# Supplementary Information Appendix

## Supplementary materials and methods

**Reagents.** Transforming growth factor-β 1(TGF-β1) and interleukin (IL)-2 were purchased from PeproTech (London, UK). Anti-CD3 antibody and fluorescence conjugated anti-CD4 (PeCy7), GATA3 (eFluor 660), FoxP3 (PeCy7) and CD69 (APC) antibodies were obtained from eBioscience (Hatfield, UK). Zombie NIR™ Fixable Viability Kit and fluorescence conjugated anti-CD4 (BV510 and PerCP-Cy5.5), IL-4 (Alexa Fluor 488), IL-17 (PE and APC), IFNγ (Alexa Fluor 647 and BV785), CD25 (PerCP-Cy5.5 and BV605), and Tbet (BV711) antibodies were obtained from BioLegend (London, UK). Fluorescence conjugated antibodies against RORγt (PE), and CD62L (PE) were purchased from BD Biosciences (Oxford, UK). Decitabine, procainamide, anti-β-actin antibody and nitrobenzylthioinosine (NBMPR) were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 and 647 conjugated anti-mouse equilibrative nucleoside transporter 1 (ENT1) antibodies and psammaplin A were purchased from Santa Cruz Biotechnology (Insight Biotechnology, Wembley, UK) and zebularine was purchased from TOCRIS bioscience (Abingdon, UK). Decitabine was dissolved in 1:1 ratio of acetic acid:PBS (25 mg/ml). Psammaplin A was dissolved in DMSO (100 mg/ml). Zebularine and procainamide were dissolved in PBS at 20 mg/ml and 2 mg/ml, respectively.

**Mice.** Wild type and IDO1$^{-/-}$ mice on a C57BL/6N.Q (H-2$^{q}$) background were bred in-house. DBA/1 mice and C57BL/6 were purchased from Envigo (Horst, Netherlands). All drug stock solutions were diluted in PBS for administration by i.p. injection. Mice were housed in ventilated cages, maintained at a 21°C ± 2°C and a 12-hour light/12-hour dark cycle, with food and water available *ad libitum*. All experimental procedures were approved by the Ethical Review Process Committee and the UK Home Office, in accordance with the Animals (Scientific Procedures) Act 1986.

**FACS.** Cells were labelled with flurochrome conjugated antibodies against cell surface markers and Zombie Fixable Viability dye. After washing, the cells were fixed and permeabilised using a mixture fixation/permeabilization solution (eBioscience). After fixation, the cells were washed and stained for intracellular markers. The labelled cells were washed in permeabilization buffer and resuspended in cell fix buffer (BD Biosciences) before running on the flow cytometer (BD Canto II or Fortessa X20). To determine cytokine expression, cells were stimulated with 20 ng/ml of phorbol myristate acetate (PMA, Calbiochem, Darmstadt, Germany) and 1 μM ionomycin (Calbiochem) in the presence of 12.5 μg/ml Brefeldin A (Sigma-Aldrich). After 3h, cells were then washed and stained with surface markers and intracellular cytokines as above.

**Western Blotting**. CD4$^{+}$ T cells (1x10$^{6}$) were lysed by RIPA buffer (Sigma-Aldrich) with protease inhibitor cocktail (1:100, Sigma-Aldrich). Around 10 μg proteins were separated by SDS-PAGE (NuPAGE Bis-Tris MOPS system, Invitrogen) and proteins of interest detected by

immunoblot and ECL (GE Healthcare Life Sciences, Amersham, UK). Immunoblot of internal control protein was probed for anti-β-actin antibody.

## Supplementary Figures

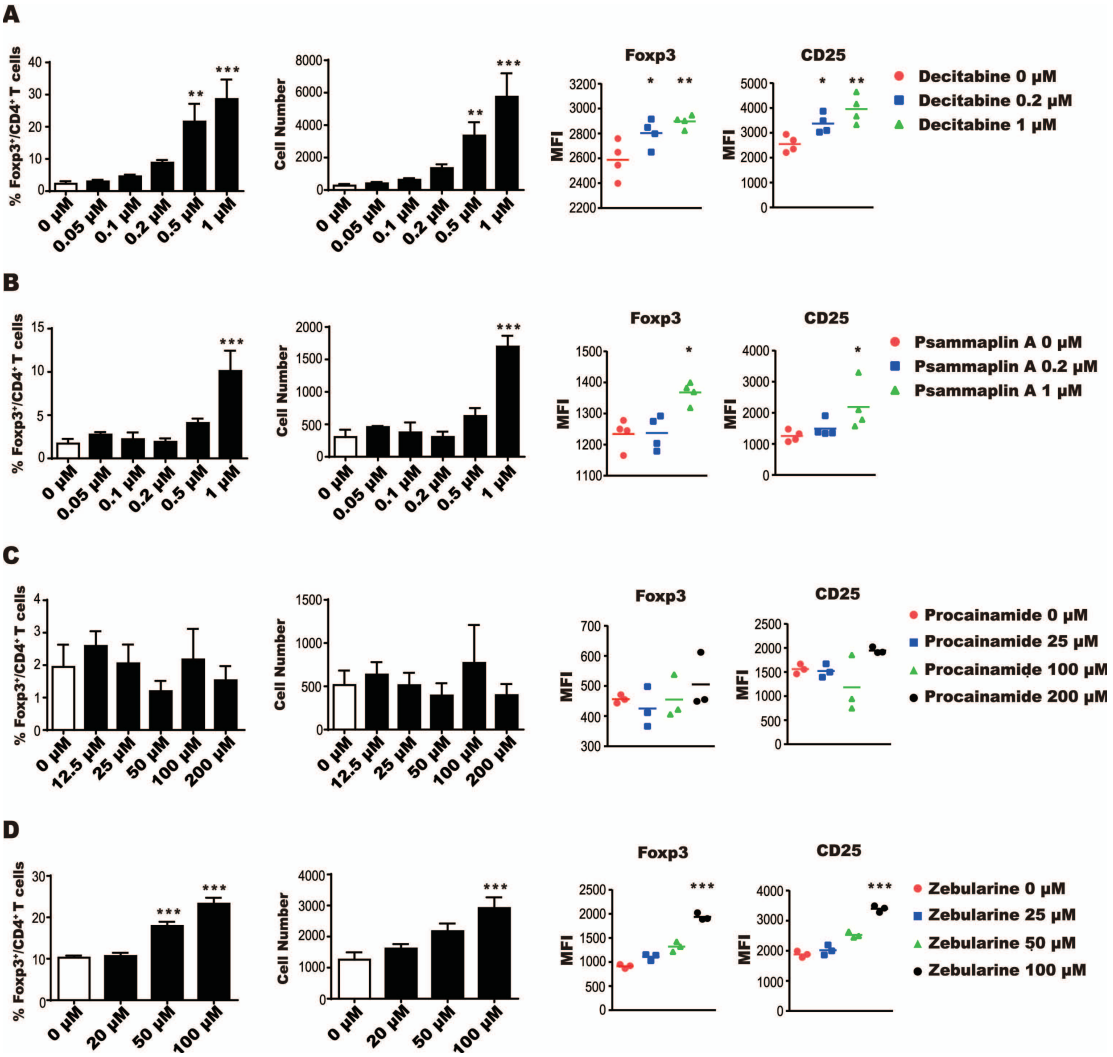


**Figure S1. DNA demethylating agents induce Tregs *in vitro*.** Naïve $CD4^+$ T cells ($CD25^-$ $CD4^+$) were isolated from spleen and LN cells 6-8 week-old mice using the MACS isolation kit. Isolated naïve cells ($4x10^6$/ml) were suspended in complete RPMI1640 with anti-CD3 antibody, IL2, TGF-β1, mitomycin treated antigen-presenting cells and decitabine **(A)**, Psammaplin A **(B)**, Procainamide **(C)** and Zebularine **(D)**. Foxp3 and CD25 expression were determined by FACS. Values are the mean ± SEM (N=3). **P*<0.05, ***P*<0.01, ****P*<0.001.

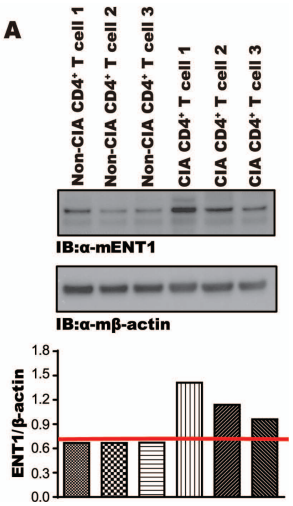


**Figure S2. Increased expression of ENT1 in CD4$^+$ T cells from mice with CIA. (A)** ENT1 expression was determined in CD4$^+$ T cells from arthritic or non-arthritic mice by Western blotting using β-actin as an internal control.

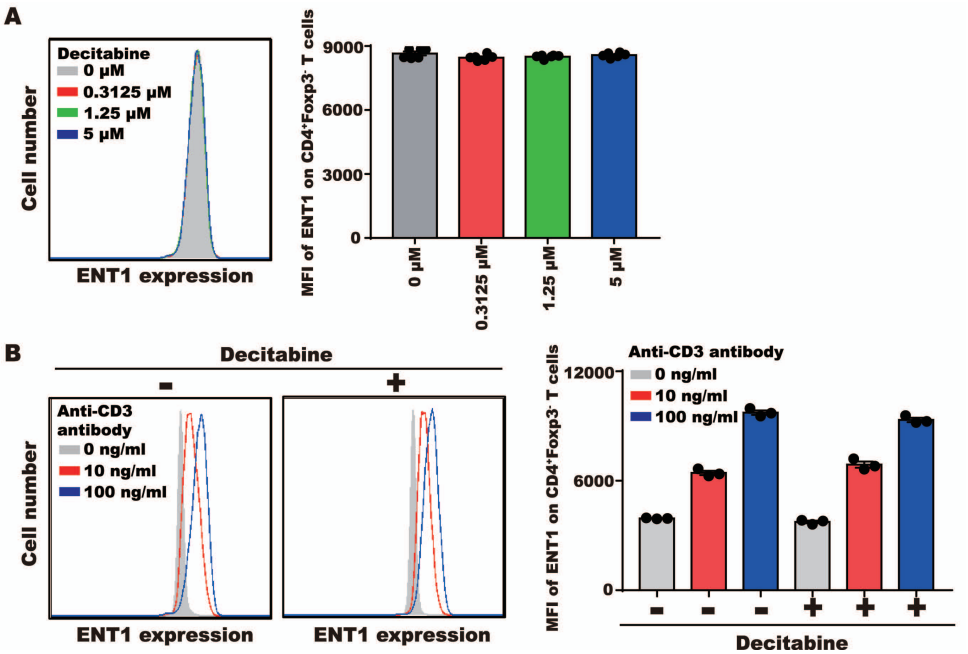


**Figure S3**. **Decitabine has no effect on binding of anti-ENT1 mAb. (A)** Spleen cells were stimulated with anti-CD3 antibody and IL-2, and then were stained with detection antibodies, CD4, CD25, Foxp3 and ENT1, in the presence of increasing doses of decitabine. **(B)** Spleen cells were stimulated with different dose of anti-CD3 antibody and IL-2, and then were stained with detection antibodies, CD4, CD25, Foxp3 and ENT1, in the presence or absence of decitabine (5 μM). As seen in the figure (right), decitabine had no effect on binding of anti-ENT1 mAb.

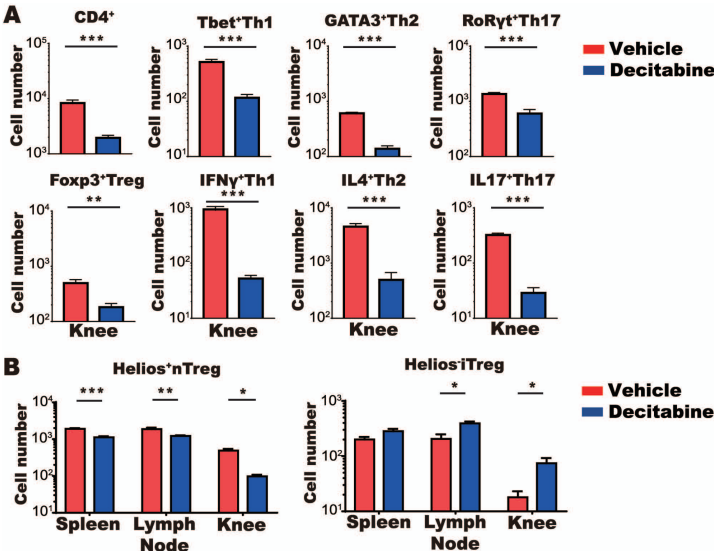


**Figure S4**. **Decitabine increases the proportion of iTreg cells in AIA.** Mice were immunized with mBSA and then treated for 5 days with decitabine (1 mg/kg/day) starting on day 10 after immunisation (mean ± SEM; N=5). Mice were given an intra-articular injection of mBSA 15 days after immunisation,. **(A)** On day 6 of arthritis, knee cells were stained with antibodies against $CD4^+$ T cell-related cytokine and lineage specific transcription factors. **(B)** Spleen, lymph node and knee cells (following stimulation with mBSA) were stained with antibodies against the nTreg cell marker, Helios. **P*<0.05, ***P*<0.01, ****P*<0.001.

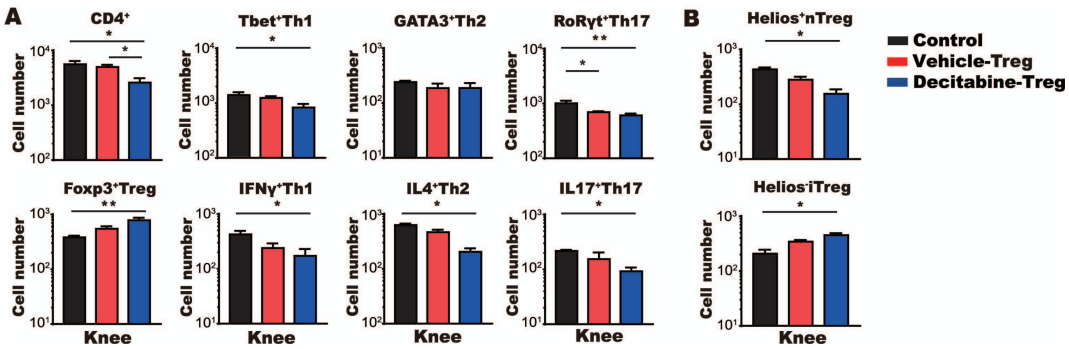


**Figure S5**. **Increased total Treg and iTreg cells in the joint following intravenous of decitabine-induced Treg cells.** Immunized mice were received Treg cells on day 10 (mean ± SEM; N=6). **(A-B)** Cells from the joint were stained with antibodies against $CD4^+$ T cell-related cytokine and lineage specific transcription factors in A, the nTreg cell marker, Helios, in B on day 6 of arthritis. **P*<0.05, ***P*<0.01, ****P*<0.001.