shown), implying that other factors including modulation of cytokine release, extracellular vesicles, and activation of intracellular pathways may lead to the enhanced cytotoxic activity of NK cells.

The ability of α-FAPx4–1BB to potentiate NK cell-mediated immune response is of great interest. Several studies have documented low levels of circulating NK cells characterized by a reduced anti-tumor activity that negatively correlates to poor prognosis [48–50]. Keruakous *et al.* demonstrated that NK cell recovery after autologous stem cell transplantation (ASCT) was associated with MRD negativity highlighting the important role of NK cells in the MM patients' outcome [51]. For these reasons, increasing efforts are currently investigating the improvement of NK cell-mediated immunotherapies, including NK cell activators, or the infusion of chimeric antigen receptor (CAR)-engineered NK cells, of *in vitro* expanded NK cells (adoptive NK cells) or of induced pluripotent stem cell (iPSC)-derived NK cells [52].

Overall, we demonstrate that the α-FAPx4–1BB DARPin may represent a valuable strategy to potentiate NK cell activity, thereby further improving the efficacy of mAbs used in clinical practice for the treatment of MM. Additionally, α-FAPx4–1BB should be able to enhance the recruitment of residual CD38$^{low/-}$ NK cells from daratumumab treated-MM patients in the BM fostering target cell engagement and NK cell cytotoxicity [53,54]. The possibility to increase the anti-myeloma activity of daratumumab by using the anti-4–1BB agonist mAb, urelumab, has been already investigated by Ochoa and colleagues [55]. They demonstrated that urelumab enhanced NK cell activation *in vitro* and delayed tumor growth *in vivo*, prolonging mice overall survival [55]. Nevertheless, urelumab has shown dose limiting toxicities (e.g. peripheral inflammation and hepatotoxicity) due to the IgG Fc-domain. These side effects have led to the development of alternative anti-4–1BB mAbs with increased tolerability (e.g. deglycosilated mAbs or the use of mAbs belonging to other IgG subclasses) [56].

In conclusion, the results of our preclinical study indicate that the α-FAPx4–1BB DARPin may improve the immune response in NK cell-defective MM patients treated with standard mAbs, promoting the recruitment of BM and peripheral NK cells towards MM FBs that conceal MM cells, fueling MRD. As MRD negativity is currently the main goal of anti-tumor therapies, α-FAPx4–1BB, acting as a bridge between tumor cells and FBs, may represent a novel approach to promote mAb-induced killing of latent tumor cells in the BM niche, eradicating minimal residual MM cells.

CRediT authorship contribution statement

Ilaria Saltarella: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Alexander Link:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Aurelia Lamanuzzi:** Methodology, Investigation, Data curation. **Christian Reichen:** Methodology, Investigation, Data curation. **Joanna Robinson:** Methodology, Investigation, Data curation. **Concetta Altamura:** Writing – review & editing. **Assunta Melaccio:** Writing – review & editing. **Antonio Giovanni Solimando:** Writing – review & editing. **Roberto Ria:** Writing – review & editing. **Maria Addolorata Mariggiò:** Writing – review & editing. **Angelo Vacca:** Writing – review & editing, Conceptualization. **Maria Antonia Frassanito:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Jean-Francois Desaphy:** Writing – review & editing.

Declaration of Competing Interest

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116877.

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