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Ig Knock-In Mice Producing Anti-Carbohydrate Antibodies: Breakthrough of B Cells Producing Low Affinity Anti-Self Antibodies

Lorenzo Benatuil,* Joel Kaye,* Nathalie Cretin,‡ Jonathan G. Godwin,* Annaiah Cariappa,‡ Shiv Pillai,‡ and John Iacomini2*

Natural Abs specific for the carbohydrate Ag Galα1–3Galβ1–4GlcNAc-R (αGal) play an important role in providing protective host immunity to various pathogens; yet little is known about how production of these or other anti-carbohydrate natural Abs is regulated. In this study, we describe the generation of Ig knock-in mice carrying functionally rearranged H chain and L chain variable region genes isolated from a B cell hybridoma producing αGal-specific IgM Ab that make it possible to examine the development of B cells producing anti-carbohydrate natural Abs in the presence or absence of αGal as a self-Ag. Knock-in mice on a αGal-deficient background spontaneously developed αGal-specific IgM Abs of a sufficiently high titer to mediate rejection of αGal expressing cardiac transplants. In the spleen of these mice, B cells expressing αGal-specific IgM are located in the marginal zone. In knock-in mice that express αGal, B cells expressing the knocked in BCR undergo negative selection via receptor editing. Interestingly, production of low affinity αGal-specific Ab was observed in mice that express αGal that carry two copies of the knocked in H chain. We suggest that in these mice, receptor editing functioned to lower the affinity for self-Ag below a threshold that would result in overt pathology, while allowing development of low affinity anti-self Abs. The Journal of Immunology, 2008, 180: 3839–3848.

Natural Abs, those produced without intentional immunization, play a major role in providing protective host immunity (1–3). However, the study of natural Abs has been difficult because in many cases their Ag specificity is unknown. The specificity of many natural Abs remains undefined; however, a significant portion are specific for carbohydrates such as blood group Ags. In addition to anti-ABO blood group Abs, natural Abs specific for a carbohydrate Ag Galα1–3β1–4GlcNAc-R (αGal), represent a significant population of natural Abs (4–11). The αGal Ag is synthesized by the glucosyltransferase UDP galactose:β-d-galactosyl-1,4-N-acetyl-d-glucosaminide α(1–3) galactosyltransferase (Enzyme classification E.C. 2.4.1.151), or αGT. All placental mammals except humans, apes and Old World monkeys express a functional αGT gene whose function appears to have been lost ~30 million years ago (5). Because these species do not recognize αGal structures as self, they consequently produce αGal-specific Abs.

αGal-specific natural Abs are estimated to comprise 1–8% of circulating Ig in humans, and ~1% of EBV-transformed peripheral blood B cells make Abs that bind αGal (6, 13). In humans, αGal-specific Abs are encoded for by a restricted set of Ig Vh genes from the V H3 family (14). Production of Abs specific for αGal is believed to be elicited in response to normal bacterial flora that colonize the human gastrointestinal tract (11, 15). The presence of anti-αGal Ab in serum and secretory fluids, such as colostrum and saliva, suggests that these Abs have evolved to play a protective role in primate immunity. Viruses produced in αGT-expressing cells that display αGal-modified glycoproteins within their envelope, such as lymphocytic choriomeningitis virus, Newcastle disease virus and vesicular stomatitis virus, as well as C-type retroviruses, have all been shown to be susceptible to inactivation by serum anti-αGal Abs (16, 17). αGal-specific Abs are therefore believed to play an important role in preventing cross-species infection by pathogens. αGal-specific Abs have also been shown to play an important role in rejection of xenogeneic tissue when transplanted into non-human primates (18–22). Despite the importance of αGal-reactive Abs to host immunity little is known about how development of B cells producing αGal-reactive or other anti-carbohydrate Abs is regulated.

αGal-deficient mutant mice lacking a functional αGT gene (GT0/0 mice) generated by gene targeting in embryonic stem cells lack expression of αGal epitopes and consequently develop αGal-specific natural Abs, the majority of which are IgM (23–25). The serum titer of αGal-specific Abs in GT0/0 mice increases in an age-dependent fashion. αGal-specific Abs in GT0/0 mice share many features with human αGal-specific Abs, including usage of related V genes (26–31). These mice therefore represent a small animal model in which αGal-reactive Abs can be studied. However, the frequency of B cells that produce αGal-specific Abs in

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Ig KNOCK-IN MICE PRODUCING ANTI-αGal Abs

these mice is low, making direct analysis of B cells producing αGal-specific Abs difficult. We and others have generated Ig transgenic mice to study regulation of B cells producing αGal-specific Abs (32, 33). However, the use of these mice to address several aspect of B cell development is limited because the Ig transgenes are randomly integrated. They are not transcriptionally regulated in an identical fashion to endogenous Ig genes, and in the case of Ig L chain transgenes, unlike endogenous self-reactive rearranged κ L chain genes, are not subject to deletion during receptor editing. Therefore, to develop a mouse model in which regulation of αGal-specific Ab production could be studied, we used gene targeting in embryonic stem cells to construct Ig gene knock-in mice. To this end, the rearranged V<sub>α</sub> and V<sub>γ</sub> regions encoding specificity for αGal were isolated from M86, a hybridoma (IgM, κ L chain), derived from GT<sup>α<sub>60</sub></sup> mice (34). M86V<sub>α</sub>V<sub>γ</sub> knock-in mice were generated on either an Ag-deficient GT<sup>α<sub>60</sub></sup> or Ag-sufficient GT<sup>α<sub>60</sub></sup> background to address fundamental issues in regulation of anti-carbohydrate natural Abs. Using these mice, we examined the source of B cells producing αGal-specific Abs and mechanisms leading to negative selection of B cells producing anti-carbohydrate Abs. Our data indicate that restricting the ability of B cells producing self-reactive anti-carbohydrate Abs to undergo receptor editing significantly affects B cell development and allows for the production of low affinity anti-self Abs.

Materials and Methods

Mice

C57BL/6 GT<sup>α<sub>60</sub></sup> mice and derivation of the colony used in these studies have been described in (35). C57BL/6 and BALB/c mice were used as controls and were obtained from The Jackson Laboratory. All mice were housed in viral Ab-free microisolator conditions. All animal experiments were conducted in accordance with Institutional guidelines.

Generation of M86V<sub>α</sub>V<sub>γ</sub> knock-in mice

The functionally rearranged M86 V<sub>α</sub>H<sub>α</sub> and V<sub>γ</sub>L<sub>γ</sub> regions were isolated using standard genomic cloning techniques. To construct Ig H chain and L chain targeting vectors the rearranged M86V<sub>α</sub>H<sub>α</sub> and V<sub>γ</sub>L<sub>γ</sub> gene segments were then cloned using standard techniques into the pVH2LNeo (36) and pVKRNeo (37) H and L chain targeting vectors kindly provided by Dr. K. Rajewsky (Immune Disease Institute, Harvard Medical School, Boston, MA). Vector integrity was confirmed by restriction mapping. Gene targeting and generation of chimeric mice was performed essentially as described (38). Briefly, each targeting vector was linearized and transfected separately into ES-J1 embryonic stem cells. Transfected cells were selected in presence of G418 (300 mg/ml) and gancyclovir (2 mM). DNA prepared from G418 resistant male and female colonies was then screened for the presence of homologous recombination by Southern blotting. ES clones containing either the targeted M86V<sub>α</sub>H<sub>α</sub> or V<sub>γ</sub>L<sub>γ</sub> regions were injected into C57BL/6 blastocysts and then transferred into (BALB/c × C57BL/6)F<sub>1</f> foster mothers. Blastocyst injection, and embryo transfer, was performed by the Massachusets General Hospital Micronjection Core Facility (Boston, MA). Chimeric mice were mated to C57BL/6 mice. DNA prepared from tail biopsy samples of resulting offspring was analyzed by Southern blotting to confirm germline transmission. Resulting knock-in mice were then crossed with cre mice (provided by the Massachusetts General Hospital Core Facility) to delete the Neo<sup>+</sup> gene targeted H chain and L chain loci. Resulting knock-in mice were then crossed to GT<sup>α<sub>60</sub></sup> mice on the C57BL/6 background to generate M86V<sub>α</sub>H<sub>α</sub>GT<sup>α<sub>60</sub></sup> and M86V<sub>γ</sub>L<sub>γ</sub>GT<sup>α<sub>60</sub></sup> mice. These mice were then bred to generate αGal expressing M86V<sub>α</sub>H<sub>α</sub>GT<sup>α<sub>60</sub></sup> and αGal-deficient M86V<sub>γ</sub>L<sub>γ</sub>GT<sup>α<sub>60</sub></sup> mice.

Flow cytometry and Abs

Single cell suspensions were prepared from blood or lymphoid tissues and then stained and analyzed by flow cytometry as described (35). αGal epitopes were detected using the Gal-specific IB lectin (Sigma-Aldrich) from Bandeiraea simplicifolia (BS-I isolectin B4) (39). The following Abs were used in this study were purchased from BD Pharmingen: RA3-6B2 (anti-CD43), and anti-Mac-1 (anti-CD11b). Goat anti-mouse IgM was purchased from Jackson ImmunoResearch Laboratories. RS.3.1 (anti-Igh-6a (40) and MB86 (anti-IgH-6b (41)) were provided by Dr. H. Wortis (Tufts University Sackler School of Biomedical Sciences, Boston, MA). B cells capable of binding αGal were detected by staining with FITC- or biotin-conjugated Gal-BSA (V-Labs).

ELISA

ELISAs were conducted as previously described (35). Briefly, ELISA plates (Corning) were coated overnight at 4°C with either αGal conjugated to BSA (Gal-BSA) or lactosamine conjugated to BSA (Lac-BSA; V-Labs) in carbonate buffer (pH 9.5), and then washed with PBS containing 0.05% Tween 20. Lac-BSA shares all determinants with Gal-BSA, except for the terminal galactose structure, and serves as a specificity control. The wells were blocked with 1% BSA in PBS-Tween for 1 h at room temperature and then washed. Serum samples were serially diluted in PBS-Tween 20, added to the plates, and incubated for 1 h at 37°C. The plates were then washed extensively with PBS-Tween 20, and bound Abs were detected using HRP-conjugated goat anti-mouse IgM (1/4000; Jackson ImmunoResearch Laboratories). To determine the relative contribution of transgene-encoded vs endogenously encoded anti-αGal, bound Abs were detected with purified biotinylated RS.3.1 or MB86, followed by HRP-conjugated streptavidin (1/800; Amersham Biosciences). The plates were incubated for 1 h at 37°C and then washed five times with PBS-Tween 20. A total of 0.01 mg/ml ω-phenylenediamine dihydrochloride (Sigma-Aldrich) in substrate buffer was then added for 20 min at room temperature to develop the assays. The reaction was terminated by adding sulfuric acid to each well, and absorbency was read at 492 nm. Background values obtained from Lac-BSA-coated plates were subtracted from those obtained using Gal-BSA-coated plates. Assays were performed in duplicate. In some instances, serum from immunized mice was used. Mice were immunized i.p. with 10<sup>7</sup> irradiated (3000 rad) pig PBMC as described (42).

Anti-αGal B cell ELISPOT assay

Multiscreen-HA plates (Millipore, Bedford, MA) were coated with 10 μg/ml of either Gal-BSA or Lac-BSA in PBS at 4°C overnight. The plates were then washed three times with PBS, allowing the plates to soak for 5 min between each wash. The plates were blocked with IMDM containing 0.4% BSA and penicillin and streptomycin for 2 h at 37°C. The blocking medium was then removed and 10-fold serial dilutions (starting at 1 x 10<sup>7</sup> cells per well) of spleen cells prepared in blocking IMDM were added to the wells. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 24, 48, or 72 h in the presence or absence of LPS (0.5 μg/ml). After culture, the plates were washed three times in PBS, followed by three additional washes in PBS-Tween 20. HRP-conjugated goat anti-mouse IgM was then added to each well and incubated for 2 h at 4°C. The plates were washed three more times with PBS-Tween 20, followed by PBS, at which point the assays were developed by adding filtered chromogen substrate (3-amino-9-ethyl carbazole) in acetate buffer (pH 5.0). Plates were incubated in the presence of chromogen substrate at room temperature for 5 min and the reaction terminated by washing the plate with water. Spots were enumerated using an automated ELISPOT reader (ImmunoSpot; Cellular Technology). In all assays, the number of background spots obtained on Lac-BSA-coated plates was subtracted from the number obtained on corresponding Gal-BSA-coated plates. All samples were plated in duplicate.

Cell culture

B cell precursors from M86V<sub>α</sub>V<sub>γ</sub> mice were grown in vitro as previously described (43, 44). Briefly, bone marrow cells were depleted of erythrocytes and were cultured in BMB220 medium (IMDM supplemented with 10% FCS, 2-ME, l-glutamine, penicillin/streptomycin, and 50–100 U/ml recombinant murine IL-7; R&D Systems) at a concentration of 2 x 10<sup>6</sup> cells/ml for 5 days. Washed cells were then cultured for 2–42 h in the absence of IL-7 in wells with irradiated (2000 rad) confluent primary adherent bone marrow-derived stroma from Ag-negative GT<sup>α<sub>60</sub></sup> or Ag-bearing wild-type C57BL/6 mice. After culturing the cells on stroma, nonadherent pre-B cells were washed and frozen at ~80°C until RNA was extracted. Stromal cultures were initiated by plating erythrocyte-depleted bone marrow cells to confluence in stromal medium (RPMI 1640, 5% FCS, 2-ME, l-glutamine, penicillin/streptomycin, sodium pyruvate, and nonessential amino acids) for 3 days, then washing away nonadherent cells and allowing the adherent stroma to grow for at least 2 wk before use. Stromal cell line were tested for the expression of αGal by staining with αGal-specific IB lectin.

Detection of RAG2 expression

RNA was prepared from cells using an RNAeasy mini kit (Qiagen). Complementary DNA was prepared from DNase-treated (Invitrogen Life
Characterization of αGal-specific Ab production in M86VHVL knock-in mice.

A. Amplification of tail DNA from M86VHVL knock-in mice and normal controls by PCR. The knocked in H chain was detected using primers that amplify the recombined VDJ segment (left panel). To analyze the presence of one or two copies, an additional primer was introduced to amplify the intron between JH3 and JH4 (JH3-JH4, middle panel) and normal controls by PCR. The knocked in H chain was detected using primers that amplify the recombined VDJ segment (right panel). Data shown are representative of multiple experiments containing at least three mice per group.

B. Anti-αGal Ab production in naive M86VHVL knock-in mice. In all cases, binding to Lac-BSA was used as a specificity control. Data shown are representative of multiple experiments containing at least three mice per group. B. Anti-αGal serum titers in naive (marked by x) and immunized M86VHVL GT\(^{+}\) (top band) and M86VHVL GT\(^{-}\) mice (bottom band). Also shown are anti-αGal serum titers in C57BL/6 mice (filled circles) and naive GT\(^{+}\) mice (open circles). C. Production of knock-in IgM\(^{+}\) or endogenous IgM\(^{+}\) anti-αGal Abs in naive M86VHVL GT\(^{+}\) mice. D. ELISPOT analysis of αGal-specific IgM production in naive M86VHVL GT\(^{+}\), M86VHVL GT\(^{-}\), and immunized GT\(^{+}\) mice. The frequency of Ab-secreting cells per 10\(^{6}\) total cells in each tissue is shown. E. Survival of GT\(^{+}\) (open triangles) or GT\(^{-}\) (open circles) hearts transplanted into naive M86VHVL GT\(^{+}\) mice.

Results

Construction of M86VHVL Ig knock-in mice

The low frequency of B cells that produce αGal-specific Abs in GT\(^{+}\) mice (25, 46, 47) limits the use of these mice to study the regulation of αGal-specific Ab production. To develop a mouse model in which B cells producing αGal-specific Abs could be directly tracked during their development, we generated Ig knock-in mice that express an αGal-specific BCR. To this end, the rearranged Ig H chain and L chain variable region segments were cloned from the hybridoma M86. M86 was derived from GT\(^{+}\) mice and produces an αGal-specific IgM Ab that uses a κ L chain (26, 32, 34). The H chain (rearranged to Jh4) and L chain (rearranged to Jk1) variable region gene segments were then cloned into the hybridoma M86. M86 was derived from GT\(^{+}\) mice and produces an αGal-specific IgM Ab that uses a κ L chain (26, 32, 34). The H chain (rearranged to Jh4) and L chain (rearranged to Jk1) variable region gene segments were then cloned into the plVhL2Neo (36) and pVKRNeo (37) H and L chains Ig targeting vectors. Each targeting vector was then electroporated separately into 129/Sv embryonic stem cells (ES-J1) as described (38). ES clones containing either the targeted M86VH or M86VL region were then injected separately into C57BL/6 blastocysts that were then transferred into (BALB/c × C57BL/6) F1 foster mothers to generate chimeric mice as described (38). ES clones containing either the targeted M86VH or M86VL region were then injected separately into C57BL/6 blastocysts that were then transferred into (BALB/c × C57BL/6) F1 foster mothers to generate chimeric mice as described (38). Offspring carrying a knocked in M86VH or M86VL allele were then crossed with Cre recombinase transgenic mice to delete the neomycin resistance gene from targeted H chain and L chain loci. Resulting knock-in mice were then crossed to GT\(^{+}\) mice on the C57BL/6 background to generate M86VHGT\(^{+}\) and M86VLGT\(^{-}\) mice (Fig. 1A) that were then bred to generate αGal-expressing M86VHVLGT\(^{+}\) mice and αGal-deficient M86VHVLGT\(^{-}\) mice.
FIGURE 2. Characterization and early B cell development in M86VHVT knock-in mice. A. Staining of bone marrow cells from C57BL/6, M86VHVT GT<sup>0/0</sup>, and M86VHVT GT<sup>100</sup> mice for expression of B220 and CD43. Shown are the frequency of B220<sup>+</sup>, CD43<sup>+</sup> B cells and B220<sup>+</sup>, CD43<sup>-</sup> B cells as determined by flow cytometry. B. Analysis of sIgMa and sIgMb expression by B220<sup>+</sup> cells in bone marrow by flow cytometry. C. Analysis of αGal-binding by sIgMa and sIgMb expressing B cells (B220<sup>+</sup>) in the bone marrow of M86VHVT GT<sup>0/0</sup> (top row) and M86VHVT GT<sup>100</sup> (bottom row) mice. D. Analysis of αGal-binding in B220<sup>+</sup>, CD43<sup>-</sup> pre-B cells and B220<sup>+</sup>, CD43<sup>-</sup> pro-B cells from M86VHVT GT<sup>0/0</sup> mice. Data shown are representative of multiple experiments containing at least three mice per group.

Characterization of αGal-specific Ab production in M86VHVT knock-in mice

Analysis of natural serum Abs revealed that M86VHVT GT<sup>0/0</sup> mice contain high titers of αGal-specific Ab in their serum (Fig. 1B). The level of αGal-specific Abs in M86VHVT GT<sup>100</sup> mice was significantly higher than that observed in GT<sup>0/0</sup> controls at all ages examined (Fig. 1B). Immunization of M86VHVT GT<sup>100</sup> mice with αGal-expressing pig cells led to an increase in the titer of αGal-specific Abs (Fig. 1B). In contrast, we were unable to detect production of αGal-specific Abs in the serum of M86VHVT GT<sup>0/0</sup> or M86VHVT GT<sup>100</sup> mice even after immunization (Fig. 1B). In M86VHVT mice the endogenous H chain locus is IgH-6<sup>b</sup> (IgM<sup>a</sup>,μ<sup>b</sup>), whereas the knocked in H chain locus is IgH-6<sup>a</sup> (IgM<sup>a</sup>,μ<sup>b</sup>), which can be detected using the anti-allotypic mAbs MB86 (41) and RS3.1 (40), respectively. αGal-specific Abs in M86VHVT GT<sup>0/0</sup> mice were encoded for by the knocked in IgM<sup>b</sup> allotype, rather than the endogenous IgM<sup>b</sup> allotype (Fig. 1C). B cells spontaneously producing αGal-specific Abs were detected in the spleen, lymph node, and bone marrow but not the peri toneum of M86VHVT GT<sup>0/0</sup> mice (Fig. 1D). Immunization with pig cells did not result in the detection of αGal-producing B cells in the peri toneum of immunized M86VHVT GT<sup>0/0</sup> mice (data not shown). The frequency of αGal-producing B cells in the spleen, bone marrow, and lymph nodes of M86VHVT GT<sup>0/0</sup> mice was at least 10-fold higher than in GT<sup>100</sup> controls (Fig. 1D). We were unable to detect B cells producing αGal-specific Abs in lymphoid tissues from M86VHVT GT<sup>0/0</sup> or M86VHVT GT<sup>100</sup> mice (Fig. 1D).

We next examined whether the titer of αGal-specific Abs in M86VHVT GT<sup>0/0</sup> mice was sufficient to induce Ab-mediated transplant rejection. MHC-matched hearts from littermate M86VHVT GT<sup>0/0</sup> or C57BL6 mice were heterotopically transplanted into the abdomen of M86VHVT GT<sup>0/0</sup> mice. Hearts from M86VHVT GT<sup>0/0</sup> and C57BL6/6 mice were uniformly rejected within 24–72 h. In contrast, hearts from M86VHVT GT<sup>100</sup> mice were accepted long-term (Fig. 1E). These data suggest that αGal-specific

FIGURE 3. Self-antigenic stimulation induces expression of RAG2 in M86VHVT GT<sup>100</sup> BM B cells. Analysis of RAG2 up-regulation by bone marrow pre-B cells upon Ag encounter. M86VHVT GT<sup>100</sup> bone marrow pre-B cells were grown in IL-7 for 5 days and then cultured on pre-established primary GT<sup>+</sup> or GT<sup>0/0</sup> bone marrow stromal cells for the times indicated. A. Real-time PCR analysis by PCR and Southern blot showed up-regulation of RAG2 mRNA in bone marrow pre-B cells cultured on GT<sup>+</sup> stroma. β-actin mRNA levels served as an internal control and were evaluated by ethidium bromide staining. Films were exposed for 2 or 24 h. B. Analysis of RAG2 expression by quantitative real-time PCR. M86VHVT GT<sup>100</sup> bone marrow pre-B cells were grown in IL-7 for 5 days and then cultured for 2, 4, and 16 h on irradiated (2000 rad) GT<sup>+</sup> or GT<sup>0/0</sup> bone marrow stromal cells. RAG2 levels were then assessed by Real-time PCR analysis by PCR and Southern blot showed up-regulation of RAG2 mRNA in bone marrow pre-B cells cultured on GT<sup>+</sup> stroma. β-actin mRNA levels served as an internal control and were evaluated by ethidium bromide staining. Films were exposed for 2 or 24 h. B. Analysis of RAG2 expression by quantitative real-time PCR. M86VHVT GT<sup>100</sup> bone marrow pre-B cells were grown in IL-7 for 5 days and then cultured for 2, 4, and 16 h on irradiated (2000 rad) GT<sup>+</sup> or GT<sup>0/0</sup> bone marrow stromal cells. Data presented are the difference in cycle threshold values normalized to GAPDH for each sample (RQ) and are the results of two different experiments. All assays were performed in triplicate. The level of RAG2 transcripts was statistically higher in pre-B cells cultured in GT<sup>+</sup> stromal cells (p < 0.05, at 2, 4 and 16 h, by Student’s t test, unpaired 95% confidence).
Abs in M86V_hV1_GT^0/0 mice are functional and of sufficient titer to induce Ab-mediated rejection.

Characterization of early lymphocyte development in M86V_hV1_LT knock-in mice

M86V_hV1_GT^0/0 and M86V_hV1_GT^+/+ mice were sacrificed and lymphoid tissues analyzed to examine lymphocyte development. αβ T cell development in the thymus was essentially normal when compared with GT^0/0 or GT^+/+ controls (data not shown). Similarly, T cell development in the spleen and lymph nodes was normal (data not shown). Characterization of B cell development in the bone marrow of M86V_hV1_GT^0/0 or M86V_hV1_GT^+/+ knock-in mice demonstrated that pro-B cells (B220^-CD43^+) and immature (B220^-CD43^-) B cells, as defined in (48, 49), were present in similar proportions to those observed in normal mice, although the frequency of each of these fractions was reduced when compared with normal controls (Fig. 2A), as observed in other Ig knock-in mice (32, 33). The frequency of pre-B cells was similar in M86V_hV1_GT^0/0 or M86V_hV1_GT^+/+ knock-in mice (data not shown).

The majority of B220^- B cells in the bone marrow of M86V_hV1_GT^0/0 and M86V_hV1_GT^+/+ mice expressed the knocked in H chain allele (B220^-, slgM^-) (40.4 ± 9.3% and 46.6 ± 10.1%, respectively) rather than endogenously encoded (B220^+, slgM^+) allele reflecting exclusion by the rearranged M86V_h_GT gene (Fig. 2B). Essentially all of the B220^-, slgM^- B cells in the bone marrow of M86V_hV1_GT^0/0 mice were able to bind αGal as determined by staining with fluorescently labeled BSA conjugated to αGal (Fig. 2C). B220^+, slgM^+ B cells able to bind αGal-BSA were not detected in M86V_hV1_GT^+/+ bone marrow cells for expression of B220 and CD43 revealed that B cells capable of binding αGal-BSA were CD43^-, pre-B or newly formed B cells (Fig. 2D). B cells able to bind αGal were not detected in the bone marrow of M86V_hV1_GT^+ mice, suggesting that B cells expressing the knocked in transgene are tolerized during their development (Fig. 2C).

Receptor editing prevents the development of B cells producing self-reactive Abs

Because the frequency of B cells expressing surface IgM^+ (slgM^+) in the bone marrow of M86V_hV1_GT^+ is relatively high (Fig. 2B) even though we were unable to detect B cells that bind αGal in the bone marrow of M86V_hV1_GT^+ mice (Fig. 2C) we reasoned that tolerance to αGal in M86V_hV1_GT^+ mice was not deletional. We
therefore examined whether tolerance was the result of receptor editing (45, 50–52). To this end, IL-7 driven pre-B cells from the bone marrow of M86V\textsubscript{HV\textsubscript{L}}GT\textsuperscript{0/0} mice were cultured on irradiated bone marrow stromal cells from GT\textsuperscript{+} and GT\textsuperscript{0/0} mice and the levels of \textit{RAG2} expression assayed by real-time PCR. Expression of \textit{RAG2} was up-regulated in M86V\textsubscript{H}\textsubscript{V}\textsubscript{L} GT\textsuperscript{0/0} pre-B cells cultured on stromal cells from GT\textsuperscript{+} cells when compared with pre-B cells from the same mice cultured on GT\textsuperscript{0/0} stromal cells (Fig. 3A). Analysis of \textit{RAG2} expression by quantitative real-time PCR confirmed this finding (Fig. 3B). These data suggest that pre-B cells producing self-reactive anti-carbohydrate Abs undergo tolerance through a mechanism that involves receptor editing.

Peripheral B cell development in M86V\textsubscript{HV\textsubscript{L}} knock-in mice

We next characterized B cell development in the periphery of M86V\textsubscript{HV\textsubscript{L}} mice. In the spleen of M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} and M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{+} mice the majority of B cells were B220\textsuperscript{+}, sIgM\textsuperscript{−} (51.8 ± 8.3% and 69.4 ± 7.6%, respectively) with the frequency of B220\textsuperscript{+}, sIgM\textsuperscript{+} B cells being slightly higher in M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{+} mice (Fig. 4A). B220\textsuperscript{+}, sIgM\textsuperscript{−} B cells were detected in both types of mice (Fig. 4A). When compared with bone marrow (Fig. 2B), the proportion of B220\textsuperscript{+}, sIgM\textsuperscript{+} B cells observed was higher in the spleen of both M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} and M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{+} mice (Fig. 4A). B cells expressing both sIgM\textsuperscript{+} and sIgM\textsuperscript{−} were not detected in either M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} or M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{+} mice, suggesting allelic exclusion by the knocked in H chain (Fig. 4B). As observed in the bone marrow, B cells capable of binding αGal were detected in the spleen of M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} but not M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{+} mice (Fig. 4C). Although we were unable to detect B cells that spontaneously secrete αGal-specific Abs in the peritoneum of M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} mice (Fig. 1D), B220\textsuperscript{+}, CD11b\textsuperscript{−}, CD5\textsuperscript{−} B cells in the peritoneum were capable of binding αGal in M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} mice (Fig. 4D). In both the spleen and peritoneum, B cells able to bind αGal were B220\textsuperscript{+}, sIgM\textsuperscript{−}. However, B cells in the peritoneum did not produce αGal-specific Abs even after stimulation with LPS (Fig. 4E). Consistent with data in Fig. 1E, B cells secreting αGal-specific Abs were only observed in the spleen, bone marrow, and lymph nodes.

Immature B cells in the bone marrow first emigrate to the red pulp in the spleen where they can be detected as sIgM\textsuperscript{high}, sIgD\textsuperscript{low}, CD21\textsuperscript{low}, CD23\textsuperscript{−} newly formed transitional or T1 cells. This emigration to the spleen is an Ag-regulated process (reviewed in Ref. 53). T1 cells then colonize the lymphoid follicles present in the spleen, and up-regulate IgD to become sIgM\textsuperscript{high}, sIgD\textsuperscript{high}, CD21\textsuperscript{int} follicular B cell precursors. These cells give rise to sIgM\textsuperscript{low}, sIgD\textsuperscript{high}, CD21\textsuperscript{int}, CD21\textsuperscript{+} naive follicular B cells, and after additional Ag stimulation, sIgM\textsuperscript{high}, sIgD\textsuperscript{high} mature follicular B cells. There is also a subpopulation of B cells that express high levels of CD21 and CD1d, and are sIgM\textsuperscript{low}, sIgD\textsuperscript{low}, CD21\textsuperscript{high}, CD23\textsuperscript{−}, CD1d\textsuperscript{high} marginal zone (MZ) B cells, which exist outside the follicle (54, 55). Analysis of the spleen of M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} mice by cell surface staining with Abs specific for IgM, IgD, and CD21 and with FITC-labeled αGal-BSA revealed that within the spleen B cells capable of binding αGal were sIgM\textsuperscript{high}, IgD\textsuperscript{low}, CD21\textsuperscript{high} MZ B cells (60 ± 22% MZ B cells are αGal specific, Fig. 5A). We were unable to detect sIgM\textsuperscript{low}, IgD\textsuperscript{high}, CD21\textsuperscript{int} follicular B cells capable of binding αGal-BSA (Fig. 5A). Analysis of tissue sections from the spleens of M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} mice revealed that essentially all B cells capable of binding αGal-BSA reside in the MZ and were excluded from the follicle (Fig. 5B). These data suggest that in M86V\textsubscript{HV\textsubscript{L}} GT\textsuperscript{0/0} mice αGal-binding B cells are committed to a MZ fate.

\textit{Increasing M86V\textsubscript{HV\textsubscript{L}} copy number alters the fate of αGal-specific B cells when self-Ag is encountered}

While breeding M86V\textsubscript{HV\textsubscript{L}} knock-in mice, we also generated mice carrying two copies of the knocked in M86V\textsubscript{H} region and either
one or two copies of knocked in M86V region (M86V2VL or M86V2VL2 mice) on a GT0/0 or GT+ background. Additionally, we generated mice carrying two copies of the knocked in M86V region (M86V2VL2GT0/0 mice) on a GT0/0 or GT+ background. In blood, the frequency of B cells in M86V2VL, M86V2VL2, and M86V2VL2GT0/0 mice on the GT0/0 background was similar to the frequency observed in M86V2VLGT0/0 mice (Fig. 6). However, the frequency of B220+ B cells in the periphery of M86V2VLGT0/0 mice (18.7 ± 2.3%) was significantly reduced when compared with the frequency in M86V2VLGT0/0 mice (24.7 ± 2.9%; p < 0.05, Student’s t test). M86V2VLGT and M86V2VL2GT0/0 mice exhibited an even greater reduction in B220+ cells (10.1 ± 0.3% and 6.6 ± 0.6%, p < 0.05) when compared with the frequency in M86V2VLGT0/0 mice (Fig. 6). These data suggest that the presence of multiple knocked in H chain or L chain alleles affects B cell development. Interestingly, the reduction in B cell numbers is observed only in mice that express αGal as a self-Ag, suggesting that the effect observed may be related to alterations in negative selection.

Production of autoreactive αGal-specific Abs in M86V2VL2GT+ knock-in mice

Our data indicate that B cells producing αGal-specific Abs were efficiently tolerated in M86V2VLGT+; however, we consistently observed the presence of αGal-specific serum IgM Abs in M86V2VL2GT+ mice (Fig. 7A). These Abs did not bind the control Ag Lac-BSA which shares all determinants with αGal-BSA except for the terminal galactose residue, indicating the binding observed was specific for αGal. Essentially all αGal-specific Abs in these mice were of the M86V-encoded IgM allele (data not shown). The titer of anti-αGal Abs in these mice was consistently lower than that observed in littermate M86V2VLGT0/0 and M86V2VL2GT0/0 mice, but was similar to the titers observed in GT0/0 control mice (Fig. 7A). Furthermore, the titers of these Abs could be boosted by pig cell immunization (Fig. 7A). To confirm these results, spleen cells from pig cell immunized mice were analyzed for their ability to produce αGal-specific Ab in Ig ELISPOT assays using αGal-BSA- or Lac-BSA-coated plates. We detected B cells producing αGal-specific IgM Abs in the spleens of M86V2VL2GT+ mice, at a frequency similar to that observed in immunized GT0/0 controls (Fig. 7B). Similar results were observed in M86V2VLGT+ mice (data not shown). These data suggest that B cells producing self-reactive anti-αGal Abs are able to develop in M86V2VL2GT+ mice.

The development of B cells producing αGal-specific Ab in M86V2VL2GT+ mice may reflect lowered affinity for Ag

The ability to detect anti-αGal Abs in M86V2VL2GT+ mice was unexpected. To begin to characterize these Abs, we analyzed the
capacity of αGal-specific Abs from these mice to bind BSA con- 
jugated to different numbers of αGal epitopes. We were readily 
able to detect binding of αGal-specific IgM from these mice when 
using ELISA plates coated with αGal-BSA conjugates in which 
the molar ratio of αGal to BSA molecules was high (15:1) (Fig. 
7C). However, the ability to detect binding of αGal in these mice 
was significantly reduced when ELISA plates were coated with 
αGal-BSA conjugates in which the molar ratio of αGal to BSA 
was lower (10:1) (Fig. 7C). Importantly, serum IgM from 
M86VHVLGT0/0 (Fig. 7C) and M86VHVLGT0/0 (data not 
shown) was able to bind to both αGal-BSA preparations to a sim-
ilar degree. These data suggested that αGal-specific Abs pro-
duced in M86VHVLGT0/2GT+ mice exhibit different binding char-
acteristics than those of littermate mice on the GT0/0 background. We suggest that B cells making self-reactive αGal-
specific Abs in M86VHVLGT0/2GT+ and M86VHVLGT0/2GT+ mice 
(data not shown) may develop because the Ab they produce 
exhibits a lowered affinity for αGal.

Discussion

Anti-carbohydrate natural Abs are thought to play a key role in 
providing protective host immunity and have been well described 
as being important in mediating transplantation rejection across 
blood group disparities as well as discordant species. Yet little is 
known about B cells that produce anti-carbohydrate Abs, in part 
due to a lack of small animal models in which the development and 
regulation of B cells producing anti-carbohydrate Abs can be stud-
ied in a physiologically relevant manner. The development of 
GT0/0 mice has made it possible to begin to address some of these 
issues. However, the frequency of B cells producing αGal specific 
Abs in these mice is too low to allow for their direct analysis. We 
and others have previously attempted to overcome this difficulty by 
generating Ig transgenic mice that express transgenes encoding 
αGal-specific Abs (32, 33). However, these models are limited 
because the expressed transgenes were not subject to regulation by 
elements within the endogenous Ig loci, making it difficult to as-
sess physiological relevance. To overcome these issues we used 
gene targeting in embryonic stem cells to construct Ig knock-in 
mice that carry rearranged H chain and L chain V regions that 
code specificity for αGal that are under regulatory control of the 
endogenous Ig H chain and L chain loci. Breeding M86VHVL 
mice to GT0/0 mice offered us the opportunity to directly examine 
the development of B cells producing αGal-specific Abs in the 
presence or absence of αGal as a self-Ag.

M86VHVLGT0 mice show high serum levels of αGal-specific 
Abs produced by the knocked in allele. The titer of these Abs was 
sufficient to mediate rejection of H-2-matched heart transplants 
from GT+ donors. The titer of αGal-specific Abs in M86VHVL 
GT0 mice could be increased by immunization. αGal-specific Abs 
were not detected in the serum of M86VHVLGT0 mice, indicating 
that expression of αGal in these mice prevented development of 
αGal-specific Abs. However, we were still able to detect sIgM+ B 
cells in the spleens of M86VHVLGT0 mice, suggesting that B 
cells expressing the knocked in M86VH region were not deleted 
during their development. Because the frequency of B cells ex-
pressing surface IgM+ in the bone marrow of M86VHVLGT0 
and M86VHVLGT0 knock-in mice was similar even though we were 
unable to detect B cells that bind αGal in the bone marrow of 
M86VHVLGT0 mice, we reasoned that tolerance to αGal in 
M86VHVLGT0 mice was most likely the result of receptor editing 
rather than deletion. Analysis of IL-7 driven pre-B cells from the 
bone marrow of M86VHVLGT0 mice revealed that exposure to 
αGal as a self-Ag led to an up-regulation of RAG2 expression in 
M86VHVLGT0 pre-B cells. These data are consistent with the
that the effect observed is related to alterations in negative selection and that increasing the copy number of knocked in alleles may alter mechanisms of negative selection by restricting receptor editing, thereby skewing tolerance toward a deletional mechanism. This would support the idea that B cells primarily use editing to eliminate self-reactive B cells, and that negative selection via deletion is secondary to receptor editing. Although B cells producing αGal-specific Abs were efficientlytolerized in M86VHγ1 mice on the GT+ background, we consistently observed the presence of αGal-specific serum IgM Abs in M86VHγ2Vλ2GT+ mice. This unanticipated finding prompted us to examine the binding characteristics of αGal specific Abs in these animals. Interestingly, although we were readily able to detect binding of αGal-specific IgM from these mice when using ELISA plates coated with αGal-BSA conjugates in which the molar ratio of αGal to BSA molecules was high, the ability to detect binding of αGal in these mice was significantly reduced when ELISA plates were coated with αGal-BSA conjugates in which the molar ratio of αGal to BSA was relatively low. These data suggest that αGal-specific Abs in M86VHγ2Vλ2GT+ mice have a lower affinity for αGal than that observed in GT- mice. Such low affinity Abs appear to require an increase in binding avidity to be detected, which can be achieved by using BSA molecules that are highly substituted with αGal epitopes. Why then would such Abs develop in mice that express αGal? Although this issue is under study, it is important to point out that production of αGal-specific Abs are produced only in mice containing two copies of the knockout in H chain allele and either one or two copies of the knock in L chain allele. In M86 mice, the knocked in H chain allele is rearranged to Jγ4, which prevents receptor editing but not V gene replacement. We suggest that in the context of multiple copies of the knocked in L chain, V gene replacement via homologous recombination may select for VH genes of similar sequences to the M86VH region, which can encode H chain variable regions that bind human anti-Gal antibodies: implications for discordant xenografting in humans. Transplant. Proc. 24: 559–562.


