mTOR Complex Signaling through the SEMA4A–Plexin B2 Axis Is Required for Optimal Activation and Differentiation of CD8$^+$ T Cells

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Mammalian target of rapamycin (mTOR) plays crucial roles in activation and differentiation of diverse types of immune cells. Although several lines of evidence have demonstrated the importance of mTOR-mediated signals in CD4$^+$ T cell responses, the involvement of mTOR in CD8$^+$ T cell responses is not fully understood. In this study, we show that a class IV semaphorin, SEMA4A, regulates CD8$^+$ T cell activation and differentiation through activation of mTOR complex (mTORC) 1. SEMA4A$^{-/-}$ CD8$^+$ T cells exhibited impairments in production of IFN-γ and TNF-α and induction of the effector molecules granzyme B, perforin, and FAS-L. Upon infection with OVA-expressing *Listeria monocytogenes*, pathogen-specific effector CD8$^+$ T cell responses were significantly impaired in SEMA4A$^{-/-}$ mice. Furthermore, SEMA4A$^{-/-}$ CD8$^+$ T cells exhibited reduced mTORC1 activity and elevated mTORC2 activity, suggesting that SEMA4A is required for optimal activation of mTORC1 in CD8$^+$ T cells. IFN-γ production and mTORC1 activity in SEMA4A$^{-/-}$ CD8$^+$ T cells were restored by administration of recombinant Sema4A protein. In addition, we show that plexin B2 is a functional receptor of SEMA4A in CD8$^+$ T cells. Collectively, these results not only demonstrate the role of SEMA4A in CD8$^+$ T cells, but also reveal a novel link between a semaphorin and mTOR signaling.
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Mammalian target of rapamycin (mTOR; also known as the mechanistic target of rapamycin) is a conserved serine/threonine kinase that plays a central role in regulation of cell growth and metabolism (1–3). mTOR forms two multiprotein complexes, mTOR complex (mTORC) 1 and mTORC2. mTORC1 contains regulatory-associated protein of mTOR (RAPTOR), which is sensitive to rapamycin, whereas mTORC2 contains the rapamycin-insensitive companion of mTOR (RICTOR), which is resistant to rapamycin. mTORC1 directly phosphorylates S6 kinase (S6K), leading to an increase in mRNA biogenesis, translational initiation and elongation, and protein synthesis (4). In the immune system, mTOR regulates the differentiation of specific types of CD4$^+$ T cells via selective activation of mTORC1- or mTORC2-mediated signals (5). For instance, T cell–specific deletion of *Mtor* results in abrogation of Th1, Th2, and Th17 cell differentiation (6). A specific deletion of *Rheb* in T cells, which leads to activation of mTORC1 signaling, results in the loss of Th1 and Th17 cell differentiation, whereas Th2 cell generation is unaffected (5). By contrast, CD4$^+$ T cells that lack RICTOR, and thus lack mTORC2 signaling, are readily skewed toward Th1 or Th17 cell lineages, but

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Abbreviations used in this article: DC, dendritic cell; IRF4, IFN regulatory factor 4; LM-OVA, OVA-expressing *Listeria monocytogenes*; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NRP, neuropilin; PLC, phospholipase C; RAPTOR, regulatory-associated protein of mTOR; RICTOR, rapamycin-insensitive companion of mTOR; shRNA, short hairpin RNA; S6K, ribosomal protein S6 kinase; TIM2, T cell Ig and mucin domain–containing protein 2; Treg, regulatory T.

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fail to differentiate into Th2 cells (5, 7). In addition, RICTOR-deficient mice are resistant to Th2 cell–mediated diseases (5, 8). These observations provide convincing evidence that mTORC1 is required for Th1 and Th17 cell differentiation, and that mTORC2 is necessary for Th2 cell development.

In contrast, only a few studies have suggested the involvement of mTORC1 signaling in $CD8^+$ T cell responses (9). For instance, T cell–specific deletion of RAPTOR abrogates $CD8^+$ T cell effector function in response to infection (10). The mTORC1–hypoxia-inducible factor 1 pathway is required to sustain glucose metabolism and glycolysis in differentiation of $CD8^+$ T cells (11). However, the mechanisms underlying the roles of mTOR-mediated signals in $CD8^+$ T cell functions remain obscure.

Semaphorins, originally identified as repulsive axon-guidance factors that participate in neuronal development (12–14), can be divided into eight classes. Invertebrate semaphorins are grouped into classes I and II; vertebrate semaphorins are grouped into classes III–VII; and virus semaphorins are grouped into class VIII (14). Semaphorins exert pleiotropic functions, playing roles in cardiogenesis (15, 16), angiogenesis (17, 18), tumor progression or suppression (19), bone homeostasis (20, 21), and immune responses (22, 23). Recent findings indicate that several semaphorins are involved in various phases of immune responses, including immune cell activation, differentiation, cell–cell interactions, and trafficking/migration (24). SEMA4A, a class IV transmembrane semaphorin, is preferentially expressed in dendritic cells (DCs) and Th1 cells (25, 26). We have previously demonstrated that SEMA4A is crucially involved not only in Ag-specific T cell priming, but also in Th1 cell and Th17 cell differentiation (26, 27). In addition, SEMA4A is required for the function and stability of regulatory T (Treg) cells (28). However, the roles of SEMA4A in $CD8^+$ T cell responses have not been determined.

Plexins (plexin A1–A4, plexin B1–B3, plexin C1, and plexin D1) and neuropilins (NRP1 and NRP2) are the primary semaphorin receptors (29, 30). In general, most membrane-bound semaphorins directly bind to plexins, whereas soluble class III semaphorins generally require NRPs as obligate coreceptors. Semaphorin–plexin signaling mediates diverse functions by regulating the activities of small GTPases and cytoplasmic/receptor-type kinases, and also regulates integrin-mediated attachment, actomyosin contraction, and microtubule destabilization (31–34). SEMA4A is bound by plexin Bs, plexin D1, T cell Ig and mucin domain–containing protein 2 (TIM2), and NRP1, and each of these receptors mediates distinct functions. For instance, via plexin D1, SEMA4A inhibits endothelial cell migration and in vivo angiogenesis by suppressing vascular endothelial growth factor–mediated activation of Rac and integrin-dependent cell adhesion (17). In the presence of the Rho family GTPase Rnd1, the binding of SEMA4A to plexin Bs induces cellular contraction through enzymatic activity of R-Ras, a GTPase-activating protein (35, 36).

In this study, we investigated the significance of SEMA4A in $CD8^+$ T cell responses. Our findings revealed that SEMA4A deficiency resulted in impaired activation and differentiation of $CD8^+$ T cells. In vitro experiments showed that SEMA4A$^{-/-}$ $CD8^+$ T cells exhibited reduced cytokine production and induction of effector molecules, and in vivo experiments showed that SEMA4A$^{-/-}$ mice exhibited impaired pathogen-specific effector $CD8^+$ T cell responses upon OVA-expressing *Listeria monocytogenes* (LM-OVA) infection. Of note, in SEMA4A$^{-/-}$ $CD8^+$ T cells, mTORC1 activity was reduced, and mTORC2 activity was elevated. We also showed that plexin B2, but not plexin B1, plexin B3, plexin D1, TIM2, or NRP1, functions as the receptor of SEMA4A in $CD8^+$ T cells.

Materials and Methods

Mice

C57BL/6J mice were purchased from CLEA Japan. C57BL/6J SEMA4A$^{-/-}$ mice [previously established (26)] were bred at the Animal Resource Center for Infectious Diseases, Research Institute for Microbial Diseases and Immunology Frontier Research Center, Osaka University. All animal procedures were conducted according to institutional guidelines.

In vitro stimulation of $CD8^+$ T cells

$CD8^+$ T cells were isolated using the Mouse CD8 T Lymphocyte Enrichment Set–DM (BD Biosciences) and cultured in RPMI 1640 medium (Nacalai Tesque) supplemented with 10% FCS and antibiotics. For in vitro generation of effector cells, naive $CD8^+$ T cells prepared from mouse spleen were stimulated for 2 d with plate-bound anti-CD3ε (2C11; BD Pharmingen) and anti-CD28 (37.51; BD Pharmingen) Abs.

Proliferation assay

For BrdU incorporation assays, cells were cultured for 24 h of culture with the indicated stimulus, supplemented with BrdU, and incubated for another 24 h. Cells were then fixed, the DNA was denatured, and BrdU content was assessed using the Cell Proliferation ELISA, BrdU (colorimetric; Roche Applied Science).

Abs

A mAb against SEMA4A that recognizes both the mouse and human proteins was generated as previously described (26). Polyclonal Abs against plexin B3 that recognize both mouse and human proteins were also generated. The following Abs were used for flow cytometry: anti-CD8α (53-6.7), anti-CD4 (GK1.5), anti-CD3ε (145-2C11), anti-CD11c (HL3), anti–IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), anti–granzyme B (NGZB), anti–T-bet (4B10), anti-Eomes (Dan11mag), anti–TCR-β (H57-597), anti-CD28 (37.51), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (HI.2F3), anti–LFA-1 (M1714), anti-CD122 (TM-B1), anti–plexin B2 (3E7), anti-TIM2 (F37-2C4), anti–H-2K^b (AF.6-88.5), and anti–p-p70-S6K (Ser^{371}); these Abs were purchased from BioLegend, eBioscience, or Cell Signaling Technology. The following Abs were used for Western blotting: anti–p-44/42-MAPK (Thr^{202}/Tyr^{204}; 197G2), anti–44/42-MAPK, anti–p-phospholipase C (PLC)-γ1 (Tyr^{783}), anti–PLC-γ1 (D9H10), anti–p-stress-activated protein kinase/JNK (Thr^{183}/Tyr^{185}), anti–stress-activated protein kinase/JNK, anti–p-ZAP70 (Tyr^{319})/Syk (Tyr^{52}), anti-ZAP70 (D1C10E), anti–β-actin (13E5), anti–p-p70-S6K (Thr^{389}; 108D2), anti–p70-S6K (49D7), anti–p-mTOR (Ser^{2448}), anti-mTOR, anti–p-AKT (Ser^{473}; D9E), anti–p-AKT (Thr^{308}), and anti-AKT (C67E7); these Abs were purchased from Cell Signaling Technology. Anti–plexin B1 and anti–plexin D1 were purchased from Abcam, and anti–IFN regulatory factor 4 (IRF4; M-17) was purchased from Santa Cruz Biotechnology.

Flow cytometry

Cells were prepared and stained for FACS analysis of cell surface markers with the following fluorochrome-conjugated Abs diluted in PBS (Nacalai Tesque) containing 2% FBS. For extracellular staining, cells were incubated with rat serum and anti-CD16/CD32, and then stained with the indicated Abs. To evaluate SEMA4A expression in specific T cell populations, we identified $CD8^+$ T cells ($CD8\alpha^+$) and $CD4^+$ T cells ($CD4^+CD82^+$) using the indicated markers. For intracellular staining, cells were incubated in the presence of brefeldin A (GolgiPlug; BD Biosciences) for 5 h. After staining of cell surface molecules, intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences). Stained cells were analyzed on a FACS Canto II (BD Biosciences), and data were processed using the FlowJo software (Tree Star).

L. monocytogenes *infection*

A *L. monocytogenes* strain expressing a secreted form of OVA (LM-OVA) was provided by M. Murakami (Neuroimmunology, Institute of Genetic Medicine, Graduate School of Medicine, Hokkaido University). For bacterial infection, mice were injected i.v. with a priming dose of 2×10^3 CFU. Spleens were analyzed 7 d postinfection. Splenocytes were stained with H-2K^{b}OVA$_{257–264}$ tetramer SIINFEKL PE (MBL). OVA-specific $CD8^+$ T cells were detected with H-2K^{b}OVA$_{257–264}$. For intracellular cytokine staining, cell suspensions were incubated for 5 h at 37°C with 100 ng/ml OVA$_{257–264}$ peptides in the presence of brefeldin A. After staining of cell surface molecules, intracellular cytokine staining was performed using the Cytofix/Cytoperm kit. Fluorochrome-labeled Abs

were obtained from eBioscience and BioLegend. The stained cells were subjected to flow cytometry on a FACS Canto II, and the data were processed using the FlowJo software.

Western blot analysis

$CD8^+$ T cells were stimulated with 1 μg/ml anti-CD3ε and 2 μg/ml anti-CD28 in RPMI 1640 medium containing 10% FCS and antibiotics. Cells were collected and lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, and 1 mM NaF). Lysates were subjected to immunoblot analyses, which were performed using standard protocols. For SDS-PAGE, samples were boiled for 5 min in SDS-PAGE sample buffer containing 0.125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 10% 2-ME. Protein samples were loaded onto NuPAGE 4–12% Bis-Tris gels (Invitrogen). Sample preparation and electrophoresis were performed according to the manufacturer's instructions. For immunoblot analysis, the gel was electroblotted onto a polyvinylidene difluoride membrane, which was blocked in 5% BSA in TBST, incubated with specific primary Ab, and then incubated with the appropriate secondary Ab.

RT-PCR analysis

Total RNA was prepared using the RNeasy mini kit (Qiagen). cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen). mRNA expression was quantitated on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primer sets were purchased from Applied Biosystems.

In vitro gene knockdown using lentivirus

Lentiviral particles expressing short hairpin RNA (shRNA) targeting plexin B2 or control shRNA were purchased from Santa Cruz Biotechnology. The infection of naive $CD8^+$ T cells with lentivirus was carried out by adding lentiviral particles to cell cultures containing anti-CD3ε (1 μg/ml), anti-CD28 (2 μg/ml), and IL-2 (100 U/ml) in the presence of puromycin. Cells were harvested 7 d postinfection.

ELISA analysis

Naive $CD8^+$ T cells (1×10^5 per well) were stimulated for 2 d with plate-bound anti-CD3ε (1 μg/ml) and anti-CD28 (2 μg/ml) Abs in flat-bottom 96-well plates. The supernatant fluid was analyzed by ELISA (IFN-γ and TNF-α; R&D Systems). For a functional rescue experiment of $SEMA4A^{-/-}$ $CD8^+$ T cells, $CD8^+$ T cells were cultured with anti-CD3 and anti-CD28 Abs for 2 d in the presence of SEMA4A-Fc recombinant protein or control human IgG-Fc proteins (Millipore). For shRNA-mediated knockdown of plexin B2 in $CD8^+$ T cells, the infection of naive $CD8^+$ T cells with lentivirus was carried out by adding lentiviral particles expressing shRNA targeting plexin B2 or control shRNA to cell cultures containing anti-CD3ε and anti-CD28 Abs for 2 d.

Cross-presentation assay

In vitro cross-presentation assays were performed, as previously described (37). Briefly, splenic DCs were isolated with $CD11c^+$ magnetic beads (Miltenyi Biotec) from wild-type or $SEMA4A^{-/-}$ mice. DCs were incubated for 15 h with or without OVA. Subsequently, DCs were stained with anti-CD16/CD32, followed by staining with anti-CD11c (HL3) and anti-H-$2K^b$–$OVA_{257–264}$ (25-D1.16), and then analyzed by flow cytometry.

Microarray data

Microarray data are accessible through Gene Expression Omnibus Series accession GSE15907 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15907).

Results

Expression patterns of SEMA4A in $CD8^+$ T cells

We first examined expression of SEMA4A in $CD8^+$ T cells. As shown in Fig. 1, SEMA4A was abundantly expressed in naive $CD8^+$ T cells, but not in naive $CD4^+$ T cells (Fig. 1A). Next, we examined whether the expression levels of SEMA4A in $CD8^+$ T cells changed during activation. After stimulation with anti-CD3 and anti-CD28, the expression of SEMA4A in $CD8^+$ T cells was transiently downregulated on days 1 and 2, but upregulated above the levels in the naive state on days 5 and 7 (Fig. 1B). To evaluate the expression of SEMA4A in $CD8^+$ T cells in vivo, we infected mice with LM-OVA, which initiates an acute infection and activates $CD8^+$ T cells (38). As shown in Fig. 1C, on day 7 postinfection, SEMA4A expression in $CD8^+$ T cells prepared from these mice was elevated. Thus, these findings not only demonstrate expression of SEMA4A in $CD8^+$ T cells, but also imply that this protein is functionally relevant to $CD8^+$ T cell responses.

$SEMA4A^{-/-}$ $CD8^+$ T cells exhibited reduced cytokine production and induction of effector molecules

We next examined the involvement of SEMA4A in proliferation and differentiation of $CD8^+$ T cells. BrdU incorporation assays and flow cytometry using propidium iodide and annexin V revealed no significant difference in proliferation or frequency of apoptosis between wild-type and $SEMA4A^{-/-}$ $CD8^+$ T cells (Fig. 2A, 2B). We then evaluated production of IFN-γ and TNF-α, cytokines that are essential for $CD8^+$ T cell differentiation, by $SEMA4A^{-/-}$ $CD8^+$ T cells. As shown in Fig. 2C and 2D, production of both IFN-γ and TNF-α was significantly impaired in $SEMA4A^{-/-}$ $CD8^+$ T cells. In addition, expression of effector molecules such as granzyme B, perforin, and FAS-L was considerably reduced in $SEMA4A^{-/-}$ $CD8^+$ T cells (Fig. 2E, 2F, Supplemental Fig. 1). Expression levels of T-bet and Eomes, transcription factors that play important roles in $CD8^+$ T cell differentiation (39, 40), were also reduced in $SEMA4A^{-/-}$ $CD8^+$ T cells (Fig. 2G, 2H). These findings indicate that SEMA4A is involved in differentiation of $CD8^+$ T cells.

However, the levels of other transcription factors such as *Bcl6*, *Prdm1*, and *Id2* were comparable between wild-type and $SEMA4A^{-/-}$ $CD8^+$ T cells (Supplemental Fig. 2A). Also, no significant difference was observed in expression of *Irf4*, which was recently reported to be crucial for $CD8^+$ T cell differentiation and expansion (41) (Supplemental Fig. 2B, 2C).

Impaired pathogen-specific effector $CD8^+$ T cell responses in $SEMA4A^{-/-}$ mice

To determine the role of SEMA4A in $CD8^+$ T cell responses in vivo, we infected wild-type and $SEMA4A^{-/-}$ mice with LM-OVA, and then tracked activation of Ag-specific $CD8^+$ T cells using H-$2K^b$ $OVA_{257–264}$ tetramer. On day 7 postinfection, the frequency of $OVA_{257–264}$-specific $CD8^+$ T cells was lower in $SEMA4A^{-/-}$ mice than in wild-type mice (Fig. 3A). In addition, the frequency of $CD8^+$ T cells producing IFN-γ or TNF-α was also considerably lower in $SEMA4A^{-/-}$ mice (Fig. 3B). These results indicated that SEMA4A is important for in vivo activation and differentiation of Ag-specific $CD8^+$ T cells in response to pathogen infection.

Because SEMA4A expressed in DCs is important for Ag-specific T cell priming (26), we examined the involvement of SEMA4A in cross-presentation of DCs. As shown in Supplemental Fig. 3, wild-type or $SEMA4A^{-/-}$ DCs were equally capable of presenting OVA peptides on MHC class I molecules, suggesting that the reduced $CD8^+$ T cell responses in $SEMA4A^{-/-}$ mice were not primarily due to impaired cross-presentation of DCs.

Decreased mTORC1 activity and increased mTORC2 activity in $SEMA4A^{-/-}$ $CD8^+$ T cells

We next investigated whether impaired activation and differentiation of $SEMA4A^{-/-}$ $CD8^+$ T cells were due to defective TCR-mediated signaling. However, expression levels of the TCR subunits TCRVα and TCRVβ, coreceptors CD3ε and CD8α, and costimulatory molecule CD28, were not reduced in $SEMA4A^{-/-}$ $CD8^+$ T cells (Fig. 4A). Also, phosphorylation of ERK, PLC-γ, JNK, and ZAP70, key molecules involved in downstream signaling pathway after TCR stimulation, was not inhibited in

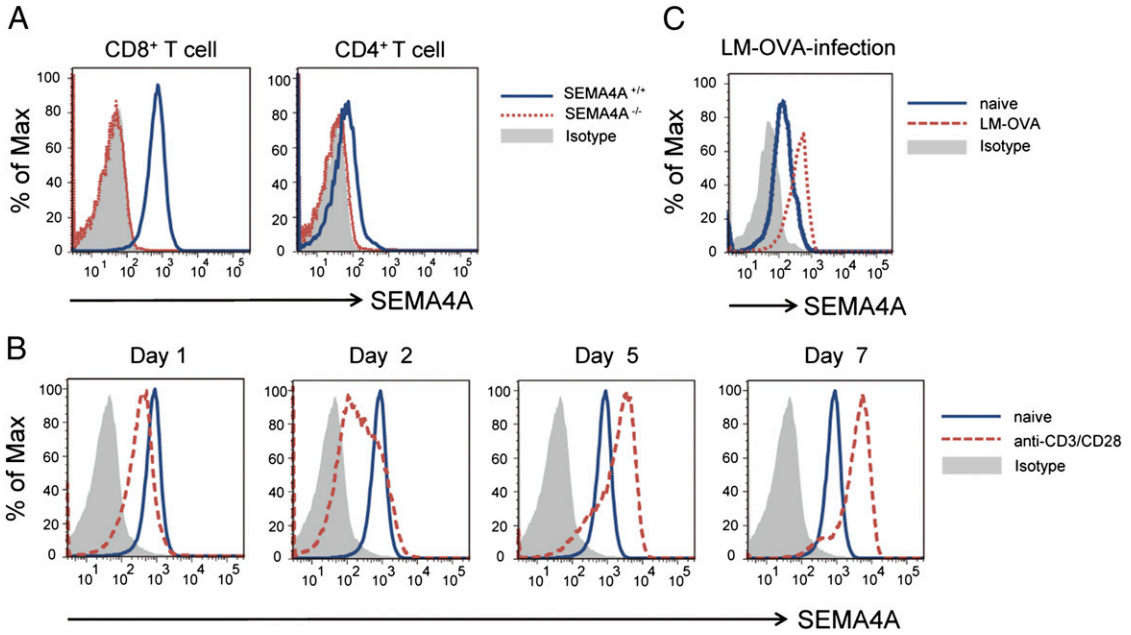

FIGURE 1. SEMA4A expression in CD8$^+$ T cells. (**A**) Flow cytometric analysis of SEMA4A expression in CD8$^+$ or CD4$^+$ T cells prepared from wild-type (blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice. Wild-type T cells were also stained with an isotype-matched control Ab (isotype, gray-filled histogram). (**B**) SEMA4A expression in CD8$^+$ T cells cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 1, 2, 5, or 7 d (activated: red dotted line). Naive CD8$^+$ T cells were also analyzed by flow cytometry (naive: blue solid line). (**C**) Wild-type mice were infected with 2×10^3 CFU LM-OVA. SEMA4A expression in CD8$^+$ T cells prepared from infected mice (activated: red dotted line) or uninfected mice (naive: blue solid line) was analyzed by flow cytometry on day 7 after infection.

SEMA4A$^{-/-}$ CD8$^+$ T cells (Fig. 4B). In addition, expression levels of other molecules associated with CD8$^+$ T cell activation and differentiation, such as CD25, CD44, CD62L, CD69, CD122, and LFA-1, were not reduced in SEMA4A$^{-/-}$ CD8$^+$ T cells (Fig. 4C). Thus, these results showed that TCR-mediated signaling pathway is not blocked in SEMA4A$^{-/-}$ CD8$^+$ T cells, suggesting that there is another mechanism that explains the impaired CD8$^+$ T cell responses in the absence of SEMA4A.

In ray precursor cells in *Caenorhabditis elegans*, the membrane-bound semaphorins SMP-1 and SMP-2 promote a shift in the TOR adaptor from RICTOR toward RAPTOR, leading to upregulation of TORC1 and downregulation of TORC2 (42). Therefore, we hypothesized that SEMA4A regulates activation of mTORC1- and/or mTORC2-mediated signals in CD8$^+$ T cells. We then compared mTORC1 and mTORC2 activity between wild-type and SEMA4A$^{-/-}$ CD8$^+$ T cells, and found that SEMA4A$^{-/-}$ CD8$^+$ T cells exhibited significantly reduced phosphorylation of mTOR and S6K, a downstream target of mTORC1 (Fig. 5A–C). By contrast, AKT Ser^{473} phosphorylation, which is a target of mTORC2 (43), was elevated in SEMA4A$^{-/-}$ CD8$^+$ T cells. In addition, AKT Thr^{308} phosphorylation by the protein kinase PDK1, which is upstream of mTORC1 signaling (44–47), was not affected (Fig. 5D). Phosphorylation of PI3K, a signaling protein upstream of AKT, was comparable between wild-type and SEMA4A$^{-/-}$ CD8$^+$ T cells (Fig. 5E). Furthermore, decreased phosphorylation of mTOR and S6K was restored by addition of SEMA4A-Fc proteins into cultures of SEMA4A$^{-/-}$ CD8$^+$ T cells, supporting the involvement of SEMA4A in activation of mTORC1 (Fig. 5F). Consistent with this, IFN-γ production by CD8$^+$ T cells was also decreased by rapamycin (Fig. 5G). Thus, mTORC1 activity is reduced and mTORC2 activity is elevated in SEMA4A$^{-/-}$ CD8$^+$ T cells, suggesting that SEMA4A mediates conversion of mTORC2 to mTORC1 signaling in CD8$^+$ T cells, as in *C. elegans*.

Plexin B2 is a receptor of SEMA4A in CD8$^+$ T cells

We next examined whether recombinant soluble SEMA4A protein binds to CD8$^+$ T cells. As shown in Fig. 6A, SEMA4A bound to activated CD8$^+$ T cells that were stimulated by anti-CD3 and anti-CD28, but did not bind naive CD8$^+$ T cells. Furthermore, reduced IFN-γ production in SEMA4A$^{-/-}$ CD8$^+$ T cells was restored by supplementation of soluble recombinant SEMA4A protein into their culture (Fig. 6B). These results suggested that SEMA4A-dependent signals are mediated via a receptor expressed on CD8$^+$ T cells.

As described above, plexin Bs, plexin D1, TIM2, and NRP1 bind to SEMA4A. We examined expression of these candidate molecules in CD8$^+$ T cells. As shown in Supplemental Fig. 4A–C, no expression of plexin B1, plexin B3, plexin D1, TIM2, or NRP1 was detected in CD8$^+$ T cells. With regard to integrins, which can bind to semaphorins and mediate their signaling (23, 48), expression levels of CD18, CD29, CD49b, CD49d, CD49e, and CD49f were comparable between wild-type and SEMA4A$^{-/-}$ CD8$^+$ T cells, either with or without stimulation by anti-CD3 and anti-CD28 (Supplemental Fig. 4D, 4E). However, we detected plexin B2 on activated CD8$^+$ T cells, and the levels of this protein were comparable between wild-type and SEMA4A$^{-/-}$ CD8$^+$ T cells (Fig. 6C, 6D). When CD8$^+$ T cells were treated with anti–plexin B2 blocking Ab, the binding of SEMA4A to CD8$^+$ T cells was significantly reduced (Fig. 6E). In addition, plexin B2–knockdown cells produced significantly less IFN-γ (Fig. 6F, 6G). These results suggest that plexin B2 is the functional receptor of SEMA4A in CD8$^+$ T cells.

Discussion

In this study, we demonstrated that by regulating mTOR signaling, SEMA4A plays an important role in activation and differentiation of CD8$^+$ T cells. SEMA4A was expressed in CD8$^+$ T cells, and deficiency in this protein resulted in impaired activation and differentiation of CD8$^+$ T cells both in vitro and in vivo. In addition, we showed that the SEMA4A–plexin B2 axis mediates conversion from mTORC2 to mTORC1 signaling in CD8$^+$ T cells.

After pathogen infection, CD8$^+$ T cells distributed in peripheral lymphoid organs transit from a quiescent state to the active and

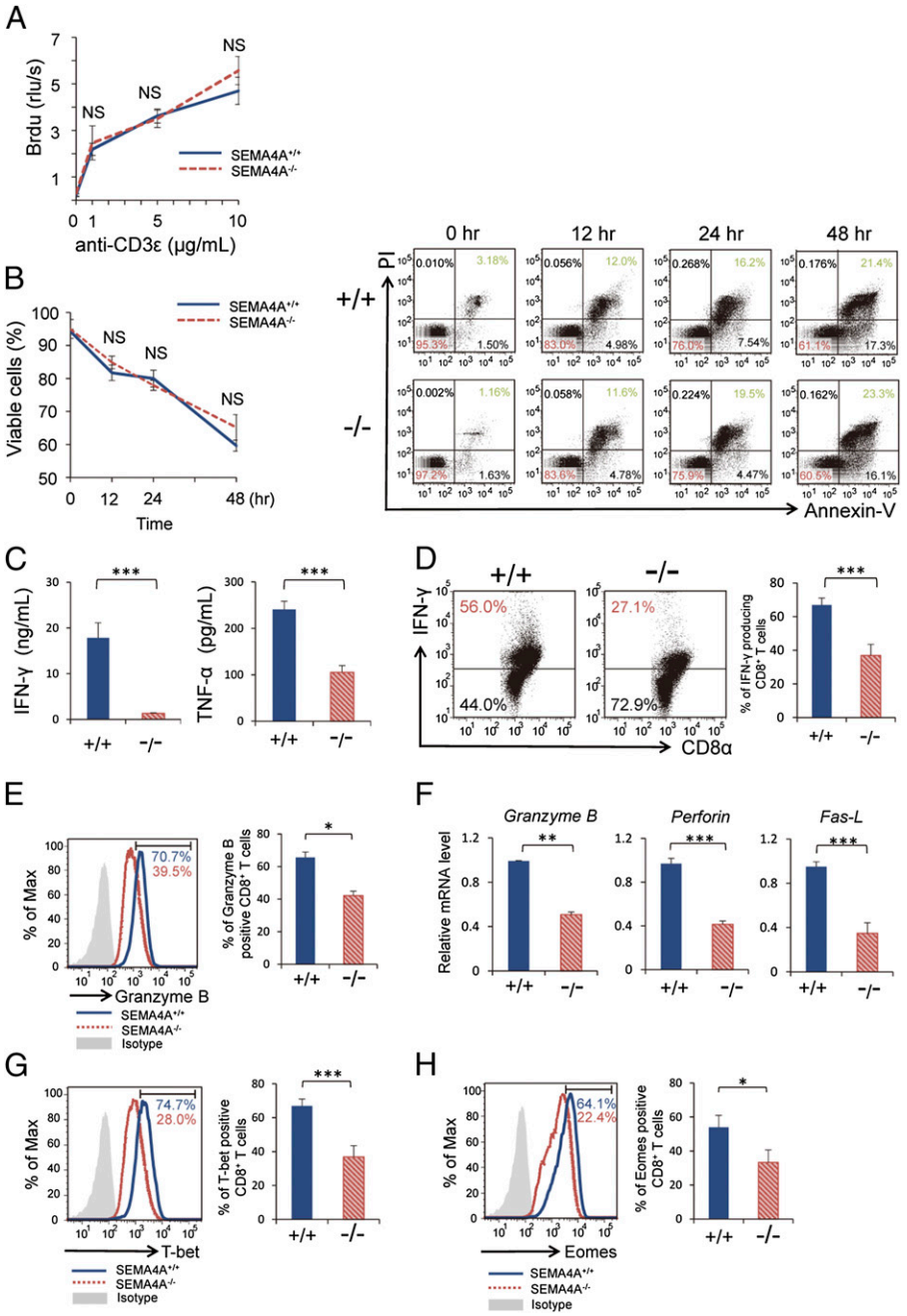

FIGURE 2. Impaired production of cytokines and decreased expression of effector molecules in SEMA4A$^{-/-}$ CD8$^+$ T cells. (**A**) CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice were cultured with anti-CD3 for 24 h, supplemented with BrdU, and incubated for another 24 h. Subsequently, proliferation of these cells was evaluated by BrdU–DNA ELISA. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). Data are representative of two independent experiments. (**B**) CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ mice (red dotted line) were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 12, 24, or 48 h. These cells or cells without stimulation (0 h) were stained with annexin V and propidium iodide. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 4$ from each group). (**C**) CD8$^+$ T cells prepared from wild-type ($^{+/+}$, blue closed bar) or SEMA4A$^{-/-}$ mice ($^{-/-}$, red hatched bar) were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. Levels of IFN-γ and TNF-α in the culture supernatants were measured by ELISA. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). ***$p < 0.001$. (**D**) The resultant cells were also analyzed by intracellular cytokine staining. The frequencies of IFN-γ–positive CD8$^+$ T cells from three independent experiments were analyzed by Student *t* test. (**E**–**H**) CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. Intracellular levels of granzyme B, T-bet, and Eomes were determined by intracellular cytokine staining. The frequencies of granzyme B, T-bet, or Eomes-positive CD8$^+$ T cells from two or three independent experiments were analyzed by Student *t* test. (F) CD8$^+$ T cells prepared from wild-type ($^{+/+}$, blue closed bar) or SEMA4A$^{-/-}$ ($^{-/-}$, red hatched bar) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. Relative expression levels of mRNAs encoding *Granzyme B*, *Perforin*, or *FAS-L* were determined by quantitative real-time PCR, with *GAPDH* as the normalization control. Data are representative of three independent experiments (see also Supplemental Fig. 1). Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

proliferating state. These cells secrete cytokines such as IFN-γ and TNF and differentiate into cytotoxic T lymphocytes, which kill infected cells through cytotoxic granules containing granzymes and perforin. During cellular activation and differentiation, expression of genes such as *T-bet*, *Eomes*, and *Irf4* changes dynamically to promote this process. A recent study using high-resolution

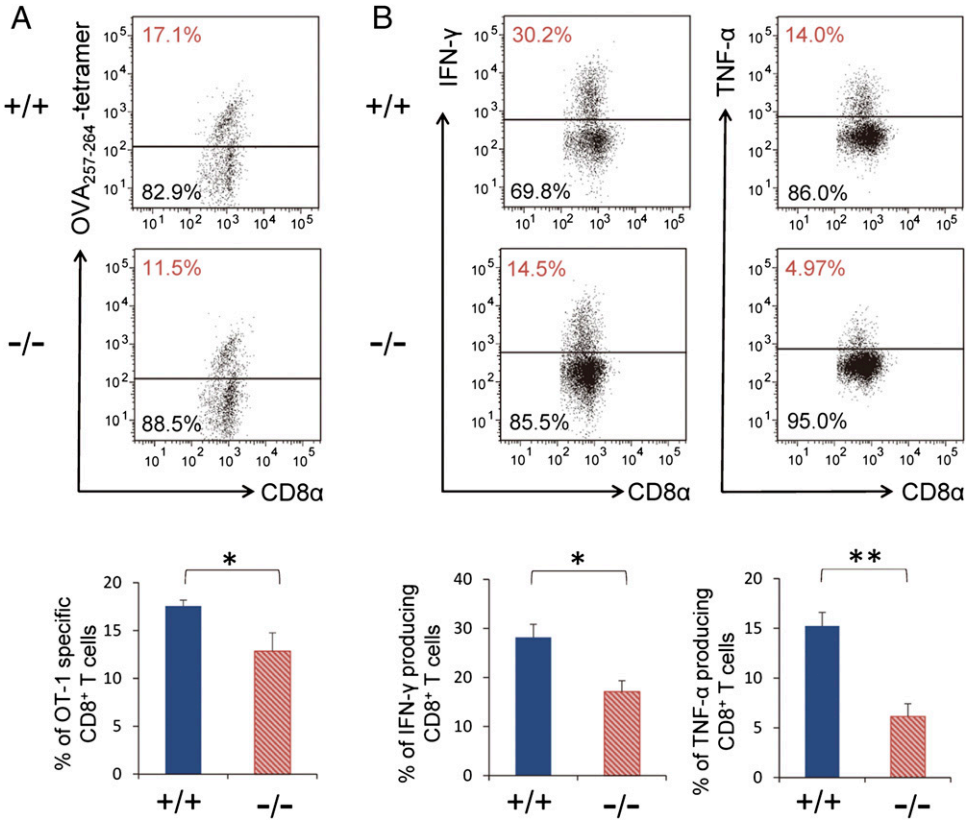

FIGURE 3. Impaired in vivo pathogen-specific effector CD8$^+$ T cell responses in SEMA4A$^{-/-}$ CD8$^+$ T cells. (**A** and **B**) For LM-OVA infection, wild-type ($^{+/+}$, blue closed bar) or SEMA4A$^{-/-}$ ($^{-/-}$, red hatched bar) mice were injected i.v. with a priming dose of 2×10^3 CFU LM-OVA. Splenocytes were analyzed by flow cytometry 7 d postinfection. CD8α-positive cells were gated, and their expression of $OVA_{257–264}$ tetramer, IFN-γ, or TNF-α was analyzed. Data are representative of two independent experiments. Data in the bar chart are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). $*p < 0.05$, $**p < 0.01$.

microarrays comprehensively defined the gene-expression profiles of CD8$^+$ T cells when mice were infected with *L. monocytogenes* (49). *Sema4A* was included in a group of genes associated with naive, late effector, or memory cells. The expression profiles of *Sema4a* presented in this work are consistent with those obtained in the microarray analysis, specifically, expression transiently decreased in the early phase, but significantly increased in the later phase of CD8$^+$ T cell activation. Indeed, phosphorylation of molecules involved in signaling pathways downstream of TCR stimulation (e.g., ERK, PLC-γ, JNK, and ZAP70) and expression

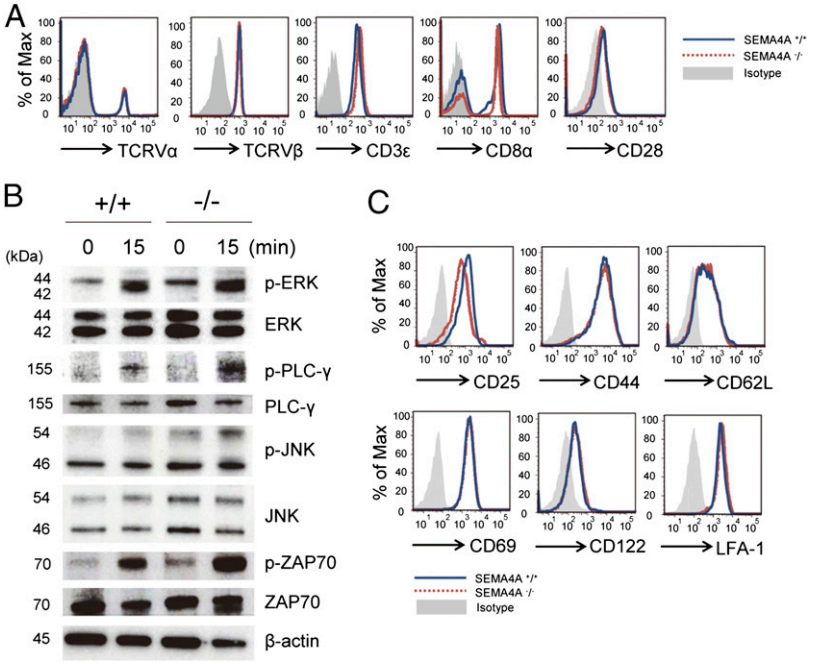

FIGURE 4. TCR-mediated signal induction is not impaired in SEMA4A$^{-/-}$ CD8$^+$ T cells. (**A**) Expression of the indicated molecules in CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice was examined by flow cytometry. Wild-type CD8$^+$ T cells were also stained with an isotype-matched control Ab (isotype, gray-filled histogram). (**B**) CD8$^+$ T cells prepared from wild-type ($^{+/+}$) or SEMA4A$^{-/-}$ ($^{-/-}$) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 15 min. Whole-cell extracts were harvested and probed by Western blotting with the indicated Abs. (**C**) CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. The levels of the indicated markers on the resultant cells were analyzed by flow cytometry. CD8$^+$ T cells prepared from wild-type mice were also stained with the isotype-matched control Ab (isotype, gray-filled histogram).

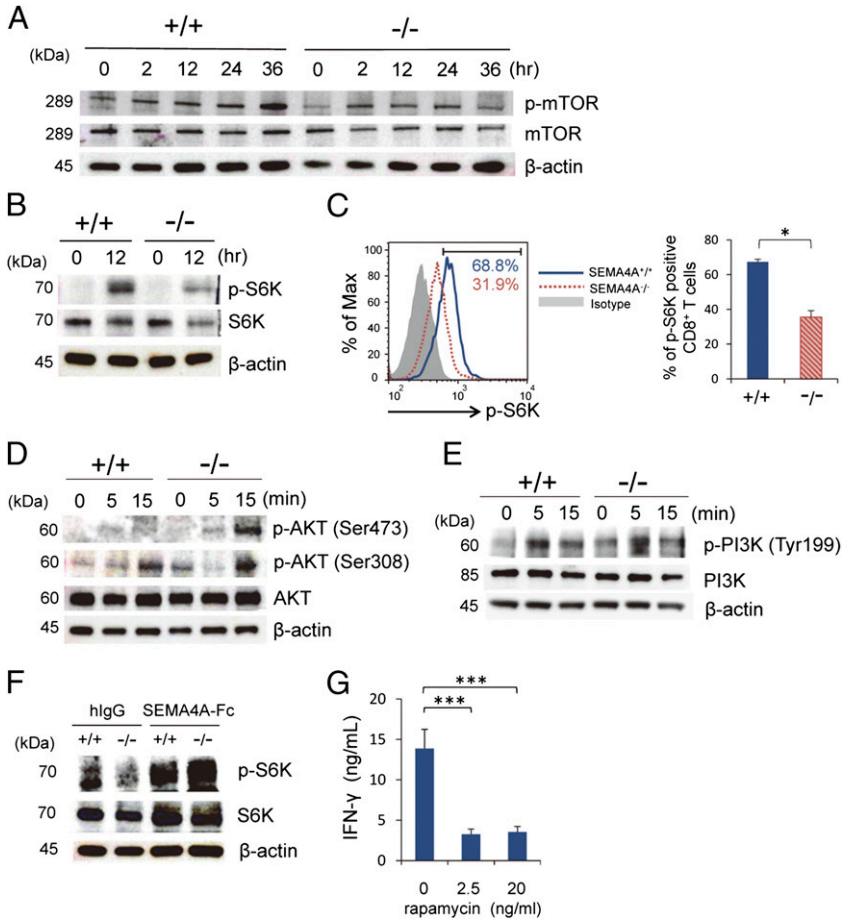

FIGURE 5. SEMA4A$^{-/-}$ CD8$^+$ T cells exhibited reduced mTORC1 activity and elevated mTORC2 activity. (**A** and **B**) CD8$^+$ T cells prepared from wild-type ($^{+/+}$) or SEMA4A$^{-/-}$ ($^{-/-}$) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2, 12, 24, or 36 h. Whole-cell extracts were harvested from these cultured cells (2, 12, 24, or 36 h) or cells without culture (0 h) and probed by Western blotting with the indicated Abs. Data are representative of three independent experiments. (**C**) CD8$^+$ T cells prepared from wild-type (SEMA4A$^{+/+}$, blue solid line) or SEMA4A$^{-/-}$ (red dotted line) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 36 h. Intracellular levels of phosphorylation of S6K were determined by intracellular staining. The frequencies of p-S6K–positive CD8$^+$ T cells from three independent experiments were analyzed by Student *t* test. Data are represented as mean ± SEM. *$p < 0.05$. (**D** and **E**) CD8$^+$ T cells prepared from wild-type ($^{+/+}$) or SEMA4A$^{-/-}$ ($^{-/-}$) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 15 min. Whole-cell extracts were harvested from these cultured cells (5 or 15 min) or cells without culture (0 min), and then probed by Western blotting with the indicated Abs. Data are representative of two independent experiments. (**F**) CD8$^+$ T cells prepared from wild-type ($^{+/+}$) or SEMA4A$^{-/-}$ ($^{-/-}$) mice were cultured with anti-CD3 (1 μg/ml) for 36 h in the presence of SEMA4A-Fc recombinant protein or control human IgG Fc proteins. Whole-cell extracts were harvested from these cultured cells (36 h) and probed by Western blotting with the indicated Abs. (**G**) CD8$^+$ T cells prepared from wild-type mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d in the presence of rapamycin. Levels of IFN-γ in the culture supernatants were measured by ELISA. Low-dose (2.5 ng/ml) rapamycin treatment preferentially inhibits mTORC1 activation, and high-dose (20 ng/ml) rapamycin treatment inhibits both the mTORC1 and mTORC2 pathway. Data are representative of two independent experiments. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). ***$p < 0.001$.

levels of other molecules associated with CD8$^+$ T cell activation and differentiation (e.g., CD25, CD44, CD62L, CD69, CD122, and LFA-1) were not reduced in SEMA4A$^{-/-}$ CD8$^+$ T cells, supporting the idea that SEMA4A is not involved in the early phases of CD8$^+$ T cell activation. Consistent with this, as shown in Fig. 2B, Sema4A$^{-/-}$ CD8$^+$ cells did not exhibit higher levels of cell death. It is noteworthy that the expression of IRF4, which is required for expansion of CD8$^+$ T cells and effector differentiation (41, 50, 51), was not affected in the absence of SEMA4A, suggesting that the activities of SEMA4A on CD8$^+$ T cells are not dependent on IRF4. This finding suggests that IRF4-dependent signal transduction alone is not sufficient for CD8$^+$ T cell differentiation, and SEMA4A-mediated signals are required in addition to these signals.

We also showed that SEMA4A plays an important role in regulation of mTOR-mediated signals in CD8$^+$ T cells, as follows: 1) SEMA4A deficiency in CD8$^+$ T cells resulted in lower phosphorylation of S6K; 2) recombinant SEMA4A protein promoted and recovered phosphorylation of S6K; and 3) the effects of SEMA4A on CD8$^+$ T cells were abolished by rapamycin treatment. These findings suggested that SEMA4A promotes a shift in mTOR-mediated signaling from mTORC2 to mTORC1 in CD8$^+$ T cells. It has been suggested that mTOR is involved in the promotion of effector CD8$^+$ T cell generation and function, as it is in the case of CD4$^+$ T cells. For instance, a recent study showed that rapamycin suppresses glycolysis in effector CD8$^+$ T cells, resulting in impairing bacterial clearance and CD8$^+$ T cell function killing during LM-OVA infection in vivo (52). However, the kinds of extracellular stimuli that lead to mTOR cascades have not been comprehensively determined. Therefore, our findings presented in this work indicate that SEMA4A plays key roles in mediating mTOR signaling in CD8$^+$ T cells. Of note, mTORC1 is important for Th1 differentiation in the case of CD4$^+$ T cells, which are characterized by their production of IFN-γ. We previously demonstrated

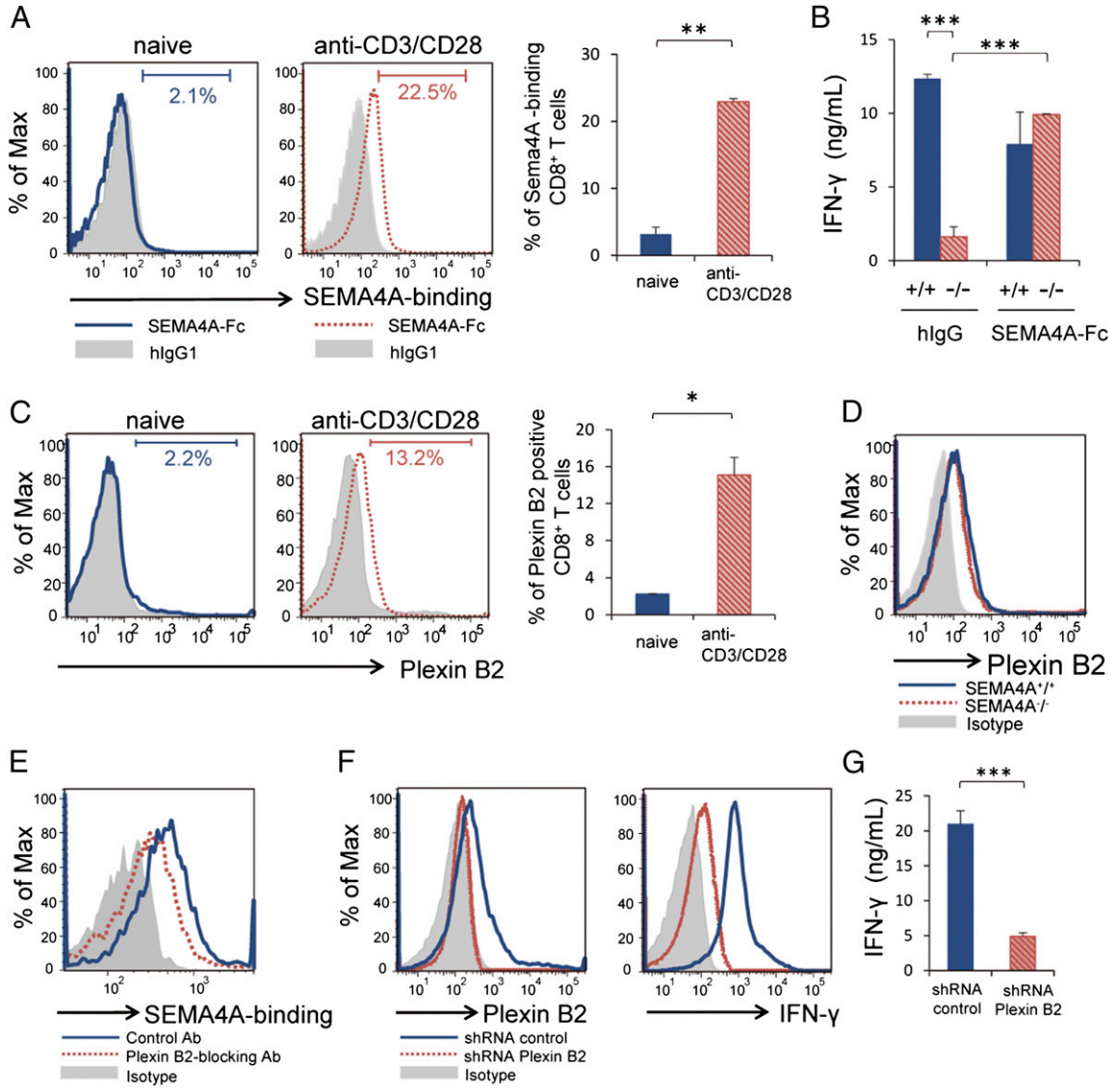

FIGURE 6. Plexin B2 expressed on $CD8^+$ T cells functions as a receptor of SEMA4A. (**A**) $CD8^+$ T cells prepared from wild-type mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. The resultant cells were collected and incubated with biotinylated SEMA4A-Fc or biotinylated control human IgG1 (hIgG). After washing, the amount of SEMA4A binding to the cell surface was evaluated by flow cytometry using streptavidin-allophycocyanin. The frequencies of allophycocyanin-positive $CD8^+$ T cells from two independent experiments were analyzed by Student *t* test. Data are represented as mean ± SEM. (**B**) $CD8^+$ T cells prepared from wild-type ($^{+/+}$, blue closed bar) or $SEMA4A^{-/-}$ ($^{-/-}$, red hatched bar) mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d in the presence of SEMA4A-Fc recombinant protein or control human IgG Fc proteins (hIgG). The levels of IFN-γ in culture supernatants were measured by ELISA. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). $^{***}p < 0.001$. (**C** and **D**) Naive $CD8^+$ T cells prepared from wild-type or $SEMA4A^{-/-}$ mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d and then stained with anti–plexin B2. Wild-type $CD8^+$ T cells were also stained with the isotype-matched control Ab (isotype, gray-filled histogram). The frequencies of plexin B2–positive $CD8^+$ T cells from two independent experiments were analyzed by Student *t* test. Data are represented as mean ± SEM. (**E**) $CD8^+$ T cells prepared from wild-type mice were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 2 d. Plexin B2 in the resultant cells was blocked by using anti–plexin B2 (plexin B2–blocking Ab, red dotted line) or control Ab (Control Ab, blue solid line). These $CD8^+$ T cells were incubated with biotinylated SEMA4A-Fc. After washing, the amount of SEMA4A binding to the cell surface was evaluated by flow cytometry using streptavidin-allophycocyanin. $CD8^+$ T cells treated with control Ab were also stained with the biotinylated control human IgG Fc protein (isotype, gray-filled histogram). (**F**) Knockdown of plexin B2 in $CD8^+$ T cells was performed by adding lentiviral particles expressing shRNA targeting plexin B2 or a control shRNA to cell cultures containing anti-CD3 (1 μg/ml), anti-CD28 (2 μg/ml), and IL-2 (100 U/ml). Seven days after transfection, the surface expression of plexin B2 and intracellular expression of IFN-γ were analyzed by flow cytometry. $CD8^+$ T cells treated with control shRNA were also stained with the isotype-matched control Ab (isotype, gray-filled histogram). (**G**) Knockdown of plexin B2 in $CD8^+$ T cells was performed by adding lentiviral particles expressing shRNA targeting plexin B2 or a control shRNA to cell cultures containing anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml). Two days after transfection, level of IFN-γ in the culture supernatants was measured by ELISA. Data are representative of three independent experiments. Data are represented as mean ± SEM. Statistical analysis used Student *t* test ($n = 3$ from each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

that expression of SEMA4A is inducible in Th1 cells and is required for the promotion of Th1 responses (26). In this context, SEMA4A might trigger mTORC1-mediated signaling in $CD4^+$ T cells, as well as in $CD8^+$ T cells, as we demonstrated in this study.

Our results demonstrated that plexin B2 is a receptor for SEMA4A in $CD8^+$ T cells, and also that the SEMA4A–plexin B2 axis is relevant to mTOR signaling. Several molecules have been reported to be involved as potential receptors in the biological

activities of SEMA4A. The binding of SEMA4A to plexin Bs in growth cones of hippocampal neurons induces growth cone collapse (36). SEMA4A associates with TIM2 and is involved in CD4$^+$ T cell activation and differentiation in the immune system (25). In the vascular system, the binding of SEMA4A to plexin D1 inhibits angiogenesis by suppressing the vascular endothelial growth factor–mediated activation of Rac and integrin-dependent cell adhesion (17). Moreover, a recent report showed that SEMA4A on effector T cells ligates to NRP1 on Treg cells, restrains Akt phosphorylation via recruitment of phosphatase and tensin homolog deleted from chromosome 10, and thereby stabilizes Treg cells (28). In CD8$^+$ T cells, we detected the expression of plexin B2, but not plexin B1, plexin B3, plexin D1, NRP1, or TIM2. When the expression of plexin B2 was suppressed by shRNA in CD8$^+$ T cells, the binding of SEMA4A to these cells was significantly inhibited and resulted in reduced IFN-γ production. Thus, these results suggest that plexin B2 is a functional receptor of SEMA4A in CD8$^+$ T cells. Although plexin B2 on myeloid and plasmacytoid DCs plays a role in production of IL-12/IL-23p40 (53), the function of plexin B2 in T cells has not been determined. To our knowledge, our study is the first to demonstrate the involvement of plexin B2–mediated signals in T cell responses. The signaling mediated by plexin B2 is involved in binding of the intracellular PDZ domain of plexin B2 to the PDZ domain of the RhoGEFs (guanine nucleotide exchange factors) PDZ-RhoGEF and leukemia-associated RhoGEF, which leads to the activation of Rho and the formation of stress fibers in fibroblasts (54). In addition, a recent study showed that, in *C. elegans*, semaphorins SMP-1 and SMP-2 activate TORC1 and simultaneously inhibit TORC2 by promoting a shift in the TOR adaptor from RICTOR to RAPTOR (42). Our findings are consistent with those results, and, to our knowledge, our study is the first to demonstrate the connection between semaphorins and mTOR signaling in the mammalian immune systems. However, this study did not reveal the precise molecular mechanisms that connect plexin B2–dependent signal transduction to mTOR-mediated signaling. This issue should be addressed in future work.

Collectively, our results revealed novel functions of SEMA4A in CD8$^+$ T cells. Specifically, SEMA4A regulates CD8$^+$ T cell functions by modulating mTOR-mediated signals. To our knowledge, our findings provide the first description of the function of an immune semaphorin in CD8$^+$ T cells, as well as demonstrate the first evidence for a connection between semaphorins and mTOR signaling in the mammalian immune systems. Further understanding of the molecular mechanisms connecting semaphorins and mTOR-mediated signaling will facilitate development of more effective treatments targeting the mTOR pathway.

Disclosures

The authors have no financial conflicts of interest.

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