

Peritoneal Cavity B Cells Are Precursors of Splenic IgM Natural Antibody-Producing Cells¹

Toshiyasu Kawahara,² Hideki Ohdan,³ Guiling Zhao, Yong-Guang Yang, and Megan Sykes⁴

Peritoneal cavity B-1 cells are believed to produce IgM natural Abs. We have used α 1,3-galactosyltransferase-deficient (*GalT*^{-/-}) mice, which, like humans, produce IgM natural Abs against the carbohydrate epitope Gal α 1,3Gal (Gal), to demonstrate that peritoneal cavity B-1b cells with anti-Gal receptors produce anti-Gal IgM Abs only after LPS stimulation. Likewise, peritoneal cavity cells of *GalT*^{-/-} and wild-type mice do not produce IgM Abs of other specificities without LPS stimulation. Development of Ab-secreting capacity is associated with loss of CD11b/CD18 (Mac-1) expression. In contrast, there are large numbers of cells producing anti-Gal and other IgM Abs in fresh splenocyte preparations from *GalT*^{-/-} and (for non-Gal specificities) wild-type mice. These cells are Mac-1⁻ but otherwise B-1b-like in their phenotype. We therefore hypothesized a pathway wherein peritoneal cavity B cells migrate into the spleen after activation in vivo and lose Mac-1 expression to become IgM Ab-producing cells. Consistent with this possibility, splenectomy reduced anti-Gal Ab production after immunization of *GalT*^{-/-} mice with Gal-positive rabbit RBC. Furthermore, splenectomized B6 *GalT*^{-/-}, Ig μ -chain mutant (μ ^{-/-}) (both Gal- and B cell-deficient) mice produced less anti-Gal IgM than nonsplenectomized controls after adoptive transfer of peritoneal cavity cells from B6 *GalT*^{-/-} mice. When sorted *GalT*^{-/-} Mac-1⁺ peritoneal cavity B cells were adoptively transferred to B6 *GalT*^{-/-}, μ ^{-/-} mice, IgM Abs including anti-Gal appeared, and IgM-producing and Mac-1⁻ B cells were present in the spleen 5 wk after transfer. These findings demonstrate that peritoneal cavity Mac-1⁺ B-1 cells are precursors of Mac-1⁻ splenic IgM Ab-secreting cells. *The Journal of Immunology*, 2003, 171: 5406–5414.

The shortage of donor organs for transplantation has become a serious problem, paradoxically, because of the success of transplantation. Xenotransplantation provides a potential solution of this organ shortage. The pig is considered to be an attractive xenograft donor, largely because of its excellent breeding characteristics and similarities in physiology and size to humans (1). In pig-to-primate species combinations, however, graft survival is limited by hyperacute rejection. Hyperacute rejection is initiated by the binding of naturally occurring Abs against the carbohydrate epitope, Gal α 1,3Gal α 1–4GlcNAc-R (Gal)⁵ on vascular endothelium of the xenograft (2–4). Humans lack a functional α 1,3Gal transferase (*GalT*) enzyme, as do *GalT* knockout (*GalT*^{-/-}) mice, which also make anti-Gal natural Abs (NABs). Both pre-existing and newly developing B cells producing anti-Gal Abs are tolerized by the induction of mixed chimerism in

GalT^{-/-} mice receiving Gal-expressing allogeneic or xenogeneic bone marrow transplants (5–8).

The repertoires of NABs may be driven by selection by self-Ags (9) or by exposure to microorganisms (10). These responses are believed to reflect activation of CD5⁺ (B-1a) B cells, which are the predominant B cell population in the peritoneal cavity (PerC) of mice (10). Because NABs against Gal are thought to develop as a result of exposure to environmental microorganisms that express this carbohydrate determinant (11), the B-1a lineage has been speculated to be the major population of anti-Gal NAB-producing cells (12). However, our previous data showed that the phenotype of B cells producing anti-Gal Abs in *GalT*^{-/-} mice was similar to that of the B-1b subset, but lacked CD11b/CD18 (Mac-1) expression, and was also distinct from marginal zone B cells (13). A similar phenotype was defined for all specificities of IgM NAB-producing cells (13). These studies demonstrated a higher frequency of B cells with anti-Gal surface IgM receptors in the PerC than in the spleen, but the PerC B cells were devoid overall of Ab-producing activity. The PerC B cells with anti-Gal Ig receptors showed phenotypic properties of B-1b cells (CD21^{-/low} IgM^{high} CD5⁻ CD43⁺ Mac-1⁺). However, PerC cells became Mac-1⁻ and developed anti-Gal and other specificities of Ab-producing activity after in vitro culture with LPS (13). These results demonstrated that differentiation to Ab-secreting cells is associated with loss of Mac-1 expression.

In contrast, there are large numbers of NAB-producing cells in fresh spleen cell preparations from *GalT*^{-/-} and wild-type mice. These cells are Mac-1⁻, but otherwise B-1b-like in their phenotype (CD21^{-/low} CD23⁻ IgM^{high} B220^{low} CD5⁻ CD43⁺ 493⁻, large size). In *GalT*^{-/-} mice, this was the major B cell subset producing anti-Gal NAB. Similarly, total IgM-producing cells in the spleen were included in the same B cell subset as anti-Gal-producing cells (13). From these results, we hypothesized that PerC B cells do not produce IgM Abs, but, following activation, migrate into the spleen and lose Mac-1 expression to become NAB-secreting cells.

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⁵ Abbreviations used in this paper: Gal, Gal α 1,3Gal α 1–4GlcNAcR; FCM, flow cytometric; *GalT*^{-/-}, α 1,3-galactosyltransferase knockout; MLN, mesenteric lymph node; PerC, peritoneal cavity; NAB, natural Ab; CXCL13, CXC chemokine ligand 13.

To clarify the role of the spleen in the production of anti-Gal NAb, we generated $GalT^{-/-}$, $\mu^{-/-}$ mice, which are both Gal and B cell deficient, for cell transfer experiments with or without splenectomy. Moreover, to assess down-regulation of Mac-1 on PerC B cells migrating to the spleen, Mac-1⁺PerC B cells from $GalT^{-/-}$ mice were sorted and adoptively transferred to B6 $GalT^{-/-}$, $\mu^{-/-}$ mice. Our results provide evidence that PerC B cells are precursors of splenic cells that produce IgM Abs against Gal and other specificities in vivo and that this phenotype is associated with down-regulation of Mac-1. Thus, PerC Mac-1⁺ B cells are precursors of Mac-1⁺ splenic IgM Ab-secreting cells.

Materials and Methods

Animals and immunization

$GalT^{-/-}$ mice were provided by Dr. J. Lowe (University of Michigan, Ann Arbor, MI) and were bred in our colony (14). Age-matched (10–12 wk old) $GalT^{-/-}$ mice on the C57BL/6 (B6) background (back-crossed seven times to B6 and designated here as B6 $GalT^{-/-}$ mice) were used for the experiments. $GalT^{-/-}$, $\mu^{-/-}$ lines were generated from the F₂ generation of crosses between B6 $GalT^{-/-}$ mice and B6 $\mu^{-/-}$ (15) mice obtained from The Jackson Laboratory (Bar Harbor, ME). B6 mice and B6-CD45.1 mice were purchased from the Frederick Cancer Research Facilities (Frederick, MD). All animals were maintained in a specific pathogen-free microisolator environment. To enhance anti-Gal Ab production, $GalT^{-/-}$ mice were immunized with Gal-bearing xenogeneic cells (i.e., 10⁹ rabbit RBC) (Cocalbio Biologicals, Reamstown, PA) 8 days before ELISPOT assays. Rabbit RBC were washed twice and resuspended at 10⁹ cells/ml in PBS before i.p. injection.

Surgical removal of spleen

After surgical skin preparation, the spleen was exteriorized through a 1-cm left subcostal incision. The splenic artery and vein were double-ligated, and the spleen was removed. The peritoneum and skin were closed in separate layers using 5.0 absorbable suture. Mice were rested for 7 days before transfer of PerC cells.

ELISA for detecting anti-Gal Ab

Anti-Gal or total IgM Ab levels in sera were quantified by ELISA according to procedures previously described (5). Briefly, ELISA plates were coated with 5 μ g/ml Gal-BSA (Alberta Research Council, Calgary, Canada) or goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL). Diluted serum samples were incubated in the plates, and bound Abs were detected using HRP-conjugated goat anti-mouse IgM-specific Ab (Southern Biotechnology Associates) (each 250 ng/ml). Color development was achieved using 0.1 mg/ml *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) in substrate buffer. The *o*-phen-

ylenediamine dihydrochloride reaction was stopped using 3 M NH₂SO₄, and OD₄₉₂ was measured.

ELISPOT for detecting anti-Gal and total IgM-secreting cells

The ELISPOT assay was performed as described previously (5, 13). Briefly, nitrocellulose membranes of a 96-well filtration plate (Millipore, Bedford, MA) were coated with 5 μ g/ml Gal-BSA (Alberta Research Council) or 5 μ g/ml goat anti-mouse IgM (Southern Biotechnology Associates) for detecting anti-Gal or total IgM-secreting cells, respectively. Nonspecific binding was blocked with 0.4% BSA in Iscove's modified DMEM. Serial dilutions of cell suspensions in Iscove's modified DMEM supplemented with 0.4% BSA, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 μ g/ml sodium selenite (all from Sigma-Aldrich), 50 μ g/ml 2-ME, and 1 μ g/ml gentamicin were added to wells in duplicate. After a 24-h culture at 37°C, bound Abs were detected using HRP-conjugated goat anti-mouse IgM Abs (Southern Biotechnology Associates), followed by color development with 3-amino-9-ethylcarbazole (Sigma-Aldrich). After the membranes were dried, red spots in each well were counted using a stereo microscope. Tiny spots without halo formation were excluded as false spots caused by cell debris or shed materials.

FCM analysis and cell sorting of IgM^{high}Mac-1⁺ PerC B cells

B cell subsets were analyzed by flow cytometric (FCM) analysis of PerC cells or spleen cells on a FACScan cytometer (BD Biosciences, Mountain View, CA) as described (16). Cells were stained with FITC-conjugated anti-Mac-1 (CD11b or control mAb HOPC1), PE-conjugated anti-CD19, and biotin-conjugated anti-mouse IgM (all from BD PharMingen, San Diego, CA). The biotinylated mAb was visualized with PE-streptavidin (for two-color FCM) or CyChrome-streptavidin (for three-color FCM). Nonspecific Fc γ R binding of labeled Abs was blocked by 2.4G2 (rat anti-mouse Fc γ R mAb). Dead cells were excluded from analysis by forward light scatter and/or propidium iodide.

Based on criteria indicated in the individual figures, cells were sorted under sterile conditions using a MoFlo high speed cell sorter (Cytomation, Fort Collins, CO). Sorted cells were reanalyzed for purity on a FACScan cytometer and were immediately resuspended in PBS and transferred into mice.

Adoptive transfer of $GalT^{-/-}$ cells into $GalT^{-/-}$, $\mu^{-/-}$ mice

Various numbers of PerC cells or spleen cells from $GalT^{-/-}$ mice were administered i.v. to $GalT^{-/-}$, $\mu^{-/-}$ recipient mice in 1 ml of PBS with or without CD4⁺ T cell depletion of the recipients. CD4⁺ T cells of $GalT^{-/-}$, $\mu^{-/-}$ mice were depleted in vivo using GK1.5 (rat IgG2b anti-mouse CD4 mAb, 1.76 mg/injection) (17) on day -5 and day -1 with respect to adoptive transfer. In some experiments, 1 \times 10⁶ IgM^{high}Mac-1⁺ PerC B cells from $GalT^{-/-}$ mice were given i.v.

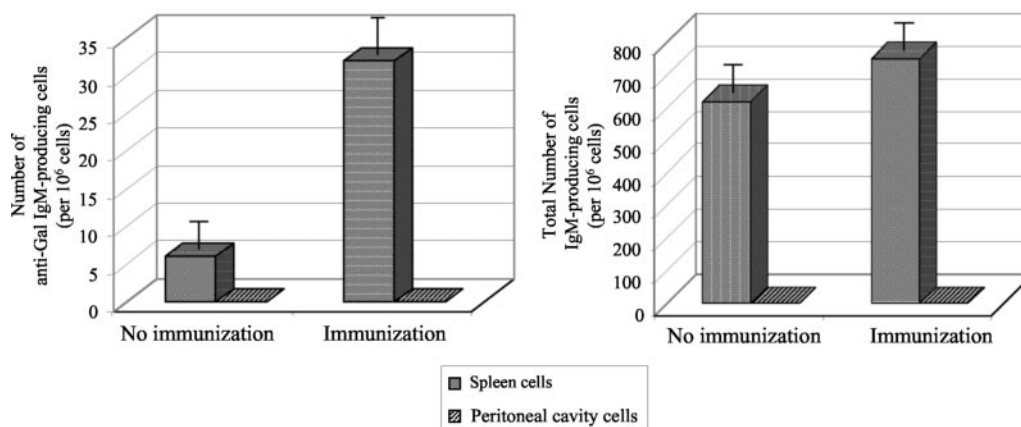


FIGURE 1. Source of IgM Abs in $GalT^{-/-}$ mice. *Left*, ELISPOT detection of anti-Gal IgM-secreting cells. Spleen and PerC cells were prepared from $GalT^{-/-}$ mice that were either untreated ($n = 5$) or immunized with rabbit RBC 8 days before the assay ($n = 5$). The pooled cells were used in ELISPOT assay to determine the frequency of anti-Gal IgM-secreting cells. The results shown are the average \pm SEM calculated from red spot number in duplicate wells with 1 \times 10⁶ cells. The results are representative of two similar experiments. *Right*, ELISPOT detection of total IgM-secreting cells. Samples from the same cell pools as those used to obtain the data in the left panel were used. The results shown are the average \pm SEM calculated from red spot number in duplicate wells containing 1 \times 10⁶ seeded cells. The results are representative of two similar experiments.

Cell migration in B6-CD45 congenic model

Spleen (1×10^7 cells/mouse) cells or PerC (5×10^6 cells/mouse) cells from B6-CD45.1 mice were transplanted into B6-CD45.2 mice i.v. PerC cells and spleen cells of recipient mice were harvested for FCM analysis at 1, 3, and 5 days after transfer.

Statistics

The results were statistically analyzed by the unpaired or paired Student *t* test or the log rank test when appropriate. *p* < 0.05 was considered to be statistically significant.

Results

Source of IgM Abs in *GalT*^{-/-} mice

Studies presented in Fig. 1 confirm our previous observation (13) that the cells actively producing anti-Gal and other IgM Abs in *GalT*^{-/-} mice are in the spleen, but not in the PerC. *GalT*^{-/-} mice were immunized by i.p. injection of 10^9 Gal-expressing rabbit RBC 8 days before analysis. The frequency of anti-Gal and total IgM-secreting cells was quantified by ELISPOT assay of spleen and PerC. As shown in Fig. 1, large numbers of anti-Gal and total IgM-secreting cells were present in the spleen, and no Ab-secreting cells were detected in the PerC of untreated or immunized mice. The absence of anti-Gal-secreting cells in the PerC was not a consequence of immunization, because Ab-secreting cells were also undetectable in the PerC of unimmunized *GalT*^{-/-} mice.

Transferred spleen cells home to the spleen, and PerC cells migrate into both spleen and PerC

To begin to address the hypothesis that PerC B cells migrate into the spleen to become Ab-secreting cells, we examined the sites to which transferred PerC and splenic B cells migrate using a CD45 congenic model. Spleen cells (1×10^7 cells/mouse) or PerC cells (5×10^6 cells/mouse) obtained from B6-CD45.1 mice were transplanted into B6-CD45.2 mice i.v. Representative flow cytometric plots obtained on Days 1, 3, and 5 posttransfer are shown in Fig. 2, and the absolute number of B cells detected in the spleen and PerC at each time point is summarized in Table I. Transferred spleen cells initially homed mainly to the spleen rather than the PerC; an almost 2000-fold greater number of B cells was detected in the spleen than in the PerC on Day 1 after transfer. Transferred PerC cells were detected in both the spleen and the PerC within 1 day after transfer (Fig. 2A), but much greater numbers (~80-fold on Day 1) appeared in the spleen than in the PerC between 1 and 5 days posttransfer (Table I). Although the difference in numbers of B cells in the spleen vs the PerC was less marked by 5 days posttransfer than on Day 1, there were still >3-fold more B cells in the spleen than the PerC at the latter time point.

Peritoneal cavity cells from *GalT*^{-/-} mice have high potential for production of anti-Gal NAb

We previously reported that PerC cells have the potential to become anti-Gal Ab secretors upon in vitro stimulation with LPS (13), which is a nonspecific polyclonal B cell activator. To compare the capacity of PerC cells and spleen cells to produce anti-Gal Abs in vivo, PerC cells or spleen cells were transferred i.v. into *GalT*^{-/-}, μ ^{-/-} mice. Because it has been reported that CD4⁺ T cells derived from μ ^{-/-} mice inhibit the development or survival of B cells in transferred PerC populations (18), we evaluated the need to deplete CD4⁺ T cells of *GalT*^{-/-}, μ ^{-/-} recipient mice in vivo, by administering GK1.5 (rat IgG2b anti-mouse CD4 mAb) to some groups of mice. We transplanted 1×10^7 spleen cells from B6 *GalT*^{-/-} mice into B6 *GalT*^{-/-}, μ ^{-/-} mice, with or without recipient CD4⁺ T cell depletion. Transplanted spleen cells led to the appearance of low levels of anti-Gal IgM in sera of both CD4⁺ T cell-depleted and undepleted recipients until 8 wk posttransfer

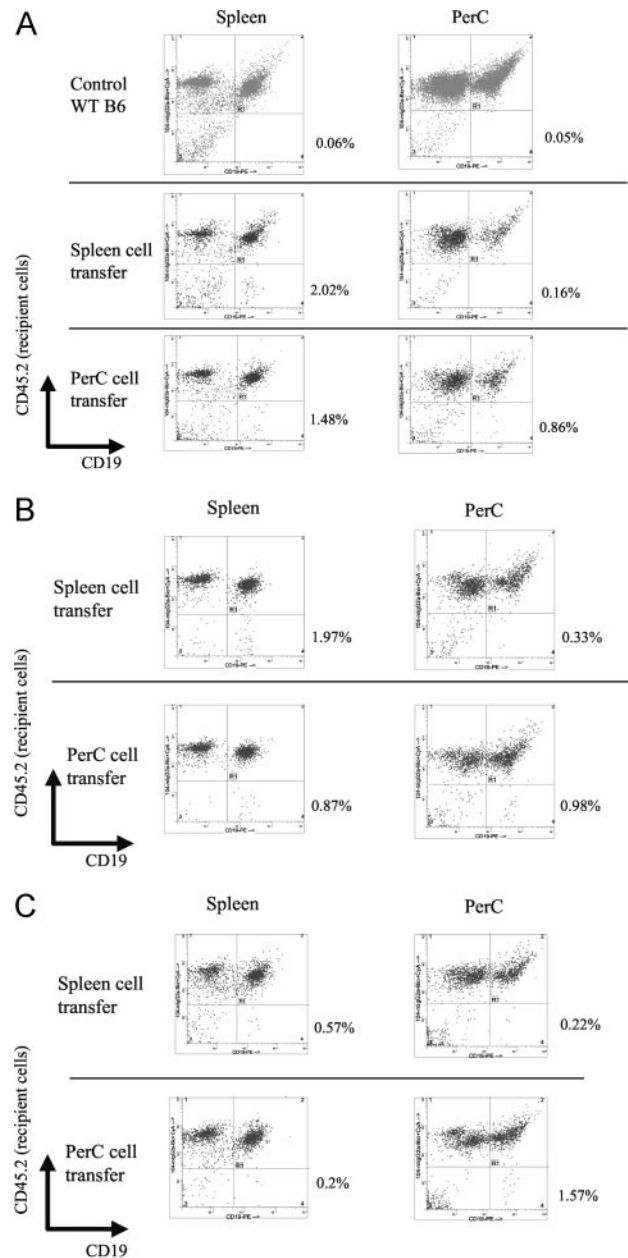


FIGURE 2. Migration patterns of transferred spleen cells and PerC cells. Spleen (1×10^7 cells/mouse) or PerC (5×10^6 cells/mouse) cells from B6-CD45.1 mice were transplanted into B6-CD45.2 mice i.v. B cells derived from donor cells were identified as CD45.2⁺CD19⁺ cells. Data are summarized in Table I. Spleen or PerC cells were obtained from adoptive recipient mice at day 1 (A), day 3 (B), and day 5 (C) after adoptive transplantation. WT, Wild type.

(Fig. 3A). However, significantly higher levels of anti-Gal IgM were observed in the CD4⁺ T cell-depleted group than in the undepleted group at 12 wk posttransfer. In CD4⁺ T cell-depleted mice, CD4⁺ T cells recovered to normal levels within 6 wk after transfer (data not shown).

Transplanted PerC cells (5×10^6 cells given i.v.) from *GalT*^{-/-} mice did not lead to anti-Gal IgM production in the undepleted recipient *GalT*^{-/-}, μ ^{-/-} mice, consistent with the results of Baumgarth et al. (18). However, CD4⁺ T cell depletion allowed transplanted PerC cells to produce high levels of serum anti-Gal IgM after adoptive transfer (Fig. 3B). Similar results were obtained when serum levels of total IgM were compared in undepleted and

Table I. Absolute number of donor B cells detected in the spleen and PerC following adoptive transfer

	No. of Donor B Cells ($\times 10^4$) \pm SD		
	Day 1	Day 3	Day 5
Spleen cell transfer (10×10^6 cells/mouse)			
Spleen	177 \pm 58	135.1 \pm 53	47.3 \pm 21
PerC	0.09 \pm 0.03	0.451 \pm 0.06	0.562 \pm 0.08
Peritoneal cavity cell transfer (5×10^6 cells/mouse)			
Spleen	80.3 \pm 9.8	42.5 \pm 5.2	11.5 \pm 1.8
PerC	1.03 \pm 0.58	2.58 \pm 1.7	3.23 \pm 0.78

CD4⁺ T cell-depleted recipients of PerC cells (data not shown), also consistent with previous studies (18). Thus, the initial presence of CD4⁺ T cells derived from $\mu^{-/-}$ mice inhibited the engraftment of PerC B cells. Most importantly, these results demonstrate that PerC cells have greater potential for the production of anti-Gal and other IgM NAb than spleen cells.

Mac-1⁺ PerC B cells 40 wk after spleen cell transfer into $GalT^{-/-}$, $\mu^{-/-}$ mice

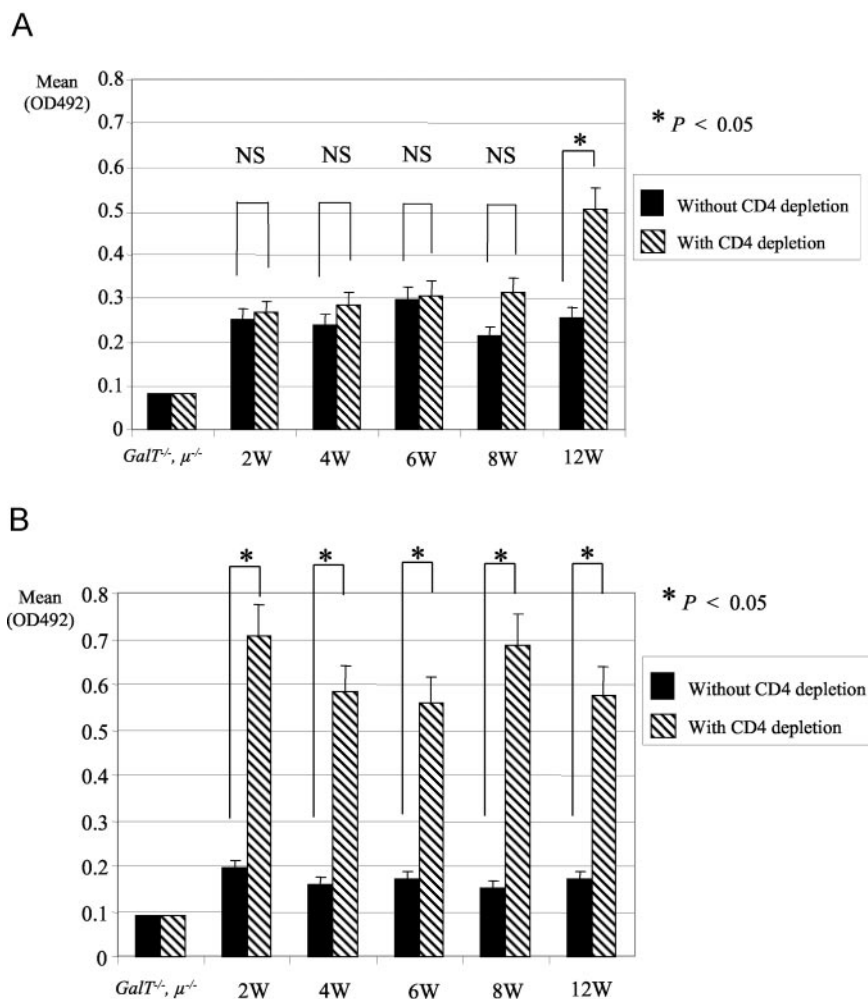
To assess the levels of Mac-1 expression on splenic B cells after adoptive transfer of splenocytes to CD4⁺ T cell-depleted $GalT^{-/-}$, $\mu^{-/-}$ recipients, the adoptive recipients were sacrificed 40 wk after transfer. Splenocytes and PerC cells of $GalT^{-/-}$, $\mu^{-/-}$ recipients of $GalT^{-/-}$ splenocytes were stained with CD19 and Mac-1 (Fig. 4). Splenic B cells from $GalT^{-/-}$ mice do not express Mac-1, as

shown in Fig. 4. Splenic and PerC B cells were reconstituted in $GalT^{-/-}$, $\mu^{-/-}$ mice 40 wk after transfer of spleen cells from $GalT^{-/-}$ mice. Mac-1 was expressed on an increased proportion of PerC B cells ($24.2 \pm 1.98\%$, $n = 3$) derived from transferred splenocytes, whereas only $2.44 \pm 0.83\%$ ($n = 3$) of splenic B cells were Mac-1⁺ in these recipients. Significantly larger numbers of Mac-1⁺ B cells were detected in the PerC of adoptive recipients than in their spleens (Table II). These results indicate that spleen cells contain progenitors of a PerC B cell population that expresses Mac-1.

Mac-1⁺ IgM^{high} PerC cells from $GalT^{-/-}$ mice can produce anti-Gal IgM after cell transfer into $GalT^{-/-}$, $\mu^{-/-}$ mice

We next investigated whether or not transplanted PerC cells could become Ab-secreting cells with reduced levels of Mac-1 expression. Mac-1⁺ IgM^{high} PerC cells were sorted from untreated

FIGURE 3. PerC cells have higher potential for anti-Gal NAb production than spleen cells. Serum anti-Gal levels were measured by ELISA. Values are average \pm SEM for the individual groups. *, $p < 0.05$ compared with adoptively transferred $GalT^{-/-}$, $\mu^{-/-}$ mice without CD4⁺ T cell depletion. A, Serum levels of anti-Gal IgM after adoptive transplantation of spleen cells from $GalT^{-/-}$ mice into $GalT^{-/-}$, $\mu^{-/-}$ mice with or without CD4⁺ T cell depletion. Number of animals in each group: adoptive recipients without CD4⁺ T cell depletion, $n = 3$; adoptive recipients with CD4⁺ T cell depletion, $n = 7$; untreated $GalT^{-/-}$, $\mu^{-/-}$ mice, $n = 3$. Although serial dilutions were performed to analyze Ab levels, data from the 1/250 dilution point are representative and are presented for simplicity. W, Weeks. B, Serum levels of anti-Gal IgM after adoptive transplantation of PerC cells from $GalT^{-/-}$ mice into $GalT^{-/-}$, $\mu^{-/-}$ mice with or without CD4⁺ T cell depletion. Number of animals in each group: adoptive recipients without CD4⁺ T cell depletion, $n = 3$; adoptive recipients with CD4⁺ T cell depletion, $n = 3$; untreated $GalT^{-/-}$, $\mu^{-/-}$ mice, $n = 3$. Although serial dilutions were performed to analyze Ab levels, data from the 1/1000 dilution point are representative and are presented for simplicity.



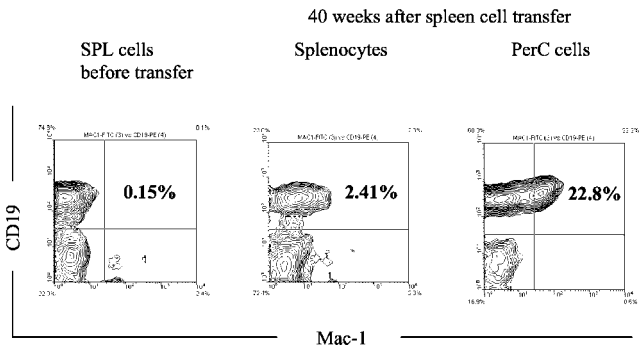


FIGURE 4. Mac-1 is expressed on PerC B cells of recipients of adoptively transferred spleen cells. FACS analysis of spleen cells or PerC cells from *GalT*^{-/-}, μ ^{-/-} recipients 40 wk after adoptive transfer of 1×10^7 spleen cells from untreated *GalT*^{-/-} mice ($n = 3$). Percentage of Mac-1⁺ cells among CD19⁺ cells is shown.

GalT^{-/-} mice, and 1×10^6 enriched Mac-1⁺IgM^{high} (93.2% pure) PerC cells were transplanted into CD4⁺ T cell-depleted *GalT*^{-/-}, μ ^{-/-} mice (Fig. 5). Serum levels of total IgM increased progressively in adoptive recipient mice until 4 wk after adoptive transfer, when anti-Gal IgM levels were still very low (Fig. 6). To enhance anti-Gal production, adoptive recipient mice were immunized with rabbit RBC at 4 wk after adoptive transplantation. Significantly higher levels of anti-Gal IgM and total IgM were detected in the serum 8 days after immunization compared to preimmunization sera and compared to untreated *GalT*^{-/-}, μ ^{-/-} mice. The anti-Gal levels measured in sera of wild-type B6 mice (Fig. 6) were consistently higher than those in sera of *GalT*^{-/-}, μ ^{-/-} mice, and may reflect the presence of cross-reactive Abs with low affinity for the Gal autoantigen.

Mac-1⁺IgM^{high} GalT^{-/-} PerC cells become Ab-secreting cells in the spleen, and this is associated with down-regulation of Mac-1

We previously reported that PerC cells became Mac-1⁻ and developed anti-Gal Ab-secreting activity after in vitro culture with LPS (13). To clarify whether or not this phenomenon is observed in vivo, spleen cells from *GalT*^{-/-}, μ ^{-/-} mice that received Mac-1⁺IgM^{high} PerC cells (enriched to 93.2%) from *GalT*^{-/-} mice were analyzed by FACS at 5 wk after transfer and 8 days after rabbit RBC immunization. Most (74.9%) of the transferred

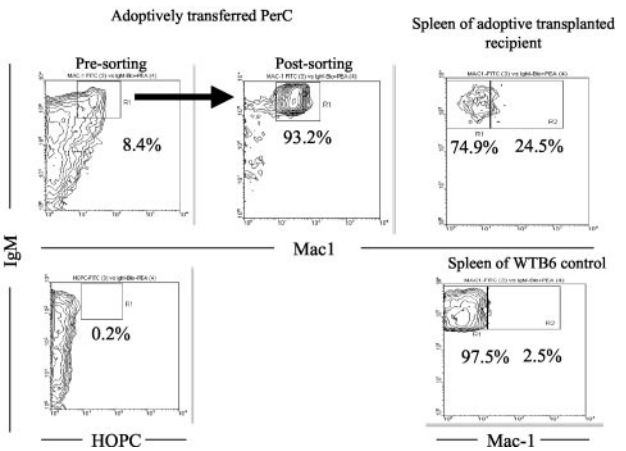


FIGURE 5. Down-regulation of Mac-1 in the spleens of adoptive *GalT*^{-/-}, μ ^{-/-} adoptive recipients of Mac-1⁺ PerC B cells. PerC cells were prepared from eight untreated *GalT*^{-/-} mice (10–12 wk of age). The pooled cells were stained with Mac-1 FITC or control HOPC1 FITC, together with biotinylated anti-mouse IgM (IgM-Bio) mAb and PE-streptavidin (PEA). The population of Mac-1⁺IgM^{high} cells was sorted as described in *Materials and Methods*. Sorted cells were reanalyzed for purity. Percentages given are of total PerC cells before transfer. Spleen cells from adoptive recipients of Mac-1⁺ PerC B cells were prepared 5 wk after transfer and stained with Mac-1-FITC, together with biotinylated anti-mouse IgM mAb and PE-streptavidin. Percentages of Mac-1⁺ and Mac-1⁻ cells among IgM⁺ cells in the adoptive *GalT*^{-/-}, μ ^{-/-} recipients are given.

Mac-1⁺ PerC B cells had become Mac-1⁻ when they were detected in the spleens of adoptive recipient *GalT*^{-/-}, μ ^{-/-} mice (Fig. 5). Moreover, both anti-Gal-secreting cells and total IgM-secreting cells were present in the spleens of the adoptive recipients (Fig. 7). These results demonstrate that Mac-1⁺IgM^{high} *GalT*^{-/-} PerC cells become Ab-secreting cells, including anti-Gal-secreting cells, in the spleen and that this development is associated with down-regulation of Mac-1.

The spleen is a major site of anti-Gal production in vivo

The majority of anti-Gal-secreting cells are found in the spleen of *GalT*^{-/-} mice (13) (Fig. 1). However, it is not known whether the

Table 2. B cells in PerC and spleens of adoptive recipients 40 wk following splenocyte transfer

	Untreated <i>GalT</i> ^{-/-} , μ ^{-/-} Mice ($n = 3$)	Untreated <i>GalT</i> ^{-/-} Mice ($n = 3$)	Spleen Cell Transfer (5×10^6 cells/mouse) ($n = 3$)
Total cell no. ($\times 10^6$) \pm SD			
Spleen	10 \pm 1.2	98.3 \pm 14.3	35 \pm 4.2
PerC	1.4 \pm 0.8	2.9 \pm 0.36	9.4 \pm 3.3
B cell no. ($\times 10^6$)			
Spleen	N/A ^a	60.2 \pm 10	21.7 \pm 2.7 ^b
PerC	N/A	1.7 \pm 0.2	5.6 \pm 1.5
Mac-1 ⁺ B cell no. ($\times 10^6$)			
Spleen	N/A	0.1 \pm 0.04	0.84 \pm 0.3
PerC	N/A	0.53 \pm 0.12	2.3 \pm 0.3

^a N/A, Not applicable.
*, $p < 0.05$.

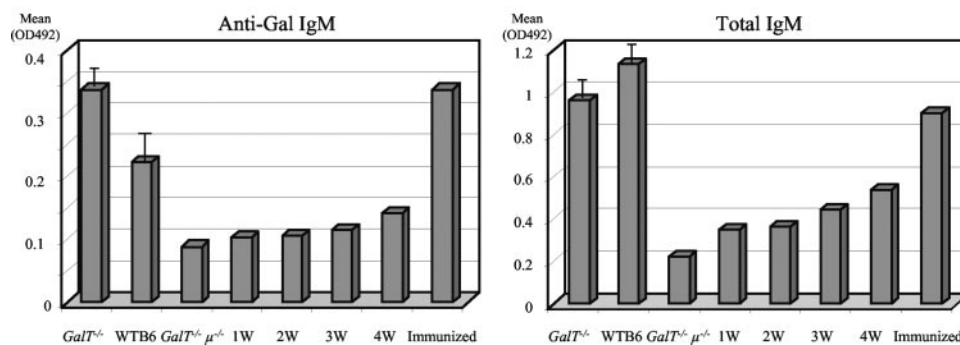
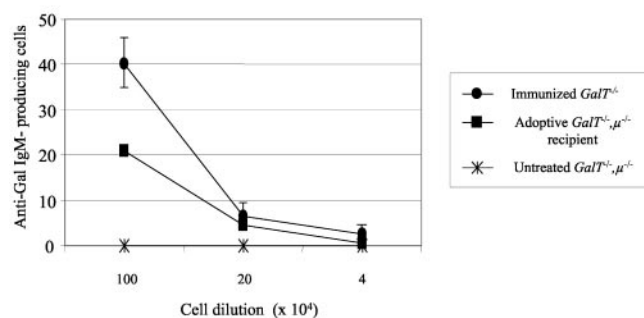


FIGURE 6. Detectable serum levels of anti-Gal IgM or total IgM in $GalT^{-/-}$, $\mu^{-/-}$ recipients ($n = 2$) after adoptive transplantation of Mac-1⁺IgM^{high} PerC cells from untreated $GalT^{-/-}$ mice. Serum Ab levels were measured by ELISA. The recipient mice were immunized with rabbit RBC at 4 wk (W) posttransfer and were sacrificed 8 days after immunization. Although serial dilutions were performed to analyze Ab levels, data from the 1/250 and 1/1000 dilution points are representative for anti-Gal and total IgM levels, respectively, and are presented for simplicity.

spleen is absolutely required for anti-Gal Ab production. To address this question, we splenectomized $GalT^{-/-}$ mice and immunized them with rabbit RBC 7 days later to enhance anti-Gal production. Eight days after immunization, serum levels of anti-Gal IgM and total IgM were examined by ELISA. Serum levels of anti-Gal IgM in splenectomized recipient $GalT^{-/-}$ mice were

markedly lower than those in nonsplenectomized controls (Fig. 8A). Serum levels of total IgM were also significantly lower in splenectomized recipient $GalT^{-/-}$ mice than in nonsplenectomized controls (Fig. 8B). These results suggest that the spleen plays an important, but not essential, role in the production of IgM Abs.

A



B

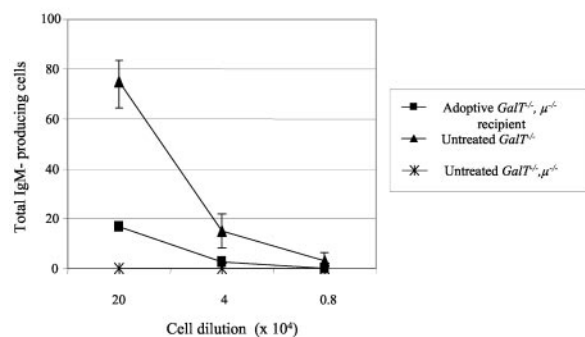


FIGURE 7. Ab-secreting cells in the spleens of $GalT^{-/-}$, $\mu^{-/-}$ adoptive recipients of Mac-1⁺ PerC B cells from $GalT^{-/-}$ mice. Adoptive recipients were sacrificed at 5 wk after transfer and 8 days after rabbit RBC immunization. A, ELISPOT detection of anti-Gal IgM-secreting cells. Spleen cells were prepared from immunized $GalT^{-/-}$ mice ($n = 3$), untreated $GalT^{-/-}$, $\mu^{-/-}$ mice ($n = 3$) and adoptive $GalT^{-/-}$, $\mu^{-/-}$ recipients ($n = 2$). The results shown are the average red spot number in duplicate wells containing the indicated number of seeded cells. B, ELISPOT detection of total IgM-secreting cells. Spleen cells were prepared from untreated $GalT^{-/-}$ mice ($n = 3$), untreated $GalT^{-/-}$, $\mu^{-/-}$ recipients ($n = 3$), and adoptive $GalT^{-/-}$, $\mu^{-/-}$ recipients ($n = 2$). Results are the average red spot number in duplicate wells containing the indicated number of seeded cells.

Peritoneal cavity B cells migrate to the spleen and become anti-Gal-secreting cells

The data presented above are also consistent with the hypothesis that PerC B cells migrate into the spleen after activation in vivo and lose Mac-1 expression to become IgM Ab-secreting cells. To address the importance of PerC B cell migration into the spleen to become Ab-secreting cells after activation in vivo, PerC cells from B6 $GalT^{-/-}$ mice were transplanted into CD4⁺ T cell-depleted B6 $GalT^{-/-}$, $\mu^{-/-}$ mice that were or were not splenectomized. At 3 wk after transfer of PerC cells from $GalT^{-/-}$ mice, serum levels of anti-Gal IgM in splenectomized recipient $GalT^{-/-}$, $\mu^{-/-}$ mice were undetectable, similar to untreated $GalT^{-/-}$, $\mu^{-/-}$ mice. In contrast, high levels of anti-Gal IgM were detected in sera of non-splenectomized control recipients of PerC cells (Fig. 9A). Furthermore, although IgM was detectable in splenectomized recipients of PerC, serum levels of total IgM in splenectomized recipient $GalT^{-/-}$, $\mu^{-/-}$ mice were lower than in nonsplenectomized controls (Fig. 9B). These results demonstrate the importance of the spleen as a site of migration of PerC cells to become IgM Ab-secreting cells.

Discussion

The B-1 subset is defined by surface expression of Mac-1, high levels of IgM, and low levels of IgD. These cells develop early during ontogeny, are found predominantly in the peritoneal and pleural cavities of mice, can self-renew, and have different receptor specificities than B-2 cells (19, 20). B-1 cells recognize common bacterial Ags, as well as a variety of self Ags, including phosphatidylcholine, Ig, DNA, and some cell membrane proteins (21). Although B-1 cells were originally defined by their expression of CD5, a population of PerC CD5⁺ B cells, referred to as B-1b cells, has been identified with a surface phenotype that otherwise resembles CD5⁺ B-1 cells, now referred to as B-1a cells (9). One of the phenotypic characteristics of both B-1a and B-1b cells is expression of the β_2 integrin Mac1 (CD11b/CD18), but the importance of this molecule in the migration and function of B-1 cells has not been defined.

FIGURE 8. Reduction of Abs in splenectomized *GalT*^{-/-} mice 8 days after rabbit RBC immunization. Splenectomy was performed 7 days before immunization. Serum levels of anti-Gal IgM and total IgM were measured by ELISA. Values are average ± SEM for the individual groups. *, *p* < 0.05 compared with non-splenectomized immunized *GalT*^{-/-} mice. *A*, Serum levels of anti-Gal IgM in controls (*n* = 5) and splenectomized *GalT*^{-/-} (*n* = 5) mice 8 days after rabbit RBC immunization. *B*, Serum levels of total IgM in controls (*n* = 5) and splenectomized *GalT*^{-/-} (*n* = 5) mice 8 days after rabbit RBC immunization.

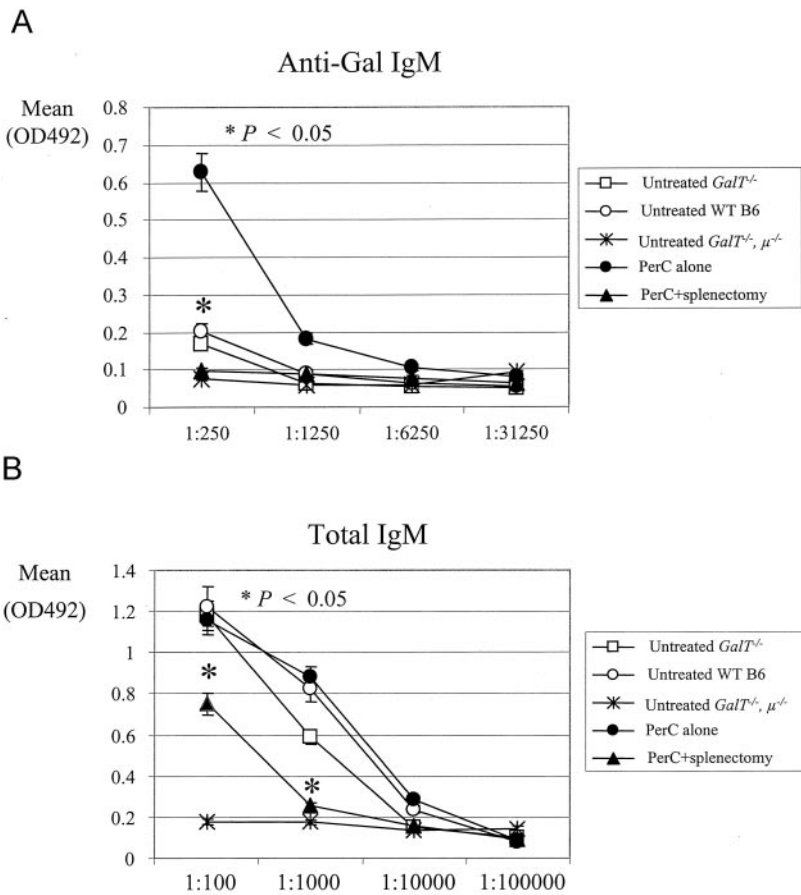
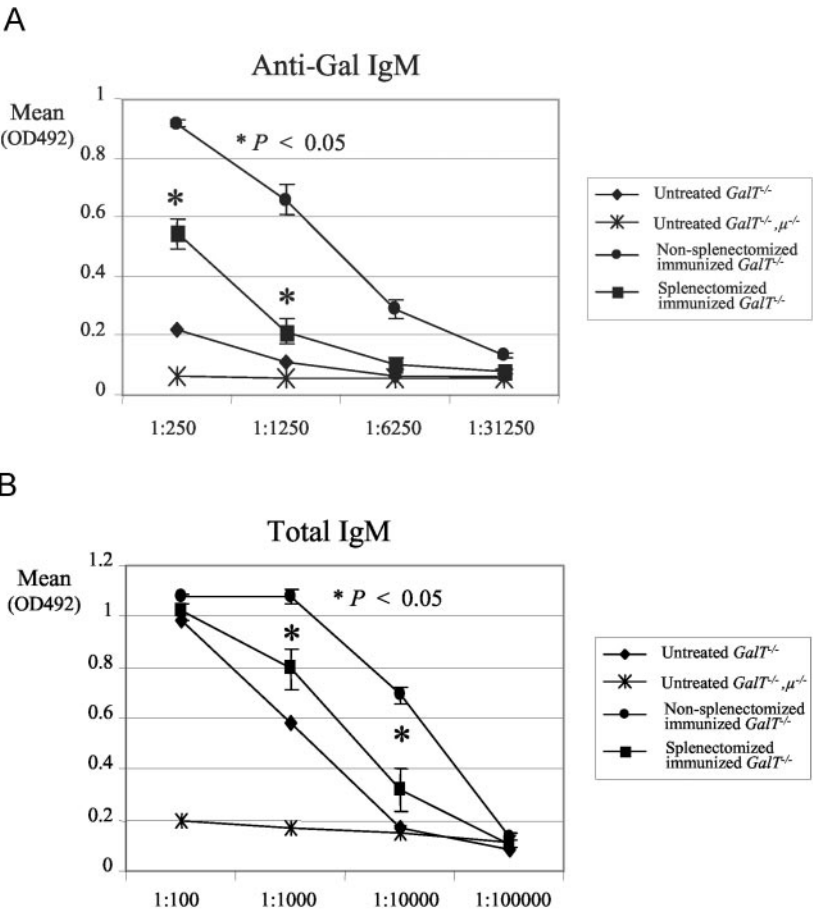


FIGURE 9. Reduced levels of Abs in splenectomized *GalT*^{-/-}, μ ^{-/-} mice 3 wk after adoptive transfer of PerC cells from untreated *GalT*^{-/-} mice. CD4⁺ T cells of *GalT*^{-/-}, μ ^{-/-} recipient mice were depleted in vivo with GK1.5 mAb treatments 1 day and 5 days before transfer. Serum levels of anti-Gal IgM and total IgM were measured by ELISA. Values are average ± SEM for the individual groups. *, *p* < 0.05 compared with no splenectomized *GalT*^{-/-}, μ ^{-/-} recipient mice. *A*, Serum levels of anti-Gal IgM were measured in control untreated *GalT*^{-/-} mice (*n* = 5), untreated B6 mice (*n* = 3), untreated *GalT*^{-/-}, μ ^{-/-} mice (*n* = 3), adoptive recipients of *GalT*^{-/-} PerC (*n* = 5), or splenectomized *GalT*^{-/-}, μ ^{-/-} recipient mice (*n* = 5) 3 wk after adoptive transfer. *B*, Serum levels of total IgM were measured in control untreated *GalT*^{-/-} mice (*n* = 5), untreated B6 mice (*n* = 3), untreated *GalT*^{-/-}, μ ^{-/-} mice (*n* = 3), adoptive recipients of *GalT*^{-/-} PerC (*n* = 5), or splenectomized *GalT*^{-/-}, μ ^{-/-} recipient mice (*n* = 5) 3 wk after adoptive transfer. WT, Wild type.

NAbs against Gal are thought to develop as a result of exposure to environmental bacteria that express this carbohydrate determinant (11, 22), and the B-1a lineage has been speculated to be the major population of anti-Gal NAb-secreting cells (12). However, we have previously demonstrated that anti-Gal and other natural IgM Ab-secreting cells are found almost exclusively within a Mac1[−]CD5[−]CD21^{−/low}CD23[−]IgM^{high}B220^{low}CD5[−]CD43⁺493[−] splenic B cell population with high forward light scatter properties (13). Except for the lack of Mac1 expression, which is known to be absent on splenic B-1 cells (9, 21), this phenotype is consistent with that of a B-1b cell population. Although there are greater numbers of anti-Gal receptor-bearing B cells in the PerC than in the SPL, Gal-binding B cells in the PerC do not produce Ab without LPS stimulation, whereas freshly isolated spleen cells of Gal knockout mice produce anti-Gal IgM (13) (Fig. 1). Similar characteristics were observed for cells in wild-type and Gal knockout mice producing IgM Abs of all specificities (13). When PerC B cells become Ab-secreting cells after *in vitro* LPS stimulation, down-regulation of Mac-1 occurs (13). These results led us to hypothesize the existence of a novel, major pathway for B cell migration and differentiation into IgM natural Ab-secreting cells. We proposed that PerC B-1 cells are in a quiescent state and, after activation, lose Mac-1 expression and migrate to the spleen, where they produce IgM Abs.

The studies described herein demonstrate the existence of such a pathway for anti-Gal and other IgM Ab-secreting cells. This pathway may partially account for the utility of splenectomy in diminishing the severity of xenograft rejection in pig-to-primate combinations (23). The evidence for this pathway includes the demonstration that PerC cells reconstituted the splenic B cell compartment in B6 *GalT*^{−/−}, μ ^{−/−} mice receiving CD4⁺ T cell-depleting Ab treatment. Furthermore, transfer of PerC cells more rapidly reconstituted anti-Gal NAb levels in double-knockout recipients than did transfer of spleen cells. These results are consistent with our previous observation that sera from SCID mice receiving PerC B cells from normal mice contained significantly higher levels of NAb at all time points than SCID recipients of splenic B cells (24). The short term adoptive transfer studies presented herein demonstrate directly that PerC B cells can home rapidly and preferentially to the spleen. The use of immunocompetent B6 CD45 congenic mice for these studies indicates that cell migration from the PerC to the spleen occurs even in the absence of stimulation for homeostatic expansion or Ab production that occurs in B cell-deficient mice (25–29). Our studies demonstrate that the spleen is the major site for production of both anti-Gal and total IgM Abs *in vivo*, because anti-Gal IgM and total IgM levels were lower in splenectomized than in control *GalT*^{−/−} mice after immunization with rabbit RBC. Furthermore, total and anti-Gal IgM Ab levels were higher in control than in splenectomized mice receiving adoptively transferred PerC B cells. Most importantly, we demonstrate that the adoptive transfer of purified Mac1⁺ PerC B-1 cells leads to the development of IgM Abs and the long term presence of Mac-1-negative, IgM-secreting, splenic B cells. These studies therefore demonstrate a previously undefined pathway of B cell migration in association with the development of IgM-secreting activity *in vivo*.

B-1 cells have been shown to contribute to the mucosal immune response, and several groups demonstrated that transfer of total PerC B cells (30, 31) or sorted B-1 cells (32) into lethally irradiated (30, 32) or *Rag2*^{−/−} (31) mice results in the appearance of donor-derived IgA-secreting plasma cells in the intestinal lamina propria and mesenteric lymph nodes (MLN). Okamoto et al. (33) showed that proliferation and differentiation of transgenic autoreactive PerC B cells to plasma cells can occur in the MLN in the presence of LPS or activated autoreactive $\gamma\delta$ T cells. In this model,

the number of PerC B-1 cells declined as IgM plasma cells appeared in the MLN (34, 35). Migration of autoreactive B-1 cells from PerC to MLN, associated with differentiation into plasma cells, was apparently induced by noncognate help from activated $\gamma\delta$ T cells. However, we previously showed that the MLN was not a major site for the production of either anti-Gal or total IgM Ab after rabbit RBC immunization *i.p.* (13). Although evidence suggests that induced anti-Gal IgM Ab responses are largely T cell dependent in Gal knockout mice (36), this response appears to be dependent on the $\alpha\beta$ TCR-bearing class of T cells (37) and not on $\gamma\delta$ T cells (A. Thall, unpublished observations). Baseline anti-Gal IgM levels in Gal knockout mice are not fully dependent on T cells (A. Thall, unpublished observations), and T cell depletion seems to actually increase the baseline anti-Gal NAb levels (38). It is not yet known whether T cells augment the induced anti-Gal IgM response by providing cognate or noncognate help. Together, our results and those of Okamoto et al. are consistent with a model wherein B-1b cells reside in the PerC in a quiescent state. After activation by T cells of either the $\alpha\beta$ or the $\gamma\delta$ class, they are capable of migrating to other lymphoid tissues and differentiating into Ab-secreting cells.

The PC to spleen pathway of B cell migration described here is the opposite of that described by several groups, wherein B-2 B cells activated by Ag in the spleen can acquire the B-1 phenotype and presumably migrate into the PerC (9, 39–41). Indeed, the spleen plays a critical role in the generation and maintenance of the B-1a PerC cell pool and in maintaining IgM NAb levels (42). Our observation that splenic B cells migrate into the PerC, where they up-regulate Mac-1, is also consistent with this pathway. However, the existence of pathways in both directions is not inconsistent with any of the previously published data or with those presented here. Autoreactive B cells have been shown to be present in the PerC (33), but we propose that they exist there in a quiescent state and that they migrate to other tissues to become Ab-secreting cells only after receiving appropriate activating stimuli. Small resting lymph node B cells have also been shown to be capable of reconstituting serum IgM after transfer to SCID mice (43). Nevertheless, our results in splenectomized mice clearly show the importance of the spleen as the site of natural and induced IgM Ab production by PerC B-1 cells.

Migration of PerC B cells requires stimuli that are largely undefined. Chemokines and their receptors probably play a critical role in these processes. B cell homing to lymphoid follicles is directed by CXC chemokine ligand 13 (CXCL13) produced by follicular stromal cells (44–47). In adoptive transfers, B-1 cells home to the omentum and the PerC in a CXCL13-dependent manner (48), and this chemokine, CXCL13, is known to be produced by cells in the omentum and by peritoneal macrophages. The possible role of chemokines in the migration of B-1 cells in the opposite direction, *i.e.*, from PerC to spleen, is currently unknown, but chemokines and adhesion molecules (perhaps Mac-1) seem likely to be involved.

Thus, we have demonstrated that PerC B cells can become Ab-secreting (including anti-Gal-secreting in Gal knockout mice) cells in the spleen and that this transition is associated with down-regulation of Mac-1. Quiescent PerC Mac-1⁺ B cells are precursors of Mac-1[−] splenic IgM Ab-secreting cells.

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