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B Cells from Mice Prematurely Expressing Human Complement Receptor Type 2 Are Unresponsive to T-Dependent Antigens

Louise Birrell,* Liudmila Kulik, † B. Paul Morgan,* V. Michael Holers,‡ and Kevin J. Marchbank2*‡

Complement receptor type 2 (CR2/CD21), in association with CD19, plays an important role in enhancing mature B cell responses to opsonized Ags. We have shown that mice expressing a human CR2/CD21 (hCR2/CD21) transgene during the CD43+/CD25– late pro-B cell stage of B cell development demonstrate marked changes in subsequent B cell ontogeny. In the present study, we show that the humoral immune response to the T cell-dependent Ag, sheep RBC, is muted severely in a manner inversely proportional to B cell expression level of hCR2. Individual Ag-specific IgG isotypes vary in the degree to which they are affected but all are reduced while IgM titers are normal. A substantial reduction in germinal centers, both in size and frequency, in the spleens of immunized hCR2 transgenic mice demonstrates a failure to maintain germinal center reaction. However, both IgM expression levels and LPS-proliferative responses appear fully intact in B cells from hCR2-positive mice, suggesting that this alteration in B cell phenotype is different qualitatively from that of specific Ag-defined anergy models. These data suggest that the unresponsiveness to T-dependent Ags displayed by hCR2-positive B cells is linked to an increase in the level of stimulus required to propel the B cell into a fully activated state and thus a normal humoral immune response to Ags. We conclude that this phenotype and these mice may offer an additional means to dissect mechanisms underlying B cell tolerance and Ag responsiveness both in bone marrow and periphery. The Journal of Immunology, 2005, 174: 6974–6982.

The production of immature B cells in the bone marrow is estimated to be in the order of 10–20 million/day. Of these, only 10–20% reach the periphery, and as few as 3% contribute to the mature B cell pool (1–4). This illustrates the fundamental mechanism that has evolved to regulate the generation of autoreactive B cells, namely negative selection through apoptosis. According to the clonal selection hypothesis, B cells that bind too strongly to self-Ags as they develop in the bone marrow will undergo apoptosis (5–7). However, the exact criteria which govern the elimination of self-reactive B cells as they develop are not yet known. Identifying these factors will be crucial to understanding the mechanisms that lie behind the breakdown of tolerance and the generation of autoimmune syndromes.

Deletion of self-reactive B cells is not the only mechanism whereby the immune system can regulate autoreactive B cells. At least two other mechanisms, receptor editing and anergy, have been shown to limit the generation of self-reactive Abs during B cell development in the bone marrow (8–12). Anergy or B cell unresponsiveness to Ags represents a means of tolerizing immature B cells that have left the bone marrow and entered the periphery. In this way, anergy may represent the last line of defense against immature B cells becoming self-reactive mature B cells and setting up an autoimmune response. The criteria that govern the decision of a B cell to become anergic or to become activated are still not fully understood. Anergy itself appears to be graded into various levels of unresponsiveness according to the situation that drives the B cell down that path. For example, Goodnow’s elegant studies using mice with B cells expressing transgenic (tg) BCR directed against hen egg lysozyme (HEL) and that also produce soluble HEL (sHEL) in the periphery illustrate one of the most extreme forms of anergy (10, 13). In this model, the B cells demonstrated a markedly reduced surface IgM expression and were found to be short lived due to their exclusion from B cell follicles. In contrast, the anti-ssDNA B cells described by Erikson and colleagues (14, 15) probably represent a far milder form of anergy. In that study, B cells do not down-regulate surface IgM, are long-lived, and can occupy B cell follicles. Examination of signal transduction potential demonstrates that the anergic B cells were still partially functional, contrasting the almost complete loss of signal transduction noted in anergic B cells from the anti-HEL/ sHEL model (14–16). Notably, it appears that all anergic B cells share an impaired ability to differentiate to Ab-secreting plasma cells in response to Ag and LPS treatment, yet anergy has been shown to be reversible, and thus, all anergic B cells have to a greater or lesser degree an ability to provide the source of autoreactive Ab that may ignite autoimmune disease (15, 17).

Expression of complement receptor type 2 (CR2) on human B lymphocytes was first studied by Tedder et al. (18), who found that CR2 was not detectable on pre-B or immature B cells or late-stage plasma cells but was easily detectable on mature B cells. Mouse

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3 Abbreviations used in this paper: tg, transgenic; HEL, hen egg lysozyme; sHEL, soluble HEL; CR2, complement receptor type 2; mCR2, mouse CR2; hCR2, human CR2; GC, germinal center; SA, streptavidin; NP-KLH, 4-hydroxy-3-nitrophenyl-keyhole limpet hemocyanin.

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CR2 (mCR2) was also found to display this tightly regulated expression pattern (19). Human CR2 (hCR2) and mCR2 are also very similar in many other respects because they share ~67% homology at the nucleotide level and 58% homology in the protein sequence (20). Both exhibit a molecular mass of ~140–150 kDa, and hCR2 binds mouse and human C3d with similar affinities (21). The importance of CR2 in promoting B cell activation to Ags coated with C3d was demonstrated clearly by Dempsey et al. (22), who found that when mice were immunized with Ags coupled to C3d, up to a 10,000-fold lower dose of coupled Ags, compared with uncoupled Ags, was required to generate a detectable Ab response. Importantly, cross-linking of CR2 in conjunction with the BCR has also been shown to rescue resting splenic B cells from apoptosis (23). These findings clearly indicated that binding of a C3d-coated immune complex through BCR and CR2 could provide the level of signal required to rescue B cells from elimination and therefore may also provide sufficient signal to reverse anergy in the periphery.

Previously, we created mice expressing hCR2 cDNA under the control of a B cell-specific λ promoter/enhancer minigene to investigate the role of CR2 in B cell fate and to create a model to investigate the use of human C3d-tagged vaccines in vivo (24). We found a 60% reduction in B cell numbers in the periphery of mice that expressed the highest levels of hCR2 protein irrespective of whether the mice did or did not express endogenous mCR2. These mice also displayed a reduction in the level of serum IgG and a marked reduction in production of specific Ig in response to the T-dependent Ag 4-hydroxy-3-nitrophenyl-keyhole limpet hemocyanin (NP-KLH) (24). These defects were all related primarily to the premature expression of CR2 on the B cell surface during the bone marrow-associated phase of B cell development. However, the limited analysis of the immune response in the previous study left several key questions regarding the effect of hCR2 transgene on B cell function unanswered. First, in the absence of endogenous mCR1/2, what effect does the level of hCR2 expression on the mouse B cell have on the immune response? Second, what effect does hCR2 expression have on production of the various IgG isotypes and the formation of a stable germinal center (GC) response? Third, does the premature expression of CR2 on the B cell alter the way it perceives Ag? To investigate these questions, we conducted detailed analysis of B cell activation markers and immune response to a second T-dependent Ag, SRBC, in three lines of hCR2-tg mice with differing hCR2 protein expression in the absence of endogenous mCR2.

### Materials and Methods

#### Cells

Blood was collected into heparin following a tail vein nick, and cells were pelleted and washed once in cold PBS. Bone marrow B cells were collected by flushing mouse femurs with cold PBS. Splenocytes were isolated from whole spleens by disrupting the spleen between two frosted glass slides in PBS buffer and transferred to 15 ml of conical tubes on ice. Large debris settled after a 10-min incubation, and the supernatant was transferred to a new tube. Cells were pelleted by centrifugation and washed once with staining buffer (PBS, 1% FCS, and 0.02% sodium azide). Samples were incubated with 0.5–1 ml of RBC lysis buffer (0.83% NH4Cl, 0.1% KCO3, and 0.1 mM EDTA) and incubated at room temperature for 1–2 min. The RBCs were pelleted and washed once in cold PBS. Bone marrow B cells were collected on a CME cryostat (Thermo Shandon) and dried at 37°C for 30 min before being fixed for 10 min in acetone. After a brief wash in PBS, endogenous peroxidase activity was quenched by incubating the section with 0.1% hydrogen peroxide in PBS for 15 min at 95°C, the samples were cooled, and 1 μg/ml proteinase K was added. Samples were incubated at 56°C, followed by an incubation at 95°C for 20 min. PCR analysis was conducted on 3 μl of detergent lysis supernatant. Confirmed founders were backcrossed subsequently onto the Cr2−/− background and followed by PCR using two independent primer sets (5’-GTGATTAAAG GCCGGACTTCATT-3’ and 5’-TTATCCCCAGTTCCATCCAC-3’). These defects were all related primarily to the premature expression of CR2 on the B cell surface during the bone marrow-associated phase of B cell development. However, the limited analysis of the immune response in the previous study left several key questions regarding the effect of hCR2 transgene on B cell function unanswered. First, in the absence of endogenous mCR1/2, what effect does the level of hCR2 expression on the mouse B cell have on the immune response? Second, what effect does hCR2 expression have on production of the various IgG isotypes and the formation of a stable germinal center (GC) response? Third, does the premature expression of CR2 on the B cell alter the way it perceives Ag? To investigate these questions, we conducted detailed analysis of B cell activation markers and immune response to a second T-dependent Ag, SRBC, in three lines of hCR2-tg mice with differing hCR2 protein expression in the absence of endogenous mCR2.

#### Antibodies

Purified and biotin conjugated mAb 171 (anti-hCR2, IgG1 isotype) (25), IgG1 isotype control, and the polyclonal anti-hCR2 Ab were produced in the laboratory following standard methods. 2G2 (anti-mCD16/mCD32, Fc block), PE-conjugated B-Ly-4 (anti-hCR2) and CD19, allophycocyanin- or FITC-conjugated RA3-6B2 (anti-mCD45R, B220), FITC-conjugated anti-B7.1 (CD80), FITC-conjugated anti-B7.2 (CD86), FITC-conjugated Gl-7, biotin-conjugated anti-CD95 (jο-1), biotin-conjugated anti-I-A2 (MHC II), biotin-conjugated anti-CD44, biotin-conjugated anti-CD25 (II-2Rα-chain), biotin-conjugated anti-CD69, and streptavidin (SA)-allophycocyanin were all obtained from BD Pharmingen. SA-FITC and SA-PE were obtained from Jackson ImmunoResearch Laboratories (Stratech). FITC-conjugated anti-IgM, purified goat anti-mouse IgGl/m, and alkaline phosphatase-conjugated goat anti-mouse IgG isotype secondaries were obtained from Callag Laboratories.

#### Transgenic mice expressing hCR2

The generation and analysis of mice expressing high (hCR2high), intermediate (hCR2int), or low levels (hCR2low) of hCR2 were described previously (24). To screen for hCR2-expressing pups, DNA was extracted from an ear punch biopsy to allow PCR analysis. The ear biopsies were incubated with detergent lysis buffer (10 mM Tris-HCl, 20 mM KCl, 5% NP40, and 5% Tween 20) for (pH 8.0)) for 15 min at 95°C, the samples were cooled, and 1 μg/ml proteinase K was added. Samples were incubated at 56°C, followed by an incubation at 95°C for 20 min. PCR analysis was conducted on 3 μl of detergent lysis supernatant. Confirmed founders were backcrossed subsequently onto the Cr2−/− background and followed by PCR using two independent primer sets (5’-GTGAATTAAG GCCGTGACTTCATT-3’ and 5’-TTATCCCCAGTTCCATCCAC-3’). These defects were all related primarily to the premature expression of CR2 on the B cell surface during the bone marrow-associated phase of B cell development. However, the limited analysis of the immune response in the previous study left several key questions regarding the effect of hCR2 transgene on B cell function unanswered. First, in the absence of endogenous mCR1/2, what effect does the level of hCR2 expression on the mouse B cell have on the immune response? Second, what effect does hCR2 expression have on production of the various IgG isotypes and the formation of a stable germinal center (GC) response? Third, does the premature expression of CR2 on the B cell alter the way it perceives Ag? To investigate these questions, we conducted detailed analysis of B cell activation markers and immune response to a second T-dependent Ag, SRBC, in three lines of hCR2-tg mice with differing hCR2 protein expression in the absence of endogenous mCR2.

#### Flow cytometry

Cells were resuspended in staining buffer (1× PBS, 1% BSA, and 0.02% sodium azide) containing 10 μg/ml 2,42G2 Ab to block FcRs. After a 15-min incubation on ice, cells were washed in staining buffer. Cells were resuspended in 100 μl of staining buffer containing the appropriate test Ab (used at between 1/50–1/400) and 1 μl of anti-B220-FITC (to label mouse B cells). Cells were incubated for 30 min on ice in the dark. After incubation, cells were washed in staining buffer two to three times and then incubated with the appropriate secondary Ab (used at 1/1000 in staining buffer). Following incubation, cells were washed as above and then resuspended in 1% paraformaldehyde. Flow cytometry was conducted using a BD FACS Calibur.

#### SRBC immunization and ELISA

SRBCs (TCS Bioscience) were washed three times with PBS, and a suspension was made containing 1× 10⁸ SRBC/ml. Mice were injected i.p. at days 0 and 28 with 500 μl of SRBC suspension. Serum was collected from tail vein bleed at days 14, 21, 28, and 36. Detection of Ab to SRBC was conducted by ELISA essentially as previously described by Heyman et al. (27). To calculate relative units, the mean OD at 405 nm from triplicate wells was compared with a semilog standard curve of OD measurements (x) vs titrated standard serum.

#### GC analysis by immunohistochemistry

Mice were injected with SRBC by the i.p. route as described above, and spleens were collected on day 8. Spleens were divided such that ~75% was mounted in OCT compound (EMS Laboratories) and snap frozen in isopentane for immunohistochemistry, and 25% was used for flow cytometric analysis in the manner outlined above. Frozen sections (10 μm) were cut on a CME cryostat (Thermo Shandon) and dried at 37°C for 30 min before being fixed for 10 min in acetone. After a brief wash in PBS, endogenous peroxidase activity was quenched by incubating the section with 0.1% H2O2 in PBS for 20 min. Biotin and avidin binding sites were then blocked using a biotin/avidin blocking kit (Vector Laboratories). Sections were incubated with 1:100 rat anti-mouse IgD (BD Pharmingen) and 1:300 biotin-conjugated PNA (Vector Laboratories) in 1% BSA/PBS for 1 h at room temperature. Slides were washed three times with PBS. Sections were then incubated with 1:300 rabbit anti-rat HRP (Stratech) and 1:400 Extra-Avidin-AP (Sigma-Aldrich) for 1 h at room temperature. Slides were washed three times in PBS. The sections were then developed using a BCIP/NBT alkaline phosphatase kit (Vector Laboratories), according to the
manufacturer’s instructions, and after a brief wash in PBS, HRP was developed using 0.05% diaminobenzidine/0.02% H₂O₂. Slides were rinsed with tap water after development, dehydrated, and mounted using Surgipath (Bretton) mounting medium. Sections were viewed using a Leica microscope with attached digital camera and analyzed using Improvision Openlab software (Improvision).

Results

Impaired humoral immune response to SRBCs (T-dependent Ags) in mice prematurely expressing hCR2

We first analyzed the immune response to the T-dependent Ag SRBCs in Cr2⁻/⁻ mice expressing hCR2 at different levels to evaluate the effect of hCR2 expression level on B cell responsiveness to Ags as measured by immune titer. Three lines of mice were investigated, which expressed hCR2 on the B cell surface in the absence of endogenous mCR1/CR2 at levels equivalent to 25–33% (hCR2high), 15% (hCR2int), and 1–2% (hCR2low) that seen on a normal human B cell. First, we found that, as previously reported (26), there is a significant reduction in the anti-SRBC IgG humoral immune response in Cr2⁻/⁻ mice as compared with Cr2⁺/⁺ wild-type mice (Fig. 1, c–f). Furthermore, all hCR2⁺ mice examined displayed a reduction in IgG isotypes titers in excess of their Cr2⁻/⁻ hCR2⁺ littermates, with the magnitude of the reduction correlating with the level of hCR2 expression (Fig. 1, c–f). Analysis of individual IgG isotypes revealed that all were similarly affected; the largest change was seen with IgG2b (Fig. 1e). The presence of hCR2 on the mouse B cells affected the generation of IgG1 and IgG2b isotypes the most at day 21 of the immune response, but IgG2a and IgG3 were reduced significantly in the hCR2high line. However, in all hCR2-expressing lines, we found that IgM and IgA serum titers in response to SRBC injection were similar when compared with hCR2⁻ littermates (Fig. 1, a and b). After a second SRBC injection at day 28 and serum collection at day 35, titers were examined again. IgM titers were slightly lower and IgA titers remained relatively unchanged after the boost in all animals examined (Fig. 2, a and b). Although a similar fold increase in IgG titers to SRBC boost was found in hCR2⁺ mice when compared with hCR2⁻ mice (Fig. 2, c–f), IgG titers still remained significantly lower than hCR2⁻ littermates. In particular, a significant reduction in the IgG2a isotype was seen in all hCR2⁺ lines at day 36 when compared with hCR2⁻ littermates at this time. Furthermore, all hCR2⁺ mice still showed a distinct correlation between increased hCR2 expression level and reduced response to SRBCs.

An impaired GC response to T-dependent Ags is found in mice prematurely expressing hCR2

The reduction in IgG titers observed in all lines of hCR2-expressing mice suggested to us that the GC response might be impaired. The general splenic architecture of unimmunized mice appeared...
unaltered in the hCR2-tg mice as compared with hCR2–/– littermates, and absolute B cell follicle numbers were found to be similar in all groups of mice analyzed (data not shown). However, wild-type mice immunized with SRBCs were found to possess large PNA-positive GCs in 66% of B cell follicles in examined sections of the spleen (Fig. 3, a and b), and in general, follicles were found to possess multiple GCs. GC number and size were found to be reduced in Cr2–/– mice as reported previously (Fig. 3b; Refs. 28–30). Furthermore, expression of hCR2 in Cr2–/– mice led to a further reduction in both the frequency and numbers of GCs per follicle when compared with their hCR2–/– littermates (Fig. 3, a and b). To further quantitate the changes in GC B cell numbers, we used flow cytometry on splenocytes and GL-7 as marker for GC B cells (31, 32). We found a significant reduction in total splenic GC B cell numbers in hCR2high- and hCR2int-tg lines in comparison to the hCR2–/– littermates (Fig. 3c and d). CD38 expression is also down-regulated on both GC B cells and mature plasma cells in the mouse (33). Therefore, to confirm the data obtained using GL-7, we also stained for CD38. In the hCR2high mice and, to a lesser extent, in both the hCR2low and hCR2int mice, we found a significant decrease in the number of B cells, which were CD38– when compared with hCR2–/– littermates, further supporting the conclusion that GC B cell numbers were reduced (Fig. 3e).

Expression of hCR2 is down-regulated during B cell development

Our initial analysis of early B cell progenitors indicated that hCR2 expression was higher on these cells when compared with mature B cells in the bone marrow and appeared to coincide with a block in B cell development (24). On further analysis, we find that hCR2 expression levels are reduced by up to 50% as B cells mature from B220+CD43– to B220–CD43+ B cells in the bone marrow of hCR2high mice (Table I). Similarly, hCR2int mice also demonstrate a small but significant reduction in hCR2 expression levels at this development phase. However, expression levels of hCR2 on B cells from hCR2low mice remain essentially unchanged in the bone marrow. Both the hCR2high and hCR2low mice displayed a further significant reduction of hCR2 expression levels on B cells isolated from the spleen, but the hCR2high mice also demonstrate a small but significant reduction in hCR2 expression levels at this development phase. However, expression levels of hCR2 on B cells from hCR2low mice remain essentially unchanged in the bone marrow. Interestingly, the hCR2low mice express higher levels of hCR2 on their splenic and peripheral blood lymphocytes when compared with B cells isolated from the bone marrow. No significant differences in CD19 and IgM expression levels
were observed between hCR2-tg and hCR2−/− littermates during B cell maturation (data not shown).

**Activation markers CD44 and MHC class II are significantly increased on the B cell surface in hCR2 high-tg mice**

Previous studies have shown that B cells can display an activated phenotype despite being unresponsive to specific Ags in models of anergy (34, 35). To investigate whether B cells expressing hCR2 adopt a similar phenotype, we examined a battery of activation markers on unstimulated B cells in the spleen and blood of hCR2-tg mice. We found that expression of hCR2 was associated with increased activation marker expression in a dose-dependent manner (Table II). MHC class II expression was consistently increased on B cells isolated from spleen and blood in the hCR2high and hCR2int lines. CD44 was also markedly up-regulated on B cells isolated from the spleen and blood of hCR2 high mice. Blood-derived B cells from hCR2int mice also demonstrated a clear increase in CD44 expression levels. In contrast, B cells from the hCR2low line exhibited a small but significant decrease in MHC II and CD44 expression levels in both compartments (Table II).

### Table I. hCR2 expression is down-regulated on mouse B cells as they mature

<table>
<thead>
<tr>
<th></th>
<th>BM CD43&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BM CD43&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCR2&lt;sup&gt;high&lt;/sup&gt;</td>
<td>59.8 ± 2.2</td>
<td>29.8 ± 3.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>24.4 ± 1.4&lt;sup&gt;**&lt;/sup&gt;</td>
<td>31.7 ± 1.1</td>
</tr>
<tr>
<td>hCR2&lt;sup&gt;int&lt;/sup&gt;</td>
<td>23.3 ± 2.4</td>
<td>20.7 ± 1.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.1 ± 0.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>21.2 ± 1.5</td>
</tr>
<tr>
<td>hCR2&lt;sup&gt;low&lt;/sup&gt;</td>
<td>7.1 ± 0.7</td>
<td>6.6 ± 1.4</td>
<td>8.2 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>hCR2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4.6 ± 0.8</td>
<td>4.6 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>4.2 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Splenocytes, bone marrow, and peripheral blood lymphocytes were collected using standard methods. Cells were counted, and flow cytometry was carried out on the lymphocytes as described in Materials and Methods. All rows show mean fluorescence units on 10,000 B220<sup>+</sup> B cells from each tissue and each group of mice. Three mice were used per group, and Student’s t test was used to establish p values compared with the adjacent tissue. **, p < 0.001, *, p < 0.05.

<sup>b</sup> BM, Bone marrow.
percentage of B cells expressing the activation markers B7.1 or B7.2 was also increased in splenic and blood-derived hCR2<sup>high</sup> B cells. A similar trend was seen in the hCR2<sup>int</sup> mice, but these increases failed to reach significance. Increased CD95/FAS expression on B cells has also been noted in certain forms of anergy (36–38). To examine whether hCR2-expressing B cells also exhibited this phenotype, CD95 expression levels were measured. The percentage of CD95-expressing B cells in hCR2<sup>high</sup> mice was increased significantly in the blood derived B cells and, although not significantly increased in the spleen, did show a trend to higher expression when compared with hCR2<sup>−</sup>tg mice (Table II).

**Table II. Activation markers are up-regulated on unstimulated B cells from hCR2 transgenic mice**

<table>
<thead>
<tr>
<th>MHC II</th>
<th>hCR2&lt;sup&gt;high&lt;/sup&gt;</th>
<th>hCR2&lt;sup&gt;int&lt;/sup&gt;</th>
<th>hCR2&lt;sup&gt;low&lt;/sup&gt;</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.7 ± 12.0***</td>
<td>70.8 ± 7.1**</td>
<td>50.6 ± 5.1</td>
<td>56.4 ± 9.1</td>
<td>110.5 ± 15.2**</td>
</tr>
<tr>
<td>CD44</td>
<td>209.3 ± 34.4*</td>
<td>219.0 ± 22.2*</td>
<td>160.7 ± 17.5*</td>
<td>184.8 ± 21.0</td>
</tr>
<tr>
<td>B7.1</td>
<td>11.1 ± 5.5*</td>
<td>9.2 ± 1.7</td>
<td>6.5 ± 1.1</td>
<td>7.9 ± 1.7</td>
</tr>
<tr>
<td>B7.2</td>
<td>2.7 ± 2.4</td>
<td>0.9 ± 0.5</td>
<td>0.9 ± 0.3</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>CD95</td>
<td>6.4 ± 2.1*</td>
<td>5.2 ± 3.8</td>
<td>3.2 ± 0.7</td>
<td>3.6 ± 0.8</td>
</tr>
</tbody>
</table>

**Table III. Expression of activation markers on B cells from hCR2<sup>high</sup> or negative mice at 72 h after LPS stimulation**

<table>
<thead>
<tr>
<th>Mock</th>
<th>Negative</th>
<th>hCR2&lt;sup&gt;high&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II</td>
<td>76.3 ± 31.0</td>
<td>144.2 ± 25.2*</td>
</tr>
<tr>
<td>CD44</td>
<td>185.8 ± 20.4</td>
<td>235.1 ± 20.1*</td>
</tr>
<tr>
<td>CD69</td>
<td>4.2 ± 2.6</td>
<td>2.28 ± 0.1</td>
</tr>
<tr>
<td>CD25</td>
<td>7.3 ± 1.8</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>slgM</td>
<td>127.4 ± 11.8</td>
<td>114.2 ± 6.5*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LPS</th>
<th>Negative</th>
<th>hCR2&lt;sup&gt;high&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II</td>
<td>149.3 ± 16.2</td>
<td>160.5 ± 16.1</td>
</tr>
<tr>
<td>CD44</td>
<td>321.0 ± 15.0</td>
<td>427.6 ± 53.5*</td>
</tr>
<tr>
<td>CD69</td>
<td>90.8 ± 12.5</td>
<td>115.5 ± 36.1</td>
</tr>
<tr>
<td>CD25</td>
<td>62.8 ± 7.6</td>
<td>53.3 ± 1.1</td>
</tr>
<tr>
<td>slgM</td>
<td>205.7 ± 14.0</td>
<td>173.3 ± 24.2</td>
</tr>
</tbody>
</table>

Isolated splenocytes were treated with LPS or just PBS for 72 h in tissue culture. Cells were counted, and flow cytometry was carried out on the splenocytes as described in Materials and Methods. All rows show mean fluorescence units on B220<sup>−</sup> B cells from each marker and each group of mice. Three mice were used per group, and Student’s t test was used to establish p values. *, p < 0.05.
The key difference between endogenous mCR2/CR1 expression and the hCR2 transgene expression is the stage in B cell development where these molecules are expressed. mCR2/CR1 is first expressed during the IgM\textsuperscript{high}IgD\textsuperscript{low} immature cell stage of development (18, 19), whereas these tg mice initiate expression of hCR2 during the CD43\textsuperscript{−}/CD25\textsuperscript{−} late pro-B cell stage of development (24). Thus, hCR2 can potentially interact with ligands during the subsequent critical stages of B cell development in which the B cell is taught the difference between self and nonself. The marked decreases in hCR2 expression levels noted on mature B cells isolated from the bone marrow of hCR2\textsuperscript{high} and hCR2\textsuperscript{int} mice suggests that B cells in these mice control the level of signal received through CR2 by modulating its expression during these stages of B cell development (Table I). Furthermore, the increased levels of the B cell activation markers B7.1 and B7.2 and the even more impressive increases in CD44 and MHC class II (Table 2) indicate that these B cells have received a potent signal during their development. Preliminary Ca\textsuperscript{2+} flux data suggests that cross-linking of hCR2 with mC3d tetramers provides a robust Ca\textsuperscript{2+} flux in peripheral B cells isolated from all lines of hCR2-tg mice (data not shown), confirming that hCR2 has the potential to signal the B cell during these critical stages of B cell development. The activation status of B cells in the hCR2\textsuperscript{high} mice appears to be similar to that noted in the modified anti-Smith Ag model of anergy; here, too, MHC class II and anti-Fas were up-regulated on B cells in the periphery (36). These authors concluded that this indicated that these cells had been marked for deletion unless a sufficient survival signal was produced; this may also be the case in the hCR2\textsuperscript{high} mice. In the previous study, we found peripheral blood B cell numbers reduced by 60% in the hCR2\textsuperscript{high} mice and by 15% in hCR2\textsuperscript{int} mice, whereas hCR2\textsuperscript{low} mice showed no difference in B cell numbers when compared with hCR2\textsuperscript{−} littermates (24). Notably, splenic B cell numbers were not affected to the same degree, suggesting that maturation of B cells through the spleen also contributes to the reduction in B cell numbers. Indeed, the fact that hCR2 expression levels on B cells isolated from hCR2\textsuperscript{high} and hCR2\textsuperscript{int} mice are further suppressed in the splenic compartment when compared with B cells in the bone marrow suggests that a second round of hCR2 modulation is required to allow these B cells to reach full maturity (Table I).

In the hCR2-tg mice, all the observed defects can be explained by considering that the early expression of CR2 in B cell development heightens the normal tolerogenic role of CR2 in the mouse and thus results firstly in a deletion of cells, which would have normally survived, and secondly induces an anergic-like phenotype on the majority of the remaining B cell population (as outlined in Fig. 4), which interacts only transiently with Ags through the BCR/CR2 complex (or possibly a pre-BCR/CR2 complex).

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![Model of effect of early expression of hCR2](image)

**FIGURE 4.** Model of effect of early expression of hCR2. The developing B cell meets a strong signal through the BCR (or pre-BCR) and undergoes apoptosis (a). The developing B cell obtains an intermediate signal from a soluble Ag and becomes anergic (b). The developing B cell meets a soluble Ag with intermediate binding through the BCR (or pre-BCR) but also gets a signal from CR2, convincing the B cell that it must undergo apoptosis (c). The B cell receives a weak or barely present signal through the BCR (or pre-BCR), but a strong signal from CR2 is enough to alter its activation potential and enter into an anergic-like state (d).

\*Y = BCR or pre-BCR
in B cell expression of CD95 indicates that the signal during B cell development that is derived through hCR2 is sufficient to earmark these cells for deletion by apoptosis. Rescue of B cells from apoptosis using complement-coated Ags has been demonstrated in the WEHI mouse B cell line (23), and thus, if a sufficient signal is received through the BCR, either with or without ligation of hCR2, then these CD95/hCR2-expressing B cells may be rescued from apoptosis. This would partly explain why a high dose of Ag is sufficient to generate an immune response in the hCR2high mice and a slightly increased boost response, even though the humoral immune response is reduced overall. Whether the use of adjuvant or even higher doses of Ags would completely overcome the observed defects remains to be formally tested. However, data from Ags plus adjuvant immunization of the CR2−/− mice suggest that a recovery in immune titers to essentially wild-type levels is possible (30) and certainly merits a detailed examination in the hCR2-tg mice. Despite the reduction in B cell numbers and the obvious functional deficits in the remaining B cell population, the hCR2-tg mice appear healthy, indicating that the deficit does not seriously affect their ability to deal with typical animal house pathogens. Whether they will display reduced ability to counter specific infections remains to be formally tested and will be the focus of subsequent experiments in these mice.

In summary, we show that hCR2-expressing murine B cells do not respond efficiently to the T-dependent Ag, SRBC, both in terms of immune titer and in the generation of an adequate GC response. The level of hCR2 expression is directly proportional to the severity of the immune deficit. B cells isolated from hCR2-tg mice have a marked increase in multiple B cell activation markers and a significant increase in CD95 expression, indicative of an increased potential to undergo deletion through apoptosis. The data suggest that B cells in the hCR2-tg mice have an altered activation state similar in many respects to Ag-specific anergy. Thus, the expression of hCR2 during early B cell development leads not only to a reduction in B cell numbers but also dramatically alters the ability of the remaining B cells to respond to Ags. The mechanism by which expression of CR2 at the pre-B cell stage can result in such far-reaching effects on B cell survival and function remains unclear. Nevertheless, the hCR2-tg mice will provide a useful model to dissect the mechanisms that govern the generation of B cell tolerance both in the bone marrow and the periphery.

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Disclosures

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