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Original Article

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A novel dual-cytokine–antibody fusion protein for the treatment of CD38-positive malignancies

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Abstract

A novel dual-cytokine–antibody fusion protein, consisting of an antibody directed against CD38 [a tumor-associated antigen mainly expressed on the surface of multiple myeloma (MM) cells], simultaneously fused to both tumor necrosis factor ligand superfamily member 10 (TRAIL) and interleukin-2 (IL2), was designed, expressed and purified to homogeneity. The novel fusion protein, termed IL2-αCD38-αCD38-scTRAIL, was able to selectively recognize its cognate antigen expressed on the surface of MM and lymphoma cell lines, as evidenced by flow cytometry analysis. Moreover, the targeted version of TRAIL was able to induce cancer cell death *in vitro*, both with MM cell lines and with fresh isolates from the bone marrow of MM patients. The experiments provide a rationale for possible future applications of IL2-αCD38-αCD38-scTRAIL for the treatment of patients with MM or other CD38-positive malignancies.

Key words: CD38, IL2, immunocytokines, multiple myeloma, TRAIL

Introduction

Hematological malignancies are cancer disorders of the blood and lymphatic organs, which can be subdivided into myeloid and lymphoid neoplasm (Gozzetti *et al.*, 2014; Smith *et al.*, 2015; Arber *et al.*, 2016; Swerdlow *et al.*, 2016). Multiple myeloma (MM) is a hematological malignancy derived from the B cell lineage, characterized by a monoclonal proliferation and accumulation of malignant plasma cells in the bone marrow. The disease typically presents with hypercalcaemia, renal insufficiency, anemia and bone lesions and may ultimately lead to death as a result of infections, bleeding, fracture complications, kidney failure or blood clots in the lung (Kyle *et al.*, 2003; Matsui *et al.*, 2004; Hideshima *et al.*, 2007; Gozzetti *et al.*, 2014; Smith *et al.*, 2015; Mai *et al.*, 2018). MM represents about 10% of hematological malignancies and, in most cases, is still an incurable disease (Sant *et al.*, 2010; Braggio *et al.*, 2015).

Conventional anticancer therapy for the treatment of MM relies on chemotherapy. In recent years, the use of drugs targeting proteasome function or acting as immunomodulatory agents (e.g. bortezomib, carfilzomib, lenalidomide and pomalidomide alone or in combination) have gained importance (Kortuem and Stewart, 2013; Murray *et al.*, 2014; Scott, 2014; Kritharis *et al.*, 2015). MM cells are more sensitive to proteasome inhibition than non-transformed lymphocytes, due to an increased cellular stress related to high-level protein production. However, usually only a small portion of patients benefit from pharmacological treatment (Gozzetti *et al.*, 2014; Braggio *et al.*, 2015; Chim *et al.*, 2017). High-dose chemotherapy with Melphalan combined with autologous stem cell transplantation represents a treatment option for eligible patients (fit and usually not older than 70 years) (Costa *et al.*, 2017). This approach resulted in prolonged survival rates, with a small proportion of patients reaching long-term remissions lasting for

many years (Gozzetti *et al.*, 2014; Chim *et al.*, 2017). However, elderly patients, which represent the majority of MM patients, are not eligible for this treatment option because of associated morbidities and for this reason, alternative therapeutic strategies are urgently required.

Immune checkpoint inhibitors are revolutionizing the field of cancer therapy and have been considered also for the treatment of hematological diseases (Sharma and Allison, 2015). In the clinic, pembrolizumab (an anti-PD-1 antibody) has been used in combination with proteasome inhibitors and immunomodulatory drugs in patients with relapsed/refractory MM, showing encouraging results (San Miguel *et al.*, 2015; Badros *et al.*, 2015). However, relevant toxicities were observed, which included neutropenia, lymphopenia, thrombocytopenia and other non-hematologic side effects.

Targeted cancer immunotherapy with monoclonal antibodies represents an attractive biomedical strategy, particularly in the field of hematological malignancies. The efficacy of therapeutic antibodies relies on the ability of the product to (i) selectively bind to their cognate antigen, (ii) induce antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity or (iii) block signaling by binding to certain receptors/growth factors. For example, treatment with rituximab (an anti-CD20 antibody) was found to be efficacious in patients with various types of B cell-derived malignancies (Subramanian *et al.*, 2017). However, B cells lose CD20 expression upon differentiation into plasma cells (de Weers *et al.*, 2011), therefore limiting the use and the efficacy of rituximab in patients with MM (Zojer *et al.*, 2006).

Recently, more specific cell surface proteins (which are preferentially expressed on MM cells) have been considered as targets for the development of therapeutic antibodies. Two membrane proteins, SLAMF7 and CD38, have been extensively investigated and validated for the generation of therapeutic antibodies against MM. Indeed, two antibody products (elotuzumab and daratumumab, directed against SLAMF7 and CD38, respectively) (de Weers *et al.*, 2011; van de Donk *et al.*, 2016) have been investigated in clinical trials for the treatment of MM (alone or in combination with chemotherapy) (Lokhorst *et al.*, 2015; Palumbo *et al.*, 2016; van de Donk *et al.*, 2016) and have received marketing authorization. Moreover, daratumumab has shown encouraging *in vitro* antitumor activities in patient-derived chronic lymphocytic leukemia samples (Matas-Cespedes *et al.*, 2017), therefore expanding the potential application of this product to other CD38-positive malignancies.

The combination of daratumumab with (i) lenalidomide and dexamethasone, (ii) bortezomib and dexamethasone or (iii) bortezomib, melphalan and prednisone showed marked increase in progression free survival and overall response rates compared to lenalidomid dexamethasone, bortezomib dexamethasone or bortezomib, melphalan and prednisone alone (Dimopoulos et al., 2016; Palumbo *et al.*, 2016; Mateos *et al.*, 2017). These results show the potential of CD38 antibodies. However, the efficacy might be further increased.

Antibody–cytokine fusion proteins ('immunocytokines') may represent an alternative to conventional immunological treatments. Interleukin-2 (IL2)-based immunocytokines, in combination with rituximab, were found to induce complete responses in rodent models of hematological diseases (Schliemann *et al.*, 2009), providing a rationale for the development of novel antibody–cytokine fusions for the treatment of MM. Our group described that the simultaneous delivery of two cytokine payloads (IL2 and TNF) to neoplastic lesions was able to induce complete responses in patients with stage IIIB/C melanoma (Danielli *et al.*, 2015). More recently, we have also described a novel class of biopharmaceutical products, named 'potency-matched dual-cytokine–antibody fusions', in which two cytokine payloads of comparable potency are fused with a tumor-homing antibody moiety (De Luca *et al.*, 2017). This novel class of biopharmaceutical products is able to induce complete responses in several immunocompetent mouse models of cancer.

Members of the TNF superfamily (including TNF, FasL, Light and TRAIL) can induce apoptosis of malignant cells by interacting with cognate cell surface receptors (Hemmerle *et al.*, 2014; Fellermeier *et al.*, 2016). However, only a modest anticancer activity has been observed so far (both *in vitro* with MM cells and *in vivo* in xenograft models) when using recombinant TRAIL as therapeutic agent (Mitsiades *et al.*, 2001). It has recently become apparent that the unstable non-covalent homotrimeric structure of TRAIL may limit pharmaceutical applications, as a result of suboptimal pharmacokinetic (Hemmerle *et al.*, 2014) and pharmacodynamic properties. For this reason, the group of Roland Kontermann engineered TRAIL mutants, connecting three TRAIL monomeric units into a single polypeptide (Siegemund *et al.*, 2016). These novel proteins showed improved thermal stability and potent anticancer activity, thus providing the basis for the development of novel tumor-homing antibody–TRAIL fusions. In addition, Apogenix and AbbVie are developing hexameric TRAIL derivatives, consisting of single-chain trimeric TRAIL units fused to a human Fc fragment, serving as homodimerization and serum half-life extension moiety (Morgan-Lappe, 2017).

In this article, we describe the generation, the characterization and the *in vitro* anticancer properties of a novel 'dual-cytokine–antibody fusion protein' based on an anti-CD38 antibody (de Weers *et al.*, 2011) fragment simultaneously fused to IL2 and to TRAIL (Siegemund *et al.*, 2016). The resulting product, termed IL2-αCD38-αCD38-scTRAIL, was able to selectively bind to MM and lymphoma cell lines *in vitro*, inducing a selective cancer cell death and providing a rationale for future therapeutic applications.

Materials and methods

Cell lines and patient samples

CHO cells, RAMOS lymphoma cells and RPMI8226 myeloma cells were obtained from the American Type Culture Collection (ATTC) between 2015 and 2017, expanded and stored as cryopreserved aliquots in liquid nitrogen. Cells were grown according to the supplier's protocol and kept in culture for no longer than 14 passages. Authentication of the cell lines also including check of post-freeze viability, growth properties and morphology, test for mycoplasma contamination, isoenzyme assay and sterility test were performed by the cell bank before shipment. Clinical bone marrow biopsies were obtained from pretreated MM patients upon written informed consent. Ethical approval was granted by the Zurich cantonal ethics committee. Mononuclear cells were isolated by density centrifugation.

Cloning, expression and protein characterization

The fusion protein IL2-αCD38-αCD38-scTRAIL contains anti-CD38 antibody-variable regions (de Weers *et al.*, 2011), in homodimeric tandem ScFv arrangement (Huston *et al.*, 1988; Pasche *et al.*, 2012), fused to human IL2 (Carnemolla *et al.*, 2002) at the N-terminus by a 12-amino acid linker and to human TRAIL in a single-chain format (scTRAIL) at the C-terminus, as described before (Siegemund *et al.*, 2016). The gene encoding for the anti-CD38 antibody, human IL2 and scTRAIL were polymerase chain reaction (PCR) amplified, PCR assembled and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) using a strategy similar to the one described before by our laboratory (Pasche *et al.*,

2011). A fusion protein in which the gene encoding for human IL2 is not present (αCD38-αCD38-scTRAIL) was cloned similarly. The fusion protein αCD38 in SIP format was cloned into the vector pcDNA3.1(+) as described before (Brack *et al.*, 2006).

The fusion proteins were expressed using transient gene expression in CHO cells as described previously (Pasche *et al.*, 2011; Rajendra *et al.*, 2011) and purified from the cell culture medium to homogeneity by protein L (GenScript) chromatography. Purified proteins were analyzed by size-exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare), SDS-PAGE and ESI-MS. The biological activity of TRAIL was determined on RAMOS cells. In 96-well plates, cells (25 000 per well) were incubated in medium supplemented with varying concentrations of the fusion proteins. After 24 h at 37°C, cell viability was determined with Cell Titer Aqueous One Solution (Promega). Results were expressed as the percentage of cell viability compared to untreated cells.

Flow cytometry

Antigen expression on RAMOS and RPMI8226 cells was confirmed by flow cytometry. Cells were centrifuged and washed in cold FACS buffer (0.5% BSA, 2 mM EDTA in PBS) and stained with IL2-αCD38-αCD38-scTRAIL (final concentration 10 μg/ml) and detected with rat anti-IL2 (eBioscience 14-7029-85) and anti-rat AlexaFluor

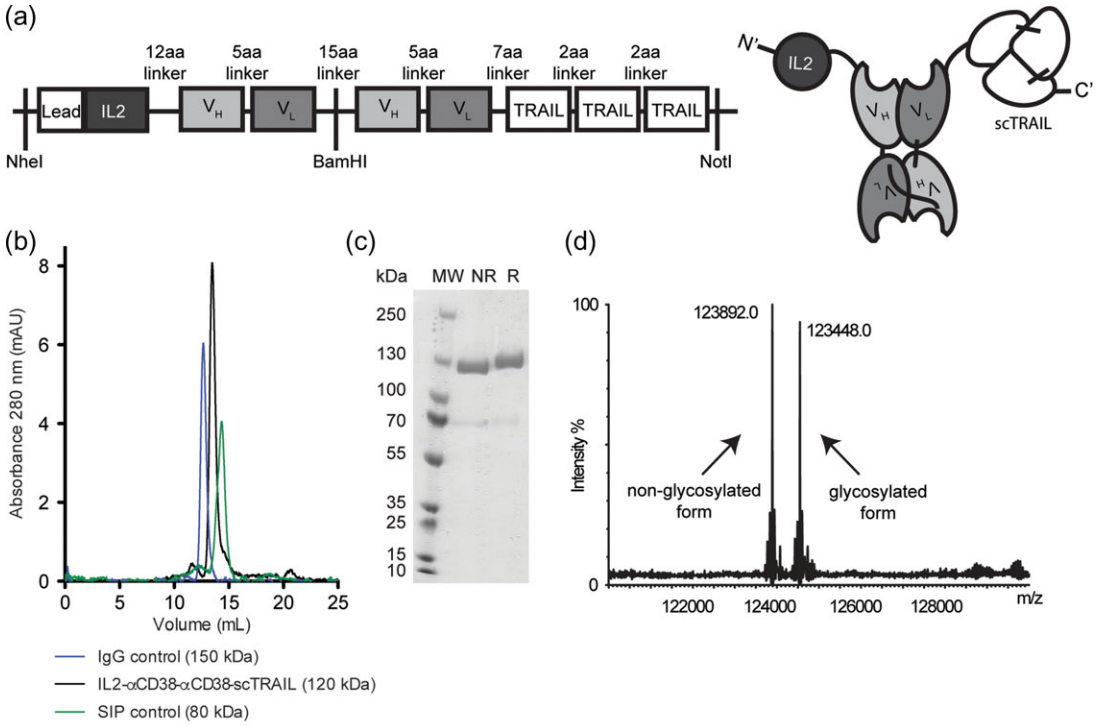

Fig. 1 Cloning, expression and characterization of IL2-αCD38-αCD38-scTRAIL. (**a**) Schematic representation of the cloning strategy and of the domain assembly. (**b**) Size-exclusion chromatography profile of IL2-αCD38-αCD38-scTRAIL (120 kDa, black line), IgG (150 kDa, blue line) and SIP (80 kDa, green line) were used as controls for the column's calibration (Gébleux *et al.* 2015). (**c**) SDS-PAGE analysis: MW, molecular weight; NR, nonreducing conditions; R, reducing conditions. (**d**) ESI-MS profile. (**e**) Amino acid sequence of IL2-αCD38-αCD38-scTRAIL. Starting from the N-terminus: human IL2, the αCD38 antibody in tandem diabody format and TRAIL in single-chain format.

488 (Invitrogen A21208). Omission of the primary antibody was used as a negative control.

The ability of IL2-αCD38-αCD38-scTRAIL to selectively kill MM was confirmed by flow cytometry on the RPMI8226 cell line. In 96-well plates, cells (25 000 per well) were incubated in medium supplemented with varying concentrations of the fusion protein. After 24 h at 37°C, cells were washed with FACS buffer and stained with anti-CD138-APC (Biolegend 352 308). Cell viability was determined with 7-AAD staining (Biolegend 420 403). All samples were analyzed on a 2-L Cytoflex Flow Cytometer (Beckman Coulter).

Isolated mononuclear cells from patients with MM were incubated with IL2-αCD38-αCD38-scTRAIL at varying concentrations in RPMI medium (supplemented with fetal bovine serum and antibiotics/antimitotic solution) and incubated 16 h at 37°C. Cells were washed with FACS buffer and stained with anti-CD138-APC (Biolegend 352 308). Cell viability was determined with 7-AAD staining (Biolegend 420 403). All samples were analyzed on a 2-L Cytoflex Flow Cytometer (Beckman Coulter).

Immunofluorescence studies

Antigen expression was confirmed on ice-cold acetone-fixed 8-µm cryostat sections of RAMOS stained with anti-CD38 (SIP) (final concentration 5 µg/ml) and detected with rabbit anti-IgE (Dako A0094) and anti-rabbit AlexaFluor 488 (Invitrogen A11008). For vascular staining, rat anti-CD31 (BD 553 370) and anti-rat AlexaFluor 594 (Invitrogen A21209) antibodies were used. Slides were mounted with fluorescent mounting medium and analyzed with Axioskop2 mot plus microscope (Zeiss).

Results

Production and characterization of an IL2/TRAIL-based dual-cytokine–antibody fusion protein

A fusion protein, consisting of an anti-CD38 antibody (de Weers *et al.*, 2011) in tandem diabody format (Pasche *et al.*, 2012) simultaneously fused by short peptide linkers to human IL2 and TRAIL (in single-chain format (Siegemund *et al.*, 2016)), was cloned for expression in mammalian cells (Fig. 1a). The resulting product, termed IL2-αCD38-αCD38-scTRAIL, was expressed in CHO cells and purified to homogeneity by affinity chromatography on a protein L resin. The fusion protein was stable and well behaved in conventional biochemical assays, as evidenced by gel filtration, SDS-PAGE and ESI-MS analysis (Fig. 1b–d). Fig. 1e reports the amino acid sequence of IL2-αCD38-αCD38-scTRAIL.

In vitro characterization on RAMOS cells

Binding of IL2-αCD38-αCD38-scTRAIL to its cognate antigen (CD38) was assessed by flow cytometry on RAMOS (CD38+) cells (Fig. 2a). A microscopic fluorescence analysis of RAMOS xenograft tumor sections, confirmed CD38 expression *in vivo* (Fig. 2b). An *in vitro*-based killing assay on RAMOS cells (Fig. 2c) confirmed the ability of TRAIL-based fusion proteins to selectively kill CD38+ lymphoma cells *in vitro* at ultra-low concentrations [IC_{50} ~ 1 pM for both IL2-αCD38-αCD38-scTRAIL and αCD38-αCD38-scTRAIL, a fusion protein produced with similar methodologies but devoid of the IL2 moiety]. In this assay, the IL2 moiety did not appear to contribute to cancer cell toxicity *in vitro*, but the payload may be important *in vivo*, contributing to a pro-inflammatory environment at the site of disease by direct activation of NK cells and T cells (Carnemolla *et al.*, 2002; Yang *et al.*, 2012).

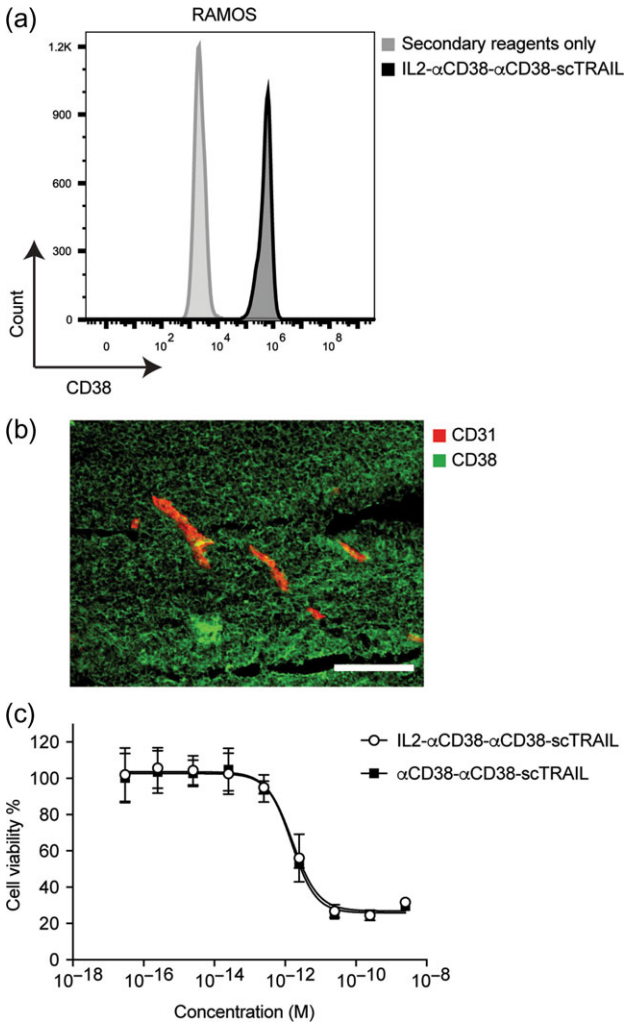

Fig. 2 *In vitro* characterization on RAMOS cells. (**a**) Flow cytometric evaluation of CD38 expression by RAMOS, detected with IL2-αCD38-αCD38-scTRAIL. (**b**) Microscopic fluorescence analysis of CD38 expression on RAMOS tumor section detected with αCD38 (SIP) (green for anti-human IgE, AlexaFluor 488) and anti-CD31 (red, AlexaFluor 594), 20× magnification, scale bar = 100 µm. (**c**) TRAIL bioactivity assay, based on the killing of RAMOS cell.

In vitro characterization on RPMI8226 cells and on patient-derived MM specimens

Binding of IL2-αCD38-αCD38-scTRAIL to a CD38+ MM cell line (RPMI8226) was confirmed by flow cytometry (Fig. 3a). The ability of the fusion protein to selectively kill MM cells (CD138+) *in vitro* was further confirmed by flow cytometry using the RPMI8226 cell line, with almost complete cell killing at 25 nM concentration of fusion protein and 24 h of incubation (Fig. 3b). Similarly, incubation of patient-derived MM cells with IL2-αCD38-αCD38-scTRAIL resulted in a selective killing of CD138+ cells (Fig. 3c).

Discussion

In this work, we have shown that the integration of IL2 and TRAIL (used as a single-chain polypeptide) into a novel antibody-

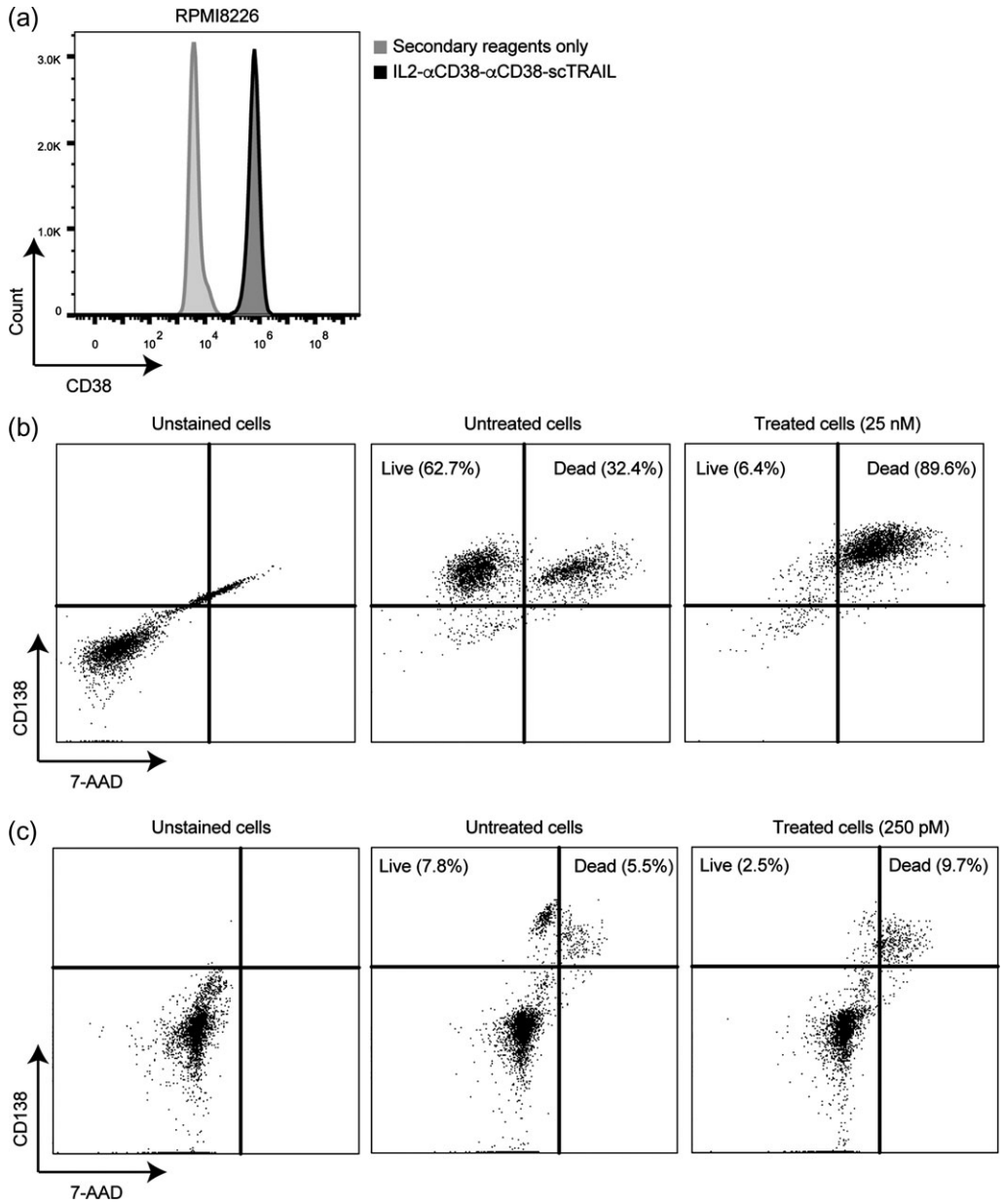

Fig. 3 Activity against MM cells. (**a**) Flow cytometric evaluation of the binding of IL2-αCD38-αCD38-scTRAIL to RPMI8226 cells, detected with an anti-IL2 reagent. (**b**) Selective killing of RPMI8226 cells 24 h after incubation with 25 nM IL2-αCD38-αCD38-scTRAIL. Dual-color flow cytometry analysis for CD138-APC and 7-AAD indicates that the fusion protein induced cell death (revealed by 7-AAD staining) in CD138-positive cells. Quadrants were set in order to differentiate CD138+ cells from unstained cells. (**c**) Selective killing of freshly isolated MM patient cells, upon 16 h of incubation with the fusion protein.

based fusion proteins specific to CD38 (a marker of MM) resulted in a novel product (IL2-αCD38-αCD38-scTRAIL) with excellent biochemical characteristics. The immunocytokine could be expressed in mammalian cells and purified to homogeneity, as revealed by SDS-PAGE, mass spectrometry and size-exclusion chromatography. The two payloads have biological activity at comparable molar concentrations and, for this reason, a tuning of the potency of the individual cytokines by mutagenesis was not required. The use of non-mutated immunostimulatory payloads may decrease the risk of immunogenicity for *in vivo* applications of the product.

The novel fusion protein retained the binding properties of the parental antibody (de Weers *et al.*, 2011), as evidenced by flow cytometry analysis on CD38-positive human cell lines. Moreover, the targeted delivery of TRAIL induced a selective cell death *in vitro* against both MM and lymphoma cell lines, as well as against bone marrow-derived MM isolates from patients. The incorporation of IL2 into the same product was not found to interfere with the activity of TRAIL *in vitro*. *In vivo*, the payload may help recruit and activate immune effector cells (e.g. T cells and NK cells), thus potentiating anticancer activity at the site of disease (Schliemann *et al.*, 2009; De Luca *et al.*, 2017).

The fully human nature of the novel IL2-αCD38-αCD38-scTRAIL fusion protein hinders a characterization of its pharmacodynamics properties in a fully syngeneic setting, since the αCD38 and the scTRAIL moieties do not cross-react with the cognate murine targets. A fully murine fusion protein, serving as a 'surrogate' for preclinical testing in immunocompetent mouse models of cancer may be needed in order to assess the therapeutic potential of the novel fusion protein. Alternatively, as previously reported for daratumumab, it may be possible to test *in vivo* activity in immunodeficient mice injected with human CD38-positive Daudi cells (de Weers *et al.*, 2011), prior to safety pharmacological assessment in non-human primates.

Conflict of interest

Dario Neri is co-founder, shareholder and member of the board of Philogen, a company working on antibody therapeutics. The authors declare no additional conflict of interest.

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