A novel protocol allowing oral delivery of a protein complement inhibitor that subsequently targets to inflamed colon mucosa and ameliorates murine colitis

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Summary

While there is evidence of a pathogenic role for complement in inflammatory bowel disease, there is also evidence for a protective role that relates to host defence and protection from endotoxaemia. There is thus concern regarding the use of systemic complement inhibition as a therapeutic strategy. Local delivery of a complement inhibitor to the colon by oral administration would ameliorate such concerns, but while formulations exist for oral delivery of low molecular weight drugs to the colon, they have not been used successfully for oral delivery of proteins. We describe a novel pellet formulation consisting of cross-linked dextran coated with an acrylic co-polymer that protects the complement inhibitor CR2-Crry from destruction in the gastrointestinal tract. CR2-Crry containing pellets administered by gavage, were characterized using a therapeutic protocol in a mouse model of dextran sulphate sodium (DSS)-induced colitis. Oral treatment of established colitis over a 5-day period significantly reduced mucosal inflammation and injury, with similar therapeutic benefit whether or not the proton pump inhibitor, omeprazole, was co-administered. Reduction in injury was associated with the targeting of CR2-Crry to the mucosal surface and reduced local complement activation. Treatment had no effect on systemic complement activity.

This novel method for oral delivery of a targeted protein complement inhibitor will reduce systemic effects, thereby decreasing the risk of opportunistic infection, as well as lowering the required dose and treatment cost and improving patient compliance. Furthermore, the novel delivery system described here may provide similar benefits for administration of other protein-based drugs, such as anti-tumour necrosis factor-α antibodies.

Keywords: colitis, complement, mouse model, oral delivery

Introduction

The complement system plays a central role in many inflammatory conditions, and there is strong evidence linking the complement cascade to the pathogenesis of inflammatory bowel disease (IBD). Clinical studies have shown that complement activation is associated with Crohn’s disease and ulcerative colitis [1–6], and the expression of complement regulatory proteins is altered in gut epithelium of patients with IBD [7–9]. In experimental models, a pathogenic role for complement in IBD has been demonstrated more directly. Mice deficient in C3 are protected from dextran sulphate sodium (DSS)-induced colitis [10], whereas deficiency of the complement inhibitor, decay-accelerating factor, leads to exacerbated inflammation and injury in this model [11]. Blocking C5 with an anti-C5 monoclonal antibody (mAb) is protective in an acute model of DSS-induced colitis [12], as is C5a receptor (C5aR) deficiency [13] and C3aR deficiency [14]. An anti-C5a mAb [15] and a C5aR antagonist [16] have also been shown to be protective in acute trinitrobenzene sulphonic acid (TNBS)-induced colitis. Conversely, there is evidence that complement has a protective role in models of IBD. Although C5aR deficiency was protective in an acute model of DSS-induced colitis it caused increased injury in a chronic model [17], and it has also been reported that
C5-deficient mice have exacerbated disease following acute DSS-induced colitis [18]. Thus, complement may have both pathogenic and protective roles in experimental IBD, and our recent study addressed more directly whether complement plays a dual role in murine colitis; we demonstrated that while complement activation was associated with DSS-induced inflammation and injury, complement also provided protection in terms of host defence and reduced serum plasma endotoxin levels following epithelial barrier loss [19].

Taken together, the above studies raise concerns regarding the potential use of systemic complement inhibition as a therapeutic modality for IBD. Indeed, other systemic anti-inflammatory drugs that are emerging as treatments for IBD, such as anti-tumour necrosis factor (TNF)–α mAb, are also immunosuppressive and increase patient susceptibility to infection [20]. We have previously demonstrated that systemic administration of a site-targeted complement inhibitor protects against acute DSS-induced colitis, while minimizing adverse effects [19]. Targeting was achieved by linking a complement inhibitor, Crry, to a fragment of complement receptor 2 (CR2, CD21) that recognizes C3 cleavage products deposited at sites of complement activation; CR2-Crry inhibits all complement activation pathways at the C3 cleavage step. Nevertheless, although CR2-Crry has a short circulatory half-life and serum complement activity is restored fairly rapidly after administration [21], it is not clear how prolonged treatment with a systemically administered, albeit targeted inhibitor, would affect outcome in the setting of IBD and ongoing inflammation. Localized delivery to the colon by oral drug administration would not only obviate the need for even temporary high circulatory levels and systemic effects, it would also provide patient convenience. However, although various formulations allow for oral delivery of small molecule drugs, protein-based drugs remain susceptible to degradation during transit through the gut.

For delivery to the colon, an orally administered drug would need a protective matrix to protect it against acidic pH and enzymatic digestion, with release from the protective matrix in the large intestine. Protective polyacrylate matrices have been developed for protecting small molecule drugs from low pH, and various acrylic co-polymers are commercially available (Evonic Industries, Tippecanoe, IN, USA). There are also delayed-release formulations that could be regarded as colon targeting, but they have not been shown to protect proteins [22]. For protection against enzymes in the small intestine, polysaccharides such as dextran have been used in matrices because they are resistant to digestion by human gut enzymes, and dextran has served as a prodrug anchor for small molecules such as naproxen [23], ketoprofen [24], and recently for glucocorticoids [25]. However, degradation of dextran occurs in the colon by bacterial dextranases, and in this study we describe a novel preparation consisting of a protein complement inhibitor loaded into a cross-linked dextran gel and coated with an acrylic polymer. We demonstrate that oral administration of this preparation effectively treats established DSS-induced colitis with localized colon release and mucosal targeting of CR2-Crry.

Materials and methods

Preparation of protective matrix containing CR2-Crry

In overview, methacrylated dextran and photoinitiator were added to CR2-Crry solutions and polymerized in droplets by ultraviolet (UV) activation. Pellets were formed from the resulting gels and were then covered with an acrylic co-polymer. In more detail, a modified dextran (MW 70 000) hydrogel containing 4 methacrylic acid residues per 100 anhydroglucoside units [DS = 4, determined by nuclear magnetic resonance (NMR) spectra] was prepared as described previously [26] and freeze-dried. To prepare pellets for oral delivery, the lyophilized modified dextran was added to a solution of CR2-Crry at 10 mg/ml in phosphate-buffered saline (PBS) to make a 25% by weight dextran solution, and polymer initiator Azobisisobutyronitrile (Sigma-Aldrich, St Louis, MO, USA) added at 0-75% of dry modified dextran weight. CR2-Crry was expressed and purified as described previously [21]. To form a pellet, 2 μl of modified dextran and CR2-Crry solution containing initiator was pipetted onto paraffin film to form a hemisphere ∼0-5 mm in diameter and polymerized by exposure to UV irradiation (365 nm, 84 watts) for 12 min. For coating the pellets with an acrylic co-polymer, a coating solution was prepared consisting of an aqueous suspension of 12-5% Eudragit L100-55 (Evonic Industries), with 6-25% talc to reduce tackiness and 1-5% triethyl citrate as a plasticizer. The dextran hydrogel pellets (with or without CR2-Crry) were coated in this suspension by quick dipping and draining of excess solution, followed by air-drying. All pellets received three coats using this method. By assaying for unincorporated protein after polymer formation, it was calculated that each pellet contained an average of 26 μg CR2-Crry.

In-vitro analysis

CR2-Crry-containing pellets were subjected to in-vitro treatments to simulate conditions encountered in the gastrointestinal tract, and protein and complement inhibitory activity were subsequently assayed. Each treatment used two pellets in a volume of 100 μl, and all treatments were performed at 37°C. Pellets were first incubated in PBS for 1 h at either pH 1-5 or 4-0 (stomach simulation, without or with antacid treatment, respectively). Pellets were then incubated in 0-05% trypsin for 5 h (small intestine simulation), followed by 3 h in 1 U/ml dextranase (colon simulation). The resulting digest was diluted in PBS and analysed for protein...
concentration (absorbance at 230 nm) and for complement inhibitory activity by measuring C3 deposition on zymosan particles as described previously [27]. Protein recovery was calculated based on a starting concentration of 26 μg protein per pellet (see above), and functional recovery calculated from a comparison of complement inhibitory activity with unincorporated CR2-Crry.

Induction of DSS-induced acute colitis and treatments

Wild-type C57BL/6 mice were used for these studies (Jackson Laboratory, Bar Harbor, ME, USA). All animal procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were given 5% (w/v) dextran sodium sulphate (DSS; MP Biomedical, Solon, OH, USA) in their drinking water for 5 days to induce acute colitis. Sham mice received normal drinking water. Following 5 days of DSS treatment, mice were given normal drinking water for 7 days, after which they were killed for analysis. Starting on day 5 (at termination of DSS treatment), two groups of mice were given a single CR2-Crry containing pellet by gavage three times per day for 5 days, with one group also receiving the proton pump inhibitor (antacid, AA), omeprazole (Sigma-Aldrich), at 10 mg/kg/day by intraperitoneal (i.p.) injection from days 4 to 9 (refer to Fig. 1). A third group of mice received no further treatment after day 5, and a sham control group received no DSS treatment. In a pilot study designed to test the feasibility of pellet treatment, mice were treated with pellets during the 5 days of DSS treatment, i.e. in a preventative protocol. Treatment groups were: 1, sham; 2, DSS only; 3, control pellet containing no protein; 4, DSS + omeprazole; and 5, DSS + omeprazole + CR2-Crry-containing pellets. Omeprazole treatment was as above on days 1–5, and pellet treatment was as above on days 1–5.

Histology

To assess histological damage and inflammation, formalin-fixed colon sections were stained with haematoxylin and eosin (H&E). Sections were scored according to a previously described scoring system [19] by a blinded observer. A cumulative scale with a maximum score of 10 was used. Three parameters were assessed: (1) severity of inflammation (0, none; 1, slight; 2, moderate; and 3, severe); (2) depth of injury (0, none; 1, mucosal; 2, mucosal and submucosal; and 3, transmural); and (3) crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; and 4, complete loss of crypt and epithelium).

Assessment of complement activation in vivo

Complement activation was assessed locally (in colon homogenates) and systemically (in serum) by analysis of the complement activation products C3d and C5a. To determine C3d deposition, immunohistochemistry was performed on paraffin-embedded colon sections as described previously (goat anti-C3d; R&D Systems, Minneapolis, MN, USA) [21]. C5a in colon homogenates and serum was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). For assay of C5a concentrations in the colon, homogenates were prepared from frozen colon samples homogenized in cell lysis buffer (Sigma-Aldrich) containing Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and stabilized with FUT-175 (BD Biosciences, San Jose, CA, USA). Homogenates were centrifuged at 10 000 g for 10 min at 4°C, and C5a levels in supernatant determined using an ELISA kit according to the manufacturer’s instructions (R&D Systems). Serum complement activity was assessed in samples taken from DSS-treated mice after 5 days of treatment with CR2-Crry-containing pellets, with or without omeprazole, and in samples from DSS-treated mice that

Fig. 1. Schematic illustrating schedule for induction of colitis and subsequent treatments. AA = antacid (Omeprazole).
received no therapy. Serum complement activity was determined using a standard assay that measures C3d deposition on zymosan particles, as described [27].

Biotin labelling and immunofluorescence analysis of colon sections

CR2-Crry was labelled with biotin to allow detection and localization of the protein in the colon following treatment with CR2-Crry-containing pellets. Biotin labelling was performed using the Anatag biotin protein labelling kit (AnaSpec, Fremont, CA, USA). Pellets were prepared with biotinylated CR2-Crry as described above for unlabelled protein. Following induction of acute colitis, mice were treated with biotinylated CR2-Crry-containing pellets in the same protocol described above, except that treatment duration was 3 days, at which time mice were killed, colons removed, formalin-fixed and sectioned. Double staining for biotinylated CR2-Crry binding and C3d deposition was performed. Goat anti-mouse C3d (1:30; R&D Systems) was applied and detected with rabbit anti-goat IgG Alexa Fluor-555 conjugate (1:100; Invitrogen, Carlsbad, CA, USA). After washing, streptavidin-AF488 (1:100; Invitrogen) was applied, followed by ToPro-3 (1:5000; Invitrogen) as a nuclear marker. Slides were coverslipped with Vector fluorescent hard mount (Vector Laboratories, Burlingame, CA, USA) and imaged on an Olympus FV10i laser scanning confocal microscope (Olympus America, Inc., Center Valley, PA, USA).

Statistical analysis

All data are represented as mean ± standard error of the mean. All data were subjected to statistical analysis using Prism Software version 5 (GraphPad, San Diego, CA, USA). Parametric analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. Non-parametric analysis (histology) was performed using one-way ANOVA with Dunn’s multiple comparison test. Comparisons between two groups were performed by Student’s t-test (parametric) or Mann–Whitney U-test (non-parametric). A P-value of <0.05 was considered significant.

Results

In-vitro analysis of CR2-Crry activity following digest simulations

To estimate release and functional recovery of CR2-Crry from pellet preparations after passage through the digestive tract, we exposed pellets to simulated in-vivo conditions and subsequently determined CR2-Crry complement inhibitory activity. Following the sequential exposure of pellets to low pH (stomach), trypsin (small intestine) and dextranase (colon) (refer to Methods), 100% of loaded protein was recovered from each pellet (Table 1). Functional recovery was determined by assaying recovered protein for complement inhibitory activity in a zymosan C3 deposition assay. CR2-Crry recovered from pellets exposed to pH 4 prior to exposure to digestive enzymes (to simulate antacid treatment) retained 80% complement inhibitory activity, and pellets first exposed to pH 1·5 (normal stomach conditions) retained 65% activity. Pellets exposed to only dextranase (colon simulation) retained 100% CR2-Crry functional activity (Table 1). These data suggest that, under in-vivo conditions, functional CR2-Crry will be delivered to the colon under normal physiological conditions, although co-treatment with an antacid may improve the delivery of functional payload.

Treatment of established acute DSS-induced colitis

Acute colitis persists for up to several weeks in C57BL/6 mice after cessation of DSS treatment, and in a therapeutic paradigm we treated mice with pellets containing CR2-Crry during a 5-day rest period following 5 days of DSS treatment. A single pellet was administered by gavage three times daily, with one group of mice also receiving omeprazole (proton pump inhibitor) for duration of pellet treatment. On day 12 (2 days after cessation of pellet treatment), mice were weighed and then killed for analysis of colon injury.

Mice treated with pellets, either with or without omeprazole, had a significantly and similarly improved outcome in terms of colon length compared to mice treated with DSS alone. There was also an apparent improvement in weight change in pellet-treated mice, but the difference did not reach significance. The outcome of pellet-treated

<table>
<thead>
<tr>
<th>Stomach simulation (1 h)</th>
<th>Small intestine simulation (5 h)</th>
<th>Colon digestion (3 h)</th>
<th>Protein recovery</th>
<th>Recovery of complement inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1 U/ml dextranase</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>PBS pH 4</td>
<td>0·05% Trypsin</td>
<td>1 U/ml dextranase</td>
<td>100%</td>
<td>80 ± 4·4%</td>
</tr>
<tr>
<td>PBS pH 1·5</td>
<td>0·05% Trypsin</td>
<td>1 U/ml dextranase</td>
<td>100%</td>
<td>65 ± 7·1%</td>
</tr>
</tbody>
</table>

PBS = phosphate-buffered saline.
mice was also improved in terms of stool consistency and the absence of faecal blood (Table 2). Sections of the distal colon were stained with H&E to assess histological damage, and sections were scored based on severity of inflammation, depth of injury and amount of crypt damage (see Methods). Pellet-treated mice had significantly improved histological scores compared to mice treated with DSS alone, with no significant difference between mice treated with or without omeprazole (Fig. 2).

Prior to the more relevant therapeutic protocol described above, we performed an initial pilot study in a preventative model to determine whether an approach of oral delivery of the therapeutic was at all feasible. For this feasibility study, we chose what we considered to be optimal conditions, i.e. treating mice with pellets during the 5-day DSS-induction of colitis (rather than after establishment of colitis) and raising stomach pH with omeprazole. Data from this experiment demonstrated that omeprazole and CR2-Crry pellet treatment reduced the severity of DSS-induced colitis and, further, that pellets containing no protein given to sham controls were safe and did not affect colon histology (Table 3 and Fig. 3).

Pellet treatment decreases local complement activation in the colon

To demonstrate that the therapeutic effect of pellet treatment was associated with local complement inhibition, complement activation was assessed in colon homogenates by analysis of C3d, a cleavage product deposited at sites of complement activation, and C5a, a soluble cleavage fragment. Immunohistochemical analysis revealed C3d staining in the colon mucosa of DSS-treated mice, with significantly lower levels of C3d staining in pellet-treated mice (Fig. 4b,c). Similarly, C5a levels in colon homogenates were significantly lower in DSS+pellet-treated mice compared to mice treated with DSS only (Fig. 4a). Administration of omeprazole did not affect C3d or C5a levels in pellet-treated mice, which correlates with the clinical and pathological data above.

To confirm that delivery of CR2-Crry directly to the colon did not have a systemic complement inhibitory effect, serum complement activity was determined in mice from the different treatment groups. As measured by C3d deposition on zymosan beads, a standard complement activity assay (see Methods), serum complement activity was similar in samples from DSS-treated mice and DSS-treated mice that received CR2-Crry pellet therapy (Fig. 5). Together, these studies show that complement activation is inhibited locally within the colon, but not systemically following treatment with CR2-Crry-containing pellets.

Targeting of CR2-Crry to the colon after oral administration

It has been shown in other models of inflammation that CR2-Crry targets to sites of C3 deposition following intra-

Table 2. Clinical effects and colon length after treatment of established colitis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight change (g)</th>
<th>Stool</th>
<th>Colon length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>+5.8 ± 0.60</td>
<td>Normal</td>
<td>6.9 ± 0.14**</td>
</tr>
<tr>
<td>DSS</td>
<td>−0.2 ± 3.15</td>
<td>Bloody diarrrhea</td>
<td>5.4 ± 0.28</td>
</tr>
<tr>
<td>DSS + pellet</td>
<td>+4.4 ± 1.82</td>
<td>Normal/soft</td>
<td>6.6 ± 0.16*</td>
</tr>
<tr>
<td>DSS + pellet + AA</td>
<td>+2.2 ± 1.16</td>
<td>Normal/soft</td>
<td>6.7 ± 0.17*</td>
</tr>
</tbody>
</table>

Mean ± standard error of the mean, n = 6. Compared to dextran sulphate sodium (DSS) treatment alone, *P < 0.05, **P < 0.01. AA = antacid.

Table 3. Clinical effects and colon length after treatment in a preventative model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight change (g)</th>
<th>Colon length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>+2.2 ± 1.41</td>
<td>6.7 ± 0.14</td>
</tr>
<tr>
<td>DSS</td>
<td>−12.07 ± 0.87</td>
<td>3.75 ± 0.18</td>
</tr>
<tr>
<td>Control pellet</td>
<td>+0.4 ± 1.39</td>
<td>5.78 ± 0.13*</td>
</tr>
<tr>
<td>DSS + AA</td>
<td>−8.88 ± 0.84</td>
<td>4.26 ± 0.28</td>
</tr>
<tr>
<td>DSS + AA + pellet</td>
<td>+0.58 ± 0.99</td>
<td>5.83 ± 0.06*</td>
</tr>
</tbody>
</table>

Mean ± standard error of the mean, n = 6. *P = not significant compared to sham control. **P = not significant compared to dextran sulphate sodium (DSS) alone, *P < 0.05 compared to DSS alone. AA = antacid.

Fig. 2. Treatment of established colitis with CR2-Crry pellets improves histological scores, with or without co-treatment with omeprazole. Mice were killed on day 12 and colons were removed, stained with haematoxylin and eosin (H&E) and scored for damage and inflammation. (a–d) Representative images of H&E staining on colons from (a) sham, (b) dextran sulphate sodium (DSS), (c) DSS + CR2-Crry pellet and (d) DSS + omeprazole (AA) + CR2-Crry pellet-treated mice. (e) Quantification of histological damage and inflammation. *P < 0.05; **P < 0.01; ***P < 0.001 versus DSS, n = 5–6.
venous injection [28], and it has been further shown that the effectiveness of CR2-Cry at inhibiting complement is largely dependent upon its targeting and binding to C3 [21]. We therefore examined whether the CR2-Cry-containing pellets, designed to release the protein in the colon, results in subsequent targeting of released CR2-Cry to the mucosal surface. Following the induction of DSS-induced colitis, mice were treated with pellets containing biotinylated CR2-Cry, and binding of CR2-Cry was visualized by subsequent streptavidin immunofluorescence analysis of colon sections. Sections were also co-stained for C3d. Biotinylated CR2-Cry was detected in the colon of DSS-treated mice in the same staining pattern as C3d (Fig. 6). Colons from sham animals (no DSS) that were treated with biotinylated CR2-Cry pellets displayed minimal streptavidin staining, marginally more than colons from sham animals not treated with pellets. The low level of streptavidin staining seen in colons from sham animals and in DSS-treated animals receiving non-biotinylated CR2-Cry pellets is probably a reflection of the presence of endogenous biotin. As expected, no C3d was detected in colons from sham animals, but was present in colons from DSS-treated mice.

**Discussion**

Although it is unclear whether IBD patients are systemically immunocompromised, studies have suggested that they have impaired innate mucosal immunity [29]. Whether or not colitis patients are inherently immunocompromised, most current treatments for IBD rely on anti-inflammatory or immunosuppressive mechanisms. Currently, one of the most widely used treatments is anti-TNF-α antibody therapy, but this must be administered intravenously and results in systemic immune suppression, with an increased risk of opportunistic infections in IBD patients [20].

![Histological injury score on day 5 after killing.](image)

**Fig. 3.** Treatment with control and CR2-Cry-containing pellets during 5 days of dextran sulphate sodium (DSS) treatment in preventative protocol. Histological injury score on day 5 after killing. AA = antacid treatment with omeprazole. Mean ± standard error of the mean, n = 6.  *P = not significant compared to sham control;  ^P = not significant compared to DSS alone;  _P < 0.05 compared to DSS alone.

![C5a levels and C3d deposition.](image)

**Fig. 4.** Treatment with CR2-Cry pellets inhibits local complement activation. Complement activation was assessed by C5a levels and C3d deposition. (a) C5a levels in colon homogenates as determined by enzyme-linked immunosorbent assay (ELISA). (b) Quantification of C3d deposition from immunohistochemistry staining. (c) Representative images of C3d staining from indicated groups.  *P < 0.05;  n = 5–6.
Complement inhibition poses a similar potential threat for treating IBD patients. While clinical and experimental evidence indicate a role for complement in the pathogenesis of IBD and mucosal injury, there is also evidence that complement has a protective as well as pathogenic role in IBD. One protective function of complement, at least in DSS-induced colitis, relates to complement-dependent host defence and protection from endotoxaemia, and we have shown that certain complement deficiencies can exacerbate DSS-induced colitis [19]. However, we have also shown that systematically (i.p.) administered CR2-Crry targets to sites of complement activation in the colon and is protective. Tissue targeting of the CR2-Crry fusion protein, and its relatively short circulatory half-life (8·7 h [21]), allows for complement inhibition at the site of injury, while allowing rapid recovery of systemic complement function. Importantly, unlike untargeted systemic complement inhibition, CR2-Crry does not increase host susceptibility to polymicrobial sepsis [21]. Even so, repeated systemic administration may have a contraindicative effect for prolonged treatment of IBD. Here, we describe a novel formulation that protects CR2-Crry during passage through the gastrointestinal tract, with release of CR2-Crry within the colon and the subsequent targeting of this protein to the inflamed mucosal surface. With this novel method of drug delivery we have demonstrated therapy of established acute DSS-induced colitis, with no detectable effect on serum complement activity. This strategy will probably not only increase the safety profile of complement inhibitors; another significant factor is patient convenience.

Oral, rather than intravenous, delivery of CR2-Crry has other potential advantages. A significant issue with anti-TNF-α therapy is the development of immunogenicity [30]. The risk of developing an immune response to anti-TNF-α antibodies is increased with increasing dose and treatment duration. Oral delivery of CR2-Crry-containing pellets will allow for localized and lower protein concentrations per treatment, which will be a benefit in terms of decreased risk of immunogenicity. In this context, we demonstrate that

![Fig. 5. Treatment with CR2-Crry pellets does not affect serum complement activity. At end of treatment protocol, complement activity was determined in serum by measuring C3d deposition on zymosan beads by flow cytometry. For an assay control, 50 nM CR2-Crry was added to normal mouse serum. Mean ± standard error of the mean, n = 3.](image-url)

![Fig. 6. Orally administered CR2-Crry targets to the inflamed colon. Images showing immunofluorescence detection of CR2-Crry and C3d after oral administration of pellets containing biotinylated CR2-Crry, or after control protocols. Representative images, n = 5.](image-url)
there is no therapeutic benefit whether pellets are administered with or without omeprazole, even though we gain higher functional in-vitro recovery of CR2-Crry at pH 4 versus 1-5 that simulates omeprazole use. This indicates that a saturating therapeutic concentration is being delivered without antacid treatment. We estimate 26 μg CR2-Crry per pellet, with an estimated 65% recovery without omeprazole without antacid treatment. We estimate 26 μg CR2-Crry per pellet, with an estimated 65% recovery without omeprazole (pH 1-5 in-vitro data), with three administrations per day, which gives an estimated delivery of 48 μg protein per day. In our previous study with systemic administration of CR2-Crry, a 250 μg dose was given [19]. With further regard to the question of immunogenicity, patients are being recruited 2 years into a Phase I trial for a human CR2-targeted complement inhibitor (TT30), in which immunogenicity is a primary outcome measure (http://clinicaltrials.gov/ct2/show/NCT01335165?term=tt30&rank=1).

In summary, although systemic administration of CR2-Crry has been shown previously to have a therapeutic benefit in DSS-induced colitis, the current study shows that packaging of CR2-Crry into modified dextran pellets provides a novel method for oral delivery of the protein. Oral delivery is preferable because it reduces systemic effects, will probably decrease the risk of opportunistic infection, lower the required dose and treatment cost, improve the safety profile of the protein and provide a significant improvement in patient convenience. Furthermore, the novel delivery system described here may provide the same benefits for administration of other protein-based drugs, especially drugs that are not site-targeted, such as anti-TNF-α antibodies.

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Disclosures

The authors declare no competing financial interests.

Author contributions

S. T., A. V. and M. E. designed the study and wrote the paper; M. E., P. B., F. Q., M. S. and C. W. performed research and collected the data; S. T., A. V. and M. E. analysed and interpreted the data.

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