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Increased Positive Selection of B1 Cells and Reduced B Cell Tolerance to Intracellular Antigens in c1q-Deficient Mice

Helen Ferry,* Paul K. Potter,† Tanya L. Crockford,* Anastasia Nijnik,* Michael R. Ehrenstein,‡ Mark J. Walport,‡† Marina Botto,‡ and Richard J. Cornall‡*†

Inherited deficiency of early components of the classical complement pathway is strongly associated with the targeting of intracellular self Ags in systemic lupus erythematosus, but the reasons for this association are debated. In this study, we show that C1q deficiency increases the positive selection of B1b B cells and IgM autoantibodies by an intracellular self Ag, which is exposed on dying cells, and decreases the negative selection of autoreactive conventional B cells by the same Ag. These effects are specific to intracellular Ag because C1q deficiency does not affect negative selection by extracellular self Ag or increase the positive selection of naive B cells. The B1-derived IgM autoantibody binds to the intracellular Ag when it is expressed on dying cells, leading to fixation of C1q and clearance of cells by phagocytosis. These findings suggest that the positive selection of autoreactive B1 cells by self Ags may contribute to the IgM and C1q-dependent clearance of dying cells in a feedback loop that limits exposure of conventional B cells to immunogenic self Ags. We show that exposure of intracellular Ag leads to the activation of conventional B cells, when there is a source of T cell help in vivo. The Journal of Immunology, 2007, 178: 2916–2922.

systemic lupus erythematosus (SLE) is a systemic autoimmune disease, characterized by autoantibodies and immune complex formation leading to widespread organ damage (1). However, the disease is clinically heterogeneous and has a complex and polygenic etiology, which is still poorly understood. For these reasons, there is great interest in studying rare monogenic disorders that also predispose to SLE. These conditions are typically due to inherited deficiency of one of the early components of the classical pathway (2), particularly C1q, which causes SLE with a 90% penetrance in affected siblings (3). Two main theories have been proposed to explain the association between inherited deficiency of early components of the classical complement pathway and SLE (2), neither of which is mutually exclusive, the first proposes that complement is required to clear immunogenic self Ags in immune complexes and dying cells (2); the second suggests that complement has a direct role in the maintenance of B cell tolerance, through the negative selection of autoreactive B cells (4).

The hypothesis that complement deficiency might predispose to autoimmunity through a failure to clear self Ags arose after it was discovered that typical lupus Ags are clustered in cell surface blebs on apoptotic cells (5, 6), and that apoptotic cells are immunogenic when injected into syngeneic hosts (7). C1q binds to the apoptotic blebs (8–10), where it activates the classical complement pathway and mediates the phagocytosis of dying cells by macrophages and dendritic cells in vitro (11–14). The strongest evidence that this mechanism may prevent the development of SLE came when it was found that C1q-deficient mice, which develop a lupus-like proliferative glomerulonephritis, are characterized by multiple apoptotic bodies (15). Although the proliferative glomerulonephritis is only apparent on lupus-prone genetic backgrounds (16), the defect in the clearance of apoptotic cells persists in C1q-deficient non-autoimmune strains such as C57BL/6 (B6) (12). This suggests that impaired clearance is a primary defect.

The alternative hypothesis that complement deficiency affects tolerance in self-reactive B cells arose from studies using the anti-hen egg lysozyme (HEL) Ig (IgHEL) transgenic model of B cell tolerance (17). When CD21/35 or C4-deficient mice on the hybrid B6/S129 genetic background were crossed to transgenic animals expressing both IgHEL and soluble HEL (sHEL), the anergic B cell phenotype differed from that in complement-sufficient mice. Anti-HEL autoantibodies remained undetectable, but the complement-deficient B cells had reduced IgM modulation and unimpaired B7.2 induction by Ag (18). On the strength of these findings, it was suggested that the attachment of C4b to self Ags and localization of these complexes to CD35 on stromal cells within the bone marrow (BM) might be critical in the negative selection of autoreactive B cells. However, in similar experiments using the same IgHEL model, C1q deficiency on a B6 genetic background had no effect on anergy, despite C1q being upstream of C4 in the classical complement cascade (19). It is not yet clear how these results might be reconciled, but differences in genetic background between the mouse strains might account for the conflicting results. Another possibility is that C4 operates independently of C1q in the HEL transgenic model.
A further significant limitation of the transgenic experiments is that sHEL is unlike the natural autoantigens targeted in SLE, which are typically cell associated and intracellular. To address this issue, we recently generated transgenic mice expressing an intracellular membrane-bound form of HEL (mHEL-KK), which is sequestered in the endoplasmic reticulum of all MHC class I-expressing cells (20). In this way, mHEL-KK has the same cellular location and systemic expression as Ags targeted in SLE. In young mice, the intracellular mHEL-KK positively selects B1 cells and high titers of IgM autoantibodies (20, 21), in contrast to HEL bound on the cell surface and HEL, which induce tolerance by deletion/editing or anergy, respectively (17). To investigate the effect of complement on the development of autoreactive B cells during intracellular Ags, we have crossed IgHEL and mHEL-KK transgenic mice to C1q-deficient animals.

Materials and Methods

Mice

Transgenic mice expressing anti-HEL Ig H and L chains (IgHEL-MD4); mHEL-KK (mHEL-KK1); and sHEL (MLS) and strains deficient in serum IgM (sldm5M-2) and C1q were genotyped by PCR, as previously described (15, 20, 22). All mice were at least seven generations backcrossed to the B6 strain, and comparisons were made between littermate and age-matched controls. No significant effect of sex or age up to 150 days was detected in any assays (ANOVA). All mice were backcrossed at least seven generations to B6 and maintained in specific pathogen-free conditions. Animals were age and sex matched and examined between the ages of 8 and 12 wk. All experiments were approved by the Oxford University Ethical Review Committee under Home Office License.

Flow cytometry and measurement of serum anti-HEL IgM and splenic IgM-secretion plasma cells

BM, spleen, thymus, mesenteric lymph node, and periportal lavage cell suspensions were stained, as previously described (20). HEL exposed on the surface of dexamethasone-treated thymocytes was detected by HyHEL9-FITC and counterstained with propidium iodide (PI) (BD Pharmingen). To demonstrate IgM binding to mHEL-KK exposed on the surface of dead cells, thymocytes were incubated for 20 min on ice with anti-HEL IgM transgenic or wild-type (WT) B6 IgM+ mouse serum. Cells were then counterstained with CD4-Tc (Caltag Laboratories), and bound IgM was detected with anti-IgM (DS-1-PE; BD Pharmingen). Dying cells were gated on forward light scatterlow (Fig. 4C) and side light scatterhigh, and live cells on FSChigh and side light scatter (Fig. 4C). All analysis was performed using a FACS caliber flow cytometer and CellQuest Pro software (BD Biosciences). Fluorescence data shown are from a single experiment, but representative of all experiments. Anti-HEL IgM+ serum titer was measured by ELISA, and anti-HEL IgM-secretion cells were measured in spot ELISA, as previously described (20).

Confocal microscopy

Thymocytes from nontransgenic or mHEL-KK mice were harvested, and washed once in 2% medium alone or medium containing 10% normal mouse serum (2% FCS, 10 mM HEPES, 2 mM glutamine, 10 μg/ml penicillin, and 10 μg/ml streptomycin). A total of 5 × 10⁶ thymocytes was incubated in poly(t-lysine) BD BioCoat tissue culture slides (BD Biosciences) at 37°C, 5% CO₂, for 4.5 h. In a 500 μl vol of 2% medium alone or medium containing 10 μM dexamethasone (Sigma-Aldrich). All subsequent steps were performed at room temperature. The slides were rinsed with PBS to remove the phenol red and dexamethasone, before fixing the cells with 4% paraformaldehyde/250 mM HEPES for 10 min and then 8% paraformaldehyde/HEPES for 10 min. After washing with PBS, the cells were incubated with 50 mM NH₄Cl for 5 min, and washed again in PBS. Some cells were then made permeable with 0.1% Triton X-100/PBS for 10 min and washed in PBS, then TBS. Sections were blocked with 5% normal goat serum (Vector Laboratories) in TBS for 30 min. To stain for HEL expression, slides were incubated with rabbit anti-HEL serum for 30 min, washed with TBS, and incubated with highly absorbed goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) for 30 min. Slides were mounted with Vectashield containing PI (Vector Laboratories). Images were collected using a ×63 objective on a Zeiss LSM510 confocal microscope.

Activation of conventional B cells by intracellular Ag in vivo

A total of 0.5 × 10⁶ IgHEL spleenocytes from C57BL/6×B10.BRh4, MD4 mice (H-2b) were injected into C57BL/6×B10.BRh4 mice (H-2b), which had been sublethally irradiated with 7Gy, with or without 7 × 10⁶ MACS purified CD4⁺ T cells (94%) from the spleen and mesenteric lymph node of 3A9 (H-2k) TCR transgenic mice. The 3A9 transgenic T cells recognize the immunodominant epitope of HEL in the context of I-Ak. Twenty-four hours later, the recipients were injected with 5 × 10⁶ untreated or dexamethasone-treated and washed thymocytes from nontransgenic or mHEL-KK transgenic mice (H-2k). Spleens were removed after 5 days, and assayed for anti-HEL secreting plasma cells by ELISPOT and up-regulation of MHC class II by flow cytometry. The short assay period limits the risk of graft-vs-host disease. All injections were 200 μl and administered via the lateral tail vein.

Isolation and culture of BM-derived macrophages and induction of apoptosis

The isolation of BM-derived macrophages was as previously described (23). Apoptotic thymocytes were prepared by incubating freshly isolated thymocytes in 0.4% BSA/RPMI 1640 medium, supplemented with 10 μM dexamethasone for ~4.5 h. This resulted in a population of cells that was ~60% apoptotic as determined by nuclear condensation, and annexin V positive, PI positive by FACS.

In vitro phagocytosis assay

Uptake of apoptotic thymocytes by BM-derived macrophages was performed, as previously described (23). Cells, induced to become apoptotic as described above, were initially incubated for 15 min at a concentration of 2 × 10⁶ cells/10 μl in a 1/32 dilution of either IgHEL/mHEL-KK, rag-/-, or rag-/- serum to allow IgM opsonization. The concentration of IgHEL/mHEL-KK-rag-/- was determined to be saturating, as follows. Monoclonal serum anti-HEL IgM (HyHEL10) from IgHEL/mHEL-KK, rag-/- mice was pooled, serially diluted, and incubated on ice with 5 × 10⁶ cell surface membrane-bound HEL-expressing splenocytes from a KLK3 transgenic mouse (17) for 25 min. The cells were washed and then stained for flow cytometry with Abs against B220 (FITC), IgM (PE), and HEL (HyHEL9-Tc). Because HyHEL10 blocked the binding of HyHEL9-Tc, the dilution of serum HyHEL10 required to saturate the surface HEL was determined as the dilution at which there was no reduction in IgM PE fluorescence and no increase in HyHEL9-Tc fluorescence compared with incubation with neat serum. The apoptotic cells were then diluted to a concentration of 2 × 10⁶ cells/ml in 10% IgM-deficient serum in RPMI 1640/0.4% BSA, to provide adequate amount of complement components for opsonization, and incubated for 15 min at 37°C. As a positive and negative control, cells were incubated in either 10% normal mouse serum or RPMI 1640/0.4% BSA, respectively. The apoptotic cells were added to the BM-derived macrophages, prepared from B6 mice, at a ratio of 2:1 (apoptotic cells to macrophages). After 30-min incubation at 37°C, phagocytosis was stopped and uningested cells were removed by washing three times in ice-cold PBS. Following Diff-Quick staining of the coverslips, the uptake of apoptotic cells was expressed as either the percentage of macrophages containing at least one apoptotic cell or as the phagocytic index (number of ingested apoptotic cells per 100 macrophages).

Statistical analysis

Column data are presented as arithmetic means with 95% confidence limits, unless otherwise stated. Comparisons between groups (n > 10) were made by unpaired Student’s t-test and computed using GraphPad Prism version 4.0 (www.graphpad.com), unless otherwise stated in the text. The Mann-Whitney test was used to compare absolute counts of peritoneal cells, which due to sampling error were not normally distributed. Comparisons between multiple groups were made by ANOVA using STATA version 7.0, and correlation was assessed using a Pearson two-tailed test.

Results

Clq deficiency increases the positive selection of IgM autoantibodies and B1 cells by intracellular self Ag

To study how Clq deficiency affects B cell selection to intracellular self Ag, mice carrying a targeted allelic of the Clq gene (Clq−/− and Clq−−/−) (15) were crossed with MD4 IgHEL transgenic mice carrying Ig H and L chain transgenes encoding high affinity IgM+ and IgD+ specific for HEL (17) and with mice expressing mHEL-KK (20). The resulting Clq−−/− I gHEL single and
FIGURE 1. Increased positive selection of IgM autoantibody and B1 cells by intracellular self Ag in the absence of C1q. A, Serum anti-HEL IgM$^\delta$ titer or B, anti-HEL IgM$^\delta$-secreting splenic plasma cells in WT/ heterozygous (⊗) and C1q-deficient (□) IgHEL mice in the presence or absence of mHEL-KK. The ELISA background is twice the level in blank wells, and is at 10 ng/mL. Columns show geometric means, and bars represent the 95% confidence limits. C, Numbers of IgM$^{\delta +}$ IgD$^+$ B1 cells in the peritoneum (PEC) of WT or C1q-deficient single (IgHEL, Ig.c1q$^{+/+}$, $n = 13$ or Ig.c1q$^{++/+}$, $n = 27$) or double (IgHEL/mHEL-KK; Dbl.c1q$^{+/+}$, $n = 18$ or Dbl.c1q$^{++/+}$, $n = 28$) transgenics. Significant difference ($p = 0.0452$; depicted by *) determined by ANOVA. D, Fluorescence of Mac-1 and CD9 on B220$^{low}$ IgM$^\delta$ IgD$^+$ lymphocytes in the peritoneum. E, Flow cytometry of peritoneal lymphocytes stained with Abs to IgM$^\delta$ and IgD$^+$. C1q$^{-/-}$ IgHEL/mHEL-KK double-transgenic mice were all at least seven generations backcrossed to B6. They bred normally, and their survival up to 200 days was no different from C1q$^{-/-}$ and C1q$^{+/+}$ WT transgenic littermates. It is already known that IgM anti-HEL autoantibodies and plasma cells are increased $>$10-fold by the presence of intracellular self Ag in IgHEL/mHEL-KK double-transgenic compared with IgHEL single-transgenic mice (Fig. 1, A and B) (20). Nevertheless, when we measured anti-HEL plasma cells and IgM autoantibodies in C1q$^{-/-}$ IgHEL/mHEL-KK double-transgenic mice, we found that C1q deficiency generated even greater autoimmunity against the intracellular Ag, as follows: $5.51 \times 10^9$ vs $2.30 \times 10^9$ plasma cells/spleen and 773 vs 293 $\mu$g/ml serum anti-HEL IgM (Fig. 1, A and B). This effect is specific to the Ag because plasma cell numbers and serum anti-HEL IgM Ab levels were unaffected by C1q in the absence of mHEL-KK (Fig. 1, A and B), which was in agreement with our previous findings (19). To establish unequivocally that effects of C1q were specific to intracellular self Ag, we repeated the experiment in which we crossed the C1q-deficient mice to ML5 transgenic mice expressing tolerogenic sHEL at 20–30 ng/ml in serum (19). Once again, we found that the induction of anergy was identical in C1q-deficient and WT IgHEL/shHEL mice (75 and 35 plasma cells/spleen and 0.5 and 0.07 $\mu$g/ml serum anti-HEL IgM titers, respectively).

C1q deficiency also increased the positive selection of IgHEL B1 cells by mHEL-KK, both in absolute terms ($33.23 \times 10^7$ vs $6.09 \times 10^7$ cells/peritoneum; Student’s $t$ test, $p = 0.0452$; Mann-Whitney $U$ test, $p = 0.0025$) (Fig. 1), and as a percentage of total peritoneal cells ($8.70 \pm 2.64\%$; Student’s $t$ test, $p < 0.0001$). These FSC$^{high}$ B220$^{low}$ IgM$^{high}$ IgD$^-$ CD5$^-$ B1b cells selected by mHEL-KK stained brightly for CD9 (Fig. 1E). Plasma cell number and IgM in individual double-transgenic mice correlated strongly with the percentage of B1 cells in the peritoneum (Pearson $r^2 = 0.258$, $p = 0.0003$, and $r^2 = 0.429$, $p < 0.0001$, respectively). Not surprisingly, this suggests that the positive selection of B1 cells by Ag and the generation of IgM autoantibody are linked. Again, the effects of C1q were specific to the intracellular Ag because there was no evidence of B1 cell selection in the absence of self Ag in C1q$^{-/-}$ or WT mice (Fig. 1, C–E), or in the presence of sHEL (data not shown).

Conventional B cells specific for intracellular self Ag survive in greater numbers in C1q-deficient mice

The phenotype of the conventional follicular (FO) and marginal zone (MZ) B cells in IgHEL/mHEL-KK double transgenics has been shown previously to be consistent with exposure to low levels of sHEL, which is cleaved from intact protein and present in the serum of mHEL-KK lines at $<10$ ng/ml (20). This level of soluble Ag is not sufficient to induce a state of anergy, but it does induce modest IgM modulation on the recirculating IgD$^+$ FO B cells and is associated with a small reduction in B cell survival (20). At the same time, CD21$^{high}$ CD23$^{low}$ MZ B cells in IgHEL/mHEL-KK double transgenics are also reduced, either due to developmental arrest or continuous depopulation in response to Ag binding (20). We now show that C1q deficiency increases the survival of auto-reactive conventional B cells in IgHEL/mHEL-KK double transgenics ($2.68 \times 10^7$ vs $4.05 \times 10^7$ splenic HEL-binding B cells; $p = 0.0051$) and reduces the level of IgM modulation on recirculating B cells (Fig. 2, A and B). The increase in B cell numbers is most evident in the FO compartment, reversing the small reduction in FO B cell numbers in WT IgHEL/mHEL-KK double transgenics compared with IgHEL$^+$ controls (Dbl.c1q$^{+/+}$, $2.11 \times 10^7$, confidence interval (CI) 1.63–2.59 × 10$^7$, $n = 8$; Dbl.c1q$^{++/+}$, $2.75 \times 10^7$, CI 2.04–3.46 × 10$^7$, $n = 13$; Ig.c1q$^{+/+}$, $3.08 \times 10^7$, CI 2.57–3.58 × 10$^7$, $n = 8$; Ig.c1q$^{++/+}$, $3.45 \times 10^7$, CI 2.64–4.26 × 10$^7$, $n = 7$) (20). The absence of CD86 (B7.2) or CD69 up-regulation is against cell survival due to increased activation by Ag (Fig. 2C).
Conventional IgHEL B cells can be activated by intracellular self Ag and T cell help

Given the large number of autoreactive conventional B cells that escape negative selection by intracellular proteins, we next wanted to see whether there were circumstances under which they could be stimulated by our intracellular Ag and differentiate into autoantibody-producing plasma cells. This is important because most pathogenic Abs in SLE have undergone T-dependent affinity maturation and class switching, and therefore have arisen from conventional rather than B1 B cells. Accordingly, we primed sublethally irradiated (B10BR\(\times\)B6)\(F_1\) H-2\(^{bb}\) mice with splenocytes from H-2\(^{kk}\) IgHEL transgenic mice, and to some of these mice we added MACS-purified H-2\(^{kk}\) 3A9 TCR transgenic CD4 T cells, which recognize the immunodominant I-A\(^k\)-restricted epitope of HEL (24). After 24 h, 5 \times 10^7 intact or late apoptotic (annexin V\(^+\), PI\(^+\)) thymocytes from mHEL-KK and nontransgenic mice (all H-2\(^{bb}\)) were injected into the recipients, and spleens were assayed for anti-HEL plasma cells at 5 days. The results show that the intracellular Ag can induce plasma cell differentiation and increase class II expression on B cells, but only when cross-presentation of the Ag can lead to T cell help (Fig. 3). The lower immunogenicity of apoptotic cells is likely to be due to increased clearance of this Ag in the circulation. These findings suggest that there may be circumstances when the failure to remove apoptotic cells increases the risk of autoimmunity against intracellular self Ags.

The positive selection of B1 cells and clearance of self Ags by C1q and IgM represent a potentially tolerogenic feedback loop

Previous studies have shown that C1q is involved in the clearance of apoptotic cells in vitro (23) and in vivo (12), and it is thought that this is the reason for the accumulation of excess apoptotic debris in the tissues of C1q-deficient mice. Therefore, we wondered whether the increased positive selection of B1 cells and a tendency to autoimmunity in the conventional B cell compartment

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FIGURE 2. Effects of C1q deficiency on conventional B cell development in the presence or absence of intracellular self Ag. A, Numbers of immature (IgM\(^{+}+/\)IgD\(^{+}+)\) and mature (IgM\(^{+}+/\)IgD\(^{+}+)\) B220\(^{+}\) lymphocytes in the BM, and mature HEL-binding B220\(^{+}\) lymphocytes in the spleen and mesenteric lymph node (MLN) of WT or C1q-deficient single (IgHEL; Ig.\(c1q^{-/+}\), \(n = 13\) or Ig.\(c1q^{-/-}\), \(n = 27\)) or double (IgHEL/mHEL-KK; Dbl.\(c1q^{-/+}\), \(n = 18\) or Dbl.\(c1q^{-/-}\), \(n = 28\)) transgenics. Significant differences in the BM (\(p = 0.0001\)), spleen (\(p = 0.0051\)), and MLN (\(p = 0.0018\)) were determined by ANOVA and depicted by ***. B, Flow cytometry of B220\(^{+}\) BM or splenic B cells, stained with Abs to IgM\(^{+}\) and IgD\(^{+}\) or CD21 and CD23, respectively. Top panel, The gate used to determine mean fluorescence intensity of IgM\(^{+}\) on recirculating B cells; Ig.\(c1q^{-/+}\) mean fluorescence intensity 200.04, CI 137.37–262.71, \(n = 4\); Ig.\(c1q^{-/-}\) mean fluorescence intensity 233.09, CI 224.05–242.14, \(n = 5\); Dbl.\(c1q^{-/+}\) mean fluorescence intensity 21.88, CI 20.15–23.60, \(n = 4\); Dbl.\(c1q^{-/-}\) mean fluorescence intensity 36.42, CI 33.11–39.72, \(n = 6\) (data representative of five separate experiments). Bottom panel, The percentage of B cells in the FO (CD23\(^{-/+}\)/CD21\(^{+}\)) and MZ (CD23\(^{+}\)/CD21\(^{+}\)) subsets (means from four experiments; Ig.\(c1q^{-/+}\), \(n = 7\); Ig.\(c1q^{-/-}\), \(n = 8\); Dbl.\(c1q^{-/+}\), \(n = 13\); Dbl.\(c1q^{-/-}\), \(n = 8\)). C, Fluorescence of CD86 (B7.2) and CD69 on B220\(^{+}\) splenocytes.
of C1q-deficient mice might be linked by a failure to clear Ag associated with apoptotic cells. To explore this, we looked first to see whether the mHEL-KK Ag would behave as a typical lupus Ag in vitro. During apoptosis, the intracellular Ags that are typically targeted in SLE translocate to the surface of the cells where they coalesce in blebs (5). Confocal microscopy of apoptotic thymocytes from mHEL-KK mice showed a similar redistribution of mHEL-KK from the endoplasmic reticulum to surface blebs (Fig. 4, A and B). The transgenic anti-HEL IgM\(^+,\) Ab in Ig\(^{HEL}\) serum is able to bind specifically to mHEL-KK, because it is exposed on the small number of dying cells isolated in samples ex vivo (Fig. 4C, upper panel). This pattern of staining is similar for polyclonal IgM\(^+\) from the serum of nontransgenic B6-Igh\(a\) mice, in which the greater intensity of fluorescence presumably reflects a greater number of IgM-targeted self Ags (Fig. 4C, lower panel).

Although the clearance of apoptotic cells depends on a variety of mechanisms, the efficient clearance of low numbers of apoptotic cells by macrophages in vitro and in vivo has been shown to depend on activation of the classical pathway by C1q binding to IgM (23). To assess the potential role of positively selected autoantibody against intracellular self Ags in this process, we mixed apoptotic mHEL-KK or nontransgenic thymocytes in vitro with BM-derived macrophages and either of the following: 1) medium alone; 2) serum from a Rag-deficient IgHEL/mHEL-KK mouse as a source of anti-HEL-only IgM (diluted 20-fold) and serum from sIgM-deficient mice as a source of fresh complement; 3) serum from sIgM-deficient mice plus serum from Rag-deficient mice as control for 2; or 4) fresh normal mouse serum. The number of

![FIGURE 3.](image)

mHEL-KK is immunogenic to conventional B cells given T cell help in vivo. A, Anti-HEL IgM\(^+\)-secreting splenic plasma cells and B, MHC class II expression in (C57BL/6 × B10.BR)F\(1\) recipients, injected with a combination of Ig\(^{HEL}\) splenocytes (B cells), purified HEL-specific CD4\(^+\) thymocytes (T cells), and untreated (med) or dexamethasone-treated (dex) thymocytes from either nontransgenic (non) or mHEL-KK transgenic (mHEL-KK) mice.

![FIGURE 4.](image)

IgM autoantibody mediates the clearance of apoptotic cells by binding to its target Ag, which is exposed on the cell surface. A, Flow cytometry of dexamethasone-treated nontransgenic (non) and mHEL-KK thymocytes, stained with PI and Abs to HEL. B, Confocal microscopy of nontransgenic and mHEL-KK thymocytes cultured in the absence (top row) or presence of dexamethasone (bottom row). Permeable cells were stained with PI (red) and anti-HEL Abs (green). Line illustrates a distance of 1 \(\mu\)m. C, Flow cytometry of dying (top panel) and live cells (bottom panel) from nontransgenic (non) and mHEL-KK (mHEL-KK) thymocytes, stained for bound IgM\(^+\), after incubation with serum from Ig\(^{HEL}\) transgenic or nontransgenic IgM\(^+\) serum. Dying (red) and live cells (green) were gated as shown. D, Phagocytosis of nontransgenic and mHEL-KK thymocytes, opsonized with different mouse serum and IgM\(^-\) serum as a source of fresh complement. The phagocytic index represents the average number of apoptotic cells ingested per 100 macrophages.
apoptotic cells ingested was scored after 30 min. As well as confirming the contribution of natural IgM Ab in normal mouse serum to the clearance of dying cells by macrophages, from both nontransgenic and mHEL-KK animals (Fig. 4D), this experiment showed an Ag-specific effect of the IgM anti-HEL Ab on the clearance of HEL-specific dying cells (ANOVA, p < 0.0001) (Fig. 4D). These findings, together with the data from the analysis of the transgenic mice, support the idea that B1-derived natural Abs might inhibit their own selection by Ag-specific clearance of positively selecting self Ag in vivo.

Discussion

In this study, we show that C1q deficiency increases the positive selection of B1b B cells by intracellular self Ag, the number of plasma cells, and the secretion of IgM autoantibodies. At the same time, negative selection of conventional B cells is reduced. There are good reasons to suppose that these effects are specific to C1q deficiency and Ag. None of the mice in our cohorts were different from WT controls in terms of survival, breeding, or general health, and C1q deficiency had no effect on B cell selection by sHEL or in the absence of Ag. We also show that the intracellular Ag behaves like a typical lupus autoantigen when it is exposed in blebs on dying cells. In this state it can bind IgM autoantibody, which can fix C1q and trigger phagocytosis and Ag clearance. The conventional B cells that escape self-tolerance can be activated by intracellular self Ag in the presence of T cell help. These findings support the idea that the expression and clearance of intracellular Ag dictate the level of positive and negative selection and risk of systemic autoimmunity.

Increased exposure to immunogenic self Ag might explain an increase in autoreactive B1b cells in C1q deficiency and would fit with a normal requirement for positive selection by Ag in this subset of B cells (25, 26). Unlike conventional B cells, B1 B cells arise more frequently in early ontogeny when their development is largely IL-7 independent (27). They are capable of self-renewal and do not require the TNF family ligand and cell activating factor belonging to the tumor necrosis factor family for continued survival (28, 29). Although they are difficult to detect by conventional staining, they recirculate continuously throughout the secondary lymphoid organs (30). B1 cells have long been associated with the secretion of normal Abs against self and foreign pathogens, which occur without an obvious inflammatory immune response (31, 32). In nontransgenic mice, B1 cells express a restricted repertoire of Ag receptors, which may reflect the effect of positive selection of a limited number by Ags, as well as evolutionary selection for particular germline specificities. Although endogenous TLR signals are not required for the positive selection of B1b cells in our model (33), there may be other as yet undefined signals that distinguish tolerogenic Ags, like cell surface HEL, from identical but positively selecting intracellular Ags, such as mHEL-KK. In these respects, it may be critical that intracellular HEL-KK is exposed on the surface of late apoptotic cells and in apoptotic vesicles.

In contrast to B1 cells, conventional B cells do not require positive selection by self Ags, and as immature cells are more liable to negative selection. Despite limited negative selection, conventional B cells survive in WT IgHEL/mHEL-KK mice and are functionally competent. The further decrease in negative selection in C1q-deficient IgHEL/mHEL-KK is consistent with the presence of higher levels of IgM autoantibody binding cleaved free sHEL and membrane-bound forms of intracellular self Ag in C1q−/− IgHEL/mHEL-KK mice. We have found recently that mHEL-KK can cause the deletion of immature IgHEL B cells from adult BM. This is blocked by the persistence of B1 cells and IgM autoantibodies that are positively selected by mHEL-KK in early ontogeny (21). Therefore, higher levels of IgM against intracellular Ags will increase the number of conventional autoreactive B cells that escape into the periphery of C1q-deficient mice. There is now good evidence that large numbers of conventional autoreactive B cells escape tolerance to systemic Ags at the immature B cell stage in humans and nontransgenic mice, and many of these cells are functional in normal adults and patients with SLE (34, 35).

The escape of immunocompetent conventional autoreactive B cells into the primary repertoire is significant because of the ability of these cells to generate pathogenic autoantibodies by class-switching and T-dependent affinity maturation. Our finding that these cells can only be activated by intracellular Ag when T cell help is present emphasizes the importance of robust T cell tolerance to ubiquitous intracellular Ags (36). Similar T cell tolerance to other intracellular self Ags could explain why nontransgenic B6 C1q−/− mice do not get overt SLE. Autoimmune disease in C1q-deficient MRL and hybrid 129 × B6 strains (16) could be due to susceptibility genes affecting this or other tolerance checkpoints. The need for B cell activation suggests that disease on a C1q-deficient background might be induced by environmental factors such as foreign Abs that break T cell tolerance by cross-presentation, or endogenous or exogenous TLR ligands (37). A future challenge lies in understanding how environmental and epigenetic factors combine with C1q deficiency to induce overt disease.

Our findings provide support for the idea that there may be a physiological role for B1 cells, C1q, and a wide spectrum of positively selected IgM autoantibodies in the rapid clearance of dying cells that might otherwise be autoinflammatory. As in the innate response against pathogens, this process could account for an evolutionary conservation of binding specificities, which are expressed without mutation by B1 cells in early ontogeny (38, 39). Like C1q deficiency, IgM deficiency causes lupus-like autoimmune disease, suggesting that it too may be protecting against autoimmune disease by increasing Ag clearance (22, 40). IgM deficiency is also associated with increased B1 cells (41). Therefore, it is possible that C1q limits the development of autoreactive B1 cells and the repertoire of natural autoantibodies against seques- tered proteins as part of a feedback loop that regulates the clearance of dying cells. This explanation could explain the Ag-specific effects of C1q deficiency. Experiments characterizing B1-derived autoantibodies in normal mouse serum against naturally occurring Ags on the surface of dying cells also support this hypothesis. The series of studies of B1-derived anti-phosphorylcholine (PC) Abs of the T15 id are particularly informative (42). Anti-PC Abs provide a first line of defense against microbial infections, such as Strep- tococcus pneumoniae, but are present in germfree mice, where they cross-react with oxidized phospholipid moieties on apoptotic cells (43). The selection of B1-derived anti-PC Ab increases in atherosclerotic apoE-deficient mice, where there is increased oxidized low-density lipoprotein target Ag (44). Natural anti-PC IgM Abs also bind to late apoptotic cells and recruit C1q, leading to C3 activation (45). This too would be expected to enhance the clearance of apoptotic cells.

In summary, this study shows that B1 cell selection and C1q are linked by positive selection, the production of IgM autoantibodies, and the clearance of intracellular self Ags. These same self Ags are potentially immunogenic to conventional B cells that escape self-tolerance to intracellular Ags. These findings provide support for the idea that C1q affects both the clearance of Ag and positive and negative selection of autoreactive B cells.
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Disclosures

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References