Despite several therapeutic advances, patients with multiple myeloma (MM) require additional treatment options since no curative therapy exists yet. In search of a novel therapeutic antibody, we previously applied phage display with myeloma cell screening and developed TP15, a single-chain fragment variable targeting intercellular adhesion molecule 1 (ICAM-1/CD54). In order to more precisely evaluate the antibody’s modes of action, fully human immunoglobulin G1 antibody variants were generated bearing the wild-type (MSH-TP15) or mutated fragment crystallizable (Fc-engineered [Fc-eng.]) region to either enhance (MSH-TP15 Fc-eng.) or prevent (MSH-TP15 Fc knockout [Fc k.o.]) Fc receptor binding. Especially MSH-TP15 Fc-eng. induced significant antibody-dependent cell-mediated cytotoxicity against malignant plasma cells by recruiting natural killer cells and engaged macrophages for antibody-dependent cellular phagocytosis of tumor cells. Binding studies with truncated ICAM-1 demonstrated MSH-TP15 binding to ICAM-1 domain 1-2. Importantly, MSH-TP15 and MSH-TP15 Fc-eng. both prevented myeloma cell engraftment and significantly prolonged survival of mice in an intraperitoneal xenograft model. In the subcutaneous model MSH-TP15 Fc-eng. was superior to MSH-TP15, whereas MSH-TP15 Fc k.o. was not effective in either of the models – reflecting the importance of Fc-dependent mechanisms of action also in vivo. The efficient recruitment of immune cells and the observed anti-tumor activity of the Fc-engineered MSH-TP15 antibody hold significant potential for myeloma immunotherapy.

Introduction

With approval of the monoclonal antibodies (mAb) daratumumab and elotuzumab in 2015, antibody-based immunotherapy has entered clinical practice for multiple myeloma (MM) patients. While both mAb show therapeutic activity in combination with standard treatment regimens, only daratumumab has significant single-agent activity and is additionally approved as front-line therapy. Increased survival rates and good tolerability are achieved with both mAb, but still not all patients benefit and first drug resistances have been reported.1,2 Thus, there is still a high unmet medical need for myeloma patients.

For homing and growth of malignant plasma cells in the bone marrow (BM), adhesion molecules are of significant importance and ‘hiding’ tumor cells in the BM are believed to induce relapse in myeloma patients.3,4 Therefore, targeting the BM microenvironment and adhesion molecules might be explicitly reasonable for MM therapy.5 For instance, intercellular adhesion molecule-1 (ICAM-1/CD54) is reported to be highly expressed on malignant plasma cells and not down-regulated under MM therapy.5 Of note, significantly higher levels of ICAM-1 are expressed on...
malignant plasma cells from MM patients treated with chemotherapy and especially in tumors with a multi-drug resistance phenotype. This is in line with the observation that adhesion molecules such as ICAM-1 are involved in macrophage-induced drug resistance and tumor escape in myeloma. In contrast, expression of ICAM-1 in healthy tissue, e.g., on endothelial cells and leukocytes, is constitutively low and increases only after stimulation with cytokines, i.e., interferon (IFN)-γ or interleukin (IL)-1β, which are up-regulated and released during inflammation and infection. Clinical trials with anti-ICAM-1 antibodies in patients with rheumatoid arthritis, renal transplants, stroke and myeloma proved safety and tolerability, but often lacked significant activity.

Most therapeutic antibodies used for cancer immunotherapy are of immunoglobulin (Ig) G1 isotype and exert their anti-tumor activity via antigen-binding fragment (Fab)- and/or fragment crystallizable (Fc)-mediated effector functions directly through antigen binding and/or interactions with the immune system. The Fc-mediated effector functions encompass antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). Over the years many efforts have been made to improve Fc-mediated effector functions of mAb to increase their cytotoxic and therapeutic activity by Fc glyco- and Fc protein-engineering techniques. Fc protein-engineering approaches make use of specific amino acid substitutions in the Fc receptors (FcγR) and C1q binding interfaces of a therapeutic antibody to specifically improve ADCC, ADCP and/or CDC activity. Lazar and colleagues identified the S239D and I332E (DE) mutations in the CH2 domain of IgG1 antibodies, which markedly enhanced binding to the activating FcγRIIa/CD16a and ADCC activity of a variety of therapeutic IgG1 antibodies. The DE mutations have been applied to generate an Fc-engineered (Fc-eng.) CD19 antibody that demonstrates promising therapeutic activity in clinical trials for relapsed/refractory B-cell non-Hodgkin lymphoma. Margetuximab, an Fc-optimized anti-HER2 antibody with reduced binding to FcγRIb/CD32b and enhanced affinity for FcγRIIa/CD16a, is currently evaluated in a phase III study for breast cancer and also highlights the potential of Fc-engineering as a strategy to improve antibodies for cancer immunotherapy.

Here, we applied Fc-engineering to MS-H-TP15, a novel, fully human anti-ICAM-1 IgG1 antibody whose variable regions are derived from TP15-Fc originally isolated by phage display and screening of myeloma cells. Fab- and Fc-mediated effector functions of MS-H-TP15 were analyzed by comparing three antibody variants that differed in their affinity for FcγR’s on immune cells. Antigen binding, direct anti-tumor effects and immune cell recruitment for tumor cell lysis by the MS-H-TP15 antibody variants were tested in vitro and in vivo against myeloma cells.

Methods

Cell separation

Mononuclear cells from peripheral blood (PBMC), pleural effusion (PE) and BM of myeloma patients and healthy donors were isolated as previously described. Samples were taken after receiving donors’ written informed consents. Experiments were in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Christian-Albrechts-University, Kiel, Germany (D442/10).

Cell lines

Ramos, CHO-K1, L5363 and U266 were obtained from DSMZ (Braunschweig, Germany), Lenti-X 293T were purchased from Takara Bio (Göteborg, Sweden), MM1.S, Raji CS and CHO-FcγRIIa-131H were gifts from YT Tai (Boston, MA, USA), MJ Glennie (Southampton, UK), and F Nimrejahn (Erlangen, Germany), respectively, and cultured as recommended. INA-6, INA-6.Tu1 and BHK-FcγRIIa-158V were established in our laboratory and cultured as previously described.

Production of MSH-TP15 antibody variants

Variable light (VL) and heavy chain (VH) sequences derived from phage PIII-1521 and BI-506522 were cloned into modified pSecTag2/HygroC vectors harboring sequences for the constant region of the human kappa light chain (LC) or either wild-type (wt) or mutated human IgG1 heavy chain (HC). Antibodies were termed MSH-TP15 (wt IgG1), MSH-TP15 Fc-engineered (Fc-eng.) and MSH-TP15 Fc knockout (Fc k.o.) and produced as described in the Online Supplementary Appendix.

Flow cytometric analyses

Immunofluorescence analyses to investigate mAb binding, induction of programmed cell death (PCD) and ADCP were performed on a Navios flow cytometer and analyzed with Kaluza software (Beckman Coulter). For details please refer to the Online Supplementary Appendix.

Co-culture experiments

BM stromal cells (BMSC) were isolated from MM patient BM aspirates and used for co-culture experiments with INA-6 cells as previously described.

Cytotoxicity assays

ADCC activity was analyzed in 3 hour (h) 51Cr release assays using PBMC and natural killer (NK) cells at E:T ratios of 80:1 and 10:1, respectively, as previously described.

Live cell imaging

INA-6 killing was analyzed over 24 h with Incucyte caspase-3/7 reagent (250 nM) in the presence or absence of NK cells (E:T ratio 10:1) and 20 μg/ml of the indicated antibodies with Incucyte live cell imaging (Essen Bioscience). Green cell counts per image were analyzed with Zoom2016A software.

Animal experiments

All animal experiments were performed according to the guidelines of the Christian-Albrechts-University Kiel along with the German Animal Protection Law. For both models 7-8 week-old female SCID/beige mice (Charles River, Sulzfeld, Germany) and red fluorescent protein-expressing INA-6.Tu1 cells (INA-6.Tu1_red; unpublished) were used. In the intraperitoneal (i.p.) model, ten mice/group were injected with 20x106 INA-6.Tu1_red cells 48 h prior start of twice weekly i.p. treatment with mAb (initial doses 10 mg/kg followed by six doses of 5 mg/kg mAb/animal) or vehicle control. In the established tumor model 5x106 INA-6.Tu1_red cells were subcutaneously (s.c.) injected into the right hind flank of five mice/group. At day 10, i.p. treatment with five doses of 10, 1 or 0.1 mg/kg mAb/mouse twice weekly was started. Tumor growth was measured by caliper and volume was calculated: (length x width^2)/2. Human IL-6 receptor was analyzed with CD126 enzyme-linked immunosorbent assay (ELISA) kit (Diaclone, Besançon, France).
Data processing and statistical analyses

Data were statistically analyzed with GraphPad Prism Software using appropriate tests (San Diego, CA, USA). Significance was accepted with $P<0.05$.

Results

Generation of MSH-TP15 IgG1 monoclonal antibodies with different FcγR binding properties

VL and VH sequences of scFv-Fc antibody PIII-15, previously selected by phage display and panning with human myeloma cell lines, were used to generate three fully human IgG1 antibody variants. Compared to the wt IgG1 MSH-TP15, the Fc-optimized MSH-TP15 Fc-eng. and the Fc k.o. variant MSH-TP15 Fc k.o. were designed to display either enhanced FcyRIIa or FcγRIIa binding to improve effector cell activation while retaining wt CDC activity or being incapable to mediate CDC and ADCC and therefore exclusively rely on Fab-mediated effector functions (Figure 1A). All proteins were produced in LentiX 293-T cells. Antibody preparations were highly pure and LC appeared at the calculated molecular masses of 25 kDa under reducing conditions. The slightly altered migration of the calculated 50 kDa HC and 150 kDa IgG1 antibodies that were observed under reducing and non-reducing conditions is most likely due to glycosylation (Figure 1B). Importantly, the three MSH-TP15 mAb showed almost identical, concentration-dependent binding to ICAM-1 with EC$_{50}$ values in the low nanomolar (nM) range, but exerted significant differences in their FcyRIIa and FcγRIIa binding (Figure 1C to E). As intended, the MSH-TP15 Fc k.o. did not bind to these FcγR on stable transfected cells, while binding of MSH-TP15 Fc-eng. was significantly enhanced for both FcγR compared to wt MSH-TP15 IgG1 (Figure 1D and E). Thus, these results confirmed the expected differences of the three antibody variants.

MSH-TP15 binds domain 1 of human ICAM-1

In order to proof that by converting the scFv to a Fab fragment, epitope specificity was not altered, L363 cells were pre-incubated with an excess of TP-15-Fc (scFv-Fc antibody) prior to staining with Alexa Fluor 755-labeled MSH-TP15 IgG1. Importantly, MSH-TP15 binding was completely inhibited indicating the same ICAM-1 binding epitope (Figure 2A). In order to more precisely localize the binding region of MSH-TP15, truncated ICAM-1 variants

Figure 1. Generation and binding characteristics of MSH-TP15 antibody variants. (A) The variable heavy (VH) (dark blue) and variable light (VL) (light blue) single-chain fragment variable (scFv) sequences of phage antibody PIII-15 were used to generate three human immunoglobulin (Ig) G1 MSH-TP15 monoclonal antibody (mAb) variants carrying either a wild-type (middle), a protein-engineered (Fc-eng.; top) or a knockout (k.o.; bottom) fragment crystallizable (Fc)-domain. Mutations in the constant region (grey) of the heavy chain (HC) are depicted in yellow, glycosylation is shown in light grey. (B) Purity and molecular masses of the produced antibodies was analyzed by sodium dodecyl sulfate polyacrylamide-based discontinuous gel electrophoresis (SDS-PAGE) and Coomassie staining under reducing (red.) and non-reducing (n.r.) conditions. ADCC: antibody-dependent cell-mediated cytotoxicity; L: molecular mass ladder; LC: light chain. (C) Intercellular adhesion molecule-1 (ICAM-1) binding was measured by flow cytometry using L363 myeloma cells and increasing antibody concentrations. CD20 antibody rituximab served as control (ctrl IgG1). *** $P<0.001$ MSH-TP15 mAb vs. ctrl IgG1. (D) Binding to human Fcγ receptor (FcγR) was analyzed with CHO cells expressing human FcγRIIa-131H or (E) BHK cells expressing either the low (FcγRIIIa-158F) or the high affinity (FcγRIIIa-158V) allelic form of human CD16a. * $P<0.05$ MSH-TP15/MSH-TP15 Fc-eng. vs. MSH-TP15 Fc k.o.; MFI: mean fluorescence intensity. Data represent mean values ± standard error of the mean of three independent experiments.
were cloned for cell surface expression on CHO-K1 cells (Online Supplementary Figure S1). Flow cytometric analyses revealed that for binding of MSH-TP15 at least ICAM-1 domain (D) 1-2 needed to be present. No binding to mouse ICAM-1 was observed (Figure 2B). For unknown reasons isolated expression of D1 was not possible. Therefore, we performed cross-blocking experiments with MSH-TP15 and the anti-human ICAM-1 mAb BI-505, 84H10 and RR1/1, both known to bind human ICAM-1 D1. As shown in Figure 2C, addition of MSH-TP15, BI-505 and 84H10 cross-blocked individual binding – suggesting that they bound to neighboring, overlapping, or even identical epitopes. This was especially observed for BI-505 and MSH-TP15 (Figure 2C). In contrast, RR1/1 binding was not cross-blocked by MSH-TP15, BI-505 or 84H10. This indicates a unique binding site for RR1/1, which was described to inhibit lymphocyte function-associated antigen-1 (LFA-1) ligand binding to ICAM-1 D1. In line with this, MSH-TP15 had no impact on LFA-1-ICAM-1 interaction (data not shown).
Direct effector functions of MSH-TP15

First, we analyzed direct effector mechanisms of MSH-TP15. MSH-TP15 did not directly inhibit cell proliferation of myeloma cell lines (data not shown), but was capable of inducing apoptosis after antibody cross-linking on the cell surface. MSH-TP15 and rituximab both induced apoptosis in Ramos lymphoma cells expressing ICAM-1 and CD20, while a control IgG1 was not effective (Figure 3A). Total percentage of Annexin V (AV)-positive cells was calculated by combining early apoptotic (AV+/7-AAD–) and dead cells (AV+/7-AAD+) as shown for one exemplified experiment in Figure 3B. Next, we analyzed the impact of MSH-TP15 on growth of IL-6-dependent INA-6 myeloma cells in the presence of BMSC isolated from myeloma patients. Growth inhibition of INA-6 cells in these co-culture experiments was observed in the presence of 10 or 100 µg/mL MSH-TP15, while no direct inhibitory effect on growth of BMSC was detectable with 100 µg/mL MSH-TP15 (Figure 3C).

MSH-TP15 Fc-engineered induces natural killer cell killing and macrophage-mediated phagocytosis of myeloma cells

In order to investigate Fc-mediated effector mechanisms of MSH-TP15, we performed chromium release assays. None of the antibody variants induced complement-dependent lysis of myeloma cells (data not shown), but significant ADCC of plasmacytoma cells could be achieved with MSH-TP15 Fc-eng. and healthy donor’s PBMC (Figure 4A). Only minimal lysis was observed with MSH-TP15 and as expected no killing was detectable with the Fc k.o. variant and control mAb. EC\textsubscript{50} values of MSH-TP15 Fc-eng. ranged from 0.36 nM (INA-6) to 3.77 nM (U266) and are summarized in Table 1. Of note, MSH-TP15 Fc-eng. also induced significant tumor cell lysis when patient-derived myeloma cells, including those from relapsed/refractory MM patients, and purified natural killer (NK) cells were used (Figure 4B, for patient details refer to Table 2). Flow cytometric analyses verified specific binding of MSH-TP15 to the CD138\textsuperscript{+} tumor cells of all patients (Figure 4B). NK cell induced killing of INA-6 myeloma cells by MSH-TP15 Fc-eng. was additionally investigated with live cell imaging (Figure 4C). Caspase activation and apoptosis of INA-6 cells was present over the entire 24 h assay time period. Exemplified microscopy images from 0, 6, 12 and 24 h are shown in Figure 4C. Quantification of the green fluorescent counts (caspase-dependent apoptosis) after 24 h revealed that significant killing of INA-6 myeloma cells occurred only in the presence of NK cells and MSH-TP15 Fc-eng. (Figure 4C). Finally, ADCP activity of the MSH-TP15 antibodies was analyzed with Raji lymphoma cells (CD19, CD20 and ICAM-1 positive) and monocyte-derived macrophages.
Figure 4. MSH-TP15 Fc-engineered efficiently recruits natural killer cells for antibody-dependent cell-mediated cytotoxicity and engages macrophages for antibody-dependent cellular phagocytosis of myeloma and lymphoma cells. (A) Antibody-dependent cell-mediated cytotoxicity (ADCC) experiments were performed as standard 3-hour chromium release assays with increasing antibody concentrations and peripheral blood mononuclear cells (PBMC) of healthy donors at an E:T ratio of 80:1. Antibody-mediated myeloma cell lysis was analyzed for MSH-TP15 (■), MSH-TP15 Fc-engineered (Fc-eng.) (▲), MSH-TP15 Fc knockout (Fc k.o.) (∇) and control monoclonal antibodies (mAb) (•, ▲) with the indicated cell lines. (B) ADCC experiments with patient-derived tumor cells from BM aspirates of multiple myeloma (MM) patients (P#1-P#4), pleural effusion of an extramedullary myeloma (EM-MM) patient (P#5) and PB of a plasma cell leukemia (PCL) patient (P#6) were performed with PBMC (80:1; black bars) or natural killer (NK) cells (10:1; grey bars) of healthy donors and 10 µg/mL of the indicated antibodies. Graph shows mean values ± standard error of the mean of six independent experiments. Histograms on the right show staining of the CD138+ malignant plasma cells of these patients (for details please refer to Table 2) with Alexa Fluor 755-labeled MSH-TP15 (grey line) or control (ctrl) IgG1 (black line). (C) Apoptosis of INA-6 myeloma cells was measured by life cell imaging over 24 hours (h). Apoptotic tumor cells became green fluorescent when intracellular caspases were activated. Right graph shows the total green counts/image measured after 24 h in the presence (black bars) or absence (grey bars) of NK cells and the indicated antibody. Pictures on the left show images taken from start of the experiment (0 h), 6, 12 and 24 h during incubation of myeloma (MM) and NK cells (NK; E:T ratio 10:1; marked with arrows in the upper left picture) with 20 µg/mL MSH-TP15 Fc-eng. (D) For antibody-dependent cellular phagocytosis (ADCP) analyses macrophages were incubated with 10 µg/ml mAb and Raji lymphoma cells at an E:T ratio of 1:3. Percentage of phagocytosed tumor cells was quantified by flow cytometry gating on CD14+, CFSE+ and CD19+ cells. Rituximab (RTX) served as positive control. Experiment was repeated six times with macrophages derived from different donors. Mean values ± standard error of the mean of phagocytosed tumor cells is summarized in the graph with significant differences between ctrl IgG1 and the other mAb marked with stars. ***P<0.001, **P<0.01 and *P<0.05 of MSH-TP15 mAb/RTX vs. ctrl IgG1 for all experiments.

Table 2. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age</th>
<th>Source of material</th>
<th>CD138+ PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>MM (IgM), initial diagnosis</td>
<td>f</td>
<td>67 y</td>
<td>BM, freshly isolated</td>
<td>30 %</td>
</tr>
<tr>
<td>#2</td>
<td>MM (IgGκ), progressive disease</td>
<td>f</td>
<td>70 y</td>
<td>BM, frozen</td>
<td>63 %</td>
</tr>
<tr>
<td>#3</td>
<td>MM (IgGλ), refractory, progress</td>
<td>m</td>
<td>56 y</td>
<td>BM, frozen</td>
<td>58 %</td>
</tr>
<tr>
<td>#4</td>
<td>EM-MM (IgGκ), active disease</td>
<td>m</td>
<td>54 y</td>
<td>BM, freshly isolated</td>
<td>35 %</td>
</tr>
<tr>
<td>#5</td>
<td>PCL (IgGκ), active disease</td>
<td>f</td>
<td>70 y</td>
<td>PE, freshly isolated</td>
<td>93 %</td>
</tr>
<tr>
<td>#6</td>
<td>PCL (IgGκ), active disease</td>
<td>f</td>
<td>67 y</td>
<td>PB, freshly isolated</td>
<td>80 %</td>
</tr>
</tbody>
</table>

MM: multiple myeloma; Ig: immunoglobulin; PC: plasma cell; PCL: plasma cell leukemia; EM: extramedullary; f: female; m: male; y: years; BM: bone marrow; PB: peripheral blood; PE: pleural effusion.
from healthy donors, which highly expressed FcγRII/CD32 and FcγRI/CD64 as well as lower levels of FcγRIIIa/CD16a (Online Supplementary Figure S2). In order to determine ADCP, CD14-stained macrophages were incubated with CFSE-labeled Raji cells at an E:T ratio of 1:3 in the presence of the indicated mAb. CD14/CFSE double positive and CD19 negative cells were defined as phagocytosed tumor cells by flow cytometry. Significant ADCP activity was observed for MSH-TP15 Fc-eng. and the positive control rituximab, while the MSH-TP15 Fc k.o. completely lacked activity and ADCP by MSH-TP15 was moderate (Figure 4D).

The significant recruitment of NK cells and macrophages for ADCC and ADCP of myeloma cells by MSH-TP15 Fc-eng. most likely reflects the improved affinity of this variant to FcγRIIIa and FcγRIIa (Figure 1D and E).

**MSH-TP15 and MSH-TP15 Fc-engineered have significant in vivo anti-myeloma activity**

The MSH-TP15 mAb variants were tested in INA-6.Tu1_red myeloma xenograft models in immunodeficient SCID/beige mice. In order to determine ADCP, CD14-stained macrophages were incubated with CFSE-labeled Raji cells at an E:T ratio of 1:3 in the presence of the indicated mAb. CD14/CFSE double positive and CD19 negative cells were defined as phagocytosed tumor cells by flow cytometry. Significant ADCP activity was observed for MSH-TP15 Fc-eng. and the positive control rituximab, while the MSH-TP15 Fc k.o. completely lacked activity and ADCP by MSH-TP15 was moderate (Figure 4D).

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**MSH-TP15 and MSH-TP15 Fc-engineered have significant in vivo anti-myeloma activity**

The MSH-TP15 mAb variants were tested in INA-6.Tu1_red myeloma xenograft models in immunodeficient SCID/beige mice. In the first experiment, 48 h after i.p. injection of tumor cells treatment with MSH-TP15 mAb or vehicle control (phosphate buffered saline [PBS]) was started. Within 65 days, control animals and MSH-TP15 Fc k.o. treated mice needed to be sacrificed due to tumor size, resulting in a median survival of 39 and 32 days, respectively (Figure 5A). Human IL-6 receptor (huIL-6R) secreted by the INA-6.Tu1_red cells was detectable in final sera of these mice and its concentration correlated with explanted tumor weight (Figure 5B). In contrast, animals treated with MSH-TP15 or MSH-TP15 Fc-eng. did not develop any tumors until the end of the experiment on day 90 (P<0.001 vs. control mice) and no huIL-6R was measured (Figure 5B). Thus, treatment with both, MSH-TP15 and MSH-TP15 Fc-eng., completely prevented the development of myeloma in this model.

Next, in order to compare the efficacy of MSH-TP15 and MSH-TP15 Fc-eng. in more detail, doses of 10, 1 or 0.1 mg/kg were administered to s.c. tumor bearing mice. MSH-TP15 Fc k.o. was given at 10 mg/kg and PBS-treated animals served as control (Figure 5C and D). Compared to the control mice that needed to be sacrificed after 22 days, MSH-TP15 Fc k.o. treated mice reached a median survival of 27 days (Figure 5C). Direct, Fab-mediated anti-myeloma effects may account for the slightly delayed tumor
growth and the survival benefit compared to control mice, but significance was not reached ($P=0.11$). Of note, treatment with 0.1 mg/kg MSH-TP15 significantly retarded tumor growth and prolonged survival (27 days; $P=0.03$ vs. control group; Figure 5C and D). Treatment with 1 mg/kg and 10 mg/kg further improved tumor control and resulted in a median survival of 53 days and 36 days, respectively ($P<0.01$ for both doses vs. control group). Importantly, with the Fc-optimized MSH-TP15 Fc-eng. survival was not only significantly prolonged compared to PBS-treated animals at doses of 0.1 mg/kg (29 days, $P=0.02$), 1 mg/kg (41 days, $P<0.01$) and 10 mg/kg (50 days, $P<0.01$), but also when compared to 10 mg/kg MSH-TP15 treated mice ($P=0.03$). This tendency of being more effective than the wt IgG1 mAb was also seen at 1 mg/kg doses, although differences did not reach statistical significance ($P=0.12$; Figure 5C). These results point to the importance of efficient effector cell recruitment for the anti-myeloma activity of MSH-TP15 in vivo.

**Discussion**

In this study, we characterized the ICAM-1 binding epitope and modes of action of the novel human IgG1 antibody MSH-TP15 originally isolated as scFv antibody by phage display and myeloma cell screening. Detailed binding analyses revealed that the MSH-TP15 epitope is located at the N-terminal D1-2 of human ICAM-1, which is a trans-membrane glycoprotein that consists of five, heavily glycosylated extracellular Ig domains. The majority of the ICAM-1 ligands bind to D1, including the β2-integrin LFA-1 (CD11a/CD18) expressed on all lymphocytes. ICAM-1 and β2-integrin interactions play a pivotal role in lymphocyte activation and adhesion, leukocyte trafficking, and cellular immune responses. In general, ICAM-1 and its ligands are expressed on lymphocytes, leukocytes and vascular endothelium, are increased upon stimulation with inflammatory cytokines like IFN-γ and mediate adhesion of immune cells to allow migration into tissue. Many anti-ICAM-1 antibodies, like RRI/1 and 84H10, bind to D1 or D2 and inhibit LFA-1-mediated adhesion of lymphocytes to ICAM-1 although they recognize different epitopes. Our cross-blocking experiments confirmed a unique ICAM-1 epitope recognized by RRI/1 distinct from that of 84H10, MSH-TP15 and BI-505. Potent blockade and thus overlapping epitopes were observed for MSH-TP15 and BI-505, while only partly or no overlapping epitopes were seen for the two human mAbs with 84H10 and RRI/1. Accordingly, MSH-TP15 was incapable of inhibiting LFA-1 and ICAM-1 interaction. Of note, MSH-TP15 and BI-505 were both identified by phage display and are derived from a fully human scFv library based on the same VH (VH-DP47), but different LC (VL-DPL3 vs. DPK9) framework. This resulted in 89% VH sequence identity between the human IgG1, mAb BI-505 and the human IgG1κ mAb MSH-TP15. Interestingly, in contrast to MSH-TP15, BI-505 was found by screening for apoptosis-inducing antibodies against Ramos Burkitt’s lymphoma cells. Its exact epitope was not determined, but our studies indicate that BI-505 and MSH-TP15 bind close epitopes of human ICAM-1 D1-2.

Despite its important role in immunological processes, ICAM-1 was found to be highly expressed on malignant plasma cells. There ICAM-1 seems to play a pivotal role in myeloma cell adherence to BMSC, and it is, thus considered to be an important molecule in the tumor-microenvironment and to be essential for immune escape of myeloma cells. In addition, ICAM-1 is thought to be involved in macrophage-induced drug resistance and was shown to be overexpressed on chemo-resistant residual myeloma cells. Furthermore, ICAM-1 expression correlates with tumor progression and metastasis in melanoma and renal cell carcinoma patients, and was recently identified as potential cancer stem cell marker in esophageal squamous cell carcinoma.

Targeting ICAM-1 for therapy was initially evaluated with mouse antibodies that were clinically tested in rheumatoid arthritis, acute stroke and renal transplantation. Production of human anti-mouse Ab caused serious problems after repeated treatment. To date, BI-505 is the only clinically tested human anti-ICAM-1 Ab tested. Of note, repeated administration of 10 mg/kg BI-505 already saturated ICAM-1 on patient’s BM myeloma cells and up to 45 mg/kg were well tolerated.

Many ICAM-1 targeting mAbs, including BI-505 and MSH-TP15, exert only little Fab-mediated anti-tumor effects. MSH-TP15 also did not directly affect myeloma cell proliferation, but was capable of inhibiting IL-6-dependent INA-6 growth in the presence of BMSC from myeloma patients. This might be an interesting function regarding the supportive role of the BM microenvironment for survival and drug resistance of myeloma cells. Additionally, MSH-TP15 induced apoptosis of ICAM-1 expressing lymphoma cells after cross-linking on the cell surface – a mechanisms which was also described for BI-505. No CDC but moderate ADCP activity and activation of human NK cells for ADCC of myeloma cells was observed for MSH-TP15. Together these data suggest engagement of NK cells and phagocytes as a predominant effector function of MSH-TP15, a fact already evident for scFv-Fc antibody TP15-Fc. In an attempt to improve the Fc-mediated effector functions of MSH-TP15, we applied Fc protein-engineering. As intended, the DE mutations introduced into the Fc domain of MSH-TP15 Fc-eng. substantially improved the antibody’s affinity for FcyRIIa and FcyRIIIa, resulting in enhanced ADCC and ADCP activity of MSH-TP15 Fc-eng. against myeloma cells in vitro. Of note, significant NK cell-mediated tumor cell lysis was also achieved for patient-derived myeloma cells. In vivo, wt and Fc-optimized MSH-TP15 controlled tumor growth in two myeloma xenograft models, while with the k.o. Ab variant no significant survival benefit compared to control mice was achieved. Myeloma cell growth was completely prevented or dose-dependently inhibited by MSH-TP15 and MSH-TP15 Fc-eng. and both antibody variants significantly prolonged survival of the mice. Importantly, improvement in tumor control and survival was also seen in vivo with the Fc-optimized MSH-TP15 Fc-eng. compared to MSH-TP15 pointing to an important role of immune cells in mice as well. Due to the fact that in SCID/bg mice NK cell function are impaired, most likely mouse myeloid effector cells (monocytes/macrophages, granulocytes) account for the observed differences. This is in line with previous findings that human IgG1 antibodies efficiently bind to mouse FcyRIV, which is the orthologue of human FcyRIIa, but which is, in contrast to the human system, restricted to mouse myeloid cells and is not expressed on mouse NK cells. Since our novel anti-ICAM-1 antib-
ies not only triggered phagocytosis with human macrophages but also significantly triggered ADCC by human NK cells in vitro this might suggest that although in the mouse model myeloid immune effector cells play a dominant role. In patients, together with macrophages also NK cells may constitute a powerful additional effector cell population, which can be recruited for tumor cell killing. Nevertheless, in the subcutaneous model, cure of the mice was not achieved. This leaves room for further improvement and allows testing of combinations of our novel anti-ICAM-1 antibodies with various established anti-myeloma treatment regimen, including proteasome inhibitors, immunomodulatory drugs and chemotherapeutic substances. Systematic testing in our model system may allow the identification of optimal combination partners for potential clinical application.

Taken together, our data indicate that predominantly Fc-mediated effector functions account for the anti-myeloma activity of MSH-T15 and that by Fc protein-engineering a significant improvement of the antibody’s in vitro and in vivo activity was particularly achieved. Particularly for myeloma therapy, where in the post-transplant setting NK cells are among the first immune cells to be present, targeting residual tumor cells in the patients RM with an Fc-eng. anti-ICAM-1 antibody like MSH-T15 Fc-eng. might be a promising strategy to improve clinical outcome.

Disclosures
No conflicts of interest to disclose.

Contributions
KK, MC, CK, TR, AO, SK and AL performed the research; KK and MP wrote the manuscript; CK, FN, TR and MG contributed to writing of the manuscript; MG and MP supervised the study

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