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Semaphorin-3A (Sema3A), a member of a large family of conserved proteins originally implicated in axon guidance, is expressed by activated T cells and downmodulates T cell activation in vitro. This study examined the effect and mechanism of action of Sema3A overexpression in a mouse model of collagen-induced arthritis. Prophylactic i.p. administration of plasmid DNA encoding Sema3A markedly reduced the incidence, disease severity, and articular inflammation compared with control plasmid without insert. Treatment of Sema3A reduced anticollagen IgG levels and suppressed collagen-specific proinflammatory cytokine (IFN-γ and IL-17) release, but increased IL-10 concentration in the serum. In line with results in arthritic mice, Sema3A expression is defective in CD4+ T cells derived from patients with rheumatoid arthritis. In contrast, increased expression of the Sema3A receptor neuropilin-1 (NP-1) is detected in the same cells. The CD4+NP-1+ T cells are a T cell subset involved in the control of the immune responses. They express greater amounts of IL-10 and show suppressive activities on autologous CD4+ T cells. Sema3A acted directly on CD4+NP-1+ T cells, because it could increase IL-10 production and influence the regulatory function on CD4+ T cell growth. Therefore, I propose that Sema3A increases the CD4+NP-1+ T cell ability to suppress alloresponses, that its transient expression is altered in rheumatoid inflammation, and that reintroduction of Sema3A is sufficient to attenuate collagen-induced arthritis, supporting its therapeutic potential in the treatment of autoimmune disorders.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: A, activating conditions; CIA, collagen-induced arthritis; DC, dendritic cell; NP, neuropilin; OA, osteoarthritis; R, resting conditions; RA, rheumatoid arthritis; Sema3A, Semaphorin-3A; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

Materials and Methods

Cell isolation

Isolation of PBMCs was performed as described previously (19). I purchased PBMCs from healthy volunteers and from individuals with RA (who had given informed consent) from a rheumatologist (A. Mancuso, San Giovanni Hospital, Rome, Italy) after approval by the Institutional Review Board of the San Giovanni Hospital, Rome, Italy. CD4+ T cells were purified from PBMCs, total spleen cells, or lymph node cells by incubating cells with anti-
CD4 mAb (GK1.5), followed by positive selection of CD4+ cells with specific T cell isolation kits (all from Miltenyi Biotec, Auburn, CA). The remaining CD4− fraction of PBMCs was then used to purify CD8+ T cells by using CD8 multisort magnetic beads (Miltenyi Biotec). Macrophages were derived from PBMCs after differentiation for 4 d with 100 ng/ml M-CSF, as previously described (25). Synovial fibroblasts were isolated from a mixed population of all synovial cell types isolated from synovial membranes obtained from patients with RA undergoing joint replacement surgery, as previously described (26). CD4+ T cells derived from PBMCs were subsequently fractionated in CD25+ (here referred as Treg) cells by using CD25 magnetic beads or in NP-1− and NP−1 cells by using BDCA-4/ NP-1 magnetic beads (all from Miltenyi Biotec). Cell purity was routinely evaluated by flow cytometry and ranged between 96 and 98%. Anti-CD4 FITC, anti-CD8 allophycocyanin, anti-CD25 PE (all from Beckman Coulter, Fullerton, CA), anti-CD3 PerCP (BD Biosciences, San Jose, CA), anti–NP-1 (BD Pharmingen, San Diego, CA), anti-Foxp3 allophycocyanin (Società Chimici Italiana), anti–IL-17–allophycocyanin (R&D Systems, Minneapolis, MD), anti–IFN-γ–PE, anti–IL-10–PE (BD Biosciences), and Biotin control Abs were used for flow cytometry analysis of relative Ags at the indicated time points according to the manufacturer’s instructions and as previously described (21, 22). The study received approval from the local ethical committee.

**CFSE labeling and cell culture**

Responder cells (CD4+ T cells) were cultured in RPMI 1640 containing 10% FBS and standard supplements (all from Sigma-Aldrich, St. Louis, MO) in 96 U-bottom multwells (5 × 10^4 cells/well/200 μl) in the presence or absence of activating anti-CD3−bound beads according to the manufacturer’s instructions (Miltenyi Biotec). In blocking studies, cell cultures were first preincubated (10 min; 4°C) with 1 μM polyclonal rabbit anti-Sema3A (H300; Santa Cruz Biotechnology, Santa Cruz, CA) or with 5 μg/ml anti–IL-10–PE (BD Biosciences, San Jose, CA). Cells were then washed before coculture, which was performed in the absence of the Abs. CD4+ T cells were incubated in 0.2 μM CFSE (Invitrogen Life Technologies, Paisley, U.K.) at 37°C for 30 min, and CD4+NP−1 T cells were left unabeled at room temperature for 3 min and extensively washed before culture. A total of 10,000 CD4+ CFSE+ cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Alternatively, supernatants were derived from PBMCs after differentiation for 4 d with 100 ng/ml M-CSF, as previously described (26). CD4+ T cells derived from PBMCs were subsequently fractionated in CD25+ (here referred as Treg) cells by using CD25 magnetic beads or in NP-1− and NP−1 cells by using BDCA-4/ NP-1 magnetic beads (all from Miltenyi Biotec). Cell purity was routinely evaluated by flow cytometry and ranged between 96 and 98%. Anti-CD4 FITC, anti-CD8 allophycocyanin, anti-CD25 PE (all from Beckman Coulter, Fullerton, CA), anti-CD3 PerCP (BD Biosciences, San Jose, CA), anti–NP-1 (BD Pharmingen, San Diego, CA), anti-Foxp3 allophycocyanin (Società Chimici Italiana), anti–IL-17–allophycocyanin (R&D Systems, Minneapolis, MD), anti–IFN-γ–PE, anti–IL-10–PE (BD Biosciences), and Biotin control Abs were used for flow cytometry analysis of relative Ags at the indicated time points according to the manufacturer’s instructions and as previously described (21, 22). The study received approval from the local ethical committee.

**Production and Purification of Sema3A and Sema3C**

Sema3A and control expression vectors have been described previously (2, 27). Mouse Sema3C cDNA (NM_013657) was cloned into pcDNA3.1 vector. I synthesized recombinant human Sema3A and Sema3C as previously described (28). The cDNAs were ligated in-frame to a FLAG epitope tag inserted in-frame before the stop codon. The cDNAs were ligated in-frame to a FLAG vector. I synthesized recombinant human Sema3A and Sema3C activity. As previously described (28), recombinant Sema3A and Sema3C were expressed in HEK293T cells. Recombinant protein was purified using Protein A affinity gel. Recombinant protein was verified by Western blot analysis. A total of 10,000 CD4+ CFSE+ cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Alternatively, supernatants were harvested on day 3 and assayed by ELISA for cytokine content.

**Induction and assessment of CIA**

CIA in DBA/1 mice was induced and scored as described previously (29). In brief, DBA/1 mice (Charles River Laboratories, Wilmington, MA) received intradermal immunization with 100 μg/mouse bovine CII (Chon- drex, Redmond, WA) emulsified in CFA containing 250 μg/mouse heat killed *Mycobacterium tuberculosis* H37Ra (BD Biosciences).Twenty-one days after immunization, mice were boosted by s.c. injection at the base of the tail with 100 μg/mouse bovine CII. For the treatment experiments, only mice with clinical arthritis (average visual arthritis score of 4) were randomized and treated. Mice were maintained according to approved home office protocols and following institute guidelines. The number of mice used in these studies was the minimum required to achieve statistical significance. Mice were scored for arthritis using the following visual scoring system: grade 0, no swelling or erythema; grade 1, mild swelling and erythema or digit inflammation; grade 2, moderate swelling and erythema confined distal to the midpaw; grade 3, more pronounced swelling and erythema with extension to the ankle; grade 4, severe swelling, erythema, and joint rigidity of the ankle, foot, and digits. Each limb was graded with a score of 0−4, with a maximum possible score of 16 for each individual mouse. Paw thickness was determined by measuring the thickness of the most severely affected hind paw with 0- to 10-mm calipers. Mice were treated with each plasmid i.p. starting on the day before CIA induction for the prevention experiments and after development of arthritis and randomized for the treatment experiments.

**Treatment protocols**

Mice (22−25 g) were treated with Sema3A plasmid (1 μg/animal; equivalent to 40 μg/kg, in 0.2 ml final volume of PBS) i.p. once every day until day 50. Control mice received psDNA at the same time points. Mice were monitored for signs of arthritis scored (29) and then killed on day 50, and sera were collected and assayed for cytokines by ELISA. The DNA dose chosen is consistent with our previous experiments in animals where I observed that Sema3A protein expression in lyesates of PBMC, spleen, and liver was upregulated by administration of Sema3A-encoding plasmid (data not shown). The level of Sema3A protein expression was assessed by Western blot in PBMCs and spleen cells freshly isolated from mice treated with Sema3A or control plasmid. Cell lysesates were separated by 10% SDS-PAGE, transferred onto membrane, and probed with anti-Sema3A Abs, as previously described (19). Histopathology studies

**Histopathology studies**

Hind limbs were fixed and decalcified in Cal-Ex II (Fischer Scientific, Milan, Italy) for 3 d before embedding in paraffin. Sections were stained with H&E and evaluated by an investigator blinded to treatment status for synovitis, pannus formation, and bone and/or cartilage destruction based on a previously described scoring system: grade 0, normal; grade 1, mild inflammation, mild hyperplasia of the synovial lining layer, mild cartilage destruction without bone erosion; grades 2−4, increasing degrees of inflammatory cell infiltrates, synovial lining hyperplasia, and pannus formation and cartilage and bone destruction (29).

**ELISA**

After compound exposure, I stimulated cells with LPS (*Escherichia coli, O111:B4; Sigma*) at 1 and 5 μg/ml, respectively, for 24 h, and analyzed supernatants for IL-23 (eBiosciences, by Prodotti Gianni, Milan, Italy), TNF-α, IL-10 (BD Biosciences), and IL-6 (BD Biosciences) by ELISA. I analyzed immune cells with Abs to CD3 and CD28 for 24 h for production of IFN-γ, IL-6, or TNF-α by ELISA (BD Biosciences).

**In vitro T cell differentiation**

CD4+CD45RA+ magnetically sorted (CD45RO depletion, MACS, according to the protocol of the manufacturer) cells were stimulated with immobilized plate-bound anti-CD3 (1 μg/ml, Okt3, IgG1) and anti-CD28 (2 μg/ml) in Th1 conditions (25 ng/ml IL-12, 5 μg/ml anti–IL-4), and R&D systems, in Th2 conditions (25 ng/ml IL-4, 5 μg/ml anti–IFN-γ, 5 μg/ml anti–IL-12; R&D systems), in Th17 conditions (5 ng/ml TGF-β; R&D Systems; and 25 ng/ml IL-21; Cell Sciences, Canton, MA) for a period of 7 d in the absence of IL-2 as previously described (30), or in Treg conditions (10 ng/ml TGF-β, 5 μg/ml anti–IFN-γ, 5 μg/ml anti–IL-12, 5 μg/ml anti–IL-4). Proliferating cells were expanded in medium containing IL-2 (30 ng/ml). For Th17, cells were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate and 200 ng/ml ionomycin in the presence of brefeldin A (Sigma).

**Real-time PCR**

Ten patients with RA and 10 patients with osteoarthritis (OA) who underwent knee joint replacement surgery and 5 control subjects without inflammatory conditions who underwent arthroscopic knee surgery were included without further selection. All patients were informed about the purpose of the study and gave written consent. Synovial tissue samples were obtained immediately after opening the knee joint capsule or during arthroscopic surgery (in control subjects). Total RNA was isolated and real-time RT-PCR analysis was done in a Chromo4 sequence detector (Bio-Rad, Milan, Italy), as previously described (19). All primers and probes were...
obtained from Applied Biosystems (Foster City, CA) and used according to standard methodologies. Details of sequences and thermal cycle conditions are available on request. Data were acquired and analyzed with the sequence detector Chromo4 software.

mAb against collagen II

CII-specific IgG (Chondrex) were measured using an ELISA kit following the manufacturer’s protocol. The levels of antitype II collagen Ab IgG isotypes in the sera of arthritic DBA/1 mice were assessed using the antitype II collagen Ab assay kit with substitution of the HRP-conjugated total IgG Ab with HRP-conjugated, isotype-specific goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Jackson ImmunoResearch Laboratories, West Grove, PA).

Statistical analyses

For comparisons of treatment groups, I performed the unpaired t test (Mann–Whitney U), paired t tests, and one-way or two-way ANOVA (where appropriate). For ANOVA, I used Bonferroni post hoc analysis to compare treatment groups. I performed all statistical analyses with GraphPad Prism Software (version 4.01). Data are expressed as the mean ± SEM, and \( p < 0.05 \) was considered significant.

### Results

**Sema3A is downregulated in T cells derived from RA patients**

Previously, I and others have reported that Sema3A is expressed in both activated DCs and T cells, and that Sema3A plays a critical role in controlling thymocyte maturation and T cell function (19–21). To explore whether Sema3A expression by T cells differs in RA patients compared with control subjects, I have isolated PBMCs, CD4+ T cells, CD8+ T cells, macrophages, and synovial fibroblasts, and performed real-time RT-PCR after stimulating conditions. In the control group, Sema3a transcript was highly upregulated in PBMCs on PHA and IL-2 administration (Fig. 1A). In the RA group, PBMCs stimulated under the same conditions did not increase Sema3a expression (Fig. 1A). Sema3a mRNA was detected in control CD4+ and CD8+ T cells after stimulation with anti-CD3/CD28 Abs (Fig. 1A). Notably, activated CD4+ T cells expressed higher amounts of Sema3a than activated CD8+ T cells. In both T cell subsets derived from patients with RA, Sema3a transcript was not induced through stimulating conditions (Fig.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** A and B, Indicated cells derived from healthy donors and patients with RA (\( n = 8 \) per group) were either unstimulated (resting conditions) or stimulated with plate-bound anti-CD3/CD28 (PBMC and T cell subsets) and LPS (macrophages and fibroblasts). Cells were harvested after 24 h, and Sema3A (A) or Sema3C (B) mRNA was quantified by real-time PCR. Reported values were expressed as relative expression to mRNAs derived from resting conditions. Bars show the mean ± SD of four independent experiments. C, PBMCs and CD4+ T cells derived from healthy donors and patients with RA were untreated or activated for 48 h with anti-CD3/CD28 Abs in vitro. Then, Sema3A protein expression was examined by Western blot. Equal loading of proteins was confirmed using GAPDH as an internal control. Blot shown is a representative from three independent experiments with similar results. D, mRNA expression of Sema3C (left) and Sema3A (right) was measured by real-time PCR in RA (\( n = 10 \)), OA (\( n = 10 \)), and control (\( n = 5 \)) synovial tissue samples. Reported values were expressed as relative expression to the housekeeping gene GAPDH (2^(-ΔΔCt) value x 1000). Horizontal lines indicate median values. \( *p < 0.05 \). A, activating conditions; R, resting conditions.
The expression of Sema3c, a member of the Sema3 family that is expressed in the synovial tissue of patients with RA (31), was slightly upregulated in activated PBMCs and T cells derived from both healthy donors and patients with RA (Fig. 1B). Finally, in purified macrophages and synovial fibroblasts, Sema3a and Sema3c mRNA levels were not detected or detected at only low levels (Fig. 1A, 1B). Stimulation with LPS significantly increased mRNA of Sema3a and Sema3c in macrophages, but not in fibroblasts. However, the expression of Sema3a and Sema3c was similarly modulated in these cells isolated from patients with RA (Fig. 1A, 1B). As expected from mRNA expression data, lower Sema3A protein levels were detected in PBMCs and CD4+ T cells derived from RA patients compared with those derived from healthy donors (Fig. 1C). Thus, Sema3A is markedly expressed in activated T cells and, to a lesser extent, in macrophages, but not in T cell subsets that are isolated from patients with RA.

I also confirmed the different expression of Sema3a and Sema3c transcript in synovial tissue samples from 10 patients with RA, 10 patients with OA, and 5 patients without inflammatory conditions. In line with a previous report (31), most OA and RA synovia expressed Sema3c, whereas all the synovial tissue specimens derived from OA and RA patients exhibited very low levels of Sema3a mRNA (Fig. 1D).

RA patients display greater percentages of CD4+ T cells that express NP-1

NP-1 and NP-2 and two plexins (Plxn-a1 and -a4) seem to act as the ligand binding and the signal transducing subunits on the surface of immune cells (18). Therefore, I analyzed whether these receptors are expressed in immune cells derived from patients with RA. Although in PBMCs, CD4+ cells, and CD8+ T cells np-1 transcript markedly increased in RA patients compared with control subjects, I did not observe significant differences in np-1 expression in purified macrophages and synovial fibroblasts (Fig. 2A). I also found np-2 expression at low levels in immune cells and no significant difference between the control and RA groups (Fig. 2A). Consistently, PBMCs derived from patients with RA had greater NP-1 protein expression in CD4+ single-positive cells compared with those derived from healthy donors (Fig. 2B). NP expression on immune cells could be upregulated by stimulating conditions (22). As observed for Sema3A, cell surface expression of NP-1 increased on CD4+ T cells during stimulation with Abs to

FIGURE 2. Gene expression profile of Sema3A receptors in immune cells derived from healthy donors and patients with RA. A. mRNA expression levels (relative to cells derived from healthy donors) of NP-1 and NP-2 in the indicated immune cells derived from patients with RA. *p < 0.05 by one-way ANOVA test. B. Freshly isolated PBMCs derived from healthy donors and patients with RA (n = 8 per group) were stained for CD4 and NP-1. Representative flow cytometric analysis of PBMCs using CD4-allophycocyanin (y-axis) and NP-1–fluorescein (x-axis) labels is shown. C. Representative example of NP-1 (left) and NP-2 (right) expression on purified CD4+ T cells stimulated with plate-bound Abs specific for CD3 and CD28 for 24 h. D. mRNA expression levels (relative to cells derived from healthy donors) of plxn-a1 and plxn-a4 in the indicated immune cells derived from patients with RA. *p < 0.05 by one-way ANOVA test.
CD3 and CD28 (Fig. 2C). In contrast, there was no significant difference in NP-2 expression in stimulated CD4+ T cells (Fig. 2C).

Good Abs to Plxn-a1 and Plxn-a4 are not yet readily available, so I compared mRNA expression of these Plxn-as using real-time RT-PCR as an indication for possible protein expression. The RA group showed significantly increased plxn-a1 expression in PBMCs, CD4+ T cells, CD8+ T cells, and macrophages respect to the control group (Fig. 2D). PBMCs and T cells also expressed higher plxn-a4 mRNA in patients with RA than in control subjects (Fig. 2D). There was very low levels of plxn-a1 and plxn-a4 mRNA and no significant difference between the control and RA groups in synovial fibroblasts (Fig. 2D). Together, these results suggested that Sema3A and NP-1 expression is induced during T cell activation, and transient expression of Sema3A is defective in T cells derived from patients with RA. Therefore, Sema3A upregulation may modify development of autoimmune arthritis in vivo.

Recombinant Sema3s and gene delivery of Sema3A in vivo
A recombinant construct of Sema3A with an Fc tag was previously found to inhibit anti-CD3–induced T cell proliferation (20). In this study, I have used a Sema3A tagged at the C terminus with a FLAG epitope tag, which I have purified on an anti-FLAG affinity resin, as previously described (28). A recombinant Sema3C was also used during in vitro experiments to determine whether Sema3A and Sema3C could act in an opposing fashion (28). Recombinant Sema3A and Sema3C, preferentially in their complete forms (~95 kDa), were detected by immunoblotting (Supplemental Fig. 1A). The degree of cleavage of semaphorins (see as a band around 65 kDa) produced by transfected HEK293 cells did not exceed 10% of the total amount of semaphorins found in the conditioned medium. The effect of Sema3A and Sema3C on the proliferation of freshly isolated T cells was then tested. The results from a representative dose–response experiment are shown in Supplemental Fig. 1B and demonstrate the negative effect of Sema3A on TCR-stimulated T cell proliferation in keeping with that described in the literature (20). In contrast, Sema3C had no effect on T cell proliferation (Supplemental Fig. 1B), possibly because these cells contain ~4 fold less NP-2 compared with NP-1 (Fig. 2A).

To characterize the expression levels of Sema3A in vivo via the Sema3A-encoding plasmid, I injected naive DBA/1 mice i.p. with Sema3A-expressing plasmid (1 µg), and protein levels were assayed at selected time points. Samples were collected from peritoneal lavages, blood, and hind paws at 1, 3, and 7 d after injection. Increased Sema3A protein levels were demonstrated by Western blot in peritoneal lavage, blood plasma, and hind-paw homogenates after i.p. Sema3A-encoding plasmid administration (Supplemental Fig. 2A). Levels of Sema3A in mice treated with control plasmid at pooled time points were not detectable.

Sema3A attenuates CIA
Next, I investigated whether overproduction of Sema3A using a Sema3A-encoding plasmid has the ability to prevent and treat the development of CIA in DBA/1 mice, a surrogate model of human RA. DBA/1 mice were immunized with CII/CFA as described in Materials and Methods. Mice began to show clinical signs of arthritis on day 20 after immunization. For the CIA prevention studies, i.p. injection of Sema3A-expressing plasmid was initiated 1 d before induction of CIA and then once every 5 d until day 50. For the CIA treatment studies, mice with established clinical arthritis (average score of 4; days 20–22 after primary immunization) were randomized and injected with Sema3A-encoding plasmid or control plasmid. There was no apparent toxicity or

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Inhibition of CIA by DNA injection with Sema3A. A and B, Prevention. Male DBA/1 mice (n = 8 per group) were injected i.p. with indicated plasmid DNA (1 µg) once daily starting 1 d before induction of CIA. Hind-paw swelling (A) and arthritis score (B) were assessed as described in Materials and Methods. C and D, Treatment. DBA/1 mice (n = 8 per group) were immunized with CII, randomized, and at onset of disease (clinical score of 4), injected i.p. once daily with plasmid DNA (1 µg) as shown. Data from one independent representative experiment of two are shown. Data shown are the means ± SEM. Where not seen, the SEM bars are smaller than the symbol used. **p < 0.01 compared with pCDNA3-treated mice.
weight loss in mice receiving DNA (Supplemental Fig. 2B). In addition, Sema3A was effectively upregulated in cell lysates of PBMCs for several days after i.p. injection of Sema3A-expressing plasmid in CIA mice (Supplemental Fig. 2C). In contrast, endogenous levels of Sema3A were very low in PBMCs and not significantly different from levels after control plasmid administration. Sema3A was also overexpressed by plasmid injection in spleen cells isolated from mice 24 h after the last DNA treatment (day 48). In contrast, a slight Sema3A expression was shown in the spleen lysates derived from arthritic mice receiving pcDNA3 (Supplemental Fig. 2D).

In the model of prevention, Sema3A overexpression demonstrated the ability of Sema3A to significantly reduce development of CIA. Indeed, I observed lower paw swelling (Fig. 3A) and clinical scores (Fig. 3B) in mice i.p. injected with Sema3A-expressing plasmid compared with those injected with control plasmid without insert (pcDNA3). Similarly, Sema3A overexpression inhibited the progression of established arthritis as assessed by both paw swelling and clinical score (Fig. 3C, 3D). The reduction in disease severity was also evident in histological examination (Supplemental Fig. 3A) and was accompanied by reduced bone erosion, synovial hyperplasia, and inflammatory infiltration into the joint compartment (Supplemental Fig. 3B).

Effects of Sema3A on the concentrations of antitype II collagen Abs and inflammatory cytokines in the sera of arthritic mice

The pathogenesis of CIA is dependent on the response of the animal to CII challenge and the subsequent generation of Abs that recognize collagen-rich joint tissue. On day 45, in arthritic mice injected with control plasmid, the concentrations of antitype II collagen Abs were greatly increased (Fig. 4A). Total antitype II collagen Ab concentrations and the serum levels of the IgG2a subclass, which is typically produced during a Th1 response, were lower in mice treated with Sema3A (Fig. 4A, 4B). Moreover, a slight but significant increase in anti-CII IgG1 levels was observed at the end of the treatment sera of DBA/1 mice injected with Sema3A in comparison with control mice (Fig. 4C).

Several studies have found that cytokines appear to influence the B cell dependent Ab generation during the progression of CIA (23). Sema3A reduced expression of IFN-γ (formally attributed to Th1 response) and IL-17 (the well-known Th17-derived cytokine) in the sera of arthritic mice (Fig. 4D, 4E). Moreover, I found that Sema3A overexpression in vivo significantly increased IL-10 production (Fig. 4F), suggesting that systemic inflammatory responses were modified.

Sema3A regulates cytokine production in a cell-type–dependent manner in vitro

I next investigated mechanisms whereby Sema3A-mediated effects were achieved. After stimulating PBMCs with Abs to CD3 and CD28 in the presence of Sema3A, IFN-γ release was inhibited up to 60% and IL-2 production was inhibited by 50% (Fig. 5A). Sema3C did not substantially affect cytokine release in CD3- and CD28-stimulated PBMCs (Fig. 5A). In parallel, I analyzed the cytokine production in endotoxin-stimulated PBMCs. Unlike Sema3C, Sema3A reduced production of IL-23 by 70% and of TNF-α and IL-6 by ∼50% (Fig. 5B). Accordingly, Sema3A blocked IL-23 production up to 80% and IL-6 and TNF-α secretion up to

![FIGURE 4.](http://www.jimmunol.org/) Serum anti-CII Abs and proinflammatory cytokines in Sema3A-treated mice. A–C, CII-specific total IgG (A), IgG1a (B), and IgG2a (C) concentration in the sera obtained from each group of mice on day 45. The titer of anti-CII IgG isotypes was evaluated by ELISA. Data were obtained from six mice per group. *p < 0.05 versus control mice. D–F, Isolated CD4+ T cells from Sema3A-treated or pcDNA3-treated mice were stimulated with CII (30 µg/ml) in the presence of irradiated spleen cells for 3 d. The IFN-γ (D), IL-17 (E), and IL-10 (F) levels in the culture supernatants (1 × 10⁶ cells) were measured by ELISA. The value of each cytokine from unstimulated CD4+ T cells from CII-immunized mice was below detection limits (data not shown). All results are representative of at least three experiments. Data are averages ± SEM from three independent experiments. *p < 0.05 versus spleen cells from control mice.
50% in stimulated PBMCs derived from individuals with active RA (Fig. 5C).

I also compared the ability of Sema3A and Sema3C to alter cytokine production on isolated PBMC subpopulations. Exogenous Sema3A suppressed IFN-γ production by CD4⁺ T cells in response to anti-CD3/CD28 Abs (Fig. 5D). Sema3C had no effect on IFN-γ secretion (Fig. 5D), probably because these cells have lower NP-2 expression levels (see Fig. 2A). Notably, neither Sema3A nor Sema3C inhibited TNF-α production in macrophages and IL-6 synthesis in synovial fibroblasts stimulated with LPS (Fig. 5D), which may be because of the lower expression of plexin-a4 observed in these cells (Fig. 2D). These results suggest that the ability to regulate cytokine production of Sema3A could reside intrinsically within the CD4⁺ T cells.

NP-1 expression identified a subgroup of CD4⁺ T cells with a Treg-like phenotype that express Sema3A and IL-10.

CD4⁺ T cells represent a series of distinct cell populations with different functions (24). In these cells, NP-1 expression has been closely associated with the function of Tregs (24). Our results indicate that NP-1 expression is induced in nonregulatory T cells (Fig. 2B), which is an important step in Treg differentiation. However, it is not known whether all CD4⁺ T cells have the same capacity to express NP-1. To investigate whether NP-1 is expressed by any T cell subset or whether expression is restricted to a distinct lineage, I analyzed NP-1 mRNA in CD4⁺ T cells driven in vitro toward Th1, Th2, Th17, or Treg phenotypes (Fig. 6A). Th2 cells were able to significantly induce NP-1 mRNA up to 10-fold on anti-CD3/CD28 stimulation and addition of TGF-β. Th1 showed only a modest increase, whereas Th17 cells stimulated under the same condition did not increase NP-1 expression. The in vitro-generated Tregs were unable to further upregulate NP-1, which was already at high levels under the resting conditions (Fig. 6A). Therefore, CD4⁺NP-1⁺ T cells may not be a distinct lineage but rather may represent a certain state of each existing lineage.

Nevertheless, most of them are likely inducible Tregs. Indeed, sorted CD4⁺NP-1⁺ T cells expressed markedly greater amounts of mRNA encoding Foxp3 (the central Treg transcription factor) and IL-10 (key cytokines for Tregs) than sorted CD4⁺NP-1⁻ T cells (Fig. 6B). Moreover, Sema3a and plexin-a4 were expressed at a substantial level in CD4⁺NP-1⁺ T cells, but not in CD4⁺NP-1⁻ T cells (Fig. 6B), thus suggesting the existence of Sema3A-dependent autocrine circuit in CD4⁺NP-1⁺ T cells. The plexin-a1 mRNA was expressed in both cell types, whereas sema3c and np-2 transcript was not detectable.

To test whether CD4⁺NP-1⁺ T cells manifest regulatory function for inhibition of CD4⁺ T cell proliferation, autologous CD4⁺ T cells were isolated and labeled with the fluorescent dye CFSE. Proliferation of CFSE-labeled CD4⁺ T cells was inhibited by coculture with CD4⁺NP-1⁺ T cells (Fig. 6C). In contrast, the

![Figure 5. Sema3A regulates cytokine synthesis in primary human cells.](http://www.jimmunol.org/)

**A**. Cytokine production by PBMCs from healthy volunteer donors (n = 5–6 donors per cytokine) after treatment with either Sema3A or Sema3C at the indicated concentrations and stimulated with plate-bound Abs specific for CD3 and CD28 for 24 h. Supernatants were analyzed for expression of INF-γ and IL-2 by ELISA. **B**. Cytokine production by PBMCs after treatment with either Sema3A or Sema3C (both at 300 ng/ml) and exposure to 1 ng/ml LPS for 24 h. Supernatants were analyzed for expression of IL-23, TNF-α, and IL-6 by ELISA. Values are presented as the mean relative level ± SEM as compared with untreated controls. *p < 0.05 versus untreated controls by paired t test of absolute cytokine levels. **C**. PBMCs from patients with RA (n = 6 per cytokine) were exposed to 300 ng/ml Sema3A and stimulated as described in **B**. Supernatants were analyzed for IL-23, TNF-α, and IL-6 by ELISA. **D**. INF-γ, TNF-α, and IL-6 synthesis by primary human CD4⁺ T cells, macrophages, and synovial fibroblasts after treatment with either Sema3A or Sema3C (both at 300 ng/ml) and exposure to anti-CD3/CD28 Abs (CD4⁺ T cells) or 1 ng/ml LPS (macrophages and fibroblasts) for 24 h. Data are shown as the mean of triplicate values ± SD from one of three representative experiments. *p < 0.05.
addition of CD4\(^+\)NP-1\(^-\) T cells to CFSE-labeled CD4\(^+\) T cells did not alter the proliferation of responder cells (Fig. 6C), thus indicating the suppressive properties of CD4\(^+\)NP-1\(^-\) cells. This inhibition of proliferation by CD4\(^+\)NP-1\(^-\) T cells was reversed by the addition of anti-Sema3A mAb (Fig. 6D), as well as anti–IL-10 mAb, but not by the addition of anti-IL-4 mAb (a central Th2 cytokine; Fig. 6D). Moreover, CD4\(^+\)NP-1\(^-\) T cells also inhibited IFN-\(\gamma\) production by CD4\(^+\) T cells in anti-CD3–driven stimulation cultures (Fig. 7A). Again, this inhibition was partially reversed by an anti-Sema3A and by anti–IL-10 mAbs (Fig. 7B). Therefore, these results demonstrate that CD4\(^+\)NP-1\(^-\) T cells have a Treg-like phenotype, and the suppressive effect is Sema3A and IL-10 dependent.

Sema3A promotes regulatory activity of CD4\(^+\)NP-1\(^+\) T cells by enhancing IL-10 production

IL-10 is a key cytokine involved in the suppressive properties of several CD4\(^+\) T cells with a Treg-like phenotype (23). Because Sema3A in vivo increased IL-10 production (Fig. 4), I tested whether Sema3A can increase endogenous levels of this cytokine in both CD4\(^+\)NP-1\(^+\) T cells and CD4\(^+\)NP-1\(^-\) T cells. After anti-CD3 stimulation, CD4\(^+\)NP-1\(^+\) T cells produced significantly more IL-10 (Fig. 7C). This production was significantly increased by Sema3A, in a dose-dependent fashion. Moreover, Sema3A did not modify IL-10 synthesis of CD4\(^+\)NP-1\(^-\) T cells (Fig. 7C), suggesting a causal relation between IL-10 and Sema3A. This relation was also supported by the findings that the anti-Sema3A Ab reduced IL-10 production in stimulated CD4\(^+\)NP-1\(^+\) T cells (Fig. 7D), and that the negative effect of this Ab on the suppressive properties of CD4\(^+\)NP-1\(^+\) cells was significantly reversed by exogenously added IL-10 (50 ng/ml). Data are representative of three experiments.

Discussion

RA is one of the most common chronic inflammatory syndromes. The immunogenetics of RA suggests a key role for aberrant pathways of T cell activation in the initiation and/or perpetuation of disease (23, 24). Recent evidence indicates that an imbalance in immune regulation caused by Treg deficiency or defective function can lead to RA. Analysis of the frequency of peripheral blood Tregs in patients with RA has, however, yielded contradictory...
results. Although some studies have reported an increased frequency of peripheral blood Tregs, others have demonstrated either no difference in the frequency of Tregs, as compared with healthy donors, or a decreased level of peripheral blood Tregs (24). I found that patients with RA showed a significantly greater percentage of CD4+ T cells that expressed NP-1 compared with control subjects. Although NP-1 is a postulated marker of Tregs, its expression may be not limited to Tregs. I observed that CD4+ T cells, CD8+ T cells, and macrophages that do not possess suppressive functions can express NP-1. Moreover, NP-1 expression correlates with in vitro proliferation of CD4+ T cells to anti-CD3/CD28 stimulation. Therefore, because patients with RA are known to exhibit high levels of T cell activation, NP-1 expression on CD4+ T cells may act as a marker of cell activation.

Notwithstanding these caveats, I decided to investigate the role of NP-1 ligand Sema3A in RA. Several lines of evidence suggest that Sema3A/NP-1/plexin-A4 functions in the T cell responses (32, 33). All these molecules are expressed in activated T cells and DCs. Both NP-1–mutant T cells, in which the Sema3A binding site is specifically disrupted, and plexin-A4–deficient T cells, exhibit enhanced in vitro proliferation after anti-CD3 Ab stimulation (32). Moreover, plexin-A4–deficient mice have enhanced T cell priming and exacerbated T cell–mediated immune responses, such as experimental autoimmune encephalomyelitis (32). I found that CD4+ T cells isolated from patients with RA are unable to up-regulate Sema3A expression after stimulating conditions, and that these cells express greater levels of plexin-A4. Therefore, the Sema3A/NP-1/plexin-A4 pathway could be pathologically relevant.

In this study, I introduced Sema3A expression vector into the T and B cell-dependent animal model of RA, namely, CIA (34). Overexpression of Sema3A was sufficient to prevent CIA and to achieve a dramatic arrest in disease progression, as judged by clinical, histopathological, and immunological manifestations of arthritis. These results extend findings demonstrating that Sema3A has a distinct role in regulating human T cell proliferation and differentiation (19–21) by showing that Sema3A regulates inflammatory responses in vivo.

FIGURE 7. Sema3A enhances IL-10 production in CD4+NP-1+ T cells. A. Polyclonal T cells (1), CD4+NP-1+ T cells (2), or CD4+NP-1- T cells (5) (all at 2 × 10^5 cells/well) were stimulated with anti-CD3. Polyclonal T cells were also cocultured in the presence of initial addition of CD4+NP-1+ T cells (3) or CD4+NP-1- T cells (4) (ratio 1:1). After 5 d, the cell-free culture supernatants were collected and analyzed for the content of INF-γ by ELISA. B. Cells were treated as indicated in A in the presence or absence of anti-Sema3A or anti–IL-10 mAbs, and the amount of INF-γ was analyzed by ELISA. Data are expressed as nanograms per milliliter and indicate the mean ± SD of three separate experiments. The relevant p values are given. C, CD4+NP-1+ T cells or CD4+NP-1- T cells were either left unstimulated or stimulated with anti-CD3 in the presence or absence of graded doses of Sema3A. Day 5 supernatants were assessed for IL-10 content. D, Isotype control or anti-Sema3A Abs were added to cultures of CD4+NP-1+ T cells stimulated with anti-CD3. IL-10 production was evaluated at day 5 by ELISA. Mean data from four experiments are shown. *p < 0.05 using two-tailed Student t test.

Sema3A probably exerts pleiotropic effects on a variety of cell types involved in RA, such as the T and B cells, macrophages, monocytes, and osteoclasts (18). Moreover, NP-1 is also expressed on endothelial cells and tumor cells, functioning as receptor for vascular endothelial growth factor (VEGF). In these cells, Sema3A and VEGF compete for binding to NP-1, and Sema3A ectopically reintroduced in cancer cells blocks tumor angiogenesis (16). Because the blockade of angiogenesis, and especially of VEGF, appears to be a promising avenue for the future treatment of RA (34), Sema3A could also inhibit development of CIA by functioning as antiangiogenic factor. However, no obvious reduction in the vessel density was observed in the Sema3A-treated mice, as demonstrated by immunostaining with the endothelial cell marker PECAM (data not shown). Despite this finding, I cannot exclude the possibility that Sema3A treatment can modify the quality of angiogenesis and perfusion occurring in different microregions within joints.

Nevertheless, I observed that IFN-γ and IL-17 were reduced in the Sema3A-treated mice, but IL-10 was increased. The major
sources of IL-10 in RA were monocytes in the lining layer and T cells in the mononuclear cell aggregates (23). IL-10 plays an essential role in the functional activities of Tregs, although different CD4+ T cell subsets can produce IL-10. Secretion of IL-10 by Th1 or Th17 cells may play an important role in limiting their own effector function. IL-10 produced by Th2 cells suppresses Th1 cell proliferation. Notably, Foxp3+ Tregs produce high levels of IL-10, suggesting that IL-10 production in Tregs is independent of Foxp3 (24). The originally described TR1 cells (IL-10–producing Tregs) include many different types of cells that are capable of producing IL-10. Because IL-10 production by all CD4+ T cells serves as a negative regulatory mechanism for limiting the immune responses and preventing host tissue damage, I hypothesized that induction of IL-10 synthesis contributed to the clinical efficacy of Sema3A therapy in CIA. Therefore, I investigated mechanisms whereby such effects were achieved at least in vitro.

In line with results in arthritic mice, Sema3A modified cytokine production, and this effect varied in a cell-type–dependent manner. I therefore decided to examine whether NP-1 was responsible for such a difference. Sema3A did not reduce proliferation of sorted CD4+NP-1+ and CD4+NP-1− T cells (data not shown), whereas sorted CD4+NP-1− T cells responded to exogenous Sema3A by increasing IL-10 production. These results indicated that Sema3A–mediated cytokine production in CD4+ T cells is NP-1 dependent. The mechanism for the induction of IL-10 by Sema3A on NP-1− T cells is currently unknown and merits further investigation. It could well involve the p38-MAPK pathway, which is activated in tumor cells by Sema3B in a NP-1–dependent manner and represents an important signaling pathway that can control cytokine expression (12).

The CD4+NP+ T cells show negative immunoregulatory effect (22). I found that already-committed cells, such as Th1 and Th17 cells, showed only moderate NP-1 induction. In contrast, Tregs expressed greater basal mRNA levels of NP-1 but were inefficient to upregulate NP-1 when treated with TGF-β. This result suggests that NP-1 expression is induced during early T cell differentiation, but in already-committed cells, it is strongly associated to Tregs. The CD4+NP+ T cells represent a previously recognized subset of Tregs derived from naive CD4+ T cells induced after TCR activation (22). These cells express Foxp3 and low levels of IL-4. In addition, they produced substantial levels of IL-10. Moreover, I found that they expressed greater Sema3A and plexin-A4 mRNA levels than CD4+NP-1− T cells, thus suggesting that Sema3A may regulate CD4+NP-1− T cell functions by autocrine mechanism. The relevance of Sema3A in CD4+NP-1− T cell activity was demonstrated by the enhanced proliferation of responder cells in cocultures when endogenously produced Sema3A was blocked with a selective Ab. It was previously shown that NP-1 expression in Tregs contributes to prolonged contact between Tregs and DCs, increasing the suppression of Th cell proliferation by Tregs (22). My results extend these data by showing that Sema3A–NP-1 interactions may play a similar role. These data also outline the complexity of NP-1 involvement in the regulation of the immune response. Indeed, whereas NP-1 participates in heterotypic interactions between DCs and T cells, because it is found polarized in DC-T cell contact zone (33), endogenous Sema3A, which does not interfere in formation of DC-T cell conjugates (20), may promote Treg function in the absence of APCs.

In conclusion, I have shown that patients with RA had significantly greater percentages of CD4+ T cells positive for NP-1 than did control subjects, and that these cells did not increase the NP-1 ligand Sema3A under stimulating conditions. The CD4+NP+ T cells are responsive to Sema3A, and addition of Sema3A-blocking Abs inhibits the CD4+NP+ T cell ability to suppress allosresponses. This is achieved by reducing IL-10 production. However, because the molecular basis and physiological role of Sema3A–NP-1 interactions in immune cells remain poorly understood, I cannot exclude the possibility that other mechanisms are involved. Furthermore, I show that upregulation of Sema3A significantly attenuated the development of CIA and proinflammatory responses when administered prophylactically. Therefore, these results provide further rationale for prospective clinical trials to determine whether Sema3A provides efficacy in RA and other autoimmune diseases.

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Disclosures

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