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Human C5aR knock-in mice facilitate the production and assessment of anti-inflammatory monoclonal antibodies

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Complement component C5a binds C5a receptor (C5aR) and facilitates leukocyte chemotaxis and release of inflammatory mediators. We used neutrophils from human C5aR knock-in mice, in which the mouse C5aR coding region was replaced with that of human C5aR, to immunize wild-type mice and to generate high-affinity antagonist monoclonal antibodies (mAbs) to human C5aR. These mAbs blocked neutrophil migration to C5a *in vitro* and, at low doses, both prevented and reversed inflammatory arthritis in the murine K/BxN model. Of ~40 mAbs generated to C5aR, all potent inhibitors recognized a small region of the second extracellular loop that seems to be critical for regulation of receptor activity. Human C5aR knock-in mice not only facilitated production of high-affinity mAbs against an important human therapeutic target but were also useful in preclinical validation of the potency of these antagonists. This strategy should be applicable to other important mAb therapeutics.

Chemoattractant receptors, a class of G protein–coupled receptors that facilitate cell migration[1,2], have attracted considerable interest, because blocking these receptors or their ligands ameliorates various inflammatory conditions in animal models[2–4]. However, development of suitable small-molecule antagonists for many chemoattractant receptors has been problematic. Recently, mAbs have been used to antagonize chemoattractant receptors and to identify important regions for ligand binding[5]. The receptor for C5a plays a critical role in numerous inflammatory conditions[6–9]. C5a is a member of the complement cascade, an important mechanism for host defense against bacteria. Increased complement activation and excessive production of C5a have been implicated in the pathogenesis of several inflammatory diseases, and considerable effort has gone into developing C5aR antagonists, including organic small molecules and peptide antagonists[10].

mAbs constitute a rapidly growing class of therapeutics, partly because of their predictable pharmacokinetic properties, their high success rate in the clinic[11,12] and their ability to antagonize interactions between large proteins such as a receptor and its protein ligand. However, poor cross-reactivity of certain mAbs between humans and rodents complicates use of the same drug both for preclinical studies involving rodents as well as for human therapies. Usually, 'surrogate' mAbs are used to examine efficacy or safety in models of disease, and results with such antibodies in mice or even primates may not always equate with the properties of the same or a different drug used in human clinical trials, as noted recently with mAbs to CD28 (anti-CD28 mAbs)[13]. We reasoned that the availability of human C5aR knock-in mice might provide an effective means to validate this receptor in mouse models of disease, using reagents that could be developed for use in clinical trials. Additionally, human C5aR knock-in mice could be a convenient means of generating very high-affinity antagonistic mAbs to human C5aR, through immunization of wild-type mice with neutrophils from human C5aR knock-in mice.

We raised mAbs to human C5aR, first by using a well-tried approach for chemoattractant receptors[5,14,15]. Mice were immunized with L1.2 cells (a mouse B-cell lymphoma line) with high expression of human C5aR (~80,000 receptors per cell). Five fusions yielded several mAbs that reacted specifically with human C5aR transfectants but not with transfectants expressing other closely related chemoattractant receptors, such as CXCR1, CXCR2 or the other C5a-binding receptor, C5L2 (ref. 16) (**Fig. 1a**). Several of the mAbs inhibited both binding of ^{125}I-labeled human C5a to human C5aR transfectants as well as chemotaxis of human neutrophils to C5a *in vitro* (see **Supplementary Fig. 1** online).

In a second approach to develop potent anti-human C5aR mAbs, we reasoned that wild-type mice immunized with neutrophils from human C5aR knock-in mice should mount a focused response to human C5aR, because it will be the only foreign antigen and is expressed highly on neutrophils (as many as 200,000 molecules per cell)[1]. 'Humanized' C5aR mice were generated by targeted-homologous recombination at the mouse C5aR gene (C5aR1). Simultaneous

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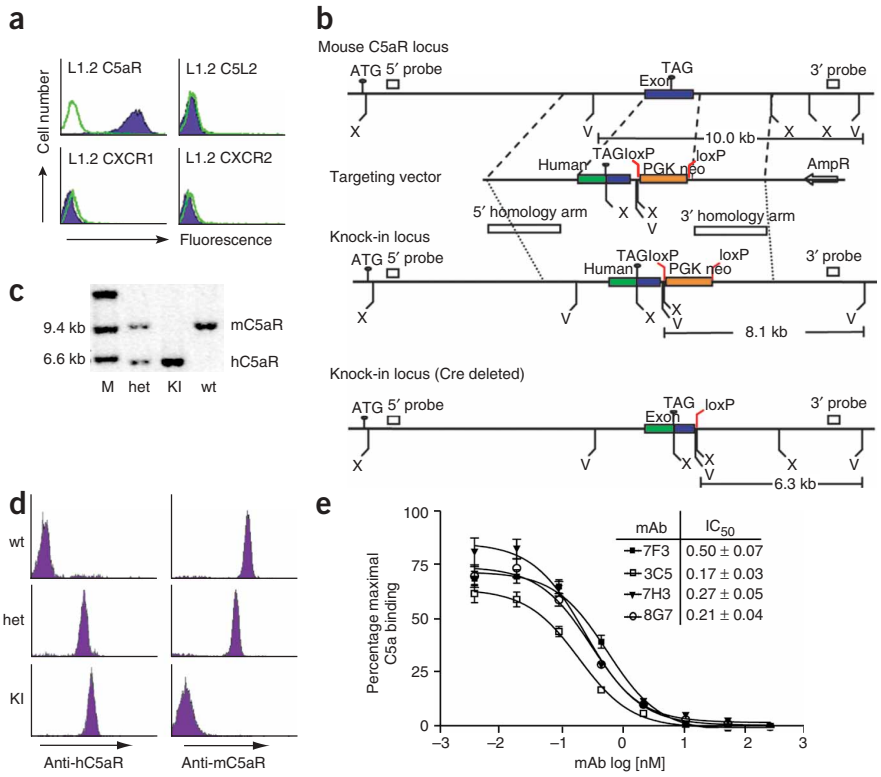

Figure 1 Generation of human C5aR knock-in mice and anti-human C5aR mAbs. (**a**) L1.2 transfectants expressing high levels of C5aR stained with a representative anti-C5aR mAb 7F3 (filled histogram) or IgG2a isotype control antibody (open histogram). mAb 7F3 stained human C5aR transfectants but not L1.2 cells transfected with CXCR1, CXCR2 or the second C5a-binding receptor C5L2. (**b**) Map of the *C5aR* locus in wild-type mice, and in human C5aR knock-in mice, and details of the targeting vector used to create human C5aR knock-in mice. X, *Xba*I; V, *Eco*RV. (**c**) Southern blot of *Eco*RV-digested genomic DNA from the tails of mice from a cross between heterozygous human C5aR knock-in mice (*hC5aR1*$^{+/-}$). M, marker; KI, *hC5aR1*$^{+/+}$; het, *hC5aR1*$^{+/-}$; wt, wild-type mice. (**d**) Expression of C5aR on neutrophils from KI, het and wt mice. Neutrophils were stained with fluorescein isothiocyanate–conjugated anti-human C5aR mAb 7F3 or anti-mouse C5aR mAb 20/70. (**e**) Anti-C5aR mAbs showed subnanomolar IC_{50} values. Antibodies generated using *hC5aR1*$^{+/+}$ mice neutrophils (3C5, 7H3 and 8G7) showed IC_{50} values two- to threefold lower than the best mAb generated using L1.2/hC5aR transfectants (7F3). IC_{50} values were determined from three or four independent competitive ^{125}I-C5a ligand-binding experiments. Data are mean ± s.e.m.

deletion of the endogenous mouse C5aR coding sequence and its replacement with human C5aR coding sequence was achieved by transfecting mouse embryonic stem cells with the targeting construct (**Fig. 1b**). Mice homozygous for the human C5aR transgene (hC5aR1$^{+/+}$) were identified by Southern blot analysis (**Fig. 1c**). Neutrophils from these mice had very high expression of hC5aR, as judged by flow cytometry using anti-human C5aR mAb 7F3, whereas neutrophils from wild-type mice were not stained by 7F3 but were stained intensely by the anti-mouse C5aR mAb 20/70 (ref. 17) (**Fig. 1d**). Human and mouse C5aRs share only 65% homology[18], but importantly for the development of human C5aR knock-in mice, mouse and human C5a bind human C5aR with similar affinities (unpublished observations). Correspondingly, human C5aR was functional in mice, in that neutrophils from hC5aR1$^{+/+}$ mice migrated in response to both human and mouse C5a, *in vitro*, in a similar fashion (data not shown).

After immunization of wild-type mice with neutrophils from hC5aR1$^{+/+}$ mice, we generated numerous human C5aR-specific mAbs. Ligand-binding assays revealed that many of these mAbs showed better inhibition of ^{125}I-labeled C5a binding to human neutrophils than did our best anti-human C5aR mAb generated using transfectants (7F3). The most potent inhibitor, mAb 3C5, had an IC_{50} of 171 pM, compared with an IC_{50} for mAb 7F3 of 503 pM (**Fig. 1e** and **Supplementary Fig. 1b** online). The anti-human C5aR mAbs generated by immunization of wild-type mice with neutrophils from hC5aR1$^{+/+}$ mice were all distinct, in that the amino acid sequences of the heavy-chain variable regions differed, indicating origins from separate clones (data not shown). In human neutrophils, anti-human C5aR mAbs 3C5 and 7F3 blocked calcium flux induced by C5a but not other chemoattractants such as IL-8, fMLP or SDF-1α (see **Supplementary Fig. 2** online). Neither 7F3 nor 3C5 showed agonistic activity in human neutrophils, as measured by calcium flux assays (data not shown). The generation of numerous high-quality and high-affinity anti-human C5aR mAbs allowed us to accurately assess C5aR expression on human leukocytes. C5aR was expressed at high levels on neutrophils, eosinophils, basophils and monocytes, but was absent from all subsets of naive, memory or effector T cells, and from most B cells (see **Supplementary Fig. 3a** online). This protein expression profile was also confirmed at the RNA transcript level, using Affymetrix Genechip profiling (see **Supplementary Fig. 3b** online), establishing C5aR as a receptor on innate and effector leukocytes but not T cells.

The use of cells expressing the entire C5aR allowed us to generate anti-human C5aR mAbs recognizing, potentially, any of the extracellular domains, as well as C5aR epitopes dependent on conformation or comprising more than one domain. The identification of regions on C5aR necessary for ligand binding or function is an important consideration for drug development. Increasingly, mAbs are proving to be useful for identifying these regions[15]. Several studies have implicated the N terminus of C5aR as an essential region for ligand binding[19–21]. We therefore determined the critical epitopes of human C5aR recognized by all of the blocking mAbs. Because these antibodies recognize human but not mouse C5aR, we constructed a panel of human/mouse C5aR chimeric receptors (**Fig. 2a**). Single or multiple extracellular domains of human C5aR were sequentially replaced by the homologous region from mouse C5aR using an overlapping extension polymerase chain reaction (PCR) method[22]. Chimeric receptors were expressed in mouse L1.2 cells, and mAb reactivity was determined by flow cytometry (**Fig. 2a**). Of the 24 antibodies tested, most bound to either the N terminus or the second extracellular loop of human C5aR. However, without exception, all antibodies with the most potent C5aR blocking activity bound to the second extracellular loop (**Fig. 2b**). To define the precise contact residues of the most potent blocking antibodies on the C5aR second extracellular loop, we performed peptide scan analyses using a set of 12-mer peptides, each overlapping by 11 amino acids, covering the second extracellular loop of human C5aR. mAbs 3C5 and 7F3 showed

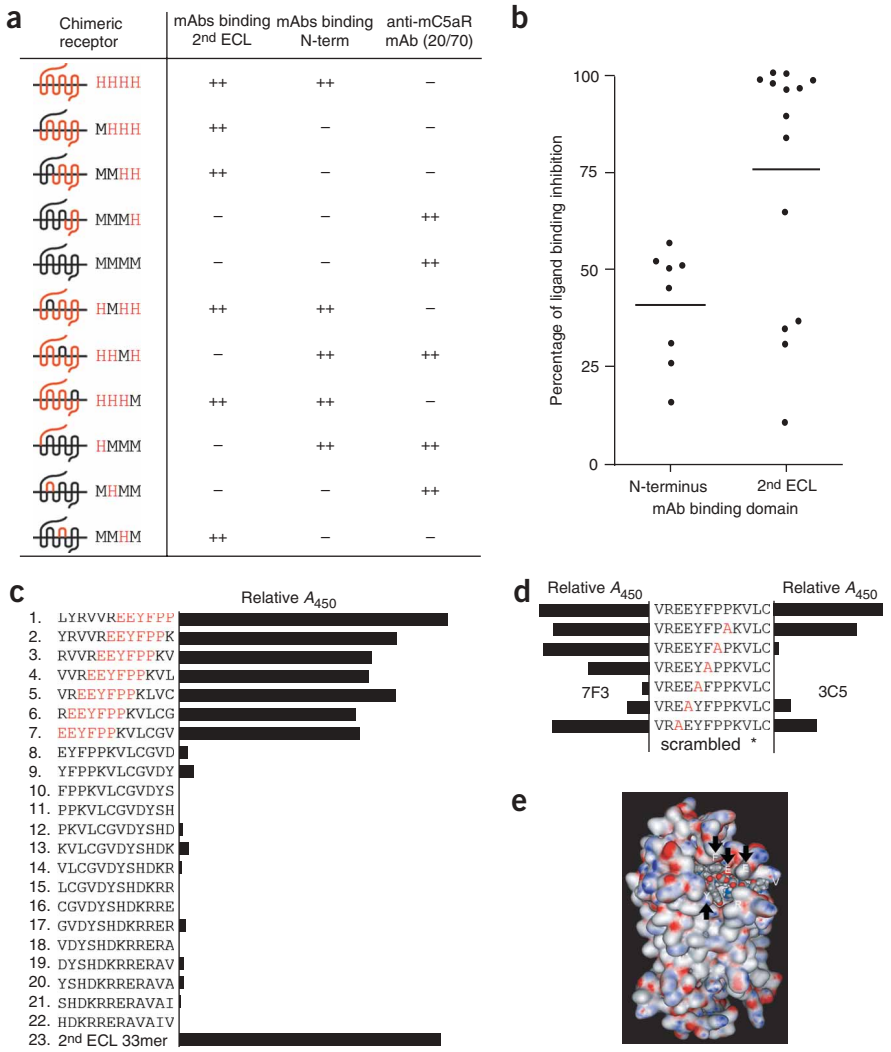

Figure 2 Potent antagonistic anti-C5aR mAbs map to a specific region on the C5aR second extracellular loop. (**a**) Binding of C5aR-specific mAbs to the chimeric human/mouse C5aR receptors. Chimeric receptors are shown schematically (regions derived from human C5aR are shown in red and from mouse C5aR in black). The origin of the four extracellular domains is designated by four-letter code (HHHH is wild-type human C5aR, mHHH has mouse N-terminal extracellular domain and human first, second, and third extracellular loops, etc.). All anti-human C5aR mAbs showed distinct, domain-restricted binding profiles, binding either to receptors containing the human C5aR N terminus or the second extracellular loop. The anti-mouse C5aR mAb 20/70 binds to chimeric receptors containing the mouse C5aR second extracellular loop. (**b**) Dot plot showing the degree to which each individual anti-human C5aR mAb inhibited C5a binding to human neutrophils. mAbs are grouped according to the receptor domain they recognized. (**c**) Mapping of antibody-binding sites on human C5aR second extracellular loop by peptide ELISA. mAbs 7F3 and 3C5 bound to all of the overlapping peptides from human C5aR second extracellular loop containing the sequence ^{179}EEYFPP^{184}. (**d**) Mapping antibody contact residues using alanine mutants of 12-mer peptides identified the critical residues on human C5aR recognized by mAbs 7F3 and 3C5. A nonspecific scrambled peptide was used as a control (asterisk, bottom peptide). (**e**) Molecular representation of the surface of human C5aR with the binding site for 7F3 and 3C5 indicated by arrows.

strong binding to the seven peptides containing the common hexapeptide sequence ^{179}EEYFPP^{184} (**Fig. 2c**). Mutagenesis studies[23] revealed that this region of human C5aR was critical for the regulation of receptor activity, possibly by stabilizing ligand-dependent activation, or regulating the activity of the receptor. Amino acid substitutions in the YFPP motif rendered C5aR constitutively active[23], presumably mimicking conformational changes that follow C5a binding to this region. The epitope ^{179}EEYFPP^{184} was further studied using alanine substitution of every amino acid spanning this peptide region. **Figure 2d** shows that amino acids Glu179, Glu180, Tyr181 and Phe182 were critical for peptide recognition by mAbs 3C5 and 7F3. The EEYFPP epitope region is shown in a predicted three-dimensional structural model of human C5aR (**Fig. 2e**), generated using the rhodopsin structure as a template, and alignment of conserved C5aR residues.

Transgenic mice that express human molecules provide a convenient means to test therapeutics intended for human use in appropriate animal models. C5aR is essential for the pathogenesis of inflammatory arthritis in mice, because C5aR-deficient mice are protected from arthritis induced by either anti-glucose 6-phosphate isomerase autoantibodies[8] or type II collagen mAbs[24]. Anti-human C5aR mAbs were tested for their ability to protect or reverse the progression of experimental arthritis in hC5aR1$^{+/+}$ mice. Transfer of serum containing autoantibodies from arthritic K/BxN mice to healthy mice induces a joint-specific inflammatory reaction that mimics the disease that develops spontaneously in K/BxN mice[25]. After K/BxN serum transfer, hC5aR1$^{+/+}$ mice pretreated with control antibody showed typical clinical arthritis with joint swelling and inflammatory infiltrates consisting mostly of neutrophils, whereas mice pretreated with an anti-human C5aR mAb were completely free of inflammation, clinically and histologically (**Fig. 3a**,**d**). There was no difference between hC5aR1$^{+/+}$ mice and control littermates in the rate of disease development (data not shown). This indicates that the human C5aR was fully functional, in that disease in the K/BxN model is dependent on C5aR (ref. 8). More importantly, when antibody was given 5 d after disease induction there was a complete reversal of inflammation (**Fig. 3b**,**c**). As little as 1 mg/kg of mAb 3C5 reversed inflammation and provided a complete and sustained inhibition of disease. A similar reversal of inflammation in arthritis models has also been observed with anti-C5 mAbs[8,26], although we saw this effect with very low amounts of anti-C5aR mAbs. mAb 3C5 and other anti-C5aR mAbs raised against human C5aR–expressing mouse neutrophils gave the greatest degree of inhibition of inflammatory arthritis in the K/BxN model, consistent with the higher affinities of these mAbs over mAbs raised against L1.2 cells expressing human C5aR (**Fig. 3c**). The most potent mAb, 3C5 (IgG3), showed no evidence of neutrophil depletion or margination, because neutrophil counts in blood were not affected by mAb treatment (see **Supplementary Fig. 4** online), and mAb 7F3 (IgG2a) showed only a modest reduction at early time points (data

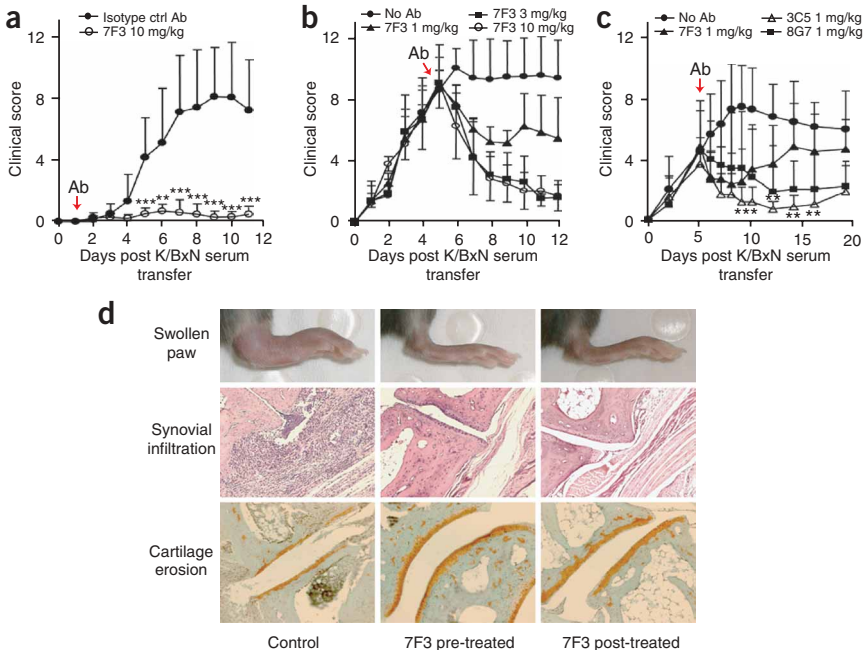

Figure 3 Anti-human C5aR mAbs prevent and reverse K/BxN serum-induced inflammation in human C5aR knock-in mice. (**a**) Preventative treatment: *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3 or isotype control antibody (each 10 mg/kg in PBS) on days −1 and 1. Clinical score data (maximum possible = 12) are mean ± s.d. ($n = 8$ per group). (**b**) Therapeutic treatment: *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3 (1, 3 or 10 mg/kg in PBS) once, on day 5 after inflammation had developed. The control group received no antibody. Data are mean clinical score ± s.d. ($n = 3$ per group). (**c**) Comparison of therapeutic efficacy of anti-human C5aR mAbs. *hC5aR1*$^{+/+}$ mice were injected i.p. with 7F3, 3C5 or 8G7 (each 1 mg/kg in PBS) once, on day 5 after inflammation had developed. Data are mean clinical score ± s.d. ($n = 6$ per group). Clinical scores and ankle thickness were measured blind. Statistical analysis compared control groups and anti-C5aR mAb-treated groups each day. $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$. (**d**) Representative photographs and sections of hind paws from *hC5aR1*$^{+/+}$ mice treated with 10 mg/kg isotype control antibody (left) or 7F3 (center) in the preventative regimen and treated with 3 mg/kg 7F3 (right) in the therapeutic regimen. Hematoxylin and eosin staining shows cellular infiltration to the joint and severity of histological arthritis. Safranin O staining shows cartilage structure. Original magnification ×100.

not shown). Anti-C5aR mAb therapy in a collagen-induced arthritis model showed similar results to that seen in the K/BxN model (data not shown). mAb 3C5 showed a longer half-life than mAb 7F3 after injection into mice (see **Supplementary Fig. 5** online), which may also account partly for the superior efficacy of mAb 3C5 *in vivo*.

The availability of human C5aR knock-in mice allowed us to generate very high-affinity anti-human C5aR mAbs. Presumably this results from the very focused immune response to human C5aR expressed at high densities on hC5aR1$^{+/+}$ mouse neutrophils. Human C5aR knock-in mice also made possible the evaluation of new therapeutics, designed ultimately for human use. In our studies, anti-human C5aR mAbs were remarkably effective in treating inflammatory arthritis in mice. The relatively low cost of creating humanized knock-in mice and their potential use in studies on safety and efficacy indicate that such mice could well become a standard component of preclinical development. However, in our studies with C5aR we were fortunate that human C5aR bound mouse C5a with high affinity, which eliminated the need to create mice expressing both human C5 and C5aR.

Another important outcome from this study was the development of a mAb that could completely prevent and reverse inflammatory arthritis. Indeed, other than anti-C5 treatment[8,26], we are unaware of any other treatment that does so as completely. Notably, anti-C5aR mAbs showed this effect at doses (1 mg/kg) considerably less than the doses of anti-C5 antibody used to inhibit arthritis in mice (~40 mg/kg)[10,26]. One reason for this may be the high concentration (~180 μg/ml) of C5 that is normally present in blood and tissue fluids. In addition, our mAbs recognize C5aR but not C5L2, the second C5a-binding receptor that provides inhibitory signals upon C5a binding[16]. Thus, some inhibition of the inflammatory response by anti-C5aR mAbs may result from uninterrupted binding of C5a to C5L2. The very rapid reversal of inflammation with anti-C5aR mAb treatment was surprising but probably relates to the critical and ongoing requirement of neutrophils and/or mast cells for perpetuating the disease process. Neutrophil depletion using a depleting mAb also reverses inflammation in the K/BxN model[27], suggesting that the continuous release of proinflammatory mediators by neutrophils is necessary to perpetuate certain inflammatory responses.

METHODS

Animal ethics. All experimental procedures involving mice were carried out according to protocols approved by the Garvan Institute–St. Vincent's Hospital Animal Ethics Committee and the Animal Resources Center–Ozgene Animal Ethics Committee.

Generation of human C5aR knock-in mice. A knockout–knock-in strategy was adopted to construct a transgenic mouse expressing human C5aR, but not mouse C5aR, under the control of the mouse C5aR gene promoter. The targeting vector comprised a 3.5-kilobase region of mouse C57BL/6 genomic DNA upstream of the C5aR gene exon 2, human C5aR gene exon 2 coding sequence, mouse C5aR gene 3′ untranslated region, PGKneo flanked by loxP sites and a 3-kilobase region of mouse genomic DNA downstream of the C5aR gene in the vector pLOz (Ozgene). Genomic DNA fragments were generated using PCR amplification. The vector was transfected into C57BL/6 Bruce4 stem cells[28], and DNA from G418-resistant colonies was screened by Southern blot. *Xba*I- and *Eco*RV-digested DNA was hybridized with 5′ and 3′ probe, respectively, to identify clones with the correct homologous recombination event at both 5′ and 3′ ends. Two embryonic stem clones out of 672 screened were identified as containing the correctly targeted human C5aR sequence. Five chimeric mice were produced from these embryonic stem cells, thus establishing the human C5aR knock-in line. Chimeras were mated with C57BL/6 females. Germline transmission of the human C5aR gene was confirmed by Southern blot of mouse tail genomic DNA (**Fig. 1d**). Mice homozygous for the human C5aR gene (*hC5aR1*$^{+/+}$) were generated, and PCR, Southern blot and flow cytometry confirmed the absence of mouse C5aR. The PGKneo gene flanked by loxP sites was deleted from the knock-in locus using a BL/6 Cre deleter strain.

Neutrophil isolation. Human neutrophils were isolated from the peripheral venous blood of healthy volunteers. Blood samples collected into ethylenediamine tetraacetic acid–coated vacutainers were centrifuged at 400 *g* for 15 min, and then the plasma and buffy coats were removed. After 1% dextran sedimentation for 30 min the white blood cells were pelleted by centrifugation at 300 *g* for 5 min and washed with phosphate-buffered saline (PBS). The cells

were then centrifuged at 500 *g* for 30 min on a cushion of 65% Percoll (density 1.093 g/ml, Amersham Bioscience). After centrifugation, the neutrophils were resuspended in PBS. Mouse neutrophils were isolated from both hind leg femurs by forcing 5 ml Dulbecco's modified Eagle medium (Gibco) with 10% fetal calf serum through the bone with a syringe. Neutrophils were separated by density centrifugation over Ficoll-Paque (Amersham Bioscience). Red blood cells were lysed using hypotonic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM ethylenediamine tetraacetic acid). Cell viability was determined by trypan blue exclusion, and the neutrophil pellet was resuspended in PBS.

Monoclonal antibody generation. C57BL/6 mice were immunized i.p. with 10^7 transfected L1.2 cells with high expression of human C5aR (ref. 29), six times at 2-week intervals, five i.p. and one i.v. for the last immunization. At 4 d after a final i.v. immunization, the spleen was removed and the cells fused with the SP2/0 cell line using standard procedures. C57BL/6 mice were immunized with 10^6 neutrophils isolated from blood of *hC5aR1*$^{+/+}$ mice, in a similar fashion. Hybridomas were grown in Dulbecco's modified Eagle medium (Gibco) containing 10% Fetalclone (HyClone) and were purified by protein A or G chromatography and concentrated. Buffer was exchanged and endotoxins removed. mAb concentration was determined using a mouse IgG enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics).

Flow cytometry. To assess reactivity of mAbs against transfected cells or leukocytes, we used indirect immunofluorescence staining and flow cytometry. Cells were washed once with PBS and resuspended in 100 µl PBS containing 2% (wt/vol) BSA and 0.1% (wt/vol) sodium azide (staining buffer), purified antibody, or 50 µl hybridoma culture supernatant. After 20 min at 4 °C, cells were washed twice with staining buffer and resuspended in 50 µl FITC-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:200 in staining buffer. After incubating for 20 min at 4 °C, cells were washed twice with staining buffer and analyzed on the FACSCalibur (Becton-Dickinson) to determine the level of surface expression. Propidium iodide staining was used to exclude dead cells.

Binding assays. Human neutrophils were washed and resuspended in binding buffer (50 mM HEPES, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% bovine serum albumin) at 1×10^7 cells/ml. For each binding reaction (in a final volume of 120 µl), 40 µl cell suspension (4×10^5 cells) with an appropriate amount of anti-human C5aR mAb, isotype-matched control mAb or unlabeled human C5a (Sigma) was incubated at 20–25 °C for 15 min. ^{125}I-labeled human C5a (Perkin Elmer) was added at a final concentration of 0.4 nM, and the reactions were incubated at room temperature for 1 h. Cells were then collected and washed thrice with binding buffer containing 150 mM NaCl. Cells were then transferred to Opti plates (Perkin Elmer) with MicroScint 20 scintillation fluid and radioactivity counted using a TopCount (Packard). Each sample was assayed in triplicate.

Construction of cell lines expressing chimeric human/mouse C5aRs. Chimeric human/mouse C5a receptors were constructed using a modified PCR-based overlap extension technique[22]. Briefly, different fragments of the human or mouse C5aR gene were amplified by PCR. Overlapping fragments were combined, denatured and re-annealed and amplified by second round of PCR. Full-length chimeric receptor sequences with appropriate restriction enzyme sites were amplified in a third PCR step and cloned into pcDNA3.1(+) (Invitrogen) for expression. PCR primers (see **Supplementary Table 1** online) were designed according to the human and mouse *C5aR* gene sequences (GenBank accession numbers M62505 and S46665, respectively).

Transfection of expression vectors into L1.2 cells. Mouse L1.2 cells were grown in RPMI 1640 (Gibco) supplemented with 10% bovine calf serum (HyClone), and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Transfected cells were used after 1 d.

Epitope analysis with synthetic peptides. Two sets of peptides with N-terminal biotin and spacer GSGS were synthesized in immobilized form on plastic pins (Mimotopes Pty Ltd.). The first set of 22 peptides contained all possible 12-mers from the human C5aR second extracellular loop, each offset by one amino acid (**Fig. 3c**). The second set were 12-mers of the sequence VREEYFPPKVLC, each with one residue substituted by alanine. Peptides were initially reconstituted in 200 µl 60% dimethyl sulfoxide and subsequently diluted in PBS to give a final concentration of 10 µg/ml for direct ELISA. The full (33-mer) amino acid sequence of the human C5aR second extracellular loop was also synthesized as above (peptide 23).

Peptide ELISA. Streptavidin-coated microtiter plates (Nunc) were coated with 10 µg/ml of peptide per well in a volume of 200 µl, and incubated at 4 °C overnight. Plates were washed three times with ELISA wash buffer (0.05% Tween 20 in PBS). mAbs were added at 2.5 µg/ml, and plates were incubated for 3 h at room temperature. Horseradish peroxidase–conjugated rabbit anti-mouse IgG antibody diluted 1:1,000 in ELISA wash buffer was used for detection. Plates were developed using TMB (3,3′,5,5′ tetramethyl benzidine; Sigma) and read at A_{450nm}.

K/BxN inflammatory arthritis model. Serum was collected from K/BxN arthritic mice as described[25]. Experimental arthritis was induced in recipient mice by injecting 150 µl serum i.p. on days 0 and 2, and disease progress was monitored as described[30]. Ankle thickness and clinical scores were determined daily. The clinical score was calculated for each mouse by summing the scores for the four paws: 0, normal joint; 1, mild-to-moderate swelling of the ankle and/or one swollen digit; 2, swollen ankle or swelling in two or more digits; 3, severe swelling along all aspects of paw or all five digits swollen. Anti-human C5aR or isotype control mAbs (1–10 mg/kg in PBS) were injected i.p. on days –1 and 1 (preventative treatment) or day 5 (therapeutic treatment). At day 12 mice were killed and paws collected for histology. Paws were fixed for 48 h in fixing solution (10% vol/vol phosphate-buffered formalin) and decalcified by treatment with 10% vol/vol formic acid in fixing solution for 5 d. Samples were then washed with PBS and embedded in paraffin. Sections of 6 µm thickness were stained with hematoxylin and eosin or Safranin O.

Statistical analysis. The statistical significance of differences between independent control and treatment groups in the K/BxN model was determined using either the Mann-Whitney test or the Kruskal-Wallis test, and post hoc analysis with Dunn's multiple comparison test.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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