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J Immunol 2007; 179:7860-7867; doi: 10.4049/jimmunol.179.11.7860
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A Complement C3 Inhibitor Specifically Targeted to Sites of Complement Activation Effectively Ameliorates Collagen-Induced Arthritis in DBA/1J Mice

Hongbin Song,2*† Fei Qiao,2* Carl Atkinson,* V. Michael Holers,‡ and Stephen Tomlinson3*

Collagen-induced arthritis (CIA) represents an animal model of autoimmune polyarthritis with similarities to human rheumatoid arthritis, and therapy with various systemic complement-inhibitory proteins has been investigated in this model with varying results. We investigated the use of complement receptor 2 (CR2)-Crry, a complement inhibitor with the ability to target C3 breakdown products deposited in a rheumatic joint. Following induction of CIA in DBA/1J mice, animals were treated with either PBS or CR2-Crry (every other day, every 4 days, or with a single injection). The severity of clinical disease was significantly reduced in all CR2-Crry-treated groups compared with controls. Joints from mice receiving multiple doses of CR2-Crry showed significantly decreased inflammatory cell infiltrate, cartilage damage, pannus formation, and bone damage. CR2-Crry treatment also significantly decreased production of anti-collagen IgG and the inflammatory cytokines TNF-α and IL-1β. IL-10 and IL-1Ra levels were increased in CR2-Crry-treated mice. CR2-Crry localized preferentially in the joints of mice with CIA. Analysis of IgG and C3 deposition in the joints of treated animals indicated that both complement regulation and the modulation of anti-collagen Ab production contributed to the protective effects of CR2-Crry. Of interest, a previous study reported that Crry-Ig, an untargeted counterpart of CR2-Crry, had minimal effect on disease, even when administered at a sufficiently high dose to maintain chronic complement inhibition. The Journal of Immunology, 2007, 179: 7860–7867.

Complement is a central component of innate and adaptive immunity and plays an important role in immune homeostasis. However, under certain disease conditions in which inappropriate or excessive complement activation occurs, complement control mechanisms breakdown or are overcome, and complement activation results in tissue injury. There is a significant amount of evidence from both clinical and animal studies indicating an important role for complement in the maintenance of the polyarticular chronic joint synovitis characteristic of human rheumatoid arthritis (RA).6 In RA patients, complement activation products are found in synovial fluid and deposited on synovial membranes (1–3). In animal models of RA, complement deficiency (4–6), complement inhibition (see below), or complement depletion (7–9) has been shown to be protective, whereas deficiency of the membrane complement inhibitor CD59 increases disease severity (10). Collagen-induced arthritis (CIA) in rodents is an established model of autoimmune polyarthritis with many similarities to human RA. CIA is induced by immunization of genetically susceptible strains with bovine type II collagen (CII). An immune response to bovine CII is followed by an autoimmune response to mouse CII and leads to synovitis and the erosion of bone and cartilage by mechanisms involving both humoral and cellular immune responses. Although evidence indicates a role for T cells in CIA, anti-collagen Abs (particularly IgG2a), complement activation and cytokine production are key mediators of the inflammatory processes that result in tissue injury (for reviews, see Refs. 11 and 12). Cytokines have been studied extensively in CIA, and they clearly influence pathophysiology of the disease. Studies using cytokine-deficient mice and cytokine inhibitors, as well as measurements of cytokine levels in CIA indicate a complex network of interactions. However, there is increased production of proinflammatory cytokines in CIA, with studies in particular indicating a role for IL-1 and TNF-α in pathogenesis (12–14). In contrast, some therapeutic strategies are associated with increased production of anti-inflammatory cytokines such as IL-1Ra and IL-10 (15, 16). An important role for complement in rodent CIA and other models of inflammatory arthritis has been established, although the relative contributions of different complement activation and effector pathways to disease progression has not been resolved. Studies with C3- and factor B-deficient mice indicate activation of both the classic and alternative pathway is important in the pathogenic process (4, 6). Complement inhibitors applied either systemically or intra-articularly have also been shown to be therapeutic in rodent models of inflammatory arthritis. With regard to systemic inhibitors, the C3 convertase inhibitors soluble complement receptor 1 (sCR1) (9) and vaccinia virus complement control protein (VCP) (17) ameliorated inflammatory arthritis. However, in another study, i.v. injection of sCR1 had no effect on the progression of Ab-induced arthritis in rats (18), and Crry-Ig did not...

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Received for publication February 15, 2007. Accepted for publication September 28, 2007.

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1 This work was supported by a fellowship from the Arthritis Foundation (to H.S.) and grants from the Alliance for Lupus Research (to S.T.), National Institutes of Health R01 AR51749 (to V.M.H.), and National Institutes of Health Grant C06 RR015455 for construction and upgrade of animal facilities.

2 H.S. and F.Q. contributed equally to this work.

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4 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, bovine type II collagen; CR1, complement receptor 1; CR2, complement receptor 2; VCP, vaccinia virus complement control protein.

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protect against CIA in mice (16). This was despite the presence of systemic complement inhibition, as measured by serum assays, and the clear benefit of chronic administration of this drug in other disease models including MRL/lpr mice (19) and anti-phospholipid Ab-mediated fetal loss (20). Cry is a structural and functional rodent analog of human CR1. Anti-C5 mAb is protective against CIA in mice (16, 21), whereas C3a receptor antagonist is protective against Ag-induced monoarticular arthritis (22) but not Ab-induced arthritis in rats (18). In the above therapeutic studies, complement inhibitor therapy was administered repeatedly over the course of the study, and systemic levels of complement inhibition were maintained. Intra-articular injection of complement inhibitors that results in local complement inhibition has also provided effective protection in rodent CIA and Ag-induced arthritis. Such an approach has been successful for decay accelerating factor (DAF-Fc fusion protein) (23) and SCR1 (24). A fragment of CR1 (25) and CD59 (26) linked to a membrane inserting tag have also suppressed disease when injected intra-articularly. In the latter approach, repeated injection was not required, and complement inhibitors were retained in the joints for up to 48 h by virtue of their lipotactic tag.

Here we investigate a targeted complement inhibitor that can be systemically (i.v.) administered and provide localized complement inhibition without systemic inhibitory effects. We characterize the effect of (complement receptor 2) CR2-Crry on inflammation, humoral immunity, and clinical disease in mouse CIA. CR2-Crry is a murine recombinant fusion protein in which the complement inhibitor Crry is linked to a targeting domain consisting of a CR2 fragment. The CR2 portion of the CR2-Crry molecule binds to long lived C3 cleavage fragments, iC3b, C3dg, and C3d, which are deposited at sites of complement activation such as the synovium in CIA (6). Importantly, CR2-Crry does not bind circulating C3 in the plasma.

Materials and Methods

Expression plasmids and protein expression
cDNA construct of the recombinant CR2-Crry fusion protein was prepared by joining the mouse CR2 sequence encoding the four N-terminal SCR units (residues 1–257 of mature protein, NCBI GenBank accession no. M35684) to a sequence encoding the extracellular region of mouse Crry (residues 1–319 of mature Crry protein sequence, NCBI GenBank accession no. NM011399). A linking sequence encoding (GGGGSG), was used for joining the two protein fragments. Details of experimental procedures for plasmid construction, recombinant protein expression, and protein purification have been described (27).

CIA model and therapy

Male DBA/1J mice between 8 and 10 wk old were injected intradermally at the base of the tail with 100 μl of an emulsion containing 200 μg of bovine CII (Sigma-Aldrich) and 200 μg of Mycobacterium tuberculosis inIFA. A booster injection of the same emulsion was administered intradermally on day 21. After the second injection of CII on day 21, mice were divided into four treatment groups. Starting on day 21, each group was injected i.v. with either 50 μl of PBS every other day (control), CR2-Crry every other day (total of 7 injections), CR2-Crry every 4 days (4 injections) or a single injection of CR2-Crry. All CR2-Crry injections were 0.25 mg in 50 μl of PBS. Starting on day 21, clinical disease activity in each paw was scored in a blinded fashion on a 3-point scale as previously described (15): 0 = normal joint; 1 = slight inflammation and redness; 2 = severe erythema and swelling affecting the entire paw, with inhibition of use; 3 = deformed paw or joint, with ankylosis, joint rigidity, and loss of function. Clinical disease activity was assessed in all 4 paws, with a maximum score of 12 for each mouse.

Histology

Both forepaws and the right hind limb (including the paw, ankle, and knee) were removed from all mice on day 35 and fixed immediately in 10% buffered formalin (Biochemical Sciences). The preparation of tissue samples and histologic analyses was performed as previously described (28). Sections were scored on a scale of 0–5 for synovial inflammation, pannus formation, and cartilage and bone damage (28). All sections were read by Dr. Martin Goodard (Papworth Hospital, Cambridgeshire, U.K.), a trained observer who was blinded to the treatment group and clinical disease activity score of each mouse.

Measurement of anti-collagen Abs

Mouse serum was obtained by retro-orbital aspiration of blood at the time of the first injection of CII (day 0) and just before second injection of CII (day 21). On day 35, serum was obtained from blood collected by cardiac puncture from all experimental mice and from 5 normal control DBA/1J mice. Anti-collagen IgG1, IgG2a, and total IgG in serum samples were assayed by ELISA as previously described (15).

Cytokine mRNA assays

Total RNA was extracted with TRIzol (Invitrogen) from isolated knee joints without dissecting out the synovium; it has been determined previously that cytokine mRNA levels in whole joints are proportional and equivalent to levels in isolated synovial samples (29). The quality of the total RNA was assessed by gel analysis. The identity of each mRNA species from total RNA samples was determined by SYBRGreen real-time PCR using the MiYQ real-time PCR detection system (Bio-Rad). Quantitative real-time PCR assays were performed by adding SYBRGreen in the reaction mix following the recommended protocol (Bio-Rad). In this study, template sets were used that included mRNA for IL-10, IL-1β, IL-1β, and TNF-α. These sets also included mRNA for the housekeeping gene GAPDH. Formation of PCR product was monitored in real time using the MiYQ real-time PCR detection system, and results are expressed as relative fold change in gene expression of the target gene normalized to the internal control gene expression and relative to the control sample (30).

Biodistribution study

CR2-Crry was labeled with 125I by the IODO-GEN method (Amersham Biosciences) as we have previously described (27). Five milligrams of 125I was used to label 100 μg of CR2-Crry protein, and iodine incorporation was in the 50–80% range. 125I-CR2-Crry (2 μg) was injected i.v. into control mice and groups of mice with CIA on day 29 (7 days after second intradermal injection of CII (n = 3–4 per group)). Groups of mice were sacrificed either 24, 48, or 72 h after 125I-CR2-Crry injection for analysis. In a separate analysis, mice with CIA were treated with therapeutic doses (0.25 μg) of CR2-Crry every other day beginning at time of second CII injection on day 21 and continuing through day 31. These mice were injected with 125I-CR2-Crry on day 33 (48 h after last therapeutic dose of CR2-Crry) and the animals sacrificed 24 h later for analysis. After sacrifice and removal of blood by cardiac puncture, animals were perfused with 10 ml of PBS and then heart, lung, spleen, liver, kidney, intestine, left joint, right joint, left ankle, right ankle, and paws were removed. Tissues were rinsed in RPMI 1640 (Invitrogen), weighed and counted with a Hewlett-Packard 5780 gammacounter at the 125I window with appropriate corrections for count decay. Results are expressed as millicuries per gram of tissue (27).

![FIGURE 1. Effect of CR2-Crry on clinical disease activity in mice with collagen-induced arthritis. After the second injection of CII on day 21, groups were injected i.v. with PBS every other day (n = 7), CR2-Crry every other day (7 injections, n = 10), CR2-Crry every 4 days (4 injections, n = 10) or with a single injection of CR2-Crry (n = 9). Clinical disease activity was assessed in each paw and scored on a 3-point scale (maximum score of 12). Mean ± SD.](http://www.jimmunol.org/)

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Immunohistochemistry

At sacrifice, the left knee, and ankle and paw joints were removed, fixed in formalin, and processed to paraffin as previously described (6). The presence of C3d and IgG were assessed immunohistochemically with polyclonal rabbit anti-human C3d that cross reacts with mouse C3d (DakoCytomation) and with rabbit anti-mouse IgG (MP Biomedicals). Primary Ab binding was visualized by standard streptavidin biotin complex immunostaining as previously described (27). Scoring for C3d and IgG was performed on the synovium and cartilage separately using a 3-point scoring system. Scoring was graded as follows: 0, absence of staining; 1, low; 2, moderate; 3, high.

Assay for anti-CR2-Crry Ab response.

The development of an immune response to CR2-Crry was assessed by ELISA. Dynatech Immulon II (Dynatech Laboratories) 96-well plates were coated with CR2-Crry (10 μg/well, 4°C overnight). Plates were then washed 4 times with PBS containing 0.05% Tween 20, and blocked for 1 h at room temperature with PBS containing 1% BSA (Sigma-Aldrich). Plates were washed four times with PBS/Tween 20 before the addition of heat-inactivated serum from control (PBS treated) mice with CIA or from mice treated with 7 injections of CR2-Crry. Serum was prepared from blood collected at the termination of experiments on day 35. Following 1 h of incubation at room temperature, plates were washed with PBS/Tween 20 and incubated with streptavidin peroxidase-conjugated anti-mouse IgM or IgG for 2 h before development with O-phenylenediamine dihydrochloride reagent (Sigma-Aldrich). Readout was performed at 492 nm.

Statistical analysis

Data are expressed as the means ± SD. Statistical analysis was conducted using StatView. Significant differences between groups were determined by ANOVA, and p < 0.05 was considered significant. All histological statistical analyses were conducted using nonparametric measures and p < 0.05.

Results

Effect of CR2-Crry on clinical disease activity in mice with collagen-induced arthritis

Immediately after the second injection of CII on day 21, mice were randomly divided into treatment groups and injected i.v. with either PBS every other day (control), CR2-Crry every other day (total of 7 injections), CR2-Crry every 4 days (4 injections) or a single injection

Table I. Effect of CR2-Crry treatment on histologic scores in mice with collagen induced arthritis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS (n = 7)</td>
</tr>
<tr>
<td></td>
<td>CR2-Crry × 7 (p) (n = 10)</td>
</tr>
<tr>
<td></td>
<td>CR2-Crry × 4 (p) (n = 10)</td>
</tr>
<tr>
<td></td>
<td>CR2-Crry × 1 (p) (n = 5)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>2.26 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.53 ± 0.5 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>0.65 ± 0.8 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.5 (0.002)</td>
</tr>
<tr>
<td>Pannus</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.42 ± 0.5 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>0.43 ± 0.5 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>1.2 ± 0.5 (0.005)</td>
</tr>
<tr>
<td>Cartilage damage</td>
<td>2.02 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.62 ± 0.4 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>0.68 ± 0.7 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.1 (0.017)</td>
</tr>
<tr>
<td>Bone damage</td>
<td>1.14 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.31 ± 0.4 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.1 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.5</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Scores based on a scale of 0–5. Shown in parentheses is the p value versus PBS treated group.

Figure 2. Representative images of the histopathology of CIA in control and CR2-Crry-treated animals. H&E staining of joints from mice treated with PBS (A), a single dose of CR2-Crry (B), four injections of CR2-Crry (C), and seven injections of CR2-Crry (D). Note the presence of extensive infiltration of inflammatory cells into the synovial cavity, pannus formation, and proliferation of the cells lining the synovial membrane seen in control animals (A) when compared with CR2-Crry-treated animals. Inflammatory cells are present in animals treated with a single dose of CR2-Crry (B) but are significantly reduced when compared with control. Images representative of n = 10; magnification, ×100.
Titers (at day 21) were 36.2 for mice receiving a single injection of CR2-Crry on day 21 (from day 21 (4 injections) (7 injections) (n = 8), mice receiving a single injection of CR2-Crry compared with mice treated with PBS (p < 0.05 from day 30). There was no significant difference in disease activity between control mice and mice treated with PBS or CR2-Crry. No anti-CII Ab Abs were detected in sera collected at day 0 from any group. Fig. 3 shows the difference in levels of Ab between days 21 and 35 and demonstrates the effect of CR2-Crry treatment on anti-CII Abs. There was an increase in anti-collagen IgG1, IgG2a and total IgG in all groups between days 21 and 35, but the increase was significantly lower in mice treated with multiple injections of CR2-Crry (both 4 and 7 injections) compared with mice treated with PBS (p < 0.05 for both treatment groups). The change in total IgG anti-CII levels was also significantly lower in mice treated with a single injection of CR2-Crry compared with mice treated with PBS (p < 0.05), but the change in IgG1 and IgG2a levels between these two groups of mice was not significantly different.

Anti-CR2-Cry Ab response was also investigated by ELISA. There was no detectable anti-CR2-Cry IgM or IgG response in mice with CIA treated with 7 injections of CR2-Crry.

**Analysis of cytokine levels**

Cytokines have been shown to play an important role in inflammation and joint destruction in rheumatoid arthritis and rodent CIA, and we therefore investigated the effect of CR2-Crry on the production of select inflammatory and anti-inflammatory cytokines in the joints. Cytokine mRNA levels were measured in isolated joints, and the data presented in Table II shows the mean fold change between control mice and mice treated with PBS or CR2-Crry. Local levels of IL-1β and TNF-α were significantly reduced and levels of IL-1Ra and IL-10 significantly increased in joints of CR2-Crry. Clinical disease activity was assessed three times a week over a period of 2 wk. Clinical disease activity was observed in all groups, but the severity of clinical disease was significantly reduced in all CR2-Crry treated groups compared with the PBS treated control group (p < 0.05 from day 25) (Fig. 1). Mice receiving multiple injections of CR2-Crry, however, developed less severe arthritis than mice receiving a single injection of CR2-Crry (p < 0.05 from day 30). There was no significant difference in disease activity between mice receiving 7 or 4 injections of CR2-Crry.

### Table II. Effect of CR2-Cry on levels of cytokine mRNA in joints of mice with collagen-induced arthritis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PBS (n = 5)</th>
<th>CR2-Cry × 7 (n = 10)</th>
<th>CR2-Cry × 1 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>2.56 ± 0.40</td>
<td>1.84 ± 0.36*</td>
<td>2.24 ± 0.41</td>
</tr>
<tr>
<td>TNFα</td>
<td>3.14 ± 0.38</td>
<td>2.11 ± 0.47*</td>
<td>2.71 ± 0.35</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>1.31 ± 0.26</td>
<td>1.79 ± 0.31*</td>
<td>1.61 ± 0.30*</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.81 ± 0.42</td>
<td>3.22 ± 0.44*</td>
<td>1.98 ± 0.34</td>
</tr>
</tbody>
</table>

*Values are mean fold difference compared to controls ± SD. *p < 0.05 versus PBS.

**Histopathology**

On day 35 after the initial CII injection both forepaws and the right hind limb (including the paw, ankle, and knee) were removed from mice in each group for histological analysis. Sections were scored on a scale of 0–5 as previously described (28). Joints from mice that received multiple CR2-Cry injections showed significantly decreased inflammatory cell infiltrate, bone damage, cartilage damage and pannus formation compared with PBS control animals (Table I and Fig. 2). There were no significant differences in the scores between mice receiving either 4 or 7 injections of CR2-Cry. Joints from mice that received a single injection of CR2-Cry also had significantly lower scores for all of the above parameters except for bone destruction, although these mice were less well protected from joint inflammation and injury than mice receiving multiple CR2-Cry injections. Joints from normal control mice that received no CII showed normal histology with no signs of inflammation or damage.

**Ab response to bovine CII**

Serum levels of anti-bovine CII IgG1, IgG2a and total IgG were determined at day 0 (before first CII injection), day 21 (before second CII injection and treatment) and day 35. Ab levels were analyzed in control mice (no CII), PBS-treated mice, and mice receiving either 7 injections of CR2-Cry, 4 injections of CR2-Cry or a single injection of CR2-Cry (Fig. 3). No anti-bovine CII Abs were detected in sera collected at day 0 from any group. Fig. 3 shows the difference in levels of Ab between days 21 and 35 and demonstrates the effect of CR2-Cry treatment on anti-CII Abs. There was an increase in anti-collagen IgG1, IgG2a and total IgG in all groups between days 21 and 35, but the increase was significantly lower in mice treated with multiple injections of CR2-Cry (both 4 and 7 injections) compared with mice treated with PBS (p < 0.05 for both treatment groups). The change in total IgG anti-CII levels was also significantly lower in mice treated with a single injection of CR2-Cry compared with mice treated with PBS (p < 0.05), but the change in IgG1 and IgG2a levels between these two groups of mice was not significantly different.

Anti-CR2-Cry Ab response was also investigated by ELISA. There was no detectable anti-CR2-Cry IgM or IgG response in mice with CIA treated with 7 injections of CR2-Cry.
from mice treated with 7 injections of CR2-Crry compared with mice treated with PBS. For mice treated with a single injection of CR2-Crry, the only significant difference compared with PBS-treated mice was an increased level of IL-1Ra.

**Localization of CR2-Crry in joints**

In a previous study, the complement inhibitor Crry-Ig, a systemic counterpart of the targeted inhibitor CR2-Crry, was not protective in the same model of CIA. A possible explanation for this difference is increased bioavailability and local retention of Crry at the joints due to the targeting of CR2-Crry. The specificity of CR2 for C3 degradation products (iC3b, C3dg, C3d) is established (32), and C3 deposition in the joints of mice with CIA has been shown previously (6, 15) (also shown below). We therefore investigated in vivo targeting of CR2-Crry by measuring the biodistribution of injected $^{125}$I-labeled CR2-Crry after the induction of CIA (at day 29). At 24 h post administration of $^{125}$I-CR2-Crry, there was a statistically significant increase ($p < 0.05$) in $^{125}$I-CR2-Crry localization in knee joints, ankles, and paws of CIA mice compared with control mice, but no significant difference in $^{125}$I-CR2-Crry localization between CIA mice and control mice in any other organ (Fig. 4A). There were also lower levels of $^{125}$I-labeled CR2-Crry in the blood of CIA mice compared with normal mice, probably reflecting the binding of CR2-Crry to sites of complement activation and inflammation and its removal from the circulation. To further investigate the local retention of CR2-Crry, biodistribution studies were also performed at 48 and 72 h after injection of $^{125}$I-CR2-Crry in animals with CIA. The data shown in Fig. 4B reveal that CR2-Crry has a relatively short retention time in the joints of CIA mice, with an approximate two thirds reduction in $^{125}$I-CR2-Crry binding between 24 and 48 h. By 72 h, $^{125}$I-CR2-Crry binding in the joints of CIA mice is almost undetectable.

In a separate analysis, mice with CIA were treated with therapeutic doses (0.25 μg) of CR2-Crry every other day beginning at time of second CII injection on day 21 and continuing through day 31. These mice were injected with $^{125}$I-CR2-Crry on day 33 (48 h after last therapeutic dose of CR2-Crry) and the animals sacrificed 24 h later for analysis. The amount of $^{125}$I-CR2-Crry localized to the joints, ankles and paws (and all other organs) in the CR2-Crry treated animals was significantly lower than in untreated animals and was indistinguishable from control animals without CIA (Fig. 4A). These data indicate CR2-Crry therapy results in reduced deposition of C3d, the targeting ligand for CR2-Crry, in the joints of CIA mice. In this experiment, $^{125}$I-CR2-Crry was administered 48 h after the last therapeutic injection of CR2-Crry, at which time there are relatively low amounts of CR2-Crry remaining in the joints (Fig. 4B). Therefore, the blocking of $^{125}$I-CR2-Crry binding by therapeutically administered CR2-Crry is unlikely to account for the level of reduced $^{125}$I-CR2-Crry binding seen in treated animals. C3d deposition was further investigated by immunohistology (below).

**IgG and C3d immunohistochemistry**

In an attempt to clarify whether the protective effects of CR2-Crry are due more to complement control or to reducing the levels of anti-collagen Abs, we examined IgG and C3 deposition in the synovium and cartilage of joints from control (PBS treated) and CR2-Crry treated mice. Formalin fixed sections of joints isolated from animals at day 35 were analyzed by immunohistochemistry. Quantification of staining intensity (see methods) indicated less IgG deposition in both the synovium and the cartilage of animals treated with multiple doses (both 4 and 7 injection groups) of CR2-Crry compared with control animals, although the differences reached statistical significance only for synovial deposition of IgG ($p < 0.05$) (Fig. 5A). Compared with IgG, there was a more significant reduction in C3d deposition in the synovium of animals treated with either 4 or 7 injections of CR2-Crry ($p < 0.01$), and there was also a significant reduction of C3d deposition in the cartilage of CR2-Crry treated animals (Fig. 5B). There were no appreciable differences in either IgG or C3d deposition between animals treated with 4 or 7 injections of CR2-Crry. A single injection of CR2-Crry did not reduce IgG or C3d deposition compared with control animals.

Representative images of IgG and C3d staining are shown in Fig. 6. In control animals, IgG staining was present in the synovium, connective tissue, within articular tissues, and was present within areas associated with inflammatory cell infiltration (Fig. 6, A and B). There is an apparent decrease in IgG deposition in the joints of animals treated with 7 injections of CR2-Crry compared with control animals (Fig. 6, C and D). There was no appreciable differences in IgG staining patterns between animals treated with 4 or 7 doses of CR2-Crry. C3d staining of joints from PBS treated controls showed a similar distribution pattern to that seen for IgG, with the exception that areas of inflammatory infiltrate were more intensely stained (Fig. 6, E and F). The joints from animals treated with 7 injections of CR2-Crry revealed that C3d deposition was almost absent from the synovium and was limited mostly to chondrocytes (Fig. 6, G and H). There was no appreciable difference in C3d staining pattern between animals treated with 4 or 7 doses of CR2-Crry. Treatment with a single dose...
transgenic animals, increased local production of mRNA for the Crry transgene was readily detected in the synovium. A mechanistic explanation for the different therapeutic effects of targeted vs systemic complement inhibition in CIA may be related to the different effect of CR2-Crry vs Crry-Ig on the humoral response to collagen and on cytokine production. Humoral autoimmunity in CIA is measured beginning by day 7–10 by the development of IgG Abs to CII that react with both bovine and murine collagen, with the major pathogenic Ab isotype being IgG2a (33–35). Multiple injections of CR2-Crry significantly suppressed anti-collagen IgG1, IgG2a and total IgG production, whereas Crry-Ig affected only IgG2a production. One possibility for the more profound effect of CR2-Crry on anti-CII Ab production may be due to its ability to target C3 opsonized Ags and block the costimulatory signal that results from the coengagement of the BCR and CR2 when it acts as the iC3b/C3dg/C3d receptor on B cells (36). Consistent with this idea, a previous study showed that treatment with CR2-Ig (which does not contain a C3 activation inhibitor) was able to block the development of a T-dependent Ag response when administered before injection of a model Ag (37). Similar effects on humoral immune responses were found when other means of blocking CR2 function on B cells was used, including the use of mAbs (38) and gene-targeting techniques (39–41). In this context, it was also recently shown that C3d-bound CII is sufficient to initiate CIA in the absence of other adjuvant immunostimulation, and mice deficient in CD21 are protected from CIA (42). Thus, CR2-Crry may demonstrate efficacy not only because of inhibition of C3 activation but also by diminishing the autoantibody response due to blocking B cell function directly. In an attempt to clarify whether the protective effects of CR2-Crry are due more to complement control or to reducing the levels of anti-collagen Abs, we examined IgG and C3 deposition in the synovium and cartilage of joints from PBS treated controls and the different groups of CR2-Crry treated mice. The significant (p < 0.05) reduction in synovial (although not cartilage) deposition of IgG in the CR2-Crry treated animals indicate that the protective effect of CR2-Crry is due, at least in part, to the inhibitory effect of CR2-Crry on anti-collagen Abs. Reduced IgG deposition can be expected to result in reduced complement activation and C3 deposition, but the highly significant reduction in C3d deposition in both the synovium and cartilage of CR2-Crry treated animals suggests that localized complement inhibitory activity of CR2-Crry also contributes to its protective effect. There is also the possibility that a contributing factor to the protective effect of CR2-Crry may be due to its ability to interfere with the recognition of iC3b by complement receptor type 3 expressed on immune effector cells. We have previously demonstrated such an effect in vitro (43).

There were also differences in local cytokine production between mice treated with CR2-Crry and Crry-Ig. In contrast to Crry-Ig, multiple injections of CR2-Crry significantly reduced the levels of the inflammatory cytokines IL-1β and TNF-α in joints, and increased the levels of IL-10 and IL-1Ra when compared with PBS treated controls. In contrast, mice expressing the Crry transgene (15) and mice treated with anti-C5 mAb (16) (both of which were protected from CIA) were found to produce IL-1β, TNF-α and IL-1Ra locally at levels that similarly reflected the changes seen in CR2-Crry treated mice. Levels of both IgG1 and IgG2a anti-collagen Abs were also significantly reduced in Crry transgenic mice and anti-C5 mAb treated mice. Thus, there does appear to be differences in the specific biologic effects of using either systemic or targeted complement inhibition by various strategies. In addition to anti-C5 mAb (16, 21), various other complement inhibitors have been shown to be protective in mouse and rat models of inflammatory arthritis. The systemic complement inhibitors sCR1 (9) and VCP (17) (functional analogues of Crry) ameliorated disease, as did intra-articular injection of CD59 and a fragment of CR1 when linked to a membrane-inserting lipid tag (25, 26). However, there are some important distinctions to be made between these

Discussion

Various complement inhibitors have shown efficacy in rodent models of inflammatory arthritis (see introduction). These inhibitors have been administered systemically resulting in systemic inhibition of the complement system, or intra-articularly resulting in localized inhibition. In the current study we investigate a complement inhibitor that targets to the joints following systemic (i.v.) administration. At a dose previously shown to have minimal effect on systemic (serum) complement activity (27), CR2-Crry provided effective protection from CIA in mice. Importantly, systemic untargeted inhibition with the same type of inhibitor (Crry-Ig) in the same aggressive model of CIA, did not result in a significant reduction in clinical disease activity or joint damage compared with control treated mice (16). Furthermore, Crry-Ig was repeatedly administered at high dose (3 mg every other day for 2 wk), whereas a single 0.25 mg dose of CR2-Crry given at the onset of clinical disease was partially protective, and the beneficial effect of using doses of 0.25 mg every fourth day were indistinguishable from every other day treatment with CR2-Crry. A possible reason for the difference between systemic and targeted inhibition is increased bioavailability of the targeted inhibitor within the joint, and we show CR2-Crry targets to the joints of mice following induction of CIA. In further support of this concept is the finding that transgenic expression of soluble Crry, unlike administered Crry-Ig, effectively reduced clinical activity and tissue damage in a mouse model of CIA (15). In the transgenic animals, increased local production of mRNA for the Crry...
previous studies and the complement inhibitory strategy used here. In the current study, local effective complement inhibition was achieved following i.v. (systemic) injection, a more preferable route of administration than intra-articular. In addition, systemic complement inhibition is not required for effective CR2-Crry treatment of CIA, a potentially important consideration for the treatment of a chronic autoimmune disease such as RA, and given the important role of complement in host defense and in the clearance of immune complexes and apoptotic cells. The dose of CR2-Crry used does not result in systemic (serum) complement inhibition (27), and the circulatory half life of CR2-Crry is short (~8 h (27)). Indeed, we have shown previously that repeated injection of CR2-Crry at the dose used in the current study does not increase susceptibility to infection in a mouse model of septic peritonitis, which was in contrast to the systemic inhibitor Crry-Ig (27). It is notable that mice given a single injection of CR2-Crry would have normal serum complement activation levels

FIGURE 6. IgG and C3d deposition in the joints of mice with CIA and treated with PBS or CR2-Crry. A–D, IgG immunohistochemistry showing IgG deposition in PBS-treated controls (A, B) and in mice treated with 7 injections of CR2-Crry (C, D). E–H, C3d immunohistochemistry showing C3d deposition in PBS-treated controls (E, F) and in mice treated with 7 injections of CR2-Crry (G, H). Representative images shown.
for 12–13 days before the experimental endpoint, and those given CR2-Cry every four days would have normal serum activity for >75% of the time. Thus, because relatively infrequentadministration of CR2-Cry is required to protect against CIA, serum complement activity will be largely maintained. The preferential retention of CR2-Cry in the joint when measured at 24 h after injection is likely an important feature of its mechanism of action. The previously characteri-zed6 inhibitors anti-C5 mAb, sCR1 and VCP required frequent in-jections to maintain systemic complement inhibition for protection against inflammatory arthritis.

Acknowledgments

We thank Dr. Martin Goodard (Papworth Hospital, Cambridge, U.K.) for histological analysis of sections.

Disclosures

V. M. Holers is a cofounder of Taligen Therapeutics, which develops complement inhibitors for therapeutic use.

References