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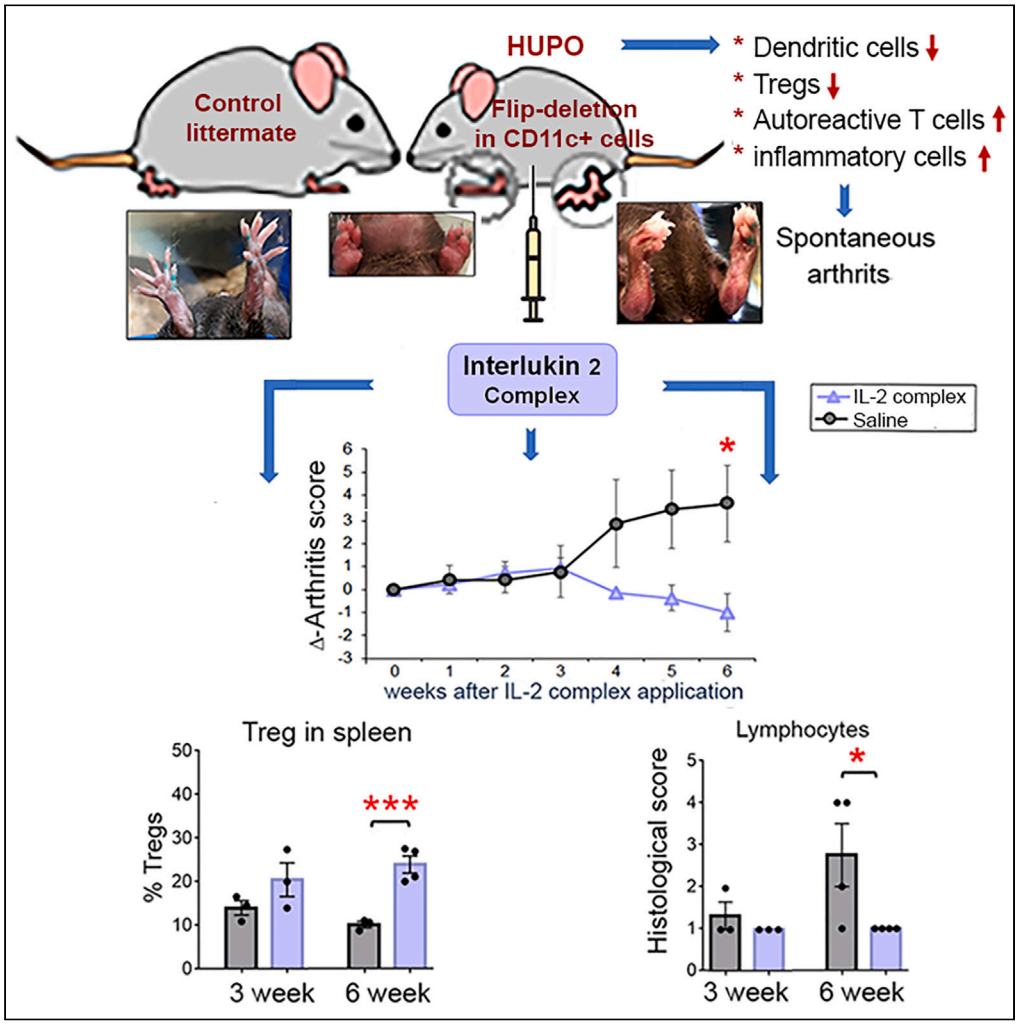

Qi-Quan Huang, Yiwei Hang, Renee Doyle, Qinwen Mao, Deyu Fang, Richard M. Pope

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Highlights

FLIP deletion in CD11c+ cells (HUPO mice) results in spontaneous, erosive arthritis

Reduced Foxp3 expression occurs before Treg loss

HUPO Treg instability is mediated by reduced DCs, IL-2, and chronic inflammation

IL-2 complex treatment increases Tregs and ameliorates HUPO arthritis

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Mechanisms regulating the loss of Tregs in HUPO mice that develop spontaneous inflammatory arthritis

Qi-Quan Huang,[1] Yiwei Hang,[1] Renee Doyle,[1] Qinwen Mao,[2] Deyu Fang,[3] and Richard M. Pope[1,4,*]

SUMMARY

T regulatory cells (Tregs) are a potential therapeutic target in many autoimmune diseases including rheumatoid arthritis (RA). The mechanisms responsible for the maintenance of Tregs in chronic inflammatory conditions such as RA are poorly understood. We employed our mouse model of RA in which, the following deletion of Flice-like inhibitory protein in CD11c$^+$ cells, CD11c-FLIP-KO (HUPO) mice develop spontaneous, progressive, erosive arthritis, with reduced Tregs, and the adoptive transfer of Tregs ameliorates the arthritis. HUPO thymic Treg development was normal, but peripheral of Treg Foxp3 was diminished mediated by reduction of dendritic cells and interleukin-2 (IL-2). During chronic inflammatory arthritis Tregs fail to maintain Foxp3, leading to non-apoptotic cell death and conversion to CD4$^+$CD25$^+$Foxp3$^-$ cells. Treatment with IL-2 increased Tregs and ameliorated the arthritis. In summary, reduced dendritic cells and IL-2 in the milieu of chronic inflammation, contribute to Treg instability, promoting HUPO arthritis progression, and suggesting a therapeutic approach in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which the release of cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) by synovial tissue macrophages is critically important.[1] The number of sublining macrophages in RA synovial tissue is an important biomarker of RA disease activity, prognosis, and response to therapy.[2] T and B lymphocytes are also critical, since autoantibodies including rheumatoid factors and anti-citrullinated protein antibodies are characteristic of the disease.[3] T regulatory cells (Tregs) are essential for suppressing autoimmunity, and a deficiency of Tregs results in fatal autoimmune disease in humans and mice.[4,5] However, how Tregs might become dysfunctional during inflammation and contribute to the pathogenesis of arthritis is incompletely understood. Patients with RA exhibit reduced Treg activity within the joints and, although not a consistent finding, the number of circulating Tregs may be reduced, inversely correlating with disease activity.[6–8] Tregs are not reduced in K/BxN arthritis and collagen induced arthritis (CIA); however, Tregs depletion exacerbates the arthritis,[9,10] suggesting a defect in the ability to promote homeostasis even though the numbers may be normal. Adoptive transfer of natural Tregs (nTregs) ameliorates CIA, while transfer retinoic acid or transforming growth factor beta (TGFβ) induced peripheral Tregs (pTregs), is more effective.[11–13] Therefore, even if Tregs are present within the normal range, this may be insufficient to suppress the inflammatory response. Further, a reduction of Treg function, or resistance of cells affected by Tregs, may also contribute to disease pathogenesis in subtle ways, since Tregs serve as a rheostat regulating autoimmunity.

Dendritic cells (DCs) may restrain autoimmunity centrally through negative selection and the generation of thymic nTregs or in the periphery by promoting CD4$^+$ T cell anergy or by generating pTregs from conventional T cells, which are recent thymic emigrants.[14] Thymic medullary conventional DCs (cDCs) may be involved in central tolerance, by virtue of cross-presentation of self-antigens from autoimmune regulator (AIRE)+ medullary thymic epithelial cells to T lymphocytes.[15–17] CD8α$^+$ cDC in the periphery contribute to the development of pTregs, which are critical for peripheral tolerance.[18,19] Most circulating Tregs are thymus-derived and their numbers are maintained by homeostatic proliferation, which is balanced by apoptosis under basal conditions.[19–21] Recently T cell receptor (TCR) signaling has also been shown to be critical for maintaining Treg homeostasis and the balance between central Tregs (cTegs), which are

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more naive and dependent on interleukin-2 (IL-2) for survival, and effector Tregs (eTregs), which are more proliferative and activated by autoantigens and less reliant on IL-2.[22,23]

Flice-like inhibitory protein (FLIP) is important in preventing death receptor mediated apoptosis and has been shown to be critical to protect DCs and macrophages from Fas-mediated cell death.[24–26] We recently examined the *in vivo* role of FLIP in CD11c-expressing cells such as DCs and a subset of macrophages, by deleting *Flip* in CD11c$^+$ cells generating CD11c-Flip-KO (HUPO) mice.[27] Splenic CD8α^+ DCs and synovial tissue-resident macrophages, but not plasmacytoid DCs, are reduced, and rather than a lupus-like autoimmunity, the mice develop a spontaneous, inflammatory, erosive arthritis, associated with the increased expression of TNFα, IL-1β and IL-6, and autoantibodies to constituents of the synovial joints, but not antinuclear antibodies. However, the antigen presenting function of HUPO DCs, when the numbers are normalized, is not different from the controls. HUPO mice are mildly lymphopenic at 4 weeks, although T cells from lymph nodes draining the inflamed joints at $\geq$ 20 weeks of age express a memory phenotype and are autoreactive. Further, the number and percent of CD4$^+$ Tregs are reduced in the spleen, and the number of splenic Tregs inversely correlates with the arthritis at $\geq$ 20 weeks, and adoptive transfer of Tregs from control mice suppresses arthritis and autoreactive T cells.[27] The mechanisms responsible for the reduction of Tregs in the periphery of HUPO mice remain to be elucidated.

In this study HUPO mice demonstrated reduced expression of Foxp3 in Tregs in the periphery, before Tregs were reduced. No reduction of hypostatic proliferation of Tregs was observed when injected into mildly lymphopenic HUPO or severely lymphopenic HUPO-*Rag*$^{-/-}$ mice. However, at 3 days post-transfer there was a reduction of the Treg mean fluorescence intensity (MFI) when T cells were transferred into HUPO-*Rag*$^{-/-}$, compared with *Rag*$^{-/-}$, mice. Employing *ex vivo* spleen cell cultures, IL-2 was significantly reduced in supernatants from HUPO, compared with littermate control, mice. Tregs were also reduced in the spleen cultures, and the addition of IL-2 or DCs normalized the Foxp3$^+$ Tregs but did not significantly increase CD4$^+$CD25$^+$Foxp3$^-$ cells. Further, exogenous antigen presentation was also reduced *in vivo* in HUPO mice and was normalized *ex vivo* by the addition of DCs. Supporting the importance of reduced IL-2 in the reduction of Tregs *in vivo*, HUPO mice demonstrated a significant reduction of cTregs and an increase of dead CD4$^+$CD25$^+$ cells, compared with littermate controls. Treatment of HUPO mice with IL-2 complexes increased Tregs and ameliorated arthritis. These observations demonstrate that the reduction of Tregs in HUPO mice results from a reduction of DCs that results in reduced basal IL-2 expression, reduced cTregs and Treg instability, and suggest that modulation of Tregs through IL-2 signaling may provide a novel pathway for the treatment of RA.

RESULTS

Normal homeostatic proliferation, but Treg instability in HUPO-Rag−/− mice

Previously, we demonstrated that the percent Tregs in the spleens of HUPO mice is reduced at $\geq$ 20 weeks, but not 4 weeks, of age.[27] Similar results were noted in the LNs draining the inflamed joints (Figure S1A). Here, we demonstrate that Treg Foxp3 expression was reduced and CD4$^+$CD25$^+$Foxp3$^-$ cells were increased, both at 4 and $\geq$ 20 weeks in the spleens of HUPO mice (Figures 1A–1C). Although peripheral blood Tregs was not reduced in mice $\geq$ 12 weeks of age, similar to the spleen, Treg Foxp3 was reduced and CD4$^+$CD25$^+$Foxp3$^-$ cells were increased (Figure S1B). In contrast, neither the percentage of thymic Tregs,[27] nor the Treg Foxp3 expression (Figures 1D and 1E) were reduced at 4 weeks. The decrease of Tregs and increase of CD4$^+$CD25$^+$Foxp3$^-$ cells may be due to reduced homeostatic proliferation of Tregs, *in vivo* activation of conventional T cells or the instability of Foxp3$^+$ nTregs. The maintenance of Tregs involves homeostatic proliferation, which is independent of IL-2 and mediated, at least in part, by interaction of CD80/86 on DCs with CD28 on T cells.[20,28,29] HUPO mice, which are mildly lymphopenic, exhibit a reduction of cDCs, particularly the CD8α^+ subset.[27] However, no difference in the homeostatic proliferation of total CD4$^+$ lymphocytes or Tregs was observed 6 days after wild-type CD45.1$^+$ carboxyfluorescein succinimidyl ester (CFSE) labeled CD3$^+$ T cells were adoptively transferred into CD45.2$^+$ HUPO or control mice (Figure S2).

Additional experiments were performed under severely lymphopenic conditions, employing HUPO-*Rag*$^{-/-}$ mice, which exhibit the HUPO phenotype of reduced cDCs.[27] Wild-type CD45.1$^+$CD3$^+$ lymphocytes were adoptively transferred into CD45.2$^+$ *Rag*$^{-/-}$ or HUPO-*Rag*$^{-/-}$ mice and harvested after 3 or 6 days (Figures 2A and 2B). As expected, Tregs demonstrated increased homeostatic proliferation at 3 days compared with conventional CD4$^+$CD25$^-$Foxp3$^-$ T cells; however, there was no difference between

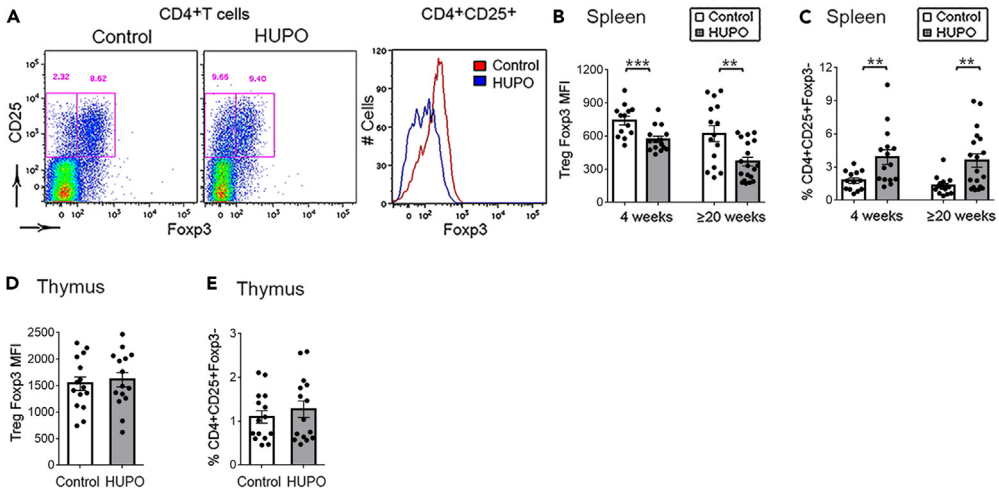

Figure 1. Reduced Foxp3 expression in Tregs and increased CD25$^+$Foxp3$^-$ cells in HUPO spleens

(A–C) The spleens of HUPO and littermate control mice were harvested at 4 or ≥ 20 weeks of age. CD4$^+$ T cells were analyzed by flow cytometry.

(A) Representative flow histograms for splenocytes from 4 weeks old mice gated on CD4$^+$ T cells (left panels) and Foxp3 expression in CD25$^+$CD4$^+$ T cells (right). Tregs were defined as the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells within the total CD4$^+$ T cell population.

(B) Splenocyte Foxp3 expression determined by the mean fluorescent intensity (MFI) for CD4$^+$CD25$^+$Foxp3$^+$ Tregs.

(C) The percentage of spleen CD4$^+$CD25$^+$Foxp3$^-$ cells of total CD4$^+$ T cells.

(D) The Foxp3 Treg MFI and (E) the percentage of CD4$^+$CD25$^+$Foxp3$^-$ cells of total CD4$^+$ T cells in the thymus of 4-week-old HUPO or control mice. The dots represent data from each individual mouse and the bars the mean ± 1 SEM, in this and all the figures. Significance was determined by unpaired 2-sided *t*-test between the same age groups.

*represents < 0.05, ** represents p < 0.01, and *** p < 0.001 between the indicated groups. See also Figure S1.

Rag$^{-/-}$ and HUPO-*Rag*$^{-/-}$mice (Figure 2B). Following the transfer of CD3$^+$ T cells into HUPO-*Rag*$^{-/-}$ mice (Figures 2C–2E), there was a modest reduction of Treg Foxp3 MFI on day 3, but not Treg frequency (Figure 2D). Further, CD4$^+$CD25$^+$Foxp3$^-$ cells were increased on day 6 after adoptive transfer (Figure 2E). These observations did not identify a defect of homeostatic proliferation but suggest instability in maintenance of Treg Foxp3 expression in recipient HUPO-*Rag*$^{-/-}$ mice.

Decreased IL-2 contributes to reduced Tregs in HUPO mice

IL-2 is critical for Treg maintenance.[20,30] *Ex vivo* spleen cell culture supernatants from HUPO mice demonstrated a reduction of IL-2, while IL-6 was increased (Figure 3A). Further, the reduction of Tregs in these cultures was abrogated by the addition of IL-2, although there was no increase of the Treg Foxp3 MFI (Figures 3B and 3C). In contrast, no significant effect of IL-2 on the CD4$^+$CD25$^+$Foxp3$^-$ population was observed for HUPO mice, although a slight increase was noted for the controls (Figure S3A). The addition of neutralizing antibodies to IL-2 resulted in reduced Tregs and Treg Foxp3 expression in control spleen cultures, while there was a marginal, further reduction of Tregs, and no further reduction of Treg Foxp3 expression in the HUPO cultures (Figure 3D), consistent with the already reduced IL-2. The neutralizing antibodies to IL-2 had no effect on the CD4$^+$CD25$^+$Foxp3$^-$ population (Figure S3B). To determine if the differences in the Tregs were due a defect of HUPO T cells, CD45.2$^+$ HUPO or littermate control spleens were depleted of CD4$^+$ T cells, which were replaced with a comparable number of wild-type CD45.1$^+$CD4$^+$ T cells. After 3 days of co-culture, IL-2 was significantly lower in culture supernatants of the HUPO spleens (Figure 3E), and Tregs were maintained by the addition of IL-2, even though IL-2 had no effect on Treg Foxp3 expression (Figure 3F). These results indicate, that in short term *ex vivo* cultures, reduced IL-2 contributed to the loss of Tregs, although IL-2 alone was insufficient to fully rescue Foxp3 expression in Tregs, together suggesting a role for DCs.

Reduced DCs contribute to decreased Tregs

In order to determine the role of the reduction of DCs for the decrease of Tregs in HUPO mice, control DCs were isolated and added back to HUPO spleen cultures. While the addition of DCs had no effect on total

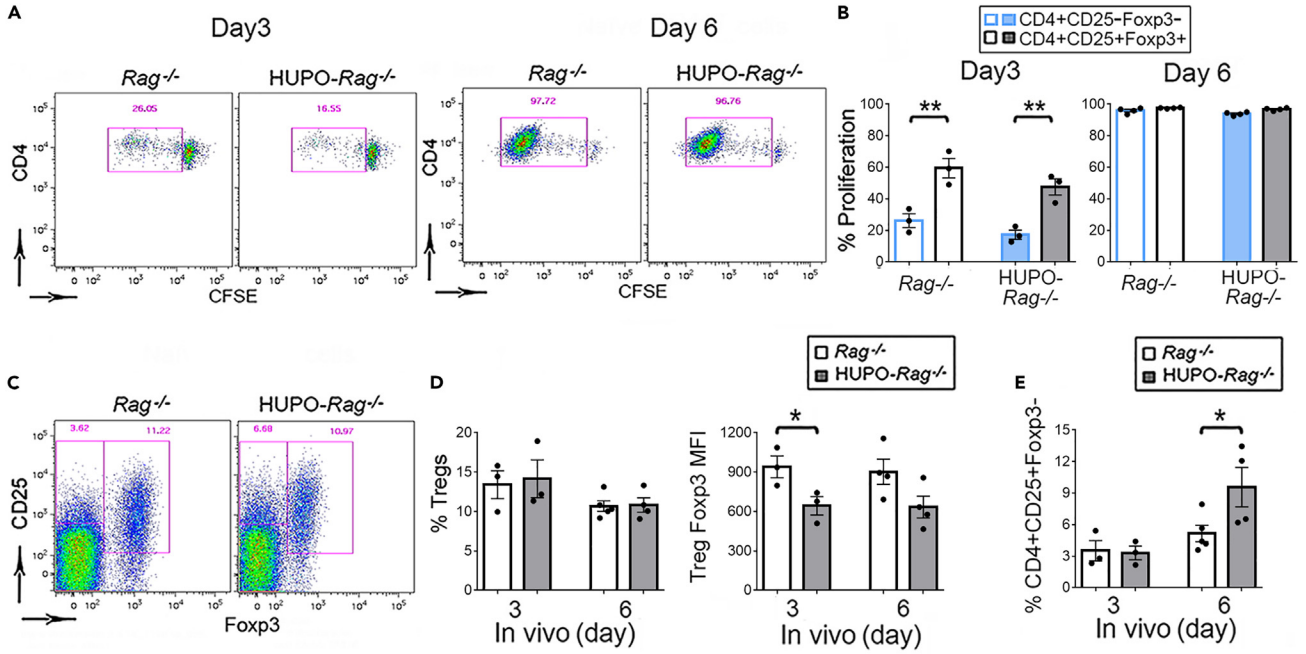

Figure 2. Normal homeostatic proliferation, but Treg instability, of wild-type T cells in HUPO-*Rag*$^{-/-}$ mice

Wild-type CD45.1$^+$CD3$^+$ T cells were purified by negative selection and adoptively transferred into CD45.2$^+$ *Rag*$^{-/-}$ or HUPO-*Rag*$^{-/-}$ recipients. The spleens of recipients were harvested at 3 or 6 days post-transfer.

(A and B) homeostatic proliferation (% proliferation) of donor CD4$^+$CD25$^-$Foxp3$^-$ cells and CD4$^+$CD25$^+$Foxp3$^+$ Tregs was determined by CFSE dilution.

(C) A representative histogram of transferred CD4$^+$ cells gated for CD25 and Foxp3.

(D) Percentage of the Tregs in CD4$^+$ T cells (left panel) and their MFI (right panel).

(E) The percent of CD4$^+$CD25$^+$Foxp3$^-$ cells of total CD4$^+$ T cells. Significance was determined by unpaired 2-sided *t*-test. *represents $p < 0.05$ and ** $p < 0.01$ between the indicated groups. See also Figure S2.

CD4$^+$ cells from HUPO mice (Figure 4A), Treg frequency was normalized by the addition of DCs in a dose dependent fashion (Figures 4B and 4C), while the CD4$^+$CD25$^+$Foxp3$^-$ population was not significantly changed (Figure S4A). Conversely, co-culture with DC-depleted splenocytes did not maintain Tregs nor increase the MFI of Foxp3 (Figure S4B), clearly indicating the importance of DCs in maintaining Tregs in HUPO mice. Although the addition of DCs did not result in a significant increase of IL-2 in the culture supernatants (Figure 4D), neutralizing anti-IL-2 antibodies added to the DC-supplemented HUPO spleen cultures resulted in a reduction of Treg frequency, but had no effect on the per cell-based Foxp3 expression (Figure 4E). In contrast, there was no difference of the CD4$^+$CD25$^+$Foxp3$^-$ population following the addition of DCs plus anti-IL-2 (Figure S4C). These observations suggest that the reduction of DCs in HUPO mice contributed to the reduction of Tregs, at least in part, due to the reduction of IL-2. This interpretation is consistent with prior studies employing wild-type mice that documented the role of DCs, particularly CD8α^+ DCs, interacting with conventional T cells, which produce IL-2, and Tregs promoting Treg homeostasis in an IL-2-dependent fashion *in vivo* and *ex vivo*.[31–34] Nonetheless, our *ex vivo* cultures failed to identify the mechanism for the reduced Foxp3 in HUPO Tregs, possibly due to the relatively short duration of the cultures, consistent with the observation that *in vivo* Foxp3 expression is stable over several weeks even in the absence of IL-2 signaling.[35–37]

Reduced DCs contribute to reduced response to exogenous antigen

Since nTregs respond to undefined self-antigens presented by DC, we examined the processing and presentation of foreign antigens in HUPO mice. First, CFSE-labeled CD4$^+$ ovalbumin-specific transgenic $\alpha\beta$ TCR-positive (OT-II) T cells were added to HUPO or control spleen cultures in the presence of OVA323-339 peptide. Proliferation was significantly reduced in the spleens of HUPO, compared to control, mice (Figure S5A). The proliferation of OT-II T cells was normalized in a dose dependent fashion by the addition of DCs (Figure S5B). Further, proliferation in the cultures without added DCs, correlated with the number of DCs presented in the spleen (Figure S5C). Finally, the *in vivo* ability of HUPO mice to process and present antigen was examined by adoptively transferring CFSE-labeled OT-II T cells into HUPO or littermate

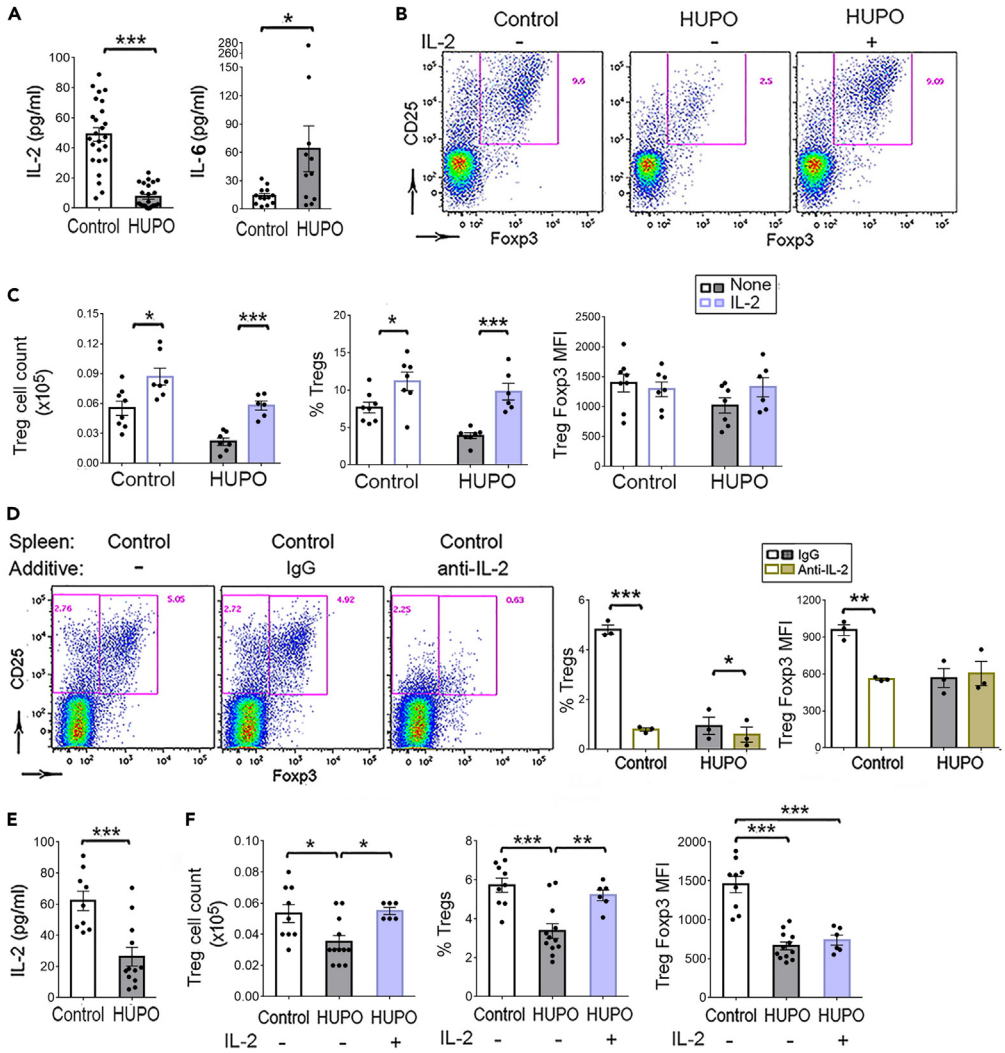

Figure 3. Decreased IL-2, and T cell extrinsic factors, contribute to reduced Tregs in HUPO mice

(A–D) IL-2 maintains HUPO Tregs. Spleen cells from 4-week-old HUPO and littermate control mice were cultured *ex vivo* for 3 days.

(A) IL-2 and IL-6 in the cultural supernatants were measured by ELISA.

(B) Representative flow histograms of $CD4^+$ T cells for CD25 and Foxp3.

(C) Recombinant IL-2 (5 units/mL) or control medium alone (none) were added to the cultures. Number and percent (%) Tregs in $CD4^+$ T cells and Treg Foxp3 expression (MFI) were determined.

(D) Control and HUPO spleen cells were cultured in the presence of neutralizing IL-2 antibody or isotype-matched control IgG. Representative flow histograms from control spleen cells gated on $CD4^+$ cells (left panel), and the percent Treg and Treg Foxp3 expression (right panels).

(E and F) The HUPO spleen milieu contributes to reduce Tregs. Control or HUPO spleens (CD45.1) with $CD4^+$ T cells depleted were co-cultured with purified wild-type $CD4^+$ T cells (CD45.2) for 3 days.

(E) ELISA measured IL-2 in the co-culture supernatants.

(F) The number and percent of $CD4^+$ Tregs and Treg MFI were measured for $CD45.2^+$ cells. Significance was determined by unpaired 2-sided *t*-test for A, C, D, E, and by ANOVA followed by Tukey's pairwise analysis for F. *Represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between the indicated groups.

control mice, followed by the injection of ovalbumin intravenously. Proliferation was significantly reduced in HUPO mice (Figure S5D). Since, we previously demonstrated normal *in vitro* antigen presentation by HUPO DCs, on a per cell basis,[27] these observations demonstrate the *in vivo* consequence of the reduction of DCs in HUPO mice.

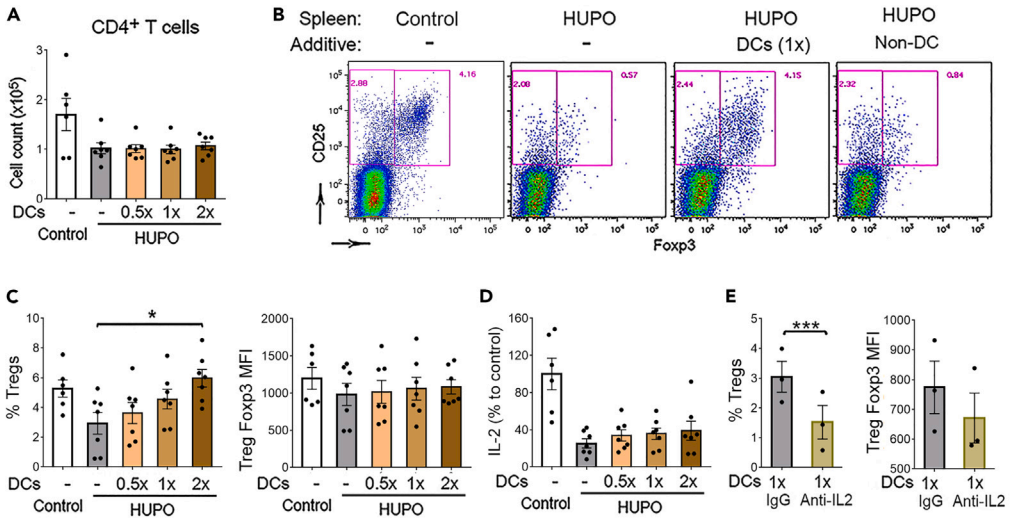

Figure 4. The loss of Tregs, but not Foxp3 destabilization in HUPO spleens is ameliorated by the addition of wild-type DCs

Spleen cells from 4-week-old littermate control mice or HUPO mice (CD45.2$^+$) were cultured alone or the HUPO cells were co-cultured *ex vivo* with wild-type (CD45.1$^+$) DCs (1×10^5 = 1x) for 3 days, followed by analysis of CD45.2$^+$ cells by flow cytometry.

(A) The number of CD45.2$^+$CD4$^+$ T cells.

(B) Representative histograms of control and HUPO CD4$^+$ T cells, gating on CD25 and Foxp3, cultured with wild-type DCs or with spleen cells depleted of DCs (Non-DC).

(C) The percent CD25$^+$CD4$^+$Foxp3$^+$ Tregs in CD4$^+$ T cells and the Treg Foxp3 MFI following the addition of wild-type DCs.

(D) IL-2 in the cultural supernatants measured by ELISA, with control spleen cultures set at 100%.

(E) Percent Tregs and the Foxp3 expression following the addition of DC for HUPO spleens cultured in the presence of neutralizing IL-2 antibody or isotype control IgG. Significance was determined by ANOVA followed by Tukey's pairwise analysis for A, C, and D comparing the 4 HUPO splenocyte cultures with and without DCs added, or by unpaired 2-sided *t*-test for E. *represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between the indicated groups. See also Figures S4 and S5.

Skewed Treg phenotype in HUPO mice

Since cTregs, but not eTregs, are highly dependent on IL-2,[29] each subset was determined in HUPO, compared with age-matched control, mice. Both number and percent of cTregs, were markedly reduced, while the eTregs were increased in the spleens of young and older HUPO mice (Figures 5A and 5B). Consistent with this skewing, inducible T cell co-stimulator (ICOS), which is important for the survival of eTregs was increased on HUPO Tregs (Figure 5C). Helios, a possible marker of thymic origin of Tregs,[38–40] was similar in HUPO mice and controls (Figure S6A). Neurophilin 1 (Nrp1), although not a reliable marker of thymic origin[38,39], but contributing to Treg stability and function through interaction with semaphorin-4a,[41] was reduced in both 4-week-old and $\geq$ 20-week-old HUPO mice (Figure 5D). Other proteins important in Treg function including cytotoxic T lymphocyte antigen 4 (CTLA4)[42] and glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR)[43] were normally expressed on HUPO Tregs, while IL-10 was slightly reduced (Figures S6B–S6D). Together these observations suggest that in HUPO mice a reduction of IL-2 and DCs resulted in a decrease of cTregs, which combined with a reduction of Nrp1, may result in increased Treg instability.

Increased Treg instability in HUPO mice

Since increased Treg apoptosis has been observed in tumors and type 1 diabetes,[44,45] and apoptosis is responsible for the reduction of Tregs over time following the deletion of CD25 in the periphery,[37] spleens from HUPO mice were examined for evidence of apoptosis. No increase of apoptosis of CD4$^+$CD25$^+$ T cells was observed in young HUPO mice and was actually decreased in the older mice (Figure 6A). Treg-specific apoptosis could not be ascertained in this study due to the fixation step required to identify Foxp3. However, since 70–76% of the HUPO CD4$^+$CD25$^+$ cells are Foxp3$^+$, the data demonstrate that apoptosis was not increased in HUPO CD4$^+$CD25$^+$ T cells. In contrast, increased cell death of CD4$^+$CD25$^+$ cells, defined as 7-aminoactinomycin D + (7ADD+), was observed, which was significantly enhanced in older HUPO mice (Figure 6A), suggesting augmented non-apoptotic cell death under chronic inflammatory conditions. In

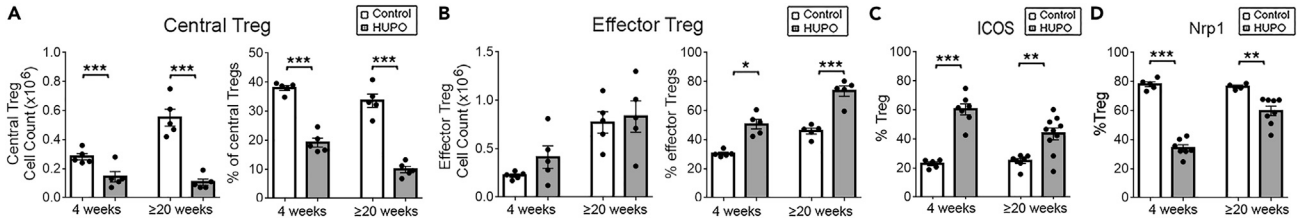

Figure 5. Central Tregs are reduced *in vivo* in young and older HUPO mice

HUPO and littermate control mice at 4 or ≥ 20 weeks of age were examined. Splenocyte Treg phenotype was characterized by flow cytometry.

(A) Central Tregs were defined as $CD44^{lo}$ $CD62^{hi}$ and (B) effector Tregs $CD44^{hi}$ $CD62^{lo}$.

(C) Treg ICOS and (D) Treg Nrp1 expression were determined. Significance was determined by unpaired 2-sided *t*-test. **represents $p < 0.01$ and *** $p < 0.001$ between the indicated groups. See also Figure S6.

order to directly assess Treg stability in HUPO mice, $CD45.1^+CD4^+CD25^+$ wild-type cells, which were 75–80% $Foxp3^+$ (Figure 6B), were adoptively transferred into $CD45.2^+$ mice (Figure 6C). Of the adaptively transferred cells, at 4 and 6 weeks, compared with controls, HUPO mice exhibited a significant reduction of $CD45.1^+CD4^+CD25^+Foxp3^+$ Tregs, without change of the Treg Foxp3 MFI (Figure 6D). Further, increased $CD45.1^+CD4^+CD25^+Foxp3^-$ cells were identified in the HUPO mice (Figure 6E). Together, these observations suggest increased Treg instability *in vivo* in HUPO mice, partially due to conversion to $Foxp3^-$ ex-Tregs, and possibly to non-apoptotic cell death.

IL-2 complexes reduce arthritis in HUPO mice

To examine the *in vivo* relevance of IL-2 to the progression of HUPO arthritis, we injected IL-2 complexes into HUPO mice with arthritis, and wild-type mice, to determine the effect on Tregs and synovitis (Figure 7A). In the control-treated HUPO mice, arthritis (inflammation score) progressed over 6 weeks compared with the baseline, while it was reduced in the IL-2 complex-treated mice at 6 weeks (Figure 7B). At week 3, the Tregs were increased in the control, but not HUPO mice (Figure 7C). However, after 6 weeks, Tregs were increased in both HUPO and control mice (Figure 7D). At both 3 and 6 weeks, there was no IL-2-mediated change in HUPO Foxp3 expression, although it was reduced in the control Tregs, possibly related to enhanced proliferation of conventional $CD4^+$ T cells (Figures 7C and 7D). No significant change in the percentage of cTregs or eTregs was observed after 3 or 6 weeks of therapy (Figure S7). Although histologically inflammation was not significantly reduced, likely due to the relatively short period of observation, at 6 weeks lymphocytes were reduced in the synovial tissue of the IL-2 treated mice (Figures 7E and 7F), consistent with our earlier observation that the adoptive transfer of Tregs suppressed the syngeneic mixed lymphocyte response in HUPO mice.[27] Together, these observations suggest that reduced IL-2 in the milieu of chronic inflammation contributes to Treg instability, which facilitates the progression of joint inflammation in HUPO mice.

DISCUSSION

Previously, we documented a reduction of Tregs in HUPO mice with active arthritis at ≥ 20 weeks of age and that the adoptive transfer of wild-type Tregs suppressed autoreactive T cell proliferation and arthritis.[27] Here, we demonstrate that even before the onset of clinical arthritis and the reduction of Tregs, the expression of Treg Foxp3 was significantly diminished. No defect in Treg homeostatic proliferation was observed. However, *ex vivo* cultures showed that decreased cDCs and IL-2 contributed to the reduction of Treg frequency, although the addition of neither DCs nor IL-2 was sufficient to rescue Treg Foxp3 expression, possibly due to the short term of observation. Further, consistent with a role for IL-2 *in vivo*, cTregs were greatly reduced in HUPO mice.[46] Supporting the effects of reduced cDC *in vivo* in HUPO mice, there was diminished proliferation of adoptively transferred antigen-specific T cells, which was corrected *ex vivo* by the addition of DCs. Since neither the percentage of HUPO thymic Tregs[27] nor the expression of thymic Foxp3 were reduced, our observations suggest that reduced DCs, lead to diminished IL-2, which together resulted in diminished Foxp3 in the periphery, sensitizing Tregs to conversion to ex-Tregs and to non-apoptotic cell death under chronic inflammatory conditions. The treatment of HUPO mice with IL-2 immune complexes resulted in increased Tregs and decreased arthritis.

Our data are consistent with the interpretation that the reduction of Tregs in HUPO mice with arthritis occurred after thymic development. There was no reduction in the percent of Tregs in the thymus or

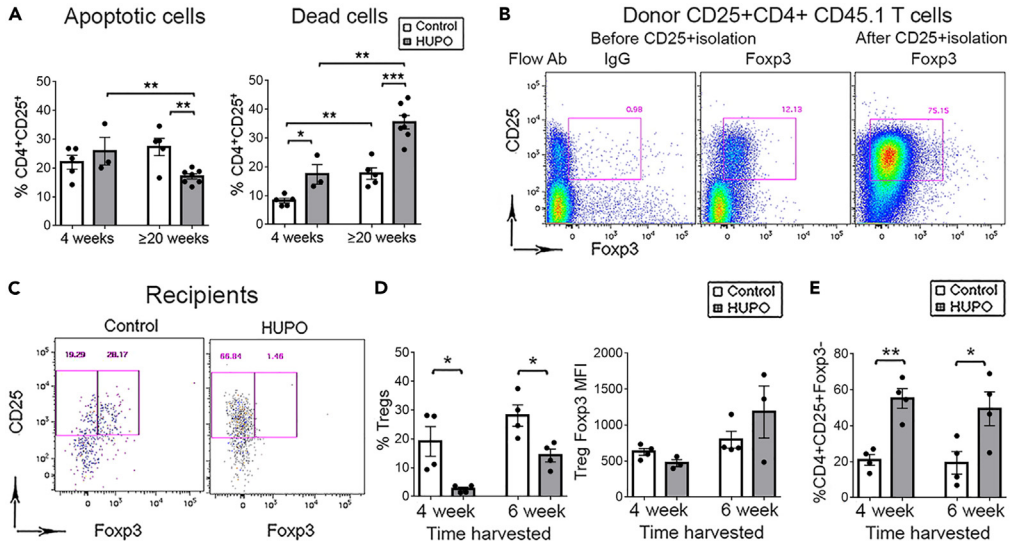

Figure 6. Increased cell death and Treg instability are responsible for the reduction of Tregs in HUPO mice

(A) $CD4^+CD25^+$ T cells in HUPO and littermate control mice at 4 weeks or ≥ 20 weeks were examined. Apoptosis was defined as Annexin V+ and 7AAD- and dead cells as positive for 7AAD.

(B) Wild-type (CD45.1) $CD4^+CD25^+$ donor T cells (~75% were $Foxp3^+$) were isolated and adoptively transferred into 4-week-old $CD45.2^+$ recipients.

(C–E) The $CD45.1^+CD4^+$donor cells present in the recipient spleens were analyzed 4 or 6 weeks post-transfer by flow cytometry.

(D) The percent $CD45.1^+$ Tregs and the Treg MFI and (E) the percent of $CD45.1^+CD4^+CD25^+Foxp3^-$ cells were determined. Significance was determined by ANOVA followed by Tukey's pairwise analysis in panel A, and by unpaired 2-sided *t*-test in panels D-E. * represents $p < 0.05$ and ** $p < 0.01$ between the indicated groups.

periphery at 4 weeks of age, but only later at ≥ 20 weeks of age, when the mice demonstrated extensive arthritis.[27] Nonetheless, the expression of splenic Treg Foxp3 was reduced even at 4 weeks of age, while at the same time point HUPO thymic Treg Foxp3 expression was normal. Under homeostatic conditions, IL-2 is important but not necessary for the induction of Foxp3 during thymic Treg development,[47–50] suggesting that in HUPO mice the reduction of IL-2 contributed to decreased Foxp3 expression in the periphery. Deletion of *IL2ra* does not abolish thymic Treg development, while *IL-2rg−/−* mice and mice deficient in IL-2 and IL-7 or 15 signaling do not generate thymic Tregs, demonstrating that signaling through the IL-2Rγ receptor by IL-2, IL-15, and IL-7, mediated by STAT 5, contribute to thymic Treg development.[49,50] While HUPO mice express less IL-2 in the periphery, they are not deficient, nor are they deficient in IL-7 or -15, likely explaining why thymic Tregs were not reduced.

The absence of DCs or IL-2 results in fatal autoimmunity.[47,51] During homeostasis, the number of Tregs is controlled in a DC-dependent manner.[51] HUPO mice have reduced, not absent DCs, which resulted in diminished IL-2, resulting in reduced Treg Foxp3, which under chronic inflammatory conditions, contributed Treg instability and the reduction of Tregs. Nonetheless, the transfer of the $CD4^+CD25^+$ cells, which were highly enriched in Tregs into HUPO mice, clearly documented enhanced conversion to ex-Tregs. In addition to the maintenance of IL-2, DCs may contribute to Treg stability through direct cell-cell interactions such as CD80/86 with CTLA4, the presentation of self-antigens or the release of mediators such as IL-10, TGFβ or indoleamine 2,3-dioxygenase.[52,53] The very low CD4+ T cells present in ankle synovium limited the further studies for DC and Treg function in the HUPO synovial tissue. Mitigating this concern, the percent Tregs in the lymph nodes draining the inflamed joints was reduced at ≥ 20 weeks of age, similar to those in the spleen at the same age,[27] and the effects of DCs on Tregs in HUPO mice is likely due to systemic changes which may be accurately assessed in the spleens.

The cTregs were reduced and eTregs increased in HUPO mice. Consistent with this observation, reduction of IL-2 signaling in mice with a hypomorphic variant of CD25, also results in reduced cTregs.[54] eTregs are more proliferative and less IL-2 dependent than cTregs, while the survival of cTregs involves IL-2.[29] Additionally, hypostatic proliferation of Tregs, which is independent of IL-2,[46] was normal in HUPO mice. The

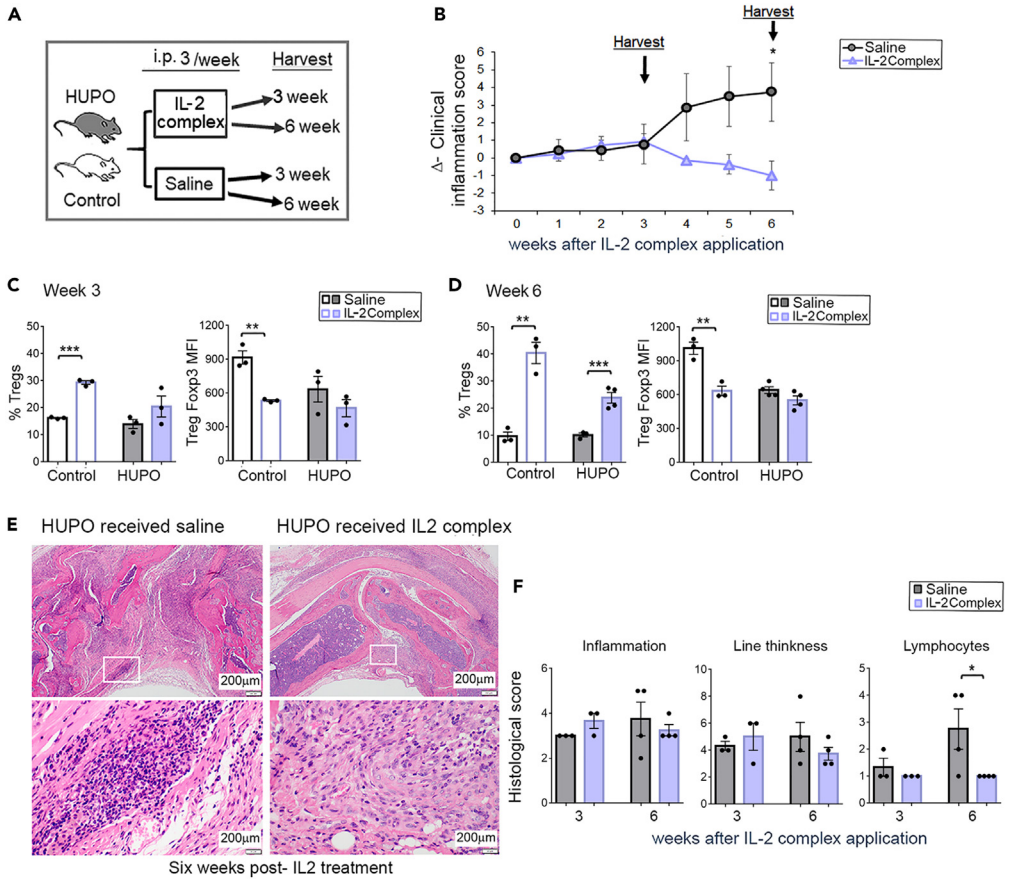

Figure 7. HUPO Tregs increase and arthritis improves following treatment with IL-2/anti-IL-2 complexes

(A) Experimental design for IL-2 complexes or saline administration intraperitoneally (i.p.), to HUPO mice with arthritis or control mice.

(B) Inflammation scores assessed for HUPO mice treated with IL-2 complexes or saline.

(C and D) Spleen Tregs examined 3 or 6 weeks post-IL-2 complex or saline treatment in HUPO and control mice.

(E and F) Histological scoring for HUPO mice treated with IL-2 complexes or saline. The areas in white boxes in upper panels in E are enlarged in panels below. Significance was determined by unpaired 2-sided *t*-test in panels B–D, by ANOVA followed by Bonferroni selected pair post-test in panel F. * represents $p < 0.05$ and ** $p < 0.01$ between the indicated groups. See also Figure S7.

survival of eTregs is enhanced by ICOS, which was increased on HUPO Tregs, which provides anti-apoptotic signals, involving B cell lymphoma 2 (Bcl-2) and myeloid leukemia cell differentiation protein 1 (Mcl-1).[29] The eTreg phenotype may also be enhanced by proliferation due to chronic inflammation.[29] Additionally, $CD4^+Foxp3^-$ T cells may be precursors of pTregs, which may represent about a third of Tregs under homeostatic conditions.[55,56] This population is enriched in $CD62L^{int}$ cells, consistent with the phenotype of eTregs.[29,56,57] Therefore, the conversion of $CD4^+Foxp3^-$ T cells into eTregs, may also contribute to the HUPO eTreg population, since the increase seems unlikely to come from cTregs, which were greatly reduced even at 4 weeks of age.

Treg Foxp3 expression is stable *in vivo* following antibody-mediated inhibition of IL-2 signaling.[35,36] Further, deletion of peripheral Treg CD25 reveals that Treg Foxp3 expression is stable for weeks, but gradually declines.[37] In mice with a hypomorphic mutation of CD25, a modest reduction of Treg Foxp3 is present and cTregs were diminished,[54] similar to HUPO mice. Consistent with the importance of IL-2 and DCs in maintaining Foxp3, T cells transferred into HUPO-*Rag*$^{-/-}$ mice demonstrated normal homeostatic proliferation, but reduced Treg Foxp3 expression and increased $CD4^+CD25^+Foxp3^-$ cells. Additionally, $CD25^+CD25^+$ T cells adaptively transferred into HUPO mice exhibited an enhanced propensity to become $Foxp3^-$. T cell proliferation in the syngeneic mixed lymphocyte response is increased in HUPO mice;

therefore, even though we documented increased Treg instability, we cannot exclude a contribution of *in vivo* activation, in part due to diminished Tregs, as a mechanism contributing to the enhanced $CD4^+CD25^+Foxp3^-$ population. Together, these observations document the delicate balance between DCs, IL-2, Tregs, and inflammation that may lead to progression to chronic, erosive synovitis, in contrast to the fatal autoimmunity that occurs following deletion of DCs, IL-2, or Tregs.

The mechanisms for Treg instability and how it contributes to disease pathogenesis is controversial,[36,58–60] and in mice may even be strain-related.[61] Previous studies demonstrated conversion of Tregs to T_H17 cells or from cTregs to eTregs under inflammatory conditions.[29,30,62] In our study, eTregs were significantly increased in both young and old HUPO mice. Adoptive transfer of $CD25^{lo}$ Tregs demonstrated conversion to T_H17 cells that exacerbated CIA, while $CD25^{hi}$ Tregs were stable phenotypically and functionally.[62] In HUPO mice, $CD25^{lo}Foxp3^+$ cells were not increased (multiple figures). Conversion of Tregs to T_H17 cells was not specifically examined in HUPO mice since T_H17 cells were neither increased in the lymph nodes draining inflamed HUPO joints, nor was IL-17 increased in those joints.[27] A variety of factors have been identified that contribute to Treg instability, including the reduced expression for Nrp-1, which was observed on HUPO Tregs.[41] Inflammation, mediated through a variety of mechanisms, has been shown to result in the reduction of Treg Foxp3 expression and function.[36,59,60] In HUPO mice, the chronic joint inflammation is mediated through inflammatory cytokines such as IL-6, IL-1β, and TNF.[27] The systemic inhibition of Janus kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3) or IL-6 signaling and local nuclear factor kappa B (NF-κB) inhibition, reduced CIA and T_H17 cells, and increased Tregs,[63–65] suggesting that NF-kB and IL-6 signaling contribute to Treg instability. Additionally, the endoplasmic reticulum stress response mediated through hydroxymethyl glutaryl coenzyme A reductase protein 1 (Hrd1) and inositol-requiring enzyme 1α (IRE-1α) may also contribute to Treg instability.[59]

Earlier studies demonstrated that following the deletion of CD25, Tregs die slowly by apoptosis and not conversion to ex-Tregs, under homeostatic conditions.[37] While no increase of HUPO Treg apoptosis was observed, which may be due to inflammation that increases the expression of anti-apoptotic molecules,[66] non-apoptotic cell death of $CD4^+CD25^+$ cells was enhanced. In the current study, we did not further characterize the potential mechanisms of Treg non-apoptotic death, but necroptosis or pyroptosis have been identified in the milieu of chronic inflammation.[67] Consistent with a potential role for pyroptosis, IL-1 is significantly increased in HUPO joints.[27]

Our observations concerning DCs are relevant to RA. Both patients with RA and HUPO mice express increased levels of circulating Flt3L.[27,68] Since reduced Flt3L results in reduced DC, and increased Flt3L increases DCs,[51] these observations are consistent with reduced DC number or function in patients with RA. Further, our observations concerning Tregs support their potential role as a therapeutic target in RA.[69] Circulating Tregs in RA have been reported to be elevated, reduced or normal.[70] Much of this confusion exists because most studies of patients with RA identified Tregs as $CD4^+CD25^+$ in the absence of staining for Foxp3. However, one study employing Foxp3 reported a reduced percentage of Tregs, and increased numbers of $CD4^+CD25^+Foxp3^-$ cells,[71] similar to the findings in HUPO mice. This observation is consistent with Treg instability in RA, although ongoing immune activation is also possible.

There are a number of approaches to enhance Treg stability, number or function. One approach genetically engineers Tregs, which may be infused for therapeutic benefit.[72] Further, low dose IL-2 has been employed to expand Tregs, with a suggestion of therapeutic benefit in variety of autoimmune diseases including RA.[73,74] A phase 2 study suggests therapeutic benefit adding low dose IL-2 to methotrexate in patients with rheumatoid arthritis.[75] Modifications of IL-2 that prolong the half-life and enhance efficiency make this approach more achievable.[74] Our prior studies demonstrate that HUPO-$Rag^{-/-}$ arthritis is initiated in the absence of B or T lymphocytes but does not progress to severe disease,[27] suggesting that the reduction of Tregs is permissive, but is not responsible for the induction of disease. In contrast, absence of Tregs results in a fatal multisystem auto-inflammatory disorder.[76,77] The observations presented here document an important role for Treg instability in the progression of chronic inflammatory arthritis, supporting Treg instability and the IL-2 signaling pathway as potential therapeutic targets.

Limitations of the study

Treg and DC functions within the synovial tissue were not examined. The mechanism responsible for the increase Treg cell death in HUPO mice was not characterized. Adoptive transfer experiments into HUPO

and control mice, employed $CD4^+CD25^+$ cells highly enriched in $Foxp3^+$ Tregs and not specifically purified $Foxp3^+$ Tregs.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse-CD4 (RM4-5)	Invitrogen	Cat# 11-0042-82 RRID: AB_464896
Anti-mouse-CD25 (PC61.5)	Invitrogen	Cat# 17-0251-82 RRID: AB_469366
Anti-mo/Rt FOXp3 (FJK-16s)	Invitrogen	Cat# 12-5773-82 RRID: AB_465936
Anti-mouse-CD8 (53-6.7)	BD Biosciences	Cat# 557654 RRID: AB_369769
Anti-CD11b (M1/70)	BD Biosciences	Cat# 557396, RRID:AB_396679
Anti- mousevCD3e (145-2C11)	Thermo Fisher Scientific	Cat# 61-0031-82, RRID:AB_2574514
Anti-mouse CD19 (1D3)	BD Biosciences	Cat# 557958, RRID:AB_396958
Anti-mouse CD45 (30-F11)	BD Biosciences	Cat# 560510, RRID:AB_1645208
Anti-mouse CD45.1 (A20)	BD Biosciences	Cat# 612811, RRID: AB_2738850
Anti-mouse CD45.2 (104)	BD Biosciences	Cat# 561874, RRID: AB_10894189
Anti-mouse CD62L (MEL-14)	BD Biosciences	Cat# 553152, RRID:AB_398533
Anti-mo-CD44 (IM7)	BD Biosciences	Cat# 553133, RRID: AB_2076224
Anti-mouse Vα2 ((B20.1)	BD Biosciences	Cat# 560623, RRID: AB_172783
Anti-mouse IL10 (JES5-16E3)	BioLegend	Cat# 505035 RRID: AB_2566030
Anti-mouse Nrp1(Neuroplilin-1, 3E12)	BioLegend	Cat# 145207 RRID: AB_2562033
Anti-mouse CD152 (CTLA4, UC10-4B9)	BioLegend	Cat# 106315 RRID: AB_2564473
Anti-mouse Helios (22F6)	eBioscience	Cat# 47-9883-41 RRID: AB_2573997
Anti-mouse CD278 (ICOS, 7E.17G9)	eBioscience	Cat# 25-9942-80 RRID: AB_2573563
Anti-mouse CD357 (GITR, DTA-1)	BioLegend	Cat#126317 RRID: AB_2563385
Anti-mouse IL2	Thermo Fisher Scientfic	Cat# 16-7022-85 RRID: AB_469207
PE-Annexin V	BD Pharmingen	Cat# 556421
7-AAD	BD Pharmingen	Cat# 559925
Chemicals, peptides, and recombinant proteins		
Collagenase D	Sigma-Aldrich	Cat#11088882001
CFSE Cell proliferation kit	ThermoFisher Scientfic	Cat# C34570
Live/Dead Fixable Aqua Dead Cell Stain Kit	Invitrogen	Cat# L34957
Recombinant Murine IL-2	PeproTech	Cat# 212-12
$OVA_{323–339}$ peptide	EZ Biolab	Cat# cp 7210
Ovalbumin	Thermo Fisher Scientific	Cat# 77120
Critical commercial assays		
EasySep Mouse CD90.2 positive selection kit	Stem Cell Technologies	Cat#: 18951
EasySep Mouse $CD4^+$ T cell isolation kit	Stem Cell Technologies	Cat#: 19852

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD25 MicroBead Kit, mouse	Miltenyi Biotec	Cat#: 130-091-072
CD11c MicroBead UltraPure, mouse	Miltenyi Biotec	Cat#: 130-125-835
Mouse IL-2 DuoSet ELISA	R&D System	Cat# DY402-05
Mouse IL-6 DuoSet ELISA	R&D System	Cat# DY406-05
Annexin V Binding Buffer (10x)	BD Pharmingen	Cat# 556454
Foxp3/transcription factor staining buffer set	Invitrogen	Cat: 00-5523-00
Experimental models: Organisms/strains		
Mouse: HUPO: *Flip*$^{flox/flox}$*CD11c*^{cre}	Huang et al. 2015 Nature Communications[27]	N/A
Mouse: CD11c-Cre-GFP line 4097: Tg(Itgax-cre,-EGFP)4097Ach	The Jackson Laboratory	Cat# JAX 007567; RRID:IMSR_JAX:007567
Mouse: B6 CD45.1: B6.SJL-Ptprca Pepcb/BoyJ	The Jackson Laboratory	Cat# JAX 002014; RRID:IMSR_JAX:002014
Mouse: RAG2 KO: B6.Cg-Rag2tm1.1Cgn/J	The Jackson Laboratory	Strain#:008449 RRID:IMSR_JAX:008449
Mouse: OTII: B6.Cg-Tg(TcraTcrb)425Cbn/J	The Jackson Laboratory	Strain #:004194 RRID:IMSR_JAX:004194
Oligonucleotides		
Primer: Flip Forward: CTTTTGGCTTTTGGATCAGTCATT	Integrated DNA Technologies	N/A
Primer: Flip Reverse: GGAACCACGAGAAGCCAACAT	Integrated DNA Technologies	N/A
Primer: Flip deleted: TCTCTAACTCATTCAGCTTCA ACCACCA	Integrated DNA Technologies	N/A
Primer: Cre Forward: CGAACATCTTCAGGTTCTGCGG	Integrated DNA Technologies	N/A
Primer: Cre Reverse: GTCGATGCAACGAGTGATCAG	Integrated DNA Technologies	N/A
Software and algorithms		
FlowJo v9.9.4	Tree Star Inc	https://www.flowjo.com
Other		
LSR II Flow Cytometer	BD Bioscience	N/A
FACSAria III Cell Sorter	BD Bioscience	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Richard M. Pope (rmp158@northwestern.edu).

Materials availability

The materials used in this study are shown in the key resources table. Any materials generated in this study will be made available, without restriction. Sperm from the HUPO mouse lines generated as part of this study have been cryopreserved at the Northwestern University Transgenic and Targeted Mutagenesis Facility, under the name of lead contact Richard M. Pope and will be made available upon request following execution of a material transfer agreement.

Data and code availability

- There is no original code associated with this paper.
- The data presented in this manuscript will be made available upon request by the lead contact.

- Any additional information required to reanalyze the data reported in this paper will be made available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse lines

We generated a mouse line with FLIP deficient in CD11c$^+$ cells (CD11c-Flip-KO), aka HUPO mice on a C57BL/6 background, genotyped as *Flip*$^{flox/flox}$*CD11c*^{cre}. HUPO mice are generated by crossing *Flip*$^{flox/flox}$ mice with CD11c-Cre-GFP transgenic mice (CD11c-Cre-GFP line 4097, Jackson stock 007567).[26,27] Control mice were littermates or age and gender matched mice without *Flip* genomic deletions, genotyping as *Flip*$^{flox/+}$CD11c^{cre} or *Flip*$^{flox/flox}$*CD11c*$^+$. We generated HUPO-*Rag*$^{-/-}$ mice line by crossing HUPO with *Rag*$^{-/-}$ mice (*RAG2 KO,* Jackson stock 008449). The HUPO or control mice on C57BL/6 CD45.1$^+$ background were generated by crossing with CD45.1 congenic strain (B6 CD45.1, Jackson stock 002014). OTII TCR-transgenic mice are from Jackson Laboratory (OTII, Jackson stock 004194). All mice were confirmed by PCR genotyping employing genomic DNA extracted from tail biopsies. Also, the CD45.1 or CD45.2 backgrounds were determined by blood flow cytometry employing antibodies to CD45.1 or CD45.2. All mice were breed on the C57Bl/6 background. All mice were bred and maintained in Northwestern University barrier animal facility and all procedures followed ethical guidelines and approved by Northwestern IACUC.

Experimental groups

HUPO or control mice age at ≥20 weeks old with arthritis and at 4-5 weeks old were used in this study unless otherwise stated. HUPO mice developed spontaneous arthritis beginning at 6 weeks of age, identified as swollen and deformed joints. Arthritis frequency and severity increased through 5 months, with no difference in incidence or severity between females and males which were both employed for these studies. For OTII TCR-transgenic mice, only males were used in experiments.

METHOD DETAILS

Sample preparation and flow cytometric immunophenotyping

Spleens, thymus, and lymph nodes, which include cervical, branchial, axillary, inguinal and popliteal lymph nodes, were combined and called mixed lymph nodes. All single cell suspensions were prepared following collagenase D (1 mg/ml) and DNase I (0.1 mg/ml) digestion for 60 min at 37°C.[27] The released cells were filtered through 40 mm nylon mesh employed for antibodies staining. Blood (100 μl) was drawn by cardiac puncture immediately after euthanizing and employed for antibodies staining.

Cell types were determined by flow cytometry employing multi-color fluorochrome-conjugated antibodies to cell surface markers, such as CD45, CD11b, CD3, CD4, CD8, CD19 and CD25 to define T or B cells. T regulatory cells (Tregs) are defined as CD4$^+$CD25$^+$and positive for the intracellular expression of Foxp3. Tregs were analyzed by their absolute number, frequency in CD4$^+$ T cells, their Foxp3 expression by mean florescence intensity (MFI). As described in the text, Tregs were analyzed for cell surface Helios, Nrp1, CTLA-4, GITR, ICOS, CD44, CD62L and intracellular IL10. CD4$^+$CD25$^+$ T cell apoptosis were determined as Annexin V+ and 7ADD-, while cell death was defined as 7AAD +. Data was acquired employing BD LSR II flow cytometer (BD FACSDIVA software) and analyzed by Flowjo (TreeStar, Inc.).

Cell isolation

The CD3$^+$ T cells or CD4$^+$ T cells were purified by positive or negative enrichment utilizing isolation kits from Stem Cell Technologies, as described for each experiment. CD4$^+$CD25$^+$ T cells were purified by CD25 positive isolation kits from Miltenyi. Total Dendritic cells were isolated by CD11c positive isolation (Miltenyi). All isolations were performed according to manufacturers' instructions. The purity of each cell type was documented by flow cytometry.

In vitro characterization of Treg

Unless otherwise stated, *ex vivo* cell cultures were performed employing RPMI 1640 medium supplemented with 10% (v/v) FBS, 100U pen-strep and 0.1% (v/v) 2-ME, at 37°C in 5% CO2 incubator. Experiments to characterize T cells and Tregs were performed employing the following experimental designs: 1) The whole spleen cell suspensions from 4-week HUPO and littermate mice were cultured at 4x10^6 total spleen

cells/ml medium, with or without supplemental recombinant IL-2 at 5 units/ml for 3 days. 2) Spleen $CD4^+$ T cells were isolated from $CD45.2^+$ control mice by negative selection. $CD4^+$ T cell depletion from whole spleen cells was performed by CD90.2 positive selection from $CD45.1^+$ HUPO or littermate control mice. The purified wild-type $CD4^+$ T cells ($4x10^5$) were co-cultured with the T cell depleted whole spleen cells ($3.6x10^6$/ml), with or without supplemental recombinant IL-2 (5 units/ml) for 3 days. 3) Whole spleen cells from 4 week $CD45.2^+$ HUPO mice ($4x10^6$ total spleen cells) were co-cultured with various numbers of wild-type DCs, or same number of DC-depleted spleen cells (CD45.1) for 3 days. 4) Whole spleen cells from 4 week HUPO mice ($4x10^6$ total spleen cells) were cultured with wild-type DCs for 3 days in the presence of neutralizing IL-2 antibody or isotype matched IgG. Supernatants, where indicated, were harvested and tested for IL-2 or IL-6 by ELISA (BD biosciences). Cells from all the cultures were analyzed by flow cytometry for T cells and Tregs.

Treg homeostatic proliferation *in vivo*

T cell homeostatic proliferation *in vivo* were determined in CD45.2 HUPO or control mice or HUPO or littermate recipients on *Rag-/-* background. The $CD3^+$ T cells were purified from CD45.1 mice by negative selection (StemCell Technologies) and CFSE labelled. Adoptive cell transfers were performed by intravenous administration into $CD45.2^+$ cells ($1x10^7$/200 μl) into recipients. Three to 6 days after transfer, spleens were harvested and T cells analyzed by flow cytometry for homeostatic proliferation by CFSE dilution (Life Technologies),[27,78] and to identify Tregs and Treg Foxp3 expression.

Antigen processing and presentation

$CD4^+$ T cells were isolated from the spleens of OTII mice by negative selection (StemCell Technologies) and CFSE-labelled. T cell proliferation were performed by co-culture the CFSE-labelled $CD4^+$ OTII ($1x10^5$) T cells with the total spleen cells from control or HUPO mice ($0.5x10^6$) in the absence or presence of $OVA_{323–339}$ peptide (EZ Biolab, 0.1 μg/ml). In some experiments, wild-type DCs were added to the HUPO spleen cell cultures. For *in vivo* antigen processing and presentation, CFSE-labelled $CD4^+$ OTII T cells were adoptively transferred into control or HUPO mice by i.v. administration ($5x10^6$/mouse) followed by ovalbumin (25 μg/mouse) i.v. administrated 24hours afterward. The spleens of recipients were harvested 3 days post-ovalbumin injection and prepared for flow analyzing. Antigen-mediated proliferation *in vitro* or *in vivo* was determined by flow cytometry, examining CFSE dilution of $CD4^+V\alpha2^+$ OTII T cells.

The Treg adaptive transfer

The $CD45.1^+$ $CD25^+$ wild-type Tregs were purified by $CD25^+$ isolation and adoptively transferred into $CD45.2^+$ HUPO or control mice. The recipient spleens are harvested at 4 or 6 weeks post-transfer. The donor Treg were analyzed by flow cytometry.

Histologic analysis

Mouse interphalangeal joints of the hind ankles were employed for histologic analysis. Ankles were fixed by 10% formalin and decalcified in 7% EDTA/10% formalin (w/v) for 2-3 weeks. After processing and paraffin embedding, sections were stained with hematoxylin and eosin (H&E), performed by Northwestern Mouse Histology and Phenotyping Laboratory. The H&E slides were scored on a scale of 0–5 for inflammation, bone erosion/pannus formation, median synovial lining thickness, neutrophil and lymphocyte infiltration, and cartilage destruction, as described.[27] All histology was scored by a pathologist blinded to the experimental design. Images were acquired by NIKON microscope (Nikon Inc.), with SPOT5.2 software (Spot imaging. Sterling Heights, Michigan).

Evaluation of arthritis and IL-2 complex treatment of HUPO mice

HUPO mice spontaneously developed arthritis.[27] The incidence and severity of arthritis was assessed by clinical scoring.[27,79,80] The clinical score was quantitated from the sum of joint swelling/inflammation (graded 0-3/each limb) and joint deformity (including toe flexion, contraction and shortening, 0-3/each limb), and the grip strength (0-4), the maximum score being 28. HUPO mice $\geq$ 20 weeks old with arthritis (inflammation score 2-6) were divided into two groups matched for age, weight, sex and inflammatory score. The mice received either IL2/anti-IL2 complex (1 μg of murine IL2 (PeproTech) with 5 μg of anti-mouse IL-2 (ThermoFisher)) in 200μL of saline per mouse or saline alone as the control. Injection was administered intraperitoneally three days each week. Three mice in each group were euthanized at week 3 and the rest at 6 weeks. At the time of termination, spleens were examined for T cells and Tregs by flow

cytometry and ankles were analyzed by histology utilizing paraffin embedded sections stained with hematoxylin and eosin.[5,6] Littermate or age and sex matched control mice were received the same treatments as the HUPO mice, and their spleens were also analyzed by flow cytometry.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed in Excel or GraphPad Prism. All quantitative data are presented as mean ± SEM. Statistical analysis between two groups was performed with unpaired 2-tailed Student's *t* test. For multiple comparisons, one way ANOVA followed by Tukey's pairwise mean comparison for multi-group analysis, or by the Bonferroni selected pair post-test. Correlations were determined by Pearsons's linear correlation. Significance levels were set at 0.05.