**IMMUNOLOGY**

cIAP1/2 inhibition synergizes with TNF inhibition in autoimmunity by down-regulating IL-17A and inducing Tregs

Joanna Z. Kawalkowska, Joy Ogbechi, Patrick J. Venables, Richard O. Williams*

IL-17 and TNF-α are major effector cytokines in chronic inflammation. TNF-α inhibitors have revolutionized the treatment of rheumatoid arthritis (RA), although not all patients respond, and most relapse after treatment withdrawal. This may be due to a paradoxical exacerbation of T$_\text{H}17$ responses by TNF-α inhibition. We examined the therapeutic potential of targeting cellular inhibitors of apoptosis 1 and 2 (cIAP1/2) in inflammation by its influence on human T$_\text{H}17$ subsets and mice with collagen-induced arthritis. Inhibition of cIAP1/2 abrogated CD4$^+$ IL-17A differentiation and IL-17 production. This was a direct effect on T cells, mediated by reducing NFATc1 expression. In mice, cIAP1/2 inhibition, when combined with etanercept, abrogated disease activity, which was associated with an increase in T$_\text{regs}$ and was sustained after therapy retraction. We reveal an unexpected role for cIAP1/2 in regulating the balance between T$_\text{H}17$ and T$_\text{regs}$ and suggest that combined therapeutic inhibition could induce long-term remission in inflammatory diseases.

**INTRODUCTION**

Inhibitor of apoptosis proteins (IAPs) are a family of proteins characterized by the presence of baculovirus IAP repeats (BIR) domains, which allow protein-protein interactions. Two members of this family, cellular IAP1 (cIAP1) and cIAP2, are ubiquitin E3 ligases and vital components of both nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (1–5). Antagonists against cIAP1/2, known as SMAC mimetics, are presently under investigation as therapeutic targets for solid tumors, hematological malignancies, and viral infections (6–12). However, the therapeutic potential of SMAC mimetics in chronic inflammatory diseases, such as rheumatoid arthritis (RA), is controversial, with reports showing that cIAP1/2 deficiency was pro-inflammatory (13, 14) and others reporting that SMAC mimetics are anti-inflammatory (1, 15), in part by reducing leukocyte infiltration and modulating endothelial cell activation (1). A more recent report suggested that cIAP1/2 antagonists induced apoptosis in synovial fibroblasts (16).

It has previously been shown that cIAP1/2 plays a role in lymphocyte cell signaling and immune responses (17, 18). For example, deletion of cIAP1/2 in CD8$^+$ T cells leads to loss of effector function during antiviral immune responses and impaired memory cell development (19, 20). Mice expressing an inactive cIAP2 mutant and human cells treated with a cIAP2 antagonist were found to be less dependent on T cell costimulatory signals for cell proliferation and cytokine production (21, 22). In contrast, B cell–specific deletion of cIAP1/2 in mice led to defective humoral responses despite an increase in B cell numbers (21, 23). cIAP1/2 are components of both NF-κB and MAPK signaling pathways, and it is therefore expected that inhibition of cIAP1/2 in vitro reduces the ability of multiple cell types to respond to tumor necrosis factor–α (TNF-α) (3, 24). cIAP1/2 also regulate multiple aspects of myeloid cell activity, including differentiation, cytokine secretion, and cell death (2, 11, 25–33).

We hypothesized that cIAP1/2 antagonism would ameliorate immune-driven inflammation, and we therefore examined the effect of a cIAP1/2 antagonist, GT13072, in collagen-induced arthritis (CIA). GT13072 mimics the endogenous inhibitor of cIAPs, SMAC (10, 34, 35). Unexpectedly, GT13072 had a remarkably selective inhibitory effect on interleukin-17A (IL-17A) production in vivo. This is of considerable translational interest, as we and others have previously reported in mice and humans that TNF-α inhibitors increase numbers of IL-17$^+$ T cells (36–40). We therefore studied the effect of treatment with GT13072 in combination with a TNF-α inhibitor and demonstrated a synergistic effect between the two treatments, leading to inhibition of T helper 17 (T$_\text{H}17$) responses and expansion of regulatory T cells (T$_\text{regs}$).

**RESULTS**

cIAP1/2 inhibition inhibits joint inflammation and reduces IL-17A$^+$ cells

We first evaluated the therapeutic potential of GT13072 (Fig. 1A) in CIA at a dose of 10 mg/kg per day (Fig. 1B). This dose was based on pilot experiments in which GT13072 produced a dose-dependent reduction in clinical severity of disease (fig. S1). As a control, a structurally related but inactive compound (GT14829) was used. Treatment with GT13072 after disease onset resulted in rapid and consistent amelioration of CIA ($P < 0.0001$) (Fig. 1C). The therapeutic effect of GT13072 was also evident in the histological analysis of arthritic paws (Fig. 1D and fig. S2A). Thus, GT13072-treated mice showed significantly lower levels of cartilage damage and cell infiltration than controls in all arthritic joints. Serum levels of TNF-α were elevated after treatment with GT13072, but this was offset by a comparable increase in IL-10 (Fig. 1E). Serum levels of IL-1β and IL-6 were unaffected by GT13072 treatment (fig. S2B).

Analysis of lymph nodes and arthritic paws revealed that cIAP1/2 inhibition led to a significant reduction in the percentage and total number of IL-17$^+$CD4$^+$ T cells (Fig. 1, F and G). There was also a reduction in IL-17A$^+$ TCRγδ T cells, while the total number of T cell...
Fig. 1. Systemic inhibition of cIAP1/2 leads to the amelioration of CIA in mice. (A) Chemical structure of the SMAC mimetic, compound GT13072. (B) Mice with CIA received daily intraperitoneal injections of GT13072 or control compound at 10 mg/kg per day. (C) All four paws were assessed daily for signs of arthritis. P values were calculated using two-way analysis of variance (ANOVA) with Tukey’s post hoc test and are not shown in the graph for clarity. (D) Histological analysis of arthritic paws from mice with CIA after 10 days of treatment with GT13072 or control compound (n = 10). (E) On day 10 of arthritis, serum samples from CIA mice were analyzed for cytokines by Meso Scale Discovery (MSD). Each data point represents one animal (n = 19 to 22). Error bars represent ±SEM. The horizontal lines represent the mean. P values were calculated using unpaired Student’s t tests. (F to I) GT13072-treated and control CIA mice were culled on day 10, and their draining lymph node and arthritic paw cells were counted and characterized by flow cytometry. P values were calculated using unpaired Student’s t tests. Each data point represents one animal (lymph node, n = 19 to 22; paw, n = 10). (F and G) The percentage of IL-17A–secreting CD4+ T cells in lymph nodes and arthritic paw cells was determined by flow cytometry. One representative dot blot or contour plot is shown. Absolute numbers of CD4+IL-17A+ cells in lymph nodes and arthritic paws are shown. (H) Absolute numbers of TCRγδ cells and IL-17+ TCRγδ T cells in arthritic paws with representative contour plot. (I) Paw cells were examined for RORγT expression.
receptor γδ (TCRγδ) T cells remained unchanged (Fig. 1H). However, GT13072 did not affect levels of RORγT (retinoic acid receptor–related orphan nuclear receptor γt), the T17 master transcription factor (Fig. 1I). Numbers of T117 and T172 cells, as well macrophages, CD8+ T cells, and B cells in lymph nodes and joints, were unaffected by GT13072 treatment (fig. S3, A to G).

Inhibition of cIAP1/2 down-regulates IL-17A production in human T cells
To establish the relevance of these findings for human disease, we first questioned whether cIAP1 and cIAP2 are expressed in human CD4+ T cells. Both cIAP1 and cIAP2 were found by Western blotting to be expressed in CD4+ T cells from healthy human donors (Fig. 2A). As expected, treatment of CD4+ T cells with GT13072 (1000 nM) induced degradation of both cIAP1 and cIAP2 within 10 min (Fig. 2A).

We next assessed the ability of GT13072 to influence IL-17A expression in human CD4+CCR6+CD161+ T cells stimulated under T117-polarising conditions in vitro. GT13072 inhibited the expression of IL-17A by 95% compared to TNF-α (IFN-γ), IL-21, or IL-22 (Fig. 2B). GT13072 did not alter the expression of TNF-α in T1717 cells, which contrasts with the increased levels of TNF-α observed in serum of mice treated with GT13072 (Fig. 1E).

The selective effect of GT13072 on IL-17A expression was confirmed by measurement of secreted cytokines in culture supernatants of human CD4+CCR6+CD161+ T cells (fig. S4A) and human CD4+ T cells (fig. S4B). The inhibitory effect of GT13072 on IL-17A expression was highly specific for T1717 cells, as no effect on cytokine expression was observed in T1711 or T1172 differentiation assays (fig. S5A). The effect of GT13072 on IL-17A secretion was found to be a class effect, as four additional cIAP1/2 inhibitors of related structure to GT13072 also inhibited IL-17A expression in human CD4+ T cells (fig. S6A).

Unexpectedly, GT13072 did not affect RORγT levels during T117 development (Fig. 2C). This led us to question whether cIAP1/2 may play a role in the regulation of IL-17A at a posttranscriptional level. However, we found that GT13072 strongly inhibited IL-17A mRNA expression by naïve human CD4+ T cells cultured in T117-inducing conditions, indicating that the compound was acting at a transcriptional level (Fig. 2D).

cIAP1/2 inhibition down-regulates NFATc1 expression in T cells
We then set out to establish the mechanism by which cIAP1/2 inhibition inhibited IL-17 production. MAPK signaling has previously been found to inhibit IL-17A production (41, 42), and we found that GT13072 inhibited the activation of p38 MAPK by TNF-α (Fig. 3A), but when CD4+ T cells were stimulated with anti-CD3/anti-CD28 in the presence of T117-polarizing cytokines MAPK activity was unaffected by GT13072 (Fig. 3B). However, the expression of NFATc1 (nuclear factor of activated T cells c1), a transcription factor known to regulate T117 differentiation (43), was profoundly reduced in differentiating T cells treated with GT13072 (Fig. 3C). GT13072 did not affect cell number or viability under these conditions (Fig. 3, D and E).

Combined inhibition of cIAP1/2 and TNF-α reduces IL-17A+ T cells and synergistically promotes Treg
As GT13072 inhibits IL-17A+ T cells, we hypothesized that the combined treatment of GT13072 with a TNF-α inhibitor (etanercept) would inhibit the increase in IL-17A+ cells induced by TNF-α blockade alone. This combination would also have the advantage of neutralizing TNF-α induced by GT13072 in vivo (Fig. 1E). As predicted, combination therapy with GT13072 and etanercept reduced numbers of CD4+IL-17A+ cells in lymph nodes and inhibited IL-17A production following restimulation in vitro compared to etanercept alone (Fig. 4A). Combined GT13072 and etanercept therapy also blocked the accumulation of IL-17A+ cells in the paws of mice with CIA (Fig. 4B). Treatment with GT13072 was extremely effective on its own, and we did not therefore observe an additive effect between GT13072 and etanercept (Fig. 4C).

Unexpectedly, there was a profound reduction in proliferative responses of CD4+ T cells from the joints of GT13072-treated arthritic mice (Fig. 4D), and this led us to speculate on the possibility of increased Treg activity in the joint. A significant increase in numbers of Treg was observed in the paws of mice treated with GT13072 plus etanercept, which was not observed in mice treated with GT13072 alone or etanercept alone (Fig. 4E). GT13072 treatment also caused a significant increase in the percentage of Treg in the lymph nodes (fig. S7, A and B). Consistent with this observation, a significant increase in IL-10 was detected in the serum of mice treated with GT13072 plus etanercept (Fig. 4F).

To confirm the significance of these findings in human lymphocytes, we activated healthy human peripheral blood mononuclear cells (PBMCs) with an anti-CD3 antibody in the presence or absence of GT13072. After 5 days of culture, we found a twofold increase in the percentage of Treg (Fig. 4G).

Dual inhibition of cIAP1/2 and TNF-α leads to sustained disease remission
On the basis of the synergistic inhibition of IL-17A responses and expansion of Treg, we hypothesized that the combination of GT13072 and etanercept would have a more durable therapeutic effect than either treatment alone. Thus, mice with CIA were treated with GT13072 alone, etanercept alone, or GT13072 plus etanercept. After 10 days, treatment was stopped, and the mice were observed daily for signs of disease relapse (Fig. 5A). After stopping treatment, there was a rapid relapse of arthritis in mice that had received GT13072 alone or etanercept alone (Fig. 5B). In contrast, mice that had received GT13072 plus etanercept did not show any significant signs of disease relapse up to day 18, when the experiment was terminated.

To determine the translational importance of these findings, we assessed the effects of cIAP1/2 inhibition ex vivo on IL-17A+ cells from the joints of RA patients. We isolated cells from the synovial membrane of patients with active RA and cultured these cells in T117-inducing conditions and in the presence of GT13072 or control compound for 6 days. Consistent with our previous results, the percentage of IL-17A+ cells was significantly reduced by the addition of GT13072, which shows that disease-relevant human T cells are targeted effectively by cIAP1/2 inhibition (Fig. 5C).

DISCUSSION
There is an increasing demand for treatments that lead to drug-free disease remission in chronic diseases like RA. The development of TNF-α inhibitors has been an important step forward, but they are not curative, and the majority of patients require regular injections to prevent flare-up of the disease (44). We found that cIAP1/2 plays a pivotal role in the secretion of IL-17A, a pro-inflammatory cytokine that contributes to the pathology of several immune-mediated
Fig. 2. cIAP1/2 antagonists inhibit IL-17A secretion by CD4+ T cells. (A) cIAP1 and cIAP2 protein expression in human CD4+ lymphocytes cultured for 10 min with GT13072 (1000 nM) or control compound. Immunoblots are representative of three independent experiments. (B and C) Human CD4+CCR6+CD161+ T cells were cultured in the presence of Th17-differentiating cytokines and anti-CD3/CD28 Dynabeads for 6 days. The effect of GT13072 or control compound (1000 nM) on cytokine secretion and RORγT was determined by flow cytometry (n = 4). Representative dot blots from four donors are shown. Bar graphs represent quantification of flow cytometry data. (D) Naïve human CD4+ T cells were cultured in the presence of Th17-differentiating cytokines and anti-CD3/CD28 Dynabeads plus GT13072 or control compound (1000 nM). After 17 hours, mRNA levels were determined by quantitative reverse transcription polymerase chain reaction. Data are expressed as the mRNA level normalized to RPLPO expression and are shown as means ± SEM from five independent human donors. RPLPO, ribosomal protein, large, P0. (B to D) Error bars represent ±SEM. P values were calculated using unpaired Student’s t tests.
Fig. 3. clAP1/2 antagonist inhibits NFATc1 expression. **(A)** Phosphorylation of p38 MAPK was determined in human CD4⁺ T cells cultured with 1000 nM GT13072 or control compound before stimulation with TNF-α at 20 ng/ml. Immunoblots are representative of two independent experiments. **(B and C)** Naïve human CD4⁺ T cells cultured with 1000 nM GT13072 before stimulation with plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (2 μg/ml), IL-6 (50 ng/ml), IL-1β (10 ng/ml), and transforming growth factor–β (TGF-β; 1 ng/ml) for the indicated durations. (B) Phospho-p38 MAPK detected by immunoblotting. Results are representative of two independent experiments. (C) NFATc1 protein detected by immunoblotting. Dotted line shows where lanes were removed from the blot. Results are representative of four independent experiments. **(D and E)** Human CD4⁺CCR6⁺CD161⁺ T cells were cultured in the presence of T helper 17–differentiating cytokines and anti-CD3/CD28 Dynabeads for 6 days. **(D)** Total number of cells on day 6 of culture. **(E)** Determination of annexin V and propidium iodide (PI) expression on day 6. Representative dot blots are shown from three donors.
Fig. 4. Synergistic effects of cIAP antagonism and TNF-α blockade. (A to F) Arthritic mice received either GT13072, etanercept, GT13072 plus etanercept, or control compound. (A) Mice were culled on day 10, and the total number of IL-17A+CD4+ T cells in lymph nodes (LN) was determined (n = 10). Lymph node cells were stimulated with anti-CD3 antibody for 48 hours, and supernatants were examined for IL-17A. (B) Total number of IL-17+CD4+ T cells in arthritic paws. ns, not significant. (C) Pooled data are from two independent experiments (n = 17). P values were calculated using two-way ANOVA with Tukey's post hoc test. (D) The percentage of proliferating CD4+ T cells was determined in arthritic paws (n = 10). (E) The total number of Tregs was determined in arthritic paws. One representative dot blot is shown. (F) Serum samples were analyzed for IL-10 by MSD (n = 10). (G) Human PBMCs were cultured with GT13072 or control compound for 6 days and analyzed for Treg. One representative dot plot is shown (n = 6). P values were calculated using unpaired Student’s t tests. (A to F) Error bars represent ±SEM. ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.
Inflammatory diseases, including RA. Thus, inhibition of cIAP1/2 led to a significant decrease in IL-17A production by T cells as well as a profound reduction in disease severity in mice with arthritis. Furthermore, dual inhibition of cIAP1/2 and TNF-α led to the induction and accumulation of Tregs in the joint and long-term disease remission.

Previous studies show that SMAC mimetics, including GT13072, induce TNF-α expression in a range of cells (6, 12, 27, 45), a finding confirmed in this study. However, it is important to emphasize that, despite increased TNF-α levels in serum, we observed a highly significant reduction in arthritis severity associated with increased levels of IL-10 and Tregs. Further analysis excluded CD4+ T cells as the primary source of the TNF-α in GT13072-treated mice.

We previously showed that TNF blockade alone paradoxically increased numbers of TTh17 cells and IL-17A expression, a finding that has been confirmed in a number of independent studies (36–40). This provides a potential explanation for the synergy observed between etanercept and GT13072, which we showed to be a potent inhibitor of IL-17A production. Although our study focused on arthritis, it is envisaged that cIAP1/2 inhibitors could also be useful in other autoimmune diseases in which IL-17A plays a pathological role, such as psoriasis or multiple sclerosis. Note that cIAP1/2 inhibitors are already available and are currently undergoing phase 1 clinical trials in patients with cancer (46–48).
signaling is important for IL-17A production (41), we found no evidence that GT13072 inhibited MAPK signaling following TCR stimulation, although it was inhibited by GT13072 following TNF receptor signaling, in agreement with previous findings (3). However, the expression of NFATc1, a transcription factor known to regulate Th17 differentiation and IL-17A expression (43, 49), was profoundly reduced in differentiating Th17 cells treated with GT13072. Previous studies have shown that cIAP1/2 are capable of “degradative” K48-linked ubiquitination, leading to protein degradation (44, 45), as well as K63-linked poly-ubiquitination (28, 50), which does not induce degradation and is essential in cell signaling cascades. In osteoclasts, NFATc1 protein stability is regulated by cIAP1/2-mediated ubiquitination (51). In our study, however, we found that T cells lower their level of NFATc1 responses to Th17-inducing stimuli after cIAP1/2 antagonism. These somewhat contradictory results could be partially due not only to cell type differences but also to different stimuli inducing NFATc1 expression in these cell types. Further studies are required to better understand the precise mechanism by which cIAP1/2 inhibition leads to decreased NFATc1 expression and IL-17A production in T cells and whether it involves the posttranslational modification of these proteins or by other means.

While the reduction in pro-inflammatory CD4⁺IL-17A⁺ and IL-17A⁺TCRγδ T cells most likely accounts for the marked therapeutic effects of GT13072 in vivo, we cannot rule out additional effects on B cells, macrophages, CD8⁺ T cells, endothelial cells, or synovial fibroblasts. Previous research has suggested that cIAP1/2 inhibitors reduce inflammation by acting on endothelial cells to inhibit leukocyte migration (1). In addition, fibroblast-like synovial cells, which play a pathological role in pannus formation, have been shown to be susceptible to SMAC mimetic–induced apoptosis (16). Our results expand on these observations and add a novel mechanism by means of which SMAC mimetics inhibit inflammation by directly targeting pro-inflammatory CD4⁺IL-17A⁺ and IL-17A⁺ TCRγδ T cells. Although we did not observe any significant effect of GT13072 on cell survival in vitro in Th17-polarizing conditions, we cannot exclude the possibility that GT13072 induces cell death in other cell types, nor can we rule out cytotoxicity as a cause of reduced cellularity in the joints and lymph nodes.

The ability of GT13072 to promote Treg cells was confirmed in vivo and in vitro, although the mechanism remains to be established. In vitro, we found that GT13072 induces Treg cells only in the presence of other immune cells. These results are in line with findings reported by Dougan et al. (22), who observed a modest increase in CD25 but not in Foxp3 expression in pure CD4⁺ T cell cultures following cIAP antagonism. Therefore, these two results suggest that the effect of SMAC mimetics on Treg is dependent on other cells, possibly monocyte-derived dendritic cells (DCs), which have been shown to enhance Treg numbers (52). In support of this conclusion is the work of Müller-Siehler et al. (25), who showed that cIAP antagonism enhanced the maturation of monocyte-derived DCs while at the same time reducing their responses to TNF-α. On the other hand, NFAT has been linked to Treg functionality (53, 54), so it could be envisioned that cIAP1/2 targeting leads to changes within Treg numbers themselves.

In summary, our research has demonstrated a novel role for cIAP1/2 in IL-17A regulation, and we have confirmed the validity of cIAP1/2 as therapeutic targets in immune-mediated joint inflammation driven by IL-17A. In addition, we have demonstrated a synergistic interaction between a cIAP1/2 inhibitor and a TNF-α inhibitor, whereby combined treatment leads to an expansion of Treg numbers in the joints, accompanied by reduced numbers of IL-17A⁺ cells and prolonged amelioration of inflammation. Thus, combining anti-TNF therapy with a cIAP1/2 inhibitor in patients with RA may have the potential to extend a potent disease-suppressive effect into long-term treatment-free remission.

METHODS

Compounds
Compounds GT13072, GT14424, GT14426, and GT14462 and an inactive control compound (GT14829) were supplied by TetraLogic Pharmaceuticals. For in vitro experiments, the compounds were dissolved in dimethyl sulfoxide (Sigma-Aldrich) to a concentration of 10 mM and stored at −20°C. The stock solution was further diluted in cell culture medium before use. For in vivo experiments, compounds were dissolved in a 50 mM citrate solution (pH 5.0).

Induction and treatment of arthritis
Arthritis was induced in DBA/1 mice, as described previously (55–57). In brief, bovine type II collagen was purified from articular cartilage and dissolved in 0.1 M acetic acid. Male DBA/1 mice (Harlan UK) received subcutaneous 100 μl injections of 200 μg of bovine type II collagen in complete Freund’s adjuvant (BD Biosciences) at the base of the tail and on the flank. After immunization, the mice were monitored daily for arthritis. Once an animal showed signs of arthritis, it was randomly assigned to a treatment group and monitored daily. Animals received GT14829 (10 mg/kg per day) or GT13072 (1, 5, or 10 mg/kg per day) intraperitoneally. Etanercept-treated animals received four doses of 100 μl (10 mg/kg) of etanercept (Pfizer) intraperitoneally on days 1, 3, 6, and 8. The experimental design is depicted in Figs. 1B and 5A. All mice with CIA were treated until day 10 of arthritis. On day 10, some animals were culled, whereas others in remission were maintained until day 18 before being culled. In both cases, peripheral blood, lymph nodes, and paws were taken. Arthritis severity was scored by an experienced nonblinded investigator as follows: 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, and 3 = ankylosis. All four limbs were scored, giving a maximum possible score of 12 per animal.

Hind paws of CIA animals on day 10 were fixed in paraformaldehyde and decalcified with EDTA. After being embedded in paraffin, the paws were sectioned and stained with hematoxylin and eosin. Each joint was scored by a researcher blinded to the study as follows: 0 = normal, 1 = cell infiltration with no signs of joint erosion, 2 = inflammation with the presence or erosion limited to discrete foci, and 3 = severe and extensive joint erosion with loss of architecture. Three hind paw joints (proximal interphalangeal, metatarsal phalan- geal, and tarsal metatarsal) were scored and a mean was calculated, giving a maximum possible score of three per animal. All procedures were approved by the Clinical Medicine Ethical Review Board and the UK Home Office.

Analysis of cellular immune responses ex vivo
A single-cell suspension was prepared from arthritic paws as follows. Each arthritic paw was digested in 1 ml of deoxyribonuclease I (0.1 mg/ml) and liberase (0.313 mg/ml) (both from Roche Diagnostics) for 90 min. Cells (2 × 10⁵ per well) were cultured in a 96-well plate in 200 μl of RPMI 1640 with 1-glutamine, 10% fetal calf serum, and penicillin/streptomycin (all from Life Technologies). To detect cytokine secretion, paw cells were stimulated with an anti-CD3e antibody (clone 145-2C11, eBioscience) for 48 hours.
Flow cytometric analysis and cell sorting

Cells were stimulated with phorbol 12-myristate 13-acetate (0.02 µg/ml), 0.4 µM ionomycin, and brefeldin A (1.25 µg/ml) (all from Sigma-Aldrich) for 4 hours. The antibodies used for surface and intracellular stainings of human and mouse cells are listed in tables S1 and S2, respectively. The samples, except for those in which IL-4 was detected, were fixed and permeabilized with the buffers provided with the FoxP3 Staining Buffer Set (eBioscience). To detect IL-4, cells were fixed with a 2% solution of formaldehyde (Merck) in phosphate-buffered saline (PBS). The permeabilization buffer contained 0.05% saponin, 0.5% bovine serum albumin (both from Sigma-Aldrich), 2 mM EDTA (Invitrogen), 0.02% sodium azide (G-Biosciences), and PBS. Data were acquired on a Canto II flow cytometer using FACSDIVA software (all BD Biosciences) and analyzed using FlowJo software (Tree Star). The gating strategy used to identify T cell subsets is described in our previous work (58).

For the human Th17 differentiation assay, PBMCs from healthy donors were stained with CD4, CCR6, and CD161 and sorted on a BD FACSAria II flow cytometer to >96% purity. For quantitative polymerase chain reaction (PCR), human PBMCs were sorted as CD4+CD45RO− lymphocytes.

Human Th17 differentiation/induction assay

Human CD4+CCR6+CD161+ T cells from healthy donors or synovial cells from RA patients were cultured in Iscove’s modified Dulbecco’s medium with KnockOut Serum Replacement (both from Life Technologies) for 6 days in the presence of Human T Cell Activator CD3/CD28 Dynabeads (Life Technologies). IL-1β, IL-6, IL-23, and transforming growth factor-β (TGF-β) (all at 10 ng/ml) were added on the first day of culture. On day 3, IL-2 (0.5 U/ml) and IL-23 were added. All cytokines were supplied by PeproTech, except IL-23, which was obtained from R&D Systems. Synovial tissue biopsies were isolated by density gradient centrifugation with Lympholyte-H (Cedarlane).

Mouse Th1 differentiation assays

CD4+ T cells from C57BL/6 mice were cultured in Iscove’s modified Dulbecco’s medium with KnockOut Serum Replacement (both from Invitrogen) for 5 days in the presence of Th1 [IL-12 (12 ng/ml) and anti–IL-4 (10 µg/ml)] or Th17 [TGF-β (3 ng/ml), IL-6 (30 ng/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml), anti–IL-4 (10 µg/ml), and anti–IFN-γ (10 µg/ml)] conditions and anti-mouse CD3 (1.0 µg/ml; clone 145-2C11, eBioscience) and anti-mouse CD28 (0.5 µg/ml; clone 37.51, eBioscience). Mouse BALB/cAnCrI CD4+ T cells were cultured in Th1 [IL-4 (40 ng/ml) and anti–IFN-γ (10 µg/ml)] conditions in RPMI 1640. Neutralizing antibodies were supplied from BioLegend. All cytokines were supplied by PeproTech, except IL-23 (R&D Systems). All mice were obtained from Harlan UK. Secreted cytokines were measured using the MSD platform according to the manufacturer’s instructions.

Human PBMC isolation

PBMCs from healthy donors were obtained from component donation cones supplied by the National Blood Transfusion Service. PBMCs were isolated by density gradient centrifugation with Lympholyte-H (Cedarlane).

Apoptosis detection

Apoptosis was detected using the Annexin V Apoptosis Detection Kit along with propidium iodide according to the manufacturer’s instructions (Sigma-Aldrich).

RNA isolation and quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) from human CD4+ CD45R0+ T cells cultured in Iscove’s modified Dulbecco’s medium with KnockOut Serum Replacement (both from Life Technologies) for 17 hours in the presence of IL-1β, IL-6, IL-23, and TGF-β (all at 10 ng/ml) and Human T Cell Activator CD3/CD28 Dynabeads (Life Technologies). Complementary DNA was transcribed using the Reverse Transcription System (Promega). For quantitative PCR, reactions were performed using TaqMan primers and probes (Applied Biosystems). Probes used are listed in table S3. The comparative threshold cycle (Ct) method (ΔΔCt) was used for relative quantification of gene expression.

Western blotting

Human lymphocytes or CD4+ T cells were cultured in RPMI 1640 or Iscove’s modified Dulbecco’s medium with KnockOut Serum Replacement (all from Life Technologies). GT13072 or GT14829 was added for 10 min. Proteins were extracted with radioimmunoprecipitation assay buffer supplemented with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail according to the manufacturer’s instructions (all from Sigma-Aldrich). Protein (20 µg) was loaded on a NuPAGE Novex 4-12% Bis-Tris gel and separated using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen). Proteins were transferred onto a 0.2-µm polyvinylidene difluoride membrane (Amersham). The antibodies used are listed in table S4. Bound antibodies were identified with the ECL Plus Western Blotting Detection Kit (GE Healthcare).

Statistical analysis

Data are presented as the arithmetic mean ± SEM. Comparisons between two groups were carried out using an unpaired two-tailed Student’s t test. Comparisons between more than two groups were carried out using an analysis of variance (ANOVA) with Dunnett’s (one-way) or Tukey’s (two-way) post hoc test. Probability values (P) of less than 0.05 were considered significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., USA).
Fig. S2. GT13072 inhibits joint destruction and induces a rise in TNF-α and IL-10 levels in serum.

Fig. S3. GT13072 has selective effect on IL-17A cells.

Fig. S4. cIAP1/2 inhibition down-regulates IL-17A secretion but has no effect on TNF-α or IFN-γ.

Fig. S5. GT13072 selectively inhibits IL-17A expression.

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/5/eaaw5422/DC1

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acknowledgments: we thank tetralogic pharmaceuticals for supplying gt13072 and gt1482, p.m. ajamdi and j. webber (university of oxford) for cell sorting, and i. parisi (university of oxford) for histology services. funding: this work was funded, in part, by a grant from the european union–funded seventh framework programme project "profiling responder in antibody therapies" (priat). author contributions: j.z.k. participated in study design; conducted experiments, data analysis, and interpretation; prepared figures; and drafted and approved the manuscript. j.o. participated in study design; conducted experiments, data analysis, and interpretation; prepared figures; and drafted and approved the manuscript. p.j.v. and r.o.w. participated in study design and data interpretation and edited and approved the manuscript. competing interests: the authors declare that they have no competing interests. data and materials availability: all data needed to evaluate the conclusions in the paper are present in the paper and/or the supplementary materials. additional data related to this paper may be requested from the authors.

submitted 3 january 2019
accepted 19 march 2019
published 1 may 2019
10.1126/sciadv.aaw5422

citation: j.z. kawalkowska, j. ogbechi, p.j. venables, r.o. williams, ciap1/2 inhibition synergizes with tnfr inhibition in autoimmunity by down-regulating il-17a and inducing \( t_{\text{reg}} \). *sci. adv.* 5, eaaw5422 (2019).
cIAP1/2 inhibition synergizes with TNF inhibition in autoimmunity by down-regulating IL-17A and inducing Tregs
Joanna Z. Kawalkowska, Joy Ogbechi, Patrick J. Venables and Richard O. Williams

Sci Adv 5 (5), eaaw5422
DOI: 10.1126/sciadv.aaw5422

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