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Treg Cell Function in Rheumatoid Arthritis Is Compromised by CTLA-4 Promoter Methylation Resulting in a Failure to Activate the Indoleamine 2,3-Dioxygenase Pathway

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Objective. **Functionally impaired Treg cells expressing abnormally low levels of CTLA-4 have been well documented in rheumatoid arthritis (RA). However, the molecular defect underlying this reduced expression is unknown. The aims of this study were to assess the role of DNA methylation in regulating CTLA-4 expression in Treg cells isolated from RA patients and to elucidate the mechanism of their reduced suppressor function.**

Methods. **CTLA-4 expression in Treg cells from RA patients and healthy controls was measured by quantitative polymerase chain reaction (PCR) and flow cytometry. Methylation of the CTLA-4 gene promoter was analyzed by bisulfite-specific PCR, followed by sequencing. Methylation-dependent transcriptional activity of the CTLA-4 gene promoter was measured by luciferase assay, and NF-AT binding to the CTLA-4 gene promoter was determined by chromatin immunoprecipitation. The role of CTLA-4 expression in controlling Teff cells was analyzed using an autologous mixed lymphocyte reaction.**

Results. **Down-regulation of CTLA-4 expression in Treg cells from RA patients was caused by methylation of a previously unidentified NF-AT binding site within the CTLA-4 gene promoter. As a consequence, Treg cells were unable to induce expression and activation of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which in turn resulted in a failure to activate the immunomodulatory kynurenine pathway.**

Conclusion. **We show for the first time that epigenetic modifications contribute to defective Treg cell function in RA through an inability to activate the IDO pathway. Therefore, this study sets a precedent for investigating potential therapeutic strategies aimed at reinforcing the IDO pathway in RA patients.**

Treg cells play an indispensable role in maintaining immune homeostasis and limiting damage mediated by pathogenic Teff cells. Dysfunctional Treg cells, lacking the capacity to control Teff cell responses, have been reported in a number of autoimmune diseases, including rheumatoid arthritis (RA), multiple sclerosis, and type 1 diabetes mellitus (1–6). In RA, there has been much confusion about whether Treg cells have an intrinsic defect, since the lack of Treg cell suppression has been attributed either to reduced responsiveness of Teff cells to Treg cell–mediated suppression (7) or to defective function of Treg cells themselves (1). More recently, however, defective Treg cell function in RA has been associated with reduced expression of the Treg cell–suppressive molecule CTLA-4 (3), which is normally constitutively expressed in healthy individuals (8).

CTLA-4 is an inhibitory molecule that plays a key role in regulating T cell function. Its expression is controlled by the transcription factor NF-AT (9), although epigenetic mechanisms have also been suggested to play a role (10). Methylation of cytosines within CpG islands has long been recognized as an epigenetic silenc-

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ing mechanism (11), as exemplified by the fact that stable FoxP3 expression requires demethylation of an intronic enhancer site within the FoxP3 gene, known as the Treg-specific demethylation region (TSDR) (12). Comparable differential demethylation regions upstream of the CTLA-4 gene locus have been reported in healthy individuals (13), raising the possibility that CTLA-4 expression is similarly influenced by DNA methylation in Treg cells from RA patients.

In this study we determined the mechanism of reduced CTLA-4 expression in Treg cells isolated from RA patients, and we examined the link between CTLA-4 and reduced Treg cell function. We demonstrated that methylation of a previously unidentified NF-AT binding site in the CTLA-4 gene promoter prevents NF-AT2 binding, resulting in reduced CTLA-4 gene transcription. This in turn leads to a failure to activate the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) in antigen-presenting cells (APCs), which accounts for the impaired suppressive capacity of Treg cells in RA.

MATERIALS AND METHODS

Patients and controls. Peripheral blood was obtained from RA patients attending rheumatology clinics at Charing Cross Hospital (London) and Northwick Park Hospital and from age- and sex-matched healthy donors. Detailed information about the patients is provided in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38715/abstract. RA was diagnosed according to the American College of Rheumatology/European League Against Rheumatism 2010 criteria (14). Peripheral blood for use in chromatin immunoprecipitation (ChIP) experiments was obtained from component donation cones supplied by the National Blood Transfusion Service. The study was approved by the London Riverside research committee (REC no. 07/H0706/81).

Cell isolation and culture. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density-gradient centrifugation, and CD4+ lymphocytes were purified by negative selection with magnetic beads (Miltenyi Biotec). Treg cells (CD25+CD127−) and Teff cells (CD25−CD127+) were sorted by fluorescence-activated cell sorting (FACS) using a FACSVantage SE (BD Biosciences). Cells were resuspended in X-Vivo 15 (Lonza) supplemented with heat-inactivated human AB serum (10%) and stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (5 μg/ml).

Treg cell suppression assay. Autologous Treg cells plus Teff cells (1:1), Treg cells alone, or Teff cells alone were stimulated in the presence or absence of mitomycin C–treated autologous APCs (CD4-depleted PBMCs). Interferon-γ (IFNγ) was measured by enzyme-linked immunosorbent assay at 72 hours, after which proliferation was measured by ^{3}H-thymidine incorporation.

Flow cytometry. Cell staining was performed at 4°C for 30 minutes. Intracellular staining was performed using a FoxP3 fix/perm buffer set (BioLegend) according to the manufacturer's instructions. A list of antibodies is available at http://www.kennedy.ox.ac.uk/supplinfo/feldmann/acribbs/arthritisrheumatology.

Construction of luciferase vector. Genomic DNA was purchased from Promega (G3041). The CTLA-4 gene promoter was amplified using specific primers (further information is available at http://www.kennedy.ox.ac.uk/supplinfo/feldmann/acribbs/arthritisrheumatology) and cloned into the pCR2.1-TOPO vector. Point mutations were introduced using a QuikChange II site-directed mutagenesis kit (Stratagene). A list of primer sequences used for site-directed mutagenesis is available at http://www.kennedy.ox.ac.uk/supplinfo/feldmann/acribbs/arthritisrheumatology. Polymerase chain reaction (PCR) products were subcloned into the pCpGL-basic vector (15). A 428-bp promoter region was excised from the pCpGL vector with restriction enzymes *Bgl* II and *Pfl* MI and incubated overnight with *M.Sssl* methylase (60 units), as recommended by the manufacturer (New England Biolabs). Following purification, methylation was confirmed using the restriction enzymes *Aci* I and *Hae* II. Equal amounts of methylated or mock-methylated DNA inserts were religated back into the pCpGL vector, and the restriction enzyme *Kpn* I was used to determine efficiency of ligation.

Luciferase assay. CD4+ lymphocytes were transfected using a T cell Nucleofector kit (Lonza) according to the manufacturer's instructions. Briefly, plasmid DNA (1 μg/ml) and a control *Renilla* plasmid pRL-CMV (2 μg/ml; Promega) were mixed with 3×10^6 cells in Amaxa mix (100 μl) and transfected by electroporation. Transfection efficiency was assessed by flow cytometry for the presence of the green fluorescent protein (GFP) control plasmid, pmaxGFP (Lonza). The cells were counted 24 hours after transfection, and 1×10^6 cells were used to determine luciferase activity by dual-luciferase reporter assay (Promega), according to the manufacturer's instructions. Firefly luciferase activity of individual transfections was normalized against *Renilla* luciferase activity.

Actinomycin D chase assay. Messenger RNA (mRNA) half-life was determined by actinomycin D chase assay. CD25+CD127− Treg cells were stimulated for 24 hours with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml). Actinomycin D (5 μg/ml) was added, and total RNA was extracted after 0, 2, and 4 hours. Reverse transcription (RT)–PCR was performed to measure abundance of transcript.

Real-time quantitative RT-PCR (qRT-PCR). Total RNA was isolated using an RNeasy mini kit (Qiagen), in accordance with the manufacturer's instructions. Reverse transcription was performed with total RNA to generate complementary DNA (cDNA) using an iScript cDNA Synthesis kit (Bio-Rad). Complementary DNA served as template for amplification of genes of interest by qRT-PCR, using a TaqMan gene expression assay (Applied Biosystems) with Fast Blue qPCR Master Mix (Eurogentec). Thermocycler conditions comprised 3 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Primers for the following genes were obtained from Applied Biosystems: *RPLPO* (4333761F), *HPRT1* (4333768F), *FoxP3* (Hs01085834_m1), *CTLA4* (Hs03044418_m1), soluble *CTLA4* (Hs03044419_m1), *IDO1* (Hs00984148_m1), *IDO2* (Hs01589373_m1), *DNMT1*

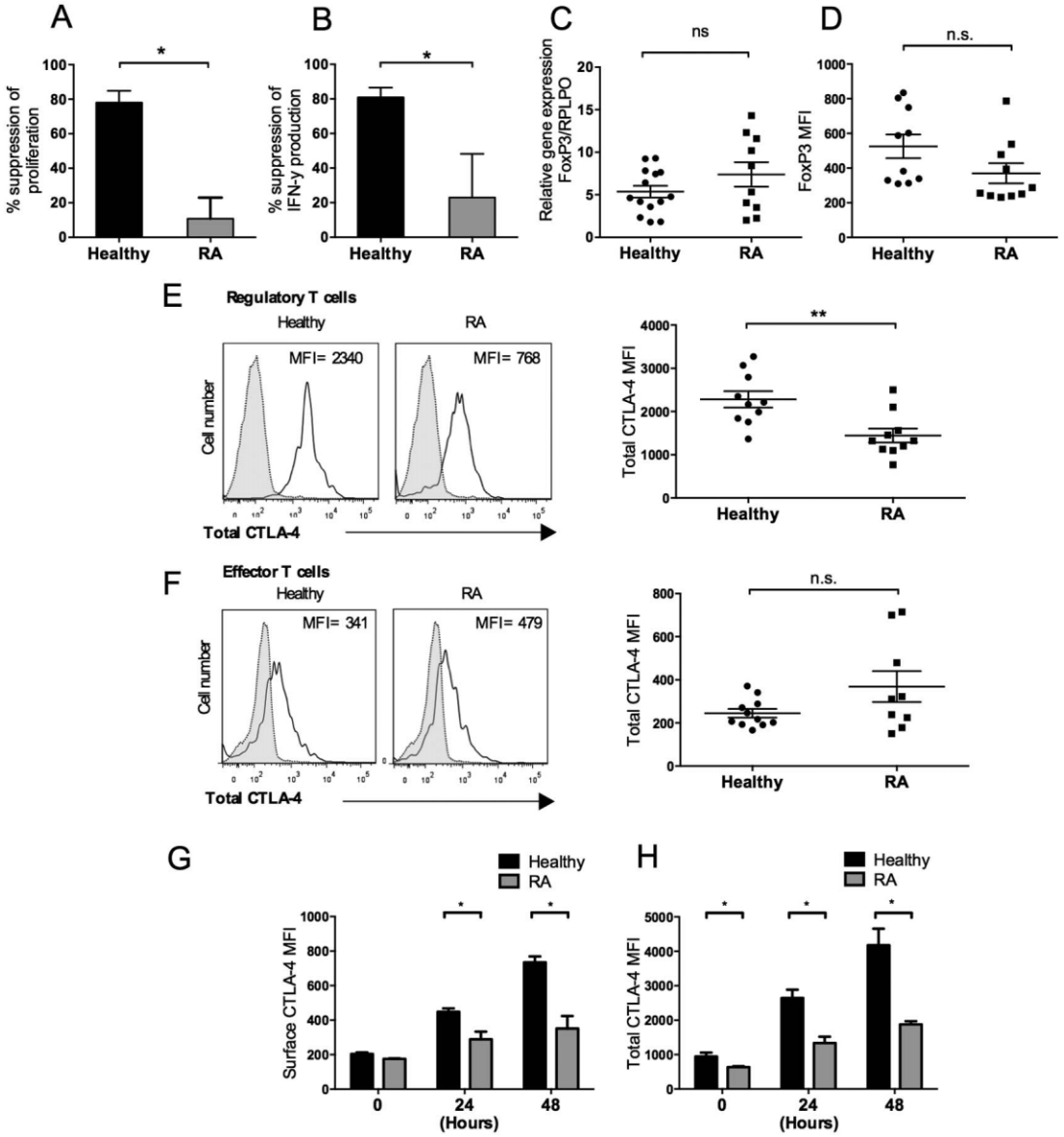

Figure 1. Treg cells isolated from rheumatoid arthritis (RA) patients display impaired function and reduced CTLA-4 expression. **A** and **B,** Shown is the percentage suppression of proliferation (**A**) and interferon-γ (IFNγ) production (**B**) from fluorescence-activated cell–sorted CD4+CD25−CD127+ (Teff) cells by CD4+CD25+CD127− (Treg) cells from RA patients and healthy controls in the suppression assay. Values are the mean ± SD of 4 individual experiments. $* = P < 0.05$ by Mann-Whitney U test. **C,** CD4+CD25+CD127− Treg cells were isolated from healthy individuals and RA patients by fluorescence-activated cell sorting, and the level of FoxP3 expression was determined by reverse transcription–polymerase chain reaction (n = 10 donors). **D–F,** CD4+ cells were fixed/permeabilized and stained intracellularly for FoxP3 and CTLA-4. Shown is the expression of FoxP3 from within gated CD4+CD25+CD127− cells from healthy individuals and RA patients (n = 10) (**D**) as well as cumulative data for total CTLA-4 expression from FoxP3+ (Treg) cells (**E**) and FoxP3− (Teff) cells (**F**) from healthy individuals and RA patients (n = 10). **G** and **H,** CD4+ cells from healthy controls and patients with new-onset RA were stimulated using antibodies to CD3 and CD28 for the indicated amounts of time. The cells were stained for surface CTLA-4, then fixed/permeabilized and stained intracellularly for FoxP3 and/or CTLA-4. The expression of surface CTLA-4 (**G**) and total CTLA-4 (**H**) was measured by gating on FoxP3+ cells. Values are the mean ± SD of 3 independent experiments. In **C–F,** symbols represent individual samples; bars show the mean ± SD. In **E,** $** = P < 0.01$ by Student's *t*-test. In **G** and **H,** $* = P < 0.05$ by one-way analysis of variance. NS = not significant; MFI = mean fluorescence intensity.

(Hs03454525_m1), *DNMT3a* (Hs00601097_m1), and *DNMT3b* (Hs01003419_m1).

Bisulfite sequencing. Genomic DNA was extracted using an Allprep DNA/RNA mini kit (Qiagen). DNA methyl-

ation was determined in T cell subsets at promoter regions of interest. DNA was bisulfite-treated using an EpiTect Plus Bisulfite conversion kit (Qiagen) according to the manufacturer's instructions. Bisulfite-specific PCR primers were designed using MethylPrimer Express software (Applied Biosystems) (further information is available at http://www.kennedy.ox.ac.uk/supplinfo/feldmann/acribbs/arthritisrheumatology). Amplification conditions were 95°C for 15 minutes; 40 cycles of 95°C for 1 minute, 55°C for 45 seconds, and 72°C for 1 minute; and a final extension step of 72°C for 10 minutes. PCR products were gel-purified using a Wizard SV gel and PCR Clean-up system (Promega) and sequenced (Eurofins MWG Operon). DNA methylation analysis was carried out using Quantification Tool for Methylation Analysis (16), and methylation was determined at each CpG dinucleotide. The frequency of conversion of C to T following the sodium bisulfite treatment and PCR was >99% at non-CpG sites.

ChIP assay. Primary lymphocytes (1×10^6 cells) were stimulated for 24 hours, harvested, and fixed for 4 minutes in 1% formaldehyde. The nuclei were sonicated using conditions optimized for primary human CD4+ lymphocytes. For normalization of samples, 10% of the volume was removed. Protein G–conjugated Dynabeads (Invitrogen) were washed and blocked in 0.5% bovine serum albumin, followed by addition of antibody overnight. Antibody complexes were washed in radioimmunoprecipitation assay buffer (0.7% sodium deoxycholate, 1% Nonidet P40, 1 m*M* EDTA, 500 m*M* LiCl, 50 m*M* HEPES-KOH, pH 7.6). DNA complexes were eluted from beads and de-crosslinked by incubation at 65°C overnight. Immunoprecipitated DNA was subjected to qPCR analysis using SYBR Premix Taq (Takara) with the primers 5′-GTTTGTCTCTGTTGAGTTAAGGC-3′ (forward) and 5′-TCCTTCTAATGGTCCCTTGACAGC-3′ (reverse). Thermocycler conditions comprised 30 seconds at 95°C followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds.

DNA binding assay and high-performance liquid chromatography measurement of kynurenine. Information on the protocols used for DNA binding assay and for determination of kynurenine levels by high-performance liquid chromatography is available at http://www.kennedy.ox.ac.uk/supplinfo/feldmann/acribbs/arthritisrheumatology.

Statistical analysis. We used GraphPad Prism software, version 6 for analysis. Group means were compared using the Mann-Whitney U test, Kruskal-Wallis test, and Student's *t*-test. Categorical data were analyzed using Fisher's exact test.

RESULTS

Functional and phenotypic analysis of Treg cells in RA. Since there has been much confusion about the suppressive ability of Treg cells isolated from RA patients, we determined their suppressive capacity in vitro. The ability of FACS-sorted CD25+CD127− Treg cells to suppress CD25−CD127+ Teff cells was compared in healthy controls and RA patients using an autologous suppression assay. To minimize the confounding effects of drug therapy, we recruited RA patients who had not been exposed to disease-modifying antirheumatic drugs or biologic agents.

The purity of Treg cells (based on FoxP3 expression) was ≥90% (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38715/abstract), and the presence of a fully demethylated TSDR confirmed that the population consisted of natural Treg cells (17) (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38715/abstract). The suppression of Teff cell proliferation and IFNγ production by Treg cells from RA patients was significantly decreased compared to that by Treg cells from healthy controls (Figures 1A and B). Despite the loss of Treg cell function in RA, no difference in FoxP3 mRNA or protein expression was observed between RA patients

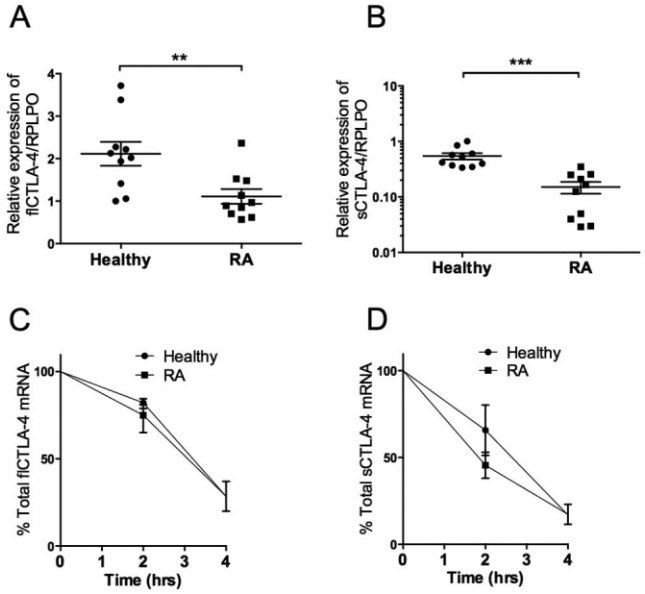

Figure 2. Reduced transcription of full-length CTLA-4 (flCTLA-4) and soluble CTLA-4 (sCTLA-4) in Treg cells isolated from rheumatoid arthritis (RA) patients. **A** and **B,** CD4+CD25+CD127− Treg cells were isolated from healthy individuals and RA patients by fluorescence-activated cell sorting. Expression levels of full-length CTLA-4 (**A**) and sCTLA-4 (**B**) were determined by quantitative reverse transcription–polymerase chain reaction (PCR) (n = 10 donors). Symbols represent individual samples; bars show the mean ± SD. In **A**, ** = $P < 0.01$ by Student's *t*-test. In **B**, *** = $P < 0.001$ by Mann-Whitney U test. **C** and **D,** CD4+CD25+CD127− Treg cells were stimulated for 24 hours with antibodies to CD3 and CD28. Actinomycin D (5 μg/ml) was added to the cultures, and the cells were harvested 0, 2, and 4 hours after treatment. Expression levels of full-length CTLA-4 (**C**) and sCTLA-4 (**D**) were determined by quantitative PCR, and half-lives were calculated. Values are the mean ± SD of 3 independent experiments.

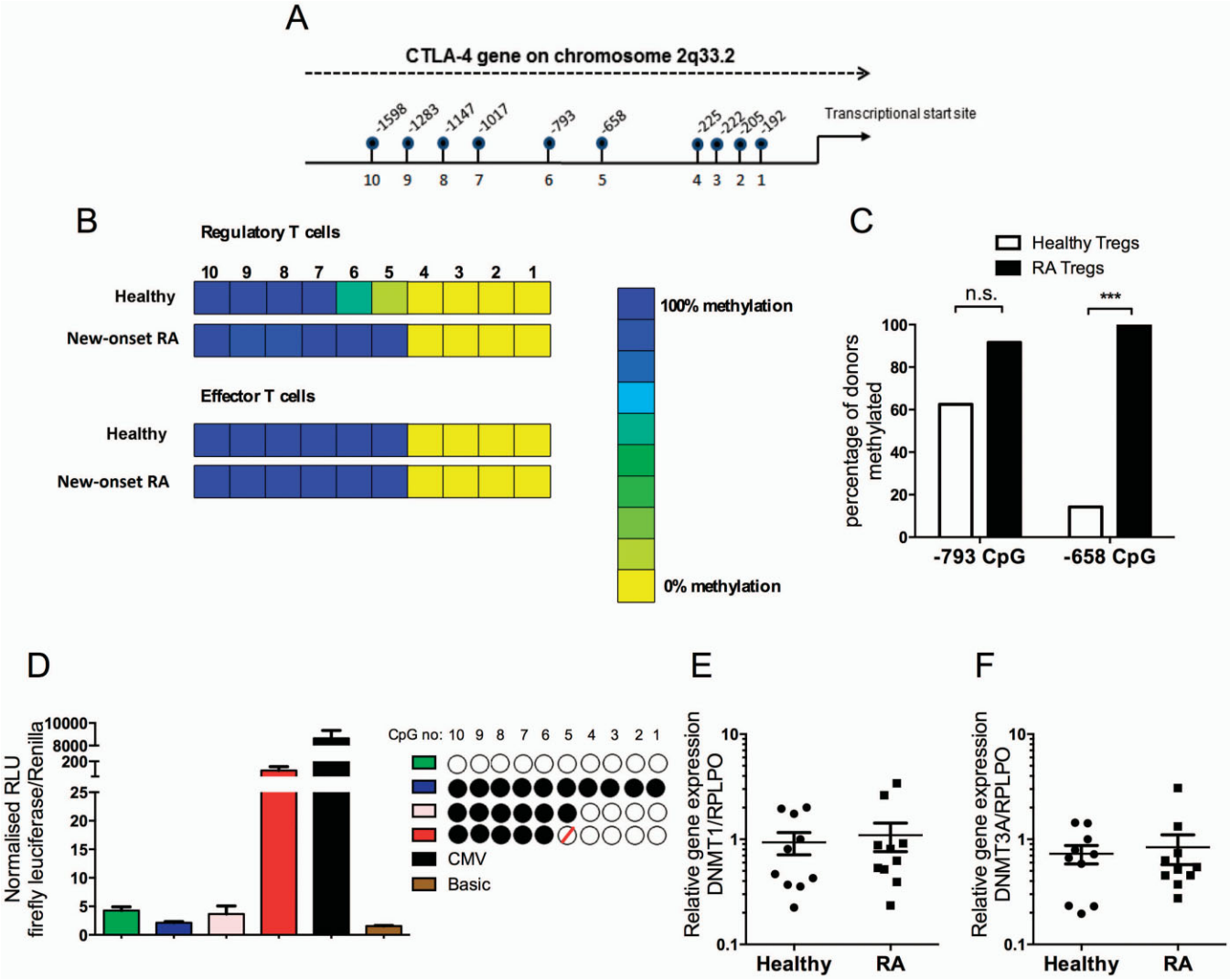

Figure 3. A CpG at position −658 upstream of the transcriptional start site in the CTLA-4 gene promoter is methylated in Treg cells isolated from rheumatoid arthritis (RA) patients. **A,** Shown is a schematic highlighting the positions of the CpGs within the CTLA-4 gene promoter. **B,** Shown is the methylation pattern of CD4+CD25+CD127− (Treg) cells and CD4+CD25−CD127+ (Teff) cells from healthy individuals and RA patients. The methylation status of the individual CpG motifs within the CTLA-4 gene promoter is color coded according to the percentage of donors that display a methylated CpG. The color ranges from yellow (0% of donors methylated) to blue (100% of donors methylated) according to the key at the right (n = 8 donors). **C,** Shown is a further analysis of the −658 and −793 CpGs in Treg cells from RA patients and healthy controls. Data from 14 healthy controls and 14 RA patients are represented. *** = $P < 0.001$ by Fisher's exact test. **D,** Primary CD4+ cells were stimulated with antibodies to CD3 and CD28, then transfected with the indicated plasmids (differentially methylated, cytomegalovirus [CMV], and basic) and a control *Renilla* plasmid and cultured for an additional 24 hours. The cells were lysed and luciferase activity was measured. Luciferase activity is shown relative to *Renilla* luciferase expression. Open circles indicate unmethylated; solid circles indicate methylated; the circle with a red slash indicates a mutated CpG. Data shown are from 1 of 3 independent experiments. Values are the mean ± SD. **E** and **F,** CD4+CD25+CD127− Treg cells were isolated by fluorescence-activated cell sorting, and the expression of genes for DNA methyltransferase 1 (DNMT-1) (**E**) and DNMT-3a (**F**) in healthy individuals and RA patients was determined by reverse transcription–polymerase chain reaction with *RPLPO* used as a comparator (n = 10 donors). In **E** and **F**, symbols represent individual samples; bars show the mean ± SD. NS = not significant; RLU = relative luminescence units.

and healthy individuals (Figures 1C and D). Similarly, there was no difference in the frequency of CD4+CD25+CD127− Treg cells between healthy individuals (mean ± SD 4.76 ± 0.9%) and RA patients (mean ± SD 6.15 ± 2.4%). However, there was a striking reduction in total CTLA-4 expression in the Treg cell population in RA that was not seen in the Teff cell population (Figures 1E and F).

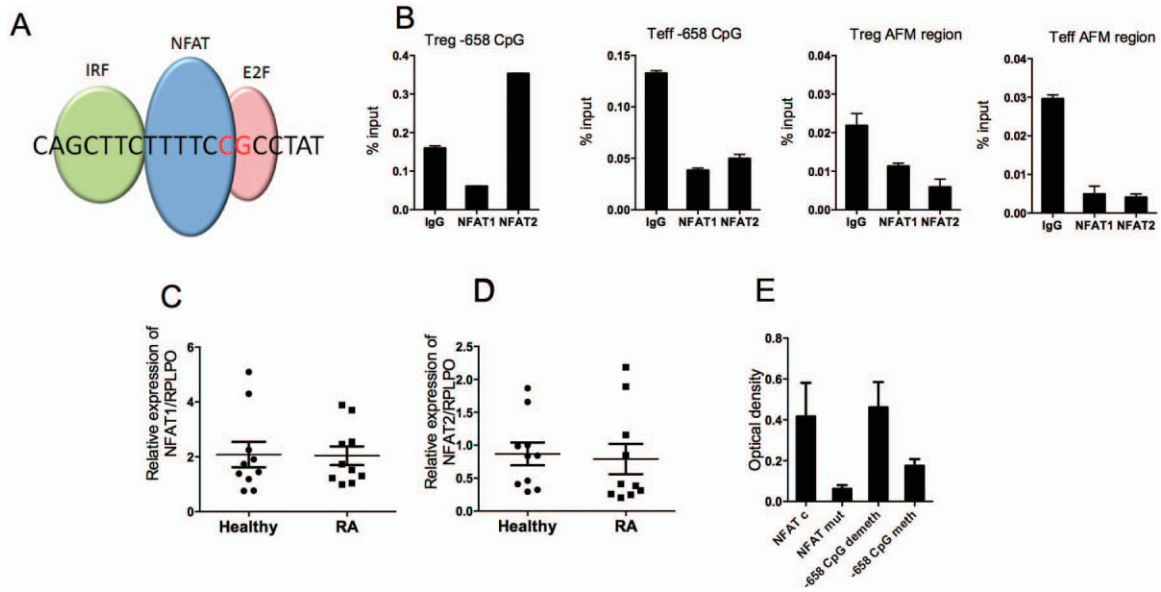

Figure 4. Identification of a methylation-sensitive NF-AT2 binding site overlapping the −658 CpG. **A,** Shown are transcription factor recognition motifs located near the −658 CpG as predicted with MatInspector. **B,** Treg cells and Teff cells were isolated by magnetic bead isolation, and chromatin immunoprecipitation was performed following 24-hour culture. Cells were then fixed for 4 minutes. DNA was immunoprecipitated with antibodies to NF-AT1 or NF-AT2 or with an anti-IgG control antibody and amplified by quantitative polymerase chain reaction (PCR) using primers specific for the region overlapping the −658 CpG or an intronic afamin (AFM) control region. Data are representative of 3 independent experiments. Values are the mean ± SD. **C** and **D,** Expression of mRNA for NF-AT1 (**C**) or NF-AT2 (**D**) in rheumatoid arthritis (RA) patients and healthy individuals was determined by reverse transcription–PCR with *RPLPO* used as a comparator (n = 10 donors). Symbols represent individual samples; bars show the mean ± SD **E,** A DNA binding assay was used to determine the relative binding of recombinant NF-AT2 to unmethylated (demeth) and methylated (meth) −658 CpG oligonucleotides. Values are the mean ± SD from 1 representative experiment of 3 independent experiments performed. IRF = interferon regulatory factor; NFATc = NF-AT consensus site; NFAT mut = NF-AT mutated consensus site. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38715/abstract.

CTLA-4 is constitutively expressed by healthy Treg cells, although its level of expression increases following stimulation (18,19). To establish whether the reduced CTLA-4 expression by Treg cells in RA was maintained following activation, surface and total CTLA-4 expression was measured following stimulation with anti-CD3 and anti-CD28. Surface and total CTLA-4 expression was reduced in Treg cells from RA patients 24 and 48 hours after stimulation (Figures 1G and H), confirming that there is an intrinsic defect in CTLA-4 expression which is independent of the state of activation.

Down-regulation of CTLA-4 occurs at a transcriptional level. Although reduced expression of CTLA-4 by Treg cells in RA has previously been reported (3), the mechanism underlying this decrease is unknown. To establish whether the reduction in CTLA-4 expression occurs at a transcriptional or posttranscriptional level, we first compared CTLA-4 mRNA levels in RA patients and healthy controls. The expression of transcripts encoding full-length CTLA-4 and soluble CTLA-4 was significantly reduced in patients with RA (Figures 2A and B). However, no differences in the stability of either CTLA-4 transcript were detected by actinomycin D chase assay (Figures 2C and D), demonstrating that the reduction in CTLA-4 protein is due to reduced transcriptional activity rather than to increased mRNA instability.

Reduced transcriptional activity of the CTLA-4 gene promoter following methylation. To test the hypothesis that methylation of the CTLA-4 gene promoter can attenuate CTLA-4 protein expression, we analyzed the methylation state of CpGs within a 1,598-bp CTLA-4 gene promoter region (Figure 3A). Of the 10 CpGs present in the promoter, significantly increased methylation ($P < 0.001$) was observed in RA only at the −658 CpG (position is relative to the ATG start codon), although a nonsignificant trend toward increased methylation was also seen at the −793 CpG (Figures 3B and C). We further investigated the effects of culturing healthy Treg cells in tumor necrosis factor α, interleukin-6 (IL-6), and IL-2 for 8 days, as previously

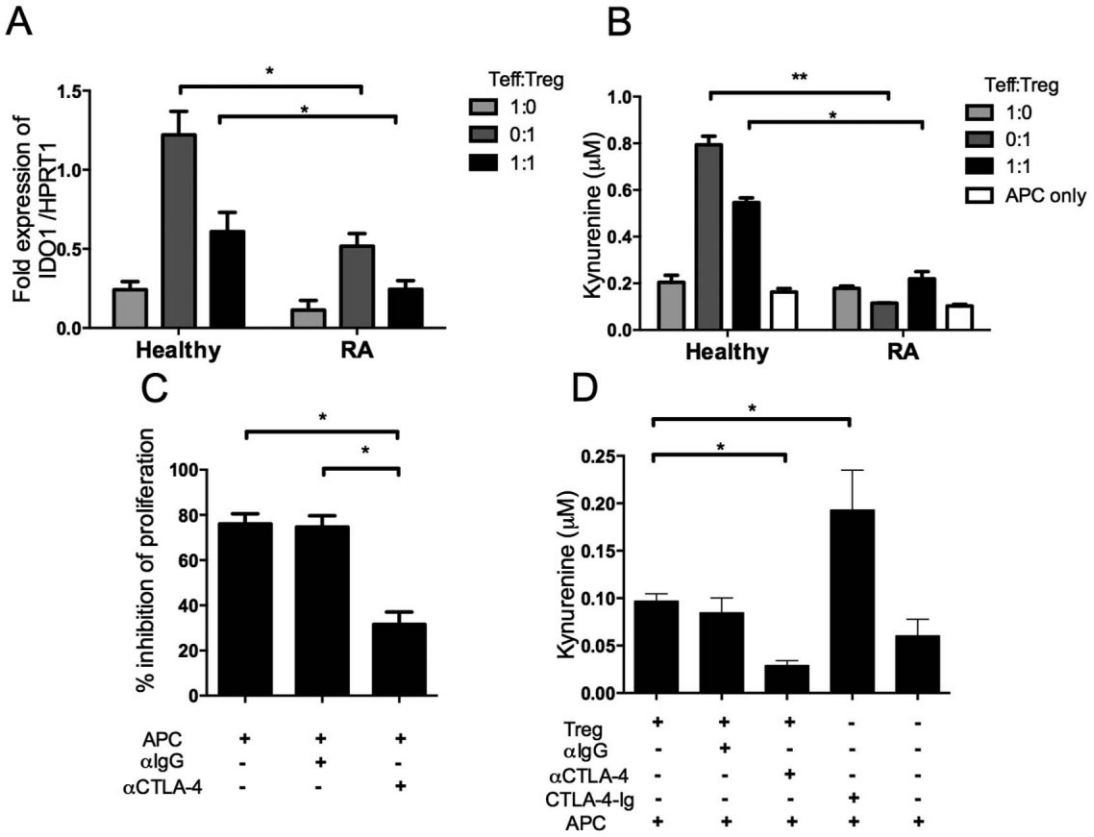

Figure 5. Reduced CTLA-4 expression by Treg cells in rheumatoid arthritis (RA) results in a failure to induce indoleamine 2,3-dioxygenase (IDO) expression. **A** and **B,** CD4+CD25+CD127− Treg cells and CD4+CD25−CD127+ Teff cells were fluorescence-activated cell sorted, cultured alone or together, and stimulated with antibodies to CD3 in the presence of autologous antigen-presenting cells (APCs) for 72 hours. **A,** Relative IDO-1 mRNA expression was assessed by quantitative polymerase chain reaction. **B,** Supernatants were harvested, and kynurenine levels were determined by high-performance liquid chromatography (HPLC). Values are the mean ± SD of 4 independent experiments. **C,** Treg cells and Teff cells isolated from healthy donors were stimulated with antibodies to CD3 in the presence of autologous APCs. Anti–CTLA-4 (10 μg/ml) or anti-IgG (10 μg/ml) was added as indicated. Percentage suppression of proliferation was measured by ^{3}H-thymidine incorporation after 4 days. **D,** APCs were cultured alone or at a 1:1 ratio with Treg cells and cultured with anti–CTLA-4 (10 μg/ml), anti-IgG (10 μg/ml), or CTLA-4Ig (50 μg/ml) as indicated. Levels of kynurenine were measured by HPLC after 96 hours. Values are the mean ± SD of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$ by Kruskal-Wallis test. HPRT-1 = hypoxanthine guanine phosphoribosyltransferase 1.

described by Beavis et al (19); however, no changes were observed in the methylation pattern at the −658 CpG (data not shown).

Since methylation of CpG sites usually precedes a reduction in transcription (20), we addressed the question of whether methylation of the −658 CpG regulates transcription of the CTLA-4 gene. To this end, the CTLA-4 gene promoter was cloned into a reporter plasmid and transfected into primary CD4+ T cells. Methylation of the −658 CpG resulted in reduced reporter activity (Figure 3D). Conversely, promoter activity increased significantly when the −658 CpG was mutated to prevent methylation, confirming the importance of this region in regulating CTLA-4 gene transcription.

In order to investigate the mechanism of this differential methylation, we determined the expression of DNA methyltransferases (DNMTs) in Treg cells isolated from healthy individuals and RA patients. There were no differences in expression of genes for DNMT-1 or DNMT-3a (Figures 3E and F), suggesting that the increased methylation seen in RA patients is independent of DNMT expression. We were unable to detect DNMT-3b mRNA in Treg cells from either healthy controls or RA patients.

Binding of NF-AT2 to the −658 CpG within the CTLA-4 gene promoter. To elucidate the molecular mechanism by which methylation of the −658 CpG reduces transcription, we first used MatInspector (21) to search in silico for transcription factor binding sites

surrounding the −658 CpG. We identified potential binding sites for a number of transcription factors, including E2F and NF-AT (Figure 4A). The NF-AT motif was of particular interest because CTLA-4 expression is dependent upon binding of NF-AT to the CTLA-4 gene promoter (9).

To demonstrate binding of NF-AT to the −658 CpG motif in T cells in vivo, ChIP assays were performed using antibodies specific for NF-AT1 or NF-AT2. Greater enrichment of NF-AT2 at the −658 CpG motif was detected in Treg cells than in Teff cells, while minimal enrichment of NF-AT1 was detected in both subsets (Figure 4B). This confirms that NF-AT2, but not NF-AT1, binds to the −658 CpG site of the CTLA-4 gene promoter in Treg cells. To rule out the possibility that reduced NF-AT expression could contribute to decreased CTLA-4 expression, we measured NF-AT1 and NF-AT2 mRNA levels in our Treg cell samples. There were no differences in the expression of mRNA for either NF-AT1 or NF-AT2 between Treg cells isolated from RA patients and those isolated from healthy controls (Figures 4C and D), indicating that CTLA-4 expression is reduced through a reduction in NF-AT binding and not through differential NF-AT expression.

We next addressed the question of whether methylation of the −658 CpG site affects NF-AT2 binding to the CTLA-4 gene promoter. NF-AT2 exhibited significantly higher binding affinity for demethylated −658 CpG than for methylated −658 CpG (Figure 4E), confirming the functional importance of the methylation state. We concluded that reduced CTLA-4 expression in RA is due to methylation of the −658 CpG in the promoter, resulting in reduced NF-AT binding.

Down-regulation of CTLA-4 in RA results in a failure to induce expression of IDO. Having shown that methylation of the CTLA-4 gene promoter reduces transcriptional activity, we next investigated the functional consequences of reduced CTLA-4 expression. It has been proposed that an important role of CTLA-4 is to up-regulate expression of the immunomodulatory enzyme IDO via ligation of CD80/CD86 on APCs (22). IDO induction was therefore compared in RA patients and healthy controls using the suppression assay. Treg cells from healthy controls, but not from RA patients, were able to activate IDO mRNA expression and increase kynurenine levels (Figures 5A and B), demonstrating defective IDO induction in RA.

To confirm the importance of CTLA-4 in regulating IDO expression and in mediating the suppressive effects of Treg cells from healthy donors, CTLA-4–neutralizing antibody was added in the suppression assay. Neutralization of CTLA-4 abrogated suppression by Treg cells and decreased levels of kynurenine (Figures 5C and D). Conversely, addition of CTLA-4Ig led to increased kynurenine levels, thus confirming the ability of CTLA-4 to activate the IDO pathway (Figure 5D). From these findings we concluded that there is a failure of IDO induction in RA as a consequence of reduced CTLA-4 expression by Treg cells.

DISCUSSION

Defects in the suppressive function of Treg cells in autoimmune diseases have been observed by others and are confirmed in this study, but the mechanism underlying the defect has remained elusive (1–6). Flores-Borja et al demonstrated that Treg cells in RA express reduced levels of CTLA-4 but failed to demonstrate the molecular defect underlying this reduced expression (3). Since differentially methylated regions in the CTLA-4 gene promoter have been identified in healthy individuals (13), we investigated whether methylation of the CTLA-4 gene promoter could contribute to dysregulated CTLA-4 expression in Treg cells isolated from RA patients. We identified an NF-AT binding site located within the CTLA-4 gene promoter that was demethylated in healthy individuals but methylated in Treg cells from RA patients. We subsequently found that methylation of this site resulted in reduced CTLA-4 gene transcriptional activity. As a consequence of reduced CTLA-4 expression in RA Treg cells, there was a failure to induce the activation of the immunosuppressive IDO pathway in APCs. Therefore, we show for the first time that epigenetic modifications contribute to defective Treg cell function in RA.

We investigated the effect of DNA methylation in the CTLA-4 gene promoter on the function of Treg cells in RA. The influence of DNA methylation on Treg cell function is an area of active research (23). Using a genome-wide approach, Schmidl et al identified more than 100 regions in CD4+ and CD4+CD25+ T cells that were differentially methylated between healthy controls and RA patients in a number of immunologically relevant genes (including the gene for CTLA-4) (13). In our study we focused on CpGs within the CTLA-4 gene promoter of Treg cells, and we identified a single CpG at position −658 from the translational start site that was unmethylated in healthy controls but methylated in RA patients. Enforced methylation at this site reduced CTLA-4 gene transcription, confirming the functional

significance of this site in regulating CTLA-4 gene transcription.

CTLA-4 expression depends on binding of FoxP3 to the CTLA-4 gene promoter in cooperation with other cofactors, particularly NF-AT (9,24,25). Therefore, the identification of an NF-AT2 binding site overlapping the −658 CpG suggested that CTLA-4 activation in Treg cells may be regulated through methylation-dependent binding of NF-AT2. Indeed, when we performed in vitro binding assays, we were able to confirm that NF-AT2 binding was methylation sensitive, a finding which has also been shown for a number of other transcription factors such as E2F, activating enhancer binding protein 2, and NF-κB (26).

Two models have been proposed to explain how DNA methylation and reduced transcription factor binding are linked. First, DNA methylation may interfere directly with transcription factor binding (27), as shown for E2F and c-Myc (28,29). Alternatively, DNA methylation may serve as the binding site for methyl-CpG–binding domains (MBDs) that recognize methylated cytosines and indirectly repress transcription through the recruitment of chromatin remodelers (30). However, our DNA binding assay was performed using recombinant NF-AT2 protein and not cell lysates, and therefore the effects of MBDs can be excluded. Thus, we suggest that reduced NF-AT binding results from direct repression by cytosine methylation.

To elucidate a potential mechanism by which methylation of the −658 CpG could directly interfere with NF-AT2 binding, we performed in silico analysis using a previously published nuclear magnetic resonance structure of NF-AT2 complexed with DNA (31) (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38715/abstract). Our analysis suggests that methylation of the −658 CpG interferes with hydrogen bond formation between DNA and Arg^{448} in the NF-AT2 binding loop, thereby preventing effective NF-AT2 binding, rather than interference with NF-AT2 binding resulting from steric hindrance due to the methyl group. However, an alteration in the mechanical properties of DNA could offer another possible explanation for the reduced NF-AT2 binding (32).

An interesting question raised by these results is what causes the methylation change to occur in the CTLA-4 gene promoter. We attempted to address this mechanistically through measuring the levels of DNMTs in RA Treg cells, but we did not identify any differences in the expression of genes for either DNMT-1 or DNMT-3a. These results suggest that the mechanism regulating the methylation of the −658 CpG in the CTLA-4 gene promoter acts specifically at this region and is independent of global differences in DNMT expression. It is widely accepted that RA synovial cells display global hypomethylated DNA that is correlated with a decrease in DNMT expression (33,34). However, while hypermethylated regions have been identified in the death receptor 3 gene in synovial cells, the mechanism by which methylation is maintained is unknown (35). These reports highlight the complex regulation of DNA methylation in RA, and they support our results showing that despite the lack of differences in DNMT expression, increased DNA methylation of specific regions can occur in a manner independent of DNMT activity.

Identification of the causative factors involved in mediating epigenetic modifications in RA is very complex. Cytokine stimulation has previously been shown to impair Treg cell function (19); moreover, dynamic DNA methylation changes can occur following cytokine stimulation (36). Since we have not been able to include another autoimmune disease control in our analysis, we are unable to rule out the possibility that the methylation change at the −658 CpG is related to general inflammation. However, it is unlikely that this modification reflects inflammation because we did not observe changes in −658 CpG methylation following culture with proinflammatory cytokines. This suggests that the methylation changes in the CTLA-4 gene promoter are mediated by one or more currently unknown epigenetic factors, which may include previously identified risk factors for developing RA, such as cigarette smoking (37), hormones (38), latent virus infection (39), or vitamin D (40). It is unlikely that a single environmental trigger is involved; most likely there is a combination of many. Support for this notion comes from studies showing that anti–cyclic citrullinated peptide autoantibodies can appear in RA patients 9 years before disease onset (41), suggesting that over time a number of epigenetic modifications contribute to the breakdown in suppressive mechanisms leading to the development of RA symptoms.

In addition to investigating the mechanism of reduced CTLA-4 expression, we investigated the consequences of reduced CTLA-4 expression in APC coculture assays. In healthy individuals CTLA-4Ig, as well as CTLA-4–expressing Treg cells, license APCs to express IDO via agonism of CD80/CD86 (22,42). In this study we demonstrate that Treg cells from RA patients fail to induce IDO expression in APCs. This inability to induce IDO was a consequence of reduced CTLA-4 expression

in RA Treg cells. Therefore, ours is the first study to show that reduced suppressive function of Treg cells in RA is due, at least in part, to a failure to activate the immunomodulatory IDO pathway. This is consistent with results of another study showing reduced numbers of IDO-expressing cells in RA (43).

In summary, we have demonstrated that defective Treg cell function in RA is due to methylation of an NF-AT binding site in the CTLA-4 gene promoter, leading to a failure to induce IDO activity. We conclude that strategies aimed at reinforcing the IDO pathway in a cell- or tissue-specific manner, as proposed by Xue et al (44), may have therapeutic potential in RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Williams had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. J Exp Med 2004;200:277–85.
2. Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, Greenstein AS, et al. Early rheumatoid arthritis is associated with a deficit in the $CD4^+CD25^{high}$ regulatory T cell population in peripheral blood. Rheumatology (Oxford) 2006;45:1210–7.
3. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci U S A 2008;105: 19396–401.
4. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971–9.
5. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in $CD4^+CD25^+$ T-cells from patients with type 1 diabetes. Diabetes 2005;54:92–9.
6. Ryba M, Marek N, Hak L, Rybarczyk-Kapturska K, Mysliwiec M, Trzonkowski P, et al. Anti-TNF rescue $CD4^+Foxp3^+$ regulatory T cells in patients with type 1 diabetes from effects mediated by TNF. Cytokine 2011;55:353–61.
7. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4+CD25+ regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50: 2775–85.
8. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006;126:375–87.
9. Gibson HM, Hedgcock CJ, Aufiero BM, Wilson AJ, Hafner MS, Tsokos GC, et al. Induction of the CTLA-4 gene in human lymphocytes is dependent on NFAT binding the proximal promoter. J Immunol 2007;179:3831–40.
10. Zhang Y, Maksimovic J, Naselli G, Qian J, Chopin M, Blewitt ME, et al. Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by FOXP3 in human regulatory T cells. Blood 2013;122:2823–36.
11. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science 1975;187:226–32.
12. Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, Baron U, et al. DNA methylation controls Foxp3 gene expression. Eur J Immunol 2008;38:1654–63.
13. Schmidl C, Klug M, Boeld TJ, Andreesen R, Hoffmann P, Edinger M, et al. Lineage-specific DNA methylation in T cells correlates with histone methylation and enhancer activity. Genome Res 2009;19:1165–74.
14. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62: 2569–81.
15. Klug M, Rehli M. Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. Epigenetics 2006;1:127–30.
16. Kumaki Y, Oda M, Okano M. QUMA: quantification tool for methylation analysis. Nucleic Acids Res 2008;36:W170–5.
17. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated $FOXP3^+$ conventional T cells. Eur J Immunol 2007;37:2378–89.
18. Perkins D, Wang Z, Donovan C, He H, Mark D, Guan G, et al. Regulation of CTLA-4 expression during T cell activation. J Immunol 1996;156:4154–9.
19. Beavis PA, Gregory B, Green P, Cribbs AP, Kennedy A, Amjadi P, et al. Resistance to regulatory T cell-mediated suppression in rheumatoid arthritis can be bypassed by ectopic foxp3 expression in pathogenic synovial T cells. Proc Natl Acad Sci U S A 2011;108:16717–22.
20. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068–70.
21. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, et al. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 2005; 21:2933–42.
22. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 2003;4:1206–12.
23. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity 2012;37:785–99.
24. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007;445:936–40.
25. Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells. Proc Natl Acad Sci U S A 2005;102:5138–43.

26. Eden S, Cedar H. Role of DNA methylation in the regulation of transcription. Curr Opin Genet Dev 1994;4:255–9.
27. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 2006;31:89–97.
28. Campanero MR, Armstrong MI, Flemington EK. CpG methylation as a mechanism for the regulation of E2F activity. Proc Natl Acad Sci U S A 2000;97:6481–6.
29. Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science 1991;251:186–9.
30. Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell 1991;64:1123–34.
31. Zhou P, Sun LJ, Dotsch V, Wagner G, Verdine GL. Solution structure of the core NFATC1/DNA complex. Cell 1998;92: 687–96.
32. Geahigan KB, Meints GA, Hatcher ME, Orban J, Drobny GP. The dynamic impact of CpG methylation in DNA. Biochemistry 2000;39:4939–46.
33. Nakano K, Boyle DL, Firestein GS. Regulation of DNA methylation in rheumatoid arthritis synoviocytes. J Immunol 2013;190: 1297–303.
34. Nakano K, Whitaker JW, Boyle DL, Wang W, Firestein GS. DNA methylome signature in rheumatoid arthritis. Ann Rheum Dis 2013;72:110–7.
35. Takami N, Osawa K, Miura Y, Komai K, Taniguchi M, Shiraishi M, et al. Hypermethylated promoter region of DR3, the death receptor 3 gene, in rheumatoid arthritis synovial cells. Arthritis Rheum 2006;54:779–87.
36. Hashimoto K, Oreffo RO, Gibson MB, Goldring MB, Roach HI. DNA demethylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes. Arthritis Rheum 2009;60:3303–13.
37. Hutchinson D, Moots R. Cigarette smoking and severity of rheumatoid arthritis. Rheumatology (Oxford) 2001;40:1426–7.
38. Cutolo M, Villaggio B, Craviotto C, Pizzorni C, Seriolo B, Sulli A. Sex hormones and rheumatoid arthritis. Autoimmun Rev 2002;1: 284–9.
39. Costenbader KH, Karlson EW. Epstein-Barr virus and rheumatoid arthritis: is there a link? Arthritis Res Ther 2006;8:204.
40. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG, et al. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis Rheum 2004;50:72–7.
41. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003;48:2741–9.
42. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol 2002;3:1097–101.
43. Furuzawa-Carballeda J, Lima G, Jakez-Ocampo J, Llorente L. Indoleamine 2,3-dioxygenase-expressing peripheral cells in rheumatoid arthritis and systemic lupus erythematosus: a cross-sectional study. Eur J Clin Invest 2011;41:1037–46.
44. Xue ZT, Sjogren HO, Salford LG, Widegren B. An epigenetic mechanism for high, synergistic expression of indoleamine 2,3-dioxygenase 1 (IDO1) by combined treatment with zebularine and IFN-γ: potential therapeutic use in autoimmune diseases. Mol Immunol 2012;51:101–11.