Human CD33 (hCD33) is an immunoglobulin (Ig) superfamily protein expressed on myeloid progenitor cells in the bone marrow and on peripheral blood monocytes. It is known to recognize α2-3- and α2-6-linked sialic acids (7, 18), which are expressed mostly at the nonreducing termini (outermost positions) of glycan chains (5, 38). Human CD33 (also known as hSiglec-3) is a member of the Siglec (sialic acid-binding Ig superfamily lectin) family of molecules defined by their mutual sequence similarity in the first two Ig-like domains and by their ability to recognize sialic acids (1, 9, 10; P. R. Crocker, E. A. Clark, M. Filbin, S. Gordon, Y. Jones, J. H. Kehrl, S. Kelm, D. N. Le, L. Powell, J. Roder, R. L. Schnaar, D. C. Sgroi, K. Stamenkovic, R. Schauer, M. Schachner, T. K. Van den Berg, P. A. Van der Merwe, S. M. Watt, and A. Varki, Letter, Glycobiology 8v, 1998). A cDNA for the putative mouse ortholog of hCD33 was cloned many years ago (33). Similarities within the extracellular domain between hCD33 and mouse CD33 (mCD33; 62% identity in amino acid sequence) (33) and similar gene structure and chromosomal position relative to adjacent genes (2) warrant its designation as the mouse ortholog of hCD33. However, the lack of sequence similarity in the cytosolic domains and difficulties in resolving phylogenetic relationships among the related Siglec molecules in humans and mice have raised questions about its functional equivalence to hCD33 (2, 4).

A subset of Siglec proteins showing relatively high sequence similarity to CD33 are called “CD33/Siglec-3-related Siglecs” (1, 9, 10). These include CD33/Siglec-3, Siglec-5, Siglec-6/OBP1, Siglec-7/AIRM1, Siglec-8, Siglec-9, Siglec-10, and Siglec-11, and a Siglec-like molecule (Siglec-L1) in humans, as well as five confirmed or putative Siglec proteins in mice (CD33, Siglec-E, Siglec-F, Siglec-G, and Siglec-H) (2). Many CD33-related Siglecs are expressed on cells involved in innate immunity. For example, hSiglec-7/AIRM-1 is expressed on natural killer cells and monocytes (14, 26); hSiglec-8 is expressed on eosinophils (17), and mSiglec-F is expressed on immature cells of the myeloid lineage (2). The cytosolic tails of most CD33-related Siglecs have two conserved tyrosine-based putative signaling motifs, one of which conforms to the immunoreceptor tyrosine-based inhibitory motif (ITIM), while the other is a putative motif of unknown function (1, 9, 10). It has been shown in some CD33-related Siglecs that the ITIM is the preferred docking site for the protein tyrosine phosphatase SHP-1 (27, 32, 36, 41), and cross-linking of these Siglecs elicits negative signaling in the cells expressing these molecules (15, 27, 36, 39, 40). Although the expression pattern of CD33-related Siglecs and their function as signaling molecules suggest their involvement in the regulation of innate immunity, their in vivo function in a model organism has not been studied so far.

Although mCD33 was cloned nearly a decade ago (33), its
protein expression pattern and ligand-binding properties have not yet been reported. In this study, we produced recombinant mCD33 and analyzed its binding properties. We also raised a monospecific antibody against mCD33 and studied its expression pattern in the intact mouse. Finally, we generated mice deficient in CD33, and analyzed CD33’s possible involvement in innate immunity. We report the initial phenotypic characterization of the CD33-deficient mice and discuss possible species differences and functional degeneracy between CD33 and other Siglecs.

MATERIALS AND METHODS

Materials. All antibodies were obtained from BD Biosciences Pharmingen, San Diego, Calif., unless otherwise stated. Ovine and porcine submaxillary mucins (OSM and PSM, respectively) were purchased from Acurate Chemical and Scientific (Westbury, N.Y.). Bovine submaxillary mucin (BSM) was prepared as described previously (24).

Production of recombinant mCD33-Fc fusion protein. The coding region for the extracellular domain of mCD33 was amplified by PCR and cloned into an expression vector, which upon expression in mammalian cell transfection produces mCD33-Fc, a recombinant fusion protein of the mCD33 extracellular domain and human IgG Fc fragment, with an enterokinase recognition sequence/FLAG tag (DYKDDDDK) in between (3, 4). CHO cells stably transfected with this vector were grown in Opti-MEM (Invitrogen) supplemented with 1.5% low-IgG fetal bovine serum (HyClone, Logan, Utah) for 5 to 7 days. The culture supernatant was collected, and the fusion protein was purified on protein A-Sepharose as described previously (3, 4). The recombinant mCD33-Fc protein was treated with *Arthrobacter ureafaciens* sialidase (AUS) while bound to protein A-Sepharose (7) to eliminate possible masking of the binding site by sialylated glycans attached to the same molecule.

Analysis of the sialic acid-binding properties of mCD33 with various cells. The full-length coding region of mCD33 (33) was amplified by reverse-transcription PCR (RT-PCR) with mouse bone marrow RNA as a template and cloned into pCDNA3.1(+) (Invitrogen). This construct was transiently transfected into COS-7 cells with a recombinant vaccinia virus containing the gene for mCD33. After 48 hours, the cells were washed and then incubated with 0.5% BSA for 30 min at room temperature. This mixture was extensively dialyzed against PBS at 4°C.

Removal of O-acetyl esters from BSM. BSM was incubated with 0.1 M NaOH for 30 min at room temperature to remove base-labile O-acetyl esters (11). Subsequently this mixture was neutralized with HCl and extensively dialyzed against PBS at 4°C. Influenza C virus hemagglutinin-esterase fused with the Fc part of human IgG (CHE-Fc) and purified as described previously (22). This O-acetyl sialic acid-specific esterase was also used to remove O-acetyl esters from the sialic acids of BSM. Ten micrograms of BSM was incubated with CHE-Fc (11.1 μg) in 300 μl of PBS for 1 h at 37°C. To remove the CHE-Fc, 35 μl of protein A-Sepharose was added to the mixture, which was then incubated at room temperature for 1 h. The protein A-Sepharose was removed, and a portion of ELISA buffer was added to the mixture. The mixture was washed and used in the subsequent ELISA. As a control, the CHE-Fc was boiled for 15 min and then incubated with BSM as described above. To confirm the effects of CHE-Fc treatment, binding was analyzed in the same ELISA using CHE-FcD (CHE-FcD is CHE-Fc treated with diisopropyl fluorophosphatase that inactivates the esterase activity, resulting in a probe that specifically binds to 9-O-acetylated sialic acids) and human CD22-Fc (binding is blocked by 9-O-acetylation of sialic acids) (20–22, 30).

Animal care. All animals used were maintained in an access-controlled barrier facility under specific-pathogen-free conditions. Studies were performed in accordance with Public Health Service guidelines and approved by the Animal Subjects Committee of the University of California, San Diego.

Production of mCD33-specific polyclonal antibody. Mice deficient in CD33, generated as described below, were immunized with 50 μg of mCD33 extracellular domain (prepared by enterokinase digestion of mCD33-Fc recombinant fusion protein and removal of the Fc portion by adsorption to protein A-Sepharose) in 400 μl of a 1:1 emulsion of PBS and Titermax Gold (Titermax USA, Norcross, Ga.) by intraperitoneal (i.p.) injection, followed by booster administration of 50 μg of mCD33 extracellular domain in 400 μl of 1:1 PBS-Titermax Gold emulsion 14 days later. After another 14 days, mice were given further boosts by i.p. injections of 10 to 20 μg of mCD33 extracellular domain in sterile PBS (200 μl) every week. Blood (~200 μl per animal) was collected from the tail vein every other week, and serum was prepared by a standard protocol. Monospecific polyclonal antibody was prepared from the collected serum by affinity purification. In brief, Siglec-9-Fc (0.5 mg) and mCD33-Fc (0.5 mg) were separately coupled to 0.5 ml of AffiGel-10 (Bio-Rad) according to the manufacturer’s instructions. Serum was first applied to a column of biotinylated 9-conjugated resin (0.25 ml) to eliminate antibodies that reacted with the enterokinase/FLAG tag or the human IgG Fc portion of the recombinant protein. The flowthrough from this column was applied to a column of mCD33-conjugated resin (0.25 ml), which was washed well, and the adsorbed antibodies were eluted with 0.1 M glycine-HCl buffer (pH 3.0) immediately followed by neutralization with 1/10 volume of 1 M Tris-HCl (pH 8.0). The affinity-purified antibody was concentrated, and the buffer was exchanged to PBS with an ultrafiltration device (UltraFree 15; molecular weight cutoff, 30,000; Millipore). A portion of this affinity-purified antibody was labeled with biotin by using EZ-Link Sulfo-NHS-biotin (Pierce) at an approximate antibody/biotin molar ratio of 1:100 according to the manufacturer’s protocol. The specificity of the antibody preparation was confirmed by an ELISA in which mCD33-Fc, mSiglec-9-Fc, and mSiglec-9-Fc (a kind gift from P. Crocker, University of Dundee) were used as test samples (data not shown).

Analysis of mCD33 expression on mouse hematopoietic cells by flow cytometry. Single-cell suspensions were prepared from thymus, spleen, bone marrow (from femurs), lymph nodes, and blood collected from anesthetized mice. Cells (10⁶ cells per sample) were first incubated with 0.5 μg of mouse Fc-Block (anti-CD16/CD32 monoconal antibody) and then with 1 μg of biotinylated anti-mCD33 antibody in 100 μl of 1% BSA-PBS at 4°C for 30 min, washed, and then incubated with phycoerythrin-conjugated streptavidin (1:100 dilution; Jackson ImmunoResearch) at 4°C for 15 min. The cells were washed again and suspended in 1 ml of 1% BSA-PBS, and analyzed with a FACSCalibur (BD Biosciences Immunocytometry Systems).

Sorting and morphological analysis of CD33-positive cells. Single-cell suspensions from peripheral blood and bone marrow were stained for CD33 antigen as described above and sorted with FACSVantage (BD Biosciences Immunocytometry Systems). The CD33-positive cell population and unsorted population were immobilized onto glass slides with a Cytospin unit (Thermo Shandon, Pittsburgh, Pa.). The cells were stained with Giemsa reagent and analyzed for their morphology under oil-immersion microscopy.

Generation of mice deficient in CD33. A genomic DNA fragment containing the mCD33 gene was obtained by screening a phage library of 129/Sv mouse genomic DNA with the mCD33 probe. DNA for the first round of ELISA as a probe. A gene-targeting construct was prepared by replacing a 3.8-kb DNA segment containing exons 1 to 5 of the CD33 gene with a pPGK-neo-pA
Hematology. Blood from the tail vein of methoxyflurane-anesthetized mice (6 to 8 weeks old) was collected into EDTA-containing polypropylene microtubes (Becton Dickinson). Analyses of erythrocytes, leukocyte count, and platelet cell numbers and morphology were carried out with a Cell-Dyn 3500 automated analyzer (Abbott Diagnostics) or Hemavet 850 FS (CDC Technologies, Oxford, Conn.), both programmed for mouse blood parameters. Differential analysis was performed manually on smears of EDTA-anticoagulated blood stained with Wright’s stain.

Serum chemistry. Blood was collected in the absence of anticoagulants by tail bleed and allowed to clot for several hours at room temperature in plastic microtubes, and the serum was collected by centrifugation. Serum chemistry analyses were performed with a Beckman CX-7 automated chemistry analyzer with a general coefficient of variation of <5%.

Analysis of hematopoietic cell populations in blood and lymphoid organs. Single-cell suspensions from thymus, spleen, lymph nodes, bone marrow, and blood were prepared from CD33-deficient mice and wild-type littermates (8 to 10 weeks old), erythrocytes were removed by lysis with ammonium chloride, and surviving cells were analyzed by flow cytometry for subpopulation distribution, using antibodies against various cell-surface markers appropriately labeled with fluorescent chromophore. The antibodies used were as follows: CD4, CD5, CD8, CD11b (Mac-1), CD21, CD22, CD23, CD24, CD25, CD28, CD40, CD43, CD44, CD11c (I-Ab), surface IgM, surface IgD, Ly6G (Gr-1), NK1.1, and TER119. Appropriate combinations were employed for multicolor analysis.

Quantification of IgS. Serum isotype-specific antibody titers were determined by sandwich ELISA with plates coated with anti-mouse isotype-specific antibodies. A standard curve was generated with purified mouse IgM, IgA (Sigma), IgG1, IgG2a, IgG2b, and IgG3 to convert optical density (OD) values to antibody concentration. Sera were diluted to 1/200 and analyzed with antimouse isotype-specific antibodies conjugated to alkaline phosphatase (IgM and IgA from Sigma; others from Pharmingen). OD at 405 nm (OD405) values were obtained with a microplate reader.

Casein-induced peritonitis. Casein (0.2% wt/vol in PBS, 1 ml per mouse; Sigma) was i.p. injected into CD33-deficient mice and wild-type littermates (8 to 12 weeks old), and the cells accumulating in the peritoneal cavity were collected by lavage with 10 ml of cold PBS containing 1% BSA and 0.5 mM EDTA at 0, 2, 4, 6, or 24 h postinjection. Peritoneal cells were stained with antibodies against Ly6G (Gr-1) and F4/80 and analyzed by flow cytometry. In some experiments, peripheral blood was collected from the retroorbital plexus of anesthetized mice prior to euthanasia and peritoneal lavage, anticoagulated with EDTA, and subjected to differential cell counting, as described above. The interleukin-6 (IL-6) concentration in EDTA-anticoagulated plasma (1:100 diluted) was measured by sandwich ELISA with monoclonal antibodies MP5-20F3 (capture antibody) and biotinylated MP5-32C11 (detection antibody), according to the manufacturer’s instructions (BD Biosciences Pharmingen). IL-6 was chosen because it is commonly elevated in a variety of inflammatory conditions.

RESULTS

Sialic acid-dependent binding by mCD33. mCD33-Fc did not show binding to any of the mouse hematopoietic cells (peripheral blood lymphocytes, bone marrow cells, and splenocytes) tested. In contrast, considerable sialic acid-dependent binding was found for hCD33-Fc, and binding was sensitive to mild periodate treatment of the cells, which truncates sialic acid side chains (data not shown). Furthermore, in contrast with hCD33, no rosettes were found to form between human erythrocytes and COS-7 cells transfected with full-length mCD33 (data not shown). These results indicate that the ligand for mCD33 is different from that for hCD33.

The sialic acid-binding abilities of mCD33-Fc were studied by ELISA with various sialylated probes. Of the biotinylated polycrylamide probes tested, sialic acid-dependent binding was only found for Neu5Aca2-6GalNAc (sialyl-Tn). While the signal for this interaction was very low, it was sensitive to mild periodate treatment (data not shown). However, no sialic acid-dependent binding was found to OSM, a mucin containing sialyl-Tn with sialic acid exclusively in the N-acetyl form (Neu5Ac) (19, 23), and very weak sialic acid-dependent binding to PSM was found (data not shown). PSM is a mucin that contains sialyl-Tn and the structures Galβ1-3[Siaα2-6]GalNAc and Fuc1-2Galβ1-3[Siaα2-6]GalNAc (23, 37), with the sialic acid almost exclusively in the N-glycolylneuraminic acid (Neu5Gc) form. mCD33-Fc did bind to BSA in a partly sialic acid-dependent manner (Fig. 1). In addition to sialyl-Tn, BSM contains Galβ1-3[Neu5Ac/Gcα2-6]GalNAc and GlcNAcβ1-3[Neu5Ac/Gcα2-6]GalNAc structures (23, 28, 35). Furthermore, BSM contains ~40% 9-O-acetylated sialic acids as determined by a chemical analysis (7). It is known that 9-O-acetylation of sialic acid reduces the binding to many Siglec, probably by blocking the glycerol side chain of sialic acid (20, 21, 30, 31). This turns out to be the case for mCD33 as well, since binding was considerably increased after removal of 9-O-acetyl esters by either base treatment or CHE-Fc treatment of
BSM (Fig. 1). As a control for the removal of 9-O-acetyl esters from BSM by CHE-Fc, binding to CD22/Siglec-2-Fc and CHE-FcD was analyzed in the same assay. This showed the expected considerable increase and decrease, respectively (data not shown). The results with the polyacrylamide probes and mucins show that the sialylated ligand for mCD33 may be similar, but not identical, to the sialyl-Tn epitope, with a possible preference for the Neu5Gc form of sialic acid. In addition, the results with the mucins indicate that it is likely that the presentation of the sialylated ligand on its protein backbone or the adjacent or underlying sugars is important for recognition by mCD33.

Mild periodate truncation of the side chains of sialic acids on BSM reduced but did not eliminate binding of the mCD33-Fc (Fig. 1, left panel). In this respect, mCD33 is again different from hCD33, which shows an absolute requirement for an intact sialic acid side chain, for binding to BSM (7).

Generation of a polyclonal mono-specific antibody against mCD33 protein. Despite three attempts in collaboration with BD Biosciences Pharmingen to generate rat monoclonal antibodies against mCD33, antibodies of the desired property and stability were not obtained. We therefore took advantage of our CD33-deficient mice (described below) and immunized them with recombinant mCD33 protein. While a strong serum response against the immunogen was seen, the group at BD Biosciences Pharmingen was again unable to isolate stable monoclonal antibodies by using the spleens from these mice. The reason for this difficulty is unclear, since the same group has had much success in generating other such monoclonal antibodies. Whatever the reason, this may explain why no other group has yet reported a monoclonal antibody against mCD33. Regardless, we were able to use the serum from the CD33-deficient mouse to affinity purify a monospecific polyclonal antibody against mCD33 and used this antibody to analyze mCD33 protein expression.

Expression of mCD33 protein on myeloid precursors and on granulocytes. Among many hematopoietic cell-types, CD33 expression was observed only on certain bone marrow and blood cells (Fig. 2). The CD33-positive cells in bone marrow were also CD11b (Mac-1) positive, indicating that mCD33 is expressed on the cells of the myeloid lineage. When sorted and examined directly after Geimsa staining, most of the CD33-positive cells in the bone marrow showed a mature granulocyte morphology under the microscope, with occasional cells showing an immature myeloid cell morphology (Fig. 2C). In peripheral blood, the CD33-positive cells were initially identified as mainly granulocytes, based on their distinctive light-scatter profile. This conclusion was supported by microscopic analysis of Geimsa-stained fluorescence-activated cell sorter (FACS)-sorted CD33-positive cells, which mostly had the morphology of mature granulocytes (Fig. 2C). The relatively low-level expression was not due to a low titer of the antibody preparation, as evident from the strong staining of 293 cells transiently transfected with mCD33 full-length expression construct (data not shown). Peripheral blood granulocytes from the CD33-deficient mice did not show any staining, confirming the specificity of the expression seen in the wild-type mice. Due to the lack of a definitive mouse monocyte marker, the paucity of monocytes in mouse blood, difficulties in morphological distinction between mouse granulocytes and monocytes, and the weak signals obtained with anti-CD33 antibody staining, we could not definitively prove or disprove mCD33 expression on circulating mouse monocytes. However, the lack of monocyte enrichment in CD33-positive sorted cells (Fig. 2) strongly suggests that mouse monocytes are negative for mCD33.

Frozen sections of pellets of transiently transfected cells

FIG. 2. CD33 expression on mouse hematopoietic cells. (A) Hematopoietic cells in bone marrow from wild-type (top panel) and CD33-deficient (bottom panel) mice were stained with the anti-CD33 antibody and analyzed by flow cytometry. The majority of Mac-1-positive cells stained weakly. (B) CD33 expression on leukocytes from various tissues. Solid bars, wild-type cells; open bars, CD33-deficient cells. MFI, median fluorescence intensity. (C) Examples of the light microscopic appearance of Geimsa-stained CD33-positive cells obtained by cell sorting from peripheral blood or bone marrow. The dominant morphology in both compartments is that of mature granulocytes.
gave a positive signal with the antibody, showing that it was suitable for immunohistochemical analyses (data not shown). Thus, we also attempted to analyze mCD33 expression in both frozen and formaldehyde-fixed paraffin-embedded sections of various organs (e.g., brain, liver, kidney, lung, gastrointestinal tract, etc.) by various immunohistochemical staining methods. However, we did not observe any signal above the background level. Thus, if cells in solid tissues (e.g., tissue macrophages) do express this molecule, they must do so at very low levels. Taken together, our data indicate that mCD33 is expressed at low levels on granulocytes and on some developing cells of myeloid lineage. This is in contrast to hCD33, which is easily detectable on immature cells of the myeloid lineage and circulating monocytes, but very weakly, if at all, on circulating granulocytes.

**Generation of CD33-deficient mice.** To investigate physiological functions of CD33 in vivo, we generated mice deficient in CD33 through conventional homologous targeting in embryonic stem cells (Fig. 3). Loss of CD33 expression in the CD33-deficient mice was confirmed by flow cytometric analysis of hematopoietic cells, as described above. In the limited-access barrier facility under specific-pathogen-free conditions, the CD33-deficient mice showed no gross morphological or histological abnormalities in many organs studied, and their growth was equivalent to that of wild-type littermates (data not shown). Also, CD33-deficient mice were fertile. Although many mice with the CD33 Δ/Δ genotype (even the offspring of C57BL/6 F10 × F10 intercross) had red eyes and a white-grayish coat, in contrast to the black eyes and black coat of wild-type and heterozygous littermates, this apparent phenotype was not strictly correlated with the CD33 genotype, in that some breeding pairs produced black pups with CD33 Δ/Δ genotype. This observation is likely to be explained by a background mutation of the gene pink-eyed dilution (gene symbol P; Mouse Genome Informatics accession identification no. MGI: 97454), which is only ~5 centimorgans apart from Cd33. The R1 ES cell line is heterozygous at the P locus because it is a hybrid between 129X1/SvJ and 129S1/Sv-p Tyr* Kitt32/-/+ (16, 29, 34), and the p allele (on 129X1-derived chromosome 7) probably coincided with the targeted CD33 allele on the same chromosome copy. We have also observed mild transmission distortion of the null allele in female F6 × F6 offspring (W/W: W/Δ:Δ/Δ = 34:64:23), but this distortion was not statistically significant (P = 0.30 for χ2 test).

### Table 1. Peripheral blood analyses of CD33-deficient mice and wild-type littermates

<table>
<thead>
<tr>
<th>Parameter observed</th>
<th>CD33−/−</th>
<th>CD33−/+</th>
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<tbody>
<tr>
<td>Erythrocytes (106/μl)</td>
<td>894 ± 41</td>
<td>765 ± 63</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>165.1 ± 11.9</td>
<td>184.4 ± 16.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>49.9 ± 4.4</td>
<td>60.3 ± 2.7</td>
</tr>
<tr>
<td>Platelets (103/μl)</td>
<td>9.0 ± 0.1</td>
<td>11.7 ± 0.9</td>
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<table>
<thead>
<tr>
<th>Differential cell counts</th>
<th>CD33−/−</th>
<th>CD33−/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes (10⁶/μl)</td>
<td>6.05 ± 0.80</td>
<td>5.55 ± 0.60</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>16.0 ± 2.6</td>
<td>21.4 ± 3.2</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>79.5 ± 2.7</td>
<td>73.8 ± 3.6</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2.0 ± 0.5</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.9 ± 0.5</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
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**Minor effect of CD33 deficiency on hematological and biochemical parameters.** Differential cell counting and analysis of blood chemistries of CD33-deficient mice and wild-type littermates were performed as described in Materials and Methods and summarized in Table 1. The only statistically significant differences between wild-type and CD33-deficient mice were a slight reduction in mean erythrocyte count and hematocrit and an increase in the mean concentration of serum glutamic-oxaloacetic transaminase (aspartate aminotransferase) in blood. Although these parameters were still within the normal range for wild-type mice, these differences may be biologically significant. Thus, the absence of CD33 has only minor hematological and biochemical consequences in mice.

**No obvious effect of CD33 deficiency on hematopoietic cell development.** Detailed analysis of the distribution and number of hematopoietic cell subpopulations in primary and secondary lymphoid organs by flow cytometry (n = 3 for each genotype) revealed no significant difference between CD33-deficient and wild-type mice (data not shown), suggesting no major nonredundant role of CD33 in the development and distribution of hematopoietic cells. Quantification of various Igs by ELISA in the serum of these mice also showed no statistically significant differences between CD33-deficient and wild-type mice (Table 2). These results suggest no major nonredundant contribution of CD33 in normal development of the humoral immune system.

**Minor effect of CD33 deficiency in casein-induced peritonitis and LPS-induced systemic inflammation.** Since the expression of CD33 was restricted to granulocytes and myeloid lineage cells, we analyzed whether CD33 is involved in the regulation of innate immunological responses. A pilot casein-
induced peritonitis experiment using a limited number of mice suggested that CD33-deficient mice might be hyporesponsive to this stimulus, showing reduced granulocyte (Gr-1⁺) infiltration at earlier stage of inflammation (up to 6 h postinjection), while the number of macrophages (F4/80⁺/Gr-1⁻) in the peritoneum was not influenced by the mCD33 genotype (data not shown). We reexamined this finding by using more mice (10 wild-type and 9 mCD33-deficient mice), while limiting the time point to only 4 h postinjection. In this study, we found no significant difference in the ratio of Gr-1⁺ to F4/80⁺ cells (granulocytes and macrophages) between wild-type and CD33-deficient mice (data not shown). The levels of IL-6 in blood induced by peritonitis (wild-type mice, 892 ± 452 pg/ml; knockout mice, 780 ± 679 pg/ml) were also not significantly different (Student’s t test, \( P = 0.68 \)). There was no significant difference between wild-type and mCD33-deficient mice in peripheral blood cell counts either, with the possible exception of the platelet count (Table 3). However, platelets are not positive for mCD33 expression (data not shown).

We then examined the effect of mCD33 deficiency on LPS-induced systemic inflammation. The IL-6 response at the peak (3 h postinjection) was somewhat weaker in CD33-deficient mice (wild-type mice, 120 ± 42 ng/ml; knockout mice, 78.4 ± 54 ng/ml), but the statistical significance of this difference was marginal (Student’s t test, \( P = 0.11 \)). Again, there was no significant difference in peripheral blood cell counts at any time points up to 24 h after LPS injection (Table 4). Along with the finding that there was no substantial difference in the ratio of Mac-1⁺ (myeloid cells) to TER119⁺ (erythroid cells) in wild-type and CD33-deficient mouse bone marrow (data not shown), these data suggest that mCD33 may not have any nonredundant functions in leukocyte trafficking nor in activity regulation of cells via common pathways involved in innate immunity.

### DISCUSSION

In this report, we have described the sialic acid-binding properties of mCD33 and its expression on bone marrow and blood granulocytes. We also generated and characterized mice deficient in CD33 and analyzed its possible involvement in innate immunity. mCD33 showed distinct binding to BSM in a sialic acid-dependent manner, and this binding was affected by 9-O-acetylation or by truncation of the glycerol-like side chain of sialic acids, confirming the designation of mCD33 as a member of the Siglec family. Although the exact glycan structure preferred by mCD33 was not identified, it is likely to be related to sialyl-Tn (Neu5Acaya2-6GalNAc) structure, as suggested by preferential binding of mCD33 to a polymeric probe with substitution of multiple copies of sialyl-Tn disaccharides. However, its preferred ligand structure may not be sialyl-Tn itself, since neither OSM nor PSM (both rich in sialyl-Tn structure) showed strong binding to mCD33. A class of glycan structures found abundantly in BSM but not in OSM or PSM is likely to explain the preferential binding of BSM. An alternative explanation is that the preferred structure is present in all mucin probes tested, but the spacing between individual glycans is not favorable in the poorer ligands.

The above results are in striking contrast to the sialic acid-
binding specificity of hCD33, which showed robust binding to α2-3- and α2-6-linked sialic acids on lactosamine units (7). The sequence identity between mouse and hCD33 is higher in the second Ig-like domain (72% identity over 93 amino acids) than in the first Ig-like domain (54% identity over 127 amino acids), which is the primary site of sialic acid recognition in Siglecs (9, 10). The striking difference in the sialic acid-binding properties between hCD33 and mCD33 is likely to be explained by these extensive sequence differences in their first Ig-like domains. Of course, it is possible that both mCD33 and hCD33 have an alternative ligand interacting with their second Ig-like domain, which has prevented the sequence divergence of the second Ig-like domain of CD33. However, curiously, this trend (lower sequence identity in the first Ig-like domain) is also observed in some other human-mouse orthologous pairs of CD33-related Siglecs (i.e., mSiglec-E/hSiglec-9 and mSiglec-G/hSiglec-10). It is tempting to interpret this trend as an indication that the first Ig-like domain of CD33-related Siglecs has been under selective pressure to undergo rapid evolution, rather than as evidence of passive decay due to functional insignificance. Such facilitated sequence evolution may be explained by its interaction with rapidly evolving pathogens, which could potentially have influenced the evolution of the first Ig-like domain of CD33-related Siglecs. Pathogens carrying sialic acids (or sialic acid-like sugars, such as legionaminic and pseudaminic acids) (5) are possible agents that could have facilitated such rapid evolution of CD33-related Siglecs via interaction with the cells expressing them.

Our study has shown that the pattern of expression of mCD33 is also not identical to that of hCD33, in that the former is expressed mostly on mature granulocytes rather than on early myeloid lineage cells in bone marrow. In contrast, hCD33 is more prominently expressed on monocytes in peripheral blood and only very weakly expressed on granulocytes, if at all. Differences in expression patterns have also been observed for another pair of putative Siglec orthologs (i.e., mSiglec-F and hSiglec-5) (2). The former is expressed on immature cells of the myeloid lineage in bone marrow (2), while the latter is prominently expressed on mature neutrophils and monocytes (8). This finding, along with the lack of a canonical ITIM in the cytosolic tail of mCD33 and difference in the sialic acid-binding properties between mCD33 and hCD33, raises the possibility that mCD33 and hCD33 are functionally not equivalent. If this is the case, it somewhat reduces the utility of CD33-deficient mice as a model to understand the function of CD33 per se in humans. Nevertheless, this is the first reported animal model in which a CD33-related Siglec was genetically deleted and can still serve as a model to study generic functions of CD33-related Siglecs.

Mice deficient in CD33 were viable and fertile, without any obvious deficiency in overall organ development and growth. Moreover, they showed no distinct difference from wild-type mice in the development and distribution of mature hematopoietic cells. These observations are not surprising, given the restricted expression pattern of mCD33. However, it was somewhat unexpected that they showed no overt phenotypical difference from wild-type mice in the models of acute inflammation, such as casein-induced peritonitis and LPS-induced systemic response. One interpretation is that CD33 does not substantially contribute to generic acute-phase inflammation. Another is that it has functional redundancy to other Siglecs known to be expressed on the same cells. A different mode of stimulus (e.g., a more sustained stimulation of the innate immune system or some specific stimulus engaging CD33) may be necessary to identify differences between wild-type and CD33-deficient mice in immune responses. In this respect, recent studies using DNA microarrays (6, 25) have shown that different bacteria induced both “stereotyped” (common to all bacteria) and “specific” (bacterial species specific) responses in human cells involved in innate immunity, suggesting involvement of both common (leading to stereotyped response) and different (leading to specific response) cellular receptors to bacterial components or products in the host recognition of bacteria. mCD33 is possibly one of these receptors involved in the specific responses. It would be interesting to know if wild-type and CD33-deficient cells respond differently to various types of bacteria (or bacterial products) by using DNA microarray analysis. We should also emphasize that the mice used in this study were maintained under specific-pathogen-free conditions, potentially masking a deficiency in innate immunity that may manifest under less controlled conditions. Transfer of these mice to a conventional vivarium and deliberate challenge with dead or live pathogens are under consideration.

Edelman and Gally (13) have noted that in many cases (up to 30%), mouse models deficient in selected gene products fail to manifest phenotypic consequences, which at least in part, is likely to be explained by the functional “degeneracy” of different gene products. The lack of a distinct phenotype in CD33-deficient mice may be also explained by the presence of another Siglec (e.g., Siglec-F) (2) in similar cell subsets that express CD33. Although the ligand-binding specificities of Siglec-F and CD33 are quite different (the former preferentially recognizes the Neu5Acα2-3Galβ1-4GlcNAc structure), some glycoproteins might express glycan structures recognized by both Siglec-F and CD33, and one of these glycoproteins may be the functional ligand for Siglec-F and CD33. In order to examine this possibility, we are currently working to generate mice deficient for Siglec-F as well as those with a combined deficiency in mCD33 and mSiglec-F. Also, there are some other mouse Siglecs (Siglec-E, -G, and -H) whose expression patterns have yet to be reported, leaving open the possibility that one (or more) of these Siglecs is coexpressed with CD33. Antibody development for these Siglecs is currently under way (P. Crocker, personal communication), and a report on the expression analysis of these Siglecs is awaited.

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