

# Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the *XID* and *CD40* deficiency genes

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## Abstract

It has been known for some time that single mutant nude or *CD40T* mice have apparently normal numbers of cells in the precursor compartments of bone marrow and the mature B cell compartments of the periphery. X-linked immunodeficiency (*XID*) mice are deficient only in some of the  $\text{slgM}^+\text{slgD}^+$  B cells. We have investigated further the contributions of the *xid* mutation, of the T cell deficiency of nude and of the inability of *CD40T* B cells to cooperate with T cells in the generation of the precursor and the mature B cell compartments in mice. Double mutant *XID/nu* and *XID/CD40T* mice have precursor B cell compartments that are no more deficient than the single mutant *XID* mice. However, the peripheral B cell compartments of both *XID/nu* and *XID/CD40T* are even more deficient than those of single mutant *XID* mice. While 10% of the peripheral B cells of wild-type or *CD40T*, one-third of *XID* and half of *XID/nu* mice turn over rapidly, as many as three-quarters of those in *XID/CD40T* are short-lived. Total numbers of  $\text{slgM}^+\text{slgD}^+$  B cells in the spleen are at best 10–15% of normal mice at 6–8 weeks of age in *XID*, *XID/nu* and *XID/CD40T* mice. They remain that low at 3 months of age in *XID/CD40T* mice, while in *XID* mice these peripheral B cells slowly build up in numbers with age. As expected, double mutant *XID/CD40T* mice do not respond to the T-dependent antigen keyhole limpet hemocyanin. Only the responses to the T-independent type I antigen, TNP-lipopolysaccharide (LPS), appear to be normal. *In vitro*, their splenic B cells respond poorly to LPS or to IgM-specific antibody in either the absence or presence of cytokines. Most notably, serum IgM, IgG2b and IgG3 levels are severely depressed, while IgG1, IgG2a and IgA levels are  $<10\ \mu\text{g/ml}$ . The results suggest a model of mature B cell development in which the peripheral, mature B cell compartments are generated in two parallel, not tandemly organized pathways. They could be selected and/or stimulated at the transition from immature to mature B cells: in *btk* controlled or in *CD40* controlled ways.

## Introduction

The discovery of X-linked immunodeficiency (*XID*) diseases in humans and mice has demonstrated the existence of genetic loci on the X chromosome which influences the growth, differentiation and function of lymphocytes (1–3). Recently, two groups have isolated the gene encoding a cytoplasmic tyrosine kinase, named Bruton's tyrosine kinase (*Btk*), which in different patients with X-linked agamma-

globulinemia (*XLA*) appears mutated in different parts of the gene encoding different domains of the *Btk* protein (4,5). On the other hand, one of the best characterized models of this disease in mice is the *CBA/N* strain that carries a *XID* gene (2). The murine *btk* gene was mapped to the *XID* locus of the murine X chromosome (6,7). A missense mutation, predicted to alter an amino acid residue (Arg28 to Cys) in the unique

N-terminal region of the Btk protein, was found in the *btk* gene of XID mice, suggesting that this mutant *btk* gene is responsible for the XID defect in CBA/N mice (6).

XID mice do not respond to thymus-independent type II (TI-II) antigens (2), respond with a lower number of B cells to lipopolysaccharide (LPS) (8), do respond to thymus-independent type I (TI-I) antigens and respond normally to thymus-dependent (TD) antigens (2,9). B cells of XID mice also do not respond to cross-linking of surface Ig (sIg) in the presence of IL-5 or IL-10 (10,11). Somewhat reduced numbers of peripheral, mature B cells, and reduced levels of IgM and IgG3 are found in the XID mice, indicating that the generation of mature B cells reactive to TI-I and TD antigens is not severely affected by the *btk* mutation.

Thymectomy of CBA/N mice abolishes the ability of these mice to populate the peripheral lymphoid compartment with T and B cells, suggesting that at least some of the peripheral B cells need T cells for their development (12).

In previous studies by Wortis and his colleagues (13), the XID mutation has been combined with the nude (*nu/nu*, called nu herein) defect leading to thymic dysplasia and failure to develop the  $\alpha\beta$  TCR-expressing lineage of T cells. The analysis of these double mutant XID/nu mice has indicated a more severe B cell dysplasia, so that B lineage precursor cell compartments in the bone marrow appeared normal at early pro/pre-B stages, but depleted of cells expressing IgM either in the cytoplasm or on their surface. The peripheral, mature B cell compartments were found severely depleted, incapable of responding to either TI or TD antigens. Concomitantly serum Ig levels were much reduced (12,14–16). The grafting of thymus into the double mutant XID/nu mice restored the peripheral mature B lymphoid compartments with cells capable of responding to TI and TD antigens (16). Again, this finding was taken as evidence that some peripheral, mature B cells are generated in thymus-dependent ways.

CD40 expressed on B cells and its ligand expressed on T cells control the interactions of helper T cells with B cells and, thus, responses of B cells to TD antigens which lead to Ig isotype switching, germinal center formation and induction of immunological memory (17–24). CD40-deficient (CD40T) mice, generated by targeted mutation, have peripheral mature B cells which respond normally to TI antigens, do respond to TD antigens with IgM production, but do not respond to TD antigens with IgG, IgA or IgE production, and do not develop germinal centers (21). This indicates that the interaction between CD40 and its ligand is essential for helper T cell-dependent Ig class switching during responses of B cells to TD antigens, but is not important for responses to TI antigens. Taken together, all these findings suggest that peripheral B cells can be generated in both *btk* controlled and CD40 controlled ways.

In this report we further investigate the contributions of the *Xid* gene to the *nu/nu* defect in the primary, B cell generating compartments of bone marrow. We have also combined the XID defect effected by the *btk* mutation with the CD40 defect in a double mutant mouse strain (called XID/CD40T) to see whether the inability of B cells to respond to some TI combined with the inability to cooperate with helper T cells controls all of the mature B cell development.

## Methods

### Mice

CBA/N mice which carry the *xid* gene were originally obtained from the Jackson Laboratory (Bar Harbor, ME). In addition, we also used XID congenic C57Bl/6 mice for some experiments. Athymic C57Bl/6 *nu/nu* mice were originally obtained from BRL (Fullinsdorf, Switzerland). *xid/xid* × *nu/nu* double mutant mice (called XID/nu in this paper) were generated as described by Wortis and his colleagues (15). The generation of CD40T mice has been described elsewhere (21).

### Generation of double mutant mice with XID and CD40 deficiency

In order to analyze the effect of a simultaneous abnormality of XID and CD40 deficiency on B cell development, we bred double mutant mice from XID and CD40T mice (XID/CD40T mice). Female CBA/N (*xid/xid*; homozygous for the mutated *btk* gene) mice or female XID congenic C57Bl/6 mice (here called XID) were mated with male CD40T mice (homozygous for the mutated CD40 gene), followed by mating of the female F<sub>1</sub> mice with male CD40T mice. In the next male offspring, double mutant XID/CD40T, single mutant CD40T, XID and wild-type mice were expected and found in a ratio of 1:4 as described below.

Mice were bled at ages between 10 and 12 weeks to measure serum Ig levels. To detect the defect of CD40, PCR analysis was done on DNA from spleen or tail as described (21). To detect the XID phenotype we analyzed peritoneal cells by FACS for the absence or presence of CD5<sup>+</sup> B cells, because lack of CD5<sup>+</sup> peritoneal B cells is one of the striking features of XID mice. To confirm the serological typing of XID mice, PCR analyses was done on DNA from spleen or tail to detect the *btk* mutation, using primers and protocols as described (7).

### Antibodies and flow cytometric analysis

FITC-conjugated mAb RA3-6B2 (anti-CD45R, B220), phycoerythrin (PE)-conjugated mAb 53-7.3 (anti-CD5), biotinylated mAb 7D4 (anti-CD25, IL-2R  $\alpha$ -chain, TAC) and mAb B3B4 (anti-CD23, IgE Fc receptor) were purchased from Pharmingen (San Diego, CA). mAb ACK<sub>4</sub> (anti-*c-kit*) (25), mAb M41 (anti-IgM) (26), mAb 1.19 (anti-IgD) (27) and mAb 7E9 (anti-CD21) (28) were purified from culture supernatants on Protein G-Sepharose (Pharmacia, Uppsala, Sweden) as recommended by the supplier, followed by conjugation with biotin according to standard protocols.

Bone marrow, spleen and peritoneal cells were cell surface stained with antibodies as described elsewhere (29–31), and stained cells were analyzed by FACScan (Becton-Dickinson, Mountain View, CA).

### Quantitation of serum Ig levels

Serum IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA levels were determined by ELISA as described elsewhere (29,31,32). Goat antibodies specific for mouse Ig classes and subclasses and mouse Ig standards were purchased from Southern Biotechnology Associates (Birmingham, AL).

### *BrdU labeling of cells*

Mice were fed with BrdU (Sigma, St Louis, MO) at 1 mg/ml in drinking water for 6 days, or for 6 days, followed by a 7 day period of feeding without BrdU (chase) before sacrifice. Blood, spleen and bone marrow lymphocytes were prepared in PBS, fixed for 1 h on ice with 0.5% paraformaldehyde in PBS. Cells were collected by centrifugation and resuspended in 1 ml of 3N HCl/0.5% Tween 20 and left for 20 min at room temperature in order to denature DNA. Cells collected by centrifugation were neutralized by resuspending them in 0.2 ml of 0.1 M borate buffer, pH 8.5, and thereafter adding 1 ml PBS-azide. Cells were washed once, resuspended in FACS buffer (PBS-azide/2% FCS) and stained with PE-labeled anti-B220 (PharMingen) and FITC-labeled anti-BrdU (Becton Dickinson Immunocytometry System, San Jose, CA) followed by analyses on a FACScan.

### *Antigens and immunization of mice*

For generation of antibody responses to keyhole limpet hemocyanin (KLH), animals were immunized i.p. with 50 µg in complete Freund's adjuvant. The anti-KLH antibody responses were measured in sera on day 14 by utilizing a KLH isotype-specific ELISA (see below).

Primary responses to TNP-LPS were generated by immunization with 50 µg TNP-LPS in PBS i.p. The IgM, IgG1, IgG2a and IgG3 anti-TNP responses were measured in sera on day 14 by a TNP-specific ELISA (see below).

### *Antigen-specific ELISA*

Microtiter plates for ELISA were coated with 10 µg/ml KLH or 5 µg/ml DNP-BSA in PBS overnight at 4°C. They were washed and blocked with PBS containing 5% FCS and sodium azide. Diluted serum samples were incubated overnight at 4°C. Samples were washed and antigen-specific antibody titers were determined with alkaline phosphatase-conjugated detection antibodies: goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA (all obtained from Southern Biotechnology Associates). After 1 h incubation at 37°C plates were washed and the ELISA was developed with the substrate 4-nitrophenyl phosphate (1 mg/ml; Sigma) in 1 M diethanolamine buffer, pH 9.8. Plates were analyzed on an ELISA reader (ThermoMax; Molecular Devices, Menlo Park, CA) at 410 and 610 nm. Units represent arbitrary values based on the titration curve of a standard immune serum from normal mice according to the procedure described by Foy *et al.* (19).

### *Cell culture and in vitro stimulation of spleen cells*

Single cell suspensions of spleen from the various mutant and wild-type mice were prepared and cultured in Iscove's modified DMEM (Gibco/BRL, Life Technologies, Paisley, UK) containing antibiotics,  $5 \times 10^{-5}$  M 2-mercaptoethanol and 2% FCS as described earlier (29). Cultures of 0.2 ml containing  $3 \times 10^5$  total spleen cells/ml were set up in 96-well flat-bottom microtiter plates and incubated for 3 days at 37°C in a 10% CO<sub>2</sub>/air atmosphere. At the onset of culture, various mitogens and/or recombinant cytokines were added to probe for B cell proliferative functions. The following mitogens were used: LPS at 20 µg/ml (from *Escherichia coli* EH100, kindly provided by Dr C. Galanos, Max Planck Institute for Immunobiology,

Freiburg, Germany), monoclonal anti-CD40 at 10 µg/ml [clone FGK 45.5 generated as elsewhere (33)], Sepharose-bound anti-IgM at 1% beads in culture [monoclonal rat anti-mouse µ-chain, clone M41 (26) bound at 1 mg/ml of packed CNBr-activated Sepharose 4B (Pharmacia, Uppsala)]. The recombinant IL-4 and IL-2 were used at 100 and 1000 U/ml respectively. They were obtained from a transfected X63-Ag8.653 cell line (34) generated in our laboratory. Cultures were pulsed with 1 µCi [<sup>3</sup>H]thymidine (sp. act. 5 Ci/mmol; Amersham Life Science, Buckinghamshire, UK) during the last 4 h of incubation and harvested onto glass fiber paper which was processed and counted in a Rack-Beta plate scintillation counter (LKB Pharmacia, Uppsala). Radioactivity recorded from triplicate cultures was expressed as the mean c.p.m. per culture. SD between triplicates never exceeded 5% of the mean.

## Results

### *B lineage precursor cells in the bone marrow of homozygous wild-type, XID, nu, CD40T, XID/nu and XID/CD40T mice*

B220(CD45R)<sup>+</sup> B lineage precursors in bone marrow of different wild-type, mutant and double mutant mice were analyzed by FACS for the expression of *c-kit*<sup>+</sup>, CD25(TAC)<sup>+</sup>, slgM<sup>+</sup>, slgD<sup>+</sup> and CD23<sup>+</sup> cells. The results of these analyses are shown in Fig. 1(a and b). The quantities of the different B-lineage subpopulations are given in Table 1. CD40T and nude mice have *c-kit*<sup>+</sup> pro/pre-B-I cells, CD25(TAC)<sup>+</sup> pre-B-II cells, slgM<sup>+</sup> and CD23<sup>+</sup> immature as well as slgD<sup>+</sup> mature B cells in numbers comparable to wild-type mice. XID mice lack a large part of the slgM<sup>+</sup>B220 high cells, of the slgD<sup>+</sup> cells and of the CD23<sup>+</sup> cells, indicating a deficit in the more mature subpopulations of B cells. XID/nu and XID/CD40T double mutant mice have normal numbers of *c-kit*<sup>+</sup> pro/pre-B-I cells. The CD25(TAC)<sup>+</sup> pre-B-II cells and slgM<sup>+</sup>, slgD<sup>+</sup> and CD23<sup>+</sup> cells are comparable in both phenotype and numbers to that of XID single mutant mice in both types of double mutant mice. The low level of CD23 expression on B cells of CD40T animals is a consistent finding and has been reported earlier (21) (also, see below).

We conclude from these analyses that the double expression of the XID and nu, respectively XID and CD40T mutations, does not impair B cell development in bone marrow beyond the defects seen with the single XID mutation. This is in contrast to previous analyses of XID/nu double mutant mice performed by Wortis and his colleagues (15).

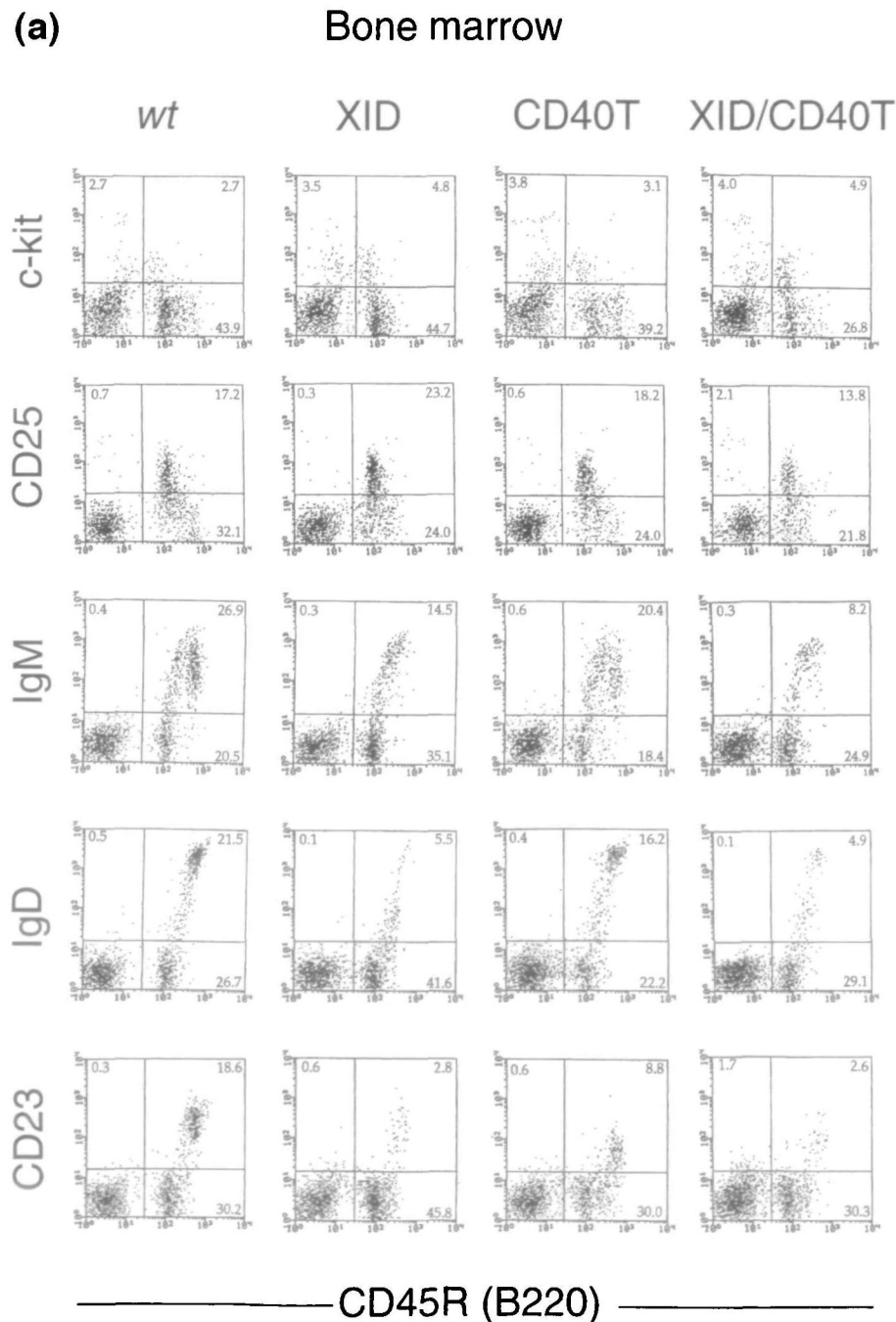
### *B lineage cells in the spleen*

B220(CD45R)<sup>+</sup> B lineage wild-type cells and the different mutant and double mutant mice were also analyzed in spleen by FACS for the expression of CD5, slgM, slgD, CD23 and CD21. These analyses were performed with mice 4–6 weeks as well as 10–12 weeks of age. The results of these analyses are shown in Fig. 2, the quantities of the different B-lineage cell subpopulations are given in Table 2. Wild-type, nu and CD40T single mutant mice have comparable numbers of B220<sup>+</sup> cells expressing slgM and slgD at both ages. Wild-type and CD40T single mutant mice have comparable numbers of CD5<sup>+</sup> and CD21<sup>+</sup> cells. Consistently, we find that the level of

CD23 expression is lower in CD40T B cells and thus the number of detectable CD23<sup>+</sup> cells is underscored in such mice. Splens of *XID/nu* and *XID/CD40T* double mutant mice at 4–6 weeks of age had sIgM<sup>+</sup> and sIgD<sup>+</sup> cells in numbers slightly less than those of single mutant *XID* mice. When the total number of CD45(B220)<sup>+</sup> sIg<sup>+</sup> cells in spleen was calculated, seven wild-type mice had an average of  $39 \pm 5 \times 10^6$  cells, 10 *XID* mice an average of  $9 \pm 4 \times 10^6$ , six

double mutant *CD40T/XID* mice an average of  $6 \pm 1 \times 10^6$  cells and five *XID/nu* mice an average of  $4 \pm 2 \times 10^6$  cells (Table 2). When the same measurements were done with splens of 10- to 12-week-old mice, the number of sIg<sup>+</sup> B cells had more than doubled in *XID* mice but remained as low as in 4- to 6-week-old mice in *XID/CD40T* mice (Table 2).

We conclude from these data that, in contrast to bone marrow, the B lineage representation in spleen of *XID/nu* and

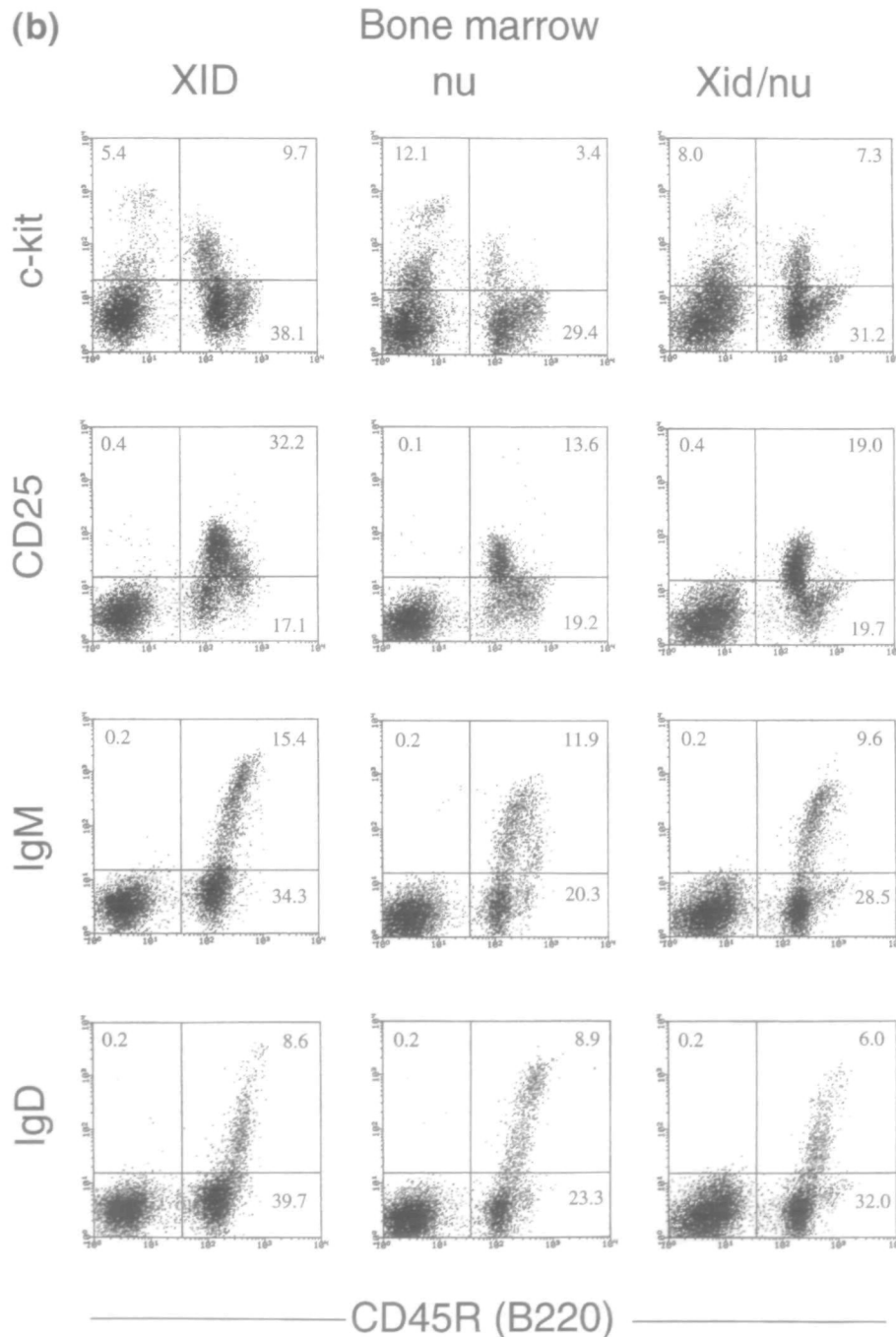


**Fig. 1.** (a) FACS analyses of bone marrow lymphoid cells of wild-type (*wt*), *CD40T*, *XID* and *XID/CD40T* mice. Horizontal axes represent fluorescence intensities of CD45R(B220) after surface labeling, while vertical axes represent fluorescence intensities after surface labeling for *c-kit* and CD25 (TAC),  $\mu$  (IgM),  $\delta$  (IgD) and CD23 as described in Methods.

*XID/CD40T* double mutant mice shows a severe reduction in the numbers of cells expressing different markers associated with mature B cells. With increasing age this defect remains severe in the *XID/CD40T* mice. Our results suggest that the simultaneous deficiency in the *XID* and *CD40* genes affects the generation of mature, peripheral B cells at the transition from the immature bone marrow compartment profoundly and much more so than does single mutations.

#### Turnover of peripheral B cells

Mice at 4–6 weeks of age were given BrdU in drinking water for 6 days, to allow incorporation into DNA of dividing cells (pulse period). Thereafter, BrdU was removed from the drinking water for 7 days (chase period) to allow analyses of the fate of labeled cells. At 6 days after the pulse, and after 7 days of the chase period, B220<sup>+</sup> cells in the spleen and blood were analyzed for BrdU label. The experimental results



**Fig. 1.** (b) FACS analyses of bone marrow lymphoid cells of *XID*, *nu* and *XID/nu* mice. Horizontal axes represent fluorescence intensities of CD45R(B220) after surface labeling, while vertical axes represent fluorescence intensities after surface labeling for c-kit and CD25 (TAC),  $\mu$  (IgM) and  $\delta$  (IgD).

**Table 1.** Total number of B cells in different subpopulations<sup>a</sup> in the bone marrow of indicated wild type, single mutant and double mutant mice

Mouse strain	Total <sup>b</sup> B220 <sup>+</sup>	B220 <sup>+</sup> / <i>c-kit</i> <sup>+</sup>	B220 <sup>+</sup> /CD25 <sup>+</sup>	B220 <sup>+</sup> /IgM <sup>+</sup>	B220 <sup>+</sup> /IgD <sup>+</sup>	B220 <sup>+</sup> /CD23 <sup>+</sup>
Wild-type	9.2 ± 0.4	0.5 ± 0.2	3.9 ± 0.4	4.6 ± 0.3	3.0 ± 0.2	2.2 ± 0.5
CD40T	7.9 ± 0.7	0.6 ± 0.1	4.7 ± 0.1	4.3 ± 0.2	3.3 ± 0.1	1.7 ± 0.6
<i>nu</i>	6.8 ± 1.2	0.8 ± 0.4	2.9 ± 0.5	2.4 ± 0.5	2.2 ± 0.2	1.8 ± 0.3
XID	10.2 ± 1.5	1.5 ± 0.6	6.5 ± 1.5	3.4 ± 0.5	2.1 ± 0.3	0.8 ± 0.1
XID/ <i>nu</i>	7.3 ± 2.3	1.3 ± 0.5	2.6 ± 2.0	1.8 ± 0.9	1.5 ± 0.2	1.3 ± 0.2
XID/CD40T	6.5 ± 1.0	0.7 ± 0.3	4.3 ± 0.2	2.9 ± 0.9	1.8 ± 0.6	0.4 ± 0.2

<sup>a</sup>Bone marrow of 4- to 6-week-old mice was prepared for FACS analyses as described in Methods. After gating out dead cells, the percentages of cells within the lymphoid cell gate of which the surface stained for the indicated markers were calculated. The total numbers of lymphoid cells contained in two femurs from individual mice were counted and they contained 22–26 × 10<sup>6</sup> cells. From that number and the percentage obtained by FACS analyses, the total number of B cells contained in the various fractions was computed. The values given represent the mean ± SD from four to six individual mice analyzed.

<sup>b</sup>Values represent number of cells (× 10<sup>6</sup>) per two femurs.

for peripheral blood B220<sup>+</sup> cells are given in Table 3, since analyses on splenic B cells gave identical results. The advantage of the analyses on blood is that the same animals can be scored both directly after the pulse of BrdU and then continuously monitored during the entire chase period. As expected from previous analyses by other laboratories (35), wild-type and *nu* mice had ~5–10% of their peripheral B cells labeled and most of the labeled cells disappeared upon a chase period of 7 days. This indicates that the labeled cells are short-lived, while the majority of unlabeled cells should be longer lived. The same extent of labeling and disappearance upon chase was observed with B lineage cells in CD40T mice. By contrast, XID mice had a much higher (30–40%) portion of short-lived cells, while XID/*nu* double mutant mice had even higher (~50%) numbers. The highest number of short-lived cells, i.e. almost three-quarters of all peripheral B lineage cells, was found in double mutant XID/CD40T mice. We conclude from these experiments that most of the B cells generated in XID/CD40T double-deficient mice are and remain short lived, indicating that long-lived B cells remain few in the periphery of these mice.

#### Response of B cells to antigenic stimulation *in vivo*

KLH was used as a TD antigen and TNP-LPS as a TI-I antigen to monitor the *in vivo* responsiveness of B cells in wild-type, single mutant CD40T or XID, and double mutant XID/CD40T mice (Fig. 3a and b). Wild-type and XID mice showed normal IgM and IgG1 responses to KLH, while CD40T mice, as expected (21), were 5-fold lower in their IgM responses, compared to the wild-type mice, and deficient in IgG1 responses. Double mutant XID/CD40T mice showed >10-fold reduced IgM responses compared to single mutant CD40T mice and showed no IgG1 (Fig. 3a), IgG3 or IgA responses (data not shown) to KLH. Wild-type, single mutant XID as well as double mutant XID/CD40T mice responded equally well to the TI-I antigen TNP-LPS (Fig. 3b)

We conclude that the double mutant XID/CD40T mice are deficient in TD responses, but do show responses to a TI-I antigen.

#### Responses of peripheral B cells to mitogenic stimulation *in vitro*

Spleen cells of wild-type, single mutant CD40T, XID and double mutant XID/CD40T, varying in B cell content by a factor of 3–5 (see preceding section), were cultured in medium alone, or in the presence of LPS, of CD40-specific mAb FGK45.5 or of mouse  $\mu$ H-chain specific mAb M41 coupled to Sepharose either alone (Fig. 4a) or in the presence of IL-4 or IL-2 (Fig. 4b). One of a series of representative experiments is shown. Splenic B cells from wild-type mice were stimulated by all three B cell mitogens. The same mitogens also stimulated spleen cells of XID mice, although ~2- to 4-fold lower responses were consistently recorded in such mass cultures, in the presence or absence of the cytokines indicated. However, as reported earlier (8), upon limiting dilution in LPS containing thymus filler cell cultures (36), spleen cells from XID mice showed 10- to 30-fold lower frequencies of LPS-reactive cells (see legend to Fig. 4). Spleen cells of CD40T mice were stimulated by LPS and IgM-specific mAb in the presence and absence of the cytokines to the same extent as wild-type spleen cells except, as expected, that the CD40-specific mAb FGK45 did not stimulate the CD40-deficient B cells.

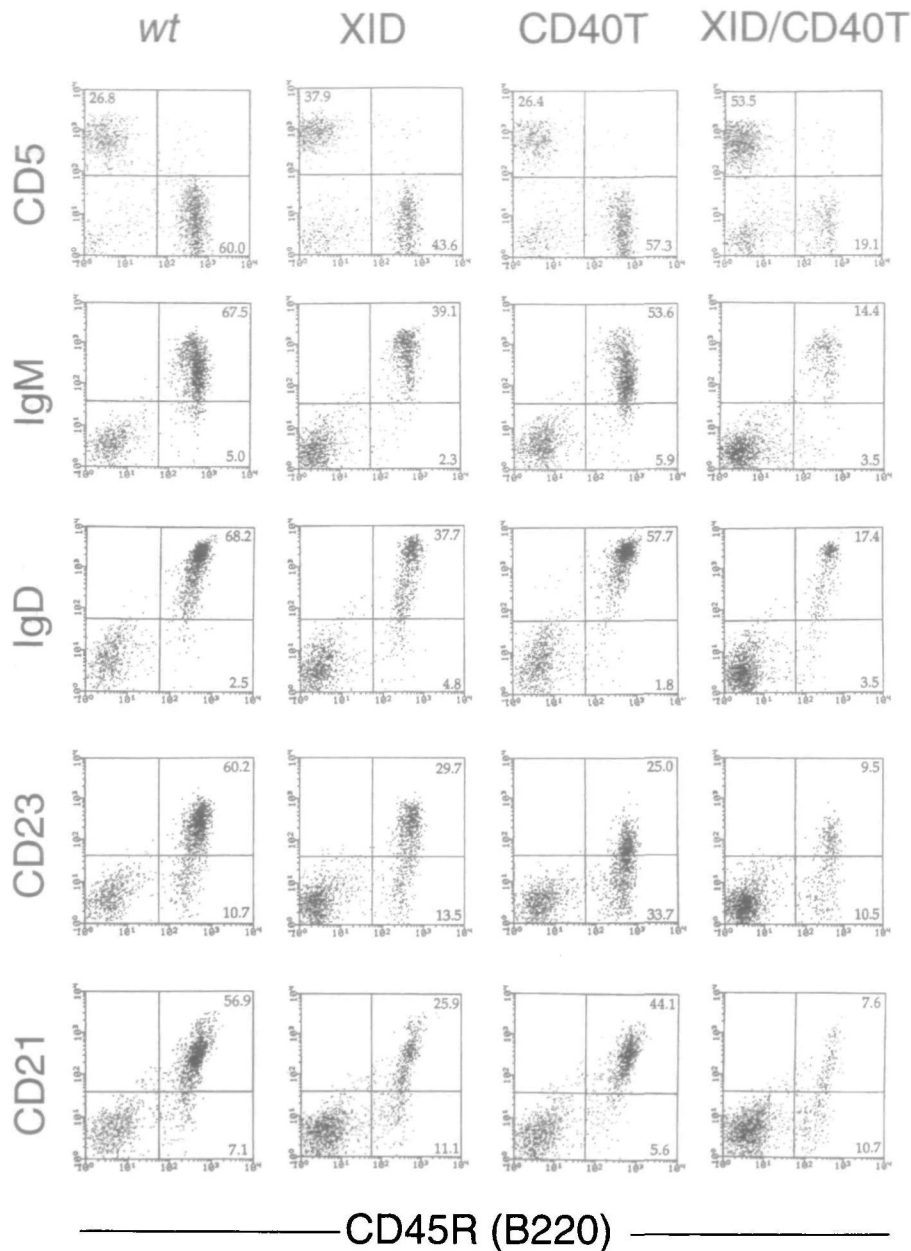
In marked contrast, spleen cells of XID/CD40T double mutant mice showed poor responses to these mitogens, either in the absence or presence of cytokines.

These results indicate that while single mutant XID or CD40T B cells retain reactivity to B cell mitogens, double mutant XID/CD40T B cells show reactivity to all mitogens tested in this study, which are less than proportional to the number of mature B cells present in their spleens.

#### Serum levels of Ig classes and subclasses

The sera of wild-type, single and double mutant XID/*nu* and XID/CD40T mice at 10–12 weeks of age were analyzed for their content of IgM, IgG1, IgG2a, IgG2b and IgG3, and, except for the XID/*nu* mice, also for their IgA content by ELISA. The results are summarized in Fig. 5. As expected (37) XID single mutant mice had lower levels of IgM and IgG3, and CD40T mice had decreased levels of IgG1 and IgA, while the other classes and subclasses of Ig were found

## Spleen



**Fig. 2.** FACS analysis of splenic lymphoid cells of wild-type (*wt*), single mutant CD40T mice, and XID mice and double mutant XID/CD40T mice. Horizontal axes represent fluorescence intensities of CD45R(B220), while vertical axes represent those of CD5, IgM, IgD, CD23 and CD21.

in concentrations comparable to those of wild-type mice. The double mutant XID/*nu* mice had depressed levels of IgM, IgG2a and IgG3. Most notably the double mutant XID/CD40T had 5- to 10-fold lower levels of IgM, while in >90% of all mice the IgG1, IgG2a, IgG2b and IgG3 as well as IgA classes and subclasses were below the detection limit of the assay. In particular, IgG1, IgG2a and IgG3 subclasses were below the detection level in all mice tested.

We conclude that the double mutant XID/CD40T mice

show a severe immunodeficiency, manifested in lower or undetectable serum Ig levels.

### Discussion

Single mutant XID, *nu* or CD40T mice show no severe deficiencies in the generation of precursor and immature B cells in the bone marrow, and in the case of CD40T and *nu* mice have normal numbers of mature B cells in bone marrow

**Table 2.** Total number of B cells in different subpopulations<sup>a</sup> in the spleen of indicated wild-type, single mutant and double mutant mice

Mouse strain	Age (weeks)	Total <sup>b</sup> B220 <sup>+</sup>	B220 <sup>+</sup> /IgM <sup>+</sup>	B220 <sup>+</sup> /IgD <sup>+</sup>	B220 <sup>+</sup> /CD23 <sup>+</sup>	B220 <sup>+</sup> /CD21 <sup>+</sup>
Wild-type <sup>c</sup>	4–6	41.2 ± 4.4	38.9 ± 5.3	38.0 ± 5.2	32.2 ± 6.5	35.9 ± 5.5
Wild type <sup>d</sup>	10–12	36.7 ± 11.8	32.1 ± 15.6	30.7 ± 12.7	36.7 ± 23.1	ND
CD40T	4–6	45.8 ± 22.7	44.5 ± 20.2	44.3 ± 23.1	18 ± 3.2	40.2 ± 20.6
CD40T	10–12	34.1 ± 14.3	35.7 ± 12.2	34.6 ± 11.2	31.8 ± 17.2	ND
nu	4–6	45.1 ± 6.2	35.7 ± 5.5	37.8 ± 6.2	ND	ND
XID	4–6	11.0 ± 6.5	8.8 ± 3.5	8.1 ± 4.3	5.1 ± 1.5	4.6 ± 0.4
XID	10–12	23.3 ± 7.2	20.2 ± 6.0	18.9 ± 5.5	15.4 ± 7.0	ND
XID/nu	4–6	9.0 ± 2.3	4.5 ± 1.9	3.8 ± 1.2	ND	ND
XID/CD40T	4–6	10.9 ± 1.0	6.1 ± 0.9	5.8 ± 1.1	2.4 ± 0.9	3.6 ± 0.3
XID/CD40T	10–12	10.2 ± 14.4	8.7 ± 8.9	8.5 ± 11.1	5.9 ± 6.3	ND

<sup>a</sup>Total splenic cells prepared and analyzed by FACS as described in Methods and in the legend of Table 1.

<sup>b</sup>Calculated numbers represent total number of spleen cells ( $\times 10^6$ ). ND, denotes not determined.

<sup>c</sup>Spleen of the same animals (4–6 weeks old) analyzed for bone marrow B cells in Table 1.

<sup>d</sup>Spleen of four to 10 individual animals 10–12 weeks old.

and the periphery. In the case of XID and CD40T mice, the peripheral, mature B cells are not reactive to TI-II antigens, respectively TD antigens, but respond normally to TD, respectively TI-II antigens. It is not clear how the peripheral B cell repertoire of short-lived and long-lived mature, antigen reactive cells is established. If it was established in ways comparable to the establishment of the peripheral T cell repertoire one might expect positive selection of immature cells through signaling via Ig which could mediate a transition without division into the mature B cell pool (reviewed in 38,39). The normal establishment of a peripheral B cell compartment in the single mutant CD40T and nu mice suggests that such antigenic selection of the B cell repertoire could occur without T cells and the interaction of CD40–CD40 ligand, while the lower number of mature B cells, of which a large fraction is short lived, in XID mice indicates the involvement of *btk* signaling for the generation of long-lived B cells.

Double mutant XID/nu and XID/CD40T mice, again, show no severe deficiencies in the generation of normal numbers of precursor and immature B cells in bone marrow but, in contrast to the single mutant mice, have severe deficiencies in the mature, peripheral B cell compartments, which in the case of XID/CD40T mice are very severe. Wortis and colleagues (15) previously described a severe B cell defect also in bone marrow of XID/nu mice, which is at variance with the results described herein. One possible explanation is the difference in nu/nu strains used for breeding, where we used C57Bl/6 and they used C3H nudes.

We find that double mutant XID/CD40T mice have decreased numbers of mature B cells, almost three-quarters of them with a high turnover, and hence show a deficiency in the generation of long-lived B cells. They have depressed levels of IgM in the serum, and lack detectable levels of IgG1, IgG2a, IgG2b, IgG3 and IgA. Furthermore, their B cells cannot be efficiently stimulated *in vivo* by TD antigens or *in vitro* by a variety of mitogenic stimuli. However, they show responses to TNP-LPS *in vivo*, although *in vitro* responses to LPS are deficient. It, therefore, appears that B cells are made in normal numbers in the bone marrow, arrive in the periphery but cannot be rendered long lived, nor reactive to TD or TI-II

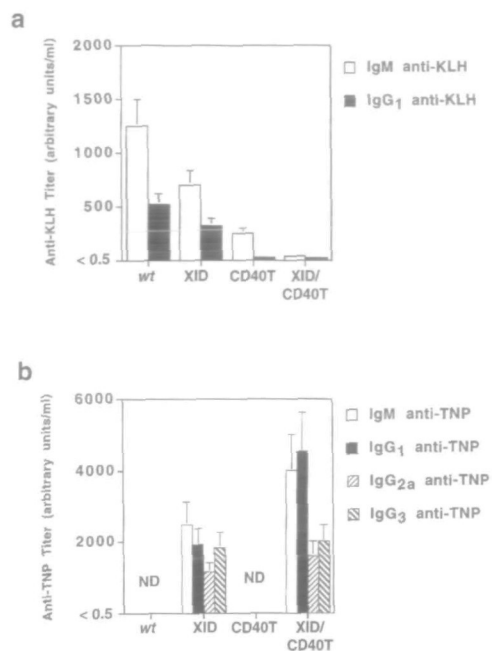
**Table 3.** Percentage of BrdU-labeled peripheral blood B cells in wild-type, single and double mutant mice

Mouse strain	BrdU-labeled cells (%) of total CD45R(B220) <sup>+</sup> cells	
	After 6 days labeling	After 7 days chase
Wild-type	10.3 ± 0.4	1.0 ± 0.5
CD40T	8.8 ± 0.5	2.0 ± 0.5
nu	7.0 ± 2.0	2.0 ± 1.2
XID	38.0 ± 1.7	6.2 ± 6.1
XID/nu	49.0 ± 3.7	5.2 ± 1.7
XID/CD40T	71.5 ± 3.0	4.8 ± 2.0

antigens. The *in vivo* responses to the TI TNP-LPS might be explained if we consider that CBA/N mice have been found to react to LPS with a 100-fold lower frequency of peripheral B cells in spleen, but still with a 100-fold higher frequency than splenic B cells of the LPS non-reactive strain C3H/HeJ (8). *In vivo* this low but measurable number of reactive B cells may be sufficient to elicit an antigen-specific response triggered in association with the mitogen, such as TNP-LPS. This may also serve as an explanation for the finding here, that approximately half of the double mutant XID/CD40T mice have IgM levels >10 µg/ml and in some cases at almost normal levels, approaching 100 µg/ml (Fig. 5).

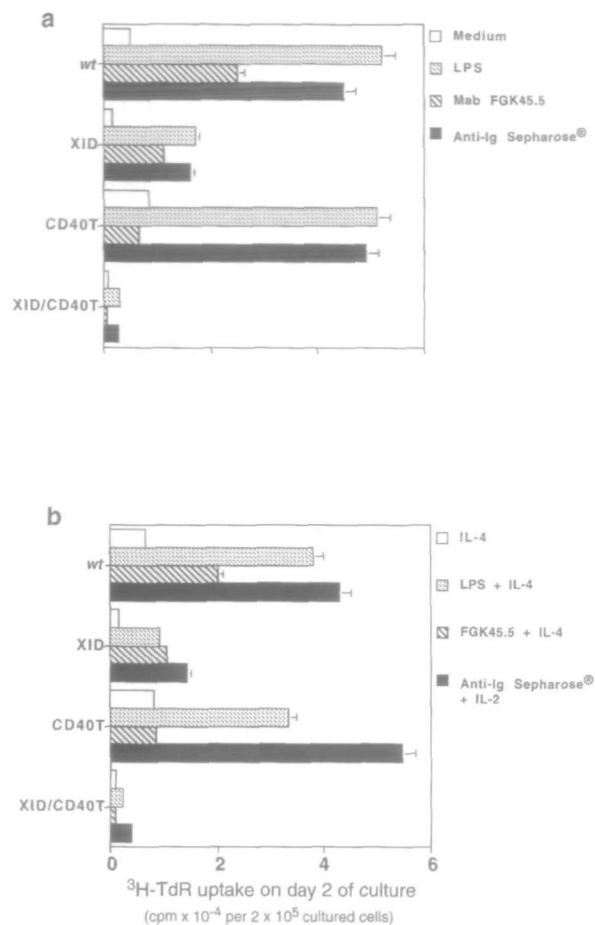
The number of peripheral, mature B cells differs by factors of 5–10 in individual XID/CD40T mice. Such fluctuations in the number of peripheral B cells might either be due to a difference in the genetic background of the mice or have epigenetic causes. It should be remembered that the XID/CD40T double mutant mice were generated by mating CBA/N with CD40T mice, the latter being a mix of the strains C57Bl/6 and 129. Hence, three different genotypes are mixed in various proportions in individual XID/CD40T mice. It remains to be investigated how such different genetic backgrounds could influence the number of short-lived and long-lived peripheral B cells.





**Fig. 3.** (a) Antibody responses to KLH of wild-type, single mutant CD40T and XID, and double mutant XID/CD40T mice. Mice were immunized with KLH in complete Freund's adjuvant (50  $\mu$ g/mouse, i.p.) and the KLH-specific serum antibody titers of IgM (open bars) and IgG1 (filled bars) were determined on day 14 after immunization by a KLH-specific ELISA (see Methods). Units represent arbitrary values based on the titration curve of a standard immune serum according to Foy *et al.* (19). The means of titers from three mice per group are shown. (b) Single mutant XID and double mutant XID/CD40T mice were immunized with TNP-LPS (50  $\mu$ g/mouse, i.p.). On day 14, mice were bled and the anti-TNP antibody titer of IgM (open bars), IgG1 (filled bars), IgG2a (light shaded bars) and IgG3 (dark shaded bars) type were determined by ELISA as described in Methods. Units represent arbitrary values based on the titration curve of a standard immune serum. The responses in wild-type and CD40T mice were similar to those of the XID and XID/CD40T [ND; data not shown, but given elsewhere (21)].

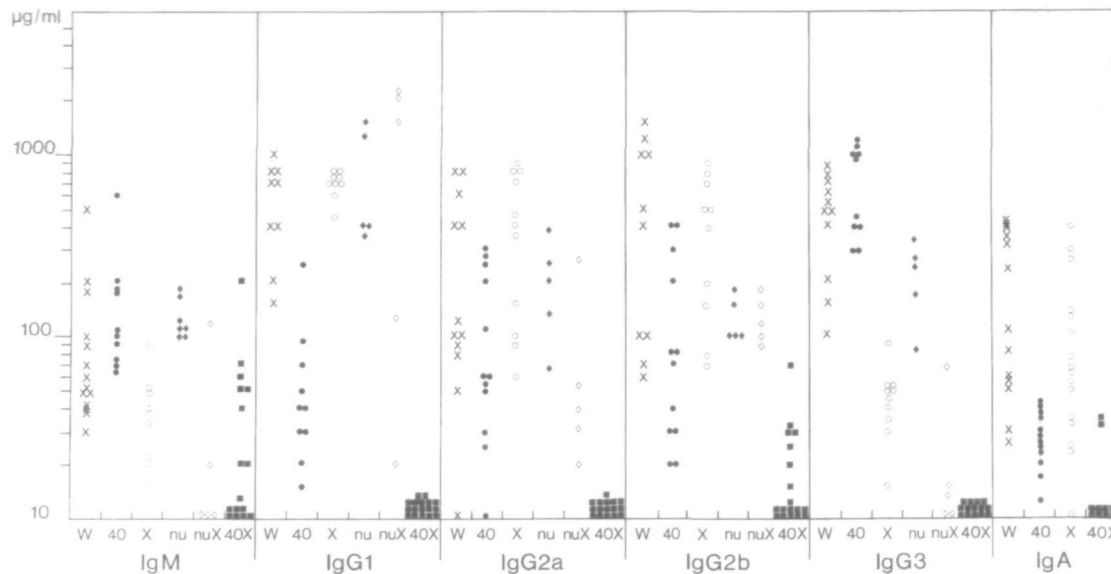
On the other hand, it is also possible that epigenetic influences, such as antigenic stimulation, could influence the level of short- and long-lived B cells in the periphery. This could either occur at the mature, resting B cell stage in the periphery or at the transition from immature to mature B cells in the bone marrow. If it were antigenic stimulation, it would have to be of a type which mediates its effects by ways different from those used by TI-II or TD antigens used in our experiments. In fact, a TI-I antigen might be a good candidate for such B cell stimulation. Influence of antigen on the development of peripheral B cells could also occur at an earlier stage. Recent experiments with Ig transgenic mice on a rearrangement-deficient (RAG-1T and RAG-2T) genetic background have shown that B cell development is arrested at the stage of an immature, slg<sup>+</sup> B cell in the bone marrow, provided that the transgenic Ig recognizes an autoantigen expressed in bone marrow (40,41). In the case of the TNP-specific Sp6 Ig transgene, arrest is likely to be mediated by double-stranded DNA present in bone marrow. It was found that injection of TNP-Ficoll, a TI-II antigen cross-reactive with



**Fig. 4.** Proliferative responses of wild-type, single mutant CD40T and XID, and double mutant XID/CD40T mice stimulated by various B cell mitogens (as described in Methods) in the absence (a) and presence (b) of cytokines. In parallel experiments the frequency of LPS-reactive cells was determined in limiting dilution analyses using rat thymus filler cells (36). The frequency of LPS-reactive cells developing into clones of IgM-secreting cells were 1:12 for wt, 1:3 for CD40T, 1:100 for XID and <1:1000 for XID/CD40T mice.

DNA, relieved this arrest of differentiation, so that B cells appeared in the periphery and could be stimulated to a response, with the development of transgenic Ig-secreting plasma cells (41).

The severely impaired establishment of the peripheral, mature B cell compartment(s) in the double mutant XID/CD40T mice suggests that CD40-dependent and *btk*-dependent ways of selection and/or stimulation of immature and/or mature B cells could control the generation and maintenance of normally-sized mature B cell pools. The fact that XID/nu mice, although deficient in the generation of mature B cells, can generate a peripheral B cell pool of which a considerably larger part is long-lived compared to that of XID/CD40T mice and the finding that slg<sup>+</sup> B cells do accumulate with age in the periphery of XID, but not of XID/CD40T mice might indicate that extrathymic components such as  $\gamma\delta$  TCR-expressing T cells might directly or indirectly contribute to this build up of a peripheral B cell pool. The *btk* gene and the CD40 ligand-



**Fig. 5.** Serum Ig levels of wild-type, single mutant CD40T and XID, and double mutant XID/CD40T and XID/nu mice. The serum levels of IgM, IgG1, IgG2a, IgG2b and IgG3 of individual wild-type (denoted W and crosses), CD40T (denoted 40 and filled circles); XID (denoted X and open circles), nu (denoted nu and filled diamonds), XID/nu (denoted nuX and open diamonds) and XID/CD40T (denoted 40X and filled squares) mice are given. Values  $<10$   $\mu\text{g/ml}$  are placed at the bottom. Serum levels of IgA were not determined in nu and XID/nu mice. Wild-type mice represent values of serum Ig levels in C57BL/6 mice and in mice of other genetic backgrounds which were pooled in this figure, since they did not significantly differ from each other.

CD40 interaction between helper T cells and B cells appear to control all of the detectable mature, long-lived B cell pools, and appear to do so in parallel and not in tandem. It remains remarkable that the *btk*-mutation alone is relatively mild (XID) in mice, but severe (XLA) in man. This might suggest that man generate peripheral B cell pools, much more so than mice, in a *btk*-dependent rather than in a CD40-dependent way.

### Acknowledgements

The able technical assistance of Ms S. Crivelli and Ms N. Staube is gratefully acknowledged. We also thank Ekaterina Platoshkina for helping in the typing of the different mouse strains. We thank Dr P. Sideras and Dr T. Winkler for critical review of this manuscript and Ms. C. Geiger for preparation of the manuscript. H. K. and T. K. are supported by grants from The Ministry of Education, Science and Culture, Japan, and J. A. is partly supported by The Swedish Medical Research Council. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd, Basel, Switzerland.

### Abbreviations

Btk	Bruton's tyrosine kinase
KLH	keyhole limpet hemocyanin
PE	phycoerythrin
LPS	lipopolysaccharide
XID	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

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