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**Generation of Mice Deficient for Macrophage Galactose- and N-Acetylgalactosamine-Specific Lectin: Limited Role in Lymphoid and Erythroid Homeostasis and Evidence for Multiple Lectins**

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Macrophage receptors function in pattern recognition for the induction of innate immunity, in cellular communication to mediate the regulation of adaptive immune responses, and in the clearance of some unsialylated cells or glycoproteins from the circulation. They also function in homeostasis by initiating the engulfment of apoptotic cells. Evidence has suggested that macrophage receptors function to recognize cells that are destined for programmed cell death but not yet overtly apoptotic. We have examined the function of a macrophage receptor specific for unsialylated glycoproteins, known as the mouse macrophage galactose- and N-acetylglactosamine-specific lectin (mMGL) (Li et al., J. Biol. Chem. 265:11295-11298, 1990; Sato et al., J. Biochem. [Tokyo] 111:331-336, 1992; Yamamoto et al., Biochemistry 33:8159-8166, 1994). With targeted disruption, we tested whether mMGL is necessary for macrophage function, controlled thymic development, the loss of activated CD8 T cells, and the turnover of red blood cells. Evidence indicates that mMGL may play a nonessential role in several of these macrophage functions. Experiments are presented that indicate the existence of another galactose- and N-acetylglactosamine-recognizing lectin distinct from mMGL. This may explain the absence of a strong phenotype in mMGL-deficient mice.

Differential sialylation is a general property of hematopoietic cell development, including lymphocytes and erythrocytes. For example, at a distinct stage in thymic development, several abundant cell surface glycoproteins, such as CD8, CD43, and CD45, are undersialylated, thus exposing terminal O-linked glycans. The extent of terminal Gal(1-3)GalNAc exposure inversely correlates with the expression of ST3Gal-I, a Gal(1-3)GalNAc-specific α2,3 sialyltransferase that is expressed primarily in medullary but not cortical thymocytes (3, 10). Upon expression of ST3Gal-I, Gal(1-3)GalNAc becomes sialylated, preventing PNA binding on these cells (23). Additionally, T cells become increasingly sialylated on N-linked Galβ1-4GlcNAc as they transit from DP to single-positive (SP) cells (36). This latter sialylation is thought to involve multiple sialyltransferases (7, 35).

Naive PNA(lo) T cells once again become PNA(hi) upon mito-
and turnover of lymphocytes during development and activation. In this report we describe the generation of mice that are deficient for mMGL. An analysis of these mice revealed that the loss of mMGL did not affect lymphoid differentiation or immune function. Further, red blood cell turnover and life span are minimally affected. We also show that mMGL is not the only Gal/GalNAc-specific lectin expressed by mouse macrophages, and its inhibition does not substantially complement the ST3Gal-I deficiency. We conclude from these studies that mMGL plays a role in T-cell and erythrocyte homeostasis but that there are other macrophage lectins with redundant or overlapping functions.

MATERIALS AND METHODS

Flow cytometry. Cells were labeled with the following monoclonal antibodies and reagents: anti-CD4-phycocerythrin (PE), anti-CD8-TC (CatTag), anti-CD5-fluorescein isothiocyanate (FITC), anti-CD3-FITC, anti-heat-stable antigen-biotin, anti-CD144-P, anti-CD25-biotin or -FITC, anti-CD62L-PE (all from Pharmingen), PNA-FITC, and Enzymuntra croquale agglutinin-biotin (Sigma Chemical Co., St. Louis, Mo.). Apoptotic cells were detected with annexin V-FITC (CatTC), or terminal deoxynucleotidyltransferase-mediated dUTP-biotin, anti-CD44-PE, anti-CD25-biotin or -FITC, anti-CD62L-PE (all from BD Pharmingen). Samples were analyzed with a FACSscan or FACSCalibur (Becton Dickinson) and CellQuest software.

Genomic mapping and knockout construct. Exons 2 and 3 encoding the translational start site, cytoplasmic domain, and the transmembrane domain of mMGL were mapped from genomic clones isolated from a strain 129SvJ library (Stratagene, La Jolla, Calif.). The probe used was a mouse full-length mMGL cDNA isolated from a subtractive hybridization experiment and identified by sequence as previously published (3). These exons were replaced with the pPGK-neo/p cassette (32), resulting in a targeting construct consisting of a 1.5-kb and a 4-kb arm to mediate homologous recombination. The construct was transfected into R1 embryonic stem (ES) cells (129 strain), and 700 G418-resistant colonies were screened for homologous recombinants. One clone, H1, was identified and injected into C57BL/6 blastocysts. Male mice obviously chimeric by coat color were crossed to C57BL/6J females to obtain F1 heterozygotes, which were then injected into C57BL/6 blastocysts. Male mice obviously chimeric by coat color were crossed to C57BL/6J females to obtain F1 heterozygotes, which were then injected into C57BL/6 blastocysts. Male mice obviously chimeric by coat color were crossed to C57BL/6J females to obtain F1 heterozygotes, which were then injected into C57BL/6 blastocysts. Male mice obviously chimeric by coat color were crossed to C57BL/6J females to obtain F1 heterozygotes, which were then injected into C57BL/6 blastocysts.

Immunohistochemistry. Tissues were collected from mice, frozen in OCT (VWR Scientific), and kept at −80°C until sectioned on a cryostat. Sections were acetone fixed, washed in Tris-buffered saline, and blocked with 2% bovine serum albumin. Biotinylated and unbiotinylated polycyclamide arrays carrying multiple copies of Galp1-3GalNAc and Galn1-3GalNAc were purchased from Glycotech (Rockville, Md.). Anti-mouse mMGL monoclonal antibody LOM-14 (13) was prepared as previously described. Briefly, mice were injected with 2 × 108 P815 tumor cells intraperitoneally. On day 10, spleens and mesenteric lymph nodes were isolated from mice, and lymphocytes were counted. Lymphocytes were added to 96-well V-bottomed plates containing 51Cr-labeled specific target cells (P815) or non-specific targets (EL-4) at various effector-to-target cell ratios. Cells were incubated for 4 h with targets, and 51Cr release was measured.

RESULTS

Generation of mMGL+/− mice. To understand the physiological role of mMGL in vivo, we targeted mMGL for germ line deletion in mice. Since mMGL is a type II transmembrane protein, we mapped the N-terminal transmembrane domain and targeted exons 2 and 3 for replacement with a neomycin resistance cassette with the vector pPGKneo (Fig. 1A). Homologous recombinants in ES cells could be identified by a 12-kb HindIII recombinant fragment, compared to a 10.5-kb endogenous fragment when probed with an upstream genomic probe. An analysis of these mice revealed that there are other macrophage lectins with redundant or overlapping functions.
The resulting chimeric mice passed the deficient allele to the first-generation progeny (Fig. 1B). Homozygous deficient mice were deficient for mMGL by Northern (Fig. 1C) as well as by reverse transcription-PCR analysis (data not shown).

mMGL expression. In order to verify the absence of mMGL expression in mMGL−− mice and further characterize mMGL expression in different tissues, immunohistochemistry was carried out with anti-mMGL monoclonal antibody (LOM14). Figure 2A depicts the staining from a cytospin of peritoneal exudate cells. As shown, a large percentage of peritoneal exudate cells from wild-type mice were strongly mMGL positive. The staining of peritoneal exudate cells from mMGL−− mice was greatly diminished, although, with purified antibody, we did detect residual staining over the rat immunoglobulin background.

We also compared the expression of mMGL to that of Mac-1 in thymus, spleen, lymph node, skin, and lung from wild-type and mMGL-deficient mice. In the thymus, we found medullary staining in the wild-type mouse that was again reduced in the thymuses from mMGL−− mice (Fig. 2B). This pattern of medullary staining is intriguing because the thymo-
cytes bearing potential ligands for mMGL, namely, unsialylated O-linked Ga\(\alpha_1\)3GalNAc, are located in the cortex, whereas cells bearing this Gal/GalNAc receptor are segregated in the medulla. In the spleen of wild-type mice, we found sparse mMGL staining compared with Mac-1 (Fig. 2C), similar to the staining found in liver (not shown). In contrast, mMGL staining in skin (Fig. 2D) and lymph nodes (Fig. 2E) was at least as frequent as Mac-1 staining. In the skin, abundant staining was seen in the dermis, and in the lymph node there was also abundant staining in the extrafollicular paracortex. These results indicate that mMGL is differentially expressed in Mac-1\(^{+}\) cells. It is definitely expressed on peritoneal macrophages; however, expression elsewhere is consistent with a subpopulation of macrophages or mature dendritic cells. While the antibody staining revealed by LOM14 is diminished in all tissues from \(mMGL^{-/-}\) mice, the possibility remains that there exists another cross-reactive mMGL-like molecule.

Normal lymphoid development and homeostasis in \(mMGL^{-/-}\) mice. We examined \(mMGL^{-/-}\)-deficient mice for lymphoid development. Our initial hypothesis was that medullary mMGL-expressing macrophages might regulate the developmental transition that occurs from the cortical, undersialylated immature thymocytes to medullary, fully sialylated mature T cells. This process can be monitored by analyzing thymocytes stained with PNA to detect O-linked terminal Gal\(\alpha_1\)3GalNAc and staining for CD4 and CD8 to detect the major thymic subpopulations (Fig. 3A). Thymocytes were separately stained with \(E.\ cristagalli\) agglutinin, which detects the terminal galactose present on N-linked branched glycoproteins (Fig. 3A).

With either of these lectins, we found that undersialylated thymocytes in wild-type mice were mainly CD4\(^{+}\)CD8\(^{-}\), the population found in the cortex, whereas the sialylated thymocytes were either CD4\(^{+}\)CD8\(^{-}\) (CD4SP) or CD4\(^{-}\)CD8\(^{+}\) (CD8SP), the mature populations found in the medulla.

We next sought to determine whether the absence of mMGL-bearing macrophages would result in an altered cellularity within the thymus. Examination of thymuses from \(mMGL^{+/+}\) and \(mMGL^{-/-}\) mice revealed that T-cell develop-
ment appears normal in mMGL-deficient mice, with thymocytes having similar subpopulations of CD4^+ CD8^- (CD4SP), CD4^- CD8^+ (CD8SP), and CD4^- CD8^- (DP) cells (Fig. 3B). The number of PNA-high thymocytes in each of the subsets was also determined. The data indicate that there is no significant increase in PNA^hi thymocytes in the mature CD4SP and CD8SP subsets (Fig. 3C). Likewise, development was normal as detected by surface expression of CD3, CD5, and heat-stable antigen (data not shown). Further, although variable, the rate of death appeared to be unaltered as measured by staining for apoptotic markers with annexin V, DiOC6, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (data not shown).

The thymus size of mMGL-deficient mice compared to heterozygous littersmates seemed slightly increased in mice aged 8 to 16 weeks (9.8 [±4.5] x 10^7 cells, n = 27 [−/−] versus 8.4 [±3.6] x 10^7 cells, n = 33 [+/+]), but due to the variability within each group, this difference was not statistically significant (P > 0.05, Student’s t test, unpaired). B-cell expression of B220 was similar among wild-type and mMGL null mice, and B-cell proliferation to anti-immunoglobulin M and lipopolysaccharide in vitro was normal (data not shown). We conclude from these data that T-cell development is grossly unaltered and that expression of mMGL is not required for normal T- or B-cell development.

We also studied the function of mMGL with respect to the induction of apoptosis in Gal/GalNAc-exposed CD4^+ CD8^- thymocytes. In the first set of experiments, we showed that fibroblasts transfected with mMGL would result in cell loss of CD4^+ CD8^- (cortical) but not CD4SP or CD8SP (medullary) thymocytes (Fig. 3D). We then sought to determine whether elicited peritoneal macrophages would mediate the loss of thymocytes in an mMGL-dependent manner. Measuring cell recovery, viability, and apoptotic indices, we found that macrophages do have a substantial effect on the recovery of thymocytes, but after extensive investigation, including numerous separate trials, we did not find a significant difference between macrophages from wild-type and mMGL-deficient mice (data not shown). Thus, despite the ability of mMGL transfectants to mediate thymocyte cell death in vitro and the demonstrated large amounts of mMGL on peritoneal macrophages (Fig. 2A), there was no indication that mMGL expression was required for thymocyte cell death.

mMGL-deficient mice were also tested for T-cell responses by measuring the cytotoxic T-cell response to major histocompatibility-disparate tumor cells. P815 tumor cells were injected intraperitoneally into mMGL^+/+ and mMGL^−/− mice. On day 10 of the response, CTL specific lysis was measured ex vivo along with the expansion of CD8 T cells. The cytotoxic T-cell responses of mMGL^+/+ and mMGL^−/− mice were similar (Fig. 4A). Similar expansion of CD8 T cells was also observed (Fig. 4B). Both mMGL^+/+ and mMGL^−/− T cells exhibited the expression of activation markers, becoming CD44^hi, CD62L^lo, and PNA^hi (data not shown). In addition, we measured the proportion of apoptotic T cells as measured by annexin V during the time course of a T-cell response in vivo and again found no significant differences in mice lacking mMGL on day 10 (Fig. 4C), day 12, or day 17 (data not shown). These results indicate that CD8 T-cell responses do not rely on mMGL for activation, effector function, or turnover of activated T cells.

**Elevated red blood cell counts but similar life span and turnover.** Mice were also subjected to extensive hematology analysis, blood coagulation, and blood chemistry tests. Only measures of erythrocyte content showed a statistically significant deviation from wild-type values. As shown in Table 1, red blood cell counts, hemoglobin, hematocrit, and mean corpuscular volume were all slightly elevated in mMGL-deficient mice. This was of potential interest because one model of erythrocyte turnover is that erythrocytes lose sialic acid as a consequence of aging, and it is possible that such aged erythrocytes would be scavenged by macrophages present in the spleen and liver (9, 14, 34). While the mean differences noted were statistically significant, the mMGL^−/− values still fell within the normal range for mice. In addition, an analysis of erythrocyte life span in vivo was carried out on mMGL^+/+ and
mMGL−/− mice. Erythrocyte life spans averaged between 36.67 and 37.27 days, respectively (Fig. 5). We conclude that there are only minor hematological consequences associated with the loss of mMGL.

**Complementation of ST3Gal-I deficiency.** In mice lacking ST3Gal-I (siat4a), there is a profound loss of CD8 T cells in the periphery (23). In these mice, there is enhanced core 2 glycosylation, revealed by the antibody 1B11, and enhanced PNA binding. Moreover, cross-linking with 1B11 on CD8 T cells from these mice induced apoptosis (23). We considered the possibility that a lack of ST3Gal-I revealed an unsialylated possibility that a lack of ST3Gal-I revealed an unsialylated from these mice induced apoptosis (23). We considered the possibility that a lack of ST3Gal-I revealed an unsialylated from these mice induced apoptosis (23). We considered the possibility that a lack of ST3Gal-I revealed an unsialylated

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mMGL+/+ (n = 11)</th>
<th>mMGL−/− (n = 10)</th>
<th>mMGL−/+ (n = 5)</th>
<th>t test</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (cells/μl)</td>
<td>7,726.6 (697.67)</td>
<td>8,260.0 (848.83)</td>
<td>8,320.0 (730.08)</td>
<td></td>
<td></td>
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<tr>
<td>Neutrophils (%)</td>
<td>8.1 (2.02)</td>
<td>11.7 (1.55)</td>
<td>9.6 (1.24)</td>
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<td></td>
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<tr>
<td>Lymphocytes (%)</td>
<td>89.1 (2.39)</td>
<td>83.2 (1.64)</td>
<td>87.1 (1.42)</td>
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<td></td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>0.4 (0.18)</td>
<td>1.4 (0.24)</td>
<td>0.8 (0.21)</td>
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</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.0 (0.00)</td>
<td>0.3 (0.12)</td>
<td>0.2 (0.08)</td>
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<td></td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>2.4 (0.42)</td>
<td>3.3 (0.25)</td>
<td>2.2 (0.22)</td>
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<tr>
<td>Erythrocytes (m/ml)</td>
<td>9.8 (0.15)</td>
<td>9.9 (0.13)</td>
<td>10.2 (0.09)</td>
<td>0.044</td>
<td>+3.5</td>
</tr>
<tr>
<td>Hemoglobin (g/liter)</td>
<td>15.2 (0.16)</td>
<td>15.4 (0.18)</td>
<td>15.8 (0.12)</td>
<td>0.009</td>
<td>+3.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.7 (0.52)</td>
<td>48.8 (0.54)</td>
<td>50.6 (0.41)</td>
<td>0.001</td>
<td>+6.0</td>
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<tr>
<td>MCV (fl)</td>
<td>48.6 (0.36)</td>
<td>49.3 (0.39)</td>
<td>49.8 (0.35)</td>
<td>0.032</td>
<td>+2.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.5 (0.10)</td>
<td>15.5 (0.12)</td>
<td>15.6 (0.10)</td>
<td></td>
<td></td>
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<tr>
<td>MCHC (g/liter)</td>
<td>31.9 (0.10)</td>
<td>31.4 (0.13)</td>
<td>31.3 (0.10)</td>
<td>0.001</td>
<td>−1.8</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>18.7 (0.22)</td>
<td>18.3 (0.45)</td>
<td>18.3 (0.18)</td>
<td></td>
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<tr>
<td>Platelets (10³/μl)</td>
<td>820.3 (48.4)</td>
<td>861.1 (55.8)</td>
<td>928.8 (29.7)</td>
<td>0.054</td>
<td>+13.2</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>7.0 (0.17)</td>
<td>7.1 (0.14)</td>
<td>7.0 (0.09)</td>
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</table>

*Data for each test are listed with the standard error of the mean in parentheses.*

**DISCUSSION**

In this report we have described the production and characterization of mice deficient in the known macrophage galactose lectin mMGL. With these mice, we addressed three questions related to a potential role for mMGL in the recognition of cell surface structures regulated by differential sialylation. First, does mMGL regulate the turnover of effector T cells that become undersialylated subsequent to activation? Second, does mMGL regulate the turnover of effector T cells that become undersialylated subsequent to activation? Third, does...
mMGL play a role in the turnover of red blood cells that lose sialylation with age?

The development and selection of thymocytes are accompanied by a high rate of cell death, so that an estimated 98% of the thymocytes produced in the thymus die prior to emigration (31). The physiological mechanisms responsible for thymocyte cell death are almost entirely uncharacterized (37). Several receptor-ligand interactions have been proposed to participate in thymocyte death (6, 16, 17, 22), but there are few examples of targeted deletions that result in thymus hyperplasia (33). Despite the many studies on the topic, there is yet no real conceptual understanding of the signaling mechanisms that distinguish positive and negative selection of developing thymocytes. As such, we wanted to study the possibility that differential sialylation during thymus development could be recognized by an endogenous lectin as a way of regulating the emergence of mature T cells. From this and previous studies, we conclude that neither the deletion of ST3Gal-I nor the deletion of mMGL appears to have a substantial effect on T-cell development (23).

The observation that activated T cells lose sialylation and following expansion show significant apoptosis prompted us to consider the possibility that an endogenous lectin could induce death based on cross-linking of undersialylated molecules. This mechanism would be consistent with the loss of CD8 T cells in ST3Gal-I-deficient mice (Onami et al., unpublished data). We saw no differences in CD8 T cell numbers for mMGL+/+ versus mMGL−/− mice following activation in vivo and similar numbers of apoptotic CD8 T cells in

FIG. 5. Erythrocyte (RBC) turnover in mMGL+/+ and mMGL−/− mice. Mean life span of red blood cells was determined in (A) mMGL+/+ and (B) mMGL−/− mice after biotinylation in vivo. The mean erythrocyte life span was 36.67 days in mMGL+/+ mice, versus 37.27 days in mMGL−/− mice.

FIG. 6. Effect of mMGL deficiency in ST3Gal-I-deficient mice. ST3Gal-I mice were crossed to mMGL-deficient mice to examine the role of mMGL in the CD8 T-cell loss in ST3Gal-I-deficient mice. Solid squares represent ST3Gal-I+/+ mice, which show normal CD8 T-cell numbers in the peripheral blood lymphocytes (PBL). Solid circles represent ST3Gal-I−/− mMGL−/− mice, which show decreased numbers of CD8 T cells. Open circles show doubly deficient mice. (Top) Female doubly deficient mice show a trend of increased CD8 T-cell numbers compared to ST3Gal-I-deficient mice, reaching statistical significance in female mice aged 3 and 5 weeks. (Bottom) Male mice showed no statistically significant changes.
these mice as well. To determine whether an mMGL gene inhibition could complement the targeted ST3Gal-I deficiency in the loss of CD8 T cells, we produced mice deficient in both genes. As shown, although there appeared to be a tendency toward higher numbers of CD8 T cells in the mice lacking mMGL, mMGL−/−ST3Gal-I−/− mice still showed a profound CD8 T-cell deficiency. Therefore, we conclude that mMGL cross-linking of undersialylated cell surface molecules is not the predominant mechanism of cell loss in ST3Gal-I−/− mice.

In addition to lymphocytes, erythrocytes undergo differential sialylation. Experiments show that sialic acids are lost as erythrocytes mature. Localized binding of the Galβ1-3GalNAc-PAA probe was observed in the splenic red pulp, and this correlated with Mac-1 staining. (B) Double staining of splenic sections from mMGL-deficient mice with Galβ1-3GalNAc-PAA and Mac-1 revealed colocalization of the lectin to a Mac-1+ cell. Galβ1-3GalNAc-PAA binding was always seen on Mac-1 binding cells but not the converse, suggesting that the lectin(s) is expressed on a subpopulation of macrophages or mature dendritic cells.

FIG. 7. Identification of Gal/GalNAc-specific lectin(s) distinct from mMGL. (A) Sialic acid (SA) binding alone. (B) Galβ1-3GalNAc-PAA. (C to E) Serial sections were probed with (C) Galβ1-3GalNAc-PAA, (D) Galβ1-3GalNAc-PAA plus Galβ1-3GalNAc, and (E) Galα1-3GalNAc plus Galα1-3GalNAc.

FIG. 8. Gal/GalNAc-specific lectin(s) is macrophage restricted. (A) Splenic sections from mMGL−/− mice were probed with biotinylated Galβ1-3GalNAc-PAA or anti-Mac-1 antibodies. Localized binding of the Galβ1-3GalNAc-PAA probe was observed in the splenic red pulp, and this correlated with Mac-1 staining. (B) Double staining of splenic sections from mMGL-deficient mice with Galβ1-3GalNAc-PAA and Mac-1 revealed colocalization of the lectin to a Mac-1+ cell. Galβ1-3GalNAc-PAA binding was always seen on Mac-1 binding cells but not the converse, suggesting that the lectin(s) is expressed on a subpopulation of macrophages or mature dendritic cells.
rocytes undergo senescence (2, 4, 14). The role of sialic acids in erythrocyte senescence is not understood, but one possibility is that it constitutes one of the signals that target aged erythrocytes for destruction (9). We considered the possibility that mMGL-bearing macrophages could recognize the undersialylated erythrocytes and execute their engulfment. There does appear to be a difference in erythrocyte numbers in mMGL−/− mice (Table 1), but the biological significance is difficult to discern. We did not find a statistically significant difference in turnover rates, leading us to believe that the increases seen in erythrocytes in mMGL−/− mice may be explained by other indirect effects. Moreover, mMGL expression is not abundant in the spleen and liver, where erythrocyte turnover takes place after desialylation.

With AFGP as a probe, we identified an additional macrophage lectin(s) or lectin-like activity that is specific for Galβ1-3GalNAc but independent of mMGL. Binding of this lectin to its ligand is not affected by EDTA, suggesting that binding is not calcium dependent, and thus it is not a C-type lectin. In future studies, this lectin can be characterized further by purifying galactose-specific binding proteins with cell lysates derived from mMGL null mice. We propose that this lectin may play a functionally redundant role to mMGL and conclude from our studies that mMGL expression is not required for normal lymphoid homeostasis.

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