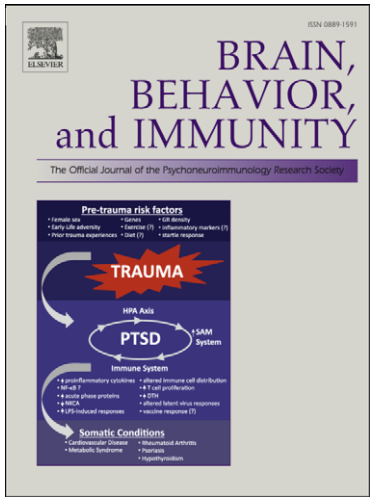

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IL-27 mediates the response to IFN-β therapy in multiple sclerosis patients by inhibiting Th17 cells

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Abstract

Interferon (IFN)-β is a commonly used therapy for relapsing remitting multiple sclerosis (RRMS). However its protective mechanism is still unclear and the failure of many patients to respond has not been explained. We have found that IFN-β suppressed IL-23 and IL-1β production and increased IL-10 production by human dendritic cells (DC) activated with the TLR2 and dectin-1 agonist zymosan. Furthermore, IFN-β impaired the ability of DC to promote IL-17 production by $CD4^+$ T cells, but did not affect IFN-γ production. IFN-β induced IL-27 expression by DC, and neutralisation of IL-27 abrogated the suppressive effects of IFN-β on zymosan-induced IL-1 and IL-23 production and the generation of Th17 cells in vitro. Complementary in vivo studies in a mouse model showed that treatment with IFN-β enhanced expression of IL-27, and reduced IL-17 in the CNS and periphery and attenuated the clinical signs of experimental autoimmune encephalomyelitis (EAE). In addition, the significant suppressive effect of IFN-β on the ability of DC to promote Th17 cells was lost in cells from IL-27 receptor deficient mice. Finally, we showed that PBMC from non-responder RRMS patients produced significantly less IL-27 in response to IFN-β than patients who responded to IFN-β therapy. Our findings suggest that IFN-β mediates its therapeutic effects in MS at least in part via the induction of IL-27, and that IL-27 may represent an alternative therapy for MS patients that do not respond to IFN-β.

Keywords: Multiple sclerosis, Th17, IL-27, IFN-β, experimental autoimmune encephalomyelitis, dendritic cell, $CD4^+$ T cell.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the CNS that results in axonal loss and cumulative disability. Interferon (IFN)-β is widely used to treat relapsing remitting MS (RRMS) and reduces the rate of relapse by approximately 30% (Panitch and Bever 1993). Although IFN-β has been shown to exert various immunomodulatory effects, the therapeutic mechanisms remain poorly understood and a high percentage of patients fail to respond to therapy. In some cases treatment failure can be attributed to the development of neutralizing antibodies (Nabs) to IFN-β (Sorensen et al. 2003). However, a proportion of patients that fail to respond to IFN-β therapy do not develop Nabs (van der Voort et al. 2009), and it is unclear why these patients are unresponsive.

Although the exact aetiology of MS is unknown, autoreactive T cells directed against myelin antigens are thought to contribute to disease pathogenesis. It was initially suggested that Th1 cells were responsible for the pathogenesis of MS. In recent years however, a new subset of pathogenic effector T cells that secrete IL-17, termed Th17 cells (Park et al. 2005), have been shown to be associated with the pathogenesis of several inflammatory diseases, including MS, experimental autoimmune encephalomyelitis (EAE), psoriasis and rheumatoid arthritis (Mills 2008). Studies in mouse models have shown that IL-17 production by T cells plays a critical pathogenic role in T cell mediated autoimmune diseases (Mills 2008; Sutton et al. 2009). In EAE, both Th1 and Th17 cells have now been shown to contribute to disease pathogenesis, with both cell types capable of inducing disease but no absolute requirement for either IL-17 or IFN-γ alone. Furthermore, Th17 cells exhibit plasticity and can convert to IL-17^{+}IFN-γ^{+} cells or cells indistinguishable from Th1 cells, which may be more encephalogenic than either cell type alone (Domingues et al.; Kroenke and Segal). Both cell types enter the CNS, expand and and release

inflammatory mediators, which help recruit other immune cells to the site of inflammation. Th1 driven disease is characterized by macrophage-rich infiltrates, prominent NOS2 up-regulation, and expression of monocyte-attracting chemokines CXCL9, 10, and 11, whereas Th17-mediated disease is associated with infiltrating neutrophils and the neutrophil-attracting chemokines CXCL1 and (Kroenke et al. 2008). Ultimately however, whether triggered by Th1 or Th17 cells, the inflammatory cascade results in myelin breakdown promoted by proteases, glutamate, reactive oxygen species and other cytotoxic agents, followed by axonal damage and neurological impairment. In some studies Th17 cells promoted atypical ataxic EAE (Domingues et al.), whereas in other cases the clinical outcome was indistinguishable from that induced by Th1 cells (Kroenke et al. 2008).

The differentiation and expansion of Th17 cells in humans and mice is mediated by pro-inflammatory cytokines, including IL-1β, IL-23, IL-21 and IL-6, secreted by innate immune cells (Mangan et al. 2006; Sutton et al. 2006; Acosta-Rodriguez et al. 2007; Wilson et al. 2007). IL-1RI deficient mice are resistant to actively induced EAE (Sutton et al. 2006; Chung et al. 2009), as are IL-23 deficient mice, and IL-1 can synergise with IL-23 to induce the production of IL-17 by $CD4^+$ T cells and γδ T cells (Sutton et al. 2006; van Beelen et al. 2007; Sutton et al. 2009). Thus IL-1 and IL-23 are important innate cytokines required for the development of pathogenic Th17 cells and autoimmunity.

IL-27, an IL-12 family member, is a heterodimeric cytokine composed of two subunits, p28, an IL-12p35 homologue and Epstein-Barr virus-induced gene 3 (EBI3), an IL-12p40 related protein (Pflanz et al. 2002). IL-27 plays a role in early Th1 cell differentiation (Takeda et al. 2003), but can also suppress Th1, Th2 and Th17 cell responses (Artis et al. 2004; Stumhofer et al. 2006; Fitzgerald et al. 2007a). IL-27 plays a protective role in EAE (Batten et al. 2006), where it

antagonises the development of Th17 cells (Fitzgerald et al. 2007a). A number of reports have suggested that IL-27 plays a role downstream of IFN-β. The IL-27p28 promoter contains two interferon-sensitive response element sites (Molle et al. 2007) and IFN-β induces the expression of IL-27p28 in human DC (Remoli et al. 2007). Furthermore, type I IFN receptor deficient mice developed more severe EAE compared with WT mice, which was reversed upon administration of recombinant IL-27 (Guo et al. 2008).

Collectively, these studies suggest that IFN-β therapy may mediate its protective effects in MS patients via the induction of IL-27, although this has not yet been established. The aim of this study was to determine the effect of IFN-β on the production of cytokines by human DC, and the subsequent ability of the DC to promote T helper cell responses. We also investigated the potential role of IL-27 in mediating the effects of IFN-β in vitro and in vivo in an animal model of CNS autoimmunity. We show that IFN-β suppresses Th17 cells either directly or through the inhibition of DC cytokines that promote the development of Th17 cells. This suppression was mediated at least in part by IL-27. Finally, using PBMC from RRMS patients, we show that the induction of IL-27 in response to *in vitro* stimulation with IFN-β is associated with response to IFN-β treatment.

2. Materials and methods

2.1 Blood samples

MS patients attending out-patient clinics at St. Vincent's University Hospital, Dublin were recruited for this study. Written informed consent was obtained from each patient and the study received ethical approval from St. Vincent's Ethics and Medical Research Committee and Trinity College Faculty of Health Sciences Research Ethics Committee. Clinical details of the MS patients are shown in Table I. Leukocyte-enriched buffy coats from anonymous healthy donors were obtained with permission from the Irish Blood Transfusion Board, St James's Hospital, Dublin and ethical approval was granted by Trinity College Faculty of Health Sciences Research Ethics Committee. PBMC were isolated by density gradient centrifugation.

2.2 Human monocyte-derived dendritic cells (DC)

$CD14^+$ monocytes were positively selected from PBMC by using anti-CD14-microbeads (Miltenyi Biotech) on an autoMACS cell sorting instrument. Monocytes were cultured at $1x10^6$ cells/ml with IL-4 (40 ng/ml; Immunotools) and GM-CSF (50 ng/ml; Immunotools) for 7 days. Every alternate day half of the medium was replaced with fresh medium supplemented with cytokines. In some experiments IFN-β (50 U/ml; PBL Interferon Source) or IL-10 (5 ng/ml; BD Pharmingen) was added at the initiation of culture. Non-adherent DC were harvested by centrifugation and cultured at $1x10^6$ cells/ml. The purity of $CD14^{lo}CD11c^+$DC-$SIGN^+$ DC was assessed by flow cytometry, and was routinely >98%. DC were cultured at $1x10^6$/ml and left unstimulated or stimulated with zymosan (Invivogen), ultrapure LPS (Invivogen) or heat killed *Mycobacterium tuberculosis* Mtb (50 μg/ml; Chondrex) in the presence or absence if IFN-β (50, 200 or 1000 U/ml) or IL-27 (25, 50 or 100 ng/ml). After 24 h supernatants were analysed by ELISA for the concentrations of IL-1, IL-12p40, IL-10 (all BD Pharmingen) IL-23 (eBioscience)

or IL-27 (R&D Systems). In some experiments, neutralising antibody to IL-27 (10 μg/ml; R&D Systems), or control antibody (10 μg/ml; R&D Systems) was added to DC 30 min prior to stimulation.

2.3 T cell assays

Total $CD4^+$ T cells were positively selected using anti-CD4-microbeads (Miltenyi Biotech) on an autoMACS cell sorting instrument, purity was assessed by flow cytometry and was routinely greater than 95%. T cells were co-cultured with allogeneic DC that had been activated with LPS (10 ng/ml; Invivogen) or zymosan (10 μg/ml; Invivogen) in the presence or absence of IFN-β (50-1000 U/ml) or IL-27 (25-100 ng/ml) for 24 hours. DC were thoroughly washed prior to addition of T cells and co-cultures were performed at a T cell:DC ratio of 10:1. In some experiments, allogeneic T cells were cultured with irradiated PBMC as APCs at a T cell:PBMC ratio of 2:1. Cell free supernatants from zymosan-activated DC were added to the above cultures with neutralising antibody IL-27 (10 μg/ml; R&D Systems), or control antibody (10 μg/ml; R&D Systems) for 5 days. Alternatively, purified $CD4^+$ T cells were stimulated with plate bound anti-CD3 (0.5 μg/ml; eBioscience) and soluble anti-CD28 (1 μg/ml; eBioscience) with or without IFN-β (50-1000 U/ml) or IL-27 (25-100 ng/ml) for 3 days. The culture supernatants were assayed for concentrations of IL-17 (R&D Systems), IFN-γ and IL-10 by ELISA (both BD Pharmingen). Cell proliferation was then measured by thymidine incorporation 18 h after addition of 1 μCi/well of tritiated thymidine.

2.4 Mice

C57BL/6 mice were purchased from Harlan, U.K. Type I interferon defective ($IFNAR^{-/-}$) mice were obtained from Paul Hertzog (Monash Institute of Medical Research, Australia) and IL-27 receptor deficient ($WSX\text{-}1^{-/-}$) mice were a kind gift from Richard Grencis (University of

Manchester, UK). Mice were bred and maintained under specific pathogen free conditions and were 6-8 weeks at initiation of experiments. All mice were maintained according to European Union regulations, and experiments were performed under license from the Department of Health and Children and with approval from the Trinity College Dublin BioResources Ethics Committee.

2.5 Murine dendritic cells and T cell assays

Bone marrow-derived DC were prepared by culturing bone marrow cells from C57BL/6, IFNAR$^{-/-}$ and WSX-1$^{-/-}$ mice in RPMI medium containing 10% FCS supplemented with 20 ng/ml GM-CSF. On day 3, fresh medium containing 20 ng/ml GM-CSF was added. On day 6, contaminating cells were discarded and semi-adherent cells collected using 0.5 mM EDTA (Sigma-Aldrich). Cells were re-cultured in medium supplemented with 20 ng/ml GM-CSF. On day 8, fresh medium containing 20 ng/ml GM-CSF was added. On day 10, loosely adherent cells were collected, washed, and used for assays. DC were cultured with LPS (100 ng/ml; Alexis), CpG (5 μg/ml) or heat-killed Mtb (50 μg/ml; Chondrex), or medium only, with or without IFN-β (50-1000 U/ml). After 24 h Supernatants were recovered and IL-1β (R&D Systems), IL-23 (eBioscience) or IL-27 (R&D Systems) concentrations determined by ELISA. For DC:T cell co-cultures, DC (1 x 10^6/ml) were cultured with Mtb (50 μg/ml) in the presence or absence of IFN-β (50-1000 U/ml) for 24 h. Cells were washed and added to MACS-purified CD4$^+$ T cells at a ratio of 1:4 (0.25 x 10^5 DC : 1 x 10^5 T cells). Supernatants were recovered after 72 h, and IL-17 (R&D Systems) concentrations determined by ELISA.

2.6 Induction, assessment and treatment of EAE

C56BL/6 mice (Harlan UK) were immunized subcutaneously (s.c.) with 150 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide (GenScript) emulsified in CFA containing 4

mg/ml (400 μg/mouse) heat killed *Mycobacterium tuberculosis (Mtb)* (Chondrex). Mice were injected intraperitoneally (i.p.) with 500 ng pertussis toxin (PT, Kaketsuken) on day 0 and 2. Mice were treated with 10,000 U of IFN-β (Avonex) or with PBS everyday from day 0. EAE was induced in SJL/J mice (Harlan UK) by s.c. injection with 80 μg of proteolipid protein $(PLP)_{139-151}$ peptide (Genscript) emulsified in CFA containing 4 mg/ml (400 μg/mouse) heat killed heat killed *Mycobacterium tuberculosis.* Mice were injected intraperitoneally (i.p.) with 500 ng pertussis toxin (PT, Kaketsuken) on day 0 and 2. After entering remission mice were treated with 10,000 U/mouse of IFN-β (PBL InterferonSource) administered i.p. every 2 days from day 24-28 post induction of EAE. Mice with EAE were monitored daily for signs of disease, and disease severity was recorded as follows: grade 0, normal; grade 1; limp tail; grade 2; wobbly gait; grade 3, hind limb weakness; and grade 4, hind limb paralysis.

2.7 Restimulation of lymphoid and brain cells from mice with EAE

Lymph node cells cells ($2x10^6$ cells/ml) from SJL mice with EAE were stimulated for 72 h with PLP (1, 2 and 25 μg/ml). IL-17 (R&D Systems) and IFN-γ (BD Biosciences) concentrations in supernatants were determined by ELISA. Mononuclear cells were isolated by percoll density centrifugation from the brains of mice perfused with PBS and stimulated for 6 h with PMA (10 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (5 μg/ml). Cells were washed and blocked with Fcγ block (BD PharMingen; 1 μg/ml) before surface staining for CD4 and CD3. Cells were then fixed and permeabilised (Intrastain; Dako), stained intracellularly for IL-17 and IFN-γ and analysed by flow cytometry on a Cyan ADP Flow Cytometer (Dako Cytomation).

2.8 Real-time quantitative PCR

Total RNA was harvested from PBMC or DC that had been stimulated with IFN-β for 4-6 hours or from brain tissue from mice with EAE using the Trizol (Invitrogen) /chloroform method. This

process was followed by reverse transcription into cDNA with Applied Biosystems High Capacity cDNA Reverse Transcriptase kit. Real time PCR for the detection of human *Il27p28* (Hs00377366_m1), *ebi3* (Hs00194957_m1), *Il10* (Hs00174086_m1), *Il6* (Hs00985639_m1); and mouse *Il17a* (Mm00439619_m1), *Il27p28* (Mm00461164_m1), *Il23* (Mm00818884_m1) and *Il1* (Mm00434228_m1) mRNA was performed with predesigned Taqman gene expression assays (Applied Biosystems) on an Applied Biosystems 7500 Fast Real Time PCR machine. Expression of mRNA was normalized relative to the endogenous control, 18s ribosomal RNA (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method* was used to calculate relative changes in gene expression determined from RT-PCR experiments ($\Delta\Delta Ct$ = (C_t target gene, treated sample - C_t 18S, treated sample) - (C_t target gene, calibrator sample - C_t 18S, calibrator sample) where Ct is defined as the cycle threshold at which amplified product is first detected). Data is expressed as fold difference relative to untreated control samples.

2.9 Statistics

Statistical analysis was performed using Graphpad Prism software. The two-tailed t test was used for statistical comparisons between two datasets where the data was normally distributed; alternatively the Mann-Whitney U-test was used for non-parametric data. The one-way ANOVA was used for comparisons between 3 or more datasets, together with Dunnett's post test for individual comparisons relative to control data.

3. Results

3.1 IFN-β inhibits IL-1β and IL-23 production by DC

Regulatory cytokines produced by DC in response to activation with pathogen-associated molecular patterns (PAMPs) play a crucial role in directing T cell responses. We therefore examined the effect of IFN-β on the secretion of cytokines by PAMP-activated DC. Monocyte derived DC were stimulated with LPS or zymosan in the presence or absence of increasing concentrations of IFN-β. After 24 h the concentrations of IL-23, IL-1β, IL-12p40 and IL-10 in the supernatants were quantified by ELISA. Both LPS and zymosan induced significant IL-23, IL-12p40 and IL-10 production from DC (Fig. 1*A*). The production of IL-23 in response to zymosan was significantly reduced in the presence of increasing concentrations of IFN-β ($p<0.05$; Fig. 1*A*). The suppression of zymosan-induced IL-23 production by IFN-β was confirmed at the transcriptional level; increasing concentrations of IFN-β significantly inhibited zymosan-induced IL-23 mRNA expression in human DC ($p<0.01$ – $p<0.001$; Fig. 1*B*). IFN-β also consistently suppressed zymosan-induced IL-1β production ($p<0.001$ and $p<0.05$; Fig. 1*C*). The suppression of zymosan-induced IL-23 and IL-1β was a consistent finding in DC from 9 donors ($p<0.01$ for untransformed data; Fig. 1*D* and *E*). In contrast, IFN-β had no significant inhibitory effect on LPS-induced IL-23 (Fig. 1*A*). In fact IFN-β enhanced LPS-induced IL-23 by DC from the majority of donors tested ($p<0.05$ for untransformed data; Fig. 1*D*). IFN-β had a small but significant inhibitory effect on zymosan-induced IL-12p40, but had no effect on LPS-induced IL-12p40 (Fig. 1*A*). In contrast, IFN-β enhanced IL-10 induced by either LPS or zymosan ($p<0.05$) in a dose dependant manner (Fig. 1*A*). The enhancement of IL-10 by IFN-β in response to zymosan stimulation was a consistent finding for the majority of donors tested

($p<0.05$ for untransformed data; Fig. 1*F*). Zymosan or LPS activation also induced high levels of IL-6, which were not altered by IFN-β (data not shown).

Having demonstrated that IFN-β inhibited TLR-2/dectin-1 but not TLR4 induced IL-1 and IL-23, we next investigated the effect of IFN-β on Mtb-induced cytokines. Mtb is the immunostimulatory component used in the induction of EAE and contains both TLR2 and TLR4 ligands. DC were stimulated with 50 μg/ml of Mtb in the presence or absence of IFN-β. Mtb induced both IL-1 and IL-23 from DC, which was significantly inhibited by IFN-β ($p<0.05$ – $p<0.01$) (Supplementary Fig. 1). These data indicate that IL-1 and IL-23 induced by Mtb, which includes TLR2 and TLR4 ligands, are inhibited by IFN-β.

Having demonstrated that IFN-β inhibited pro-inflammatory cytokine production by DC when added at their maturation stage, we next investigated the effect of IFN-β when added during DC differentiation. DC were differentiated from monocytes using GM-CSF and IL-4 in the presence or absence of 50 U/ml IFN-β. After 7 days the culture supernatants were analysed for the concentrations of IL-12p40, IL-23 and IL-10. DC differentiated in the absence of IFN-β spontaneously produced IL-12p40, which was abolished in the presence of IFN-β ($p<0.001$) (Supplementary Fig. 2A). IL-23 was barely detectable in the absence of IFN-β, and this was not significantly altered by addition of IFN-β. Culture in the presence of IFN-β resulted in a small but significant induction of IL-10 (Supplementary Fig. 2A). DC that had been differentiated in the presence or absence of IFN-β were washed and stimulated with zymosan or medium only. The concentrations of IL-12p40, IL-23 and IL-10 were measured in the supernatants after 24 hr. Cytokines were undetectable in unstimulated DC, however DC stimulated with zymosan produced IL-12p40, IL-23 and IL-10, all of which were significantly inhibited by prior differentiation in the presence of IFN-β ($p<0.01$-0.001) (Supplementary Fig 2B). These data

indicate that IFN-β added to DC at the differentiation stage suppressed their ability to produce cytokines in response to subsequent stimulation with zymosan.

3.2 IFN-β inhibits Th17 cells directly or via DC

Since both IL-1 and IL-23 have been shown to be important in the development of Th17 cells, we next investigated the effect of IFN-β on IL-17 production by $CD4^+$ T cells. DC stimulated by zymosan in the presence or absence of IFN-β for 24 h were washed and then used to stimulate allogeneic $CD4^+$ T cells. After 5 days the supernatants were analysed for IL-17, IFN-γ and IL-10. Treatment of DC with 50 or 200 U/ml of IFN-β significantly reduced their ability to promote IL-17 production from allogeneic T cells ($p<0.05$; Fig. 2*A*). In contrast, treatment of DC with IFN-β had no significant effect on their ability to promote IFN-γ or IL-10 production by $CD4^+$ T cells (Fig. 2*A*).

We next examined the effect of IFN-β added to DC during differentiation on their subsequent ability to stimulate T cell responses in vitro. DC differentiated in the presence or absence of 50 U/ml of IFN-β for 7 days were washed, stimulated with zymosan for 24 h and then washed and added to allogeneic $CD4^+$ T cells. After 5 days the supernatants were analysed for IL-17, IFN-γ and IL-10. The results showed that addition of IFN-β during the differentiation of DC significantly inhibited their ability to stimulate IL-17 production by $CD4^+$ T cells, but had no effect on IFN-γ or IL-10 ($p<0.05$ - $p<0.001$; Fig. 2*B*).

Having shown that IFN-β inhibited Th17 indirectly via the DC, we next investigated the possibility that IFN-β may have direct effects on T cells. $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence or absence of IFN-β. After 72 h IL-17, IFN-γ and IL-10 production was quantified by ELISA and proliferation was determined by ^{3}H-thymidine

incorporation. IFN-β induced dose dependant and significant suppression of proliferation by $CD4^+$ T cells ($p<0.01$- $p<0.001$; Fig. 2*C*). IFN-β also significantly reduced IL-17 production by $CD4^+$ T cells, at all concentrations tested ($p<0.01$ - $p<0.001$; Fig. 2*C*). IFN-γ production was also reduced in response to IFN-β, although not as strikingly ($p<0.01$; Fig. 2*C*), but IL-10 production was unaffected. Thus in addition to inhibiting Th17 cells via the DC, IFN-β also directly inhibited $CD4^+$ T cell proliferation, IL-17 secretion, and to a lesser extent IFN-γ production.

3.3 IFN-β induces IL-27 expression by DC

IL-27 has been shown to suppress Th17 responses and to attenuate EAE (Stumhofer et al. 2006; Fitzgerald et al. 2007a; Fitzgerald et al. 2007b). Furthermore, interferon sensitive response element sites have been identified in the IL-27 promoter (Molle et al. 2007; Pirhonen et al. 2007), suggesting possible responsiveness to IFN-β. To investigate a possible role for IL-27 in the immunosuppressive effects of IFN-β, we first examined the capacity of IFN-β to induce IL-27 production by DC. Human DC were stimulated with increasing concentrations of IFN-β and IL-27 expression was quantified by ELISA or real time PCR after 24 h or 6 h respectively. We observed induction of both IL-27 protein (Fig. 3*A*) and IL-27p28 and EBI3 mRNA (Fig. 3*B*) in response to IFN-β. IFN-β-induced IL-27p28 mRNA expression peaked at 4 h, while EBI3 was induced to a much lesser extent and peaked at 6 h (Fig. 3*C*). IFN-β also induced IL-10 mRNA expression in a dose dependant manner (Fig. 3*B*).

3.4 IL-27 modulates TLR-induced cytokine production by DC and inhibits induction of Th17 cells

Having shown that IFN-β induces IL-27 production by DC, we next investigated the effect of IL-27 on regulatory cytokine production by DC and their ability to promote Th17 cells. We found

that incubation of DC with IL-27 significantly inhibited zymosan-induced IL-1β ($p<0.05$ - $p<0.01$) and IL-23 ($p<0.05$), but had no effect on IL-10 production (Fig. 4*A*). The DC were then washed and used to stimulate allogeneic T cells. On day 5, the concentrations of IL-17, IFN-γ and IL-10 were quantified in the supernatants by ELISA. Treatment of zymosan-stimulated DC with IL-27 significantly inhibited their ability to induce IL-17 from allogeneic T cells ($p<0.05$ - $p<0.01$). In contrast, IFN-γ production was not affected by IL-27 (Fig. 4*B*), and IL-10 was increased in a dose dependant, but not significant, manner (Fig. 4*B*). Having shown that IL-27 could inhibit Th17 cells via the modulation of DC, we next investigated the direct effects of IL-27 on T cells. $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence or absence of IL-27. After 72 h IL-17, IFN-γ and IL-10 production was quantified by ELISA. IL-27 significantly inhibited the production of IL-17 by $CD4^+$ T cells in a dose dependant manner ($p<0.01$; Fig. 4*C*). In contrast, IFN-γ was slightly increased ($p<0.05$; Fig. 4*C*), and IL-10 was significantly enhanced ($p<0.05$ – $p<0.01$; Fig. 4*C*). Our findings demonstrate that IL-27 can inhibit the induction of human Th17 cells, by acting directly on the T cell and also by modulating innate cytokine production by DC.

3.5 IL-27 mediates the suppressive effects of IFN-β on Th17 cells

Since IFN-β and IL-27 exerted similar effects on DC and T cell cytokines, we next investigated whether IFN-β might mediate its effects via the induction of IL-27. DC were treated with neutralising antibody specific for IL-27 prior to stimulation with zymosan in the presence or absence of IFN-β. After 24 h the concentrations of IL-23 and IL-1β were measured in the supernatants. Consistent with the data in Fig. 1, IFN-β inhibited zymosan-induced IL-23 production ($p<0.05$; Fig 5*A*). This inhibition was significantly reversed by blocking IL-27 ($p<0.01$ - $p<0.001$; Fig. 5*A*). Similarly, the inhibitory effect of IFN-β on zymosan-induced IL-1β

($p<0.05$ – $p<0.01$; Fig. 5*A*) was abrogated by anti-IL-27 antibody ($p<0.05$ - $p<0.01$; Fig. 5*A*). These data indicated that the inhibitory effect of IFN-β on zymosan-induced IL-1 and IL-23 production by DC was mediated via IL-27.

We next determined whether IL-27 was responsible for the inhibitory effect of IFN-β on the ability of zymosan-activated DC to promote IL-17 production by $CD4^+$ T cells. DC were pre-treated with neutralising antibody specific for IL-27 prior to stimulation with zymosan in the presence or absence of IFN-β. After 24 h the supernatants were removed from the DC and added to $CD4^+$ T cells that were stimulated with irradiated allogeneic PBMC. After 5 days the culture supernatants were analysed for IL-17 and IFN-γ. IL-17 production by T cells was inhibited when DC had been treated with 1000 U/ml of IFN-β ($p<0.0001$; Fig. 5*B*), and this inhibition was significantly reversed by addition of anti IL-27 ($p<0.01$; Fig. 5*B*). In contrast, IFN-γ production by T cells in response to zymosan activated DC was unaffected by treatment of DC with IFN-β (Fig. 5*B*). These findings suggest that the inhibitory effect of IFN-β on the ability of zymosan-activated DC to induce Th17 responses was mediated via IL-27.

3.6 IFN-β inhibits Mtb-induced IL-1 and IL-23 in murine DC, and inhibits Th17 cells via IL-27

Prior to investigating the effect of IFN-β in vivo in the EAE model, we first established whether IFN-β could inhibit IL-1 and IL-23 production by murine BMDC as well as human DC. Mtb was used to stimulate DC, since it represents the immunostimulatory component of CFA used to induce EAE. Murine BMDC from C57BL/6 mice were stimulated with 50 μg/ml Mtb in the presence or absence of IFN-β. After 24 hr the supernatants were analysed by ELISA for the concentrations of IL-1 and IL-23. Both IL-1 and IL-23 were significantly inhibited by the addition of IFN-β at different concentrations ($p< 0.05$-$p<0.001$; Figure 6*A*). We next investigated

the role of type I interferon in induction of IL-27, by comparing the production of IL-27 in response to TLR stimulation in IFNAR$^{-/-}$ versus wild type C57BL/6 (WT) mice. BMDC from WT or IFNAR$^{-/-}$ mice were stimulated with TLR agonists LPS or CPG and after 24 hr the supernatants were analysed for the concentration of IL-27 by ELISA. IL-27 was induced by LPS, and to a lesser extent CpG, in WT BMDC, however, IL-27 was virtually undetectable in BMDC from IFNAR$^{-/-}$ mice, indicating that induction of IL-27 by TLR stimulation is dependent on type I IFN signalling (Fig. 6*B*). Having shown that IFN-β could inhibit the ability of human DC to promote Th17 responses, we next determined whether it had a similar effect on murine BMDC stimulated with Mtb, and whether this may be dependent on IL-27. BMDC from WT or WSX-1$^{-/-}$ mice were stimulated with Mtb in the presence or absence of IFN-β for 24 hr, washed and used to stimulate CD4 T cells. After 3 days the supernatants were analysed by ELISA for the concentration of IL-17. Treatment of Mtb-activated WT DC with IFN-β significantly inhibited IL-17 production by T cells ($p<0.01$-0.001; Fig. 6*C*). In contrast, IFN-β only marginally though not significantly inhibited IL-17 induced by Mtb-activated DC from WSX-1$^{-/-}$ mice (Fig. 6*C*). These data indicated that the IFN-β treatment of murine DC stimulated with Mtb inhibited their ability to promote Th17 responses, and that this effect was largely dependent on IL-27 signalling.

3.7 In vivo treatment with IFN-β inhibits Th17 cells and attenuates EAE

Having shown that IFN-β inhibited the induction of human and murine Th17 cells in vitro, we next investigated whether IFN-β could attenuate EAE by suppressing Th17 cells in vivo. Chronic EAE was induced in C57BL/6 mice with MOG_{35-55} peptide and CFA and either IFN-β, or PBS as a control, was administered every day from induction and mice were monitored daily for clinical signs of EAE. Treatment of mice with IFN-β significantly reduced the severity of disease (day 18-22, $p<0.05$; Fig. 7*A*). At the peak of disease, there were significantly fewer infiltrating

IL-17$^+$CD4$^+$ and IL-17$^+$IFN-γ$^+$CD4$^+$ T cells in mice in the brains of mice that had been treated with IFN-β (Fig. 7*B* and *C*), and also fewer mean total cells in the brain (9 x 10^4 versus 17 x 10^4 in untreated mice). In contrast there was no significant difference in the frequency of CD4$^+$ IFN-γ or IL-10 secreting cells in the brain between control and treated mice (Fig. 7*B*).

We also investigated the effects of IFN-β treatment on relapsing remitting EAE induced in SJL/J mice. Here IFN-β was administered therapeutically after the peak of disease. Relapse was delayed in mice treated with IFN-β, and they exhibited significantly reduced clinical scores ($p<0.05$ day 25-27) (Fig. 7*D*). Antigen-specific IL-17 responses to $PLP_{139-151}$ peptide were significantly reduced in the lymph nodes (Fig. 7*E*) of IFN-β-treated mice ($p<0.05$-$p<0.001$). In contrast, PLP-specific IFN-γ was not inhibited (Fig. 7*E*). IL-27 mRNA was induced in the cerebellum of mice treated with IFN-β ($p<0.05$; Fig. 7*F*). Furthermore, IL-1β, IL-23 and IL-17 expression was significantly inhibited in the cortex of IFN-β treated mice ($p<0.05$-0.01; Fig. 7*F*). These data indicated that IFN-β attenuates the course of chronic and relapsing EAE in mice and has similar effects in vivo to those observed in vitro with human cells, inducing IL-27 and inhibiting IL-17 production.

3.8 Response to IFN-β treatment in MS patients is associated with induction of IL-27

Since IL-27 appears to mediate the inhibitory effect of IFN-β on IL-1β and IL-23 production by DC, and the consequent development of Th17 cells in vitro, we examined the possibility that IL-27 may mediate the protective effect and responsiveness to IFN-β therapy in RRMS patients. We examined the induction of IL-27 by IFN-β in RRMS patients, and compared the induction of IL-27 between those who responded or failed to respond to IFN-β treatment (patient details in Table I). DC from RRMS patients were treated with IFN-β for 6 h and then analysed for IL-27p28,

EBI3 and IL-10 mRNA expression. Consistent with the results for healthy controls (Fig. 3), treatment of DC with IFN-β induced expression of IL-27p28 (Fig. 8*A*). IFN-β also induced expression of EBI3 and IL-10, although to a much lesser extent than IL-27p28 (Fig. 8*A*). We next compared PBMC from IFN-β responder and non-responder patients for IL-27 expression in response to IFN-β or LPS. IFN-β induced IL-27p28 mRNA expression by PBMC from a responder patient (Fig. 8*B*). LPS induced lower expression of IL-27p28 from the same cells. In contrast, PBMC from the non-responder patient showed very little induction of IL-27p28 in response to IFN-β or LPS (Fig. 8*B*). In order to confirm these findings with a larger number of patients, we examined the induction of IL-27p28 in frozen PBMC samples from responder and non-responder patients. IFN-β (50 or 1000 U/ml) induced significant IL-27p28 expression in PBMC from all but one of the responder patients tested ($p<0.05$; Fig. 8*C*). In contrast, the induction of IL-27p28 was significantly lower in patients that had not responded to IFN-β treatment ($p<0.05$; Fig. 8*C*). Conversely, the expression of IL-6 in response to Poly I:C was not defective in non-responder patients, suggesting that the lower IL-27 was not due to general un-responsiveness of the PBMC from IFN-β non-responder patients (Fig. 8*D*). These findings suggest that responsiveness to IFN-β therapy is in part determined by the ability to produce IL-27.

4. Discussion

IFN-β is commonly used as a first line immunomodulatory therapy for RRMS. However its therapeutic mechanism in MS remains to be elucidated. Furthermore, a high proportion of MS patients are unresponsive to IFN-β therapy. In this study we aimed to determine the mechanism of action of IFN-β treatment in MS. We provide evidence that IFN-β mediates its therapeutic effects in MS at least in part by inducing the production of IL-27, which inhibits Th17 cells both directly and through inhibition of cytokines that promote the development of these pathogenic T cells. Complementary studies in the EAE model showed that the protective effect of IFN-β was associated with enhanced IL-27 and reduced IL-17, and the inhibition of Th17 cells by IFN-β was reversed in cells from IL-27 receptor deficient mice. Furthermore, we showed for the first time that the induction of IL-27 by IFN-β has clinical and therapeutic relevance. MS patients that failed to respond to IFN-β therapy produced significantly less IL-27 in response to in vitro stimulation with IFN-β than responder patients.

Our finding that IFN-β inhibits Th17 cells in vitro and in vivo is clearly applicable to MS and EAE, since there is now convincing evidence that Th17 cells mediate autoimmune inflammation in the CNS. IL-17 has been detected in the brain lesions and CSF of MS patients and the percentage of Th17 cells was increased during relapse (Matusevicius et al. 1999; Lock et al. 2002; Durelli et al. 2009). In the EAE model, numerous studies have highlighted a central role for Th17 cells, although Th1 cells can also play a role (Fletcher et al.) Th17 cells can exhibit functional plasticity and cells producing both IL-17 and IFN-γ are enriched in the CNS during MS and EAE, suggesting a role for IL-17$^+$IFN-γ$^+$ cells in pathology (Kebir et al. 2009; Murphy et al. 2010). Interestingly, we observed inhibition of both IL-17$^+$ and IL-17$^+$IFN-γ$^+$, but not IFN-γ$^+$ cells in the brains of mice with EAE that had been treated with IFN-β, indicating that IFN-β

has a preferential effect on IL-17 producing cells. Similarly, IFN-β inhibited human Th17 cells in vitro, both directly and indirectly via effects on the DC. However, the deletion of IFNAR on myeloid cells, but not on T cells, exacerbated EAE in conditional IFNAR$^{-/-}$ mice (Prinz et al. 2008), suggesting that in vivo the effects of IFN-β may be mediated mainly via the DC. Our data contrasts a recent study which showed that IFN-β suppressed EAE induced by transfer of Th1, but not Th17 cells (Axtell et al.).

Stimulation of DC with various PAMPs, including TLR ligands, results in different cytokine profiles that can drive particular Th responses; IL-1, IL-23 and IL-6 promote Th17 responses, whereas IL-12 promotes Th1 cells. In the present study we focused on the effects of IFN-β on DC stimulated with zymosan, a TLR2/dectin-1 agonist, the TLR4 agonist LPS, as well as Mtb, which is the immunostimulatory component used in the induction of EAE and contains both TLR2 and TLR4 ligands. DC stimulated with either zymosan or Mtb produced the Th17 promoting cytokines IL-1 and IL-23, which were inhibited by IFN-β. Furthermore, prior treatment of DC with IFN-β inhibited their ability to induce IL-17 production by CD4 T cells. IL-1 and IL-23 have been shown to be crucial cytokines for promoting IL-17 production by both human and mouse T cells (Sutton et al. 2006; van Beelen et al. 2007; Wilson et al. 2007). Thus, our data suggested that the inhibition of Th17 cells by treatment of DC with IFN-β was mediated through the inhibition of IL-1 and IL-23. IFN-β also modulated spontaneous cytokine production by DC when added during the differentiation stage, and inhibited the ability of these DC to subsequently promote Th17 responses.

We found that IFN-β was most effective in modulating IL-1 and IL-23 produced by DC in response to zymosan. Zymosan binds dectin-1 as well as TLR2, two PRRs that have been associated with Th17 responses and autoimmunity (Yoshitomi et al. 2005; Farez et al. 2009;

Reynolds et al. 2010). Although the TLR2/1 and TLR 2/6 agonists Pam_3CSK and Malp-2 respectively were less effective than zymosan in driving IL-23, we found that IFN-β could also suppress IL-23 induced by Pam_3CSK and Malp-2, suggesting that IFN-β can broadly modulate TLR2-induced responses. The effects of IFN-β on IL-1β and IL-23 induced by activation of TLR2, but not other TLRs may be due to the fact that unlike TLR3, TLR4, TLR7, TLR8 and TLR9, TLR2 activation does not result in production of IFN-β (Toshchakov et al. 2002). TLR2 signalling is involved in the progressive phase of EAE and elevated levels of a TLR2 agonist was detected in secondary progressive MS but not RRMS patients (Farez et al. 2009). In addition, TLR2 signalling in T cells promotes the development of Th17 cells, and mice deficient in TLR2 expression on T cells are resistant to EAE (Reynolds et al. 2010). Thus, inhibition of cytokine production in response to TLR2 and dectin-1 by IFN-β may be of benefit in MS by decreasing Th17 cell differentiation and CNS inflammation.

It has been reported that IFN-β suppresses LPS-induced IL-23 (Ramgolam et al. 2009), however, we found more variable effects of IFN-β on LPS-induced IL-23 and in fact observed enhancement in many donors. The explanation for this difference in the effect of IFN-β on TLR2 versus TLR4 activation may lie in the fact that in contrast to TLR2 activation, stimulation via TLR4 results in endogenous IFN-β production (Toshchakov et al. 2002), which would mask the effect of exogenous IFN-β. However, in EAE and in vivo in general, stimulation by combinations of TLR agonists, such as Mtb, may be more relevant than single agonists. Interestingly, in contrast LPS-induced responses which were not inhibited by IFN-β, we found that IFN-β inhibited IL-1 and IL-23 induced by Mtb, which contains both TLR4 and TLR2 ligands. It has been shown that co-activation of TLR4 and TLR2 abolished the type I IFN amplification loop induced by TLR4 alone and promoted Th17 responses (Wenink et al. 2009),

explaining why IFN-β may act differently after TLR2 and TLR4 co-activation compared with TLR4 activation alone.

We found that IFN-β promoted IL-27 production by human DC, and in addition, DC from IFNR$^{-/-}$ mice failed to produce IL-27 after TLR activation, indicating that induction of IL-27 is mediated via type I IFN. Consistent with our findings, studies in humans and mice have shown that type I IFN induces IL-27 production by innate immune cells (Pirhonen et al. 2007; Remoli et al. 2007; Ramgolam et al. 2009). It has recently been shown that IRF-1, IRF-3 and IRF-9 as part of the ISGF3 complex, all co-operate for the optimal induction of IL-27p28 by TLR3 or TLR4 signalling (Molle et al.). Since IFN-β signalling can activate IRF-9 in the absence of TLR signalling, it is likely that the induction of IL-27p28 by IFN-β that we observed in the absence of TLR signalling was mediated via IRF-9. Although the IL-12 genes can be induced by IRF-1 and IRF-3, IRF-9 oes not appear to be required, suggesting that unlike IL-27p28, IL-12p70 would not be induced by IFN-β alone in the absence of TLR signalling.

IL-27 had very similar inhibitory effects to IFN-β on IL-1 and IL-23 production by DC and consequently the ability of these DC to promote Th17 responses, suggesting that the immunomodulatory effects of IFN-β may be mediated through IL-27 production. Evidence in support of this conclusion was provided by our demonstration that neutralisation of IL-27 abrogated the suppressive effects of IFN-β on the induction of Th17 cells by zymosan-activated DC. Furthermore, the significant suppressive effect of IFN-β on the ability of DC to promote Th17 cells was lost in cells from IL-27 receptor deficient mice. In support of our findings in human cells, studies using IL-27 receptor deficient mice in the EAE model have demonstrated a protective role for IL-27, which is mediated via the inhibition of Th17 cells (Batten et al. 2006; Stumhofer et al. 2006; Fitzgerald et al. 2007a; Fitzgerald et al. 2007b; Diveu et al. 2009).

Furthermore, a protective role for IFN-β-induced IL-27 in vivo is supported by the observations that the exacerbated EAE observed in IFNAR$^{-/-}$ mice could be reversed by administration of recombinant IL-27 (Guo et al. 2008).

Finally we examined the ability of PBMC from RRMS patients to produce IL-27 in response to IFN-β stimulation, and found that IFN-β responder patients produced significantly more IL-27 in response to IFN-β than non-responders. This study is the first to show an association between the clinical response to IFN-β therapy and induction of IL-27 by IFN-β. IFN-β responder patients were defined as those who experienced a maximum of one relapse during the 2 yr after IFN-β therapy and had no sustained disease progression, while those who had two or more relapses on IFN-β therapy over 24 months were defined as having failed therapy. There was no significant difference in disease duration between the two groups of patients, however responders were on average significantly older than non-responders ($p<0.05$), consistent with the fact that responders remain on treatment indefinitely, whereas non-responders are transferred to alternative therapy after treatment failure. The precise nature of the defect in IFN-β-induced IL-27 production in some individuals and not in others is unclear. SNPs in the IL-27 gene have been associated with asthma and IBD (Chae et al. 2007; Li et al. 2009), and it is possible that such a functional SNP could account for reduced induction of IL-27 by IFN-β. Altered expression of other IFN-stimulated genes such as STAT1 (Feng et al. 2002), or in the type I IFN receptor (Gilli et al. 2008) have been described in MS patients, and may also contribute to the suboptimal response to IFN-β in some MS patients.

In summary, we have shown both in vitro and in vivo that IFN-β induces IL-27 which inhibits Th17 cells. Furthermore, induction of IL-27 by IFN-β was associated with response to IFN-β therapy in MS patients. However a proportion of patients fail to respond to treatment and

these have a limited ability to produce IL-27 in response to IFN-β. Therefore, administration of IL-27 to these patients may represent an alternative therapeutic approach to IFN-β.

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Figure Legends

Fig. 1. IFN-β inhibits zymosan-induced IL-1β and IL-23 production by human DC. Monocyte derived DC were stimulated with LPS or zymosan in the presence or absence of IFN-β. After 24 hr, the concentrations of IL-23, IL-12p40 and IL-10 were measured by ELISA (A). Alternatively, after 6 hr IL-23 mRNA expression was evaluated by real time-PCR and is shown as fold induction (FI) relative to the unstimulated control (B). DC were stimulated with zymosan in the presence or absence of IFN-β (50, 200 or 1000 U/ml), after 24 hr supernatants were harvested and the concentration of IL-1β was measured by ELISA (C). DC were treated as in (A) and data from multiple donors are shown as % of the medium control for IL-23 (D). DC were treated as in (C) and data from multiple donors are shown as % of the medium control for IL-1β (E) and IL-10 (F). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β versus medium control (one-way ANOVA test).

Fig. 2. IFN-β inhibits Th17 cells directly or via the DC. (A) Monocyte derived DC were stimulated with zymosan in the presence or absence of IFN-β (50, 200 or 1000 U/ml). After 24 hr DC were washed and used to stimulate allogeneic T cells. After 5 days supernatants were analysed for IL-17, IFN-γ and IL-10. (B) DC were differentiated in the presence or absence of IFN-β (50 U/ml) or IL-10 (5 ng/ml) for 7 days, then stimulated with zymosan for 24 hr. DC were washed and used to stimulate allogeneic T cells. After 5 days the concentrations of IL-17, IFN-γ and IL-10 in the supernatants were analysed by ELISA. (C) Purified $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of IFN-β (50, 200 or 1000 U/ml). After

72 hr supernatants were recovered and concentrations of IL-17, IFN-γ and IL-10 determined by ELISA. Proliferation was assessed by thymidine incorporation. Data are representative of at least three separate experiments using different donors.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β versus medium control (One-way ANOVA test).

Fig. 3. IFN-β induces IL-27 expression by DC. (A) DC were stimulated with IFN-β for 24 hr, and the concentration of IL-27 in the supernatant was determined by ELISA. * $p<0.05$ for IFN-β compared to control (one-way ANOVA test). (B) DC were stimulated with IFN-β for 6 hr, cells were harvested and expression of IL-27p28, EBI3 and IL-10 were determined by real time-PCR. (C) DC were stimulated with IFN-β (200 U/ml) for various times and the expression of IL-27p28 and EBI3 were determined by real time-PCR.

Fig. 4. IL-27 modulates cytokine production by DC and inhibits induction of Th17 cells. (A) DC were stimulated with zymosan in the presence or absence of IL-27 (25, 50 or 100 ng/ml). After 24 hr supernatants were analysed for IL-1β, IL-23 and IL-10 by ELISA. (B) DC were washed and used to stimulate allogeneic T cells. After 5 days supernatants were analysed for IL-17, IFN-γ and IL-10 by ELISA. (C) Purified $CD4^+$ T cells were stimulated using anti-CD3 and anti-CD28 in the presence or absence of IL-27 (25, 50 and 100 ng/ml). After 72 hours IL-17, IFN-γ and IL-10 concentrations were measured in supernatants by ELISA. Data are representative of at least three separate experiments using different donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β versus medium control (one-way ANOVA test).

Fig. 5. IL-27 mediated the suppressive effects of IFN-β on Th17 cells. DC were pre-treated with anti-IL-27 neutralising antibodies prior to stimulation with zymosan in the presence or absence of 50, 200 or 1000 U/ml IFN-β. (A) After 24 hr supernatants were analysed for IL-23, and IL-1. (B) Supernatants from DC were added to T cells stimulated with irradiated allogeneic PBMC. After 5 days IL-17 and IFN-γ concentrations were measured in supernatants by ELISA. Data are representative of three independent experiments using different donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β compared to medium control; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ for IFN-β plus neutralising antibody compared to IFN-β alone (one-way ANOVA test).

Fig. 6. IFN-β inhibits Mtb-induced IL-1 and IL-23 in murine DC, and inhibits Th17 cells via IL-27. (A) Murine BMDC were stimulated with 50 μg/ml Mtb in the presence or absence of IFN-β (50-1000 U'ml). After 24 hr the supernatants were analysed by ELISA for the concentrations of IL-1 and IL-23. (B) BMDC from WT or $IFNAR^{-/-}$ mice were stimulated with LPS (100 ng/ml) or CpG (5 μg/ml) and after 24 hr the supernatants were analysed for the concentration of IL-27 by ELISA. (C) BMDC from WT or $WSX\text{-}1^{-/-}$ mice were stimulated with Mtb (50 μg/ml) in the presence or absence of IFN-β for 24 hr, washed and used to stimulate $CD4^+$ T cells. After 3 days the supernatants were analysed by ELISA for the concentration of IL-17. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β compared to medium control (one-way ANOVA test).

Fig. 7. Treatment of EAE with IFN-β induces IL-27 in vivo and inhibits IL-17 production by T cells. EAE was induced in C57BL/6 mice and IFN-β was administered every day from induction onwards. (A) Clinical scores for control (PBS) or IFN-β treated mice are shown as the mean

score ± SEM (n=5-10). (B) At disease peak (day 22) mice were sacrificed. Mononuclear cells were isolated from the brain, stimulated with PMA and ionomycin and analysed by flow cytometry for intracellular expression of IL-17, IFN-γ and IL-10, representative dot plots are shown in (C) * $p<0.05$; t-test. EAE was induced in SJL mice and IFN-β or PBS was administered every second day from day 24-28 post induction as indicated. (D) Clinical scores of control (PBS) and IFN-β treated mice are shown as the mean score ± SEM (n=5-10). Mice (n=5) were sacrificed on day 29. (E) lymph nodes were harvested and restimulated with 1, 5 or 25 μg/ml $PLP^{139-151}$ peptide or were left unstimulated. The concentrations of IL-17, IFN-γ and IL-10 were determined after 72 hr. ** $p<0.01$, *** $p<0.001$ for PBS versus IFN-β; one-way ANOVA. (F) The expression of IL-27p28, IL-1β, IL-23 and IL-17 was determined in the cortex by real time PCR. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for IFN-β treated versus PBS control mice. $P<0.05$-0.01; t test.

Fig. 8. Response to IFN-β treatment in MS is associated with induction of IL-27. (A) Monocyte derived DC from an IFN-β responder MS patient were stimulated with IFN-β and after 6 hr the expression of IL-27p28, EBI3 and IL-10 were determined by real time PCR. * $p<0.05$, ** $p<0.01$ for IFN-β versus control; one-way ANOVA (B) Freshly isolated PBMC from an IFN-β responder (Rsp) and a non-responder (N-Rsp) MS patient were stimulated with LPS, IFN-β (50, 200 or 1000 U/ml) or medium (Med) only for 6 hr and expression of IL-27p28 was measured by real time PCR. (C) Cryopreserved PBMC from IFN-β responder or non-responder MS patients were treated with IFN-β (50 U/ml and 1000 U/ml) for 4-6 hr and expression of IL-27p28 was measured by real time PCR, expressed as fold induction (FI). Alternatively cells were stimulated

with Poly I:C for 6 hr and expression of IL-6 was measured by real time PCR (D). * $p<0.05$; Mann-Whitney U-test.

Table I

Clinical details of MS patients

Age (years)	Sex	Disease duration (years)	EDSS[a]	IFN-β Treatment	Relapses post IFN-β treatment[b]	Response to IFN-β [c,d]
41	F	13	1.5	Avonex	0	Responder
42	F	20	1.5	Avonex	0	Responder
55	F	6	1.5	Avonex	0	Responder
42	F	6	1	Avonex	1	Responder
42	F	5	1	Avonex	0	Responder
30	F	10	2	Avonex	1	Responder
39	F	14	1.5	Avonex	0	Responder
32	F	4	1.5	Avonex	0	Responder
59	F	13	1.5	Betaferon	0.5	Responder
51	M	5	1	Rebif	0	Responder
24	F	3	1.5	Betaferon	1	Responder
39	F	6	3.5	Betaferon	1	Responder
46	F	6	3	Betaferon	0	Responder
30	F	3	2	Rebif	0	Responder
Ave: 40		**Ave: 7.9**	**Ave: 1.7**		**Ave: 0.4**	
30	F	6	3	Rebif	2	Non- responder
42	M	13	2	Avonex	2	Non-responder
29	M	4	3	Avonex	4	Non-responder
22	F	3	1.5	Avonex	2	Non-responder
47	F	12	5	Betaferon	6	Non-responder
24	F	1	1	Rebif	6	Non-responder
32	F	7	1.5	Betaferon	2	Non-responder
35	M	1	4	Avonex	4	Non-responder
29	M	3	1.5	Rebif	1	Non-responder [e]
Ave: 32*		**Ave 5.0**	**Ave: 2.5**		**Ave: 3.2*****	

[a] Expanded Disability Status Scale (EDSS) was assessed when blood samples were taken

[b] Number of relapses during the first 2 yr on IFN-β treatment

[c] IFN-β responders were defined as those who experienced a maximum of one relapse during the 2 yr after IFN-β therapy and had no sustained disease progression. Patients who had two or more relapses on IFN-β therapy over 24 months, or with or without sustained disability progression were defined as having failed therapy

[d] Non-responder patients who had tested positive for Nabs were excluded from the study

[e] Patient had sustained disease progression by MRI

* p=0.04, *** p<0.0001 responder versus non-responder (unpaired t-test)

Figure 1: Sweeney et al., IFN-β inhibits Th17 cells via IL-27

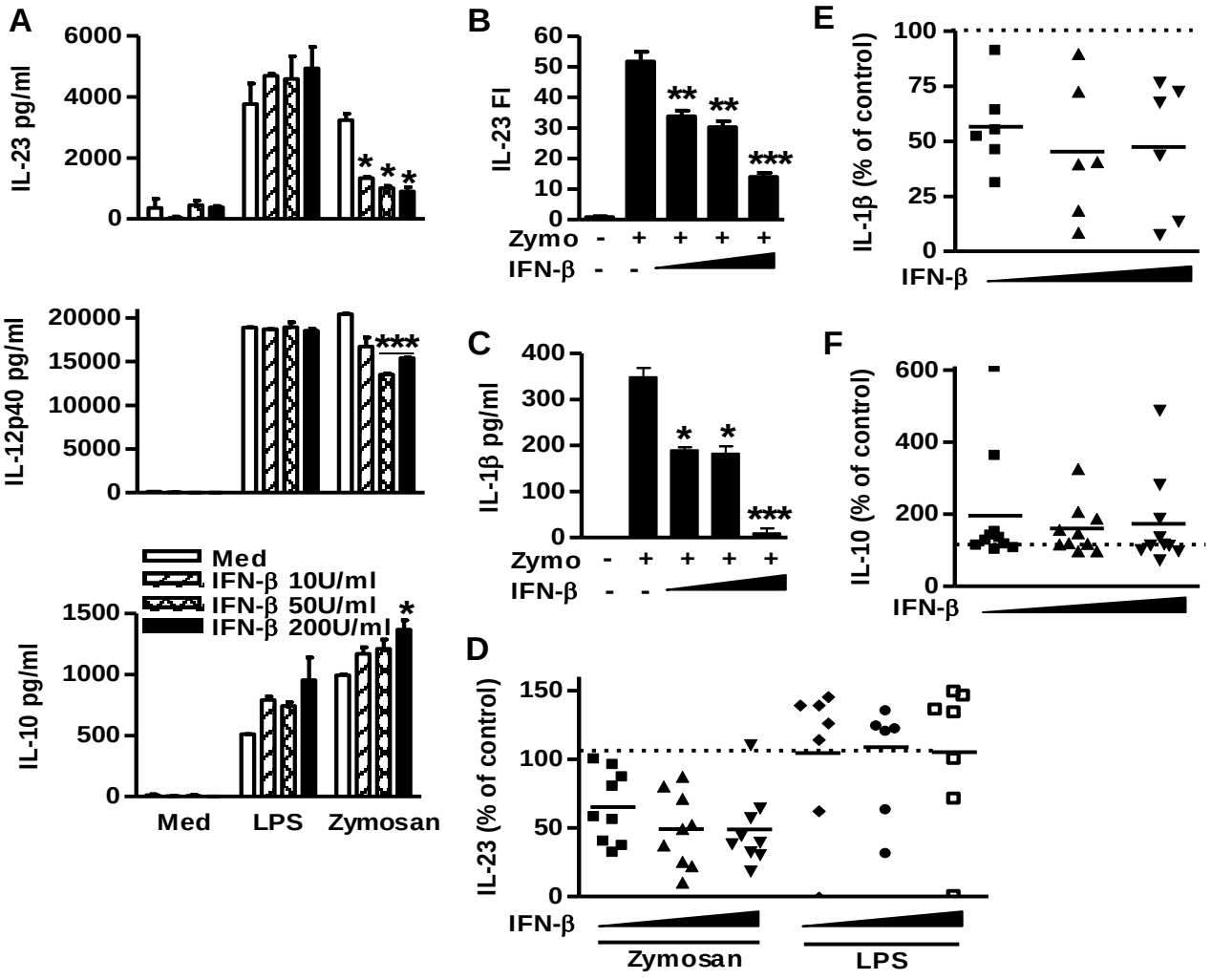


Figure 2: Sweeney et al., IFN-β inhibits Th17 cells via IL-27

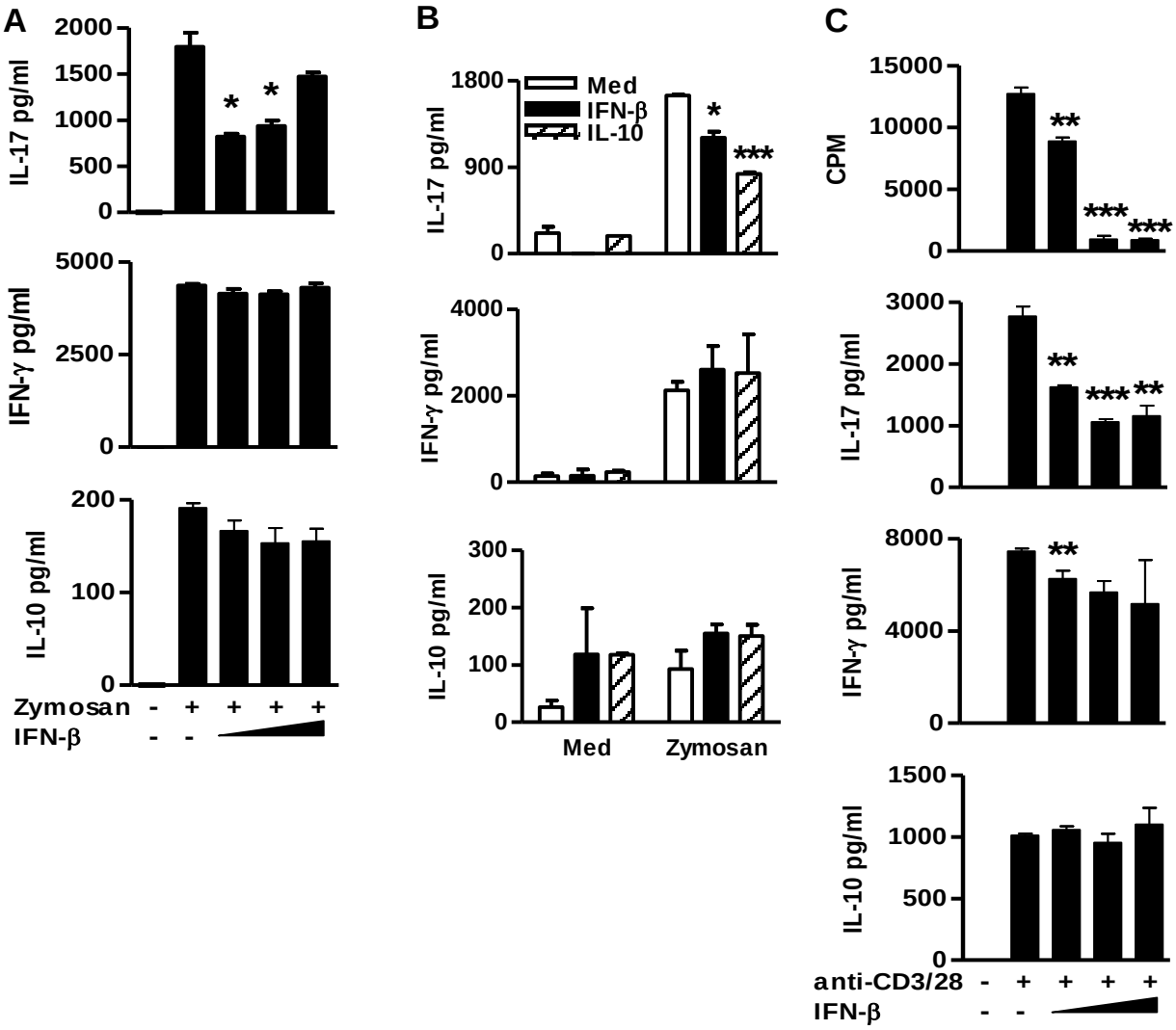


Figure 3: Sweeney et al., IFN-β inhibits Th17 cells via IL-27

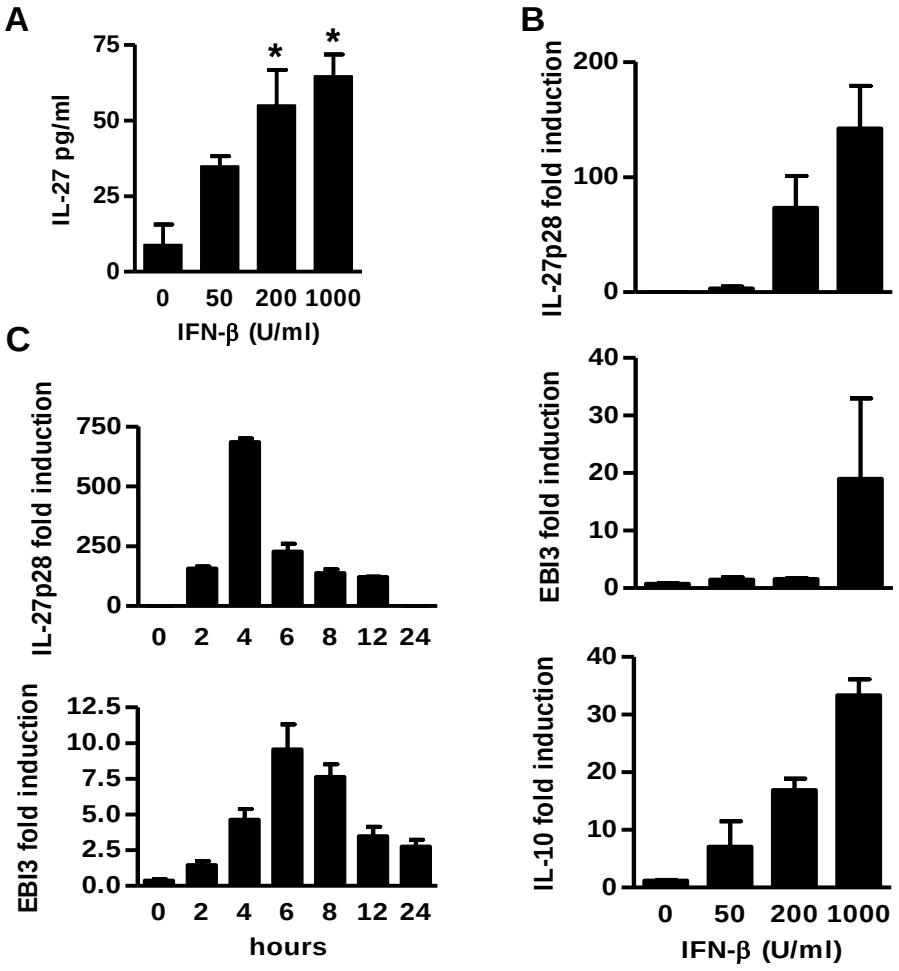


Figure 4: Sweeney et al., IFN-β inhibits Th17 cells via IL-27

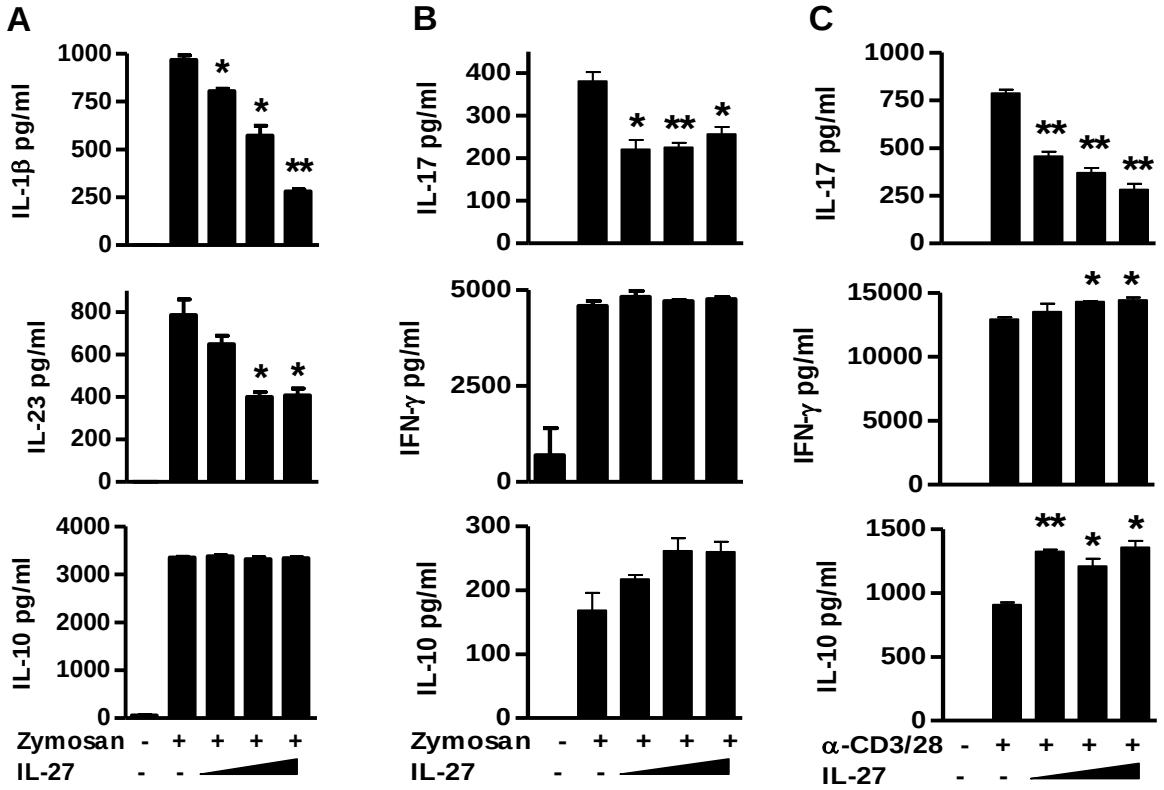
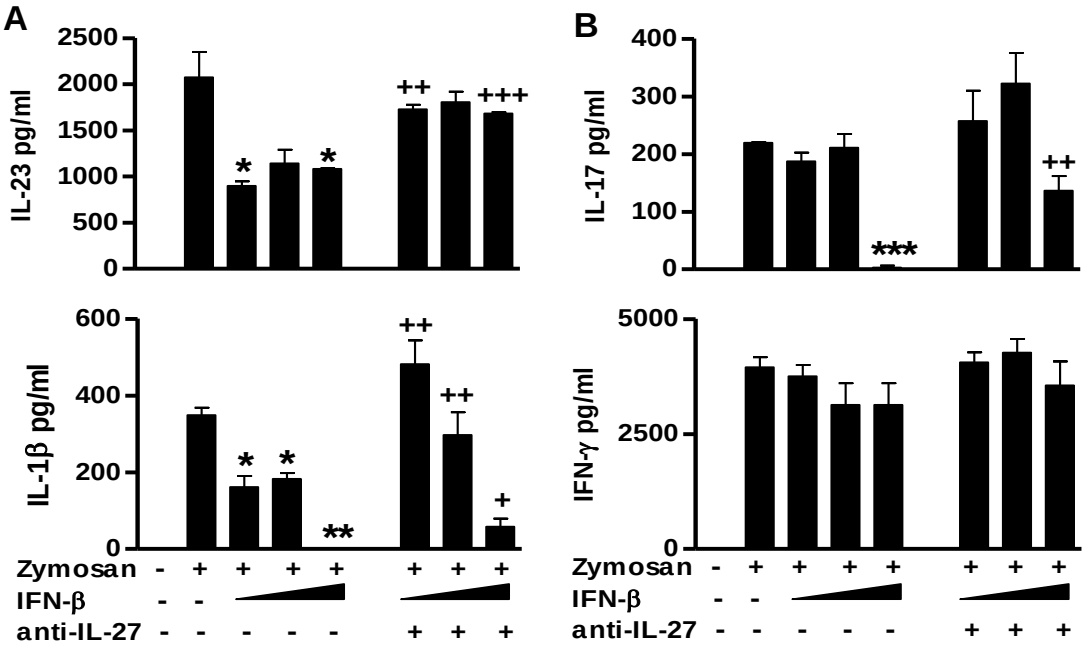


Figure 5: Sweeney et al., IFN-β inhibits Th17 cells via IL-27



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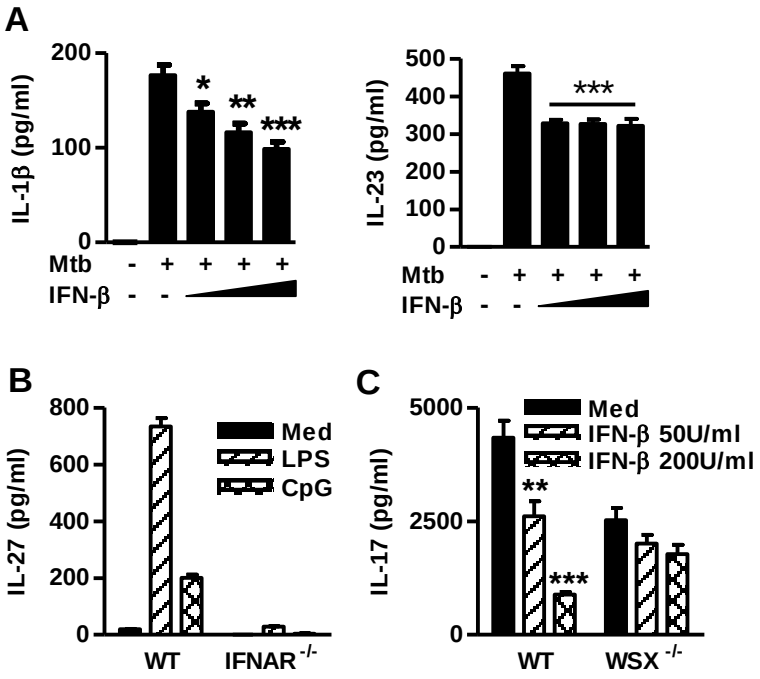


Figure 7: Sweeney et al., IFN-β inhibits Th17 cells via IL-27

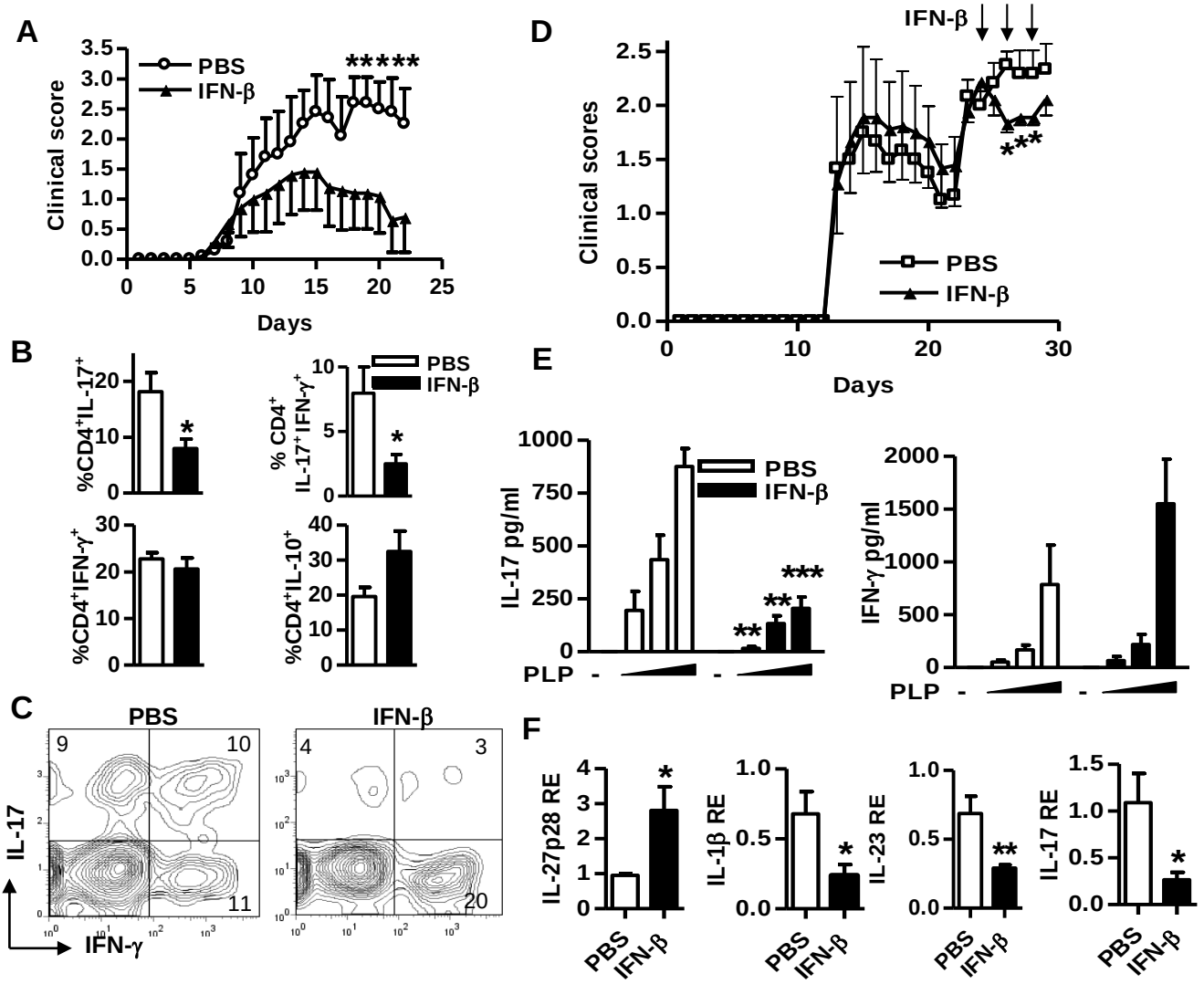
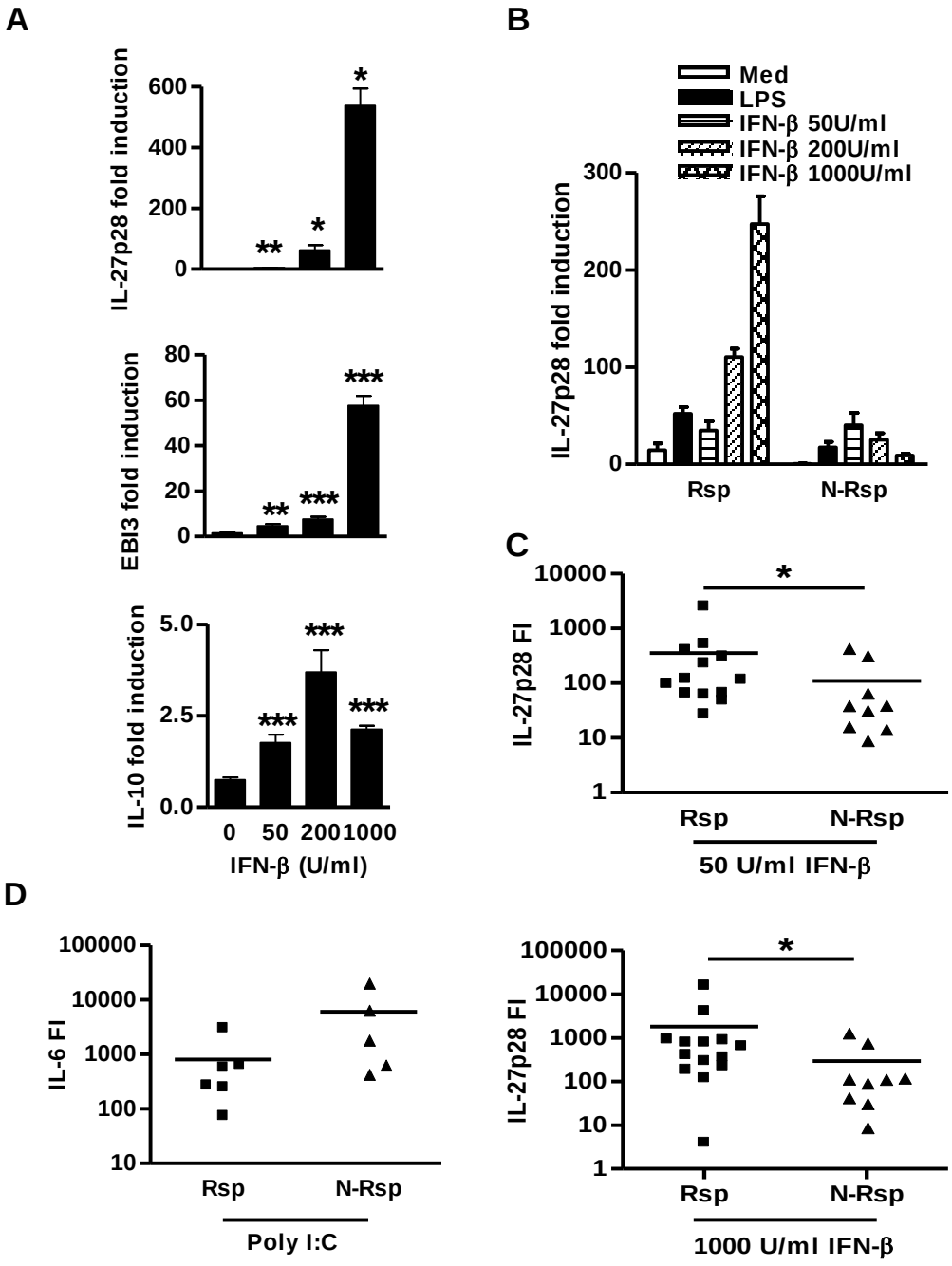


Figure 8: Sweeney et al., IFN-β inhibits Th17 cells via IL-27



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IFN-β therapy induces IL-27, which inhibits pathogenic Th17 cells that mediate autoimmunity in MS and EAE.