Therapeutic effect of CD137 immunomodulation in lymphoma and its enhancement by $T_{reg}$ depletion

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Running title: Anti-CD137 antibody therapy in lymphoma
Abstract

Despite the success of passive immunotherapy with monoclonal antibodies (mAbs), many lymphoma patients eventually relapse. Induction of an adaptive immune response may elicit active and long-lasting antitumor immunity, thereby preventing or delaying recurrence. Immunomodulating mAbs directed against immune cell targets can be used to enhance the immune response in order to achieve efficient antitumor immunity. Anti-CD137 agonistic mAb has demonstrated antitumor efficacy in various tumor models and has now entered clinical trials for the treatment of solid tumors. Here, we investigate the therapeutic potential of anti-CD137 mAb in lymphoma. We found that human primary lymphoma tumors are infiltrated with CD137+ T cells. We therefore hypothesized that lymphoma would be susceptible to treatment with anti-CD137 agonistic mAb. Using a mouse model, we demonstrate that anti-CD137 therapy has potent anti-lymphoma activity in vivo. The antitumor effect of anti-CD137 therapy was mediated by both NK and CD8 T cells and induced long-lasting immunity. Moreover, the antitumor activity of anti-CD137 mAb could be further enhanced by depletion of T_{reg} cells. These results support the evaluation of anti-CD137 therapy in clinical trials for patients with lymphoma.
Introduction

Lymphoma is responsive to immunotherapy\(^1\). Despite the success of passive immunotherapy with monoclonal antibodies (mAb) directed against tumor cells (e.g. anti-CD20, rituximab), many lymphoma patients eventually relapse. Active immunotherapy for the treatment of lymphoma aims to induce an adaptive and long-lasting antitumor immune response to prevent or prolong time to recurrence. Although antitumor immune cells can be found in cancer patients, these cells may be rendered ineffective in eradicating cancer due to tumor-induced immunosuppression\(^2\). Monoclonal antibodies that target and modulate the function of tumor-reactive immune cells may enhance antitumor immune responses to therapeutic levels\(^3\). Targeting the immune environment of the tumor as opposed to the malignant cells presents unique advantages. Whereas targeting tumor cells with mAbs requires a tumor-specific antigen (Ag), stimulating or inhibiting non-malignant immune cells is expected to be applicable across different patients and cancer types. Further, unlike tumor cells that may mutate into therapy-resistant clones under selective pressure of mAb treatment, normal immune cells are not expected to be clonally selected for such mutations. Finally, whereas tumor-directed mAbs are considered passive immunotherapy and therefore only offer transient efficacy, targeting the immune system with mAbs aims to induce and/or potentiate an active and long-lasting immune response against cancer.

Antibody-mediated T cell modulation can be accomplished in several ways, including: (1) enhancing co-stimulation on conventional T cells (T\(_{\text{conv}}\)) (e.g., agonistic anti-CD137 and anti-OX40 mAbs); (2) blocking negative signals on T\(_{\text{conv}}\) (e.g. antagonistic anti-CTLA4 mAb); or (3) abrogating regulatory T cell (T\(_{\text{reg}}\))-mediated suppression (e.g. agonistic anti-GITR mAb)\(^3,4\). Some of these mAbs are thought to have multiple effects. For example, anti-OX40 and anti-GITR mAbs trigger costimulatory molecules on T\(_{\text{conv}}\) as well as block the suppressive function of T\(_{\text{reg}}\)\(^5\)-\(^9\).

CD137 (4-1BB) is a surface glycoprotein that belongs to the tumor-necrosis factor receptor superfamily (TNFRSF). CD137 is broadly inducible on immune cells including activated CD4 and CD8 T cells, T\(_{\text{reg}}\) cells, natural killer (NK) cells, NK-T cells, monocytes, neutrophils, and dendritic cells\(^10\). On T cells specifically, CD137 functions as a co-stimulatory receptor induced upon T cell receptor (TCR) activation. Binding of CD137 to its ligand leads to increased T cell proliferation, cytokine production, functional maturation, and prolonged CD8 T cell survival\(^10\). The effect of CD137 ligation on T\(_{\text{reg}}\) is not as clearly understood, with conflicting reports showing both stimulation and inhibition of the immunosuppressive functions of these cells\(^10\)-\(^14\). Consistent with the co-stimulatory function of CD137 on T\(_{\text{conv}}\), 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 tumor models including sarcoma, mastocytoma, glioma, colon carcinoma, and myeloma\(^3,15\).

Anti-CD137 mAb has now entered clinical trials for solid tumors (melanoma, renal cell carcinoma, lung cancer and ovarian cancer) but little is known about its potential therapeutic effect in lymphoma. In this study, we investigated the potential relevance of anti-CD137 therapy in lymphoma. We found that bulk tumor specimens from lymphoma patients over-expressed CD137 mRNA compared to other tumor types. Single cell analysis performed on primary lymphoma samples of various histologies demonstrated that CD137 was not expressed on the tumor cells but by
tumor-infiltrating T cells. This suggested that the target of anti-CD137 mAb was present and selectively expressed on cells with potential antitumor activity in lymphoma patients. Using a mouse model, we next examined the antitumor effect of anti-CD137 agonistic mAb in vivo and investigated the contribution of various immune cell types to the therapy, including regulatory T cells. Our findings support the evaluation of anti-CD137 agonistic mAb in clinical trials for patients with lymphoma and suggest approaches to optimize this therapy.

Materials and Methods

Analysis of Microarray Gene Expression Data for CD137 mRNA expression

CD137 mRNA (TNFRSF9) expression data were analyzed across diverse histopathological tumor groups in two independent microarray datasets (Ramaswamy et al.16; expO intgen URL https://expo.intgen.org/geo/) obtained from Oncomine17. Using a 2-tailed Student’s t-test, log2 relative gene expression measurements were analyzed for tumor specific patterns of expression in the primary dataset (n=184, U03397_s_at, Ramaswamy et al.16). After identification of biased over-expression of TNFRSF9 in follicular lymphoma (FL, n=11) and diffuse large B cell lymphoma (DLBCL, n=11), over-expression of TNFRSF9 in a second cohort of lymphomas (n=21) was independently validated in a separate microarray dataset (207536_s_at; NCBI GEO GSE2109).

CD137 expression on Human Primary Lymphoma Specimens

Tumor biopsies were obtained from patients with FL or mantle cell lymphoma (MCL) at Stanford Hospital or from patients with de novo DLBCL or MCL at the Norwegian Radium Hospital. Biopsies were obtained with informed consent in accordance with the Declaration of Helsinki and this study was approved by Stanford University's Administrative Panels on Human Subjects in Medical Research and the regional Committee for Medical Research Ethics, Region Eastern Norway, University of Oslo. Tumor specimens were obtained before patients had received any treatment and single cell suspensions were prepared and frozen as previously18,19. PBMC from healthy individuals were isolated using density gradient separation Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and subsequently analyzed as described for FL specimens. For immunophenotyping, an individual cryotube was thawed, washed, and resuspended in RPMI + 10% fetal bovine serum. Thawed cells were allowed to rest at 37 °C for 15 minutes in a 5% CO2 tissue culture incubator, before staining with mAbs and FACS analysis.

Cell lines and mice

The A20 cell line, a BALB/c B cell lymphoma expressing MHC class I and class II H-2d molecules, was obtained from the American Type Culture Collection. Tumor cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone Laboratories), 100U/mL penicillin, 100µg/ml streptomycin (both from Invitrogen Life Technologies), and 50µM 2-ME (Sigma-Aldrich). Cells were grown in suspension culture at 37°C in 5% CO2.

Eight to ten weeks old female BALB/c mice were purchased from Charles River Laboratories and were housed at the Laboratory Animal Facility at the Stanford University Medical Center (Stanford, CA). All experiments were conducted in accordance with Stanford University Animal Facility and National Institutes of Health guidelines.
Tumor transplantation and immunotherapy

A20 lymphoma cells were implanted into 8-10 weeks old female BALB/c mice at a dose of 5x10^6 cells in 50μL of RPMI by the subcutaneous (s.c.) route into the abdomen. In vivo systemic administration of monoclonal antibodies against T cell targets was performed by intra-peritoneal (i.p.) injections. Anti-CD137 (4-1BB) mAb (Rat IgG2a, clone 2A20), anti-OX40 (CD134) mAb (Rat IgG1, clone OX86; European Collection of Cell Cultures), anti-CTLA4 (CD152) mAb (Hamster IgG, clone 9H10; kind gift from Dr. J. Allison), anti-GITR mAb (Rat IgG2b, clone DTA-1; kind gift from Dr. S. Sakaguchi), and anti-FR4 mAb (Rat IgG2a, clone TH6; kind gift from Dr. S. Sakaguchi) were produced from ascites in SCID mice. None of the targeted molecules (CD137, OX40, CTLA4, GITR, FR4) was expressed on the surface of A20 tumor cells (data not shown). IgG from rat serum was used as control antibody for anti-CD137 mAb and obtained from Sigma-Aldrich. Antibodies used for T cell modulation in Figure 2 were given i.p. on days 5 and 10 post tumor inoculation at what is generally considered to be the optimal dose according to prior publications: 150μg per injection for anti-CD137, 400μg per injection for anti-OX40, 100μg per injection for anti-CTLA4, and 500μg per injection for anti-GITR. In all other experiments, anti-CD137 mAb was given as a single i.p. injection on day 6 at a dose of 150ug per mouse unless otherwise specified. The growth of tumor was monitored by a caliper twice a week, and expressed as length by width in square centimeters. Mice were sacrificed when tumor size reached 4cm^2 or when tumor sites ulcerated.

Detection of tumor-reactive T cells

BALB/c mice that have been cured with anti-CD137 therapy for more than 100 days were re-challenged s.c. with A20 tumors. A week later, splenocytes from αCD137-cured mice or control splenocytes from naïve mice were harvested, made into single-cell suspensions, and the red blood cells were lysed. A total of 5x10^5 splenocytes were cocultured with 5x10^5 irradiated A20 cells for 24 hours at 37ºC and 5% CO_2. Monensin was added during the last 8 hours of culture. Afterwards, cells were washed and stained with anti-mouse mAbs as indicated.

Depletion of NK cells, CD4 T cells, CD8 T cells, and T_{reg} cells

Ascitic fluid was harvested from SCID mice bearing hybridoma GK1.5, 2.43 and TH6 producing anti-CD4 (Rat IgG2b), anti-CD8 (Rat IgG2b) and anti-FR4 (Rat IgG2a, clone TH6; kind gift from Dr. S. Sakaguchi) mAb, respectively. The ascites were diluted in sterile PBS. Anti-Asialo GM1 antiserum was used to deplete NK cell activity in vivo and was purchased from Wako. Depleting antibodies were injected i.p. on day -1, day 0 of tumor inoculation and every 5 days (or every 4 days for anti-FR4 mAb) thereafter for 3 weeks at a dose of 50uL per injection for anti-Asialo GM1, 500μg per injection for anti-CD4 and anti-CD8, and 100ug per injection for anti-FR4. The depletion conditions were validated by flow cytometry of peripheral blood showing more than 95% depletion of CD4 and CD8 T cells. T_{reg} depletion following anti-FR4 treatment was confirmed by flow cytometry of peripheral blood showing ~80% depletion, similar to previous publication.

Antibodies and FACS analysis

The following mAbs to human antigens were used for staining of human primary lymphoma specimens: CD4 Pacific Blue, CD8 FITC, CD20 APC-Cy7, CD25 PE and CD45RO PE-Cy7 (all from Becton Dickinson Biosciences (BD), CA), CD3 QD605 from Invitrogen, CD137 APC (clone 4B4-1) from Biosource, and FoxP3 (clone PCH101) from eBioscience. The following mAbs to mouse antigens were used: anti-CD8 FITC, anti-CD4 PerCP, and anti-
CD44 APC mAb (BD). Intracellular IFN-γ expression was assessed using BD Cytofix/Cytoperm kit (catalog no. 554722) as per instructions. To detect FoxP3, single cell suspensions from spleens or tumors were stained with CD25, CD3, CD4 and CD8 (all BD) in addition to CD137 (eBioscience). Cells were washed and then fixed, permeabilized and stained with anti-FoxP3 (eBioscience, clone FJK-16s), using the eBioscience protocol. Stained cells were collected on a FACSCalibur or a LSRII three-laser cytometer (BD) and data were analyzed using Cytobank (http://www.cytobank.org).

Statistical analysis

Prism software (GraphPad software) was used to analyze tumor growth and to determine statistical significance of difference between groups by applying an unpaired Student’s t test. Kaplan-Meier plots were used to analyze survival. 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.

Results

Tumor-involved lymph nodes from lymphoma patients express high levels of CD137 mRNA and are infiltrated by CD137+ T cells

To investigate whether anti-CD137 mAb would be a clinically translatable therapy for patients with lymphoma, we first examined expression of CD137 mRNA (TNFRSF9) in bulk tumor specimens from lymphoma patients and compared it to a variety of other tumors. Using publicly available microarray gene expression data, we found that bulk tumor specimens from patients with follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) significantly over-expressed TNFRSF9 when compared to non-lymphoma tumor specimens (Fig. 1A, p<0.0001 for each comparison). Aside from FL and DLBCL, no other histopathological group significantly over-expressed TNFRSF9 when compared to other tumor types. The over-expression of TNFRSF9 in non-Hodgkin’s lymphomas (n=21) as compared with a diverse group of tumors from 1898 patients was validated in an independent dataset (p<0.0001).

Lymphoma tumors contain significant numbers of tumor-infiltrating non-malignant T cells. In order to identify which cells within the tumor specimens expressed CD137, we used flow cytometry to examine lymph node biopsies from untreated lymphoma patients. We did not observe any significant expression of CD137 on the tumor cells in any non-Hodgkin lymphoma (NHL) samples tested (Fig. 1B and data not shown): FL (n=35), mantle cell lymphoma (MCL, n=29), and DLBCL (n=18). In contrast, we found a significant percentage of CD137+ cells among tumor-infiltrating T cells in all lymphoma subtypes tested (Fig. 1B and C, and Suppl. Fig. 1). This percentage was significantly higher as compared to T cells from peripheral blood of healthy individuals (p<0.0001 for all NHL). This difference remained significant when CD4 and CD8 T cell subsets were analyzed separately (p<0.0001 for both). However, the percentage of CD137+ tumor-infiltrating T cells was generally higher in the CD8 compartment than in the CD4 compartment (Fig. 1B and C, and Suppl. Fig. 1B). In the CD8 compartment, the mean percentage of CD137+ cells in healthy PBMC, FL, MCL, and DLBCL was 0.9%, 6%, 15.4% and 18.7%, respectively (Fig. 1C). CD137+ tumor-infiltrating T cells were almost exclusively confined to the CD45RO+ memory compartment (Fig. 1B). These results show that lymphoma tumor specimens over-express CD137
compared to other tumor types, presumably not due to the tumor cells themselves but to the presence of CD137+ tumor-infiltrating T cells. Our observation suggests that lymphoma patients may therefore be sensitive to anti-CD137 therapy.

**Anti-CD137 agonistic mAb has potent anti-lymphoma activity *in vivo***

Based on our observations in human primary lymphoma samples, we next assessed the therapeutic potential of anti-CD137 agonistic mAb *in vivo* using the A20 mouse model of lymphoma. In this model, we have previously reported that anti-OX40, anti-CTLA4, and anti-GITR mAbs exhibit antitumor activity when administered in combination with a CpG-based vaccine. We compared these mAbs to anti-CD137 mAb as monotherapy for the treatment of established A20 tumors. A20 lymphoma cells (5x10⁶ per mouse) were inoculated s.c. in BALB/c mice on day 0 and the corresponding mAb was injected i.p. on days 5 and 10. At the time of treatment, the tumor was well established with the largest diameter measuring about 5mm. At 100 days post tumor inoculation, 60% of the mice treated with anti-CD137 mAb had no evidence of tumor whereas only 10% of animals treated with anti-OX40, anti-CTLA-4 or anti-GITR mAbs remained tumor-free (Fig. 2A and B). Antitumor effects were not due to direct targeting of the tumor as the A20 tumor cells did not express CD137, OX40, CTLA4, nor GITR (data not shown).

**Anti-CD137 mAb efficacy is time dependent**

To determine the optimal schedule for administering anti-CD137 mAb, BALB/c mice were inoculated s.c. with A20 lymphoma cells and subsequently treated with a single injection of anti-CD137 mAb at various time points after tumor challenge. Surprisingly, treatment at early time points (days 0 and 3) resulted in no tumor rejection whereas treatment at later time points (days 6 and 9) resulted in significant tumor regression and increased overall survival curing 30-40% of the mice for greater than 100 days (Fig. 3). Treatment at day 12 or later failed to cure the mice (data not shown), potentially due to excessive tumor burden.

**Mice cured with anti-CD137 mAb develop long-lasting antitumor immunity**

To determine whether anti-CD137 therapy elicits long-lasting antitumor immunity, mice that had been successfully treated with anti-CD137 mAb were re-challenged s.c. with A20 lymphoma cells (5x10⁶ per mouse) at a different site (on the back) from original tumor challenge. One hundred days after successful treatment, mice rejected this tumor re-challenge while naïve mice all died from progressive tumor growth (Fig. 4A and B).

We also observed that mice cured with anti-CD137 treatment contained antitumor IFN-γ producing CD8 T cells. Splenocytes from cured mice and naïve control mice were re-stimulated with irradiated A20 tumor cells for 24h. CD8 T cells from treated but not from naïve spleens produced IFN-γ in response to re-stimulation (Fig. 4C). IFN-γ producing CD8 T cells were restricted to the CD44hi memory T cell population (Fig. 4D), consistent with the finding that these mice elicited long-lasting protection against the tumor.

**Anti-CD137 therapy requires NK cells and CD8 T cells and is enhanced by CD4 T cell depletion**

We next investigated the requirements for the antitumor response generated by anti-CD137 mAb. To identify the immune cells responsible for the antitumor activity of anti-CD137 mAb, we carried out *in vivo* leukocyte subset depletion prior to anti-CD137 treatment. Depletion of either NK cells or CD8 T cells significantly impaired the
therapeutic effect of anti-CD137 treatment (Fig. 5A and B). In contrast, CD4 T cell depletion significantly improved efficacy of anti-CD137 therapy (90% vs. 20% overall survival at day 100, p=0.0018).

To understand the changes induced by anti-CD137 therapy, we analyzed tumors and spleens from tumor-bearing mice 5 days after anti-CD137 treatment and compared them to untreated mice. We found that anti-CD137 therapy was accompanied by a significant reduction of Treg cells (Fig. 5C and D, p=0.004) and an increase of CD8 T cells at the tumor site (Fig. 5E). This result further confirmed the importance of CD8 T cells in mediating the antitumor effect of anti-CD137 mAb.

**Selective depletion of Treg cells enhances anti-CD137 therapy**

The finding that CD4 T cell depletion enhanced anti-CD137 therapy led us to hypothesize that CD25+FoxP3+ Tregs contained in the CD4 compartment may impair the efficacy of anti-CD137 therapy. Anti-Folate Receptor 4 (FR4) mAb has been described to selectively deplete Treg cells while sparing other cell populations. Using this mAb, we selectively depleted Treg cells and determined whether this treatment improved anti-CD137 therapy. Treg depletion was measured in peripheral blood as described in the original publication. Anti-FR4 mAb depleted ~80% of the FoxP3+ Treg population from the blood and only removed a minority of FoxP3- CD4 Tconv (<25%). Anti-FR4 mAb also preserved CD8 T cells as no CD8 depletion was observed (data not shown). Following FR4 depletion, mice received a single dose of anti-CD137 mAb at day 6 or control rat IgG. Selective Treg depletion significantly enhanced the efficacy of anti-CD137 therapy, raising the overall survival from 40% to 100% (p=0.004) after 70 days (Fig. 6). Although CD137 could be expressed on Treg cells in tumor-bearing mice, there was no increase in their suppressive capacity or their ability to proliferate in vitro following treatment with anti-CD137 mAb (data not shown). This result is consistent with the observation that Treg cells were not increased (but instead decreased) at the tumor site following anti-CD137 therapy in vivo (Fig. 5C and D). These results suggest that Tregs did not directly benefit from anti-CD137 therapy but impaired the efficacy of anti-CD137 therapy due to independent immune suppression.

**Discussion**

Immunomodulation with anti-CD137 agonistic mAb has demonstrated efficacy in several tumor models and has been translated to Phase I and II clinical trials for solid tumors (NCT00309023, NCT00612664). Application of this novel therapy has not been previously tested in lymphoma. In the present study, we used human primary lymphoma samples as well as an in vivo mouse model to assess the potential relevance of anti-CD137 therapy in lymphoma.

We found that bulk tumor specimens from patients with lymphoma significantly over-expressed CD137 mRNA when compared to non-lymphoma tumor specimens. Using a single cell approach, we showed that the tumor B cells are uniformly negative for CD137 while there is a significant population of CD137+ tumor-infiltrating T cells (Fig. 1). These findings have important consequences. First, the absence of CD137 expression on the tumor cells addresses the concern that anti-CD137 agonistic mAb might stimulate the malignant cells. Second, the presence of a significant amount of CD137+ T cells at the tumor site as seen in all three NHL subtypes tested (FL, MCL,
and DLBCL) is suggestive of a therapeutic target for anti-CD137 mAb. Indeed, CD137+ tumor-infiltrating T cells are thought to be tumor-reactive cells that can potentially be targeted and stimulated by the agonistic mAb. More specifically, the number of CD137+ tumor-infiltrating T cells was particularly high in the CD8 compartment – an observation that is important as it has been shown that the therapeutic effect of anti-CD137 mAb is mediated through the engagement and stimulation of CD8 T cells in most, if not all, preclinical tumor models3, including ours. We hypothesize that a correlation will exist between the amount of CD137 expressing T cells and the response to therapy in anti-CD137 treated cancer patients. Both ongoing and future clinical trials with anti-CD137 mAb should investigate this relationship. Third, over-expression of CD137 mRNA in lymphoma patients (presumably due to the presence of CD137+ tumor-infiltrating T cells) compared to other tumor types suggests that lymphoma may be particularly sensitive to anti-CD137 therapy.

Having shown that lymphoma patients harbor CD137+ tumor-infiltrating T cells that could be targets for anti-CD137 therapy, we next evaluated the therapeutic effect of anti-CD137 mAb in vivo. Using a mouse model of lymphoma, we found that anti-CD137 agonistic mAb had potent antitumor activity in vivo, curing large and established tumors (Fig. 2). The antitumor effect was not due to direct targeting of malignant cells as the murine tumor cells did not express CD137 but instead to immunomodulation of antitumor immune cells. In our model, anti-CD137 mAb appeared to be significantly more potent as a monotherapy than other immunomodulating mAbs previously studied (anti-OX40, anti-CTLA4, anti-GITR). However, this observation should not preclude the potential interest of these mAbs in lymphoma as we have previously shown that they could be successfully used in combination strategies26. We also found that anti-CD137 treatment was most effective when administered after tumors had become established (Fig. 3). This temporal dependency of anti-CD137 therapy may suggest that a critical tumor burden or “tumor antigenic load” must be present, likely for the purpose of immune cell activation upon which these cells up-regulate CD137 and become sensitive to stimulation with the mAb. Consistent with this hypothesis, we showed that anti-CD137 therapy required NK and CD8 T cells. Additionally, it has been shown that administration of anti-CD137 mAb during early infection of LCMV induces suppression and death of virus-specific T cells while injection of anti-CD137 mAb in later stage enhances CD8 T cell-mediated antiviral immunity27, suggesting that CD8+ T cells are programmed differently in the early and late responses in responding to anti-CD137 mAb. This result may have clinical relevance, as patients present with pre-existing and sometimes large tumor burden. Anti-CD137 therapy also induced long-lasting immunologic memory as successfully treated mice harbored antitumor IFN-γ producing memory CD8 T cells and were protected from tumor re-challenge more than 100 days later (Fig. 4). This finding is of consequence as an important aim of active immunotherapy is the induction of prolonged immune protection against cancer to prevent recurrence in patients. Also, the crucial role of CD8 T cells in the antitumor response induced by anti-CD137 therapy in mice - both at the early (tumor regression) and late stage (tumor protection) - is encouraging as we found that CD137 was expressed on a significant number of tumor-infiltrating CD8 T cells in patients with lymphoma.

Similar to the majority of other murine tumor models10, we found that NK and CD8 T cell depletion completely abrogated the effect of anti-CD137 therapy (Fig. 5). The mechanism by which NK cells participate in tumor eradication following anti-CD137 therapy has been investigated in other studies28,29. These studies suggested that NK cells promote T cell help rather than mediating direct tumor cytotoxicity. Surprisingly, we found that CD4
depletion significantly improved the therapeutic effect of anti-CD137 mAb (Fig. 5). Our laboratory\textsuperscript{30} and others have shown that CD4 T cell help can support lymphoma growth of primary tumor cells. This phenomenon might account, at least in part, for increased efficacy of anti-CD137 therapy following CD4 T cell depletion in our model. In this study, we hypothesized that T\textsubscript{reg} might be responsible for this effect. While others have reported similar enhancement of anti-CD137 therapy with CD4 depletion in a different tumor model\textsuperscript{31}, there has been no definitive proof that T\textsubscript{reg} were responsible for this effect. In fact, some studies have reported that high numbers of T\textsubscript{reg} correlate with better clinical outcome in patients with lymphoma\textsuperscript{32,33}, putatively due to direct suppression or killing of tumor B cells by T\textsubscript{reg}\textsuperscript{34,35}. In this study, using a mAb (anti-FR4) capable of selectively depleting T\textsubscript{reg} cells\textsuperscript{24}, we demonstrate a synergistic enhancement when combining T\textsubscript{reg} depletion with anti-CD137 therapy (Fig. 6). This result suggests that T\textsubscript{reg} interfere with anti-CD137 therapy, presumably through inhibition of antitumor immune cells. As suggested by our data, it may be necessary to remove T\textsubscript{reg} in order for anti-CD137 therapy to function more effectively. However, clinical translation of this finding will require elucidation of the optimal timepoint for T\textsubscript{reg} depletion as this has been shown to be important for lymphoma rejection\textsuperscript{36}, and identification of an anti-human CD137 mAb with similar biological activity and therefore, hopefully, antitumor efficacy as observed in the mouse model.

In conclusion, lymphoma appears to be a promising candidate for anti-CD137 therapy. First, lymphoma has proven to be responsive to immunotherapy\textsuperscript{1}. Second, human primary lymphoma tumors over-express CD137 mRNA compared to other tumors, presumably not due to the tumor cells themselves but to the presence of CD137+ tumor-infiltrating T cells. These CD137+ T cells are thought to be tumor-reactive cells whose function could be further stimulated with anti-CD137 agonistic mAb. Third, we showed that anti-CD137 agonistic mAb has potent anti-lymphoma activity \textit{in vivo}. This effect can be further improved by T\textsubscript{reg} depletion, leading to spectacular tumor regression and prolonged remission in mice. In addition, lymphoma offers the possibility to combine anti-CD137 mAb with other lymphoma-specific therapies such as Rituximab. Due to the presumed role of NK cells in both anti-CD137 (shown here in Fig. 5) and anti-CD20 mAb therapies (the latter through antibody-dependent cell cytotoxicity), we hypothesize that the combination of a tumor-directed mAb (Rituximab) and anti-CD137 mAb will be synergistic. This hypothesis is currently under investigation in our laboratory.

Overall, our study demonstrates the promise of anti-CD137 agonistic mAb for the treatment of lymphoma. Our results identify important insights into the therapeutic use of anti-CD137 mAb and support the testing of anti-CD137 agonistic mAb in clinical trials for patients with lymphoma.

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Authorship

Contribution: R.H. designed and performed experiments, analyzed data and wrote the paper. M.J.G., H.E.K., J.H.M., A.A.A., J.T.L., J.M.I. and J.T. designed and performed experiments, analyzed data and reviewed the paper. A.K. collected and provided the human lymphoma biopsies from Norway. L.C. provided the 2A hybridoma, and reviewed data and the paper. R.L. designed experiments, and reviewed data and the paper.

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References

Figure Legends

**Figure 1** – Tumor-involved lymph nodes from lymphoma patients express high levels of CD137 mRNA and are infiltrated by CD137+ T cells.

(A) Publicly available microarray gene expression data\textsuperscript{16,17} from bulk tumor specimens of 184 patients were analyzed for the expression of CD137 across histopathological groups. DLBCL and FL specimens significantly over-expressed CD137 when compared to non-lymphoma specimens (p<0.0001 for each comparison). Aside from FL (n=11) and DLBCL (n=11), no other histopathological group significantly over-expressed CD137 when compared to other tumor types. CA, carcinoma; NSCLC, non-small cell lung adenocarcinoma, CRC, colorectal adenocarcinoma, TCC, Transitional cell carcinoma; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

(B-C) Tumor-involved lymph nodes from untreated lymphoma patients (FL, MCL, and DLBCL) and PBMC from healthy donors were analyzed by flow cytometry for CD137 expression on B and T cells. (B) shows CD137 expression on tumor B cells, CD4 and CD8 T cells for one representative lymphoma sample (DLBCL). (C) shows the percentage of CD137+ cells among CD8 T cells in healthy PBMC and lymphoma samples from different histologies.

**Figure 2** – Anti-CD137 agonistic mAb has potent anti-lymphoma activity *in vivo*.

BALB/c mice were inoculated s.c. with 5x10\textsuperscript{6} A20 tumor cells. Mice received either no treatment (●) or two i.p. injections of mAb anti-OX40 mAb (▲), anti-CTLA4 mAb (▼), anti-GITR mAb (♦), or anti-CD137 mAb (■) at day 5 and 10 post tumor inoculation as described in Materials and Methods. Mice (10 per group) were then monitored for tumor growth (A, mean+/−SEM) and overall survival (B).

**Figure 3** – Anti-CD137 mAb efficacy is time dependent.

BALB/c mice were inoculated s.c. with 5x10\textsuperscript{6} A20 tumor cells. Mice received either no treatment (● and dashed line) or one i.p. injection of anti-CD137 mAb (■ and continuous line) at various time points following tumor inoculation: day 0, day 3, day 6 or day 9. Mice (10 per group) were monitored for tumor growth (A, mean+/−SEM) and overall survival (B).

**Figure 4** – Mice cured with anti-CD137 mAb develop long-lasting antitumor immunity.

(A-B) BALB/c mice treated and cured by anti-CD137 mAb therapy for more than 100 days (■) or naïve mice (●) were (re-)challenged s.c. with 5x10\textsuperscript{6} A20 tumors at a different site from original tumor challenge. Mice were then monitored for tumor growth (A, mean+/−SEM) and overall survival (B).

(C-D) Following tumor re-challenge, splenocytes from αCD137-cured mice or control splenocytes from naïve mice were harvested, re-stimulated *in vitro* with irradiated A20 tumor cells for 24 hours, and assessed for intracellular
IFN-γ secretion by flow cytometry. Dot plots from FACS analysis show the proportion of IFN-γ positive cells among all CD3+ T cells (C) and CD8 T cells (D).

**Figure 5 – Anti-CD137 therapy requires NK and CD8 T cells and is enhanced by CD4 T cell depletion.**

(A-B) BALB/c mice were inoculated s.c. with 5x10⁶ A20 tumor cells. Depletion was initiated prior to tumor challenge as described in Materials and Methods. Mice received one i.p. injection of either or anti-CD137 mAb at day 6 post tumor inoculation. Groups include: rat IgG alone (●), anti-CD137 alone (■), CD4 depletion + anti-CD137 (▲), CD8 depletion + anti-CD137 (▼), or Asialo GM1 depletion + anti-CD137 (♦). Mice were monitored for tumor growth (A, mean+/−SEM) and overall survival (B).

(C-E) BALB/c mice were inoculated s.c. with 5x10⁶ A20 tumor cells and received either no treatment (Tx) or one i.p. injection of anti-CD137 mAb at day 8 post-tumor inoculation. Five days after treatment, mice were sacrificed and tumors and spleens were collected for analysis. (C) shows representative data of the percentage of CD25+FoxP3+ Treg among CD4 T cells in both spleen and tumor from untreated and treated groups. (D-E) shows the average percentage of CD25+FoxP3+ Treg among CD4 T cells (D) and CD8 T cells among total lymphocytes (E) between the untreated and treated groups (3 mice per group).

**Figure 6 – Selective depletion of Treg cells enhances anti-CD137 therapy.**

BALB/c mice were inoculated s.c. with 5x10⁶ A20 tumor cells. Mice then received one i.p. injection of either rat IgG or anti-CD137 mAb at day 6 post tumor inoculation. FR4 depletion was initiated prior to tumor challenge as described in Materials and Methods. Groups include: Rat IgG alone (●), Rat IgG + anti-FR4 (■), Anti-CD137 mAb alone (▲), anti-CD137 mAb + anti-FR4 (▼). Mice were then monitored for tumor growth (A) and overall survival (B).
FIGURE 2

A

- No treatment
- αOX40
- αCTLA4
- αGITR
- αCD137

Tumor size (cm²)

Days after tumor inoculation

B

Percent survival

Days after tumor inoculation

p<0.001

p=0.002
FIGURE 3

A

Day 0

Day 3

Day 6

Day 9

Tumor size (cm²)

αCD137

B

Day 0

Day 3

Day 6

Day 9

Percent survival

Days after tumor inoculation

αCD137
FIGURE 6

A

Rat IgG
Rat IgG+αFR4
αCD137
αCD137+αFR4

Tumor size (cm²)

Days after tumor inoculation

B

Percent survival

Days after tumor inoculation

Rat IgG
Rat IgG+αFR4
αCD137
αCD137+αFR4

P=0.004

P=0.0001

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