Membrane Cholesterol Content Accounts for Developmental Differences in Surface B Cell Receptor Compartmentalization and Signaling*

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Recent studies argue for an important role for cholesterol in maintaining plasma membrane heterogeneity and influencing a variety of cellular processes, including signaling, adhesion, and permeability. Here, we document that tolerance-sensitive transitional immature B cells maintain significantly lower membrane unesterified cholesterol levels than mature-stage splenic B cells. In addition, the relatively low level of cholesterol in transitional immature B cells impairs compartmentalization of their B cell receptor (BCR) into cholesterol-enriched domains following BCR aggregation and reduces their ability to sustain certain aspects of BCR signaling as compared with mature B cells. These studies establish an unexpected difference in the lipid composition of peripheral transitional immature and mature B cells and point to a determining role for development-associated differences in cholesterol content for the differential responses of these B cells to BCR engagement.

The spatial relationships between proteins in the inner and outer leaflet of the plasma membrane play important roles in a number of diverse biological processes, including the initiation and transduction of receptor generated signals, protein sorting, viral entry, and membrane internalization (1–4). Organization of the plasma membrane into discrete compartments determined by lipid content provides additional regulation for protein-protein and protein-lipid interactions. The primary classes of lipids that reside in the plasma membrane are glycerophospholipids, sphingolipids, and membrane-active sterols (5). Glycerophospholipids contain two acyl chains, one of which is often unsaturated. In contrast, sphingolipids contain two acyl chains, which are both larger and saturated. Membrane-active sterols, mainly represented by cholesterol in mammals, are defined by a flat fused-ring system. Cholesterol has a greater affinity for the saturated acyl chains of sphingolipids and its incorporation into membranes provides rigidity via its four-ring structure. The higher concentration of cholesterol within the membrane that are resistant to some non-ionic detergents is termed lipid rafts. Rafts are operationally defined as regions of membrane microdomain formation within the plasma membrane, as opposed to disordered, acyl-containing membrane domains (6, 7).

Many recent studies have focused on these higher ordered sphingolipid/cholesterol-enriched membrane microdomains, termed lipid rafts. Rafts are operationally defined as regions of the membrane that are resistant to some non-ionic detergents and are enriched in the ganglioside GM1 (8). It has been suggested that raft domains play important roles in signaling, because several receptors, including the B cell receptor (BCR) (2), T cell receptor (9), FcεR (10), and insulin receptor (11), are observed to redistribute from the detergent-soluble, lipid-disordered to the GM1-enriched, detergent-insoluble compartment upon ligand binding.

B lymphocyte responses to similar stimuli differ dramatically at discrete stages of maturation and in different microenvironments. Notably, bone marrow and peripheral immature B cells are not activated by signals generated through the BCR but rather undergo apoptosis (deletion) or receptor modification (editing) (12). Transitional immature B cells are a population of peripheral immature B cells that have recently left the bone marrow and are a critical link to immunocompetent mature B cells (13–15). Our laboratory has previously shown that, upon BCR cross-linking, transitional immature B cells undergo apoptosis, whereas mature B cells enter and progress through cell cycle, up-regulate proteins necessary to activate T cells, and manifest other processes associated with activation (12, 16, 17). Importantly, the ligand-induced redistribution of the BCR is developmentally regulated, such that, although it preferentially associates with the lipid raft marker GM1 in mature cells after BCR aggregation, this inducible redistribution does not occur in transitional immature B cells (2, 14). The relationship between these responses and plasma membrane composition has not been explored. We speculated that this phenomenon might be related to differential membrane composition. Because of the unique function of cholesterol to facilitate membrane microdomain formation within the plasma membrane, we have compared cholesterol levels between transitional immature and mature B cells and have determined the association of membrane cholesterol levels to BCR proximal signaling and lipid raft compartmentalization.

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1 The abbreviations used are: GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)-Galβ1,4Glc-ceramide; MCD, methyl-β-cyclohexatin; BCR, B cell receptor; PBS, phosphate-buffered saline; MES, 2-(morpholinoo)ethanesulfonic acid; DS, detergent-soluble fraction; DI, detergent-insoluble fraction; PLCγ2, phospholipase Cγ2.

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Cholesterol and Developmental Differences in B Cells

MATERIALS AND METHODS

Isolation and Definition of Mature and Transitional Immature B Cell Subsets—Transitional immature B cells were isolated from spleens of 6- to 10-week-old sublethally irradiated, auto-reconstituting BALB/c mice 14 days post sub-lethal irradiation at 500 rads, as described previously (18). A 1:1 mixture was applied to an irradiated mice bone marrow population enriched in mature B cells. For these studies, the isolated populations ranged between 94 and 98% B cells, as assessed by B220 expression. Non-sorted mature populations generally contain 10–15% transitional immature B cells (19). The non-sorted transitional immature populations used in these studies were devoid of detectable mature B cells, as assessed by the absence of sIgMpos, B220pos, and AA4.1hi cells.

Flow-cytometry-Cell Sorting and Filipin Staining—For flow-cytometry-cell Sorting and Filipin Staining, the expression of F(ab’)2 anti-IgM (Jackson ImmunoResearch) and anti-transferrin receptor (Zymed Laboratories Inc., and detected by ECL.

RESULTS

BCR Aggregates Co-polarize with Cholesterol in Mature B Cells—We have previously shown that anti-BCR-induced BCR aggregates in mature B cells (B220pos, CD23pos, and AA4.1pos) co-polarize with the lipid raft marker GM1 to a significantly greater extent than do those in transitional immature B cells (B220pos and AA4.1neg) (14). Because cholesterol is a key component in lipid rafts, we wanted to determine if anti-BCR-induced BCR aggregates localized to areas relatively enriched in unesterified cholesterol in transitional immature and mature B cells. We used filipin as a marker for unesterified (free) cholesterol (22–24). In both unstimulated mature and transitional immature B cells, cholesterol and the BCR were evenly distributed around the cells. Anti-BCR-induced BCR aggregates in mature B cells co-polarized with cholesterol in 48 ± 4% of observed cells (Fig. 1A, top panel, and Table I). Anti-BCR-induced BCR aggregates in transitional immature B cells co-polarized with cholesterol in 16 ± 3% of observed cells (Fig. 1B, bottom panel, and Table I). Therefore, in agreement with the observed co-polarization of BCR aggregates with GM1, BCR aggregates co-polarized with cholesterol to a greater extent in mature than in transitional immature B cells.

Cholesterol Content of Transitional Immature B Cell Subsets Is Equivalent and Different from Mature Splenic B Cells—We next wanted to determine if the relative inability of BCR aggregates to co-polarize with cholesterol in transitional immature B cells was due to lower levels of cholesterol in their plasma membrane. In the studies to follow, the fluorescence emission of filipin bound to cholesterol was utilized to detect free cholesterol in fixed cells while allowing for the simultaneous measurement of other markers by flow cytometry, thereby permitting subpopulation analysis in heterogeneous populations.

Cholesterol levels were first determined by filipin binding to...
transitional immature and mature B cells (Fig. 2A). We observed that the mature population binds ~2–3 times the amount of filipin as the transitional immature population. Notably, filipin binding to mature B cells was bimodal. Subsequent gating and phenotypic analysis of the lower intensity population indicated that this peak represents the transitional immature and mature B cells (Fig. 2A). Therefore, differences in the cholesterol content between these populations are not likely attributable to differences in plasma membrane area. Of note, the total BCR expression (IgM and IgD combined) are equivalent for mature and transitional immature and mature B cells (data not shown). Importantly, in all our studies we are stimulating through BCR using an antibody that reacts with the light chain component common to both isoforms so that all surface BCR complexes are engaged.

The result depicted in Fig. 2A indicates that transitional immature B cells contain less unesterified cholesterol than do mature B cells. To corroborate this finding more quantitatively and to address the issue that the cholesterol content of the plasma membrane in these cells may not be at equilibrium, we determined the relative levels of unesterified (free) and total cholesterol by gas-liquid chromatography from both populations (Table II). Esterified cholesterol does not have the ability to bind to the plasma membrane, and it is typically found in cells such as hepatocytes that store cholesterol (25). Lymphocytes do not contain appreciable amounts of esterified cholesterol (26–29). As in previous studies by others (22, 24), we observed a close agreement in the cholesterol levels determined by biochemical analysis and filipin binding. Regardless of whether the results were normalized to cell number or protein level, the mature B cells contained 2–3 times the amount of cholesterol relative to that found in transitional immature B cells. In addition, this analysis confirmed that total and free cholesterol pools were identical, indicating that all the cholesterol in these cells is unesterified. Therefore, the cholesterol levels quantitated by filipin binding levels represent plasma membrane or plasma membrane precursor pools and strongly support the interpretation that the cholesterol content of the transitional immature B cell plasma membranes is reproducibly lower than in mature B cells.

**Manipulation of the Cholesterol Content of Transitional Immature B Cells Facilitates Anti-BCR Induced Co-localization of Cholesterol-enriched Compartments with Aggregated BCR Complexes—MβCD is a carbohydrate that binds selectively and reversibly to cholesterol. Most studies have utilized MβCD to deplete plasma membrane cholesterol. However, it can also be used to transfer cholesterol from a high cholesterol-containing medium into the relatively low cholesterol-containing plasma membrane.** Fig. 3A shows the effect of incubating transitional immature B cells with varying levels of extracellular free cholesterol at 37 °C in the presence of MβCD at various times of incubation at each condition. The results depicted in Fig. 3A indicate a dose-dependent increase in membrane-associated cholesterol, as determined by filipin binding. At 5 mM MβCD with 300 μg/ml cholesterol, transitional immature cells exhibited similar filipin binding to mature cells. This addition of cholesterol was dependent upon the presence of MβCD, because transitional cells treated with cholesterol alone showed no increase in filipin binding (second panel from left). To determine if the filipin binding analyzed by fluorescence-activated cell sorting was at the limit of detection, we added cholesterol to mature B cells, and the results showed that the cholesterol level of mature cells could be detectably increased (data not shown). Therefore, the identical filipin binding of MβCD/cholesterol-treated transitional immature cells and untreated mature cells was not due to the limit of detection of filipin binding. Furthermore, we noted no detectable change in the level of BCR expression on transitional immature B cells after cholesterol addition (data not shown).
One characteristic of cholesterol-enriched domains is that they are resistant to solubilization in 1% Triton X-100 at 4 °C. We next determined if the addition of MβCD/cholesterol to transitional immature B cells affected the distribution of proteins in 1% Triton X-100-soluble and -insoluble domains. Primary mature, transitional immature, and transitional immature B cells treated with MβCD/cholesterol were lysed in 1% Triton X-100. The cells were then centrifuged at 100,000 g for 1 h at 4 °C, and the resultant supernatant was labeled DS. The 100,000 g pellet was resuspended in octylglucoside and then centrifuged at 10,000 g for 12 min at 4 °C. The resultant supernatant was labeled Triton X-100 DI. Equivalent cell amounts were loaded onto SDS-PAGE, and the proteins were detected with silver stain. Fig. 3B indicates that the protein profile in the DS fraction is similar in each sample tested. However, there is less protein detected in transitional DI fraction than the mature DI fraction. MβCD/cholesterol treatment of transitional immature B cells resulted in a similar protein profile in the DI fraction as compared with the mature DI fraction.

Addition of Cholesterol to the Immature WEHI-231 B Cell Line Promotes Localization of BCR to Detergent-insoluble Membrane Fractions—It is not possible to recover the BCR from primary B cells on sucrose gradients after stimulation, owing to its strong association with the cytoskeleton. However, it has previously been shown that the BCR in the mature B cell line CH27 is present in the detergent-insoluble raft fractions following identical stimulation (30). Together, these data suggest that MβCD/cholesterol treatment of transitional immature B cells results in similar content and distribution of protein in DI fractions and similar BCR aggregate co-polarization with cholesterol as compared with mature B cells (47 ± 3% versus 48 ± 4%).

| Table II Differences in Unesterified Membrane Cholesterol Content Distinguish Mature and Transitional Immature B Cells |
|---------------------------------|
|                               | Mature | Transitional immature |
| Freea                          | Per 10^6 cells 0.31 ± 0.02 | 0.10 ± 0.01 |
| Per mg protein                 | 13.62 ± 1.00 | 7.02 ± 0.76 |
| Totalb                         | Per 10^6 cells 0.29 ± 0.03 | 0.11 ± 0.02 |
| Per mg protein                 | 13.45 ± 1.20 | 7.21 ± 1.21 |

a Unesterified cholesterol (micrograms).
b Unesterified plus esterified cholesterol (micrograms).

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MβCD/cholesterol addition to WEHI-231 B cells resulted in BCR isolation in detergent-insoluble fractions after BCR stimulation (Fig. 4). Isolation of the BCR in detergent-insoluble...
FIG. 4. Reconstitution of BCR translocation to detergent-insoluble fractions following cholesterol enrichment of the immature B cell line WEHI-231. WEHI-231 cells, WEHI-231 cells treated with MβCD/cholesterol for 90 min, and CH27 cells were lysed in 1% Triton X-100 at 4 °C and subjected to sucrose gradient ultracentrifugation. Sucrose gradients were divided into 12 fractions and separated on SDS-PAGE. Immunoblots were probed with antibodies against IgM and Tfr. Lanes 2–4 represent the detergent-insoluble fractions, whereas lanes 7–12 represent the detergent-soluble fractions. Untreated WEHI-231 cells do not inducibly translocate the BCR into detergent-insoluble fractions following BCR cross-linking. Addition of cholesterol restores inducible BCR translocation to detergent-insoluble fractions. The IgM levels in the raft fractions of anti-BCR stimulated WEHI-231 cells with added cholesterol were 39-fold higher than for the identical stimulated WEHI-231 cells without additional cholesterol. Sucrose gradients from untreated and anti-BCR-treated mature B cell line CH27 are shown for comparison.
fractions was dependent on BCR stimulation, because the BCR was not isolated in the detergent-insoluble fractions of resting WEHI-231 B cells treated with MβCD/cholesterol. Sucrose gradients of CH27 cells were used as a comparison for mature B cells. The detergent-soluble marker transferrin receptor was not detected in any of the detergent-insoluble fractions tested. One potential caveat with sucrose gradient isolation of cholesterol-enriched domains is that some internal membranes are detergent-insoluble. To determine if the BCR isolated in the detergent-insoluble fraction of cholesterol-enriched WEHI-231 cells was located at the cell surface, we biotinylated WEHI-231 cells after cholesterol addition. The results indicate that the BCR found in the detergent-insoluble fraction was on the surface of the cell (data not shown).

**Inducible BCR Co-localization with Cholesterol-enriched Compartments Results in Sustained Signaling Resembling That of BCR-triggered Mature B Cells**—Our laboratory and others have shown that signals generated through the BCR in mature and transitional immature B cells differ in both intensity and duration, specifically hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of inositol 3,4,5-trisphosphate (12, 31–33). Because cholesterol levels are different in transitional immature and mature B cells, and because cholesterol-enriched domains have been implicated in amplifying and/or sustaining signaling, we investigated if cholesterol addition to transitional immature B cells affects downstream signaling. In mature B cells, anti-BCR-induced PLCγ2 phosphorylation peaked at 2 min and then remained phosphorylated through 60 min (Fig. 5A). Although transitional B cells can maintain anti-BCR-induced phosphorylation through 60 min, the initial peak activation at 2 min was reduced compared with mature B cells. Cholesterol addition to transitional immature B cells resulted in a peak activation of anti-BCR-induced PLCγ2 phosphorylation that was similar to mature B cells at 2 min and was maintained through 60 min. Therefore, cholesterol addition to transitional immature B cells results in a signal intensity similar to that seen in mature B cells.

Recent studies have shown that cholesterol-enriched domains play a role in NFκB signaling (13). This signaling cascade is initiated by PLCγ2 hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in protein kinase Cβ activation of IkB kinases that leads to ubiquitination and subsequent degradation of IkBα. In particular, induction of c-myc expression has been shown to be up-regulated by BCR signaling through this pathway (34). Furthermore, previous studies have shown that the BCR-induced increase in expression of c-myc transcripts in the immature B cell line WEHI-231 are transient compared with mature cell lines (35), suggesting lack of sustained signaling through this pathway in immature B cell lines. We therefore wanted to determine levels of c-myc transcript expression in primary transitional immature and mature B cells following BCR stimulation. For both transitional immature and mature B cells, there was a clear increase in c-myc expression after 1 h (Fig. 5C). However, although the anti-BCR stimulated mature B cells maintained expression through 4 h (74% of peak), levels decreased significantly in the anti-BCR stimulated transitional immature B cells (27% of peak). Thus, as compared with mature B cells, these results argue that transitional immature B cells are unable to sustain signals leading to c-myc expression following BCR aggregation. We next evaluated if MβCD/cholesterol addition to transitional immature B cells leads to sustained c-myc mRNA expression levels. The addition of MβCD/cholesterol to transitional immature B cells rescues c-myc expression after 4 h of anti-BCR treatment (63% of peak). Therefore, MβCD/cholesterol addition to transitional immature B cells not only results in the association of BCR with cholesterol-enriched domains following BCR aggregation but also converts the signaling phenotype of transitional immature B cells to the sustained signaling phenotype of mature B cells.

**DISCUSSION**

BCR-linked differences between immature and transitional immature B cells and mature B cells have been documented through work from our laboratory (12, 14, 16) and others (15, 32, 33, 36). Moreover, several of these studies (37, 38) implicate these differences in the regulation of the responses of immature and mature B cells to BCR cross-linking. In general, these
studies have implicated differences in the ability to engage or maintain the phosphatidylinositol 4,5-bisphosphate hydrolysis → protein kinase Cβ → 1xK kinase → NFκβ → c-myc pathway leading to differential survival/cell cycle progression or apoptosis. The mechanistic basis for the differential regulation of specific aspects of BCR signaling at these two developmental stages remains unresolved. However, studies documenting the differential ability of the BCR in mature and transitional immature B cells and transformed cell line models to translocate into detergent-insoluble (1, 2) and GM1-enriched (14) microdomains are provocative. The studies reported here document distinct and biologically significant differences in the membrane cholesterol content of transitional immature and mature B cells. This observation was unexpected, because there are no other examples where the unesterified cholesterol content of mammalian cells is developmentally regulated. More remarkably, these differences integrate the previous observations regarding BCR signaling at these stages.

We show that cholesterol levels between transitional immature and mature B cells differ. Related studies using neurons have shown that variations in cholesterol levels within individual cells result in signaling changes important for neuronal function (39). Therefore, we hypothesized that the difference in cholesterol content between transitional immature and mature B cells may play a role in the developmentally regulated differences in BCR signal transduction observed at these developmental stages. It is possible that some of the cholesterol measured in these cells will likely be associated with endoplasmic reticulum, mitochondrial, and Golgi membrane fractions. However, in mammalian cells these pools represent a relative small fraction, as little as 10%, of the membrane-associated cholesterol (28, 29). We believe that the most likely interpretation of our data is that the differences observed reflect differences in plasma membrane cholesterol.

Because cholesterol plays a role in maintaining membrane heterogeneity, we analyzed the partitioning of the BCR within the membrane in transitional immature and mature B cells. We have previously shown that BCR stimulation of mature B cells leads to receptor aggregation and co-localization with aggregated GM1 by the use of cholera toxin staining. However, although BCR stimulation of transitional immature B cells results in receptor aggregation, the GM1 on BCR-stimulated transitional immature B cells does not aggregate. Because studies have shown that cholera toxin binding is not limited to GM1 and insufficient fixation can result in receptor redistribution, we fixed cells in 4% paraformaldehyde with 0.1% glutaraldehyde and stained with the cholesterol marker filipin. Our initial results indicated greater cholesterol co-localization with the BCR in BCR-aggregated mature cells. The distinction between cholera toxin staining and filipin staining as a marker for membrane heterogeneity is important, because we are primarily interested in cholesterol variances between transitional immature and mature B cells. Inducible BCR co-localization with cholesterol supports the model that membrane cholesterol mediates membrane heterogeneity and that signals transmitted through the BCR may be modulated by differential localization within membrane microdomains.

Instead of basing our studies on a loss of function with the depletion of cholesterol, we determined if cholesterol addition to transitional cells results in a gain of function. To add cholesterol to the plasma membrane, we utilized MβCD medium containing excess levels of cholesterol to shuttle cholesterol into the plasma membrane. Although the treatment of cells with MβCD alone results in membrane perturbation and cell death, cell viability is generally not compromised when cells are treated with MβCD and excess levels of cholesterol (40, 41). Therefore, using this treatment we can increase the levels of cholesterol in transitional B cells and look for phenotypic differences in BCR receptor partitioning in the plasma membrane. Our results show that the low level of cholesterol in transitional immature B cells does not result in significant enrichment of cholesterol in BCR aggregates. However, treatment of transitional immature B cells with MβCD/cholesterol restores enrichment of cholesterol within BCR aggregates.

The implications of these studies extend beyond the regulation of BCR proximal signaling. Together with our previous studies, these results suggest that cholesterol content plays a determining role in the fate decisions of transitional immature and mature B cells in response to antigen. One would predict that individuals with genetic defects affecting cholesterol production, uptake, and/or turnover in the plasma membrane might generate B cells with defective responses to antigen. For example, immature B cells with incrementally elevated plasma membrane cholesterol content might be less sensitive to BCR-induced apoptosis and, therefore, exhibit impaired negative selection. In these individuals, polymorphisms in genes regulating these processes would be considered genetic risk factors for autoimmunity. Similarly, mature B cells with incrementally lower cholesterol levels, as might occur in individuals treated with statins to lower serum cholesterol, could be compromised in their ability to respond to antigen. Our current studies point to these issues as important directions for future studies, including the relevance of these findings to human disease and potential therapeutic targets for modulating B cell responsiveness in autoimmune disorders and allogeneic transplants.

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