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Humanized Mice as a Model for Aberrant Responses in Human T Cell Immunotherapy

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Immune-deficient mice, reconstituted with human stem cells, have been used to analyze human immune responses in vivo. Although they have been used to study immune responses to xenografts, allografts, and pathogens, there have not been models of autoimmune disease in which the mechanisms of the pathologic process can be analyzed. We have found that reconstituted “humanized” mice treated with anti–CTLA-4 Ab (ipilimumab) develop autoimmune disease characterized by hepatitis, adrenalitis, sialitis, anti-nuclear Abs, and weight loss. Induction of autoimmunity involved activation of T cells and cytokine production, and increased infiltration of APCs. When anti–CTLA-4 mAb–treated mice were cotreated with anti-CD3 mAb (teplizumab), hepatitis and anti-nuclear Abs were no longer seen and weight loss did not occur. The anti-CD3 blocked proliferation and activation of T cells, release of IFN-γ and TNF, macrophage infiltration, and release of IP-10 that was induced with anti–CTLA-4 mAb. We also found increased levels of T regulatory cells (CD25+/C211−) in the spleen and mesenteric lymph nodes in the mice treated with both Abs and greater constitutive phosphorylation of STAT5 in T regulatory cells in spleen cells compared with mice treated with anti–CTLA-4 mAb alone. We describe a model of human autoimmune disease in vivo. Humanized mice may be useful for understanding the mechanisms of biologies that are used in patients. Hepatitis, lymphadenopathy, and other inflammatory sequelae are adverse effects of ipilimumab treatment in humans, and this study may provide insights into this pathogenesis and the effects of immunologies on autoimmunity. The Journal of Immunology, 2014, 193: 587–596.

Efforts to develop new treatments for human autoimmune diseases have been hampered by the lack of small-animal models of the disease states and the inherent differences between immune responses of human and murine cells. Studies of human autoimmune diseases have relied on the analysis of rodent models or characterization of human cells ex vivo. However, studies of human cells in vitro cannot re-create the cellular dynamics that may be important in the initiation, progression, and regulation of disease. Murine models have been relied on to provide insights into the pathologic mechanisms, but there are many shortcomings of these models because of intrinsic differences between rodent and human immune systems (1). In some cases, the failure to recognize the differences between rodent and human immune responses has resulted in significant harm to clinical trial participants (1, 2). Thus, an animal model system of autoimmunity with human immune cells would be optimal.

Many investigators have used “humanized” mice to study human immune responses in vivo. These model systems have generally involved transfer of mature human T cells or alternatively murine cells that express human genes, but these systems do not permit the study of human immune cells and responses after perturbations of cells that develop in and are tolerant to the host (3–9). A model system that can be used to study human autoimmune disease in vivo has not been described.

After activation, T cells express CTLA-4, a transmembrane glycoprotein of the Ig superfamily. Its engagement delivers negative signals that circumvent the actions of the CD28-mediated costimulatory pathway. CTLA-4 was first identified during a search for cytotoxic genes using subtractive hybridization, and subsequent studies in knockout mice showed its pivotal role in maintaining peripheral tolerance (10–13). CTLA-4−/− mice develop an autoimmune lymphoproliferative disorder characterized by infiltration of CD4+ T cells leading to multiorgan tissue destruction within 3–4 wk of age. A number of molecular mechanisms have been proposed for the functions of CTLA-4–mediated negative regulation in T cells, including competition for CD28 ligands, disruption of CD28 localization in the immunological synapse, decreased TCR signaling by PP2A and SHP-2, and limited engagement of T cells with APCs (14–18). These cell intrinsic mechanisms have been thought to account for the widespread activation and proliferation of T cells that is seen in CTLA-4−/− mice, which may include cells with self-reactive TCRs leading to autoimmune disease (19–21).

In addition, cell extrinsic factors may also account for autoimmunity in the setting of CTLA-4 blockade. CTLA-4 is constitutively expressed on CD4+ T regulatory cells (Tregs) (22), whereas it is rapidly induced on T conventional cells (Tconvs) after TCR en-
gagement (23), suggesting that CTLA-4 has an important role in both Treg and Tconv function. Recently, dual function of CTLA-4 in Tregs and T effector cells (Teffs) to prevent autoimmunity has been proposed (24). Yamaguchi et al. (25) showed that a triad of IL-2 repression, CTLA-4 expression, and antigenic stimulation is a minimalistic requirement for conferring Treg-like suppressive activity on Tcons.

Because of its role in maintaining tolerance and the enhanced cellular immune responses in its absence, blockade of CTLA-4 with an mAb has been developed for the treatment of malignancies to activate tumor-specific effector cells (26). The anti-human CTLA-4 mAb, ipilimumab, has been approved for the treatment of metastatic melanoma and is also being tested in combination with other agents for treatment of refractory tumors. Blockade of CTLA-4 pathway resulted in an increased ratio of effector to Tregs (27), which has been proposed to account for the enhanced killing of tumors in anti–CTLA-4 mAb–treated patients. However, in addition to these antitumor effects, ipilimumab-treated patients also develop immune-mediated adverse events including dermatitis, colitis, hepatitis, pancreatitis, hypophysitis, and uveitis (28). These immune-mediated adverse effects are also thought to be due to the release of T cell inhibition, but there have not been studies that have analyzed the immune mechanisms at the sites of pathology. Moreover, hyperproliferation of CD4+ T cells is thought to drive autoimmune tissue pathology in other settings such as in human systemic lupus erythematosus (SLE) (29). SLE is characterized by the presence of anti-nuclear Abs (ANAs).

We therefore have studied the effects of ipilimumab in humanized mice that were created by reconstitution of immune-deficient recipients with human CD34+ stem cells. We found that the drug induced autoimmune disease characterized by weight loss, hepatitis, and detectable ANAs. To define the mechanisms of the disease and tissue pathology, we treated mice with another human biologic, teplizumab, which is a non-FcR binding anti-CD3 mAb that has been used to treat patients with new-onset type 1 diabetes (30–34). Coadministration of anti-CD3 with anti–CTLA-4 reversed all autoimmune manifestations induced by anti–CTLA-4 Ab. Our studies suggest three mechanisms that account for the development of autoimmunity: T cell activation and proliferation, impaired localization of Tregs to pathological sites, and recruitment and interaction with APCs. These findings were reversed by anti-CD3 mAb treatment.

Materials and Methods

Mice

NOD.SCID IL-2rg-/- (NSG) mice were purchased from The Jackson Laboratory, bred at our animal facility, and maintained under specific pathogen-free conditions. Experiments were approved by the Institutional Animal Care and Use Committee of Yale University. Human fetal liver was obtained from Advanced Bioscience Resources (Alameda, CA). Approval was obtained from the Institutional Review Board.

Isolation of human CD34+ stem cells and reconstitution of mice

CD34+ cells were isolated from human fetal liver from six donors on a discontinuous density gradient of Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA) and purified with magnetically labeled microbeads conjugated to anti-human CD34 (Miltenyi Biotec, San Diego, CA). Preparations of the CD34+ cells that showed at least 80% purity by FACS were used for reconstitution of mice. The CD34+ cells (2 × 106) were injected intrahepatically into 1- to 2-d-old NSG pups that had been irradiated (1 Gy). The reconstitution of these mice with human CD34+ (hCD34+) cells was determined in peripheral blood at 8 wk by flow cytometry (FACSFortessa; Becton Dickinson, San Jose, CA). On average, 91% of the cells within the scatter gates were hCD45+. All FACS staining was performed in PBS supplemented with 2% heat-inactivated FCS (Atlanta Biologicals, Flowery Branch, GA) and 0.01% sodium azide (Sigma-Aldrich, St. Louis, MO). The FACS data were analyzed with FlowJo software (version 7.6.3).

Treatment of mice

Each reconstituted mouse was given 500 μg ipilimumab (Bristol-Myers Squibb, New York, NY) or human Ig (hlg; Sigma, St. Louis, MO) with or without administration of teplizumab (MacroGenics; 5 μg) beginning the day before ipilimumab i.p. every 4 d for a period of 40 d (35, 36). The mice were weighed regularly, and those that lost 15% of their baseline body weight were sacrificed.

Histology and immunohistochemistry

The experimental mice were sacrificed after they had lost 15% of their body weight or after 4 wk. Paraffin-embedded sections were prepared as previously described (36). For immunohistochemistry, frozen sections were incubated for 15 min with 1% goat serum in PBS followed by avidin/biotin blocking as per the manufacturer’s protocol (Vector Laboratories, Burlingame, CA). Anti-human CD4 (4B12), CD8 (CD4/14B), CD19 (LE-CD19), and CD68 (L1P1) from Dako (Glostrup, Denmark) were applied to contiguous slides overnight followed by staining with biotinylated goat anti-mouse Ab (Dako) and staining with peroxidase substrate (Vector laboratories). Murine macrophages were stained with pan macrophage marker F4/80 (BM8). Hematoxylin counterstain was applied. Immunofluorescent staining for human macrophages was performed on frozen liver sections. In brief, tissue sections were treated with citrate buffer and then heated by microwave. The slides were then blocked with 4% BSA and 5% BSA in PBS for 1 h. Anti–CD68 and F4/80 Abs were diluted in blocking buffer and added to the slides for 2 h followed by washing and staining with goat anti-mouse Alexa flour 546 red (1:300) and DAPI.

Extraction, purification, and FACS analysis of human lymphocytes

Cells were isolated from the spleen, peripheral and mesenteric lymph nodes, liver, and adrenal glands by tissue homogenization. The liver-infiltrating cells were purified by Ficoll density gradient centrifugation. The cells from spleen and liver were treated with ACK lysing buffer (Lonza, Walkersville, MD) to remove RBCs. Some splenocytes were studied immediately, and others were frozen and analyzed later. The following conjugated mAbs were purchased from Biolegend (San Diego, CA) and BD (San Jose, CA) and used for flow cytometry analysis to phenotype lymphocytes: CD45 (H100) allophycocyanin, anti-mouse CD45 (30-F11) Pacific Blue, CD19 (S25C1) PE, CD3 (OKT3) FITC, CD4 (RPA-T4) allophycocyanin-H7, CD8 (RPA-T8) PerCP-Cy5.5, CD45RO (UCHL-1), CD62L (DREG-56) PE-Cy7, CCR7 (G043H7) PE, CD45RA (HI100) allophycocyanin-H7, CXXC3(402SH7) Alexa 647, CCR6 (11A9), CD127(A019D5) Brilliant Violet 421, ICOS (9C9.4A) PerCP-Cy5.5, CD25 (2A3) PE-Cy7, PD-1 (EH12.2H7) Alexa Fluor 647. CD4+ T cell phenotype was determined using CD45RA, CD45RO, and CCR7 markers: naive: CD45RA+CCR7+; effector: CD45RA-CCR7-; effector memory RA: CD45RA-CCR7+; central memory: CD45RA+CCR7+. To identify Tregs, we fixed the cells with intracellular fix/perm buffer for 20 min at room temperature, followed by permeabilization buffer and staining with anti-FOXP3 (29D9) Alexa Fluor 647 (Biolegend) and anti–CD3 (OKT-3) FITC, CD4 (RPA-T4) allophycocyanin-H7, CD25(2A3)-PE-Cy7, CD127 (A019D5). For FACS analysis, an electronic gate was placed on live hCD45 cells. Human T and B cell lymphocyte numbers were enumerated and expressed as the total cell counts or percent of hCD45+ cells. Viability of cells was confirmed by use of a Live/Dead fixable dead stain kit (Invitrogen, Grand Island, NY). FACS analysis was performed on an LSRII (BD Biosciences).

Intracellular cytokine staining

Frozen spleen cells were thawed and the cells were counted. The cells were then stimulated with PMA (10 ng/ml) and Ionomycin (500 ng/ml) in the presence of GolgiStop (BD Biosciences) for 5 h. The cells were washed with FACS staining buffer and stained first with the live/dead marker followed by staining with surface markers CD3 (OKT-3) Brilliant Violet 650, CD4 (RPA-T4), CD8 (HT1A) for 30 min at 4°C. Cells were fixed and permeabilized and stained with anti–IFN-γ (4S.B3) and IL-5 (JESI-39D10) PE, TNF-α (MAb11) PerCP-Cy5.5, IL-10 (JESI-9D7) PE-Cy7, and IL-17A (SCP-1362) Alexa Fluor 647 Abs. The cells were incubated at 4°C for 30 min, washed, and resuspended in the staining buffer.

Stat5 phosphorylation in T cells

STAT5 phosphorylation was also measured by flow cytometry in CD3+, CD4+, CD8+, and Tregs from the spleen ex vivo and after stimulation with IL-2 in vitro (100 U/well). Splenocytes were cultured overnight in serum-free medium (AIM-V from Invitrogen, Carlsbad, CA) followed by incu-
bation with 100 U recombinant human IL-2 for 10^5 cells for 20 min at 37°C. The cells were stained with anti-CD3 PE/Cy7 (clone UCHT1), anti-CD4 Alexa700 (RPA-T4), and anti-CD8a allophycocyanin-Cy7 (clone HIT8a). Cells were incubated for 15 min at 4°C and immediately fixed with 2% PFA at 37°C for 10 min followed by permeabilization procedure as described previously (37). The anti-STAT5 Ab (y694)-Alexa 488 was added and incubated for 1 h at room temperature in the dark, followed by washing with staining buffer. Cells were analyzed by flow cytometry using a FACSaria (BD Biosciences).

**Serum cytokine analysis**

Human cytokines were measured in serum samples with an ultrasensitive Milliplex xMAP assay (Millipore, Billerica, MA). The lower limit of detection for this assay was 1 pg/ml.

**ANA staining**

ANA testing was performed with HEp-2 slides that were incubated with a 1:40 dilution of serum samples collected from both control and experimental mice. The serum was incubated with the slides for 1 h at room temperature in a humidifying chamber. Excess unbound Abs were washed for 5 min in PBS. The slides were incubated with secondary Ab at a 1:100 dilution in PBS for 1 h. They were washed in PBS for 5 min and mounted. Images were rendered using an Olympus BX40 microscope. They were scored from +1 to +4 depending on the intensity of staining for ANA.

**Statistical analyses**

Unless indicated, the mean ± SEM is reported. The analyses were performed with GraphPad software.

**Results**

**Weight loss in reconstituted humanized mice treated with anti-CTLA-4 mAb**

We screened the reconstituted mice for hCD45^+ cells in peripheral blood by flow cytometry at 8 wk of age. On average, 91% of the cells within the scatter gates were hCD45^+; 25.5% were CD3^+ and 60.6% were CD19^+. The reconstituted mice were treated with ipilimumab or hlg every 4 d at a dose that has been used to treat patients with malignant melanoma (20 mg/kg). Beginning at day 7, the ipilimumab-treated mice began to lose weight; by day 30, 80% of the mice had lost at least 15% of their pretreatment body weight or an average of 14 g (Fig. 1; p < 0.05 versus baseline weight). The majority of autoantibodies were IgM isotype, but we also found autoantibodies against IgG in 2 of 10 mice (Fig. 5A). The serum cortisol levels were not reduced.

**Autoimmunity in humanized mice treated with anti-CTLA-4 mAb**

These findings showed that treatment with anti–CTLA-4 mAb induced activation of T cells resulting in cytokine production and heightened cytokine signaling. To determine whether the T cell activation observed after treatment of humanized mice with anti–CTLA-4 mAb led to organ-specific damage, we examined the liver for inflammatory lesions because damage of this organ might have contributed to the observed weight loss. We found inflammatory infiltrates in the liver, which were composed of human CD4^+ and CD8^+ T cells, B cells (CD19^+), and both human (CD68^+; Fig. 4A) and mouse (F4/80^+; macrophages (Fig. 4B). The serum levels of alanine aminotransferase (ALT) were elevated in the ipilimumab-treated mice, and the serum albumin was decreased, indicating hepatocellular damage with decreased synthetic function (Fig. 4C).

We also found cellular infiltrates in the adrenal cortex and salivary glands of the ipilimumab-treated mice (Supplemental Fig. 2). However, the levels of serum cortisol were not reduced.

We then tested the mice for the development of human ANAs using a standard clinical assay with HEp-2 cells. Positive ANA staining (scale +1 to +4) of at least grade +2 was found more frequently in serum (7/11) of ipilimumab-treated mice compared with (1/10) hlg-treated mice (p = 0.02; Fig. 5B) and with higher average titer (p < 0.05; data not shown). The majority of autoantibodies were IgM isotype, but we also found autoantibodies against IgG in 2 of 10 mice (Fig. 5A).

**Effects of ipilimumab on T cell differentiation and Tregs**

In general, the development of T-dependent immune responses in humanized mice has been difficult to achieve, but the finding of IgG ANAs suggested this may be occurring in the ipilimumab-treated mice. We therefore determined whether there was differentiation of T cells in the organs with immune infiltrates. The proportion of CD3^+ T cells among the total lymphocytes was increased in the liver and spleen from the anti–CTLA-4 mAb–treated compared with hlg-treated mice. The increase in T cells was accounted for primarily by an expansion of the CD4^+ T cell (CCR7^+CD45RO^+). Expansion and activation of human T cells with anti–CTLA-4 mAb treatment. To determine the effects of the anti–CTLA-4 mAb treatment on immune cells, we analyzed the cellular composition of the lymphoid organs. In general, NSG mice that are reconstituted with fetal liver CD34^+ cells show a reduced number of splenocytes (~18 × 10^6) compared with the number of murine lymphocytes in the spleen of normal mice. However, in mice reconstituted with fetal liver cells and treated with ipilimumab, the spleens were enlarged (Fig. 2A, 2B), with a 2-fold increase in the number of hCD45^+ splenocytes. Moreover, the mesenteric and other peripheral lymph nodes were enlarged and showed infiltration with CD4^+, CD8^+, and CD19^+ lymphocytes (Fig. 2C). However, the lymph nodes did not show evidence of organization (Fig. 2Ca, b).

We analyzed activation markers on the lymphocytes in the spleen, as well as the levels of cytokines in the circulation. The CD4^+ splenocytes in the ipilimumab-treated mice expressed higher levels of CXCR3, PD-1, ICOS, and CD25, and the levels of IFN-γ and TNF were increased in the serum of the mice, compared with the hlg-treated mice (Fig. 3A, 3B). Furthermore, >12% of the CD4^+ cells expressed both IFN-γ and TNF after PMA/Ionomycin stimulation (Fig. 3C, 3D).

To understand the effects of the mAb on T cell expansion and activation, we measured STAT5 phosphorylation (pSTAT5) in T cells after stimulation with IL-2. pSTAT5 levels were increased after culture with IL-2 in CD4^+ and CD8^+ (Fig. 3E) Teffs from ipilimumab-treated mice.

**Figure 1.** Systemic effects of ipilimumab (anti-CTLA-4 mAb) treatment on humanized mice. Mice treated with ipilimumab (circles) had median survival of 26 d (versus hlg [squares]; p = 0.0002, n = 12 in each group).
were significantly increased in the liver \((p < 0.05)\). These findings suggest that there had been T cell activation and memory cell development by CD4\(^+\) cells in the liver and spleen.

Similar to the expansion of CD4\(^+\) Teff cells, there was an increase in the total number of Tregs (CD4\(^+\)CD25\(^+\)CD127\(^lo\)FOXP3\(^+\)Helios\(^+\)) overall. However, these cells showed different proportions and distri-

FIGURE 2. Lymphoproliferation in humanized mice treated with ipilimumab. (A) Hypersplenism was seen in the ipilimumab-treated mice after 4 wk of treatment. A single pair of 11 is shown. (B) The increase in the size of the spleen was associated with an increase in the total number of lymphocytes in the organ \((37 \pm 7.1 \times 10^6 [n = 11] \text{ vs. } 14.58 \pm 1.9 \times 10^6 [p = 0.0006])\). (C) The lymph nodes were also enlarged in the ipilimumab versus control Ig-treated mice. Axillary nodes are shown. Immunohistochemical staining of axillary lymph nodes from ipilimumab-treated humanized mice: (a) H&E staining showing lymph node without evidence of organization (original magnification \(\times 20\)). (b) H&E higher magnification image (original magnification \(\times 40\)). (c) Human CD4 within lymph node (brown stain, red arrows). (d) CD8. (e) CD19. (f) There was little staining for hCD68 (all original magnifications \(\times 40\)). An individual animal is shown that is representative of six in each group. (D) Immunohistochemical staining of axillary lymph nodes from hIg-treated mice: (a) CD4, (b) CD8, and (c) human CD68. ***\(p < 0.001\) (p<0.001).

FIGURE 3. Phenotype of CD4\(^+\) T cells after ipilimumab treatment. (A) The expression of CXCR3, PD-1, ICOS, and CD25 are shown on CD4\(^+\) splenocytes after 4 wk of treatment. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). (B) Proinflammatory cytokines IFN-\(\gamma\) and TNF are elevated in the systemic circulation after treatment with ipilimumab (hIg: \(n = 14\), ipilimumab: \(n = 13\)). (C) IFN-\(\gamma\) and TNF production in mice treated with hIg or anti–CTLA-4 mAb after stimulation with PMA/Ionomycin. There were more T cells that coexpressed IFN-\(\gamma\) and TNF in CD4\(^+\) T cells of spleen in the ipilimumab-treated mice (\(p < 0.05\) (D). (E) Spleen cells stimulated with IL-2 (100 U/10\(^6\) cells) showed enhanced phosphorylation of STAT5 in CD4\(^+\) and CD8\(^+\) T cells (\(p < 0.05\), **\(p < 0.01\); each dot represents an individual mouse).
bution in secondary lymph structures and tissue. There was a 4.5-fold increase in the percentage of Tregs in the lymph nodes, but not in the liver (Fig. 7A; \( p < 0.01 \)). In addition, the ratio of the Treg/Teff was 3.5-fold lower in the livers from mice that lost weight compared with those that did not (Fig. 7B).

Effects of anti-CD3 mAb treatment on autoimmunity induced with anti–CTLA-4 mAb. Our findings suggested that the autoimmunity in the ipilimumab-treated humanized mice was due to the activation of the human T cells. To test this hypothesis, we cotreated mice with ipilimumab and FcR nonbinding anti-CD3 mAb (teplizumab) with the equivalent dose (5 \( \mu \text{g} \)) as that used to treat patients with new-onset type 1 diabetes (30–34). The mice treated with both teplizumab and ipilimumab did not lose weight and showed improved survival compared with mice treated with anti–CTLA-4 alone (\( p = 0.0005 \); Fig. 8A). Anti-CD3 treatment reduced the infiltration of T cells in the liver to levels that were similar to hIg-treated mice (Fig. 8B). In addition, the increased levels of ALT triggered by anti-CTLA4 were reduced and liver function was restored by anti-CD3, as measured by albumin levels (Fig. 8C and 8D). In addition, the production of inflammatory mediators, IFN-\( \gamma \) and TNF, was reduced by anti-CD3 mAb treatment (\( p < 0.05; \)

FIGURE 4. Ipilimumab-treated humanized mice show cellular infiltrates in the liver and development of autoimmune hepatitis. (A) Histology and immunohistochemistry of liver showing human lymphocytic infiltration of liver in ipilimumab-treated (c–h) but not hlg-treated (a, b) animals. The liver was heavily infiltrated with lymphocytes (arrows) in mice treated with ipilimumab seen with H&E staining [arrows; original magnification \( \times 20 \) (c); \( \times 40 \) (d)] when compared with hlg-treated controls [original magnification \( \times 20 \) (a), \( \times 40 \) (b)]. There was lobar hepatitis accompanied by ballooning and rosetting of peribiliary hepatocytes. There was infiltration of human monocytes (CD68) that had formed multinucleated giant cells (e, original magnification \( \times 40 \)) and an infiltrate of human CD4 (f) and CD8 cells (g) with limited evidence of B lymphocytes (CD19) (h, original magnification all \( \times 40 \)). (B) Murine macrophages (F4/80\(^+\)) are infrequent in hlg-treated mice but were found (arrow) in the liver of anti–CTLA-4–treated mice. (C) Serum levels of liver enzyme (ALT) and albumin. Each point represents an individual mouse (*\( p = 0.01 \) and 0.009, respectively). (D) There were very few CD4\(^+\) (a), CD8\(^+\) (b), and macrophages (arrow) (c) in the livers of the hlg-treated animals.

FIGURE 5. Ipilimumab-treated humanized mice develop ANA. (A) IgM and IgG ANAs were tested using standard laboratory methods. Identification of IgM (a) and IgG (b) ANAs on HEP2 cells. (c) Staining for hlgM Abs in an hlg-treated mouse. Original magnification \( \times 40 \). (B) There was a significantly greater proportion of ANA scores \( >1 \) in the anti–CTLA-4 mAb–treated mice (\( p = 0.02 \)). *\( p < 0.05 \).
FIGURE 6. Number and phenotype of infiltrating T cells after ipilimumab treatment. (A) FACS analysis of human lymphocytes isolated from liver and spleen. There was a relative increase in CD4+ T cells and a decrease in B cells at the three sites (*p < 0.05, **p < 0.01, ***p < 0.001; n = 6 hlg and 11 anti–CTLA-4 mAb). (B) Ipilimumab treatment decreased the proportion of naïve CD4+ T cells and increased the proportion of Teffs in the liver and spleen. The proportion of central memory CD4+ T cells increased significantly in the liver.

Fig. 8E and 8F). There was also a reduction in the levels of anti-hIgM ANAs (p < 0.05) with no detection of IgG ANAs (Fig. 8G).

The frequency of hCD68+ cells was reduced in the livers of mice treated with both mAbs (Fig. 9A); in addition, the levels of circulating IP-10 were reduced, consistent with reduced activation of mononuclear cells (Fig. 9B). Finally, we compared the frequency of Tregs in the livers of the mice treated with anti–CTLA-4 and anti–CTLA-4+ anti-CD3 mAbs. The frequency of CD4+CD25+CD127− Tregs was increased in the spleen in the mice treated with both mAbs, and these cells showed increased constitutive phosphorylation of STAT5, suggesting in vivo activation (Fig. 9D).

Discussion

Efforts to develop new treatments for human autoimmune disease have been hampered by the lack of small-animal models that can predict the safety and efficacy of interventions in humans. The ability of anti–CTLA-4 mAbs to prevent negative costimulation has led to its development as a biologic to enhance immune-mediated killing of tumors such as malignant melanoma (28, 38, 39). At the same time, the frequency of immune-related adverse events has been reported as high as 64.2% in patients receiving ipilimumab. Common findings are enterocolitis, dermatitis, hepatitis, hypophysitis, adenopathy, as well as adrenalitis (38, 40, 41). Similarly, our humanized mouse model treated with ipilimumab recapitulated some of these pathologies found in treated humans. Thus, the present model is relevant to define immunologic pathways of untoward effects of this therapeutic modality.

In our studies, we exploited the known adverse effects of anti–CTLA-4 to develop a model of autoimmune disease caused by human immune cells. The critical role of human T cells in this pathology is illustrated by cotreatment with teplizumab, a non-FcR binding anti-CD3 mAb that has been shown to ameliorate the progression of type 1 diabetes. The anti–CTLA-4 mAb caused weight loss and autoimmune hepatitis associated with the devel-

FIGURE 7. Reduced Tregs in liver with weight loss. (A) The relative proportion of Tregs (CD4+CD25+CD127−FOXP3+) in the mesenteric lymph nodes and liver is shown (**p < 0.01, ***p < 0.001, n = 5 hlg and 9 anti–CTLA-4 mAb–treated mice). (B) The ratio of Treg/Teff was relatively greater in the anti–CTLA-4 mAb–treated mice that did not lose weight.
development of ANAs. We found no evidence of arthritis or renal disease, which are also rare findings in humans treated with anti–CTLA4. Both IgG and IgM ANAs were identified in the ipilimumab-treated mice, and this classical clinical autoimmune manifestation appeared to be dependent on T cells because treatment with anti-CD3 mAb prevented their induction. We also found infiltration of human T cells in the adrenals and salivary glands. The increase in Th1 cytokines in the anti–CTLA-4 humanized was associated with a general enhancement in T cell responsiveness indicated by increased STAT5 phosphorylation in response to IL-2. The human cells also displayed increased expression of CXCR3, CD25, and PD-1 consistent with activation and a Th1 phenotype. These findings indicate generalized activation of T cells, but the pathologic changes were limited to specific tissues, which suggest that the development of autoimmune disease does not simply reflect T cell activation. Likewise, the pathology was not due to general loss of immune tolerance because we did not detect signs of GVHD in the gut, which is a common finding in NSG mice that receive mature human T cells (43).

We uncovered three mechanisms involved in the development of autoimmunity: general activation of T cells leading to cytokine release and lymphoproliferation with enlarged secondary lymph nodes, the association of lymphocytes with macrophages at the site of tissue destruction, and failure to localize Tregs to the tissues. These studies are supported by related systemic autoimmune syndromes (SLE) in humans and mouse models that are marked by aberrant T cell hyperproliferation and ANAs, both diagnostic of this syndrome (29, 44, 45). To our knowledge, this is the first described model more closely recapitulates human clinical disease than the CTLA-4–deficient mice.

The reduction in Treg/Teff cells that we observed may shift the balance to promote autoimmune pathology. This notion is supported by the finding that the ratio of Treg/Teff was reduced in mice that lost weight after ipilimumab treatment compared with those that did not. Moreover, there was an increase in the total number of Tregs and as a proportion of the total CD4+ cells in the lymph nodes, but not in the liver, suggesting that the Tregs failed to migrate to the liver. Blockade of CTLA-4 signaling may interfere
with the generation of both CD4+ and CD8+ T cells (47, 48), as well as the CD28-driven migration of memory T cells to extra-lymphoid tissue (49). Our findings support a similar regulation of migration of Tregs, thus explaining their failure to migrate to the liver. In addition, it is possible that increases in the proinflammatory cytokine TNF interfere with Treg suppressive function, as has been described in rheumatoid arthritis (50) and antibiotic refractory Lyme arthritis (51). Finally, teplizumab treatment appeared to increase Treg number and restore function in a manner not unlike we and others previously demonstrated in treated human patients (52–54). We found that there were increased numbers of Tregs and enhanced function reflected by the increase in constitutively phosphorylated STAT5. We previously have reported that teplizumab induces Tregs in humanized mice via transit of the cells through the gut. Consistent with this mechanism, we found increased expression of CCR6 on CD4+ and CD8+ Teffs in the periphery (data not shown).

Tissue destruction appeared to depend on the recruitment of macrophages to the sites of pathology and their interaction with the human T cells. These macrophages are the likely source of CXCL10 that we found was elevated in the ipilimumab-treated mice and reduced when these mice were treated with teplizumab. A limitation for modeling human immune responses in NSG mice in general has been their failure to reconstitute human innate immune cells (55). The recruitment of macrophages to the liver of the ipilimumab-treated mice is most likely due to products elaborated by activated T cells. Indeed, we did not identify infiltration of human CD68+ cells in the livers of mice that had not been treated with ipilimumab or were cotreated with teplizumab. Interestingly, we also found an increased proportion of murine macrophages in the livers of ipilimumab-treated mice and a reduced frequency when they were treated with teplizumab, suggesting there may be productive interactions between human cells and murine macrophages because many of the murine ligands can cross react with their receptors on T cells such as B7.1 and B7.2 (56).

In conclusion, we report the development of a small-animal model of human autoimmune hepatitis that could be controlled by teplizumab. Our studies have identified several mechanisms associated with disease, including T cell activation and expansion, cytokine release, a failure of Tregs to migrate to tissues, and participation of murine macrophages. These studies suggest potential therapeutic strategies to prevent tissue damage or enhance Treg activity in patients treated with ipilimumab, and the model may also be useful for testing agents that may be used for the treatment of human autoimmune hepatitis. Further studies in these mice may enhance our understanding of the mechanisms of human immune-mediated autoimmune disease.

Disclosures
The authors have no financial conflicts of interest.

References


