IL-6 receptor blockade corrects defects of XIAP-deficient regulatory T cells

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X-linked lymphoproliferative syndrome type-2 (XLP-2) is a primary immunodeficiency disease attributed to XIAP mutation and is triggered by infection. Here, we show that mouse Xiap−/− regulatory T (Treg) cells and human XIAP-deficient Treg cells are defective in suppressive function. The Xiap−/− Treg cell defect is linked partly to decreased SOCS1 expression. XIAP binds SOCS1 and promotes SOCS1 stabilization. Foxp3 stability is reduced in Xiap−/− Treg cells. In addition, Xiap−/− Treg cells are prone to IFN-γ secretion. Transfer of wild-type Treg cells partly rescues infection-induced inflammation in Xiap−/− mice. Notably, inflammation-induced reprogramming of Xiap−/− Treg cells can be prevented by blockade of the IL-6 receptor (IL-6R), and a combination of anti-IL-6R and Xiap−/− Treg cells confers survival to inflammatory infection in Xiap−/− mice. Our results suggest that XLP-2 can be corrected by combination treatment with autologous iTreg (induced Treg) cells and anti-IL-6R antibody, bypassing the necessity to transduce Treg cells with XIAP.

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XIAP-deficiency decreases Foxp3 stability in Treg cells. We next examined why the inhibitory activities of XIAP−/− Treg cells were defective. XIAP−/− Treg cells were stimulated through TCR/CD28 in the presence of IL-2, and the expression of Foxp3 was determined. Total numbers of Foxp3+ T cells were reduced in specific genes can be corrected by re-introduction of the respective wild-type gene.

In the present study, we demonstrate that XIAP is required for the suppressive function of Treg cells. XIAP-deficient Treg cells are ineffective in inhibiting inflammation. SOCS1 expression is reduced in XIAP-deficient Treg cells. XIAP promotes SOCS1 K63 ubiquitination and maintains SOCS1 protein stability. Transfer of wild-type Treg cells partly suppresses infection-induced inflammation in XIAP−/− mice. Moreover, a combination of XIAP−/− Treg cells and anti-IL-6R corrects the vulnerability of XIAP−/− mice to infection. Our results provide evidence of a mechanism underlying the generation of XLP-2 syndromes in XIAP mutant patients. Furthermore, we demonstrate the therapeutic feasibility of combining autologous Treg cells and anti-IL-6R for the treatment of primary immunodeficiency diseases such as XLP-2.

Results

Impaired inhibitory activity of XIAP−/− Treg cells. A previous study showed that XIAP-deficiency did not affect the development of iTreg cells and that the frequency of CD4+Foxp3+ cells was comparable between XIAP−/− mice and littermate control (WT) animals (Fig. 1a)15. Generation of iTreg cells was similar between WT and XIAP−/− naive CD4+CD25+ T cells when and dosage of TGF-β were optimized (Fig. 1a). We also determined the expression of several Treg cells-associated molecules in isolated WT and XIAP−/− iTreg cells (Supplementary Figure 1) and found that expressions of CTLA-4, GITR, LAG3, and FR4 were comparable between WT and XIAP−/− iTreg cells (Fig. 1b).

Production of IL-10 and TGF-β in XIAP−/− iTreg cells was indistinguishable from that in WT iTreg cells (Fig. 1c). Despite having normal phenotypes, XIAP−/− iTreg cells were less effective than WT Treg cells in suppressing the proliferation of CD4+CD25+ effector T cells (Fig. 1d). Inhibition of CD4+CD25+ T cell activation by XIAP−/− iTreg cells was also compromised (Supplementary Figure 2). Since XIAP is an anti-apoptotic and signaling protein, the proliferation and viability of XIAP−/− Treg cells were examined and found to be comparable with WT iTreg cells (Supplementary Figure 3). We also used human XIAP-knockdown Treg cells to mimic Treg cells of XLP-2 patients (Fig. 1c). The differentiation of human XIAP-deficient iTreg cells was similar to that of control iTreg cells (Supplementary Figure 4). However, the suppressive activity of human XIAP-knockdown iTreg cells was impaired relative to control iTreg cells (Fig. 1f, Supplementary Figure 5).

XIAP−/− iTreg cells also displayed a diminished capacity to suppress T cell activation in an in vivo functional assay. Colitis was induced in Rag1−/− mice by administration of CD4+CD25+ T cells, leading to body weight loss (Fig. 1g), rectal prolapse and diarrhea. Tissue sections of inflamed colon revealed inflammatory infiltrate, crypt cell damage and goblet cell damage (Fig. 1h, Telf).

Co-administration of WT Treg cells effectively suppressed the induction of colitis by CD4+CD25+ T cells. In contrast, co-administration of XIAP−/− Treg cells did not prevent the colitis-associated pathogenesis (Fig. 1g and h), suggesting that the absence of XIAP greatly diminished the suppressive activities of Treg cells in vivo. Therefore, even though XIAP is not involved in the development of iTreg cells or iTreg cell differentiation, XIAP is essential for the suppressive function of Treg cells in vitro and in vivo.
Xiap−/− iTreg cells (Fig. 2a and b). We also determined Foxp3 levels in the adoptively transferred CD45.1+tTreg cells isolated from Rag1−/− mice (Fig. 1f). Figure 2c illustrates that the Foxp3high fraction in Xiap−/− iTreg cells activated in vivo were lower than those in WT iTreg cells. Quantitation of the Foxp3+ population confirmed a nearly two-fold reduction in the Foxp3high fraction in Xiap−/− iTreg cells (Fig. 2d). Thus, XIAP-deficiency decreases Foxp3 stability in Treg cells in vitro and in vivo.

XIAP interacts with SOCS1 and increases SOCS1 expression. A recent report indicated that SOCS1 is essential for Foxp3 stability and its suppressive function.36 We examined IL-2-stimulated SOCS1 expression in WT and XIAP-deficient T cells. SOCS1 was decreased in IL-2-treated Xiap−/− T-cells (Fig. 3a). XIAP-knockdown also decreased SOCS1 levels in human iTreg cells (Fig. 3b). IL-2-induced Socs1 transcript levels in T cells were not affected by XIAP-deficiency (Supplementary Figure 6a). In contrast, the protein stability of SOCS1 was dependent on the presence of XIAP (Supplementary Figure 6b), which is supported by the enhanced SOCS1 expression under increased levels of XIAP (Fig. 3c) and that XIAP knockdown in human iTreg cells increased cycloheximide-induced SOCS1 degradation (Supplementary Figure 6c). These results suggest that XIAP regulates SOCS1 expression by maintaining SOCS1 protein stability.

We found an association between SOCS1 and XIAP. Immunoprecipitation of SOCS1-HA brought down XIAP-FLAG (Fig. 3d), and precipitation of endogenous SOCS1 pulled down endogenous XIAP in T cells (Fig. 3e). XIAP consists of N-terminal baculovirus IAP (BIR) 1, BIR2 and BIR3, as well as...
a C-terminal really interesting new gene (RING)-finger domain. Using different truncated forms of FLAG-tagged XIAP, we mapped the BIR1 domain of XIAP as being the SOCS1-interacting region (Fig. 3f). For SOCS1, which comprises an N-terminus, a central Src homology 2 (SH2) domain and a C-terminal SOCS-BOX domain, we found the SH2 domain to be the XIAP-binding region (Fig. 3g).

**XIAP promotes SOCS1 K63 ubiquitination.** Previous reports have found that SOCS1 is associated with the Elongin B/C complex, which functions as an E3 ligase. Immunoprecipitation of overexpressed Elongin B/C brought down SOCS1-HA (Fig. 4a). Notably, co-expression of full-length XIAP-FLAG increased the association of SOCS1-HA with the Elongin B/C-Myc complex (Fig. 4a). By contrast, ΔRING-XIAP did not enhance association of SOCS1 with Elongin B/C (Fig. 4a). We also determined whether XIAP promoted SOCS1 poly-ubiquitination. Co-expression of XIAP enhanced the addition of WT ubiquitin or K63 ubiquitin, but not K48 ubiquitin, to SOCS1 (Fig. 4b). In an in vitro ubiquitination analysis, addition of recombinant XIAP (but not XIAPPARF) to reaction mixtures containing ubiquitin, E1, E2 (UBC13), Elongin B/C and recombinant SOCS1 increased K63 ubiquitination of SOCS1 (Fig. 4c). Together, these results suggest that XIAP binds SOCS1 and promotes SOCS1 K63 polyubiquitination, likely contributing to the increased protein stability of SOCS1.

**XIAP−/− Treg cells are prone to IFN-γ and IL-17 production.** We examined whether reduced SOCS1 in XIAP−/− Treg cells conferred on them the susceptibility to produce inflammatory cytokines, similar to Socs1−/− Treg cells. Foxp3-GFP tagged WT and XIAP−/− tTreg cells, isolated by GFP expression, were activated by CD3/CD28 plus IL-2, with the additional presence of IL-12 or IL-1α/IL-1β/IL-6. We found a smaller increase in IFN-γ expression for XIAP−/− tTreg cells after TCR re-stimulation (Fig. 5a). The presence of IL-12 substantially increased the fraction of IFN-γ-expressing XIAP−/− tTreg cells relative to WT tTreg cells (Fig. 5a and b), and elicited a significant increase in IFN-γ secretion by reactivated XIAP−/− tTreg cells (Fig. 5c). Similarly, human XIAP-knockdown iTreg cells generated more IFN-γ after IL-12 co-stimulation than WT iTreg cells (Fig. 5d and e). Co-treatment with IL-6 also led to enhanced production of IFN-γ in human XIAP-deficient tTreg cells (Fig. 5d).

Enhanced generation of IL-17 was also detected in XIAP−/− tTreg cells compared to WT tTreg cells after being co-stimulated with TCR and IL-6/IL-1 (Fig. 5f). We further examined the cytokine profile of tTreg cells isolated from Rag1−/− mice after induction of colitis by effector T cells. The recovered CD45.2+ T cells, representing the transferred tTreg cells, were assessed for expression of IFN-γ and IL-17A (Fig. 5g and h). Increased IFN-γ and IL-17A expression was observed in XIAP−/− Treg cells isolated from Rag1−/− mice, illustrating the enhanced plasticity of XIAP−/− tTreg cells in vivo.

**Increased STAT1 and STAT3 activation in XIAP−/− Treg cells.** Inflammatory cytokines including IL-12, IL-1, and IL-6 trigger JAK-STAT signaling in T cells. SOCS1 is a negative regulator of JAK-STAT signaling, so we determined whether the reduced SOCS1 in XIAP−/− iTreg cells led to an enhanced response to inflammatory cytokines. WT and XIAP−/− iTreg cells were stimulated with IL-12 or IL-1 plus IL-6, and we found increased phosphorylation of STAT1 or STAT3 in XIAP−/− iTreg cells relative to WT iTreg cells, in the context of comparable levels of STAT1 and STAT3 (Supplementary Figure 7a and b). Therefore, XIAP deficiency leads to enhanced activation of Treg cells in

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**Fig. 2** Foxp3 instability in activated Xiap−/− Treg cells. **a, b** Reduced Foxp3+ population in activated Xiap−/− tTreg cells. WT and Xiap−/− tTreg cells were stimulated with anti-CD3/CD28 in the presence of IL-2 for 4 days. Foxp3 expression of activated tTreg cells was determined by intracellular staining and analyzed by flow cytometry. The gated section represents the Foxp3+ population and the number indicates the percentage of each population (a). The Foxp3+ fractions in WT and Xiap−/− tTreg cells were quantitated (b), n = 5. **c, d** Diminished Foxp3high population in adoptively transferred Xiap−/− tTreg cells. CD45.2+ tTreg cells were recovered from CD45.1+ Rag1−/− mice into which CD45.2+ tTreg cells and CD45.1+ CD4+CD25− T cells had been transferred a month earlier. The isolated CD45.2+ CD4+ T cells were reactivated with TPA/A23187 and Foxp3 expression was determined by intracellular staining (c). The Foxp3+ fractions in the transferred WT and Xiap−/− tTreg cells re-isolated from Rag1−/− mice were quantitated (d), n = 9. *P < 0.05 for unpaired t-test.
response to inflammatory cytokines, similar to Sox1−/− Treg cells. Since SOCS1 inhibits the activation of IL-2-STAT5, IL-2-induced phosphorylation of STAT5 was also increased in Xiap−/− iTreg cells (Supplementary Figure 8).

**Introduction of SOCS1 corrects defects in Xiap−/− Treg cells.**

This next examined whether expression of SOCS1 restored the function of Xiap−/− Treg cells. HA-SOCS1 was re-introduced into Xiap−/− Treg cells by retroviral transduction (Supplementary Figure 9a). Expression of HA-SOCS1 restored the impaired activity of Xiap−/− Treg cells (Supplementary Figure 9b). The enhanced production of IFN-γ stimulated by IL-12 or IL-2/IL-1, as well as the increased generation of IL-17 activated by IL-1/IL-6, in Xiap−/− Treg cells was suppressed by the re-introduction of HA-SOCS1 (Supplementary Figure 9c and d). Expression of SOCS1 also increased FOXP3 in Xiap−/− iTreg cells to levels comparable to WT iTreg cells (Supplementary Figure 9e), indicating enhanced Foxp3 stability. IL-12-mediated IFN-γ production in Xiap−/− Treg cells was similarly inhibited by SOCS1 re-introduction (Supplementary Figure 9f). Altogether, these results suggest that SOCS1 is one of the major functional targets of XIAP in Treg cells and that re-introduction of SOCS1 corrects the functional defects and plasticity of Xiap−/− iTreg cells.

**WT Treg cells rescue Xiap−/− mice from inflammatory death.**

It has previously been demonstrated that Xiap-deficient mice are highly susceptible to infection by selective pathogens such as *C. albicans*. With resulting syndromes analogous to XLP-2. To determine whether impaired Xiap−/− Treg cells activity contributed to the sensitivity of Xiap−/− mice to infections, we analyzed Treg cells isolated from mice infected with *C. albicans.*
XIAP increases the association of Elongin B/C with SOCS1 and promotes SOCS1 K63 ubiquitination. a XIAP increases the association of Elongin B/C with SOCS1. Full-length, ΔRING (ΔR) or C-terminal (C) XIAP-FLAG was co-transfected with SOCS1-HA and Elongin B/C-Myc into HEK293T cells. Elongin B/C-Myc in cell lysates was pulled down by anti-Myc and the presence of SOCS1 and XIAP in the precipitates and lysates was determined by Western blotting. SOCS1-HA was pulled down from the rest of the reaction mixture and ubiquitination of the SOCS1 complex was determined. Experiments were repeated three times (a). b XIAP enhances the addition of K63 ubiquitin to SOCS1 in vitro. Recombinant K63 ubiquitin-HA, E1, E2 (UBC13), SOCS1-HA (on Mag beads), Elongin B/C-Myc, or XIAP-FLAG was co-transfected with SOCS1-HA and Elongin B/C-Myc into HEK293T cells. Elongin B/C-Myc (EloB/C) or SOCS1 (SOCS1) were immune-precipitated, and the association of ubiquitin type determined. c XIAP promotes SOCS1 K63 poly-ubiquitination in vitro. Recombinant K63 ubiquitin-HA, E1, E2 (UBC13), SOCS1-HA (on Mag beads), Elongin B/C-Myc, or XIAP-FLAG was added as indicated to a ubiquitination reaction conducted at 30 °C for 1 h. Around 10% of the reaction mixture was used for Western blotting. SOCS1-HA was pulled down from the rest of the reaction mixture and ubiquitination of the SOCS1 complex was determined. Experiments were independently repeated three times (a, c) or twice (b).

Low-dose (1 x 10^5) C. albicans infection killed Xiap^-/- mice after 10 days, but did not affect the viability of most WT mice. We isolated Treg cells from WT and Xiap^-/- mice 10 days after C. albicans infection. CD4^+Foxp3^+ cell frequency was comparable between infected WT and Xiap^-/- mice (Fig. 6a). However, the fractions of IFN-γ^+Foxp3^- and IL-17A^-Foxp3^- Treg cells in Xiap^-/- mice were higher than those in WT mice (Fig. 6a), suggesting an increased conversion of Xiap^-/- Treg cells to IFN-γ^- and IL-17-secreting cells after C. albicans infection.

To determine whether impaired Treg cells activity contributed to the sensitivity of Xiap^-/- mice to infections, WT iTreg cells were transferred into Xiap^-/- mice after C. albicans infection. Administration of WT iTreg cells 2 days after low-dose C. albicans infection partly rescued the survival of Xiap^-/- mice; 60% of infected Xiap^-/- mice that received WT iTreg cells survived 40 days after infection, whereas all untreated Xiap^-/- mice had died by 18 days post-infection (Fig. 6b). WT iTreg cells transfer also alleviated kidney inflammation in infected Xiap^-/- mice, as demonstrated by diminished kidney neutrophil infiltration (Fig. 6c). In addition, the elevated levels of serum inflammatory cytokines in untreated infected Xiap^-/- mice were profoundly suppressed by adoptive transfer of WT iTreg cells (Fig. 6d). These results suggest that deficient inflammation control in Xiap^-/- mice is partly due to impaired Treg cells functioning and that XIAP-intact iTreg cells restore the ability of Xiap^-/- mice to respond to infection-induced inflammation.
Anti-IL-6R rescues defects in Xiap−/− Treg cells. Among the serum inflammatory cytokines analyzed in C. albicans-infected Xiap−/− mice, the high levels of IL-6 were particularly noticeable (Fig. 6d). Therefore, we examined whether the impaired functioning of Xiap−/− Treg cells was associated with IL-6. IL-6 is generated by many different types of cells. Co-stimulation with IL-12 also induced production of IL-6 from human XIAP-deficient iTreg cells (Supplementary Figure 10). Inclusion of anti-IL-6R effectively suppressed TCR/CD28-induced IFN-γ production in Xiap−/− iTreg cells with or without IL-12 (Fig. 7a), and reduced the fraction of IFN-γ-expressing Xiap−/− iTreg cells activated through TCR and IL-12 (Fig. 7b). The effectiveness of IL-6R blockage in inhibiting conversion into IFN-γ+ iTreg cells was also confirmed in Foxp3-GFP-tagged Xiap−/− iTreg cells (Supplementary Figure 11). In addition, inclusion of anti-IL-6R decreased the expression and secretion of IL-6 in Xiap−/− Treg cells induced by IL-12 and/or TCR/CD28 (Supplementary Figure 12). Inclusion of anti-IL-6R also inhibited the expression and secretion of IFN-γ in human control and XIAP-knockdown iTreg cells (Fig. 7c and d). The production of IL-17 and IL-16 in human control and XIAP-deficient iTreg cells were similarly suppressed by anti-IL-6R (Supplementary Figure 13). Thus, anti-IL-6R effectively suppresses IFN-γ, IL-6, or IL-17 generation in XIAP-deficient Treg cells stimulated by TCR/CD28 with IL-12 or IL-6.

We also examined whether blocking other inflammatory cytokines, such as TNF or IL-1β, reversed the plasticity of Xiap−/− iTreg cells. Both anti-TNF and anti-IL-1R failed to antagonize activation- or IL-12-induced IFN-γ secretion in Xiap−/− iTreg cells. However, anti-IL-6R rescued the defects in XIAP-knockdown iTreg cells (Fig. 7e), indicating that IL-6R blockage may be a promising strategy to reverse Treg cell plasticity.
Cells upon T cells were isolated from spleen, and the frequencies of CD4+Foxp3+, Foxp3+IFN-γ, and Foxp3+IL-17 were quantitated by intracellular staining. Transfer of WT iTreg cells partially rescues inflammation in C. albicans-infected Xiap−/− mice with WT iTreg cells transfer. Kidney was isolated from Xiap−/− mice 10 days after C. albicans injection and infiltrated neutrophil contents were determined. Reduced inflammatory cytokine production in C. albicans-infected Xiap−/− mice into which WT iTreg cells had been transferred. Serum from mice was collected at the indicated time-points after C. albicans injection, and the levels of IL-6, TNF, MCP-1, and IL-12 were determined, n = 6. Values (a, d) are mean ± SD of samples. *P < 0.05, **P < 0.01, ***P < 0.001 for unpaired t-test.

Fig. 6 Transfer of Treg cells rescues Xiap−/− mice from lethal C. albicans infection. a Increased expression of IFN-γ and IL-17+ cells in Xiap−/− Treg cells upon C. albicans infection. WT and Xiap−/− female mice were intravenously infected with C. albicans (1 x 10^6), and mice were killed at day 10. T cells were isolated from spleen, and the frequencies of CD4+Foxp3+, Foxp3*IFN-γ* and Foxp3*IL-17* were quantitated by intracellular staining. b Transfer of WT iTreg cells partially rescues Xiap−/− mice from C. albicans-induced lethality. WT and Xiap−/− female mice were intravenously administered with C. albicans (1 x 10^5) and a group of Xiap−/− mice also received WT iTreg cells (1 x 10^6) at day 2. Survival of mice was monitored and is presented as a Kaplan–Meier survival curve (n = 7 for each group). **P < 0.01. c Reduced kidney inflammation in C. albicans-infected Xiap−/− mice with WT iTreg cells transfer. Kidney was isolated from Xiap−/− mice 10 days after C. albicans injection and infiltrated neutrophil contents were determined. d Reduced inflammatory cytokine production in C. albicans-infected Xiap−/− mice into which WT iTreg cells had been transferred. Serum from mice was collected at the indicated time-points after C. albicans injection, and the levels of IL-6, TNF, MCP-1, and IL-12 were determined, n = 6. Values (a, d) are mean ± SD of samples. *P < 0.05, **P < 0.01, ***P < 0.001 for unpaired t-test.

Anti-IL-6R restores the function of Xiap−/− Treg cells. We further examined whether a combination of anti-IL-6R and Xiap−/− Treg cells restored the resistance of Xiap−/− mice to infection-induced inflammation and lethality. Xiap−/− mice were infected with a lethal dose of C. albicans that did not affect WT mice. Xiap−/− iTreg cells were administered 2 days after infection, with or without by anti-IL-6R. Either Xiap−/− iTreg cells or anti-IL-6R partly increased the survival of Xiap−/− mice, though the increase was not statistically significant (Fig. 8a). Combinatory treatment of anti-IL-6R and Xiap−/− iTreg cells effectively rescued Xiap−/− mice from inflammatory infection (Fig. 8a). The elevated serum IL-6 levels in untreated infected Xiap−/− mice were suppressed by the combination of Xiap−/− iTreg cells and anti-IL-6R (Fig. 8b). We further traced the fate of CD45.1+ Xiap−/− iTreg cells after their transfer into infected CD45.2+ Xiap−/− mice. CD45.1+ Xiap−/− iTreg cells became inflammatory, with abundant IFN-γ expression in Xiap−/− mice pre-infected with C. albicans (Fig. 8c and d). The administration of anti-IL-6R inhibited the conversion of CD45.1+ Xiap−/− Treg cells into IFN-γ+Foxp3+ cells (Fig. 8c and d). The additive therapeutic effect of anti-IL-6R application is demonstrated by the fact that a combination of anti-IL-6R and Xiap−/− Treg cells reduced kidney neutrophil infiltration more effectively than either treatment alone (Fig. 8e). Notably, a significant decrease in kidney fungal inflammation and lethality.
times with similar results

activity of triplicate samples in an experiment. Treg cells co-transferred with TPA/A23187 for 5 h and the expressions of Foxp3 and IFN-γ were determined by intracellular staining (b). c, d Anti-IL-6R inhibits IFN-γ expression in human Treg cells. Control and human XIAP-knockdown iTreg cells were stimulated as in (a, b), with additional IL-6 as indicated, and secretion (c) or intracellular expression (d) of IFN-γ was determined. e Inability of anti-TNF or anti-IL-1R to inhibit the production of IFN-γ in activated Xiap−/− Treg cells. WT and Xiap−/− iTreg cells were stimulated, as described in (a), in the presence or absence of anti-TNF or anti-IL-1R (50 µg ml⁻¹ each) for 4 days. Treg cells were re-stimulated with TPA/A23187 for 24 h and the secreted IFN-γ was determined by ELISA. f Anti-IL-6R rescues the impaired suppressive activity of Xiap−/− iTreg cells in vivo. CD45.2 WT or Xiap−/− iTreg cells were co-transferred with CD45.1+ CD4+CD25- effector T cells into male CD45.1+ WT CD45.1+CD25- T cells. The body weights of mice were monitored at the indicated time-points. ***P < 0.001 by two-way ANOVA. g, h Anti-IL-6R restores Foxp3 stability and reduces IFN-γ expression in Xiap−/− Treg cells transferred in vivo. Lymphocytes were isolated from spleen and mesenteric lymph nodes of mice in (f) and reactivated with TPA/A23187. The expressions of Foxp3 (g) and IFN-γ (h) in CD45.2+ T cells were determined by intracellular staining. Values are mean ± 5D of triplicate samples in an experiment. *P < 0.05, **P < 0.01, ***P < 0.001 for unpaired t-test (a, c, g, h). Experiments (e-a) were independently repeated three times with similar results.

titers was detected for Xiap−/− Treg cells treatment, with or without anti-IL-6R (Fig. 8f). We further investigated whether the presence of anti-IL-6R dampened the T helper 17 responses, since Th17 plays a prominent role in resolving C. albicans infection. Figure 8g illustrates that IL-17 production by T cells isolated from infected mice was not affected by anti-IL-6R or anti-IL-1R plus Xiap−/− Treg cells. These results suggest that inhibition of Treg cells re-programming by anti-IL-6R significantly increased the functioning of XIAP-deficient Treg cells in vivo. Moreover, we have illustrated, likely for the first time, that in conjunction with anti-IL-6R, functionally unstable Treg cells could be used to treat inflammatory diseases.

Discussion

XIAP is a caspase-binding anti-apoptotic protein critical for cell survival. Although XIAP-knockout did not affect Treg cells development, the expression of Treg cells markers or the production by Treg cells of IL-10 and TGF-β, XIAP-deficient Treg cells were defective in their suppressive activity both in vitro and in vivo (Fig. 1). We found that SOCS1 was specifically reduced in XIAP-deficient T cells (Fig. 3). We also demonstrate that XIAP binds to SOCS1 and increases the protein stability of SOCS1, likely by promoting its K63 ubiquitination (Fig. 4). In contrast, K48 ubiquitination of SOCS1 was not affected by XIAP. Since SOCS1 is critical to maintaining the inhibitory activity of Treg cells, XIAP-deficient Treg cells exhibited a compromised suppressive ability. We further demonstrate that re-introduction of SOCS1 corrects defects of Xiap−/− Treg cells (Supplementary Figure 9). Thus, the impaired Treg cells functioning in Xiap−/− Treg cells could be partly attributed to a downregulation of SOCS1. Our results reveal an unexpected requirement for XIAP in Treg cells.

We have identified two different defects that account for the diminished suppressive activity in Xiap−/− Treg cells. The stability
of Foxp3 protein was reduced in activated Xiap−/− Treg cells (Fig. 2). Since persistent expression of Foxp3 is required to maintain the suppressive activities of Treg cells26, 27, the impaired functioning of Foxp3−/− Treg cells may be partially attributable to decreased Foxp3 presence. Notably, even though IL-2 is involved in Foxp3 stabilization39, 40, the reduced Foxp3 stability in Foxp3−/− Treg cells is IL-2-independent. IL-2-induced STAT5 activation was enhanced in Xiap−/− Treg cells (Supplementary Figure 8), similar to those in Socs1−/− T cells31.

A more profound defect of XIAP-deficient Treg cells was the increased conversion into IFN-γ−, IL-6−, and IL-17-producing Treg cells (Fig. 5). IL-12 is known to suppress Treg cells and converts Treg cells into Th1-type cells52. Deletion of SOCS1 elicits excess activation of the IFN-γ-STAT1 axis36, 37. Consistent with these observations, we found that IL-12 enhances WT and XIAP-deficient Treg cells to produce IFN-γ (Fig. 5). Previous studies have shown that pathogenic Treg cells lose Foxp3 expression and produce IFN-γ28, 53. IFN-γ promotes tissue inflammation, and IFN-γ-expressing Xiap−/− Treg cells likely

Fig. 8 Combination anti-IL-6R and Xiap−/− Treg cells rescue mice from infection-induced inflammation. a, b Xiap−/− iTreg cells plus anti-IL-6R rescue Xiap−/− mice from C. albicans infection. Male Xiap−/− mice (KO) were intravenously administered with C. albicans and Xiap−/− iTreg cells (1 × 10⁶) or anti-IL-6R antibody (500 μg) at day 2, or both, as indicated. Survival of mice is presented as a Kaplan–Meier survival curve (a). n = 11 for KO, n = 10 for KO + KO iTreg, n = 6 for KO + Ab, n = 9 for KO + KO iTreg + Ab. **P < 0.001 for Log-rank (Mantel–Cox) Test (a). n.s., not significant. The serum level of IL-6 was determined on day 14 after infection (b). *P < 0.05 for unpaired t-test (b). c, d Anti-IL-6R prevents the conversion of the transferred Xiap−/− Treg cells. CD45.1+ Xiap−/− iTreg cells were transferred into CD45.2+ mice infected with C. albicans as in (a). CD45.1+ T cells were recovered at day 10 post-infection, and the expression of Foxp3 and IFN-γ was determined (c). The Foxp3+IFN-γ− fractions in the CD45.1+ Treg cells were quantitated (d), n = 5. **P < 0.01 for unpaired t-test (d). e Anti-IL-6R reduces kidney neutrophil infiltration and fungal load in C. albicans-infected Xiap−/− mice with KO iTreg cells transfer. Male Xiap−/− mice were infected with C. albicans, as described in (a). Kidneys were isolated 14 days post-infection and neutrophils (e) and C. albicans titres (f) were quantitated. *P < 0.05, **P < 0.01 for unpaired t-test. n.s., not significant. g Anti-IL-6R does not affect IL-17 production. Male CD45.1+ Xiap−/− mice were infected with C. albicans and treated with anti-IL-6R and anti-IL-6R plus CD45.2+ KO iTreg cells. Spleen T cells and B cells were isolated 7 days after treatments. CD45.2+ iTreg cells were depleted. T cells were incubated with B cells and heat-killed C. albicans (HKCA) for 4 days, and the production of IL-17A after stimulation with TPA/A23187 (10/100 ng ml⁻¹) was determined by ELISA47, 48. Values are mean ± SD of triplicate samples in an experiment. Experiments were independently repeated three times with similar results.
contribute to inflammatory pathology. XIAP-deficiency also conferred on Treg cells an enhanced production of IL-6 and IL-17 (Fig. 5f, Supplementary Figures 10, 12, 13). The increase in IFN-γ generation is more pronounced than for enhancement of IL-17 expression in XIAP-deficient Treg cells in vitro (Fig. 5c vs. 5f, Fig. 5d vs. Supplementary Figure 13b) and in vivo (Fig. 5g and h), suggesting that XIAP-deficiency preferentially re-programs the development of Treg cells into Th1-like cells. IL-2 inhibits Th17 differentiation54, whether enhanced IL-2 responses in Xiap−/− Treg cells contributes to preferential Th1 over Th17 reprogramming is currently being investigated.

The critical role of SOCS1 in maintaining Treg stability has been demonstrated55, 56. In the present study, the defective activity of Xiap−/− Treg cells could be partly attributed to a loss of SOCS1. Overexpression of SOCS1 in Xiap−/− Treg cells prevented their conversion into IFN-γ-expressing T cells and restored the stability of Foxp3 (Supplemental Figure 9). We also demonstrate that XIAP increases the stability of SOCS1. SOCS1 is an E3 ligase known to target signaling molecules, such as VAV1 and Jak2, for ubiquitination and degradation. Our results indicate that, in the absence of XIAP, SOCS1 protein itself is subjected to degradation. Together with the capacity of XIAP to promote SOCS1 K63 polyubiquitination, our results suggest that ubiquitination of SOCS1 likely represents a new level of regulation responsible for the stability and function of the SOCS1 E3 ligase.

Despite the similar susceptibility between Socs1fl/fl LckCre− Treg cells56 and Xiap−/− Treg cells to generate IFN-γ and their defective abilities to inhibit colitis (Fig. 1g and h), Treg-specific-knockout of SOCS1 (Socs1fl/fl Foxp3Cre−) generates Treg cells that can still suppress colitis in Rag2−/− mice55. It has been suggested that environmental inflammatory cues prime Socs1−/− Treg cells to become IFN-γ-producing cells.55 We have previously shown that myeloid components in Xiap−/− mice become inflammatory upon infection15, suggesting a possibility that Xiap−/− myeloid cells prime Xiap−/− Treg cells for fragility. We plan to investigate whether the conversion of Xiap−/− Treg cells into inflammatory ex-Treg cells involves conditioning from Xiap−/− myeloid cells using mice with Treg-specific knockout of XIAP.

Our results also illustrate that defects in XIAP-deficient Treg cells are manifested by inflammatory cytokines. TCR/CD28 activation induces low-level production of IFN-γ, IL-6 and IL-17 in XIAP-deficient Treg cells (Fig. 5 and Supplementary Figures 10–13). Only upon co-stimulation with IL-12 or IL-1/IL-6 does the expression of IFN-γ, IL-6 or IL-17 become prominent in XIAP-deficient Treg cells. Therefore, Treg cells do not manifest pathogenic consequences in Xiap−/− mice before any inflammatory stimulation. We found that elevated serum IL-6 was the most prominent among the several inflammatory cytokines we measured upon C. albicans lethal infection in Xiap−/− mice (Fig. 6). IL-6 affects the stability of Treg cells56, and blockade of IL-6 increases the population of Treg cells57, 58. We further demonstrate that anti-IL-6R effectively inhibited IL-12-induced production of IFN-γ, while maintaining Foxp3 expression in Xiap−/− Treg cells (Fig. 7). Surprisingly, other anti-inflammatory biologics, i.e., anti-TNF, anti-IL-1R, or anti-IFN-γ did not effectively prevent IFN-γ generation in Xiap−/− Treg cells (Fig. 7e, Supplementary Figures 14 and 15a), illustrating the distinct role of IL-6R in Xiap−/− Treg cells conversion. Anti-IL-6R would be expected to prevent conversion of Treg cells into Th17-like cells, but our results illustrate that anti-IL-6R is unique in maintaining Treg cells stability in a Th1-prone environment.

Among the possible complications of anti-IL-6R is the increased susceptibility to infection and reduced generation of Th17 cells. In the present experiment, anti-IL-6R was administered 2 days after C. albicans infection. Notably, anti-IL-6R decreased inflammation, evidenced by both inflammatory cytokine levels and kidney neutrophil infiltration (Fig. 8b and e). Fungal load was actually reduced in mice treated with anti-IL-6R or anti-IL-6R plus Xiap−/− (Fig. 8f). In addition, anti-IL-6R did not affect antifungal Th17 responses (Fig. 8g). We speculate that a single dose of anti-IL-6R acts to prevent the conversion of Xiap−/− Treg cells and to attenuate inflammation, but it is not sufficient to attenuate antifungal immunity nor to suppress Th17 responses.

XIAP-deficiency leads to XLP-2 in patients, who exhibit over-activation of macrophages and lymphocytes. Xiap−/− mice succumb to infection by various pathogens12-15. Our results and those of others suggest that XIAP-deficient individuals are unable to clear infection due to primary immunodeficiency and persistent inflammation, leading to XLP-2 and lethality. As a primary immunodeficiency disease, the excess activation of lymphocytes in XLP-2 is likely a consequence of myeloid cell over-activation. Here, we have identified a subtle defect in the adaptive immunity of Xiap−/− mice related to their Treg cells. Xiap−/− Treg cells were mostly normal before infection, but were converted into IFN-γ-, IL-6-, and IL-17-expressing T cells in an inflammatory environment. Normal Treg cells are known to inhibit various immune cells59. Our observation that transfer of WT Treg cells effectively prevented infection-induced inflammation and conferred survival after an otherwise lethal infection in Xiap−/− mice (Fig. 6b–d) supports the notion that Treg cells are defective in Xiap−/− mice and XLP-2 patients, and that correction of XIAP-deficiency restores functional Treg cells. Our findings may also explain how lymphocytes become over-activated in XLP-2 patients, as Treg cells are required to keep lymphocyte activation in check. We propose a scenario whereby Xiap−/− mice are unable to control early infection as a consequence of impaired innate immunity, and inflammation caused by persistent pathogen presence primes for aberrant inflammatory Treg cells activation, leading to further escalated lymphocyte activation in Xiap−/− mice.

As a primary immunodeficiency disease, the only curative treatment for XLP-2 patients is allogeneic hematopoietic cell transplantation (HCT) to restore expression of XIAP in hematopoietic stem cells (HSCs), yet outcomes are limited by the toxicity associated with transplantation60. Even though transplantation of the WT gene into hematopoietic progenitor cells from patients would in theory rescue the genetic defect61, autologous HSC transplantation in XLP-2 patients is difficult given the treatments required to eradicate endogenous hematopoietic progenitor cells in these ill pediatric patients. As an alternative, adoptive T cell immunotherapy has been explored for treatment of primary immunodeficiency diseases linked to viral infection62. Our study illustrates another possibility of using Treg cells in the treatment of inflammatory diseases caused by innate immunodeficiency. Our results (Fig. 6) suggest that administration of XIAP-reconstituted Treg cells could help reduce the excess immuno-related inflammation seen in XLP-2 patients. XIAP could be re-introduced into T cells from XLP-2 patients, and these XIAP-restored T cells could then be differentiated into Treg cells in vitro in sufficient quantities. Expression of XIAP in T cells could thus be considered an improvement over transduction of XIAP in HSCs from XLP-2 patients. It may be noted that, due to the low proliferative ability of mouse Treg cells, we used mouse iTreg cells to test such a possibility (Fig. 6). Given that iTreg cells are more stable than rTreg cells, human iTreg cells are expected to be more effective than the rTreg cells shown in Fig. 6 in suppressing infection-induced inflammation.

We further advanced our therapeutic effect in mice by using a combination of anti-IL-6R and XIAP-deficient Treg cells. We illustrate that anti-IL-6R prevents the re-programming of Xiap−/− Treg cells into inflammatory Treg cells, and that Xiap−/− Treg cells become effective inhibitors of inflammation in the presence...
of anti-IL-6R (Fig. 8). Inflammation-induced Treg cells reprogramming is a major cause of Treg cells inactivation in vivo. Our results suggest that even if Treg cells functioning is substantially impaired by primary mutation, suppression of inflammation by co-administration of anti-IL-6R effectively restores the functioning of Treg cells. Therefore, a combination treatment of anti-IL-6R with autologous Treg cells should be effective in treating the inflammation and pathology of primary immunodeficiency diseases, bypassing the need to re-introduce the respective WT version of the defective gene into Treg cells.

A recent study revealed that innate immunodeficiency could be rescued by adaptive immunity, evidencing that an impaired response to Staphylococcus infection caused by TLR2 adapter deficiency could be rescued by antibodies against staphylococcal lipoteichoic acid. Our results may be viewed as another way of rescuing the immunity of Treg cells, evidencing that an impaired function of Treg cells functioning is sub-

We further used Xiap−/− mice to demonstrate that XLP-2 could be treated through combinatory use of ex vivo-expanded Xiap−/− Treg cells and anti-IL-6R. Our results further suggest, most likely for the first time, the possibility of treating primary immunodeficiency diseases and inflammatory diseases by simultaneous use of autologous Treg cells and anti-IL-6R. Further characterizations may help establish the protocol for clinical applications.

Methods

Antibodies and reagents. Antibodies against pSTAT3Tyr705 (#9167, 58D6, 1:1000), pSTAT3Tyr604 (#9395, CI15C, 1:1000), STAT1 (139122, 1:2000), SOCS3 (39505, A15E, 1:1000), Myc-tag (#2276, 9B11, 1:1000), and Myc-tag-HPR (#20405, 9B11, 1:2000) were purchased from Cell Signaling (Beverly, MA). Antibodies against GAPDH (sc-23223, 6C5, 1:4000), ElonginB (sc-133090, D5, 1:1000) and normoxia-gr (sc-20650) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-Flag (F1804, M2, 1:2000), anti-Flag-HRP (A8592-2MG, M2, 1:1000), anti-HA (HA9658, HA-7, 1:1000) and anti-HA-HPR (H6533, HA-7, 1:4000) were obtained from Sigma (St Louis, MO). Anti-ubiquitin (MAB1510, a0249b1, 1:1000) and anti-Actin (A1978, C4, 1:4000) were purchased from Sigma-Aldrich (St Louis, MO). Anti-STAT3 (AB1812, 1:2000) was purchased from Invitrogen (Waltham, MA) and anti-STAT3s (13-3600, 1:2000) was purchased from Zymed (ThermoFisher Scientific). Anti-STAT3 (ARG54150, AG4C8-10H-8, 1:2000) was purchased from Arigo (Basiglio, IT). Anti-SOCS1 (ab68760, 1.0 μg per test for IP) was purchased from Abcam (Cambridge, UK). Anti-beta-actin (A2228, 1:1000), anti-Gr-1-PE (RB6-8C5, 1:1000), anti-human CD127-AF647 (HIL-FLAX, 1:100), anti-human CD45.1-BV421 (A20, 110732, 1:100), anti-CD45.2-FITC (104, 109806, 1:100), anti-human CD4-FITC (OKT4, 317408, 1:100), anti-human CD45RA-Paci (12-13489), anti-human CD11c-PE (HLA-DR, 88-7066-86), human IL-6 (88-7176-88), and human IL-6 (88-7066-86) were purchased from eBioScience. LEAF-purified anti-mouse IFN-γ (R4-6A2, 505707) was purchased from BioLegend. Recombinant mouse or human IL-12, IL-10 and IL-1p were purchased from R&D (Minneapolis, MN). Recombinant mouse IL-2, human IL-2 and mouse IL-6 were purchased from eBioScience. Recombinant human IL-6 and human TGF-β were purchased from Peprotech (Rocky Hill, NJ). C57 (65-0850-84) and Violet-Tag (425101) were purchased from eBioscience and BioLegend. Heat-killed C. albicans was obtained from InvivoGen (San Diego, CA). Recombinant human E1, E2 (UBC13) and Ubiquitin (88-8088-88) were purchased from Nod. E1, E2, and Ubiquitin were isolated from Bovine milk (Cambridge, MA). The primer sets used in RT-PCR were previously described. Mouse cDNA was isolated from DO11.10 cells by RT-PCR. PRKs-HA WT U6, K63 U6, and K48 U6 were purchased from Addgene (Cambridge, MA). Sequences of the primers used for cloning of Eolingon/β, XIAP fragments and SOCS1 fragments are listed in Supplementary Table 1.

Cell cultures. HEK293T (ATCC CRL-3216) cells were obtained from ATCC. Cell lines were examined for mycoplasma contamination using a Mycoplasma Detection Kit (R&D). Primary mouse T cells and human CD14+ cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA). 10 mM glutamine, 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, and 50 μM 2-mercaptoethanol (referred to as complete RPMI medium). Human Treg cells were cultured in X-VIVO 15 medium with supplements identical to complete RPMI medium (complete X-VIVO15 medium). HEK293T cells were cultured in DMEM medium with the same supplements as for complete RPMI medium.

Mouse Treg cell isolation and iTreg cell differentiation. Mouse total T cells were isolated from spleen and lymph nodes using anti-mouse Ig panning. T cells were cultured in complete RPMI 1640 medium. Mouse iTreg cells were purified from total T cells by sorting on a MoFlo Astrios system (Beckman Coulter) or by using a MACS CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotech, Germany) and confirmed by FACS (Supplementary Figure 1). The purity of 100% and Foxp3 expression >98.6% (Supplementary Figure 1). For WT and Xiap−/− mice carrying Foxp3-GFP, iTreg cells were isolated by sorting based on GFP+. IL-10 and TGF-β secretion from iTreg cells were determined by having CD25 staining of 100% and Foxp3 expression >98.6% (Supplementary Figure 1). For WT and Xiap−/− mice carrying Foxp3-GFP, T reg cells were isolated by sorting based on GFP+. IL-10 and TGF-β secretion from iTreg cells were determined by having CD25 staining of 100% and Foxp3 expression >98.6% (Supplementary Figure 1). For WT and Xiap−/− mice carrying Foxp3-GFP, T reg cells were isolated by sorting based on GFP+. IL-10 and TGF-β secretion from iTreg cells were determined by having CD25 staining of 100% and Foxp3 expression >98.6% (Supplementary Figure 1).

In vitro Treg cell suppression assay. T eff (4 × 106) and antigen-presenting cells (APC, mitomycin C-treated T-depleted splenic cells, 1.2×106) were co-cultured with iTreg cells or iTreg cells in 200 μl complete RPMI medium containing 2 μg ml−1 of anti-CD3 in U-bottomed 96-well plates. The ratio of iTreg: T eff were 1:1, 1:2, 1:4, and 1:8. After 72 h, 0.5 μM H3-thymidine was added into culture medium for 6 h and T eff proliferation was determined. CESE halving was used for the human Treg cells suppressive assay. Human Treg cells were labeled with 5 μM CESE and incubated with different amounts of Treg cells as described above. The intensity of CESE in CD4 T eff cells was analyzed by flow cytometry at 72 h.

In vivo Treg cell suppression assay. CD4+CD25− T cells (4 × 106) from CD45.1+ female mice were administered by intraperitoneal injection into CD45.1+ Rag1−/− females (8–10-weeks old) with or without 1 × 106 iTreg cells from CD45.2 WT or...
**Human peripheral blood mononuclear cell and T cell isolation.** Exemplar human whole blood (WBC) concentrations were obtained from the Taipei Blood Bank with approval from the institutional review boards of Taipei Blood Bank and Academia Sinica. WBC concentrations were diluted with 1× HBSS, overlaid on Ficoll-Paque, and centrifuged at 400 × g for 20 min. The interphase cells were collected and washed with 5% RPMI medium. The obtained peripheral blood mononuclear cells (PBMCs) were re-suspended in MACS® buffer (PBS containing 0.5% FBS and 2 mM EDTA). Naive human CD4+ T cells (CD4+CD45RA+CD45RO−) were isolated by human naive T cells and tTreg cells were activated for 24 h by plate-bound anti-CD3 (2 μg psPAX2 and 3 μg pMD2G). Naive human naive T cells and Treg cells were activated for 24 h at plate-bound anti-CD3 (2 μg ml−1) and anti-CD28 (2 μg ml−1), infected with recombinant lentivirus, and the Thy1.1-expressing cells were isolated by MACS® beads 96 h later. T cells were rested for 4 days before re-stimulation with Thy1.1+ T cells by MACS®. IL-2 (50 ng ml−1) was present during the activation, infection and resting of T cells. Flow cytometry confirmed that human naive T cells remained CD4+CD45RA+CD45RO− after infection and resting, and were used for iTreg cell differentiation.

**XIAP knockdown in human T cells.** The XIAP knockdown lentiviral construct was generated by cloning human XIAP-specific shRNA (5′-CCA-GAATGCTGACTACAA-3′) into pLKO.3-Thyl.1 vector. Lentiviruses were harvested from the culture supernatant of HEK293T cells transfected with 10 μg pLKO.3-Thyl.1 or pLKO.3-Thyl.1-hXIAI shRNA. 7.5 μg psPAX2 and 3 μg pMD2G. Human naive T cells and Treg cells were activated for 24 h by plate-bound anti-CD3 (2 μg ml−1) and anti-CD28 (2 μg ml−1), infected with recombinant lentivirus, and the Thy1.1-expressing cells were isolated by MACS® beads 96 h later. T cells were rested for 4 days before re-stimulation with Thy1.1+ T cells by MACS®. IL-2 (50 ng ml−1) was present during the activation, infection and resting of T cells. Flow cytometry confirmed that human naive T cells remained CD4+CD45RA+CD45RO− after infection and resting, and were used for iTreg cell differentiation.

**Transient transfection.** HEK293T cells were transfected with plasmid DNA by Lipofectin (Invitrogen). Cells (4 × 106) were seeded onto 10 cm Petri-dishes overnight and transfected with 1 μg DNA combined with 2 μl transfection reagent. Cells were collected and lysed by 200 μl WCE lysis buffer containing 10 μg pLKO.3-Thyl.1 or pLKO.3-Thyl.1-hXIAI shRNA, 7.5 μg psPAX2 and 3 μg pMD2G. Naive human naive T cells and Treg cells were activated for 24 h by plate-bound anti-CD3 (2 μg ml−1) and anti-CD28 (2 μg ml−1), infected with recombinant lentivirus, and the Thy1.1-expressing cells were isolated by MACS® beads 96 h later. T cells were rested for 4 days before re-stimulation with Thy1.1+ T cells by MACS®. IL-2 (50 ng ml−1) was present during the activation, infection and resting of T cells. Flow cytometry confirmed that human naive T cells remained CD4+CD45RA+CD45RO− after infection and resting, and were used for iTreg cell differentiation.

**Human dendritic cell differentiation.** Human CD14+ cells were isolated from PBMCs by CD14 microbeads on a MACS® Separator (Miltenyi Biotec), and were differentiated into dendritic cells (DCs) in complete RPMI medium containing GM-CSF (100 ng ml−1) and IL-4 (50 ng ml−1) for 7 days, with medium being replenished every 3 days. DCs were then activated by 10 ng ml−1 TNF-α for 1 day and the mature DCs were used as antigen-presenting cells for human Treg cells differentiation and activation.

**Cell lysates and immunoprecipitation.** Stimulated cells used for phosphorylation analysis were lysed by p-signal cell lysis buffer (25 mM pH 7.7 HEPES, 300 mM NaCl, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1 mM Na3VO4, 50 mM NaF, 0.5 mM DTT and 10% glycerol) 24 h after transfection. HEK293T cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with supplements identical to complete RPMI medium (referred to as complete X-VIVO15 medium). The XIAP knockdown lentiviral construct was generated from human naive T cells with approval from the institutional review boards of Taipei Blood Bank and Academia Sinica. WBC concentrations were diluted with 1× HBSS, overlaid on Ficoll-Paque, and centrifuged at 400 × g for 20 min. The interphase cells were collected and washed with 5% RPMI medium. The obtained peripheral blood mononuclear cells (PBMCs) were re-suspended in MACS® buffer (PBS containing 0.5% FBS and 2 mM EDTA). Naive human CD4+ T cells (CD4+CD45RA+CD45RO−) were isolated from human PBMCs by sorting on a MoFlo Astrios system. Human Treg cells (CD4+CD127lowCD25+) and Teff (CD4+CD25−) cells were similarly isolated from PBMCs. Naive human T cells were cultured in complete RPMI medium. Human tTreg cells were cultured in X-VIVO15 medium with supplements identical to complete RPMI medium (referred to as complete X-VIVO15 medium). XIAP knockdown in human T cells. The XIAP knockdown lentiviral construct was generated by cloning human XIAP-specific shRNA (5′-CCA-GAATGCTGACTACAA-3′) into pLKO.3-Thyl.1 vector. Lentiviruses were harvested from the culture supernatant of HEK293T cells transfected with 10 μg pLKO.3-Thyl.1 or pLKO.3-Thyl.1-hXIAI shRNA, 7.5 μg psPAX2 and 3 μg pMD2G. Naive human naive T cells and Treg cells were activated for 24 h by plate-bound anti-CD3 (2 μg ml−1) and anti-CD28 (2 μg ml−1), infected with recombinant lentivirus, and the Thy1.1-expressing cells were isolated by MACS® beads 96 h later. T cells were rested for 4 days before re-stimulation with Thy1.1+ T cells by MACS®. IL-2 (50 ng ml−1) was present during the activation, infection and resting of T cells. Flow cytometry confirmed that human naive T cells remained CD4+CD45RA+CD45RO− after infection and resting, and were used for iTreg cell differentiation.

**In vitro ubiquitination assay.** The reaction mixture containing 1 μg E1, 0.2 μg E2 (UBC13), 0.1 μg ubiquitin-K63, 0.1 μg E3 (XIAP or XIAP(Ring)), 0.1 μg substrate SOCS1-HA-Mag beads, 0.1 μg recombinant hEloB and hEloC in reaction buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 2 μM Aprotinin, 0.5 mM DTT) in a final volume of 40 μl was incubated at 30 °C for 1 h. A 4 μl sample was used to verify the protein components after the reaction. The complex on SOCS1-HA-Mag beads was pulsed down in a magnetic rack. The extent of ubiquitination was quantified by western blotting.

**Generation of GFPVR retroviral supernatants.** SOCS1-Δ was digested from SOCS1-ΔHA-pCDNA4 with BamH1 and PsnI, and then subcloned into GFPVR to generate GFPVR-SOCS1-ΔHA. GFPVR or GFPVR-SOCS1-ΔHA (5 μg) was cotransfected with 3 μg psPAX2 and 2 μg pMD2G into HEK293T cells. Culture medium was collected and replaced every day. The culture media collected at day 2, 3, and 4 were combined and concentrated by ultrafiltration through 26000 rpm on a Beckman SW28 rotor for 2 h. Pellets were resuspended in 5% BSA/PBS overnight at 4 °C and stored at −80 °C.

**SOCS1 overexpression in primary iTreg and tTreg cells.** (EUB) cells, 0.1 μg E3 (XIAP or XIAP(Ring)), 0.1 μg substrate SOCS1-HA-Mag beads, 0.1 μg recombinant hEloB and hEloC in reaction buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 2 μM Aprotinin, 0.5 mM DTT) in a final volume of 40 μl was incubated at 30 °C for 1 h. A 4 μl sample was used to verify the protein components after the reaction. The complex on SOCS1-HA-Mag beads was pulsed down in a magnetic rack. The extent of ubiquitination was quantified by western blotting.
incubated with PE Detection Reagent (BD Biosciences) in the dark at room temperature. The mixtures were washed and the fluorescence of the re-suspended beads was determined in a FACSCalibur system (BD Biosciences). The acquired data were analyzed with FCAP Array software (BD Biosciences).

**Statistics.** Data in this study were randomly collected but were not blinded. No data were excluded in this study. Our data mostly meet the assumption of the tests (normal distribution). GraphPad Prism 5 and Microsoft Office Excel and were used for data analysis. Unpaired two-tailed Student t-tests were used to compare results from between two groups. Data were presented as mean with standard deviation (s.d.) or standard error of the mean (s.e.m.), as indicated in the figure legend. Weight loss was analyzed by two-way ANOVA for multiple comparisons. Survival curves were plotted with Kaplan–Meier survival curve analysis and analyzed by the log rank test (Mantel–cox). P values ≤0.05 were considered significant.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary files, or are available from the authors upon request.

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Author contributions

W.C.H., acquisition of data, analysis and interpretation of data, study design, statistical analysis; S.T.H., data presentation, key materials support; Y.J.C., key materials support; M.Z.L., study concept and design, study supervision, drafting of the manuscript.

Additional information

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