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

Matthew J. Goldstein, Holbrook E. Kohrt, Roch Houot, Bindu Varghese, Jack T. Lin, Erica Swanson, and Ronald Levy

Abstract

Adoptive immunotherapy with antitumor T cells is a promising novel approach for the treatment of cancer. However, T-cell therapy may be limited by the cotransfer of regulatory T cells (Treg). Here, we explored this hypothesis by using 2 cell surface markers, CD44 and CD137, to isolate antitumor CD4 T cells while excluding Treg. In a murine model of B-cell lymphoma, only CD137\textsuperscript{neg}CD44\textsuperscript{hi} CD4 T cells infiltrated tumor sites and provided protection. Conversely, the population of CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells consisted primarily of activated Treg. Notably, this CD137\textsuperscript{pos} Treg population persisted following adoptive transfer and maintained expression of FoxP3 as well as CD137. Moreover, \textit{in vitro} these CD137\textsuperscript{pos} cells suppressed the proliferation of effector cells in a contact-dependent manner, and \textit{in vivo} adding the CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 cells to CD137\textsuperscript{neg}CD44\textsuperscript{hi} CD4 cells suppressed the antitumor immune response. Thus, CD137 expression on CD4 T cells defined a population of activated Treg that greatly limited antitumor immune responses. Consistent with observations in the murine model, human lymphoma biopsies also contained a population of CD137\textsuperscript{pos} CD4 T cells that were predominantly CD25\textsuperscript{pos}FoxP3\textsuperscript{pos} Treg. In conclusion, our findings identify 2 surface markers that can be used to facilitate the enrichment of antitumor CD4 T cells while depleting an inhibitory Treg population. Cancer Res; 72(5): 1239–47. ©2012 AACR.

Introduction

Immunotherapy for cancer has included nonspecific 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 antitumor T cells \textit{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 antitumor 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 \textit{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 cotransfer of regulatory T cells (Treg). Here, we have identified 2 surface markers—CD44 and CD137—that can be used to isolate a population of antitumor CD4 T cells while excluding a population of inhibitory Treg. Adoptive transfer of CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells provided significant protection from B-cell lymphoma. CD137\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells were predominantly Treg and suppressed both effector cell proliferation and antitumor activity.

Materials and Methods

Reagents

CpG 1826 with sequence 5’-TCCATGACGTTCCTGACGTT-3’ was provided by Coley Pharmaceutical Group (Ottawa, Canada). Fluorescein isothiocyanate–conjugated CpG 1826 was purchased from InvivoGen. The following monoclonal antibodies (mAb) were used for flow cytometry: anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD44, anti-mouse CD137, anti-mouse IFN-γ, 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 CD45R0, anti-human CD19, anti-human CD44, anti-human CD40, anti-human CD80, and anti-human CD86.

Received May 26, 2011; revised September 20, 2011; accepted October 17, 2011.

Published OnlineFirst January 9, 2012; DOI: 10.1158/0008-5472.CAN-11-3375
CD137, anti-human CD25, anti-human FoxP3, and isotype control. Antibodies were purchased from either Becton Dickinson (BD) Biosciences or eBioscience.

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 10% fetal calf serum (Thermo Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (both from Invitrogen Life Technologies), and 50 μmol/L 2-ME (Sigma-Aldrich). This cell line was previously used in studies from our laboratory (2) and was tested for uniformity of cell-surface markers (B220<sup>+</sup>CD43<sup>-</sup> IgM<sup>-</sup>IgD<sup>-</sup>) by fluorescence-activated cell sorting (FACS). Six- to 8-week-old female Thy1.1, CD45.1, and wild-type C57BL/6 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 3 μg/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 3 times with wash buffer to remove any unbound, residual CpG. H11 cells that were loaded with CpG (CpG/H11) were irradiated at 50 Gy and used to vaccinate C57BL/6 donor mice s.c. for 5 consecutive days. On the 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, 15 mA) along with 1 × 10<sup>6</sup> irradiated CpG/H11 tumor cells as a posttransplant "booster" vaccine that was prepared in the same fashion as above (1). In studies previously used in studies from our laboratory (2) and was tested for uniformity of cell-surface markers (B220<sup>+</sup>CD43<sup>-</sup> IgM<sup>-</sup>IgD<sup>-</sup>) by fluorescence-activated cell sorting (FACS). Six- to 8-week-old female Thy1.1, CD45.1, and wild-type C57BL/6 mice were purchased from Jackson Laboratories. All studies were approved by the Stanford Administrative Panel on Laboratory Animal Care.

In vitro assays
IFN-γ production assay. Peripheral blood mononuclear cells (PBMC) were collected from tail vein, anticoagulated with 2 mmol/L ethylenediaminetetraacetic acid in PBS, then diluted 1:1 with Dextran T500 (Pharmacosmos) 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). PBMCs were then cocultured with 1 × 10<sup>6</sup> irradiated H11 cells for 24 hours with 0.5 μg anti-mouse CD28 mAb (BD Pharmingen) and in the presence of monensin (Golgistop; BD Biosciences) for the last 5 hours at 37°C and 5% CO₂. Intracellular IFN-γ expression was assessed with BD Cytofix/Cytoperm Plus Kit per instructions.

Statistical analysis
Prism software (GraphPad) was used to analyze tumor growth and determine statistical significance of differences between groups by applying an unpaired Student t test. P < 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 antitumor T cells are generated in vivo through vaccination with a CpG-loaded whole-cell vaccine (CpG/H11; ref. 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<sup>T</sup> cells was tumor reactive. CD4 or CD8<sup>T</sup>-cell subsets were negatively isolated from CpG/H11-vaccinated, Thy1.2 congenic, donor mice. These purified subsets were transferred into lethally irradiated, Thy1.2 congenic recipients. Three days posttransfer, recipients were challenged s.c. with 1 × 10<sup>7</sup> H11

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, resuspended in media composed of 90% FBS (HyClone) and 10% dimethyl sulfoxide (Sigma), frozen slowly in the vapor phase of liquid nitrogen in multiple cryotubes, and stored in liquid nitrogen. PBMCs from healthy individuals were isolated with density gradient separation Ficoll-Paque PLUS (Amersham Biosciences) and subsequently stained with antibodies from Becton Dickinson or eBioscience as above.

Flow cytometry
Cells were surface stained in wash buffer (PBS, 1% 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 with FlowJo (Tree Star). Flow-cytometric cell sorting was used to purify T-cell subsets from splenocytes of CpG/H11-vaccinated mice.

Statistical analysis
Prism software (GraphPad) was used to analyze tumor growth and determine statistical significance of differences between groups by applying an unpaired Student t test. P < 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 antitumor T cells are generated in vivo through vaccination with a CpG-loaded whole-cell vaccine (CpG/H11; ref. 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<sup>T</sup> cells was tumor reactive. CD4 or CD8<sup>T</sup>-cell subsets were negatively isolated from CpG/H11-vaccinated, Thy1.2 congenic, donor mice. These purified subsets were transferred into lethally irradiated, Thy1.2 congenic recipients. Three days posttransfer, recipients were challenged s.c. with 1 × 10<sup>7</sup> H11
tumor cells (Fig. 1A). Purified CD4 T cells from vaccinated donors were sufficient to protect 80% of recipient mice for more than 50 days (Fig. 1B). Conversely, purified CD8 T cells were comparable with splenocytes from unvaccinated donor mice 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 natural killer (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 posttransfer, recipients were challenged s.c. with 1 × 10^7 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 antitumor effect in 6 of 10 recipient mice (Fig. 1C). Reconstitution of the endogenous,
host CD8 T cell compartment can be observed as early as 10 days posttransplant (Supplementary Fig. 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.

Antitumor immune responses in the recipient mice were analyzed 15 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 more than 99% pure populations of either CD4 or CD8 T cells (Supplementary Fig. S2). Previously, we showed that CD4 antitumor 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 (PBL) 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 recipient cells (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 Thy1.1neg CD4 T cells were IFN-γpos (Fig. 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 assay. 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 among the IFN-γ-producing, donor T cells (Fig. 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.

**CD137posCD44hi CD4 T cells are expanded in vaccinated donor mice**

We sought to identify the CD4 T cell subset necessary for mediating antitumor 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 (Supplementary Fig. 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 (Fig. 2). The CD137pos population was predominantly within the CD44hi subset, similar to IFN-γ-producing cells shown in Fig. 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, 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 shown that agonistic mAbs against CD137 (4-IBB) can provoke CD8 T-cell antitumor responses capable of eradicating established tumors in a range of murine tumor models (20–22).

**CD137 identifies a population of activated Tregs**

We characterized the population of CD137pos 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 physiologic ratio of approximately 5% to 15% of CD4 T cells as Tregs in donor mice (23). Surprisingly, in vaccinated mice, CD137 expression marked more than 65% of this Treg population. Conversely, in unvaccinated donors or donors vaccinated with CpG alone, CD137 identified less than 35% of Tregs (Fig. 3A and Supplementary Fig. S4). Therefore, in the context of our vaccination, CD137 identifies an expanded subset of FoxP3pos CD4 T cells.

We compared CD137pos and CD137neg Tregs from vaccinated donors for expression of traditional Treg markers including CD25, GITR, and CD103 as well as memory/activation markers including CD44, CD62L, and CD69 (Fig. 3B). Compared with CD137neg Tregs, the CD137pos 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 CD137neg counterparts. The phenotype of CD44hiCD62Llow is characteristic of an effector memory population.

**CD137posCD44hi and CD137negCD44hi CD4 T cells maintain expression of both CD137 and FoxP3 after adoptive transfer**

As they seem to have an effector memory phenotype, we asked whether CD137posCD44hi Tregs persisted following adoptive transfer and whether they maintained expression of CD137 and FoxP3. With FACS, we isolated CD137posCD44hi and CD137negCD44hi CD4 T cells from vaccinated, congenic (CD45.1pos) donors to purity of greater than 98% (Fig. 4A). We transfected these purified populations into lethally irradiated...
recipients. Ten days after transfer, recipient mice were sacrificed and splenocytes were analyzed for the presence of T cells expressing the congenic marker, CD45.1. Both CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} donor cells were present in the spleens of recipient mice. Furthermore, each transferred subset retained its original phenotype: both CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} donor cells maintained their original expression levels of both CD137 and FoxP3 (Fig. 4B).

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

We investigated whether CD137^{pos}CD44^{hi} CD4 T cells were capable of infiltrating sites of tumor. CD137^{pos}CD44^{hi} and CD137^{neg}CD44^{hi} CD4 T cells were isolated from vaccinated, congenic (CD45.1^{pos}) donors by FACS and adoptively transferred into lethally irradiated recipients. Three days posttransfer, recipients were challenged s.c. with 1 × 10^7 H11 tumor cells. Approximately 7 days after transfer, tumor size averaged approximately 0.5 cm^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^{pos} versus CD137^{neg} subsets. CD137^{pos}CD44^{hi} cells made up only 14.4% of total tumor-infiltrating T cells, whereas CD137^{neg}CD44^{hi} cells made up

Figure 3. CD137 identifies a population of activated Tregs. 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 3 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 carried out 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 Treg phenotype of these 2 subpopulations. B, CD137^{pos} and CD137^{neg} subpopulations were adoptively transferred into CD45.1^{neg} recipients. Seven 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 3 individual mice per group.
57.6% of total tumor-infiltrating T cells (Fig. 5A). CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells infiltrate sites of tumor and facilitate tumor rejection while CD137\textsuperscript{neg}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 (Fig. 5B, left). Similarly, in mice receiving CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells, 18.8% of splenic T cells were of donor origin (Fig. 5B, right). 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\textsuperscript{pos}CD44\textsuperscript{hi} CD4 T cells have T\textsubscript{reg} function and suppress the antitumor activity of CD137\textsuperscript{neg}CD44\textsuperscript{hi} effector cells.**

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

Next, we tested whether CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells could suppress the antitumor response mediated by CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells. Previously, when we transferred whole CD4 T cells we observed impressive tumor rejection (2; Fig. 1). In this bulk CD4 population, the ratio of CD137\textsuperscript{pos}CD44\textsuperscript{hi} to CD137\textsuperscript{neg}CD44\textsuperscript{hi} was approximately 3:1. To determine whether CD137\textsuperscript{pos}CD44\textsuperscript{hi} could suppress antitumor immune responses in vivo, we isolated CD4 T cells by FACS from vaccinated donors to greater than 98% purity. Either CD137\textsuperscript{pos} cells alone (2 × 10\textsuperscript{6} per mouse) or a combination of CD137\textsuperscript{pos} (2 × 10\textsuperscript{6} per mouse) and CD137\textsuperscript{neg} (4 × 10\textsuperscript{5} per mouse) cells at a ratio of 1:2 were adoptively transferred into lethally irradiated recipients. A lethal challenge of 1 × 10\textsuperscript{6} H11 tumor cells was given s.c. on day 3 following transfer. CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells were sufficient to protect 100% of recipient mice from lethal tumor challenge. When CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells cotransferred, this antitumor effect was blocked and 0% of recipient mice survived longer than 20 days (Fig. 6C). Lower numbers of CD137\textsuperscript{pos}CD44\textsuperscript{hi} cells did not inhibit tumor rejection. On day 15 after transfer, PBLs from recipient mice were collected and placed in coculture with irradiated tumor cells for 24 hours. IFN-γ production was measured by intracellular flow cytometry. In mice receiving CD137\textsuperscript{pos} and CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells, only 0.9% of CD4 T cells produced IFN-γ (Fig. 6D). Conversely, in recipient mice receiving CD137\textsuperscript{neg}CD44\textsuperscript{hi} cells alone, 4.7% of CD4 T cells produced IFN-γ.

In human lymphoma-involved lymph nodes, CD137 identifies a population of activated T\textsubscript{reg}.

Recent studies have identified cell surface markers that define a discrete subset of CD25\textsuperscript{pos}FoxP3\textsuperscript{pos} T\textsubscript{reg} 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\textsuperscript{pos} CD4 T cells were present in tumor-involved spleen. This CD137\textsuperscript{pos} population was overwhelmingly CD25\textsuperscript{pos}FoxP3\textsuperscript{pos} T\textsubscript{reg} (Fig. 7A). We examined human lymphoma biopsies for whether CD137 similarly served as a marker for CD25\textsuperscript{pos}FoxP3\textsuperscript{pos} T\textsubscript{reg}. 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 (Fig. 7B). CD137\textsuperscript{pos}CD45RO\textsuperscript{pos} CD4 T cells were present in FL (mean = 5.14% ± SEM 0.34%) and MCL (mean = 8.24% ± SEM 1.68%) (Fig. 7C, left). As in the murine system, this CD137\textsuperscript{pos} population was predominantly CD25\textsuperscript{pos}FoxP3\textsuperscript{pos} (FL: mean = 68.65% ± SEM
3.28% CLL: mean = 54.73% ± SEM 5.70%; Fig. 7C, right). Therefore, CD137 may serve as an effective marker for CD25posFoxP3pos Tregs in human lymphoma patients as well.

Discussion

Our results indicate that 2 markers—CD44 and CD137—can be used to isolate a population of antitumor CD4 T cells while effectively excluding Tregs. Here, we show that (i) CD137posCD44hi expression defines a population of activated CD4 Tregs that can suppress the proliferation of effector cells in a contact-dependent manner, (ii) the CD137posCD44hi Treg population persists following adoptive transfer and can inhibit tumor rejection in vivo, and (iii) vaccination with CpG-activated lymphoma induced CD137negCD44hi CD4 T cells can transfer protection from B-cell lymphoma tumor challenge.

Rosenberg and colleagues have shown impressive clinical outcomes using in vivo generated T cells in adoptive immunotherapy for metastatic melanoma (3–5). Our prior work established that a CpG-loaded whole-cell vaccine induced antitumor CD4 T cells that were both necessary and sufficient to adoptively transfer antitumor 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 antitumor immunity have also been shown (8–11). Hunder and colleagues showed durable clinical remissions in melanoma patients treated with ex vivo expanded, antigen-specific CD4 T cells (8). Recently, studies by Xie and colleagues (10) and Quezada and colleagues (9) have shown 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 antitumor CD4 T cells can be induced in vivo, isolated, and used in adoptive immunotherapy of established lymphoma tumors. Our results suggest that these antitumor CD4 T cells produce IFN-γ and recruit a long-lasting antitumor 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 in which antitumor T cells are isolated following in vivo induction. Hunder and colleagues addressed this issue by selecting tumor reactive clones and expanding them ex vivo. Quezada and colleagues 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
T_{reg} that can facilitate their removal prior to adoptive transfer or allow their targeting with mAbs in the posttransplant recipient. Specifically, CD137 and CD44 can be used to distinguish antitumor CD4 T cells from T_{reg}. To our knowledge, this is the first report of CD137 expression being used to distinguish these 2 populations.

Surface marker identification of T_{reg} is not a novel pursuit and a number of studies have claimed to identify T_{reg} by expression of surface markers including MHC-II, CD45RO/RA, CCR7, and ICOS (25–28). Most recently, Camisaschi and colleagues (24) have observed that the expression of LAG-3 on human CD4 T cells defines a discrete subset of CD25^{+/-}FoxP3^{+/-}T_{reg} that is preferentially expanded in patients with cancer. Although 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 T_{reg}.

In summary, the work reported here shows that CD137 expression on CD4 T cells identifies a population of activated T_{reg} that suppress proliferation and antitumor immune responses. Importantly, the combination of cell surface markers CD44 and CD137 can be used to isolate a population of antitumor CD4 T cells while excluding a large, activated subset of T_{reg} for the purpose of adoptive immunotherapy of lymphoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors thank K. Sachen and D. Czerwinski for their review of this manuscript.
Grant Support
This work was supported by grants from the NIH (grants CA13399 and CA34233). M.J. Goldstein is supported by the Ruth L. Kirschstein National Research Service Award (F30HL103193). R. Levy is an American Cancer Society Clinical Research Professor.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked.

References
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.