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Treml4, an Ig Superfamily Member, Mediates Presentation of Several Antigens to T Cells In Vivo, Including Protective Immunity to HER2 Protein

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Members of the triggering expressed on myeloid cells (Trem) receptor family fine-tune inflammatory responses. We previously identified one of these receptors, called Treml4, expressed mainly in the spleen, as well as at high levels by CD8α+ dendritic cells and macrophages. Like other Trem family members, Treml4 has an Ig-like extracellular domain and a short cytoplasmic tail that associates with the adaptor DAP12. To follow up on our initial results that Treml4-Fc fusion proteins bind necrotic cells, we generated a knockout mouse to assess the role of Treml4 in the uptake and presentation of dying cells in vivo. Loss of Treml4 expression did not impair uptake of dying cells by CD8α+ dendritic cells or cross-presentation of cell-associated Ag to CD8+ T cells, suggesting overlapping function between Treml4 and other receptors in vivo. To further investigate Treml4 function, we took advantage of a newly generated mAb against Treml4 and engineered its H chain to express three different Ags (i.e., OVA, HIV GAGp24, and the extracellular domain of the breast cancer protein HER2). OVA directed to Treml4 was efficiently presented to CD8+ and CD4+ T cells in vivo. Anti–Treml4-GAGp24 mAbs, given along with a maturation stimulus, induced Th1 Ag-specific responses that were not observed in Treml4 knockout mice. Also, HER2 targeting using anti-Treml4 mAbs elicited combined CD4+ and CD8+ T cell immunity, and both T cells participated in resistance to a transplantable tumor. Therefore, Treml4 participates in Ag presentation in vivo, and targeting Ags with anti-Treml4 Abs enhances immunization of otherwise naive mice. The Journal of Immunology, 2012, 188: 1147–1155.

The online version of this article contains supplemental material.

Abbreviations used in this article: BFA, brefeldin A; DC, dendritic cell; ECD, extracellular domain; ES, embryonic stem; α-GalCer, 2S, 3S, 4R-1-O-(α-galactopyranoxy)-2(N-hexacosanoylamino)-3,4-octadecanetriol; KO, knockout; LN, lymph node; poly-IC, polyinosinic-polycytidylic acid; Trem, triggering expressed on myeloid cell; WT, wild-type.

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found that anti-Trem4 mAb binds to appropriate DC, macrophage, and monocyte subsets in the spleen. Also, we considered whether Trem4 has the capacity to initiate Ag uptake, processing, and presentation on MHC class I and II using a novel approach that involves delivery of Ags coupled to mAbs. This approach was shown to increase the efficiency of Ag presentation on MHC class I and II molecules ~100-fold, and it allows T cell immunization (13–15). However, many of the receptors targeted to date belong to the C-type lectin family, which are probably involved in the physiological capture of pathogens and subsequent Ag presentation. In this study we show with three different protein Ags that, similar to C-type lectin receptors, an Ig superfamily member called Trem4 can bring about Ag presentation and priming of CD4+ and CD8+ T cells in vivo.

Materials and Methods

Mice

We purchased C57BL/6j (B6), BALB/c1, and FVB/Nj mice from The Jackson Laboratory. BALB/c × C57BL/6j (C6B6) F1 mice were from Harlan. OVA-specific TCR transgenic mice OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J) and OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J), and TAP Harlan. OVA specific TCR transgenic mice OT-I (C57BL/6-Tg(TcraTcrb)425Cbn/J) with 20% FBS (Life Technologies, Invitrogen), 1%L-glutamine, 1%Breast Media, which consisted of RPMI 1640 (Life Technologies, Invitrogen) for 10 min at 37˚C. Twenty-four hours later, 3M CFSE-labeled anti-CD8 (2.43, for OT-II preparation). T cell proliferation was evaluated by injecting individual animals with either 2–3×10^6 OT-II cells, labeled at 10^7/ml with 5×10^9 CD8+ T cells or 10^9 CD4+ T cells from lymph nodes (LNs) and spleen cells of experimental mAbs, for OVA-specific T cell proliferation responses. CD90+ CD4+ or CD90− CD8+ T cells were purified from lymph nodes (LNs) and spleen cell suspensions of OT-I and OT-II mice, respectively, by negative selection using anti-F4/80, anti-MHCII (TIB120), anti-B220 (RA3-6B2), anti-NK1.1 (PK136), and anti-CD4 (GK1.5, for OT-II preparation). T cell proliferation was evaluated by injecting individual animals with either 2–3×10^6 OT-I cells or 3.5–10^6 OT-II cells, labeled at 10^6/ml with 5 μM CFSE (Invitrogen) for 10 min at 37˚C. Twenty-four hours later, 3 μg anti-receptor mAbs conjugated with OVA were injected s.c. into the footpad. OVA-specific transgenic CD8+ or CD4+ T cells were purified from lymph nodes (LNs) and spleen cell suspensions of OT-I and OT-II mice, respectively, by negative selection using anti-F4/80, anti-MHCII (TIB120), anti-B220, anti-I-Ab (24D2; BioLegend, San Diego, CA), and anti-CD45.1 mice in house. The targeting vector for Trem4 KO mice was designed by replacing a 1.7-kb fragment including exon 1 and 2 with an ACN cassette. The ACN cassette contains the sequences encoding in-frame nonsense under the control of the RNA polymerase II promoter and Cre recombinase gene under the control of angiotensin-converting enzyme promoter, flanked by loxp sites (16). Cre-mediated recombination during spermatogenesis removed the cassette, leaving one loxp (Fig. 1A). The targeting construct was transfected into B6 embryonic stem (ES) cells (Cy2.4). Targeted ES cells were screened by Southern blotting and subsequently injected into SCID embryos. The resulting male chimeric mice were bred to female B6 or C57BL/6-Tyrc-2J mice to obtain chimeric mice. DNA for OVA, HIV GAGp24 (aa 133–363 derived from HIV isolate BH10), and HER2 (aa 22–653) was cloned in frame into the C terminus of the H chain of anti–mouse DEC205- Trem4, and control Ig, as previously described (15, 20). An OLLAS epitope sequence was inserted between the H chain and HIV GAGp24 or HER2 sequences (B, Wang, N. Zaidi, L.Z. He, K. Zhang, J.M.Y. Kuroiwa, T. Keiter, and R.M. Steinman, submitted for publication) (15, 18). Fusion mAbs were expressed by transient transfection (calcium phosphate) in 293T cells in serum-free DMEM supplemented with Nutridoma SP (Promega, Madison, WI). PCR was performed, and then the products were cloned and sequenced. Primers used to amplify IgG1 H chain cDNA were 5’-GCT CAA TGG CAG GAC GTT CAG ATG-3’ and 5’-GTC TAA CAC TCA TCT-3’ to synthesize the Superscript first-strand cDNAs (Invitrogen). After circularization of the cDNAs with RNA ligase (Promega, Madison, WI), PCR was performed, and then the products were cloned and sequenced. Western blotting using anti-mouse IgG1-HRP (Southern Biotech, Birmingham, AL), HRP-anti-GAGp24 (ImmunoDiagnostics, Woburn, MA), HRP-anti-OVA (Research Diagnostics), biotin-anti-HER2 (clone 42; BD Transduction Laboratories, San Jose, CA), and biotin-anti-OLLAS (produced in house), followed by HRP-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Receptor binding was verified using CHO-transfected cells by FACs analysis staining with Alexa Fluor 647-labeled anti-OLLAS, PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), or Alexa Fluor 488-conjugated anti-HER2 mAb (clone 2D2; BioLegend, San Diego, CA).

Cell preparation

For analysis of different leukocyte populations, spleens were cut into small pieces and incubated in a Hanks buffer (Life Technologies) solution containing 400 U/ml Collagenase D (Roche) and 50 μg/ml DNase I (Roche) for 25 min at 37˚C. Five milliliter of EDTA (Life Technologies) was added for the last 5 min of incubation, and the cell suspension was passed through a 70-μm cell strainer. In some experiments, cells were enriched by negative selection using anti-CD19 magnetic beads and passed through LS columns (Miltenyi Biotec, Auburn, CA). For analysis of intracellular cytokine production by T cells, spleens were force-passed through a 70-μm cell strainer to obtain a homogeneous cell suspension without enzymatic digestion. BRCs were lysed by incubating with RBC Lysis Buffer (BFA; 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA) and then processed as previously described (15, 20). An OLLAS epitope sequence was inserted between the H chain and HIV GAGp24 or HER2 sequences (B, Wang, N. Zaidi, L.Z. He, K. Zhang, J.M.Y. Kuroiwa, T. Keiter, and R.M. Steinman, submitted for publication) (15, 18). Fusion mAbs were expressed by transient transfection (calcium phosphate) in 293T cells in serum-free DMEM supplemented with Nutridoma SP (Promega, Madison, WI). PCR was performed, and then the products were cloned and sequenced. Primers used to amplify IgG1 H chain cDNA were 5’-GCT CAA TGG CAG GAC GTT CAG ATG-3’ and 5’-GTC TAA CAC TCA TCT-3’ to synthesize the Superscript first-strand cDNAs (Invitrogen). After circularization of the cDNAs with RNA ligase (Promega, Madison, WI), PCR was performed, and then the products were cloned and sequenced. Western blotting using anti-mouse IgG1-HRP (Southern Biotech, Birmingham, AL), HRP-anti-GAGp24 (ImmunoDiagnostics, Woburn, MA), HRP-anti-OVA (Research Diagnostics), biotin-anti-HER2 (clone 42; BD Transduction Laboratories, San Jose, CA), and biotin-anti-OLLAS (produced in house), followed by HRP-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Receptor binding was verified using CHO-transfected cells by FACs analysis staining with Alexa Fluor 647-labeled anti-OLLAS, PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), or Alexa Fluor 488-conjugated anti-HER2 mAb (clone 2D2; BioLegend, San Diego, CA).

Adoptive transfer and in vivo T cell-proliferation responses

OVA-specific transgenic CD8+ or CD4+ T cells were purified from lymph nodes (LNs) and spleen cell suspensions of OT-I and OT-II mice, respectively, by negative selection using anti-F4/80, anti-MHCII (TIB120), anti-B220 (RA3-6B2), and anti-CD4 (GK1.5, for OT-II preparation). T cell proliferation was evaluated by injecting individual animals with either 2–3×10^6 OT-I cells or 3.5–10^6 OT-II cells, labeled at 10^6/ml with 5 μM CFSE (Invitrogen) for 10 min at 37˚C. Twenty-four hours later, 3 μg anti-receptor mAbs conjugated with OVA were injected s.c. into the footpad. CFSE dilution was evaluated 3 d later by FACs of spleen and pooled skin-draining LN (popliteal, inguinal, axillary, brachial, and cervical) after surface staining for CD4 (OT-II) or CD8 (OT-I) and Cy2 (OVA-specific TCR; clone B20.1). Fold clonal expansion of primed T cells was calculated as the ratio of the number of cells with more than one division/number of undivided cells in the control group, as previously described (20).
Uptake of dying cells and cross-presentation assay

To evaluate uptake of dying cells, splenocytes from B6 mice were labeled with 5 μM CFSE for 10 min at 37°C and then cultured to undergo apoptosis by osmotic shock (21). A total of 2 × 10^6 CFSE-labeled dying cells was inoculated i.v. for 2 h, subsequently, spleens were collected, digested with Collagenase D, and analyzed by FACS for DCs containing CFSE. To measure presentation of cell-associated Ags, mice were similarly injected with 2 × 10^7 OVA-pulsed and osmotically shocked TAP^-/- splenocytes (TAP^-/-/OVA), followed by 4% poly-L-lysine and carbboxymethylcellulose stabilized poly-IC (Oncovir, Washington, DC) as adjuvant (22). Immune responses to OVA were measured 1 wk later by restimulating splenocytes for 6–12 h with OVA peptide (SIINFEKL) in the presence of BFA (23).

Mice inoculations

Mice were immunized i.p once with fusion mAbs with a stimulus for DC maturation, which was 50 μg polyinosinic-polycytidylic acid (poly-IC; InVivoGen, San Diego) together with 25 μg IC10 agonistic anti-CD40 mAb (15). In other experiments, we used a prime/boost regimen in which anti-CD40 was omitted and 50 μg poly-IC (Thermo Scientific, Waltham, MA) or poly-L-lysine and carboxymethylcellulose stabilized poly-IC (Oncovir, Washington, DC) was the adjuvant for both prime and boost immunizations given 1 mo apart. In tumor-protection experiments, 10^6 NT2.5 neu-expressing tumor cells in the shaved right flank. Tumor size was measured three times every week using a caliper. Tumor volumes were estimated according to the formula: length × (width)^2 × 0.5. When animals seemed stressed or exhibited tumor ulceration or the tumor diameters exceeded 12 mm, the mice were sacrificed, and this was recorded as the date of death. For Ab depletion, 200 μg anti-CD4 (GK1.5) or anti-CD8 (2.43) mAb or both were given to mice i.p after boost immunization at 9, 6, and 3 d prior to tumor challenge. Rat IgG (GL117) was given as the control mAb. Efficiency of depletion, defined as a reduction of >90% of the targeting cell subtypes, was confirmed by FACS analysis of peripheral blood cells. For in vivo-targeting experiments, mice were injected i.p. for 1 h with 10 μg anti-Treml4 mAb labeled with Alexa Fluor 647.

Intracellular cytokine staining

Bulk splenocytes were restimulated with 15-mer peptide mix from HIV GAGp24 (1 μg/ml), nonreactive HIV GAGp17 (1 μg/ml), the ECD of HER2, or neu peptides (1 μg/ml) in the presence of 2 μg/ml costimulatory anti-CD28 (clone 37.51; American Type Culture Collection) for 6 h at 37°C, adding 10 μg/ml BFA for the last 5 h to allow accumulation of intracellular cytokines. Cells were washed, incubated for 10 min at 4°C with 2.4G2 mAb (American Type Culture Collection) to block FcγRs, and stained with mAbs against surface molecules for 20 min at 4°C. Cells were then fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained with mAbs against cytokines. A total of 1–3 × 10^6 live CD3^+ cells was acquired on a BD LSR II flow cytometer, and data were analyzed with FlowJo Software (Tree Star, San Carlos, CA).

FIGURE 1. Generation of Treml4 KO mice. A, Schematic diagram of the mouse Treml4 WT allele, targeting vector, mutated allele in ES cells, and mutated allele in Treml4-deficient mice. Filled and open boxes denote coding exons and noncoding exons, respectively. B, Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with SphI, electrophoresed, and hybridized with the radiolabeled probe indicated in A. WT and mutated alleles of Treml4 gene were at 9.1- and 7.4-kb, respectively. C, Northern blot analysis of whole splenocytes. Total RNA (10 μg) was electrophoresed, transferred to a nylon membrane, and hybridized with Treml4 cDNA or β-actin cDNA fragment as a probe. D, Representative data of the immunoblot signals for Treml4 in B6 WT and Treml4 KO mice. Spleens were lysed, 25 μg total protein was separated by SDS-PAGE, and Treml4 expression was assayed by Western blot using anti-Treml4 mAb, followed by peroxidase-conjugated anti-mAb. To control for protein loading, the membrane was incubated with stripping buffer and immunoblotted with anti-β-actin-HRP mAb. Each experiment is representative of two with similar results. E, Uptake of dying cells by DCs. A total of 20 × 10^6 CFSE-labeled dying splenocytes was injected i.v. to WT littermates (Treml4^+/-) or Treml4 KO (Treml4^-/-) mice. Two hours after injection, spleens were harvested, and uptake of CFSE^+ cells was monitored in CD8α^- and CD8α^- DC subsets. Plots show CD11c^+ gated cells and are representative of two independent experiments. F, Cross-presentation of cell-associated Ag. Control littermates (Treml4^+/-) or Treml4 KO (Treml4^-/-) mice were immunized i.v. with 20 × 10^6 dying TAP^-/- splenocytes loaded with OVA (TAP^-/-/OVA), in the presence or absence of the adjuvant α-GalCer. Seven days later, splenocytes from immunized mice were restimulated with OVA peptide (SIINFEKL) for 12 h with BFA, and the presence of CD8^+ T cells producing IFN-γ was evaluated by flow cytometry after intracellular cytokine staining. Plots shown CD3e^- gated T cells. G, As in F, but the percentage of IFN-γ^+ CD8^+ T cells of CD3e^+ splenocytes is shown as the mean of three independent experiments. Each symbol represents an individual mouse, and the horizontal line denotes the mean.
Immunofluorescence staining

B6 or Treml4 KO mice were inoculated i.p. with 25 μg purified anti-Treml4 mAb 3 h later, and spleens were harvested and frozen (−80°C) in Tissue-Tek OCT (Sawura). Ten- to twelve-micrometer spleen sections were fixed for 15 min in cold acetone, rehydrated in PBS, and blocked with 5% mouse serum in FACs buffer (PBS/2% FBS) for 1 h at room temperature. Sections were stained in a humidified chamber overnight at 4°C with anti-rat Alexa Fluor 555 (Invitrogen), washed in FACs buffer, and stained with secondary mAb. Marginal metallocipic macrophages were detected with FITC-labeled anti-CD169 (AbD Serotec), followed by anti-FITC Alexa Fluor 488 (Invitrogen). Marginal zone macrophages were stained with purified with anti–SIGNR1 (in house), followed by anti-hamster FITC (Jackson ImmunoResearch) and anti-FITC Alexa Fluor 488. In all cases, B cells were detected by staining sections with Alexa Fluor 647-labeled anti-B220 (eBioscience). In all cases, B cells were detected by staining sections with Alexa Fluor 647-labeled anti-B220 (eBioscience). Sections were mounted in Aqua-Poly Mount (Polysciences), examined on a Zeiss LSM 510 system (Carl Zeiss) at The Rockefeller University Bio-Imaging Resource Center, and analyzed with ImageJ (National Institutes of Health).

Statistical analysis

Data were analyzed and charts were generated using Prism 5 GraphPad software (San Diego, CA). The Student t test (between two groups or conditions) was applied to compare statistical significance between peptide-specific responses. A two-way ANOVA test was applied to compare differences between treated group and control groups. Results were considered statistically significant when \( p < 0.05 \).

Results

Treml4-deficient mice do not exhibit changes in the uptake and cross-presentation of dying cells

We used gene targeting to generate Treml4 KO mice for these studies. The targeting vector used to generate Treml4 KO mice was constructed to replace exon 1 and 2 with a self-excising ACN cassette (Fig. 1A). Correctly targeted ES cells were injected into B6 blastocysts, followed by germline transmission of the mutated allele (Fig. 1B–D). Treml4 KO mice were born at the expected Mendelian frequency, were fertile, and appeared to be healthy over 6 mo of observation. Treml4 is primarily expressed in the spleen (4), but Treml4 KO mice had normal numbers of splenic leukocytes, including T cells, B cells, NK cells, granulocytes, monocytes, DCs, and red pulp macrophages (data not shown).

To investigate the in vivo function of Treml4, we evaluated whether Treml4 is involved in the uptake of dying cells and cross-presentation of cell-associated Ags. B6 splenocytes were labeled with CFSE, induced to undergo apoptosis by osmotic shock (21), and then injected i.v. into wild-type (WT) littermates (Treml4+) or Treml4 KO (Treml4−/−) mice. Two hours later, the uptake of CFSE+ cells by CD11c+ DCs was monitored by FACS. We detected uptake of CFSE+ dying cells in CD8a+ classical DCs in both WT littermates (Treml4+) and Treml4 KO (Treml4−/−) mice, and the frequency was not different between the strains of mice, which suggested that Treml4 is not essential for uptake of dying cells by DCs in vivo (Fig. 1E). To monitor Ag cross-presentation in vivo, WT littermates or Treml4 KO mice were injected i.v. with dying TAP−/− splenocytes loaded with OVA (TAP−/−OVA) in the presence or absence of an adjuvant (α-GalCer). After 7 d, Ag presentation and priming of OVA-specific CD8+ T cells was monitored after in vitro restimulation with OVA peptide in the presence of BFA and staining of intra-cellular IFN-γ. As shown in Fig. 1F and 1G, Ag-specific IFN-γ–producing CD8+ T cells were generated to comparable extents in Treml4 KO (Treml4−/−) mice as in control littermates (Treml4+), indicating that cross-presentation was independent of Treml4 in this system. Thus, Treml4 is not essential for uptake and cross-presentation of dying cells in vivo.

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** mAb against Treml4 labels CD8α+ DCs, red pulp macrophages (RPM), and Ly6Chigh monocytes. A, C57BL/6 (top panel), BALB/c (middle panel), or CxB6 F1 (bottom panel) splenocytes were analyzed by multicolor flow cytometry for the expression of Treml4 in different subsets of leukocytes (24). For control, we used rat IgG1 isotype control (blue graphs) or Treml4 KO mice on B6 background (gray graphs). B, CD115+ splenic monocytes from B6 or Treml4 KO mice were further gated on the expression of Ly6C. Ly6Clow and Ly6Chigh monocytes were analyzed for the expression of Treml4. C, As in B, but expression of Treml4 was evaluated in Ly6Chigh and Ly6Chigh blood monocytes. In all cases, one experiment of two with similar results is shown. PDCs, plasmacytoid DCs.
Treml4 is mainly expressed on CD8α+ DCs, tissue macrophages, and monocytes

To examine the pattern of expression of Treml4 in different mouse tissues, we used a previously described multicolor flow cytometry strategy that facilitates side-by-side evaluation of distinct splenic leukocytes populations (15, 24). Consistent with our previous results (4), Treml4 was abundantly expressed on splenic CD8α+ DCs and splenic red pulp macrophages of all mouse strains analyzed (C57BL/6, BALB/c, and CxB6 F1 mice) but not on B cells, T cells, NK cells, or granulocytes (Fig. 2A). We found that splenic CD8α− DCs expressed low levels of Treml4, but plasmacytoid DCs were negative for this receptor. As previously reported, Treml4 was also found to be a good marker for Ly6Clow monocytes both in the spleen and blood (Fig. 2B, 2C, respectively) (25). Importantly, no staining with anti-Treml4 mAb was observed on any leukocytes from Treml4 KO mice. Therefore, mouse Treml4 is highly expressed on CD8α+ DCs, spleen macrophages, and Ly6Clow monocytes.

Injected anti-Treml4 mAb rapidly labels CD8α+ DCs, tissue macrophages, and monocytes in mice

To assess whether specific targeting of anti-Treml4 mAb took place in vivo, B6 or Treml4 KO mice were inoculated i.p. with 10 μg Alexa Fluor 647-labeled mAb. The uptake of labeled mAb was evaluated by multicolor flow cytometry, as above, 1 h postinoculation. The injected anti-Treml4 mAb labeled most CD8α+ DCs and red pulp macrophages, but it only slightly labeled the CD8α− DCs (Fig. 3A). This labeling was specific because none was observed in Treml4 KO mice (Fig. 3A). In addition, Treml4 mAb was taken up by splenic monocytes, especially Ly6Clow ones (Fig 3B). Immunofluorescence staining of tissue sections showed high uptake of anti-Treml4 mAb by CD169+ marginal metallophilic macrophages (Fig. 3C) but only weak capture by SIGNR1+ marginal zone macrophages (Fig. 3D), which corresponds to the described expression of Treml4 by these macrophage populations (4). Altogether, these results indicated that i.p. inoculation of anti-Treml4 mAb faithfully targets in vivo to cells that express the receptor.

FIGURE 3. Anti-Treml4 mAb is mainly captured by CD8α+ DCs, splenic macrophages, and Ly6Clow monocytes in vivo. A, B6 or Treml4 KO mice were inoculated i.p. with 10 μg Alexa Fluor 647-labeled anti-Treml4 mAb. Uptake of labeled mAb by different splenic DC subsets and leukocyte populations was evaluated 1 h after inoculation by multicolor flow cytometry (15). B, As in A, but two populations of CD11b+CD115+ splenic monocytes, Ly6Clow (left panel) and Ly6Chigh (right panel), were further analyzed for the uptake of Alexa Fluor 647-labeled anti-Treml4 mAb. C and D, B6 or Treml4 KO mice were inoculated i.p. with 25 μg purified anti-Treml4 mAb. Three hours postinoculation, dissected spleens were cryopreserved, sectioned, and stained with anti-rat Alexa Fluor 555 (red) to detect the inoculated mAb. Marginal metallophilic macrophages (arrows) were identified by staining with anti-CD169 (C, green), and marginal zone macrophages (dots) were identified by staining with anti-SIGNR1 (D, green). To identify B cell follicles (B), sections were stained with B220 (blue). Scale bars, 100 μm. In all cases, one representative experiment of two or three with similar results is shown. PDCs, plasmacytoid DCs; RP, red pulp; RPM, red pulp macrophage.
Anti-Treml4 mAb delivers OVA Ag for efficient presentation in vivo

To examine whether Treml4 could be used to enhance Ag presentation in vivo, we genetically engineered the H chain of the mAb against Treml4 (32D11) (4) to express OVA. As previously described (20), we added a tag sequence called OLLAS between the H chain and the Ag to facilitate tracking of the mAb. For comparison, we used a previously described anti-DEC205-OVA mAb, as well as a control Ig-OVA fusion mAb that has no receptor affinity (GL117) (20). The engineered H chains had a molecular mass ∼110 kDa (Supplemental Fig. 1A) and reacted with anti-mouse IgG1, anti-OVA, and anti-OLLAS by Western blotting (Supplemental Fig. 1B) (20). By FACS, anti-Treml4-OVA bound appropriately to CHO transfectants expressing Treml4 but not to untransfected CHO/NEO cells (Supplemental Fig. 1C).

We used CD8+ OT-I and CD4+ OT-II transgenic T cells as reporters to evaluate the capacity of Treml4 to mediate presentation of OVA through the MHC I and MHC II pathway. B6 mice were injected s.c. with 3 μg anti-Treml4-OVA in the footpad 1 d after inoculating CFSE-labeled OT-I CD8+ T cells or OT-II CD4+ T cells. After 3 d, spleen and skin-draining LNs were harvested, and expansion of CD8+ or CD4+ T cells was evaluated by production of IFN-γ, TNF-α, and IL-2 in response to HIV GAGp24 peptides by multicolor flow cytometry. As shown in Fig. 4, Treml4 mediated efficient Ag presentation to CD8+ T cells (Fig. 4A) and CD4+ T cells (Fig. 4B). Virtually all T cells entered the cell cycle and underwent four to six cell divisions after a single dose of anti-Treml4-OVA mAb, whereas control Ig-OVA elicited no cell division. As quantified in Fig. 4C, anti-Treml4-OVA Ag delivery was less efficient than was anti–DEC-OVA for cross-presentation to CD8+ T cells (Fig. 4C, left panel) but was more efficient for Ag presentation to CD4+ T cells (Fig. 4C, right panel). Taken together, Treml4 efficiently brought about the presentation of OVA Ag on MHC I and MHC II products.

Treml4 HIV GAGp24 fusion mAb primes Th1 CD4+ T cells

To examine the capacity of anti-Treml4 mAb to immunize scarce microbial-specific T cells in the polyclonal repertoire, we introduced HIV GAGp24 (p24) by engineering the H chain. As controls, we again used anti–DEC-p24, as well as a control Ig-p24 mAb without receptor affinity, as previously described (15). As shown in Supplemental Fig. 1, anti–Treml4-p24 had a molecular mass ∼75 kDa (Supplemental Fig. 1D), reacted with anti-mouse IgG1 and anti-p24 by Western blotting (Supplemental Fig. 1E), and bound to CHO cells expressing the Treml4 receptor but not to untransfected CHO/NEO cells (Supplemental Fig. 1F). Thus antitrem4 mAb, like several other mAbs, can be successfully engineered to express the HIV GAGp24 Ag.

To evaluate whether Treml4 could mediate priming of CD4+ T cells in vivo, we immunized B6 mice with 5 μg anti-Treml4-, anti-DEC-, or control Ig- mAbs coupled with HIV GAGp24, along with 25 μg anti-CD40 and 50 μg poly-IC as a maturation stimulus or adjuvant. Two weeks after immunization, Ag-specific responses were evaluated by production of IFN-γ, TNF-α, and IL-2 in response to HIV GAGp24 peptides by multicolor flow cytometry. As shown in Fig. 5A–D, anti–Treml4-p24 induced significantly stronger gag-specific T cell responses compared with control Ig-p24, with frequencies >5–6% of IFN-γ+, TNF-α+, and IL-2–producing CD3+ CD4+ T cells after a single i.p. injection. Importantly, this Ag-specific response induced by delivery of p24 using anti-Treml4 mAb was entirely Treml4 receptor dependent, as shown by the lack of CD4+ T cell priming when Treml4 KO mice were used (Fig. 5G).

To evaluate whether the immune CD4+ T cells could proliferate in response to the reactive GAG peptide pool, we labeled splenocytes with CFSE to follow the successive halving of CFSE/cell with each division. CFSE-labeled splenocytes from mice primed for 2 wk with anti–Treml4-p24 in the presence of 25 μg anti-CD40 + 50 μg poly-IC were cultured for 4 d in the presence of p24 reactive peptide or p17 nonreactive peptide, followed by challenge with p24 peptides. As illustrated in Fig. 5E, and quantified in Fig. 5F, the T cells responded by proliferation and IFN-γ production only to the p24 peptide mix and not to the control p17 peptide mix. We concluded that targeting vaccine proteins with anti-Treml4 mAb was effective at initiating Th1 CD4+ T cell responses.

Similar results also were obtained when we used a prime/boost regimen for immunization in the absence of anti-CD40 in B6 mice (Supplemental Fig. 2).

Treml4 coupled with the breast cancer Ag HER2 elicits T cell immunity and tumor protection

To examine whether Treml4 could mount an immune response against the clinically relevant tumor Ag HER2, we genetically
engineered anti-Trem4 to express the ECD of the breast cancer protein HER2 (Wang et al., submitted for publication). SDS-PAGE and Western blotting expectedly showed that the fusion H chain was 120 kDa and that the fusion mAb reacted to both anti-mouse IgG and anti-HER2 (data not shown). Also, similarly to anti-Trem4 mAbs conjugated with OVA and HIV GAGp24, the fusion mAb bound to CHO cells expressing Trem4 but not to CHO NEO cells (data not shown).

To determine the immunogenicity of the anti–Trem4-HER2 mAb in vivo, FVB/N mice were immunized in a prime-boost regimen consisting of two doses, administered 4 wk apart, of 5 μg anti–DEC205 or anti-Trem4 mAbs conjugated with HIV GAGp24 (p24) in the presence of 50 μg poly-IC and 25 μg anti-CD40 (Adj). A mAb conjugated with HIV GAGp24 without receptor affinity was used as a control (control-p24). Fourteen days after inoculation, splenocytes were restimulated in vitro with HIV GAGp17 (p17, negative control) or HIV GAGp24 peptide mix (p24) in the presence of BFA for 6 h. A. Intracellular staining was performed to detect the percentage of IFN-γ in CD3+ CD4+ gated T cells. B. Percentage of IFN-γ+ CD4+ T cells is shown as mean ± SD of four to six experiments with two or three mice/group. As in B, but TNF-α (C) and IL-2 (D) cytokine production was evaluated by intracellular cytokine staining in the CD3+ CD4+ gated T cells in response to HIV GAGp17 or HIV GAGp24 peptide mix. Mean ± SD from two experiments with eight mice total is shown. E. Bulk splenocytes were labeled with CFSE and stimulated with HIV GAGp24 peptide mix (p24) or control HIV GAGp17 peptide mix (p17) for 4 d, wherein the cells were restimulated for 6 h with HIV GAGp24 peptides in the presence of BFA to detect IFN-γ in proliferated CFSE™ T cells. Panels show the percentage of IFN-γ+ or IFN-γ- CD3+ CD4+ proliferating T cells (top row and bottom row, respectively) from one of two similar experiments with four mice/group. F. As in E, but the percentage of CFSElow IFN-γ+ CD4+ T cells is shown as mean ± SD of eight animals in two different experiments. G. B6 or Trem4 KO mice were immunized with 5 μg anti–DEC205 or anti-Trem4 mAbs conjugated with HIV GAGp24 (p24) in the presence of 50 μg poly-IC and 25 μg anti-CD40 (Adj) and analyzed 14 d after restimulation in vitro with HIV GAGp17 (p17, negative control) or HIV GAGp24 peptide mix (p24) in the presence of BFA for 6 h. Percentage of IFN-γ+ CD4+ T cells is shown as mean ± SD of two experiments, with a total of four mice/group.

We extended the experiments to determine whether anti-Trem4-HER2 could be used to induce an antitumor response in vivo. We tested tumor protection in a neu-expressing mouse transplantable-tumor model. In a different study, we showed that targeting HER2 to DEC205 receptor was able to mediate efficient tumor protection by a T cell-dependent mechanism (Wang et al., submitted for publication). To this end, we primed and boosted FVB/N mice with anti–Trem4-HER2, along with poly-IC as an adjuvant, as above. Ten days after the boost vaccination, mice were injected with 1 × 10^6 neu-expressing NT2.5 tumor cells. Mice were monitored for tumor growth every other day for 35 d. As shown in Fig. 6C, mice that received anti–Trem4-HER2 mAb showed significantly smaller tumor volumes compared with those injected with control HER2, poly-IC alone, or PBS (p < 0.01) (Fig. 6C). This protection was mediated by both CD4+ and CD8+ T cells, because depletion of either (or both) T cells strongly abolished tumor protection (Fig. 6D). Thus, directing HER2 to Trem4-expressing cells resulted in significant tumor protection in vivo.

Discussion

We used Ag targeting with anti-mouse Trem4 mAbs to determine the capacity of this receptor for dying cells to mediate Ag presentation and immunization of naïve mice. It was feasible to engineer three proteins—OVA, HIV GAGp24, and the extracellular
domain of the oncogene HER2—into the H chain of cloned anti-Trem4 mAbs. With OVA, we studied clonal expansion of CD4+ and CD8+ TCR-transgenic T cells 3 d after a s.c. dose of 3 μg fusion mAbs was inoculated into the footpad. When we compared Ag presentation after targeting OVA using anti-Trem4 or anti-DEC mAbs, we found that anti-Trem4-OVA was more efficient at inducing CD4+ T cell expansion, whereas anti-DEC-OVA was more efficient for CD8+ T cells. Accordingly, anti–Trem4-OVA was comparable in bringing about Ag presentation to another mAb we studied that reacts with CD8α+ DCs (i.e., anti-langerin; data not shown) (20). With HIV GAGp24 and HER2, we studied immunization of naïve mice. For this to take place, it was necessary to coadminister with the fusion mAb a stimulus for innate immunity; in our experiments, this was poly-IC administered alone or in combination with anti-CD40. As in prior work on Ag targeting to CD8α+ DCs via mAbs against DEC205, langerin, and Clec9A (15), targeting to the Ig superfamily member Trem4 greatly enhanced immunity relative to a nonreactive Ig control. The targeting was receptor specific, because immunization was ablated in Trem4 KO mice.

Trem4 is an unusual receptor for dying cells, because it is primarily expressed in spleen and not other lymphoid or nonlymphoid tissues (4). The high expression in spleen was previously attributed to high expression in red pulp macrophages and CD169+ marginal metallophilic macrophages (4). We extended the analysis of Trem4 expression to a large panel of cell types in spleen. The heightened expression on CD8α+ DCs and on macrophages was confirmed. In addition, expression on splenic monocytes was noted. Blood monocytes were also positive for Trem4, particularly the Ly6Cd+ subset. However, peritoneal macrophages were Trem4+, as were liver and LN CD169+ sinus macrophages, as documented in our initial report (data not shown) (4). Because blood monocytes express this dying cell receptor, but spleen is the main site for Trem4+ macrophages, we suspect the spleen sustains Trem4 on monocytes that become splenic macrophages, whereas other tissues do not. Alternatively, Ly6Cd+ monocytes may primarily be giving rise to splenic, but not other, macrophages in the steady state.

We did not detect major differences in immunization outcome via Trem4 targeting relative to targeting DEC205, which is highly expressed on the CD8α− subset of DCs. Furthermore, Trem4 was clearly able to mediate induction of an integrated and protective CD4+ and CD8+ T cell response, as noted with HER2-specific immune responses in FVB mice.

Trem4 is only one of several receptors that DCs express with the potential to react with dying cells. Others include CD36 (26), αvβ3 and αvβ5 (27, 28), and CLEC 9A (29). Of note, these receptors are specific for the CD8α+ DC subset, which, in contrast to CD8α− DCs, can selectively take up and present dying cells (Fig. 1) (30). CLEC9A has been studied most extensively for cross-presentation of dying cell-associated Ags. The data showed that CLEC9A is not required for phagocytosis but instead contributes to cross-presentation following the uptake of dead cell-associated Ags (29). In contrast, CD36, αvβ5, and αvβ3 do not appear to...
contribute to the superior cross-presentation abilities of CD8+ DCs (31). We were unable to detect alterations in either uptake or cross-presentation of cell-associated OVA in Trem4 KO mice relative to WT mice. This result suggested overlapping function between Trem4 and other dying cell uptake receptors in vivo. Alternatively, it is possible that dying cells used in experimental studies are not the best physiological representatives of dying cells in vivo.

As of now, a human anti-Trem4 mAb has not been produced. Thus, the function of this molecule and its clinical application in humans remains unclear. According to sequencing data (GenBank), the human Trem4 gene contains a stop codon in the transmembrane region, which could be consistent with its release as a soluble protein (4). Trem4 also shares functional similarities with another Trem family member, Trem2; although the expression of Trem4 has not been mapped in humans, Trem2 is known to be expressed on human macrophages and monocyte-derived DCs (4). It was also shown that mouse Trem2 is involved in the clearance of apoptotic neurons by microglia (8). Likewise, we reported that soluble Trem4 has a strong affinity for dead cells, suggesting a functional similarity between the respective molecules (4). Therefore, it is possible that Trem2 and Trem4 may have compensatory functions, warranting further studies of both molecules in humans.

C-type lectin-like receptors have been emphasized for receptor-mediated Ag targeting within mAbs. Trem4 is unique, in that it belongs to the Ig superfamily. The use of mAb-based Ag targeting allowed the faithful delivery of Ag to the cells that express the receptor (i.e., Trem4), and it enabled an assessment of the ability of that receptor to enhance Ag presentation and immunization in an intact animal. Specifically, we showed that a receptor previously documented to engage with necrotic cells can capture, present Ags, and, in the presence of an innate stimulus like poly-IC, induce Th1 CD4+ and CD8+ T cell immunity that promotes tumor protection.

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Disclosures
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