

RANKL Expression, Function, and Therapeutic Targeting in Multiple Myeloma and Chronic Lymphocytic Leukemia

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Abstract

Bone destruction is a prominent feature of multiple myeloma, but conflicting data exist on the expression and pathophysiological involvement of the bone remodeling ligand RANKL in this disease and the potential therapeutic benefits of its targeted inhibition. Here, we show that RANKL is expressed by primary multiple myeloma and chronic lymphocytic leukemia (CLL) cells, whereas release of soluble RANKL was observed exclusively with multiple myeloma cells and was strongly influenced by posttranscriptional/posttranslational regulation. Signaling via RANKL into multiple myeloma and CLL cells induced release of cytokines involved in disease pathophysiology. Both the effects of RANKL on osteoclastogenesis and cytokine production by malignant cells could be blocked by disruption of RANK–RANKL interaction with denosumab. As we aimed to combine neutralization of RANKL with induction of antibody-dependent cellular cytotoxicity of natural killer (NK) cells against RANKL-expressing malignant cells and as denosumab does not stimulate NK reactivity, we generated RANK-Fc fusion proteins with modified Fc moieties. The latter displayed similar capacity compared with denosumab to neutralize the effects of RANKL on osteoclastogenesis *in vitro*, but also potently stimulated NK cell reactivity against primary RANKL-expressing malignant B cells, which was dependent on their engineered affinity to CD16. Our findings introduce Fc-optimized RANK-Ig fusion proteins as attractive tools to neutralize the detrimental function of RANKL while at the same time potently stimulating NK cell antitumor immunity. *Cancer Res*; 73(2); 683–94. ©2012 AACR.

Introduction

RANK (TNFRSF11A), osteoprotegerin (OPG, TNFRSF11B), and their ligand (RANKL, TNFSF11) are key regulators of bone remodeling (1). RANKL may further influence progression of B-cell–derived malignancies such as chronic lymphocytic leukemia (CLL) or multiple myeloma (2, 3). In CLL, RANKL mediates release of IL-8, which contributes to disease pathophysiology (2). In multiple myeloma, the balance of RANKL and OPG is disrupted causing activation of osteoclasts and bone destruction, and RANKL neutralization delayed multiple myeloma progression in mice (3–6). Elevated levels of soluble RANKL

(sRANKL) in patients with multiple myeloma were shown to be associated with disease activity and prognosis (7), but the origin of the elevated RANKL levels is still unclear. Both RANKL release by multiple myeloma cells themselves and indirect effects of the malignant B cells on stromal cells causing an imbalance of the RANKL/OPG ratio in the bone marrow have been implicated (3, 8–10).

Recently, a monoclonal antibody capable of blocking RANKL (denosumab) was proven to be effective for the treatment of nonmalignant and malignant osteolysis (11, 12). In patients with multiple myeloma, denosumab reduced bone turnover (13), but did, in contrast to RANKL neutralization with RANK-Fc and OPG-Fc fusion proteins in mouse models, not significantly decrease disease burden (3–5). Notably, denosumab was developed to neutralize RANKL without inducing complement activation and antibody-dependent cellular cytotoxicity (ADCC; ref. 14). As malignant cells are thus not targeted for destruction by immune effector mechanisms, denosumab differs from "classical" antitumor antibodies such as rituximab, which meanwhile is an essential component of most treatment strategies for B-cell non-Hodgkin lymphoma (15). The therapeutic activity of this antibody is largely attributed to its capacity to trigger immune effector mechanisms such as ADCC (16). Multiple efforts are presently made to enhance the efficacy of this and other antitumor antibodies by increasing their affinity to the Fc receptor IIIa (CD16; ref. 17). Several Fc-engineered antilymphoma antibodies that mediate markedly enhanced ADCC are presently in preclinical and early clinical

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development, and it is hoped that their therapeutic activity is increased accordingly (18, 19). As multiple myeloma cells do not express CD20, the target antigen of rituximab and its successors, novel antibodies directed to multiple myeloma antigens are presently being developed, and recently an Fc-modified antibody that potently targets multiple myeloma cells for NK cell reactivity was reported (20, 21).

As (i) multiple myeloma and CLL cells may express RANKL (2, 8, 9), and (ii) neutralization of RANKL by fusion proteins containing immunostimulatory Fc parts delayed progression of multiple myeloma, but the clinically available denosumab does not induce antitumor immune effector mechanisms (3–5, 14), and (iii) techniques to increase the affinity of Fc parts to CD16 resulting in enhanced NK reactivity are meanwhile available (22), we here studied RANKL expression, release, and function in multiple myeloma and also CLL cells. After defining RANKL expression as frequent feature of these malignancies and gathering evidence for the involvement of RANKL in disease pathophysiology, we developed an Fc-engineered RANKL-Fc fusion protein that, beyond its ability to neutralize RANKL, effectively targets the malignant B cells for destruction by ADCC.

Materials and Methods

Patients

Peripheral blood mononuclear cells (PBMC) and bone marrow cells of patients and healthy donors were isolated by density gradient centrifugation after informed consent in accordance with the Helsinki protocol. The study was conducted according to the guidelines of the local ethics committee.

Transfectants and cell lines

The RANKL transfectants (L-RANKL) and parental controls (L cells) were previously described (23). RAW264.7 cells were from American Type Culture Collection (ATCC). Multiple myeloma cell lines were obtained internally or purchased from DSMZ or ATCC. Authenticity was determined by validating the immunophenotype described by the provider using fluorescence-activated cell sorting (FACS) every 6 months and specifically before use in experiments.

Antibodies and reagents

The monoclonal antibodies (mAb) against RANKL (MIH23 and MIH24) were previously described (23). Anti-RANK mAb (clone 80704) was from R&D Systems. Anti-mouse Ig-PE conjugate and phycoerythrin (PE)-conjugated streptavidin were from Jackson ImmunoResearch, anti-human IgG1-PE and anti-mouse IgM-HRP were from Southern Biotech. All other antibodies were from BD Biosciences. RANKL (rRANKL) and GITRL were from ImmunoTools GmbH. The IgG2 antibodies denosumab and panitumumab as isotype control were obtained from Amgen.

Production and purification of RANKL-Fc fusion proteins and isotype controls

SP2/0-Ag14 cells (ATCC) were transfected with vectors coding for the different RANKL-Fc fusion proteins or Fc parts as controls by electroporation. Protein was purified from culture supernatants by Protein A affinity chromatography (GE Health-

care). Purity was determined by SDS-PAGE and size exclusion chromatography using a Superdex 200 PC3.2/30 column (SMART System, GE Healthcare). Biotinylation was conducted using the Biotin conjugation kit from Innova Biosciences. Endotoxin levels were less than 1 EU/mL for all proteins.

Flow cytometry

FACS was conducted using specific mAb, RANKL-Fc fusion proteins, and isotype controls at 10 µg/mL followed by species-specific PE conjugates (1:100). Analysis was conducted using a FC500 (Beckman Coulter) or FACSCanto II (BD Biosciences). Where indicated, specific fluorescence indices (SFI) were calculated by dividing median fluorescences obtained with specific mAb by median fluorescences obtained with isotype control. To exclude potential artifacts due to unspecific antibody binding, a threshold for defining surface positivity was set at SFI ≥ 1.5.

PCR analysis

Reverse transcription (RT)-PCR was conducted as described previously (24). The following primers were used for Nested PCR of RANKL splice variants: membrane-bound RANKL (NM_003701): 5'-cgtcgccctgttcttatt-3' and 5'-tatgggaacacatgatgggatg-3' (step 1; 353 bp) and 5'-tcagaagatggcactcactg-3' and 5'-tgagatgagcaaaaggctga-3' (step 2; 268 bp); soluble RANKL (NM_033012): 5'-cttagaagccacaaagaattg-3' and 5'-tatgggaaccagatgggatg-3' (step 1; 347 bp) and 5'-tcagaagatggcactcactg-3' and 5'-tgagatgagcaaaaggctga-3' (step 2; 268 bp); 18S rRNA, 5'-cggctaccacatccaaggaa-3' and 5'-gctggaattaccggcgt-3' (186 bp).

Determination of soluble RANKL

RANKL levels in supernatants were analyzed by ELISA after 72 hours of culture. In brief, 96-well plates were coated with mAb MIH24 (2 µg/mL), blocked with 7.5% bovine serum albumin (BSA)-PBS, and washed. Afterwards, serial dilutions of rRANKL as standard and supernatants were added. After incubation, plates were washed and mAb MIH23 (2 µg/mL) in 3.75% BSA-PBS, followed by anti-mouse IgM-HRP (1:5,000 in 3.75% BSA-PBS), was added. Plates were developed using the TMB substrate system (KPL). Absorbance was measured at 450 nm. Sensitivity and specificity are shown in Supplementary Fig. S1.

Determination of cytokines

Levels of TNF, interleukin (IL)-6, and IL-8 in culture supernatants were determined by ELISA using OptEIA sets from BD Biosciences according to manufacturer's instructions.

Osteoclast differentiation assay

RANKL-induced osteoclastogenesis of RAW264.7 cells was determined by measuring tartrate-resistant acid phosphatase (TRAP) activity (25). A total of 1×10^4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing rRANKL (0.1 µg/mL). Medium was replaced at day 3. On day 6, cells were fixed and incubated with TRAP substrate solution [50 mmol/L sodium tartrate and 100 mmol/L sodium acetate (pH 5.0) supplemented with 2 mg/mL nitrophenol phosphate] for 30 minutes at 37°C before addition of 0.1 mol/L NaOH and absorbance was measured at 405 nm.

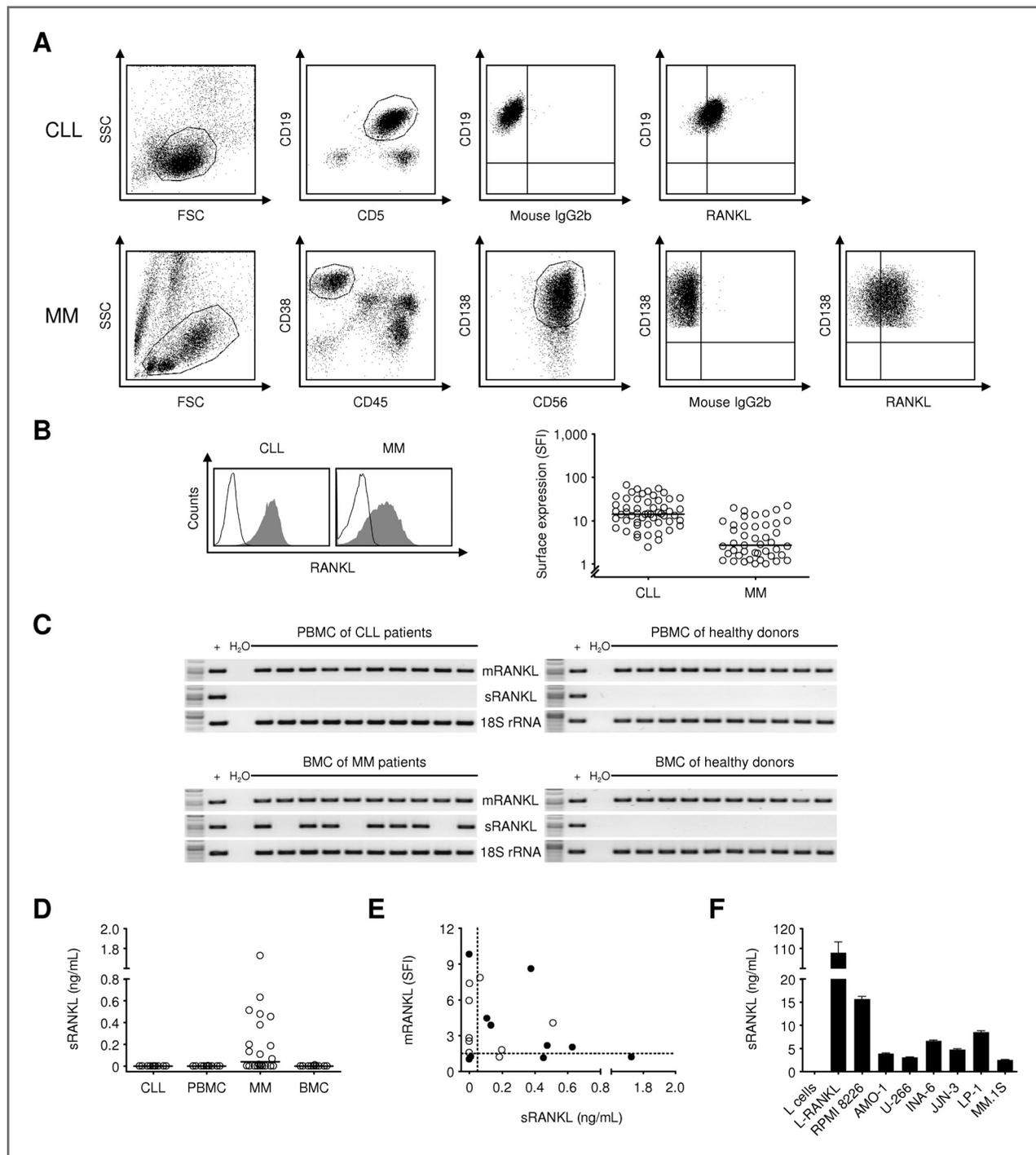


Figure 1. Expression and release of RANKL by CLL and multiple myeloma cells and healthy controls. A and B, RANKL expression on primary CLL and multiple myeloma cells ($CD19^+CD5^+$ and $CD38^+CD138^+CD45^{low}CD56^+$, respectively) was investigated by FACS using the RANKL mAb MIH24 with mouse IgG2b as isotype control. A, the gating strategy is shown for one representative patient each. B, left, histograms depicting representative results from exemplary patients; right, SFI levels of 54 CLL and 44 multiple myeloma patient samples with medians of results. C, PBMC and bone marrow cells (BMC) of CLL and multiple myeloma patients (>80% content of malignant cells each), respectively, and healthy controls were investigated for RANKL mRNA expression by RT-PCR with 18S rRNA serving as control. D, levels of sRANKL in supernatants of primary CLL cells and healthy PBMC ($n = 10$ each) as well as primary multiple myeloma cells ($n = 22$) and healthy bone marrow cells (BMC; $n = 10$); results obtained with single patients and medians of all measurements are depicted. E, RANKL expression was correlated with release of sRANKL according to absence (open circles) or presence (filled circles) of sRANKL mRNA in each of a total of 19 bone marrow samples from different patients with multiple myeloma. Dotted lines indicate ELISA detection limit (0.05 ng/mL, x-axis) and SFI 1.5 as defined threshold for surface positivity (y-axis). F, sRANKL levels in supernatants of RANKL transfectants and the indicated multiple myeloma cell lines obtained after 72 hours of culture.

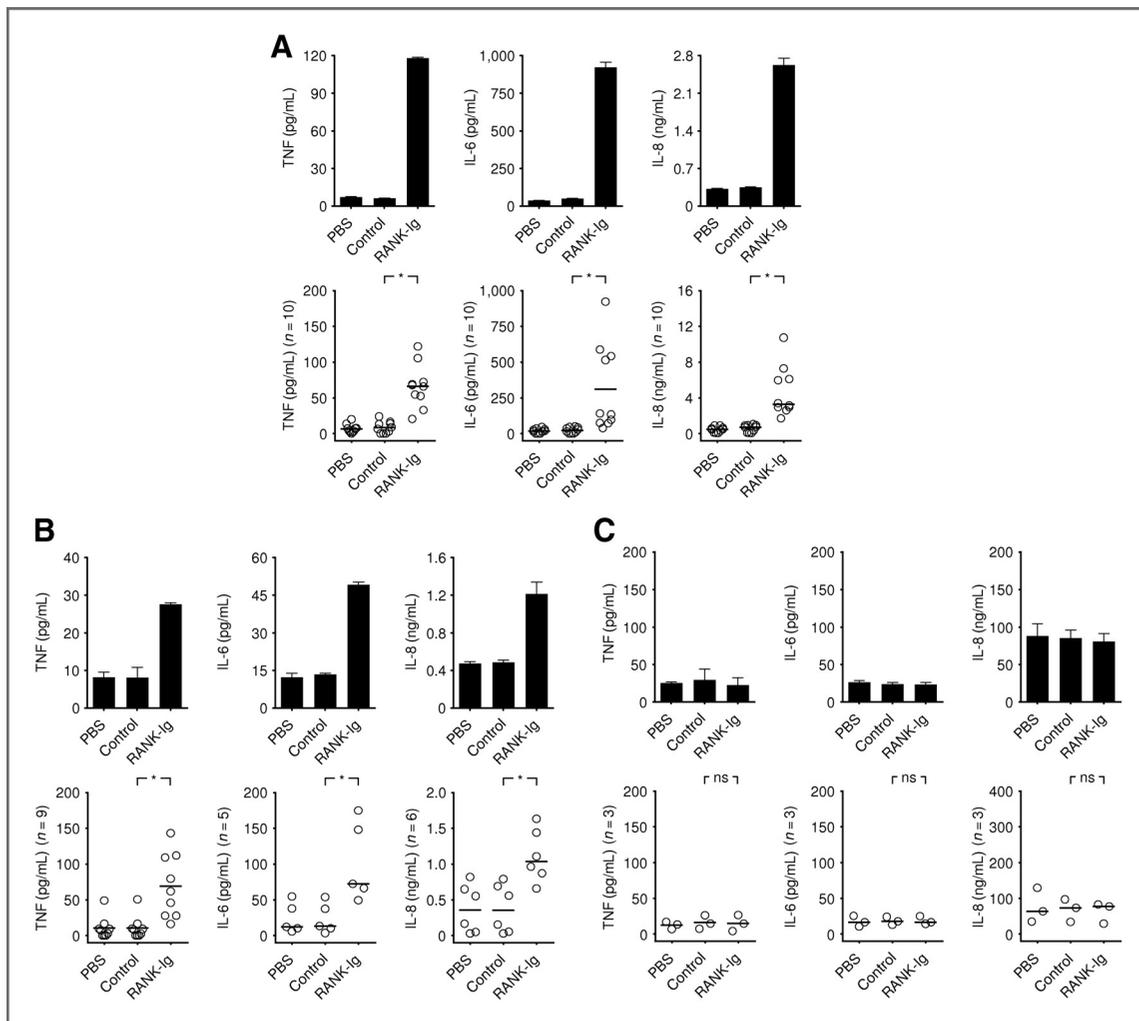


Figure 2. RANKL signaling induces release of TNF, IL-6, and IL-8. PBMC of CLL patients (A) and BMC of multiple myeloma patients (B and C) with more than 80% content of malignant cells were cultured alone, on immobilized RANK-Ig or human IgG1 as control. Levels of TNF (after 6 hours), IL-6, and IL-8 (both after 24 hours) in supernatants were determined by ELISA. Top, exemplary results; bottom, combined analysis of at least 5 experiments with CLL cells and RANKL-positive multiple myeloma cells (A and B) and 3 experiments with RANKL-negative multiple myeloma cells (C). Statistically significant differences (all $P < 0.05$, Mann-Whitney U test) are indicated by *; ns, not significant.

Preparation of polyclonal NK cells

Polyclonal NK cells were generated by incubation of non-plastic-adherent PBMC with irradiated RPMI-8866 feeder cells and IL-2 (50 U/ml) as previously described (26). Experiments were carried out when purity of NK cells ($CD56^+CD3^-$) was more than 90% as determined by flow cytometry.

NK cell degranulation, activation, cytotoxicity, and cytokine production

CD107a and CD69 as markers for NK cell degranulation and activation, respectively, were analyzed by FACS. NK cells were selected by staining for $NKp46^+CD3^-$ or $CD56^+CD3^-$. Cytotoxicity was analyzed by 2 hour BATDA Europium release assays (27). IFN- γ production was analyzed using the ELISA mAb set from Thermo Scientific according to manufacturer's instructions. Lysis rates and cytokine concentrations in supernatants are shown as means of triplicate measurements in each experiment.

Results

Expression and release of RANKL in CLL and multiple myeloma

As a first step, we confirmed that the RANKL antibodies MIH23 and MIH24 specifically bound to RANKL protein (Supplementary Fig. S1). Then, we used FACS analysis to determine RANKL expression on CLL ($CD19^+CD5^+$) and multiple myeloma ($CD38^+CD138^+CD45^{low}CD56^+$) cells within PBMC and bone marrow cells (BMC) of patients (Fig. 1A). Substantial expression (SFI ≥ 1.5) was detected on all 54 investigated CLL samples and in 35 of 44 (80%) multiple myeloma cases (Fig. 1B). Next, we studied RANKL mRNA expression in samples containing more than 80% malignant cells and PBMC and BMC of healthy donors by RT-PCR. Amplicons of membrane-bound RANKL (mRANKL) were detected in all investigated CLL and multiple myeloma samples, but also in PBMC and bone

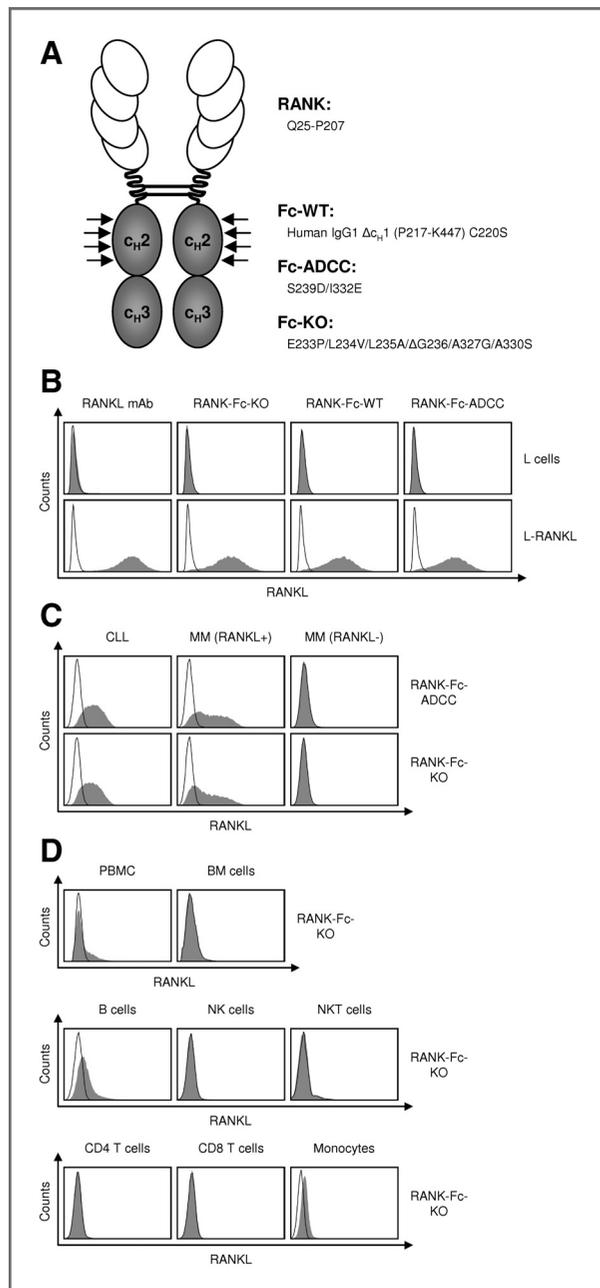


Figure 3. Generation and binding characteristics of RANK-Fc fusion proteins. A, RANK-Fc fusion proteins were generated as dimeric constructs containing the extracellular domain of RANK (Q25-P207) and a human Fc tail (P217-K447 with C220S) with wild-type amino acid sequence or modifications affecting recognition by CD16. B, binding of the RANK-Fc fusion proteins to surface-expressed RANKL was determined by FACS using RANKL transfectants and control cells (L cells). RANKL mAb served as control. Shaded peaks, specific mAb or fusion proteins; open peaks, isotype controls. C, binding of RANK-Fc-ADCC and RANK-Fc-KO to primary CLL and multiple myeloma cells was analyzed as described in B. D, binding of RANK-Fc-KO to PBMC and BMC of healthy donors was analyzed as described in B. Cellular subpopulations within PBMC were identified by counterstaining for CD19⁺ (B cells), CD56⁺CD3⁻ (NK cells), CD56⁺CD3⁺ (NKT cells), CD3⁺CD4⁺ (CD4 T cells), CD3⁺CD8⁺ (CD8 T cells), and CD14⁺ (monocytes). Data of one representative experiment out of at least 3 with similar results are shown (B–D).

marrow cells of all healthy donors, which may be due to RANKL expression, for example, in healthy B or T cells (2, 23). The splice variant coding for the soluble form of RANKL (sRANKL) was detected exclusively in samples of patients with multiple myeloma, where it was expressed in 7 of 10 investigated cases (Fig. 1C). ELISA of supernatants from PBMC and BMC of patients with CLL and multiple myeloma, respectively, as well as healthy controls showed sRANKL solely in supernatants of multiple myeloma cells (Fig. 1D). Combined analysis of 19 multiple myeloma samples revealed that mRANKL amplicons were present in all cases, whereas relevant surface expression (SFI ≥ 1.5) was only observed with 14 samples (74%). Release of sRANKL protein was observed in 11 cases (58%), of which only 7 displayed sRANKL mRNA. In total, 10 samples exhibited positivity for sRANKL mRNA, but release of sRANKL protein was detected only in 7 of the sRANKL mRNA-positive cases (Fig. 1E). As contamination with healthy cells that express RANKL mRNA or release the soluble protein could have influenced these results, we next studied RANKL in multiple myeloma cell lines. Both the mRNA encoding for mRANKL and sRANKL were detected in each of the 7 investigated cell lines. While release of sRANKL protein was observed in all cases, none of the multiple myeloma cell lines displayed RANKL surface expression (Fig. 1F and Supplementary Fig. S1). While this confirmed that multiple myeloma cells in fact can express RANKL mRNA and protein, it also shows that mRNA and protein expression, both with regard to mRANKL and sRANKL, do not necessarily correlate.

RANKL stimulates cytokine release of CLL and multiple myeloma cells

Next, we studied whether the RANKL expressed by primary CLL and multiple myeloma cells was capable to transduce reverse signals that influence the release of cytokines associated with disease pathophysiology (28–31). PBMC and bone marrow samples of patients with CLL and multiple myeloma were cultured alone, on isotype control, or immobilized RANK-Ig, which enables RANKL multimerization. Subsequent analysis of supernatants by ELISA revealed that RANKL signaling significantly enhanced the release of TNF, IL-6, and IL-8 (Fig. 2A and B). No effects were observed when RANKL-negative multiple myeloma patient samples were used, which substantiates the role of RANKL for mediating cytokine release (Fig. 2C). Notably, substantial interindividual differences concerning the response to RANKL signaling seem to exist, as in several RANKL-positive CLL and multiple myeloma cases, patient cells released only one or two of the investigated cytokines (data not shown). RANKL may thus (variably) contribute to the cytokine milieu that is associated with multiple myeloma and CLL pathogenesis.

Generation and functional characterization of Fc-engineered RANK-Fc fusion proteins

To generate Fc-modified RANK-Ig fusion proteins that can neutralize RANKL and at the same time target RANKL-expressing CLL and multiple myeloma cells for ADCC, the extracellular domain of RANK (Q25-P207) was fused to the Fc part of human IgG1 (P217-K447) lacking the C_H1 domain and containing a Cys to Ser substitution at position 220 (RANK-Fc-WT). To

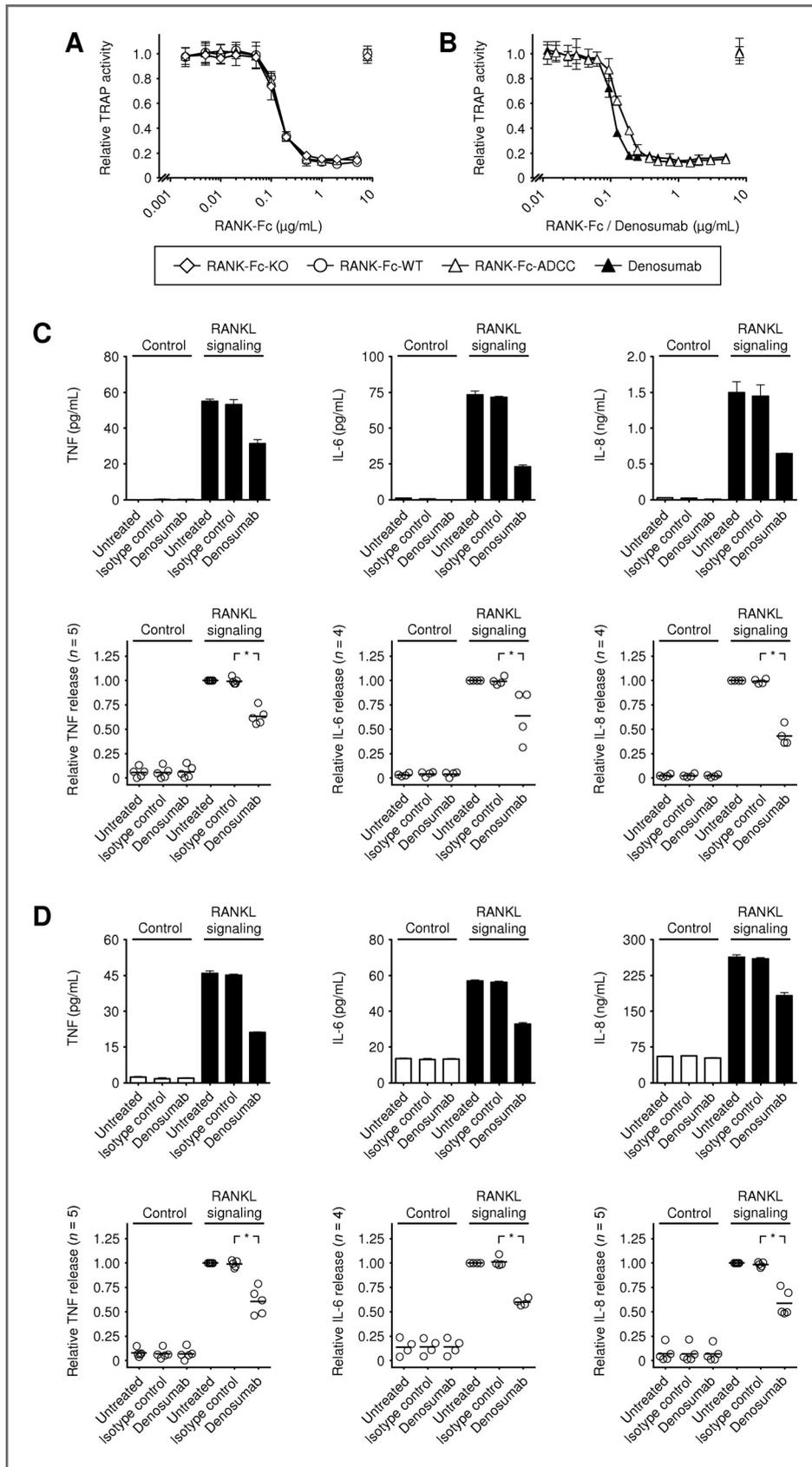
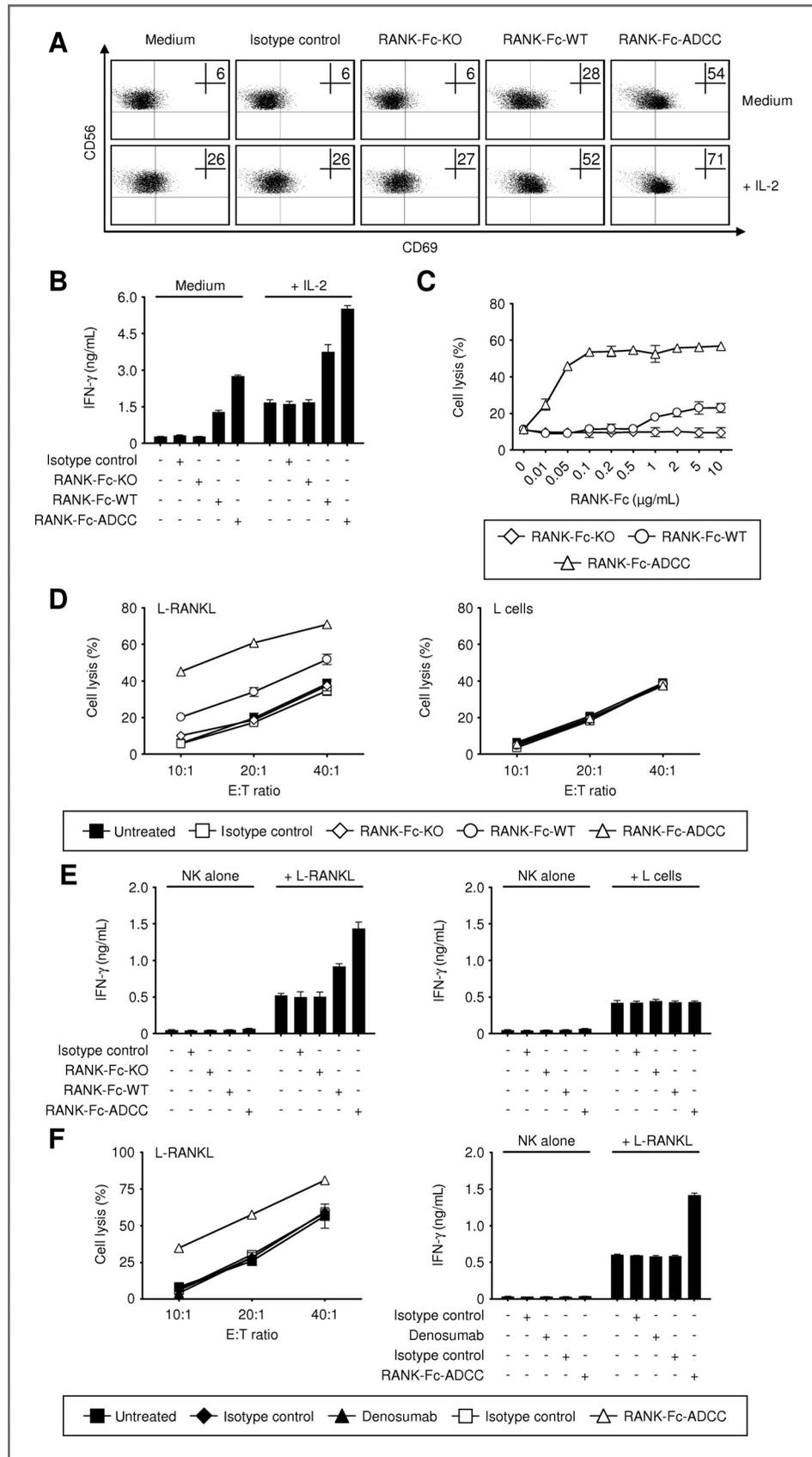
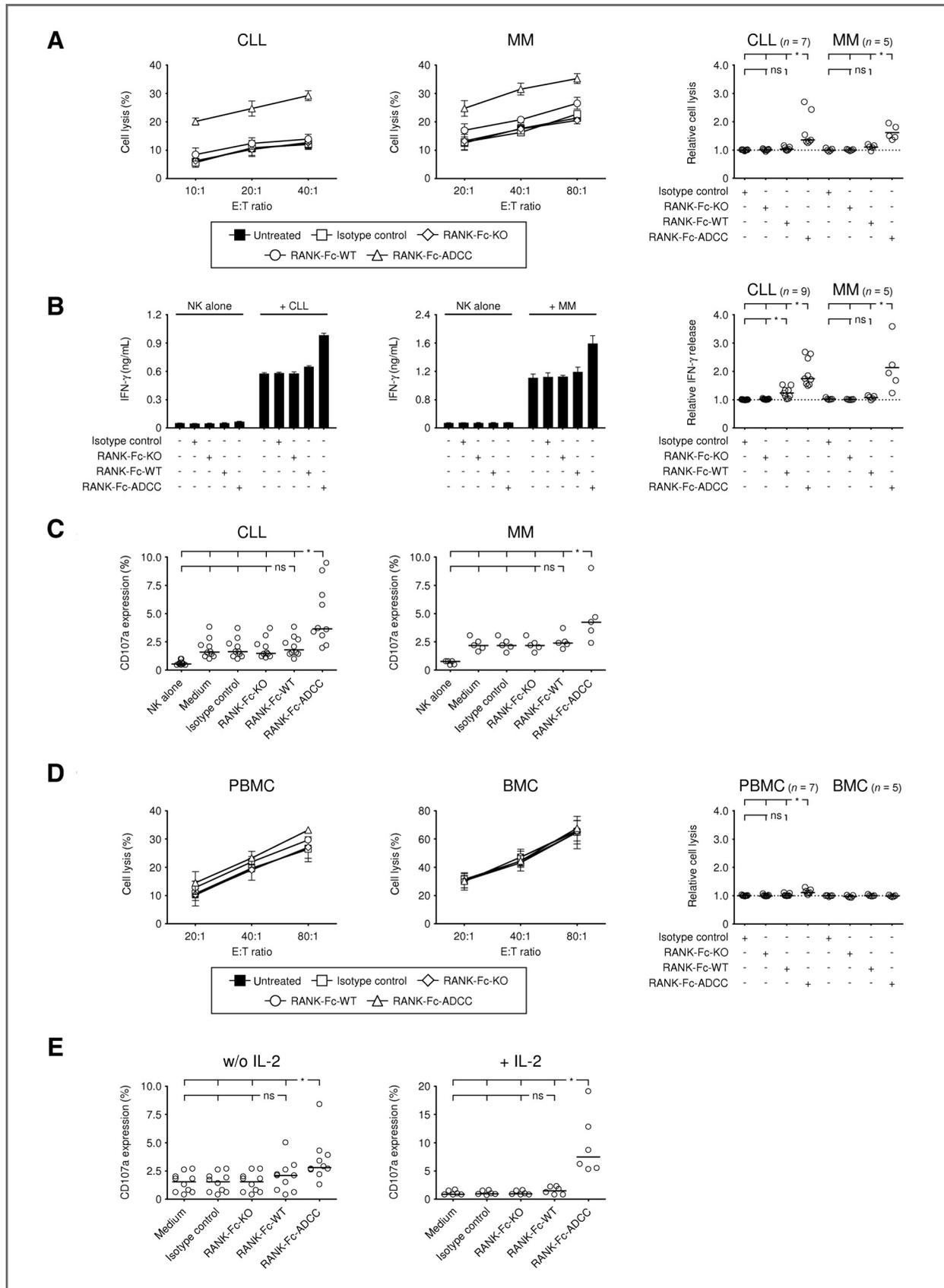


Figure 4. RANK-Fc fusion proteins inhibit the biologic functions of RANKL. A and B, RANKL-induced osteoclastogenesis was determined in the presence of the indicated concentrations of the different RANK-Fc fusion proteins (A) or RANK-Fc-ADCC and denosumab (B). Results upon addition of the respective isotype controls (10 μg/mL each) are depicted at the top right side. Data represent means of triplicates with SDs. C and D, PBMC of CLL patients (C) or BMC of multiple myeloma patients (D; >80% content of malignant cells each) were incubated for 1 hour alone, with denosumab or isotype control (10 μg/mL each) followed by washing. Afterwards, cells were cultured on immobilized RANK-Ig to induce RANKL signaling (black bars) or human IgG1 as control (white bars). TNF (after 6 hours), IL-6, and IL-8 (both after 24 hours) levels in supernatants were determined by ELISA. Top, exemplary results; bottom, combined analysis of at least 4 independent experiments. Statistically significant differences (all $P < 0.05$, Mann-Whitney U test) are indicated by *.

Figure 5. Influence of the Fc modifications on NK cell reactivity and target antigen restriction of the RANK-Fc fusion proteins. A and B, NK cells were cultured for 24 hours in the absence (medium) or presence of 100 U/mL IL-2 (+IL-2) on immobilized RANK-Fc fusion protein or isotype control (10 μg/mL each). A, the percentage of CD69-positive NK cells as determined by FACS is indicated. B, IFN-γ production was determined by ELISA. C, NK cell lysis of L-RANKL (E:T ratio 20:1) in the presence of the indicated concentrations of the RANK-Fc fusion proteins. D and E, NK cells were cultured with L-RANKL or RANKL-negative L cells with or without 10 μg/mL of the different RANK-Fc fusion proteins or isotype control and cytotoxicity and IFN-γ production after 24 hours were determined. F, cytotoxicity and IFN-γ production of NK cells cultured with RANKL transfectants in the presence or absence of RANK-Fc-ADCC, denosumab, or isotype controls (all 10 μg/mL). One representative experiment of a total of at least 3 is shown.





obtain RANK-Fc fusion proteins with highly enhanced and abrogated affinity to CD16, we modified the Fc part by the amino acid substitutions S239D/I332E and E233P/L234V/L235A/ Δ G236/A327G/A330S (RANK-Fc-ADCC and RANK-Fc-KO, respectively) as previously described (Fig. 3A; refs. 22, 32). The different fusion proteins and also Fc-specific controls were then produced as described in the Methods section.

All 3 fusion proteins comparably bound to our RANKL transfectants but not to the controls (Fig. 3B). Moreover, all RANK-Fc fusion proteins bound to primary CLL cells and patient multiple myeloma cells, which had displayed RANKL expression in analyses with RANKL antibody. RANK-Fc-ADCC and RANK-Fc-KO yielded comparable stainings, which confirmed that they bound to RANKL and not, at least not in great part, to Fc receptors potentially expressed by the malignant B cells (Fig. 3C and data not shown). Analyses with resting PBMC of healthy donors revealed weak binding to B cells and monocytes, whereas no relevant binding to BMC of healthy donors was detected (Fig. 3D). Thus, our RANK-Fc fusion proteins specifically bind to RANKL, which is overexpressed on malignant B cells (2).

RANK-Fc fusion proteins and the clinically available RANKL antibody denosumab display comparable capacity to neutralize RANKL

Next, we characterized the ability of our fusion proteins to neutralize the effects of RANKL in osteoclast differentiation assays (25). All 3 constructs comparably reduced RANKL-induced TRAP activity of RAW264.7 cells in a dose-dependent manner, confirming that RANKL binding was not affected by the Fc modifications (Fig. 4A). The neutralizing capacity of our RANK-Fc fusion proteins was slightly lower than that of denosumab at concentrations between 0.06 and 0.2 μ g/mL, but comparable in higher concentrations (Fig. 4B). Next we treated RANKL-expressing primary CLL and multiple myeloma cells with denosumab before induction of RANKL signaling by immobilized RANK-Fc, which significantly reduced the release of TNF, IL-6, and IL-8 by the malignant B cells (Fig. 4C and D). Considering the comparable ability of denosumab and our RANK-Fc fusion proteins to block osteoclastogenesis (Fig. 4B), it seems likely that not only denosumab, but also our fusion proteins may prevent RANKL signaling into malignant B cells.

Modulation of NK cell reactivity by the engineered Fc parts

Next, we compared the capacity of our constructs to trigger CD16 on NK cells. RANK-Fc-KO had no effect, whereas RANK-

Fc-WT enhanced expression of the activation marker CD69 and IFN- γ release. RANK-Fc-ADCC caused substantially more pronounced NK activation than RANK-Fc-WT. Additional stimulation of NK cells with IL-2 generally increased NK cell reactivity without affecting the differential effects of the fusion proteins (Fig. 5A and B).

Next, we conducted dose titrations with the constructs in cultures of NK cells with RANKL transfectants. In concentrations up to 10 μ g/mL, RANK-Fc-KO did not influence target cell lysis. RANK-Fc-ADCC profoundly stimulated NK reactivity at concentrations as low as 0.01 μ g/mL, and maximal effects occurred at about 0.1 μ g/mL. RANK-Fc-WT did not alter NK reactivity at concentrations less than 1 μ g/mL while inducing ADCC at higher concentrations with maximal effects occurring at 5 μ g/mL. Even at high concentrations, its effects were rather marginal compared with that mediated by RANK-Fc-ADCC. In cultures with RANKL-negative targets, none of the fusion proteins altered NK cytotoxicity or IFN- γ production, which constitutes a second major effector mechanism by which NK cells mediate antitumor immunity, thereby confirming that stimulation of NK reactivity required binding of our constructs to surface-expressed RANKL (Fig. 5C–E). Neither cytotoxicity nor cytokine production of NK cells were altered by denosumab (Fig. 5F). Thus, RANK-Fc-ADCC, in contrast to denosumab, profoundly induces antigen-restricted NK reactivity depending on the modifications in its Fc part.

Induction of ADCC against primary malignant B cells

Finally, we determined how our fusion proteins influenced NK reactivity against RANKL-expressing CLL or multiple myeloma cells of patients. With both target cell types, the effects observed in independent experiments varied substantially. Overall, both NK cytotoxicity and cytokine production were potently and significantly enhanced by RANK-Fc-ADCC. RANK-Fc-KO and isotype controls did not influence NK reactivity. With RANK-Fc-WT, only weak effects were observed that, solely in analyses of cytokine release with CLL cells, reached statistical significance (Fig. 6A and B). Comparison of results obtained with multiple myeloma cells that do versus do not release sRANKL revealed no influence of this characteristic on the effects of RANK-Fc-ADCC in our experimental system, which can be attributed to the washing of the multiple myeloma cells before functional experiments resulting in removal of sRANKL, the excess of fusion protein and the short assay time. Moreover, experiments with RANKL transfectants revealed that the induction of NK reactivity by RANK-Fc-ADCC, at the used concentrations, was not affected by sRANKL

Figure 6. Targeting primary malignant B cells and healthy cells for NK reactivity by RANK-Fc fusion proteins. A–C, NK cells were cultured with PBMCs of CLL or BMC of multiple myeloma patients (>80% lymphocyte or plasma cell count) with or without the indicated RANK-Fc fusion proteins or isotype control (10 μ g/mL each). Note that target cells were washed according to the technical requirements of the experiments, which removes potentially released sRANKL. A and B, one representative experiment (left) and combined results (right) of the indicated number of independent experiments (data obtained with untreated target cells were set to 1 in each experiment to enable statistical analysis) are shown for analyses of cytotoxicity (A) and IFN- γ production (B). C, percentages of CD107a-positive NK cells in analyses with CLL (left, $n = 10$) and multiple myeloma (right, $n = 5$) samples. D, modulation of NK cytotoxicity by the constructs with PBMC (left) and BMC (right) of healthy donors serving as targets. Left, one representative experiment; right, combined results obtained as described in A. E, PBMCs of patients with CLL were used directly (left, $n = 10$) or exposed to IL-2 (100 U/mL) for 12 hours (right, $n = 6$) before addition of the indicated constructs (10 μ g/mL each) and determination of CD107a upregulation on autologous NK cells. Percentages of CD107a-positive NK cells in individual samples and medians of results are shown. Statistically significant differences ($P < 0.05$, Mann-Whitney U test) are indicated by *; ns, not significant.

up to concentrations more than 100-fold exceeding that detectable in culture supernatants of multiple myeloma cells. In addition, no clear correlation between RANKL expression on CLL and multiple myeloma cells and the induced level of NK reactivity was observed. No relevant effects of RANK-Fc-ADCC were observed when RANKL-negative multiple myeloma cells were used as targets, which confirmed further that induction of NK reactivity was dependent on target antigen expression (Supplementary Fig. S2). To implement determination of degranulation for subsequent analyses with autologous NK cells, we studied CD107a expression on allogeneic NK cells in cultures with primary malignant B cells. Analyses with CLL and multiple myeloma cells of 10 and 5 different patients, respectively, again revealed that the effects of our constructs varied substantially, and statistically significant induction of NK reactivity occurred solely with RANK-Fc-ADCC (Fig. 6C).

Next, we evaluated how our fusion proteins affected NK reactivity against healthy PBMC and BMC. Weak but statistically significant ADCC against resting allogenic PBMC was observed with RANK-Fc-ADCC, which is most likely due to RANKL expression on B cells and monocytes. Significant ADCC against BMC (that contain a smaller subset of B cells and monocytes, not shown) was not observed (Fig. 6D).

Finally, we determined the capacity of our fusion proteins to induce reactivity of patient NK cells in an autologous setting by degranulation assays. These analyses were limited to PBMCs of patients with CLL, as experiments with multiple myeloma patient samples were prevented by low cell numbers and overall availability of bone marrow material. Again, the effects of the fusion proteins on NK cell reactivity varied substantially among different patients. RANK-Fc-WT induced only minor and statistically not significant effects, whereas RANK-Fc-ADCC substantially enhanced NK degranulation in all experiments. Significant induction of NK reactivity was observed both in the absence and presence of IL-2, with the effects being more pronounced with cytokine-activated NK cells (Fig. 6E).

Together, these data show that our ADCC-optimized RANK-Fc fusion protein is capable to profoundly induce ADCC against RANKL-expressing primary CLL and multiple myeloma cells due to the engineered Fc modification.

Discussion

RANKL may, beyond influencing bone metabolism, also contribute to the pathophysiology of multiple myeloma and CLL (2, 3). However, while some investigators reported that primary multiple myeloma cells express RANKL themselves, others attributed RANKL expression to other cell types in bone marrow of patients with multiple myeloma (3, 8–10). In our study, we observed substantial RANKL surface expression and release in 80% and 50%, respectively, of the investigated multiple myeloma patient samples. While mRNA for mRANKL was always present, sRANKL mRNA was detected only in 53%. Notably, release of sRANKL protein was also observed with patient cells not displaying mRNA for the soluble splice variant, likely due to shedding of RANKL from the cell surface (33). This could also explain the surface negativity of some samples with

positivity for mRANKL mRNA. Moreover, presence of sRANKL mRNA did not always correlate with release of sRANKL protein. It cannot be excluded that limited sensitivity of our ELISA or contaminating RANKL-positive healthy cells may have influenced these results, especially with multiple myeloma cells that may alter RANKL expression of healthy bone marrow cells in their microenvironment (34). Notably, RANKL surface expression was clearly attributable to multiple myeloma cells by our FACS analyses. We conclude that expression and release of RANKL is influenced by posttranscriptional and/or post-translational mechanisms in individual patients. The fact that RANKL mRNA and soluble protein was observed in all multiple myeloma cell lines, whereas none of them displayed RANKL surface expression, confirms this notion, which may also explain discrepancies of previous studies regarding detection of RANKL in multiple myeloma cells (3, 8–10). In contrast to multiple myeloma, all investigated CLL samples displayed mRANKL protein and mRNA, whereas release of sRANKL or mRNA for sRANKL, like with PBMC and BMC of healthy donors, was never observed. This points to a particular regulation/relevance of RANKL in multiple myeloma.

Reverse signaling via RANKL stimulated the release of TNF, IL-6, and IL-8 by multiple myeloma and CLL cells, which is in line with available data that RANKL signals bidirectionally in healthy cells (2, 35, 36). The induced cytokines were described as autocrine/paracrine growth and survival factors in B-cell malignancies and contribute to bone destruction in multiple myeloma (28–31, 37). Of note, previous studies reported that some, but not all, investigated primary multiple myeloma cells produce TNF and IL-6 (38, 39). This is in agreement with our finding that RANKL did not induce cytokine release with all patient samples and could be due to a regulatory or mutational blockade associated with development and progression of disease.

Preventing release of cytokines involved in disease pathology may improve the clinical course of B-cell malignancies. Our data indicate that this can be achieved by blocking RANKL on multiple myeloma and CLL cells with denosumab. However, denosumab did not influence the course of disease in patients with multiple myeloma, indicating that blocking RANK–RANKL interaction alone may be therapeutically not sufficient (13). Denosumab does not induce Fc receptor-mediated effects (14) that could have contributed to reported antitumor effects of RANK-Fc or OPG-Fc fusion proteins in mouse models (3–6). However, the potential role of Fc-mediated effects was not studied in the animal models, and, maybe more importantly, OPG-Fc or RANK-Fc were administered as early therapeutic intervention (3–6), whereas patients with multiple myeloma treated with denosumab suffered from refractory or relapsed disease. Nevertheless, the above described data lead us to generate RANK fusion proteins that are capable to stimulate CD16. In osteoclastogenesis assays, the activity of our constructs to neutralize RANKL was comparable with that of denosumab except at concentrations below 0.2 $\mu\text{g}/\text{mL}$. With regard to affinity, differences between denosumab and RANK-Ig may be somewhat more pronounced because of the lower molecular weight of the fusion proteins. As no difference with regard to neutralizing capacity was observed at higher concentrations corresponding to that achieved with other

clinically used fusion proteins (40) or denosumab in the setting of malignant disease (41), both compounds likely are effective for RANKL neutralization *in vivo*.

In contrast to denosumab, our constructs also induced NK cell reactivity against RANKL-expressing target cells. NK cells are components of innate immunity and play a crucial role in antitumor immunity by mediating cellular cytotoxicity and by releasing cytokines that shape subsequent adaptive immune responses (42). Numerous attempts thus presently aim to use NK cells for cancer treatment (43). Induction of ADCC with antitumor antibodies like rituximab constitutes such an approach and is clinically highly successful. Multiple efforts are presently made to improve the efficacy of antitumor antibodies by increasing their affinity to CD16 resulting in enhanced ADCC (17). This can be achieved by amino acid substitutions in the Fc part and was used here to generate Fc-engineered RANK-Fc fusion proteins (22, 32). RANK-Fc-KO did not alter NK reactivity, RANK-Fc-WT displayed weak effects, and RANK-Fc-ADCC induced by far stronger NK reactivity than the other constructs. When primary RANKL-expressing multiple myeloma and CLL cells were used as targets, weak effects that mostly did not reach statistical significance were observed with RANK-Fc-WT, whereas RANK-Fc-ADCC always induced significant ADCC. Substantial donor variation with regard to the effects of the fusion proteins was observed that was not dependent on RANKL surface levels or the ability of multiple myeloma cells to release sRANKL. Notably, the number of independent experiments available for such analyses was rather limited, and the influence of RANKL expression levels in the different patients and/or low levels of sRANKL may have been concealed by other factors like KIR mismatch, differential expression of ligands for activating/inhibitory NK receptors, or F158V polymorphisms in CD16 (44) that largely influence NK cell reactivity and were not accounted for in our experimental models. The improved immunostimulatory properties of RANK-Fc-ADCC may be especially relevant in multiple myeloma and CLL, where NK cell reactivity has been reported to be impaired (45, 46).

Recently, induction of ADCC was shown to cause exhaustion of NK cells, but their reactivity could be restored by IL-2 (47). Moreover, combined application of rituximab and IL-2 resulted in enhanced antitumor effects in clinical trials (48). In our study, the effect of RANK-Fc-ADCC on NK cell reactivity was clearly enhanced by concomitant application of IL-2, which is in line with the fact that NK reactivity is governed by multiple activating and inhibitory receptors (49).

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With regard to a future clinical application, it needs to be considered that RANKL expression is not restricted to malignant cells. In line, we observed binding of our fusion proteins to B cells and monocytes among healthy PBMC that, in case of RANK-Fc-ADCC, resulted in weak but statistically significant induction of ADCC of allogenic NK cells. Notably, healthy B cells are eliminated upon therapeutic application of rituximab, but reconstitute after about 9 to 12 months (50). Side effects of RANK-Fc-ADCC on healthy B cells could also be expected to be temporary of nature, especially as we did not detect relevant binding to healthy BMC, and application of RANK-Fc-ADCC did not induce bone marrow cell lysis. Moreover, malignant B cells express substantially higher levels of RANKL than their healthy counterparts (2). At present, however, it remains unclear which potential toxicity may occur upon *in vivo* application of RANK-Fc-ADCC, as different other tissues like bone marrow stroma, lymph nodes, gut cells, and mammary epithelium also express RANKL (6). Further analyses to determine the safety and efficacy of RANKL targeting by RANK-Fc-ADCC, among others in suitable animal models, are certainly required and will provide essential information for the further development of RANK-Fc-ADCC for NK cell-based immunotherapy of multiple myeloma and CLL in humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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RANKL Expression, Function, and Therapeutic Targeting in Multiple Myeloma and Chronic Lymphocytic Leukemia

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