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An Antibody-based Multifaceted Approach Targeting the Human Transferrin Receptor for the Treatment of B-cell Malignancies

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Summary

We previously developed an antibody-avidin fusion protein (ch128.1Av) targeting the human transferrin receptor 1 (TfR1, also known as CD71), which demonstrates direct in vitro cytotoxicity against malignant hematopoietic cells. This cytotoxicity is attributed to its ability to decrease the level of TfR1 leading to lethal iron deprivation. We now report that ch128.1Av shows the ability to bind the Fc γ receptors and the complement component C1q, suggesting that it is capable of eliciting Fcmediated effector functions such as antibody-dependent cellmediated cytotoxicity and complement-mediated cytotoxicity. In addition, in 2 disseminated multiple myeloma xenograft mouse models, we show that a single dose of ch128.1Av results in significant antitumor activity, including long-term survival. It is interesting to note that the parental antibody without avidin (ch128.1) also shows remarkable in vivo anticancer activity despite its limited in vitro cytotoxicity. Finally, we demonstrate that ch128.1Av is not toxic to pluripotent hematopoietic progenitor cells using the long-term cell-initiating culture assay suggesting that these important progenitors would be preserved in different therapeutic approaches, including the in vitro purging of cancer cells for autologous transplantation and in vivo passive immunotherapy. Our results suggest that ch128.1Av and ch128.1 may be effective in the therapy of human multiple myeloma and potentially other hematopoietic malignancies.

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Conflicts of Interest: None.

Keywords

transferrin receptor; CD71; antibody fusion protein; avidin; multiple myeloma

Multiple myeloma (MM) is a clonal B-cell malignancy that accounts for 10% to 15% of hematopoietic malignancies and 2% of all cancer deaths in the United States.[1] In 2010, there was an estimated 20,180 new cases of MM and 10,650 myeloma-associated deaths.[1] Within the past decade the overall survival rate of MM has increased dramatically due to the utilization of thalidomide and its derivative lenalidomide, and the proteosome inhibitor bortezomib.[2] Combination therapies of these new drugs with dexamethasone, prednisone, melphalan, or anthracyclines (with or without autologous stem cell transplantation) have dramatically improved the survival of patients with MM.[2,3] However, MM remains incurable and novel therapies are still needed.[2] In addition, there are no Food and Drug Administration-approved antibody-based therapies for the treatment of MM. A successful antibody-based therapy for other B-cell malignancies is rituximab (Rituxan), a chimeric IgG1 that targets CD20.[4] This antibody alone or combined with various forms of chemotherapy is Food and Drug Administration approved for the treatment of B-cell malignancies, including chronic lymphocytic leukemia and non-Hodgkin lymphoma (NHL). Owing to the small percentage of MM that are $CD20^+$ (13% to 22%), rituximab is not a common treatment for MM.[5] In addition, only 10% of $CD20^+$ MM patients have shown a partial response to rituximab treatment.[5] In lymphoma patients in whom rituximab has significantly increased survival, resistance to treatment continues to be a problem.[4,6] Therefore, there is still a need for the development of new therapies for the treatment of B-cell malignancies as a whole, and for MM in particular.

Iron is involved in many cellular processes, including metabolism, respiration, and DNA synthesis.[7] The most common pathway of iron uptake by cells is through the interaction of iron-loaded transferrin (Tf) with its receptor, the transferrin receptor 1 (TfR1, also known as CD71).[7] This receptor is constitutively internalized and recycled back to the cell surface. Generally, it is expressed at low levels on normal cells, but can be expressed at higher levels on cells with a fast rate of proliferation or an increased need for iron.[7] In addition, TfR1 expression is dramatically increased on a wide variety of cancer cells, including lymphocytic leukemia and NHL, its expression can be correlated with tumor stage or prognosis.[7] Due to its increased expression on cancer cells, its extracellular accessibility, its central role in cancer pathology, and its ability to internalize, the TfR1 has been used extensively as a target for cancer therapy.[7,8] In fact, a recent study showed that Tf-targeted nanoparticles carrying a small interfering ribonucleic acid could be systemically delivered to patients affected with melanoma resulting in specific gene inhibition within the tumor.[9]

To target human cancer cells that overexpress the TfR1 we previously developed an antibody fusion protein that consists of a mouse/human chimeric IgG3 specific for the TfR1 with avidin genetically fused to the C_H3 domains of the antibody (ch128.1Av; previously known as anti-human TfR IgG3-Av).[10] This fusion protein was originally designed as a universal delivery system to carry biotinylated molecules into cancer cells. It was shown that ch128.1Av does not inhibit the binding of Tf or hemochromatosis protein (HFE) to the TfR1.[11,12] In addition, the fusion protein targets the human TfR1 without crossreacting with TfR2,[11,12] although the 2 receptors share 66% similarity in their extracellular domains.[7] It was also found that ch128.1Av exhibits direct in vitro antiproliferative/proapoptotic activity against a variety of malignant B cells, including MM and NHL cells, and when compared to the parental antibody ch128.1 (previously known as antihuman TfR IgG3) this activity was found to be greater than that of ch128.1.[11,13] Binding of ch128.1Av to the TfR1 results in the alteration of its cycling and ultimately in a decreased level of TfR1 and lethal iron

deprivation.[11,13] Conjugation of ch128.1Av with a biotinylated anticancer agent, such as the plant toxin saporin, results in an increased cytotoxic effect due to the delivery of this biotinylated cargo.[14] In fact, this immunotoxin was effective in cells that show resistance to ch128.1Av alone. The cytoxicity can also be enhanced by the combination of the fusion protein with nonbiotinylated anticancer agents, such as gambogic acid [13] and cisplatin.[15] Importantly, ch128.1Av alone was recently shown to block the Akt and NF-κB survival pathways that are constitutively active inMMcells.[15] In fact, NF-κB plays a major role in myeloma development and progression.[16]

In summary, our previous studies show that ch128.1Av is capable of a 2-pronged attack against malignant B cells by its direct cytotoxic activity, and its capability of delivering biotinylated anticancer agents. We now show, for the first time, the ability of ch128.1Av to interact with Fc γ receptors (FcγRs) and the complement component C1q, as well as the in vivo efficacy of both ch128.1Av and its parental antibody ch128.1 in 2 disseminated models of MM. Importantly, we also show a lack of toxicity of ch128.1Av against pluripotent hematopoietic progenitor cells. Taken together, our results suggest that both ch128.1Av and ch128.1 are promising therapeutics that can be used alone or potentially in combination with existing treatments for MM and other B-cell malignancies.

DESIGN AND METHODS

Human Cell Lines

IM-9 (Epstein-Barr virus-transformed lymphoblastoid cells), ARH-77 (Epstein-Barr virus-transformed lymphoblastoid cells), U266 (myeloma cells), HL-60 (acute promyelocytic leukemia cells), Ramos (North American Burkitt lymphoma cells), and U-937 (monocytes derived from the pleural effusion of a patient with histiocytic lymphoma[17]) were all purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Invitrogen Corporation, Carlsbad, CA). KMS-11 human myeloma cells were a kind gift from Lawrence Boise (Emory University) and were cultured in Iscove's Modfied Dulbecco's Medium (Invitrogen). All cell lines were grown in media supplemented with 100 U/mL penicillin, 10 μg/mL streptomycin, and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) in 5% CO_2 at 37°C.

Recombinant Antibodies and Antibody Fusion Proteins

ch128.1, ch128.1Av, and IgG3-Av (isotype control fusion protein specific for the hapten dansyl: 5-dimethylamino naphthalene-1-sulfonyl chloride) have been described previously.[10,11] ch128.1 and ch128.1Av contain the variable regions of the murine monoclonal anti-human TfR IgG1 antibody 128.1.[18] The IgG3 (specific for HER2/*neu*)[19] was used as an isotype control for ch128.1. All contain κ light chains, were expressed in murine myeloma cells, and were purified from cell culture supernatants as described.[20] In addition, rituximab (mouse/human chimeric anti-CD20 IgG1) was purchased from Biogen IDEC, Inc. (Cambridge, MA).

Binding to FcγRs

U-937 cells (5×10^5) were incubated with 1 μg of the isotype controls (IgG3-Av or IgG3) in RPMI containing 10% FBS for 2 hours on ice. Binding was detected using a fluorescein isothiocyanate (FITC)-conjugated anti-human κ antibody (BD Biosciences, San Jose, CA). Unstained cells were incubated in media alone. For inhibition studies, the test antibodies were preincubated with 2 μg soluble FcγRI (sCD64; R&D Systems, Minneapolis, MN) for 30 minutes on ice before the incubation with U-937 cells. In another approach, U-937 cells were preincubated with human FcBlock (Miltenyi Biotec, Auburn, CA) for 30 minutes at 4°C before the addition of antibodies. When FcBlock was used, binding was detected using

an anti-human IgG3- FITC (Sigma Aldrich, St Louis, MO) as the FcBlock reagent consists of pooled human IgG. In all cases cells were washed with buffer [0.5% bovine serum albumin, 2mM ethylenediaminetetraacetic acid in phosphate buffered saline (PBS)], fixed with 2% paraformaldehyde in PBS and analyzed on a Becton Dickinson BD-FACScan Analytic Flow Cytometer in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility. Ten thousand events were recorded and histograms were created using the FCS Express V3 software (De Novo Software, Los Angeles, CA).

Complement Binding Assay

Target cells (4×10^5) were incubated with 5 μg/mL rituximab, ch128.1, or ch128.1Av in serum-free RMPI 1640 for 30 minutes at room temperature. As a source of human complement, 20% "cold," not heat inactivated, normal human serum (Atlanta Biologicals) was added and the incubation continued for an additional 15 minutes at 37°C. Cells were washed and incubated with a FITC-conjugated rabbit anti-human C1q (Dako, Carpinteria, CA) for 30 minutes on ice. Cells were washed, fixed with 2% paraformaldehyde in PBS, and analyzed by flow cytometry as described above.

Detection of CD71 Surface Expression and Proliferation Assay

KMS-11 or ARH-77 cells (5×10^5) were incubated for 30 minutes on ice with either phycoerythrin (PE)-conjugated mouse IgG2a isotype control or PE-conjugated mouse anti-human CD71 (hTfR1) monoclonal antibodies (both from BD Biosciences). After staining, all cells were washed, fixed, and analyzed by flow cytometry as described above. For the proliferation assay, cells were treated in triplicate with various concentrations of ch128.1, ch128.1Av, or IgG3-Av for 96 hours. Proliferation was monitored using the [^{3}H]-thymidine incorporation assay as described previously.[14]

In Vivo Antitumor Activity

All experimental protocols were approved by the UCLA Institutional Animal Care and Use Committee, and all local and national guidelines on the care of animals were strictly adhered to. C.B-17 severe combined immune deficiency (SCID)-Beige mice were obtained and housed in the Defined-Flora Mouse Facility in the Department of Radiation Oncology at UCLA. Eight to 12 weeks old female mice were exposed to 3 gray total body irradiation using a MARK-1-30 irradiator (^{137}Cs source, J.L. Shepherd & Associates, San Fernando, CA) on the day before tumor challenge. Tumor challenge consisted of intravenous injection of 5×10^6 ARH-77 or KMS-11 cells in Hanks' balanced salt solution by the tail vein. Mice were randomized into treatment groups and treatments were given intravenously 2 days after tumor challenge also in Hank's balanced salt solution. Survival was based on the time from tumor challenge to the development of hind limb paralysis, when mice were euthanized. Survival plots were generated using GraphPad Prizm Version 5 (GraphPad Software, Inc., La Jolla, CA). Median survival and differences in survival (log rank test) were determined using the same software. Results were considered significant if $P<0.05$.

Long-term Culture-initiating Cell Assay

Bone marrow mononuclear cells (StemCell Technologies, Vancouver, Canada) were treated with 1 or 10 nM ch128.1Av for 1 hour in Iscove's Modfied Dulbecco's Medium with 2% FBS. Cells were then washed 3 times and and the assay was carried out as recommended in the LTCIC Procedure Manual (StemCell Technologies). In brief, treated cells were seeded on a M2-10B4 (American Type Culture Collection, Manassas, VA) murine fibroblast feeder layer in human long-term medium (StemCell Technologies). Cells were cultured for 5 weeks with half media changes weekly. Both nonadherent and adherent cells were harvested and

seeded in quadruplicate in MethoCult GF+H4435 ("Complete PLUS" methylcellulose medium with recombinant cytokines and erythropoietin; StemCell Technologies). After an 18-day incubation total colony numbers were determined using an Olympus CK2 inverted microscope (Olympus America Inc., Center Valley, PA).

RESULTS

Binding Analysis of the Fc Region of ch128.1Av

As avidin was fused to the C-terminus of the antibody, we wanted to determine if ch128.1Av is able to bind the FcγRs. We used U-937 cells for this purpose as they express FcγRI, II, and III.[21] However, the TfR1 is also expressed on this immortalized cell line. Therefore, we tested the binding of the isotype control IgG3-Av as it is identical to ch128.1Av except for its specificity. Both the IgG3-Av fusion protein and an antibody of the same isotype without avidin (IgG3) showed similar binding to U-937 cells (Fig. 1). However, the binding signal of the fusion protein was slightly higher compared with that of the antibody alone. Importantly, this binding could be blocked with the addition of either soluble FcγRI (sCD64; Fig. 1A) or human FcBlock (Fig. 1B) indicating that IgG3-Av bound to U-937 cells through the interaction of its Fc region with FcγRs. These data show that even with avidin fused to the C-terminus, ch128.1Av is still capable of binding FcγR and suggests that it should maintain its ability to elicit Fc effector functions mediated by these receptors including antibody-dependent cell-mediated cytotoxicity (ADCC).

Complement-dependent cytotoxicity (CDC) is an additional anticancer effector function that can be mediated by antibodies.[22,23] To determine if ch128.1Av can potentially activate the complement system, we evaluated the ability of the fusion protein to bind the first protein in the complement cascade (C1q) in human serum. Rituximab was used both as a positive and negative control depending on the cell line. IM-9 ($CD20^+/TfR1^+$), U266 ($CD20^-/TfR1^+$), HL-60 ($CD20^-/TfR1^+$), and Ramos ($CD20^+/TfR1^+$) cells were used as target cells for this assay. As expected, rituximab-mediated C1q binding on the surface of target cells, as evidenced by an increase in fluorescence in the FL-1 channel, was detected on IM-9 and Ramos cells but not on $CD20^-$ cells, U266 and HL-60 (Fig. 2). Binding of C1q on the surface of $TfR1^+$ cells was also observed with both ch128.1Av and ch128.1 although the binding signal of the former was superior (Fig. 2). These results suggest that the fusion protein can activate the complement system.

In Vitro and In Vivo Efficacy of ch128.1Av and ch128.1

To study the in vivo efficacy of ch128.1Av we used 2 cell lines with varying levels of in vitro sensitivity to ch128.1Av-mediated cytotoxicity in xenograft models of MM. We have previously shown that the human Blymphoblastoid cell line ARH-77 is highly sensitive to the direct in vitro cytotoxicity of ch128.1Av.[11] In addition, this cell line has been used as a model of MM as intravenous injection to SCID mice leads to the development of a disease that mimics human MM with mice developing hypercalcemia, lytic bone lesions, and hind limb paralysis. [24,25] We also utilized the human myeloma cell line KMS- 11[26] that has been used to evaluate the in vivo efficacy of several therapeutics against MM including milatuzumab and bortezomib.[27] We found that this cell line has low in vitro sensitivity to ch128.1Av under the conditions tested, despite high expression levels of the TfR1 (Fig. 3). Compared with ARH-77 cells, 500 fold more of ch128.1Av was required to observe a similar effect in KMS-11 cells (Fig. 3). There is no antiproliferative effect with ch128.1, even with the use of high concentrations (500 nM).

For all in vivo efficacy studies, SCID-Beige mice were challenged with a lethal dose of either ARH-77 or KMS-11 cells on day 0 and treated on day 2. For each cell line, the

experiment was carried out twice. In the first experiment using ARH-77, both ch128.1Av and ch128.1 prolonged survival compared with controls (Fig. 4A and Table 1). There was 1 long-term survivor (>100 days) in the ch128.1Av-treated group and 2 in the ch128.1-treated group. Despite the prolonged median survival in the ch128.1-treated group (Table 1), there was no significant difference in survival in this experiment between ch128.1Av and ch128.1. Isotype controls were then tested to confirm that targeting by the antibodies was necessary for the antitumor effect. Both ch128.1 and ch128.1Av exhibited protection compared with either buffer alone or their matched isotype control (Fig. 4B). There were 3 long-term survivors in the group treated with ch128.1 in this experiment. In addition, ch128.1 prolonged survival compared with ch128.1Av ($P<0.01$; Fig. 4B). The median survival for both experiments using ARH-77 combined are shown in Table 1. These studies demonstrate that despite the fact that ch128.1Av shows stronger in vitro activity compared with ch128.1, a single dose of either ch128.1 or ch128.1Av resulted in dramatic antitumor effects in vivo against cells that are highly sensitive to both agents in vitro.

Using KMS-11 cells, both ch128.1Av and ch128.1 significantly prolonged survival compared with buffer alone (Fig. 4C, Table 1). There were 6 long-term survivors in the group treated with ch128.1, but none in the group treated with the fusion protein. Thus, ch128.1 showed significantly better protection compared with the fusion protein ($P<0.01$). Again, isotype controls were tested and data from this second experiment showed results very similar to the first (Fig. 4D, Table 1). Both ch128.1 and ch128.1Av significantly prolonged survival compared with controls, and ch128.1 demonstrated a stronger effect compared with ch128.1Av (Fig. 4D, Table 1). It is interesting to note that all ch128.1-treated animals were long-term survivors and showed no evidence of disease. The median survival of both experiments with KMS-11 cells are shown in Table 1. These studies show that both ch128.1Av and ch128.1 have strong in vivo antitumor activity, despite minimal in vitro activity against this cell line. In addition, in 3 of the 4 in vivo experiments with the 2 cell lines, ch128.1 demonstrated significantly stronger in vivo anticancer activity compared with ch128.1Av.

In Vitro Toxicity to Normal Pluripotent Hematopoietic Stem Cells

As we are evaluating ch128.1Av as a potential therapeutic for hematopoietic malignancies, we addressed the concern of potential toxicity to normal hematopoietic progenitors, using the LTC-IC assay. This assay is used to enumerate the number of noncommitted or pluripotent stem/progenitor cells within a given sample and has been used to evaluate the toxicity of potential anti-myeloma therapeutics on this population of cells.[28] This is in contrast to the more common colony forming assays that detect toxicity against committed hematopoietic progenitors. As ch128.1Av showed a significantly higher level of in vitro toxicity compared with ch128.1 against the malignant cell lines, we evaluated its toxicity on pluripotent progenitor cells from bone marrow-derived mononuclear cells from 3 different healthy human donors. The concentrations used for this assay are equivalent to those that show dramatic in vitro antitumor effects in sensitive cells, such as ARH-77. The number of colonies at the end of the assay correlates with the number of pluripotent progenitor cells that resulted after treatment. In the case of each donor, exposure to ch128.1Av did not result in a reduction in colony number (Table 2), suggesting ch128.1Av is not toxic to these progenitors.

DISCUSSION

We have previously studied the mechanism of direct in vitro cytotoxicity of ch128.1Av;[11,12,14,15] however, the binding ability of the Fc region and the in vivo activity of the fusion protein have never been evaluated. The anticancer effects of many recombinant antibodies in vivo depend, at least in part, on Fc effector functions, including ADCC and

CDC.[22,23] In this study we show that a fusion protein composed of avidin genetically fused to the C-terminus of an IgG3 is able to bind the FcγRs and the complement protein C1q, suggesting that antibody fusion proteins with this structure are also capable of eliciting Fc effector functions. The superior binding signal exhibited by ch128.1Av compared to ch128.1 to C1q and to a lesser extent FcγRs may be explained by their differences in structure. One avidin monomer was genetically fused to each of the 2 heavy chains of IgG3. Thus, the fusion protein as a monomer contains 2 avidin moieties. However, fast protein liquid chromatography analysis has shown that ch128.1Av has a molecular weight consistent with a noncovalent dimer composed of 2 fusion protein monomers.[10] This structure is expected as in nature avidin is a homotetrameric protein comprised of 4 noncovalently linked monomers[29] and each antibody-avidin fusion protein contains 2 molecules of avidin (1 genetically fused at the C-terminus of each heavy chain). The parental antibody as an IgG3 molecule is monomeric. Thus, it is unclear as to whether the increased signal of ch128.1Av is due to an increase in avidity because of its dimeric structure and/or whether it is simply due to an amplification in signal intensity due to the increased binding of the labelled antibody.

In this study we also show that despite the fact that ch128.1Av demonstrates stronger in vitro activity compared with ch128.1, a single relatively low dose of either resulted in dramatic antitumor effects in vivo in ARH77 and KMS-11 xenograft models. This was particularly surprising given the lack of in vitro sensitivity of KMS-11 to ch128.1. The superior protection exhibited by ch128.1 may be explained by a lower bioavailability of ch128.1Av, which contains avidin, and therefore it is expected to clear faster from the blood and accumulate in the liver.[30] In fact, in studies conducted in a 1-week time frame we observed that ch128.1Av clears from blood much faster compared to ch128.1 (Daniels et al, unpublished results) suggesting that ch128.1Av is highly effective as an antitumor agent in vivo and that at higher doses and/or multiple administrations it might be possible to achieve an antitumor effect that cannot be matched by the antibody alone under certain conditions. This protective effect may also be potentially increased by conjugating ch128.1Av with biotinylated drugs. However, this analysis is beyond the scope of these studies. Given the species specificity of ch128.1Av, adequate safety testing in murine models based on this antibody is difficult. This is a common issue for antibody-based therapies, which will ultimately necessitate testing in nonhuman primates and/or in clinical trials. Certainly, further studies are needed to evaluate the overall safety of ch128.1Av and ch128.1.

The mechanism of the in vivo antitumor activity exhibited by ch128.1Av and ch128.1 is unclear at this moment. Direct cytotoxicity through iron deprivation as we have previously demonstrated in vitro,[11,13] may be a mechanism. Even though limited direct cytotoxicity was observed in vitro, especially in KMS-11 cells, it is possible that the in vivo environment makes the cells particularly sensitive to iron starvation. Other mechanisms, including the induction of Fc effector-mediated functions, cannot be ruled out. However, it is important to note that the SCID-Beige mice used for these studies have a limited effector cell repertoire. In fact, the beige mutation results in impairment of natural killer function, even though a normal natural killer cell population is present,[31] and these mice also have impaired neutrophil activity.[32] SCID-Beige mice are reported to maintain a relatively normal macrophage population.[33] Thus, it is possible that ch128.1 and ch128.1Av act, at least in part, through Fc effector functions mediated by macrophages, such as ADCC and/or antibody-dependent cellmediated phagocytosis. Whether or not the effector cells are capable of eliciting ADCC and/or antibody-dependent cellmediated phagocytosis in SCID-Beige mice, it is also possible that the anticancer effect is due to extensive crosslinking that might occur if ch128.1Av or ch128.1 binds to the FcγRs on the surface of effector cells in the tumor microenvironment. This extensive crosslinking may affect the physiology of TfR1 function, including its internalization, resulting in iron starvation and cell death. Finally, we

cannot rule out the possibility that CDC may also be involved. Importantly, our results should serve as a cautionary note that the in vitro activity of an antibody targeting TfR1 might not necessarily predict its in vivo activity. Evaluation of potential therapeutics based only on in vitro data may not result in identification of the best candidates for effective therapeutics, as other parameters may be involved in vivo. Further studies are needed to determine the mechanism of in vivo antitumor activity of both ch128.1 and ch128.1Av.

We used an in vitro assay system to explore the potential toxicity to pluripotent hematopoietic progenitor cells. Previously, we have shown that at concentrations of 1 nM and above ch128.1Av significantly reduces the number of CFU-E, BFU-E, and CFU-GM colonies in the human progenitor assay.[13] This indicates that the fusion protein is toxic to late hematopoietic progenitor cells at these concentrations. Many of these cells are known to express high levels of TfR1 at various stages of differentiation, [7] thus, such toxicity is not surprising. However, pluripotent progenitor cells express low levels of the TfR1 or no TfR1,[34] suggesting low to no toxicity to this cell population would be observed in vivo. We now show that ch128.1Av is not toxic to these cells, which suggests that even though committed progenitors might be affected, primitive progenitors may be able to repopulate these cells. Autologous stem cell transplantation remains a viable therapeutic option for younger MM patients. However, myeloma cell contamination within these grafts is often present and may shorten progression-free survival.[35,36] Thus, new purging strategies would be beneficial for MM treatment. ch128.1Av used alone or combined with a biotinylated cytotoxic agent may potentially contribute to this purpose.

In addition to our antibody and antibody fusion protein, other antibodies targeting the TfR have shown anticancer activity against hematologic malignancies.[7] A murine monoclonal anti-human TfR antibody 42/6 (IgA) has been evaluated in a phase I clinical trial.[37] The treatment was well tolerated and 3 patients with hematologic malignancies showed transient mixed responses. This limited response can be attributed to the rapid clearance of the murine IgA and the generation of a humoral immune response against the antibody that may have neutralized the treatment. More recently, the murine anti-human TfR antibody A24 (IgG2b) has demonstrated both direct in vitro cytotoxicity and in vivo efficacy in a local (subcutaneous) model using mantle cell lymphoma cells with a single injection of 40 mg/kg of the antibody.[38] Like ch128.1Av, and to a much lesser extent ch128.1, in vitro incubation with A24 leads to degradation of TfR1.[38] However, unlike ch128.1Av and ch128.1, A24 (and 42/6 described above) is a receptor neutralizing antibody in that it inhibits the binding of Tf to TfR1.[39] We show in this study in vivo efficacy for treatment of a disseminated disease using our antibodies at a dose that is several fold lower than what was used for A24. In addition, ch128.1 and the fusion protein consist of a mouse/human chimeric antibody that will be less immunogenic than a murine antibody and for which the Fc fragments will be active in humans. The avidin moiety of ch128.1Av is of chicken origin and may potentially be immunogenic. However, avidin was chosen for the construction of the fusion protein as it is less immunogenic than its bacterial counterpart streptavidin. As oral antigens induce tolerance,[40] the decreased immunogenicity of avidin may be due to the induction of a tolerogenic response resulting from the frequent exposure to avidin through the consumption of eggs. This is supported by a study that evaluated a radioactive avidin conjugate as an imaging method for prosthetic vascular graft infection in which none of the patients developed a humoral immune response to avidin.[41] In addition, this immunogenicity might not be a major issue as patients with hematopoietic malignancies, includingMM and NHL, often show an impaired immune response.[42,43]

A mouse/human chimeric antibody D2C specific for the human TfR has also been developed and shown to mediate both ADCC and CDC activity.[44] Thus, even though the antibodies specific for the TfR1 are internalized, still there is a sufficient amount of these

molecules on the cell surface to elicit ADCC and CDC. Antibodies targeting other cell surface antigens that are internalized, such as trastuzumab,[45] which is specific for HER2/*neu*, have also shown the ability to elicit ADCC.[46]

Many antibody-mediated therapies targeting other tumor-associated antigens are currently being explored for the treatment of MM. There are ongoing clinical trials of recombinant antibodies alone or combined with bortezomib for the treatment of relapsed and/or refractory MM.[47,48] These include elotuzumab (anti-CS1), mapatumumab (anti-TRAIL-R1), CNTO-328 (anti-IL-6), and milatuzumab (anti-CD74). All of these results using recombinant antibodies are promising; however, it should be noted that our antibody and fusion protein are directed against another site of myeloma cell vulnerability (TfR1/CD71) and show that a single treatment achieved significant antitumor effects including long-term survival at a lower dose compared with those commonly used for the above mentioned therapeutic antibodies when they were used in human xenograft models. However, we would like to stress that our antibody and fusion protein would not necessarily be a replacement for the conventional chemotherapy or other MM therapies (including those described above), but instead may provide an alternative therapy to be used in combination with other anticancer approaches.

In summary, this study shows that ch128.1Av has the ability to interact with the FcγRs and the complement protein C1q and that both the fusion protein and ch128.1 have remarkable anticancer activity in 2 xenograft models of MM. This study also shows that ch128.1Av is not toxic to early hematopoietic progenitor cells suggesting that this relevant population of cells will be preserved in vivo. Both ch128.1Av and ch128.1 show potential as therapeutics for B-cell malignancies including MM. In addition, the fusion protein is a molecule that may be cytotoxic to cancer cells in the following ways: (1) through direct cytotoxicity mediated mostly by lethal iron deprivation, (2) through delivery of a wide variety of biotinylated anticancer agents, and (3) through Fc-mediated effector functions, including ADCC and CDC. Therefore, together ch128.1 and ch128.1Av represent a multifaceted approach for the treatment of B-cell malignancies.

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References

1. Jemal A, Siegel R, Xu J, et al. Cancer statistics. CA Cancer J Clin. 2010; 60:277–300. [PubMed: 20610543]
2. Laubach JP, Richardson PG, Anderson KC. The evolution and impact of therapy in multiple myeloma. Med Oncol. 2010; 27:S1–S6. [PubMed: 20169425]
3. Palumbo A, Rajkumar SV. Treatment of newly diagnosed myeloma. Leukemia. 2009; 23:449–456. [PubMed: 19005483]
4. Bello C, Sotomayor EM. Monoclonal antibodies for B-cell lymphomas: rituximab and beyond. Hematology Am Soc Hematol Educ Program. 2007:233–242. [PubMed: 18024635]
5. Kapoor P, Greipp PT, Morice WG, et al. Anti-CD20 monoclonal antibody therapy in multiple myeloma. Br J Haematol. 2008; 141:135–148. [PubMed: 18318769]

6. Oflazoglu E, Audoly LP. Evolution of anti-CD20 monoclonal antibody therapeutics in oncology. MAbs. 2010; 2:14–19. [PubMed: 20081379]
7. Daniels TR, Delgado T, Rodriguez JA, et al. The transferrin receptor part I: biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol. 2006; 121:144–158. [PubMed: 16904380]
8. Daniels TR, Delgado T, Helguera G, et al. The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin Immunol. 2006; 121:159–176. [PubMed: 16920030]
9. Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010; 464:1067–1070. [PubMed: 20305636]
10. Ng PP, Dela Cruz JS, Sorour DN, et al. An anti-transferrin receptor-avidin fusion protein exhibits both strong proapoptotic activity and the ability to deliver various molecules into cancer cells. Proc Natl Acad Sci U S A. 2002; 99:10706–10711. [PubMed: 12149472]
11. Ng PP, Helguera G, Daniels TR, et al. Molecular events contributing to cell death in malignant human hematopoietic cells elicited by an IgG3-avidin fusion protein targeting the transferrin receptor. Blood. 2006; 108:2745–2754. [PubMed: 16804109]
12. Rodriguez JA, Helguera G, Daniels TR, et al. Binding specificity and internalization properties of an antibody-avidin fusion protein targeting the human transferrin receptor. J Control Release. 2007; 124:35–42. [PubMed: 17884229]
13. Ortiz-Sanchez E, Daniels TR, Helguera G, et al. Enhanced cytotoxicity of an anti-transferrin receptor IgG3-avidin fusion protein in combination with gambogic acid against human malignant hematopoietic cells: functional relevance of iron, the receptor, and reactive oxygen species. Leukemia. 2009; 23:59–70. [PubMed: 18946492]
14. Daniels TR, Ng PP, Delgado T, et al. Conjugation of an anti transferrin receptor IgG3-avidin fusion protein with biotinylated saporin results in significant enhancement of its cytotoxicity against malignant hematopoietic cells. Mol Cancer Ther. 2007; 6:2995–3008. [PubMed: 18025284]
15. Suzuki E, Daniels TR, Helguera G, et al. Inhibition of NF-kB and Akt pathways by an antibody-avidin fusion protein sensitizes malignant B cells to cisplatin-induced apoptosis. Int J Oncol. 2010; 36:1299–1307. [PubMed: 20372806]
16. Li ZW, Chen H, Campbell RA, et al. NF-kappaB in the pathogenesis and treatment of multiple myeloma. Curr Opin Hematol. 2008; 15:391–399. [PubMed: 18536579]
17. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer. 1976; 17:565–577. [PubMed: 178611]
18. White S, Taetle R, Seligman PA, et al. Combinations of antitransferrin receptor monoclonal antibodies inhibit human tumor cell growth in vitro and in vivo: evidence for synergistic antiproliferative effects. Cancer Res. 1990; 50:6295–6301. [PubMed: 2400993]
19. Huang TH, Morrison SL. A trimeric anti-HER2/neu ScFv and tumor necrosis factor-alpha fusion protein induces HER2/neu signaling and facilitates repair of injured epithelia. J Pharmacol Exp Ther. 2006; 316:983–991. [PubMed: 16291729]
20. Helguera G, Penichet ML. Antibody-cytokine fusion proteins for the therapy of cancer. Methods Mol Med. 2005; 109:347–374. [PubMed: 15585931]
21. Schiller C, Spittler A, Willheim M, et al. Influence of suramin on the expression of Fc receptors and other markers on human monocytes and U937 cells, and on their phagocytic properties. Immunology. 1994; 81:598–604. [PubMed: 8039810]
22. Campoli M, Ferris R, Ferrone S, et al. Immunotherapy of malignant disease with tumor antigen-specific monoclonal antibodies. Clin Cancer Res. 2010; 16:11–20. [PubMed: 20028761]
23. Helguera, G.; Daniels, TR.; Rodriguez, JA., et al. Monoclonal antibodies, human engineered. In: Flickinger, MC., editor. Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology. Vol. 5. Hoboken, New Jersey: John Wiley & Sons, Inc; 2010. p. 3526-3542.
24. Gado K, Silva S, Paloczi K, et al. Mouse plasmacytoma: an experimental model of human multiple myeloma. Haematologica. 2001; 86:227–236. [PubMed: 11255268]
25. Alsina M, Boyce B, Devlin RD, et al. Development of an in vivo model of human multiple myeloma bone disease. Blood. 1996; 87:1495–1501. [PubMed: 8608240]

26. Namba M, Ohtsuki T, Mori M, et al. Establishment of five human myeloma cell lines. In Vitro Cell Dev Biol. 1989; 25:723–729. [PubMed: 2768132]
27. Stein R, Smith MR, Chen S, et al. Combining milatuzumab with bortezomib, doxorubicin, or dexamethasone improves responses in multiple myeloma cell lines. Clin Cancer Res. 2009; 15:2808–2817. [PubMed: 19351768]
28. Servida F, Soligo D, Delia D, et al. Sensitivity of human multiple myelomas and myeloid leukemias to the proteasome inhibitor I. Leukemia. 2005; 19:2324–2331. [PubMed: 16224484]
29. Green NM. Avidin. Adv Protein Chem. 1975; 29:85–133. [PubMed: 237414]
30. Rosebrough SF, Hartley DF. Biochemical modification of streptavidin and avidin: in vitro and in vivo analysis. J Nucl Med. 1996; 37:1380–1384. [PubMed: 8708779]
31. Roder JC. The beige mutation in the mouse. I. A stem cell predetermined impairment in natural killer cell function. J Immunol. 1979; 123:2168–2173. [PubMed: 489978]
32. Jones-Carson J, Vazquez-Torres A, Balish E. Defective killing of Candida albicans hyphae by neutrophils from beige mice. J Infect Dis. 1995; 171:1664–1667. [PubMed: 7769315]
33. Roder JC, Lohmann-Matthes ML, Domzig W, et al. The beige mutation in the mouse. II. Selectivity of the natural killer (NK) cell defect. J Immunol. 1979; 123:2174–2181. [PubMed: 158612]
34. Gross S, Helm K, Gruntmeir JJ, et al. Characterization and phenotypic analysis of differentiating CD34+ human bone marrow cells in liquid culture. Eur J Haematol. 1997; 59:318–326. [PubMed: 9414644]
35. Bashey A, Perez WS, Zhang MJ, et al. Comparison of twin and autologous transplants for multiple myeloma. Biol Blood Marrow Transplant. 2008; 14:1118–1124. [PubMed: 18804041]
36. Vogel W, Kopp HG, Kanz L, et al. Myeloma cell contamination of peripheral blood stem-cell grafts can predict the outcome in multiple myeloma patients after high-dose chemotherapy and autologous stem-cell transplantation. J Cancer Res Clin Oncol. 2005; 131:214–218. [PubMed: 15616828]
37. Brooks D, Taylor C, Dos Santos B, et al. Phase Ia trial of murine immunoglobulin A antitransferrin receptor antibody 42/6. Clin Cancer Res. 1995; 1:1259–1265. [PubMed: 9815920]
38. Lepelletier Y, Camara-Clayette V, Jin H, et al. Prevention of mantle lymphoma tumor establishment by routing transferrin receptor toward lysosomal compartments. Cancer Res. 2007; 67:1145–1154. [PubMed: 17283149]
39. Moura IC, Lepelletier Y, Arnulf B, et al. A neutralizing monoclonal antibody (mAb A24) directed against the transferrin receptor induces apoptosis of tumor T lymphocytes from ATL patients. Blood. 2004; 103:1838–1845. [PubMed: 14592824]
40. Weiner HL. Oral tolerance. Proc Natl Acad Sci U S A. 1994; 91:10762–10765. [PubMed: 7971958]
41. Samuel A, Paganelli G, Chiesa R, et al. Detection of prosthetic vascular graft infection using avidin/indium-111-biotin scintigraphy. J Nucl Med. 1996; 37:55–61. [PubMed: 8544003]
42. Grulich AE, Vajdic CM, Cozen W. Altered immunity as a risk factor for non-Hodgkin lymphoma. Cancer Epidemiol Biomarkers Prev. 2007; 16:405–408. [PubMed: 17337643]
43. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol. 2007; 138:563–579. [PubMed: 17686051]
44. Qing Y, Shuo W, Zhihua W, et al. The in vitro antitumor effect and in vivo tumor-specificity distribution of human-mouse chimeric antibody against transferrin receptor. Cancer Immunol Immunother. 2006; 55:1111–1121. [PubMed: 16341531]
45. Hurwitz E, Stancovski I, Sela M, et al. Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc Natl Acad Sci U S A. 1995; 92:3353–3357. [PubMed: 7724565]
46. Gennari R, Menard S, Fagnoni F, et al. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. Clin Cancer Res. 2004; 10:5650–5655. [PubMed: 15355889]
47. Wright JJ. Combination therapy of bortezomib with novel targeted agents: an emerging treatment strategy. Clin Cancer Res. 2010; 16:4094–4104. [PubMed: 20682705]

48. Berkova Z, Tao RH, Samaniego F. Milatuzumab: a promising new immunotherapeutic agent. Expert Opin Investig Drugs. 2010; 19:141–149.

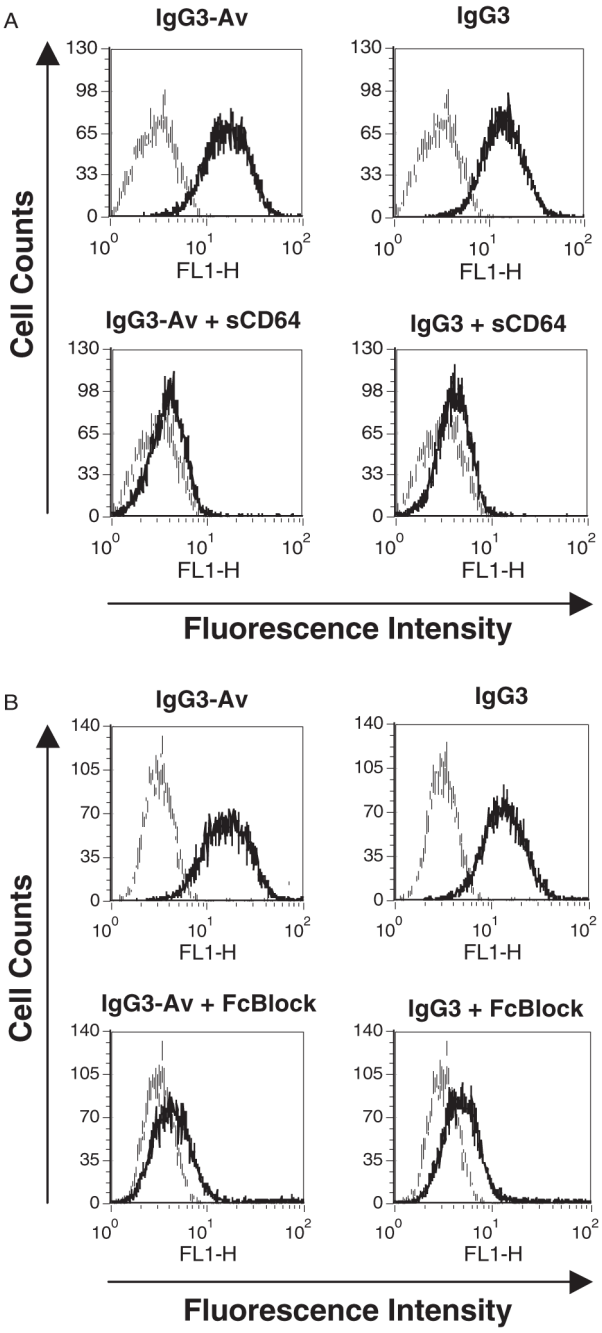

FIGURE 1.

Binding of the avidin fusion protein to FcγRs on the surface of U-937 cells. Cells were incubated with 1 μg IgG3-Av or IgG3 for 2 hours on ice. Binding was detected using an antihuman κ or anti-human IgG3-FITC conjugate and flow cytometry analysis. Specificity of binding was shown through inhibitory assays using A, soluble FcγRI (sCD64) or B, human FcBlock. Gray lines represent cells stained with secondary antibody alone, whereas black lines denote test antibody alone (top panels) or antibody plus inhibitor (bottom panels). Data are representative of 2 independent experiments.

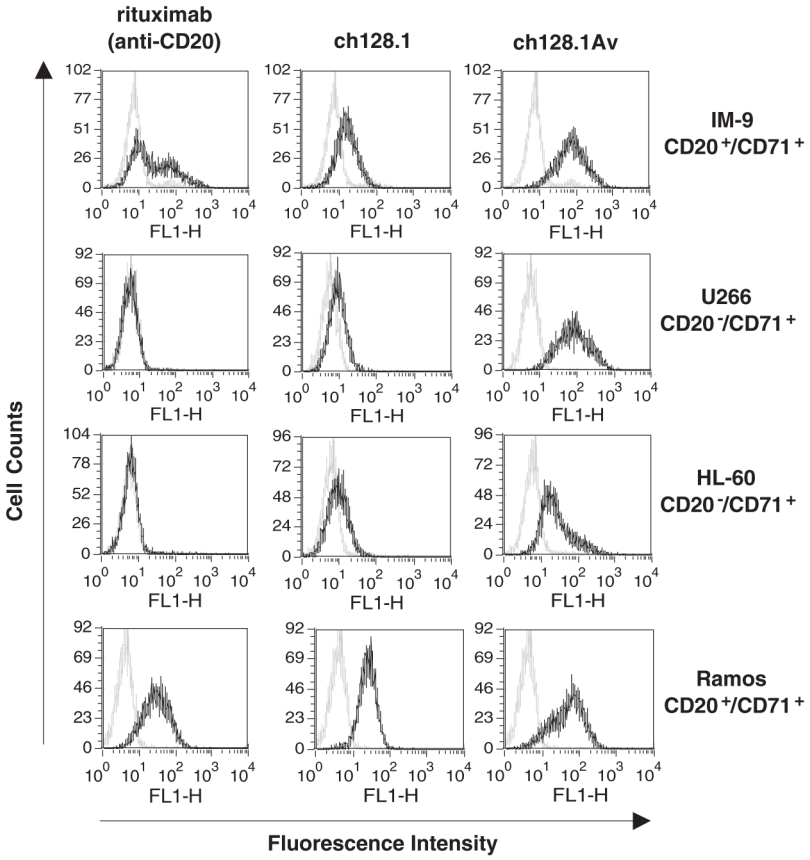

FIGURE 2.

Antibody-mediated C1q binding on the surface of target cells. IM-9, U266, HL-60, or Ramos cells were incubated with 5 μg/mL of rituximab, the parental ch128.1, or the fusion protein ch128.1Av for 30 minutes at room temperature. Samples treated with 20% cold human serum (black lines) or without human serum (gray lines) are shown. Binding of the antibodies the complement protein C1q on the surface of target cells was detected by flow cytometry using an anti-human C1q-FITC conjugate. Data are representative of 3 independent experiments.

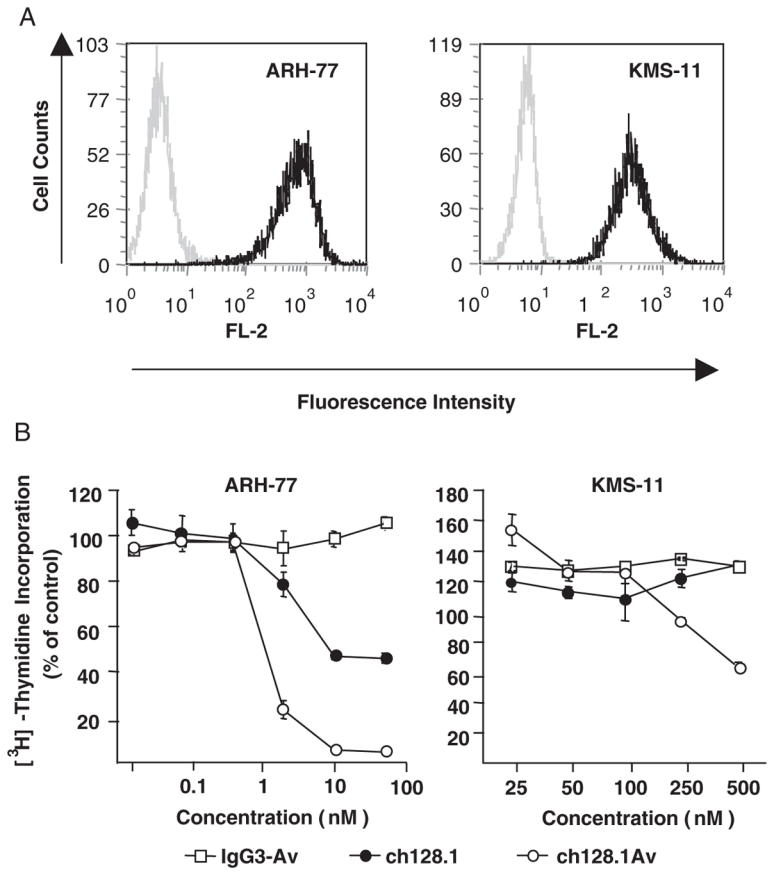

FIGURE 3.

CD71 cell surface expression and in vitro cytotoxicity of ch128.1Av and ch128.1 against KMS-11 and ARH-77 cells. A, Expression of CD71 on the surface of KMS-11 and ARH-77 cells as determined by flow cytometry. Cells were labeled with either an isotype PE-conjugated control antibody (gray line) or an anti-CD71 PE-conjugated antibody (black line). B, Cells were treated with various concentrations (ranging from 0.016 to 50nM for ARH-77 cells and 25 to 500nM for KMS-11 cells) of ch128.1, ch128.1Av, or IgG3-Av for a total of 96 hours. Cytotoxicity was measured using the [$_3$H]-thymidine incorporation assay. The average of triplicate wells is shown with the standard deviation. Data are presented as a percent of radioactivity incorporated into control cells and are representative of 3 independent experiments.

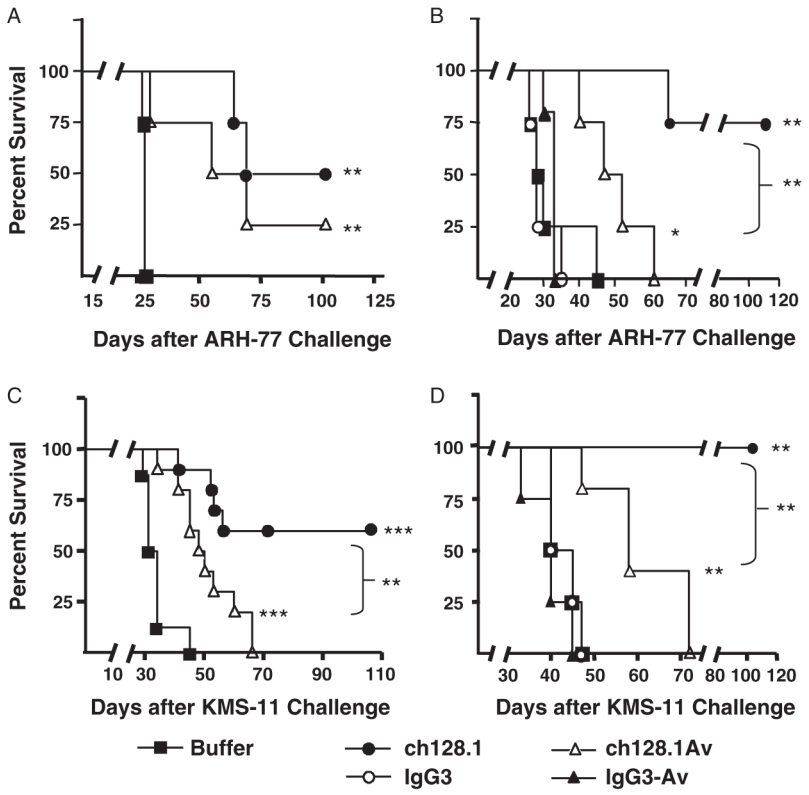

FIGURE 4.

In vivo efficacy of ch128.1Av and ch128.1 in 2 disseminated models of MM. Kaplan-Meier plots indicating survival of SCIDBeige challenged intravenously with 5×10^6 ARH-77 (A and B) or KMS-11 (C and D) cells. For experiments with ARH-77 cells (A and B) 100 μg of each treatment was injected intravenously 2 days after tumor challenge, whereas 125 μg of each treatment was used for the KMS- 11 studies. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as determined by the log rank test.Median survival for each experiment are given in Table 1.

TABLE 1

Median Survival of SCID-Beige Mice Treated With ch128.1 or ch128.1Av

	ARH-77 (#1)	ARH-77 (#2)	KMS-11 (#1)	KMS-11 (#2)
Buffer	28 (n=4)	29 (n=4)	33 (n=8)	43 (n=4)
ch128.1	85 (n=4)	>100 (n=4)	>100 (n=10)	>100 (n=5)
ch128.1Av	62 (n=4)	50 (n=4)	49 (n=10)	58 (n=5)
IgG3	NT	28 (n=4)	NT	43 (n=4)
IgG3-Av	NT	33 (n=5)	NT	40 (n=4)
	ARH-77 (#1 and #2)		KMS-11 (#1 and #2)	
Buffer	28 (n=8)		34 (n=12)	
ch128.1	>100 (n=8)		>100 (n=15)	
ch128.1Av	54 (n=8)		53 (n=15)	
IgG3	28 (n=4)		43 (n=4)	
IgG3-Av	33 (n=5)		40 (n=4)	

Median survival is given in days. Data are from the same experiments as those shown in Figure 4.

NT indicates not tested; SCID, servere combined immune deficiency.

TABLE 2

Effect of ch128.1Av on Normal Pluripotent Hematopoietic Progenitor Cells From 3 Separate Donors as Determined by the LTC-IC Assay

	Donor #1	Donor #2	Donor #3
Untreated	7±0.96	1±0.82	13±1.71
Buffer	13±1.00	3±1.83	ND
10 nM ch128.1Av	15±2.20	3±1.50	13±2.16
1 nM ch128.1Av	17±3.32	8±1.50	17±2.06

Data represent the mean of quadruplicates ± the standard deviation.

LTC-IC indicates long-term cell-initiating culture; ND, not determined.