Characterization and Cloning of a Novel Glycoprotein Expressed by Stromal Cells in T-dependent Areas of Peripheral Lymphoid Tissues

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Summary

A novel glycoprotein (gp) expressed by stromal cells of peripheral lymphoid tissue has been characterized immunohistochemically, biochemically, and at the molecular level. This molecule, gp38, was identified with a monoclonal antibody (mAb) (clone 8.1.1) previously shown to react with a subpopulation of thymic epithelium. This mAb generated a reticular labeling pattern in medullary and paracortical areas of lymph nodes and in splenic white pulp. At the ultrastructural level, labeling by the 8.1.1 mAb was restricted to fibroblastic reticular stromal cells. Serial sections of lymph node and spleen labeled with anti-CD3, anti-B220, and 8.1.1 mAbs clearly showed that the 8.1.1$^+$ cells were associated with T cell–dependent areas. In severe combined immunodeficiency (SCID) or Nu/Nu mice, splenic white pulp also exhibited reticular labeling with the 8.1.1 mAb in the absence of detectable numbers of T cells, indicating that the appearance of 8.1.1-reactive stromal cells in discrete areas of peripheral lymphoid tissue was T cell independent. The cDNA encoding this stromal cell molecule was cloned by direct expression in COS cells and found to encode a 172 amino acid sequence with the typical features of a type I integral membrane protein. COS cells transfected with the gp38 clone direct the expression of an approximately 38-kD protein that reacts with the 8.1.1 mAb but not with isotype-matched controls. Comparison of the predicted amino acid sequence of 8.1.1 mAb but not with isotype-matched controls. Comparison of the predicted amino acid sequence of 8.1.1 with proteins in the National Biomedical Research Foundation (NBRF) data base showed that gp38 is very closely related to the early response protein OTS-8 obtained from a cDNA library of tumor promoting agent (TPA)-induced murine osteoblastic cell line, MC3T3-E1.

Peripheral lymphoid tissues are very compartmentalized with respect to the distribution of different lymphocyte populations (1, 2). The basis for this compartmentalization of lymphocyte populations within peripheral lymphoid tissue is poorly understood. Observations that different populations of nonlymphoid cells also exhibit differential localization within lymphoid tissues has led to the notion that the observed compartmentalization of lymphocyte subpopulations may be dependent on lymphocyte interactions with different populations of nonlymphoid cells located within distinct anatomic compartments of lymphoid tissue (3, 4). At least four major types of nonlymphoid stromal cells have been identified within peripheral lymphoid tissue on the basis of distinctive morphologic and phenotypic criteria: follicular dendritic reticular cells (5, 6), cells of the mononuclear phagocyte lineage (7, 8), interdigitating or dendritic cells (9–11), and extrafollicular reticular cells (11–13).

In this report, we describe the cloning and characterization of a cell surface glycoprotein, gp38, that is preferentially expressed by stromal cells in T cell–dependent areas of peripheral lymphoid tissue and has been previously shown to be expressed by a subset of thymic epithelial cells as well (14).

Materials and Methods

Mice and Hamsters. Male and female BALB/c mice were purchased from Bantin and Kingman, Inc. (Fremont, CA) and maintained in the Department of Biological Structure vivarium. For timed pregnancies, the appearance of a vaginal plug was considered to be day 0 of gestation. Adult tissue was obtained from 4–8-wk-old mice. SCID mice (15) were generously provided by Dr. F. Smith (Fred Hutchinson Cancer Research Center, Seattle, WA). All procedures involving animals followed institutional guidelines established by the Department of Comparative Medicine at the University of Washington.

Reagents and Cell Lines. The following mAbs were used in the form of culture medium from exhaustively grown hybridomas, or purified and conjugated with biotin or horseradish peroxidase as previously described (16): anti-rat κ chain, clone RG7/9.1 (17); anti-IA$^{d/b}$, clone 34.5.3 (18); anti-B220, clone 14.8 (19); anti-CD3, clone 500A2 (20); and 8.1.1, a hamster mAb reacting with subcapsular and medullary thymic epithelium, as well as epithelial and mesenchymal cells of multiple tissues (14). Biotinylated and peroxidase-labeled goat anti–hamster IgG antibodies were purchased from Caltag Laboratories (South San Francisco, CA). A hamster mAb, clone F531 (kindly provided by Dr. J. Allison, University of California, Berkeley) was used as a negative control in these studies. Goat anti–hamster antibodies were purchased from Caltag Laboratories and iodinated using iodogen (Pierce Chemical Co., Rockford, IL). Na^{125}I was purchased from New England Nuclear (Boston, MA). Except where noted, other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

The cell lines utilized in this study included the thymic lymphoma, EL-4 (21), the keratinocyte cell line, PAM-212 (22), the medullary thymic epithelial cell line, TE-71 (23), and a panel of thymic stromal cells derived from transgenic mice bearing the SV40 large T antigen driven by the *lck* promoter (24).

Immunohistochemistry and Biochemistry. The techniques used for light and electron microscopic immunohistochemistry have been described in detail (14). For immunoblot analysis of 8.1.1 reactivity, neonatal thymuses or pooled axillary and mesenteric lymph nodes from 6–10-wk-old mice were minced with a razor blade to generate tissue fragments. After washing repeatedly with HBSS using unit gravity sedimentation to remove lymphocytes, stroma fragments were processed for immunoblot analysis as described previously (14).

Cloning and Nucleic Acid Analyses. Protocols for the preparation of a TE 71cDNA library and the expression cloning system utilized have been previously described (25, 26).

Probing of mRNA and genomic DNA prepared from cell lines and tissue utilized standard methods. A single-stranded probe complimentary to gp38 transcript was made from single-stranded CDM8 vector containing gp38 cDNA. By using a primer 5' of the gp38 insert and limiting amounts of dCTP, only the CDM8 vector was labeled (27). For Southern and Northern blot hybridization of

tissues, the gp38 insert was excised from the CDM8 vector by an XbaI digest and purified from a low melting temperature agarose gel using Gene Clean (Bio 101, Inc., La Jolla, CA). The probe was then labeled via random primer extension. To confirm equal loading of RNA, a probe was made from rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (28) via random primer extension (Boehringer Mannheim Corp., Indianapolis, IN).

Results and Discussion

Immunohistology of Peripheral Lymphoid Tissue. In a previous study we have identified a 36–40 kD glycoprotein expressed by medullary thymic epithelial cells with the mAb 8.1.1 (14). Here we have shown this molecule is also expressed by stromal cells in T cell–dependent areas of peripheral lymphoid tissues. The staining pattern of the 8.1.1 mAb in lymph nodes was reticular within discrete areas. Follicular areas of the lymph node contained only a few scattered 8.1.1$^+$ cells, whereas the paracortical areas and medullary cords were well decorated with peroxidase reaction product (Fig. 1 *a*).

In the spleen, the antigen detected by the 8.1.1 mAb was preferentially associated with the splenic white pulp, with the most intense labeling associated with the periarteriolar lymphatic sheath (PALS) and adjacent reticular cells (Fig. 1 *b*). Although this mAb may react with cells in the splenic red pulp, the intensity of labeling in red pulp resembled background staining observed with an irrelevant hamster primary antibody or the anti–hamster Ig secondary antibody alone (Fig. 1 *c*). Mesothelium covering the capsule of the spleen was also labeled (data not shown).

The association of 8.1.1$^+$ stromal cells with T cell–dependent areas of the lymph node was clearly revealed when T and B cell–dependent areas of spleen and lymph node were identified in frozen sections serial to sections labeled with 8.1.1 mAbs, using anti-CD3 and anti-B220 mAbs, respectively (Fig. 2). As shown in Fig. 2 *a*, and in agreement with
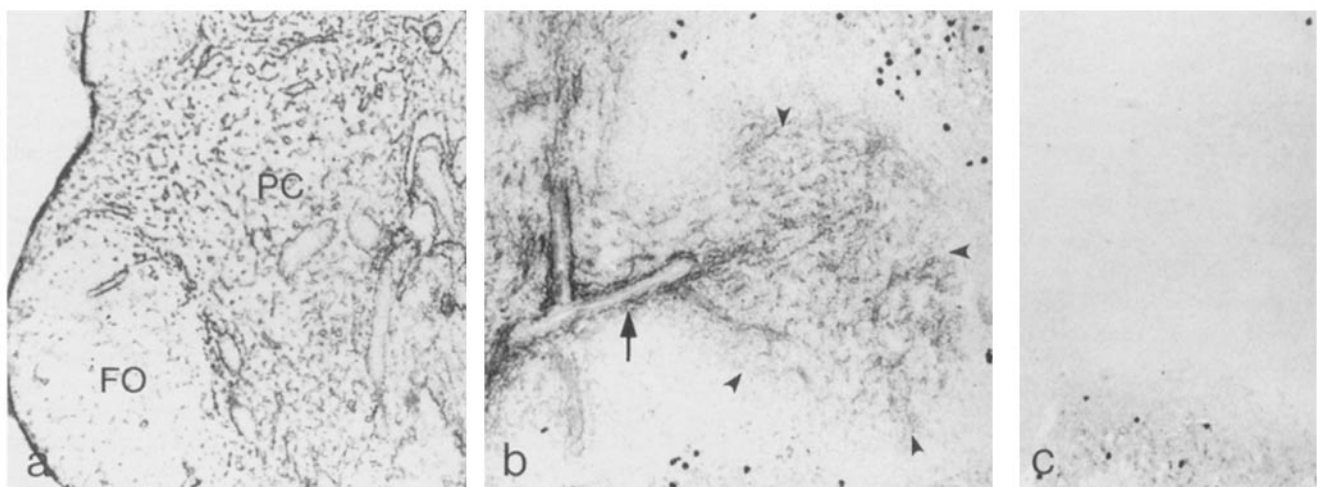

Figure 1. Reactivity of the 8.1.1 mAb with peripheral lymphoid tissue. (*a*) Lymph node cortex and paracortex. (*FO*) follicle; (*PC*) paracortex. (*b*) White pulp of spleen. (*Arrow*) Obliquely sectioned central artery. (*Arrowheads*) Perimeter of 8.1.1 labeling in white pulp. (*c*) Background labeling of spleen with irrelevant hamster Ig followed by goat anti–hamster IgG antibodies. Note the background reaction product in red pulp area at bottom of the field. ×60–70.
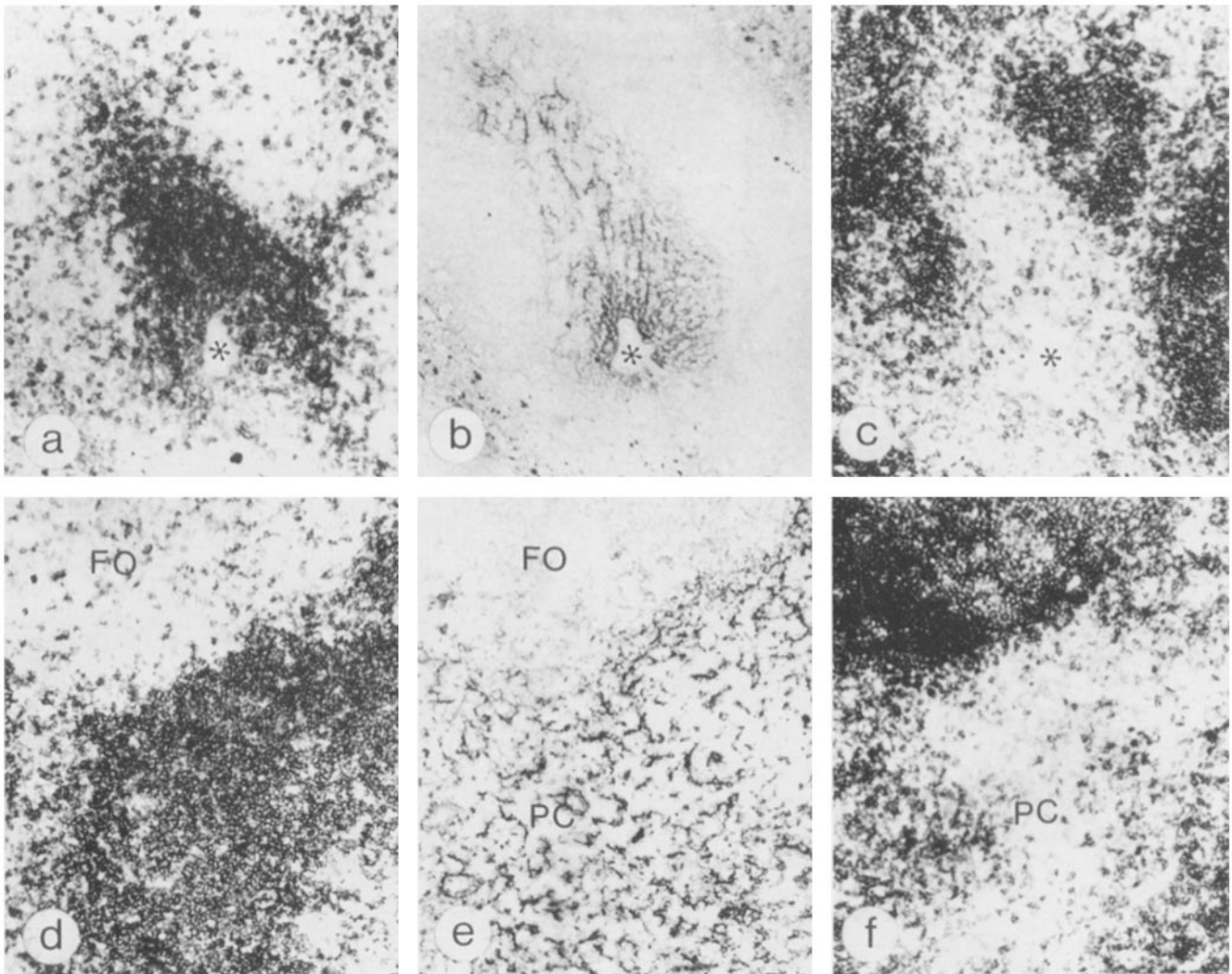
Figure 2. Association of 8.1.1⁺ stromal cells with T cell–dependent areas of lymph node and spleen. In *a–c*, adjacent serial sections of spleen were labeled with anti-CD3, 8.1.1 mAb, and anti-B220, respectively. The central artery (*) serves as a point of reference in the three sections. In *d–f*, adjacent serial sections of lymph node were labeled with anti-CD3, 8.1.1 mAb, and anti-B220, respectively. (*FO*) Follicle; (*PC*) paracortex. ×80.

previous descriptions of splenic white pulp (29), T cells were concentrated in a perivascular location adjacent to the central artery of the white pulp. The reactivity of the 8.1.1 mAb with the adjacent serial section (Fig. 2 *b*) revealed a reticular

organization of stromal cells which corresponded well to the T cell–dependent area of the white pulp. Areas of the white pulp largely devoid of 8.1.1 stromal cells corresponded to B cell–dependent areas, as indicated by reactivity of adjacent
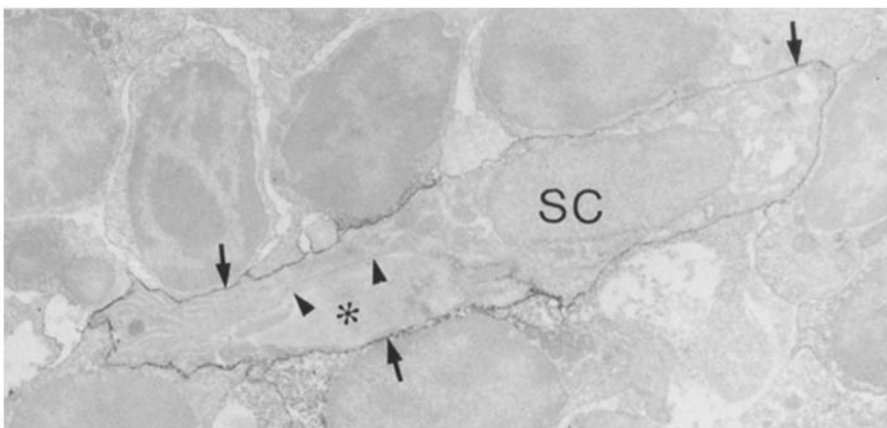

Figure 3. Ultrastructure of 8.1.1⁺ cell in lymph node paracortex. Stromal cell surface in contact with lymphoid cells bears reaction product (*arrows*). (*Arrowheads*) Stromal cell membrane investing a reticular fiber bundle (*) is not labeled. (*SC*) Stromal cell. ×4,500.
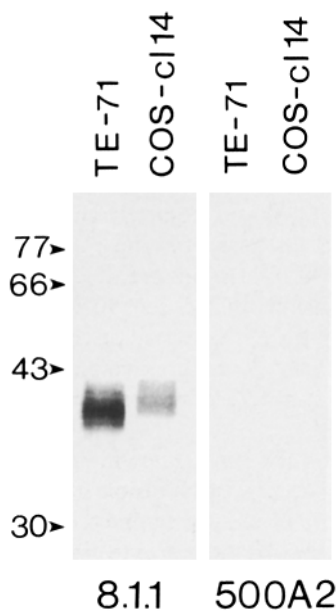
Figure 4. gp38 cDNA encodes a protein detected by 8.1.1 mAb. Immunoblot comparison of TE-71 with COS cells transfected with gp38 cDNA (clone 14). Samples were probed with 8.1.1 mAb and an irrelevant mAb (*500A2*). Molecular weights of standard proteins are shown in kD.

serial sections with an anti-B cell reagent antibody (Fig. 2 *c*). Similar studies of lymph node tissue (Fig. 2, *d–f*) demonstrated a relative paucity of 8.1.1$^+$ stromal cells in the B cell–dependent cortical follicles. Reactivity of lymph node or splenic white pulp with peroxidase labeled goat anti–hamster or goat anti–rat IgG was minimal and resembled the endogenous peroxidase controls (data not shown).

At the ultrastructural level, labeling by the 8.1.1 antibody was restricted to reticular stromal cells in both spleen and lymph node (Fig. 3). No reaction product was detected in association with lymphoid cells, macrophages, or dendritic cells. Occasional perivascular stromal cells exhibited polarized reactivity with the 8.1.1 antibody and were devoid of reaction product at areas in contact with the basal lamina (data not shown). The stromal cell nature of the labeled cells was reflected by their investment around bundles of reticular fibers. As with the perivascular reticular cells, the segments of the stromal cell membranes investing the extracellular matrix of the reticular fibers were not labeled with the 8.1.1 antibody.

In terms of other mAbs reacting with extrafollicular stromal cells of peripheral lymphoid tissue, the localization pattern observed with 8.1.1 is more restricted than reported for ER-TR7 (12) or for ED11–15 (13) mAbs. The 8.1.1 labeling patterns in peripheral lymphoid tissue, thymus (14), and bone marrow (data not shown) closely resemble those described in the rat by Van den Berg et al. (13) for the ED10 mAb.

The presence of an 8.1.1$^+$ stromal cell population in periarteriolar areas of splenic white pulp from SCID or Nu/Nu mice (data not shown) indicate that the development of this stroma cell population occurs independently of T cells. Our results also indicate that expression of gp38 by this population of stromal cells is not dependent on interactions with T cells and/or their products. Consistent with this latter observation, we have observed little if any effect of cytokines (IL-1, TNF-α, IFN-γ, or IL-4) on the reactivity of thymic stromal cell lines with 8.1.1 antibody in vitro (data not shown).

Molecular Characterization of gp38. Cloning of the gp38 revealed a 172-amino acid-long type I membrane protein with a putative 20-amino acid-long amino terminal secretory signal sequence, followed by an extracellular domain rich in both Ser and Thr residues and containing one potential N-linked glycosylation site, a hydrophobic transmembrane domain, and a short cytoplasmic domain (EMBL accession no. M96645). Although the predicted size of the protein encoded by the gp38 cDNA is 16 kD, transfection of this cDNA into COS cells directs the expression of a 40 kD protein which is recognized by the 8.1.1 mAb (Fig. 4). The large discrepancy between the M_r and the predicted molecular mass of 16 kD might be due to the presence of N-linked and/or O-linked carbohydrates on the mature protein. The broad banding pattern exhibited by this molecule in immunoprecipitation studies with TE-71 cells and COS cell transfectants and immunoblot analysis of lymph node stroma (data not shown) suggests that gp38 is variably glycosylated. The presence of multiple Ser and Thr residues in the extracellular domain of gp38 and our previous observations that gp38 binds wheat germ lectin and that N-glyconase treatment failed to alter the M_r of gp38 (14) would be consistent with the notion that gp38 bears O-linked carbohydrates. The apparent discrepancy in size may also reflect other posttranslational modifications, such as myristylation, sulfation, or phosphorylation (30–32). Similar discrepancies in size between the M_r of deglycosylated proteins and their predicted M_r have been reported for N-CAM and MRC OX-2 (33, 34).

The gp38 probe detected a single species of RNA with a molecular size of 2 kb in RNA samples isolated from murine fibroblast 3T3 cells and several thymic stromal cell lines (186CC, Z172, Z199, Z210, Z210R.1.7, and TE-71), but not with poly(A) RNA isolated from a T cell lymphoma or keratinocyte cell line. A similar sized RNA species from thymus, spleen, lymph node, and skin also hybridized with the gp38 probe (data not shown). Blot hybridization analysis of genomic DNA isolated from the murine cell lines TE-71 and PAM 212 showed identical patterns after digestion with BamHI, EcoRI, HindIII, KpnI, PvuII, PstI, ScaI, or XbaI restriction enzymes and binding to a radiolabeled gp38-specific probe (data not shown).

Although RNA blot analysis indicates that the size of the gp38 cDNA described here is significantly smaller than the mRNA found in cell lines known to express gp38 (data not shown), the finding that this cDNA directs the expression of an 8.1.1-reactive molecule with the same molecular weight as that obtained from cell lines that express gp38 indicates that this cDNA contains all or almost all of the gp38 coding sequence. Further work is needed to isolate a full-length copy of the gp38 transcript and verify that the cDNA described here does contain the complete gp38 coding sequence. DNA blot analysis with genomic DNA isolated from 8.1.1$^+$ and 8.1.1$^-$ cell lines suggest that gross genomic rearrangements are not involved in controlling gp38 expression (data not shown).

Comparison of the predicted amino acid sequence of gp38 with those in the National Biomedical Research Foundation

(NBRF) data base using the FASTP algorithm contained in the University of Wisconsin Genetic Computing Group (GCG) software (35) package showed that gp38 was closely related to the TPA-inducible gene, OTS-8 isolated from MC3T3-E1 cells, a murine osteoblastic cell line (36). Five differences in the nucleotide sequences were observed in the 5' end of the gp38 coding sequence. These occur at nucleotide positions 160, 164, 167, 189, and 191 (all nucleotide numbering here is based on the gp38 cDNA). In each case, the gp38 cDNA contains adenine nucleotides in these positions, whereas the OTS-8 cDNA contains guanine nucleotides in these positions. These differences resulted in changes in the predicted amino acid sequence from K, N, N, E, and N in gp38 to E, D, D, G, and D in OTS-8 for residues 9, 10, 11, 18, and 19, respectively (all amino acid numbering is based on the mature gp38 protein). One additional difference was found at the 3' end of the gp38 coding sequence which contained a thymine nucleotide at position 585 absent in the OTS-8 cDNA (nucleotide numbering is based on the gp38

cDNA). This results in a change of frame that alters amino acids 151-153 and also moves the stop codon position, such that the cytoplasmic domain of the OTS-8 protein was 33 residues longer than that of gp38 (amino acid numbering is based on the mature gp38 protein).

Although it was predicted that the OTS-8 gene product would be a secreted protein (36), flow cytometric analyses of 8.1.1 reactivity with stromal cell lines (14, and data not shown) and the fact that the gp38 cDNA directs the cell surface expression of gp38 by transfected COS cells (data not shown) clearly demonstrate that gp38 is expressed on the cell surface. It remains to be determined how these two proteins are functionally related and if they represent members of a new gene family.

At this time the function of gp38 is not known. We are presently constructing gp38-Ig recombinant fusion protein to identify possible ligands for gp38 and to examine the possible involvement of this molecule in T lymphocyte differentiation and/or functional organization of lymphoid tissue.

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