Journal of Crohn's and Colitis, 2015, 647–658
doi:10.1093/ecco-jcc/jjv089
Advanced Access publication May 18, 2015
Original Article

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Oral Delivery of Particulate Transforming Growth Factor Beta 1 and All-Trans Retinoic Acid Reduces Gut Inflammation in Murine Models of Inflammatory Bowel Disease

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Abstract

Background and aims: We investigated oral delivery of transforming growth factor beta 1 [TGFβ]- and all-trans retinoic acid [ATRA]-loaded microspheres as therapy for gut inflammation in murine models of inflammatory bowel disease [IBD].
Methods: ATRA and TGFβ were separately encapsulated in poly [lactic-co-glycolic] acid or polylactic acid microspheres [respectively]. TGFβ was encapsulated using proprietary phase-inversion nanoencapsulation [PIN®] technology.
Results: PIN® particles provided sustained release of bioactive protein for at least 4 days and were stable for up to 52 weeks when stored at either 4⁰C or -20⁰C. In the SCID mouse CD4 + CD25-T cell transfer model of IBD, oral treatment starting at disease onset prevented weight loss, significantly reduced average disease score [~ 50%], serum amyloid A levels [~ 5-fold], colon weight-to-length ratio [~ 50%], and histological score [~ 5-fold].
Conclusions: Both agents given together outperformed either separately. Highest TGFβ doses and most frequent dose schedule were most effective. Activity was associated with a significant increase [45%] in Foxp3 expression by colonic lamina propria CD4+ CD25+T-cells. Activity was also demonstrated in dextran sulphate sodium-induced colitis. The data support development of the combination product as a novel, targeted immune based therapy for treatment for IBD.

Keywords: IBD; TGFβ; ATRA

1. Introduction

Conventional therapies for inflammatory bowel disease [IBD], including Crohn's disease [CD] and ulcerative colitis [UC], fall into three groups: salicylates, immunosuppressants, and antibiotics. Although these are effective in treating active disease, remissions are often short-lived and associated with significant side effects. Despite therapy, 80% and 45% of CD and UC patients [respectively] require surgery.[1,2,3,4,5,6]

More recently, the so-called biological response modifiers or 'biologicals' have emerged: macromolecules that target inflammatory lymphocytes or the cytokines they produce.[7] One such molecule, infliximab, a chimeric anti-human tumour necrosis factor alpha [TNFα]

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antibody, earned Food and Drugs Administration [FDA] approval in 1998 with a high response rate, significant mucosal and fistula healing, and long-term remissions in Crohn's disease. Other emerging biologicals include anti-p40, anti-p19, anti-IL-12, anti-IL-17, and anti-alpha 4 integrin antibodies.[7,8] However, an estimated 30% of patients will not respond to biologicals and of those who initially respond, 50% relapse within a year. Biologicals have had only modest impact on surgical intervention rates.[9] Thus, the need for a deeper understanding of pathogenesis and novel, targeted therapies remains acute.

Although aetiologies are incompletely understood, genetic, immunological, and environmental factors all make significant contributions. Human and animal studies implicate abnormal responses to commensal microflora and perturbed local immune homeostasis. In CD, activated Th1 cells have been implicated, although emerging evidence suggests important roles for Th17 cells. In UC, on the other hand, IL-1, IL-5, IL-6, IL-8, and TNF-α responses are increased, but not IL-4 or IFNγ, suggesting abnormal macrophage and monocyte activity.[1,2,10,11,12,13]

Regulatory T-cells [Treg] and mucosal dendritic cells [DC] help maintain gut immune homeostasis and represent attractive therapeutic targets.[13,14,15,16,17] Adoptively transferred CD4+ CD25+ Foxp3+ TGFβ-dependent Tregs prophylactically and therapeutically limit IBD via direct suppression of T-effector priming activity and innate effector function.[18,19,20] Separately, mucosal DCs produce retinoic acid, an essential cofactor in TGFβ-dependent Treg generation in the gut.[16,17] This retinoic acid requirement was linked to induction of Foxp3 expression in T cells.[21] Thus, these molecules represent potential therapeutic agents to treat IBD by activation and expansion of gut Tregs.

However, acid-induced hydrolysis and enzymatic degradation often prevent effective oral delivery of bioactive macromolecules, such as TGFβ.[22,23] Encapsulation of labile biologicals into biodegradable polymer-based microspheres provides protection from stomach acids and proteases, allowing safe passage into the lumen of the gastrointestinal [GI] tract for uptake.[24,25] Although the use of polymeric particles for oral drug delivery to the inflamed colon is not a new concept,[26] the utility of this technology in delivery of labile protein-based therapeutics to gut tissues is yet to be fully determined. Phase inversion nano-encapsulation [PIN®] utilizes a non-mechanical encapsulation approach that preserves the structural integrity and biological activity of macromolecules, provides extended shelf life at room temperature [years] and allows effective delivery of protein therapeutics to the intestinal immune structures.[28,29]

In the present studies, we investigated whether oral administration of TGFβ- and ATRA-loaded PIN® microspheres could ameliorate disease in murine models of IBD.

2. Methods

2.1. Preparation and characterization of ATRA- and TGFβ-loaded microspheres

2.1.1. Microsphere preparation

ATRA was encapsulated into poly [lactic-co-glycolic] acid microspheres [1 mg of ATRA per gram of particles] using a modification of the solvent evaporation technique.[27] Briefly, 5.0 mg of ATRA [Sigma-Aldrich] was dissolved in 20 ml dichloromethane [DCM] to produce a 250 mg/ml solution in an amber vial; 8 ml of this solution was added to 1000 mg of 503H polymer [Resomer] dissolved in 32 ml DCM to produce the oil phase; 20 µl of Span 80 was also pipetted into this solution. To prepare the water phase, 600 ml of 1% 25K polyvinyl alcohol [PVA] was added to a glass beaker placed on ice within a stainless steel container. The water phase was then set under a Silverson mixer at a shear rate of 4000 rpm for 75 min as the oil phase was added via a glass pipette. The emulsion was then transferred to the overhead mixer with an additional litre of double-distilled water and spun for 225 min. The microspheres were washed, centrifuged, and lyophilized for 48 h.

TGFβ was encapsulated into polylactic acid [PLA] microspheres [0.285 mg TGFβ per gram of particles] using PIN® as described previously.[28]

2.1.2. *In vitro* drug release

Human recombinant TGFβ [Peprotech, Rocky Hill, NJ] was encapsulated into microspheres and release tested using an *in vitro* release assay described previously.[29] Briefly, 10 mg of TGFβ PIN® particles were suspended in 1.0 ml release buffer and 0.2 ml transferred to the wells of a 96-well plate in triplicate. The plate was incubated at 37°C in 5% CO_2, the supernatant replaced daily and stored at -20°C for analysis in TGFβ ELISA [Pierce-Endogen, Thermo-Fisher Scientific Inc., Rockford, IL] and bioassay [see below].

2.1.3. Stability studies

TGFβ particles were stored at 4 or -20°C, and samples removed at selected time points. For each time point, an overnight release assay was performed as above. TGFβ concentration and bioactivity values in supernatants were then compared with those observed for the same samples prior to storage [unstored]. Final values for specific activity were expressed as % recovered bioactivity divided by % recovered protein.

TGFβ biological activity was tested using the TGFβ sensitive mouse lymphoblasT-cell line HT-2. Cells were plated at 1.5 X 10^4 cells/well in complete media containing 15 ng/ml murine IL-4. Standards and samples were added to the wells to produce a final volume of 200 µl/well. The 96 well plates were incubated at 37°C in 5% CO_2 for 67 h. Cell numbers were determined using Promega Cell titre 96 Aqueous One Solution Reagent [Promega, Madison, WI] as per the manufacturer's instructions.

2.2. SCID mouse CD4+ T-cell transfer colitis model

2.2.1. Animals

Mice 6 to 8 weeks old (BALB/c and CB-17 scid [SCID] mice [males and females]) were purchased from Taconic Farms [Germantown, NY]. The mice were kept under standard laboratory conditions with free access to food and water. They were allowed to adapt for 1 week before starting the study. The care and use of laboratory animals was in accordance with a University at Buffalo IACUC-approved animal use protocol.

2.2.2. Isolation of CD4+ CD25- T-cells

CD4+ CD25- T-cells were purified from the spleens of naïve BALB/c mice by magnetic bead separation using MACS® column and separator according to manufacturer's instructions [Miltenyi Biotech, San Diego, CA]. Purity and viability [> 95%] were assessed by flow cytometry [FACScan, Becton Dickinson, San Jose, CA].

2.2.3. Induction of colitis

Purified CD4+ CD25- T-cells were adoptively-transferred to SCID recipients [4 x 10^5 cells per mouse, *IP*]. Mice were monitored twice daily for disease development and scored according to criteria in Table 1. Treatment started when mice reached an average score of 8–9 out of 25 [a score of 5 considered normal].

Table 1. Disease scoring criteria.

Score	Dehydration	Activity	Coat condition	Posture	Faecal consistency
1	Normal skin tent	Explores environment	Well groomed coat	Normal posture	Normal stool; small, firm and dry
2	Skin less elastic	Slight reduction in activity	Slightly ruffled coat	Occasional hunching	Small, firm, moist, adherent
3	Skin tent	Moderate reduction in activity	Moderately ruffled coat	Moderately hunched, mobile	Larger, pliable, very adherent stool
4	Skin tent+ sunken eyes	No movement w/o stimulation	Piloerected/poorly groomed	Severely hunched, lethargic	Uniform liquid stool
5	Persistent skin tent + sunken eyes	Does not respond to physical stimulation	Completely hunched, peri-anal dermatitis	Piloerection, no movement	Liquid stool with mucus or blood

w/o, without.

2.2.4. Isolation of lymphocytes from lamina propria

Colonic lamina propria [LP] lymphocyte populations were isolated by enzymatic digestion of minced colonic sections followed by purification on lympholyte as described.[30] The CD4+ CD25+ T-cells were then analysed for Foxp3 expression by flow cytometry.[31]

2.2.5. Histology

Ascending, transverse, and descending colon from treated and control mice were fixed in 10% formalin, embedded in paraffin, sectioned [5 µm] and stained with haematoxylin and eosin [H & E]. Five randomly-selected sections from each mouse were scored for disease severity using the scoring method outlined in Table 2.

2.2.6. Serum amyloid A levels.

Serum levels of amyloid A [SAA] were measured using an ELISA kit [BioSource, Inc. Camarillo, CA].

2.3. Dextran sodium sulphate-induced colitis

2.3.1. Animals

Mice 6 8-weeks old (C57Black6 mice [males: n = 6; females: n = 6; 12 per group]) were purchased from Harlan Nossan, San Pietro al Natisone, Udine, Italy, and housed in a vivarium under pathogen-free conditions at the Department of Biomedical Sciences in the School of Medicine at the University of Catania [Catania, Italy]. The mice were kept under standard laboratory conditions with free access to food and water. They were allowed to adapt for 1 week before starting the study. The care and use of laboratory animals was in accordance with a University of Catania IACUC-approved animal use protocol.

2.3.2. Induction of colitis and treatment

Colitis was induced with 3% dextran sodium sulphate [50 kDa; Sigma-Aldrich] in drinking water for 4 days. The first treatment was given 24 h before induction of colitis [Day -1] and continued every other day for 6 days.

2.3.3. Disease score, SAA levels and histology

Disease score, SAA levels and histological analysis were performed as above.

2.4. Statistical analysis

Significance [$p \leq 0.05$] between experimental and control groups was determined using Student's t-test analysis. In experiments with multiple groups, homogeneity of inter-group variance was analysed by ANOVA.

Table 2. Histology scoring criteria.

Severity of inflammation	
0	None
1	Mild lymphoid infiltration
2	Lymphoid infiltration / focal crypt degeneration
3	Multifocal crypt degeneration and/or erosions
Extent of inflammation	
0	None
1	Mucosal
2	Submucosal
3	Transmural
Amount of mucus	
0	Normal
1	Slight decrease of mucus
2	Moderate decrease. Focal absence of mucus
3	Severe depletion of mucus
4	Total absence of mucus
Degree of proliferation	
0	None
1	Mild increase in cell numbers and crypt length
2	Moderate increase. Focally marked increase
3	Marked increase: entire section

3. Results

3.1. TGFβ is released from PIN® microspheres over 4 days

A number of technologies encapsulate small molecules such as ATRA, achieving slow release.[27,32] The gentle encapsulation of labile proteins, protecting them from degradation and retaining bioactivity and shelf stability, has been more elusive. Thus, the present studies focus on PIN® encapsulated TGFβ. The 4-day release profiles for human TGFβ from PIN® particles is shown in Figure 1A. These data demonstrate that an initial burst was followed by sustained cytokine release for at least 4 days. To determine whether the released TGFβ was active, we evaluated the 15-min time point samples for bioactivity. In this assay, the activity of the released protein was expressed as the concentration equivalent of unencapsulated TGFβ inducing that same amount of cell proliferation. Comparison of TGFβ concentrations as determined by bioassay versus ELISA revealed a high correlation [r^2 = 0.823] between the two assays, demonstrating that the activity of the encapsulated cytokine was fully preserved [Figure 1B].

3.2. Bioactive TGFβ is released from PIN® microspheres after 1 year of storage at either 4°C or -20°C

The bioactivity of the TGFβ released from PIN® microspheres stored for up to 1 year at 4°C and -20°C did not significantly differ from the bioactivity of the TGFβ released from fresh, unstored particles [Figure 2]. Overall these results show that TGFβ-loaded PIN® microspheres can be stored for at least 1 year at 4°C or -20°C with no significant reduction in the biological activity of encapsulated TGFβ.

3.3. TGFβ- and ATRA-loaded PIN® microspheres have optimal therapeutic effects on IBD when given in combination

We first sought to determine if either ATRA- or TGFβ-loaded PIN® microspheres alone could ameliorate disease as effectively as the two in combination. Mice with established disease were fed with blank microspheres [control], ATRA-loaded microspheres alone, TGFβ-loaded microspheres alone, or with the combination. Treatment was three times a week for 2 weeks. Mice were sacrificed 4 days after last treatment for analysis of colons and sera.

In contrast to single-agent treatment groups, differences between control and TGFβ/ATRA treatment groups, in terms of percent body weight change [Figure 3 panel A], changes in colon weight-to-length ratio [Figure 3 panel B] and reduced SAA levels [Figure 3 panel C] were significant. In terms of histological scoring, the combination group performed significantly better than the control or either agent alone [Figure 3 panel D]. Whereas ATRA or TGFβ alone appeared to have modest effects overall, these were not statistically significant in most cases, suggesting that combined administration of TGFβ and ATRA is essential to optimal amelioration of established disease.

3.4. Effect of TGFβ or ATRA dose on therapeutic efficacy

In the next set of experiments, using the same CD4+ CD25- T-cell transfer model of IBD, dose-response analysis was first performed for TGFβ. Mice with established disease were treated orally with a constant dose of ATRA [20 μg]-loaded PIN® microspheres and increasing doses of TGFβ-loaded PIN® microspheres. Selected disease markers were then monitored. The differences between control [blank microspheres] and treatment groups in terms of % body

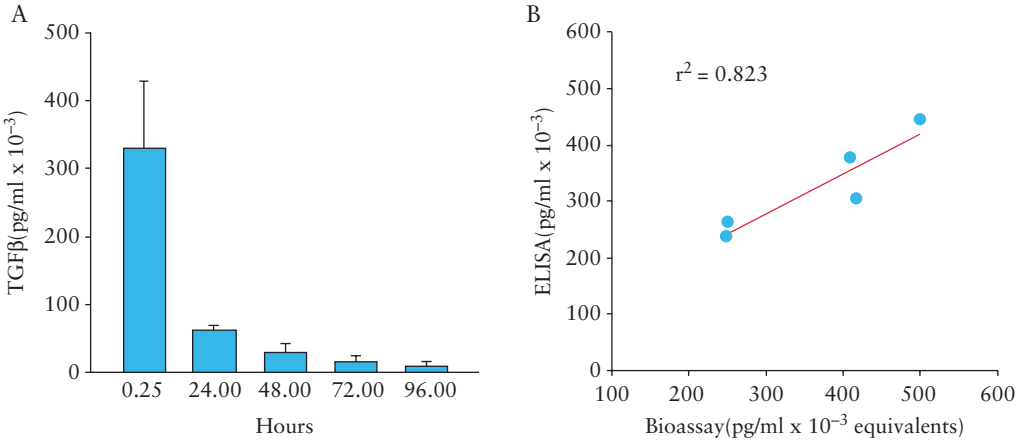

Figure 1. *In vitro* release profile and bioactivity of TGFβ over 4 days. [A] TGFβ 4-day release profile was determined by ELISA as described in Methods. Data are averages of five different batches. Error bars = standard deviation [SD]. [B] The 15-min release samples from [A] were analysed for TGFβ bioactivity as described in Methods. Bioactivity is expressed as the equivalent of unencapsulated recombinant TGFβ concentration.

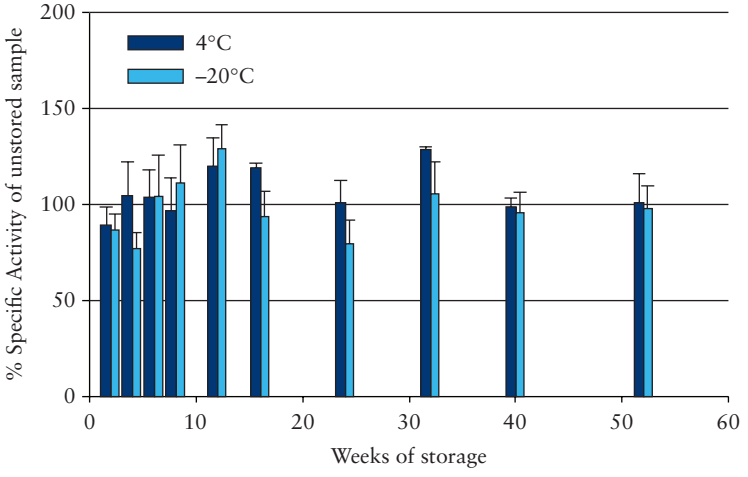

Figure 2. Effect of storage time and temperature on TGFβ biological activity. Samples stored at 4°C or -20°C were assayed for bioactivity. The bioactivity in stored samples was compared with that observed in a release sample from the same microsphere preparation assayed before storage [un-stored]. Data are averages of triplicates from one of at least three experiments with similar results and are expressed as % activity of the un-stored sample. There were no statistically significant differences between any of the time points. Error bars = standard error [SE].

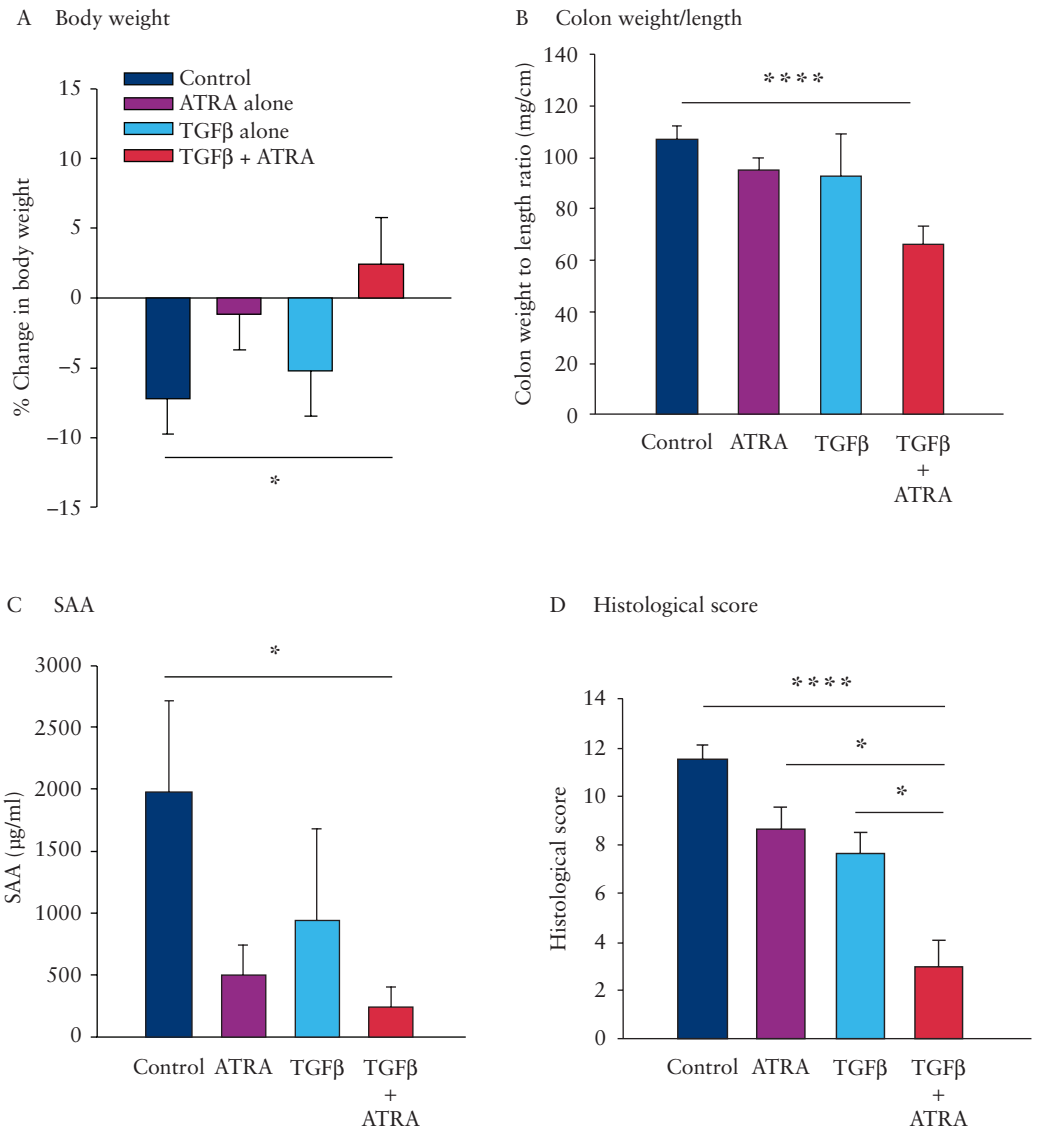

Figure 3. Therapeutic activity of oral TGFβ or ATRA-loaded particles given alone versus together in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease [IBD]. Mice with established disease [see Methods] were weighed on Day 0 and fed TGFβ [5 μg] and/or ATRA [20 μg] microspheres [17.5 mg and 20 mg, respectively], alone or in combination, or with blank microspheres [37.5 mg, control] in 0.2 ml water three times per week. After 2 weeks, mice were weighed, sacrificed, sera were taken and colons samples prepared for histological analysis [five randomly selected sections from each mouse]. Data are expressed as [A] % change in body weight relative to day of first treatment; [B] colon weight/length; [C] serum amyloid A [SAA] μg/ml as determined by ELISA; or [D] histological score. Error bars = standard error [SE], $n = 4$ per group. Significance: *, **, ***, and **** denote $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

weight change [Figure 4, panel A], colon weight-to-length [Figure 4, panel B] and SAA levels [Figure 4 panel C] were significant at all dose levels. However, by all three measures, the 10-μg TGFβ dose appeared most effective.

Next, keeping the TGFβ dose at10 μg, dose-response analysis was performed for ATRA [Figure 5]. All doses of ATRA, except the highest dose tested, appeared to slow weight loss [Figure 5, panel A] although the 10- and 20-μg doses seemed most effective. In terms of colon weight-to-length ratio [Figure 5, panel B], an apparent dose response was observed except at the 40-μg dose, which was no more effective than the 20-μg dose. All doses of ATRA significantly reduced SAA levels [Figure 5 panel C], with the exception of the 40-μg dose. Differences between treatment groups were not generally significant. These observations suggest that in the context of 10 μg TGFβ1, 20 μg ATRA is optimal in this model.

3.5. Effect of dose schedule on TGFβ/ATRA therapeutic activity

Having established effective doses for TGFβ/ATRA, the next parameter to be established in this model was the optimal treatment schedule. The same CD4+ CD25- T-cell transfer method was used to induce IBD in SCID mice. When the mice became symptomatic for IBD, they were separated into groups as follows: Group 1: untreated controls; Group 2: mice that received oral TGFβ/ATRA three times per week [3x/wk]; Group 3: treatment two times per week [2x/wk]; Group 4: one treatment per week [1x/wk]; and Group 5: one treatment over 2 weeks [1x/2wk]. Each treatment consisted of the effective doses levels for TGFβ [10 μg] and ATRA [20 μg] established in our previous studies [Figures 4 and 5]. Selected disease markers were then monitored. In terms of maintenance of body weight [Figure 6 panel A], the 3x/wk dosing schedule was significantly different from control and

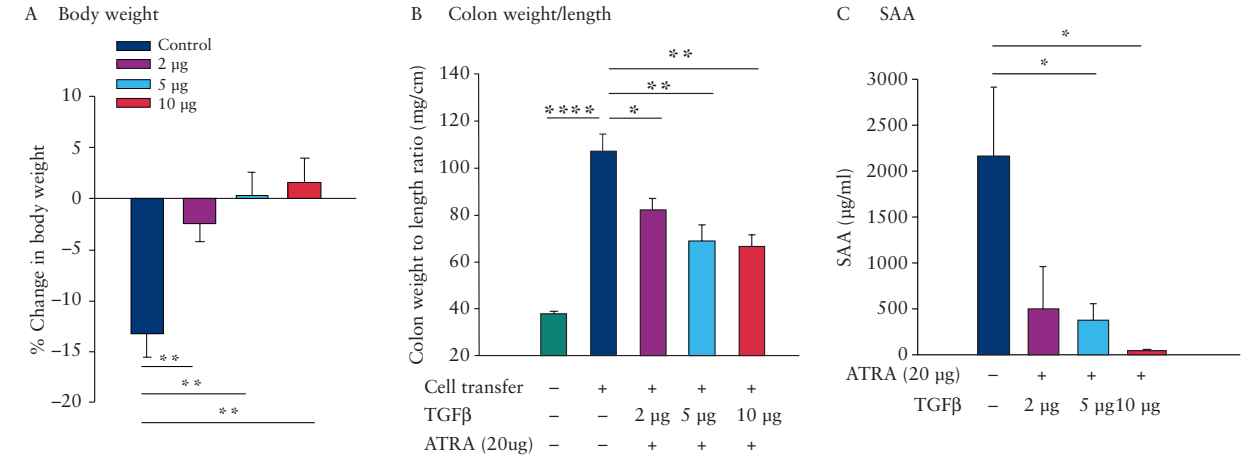

Figure 4. Dose-response of TGFβ in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease [IBD]. Mice with established disease were weighed [day 0] and fed TGFβ [2, 5, or 10 µg] and ATRA [20 µg] microspheres, or blank microspheres [control] in 0.2 ml water three times per week. After 2 weeks, mice were weighed, sacrificed, sera were taken and colons weighed and measured. Data are expressed as: [A] % change in body weight relative to day of first treatment; [B] colon weight/length; or [C] serum amyloid A [SAA] µg /ml as determined by ELISA. Error bars = standard error [SE], n = 4 to 5 per group. Significance: *, **, ***, and **** denote $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

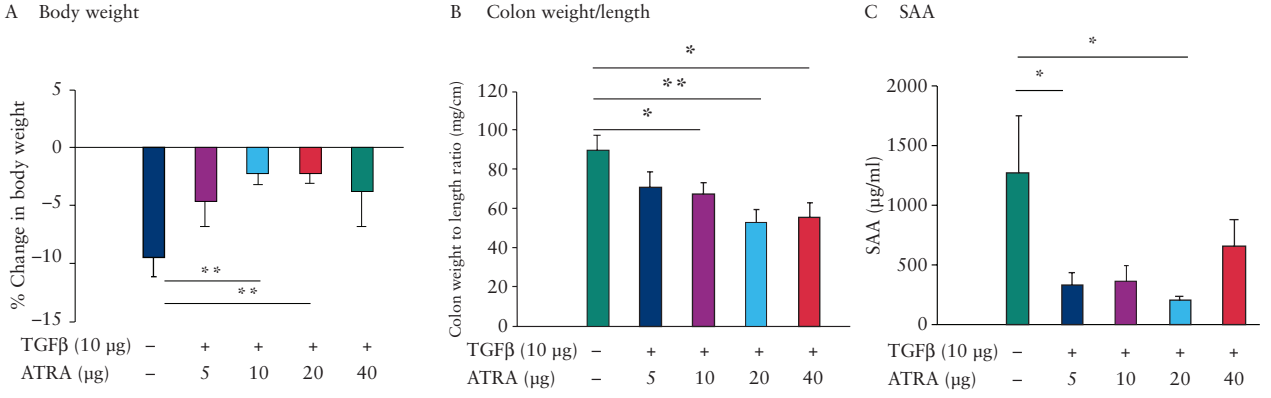

Figure 5. Dose-response of ATRA in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease [IBD]. Mice with established disease were weighed [Day 0] and fed TGFβ [10 µg] and ATRA [5–40 µg] microspheres, or blank microspheres [control] in 0.2 ml water three times per week. After 2 weeks, mice were weighed, sacrificed, sera were taken and colons were weighed and measured. Data are expressed as: [A] % change in body weight relative to day of first treatment; [B] colon weight/length; or [C] serum amyloid A [SAA] µg /ml as determined by ELISA. Error bars = standard error [SE], n = 5 per group. Significance: * and ** denote $p < 0.05$ and 0.01, respectively.

performed best. A dosing schedule consisting of three treatments per week was also found to be superior by colon weight-to-length ratio [Figure 6 panel B] and reduced SAA levels [Figure 6 panel C].

3.6 Treatment of established IBD disease with TGFβ/ATRA-loaded microspheres

To more fully test the optimised ability of oral TGFβ/ATRA-loaded microspheres to ameliorate established IBD, a larger cohort study with frequent monitoring of five independent parameters was undertaken. TGFβ/ATRA-loaded [10 and 20 ug] or blank microspheres [ACT; adoptive cell transfer] were administered orally three times per week for 2 weeks, and mice were monitored for overall disease score [Table 1] and weight loss three times a week. At the end of 2 weeks, animals were sacrificed and SAA levels, colon weight-to-length ratios and colon histology scores determined.

Treatment reversed disease rapidly as overall disease score declined within 4 days of treatment and remained low, whereas it increased from an average of 8 or 9 to over 12 in control [ACT alone] mice during the 14-day treatment interval [Figure 7 panel A]. Similarly, whereas the mice in the control group progressively lost weight [average of 17%] during this period, the weight remained stable in the treated group [Figure 7 panel B].

Mice were sacrificed on day 18 after first treatment [4 days after last treatment] and levels of SAA determined [Figure 7 panel C]. Age-matched naive SCID mice [no adoptive cell transfer] were used to establish the baseline SAA levels [~ 14 µg/ml in naive SCID]. These observations confirmed the above pattern of reduced disease score and suggested an even more dramatic reversal of disease. In control mice [ACT; fed blank particles alone] SAA levels increased over 50-fold to an average of ~ 850 µg/ml. In contrast, TGFβ/ATRA treatment reduced SAA levels to ~ 39 µg/ml. Differences between naive and control, and naive and TGFβ/ATRA-treated, groups were significant. Importantly, the difference between the control and the TGFβ/ATRA group was also significant. The colons of sacrificed mice were also analysed for colon weight-to-length ratio [Figure 7 panel D]. Colon weight-to-length ratio increased 2.7-fold in the control group in contrast to only a 1.6-fold increase in treated mice when compared with naive SCID mice. Treatment resulted in a 45% decline in colon thickening.

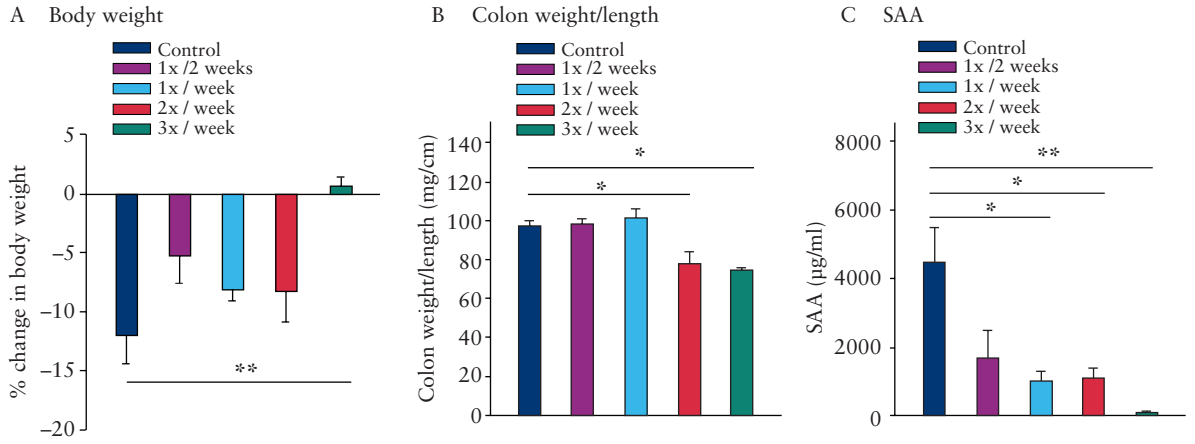

Figure 6. Effect of dose schedule on TGFβ/ATRA therapeutic activity in the SCID mouse adoptive CD4+ CD25-T-cell transfer model of inflammatory bowel disease [IBD]. Mice with established disease were weighed [day 0] and fed TGFβ [10 µg] and ATRA [20 µg] microspheres, or blank microspheres [control] in 0.2 ml water once, twice, or three times per week. After 2 weeks, mice were weighed, sacrificed, sera were taken and colons were weighed and measured. Data are expressed as: [A] % change in body weight relative to day of first treatment; [B] colon weight/length; or [C] serum amyloid A [SAA] µg /ml as determined by ELISA. Error bars = standard error [SE], $n = 4$ per group. Significance: * and ** denote $p < 0.05$ and 0.01, respectively.

Next, ascending, transverse, and descending colon from naive SCID TGFβ/ATRA-treated and control [ACT alone; fed blank particles] mice were fixed in 10% formalin, embedded in paraffin, sectioned [5 µm], and stained with H & E. Five randomly-selected sections from each mouse were scored [Table 2] for disease severity. A 22-fold increase in disease score was observed in control mice compared with naive SCID mice, whereas the severity of disease was increased only by 6-fold in the treated group [Figure 7 panel E]. The difference between naive and control was highly significant. More importantly, the difference between control and TGFβ/ATRA-treated mice was highly significant whereas naive and TGFβ/ATRA-treated mice were not significantly different.

3.7. Efficacy and toxicity of long-term TGFβ/ATRA microsphere therapy

IBD was established in SCID mice using the same adoptive cell transfer model as before. Mice were considered to have IBD when they attained a disease score of 9 or above. At that time, mice were entered into control or treatment groups and treatments initiated. Treatments were administered for 8 weeks and consisted of three treatments each week of encapsulated TGFβ [10 µg] and ATRA [20 µg]. Mice were sacrificed if they developed rectal prolapse, their disease score exceeded a total of 17, or if they attained a score of 5 in any scoring category. The study was concluded and all remaining mice were sacrificed at 8 weeks [after 24 treatments].

The colon weight-to-length ratio of the control [ACT alone] mice was similar to that seen in control animals in earlier experiments regardless of the length of time with IBD [Figure 8, panel A]. Similarly, the mice receiving TGFβ/ATRA treatment for an extended 8-week period maintained the same colon weight-to-length ratio that is seen in mice that were treated for only 2 weeks. Figure 8, panel B, shows a clear survival advantage in mice receiving TGFβ/ATRA treatments with 83% of mice receiving TGFβ/ATRA therapy surviving to the study's end point of 8 weeks as compared with just 10% of mice treated with blank microspheres [control].

Lungs from mice receiving TGFβ/ATRA treatment for 8 weeks showed no statistically significant [$p = 0.84$] difference in the lung-to-body weight ratio compared with untreated mice with IBD [data not shown], arguing against any fibrosis or systemic effect and for only therapeutic action.

3.8. Increased Foxp3+ expression in colonic lamina propria CD4+ CD25+ T-cells from treated animals

Oral treatment with TGFβ/ATRA particles induced a 45% increase [versus animals given blank particles] in Foxp3 mean florescence intensity [MFI] in CD4+ CD25+ colonic lamina propria regulatory T-cells [Figure 9]. This is consistent with the current paradigm that TGFβ and retinoic acid synergise in inducing T-regulatory cell phenotype and function and provides support for the hypothesis that oral administration of sustained-release TGFβ and ATRA ameliorates established disease by shifting the CD4+ effector/Treg balance in favour of the regulatory phenotype in the GI tract.

3.9. TGFβ/ATRA-loaded microspheres attenuate DSS-induced colitis

To more broadly test the ability of oral TGFβ/ATRA-loaded microspheres to ameliorate IBD, a second model, the DSS-induced colitis model, was employed. TGFβ/ATRA-loaded [10 and 20 µg, respectively] or blank microspheres [control] were fed [gavage] to mice [$n = 12$ per group] 24 h before induction of colitis [Day -1] and then every other day for 6 days. As a positive control group, eight additional mice were treated orally with sulphasalazine at the dose of 50 mg/kg daily for 7 consecutive days starting from Day 2. Mice were monitored daily for overall disease score and weight loss. At the end of 7 days, animals were sacrificed and SAA levels, colon weight-to-length ratios, and histology scores determined.

TGFβ/ATRA treatment attenuated disease as evidenced by significantly [$p < 0.001$] reduced cumulative disease score compared with either untreated animals, or mice fed blank particles [control; Figure 10, panel A] however, neither sulphasalazine nor TGFβ/ATRA treatment was able to reduce weight loss in this model [Figure 10, panel B].

Mice were sacrificed 7 days after first treatment and levels of SAA determined [Figure 10, panel C]. Naive C57Black6 mice [no disease] were used to establish baseline SAA levels [~ 20 µg/ml]. These observations confirmed the above pattern of reduced disease score. In control groups [mice left untreated or fed blank particles] SAA levels increased over 10-fold to an average of ~ 350 µg/ml. In contrast, TGFβ/ATRA treatment reduced SAA levels to less than 300 µg/ml. Differences between no treatment and suphasalazine- and TGFβ/ATRA-treated groups were significant. Importantly, the

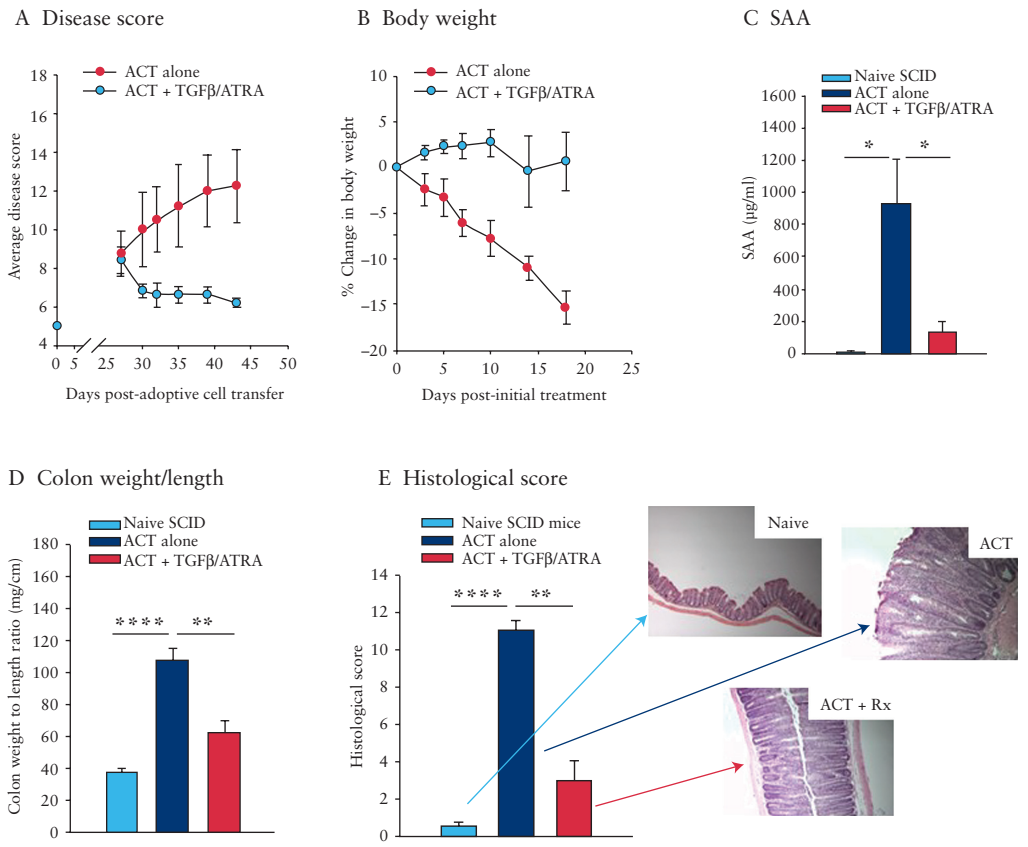

Figure 7. Therapeutic activity of dose and schedule-optimised TGFβ/ATRA-loaded particles in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease [IBD]. Mice [n = 6–9 per group] with established disease were weighed [Day 0] and fed TGFβ [10 μg] and ATRA [20 μg] microspheres, or blank microspheres [adoptive cell transfer [ACT] alone; control] in 0.2 ml water three times per week for 2 weeks. Mice were monitored for overall disease score and weighed three times per week during treatment. Mice were sacrificed 4 days after the last feeding, sera were taken colons weighed and measured and colon samples prepared for histological analysis [five randomly selected sections from each mouse]. Data are expressed as: [A] average disease score; [B] % change in body weight relative to day of first treatment; [C] serum amyloid A [SAA] μg/ml as determined by ELISA; [D] colon weight-to-length ratio; or [E] histological score with representative colon histology for each group shown. Micrographs = 100 X magnification. Panel A. Differences between the treated and untreated groups on Days 32–43 were significant [$p < 0.049$]. Panel B. Differences between groups were significant between Days 5 and 18 [$p < 0.039$]. Error bars = standard error [SE]. Significance: *, **, ***, and **** denote $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

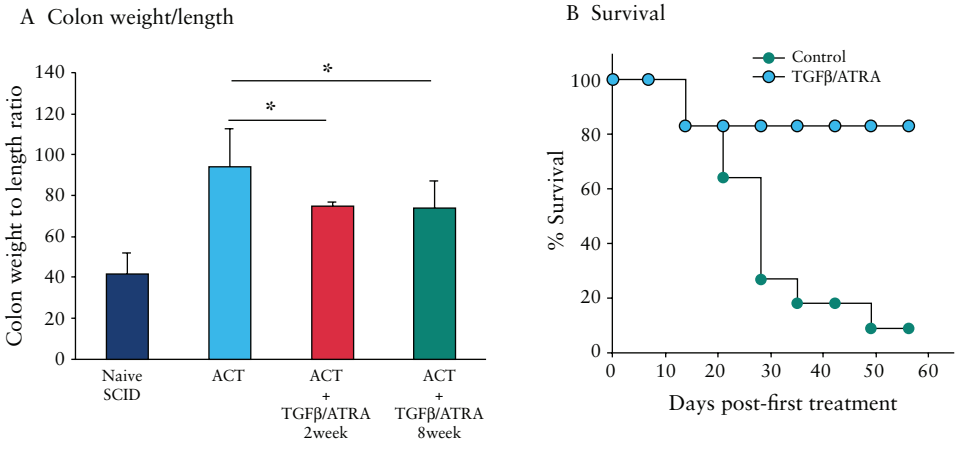

Figure 8. Efficacy and toxicity of long term TGFβ/ATRA microsphere therapy. Mice with established disease [ACT] were treated either with control [ACT only] or TGFβ/ATRA particles for 2 weeks or 8 weeks and monitored for survival. All mice in the 2-week treatment group were euthanised at week 2. Mice that were in the control or 8-week treatment groups were euthanised upon reaching terminal disease criteria or at week 8 [see text]. [A] Colon weight-to-length ratio. Colons were harvested at euthanasia (between Weeks 4–8 in the control [ACT] group, at Week 2 in the 2-week treatment group and at Week 8 in the long-term treatment group) and analysed for weight-to-length ratio. Values from age-matched naive controls are shown for comparison. Error bars = standard error [SE], n = 5, 6, 6, 7 for naive, untreated [ACT], 2-week treatment, and 8-week treatment groups, respectively. Significance: * denotes $p < 0.05$. [B] Survival: the 8-week survival for control [ACT only] and 8-week TGFβ/ATRA groups are shown. The difference between the groups was significant [$p = 0.024$] by log-rank analysis [n = 11 and 7 for control and TGFβ/ATRA groups, respectively].

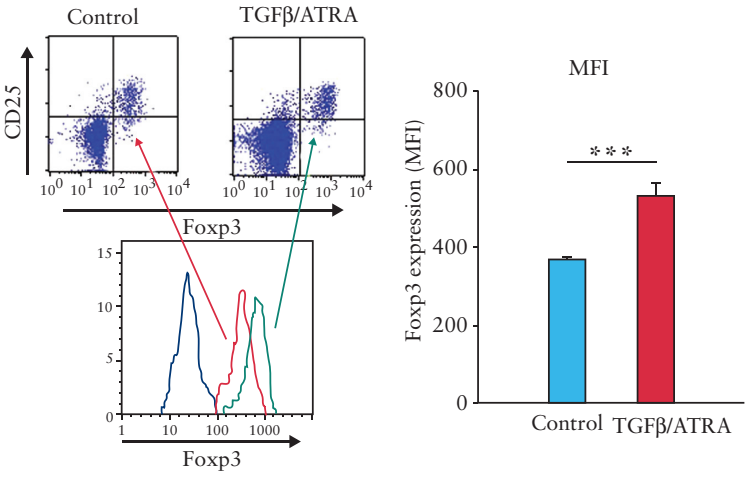

Figure 9. Effect of TGFβ/ATRA treatment on CD4+ CD25+ Foxp3+ expression by lamina propria T-cells in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease [IBD]. Mice in control and TGFβ/ATRA-treated groups were sacrificed following standard 2 weeks of treatment and lamina propria CD4+ CD25+ T-cells were analysed for Foxp3 expression by flow cytometry. CD4+ T-cells were gated on and analysed for CD25 and Foxp3 expression [dot plots]. Histogram: blue = CD4+ CD25- cells; red = CD4+ CD25+ cells from control mice; green = CD4+ CD25+ cells from TGFβ/ATRA-treated mice. The average mean fluorescent intensity [MFI] per group is shown in the bar graph. Error bars = standard error [SE], $n = 5$ per group. Significance: $p = 0.0015$.

difference between the control and the TGFβ/ATRA group was also highly significant [$p < 0.001$]. The colons of sacrificed mice were also analysed for colon weight-to-length ratio [Figure 10 panel D]. Colon weight-to-length ratio increased to almost 40 mg/cm in the control groups in contrast to only 35 mg/cm in sulphasalazine- and TGFβ/ATRA-treated mice. This difference was also significant [$p < 0.01$].

Next, ascending, transverse, and descending colon from naive C57Black6 [no disease], untreated, TGFβ/ATRA-treated, and control [fed blank particles] mice were fixed in 10% formalin, embedded in paraffin, sectioned [5 µm] and stained with H & E. Five randomly-selected sections from each mouse were scored [Table 2] for disease severity. Cumulative histological score was nearly 12 in untreated and control mice, but was reduced to less than 10 in the treated groups [Figure 10 panel E]. The differences were highly significant [$p < 0.001$]. Importantly, TGFβ/ATRA performed as well as the positive control drug sulphasalazine in this model.

4. Discussion

We have shown that TGFβ-loaded PIN® microspheres provide sustained release of bioactive protein *in vitro* for at least 4 days and that the formulation is stable at -20 and 4°C for at least 52 weeks. The combination of TGFβ and ATRA diminished the symptoms of established IBD in both CD4+ CD25- T-cell transfer and DSS-induced colitis models as evidenced by significant changes in multiple disease markers. Higher and more frequent doses were more effective and both were required to achieve maximal disease amelioration, which was associated with enhanced Foxp3 expression in lamina propria CD4+ CD25+ T-cells. TGFβ is an acid labile protein and, working under the assumption that encapsulation would improve efficacy and enhance stability and storage options, we chose to focus our initial studies on this macromolecule.

The systemic or local administration of TGFβ protects in several autoimmune disease models [33,34,37,38] but requires microgram doses. Gene therapy approaches have economically produced effective amounts [33,35] but high systemic levels raise serious safety concerns including pulmonary fibrosis and scleroderma,[39,40,41,42,43] chronic graft vs host disease[44] and glomerulonephropathies.[45] Thus, fibrosis was an expected treatment side effect. However, surprisingly, our results suggested that any fibrosis was limited to that caused by disease at the time of treatment initiation. This is evidenced by our observation that long-term oral treatment with TGFβ/ATRA combination did not lead to an increased colon weight-to-length ratio, as would be expected in the case of treatment-induced fibrosis. Instead, results indicated that long-term treatment with TGFβ/ATRA slowed disease progression, prolonged survival, and did not increase intestinal fibrosis. Additionally, we found that long-term oral treatment with TGFβ/ATRA did not induce lung fibrosis as evidenced by no increase in lung-to-body weight ratio compared with untreated mice with IBD [data not shown].

Oral delivery of low doses of TGFβ targeted to the GI tract could significantly improve the efficacy of TGFβ treatment in IBD patients while avoiding the potential problems associated with gene therapy and toxicities associated with systemic TGFβ administration. In this regard, we conducted a bioavailability and pharmacokinetic study of TGFβ and ATRA following a single oral administration of PIN®-encapsulated TGFβ and ATRA in male rats [manuscript in preparation]. Results argued against significant systemic TGFβ exposure after oral administration. After intravenous [IV] injection of soluble protein [0.04 mg/kg], serum levels of TGFβ peaked [35 000 pg/ml] at 5 min and declined rapidly over the first few minutes [t1/2 = 5 min]. Area under the curve [AUC] was 10 ng–hr/ml. These observations generally agree with the literature.[46] In contrast, for the oral TGFβ encapsulated group [100 mg/kg], very little TGFβ [~ 500 pg/ml] was observed in serum and quickly declined to undetectable levels [C_{max} ~ 30 min]. For ATRA, serum levels [~ 3000 ng/ml] peaked at 5 min after IV injection of soluble drug [1.6 mg/kg] and declined rapidly over the first hour [$t_{1/2}$ = 34 min]. AUC was 3.5 ug–hr/ml. These observations also generally agree with the literature.[47] The oral encapsulated ATRA group [18 mg/kg] also differed from the IV administration group: only a small amount [~ 20 ng/ml] appeared in blood. Levels peaked at 60 min and rapidly declined with a $t_{1/2}$ of 143 min [Auci *et al.*, unpublished observations]. These findings are consistent with the conspicuous lack of fibrosis in the lungs of mice receiving long-term TGFβ/ATRA treatment. Nevertheless, risk

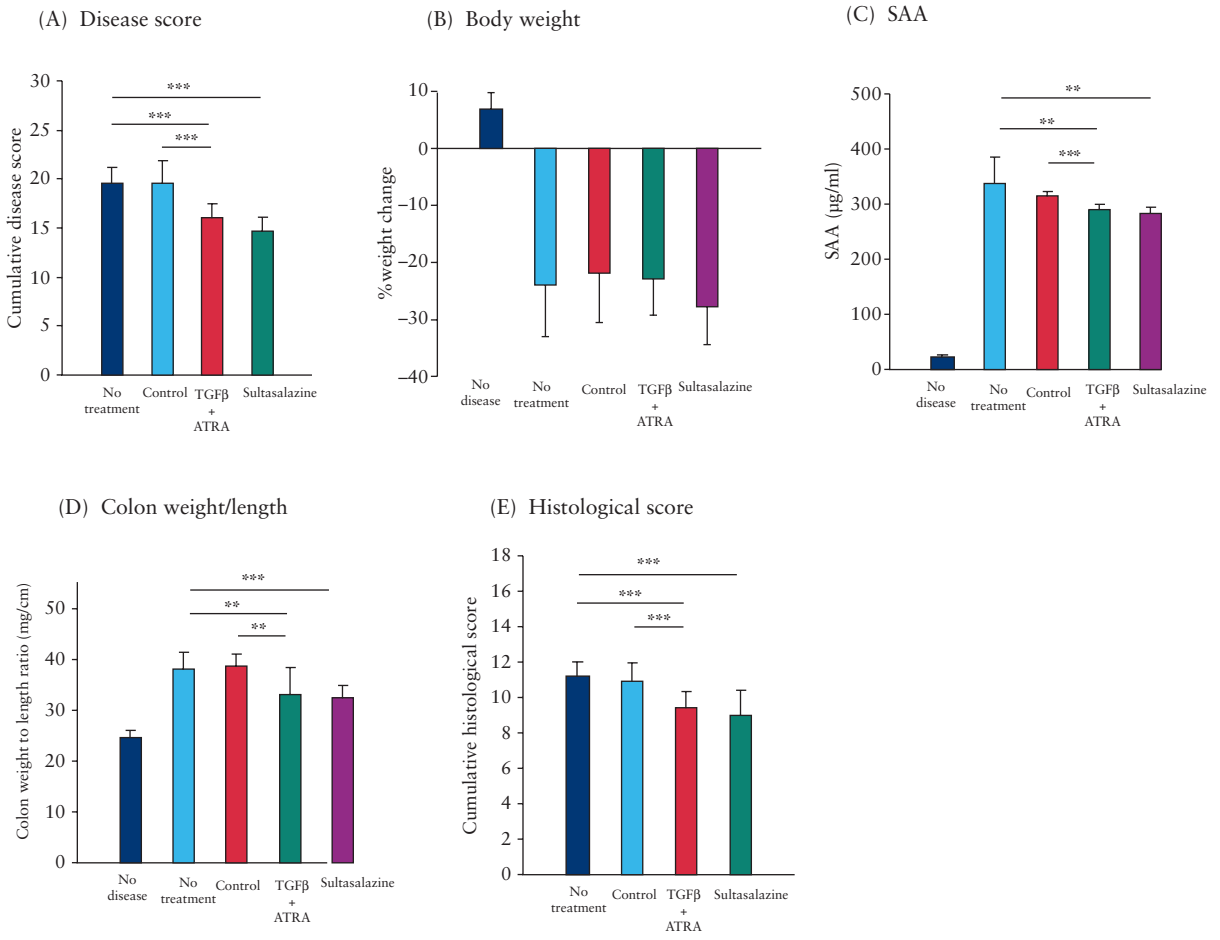

Figure 10. Therapeutic activity of TGFβ/ATRA-loaded particles in the murine model of dextran sodium sulphate- [DSS]-induced colitis. Acute colitis was induced in C57Black6 mice [equal numbers of males and females, 8–12 per group] with 3% DSS[50 kDa] in drinking water for 4 days. The first treatment with TGFβ/ATRA [10/20 μg], sulfasalazine [50 mg/kg], or with blank microspheres [control] was given [gavage] in 0.2 ml water 24 h before induction of colitis [Day -1] and continued every other day for 6 days. Body weights were taken daily starting on Day 0. Mice were sacrificed, sera taken and colons harvested, weighed, and measured on Day 7 [24 h after final treatment]. Colon samples were then prepared for histological analysis [five randomly selected sections from each mouse]. Data are expressed as: [A] average cumulative disease score; [B] % change in body weight relative to day of first treatment; [C] serum amyloid A [SAA] μg/ml as determined by ELISA; [D] colon weight-to-length ratio; or [E] histological score. Error bars = standard error [SE]. Significance: *, **, ***, and **** denote $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

remains that oral TGFβ could be responsible for the development of intestinal fibrosis during treatment for chronic colitis.

In contrast to TGFβ, ATRA is a small molecule with significant oral bioavailability and wider storage options.[48] The ability of nanoparticulate ATRA to suppress Th17 cells and induce regulatory T-cells *in vitro* has already been reported.[49] In addition, the efficacy of ATRA alone in IBD models is well established. By using mice that overproduce tumour necrosis factor [TNF] and develop chronic ileitis [TNFΔARE mice], Collins and colleagues[50] showed that twice-weekly ATRA injections (300 μg given intraperitoneally [IP]) significantly attenuated established disease by increasing the number and function of CD103+ DCs and regulatory T-cells while reducing Th17 cells. Bai and colleagues[51] showed that IP treatments of mice with ATRA ameliorated disease in the TNBS colitis model as judged by improved body weight and colon inflammation. Hong and colleagues[52] showed similar efficacy for ATRA in the mouse model of DSS-induced colitis. However, studies by Sanders[53] and colleagues suggested complexity in human tissue. Macrophages from the intestinal mucosa of Crohn's disease patients showed an increased capacity to generate ATRA, where it increased pro-inflammatory phenotypes. Initial reports suggested that ATRA might serve as a trigger for IBD, especially UC.[54,55] However, a series of recent clinical studies found no association between ATRA use and IBD, and even indicated decreased risk.[56,57]

ATRA is used to treat severe cystic acne [also known as nodular acne] that has not responded to other treatment[58] as well as acute promyelocytic leukemia [APML].[59,60,61] Despite its effectiveness, it has been associated with depression, suicidality, and teratogenicity.[59] It has been suggested that this systemic toxicity could be avoided by targeting administration to the desired tissue site. Examples of this approach include immune-targeting of liposomal retinoids[62] or retinoid aerosolisation.[63] One study sought to suppress epithelial carcinogenesis by delivering ATRA micro-particles via a subcutaneous injection. The slow release of ATRA from these particles resulted in a therapeutically significant release of ATRA without significant toxicity.[64] From those studies it appears that targeting ATRA to the inflamed bowel in IBD should result in a significant reduction in the amount of drug needed while decreasing the possibility of side effects. Thus, the oral delivery of encapsulated ATRA should have a therapeutic advantage over parenteral or intravenously administered drug.

We found that ATRA- or TGFβ-loaded microspheres given alone had only modest effects that were not statistically significant

in most cases. Both were required for broadly significant benefit, which was associated with enhanced Foxp3 expression by the colonic lamina propria CD4+ CD25+ T-cells. We did not perform functional assays, but these findings are consistent with numerous reports supporting the requirement for ATRA in TGFβ-driven development of regulatory T-cells[17,65,66,67] and suggesting that ATRA is needed as a co-factor in TGFβ-based IBD therapy. In the presence of ATRA and TGFβ, antigen-activated naive T-cells become regulatory T-cells[16,17,66,68] while in the presence of IL-6, TGFβ promotes Th-17 T-cells.[67] Mechanistically, ATRA increases FoxP3 expression by Treg cells and inhibits the development of Th17 cells by enhancing TGFβ-driven Smad3 signalling and inhibiting IL-6 and IL-23 receptor expression.[69] This also suggests that another advantage to the combined product may be that ATRA tends to reduce the side effects of TGFβ.

Encapsulation of labile biologicals into biodegradable polymers provides protection from the acidic environment and the proteases of the stomach and allows safe passage through the lumen of the GI tract for uptake.[70] Until recently, this technology primarily targeted small-molecule drugs and peptides because larger bioactive macromolecules are generally more highly sensitive to organic solvents and mechanical agitation techniques that are used during encapsulation. PIN® technology has now made it possible to encapsulate biologically active macromolecules into biodegradable polymer microspheres while preserving biological activity. We have not strictly demonstrated targeted delivery of TGFβ/ATRA to the gut in these series of studies, but our previous work showed that orally administered protein-loaded PIN® microspheres are efficiently taken up in Peyer's patches and mesenteric lymph nodes.[71]

Three different strategies to locally deliver biopharmaceutical drugs to the gut using oral administration of nanoparticles have recently been compared.[26] PLGA-PEG-mannose nanoparticles achieved highest accumulation in inflamed tissue. No such studies with PIN® encapsulated TGFβ have been performed, but our observations suggest that oral TGFβ/ATRA-loaded microspheres reduce gut inflammation through local induction of regulatory T-cells. Preservation of biological activity and improved stability are what makes PIN® a novel, unique and proprietary process and support continued development of the combination TGFβ/ATRA product as an oral immune-based therapy for IBD.

Acknowledgements

All authors contributed to the analysis of data and drafting the article and revising it critically for important intellectual content. TFC, NKE, and EM contributed to conception of study design; TFC, LH, and SF contributed to the acquisition of data.

This work was supported by the National Institutes of Health [5R44AI080009-04] to TFC.

Conflict of Interest

NKE and DLA have equity stakes in TherapyX^{inc}.

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