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Mannose Receptor Ligand-Positive Cells Express the Metalloprotease Decysin in the B Cell Follicle[1]

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Decysin, a gene encoding a disintegrin metalloprotease, is transcribed in human dendritic cells (DC) and germinal centers (GC). We have cloned its murine homologue and show that it is processed by the endoprotease furin before secretion of the catalytic domain. We have defined the cell types that express decysin in mouse spleen in the course of an immune response to T cell-dependent Ags. Like in humans, decysin is transcribed by activated CD11c^{+} DC that enter the T cell zone from the marginal zone (MZ). In the GC, decysin is expressed by follicular DC and tingible body macrophages. In addition, a MZ cell population expresses decysin and appears to migrate into the B cell follicle. The majority of these follicle-homing cells express the mannose receptor ligand, a marker for the macrophage-like MZ metallophils. The follicle-homing cells are M-CSF dependent, as they are absent in *op/op* mice that lack functional M-CSF. This suggests that mannose receptor ligand^{+} MZ metallophils differentiate into cells that migrate from the MZ into the B cell follicle. Decysin represents the first marker for this previously unrecognized cell population of the mouse spleen, which may represent a precursor for GCDC and may be specialized in the transport of unprocessed Ag from the MZ into developing GC. *The Journal of Immunology,* **2001, 167: 5052–5060.**

An efficient immune reaction depends on the successful capture and transfer of Ag to B and T cells, which are concentrated into microanatomical compartments in the secondary lymphoid organs. In the spleen, blood-borne Ags drain into the sinus of the marginal zone (MZ),[3] which surrounds the white pulp comprising the B cell follicle and the T cell-rich periarteriolar lymphoid sheath (PALS). The MZ contains the MZ macrophages that phagocytose Ag, and may play a role in presenting T cell-independent Ags to MZ B cells (1, 2). Along the inner border of the MZ are located the MZ metallophils, also known as metallophilic macrophages, which are poorly phagocytic and express little MHC-II Ag (3). Their function is still unclear. The MZ also comprises CD11c^{+} dendritic cells (DC) that are grouped around the bridging channels, adjacent to the PALS.

DC are a family of professional APCs that reside in peripheral tissue in an immature state, optimal for Ag capture. In response to microbial stimuli and inflammatory cytokines, which are most likely produced by T cells and macrophages communicating with the immature DC, the DC mature and migrate to the T cell zone of secondary lymphoid organs (4, 5). Mature DC express high levels of costimulatory molecules and antigenic peptides, bound to MHC class I and II molecules, and are highly efficient T cell stimulators. Shortly after immunization, splenic CD11c^{+} DC migrate from the MZ bridging channels into the PALS, suggesting that immature DC reside at the bridging channels, and mature DC in the PALS (5, 6). DC have also been implicated in the transport of unprocessed Ag to B cells (7, 8). B cells would subsequently present antigenic peptides to T cells and receive T cell-encoded stimuli in form of cytokines and CD40 ligand (9, 10).

About 4 days following immunization, activated B cells migrate into the follicles to form germinal centers (GC), where they undergo affinity maturation and isotype switch (11, 12). Besides B cells, the GC reaction involves at least three additional cellular components: follicular DC (FDC), Ag-specific Th cells, and tingible body macrophages that act as scavengers for apoptotic lymphocytes. FDC are nonphagocytic and trap native Ag in form of immune complexes on Fc and complement receptors (13–15). Retention of immune complexes is necessary for a productive GC, as mutations that abolish immune complex retention can severely impair GC reaction. Studies of the mode of transport of immune complexes have suggested that Ag-transporting cells carry immune complexes on the cell surface from the subcapsular sinus to developing GC (16). Using an Fc chimeric protein, containing the cysteine-rich domain of the mouse mannose receptor (CR-Fc), it was observed that mannose receptor ligand^{+} (MR-L)$^{+}$ cells migrate from the subcapsular sinus into the developing GC (17). In addition, using the same probe, Berney et al. (8) have shown that MR-L^{+} subcapular sinus macrophages migrate to the interface of the T cell zone and the follicle and that purified MR-L^{+} cells differentiate into T and B cell stimulatory DC. Therefore, it appears that MR-L^{+} subcapular sinus macrophages can differentiate into GC-homing putative Ag-transporting cells and bona fide DC. The splenic counterpart to the subcapsular sinus macrophages appears to be the MZ metallophils, as they also express MR-L (17,

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[3] Abbreviations used in this paper: MZ, marginal zone; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CR-Fc, cysteine-rich domain of the mouse mannose receptor; DAB, diaminobenzidine; DC, dendritic cell; EST, expressed sequencing tag; GC, germinal center; MR-L, mannose receptor ligand; NBT, nitroblue tetrasodium; pAb, polyclonal Ab; PALS, periarteriolar lymphoid sheath; PNA, peanut lectin.

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18). However, in response to immunization, MZ metallophils appear not to migrate into the B cell follicle or to the T cell zone (17).

Studies on human tonsils have suggested the presence of T and B cell stimulatory DC in GC (19–21). However, the cellular and microanatomical origin of GCDC is currently unclear. In the mouse, GCDC have not yet been identified. Decysin, a novel disintegrin metalloprotease, has been cloned from a cDNA library made from ex vivo purified human GCDC. In situ hybridization revealed transcription of decysin in human GC, suggesting that decysin is a novel marker for this specialized DC subset (22). In this study, we have cloned the mouse homologue of decysin and show that in immunized mice it is expressed by CD11c$^+$ DC, FDC, and tingible body macrophages. Strikingly, following immunization, MR-L$^+$ MZ metallophils express decysin and appear to migrate into the developing GC in the course of the immune response. These MZ-derived decysin$^+$ cells are absent in *op/op* mice that lack functional M-CSF, supporting the idea that the M-CSF-dependent MZ metallophils migrate into the follicle. MZ metallophils may be a precursor for the murine GCDC, and could carry immune complexes from the marginal sinus into the developing GC.

Materials and Methods

Animals

BALB/c mice were purchased from Iffa Credo (L'Arbresle, France) and used at 8–12 wk of age. A total of 4×10^7 SRBC (Sanofi Diagnostics Pasteur, Paris, France) was injected i.p. Osteopetrotic *op/op* and control +/? mice were used at 3 wk of age and were immunized i.p. with 10 μg alum-precipitated chicken OVA. BALB/c mice were injected s.c. into hind footpads with 20 μg alum-precipitated OVA (grade V; Sigma, St. Louis, MO) and boosted 2 wk after. Popliteal lymph nodes were removed before secondary immunization (day 0) and 2 days after secondary injection. For each time point, three BALB/c mice and two *op/op* mice were used, and multiple sections were analyzed with identical results.

Cloning of mouse decysin and plasmid constructions

cDNA was amplified from reverse-transcribed BALB/c mouse spleen mRNA (Clontech, Palo Alto, CA) in a PCR using a primer derived from expressed sequencing tag (EST) AA608260 and an upstream primer derived from human decysin. The product was cloned into a T/A vector (pCR2.1; Invitrogen, San Diego, CA) and sequenced. A primer derived from this sequence was used to amplify the 5′ untranslated region using Marathon rapid amplification of cDNA ends (Clontech). The PCR product was directly sequenced from both strands. To obtain the full-length mouse decysin-coding cDNA, the upstream primer (5′-ctcgagaccatgctgcctgggactt ctcggc) and the downstream primer (5′-ctcgagttctgtgatgtggtgg) were used in a PCR reaction with DNA polymerase *Pfu*Turbo (Stratagene, La Jolla, CA) and reverse-transcribed BALB/c spleen mRNA as template. After addition of 3′-adenosine overhangs by *Taq* polymerase (AmpliTaq; PerkinElmer Roche, Norwalk, CT), the PCR product was cloned into the T/A vector (pCR 2.1; Invitrogen) and sequenced.

The insert was liberated at *Xho*I sites, introduced on the primers, and subcloned into plasmid pIg (R&D Systems, Minneapolis, MN), which directs expression of a fusion protein with the human IgG1 Fc tail. The *Xho*I restriction fragment was also subcloned into pCDNA3.1 (Invitrogen) in frame with a carboxyl-terminal *myc* epitope and a 6× histidine (His) tag. Nucleotide sequence of all constructs was verified by double-stranded sequencing.

In vitro expression and furin convertase cleavage

Myc/6× His-tagged decysin was transcribed with T7 RNA polymerase and translated in rabbit reticulocyte nuclear lysate using the TnT-coupled reticulocyte system (Promega, Madison, WI) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech, Piscataway, NJ). To purify decysin, lysates were added to 200 μl binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing preequilibrated Ni^{2+}-NTA slurry (Qiagen, Chatsworth, CA). After binding for 1 h at room temperature, beads were pelleted, washed in buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole), and resuspended in 20 μl furin convertase cleavage buffer (50 mM HEPES, pH 7.5, 1 mM $CaCl_2$, 0.5% Nonidet P-40). Ten microliters were treated with 2 U of purified recombinant human furin convertase (Alexis Biochemicals, San Diego, CA) at 30°C for 1 and 3 h. Proteins were resolved on a SDS-PAGE and visualized by fluorography.

Expression of Fc-tagged recombinant decysin

A total of 3×10^5 Cos-7 cells was transiently transfected (lipofectamin; Life Technologies, Rockville, MD) with plasmid pIg decysin. Two days later, cells were preincubated for 15 min in methionine/cysteine-free RPMI medium (ICN, Costa Mesa, CA) in the presence of 10% FCS (Valbiotech, Paris, France) and then labeled for 2 h with 150 μCi/ml pro-mix L-^{35}S (Amersham Pharmacia Biotech) (70% [^{35}S]methionine, 30% [^{35}S]cysteine). After a chase of 1 h in RPMI 1640 medium (Life Technologies) containing 10% FCS, pulse and chase growth medium were pooled, and protease inhibitors were added. Cells were washed twice in cold PBS and lysed at 4°C in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, aprotinin, PMSF, leupeptin). Cleared cell lysates and growth medium were incubated with protein A-Sepharose CL-4B (Sigma) and then washed with RIPA buffer. Precipitated proteins were resolved by SDS-PAGE and visualized by fluorography.

RNA isolation and RT-PCR analysis

To isolate fresh DC, spleen cells from naive or SRBC-immunized BALB/c mice were isolated by mechanical disruption in cold Mg^{2+}- and Ca^{2+}-free PBS and loaded on a Ficoll gradient (Roche Molecular Biochemicals, Basel, Switzerland). Mononuclear cells were collected and allowed to settle on plastic. After 2 h, nonadherent and loosely adherent cells were detached by washing, incubated with biotinylated anti-B220 mAb RA3-6B2, washed, and incubated with streptavidin microbeads (Miltenyi Biotec, Auburn, CA). After two passages over a MiniMacs column (Miltenyi Biotec), B cell-depleted cells were then positively enriched for CD11c$^+$ DC by incubating cells with biotinylated N418 mAb, streptavidin microbeads, and passage over a MiniMacs column (Miltenyi Biotec). RNA was isolated using RNA^{Plus} (Quantum, Durham, NC) and reverse transcribed using the Ready To Go kit (Amersham Pharmacia). RNA from untreated, LPS-, and TNF-α-stimulated DC line D1 (23) were kindly provided by S. Amigorena (Institut Curie, Paris, France). Mouse decysin was amplified by primers 5′-gaggaatgtaccaatctt (sense) and 5′-tcaccaggattcggctcc (antisense), which generate a 497-bp product. G3PDH primers were from Clontech.

Antibodies

Anti-decysin rabbit polyclonal Abs (pAb) were raised against the prodomain peptide 69-NQTERYGKEEKYAPEV-74 and affinity purified (CovalAb, Ouillins, France). Remaining Abs are listed in Table I. Isotype-matched control Abs from PharMingen were used in preliminary experiments to validate the specificity of the staining. Decysin and FDC-M2 double-labeling analyses were done in the presence of 10 μg/ml Fc receptor-blocking Ab 2.4G2 (own production).

Immunohistochemistry

Acetone-fixed spleen sections were incubated with anti-decysin pAb (0.5 μg/ml) overnight at 4°C in PBS/2% goat serum, or with the following Ab for 1 h at room temperature in PBS/2% goat serum: N418 (5 μg/ml), Moma-1 (1/25 dilution), biotinylated RA3-6B2 (2.5 μg/ml), biotinylated peanut lectin (PNA; 2.5 μg/ml), FDC-M2 (1/200 dilution), FA11 (1/100 dilution). Decysin was revealed by biotinylated goat anti-rabbit Ab (Vector, Burlingame, CA) and alkaline phosphatase (AP)-conjugated avidin-biotin complex (Vector). Color development was in Fast Blue (Vector). For N418, FDC-M2, and FA11, the secondary Ab was HRP-conjugated goat

Table I. *Abs/probes used in this study*

Ab/Probe	Murine Ag	Ref./Supplier
N418	CD11c, DC	6
Moma-1	MZ metallophils	BMA Biomedicals
CR-Fc (probe)	MR-L, MZ metallophils Subcapsular sinus macrophages	17
RA3-6B2	B220, B cells	BD PharMingen
PNA	GC B cells	Vector
FDC-M2	FDC	12
FA11	Macrosialin/CD68, tingible body macrophages	Serotech

anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL) and developed in diaminobenzidine (DAB; DAKO, Carpenteria, CA). For Moma-1, the secondary Ab was AP-conjugated goat anti-rat IgG (Southern Biotechnology Associates), and color development was in Fast Red (Sigma). Biotinylated RA3-6B2 and PNA were revealed by HRP-conjugated streptavidin, and development was in DAB (DAKO).

In situ hybridization

The protocol is from reference (24). Mouse decysin-coding cDNA was transcribed in antisense direction by T7 RNA polymerase in presence of digoxigenin UTP. Sense probes were used as control and produced unspecific background staining (not shown). The RNA probe was separated from unincorporated label by gel filtration chromatography (Chroma Spin; Clontech) and used for hybridization without prior alkaline hydrolysis. Acetone-treated cryostat sections were fixed in PBS-buffered 4% paraformaldehyde for 10 min at room temperature. RNases were inactivated in PBS containing 1/1000 vol diethylpyrocarbonate (Sigma) for 30 min at room temperature. Sections were washed in 4× SSC and prehybridized for 4 h at 60°C in 4× SSC/50% deionized formamide/10% dextran sulfate/40 μg/ml salmon sperm DNA. The prehybridization solution was then replaced with fresh solution containing the RNA probe and covered with a hydrophobic coverslip (Grace Biolabs, Bend, OR). After overnight incubation at 60°C, sections were washed at 65°C in 2× SSC for 1 h and in 0.1× SSC for 1 h. Sections were then incubated at room temperature for 2 h with anti-digoxigenin AP-conjugated Fab (Roche Molecular Biochemicals) diluted 1/5000 in buffer A (0.1 M Tris-HCl, pH 7.5/150 mM NaCl) containing 0.5% blocking agent (Roche Molecular Biochemicals). After washing in buffer A, development was done in 50 ml buffer B (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) containing 175 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 225 μl nitroblue tetrasodium (NBT; Kirkegaard & Perry, Gaithersburg, MD) for between 1 and 2 days at room temperature in the dark. The sections were either counterstained with Fast Nuclear Red (Certistain; Merck, West Point, PA) or subjected to immunohistochemistry. After reequilibration in PBS, sections were incubated with biotinylated PNA (5 μg/ml) in PBS/2% sheep serum, followed by HRP-conjugated streptavidin, and developed in DAB (DAKO). Alternatively, sections were incubated with 6 μg/ml chimeric probe CR-Fc in PBS/2% sheep serum, followed by biotinylated goat anti-human IgG (Southern Biotechnology Associates) and HRP-conjugated avidin-biotin complex (Vector). Development was with DAB (DAKO). All images were recorded by a 3CCD camera (Hamamatsu, Massy, France) and assembled using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Results

Cloning of mouse decysin

Database searching for EST with significant homology to human decysin identified a nucleotide sequence from mouse colon. The missing open reading frame was cloned from mouse spleen cDNA using a primer derived from the EST sequence and a primer of the 5′ end of human decysin. Comparison of the deduced amino acid sequence with human decysin showed 78% similarity and 65% identity between the two proteins (Fig. 1). Like human decysin, the mouse sequence comprises a peptide leader sequence and a recognition site for furin endopeptidases, which would separate the N-terminal prodomain from the catalytic domain. The catalytic domain contains the zinc binding site and a short disintegrin domain. The zinc binding sites of both sequences comprise aspartate at position 361 (boxed residue), which is a unique feature of decysin among all other mammalian disintegrin metalloproteases that comprise a histidine residue at this position (25).

Decysin is processed by furin convertase and secreted

To verify that decysin is cleaved by furin convertase, *myc*/6× His-tagged mouse decysin was synthesized in vitro in the presence of [^{35}S]methionine and purified by Ni^{2+}-chelate affinity chromatography. The labeled protein was mock treated or incubated with recombinant human furin convertase, and cleavage products were analyzed by SDS-PAGE. As shown in Fig. 2*A*, decysin was cleaved by furin convertase in a time-dependent manner, resulting in two clearly visible protein bands corresponding to the 54-kDa full-length protein and the cleaved 32-kDa catalytic domain. The 22-kDa prodomain was further processed at an internal cleavage site (Fig. 1) and migrated with an apparent molecular mass of 18 kDa. It was poorly visible, as it contains only two labeled methionine residues.

To test whether mouse decysin is secreted after furin processing, it was expressed in Cos-7 cells as an Fc-fusion protein. Following metabolic [^{35}S]methionine labeling, decysin Fc was precipitated

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                           Peptide leader
Mouse    1 MLPGTSRLPTEASMSWVLLSVLWLIIQIQVIDATLTPELKPHEIVRPKKLPISQKRGLENN 61
           ML G S+LP  A+MSWVLL VLWLI+Q Q I    TPEL  HEIV PKKL I  KR ++NN
Human    1 MLRGISQLPAVATMSWVLLPVLWLIVQTQAIAIKQTPELTLHEIVCPKKLHILHKREIKNN 61

Mouse   62 QTERYGKEEKYAPEVQYQIILNGEEIVFHLKRTKHLLGPDYTETSYSPRGEESTRHSQDVK 122
           QTE++GKEE+Y PEVQYQ+ILNGEEI+  L++TKHLLGPDYTET YSPRGEE T   ++++
Human   62 QTEKHGKEERYEPEVQYQMILNGEEIILSLQKTKHLLGPDYTETLYSPRGEEITTKPENME 122
                                 Furin convertase consensus
Mouse  123 PCYYEGHIQNARGSLARISTCDGLRGYFTHRDQRYQIKPLQSTDEGEHAVLPYSWKGQDTV 183
            CYY+G+I N + S+A ISTCDGLRGYFTH  QRYQIKPL+STDE EHAV   + + QD
Human  123 HCYYKGNILNEKNSVASISTCDGLRGYFTHHHQRYQIKPLKSTDEKEHAVFTSNQEEQDPA 183
                  Furin convertase consensus
Mouse  184 HDKDAEKQVVRKRSHLRTSRSLKNPN-EDLLQGQKYIGLFLVLDNAYYKLYNGNVTQMRTF 243
           +     K    K+  +R SRSLK+P  ED L+ QKYI L+LVLDNA+YK YN N+T+R+ F
Human  184 NHTCGVKSTDGKQGPIRISRSLKSPEKEDFLRAQKYIDLYLVLDNAFYKNYNENLTLIRSF 244

Mouse  244 LFKVLNLLNMIYKTINIQVSLVGMEIWSDQDKIKVEPNLGATFTHFMRWHYSNLGKRIHNH 304
           +F V+NLLN+IY TI++QV+LVGMEIWSD DKIKV P+   TF +F+RWH SNLGK+IH+H
Human  245 VFDVMNLLNVIYNTIDVQVALVGMEIWSDGDKIKVVPSASTTFDNFLRWHSSNLGKKIHDH 305
                                                  Zinc binding site
Mouse  305 AQLLSGASFRHGRVGMAAGNSFCTTSSVSVIEAKKKNNVALVALMSHELGHALGMKDVPYY 365
           AQLLSG SF + RVG+AA NS C+ SSV+VIEAKKKNNVALV +MSHELGH LGM DVP+
Human  306 AQLLSGISFNNRRVGLAASNSLCSPSSVAVIEAKKKNNVALVGVMSHELGHVLGMPDVPFN 366
                                                 Disintegrin domain ➔
Mouse  366 TKCPSGSCVMNQYLSSKFPKDFSTVSRSHFQGFLSSRNARCLLLAPDPKNIIK-PTCGNQV 425
           TKCPSGSCVMNQYLSSKFPKDFST  R+HF+ +L S+  +CLL AP P NI+  P CGN +
Human  367 TKCPSGSCVMNQYLSSKFPKDFSTSCRAHFERYLLSQKPKCLLQAPIPTNIMTTPVCGNHL 427

Mouse  426 LDVGEECDCGSPEECTNLCCEPLTCRLKSQPDCS-EASNHITE 467
           L+VGE+CDCGSP+ECTNLCCE LTC+LK   DC  +A NH TE
Human  428 LEVGEDCDCGSPKECTNLCCEALTCKLKPGTDCGGDAPNHTTE 470
```

FIGURE 1. Amino acid sequence comparison of mouse and human decysin. The peptide leader sequences are underlined. Highlighted in bold type are the furin convertase cleavage sites, the zinc binding sites, and the disintegrin domains. The unique aspartate residue in the zinc binding site is boxed. The mouse decysin sequence has been deposited in EMBL/GenBank/DDBJ under the accession number AJ242912.

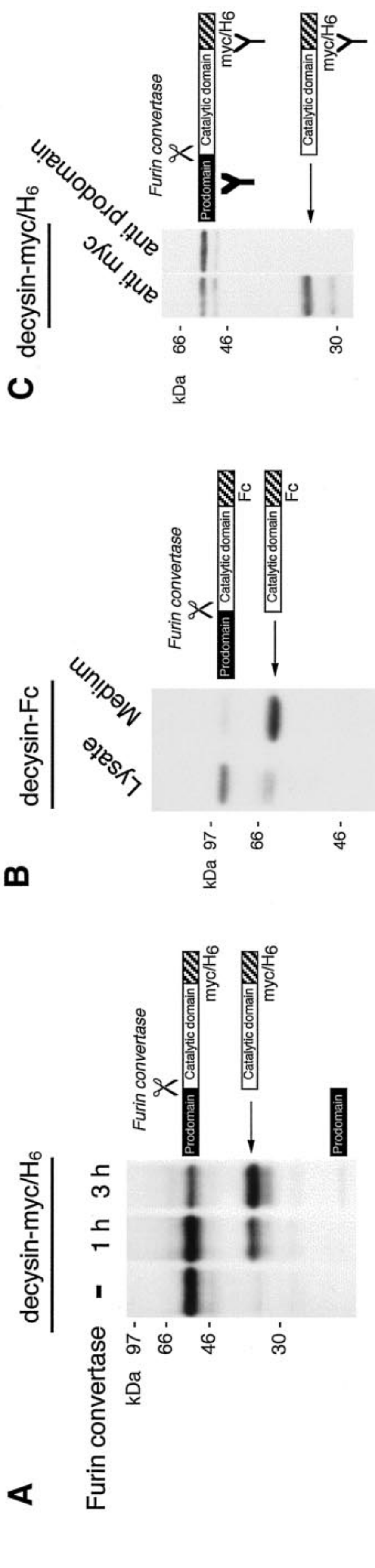

FIGURE 2. Decysin is processed by furin convertase and secreted. *A*, Mouse decysin tagged at the C terminus with a *myc* epitope and six histidine residues was synthesized in vitro in the presence of [^{35}S]methionine. The labeled decysin was purified by Ni^{2+}-chelate affinity and mock treated or incubated with recombinant furin convertase for 1 and 3 h. Unprocessed decysin and proteolytic fragments were resolved by SDS-PAGE and visualized by fluorography. *B*, Fc-tagged mouse decysin was expressed in Cos-7 cells and metabolically labeled. Cell extracts and culture supernatant were incubated with protein A, and bound proteins were resolved by SDS-PAGE and visualized by fluorography. The expected sizes of unprocessed and processed decysin are 79 and 57 kDa, respectively. The size difference is most likely due to posttranslational modifications. *C*, Mouse decysin tagged at the C terminus with the *myc* epitope and six histidine residues was expressed in *E. coli* and purified by Ni^{2+}-chelate affinity under denaturing conditions. The anti-*myc* mAb recognized both forms, whereas the decysin anti-prodomain pAb recognized only full-length decysin.

with protein A from cell lysates and culture supernatants and analyzed by SDS-PAGE (Fig. 2*B*). Cell lysates contained predominantly the unprocessed form, and cell culture supernatants contained almost exclusively the processed catalytic domain. This provides evidence that the decysin catalytic domain is secreted while unprocessed decysin remains intracellular.

A rabbit antiserum was raised against a prodomain peptide and affinity purified. Western blot of *myc*/6× His-tagged mouse decysin produced in *Escherichia coli* showed that the anti-peptide Ab recognizes the unprocessed protein, but not a shorter fragment that migrated with the apparent m.w. expected of the catalytic domain (Fig. 2*C*). The anti-*myc* Ab recognized both forms.

Decysin is transcribed by DC of the T cell zone

We wondered whether, like its human homologue, mouse decysin is transcribed by mature DC. The mouse DC line D1 (23) was stimulated by LPS or TNF-α, and the expression of decysin mRNA was tested by RT-PCR. LPS and TNF-α had previously been found to be effective maturation agents for this cell line (23). As shown in Fig. 3*A*, the message was absent in immature D1 cells, weakly induced by LPS, and strongly induced by TNF-α. Next, CD11c$^+$ DC were purified from spleens of naive and immunized mice and tested for the presence of decysin message. Decysin was transcribed in CD11c$^+$ DC isolated from naive mice, but its message was up-regulated 1 day after immunization with SRBC (Fig. 3*A*). The presence of decysin mRNA in DC from naive mice was most likely due to DC activation in the course of cell manipulation. To further analyze the induction of the decysin message in DC as well as to explore its expression profile in situ, we performed in situ hybridization on spleen sections from naive mice and 1 day after immunization with SRBC. As shown in Fig. 3*B*, in naive spleen, no decysin transcripts were detected in the white pulp, comprising the PALS, that surrounds the central arteriole, and the follicles. Also the MZ, which delineates the white pulp, and the bridging channel (asterisk) showed no significant signal above background. A weak signal was visible in the red pulp close to the MZ, which may reflect a low level of transcription by red pulp macrophages. The expression profile drastically changed 1 day after immunization (Fig. 3*C*). Now, cells in the PALS clearly transcribed decysin, which correlated with the immigration of CD11c$^+$ DC from the bridging channels (asterisk) into the PALS (Fig. 3*D*). In contrast, the CD11c$^+$ DC residing at the bridging channel expressed little, if any, decysin mRNA. Taken together, these data provide evidence that decysin transcription is induced by immunization, and strongly suggest that decysin is transcribed by mature mouse CD11c$^+$ DC.

MR-L$^+$ decysin$^+$ cells enter the B cell follicle from the MZ

At day 1, a cell population located in the Moma-1$^+$ MZ also transcribed decysin (Fig. 3, *C* and *D*). To test whether these cells were MZ metallophils, spleen sections 1 day after immunization were double stained for decysin mRNA and MR-L, a marker for MZ metallophils (17). As shown in Fig. 4*A*, almost all decysin-transcribing cells expressed MR-L and were located at the inner MZ. Few decysin$^+$ MR-L$^+$ cells were found within the follicle (arrowheads). To further assess whether these cells migrate into the B cell follicle during the immune response, spleen sections 2 days after immunization were stained for decysin mRNA and MR-L (Fig. 4*B*). Now, cells expressing decysin mRNA and MR-L were clearly seen in the B cell follicle (arrowheads). To analyze whether the decysin$^+$ cells migrate toward a developing GC, spleen sections taken from mice 2 and 4 days after immunization were stained for decysin using the anti-decysin anti-peptide pAb and PNA. Two

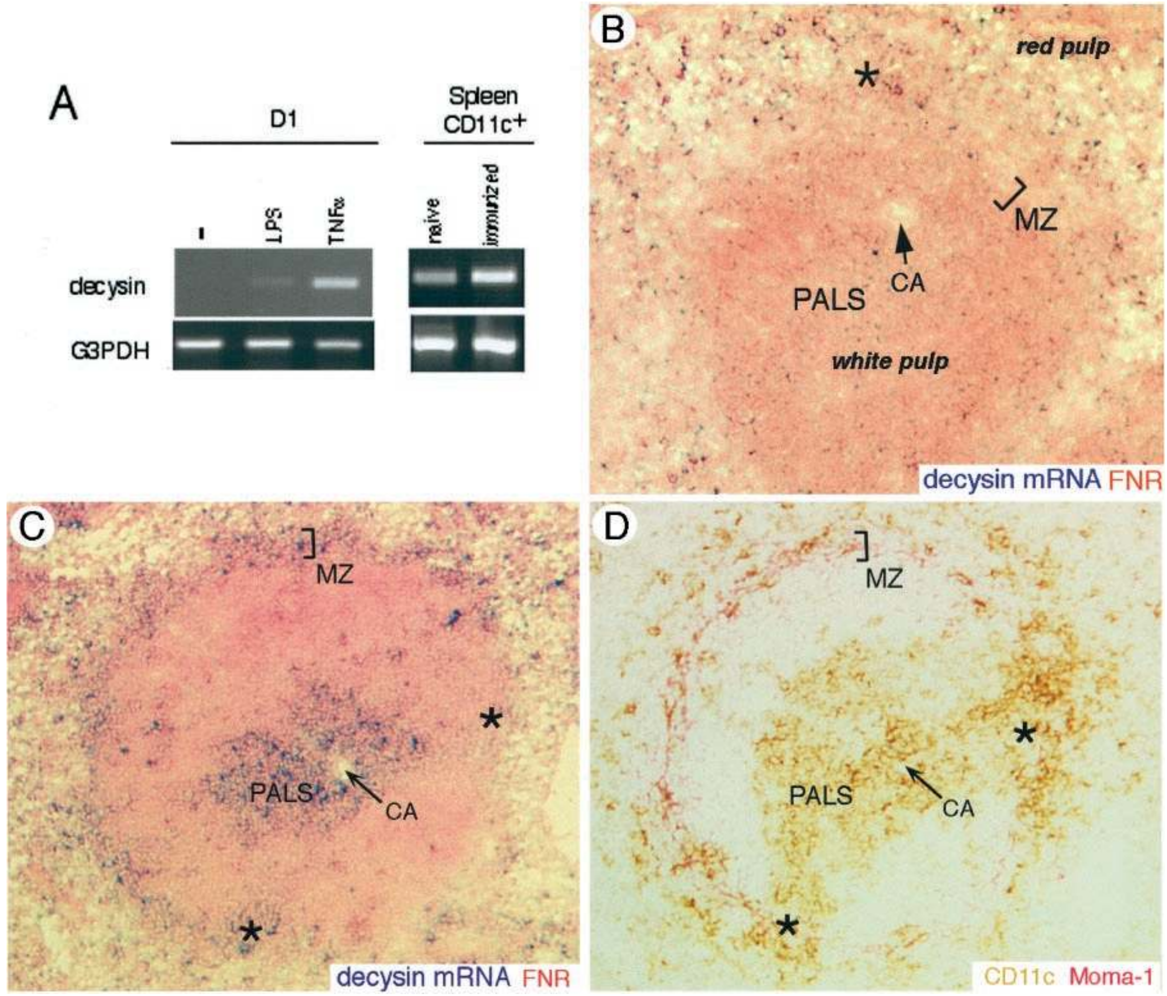

FIGURE 3. Decysin is expressed in CD11c$^+$ DC in response to immunization. *A*, RT-PCR with decysin- and G3PDH-specific primers on untreated, LPS-, or TNF-α-activated D1 cells, as well as CD11c$^+$ DC, purified from spleens of naive and immunized BALB/c mice. *B* and *C*, In situ hybridization with digoxigenin-labeled antisense mouse decysin of a BALB/c spleen section taken from a naive mouse (*B*) and 1 day after immunization with SRBC (*C*). The signal was visualized with NBT/BCIP (blue). The sections were counterstained with Fast Nuclear Red (FNR). *D*, A serial section of the spleen 1 day after immunization was stained for CD11c$^+$ DC (DAB, light brown) and Moma-1$^+$ MZ metallophils (Fast Red). CA, central arteriole; ∗, indicates the position of the MZ bridging channel. Original magnifications: ×80.

days after immunization, decysin$^+$ cells were positioned between the MZ and a developing PNA$^+$ GC (between arrowheads) (Fig. 4*C*). Four days after immunization, the decysin$^+$ cells were no longer seen at the MZ, but were distributed within the GC (arrowheads) (Fig. 4*D*). Taken together, these data suggest that shortly after immunization, MR-L$^+$ MZ metallophils express decysin and home into the developing GC in the course of the immune response.

In the osteopetrotic *op/op* mice, MZ metallophils are missing due to the lack of functional M-CSF (26). To verify that the decysin$^+$ cells in the follicle were derived from MZ metallophils, *op/op* and +/? littermates were immunized with OVA, and spleen sections were stained for decysin and B220 2 days later. While in the +/? littermates decysin$^+$ cells were present in the MZ and the outer follicle (Fig. 4*E*), these cells were absent in *op/op* mice (Fig. 4*F*). The remaining decysin$^+$ cells were FDC (see below). Thus, the decysin$^+$ follicle-homing cells are M-CSF dependent, supporting the notion that they are derived from MZ metallophils.

To further substantiate these findings, we investigated whether in the lymph node decysin is transcribed by the MR-L$^+$ subcapsular sinus macrophages (8, 17). Lymph nodes were taken from BALB/c mice before (Fig. 4*G*) or 2 days after (Fig. 4*H*) secondary immunization with OVA and stained for decysin mRNA and MR-L. Before immunization, the decysin-transcribing cells were almost exclusively localized in the subcapsular sinus area, and the majority expressed MR-L (Fig. 4*G*). Occasionally, some decysin mRNA$^+$ cells were seen in the follicle (arrowheads). Two days after immunization, the subcapsular sinus macrophages translocated from the subcapsular sinus toward the center of the lymph node and continued to transcribe decysin (Fig. 4*H*, arrowheads). Staining of adjacent sections confirmed that the MR-L$^+$ decysin$^+$ had entered the B cell follicle (data not shown). These results extend the observations made in the spleen by showing that also in the draining lymph node, decysin is transcribed by the specialized MR-L$^+$ macrophages that home into the B cell follicle in response to immunization with T cell-dependent Ags.

Decysin is expressed by FDC and tingible body macrophages

Immunized *op/op* mice showed decysin expression in the follicular center (Fig. 4, *E* and *F*). To address the question of whether activated FDC express decysin, spleen sections of BALB/c mice 4–6 days after immunization were analyzed for decysin mRNA and protein expression. In situ hybridization revealed, besides decysin

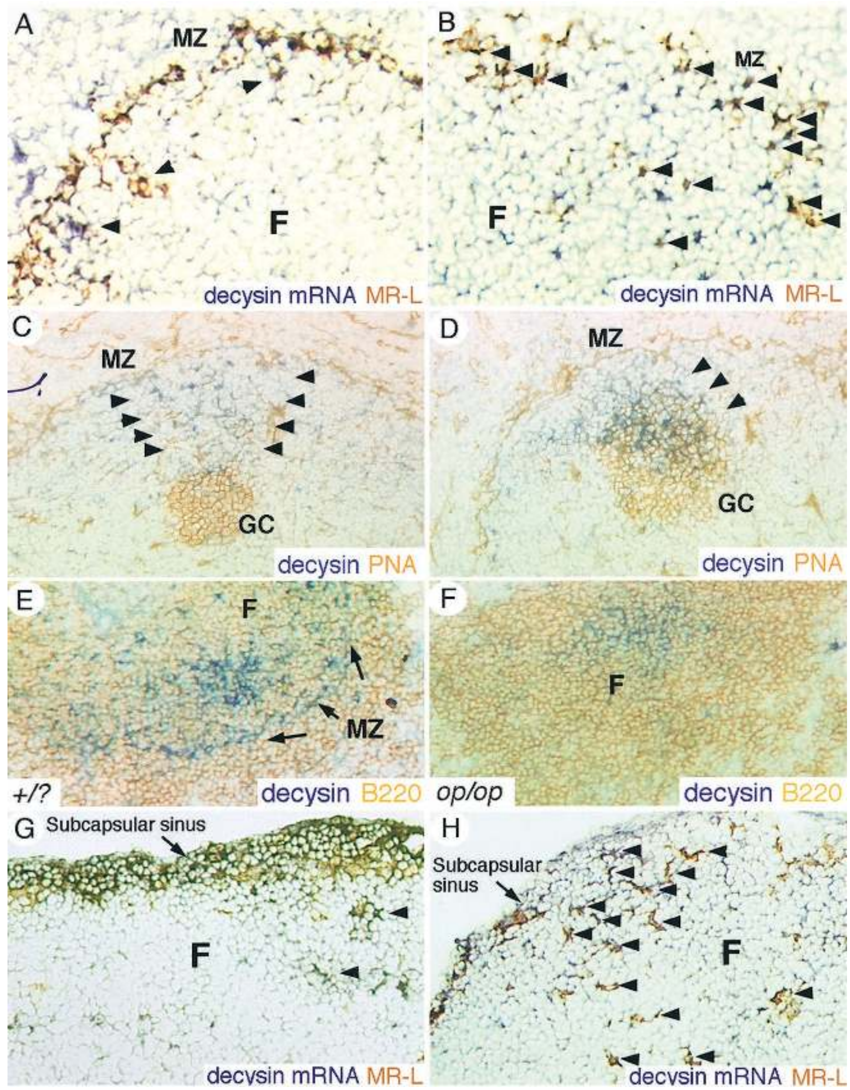

FIGURE 4. Decysin is expressed by MR-L-positive cells in the B cell follicle. *A* and *B*, In situ detection of decysin mRNA (NBT/BCIP) and MR-L (DAB) in BALB/c spleen sections, 1 day (*A*) and 2 days (*B*) after immunization with SRBC. Cells that coexpress decysin mRNA and MR-L in the follicle are indicated by arrowheads. *C* and *D*, Immunohistochemical staining of decysin using the anti-prodomain pAb (Fast Blue) and PNA (DAB) in spleen sections 2 days (*C*) and 4 days (*D*) after immunization. Decysin$^+$ cells are indicated by arrowheads. *E* and *F*, Immunohistochemical staining of decysin using the anti-prodomain pAb (Fast Blue) and B220 (DAB) in spleen sections 2 days after immunization taken from +/? littermates (*E*) and *op/op* mice (*F*). *G* and *H*, In situ detection of decysin mRNA (NBT/BCIP) and MR-L (DAB) in BALB/c popliteal lymph node sections, before (*G*) and 2 days after (*H*) footpad immunization with OVA. Decysin mRNA and MR-L-coexpressing cells in the follicle are indicated by arrowheads. F, follicle. Original magnifications: *A* and *B*, ×150; *C–F*, ×80; *G* and *H*, ×100.

transcription around the central arteriole, decysin mRNA in distinct areas close to the periphery of the white pulp, where GC were expected (Fig. 5*A*). Immunohistochemical staining with the anti-decysin pAb and anti-B220 showed decysin$^+$ cells in the B cell follicle (Fig. 5*B*), and further double-label analysis showed decysin$^+$ cells closely associated with PNA$^+$ GC B cells (Fig. 5*C*). The decysin-expressing cells were tightly associated with GC B cells and formed a network throughout the outer side of the GC, which was reminiscent of FDC. Double-label analysis with the FDC-specific marker FDC-M2 confirmed that decysin was expressed by FDC, creating a black overlay staining in the center of the FDC-M2$^+$ network (Fig. 5*D*).

In some PNA$^+$ GC, decysin mRNA was detected in cells, which did not resemble FDC (arrows, Fig. 5*E*). To test whether these cells were GCDC (19) or tingible body macrophages, GC were double stained for decysin and macrosialin (CD68), a protein expressed by tingible body macrophages, but not GCDC (19). With exception of the FDC network, all decysin$^+$ cells expressed macrosialin (arrows, Fig. 5*F*). Moreover, the double-labeled cells were large cells and appeared to contain a number of apoptotic bodies, not stained by the Abs, which was a typical feature of tingible body macrophages. The tingible body macrophages did not express CD11c (data not shown).

Discussion

Previously, human decysin had been cloned from a cDNA library made from GCDC (22). These DC had been identified in tonsilar GC by virtue of their CD4/CD11c expression (19, 20). In this study, we cloned the murine homologue of decysin (Fig. 1) and showed that, like genuine metalloproteases, it is processed by furin proteases (Fig. 2). However, decysin is distinct from the many other mammalian disintegrin metalloproteases 1) by the replacement of the conserved histidine residue by an aspartate in the catalytic site and 2) by its being secreted.

Human decysin is transcribed by mature DC and in tonsilar GC (22). By the use of in situ mRNA hybridization and immunohistochemistry with a pAb specific for the intracellular prodomain, we have identified the cell types that express decysin during an immune response in the mouse (Table II). In the spleen of nonimmunized mice, decysin is not transcribed in the white pulp or the MZ. However, 1 day after immunization, decysin is clearly transcribed in the PALS (Fig. 3). It had previously been shown that the majority of CD11c$^+$ DC presenting a peptide derived from hen egg lysozyme bound to MHC-II are located in the PALS, whereas DC of the MZ present little peptide (27). This observation suggests that immature DC process Ag at the MZ bridging channels, migrate into the PALS where they mature, and present antigenic peptides

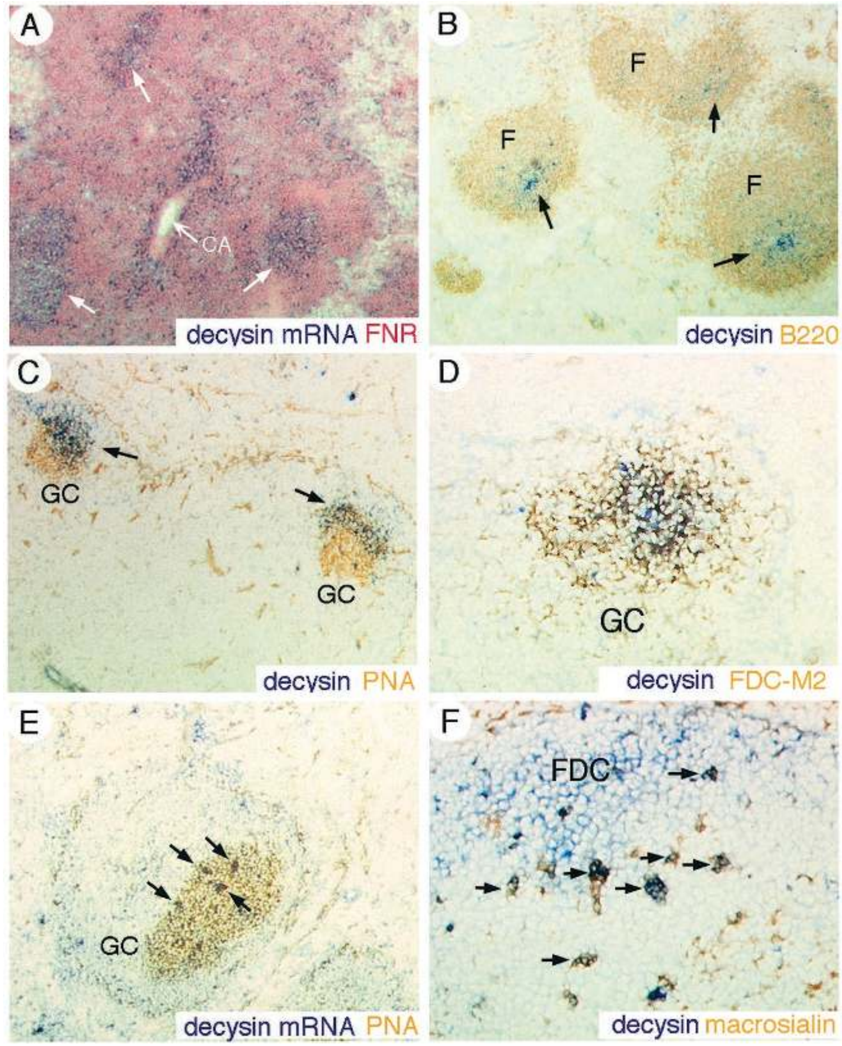

FIGURE 5. Decysin is expressed by FDC and tingible body macrophages. *A*, In situ detection of decysin mRNA (NBT/BCIP) with counter coloration in Fast Nuclear Red (FNR). *B–F*, Immunohistochemical staining for decysin using the anti-prodomain pAb (Fast Blue) and for the indicated Ags (DAB). Spleen sections were from mice 4 days (*A–D*) and 6 days (*E* and *F*) after immunization with SRBC. Arrows point to decysin$^+$ cells. CA, central arteriole; F, follicle. Original magnifications: *A–C* and *E*, ×40; *D*, ×125; *F*, ×150.

to T cells. The decysin transcription pattern correlates with the migration of mature DC from the MZ bridging channels and suggests that decysin is expressed by mature CD11c$^+$ DC. In concordance, the unstimulated DC cell line D1 does not transcribe decysin, but its transcription is up-regulated after maturation with LPS or TNF-α. Maturation of DC is induced by microbial products and by cytokines such as IFN-γ, IL-1, GM-CSF, and TNF-α, which are most likely produced by T cells and macrophages, communicating with the immature DC.

In addition to CD11c$^+$ DC of the PALS in immunized mice, we observed a cell population that expressed decysin in the MZ (Figs. 3 and 4). In the course of the immune response, these MZ decysin$^+$ cells appeared to migrate into the B cell follicle (Fig. 4). The majority of the MZ decysin$^+$ cells carry MR-L, recognized by the CR-Fc probe, which is expressed by MZ metallophils (8, 17, 18). *op/op* mice, which lack functional M-CSF, are devoid of decysin expression in the MZ, which is in accordance with decysin being expressed by the M-CSF-dependent MZ metallophils (Fig. 4) (26). However, as some MZ-derived decysin$^+$ cells lacked CR-Fc binding, we cannot exclude that other cells, such as MZ macrophages, express decysin. On the other hand, it might be that MR-L is lost in the course of the immune response to release mannosylated Ag bound to the MR-L for uptake by B cells or FDC. In the lymph node, decysin is expressed by MR-L$^+$ subcapsular sinus macrophages, which also migrate into the follicle in response to immunization (Fig. 4) (8, 17).

Table II. *Decysin-expressing cells in the mouse spleen or draining lymph node*

Cell Type	Localization Before→After Immunization	Markers
DC in the PALS	MZ bridging channel→PALS	CD11c
MZ metallophils	MZ→follicle	MR-L
Subcapsular macrophages	Lymph node subcapsular sinus→follicle	MR-L
FDC	Follicle/germinal center	FDC-M2
Tingible body macrophages	Follicle/germinal center	CD68

Berney et al. (8) have shown that MR-L$^+$ subcapsular sinus macrophages expressed low levels of CD11c. Upon immunization, the cells migrate toward the outer B cell follicle and associate with B and T lymphocytes. Purified and reinjected into mice, they were able to prime T cells and induce the production of Ag-specific IgM and IgG1 (8). This suggests that lymph node MR-L$^+$ subcapsular sinus macrophages can differentiate into T and B cell stimulatory DC, features previously described for human interdigitating DC and GCDC (20, 28). This raises the interesting possibility that also splenic MR-L$^+$ decysin$^+$ metallophils differentiate into DC in the outer follicle and GC. However, we could not use decysin as a specific marker for these DC, as tingible body macrophages and

FDC also expressed this gene (Fig. 5), thus masking detection of potential murine GCDC.

It has recently been observed in TNF-α- and p55TNF-R-deficient mice that the FDC-M2 mAb stained a cell population in the MZ, and that it is capable of binding immune complexes (29). The authors proposed the existence of a MZ-derived FDC population. It is possible that the decysin$^+$ MZ-derived cells may be identical to these putative FDC precursors.

It has been suggested that cells close to the subcapsular sinus are involved in the transport of immune complexes into the developing GC (16). The cells are likely to be nonphagocytic, as they carry immune complexes on the cell surface. It is probable that these Ag-transporting cells are derived from the MR-L$^+$ decysin$^+$ subcapsular sinus macrophages, as they home into the follicle in response to immunization (Fig. 4) (8, 17). MZ metallophils are poorly phagocytic and are ideally positioned at the MZ-follicle interface to transfer antigenic material from the marginal sinus into the follicles (3). The obervation that MR-L$^+$ MZ metallophils express decysin and migrate into the follicle in response to immunization raises the possibility that MZ metallophils may transport Ag into the GC. Whether the cells then differentiate into DC or FDC-like cells remains to be determined. As mice commonly contain natural Abs that recognize hemagglutinins, the immunogen (SRBC) is likely to be transported in the form of immune complexes bound to Fc or complement receptors. It would be interesting to investigate the role of MZ metallophils in Ag transport using *op/op* mice.

In addition to DC and MZ metallophils, we identified two further cell types that expressed decysin in response to immunization: FDC and tingible body macrophages (Fig. 5). As decysin is not expressed by FDC in naive mice, and few genes are known to be expressed by murine FDC, decysin may represent a useful marker for activated mouse FDC. Our inability to detect mouse GCDC using decysin as a probe could be due to their masking by the FDC network and tingible body macrophages. It may also be that GCDC are restricted to human GC, which are much larger and may require DC to stimulate T and B lymphocytes in GC.

The anti-decysin pAb specific for the prodomain recognizes decysin expressed in the MZ-derived cells, FDC, and tingible body macrophages, but not in DC of the PALS (data not shown). We have verified the specificity of the anti-decysin pAb in mice deficient for decysin, in which the Ab does not produce any cell staining (data not shown). As the prodomain may be rapidly degraded after furin-dependent processing of the zymogen, it is possible that the anti-prodomain Ab only recognizes unprocessed decysin. This may imply that DC process decysin more effectively and/or degrade the prodomain more rapidly than other cells, a likely feature of DC, given their efficient endoprotease and exoprotease machinery involved in Ag processing and presentation. Whether decysin itself plays a role in Ag processing is not known. Alike other members of its family (30), it is probable that decysin forms heterodimers with other disintegrin metalloproteins. This would enable it to exert different functions, specific for the different cell types that express decysin. For example, the disintegrin metalloprotease TNF-α-converting enzyme (ADAM 17) not only cleaves TNF-α, but also TGF-α, and may be involved in the processing of L-selectin and amyloprotein precursor (31–33). Interestingly, disintegrin metalloproteases have been shown to cleave complement component C3 (34), which plays a key role in GC formation (35–37). An attractive hypothesis would be that after secretion into the GC environment, decysin cleaves complement component C3 and thus terminates GC reaction.

Despite the key role that GC play in shaping the humoral immune response, exemplified by the susceptibility of hyper IgM patients to infectious diseases (38), GC formation still remains far from being fully understood. Efforts aimed at defining the molecules that regulate cellular interactions in the GC reaction should eventually provide us with a molecular framework necessary to understand the complex cellular cross-talk involved in the formation of the secondary B cell response.

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