Immunoregulatory role of IL-35 in T cells of patients with rheumatoid arthritis

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Abstract

Objective. IL-35 is the most recently identified member of the IL-12 family. It consists of EBV-induced gene 3 (EBI3) and IL-12α chain p35. We investigated whether IL-35 enhances the in vitro immunosuppressive function of peripheral blood isolated from patients with RA.

Methods. Peripheral blood was harvested from 17 active and 10 inactive RA patients and IL-35 concentrations were quantified using an ELISA. An expression vector containing IL-35 with a FLAG tag at the carboxyl-terminus was constructed by covalently linking EBI3 and IL-12α (p35). The function of IL-35 was then evaluated in a suppression assay using T cells isolated from human RA patients with CD2, CD3 and CD28 antibodies.

Results. Serum IL-35 levels and the number of Treg were decreased significantly in patients with active RA. There was a significant correlation between serum IL-35 and the 28-joint DAS with ESR (DAS28-ESR) in patients with active RA. IL-35 treatment enhanced the regulatory function, suppressing the levels of inflammatory cytokines such as IL-17 and IFN-γ and the cellular growth of effector T cells stimulated by conjugation with CD2, CD3 and CD28.

Conclusion. These data revealed that IL-35 might suppress T cell activation during the peripheral immune responses of RA. Therefore our data suggest that IL-35 might have multiple therapeutic targets.

Key words: rheumatoid arthritis, regulatory T cells, IL-35, DAS28-CRP, IFN-γ, IL-17, suppressive function.

Introduction

Treg are a unique subset of CD4+ T cells that are essential for regulating autoimmune disease. Naturally occurring Treg (nTreg) are a small population of thymus-derived CD4+ T cells that are characterized by the constitutive expression of the IL-2Rα chain (CD25) without antigen responses [1]. Recently the lineage-specific transcription factor forkhead box P3 (FoxP3) was determined to be a key hallmark of nTreg, functioning as the master regulatory gene during their development and function [2–4]. Although nTreg can suppress the responses of effector T cells [5, 6], their underlying mechanism of action remains unclear.

Recently a novel member of the IL-12 family of cytokines, IL-35, was found to be involved in the enhanced function of Treg in vitro and in vivo [7–9]. IL-35 is a heterodimeric cytokine composed of the EBV-induced gene 3 (EBI3) and the p35 subunit of IL-12. It is constitutively expressed by mouse Treg, but not by CD4+CD25+ conventional responder T cells (Tres) [8, 9]. Maximal expression and secretion of IL-35 requires cell–cell contact between Treg and Tres. The loss of IL-35 expression reduces the in vivo suppressive capacity of Treg.
In addition, recombinant mouse IL-35 enhanced the proliferation of Treg and suppressed the proliferation of Tres when cultured with soluble anti-CD3 antibody and antigen presenting cells (APCs) in vitro [7]. The IL-35 receptor is IL-12Rβ2 and gp130, and its signalling cascade is known [10]. Therefore IL-35 plays a crucial role in Treg-mediated immune suppression.

To explore the possible involvement of IL-35 in autoimmune disease, Niedbala et al. [7] showed that recombinant IL-35 effectively attenuated CIA, a mouse model of RA, via mechanisms involving the suppression of T cell proliferation and Th17 cell differentiation. Kochetkova et al. [11] also reported that recombinant IL-35 treatment inhibited the progression of murine CIA and that Treg stimulated with IL-35 produced IL-10 in vitro. These data suggest that enhanced production of IL-10 might play a role in the effects of IL-35 on CIA. This same study also reported that IL-35 suppressed T cell proliferation and Th17 differentiation [11]. In an additional study, Valencia et al. [12] reported that Treg isolated from patients with active RA expressed reduced levels of FoxP3 and poorly suppressed effector T cells; however, their suppressive function was restored after treatment with an anti-TNF antibody in vitro [12]. Taken together, these studies suggest that Treg from patients with RA exhibit reduced ability to suppress the production of two principal pro-inflammatory cytokines by effector T cells [13].

However, little is known regarding the definitive mechanisms of IL-35 in autoimmune disease. Therefore we investigated the immunoregulatory role of IL-35 in patients with RA.

### Patients and methods

#### Patients and clinical data assessments

Peripheral blood was obtained from 17 patients with active RA, 11 with inactive RA (Table 1) and 20 healthy controls. All RA patients met the 1987 ACR criteria [14].

The following clinical parameters were assessed: RF, MMP-3, ACPA and the 28-joint DAS based on CRP (DAS28-CRP). RA disease activity can be interpreted as low (DAS28-CRP <2.0), moderate (DAS28-CRP 2.04–2.7), moderate (DAS28-CRP 2.7–4.1) or high (DAS28-CRP >4.1) [15]. Moderate and high disease activity patients were defined as having active RA, whereas those with low disease activity had inactive RA. This study was approved by the ethics committee of Juntendo University and was performed in accordance with the principles outlined in the Declaration of Helsinki. Signed informed consent was obtained from all patients and healthy controls prior to starting the study.

#### Antibodies and reagents

Mouse monoclonal FLAG (M2 antibody) and fluorescein isothiocyanate-conjugated M2 antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal antibodies against β-actin (H-196) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phycoerythrin-cyanin 5-labelled anti-human CD4 (RPA-T4) antibodies were purchased from eBioscience (San Diego, CA, USA). PE-conjugated anti-human CD25 (2A3) and Alexa Fluor 647-labelled anti-human CD127 (A7R34) antibodies were obtained from BD Pharmaning (San Diego, CA, USA). 5,6-carboxy fluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, OR, USA). Anti-biotin MACSibead particles pre-loaded with biotinylated CD2 (LT2), CD3 (OKT3) and CD28 (15E8) antibodies (Treg Suppression Inspector) were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). All DNA oligonucleotides (oligos) were obtained from Hokkaido System Science (Sapporo, Hokkaido, Japan).

#### Construction of IL-35 expression vector

IL-35, FLAG-tagged at the C-terminus, was constructed using the p3×FLAG-CMV-13 vector (Sigma-Aldrich). A pre-protrypsin leader sequence (PPTLS) was added to the N-terminus of FLAG-tagged IL-35 to trigger spontaneous secretion. The covalent linkage of human EBi3 and IL-12/p35 was mediated by self-cleaving 2A peptide (VKQLNFDLDLKLAGDVE150SPG) [16] or the linker peptide (3×GGGGS) [7, 8]. The IL-35 expression constructs were created as described below. Annealed double-stranded oligos encoding a linker peptide were subcloned into the p3×FLAG-CMV-13 vector at the EcoRI and Clal restriction enzyme sites. Human full-length DNAs encoding

### Table 1 Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Active RA patients (n=17)</th>
<th>Inactive RA patients (n=11)</th>
<th>P-value</th>
<th>RA patients total (n=28)</th>
<th>Healthy controls (n=20)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Age, mean (s.d.), years</td>
<td>46.9 (12.1)</td>
<td>55.3 (11.1)</td>
<td>0.06</td>
<td>50.1 (12.2)</td>
<td>50.2 (15.1)</td>
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<td>Sex, males/females, n</td>
<td>7/11</td>
<td>5/6</td>
<td>0.727</td>
<td>12/17</td>
<td>7/13</td>
<td>0.65</td>
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<td>Duration of RA, mean (s.d.), years</td>
<td>6.0 (7.52)</td>
<td>9.8 (5.13)</td>
<td>0.014</td>
<td>7.5 (6.72)</td>
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<tr>
<td>DAS28-CRP, mean (s.d.)</td>
<td>4.66 (1.25)</td>
<td>2.04 (0.59)</td>
<td>0.0001</td>
<td>3.62 (1.62)</td>
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<tr>
<td>MTX dose, mean (s.d.), mg</td>
<td>6.94 (1.23)</td>
<td>6.54 (2.97)</td>
<td>0.84</td>
<td>6.78 (2.42)</td>
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</tr>
<tr>
<td>Biologic therapy, n (%)</td>
<td>4 (23.5)</td>
<td>9 (81.8)</td>
<td>0.0025</td>
<td>13 (46.4)</td>
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</tbody>
</table>

DAS28-CRP: 28-joint DAS based on CRP.
EBI3 and IL-12/p35 were amplified by PCR from a human spleen cDNA library using Phusion Hot Start DNA Taq polymerase (Finnzymes, Espoo, Finland). The primer sequences were as follows: EBI3 sense 5'-ggatccagctggccgcccctgaggg-3' and anti-sense 5'-ggatccgcggccgcccctgaggg-3', and IL-12/p35 sense 5'-ggatccatcgataacccgctgagggc-3' and anti-sense 5'-ggatccatcgataacccgctgagggc-3'. HindIII and NotI restriction sites were introduced into EBI3 and a CiaI site was introduced into IL-12/p35. The PCR fragments containing the EBI3 and IL-12/p35 sequences were then serially subcloned into the p3XFLAG-CMV-13 vector to form a pPPTLS-EBI3-linker-IL-12/p35-3′ × FLAG expression construct. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). In-frame expression was confirmed by sequencing.

Preparation of human IL-35

The FLAG-tagged human IL-35 expression construct was transfected into HEK293 cells using the calcium phosphate method. A p3XFLAG-CMV-13 vector containing only a portion of the linker peptide was also used as a control. The culture medium was switched to X-VIVO15 (Cambrex Bio Science, Walkersville, MD, USA) 24 h after transfection; IL-35 supernatants were then harvested after a 3-day incubation.

Immunoblotting

Immunoblotting was performed according to standard methodologies. Briefly, cells were lysed gently in ice-cold buffer containing 1% Nonidet P-40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and 30 mM NaF supplemented with protease inhibitors (Protease Inhibitor Cocktail Set V; EMD Chemicals, Darmstadt, Germany). Lysates were clarified by centrifugation at 10 000 g at 4°C. Extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with anti-FLAG M2 antibodies and then incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Arlington Heights, IL, USA). Blots were developed using an enhanced chemiluminescent substrate (Super Signal West Pico; Pierce, Rockford, IL, USA) and visualized using a LumiVision analyser (Taitec, Tokyo, Japan).

Isolation of T cell subsets

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and patients with RA using Ficoll-Hypaque density gradient centrifugation. Monocytes were depleted by adherence to the plastic surfaces of the culture dishes. Next, 1 × 10⁷ lymphocytes were resuspended in 600 μl of PBS containing 3% fetal calf serum and labelled with 5 μl of phycoerythrin–cyanin 5 conjugated anti-human CD4, 5 μl of phycoerythrin conjugated anti-human CD25 and 5 μl of Alexa Fluor 647 anti-human CD127 antibodies. After incubation for 20 min at 4°C, fluorochrome-labelled T cells were separated into CD4⁺ CD127⁻ CD25⁺ Treg and CD4⁺ CD127⁺ CD25⁺ Tres using a FACSARia cell sorter (Becton Dickinson, Mountain View, CA, USA). The resultant purity was >90% for each T cell subset.

IL-35 mRNA analysis

Total RNA was isolated from 1 × 10⁶ Treg, Tres and PBMCs using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcriptase (RT) reactions were performed using the High Capacity cDNA Reverse Transcriptase Kit (Applied BioSystems, Foster City, CA, USA) according to the manufacturer’s protocol. Semi-quantitative real-time PCR was performed in a 25 ml reaction volume containing 12.5 ml of Power SYBR Green Master Mix (Applied BioSystems), 10 nM forward and reverse primers for human FoxP3 (forward 5'-aaggtctcatctgtgcatcatc-3' and reverse 5'-tctctcgctcatccacgg-3'), EBI3 (forward 5'-gtgccgctctgatagggc-3' and reverse 5'-gccagctttgctttgaggg-3'), IL-12/p35 (forward 5'-aacaccttcctggcggcactc-3' and reverse 5'-gaacgttcgccagctttgaggg-3') and β-actin (forward 5'-caagcccaacctgagaaagac-3' and reverse 5'-aggagggagatgggtaaggg-3') and 1 μg of cDNA. The cycling conditions were denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min for annealing and extension. The expression of EBI3 and IL-12/p35 was measured on an ABI Prism 7500 Sequence Detection System (Applied BioSystems). PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The levels of EBI3 and IL-12/p35 in each sample were normalized to β-actin.

Measuring serum IL-35 and IL-10

Human IL-35 concentrations were quantified using an ELISA kit (LEGEND MAX Human IL-35 Heterodimer ELISA Kit with pre-coated plates; BioLegend, San Diego, CA, USA) following the manufacturer’s instructions. The concentrations of human IL-10 (R&D Systems, Minneapolis, MN, USA) were quantified by ELISA following the manufacturer’s instructions.

In vitro suppression assay

A CFSE-based assay for measuring the regulatory function of CD4⁺ CD127⁻ CD25⁺ Treg was performed as described previously [17]. CD4⁺ CD127⁺ CD25⁻ Tres (1 × 10⁶/ml) were suspended in PBS/0.1% BSA and incubated with 10 μM CFSE for 10 min at 37°C. Ice-cold X-VIVO15 was then added to the cell suspensions, which were incubated on ice for 5 min to quench the staining. CFSE-labelled Tres were washed three times and resuspended in X-VIVO15. Before plating the cells, 10 μl of AB serum (TaKaRa BIO, Shiga, Japan) was added to 96-well round-bottom plates (Iwaki, Tokyo, Japan). CFSE-labelled Tres cells (3 × 10⁵/well) were then co-cultured with 1 × 10⁶ Treg cells (Tres:Treg ratio 3:1) in 200 μl of solution in the presence of 5 μl of Treg Suppression Inspector (bead:Tres ratio 1:1) and 50 μl of IL-35 or control supernatant (25% v/v). After 5 days of culture, proliferation was assessed by measuring the CFSE signals using flow cytometry. The concentrations
of human IL-17, IFN-γ and IL-10 (R&D Systems, Minneapolis, MN, USA) were quantified by ELISA following the manufacturer’s instructions.

Statistical analysis

All data were expressed as mean (±D). Statistical analyses were performed using the Mann–Whitney U-test, Steel-Dwass method and Pearson’s correlation coefficient. Statistical significance was defined as \( P < 0.05 \).

Results

Construction of a human IL-35 expression vector

We devised a construct encoding human IL-35 by covalently linking intact EBI3 with IL-12α/p35-FLAG protein, as described previously for mouse IL-35 [7, 8]. A secretory signal sequence (PPTLS) was added to the N-terminus of EBI3 to promote spontaneous secretion and EBI3 was linked to IL-12α/p35 with two different types of linker: native IL-35 (self-cleaving 2A peptide) and single chain IL-35 (3 × GGGGS linker peptide) (Fig. 1A). Harvested supernatants from HEK293 cells transfected with these expression vectors showed a high yield and enriched soluble production of human IL-35 (Fig. 1B).

Expression of IL-35 in Treg isolated from RA patients

Next we determined the Treg:Tres ratio and mRNA expression level of p35 and EBI3, the two components of IL-35, in peripheral blood isolated from patients with active RA. However, there were no significant differences in the expression of IL-35 in Treg cells from patients with active RA vs healthy donors (Fig. 2A) and active RA vs inactive RA (data not shown). The ratio of CD4+CD127−CD25+ Treg was decreased in patients with active RA compared with that in RA patients with stable disease whose symptoms had been suppressed by treatment (Fig. 2B).

Expression of serum IL-35 in RA patients and correlations with clinical data

We examined serum IL-35 levels in patients with RA and found the levels were significantly decreased in these patients (*\( P < 0.05 \), **\( P < 0.01 \); Fig. 3A). Moreover, serum IL-35 levels were significantly higher in patients with inactive vs active RA (*\( P < 0.05 \), **\( P < 0.01 \)).

Fig. 1 Construction and production of recombinant human IL-35

(A) Schematic diagram of the IL-35 construct. IL-35, tagged with 3 × FLAG at the C-terminus, was used to covalently link EBV-induced gene 3 (EBI3) and IL-12α chain p35 (IL-12α/p35) by alternately employing two linker peptides: a self-cleaving 2A peptide (VKQTLNFDLLKLAGDVESNPGP) and a linker peptide (3 × GGGGS). Pre-protrypsin leader sequence (PPTLS) was added to the N-terminus of IL-35 to allow spontaneous secretion. (B) Western blotting of recombinant IL-35. 293EBNA cells were transfected with two types of IL-35-encoding expression vector or p3XFLAG-CMV13 empty vector. Twenty-four hours after transfection, the culture medium was switched from DMEM containing 10% fetal calf serum to serum-free X-VIVO15 medium. Supernatants were harvested after 3 days of culture and then analysed by western blotting using anti-FLAG antibodies.
Fig. 2 Reduced expression of IL-35 mRNA in Treg

(A) PBMCs and purified responder T (Tres) and regulatory T (Treg) cells were analysed for the expression of FoxP3, EBI3 and IL-12α/p35 using RT-PCR. β-actin was used as an internal control to ensure that there were equal amounts of cDNA in each fraction. NC: healthy donors. IL-35 positive control: WEHI3B human IL-35 transfectant. (B) CD4+CD127−CD25+ Treg cells from a patient with RA and healthy control peripheral blood mononuclear cells (PBMCs) were examined by flow cytometry. The number of cells in each quadrant was assessed.

(A) Healthy Donor | RA patient | WEHI3B hIL-35 transfectant
---|---|---
Treg | Tres | Treg | Tres
IL-12α/p35 (214bp) | | |
EBI3 (237bp) | | |
FOXP3 (233bp) | | |
β-actin (212bp) | | |

(B) RA | NC | RA | NC
EBI3 | | |
IL-12α p35 | | |
However, serum IL-10 levels were not detected in patients with RA (data not shown).

Next we investigated the relationship between serum IL-35 levels in patients with RA and clinical parameters (DAS28-CRP, RF, MMP-3 and ACPA) and demonstrated a significant inverse correlation with DAS28-CRP (Fig. 3B; \( P < 0.01, R = -0.794 \)). In contrast, RF, MMP-3 and ACPA were not correlated with IL-35 (data not shown). It was dispersion data on CRP and ESR (data not shown) in patients with RA, therefore CRP and ESR were not correlated with IL-35. Moreover, serum IL-10 levels were not correlated with serum IL-35 levels.

**Effect of IL-35 on the regulatory potential of Treg**

To examine the suppressive effect of human IL-35 on the regulatory capacity of nTreg, FACS-sorted Treg and autologous Tres from patients with RA were co-cultured with human IL-35 in vitro. Isolated Tres were labelled with CFSE to allow Tres and Treg to be easily distinguished; non-labelled CD25+ Treg could be excluded from the analysis because they did not emit the CFSE signal. The CFSE division profile of Tres activated by anti-CD2-, anti-CD3- and anti-CD28-coated microbeads was then assessed over a 5 day incubation. There were up to four discrete division cycles of control cells, which was clearly reduced to two division cycles by co-culture of activated Treg with Tres at a ratio of 1:3 (Fig. 4A). Culture supernatants containing human full-length IL-35, not the linker peptide without EBI3 and IL-12α/β35, significantly enhanced the suppressive capacity of Treg with an increase in non-divided Tres (Fig. 4B). In contrast, there were no differences in the population of non-divided Tres that were co-cultured with Treg between native 2A and linker IL-35 supernatants (data not shown). IL-35 supernatants had no effect on the proliferation of activated Tres in response to anti-CD3/CD28 in the absence of Treg (data not shown). Therefore activated Tres in patients with RA were more differentiated to Th1 or Th17 and up-regulated IFN-\( \gamma \) and IL-17 production. In cytokine analysis, IL-35 treatment significantly suppressed the levels of TNF-\( \alpha \), IL-17 and IFN-\( \gamma \) in a dose-dependent manner, and the greatest suppression was observed at 500 pg/ml IL-35 (Fig. 4C). In contrast, IL-10 was not significantly affected by IL-35 treatment (data not shown).

**Fig. 3 Expression of IL-35 in the serum of RA patients and correlations with clinical parameters**

(A) Serum IL-35 levels were measured using ELISA in 28 patients with RA and 20 normal controls. Serum IL-35 levels were significantly decreased (*\( P < 0.05 \), **\( P < 0.01 \)) in RA patients. (B) Serum IL-35 levels correlated with the 28-joint DAS based on CRP (DAS28-CRP) in patients with RA (*\( P < 0.01 \), \( R = -0.794 \)).
of IL-17 (Fig. 4C) and IFN-γ (Fig. 4D), but not IL-10 (data not shown). These data reveal the immunosuppressive properties of IL-35, whereby it enhances Treg activity in patients with RA (see supplementary Fig. S1A and S1B, available at Rheumatology Online).

Discussion

In the current study we demonstrated a potential role for IL-35 as a negative regulator of peripheral immune responses in patients with RA. First, we assessed the levels of IL-35 in the serum of patients with RA and SLE and found that IL-35 production was significantly reduced in RA compared with healthy controls and patients with SLE. Moreover, there was preferential expression of EBI3 in natural Treg isolated from the peripheral blood of RA patients. We constructed a novel expression vector encoding FLAG-tagged EBI3 linked to IL-12/λ/p35 and identified the selective binding of human IL-35 to activated Treg (see supplementary Fig. S1A and S1B,
available at *Rheumatology* Online). IL-12α/p35 is constitutively expressed in most tissues, whereas EBI3 is expressed mainly in haematopoietic cells. Bardel et al. [18] used human lymphoid tissues to demonstrate that no EBI3 message was detected in FoxP3⁺ Treg isolated from lymph nodes, tonsils, the spleen or intestines. This suggests that Treg exert their regulatory function even in the absence of IL-35 and the 28-joint DAS based on CRP (DAS28-CRP) in humans. In the present study, Treg isolated from the PBMCs of patients with RA showed the selective expression of EBI3 and IL-12α/p35, as assessed by RT-PCR. Interestingly, previous studies revealed that contact-independent Treg-mediated suppression was IL-35 dependent and did not require either IL-10 or TGF-β [19, 20].

The current study also revealed that serum IL-35 levels were significantly lower in patients with active RA compared with RA where disease activity was low. Moreover, there was a correlation between the expression of serum IL-35 and DAS28-ESR in RA patients. This suggests that the numbers of Treg and levels of IL-35 may fluctuate depending on the disease status of RA and therefore might reflect RA disease activity. We found previously that IL-35 was expressed by naturally occurring CD4⁺CD25⁺ Treg isolated from human PBMCs, but not by CD4⁺CD25⁻ Tres [21]. Recent reports describing the treatment of RA patients with TNF inhibitors suggested that TNF down-regulates the function of human CD4⁺CD25⁺ Treg [22-24]. In addition, the number of Treg was reported to be significantly lower in patients with RA compared with healthy controls [25]. The results of the current study suggest that IL-35 is involved in the Treg-mediated suppression of autoimmunity in RA. However, the IL-35 receptors and downstream intracellular signalling pathways have not yet been identified. Therefore further studies are needed to elucidate the molecular mechanisms underlying the effects of IL-35 in humans.

We also revealed that recombinant human IL-35 enhanced natural Treg function in vitro and suppressed Tres proliferation and inflammatory cytokines such as IL-17 and IFN-γ in RA patients. Although IL-35 is known to suppress Tres responses, it remains unclear whether the suppressive function of Treg is derived from IL-35 or secondary responses such as the production of other cytokines by APCs. We demonstrated that IL-35 could itself enhance Treg function since the co-cultures used in the in vitro assay had no APCs. Consistent with a previous report, we used an assay to detect Treg-mediated suppression of Tres responses using CFSE-labelled Tres to determine when both cell types divide in response to a stimulus. If IL-35 leads to the expansion of Treg, a standard proliferation assay using [³H]thymidine incorporation will provide misleading data indicating that IL-35 inhibited Treg-mediated suppression of the Tres response. The activation of TCR signalling in Treg is required for affinity to IL-35. This suggests that IL-35 might participate in a positive feedback loop to promote the functional activity of Treg by up-regulating the IL-35 receptor, reducing the threshold for muting antigen-specific Tres responses. However, the addition of IL-35 to the Tres suppression assay did not completely inhibit the proliferation of Tres, suggesting that there may be a lag in response until the IL-35 receptor is up-regulated. As such, further analysis assessing the contribution of IL-35 to Treg function will require the development of neutralizing antibodies and the characterization of its receptor.

Our CFSE-based Treg suppression assay revealed that the regulatory function of Treg was significantly enhanced by high doses of IL-35. This suggests that naturally occurring Treg in peripheral blood are not fully functional and that high local IL-35 concentrations that increase cell–cell contact might be required for the suppressive effects of Treg. Furthermore, the binding of IL-35 to Treg required activation, suggesting that an unknown IL-35 receptor was needed by FoxP3 to sustain the suppressive effects. Collison et al. [8] demonstrated that Treg produce IL-35 and that FACS-purified Treg from EBI3-deficient mice are functionally defective. Consistent with this, we demonstrated that endogenous IL-35 in Treg is crucial for their suppressive functions. Therefore it is possible that IL-35 production in Treg during RA is crucial for the suppression of T cell responses.

Our study also suggests that the antigens that activate Treg via TCR and CD28 signalling are highly sensitive to IL-35 and enhance Treg-mediated suppression of activated helper T cells in an autocrine manner. Consistent with this, IL-35-deficient Treg failed to control the homeostatic expansion of Tres and were less able to exert effects in established colitis compared with wild-type Treg [26]. Interestingly, IL-35-deficient Treg isolated from either Ebi3⁻/⁻ or Il12a⁻/⁻ mice failed to enhance suppression [27]. Under conditions such as chronic infection or inflammation, we suggest that IL-35 enhances Treg function as part of the peripheral immune response to prevent the onset of excessive autoimmune responses in Treg.

Although many previous studies have implicated Treg in the pathogenesis of human autoimmune diseases, none has examined the role of IL-35 specifically. This is the first study to suggest the possible involvement of IL-35 in the pathophysiology of RA (see supplementary Fig. S2, available at *Rheumatology* Online). Although current treatment strategies for RA, including anti-TNF therapy, aim mainly to control inflammation, our data suggest that IL-35 and Treg might have multiple therapeutic targets.

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