Therapeutic Antitumor Efficacy of Anti-CD137 Agonistic Monoclonal Antibody in Mouse Models of Myeloma

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Abstract

Purpose: Eradication of post-treatment residual myeloma cells is needed to prevent relapses, and immunostimulatory monoclonal antibodies (mAb) such as anti-CD137, CTLA-4, CD40, etc., which enhance the immune response against malignancies, represent a means of achieving this purpose. This study explores anti-CD137 mAbs for multiple myeloma treatment in preclinical models of the disease because they safely augment tumor immunity and are in clinical trials for other cancers.

Experimental Design: The antitumor effect of anti-CD137 mAb on mouse plasmacytomas derived from HOPC and NS0 cell lines was studied and compared with that of anti-CTLA-4, anti-CD40, and anti-ICAM-2 mAbs. The antitumor effect of anti-CD137 mAb was also examined in a mouse syngeneic disseminated myeloma (STGM1) model, which more closely resembles human multiple myeloma. Depletions of specific cell populations and gene-targeted mice were used to unravel the requirements for tumor rejection.

Results: Agonistic mAb against CD137 and blocking anti-CTLA-4 mAb showed activity against i.p. HOPC tumors, resulting in extended survival of mice that also became immune to rechallenge. Anti-CD137 mAbs induced complete eradications of established s.c. NS0-derived tumors that were dependent on IFN-γ, natural killer cells, and CD8+ T lymphocytes. Natural killer cell accumulation in tumor draining lymph nodes and showed increased IFN-γ production. Antitumor efficacy of anti-CD137 mAb was preserved in CD28-deficient mice despite the fact that CD28 signaling increases the expression of CD137 on CD8+ T cells. Importantly, anti-CD137 mAb treatment significantly decreased systemic tumor burden in the disseminated STGM1 model.

Conclusions: The immune-mediated antitumor activity of anti-CD137 mAb in mouse models holds promise for myeloma treatment in humans.

Multiple myeloma is a fatal neoplasm characterized by the uncontrolled proliferation of monoclonal plasma cells (1). Currently, the two most efficacious treatment options for patients with multiple myeloma are tandem high-dose chemotherapy, followed by autologous stem cell infusion, or allogeneic hematopoietic stem cell transplantation after myeloablative therapy or reduced-intensity conditioning (1, 2). New drugs have recently been incorporated in our armamentarium including the proteasome inhibitor bortezomib (Velcade; ref. 3) and thalidomide derivatives that act as immunomodulators (4). Nevertheless, cure is very rarely achieved due to persistence of residual disease. Therefore, new therapeutic approaches to control or even eradicate residual tumor cells are definitely needed, opening an opportunity for immunotherapy (5).

Over the last few years, cancer immunotherapy has emerged as a novel experimental treatment modality in multiple myeloma (6). This approach harnesses the potential of the host immune system to recognize and eradicate neoplastic tissue. Therefore, the success of cancer immunotherapy depends on the efficient induction and maintenance of endogenous antitumor immune responses mediated by innate immunity and other factors (7-9). The immune-mediated antitumor activity of anti-CD137 mAb in mouse models holds promise for myeloma treatment in humans.
Translational Relevance

Multiple myeloma treatment is fast improving. New therapeutic agents such as bortezomib and leniolinomide have been introduced with impressive results, albeit a curative treatment constitutes an unmet need. This article explores at the preclinical level the use of immunostimulatory mAbs for myeloma treatment. These agents act augmenting the antitumor immune response acting on molecules of the immune system. Such mAbs either release the brakes of inhibitory activities or agonistically enhance tumor-rejecting functions. This article focuses on agonist anti-CD137 mAbs that are known to enhance immunity against several tumor types in mice and are currently undergoing phase I and II clinical trials in patients with other malignancies. The in vivo effects of these antibodies in various myeloma models indicate the suitability of these agents for clinical trials in multiple myeloma.

and adaptive immune cells, which in the case of myeloma are counterbalanced by immunosuppressive factors produced by the tumor (6).

Immunostimulatory monoclonal antibodies (mAb) represent a new and exciting strategy in cancer immunotherapy to potentiate the immune responses of the host against the malignancy (7). Such agonistic or antagonistic mAbs bind to key receptors in cells of the immune system to enhance antigen presentation (e.g., anti-CD40), to provide costimulation (e.g., anti-CD137), or to counteract immunoregulation (e.g., anti-CTLA-4). The aim is to boost weak, ineffectual, endogenous antitumor immunity to therapeutic levels. This potential has been shown in animal models with some of these mAbs showing impressive therapeutic activity in preclinical settings (7, 8). Anti-CTLA-4 mAbs are in advanced clinical trials for melanoma and other indications (8, 9). However, a possible obstacle to the clinical development of some of the immunostimulatory mAbs is their associated toxicity, most commonly reversible autoimmunity and/or systemic inflammatory reactions (7). In this regard, anti-CD137 is one of the most interesting immunostimulatory mAbs tested for cancer therapy (10–12), because the very same anti-CD137 mAbs that potently enhance tumor rejection are capable of reducing the incidence and severity of experimental autoimmune diseases (12–16).

CD137 (also called 4-1BB) is a T-cell costimulatory receptor induced on TCR activation (11, 17). In addition to its expression on activated CD4+ and CD8+ T cells, CD137 is also expressed on CD4+CD25+ regulatory T cells, natural killer (NK) and NK-T cells, monocytes, neutrophils, and dendritic cells. Its natural ligand, CD137L, has been described on antigen-presenting cells including B cells, monocyte/macrophages, and dendritic cells (17). On interaction with its ligand, CD137 leads to increased TCR-induced T-cell proliferation, cytokine production, functional maturation, and prolonged CD8+ T-cell survival (11, 17). Moreover, ligation of CD137 increases the proliferation and IFN-γ secretion of NK cells in response to IL-2 (18). Consistent with the costimulatory function of CD137, agonistic mAbs against this receptor have been shown to provoke powerful tumor-specific T-cell responses capable of eradicating tumor cells in a variety of murine syngeneic tumor models leaving the animal immune to rechallenge (10, 19). Depletion and functional experiments indicate that CD8+ T and NK cells are the most consistent protagonists of the immune rejection process (10, 11, 19–21).

However, little is known about the potential therapeutic effect of this and other immunostimulatory mAbs in multiple myeloma. In this study, we examined and compared the antymyeloma effect of various immunostimulatory mAbs including anti-CD137 in two distinct mouse plasmacytoma models and investigated the requirements for the antitumor response generated by anti-CD137 mAb in these models. Finally, we have corroborated the antitymoma effect of anti-CD137 mAb in a disseminated myeloma model transplantable to immunocompetent mice that more closely resembles many features of the corresponding human disease.

Materials and Methods

Mice and cell lines. Female BALB/c wild-type (WT) mice (5–6 weeks old) were purchased from Harlan Laboratories. Female C57BL/KaLwRijHsd mice (6–8 weeks old) were from Harlan. IFN-γ−/− (C3.H10T1/2.Ne3) and CD28−/− (C3.H10T1/2.Ne3 CD28−/−) mice and respective WT littermates, both on the C57BL/6J background, were obtained from Jackson Laboratories. NS0 cells were obtained from the American Type Culture Collection. CT26 cells were received from Dr. T.P. Colombo. Cell lines were maintained in complete RPMI (RPMI 1640 with Glutamax Life Technologies/Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin and 100 μg/mL streptomycin (BioWhittaker), and 5 × 10−5 mol/L 2-mercaptoethanol (BioTechnologies). Mouse STG1M myeloma cells expressing enhanced green fluorescent protein (EGFP; STG1M-EGFP, ref. 22) were generated from the parental STG1M cell line (23), which was in turn established from the transplantable mouse ST333 myeloma (24).

Antibodies and reagents. The hybridoma cell lines anti-CD40 (FGK-45), anti-CD4 (GK 1.5), anti-CD8 (H129.19), and anti-IFN-γ (HB170) were obtained from the American Type Culture Collection; the anti-4-1BB (2A) and anti-CTLA-4 (9H10) hybridomas were kind gifts from Dr. L. Chen (Johns Hopkins; ref. 25) and Dr. J. Allison (Memorial Sloan Kettering; ref. 26), respectively. Anti-ICAM 2 (4G8) mAb was produced in our laboratory as described before (27, 28). The mAbs produced by these hybridomas were purified from respective culture supernatant by affinity chromatography in Sepharose protein G columns according to manufacturer’s instructions (GE Healthcare Biosciences AB). IgG from rat serum was used as control antibody and obtained from Sigma-Aldrich. Anti-asialoGM1 antiserum was used for in vivo NK cell depletion and purchase from Wako. Poly(dI-dC) was purchased from Pharmacia.

In vivo tumor growth and depletion of lymphocyte subsets. For the i.p. myeloma models, BALB/c mice received an i.p. injection of either HOPC or NS0 cells (5 × 105 per mouse) on day 0 and on days 4 and 7 were treated i.v. with the corresponding mAb at 100 μg/injection. These mice were examined weekly for palpable abdominal tumors or ascites.

For the s.c. myeloma model, BALB/c mice received a s.c. injection of 5 × 105 NS0 cells on day 0 and on days 9, 11, 13, and 15 were treated i.p. with either anti-CD137 mAb or the control rat IgG at 100 μg/injection. Tumor diameters were measured using a electronic caliper every 2 to 4 days, and tumor size was determined by multiplying perpendicular diameters.
For in vivo leukocyte subset depletion, mice bearing N50 s.c. tumors were injected with either depleting CD4 or CD8-specific mAbs (200 μg/dose) or anti-asialo GM1 antiserum (50 μl/dose) before anti-CD137 treatment. Both depleting mAbs and anti-asialo GM1 antiserum were administered daily for 5 consecutive days beginning 3 days before treatment onset and then every 4th day for the remainder of the experiment.

In vivo IFN-γ blockade, mice bearing s.c. N50 tumors were given 200 μg neutralizing anti-IFN- γ 1 day after treatment onset and every 4th day thereafter for the next 2 weeks.

**Experiments with the STGM1 multiple myeloma model.** STGM1-GFP cells (10⁶ per mouse) were i.v. inoculated via tail vein, into 6- to 8-week-old female C57BL/KaLwRijHsd mice. Immediately following tumor cell inoculation, mice were randomly assigned to one of four different groups (n ≥ 8 per group) and treated thereafter for 28 days by i.p. injection according to the following protocol: group I, vehicle (PBS); group II, hortezomib (1 mg/kg body weight three times a week); group III, rat IgG (control IgG; 100 μg on days 4, 8, 14, and 18 post-tumor cell inoculation); and group IV, anti-CD137 mAb (100 μg on days 4, 8, 14, and 18 post-tumor cell inoculation). Body weights were obtained at baseline and at weekly intervals thereafter. At the end of the experiment, mice were sacrificed and whole skeletons and visceral organs (spleens, livers, kidneys, ovaries, brains, lungs, and hearts) were harvested and immediately imaged for fluorescent tumor foci as described previously (22). Briefly, selective excitation of EGFP was achieved using an Illumatools fiber-optic fluorescence lighting system (Epi model LT-9500; Lighthools Research) with a 470/40 nm band-pass filter and a dichroic mirror. Emitted fluorescence was collected through the long-pass. Filter at 515 nm with a MagnaFire SP cooled color charge-coupled device camera (Optronics) with an 11 to 48 mm zoom lens, with same exposure times. High-resolution images (1,300 × 1,030 pixels) were captured directly on a Macintosh laptop computer and are presented here unadjusted for contrast or brightness.

Tumor burden was also assessed by assaying STGM1-specific monoclonal paraprotein (IgGκ,κ) in sera prepared from whole blood obtained by retro-orbital sinus bleed of tumor-bearing mice just before sacrifice under light methoxyflurane-induced anesthesia. Mouse IgGκ,κ levels were assayed using a specific in-house sandwich ELISA as described previously (23), with a rat IgG1 antibody and a horseradish peroxidase-conjugated rat IgG1 that binds mouse IgGκ light chain (clone LO-MG2b-2; Research Diagnostics) as the capture antibody and a biotinylated antibodies. mAbstothefollowingmouseantigensconjugatedtoFITC, heavy chain (clone LO-MG2b-2; Research Diagnostics) as the capture antibody and a horseradish peroxidase-conjugated rat IgG1 that binds mouse IgGκ light chain (clone LO-MK-2; BDBiosciences) and an isotype control antibody. In this ELISA, there is no species cross-reactivity or cross-reactivity to other mouse immunoglobulins.

**Isolation of mononuclear cells from lymph nodes and tumors.** At the indicated time points, tumor draining lymph nodes (TDLN) or minced tumor nodules were harvested from tumor-bearing mice that had been treated with either anti-CD137 mAb or the control Ab. Both axillary and inguinal TDNLs from individual mice were pooled. Then, the lymph nodes were incubated in collagenase and DNase (Roche) for 15 min at 37°C and pressed between two semifrosted microscopic slides. Finally, the dissociated cells were passed through a 70 μm nylon mesh filter (BD Falcon, BD Biosciences) and washed further before use.

**Fluorescence-activated cell sorting analysis and cytotoxicity experiments.** Cells were resuspended in PBS with 5% fetal bovine serum and pretreated with Fc-block (anti-CD16/32; eBioscience) to reduce the nonspecific staining. Afterwards, cells were incubated with the staining antibodies. mAbs to the following mouse antigens conjugated to FITC, phycoerythrin, allophtocyanin, or biotin were used: CD40, CD137, ICAM 2, CD80, CD86, IAα, H2K, CD49b, CD3, CD4, CD8, IFN-γ, and CD69. These specific mAbs and their corresponding isotype controls were obtained from BD PharMingen. Biotinylated antibodies were visualized with streptavidin-FITC (Sav-FITC; BD PharMingen). A FACScalibur (BD) was used for cell acquisition and data analysis was carried out using CellQuest Pro (BD) or FlowJo (Tree Star) software. Eight-color flow cytometry. Five-hour ⁵¹Cr release assays were done to measure NK and CTL activity in spleen and lymph node cell suspensions as described previously (20, 27). For CTL activity, 5-day restimulation cocultures with irradiated (120 Gy) N50 cells were done as described (27, 29).

**Intracellular cytokine staining.** Lymphocytes from TDNLs were resuspended in complete RPMI, set up in 96-well plates at a concentration of 10⁶ cells per well, and incubated with PMA (5 ng/mL) and ionomycin (500 ng/mL) for 5 h. After 1 h incubation, brefeldin A was added at 10 μg/mL and monensin at 5 μg/mL. Finally, the cells were surface stained using NK markers, processed with the Fix & Perm kit (BD Biosciences), and stained for the intracellular cytokine IFN-γ. In case of T cells restimulated with irradiated N50 cells, a similar protocol was used, but surface staining with FITC-tagged anti-CD107a mAb (BD) was done before intracellular staining.

**In vitro antibody stimulation.** Naïve CD8+ lymphocytes were prepared from spleen cells from naive BALB/c mice by generating a single-cell suspension, which was enriched in CD8+ T cells by negative selection using immunomagnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). For stimulation with anti-CD3, 96-well plates were coated with 0.1 mL/well anti-CD3 mAb (145.2C11) at 1 μg/mL for 3 h at 37°C. When anti-CD28 (BD Biosciences) was used, it was added at 5 μg/mL. After 4-day incubation, CD137 expression was analyzed on CD3+ CD8+ population by fluorescence-activated cell sorting.

**Statistical analysis.** Kaplan-Meier plots were used to analyze survival. Prism software (GraphPad Software) was used to analyze tumor growth and the percentages of NK cells and to determine statistical significance of difference between groups by applying an unpaired Student’s t test. Comparison of survival curves was made by the log-rank test. P values < 0.05 were considered significant. For tumor burdens, comparison of means was done by ANOVA and post hoc analysis by Fisher’s PLSD test using Statview Software (SAS Institute).

**Results**

**Anti-CD137 mAb increases overall survival in aggressive plasmacytoma models, inducing long-lasting tumor-specific immunity.** Anti-CTLA-4, anti-CD40, and anti-CD137 are some of the most efficacious immunostimulatory mAbs (7). In numerous animal models, they have been shown to promote powerful tumor-specific T-cell responses capable of clearing established tumors. In addition, we have observed previously that ICAM-2-specific mAb exhibited antitumor activity in the CT26 mouse colon cancer model that is mediated by inhibition of activation-induced cell death in T lymphocytes (27, 28).

In this study, we examined and compared the therapeutic efficacy of these four immunostimulatory mAbs in the highly tumorigenic HOPC myeloma model. To this end, HOPC cells (5 × 10⁵ per mouse) were inoculated in the peritoneal cavity of BALB/c mice on day 0 and on days 4 and 7 the corresponding mAb was i.v. injected. In this experimental system, we found that both anti-CD137 and anti-CTLA-4 mAbs showed a clearly defined antymyeloma effect, with 40% to 50% of animals surviving long-term (>120 days), whereas anti-ICAM-2 and anti-CD40 mAbs at these dose regimes showed little and no therapeutic activity, respectively (Fig. 1A). It is noteworthy that whereas CD137, CTLA-4, and CD40 are absent from the plasma membrane of tumor cells, ICAM-2 is readily expressed (Fig. 1B).

To determine whether treatment with anti-CD137 or anti-CTLA-4 mAbs concomitantly elicits antitumor immunity that is long-lasting, mice that had been cured of the HOPC tumor by treatment with these two immunostimulatory mAbs were rechallenged s.c. with HOPC cells. We found that whereas
cured mice did not develop palpable HOPC tumors, all naive mice showed progressive tumor growth (Fig. 1C). In parallel, we observed that the long-lasting immune memory developed in mice cured by anti-CD137 or anti-CTLA-4 mAbs was specific for HOPC, because these mice did not reject the syngeneic CT26 tumor cell line inoculated on their opposite flank (Fig. 1C). These findings indicate that both anti-CD137 and anti-CTLA-4 mAbs may be useful for myeloma therapy.

Although there was previous published information about anti-CTLA-4 mAbs in plasmacytoma models (30), the potential of anti-CD137 mAbs that have a safer preclinical profile (31, 32) remains unexplored. Therefore, we next confirmed the antitumor effect of anti-CD137 mAb in another experimental plasmacytoma model. With this aim, NS0 myeloma cells ($5 	imes 10^5$) were inoculated i.p. in BALB/c mice on day 0, and on days 4 and 7 post-tumor cell inoculation, anti-CD137 mAb or the control rat IgG were i.v. injected. In this experimental system, we found that anti-CD137 mAb showed a potent therapeutic effect, with 70% to 80% of animals surviving long-term (>120 days; Fig. 2A).

Interestingly, neither HOPC cells nor NS0 cells expressed the CD137 molecule on their plasma membranes, indicating that the therapeutic effect of the agonistic mAb is not mediated via direct targeting of the malignant plasma cells (Figs. 1B and 2B).

We also evaluated the therapeutic effect of anti-CD137 mAb in established s.c. NS0 tumors that were clearly palpable before commencement of treatment. To this end, mice received a s.c. injection of $5 	imes 10^5$ NS0 cells on day 0 and on days 9, 11, 13, and 15 post-tumor cell inoculation were treated i.p. with either anti-CD137 mAb or control rat IgG. Consistent with our previous results, anti-CD137 mAb treatment resulted in profound inhibition of tumor growth and >60% of mice bearing NS0 tumors were completely cured (Fig. 2C). This robust therapy model was thereafter chosen to study the mechanistic requirements behind the therapeutic effects of anti-CD137 mAb.
NSO tumor rejections are accompanied by CTL induction. The spleen and TDLNs from the mice that had been cured from NS0 s.c. tumors by anti-CD137 treatment contained cells that on 5-day restimulation in culture with irradiated NS0 cells showed tumor specific cytolytic activity against NS0 cells in 51Cr release assays (Fig. 2D, right). Moreover, these cocultures contained CD8+ T cells that up-regulated intracellular IFN-γ and showed degranulation (measured as surface CD107a) specifically on reexposure to NS0 cells but not CT26 cells (Fig. 2D, left, dot plots). This indicates that CD8 T cells can recognize tumor antigens on NS0 cells.

Both NK and CD8+ T cells are required for tumor rejection. To identify the cell types responsible for the antitumor activity of anti-CD137 mAb, we carried out in vivo leukocyte subset depletion before anti-CD137 treatment. As shown in Fig. 3A, depletion of either NK cells or CD8+ T cells significantly impaired the therapeutic effect of the treatment. In this regard, we found that NS0 cells are almost as susceptible to NK-mediated lysis as the sensitive YAC-1 cells (Fig. 3A, inset, NK depletion) despite of the fact that NS0 cells intensely express surface MHC class I (Fig. 2B).

In vitro NK cytotoxicity was observed with NK cells obtained from poly(dI:dC)-preinjected
**Fig. 3.** Absolute requirements of IFN-γ, NK cells, and CD8+ T lymphocytes for eradication of NS0 plasmacytomas after anti-CD137 mAb treatment. 

**A,** involvement of CD4+ cells, CD8+ T cells, and NK cells in the eradication of tumors after anti-CD137 treatment as in Fig. 2C. BALB/c mice, in groups of 6, bearing s.c. NS0 tumors were injected i.p. with either anti-CD4 or anti-CD8 mAbs or i.v. with anti-asialo GM1 antisera. A total of 200 μg/dose of each mAb were injected into recipient mice for depleting CD4+ and CD8+ T cells and 50 μL/dose anti-asialo GM1 was administered for depleting NK cells. Both CD4- and CD8+-specific mAbs and anti-asialo GM1 antisera were administered as described in Materials and Methods. Fraction of surviving tumor-free mice is provided in each graph. Inset, NK cell depletion, specific lysis (mean ± SE) in 51Cr release assays showing the sensitivity of NS0 cells to killing by activated DX5 + NK cells isolated from the spleens of Rag1−/− mice that had been pretreated 18 h earlier with 50 μg poly(dI:dC) i.p. Lysis was compared with those achieved against YAC-1 cells and P815 targets. 

**B,** to determine whether IFN-γ was required for eradication of NS0-derived tumors after anti-CD137 mAb treatment, WT or IFN-γ−/− (IFN-γ KO) BALB/c mice (6 per group) were inoculated s.c. with 5 × 10^5 NS0 viable cells and then treated with either anti-CD137 mAb or control antibody. Alternatively, tumor-bearing mice (n = 6) were treated with anti-CD137 mAb and subsequently given 200 μg neutralizing anti-IFN-γ as described in Materials and Methods. Statistical analyses were done using the t test. These experiments were done at least twice, yielding similar results. Representative data. Compiled data for statistical analysis are presented in separate graphs for A and B.
Rag-II syngeneic mice. These NK cells unsuccessfully killed NK-resistant P815 cells in the same assays (Fig. 3A, inset, NK depletion graph).

In contrast, CD4+ T-cell depletion had no significant effect on tumor rejection. These results indicate that both NK and CD8+ T cells, but not CD4+ T cells, are required for tumor rejection. It is noteworthy that depletion of CD8+ subset was done with an anti-CD8α-depleting antibody to ensure that only peripheral CD8+ T cells but not CD8α+ dendritic cells were affected.

Normal function of IFN-γ is an absolute requirement for tumor rejection. IFN-γ production is critical for the cell-mediated antitumor immune response. Here, we examined whether IFN-γ was required for the antmyeloma effect of anti-CD137 treatment as described for other tumor models (33). To this end, both WT and IFN-γ-deficient mice were inoculated with NS0 cells and treated with anti-CD137 mAb or the control IgG as described in Materials and Methods. Whereas tumors in the WT BALB/c mice regressed after treatment, all of IFN-γ-deficient mice developed progressively growing tumors (Fig. 3B). Similarly, tumor regression was significantly impaired in mice that received a neutralizing anti-IFN-γ mAb (Fig. 3B). The fact that the anti-IFN-γ mAb did not completely abolish the therapeutic effect of anti-CD137 mAb may simply be a result of incomplete blockade. Therefore, tumor eradication after treatment with agonistic anti-CD137 mAb is dependent on IFN-γ.

Anti-CD137 mAb induced NK cell augment in TDLNs and IFN-γ production by NK cells. The abolishment of efficacy on NK cell depletion was remarkable (Fig. 3A). At the time when tumor rejections were first observed (8-10 days after treatment onset), both inguinal and axillary TDLNs had increased relative and absolute numbers of NK cells (CD3-DX5+ cells) when the mice had been treated with anti-CD137 mAb (Fig. 4A). We wondered if such NK cells under anti-CD137 treatment would also show a higher degree of activation and we found that DLN NK cells showed similar levels of CD69 expression regardless of the antibody used for treatment. However, anti-CD137 treatment up-regulated the capability of NK cells in DLN to produce IFN-γ as assessed by intracellular staining (Fig. 4B). These effects on NK biology at the DLN are likely involved in the absolute requirement of NK cells for the antitumor effects.

Tumor infiltrate of NSO tumors that are responding to anti-CD137 treatment are enriched in CD8 T cells. Cell suspensions from NSO tumors taken from mice that have been treated with anti-CD137 and showed signs of growth delay or shrinkage were obtained to study their lymphocyte content. It was found that there was an increase of CD8 T cells, whereas NK and CD4 cells were not increased in the tumor rejecting infiltrate.
(Fig. 4C). These data indicate that the main role at the final execution of tumor rejection corresponds to CD8 T cells that abundantly populate the tumor lesion. This does not preclude that NK cells could be playing a role at earlier stages or that they cooperate with CTLs in an orchestrated fashion (29). However, percentage and absolute numbers (data not shown) of intratumor leukocytes provide evidence indicating that CTLs are the main players when rejections become clinically meaningful.

**Antitumor effect of anti-CD137 is independent of CD28.** To determine whether costimulation through CD28 is a prerequisite for CD137 signaling, we first investigated whether triggering the CD28 pathway contributes to the up-regulation of CD137 on naïve T cells. To this end, purified CD8+ T cells were stimulated in vitro with plate bound anti-CD3 mAb in the presence or absence of soluble anti-CD28 mAb. We observed that TCR triggering by anti-CD3 mAbs in combination with CD28 costimulation was much more efficient at inducing surface expression of CD137 on CD8+ T cells than TCR-CD3 triggering alone (Fig. 5A).

Based on these results, we hypothesized that the in vivo blockade of the CD28 pathway would abrogate the antmyeloma effect of anti-CD137 mAb. To test this hypothesis, both WT and CD28-deficient mice were inoculated with NS0 cells and treated with anti-CD137 mAb or the control rat IgG as described before. Surprisingly, the antmyeloma effect of anti-CD137 mAb was preserved in CD28-deficient mice. Thus, although CD28 signaling increases the expression of CD137 on CD8+ T cells in vitro, the therapeutic effect of anti-CD137 is independent of CD28 (Fig. 5B).

**Anti-CD137 decreased tumor burden in 5TGM1 myeloma-bearing mice.** To extend our observations about the antiplasmacytoma effect of anti-CD137 to a multiple myeloma model considered more relevant to the human disease, we used the 5TGM1 cell line transfected with the EGFP gene to accurately monitor tumor progression (22). This cell line does not express detectable levels of CD137 on its plasma membrane on fluorescence-activated cell sorting analysis (data not shown). I.v. injection of these tumor cells in naïve syngeneic C57BL/KaLwRijHsd mice gives rise to a disseminated tumor model in which fluorescent tumor cells can be visualized as tumoral foci in multiple organs in a distribution that very closely resembles severe cases of multiple myeloma in humans. This is true particularly with regard to skeletal involvement (Fig. 6A and B). An anti-CD137 mAb treatment course started 4 days after tumor cell inoculation clearly reduced multiorgan tumor burden in a significant proportion of mice (Fig. 6A and B), although it did not attain the efficacy achieved by a course of bortezomib in a similar experimental schedule (Fig. 6A). Anti-CD137 mAb-mediated reduction of tumor burden was evident in the skeleton of 7 of 10 mice, and although tumor disappearance was complete in only 3 of 7 mice, the tumor fluorescent foci in the remaining four mice was nonetheless significantly reduced and comparable with those seen in bortezomib-treated mice. The efficacy of anti-CD137 mAb treatment was even more dramatic in visceral
Fig. 6. Anti-CD137 mAb treatment significantly decreases tumor burden in a disseminated multiple myeloma model. To investigate the antitumor efficacy of anti-CD137 mAb in a model of myeloma with widespread skeletal involvement, 5TGM1-GFP myeloma cells (10^5) were i.v. injected into syngeneic C57BL/KaLwRijHsd mice (≥8 per group) and then randomly assigned to four groups, which received either vehicle, bortezomib, control rat IgG, or anti-CD137 mAb for 4 wk (see Materials and Methods for dosing protocol). A, fraction of mice with detectable EGFP+ foci in the indicated organs of mice immediately after sacrifice on day 30. Tissues were optically imaged, as described in Materials and Methods, in anteroposterior and posteroanterior orientations and then scored. Percentage of mice in each group with ≥2 fluorescent foci in both orientations in ≥3 bones. B, representative pictures of green fluorescence emitted (anteroposterior and posteroanterior views) of mice from A that had been eviscerated during necropsy and the corresponding pictures from the explanted organs. In the case of anti-CD137 mAb, the mice photos shown are from representative animals displaying either fluorescence under the threshold of detection (representing completely eradicated tumor/no residual disease) or reduced but detectable fluorescence (representing some residual disease) as indicated in the figure. C, serum concentrations (mean ± SE) of the 5TGM1 monoclonal paraprotein (IgG2b) from mice in the different treatment groups measured 30 d after tumor cell inoculation.
is clearly efficacious in this multiple myeloma model even as monotherapy and further suggest the clinical potential of the approach.

Discussion

This study shows that pharmacologic agonistic manipulation of CD137 with a specific mAb shows benefit in various murine myeloma models and provides important clues on the involvement of CD8+ T lymphocytes and NK cells in the therapeutic immune response. The effects of anti-CD137 mAb are not directed against the malignant plasma cells but indirectly mediated by immune regulation as suggested by the absence of CD137 from the cell membrane of the different plasmacytomas used.

It has been postulated that the antibody engages CD137 expressed on activated T cells and maybe on some activated NK cells in the tumor-bearing mice (7). CD137 induction on CD8+ T lymphocytes must be the result of tumor antigen presentation by either tumor cells or professional antigen-presenting cells. The advantageous effects of CD137 ligation on CTL differentiation and memory have been extensively reported (34–37). However, the direct or indirect pathway that leads in vivo to the activation of NK cells, as we have observed in DLN, is still unclear (38). We are investigating whether ligation of the CD137 molecules expressed on NK cells plays a role in tumor rejection. In this regard, we have recently reported that tumor rejection is often the result of the concerted action of multiple leukocyte partners, including NK cells, which play a pivotal role and can be found to be producing IFN-γ in vivo (29). The finding of the sensitivity of NSO cells to autologous NK cells despite high-surface MHC class I indicates the expression of ligands for NK activatory receptors (38).

NK-cell production of IFN-γ at sentinel lymph node have been reported to be important in the initiation of T-cell immunity, and tumoricidal NK cells have been detected in tumor rejecting infiltrates (38). In our case, NK cells at TDLN show activation features and numeric increases that could be dependent on either recruitment or local proliferation of NK cells. In spite of the absolute need of NK cells to achieve efficacy, it is clear that, at the time when the tumors start to be clinically controlled by treatment, the rejecting infiltrate is dominated by CTLs. More investigation is needed to precisely define the train of events that leads to tumor rejection through the cooperative crosstalk between NK and CTLs. Our results further highlight an important role for NK cells operating at the draining lymph node (38).

IFN-γ is a pleiotropic cytokine and it has been reported to induce changes in tumor endothelial cells, thereby facilitating homing of effector T cells into solid tumors, and to inhibit angiogenesis. In addition, IFN-γ may damage tumor cells by means of antiproliferative and proapoptotic signaling pathways (39) while up-regulating antigen presentation as well as CTL and macrophage activity (40). In our hands, recombinant IFN-γ had no effect on the proliferation and survival of NS0 plasma cells in culture (data not shown), strongly suggesting that it plays an indirect role in the efficacy of anti-CD137 mAb treatments. The critical requirement of IFN-γ for tumor rejection suggests its potential as a biomarker to correlate with efficacy in eventual clinical trials for myeloma.

The injected therapeutic antibody can bind CD137 on other leukocyte and endothelial cell lineages (17). The role of these interactions at inducing tumor immunity remains unexplored. We can conclude from selective depletions that intervention of CD4+ T cells is dispensable at least in the NS0 model. In this sense, it has been recently reported that, in mouse melanoma, CD4 depletion is even beneficial for antitumor effects (41).

We confirm that anti-CTLA-4 mAb can also have a role in the treatment of myeloma (30) in a fashion that looks comparable with the effects anti-CD137 mAb as observed in an i.p. plasmacytoma model in which suboptimal efficacy is attained by both antibodies. The mechanism of action of anti-CTLA-4 has been described by the group of Allison (42) and in essence involves blockade of anti-CTLA-4-negative signals, in such a way that the costimulatory activity of CD28 is liberated. Therefore, the mechanism of action of anti-CTLA4 mAb is different from that of anti-CD137 mAb, although both converge in the generation of a tumor-rejecting lymphocyte infiltrate. It is noteworthy that tumor cells used in this work express CD80 that may bind CTLA-4 and CD28 on activated T cells. Thus, in these cases, CTLA-4 mAb could block this inhibitory interaction on in vivo lymphocyte-tumor cell encounter (43).

Over the last few years, there has been intense interest in whether the CD80/86-CD28 costimulatory pathway is required for CD137 mAb activity. In 1997, DeBenedette et al. reported that ligation of CD137 can costimulate human and mouse CD28− T cells (44), suggesting that CD137 triggering can take place independently of the CD28 pathway (45). This group also showed that enforced costimulation through CD137 by CD137L could promote antitumor responses in the presence or in the absence of CD28 (46). However, Diehl et al. (47) recently reported that blockade of the costimulatory pathway abrogated the capacity of agonistic anti-CD137 mAb to trigger CTL immunity in response to the Ad5E1A peptide vaccine. This was probably because, under weaker TCR signals, CD137 expression on naïve T cells is not attained; therefore, the susceptibility of these cells to anti-CD137 mAbs required both stimulation of the TCR and CD28-dependent costimulation. However, in our hands, CD28 function is dispensable for CD137 activity, albeit it is easy to find CD8+ T-cell activation conditions in which CD28 costimulation results in much higher CD137 expression by CD8+ T cells in vitro as suggested previously by experiments with the natural ligand (48).

This study describes the therapeutic effects of anti-CD137 mAb as monotherapy in myeloma, but it is clear that CD137-targeted therapy would be most efficacious in combination immunotherapy. Combinations of tumor vaccines, adoptive T-cell therapy, chemotherapy, and/or radiotherapy are known to display additive and synergistic effects in cancer, and combination therapies of anti-CD137 mAb and other immunostimulatory mAbs have shown remarkable activity in other tumor models (7, 32, 49, 50). However, for practical reasons, work on myeloma models should first define anti-CD137 combination potential with standard treatments for multiple myeloma such as bone marrow transplantation and chemotherapy.

The significant reduction of tumor burden in the experiments done with the 5TGM1 model is remarkable due to the nature of the systemic dissemination of this experimental disease. The comparison of anti-CD137 mAb with bortezomib seems to
favor the latter agent but should be considered with caution. On the one hand, bortezomib has direct cytotoxic effects on myeloma cells and likely acts immediately on the tumor cells in vivo, whereas the full effect of the anti-CD137 mAb would need a latency period until the immune response is sufficiently up-regulated. On the other hand, the full potential of anti-CD137 mAb has yet to be exploited, in particular with regard to combination strategies either with a tumor vaccine (25) or an intervention to increase tumor immunogenicity. There is also the possibility of combining suboptimal doses of bortezomib with anti-CD137 mAb to reduce some of the well-known side effects of bortezomib such as neuropathies. The use of the 5TGM1 myeloma model for such combination therapy studies in the future has obvious advantages because it uses immunocompetent mice and accumulation of the monocalonal paraprotein in serum accurately reflects tumor burden. In addition, transfection of 5TGM1 cells with EGFP permits image assessment (22) of tumor load in real-time without increasing immunogenicity, because CTLs do not recognize EGFP as foreign in the H-2^d class I molecules (29). There are several rationale for proposing clinical trials with agonist anti-CD137 mAb in myeloma: (a) the results of the present study on three different myeloma mouse models, including the disseminated 5TGM1 model that shares many characteristic features with the human disease; (b) a safe clinical leading profile (ASCO 2008, abstract 3007, and ref. 31); (c) the abundance of NK cells and memory T cells in the bone marrow, which is the most common primary site for malignant plasma cells in myeloma (1); and (d) an agent of this kind (BMS663513) is already undergoing phase I and II trials for melanoma, renal cell carcinoma, lung cancer, and ovarian cancer. The effects of the anti-CD137 mAb on the disseminated 5TGM1 model reported herein provide a baseline to further optimize combination strategies and improve preclinical efficacy to guide future clinical trial design.

Disclosure of Potential Conflicts of Interest

I. Melero has received research grants and other research support and is on the speakers’ bureau.

Acknowledgments

We thank Dr. L. Chen both for providing an agonist anti-CD137 producing hybridoma as well as for helpful criticism and discussion; Dr. James Allison for providing anti-CTLA-4 mAb-producing hybridoma cells; Drs. Jesús Prieto, Ascensión Lopez-Díaz de Cerio, Mercedes Rodríguez-Calvillo, Maurizio Bendandi, and Juan José Lasarte for support and scientific discussion; and Elena Cordia, Javier Guillén, Juan Peraez, and Eneko Elizalde for excellent animal care.

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