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CD137-mediated signals costimulate T cells and protect them from activation-induced apoptosis; they induce curative antitumor immunity and enhance antiviral immune responses in mice. In contrast, anti-CD137 agonistic mAbs can suppress T-dependent humoral immunity and reverse the course of established autoimmune disease. These results have provided a rationale for assessing the therapeutic potential of CD137 ligands in human clinical trials. In this study, we report that a single 200-μg injection of anti-CD137 given to otherwise naive BALB/c or C57BL/6 mice led to the development of a series of immunological anomalies. These included splenomegaly, lymphadenopathy, hepatomegaly, multifocal hepatitis, anemia, altered trafficking of B cells and CD8 T cells, loss of NK cells, and a 10-fold increase in bone marrow (BM) cells bearing the phenotype of hemopoietic stem cells. These events were dependent on CD8 T cells, TNF-α, IFN-γ, and type I IFNs. BM cells up-regulated Fas, and there was a significant increase in the number of CD8+ T cells that correlated with a loss of CD19+ and Ab-secreting cells in the BM. TCR Vαβ usage was random and polyclonal among liver-infiltrating CD8 T cells, and multifocal CD8+ T cell infiltrates were resolved upon termination of anti-CD137 treatment. Anti-CD137-treated mice developed lymphopenia, thrombocytopenia, and anemia, and had lowered levels of hemoglobin and increased numbers of reticulocytes. The Journal of Immunology, 2007, 178: 4194–4213.
settings, anti-CD137 mAbs exacerbate acute graft-vs-host disease and accelerate skin and cardiac allograft rejection (29). Paradoxically, the same reagents enhance the survival of intestinal allografts (4), and when administered during Ag priming, anti-CD137 mAbs effectively suppress T-dependent humoral immunity (30). The latter observation led to the testing of, and the realization that, anti-CD137 mAbs could suppress and reverse the development of autoimmune diseases such as experimental autoimmune encephalomyelitis, systemic lupus erythematosus, and collagen-induced arthritis (5–8). These outcomes have been attributed to enhanced CD8 T cell survival (27), induction or suppression of CD4 T cell help (30–33), CD8 regulatory T cells (34, 35), Th1 cytokine production (29), enhanced CD8 T cell and NK cell function (18), or regulation of Ag priming by DC (19).

In this study, we report that anti-CD137 mAbs induced multifocal mononuclear cell infiltrates in the livers of BALB/c and C57BL/6 mice. These infiltrates were composed primarily of CD8 T cells having a diverse TCR Vβ phenotype. C57BL/6 mice also displayed evidence of dysregulated hemopoiesis in the spleen, lymphocyte trafficking, lymphopenia, thrombocytopenia, and anemia in an anti-CD137 dose-dependent (200-10 μg/mouse) manner. All of these outcomes were T cell and anti-CD137 dependent because they did not occur in SCID, nude, Rag-/-mice, or CD137-/- mice. Anti-CD137-treated, spleen cell-reconstituted, but not B cell-reconstituted Rag-/-mice led to the same outcomes observed in naive BL/6 mice, and identical results were obtained in anti-CD137-injected 4-1BB ligand-/-mice. CD8 T cells, TNF-α, type I IFNs, and IFN-γ were essential for the induction of various aspects of pathology. Our findings suggest that anti-CD137-induced activation leads to the production of proinflammatory cytokines, and they in turn induce many, if not all, of the observed anomalies found in anti-CD137-treated mice.

Materials and Methods

Mice and reagents

Female BL/6 mice were purchased from The Jackson Laboratory. CD137-deficient BL/6 mice were generated, as previously described (36). BL/6 4-1BB ligand-deficient mice were obtained from C. Larsen (Department of...
Surgery, Emory University, Atlanta, CA) and were initially provided by J. Peschon (Amgen, Seattle, WA). All mice were housed at the Yerkes National Primate Research Center vivarium, where they were maintained according to Institutional Animal Care and Use Committee regulations. The generation of anti-CD137 mAbs has been described (29). Anti-4-1BB ligand mAbs were generated by immunizing Lewis rats with a 4-1BB ligand-human CD8 fusion protein (37), and hybridomas were produced, as previously described (29). Anti-human CD137 mAbs were produced by immunizing Lewis rats with a fusion protein consisting of the extracellular domain of human CD137 with the human IgG1 H chain C domain (37).

Peripheral blood complete blood counts (CBC)

CBC were conducted on peripheral whole blood collected in heparinized Pasteur pipettes by retro-orbital venipuncture. Analysis was conducted using a Sysmex KX-21 automated hematology analyzer (Roche Diagnostics).

Flow cytometry

Fluorochrome-conjugated anti-mouse CD3e (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD45 (2D1), CD117 (c-kit, 2B8), pan NK, NK (DX5), anti-sca-1 (E13-161.7), and anti-CD117 (Fas) (Jo2) were purchased from BD Pharmingen. Cells were preincubated with anti-Fc mAb (2.4G2) and a 10-fold excess of mouse IgG for 10 min before staining. Ab incubations were done on ice for 30 min in PBS containing 1% BSA and 0.05% NaN₃. Cells were either acquired immediately or fixed with 1% paraformaldehyde and acquired the following day. FACS analysis was conducted using a BD Pharmingen multicolor parameter LSR-II or BD Pharmingen FACSCalibur flow cytometers. Data analysis was conducted using FlowJo software (Tree Star). Single-cell suspensions of spleen, lymph nodes (LNs), and thymus were prepared by passage through 70-μm cell strainers (BD Pharmingen). Liver and lung were pretreated with digestion buffer (1× HBSS, 20 mM HEPES (pH 7.4), 0.0175% collagenase IV, 5 mM CaCl₂, 0.0005% DNase I, and 0.0025% hyaluronidase) for 90 min at room temperature. Dead cells, debris, and liver and lung cells were then removed by Ficoll-Hypaque discontinuous density-gradient centrifugation. Bone marrow (BM) was obtained by irrigating femurs and tibias with cold PBS using a 25-gauge needle. RBC were lysed with ammonium chloride RBC lysis buffer containing the following: 8.29 g/L NH₄Cl, 1.00 g/L KHCO₃, and 0.037 g/L EDTA (pH 7.27).

Anti-CD137 treatment of mice

Eight- to 12-wk-old BL/6 female mice, CD137-deficient, or 4-1BB ligand-deficient mice were injected i.p. with 200 μg of rat anti-CD137 (3H3), rat anti-human CD137 (26G6), or rat IgG (Sigma-Aldrich) once weekly, as indicated. All Abs were standardized to a concentration of 1 mg/ml, and endotoxin levels were nil.

ELISA and ELISPOT

NUNC maxisorp 96-well plates were coated with Sigma-Aldrich rat IgG (I-4131) at 20 μg/ml at 4°C overnight. They were then washed with PBS with 0.05% Tween 20. They were blocked with 2% BSA in PBS overnight at 4°C. After washing, serum samples were serially diluted with PBS containing 1% BSA and 0.05% Tween 20 and incubated for 3 h at room temperature. Anti-rat IgG (H+L) was used as a standard (Biomedia). After extensive washing, goat anti-mouse IgG (H+L) HRP (Invitrogen Life Technologies) was added and incubated at room temperature for 2 h. Plates were then washed and developed with urea-buffered PBS containing hydrogen peroxide and o-phenylenediamine. The reaction was stopped with 50 μl of H₂SO₄ per well. Plates were read at 490 nm with a Spectra Max

FIGURE 1. (continued)
For ELISPOT assays, Millipore MAHA N4510 plates (Millipore) were coated with goat anti-mouse IgG, IgM, and IgA (Invitrogen Life Technologies) at 100 μg/ml/well, 5 μg/ml overnight at 4°C. They were blocked with RPMI 1640 complete medium for 2 h. Splenocytes were plated at 10⁶ in 100 μl of RPMI 1640 complete medium, serially diluted 3-fold, and incubated for 5 h at 37°C. Biotinylated goat anti-mouse IgG (Invitrogen Life Technologies) was added, and plates were incubated overnight at 4°C. Plates were developed with HRP-conjugated avidin-D (Vector Laboratories) at 5 μg/ml for 1 h and 3-amino-9-ethylcarbazole substrate. Spots were read using ImmunoSpot ELISPOT reader and software (Cellular Technology).

Histology
Organs were fixed in 10% formalin overnight and paraffin embedded. Slices (5 μm) were H&E stained. Photomicrographs were taken with a Zeiss Axioskop 2 microscope.

CBCs
Mice were anesthetized with isoproterenol and bled retro-orbitally through heparinized capillary tubes into microtainers containing EDTA (BD Pharmingen). A total of 50 μl of blood was analyzed with a blood cell analyzer (Kx-21; Sysmex).

FIGURE 2. Spleen and LN cell numbers. a, Total spleen cell numbers (rectangle represents mice within spleen cells within the normal range) as well as T and B cell numbers from spleens and inguinal LNs obtained from anti-CD137- or rat IgG-injected mice for the indicated periods were determined following FACS analysis. CD137- or rat IgG-injected mice were collected 1 wk after the indicated number of injections. Cell numbers in lymphoid tissues of rat IgG-injected mice varied by <10% over the course of three weekly injections, and the time point shown in all figures is of mice that received three injections. b–e, Single-cell suspensions of spleen, LN, liver, and lung, respectively, from anti-CD137- or rat IgG-injected mice were phenotyped 1 wk following three weekly injections for T, NK, and NKT cells; CD44, CD62L, CD45RB, and CD69 expression was measured on T cells.
Statistical analysis

Statistical significance at the 95% confidence interval was conducted using unpaired, two-sided Student’s $t$ test, or one-way ANOVA, depending on the nature of the study groups.

CFSE labeling and BrdU incorporation

Lymphocytes were resuspended at $2 \times 10^7$/ml in PBS containing 2.5 mM CFSE (Molecular Probes) and incubated at 37°C with periodic shaking for 5 min. The suspension was washed twice with 15 ml of cold PBS and
BrdU labeling, mice were injected i.p. with 200 μl of a 10 mg/ml solution of BrdU in sterile Dulbecco’s PBS. Four hours later, the mice were euthanized and single-cell suspensions of lymphocytes were intracellular stained with FITC anti-BrdU following the manufacturer’s recommendations (BD Immunocytometry Systems).

Results

Gross pathology and histopathology

The fact that anti-CD137-injected NZB/W F1 autoimmune mice developed hepatomegaly was of immediate concern. However, this outcome may have been related to their predisposition to develop autoimmune disease. Therefore, we injected naive BALB/c mice and 4-1BB ligand-deficient mice that were on the BL/6 background. Because both strains of mice developed indistinguishable pathology, this report focuses solely on data obtained from anti-CD137- or rat IgG-injected BL/6 mice. Mice were euthanized 1 wk following the indicated number of weekly 200-μg i.p. Ab injections. Spleens, livers, and left and right inguinal LNs obtained from anti-CD137-injected mice were markedly enlarged, and this correlated with elapsed time of treatment (Fig. 1a). Rat IgG-injected mice exhibited minor enlargement of the spleen and LNs, but this was generally small (<10%). In contrast to splenomegaly and lymphadenopathy that continued to increase over the treatment period, hepatomegaly plateaued within 2 wk of treatment (Fig. 1a). Mice given a single 200-μg dose of anti-CD137 and assayed 5 wk later also developed splenomegaly, hepatomegaly, and lymphadenopathy (Fig. 1b). However, in this instance, the level of splenomegaly and hepatomegaly was less severe than that seen following multiple injections of anti-CD137. Similarly, five weekly 10 μg doses of the 3H3 anti-CD137 mAb, or any of several independently generated anti-CD137 mAbs of varying isotype and fine specificity were sufficient to cause the same effects (Fig. 1c). In contrast, a single 10- or 30-μg injection of anti-CD137 was without effect (data not shown). These data indicate that the specificity or isotype of the 3H3 mAb used in these studies was not the cause of these events. All Abs were endotoxin free, as judged by standard Limulus agglutination assays. The 3H3 is a rat IgG2a isotype Ab having a serum t1/2 in mice of 7 days. Histology was performed on all tissues from anti-CD137- or rat IgG-injected mice 1 wk following the fifth weekly 200-μg injection. In all instances, spleen, liver, and lungs from rat IgG-injected mice appeared normal (Fig. 1d, left panels), whereas the LNs, liver, and lungs of anti-CD137-injected mice displayed mononuclear cell infiltrates (Fig. 1d, middle and right panels), and in the case of the spleen dissolution of the typical histological architecture and marked evidence of extramedullary hemopoiesis (Fig. 1d, middle and right panels). Livers from rat IgG-treated mice were normal (Fig. 1d, left panel), whereas livers from anti-CD137-treated mice revealed evidence of multifocal hepatitis (Fig. 1d, middle panel) with prominent perivascular accumulation of mononuclear cells and the presence of apoptotic hepatocytes around the vasculature (Fig. 1d, middle right panel). Indicative of possible liver inflammation, we observed a 2-fold elevation in serum glutamic oxaloacetic transaminase and serum glutamic pyruvate transaminase levels in the serum of the mice within 2 wk of treatment (Fig. 1e). Although the increase in transaminase levels can be caused by other factors, and was only 2-fold over controls, the observation was highly reproducible and no other signs of pathology could explain this occurrence. Furthermore, FACS analysis revealed that >30% of liver-infiltrating CD4+ T cells and ~15% of infiltrating CD8+ T cells expressed Fas ligand, suggesting that these T cells could induce apoptosis of hepatocytes that express Fas (CD95) on their surface. Although the lungs of anti-CD137-treated mice were normal on gross inspection, they contained a 10-fold increase in the number of infiltrating peribronchiolar mononuclear cells (Fig. 1d, middle panel), and these cells were observed migrating into the bronchial lumen (Fig. 1d, right panel).

In the spleen, we found a direct correlation between spleen weight and cell number during the first 21 days of treatment (Figs. 1a and 2a). However, by day 28 we observed a decreasing trend in spleen cell numbers (Fig. 2a). However, this decrease did not reach statistical significance until after week 4 for CD8+ T cells (Fig. 2a). In contrast, we observed a more rapid loss of CD19+ B cells and CD4+ T cells by weeks 2 and 3, respectively (Fig. 2a). This was not due to CD19 down-regulation because there was a concomitant loss of B220 and surface IgM+B cells (data not shown). Thus, splenic B cells were either being deleted or trafficking elsewhere. This is in contrast to what we observed in collagen-induced arthritis-susceptible DBA/1J mice, in which spleen cell numbers actually increased at ~30 days of anti-CD137 treatment (6). The reason for this discrepancy is not immediately evident and may simply reflect, for reasons unknown, a difference in the kinetics of...
lymphocyte trafficking in the two strains of mice. It should also be pointed out that DBA/1J mice had far fewer spleen cells at basal levels than did BALB/c or C57BL/6 mice. In addition to the loss of splenic B cells, we observed a near complete loss of NK and NKT cells in the spleens of anti-CD137-injected mice (Fig. 2b). Phenotypic analysis of splenic T cells for CD44 and CD62L expression revealed a small increase in the frequency of CD44\textsuperscript{high}CD62L\textsuperscript{low}/H11002 memory T cells in anti-CD137-treated mice; however, the majority of cells expressed high levels of CD62L (Fig. 2b). Furthermore, we observed an increase in the frequency of T cells that had down-regulated their expression of CD45RB and did not express the early activation marker CD69 (Fig. 2b). Phenotypic analysis of LN cells to a large degree mirrored what was seen in the spleen. In this instance too, there was an absence of NK and NKT cells. With regard to their activation state, LN T cells significantly down-regulated CD45RB expression with no change in memory phenotype (Fig. 2c). One means by which lymphocytes are retained within LNs is by up-regulating expression of CD69. Studies by Shiow et al. (38) have shown that the activation Ag CD69 complexes with sphingosine-1-phosphate (S1P) receptor 1 (S1P\textsubscript{1}) and has an antagonistic effect on S1P\textsubscript{1}-mediated egress of lymphocytes from the LNs and thymus. However, we did not find evidence of CD69 expression on LN lymphocytes from anti-CD137-treated mice.

Within the livers of rat IgG-injected mice, we found very high frequencies of NKT cells (\textsuperscript{30%}) and relatively high frequencies of NK cells (Fig. 2d). In contrast, we found a 2-fold reduction in the frequency of liver NKT cells, but little change in the frequency of NK cells in the livers of anti-CD137-injected mice, although the intensity of NK1.1 expression on these cells had decreased.

FIGURE 3. Phenotypic analysis of T cell subsets. CD3\textsuperscript{CD4}\textsuperscript{CD8}, CD3\textsuperscript{CD4}CD8\textsuperscript{CD8}, and CD3\textsuperscript{CD4}CD8\textsuperscript{CD8} T cell subsets from the spleen (a), LNs (a), livers (b), and lungs (c) of anti-CD137- or rat IgG-injected mice (weekly \times 3) were phenotyped 1 wk following the final injection of Ab. The data are representative of one of five mice/group, and the experiment was repeated twice.
However, when we determined the absolute numbers of liver NKT and NK cells in rat IgG- and anti-CD137-treated mice, there was no difference in NKT cell number between the two groups of treated mice, whereas there were increased numbers of NK cells in anti-CD137-injected mice that almost reached statistical significance (Fig. 2d). NK cells in mice and in humans display different phenotypic markers, such as CD16 and CD56 in humans. In mice this is true for the IL-7Rα (CD127) expression and the requirement for GATA-3, depending upon whether they are BM or thymus derived. Vosshenrich et al. (39) have found that splenic NK cells do not express GATA-3, whereas BM and liver NK cells express low levels of

FIGURE 4. B cells, BM, and ASC. BM cells were obtained from the femurs of rat IgG- and anti-CD137-treated mice that had received weekly injections of 200 μg of Ab over a 5-wk period. The mice were euthanized 1 wk later, and single-cell suspensions were prepared by passage through 70-mesh nylon screens, counted for cell viability, stained with the indicated fluorochrome-conjugated mAbs, and analyzed by flow cytometry. The absolute number of a, CD19+ cells; b, CD8+ cells; c, c-kit− Sca-1− Lin− hemopoietic stem cells (KSL); and d, Fas+ cells was determined by FACS analysis. e, The number of CD19+ BM cells/femur was determined in C57BL/6 IFN-γ-deficient mice injected with either rat IgG or anti-CD137, as described above. f, The number of IgG-secreting BM cells/femur of Ab-treated mice was determined by ELISPOT assay.
GATA-3, but thymic NK cells express high levels of GATA-3. Both BM- and thymus-derived NK cells can be found in the LNs of normal mice. Because we do not find any NK cells in the LNs of anti-CD137-treated mice, we can conclude that both BM- and thymus-derived NK cells are equally affected by anti-CD137 mAb treatment. Thus, it is interesting to note that anti-CD137 treatment fails to eliminate NK cells from the liver; instead, it modestly increased their numbers. In the lungs, the frequency (Fig. 2e) and absolute number of NK cells (data not shown) increase dramatically in anti-CD137-treated mice, and red histograms represent rat IgG-treated mice. The percentages shown for each histogram represent the frequency of nondividing cells in each gated population denoted by the horizontal bar intersecting the histogram peak. b, Inguinal LN B and T cell proliferation was also determined by BrdU short pulse labeling for 4 h following the third weekly injection of anti-CD137 or rat IgG.

FIGURE 5. a, In vivo proliferation of LN CD8 T cells and B220 B cells was conducted by adoptively transferring CFSE-labeled BL/6 CD45.1 congenic spleen cells into wild-type CD45.2 BL/6 mice before treating the mice with five weekly injections of 200 μg of anti-CD137 or rat IgG. FACS analysis was performed at week 6. CD45.1 CD3+ CD8+ T cell and CD19+ B cell proliferation was determined by CFSE dilution. Black histograms represent CD137-treated mice, and red histograms represent rat IgG-treated mice. The percentages shown for each histogram represent the frequency of nondividing cells in each gated population denoted by the horizontal bar intersecting the histogram peak. b, Inguinal LN B and T cell proliferation was also determined by BrdU short pulse labeling for 4 h following the third weekly injection of anti-CD137 or rat IgG.
from rat IgG-injected mice, with the exception of CD4+ T cells, consisted of CD45RBhigh cells (Fig. 3b). In contrast, in anti-CD137-injected mice, CD45RB expression was highly down-regulated on liver CD8+ T cells and Dbl. Neg. T cells (Fig. 3b), whereas CD62L down-regulation was most evident on Dbl. Neg. and Dbl. Pos. T cells in these mice (Fig. 3b). Analysis of lung-derived T cells revealed that anti-CD137 treatment blocked CD45RB down-regulation on CD4+ and CD8+ T cells, and to some extent, on Dbl. Pos. T cells. In contrast, we observed a mixed phenotype within the Dbl. Neg. T cell subset, in which both reduced and increased CD45RB expression was found on populations of these cells (Fig. 3c). CD44 expression was down-regulated on the Dbl. Neg. T cell population of anti-CD137-treated mice, up-regulated on the Dbl. Pos. T cell population, highly up-regulated on CD8+ T cells, and modestly up-regulated on CD4+ T cells (Fig. 3c). CD62L expression, in contrast, was up-regulated on CD8+ T cells and Dbl. Pos. T cells in the lungs of anti-CD137-injected mice, whereas CD69 expression was either unchanged or modestly down-regulated on these T cell subsets (Fig. 3c).

We observed three striking features in the BM of anti-CD137-treated mice. The first was the marked loss of CD19+ cells (Fig. 4a); the second was a 6- to 10-fold increase in the number of infiltrating CD8+ T cells (Fig. 4b); and the third, and perhaps most striking, was a >10-fold increase in CD45+ KSL, i.e., c-kit+ lin- hematopoietic stem cells (Fig. 4c). It has been shown that the regulation of hemopoiesis and cellular proliferation in the BM is in part controlled by TNF-α, in which TNF-α potently suppressed the development of primitive and committed BM progenitors in both mice and humans (45, 46). It has also been shown that Fas-mediated apoptosis regulates BM progenitor cell development (47), and that TNF-α and IFN-γ induce Fas expression on T cells (48). We, therefore, examined the role of CD8 T cells, Fas (CD95) expression, and TNF-α on the loss of CD19+ BM cells from rat IgG- or anti-CD137-injected mice 1 wk following the last of five weekly injections of Ab. We first assessed the role of CD8 T cells in the loss of CD19+ BM progenitor cells by injecting CD8 T cell-deficient mice with rat IgG or anti-CD137. However, these studies ruled out CD8+ T cells as mediators of CD19+ BM cell loss because we found the same degree of CD19+ BM cell depletion in CD8 T cell-deficient mice with rat IgG or anti-CD137. However, these studies ruled out CD8+ T cells as mediators of CD19+ BM cell loss because we found the same degree of CD19+ BM cell depletion in CD8 T cell-deficient mice in wild-type mice (data not shown). We next determined whether TNF-α was responsible for the loss of B cell progenitors. BL/6 mice were injected with anti-CD137 or rat IgG once weekly with or without 200 μg of a neutralizing anti-TNF-α mAb for 5 wk, and 1 wk later the mice were euthanized and their BM collected and FACS analyzed. In addition, TNF-α-deficient mice were also treated with anti-CD137 or rat IgG. In both sets of experiments, we found the same degree of CD19+ BM cell and Ab-secreting cell (ASC) loss as that seen in
wild-type mice (data not shown). We next examined, and found, marked up-regulation of Fas expression on BM cells, and this correlated with the concomitant loss of CD19− BM cells (Fig. 4d), suggesting that the loss of these cells and perhaps other progenitor cells may occur in part through Fas-mediated signaling. However, when we treated MRL-lpr mice with anti-CD137, we still found the same level of CD19− BM cell loss (data not shown). Previous studies have reported the suppression of B cell development in a BM transplantation model in which the donor BM differed from the recipient at several minor MHC loci. Suppression of B cell development was caused by donor T cell-mediated graft-vs-host disease reactions and was dependent upon the production of IFN-γ by these alloreactive T cells (49). Because anti-CD137 stimulation induces the production of high levels of IFN-γ, we tested whether this cytokine played a role in the loss of CD19− cells by using IFN-γ-deficient mice or BL/6 mice injected weekly with neutralizing anti-IFN-γ mAbs. In contrast to all other strains tested, BM CD19− progenitors in anti-CD137-injected, IFN-γ-deficient mice were the same in number as those found in rat IgG-injected IFN-γ-deficient mice, or wild-type mice (Fig. 4e). To determine whether BM ASC were affected by anti-CD137 treatment, mice were injected with PBS, rat IgG, or anti-CD137, as described above. BM was collected and assayed by overnight culture on ELISPOT plates coated with sheep anti-mouse IgG. Whereas PBS-injected mice had on average 280 ASC/10⁶ BM cells, anti-CD137-injected mice had <40 ASC/10⁶ BM cells (Fig. 4f). These data are in keeping with our previous studies showing that anti-CD137 mAbs suppress the induction of T-dependent humoral immunity in mice (5, 6, 30), and important to this study, to itself.

LN CD8+ T and B cell proliferation

In contrast to splenic lymphocytes, CD4+ and CD8+ T cells and CD19+ B cells in the inguinal LNs significantly increased in number following anti-CD137 treatment (Fig. 2a). To assess whether this was due to lymphocyte trafficking, proliferation, or both, we injected 1–2 × 10⁶ CFSE-labeled BL/6 CD45.1 congenic splenocytes i.v. into naive BL/6 CD45.2 mice before beginning anti-CD137 treatment 10 days later. Twenty-one days after the last of
three weekly injections of anti-CD137, the mice were euthanized and inguinal LNs were removed. LN CD45.1+ CFSE-labeled T and B cells were counted and proliferation was measured by FACS. Over 50% of CD8+ T cells from anti-CD137-treated mice (black histogram) had undergone multiple rounds of cell division compared with 17% for rat IgG (red histogram)-treated mice (Fig. 5a, left panel). Thus, we could not determine whether CD8 T cells in anti-CD137-injected mice were proliferating within, or trafficked to the LNs following proliferation, and this was true for CD4 T cells (data not shown). However, this was not the case for B cells, in which <30% (mean of 27 ± 4%; n = 4) of CD45.1+CD3- B220+ B cells (black histogram) had undergone cell division, a frequency that was comparable with that observed in rat IgG (red histogram)-injected mice (Fig. 5a, right panel). Still, there was a 3- to 7-fold increase in the total number of LN B cells in anti-CD137-treated mice (Fig. 2a), suggesting that B cells had emigrated to the inguinal LNs. To further address this issue, and to determine the level of in situ proliferation at various time points following anti-CD137 treatment, we i.p. injected anti-CD137-treated and rat IgG-treated mice with BrdU 1 day following the second weekly injection of Ab. Four hours later, the mice were euthanized and inguinal LNs were collected (as were other tissues), and single-cell suspensions were stained with fluorochrome-conjugated mAbs specific for CD3, CD4, CD8, and CD19. The cells were permeabilized and stained with a fluorochrome-conjugated anti-BrdU mAb (BD Immunocytometry Systems) and analyzed by FACS. However, although high levels of proliferation were seen in the BM and thymus, there was no difference between the two groups of treated mice, and there was little evidence of lymphocyte proliferation in any of the other tissues studied (data not shown). Therefore, a second experiment was performed in which the mice were given two weekly injections of Ab. One day following the third weekly injection, the mice were injected with BrdU, and 4 h later euthanized. Inguinal LN cells (Fig. 5b) as well as spleen, liver, and lung were analyzed, as described above (Fig. 6c). Analysis of LN B and T cells from rat IgG- or anti-CD137-injected mice showed similar levels of BrdU incorporation, suggesting that the major cause of the increase in absolute numbers of these cells in the LNs of anti-CD137-treated mice was caused by cell trafficking and not proliferation.

Splenic T cells and lung- and liver-infiltrating T cells
Lung and liver mononuclear cells from mice that received two to three injections of 200 μg of anti-CD137 mAbs or rat IgG were...
stained with fluorescence-conjugated mAbs, and the frequency of cell lineages was determined by FACS analysis. The number of non-T cells in lungs and livers from anti-CD137-treated mice was equal to controls (data not shown). In the lungs, there was no change in the frequency (data not shown) or absolute numbers of CD4\(^+\) T cells (Fig. 6a), whereas CD8\(^+\) T cell number increased almost 10-fold (Fig. 6a). In the liver, there was a dramatic increase in both CD4\(^+\) and CD8\(^+\) T cells (Fig. 6a). Proliferation of spleen-, liver-, and lung-infiltrating CD8 T cells was determined by CFSE dilution, as described above, in mice given 1 × 3 weekly 200-µg injections of anti-CD137 or rat IgG. Whereas a significant number of CD8 T cells in the spleen and virtually all CD8 T cells in the livers of anti-CD137-injected mice were proliferating, CD8 T cells in the lungs from anti-CD137- or rat IgG-injected mice had undergone a couple of cell divisions either before or following their entry into the lungs (Fig. 6b). Regardless, there was no notable change between treated and control groups. CD8\(^+\) T cell proliferation was then determined by BrdU incorporation, as described above, 24 h following the last of three weekly injections of either anti-CD137 or rat IgG. Again, although massive proliferation was observed in the BM of both groups of mice, and was in fact increased in anti-CD137-injected mice and in the thymus, in which no differences in proliferation between the groups were seen, we observed only a slight difference in BrdU incorporation by liver CD8\(^+\) T cells obtained from anti-CD137-treated mice (Fig. 6c).

Lymphocyte proliferation

Lymphocyte proliferation was studied in greater detail to determine whether the observed changes in cell number in various tissues were a result of proliferation or trafficking. In addition to B

FIGURE 8. TCR V\(\alpha\) and V\(\beta\) usage by liver-infiltrating CD8 T cells and liver NK/NKT cells. a and b, TCR V\(\alpha\) and V\(\beta\) usage, respectively, by liver-infiltrating CD8 was determined in three separate experiments by staining CD8 T cells with fluorochrome-conjugated mAbs specific for individual V\(\alpha\) and V\(\beta\) gene products, followed by FACS analysis 1 wk following the last of three weekly 200-µg injections of anti-CD137 or rat IgG. c, The absolute number of liver-infiltrating NK and NKT T cells was determined by flow cytometry following staining with anti-CD3 and NK1.1.
cells and single-positive T cells, we examined CD3\(^{-}\)CD4\(^{+}\)CD8\(^{-}\) subsets. In the thymus, we did not observe any changes in the frequency or absolute number of proliferating mature T cells, B cells, or among thymocyte subpopulations in mice injected with anti-CD137 or rat IgG (data not shown). In the BM, we observed a slight, but reproducible increase in the proliferation of CD3\(^{-}\)CD19\(^{+}\) cells in anti-CD137-injected vs rat IgG-injected mice (46.2 vs 34.7%). In the spleens, we observed a slight decrease in proliferation within the CD3\(^{-}\)CD4\(^{+}\)CD8\(^{-}\) population and a 3-fold increase in CD3\(^{-}\)CD19\(^{+}\) lymphocytes (Fig. 7a), whereas in the LNs we observed an increase in the proliferative rate of CD3\(^{-}\) Dbl. Neg. lymphocytes (Fig. 7b). In the livers, we did not observe any significant change in the proliferation of any subset examined (Fig. 7c), whereas we found a 2-fold increase in the frequency of CD4\(^{-}\) T cell proliferation in the lungs and a 14% increase in proliferation of CD3\(^{-}\) Dbl. Neg. lymphocytes (Fig. 7d). From these data we can conclude that the major changes in lymphocyte numbers in the LNs, livers, and lungs, and CD8 T cell accumulation in the BM of anti-CD137-injected mice are largely the result of altered lymphocyte trafficking.

**TCR specificity and pathophysiology**

T cells are essential for the manifestation of the pathologies found in anti-CD137-treated mice because no changes were seen in anti-CD137-treated Rag\(^{-/-}\) mice, the mice developed all of the pathologies observed in BL/6 mice. TCR specificity and the state of T cell activation in anti-CD137-treated mice were studied by injecting naive lymphocytic choriomeningitis virus gp33–41-specific BL/6 P14 TCR Rag\(^{-/-}\) transgenic, and BL/6 OT-II OVA-specific TCR transgenic Rag\(^{-/-}\) mice weekly, over a 5-wk period, with anti-CD137 mAbs. In neither case did we observe any effect of anti-CD137, including activation markers or CFSE dilution (data not shown), suggesting that naive T cells are not targets of anti-CD137 mAbs. To determine whether Ag-activated P14 CD8\(^{+}\) T cells could generate the observed pathologies, we activated P14 cells in vitro for 5 days with gp33–41 peptide-pulsed DC in the presence of IL-2 and transferred them along with syngeneic B cells into recipient Rag\(^{-/-}\) recipients, and the mice were injected weekly with anti-CD137 over a 4-wk period. The T cells continued to proliferate in anti-CD137-treated mice and increased in number at a rate faster than that seen in homeostatic proliferation (data not shown). Nevertheless, the mice developed none of the pathologies seen in anti-CD137-treated normal naive mice (data not shown), suggesting that activated P14 CD8 T cells alone were insufficient to induce pathology, or that TCR specificity was an important component to the establishment of pathology. To address potential autoreactivity and TCR clonality of CD8 T cells in the livers of anti-CD137-injected mice, we injected naive mice with anti-CD137 or rat IgG once weekly for 5 wk. Livers were removed 1 wk after the final Ab injection and mononuclear cells
were isolated. Because CD8 T cell-deficient mice do not develop hepatitis and CD4 T cell-deficient mice do (see below), we only examined the frequency and number of CD8\(^+\) T cells bearing different V\(\alpha\) and V\(\beta\) specificities. Data from three experiments showed increased frequencies of T cells bearing diverse TCR V\(\alpha\) and V\(\beta\) gene products, indicating a lack of clonality and, by extension, a low probability that the expansion of CD8 T cells represented an autoimmune reaction (Fig. 8). Indeed, mice given anti-CD137 for 26 wk never developed signs of autoimmune disease; once anti-CD137 injections were terminated, the livers of these returned to normal, suggesting that hepatitis may be caused primarily by proinflammatory cytokines.

**Peripheral mononuclear cells and blood elements**

Anti-CD137-treated mice developed evidence of extramedullary hemopoiesis in the spleen. To determine whether the hemopoietic system in these mice was under stress, we studied the effect of anti-CD137 treatment on PBMC number. Mice were injected weekly with 200 \(\mu\)g of anti-CD137 or rat IgG and bled every 10–14 days over a 4-mo time span. White blood cell counts

**FIGURE 10.** Anti-CD137 treatment of CD8- or CD4-deficient mice. a and b, Spleens, inguinal LNs, and livers from C57BL/6 CD8- or CD4-deficient mice, respectively, were collected 1 wk following the fifth weekly injection of anti-CD137 or rat IgG mAb and weighed. BM from the femurs was prepared and phenotyped by FACS, and the number of BM CD19\(^+\) and KSL (c-kit\(^+\) Sca-1\(^+\) Lin\(^-\)) cells was determined.
dropped precipitously within 2 wk of treatment (Fig. 9a) due to the onset of lymphopenia (Fig. 9b), whereas the number of monocytes increased 2- to 3-fold (Fig. 9c). By the fifth week, anti-CD137-treated mice developed marked thrombocytopenia (Fig. 9d). Platelet consumption can occur in response to high systemic levels of TNF-α (50, 51), and we have found that anti-CD137 induces TNF-α production (our unpublished observations). The onset of thrombocytopenia in anti-CD137-injected mice is noteworthy because platelets are a major source of S1P (52–54). High S1P levels in the blood relative to the tissues are essential for the formation of a concentration gradient needed for the egress of S1P1-expressing lymphocytes from the thymus and LNs (55). As shown above, we find a significant number of nonproliferating B cells as well as T cells in the LNs of anti-CD137-injected mice, suggesting that the loss of platelets in these mice may be responsible for lowering serum S1P levels. As an indirect indicator that this may be the case, we find that S1P1 mRNA is up-regulated on lymphocytes in anti-CD137-injected mice by a factor of 2 compared with rat IgG-treated controls (data not shown). This being the case, one would expect that these cells would rapidly egress from this tissue, but they do not. Other changes in the CBC of anti-CD137-injected mice included an increase in the number of reticulocytes (Fig. 9e), an observation that correlated with a 25% decrease in hemoglobin levels (Fig. 9f) and a low hematocrit (data not shown). Spleens from anti-CD137-injected mice, rather than having a deep dark red color, were pink and pale in color (data not shown). Reduced CBC could be accelerated in anti-CD137-treated, but not rat IgG-injected mice by bleeding them every other day (100 μl) for 1 wk. This stress led to a severely diminished hematocrit and death of 90% of the mice (n = 10) within 1 wk.

**CD8 and CD4 T cell-mediated pathology**

We determined whether CD8– and/or CD4+ T cells were required for the onset of pathology. CD8 T cell−/− and CD4 T cell−/− mice received 200 μg of anti-CD137 mAbs or rat IgG once weekly for 5 wk and were euthanized 1 wk later, and lymphoid tissues and selected organs were removed and analyzed. CD8 T cell−/− mice developed splenomegaly and lymphadenopathy (Fig. 10a), suggesting that CD4 T cells and/or NK cells could induce these reactions. Whereas NK cells may play a role in the induction of hepatomegaly, it is unlikely they induce splenomegaly or lymphadenopathy, because virtually all NK cells are depleted from the spleens and LNs of anti-CD137-injected mice. However, CD8−/−

**FIGURE 11.** Anti-CD137 treatment of TNF-α−/− mice. BL/6 TNF-α−/− mice were injected i.p. with 200 μg of rat IgG or anti-CD137 mAbs 1 × weekly for 3 wk. The following week, the mice were bled and euthanized. Spleen, inguinal LNs, and livers were weighed (a–c), and tissues were processed into single-cell suspensions, stained for CD8 T and CD19-positive B cells, and analyzed by FACS. From FACS frequency plots and microscopic count of viable cells, absolute numbers of liver and LN CD8 T cells were determined (d and e, respectively), and LN and splenic CD19 B cells were determined (f and g, respectively).
mice did not develop hepatomegaly (Fig. 10a) or multifocal hepatitis (data not shown). CD8 T cell/H11002 mice nevertheless had reduced numbers of CD19/H11001 BM cells (Fig. 10a) and fewer splenic CD19− cells, KSL cell, and CD8+ T cell numbers were determined in single-cell suspensions stained with fluorochrome-conjugated anti-CD19, anti-CD3, anti-CD8, anti-c-kit, anti-Sca-1, and lineage-specific mAbs. B cells, KSL cells, and CD8 T cells were enumerated following FACS analysis.

Cytokine-deficient mice

We have found that TNF-α serum levels are elevated and prolonged following anti-CD137 treatment of lymphocytic choriomeningitis virus-infected mice (C. H. Maris, B. Zhang, I. Foell, J. Whitmire, L. Niu, J. Song, J. Tan, E. Sotomayor, B. S. Kwon, A. T. Vella, J. Jacob, R. Ahmed, and R. S. Mittler, manuscript in preparation). Therefore, we assessed the contribution of TNF-α in promoting anti-CD137-induced pathologies. Anti-CD137-treated BL/6 TNF-α−/− mice did not develop splenomegaly, lymphadenopathy, or hepatomegaly (Fig. 11, a–c, respectively). Furthermore, the mice did not develop lymphopenia (data not shown). This is in keeping with previous reports showing that mediators of innate immunity, including type I IFNs and TNF-α, can shut down systemic shutdown of egress of lymphocytes from secondary lymphoid tissues, and as a result, a loss of these cells in the circulation (56–58). Whereas the livers of anti-CD137-injected mice had a 4-fold increase in numbers of CD8 T cells (Fig. 11d), they did not show any signs of liver pathology or elevated levels of transaminases (data not shown). Consistent with data from CD8−/− mice, the absence of TNF-α did not alter the loss of CD19− B cells from the spleen (Fig. 11e), nor the increase in the number of B cells and CD8 T cells in the LNs (Fig. 11, f and g, respectively). The observations observed in TNF-α-deficient mice were also observed by injecting neutralizing anti-TNF-α mAbs into anti-CD137-treated BL/6 mice (data not shown).

Because we had shown previously that anti-CD137 enhances production of proinflammatory cytokines such as IFN-γ by CD8−

FIGURE 12. Anti-CD137 treatment of IFN-γ- and IFN-αR-deficient mice. a, Inguinal LNs from IFN-γ-deficient mice were collected 1 wk following the third weekly injection of anti-CD137 or rat IgG, weighed and processed into single-cell suspensions, and phenotyped by FACS to determine total B cell and CD8 T cell numbers. b, BM, inguinal LNs, and spleens were collected from IFN-αR-deficient mice, as above. CD19− cell, KSL cell, and CD8+ T cell numbers were determined in single-cell suspensions stained with fluorochrome-conjugated anti-CD19, anti-CD3, anti-CD8, anti-c-kit, anti-Sca-1, and lineage-specific mAbs. B cells, KSL cells, and CD8 T cells were enumerated following FACS analysis.
T cells (29), we assessed the effects of anti-CD137 mAb injection of BL/6 IFN-γ−/− mice. Although IFN-γ−/− mice developed splenomegaly, hepatomegaly, multifocal hepatitis (data not shown), lymphopenopathy (Fig. 12a), and an increase in the numbers of BM KSL cells (data not shown), we found no change in the number of LN CD19+ B and CD8+ T cells (Fig. 12a). These outcomes were also observed in anti-CD137-injected BL/6 mice coinjected with neutralizing anti-IFN-γ mAbs (data not shown).

Plasmacytoid DC are efficient producers of type I IFNs (59). We, and others, have shown that DC can express CD137 (19, 20). Among DC subpopulations, we found that plasmacytoid express the highest density of CD137 receptors on their surface, and as a population, they contain the highest frequency of CD137-positive DC (our unpublished observation). We considered that cross-linking CD137 on pDC might contribute to the anti-CD137-induced phenotype through up-regulation of type I IFN production. Because type I IFNs bind a common receptor (59), we used type I IFNR−/− mice to assess whether these cytokines were required for anti-CD137-induced pathology. The mice were injected once weekly for 3 wk with 200 μg of anti-CD137 or rat IgG. One week after the last injection, the mice were euthanized, and organs and cells were analyzed. All of the anti-CD137-treated mice developed splenomegaly, lymphopenopathy, hepatomegaly, and multifocal hepatitis (data not shown). However, unlike that seen in other mice, the frequency and absolute number of CD19+ BM cells were normal (Fig. 12b). Most striking, these mice had normal frequencies and absolute numbers of KSL+ BM cells (Fig. 12b), and normal numbers of CD19+ B cells and CD8 T cells in the LNs (Fig. 12b). Furthermore, the number of splenic CD19+ B cells was greatly increased (Fig. 12b).

Discussion

Based on encouraging preclinical data from mouse studies, anti-human CD137 mAbs are entering clinical trials for treating cancer, rheumatoid arthritis, and systemic lupus erythematosus. However, little is known regarding potential adverse events associated with repeated in vivo administration of anti-CD137. Having knowledge of such reactions, should they occur, would be of considerable value in guiding the design of clinical trials, especially because earlier studies of several receptor-ligand pairs of the TNFR-TNF superfamilies such as TRAIL-DR5 (60, 61), Fas ligand-Fas (62, 63), and CD70-CD27 (64) have been found to induce severe toxicity when given in vivo, or during chronic stimulation.

The results of our study demonstrate that although the CD137 signaling pathway is another example in which its therapeutic manipulation holds promise for its clinical applicability, such manipulation can lead to unexpected adverse events, potential pathology, and the alteration of normal immune system or organ function. One injection of anti-CD137 mAb at 10 or 0.5 mg/kg given once weekly over a 5-wk period induced a series of adverse events in normal mice that appear to be confined to the immune and hemopoietic systems. These occurrences may ultimately adversely affect the normal function of several major organs, including the liver, lungs, spleen, and BM. CD137 receptor cross-linking, CD8+ T cells, and the production of TNF-α, IFN-γ, and IFN-α were found to be essential for the induction of these adverse events. It is unclear whether T cells are the direct targets of anti-CD137 mAbs, or whether they are indirectly affected by cytokines, or chemokines produced by other lineages of CD137-expressing cells. Studies addressing this question are now in progress. Liver-infiltrating CD8+ T cells and TNF-α were essential for the induction of hepatitis, and the absence of either prevented disease in anti-CD137-injected mice. As best we could discern, these liver CD8+ T cells were not Vαβ clonally restricted or oligoclonal in nature. Rather, Vαβ us-
activating, to see whether they induce similar responses to those observed in mice.

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Disclosures

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