Transitional B Cells Are the Target of Negative Selection in the B Cell Compartment

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Summary

B lymphocytes recognize antigen through membrane-bound antigen-receptors, membrane IgM and IgD (mIgM and mIgD). Binding to foreign antigens initiates a cascade of biochemical events that lead to activation and differentiation. In contrast, binding to self-antigens leads to death or to inactivation. It is commonly believed that the B cells acquire the ability to discriminate between self and nonself in the early phases of development. We report here that immature B cells, which have just emerged from the mIgM\textsuperscript{neg} pool, are not deleted upon binding of self-antigen. In vivo, developing B cells become sensitive to tolerance induction in a relatively late window of differentiation, when they are in transition from the immature (HSA\textsuperscript{bright}, B220\textsuperscript{null}) to the mature (HSA\textsuperscript{null}, B220\textsuperscript{bright}) stage. In the transitional B cells, early markers of differentiation such as Pgpl (CD44) and ThB reach the highest level of expression, while the expression of CD23 and mlgD, late markers of differentiation, and expression of class II MHC, progressively increases. Most of the transitional B cells, but only few of the mature and of the immature B cells, express the fas antigen, while mature B cells, but not immature and transitional B cells, express bcl-2 protein. mlgM is present in low amounts in immature B cells, reaches the highest level of expression in transitional B cells and is down-regulated in mature resting B cells, where it is coexpressed with mlgD. The high expression of mlgM, the presence of the fas antigen and the absence of bcl-2 protein is compatible with the high sensitivity of transitional B cells to negative selection. In vitro, immature B cells die rapidly by apoptosis after cross-linking of mlgM. This result, combined with the resistance of immature B cells to elimination in vivo, suggests that early in development the stroma cell microenvironment modulates signals transduced through mlgM. The functional and phenotypic division of IgM\textsuperscript{pos} bone marrow B cells in three compartments not only allows to define the target population of physiological processes like negative selection, but will also be a helpful tool for an accurate description of possible developmental blocks in mutant mice.

Throughout life, lymphocyte cells of the B lineage are generated and develop in the bone marrow, where they can be found in all maturation stages. They can be discriminated on the basis of their phenotype and of their functional characteristics (1, 2).

The first major population that can be identified corresponds to the pro-B lymphocytes. They are large proliferating cells that are characterized by a low expression of B220, the B cell-specific splice variant of the surface marker CD45 and by a high expression of the surface marker CD24 (heat-stable antigen [HSA]). These cells start to rearrange their Ig heavy chain (HC) genes. After a productive rearrangement, they can express μ chains in the cytoplasm and are called pre-B cells.

Pre-B cells do not proliferate and are smaller in size. Recombination stops at the HC locus and starts at the light chain locus (3, 4). With the successful rearrangement of the light chain locus, a complete IgM molecule can be produced and expressed on the cell surface. Membrane (m)IgM\textsuperscript{pos}, HSA\textsuperscript{bright}, B220\textsuperscript{null} cells are considered immature B lymphocytes. Further differentiation leads to down-regulation of HSA and to up-regulation of B220. B cells now become independent of the stromal cells and migrate towards the sinusoids to leave the bone marrow (5, 6).

Before and during HC rearrangement, under the influence of stromal cells and their factors, the pool size of the B lineage increases (1). Later, progression along the developmental pathway is guided by the presence of Ig molecules (7).

At two differentiation steps after rearrangement, extensive cell loss occurs. 70% of the pre-B cells that are produced every day in the bone marrow die by apoptosis (8–10). The survivors are the immature B cells. Only 10–15% of them reaches the pool of mature B cells in the spleen (11, 12). Cell death could either eliminate the cells at random or specifically. Similarly to B cells, T cells construct their antigen receptor
through the rearrangement of different DNA segments. The mature T-cell repertoire is shaped by positive and negative selection in the thymus (13). Likewise, the emerging repertoire of the B lineage differs from the mature repertoire (14-16). The extensive loss of B cells belonging to defined developmental stages in the bone marrow, combined with the selective enrichment for certain VH regions in the periphery, suggests that also B lymphocytes are subject to selection processes.

Tolerance induction (negative selection) in the B cell compartment has been shown in normal mice many years ago (17) and was confirmed recently in transgenic mouse models (reviewed in references 18 and 19). A widely accepted notion describes tolerance to self-antigen as a property that the immune system acquires early in development. In the neonatal animal, all B cells are immature and are considered sensitive to tolerance induction, while in the adult, only the immature B cells in the bone marrow are considered the target of negative selection (17). We have reported (20) that B cells of mice transgenic for IgM (μ and κ transgenes) against the hapten trinitrophenyl (TNP) undergo apoptosis upon administration of TNP conjugates. If mlgD is coexpressed with mlgM (in mice carrying μ, κ, and δ transgenes), deletion is limited to a few cells that have a high density of mlgM. We concluded that in the absence of T cell help, the extent of mlgM cross-linking determines whether B cells are deleted when they encounter antigen. The engagement of mlgD together with mlgM protects the B cells from a rapid death. In normal mice, mature resting B cells coexpress mlgM with an average of 3-10-fold excess of mlgD (21). This phenotype guarantees that B cells can bind antigen with high avidity and can be recruited in the immune response. The phenotype of immature B cells (mlgM<sup>high</sup>, mlgD<sup>low</sup>) favors their elimination upon antigen binding.

Little is known about the molecular mechanisms involved in the execution of programmed cell death. Apoptosis can be induced, among others, by members of the TNF-receptor family. A prominent representative of this family is the fas/APO-1 antigen (reviewed in 22). A major role to prevent apoptosis has been ascribed to members of the bcl-2 family (23). Bcl-2 can, for instance, protect a cell from fas-induced cell death (24, 25), it cannot, however, prevent negative selection in the B and T cell compartments (26-28).

In this study, we characterize the population of B cells that can be negatively selected upon cross-linking of their surface-antigen receptor in transgenic and normal mice. In contrast to previous observations (17), this population does not coincide with the B cells that appear very early in ontogeny and development. Engagement of mlgM causes apoptotic cell death only when B cells are in transition from the immature to the mature stage. On most of these cells, the fas antigen is expressed, while the bcl-2 protein is absent.

### Materials and Methods

**Mice.** Mice were obtained from our breeding facility. Homozygous adult (7-10 wk old) mice of the SP6 μ,κ transgenic mouse line (29, 30) on the BALB/c background, and normal BALB/c, C57BL/6 and (BALB/c x C57BL/6) F1 mice were used for most experiments. Newborn BALB/c mice from the same litter were analyzed in pools of two to three pups.

**Tolerogens.** TNP was coupled to amino-dextran (mol wt = 70,000; Molecular Probes, Inc., Eugene, OR) as described (20). The degree of substitution was between 17 and 23. TNP-dextran (800 μg in 100 μl PBS) was injected i.v. in the tail veins of adult mice 24 h before analysis. As control FITC-dextran (mol wt = 70,000, degree of substitution = 15-20; Molecular Probes) or PBS was used, with identical results (see also reference 20). Rat monoclonal anti-IgM (clone 2911) and anti-IgE (clone 37.1949, a gift of Dr. I. Aarden, CLB, Amsterdam, The Netherlands), both of the γ1 subclass, were administered in 100 μl (500 μg/ml PBS) i.p. to newborn mice.

**Flow Cytometric Analysis and Cell Sorting.** Flow cytometric analysis was performed as described before (20). Lymphocytes were identified on the basis of small angle vs orthogonal light scatter characteristics. Data were collected from 2-3 x 10<sup>6</sup> cells on a FACScan® flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Four-color fluorescence analysis and cell sorting was carried out on a FACStar® Plus flow cytometer. Data are represented as 5% equal-probability double-contour plots, as dot plots, or as histograms. Measurement of fluorescence was on a logarithmic scale. In selected experiments, propidium iodide was used for live-dead cell discrimination. For staining of intracellular proteins, cells were permeabilized with saponin after staining for extracellular determinants. Briefly, antibodies were diluted in FACS® buffer (PBS, 0.1% azide, 3% dialyzed FCS) containing 0.03% saponin. Cells were washed extensively in FACS® buffer containing 0.01% saponin. DNA staining was performed as described (31).

The following monoclonal antibodies (species; specificity) were used: M1/69 (rat; HSA/CD24), RA3-6B2 (rat; B220/CD45), X36 (rat; kappa [Becton Dickinson]), 2911 (rat; third constant domain of the μ chain), B3B4 (rat; CD22), KM81 (rat; Fgp1/CD44), 49h4 (rat; Th1, a gift of Dr. L. Eckhardt, CUNY, New York), 11-26c (rat; δ chain, a gift of Dr. J. Kearney, University of Alabama at Birmingham, Birmingham, AL), 3F11 (hamster, bel-2, a gift of Dr. S. Korsmeyer, Washington University School of Medicine, St. Louis, MO). These antibodies were in our collection or obtained from the American Type Culture Collection (Rockville, MD). Antibodies were coupled to amino-hexanoyl-biotin-N-hydroxysuccinimide ester (long-arm biotin; Molecular Probes) or to FITC (Molecular Probes) as described (30). Monoclonal hamster anti-fas antigen, coupled to PE, was bought from Pharmingen (San Diego, CA). Goat anti-mouse IgM coupled to PE and to Cy5 were purchased from Medac (Hamburg, Germany) and from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively. A rat monoclonal anti-hamster cocktail labeled with biotin was obtained from PharMingen (San Diego, CA). Goat anti-mouse IgM coupled to PE and to Cy5 were purchased from Medac (Hamburg, Germany) and from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). A rat monoclonal anti-hamster cocktail labeled with biotin was obtained from Pharmingen. RA3-6B2 labeled with PE, and with the tandem conjugates PE-Texas red (Red613) or PE-Cy5 (Red670) were obtained from GIBCO BRL (Gaithersburg, MD). Biotin-labeled antibodies were revealed with streptavidin-PE (Southern Biotechnology Associates, Birmingham, AL), with streptavidin-Cy5 (GIBCO BRL), or with streptavidin-Cy5 (Jackson ImmunoResearch Laboratories).

**In Vitro Cultures.** Bone marrow cells of adult mice and splenic cells of neonatal mice were depleted of erythrocytes by isotonic lysis (20). The cells were then cultured at 10<sup>4</sup> cells/ml in Iscove's medium supplemented with a selected nonmitogenic FCS (10%) in 24-well plates (Costar Corp., Cambridge, MA) with the indicated substances for the indicated time.

### Results

**Immature B Cells are Resistant to Negative Selection.** Based on the effects observed after treatment of mice which carried
either μ and κ (Sp6 μ,κ mice) or μ,κ and δ transgenes (Sp6 μ,κ,δ mice) with specificity for the hapten TNP, with TNP conjugates, we previously concluded that engagement of mlgM on mature B lymphocytes is an apoptotic signal, unless other signals (e.g., engagement of mlgD) interfere with this default pathway (20). Immature B cells in the bone marrow were resistant to deletion both in μ,κ and in μ,κ,δ mice (20). This is surprising because immature B cells in the marrow are supposed to be the target of tolerance induction (17). Since immature B cells were resistant to deletion when they coexpressed mlgM and mlgD, but also when they only expressed mlgM, the type of antigen receptor does not play a role in this stage of differentiation. We therefore investigated in more detail the results of cross-linking of mlgM in immature B cells of both transgenic and normal mice.

Bone marrow mlgMpos B cells are usually divided in two populations: immature B cells, which are dull for B220, and mature B cells, which are bright for B220. Immature B cells (Fig. 1 a, left arrow) derive from mlgMneg, B220pos precursors, while the B220bright, mlgMpos cells (Fig. 1 a, right arrow) are mature, recirculating B lymphocytes (1). Although based on these two markers this classification is justified, the functional analysis discussed below is more compatible with a division in three populations. In Fig. 1, a and b, we call immature B cells only those in gate G1 (Fig. 1, a and b). They express low amounts of mlgM and are dull for B220. In the most mature B cells (gate G2 in Fig. 1, a and b), B220 expression is high. mlgMbright B cells are represented as a separate population (in gate G3). They are either dull (more immature) or bright (more mature) for B220, as is indicated by the vertical arrows. We based this seemingly arbitrary division on the effects observed after treatment with the antigen TNP-dextran. 77% of the cells in gate G2 (mature cells) were deleted after treatment with TNP-dextran in vivo (Fig. 1 a [sham treatment] vs b [TNP-dextran treatment]). These cells express CD23, the low-affinity receptor for IgE, a marker that normally appears on the surface of more mature B cells at the same time as IgD (Fig. 1, c and d). The immature B cell pool remained intact after treatment (94% survival, compare the cells contained in gate G1 a and b). We have shown that in mature B cells, the density of mlgM and the extent of mlgM cross-linking determines whether B cells are deleted (20). Since the amount of mlgM on most of the immature cells was similar to the amount of mlgM on the more mature population (compare the staining for IgM in gates G1 and G2 of Fig. 1 a), we tentatively concluded that resistance to negative selection is an intrinsic property of the more immature bone marrow B cells. mlgMbright B cells, contained in gate G3 of Fig. 1, a and b, were deleted after TNP-dextran treatment independently of their staining profile for B220: 70% of the more immature (B220pos) and 88% of the more mature (B220bright) cells were eliminated (Fig. 1 e). In absolute numbers (Fig. 1 f), roughly 4 times more cells were deleted from the mlgMbright population than from the immature B cell pool.

We conclude that of the three populations of B lymphoid cells that we have defined in the bone marrow of Sp6 μ,κ mice, the most immature population (in gate G1) is resistant to negative selection and that the two more mature cell types (in gates G2 and G3) are about equally sensitive.

**Transitional B Cells of Normal Mice.** B cell populations equivalent to those described in Fig. 1 in transgenic mice can be identified in the bone marrow of normal mice (Fig. 2 a). Here the mature cells, which are found in gate G2, express mlgM and mlgD. We have shown that cells with this phenotype are protected from deletion (20). The physiological target of negative selection could therefore coincide with the mlgMbright population (in gate G3). In this section we describe this population in normal mice.

In the bone marrow of normal mice, mlgMbright cells (see Fig. 2 a, in gate G3, red) represent 12–20% of all mlgMpos cells (contained in gates G1, G2, and G3). By using HSA as an additional marker, we have located the mlgMbright population along the B cell differentiation pathway. In Fig. 2 b, the staining profile for HSA and B220 of whole bone marrow is shown, while in panel c, only the mlgMbright cells, contained...
Figure 2. Transitional B cells. Bone marrow cells from adult BALB/c mice were analysed by multiparameter flow cytometry. (a) Cells were stained with anti-B220 and anti-IgM Abs, and divided in three populations contained in gates G1, G2, and G3. G1: IgM<sub>neg</sub>, B220<sub>dim</sub> immature B cells, dark blue; G2: IgM<sub>neg</sub>, B220<sub>bright</sub> mature B cells, green; G3: IgM<sub>bright</sub> transitional B cells, red. (b and c) The use of HSA as additional marker allows a better definition of IgM<sub>bright</sub> B cells. (c) Staining pattern for B220 and HSA of IgM<sub>bright</sub> cells. For comparison, all bone marrow cells are shown in panel b. (d-h) Expression pattern of other differentiation markers. The gates in panels d-h show the localization of transitional B cells (defined as IgM<sub>bright</sub>, HSA<sub>bright</sub>, B220<sub>dim</sub> in respect to the whole bone marrow. The color code is the same as above, other bone marrow cells (B220<sub>dim</sub> and B220<sub>neg</sub>) are shown in light blue.
cells is given. The percentages of cells in the respective gates are represented beside the gates, considering the total number of B220<sup>pos</sup> cells (a-c and d-f) or of kappa<sup>pos</sup> cells (d-f and j-l) as 100%. During the first 2 wk of life, the number of cells in the spleen increases 10-fold and in the bone marrow 4-fold.

**Figure 3.** Development of transitional B cells in the newborn mouse. Bone marrow and spleen cells of BALB/c mice of the indicated age (in days after birth) were stained with anti-B220, anti-HSA, and anti-kappa Abs for flow-cytometric analysis. Staining patterns for B220 and HSA are depicted in a-c for bone marrow cells and in g-i for spleen cells. In d-f (bone marrow) and j-l (spleen), the staining pattern for B220 and HSA of kappa<sup>pos</sup> cells is shown. In a-c and g-i, the percentage of B220<sup>pos</sup> cells is given; in d-f and j-l, the fraction of kappa<sup>pos</sup> cells of B lineage (B220<sup>pos</sup>) cells is given. The percentages of cells in the respective gates are represented beside the gates, considering the total number of B220<sup>pos</sup> cells (a-c and d-f) or of kappa<sup>pos</sup> cells (d-f and j-l) as 100%. During the first 2 wk of life, the number of cells in the spleen increases 10-fold and in the bone marrow 4-fold.

We labeled the cells with anti-HSA and anti-B220 Abs and identified mlg<sup>pos</sup> cells by staining with anti-kappa Abs. Staining with anti-IgM Abs gave identical results (not shown). In the bone marrow on day 2 after birth, 15% of the cells in the lymphocyte gate are B220<sup>pos</sup> and have an immature phenotype (HSA<sup>bright</sup>; B220<sup>dim</sup>; Fig. 3 a). Only 10% of these cells express mlg (Fig. 3 d, showing staining profiles for HSA and B220 of kappa<sup>pos</sup> cells). The percentage of B220<sup>pos</sup> cells rapidly increases and reaches 66% on day 14 (Fig. 3 c). At this time, some of the kappa<sup>pos</sup> cells have down-regulated HSA expression and start to up-regulate B220 (Fig. 3 f). Mature, IgD<sup>pos</sup> transitional B cells (Fig. 3). We labeled the cells with anti-HSA and anti-B220 Abs and identified mlg<sup>pos</sup> cells by staining with anti-kappa Abs. Staining with anti-IgM Abs gave identical results (not shown). In the bone marrow on day 2 after birth, 15% of the cells in the lymphocyte gate are B220<sup>pos</sup> and have an immature phenotype (HSA<sup>bright</sup>; B220<sup>dim</sup>; Fig. 3 a). Only 10% of these cells express mlg (Fig. 3 d, showing staining profiles for HSA and B220 of kappa<sup>pos</sup> cells). The percentage of B220<sup>pos</sup> cells rapidly increases and reaches 66% on day 14 (Fig. 3 c). At this time, some of the kappa<sup>pos</sup> cells have down-regulated HSA expression and start to up-regulate B220 (Fig. 3 f). Mature, IgD<sup>pos</sup> cells are still rare (not shown). In the spleen B cells are in more advanced stages of differentiation (Fig. 3, g-i). In particular, transitional B cells are easily recognized on day 2 (Fig. 3 f). The percentage of B220<sup>pos</sup> cells, low on day 2 (15% of lymphoid cells), reaches adult levels (59%) at 2 wk of age, when most of the B cells are, however, still immature (HSA<sup>bright</sup>; B220<sup>dim</sup>). About half of the cells express now IgD<sup>pos</sup> (not shown). Fig. 5 a shows that IgM<sup>bright</sup> splenic B lymphocytes of neonatal mice resemble the transitional B cells found in the bone marrow of adult mice. They are clustered at the passage from the immature (HSA<sup>bright</sup>; B220<sup>dim</sup>) to the mature (HSA<sup>dim</sup>; B220<sup>bright</sup>) B cell stage (Fig. 5 c).

Both the phenotype and the time point of first appearance suggest that transitional B cells represent a relatively late stage of differentiation. They derive from the immature B cells and proceed to become mature B cells.

**In the Adult, Bone Marrow Transitional B Cells Derive from HSA<sup>neg</sup>, B220<sup>dim</sup>, IgM<sup<neg</sup> Precursors.** To demonstrate that also in the adult bone marrow transitional B cells derive from B220<sup>pos</sup>, IgM<sup>neg</sup> precursors, we have followed the development of B220<sup>pos</sup>, IgM<sup>Neg</sup> bone marrow cells in vitro (Fig. 4). Bone marrow from adult BALB/c mice was depleted of mlgM<sup>pos</sup> B cells by cell sorting. Staining profiles for mlgM and mlgD before and after depletion are shown in Fig. 4, e and f, and i and j, respectively. The staining profile for HSA and B220 of IgM<sup>pos</sup> cells is shown in Fig. 4, a and h Immature B cells, using the definition introduced in Figs. 1 and 2, are depicted in blue, transitional B cells in red, and mature B cells in green. After 1 d in culture, 24% of the cells had acquired mlgM (Fig. 4 g), but most of them were still in the immature B cell gate (Fig. 4 c). After 36 h, 39% of the cells expressed mlgM (Fig. 4 h). 15% of the B cells were in transition from the immature to the mature stage (Fig. 4 d). The expression of modest amounts of mlgD after in vitro culture (Fig. 4, j-l) was in agreement with the progression from the immature to the transitional B cell stage. In vitro culture systems support differentiation up to the mlgM<sup>pos</sup> stage, but are insufficient to support differentiation into mature cells (32). These observations suggest that the final B cell maturation step is dependent on factors or interactions found in vivo, but not in vitro cultures. We conclude that also in adult bone marrow, pre-B cells evolve to become immature B cells and subsequently transitional B cells.

**Transitional B Cells Are the Target of Negative Selection in Normal Mice.** Antigen-specific negative selection is difficult to study in normal mice because the frequency of target cells is very low. Therefore, anti-IgM Abs have been used as a surrogate universal antigen/tolerogen for B cells. We verified whether also in normal mice transitional B cells are the target of negative selection by treating newborn mice with a rat mAb to IgM. We reasoned that the binding of a mAb to IgM more closely resembled the interaction of an antigen with its specific binding site than the "super" cross-linking achieved by polyclonal Abs, and that in very young mice, interference with circulating IgM and with mature B cells could be avoided. The time of injection had to coincide with...
the first appearance of transitional B cells because we wanted to compare directly immature and transitional B cells.

A single i.p. injection of 50 µg of either anti-IgM (mAb 2911) or of an isotype control mAb (37.1949, anti-mouse IgE) was given to 1-d old BALB/c mice. 1 d later, spleen cells were prepared, counted, and analyzed by flow cytometry (Fig. 5). In the spleen, 98% of mlg<sup>bright</sup> cells were deleted after anti-IgM treatment (compare the populations contained in gate G3 of Fig. 5 a [anti-IgE treatment] with those of Fig. 5 b [anti-IgM treatment]). This is reflected in Fig. 5, c and d, showing the staining profiles for HSA and B220 of the mlg<sup>bright</sup> cells. The cells that were deleted after treatment with anti-IgM, stained very brightly for Pgpl, confirming that transitional B cells are the target of negative selection (Fig. 5 e). The mlg<sup>dull</sup> B cells (in gate G1 of Fig. 5, a and b) were largely spared from deletion. The disappearance of mlg<sup>bright</sup> B cells could not be explained by modulation of mlgM. First, the loss of mlg<sup>bright</sup> B cells was not compensated by a proportional increase of mlg<sup>dull</sup> or mlg<sup>−</sup> B220<sup>pos</sup> cells. Indeed, after anti-IgM treatment, the percentage of B220<sup>pos</sup> cells was reduced in the spleen (compare histograms in Fig. 5 f). Second, signs of apoptosis were evident in the spleen of mice injected with anti-IgM, but not in the spleen of mice treated with anti-IgE, as revealed by cell cycle analysis. Upon anti-IgM treatment, a new peak appeared in the DNA profile to the left of the G1 peak (Fig. 5 h, APO). These cells that have less than 2N DNA, are undergoing apoptosis (31). The number of the apoptotic cells does not perfectly correspond to the extent of deletion. The cells that underwent apoptosis first (~6 h after contact with anti-IgM) are completely lysed at the time point of analysis and appear as cell debris outside the lymphocyte gate (not shown). Other physical signs of apoptosis were also seen:
blebbing of the nucleus and ruffling of the cell membrane (not shown).

**Transitional B Cells Express the fas Antigen, but Not the bcl-2 Protein.** Since the presence of the fas antigen marks the cells that are susceptible to the induction of apoptosis by the fas ligand (22), we examined the expression of the fas antigen on B cells in the bone marrow. B220<sup>−</sup> cells were divided into pro- and pre-B cells, immature, transitional, and mature B cells (Fig. 6a). Fig. 6c shows that most of the transitional, but few of the immature or mature B cells, express the fas antigen. We further examined the cells for the presence of the bcl-2 protein, which can protect cells from apoptosis. Mature, but not immature or transitional B cells, stain positively for bcl-2 protein (Fig. 6b).

We conclude that in transitional B cells, the presence of the fas antigen and the absence of the bcl-2 protein correlates with their susceptibility for negative selection.

**In Vitro, Immature B Cells Are Not Resistant to Negative Selection.** We have shown that B cells become sensitive to tolerance induction relatively late in development, while immature B cells are surprisingly resistant to deletion. Resistance and sensitivity to deletion could be intrinsic characteristics of immature and transitional B cells, respectively. It is, however, also possible that the contact to stroma cells protects the immature B cells from deletion, but not the more mature B cells, that have left their nursing micro environment (1, 5, 6). Furthermore, the assessment of susceptibility for tolerance induction in previous studies was routinely carried out in in vitro systems (17).

To address these questions, we have repeated some of the experiments in vitro. In this approach, the intimate contact between immature B cells and stroma cells is disrupted. Bone marrow cells of adult Sp6 μ,κ mice were cultured in normal medium or in the presence of TNP-dextran. After 24 h in culture, the cells were counted, stained with anti-IgM, anti-HSA, and anti-B220 Abs and analysed by flow cytometry. About 80% of the transitional B cells and 70% of the mature B cells were deleted (see Fig. 7a, white columns). Immature B cells, which were not affected by the challenge with TNP-dextran in vivo, were deleted in vitro. We found 60% deletion in vitro vs 6% deletion in vivo.

We also treated cells of normal mice in vitro with the mAb 2911. In vitro, we could study the effect of mlgM cross-linking on B cells from neonatal spleen and adult bone marrow. The different B cell maturation stages could not be identified using mlgM as a marker because in vitro, in contrast to in vivo, mlgM is modulated upon cross-linking with anti-IgM Abs (not shown). Staining with anti-Pgp1 and anti-B220 Abs, however, allowed a discrimination between transitional and mature B cells. 70–80% of the transitional and mature B cells were eliminated in normal mice (Fig. 7b; the white columns correspond to neonatal splenic B cells and the filled columns to adult bone marrow B cells). The compartment of the immature cells was reduced by 30–40%. This limited cell loss does not reflect a particular resistance to negative signals, and can easily be explained considering that only 30–40% of the B220<sup>−</sup>, Pgp1<sup>−</sup> pool expresses mlgM, as we could calculate from the staining of control cells.

We conclude that the resistance to deletion demonstrated in vivo of the pool of the immature B cells can not be confirmed in vitro. In the hemopoietic organs, most probably the local microenvironment plays a fundamental role in modulating signals conveyed through the B cell receptor (BCR). The notion that the immature B cell is the target for negative selection, derives from in vitro experiments. We have shown that these experiments can not be interpreted as representative of the in vivo situation.

**Discussion**

In this paper, we have shown that within the B cell compartment, cross-linking of the membrane-bound BCR causes negative selection when B cells are in a defined and narrow window of differentiation that is situated between the immature and the mature stages. We have called B cells in this window of differentiation transitional B cells. In vivo, both immature B cells and mature B cells (20) are protected from negative selection.

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**Figure 6.** Expression patterns of the fas antigen and of bcl-2 protein in bone marrow cells. Bone marrow cells were stained for mlgM, B220, and fas antigen or intracellular bcl-2 protein, respectively. (a) The staining profile for mlgM and B220 is shown and the division in compartments based on this staining pattern. (b) Staining for bcl-2 of the cells in the different compartments is shown; (c) staining profiles for the fas antigen are depicted. Staining patterns represented in c were obtained from a different mouse than used for the generation of a and b.
mental stages were identified on the basis of Pgp1 and B220 expression.

Cells from four equally treated wells were stained with anti-HSA, anti-B220, and anti-IgM Abs for flow cytometric analysis and the absolute number of cells in the immature, mature, and transitional B cell pools was calculated. Results are given as percentage of surviving cells, with the number obtained from control wells set at 100. Similar results were obtained at other time points (36 and 60 h, not shown). (b) Splenic cells of 20 normal (BALB/c) newborn mice or bone marrow cells of 2 normal adult mice (7 wk old) were cultured in medium or in the presence of anti-IgM (mAb 291-1, 1 µg/ml) at 10^6 cells/ml. After 24 h, pooled cells from four identically treated wells were counted and stained for flow cytometric analysis. The different developmental stages were identified on the basis of Pgp1 and B220 expression (see Fig. 2). On control cells (cultured with medium only), IgM was used as additional marker.

Identification of Transitional B Cells. After antigen administration, not only the mature B cell population, but also another group of cells was deleted in the bone marrow of Sp6 mice. These cells stain brightly for IgM and, based on the expression of HSA and B220, appear to be in transition from the immature to the mature B cell pool. We studied the expression of other differentiation markers in order to identify this population more precisely (Fig. 2). Both Pgp1 and ThB, which appear on the cell surface early in development, are expressed at the maximum level in transitional B cells. In contrast, the expression of MHC class II and CD23 gradually increases in this population without reaching the levels found on mature B cells. mIgM reaches the highest levels of expression in transitional B cells and it is down regulated in peripheral B cells. In normal, but not in μ, κ transgenic mice, mIgD, which is present in excess over mIgM in mature B cells, just starts to appear on the surface of transitional B cells. For us, the most intriguing feature of these cells is that in normal mice they express high amounts of mlgM and very little if any mlgD. This phenotype corresponds perfectly to the phenotype of the cells which, as we showed (20), are maximally sensitive to negative selection. Transitional B cells represent an intermediate stage of differentiation between the immature and the mature population, and they form ~12-20% of the B220^hi cells in the adult bone marrow. Accordingly, transitional B cells are found after

B Cell Tolerance: Old Models and New Facts. Although antibodies with auto-specificity are produced by rearrangement of Ig genes, the mature B cell pool normally reacts to foreign antigens, but is tolerant towards itself. A widely accepted notion states that tolerance is induced early in development, at the transition from the pre-B to the immature B cell stage (17), and that resistance to tolerance induction progressively increases during differentiation to the mature stage (17). Antigens encountered early in development would therefore be recognized as self and cause deletion of immature B cells if present in large amounts, or cause anergy if present in low concentration (17). This theory postulates that negative signals are transmitted through mlgM early in development, but positive signals are transmitted when B cells reach the mature stage (17). Recent studies, however, have suggested that regulation of life and death in B lymphocytes is not this simple.

Thanks to transgenic mice, it has been confirmed that the contact with self-antigens leads to clonal deletion (18-20) of autoreactive B cells. Anergy has been considered a major mechanism for the maintenance of tolerance in the B cell compartment (17, 18). Anergic B cells, however, have such a reduced life-span that anergy can be considered a prerequisite for elimination (33).

In disagreement with the commonly accepted theory, transgenic mouse models have shown that also mature B cells can be easily tolerized (reviewed in references 18 and 19), even when they first encounter antigen in the periphery. We have recently shown that in vivo mature B cells undergo apoptosis upon contact with a multivalent antigen if they express only mlgM, but not if they express mlgM and mlgD (20).

Surprisingly, immature B cells from the bone marrow, which are supposed to be the target of tolerance induction (17), are rather insensitive to deletion (20, 28) upon contact with self-antigens in vivo, independent of whether they express mlgM and mlgD (in μ, κ, δ mice) or only mlgM (in μ, κ mice).

In this study, we have identified the primary target of negative selection in the bone marrow of normal mice. We reasoned that, since most of the immature B cells are resistant to negative selection and mature B cells can be deleted only when they express mlgM, but not mlgD, the physiological target of negative selection should be a cell type that is relatively mature but still lacks mlgD.
36 h of in vitro culture of sorted mIgM{{\textsuperscript{neg}}}, B220{{\textsuperscript{null}}}, and immature B220{{\textsuperscript{null}}} cells from the bone marrow (Fig. 4). After 24 h of culture, only immature cells were seen. Furthermore, transitional B cells are rare in the bone marrow of newborn mice before 6 d of age. They are, however, already evident in the spleen 2 d after birth (Fig. 3).

Transitional, but not immature, B cells are the target of negative selection. Upon injection of anti-IgM Abs, transitional B cells completely disappear from the spleen of normal neonatal mice, while most of their more immature HSA{{\textsuperscript{bright}}} B220{{\textsuperscript{null}}}, IgM{{\textsuperscript{low}}} precursors survive (Fig. 5). DNA staining of B220{{\textsuperscript{positive}}} splenocytes of neonatal mice demonstrated that cross-linking of mIgM had initiated a signaling pathway leading to programmed cell death (Fig. 5). Our findings confirm that also in normal mice, IgM{{\textsuperscript{bright}}} transitional B cells are the target of negative selection, while immature B cells can not be deleted. This conclusion contrasts with current models of negative selection, suggesting that immature B cells to transduce mIgM-derived signals. Cross-linking of mIgM normally results in the rapid phosphorylation of tyrosine residues of different substrates, among which is phospholipase C-\gamma. Activated phospholipase C-\gamma initiates a signaling pathway leading to Ca\textsuperscript{2+} influxes and to protein kinase C activation (34, 35). We compared Ca\textsuperscript{2+} influxes induced by anti-IgM or by antigen in immature and in mature B cells of normal and of Sp6 \(\mu.K\) mice. Cross-linking of mIgM Abs induced Ca\textsuperscript{2+} influxes both in immature and mature B cells. These unpublished observations (Carsetti, R.) do not exclude that the still largely unknown late events of activation may differ in the two differentiation stages. They, however, certainly exclude that immature B cells have a major defect in coupling of the mBCR to its "immediate" (tyrosine kinases) and "early" (Ca\textsuperscript{2+}) second messengers.

Most of the data reporting about the extreme sensitivity of immature B cells from the bone marrow to negative selection derive from experiments done in vitro. This experimental design could be the cause for the discrepancy with our results. Indeed, in vitro, all B cells populations are deleted to the same extent after cross-linking of mIgM with antigen (in Sp6 \(\mu.K\) mice) or with anti-IgM Abs (Fig. 7). In contrast, deletion of immature B cells was not observed in vivo, even at concentrations in four- to fivefold excess over the amount of ligand given in vitro. The results could be explained by a constant feeding from the pre-B precursor pool into the immature B cell pool in vivo, but not in vitro. However, also in vitro B220{{\textsuperscript{null}}}, mIgM{{\textsuperscript{null}}} cells mature into mIgM{{\textsuperscript{null}}} cells (Fig. 4, and Lamers, M. C., unpublished observations). This suggests that mIgM transmits an apoptotic signal also in immature B cells. Stromal micro-environment in the early phases of development most likely modulate the effects of mIgM cross-linking.

We have shown that negative selection can happen in the B cell compartment in a defined window of differentiation that is situated between the immature and the mature stage. We have called B cells in this window transitional B cells. In this population, the presence of high amounts of mIgM in absence of mIgD results in the transmission of signals that, by default, lead to apoptosis and cell death.

The expression pattern of two proteins that are directly involved in the induction or prevention of apoptosis, the fas antigen and bcl-2, respectively, support this notion. Engagement of the fas antigen, a member of the TNF-cytokine receptor family, by its ligand can induce apoptosis in the target cell (22). The expression of the bcl-2 protein, on the other hand, is correlated with an albeit not absolute protection from apoptosis (26–28) and can prevent fas-mediated apoptosis (24, 25). Transitional B cells are largely positive for the fas antigen, but negative for the bcl-2 protein (Fig. 6). This phenotype would make them exquisitely susceptible for deletion. Immature B cells express neither the fas antigen, nor the bcl-2 protein, while mature B cells express only the bcl-2 protein. Mature B cells are not immune to deletion if only mIgM is engaged by antigen, as we have shown (20), and even the presence of a constitutively expressed bcl-2 transgene does not protect from negative selection (28). In this context, the model that was recently proposed by Oltvai and Korsmeyer (23) is of interest. It postulates that an apoptotic signal can be modulated at multiple checkpoints, before it is executed. This nicely explains the phenomena that occur during selective processes. In wild-type animals, mature B cells are relatively resistant to apoptosis induced by cross-linking of mIgM. First, the bcl-2 protein postpones the execution of programmed cell death. Second, other signals, for instance those generated by mIgD, by accessory molecules such as CD40 and by T cell-derived cytokines can modulate the signals conveyed through mIgM in a longer time frame. In vivo, immature B cells could be protected by the stroma cell environment and the expression of other members of the bcl-2 family, like bcl-x, that has similar properties as bcl-2 (36) and is abundantly expressed in bone marrow (37). In the absence of bcl-2 and of bcl-x (as we presume), the possibility for modulation of the apoptotic signal is lacking in transitional B cells.

Recently, Hartley et al. (28) have also observed that immature IgM{{\textsuperscript{low}}} B cells of anti-HEL transgenic mice are not eliminated in the presence of the corresponding auto-antigen, while mature B cells can not be found. The authors have suggested that, while mature cells are deleted, immature B cells become developmentally arrested when they bind antigen in the bone marrow. They showed that IgM{{\textsuperscript{null}}} B cells of double transgenic mice developed into IgM{{\textsuperscript{high}}} B cells if cultured in vitro in absence of self-antigen. A simpler interpretation of their results is that immature, IgM{{\textsuperscript{low}}} B cells do not undergo programmed cell death when they bind auto-antigens, but proceed along the developmental pathway to become transitional B cells. At this stage, they are eliminated when they encounter antigen. The in vitro data of Hartley et al. (28) indeed support our interpretation: only immature, IgM{{\textsuperscript{low}}} B cells survived after culture in the presence of antigen. Cell loss happened independently of whether the cells had or had not met the antigen in vivo. In addition, no accumulation of immature developmentally arrested B cells was seen in the bone marrow of double transgenic mice.

**The Role of mIgMolecules in B Cell Development: A Model.** In the bone marrow, cells of the B lineage can be grouped...
Figure 8. A model of B cell development. Three populations of cells are represented, based on the staining pattern for HSA and B220. HSA\textsuperscript{high}, B220\textsuperscript{low} B cells include pro-, pre-, and immature B cells. They develop into HSA\textsuperscript{high} B220\textsuperscript{null} a high transitional B cells. HSA\textsuperscript{null}, B220\textsuperscript{high} cells correspond to mature cells. In the lower part of the figure the expression pattern of other developmentally regulated markers is listed. Cross-linking of mlgM has positive (further differentiation) or negative (negative selection) effects during development.

in three partially overlapping populations (Fig. 8). In our model, signalling through the BCR has varying effects depending on the developmental stage and on the microenvironment. In the early stages of differentiation, the contact to stroma cells and the presence of functional Ig molecules are necessary to guide B cells along the differentiation pathway (7) from pro-B to immature B cell.

Immature B cell proceeding to the transitional B cell stage leave the stroma cell microenvironment and pause in the bone marrow sinusoids before migrating to the periphery (5, 6). It is not known whether the differentiation step from the immature to the transitional B cell stage happens by default or whether it is an induced process. The availability of mutant mice that are arrested in the immature stage would clarify this point. We have recently generated transgenic mice with a mutant IgM receptor, in which the B cells are unable to progress to the transitional B cell stage (Carsetti, R., M. C. Lamers, manuscript in preparation).

Transitional B cells express a high amount of mlgM, are largely positive for the fas antigen, and are negative for bcl-2 expression. Signaling through mlgM is not modulated and leads to programmed cell death. The population of recent bone marrow emigrants described by Allman et al. (12) in the spleen is identical to the population of transitional B cells. The authors suggest that these cells may be the targets of negative selection in the periphery (12). The continuity of the transitional phenotype into the periphery would guarantee that tolerance can be induced to antigens that are expressed exclusively in the periphery (peripheral tolerance).

The last step of differentiation from transitional B cells to mature B cells probably happens in peripheral lymphoid organs (1, 12). The existence of mutant mouse lines in which this step does not occur would help to clarify the molecular mechanisms involved. Using the division of bone marrow B cells in three populations as we have presented in this paper, we were able to identify two mutant mice lines with a defect in the final B cell maturation: the CBA/N mouse, which has an X-linked immunodeficiency caused by a mutation in Bruton's tyrosine kinase (38, 39), and the CD45 knock-out mouse (40), which lacks the expression of a phosphotyrosine phosphatase (in collaboration with Drs. C. Paige, The Wellesley Hospital Research Institute, Toronto, Canada, and T. Mak, Ontario Cancer Institute, Toronto, Canada). Also at the mature B cell stage, mlgM transmits negative signals (20). Binding to antigen, however, can result in activation thanks to the combined action of mlgD, T cell—derived factors, and accessory molecules. A role for mlgD has already been implied in the protection of mature resting B cells from tolerance induction and in favoring antigen-induced activation (41, reviewed in reference 42). We have confirmed this hypothesis in IgM, IgD double-transgenic mice (20). In addition, we now have evidence that the components of the IgM-BCR and of the IgD-BCR are not identical. mlgM and mlgD associate with the classical coreceptor proteins Igα and Igβ (35). However, mlgM, but not mlgD, is found to be associated with at least two additional proteins: BAP32/prohibitin and BAP37/prohibitone (43). Prohibitin was originally cloned based on its growth-arresting property and was suggested to be a tumor suppressor (44). The antiproliferative activity of prohibitin could quite well explain the functional difference between the IgM- and IgD-BCR.

Finally, the division of bone marrow B cells in three compartments is based both on functional and phenotypic criteria. The description of the transitional B cell allows for a better assessment of the differentiation stage of B cells, and it is of utmost importance in the evaluation of mutant mouse models.

We thank Drs. Peter J. Nielsen and Lars Nitschke for their critical reading of the manuscript, and Hubertus Kohler for his help in the flow cytometric analyses.
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