Tolerization of Anti–Galα1-3Gal Natural Antibody–forming B Cells by Induction of Mixed Chimerism

By Yong-Guang Yang,* Emil deGoma,* Hideki Ohdan,* Jennifer L. Bracy,* Yuanxin Xu,‡ John Iacomini,* Aron D. Thall,‡ and Megan Sykes*

*From the *Transplantation Biology Research Center, Surgical Service, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts 02129; and ‡BioTransplant, Inc., Charlestown, Massachusetts 02129*

Summary

Xenotransplantation could overcome the severe shortage of allogeneic organs, a major factor limiting organ transplantation. Unfortunately, transplantation of organs from pigs, the most suitable potential donor species, results in hyperacute rejection in primate recipients, due to the presence of anti–Galα1-3Gal (Gal) natural antibodies (NAbs) in their sera. We evaluated the ability to tolerize anti-Gal NAb–producing B cells in α1,3-galactosyltransferase knockout (GalT KO) mice using bone marrow transplantation (BMT) from GalT$^{+/+}$ wild-type (WT) mice. Lasting mixed chimerism was achieved in KO mice by cotransplantation of GalT KO and WT marrow after lethal irradiation. The levels of anti-Gal NAb in sera of mixed chimeras were reduced markedly 2 wk after BMT, and became undetectable at later time points. Immunization with Gal$^{+/+}$ xenogeneic cells failed to stimulate anti-Gal antibody production in mixed chimeras, whereas the production of non–Gal-specific antixenoantigen antibodies was stimulated. An absence of anti-Gal–producing B cells was demonstrated by enzyme-linked immunospot assays in mixed KO+WT→KO chimeras. Thus, mixed chimerism efficiently induces anti-Gal–specific B cell tolerance in addition to T cell tolerance, providing a single approach to overcoming both the humoral and the cellular immune barriers to discordant xenotransplantation.

Although modern immunosuppressive therapies have improved the success of clinical organ transplantation, a severe shortage of allogeneic organs currently limits the number of transplants performed (1, 2). Given the urgent need for donor organs and the problems associated with the use of nonhuman primates, interest has become focused on the potential use of nonprimates as organ donors for humans. The species generally believed to be most suitable for this purpose is the pig (2–4). However, xenotransplantation from evolutionarily distant species such as the pig poses formidable obstacles. One particularly imposing challenge arises from the presence of preexisting, or "natural," xenoreactive antibodies (NAbs)[1] at high titers in the sera of all humans (5). These NAbs are major effectors of hyperacute rejection. Furthermore, even if NAbs are absorbed or in some way removed before xenogeneic organ transplantation, their return is associated with the phenomenon of delayed xenograft rejection/acute vascular rejection (2, 6–8).

Most of the NAb activity against porcine cells in human sera is directed against the Galα1-3Galβ1-4GlcNAc-R epitope, which is widely expressed on glycoproteins and glycolipids of most mammalian species, including swine (9–11). Although a number of strategies have been used to promote successful xenotransplantation by targeting different steps in the progression of NAb-mediated rejection, none has proved entirely successful. The elimination of anti-Gal NAb from recipients by immunoabsorption by donor-species organ hemoperfusion (12) or immunoaffinity columns of synthetic oligosaccharides (13, 14), or by treatment with anti-Ig antibodies (15) has been found effective in preventing or delaying hyperacute rejection (13–15). However, the efficiency of such treatments is short-lived, as NAb levels return rapidly to their original levels and participate in the delayed rejection of xenografts. In view of the ability of NAb to initiate complement-independent changes in endothelium, to participate in antibody-dependent cell-mediated cytotoxicity against porcine

[1]*Abbreviations used in this paper:* BMC, bone marrow cells; BMT, bone marrow transplantation; ELISPOT, enzyme-linked immunospot; FCM, flow cytometric; Gal, Galα1-3Gal; GalT, α1,3-galactosyltransferase; KO, knockout; NAb, natural antibody; RAG, recombination activating gene; TCD, T cell depleted; WT, wild-type.

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cells, and to play a role in delayed xenograft rejection/acute vascular rejection, inactivation of the recipient complement system (2, 8, 10) or approaches to reducing Gal epitope density (16–18) alone would be unlikely to permit long-term xenoengraftment.

Previous studies have demonstrated that induction of a state of mixed hematopoietic chimerism can lead to permanent tolerance of T cells to allogeneic and concordant xenogeneic antigens, with excellent immunocompetence (19–23). In addition, reductions in mouse IgM NAbs capable of binding to rat bone marrow cells (BMC) were observed in rat→mouse mixed chimeras (24), suggesting that NAb-forming B cells might also be tolerized by this approach. Recently, mice homozygous for a null α1,3-galactosyltransferase allele (GalT KO) have been generated by targeted disruption of the murine α1,3GalT gene (25). As in humans, sera of these animals contain anti-Gal NAb, thus providing a model in which to evaluate methods of inducing anti-Gal NAb tolerance. We have now evaluated the possibility of achieving mixed chimerism in GalT KO mice by transfer of $GalT^{+/+}$ wild-type (WT) mouse BMC with GalT KO mouse marrow to lethally irradiated GalT KO mouse recipients, in order to determine the potential of mixed chimerism to tolerize anti-Gal NAb–producing B cells.

Materials and Methods

Animals. GalT KO ($Gal^{-/-}$, H-2^d) and $GalT^{+/+}$ WT (H-2^{bxd}) mice were derived from hybrid (129SV × DBA/2 × C57BL/6) animals (25). C.B-17 *scid/scid* (C.B-17 *scid*) and recombination activating gene 1 (RAG-1)–deficient (mixed B6 and 129 background) mice were purchased from the Department of Radiation Oncology of Massachusetts General Hospital (Boston, MA), and The Jackson Laboratory (Bar Harbor, ME), respectively. Mice were housed in sterilized microisolator cages and received autoclaved feed and autoclaved, acidified drinking water.

Bone Marrow Transplantation. BMC were T cell–depleted (TCD) using anti-CD4 and anti-CD8 mAbs and rabbit complement as described (26, 27). Recipients were treated with 9.75 Gy whole body irradiation from a ^{137}Cs source (0.97 Gy/min), followed within 4–6 h by i.v. infusion of TCD BMC.

Flow Cytometric Analysis of Chimerism. Single-cell suspensions were incubated with anti–WT mouse H-$2K^b$ 5F1-FITC and PE-labeled anti-CD19 (for B cell chimerism), or anti–CD4-PE plus anti–CD8-PE (for T cell chimerism) (for two-color flow cytometric [FCM] analysis), or with 5F1-FITC, anti–CD19-PE, and anti–CD4-Bio plus anti–CD8-Bio (for three-color FCM analysis) mAbs (PharMingen, San Diego, CA) as described (27). Nonspecific FcγR binding was blocked with rat anti–mouse FcγR mAb 2.4G2 (28) as described (27). FITC-labeled and biotinylated mouse IgG2a mAb HOPC-1 and PE-labeled rat IgG2a mAb (PharMingen) were used as nonstaining negative control antibodies. FCM analysis was performed on a FACScan® cytometer (Becton Dickinson, Mountain View, CA).

ELISA for Detecting Mouse NAb Reactive with Gal. 96-well microtiter ELISA plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with 100 μl of 5 μg/ml of αGal(1→3)βGal (1→4) conjugated to BSA (Alberta Research Council, Alberta, Canada) in bicarbonate buffer (0.05 M $NaHCO_3$, pH 9.6). The wells were washed five times with PBS/Tween-20 (0.05% Tween-20). Plates were blocked with 200 μl/well of 1% BSA in PBS/Tween-20 for 1 h at 37°C and washed five times. Serum samples were serially diluted in PBS/Tween-20, and triplicate samples of 100 μl/well were added to wells. Plates were incubated for 1 h at 37°C, then washed five times with PBS/Tween-20. Bound antibodies were detected using 100 μl/well of horseradish peroxidase–conjugated polyclonal donkey anti–mouse IgM and IgG antibodies (Accurate Chemical and Scientific Corp., Westbury, NY) at 250 ng/ml. Plates were incubated for 1 h at 37°C, and washed five times with PBS/Tween-20. Color development was achieved using 100 μl of 0.01 mg/ml *o*-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co., St. Louis, MO) in substrate buffer (0.1 M sodium citrate, 0.012% H_2O_2, pH 4.5). After 30 min of incubation at room temperature and in complete darkness, the OPD reaction was stopped using 50 μl of 3 M NH_2SO_4, and absorbance at 492 nm was measured using an ELISA reader (SLT Labinstruments, Vienna, Austria).

FCM Analysis of Anti-Gal, Anti–rabbit RBC, and Anti–pig PBMC Antibodies. Indirect immunofluorescence staining of WT mouse cells after incubation with $Gal^{-/-}$ mouse serum provides an accurate method for detecting the presence of anti-Gal–specific NAb. However, the presence of surface Ig^+ B cells in immunocompetent WT mice complicates the detection of mouse IgM NAb in sera. Therefore, we elected to use immunodeficient (*scid* or RAG-$1^{-/-}$) mouse spleen cells and BMC, which lack surface Ig^+ cells, as a source of WT cells for staining with murine sera. Rabbit RBCs (Sigma Chemical Co.) and pig PBMCs were used to detect serum levels of anti–rabbit and anti–pig antibodies, respectively. 10^6 cells were stained for 30 min at 4°C with 10 μl of serially diluted mouse serum, washed, then incubated with FITC-conjugated rat anti–mouse IgM for 30 min at 4°C. The Gal specificity of GalT KO mouse anti-*scid* (or anti–RAG-$1^{-/-}$) antibodies was verified by a Gal NAb–specific ELISA assay, and levels of anti–*scid*/RAG-$1^{-/-}$ antibodies detected by immunofluorescence correlated with titers of anti-Gal Ig observed in the ELISA assay ($r > 0.9$).

Enzyme-linked Immunospot Assay for Detecting Anti-Gal Antibody–producing Cells. Enzyme-linked immunospot (ELISPOT) assays were performed as described (Xu, Y. and A.D. Thall, manuscript in preparation). In brief, cell suspensions were serially diluted (four fivefold dilutions, beginning with 8 × 10^5 cells/well) and placed in triplicate wells in MultiScreen-HA plates (Millipore Corp., Bedford, MA) precoated with αGal-BSA at 4°C overnight. Plates were incubated at 37°C overnight, and bound antibodies were detected using horseradish peroxidase–conjugated goat anti–mouse IgM plus IgG (Southern Biotechnology Associates Inc., Birmingham, AL). The spot number in each well was counted by an observer unaware of the treatments administered to each sample donor.

Statistical Analysis. Student's *t* test for comparison of means was used for statistical analysis. A P value <0.05 was considered to be significant.

Results

Establishment of Mixed Chimerism by Cotransplantation of GalT KO and $Gal^{+/+}$ WT BMC to Lethally Irradiated GalT KO Mice. To determine whether anti-Gal NAb–producing B cells in GalT KO mice would be rendered tolerant in the presence of WT hematopoietic cells, experiments were conducted in which GalT KO mice were lethally irradiated (9.75 Gy) and reconstituted with 26 × 10^6 TCD WT BMC

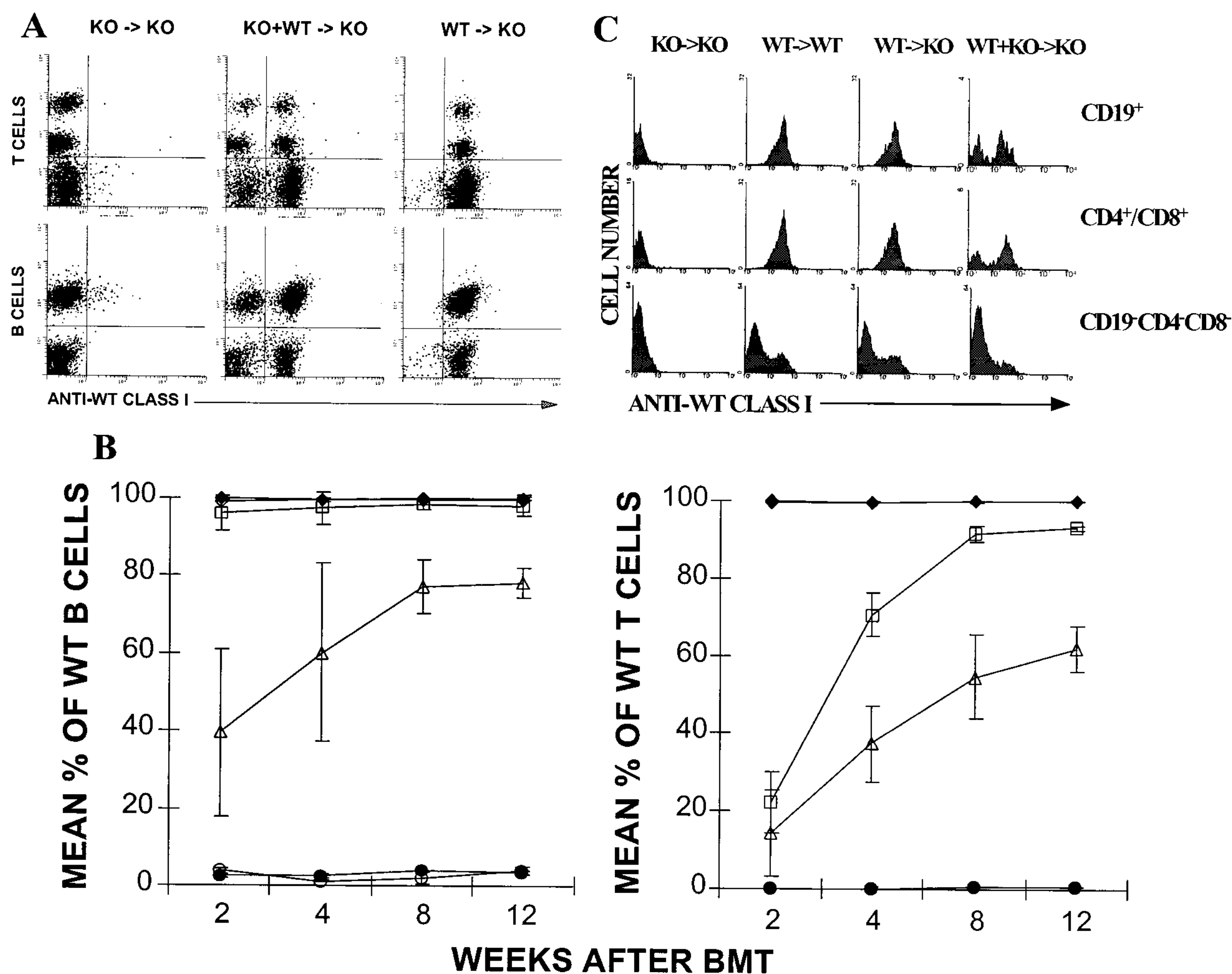

Figure 1. Stable mixed chimerism in GalT KO recipients of mixed WT and KO BMC. PBLs were stained with anti–WT H-$2K^b$ mAb 5F1-FITC and anti–CD19-PE or anti–CD4-PE plus anti–CD8-PE. Both WT (H-2^{bxd}) and KO (H-2^d) cells were detected in GalT KO recipients of mixed WT and KO BMC. (*A*) Representative FACS® profiles of PBLs from mixed and fully allogeneic chimeras at 12 wk after BMT. The two T cell populations of lower and higher intensity represent $CD4^+$ and $CD8^+$ T cells, respectively. (*B*) Percentages of WT B and T cells in the PBLs of untreated GalT KO mice (●, $n = 3$), WT mice (◆, $n = 2$), GalT KO mouse recipients of KO BMC (○, $n = 3$), WT BMC (□, $n = 5$), or mixed KO plus WT BMC (△, $n = 5$), and WT mouse recipients of WT BMC (◇, $n = 3$). Average (± SD) net percentages (after subtraction of background staining) of WT B cells (percentage $5F1^+CD19^+$ cells/total B cells) and T cells (percentage $5F1^+CD4/8^+$ cells/total T cells) are shown at the indicated time points. (*C*) BMC were prepared from GalT KO recipients of KO BMC (*KO→KO*), WT BMC (*WT→KO*), or WT plus KO BMC (*WT+KO→KO*), or WT recipients of WT BMC (*WT→WT*) 19 wk after BMT, and were stained by FITC-conjugated anti–WT H-$2K^b$ mAb 5F1, anti–CD19-PE, and anti–CD4-Bio plus anti–CD8-Bio, followed by incubation with Cy-chrome-streptavidin. Typical histogram representation of WT ($5F1^+$) cells on gated B cells ($CD19^+$, *top*), T cells ($CD4^+/CD8^+$, *middle*), and nonlymphoid cells ($CD19^-CD4^-CD8^-$, *bottom* [most of these were Mac-1^+]) are shown. Similar results were observed for spleen and intraperitoneal cells (data not shown).

alone, or with 6.5×10^6 TCD GalT KO plus 26×10^6 TCD WT BMC. GalT KO mice injected with 6.5×10^6 TCD GalT KO BMC, WT mice injected with 26×10^6 TCD WT BMC, and uninjected GalT KO and WT mice served as control groups. Mixed chimerism was detected in GalT KO recipients of a mixture of WT and KO BMC at all time points studied. The proportion of WT donor cells increased in these mice between 2 and 8 wk after bone marrow transplantation (BMT), and was subsequently maintained at a steady state. As is shown for a representative chimera in Fig. 1 *A* and summarized in Fig. 1 *B*, mixed chimerism was observed among both B and T cells in the PBLs of these animals. Mixed chimerism, including B and T as well as myeloid lineages, was also detected in the bone marrow, spleen, and peritoneal cavity at the time of killing 19 wk after BMT (Fig. 1 *C*, and data not shown). As expected, at each time point, T and B cells in PBLs of syngeneic BMT recipients of KO marrow alone (KO→KO) or WT marrow alone (WT→WT) were fully of KO and WT origin, respectively. B cells in GalT KO recipients of WT BMC alone (WT→KO) were almost fully WT in origin (Fig. 1).

Serum Levels of Anti-Gal NAb in Nonreconstituted, Lethally Irradiated αGalT KO Mice. To determine whether or not anti-Gal NAb persisted in the serum after lethal irradiation, GalT KO mice were lethally irradiated, and their serum

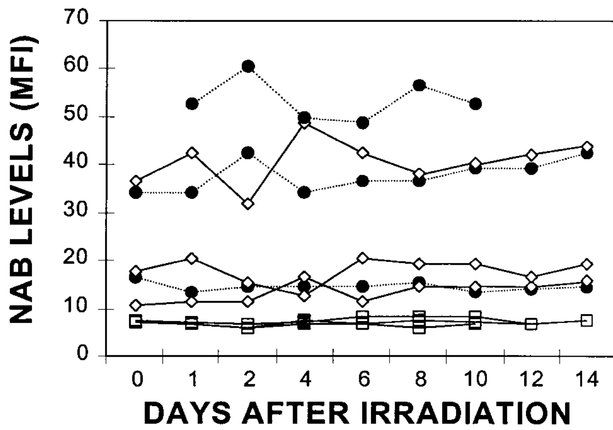

Figure 2. Serum levels of anti-Gal NAb in lethally irradiated GalT KO mice. Peripheral blood samples (40 μl) were collected from lethally irradiated (9.75 Gy) GalT KO mice that did (◇) or did not (●) receive TCD GalT KO BMC (5 × 10^6 cells/mouse), and from 9.75 Gy–irradiated WT mice (□) at the indicated times, and serum levels of anti-Gal NAb were measured by FCM. WT *scid* mouse cells were stained with 10 μl serum, and NAbs were detected using rat anti–mouse IgM-FITC as secondary mAb. The anti-Gal NAb (*NAB*) levels are presented as median fluorescence intensity (*MFI*). Each line represents an individual animal.

levels of anti-Gal NAb were measured at various times. As is shown in Fig. 2, no decline in the serum levels of anti-Gal NAb was observed in irradiated GalT KO mice, even in animals not receiving BMT, by 14 d after irradiation. Since all lethally irradiated mice not receiving BMT appeared sick and began to succumb by 10 d after irradiation, no data were obtained beyond 2 wk after irradiation. The absence of a significant difference in the levels of anti-Gal NAb between lethally irradiated GalT KO mice that did or did not receive reconstituting KO BMC (Fig. 2) suggests that newly developed B cells derived from BMT inocula were not a major source of NAb at early time points up to 14 d after irradiation. The relatively constant levels of anti-Gal NAb in sera of these mice suggest either that the anti-Gal NAbs are long-lived Igs, or that the irradiation dose of 9.75 Gy does not eradicate all host anti-Gal NAb–producing B cells.

Disappearance of Anti-Gal NAb in Sera of Mixed Chimeras. Levels of anti-Gal NAb were determined by Gal-specific ELISA assay and FCM analysis. Similar results were obtained with both assays. As is shown in Fig. 3, sera of GalT KO mice receiving KO BMC contained levels of anti-Gal NAb that were similar to those of untreated KO mice at all time points studied. In contrast, levels of anti-Gal NAb in sera of WT+KO→KO recipients were reduced significantly by 2 wk, and declined further to become undetectable by 4 wk after BMT (Fig. 3 *B*). A similar reduction in serum levels of anti-Gal NAb was also observed in lethally irradiated WT→KO recipients. As expected, sera of WT→WT recipients and of normal WT control mice did not contain anti-Gal NAb (Fig. 3). Similar results were observed in two repeat experiments in which anti-Gal NAb became undetectable in sera of 12 of 12 le-

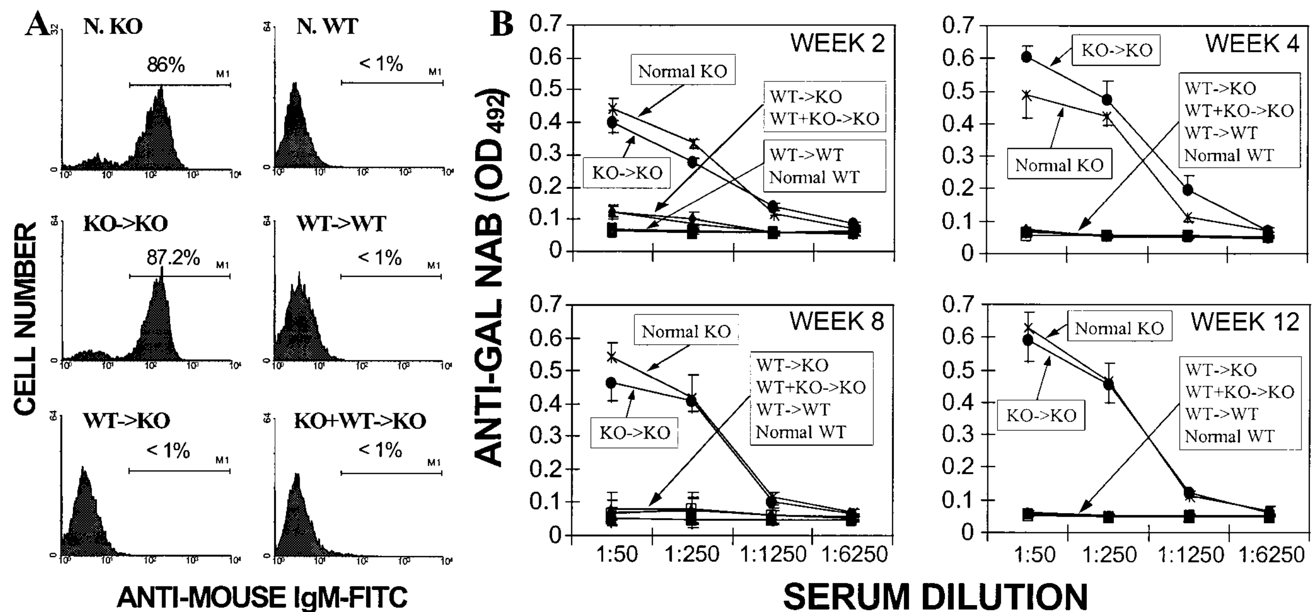

Figure 3. Reduced anti-Gal NAb levels in sera of mixed WT+KO→KO chimeras. (*A*) Representative histograms obtained by FCM analysis show an absence of anti-Gal NAb in mixed and fully WT→KO chimeras. WT RAG-$1^{-/-}$ mouse cells were stained with sera from normal KO and WT mice and BMT recipients, and NAbs were detected using rat anti–mouse IgM-FITC as secondary mAb. Typical histogram appearances for normal KO and WT mice, and for BMT recipients at 4 wk after BMT are shown. (*B*) Kinetics of serum anti-Gal NAb levels measured by Gal-specific ELISA assay. Sera were collected from normal KO and WT control mice and BMT recipients at 2, 4, 8, and 12 wk after BMT, and anti-Gal NAb (IgM plus IgG) levels were determined. Average and SDs for the individual groups are shown. Number of animals in each group: normal KO (x), 3; normal WT (■), 2; KO→KO (●), 3; WT→WT (□), 3; WT→KO (▲), 5; WT+KO→KO (◆), 5.

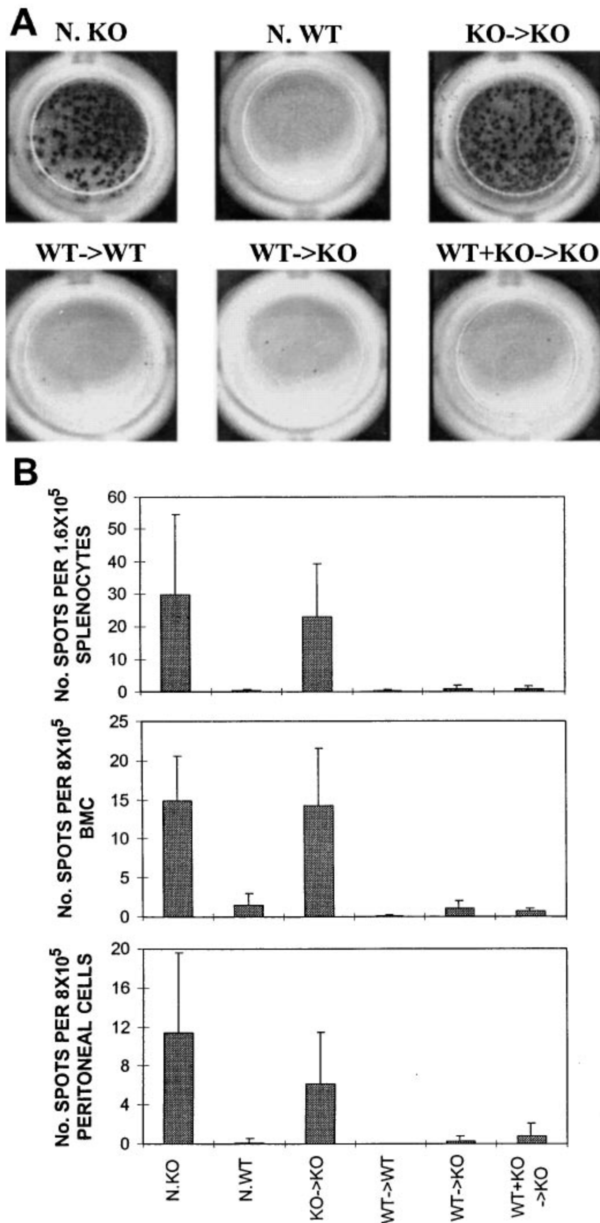

Figure 4. Reduced number of B cells producing anti-Gal antibodies (IgM plus IgG) in mixed chimeras. Spleen cells, BMC, and intraperitoneal cells prepared from BMT recipients or normal (*N.*) KO or WT mice 8 d after sensitization with rabbit RBC were serially diluted and used in ELISPOT assays. (*A*) Typical ELISPOT wells of spleen cells from indicated mice. (*B*) Average number of spots (± SD) (anti-Gal Ig–producing B cells) in spleen (*top*), BMC (*middle*), and intraperitoneal cells (*bottom*) of the mice killed 8 d after rabbit RBC immunization.

thally irradiated GalT KO recipients of mixed WT and KO BMC, all of which showed mixed chimerism, with proportions of PBL B cells that were WT ranging from 1 to 50% (data not shown).

Tolerance of Anti-Gal–producing B Cells in Mixed Chimeras. The reduced NAb levels detected in mixed chimeras could be due to downregulation of anti-Gal NAb production by GalT KO B cells in the presence of WT hematopoietic cells, or might reflect adsorption of NAb onto WT hematopoietic cells. FCM analyses did not provide any evidence for absorption of NAb on WT hematopoietic cells in chimeras (data not shown). Furthermore, we looked directly for the presence of anti-Gal NAb–producing B cells in these animals using an ELISPOT assay. To increase the sensitivity of the assay and to determine whether or not B cells of mixed chimeras were tolerant to the Gal epitope on xenogeneic cells, animals were immunized by intraperitoneal injection of 10^9 rabbit RBCs, which express large amounts of Gal, 19 wk after BMT. Spleen cells, BMC, and peritoneal cavity cells were analyzed 8 d later for the capacity to produce anti-Gal antibodies as measured by ELISPOT assay. B cells producing anti-Gal antibodies (both IgM and IgG) were undetectable in all three tissues of mixed chimeras, whereas large numbers of these cells were detected in normal GalT KO mice and GalT KO recipients of KO BMC. Results in mixed chimeras resembled those from WT→KO recipients and, most importantly, those from normal WT mice (Fig. 4) in which anti-Gal–forming B cells were not detected. These results show definitively that mixed WT+KO→KO chimeras are fully tolerant of the Gal epitope at the B cell level, and rule out the possibility that the reduced anti-Gal NAb levels in sera of mixed or fully WT→KO chimeras were caused by adsorption of the NAb on WT cells.

Specificity of Tolerance of Anti-Gal NAb–producing B Cells in Mixed Chimeras. To determine whether or not mixed chimeras were capable of producing antibodies against antigens other than Gal, sera from rabbit RBC–immunized mixed chimeras were tested for the development of anti–rabbit antibodies. As is shown in Fig. 5 *A*, sera of KO→KO recipients but not of WT mice or chimeras contained both anti-Gal and anti–rabbit RBC antibodies after immunization. However, rabbit RBC–immunized WT mice, WT→KO chimeras, and WT+KO→KO chimeras showed an increase in the level of anti–rabbit RBC serum antibodies, but not with anti-Gal specificity (Fig. 5 *A*).

Since expression of Gal on porcine cells is a major barrier to xenotransplantation from pigs to humans, we evaluated the ability of mixed chimeras to remain tolerant to Gal after immunization with pig cells. Normal GalT KO mice, mixed KO+WT→KO chimeras, and control KO→KO and WT→KO BMT recipients were immunized three times with 10^6 pig PBMCs at 15, 16, and 22 wk after BMT. Serum levels of anti–pig and anti-Gal antibodies were determined 3 wk after the last injection by FCM and ELISA, respectively. Again, anti-Gal IgM were detected only in sera of normal GalT KO mice and KO→KO recipients but not in sera of mixed or fully WT chimeras. In contrast, increased serum levels of anti–pig IgM were observed in all pig PBMC–sensitized mice (Fig. 5, *B* and *C*). An absence of functional anti-Gal–forming B cells in these mixed chimeras after immunization with pig PBMCs was further demonstrated by ELISPOT assays (data not shown). These results confirm that B cells recovering in mixed WT+KO→KO chimeras are functional and specifically tolerant to the Gal epitope.

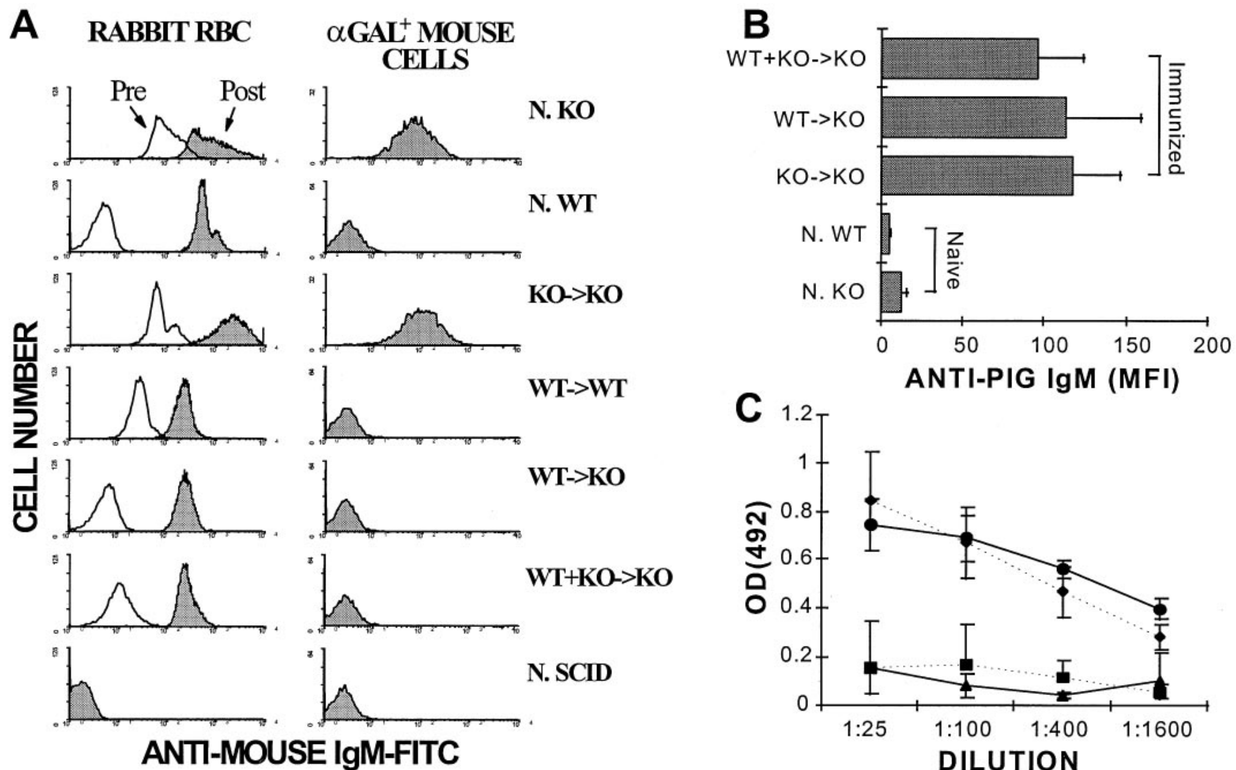

Figure 5. Absence of anti-Gal antibodies in sera of xenogeneic cell–sensitized mixed chimeras, and evidence of sensitization to xenoantigens. (*A*) Serum levels of anti–rabbit and anti-Gal antibodies in rabbit RBC–sensitized mice. Anti–rabbit RBCs and anti-Gal antibodies were detected by staining rabbit RBC and WT *scid* mouse cells, respectively, with serially diluted serum, followed by incubation with FITC-conjugated anti–mouse IgM. *Left column,* Rabbit RBCs stained with sera of indicated mice collected before (*Pre*) (10 μl of undiluted serum) and 8 d after (*Post*) sensitization (10 μl of twofold diluted serum). *Right column,* WT *scid* cells stained with sera collected 8 d after sensitization with rabbit RBC (10 μl of 50-fold diluted serum). Sera from *scid* mice (*N. SCID*) were used as negative controls. (*B* and *C*) Serum levels of anti–pig and anti-Gal antibodies in pig PBMC–sensitized mice. Lethally irradiated BMT recipients of KO→KO, WT→KO, or WT+KO→KO and normal GalT KO mice were immunized three times by intraperitoneal injection of 10^6 pig PBMCs at 15, 16, and 22 wk after BMT, and sera were collected 3 wk after the last injection. Sera from unimmunized normal WT (*N. WT*) and GalT KO (*N. KO*) mice were used as controls. (*B*) Serum levels of anti–pig PBMC IgM measured by FCM analysis. Pig PBMCs were stained with sera (10 μl of undiluted serum) collected from indicated groups (four mice for each group), and anti–pig IgM was detected using FITC-conjugated anti–mouse IgM. The anti–pig PBMC IgM levels are presented as median fluorescence intensity (*MFI*). (*C*) Serum levels of anti-Gal antibodies in pig PBMC-sensitized normal GalT KO mice (n = 4; ◆, *dotted line*) and BMT recipients of KO→KO (n = 5; ●, *solid line*), WT→KO (n = 6; ■, *dotted line*), or WT+KO→KO (n = 8; ▲, *solid line*) detected by ELISA assay. Average and SDs for the individual groups are shown.

Discussion

While the broad species distribution of the α-galactosyl carbohydrate residue (Gal) has previously limited the analysis of anti-Gal NAb to primate species, the recent development of GalT KO mice, which do not express Gal, now permits evaluation of anti-Gal NAb in a small animal model system. We have used GalT KO mice to explore the possibility that mixed chimerism could induce specific tolerance of anti-Gal–producing B cells. We have demonstrated recently that xenoreactive anti–pig NAb can be rapidly restored in C.B-17 *scid* mice by transfer of immunocompetent adult mouse BMC, indicating that marrow-derived B cells are efficient producers of IgM NAb (28a). Therefore, in this study, we reconstituted lethally irradiated GalT KO mice with GalT KO mouse BMC to restore the potential to produce anti-Gal NAb, and to evaluate the potential of cotransplanted WT marrow to tolerize anti-Gal–producing B cells.

The results of our studies demonstrate that successful induction of mixed bone marrow chimerism leads to tolerance of anti-Gal NAb–producing B cells. This conclusion was supported by the results of ELISPOT assays, which demonstrated definitively the absence of functional anti-Gal–producing B cells in mixed chimeras. Furthermore, B cells in these animals are capable of producing Ig with specificities other than anti-Gal, indicating that specific tolerance of anti-Gal–producing B cells was achieved by the induction of mixed chimerism.

Our ELISPOT data are consistent with the possibility that tolerance of anti-Gal–producing B cells was induced by either clonal deletion or anergy, or by both mechanisms. Experiments using transgenic mice have shown that immature self-reactive B cells can be eliminated by apoptosis (clonal deletion) and/or alteration of receptor antigenic specificity (receptor editing) via signals induced through

surface Ig cross-linking (29–34). Since the Gal epitope is recognized as a self antigen in mixed GalT WT+KO→KO chimeras, the above mechanisms of tolerance induction of self-reactive B cells would explain the observed tolerance among NAb-producing B cells resulting from induction of mixed chimerism. These studies of the important Gal specificity provide the first demonstration that BMT can induce B cell tolerance among a polyclonal population of nontransgenic NAb-producing B cells with a known specificity.

Although the above mechanisms of B cell tolerance appear to depend on a signal induction cascade applicable to immature but not mature B cells, cell surface–associated antigens are also capable of inducing tolerance among peripheral mature B cells (29–32, 35–37). Experiments using transgenic mice have shown that cross-linking of cell surface IgM is able to induce mature B cell tolerance via apoptotic cell death (deletion) (30, 37). In the present study, because BMT recipients were lethally irradiated before BMT and only a limited number of mature $Gal^{-/-}$ B cells was included in the BMT inoculum, the majority of GalT KO B cells in tolerized mixed chimeras developed in the presence of WT hematopoietic cells. However, the persistence of anti-Gal IgM NAb in nonreconstituted, irradiated GalT KO mice (Fig. 2) suggests that anti-Gal NAb–producing B cells might be radioresistant. Since IgM has an average half-life of only 2 d in the serum of adult mice (38), the constant level of anti-Gal NAb in these mice over a 2-wk period likely reflects the ongoing production of these NAb by radioresistant B cells. The reduction in anti-Gal NAb levels observed as early as 2 wk after BMT in recipients of WT BMC is consistent with the possibility that preexisting anti-Gal NAb–forming B cells were also tolerized in these mice. To address the possibility that mixed chimerism can lead to tolerance of preexisting mature B cells, mixed Gal chimerism is now being induced in mice conditioned with a nonmyeloablative conditioning regimen.

These studies demonstrate that mixed chimerism has the potential to induce specific tolerance of anti-Gal NAb–producing B cells, in addition to the T cell tolerance to xenoantigens demonstrated previously (21, 39). To our knowledge, mixed chimerism is the first approach shown to achieve efficient and permanent inhibition of polyclonal antidonor NAb production. These findings suggest that this approach may ultimately contribute to the successful use of discordant xenogeneic organs in clinical transplantation. The potential of this strategy to induce both B and T cell tolerance, and thus to permit solid organ xenograft acceptance in a pig-to-primate species combination, is currently under investigation.

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Address correspondence to Megan Sykes, Bone Marrow Transplantation Section, Transplantation Biology Research Center, Massachusetts General Hospital, MGH East, Bldg. 149-5102, 13th St., Boston, MA 02129. Phone: 617-726-4070; Fax: 617-724-9892; E-mail: sykes@helix.mgh.harvard.edu

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