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Synergy of Transforming Growth Factor Beta 1 and All Trans Retinoic Acid in the Treatment of Inflammatory Bowel Disease: Role of Regulatory T cells

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Introduction

Gut immune homeostasis breakdown is central to the pathogenesis of both Crohn's disease (CD) and ulcerative colitis (UC). Apprehension of local imbalances in pro- and anti-inflammatory signaling common to both led to the collective term '*inflammatory bowel disease*' (IBD), a malady afflicting one and one-half million Americans.

Three groups of conventional therapies include salicylates, immunosuppressants and antibiotics. Yet relief is often short-lived and comes with significant side effects. 80% and 45% of CD and UC patients (Respectively) will still require surgery [1–6]. Biological response modifiers or 'biologics', macromolecules that target inflammatory lymphocytes or the cytokines they produce [7], have more recently emerged as another highly effective therapeutic class. In 1998, the FDA approved infliximab, with a high response rate, significant mucosal and fistula healing and long-term remissions in Crohn's disease. Other biologics targeting p40, p19, IL-12, IL-17 and anti-alpha 4 integrin [7, 8] are either marketed or in various development stages. However as many as 30% of patients will not respond to biologics and half of those who initially respond, will relapse within a year. None have significant impact on surgical intervention rates [9].

Thus, the need for novel targeted therapies remains acute and will likely depend on a deeper understanding of chronic gut inflammation.

While etiologies remain incompletely understood, both genetic and epigenetic elements predominate [10]. Rising incidences coeval with industrialization focused suspicions on pollutants [11], refrigeration [12], dietary changes [13], improved hygiene and decreased helminthic infestations [14, 15]. More recent human and animal studies implicate abnormal

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gut immune responses to microbiota [1, 2, 16–21]. Gut microbiota aid digestion, synthesize essential nutrients and contribute to host defense [22–25], but in susceptible individuals, drive chronic inflammation *via* untoward effects on epithelial and mucosal barriers [26, 27] as well as gut associated lymphoid tissue (GALT) [28–30].

Key regulators of chronic gut inflammation include the regulatory cytokine transforming growth factor beta 1 (TGFβ) and all trans retinoic acid (ATRA), the signaling form of vitamin A. Together, they drive the differentiation, activation and stability of gut regulatory T cells (T_{regs}), a specialized T cell population that temper the intense and persistent pro-inflammatory signals created by microbiota and help maintain gut immune homeostasis and tolerance [19, 31–35]. In these review roles of TGFβ and ATRA in gut immune homeostasis and related therapeutic potentials will be considered separately followed by the potential to exploit synergy in treatment of gut inflammation.

Role of TGFβ in gut immune homeostasis

TGFβ is a key anti-inflammatory and regulatory cytokine that suppresses the production of pro-inflammatory signals such as TNFα, IFNγ, IL-1β. Its central anti-inflammatory role was evidenced by gene knockout [36, 37], receptor blockade [38] and signal disruption studies in rodents [39], and all reporting chronic inflammation in multiple tissues including gut. Its significance in gut inflammation has recently been reviewed [40]. In rodents, treatment of TNBS-induced colitis by induction of oral tolerance with haptenized colonic proteins was associated with increased TGFβ production [41]. Treating healthy human colon tissue with anti-TGFβ antibodies decreased effector T-cell apoptosis and increased production of pro-inflammatory cytokines supporting TGFβ's anti-inflammatory role in the human GI tract [42]. However, increased TGFβ levels and defective signal transduction found in UC and CD gut tissues underscore disease complexity [43, 44].

TGFβ-dependent T_{regs} in gut prophylactically and therapeutically limit IBD *via* direct suppression of local T-effector priming activity and innate effector function [45, 46, and 47]. However, in context of pro-inflammatory signals like IL-6, TGFβ drives the differentiation of pathogenic Th-17 cells, reflecting the importance of secondary signals and T cell priming by local DC populations [16].

Therapeutic potential of TGFβ in IBD

Disease complexity and the pluripotent nature of TGFβ signaling not withstanding, the systemic or local administration of TGFβ protects in animal models of immune mediated inflammatory diseases [48–53]. Unfortunately systemic treatment is associated with severe dose limiting toxicities, including pulmonary fibrosis and scleroderma [54–58] chronic GVHD [59] and glomerulonephropathies [60]. To circumvent these issues, local treatment of GALT has been proposed. But because acid-induced hydrolysis and enzymatic degradation in the stomach prevent direct oral delivery of TGFβ to the gut [61, 62] only a few, complex gene therapy approaches have been employed to achieve locally effective TGFβ signaling. For example, therapy with TGFβ gene-modified immature dendritic cells delayed rodent

DSS-induced colitis [63]. Interestingly, TGFβ gene therapy approaches also show efficacy in rodent models of arthritis and type-1 diabetes [48, 50].

Role of Retinoic Acid in gut immune homeostasis

RA is the signaling form of vitamin A, regulating the transcription of many genes [64]. A product of mucosal DCs, it is a key regulator of gut inflammation. Supplementation and deficiency studies have revealed its pro-inflammatory potential *via* effects on frequencies and function of immune cells [65–67], prevention of apoptosis [68], increased gut homing receptor expression [69, 70], antibody production [71–73], enhanced phagocytosis, resistance to bacterial infection [74], as well as maintenance of DTH responses and Th1 cell function [75]. Anti-inflammatory potential was indicated by suppression of cell proliferation [76], cytokine (TNFα and IL-12 *vs* IL-10) [77] production and experimental autoimmune encephalomyelitis [78]. Its central role in the ontogeny of mucosal DCs was recently reviewed [79]. Mucosal DCs are key antigen presenting cells in the gut. They generate RA from vitamin A and are uniquely primed by it to modulate lymphocyte activation, trafficking and differentiation [80, 81].

Therapeutic potential of RA in IBD

A wide literature reports RA activity in various rodent models of IBD. In TNFΔARE mice (animals that overproduce TNFα and develop chronicileitis) twice weekly RA injections (300 μg given *i.p.*) significantly attenuated established disease by increasing the number and function of CD103+ DCs and T_{reg} cells while reducing Th17 cells [82]. In the murine TNBS-induced colitis model *i.p.* RA treatments improved body weight and reduced colon inflammation [83]. Similar efficacy was shown against murine DSS-induced colitis [84]. However, once again, observations in diseased tissues suggest complexity. CD patient's mucosal macrophages showed expanded pro-inflammatory phenotypes and a heightened ability to generate RA [85]. Indeed, initial clinical observations suggested that RA treatments might have triggered UC [86, 87]. However, more recent and larger studies found no association between RA use and IBD, and even reported decreased risk. ATRA is marketed as *Tretinoin*® and is used to treat acute promyelocytic leukemia and severe cystic acne unresponsive to other treatment [90–93]. However, systemic use is associated with severe side effects including depression, suicidality and teratogenicity [90]. As with TGFβ efforts to circumvent these systemic toxicities with targeted administration have included liposomes [94] and aerosolization [95].

RA and TGFβ synergistically generate uniquely stable regulatory T cells from naïve T cells

T_{reg} cells arise either in thymus or peripheral lymphoid tissues. The former require T cell receptor interaction with ligands on specific thymic epithelial cells but not antigen, TGFβ or intact TGFβ signaling [96]. The latter are derived from naive T cells in an antigen dependent fashion that includes an obligatory role for TGFβ and intact TGFβ signaling [97, 98]. GALT is more effective at generating antigen dependent T_{reg} cells from naive T cell precursors than other peripheral lymphoid tissues, a bias central to both oral tolerance and gut homeostasis

and dependent upon DC-derived RA [33, 34]. DC derived RA acts in concert with TGFβ to promote the conversion of naïve T cells into T_{regs} cells that help maintain gut tolerance [79]. In the presence of both RA and TGFβ, mucosal DCs efficiently induce T_{reg} cells. Interestingly, even in the context of IL-6 and TGFβ, they are comparatively poor inducers of pro-inflammatory IL-17 producing T cells, perhaps reflecting the tolerogenic and immune homeostatic bias of the gut [34]

Initial experiments revealed RA/TGFβ synergy *in vitro*, the combination potently driving the generation of gut-homing T_{reg} cells from naïve precursors [35]. The surprising inability of RA to achieve this alone revealed an exclusive dependence on exogenous TGFβ. Importantly, RA/TGFβ was fully capable of inducing gut homing T_{reg} cells even in the presence of high levels of co-stimulation signals. Confirmation of RA/TGFβ synergy was provided by other *in vitro* studies aimed at the generation of TCR-transgenic regulatory T cells with gut homing potential under conditions of low-peptide stimulation [99]. In those studies, numbers of T_{reg} cells generated in cultures containing both RA and TGFβ were 3-fold greater than in cultures with TGFβ alone.

Studies focused on other indications also support RA/TGFβ synergy in generation of stable, protective T_{reg} cells. Kishi and colleagues showed that T_{regs} generated *in vitro* by co-culture with TGFβ and ATRA completely prevented diabetes when transferred to NOD-scid mice [100]. Similar observations have been extended to human T_{regs}. Culture with ATRA and TGFβ enabled CD4+CD25RA+ cells to express gut trafficking receptors and a phenotype and similar to natural T_{regs} [101]. Importantly, such cells appeared particularly resistant to effector conversion by pro-inflammatory signals.

Mechanisms of synergy

Mechanisms involved in RA/TGFβ T_{reg} generating synergy are incompletely understood. RA expands T_{reg} cells and inhibits the development of Th17 cells by enhancing intracellular TGFβ signaling and inhibiting IL-6 and IL-23 receptor expression [102].

Transduction of TGFβ signal following interaction with its receptor is accomplished through the Smad family of intercellular proteins, as has recently been reviewed in the context of IBD [103]. Activation and phosphorylation cascades lead to Smad2, Smad3 and Smad4 hetero-complex formation, translocation into the nucleus and transcriptional regulation of target genes [104]. Another member of the Smad family, Smad7 blocks hetero-complex formation and attenuates the TGFβ signal [104, 105]. Smad7 was found to be over expressed in mucosa and purified T cells taken from CD and UC patients as compared to healthy controls [44], explaining the persistence of IBD even in context of high TGFβ tissue levels [43, 44].

Novel therapeutic implications

i. Targeting TGFβ signaling

These observations suggested an IBD therapeutic strategy aimed at perturbed TGFβ signal transduction. Boirivant and colleagues showed that oral administration of Smad7 antisense

oligonucleotide restored TGFβ signaling and ameliorated inflammation in hapten-induced colitis [106]. TGFβ signal transduction manipulation in the form of a novel IBD treatment called *GED0301* has recently been reviewed by the same group [107]. They caution that despite the wealth of human and animal studies supporting the therapeutic potential of TGFβ in IBD [108, 109] TGFβ delivery alone may not be sufficient to effectively and broadly treat IBD; another signal might be required [103].

ii. RA and TGFβ combination therapy

The hypothesis that RA and TGFβ given together would be more effective at treating IBD than either alone was recently tested *in vivo* using an orally delivered combinatorial product called *TGFβ Nanocap*®. This product contains both TGFβ and RA in a 1:2 *w/w* ratio [110]. TGFβ was encapsulated in poly-lactic acid microspheres manufactured using a proprietary phase-inversion nanoencapsulation (PIN®) technology. PIN® preserved the structural integrity and biological activity of TGFβ, provided extended shelf-life (years) and allowed effective delivery of protein to intestinal immune structures [111, 112]. RA was encapsulated in poly-lactic-co-glycolic acid microspheres manufactured using the solvent evaporation technique [113]. In addition to improved storage stability, protection from hydrolysis and targeted drug delivery, encapsulation also achieved local and sustained release of both signals over extended periods.

In the SCID mouse CD4+CD25− T-cell transfer model of IBD, oral treatments with *TGFβ-Nanocap*® starting at disease onset prevented weight loss and dramatically reduced average disease score. Significant improvements in several markers of disease were also reported including decreased serum amyloid A levels, colon weight-to-length ratio and histological score. Both agents given together outperformed either separately. Highest doses and most frequent dose schedule were most effective. Importantly, activity was associated with a significant increase in Foxp3 expression by colonic lamina propria CD4+ CD25+ T cells, suggesting the predicted generation of conversion resistant T_{reg} cells in gut. Significant activity was also reported in the murine model of DSS-induced colitis. Preliminary pharmacokinetic studies in rats indicated minimal systemic exposure of either TGFβ or RA after treatment [110]. Concerns over oral TGFβ therapy potential side effects include intestinal fibrosis and stricture formation, common and serious IBD complications related to over production of TGFβ in the context of impaired signal transduction [103, 114, 115]. Surprisingly, even long term oral treatment (up to 8 weeks) of mice with *TGFβ-Nanocap*® did not increase fibrosis in intestines or lungs above that normally associated with disease [110]. Interactions between RA and TGFβ signaling pathways may help explain this unexpected, fortuitous result and bear on RA/TGFβ synergy.

iii. Mechanisms of combination therapy

While precise mechanisms remain unknown, cross-talk between RA and TGFβ signaling pathways mediated *via* Smad3 [116] have previously been reported, as have observations that RA receptor agonists and antagonists repressed and potentiated (respectively) Smad3/Smad4 driven transcription [117].

A number of subsequent reports using various cell types have confirmed RA modulation of TGFβ signaling, but with varying results. RA up-regulated Smad proteins in chicken chondrocytes [118] but lowered TGFβ induced levels of nuclear Smad complexes in HL60 cells, skewing their differentiation towards monocytic end points [119]. In one report, RA reduced proliferation of mouse mesenchymal stem cells by down regulating TGFβ-induced Smad signaling [120] but in another stimulated Smad3 expression [121]. Importantly, in hyperoxic mouse lungs, RA reduced Smad4 mRNA and increased Smad7 protein expression, helping to repair Smad3/TGFβ1-induced fibrosis [122]. This observation in particular may help explain the lack of fibrosis after *TGF*β*-Nanocap*® treatment. However, studies in xenopus and chick embryos found Smad7 activity to be unexpectedly complex and suggested that experiments involving Smad7 inhibition of the TGFβ signaling pathway must be interpreted "with considerable caution"[123]. The precise effects of RA on TGFβ Smad signaling in the context of IBD remain unknown but the potent efficacy of *TGFβ* Nanocap® in both SCID transfer and DSS mouse models of IBD suggest therapeutically relevant impact.

Forward

The comparative abilities of novel IBD therapies like the *TGFβ-Nanocap*® and the Smad7 silencing antisense oligonucleotide product *GED030* alone and in combinations, to provide benefit to patients must ultimately be determined in the clinic. Preliminary phase I studies with oral *GED0301* in patients with active CD indicate the drug is generally safe, well-tolerated and biologically active [124]. At this writing, it remains in phase I development. *TGFβ Nanocap*® is in late preclinical development. Studies in rodents have confirmed that repeated oral dosing is safe and well tolerated (Auci, *et al,* unpublished observations).

Several reports already cited indicate that $CD103^+$ DCs in GALT are specially equipped for converting antigen-specific T cells into $Foxp3^+$ T_{reg} cells in a TGFβ/RA dependent manner [33, 34, 35]. Indeed, a crucial role for RA in oral tolerance has been postulated [125], perhaps involving an anatomically central "immune fire wall"-like function for MLN that can transduce oral tolerance systemically [126]. We have already alluded to the potential of RA/TGFβ based Treg conversion as a strategy aimed at T1DM [100, 101]. However, a number of reports suggest the potential for an even more general expansion to other indications including rheumatoid arthritis [127], multiple sclerosis [128] and allergic disease [129].

Aiming for synergistic effects when novel products are combined may hold out the greatest hope for effective long-term treatment of immune mediated inflammatory diseases.

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