

## DIFFERENTIAL SUSCEPTIBILITY TO ANTI-RECEPTOR INDUCED APOPTOSIS IN ADULT MURINE B-CELLS: ROLE OF B1 CELLS

Bourke R. Maddox and David W. Scott<sup>1</sup>

Immunology Department, American Red Cross Holland Laboratory, Rockville, MD 20855 USA

### ABSTRACT

We and others have recently found that mature murine B cells can be induced to undergo apoptosis, *in vitro*, in a dose-dependent manner, by extensive crosslinking of membrane IgM with polyclonal anti- $\mu$ . During the analysis of tolerance in transgenic mice expressing rearranged IgM or IgM<sup>+</sup> IgD receptors, we observed that, Sp6 anti-TNP Ig and anti-MHC transgenic splenocytes, would undergo receptor-mediated apoptosis *in vitro* just like their normal, non-transgenic littermates. However, transgenic mice expressing rearranged receptors typical of B1 cells, not only contained large numbers of CD5<sup>+</sup> cells in their spleens, but these cells failed to undergo apoptosis under conditions that led to programmed cell death in normal splenocytes. B1 spleen cells also failed to proliferate with anti-IgM, although the responsiveness of cells from the other transgenic lines varied depending on the background strains. These differences are due in part, to strain differences, but they also imply that the response pattern of transgenic B cells reflects not only the subset composition in this organ, but also the transgenic specificity of the receptor.

### INTRODUCTION

Two distinct subsets of B cells have been described in mice and humans: the conventional B cell, predominant in the spleen and lymph nodes, and the B1 (CD5<sup>+</sup>) cell, which comprises the majority of B cells in the peritoneum ( $\geq 85\%$ ). B1 cells, which are prevalent early in ontogeny and possibly play a role in B-cell repertoire maturation, have been shown to express polyvalent, low affinity antibodies with self-reactivity (1) and have been linked to several autoimmune diseases (2). We (3,4) have previously shown that B1 cells are resistant to tolerance protocols involving crosslinking of sIgM that lead to hyporesponsiveness of conventional B cells. B1 cells, however, can be rendered hyporesponsive to LPS challenge by ionomycin or activation of protein kinase C with phorbol esters, for example, suggesting that

B1 cells are defective in a calcium-dependent protein kinase C pathway (4, 5). In this communication, we compare the ability of B1 and conventional B cells to undergo programmed cell death (PCD) via apoptosis, and proliferation in response to sIgM crosslinking. To examine this, we used transgenic mice that either express rearranged receptors typical of the B1 repertoire, or rearranged receptors typical of conventional B cells; spleens from the B1 transgenic mice contain primarily cells of the B1/CD5<sup>+</sup> type, unlike their normal counterparts. We have previously shown that the defect in signaling in murine B1 cells is not related to their location *in vivo*, but rather to differences in membrane signaling (4). It is these differences, and how they relate to PCD, that are of interest.

### MATERIALS AND METHODS

#### Animals.

All transgenic animals used are lines previously described (4). Briefly, line 6.1 V<sub>H</sub>12 transgenic and non-transgenic littermates are identical to C57BL/10.A at the MHC locus (H-2<sup>a</sup>), but carry the IgMb locus of the C57Bl/6 background. Ninety six to one hundred percent of splenic B cells from this line expressed the transgenic V<sub>H</sub>12 idiotype (Id) found in the CH27 lymphoma (4, 6), and significant serum levels of Id were present by 4 weeks of age; ~66-75% of their B cells were CD5<sup>+</sup>. Anti-MHC class 1 transgenic mice (3.83; ref. 7), which possess rearranged IgM and IgD receptors specific for mouse H-2K<sup>k</sup>, were originally provided by Dr. David Nemazee (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Transgenic and non-transgenic, normal littermates were obtained through backcrossing with BALB/cByJ from the Jackson Laboratory as previously published. The Sp6 ( $\mu + \kappa$ ) anti-trinitrophenyl (anti-TNP) homo-zygous transgenic (Sp6; ref. 8) mice were generously provided by Dr. Rinus Lamers and the late Prof. Georges Köhler (Max Planck Institute for Immuno-biology, Freiburg, Germany) and have been backcrossed to C57Bl/6 or Balb/c.

#### Antibodies and other reagents.

Goat anti-mouse IgM,  $\mu$  heavy chain-specific (Southern Biotech Associates, Inc. Birmingham, AL), was used for all apoptosis and proliferation assays. Cells were cultured in RPMI-1640 (Bio-Whittaker, Walkersville, MD) with 5% heat inactivated fetal bovine serum (FBS, Sigma Chemical Company, St. Louis, MO) and  $5 \times 10^{-5}$  M

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<sup>1</sup>To whom correspondence should be addressed, at American Red Cross Holland Lab, 15601 Crabbs Branch Way, Rockville, MD 20855 USA Tel #: (301) 517-0335, Fax #: (301) 517-0344, Email: scottdd@hlsun.red-cross.org

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2-mercaptoethanol. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 and pokeweed mitogen (PWM) were both purchased from Sigma.

### Cell preparation, proliferation and apoptosis assays.

Splenic single cell suspensions were obtained and red cells removed as described previously (3-5). Viable cell counts were performed using trypan blue exclusion, and suspensions were normalized to  $5 \times 10^6$  cells per ml. Unless otherwise noted, all spleens were processed individually, in parallel, on a given day.

Proliferation assays were performed as described earlier (5). Briefly, cells were cultured in 96 well tissue culture plates at 37 °C, 7% CO<sub>2</sub>, at  $5 \times 10^5$  cells per well in 200 µl complete medium. The indicated doses of anti-IgM (LPS or PWM; not shown) were added at time zero, and the cells pulsed at 44-50 hours with 0.5 µCi [methyl-3H]-thymidine (Amersham Life Sciences, Inc.); plates were harvested at 50 hours on a Filtermate 196 harvester (Packard Instrument Company, Downers Grove, IL) onto glass fiber filters. Filters were allowed to air dry overnight and then read on a Matrix 9600 Direct Beta Counter (Packard).

For apoptosis (9-11), spleen cells were cultured for 24 hours at 37 °C, 7% CO<sub>2</sub>, in 24 well tissue culture plates. Doses of anti-IgM, as indicated, were added at time zero to one ml of  $5 \times 10^6$  cells/ml per well. Pellets were washed twice with cold PBS and then resuspended in 70% ethanol and maintained at 0 °C for a minimum of one hour to fix. When noted, B cells were labeled with FITC-anti-B220 before fixation and the positive cells gated in order to only analyze B220<sup>+</sup>, IgM<sup>+</sup> populations. After fixation, cells were then washed twice with cold PBS and resuspended in one ml PBS containing 10 µg/ml RNase (Sigma) for 30 minutes at 37 °C. Propidium iodide was added to a final concentration of 50 µg/ml, and allowed to equilibrate for ten minutes. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), using FL2 channel and low flow rate. FL2 area and width were used to gate out doublets, and front scatter and FL2 height were used to gate out debris; a minimum of 10,000 gated cells were collected for each sample. Final data was prepared using gated cells on an FL2 height histogram, with apoptotic cells appearing as a sub 2N population to the left of G<sub>0</sub>/G<sub>1</sub> peak (9).

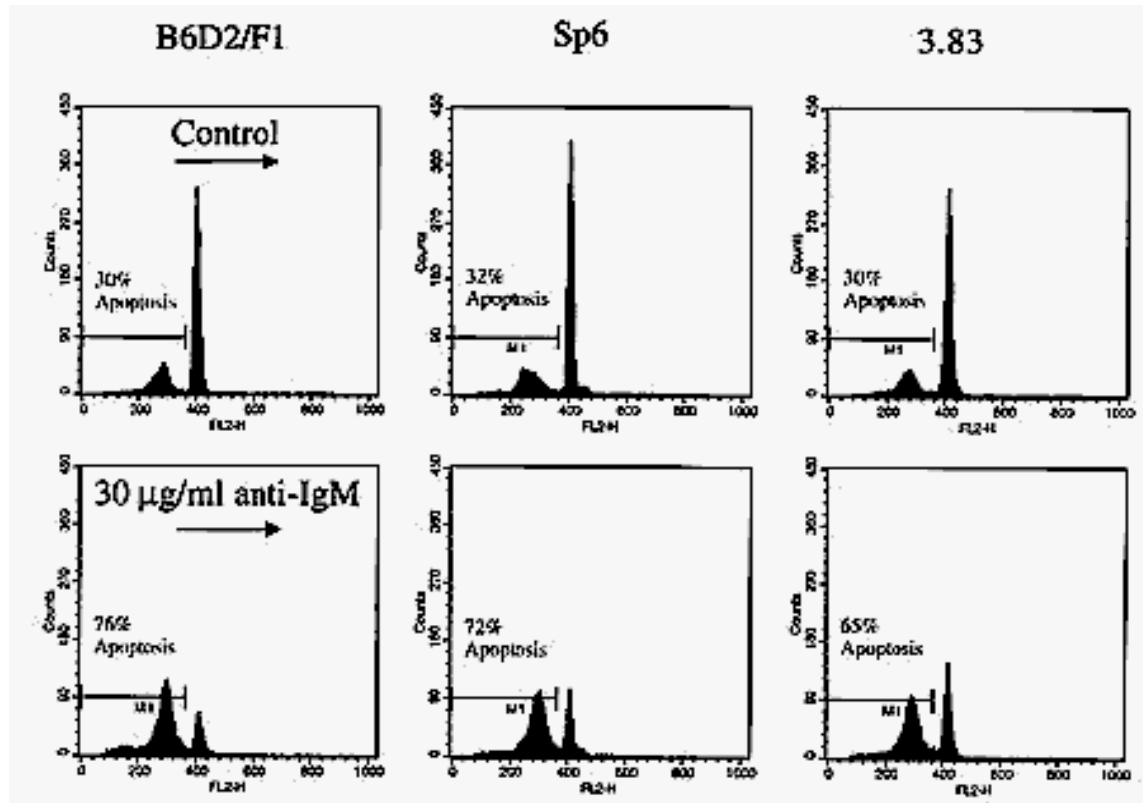
It should be noted that the numbers of B cells in each line was relatively constant, ranging from 38-65%, although the 6.1 line possessed a large number of B220<sup>low</sup> cells, typical of the CD5 subset.

## RESULTS

Transgenic mice, expressing IgM receptors typical of either B1 or conventional B cells, were used to compare susceptibility of these B cell types to the induction of PCD by anti-IgM. We used three lines of transgenics, one expressing IgM receptors typically of the B1 repertoire, and two lines expressing receptors typical of conventional B cells. In each case, we used non-transgenic littermates for each line as controls, as well as normal C57Bl/6 x DBA/2J F1 (B6D2) mice. In particular, line 6.1 spleen cells expressed >96% of the CH27 V<sub>H</sub>12 idiotype seen in peritoneal B1 cells, whereas the 3.83 and Sp6 lines contained primarily conventional (B2) cells in the spleen. Importantly, more than 66-75% of splenic B cells in line 6.1 mice are CD5<sup>+</sup>, the remainder having the characteristics of B1 "sister cells" (4). Therefore, this allows us to compare splenic populations from different IgM transgenic lines in terms of their susceptibility to anti-IgM-induced apoptosis and eliminates problems associated with purification of peritoneal B cells. The standard protocol of hyper-crosslinking sIgM with anti-µ specific goat antibodies, followed by a 24 hour incubation period (9, 10) was employed. Cultures were then examined by FACScan® for apoptotic B-cell populations after gating on B220<sup>+</sup> cells. The results in Figure 1-2 summarize the observed differences.

### Susceptibility of Sp6 anti-TNP transgenic B cells to programmed cell death with anti-µ.

It has been reported that the majority of splenic B cells in the Sp6 anti-TNP line are transgenic, CD5<sup>+</sup>, and express IgM and B220 at levels typical of normal splenic B cells. This phenotype suggests that these transgenic B cells are of the conventional B2 cell type (4, 8, 12), except that they do not express sIgD due to lack of endogenous rearrangements. We have shown in prior studies that splenic B cells from these animals can be rendered unresponsive to subsequent challenge through sIgM crosslinking in a manner similar to non-transgenic conventional B cells (4). Moreover, Carsetti and colleagues demonstrated that Sp6 B cells would undergo programmed cell death, *in vivo* and *in vitro*, with a specific antigen like TNP-dextran (12). This suggests an intact and functioning membrane IgM signaling pathway. We report here that Sp6 splenic B cells also undergo induced PCD in a normal, dose-dependent manner (Figure 1 middle). In repeat experiments, the dose response curves for Sp6 transgenic B cells were similar to both non-transgenic littermates and B6D2 controls (Figure 1 left and data not shown).



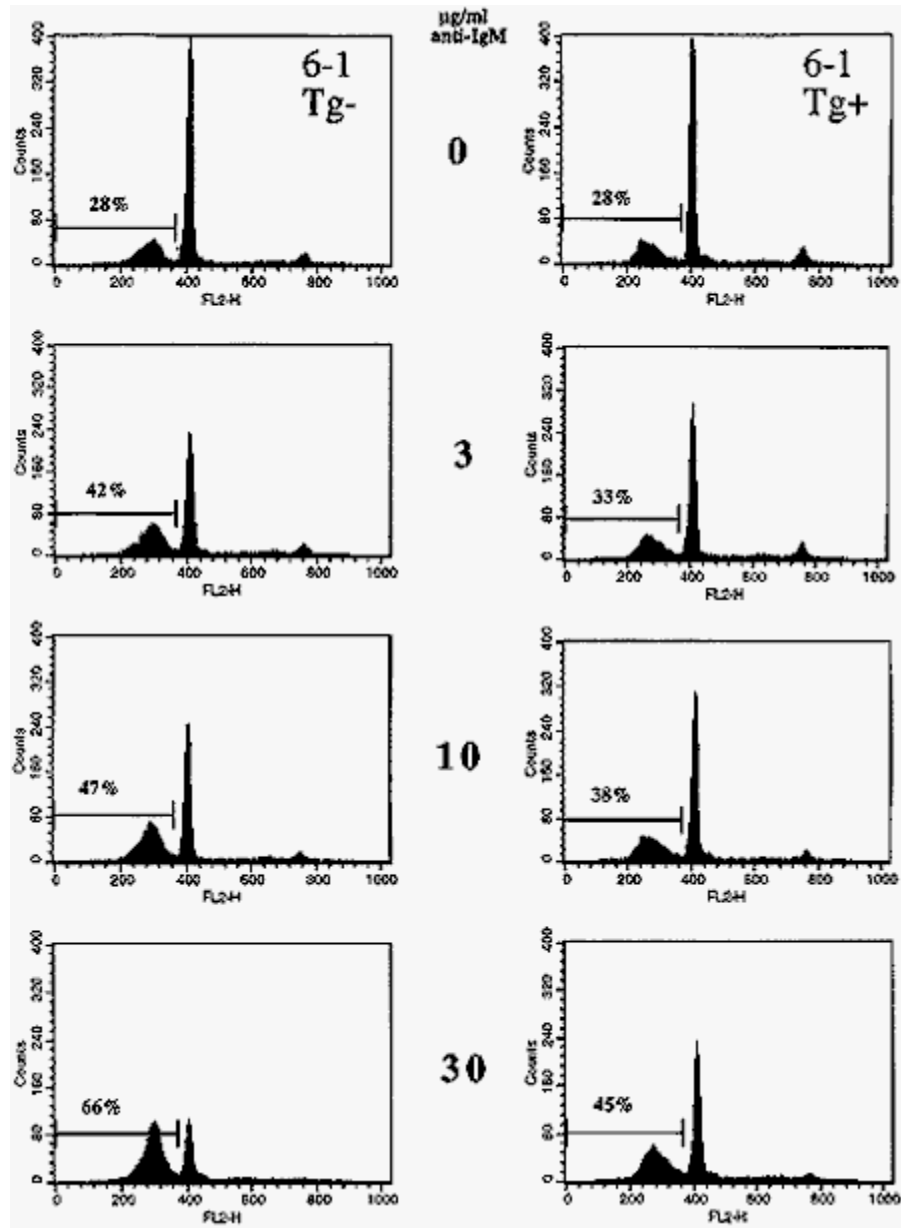
**Figure 1.** Induction of apoptosis by anti-IgM in non-transgenic and transgenic B cells. Assay for apoptotic populations was performed with propidium iodide staining and FACS analysis as described in Materials and Methods. Splenic B cells from B6D2/F1, Sp6 (anti-TNP), and 3.83 (anti-MHC) mice were analyzed for spontaneous and inducible apoptosis. Top panels contain background apoptosis controls (medium alone); the bottom panels show apoptosis induced with 30  $\mu\text{g/ml}$  anti-IgM. Histograms are from a single experiment, but are representative of at least three similar experiments. These data show that Sp6 and 3.83 transgenic B cells exhibit similar levels of apoptosis when compared to normal B6D2/F1 control mice, and non-transgenic littermates (data not shown).

#### Susceptibility of 3.83 anti-MHC transgenic B cells to programmed cell death with anti- $\mu$ .

The results above suggest that transgenic anti-TNP specific B cells respond normally to hyper-crosslinking signals with anti- $\mu$ . It was important to repeat these experiments in another transgenic strain expressing Ig receptors of a different specificity. We chose to use the 3.83 anti-MHC transgenic line both because it had been used previously to demonstrate deletion of the developing B-cell repertoire (7) and to be susceptible to tolerance *in vitro* in our anti- $\mu$  protocol (4). Spleen cells from 3.83 transgenic and non-transgenic control littermates were treated with anti- $\mu$  as above and analyzed for apoptosis. The results in Figure 1 (right) indicate that the anti-MHC transgenics also responded normally to hyper-crosslinking with anti- $\mu$  by undergoing PCD, as was seen in B6D2 and Sp6 conventional splenocytes.

#### V<sub>H</sub>12 anti-phosphatidyl choline transgenic B cells are resistant to programmed cell death with anti- $\mu$ .

We have previously reported that V<sub>H</sub>12 transgenic mice, as well as CH12 transgenics, are resistant to tolerance induction with anti- $\mu$  (4). Both strains contain large numbers of B1 cells, either CD5<sup>+</sup> or CD5<sup>-</sup> "sister" cells in their spleens. To establish the susceptibility of V<sub>H</sub>12 transgenic B cells to apoptosis, we repeated the anti- $\mu$  protocol above. The results in Figure 2 show that V<sub>H</sub>12 transgenic B1 B cells are relatively resistant to anti- $\mu$ -driven PCD. Note that non-transgenic control spleen cells, which possess similar numbers of B cells, examined simultaneously were susceptible to PCD just like the previous conventional strains tested (see Figure 1). Moreover, this is not simply a difference in dose response curves because a full range of anti- $\mu$  concentrations was done in each experiment (Figure 2). This suggests that one explanation of the resistance to tolerance of B1 cells is their failure to process the hyper-crosslinking signals that lead to apoptotic cell death.

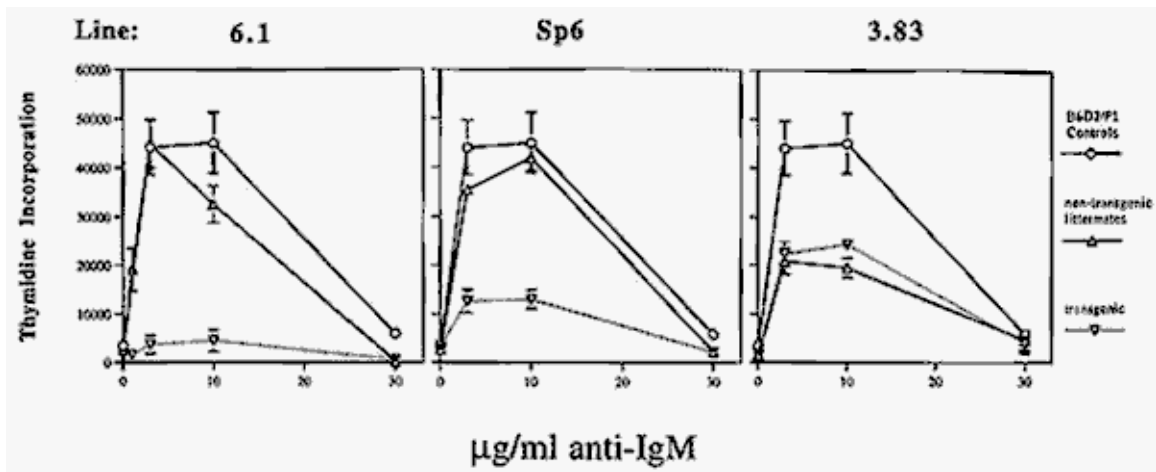


**Figure 2.**  $V_{H12}$  line 6.1 transgenic exhibit an impaired ability to undergo induced apoptosis via hypercrosslinking of sIgM. Dose responses to anti-IgM treatment of non-transgenic littermate (left) versus 6.1 transgenic B cells are shown. Methods were identical to those used Figure 1.

**Strain differences in the proliferative response to anti- $\mu$ .**

We previously suggested that initial signaling differences between  $V_{H12}$  (B1) and the conventional transgenic B cells tested could account for differential susceptibility to tolerance induction (4). Moreover, if one bypassed the Ig receptors using phorbol esters or ionomycin (or both), then unresponsiveness ensued. In these experiments, it was not known whether  $V_{H12}$  transgenic B cells (or the other strains tested) were able to

proliferate normally to anti- $\mu$ . Therefore, we examined splenocytes from all three strains and normal B6D2 F1 mice for their ability to proliferate with polyclonal anti- $\mu$ . In these studies, spleen cells are cultured with 3, 10 and 30  $\mu\text{g/ml}$  anti- $\mu$  for 44-50 hours in order to drive significant numbers of B cells into cycle. The results in Figure 3 show that  $V_{H12}$  transgenic B cells failed to proliferate with anti- $\mu$ , whereas the other transgenic lines were more responsive with peak proliferation at 10  $\mu\text{g/ml}$ . However, it should be



**Figure 3.** Proliferation of splenic B cells in response to anti-IgM treatment. Lines 6.1 and Sp6 both exhibit inhibited proliferation when compared to non-transgenic littermates and B6D2/F1 controls. Line 3.83 shows a strain related reduction in proliferation, though proliferation appears normal when compared to non-transgenic littermates.

noted that Sp6 transgenic B cells (which represent conventional B2 cells) responded less well than their non-transgenic counterparts and below the levels of B6D2 control B cells. Importantly, V<sub>H</sub>12 B cells failed to proliferate at any dose of anti- $\mu$ .

## DISCUSSION

Adult murine spleen cells will undergo dose-, time- and temperature-dependent apoptosis *in vitro* if sufficient crosslinking of membrane IgM occurs via anti-IgM treatment (9-11). The data presented herein confirm that mature splenic B cells will be driven to PCD (apoptosis), but differences in the susceptibility of B-cell subsets to apoptosis can be seen in this system. These results are reminiscent of our recent observation of resistance to tolerance induction in murine B1 cells in the peritoneum (3) and the spleen (4). Indeed, these results suggest that the differential susceptibility to tolerance of murine B cells may reflect these signaling differences for apoptosis. Immunoglobulin receptor crosslinking also is important as the first step in B-cell activation for cell cycle entry (13, 14). Clearly, the maturity and lineage of the target B cells, as well as second signals like CD40 and cytokines, play a role in driving B-cell proliferation (15, 16). The data presented herein indicate that strain and subset differences also play a role in B-cell apoptosis.

Crosslinking of membrane IgM causes initial tyrosine phosphorylation of a number of substrates, as well as phosphatidyl inositol hydrolysis, calcium mobilization and downstream effector events to lead to exit from G<sub>0</sub> and entry into the cell cycle (17). In our experience, the ability to drive B-cell proliferation and apoptosis generally do not correlate. For example, mitogenic monoclonal anti- $\mu$

reagents do not induce apoptosis unless hyper-crosslinked (9, 10), yet such antibodies can drive resting B cells into cycle reproducibly. Moreover, CD45 knockout mice (18), which can not be driven into cycle with any form of anti- $\mu$ , are nonetheless susceptible to apoptosis induced by goat anti- $\mu$ . (9). Finally, *xid* mice show the same separation of proliferation and apoptosis: anti- $\mu$  induces PCD but can not drive *xid* B cells into S (19). Therefore, the ability to drive apoptosis and cell cycle entry, even abortively, does not correlate.

Interestingly, in CD45 knockout and *xid* spleen cells (9,19), anti- $\mu$  causes a reduction in the p27 kinase inhibitor (20) known to regulate cell cycle entry in other cell types. It will be important to discern whether the anti- $\mu$  signaling defect is upstream or downstream from p27 modulation in 6.1 transgenic B cells. These studies are planned.

Our results are contrary to previously published data (21) suggesting normal proliferation in response to soluble anti-IgM in Sp6, but not M167 transgenic mice, which are predominantly B2-like in the spleen. Several differences between these studies should be noted. First, very high doses of different antibodies were used to induce proliferation in their system (21); indeed, in our hands, polyclonal anti- $\mu$  at >10  $\mu$ g/ml induces extensive B-cell PCD and minimal proliferation. However, a more likely explanation is that the background genes for these transgenic strains may differ (C57Bl vs. Balb/c). In both their studies and ours, transgenic B cells did proliferate normally in response to LPS and PWM. These data suggest that the differences noted may be downstream from initial tyrosine phosphorylation events (3, 4) since

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this event is relatively normal in Sp6 and 3.83 transgenics, and only modestly affected in 6.1 transgenic B cells (4).

Clearly, further studies are necessary to pinpoint the defect(s) leading to diminished B-cell apoptosis and proliferation in 6.1 transgenic B1-type cells compared to conventional B cells. Nonetheless, our results suggest that one reason for the resistance of B1 cells to tolerance induction is a failure to undergo apoptosis when their surface IgM is sufficiently crosslinked.

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