The commitment of a pluripotent stem cell to the B-cell lineage and the progression of the committed B-cell precursor to a mature, immunocompetent B-cell is a highly regulated process. Extrinsic processes and internal programmes initiate and regulate the changes in gene expression that alter the phenotype and functional characteristics of the B-cell as it proceeds through its maturation. Many of these processes are only now beginning to be recognized and some have begun to be defined. This level of regulation of differentiation and cellular development is common to many if not all cellular lineages. In addition, B (and T) lymphocytes have superimposed on this developmental process another level of regulation. This level involves signals generated through the antigen receptor and exists to select for lymphocytes expressing receptors with certain characteristics and specificities. This selection process not only serves to encourage development of B-cells with functional antigen receptors (positive selection) but also provides a mechanism of elimination of clones able to respond to endogenous or self antigens (negative selection). Thus signalling through the antigen receptor at defined stages of B-cell development provides checkpoints for ordered B-cell maturation and further allows the shaping of the repertoire that will be represented among the mature, immunocompetent B-cells.

Although the importance of the B-cell antigen receptor (BCR) expressed on the surface of mature B-cells has been recognized for some time, it is only relatively recently that we have begun to recognize a role for this receptor (or forms of this receptor) in fate determination at other stages of B-cell development. In Figure 1 is a simplified diagram of B-cell development, highlighting antigen receptor expression and the functional outcome of BCR engagement.

In mature B-cells the BCR is composed of a surface form of immunoglobulin (Ig) in association with homodimers of two transmembrane signal transduction proteins, Igα (CD79a) and Igβ (CD79b). Although Igα and Igβ are expressed as early as the pro-B-cell stage, a surface form of the BCR is not expressed until the pre-B-cell stage. The pre-B-cell receptor is composed of a homodimer of the μ heavy chain non-covalently associated with a surrogate light chain composed of protein products of the λ5 and VpreB genes. Expression of this receptor on the surface is transient and might be important for the development and/or expansion of pre-B-cells [1–4]. The mature form of the antigen receptor is first expressed on the surface at the immature B-cell stage. Immature-stage B-cells in the bone marrow are characterized by expression of the IgM isotype. Mature B-cells co-express IgM and IgD. Transitional B-cells, representing newly emigrating B-cells from the bone marrow, co-express IgM and IgD and are functionally equivalent to immature-stage B-cells [5–8]. Despite the expression of the BCR on immature/transitional and mature-stage B-cells, engagement of this receptor on these two populations of B-cells results in very different responses.

Studies by Metcalfe and Kinman [9] and Nossal et al. [10] demonstrated that interactions mediated through the antigen receptor on immature-stage B-cells in vitro resulted in the functional silencing of these cells on transfer into naive animals and rechallenge with antigen. The interpretation of these studies was that exposure
of antigen-reactive immature-stage B-cells to antigen leads to clonal inactivation through functional or physical elimination. Furthermore these studies documented the specific sensitivity of immature-stage B-cells (relative to mature) for inducible clonal inactivation and elimination. More recent studies in immunoglobulin transgenic animals have also shown functional (anergy) or physical (deletion) elimination of B-cells reactive to endogenous antigen when they are exposed to these antigens during the immature B-cell stage.

For some time we have been interested in the molecular regulation of different B-cell fates as a consequence of BCR signalling at different stages of development and activation. In particular we have focused our attention on understanding the molecular basis for the sensitivity of immature B-cells to tolerance induction. In this regard we have studied the cellular and molecular basis for the differential responsiveness of immature- and mature-stage B-cells to BCR cross-linking.

Our initial studies confirmed and extended previous studies [11] with anti-Ig antibodies to cross-link the BCR on isolated immature- and mature-stage B-cells from mice. We found that whereas anti-Ig induced the cross-linking of mature B-cells, causing them to be activated as determined by proliferation, immature-stage B-cells from either the neonatal mouse spleen or the adult mouse bone marrow failed to be activated by anti-Ig [12,13]. More interestingly, we have subsequently observed that the immature-stage B-cells do enter the G1 phase of the cell cycle [14] but are subject to an abortive activation response associated with induced cell death involving an apoptotic mechanism [13,14].

The use of polyclonal anti-Ig in our studies facilitates extensive cross-linking of the BCR, forming large aggregates of clustered antigen receptors. In this regard, these studies provide information relevant to differential responses of immature and mature B-cells as a consequence of relatively strong signals induced by the BCR. Our studies are probably most analogous to studies in vivo in which high-affinity receptors are engaged or when the BCR is aggregated by interaction with antigen expressed on the surface of a cell [15,16].

The observed differential sensitivity of the immature and transitional B-cell to tolerance induction could involve mechanisms extrinsic or intrinsic to the B-cell. Extrinsic factors could
include the unavailability of T-cell help in micro-environments inhabited by immature-stage B-cells. By a related mechanism, immature and transitional B-cells might be unable to receive T-cell-derived help signals necessary for their survival and subsequent activation. Alternatively, the microenvironment could play a determining role in the fate of the immature B-cell after BCR engagement. Cell-bound molecules such as Fas ligand in the bone marrow or in the periphery could eliminate Fas-positive immature and transitional B-cells. In this regard it is interesting that both mature- and immature-stage B-cells up-regulate Fas expression upon BCR cross-linking. Finally, immature B-cells might inducibly secrete negative autocrine factors, or alternatively fail to secrete survival cytokines, after antigen receptor engagement and cross-linking.

Our previously published studies [13] would seem to rule out extrinsic factors of the above kind as possible mechanisms for regulating the differential sensitivity of immature B-cells to BCR-induced negative selection. Anti-Ig-induced cross-linking of BCR on isolated and purified mature- and immature-stage B-cells shows that immature B-cells are induced to undergo apoptosis, whereas mature B-cells are induced to enter and proceed through the cell cycle. The fact that these differential responses proceed in the absence of demonstrable T-cells indicates that lack of T-cell help is not a determining factor. Furthermore, interleukin 4 [13,14] or soluble CD40 ligand (R. Sater and J. G. Monroe, unpublished work) protects immature and transitional B-cells from anti-Ig-induced apoptosis, indicating that these cells are responsive to some forms of T-cell help. Importantly, anti-Ig-induced apoptosis is seen in immature B-cells isolated from Fas-defective lpr mice (A. Norvell and J. G. Monroe, unpublished work), negating a role for Fas/Fas ligand in determining the outcome of the immature B-cell to BCR cross-linking. Finally, experiments in which BCR cross-linking was accomplished in populations containing a mixture of both immature and mature B-cells [13] argue against the notion that positive or negative autocrine factors play a role in fate determination in these responses.

The above studies have supported the idea that the differential responsiveness of immature and mature B-cells to BCR engagement must reflect a characteristic that is intrinsic to these cells. Among the intrinsic factors that we have considered and studied with regard to the regulation of the differential responsiveness of these two developmental populations are: differential expression of IgM and IgD, receptor sensitivity, and qualitative differences in BCR signal transduction.

Because immature-stage B-cells are characterized by a relative lack of IgD expression, the hypothesis has been put forward [17–19] that the differential effects of BCR engagement on mature and immature B-cells reflect the isotype engaged. By this hypothesis, IgM would be expected to generate a negative or tolerogenic signal, whereas IgD would be predicted to generate activation signals. In this model, the activating signals generated by IgD would be expected to be dominant over the tolerogenic signals of IgM on IgD⁺IgM⁻ mature B-cells. We tested this model by comparing the fate of co-expressing transitional immature B-cells and mature B-cells to anti-δ and anti-μ-mediated receptor engagement. Our studies [7] clearly showed that both IgM and IgD initiated the negative selection of transitional immature B-cells as determined by induced apoptosis, whereas either isotype generated only positive proliferative responses in mature B-cells. Our conclusion from these studies was that it is the developmental stage that determines the fate of the B-cell to BCR signalling rather than the particular isotype engaged. Most [20–22] but not all [18] immunoglobulin transgenic studies for tolerance and activation are consistent with this interpretation.

Studies by Parry et al. [23] have suggested that hyper-cross-linking of the BCR on mature-stage B-cells can induce apoptosis. This experiment suggested to us that a strong BCR-mediated signal might be interpreted by the B-cell as a signal for apoptosis and negative selection. Interesting in this regard is the observation that immature B-cells express relatively low levels of CD22 (R. J. Wechsler-Reya, A. L. Sillman and J. G. Monroe, unpublished work). Their expression of this surface molecule is roughly 10–20% that of mature B-cells. Given that studies by Doody et al. [24] have indicated that CD22, through its association with the protein tyrosine phosphatase SHP-1, can negatively affect the level of BCR signalling, we considered that altering the threshold of BCR cross-lining on mature B-cells by sequestration of CD22 away from the antigen receptor might alter the fate of receptor engagement. However, like the elegant studies from Doug Fearon’s laboratory, we observed an enhanced activation response by
these mature B-cells. This enhanced response was observed even at concentrations of anti-Ig that trigger apoptosis by the CD22\textsuperscript{low} immature and transitional B-cells. In confirmation of this response, there was no measurable increase in the frequency of apoptotic cells among the population of mature B-cells with sensitive antigen receptors. Despite this result, we still believe that the relative lack of CD22 expression is relevant to the biology of the immature B-cell. The enhanced inherent sensitivity of the BCR in the low-CD22-expressing cells would allow them to trigger signals at ligand–receptor interactions at much lower affinity than could be accomplished by mature-stage B-cells. Thus the threshold at which negative selection is accomplished would be lower than that necessary to trigger an activation response later in development of a particular clone. In this way, this process obviates the concern that antigen–receptor engagements that escape negative selection would not be able to trigger a positive activation response later on in development.

The studies described above lead one to the conclusion that the differential responses of immature and mature B-cells to BCR engagement is due to an intrinsic property of these two populations of B-cells. Therefore the mechanism regulating this intrinsic property must be developmentally associated. Moreover, because this differential responsiveness is observed between highly purified populations, representing these stages of B-cell development in response to direct stimulation through the BCR, we have postulated that biochemical differences in BCR signal transduction determine the developmentally associated differences in B-cell responsiveness inherent at the immature to mature B-cell transition.

In searching for the underlying molecular difference in receptor signalling in mature compared to immature B-cells, several observations have been made. One difference, noted first in immature B-cells from the spleens of neonatal mice, is that these cells are defective in their ability to hydrolyse phospholipids [phosphoinositol (PI) hydrolysis] in response to BCR cross-linking [12]. Interestingly, these cells still exhibited BCR-induced release of intracellular Ca\textsuperscript{2+} influx despite the lack of detectable inositol 1,4,5,-trisphosphate (IP\textsubscript{3}). However, in keeping with the lack of PI hydrolysis in BCR-stimulated immature B-cells, protein kinase C (PKC)-linked downstream events such as c-myc induction are also impaired at this stage of development [14]. Furthermore, unless PKC is activated pharmacologically, immature B-cells enter the early G\textsubscript{1} phase of the cell cycle but fail to express cyclin E, which is required for progression through late G\textsubscript{1} and into the S phase [14]. Recent experiments suggest that the apparent lack of PKC activation, coupled with the elevation of intracellular Ca\textsuperscript{2+}, is necessary and sufficient to trigger an apoptotic response by immature-stage B-cells. Moreover, apoptosis is induced by BCR cross-linking in mature B-cells depleted of diacylglycerol-regulated PKC isoforms. These results suggest that differential coupling to PI hydrolysis might be the central mechanism responsible for regulating the process of negative selection during early B-cell development. Proof of this hypothesis will await the ability to alter BCR signalling genetically at the mature and immature stages of development \textit{in vivo}.

In the search for membrane-proximal biochemical differences in the mature compared with the immature B-cell antigen receptor, which might be related to BCR uncoupling from PI hydrolysis or to the more generalized functional differences between these cells, the expression and activation of Src family kinases were assessed in primary immature and mature B-cells [25]. It was noted: first, that the Src-family tyrosine kinase Fgr is expressed by adult murine B-cells; and second, that expression of this kinase is developmentally regulated such that Fgr was present at very low levels, if at all, in immature B-cells. Wechsler and Monroe [25] showed a progressive increase in Fgr protein expression during B-cell development, correlated with the ability to respond positively to BCR cross-linking. The relationship of Fgr expression and activation to regulation of phospholipase C-regulated PI hydrolysis is currently under investigation.

In summary, antigen engagement with B-cells at the immature stage of development does not lead to an activation response like that observed for the similar stimulation of mature-stage B-cells. Rather, strong signals generated by the BCR cross-linking of immature B-cells leads to the induction of an apoptotic programme. We believe that \textit{in vivo} this response corresponds to the deletion of antigen-reactive cells in response to extensive receptor aggregation. Such a situation might be expected to occur for B-cells reactive to cell-bound antigens or those associated with an insoluble matrix. Extracellular matrix
proteins or membrane proteins such as MHC antigens as well as phospholipids, carbohydrates and nucleic acids could all fall into this category and therefore reactivities to these antigens could be negatively selected by this process.

Importantly, both immature-stage and transitional-immature B-cells respond identically in our studies. Furthermore both populations seem to be under the same biochemical regulation with respect to induced apoptosis, as described above. This characteristic predicts that negative selection of the type described here could operate on B-cells both in the bone marrow as well as in the periphery, before the transitional cells have transited into the mature B-cell stage. Our studies have also provided strong support for the conclusion that the apparent sensitivity of immature B-cells to negative selection is a consequence of factors intrinsic to these cells. Our biochemical studies are consistent with this conclusion and have made the first steps towards a molecular explanation of the mechanism regulating the differential sensitivity of immature and mature B-cells to negative selection. Finally, our studies have not specifically addressed the consequence of lower-avidity antigen interactions with immature-stage B-cells nor the mechanisms regulating peripheral tolerance induction in mature B-cells. Our feeling at this point is that these processes are unrelated to the developmentally regulated processes discussed here. For these latter phenomena, extrinsic processes are likely to be important.

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