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# Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81

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**CD81 (TAPA-1) is a member of the transmembrane 4 superfamily (TM4SF) which is expressed on the cell surface of most cells of the body throughout their cellular differentiation. It has been recognized in several cell surface complexes of lymphocytes, suggesting that it may have diverse roles in lymphocyte development and activation regulation. Mice with a CD81 null mutation revealed normal T- and conventional B-cell development, although CD19 expression on B cells was dull and B-1 cells were reduced in number. However, both T and B cells of mutant mice exhibited strikingly enhanced proliferation in response to various types of stimuli. Interestingly, while proliferative responses of T cells following T-cell antigen receptor (TCR) engagement was enhanced in the absence of CD81, B-cell proliferation in response to B-cell antigen-receptor (BCR) cross-linking was severely impaired. Despite these altered proliferative responses, both tyrosine phosphorylation and intracellular calcium flux in response to cross-linking of cell surface antigen receptors were normal in mutant mice, reflecting apparently normal initial signaling of antigen receptors. In conclusion, though CD81 is not essential for normal T- and conventional B-cell development, it plays key roles in controlling lymphocyte homeostasis by regulating lymphocyte proliferation in distinct manners, dependent on the context of stimulation.**

*Keywords*: CD81/knockout mice/proliferation/thymus

## Introduction

The recently discovered transmembrane 4 superfamily (TM4SF, also called the tetraspans superfamily) proteins are cell surface molecules which are characterized by the presence of four hydrophobic domains, each presumed to be membrane-spanning (reviewed by Wright and Tomlinson, 1994). The TM4SF comprises up to 15 members that are differentially expressed on lymphocytes as well as on a variety of other cells types. It is well known that these TM4SF proteins are associated with various molecules, which include other TM4SF proteins themselves, on the cell surface (Angelisova *et al.*, 1994; Tedder *et al.*, 1994; Imai *et al.*, 1995). Rubinstein *et al.* (1996) recently reported that CD9, CD63, CD81 and CD82, members of the TM4SF, form a network connected to major histocompatibility complex (MHC) class II molecule and very late antigen (VLA) integrins. Although such evidence of their expression and distribution, as well as results of *in vitro* functional studies (see review by Wright and Tomlinson, 1994), has implicated the TM4SF proteins in the regulation of cell growth, motility and signaling, their functions *in vivo* remain elusive.

CD81 is a broadly expressed molecule which was identified originally as the target of an anti-proliferative antibody (TAPA-1) that inhibited *in vitro* cellular proliferation (Oren *et al.*, 1990). In lymphocytes, CD81 has been believed to be essential for B-cell development as well as regulating activation because it is expressed early and associates with CD19, CD21 and leu13 to form a signal transduction complex (Matsumoto *et al.*, 1991; Bradbury *et al.*, 1992). Co-ligation of this CD81-containing complex and surface IgM/B-cell antigen receptor (BCR) resulted in enhanced proliferation of B cells (Carter and Fearon, 1992). More recently, Boismenu *et al.* (1996) demonstrated that a monoclonal antibody (mAb), which could block thymocyte development in a fetal thymus organ culture (FTOC) system, recognized CD81, concluding that CD81 is also essential for thymocyte development. They supported this possibility by showing that re-aggregation of fetal thymocytes with CD81-transfected CHO cells, but not non-transfected CHO cells, could induce thymocyte maturation.

Thus, the accumulating evidence indeed implicates the CD81 molecule as being involved broadly in several functions of lymphocytes. However, the roles of CD81 *in vivo* still remain controversial. Therefore, we have generated mice incapable of making the CD81 molecule ($CD81^{-/-}$) by mutating the *CD81* gene via homologous recombination in embryonic stem (ES) cells. Here we present a characterization of these animals, concentrating on their lymphocyte development and peripheral lymphocyte activation.

## Results

### *Generation of $CD81^{-/-}$ mice*

A targeting vector was constructed by replacing all of exons 3–7 and part of exon 8, which encode the second extracellular loop, the second to fourth membrane-spanning domains and the entire C-terminal intracellular domain (Andria *et al.*, 1991), with the neomycin resistance gene (Figure 1A). Resulting $CD81^{-/-}$ mice were born at the expected Mendelian frequency and raised normally until at least 18 weeks of age under germ-free conditions. No obvious behavioral abnormalities were detected. The abolished expression of CD81 in homozygous mutants was determined by Northern blotting (Figure 1B) and RT–PCR (Figure 1C) analysis either of spleen or thymus RNA, and the absence of CD81 protein production was

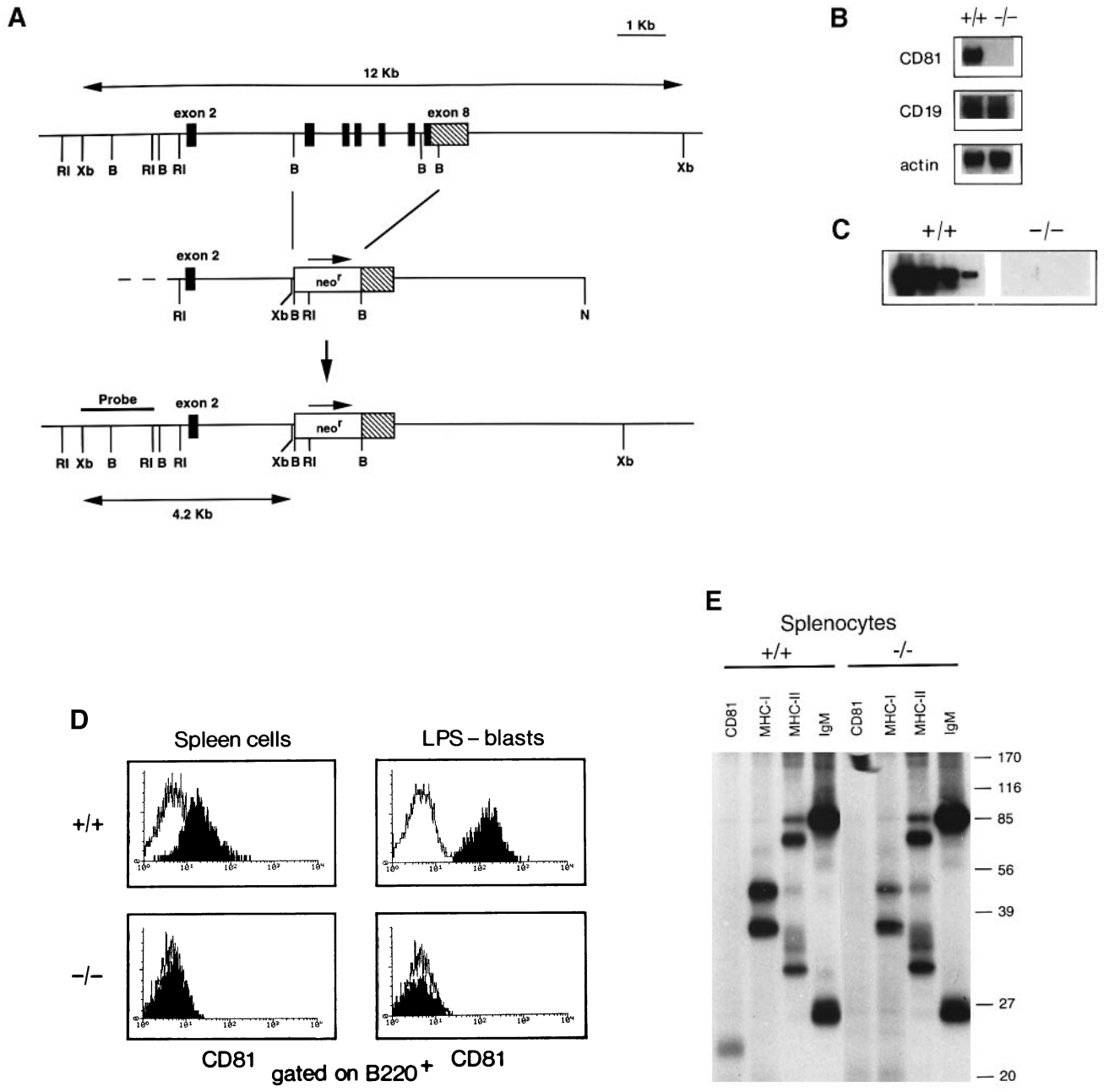


**Fig. 1.** *CD81* gene inactivation. (**A**) Knockout strategy. Restriction maps are shown for the wild-type *CD81* gene locus (top), targeting vector (middle) and recombinant gene locus (bottom). Exons are indicated as black boxes with hatched shading of the untranslated region. Neo$^r$; neomycin resistance gene. Restriction sites: B, *Bam*HI; RI, *Eco*RI; Xb, *Xba*I; N, *Not*I. The *Not*I site in the targeting vector is artificial, derived from the original λ clone from which this pBSK subclone was derived. The probe DNA fragment for Southern blotting is indicated, as are the 12 and 4.2 kb *Xba*I hybridizable fragments in wild-type and mutant DNA, respectively. (**B**) Northern blotting. Fifteen μg of total spleen RNA either from wild-type (+/+) or mutant (–/–) littermates was hybridized with either CD81, CD19 or β-actin cDNA fragments. The same RNA filter was hybridized with each probe after stripping the previous probe. (**C**) RT–PCR for CD81 mRNA. *CD81* gene expression in total thymus RNA of wild-type (+/+) or mutant (–/–) littermates was assessed by semi-quantitative RT–PCR. The amount of template cDNA for PCR corresponds to 50, 5, 0.5 or 0.05 ng from the left lane to the right lane. (**D**) Flow cytometric analysis of CD81 expression. Fresh spleen cells or LPS-blasts were stained for CD81 and B220 (by anti-CD81 mAb and FITC-conjugated secondary antibody, as well as PE-conjugated anti-B220). Histograms show the CD81 expression on B220-positive cells. In all panels, white histograms represent control staining of the indicated cells, stained only with secondary antibody and PE–anti-B220. All mAbs used here were obtained from Pharmingen. (**E**) Splenocytes were radioiodinated with Bolton–Hunter reagent, lysed in RIPA buffer, and the indicated proteins were immunoprecipitated. Immunoprecipitating antibodies were 2F7 (anti-CD81), M1/42.3.9.8 (anti-MHC class I), M5/114.15.2 (anti-MHC class II), and b-7-6 (anti-IgM). Migration of molecular mass markers is indicated in kDa. CD81 migrates as expected at ~25 kDa. Though the level of class I MHC appears less in mutant mice, we could not detect any difference in the amount of class I MHC in separate experiments or when we used digitonin buffer lysis in the same experiment. Moreover, surface staining of class I MHC on thymocytes did not reveal any differences between wild-type and mutant mice (data not shown).

confirmed by cytofluorometric analysis of resting and lipopolysaccharide (LPS)-activated splenic B cells (Figure 1D), as well as immunoprecipitation of CD81 protein from spleen cells (Figure 1E), thymocytes and thymic capsules (data not shown), using 2F7, a mAb against CD81 (Boismenu *et al.*, 1996).

### *T-cell development*

As previously mentioned, Boismenu *et al.* (1996) recently demonstrated that the mAb 2F7, which we used for the protein analysis above, could block thymocyte development in a FTOC system and concluded that CD81 is essential for thymocyte development. Therefore, we examined T-cell development in $CD81^{-/-}$ mice by cytofluorometric analysis of the thymic and peripheral T-cell compartments. Total thymocyte cell numbers were slightly increased in mutant animals as compared with those of wild-type animals [$1.2 \pm 0.5 \times 10^8$ in +/+, $1.6 \pm 0.4 \times 10^8$ in –/– ($n = 10$)]. Staining of CD4 and CD8 surface markers provides a standard profile of thymocyte maturation. Typical staining profiles are presented in Figure 2A. Surprisingly, the CD4/CD8 profiles of $CD81^{-/-}$ thymus were indistinguishable from those of $CD81^{+/+}$ thymus (Figure 2A, upper panels). αβ T-cell antigen receptor (TCR) and heat-stable antigen (HSA) expression were analyzed as maturation markers, but no differences were detected in $CD81^{-/-}$ and $CD81^{+/+}$ mice (Figure 2A, lower panels). Histologically, the structural integrity of the cortex/medulla in the thymic tissue was indistinguishable between mutant and wild-type animals (data not shown). We also analyzed CD25/CD44 and γδTCR expression in the less mature CD4/CD8 double-negative (DN) population, but again no differences were observed (data not shown). We examined thymi from embryos at day 17.5 when CD4/CD8 double-positive (DP) cells grossly develop (Rodewald *et al.*, 1994), but again, there was no detectable difference between $CD81^{-/-}$ and $CD81^{+/+}$ mice (Figure 2B), indicating that maturation also occurs with normal kinetics in the mutant mice.

Total numbers of mature T cells in the lymph nodes and spleen were comparable in the two types of mice, and the relative contributions of the different populations were essentially the same in mutant and wild-type animals. Expression patterns of the T-cell activation markers CD25 and CD44 were also comparable in each population from mutant and wild-type mice (data not shown).

Positive and negative T-cell selection processes are critical stages in the development of mature T cells that can recognize foreign antigen in the context of self-MHC molecules yet are not reactive to endogenous self-antigen peptides. These processes were examined by breeding the $CD81^{-/-}$ mutation into transgenic mice expressing the αβTCR against male antigen (H-Y). Thymocytes expressing the H-Y-specific αβTCRs are positively selected in female H-$2^b$ mice and negatively selected in male H-$2^b$ mice (Kisielow *et al.*, 1988; von Boehmer, 1990). In both females and males, thymocyte phenotypes were comparable in $CD81^{-/-}$ and $CD81^{+/+}$ transgenic H-$2^b$ mice as defined by staining for CD4, CD8 and the transgenic TCR α-chain (Figure 2C), indicating that both selection processes occur normally even in the absence of CD81.

Together, contrasting sharply with the reported results from organ culture experiments, these data strongly suggest that T cells can develop normally in the absence of CD81 molecules.

### *B-cell development*

Development of B-lineage cells was determined by cytofluorometric analysis of bone marrow (BM), spleen and peritoneal cavity cells. BM and spleen cells were double-stained with an antibody to the B cell-specific marker B220 in combination with anti-IgD, A4.1, Gr-1, anti-MHC class II, anti-CD34, anti-CD5, anti-c-Kit, anti-CD43, anti-CD25, anti-IL-7 receptor, anti-CD19, anti-CD23, anti-CD40, anti-IgM and anti-CD21 mAbs, respectively (here we show CD19/B220 and IgM/B220 for BM cells, CD19/B220, CD21/B220, IgM/B220 and IgD/B220 for spleen cells in Figure 3A and B). Despite the early expression and signaling potential of CD81, this analysis of BM cells from mutant animals revealed grossly normal development and expansion of B-lineage cells, except for CD19 expression. As indicated in Figure 3A, mutant BM cells had much duller cell surface expression of CD19 as compared with wild-type BM cells, and there was a distinct compartment characterized as $B220^+CD19^{\text{very low or negative}}$. Splenic B cells from $CD81^{-/-}$ mice were also CD19 dull (Figure 3B), although expression of other surface markers in the B-cell compartment, including CD21, and the antigen receptors IgM and IgD (and others; see above), was similar to that in $CD81^{+/+}$ mice (Figure 3B). Slightly decreased CD19 expression was detected even in heterozygote ($CD81^{+/-}$) mice, in which CD19 expression was at an intermediate level between $CD81^{-/-}$ and $CD81^{+/+}$ mice (data not shown). This low CD19 expression is not due to down-regulated *CD19* gene expression, because CD19 mRNA levels in $CD81^{-/-}$ splenic B cells were indistinguishable from those in $CD81^{+/+}$ splenic B cells (Figure 1B, Northern blot). These observations indicate that CD81 might be involved in efficient cell surface expression of the CD19 molecule. However, one cannot exclude the possibility that lack of CD81 might alter the conformation of the CD19/CD21/CD81/leu13 signaling complex, resulting in loss of epitope recognized by the mAb. Unfortunately, it is difficult to examine this possibility because this is the only anti-CD19 mAb available for mouse B cells. Interestingly, CD21 expression was normal in the absence of CD81, although CD21, like CD19, comprises another element of this common signaling complex on B cells. Similarly, it is reported that CD21 expression was also not affected in $CD19^{-/-}$ mice (Engel *et al.*, 1995; Rickert *et al.*, 1995).

B-1 cells, which predominate in the peritoneal cavity, account for most serum IgM and are distinguished from conventional (B-2) B cells by distinctive cell surface expression of several markers, antigen specificity, signaling and growth properties (reviewed by Hardy and Hayakawa, 1994). Interestingly, B-1 cell formation in $CD19^{-/-}$ mice was reported to be severely affected (Engel *et al.*, 1995; Rickert *et al.*, 1995). In $CD81^{-/-}$ mice, B-1 cells which exhibit the $CD5^+CD23^-IgM^+$ phenotype were also reduced in the peritoneal cavity, but not as drastically as reported for $CD19^{-/-}$ mice (Figure 3D). This slight reduction of B-1 cells in $CD81^{-/-}$ mice could be explained by the dull expression of CD19, although a direct effect through absence of CD81 cannot be excluded. The reduction of serum IgM, which was reported in $CD19^{-/-}$ mice, was not detected in $CD81^{-/-}$ mice. In addition, basal levels of every isotype of serum IgG were comparable in $CD81^{-/-}$ and $CD81^{+/+}$ mice (data not shown).

### *Enhanced T-cell proliferation responses*

To determine peripheral T-cell functions, we evaluated proliferation of T cells from $CD81^{-/-}$ and $CD81^{+/+}$ mice

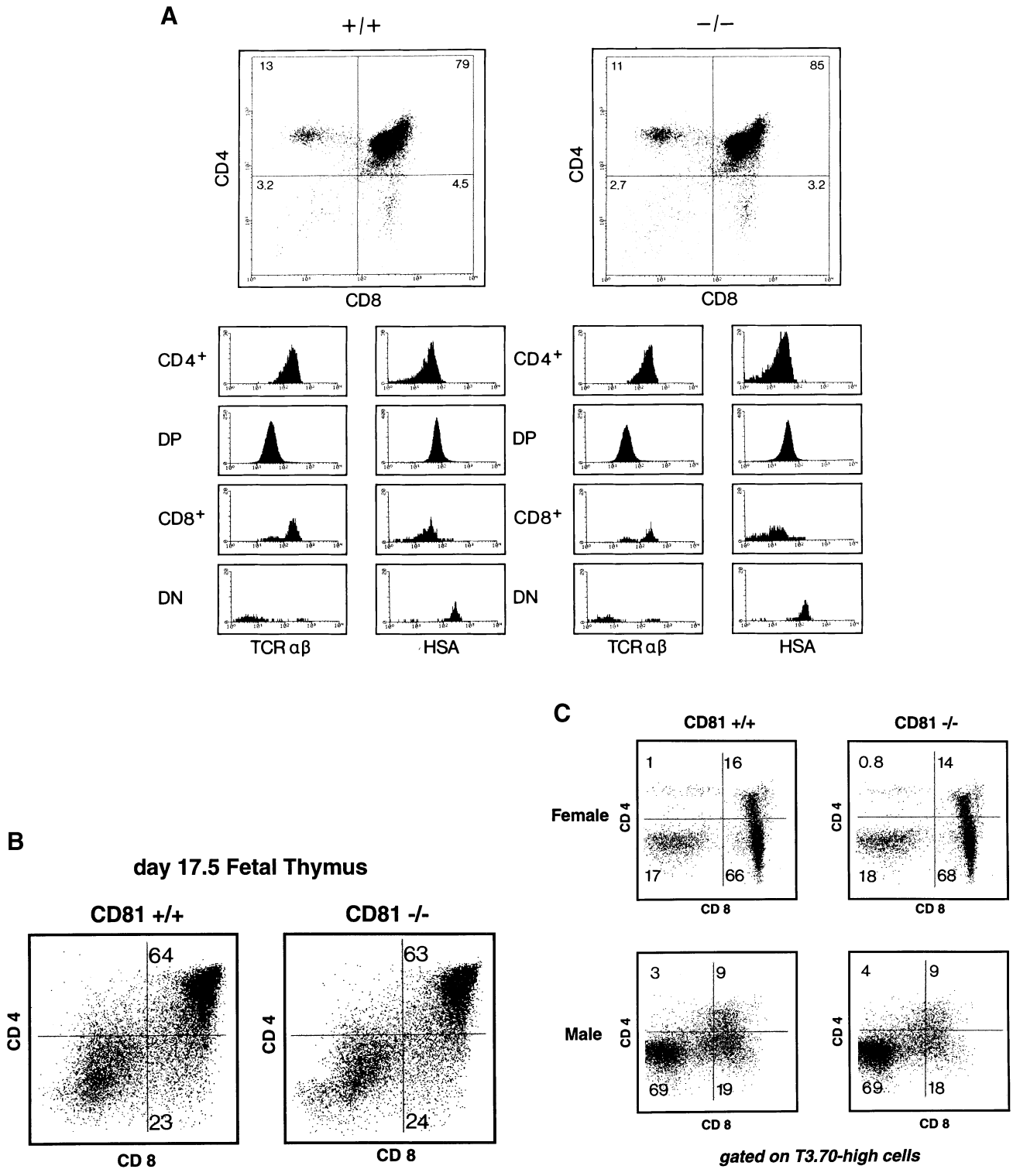


**Fig. 2.** Normal T-cell development in CD81$^{-/-}$ mice. Thymocyte suspensions from (**A**) adult or (**B**) day 17.5 fetal wild-type (+/+) or mutant (–/–) mice were stained and analyzed by flow cytometry. The upper panels display the CD4/CD8 profiles. Numbers indicate the relative percentages of positive cells within a quadrant. αβTCR and HSA histograms of CD4$^+$, double-positive (DP), CD8$^+$ or double-negative (DN) cells are shown in the lower panels of (A). (**C**) Thymocytes from CD81$^{-/-}$ and CD81$^{+/+}$ H-Y TCR-transgenic mice (H-Y Tg$^+$) in a positive-selecting background (H-2$^b$ female) or negative-selecting background (H-2$^b$ male) were stained for CD4, CD8 and anti-transgenic TCR-α chain (T3.70), and analyzed by flow cytometry. CD4/CD8 plots of T3.70-high cells are presented. For all experiments, at least 10 pairs of mutant and wild-type mice were examined.

in response to various stimuli. As shown in Figure 4A, T cells from CD81$^{-/-}$ mice proliferated much better in response to either anti-CD3 mAb, which cross-links the signaling chains of the TCR complex, or concanavalin A (ConA), a lectin which is known to activate T cells. In particular, at 1 μg/well of anti-CD3 mAb, the proliferation of mutant T cells was ~3- to 5-fold more than that of wild-type T cells. When T cells were co-stimulated with

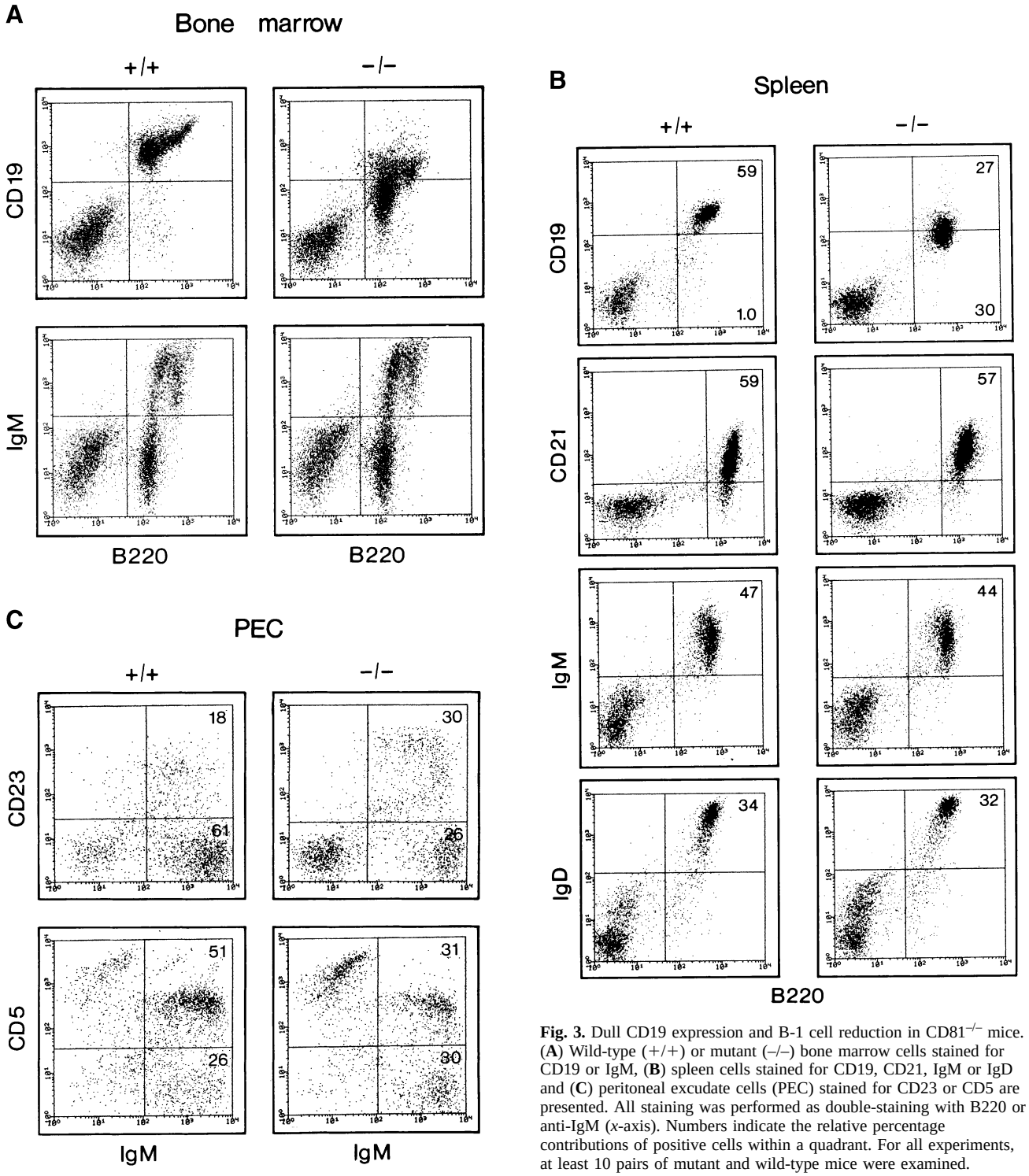


**Fig. 3.** Dull CD19 expression and B-1 cell reduction in CD81$^{-/-}$ mice. (**A**) Wild-type (+/+) or mutant (–/–) bone marrow cells stained for CD19 or IgM, (**B**) spleen cells stained for CD19, CD21, IgM or IgD and (**C**) peritoneal excudate cells (PEC) stained for CD23 or CD5 are presented. All staining was performed as double-staining with B220 or anti-IgM (*x*-axis). Numbers indicate the relative percentage contributions of positive cells within a quadrant. For all experiments, at least 10 pairs of mutant and wild-type mice were examined.

anti-CD28 mAb and either anti-CD3 mAb or ConA, proliferation responses of both CD81$^{-/-}$ and CD81$^{+/+}$ T cells were increased and, even then, CD81$^{-/-}$ T cells showed better proliferation. This observation may exclude the possibility that CD81$^{-/-}$ T cells are already primed for activation, resulting in the enhanced proliferation in response to anti-CD3 or ConA stimulation. A similar increased response of mutant T cells was also elicited by allo-MHC stimulation. Figure 4B depicts a representative mixed lymphocyte reaction (MLR) experiment, illustrating that lymph node T cells from CD81$^{-/-}$ were unusually good responders. T cells from CD81$^{-/-}$ mice showed ~3- to 4-fold increased response to allo-stimulation by antigen-presenting cells (APCs) from B6.H-2$^{bm12}$, a congenic strain with C57Bl/6 (B6) bearing mutations in the class II gene (Figure 4B, left), while these T cells did not react to APCs from mutant animals lacking both MHC class I and class II molecules (Figure 4B, right).

### *Variably altered B-cell proliferation responses*

Like T cells, B cells from CD81$^{-/-}$ mice exhibited enhanced proliferation in response to either anti-CD40 mAb or LPS

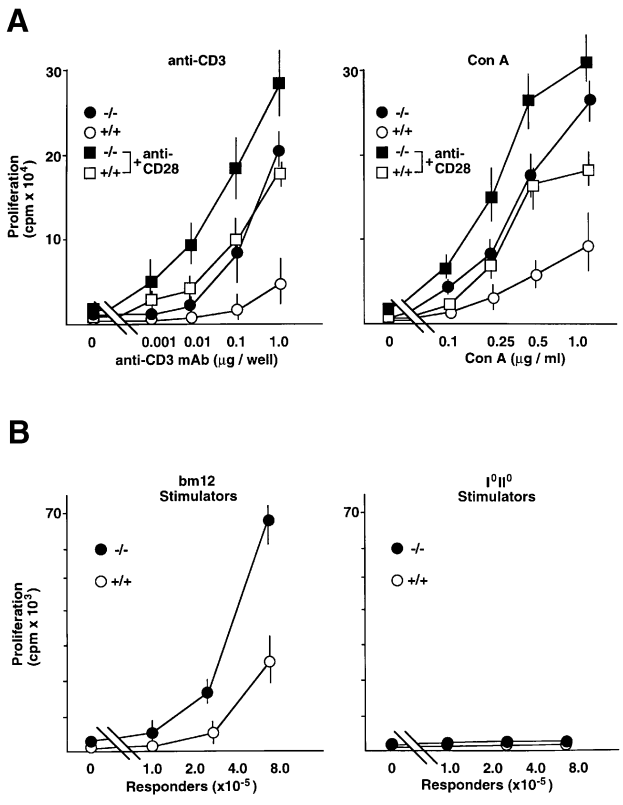


**Fig. 4.** Enhanced T-cell proliferation in CD81$^{-/-}$ mice. (**A**) Lymph node (LN) cells from wild-type and mutant mice were stimulated by culture with either anti-CD3 mAb or ConA in the presence or absence of 50 ng/well of anti-CD28 mAb (○, +/+ mice with anti-CD3 only; ●, –/– mice with anti-CD3 only; □, +/+ mice with anti-CD3 + anti-CD28; ■, –/– mice with anti-CD3 + anti-CD28), or (**B**) in MLR cultures by 2000 rad-irradiated spleen cells from B6.H-2$^{bm12}$ (bm12) or MHC class I$^{-/-}$ class II$^{-/-}$ mice (I$^{0}$II$^{0}$). In all experiments, proliferation was assessed by duplicate [$^{3}$H]thymidine incorporation. In MLRs, spontaneous proliferation-derived counts of corresponding cell numbers of lymph node cells (without APCs) were subtracted from each value. All experiments were repeated with at least four pairs of CD81 +/+ or –/– littermates. In all panels, each value represents the mean of all results and the ranges of data are shown by bars.

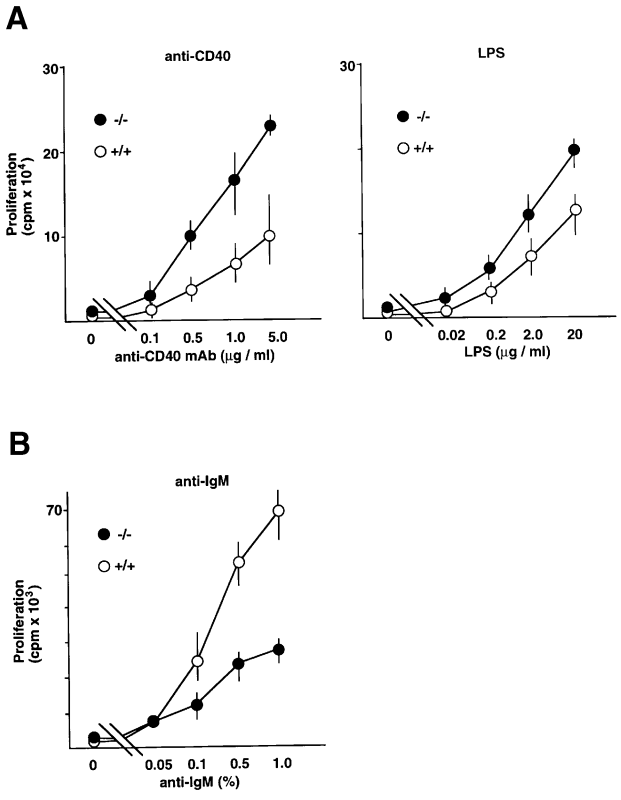


**Fig. 5.** Differentially altered B-cell proliferation in CD81$^{-/-}$ mice. (**A**) Spleen cells from wild-type or mutant mice were stimulated by anti-CD40 mAb or LPS, or (**B**) Sepharose-conjugated anti-IgM mAb. In all experiments, proliferation was assessed by duplicate [$^{3}$H]thymidine incorporation. All experiments were repeated with at least four pairs of CD81 +/+ or –/– littermates. In all panels, each value represents the mean of all individual experiments and the ranges of data are shown by bars.

(Figure 5A). When compared with CD81$^{+/+}$ B cells, their proliferation was increased by ~2- to 3-fold in response to anti-CD40 stimulation and by 1.5-fold in response to LPS stimulation. In contrast, and very importantly, B-cell proliferation following surface IgM (BCR) cross-linking was impaired in CD81$^{-/-}$ mice; it was in fact reduced by up to 40–50% when compared with CD81$^{+/+}$ mice (Figure 5B).

### *T-cell-dependent B-cell activation*

T-cell-dependent B-cell activation was analyzed *in vivo* by antibody production against a T-dependent antigen, keyhole limpet hemocyanin (KLH), and germinal center (GC) formation in swelling lymph nodes after immunizing with ovalbumin (OVA). Although grossly normal primary antibody production by B cells against KLH did result in CD81$^{-/-}$ mice (data not shown), the titer of $IgG_1$-isotype of KLH-specific antibody in the secondary responses appeared partially reduced by up to 50% on average of that of wild-type littermates (Figure 6). In line with this partially impaired T-cell-dependent B-cell activation, GC formation occurred in lymph nodes after immunization

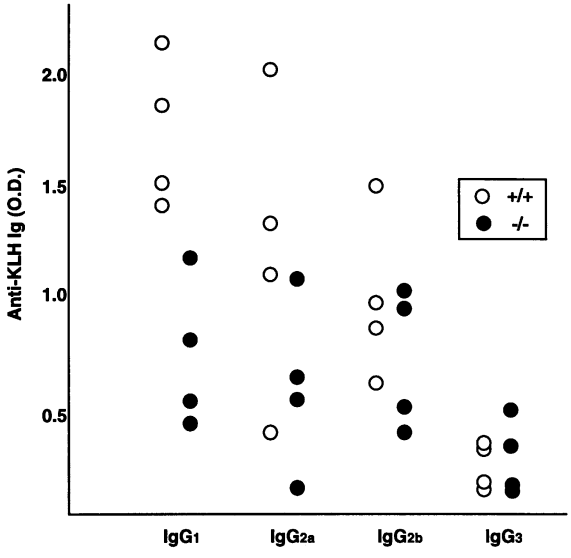


**Fig. 6.** Partially impaired T-cell-dependent B-cell activation. T-cell-dependent antibody production by B cells. Secondary antibody responses are demonstrated. Wild-type (○) and mutant (●) littermates were immunized with 100 μg of KLH in CFA by i.p. injection and boosted 16 days later with 10 μg of KLH in PBS. Serum was collected 4 days after the boost and the content of KLH-specific IgG was measured by KLH-specific ELISA. Each dot represents the response of an individual animal. Sera from unimmunized controls gave OD values of <0.01 in these assays.

with OVA in CD81$^{-/-}$ mice, although the numbers of GC in the swelling lymph nodes were reduced in comparison with those in CD81$^{+/+}$ mice (7–10 per section in +/+ versus 1–4 per section in –/–; data not shown).

### *Intact tyrosine phosphorylation and calcium flux following TCR or BCR engagement*

The differential proliferative responses of antigen receptors between T and B cells begged the question as to whether the absence of CD81 might directly modulate the signaling responsiveness of these receptors. We addressed this question first by evaluating the tyrosine phosphorylation of cellular proteins in lymphocytes following cross-linking of either BCR or the CD3 components of the TCR, expecting that this assay would reflect the initial signal transduction capacities of the receptors (Robey and Allison, 1995). Interestingly, in both cases, the pattern, degree and kinetics of phosphorylated substrates were essentially comparable in mutant and wild-type mice (data not shown). Thus, the tyrosine phosphorylation profiles from CD81$^{-/-}$ mice did not mirror the decreased and increased proliferative responses of B and T lymphocytes, respectively. Similarly, intracellular calcium flux following antigen receptor cross-linking was also evaluated and, again, no detectable differences were observed in kinetics, magnitudes and dose responses (data not shown).

## Discussion

### *CD81 is not essential for early T-cell development*

The potential involvement of CD81 in *in vivo* T-cell development has drawn considerable interest following a recent report which implicated CD81 as an important mediator of the pre-TCR-dependent development of DP thymocytes in *in vitro* FTOC experiments (Boismenu *et al.*, 1996). Two kinds of *in vitro* experiments were provided in that report to reach this conclusion. First, an mAb, 2F7, which recognizes CD81 could block thymocyte development in an FTOC system. Second, addition of CD81-transfected CHO cells allowed progression of DN precursor T cells to the DP developmental stage in a reaggregation culture system. Therefore, it was surprising that T cells developed normally in our CD81$^{-/-}$ mice. Having comparable developmental phenotypes in wild-type and mutant animals both in fetal and adult thymi, it appears unlikely that CD81 is required specifically for development of fetal thymocytes.

One could argue that the 2F7 mAb might affect *in vitro* thymocyte development by generating an inhibitory signal via cross-linking a distinct CD81-containing receptor complex on thymocytes. This argument might explain the results of the FTOC experiments in which 2F7 could block the thymocyte development, although it contrasts with the results of reaggregation experiments with CD81-transfected CHO cells, which implicated CD81-mediated signals as a positive effector for early T-cell development.

An alternative explanation for the discrepancy in the results between the knockout mice and the organ cultures is that the mAb used in the blocking experiments might cross-react with other molecules which are essential for thymocyte development. This appears unlikely, however, because cytofluorometric analysis using the same mAb did not detect any cross-reactive surface protein on CD81$^{-/-}$ spleen cells. Moreover, in immunoprecipitation from spleen cells, thymocytes and thymic capsules of CD81$^{-/-}$ mice or CD81$^{+/+}$ mice with the 2F7 mAb, no additional specific signals could be detected.

Alternatively, one might expect that other TM4SF proteins which are also expressed in the thymus, such as CD63 and CD82 (Nagira *et al.*, 1994), could take over the role of CD81 in thymocyte development. This possibility cannot be excluded.

Thus, although we cannot definitively exclude the possibility that CD81 might have some roles in thymocyte development, thymocytes can develop normally even in the absence of CD81 expression.

### *CD81 potently regulates lymphocyte proliferation in a multipronged manner*

CD81 originally was named as the target of anti-proliferative antigen (TAPA-1) because anti-CD81 antibodies inhibit the proliferation of many lymphoid cell lines. It has been controversial, however, whether these anti-CD81 antibodies induce negative signals to the target cells or interfere with the ligation of CD81 and its potential ligand(s). Our results show that CD81 acts as a negative or positive mediator of lymphocyte proliferation, dependent on the types of stimuli. In the absence of CD81, lymphocytes exhibited enhanced proliferation in response to CD3 cross-linking, ConA, allo-MHC, CD40 cross-linking or LPS, whereas proliferation in response to IgM/BCR cross-linking was impaired. These diverse alterations of proliferation responses in T and B lymphocytes from CD81$^{-/-}$ mice suggest the involvement of CD81 in disparate signaling pathways for lymphocyte proliferation. Potential signaling mechanisms through CD81 are as yet undefined, because the three putative intracellular domains of this TM4SF protein are each only 10 amino acids long and contain no known motifs for signal transduction (Andria *et al.*, 1991). Since CD81 is associated with various molecules on the cell surface (reviewed by Fearon, 1993; Tedder *et al.*, 1994; Imai *et al.*, 1995; Rubinstein *et al.*, 1996; Szollosi *et al.*, 1996), one could envisage that the individual complexes of CD81 and varied associated molecules may provide distinct intracellular signals in different contexts. In this scheme, the multifaceted regulation of lymphocyte proliferation by CD81 might be due to its varied coupling partners.

Decreased proliferative response of CD81$^{-/-}$ B cells following IgM cross-linking could be due to the low CD19 expression, although the enhanced proliferation in response to anti-CD40 mAb or LPS cannot be explained in this manner. The effect of CD19 in B-cell proliferation is still controversial because one group reported normal proliferation of B cells in response to IgM cross-linking in CD19$^{-/-}$ mice (Rickert *et al.*, 1995) and another group reported an impaired proliferation response (Engel *et al.*, 1995). Moreover, despite the impaired B-cell proliferation following IgM cross-linking, antibody production by B cells against the T-dependent antigen *in vivo* was not strikingly affected in CD81$^{-/-}$ mice, with only a partial decrease in the production of the $IgG_1$ isotype. In contrast, it was severely impaired in CD19$^{-/-}$ mice. Measuring a T-dependent B cell response in CD81$^{-/-}$ mice, however, might be complex. One could argue that the hyper-responsiveness of T cells might compensate for the

impaired B-cell activity. Further studies are necessary to dissect these apparently complex phenotypes.

Although both proliferation responses following either TCR or BCR were affected in CD81$^{-/-}$ mice, both tyrosine phosphorylation and intracellular calcium flux in lymphocytes following the engagement of these cell surface receptors were entirely comparable with those in wild-type mice. These results clearly demonstrate that the initial signaling events of both TCR and BCR/surface IgM were not altered in CD81$^{-/-}$ lymphocytes, suggesting that CD81 might differentially modify the cell proliferation responses at some point downstream of these membrane-proximal events. Consistent with this conclusion, there is no evidence suggesting direct associations between CD81 and either TCR or BCR. The absolute biochemical mechanisms through which CD81-mediated signals affect the regulation of lymphocyte proliferation are still unclear, and further studies are required. Finally, these CD81-mediated modulations of cell proliferation events might occur in later stages of cell activation, particularly in view of the increased expression of CD81 after activation of B cells, T cells and thymocytes (B cells, Figure 1D; T cells and thymocytes, T.Miyazaki, unpublished data).

### Perspective

Newly generated CD81 knockout mice have provided novel insights, two points of which are worth re-emphasizing. First, expression of CD81 is not necessary for normal T-cell development. Second, CD81 potently regulates lymphocyte proliferation responses in a multipronged manner. These mutant mice may provide clues to understanding some lymphoproliferative disorders, and underscore the need for further dissection of the varied roles of CD81 in proliferation of both lymphoid and non-lymphoid cells.

## Materials and methods

### Generation of CD81$^{-/-}$ mice

A mouse genomic DNA clone containing exons 2–8 of the *CD81* gene was isolated by screening a genomic DNA library derived from the 129/sv mouse strain. The targeting vector was prepared using a 10 kb *Eco*RI–*Not*I (the *Not*I site was in the phage arm) fragment containing exons 2–8 of the *CD81* gene, pMC1-neo-polyA (Stratagene) plasmid and the pBluescript (pBSK) plasmid (Stratagene). This construct was designed to delete a 2.5 kb *Bam*HI fragment from CD81, encompassing exons 3 to part of exon 8 (including the TGA stop codon) as schematized in Figure 1A. The *Not*I-linearized targeting vector was electroporated into the E-14.1 ES line as previously described (Miyazaki *et al.*, 1996a). G418-resistant clones were isolated and screened by Southern blotting. Four of 146 G418-resistant clones had undergone the desired homologous recombination. Positive clones were injected into B6 blastocysts, and chimeric offspring were mated to B6 females. Mice carrying the mutation in the heterozygous state (CD81$^{+/-}$) were inter-crossed to produce homozygous mutants (CD81$^{-/-}$) and 4- to 8-week-old mice were used for analysis.

### RNA analysis

Northern blotting was performed using total RNA of spleens from wild-type or mutant littermates using standard conditions. Probes for hybridization were: for a CD81 cDNA fragment (positions 67–647; numbering 1 at the ATG start codon), for a CD19 cDNA fragment (positions 1–417; numbering as for CD81) and a mouse β-actin full-length cDNA fragment. All probe cDNAs were amplified by RT–PCR from mouse spleen RNA and sequenced. RT–PCR was performed using total thymus RNA as template, as described (Miyazaki *et al.*, 1996b). Primers used for PCR were 5′-GAGGGCTGCACCAAATGCATC (positions 10–30 in exon 2) and 5′-TCTCATGGAAAGTCTTCACCA (positions 457–437 in exon 5). Ten percent of each PCR product was loaded on a gel, blotted and hybridized with the CD81 cDNA fragment.

### Cell surface staining and flow cytometry

The mAbs for staining T cells: anti-CD4 [phycoerythrin (PE)-conjugated], anti-CD8 [fluorescein isothiocyanate (FITC)-conjugated or biotinylated], anti-αβTCR (FITC-conjugated), anti-γδTCR (FITC-conjugated), anti-HSA (biotinylated), anti-Vβ8 (FITC-conjugated), anti-CD44 (FITC-conjugated), anti-CD25 (PE-conjugated) and streptavidin-tricolor were all purchased from Pharmingen. Anti-CD81 (2F7, hamster IgG anti-mouse) and FITC-conjugated anti-hamster IgG were also obtained from Pharmingen. The mAbs for staining B-lineage cells: anti-IgD, A 4.1, Gr-1, anti-MHC class II, anti-CD34, anti-CD5, anti-c kit, anti-CD43, anti-CD25, anti-IL-7 receptor, anti-CD19, anti-CD23, anti-CD40, anti-IgM and anti-CD21 (all biotinylated) were kindly provided by Dr R.Ceredig, Basel. PE-conjugated B220 and streptavidin–FITC were purchased from Pharmingen. Cells were stained with saturating levels of mAbs and analyzed using a FACScan cytometer.

### In vitro proliferation assays

Lymph node cells ($5\times10^5$/well) were cultured in the presence of various concentrations of either immobilized anti-CD3 mAb (KT-3; kindly provided by Dr C.Benoist, Strasbourg) or ConA (Sigma) in the presence or absence of anti-CD28 mAb (Pharmingen; 50 ng/well). The KT-3 mAb was immobilized by pre-incubation of wells at 4°C overnight. Spleen cells were cultured in the presence of various concentrations of either LPS (Sigma), anti-CD40 mAb (FGK-45.5; kindly provided by Dr J.Andersson, Basel) or Sepharose-conjugated anti-IgM (M41). All cultures were performed for 3 days and proliferation was assessed by [$^3$H]thymidine incorporation in the last 16 h of cultures.

### MLRs

Responders (total lymph node cells) were cultured with stimulators (2000 rad γ-irradiated spleen cells) at various ratios for 5 days. Proliferation was assessed by [$^3$H]thymidine incorporation in the last 16 h of cultures. MHC class I$^{-/-}$ class II$^{-/-}$ mice were kindly provided by Dr C.Benoist.

### T-cell-dependent antibody production assay

Mice were immunized by i.p. injection with 100 μg of KLH (Calbiochem, USA) in complete Freund's adjuvant (CFA) and, 16 days later, mice were boosted with 10 μg of KLH in phosphate-buffered saline (PBS). Blood serum was taken at day 20 (4 days after the second immunization). The titers of anti-KLH antibody were measured by enzyme-linked immunosorbent assay (ELISA), as described previously (Le Meur *et al.*, 1985).

### GC formation

Mice were immunized with 50 μg of OVA protein (Sigma) in CFA (Sigma) subcutanously in the hind foot pads. Swelling knee lymph nodes were isolated 10 days later. Serial sections of lymph nodes were stained with biotin–peanut agglutinin (PNA) (Sigma) or biotin–anti-IgD (kindly provided by Dr R.Ceredig, Basel). Signals were detected with streptavidin–alkaline phosphatase and developed with Alkaline Phosphatase Substrate Kit (Vector Labs). Samples were counterstained with hematoxylin by standard procedures.

### Cell surface radioiodination and immunoprecipitation

Splenocytes ($3\times10^7$), thymocytes ($5.5\times10^7$) and a thymic capsule were isolated from crushed organs, and red blood cells were lysed with 155 mM $NH_4Cl$ and 17 mM Tris (pH 7.6; 10 min at 24°C). Cells were surface radiolabeled with $^{125}$I using the Bolton–Hunter reagent (Sulfo-SHPP; Pierce Chemical Co.) and IODO-GEN (Pierce). Labeled cells were lysed on ice with RIPA buffer containing 1% Triton X-100 (Pierce), 0.1% SDS, 0.1% Na deoxycholate, 10 mM Tris (pH 7.4), 150 mM NaCl, 0.4 mM EDTA, 10 mM NaF, 2 mM Na orthovanadate, 1 mM Pefabloc SC (Boehringer) and 1 μg/ml each of soybean trypsin inhibitor, aprotonin and leupeptin (all from Sigma). Lysates were centrifuged at 15 000 r.p.m. for 15 min, pre-cleared overnight at 4°C with 3 μg of anti-rat CD8α antibody (OX-8; mouse $IgG_1$; Pharmingen) and 30 μl of protein G–Sepharose (Pharmacia), and aliquots were immunoprecipitated for 4 h with protein G–Sepharose which was pre-coupled with 2.5 mg of either anti-mouse CD81 antibody (2F7), anti-mouse H-2 K (M1/42.3.9.8; rat $IgG_{2a}$), anti-mouse class II (M5/114.15.2; rat $IgG_{2b}$) or anti-IgM (b-7-6; rat $IgG_1$; all were provided by Dr J.Cambier, Denver). Immunoprecipitates were washed six times with RIPA buffer (as above), denatured in reducing Laemmli sample buffer and separated on a 12.5% SDS–PAGE gel that was dried and subjected to autoradiography.

### *Anti-phosphotyrosine immunoblotting of cell lysates*

Thymocytes ($10^6$/0.5 ml PBS) were pre-incubated with or without 10 μg/ml anti-CD3ε (145-2C11; hamster IgG; Pharmingen) on ice for 30 min, flash-microfuged, and pelleted cells were all resuspended in pre-warmed PBS (0.5 ml) at 37°C which contained 5 μg/ml anti-hamster IgG (mouse $IgG_1$ and $IgG_{2b}$ cocktail, non-reactive to mouse Ig; Pharmingen). Splenocytes were pre-warmed at 37°C for 15 min prior to stimulation with or without goat anti-mouse IgG and IgM (Fab′, H and L chain-specific, 10 μg/ml; Jackson ImmunoResearch Labs Inc.). After stimulation for 1, 5 or 15 min on a shaking heat block, all samples were flash-microfuged, and pelleted cells were lysed in reducing Laemmli sample buffer, immediately frozen in liquid $N_2$, stored at –70°C, thawed at 95°C for 3 min, probe-sonicated for 7 s, separated on 11% SDS–PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were immunoblotted with horseradish peroxidase-conjugated 4G10 antibody (Upstate Biotechnology) as previously described (Campbell *et al.*, 1996).

### *Analysis of intracellular calcium*

Splenocytes were isolated from mutant and normal mice and red cells lysed as for radioiodination, above. Cells were divided in half for immunofluorescence staining of (i) B cells and NK cells [PE-conjugated anti-B220 and anti-CD16/CD32 (2.4G2); Pharmingen] or (ii) T cells (PE-conjugated anti-CD3ε) at saturating mAb concentrations. Cells were then loaded with indo-1 AM (5 μM in IMDM medium at $5\times10^6$/ml; Molecular Probes) for 30 min at 37°C, washed and resuspended in IMDM at room temperature ($2\times10^6$/ml). Immunofluorescent stained populations were gated out of the analysis on FACS to monitor separately the stimulation of (i) T-cell or (ii) B-cell populations, respectively, to minimize prior manipulation of the splenocytes. Intracellular calcium concentrations were determined from emission ratio analysis using a Becton Dickinson FACS Vantage cytofluorimeter and WinMDI software (version 2.4 by Joseph Trotter, Scripps Institute). After a 30 s baseline was achieved, T cells were stimulated with either 2 or 20 μg/ml of anti-CD3ε (145-2C11; kindly provided by Dr Thomas Bäckström, Basel) and B cells with 1 or 10 μg of anti-IgM (b-7-6) and analyzed for 6.5 min.

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