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Immunology

Intrinsic B-cell hyporesponsiveness accounts for self-tolerance in lysozyme/anti-lysozyme double-transgenic mice

(immunoglobulin-transgenic/antibody response/autoantibody)

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ABSTRACT **In double-transgenic mice expressing a gene construct encoding hen egg lysozyme as well as rearranged anti-lysozyme antibody genes, large numbers of anti-lysozyme B cells are present in peripheral lymphoid tissues but are profoundly tolerant. The cellular basis for this form of nondeletional self-tolerance was explored. The tolerant anti-lysozyme B cells from double-transgenic mice were found to produce much less antibody than nontransgenic controls in T-cell-dependent antigen-specific responses, in adoptive transfer *in vivo*, and in hanging-drop cultures *in vitro*, as well as in response to stimulation with the nonspecific mitogen lipopolysaccharide. The diminished responsiveness of the tolerant B cells was not due to a reduction in the number of responding B-cell precursors *per se* nor were suppressor cells detected in titration, depletion, or mixing experiments. Nondeletional tolerance in this model, therefore, appears to result from an intrinsic functional change in the self-reactive B cells themselves.**

The ability to discriminate between self and foreign antigens is essential for the maintenance of immunological homeostasis. To explain the absence of immune responses to self antigens, it has been hypothesized that self-reactive T or B lymphocytes are intrinsically inactivated [for example, by physical elimination (clonal deletion) (1, 2) or by functional down-regulation (clonal anergy) (3–5)] or that such cells although functionally normal are continuously inhibited through interaction with suppressor T cells (6, 7) or anti-idiotypic antibodies (8). Determining the actual fate of self-reactive T or B cells has proved to be difficult, however, since cells with receptors specific for any particular antigen, including self antigens, are normally produced at very low frequency and with a wide range of receptor affinities.

Transgenic mice carrying rearranged immunoglobulin or T-cell receptor transgenes provide an opportunity to follow the development and selection of antigen-specific lymphocytes *in vivo*, as they produce large numbers of B or T cells expressing only the transgene-determined antigen specificity (9–17). In transgenic mice expressing self-reactive receptor specificities, it has been possible to show that clonal deletion constitutes one means of achieving self-tolerance in both T cells and B cells (13–15), thereby confirming and extending earlier studies of T-cell tolerance to autologous "superantigens" such as Mls (18–20). By using the same type of approach, equally convincing evidence for nondeletional mechanisms of self-tolerance has also been obtained. Thus, in double-transgenic (Dbl-Tg) mice carrying a gene construct encoding hen egg lysozyme (HEL) as well as rearranged anti-lysozyme antibody genes, anti-lysozyme antibody secretion is reduced by a factor of 200 compared to that of littermates carrying only the immunoglobulin transgenes, yet the frequency of peripheral B cells expressing high-affinity anti-lysozyme IgM and IgD antigen receptors is reduced by less than a factor of 2 (16, 17). Similarly, nondeletional tolerance mechanisms affecting the T-cell repertoire have been described in situations where self-antigen is encountered either exclusively on radioresistant components within the thymus (21) or only outside of the thymus (22–26).

A key issue raised by these findings concerns the extent to which nondeletional mechanisms of self-tolerance involve either intrinsic changes in the self-reactive B or T cells themselves or active inhibition by suppressor T cells or anti-idiotypic antibodies. The aim of the current paper is to examine the role of these alternative mechanisms in maintaining the unresponsive state of self-reactive B cells in lysozyme/anti-lysozyme Dbl-Tg mice.

MATERIALS AND METHODS

Transgenic Mice. Dbl-Tg mice were produced by matings between hemizygous C57BL/6 lysozyme-transgenic mice (line ML-5) and hemizygous C57BL/6 immunoglobulin-transgenic (Ig-Tg) mice from lines MD-3 and MD-4 (16, 17) (equivalent results were obtained with both lines) or by mating Dbl-Tg mice with nontransgenic C57BL/6 mice.

Adoptive Transfer. Adoptive transfer was as described (16).

Hanging-Drop Cultures. Cell suspensions from spleen or mesenteric lymph node were prepared by gentle sieving into RPMI 1640 medium with 5% (vol/vol) fetal calf serum (FCS; 5% FCS/RPMI). Erythrocytes were lysed in 17 mM Tris·HCl/144 mM NH_4Cl, pH 7.2, and the cells were washed twice in 5% FCS/RPMI and resuspended in RPMI containing 10% FCS, 2 mM glutamine, and 50 μM 2-mercaptoethanol. The indicated number of spleen or lymph node cells from transgenic mice was cultured with 3×10^4 spleen cells from sex-matched nontransgenic mice primed previously to sheep erythrocytes (SRBCs) and 1×10^4 HEL-coated SRBCs, in 20-μl volumes dispensed in Microtest plates (Nunc). The plates were incubated inverted in humid chambers at 37°C in an atmosphere of 5% CO_2/95% air for 8 days, and secreted antibody was measured by ELISA (16).

Lipopolysaccharide (LPS)-Stimulated Cultures. For short-term antibody responses, 1×10^5 spleen cells per well were cultured for 3 days in flat-bottomed microtiter trays (Flow Laboratories) in 0.2 ml of 10% FCS/RPMI supplemented with 2 mM glutamine and 50 μM 2-mercaptoethanol. LPS (*Escherichia coli* O111:B4; Difco) was added at 20 μg/ml. Secreted antibody was measured in the medium from triplicate wells by an ELISA (16, 27), and antibody-forming cells were enumerated by a spot ELISA (27, 28). For limiting dilution analysis graded numbers of transgenic spleen cells

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Abbreviations: Dbl-Tg, double-transgenic; HEL, hen egg lysozyme; HRBC or SRBC, horse or sheep erythrocyte; Ig-Tg, immunoglobulin-transgenic; LPS, lipopolysaccharide; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter.

were cultured as above, except that 2×10^6 C57BL/6 thymocytes were added to each culture, since this was found to greatly improve LPS-induced antibody responses from Ig- and Dbl-Tg cultures containing $<10^3$ spleen cells, as described for nontransgenic spleen cells (29). The culture supernatants were tested after 7 days of incubation to maximize sensitivity of detecting the response from individual clones; the inner 60 wells of 96-well plates were analyzed at each cell input.

B- or T-Cell Depletion. B cells were depleted using magnetic beads coated with sheep anti-mouse immunoglobulin (Dynabeads M450; Dynal, Oslo) according to the manufacturer's instructions, by adding an equal volume of beads at 4×10^7 beads per ml to 2×10^7 spleen cells per ml in 2% FCS/RPMI. Cells were analyzed by monoclonal antibody staining and fluorescence-activated cell sorter (FACS) analysis as described (16); CD4 and CD8 cells were detected with phycoerythrin-conjugated GK1.5 and fluorescein-conjugated anti-Lyt2 monoclonal antibodies (Becton Dickinson). T cells were depleted by staining cells with biotinylated monoclonal antibody RA36B2 (30) and streptavidin-phycoerythrin and sorting the stained cells on a FACS 440. Sorted cells were at least 90% $B220^+$ and 95% viable, as assessed by fluorescence microscopy.

RESULTS

B Cells from Dbl-Tg Mice Respond Poorly to Antigen-Specific Stimuli *in Vivo*. The marked difference in antibody-producing capacity between the Dbl-Tg mice and their Ig-Tg littermates is to a large extent maintained after adoptive transfer of their spleen cells into nontransgenic recipients (16). Since 1×10^5 spleen cells were transferred into each recipient mouse, however, it was not possible to determine whether the poor adoptive response of cells from the Dbl-Tg mice was due to the presence of regulatory cells in the transferred inoculum or to an intrinsic change in the B cells themselves. To overcome this problem, graded numbers of unfractionated spleen cells from Dbl-Tg mice or Ig-Tg littermates were adoptively transferred with a constant number of spleen cells from nontransgenic mice primed previously to horse erythrocytes (HRBCs) and HEL conjugated to HRBCs (HEL-HRBCs). The amount of antibody produced by both Ig- and Dbl-Tg B cells was maximal and constant in recipients receiving between 5×10^3 and 1×10^6 spleen cells (Fig. 1 and data not shown). Less than 1×10^3 lysozyme-specific B cells per mouse, therefore, constitutes a saturating dose, suggesting that some factor, such as T-cell help, may become limiting above this dose or that regulatory mechanisms may exist to limit the response within a defined range. The magnitude of the response began to titrate out at 5×10^2 spleen cells (≈100

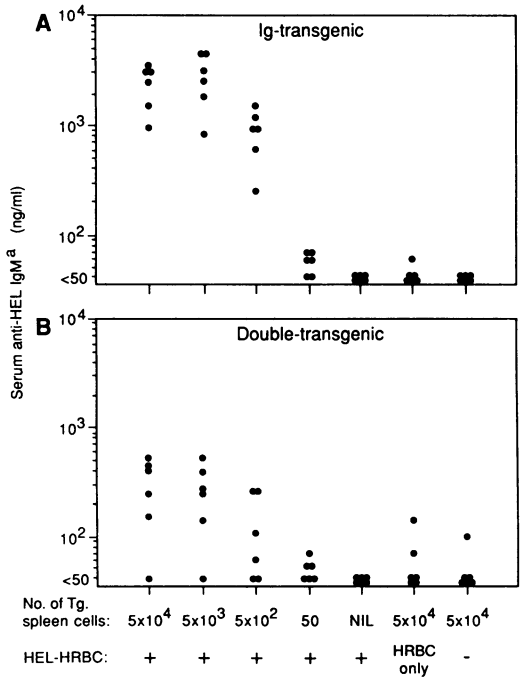

FIG. 1. Titration of spleen cells from Ig-Tg (*A*) and Dbl-Tg (*B*) mice in adoptive transfer. The indicated numbers of spleen cells from MD3 × ML5 Ig-Tg or Dbl-Tg littermates were injected intravenously into irradiated nontransgenic recipients with 5×10^6 spleen cells from HRBC-immunized nontransgenic mice and 2×10^8 HEL-HRBCs or unconjugated HRBCs. Antibody responses in individual recipients, indicated by dots, were measured 7 days after transfer by quantitating lysozyme-binding a-allotype IgM in serum by ELISA (16, 27). In this experiment, B cells accounted for 35% of Ig-Tg and 25% of Dbl-Tg spleen cells.

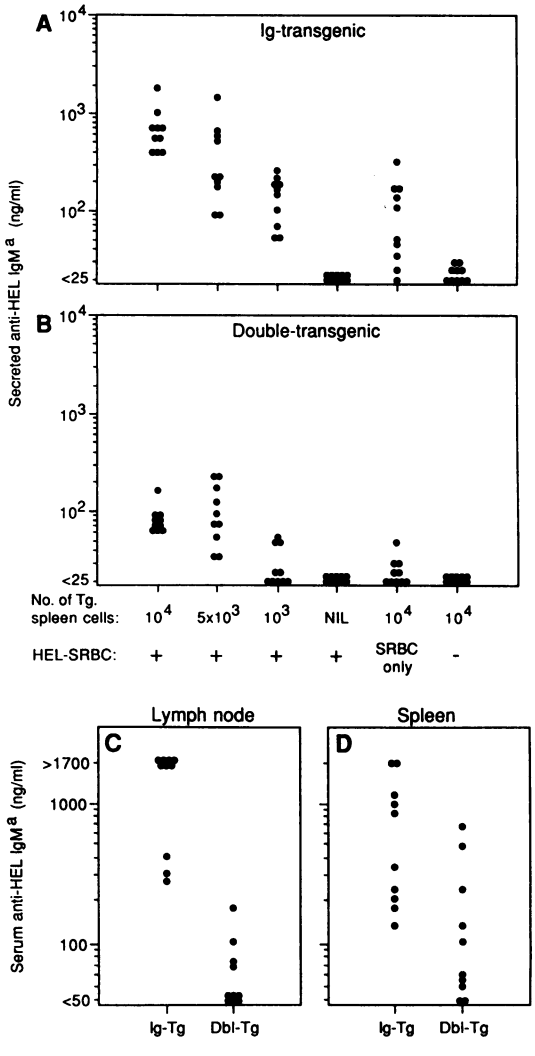

FIG. 2. Antigen-specific *in vitro* antibody responses of spleen (*A*, *B*, and *D*) or lymph node (*C*) cells from Ig- and Dbl-Tg mice. The indicated numbers of spleen cells from MD3 × ML5 Ig-Tg (*A*) or Dbl-Tg (*B*) littermates were cultured in hanging drops with 3×10^4 spleen cells from SRBC-immunized nontransgenic mice as a source of T-cell help and HEL-SRBCs or SRBCs as antigen. Secreted anti-lysozyme IgM antibody in individual cultures, indicated by dots, was measured at 8 days of culture by ELISA. FACS analysis showed that B cells accounted for 27.5% of Ig-Tg and 15.3% of Dbl-Tg spleen cells. (*C* and *D*) In spleen or lymph node cells (1×10^4 cells) from MD4 × ML5 Ig- or Dbl-Tg littermates were cultured and analyzed in the same way as above. The proportion of B cells in various cell populations was as follows: Ig-Tg spleen, 32.2%; Dbl-Tg spleen, 32.7%; Ig-Tg lymph node, 6.3%; Dbl-Tg lymph node, 6.8%.

B cells per mouse) with trace amounts of antibody still being detectable in recipients of ≈10 B cells. Most importantly, although the titration curves of both spleen populations paralleled one another, the tolerant Dbl-Tg cells gave rise to ≈10 times less antibody at all cell doses tested (Fig. 1), thus providing no indication of a titratable regulatory cell.

B Cells from Dbl-Tg Mice Respond Poorly to Antigen-Specific Stimuli *in Vitro*. The complexity of events in the intact host makes it difficult to rely exclusively on adoptive transfer systems for investigating the mechanism of self-tolerance in the Dbl-Tg model. To simplify analysis, a hanging-drop culture system was developed to enable antigen-specific antibody responses to be measured *in vitro*, using carrier-primed (HRBC or SRBC primed) nontransgenic spleen cells as a source of T-cell help and lysozyme conjugated to HRBCs or SRBCs as antigen.

Comparison of *in vitro* anti-lysozyme antibody responses from Ig- and Dbl-Tg spleen cells gave similar results to those observed after adoptive transfer *in vivo*. Thus, when graded numbers of unfractionated Ig- or Dbl-Tg spleen cells were cultured in the presence of carrier-primed spleen cells and HEL-SRBCs, the tolerant B cells from Dbl-Tg mice produced 5–20 times less antibody at each cell dose tested, despite the presence of similar numbers of anti-lysozyme B cells (Fig. 2 *A* and *B*). Similar differences were observed in cultures of mesenteric lymph node cells from Ig- and Dbl-Tg mice (Fig. 2 *C* and *D*).

B Cells from Dbl-Tg Mice Respond Poorly to Nonspecific Stimuli *in Vitro*. To focus more selectively on the intrinsic ability of the B cells to proliferate and differentiate into antibody-secreting cells, spleen cells from Ig- or Dbl-Tg mice were stimulated *in vitro* with the nonspecific B-cell mitogen LPS, which induces B-cell proliferation and antibody secretion with little or no need for cellular interactions (29, 31). When 1×10^5 unfractionated spleen cells from Ig- or Dbl-Tg mice were cultured for 3 days with LPS in microtiter wells, the Dbl-Tg cells secreted 10–20 times less antibody (Fig. 3*A*) and generated a similarly reduced number of antibody-secreting cells (Fig. 3*B*). The markedly diminished antibody response from the tolerant Dbl-Tg B cells was not due to a difference in the number of B cells responding to LPS, however, since limiting dilution analysis of the same spleen cell suspensions yielded a responder frequency of 1/5.5 B cells and 1/10 B cells from Ig- and Dbl-Tg mice, respectively (Fig. 3 *C* and *D*). There was no detectable deviation from linearity such as might be expected if tolerance were due to inhibition by regulatory cells. The slightly lower responder frequency in Dbl-Tg spleens may reflect a decreased sensitivity in detecting responding clones, because the Dbl-Tg B cells secreted less antibody per clone (Fig. 3 *E* and *F*). Interestingly, the difference in antibody secretion between Ig- and Dbl-Tg spleen cells was less marked in 7-day bulk cultures (data not shown) and at limiting dilution (Fig. 3 *E* and *F*) when compared to that observed in 3-day bulk cultures (Fig. 3 *A* and *B*). This may indicate either more rapid exhaustion of the antibody response in the Ig-Tg cell cultures or, alternatively, a gradual reversal of tolerance in the Dbl-Tg cells during prolonged culture with LPS. The antibody secreted by the responding Dbl-Tg B cells was qualitatively identical to that secreted by nontolerant Ig-Tg B cells, in that it carried the transgene-encoded a-allotype and bound lysozyme in proportion to the amount of constant region allotype (Fig. 3 *E* and *F*).

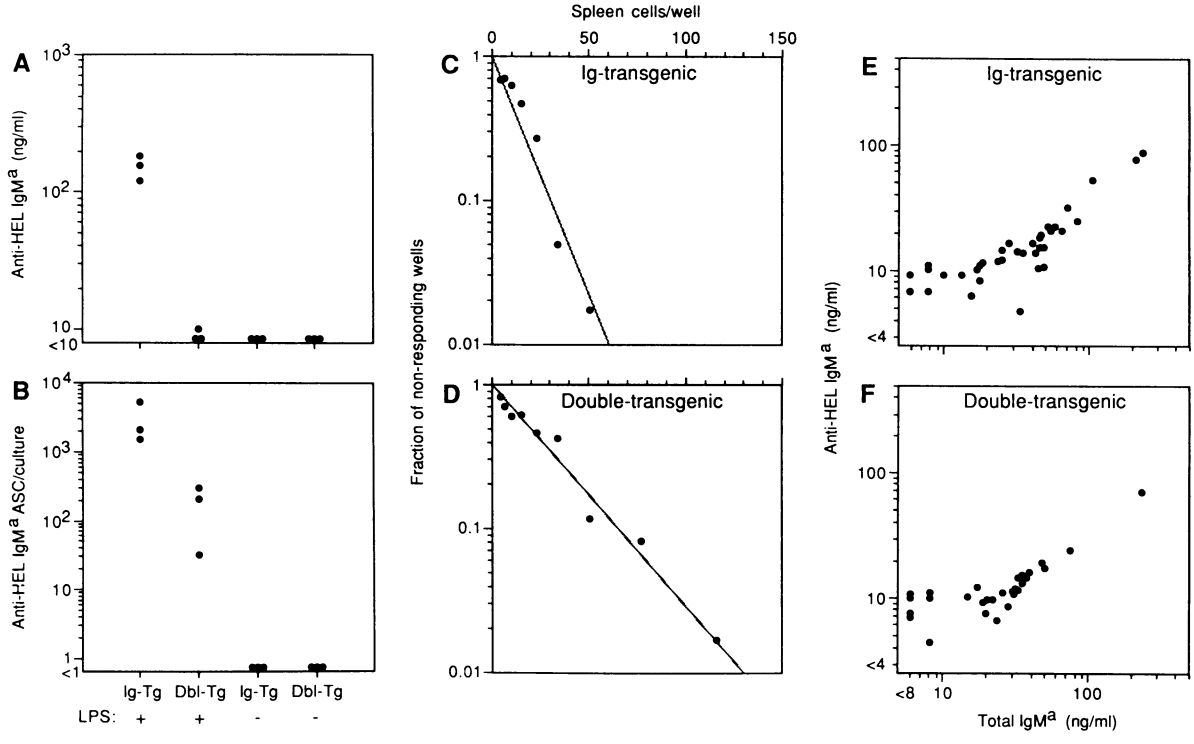

FIG. 3. LPS-induced *in vitro* antibody responses from Ig- or Dbl-Tg spleen cells. Spleen cells from MD4 × ML5 Ig- or Dbl-Tg littermates were cultured in triplicate at 1×10^5 cells per well for 3 days in microtiter trays with or without LPS, and secreted anti-lysozyme IgM^a was quantified in the medium of individual cultures by ELISA (*A*) or the number of anti-lysozyme IgM^a antibody-secreting cells (ASC) in each culture was determined by a spot ELISA (*B*). No antibody or antibody-forming cells were detected in parallel cultures of nontransgenic spleen cells. The frequency of LPS-responding B cells was estimated by limiting dilution from 7-day cultures of the same spleen-cell suspensions (*C* and *D*). Medium from 60 wells was assayed for anti-lysozyme IgM^a by ELISA, and wells with detectable antibody (>6 ng/ml) were scored as positive. Straight lines were fitted by linear regression, and precursor frequencies were derived by interpolating to 37% nonresponding cultures (32); the precursor frequencies obtained for Ig- and Dbl-Tg cultures were 1/13 and 1/28 spleen cells, respectively. Dividing these values by the proportion of B cells at the start of culture (Ig-Tg, 42.5%; Dbl-Tg, 35.9%) yields responder frequencies of 1/5.5 and 1/10 B cells, respectively. The antibody secreted in individual positive cultures derived from the two most dilute sets of cultures in *C* and *D*, where 85% of the responding clones would be expected to be clonal (32), was further analyzed by quantitating lysozyme-binding IgM^a versus total IgM^a (*E* and *F*).

Poor Response of Dbl-Tg B Cells Is Not Associated with Detectable Suppression. In each of the assay systems described above, stimulation of limiting numbers of Dbl-Tg spleen cells failed to reveal evidence for a titratable regulatory cell subset responsible for maintaining self-tolerance. Nevertheless, cells with either anti-idiotypic specificity or suppressor function could in theory be present at higher frequencies than the anti-lysozyme B cells themselves and thus fail to be diluted out except at extremely low cell inputs. On FACS analysis of Dbl-Tg spleen cells <10% of the B cells or 1–3% of all spleen cells express endogenous immunoglobulin heavy chains (16, 17), thus making it unlikely that interaction with anti-idiotypic B cells could be responsible for maintaining self-tolerance in the Dbl-Tg mice. A possible role for T-cell-dependent suppression was more plausible, however, since T cells were present in excess of B cells in the Ig- and Dbl-Tg mice. Thus, T cells accounted for 30–70% of spleen cells in the transgenic mice, although the overall number and ratio of $CD4^+$ and $CD8^+$ T-cell subsets was within the normal range (Fig. 4*A*). To test the possibility that suppressor T cells were responsible for the poor response by the tolerant Dbl-Tg B cells, T-cell-depletion and cell-mixing experiments were performed. As shown in Fig. 4*B*, depletion of T cells and accessory cells from Dbl-Tg spleen cell suspensions by FACS sorting for cells bearing the B-lineage-associated (30) B220 antigen failed to reverse the low response from Dbl-Tg cells in adoptive transfer. Conversely, addition of up to a 100-fold excess of Dbl-Tg T cells to the adoptively transferred inoculum did not reduce the response of nontolerant B cells from Ig-Tg mice (Fig. 4*C*).

DISCUSSION

The presence of large numbers of functionally silent self-reactive B cells in the lysozyme/anti-lysozyme Dbl-Tg mice provides an opportunity for studying nondeletional mecha-

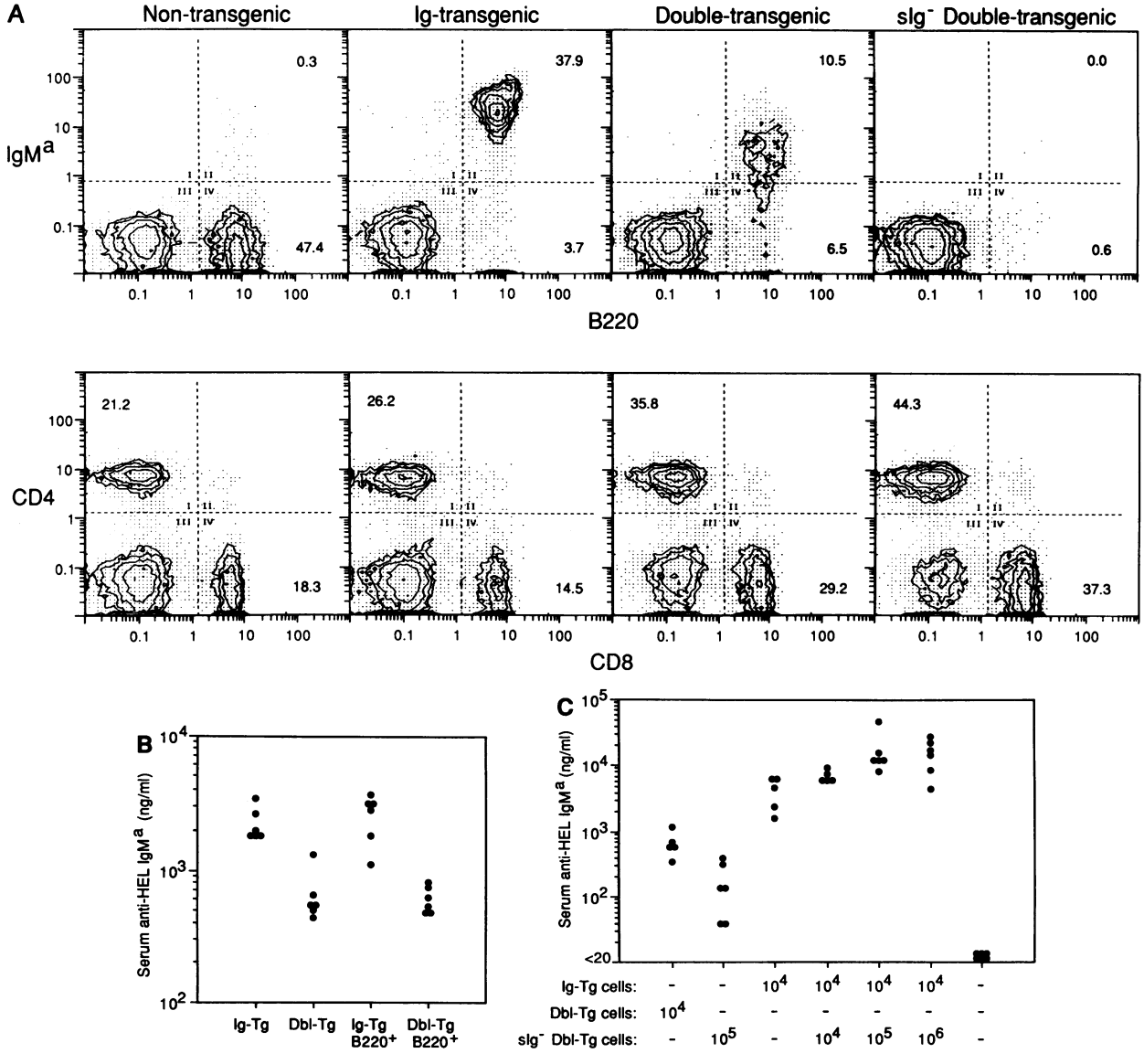

FIG. 4. Absence of detectable suppressor cells in Dbl-Tg mice. (*A*) FACS analysis of B and T cells from spleens of MD4 × ML5 littermate nontransgenic mice, Ig- and Dbl-Tg mice, and Dbl-Tg spleen cells depleted of B cells (sIg^-) with anti-immunoglobulin magnetic beads. The mean number of $CD4^+$ and $CD8^+$ cells in the spleen of two mice of each type was as follows ($\times 10^7$): nontransgenic, 1.34 CD4/1.14 CD8; Ig-Tg, 1.12 CD4/0.5 CD8; Dbl-Tg, 1.10 CD4/0.75 CD8. (*B*) Adoptive transfer of unfractionated spleen cells or FACS-sorted $B220^+$ spleen cells from MD3 × ML5 Ig- and Dbl-Tg mice into irradiated nontransgenic mice with HRBC-primed spleen cells and HEL-HRBCs. Serum anti-lysozyme IgM in individual recipients (dots) was measured 7 days later. (*C*) *In vivo* adoptive response of Ig-Tg spleen cells transferred alone or mixed with a 100-fold or 10-fold excess or an equal number of B-cell-depleted Dbl-Tg spleen cells. For comparison, the response of 1×10^4 undepleted or 1×10^5 B-cell-depleted Dbl-Tg cells is shown.

nisms of self-tolerance. After removal from the tolerant environment of the Dbl-Tg mice, the self-reactive B cells were found to produce 5–20 times less antibody than did nontolerant B cells from Ig-Tg controls when provided with a source of T-cell help and antigen *in vivo* or *in vitro* (Figs. 1 and 2) or when stimulated with LPS *in vitro* (Fig. 3). The diminished antibody response from the tolerant B cells was not due to a comparable reduction in the number of responding precursors since the responses of Ig- and Dbl-Tg cells show similar cell dose–response curves *in vivo* and *in vitro* (Figs. 1–3). Moreover, the fact that the same differences between nontolerant and tolerant B cells are observed in three cellular assays, and in cells from spleen, lymph node (Fig. 2 and ref. 17), and bone marrow (D. Y. Mason and C.C.G., unpublished results), argues strongly against the possibility that these differences simply reflect changes in the anatomical distribution or numbers of various B-cell subsets. Thus, the nondeletional form of tolerance observed in the lysozyme Dbl-Tg mice is associated with a persistent decrease in the ability of most if not all of the B cells to secrete antibody. This functional change appears to be intrinsic and does not require continued interactions with suppressor T cells or anti-idiotypic B cells for its persistence, as indicated by the failure to detect any inhibitory activity in cell titration, depletion, or mixing experiments. It remains possible that interactions with these regulatory elements are necessary for the initial induction of tolerance in the Dbl-Tg mice, although the lack of any demonstrable suppressive activity after cell mixing (Fig. 4*B*) coupled with the previous demonstration that tolerance can be rapidly induced on transfer of nontolerant cells into heavily irradiated lysozyme-transgenic recipients (27) tend to argue against such a possibility.

The precise nature of the response defect in the tolerant Dbl-Tg B cells is not yet known. Generation of a measurable antibody response in all three of the assay systems studied here requires B-cell activation, proliferation, and differentiation into antibody-secreting cells; thus a block at any or all of these steps could give rise to the observed diminution in the antibody response. Other experimental models, involving B cells or B-lymphoma cells exposed to anti-immunoglobulin *in vitro* (33–40) or B cells exposed to hapten-coupled deaggregated γ-globulin *in vivo* (41, 42), indicate that each of these steps can potentially be a site for negative regulatory effects.

Although the 5–20 times reduction in antibody response from the Dbl-Tg B cells provides good evidence for a long-lasting functional change in the B cells, it is intriguing to note that much greater differences in antibody secretion, typically 50- to 500-fold, are observed between Ig- and Dbl-Tg B cells *in situ* within the donor mice (16), or when Ig-Tg cells are transferred and challenged *in vivo* in nontransgenic versus lysozyme-transgenic mice (27). The appreciable antibody response from the Dbl-Tg B cells observed in each of the assay systems described here is not due to low-affinity nontolerant B-cell variants expressing endogenous (nontransgene encoded) heavy or light chains, as the antibody produced carries the transgene-encoded heavy chain allotype and binds lysozyme in proportion to the amount of allotype present (Fig. 3 *E* and *F* and data not shown). Residual antibody secretion by the Dbl-Tg spleen cells is also not due simply to the presence of nontolerant pre-B cells in the transferred or cultured inocula, in that 70–90% of this response can be abolished by depleting surface immunoglobulin-bearing cells with anti-immunoglobulin magnetic beads (Fig. 4*C* and data not shown). Since each of the systems employed in the current study involves removal of the Dbl-Tg B cells to an environment that lacks tolerogenic concentrations of lysozyme, the limited antibody responses observed may indicate that the tolerant state of the cells exhibits some degree of reversibility.

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