Adoptive Cell Therapy for Lymphoma with CD4 T Cells Depleted of CD137 Expressing Regulatory T Cells

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Abstract

Adoptive immunotherapy with anti-tumor T cells is a promising novel approach to the treatment of cancer. However, T cell therapy may be limited by the co-transfer of regulatory T cells (Tregs). Here we explored this hypothesis by using two cell surface markers, CD44 and CD137, to isolate anti-tumor CD4 T cells while excluding Tregs. In a murine model of B cell lymphoma, only CD137negCD44hi CD4 T cells infiltrated tumor sites and provided protection. Conversely, the population of CD137posCD44hi CD4 T cells consisted primarily of activated Tregs. Notably, this CD137pos Treg population persisted following adoptive transfer and maintained expression of FoxP3 as well as CD137. Moreover, in vitro these CD137pos cells suppressed the proliferation of effector cells in a contact-dependent manner, and in vivo adding the CD137posCD44hi CD4 cells to CD137negCD44hi CD4 cells suppressed the anti-tumor immune response. Thus, CD137 expression on CD4+ T cells defined a population of activated Tregs that greatly limited anti-tumor immune responses. Consistent with observations in the murine model, human lymphoma biopsies also contained a population of CD137pos CD4 T cells that were predominantly CD25posFoxP3pos Tregs. In conclusion, our findings identify two surface markers that can be used to facilitate the enrichment of anti-tumor CD4 T cells while depleting an inhibitory Treg population.

Precis

This important study provides knowledge which can immediately be translated into the clinical setting to optimize cell-based cancer immunotherapies based on adoptive transfer of CD4+ T lymphocytes.

Introduction
Immunotherapy for cancer has included non-specific stimulation of the immune system, active immunization with tumor-specific antigens, and adoptive cell therapy—the transfer of tumor-specific T cells. We have investigated adoptive cell therapy for treating lymphoma(1, 2). Historically, adoptive cell therapy for cancer has focused on isolating and expanding populations of T cells that have direct cytotoxic effects on tumors(3-6). We use active immunization to generate anti-tumor T cells in vivo and transfer these T cells into lymphodepleted recipient mice. The efficacy of this maneuver is impressive and cures large tumors(1, 2). Specifically, our early work showed that CD8 T cell anti-tumor immunity can be induced by the combination of cytotoxic chemotherapy with local, intratumoral injection of CpG(1, 7). Recently, we have extended the use of CpG as an immunotherapy by exposing tumor B cells to CpG ex vivo and subsequently injecting them into the host as a whole tumor cell vaccine(2). This approach obviates the need for an accessible, injectable, tumor site. Importantly, vaccination with such CpG-loaded tumor B cells induces a tumor-specific CD4 and not CD8 T cell response. Relatively small numbers of these CD4 T cells were sufficient to cure large and established lymphoma tumors.

This and other recent reports have suggested that CD4 T cells can be effective in adoptive immunotherapy(2, 8-11). However, a relevant concern in using CD4 T cells for adoptive therapy is the potential for co-transfer of Tregs. Here, we have identified two surface markers—CD44 and CD137—that can be used to isolate a population of anti-tumor CD4 T cells while excluding a population of inhibitory Tregs. Adoptive transfer of CD137negCD44hi CD4 T cells provided significant protection from B cell lymphoma. CD137posCD44hi CD4 T cells were predominantly Tregs and suppressed both effector cell proliferation and anti-tumor activity.
Methods

Reagents. CpG 1826 with sequence 5'-TCCATGACGTTCCTGACGTT was provided by Coley Pharmaceutical Group (Ottawa, Canada). FITC-conjugated CpG 1826 was purchased from InvivoGen (San Diego, CA). The following monoclonal antibodies (mAbs) were used for flow cytometry: anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD44, anti-mouse CD137, anti-mouse IFNγ, anti-mouse CD25, anti-mouse FoxP3, anti-mouse CD62L, anti-mouse CCR7, anti-mouse CD103, anti-mouse CD69, anti-mouse GITR, anti-mouse Thy1.1, anti-mouse CD45.1, anti-human CD3, anti-human CD4, anti-human CD8, anti-human CD45RO, anti-human CD137, anti-human CD25, anti-human FoxP3, and isotype control. Antibodies were purchased from either Becton Dickinson (BD) Biosciences (Franklin Lakes, NJ) or eBioscience (San Diego, CA).

Cell lines and mice. H11 is a pre-B cell line in the C57BL/6 background generated as follows: Primary bone marrow cells were isolated from C57BL/6 mice and infected with the retrovirus vector MSCV-neo/p190Bcr-Abl, which carries the oncogene Bcr-Abl(12) (A gift from Drs. M. Cleary and K. Smith, Stanford School of Medicine). Tumor cells were cultured in complete RPMI 1640 (cRPMI) medium (Invitrogen Life Technologies) containing with 10% FCS (Thermo Scientific; Waltham, MA), 100U/ml penicillin, 100μg/ml streptomycin (both from Invitrogen Life Technologies), and 50μM 2-ME (Sigma-Aldrich; St. Louis, MO). This cell line was previously used in studies from our lab (2) and was tested for uniformity of cell-surface markers (B220<sup>pos</sup>CD43<sup>neg</sup>IgM<sup>neg</sup>IgD<sup>neg</sup>) by FACS. Six- to eight-week-old female Thy1.1, CD45.1, and wild-type C57BL/6J mice were purchased from Jackson Laboratories. All studies were approved by the Stanford Administrative Panel on Laboratory Animal Care.
**Tumor inoculation and animal studies.** H11 tumor cells were incubated in the presence of 3μg/ml of CpG at 37°C and 5% CO₂. As shown previously, CpG is taken-up by H11 cells during this incubation(2). After 24 hours, cells were vigorously washed three times with wash buffer to remove any unbound, residual CpG. H11 cells that were loaded with CpG (CpG/H11) were irradiated at 50Gy and used to vaccinate C57BL/6 donor mice subcutaneously (s.c.) for five consecutive days. On Day 13, bone marrow and splenocytes of donor mice were transferred by i.v. injection into irradiated C57BL/6 recipient mice (9.5Gy TBI, Phillips x-ray unit, 250 kV, 15mA) along with 1x10⁶ irradiated CpG/H11 tumor cells as a post-transplant ‘booster’ vaccine that was prepared in the same fashion as above(1). In studies using congenic donors, transferred bone marrow was from wild-type, non-congenic mice to allow precise tracking of transplanted donor cells. Recipient mice were challenged with H11 tumor cells s.c. at a dose of 1x10⁷ cells in 50μl of serum-free RPMI on Day 16. Tumor growth was monitored by caliper measurement (Figure 1A).

**In vitro assays.** IFNγ production assay: PBMCs were collected from tail vein, anticoagulated with 2 mM ethylenediaminetetraacetic acid in PBS, then diluted 1:1 with Dextran T500 (Pharmacosmos,Holbaek, Denmark) 2% in PBS and incubated at 37°C for 45 minutes to precipitate red cells. Leukocyte-containing supernatant was removed and centrifuged, and remaining red cells were lysed with ammonium chloride potassium buffer (Quality Biological, Gaithersburg, MD). PBMCs were then cocultured with 1x10⁶ irradiated H11 cells for 24 hours with 0.5μg anti-mouse CD28mAb (BD PharMingen) and in the presence of monensin (Golgistop; BD Biosciences, San Jose, CA) for the last 5 hours at 37°C and 5% CO₂. Intracellular IFNγ_expression was assessed using BD Cytofix/Cytoperm Plus Kit per instructions. Treg Suppression Assay: CD137⁺ and CD137⁻ cell populations were isolated by FACS and mixed at various ratios in a 96-
well round bottom plate. Cells were stimulated in with equal numbers of polystyrene latex
beads (Interfacial Dynamics) coated with 1.0 μg/ml anti-CD3 (145-2C11; eBioscience)
and 0.5μg/ml anti-CD28 (37.51; eBioscience). Cells were pulsed with 1 μCi of methyl-
[^3]H]thymidine (Amersham Biosciences) for 6 h during the last 72 h of stimulation and
harvested onto filters (Wallac). Filters were wetted with Betaplate scintillation
fluid (PerkinElmer) and counts per minute read on a 1205 Betaplate liquid scintillation
counter (Wallac).

**Flow cytometry.** Cells were surface-stained in wash buffer (phosphate buffered saline,
1% fetal bovine serum (FBS), and 0.01% sodium azide), fixed in 2% paraformaldehyde
and analyzed by flow cytometry on a BD FACS Calibur, LSR II, or FACS Aria System.
Data were analyzed using Cytobank(13). Flow cytometric cell sorting was used to purify
t cell subsets from splenocytes of CpG/H11-vaccinated mice.

**Primary Human Lymphoma Specimens.** Tumor specimens were obtained with
informed consent in accordance with the Declaration of Helsinki and with approval by
Stanford University’s Administrative Panels on Human Subjects in Medical Research.
Samples were transferred directly from the operating room to the laboratory and used for
the preparation of viable, sterile single-cell suspensions. Lymph node tissue was
disaggregated, filtered through a metal sieve, washed, re-suspended in media
composed of 90% FBS (HyClone) and 10% DMSO (Sigma), frozen slowly in the vapor
phase of liquid nitrogen in multiple cryotubes, and stored in liquid nitrogen. PBMCs from
healthy individuals were isolated using density gradient separation FicollPaque Plus
(Amersham Biosciences) and subsequently stained with antibodies from Becton
Dickinson or eBioscience as above.
Statistical analysis. Prism software (GraphPad; La Jolla, CA) was used to analyze tumor growth and determine statistical significance of differences between groups by applying an unpaired Student’s t-test. P values <0.05 were considered significant.

Results

Vaccine-induced CD4 T cells are necessary and sufficient for tumor rejection.

We have developed a model for adoptive cell therapy of lymphoma whereby anti-tumor T cells are generated in vivo through vaccination with a CpG-loaded whole cell vaccine (CpG/H11)(2). These vaccine-induced cells are tumor-specific and cure large and established tumors when isolated and transferred into lethally irradiated, syngeneic recipient mice(2). We investigated whether a specific subset of CD4 T cells was tumor-reactive. CD4 or CD8 T cell subsets were negatively isolated from CpG/H11-vaccinated, Thy1.1 congenic, donor mice. These purified subsets were transferred into lethally irradiated, Thy1.2 congenic recipients. Three days post-transfer, recipients were challenged s.c. with 1x10^7 H11 tumor cells (Figure 1A). Purified CD4 T cells from vaccinated donors were sufficient to protect 80% of recipient mice for greater than 50 days (Figure 1B). Conversely, purified CD8 T cells were comparable to splenocytes from unvaccinated donors and had no effect on tumor growth rate.

We investigated the mechanism for CD4-mediated tumor cell killing. As we described previously, the H11 tumor cell line is MHC Class II negative, therefore CD4 T cells cannot kill tumor cells directly(2). We tested the role of other effector cell populations by treating recipient mice with purified CD4 T cells plus a depleting antibody for NK cells (anti-NK1.1), CD8 T cells (anti-CD8), or a blocking antibody against IFN-γ (anti-IFN-γ). Depletion of NK cells and CD8 T cells were confirmed by flow cytometry. Three days post-transfer, recipients were challenged s.c. with 1x10^7 H11 tumor cells.
Depletion of NK cells had no effect on tumor rejection. However, depletion of CD8 T cells resulted in late relapses with tumors beginning to grow 20 days after tumor challenge. Blockade of IFN-γ eliminated the anti-tumor effect in 6/10 recipient mice (Figure 1C). Reconstitution of the endogenous, host CD8 T cell compartment can be observed as early as 10 days post-transplant (Figure S1), these results suggest that CD4-mediated IFN-γ production is a critical early mediator in inducing a host CD8 T cell response that is ultimately necessary for lasting tumor rejection.

Anti-tumor immune responses in the recipient mice were analyzed fifteen days after transfer. Phenotypic fidelity of the transferred T cell subsets was confirmed and donor-derived cells (identified by expression of the Thy1.1 marker) were >99% pure populations of either CD4 or CD8 T cells (Figure S2). Previously, we demonstrated that CD4 anti-tumor immune responses were tumor specific(2). Here, we evaluated by in vitro IFN-γ production assay whether tumor-reactivity was limited to donor cells. Whole peripheral blood lymphocytes (PBLs) from recipient mice were collected, placed in coculture with irradiated tumor cells, and IFN-γ production was measured by intracellular flow cytometry. Using the Thy1.1 marker, we were able to distinguish whether donor-derived (Thy1.1pos) or reconstituted recipient cells (Thy1.1neg) were producing IFN-γ. At day 15, only a subset of donor-derived CD4 T cells produced IFN-γ: 5.1% of Thy1.1pos CD4 T cells were IFN-γpos (Figure 1D). Conversely, only 0.6% of donor-derived (Thy1.1pos) CD8 T cells were IFN-γpos.

Because it has been reported that homeostatic proliferation induces a memory phenotype in transferred T cells(14, 15), we assayed for CD44 expression on tumor-reactive CD4 and CD8 T cells in an in vitro IFN-γ production assays. CD44, the hyaluronic acid receptor, identifies antigen-experienced CD4 T cells(16). In recipients transplanted with vaccine-induced T cells, there was evident increased expression of
CD44, specifically amongst the IFN-γ producing, donor T cells (Figure 1D). This suggested that expression of CD44 might serve as one potential marker for the identification and eventual isolation of a tumor-reactive subset of CD4 T cells.

**CD137^{pos}CD44^{hi} CD4 T cells are expanded in vaccinated donor mice.**

We sought to identify the CD4 T cell subset necessary for mediating anti-tumor immune responses. We compared vaccinated and unvaccinated donor mice for expression of memory and activation markers including CD44, CD62L, and CCR7(14-18). CD44 expression was increased on CD4 T cells in vaccinated mice. However, we observed no differences between vaccinated and unvaccinated mice in any of the other markers or combinations thereof (Figure S3).

When comparing expression of CD137 in vaccinated and unvaccinated donor mice, we observed a distinct increase in the expression of CD137 on CD4 T cells in vaccinated animals (Figure 2). The CD137^{pos} population was predominantly within the CD44^{hi} subset, similar to IFN-γ producing cells shown in Figure 1. Based on these observations, we initially hypothesized that CD137 expression on CD4 T cells in vaccinated mice may identify a tumor reactive population. To further support this hypothesis, studies have shown that CD137 is an activation marker on many immune cell types including CD4 and CD8 T cells, B cells, natural killer (NK) cells, NK-T cells, monocytes, neutrophils, and dendritic cells(19). On CD8 T cells, binding of CD137 leads to proliferation, cytokine production, functional maturation, and prolonged survival(19).

Recent work from our group and others has demonstrated that agonistic mAbs against CD137 (4-1BB) can provoke CD8 T cell anti-tumor responses capable of eradicating established tumors in a range of murine tumor models(20-22).
CD137 identifies a population of activated, regulatory T cells.

We characterized the population of CD137^{pos} CD4 T cells further and analyzed whole splenocytes from vaccinated and unvaccinated donor mice for expression of CD44, CD137, and FoxP3. As expected, FoxP3 staining identified the natural physiological ratio of approximately 5-15% of CD4 T cells as T_{reg} in donor mice(23). Surprisingly, in vaccinated mice, CD137-expression marked >65% of this T_{reg} population. Conversely, in unvaccinated donors or donors vaccinated with CpG alone, CD137 identified <35% of T_{reg} (Figure 3A and Figure S4). Therefore, in the context of our vaccination, CD137 identifies an expanded subset of FoxP3^{pos} CD4 T cells.

We compared CD137^{pos} and CD137^{neg} T_{reg} from vaccinated donors for expression of traditional T_{reg} markers including CD25, GITR, and CD103 as well as memory/activation markers including CD44, CD62L, and CD69 (Figure 3B). Compared to CD137^{neg} T_{reg}, the CD137^{pos} subset is distinct in its expression of CD69 (high), CD44 (high), and CD62L (low). High CD69 expression suggests that these cells are more activated than their CD137^{neg} counterparts. The phenotype of CD44^{hi}CD62L^{low} is characteristic of an effector memory population.

CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells maintain expression of both CD137 and FoxP3 after adoptive transfer.

As they appear to have an effector memory phenotype, we asked whether CD137^{pos}CD44^{hi} T_{reg} persisted following adoptive transfer and whether they maintained expression of CD137 and FoxP3. Using FACS, we isolated CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells from vaccinated, congenic (CD45.1^{pos}) donors to purity of greater than 98% (Figure 4A). We transferred these purified populations into lethally irradiated recipients. 10 days after transfer, recipient mice were sacrificed and splenocytes were analyzed for the presence of T cells expressing the congenic marker,
Both CD137\textsuperscript{pos}CD44\textsuperscript{hi} and CD137\textsuperscript{neg}CD44\textsuperscript{hi} donor cells were present in the spleens of recipient mice. Furthermore, each transferred subset retained its original phenotype: both CD137\textsuperscript{pos}CD44\textsuperscript{hi} and CD137\textsuperscript{neg}CD44\textsuperscript{hi} donor cells maintained their original expression levels of both CD137 and FoxP3 (Figure 4B).

CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells are present in spleen but do not home to tumor sites.

We investigated whether CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells were capable of infiltrating sites of tumor. CD137\textsuperscript{pos}CD44\textsuperscript{hi} and CD137\textsuperscript{neg}CD44\textsuperscript{hi} CD4 T cells were isolated from vaccinated, congenic (CD45.1\textsuperscript{pos}) donors by FACS and adoptively transferred into lethally irradiated recipients. Three days post-transfer, recipients were challenged s.c. with 1x10\textsuperscript{7} H11 tumor cells. Approximately seven days after transfer, tumor size averaged approximately 0.5cm\textsuperscript{2} in both groups. Mice were sacrificed and both spleen and tumor analyzed for T cell infiltration. Transferred donor cells were identified by surface expression of the congenic marker, CD45.1.

T cell infiltration of tumors was markedly different in mice that received CD137\textsuperscript{pos} vs. CD137\textsuperscript{neg} subsets. CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells made up only 14.4% of total tumor-infiltrating T cells while CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells made up 57.6% of total tumor-infiltrating T cells (Figure 5A). CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells infiltrate sites of tumor and facilitate tumor rejection while CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells do not. However, both subsets of donor-derived CD4 T cells were similarly present in recipient spleens. In mice receiving CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells, 14.5% of splenic T cells were of donor origin (Figure 5B, left panel). Similarly, in mice receiving CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells, 18.8% of splenic T cells were of donor origin (Figure 5B, right panel). Recipient bone marrow and lymph node compartments were not studied. These findings suggest that the suppressive effect of the CD137\textsuperscript{pos} population takes place in the spleen or other lymphocyte compartments but not in the tumor itself.
CD137^{pos}CD44^{hi} CD4 T cells have T_{reg} function and suppress the anti-tumor activity of CD137^{neg}CD44^{hi} effector cells.

We sought to confirm the suppressive function of CD137^{pos}CD44^{hi} CD4 T cells both in vitro and in vivo. CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells were isolated by FACS from vaccinated donors to greater than 98% purity. We co-cultured CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells at ratios of 0:1, 1:1, 1:2, 1:4, and 1:8 (CD137^{pos}:CD137^{neg}) and stimulated them with bead-bound anti-CD3/anti-CD28. CD137^{pos}CD44^{hi} cells inhibited the proliferation of CD137^{neg}CD44^{hi} cells in a dose-dependent manner (Figure 6A). To address whether this suppressive effect required cell-to-cell contact, we performed a similar assay but separated CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} cells across a transwell. The suppressive effect of CD137^{pos}CD44^{hi} cells was eliminated in the absence of cell-to-cell contact. In co-culture across the transwell, proliferation of CD137^{neg}CD44^{hi} cells was similar to controls (Figure 6B).

Next, we tested whether CD137^{pos}CD44^{hi} cells could suppress the anti-tumor response mediated by CD137^{neg}CD44^{hi} cells. Previously, when we transferred whole CD4 T cells we observed impressive tumor rejection(2) (Figure 1). In this bulk CD4 population, the ratio of CD137^{neg}CD44^{hi} to CD137^{pos}CD44^{hi} was approximately 3:1. To determine whether CD137^{pos}CD44^{hi} could suppress anti-tumor immune responses in vivo, we isolated CD4 T cells by FACS from vaccinated donors to greater than 98% purity. Either CD137^{neg} cells alone (2x10^6/mouse) or a combination of CD137^{neg} (2x10^6/mouse) and CD137^{pos} (4x10^6/mouse) cells at a ratio of 1:2 were adoptively transferred into lethally irradiated recipients. A lethal challenge of 1x10^7 H11 tumor cells was given s.c. on day 3 following transfer. CD137^{neg}CD44^{hi} cells were sufficient to protect 100% of recipient mice from lethal tumor challenge. When CD137^{pos}CD44^{hi} cells we co-transferred, this anti-tumor effect was blocked and 0% of recipient mice survived.
longer than 20 days (Figure 6C). Lower numbers of CD137^{pos}CD44^{hi} cells did not inhibit tumor rejection. On day 15 after transfer, PBLs from recipient mice were collected and placed in co-culture with irradiated tumor cells for 24 hours. IFN-\(\gamma\) production was measured by intracellular flow cytometry. In mice receiving CD137^{pos} and CD137^{neg}CD44^{hi} cells, only 0.9\% of CD4 T cells produced IFN-\(\gamma\) (Figure 6D). Conversely, in recipient mice receiving CD137^{neg}CD44^{hi} cells alone, 4.7\% of CD4 T cells produced IFN\(\gamma\).

In human lymphoma-involved lymph nodes, CD137 identifies a population of activated, regulatory T cells.

Recent studies have identified cell surface markers that define a discrete subset of CD25^{pos}FoxP3^{pos} T_{regs} preferentially expanded in patients with cancer(24). We investigated whether our observations about CD137 in a murine tumor model were consistent in the human tumor samples. In tumor-bearing mice, we observed that a large percentage of CD137^{pos} CD4 T cells were present in tumor-involved spleen. This CD137^{pos} population was overwhelmingly CD25^{pos}FoxP3^{pos} T_{regs} (Figure 7A). We examined human lymphoma biopsies for whether CD137 similarly served as a marker for CD25^{pos}FoxP3^{pos} T_{regs}. Follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL) were analyzed by flow cytometry. A representative flow plot shows the gating strategy used to define these populations (Figure 7B). CD137^{pos}CD45RO^{pos} CD4 T cells were present in FL (mean = 5.14\% ± SEM 0.34\%) and MCL (mean = 8.24\% ± SEM 1.68\%) (Figure 7C, left panel). As in the murine system, this CD137^{pos} population was predominantly CD25^{pos}FoxP3^{pos} (FL: mean = 68.65\% ± SEM 3.28\%; CLL: mean = 54.73\% ± SEM 5.70\%) (Figure 7C, right panel). Therefore, CD137 may serve as an effective marker for CD25^{pos}FoxP3^{pos} T_{regs} in human lymphoma patients.
as well.

Discussion

Our results indicate that two markers—CD44 and CD137—can be used to isolate a population of anti-tumor CD4 T cells while effectively excluding T regs. Here, we demonstrate that (1) CD137^{pos}CD44^{hi} expression defines a population of activated CD4 T_{regs} that can suppress the proliferation of effector cells in a contact-dependent manner; (2) the CD137^{pos}CD44^{hi} T_{reg} population persists following adoptive transfer and can inhibit tumor rejection \textit{in vivo}; and (3) vaccination with CpG-activated lymphoma induced CD137^{neg}CD44^{hi} CD4 T cells can transfer protection from B cell lymphoma tumor challenge.

Rosenberg and colleagues have demonstrated impressive clinical outcomes using \textit{ex vivo} generated T cells in adoptive immunotherapy for metastatic melanoma(3-5). Our prior work established that a CpG-loaded whole cell vaccine induced anti-tumor CD4 T cells that were both necessary and sufficient to adoptively transfer anti-tumor immunity. To date, the field of adoptive cell therapy has focused primarily on CD8 CTLs(3, 5, 6). However, important roles for CD4 T cells in anti-tumor immunity have also been shown(8-11). Hunder and colleagues demonstrated durable clinical remissions in melanoma patients treated with \textit{ex vivo} expanded, antigen-specific CD4 T cells(8). Recently, studies by Xie et al.(10) and Quezada et al.(9) have demonstrated in a TCR transgenic model that naive tumor-specific CD4 T cells are able to induce regression of established tumors in lymphopenic hosts. Our findings support this prior body of work and suggest that anti-tumor CD4 T cells can be induced \textit{in vivo}, isolated, and used in adoptive immunotherapy of established lymphoma tumors. Our results suggest that these anti-tumor CD4 T cells produce IFN-\gamma and recruit a long-lasting anti-tumor
response mediated by host CD8 T cells. Importantly, our work does not rule out other sources of IFN-γ including, but not limited to, NKT cells.

A concern in any CD4 T cell-based therapy is the potential for contamination by Tregs. This is particularly relevant to our system where anti-tumor T cells are isolated following in vivo induction. Hunder et al. addressed this issue by selecting tumor reactive clones and expanding them ex vivo. Quezada et al. combined CD4 adoptive transfer with blockade of CTLA-4 to inhibit the function of Tregs. These approaches have led to impressive outcomes in human melanoma and mouse melanoma tumor models, respectively. We have defined a surface marker for a population of activated Tregs that can facilitate their removal prior to adoptive transfer or allow their targeting with mAbs in the post-transplant recipient. Specifically, CD137 and CD44 can be used to distinguish anti-tumor CD4 T cells from Tregs. To our knowledge, this is the first report of CD137 expression being used to distinguish these two populations.

Surface marker identification of Tregs is not a novel pursuit and a number of studies have claimed to identify Tregs by expression of surface markers including MHC-II, CD45RO/RA, CCR7, and ICOS(25-28). Most recently, Camisaschi et al.(24) have observed that the expression of LAG-3 on human CD4 T cells defines a discrete subset of CD25posFoxP3pos Tregs that is preferentially expanded in patients with cancer. While LAG-3 was not included in our evaluation of human lymphoma biopsies, we anticipate that both CD137 and LAG-3 may be complementary in identifying tumor-reactive Tregs.

In summary, the work reported here demonstrates that CD137 expression on CD4 T cells identifies a population of activated Tregs that suppress proliferation and anti-tumor immune responses. Importantly, the combination of cell surface markers CD44 and CD137 can be used to isolate a population of anti-tumor CD4 T cells while excluding a large, activated subset of Tregs for the purpose of adoptive immunotherapy of lymphoma.
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Literature Cited


**Figure 1.** Vaccine-induced CD4 T cells are necessary and sufficient for tumor rejection. (A) Vaccination and adoptive therapy schema. (B) Recipient mice received purified CD4 or CD8 T cells from vaccinated donor mice or complete splenocytes from unvaccinated donor mice. Arrow indicates day of tumor challenge. (C) Recipient mice received purified CD4 T cells as above and were treated with depleting antibodies. Recipient mice were followed for tumor growth (n; 10 mice/group). (D) PBLs were isolated from recipient mice 10 days after transfer and assayed for IFN-γ production. Plots are representative of three independent samples per group. N/A; only T cells of the transferred subset were present in recipient mice.

**Figure 2.** CD137^{pos}CD44^{hi} CD4 T cells are expanded in vaccinated donor mice. Vaccination performed as described. CD4 T cells from vaccinated or unvaccinated donor mice were analyzed for expression of CD44 and CD137. Plots are representative of three independent samples per group.

**Figure 3.** CD137 identifies a population of activated, regulatory T cells. (A) CD4 T cells from vaccinated or unvaccinated donor mice were analyzed for expression of CD137 and FoxP3. (B) CD137^{pos} and CD137^{neg} FoxP3^{pos} T cells from vaccinated donor mice were compared for expression of CD25, CD44, CD62L, CD103, GITR, and CD69. Plots are representative of three independent samples per group.

**Figure 4.** CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells maintain expression of both CD137 and FoxP3 after adoptive transfer. Vaccination and adoptive transfer were performed as described. (A) Sort strategy and results for isolation of CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells from vaccinated CD45.1^{pos} donor mice. CD25 and FoxP3 expression were used to characterize the T_{reg} phenotype of these two subpopulations. (B) CD137^{pos} and CD137^{neg} subpopulations were adoptively transferred into CD45.1^{neg} recipients. 7 days after transfer, recipient mice were sacrificed. In recipients, transferred donor cells were identified by expression of CD45.1. CD45.1^{pos} cells were analyzed by flow cytometry for expression of CD137 and FoxP3. Plots are representative of three individual mice per group.

**Figure 5.** CD137^{pos}CD44^{hi} CD4 T cells are present in spleen but do not home to tumor sites. CD137^{pos} and CD137^{neg} populations were isolated by FACS from vaccinated, CD45.1^{pos} donors and adoptively transferred into tumor challenged, lethally irradiated CD45.1^{neg} recipients. Spleen and tumor were analyzed for total T cell populations by surface CD3 and CD4 expression. Transferred donor cells were identified by surface expression of the congenic marker CD45.1. Tissues were analyzed by flow cytometry from recipients of either CD137^{pos} or CD137^{neg} populations. Plots showing (A) tumor infiltrating CD3^{pos} T cells from tumor biopsies and (B) splenic CD3^{pos} T cells. All plots are representative of five individual mice per group.

**Figure 6.** CD137^{pos}CD44^{hi} CD4 T cells suppress the proliferation and anti-tumor activity of CD137^{neg}CD44^{hi} effector cells. T_{reg} suppression assays: CD137^{pos} and CD137^{neg} populations were isolated from vaccinated mice by FACS. (A) CD137^{pos} cells were co-cultured with purified CD137^{neg} cells at ratios of 0:1, 1:1, 1:2, 1:4, and 1:8 (CD137^{pos}:CD137^{neg}). Proliferation was assessed by incorporation of ^{3}H (cpm). All conditions were performed in triplicate. (B) CD137^{pos} and CD137^{neg} populations were plated across a transwell. (C) CD137^{pos} and CD137^{neg} populations were isolated and
transferred into lethally irradiated recipient mice (n; 5 mice/group). Mice were followed for tumor growth and overall survival. Arrow indicates day of tumor challenge. (D) PBLs were isolated from recipient mice 10 days after transfer and assayed for IFN-γ production following 24-hour co-culture with irradiated tumor cells. Plots are representative of three independent samples. ** indicates p < 0.001; *** indicates p < 0.0001.

Figure 7. CD137 identifies a population of activated, regulatory T cells in human lymphoma. (A) Splenocytes from a 10-day tumor-bearing mouse were analyzed by flow cytometry for CD44, CD137, CD25, and FoxP3 expression. Plots are representative of 3 independent animals. (B,C) Human tumor biopsies and PBMCs were analyzed by flow cytometry for CD45RO, CD137, CD25, and FoxP3 Expression. (B) A representative plot of a follicular lymphoma (FL) tumor biopsy demonstrating the gating hierarchy. (C) Summary plots of flow cytometry analyses.
FIGURE 3

A. Unvaccinated  CpG/H11 Vaccinated

B. CpG/H11 Vaccinated:
   CD137^{pos} FoxP3^{pos}
   CD137^{neg} FoxP3^{pos}

CD25  CD44  CD62L  CD103  GITR  CD69
FIGURE 4

A. DONOR

Pre-Sort Population

Sort Results

$\text{CD}^{137^{\text{pos}}\text{CD44}^{\text{hi}}}$

$\text{CD}^{137^{\text{neg}}\text{CD44}^{\text{hi}}}$

T$_{\text{reg}}$ Phenotype

$\text{CD}^{137^{\text{pos}}\text{CD44}^{\text{hi}}}$

$\text{CD}^{137^{\text{neg}}\text{CD44}^{\text{hi}}}$

B. RECIPIENT

CD137$^{\text{pos}}$CD44$^{\text{hi}}$ Subset

82.0%

3.5%

9.1%

CD137$^{\text{neg}}$CD44$^{\text{hi}}$ Subset

1.5%

67.3%

7.2%
FIGURE 5

A. Tumor Biopsy: TILs

Received:
CD137^{\text{pos}} CD44^{\text{hi}} CD4 T Cells

- 35.2%
- 14.4%

Received:
CD137^{\text{neg}} CD44^{\text{hi}} CD4 T Cells

- 18.8%
- 57.6%

B. Spleen: Splenic T Cells

Received:
CD137^{\text{pos}} CD44^{\text{hi}} CD4 T Cells

- 59.4%
- 14.5%

- 25.6%

Received:
CD137^{\text{neg}} CD44^{\text{hi}} CD4 T Cells

- 49.2%
- 18.8%

- 30.9%
FIGURE 7.

A. Murine: Tumor-Involved Spleen

- CD137
- CD44
- CD4 T Cells
- 23.3%
- CD137
- CD25 T Cells
- FoxP3
- 92.7%
- 5.0%

B. Human: Lymphoma-Involved Lymph Node

- CD137
- CD45RO T Cells
- 75.5%
- CD25
- CD137pos T Cells
- FoxP3
- 86.9%
- 11.0%

C. Diagram of % CD45RO CD137pos CD4 T Cells and % of CD137pos that are FoxP3pos

- PBMC
- FL
- CLL
- MCL

- % CD45RO CD137pos CD4 T Cells
- % of CD137pos that are FoxP3pos
Adoptive Cell Therapy for Lymphoma with CD4 T Cells Depleted of CD137 Expressing Regulatory T Cells

Matthew J Goldstein, Holbrook E Kohrt, Roch Houot, et al.

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