Immunoglobulin M and D Antigen Receptors are Both Capable of Mediating B Lymphocyte Activation, Deletion, or Anergy After Interaction with Specific Antigen

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Summary

A series of immunoglobulin (Ig)-transgenic mice were generated to study the functional capabilities of the IgM and IgD classes of B lymphocyte antigen receptor in regulating both cellular development and responses to specific antigen. B cells from Ig-transgenic mice expressing either hen-egg lysozyme (HEL)-specific IgM or IgD alone were compared with B cells from mice that coexpressed IgM and IgD of the same anti-HEL specificity. In all three types of Ig-transgenic mice, conventional B cells specific for HEL exhibited exclusion of endogenous Ig expression and matured to populate the usual microenvironments in peripheral lymphoid tissues. These peripheral B cells could be stimulated by HEL through either IgM or IgD antigen receptors to generate T cell dependent antibody production in vivo or to enhance T cell independent proliferative responses to lipopolysaccharide in vitro. Conversely, when HEL was encountered in vivo as a self-antigen, B cells expressing HEL-specific IgM or IgD alone were both rendered tolerant. In each case this occurred by clonal anergy in response to soluble autologous HEL, and clonal deletion when HEL was recognized as a membrane-bound self-antigen. Taken together, these findings indicate that IgM and IgD antigen receptors expressed alone on conventional B cells can support normal differentiation, antigen-dependent activation, and induction of self-tolerance, the only overt difference lying in a greater degree of receptor downregulation for IgM relative to IgD after induction of clonal anergy by soluble HEL.

The various classes of Ig produced by mammalian B lymphocytes have the dual function of serving as surface receptors for antigen and as effector molecules secreted in response to antigenic stimulation. Whereas distinct effector roles appear to account for the existence of IgM, IgG, IgA, and IgE, a unique function for IgD has remained obscure. Although little secreted IgD can be detected (1, 2), cell-surface IgD is coexpressed with IgM on the majority of mature B cells in peripheral lymphoid tissues of mice and humans (3–5). IgM and IgD molecules present on individual B cells share identical V regions and antigen binding sites (6–8), and are coexpressed by differential splicing of a common VDJ exon to either $C\mu$ or $C\delta$ C region exons after transcription of the V_H-μ-δ locus in the cell's functionally rearranged Ig H chain gene (9). Despite the fact that IgD is an abundant receptor on most B cells found in follicular mantle zones of lymphoid tissues and in peripheral blood, its expression appears to be restricted to this single stage during B cell development. Thus, only μ H chains are synthesized in pre-B cells, and IgM is expressed without IgD on the surface of newly differentiated, immature B cells in the bone marrow (7, 10, 11). Similarly, IgD is expressed at only very low levels on the IgM^{high} B cells in the splenic marginal zone (12) and on IgM^{high} cells belonging to the B1 (Ly-1/CD5) B cell lineage (13, 14), and is absent from high affinity memory B cells which have switched to expression of downstream H chain isotypes (15, 16).

The characteristic pattern of expression exhibited by IgD has focused attention on the possibility that this class of antigen receptor may have signaling properties distinct from

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those of IgM, thereby altering the responsiveness of B cells coexpressing IgM and IgD relative to those bearing IgM alone. In particular, previous evidence supported unique roles for IgM and IgD in determining whether B cell tolerance or activation occurred, since antigen binding to IgM alone on immature IgD$^-$ B cells induced tolerance, whereas binding of antigen by mature B cells generally provided stimulatory signals (for review see reference 17). Consistent with these findings, more recent studies of immature B cell lymphomas expressing IgD after transfection have shown that antibody-mediated crosslinkage of membrane IgD is incapable of inducing the growth-arrest normally observed after IgM crosslinkage (18, 19). By contrast, the IgM and IgD antigen receptors on B cells have appeared functionally similar in one study of lymphoma growth arrest (20) and in many studies of antigen presentation and T cell dependent activation (21–25), T cell independent activation (26), and elicitation of intracellular second messages (27, 28).

To date, much of the information on class-specific functions of IgM and IgD antigen receptors has been obtained using anti-μ and -δ chain antibodies to simulate the binding of antigen. Extrapolation of these results to responses induced by bona fide antigens, however, is complicated by the fact that isotype-specific antibodies do not interact with the normal antigen binding site of membrane Ig, and that the consequences of their interactions with the receptor may therefore be dependent on the site, affinity, or avidity of binding rather than isotype specificity per se (29–31). As an alternative approach to studying the functions of IgM and IgD antigen receptors, we have produced two parallel sets of Ig-transgenic mice in which most B cells express either transgene-encoded IgM or IgD alone, each possessing identical specificity for the well-characterized protein antigen, hen-egg lysozyme (HEL)[1]. Consequently, the development and responsiveness of IgM-only or IgD-only HEL-specific B cells could be followed in vivo either in the absence of immunization, after challenge with HEL as an immunogenic foreign antigen, or after encounter with HEL as a soluble or membrane-bound self-antigen. Assayed in this way, IgM and IgD antigen receptors were indistinguishable with respect to exclusion of endogenous Ig expression, promotion of B cell maturation, and in the signaling of B cell activation, anergy, and deletion.

Materials and Methods

Mice. Conventional inbred C57BL/6 and CBA mice, (C57BL/6 $\times$CBA)F_1 mice, and transgenic mouse lines were maintained in the Blackburn Animal Facility, University of Sydney. Hemizygous Ig-transgenic and HEL-transgenic mouse lines were maintained on a C57BL/6 background by backcrosses with nontransgenic and transgenic C57BL/6 mice.

Gene Constructs. The original MD (IgM + IgD) series of Ig-transgenic mice was produced by cointroducing H chain (V_H10-μ-δ) and L chain (V_κ10-C_κ) Ig gene constructs into the germline of C57BL/6 (H chain b-allotype, IgH^b) mice (32). These constructs together encode IgM and IgD (H chain a-allotype, IgH^a) carrying the antigen binding site of the high-affinity (1.5×10^9 M^{-1}) anti-HEL mAb HyHEL10 (33, 34). The V_H10-μ H chain gene construct comprising the HyHEL10 VDJ segment and μ H chain sequences was assembled in the EcoRI site of the pSVG-gpt vector during production of the pSVG-V_H10-μ-δ plasmid (32). The pSVG-V_H10-δ plasmid was produced by partial EcoRI digestion of pSVG-V_H10-μ-δ to remove the 12.4-kb EcoRI fragment containing the μ H chain switch recombination and C region coding sequences (Fig. 1). Construction of V_κ10-C_κ has been described previously (32). All these gene constructs were prepared using standard recombinant DNA techniques (35).

Transgenic Lines. For microinjection, gene constructs were separated from vector sequences by digestion with SalI and purified as described (32). MM (V_H10-μ + V_κ10-C_κ) and DD (V_H10-δ + V_κ10-C_κ) founder Ig-transgenic mice were generated after microinjection of C57BL/6 fertilized eggs with an equimolar mixture of H and L chain constructs as described (32, 36). Screening for founder Ig-transgenic mice and their progeny was performed initially by Southern blot analysis of tail DNA (36), but subsequently by ELISAs detecting serum anti-HEL IgM^a or anti-HEL IgD^a (see below). Southern blot analyses revealed the approximate numbers of transgene copies in the Ig-transgenic lines used in this study to be: MD-4 = 6–8 $\mu\delta$, 4–6 κ; MM-4 = 4–6 μ, 2–3 κ; MM-7 = 3–5 μ, 3–5 κ; DD-1 = 6–8 δ, 2–3 κ, and DD-6 = 2–3 δ, 3–5 κ.

Transgenic mice expressing soluble HEL (ML-5 line) or membrane-bound HEL (KLK-3 line) (32, 37) were screened serologically using a HEL-specific ELISA (see below). Double-transgenic mice inheriting Ig and soluble-HEL transgenes were produced by crossing Ig-transgenic and ML-5 HEL-transgenic mice, or by mating existing double-transgenic with nontransgenic mice. Progeny were screened by serum ELISAs detecting HEL and either IgM^a, anti-HEL IgM^a, or anti-HEL IgD^a (see below). Ig-transgenic and double-transgenic mice were analyzed when aged 5–15 wk.

mAbs. The following mAbs were used (specificities bracketed): RS-3.1 (IgM^a; 38); AMS-15.1 (IgD^a; 39); AF6-78.25 (IgM^b; 39); RA3-6B2 (B220; 40); M1-69 (heat stable antigen [HSA]; 41); 53-7 (CD5; 42); HyHEL5 (HEL; 43); and HyHEL10 (HEL; 33). Purified antibodies were labeled with biotin or fluorescein as previously indicated (32). Biotinylated 53–7 was purchased from Becton Dickinson Immunocytometric Systems (Mountain View, CA) and PE-labeled RA3-6B2 from Caltag Laboratories (South San Francisco, CA).

FACS® Analysis. Spleen, lymph node, and bone marrow cells were prepared and stained for FACS® analysis as previously indicated (32). For two-color analysis, cells were incubated with both directly fluoresceinated and directly biotinylated reagents, and binding of biotin-labeled reagents revealed with streptavidin/PE (Caltag Laboratories). HEL binding was detected with 200 ng/ml HEL followed by biotinylated HyHEL5 (32), or with HEL/fluorescein. For three-color analysis, staining by biotinylated antibodies was revealed with streptavidin/RED613 (Gibco BRL, Gaithersburg, MD). Fluorescence and light scatter data from stained cells were acquired on either a FACS® 440 or FACScan® flow cytometer (Becton Dickinson & Co.), and data from cells exhibiting a lymphocyte scatter profile displayed. FACS® 440 fluorescence data were displayed in two-dimensional contour plots (32), and FACScan® fluorescence data were displayed in two-dimensional dot plots.

ELISAs. The ELISA for detecting anti-HEL IgM^a was performed using HEL-coated microtitre plates as described (44), and modified to detect anti-HEL IgD^a using biotinylated AMS-15.1.

[1] *Abbreviations used in this paper:* HEL, hen-egg lysozyme; HSA, heat stable antigen.

Standard solutions consisted of culture supernatants derived from a hybridoma secreting transgene-encoded anti-HEL IgM^a (J. Crosbie, unpublished data) and from transfected Sp2/0 myeloma cells secreting transgene-encoded anti-HEL IgD^a. ELISAs detecting serum HEL and total IgM^a were performed as described (33, 44).

Immunohistology. Mouse spleens were prepared and stained as described (45).

Adoptive Transfer. Spleen cells were prepared from Ig-transgenic and double-transgenic donors and from 2–6-mo-old nontransgenic C57BL/6 male mice primed with 2×10^8 SRBC intraperitoneally 1–4 mo earlier. HEL was covalently coupled to SRBC as previously indicated (32), while control SRBC were mock-labeled in the absence of HEL. 3–6-mo-old nontransgenic C57BL/6 male recipients irradiated with 750 rad were injected intravenously with a single inoculum containing 10^4 Ig-transgenic or double-transgenic spleen cells, 5×10^6 SRBC-primed spleen cells, and 2×10^8 HEL-SRBC or control SRBC. Recipients were bled 7 d after cell transfer and serum levels of anti-HEL IgH^a measured by ELISA (see above).

In Vitro Stimulation with LPS. Spleen cells were cultured with LPS (20 μg/ml) ± HEL (100 ng/ml) and [^{3}H]thymidine incorporation was measured after 64 h as described (46).

Bone Marrow Chimeras. Nontransgenic (C57BL/6) and KLK-3 membrane-HEL transgenic recipients aged between 4 and 10 mo were irradiated with 950 rad and injected intravenously with 3×10^6 bone marrow cells from Ig-transgenic donors. Chimeras were allowed to reconstitute for 6 wk before analysis.

Results

Production of IgM-only and IgD-only Anti-HEL Ig-transgenic Mice. To generate transgenic mice with B cells expressing anti-HEL IgM or IgD alone, the V_H10-μ-δ construct used to produce the original MD (IgM + IgD) series of Ig-transgenic mice (32) was modified to yield two different H chain constructs, V_H10-μ and V_H10-δ (Fig. 1). Although both V_H10-μ and V_H10-δ retained the same transcriptional control elements and VDJ exon, in V_H10-μ the μ-δ locus was truncated 3′ to the Cμ constant region exons to exclude the Cδ exons and eliminate the possibility of IgD expression. In V_H10-δ, an internal deletion was made from V_H10-μ-δ that removed the Cμ exons and placed the Cδ exons immediately downstream of the VDJ region, analogous to the arrangement of the Ig H chain gene in IgD-secreting plasmacytomas (47). Coinjection of the $V_κ10$-$C_κ$ L chain construct (32) into C57BL/6 embryos with either V_H10-μ or V_H10-δ yielded eight μ + κ (MM) and four δ + κ (DD) founder Ig-transgenic mice carrying multiple cointegrated copies of H and L chain gene constructs. In the current paper, four representative Ig-transgenic lines (MM-4, MM-7, DD-1, and DD-6) derived from founder mice that carried low numbers of transgene copies (see Materials and Methods) were selected for study and analyzed in conjunction with the previously characterized MD-4 Ig-transgenic line (45).

Ig Expression by Ig-transgenic Mice. Expression of the Ig transgenes was monitored by ELISA and FACS® analysis that detected both transgene-encoded H chain (a-allotype, IgH^a) and antigen specificity (HEL-binding). In lymphoid tissues of the representative Ig-transgenic lines, cell surface expression of IgH^a was confined to cells of the B lineage as judged by coexpression of the B220 isoform of CD45 (40) (Figs. 2 and 9 *A*). The great majority of mature splenic B cells in MM and DD Ig-transgenic mice expressed high levels of IgM^a and IgD^a, respectively, but no detectable IgM carrying endogenous (C57BL/6) b-allotype μ chain (IgM^b) (Fig. 3). This is consistent with previous findings that rearranged δ as well as μ H chain transgenes inhibit expression of endogenous H chain genes (48–50). The small numbers of B cells that did express IgM^b stained very weakly for transgene-encoded IgH^a (Fig. 3), which suggests that these cells may express the Ig transgenes at low levels. Alternatively, this observation is consistent with cytophilic binding of transgene-derived Ig since similar levels of IgH^a staining are found on IgH^{b+} cells (and vice versa) in nontransgenic (C57BL/6 × CBA)F_1 animals (Fig. 3).

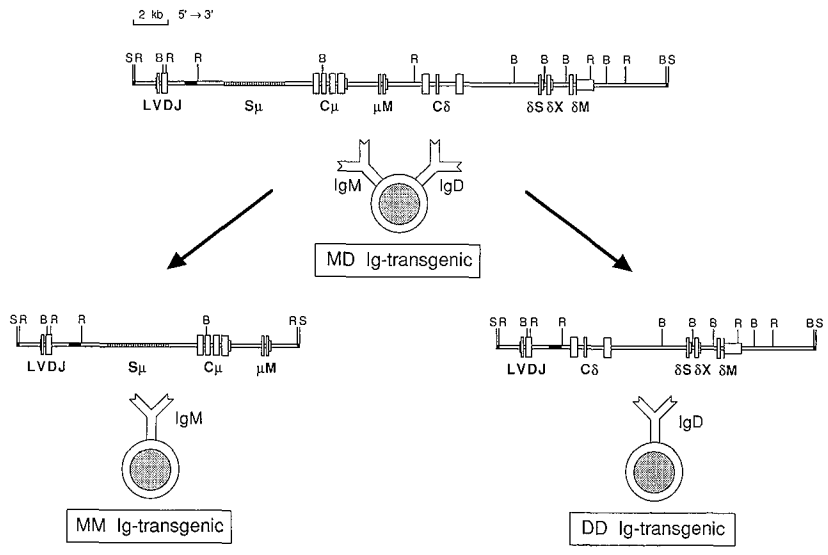


Figure 1. Structure of Ig H chain gene constructs. MD Ig-transgenic mice in which B cells coexpressed anti-HEL IgM and IgD had been produced previously (32) using the V_H10-μ-δ H chain construct (*top*). V_H10-μ (*bottom left*) and V_H10-δ (*bottom right*) H chain constructs were derived from V_H10-μ-δ and microinjected to produce MM and DD Ig-transgenic mice expressing anti-HEL IgM or anti-HEL IgD alone. (*Thin open bars*) Flanking or intronic DNA sequences; (*intermediate open bars*) 5′ and 3′ untranslated sequences; (*thick open bars*) coding sequences. Exons encoding the constant domains (*C*) and membrane termini (*M*) of the μ and δ H chains are indicated, as are those encoding the secretory terminus (*S*) and potential secretory terminus (*X*) (9) of the δ chain. The rearranged VDJ exon of the HyHEL10 hybridoma and the accompanying leader (*L*) sequence are also included. (*Thin striped bar*) Switch recombination sequences of the μ H chain gene ($S_μ$), (*thin black bar* 3′ of the VDJ exon) H chain enhancer element. Restriction endonuclease recognition sites are indicated for EcoRI (*R*), BamHI (*B*), and SalI (*S*).

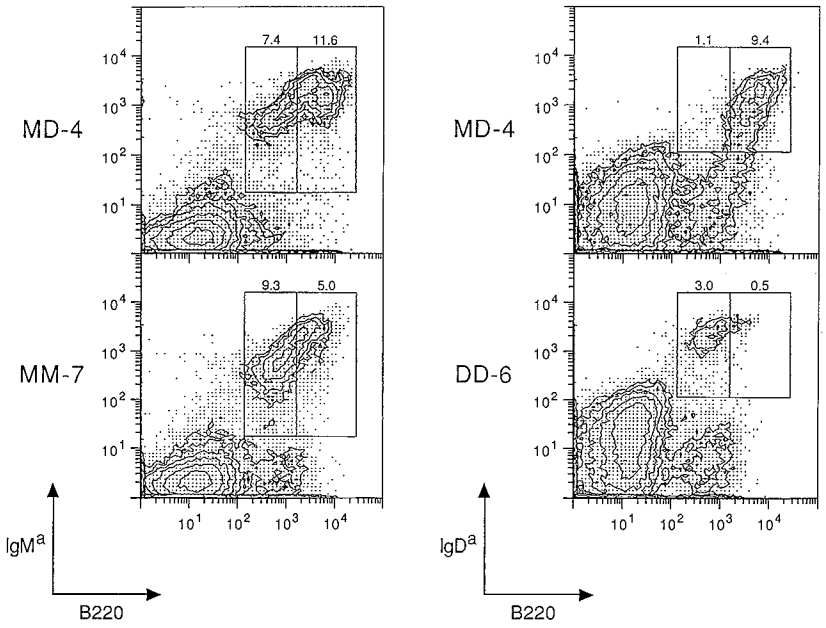


Figure 2. Expression of transgene-encoded IgM or IgD on bone marrow cells from Ig-transgenic mice. Bone marrow cells from MD-4, MM-7, and DD-6 Ig-transgenic mice were stained with fluoresceinated antibody to B220 and biotinylated antibody recognizing either IgM^a (*left*) or IgD^a (*right*) followed by streptavidin/PE. Rectangular windows delineating $B220^{low}$ (immature) and $B220^{high}$ (mature) B cells expressing IgM^a or IgD^a were set by reference to equivalently stained and analyzed C57BL/6 nontransgenic bone marrow cells.

Counterstaining of spleen cells from MM and DD Ig-transgenic mice for HEL-binding sites revealed that the majority of IgH^{a+} cells bound HEL in proportion to their surface level of transgene-encoded H chain (Fig. 3). Since MD Ig-transgenic mice require expression of both L and H chain transgenes to produce high-affinity HEL-specific Ig (32), the antigen receptor phenotype of these "on-diagonal" B cells demonstrated that they expressed transgenic κ chain as their

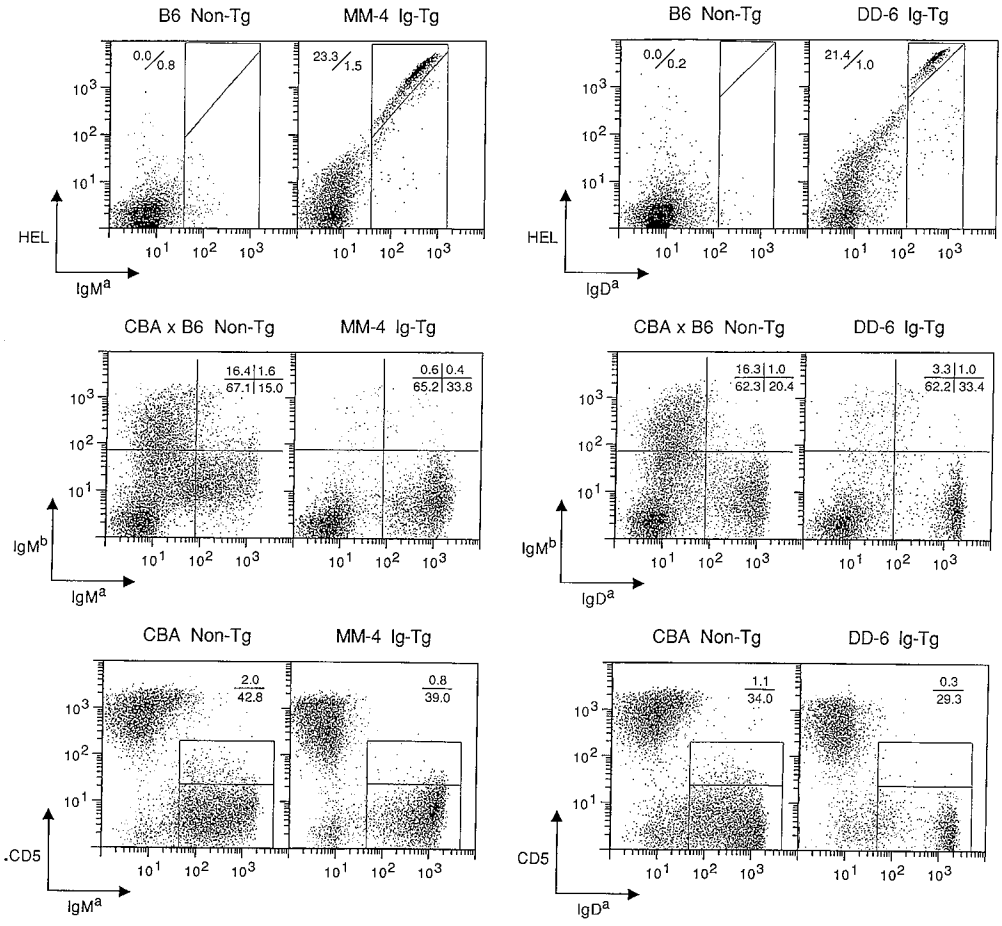


Figure 3. Expression of transgene-encoded and endogenous Ig and CD5 on spleen cells from nontransgenic and MM and DD Ig-transgenic mice. Spleen cells from MM-4 Ig-transgenic (*Ig-Tg*) mice, as well as nontransgenic (*non-Tg*) C57BL/6 (B6; IgH^{b+}), CBA (IgH^{a+}), and (C57BL/6 × CBA)F_1 mice were stained with fluoresceinated antibody to IgM^a (*left*), and DD-6 Ig-transgenic and nontransgenic spleen cells were stained with fluoresceinated antibody to IgD^a (*right*). Cells were counterstained with biotinylated antibodies detecting either HEL-binding (*top*), IgM^b (*middle*), or CD5 (*bottom*) followed by streptavidin/PE. In the top panels, on-diagonal Ig-transgenic B cells expressing proportional levels of IgH^a and HEL-binding sites are indicated within the top windows.

predominant L chain species. The on-diagonal B cells of MM and DD Ig-transgenic mice therefore appeared to express transgene-encoded HEL-specific Ig as their sole species of antigen receptor. Moreover, the titration curves for binding of fluoresceinated HEL to MM and DD Ig-transgenic B cells were identical (Fig. 4), indicating that the transgene-encoded IgM and IgD antigen receptors expressed by these cells had an equivalent affinity for HEL. In addition to on-diagonal B cells, MM and DD Ig-transgenic mice also contained low numbers of IgH^{a+} cells which bound less than proportional amounts of HEL (Figs. 3 and 8), presumably because of expression of either endogenously encoded L chains or of transgenes with somatically mutated V region sequences.

In both MM and DD Ig-transgenic mice, the majority of splenic IgH^{a+} B cells did not appear to belong to the B1 (Ly-1/CD5) lineage (13, 14) since they did not express CD5 (Fig. 3) and displayed high levels of B220 characteristic of mature conventional B cells (Fig. 9 *A*) (11, 13). Moreover, radiation chimeras that were produced by injecting nontransgenic mice with adult bone marrow (which lacks B1 lineage precursors; 51) from MM and DD Ig-transgenic donors were efficiently reconstituted with IgH^{a+} B cells (Fig. 9 *A*). These findings were consistent with previous analyses of MD Ig-transgenic mice (45, 52), but contrasted with the preferential development of B1 lineage cells described in some lines of Ig-transgenic mice (53).

The pattern of expression of IgM by MM Ig-transgenic mice was very similar to that reported previously for MD Ig-transgenic mice (32, 45). Thus transgene-encoded IgM^a was present at high levels on immature $B220^{low}$,HSA^{high} B lineage cells in bone marrow from MM as well as MD Ig-transgenic mice (Figs. 2 and 9 *B*), which is consistent with rapid expression of the already rearranged Ig transgenes during the pre-B cell stage of B lymphopoiesis (11, 45, 54). In addition, constitutive secretion of anti-HEL IgM^a occurred in both types of unimmunized Ig-transgenic mice, with serum concentrations ranging from 2 to 50 μg/ml (Fig. 7 *A*). In DD Ig-transgenic mice, however, the pattern of IgD expression differed from that seen in MD Ig-transgenics and appeared more typical of the pattern usually associated with IgM. First, in contrast to MD Ig-transgenic and nontransgenic mice in which surface expression of IgD was confined to mature $B220^{high}$,HSA^{low} B cells (Figs. 2 and 9 *B*) (11, 45), IgD was also expressed on immature $B220^{low}$,HSA^{high} B cells in the bone marrow of DD Ig-transgenic mice (Figs. 2 and 9 *B*). Second, whereas <0.1 μg/ml of secreted anti-HEL IgD^a could be detected in the serum of MD Ig-transgenic mice (data not shown), levels of up to 100 μg/ml were constitutively present in DD Ig-transgenic sera (Fig. 7 *A*), and up to 1,000 μg/ml could be elicited in adoptive transfer recipients of DD Ig-transgenic spleen cells challenged with HEL in immunogenic form (Fig. 6 *A*). The observation in DD Ig-transgenic mice of both an early onset of δ chain expression in pre-B/immature B cells and a persistence of IgD expression in Ig-secreting plasma cells is consistent with previous data from δ chain transgenic mice (50), and presumably reflects the absence of the Cμ region in the V_H10-δ gene construct since transcription of Cδ is normally prevented at both these stages of B cell differentiation by termination immediately 3′ to Cμ (55, 56).

Development and Distribution of B Cells in Ig-transgenic Mice. Since coexpression of IgM and IgD is characteristic of normal B cell development, it was interesting to see if the enforced expression of only one class of Ig would reveal any phase of development that was absolutely reliant on the presence of either IgM or IgD. FACS® analyses of lymphoid tissues from MM and DD Ig-transgenic mice indicated that B cells expressing either IgM or IgD alone could mature into conventional $B220^{high}$,HSA^{low} B cells with the capacity to populate peripheral lymphoid tissues and to circulate back to the bone marrow (Figs. 2, 3, and 9). Expression of δ chain/IgD from the pre-B cell stage in DD Ig-transgenic mice therefore sustained the early development of B lineage cells within the bone marrow, a process which, under normal circumstances, relies on expression of functional μ chain/IgM (57). Moreover, the maturation of surface Ig^+ B cells, including their migration to peripheral lymphoid tissues and circulation back to the bone marrow, did not appear to depend on specific expression of either IgM or IgD.

The pattern of surface IgM and IgD expression not only varies during normal B cell maturation, but also differs between B cells occupying physically distinct compartments within peripheral lymphoid tissues. In particular, B cell areas in the white pulp of the spleen can be resolved histologically into the follicular mantle zone, where B cells normally exhibit an IgM^{low},IgD^{high} surface phenotype, and the surrounding marginal zone in which the B cells are characteristically IgM^{high},IgD^{low} (12, 45). To establish whether development of these distinct subpopulations of B cells depends on expression of either IgM or IgD, spleens from MM and DD Ig-transgenic mice were examined immunohistologically.

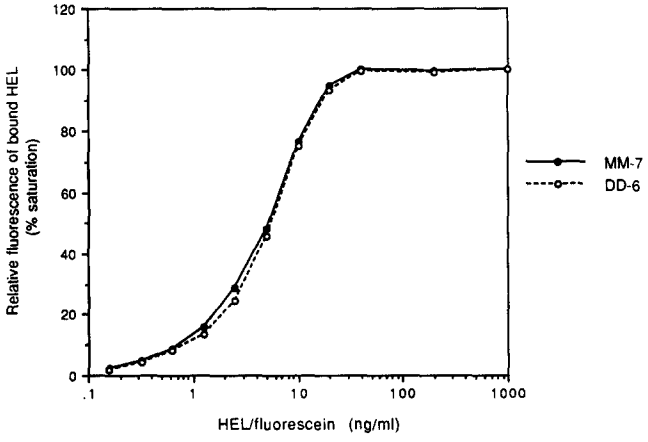
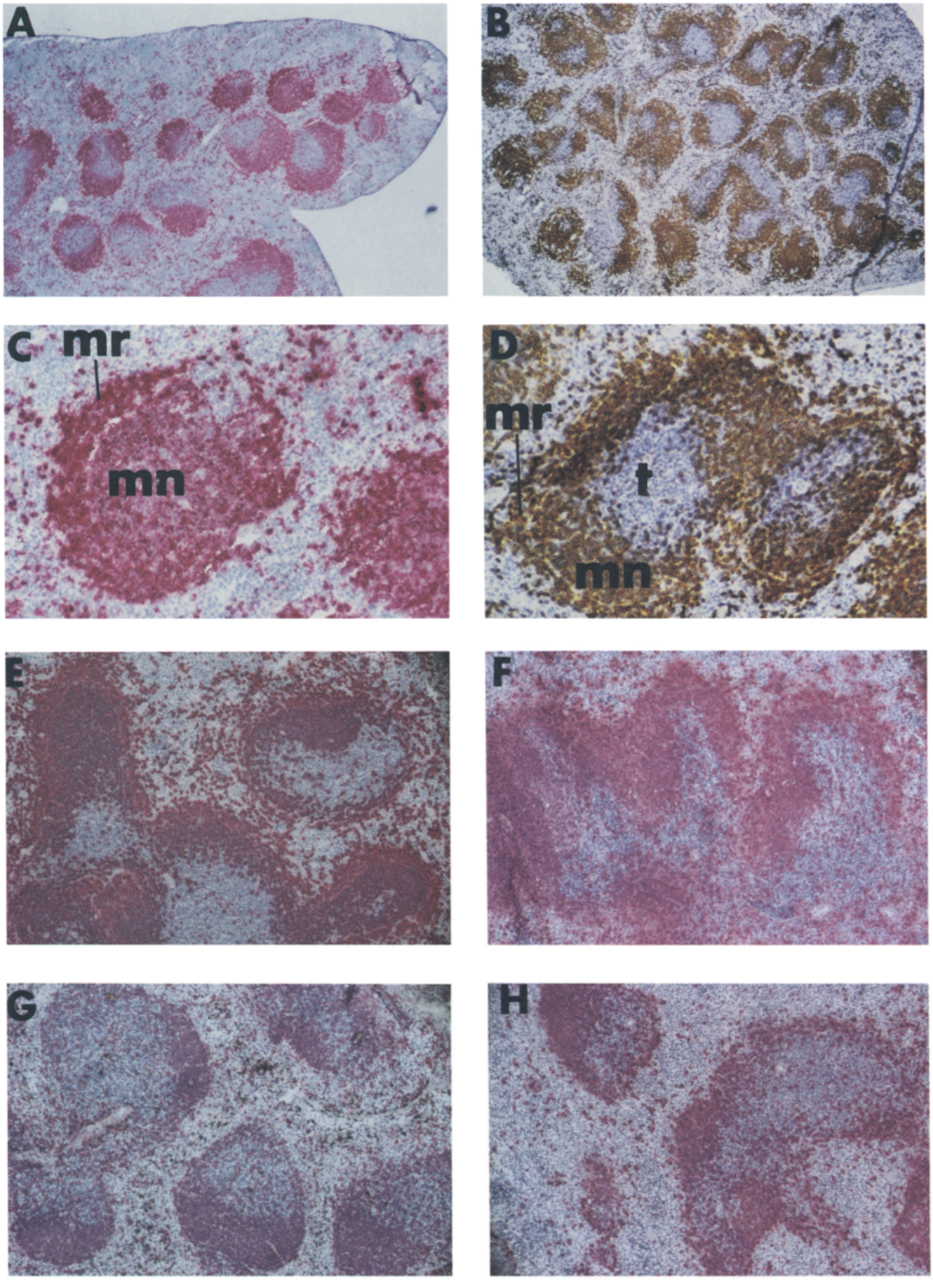


Figure 4. Titration of HEL-binding by MM-7 and DD-6 Ig-transgenic spleen cells. Equal numbers of spleen cells from MM-7 and DD-6 Ig-transgenic mice were combined, stained with various concentrations of HEL/fluorescein, and binding to IgM^{a+} or IgD^{a+} B cells resolved by counterstaining with specific biotinylated antibodies followed by streptavidin/PE. Mean green fluorescence of on-diagonal B cell populations (see Fig. 3) was measured and expressed as a percentage of the value obtained for cells stained with saturating (1,000 ng/ml) HEL/fluorescein.


A
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Staining revealed that cells expressing transgene-encoded IgH^a (Fig. 5, *A–D*) and exhibiting HEL-binding activity (Fig. 5, *E* and *F*) were readily identifiable in both the mantle and marginal zones of IgM-only and IgD-only transgenic spleens. Taken together, therefore, these results argue against an absolute requirement for either IgM or IgD in the development of B cells, or in their localization to the B cell microenvironments within peripheral lymphoid tissues.

Antigenic Stimulation of B Cells Expressing IgM or IgD Alone. The observation that B cells expressing IgM or IgD alone developed and populated peripheral lymphoid tissues raised the question whether B cells expressing one or other class of antigen receptor could also be activated by antigen. Initially, the function of MM and DD Ig-transgenic B cells was assessed by measuring their T cell–dependent antibody responses to HEL in vivo. To obviate the low responder status of C57BL/6 mice to HEL, it was necessary to challenge the B cells with HEL coupled to an immunogenic carrier and to provide a source of T cell help (32). For this purpose an adoptive transfer system was employed in which small numbers of Ig-transgenic spleen cells were transferred into irradiated nontransgenic recipients together with HEL coupled to the foreign carrier SRBC (HEL-SRBC) and spleen cells from SRBC-primed nontransgenic mice. Transgene-encoded antibody production in recipient mice was subsequently measured by serum ELISA for anti-HEL IgM^a (MD and MM Ig-transgenic donors) or anti-HEL IgD^a (DD Ig-transgenic donors). As shown in Fig. 6 *A*, B cells from MM and DD, as well as MD Ig-transgenic mice all generated substantial HEL-specific antibody responses in the adoptive recipients 7 d after challenge with HEL-SRBC. By contrast, immunization with uncoupled SRBC elicited 100-fold lower antibody responses, demonstrating that the responses to HEL-SRBC were primarily dependent on specific binding of the SRBC carried by the antigen receptors of the HEL-specific B cells. In addition, 10-fold-less antibody was produced if carrier-primed

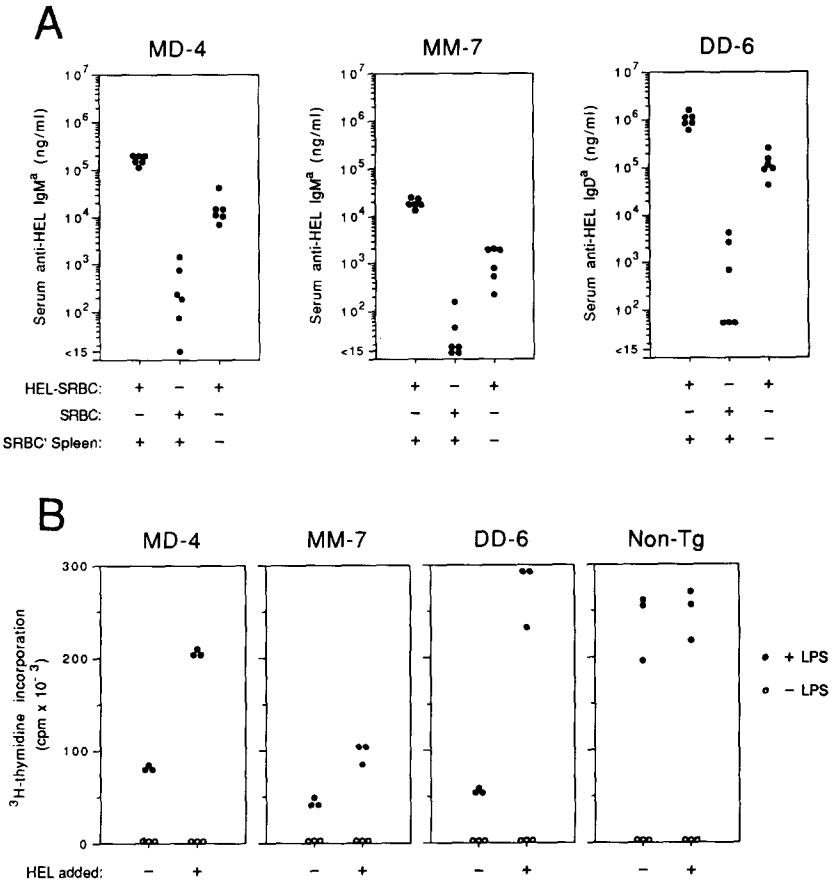

Figure 6. Responsiveness of IgM-only and IgD-only B cells to antigen in immunogenic form. (*A*) T cell dependent antibody responses of Ig-transgenic B cells. Spleen cells from MD-4, MM-7, and DD-6 Ig-transgenic mice were transferred into irradiated nontransgenic recipients, with or without carrier primed (*SRBC'*) spleen cells, and challenged with HEL-coupled carrier (*HEL-SRBC*) or carrier alone (*SRBC*). (*Dots*) Serum levels of anti-HEL antibody as determined by ELISA in individual recipients 7 d after transfer and challenge. Recipients of nontransgenic C57BL/6 donor spleen cells exhibited undetectable levels (<15 ng/ml) of both anti-HEL IgM^a and anti-HEL IgD^a. (*B*) Augmentation by HEL of in vitro proliferative responses of Ig-transgenic B cells to LPS. Spleen cells from nontransgenic C57BL/6 (*non-Tg*) and MD-4, MM-7, and DD-6 Ig-transgenic mice were stimulated with LPS ± HEL in vitro and pulsed with [^{3}H]thymidine. (*Dots*) Radioactivity incorporated in individual cultures. Increases in mean proliferation observed in cultures stimulated with LPS + HEL relative to those stimulated with LPS alone were: 153% (*MD-4*), 120% (*MM-7*), 380% (*DD-6*), and 4.5% (*non-Tg*).

Figure 5. Localization of transgene-expressing B cells in spleens from MM and DD Ig-transgenic mice and double-transgenic mice expressing soluble HEL. Frozen sections of spleens from MM-7 (*A* and *C*), MM-4 (*E*), and DD-6 (*B*, *D*, and *F*) Ig-transgenic mice as well as MM-4 × ML-5 (*G*) and DD-6 × ML-5 (*H*) double-transgenic mice were stained to reveal expression of IgM^a (*A* and *C*), IgD^a (*B* and *D*), or HEL-binding (*E–H*) using biotinylated antibodies and either avidin/alkaline phosphatase (*A*, *C*, and *E–H*) or avidin/horseradish peroxidase (*B* and *D*). Stained sections were photographed at ×10 (*A* and *B*), ×100 (*C* and *D*), or ×40 (*E–H*). Examples of the marginal zones (*mr*), mantle zones (*mn*), and T cell areas (*t*) of lymphoid follicles are indicated. Note the absence of marginal zone staining in the spleens from double-transgenic mice (*G* and *H*).

spleen cells were omitted from the transfer inoculum, which is consistent with the T cell dependence of these antibody responses.

According to previous data, signaling through the antigen receptors of MD Ig-transgenic B cells after binding of soluble HEL in culture synergizes with the T cell independent proliferative stimulus provided by LPS (46). To examine the relative abilities of IgM and IgD to provide a comitogenic signal for LPS-induced activation of B cells, MM and DD, as well as MD Ig-transgenic spleen cells, were cultured with LPS in the presence or absence of soluble HEL (Fig. 6 *B*). Augmentation of LPS-induced proliferation by HEL was again observed in each case, indicating that antigenic signaling through either IgM or IgD can synergize with LPS in inducing T cell independent B cell proliferation.

Induction of Tolerance in B Cells Expressing IgM or IgD Alone. Previous experiments involving Ig-transgenic models of self-tolerance in the B cell repertoire have demonstrated unresponsiveness in autoreactive B cells because of both clonal deletion and clonal anergy (32, 37, 58–60). In the anti-HEL Ig-transgenic model, B cells specific for autologous cell membrane HEL are eliminated from peripheral lymphoid tissues, whereas those recognizing the same self-antigen in soluble form persist in a functionally silent (anergic) state characterized by downregulation of surface IgM (32). Although both anergy and deletion operate on IgM^+ B cells irrespective of the presence of surface IgD (32, 37, 44, 60), the ability of the IgD antigen receptor to mediate these forms of B cell tolerance remains unknown. To resolve this issue, we have compared the development and function of DD Ig-transgenic B cells with those of MD and MM B cells in mice expressing HEL as either a soluble or cell surface self-antigen.

In the first set of experiments, double-transgenic offspring were produced by mating the three types of Ig-transgenic mice with the transgenic line ML-5, mice from which express HEL in soluble form at concentrations sufficient to induce anergy in MD Ig-transgenic B cells (32). When in situ levels of constitutively secreted anti-HEL IgH^a were measured in MM and DD double-transgenic mice and their Ig-transgenic littermate controls, reductions in antibody levels comparable with those observed for the MD double-transgenic combination were found (Fig. 7 *A*). Since immune complex formation has been formally excluded previously as a possible explanation for the low antibody levels observed in the sera of double-transgenic mice (32, 44), these results are consistent with induction of tolerance in the B cells from all three double-transgenic combinations.

The mechanism responsible for self-tolerance in the MM × ML-5 and DD × ML-5 double-transgenic mice proved to be similar to that operating in the original MD × ML-5 double-transgenic combination, according to a number of functional and phenotypic criteria. First, autoreactive B cells were not deleted. Rather, mature on-diagonal HEL-binding B cells persisted, albeit at reduced frequencies, in the spleen, lymph nodes, and bone marrow of both MM and DD double-transgenic animals (Fig. 8 and Table 1, data not shown). Second, antigen receptor levels were reduced on the surface of HEL-binding B cells derived from all three double-transgenic combinations. As shown previously (32), receptor downregulation on MD double-transgenic B cells was confined to IgM

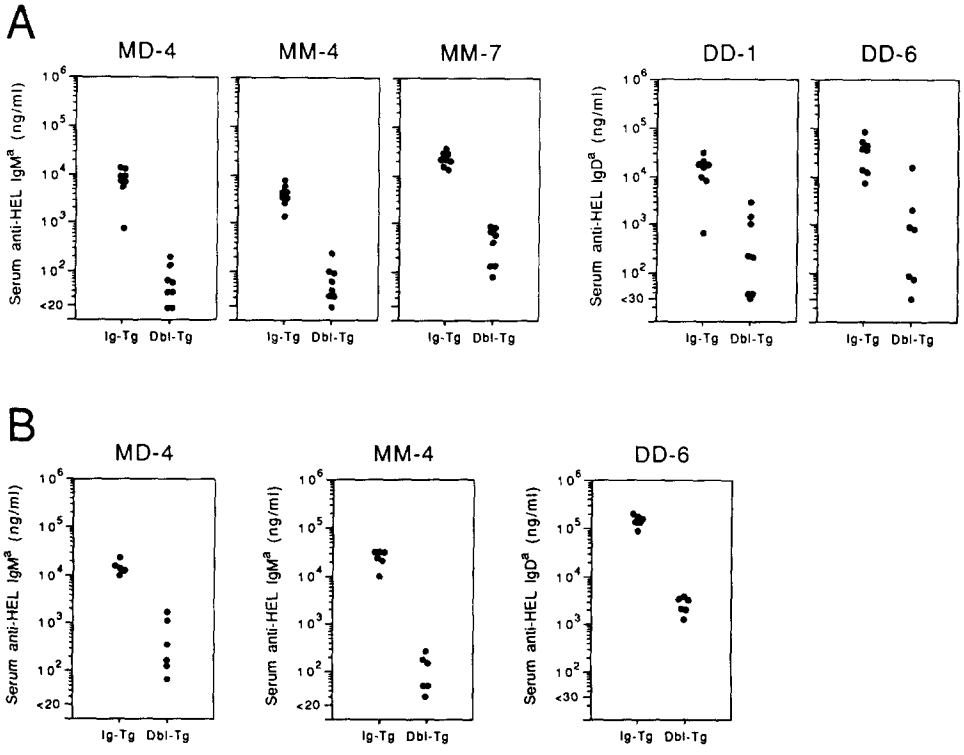

Figure 7. Functional silencing of anti-HEL B cells in double transgenic mice expressing soluble HEL. (*A*) Decreased levels of transgene-encoded anti-HEL antibody in sera from unimmunized double-transgenic (×ML-5) mice (*Dbl-Tg*) relative to Ig-transgenic littermates (*Ig-Tg*). Antibody levels were measured by ELISA and were below the limits of detection (<20 ng/ml for anti-HEL IgM^a and <30 ng/ml for anti-HEL IgD^a) in both nontransgenic and HEL-transgenic sera. (*B*) Decreased responsiveness of spleen cells from double-transgenic (×ML-5) relative to Ig-transgenic mice after adoptive transfer into irradiated nontransgenic recipients together with HEL-SRBC and SRBC' spleen cells. Anti-HEL antibody responses in recipients of Ig-transgenic and double-transgenic cells were measured as for Fig. 6 *A*. The percentages of HEL-binding B cells in the donor spleen cell populations were: MD-4 Ig-Tg, 27.6%; MD-4 Dbl-Tg, 26.7%; MM-4 Ig-Tg, 10.4%; MM-4 Dbl-Tg, 12.0%; DD-6 Ig-Tg, 12.5%; and DD-6 Dbl-Tg, 10.2%.

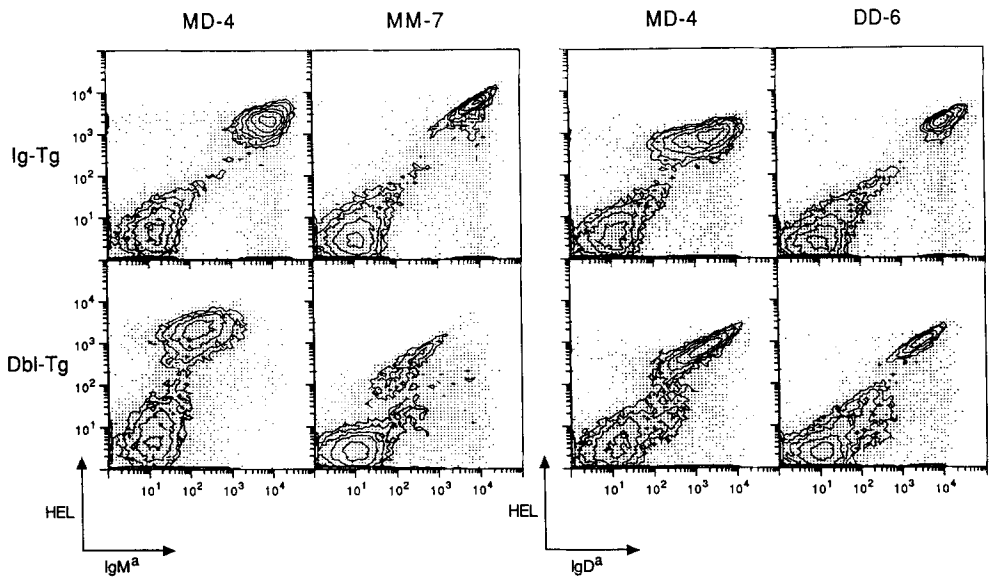

Figure 8. Reduced expression of surface Ig on spleen cells from double-transgenic mice expressing soluble HEL. Spleen cells from Ig-transgenic mice and double-transgenic (×ML-5) littermates were stained with fluoresceinated antibodies to IgM^a (*left*) or IgD^a (*right*) and counterstained with HEL followed by HyHEL5/biotin and streptavidin/PE.

(Fig. 8 and Table 1). Reduced expression of IgM was also observed on HEL-binding B cells from MM double-transgenic mice, although the magnitude of downregulation appeared slightly less (10–40-fold compared with 20–100-fold in MD transgenic animals; Fig. 8 and Table 1). Downregulation of IgD expression did occur on HEL-binding cells from DD double-transgenic mice although this was limited to between a two- and fivefold reduction (Fig. 8 and Table 1). Third, the B cells from MM and DD, as well as MD double-transgenic mice proved to be intrinsically anergic. This was formally

Table 1. *Splenic B Cell Frequency and Antigen Receptor Downregulation in Double-transgenic Mice Expressing Soluble HEL*

		B220$^+$ cells		HEL-binding cells		Receptor downregulation	
Ig-Tg line	Tg genotype	%	No. (× 10^{-6})	%	No. (× 10^{-6})	IgM^a	IgD^a
MD-4 ($n=4$)	Ig-Tg	55.5	47.7	49.2	42.3		
	Dbl-Tg	42.7	37.9	37.2	33.0	75-fold	1.4-fold
MM-4 ($n=1$)	Ig-Tg	34.3	22.6	26.0	17.2		
	Dbl-Tg	18.5	12.0	13.7	8.9	8.9-fold	-
MM-7 ($n=3$)	Ig-Tg	31.4	17.8	26.2	14.8		
	Dbl-Tg	20.8	9.4	12.7	5.8	34-fold	-
DD-1 ($n=2$)	Ig-Tg	28.8	21.8	20.0	15.0		
	Dbl-Tg	24.2	16.5	19.1	13.0	-	3.8-fold
DD-6 ($n=6$)	Ig-Tg	29.7	14.8	17.4	8.7		
	Dbl-Tg	18.4	9.1	9.4	4.6	-	4.3-fold
-	Non-Tg	60.0	70.0	0.2	0.2	-	-

Spleen cells from littermate pairs of Ig-transgenic (Ig-Tg) and double-transgenic (Dbl-Tg) (× ML-5) mice were stained for HEL-binding sites, counterstained for IgM^a, IgD^a, or B220, and analysed by FACS®. Percentages of B220$^+$ and HEL-binding cells were derived from FACS® plots and numbers per spleen calculated using the total number of nucleated cells recovered from each spleen. Values displayed represent means of data from n pairs of mice. For MM and DD mice, HEL-binding cells represent those in the on-diagonal population (see Fig. 3). Antigen receptor downregulation was estimated for each simultaneously analyzed pair by dividing mean IgM^a or IgD^a, fluorescence of double-transgenic HEL-binding cells by that of Ig-transgenic HEL-binding cells. The inverse of the mean of data obtained from n pairs of mice was used to give the degree of downregulation. Data for nontransgenic mice represent mean values obtained from four C57BL/6 mice.

demonstrated by transferring the cells out of their tolerant environment into irradiated recipients and stimulating them with HEL-SRBC in the presence of SRBC-primed T helper cells. 10–100-fold less HEL-specific antibody was produced in recipients of MM and DD double-transgenic B cells than in recipients of cells from nontolerant Ig-transgenic controls (Fig. 7 *B*), thereby confirming that the self-reactive B cells expressing IgM or IgD alone were functionally silenced by soluble HEL. Finally, tolerant HEL-binding B cells from MM and DD double-transgenic mice populated the follicular mantle zones in the spleen normally, but were absent from the splenic marginal zones (Fig. 5, *G–H*) as was found previously in MD double-transgenic animals (45). The reductions in splenic B cell numbers observed in double-transgenic relative to Ig-transgenic mice (Table 1) are presumably due, at least in part, to depletion of marginal zone HEL-binding B cells.

These experiments established that signaling through IgD as well as IgM could induce clonal anergy in self-reactive B cells after exposure to soluble self-antigen. To determine whether clonal deletion could also be mediated by both classes of antigen receptor, a different HEL-transgenic line (KLK-3) was used in which HEL is expressed on the surface of a number of cell types, including bone marrow and peripheral lymphocytes (37). To avoid the technical complication of membrane HEL expression by HEL-specific B cells, tolerance was examined in a series of bone marrow chimeras rather than double-transgenic mice per se. For this purpose, bone marrow cells from each of the three types of Ig-transgenic mice were used to reconstitute lethally irradiated KLK-3 membrane HEL-transgenic, or nontransgenic mice. 6-wk later, spleen and bone marrow cells from the chimeric mice were subjected to FACS® analysis. As expected, the spleens from nontransgenic recipients

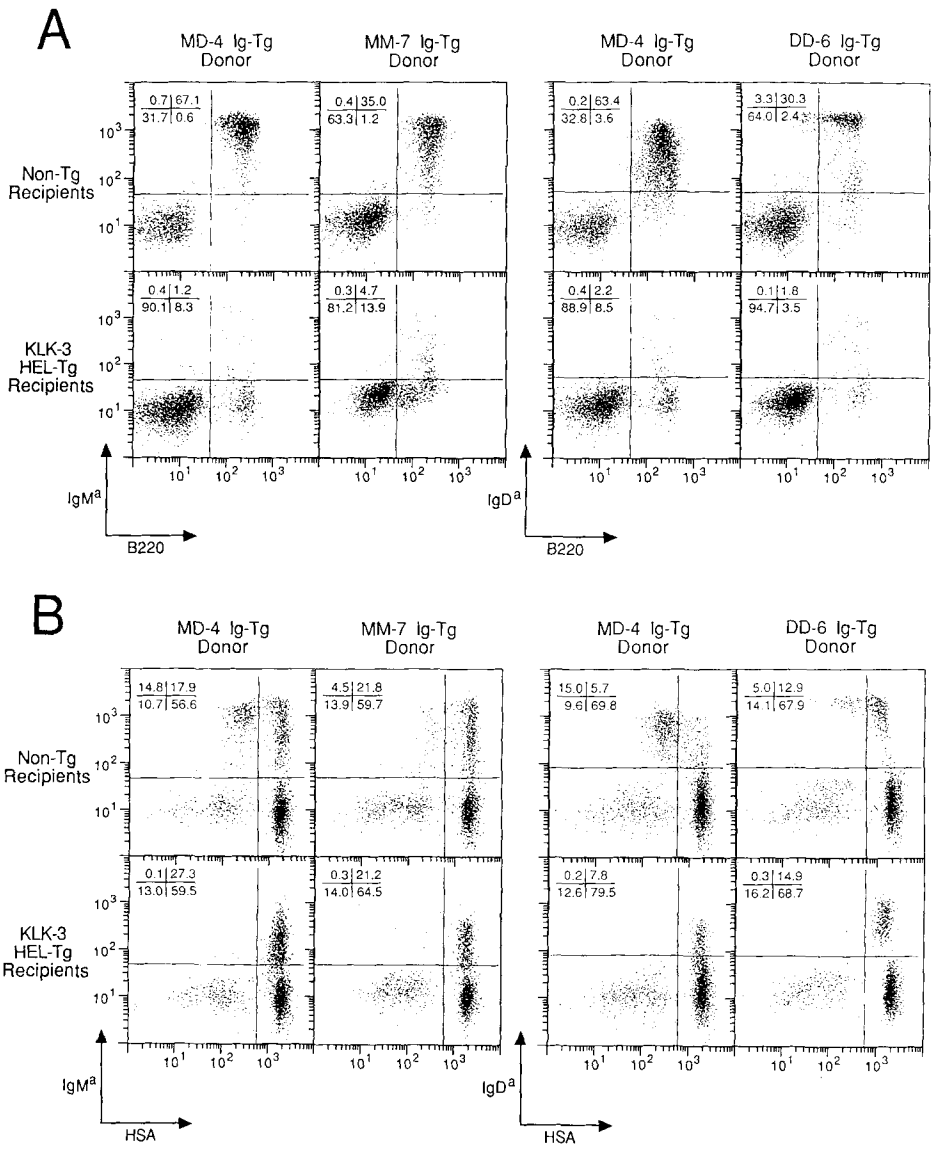

Figure 9. Clonal deletion of anti-HEL IgM-only or IgD-only B cells in radiation chimeras expressing membrane-bound HEL. Lethally irradiated nontransgenic C57BL/6 mice (*non-Tg*) or KLK-3 transgenic mice expressing cell surface HEL (*KLK-3 HELTg*) were reconstituted with bone marrow cells from MD-4, MM-7, or DD-6 Ig-transgenic donors. Spleen cells (*A*) and bone marrow cells (*B*) from the chimeras were analyzed 6 wk after reconstitution. Cells were stained for B220 with RA3-6B2/PE, for HSA with M1-69/biotin followed by streptavidin/RED613, and for either IgM^a with RS-3.1/fluorescein (*left*) or for IgD^a with AMS-15.1/fluorescein (*right*). (*A*) In the spleen, very few IgM^{a+} or IgD^{a+} B cells remained in the KLK3 HEL-transgenic recipients. The $B220^+$, IgH^{a-} cells remaining were shown in parallel stains to express IgM^b. Fewer spleen cells were recovered from all KLK3 HEL-transgenic relative to nontransgenic recipients, the respective numbers ($\times 10^{-6}$) being: 61 vs. 119 (*MD-4 donor*), 53 vs. 100 (*MM-7*), and 40 vs. 67 (*DD-6*). (*B*) In the bone marrow, mature (HSA^{low}) B cells expressing IgM^a or IgD^a were also absent in KLK3 HEL-transgenic recipients. By contrast, immature (HSA^{high}) B cells expressing IgM^a or IgD^a persisted but with reduced levels of surface Ig.

of each type of Ig-transgenic bone marrow contained large numbers of B cells expressing the transgenic antigen receptor (IgH^a) (Fig. 9 *A*). By contrast, KLK-3 membrane HEL-transgenic recipients contained very few IgH^{a+} B cells in their peripheral lymphoid tissues (Fig. 9 *A*), the $B220^+$ cells that did persist consisting mainly of those expressing endogenously encoded IgM^b (data not shown). This effect occurred in recipients of bone marrow not only from MD and MM Ig-transgenic donors (37), but also in those given DD Ig-transgenic cells. Moreover, as was reported previously for MD and MM chimeras (37), depletion of transgene-expressing B cells from the periphery of the membrane HEL expressing DD chimeras was only partially reflected in the bone marrow of these mice. Thus, although there was complete depletion of mature HSA^{low},IgD^{a+} B cells in the bone marrow, the frequencies of immature HSA^{high},IgD^{a+} B cells were not reduced in KLK-3 compared with nontransgenic recipients (Fig. 9 *B*). Since the mature $B220^{high}$,HSA^{low} B cells of the bone marrow appear to represent cells that have recirculated from the periphery (11), the results from these chimeras suggest that despite encountering HEL in the bone marrow (as reflected in their reduced levels of antigen receptor; Fig. 9 *B*), the majority of transgene-expressing B cells generated in membrane HEL-bearing mice die in transit between the bone marrow and peripheral lymphoid tissues. Irrespective of the precise mechanism of peripheral depletion of HEL-binding B cells, these results clearly indicate that both IgM and IgD antigen receptors can mediate this process after recognition of membrane-bound self-antigen.

Discussion

Transgenic mice with B cells expressing IgM or IgD as their sole species of surface Ig constitute a useful system for exploring the relative capabilities of these classes of antigen receptor in regulating B cell development and in mediating specific responses to antigen. In this report, IgM-only (MM) and IgD-only (DD) transgenic mice were produced in which the majority of B cells lacked surface expression of endogenously encoded Ig (Fig. 3) and bound HEL through their transgene-encoded IgM or IgD with homogeneous and identical affinity (Fig. 4). Thus it was possible to compare the function of the two classes of antigen receptor during normal B cell development and upon exposure to foreign or self antigen in vivo.

As has been found previously (48–50), expression of productively rearranged μ or δ transgenes in developing B lineage cells facilitated exclusion of endogenous Ig H chain expression (Fig. 3) and promoted maturation of pre-B cells into immature B cells (Figs. 2 and 9 *B*). Although the signaling events involved in triggering these steps in normal B cell differentiation remain unclear, these findings demonstrate that δ-membrane H chain can substitute for the μ chain transmembrane region which is normally required to mediate these functions (48, 57). Similarly, no absolute requirement for IgM or IgD expression was found in the later stages of B cell maturation and migration since HEL-binding B cells populated the spleens and lymph nodes of IgM-only and IgD-only Ig-transgenic mice and were found in both the mantle and marginal zones of the spleen (Fig. 5). It is nevertheless difficult to exclude the existence of quantitative differences between IgM and IgD in supporting B cell development. Consistent with such a difference was the presence of fewer B cells in the spleens of IgM-only and IgD-only transgenic mice compared with IgM + IgD transgenic animals (Figs. 8 and 9 *A*, and Table 1) suggesting that expression of both IgM and IgD is required for B cell development to operate at optimal efficiency. On the other hand, B cells unable to produce δ chain as a result of specific gene targeting appear to mature normally (61) suggesting that the maturation of B cells can operate optimally in the complete absence of IgD expression.

The capacity of B cells expressing only anti-HEL IgM or only anti-HEL IgD to mature in a similar fashion allowed these cells to be compared directly in terms of their reactivity to antigen during T cell dependent and T cell independent responses. Antigen binding to either surface IgM and IgD was capable of augmenting T cell independent B cell proliferation in the presence of LPS (Fig. 6 *B*), which is consistent with previous findings since dextran-conjugated anti-μ or anti-δ antibodies are both efficient T cell independent mitogens for B cells (26). In addition, both IgM-only and IgD-only Ig-transgenic splenic B cells responded well to HEL-coupled SRBC in adoptive transfer (Fig. 6 *A*). These responses were primarily T cell dependent since 10-fold less antibody was produced in the absence of SRBC-primed spleen cells (Fig. 6 *A*), although it is difficult to know to what extent they were dependent on cognate T–B cell interactions, or on T cell independent type II activation rendering the B cells responsive to T cell–derived cytokines (62). The responsiveness of both IgM-only and IgD-only B cells is nevertheless consistent with previous evidence that these classes of antigen receptor are indistinguishable in their ability to internalize antigen for processing and presentation to T helper cells (25).

Although more anti-HEL antibody was usually produced by IgD-only than by IgM-only B cells in adoptive transfer, the specific IgM responses of IgM + IgD B cells were essentially comparable with those of B cells from IgM-only mice (Figs. 6 *A* and 7 *B*). In other words, the production of higher levels of antibody by IgD-only compared with IgM-only B cells cannot be explained in terms of a more active role for IgD than IgM in mediating T cell dependent antibody responses. Rather, it is more likely that the production and secretion of IgD antibody per se is more efficient than that of IgM, perhaps because of the different splicing events required to produce secreted μ and δ chains (9), or to the additional steps involved in the production of the secreted IgM pentamer.

In contrast to B cell activation, where signaling through membrane Ig must often be accompanied by other signals, induction of B cell tolerance appears to be mediated primarily, if not solely, via membrane Ig signaling (17, 63). In the experiments reported here, two distinct mechanisms of tolerance shown previously to operate for IgM + IgD transgenic B cells were both found to be triggered efficiently through

either IgM or IgD alone. First, IgM-only and IgD-only HEL-binding B cells were deleted from the peripheral lymphoid tissues of transgenic mice expressing HEL as an integral membrane protein (Fig. 9). Second, when B cells expressing either class of antigen receptor alone developed in transgenic mice expressing HEL in soluble form, they matured to populate the mantle zones of peripheral lymphoid follicles, but were in each case rendered anergic and excluded from the follicular marginal zones (Figs. 5 and 7). Based on the differential induction of deletion and anergy by multivalent membrane-bound HEL versus oligovalent/monovalent soluble HEL, we have previously suggested that two distinct Ig-signaling thresholds may govern which of these processes is employed to induce B cell self-tolerance (37). The finding here that IgM and IgD appear equivalent in mediating deletion and anergy suggests that any differential signaling based on the degree of receptor crosslinkage is manifest through either class of antigen receptor.

The observation that IgM and IgD can function interchangeably in many of the in vivo events involved in B cell development is consistent with much of the previous evidence for the functional equivalence of these two classes of antigen receptor. These findings are also consistent with current structural data on membrane Ig, since both IgM and IgD are known to be noncovalently associated with the CD3-like MB-1 (Ig-α) and B29 (Ig-β) molecules (64, 65) which are believed to provide the links between B cell antigen receptors and intracellular signaling pathways (66). It is difficult, however, to reconcile the efficient induction of B cell anergy and deletion through either IgM or IgD with previous findings that negative signaling in certain B cell lymphomas is specifically associated with crosslinkage of IgM but not IgD antigen receptors (18, 19). Although in vitro growth inhibition of B lymphoma cells by anti-Ig antibody may involve signaling mechanisms distinct from those responsible for antigen-induced B cell tolerance in vivo, it is also possible that quantitative signaling differences between IgM and IgD may not have been detected in the in vivo assays of B cell tolerance used here. This may apply particularly in the case of clonal deletion since recent data suggest that deletion of IgM-expressing B cells by membrane-bound HEL is equally efficient when the affinity of the antigen receptor for HEL is over 100-fold lower than in the experiments described here (S. B. Hartley & C. C. Goodnow, unpublished observations). On the other hand, the level of soluble HEL used here to induce anergy in vivo is only just sufficient for tolerance induction (44), suggesting that quantitative differences of less than 10-fold in the signaling efficiency of IgD versus IgM would be readily detectable. One intriguing explanation for the discrepancy in IgD function between these two models of B cell tolerance may lie in the fact that IgM was coexpressed with IgD on the surface of each of the B lymphoma lines examined (18, 19), whereas HEL-specific B cells from the IgD-only transgenic mice expressed IgD in the absence of IgM. The implication that IgD antigen receptor function may be significantly modified by coexpression of membrane IgM should be readily testable in the anti-HEL Ig-transgenic system.

Overall, only one consistent difference in the behavior of IgM and IgD antigen receptors was observed in the experiments described here. Thus, in B cells rendered anergic by autologous soluble HEL, the degree of antigen receptor downregulation accompanying tolerance induction (Fig. 8 and Table 1) was invariably greater for IgM (10–100-fold) than for IgD (less than twofold in IgM + IgD mice and two- to five-fold in IgD-only mice). Since receptor downregulation on anergic B cells is not accompanied by reductions in the steady state levels of mRNA encoding μ or δ membrane H chains (67, R. Brink, unpublished observations), the changes in surface Ig expression and differences in the magnitudes of IgM and IgD downregulation must reflect translational or posttranslational events within the cells. Whatever the precise molecular explanation of downregulation, the observation of greater decreases in antigen receptor levels on IgM-only compared with IgD-only anergic B cells confirms that the original observation of preferential IgM downregulation on IgM + IgD anergic B cells (32) represents a class-specific difference between IgM and IgD which is not dependent on the coexpression of IgD with IgM, nor on the expression of IgD late in B cell maturation. The significance of this relative resistance of IgD to downregulation on anergic B cells nevertheless remains unclear.

The finding that IgM and IgD appear to be functionally interchangeable in vivo makes the role of IgD all the more perplexing. On the one hand, it must be considered possible that IgD performs no unique function and is instead either redundant or evolutionally obsolete. On the other hand, the IgD intron/exon organization and expression pattern are so distinctive (9) that it seems unlikely to have evolved without selection. Presumably unequivocal identification of a unique role for IgD will require more detailed investigation of processes not examined in this report such as the generation and affinity maturation of memory B cells (15), or the modulation of antibody responses by T helper cells expressing IgD-binding molecules (68). These possibilities are all amenable to further exploration using either the Ig-transgenic approach employed here, or IgD-deficient mice generated by gene targeting (61).

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