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Engineered Regulatory T Cells Coexpressing MHC Class II: Peptide Complexes Are Efficient Inhibitors of Autoimmune T Cell Function and Prevent the Development of Autoimmune Arthritis

Zhaohui Qian,* Kary A. Latham,* Karen B. Whittington,† David C. Miller,*‡ and Edward F. Rosloniec*†‡

Regulatory T cells (Tregs) are critical homeostatic components in preventing the development of autoimmunity, and are a major focus for their therapeutic potential for autoimmune diseases. To enhance the efficacy of Tregs in adoptive therapy, we developed a strategy for generating engineered Tregs that have the capacity to target autoimmune T cells in an Ag-specific manner. Using a retroviral expression system encoding Foxp3 and HLA-DR1 covalently linked to the immunodominant peptide of the autoantigen type II collagen (DR1-CII), naive T cells were engineered to become Tregs that express DR1-CII complexes on their surface. When these cells were tested for their ability to prevent the development of collagen induced arthritis, both the engineered DR1-CII-Foxp3 and Foxp3 only Tregs significantly reduced the severity and incidence of disease. However, the mechanism by which these two populations of Tregs inhibited disease differed significantly. Disease inhibition by the DR1-CII-Foxp3 Tregs was accompanied by significantly lower numbers of autoimmune CIA-specific T cells in vivo and lower levels of autoantibodies in comparison with engineered Tregs expressing Foxp3 alone. In addition, the numbers of IFN-γ– and IL-17–expressing T cells in mice treated with DR1-CII-Foxp3 Tregs were also significantly reduced in comparison with mice treated with Foxp3 engineered Tregs or vector control cells. These data indicate that the coexpression of class II autoantigen–peptide complexes on Tregs provides these cells with a distinct capacity to regulate autoimmune T cell responses that differs from that used by conventional Tregs. The Journal of Immunology, 2013, 190: 000–000.

Regulatory T cells (Tregs) are CD4+,CD25+,Foxp3+ cells capable of suppressing the function of T effector cells to enforce immunological homeostasis. The genetic absence of Tregs results in widespread deregulation of the adaptive immune response in both humans and animal models (1, 2). In humans, mutations in the Foxp3 gene, a key transcriptional regulator for the differentiation and function of Tregs (3), has been linked with immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, which manifests as a myriad of autoimmune disorders including diabetes, thyroiditis, and colitis (for a review, see Ref. 4). Similarly, scurfy mice, which carry an insertion mutation in the Foxp3 gene that results in a nonfunctional protein, also develop a variety of autoimmune disorders (3, 5). The widespread lymphoproliferative and autoimmune disorder that develops in the absence of Tregs has suggested that therapeutic use of Tregs might be a viable approach for the treatment of autoimmune diseases. Indeed, transfer of thymic-derived Foxp3+ cells into neonatal scurfy mice prevents development of the lymphoproliferative and autoimmune disorders that normally develop in these mice (3, 6).

Several studies have shown that adoptive transfer of Tregs offers promise for immunotherapy of autoimmune diseases. Adoptively transferred Tregs have been shown to alter the development of disease in several mouse models, including colitis (3, 7), experimental autoimmune encephalitis (8–10), arthritis (11–13), diabetes (14, 15), and lupus (16). In most cases, these studies have used polyclonal Tregs to inhibit the initiation of autoimmunity and most have demonstrated only a reduction in severity of disease, although some success has been achieved using Tregs to alter established disease (17). In attempts to increase the efficacy of adoptive Treg therapy in autoimmune diseases, several investigators have examined the effect of Ag-specific Tregs on autoimmune responses. Several studies have demonstrated that Ag-specific Tregs may be more effective than polyclonal Tregs in ameliorating or preventing autoimmunity in arthritis (18, 19), autoimmune gastritis (20), and type I diabetes models (15). Whereas polyclonal Tregs were only minimally effective in the treatment of type I diabetes in mice, autoantigen-specific Tregs effectively suppressed the disease (14, 15). Although the results from autoantigen-specific Treg treatments are promising, the possibility of contamination of these cells with autoimmune T effector cells during the preparation of the therapeutic Tregs remains a concern.

Another means by which Tregs may target pathogenic T cells is via their expression of class II molecules. Although the mouse is one of few mammalian species that do not express class II on activated T cells (21), it has been demonstrated that human Tregs...
can express HLA class II, and those that do, express higher levels of Foxp3 and appear to be more efficacious in inhibiting T cell responses in vitro (22). However, it remains unclear whether these class II–expressing Tregs have therapeutic potential in vivo. Given the evidence for increased Treg efficacy through class II expression, and the ability to produce large numbers of Tregs via transduction of Foxp3 (3, 15, 19, 23), we engineered targeted Tregs by transducing naive CD4+ T cells with a construct carrying both Foxp3 and HLA DR1 class II covalently linked to an autoantigenic peptide that drives the development of collagen-induced arthritis (CIA). This approach provides the Tregs with both membrane expressed HLA class II and via the autoantigenic peptide a means to specifically target the pathogenic T cells driving the autoimmune response. Our goal was to develop a system for producing large numbers of Tregs for therapeutic studies and provide a targeting mechanism that would enhance the interaction of the engineered Treg with the pathogenic T cells in a DR1-humanized autoimmune arthritis mouse model. In the following studies we demonstrate that adoptive transfer of these HLA class II–targeted, engineered Tregs effectively inhibits an established autoimmune response and prevents the development of autoimmune arthritis in DR1 transgenic (Tg) mice.

In comparison with engineered Tregs expressing only Foxp3, the class II–targeted Tregs were more efficient and appeared to use a different mechanism to alter the autoimmune T cell response by both inhibiting T cell function as well as altering the functional phenotype of the autoimmune T effector cells.

Materials and Methods

Mice

The development of the B10M-DR1 (DRB1*0101)–humanized mouse strain has been described previously (24). These mice express a chimeric class II molecule, with the second domains derived from I-E, enabling interaction with murine CD4 (25). The B10.M-DR1-TCR Tg mice were obtained from Dr. L. Myers (University of Tennessee Health Science Center, Memphis, TN). The TCR transgene was established by cloning of the V\textalpha2-j2c and V\textbeta8.1B2.4 genes from a DR1–restricted, CII259-270–specific T cell hybridoma (E168) into the pV\textalphaV and pV\textbetaV cassette vectors (a gift from Dr. V. Kouskoff, Paterson Institute for Cancer Research, Manchester, U.K.), respectively, and injection of the DNA into fertilized eggs of FVB/N mice. Tg founders were identified by PCR analysis and flow cytometry, and the transgene was backcrossed onto the B10.M-DR1 strain. These mice, named TCR-E168 DR1, express this CII-specific, DR1-restricted TCR on the majority of their CD4+ T cells, and as naïve T cells they respond to CII peptide stimulation. All mice were maintained in a pathogen-free environment in accordance with institutional guidelines.

Induction of CIA

Autoimmune arthritis was induced in 8- to 10-wk-old mice by immunization at the base of the tail with 100 \mu g bovine CII emulsified in CFA (Difco) containing 4 mg/ml Mycobacterium (26). Severity of disease was assessed by visual scoring of each limb three times per week and assigning a score of 0–4 as described previously (26). For treatment with the engineered Tregs, mice were injected with 5 x 10^6 GFP+ cells per each time point via the tail vein.

RNA isolation and RT-PCR

For amplification of the Foxp3 gene, CD4+CD25+ T cells were enriched from the spleens of B10M-DR1 mice by using a CD4+CD25+ isolation kit from Miltenyi Biotec. Briefly, 5 x 10^7 cells were incubated with biotinylated anti-CD8 and CD19 Abs for 10 min at 4°C, followed by the addition of avidin-conjugated microbeads and anti–CD25-PE. A two-step procedure consisting of negative selection for CD4+ T cells followed by positive selection of the CD25+ cells using anti-PE microbeads was performed using an AutoMACS Pro (Miltenyi Biotec) according to the manufacturer’s guidelines. Total RNA was isolated from 1 x 10^6 CD25+CD4+ cells using the RNeasy Minikit (Qiagen), according to the manufacturer’s instructions. cDNA was synthesized from the RNA using SuperScript II RT (Invitrogen).

The full-length Foxp3 cDNA was amplified using a Foxp3 forward primer 5’-CTC CGG CCA ACT TCT CCT GAC-3’ and a reverse primer 5’-TCA AGG GGA GAT TGA GCA CT-3’. PCR was performed using the Phusion kit (New England Biolabs) in a 50-μl reaction with 2 μl cDNA, 10 μl 5× Phusion HF buffer, 1 μl 10 mM 2’-deoxynucleoside 5’-triphosphates, 2.5 μl 10 μM Foxp3 forward primer, 2.5 μl 10 μM Foxp3 reverse primer, 0.5 μl 2 U/μl Phusion DNA polymerase, and 33.5 μl H2O. Following a 30-s initial denaturation at 98°C, the Foxp3 cDNA was amplified by 30 PCR cycles, at 98°C 15 s, 55°C 15 s, and 72°C 1 min, followed by a final 7-min extension at 72°C. The PCR products were purified and incubated with TaqMan to polyadenylate the PCR product to facilitate cloning. The final PCR products were gel purified using Gene Clean (Bio101) and cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen). The sequence of Foxp3 (pCR2.1-Foxp3) was verified by DNA sequencing.

Retroviral constructs

The constructs for retroviral expression of the Foxp3 and DRB1*0101 and DRB1*0101 genes were inserted into the EcoRI site of the mouse stem cell virus (MSCV) retroviral vector (27). To generate the DR1-CII retroviral construct, total RNA was isolated from the spleen of a B10.M-DR1 mouse, reverse transcribed into cDNA, and used as a template to amplify the full length DR1α chain and DR1β chain by PCR. The primers for the DR1α chain are 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC TGT TGG CTG-C-3’. The primers for the DR1β chain are 5’-AAA ATG GTG TGC CTC CCC AG-3’ and 5’-TCA GCT CAG TGC TGT TGG CTG-C-3’. To construct the full length DR1β chain with the CII peptide, two rounds of PCR were performed. The DNA fragment containing the extracellular domain of DR1β with the CII peptide was generated using pRksnsb-DR1-CII as template and using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-AAA ATG TGC GTG CTC CCC AG-3’ as primers. These two DNA fragments served as templates for the second round of PCR using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers. The PCR products were inserted into the pCR2.1 vector to generate pCR2.1-DR1-CII and pCR2.1-DR1α. Their sequences were verified by DNA sequencing.

To enable expression of multiple genes off the same MSCV promoter, the DR1 and Foxp3 genes were concatenated using T2A sequences to separate the genes (Fig. 1A) (28). This approach allows for the expression of multiple proteins from the same transcript with similar efficiencies for each gene (20). pCR2.1-DR1 as a DR1-CII and pCR2.1-DR1α as DR1-CII were used as templates to generate DR1-CII-T2A-DR1α. The DR1-CII-T2A fragment was amplified by using pCR2.1-DR1-CII as the template and using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers; the other DNA fragment containing the membrane domain and intracellular domain of DR1β was amplified using full-length DR1β as the template and using 5’-CCG AAA TGG AGA CTG GAC CTT C-3’ and 5’-TGA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers. These two DNA fragments were used as templates for the second round of PCR using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers. The PCR products were inserted into the pCR2.1 vector to generate pCR2.1-DR1-CII and pCR2.1-DR1α. Their sequences were verified by DNA sequencing.

The DR1-CII-Foxp3 construct was produced by combining the DR1-CII-T2A-DR1α construct and the Foxp3 cDNA by another T2A sequence. The DR1-CII-T2A-DR1α – T2A fragment was produced using MSCV-DR1-CII as the template and using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers; the other DNA fragment containing the membrane domain and intracellular domain of DR1β was amplified using full-length DR1β as the template and using 5’-CCG AAA TGG AGA CTG GAC CTT C-3’ and 5’-TGA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers. The final PCR product was then cloned into pCR2.1, verified by DNA sequencing, and inserted into the EcoRI site of MSCV.

The DR1-CII-Foxp3 construct was produced by combining the DR1-CII-T2A-DR1α construct and the Foxp3 cDNA by another T2A sequence. The DR1-CII-T2A-DR1α – T2A fragment was produced using MSCV-DR1-CII as the template and using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers; the other DNA fragment containing the membrane domain and intracellular domain of DR1β was amplified using full-length DR1β as the template and using 5’-AAA ATG TGC GTG CTC CCC AG-3’ and 5’-TCA GCT CAG TGC CTT TGT TGG CTG-C-3’ as primers. The final PCR product was then cloned into pCR2.1, verified by DNA sequencing, and inserted into the EcoRI site of MSCV.

Production of retrovirus and infection of T cells

The retroviral constructs were cotransfected into 293T cells with two helper DNAs, pcDNA-gag-pol, and pcDNA-Env (gifts from Dr. L. Albrition, University of Pennsylvania). Following transfection, the supernatants were collected, concentrated, and used to transduce naïve CD4+CD25+ T cells with a retroviral vector containing a puromycin resistance gene. The cells were selected for puromycin resistance for 10 days before analysis.
Suppression assay

Effector CD4+ T cells were enriched from the spleens of TCR-E168 DR1 Tg mice using a CD4+ T cell isolation kit (Miltenyi Biotec) and were seeded in 96-well plates at a density of 5 × 10^4 cells/well. These wells were mixed with 5 × 10^5 irradiated APC and various numbers of engineered Tregs, and incubated for 4 d in the presence of 1 μg/well of the CII (257–274) peptide. One microcurie of [3H]thymidine was added for the last 16 h of incubation. Cells were then harvested onto filters, and the incorporation of [3H]thymidine was measured by a Matrix 96 direct ionization beta counter (Packard Instrument).

CII Ab measurements

Sera from mice were collected at days 28 and 42 postimmunization, and the quantity of anti-CII IgG Abs was determined using a solid-phase ELISA as described previously (24). Briefly, microtiter plates were coated with 500 ng bCII at 4°C overnight. After extensive washing with 0.5 M saline/0.1% Tween 20, wells were blocked by the addition of 2% BSA for 30 min at 4°C and washed. A 1:2000 dilution of the mouse sera in 2% goat serum/1% Tween 20, a HRP-labeled goat anti-mouse IgG (1:10,000 dilution; Southern Biotechnology Associates) was added. After 2 h, the plates were washed and developed by the addition of o-phenylenediamine (Sigma-Aldrich). After stopping the reaction with 2.5 N H4SO4, the degree of color development was measured using a spectrophotometric plated reader (Spectra Max; Molecular Devices). Data are expressed as OD at 490 nm with background absorbance of 650 nm subtracted.

Results

Generation of retrogenic Tregs expressing MHC class II

Targeted Tregs where engineered using a replication deficient retrovirus encoding Foxp3 and an HLA-DR1 peptide–ligand complex (constructs shown in Fig. 1A). This DR1-CII peptide complex in tetrameric form has been previously shown to bind selectively to the TCR of CII-specific, CD4+ pathogenic T cells (30, 34). CDNA encoding the immunodominant CII peptide and a linker sequence were inserted near the N terminus of the DRB1 chain to covalently link the CII peptide to the DR1 molecule (30, 35). Empty vector (MSCV) and vector encoding only the DR1-CII construct were used as controls (Fig. 1A), and all of the vectors expressed GFP in a second cistron using an IRES promoter.

Flow cytometric analysis of the transduced T cells indicated that nearly all GFP+ cells expressed the retroviroly encoded genes (Fig. 1B). To determine whether the Foxp3 and DR1-CII proteins expressed by the retrovirus were functional, Ag presentation experiments and Treg suppression assays were performed (Fig. 1C, 1D). As shown in Fig. 1C, T cells transduced with a construct containing DR1-CII-Foxp3 are highly efficient in their ability to stimulate DR1-resticted, CII-specific T cell hybridomas to produce IL-2. The inclusion of the Foxp3 construct had no effect on the T hybridomas as these cells are not susceptible to Treg function (data not shown). In comparison, T cells transduced with the empty MSCV control vector (Fig. 1C) or Foxp3 alone vector (data not shown) were incapable of stimulating the T hybridoma cells. These data indicate that the DR1 molecule is expressed as a functional molecule and that the covalently linked CII peptide is properly folded into the DR1 binding pocket. To determine whether the Foxp3 gene is functional and converts naive CD4+ T cells to Tregs, T cells transduced with either the control MSCV vector or the vector containing the DR1-CII-Foxp3 construct were tested for their ability to suppress the in vitro proliferative response of DR1-restricted, CII-specific T cells recovered from TCR-E168 DR1 mice. As shown in Fig. 1D, T cells transduced by the control MSCV vector did not inhibit the proliferation of CII-specific T cells at any ratio of engineered Treg to T cell tested, nor did cells transduced with DR1-CII alone (data not shown). In con-

University of Tennessee Health Science Center) at a molar ratio of 1:1:1 by using Fugene HD, according to the manufacturer’s instructions (Roche). Briefly, 10 μg DNA were diluted into 500 μl Opti-MEM and then 40 μl Fugene HD was added to the DNA solution. After a 15-min incubation, the DNA-Fugene mixture was added to the 293T cells by drop. Sixteen hours later, the DNA-Fugene containing medium was replaced with fresh EHAA medium with 10% FBS and penicillin/streptomycin. Viral supernatant was harvested 24 and 48 h later.

Before transduction, T cells from the spleens of DR1 mice were activated by plate-bound anti-CD3 (clone 145-2C11, 5 μg/ml) and anti-CD28 (clone PV1, 5 μg/ml) and cultured in EHAA medium with 10% FBS, penicillin/streptomycin, 50 μM 2-ME, and 15 U/ml IL-2 for 2 d. Cells were washed twice with EHAA medium without 2-ME and resuspended into freshly harvested viral supernatant with 10 μg/ml polybrene (Sigma-Aldrich) and 15 U/ml IL-2 at a density of 1 × 10^6 cells/ml viral supernatant. Infection was aided by spin inoculation at 700 × g for 90 min at room temperature, followed by overnight incubation at 37°C. The infection procedure was repeated 24 h later with fresh viral supernatant. After a second 24-h incubation period, cells were collected, their efficiency of transduction evaluated by analyzing GFP expression using flow cytometry (FACS Calibur or LSRII; BD Biosciences) and used in experiments. Transduction efficiency averaged 60% for cells used in experiments.

Immunoﬂuorescence and flow cytometry

Nuclear staining of Foxp3 was performed using a mouse Foxp3 staining kit (eBioscience) according to the manufacturer’s instructions. Prior to nuclear staining, cells were stained with the anti-DR Ab L243. Cells were analyzed by flow cytometry with a minimum of 10,000 events collected.

For IL-17 and IFN-γ staining, cells from draining lymph nodes of CII-immunized mice were seeded into 48-well plates at a density of 2 × 10^6 cells/well and incubated with 400 ng/well CII peptide 257–274 overnight. The next day, PMA, ionomycin, and monensin were added at concentrations of 5 ng/ml, 500 ng/ml, and 0.66 μg/ml, respectively. Cells were incubated for 4 h at 37°C. Cells were then washed and stained with anti-CD4 FITC, anti-CD8-PerCP, and CD19-PerCP-conjugated Abs for 30 min at 4°C. After this incubation, cells were washed twice with PBS with 2% FBS, resuspended into 500 μl Cytofix/Cytoperm (BD Biosciences), incubated for 20 min at 4°C, and washed twice with 1× perm/wash (BD Biosciences). Cells were then incubated with anti–IFN-γ–allophycocyanin and anti–IL17A-PE (BD Biosciences) Abs for 30 min at 4°C and analyzed by flow cytometry.

For staining with the DR1-CII tetramer, 1 × 10^6 cells from draining lymph nodes were incubated with 1 μg of DR1 tetramer in 50 μl complete medium with 5 mM NaCN as described previously (30). After 2.5 h of incubation at 37°C, anti–CD4–APC, anti–CD8–PerCP, anti–CD28–APC, and anti–CD19–PE were added, cells were incubated for 30 min at 4°C, and analyzed by flow cytometry. For tetramer studies, a minimum of 100,000 cells were analyzed from each sample.
contrast, cells transduced with the DR1-CII-Foxp3 vector significantly suppressed the proliferation of the CII-specific T cells and suppression was still evident at a ratio of 1:8, Treg:T responder cell, indicating that the Foxp3 expressed by the construct is functional and transduction of naive CD4+ T cells with this construct produces cells with Treg function.

FIGURE 1. Transduction of T cells with retroviral constructs encoding Foxp3 and DR1-CII. (A) Constructs used to create replication-deficient retrovirus for infection of T cells. Concatenated genes (DR1a, DR1b, and Foxp3) were separated by picorna virus T2A sequences, allowing translation of all three genes from a single transcript driven by a single promoter. cDNA encoding the CII(257–274) peptide was integrated into the DR1b chain cDNA sequence to ensure that the expressed DR1 molecules were bound to the CII peptide. (B) Expression of HLA-DR1-CII and Foxp3 by GFP+ T cells infected with engineered replication-deficient retrovirus. Transduced T cells were stained with Abs specific for HLA-DR and Foxp3. Data shown are gated on GFP+ cells. (C) HLA-DR1-CII expressed by retroviral-infected T cells is functional as an Ag presentation molecule. The ability of CII-specific T cell hybridomas to be stimulated by the transfected T cells expressing DR1-CII was tested in vitro in an Ag presentation assay. Transduced T cells functioning as APCs and T-hybridoma cells were incubated in 96-well plates, and after 24 h, the supernatant was collected and the quantity of IL-2 produced was measured in a bioassay as described in Materials and Methods. (D) Expression of Foxp3 confers regulatory function on the transduced T cells. The ability of the engineered Tregs transduced with Foxp3-encoded constructs to inhibit the proliferation of CII-specific T cells was tested in vitro. T cells expressing DR1-CII-Foxp3 were mixed at various ratios with CII-specific T cells from a TCR Tg mouse in 96-well plates containing 5 × 10^4 responder cells, 2 × 10^5 irradiated APCs, and 1 μg/well CII peptide. Proliferation was measured using [3H]thymidine incorporation in a 4-d assay.
Retrogenic Tregs expressing HLA-DR1-CII suppress autoimmune arthritis in DR1 Tg mice

To determine whether the engineered Tregs could alter the development of autoimmune arthritis, humanized DR1 Tg mice were immunized with bCII and treated with $3 \times 10^6$ GFP+ transduced T cells i.v. on days 7, 14, and 21 postimmunization. As shown in Fig. 2, engineered Tregs expressing the DR1-CII-Foxp3 construct were highly efficient in inhibiting the development of autoimmune arthritis (Fig. 2A). High incidences of disease were observed in both groups of control animals that received either T cells transduced with the MSCV vector (80% incidence) or T cells transduced with the vector containing only the DR1-CII construct (100% incidence). In contrast, mice receiving T cells transduced with either Foxp3 alone or DR1-CII-Foxp3 had significantly reduced incidences of disease ($p \leq 0.03$; Fig. 2A). Although the arthritis incidence was reduced to 40% for mice receiving the Foxp3-transduced T cells, when DR1-CII and Foxp3 were coexpressed by the engineered Tregs, only 20% of the mice developed arthritis. Although this difference did not reach statistical significance within a single experiment, this trend was observed in multiple experiments. Of the few mice that did develop arthritis in the Foxp3 and DR1-CII-Foxp3 groups, the onset of disease was delayed (mean of 33.75 and 38.5 d, respectively; $p < 0.02$) in comparison with the control group (mean of 28.1, MSCV group), and the severity of disease was reduced at the early stages of disease (Fig. 2B). However, as disease progressed in these mice, no differences in severity were observed in comparison with the control groups. Thus, these data indicate that coexpression of Foxp3 and DR1-CII by the Tregs enhances their efficacy in inhibiting the development of arthritis in this model.

Because a delayed onset and a prolonged inhibition of arthritis in animals treated with the T cells transduced with the Foxp3 or DR1-CII-Foxp3 construct was observed, we sought to determine how long these engineered Tregs persisted in the mouse after transfer. Mice were injected with a single dose of $3 \times 10^6$ engineered Tregs expressing DR1-CII-Foxp3 and peripheral blood was collected at several time points after, and the percentage of GFP+ cells present was assessed by flow cytometry. As shown in Fig. 3, the engineered Tregs survived well beyond the time frame during which arthritis developed in all four groups (Fig. 2A). Five days after injection, GFP+ (transduced) cells comprised ∼1.8% of PBls and persisted at low levels at least through day 32 (0.3%). Although it is not clear whether the regulatory function of these cells is maintained over this time, these data are consistent with the sustained inhibition of arthritis observed in Fig. 2A.

Because autoantibodies to CII are a major component of the arthritis pathogenesis in this model, we sought to determine whether inhibition of disease by the engineered Tregs was accompanied by changes in the anti-CII IgG response in these mice. Sera from mice treated with the engineered Tregs, DR1-CII alone, and MSCV control cells were collected at days 28 and 42 postimmunization, and the quantity of CII-specific Ab was measured using a solid-phase ELISA. As is shown in Fig. 4, mice injected with DR1-CII-Foxp3-transduced T cells produced significantly less CII-specific IgG on days 28 and 42 postimmunization compared with all other groups. The levels of anti-CII Ab production in the MSCV and DR1-CII control groups correlated with the high level of disease observed in these mice (Fig. 2A). Interestingly, although mice injected with the Foxp3-transduced T cells had significantly lower arthritis incidence (40%) than mice treated with the MSCV of DR1-CII control T cells (80 and 100%, respectively), anti-CII Ab levels were similar between these three groups. These data suggest that
different mechanisms are used between the Foxp3- and DR1-CII-
Foxp3–engineered Tregs and imply a role for pathogenic T cells in
addition to autoantibody in this model.

**DR1-CII-Foxp3 Tregs reduce the number and function of
CII-specific autoimmune T cells in vivo**

Because CII-specific Ab concentrations in the sera were sig-
nificantly lower in the DR1-CII-Foxp3 group, we examined the
magnitude and functional state of the CD4+ CII–specific T cell
response in mice treated with the engineered Tregs. To determine
the magnitude of the T cell response in vivo, mice were immu-
nized with CII and treated on days 1 and 7 after immunization
with T cells transduced with MSCV, Foxp3, or DR1-CII-Foxp3. On
day 10, the peak of the CII-specific T cell response in vivo (30), cells
from the draining lymph nodes were recovered and labeled with a PE-labeled HLA-DR1-CII tetramer and fluorochrome-
labeled Abs specific for CD4, CD8, and CD19. Cells were then
analyzed by flow cytometry, and the data were analyzed by nega-
tive gating on CD8 and CD19 expression. As shown in Fig. 5,
CII-specific autoimmune T cells comprised 1% of the CD4+ T
cells from the draining lymph nodes of immunized mice treated
with MSCV-transduced T cells and averaged 0.8% from mice
treated with Foxp3-engineered Tregs. In contrast, the mice treated
with the DR1-CII-Foxp3–engineered Tregs had 50% fewer CII-
specific T cells, comprising <0.5% of the CD4+ population. These
data indicate that one mechanism governing the effectiveness
of these engineered Tregs appears to be inhibition of the expansion
of the autoimmune CII-specific T cells in vivo.

Although the CII-T cell response was reduced in the mice treated
with the DR1-CII-Foxp3 Tregs, a 0.5% population still represents
the presence of a significantly expanded population of T cells
driven by Ag stimulation in vivo. This raised the questions as to
whether these CII-T cells were not only reduced in number but also
rendered functionally inactive by the engineered Tregs. To assess
their functional state, cells were recovered from the draining lymph
nodes of CII-immunized mice that had been treated with engi-
nereed T cells, and the CII-specific T cells present were tested
for their ability to proliferate in response to stimulation by the
immunodominant CII peptide in vitro. When T cells from mice
immunized with MSCV- or Foxp3-transduced T cells were stimulated
with the CII peptide, a strong proliferative response was ob-
served in each case (Fig. 6). However, T cells from mice treated
with DR1-CII-Foxp3 Tregs were highly refractory to CII peptide
stimulation, responding minimally above the background level of
unstimulated cells (Fig. 6). The lack of a response by the T cells

![FIGURE 4. Decrease in CII-specific autoantibody in mice treated with engineered Tregs. Sera were obtained from the mice on days 28 and 42
after immunization, and the quantity of CII-specific Ab measured using
a CII-specific ELISA. Data are expressed as OD at 490-nm absorbance
minus absorbance at 650 nm. *p < 0.05 between DR1-Foxp3 and MSCV
groups at day 28 and p < 0.00005 at day 42.](http://www.jimmunol.org)

![FIGURE 5. Treatment of mice with engineered Tregs reduces the number of CII-specific T cells in vivo. Mice were immunized with CII/CFA, treated
with the engineered Tregs on days 1 and 7 postimmunization, and on day 10, draining lymph node cells were recovered and stained with Abs specific for
CD4, CD8, and CD19 and with an HLA-DR1-CII tetramer. Naive T cells from an unimmunized DR1 Tg mouse was used as a control for tetramer staining.
Data shown are based on negative gating of CD8- and CD19-expressing cells. (A) Representative experiment of tetramer staining by CD4+ T cells. (B)
Cumulative data represented by average percent of CD4+, DR1-CII tetramer+ T cells, compiled from three or more experiments. *p < 0.002 relative to
MSCV control.](http://www.jimmunol.org)
from these mice is not a simple result of the reduced numbers of CII-specific T cells, because we have found that as few as 0.1% tetramer-positive cells will still generate an appreciable Ag-specific, T cell–proliferative response in vitro (E.F. Rosloniec, unpublished observations). Thus, these data indicate that treatment of mice with the DR1-CII-Foxp3 Tregs renders the autoimmune T cells unresponsive to Ag stimulation and that this unresponsive state is likely playing a role in the suppression of the autoimmune arthritis.

*Retrogenic Tregs alter the cytokine phenotype of autoimmune T cells*

To determine whether treatment of the mice with the engineered Tregs altered the Th1 and Th17 T cell response in these mice, we measured the number of CD4+ T cells producing IFN-γ and Th17 in mice treated with the engineered T cells. On the basis of our observation that the engineered Tregs expressing only Foxp3 inhibited arthritis development without changes in Ab production or T cell responses, we hypothesized that reductions in proinflammatory Th1 and Th17 T cells may be associated with this inhibition of arthritis. To test this concept, mice were immunized with CII and treated with engineered Tregs, and 10 d later, lymph node cells were recovered, restimulated with CII peptide in vitro, and analyzed for intracellular expression of IL-17 and IFN-γ by flow cytometry. Day 10 was chosen for analysis because it is the peak of the CD4+ T cell response in this model (30). As shown in Fig. 7A and 7B, Th17 cells from immunized mice receiving MSCV control T cells comprised 1% of the CD4+ T cells (Fig. 7B) and IFN-γ+ Th1 cells were 1.6% (Fig. 7C). In contrast, mice receiving the Foxp3- or DR1-CII-Foxp3-engineered Tregs had significant decreases in IFN-γ+ Th1 cells or Th1 and Th17 T cells, respectively. Although the Foxp3-transduced Tregs had similar numbers of Th17 cells (1.2%) compared with the controls (Fig. 7B), the IFN-γ+ Th1 cells comprised only 0.8% of the CD4+

![Graphs and diagrams related to the text]

**FIGURE 6.** Proliferative responses of CII-specific T-cells recovered from the draining lymph nodes of immunized mice treated with engineered Tregs. Mice were immunized with CII/CFA and treated twice (days 1 and 7) with the engineered Tregs, and lymph node cells were recovered 10 d post-immunization and tested for their ability to proliferate in vitro when stimulated with the CII peptide. Proliferation was measured by [H]thymidine incorporation after 4 d of culture.

**FIGURE 7.** Treatment of mice with engineered Tregs expressing DR1-CII and Foxp3 inhibits the development of IL-17− and IFN-γ−expressing CD4+ T cells. Mice were immunized with CII/CFA and treated with engineered Tregs on days 1 and 7 postimmunization. On day 10 postimmunization, cells from draining lymph nodes were recovered, stimulated overnight with CII peptide, and stained with Abs specific for CD4, CD8, CD19, IL-17, and IFN-γ. Data are based on negative gates for CD8- and CD19-expressing cells and CD4+ cells. GFP+ engineered Tregs were removed from the data analysis by negative gating. (A) Representative experiment measuring Th17 and IFN-γ expression by CD4+ T cells. (B and C) Cumulative data from three or more experiments represented by average percentage of CD4+ T cells expressing IL-17 (B) or IFN-γ (C). *p < 0.05 relative to MSCV control.
T cells, a significant decrease of 50% in comparison with the control mice (Fig. 7C). Interestingly, although fewer mice in both the Foxp3 group and the DR1-CII-Foxp3 group developed arthritis relative to the vector control group, only the DR1-CII-Foxp3–treated group had reduced percentages of Th17 and Th1 cells. The DR1-CII-Foxp3 group had the lowest percentage of IFN-γ+ Th1 cells (0.6%) and a 50% decrease in Th17 cells (0.5%) compared with the control group. Unlike the relationship among these three groups observed with the Th17 data, the percentages of IFN-γ+ Th1 cells (Fig. 7C) were directly proportional to the arthritis incidence in Fig. 2. The significant decrease in both IFN-γ+ cells and IL-17+ cells in the DR1-CII-Foxp3 group suggests that part of the mechanism mediating the enhanced inhibition of arthritis by these cells may involve interference or redirection of the generation of both Th1 and Th17 cells.

Discussion

Although the potential of adoptively transferred Tregs to be used therapeutically for autoimmunity and transplantation is promising, there are a number of obstacles to overcome, including generation of sufficient numbers of autologous Tregs and improved efficacy in ameliorating the disease. Given that the total absence of Tregs results in profound autoimmunity in both human and mouse models (1, 2), it is clear that Tregs play a significant role in homeostasis of the immune system and prevent the onset of multiorgan autoimmunity. Although most adoptive transfer studies of Tregs in animal models have focused on the use of polyclonal Tregs, there is some evidence that monoclonal Tregs expressing TCR specific for the autoantigen are more efficacious in inhibiting autoimmune responses via the “targeting” capabilities of these cells (15, 18–20). In the study described in this paper, we engineered targeted Tregs using a complex of the MHC ligand and peptide Ag that drives the pathogenic T cell response in an autoimmune arthritis model. These targeted engineered Tregs were produced by transduction of naive CD4+ T cells with Foxp3 and HLA-DR covalently linked to an immunodominant peptide derived from CII that drives the pathogenic T cell response in an autoimmune arthritis model. This approach generated engineered T cells with both the functional properties of a Treg as well as a targeting mechanism (DR1-CII) to promote their interaction with the CII-specific, pathogenic T cells. Although it is generally accepted that functional maturation of Tregs requires stimulation via the TCR, it appears that anti-CD3/CD28 activation of the naive T cells just prior to transduction with Foxp3 suffices to create fully functional Tregs (3, 36). When these engineered Tregs were used as an adoptive therapy after the initiation of an autoimmune response in the arthritis model, they significantly reduced the incidence of disease and were consistently more effective than engineered Tregs expressing Foxp3 alone. Not only were the class II–expressing Tregs effective in preventing the autoimmune response from becoming overt disease, but the mechanism by which they inhibited the autoimmune response appears to be very different from that of the classical Tregs expressing Foxp3 only. Unlike the Foxp3 only Tregs, the DR1-CII-Foxp3 Tregs inhibited the expansion of the T effector response to the autoantigen in vivo, significantly reduced the autoantibody response, and prevented the development of both Th1 and Th17 cells. None of these mechanisms were evident in the mice treated with the Foxp3 only engineered Tregs, even though they were efficient inhibitors of autoimmune arthritis. Thus, the expression of the HLA-DR:peptide ligand by the engineered Tregs generated a highly effective regulatory cell with additional mechanistic capabilities of altering an autoimmune response.

Although mouse T cells do not normally express MHC class II molecules, the expression of class II molecules by activated T cells is normal in most all other species (21, 37). It has been recognized for many years that activated human T cells express class II, and most recently, it was demonstrated that this extends to at least a subpopulation of human Tregs (22). These DR+ Tregs express high levels of Foxp3 and appear to be a functionally distinct lineage of CD4+CD25+ T cells based on their capacity to inhibit T cell proliferative responses in vitro. Whether or not the DR expression indicates that these Tregs are Ag-experienced iTregs is not clear, but like our engineered DR-CII-Foxp3 Tregs, the DR+ human Tregs have functional mechanisms that differ from DR− Tregs. Functionally, there are a number of similarities between the human DR+ Tregs and our engineered murine DR1+ Tregs. In our studies, autoantigen–specific T cells from mice treated with the DR1-CII-Foxp3–engineered Tregs were completely unresponsive to stimulation with Ag in vitro, whereas the autoantigen–specific T cells from mice treated with “conventional” Foxp3 only Tregs responded vigorously. This is similar to the studies of human Tregs where DR1+ Tregs strongly inhibited the anti-CD3/CD2 response of naive T cells whereas DR1− Tregs did not (22). In addition, treatment with the DR1+ engineered Tregs caused a decrease in the number of Th1 cells producing IFN-γ as did human DR+ Tregs but not the DR− Tregs in both cases. Thus, it appears that Tregs that express class II molecules represent a functionally distinct subset of Tregs responsible for immune homeostasis in humans.

The mechanisms by which our Foxp3 only and DR1-CII-Foxp3–engineered Tregs function appear to be significantly different. Although the Foxp3 only Tregs significantly reduced the incidence of arthritis, the changes in the autoimmune response brought about by these cells were difficult to identify. No significant changes in autoantibody levels, numbers of activated CII-specific T cells, nor the ability of these T cells to be stimulated by Ag in vitro were observed. Numbers of Th17 T cells produced in mice treated with Foxp3–engineered Tregs were also unchanged, although there was a significant decrease in Th1 IFN-γ-producing cells. Surprisingly, this is not an uncommon observation in studies where adoptive transfer of syngeneic Tregs were used to successfully inhibit the development of an autoimmune disease. It has been suggested that the mechanism of inhibition for Tregs occurs at the site of inflammation (38), and this may explain the apparent lack of effect in peripheral lymphoid organs. In contrast to the Foxp3-engineered Tregs, the DR1-CII-Foxp3–engineered Tregs drastically altered the autoimmune response in the periphery. Significant decreases in autoantibody levels were observed, the development of the autoimmune T cells response in draining lymph nodes was reduced, and the CII-specific T cells that were present were totally refractory to stimulation by autoantigen in vitro. In addition, adoptive transfer of the DR1-CII-Foxp3 Tregs reduced the numbers of both Th17 and Th1 T cells in the recipient mice to nearly background levels. How the expression of the DR1-CII ligand on the Tregs confers this regulatory mechanism is not clear. Although the expression of class II in the absence of costimulatory molecules would be expected to induce a negative signal in naive T cells, the engineered Tregs were adoptively transferred into the DR1 Tg mice after the initiation of the autoimmune response, a time when the autoimmune T cells should no longer be dependent on costimulation. However, the function of class II expression by T cells is still unknown, and it remains possible that an interaction between a T effector cells and class II+ T cell results in a negative signal to the T effector cell regardless of its activation state (39), perhaps through an undefined costimulatory/regulatory receptor/ligand interaction. In our sys-
immune system or simply represents a therapeutic exploit remains to be determined. Regardless, these data imply that treatment of autoimmune diseases by expansion of endogenous class II Tregs may be an effective therapeutic approach. Development of a means of stimulating their expansion in vivo or expansion in vitro followed by peptide loading and transfer back to the patient are attractive approaches for novel therapy in autoimmunity.

Disclosures

The authors have no financial conflicts of interest.

References


