## **Supporting Information**

## Beavis et al. 10.1073/pnas.1112722108



**Fig. S1.** Regulatory T cells used in the rheumatoid arthritis synovial mononuclear cell (RA MNC) cultures are capable of suppressing both syngenic and allogenic T effector cells (Teffs).  $CD4^+CD25^+$  regulatory T cells (Tregs) isolated from the peripheral blood of healthy donors were co-cultured with  $1 \times 10^5$  syngenic (A) or allogenic (B)  $CD4^+CD25^-$  Teffs isolated from the peripheral blood in the presence of 1 µg/mL soluble anti-CD3 and  $5 \times 10^4$  irradiated antigen presenting cells (APCs) for 72 h. Cells were pulsed with 0.5 µCi 3H-thymidine for the last 18 h of culture. Ratios indicate the proportion of Teff:Treg. Data from a representative donor are shown as mean  $\pm$  SD of triplicate cultures and normalized to the proliferation of Teffs cultured alone. In a separate experiment in which the addition of peripheral blood CD4<sup>+</sup>CD25<sup>+</sup> Tregs to RA mononuclear cell (MNC) cultures resulted in inhibition of TNF- $\alpha$  production, it was determined whether the phenotype of the synovial CD14<sup>+</sup> (macrophage) population was modulated. Phenotyping was performed by flow cytometry on the CD14<sup>+</sup> subset of the CD45<sup>+</sup>-gated RA MNC population (C) and expression of CD86, HLA-DR, and CD80 was determined in RA MNC macrophages alone (red bars) and in those RA macrophages co-cultured with activated Tregs (blue bars) (D).



**Fig. 52.** Cytokine-activated CD4<sup>+</sup>CD45RO<sup>+</sup> contain a foxp3<sup>hi</sup> T regulatory cell (Treg) population that remains unable to secrete IL-2 and IFN- $\gamma$ . The phenotype of CD4<sup>+</sup>CD45RO<sup>+</sup> cells was determined before and after (8 d) stimulation with the cytokine-activated T cells (Tck) mixture by flow cytometry (*A*). The indicated gates were used to assess Treg phenotype, cytokine production, and number before and after cytokine stimulation. CD4<sup>+</sup>CD45RO<sup>+</sup> cells were stimulated with phorbol 12-myrisate 13-acetate (PMA) and ionomycin for 4 h in the presence of brefeldin A before and after cytokine stimulation. The proportion of foxp3<sup>+</sup> and foxp3<sup>-</sup> cells producing IFN- $\gamma$ , IL-2, and IL-17 was determined. (*B* and *C*) Representative donor (*B*) and pooled donors (*C*).



**Fig. S3.** IL-2 but not TNF- $\alpha$  or IL-6 abrogate T regulatory cell (Treg)-mediated suppression. CD4<sup>+</sup>CD45R0<sup>+</sup>CD25<sup>-</sup> (T effector) cells were cultured in the presence (gray bars) or absence (black bars) of CD4<sup>+</sup>CD45R0<sup>+</sup>CD25<sup>+</sup> (Treg) cells at a 1:1 ratio and stimulated with anti-CD3 (1  $\mu$ g/mL)/anti-CD28 (2.5  $\mu$ g/mL) and indicated cytokines at the following concentrations: IL-2 (25 ng/mL), IL-6 (100 ng/mL), TNF- $\alpha$  (25 ng/mL). Proliferation was assessed by measuring the incorporation of 0.5  $\mu$ Ci<sup>3</sup>H-thymidine between 102 and 120 h. Data are presented as the mean  $\pm$  SEM of three individual experiments. \**P* < 0.05, \*\**P* < 0.01. n.s., not significant.



**Fig. 54.** Ectopic expression of foxp3 induces a T regulatory cell (Treg) phenotype. (A) Percentage CD4<sup>+</sup> lymphocytes expressing truncated nerve growth factor receptor ( $\Delta$ NGFR) and foxp3 transduced with pCCL or pCCL-foxp3 lentivirus as per *Methods* (main text) was determined by flow cytometry. (*B*) Histogram analysis indicates comparative levels of Foxp3 expression in the  $\Delta$ NGFR<sup>+</sup> gated population of pCCL-foxp3 (green) or pCCL-transduced (red) or non-transduced/ unstained (black) cells. Foxp3 expression in peripheral blood CD4<sup>+</sup>CD25<sup>+</sup> (endogenous Treg) cells is shown as a comparison (blue) (representative of *n* = 8). Expression of cell surface markers CD25, CTLA-4, CD39, and CD127 on the  $\Delta$ NGFR<sup>+</sup> subset of pCCL-foxp3 (green) and pCCL (red) transduced cells. Non-transduced cells (black) (*C*).



**Fig. S5.** Ectopic foxp3 expression does not recapitulate the TNF-R11, CD126 or CD130 expression profiles of Tregs. Expression of TNF-R11, CD126, and CD130 on pCCL (red), pCCL-foxp3 (green) transduced and non-transduced (black) CD4<sup>+</sup> lymphocytes (A); endogenous CD4<sup>+</sup> Foxp3<sup>+</sup> (Treg-green) and CD4<sup>+</sup> Foxp3<sup>-</sup> (Teff-red) or unstained cells (black) (B) derived from the peripheral blood of healthy donors (representative n = 6).



Fig. S6. Ectopic foxp3 expression does not affect STAT-5 phosphorylation in CD4<sup>+</sup> lymphocytes' response to activation. The kinetics of STAT-5 phosphorylation was determined in control (pCCL) and ectopic foxp3 (pCCL-foxp3) transduced cytokine-stimulated T effector cells by flow cytometry (mean  $\pm$  SEM of n = 3).

	Anti	-CD3	IL-2/IL-6/TNF-α		
Variable	Teff (foxp3 <sup>-</sup> )	Treg (foxp3 <sup>+</sup> )	Teff (foxp3 <sup>-</sup> )	Treg (foxp3 <sup>+</sup> )	
CTLA-4	3.1 ± 0.6	4.7 ± 1.2	2.6 ± 0.2	1.7 ± 0.2	
CD69	9.7 ± 2.2	14.6 ± 5.1	10.3 ± 0.9	$2.2 \pm 0.3$	
CD18	1.4 ± 0.1	3.7 ± 0.6	$4.0 \pm 0.6$	1.8 ± 0.2	
CD11a	0.7 ± 0.2	1.8 ± 0.3	$2.0 \pm 0.3$	$1.0 \pm 0.1$	
HLA-DR	1.2 ± 0.2	1.7 ± 0.2	9.8 ± 2.6	1.9 ± 0.2	
CD25	14.6 ± 2.8	10.6 ± 2.4	7.3 ± 0.7	$4.9 \pm 0.9$	

Table S1. Treg effector molecules and activation markers are not up-regulated on Tregs after activation with IL-2/IL-6/TNF- $\alpha$ 

Peripheral blood CD4<sup>+</sup>CD45RO<sup>+</sup> lymphocytes were isolated and stimulated with either anti-CD3 (48 h) or IL-2/IL-6/TNF- $\alpha$  (8 d). The expression of indicated ligands was determined on the foxp3<sup>-</sup> gated and foxp3<sup>+</sup> gated populations. Values indicate the average fold change in mean fluorescence intensity in expression before and after activation ( $n \ge 5$ ). Treg, T regulatory cells; Teff, T effector cells.

Table S2. Patient demographics showing age, sex, and duration of disease

Patient no.	Age (y)	Sex	Duration (y)	RhF	ESR	Joint	DMARD	TNF blockers
1	69	F	1	Positive	33	Wrist	Yes	No
2	79	F	5	Positive	56	NA	Yes	No
3	67	F	12	Positive	21	Knee	Yes	No
4	66	F	2	NA	NA	Hip	Yes	No
5	31	М	1	Negative	Normal	Knee	Yes	No
6	27	М	10	Positive	85	Knee	Yes	Yes
7	59	F	NA	Positive	80	Knee	Yes	Yes
8	46	М	13	Positive	13	Wrist	Yes	No
9	49	F	NA	NA	NA	Knee	Yes	No
10	67	F	25	Positive	NA	Knee	Yes	No
11	49	F	NA	Positive	21	Knee	Yes	No
12	54	F	NA	NA	NA	Hip	Yes	No
13	64	М	NA	NA	NA	Wrist	Yes	No

Demographics for all rheumatoid arthritis (RA) clinical synovial samples used in this study. All patients had RA. Where available, rheumatoid factor status and erythrocyte sedimentation rate were obtained. Joint indicates source of synovial material. DMARD indicates more than one drug for >12 mo; TNF blockers refer to antibiological therapy >12 mo. NA, not available.

PNAS PNAS