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Does CD40 Ligation Induce B Cell Negative Selection?1

Jesús Martínez-Barnetche,* Vicente Madrid-Marina,† Richard A. Flavell,‡ and José Moreno*2

Binding of CD154 to its receptor, CD40, provides costimulation for mature B cell activation and differentiation in response to Ag receptor signals. In mice, early B cell precursors express CD40, but its function at this stage is unknown. We examined the effects of CD40 ligation during B cell ontogeny in transgenic mice constitutively expressing CD154 on B cells (κEP-CD154). Precursors beyond pro-B cells were absent in adult bone marrow but were increased in the fetal liver. Newborn κEP-CD154 mice had largely increased numbers of peripheral B cells, which were CD154+, and that 36 h after birth expressed high surface levels of CD23 and MHC class II, resembling activated mature B cells. Nevertheless, κEP-CD154 mice were hypogammaglobulinemic, indicating that the expanded population of apparently activated B cells was nonfunctional. Further analysis revealed that soon after birth, κEP-CD154 mice-derived B cells became CD5+/Fas−, after which progressively decreased in the periphery in a CD154-CD40-dependent manner. These results indicate that CD40 ligation during B cell ontogeny induces negative selection characterized by either hyporesponsiveness or an arrest in maturation depending on the time of analysis and the anatomic site studied. The Journal of Immunology, 2002, 168: 1042–1049.

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cell-dependent B cell activation begins with B cell Ag receptor (BCR)3 cross-linking, followed by costimulatory molecule and cytokine signals (1–3). The best characterized co-stimulatory signal for B cells is delivered by activated T cells by means of the TNF family molecule CD154 to CD40, a TNFR family member (4–7). The biological effects induced by CD40 ligation on B cells include: proliferation (8, 9), differentiation into Ab-secreting cells (7, 10, 11), and isotype-switching (7, 12–14).

During ontogeny, B cell precursors sequentially express several genes and receptors, some of which are involved in their maturation. The CD40 receptor is expressed during B cell ontogeny but its possible function in early B cell precursors is unknown. In mice, CD40 expression was first detected at the pre-B cell stage (15), differing from humans, where CD40 is first seen on pre-B cells (4).

The majority of information on the role of CD40 in B cell function relates to its positive effects on proliferation and differentiation. However, depending on the stage of differentiation and/or functional state, CD40 can also negatively influence B cells (16, 17). Thus, CD40 plus BCR signaling on immature B cells leads to tolerance and Fas-FasL dependent exclusion from germinal centers (16). Moreover, under certain circumstances, CD40 ligation stops terminal B cell differentiation in mice (17) and can also inhibit Ig secretion by human tonsilar IgD+ memory B cells (18).

In normal individuals, autoactive B cells are inactivated in different ways leading to tolerance. After BCR signaling, mainly in the periphery, immature IgM+B cells follow one of three fates: receptor editing (19, 20), clonal deletion (21), or clonal anergy (22). Although CD40 appears to participate in B cell clonal anergy (23, 24), its possible role in other forms of B cell tolerance has not been clarified.

However, abnormal CD154 expression can induce a pathological state. Thus, constitutive CD154 expression in the skin results in T cell-mediated local autoimmune disease, through activation of Langerhans dendritic cells, in addition to autoantibody-mediated systemic connective tissue disease (25). The latter could be due to a direct effect of CD154 on B cells, leading to B cell hyperresponsiveness to BCR signaling, which in the case of self-reactive B cells would result in autoantibody switch recombination and secretion (7, 14). Most pathogenic autoantibodies bear isotypes which are dependent of CD40-CD154 interactions such as IgG. It was reported that peripheral blood B cells of systemic lupus erythematosus patients (27–29), as well as mice models of systemic lupus erythematosus (30) constitutively express CD154. However, its possible role in the pathogenesis of autoantibody production was not examined. Although normal B cells have also been found to express CD154 (31,32), its meaning is unknown.

CD40 ligation could also influence B cell fate during positive and/or negative selection, as suggested by the increase in autoreactive B cells in human CD154 deficiency (33). Thus, while CD154-CD40 is necessary for terminal B cell differentiation and pathogenic autoimmunity, its absence alters shaping of the B cell repertoire.

To gain insight on the effects of augmented CD40 ligation on B cells, and to examine a possible role of CD154-CD40 in B cell ontogeny in vivo, we generated transgenic mice (tgM) with selective expression of CD154 by B cells. Although these mice have an early expansion of B cells, adult mice show a profound reduction of the peripheral B cell compartment due to both peripheral loss and failure of B cell maturation during early stages of bone marrow (BM) (2) lymphopoiesis.
Materials and Methods

Plasmids and constructs

The CD154 cDNA (kindly provided by Dr. M. Berton, University of Arizona, Tuscon, AZ) was inserted in a plasmid containing the K14 β-globin cassette (kindly provided by Dr. E. Fuchs, University of Michigan, Ann Arbor, MI), where the K14 promoter was substituted by a 1.6-kb κ fragment of the plasmid pKenVpr, a gift of Dr. U. Storb (University of Chicago, Chicago, IL; Fig. 1a), which contains a cassette with the Vκ intronic enhancer, linked to the Vκ promoter. To excise the entire κ enhancer/promoter cassette, a second HindIII site of pKenVpr located between the enhancer and promoter was eliminated by partial HindIII digestion, followed by isolation of the linearized plasmid, which was then filled-in with Klenow DNA polymerase and re-ligated with T4 DNA ligase (Life Technologies, Rockville, MD). The κ enhancer/promoter cassette was subchon by blunt ligation of a filled-in HindIII-Xhol fragment into a filled-in Aval site of the CD154/K14 plasmid, where the 0.8-kb human β-globin intron was left downstream of the promoter as a splicing donor, followed by CD154, and the 3’ untranslated region of the human K14 gene, respectively. We refer to this construct as eEP-CD154.

Mice

tgM expressing CD154 on B cells (xEP-CD154 mice) were generated by microinjection of (C57BL/6xC3H)F1 zygotes with a K14 promoter cassette, a second HindIII site of pKenVpr located between the enhancer and promoter was eliminated by partial HindIII digestion, followed by isolation of the linearized plasmid, which was then filled-in with Klenow DNA polymerase and re-ligated with T4 DNA ligase (Life Technologies, Rockville, MD). The κ enhancer/promoter cassette was subchon by blunt ligation of a filled-in HindIII-Xhol fragment into a filled-in Aval site of the CD154/K14 plasmid, where the 0.8-kb human β-globin intron was left downstream of the promoter as a splicing donor, followed by CD154, and the 3’ untranslated region of the human K14 gene, respectively. We refer to this construct as eEP-CD154.

Serum IgG

These were determined by an ELISA method with biotinylated rabbit anti-mouse isotype-specific Abs followed by HRP-labeled streptavidin in poly- styrene plates (Nunc, Roskilde, Denmark) coated with polyclonal goat anti-mouse Ig. Plates were read in an automated ELISA reader (Labsystems, Helsinki, Finland) at 492 nm. All ELISA reagents were purchased from Zymed Laboratories (San Francisco, CA).

Results

xEP-CD154 mice

Six of 10 xEP-CD154 transgenic founders were rusted, and died before 4 wk of age, which precluded further analysis. The remaining four founders were backcrossed to C57BL/6 mice for further analysis. Semi quantitative RT-PCR showed maximal transgene expression in the spleen, followed by lymph nodes (LN), thymus, lung, and adult liver (Fig. 1c).

Decreased number of peripheral B cells in adult xEP-CD154 mice

Preliminary flow cytometric analysis aiming at defining transgene expression in 8-wk-old xEP-CD154 mice revealed a marked decrease of B220+ cells in peripheral blood, LN, spleen, BM, and peritoneal cavity (not shown). The microscopic architecture of LN and spleen was abnormal with increased white pulp, poorly defined follicles, and absent germinal centers (Fig. 2). As these findings were consistent in all surviving transgenic lines, the results presented hereafter derive from analysis of one representative line, 28.

Although spleens of 10- to 12-wk-old xEP-CD154 mice had about half the total cell number as nontransgenic littermates, B cells were decreased by at least 15-fold (Fig. 3a). This was more evident in LN, where B220+ cells were less than 3% (data not shown). The few remaining B220+, but no other cells, in the spleens of xEP-CD154 mice expressed low density CD154 (Fig. 1b). Additional findings included slightly increased T cell numbers and greatly increased Mac-1+ /Gr-1+ cells in spleens and LN of 10- to 12-wk-old xEP-CD154 mice (data not shown). The B cell defect in xEP-CD154 mice was due to CD154-CD40 ligand-receptor interaction, as B cell numbers in adult xEP-CD154 transgenic-CD40−/− mice (35) were normal (Fig. 2b).

Semiquantitative southern RT-PCR

Total RNA was obtained from tissue homogenates in TRIzol reagent (Life Technologies) according to manufacturer instructions with a second extraction to exclude elimination of genomic DNA. A total of 5 μg RNA was reverse-transcribed with Superscript II RT (Life Technologies) and 1/25 reaction volume was amplified by PCR with the oligonucleotide pairs: CD154 Gp39F (363–385); 5’-GGA TCC TCA AAT TGC AGC ACA CG-3’ and CD154 Isser (891–917); 5’-CTC TCA GAT CCA ATG TGG GCC AGG ACC ACC GCC AGG ACC TGA C-3’ and 5’-AGG CAT GA TGT GGT GCC GCC AGA C-3’. The CD154 primers amplify a 255 bp fragment spanning exons 4 and 5, which in wild-type (WT) mice allows the distinction between genomic DNA from cDNA. After a 15 cycle amplification, samples were run in 2% agarose gels and transferred onto nylon membranes (HyBond N; Amersham Pharmacia, Buckinghamshire, U.K.) in 20 X SSC, then hybridized at 60°C with alkaline phosphatase-labeled CD154 and β-actin cDNA probes, detected with a chemiluminescent kit according to man-ufacturer instructions (AlkPhos; Amersham Pharmacia) and exposed for 1 h to Hyperfilm ECL (Amersham Pharmacia).

Lymphoid cell preparations

Spleen cells were obtained in FACS buffer (PBS, pH 7.4, 2% FBS, 5 mM EDTA, 1% rabbit serum, and 0.1% NaN3) by dissociation of spleens between frosted glass slides. BM cells were obtained by injecting 0.75 ml of FACS buffer in one end of the femur, and collecting the cells at the other end. Fetal liver cells were obtained from timed matings of the different mouse combinations, as described for spleen cells. Contaminating RBC in all cell suspensions were eliminated with 0.16 M NH4Cl buffer, and then washed twice in FACS buffer. The total cell number in each suspension was estimated by counting in a hemocytometer.

Cell staining and flow cytometry analysis

A total of 1 × 10^6 cells were reacted in FACS buffer for 20 min on ice with combinations of the following conjugated mAb: anti-B220/CyChrome or FITC, anti-CD43 (S7)-biotin, anti-CD24-biotin (30-F1), anti-IgM (LoMe9)-FITC, anti-IgD-biotin, anti-CD40-FITC, anti-CD154 (MR1)-biotin, anti-CD5-PE, and anti-Thy-1.2-biotin (BD Pharmingen). In experiments with MD4 tGM, specific B cells were labeled with biotinylated HEL. Streptavidin-FITC, streptavidin-PE or streptavidin-PerCP (BD Pharmingen) were used as a second label for biotin-conjugated reagents. Samples were run on a dual laser FACS Calibur and analyzed with CellQuest software (BD Immunocytometry Systems, Mountain View, CA).

Effect of CD154 on peripheral B and T cell populations

Expression of CD154 on peripheral B cells was substantially reduced in xEP-CD154 mice (Fig. 1c). As a consequence, all cell types that express CD154 on B cells, including T cells and dendritic cells, were decreased in xEP-CD154 mice. The most obvious change was in the numbers of T cells, which were reduced by at least 20-fold. The decrease in T cells was not due to a decrease in the numbers of CD4+ or CD8+ T cells, as shown by flow cytometry (Fig. 1c). Similarly, the decrease in T cells was not due to a decrease in the numbers of CD4+ or CD8+ T cells, as shown by immunohistochemistry (Fig. 2). The lack of CD154 expression on T cells in xEP-CD154 mice suggests that the decrease in T cells is due to the absence of CD154 on B cells. The decrease in T cells is likely due to the absence of CD154 on B cells, as shown by flow cytometry (Fig. 1c). The decrease in T cells is likely due to the absence of CD154 on B cells, as shown by immunohistochemistry (Fig. 2).
Ten to 12-wk-old \( \kappa \)EP-CD154 mice had few peripheral B cells, which in older mice were only vestiges. Therefore, we examined the \( \kappa \)EP-CD154 B cell compartment from newborn to adult animals in greater detail. Newborn \( \kappa \)EP-CD154 spleens, had twice total B cell numbers as control mice (Fig. 3c). During fetal life in mammals, B cell lymphopoiesis occurs in the liver, although after birth it takes place in BM (37). Fetal \( \kappa \)EP-CD154 mice appeared to have a spared B cell lymphopoiesis as indicated by the B cell expansion from birth to 2 wk of age. However, the inability to replenish the B cell compartment after this time suggested that in addition to the peripheral B cell loss, \( \kappa \)EP-CD154 BM was unable of generating new B cells. Hence, \( B_{220}^+\) cells in BM or in newborn livers of \( \kappa \)EP-CD154 and nontransgenic

**Deficient B cell maturation in the BM but not in the fetal liver of \( \kappa \)EP-CD154 mice**

During fetal life in mammals, B cell lymphopoiesis occurs in the liver, although after birth it takes place in BM (37). Fetal \( \kappa \)EP-CD154 mice appeared to have a spared B cell lymphopoiesis as indicated by the B cell expansion from birth to 2 wk of age. However, the inability to replenish the B cell compartment after this time suggested that in addition to the peripheral B cell loss, \( \kappa \)EP-CD154 BM was unable of generating new B cells. Hence, \( B_{220}^+\) cells in BM or in newborn livers of \( \kappa \)EP-CD154 and nontransgenic

**FIGURE 1.** Generation and characterization of \( \kappa \)EP-CD154 tgM. a, Construct used to generate \( \kappa \)EP-CD154 mice and primers used to amplify CD154 message by RT-PCR. The upper part shows primer annealing with CD154 exons four and five. b, FACS analysis with biotin-anti-CD154, followed by streptavidin-PE of splenocytes from 4- and 8-wk-old \( \kappa \)EP-CD154 (dotted lines) or normal (shaded histograms) mice, gated on \( B_{220}^-\) cells (FITC-B220). c, Semiquantitative RT-PCR analysis of total RNA obtained from the indicated tissues of \( \kappa \)EP-CD154 or control mice amplified with primers for CD154 (\( \Box \)) or \( \beta \)-actin and hybridized with equivalent cDNA probes. Lanes: 1) spleen; 2) LN; 3) BM; 4) liver; 5) kidney; 6) CNS; 7) skeletal muscle; 8) lung.

**FIGURE 2.** Retarded growth, splenomegaly, and abnormal lymphoid tissue in \( \kappa \)EP-CD154 tgM. a, Macroscopic view of a \( \kappa \)EP-CD154 mouse (left) and control littermate (right), showing the retarded growth and the enlarged, whitish spleen. b, Panoramic view of \( \kappa \)EP-CD154 spleen revealing an altered organization and predominant white pulp (H&E \( \times 10 \), Opto 2.5). c, A closer view of the spleen showing the increase of proliferating cells (H&E \( \times 20 \), Opto 3.3). d, Tracheal LN of \( \kappa \)EP-CD154 mouse, showing diffuse hyperplasia, disruption of normal architecture of the follicular areas, and virtual absence of germinal centers (H&E \( \times 10 \), Opto 4). e, \( \kappa \)EP-CD154 liver showing nodular lymphoid infiltrates and hematopoietic foci in portal spaces and acini (H&E, \( \times 10 \), Opto 4).

**FIGURE 3.** a, B cell deficiency in adult \( \kappa \)EP-CD154. Absolute number of splenocytes and \( B_{220}^+\) cells from 10- to 12-wk-old \( \kappa \)EP-CD154 or normal mice. Results represent the mean ± SEM of five independent analysis, WT, \( n = 10 \); \( \kappa \)EP-CD154, \( n = 9 \). b, CD40-CD154-dependent B cell loss in \( \kappa \)EP-CD154. Absolute B cell counts in spleens of \( CD^{40/-} \), \( CD^{40/-}/\kappa \)EP-CD154, \( CD^{40/-}/\kappa \)EP-CD154, and \( CD^{40/-}/\kappa \)EP-CD154. Mean ± SEM of two independent experiments are shown. c, Peripheral B cell compartment in \( \kappa \)EP-CD154 is expanded during early life followed by rapid and progressive B cell loss. Total \( B_{220}^-\) cells in spleens from 1–2 days, and 2–4, 10–12, and 32-wk-old \( \kappa \)EP-CD154 (■), or littermate control mice (□). d, B lymphopoiesis in \( \kappa \)EP-CD154 is enhanced in neonatal liver but suppressed in adult BM. Total B (\( B_{220}^-\) cells) (squares) and \( B_{220}^-IgM^-\) (pro-B + pre-B, circles) cells in 1- to 2-day-old liver and 2–4, 10–12, and 32-wk-old mice BM. Data represent the mean ± SEM of 14 independent analysis (WT, \( n = 29 \), \( \kappa \)EP-CD154, \( n = 27 \)).
littermates, were examined by flow cytometry (Fig. 3d). As seen, while B220<sup>+</sup> cell numbers in newborn kEP-CD154 mice livers were increased when compared with nontransgenic littermates, from 2 to 32 wk of age (the earliest and latest ages examined), total B220<sup>+</sup> cells in BM of kEP-CD154 mice were markedly decreased. Thus, in kEP-CD154 mice, B cell lymphopoiesis is enhanced in the fetal liver, but it is profoundly impaired in the BM. Of note, both IgM<sup>+</sup>/B220<sup>+</sup> and IgM<sup>−</sup>/B220<sup>+</sup> cells were increased in the livers of newborn kEP-CD154 mice (see below).

**B cell maturation in the BM of kEP-CD154 mice halts during or after the pro-B cell stage**

B cell ontogeny in the liver and BM, in mice, are slightly different (37). However, in both sites, the earliest identifiable B cell precursor is B220<sup>low</sup>/CD43<sup>+</sup> (pro-B), whereas pre-B cells (the next stage of differentiation) are B220<sup>+</sup>/CD43<sup>−</sup> (38). Therefore, we conducted a detailed two-color FACS analysis of B cell precursor profile in newborn liver and adult BM of kEP-CD154 mice to define at which stage was B cell ontogeny impaired in kEP-CD154 mice, and to establish possible differences between adult BM and newborn liver.

As shown in Fig. 4a, at all ages studied, the BM of kEP-CD154 mice had an almost complete absence of B220<sup>low</sup>/CD43<sup>+</sup> pre-B cells, but not of B220<sup>high</sup>/CD43<sup>−</sup> pro-B cells. In contrast, and as shown above, B220<sup>+</sup> cells were increased in the livers of newborn kEP-CD154 mice. Indeed, all B cell precursors, regardless of their stage of maturation, were increased in the livers of newborn kEP-CD154 mice (Figs. 3b and 4b).

B220 levels on newborn kEP-CD154 liver B cells (which in normal newborn mice are immature B cells) were higher than in controls (data not shown), resembling the phenotype of mature B cells. Control 32-wk-old BM had a reduced number of B cell precursors, which is age-related (37); yet, kEP-CD154 BM had one-tenth the number of pre-B cells. Finally, young kEP-CD154 mice had a considerable number of B220<sup>highb</sup>/CD43<sup>−</sup> cells in the BM, which have been considered by some (38) as recirculating B cells.

Taken together, these results indicate that transition from pro-B to pre-B takes place in neonatal kEP-CD154 livers and their B220 levels resemble recirculating B cells (38), suggesting an accelerated rate of maturation, whereas in kEP-CD154 BM, B cell development stops during pro-B to pre-B cell transition. The fact that IgM<sup>−</sup>/B220<sup>+</sup> cells, representing all B cell precursor stages before immature B cells, are also increased in the fetal liver, indicates that B cell expansion in fetal and newborn kEP-CD154 mice is due to a signal delivered in the fetal liver at either pro-B or pre-B cell stages. As the only difference between kEP-CD154 and normal mice is the transgene (CD154), it seems reasonable to conclude that CD40 signaling at early stages of B cell ontogeny in the fetal liver or in the BM has distinct effects. Hence, CD40 ligation in the BM prevents B cell precursor maturation beyond the pro-B cell stage, whereas in the fetal liver, the same signal induces B cell expansion followed by early maturation and apparent elimination in the periphery.

**Arrest of B cell differentiation in the BM and expansion in the fetal liver of kEP-CD154 mice occurs at a late pro-B cell stage**

Expression by B220<sup>+</sup>/CD43<sup>−</sup> cells of the heat-stable Ag (CD24) and Ly51 (BP-1) subdivide pro-B cells into three maturational stages (38). The earliest (fraction A) is CD24<sup>+</sup>/BP-1<sup>+</sup>, fraction B is CD24<sup>−</sup>/BP-1<sup>−</sup>, and fraction C is CD24<sup>−</sup>/BP-1<sup>+</sup>. To examine at which pro-B cell stage B cell ontogeny was arrested in kEP-CD154, BM cells were examined by four-color FACS analysis for B220, CD43, CD24, and BP-1 expression. Fig. 5a shows that A and B fractions (CD24<sup>−</sup>/BP-1<sup>−</sup> and CD24<sup>+</sup>/BP-1<sup>+</sup>, respectively) were present in the BM of 6- to 8-wk-old mice, whereas late pro-B cells (fraction C) were absent. Further analysis of B220<sup>+</sup> cells with CD25 and c-Kit, as defined by Melchers and Rolink (Ref. 37; Fig. 5b) confirmed the marked decrease of B cell precursors after the small pre-B1 stage (CD25<sup>+</sup>/c-Kit<sup>−</sup>; see Ref. 37). This indicates that B cell precursor development in BM of kEP-CD154 mice halts after the first heavy chain gene rearrangement (D-J).

Similar to the findings by two-color FACS analysis of liver B cells with B220 and CD43, two-color analysis of kEP-CD154 liver cells with C-Kit and B220 revealed an increase of B220<sup>+</sup>/c-Kit<sup>−</sup> B cells that include all stages of B cell differentiation beginning with small pre-B1 cells, further suggesting that the signal responsible for B cell expansion in the liver of kEP-CD154 mice is the same as the signal that prevents B cell maturation in the BM, most likely CD154/CD40.

**B cell maturation in BM and in the fetal liver of kEP-CD154 mice is disturbed at the time of CD154/CD40 expression**

As it was clear that changes in B cell ontogeny in newborn liver and adult BM kEP-CD154 were occurring at a discrete stage during late pro-B cell differentiation, we examined the expression of CD154 in kEP-CD154 mice and CD40 in normal B6 mice by three-color FACS analysis with B220 and CD43 plus CD40 or CD154. This was important because it was reported that, in mouse, CD40 is expressed at the pre-B cell stage, whereas in the human, CD40 is first seen on pro-B cells. Fig. 6a shows that all B cell precursors in normal mice, including CD43<sup>−</sup>/B220<sup>+</sup> cells

FIGURE 4. B cell lymphopoiesis is enhanced in the liver of kEP-CD154 mice, but arrested in the BM at the pro-B cell stage. *a*, FACS analysis of 2-, 4-, and 32-wk-old mouse BM-stained with anti-B220-FITC and anti-CD43-PE. B220<sup>+</sup>/CD43<sup>−</sup> pro-B cells, B220<sup>low</sup>/CD43<sup>−</sup> pre-B cells, and B220<sup>high</sup>/CD43<sup>−</sup> recirculating mature B cells are boxed and percentages are given. *b*, Representative two-color histograms of FACS analysis of newborn kEP-CD154 mouse or control littermate liver cells stained as in *a.*
inducing expansion of B cell precursors, which could no longer be found in the periphery beyond four wk of age. Therefore, it was important to examine the functional and phenotypic consequences of CD40 signaling during fetal B cell ontogeny in newborn and young adult κEP-CD154 in peripheral B cells. Initial analysis of the cell cycle in 7-wk-old κEP-CD154 mice showed that among the few B cells remaining in their spleens, the proportion of cells in S, G2, and M phases of the cell cycle was higher than in normal controls (data not shown). Fig. 7, a and b, show that, while at birth, splenic κEP-CD154 B220+ cells had the same phenotype as controls. As early as 36 h later, κEP-CD154 B cells expressed continuously increasing levels of CD23 and MHC class II. Moreover, their cell size was increased as demonstrated by a higher forward scatter (data not shown). These results indicate that perinatal engagement of CD40 on κEP-CD154 B cells or their precursors, most likely in the fetal liver, induce early maturation and expression of activation markers. Thus, homotypic pro-B or pre-B cell CD40-CD154 interaction in fetal κEP-CD154 mice liver appears to induce functional changes on their progeny reminiscent of B cell activation, followed by peripheral loss.
The activated B cells of young κEP-CD154 mice are not functional

Anergic B cells, before their elimination, express activation markers and are excluded from entering the spleen follicles, after which they are eliminated (16). The activated phenotype of the increased B cells found in young κEP-CD154 mice suggested that these cells could be undergoing clonal inactivation before being apparently eliminated in the spleen. Therefore, we indirectly examined the functional status of κEP-CD154 mice B cells in vivo by measuring serum Ig levels. Two-week-old κEP-CD154 mice had slightly increased IgM, but normal or decreased levels of all other Ig isotypes (Fig. 8). These were even lower when corrected for the total B cell number at each stage (data not shown), and considering that, at least part of the Ig detected was maternally-derived. Thus, despite the high numbers of activated B cells present, young κEP-CD154 mice do not have increased serum IgS, indicating that B cells in young κEP-CD154 mice are not functional, probably anergic.

Peripheral κEP-CD154 B cells acquire expression of Fas and CD5, after which they can no longer be detected

Peripheral elimination of anergic B cells is mediated by T cells in a CD95 (Fas)-CD40L-BCR-dependent manner (16). Moreover, other studies have indicated that B-2 type B cells can express CD5 (39), which has been associated to tolerance induction (40). Therefore, we examined the expression of CD95 and CD5 by κEP-CD154 B cells. As seen in Fig. 7, c and d, in parallel with their acquisition of activation markers, κEP-CD154 B cells became Fas−/CD5+, remaining positive as they progressively decreased. Thus, the expanded B cell population in κEP-CD154 mice express surface molecules, which are involved in deletion or tolerance induction and resemble clonally anergic B cells.

CD5+ B cells in κEP-CD154 are B-2 type B cells

Many authors have long considered CD5 as a marker for the B-1 type B cell subset, which uses different sets of H and L chain V region segments (41). Transgenic expression of rearranged Ig genes that use V regions belonging to either B-1 or B-2 type B cells leads to development of only the B cell subset corresponding to the V region in the rearranged gene (42). To determine whether the CD5+ B cell population present in κEP-CD154 mice represented B-1 or B-2 type B cells, κEP-CD154 mice were crossed to mice carrying rearranged transgenic B-2 type Ig H and L chain genes specific for HEL (22). This experiment was important for two reasons: first, to define if expression of a rearranged Ag receptor before CD40 ligation could rescue B cell precursors; second, because these mice should only develop B-2 type B cells, and therefore, if CD5 were only a B-1 cell marker, no CD5+ cells would be found in double tgM for anti-HEL-κEP and CD154. Fig. 9 shows that HEL-binding B cells in anti-HEL-κEP-CD154 double tgM were CD5+ with no remarkable differences to κEP-CD154 mice. These results indicate that CD5+ B cells in both κEP-CD154 and anti-HEL-κEP-CD154 double tgM are B-2 type B cells and are, therefore, in agreement with observations, that CD5 expression by B cells is not restricted to the B-1 subset (39). Moreover, no B-1 type Ab specificities were detected in κEP-CD154 sera (data not shown). Although, Ig transgenes did not rescue anti-HEL-κEP-CD154 double tgM B cells from deletion, this was slightly delayed (not shown).

Discussion

We started these studies with the hypothesis that constitutive expression of CD154 by B cells would lead to B cell hyperactivity at least at two levels. During ontogeny, CD40-ligation could bypass tolerance induction in immature IgM+ B cells. On mature B cells, CD40-ligation could lead to hyperactivity and, possibly, autoimmunity. Unexpectedly, the result was the opposite, transgenic CD154 expression by B cells drastically decreased B cell numbers in adult mice. In contrast, during fetal life, CD154-CD40 interaction induced B cell expansion and expression of CD23, MHC-II, CD5, and Fas. These cells were functionally-impaired and were apparently deleted in the periphery.

The essential role of CD40 in mature B cell differentiation and isotype switching is beyond controversy. In addition, CD40 ligation rescues B cells from apoptosis in germinal centers during affinity maturation (43–46). However, the later is not so straightforward, as CD40 can also facilitate elimination of tolerant B cells in a T cell- and Ag-dependent manner (16–18). Ligation of CD40 plus Fas on the surface B cells rendered tolerant by BCR-signaling...
when immature leads to their elimination (16), indicating that timing of BCR ligation is critical for the outcome of CD40-signaling. Moreover, CD40 ligation can inhibit proliferation and Ig secretion by normal human B cells (18), human or mouse B cell lymphomas (47), and hybridomas (48). Thus, CD40-signaling can mediate either B cell survival or death in different situations.

Studies on the role of CD40 during early B cell ontogeny are scarce and in some cases conflicting. In one study (49), CD40 ligation on human fetal BM pro-B cells (CD34+/CD10+) inhibited IL-7-induced proliferation in vitro, whereas in another, it induced surface Ig negative human B cell precursors to proliferate, acquire expression of CD23, and to become CD10+/surface Ig+, in response to IL-3 and other cytokines (50). Although it was reported that CD40 expression both at the cell surface and mRNA in mice begins in pre-B cells, which are refractory to in vitro CD40-stimulation (51), in the current studies, CD40 expression was evident from the pro-B cell stage both in normal and in κEP-CD154 tgM and was functional in vivo, as demonstrated by the CD154-CD40-dependent B cell depletion in κEP-CD154. It is unlikely that this merely reflects an increased susceptibility of pro-B cells to signal-induced cell death, as newborn κEP-CD154 B220+ cells in liver and peripheral lymphoid tissues were expanded. Thus, the profound B cell deficiency in adult κEP-CD154 mice was due to a halt on B cell ontogeny during pro-B to pre-B cell transition in the BM as well as to peripheral loss of fetal liver-derived B cells which resembled the phenotype found in anergic B cells.

CD40-mediated apoptosis has been described for transformed nonlymphoid cells (52), but the mechanism involved is unclear since the cytoplasmic tail of CD40 lacks the TNFR family death domains. We found that B cell precursors in adult BM, but not in the liver of newborn κEP-CD154 mice, expressed Fas (not shown). However, in the latter, peripheral B cells expressed maturation and activation markers, and became Fas+ 36 h after birth. Although we did not show the involvement of Fas in the loss of B cells and their precursors in κEP-CD154 mice, taken together the data suggests that Fas is involved in the apparent B cell deletion in this model, and could explain why this loss is delayed in liver-derived, but immediate in BM-derived B cells. As in mature B cells, CD40 stimulation of early B cell precursors could induce Fas expression, resulting in increased susceptibility to apoptosis (53, 54). Overall, what this implies is not readily apparent, but suggests that B cell development in the BM and in the liver, is governed by different signals.

The possibility that κEP-CD154 B cells did not undergo CD40 ligation before leaving the liver and the CD154-CD40 signal was instead delivered in the periphery is unlikely for at least two reasons. First, liver B cells were expanded in κEP-CD154, and the only possible explanation for this was an increased CD154-CD40 interaction. The second explanation requires the assumption that only B cell precursors receiving two signals were the targets of elimination/expansion, and if the same stimulus led to both events. The first signal is mediated through the BCR, and the second through CD40. If this is true, κEP-CD154 pre-B cells would receive the dual signaling event. Although the signaling events following CD40 ligation in mature B cells are synergistic to BCR signaling, leading to a fully activated state; in immature B cells, BCR signaling leads to anergy or cell death (reviewed in Ref. 39).

What could the current findings mean in normal mice? The pre-BCR is a constitutively signaling molecule (37) that plays a role in positive selection of pre-B cells expressing a functional H chain, apparently without additional signals. In contrast, the BCR on immature B cells signals only after Ag-binding (which is predominantly the case for self-reactive B cells). This takes place in the periphery, in the presence of CD154 expressing cells, which do not normally appear to be present in the BM or liver. Hence, in the periphery, only autoreactive, immature B cells would concomitantly signal through the BCR plus CD40. Such dual signaling would delete immature B cells, whereas CD40-only signaling allows immature B cells to continue their development into mature μδ B cells and further (55, 56). This hypothesis is in agreement with the finding that CD154-defective individuals have an expanded B cell repertoire and persistence of many autoreactive B cells (33). As there is no information in the literature concerning CD154 expression in the normal BM and in the present study, normal mouse BM did not contain CD154 mRNA (Fig. 1), we propose that this mechanism takes place mainly in the periphery. In κEP-CD154 mice, B cell precursors undergo homotypic CD40-CD40 interaction at the time their pre-B cell receptor starts signaling. Like κEP-CD154 mice, all cells with a functional pre-BCR receive both signals. We interpret this as that they are suffering the same fate as immature autoreactive B cells in the periphery of normal mice. The nature of the second signal could not be only restricted to CD40, as expression of other surface molecules, including other TNF-TNFR family members (57) can produce a similar effect. This could also have implications in pathologic states of immune stimulation, where activated T cells could enter the BM, thus providing CD40 signaling, which could impair B lymphopoiesis.

The failure of a functional BCR to rescue B cells in anti-HEL-κEP-CD154 double tgM is not clear, as these mice should not have suffer B cell loss in the absence of their specific Ag or Ig crosslinking, unless low affinity BCR-Ag interactions were taking place in the BM, according to our hypothesis that BCR signaling plus co-stimulatory signals eliminate B cell precursors. The delayed B cell deletion seen in κEP-CD154 mice suggest that additional mechanisms are involved.

One last relevant observation of the current studies was the finding that most B cells in κEP-CD154 were CD5+, and that a transgenic B-2 type BCIR did not prevent the expression of CD5. According to the hypothesis that B-1 or B-2 type phenotypes are dictated by the BCR V region usage, this indicates that these B cells are indeed B-2 and not B-1 type cells. CD5 expression by B cells is not restricted to the B-1 subset, as activated B-2 type B cells can express CD5 (39), which could be a marker of self-reactive B cells undergoing tolerance induction, as this phenotype resembles clonally inactivated B cells (16, 40), and is compatible with the hypogammaglobulinemia seen in young κEP-CD154 mice. Thus, CD40 ligation on late pro-B or, perhaps, early pre-B cells prevents their maturation, which is immediate in the BM but delayed in the liver. Fetal liver-derived B cells in κEP-CD154 mice move into in the periphery, after they further expand until 4 wk of age, after which they can no longer be found. Such expansion and apparent elimination is likely to take place in the spleen. The cellular mechanism responsible for this event was not defined, but it has been suggested that T cells are responsible for the elimination of clonally anergic B cells (16), which could be similar to our findings if the expanded B cell population in κEP-CD154 indeed represents clonally anergic B cells. The roles of Fas and CD5 expression on B cell fate and function in κEP-CD154 mice, respectively, deserve further studies.

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References


