

Interleukin-13-mediated colitis in the absence of IL-4R α signalling

Short title: IL-13 induced colitis independent of IL-4R α .

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Abbreviations: interleukin (IL); Interleukin-4 receptor-alpha (IL-4R α); ethanol (etoh), oxazolone (oxa); Extracellular signal-regulated kinases (ERK); Mitogen-activated protein kinases (MAPK).

Sufficient evidence points to interleukin (IL)-13 as an important pathological factor in ulcerative colitis and raises hopes to a promising new treatment strategy, [1-3]. However, the outcome of two recent clinical trials, both published in *Gut* 2015 suggest otherwise, [4, 5]. A commentary published in the same issue, described these results as crushing the enthusiasm for anti-IL-13 treatment in ulcerative colitis, [6]. In this letter we show evidence that the disease outcome is determined by the type of signalling pathway utilized by IL-13 in mice. Therefore we suggest that directly blocking IL-13 remains a potential treatment strategy for a subset of ulcerative colitis patients that have elevated tissue IL-13 production.

In the first clinical study using Anrukinzumab treatment, which targets the IL-4 receptor-alpha (IL-4R α) and not IL-13 directly, had no effect on improving the clinical response or remission rates of ulcerative colitis patients, [4]. In contrast, although patients treated with Tralokinumab, which blocks IL-13 directly, showed no improved clinical response rate, there was a significantly improved clinical remission rate and partial Mayo score at week 4 in these patients [5]. Here it was suggested that the ability of patients to respond to Tralokinumab depends on baseline levels of colonic mucosal IL-13 and that targeting a sub-group of patients with high levels of mucosal IL-13 may prove to be beneficial in these patients. Highlighting the mode of action of the therapies above, IL-4 and IL-13 both signal through the IL-4R α . This receptor is part of the type I (IL-4R α / γ c) and type II (IL-4R α /IL-13 α 1) receptor complexes. While Anrukinzumab only blocks signalling of IL-13 through IL-4R α , Tralokinumab blocks IL-13 itself, and would therefore prevent IL-13 from binding any other potential receptors.

Using a mouse model of acute oxazolone-induced colitis [7, 8], we confirmed that IL-13-deficient mice are protected from developing colitis (Table 1, see IL-13^{-/-}, Supplementary figure 1 and Supplementary figure 2).

Table 1: Disease activity in oxazolone treated gene-deficient or transgenic BALB/c mice.

Groups	Body weight ^a	Distress Score ^b	Colon length (cm)	Colitis Score ^c	Previously published
BALB/c etoh	98.19±1.6	3.33±0.2	8.23±0.5	2.78±0.5	BALB/c [2]
BALB/c oxa	91.66±1.8 *	9.38±0.9 ***	6.81±0.4 *	8.33±0.8***	BALB/c [2]
BALB/c IL-4 ^{-/-}	89.85±1.4 **	9.34±1.2 **	7.84±0.6	6.78±1.3*	SJL/J anti-IL-4 [9]
BALB/c IL-13 ^{-/-}	98.74±1.5 #	5.50±0.5 #	9.53±0.6	5.50±0.6 #	BALB/c IL-13 ^{-/-} [2] C57Bl/10 anti-IL-13 [7]
BALB/c IL-13 trans	88.50±1.2 #	12.33±1.2 ###	6.50±0.6 **	12.00±0.5###	
BALB/c IL-4Rα ^{-/-} /IL-4 ^{-/-}	87.21±2.2 **	9.00±1.4 *	7.47±0.1 *	9.57±0.5***	

* = p <0.05, ** = p <0.01 and *** = p <0.001 vs BALB/c etoh control.

= p <0.05 and ### = p <0.01 BALB/c oxa vs. genetically modified mice.

^a Body weight is shown as percentage starting weight 2 days post-challenge.

^b Distress is scored according to appearance, clinical signs, natural and provoked behaviour.

^c Colitis score out of 15 (Supplementary methods).

In striking contrast, mice deficient for IL-4Rα, [10] are highly susceptible to disease onset and develop an exacerbated pathology compared to BALB/c wild-type control mice (Figure 1A-D, Supplementary methods). Rapid onset colitis was marked by weight loss and an increased distress score. Macroscopic examination revealed severe colitis limited to the distal half of the colon, with inflammation-associated colon shortening. Microscopic examination showed superficial inflammation scored according to the presence of mononuclear cells, oedema, infiltration of granulocytes, epithelial layer disruption and loss of mucus production. The IL-13-driven pathology can be reverted to protection in IL-4Rα/IL-13-double deficient mice (Figure 1A-D). Hence, the protection in these mice and the detectable level of IL-13 in IL-4Rα-deficient mice suggests a potential role of IL-13 signalling in mediating colitis, independently of the IL-4Rα.

Although further investigation is required to confirm a mechanism of the IL-4R α -independent signalling pathway of IL-13, our results are important to consider when designing new therapeutic strategies against T helper type 2-mediated inflammation. This data also suggests that attempts to neutralize IL-4R α may indeed have a detrimental outcome on disease. Furthermore, results from our previous work in an ovalbumin-induced dermatitis model suggest that this mechanism may not be limited to ulcerative colitis [11]. In dermatitis, the complete protection of IL-4R α /IL-13-double deficient but not IL-4R α mice was associated with a decrease in Extracellular signal-regulated kinases (ERK) phosphorylation of the Mitogen-activated protein kinases (MAPK) signal transduction pathway. This alludes to the possible involvement of IL-13 signalling through the IL-13R α 2, or theoretically an unknown IL-13 receptor. In summary, we conclude that IL-13 signalling is able to drive colitis in the absence of IL-4R α and suggest that anti-IL-13 treatment but not anti-IL-4R α treatment strategies could still be beneficial in a subset of ulcerative colitis patients with increased IL-13.

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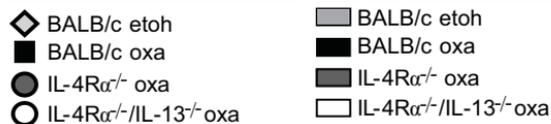
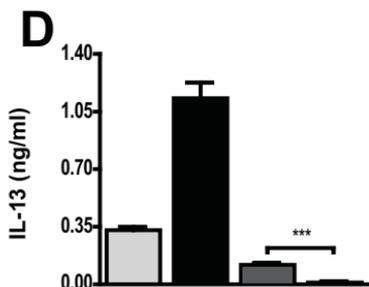
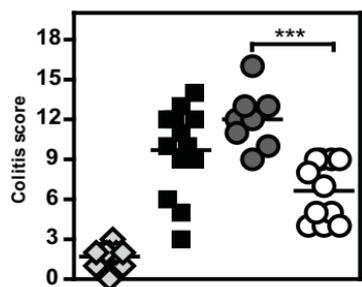
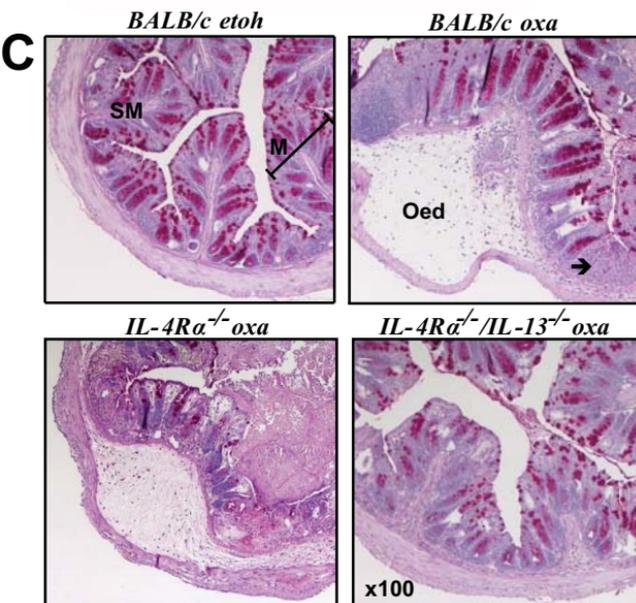
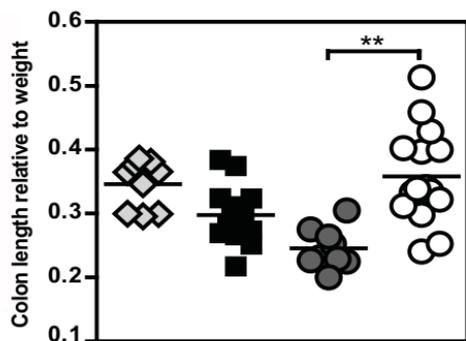
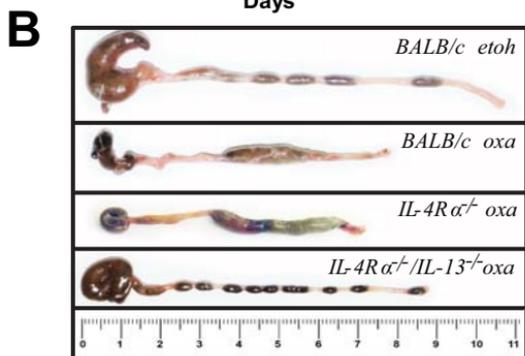
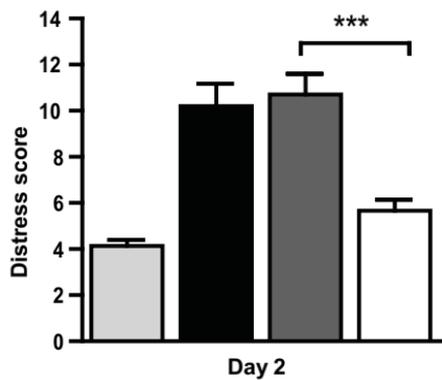
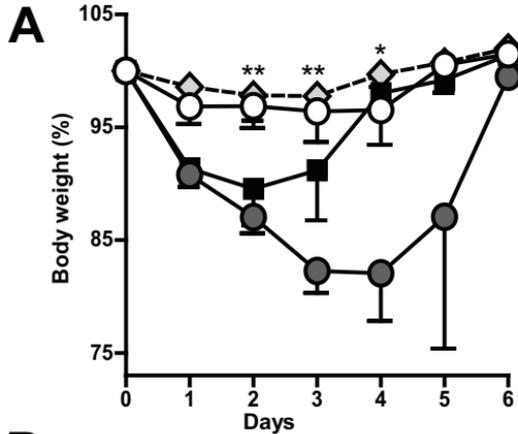
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FIGURE LEGEND

Figure 1

Deletion of IL-4R α exacerbates oxazolone-induced colitis which is rescued in the absence of IL-13

(A) Body weight (day6) and distress score (day2). (B) Macroscopic appearance and colon length. (C) PAS-stained distal colon and colitis score. (D) Mesenteric lymph node IL-13 production. Data represents 2-3 individual experiments (n=4-10 mice) and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, IL-4R $\alpha^{-/-}$ oxa vs. IL-4R $\alpha^{-/-}$ /IL-13 $^{-/-}$ oxa mice.



SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

IL-4/IL-13 mediates oxazolone-induced colitis in BALB/c mice

(A) Body weight (day7) and distress score (day2). (B) Macroscopic appearance and colon length. (C) PAS-stained distal colon sections and colitis score. SM=submucosa, M=mucosa, →infiltrating mononuclear cells, Oed=oedema (D) Systemic cytokine production. Data represents >3 individual experiments (n=4-10 mice) and *=p<0.05, **=p<0.01 and ***=p<0.001 BALB/c oxa vs. BALB/c etoh-only control mice and #=p<0.05 and ##=p<0.01 BALB/c oxa vs. IL-4/IL-13^{-/-} oxa.

Supplementary Figure 2

IL-13 mediates oxazolone-induced colitis in BALB/c mice

(A) Body weight (day3) and distress score (day2). (B) Macroscopic appearance and colon length. Data represents 2 individual experiments (n=7-10 mice) and *=p<0.05, **=p<0.01 and ***=p<0.001 BALB/c oxa vs. IL-13^{-/-} oxa.

SUPPLEMENTARY METHODS

Mice

Male BALB/c mice (6-8 weeks old) and IL-4^{-/-}, IL-13^{-/-}, IL-4R α ^{-/-} and IL-13 transgenic [IL-13^{trans}] mice on a BALB/c background were used in the experiments, [1-5]. Furthermore, IL-4^{-/-}, IL-13^{-/-}, IL-4R α ^{-/-} and IL-13^{trans} mice were inter-crossed to generate IL-4/IL-13, IL-4R α /IL-4, IL-4R α /IL-13 double-deficient and IL-4R α ^{-/-}/IL-13^{trans} strains. All mice were housed in specific pathogen-free conditions at the University of Cape Town, South Africa and experiments were approved by the University's Animal Ethics Committee.

Induction of colitis by haptening agent oxazolone

Oxazolone-mediated colitis was induced in BALB/c mice by modifying previously described methods, [6]. Anesthetized mice were sensitized on the shaved abdomen by application of 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich) in 100% ethanol (150µl) followed 7 days later by intra-rectal administration of 1% oxazolone in 50% ethanol (150µl). Control mice were sensitized and challenged with ethanol only for all groups. Mice were weighed and monitored daily and killed 3 days post-challenge for immunopathological analyses or 6 days post-challenge for survival studies.

Disease activity index

Oxazolone treated BALB/c mice develop rapid-onset colitis marked by weight loss and distress score. Disease progression was determined as previously described, [6]. Briefly, weight loss was measured as a percentage of starting weight and distress was scored at day 2 post-challenge. Colon length was measured from the anus to the caecum and recorded as an indication of inflammation.

Histological assessment of colitis

Colon sections taken 1 cm from the anus were processed as previously described and stained with haematoxylin and eosin (H&E) for inflammatory cells or Periodic acid-Schiff reagent (PAS) for mucus producing goblet cells. Semi-quantitative histopathological grading of oxazolone-induced colitis was determined as previously described, [6]. Mice were graded on 5 criteria, 1) presence of mononuclear cells, 2) reduced goblet cells, 3) epithelial injury, 4) granulocyte infiltration and 5) oedema. Each criteria were scored from 0-3 and the total score was added resulting in a total additive score of between 0 (no colitis) and 15 (maximal colitis activity). Histology sections were processed and stained by the Department of Surgery, Groote Schuur Hospital.

IL-13 production by mesenteric lymph nodes

Individual mesenteric lymph nodes were collected from oxazolone treated mice respectively in complete Iscove's Modified Dulbeccos Medium (IMDM) supplemented with; 10% FCS (Gibco Life Technologies), penicillin, streptomycin, 1mM sodium pyruvate, 50µM B2-ME (Sigma) and NEAA (Invitrogen). Single cell suspensions were obtained by pressing lymph nodes through a 70µm cell strainer (Falcon) and centrifugation at 1200rpm for 5min at 4°C in a 15ml tube. The pelleted cells were depleted of red blood cells by hypotonic lysis in Red Cell Lysis Buffer and the centrifugation step was repeated. Cells were resuspended in 2ml complete IMDM and stained with Trypan Blue (Sigma-Aldrich) to count viable cells in a haemocytometer. Cells were diluted to 1×10^6 cells/ml and cultured in a 96-well plate in complete IMDM with anti-CD3 (clone: 145-2C11, 10µg/ml) for 48h at 37°C and 5% CO₂. Supernatants were collected and stored at -80°C.

Cytokine ELISA

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Maxisorp microtitre plates (Nunc) were coated with purified anti-IL-13 (clone: 38213, 1µg/ml, BD Pharmingen), diluted in phosphate buffered saline (PBS, pH 9.5) and incubated overnight at 4°C. Plates were blocked with milk powder in PBS for 3h at 37°C and serially diluted standards, purified recombinant IL-13 (BD Pharmingen) was added. Cell supernatants were added, diluted in ELISA dilution buffer containing bovine serum albumin in PBS and incubated overnight at 4°C. Biotinylated anti-mouse secondary antibody for IL-13 (goat anti-mouse, 0.5µg/ml, BD Pharmingen) was added for 3h at 37°C followed by HRP labelled streptavidin. Subsequently, plates were incubated with TMB Peroxidase Substrate (KPL), the colour reaction stopped with 2M H₂SO₄ and absorption measured at 450nm. ELISA wash buffer was used to wash plates 4x between each step.

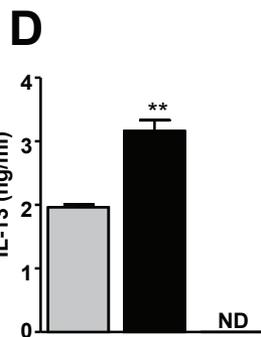
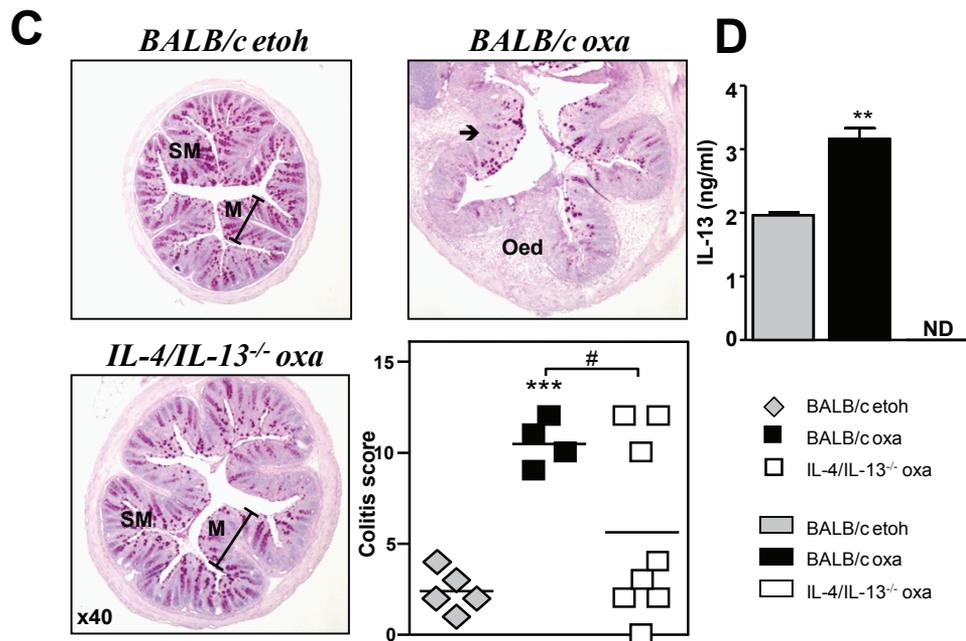
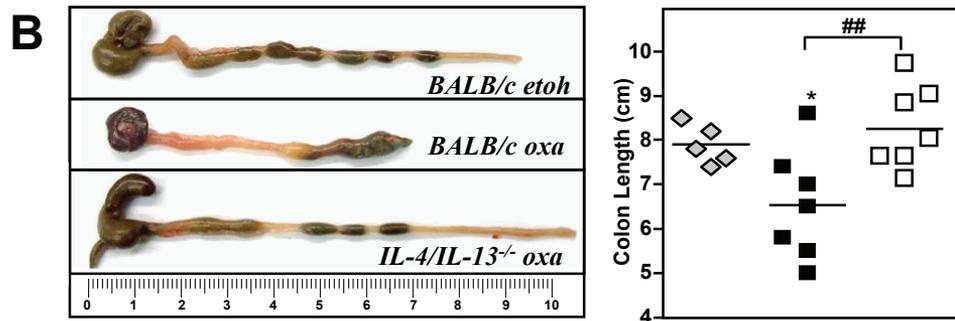
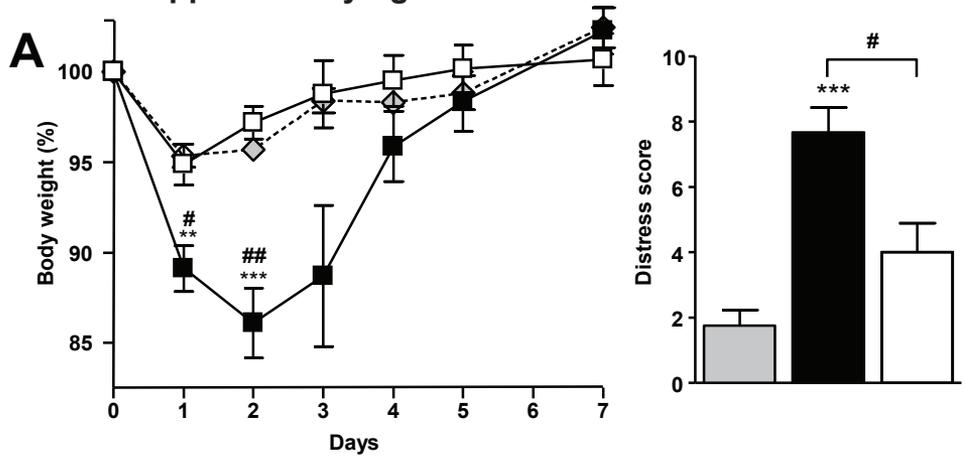
Statistical analysis

Values are given as mean \pm SEM, and significant differences were determined using unpaired two-tailed students *t* test or one-way ANOVA using a Bonferroni post-test (GraphPad Prism). Values of $p < 0.05$ were considered significant.

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Supplementary figure 1



- ◇ BALB/c etoh
- BALB/c oxa
- IL-4/IL-13^{-/-} oxa
- BALB/c etoh
- BALB/c oxa
- IL-4/IL-13^{-/-} oxa

Supplementary figure 2

