

Pharmacological modulation of T cell immunity results in long-term remission of autoimmune arthritis

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Significance Statement

Chronic inflammatory diseases are characterized by an imbalance between pathogenic T effector cells and regulatory T cells. In this report we demonstrate that decitabine, a cytosine analogue, has a rapid and sustained therapeutic effect in animal models of rheumatoid arthritis. Decitabine selectively targets T effector cells expressing ENT1, a nucleoside transporter, and generates robust regulatory T cells with the capacity to suppress immune-driven inflammation. This study identifies a path towards resetting tolerance in autoimmune disease using a repurposed drug.

Abstract

Chronic inflammatory diseases like rheumatoid arthritis are characterized by a deficit in fully-functional regulatory T cells. DNA methylation inhibitors have previously been shown to promote regulatory T cell responses and in the present study we evaluated their potential to ameliorate chronic and acute animal models of rheumatoid arthritis. Of the drugs tested, decitabine was the most effective, producing a sustained therapeutic effect that was dependent on indoleamine 2,3-dioxygenase (IDO) and was associated with expansion of induced regulatory T cells, particularly at the site of disease activity. Treatment with decitabine also caused apoptosis of Th1 and Th17 cells in active arthritis in a highly selective manner. The molecular basis for this selectivity was shown to be ENT1, a nucleoside transporter, which facilitates intra-cellular entry of the drug and is upregulated on effector T cells during active arthritis. It was further shown that short-term treatment with decitabine resulted in the generation of a population of regulatory T cells that were able to suppress arthritis upon adoptive transfer. In summary, a therapeutic approach using an approved drug is described that treats active inflammatory disease effectively and generates robust regulatory T cells with the IDO-dependent capacity to maintain remission.

Key words: autoimmunity; DNA methylation inhibitor; rheumatoid arthritis; indoleamine 2,3-dioxygenase

Introduction

Despite recent advances in the therapy of chronic inflammatory diseases such as rheumatoid arthritis (RA), the induction of drug-free disease remission remains an elusive goal for the majority of patients. One reason for this is the lack of available drugs with the capacity to target pathogenic T effector (Teff) cell subsets selectively, whilst sparing or increasing the activity of regulatory T (Treg) cells.

In health, Treg cells play a non-redundant role in maintaining self-tolerance by controlling the activity of Teff cells. However, chronic inflammation is associated with decreased Treg cell function, a phenomenon that has been documented in a number of autoimmune diseases, including RA, systemic lupus erythematosus and type 1 diabetes (1-4). In the case of RA, we and others have previously shown that loss of Treg cell function is accompanied by reduced expression of CTLA-4 due to aberrant CpG methylation in the *cis*-regulatory regions of the gene (5). In addition, the importance of DNA demethylation at the *FOXP3* locus in determining Treg cell function has been confirmed in a number of independent studies (6-12).

These findings led us to question whether DNA demethylating agents could promote Treg cell responses in chronic inflammatory diseases. There are two broad classes of DNA demethylating agents: nucleoside analogues and non-nucleoside analogues. Two nucleoside analogues, decitabine and azacytidine are approved for use in cancer due to their ability to induce cell death at high-dose and promote re-expression of silenced tumour suppressor genes at low-dose (13). Serendipitously, it was observed that numbers of circulating Treg cells are increased in the peripheral blood of patients following treatment with azacytidine (14-16). In addition, nucleoside analogue demethylating agents have been reported to promote the generation and suppressive function of induced Treg (iTreg) cells *in vitro* (14, 17, 18) and to protect against experimental autoimmune encephalomyelitis and allogeneic cardiac transplant rejection *in vivo* (19).

Against this background we compared the ability of nucleoside and non-nucleoside based DNA demethylating agents to promote induction of Treg cells in animal models of RA. We found that short-term treatment with the cytosine analogue, decitabine, depleted pathogenic Teff cells and promoted Treg cell responses, leading to lasting disease remission.

Results

1. DNA demethylating drugs promote generation of Treg cells *in vitro*

We first assessed the ability of nucleoside- and non-nucleoside based DNA demethylating drugs to promote the generation of iTreg cells by stimulating naïve CD4⁺ T cells with anti-CD3 antibody under Treg cell inducing conditions (Table 1). Treatment with decitabine, psammaplin A or zebularine resulted in a dose-dependent increase in the percentage and total number of iTreg cells *in vitro*, as well as increased FoxP3 and CD25 expression (SI Appendix, Fig. S1A, B and D). A fourth drug, procainamide, failed to increase numbers of iTreg cells (SI Appendix, Fig. S1C).

2. Decitabine induces long-term remission of collagen-induced arthritis (CIA)

We next evaluated the efficacy of the DNA demethylating drugs in CIA, as a model for RA. DBA/1 mice were immunized with bovine type II collagen and treated with decitabine, zebularine or psammaplin A at pre-determined doses for 4 days only, starting after arthritis onset. There was a rapid reduction in clinical score (Fig. 1A) in mice treated with decitabine, and to a lesser extent zebularine, versus control mice that was sustained until the end of the experiment on day 10. Psammaplin A was also effective in reducing arthritis severity but the therapeutic effect was sustained only until day 8 (Fig. 1A). Thus, decitabine exhibited a superior therapeutic effect to zebularine and psammaplin A and was therefore used for all subsequent experiments. In a follow-up experiment, we demonstrated the ability of decitabine to maintain remission at least up to day 20, when the experiment was terminated (Fig. 1B). Histological assessment also confirmed the ability of decitabine to provide long-term protection against joint inflammation and erosion of cartilage and bone (Fig. 1C).

To establish its mechanism of action, type II collagen-immunized mice were treated with decitabine or vehicle for 4 days as in the previous experiment. Measurement of T cell subsets revealed a profound reduction in numbers of Th1 (IFN γ ⁺CD4⁺ and Tbet⁺CD4⁺) and Th17 (IL-17⁺CD4⁺ and RoR γ t⁺CD4⁺) cells in decitabine-treated mice (Fig. 1D-E). In addition, the number of proliferating FoxP3-CD4⁺ T cells from decitabine-treated mice *ex vivo* was markedly decreased compared to controls (Fig. 1F). Numbers of Treg (FoxP3⁺CD4⁺) cells were comparable in treated and control mice (Fig. 1E). However, the percentage of induced Treg (iTreg) cells (as defined as Helios⁻FoxP3⁺CD4⁺ cells) within the total Treg cell population was increased in decitabine-treated versus control mice (Fig. 1G). Moreover, the suppressive activity of Treg cells from decitabine-treated mice was significantly increased compared to controls (Fig. 1I), which is in agreement with previous findings (18). Increased Treg cell function was consistent with the increased expression of CD25 on Treg cells from decitabine-treated mice (Fig. 1H). It was concluded that decitabine caused increased Treg cell activity as well as a reduction in pathogenic Th1/Th17 cells.

3. Indoleamine 2,3-dioxygenase (IDO) is required for sustained remission

IDO1 is important for maintaining robust Treg cell responses and its expression is also known to be suppressed by DNA methylation (20-22). This led us to investigate whether decitabine promotes IDO1 expression within the context of inflammation. Decitabine and IFN γ were found to act synergistically in the upregulation of IDO1 expression in BMDCs *in vitro*, confirming previous findings (23) (Fig. 2A). In addition, increased IDO1 expression was observed in spleens and lymph nodes of type II collagen immunized mice treated with decitabine (Fig. 2B). Thus, we further assessed whether the therapeutic effects of decitabine in CIA can be attributed to increased IDO1 expression. Arthritis was induced in *Ido1*^{-/-} and wild-type mice, which were then treated with decitabine or vehicle for four days, as described above. Initially, decitabine had the same therapeutic effect in both groups but disease rapidly relapsed in *Ido1*^{-/-} mice but not wild-type mice (Fig. 2C-D). This was consistent with the fact that numbers of Treg cells and the expression of FoxP3 and CD25 in FoxP3⁺ Tregs were all significantly decreased in *Ido1*^{-/-} mice compared to wild-type mice (Fig. 2E-F).

4. Decitabine selectively targets ENT1⁺ T cells

Decitabine is known to enter cells via the equilibrative nucleoside transporter 1 (ENT1), which is also known to be upregulated on proliferating cells (13). We reasoned that this could explain the selective action of decitabine on Teff cells (Fig. 1). Indeed, ENT1 expression was higher in CD4⁺ T cells from type II collagen-immunised mice with active arthritis *versus* immunised mice without arthritis (Fig. 3A and SI Appendix, Fig. S2A). We also found a higher level of ENT1 expression in activated CD62L⁻CD69⁺CD4⁺ T cells compared to CD62⁺CD69⁻CD4⁺ non-activated T cells (Fig. 3B). Moreover, ENT1 expression in activated CD62L⁻CD69⁺CD4⁺ T cells from arthritic mice was greater than activated CD62L⁻CD69⁺CD4⁺ T cells from non-arthritic mice (Fig. 3B). These results suggest that pathogenic Teff cells increase expression of ENT1 during active disease. We further showed that treatment with decitabine at 0.5 mg/kg led to complete elimination of ENT1⁺CD4⁺ cells from draining lymph nodes of arthritic mice (Fig. 3C). We ruled out the possibility that loss of ENT1⁺ cells was due to masking of ENT1 by decitabine, thereby preventing binding of the detection antibody (SI Appendix, Fig. S3). In addition, *ex vivo* stimulation with bovine type II collagen showed that decitabine treatment led to a dose-dependent reduction in antigen-stimulated proliferation of CD4⁺ T cells from arthritic mice, without affecting the basal rate of proliferation (Fig. 3D), thereby confirming the selective action of decitabine. Further analysis of CD4⁺ T cell subsets revealed that decitabine suppressed antigen-stimulated proliferation of Th1, Th2 and Th17 cells (but not Treg cells) from arthritic mice but had no effect on T cells from non-arthritic mice (Fig. 3E-F). Taken together, these results demonstrate that ENT1 is upregulated in pathogenic Teff cells during active disease and decitabine selectively depletes these cells.

5. Depletion of Teff cells by decitabine is dependent on ENT1

We next set out to address the mechanism by which decitabine depletes Teff cells. We first showed that proliferative responses of FoxP3⁻CD4⁺ T cells from arthritic mice were significantly more sensitive to the inhibitory effects of decitabine than those of non-arthritic mice (Fig. 4A). This was not due to an increased rate of proliferation in FoxP3⁻CD4⁺ T cells in arthritis because numbers of proliferating cells were similar between arthritic and non-arthritic mice without decitabine treatment (Fig. 4A). Rather, we attributed the greater sensitivity of FoxP3⁻CD4⁺ T cells from arthritic mice to increased expression of ENT1 (Fig. 4A). The involvement of ENT1 was subsequently confirmed by the fact that nitrobenzylthioinosine (NBMPR), a specific inhibitor of ENT1, significantly reduced the anti-proliferative effect of decitabine but had no effect on proliferation in the absence of decitabine (Fig. 4B).

To determine the mechanism by which decitabine reduces numbers of proliferating T cells, we looked for evidence of DNA fragmentation and apoptosis in the T cell population using the comet assay and annexin V/propidium iodide staining, respectively. Decitabine increased DNA fragmentation and annexin V staining of CD4⁺ T cells in a dose-dependant manner (Fig. 4C) and the percentage of apoptotic T cells correlated positively with ENT1 expression following anti-CD3 antibody stimulation (Fig. 4D). Furthermore, decitabine not only reduced the proliferation of FoxP3⁻CD4⁺ T cells, but also enhanced the generation of iTreg cells under the conditions of anti-CD3 antibody stimulation (Fig. 4E). Taken together, the results are consistent with previous reports (13), and our own *in vivo* findings (Fig. 1), that decitabine depletes proliferating cells by DNA fragmentation and apoptosis whilst expanding iTreg cells, potentially by promoting the expression of regulatory genes previously silenced by DNA methylation.

6. Localisation of decitabine-induced iTreg cells at the site of disease activity

Administration of decitabine to mice with CIA led to an increase in the proportion of iTreg cells in draining lymph nodes (Fig. 1G). In order to assess the pathophysiological importance of this finding, we used the antigen-induced arthritis (AIA) model in which phenotypic analysis can be performed on cells extracted from inflamed knee joints *ex vivo*. C57BL/6 mice were immunised with mBSA, then treated with decitabine for five days prior to intra-articular injection of mBSA. The severity of arthritis was profoundly reduced in decitabine-treated mice, as indicated by significant reductions in knee-swelling, cathepsin activity and inflammatory cell infiltration (Fig. 5A-C). Phenotypic analysis of cells in the knee joints of decitabine-treated mice revealed reductions in CD4⁺ T cells, Th1, Th2, Th17 and Treg cells (Fig. 5D and SI Appendix, Fig. S4A). However, within the Treg cell population, there was a marked increase in the proportion of iTreg cells (as defined by Helios⁻FoxP3⁺CD4⁺ cells), particularly in the joints but also in the spleens and lymph nodes (Fig. 5E and SI Appendix, Fig. S4B). The expression of CD25 and Foxp3 was significantly increased in total Treg cells from decitabine-

treated mice (Fig. 5F). Thus, decitabine treatment reduced inflammatory cell accumulation in the joint and increased the proportion of iTreg cells locally and systemically.

7. Decitabine-induced Treg cells possess anti-arthritis activity

Finally, we confirmed the anti-arthritis activity of decitabine-induced Treg cells by adoptive transfer from decitabine-treated donor mice to untreated, arthritic recipient mice. Treg cells were collected from spleens and lymph nodes of mBSA-immunised donor mice that had been treated previously with decitabine or vehicle alone. Treg cells (3×10^5 cells/mouse) were then injected intravenously into mBSA immunised recipient mice prior to intra-articular injection of mBSA. Treg cells from decitabine-treated mice, but not control mice, significantly reduced inflammatory cell infiltration with significant reductions in CD4⁺ T cells, Th1, Th2 and Th17 cells (Fig. 6A-D and SI Appendix, Fig. S5A). Moreover, numbers of Treg cells were increased in the arthritic joints of decitabine Treg-treated mice (Fig. 6D) and the MFI of CD25 was significantly increased on Treg cells from the knees of decitabine Treg-treated mice (Fig. 6F). The percentage and numbers of iTreg cells (Helios⁻FoxP3⁺CD4⁺) within the total Treg cell population were also significantly increased in the knees of mice that had received Treg cells from decitabine-treated mice versus control mice (Fig. 6E and SI Appendix, Fig. S5B). Hence, administration of decitabine-induced Treg cells had a profound and durable protective effect on development of arthritis.

Discussion

Chronic inflammation is associated with loss of Treg cell function due, at least in part, to DNA methylation at regulatory regions of functionally important genes, including FoxP3 and CTLA4 (1-5). This led us to question whether DNA methylation inhibitors would be effective in re-establishing tolerance in autoimmunity by augmenting Treg cell activity. We therefore evaluated the effect of short-term treatment of arthritis with three DNA methylation inhibitors and found that one in particular, decitabine, was remarkably effective in suppressing disease activity and maintaining long-term remission. Amelioration of arthritis was accompanied by increased expression of Treg signature genes, CD25, increased Treg cell function and reduced numbers of Th1 and Th17 cells. Importantly, decitabine-induced Treg cells were shown to be functionally active, as evidenced by their ability to suppress arthritis upon adoptive transfer. Phenotypic analysis of Treg cells in the joints and lymphoid organs of decitabine treated mice revealed an increase in the proportion of iTreg cells (defined as Helios⁻FoxP3⁺CD4⁺), although total numbers of Treg cells (FoxP3⁺CD4⁺) were unchanged. On this basis it is proposed that treatment with decitabine increases the ratio of iTreg:nTregs but does not alter the size of the Treg cell pool.

It has been suggested that iTreg cells have a less stable phenotype than nTreg cells and can lose FoxP3 expression under inflammatory conditions (24). We found, however, that short-term treatment with decitabine induces Treg cells, expressing high levels of FoxP3 and CD25, even in inflammation. Furthermore, the increased expression of CD25 remained stable despite adoptive transfer to arthritic recipient mice. Thus, administration of decitabine to mBSA-immunised mice generated a robust and durable Treg cell population that was able to protect against disease upon transfer to mBSA-immunised mice, injected intra-articularly with the same antigen. Several possible therapeutic strategies have been proposed to exploit the homeostatic potential of Treg cells, including administration of low dose IL-2, expansion of autologous Treg cells *in vitro* and generation of iTregs by TCR stimulation in the presence of IL-2 and TGF- β (25-29). However, functional instability of Treg cells is a major problem encountered with all of these approaches; therefore the finding that decitabine treatment produces a phenotypically robust Treg cell population is noteworthy.

Given the importance of IDO in generation and amplification of Treg cell responses (20, 21), we hypothesised that deletion of IDO would reduce the efficacy of decitabine therapy. Indeed, this was confirmed by the fact that treatment of arthritic *Indo1*^{-/-} mice failed to provide stable remission and was associated with reduced numbers of Treg cells and increased numbers of Th1 and Th17 cells. It is noteworthy that the induction of tolerogenic responses in macrophages by phagocytosis of apoptotic cells is dependent on IDO (30). Hence, it is possible that the tolerogenic uptake of apoptotic Teff cells following treatment with decitabine played a role in maintaining long-term remission of arthritis. Another consideration is that a related drug, 5-

azacytidine, has recently been shown to mediate hematopoietic stem cell depletion (31), which could potentially allow for the subsequent re-emergence a more robust Treg cell population.

In addition to its effect on Treg cells, decitabine also caused rapid depletion of Teff cell populations. Whilst this may have been caused partly by the increase in Treg cell activity, decitabine also had a direct effect on Teff cells, by inducing DNA damage and apoptotic cell death at sub-micromolar concentrations. This is consistent with the observations of Agrawal *et al*, who showed that high dose decitabine causes cell death, an effect that masks any effects on DNA methylation (13). The intracellular entry point for decitabine is the nucleoside channel, ENT1, and blockade of this channel impaired decitabine's capacity to inhibit Teff cell proliferation. This provides an explanation for decitabine's selectivity, as ENT1 expression was increased in Teff cells. Furthermore, ENT1 expression in Teff cells was elevated in arthritic mice compared to non-arthritic mice, despite the fact that T cell proliferation was comparable between the two groups. This suggests that there was an association between ENT1 expression and pathogenic T cells, although this remains to be characterised more fully.

A major obstacle to the therapeutic use of nucleoside analogue-based drugs, like decitabine, in a non-fatal disease like RA is lack of specificity giving rise to off-target effects. Nevertheless, a degree of specificity is provided since decitabine incorporates into the DNA of cells in S phase (32), hence selectively targeting proliferating cells. More importantly, as discussed above, potentially pathogenic T cells from arthritic mice were more sensitive to the effects of decitabine than T cells from non-arthritic mice. Thus, the selective activity of decitabine is controlled at two levels: cellular proliferation and expression of ENT1. This potentially provides a therapeutic window which could allow immunomodulation with minimal side-effects. It is worth emphasising that no clinical signs of toxicity were observed in mice treated with decitabine at a dose of 1 mg/kg. Furthermore, a prospective, multicenter study of low-dose decitabine in adults with refractory immune thrombocytopenia has recently been performed with no serious adverse events being recorded (33).

In summary, we have shown that a brief period of treatment with decitabine generates anti-arthritic Treg cells, depletes Teff cells and induces IDO-dependent disease remission in a validated model of RA. In view of its proven safety, and with further data on its effects on host immunity and gene expression, it should be possible to pursue this approach in human autoimmune disorders.

Materials and methods

Study design. Studies were primarily designed to test the long-term therapeutic effects of short-term treatment of DNA demethylating agents in immune-driven inflammation. All experiments were performed in a blinded fashion and key experiments were repeated to ensure reproducibility. For experiments involving genetically-modified mice, age-matched littermates were housed together and randomly assigned to drug or vehicle treatment groups, thereby avoiding cage effects, body weight effects and genetic drift. In general, animal numbers were determined on the basis of previous experience and from pilot studies. Clinical scores and paw- or joint-swelling were monitored daily after onset of arthritis. In accordance with our ethical approval, mice with CIA were euthanized 10 days after onset of arthritis. However, mice deemed to be in remission (defined as a clinical score of less than 2) were kept until day 20 or until they relapsed. For *ex vivo* studies, experiments were performed with at least three mice per study in addition to pilot optimization studies for dose and time-point determination.

Reagents. Full details are provided in SI Appendix.

Mice. Full details are provided in SI Appendix.

CIA. Immunization for CIA was carried out as previously described (34). In brief, a solution of bovine type II collagen (4 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant (CFA; BD Biosciences). DBA/1 mice (or wild type and *Ido1*^{-/-} mice on a C57BL/6N.Q, H-2^q background) were immunized by subcutaneous injection of 100 μ l emulsion.

The clinical severity of CIA was scored in each paw as follows: 0=normal, 1=slight swelling and/or erythema, 2=marked swelling, 3=ankylosis. Hindpaw thickness was measured using microcalipers (Kroeplin, Schluchtem, Germany). For histological assessment, paws were fixed in 10% neutral buffered formalin followed by decalcification with 5.5% EDTA in buffered formalin. Fixed paws were embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. Histopathologic changes in the joints were scored on a scale of 0–3, where 0=normal, 1=minimal synovitis without cartilage/bone erosion, 2=synovitis with some marginal erosion but joint architecture maintained and 3=severe synovitis and erosion with loss of normal joint architecture.

Antigen-induced arthritis (AIA). AIA was induced as previously described (35). Briefly, on day 0, 8-10 week old male C57BL/6 were anaesthetised with 2% isoflurane and immunised subcutaneously with 100 μ g of methylated bovine serum albumin (mBSA) in CFA. Where indicated, mice were administered with 1 mg/kg of decitabine or vehicle intraperitoneally from day 1 to day 5 for Treg purification or from day 10 to day 14 for normal AIA experiments. For adoptive transfer experiments, Treg cells from decitabine- or vehicle-treated mice were purified by FACS sorting, and then cultured overnight with 10 ng/ml IL-2 and 20 ng/ml IL-7. Recipient mice received an intravenous injection of 3×10^5 purified Treg cells on day 10 after immunization with mBSA. On day 15, mice were anaesthetised with 2% isoflurane, both knees were shaved and 125 μ g of mBSA was administered intra-articularly in the right knee joint.

Left knee joints received a vehicle control injection. Knee swelling was monitored daily using digital calipers for 5 days.

In Vivo Imaging System (IVIS). On day 1 of AIA, mice received an IV injection of 4 nmol ProSense 750 FAST imaging probe (PerkinElmer). Mice were imaged 20 hours post IV injection using the IVIS Spectrum (Perkin Elmer, Waltham, Massachusetts, USA) with an excitation wavelength of 745 nm and an emission wavelength of 800 nm, automatic exposure and medium binning. Images were analysed using Living Image 4.7 software (Perkin Elmer, Waltham, Massachusetts, USA) to obtain the average fluorescence intensities of a circular region of interest encompassing the knee joint.

FACS. Full details are provided in SI Appendix.

Proliferation/viability assay. Spleen/LN cells (1×10^7) were labelled with the CellTrace CFSE Cell Proliferation Kit (5 μ M; Invitrogen, Paisley, UK) and stimulated with anti-CD3 antibody (100 ng/ml or 1 μ g/ml) and IL-2 (10 ng/ml) in the presence or absence of decitabine for 72h. The cells were then stained with PerCP-Cy5.5 conjugated anti-CD4 antibody, PeCy7 conjugated anti-FoxP3 antibody and Zombie Fixable Viability dye, washed and analysed by FACS to determine the proliferation of FoxP3⁻CD4⁺ T cells. For proliferation of naïve CD4⁺ T cells purified by MojoTM Mouse CD4 Naïve T Cell Isolation Kit (Biolegend), 1×10^7 cells were labelled with the CellTrace CFSE Cell Proliferation Kit and stimulated with different doses of anti-CD3 antibody (1-100 ng/ml) and IL-2 (10 ng/ml) in the presence or absence of different dose of decitabine (0-0.5 μ M) for 72h. The cells were then stained with PerCP-Cy5.5 conjugated anti-CD4 antibody, AF-647 conjugated anti-ENT1 antibody, PeCy7 conjugated anti-FoxP3 antibody and Zombie Fixable Viability dye, washed and analysed by FACS to determine the proliferation and numbers of CD4⁺ Teff cell and generation of FoxP3⁺CD4⁺ Treg cells.

Treg suppression assay. Naïve CD4⁺ T cells (CD25⁻CD4⁺) and CD25⁺CD4⁺ T (Treg) cells were isolated from spleen/LN pooled cell suspension using the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instructions. The remaining CD4⁺ cells were treated with mitomycin C (20 μ g/ml, Sigma-Aldrich) for 30 min and used as APCs. Naïve CD4⁺ T cells were labeled with CFSE (as described above) and cultured with mitomycin C-pretreated APCs and Treg cells in the presence of anti-CD3 antibody (1 μ g/ml) and IL-2 (10 ng/ml) for 72h. The ratio of Treg:Tnaive:APC ranged from 2:1:1 to 0.0675:1:1. Proliferation of FoxP3-CD4⁺ T cells was analyzed and determined by flow cytometry and flowjo (V10).

Western Blotting. Full details are provided in SI Appendix.

DNA fragmentation and apoptosis assay. Single spleen cell suspensions were used to isolate naïve CD4⁺ T cells by MojoTM Mouse CD4 Naïve T Cell Isolation Kit. Cells were then stimulated with anti-CD3 antibody (1, 10 and 100 ng/ml) and IL-2 (10 ng/ml) in the presence or absence of decitabine (0.125 and 0.5 μ M) for 24h, 48h or 72h. A fraction of the cells were

removed to perform the DNA fragment assay using the Comet Assay Kit (abcam) according to the manufacturer's instructions, and then analysed using a Lecia microscope. The rest of cells were stained by using PB-Annexin V Apoptosis Detection Kit with PI (Biolegend) according to the manufacturer's instructions, and then analysed by FACS to determine the degree of apoptosis of CD4⁺ T cells.

Induced Treg (iTreg) cell differentiation assay. Pooled single cell suspension from spleens and lymph nodes was used to isolate Treg cells, Naïve CD4⁺ T cells and APCs as described above. A 1:1 ratio of mitomycin C-pretreated APCs and Naïve CD4⁺ T cells were cultured in a 96 well U-bottom tissue culture plate in the presence of anti-CD3 antibody (1 µg/ml) or OVA peptide (0-1 µM) with TGFβ (5 ng/ml) and IL-2 (10 ng/ml). DNA demethylating agents were added in serial concentration. Numbers of iTreg cells were measured after 72h by FACS.

Reverse Transcription qPCR. Cell samples were lysed using RLT buffer (Qiagen, Crawley, UK) with 1 in 100 dilution of 2-mercaptoethanol (Sigma-Aldrich). RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and stored at -80°C until conversion to cDNA. Reverse transcriptase qPCR were carried out as previously described (36).

Statistical Analysis. Clinical and histological scores were compared unpaired t-test or two-way ANOVA with Tukey's or Sidak's multiple comparison test, as appropriate. Numbers of relapsing mice were compared by survival curve comparison test. Phenotypic comparisons of T cell subsets were achieved using one-way ANOVA with Tukey's multiple comparison or unpaired t-test. Proliferative responses were compared by two-way ANOVA with Sidak's multiple comparison test, one-way ANOVA with Tukey's multiple comparison test or unpaired t-test, as appropriate. All calculations were performed using GraphPad Prism 7 software. A P-value less than 0.05 was considered significant.

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Author contributions

YSH, WYT, FLC, LMT, JO, KM, DP, NYC and PE performed experiments and analyzed data. TWS, HHL, SFL, AS, PE, ZX, LGS, HOS and ROW helped in design, analysis and interpretation of the data. YSH, TWS, HHL, SFL and ROW helped in drafting the manuscript.

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Figure legends

Figure 1. Decitabine has a sustained therapeutic effect in CIA. Mice with established arthritis were treated for 4 days with decitabine (1 mg/kg/day), psammaplin A (10 mg/kg/day) or zebularine (400 mg/kg/day). **(A)** Clinical scores (mean \pm SEM; N=10). **(B)** Clinical scores up to day 20 of mice treated with decitabine on day 1-4 (mean \pm SEM; N=7). **(C)** Representative images of proximal interphalangeal joint sections from mice treated with vehicle and decitabine are shown. Arrows indicate bone loss. Asterisks indicate damage of articular cartilage (scale bar represents 100 μ M; S=synovial membrane; JS=joint space). Graph shows histological scores (mean \pm SEM; N=10). **(D-H)** Phenotypic characterisation of Teff and Treg cells on day 10 of arthritis. Lymph node cells were stained with antibodies against cytokines (following stimulation with PMA/ionomycin) in D, lineage specific transcription factors in E or the nTreg cell marker, Helios in G. Proliferation of FoxP3⁺CD4⁺ T cells was determined by CFSE labelling in F. Expression of FoxP3 and CD25 was quantified based on MFI in H. **(I)** Suppressive function of Treg cells was determined by culture with a fixed number of CFSE-labeled naïve CD4⁺ T cells (CD25⁻CD4⁺) with mitomycin C treated APCs from control mice under anti-CD3 antibody and IL-2 stimulation. Proliferation was determined by FACS (mean \pm SEM; N=3). * P <0.05, ** P <0.01, *** P <0.001.

Figure 2. IDO is induced by decitabine and is required for sustained disease remission. **(A)** *IDO1* gene expression of BMDCs of C57BL/6 mice treated with IFN- γ and/or decitabine was determined by qPCR. Values are the mean \pm SEM (N=3). **(B)** C57BL/6 mice were immunized with bovine type II collagen in CFA and treated with decitabine (1 mg/kg/day) or vehicle for 4 days. Spleens and LNs were harvested on day 11 after immunization. *IDO1* gene expression was determined by qPCR. Values are the mean \pm SEM of 3 mice. **(C-D)** Wild-type and *Ido1*^{-/-} mice with CIA were treated for 4 days with decitabine (1 mg/kg/day) and monitored for 20 days. **(A)** Clinical scores, **(B)** % relapsed mice. **(E-F)** Wild-type and *Ido1*^{-/-} mice treated with decitabine in A were culled on day 20. LN cells were stained with lineage specific transcription factors **(C)** and MFI of Treg immunoregulatory markers was determined by gating on Treg cells in **(D)**. * P <0.05, ** P <0.01, *** P <0.001.

Figure 3. Decitabine depletes ENT1⁺ cells. **(A)** ENT1 expression was determined in CD4⁺ T cells from LN of non-arthritis mice (red) versus arthritis mice (blue) by FACS. **(B)** ENT1 expression was determined in non-activated CD4⁺ T cells (red, CD62L⁺CD69⁻) and activated CD4⁺ T cells (blue, CD62L⁻CD69⁺) from LN of non-arthritis mice versus arthritis mice by FACS. **(C, D)** Arthritis mice were treated for 4 days with decitabine (0.1 or 0.5 mg/kg/day). **(C)** The ratio of ENT1⁺:ENT1⁻ CD4⁺ T cells was determined in LN by FACS. **(D)** LN cells were stimulated with or without bovine type II collagen (BCII) and IL-2; % CD4⁺ T cells in LN was determined by FACS. **(E, F)** LN cells from non-arthritis mice (red) or arthritis mice (blue) were

stimulated with or without BCII, IL-2 and decitabine. % T cell subsets, Th1, Th2, Th17 and Tregs (E) or ENT1 expression (F) in CD4⁺ T cells was determined by FACS. * P <0.05, ** P <0.01, *** P <0.001.

Figure 4. Decitabine inhibits proliferation of Teff cells and generates iTreg cells. (A) CFSE-stained spleen cells were treated with anti-CD3, IL-2 and decitabine. Proliferation of FoxP3⁻ CD4⁺ T cells was determined by FACS. (B) CFSE-stained spleen cells from arthritic mice were stimulated with anti-CD3, IL-2 and decitabine +/- ENT1 inhibitor (NBMPR; 100 μ M). Proliferation of CFSE-labeled FoxP3-CD4⁺ T cells was determined by FACS. (C) Naïve CD4⁺ T cells were treated with anti-CD3, IL-2 and decitabine. DNA fragmentation and apoptosis were determined by comet assay and Annexin V/PI apoptosis kit. (D) Naïve CD4⁺ T cells were treated with anti-CD3, IL-2 and decitabine (0.5 μ M). ENT1 expression of naïve CD4⁺ T cells was determined by FACS and apoptosis was determined by Annexin V/PI apoptosis kit. (E) Naïve CD4⁺ T cells treated with decitabine and anti-CD3/IL-2. Treg and non-Treg cells were quantified by FACS. * P <0.05, ** P <0.01, *** P <0.001.

Figure 5. Accumulation of iTreg cells in the joint following treatment with decitabine. (A-C) Mice were immunized with mBSA and then treated for 5 days with decitabine (1 mg/kg/day) starting on day 10 after immunisation (mean \pm SEM; N=5). Mice were given an intra-articular injection of mBSA 15 days after immunisation. Knee-swelling was monitored for 5 days (A). Cathepsin activity was measured using a fluorescent probe and detected using the *In Vivo* Imaging System (IVIS) on day 2 of arthritis (B) and cell numbers in spleen, lymph node and knee were counted on day 6 of arthritis (C). (D) Cells from arthritic joints were stained with antibodies against CD4⁺ T cell cytokines and lineage specific transcription factors. Total numbers of cells are shown in SI Appendix, Fig. S3A. (E) Spleen, lymph node and knee cells (following stimulation with mBSA) were stained with antibodies against the nTreg cell marker, Helios. Total numbers of cells are shown in SI Appendix, Fig. S3B. (F) Expression of FoxP3 and CD25 of Treg cells gated from D was quantified based on MFI. * P <0.05, ** P <0.01, *** P <0.001.

Figure 6. Decitabine-induced Treg cells ameliorate arthritis. (A-C) Mice received Treg cells intravenously 10 days after immunisation with mBSA (mean \pm SEM; N=6). Knee swelling was monitored for 5 days (A), cathepsin activity measured using a fluorescent probe and detected by IVIS on day 2 of arthritis (B) and cell numbers of spleen, lymph node and knee were counted on day 6 of arthritis (C). (D-E) Cells from arthritic joints were stained with antibodies against CD4⁺ T cell cytokines and lineage specific transcription factors in D, the nTreg cell marker, Helios, in E. Total numbers of cells are shown in SI Appendix, Fig. S4. (F) Expression of FoxP3

and CD25 of Treg cells gated from D was quantified based on MFI. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1. Potency of DNA-demethylating agents at optimal doses for induction of Treg cells *in vitro*

	%Treg cells	Total no. of Treg cells	MFI of Foxp3	MFI of CD25
Decitabine (1 μM)	30 \pm 11	5953 \pm 2795	2897 \pm 48	3958 \pm 535
Psammaphin A (1 μM)	10 \pm 4	1697 \pm 311	1369 \pm 32	2129 \pm 394
Procainamide (200 μM)	1 \pm 1	398 \pm 303	505 \pm 83	1895 \pm 118
Zebularine (100 μM)	23 \pm 4	2920 \pm 851	1939 \pm 67	3295 \pm 198

Data are expressed as means \pm SD