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Characterization of a B Cell Surface Antigen with Homology to the S100 Protein MRP8

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The S100 proteins comprise a large sub-family of the EF-hand calcium-binding proteins. Here we describe a novel monoclonal antibody recognizing a B cell surface antigen. This monoclonal antibody immunoprecipitates three proteins in the 12–18 kDa range and the smallest of these proteins has a striking homology at its amino-terminus to human MRP8, a myeloid specific member of the S100 family. Similarly to MRP8 in myeloid cells, this antigen is expressed in the cytoplasm of B cells and is secreted by LPS-induced activated B cells. This surface antigen is not B cell specific. Since MRP8 is not expressed by lymphoid cells, however, this antibody appears to recognize a new member of the S100 family.

The S100 family of proteins is comprised of small (6–14 kDa) acidic proteins containing two calcium-binding EF hands. The functions of S100 proteins are thought to include a role in cell growth, cell cycle regulation, cytoskeleton development, and inflammation [for reviews see (1–4)]. To date, 18 members of this family have been identified in humans displaying 25–65% homology at the amino acid level. Thirteen of these genes map to a cluster on human chromosome 1 (5, 6) and many of the murine homologs map to a syntenic region on mouse chromosome 3 (7). S100 proteins exist as monomers or homodimers and some members form heterodimers with other S100 family members (8, 9).

The structure of several S100 proteins has been determined by NMR spectroscopy or X-ray crystallography (10–13). The EF hands are comprised of a helix-loop-helix structure; are flanked by amino- and carboxy-terminal hydrophobic regions; and are separated by a hinge region which is the least conserved region among the family members. The C-terminal EF hand has a canonical calcium binding loop of 12 amino acids which undergoes a conformational change upon calcium binding, resulting in the exposure of amino acid residues in the hinge region and C-terminus of the protein (10). These newly exposed amino acids are thought to be involved in protein-protein interactions with target molecules such as annexins I (14), II (15) and VI (16); cytoskeletal proteins such as tubulin (17, 18), cytokeratins (19), and type III intermediate filaments (16, 20); kinases such as the nuclear serine/threonine kinase ndr (21), and the myosin-associated giant kinase, twitchin (22); and the tumor suppressor protein p53 (23, 24).

S100 proteins are expressed in a wide variety of tissue types with each individual gene expressed in a tissue and cell specific manner. MRP8 and MRP14, for example, are expressed in monocytes and neutrophils, but not in mature tissue macrophages (25, 26). They are also expressed in epithelial cells (27) and recently have been shown to be expressed in microvascular endothelial cells (28). Elevated levels of S100 family members are associated with a variety of diseases or inflammatory conditions including cancer, cystic fibrosis, rheumatoid arthritis, psoriasis, and Alzheimer's disease (1, 29, 30).

Little is known about the expression of S100 proteins in lymphocytes. The presence of S100A1, S100B, and S100A4 has been reported in lymphocytes but their role has not been studied (31, 32). In the course of characterizing a monoclonal antibody (mAb) raised against murine spleen cells, we identified a cell surface determinant expressed on B-lymphocytes. Immunoprecipitation experiments revealed three proteins in the 12–18 kDa range. Here we report that N-terminal amino acid sequencing of the smallest of these proteins shows homology to human MRP8. Because MRP8 is not expressed by lymphocytes (25), this antibody appears to recognize a new member of the S100 family.

MATERIALS AND METHODS

Mice and cell culture. BALB/c or DBA/2 female mice, 4–12 weeks old, (NCI-DCT, Frederick, MD) were housed in a clean facility at CBER. Experiments were performed under an IACUC approved

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protocol. 70Z/3 pre-B cells or freshly isolated spleen cells were incubated in complete RPMI (RPMI 1640 with glutamine, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids, 50 μM 2 β-mercaptoethanol, and 2 mM glutamine) at 37°C with 5% CO_2. Supernatants were separated from cells by centrifugation and concentrated approximately 10-fold by spinning over Centricon concentrators (Amicon, Beverly, MA) with a 10K molecular weight cut-off. Spleen cells were cultured for 24 to 48 h in complete RPMI with 50 μg/ml LPS (*E. coli* serotype 0111:B4, Sigma, St. Louis, MO).

Monoclonal antibody 90.12. The generation and characterization of mAb 90.12 will be described elsewhere (manuscript submitted). Briefly, C57BL/6 mice were hyperimmunized with DBA/2 spleen cells and rested for at least 2 months before a final boost. Fusions with Sp2/0 (ATCC, Rockville, MD) were performed three days later. Hybridoma supernatants were screened for reactivity with B lymphocytes by flow cytometry. 90.12 antibody from hybridoma supernatants was purified and labeled with biotin for use in flow cytometry or coupled to CNBr-activated Sepharose 4B for immunoprecipitations. MAb 90.12 is an IgG2b, κ antibody. An IgG2b, κ isotype control or normal mouse Ig were used as controls in all experiments.

Immunoprecipitation of cell lysates and culture supernatants. Lysates were prepared as previously described (33). Briefly, 2×10^8 cells/ml in PBS were surface labeled with 1 mM sulfo-LC-NHS-biotin (Pierce, Rockford, IL) for 30 minutes at room temperature. Cells were lysed for 1 h at 4°C in lysis buffer (20 mM Tris-HCl pH 7.0, 0.5%, 150 mM NaCl, 5 mM EDTA, 0.5%Tween 20, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Following centrifugation at $3000 \times$ g for 15 minutes at 4°C, 250 μl aliquots of lysate were transferred to microcentrifuge tubes containing either 25 μl 90.12-Sepharose 4B beads or normal mouse IgG-Sepharose 4B beads and rocked overnight at 4°C. Immunoprecipitates were washed twice in 600 mM NaCl lysis buffer, twice in 300 mM NaCl lysis buffer, and twice in 150mM NaCl lysis buffer. Immunoprecipitates were heated with reducing sample buffer for 5 minutes at 95°C and run on 10–20% Tris-glycine precast gels (Bio-Rad, Hercules, CA) with Laemmli running buffer. Proteins were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA) and blocked overnight with I-Block buffer (Tropix, Bedford, MA). Detection of the biotin-labeled 90.12 antigen was performed using the Western-Light Plus kit (Tropix) with the chemiluminescent CSPD substrate.

Alternatively, lysates were prepared as above from cells without surface biotin labeling. Immunoprecipitates from cell lysates and from 10$\times$ concentrated tissue culture supernatants were heated with NuPage SDS reducing sample buffer (Novex, San Diego, CA) for 5 minutes at 95°C and run on precast NuPage 10% bis-Tris gels with 2(N-morpholino) ethane sulfonic acid (MES)/SDS running buffer.

Western blotting. Whole cell lysates were run on a 10–20% Tris-glycine precast gradient gel and transferred to an Immobilon PVDF membrane for Western blotting. Strips were developed with mAb 90.12, normal mouse IgG or the secondary reagent anti-mouse IgG only, using Protoblot Western Blot, AP System for mouse (Promega, Madison, WI).

Tunicamycin treatment. BALB/c spleen cells were harvested and cultured for 36 h with either 50 μg/ml LPS alone or with LPS and 7.5 μg/ml tunicamycin (Calbiochem, San Diego, CA). Lysates prepared from surface biotin-labeled cells were immunoprecipitated and transferred to Imobilon membranes as described above. The membrane was incubated with streptavidin-horseradish peroxidase in PBS with 0.1% Tween 20 for one h. The blot was washed five times with PBS-Tween, incubated for one minute with ECL reagent (Amersham Pharmacia Biotech, Piscatway, NJ) and bands visualized by chemiluminescence.

Pronase treatment. BALB/c spleen cells were T cell depleted and cultured in complete RPMI with 50 μg/ml LPS for 24 h. Cells at 1.6×10^6/ml in HBSS were incubated with 25% pronase (Sigma) for 30 minutes at 37°C or left untreated. For analysis by flow cytometry, all incubations and centrifugations were performed at 4°C. Cells were treated with 2.4G2 Fc receptor block (Pharmingen, San Diego, CA) for 5 minutes and surface receptors were double-stained for 20 minutes with B220-FITC (Pharmingen) and either biotin-90.12, biotin-IgG2b isotype control (Pharmingen), or PE-Ia (Pharmingen). Cells were washed twice and those treated with biotinylated primary antibody were incubated with PE-streptavidin (Molecular Probes, Eugene, OR) for 20 minutes. Cells were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using Forward scatter (FSC) versus Side scatter (SSC) gating. Quadrants were set by comparision with the IgG2b isotype control. Data was analyzed using CELLQuest software (Becton Dickinson).

N-terminal amino acid sequencing. Immunoprecipitates of 70Z/3 cell lysates were run on a precast 4–12% bis-Tris gradient gel (Novex) under reducing conditions. Following electrophoresis, proteins were electroblotted onto an Immobilon PVDF membrane in NuPage bis-Tris-bicine transfer buffer. The membrane was stained with 0.1% Coomassie Brilliant Blue 250. Relevant bands were excised and analyzed in a gas-phase sequenator (Applied Biosystems model 494A, Foster City, CA). Sequencing was performed by the Core Facility for Biotechnology Resources at CBER.

Cytoplasmic staining. Spleens from BALB/c mice were treated with ACK lysing buffer to lyse red blood cells and the remaining cells were washed in PBS and resuspended at 2×10^7 cells/ml in Dulbecco's PBS (DPBS) without Mg^{++} or Ca^{++}, 1% FCS, and 0.1% sodium azide. Cells were double-stained for surface markers using FITC-B220 and either PE-Ia, biotin-90.12, or biotin-IgG2b isotype control. In parallel, cells were surface stained with FITC-B220, fixed with 4% paraformaldehyde in DPBS for 20 minutes, washed twice, and resuspended in permeabilization buffer [DPBS, 1% FCS, 0.1% sodium azide, and 0.1% saponin]. Cytoplasmic staining of cells utilized either PE-Ia, biotin-90.12, or biotin-IgG2b isotype control for 20 minutes. Cells were washed twice with permeabilization buffer and biotin-90.12 and IgG2b treated-cells were then stained 20 minutes with PE-streptavidin conjugate. Cells were analyzed as described above.

RESULTS

Initial experiments indicated that mAb 90.12 recognized a cell surface determinant which is upregulated on B cells upon LPS stimulation. The 90.12 antigen can be detected on 5–20% of resting B lymphocytes and on >95% of LPS-stimulated lymphocytes (manuscript submitted). Figures 1A and 1B show an immunoprecipitation and Western blot of the 90.12 antigen from LPS-stimulated DBA/2 spleen cells. Immunoprecipitation with mAb 90.12 (Fig. 1A) results in three bands in the 12–18 kDa size range with the upper two bands running as a doublet. On Western blot analysis (Fig. 1B) of whole cell lysates, mAb 90.12 clearly reacts with the two lower bands. The upper band in Fig. 1B may also be a doublet as other Western blot experiments have shown three bands.

Several lymphocyte/leukocyte cell surface determinants such as ThB and Thy-1 are in the 12–18 kDa size range and are linked to the membrane via a phosphatidyl inositol (PI) linkage. Using PI-phospholipase C under conditions where ThB or Thy-1 are removed from cells, the 90.12 antigen was not and therefore, is not linked to the membrane via phosphatidyl inositol (data not shown). To further characterize this antigen,

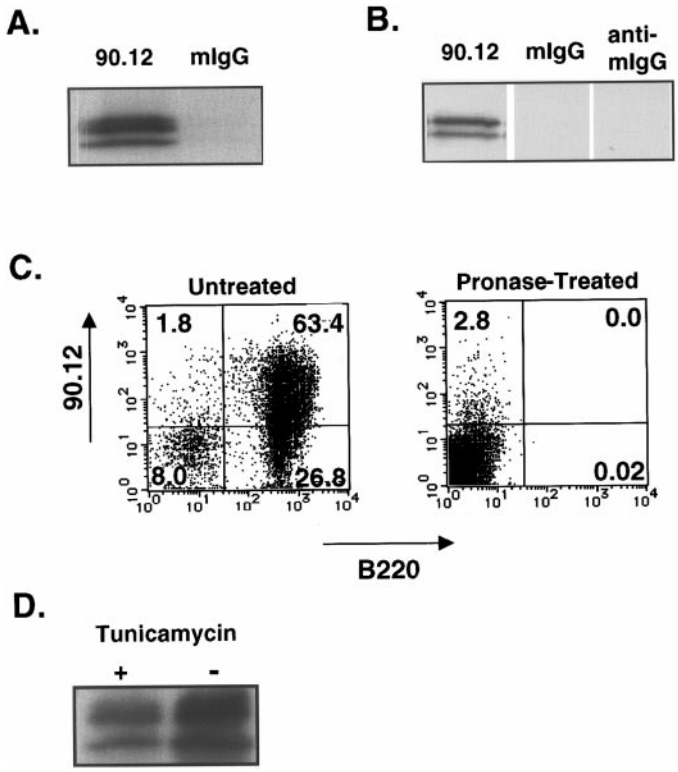

FIG. 1. Characterization of the 90.12 antigen. (A) Lysates from LPS-stimulated, biotin-labeled DBA/2 spleen cells were immunoprecipitated with 90.12- or mouse IgG-Sepharose beads. (B) LPS-stimulated DBA/2 whole cell lysates (unlabeled) were run on SDS-PAGE, transferred to a PVDF membrane and probed with mAb 90.12, mouse IgG control, or no primary antibody. Anti-mouse IgG secondary antibody was used to develop the strips. (C) BALB/c spleen cells were T-cell depleted and cultured for 24 h with 50 μg/ml LPS to induce 90.12 expression. Pronase treatment was prior to surface staining for B220 and 90.12. (D) BALB/c spleen cells were cultured for 36 h with 50 μg/ml LPS or with 50 μg/ml LPS and 7. 5 μg/ml tunicamycin to block N-linked glycosylation. Biotin-labeled cell lysates were immunoprecipitated with 90.12-Sepharose beads.

splenic B cells were grown in the presence of LPS to upregulate 90.12 surface expression. Cells were then treated with pronase to determine whether the epitope recognized was protein-associated. Figure 1C shows that treated cells lost 90.12 surface expression and therefore, the epitope is at least protein associated if not part of a protein itself. As seen in Fig. 1D, there is no difference in the immunoprecipitated proteins from cells grown in the presence of tunicamycin compared to those grown in its absence. Thus, the 90.12 antigen is not N-glycosylated.

Lysates from 1.8–3 $\times$ 10^8 70Z/3 cells were immunoprecipitated and proteins transferred to PVDF membranes, stained with Coomassie blue, and excised for N-terminal amino acid sequencing. Sequence information was obtained only from the smallest molecular weight band and was consistent over three independent sequencing runs. The longest sequence determined was 16 residues and a database search using the BLASTP algorithm (34) revealed significant homology with human MRP 8. Figure 2 shows an alignment of the 90.12 antigen N-terminal sequence with human and murine MRP8 as well as S100A1, S100B and S100A4 proteins. The 90.12 antigen sequence matches human MRP8 in 11 of 16 positions and in 13 of 16 positions if the alanine and leucine residues which were also detected in positions 8 and 9 are considered. Comparison with murine MRP8 shows 7–10 matches while S100A1, B and A4 show only 3–7 matches. Since MRP8 is not expressed in lymphocytes (25), it is possible that a new member of the S100 protein family has been identified.

MRP8 is prevalently cytoplasmic and with MRP 14, comprises up to 40% of the total cytoplasmic protein in neutrophils (35). Since the 90.12 antigen was originally detected as a cell surface antigen, it was surprising to find homology with such an abundant cytoplasmic protein. Therefore, we examined surface versus cytoplasmic expression of 90.12 on freshly isolated $B220^+$ B cells by flow cytometry. Ia, a class II MHC antigen expressed on all B cells, as well as macrophages, was used as a control. Ia molecules continually turn over such that the intracellular component should be roughly equivalent to that on the surface. Figure 3 shows surface and cytoplasmic staining histograms. Total $B220^+$ B cells are shown in the upper and lower right quadrants. $B220^+$ B cells co-staining with either 90.12 or Ia are within the upper right quadrant. As seen in Fig. 3B and 3D, >95% of the $B200^+$ B cells express Ia both on the surface and in the cytoplasm. In contrast, only 11% of the B cells express 90.12 on the surface while 90% express it in the cytoplasm (Fig. 3A and 3C). Furthermore, 90.12 is also detected in the cytoplasm of 66% of the non-B cells present in the spleen (see upper left quadrant, 3C). Thus, the 90.12 antigen is expressed in the cytoplasm of 80% of all spleen cells.

MRP 8 and 14 can be detected in extracellular fluids. Therefore, we examined whether the 90.12 antigen was also secreted. Figure 4 compares the 90.12 immunoprecipitation profiles from 70Z/3 lysates to concentrated tissue culture supernatant from LPS-stimulated

	AL L K
90.12	XLTELEKSGNGIXDVY
hMRP8	MLTELEKALNSIIDVYHKYSL
mMRP8	PSELEKALNSLIDVYHNYSN
mS100A1	MGSELESAMETLINVFHAHSG
mS100B	MSELEKAMVALIDVFHQYSG
mS100A4	MARPLEEALDVIVSTFHKYSG

FIG. 2. Alignment of murine 90.12 antigen with S100 family members. Residues listed above the sequence were detected at a lower level in that position. Comparisons are made to human MRP 8 (accession number 115442), murine MRP 8 (accession number 1173338), murine S100A1 (accession number 3746892), murine S100B (accession number 1710815), and murine S100A4 (accession number 4506765).

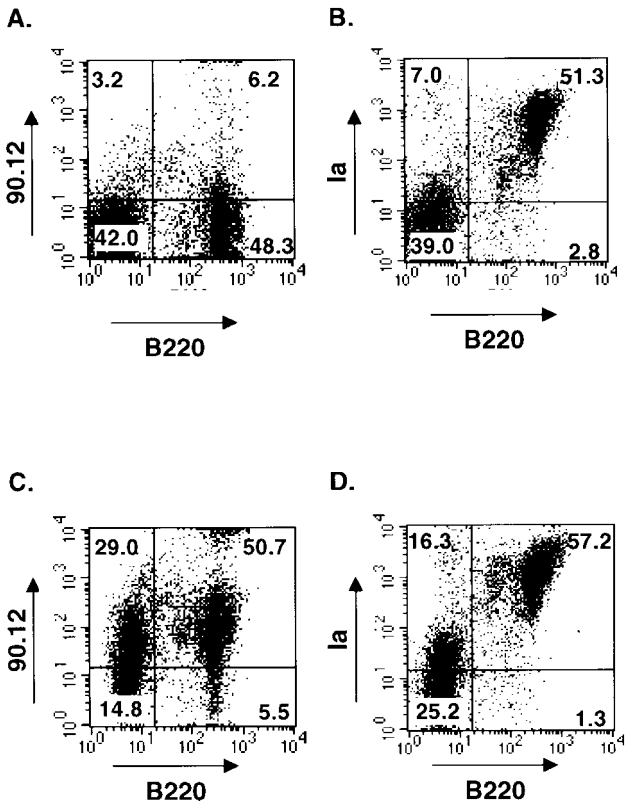

FIG. 3. 90.12 antigen is expressed in the cytoplasm of spleen cells. Total BALB/c spleen cells were stained for surface and cytoplasmic expression of 90.12 or Ia (Y axis, upper quadrants) and analyzed by flow cytometry. B cells were defined as $B220^+$ cells (X axis, right quadrants). Surface expression of (A) 90.12. (B) Ia. Cytoplasmic staining of (C) 90.12. (D) Ia. Numbers represent percentage of total cells in each quadrant. The sum of cells in the upper and lower right hand quadrants represents total B cells while cells in the upper right hand quadrant are 90.12^+, $B220^+$ B cells or Ia^+, $B220^+$ B cells.

murine spleen cells and concentrated serum-containing tissue culture medium. The 3 band pattern is observed in the 70Z/3 lysate while a similar pattern with an additional protein is found in the supernatant from LPS-stimulated spleen cells. No proteins were immunoprecipitated from serum-containing concentrated tissue culture medium. Thus, similar to MRP8 and MRP14, the 90.12 antigen is secreted.

DISCUSSION

The functions of the S100 proteins are not known but they have been implicated in cell growth, cell cycle regulation, cytoskeleton development, and inflammation. MRP8 and MRP14, myeloid specific members of this family, are thought to have both intracellular and extracellular functions. Extracellularly, murine MRP 8 has been shown to be a potent chemoattractant for myeloid cells (36) and human MRP14 has been shown to play a role in Mac-1 mediated adhesion of neutrophils (37). The MRP8/14 complex also has an extracellular function in that it has been shown to have antimicrobial activity (38–40). Intracellularly, MRP8 and MRP14 have been shown to inhibit casein kinase I and II (41), are phosphorylated and translocated to the membrane upon neutrophil activation (26, 42–44), and are dependent upon calcium binding for complex formation (45).

In the course of studying B lymphocyte surface antigens, we identified a murine monoclonal antibody raised against murine splenocytes which recognizes an activation-induced antigen on B cells upon LPS stimulation. This mAb is not B cell specific as it also reacts with all hematopoietic cell types. This antibody immunoprecipitates 3 proteins in the 12–18 kDa range from LPS-stimulated but not untreated B cells (manuscript submitted). N-terminal amino acid sequence of the smallest protein shows significant homology (62%–75% identity over 16 amino acids) to human MRP8. As MRP8 is not expressed by B lymphocytes (25), we have potentially identified a new member of this protein family. Sequencing of the larger protein bands resulted in either a mixed sequence or too short a sequence to productively compare to other proteins. This is likely due, in part, to poor separation of the larger proteins and to the possibility that the other proteins may have blocked amino-termini as does MRP14 (35). It remains to be seen whether these proteins also share homology with the S100 family.

S100 proteins are not usually considered cell surface antigens. MRP8 and MRP 14 have been demonstrated, however, to appear as heterodimers on surfaces of subsets of monocytes and macrophages in acute inflammatory lesions (46, 47). Non-myeloid cells such as Raji (a Burkitt's lymphoma-derived cell line) and MOLT-4 (a T-cell lymphoma line) have MRP8/14 heterodimer binding sites on their surfaces where binding to the cell surface is MRP8 driven in a calcium independent manner (48). In these cases, MRP8/14 surface expression is through extracellular binding of the heterodimer.

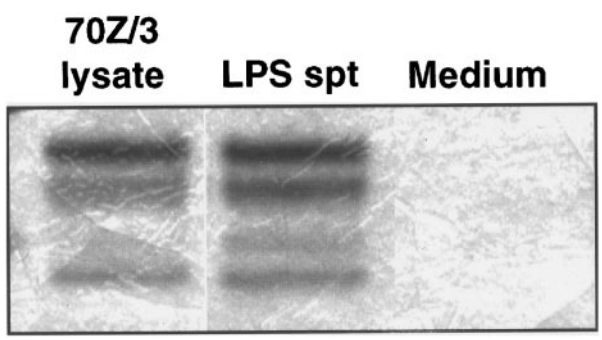

FIG. 4. The 90.12 antigen is secreted. BALB/c spleen cells were cultured for 24 h with 50 μg/ml LPS. Culture supernatants were collected and concentrated approximately 10-fold. A lysate from 70Z/3, concentrated LPS supernatant, and tissue culture medium were immunoprecipitated with 90.12-Sepharose beads.

Since we have shown the 90.12 antigen to be present on the cell surface of B cell lines as well as on freshly isolated B cells, and since it cannot be immunoprecipitated from serum-containing concentrated tissue culture medium, it is unlikely that the 90.12 antigen is expressed on B cell surfaces through the formation of complexes of extracellular 90.12 derived from non-B cells. It is unclear, however, how S100 proteins are expressed on the cell surface as they have neither leader peptide nor a transmembrane domain.

Many other features of the 90.12 antigen are consistent with features of MRP8 and MRP14. In addition to being in a similar molecular weight range, the 90.12 antigen is also not N-glycosylated. The 90.12 antibody immunoprecipitates at least 3 proteins as does the Mac 387 monoclonal antibody which is specific for MRP 14 (49–51) and a polyclonal rabbit antiserum raised against purified MRP8 (44). It may be that the 90.12 antibody recognizes an epitope on one of the proteins which immunoprecipitates a complex of the proteins. Western blot results however, suggest that the 90.12 antibody recognizes a common epitope on at least two of the three immunoprecipitated proteins.

MRP8 and MRP14 are abundantly expressed in the cytoplasm, are secreted, and can be detected on the cell surface of a subpopulation of myeloid cells. We have shown that 90% of freshly isolated unactivated splenic B cells and 80% of all splenic cells express the 90.12 antigen intracellularly. Future experiments will determine whether the 90.12 antigen, like MRP8 and MRP14, is alsotranslocated to the membrane upon B cell activation and whether or not this is calcium dependent.

We have also shown that the 90.12 antigen is secreted by LPS-stimulated total spleen cells. Surface expression of the 90.12 antigen is upregulated by LPS and anti-IgM plus IL4 and IL5 (manuscript submitted). LPS has been shown to upregulate the surface expression of the MRP8/MRP14 complex on monocytes (52) as well as the expression of MRP8 mRNA and protein in myeloid lineage cell lines and elicited peritoneal macrophages (53). Interestingly, another study showed that pokeweed mitogen stimulated the secretion of MRP14 and MRP8/14 complex from monocytes and that the Th2 cytokines IL10 and IL4 downregulated this secretion (54). It will therefore be of interest to look at the simultaneous effect of anti-IgM, IL4 and IL5 on both surface expression and secretion of the 90.12 antigen. Finally, since the 90.12 antigen may represent a new S100 family member, we will further sequence these proteins and clone cDNAs from a B cell library.

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