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Th1 cells promote neurite outgrowth from cortical neurons via a mechanism dependent on semaphorins

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ABSTRACT

The roles of T lymphocytes in the central nervous system (CNS) are diverse; their roles in the injured CNS have been reported to be both detrimental and advantageous. Hence, an investigation of the effects of specific subsets of T cells on neurons may provide an insight into the interaction between the nervous system and the immune system. In the present study, we demonstrate that a specific subset of T lymphocytes enhanced neurite outgrowth in vitro. When cultured T helper type 1 (Th1) cells were co-cultured with cortical neurons, neurite outgrowth from neurons was enhanced; however, the same was not observed when Th2 or naïve T cells were used. We observed that the promotion of neurite outgrowth by Th1 cells was completely inhibited by anti-interferon γ (IFN-γ) neutralizing antibody, but that IFN-γ did not directly promote neurite growth. Furthermore, experiments using knockout mice revealed that semaphorin 4A (Sema4A) but not Sema7A was required for the effect produced by Th1 cells. These results demonstrate that Sema4A and IFN-γ expressed in Th1 cells play a critical role in enhancing neurite outgrowth from cortical neurons.

1. Introduction

Immune reactions after central nervous system (CNS) trauma have been thought to be harmful for axonal regeneration and functional recovery [1–3]. However, during the last decade, it has been reported that the transfer of autoimmune T cells or active immunization with T cells promoted functional recovery after CNS injury [4–9]. In contrast, others reported that T lymphocytes caused axonal damage after the CNS injury [3,10–12]. It could be assumed that these contradictory results may be explained by distinct roles of subsets of T cells such as T helper type 1 (Th1) or Th2 cells.

Subsets of helper T cells have been suggested to be involved in the etiology of CNS diseases. Interferon γ (IFN-γ)-producing Th1 cells and interleukin (IL)-17-producing helper T (Th17) cells are associated with the onset and progression of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis [13,14]. Interleukin-4-producing Th2 cells were considered to promote functional recovery after the CNS injury [15]. However, the role of each subset of T cells had not been explored in depth, and whether a specific subset of T cells was beneficial to CNS injury remained elusive. Contradictory reports on the role of T cells in vivo after a CNS injury may be because of the distinct roles of helper T cell-subsets. Hence, it is important to dissect out the precise effects of each T cell subset on neurons. For this purpose, we performed a neurite outgrowth assay using cortical neurons in vitro because this assay is considered to reveal the ability of neurons to regenerate axons in vivo. We report that Th1 cells but not Th2 cells enhanced neurite outgrowth from embryonic cortical neurons. Furthermore, we explored the molecular mechanism underlying the promotion of neurite outgrowth by Th1 cells. We investigated whether IFN-γ, which is mainly secreted by Th1 cells or semaphorins (axon guidance molecules as well as immunomodulators expressed on immune cells) [16], were required for the effects produced by Th1 cells. Our results demonstrated that Sema4A but not Sema7A was required for the effects produced by Th1 cells. Further, IFN-γ acting on Th1 cells was also required for producing these effects.

Abbreviations: CNS, central nervous system; Th1, T helper type 1; IFN-γ, interferon γ; Sema4A, semaphorin 4A; IL, interleukin; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; APC, allophycocyanin.

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2. Materials and Methods

2.1. Mice

C57BL/6 mice were purchased from Charles River. Sema7A -/- mice on the C57BL/6 background were used [17]. Sema4A -/- mice were generated as described previously [18]. All mice used in this study were housed in specific pathogen-free conditions. All the experimental procedures were approved by the Institutional Committees of Chiba University and Osaka University.

2.2. Differentiation of $CD4^+$ T cells

Spleens were collected from C57BL/6j female mice, and single-cell suspensions were prepared by mechanical disruption in RPMI-growth medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μM sodium pyruvate, and 2.5 μM ß-mercaptoethanol. $CD4^+$ T cells were isolated by magnetic sorting with anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were stimulated with anti-CD3ε antibodies (BD Biosciences, Franklin Lakes, NJ) coated on 24-well plates (Greiner Bio-One, Kremsmünster, Austria) at a concentration of 10 μg/ml. Th1 cells were differentiated by the addition of recombinant IL-2 (25 U/ml; R&D Systems, Minneapolis, MN), IL-12 (10 U/ml; R&D Systems), and anti-IL-4 antibodies (25% culture supernatant of hybridoma; clone HB-188; American Type Culture Collection, Rockville, MD). Th2 polarization was initiated by the addition of recombinant IL-2 (25 U/ml; R&D Systems), IL-4 (100 U/ml; Peprotech, Rocky Hill, NJ) and anti-IFN-γ antibodies (1% culture supernatant of hybridoma; clone CRL-1975; American Type Culture Collection). These differentiated $CD4^+$ T cells were diluted 1:3 for passage on day 2, and cultured with IL-2 and IL-12 for Th1 cells, or IL-2 and IL-4 for Th2 cells. The concentration of each cytokine used was the same as mentioned above.

2.3. Intracellular cytokine staining

Harvested $CD4^+$ T cells were stimulated for 6 hours with 2 μM monensin (Funakoshi, Tokyo, Japan) and anti-CD3ε antibodies (BD Biosciences) that were coated on 24-well plates at a concentration of 10 μg/ml for 6 hours. After washing the cells twice with cold phosphate-buffered saline (PBS), the T cells were fixed with 4% (wt/vol) paraformaldehyde for 10 minutes at room temperature. The cells were then washed twice with fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin (BSA) in PBS) and permeabilized with permeabilization buffer (50 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 0.5 % Triton X; pH 7.5) for 10 minutes on ice. After washing the cells twice with FACS buffer, the cells were blocked with 3% BSA in PBS for 15 minutes on ice. The cells were again washed with FACS buffer, and subjected to intracellular staining using anti-IFN-γ-fluorescein isothiocyanate (FITC) and anti-IL-4- allophycocyanin (APC) antibodies (BD Biosciences).

2.4. FACS analysis

FACS analysis was performed on a FACSCaliber (BD Biosciences) and analyzed using ProQuest software (BD Biosciences).

2.5. Co-culture of neurons and lymphocytes

Cortical neurons from the cerebral cortex were obtained from mice on embryonic day 15 and 16; they were dissociated by trypsinization (0.25% trypsin in PBS for 15 min at 37 °C), resuspended in a serum-containing medium, triturated, and washed 3 times with PBS. The dissociated neurons were plated at 1.5×10^4 cells/cm^2 into Lab-Tek 4-well chamber slides (Thermo Fisher Scientific, Waltham, MA) coated with poly-l-lysine in serum-free Neuro-basal medium (200 μl per well; Invitrogen) supplemented with B27 (Invitrogen), and l-glutamine (Nacalai Tesque, Kyoto, Japan). Cultured Th1/Th2 $CD4^+$ T cells were diluted 1:2 on day 4, and cultured for 1 day with IL-2 (25 U/ml) and IL-12 (10 U/ml) for Th1 cells, and IL-2 (25 U/ml) and IL-4 (100 U/ml) for Th2 cells. These cells were harvested on day 5 and plated (1.5×10^4 cells/cm^2) with 200 μl conditioned RPMI-growth medium per well in chamber slides containing cortical neurons. $CD4^+$ T cells isolated by magnetic sorting with anti-CD4 magnetic beads were used as naïve helper T cells. The cortical neurons and the T cells were co-cultured for 24 hours. As control, 200 μl RPMI-growth medium lacking the cells was added to a chamber in which cortical neurons were plated. Where indicated, recombinant IFN-γ (20 ng/ml; R&D) was added in the culture medium of the cortical neurons. In addition, anti-IFN-γ antibodies (1% culture supernatant of hybridoma; clone CRL-1975; American Type Culture Collection) were added into the co-culture 1 hour prior to the addition of T cells into the neuronal culture. Th1-conditioned cells (in the presence of IL-12 plus anti-IL-4) were differentiated from $CD4^+$ T cells derived from spleens of semaphorin-knockout mice according to the same protocol as mentioned above. Sema7A (-/-) and Sema4A (-/-) mice were used.

2.6. Measurement of neurite length from cortical neurons

The co-cultured neurons were fixed in 4% (wt/vol) paraformaldehyde and immunostained with a monoclonal antibody (TuJ1) that recognized the neuron-specific ß tubulin III protein (1:1000; Covance, Princeton, NJ). The neurons were viewed under an inverted light microscope equipped with epifluorescence optics

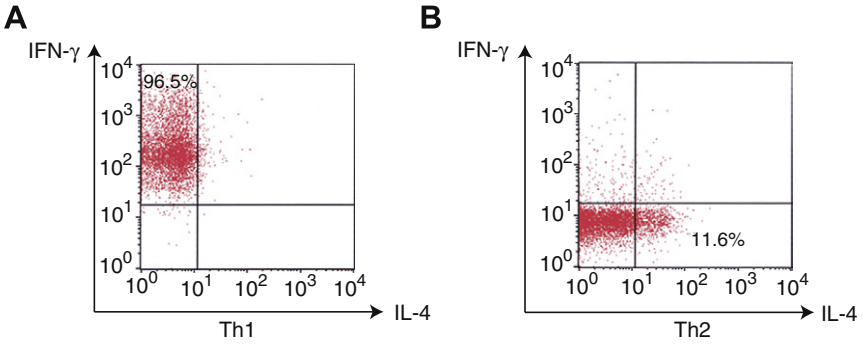

Fig. 1. Isolated naïve $CD4^+$ T cells differentiated into Th1 and Th2 cells. (A) Representative dot plots of FACS analysis. Most of the cultured $CD4^+$ T cells, which were treated with IL-2, IL-12, and anti-IL-4 antibodies, secreted IFN-γ; this is a distinct characteristic of differentiated Th1 cells. (B) Cultured $CD4^+$ T cells treated with IL-2 and IL-12 secreted IL-4 that is a distinct characteristic of differentiated Th2 cells.

and a dry condenser for phase-contrast microscopy (DP70, Olympus). The length of the longest neurite was measured for each TuJ1-positive neuron using ImageJ (National Institutes of Health, USA). In each set of experiments, more than 80 neurites were measured, and their mean length was calculated.

3. Results

3.1. Cultured Th1 cells enhance neurite outgrowth

In order to assess the functional interaction between neurons in the CNS and T cells, we performed a neurite growth assay. We examined neurite outgrowth from cortical neurons in the cerebral cortex of mice on embryonic day 15 and 16 for 24 hours. The effect of helper T cells on the neurons was examined by co-culture. For this experiment, $CD4^+$ naïve helper T cells isolated ex vivo from mice spleens between postnatal weeks 7 and 9 were differentiated into Th1 or Th2 cells. First, we confirmed successful differentiation of the $CD4^+$ naïve helper T cells into Th1 (Fig. 1A) or Th2 (Fig. 1B) cells by quantifying the amount of IFN-γ and IL-4 with FACS analysis. The differentiated helper T cells were then added to the neuron culture (Fig. 2, schematic figure on top). After 24 hours, these co-cultured cells were fixed, and immunostained with the monoclonal antibody (TuJ1) that recognizes the neuron-specific ß tubulin III protein in order to label the neurites of neurons. Neurite outgrowth from the cortical neurons was significantly boosted when the neurons were co-cultured with Th1 cells as compared to that observed in the controls (Fig. 2A and B). Naïve helper T cells or Th2 cells did not modulate the neurite growth rate (Fig. 2A and B). The mean neurite length of the cortical neurons maintained without co-culture (RPMI-growth medium was used in a control) and of those co-cultured with naïve helper T cells, and of Th1 or Th2 cells was 54.6 μm, 60.2 μm, 73.1 μm, and 55.2 μm, respectively. Therefore, Th1 cells but not naïve helper T cells or Th2 cells promoted neurite growth from the cortical neurons in vitro.

3.2. IFN-γ is associated with the promotion of neurite outgrowth by Th1 cells

Next, we tried to explore the molecular mechanism underlying the promotion of neurite elongation by Th1 cells. We first investigated whether IFN-γ, which is one of the major cytokines secreted from Th1 cells, directly acted on the cortical neurons to promote neurite outgrowth. To test this hypothesis, we treated the cortical neurons with recombinant IFN-γ at a concentration of 20 ng/ml, and measured neurite growth. However, IFN-γ appeared to inhibit neurite growth (Fig. 3). This result excludes the possibility that IFN-γ secreted by Th1 cells acted directly on the neurons to enhance neurite elongation. Then, we determined whether endogenous IFN-γ acted on Th1 cells in an autocrine manner and indirectly produced the effect on neurons. For this purpose, anti-IFN-γ neutralizing antibody was added to the co-culture. Interestingly, we observed that the promotion of neurite outgrowth by Th1 cells was completely diminished after treatment with the anti-IFN-γ neutralizing antibody (Fig. 3). These results demonstrate that the effect of IFN-γ on Th1 cells was necessary for the promotion of neurite growth by Th1 cells. IFN-γ may act on Th1 cells but not on the neurons, thereby resulting in enhanced neurite growth. Other factors secreted by the Th1 cells may be responsible for the effect produced by the Th1 cells on neurons.

3.3. Sema4A but not Sema7A is required for Th1 cell-enhanced neurite outgrowth

To identify the molecules secreted from Th1 cells, we focused on semaphorin family members. Semaphorin family of membrane

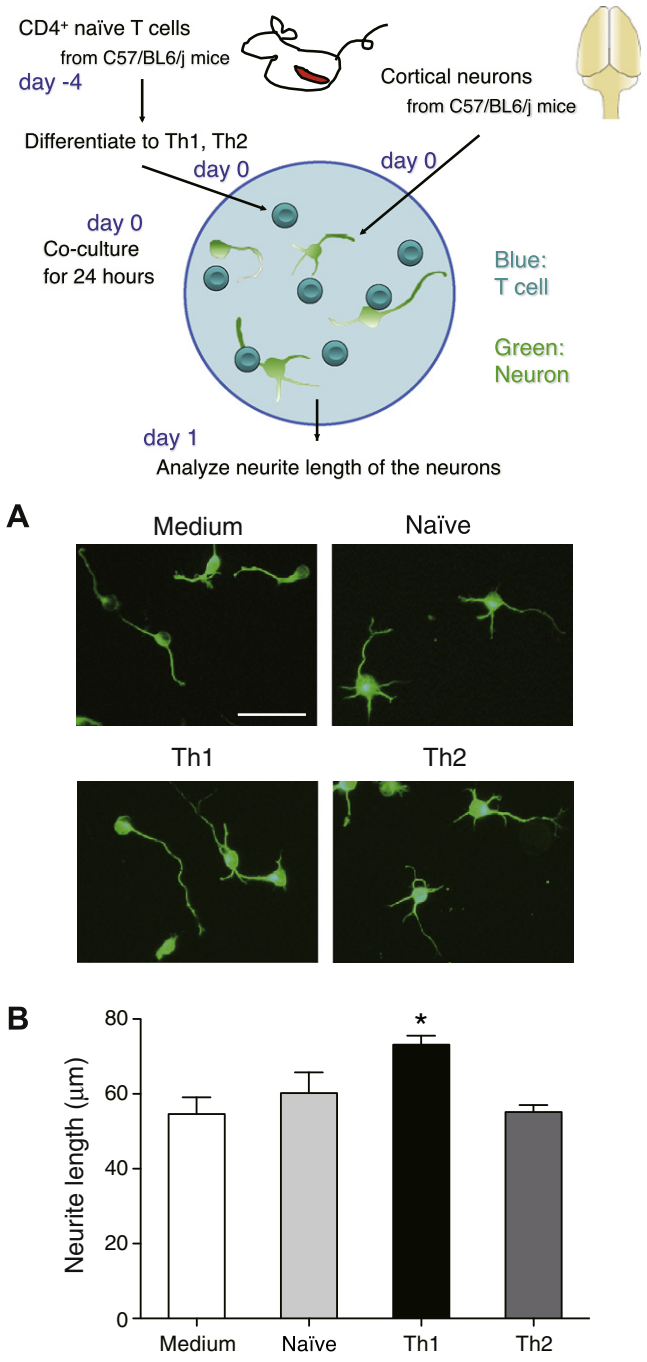

Fig. 2. Cultured Th1 cells but not Th2 cells promoted neurite outgrowth from cortical neurons in vitro. (Upper panel) Schematic figure indicating the protocol for the co-culture of helper T cells and cortical neurons. (A) Representative images of Tuj1-labeled neurons. Scale bar = 50 μm (B) Quantification of neurite length after co-culture for 24 hours. Medium: Control, n = 4; Naïve, n = 4; Th1, n = 4; Th2, n = 4. Values are the mean (SEM) $^*P < 0.05$ versus control group (one-way ANOVA with Dunnett's post-test).

proteins plays a role in not only axon guidance of developing neurons but also modulation of the immune system [16]. Among the family members, Sema7A was reported to be expressed in T cells [19] and promote axon growth in olfactory neurons [17]. We obtained T cells derived from spleens of Sema7A-knockout mice, and induced differentiation of these cells in the presence of IL-12 plus anti-IL-4 (Th1-skewing conditions). The results demonstrated that Th1-conditioned cells lacking Sema7A promoted neurite growth to the same extent as Th1 cells from wild-type mice (Fig. 3). Hence, it was inferred that Sema7A was not required for

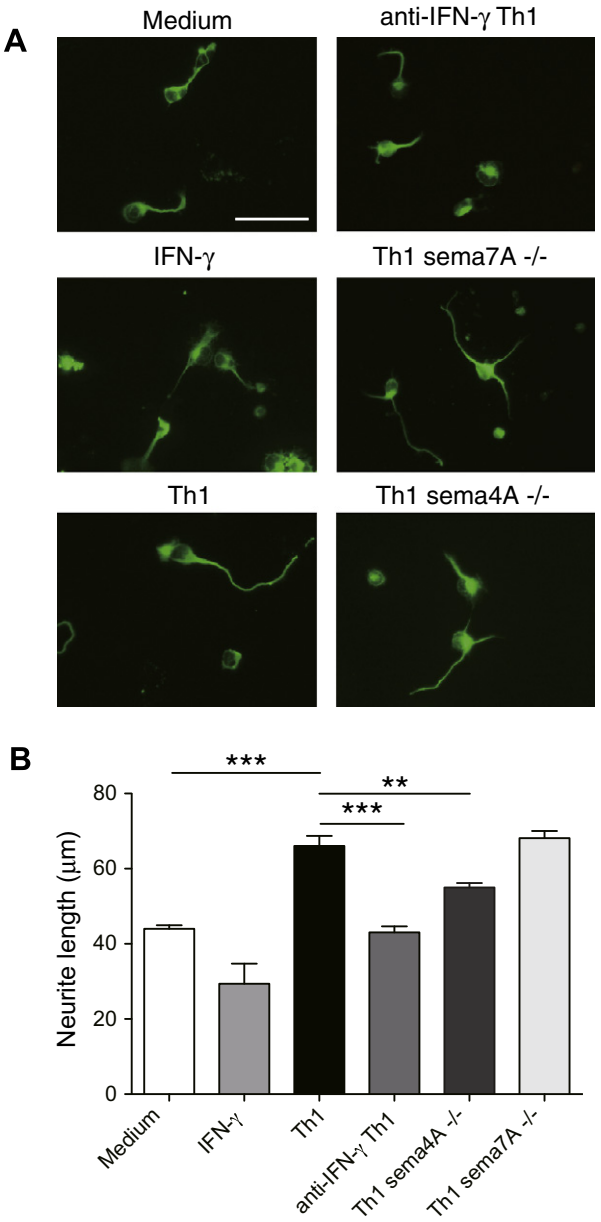

Fig. 3. Th1 cells required IFN-γ and Sema4A for the promotion of neurite outgrowth. (A) Representative images of Tuj1-labeled neurons. Scale bar = 50 μm (B) Measurement of neurite length after 24-hour culture of the cortical neurons. Recombinant IFN-γ or neutralizing antibody to IFN-γ was added to the culture medium. Th1-conditioned cells were differentiated from $CD4^+$ T cells derived from spleens of wild-type, Sema7A -/-, or Sema4A -/- mice, and added into the neuronal culture. Medium: Control, n = 4; IFN-γ, n = 2; Th1, n = 4, anti-IFN-γ: IFN-γ-neutralizing antibodies, n = 3; Th1 Sema4A -/-, n = 4; Th1 sema7A -/-, n = 3. Values are the mean (SEM) $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA with Bonferroni's post-test).

producing the effect of Th1 cells on neurons. Finally, we examined whether Sema4A, another member of the semaphorin family, was involved in producing the effect of Th1 cells because Sema4A is also expressed in T cells [20] and induces growth cone collapse of hippocampal neurons [21]. The neurite growth-promoting effect of Th1-conditioned cells lacking Sema4A was significantly lower than that of wild-type Th1 cells (Fig. 3). These results demonstrate that Sema4A is necessary for the effects of cultured Th1 cells. However, it should be noted that the effect of Th1 cells was not completely abolished when Th1-conditioned cells lacking Sema4A were used. Therefore, other molecules such as neurotrophic factors may also play an additional role in promoting neurite growth from cortical neurons.

4. Discussion

There have been controversies concerning the efficacy of adoptive transfer of T lymphocytes during CNS trauma [15]. Critically, no experiments focusing on specific T cell subsets with regard to these conflicting results have been reported. In the present study, we investigated the effects of specific T cell subsets on neurite outgrowth to evaluate neuronal regeneration in vitro. Unexpectedly, Th1 cells, which were suggested to be detrimental during CNS injury [15] and pathogenic during EAE [13,14], enhanced neurite outgrowth from cerebral cortical neurons, although naïve helper T cells or Th2 cells did not have such effects. These results are seemingly inconsistent with a previously reported hypothesis [15]. However, IFN-γ that is dominantly secreted from Th1 cells was reported to enhance neurogenesis in the brain [22] and be protective in EAE [23]. Indeed, we identified that IFN-γ was required by Th1 cells for enhancing neurite outgrowth, although IFN-γ itself appeared to act on Th1 cells but not on the neurons directly. In addition, experiments with cultured T cells derived from Sema4A-knockout mice revealed that Sema4A in Th1 cells was required by Th1 cells for promoting neurite outgrowth. Because Sema4A is a membrane-spanning molecule that differs from the secretory cytokine IFN-γ, it is possible that there was direct interaction between Th1 cells and the cortical neurons, and thus the Sema4A signal was transduced to the cortical neurons. However, it was reported that Sema4A induced growth cone collapse of other types of neurons, including hippocampal neurons [21]. Thus, the function of Sema4A in the CNS may be cell context dependent. Another possibility is that Th1-conditioned cells lacking Sema4A may secrete trophic factors at a lower level than wild-type Th1 cells because of the defective T cell differentiation. Because the production of IFN-γ by Th1-conditioned cells from Sema4A-deficient mice was severely impaired compared to that in wild-type mice [18], it is also likely that the reduction of IFN-γ due to the absence of Sema4A leads to the attenuation of enhanced neurite growth by Th1 cells.

In accordance with our results, it could be assumed that the reported beneficial effects of T cells transferred into animals after CNS injury may be attributed to the Th1 subset. Inconsistent results obtained in vivo could be explained by the diversity in the ratio of specific subsets of T cells. Therefore, the precise in vivo role of specific subsets of T cells should be determined, presumably by the transfer of each subset of the T cells. This topic should be explored in future studies.

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