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Longitudinal interferon-β effects in multiple sclerosis: Differential regulation of IL-10 and IL-17A, while no sustained effects on IFN-γ, IL-4 or IL-13

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ABSTRACT

Background: Recent studies in experimental models and in vitro indicate lowering of IL-17/Th17 as an important mechanism of interferon-beta (IFN-β) treatment in multiple sclerosis (MS).
Material and methods: In this longitudinal study of MS patients (n = 25), spontaneous and myelin antigen-induced secretion of IL-4, IFN-γ and IL-10 (ELISPOT), mitogen stimulated secretion of IL-13 and IL-17A (ELISA) and circulating cytokine levels (Luminex) were recorded at inclusion and after 1.5, 3, 6 and 12 months of IFN-β treatment.
Results: Early changes were noted for IL-4, while after one year of treatment the only recorded significant effects were a decrease in secreted IL-17A levels and an increase in IL-10 secreting cells. While IL-17A levels tended to be higher in non-responders (n = 8), the decrease in IL-17A levels seemed to be more pronounced in responders (n = 17) showing significantly lower IL-17A levels after one year as compared with non-responders.
Conclusion: IFN-β treatment seems to mainly affect IL-17/IL-10-associated pathways rather than the IFN-γ/IL-4 axis.

1. Introduction

T helper (Th) cells and cytokines play major roles in the development and maintenance of the inflammatory process in multiple sclerosis (MS). Th cells are divided into functional subsets defined according to their cytokine profile and pathogenic relevance. Originally, MS was regarded as a Th1-mediated disease, underscored by the observation that treatment with interferon-γ, the Th1 signature cytokine, in fact worsened the clinical course of MS [1]. In contrast, Th2 immunity, including IL-4 and IL-13, was ascribed a protective role in MS by counteracting Th1 immunity, although this paradigm has been somewhat questioned [2]. More recently, Th17 immunity, named after the signature cytokine IL-17, has been linked to MS pathogenesis [3] while regulatory T (Treg) cells were considered protective [4]. Treg cells dampen inflammation and counteract autoimmunity in several ways, one being through secretion of IL-10, a cytokine in general showing anti-inflammatory actions [5]. A dysfunction of Treg cells and imbalance of suppressive Th subsets have been suggested in MS [6,7].

Despite the launching of new drugs, interferon-beta (IFN-β) and glatiramer acetate are still used as first line treatments in many patients with relapsing remitting (RR) MS. IFN-β is usually well tolerated, reduces the relapse frequency by approximately 30% and has long-term beneficial effects on disability [8]. However it is well known that side-effects are sometimes prominent and that a substantial portion of patients (10–50%) do not respond to treatment, which is not only due to the induction of antibodies against the drug [9]. Thus, a possibility to predict the treatment response is therefore much wanted and it would therefore be important to better understand the mechanisms of action for IFN-β in relation to disease development. Although several mechanisms of IFN-β were indicated, including anti-inflammatory effects, diminished trafficking of T cells into the central nervous system (CNS) and effects on antigen-presenting cells, the precise effects are not fully understood [8].

Interestingly, the involvement of Th17 mediated immunity has come in focus not only for MS development but also because it's potential role in IFN-β therapy. Thus, IFN-β was shown to reduce IL-17 production directly or indirectly by reduction of osteopontin and induction of IL-27 [10]. Consequently, studies have demonstrated that IFN-β may exert its effect in MS by reduction of IL-17-associated immunity [11–14]. In contrast, it has been proposed that IFN-β may worsen disease outcome in a subgroup of MS patients [15], based on findings that IFN-β worsened Th17-induced EAE, while it was beneficial in Th1-induced EAE. These data were in accordance with higher serum levels of IL-17F in a group of non-responder MS patients [16]. It has also been shown that IL-17A levels in blood were numerically higher at baseline and throughout the course of IFN-β treatment in a

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subset of RRMS patients with relapses compared to those without relapses [17]. A recent study, however, showed that IL-17F does not predict poor response to IFN-β in RRMS [18]. Although consistent data indicate that IFN-β acts by reduction of Th17-mediated immunity, divergent results concerning IL-17 as a predictor of response to IFN-β thus exist. In this prospective study we report longitudinal changes in Th1, Th2 and Th17 associated cytokines during the first year of IFN-β therapy. We also evaluated if pre- or post-treatment IL-17A levels were associated with response to treatment.

2. Materials and methods

2.1. Patients

Twenty-five consecutive patients with MS, diagnosed according to the McDonald criteria [19] were included (Table 1). The inclusion criteria were initiation of IFN-β therapy and no other present immunomodulatory drug treatment. Thirteen patients had RR MS and 12 patients had a secondary progressive (SP) MS with relapses or contrast enhancing lesions on magnetic resonance imaging (MRI) indicating an inflammatory component. The patients were admitted to the Department of Neurology, University Hospital of Linköping, Sweden. None of the patients had any relapse within the last month prior to inclusion, and none received immunomodulating therapy within 6 months before onset. The patients received treatment with either IFN-β 1a (n = 12) (Avonex, BiogenIdec; Rebif, Merck Serono) or IFN-β 1b (n = 13) (Betaferon, Schering). Patients without clinical relapse during one year of treatment were judged to be responders (n = 17), whereas patients with one or more relapses (n = 2), or increased disability according to Kurtzke Expanded Disability Status Scale (EDSS) of at least 0.5 step (n = 6), were judged as non-responders. Three patients interrupted the treatment during the study period because of adverse effects. Peripheral blood was collected before onset of treatment and 6 weeks, 3 months, 6 months, and 12 months after. Some patients were lost to follow-up because they had moved from the catchment area or denied further extra blood sampling. Also some blood samples were lost due to technical laboratory difficulties or shortage of sample volume or cell numbers. Therefore the number of observations ranged between 14 and 24 pre-treatment and between 9 and 17 after one year. The samples were drawn at least 9 h after the last injection. The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping.

2.2. Antigens and mitogens

The myelin antigens used were two peptides from myelin oligodendrocyte glycoprotein (MOG) (synthesized by Dr Åke Engström, Department of Medical and Physiological Chemistry, Uppsala, Sweden) amino acid sequence 14–39 and 63–87, respectively, and bovine myelin basic protein (MBP) (Sigma-Aldrich, St. Louis, MO, USA). It has previously been shown that peripheral blood mononuclear cells (PBMC) from MS patients secrete cytokines in response to both MBP [20], MOG [21], as well as the above MOG peptides [22]. The mitogen phytohemagglutinin (PHA) (Sigma-Aldrich) was used as a positive control in the enzyme-linked immunospot (ELISPOT) assay, always eliciting strong responses of several hundreds of spots for all the cytokines analysed. PHA was also used to induce secretion of IL-10 and IL-17A to be quantified by ELISA in cell culture supernatants. The MOG peptides, MBP and PHA were all used in the final concentration of 10 μg/mL. Keyhole limpet hemocyanin (KLH) (Calbiochem, Lab Kemi, Stockholm, Sweden) was used as a negative control (irrelevant antigen) in the ELISPOT assay at a final concentration of 100 pg/mL. No increases in the number of spots in the KLH-stimulated wells were seen for any of the cytokines compared with the non-stimulated wells. As an additional negative control, some wells on each plate were incubated with culture medium without cells, otherwise treated as the other wells.

Table 1
Demographic and clinical data for patients with multiple sclerosis; the whole group and stratified according to clinical response.

	All	Responder	Non-responder[a]
Female/male (n)	18/7	13/4	5/3
Age [mean (range); y]	46 (30–66)	44 (30–66)	48 (33–58)
MS duration [mean (range); y]	13 (1–38)	12 (1–38)	16 (1–27)
MS phase (RR/SP; n)	13/12	11/6	2/6
EDSS at start [median (range)]	2 (0–6.5)	2 (0–6)	3 (1–6.5)
EDSS at 12 months [median (range)]	2.5 (0–7.5)	1.5 (0–6)	4 (1–7.5)

MS = multiple sclerosis; EDSS = Expanded Disability Status Scale; RR = Relapsing-Remitting; SP = Secondary Progressive; y = years.
[a] Non-responders were defined as at least one relapse or an increase of at least 0.5 step in EDSS during one year of treatment.

2.3. Cell preparation

PBMCs were separated from heparinised venous blood by gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway), according to Bøyum [23]. The PBMCs assayed by ELISPOT were diluted in tissue culture medium (TCM) consisting of Iscove's modification of Dulbecco's medium (IMDM) (Gibco BRL, Paisley, Scotland) in accordance with previous description [24]. The cells were counted under a phase contrast microscopy. Since mainly lymphokines were studied, the cell density was adjusted to a fixed number of lymphocytes (1×10^6/mL), implying that a number of monocytes were present in addition to the lymphocytes.

2.4. ELISPOT

ELISPOT for detection of IFN-γ, IL-4- and IL-10-secreting cells was performed as previously described [25]. All antibodies were purchased from Mabtech (Stockholm, Sweden). PBMCs were incubated with or without antigens for 48 h. Mean values of triplicates were calculated for the spontaneous secretion and for antigen-induced secretion. Spots were counted under a dissection microscope. To obtain the number of antigen-specific cells, the values of the non-stimulated cells were subtracted from the values of the stimulated cells. Some wells on each plate were incubated with TCM only (without cells) but otherwise treated likewise. No or occasionally a few spots were seen in these wells.

2.5. ELISA

PBMCs were incubated with or without antigens for 48 h before supernatants were collected and stored at −70 °C until analysis. The ELISA was performed as described previously [26]. Microtitre plates (Costar, Cambridge, MA, U.S.) were coated (50 μL/well) with 4 μg/mL of anti-human IL-13 or IL-17A antibody, respectively (R&D Systems, Abingdon, UK) in carbonate buffer (pH 9.6), over night in room temperature. The plates were washed 4 times with PBS-Tween, followed by incubation on a plate shaker for 1 h at room temperature with 100 μL/well of 2% low-fat milk in PBS, to block non-specific protein binding sites. After additional washings, samples and standard curves were plated (50 μL/well in duplicate) and incubated for 1 h at room temperature. Different dilutions in TCM of recombinant human IL-13 (R&D Systems; range 62.5–1500 pg/mL), or IL-17A (R&D Systems; range 7.8–500 pg/mL) provided standard curves. After repeated washings, biotinylated antibodies diluted in High Performance ELISA (HPE) buffer (CLB, Amsterdam, Netherlands) were added (50 μL/well) at the following concentrations: 500 ng/mL of anti-IL-13, or 75 ng/mL of anti-IL-17A (R&D Systems), followed by incubation on a plate shaker for 1 h at room temperature. The plates were again washed 4 times

with PBS-Tween and then Streptavidin-HRP (CLB), diluted 1:10,000 in HPE buffer, was added (50 μL/well) and incubated for 30 min on a plate shaker at room temperature. Subsequent washing 4 times with PBS-T, was followed by adding 50 μL/well of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) and incubation on a plate shaker for 30 min in dark at room temperature. Stop solution (1.8 M H_2SO_4) was added (50 μL/well) and the optical densities at 450 nm were read within 30 min.

2.6. Luminex

Serum samples were stored in −70 °C until use. After thawing, the sera were analysed with Human Cytokine LINCO*plex* Kit, for IL-17A, and with High Sensitivity Human Cytokine LINCO*plex* Kit, for IFN-γ, IL-10 and IL-13 (LINCO Research, St. Charles, MO, USA). The two kits were assayed separately, according to the manufacturer's instructions. The data were analysed with StarStation software.

2.7. Statistics

Non-parametric tests were chosen because of the non-normally distributed data. The Mann–Whitney *U*-test was used to compare data from each follow-up time point with pre-treatment values (comparisons across follow-up time points were not considered relevant) as well as for comparing responders to non-responders. Chi-2 test was used to compare the distribution of demographic and clinical data between responders and non-responders. The statistical analyses were performed with GraphPad Prism 5. A two-tailed p-value<0.05 was considered significant.

3. Results

3.1. ELISPOT detection of IFN-γ, IL-4 and IL-10 secreting cells

ELISPOT was used to enumerate numbers of cytokine secreting cells in patients treated with IFN-β during one year (Fig. 1). The number of cells spontaneously secreting IL-10 increased after 12 months compared with pre-treatment levels ($p=0.04$). The number of myelin antigen-induced IL-4 secreting cells was reduced after 3 months (MOG 14-39-stimulated cells, $p=0.01$, and MOG 63-87-stimulated cells, $p=0.04$). The reduction did not persist after 6 months and had returned to pre-treatment values after one year. No significant changes were seen in the secretion of IFN-γ during the treatment period. We did not find any differences in IFN-γ, IL-4 and IL-10 secreting cells between responders versus non-responders to treatment.

3.2. Serum levels of IFN-γ, IL-13, IL-10 and IL-17A

Multiple bead technology was used to measure serum levels of cytokines (Fig. 2). There were no significant changes in the serum concentrations of any of the cytokines assayed during the treatment period, compared with pre-treatment values.

3.3. ELISA detection of ex vivo IL-13 and IL-17A secretion

PHA-stimulated cytokine secretion was measured in cell culture supernatants. No changes were seen in the secretion of IL-13 (data not shown). A reduction in the secretion of IL-17A was seen after 12 months compared with pre-treatment values (median 186 pg/mL [pre-treatment] compared with 113 pg/mL [12 months], $p=0.04$, Fig. 3A). In considering the secretion of IL-17A in relation to clinical response, non-responders (one or more relapses or an increase of at least 0.5 step in EDSS during one year of treatment) tended to have higher values pre-treatment ($p=0.05$) compared to responders (no relapse during one year of treatment or stable EDSS). As shown in Fig. 3B, a significant decrease was seen in the responder group after 12 months compared to pre-treatment values (median 126 pg/mL [pre-treatment] compared to 80 pg/mL [12 months], $p=0.03$). A reduction was also seen in the non-responder group, although not statistically significant ($p=0.09$). At the end of the treatment period, significantly lower values of IL-17A were detected in clinical responders compared to non-responders (median at 12 months 80 pg/mL [responders] compared to 163 pg/mL [non-responders], $p<0.002$, Fig. 3B). In serum, a similar pattern in IL-17A concentrations was suggested, both considering the whole group (Fig. 2D) and according to clinical response (Fig. 3C), although no statistically significant changes were found. For cytokines other than IL-17A, there were no differences when comparing responders and non-responders at baseline or follow-up.

One potential confounder of IL-17A responses related to clinical response would be if responders and non-responders differed in any respect. RR MS seemed to be more common in responders (Table 1), although the frequency did not statistically differ from that in non-responders ($p=0.10$). Even more important, IL-17A levels were similar in responders versus non-responders (no significant difference was found, $p=0.3$–0.6 at different time points). All other parameters (Table 1) were similar across the groups. No significant differences in IL-17A secretion occurred between IFN-β 1a and IFN-β 1b treated patients.

4. Discussion

The main aim of the present study was to prospectively assess longitudinal changes in Th1, Th2 and Th17-associated cytokine secretion in patients treated with IFN-β. In general, few changes occurred during the course. After one year of treatment, the only significant changes were a reduction in IL-17A secretion and an increase in the number of IL-10 secreting cells. Interestingly, these findings mirror changes as predicted by detailed in vitro mechanistic studies of IL-17 [9–12,14] and IL-10 [27]. Another main finding of our study was the potential difference between responders and non-responders in IL-17A levels before and after treatment.

Th17-mediated immunity has come into focus in MS because of its involvement in EAE as well as in MS pathology [16]. Interestingly, a reduction in IL-17 production was recently reported as an important mechanism of action for IFN-β [10]. Also, Th17 cells showed a higher expression of type-1-interferon receptors as compared with Th1 cells, suggesting Th17 cells to be selectively targeted by IFN-β [12]. The detailed mechanisms involved in IFN-β actions were recently reported including the ability of IFN-β to inhibit human Th17 cell differentiation [13], to inhibit IL-17- as well as osteopontin production in human CD4+ cells [11], and to inhibit IL-17 production by dendritic cells in MS patients, the latter mechanism probably mediated through IL-27 induction [14]. We here report the longitudinal effects on IL-17A during INF-β treatment. We show that ex vivo induced IL-17A secretion was decreased after one year of treatment. In addition to the effect on IL-17A, we also noted an increase in the number of IL-10 secreting cells, which is in line with previous reports [28–31]. IL-10 is an immunomodulatory cytokine with predominantly suppressive actions that is produced by many cell types including regulatory T cells [5] as well as regulatory B cells [32]. Interestingly, mechanistic studies recently showed that IFN-β induced IL-10 in CD4 cells [13,16]. Collectively, data indicate that reduction of IL-17 and induction of IL-10 seem to be important effects induced by IFN-β.

Regarding Th1 and Th2 associated cytokines we did not see any differences after 12 months compared with pre-treatment levels. During the course, myelin antigen-induced IL-4 secretion was reduced after three months, which corroborates some previous findings of decreased IL-4 during IFN-β treatment [33,34], while other studies showed no difference [25,35], or increased IL-4 [28,36]. The inconsistencies in previous data surely depend on differences in methodology as well as in time points of sampling. For the Th1-associated cytokine IFN-γ we did not find any changes during the course of treatment,

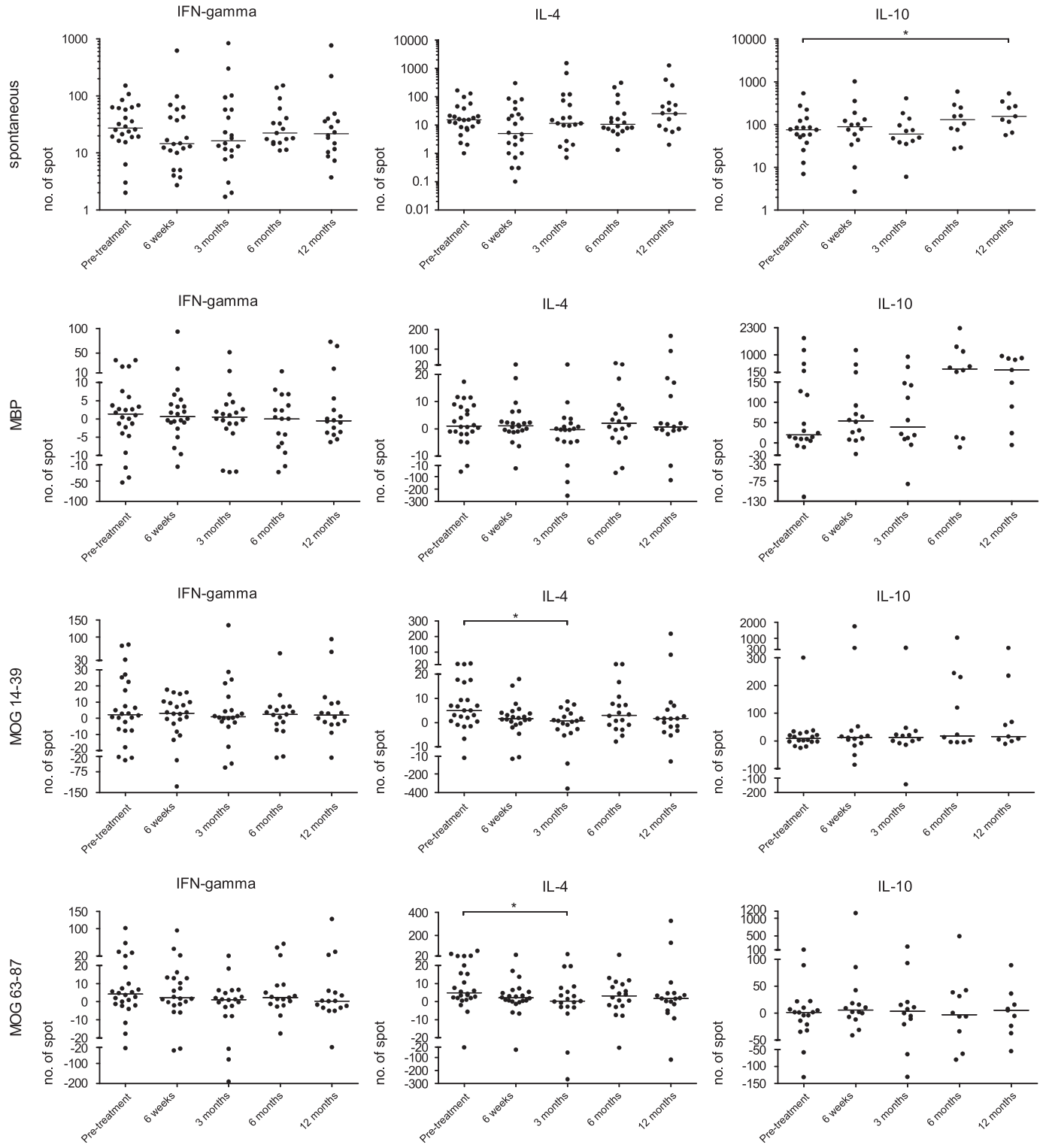

Fig. 1. The number of cells secreting IFN-γ, IL-4 and IL-10, respectively, assayed with ELISPOT. The values for spontaneous (non-stimulated) and for myelin-stimulated secretion are shown. Note the different scales. The values for myelin-stimulated cells are shown as net-secretion, i.e. after subtraction of the spontaneous number of cytokine secreting cells. The Mann–Whitney *U*-test was used to compare each follow-up time with pre-treatment values. The medians are indicated. * = $p<0.05$.

which corroborates some previous studies [35,37], while others showed a decrease [25,28,33,36] or an increase [38]. Thus, previous data are inconsistent also regarding Th1 cytokines. Taken together, there is no consensus regarding IFN-β effects on Th1/Th2 associated cytokines. In that sense, our findings of no sustained changes in Th1/Th2 cytokines after one year of treatment would be expected. We therefore propose that IFN-β may preferentially induce long-term changes in the IL-17/IL-10 axis rather than in the IFN-γ/IL-4/IL-13 axis.

Since there is a substantial portion of patients that do not respond to IFN-β treatment (in addition to those caused by antibodies against IFN-β), a possibility to predict treatment outcome would be of great

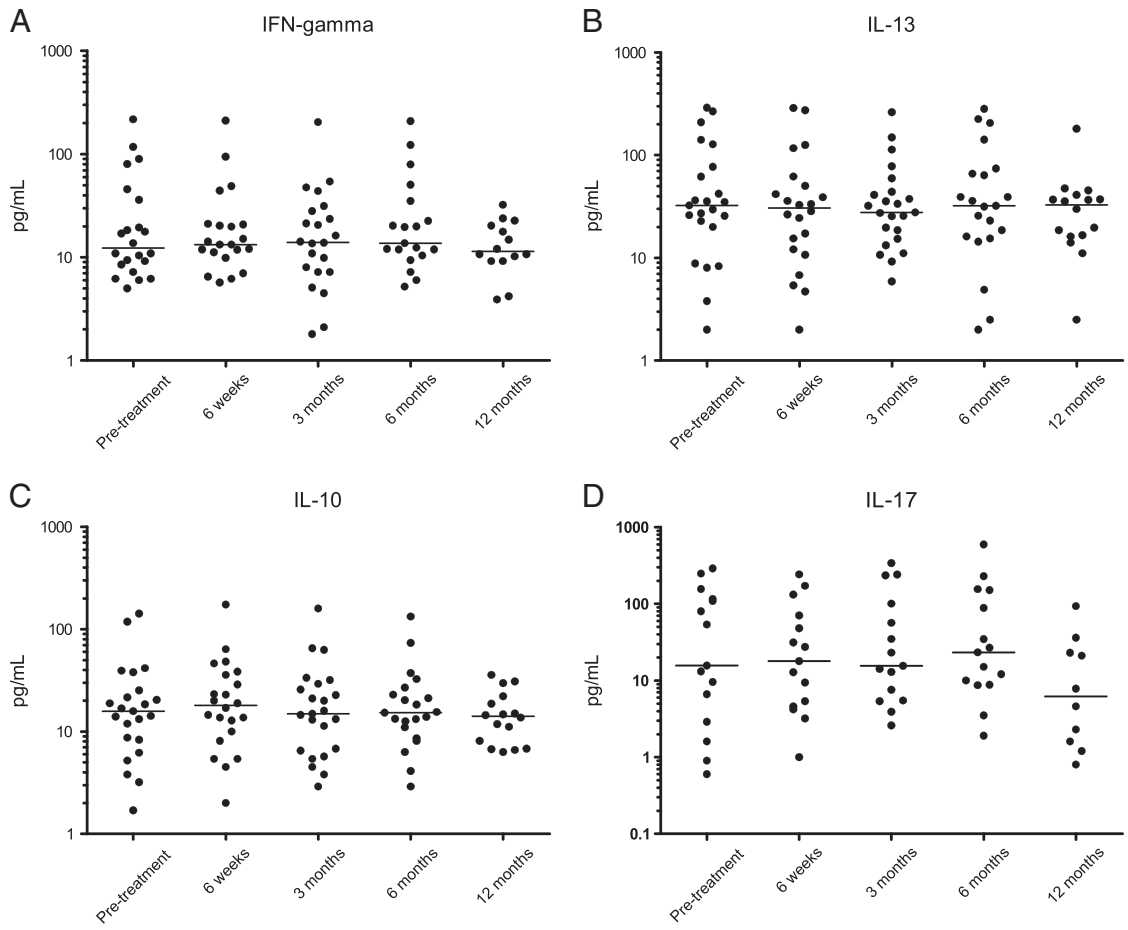

Fig. 2. The concentrations of IFN-γ, IL-13, IL-10 and IL-17A in serum, detected with Luminex. Note the logarithmic scale. The Mann–Whitney *U*-test was used to compare each follow-up time with pre-treatment values. The medians are indicated. No significant changes could be seen during the treatment period.

value. It was recently proposed that IFN-β therapy was beneficial in EAE associated with Th1 pathology, while in Th17 pathology IFN-β treatment could even worsen disease outcome [16]. Consequently, it was noted that higher pre-treatment IL-17F levels were found in a subgroup of non-responder MS patients, which is in line with a lack of effect in individuals with Th17 associated pathology. In addition, IL-17A levels were reported to be numerically higher in relapsing versus non-relapsing RRMS patients at base-line and during one year follow-up of IFN-β treatment, reaching statistical significance at 6 months [17]. However, in another and larger cohort of RRMS patients, IL-17F levels at baseline did not differ between good- and poor responders [18]. Regarding IL-17A levels before treatment, we found that IL-17A levels tended to be higher in our cohort of non-responders compared with responders. We also measured IL-17A levels after one year of treatment and found that the responders showed a significant reduction after one year, while non-responders only tended to have a reduction, leading to significantly lower IL-17A levels in responders versus non-responders after one year. Thus, a clear reduction in IL-17A levels in responders would rather indicate that patients with Th17 mediated pathology are those that respond better to treatment. The somewhat different results obtained regarding IL-17 levels and response to IFN-β treatment in MS patients may be explained by differences in cohorts and by methodological factors including the measuring of IL-17A (17 and present study) or IL-17F [16,18] and measuring induced secretion (17 and present study) or circulating levels in serum [16,18]. In line with our findings, it was reported that IFN-β treatment in MS patients induced higher PBMC production of IL-27 in responders as compared with non-responders [14]. Since IL-27 may act by inhibiting IL-17, these data corroborate our findings of lowered IL-17A levels in particular in responders. Clearly more studies are needed to prove whether IL-17-family molecules can serve as biomarkers for prediction of treatment response.

A limitation of is the size of the study, in particular during follow-up. However, the missing values occurred at random, thus not skewing the results. Furthermore, the significant changes that occurred were reasonable in considering reported mechanisms of IFN-β. The study included patients with both RR and SP MS with possible different disease mechanisms. The decision to include both RR and SP MS patients was made upon the assumption that SP patients with relapses or MRI activity were still in the inflammatory phase of the disease. Responders were judged from clinical criteria (the absence of relapses or a stable EDSS), possibly missing occasional patients with MRI activity. MRI, however, was not used to follow-up the treatment routinely. When subgrouping the results from patients with either IFN-β 1a or IFN-β 1b treatment the only significant difference was found for spontaneous IFN-γ secretion which was lower in patients treated with IFN-β 1b after one year of treatment. The groups are small, however, and the difference between the treatment groups should be interpreted carefully. The strength of the study was the prospective design as well as the broad covering of cytokines associated with Th1, Th2, and Th17 and immunosuppressive IL-10.

In conclusion, we show that IL-17A secretion of blood cells is decreased after one year of IFN-β treatment, which reflects recent in vitro studies of IFN-β effector mechanisms. Further, the decrease in IL-17A levels seemed to occur in both responders and non-responders, although the decrease was more pronounced in the responder group,

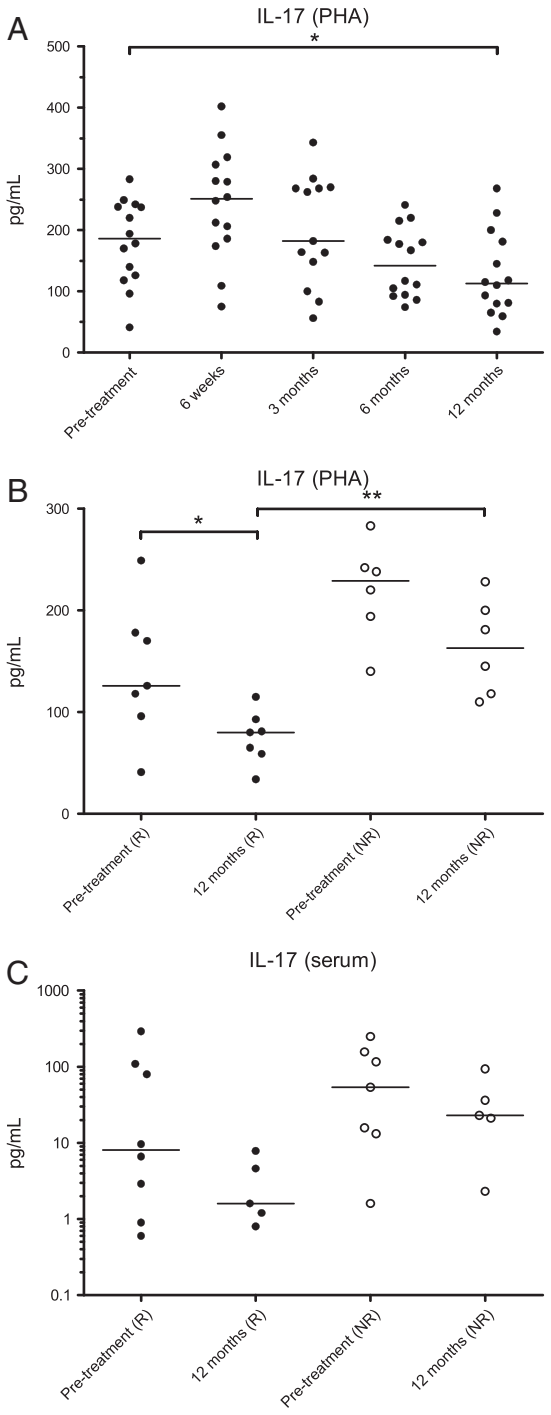

Fig. 3. PHA-stimulated secretion of IL-17A as measured by ELISA in cell culture supernatants, for all patients (A) and separated according to clinical response (B). Serum concentrations of IL-17A as measured by Luminex are displayed in (C). Pre-treatment values and values after 12 months of treatment are given for responders (R, filled dots) and non-responders (NR, open dots). The medians are indicated. * = $p < 0.05$. ** = $p < 0.005$.

suggesting that also individuals with Th17-mediated disease can respond to this treatment. The number of cells secreting the immunosuppressive IL-10 cytokine was increased after one year of treatment. In contrast, changes in Th1/Th2-associated cytokines were recorded early, but no sustained effects were observed after one year. Thus, the effects of IFN-β treatment seem to be more related to the IL-17/IL-10 axis than the IL-4/IFN-γ axis.

Conflict of interest

MV has received speaker honoraries from Biogen Idec and Merck Serono and an unrestricted research grant from Biogen Idec. The other authors declare that they have no competing interests.

Authors' contributions

MK and JY performed the immunological analyses. MK prepared the initial manuscript and JY added further data and prepared the figures. CE, MV and JE planned the study, oversaw the experiments, and edited the manuscript. All authors read and approved the final manuscript.

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