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A New Triggering Receptor Expressed on Myeloid Cells (Trem) Family Member, Trem-Like 4, Binds to Dead Cells and Is a DNAX Activation Protein 12-Linked Marker for Subsets of Mouse Macrophages and Dendritic Cells[1]

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Dendritic cells (DCs) are professional APCs that can control immune responses against self and altered self, typically foreign, determinants. DCs can be divided into several subsets, including CD8α^+ and CD8α^- DCs. These subsets possess specific functions. For example, mouse splenic CD8α^+, but not CD8α^- DCs selectively take up dying cells and cross-present cell-associated Ags to naive T cells. In this study, we identified genes that were more expressed in CD8α^+ than CD8α^- DCs by microarray analysis. Only one of these genes, when the extracellular domains were linked to human IgG Fc domain, could bind to late apoptotic or necrotic cells. This gene was a new member of the triggering receptor expressed on myeloid cells (Trem) family, Trem-like 4 (Treml4). Treml4 mRNA and protein, the latter detected with a new mAb, were predominantly expressed in spleen. Treml4, like other Trem family members, could associate with the adaptor molecule DNAX activation protein 12 kDa, but neither DNAX activation protein 10 kDa nor FcRγ. Consistent with the microarray data, we confirmed that Treml4 protein was more expressed on CD8α^+ than CD8α^- DCs, and we also found that Treml4 was expressed at high levels on splenic macrophages in spleen, particularly red pulp and marginal metallophilic macrophages. In addition, Treml4 expression on DCs was not changed after maturation induced by TLR ligands. Thus, Treml4 is a new Trem family molecule that is abundantly expressed on CD8α^+ DCs and subsets of splenic resident macrophages, and can recognize dead cells by different types of phagocytes in spleen. *The Journal of Immunology,* **2009, 182: 1278–1286.**

Dendritic cells (DCs)[3] consist of several subsets that are classified according to their expression of select cell surface molecules and distinct functions. In mouse spleen, for example, there are CD8α^+ DEC-205$^+$ CD11c$^+$ and CD8α^- DCIR2$^+$ CD11c$^+$ classical DCs, and B220$^+$ CD11c^{dull} plasmacytoid DCs. CD8α^+ spleen DCs are the predominant producers of IL-12 upon stimulation with *Toxoplasma gondii* (1, 2), and selectively engulf different types of dying cells, including allogeneic cells killed by NK cells, irradiated tumor cells, and virus-infected cells (3, 4). Furthermore, CD8α^+ DCs are superior cells for the cross-presentation of Ags on MHC class I molecules (5–7). Molecular mechanisms for these distinct functions are starting to be identified, e.g., CD8α^+ DCs express higher levels of mRNA transcripts and proteins involved in the MHC class I processing pathway (7).

Apoptotic cells are generated continually in mammals and other species, and cell death is increased further during embryo development, tissue remodeling, and inflammation. Potentially harmful cells that are eliminated by apoptosis include self-reactive T and B cells, tumor cells, and cells infected with viruses and some bacteria. Engulfment of apoptotic cells in some instances suppresses production of inflammatory cytokines from activated macrophages (8). When dying cells are engulfed by DCs in the steady state in the absence of infection or immunological adjuvants, Ag-specific tolerance to Ags in the dying cells can be induced (3). In contrast, if mice are given dying cells with adjuvants, Ag-specific T cell responses develop (3, 9). Analyses of genetically modified mice have shown that increased accumulation of apoptotic cells induces the development of autoimmune diseases (10–12). Thus, accumulating evidence indicates that the uptake of apoptotic cells is not only a clearance mechanism, but is also capable of suppressing inflammation and modulating immune responses from Ag-specific tolerance to resistance (13).

In vitro studies indicate that many receptors can be involved in the uptake of dying cells by macrophages, including scavenger receptors, the phosphatidylserine receptor, integrins, CD14, C1q, C1qR, and CD36 (14–21). Recently, the T cell Ig and mucin domain-containing molecules, Tim-1 and Tim-4,

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Received for publication August 19, 2008. Accepted for publication November 24, 2008.

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[1] This work was supported by National Institutes of Health Grant AI13013 (to R.M.S.) and in part by the Program for Improvement of Research Environment for Young Researchers from Special Coordination Funds for Promoting Science and Technology commissioned by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Grant-in-Aid for Young Scientists (19890063 and 20790299, to H.H.) from Ministry of Education, Culture, Sports, Science, and Technology of Japan. H.H. was a Japan Society for the Promotion of Science Postdoctoral Fellow for Research Abroad.

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[3] Abbreviations used in this paper: DC, dendritic cell; DAP10, DNAX activation protein 10 kDa; DAP12, DNAX activation protein 12 kDa; Flt3L, Fms-like tyrosine kinase 3 ligand; HEK, human embryonic kidney; Mertk, *Mer* tyrosine kinase; PI, propidium iodide; poly(I:C), polyinosine-polycytidylic acid; Trem, triggering receptor expressed on myeloid cells; Treml4, Trem-like 4; SIGN-R1, specific intracellular adhesion molecule-grabbing nonintegrin receptor 1.

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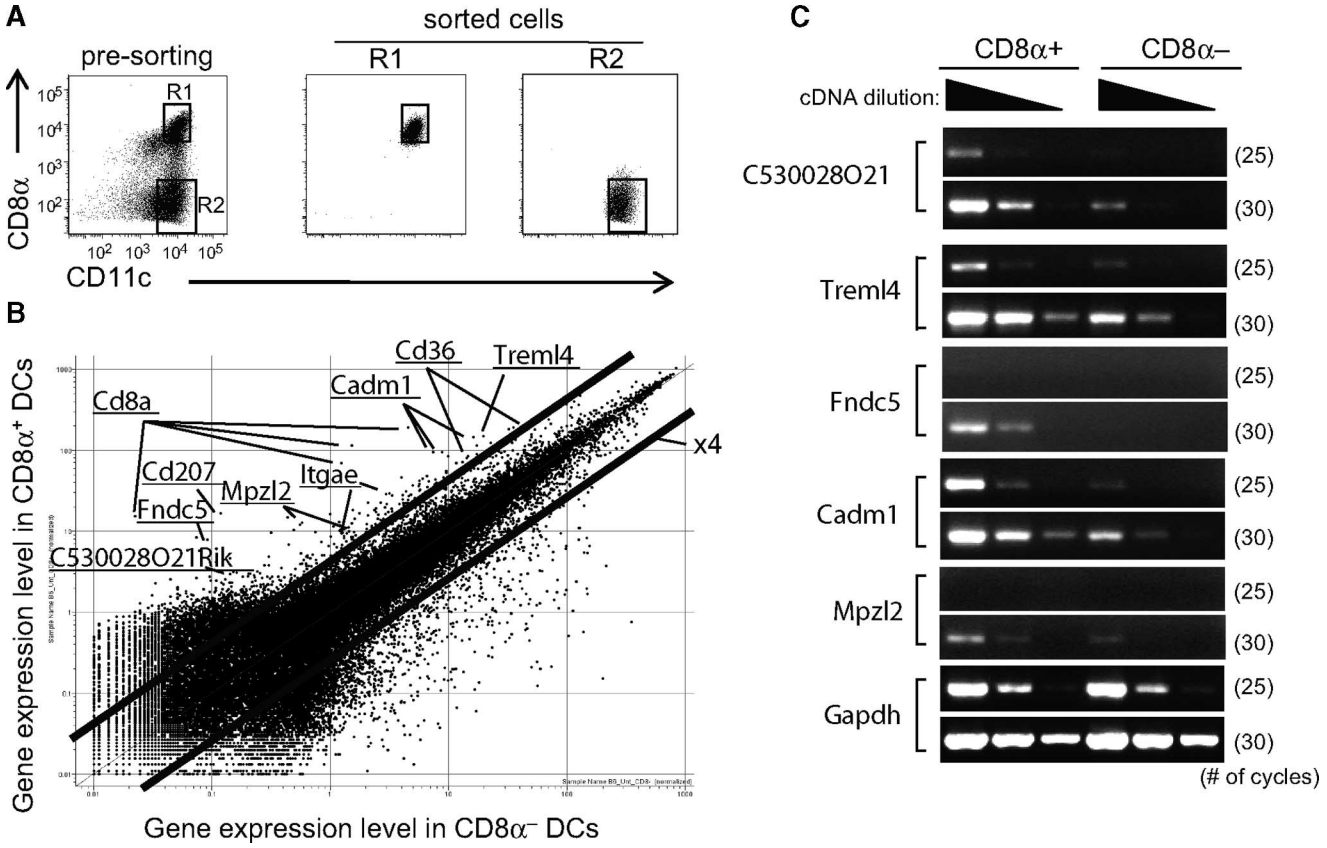

FIGURE 1. Microarray analysis of two Flt3L-expanded DC subsets from mouse spleen. *A*, In vivo expanded mouse splenic DCs (*Materials and Methods*) were sorted from CD3ε^- B220$^-$ CD11c$^+$ fractions (*left panel*). The purities of CD8α^+ and CD8α^- DCs were 94 and 91%, respectively (*middle* and *right panels*). *B*, DNA microarray analysis was performed using RNAs from CD8α^+ and CD8α^- DCs. Names of representative genes are indicated. Cadm1, cell adhesion molecule1; Itgae, integrin α_E; Mpzl2, myelin protein zero-like 2; Fndc5, fibronectin type III domain containing 5. *C*, A semiquantitative RT-RCR was conducted in two DC subsets. The numbers in parentheses indicate the number of PCR cycles.

were shown to recognize phosphatidylserine and mediate apoptotic cell uptake (22–24). Recent in vivo studies have shown that the *Mer* tyrosine kinases (Mertk) and milk fat globule epidermal growth factor 8 contribute to the engulfment of apoptotic cells by macrophages (11, 12). Sen et al. (25) also reported that Mertk mediates suppression of NF-κB activation in DCs that take up dying cells. Mertk also suppresses the maturation and cytokine production by DCs taking up apoptotic cells, and mediates induction of immunological tolerance (25, 26). CD36 and DEC-205 (CD205) are two receptors that are expressed more abundantly on CD8α^+ than CD8α^- DC subset in mouse spleen, but each of these molecules is dispensable for uptake of dying cells by CD8α^+ DC in vivo (3, 27, 28).

To identify gene(s) involved in dying cell uptake and signaling by DCs, we compared gene expression by DNA microarrays from mouse splenic CD8α^+ and CD8α^- DCs, where the former are specialized to clear apoptotic cells. Several transmembrane genes were more expressed by CD8α^+ DCs, but the soluble form of only one of them, the triggering receptor expressed on myeloid cells (Trem)-like 4 (Treml4), exhibited binding to dead cells. We will describe these findings as well as the capacity of Treml4 to associate with an adaptor molecule DNAX activation protein 12 kDa (DAP12) and to serve as a marker for select groups of both DCs and macrophages. Surprisingly, macrophages in spleen, but not several other organs, expressed Treml4. Thus, we have identified a new type of molecule with the potential to recognize dead cells and signal different types of splenic phagocytes.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories, Taconic Farms, or CLEA Japan. All mice were maintained under specific pathogen-free conditions and used at 6–12 wk of age under institutional guidelines of the Rockefeller University and Tokyo Medical and Dental University.

Cell lines

B16.Flt3L cells, which produce mouse Fms-like tyrosine kinase 3 ligand (Flt3L), were established via retroviral gene transfer to B16 melanoma cell line by Dranoff et al. (29) and provided by L. Santambrogio (Albert Einstein College of Medicine, New York, NY). A hybridoma clone, 2.4G2, was from American Type Culture Collection. Human embryonic kidney (HEK) 293A and 293T cells were cultured in DMEM supplemented with 10% FBS and 10 mM HEPES.

Abs and reagents

Purified anti-CD16/32 (clone 2.4G2) or FITC-, PE-, allophycocyanin-, or biotin-conjugated anti-CD3ε (145-2C11), anti-B220 (RA3-6B2), anti-CD11c (HL3), and anti-CD8α (53-6.7) were from BD Biosciences. Anti-CD11c beads (N418) and anti-PE beads were from Miltenyi Biotec. FITC-conjugated anti-human IgG (H + L) was from Jackson ImmunoResearch Laboratories. Purified anti-Treml4 mAb was biotinylated with EZ-link NHS-biotin reagent (Pierce), according to manufacturer's instructions. LPS (O55:B5) was from Sigma-Aldrich. Polyinosine-polycytidylic acid (poly(I:C)) was from GE Healthcare or InvivoGen. Phosphorothioate-stabilized CpG deoxyoligonucleotides (CpG DNA, 5′-TCCATGACGTTCCTGATGCT-3′) were purchased from Genelink or Qiagen.

Cell sorting

B16.Flt3L cells were cultured with DMEM containing 10% FBS, and 5×10^6 cells were injected into the backs of mice. After 10–12 days, when the secreted Flt3L had greatly expanded the numbers of all major DC subsets (30), the spleens were collected and cut into small fragments. Single-cell suspensions were prepared by treating with collagenase D (Roche Applied Science). After adding anti-CD16/32 mAb or 2.4G2 hybridoma supernatant to block nonspecific binding of Abs, the cells were stained with FITC-conjugated anti-CD3ε and anti-B220, PE-conjugated anti-CD11c, and allophycocyanin-conjugated anti-CD8α. After washing, CD11c$^+$ DCs were enriched by positive selection using anti-PE magnetic beads and MACS columns (Miltenyi Biotec). CD8α^+CD11c$^+$ or CD8α^-CD11c$^+$ cells were then sorted from the electronically CD3ε- and B220-negative fractions by FACSVantage (BD Biosciences). The purity of each fraction was more than 98%.

Microarray analysis

Total RNA was isolated from the sorted cells by TRIzol (Invitrogen) and subjected to microarray analysis using the mouse genome 430 2.0 array (Affymetrix). The data were analyzed by GeneSpring software (Agilent). Microarray data were deposited in National Center for Biotechnology Information's Gene Expression Omunibus with the accession number GSE13250.

RT-PCR and real-time PCR

Total RNA was isolated from sorted cells or tissues by TRIzol and used to synthesize cDNA with Superscript III (Invitrogen). Semiquantitative PCR was performed using primer pairs indicated in Supplemental Table I.[4] Real-time quantitative PCR was performed with the iCycler iQ real-time PCR detection system (Bio-Rad).

Cloning of Treml4

Mouse Treml4 cDNA was amplified by PCR, cloned into pCR2.1 vector (Invitrogen), and sequenced. The sequence of cloned Treml4 open reading frame was identical with a clone registered on GenBank (accession number BC117091). PCR primers were sense, 5′-GACTGGTATGGCCTGGAG GTACTC-3′ and antisense, 5′-GCCTGTTCTGCCTTAGTACCAGTT-3′.

Production of soluble proteins

To produce soluble forms of several transmembrane proteins, cDNA fragments encoding the extracellular region of each gene were amplified by PCR and cloned into an expression vector containing the exons for hinge, C_H2, and C_H3 region of human IgG1 (31). The fusion proteins were produced by transient transfection of HEK293T cells using calcium phosphate. Cells were cultured in serum-free DMEM supplemented with 1% Nutridoma SP (Roche Applied Science) for 4–5 days. The fusion proteins were purified from culture supernatants using protein G-Sepharose beads (GE Healthcare Bioscience) after ammonium sulfate precipitation.

Preparation of dying or dead cells

Splenocytes or thymocytes were prepared from C57BL/6 mice, irradiated with γ-ray (1500 rad), and cultured for 5 h to induce apoptosis. To prepare necrotic cells, splenocytes or thymocytes were incubated at 56°C for 30 min or 100°C for 10 min. These dying or necrotic cells were incubated with propidium iodide (PI; BD Biosciences) or 7-aminoactinomycin D (BD Biosciences) together with annexin V (BD Biosciences), according to manufacturer's instructions.

Flow cytometry

Dying or dead cells were incubated with anti-CD16/32 Ab to block nonspecific Fc binding, and then incubated with soluble fusion proteins. After washing, binding to dying or necrotic cells was detected by incubating with FITC-conjugated anti-human IgG Ab (Jackson ImmunoResearch Laboratories) and analyzed on a FACSCalibur (BD Biosciences).

To check for expression of Treml4 on splenic CD11c$^+$ DCs, whole splenocytes, or lymph node cells, each population was first incubated with biotinylated anti-Treml4, the indicated fluorescent Abs, and exposed allophycocyanin- or PE-conjugated streptavidin (BD Biosciences), and analyzed on a FACSCalibur or Guava EazyCyte Mini (Guava Technologies). Data were analyzed by FlowJo software (Tree Star).

[4] The online version of this article contains supplemental material.

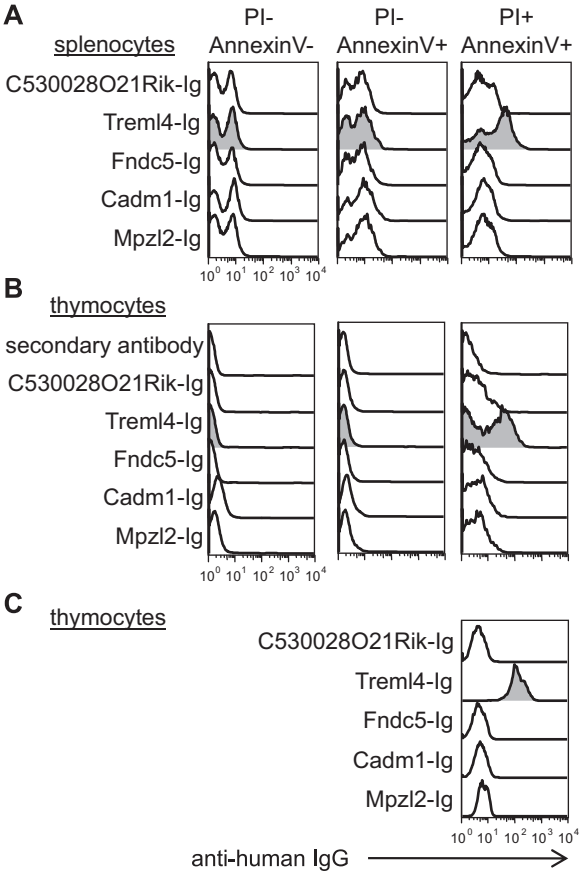

FIGURE 2. Binding of chimeric fusion proteins to apoptotic or necrotic cells. Splenocytes (*A*) or thymocytes (*B*) were exposed to γ-irradiation to induce apoptosis, and then stained with the indicated chimeric fusion proteins. The binding of fusion proteins was evaluated by FACS. Apoptosis was monitored by staining with PI and annexin V. *C*, Thymocytes were incubated at 100°C for 10 min to induce necrosis, stained with fusion proteins, and then analyzed by FACS. Representative data from at least two independent experiments are shown.

Coimmunoprecipitation assay and Western blotting

To construct the expression vectors for FLAG-tagged Treml4 and *myc*-tagged DNAX activation protein 10 kDa (DAP10), DAP12, and FcRγ, each cDNA was amplified by PCR, sequenced, and inserted into pcDNA3.1 vector (Invitrogen). HEK293A cells were transiently transfected with the indicated combination of expression vectors using Lipofectamin2000 (Invitrogen). Whole-cell lysates were prepared 36 h after transfection with lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, and 10% glycerol, and precipitated with anti-FLAG M2 Ab (Sigma-Aldrich) and protein G-Sepharose beads (GE Healthcare Bioscience). The immunoprecipitates were separated on a 5–20% polyacrylamide gradient gel (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with HRP-conjugated anti-*myc* mAb (9E10; Roche). Peroxidase activity was detected with the ECL Plus System (GE Healthcare Bioscience).

For Western blotting of tissue lysates, spleen, liver, lung, bone marrow, peritoneal lavage, and peripheral lymph nodes (popliteal, cervical, inguinal, axillary, and brachial) were collected. Organs were meshed with two frozen slides. The cells were collected and solubilized in radioimmunoprecipitation assay buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 5% protease inhibitor mixture; Sigma-Aldrich) for 15 min at 4°C. Cell lysates were sonicated to homogenize and then quantified by DC protein assay (Bio-Rad). Equal amounts of protein were separated by 4–12% polyacrylamide NuPage bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were incubated with the primary Ab, washed, and incubated with peroxidase-conjugated species-specific Ab (Supplemental Table II*a*).[4] After washing, membranes were developed with ECL Plus System. To control for protein loading, membranes were incubated in Restore Western

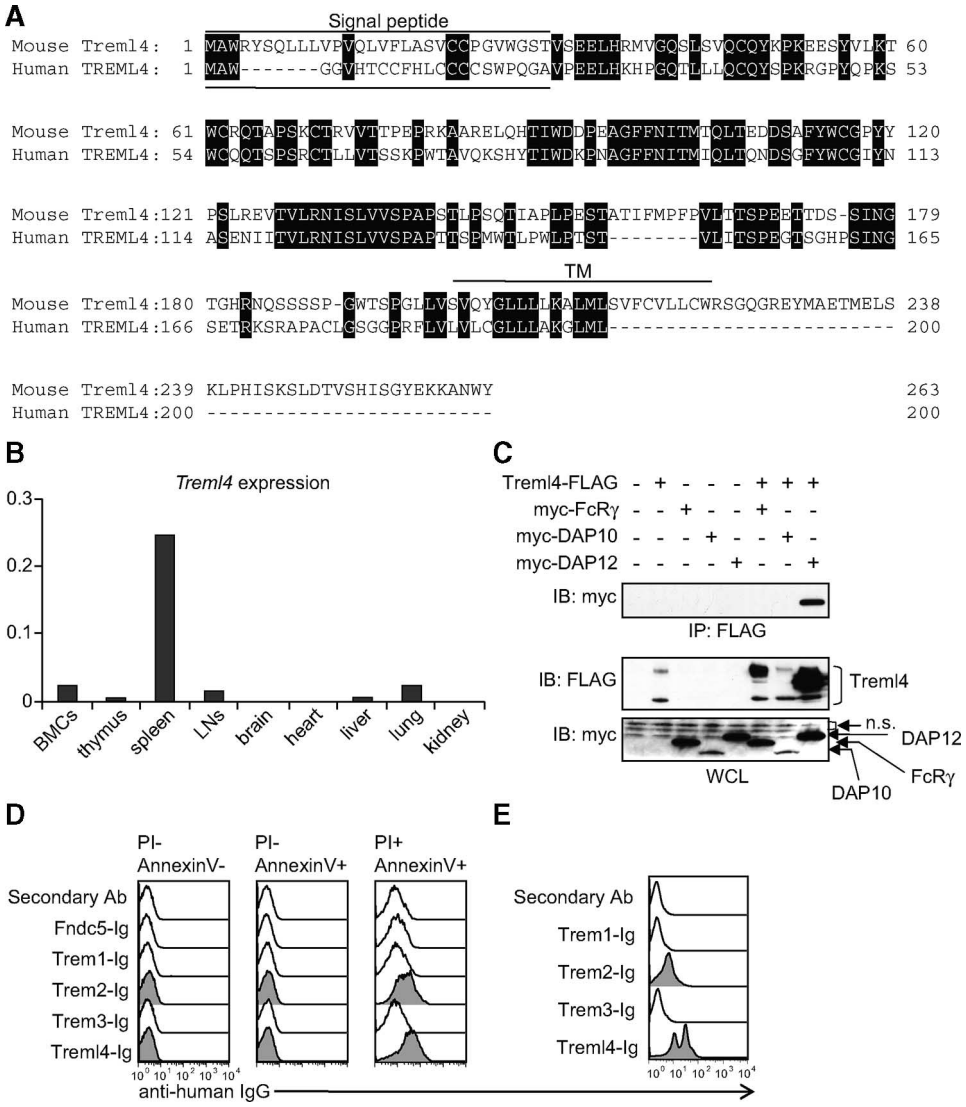

FIGURE 3. Association of Treml4 with DAP12. *A*, Amino acid sequence alignment of human and mouse Treml4. Human (GenBank accession number NM198153) and mouse Treml4 share an overall amino acid identity of 39.9%. The predicted signal peptide (mouse, residues 1–30; human, 1–23) and transmembrane segments (TM; mouse, 200–333) are indicated. *B*, Real-time RCR analysis of Treml4 mRNA. Values are relative to expression of the gene encoding GAPDH. BMCs, bone marrow cells; LNs, lymph nodes. *C*, HEK293 cells were transiently transfected with the indicated combination of Treml4-FLAG, *myc*-FcRγ, *myc*-DAP10, and *myc*-DAP12 (*above lane*). After 36 h, cell lysates were immunoprecipitated (IP) with anti-FLAG Ab, followed by blotting (IB) with anti-*myc* Ab. WCL, whole-cell lysate; n.s., nonspecific bands. *D*, Thymocytes were exposed to γ-irradiation to induce apoptosis, and then stained with the indicated chimeric fusion proteins. *E*, Thymocytes were incubated at 56°C for 30 min and then stained with fusion proteins and analyzed by FACS. Representative data from at least two independent experiments are shown.

Blot Stripping Buffer (Thermo Scientific), washed, and immunoblotted using an anti-actin mAb (Sigma-Aldrich). Developed films were analyzed by densitometry using computerized image analysis software (MCID-M5; Imaging Research), and the data were normalized as follows: protein band (density × area)/actin (density × area). Student's *t* test was applied to reveal significant differences in protein expression between BALB/c and C57BL/6 mice.

Production of anti-Treml4 mAb

A chimeric protein consisting of Treml4 extracellular portion and human IgG1 Fc portion was used to immunize Wistar rats four times. One month after fourth immunization, purified FLAG-tagged extracellular domain of Treml4 was injected for a final boost. Three days later, splenocytes were fused with myeloma cell line P3 × 63.Ag14. Production of mAb against Treml4 was screened by ELISA using Treml4-FLAG protein. Two hybridoma clones, named 16E5 and 32D11, were established, but they produced the identical Ab (IgG1, κ L chain).

Immunofluorescence staining

Spleens were harvested and frozen in Tissue-Tek OCT compounds (Sakura Finetek). Frozen tissue was sectioned at 10 μm in thickness on a microtome (Microm Laborgeräte), fixed for 15 min with cold acetone, rehydrated in PBS, and blocked with avidin/biotin-blocking reagents (Zymed Laboratories), according to the manufacturer's instructions. Sections were first blocked with 5% mouse serum in FACS buffer (2% FCS in PBS) for 60 min at room temperature, and then stained in humidified chamber overnight at 4°C with primary Abs (Supplementary Table II*b*). After washing in FACS buffer, sections were stained with anti-FITC Alexa 488 and streptavidin Alexa 555 (Molecular Probes, Invitrogen) diluted in FACS buffer/2% mouse serum. Specific intracellular adhesion molecule-grabbing nonintegrin receptor 1 (SIGN-R1) staining was performed using anti-hamster FITC (Jackson ImmunoResearch Laboratories), followed by anti-FITC Alexa 488. Sections were mounted in Aqua-Poly Mount (Polysciences) and were stored at 4°C until microscopic examination. The images were acquired with a Zeiss LSM 510 system (Carl Zeiss MicroImaging) at the Rockefeller University Bio-Imaging Resource Center.

Results

Microarray analysis of mouse splenic DC subsets

To identify gene(s) involved in the uptake and signaling of dying cells by DCs, we conducted a DNA microarray analysis using sorted mouse splenic CD8α^+ and CD8α^- DCs. Because the number of splenic DCs is quite low, we initiated s.c. tumors with B16.Flt3L cells that had been retrovirally transduced to express mouse Flt3L (29) to expand DCs. These in vivo expanded DCs showed similar phenotypes to that observed in nontumor-carrying animals, and functioned similarly in terms of cytokine production in response to bacterial products, stimulation of T cells in the MLR, and uptake of dying cells by CD8α^+ DCs (data not shown). Ten to 12 days after injection of Flt3L tumor cells, splenic CD11c$^+$ DCs were enriched by MACS and further purified by FACS cell sorter into CD8α^+ and CD8α^- subsets. Their purities were more than 90% (Fig. 1*A*). On microarray, several genes for predicted transmembrane proteins were differentially expressed by

the CD8α^+ subset that takes up dying cells (Fig. 1*B*). We selected genes showing greater than 4-fold mRNA abundance in CD8α^+ cells for further investigation, e.g., CD36 and CD207, which were previously reported to be more abundantly expressed in CD8α^+ DCs (3, 27, 28, 32). We confirmed the gene expression results either by FACS analysis (CD36, CD103, and CD207; data not shown) or semiquantitative RT-PCR (*C530028O21Rik*, *Fndc5*, *Cadm1*, *Mpzl2*, and *Treml4*; Fig. 1*C*). Thus, several transmembrane proteins were available as potential receptors on CD8α^+ DCs for dying cells.

Binding of Fc fusion proteins to apoptotic or necrotic cells

To determine whether the identified CD8α^+ DC proteins could recognize apoptotic or necrotic cells, we generated soluble chimeric fusion forms of C530028O21Rik, Fndc5, Cadm1, Mpzl2, and Treml4 proteins. These fusion proteins consisted of their extracellular domain fused to the human IgG1 Fc domain and were produced mainly as dimers (data not shown). Apoptosis of splenocytes was induced by γ-ray irradiation, and then these dying cells were incubated with the individual chimeric proteins. As shown in Fig. 2*A*, we could not detect any binding of fusion proteins to living (PI$^-$ annexin V$^-$) or early apoptotic (PI$^-$ annexin V$^+$) cells. Interestingly, Treml4 bound to late apoptotic (PI$^+$ annexin V$^+$) splenocytes. Also, Treml4 bound to various other sources of dying cells, including thymocytes and the 293, EL4, and Chinese hamster ovary tumor cell lines (Fig. 2*B* and data not shown). Because late apoptotic cells, also called secondary necrotic cells, show similar membrane integrity to necrotic cells, we next investigated whether these fusion proteins bound to necrotic cells. Thymocytes were incubated at 56°C for 30 min or 100°C for 10 min, and then stained with these fusion proteins. Only Treml4, but not the other fusion proteins, bound to necrotic cells that were positive for PI staining (Fig. 2*C* and data not shown). Thus, a chimeric Treml4 fusion protein binds to PI-positive, late apoptotic, and necrotic cells, but not living or early apoptotic cells.

Treml4 associates with DAP12

Mouse Treml4 showed similar structural features to other members of the Trem family (reviewed in Ref. 33), with a single V-type Ig-like motif in the extracellular portion and a short cytoplasmic tail (Fig. 3*A*). Treml4 mRNA was predominantly expressed in spleen (Fig. 3*B*). Trem1, 2, 3, and PDC-Trem are known to associate with and transduce signals through an adaptor molecule DAP12 (34–37). This association is mediated by a cationic lysine residue in the transmembrane domain, which was also found in the transmembrane region of Treml4. To determine whether Treml4 associated with DAP12 or other adaptor molecules such as DAP10 and FcRγ, HEK293A cells were transiently transfected with an expression vector for FLAG-tagged Treml4 together with expression vectors for *myc*-tagged DAP12, DAP10, or FcRγ, and then their association was evaluated by coimmunoprecipitation assay. As shown in Fig. 3*C*, we found that Treml4 associated with DAP12, but not DAP10 nor FcRγ.

Although it is well known that Trem members can modulate cellular responses to bacterial products (reviewed in Ref. 33), the identity of natural ligands for them is largely unknown. It has been reported that Trem2 recognizes anionic ligands expressed on bacteria (38), and more recently Takahashi et al. (39) reported that Trem2 is involved in the clearance of apoptotic neurons by microglia. Therefore, we purified soluble forms (Fc fusion proteins) of Trem1, 2, and 3, to investigate whether they could bind to apoptotic or necrotic cells. We found that Trem2, like Treml4, bound to late apoptotic and necrotic cells, whereas Trem1 and Trem3 did not exhibit detectable binding. Thus, Trem2- and Treml4-Fc fusion proteins can bind to dead cells (Fig. 3, *D* and *E*).

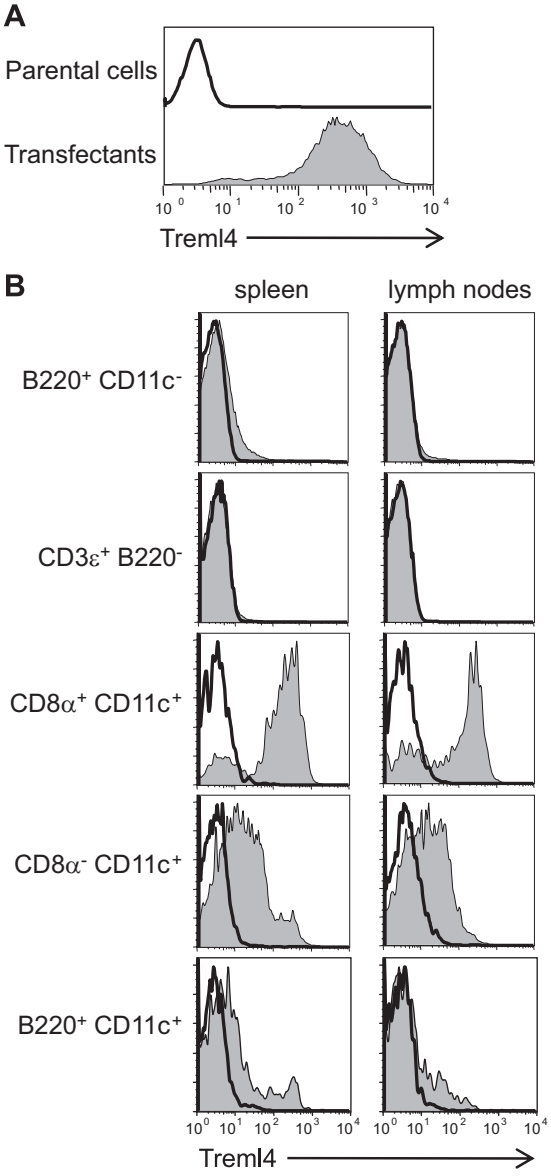

FIGURE 4. Distribution of Treml4 by FACS. *A*, Parental Chinese hamster ovary cells or Treml4 stable transfectants were stained with anti-Treml4 mAb. *B*, Splenocytes or lymph node cells were prepared with collagenase digestion, and the cells were stained with control Ab (open histogram) and anti-Treml4 Ab (gray histogram). The expression of Treml4 was monitored by FACS on B220$^+$ B and CD3ε^+ T cells, CD8α^+ and CD8α^- DCs (CD8α^+CD11c$^+$ and CD8α^-CD11c$^+$, respectively), and B220$^+$ plasmacytoid DCs (B220$^+$ CD11c$^+$).

Treml4 is expressed on a subset of DCs: FACS analyses

To further pursue the expression and biology of Treml4, we generated an anti-Treml4 mAb (Fig. 4*A*). We first investigated the types of leukocytes that express Treml4 by FACS analysis. CD3ε^+ T cells or B220$^+$ B cells in spleen or lymph nodes did not express Treml4 (Fig. 4*B*). For splenic DCs, CD11c$^+$ cells were enriched with anti-CD11c beads. Treml4 was more abundantly expressed on CD8α^+ DCs than CD8α^- DCs, which was consistent with results from the microarray analyses and RT-PCR of splenic DCs (Fig. 4*B*). Another DC subset, plasmacytoid DCs that were B220$^+$ CD11c$^+$ cells, showed at best low expression of Treml4 (Fig. 4*B*). A similar expression pattern was observed with DCs from lymph nodes (Fig. 4*B*). Thus, Treml4 is expressed on subsets of DCs, especially on CD8α^+ DCs, but not on lymphocytes.

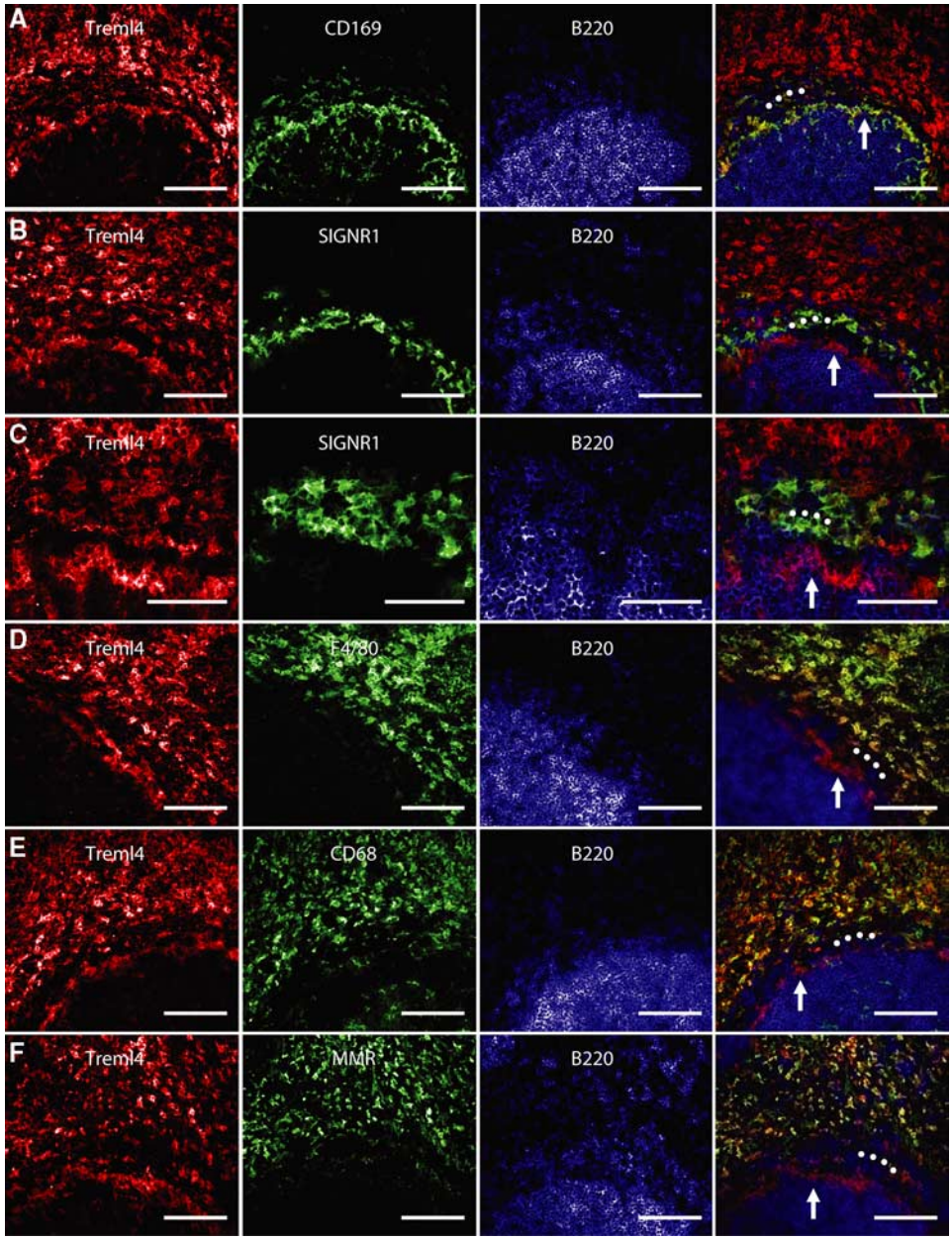

FIGURE 5. Views of the border of red pulp (*top*) and white pulp (*bottom*) of BALB/c mouse spleen. The B cell follicle of the white pulp was marked in blue by the anti-B220 mAb, whereas the macrophages of the red pulp and marginal zones were stained in green for the indicated molecules. The signal for Treml4 was stained in red. Between the B cell follicle and red pulp are concentric layers of two types of macrophages: the marginal metallophilic macrophages (white vertical arrows), stained in green for CD169 (*A*), and the SIGNR1$^+$ marginal zone macrophages (*B* and *C* for higher magnification; ○), which have weak staining for Treml4. *D*, F4/80 Ag (green) is found on red pulp macrophages, but not marginal metallophilic (white vertical arrow) and marginal zone macrophages (○). *E*, CD68 is abundant on red pulp macrophages (green), but not on Treml4$^+$ (red) marginal metallophilic macrophages (white vertical arrows) or marginal zone macrophages (○). *F*, As in *E*, but MMR (green) is abundant on red pulp macrophages, and not in marginal metallophilic (white arrows) or marginal zone macrophages (○). Scale = 100 μm, but *C*, where scale = 50 μm. MMR, macrophage mannose receptor.

Treml4 is abundant on two groups of macrophages in tissue sections of spleen

To investigate in more detail the distribution of Treml4, we stained sections from spleen and lymph node with mAbs to Treml4 and various macrophage markers. It is known that there are several distinct types and locations of macrophages in spleen. Red pulp macrophages express F4/80, CD68, and the macrophage mannose receptor/CD206, and marginal zone macrophages are positive for SIGN-R1/CD209b$^+$, whereas marginal metallophilic macrophages express sialoadhesin/CD169 (reviewed in Ref. 40). Double staining for Treml4 mAb and Abs to either F4/80, SIGN-R1, or CD169 showed that Treml4 was expressed on F4/80$^+$ red pulp and CD169$^+$ marginal metallophilic macrophages, but only very weakly on SIGN-R1$^+$ marginal zone macrophages (Fig. 5). In lymph node, however, we observed little, if any, staining of macrophages for Treml4, indicating again that Treml4 is restricted to selected populations of macrophages (data not shown). These data indicate that Treml4 is primarily expressed in spleen, consistent with the mRNA analyses (Fig. 3*B*), and in spleen, Treml4 is predominantly expressed on macrophages, but also some DCs.

Treml4 is expressed primarily in spleen: Western blotting

We further investigated the tissue distribution of Treml4 by Western blotting. We developed a protocol (see *Materials and Methods*) to homogenize various tissues for quantitative blotting studies. Endogenous Treml4 protein was readily detected in a spleen lysate (Fig. 6*A*). However, the expression of Treml4 in lymph nodes was less than that in spleen, because larger samples and longer exposure times for development were required to obtain even a weak Western blotting signal, whereas other macrophage Ags like CD68 and F4/80 were readily detected in lymph node (Fig. 6*B*). Among several tissues, Treml4 protein was clearly detected only in spleen, even though other macrophage markers were observed in all tissues investigated, suggesting that Treml4 protein, as consistent with RT-PCR analysis, is predominantly expressed in spleen (Fig. 6*C*). Moreover, although CD68 expression was not different between two strains

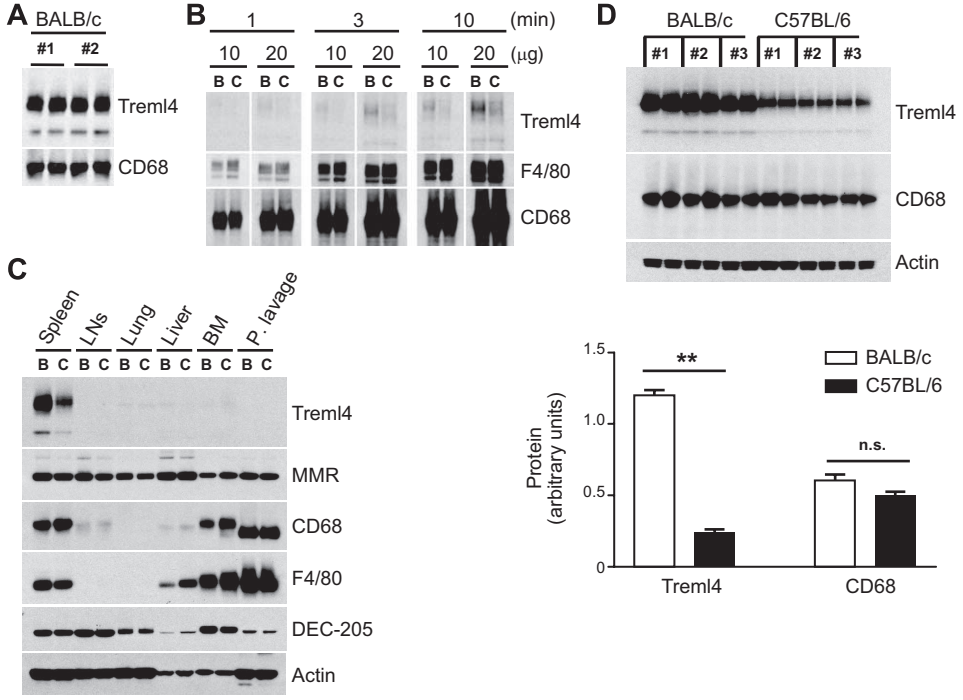

FIGURE 6. Tissue distribution of Treml4 by Western blotting. *A*, Representative data of the immunoblot signals for Treml4 and CD68 in BALB/c spleen lysates from two mice (*1* and *2*). *B*, Expression of Treml4, F4/80, and CD68 was assayed in lymph nodes of BALB/c (*B*) or C57BL/6 (*C*) mice using different amounts of protein (10 or 20 μg) and different film exposure times (1, 3, or 10 min) to illustrate that signals were acquired under limiting conditions for quantification. *C*, The expression of the indicated molecules was monitored by Western blotting. The indicated organs from BALB/c (*B*) or C57BL/6 (*C*) mice were lysed, and 10 μg of protein (except for CD68 and F4/80 markers in peritoneal lavage; 2 or 1 μg of protein was loaded, respectively) was separated by SDS-PAGE. LNs, lymph nodes; BM, bone marrow; P. lavage, peritoneal lavage. *D*, Treml4 and CD68 were assayed by Western blot (*upper panel*) and densitometry (*lower panel*) from three BALB/c (*B*; *1*, *2*, and *3*) and three C57BL/6 (*C*; *1*, *2*, and *3*) mice. Asterisk represents statistical difference between both mice strains: **, $p < 0.01$.

of mice, BALB/c and C57BL/6, Treml4 was more abundantly expressed in the spleen from BALB/c than C57BL/6 mice (Fig. 6*D*). Thus, Treml4 is a distinctive marker in that it is peculiarly abundant in spleen rather than other organs.

Treml4 expression is not altered after DC maturation

Bacterial products are reported to up-regulate expression of TREM1 on human monocytes and neutrophils (34, 41), whereas maturation stimuli down-regulate expression of human TREM2 on

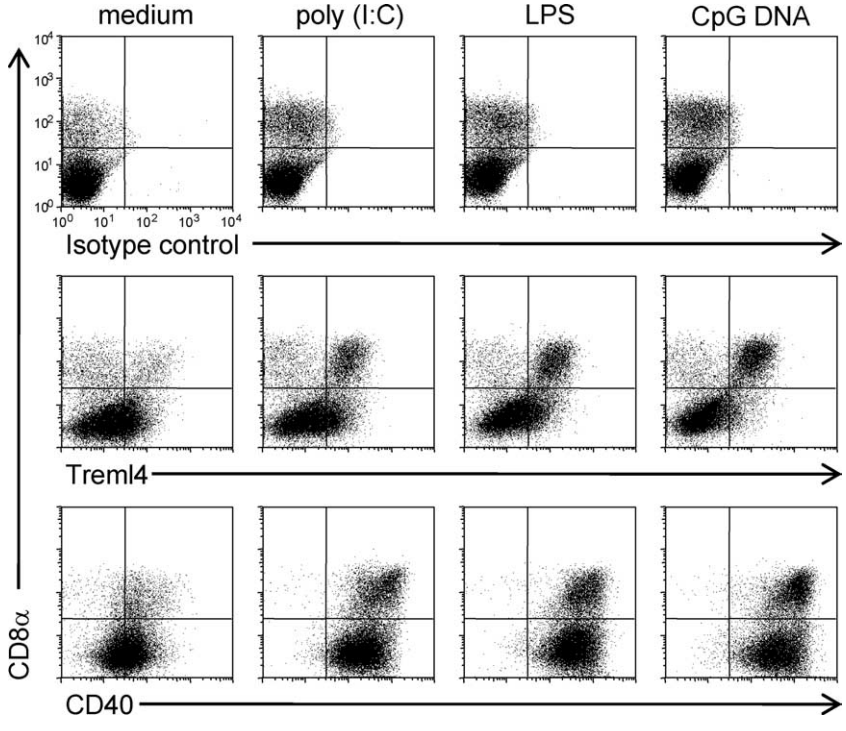

FIGURE 7. Regulation of Treml4 expression on DCs. MACS-enriched CD11c$^+$ splenic DCs were left unstimulated (medium) or stimulated with poly(I:C) (10 μg/ml), LPS (1 μg/ml), or CpG DNA (1 μM). Twenty-four hours later, the expression of Treml4 and CD40 was monitored by FACS. CD40 was used as a marker for DC activation by the TLR ligands.

immature DCs or mouse Trem2 on bone marrow-derived macrophages (42). Recently, a new Trem family member, termed PDC-Trem, was found to be expressed on activated plasmacytoid DCs, but not immature plasmacytoid DCs (37). Thus, the expression of Trem family members can be differentially regulated. We therefore investigated Treml4 expression after DC activation. MACS-enriched splenic CD11c$^+$ DCs were stimulated with TLR ligands such as poly(I:C) (TLR3), LPS (TLR4), or CpG DNA (TLR9) to activate DCs, and the expression of Treml4 on DCs was monitored 24 h after stimulation. The expression of Treml4 was clearly detected on freshly isolated DCs. Although the relative frequency of CD8α^+ DCs was slightly reduced when the cells were cultured without any stimulus (medium in Fig. 7), the expression of Treml4 on CD8α^+ DCs was retained. Treml4 on CD8α^+ DCs was neither down- nor up-regulated, even though DCs were activated by some TLR ligands, as indicated by up-regulation of CD40 (Fig. 7). Thus, Treml4 expression was regulated distinctly from other Trem family members.

Discussion

We have identified a new member of the Trem family in mice and found that it has a distinctive tissue distribution being primarily expressed on subsets of splenic macrophages (red pulp and marginal metallophilic macrophages) and DCs (CD8α^+ DCs). We also found that the soluble form of Treml4 has binding affinity to necrotic cells.

DNA microarray analysis revealed that many genes were differentially expressed between splenic CD8α^+ and CD8α^- DCs, where the former DCs selectively take up dying cells in vivo (3). Consistent with previous reports, our microarray data indicated that CD36, CD205 (DEC-205), CD207 (Langerin), and CD103 (α_E integrin) mRNA were each more abundantly expressed in CD8α^+ DCs. However, it had been reported that CD36- or DEC205-deficient DCs could take up dying cells in vivo (3, 27, 28). Because CD207 is weakly expressed on the surface of splenic DCs from C57BL/6 mice (43), CD207 was not likely to be required for dying cell uptake. Thus, none of these molecules expressed by CD8α^+ DCs seemed essential for their uptake of dying cells.

We further considered the *C530028O21Rik*, *Fndc5*, *Cadm1*, *Mpzl2*, and *Treml4* genes that were more abundantly expressed in CD8α^+ than CD8α^- DCs. Functional analyses of *Cadm1* and *Mpzl2* knockout mice had not assessed uptake of dying cells (44–47). When we prepared Fc fusion proteins of each of these five genes, to see whether they could bind to dying cells, only Treml4 showed binding to necrotic PI$^+$ annexin V$^+$ cells, but not early apoptotic cells in our study.

The predicted amino acid sequence of Treml4 showed characteristic features of the Trem family, including the presence of a V-type Ig-like motif in the extracellular portion and a short cytoplasmic tail (reviewed in Ref. 33). Many Trem members, including Trem1, 2, 3, and PDC-Trem, have a cationic lysine residue in their transmembrane domain, which is essential for association with ITAM-bearing adaptor molecule, DAP12. This lysine residue was also observed in the transmembrane domain of Treml4, and consistent with this, we observed a Treml4/DAP12 association by immunoprecipitation assay.

To begin to study the biological features of Treml4, we found a unique expression pattern. Real-time PCR and Western blotting analysis indicated that Treml4 was predominantly expressed in the spleen, and its expression was more abundant in BALB/c than C57BL/6 spleen. Immunofluorescent staining of tissue sections and FACS analysis of cell suspensions from spleen revealed that Treml4 was expressed on DCs as well as spleen resident macrophages, especially in red pulp and marginal metallophilic macrophages. Our quantitative Western blotting data indicated that the expression of Treml4 was primarily confined to macrophages in spleen, which is an unusual feature relative to many other known macrophage products that we studied, such as F4/80, CD68, CD169, and CD206.

Based on genomic sequencing data, the human Treml4 sequence seems to be truncated around the predicted transmembrane domain of its mouse counterpart (Fig. 3*A*, and reviewed in Ref. 33), suggesting that human Treml4 may be produced only as soluble form and/or is not functional. Mouse Trem2 is reported to be involved in the clearance of apoptotic neurons by microglia (39). Human Trem2 is expressed on macrophages and monocyte-derived DCs, but mouse Trem2 is not expressed on splenic DCs (data not shown). In our experiments, Trem2-Fc fusion protein could bind to annexinV$^+$ PI$^+$ late apoptotic or necrotic cells just like Treml4-Fc. At present, the ligand for Treml4 is not yet identified, and we do not know whether Trem2- and Treml4-Fc fusion proteins bind to the same molecules. A report suggests that ligands for Trem2 are found on the surface of microbes and they are anionic molecules (38). However, Treml4-Fc fusion protein did not bind to *Escherichia coli* or *Staphylococcus aureus* (supplemental figure).[4] However, Trem2- and Treml4-Fc fusion protein bound to necrotic cells that were incubated at 100°C, indicating that the ligands for Trem2 and/or Treml4 in necrotic cells are thermostable substances. Therefore, we cannot rule out a possibility that Trem2 and Treml4 may compensate functions for each other in mice.

It has been reported that signals from Trem members affect the activation status of DCs and macrophages (33). Although both Trem1 and Trem2 associate with DAP12, they can have contrasting effects on immune cells. For example, whereas activation of Trem1 showed a synergistic effect with TLR ligands (41), Trem2-deficient macrophages show enhanced cytokine production in response to TLR ligands (42). Furthermore, DAP12-deficient cells showed enhanced or reduced cytokine production in response to TLR ligands depending on the cell type or experimental conditions (48, 49). It remains unclear whether activation of Treml4 can affect cellular activation of DCs and, reciprocally, whether TLR ligands intersect with Treml4 function on these cells.

Further studies are required to clarify whether Treml4 is involved in the uptake of dying or necrotic cells and mediates immune recognition in vivo. It has been reported that some C-type lectins, including Lox-1, mannose-binding lectin, and Mincle, can recognize dead cells or molecules leaked from them (50–52). Trem2, which showed affinity to dead cells in our experiments (Fig. 3*E*), is expressed on DCs and macrophages such as GM-CSF-induced bone marrow-derived DCs and bone marrow-derived or thioglycolate-elicited peritoneal macrophages in mice (42, 53). Therefore, it is difficult to discriminate a role of Treml4 engagement from the activation of other C-type lectins by dead cells in wild-type DCs and macrophages. Treml4-deficient mice and cells will help to clarify these points.

Acknowledgments

We thank Dr. Takashi Suda (Kanazawa University, Kanazawa, Japan) for providing the expression vector for human IgG1 Fc fusion protein, and Dr. Laura Santambrogio (Albert Einstein College of Medicine, New York, NY) for B16.Flt3L cells, Klara Velinzon for expert sorting, Alison North and the Bio-Imaging Resource Center of Rockefeller University, Margarita Oks for technical help, and Judy Adams for help with figures. We also thank Drs. Kayo Inaba (Kyoto University, Kyoto, Japan), Yoshiko Iwai (Tokyo Medical and Dental University, Tokyo, Japan), Olga Mizenina, Sayuri Yamazaki, Anna Charalambous, and Godwin Nchinda for valuable discussions. We gratefully acknowledge Jay Overholser and Dr. Frances Weis-Garcia (Sloan-Kettering Cancer Center, New York, NY) for their help with hybridoma production, and Dr. Bruz Marzolf (Institute for Systems Biology, Seattle, WA) for DNA microarray analysis.

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