Expression of Human Complement Receptor 2 (CR2, CD21) in Cr2−/− Mice Restores Humoral Immune Function

Kevin J. Marchbank, Clay C. Watson, David F. Ritsema and V. Michael Holers

*J Immunol* 2000; 165:2354-2361; doi: 10.4049/jimmunol.165.5.2354

http://www.jimmunol.org/content/165/5/2354

---

**Why *The JI*?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References** This article cites 40 articles, 28 of which you can access for free at: [http://www.jimmunol.org/content/165/5/2354.full#ref-list-1](http://www.jimmunol.org/content/165/5/2354.full#ref-list-1)

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions** Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Expression of Human Complement Receptor 2 (CR2, CD21) in Cr2−/− Mice Restores Humoral Immune Function

Kevin J. Marchbank, Clay C. Watson, David F. Ritsema, and V. Michael Holers

Complement receptor type 2 (CR2, CD21) is expressed by both human and murine B cells and has been demonstrated to play a pivotal role in the humoral immune response. We have reconstituted Cr2−/− mice with an 80-kb human genomic fragment (designated P1-5) containing the full-length human CR2 (hCR2) gene. Transfection of P1-5 into the mouse A20 B cell line confirmed that it would direct expression of the hCR2 protein in mouse B cells. Immunoprecipitation analysis in these cells revealed that hCR2 coassociates with mouse CD19. After creation of transgenic mice using P1-5, we found significant expression of hCR2 on peripheral blood and splenic B cells by flow cytometric analysis. RT-PCR analysis of tissues and purified cell populations from transgene-positive mice revealed that hCR2 expression was restricted to B cells and the spleen in a pattern that matches mouse CR2. To rigorously assess the functional capabilities of hCR2, the transgene was bred onto Cr2−/− mice, which have a notable defect in response to SRBC Ag. We found that Cr2−/− mice expressing hCR2 had a substantial restoration of the humoral immune response to SRBC as compared with nontransgenic Cr2−/− littermate controls. Overall, this study suggests that hCR2 is able to substitute for mouse CR2 in the murine immune system. Therefore, hCR2-transgenic mice offer a valuable model system to further examine immunologic roles as well as structure-function relationships important for hCR2 function in primary cells in vivo.


E

fficient activation of B cells in response to an Ag bound to the B cell receptor is in large part due to an accessory signal mediated through complement receptor type 2 (CR2, CD21) in the context of the CR2/CD19/CD81 membrane protein complex on the B cell (reviewed in Refs. 1–3). CR2 is a member of the regulators of complement activation gene family (4). The predominant characteristics of proteins encoded by this family are their ability to bind the activation products of the C3 and C4 components of the complement cascade and the presence of repeat motifs of 60–70 aa, termed short consensus repeats (SCRs), in their structure (4).

Human CR2 (hCR2) is the product of a separate gene from that of human CR1 (hCR1), whereas mouse CR2 (mCR2) and mCR1 are products of a single alternatively spliced gene (5–7). Despite this fundamental difference at the genomic level, hCR2 and mCR2 are very similar in many other respects. They share ~67% homology at the nucleotide level and 58% homology in the protein sequence (8), and both consist of an extracellular domain comprised entirely of 15–16 tandemly arranged SCRs followed by a transmembrane domain and a short intracellular domain (6, 8). Both exhibit a M, of ~140–150 kDa, and hCR2 binds mouse and human C3d with similar affinities (9). Both exhibit a highly regulated expression pattern being expressed solely or primarily on the surface of B cells and follicular dendritic cells (FDC) for mCR2 and hCR2, respectively (10–14). Notably, hCR2 is also found on thymocytes and a subset of T cells (15, 16) in addition to epithelial cells (17). hCR2 also binds CD23 (18) and is the receptor for the EBV membrane protein gp350/220 allowing the specific infection of B lymphocytes in humans (19, 20). mCR2 does not bind EBV (21).

The concept that CR2 binding to C3d is involved in the regulation of the humoral immune response came directly from the work of Pepys (22) who first noted that mice depleted of C3 using cobra venom factor had diminished Ab responses following immunization with SRBC. Several subsequent studies in humans, guinea pigs, and dogs with genetically determined deficiencies of the early complement components confirmed the hypothesis that C3 is required for a robust immune response to T-dependent and T-independent Ags (5). In an elegant study where mice were immunized with Ag coupled to the CR2 ligand C3d, it was found that 10,000-fold less coupled Ag was required to generate a detectable Ab response than uncoupled Ag (23). This study clearly demonstrated the importance of CR2 in the development of an immune response to a complement-activating Ag.

hCR2 has been shown to be noncovalently associated with human CD19 (hCD19), a B cell-restricted protein (24). Unpublished results by Sato et al. (25) have suggested that this is also true for hCD19 and mCR2. It is believed that the association with CD19 is what links CR2 ligand binding to the downstream signaling events leading to enhanced B cell activation and proliferation. This concept is supported by the fact that CD19 has a long intracytoplasmic tail that interacts with protein tyrosine kinases, Vav, phosphatidylinositol 3-kinase, and in particular the Src-type B cell receptor-associated protein tyrosine kinase Lyn (1, 3). CR2 has a short
cytoplasmic tail and is attributed with only a few signaling capabilities by itself. However, it should also be noted that CD81, another member of this complex that is widely distributed, has been shown to be responsible for signaling homotypic adhesion and can contribute to Ca\(^{2+}\) release upon cross-linking (3). In addition, CR2 can induce homotypic adhesion in a CD19-independent manner (26). As CD81 is widely distributed, and CD81-deficient mice exhibit alterations in humoral immunity (27, 28), the relative contribution of CD19 and CD81 to all CR2-mediated B cell responses is not yet clear.

In two independent studies, mice were made deficient for mCR1 and mCR2 through insertional mutagenesis of the Cr2 gene (29, 30). These mice showed no obvious phenotypic abnormalities, and B2 B cell numbers were essentially unaltered. B1 B cells were decreased in one study (30), but not in the other (29). However, Cr2\(^{-/-}\) mice in both studies demonstrated a marked decrease in their humoral immune response to T-dependant Ags. This effect was found both in the primary and secondary responses, and is consistent with the studies outlined above. To examine the relative role played by CR2 expression on B cells vs FDC, transplantation of bone marrow from mCR2-positive mice was used to partially reconstitute Cr2\(^{-/-}\) Rag\(^{-/-}\) mice (30). In these mice, where CR2 was expressed only on B cells and not FDC, the primary immune response was returned to normal levels. This result suggested that FDC expression plays a less dominant role in the generation of the immune response to Ag than B cells. However, in reciprocal bone marrow transplants, FDC and B cell CR2 were both shown to be essential for immune complex trapping and humoral responses to SRBC and keyhole limpet hemocyanin (31). Despite these differences, both studies underscore the important role of CR2 in establishing a robust humoral immune response in mice.

To further investigate the role of CR2 as well as create a model of hCR2 expression in primary cells in which structure-function relationships can be studied in vivo, we have created mice containing the whole hCR2 gene encoded within a single P1 phage clone. We have found expression of hCR2 in mouse B lymphocytes by flow cytometric and RT-PCR analysis. Expression of hCR2 using this strategy was restricted to appropriate tissues and cytes by flow cytometric and RT-PCR analysis. Expression of hCR2 was found both in the primary and secondary responses, and is consistent with the studies outlined above. To examine the relative role played by CR2 expression on B cells vs FDC, transplantation of bone marrow from mCR2-positive mice was used to partially reconstitute Cr2\(^{-/-}\) Rag\(^{-/-}\) mice (30). In these mice, where CR2 was expressed only on B cells and not FDC, the primary immune response was returned to normal levels. This result suggested that FDC expression plays a less dominant role in the generation of the immune response to Ag than B cells. However, in reciprocal bone marrow transplants, FDC and B cell CR2 were both shown to be essential for immune complex trapping and humoral responses to SRBC and keyhole limpet hemocyanin (31). Despite these differences, both studies underscore the important role of CR2 in establishing a robust humoral immune response in mice.

To further investigate the role of CR2 as well as create a model of hCR2 expression in primary cells in which structure-function relationships can be studied in vivo, we have created mice containing the whole hCR2 gene encoded within a single P1 phage clone. We have found expression of hCR2 in mouse B lymphocytes by flow cytometric and RT-PCR analysis. Expression of hCR2 using this strategy was restricted to appropriate tissues and cytes by flow cytometric and RT-PCR analysis. Expression of hCR2 was found both in the primary and secondary responses, and is consistent with the studies outlined above. To examine the relative role played by CR2 expression on B cells vs FDC, transplantation of bone marrow from mCR2-positive mice was used to partially reconstitute Cr2\(^{-/-}\) Rag\(^{-/-}\) mice (30). In these mice, where CR2 was expressed only on B cells and not FDC, the primary immune response was returned to normal levels. This result suggested that FDC expression plays a less dominant role in the generation of the immune response to Ag than B cells. However, in reciprocal bone marrow transplants, FDC and B cell CR2 were both shown to be essential for immune complex trapping and humoral responses to SRBC and keyhole limpet hemocyanin (31). Despite these differences, both studies underscore the important role of CR2 in establishing a robust humoral immune response in mice.

Materials and Methods

Cells and Abs

The mouse B cell line A20 was cultured in RPMI 1640 medium plus 10% FCS, 4 mM L-glutamine, 2 mM sodium pyruvate, 100 IU/ml penicillin, and 100 IU/ml streptomycin. PBL from mice were collected into 20 μl heparin via a tail-vein bleed, washed once in cold PBS, and resuspended in 0.5 ml RBC lysis buffer. After a brief incubation at room temperature, 1 ml PBS was added, and the cells were pelleted by 5-min centrifugation at 1000 rpm, 2.4G2 (anti-mCD16/mCD32), CD3e-FITC, and RA3-6B2-FITC (anti-mCD43R/B220) were obtained from Pharmingen (San Diego, CA). Bio- tinylated and FITC-conjugated HB5 (anti-hCR2), and purified and biotin- labeled anti-B220-FITC where appropriate. Cells were incubated for 30 min on ice, electroporated, and returned to ice for 5–10 min. The cells were then transferred to Eppendorf tubes (Eppen- dorf Scientific, Westbury, NY) and centrifuged for 3 min at 1000 rpm. The supernatant was discarded and the cells were placed in 10 ml prewarmed complete media for 48 h in 5% CO2. At 48 h after transfection, cells were pelleted, resuspended in 12–14 ml complete media containing 400 μM hygromycin (Calbiochem, San Diego, CA) or 1 mg/ml G418 (Mediatech, Herndon, VA) where appropriate, and plated in six-well plates. At 1 wk, cells were plated onto 96-well plates using serial dilutions to produce sin- gle-cell-derived A20 clones. Surviving cells were analyzed by flow cytometry and RT-PCR at 3 wk after placement in hygromycin or G418.

Flow cytometry

Cells were resuspended in 500 μl of 10 μg/ml 2.4G2 Ab to block Fc receptors. After a 15-min incubation on ice, cells were washed in staining buffer (PBS, 1% FBS, 0.02% sodium azide). Cells were resuspended in 100 μl staining buffer containing biotinylated anti-hCR2 (HB5 or 171) or anti- mCR2 (7E9) and 1 μl anti-B220-FITC appropriate. Cells were incu- bated for 30 min on ice in the dark. After incubation, cells were washed in staining buffer two to three times and then incubated with SA-PE to detect biotin-labeled primary Abs. Following incubation, cells were washed as described above and then resuspended in 1% paraformaldehyde. Flow cytometry was conducted at the UCHSC Flow Cytometry Core Facility using a Beckman Coulter XL (Hialeah, FL).

Splenocyte analysis

Mice were sacrificed and spleens were harvested into RPMI 1640. The tissue was ground into single-cell suspensions using frosted glass slides and transferred to 15-ml conical tubes on ice. After 10 min incubation, the supernatant was transferred to a new tube, and the cells were pelleted and washed once with staining buffer. The pellet was resuspended in 0.5 ml RBC lysis buffer and incubated at room temperature for 1–2 min. The cells were then washed with 1 ml staining buffer one to two times. Cells were then counted and 0.5–1 × 10^6 cells were used per analysis. Cells were stained as described above.

Creation and screening of transgenic mice

To create transgenic mice, the hCR2 genomic fragment was released from the SacII vector backbone of P1-5 using SalI and NotI. The hCR2 gene fragment was separated from the vector using low-melt-point agar (FMC, Rockland, ME) and a Geneline II (Beckman Coulter) transverse alternating field electrophoresis gel apparatus. The gene fragment was excised from the gel and was then dialyzed in injection buffer (mTE) for 24 h with three changes of buffer. The gel slice was melted, and the agar was digested with Gelase (Episcience Technologies, Madison, WI) according to the manufactur- er’s instructions. After digestion, the sample was centrifuged at 3000 rpm in an Eppendorf centrifuge (Eppendorf Scientific). The pellet was discarded. The purified hCR2 gene fragment was then injected directly into fertilized ova by the UCHSC Transgenic Core Facility using standard techniques.

To screen for potential P1-5 (hCR2) founders in the population of put- tative transgenic pups, tail DNA was purified and tested by Southern blot analysis using a variety of informative probes illustrated in Fig. 1.

Transfection

Approximately 48 h before transfection, A20 cells were fed and resus- pended in media at a density of 0.1 × 10^6 cells/ml. Plasmid and P1-5 DNA were first purified by CsCl3 or Qiagen maxi prep (Qiagen, Valencia, CA). Plasmid DNA was then linearized by restriction enzyme digest and re- purified by phenol-chloroform extraction and ethanol precipitation, fol- lowed by resuspension in sterile Tris-EDTA (TE). P1-5 DNA was resus- pended directly in sterile TE after CsCl3 purification to minimize degradation caused by handling of this large DNA fragment. P1-5 (100 μg) was cotransfected with 1 μg linearized p REP (hygromycin resistance plas- mid; Stratagene, La Jolla, CA) using a BTX electroporator (San Diego, CA) at 950 μF and 300 volts. As a positive control, A20 cells were trans- fected with 1 μg of the hCR2 DNA containing construct pSFV-neo-CR2 (Ref. 21; selected clone was designated A20-2A3), and as neg- ative controls, A20 were transfected with 1 μg of the hygromycin or neomycin (RSV-neo; Ref. 32) resistance plasmids only.

A20 cells were washed twice in serum-free RPMI 1640 and resus- pended at 1 × 10^7 cells/ml in 400 μl serum-free RPMI 1640. A total of 100 μl DNA solution was added in a 1 × HeBS/TE buffer. The cell/DNA mix- ture was incubated on ice for 5–10 min, electroporated, and returned to ice for 5–10 min. The cells were then transferred to Eppendorf tube (Eppen- dorf Scientific, Westbury, NY) and centrifuged for 3 min at 1000 rpm. The supernatant was discarded and the cells were placed in 10 ml prewarmed complete media for 48 h in 5% CO2. At 48 h after transfection, cells were pelleted, resuspended, and 12–14 ml complete media containing 400 μM hygromycin (Calbiochem, San Diego, CA) or 1 mg/ml G418 (Mediatech, Herndon, VA) where appropriate, and plated in six-well plates. At 1 wk, cells were plated onto 96-well plates using serial dilutions to produce sin- gle-cell-derived A20 clones. Surviving cells were analyzed by flow cytometry and RT-PCR at 3 wk after placement in hygromycin or G418.
or PCR analysis. For Southern blot analysis, tail DNA was digested with EcoRI and hybridized with a 1.5 kb EcoRI-HindIII fragment from the gh-3 plasmid (probe pIII, Fig. 1). Confirmed founders were then subsequently backcrossed onto the Cγ2−/− knockout mice and followed by PCR using two independent primer sets (5′-CCTGGCTACAGCTGGTTGC-3′ and 5′-GAGAGCCAAGAAAACCCCGA-3′) at 94°C for 1 min, 58°C for 1 min, and 72°C for 30 s, and 5′-AGATGGGCTTAGATACCGG-3′ and 5′-TGGCCTCTTGGTGGTAAT-3′ at 94°C for 56°C for 1 min, and 72°C for 1 min). Both PCR were conducted for 33 cycles. Genotyping was also confirmed by flow cytometric analysis. F1 to F3 backcross mice as designated were used in these studies. Estimation of the P1 copy number in transgenic mice was conducted by PCR analysis using standard curves generated with increasing copies of purified P1 DNA added to normal mouse genomic DNA. PCR analysis for hCR2 was conducted on transgenic mice using doubling dilutions of tail DNA, and densitometry was used to calculate the copy number relative to the standard curve and known DNA concentration. Both primer sets outlined above were used, and several independent members of each transgenic line were examined to control PCR and sample variation.

RT-PCR analysis

To analyze hCR2 gene mRNA expression in individual murine tissues and A2O clones, RNA was first isolated using the Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. For analysis of T and B cell subsets from the spleen, splenocytes were collected as outlined above and stained with 10 μg/ml CD3e-FITC and B220-FITC. Splenocytes were sorted into culture media using a MoFlo Multi-laser sorter flow cytometer (Cytomation, Fort Collins, CO) into PE or FITC (T cell) samples. An aliquot of the sorted cells was re-analyzed by flow cytometry to confirm the purity of the population. Cells were pelleted and resuspended in Trizol reagent. hCR2 mRNA expression was analyzed using RT-PCR. Two micrograms of total RNA from each tissue was first treated with 10 U DNase I (AmpGrade, Life Technologies) for 15 min at room temperature, followed by incubation with 1 μl of 25 mM EDTA for 10 min at 65°C. One microgram was then used in each RT-PCR. Reverse transcriptase reactions were primed with random primers according to manufacturer’s specifications (Life Technologies). Parallel samples with and without murine leukemia virus reverse transcriptase were used to assure that templates were mRNA- and not DNA-derived (not shown). PCR was performed using the GeneAmp PCR kit (Perkin-Elmer, Norwalk, CT) according to the manufacturer’s directions using 2 mM MgCl2 and was amplified 25 cycles using oligonucleotides 5′-ATGAGGGGCAGGTTGGCT and 3′-TGTGCCTCATTGCTTGGAAT. Both PCR were conducted for 33 cycles. Genotyping of the P1 clones containing the entire hCR2 gene (Ref. 33, Fig. 1). The positive control for endogenous murine RNA quality and relative quantity for these studies is Cγryp65 (34), a gene expressed in all murine tissues (34, 35) that was detected using the oligonucleotides 5′-CCTGTC CCAGCCCCATCAC-3′ derived from SCR 1 in the cDNA sequence and 5′-CGAGATACAGTTTGGACGAG-3′ derived from SCR 5 in the cDNA sequence (34). PCR conditions were identical except for a hybridization temperature of 58°C. This oligonucleotide pair resulted in the creation of a 517-bp spliced PCR product from mRNA that extends over the intron that separates exons containing SCR 14 and 15 of the hCR2 gene (Ref. 33, Fig. 1). The positive control for endogenous murine RNA quality and relative quantity for these studies is Cγryp65 (34), a gene expressed in all murine tissues (34, 35) that was detected using the oligonucleotides 5′-CCTGTC CCAGCCCCATCAC-3′ derived from SCR 1 in the cDNA sequence and 5′-CGAGATACAGTTTGGACGAG-3′ derived from SCR 5 in the cDNA sequence (34). PCR conditions were identical except for a hybridization temperature of 58°C. This oligonucleotide pair resulted in the creation of a 517-bp spliced PCR product from mRNA that extends over the intron that separates exons containing SCR 14 and 15 of the hCR2 gene (Ref. 33, Fig. 1).

Immunoprecipitation

Cells were washed once in PBS before counting. After counting, cells were pelleted, resuspended in 1% digitonin/PBS containing a protease inhibitor mixture (Minitablet, Roche Molecular Biochemicals, Palo Alto, CA) at 107/ml, and incubated on ice for 20 min. Cell lystate was collected after a 5-min centrifugation at 14,000 rpm in an Eppendorf centrifuge. The lysate was then precleared by incubation with 2× 50 μl protein G-Sepharose beads for 30 min by mixing at 4°C. The Sepharose beads were then pelleted at 5,000 rpm for 3 min, and the supernatant was taken to a fresh Eppendorf. Ab (10 μg) was then added, followed by 100 μl protein G-Sepharose. This mixture was incubated for 60 min as described above. The Sepharose beads were pelleted as in previous steps, and the supernatant was discarded. The beads were washed three times with 0.3% digitonin/PBS/protease inhibitor solution. Finally, the beads were incubated at 95°C in 5× SDS-PAGE sample buffer on a hot plate for 5 min. Supernatants were isolated and samples were frozen at −20°C until used.

SDS-PAGE and Western blot analysis

Samples were run on 7.5% SDS-PAGE gels under nonreducing conditions, blotted onto nitrocellulose, and blocked with 5% dried milk/PBS. The blots were incubated overnight at 4°C with polyclonal rabbit anti-mCD19 (1/5000 in 5% dried milk/PBS), a generous gift of Dr. John Cambier (National Jewish Center, Denver, CO), washed three times in PBS/0.1% Tween 20, incubated with goat anti-rabbit-HRP (1/1000 in 5% dried milk/PBS), washed twice with PBS/0.1% Tween 20, and washed twice with PBS. Blots were developed using an enhanced chemiluminescence reagent (Amer sham, Picataway, NJ) according to the manufacturer’s specifications.

SRBC immunization and ELISA

SRBC (Colorado Serum, Denver, CO) were washed three times with PBS, and a suspension was made containing 1 × 107 SRBC/ml. Mice were injected i.v. at day 0 and day 29 with 100 μl of SRBC suspension. Serum was collected from tail-vein bleed at day 36. Detection of Ab to SRBC was conducted by ELISA essentially as described by Heyman et al. (36). To calculate relative units, the mean OD at 405 nm from triplicate wells were compared with a semilog standard curve of OD measurements vs titrated standard serum.

Results

Selection and analysis of the P1 clones containing the entire hCR2 gene

PCR-based screening of a human PAC library was conducted using two primers that were specific for SCR 14 of hCR2. This strategy yielded two candidate PACs, both ~80 kb in size. These clones were designated P1-4 and P1-5. After extensive Southern blot-based mapping studies were performed on these clones using several plasmid and PCR probes (Table I), it was found that P1-4 was devoid of coding sequence for the intracytoplasmic domain and the 3′ untranslated region of hCR2 (determined by lack of binding of both probes, pII and pVII; Fig. 1). Using the unique EcoRI restriction site within the first exon of the hCR2 gene and pv, we determined that P1-4 extended ~35 kb upstream of the hCR2 transcriptional start site. The lack of the 3′ end of the hCR2 gene in P1-4 ruled out its use in these studies. In contrast, analysis of clone P1-5 revealed the binding of all probes used and, therefore, that it encoded all of the known sequence for hCR2 including the transcriptional start site. The lack of the 3′ end of the hCR2 gene in P1-4 ruled out its use in these studies. In contrast, analysis of clone P1-5 revealed the binding of all probes used and, therefore, that it encoded all of the known sequence for hCR2 including the recently described CRS (CR2 Silencer), which controls stage- and cell-specific expression of hCR2 (32). Use of informative EcoRI and EcoRI digests revealed that P1-5 contained ~10–15 kb of additional genetic material flanking the previously known 5′ and 3′ coding sequences of the hCR2 gene, respectively.
Expression of hCR2 on a B cell line

To establish that P1-5 would direct expression of hCR2 in a mouse B cell, it was transfected into mouse A20 B cells. Flow cytometric analysis of the A20 cell line 3 wk after transfection with P1-5 revealed the presence of hCR2 (Fig. 2). This expression was clearly detectable, but less than that produced by parallel transfection of the eukaryotic expression vector pSFFV-Neo, containing CR2 cDNA, into A20 cells (designated A20-2A3). mCR2/mCR1 expression on these cells was comparable to hCR2 expression seen on A20-2A3 (data not shown). Overall, this demonstrates that the P1-5 clone can be transcribed in mouse B cells and direct expression of hCR2 protein. However, the relative level of expression produced from P1-5 is less than by the SFFV retroviral long terminal repeat promoter.

hCR2 associates with mCD19 in mouse B cells

In order for hCR2 to function in mouse B cells, it likely must associate with the mCD19 signaling complex. Expression of hCR2 on the A20 line allowed us to test for the coassociation of mCD19 with hCR2 (Fig. 3). Using mAb to hCR2 (171) we were able to coprecipitate mCD19 (lane 2) only from the A20-2A3 line (expressing hCR2) and not from either the parental A20 cells (lane 1) or the vector-control transfected (A20-pRep) cell line (lane 3). This indicates that hCR2 associates with mCD19 on the B cell membrane. As expected, coprecipitation of mCD19 with a mAb directed to mCR2/mCR1 (7E9, lanes 1–3) was also achieved irrespective of the cell line used. The levels of hCR2 coprecipitated with mCR2 appeared to be severalfold more than that observed for hCR2. This may be a result of several parameters, including the relative amount of hCR2 and mCR2 on the B cell membrane, differences in Ab binding capacity, or the strength of the association of hCR2 vs mCR2 with mCD19. It is also evident that neither Abs to mCR2/mCR1 or hCR2 coimmunoprecipitated all of the CD19 available in the cell lysate (1D3, lanes 1–3). This result suggests a significant amount of CD19 is not complexed with either mCR2 or hCR2, which is supported by previous studies (1). Of note, expression of hCR2 on the A20 B cell line did not effect the levels of either mCR2/mCR1 or mCD19 as assessed by flow cytometry (data not shown).

Creation of transgenic mice expressing hCR2

The P1-5 clone was injected into fertilized mouse ova using standard techniques. Southern blot analysis of tail DNA from the resulting 22 mice revealed the presence of six genotype-positive mice. Analysis of PBL was conducted by flow cytometry. Lymphocytes were selected by forward and side scatter, and B cells were detected using B220. Four mice exhibited definite staining for hCR2 on their B cells, two of which, designated 319 and 322, are shown in Fig. 4a. The expression levels of hCR2 on the B cell population was relatively low for both of these mice when compared with the expression of mCR2/mCR1. The B220-negative population (primarily T cells) showed no staining above background for hCR2, indicating that these cells did not express detectable amounts of hCR2. In concurrence with the data for the A20 cell line, mCR2/mCR1 levels appeared unaltered on B cells, which also expressed hCR2 on their surface.

Of the original six genotype-positive mice, five successfully transmitted the transgene (Tg). Of those, two lines of mice (319 and 322) were chosen based on the level of hCR2 surface expression displayed for more extensive analysis. The Tg copy number in both lines was estimated by PCR to be no more than one to three copies. The activation state and B cell numbers appeared unaltered in both lines (primarily T cells) showed no staining above background for hCR2, indicating that these cells did not express detectable amounts of hCR2. In concurrence with the data for the A20 cell line, mCR2/mCR1 levels appeared unaltered on B cells, which also expressed hCR2 on their surface.
exception of expression of hCR2 in the kidney (Fig. 5d). Immunohistochemistry of kidneys from the 319 line revealed the presence of hCR2 in the glomeruli (data not shown). Subsequent RT-PCR analysis conducted on one of the other Tg+/+ P1 lines (646) that expressed hCR2 on its B cells at comparable levels to the 322 and 319 lines revealed that the hCR2 expression was restricted to the lymphoid compartment (data not shown), which was identical with the 322 line. Therefore, the kidney expression displayed by the 319 line alone is likely to be related to the insertion site of the P1 DNA, and hCR2 expression directed by P1-5 is regulated in a very similar manner to mCR2/mCR1 within these mice.

**Functional restoration of the humoral immune response to SRBC in Cr2−/− mice**

To rigorously assess the functional capabilities of hCR2 expressed in mice, both the 319 and 322 lines were bred three generations back onto Cr2−/− mice (29), mCR2/mCR1-deficient mice have a notable defect in the switched IgG humoral response to the T cell-dependent Ag SRBC, and because of that we chose this response for initial analysis as the one most likely to be informative. To control for background genes, hCR2-transgenic mice were compared with littermate controls at the F3 generation. The Cr2−/− genotype was confirmed at both the F2 and F3 generation by flow cytometry (data not shown). Following immunization, Tg+/− mice (Cr2−/−; hCR2+/−) demonstrated substantially higher IgG1 titers to SRBC than Tg+/− (Cr2−/−; hCR2−/−) littermates (Fig. 6). Results shown are of a combined analysis of both transgenic lines, but each demonstrated an enhanced humoral response. On the average, Tg+/− mice did not exhibit titers equivalent to age-matched wild-type Cr2+/+ mice, indicating only a partial recovery in response to SRBC. Examination of the means of the responses, data for which is also included in Fig. 6, reveals that the response in Tg+/− mice is ~50% of normal (16,745 for wild-type Cr2+/+ vs 2,748 for Tg−/−, and 10,773 for Tg−/− mice). However, it is striking that even the modest levels of hCR2 in these mice were sufficient to restore a substantial degree of the humoral immune response to SRBC.

Two issues regarding this analysis deserve additional comment. First, the range of responses in the Cr2−/− hCR2+/+ mice is relatively broad, even more so than wild-type C57BL/6 (Cr2+/+) mice where responses vary by 3- to 4-fold. We believe this is due to experimental variation at this still relatively early backcross point. Because of this, though, we also performed a statistical analysis after removing the three highest responders from the Cr2−/− hCR2+/+ group and the one highest from the Cr2−/− hCR2−/− littermate control group, and still there was statistical significance at the p < 0.01 level by the Wilcoxon test (data not shown). Second, we also considered whether the highest responders in the Cr2−/− hCR2+/+ group exhibited higher levels of hCR2, but this was not evident on analysis of peripheral blood B cells.

**Discussion**

We have created mice that express hCR2 in a tissue- and cell-specific manner. We first used PCR primers to screen a human PAC library, and we identified two clones. After extensive mapping studies, one of these clones (P1-5) was found to contain all of the known elements required for transcription and translation of hCR2. Transgenic mice were then created using the 80 kb fragment insert from P1-5. B cell surface expression of hCR2 was detected on several founders, but at levels that were relatively low compared with that of mCR1/mCR2. Expression of hCR2 at these levels had no substantial phenotypic effect on the mice or on B cell numbers and expression levels of the CR2-associated protein, CD19 (data not shown). CD81 expression levels were not assessed.

RT-PCR analysis confirmed that hCR2 expression was restricted to B cells. No expression was noted in the thymus or on T cells. It appears that expression patterns of hCR2 in the mouse when driven by its endogenous promoter and silencer (within the
80-kb genomic fragment) resemble the expression pattern of mCR2/mCR1 and do not exhibit the marginally broader expression pattern for hCR2 observed in humans. In a previous study (32), where the Neo reporter gene was driven by 25-kb promoter of hCR2 plus 2.4 kb of the intronic silencer, designated CRS, expression of Neo was found in T cells and thymocytes. One possible explanation for this difference is that elements in the gene fragment outside of the 25 kb promoter and CRS used in the previous study but carried by P1-5 may restrict expression of hCR2 in the murine thymus. This would suggest additional areas that control the expression of hCR2 are present that have not yet been identified. The expression in the kidney of the 319 line is probably a result of the specific insertion site of the hCR2 gene into the mouse genome as progeny derived from two other founders did not demonstrate this phenotype.

The observed low levels of hCR2 expression noted on the B cells may be a function of the integration sites, assuming that hCR2 would be expressed at similar levels as mCR2/CR1 in the mouse under ideal conditions. Alternatively, it is also possible that the human promoter does not function as well in the mouse because of interspecies differences. Expression of hCR2 from P1-5 in the A2O mouse B cell line was also found to be low in comparison to endogenous mCR2/CR1 (data not shown), which lends support to the latter explanation.

The expression of hCR2 on a mouse B cell line allowed us to examine an important question regarding the ability of hCR2 to substitute for mCR2 on B cells. The significance of B cell signaling events through hCR2 in humans has been clearly established (1–3). hCR2 primarily mediates its signaling through association with hCD19. Therefore it was of interest to establish whether or not hCR2 would associate with mCD19. We were able to show clearly that mCR2 does associate with mCD19 (Fig. 3) as had been previously reported (37) and, of great significance to these and subsequent studies, we also found that hCR2 associates with mCD19 (Fig. 3). The levels of mCD19 associating with hCR2 were less than mCD19 with mCR2. Whether this reflects a true decreased affinity of hCR2 for mCD19 in competition with mCR2/mCR1 is not yet known but is an important question for future study. It also appeared that not all of the mCD19 available on the A20 cell was associated with mCR2 or hCR2. If this a true reflection of the normal situation on the mouse B cell it indicates that, as with humans (1), mCD19 is in excess to mCR2 (and hCR2 on these cells) and a population of molecules resides independently on the cell membrane.

Arguably the most sensitive assay of hCR2 function is the ability to activate the B cell in vivo in response to Ag. In mCR1/mCR2-deficient mice the IgG1 response to SRBC is dramatically reduced when compared with normal littermates (29). Importantly,
The Wilcoxon test was used to determine the p values. We found that even with the low expression of hCR2 observed on the B cell population in these mice, the defect in immune response to SRBC was substantially recovered in Tg+ mice when a Cr2−/− background.

It was noted in the original experiments in Cr2−/− mice that heterozygous mice with 50% of normal mCR2/mCR1 levels produced intermediate IgG titers (29). This current study in which suboptimal immune responses are present in a setting of low receptor expression further underlines the possible link between CR2 levels and the magnitude of IgG anti-SRBC titers reached in mice after immunization with SRBC Ag. These results in toto suggest that CR2, like CD19, may act as a B cell response modifier. In contrast, it is also possible that CR1 expression is required for optimal B cell responses, perhaps as previously suggested by serving as a cofactor for conversion of C3b to C3d, a better ligand for CR2 (38), or by enhancing the differentiation of B cells into Ab-secreting cells (39, 40). Future studies in which comparison of lines expressing hCR2 at different levels, with and without CR1, should provide important insights into these questions.

Previous work by Fang et al. (31) has demonstrated that full recovery of IgG responses to Ag is dependent on FDC expression of CR2. One of the reasons for using a large genomic fragment containing the whole hCR2 gene was to re-create the appropriate functional, tissue- and stage-specific expression of hCR2 within the mouse. We are currently addressing the question of whether hCR2 is expressed on FDC within P1-5-transgenic mice and at which stage during mouse B cell development hCR2 expression appears. However, the clear demonstration that hCR2 is functional and exhibits the tightly restricted tissue-expression pattern associated with this gene indicates that the use of a large human genomic DNA fragment has proved successful in reaching our primary aims.

hCR2 expression has long been associated with infection with EBV, (19, 20) as hCR2 binds strongly to gp350/220 on the EBV membrane. The generation of a mouse model of EBV infection would substantially aid the understanding of the disease associated with the virus infection. mCR2 does not bind EBV (21), which is the initial hurdle to overcome if a mouse model for EBV infection is to be established. The creation of hCR2-expressing mice reported herein allows us to further pursue the reasons underlying the lack of susceptibility of mice to EBV infection. It is important to note that although stable transfection of mouse L cells with hCR2 was shown to allow binding of EBV to these cells and transient expression of EBV genes, it did not lead to stable infection with EBV. Thus, it is clear that expression of hCR2 alone on mouse fibroblasts is not sufficient to confer susceptibility to EBV infection. The magnitude and molecular events that will occur in B cells from our hCR2-expressing transgenic lines following EBV infection either in vitro or in vivo remains to be seen. However, a recent study has determined that HLA class II molecules are also involved as cofactors in EBV internalization and infection of human B cells (41). This finding offers an explanation for the earlier results in mouse fibroblasts and suggests that mouse B cells expressing both HLA class II and hCR2 would provide a better target for EBV infection. As transgenic mice that express human HLA class II molecules already exist, we will be able to directly test this hypothesis as well as others.

Finally, expression of mutated forms of hCR2 in Cr2−/− mice will offer the unique opportunity to analyze in primary cells the structural components required for hCR2 to associate with CD19, bind and internalize its various ligands, and amplify the humoral immune response to Ag. Overall, the data presented herein suggest that hCR2 can substitute for mCR2 in enhancing the murine immune response to foreign Ag, and thus these and other hCR2-transgenic mice should provide an invaluable in vivo model system to examine these questions.

Acknowledgments

We thank Joe Anderson and Rachel Henderson of the University of Colorado Cancer Center Transgenic Core Facility as well as Karen Helm, Pat Schor, and Mike Ashton of the University of Colorado Cancer Center Flow Cytometry Core Facility for all the guidance given while carrying out this study.

References


