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ORIGINAL ARTICLE

The IL-27/IL-27R axis is altered in CD4$^+$ and CD8$^+$ T lymphocytes from multiple sclerosis patients

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Abstract

Objectives. Pro- and anti-inflammatory properties have been attributed to interleukin-27 (IL-27). Nevertheless, the impact of this cytokine on chronic inflammatory diseases such as multiple sclerosis (MS) remains ill-defined. We investigated the biology of IL-27 and its specific receptor IL-27Rα in MS patients. **Methods.** Levels of IL-27 and its natural antagonist (IL-27-Rα) were measured by ELISA in biological fluids. CD4$^+$ and CD8$^+$ T lymphocytes were isolated from untreated relapsing–remitting MS patients and healthy donors. Transcriptome-wide analysis compared T-cell subsets stimulated or not with IL-27. Expression of the IL-27Rα, key immune factors, STAT phosphorylation and cytokine production was assessed by flow cytometry. **Results.** We observed elevated levels of IL-27 in the serum and cerebrospinal fluid of MS patients compared with controls. Moreover, we show that specific IL-27-mediated effects on T lymphocytes are reduced in MS patients including the induction of PD-L1. IL-27-triggered STAT3 signalling pathway is enhanced in CD4$^+$ and CD8$^+$ T lymphocytes from MS patients. Elevated IL-27Rα levels in serum from MS patients are sufficient to impair the capacity of IL-27 to act on immune cells. We demonstrate that shedding of IL-27Rα by activated CD4$^+$ T lymphocytes from MS patients contributes to the increased IL-27Rα peripheral levels and consequently can dampen the IL-27 responsiveness. **Conclusion.** Our work identifies several mechanisms that are altered in the IL-27/IL-27R axis in MS patients, especially in T lymphocytes. Our results underline the importance of characterising the biology of cytokines in human patients prior to design new therapeutics.

Keywords: cytokines, IL-12, neuroimmunology, PD-L1, STAT signalling, T cells

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterised by demyelination and neuronal loss.[1,2] It is well established that the immune system participates in the pathobiology of MS.[1,2] Pro-inflammatory T lymphocytes infiltrating the CNS play a central role in driving inflammation in both MS patients and the animal model experimental autoimmune encephalomyelitis (EAE).[2,3] Multiple cytokines can shape the phenotype and functions of T lymphocytes. Notably, an increasing number of publications support the role of interleukin-27 (IL-27) in shaping inflammation in MS and other autoimmune diseases.[4–6] Whereas IL-27 can limit EAE,[7–13] its role in the human disease, MS, remains ill-defined.[6]

IL-27 belongs to the IL-12 cytokine family and is composed of two subunits: the Epstein–Barr virus-induced gene 3 (EBI-3) and the p28 subunits. Two chains form the IL-27 receptor (IL-27R): IL-27Rα and glycoprotein 130 (gp130), the latter being shared with other cytokines such as IL-6.[14,15] The IL-27R is expressed by different subsets of leucocytes, including T lymphocytes.[14,16] Upon binding to these lymphocytes, IL-27 triggers mainly STAT1 and STAT3 signalling.[17–19] Several studies indicate that IL-27 can exhibit pro- and anti-inflammatory effects on T lymphocytes.[6,20] For instance, IL-27 can promote the differentiation of naïve $CD4^+$ or $CD8^+$ T lymphocytes into Th1 and Tc1 cells[15,21–23] and block the development of $FoxP3^+$ regulatory T lymphocytes.[24] However, this cytokine dampens the proliferation and cytokine production of established Th1 and Th17 cells[25,26] and favors the development of $FoxP3^-$ regulatory T lymphocytes Tr1 that produce anti-inflammatory molecules such as IL-10.[20]

An increasing body of evidence indicates that IL-27 expression is elevated in the CNS and periphery of MS patients. We and others have established that IL-27 is elevated in MS brains.[27,28] We have also shown that CNS-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes express IL-27R in MS brains.[27] Cerebrospinal fluid levels of IL-27 are higher in relapsing–remitting MS (RRMS) patients compared with controls.[28] Contradictory results have been reported for IL-27 serum levels in MS patients; while one study reported similar levels in untreated and treated MS patients compared with HC,[28] other studies showed higher IL-27 serum levels in MS patients correlate with positive clinical response to disease-modifying therapies.[29–32] Accumulating evidence from multiple physiological states supports the notion that the impact of IL-27 is shaped by specific conditions.[6] Whether MS pathobiological conditions influence the IL-27-mediated effects has not been previously studied.

We compared the impact of IL-27/IL-27R pathway on T lymphocytes from MS patients and age/sex-matched healthy controls (HC). First, we show that IL-27 levels are elevated in biological fluids (serum and cerebrospinal fluid) from MS patients. Based on microarray data, we demonstrate that IL-27 increases the expression of key immune factors such as PD-L1, Fas and ICAM-1 by T lymphocytes from both MS patients and HC. However, the IL-27-mediated induction of PD-L1 is less efficient in T lymphocytes from MS patients. Second, we report that IL-27 triggers STAT3 phosphorylation in greater proportions of $CD4^+$ and $CD8^+$ T lymphocytes from MS compared with HC's counterparts. However, such enhanced signalling is not because of elevated expression of IL-27R by T lymphocytes from MS patients. Notably, greater serum levels of the soluble form of the IL-27Rα chain, a natural antagonist of IL-27, are detected in MS patients compared with HC. Finally, we show that activated $CD4^+$ T lymphocytes from MS patients shed greater amounts of IL-27Rα upon activation than those from HC. Moreover, we validated that these amounts can impair IL-27-mediated effects in T lymphocytes. Overall, our results underscore that the IL-27/IL-27R pathway is altered in T lymphocytes from MS patients.

RESULTS

Elevated IL-27 levels in serum and CSF from MS patients compared with controls

We have previously shown that IL-27 is upregulated in post-mortem brain tissues from MS patients.[27] To validate whether IL-27 levels are elevated in biological fluids from MS patients,[28–32] we measured this cytokine in sera and cerebrospinal fluid (CSF) from untreated MS patients. We detected significantly higher levels of IL-27 in sera from MS patients than from HC (Figure 1a). Moreover, IL-27 levels in CSF collected at time of diagnosis from MS

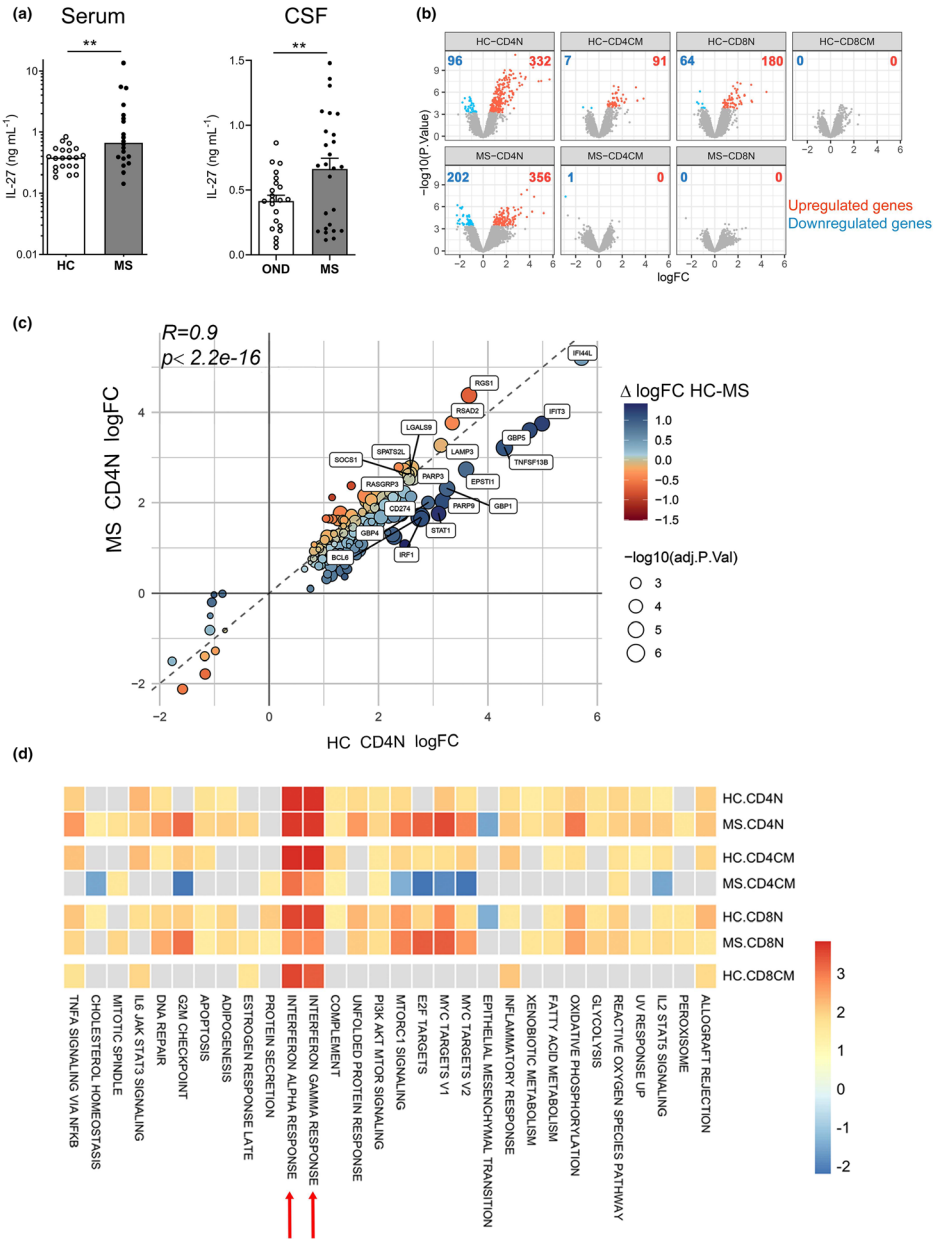

Figure 1. Elevated IL-27 levels in serum and CSF from MS patients, and IL-27 triggers multiple genes in human T-cell subsets. **(a)** IL-27 levels in serum (left panel) and CSF (right panel) from untreated MS patients and age/sex-matched HC or patients with other neurological diseases (OND); each dot represents one donor. Serum data are shown as median; the Mann–Whitney test comparing MS patients vs. and HC (n = 20). Data for CSF IL-27 levels are shown as mean ± SEM; an unpaired t-test comparing MS (n = 26) vs. OND (n = 22), $^{**}P < 0.01$. **(b)** Volcano plots depicting the impact of IL-27 on the gene expression profiles of αCD3/αCD28-activated naïve and central memory (CM) $CD4^+$ and $CD8^+$ T lymphocytes from HC and MS patients, n = 3 per group. Genes were identified as significantly differentially expressed when the adjusted P-value ≤ 0.05 and the absolute fold change > 1.3. Number of genes downregulated (blue) or upregulated (red) is indicated for each subset. Log fold change (logFC) shown according to $-\log_{10}$ of P-values ($-\log_{10}P$-value) for each subset. **(c)** Correlation of log FC for genes identified as modified by IL-27 in activated naïve CD4 T cells from MS (y-axis: MS CD4N logFC) and naïve CD4 T cells from HC (x-axis: HC CD4N logFC). Δ logFC (HC CD4N logFC-MS CD4N logFC) for each gene is colour-coded according to the scale (blue greater logFC in HC cells and red greater logFC in MS cells); $-\log_{10}$(adjusted P-value) is depicted by different circle size. **(d)** Enrichment for general pathways induced by IL-27 in activated naïve $CD4^+$ T cells (CD4N), central memory $CD4^+$ (CD4CM), naïve $CD8^+$ T cells (CD8N) and central memory $CD8^+$ T cells (CD8CM) from HC and MS using the R package fgsea v1.11.0 on the curated hallmark database. Genes were ranked according to the t-statistic of the corresponding comparison. Only terms significant (adjusted P-value < 0.1) in at least one contrast are shown according to the colour code. Grey boxes indicate that no change was observed in response to IL-27 in this T-cell subset.

patients were significantly greater than in samples from patients with other neurological diseases (Figure 1a). IL-27 levels in CSF samples from MS patients could be divided into low- and high-level groups, but such difference could not be linked to the sex of patients. Nevertheless, younger patients tended to exhibit higher levels than older patients without reaching statistical significance. Our results show that IL-27 is an immune mediator present in elevated quantities in both the periphery and CNS of MS patients.

IL-27 induces the expression of a broad spectrum of genes in $CD4^+$ and $CD8^+$ T lymphocytes

As T lymphocytes play a central role in the pathobiology of MS[1,33] and that these cells can respond to IL-27,[6] we examined the broad impact of this cytokine on human T lymphocytes. We performed a microarray analysis assessing 20 800 well-annotated genes on purified naïve ($CD45RA^+CD45RO^-CCR7^+$) and central memory ($CD45RA^-CD45RO^+CCR7^+$) $CD4^+$ and $CD8^+$ T cells from HC and untreated MS patients that were activated *in vitro* with αCD3/αCD28 in the presence or absence of IL-27 (10 ng mL^{-1}). Unfortunately, it was not possible to obtain a sufficient number of central memory $CD8^+$ T cells from MS donor samples to perform this assay. Genes were identified as significantly differentially expressed when they showed an adjusted P-value < 0.05 and an absolute log fold change > 1.3. Our results show that addition of IL-27 to activated T cells triggered a transcriptional response that was most prominent in naïve T cells, especially in $CD4^+$ T cells (Figure 1b). The majority of differentially expressed genes (DEGs) were upregulated upon stimulation, suggesting a transcriptional activation in response to IL-27. When comparing the fold changes associated with DEGs in response to IL-27 by αCD3/αCD28-activated naïve CD4 T cells from HC and untreated MS patients, we found a strong correlation ($R = 0.9$, $P < 2.2 \times 10^{-16}$) between both groups (Figure 1c). While some genes showed slightly higher or lower fold change in either donors' groups, the general transcriptional response was similar. Almost no gene was identified as being significantly altered in central memory $CD4^+$ and naïve $CD8^+$ T cells from MS patients compared with HC counterparts, suggesting a more consistent response to IL-27 in HC. To examine which biological pathways are involved in the response to IL-27, we performed a Gene Set Enrichment Analysis (GSEA) using the fgsea package in R. This allowed a single-list approach where all genes are ranked according to their t-statistic, taking into account both the significance and the directionality of the statistical test. To have the broadest view, we used the hallmark gene sets of MSigDB, which contain a total of 50 general pathways. We found that the most enriched pathways were associated with interferon response (interferon-α/γ) and that these effects were similar in HC and MS patients. Despite having less significant changes, this approach shows that genes associated with interferon-α and interferon-γ responses were also enriched in the transcriptional variation induced by IL-27 in central memory $CD4^+$ and $CD8^+$ T-cell subsets (Figure 1d).

The capacity of IL-27 to shape $CD4^+$ and $CD8^+$ T lymphocyte properties is altered in MS patients

Amongst the genes increased by IL-27 in T lymphocytes, we selected a subset of immune mediators (Table 1) that have been previously implicated in MS pathobiology for validation at the protein level; those included CD274 (PD-L1), Fas (CD95) and ICAM-1 (CD54).[34–40] Moreover, to investigate whether IL-27 has similar effects on cells from MS and HC, we compared the impact of this cytokine on $CD4^+$ and $CD8^+$ T lymphocytes from both groups. We confirmed by flow cytometry that IL-27 significantly increased the percentage of activated $CD4^+$ and $CD8^+$ T lymphocytes expressing PD-L1, CD95 or CD54 (Figure 2) in all donors. Since the proportion of $CD4^+$ (HC, 6.5%; MS, 3.0%) and $CD8^+$ (HC, 11.4%; MS, 4.6%) T lymphocytes expressing PD-L1 in the absence of IL-27 was lower in MS vs. HC, we compared the increased percentage of PD-L1-positive cells induced by IL-27 (Figure 2c). We observed that IL-27 had significantly less impact on the proportion of $PD\text{-}L1^+$ cells in $CD4^+$ T lymphocytes from MS patients compared with HC (Figure 2b and c). Notably, IL-27 triggered a more modest increase in PD-L1 mRNA levels in naïve $CD4^+$ T lymphocytes from MS patients compared with HC's counterparts (Table 1). Hence, our results establish that IL-27 has a reduced capacity at inducing an inhibitory pathway (e.g. PD-L1) in $CD4^+$ T lymphocytes from MS patients.

Table 1. IL-27-triggered upregulation of gene expression in activated naïve and central memory $CD4^+$ and $CD8^+$ T cells

		CD4 naïve				CD4 CM				CD8 naïve				CD8 CM	
		HC		MS		HC		MS		HC		MS		HC	
Gene symbol	Protein name	logFC	Adj. *P*-value	logFC	Adj. *P*-value	logFC	Adj. *P*-value	logFC	Adj. *P*-value	logFC	Adj. *P*-value	logFC	Adj. *P*-value	logFC	Adj. *P*-value
CD274	CD274 (PD-L1)	2.59	5.79E-04	2.14	0.0093	1.46	0.26	0.17	0.96	1.76	0.06	0.81	0.99	0.65	0.99
Fas	Fas (CD95)	2.26	9.60E-06	2.12	0.05	0.98	0.12	0.62	0.87	1.74	1.32E-03	1.03	0.99	0.43	0.99
ICAM1	ICAM-1 (CD54)	2.26	2.75E-05	1.3	0.12	0.96	0.28	0.15	0.97	1.95	1.41E-03	0.66	0.99	0.38	0.99
IL12RB1	IL-12Rβ1	1.63	1.77E-05	0.97	0.13	0.63	0.38	−0.003	0.99	0.59	0.24	0.32	0.99	0.45	0.99
IL12RB2	IL-12Rβ2	2.65	0.02	4.21	0.0055	2.24	0.17	1.00	0.87	1.88	0.20	2.66	0.99	1.46	0.99
IL23R	IL-23R	−0.17	0.98	0.2	0.92	−0.09	0.99	−0.18	0.97	0.13	0.96	0.18	0.99	−0.17	0.99

$CD4^+$ and $CD8^+$ T-cell subsets isolated from three healthy donors and three untreated MS patients were stimulated with αCD3 and αCD28 ± IL-27 for 3 days, and then, transcriptomic profiles were analysed by microarray.

The capacity of IL-27 to enhance T-cell response to IL-12 is reduced in MS patients

Pro-inflammatory Th1/Tc1 and Th17/Tc17 subsets contribute to the pathobiology of MS and EAE.[2] The capacity of IL-27 to modulate T-cell polarisation in animal models of MS has been shown.[8–12] Notably, our microarray data showed IL-27-mediated upregulation of receptor chains of key cytokines involved in T-cell polarisation such as IL-12 receptor chains: *IL12RB1* and *IL12RB2* genes in naïve CD4 T lymphocytes from HC, while mRNA levels for *IL12RB2* but not *IL12RB1* were increased in MS counterparts (Table 1). We observed that IL-27 significantly enhanced IL-2Rβ1 expression at the protein levels by $CD4^+$ and $CD8^+$ T lymphocytes from both MS and HC donors (Figure 3a and b), despite no significant changes at the mRNA levels in MS' T cells. Unfortunately, commercially available antibodies did not allow the robust detection of the IL-12Rβ2 chain despite its upregulation at the mRNA detected in our microarray data. The IL-12Rβ1 chain is shared by the receptor complex of IL-12 and IL-23. We thus analysed whether the IL-27-mediated upregulation of IL-12Rβ1 led to an enhanced susceptibility to IL-12 and/or IL-23 by assessing the main STAT pathway triggered by these cytokines. Induction of STAT4 and STAT3 phosphorylation by IL-12 and IL-23, respectively, was compared in $CD4^+$ and $CD8^+$ T lymphocytes that have been pre-activated in the presence or absence of IL-27 (Figure 3c). We observed that pre-exposure to IL-27 increased the capacity of $CD4^+$ and $CD8^+$ T lymphocytes to respond to IL-12 (Figure 3d and e). Notably, the impact of IL-27 was significantly less potent on $CD4^+$ and $CD8^+$ T lymphocytes from untreated MS patients compared with HC (Figure 3e). In contrast, the IL-27 pre-activation of $CD4^+$ and $CD8^+$ T lymphocytes had no impact on the IL-23 response as assessed by STAT3 phosphorylation (Figure 3f). Our results suggest that IL-27 has a diminished capacity at inducing an IL-12 responsiveness in $CD4^+$ and $CD8^+$ T lymphocytes from MS patients.

IL-27 decreases GM-CSF and IL-17 production by $CD4^+$ and $CD8^+$ T lymphocytes

GM-CSF, IL-17 and IFN-γ are crucial inflammatory cytokines produced by T lymphocytes in the context of MS and its animal models.[41–46] We assessed the impact of IL-27 on the production of these

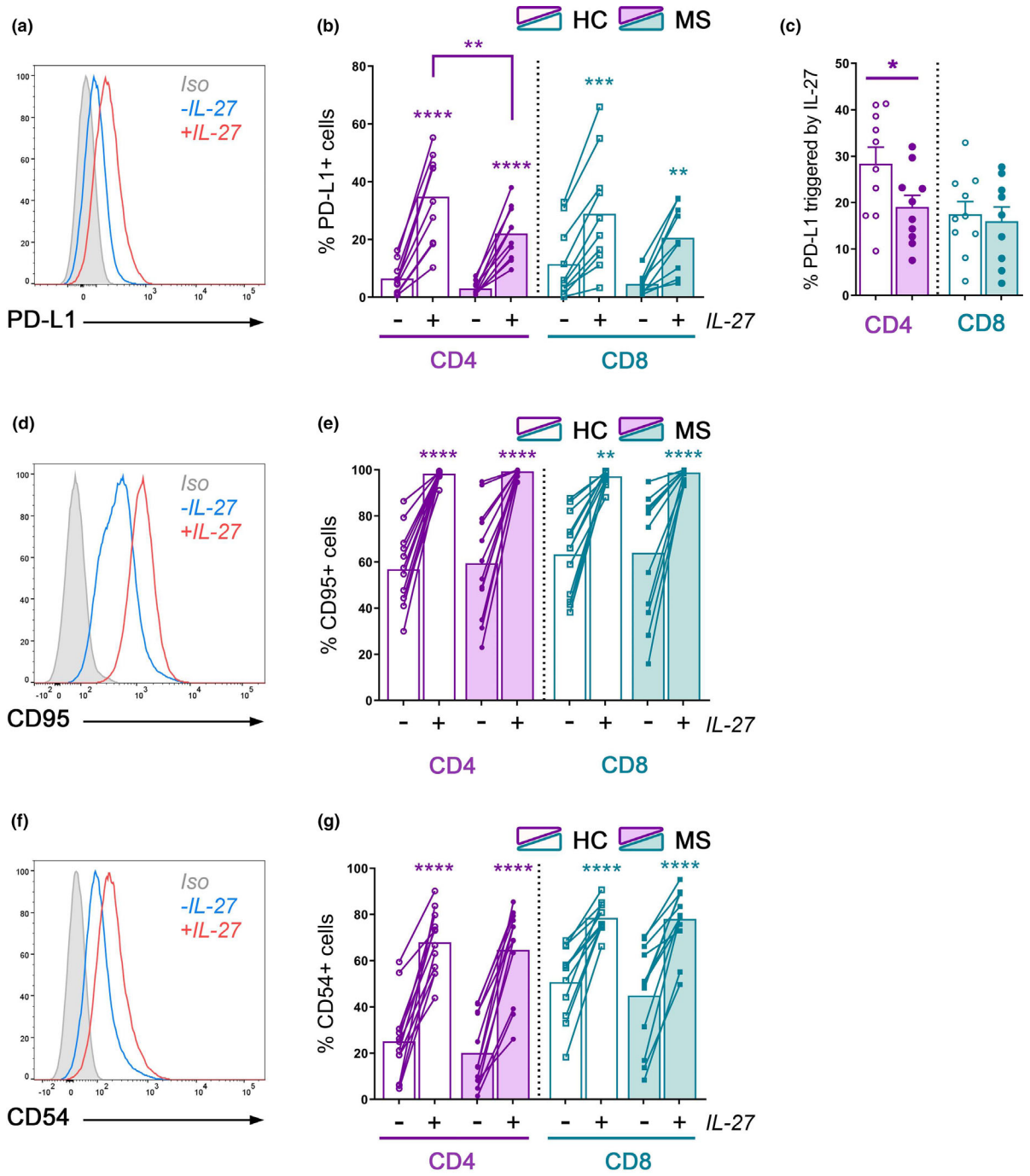

Figure 2. IL-27 induces PD-L1 with less efficiently on T lymphocytes from MS patients. Isolated $CD4^+$ (magenta) and $CD8^+$ (aqua) T cells from untreated MS patients and age/sex-matched HC were stimulated during 4 days with αCD3/αCD28 in the absence or presence of IL-27 (100 ng mL^{-1}) and then analysed for the surface expression of PD-L1, CD95 (Fas) and CD54 (ICAM-1) by flow cytometry. Representative histograms for the detection of PD-L1 **(a)**, CD95 **(d)** and CD54 **(f)** for activated $CD4^+$ T cells in the absence (blue) or presence of IL-27 (red) compared with isotype control (grey) are shown. Percentage of positive cells for $CD4^+$ and $CD8^+$ T cells (n = 9–12) are shown as mean **(b, g)** or median **(e),** and each dot represents one donor, for PD-L1 **(b)**, CD95 **(e)** and CD54 **(g)**. **(c)** Increased percentage of PD-$L1^+$ cells triggered by IL-27 (percentage of PD-L1+ cells in IL-27 treated sample – percentage of PD-$L1^+$ cells in IL-27 untreated sample) for each subset and donors' group shown as mean ± SEM. One-way paired ANOVA followed by Fisher's LSD test **(b, g)** or the Friedman test **(e)** comparing T-cell subsets in the presence vs. absence of IL-27 from each donors' group or one-way unpaired ANOVA **(b, c)** comparing MS vs. HC (P-value above lines) for the same subset and condition. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

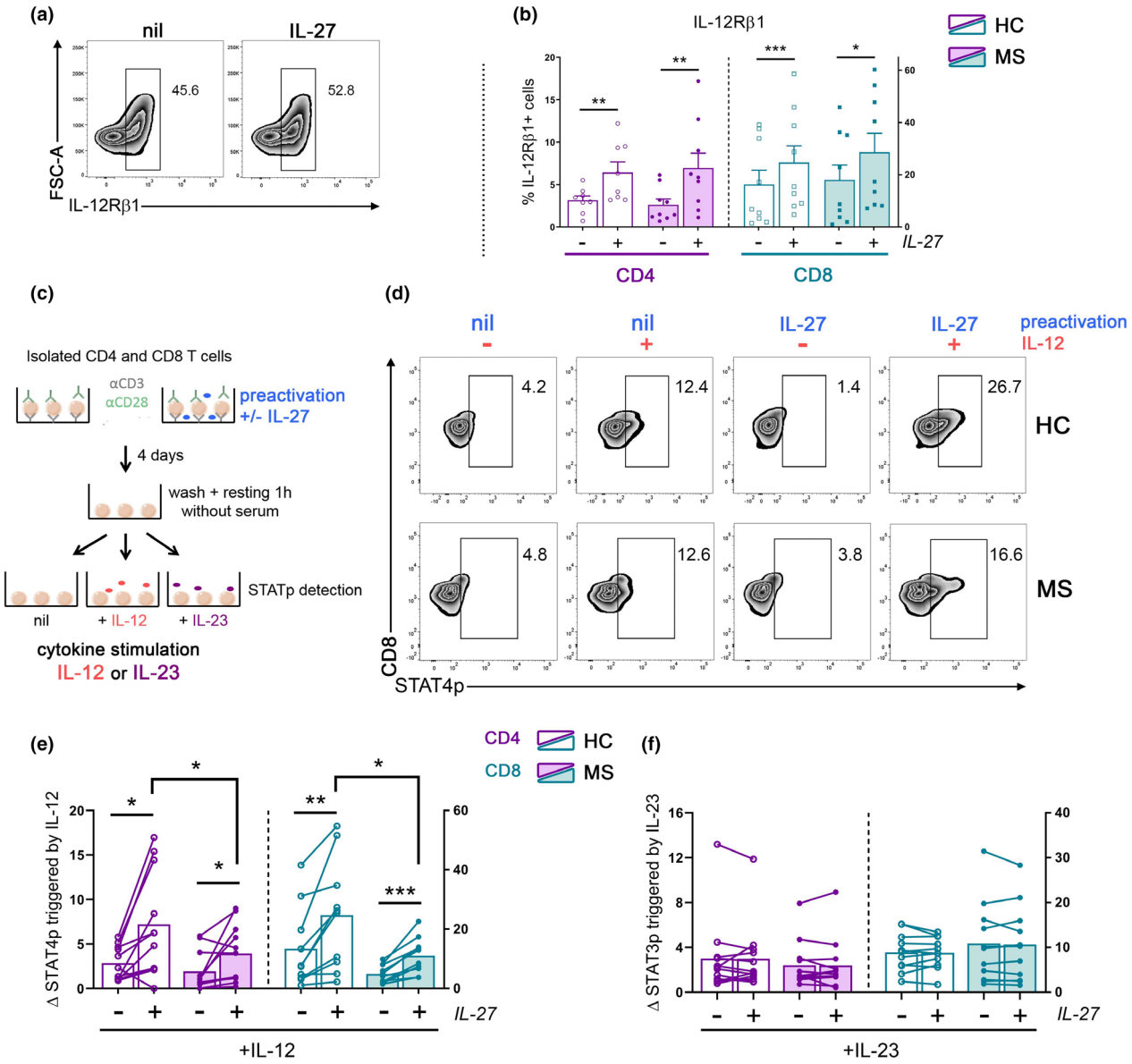

Figure 3. IL-27 pretreated T lymphocytes from MS patients exhibit lower responsiveness to IL-12. Isolated $CD4^+$ (magenta) and $CD8^+$ (aqua) T cells from untreated MS patients and age/sex-matched HC were stimulated during 4 days with αCD3/αCD28 in the absence or presence of IL-27 (100 ng mL^{-1}) and then analysed by flow cytometry for their expression of IL-12Rβ1 **(a, b)** and their response to IL-12 (25 ng mL^{-1}, 30 min) and IL-23 (20 ng mL^{-1}, 15 min) as assessed by phosphorylation of STAT4 and STAT3, respectively **(d–f)**. **(a)** Representative contour plots of $CD8^+$ T cells from one MS patient showing typical surface IL-12Rβ1 expression. **(b)** Proportion of $CD4^+$ and $CD8^+$ T cells expressing IL-12Rβ1 shown as mean ± SEM (n = 8), each dot represents one donor. One-way paired ANOVA comparing T-cell subsets in the presence vs. absence of IL-27 from MS patients and HC. *P < 0.05, **P < 0.01 and ***P < 0.001. **(c)** Isolated $CD4^+$ and $CD8^+$ T lymphocytes cultured with αCD3/αCD28 in the presence or not of IL-27 during 4 days were washed, rested without serum and then exposed to IL-12 or IL-23 for 30 and 15 min, respectively. **(d)** Representative contour plots of STAT4p detection in $CD8^+$ T cells from one HC and one untreated MS patient in response to IL-12 after pre-activation with or without IL-27. **(e, f)** Percentages of $CD4^+$ or $CD8^+$ T cells expressing STAT4p after IL-12 stimulation (n = 10) **(e)** or expressing STAT3p after IL-23 stimulation (n = 9) **(f)**. Results are shown as median, and each dot represents one donor. One-way paired ANOVA comparing T-cell subsets in the presence vs. absence of IL-27 from MS patients and HC; a paired t-test comparing without vs. with IL-27 for the same subset and the donors' group. *P < 0.05, **P < 0.01 and ***P < 0.001.

cytokines by T lymphocytes from MS patients. We activated $CD4^+$ or $CD8^+$ T lymphocytes with αCD3/αCD28 in the presence or not of IL-27 and assessed their cytokine production by flow cytometry and ELISA. As shown by others,[47,48] a greater proportion of $CD4^+$ T cells from MS patients produced IL-17A, GM-CSF or IFN-γ than from the HC in the basal activation conditions in the absence of IL-27 (Figure 4). Moreover, we measured elevated amounts of IL-17 and IFN-γ in supernatants from $CD4^+$ T lymphocytes from MS patients compared with cells from HC (Figure 4a and c). We observed

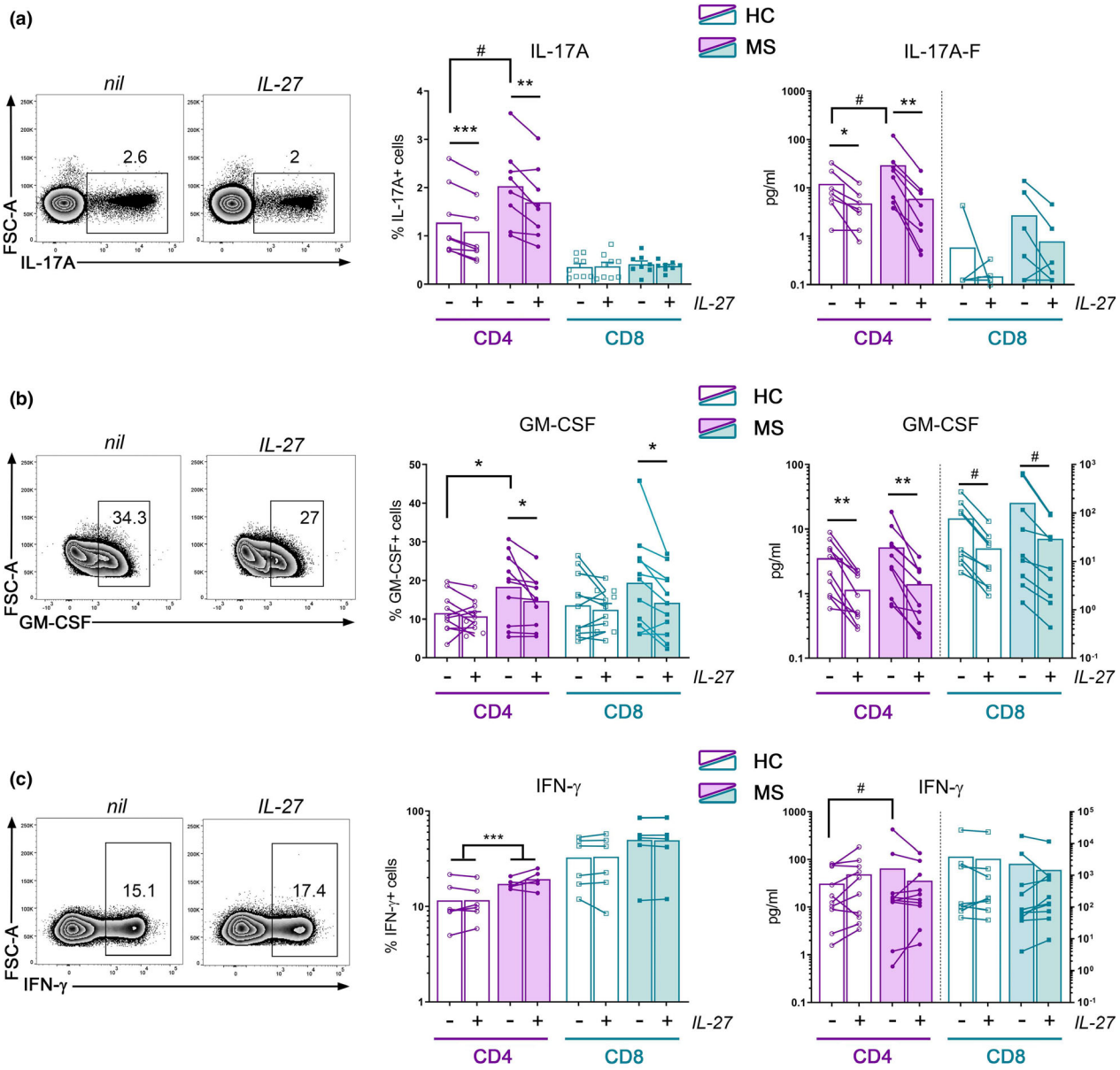

Figure 4. IL-27 decreases the percentages of IL-17- and GM-CSF- producing $CD4^+$ T cells from MS patients. Isolated $CD4^+$ (magenta) and $CD8^+$ (aqua) T cells from MS patients and age/sex-matched HC were stimulated during 4 days with αCD3/αCD28 in the absence or presence of IL-27 (100 ng mL^{-1}). Supernatants were collected for ELISA and cells shortly stimulated for intracellular detection by flow cytometry of IL-17A **(a)**, GM-CSF **(b)** and IFN-γ **(c)**. Contour plots from one MS patient gated on $CD4^+$ T lymphocytes illustrating the detection of each cytokine (left panels), compiled data for the flow cytometry detection of cytokine (middle panels) and compiled data for ELISA measures of cytokine (right panels). Results are shown as mean, each dot represents one donor (n = 6–11). One-way ANOVA comparing T-cell subsets in the presence vs. absence of IL-27 for MS patients or HC or comparing without vs. with IL-27 for the same subset and the donors' group. $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

that addition of IL-27 to activated T lymphocytes from both MS patients and HC significantly decreased the percentages of IL-17A-producing $CD4^+$ T lymphocytes and the secreted amounts of this cytokine by $CD4^+$ T lymphocytes (Figure 4a). The addition of IL-27 to activated $CD4^+$ or $CD8^+$ T lymphocytes significantly reduced the amounts of secreted GM-CSF in both donors' groups and significantly diminished the percentage of $CD4^+$ and $CD8^+$ T lymphocytes from MS patients producing this cytokine (Figure 4b). We did not detect significant impact of IL-27 on the production of IFN-γ (Figure 4c). Overall, our results suggest that IL-27 exhibits a similar capacity in MS patients and HC to decrease the production of both IL-17 and GM-CSF by activated T lymphocytes.

IL-27 induces higher STAT3p response in $CD4^+$ and $CD8^+$ T-cell subsets from MS patients

We and others have previously shown that T lymphocytes from MS patients exhibit enhanced response to cytokines.[49,50] Since we observed that specific effects of IL-27 on $CD4^+$ and $CD8^+$ T lymphocytes (i.e. PD-L1 and IL-12 response) were altered in MS patients compared with HC, we investigated whether such observation could be explained by dysregulation in the upstream IL-27-triggered signalling pathways. We compared the induction of STAT1 and STAT3 phosphorylation, the main IL-27-triggered signalling pathways,[17–19] in T lymphocytes from untreated MS and age/sex-matched HC. *Ex vivo* PBMCs were exposed to IL-27 (10 ng mL^{-1}) for 15 min, corresponding to the peak of response as previously observed,[22,51] and analysed by flow cytometry for the different $CD4^+$ and $CD8^+$ T-cell subsets based on the expression of CCR7, CD45RA and CD45RO: naïve ($CD3^+CCR7^+CD45RA^+CD45RO^-$), central memory (CM: $CD3^+CCR7^+CD45RA^-CD45RO^+$) and effector memory (EM: $CD3^+CCR7^-$) subsets. IL-27 triggered STAT1p in similar proportions of $CD4^+$ and $CD8^+$ T-cell subsets from untreated MS patients and HCs (Figure 5b); average density of STAT1p as assessed by median fluorescence intensity (MFI) was also similar between donors' groups (data not shown). In contrast, we found a significantly elevated percentage of $STAT3p^+$ cells in all $CD4^+$ and $CD8^+$ T-cell subpopulations from untreated MS patients compared with HC (Figure 5c). Moreover, the average density (MFI) of STAT3p in T lymphocytes was increased in MS patients compared with HC (data not shown). The availability of total STAT1 and STAT3 proteins in T-cell subsets was not responsible for such differences as similar or lower levels were detected in MS samples (Supplementary figure 1). Moreover, we tested all combinations of antibodies to detect total and phosphorylated forms to rule out any steric hindrance. Our results establish that IL-27-triggered STAT3 signalling pathway is enhanced in $CD4^+$ and $CD8^+$ T lymphocytes from MS patients, which may explain the altered effects of IL-27 on these immune cells.

IL-27R is differentially expressed on $CD4^+$ T lymphocytes from MS patients

To determine whether the altered impact of IL-27 on T lymphocytes from MS patients compared with HC is influenced by the expression levels of IL-27R, we assessed the expression of both chains gp130 and IL-27Rα on *ex vivo* PBMCs by flow cytometry. We detected lower proportions of naïve, CM and EM $CD4^+$ T cells expressing IL-27R ($gp130^+$IL-27Rα) in MS patients than in the HC group (Figure 6b). In contrast, we did not observe significant differences for $CD8^+$ T-cell subsets between donors' groups (Figure 6b). We also assessed gp130 expression regardless of IL-27Rα chain and confirmed that less memory (CM and EM) $CD4^+$ and $CD8^+$ T lymphocytes carried the gp130 subunit (Figure 6c).[52] The gp130 expression was similar between MS and HC, demonstrating that the differences we observed between MS patients and HC were specific to the IL-27Rα chain (Figure 6d). Overall, our results showed that cell surface IL-27R expression is decreased in $CD4^+$ T lymphocytes from MS patients compared with HC.

Elevated sIL-27Rα serum levels in MS patients

A soluble form of the IL-27Rα (sIL-27Rα) receptor chain has been identified as a natural antagonist of IL-27 in human serum.[53] This soluble form captures IL-27 and prevents its binding to the receptor expressed on cells (Figure 6e). We detected significantly greater levels of the sIL-27Rα in sera from untreated MS patients than in sera from the HC (Figure 6f). These results suggest that in the periphery of MS patients, IL-27 can be impaired from acting on its targets because of the presence of this natural antagonist.

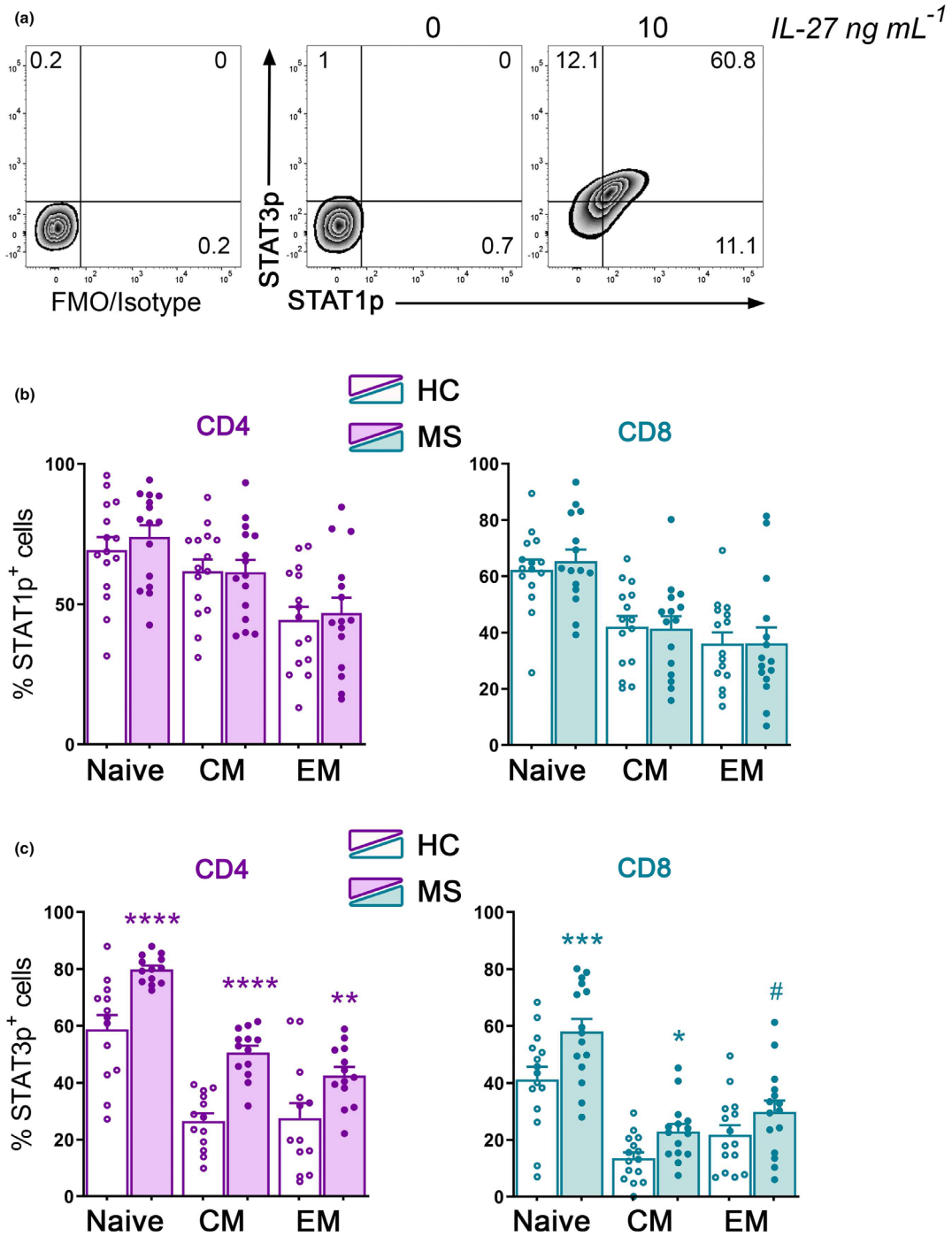

Figure 5. IL-27-signalling pathways are altered in MS patients. $CD4^+$ (magenta) and $CD8^+$ (aqua) T-cell subsets from untreated MS patients were compared with age/sex-matched HC for their capacity to respond to IL-27. *Ex vivo* PBMCs incubated in the absence or presence of IL-27 (10 ng mL^{-1}) were stained for CD3, CD14, CD4, CD8, CD45RA, CD45RO, CCR7, STAT1p and STAT3p and analysed by flow cytometry. **(a)** Representative contour plots illustrating typical STAT1p and STAT3p expression gated on central memory (CM) from one HC either untreated or stimulated with IL-27 for 15 min. Percentage of naïve, CM or effector memory (EM) $CD4^+$ and $CD8^+$ T cells expressing STAT1p **(b)** and STAT3p **(c)** following IL-27 stimulation for 15 min. Each dot represents one donor. Graphs show mean ± SEM (n = 15). One-way ANOVA followed by the uncorrected Fisher's LSD as a *post hoc* test comparing T-cell subsets from MS patients to HC. $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

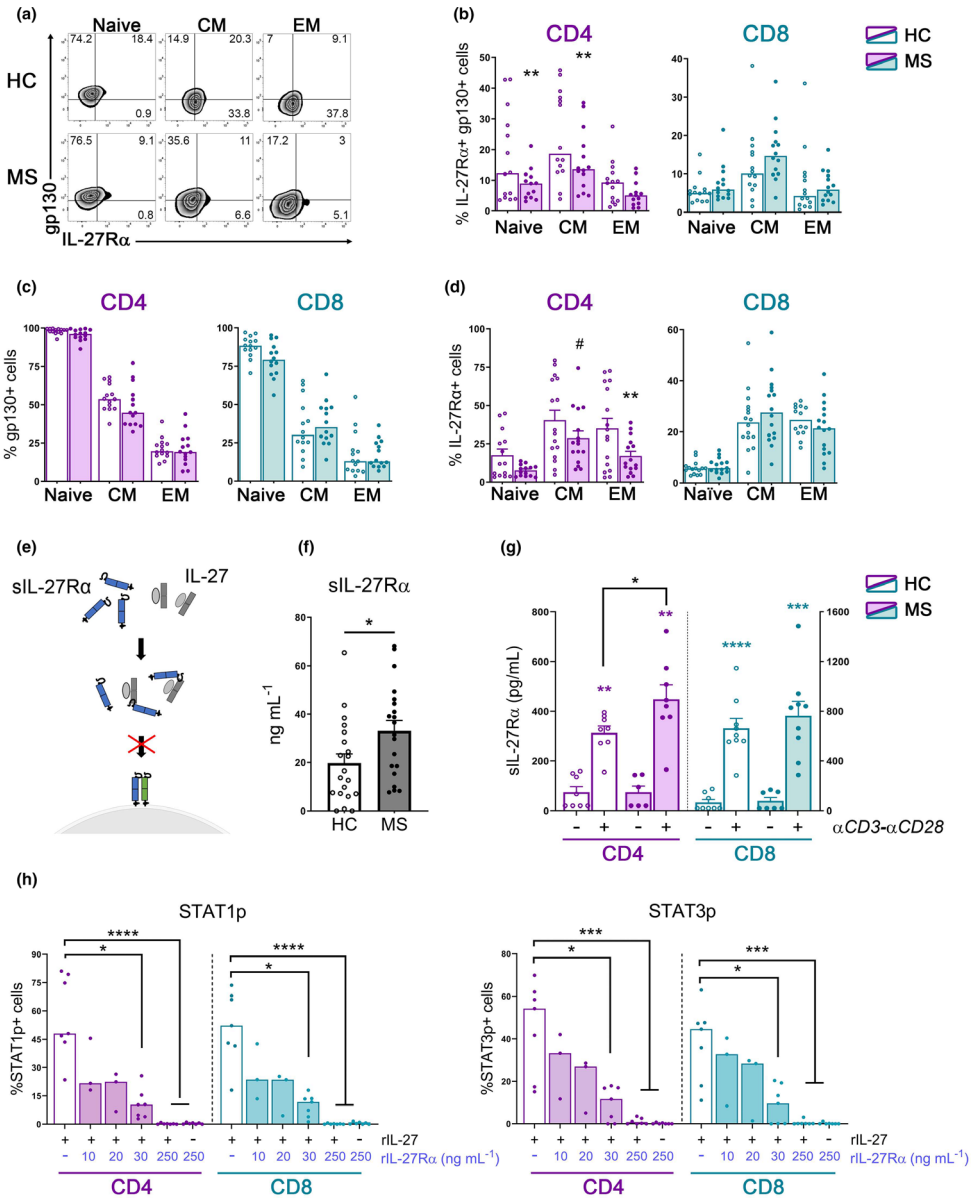

Figure 6. Altered peripheral IL-27R and sIL-27Rα levels in MS patients, and sIL-27Rα shed by CD4 T cells impairs IL-27 signalling. **(a–d)** *Ex vivo* PBMCs from MS patients and age/sex-matched HC were stained for IL-27Rα, gp130 and other surface markers and analysed by flow cytometry. **(a)** Representative contour plots gated on naïve, central memory (CM) and effector memory (EM) $CD4^+$ T cells illustrating IL-27Rα and gp130 expression are depicted. Percentage of $CD4^+$ (magenta) and $CD8^+$ (aqua) T cells expressing both IL-27Rα and gp130 chains **(b)**, gp130 **(c)** or IL-27Rα **(d)**. Each dot represents one donor; results are shown as median (b, c, d (CD8)) or mean ± SEM (d (CD4)) (n = 15). The Kruskal–Wallis test followed by an uncorrected Dunn's test as a *post hoc* test (b) or one-way ANOVA (**d**) comparing CD4 T-cell subsets from MS patients to HC: $^{\#}0.05 < P < 0.1$, $^{**}P < 0.01$. **(e)** The soluble form of the IL-27Rα receptor chain, sIL-27Rα, can capture IL-27 and block its binding to cells expressing the surface IL-27R. **(f)** Serum levels of sIL-27Rα in MS patients and age/sex-matched HC; each dot represents one donor (n = 20/group). Results are shown as mean ± SEM; an unpaired t-test comparing MS vs. HC $^{*}P < 0.05$. **(g)** Isolated $CD4^+$ (magenta) and $CD8^+$ (aqua) T cells from MS patients and age/sex-matched HC were cultured in the absence or presence of αCD3/αCD28 during 6 days and cell culture supernatants assessed for sIL-27Rα (n = 8). Results are shown as mean ± SEM, each dot represents one donor. One-way ANOVA comparing each T-cell subsets with vs. without αCD3/αCD28 and comparing MS patients with HC for the same subset and condition. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. **(h)** Recombinant IL-27 (2 ng mL^{-1}) and IL-27Rα (10, 20, 30 or 250 ng mL^{-1}) were pre-incubated during 30 min and then added to PBMCs for 15 min prior to flow cytometry analysis for STAT1p and STAT3p expression (n = 3–7). Results are shown as median. The Kruskal–Wallis test followed by an uncorrected Dunn's test as a *post hoc* test comparing each T-cell subset for IL-27 alone vs. all other conditions, $^{*}P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

Increased shedding of IL-27Rα in MS patients can impair IL-27 signalling

To identify potential sources of sIL-27Rα, we cultured $CD4^+$ and $CD8^+$ T lymphocytes from MS patients and HC with or without αCD3-αCD28 for 6 days and assessed the release of sIL-27Rα in supernatants. As previously shown,[53] the αCD3/αCD28 activation increased the quantities of sIL-27Rα shed by $CD4^+$ and $CD8^+$ T lymphocytes. Notably, the amounts released by activated $CD4^+$ T lymphocytes from MS patients were significantly higher than those observed for cells from the HC (Figure 6g). Such enhanced release of sIL-27Rα by $CD4^+$ T lymphocytes from MS patients could partially explain the lower proportions of $CD4^+$ T lymphocytes expressing surface IL-27Rα observed in these patients (Figure 6b). $CD8^+$ T lymphocytes from MS patients did not release higher levels of IL-27Rα than those from HC (Figure 6g) nor did they exhibit decreased expression *ex vivo* (Figure 6b).

To assess whether the measured serum levels of sIL-27Rα could have a biological impact, we added sIL-27Rα to similar concentrations (10, 20 and 30 ng mL^{-1}) measured in HC and MS' sera to block the effect of IL-27 on T lymphocytes and used the induction of STAT1 and STAT3 pathways as a read-out in *ex vivo* PBMCs. As expected, addition of recombinant sIL-27Rα alone did not trigger STAT1 and STAT3 phosphorylation on CD4 and CD8 T lymphocytes (Figure 6h), whereas IL-27 alone potently induced STAT1 and STAT3 phosphorylation. Addition of 10 and 20 ng mL^{-1} of sIL-27Rα modestly reduced the proportions of $STAT1p^+$ and $STAT3p^+$ T lymphocytes without reaching statistical significance (Figure 6h), whereas 30 ng mL^{-1} of sIL-27Rα significantly reduced both STAT1 and STAT3 phosphorylation in response to IL-27 in $CD4^+$ and $CD8^+$ T lymphocytes. Notably, the 250 ng mL^{-1} dose, reported to be the highest concentration in serum from Crohn' disease patients,[53] completely abolished the impact of IL-27 (Figure 6h). Overall, our results support the notion that elevated levels of sIL-27Rα in serum of MS patients, originate, at least in part, from the shedding of activated CD4 T lymphocytes and are sufficient to significantly impair the capacity of IL-27 to act on immune cells.

DISCUSSION

Pro- and anti-inflammatory properties have been attributed to IL-27 in several chronic inflammatory and autoimmune diseases.[6,54] Although IL-27 can dampen the severity of the disease in EAE mouse models, the role of IL-27 in the human disease, MS, is still unclear. In this study, we demonstrate that (1) IL-27-mediated effects are altered in $CD4^+$ and $CD8^+$ T lymphocytes from MS patients, (2) IL-27-triggered STAT3 signalling pathway is enhanced in $CD4^+$ and $CD8^+$ T lymphocytes from MS patients, (3) peripheral levels of both IL-27 and sIL-27Rα are elevated in MS patients, and (4) the physiological amounts of sIL-27Rα in sera from MS patients can impair the capacity of IL-27 to act on immune cells.

Elevated levels of IL-27 have been reported in biological fluids from MS patients. The higher amounts of this cytokine we detected in CSF from RRMS patients compared with other neurological diseases (Figure 1a) correlate with the results reported by Lalive and colleagues.[28] We can speculate that astrocytes and macrophages/microglia, which are the primary producers of IL-27 in post-mortem MS brain tissues,[27] represent potential sources of these enhanced levels in the CSF. Moreover, the amounts of IL-27 we detected in sera from untreated RRMS patients (mean 1951 pg mL^{-1}; median 670 pg mL^{-1}, Figure 1a) represent meaningful concentrations as we can detect IL-27-induced STAT1 and STAT3 phosphorylation in human T lymphocytes at both 500 and 1000 pg mL^{-1} doses (Supplementary figure 1e and f). Other groups reported either higher or lower peripheral levels of IL-27 in MS patients than in controls.[18,28,55] Differences could be because of the type of biological fluids analysed (plasma versus serum), the cohort of patients studied, and the sensitivity of each assay and not perfectly matched age/sex cohorts. Several groups reported increased IL-27 serum or mRNA levels in PBMCs from treatment (glatiramer acetate, alemtuzumab, IFN-β) responder MS patients compared with non-responders[29–32] suggesting that increased peripheral IL-27 levels are beneficial.

We subsequently investigated the impact of IL-27 on human T lymphocytes. We selected IL-27 doses for each assay based on our preliminary data, identifying an intermediate dose that induced a robust effect but did not reach the plateau (data not shown). Notably, other groups have used doses of IL-27 ranging from 10 to 200 ng mL^{-1} to treat human cells *in vitro*.[23,56–59] We showed that IL-27 increases the expression of key immune factors implicated in the activation, regulation and/or migration of $CD4^+$ and $CD8^+$ T

lymphocytes such as PD-L1, CD95 (Fas) and CD54 (ICAM-1) (Figure 2). The lower basal levels of PD-L1 we detected on $CD4^+$ T lymphocytes from MS patients than from healthy donors correlate with the decreased mRNA levels reported in PBMCs from MS patients.[60] PD-L1 expression by murine $CD4^+$ and $CD8^+$ T lymphocytes can limit both their response and survival.[61] CD80 (B7-1) has been identified as a PD-L1 binding partner reducing the activation and cytokine production of murine PD-$L1^+$ T lymphocytes.[62] IL-27-treated murine $CD4^+$ T lymphocytes carrying PD-L1 can act in trans on other $CD4^+$ T lymphocytes, block the activation of Th17 cells and prevent the development of EAE.[63] Moreover, PD-L1 blockers have been shown to be more efficient than PD-1 blockers as cancer therapies supporting the contribution of PD-L1's interactions with other binding partners than PD-1.[64] Finally, a proportion of cancer patients treated with immune checkpoint inhibitors such as PD-1 and PD-L1 blockers develop autoimmune disorders supporting the key role of PD-L1 in restricting autoimmunity.[65] Whether the impaired capacity of IL-27 to enhance PD-L1 on MS $CD4^+$ T lymphocytes contributes to favoring autoreactivity will need to be further investigated.

Although IL-27 shapes the polarisation of T cells in MS mouse models[8–12] and that both Th1/Tc1 and Th17/Tc17 subsets play crucial roles in the pathobiology of MS,[2,66–69] the impact of IL-27 on these T-cell subsets in MS patients is not completely resolved. We demonstrated that IL-27 has multiple effects on human $CD4^+$ and $CD8^+$ T lymphocytes such as augmenting the expression of IL-12Rβ1, the responsiveness to IL-12 and decreasing the production of IL-17 and GM-CSF (Figures 3 and 4) on human $CD4^+$ and $CD8^+$ T lymphocytes, similar to what has been shown for mouse T cells.[70] Despite the expected[47,48] greater proportions of $CD4^+$ T lymphocytes from MS patients producing IL-17A, GM-CSF or IFN-γ compared with HC (Figure 4), IL-27 similarly decreased the production of IL-17A and GM-CSF by $CD4^+$ T cells from MS patients and HC (Figure 4). However, IL-27's impact on T lymphocytes from MS patients was significantly less potent than on HC's cells for specific immune functions (IL-12 responsiveness; Figure 3). Others have shown that IL-6 triggers STAT3 phosphorylation in a greater proportion of $CD4^+$ T lymphocytes from untreated RRMS patients than from controls.[49] Moreover, they suggested that an elevated expression of the IL-6-specific receptor chain IL-6Rα, but not the shared gp130 chain, was involved in such augmented STAT3 activation.[49]

We investigated whether the altered impact of IL-27 on MS T lymphocytes could be explained by upstream events. IL-27 can induce STAT1 and STAT3 homodimers and STAT1/STAT3 heterodimers.[71] We observed a striking elevated STAT3 phosphorylation induced by IL-27 in all T-cell subsets from MS patients compared with HC but no difference for STAT1 (Figure 5). Studies performed in mice highlighted a cross-regulation between each STAT protein to achieve specific biological functions.[72,73] The cellular STAT3:STAT1 ratio plays a central role in murine Th17 differentiation downstream of IL-6 family members.[51] The IL-27-mediated upregulation of PD-L1 on $CD4^+$ T lymphocytes has been shown to be STAT1-dependent.[63] Zhang *et al.*[74] demonstrated that STAT3 loss of function and STAT1 gain of function enhanced PD-L1 expression in T lymphocytes. Moreover, the IL-27-induced IL-12Rβ1 upregulation in naïve $CD4^+$ T cells is achieved via the STAT1 pathway.[75] These publications support the notion that the increased STAT3 signalling triggered by IL-27 we detected in T lymphocytes from MS patients compared with HC most likely contributes to the reduced PD-L1 upregulation and response to IL-12, two pathways related to STAT1-STAT3 balance (Figures 2 and 3). We did rule out that the enhanced IL-27-triggered STAT3 response in T cells from MS patients was because of a greater abundance of this signalling protein (Supplementary figure 1) or enhanced IL-27R expression by MS T cells (Figure 6). The mechanisms underlying the elevated IL-27-induced STAT3 phosphorylation in MS T lymphocytes will require additional investigations.

In the current manuscript, we report that IL-27-mediated effects are altered in T lymphocytes from untreated RRMS patients compared with those from HC. We establish that one of the mechanisms contributing to these altered responses is a shift in the balance of STAT1-STAT3 triggered signalling with a bias for an enhanced STAT3 response in MS T lymphocytes. Moreover, we demonstrated that in the peripheral compartment, the beneficial *in vivo* anti-inflammatory effects of IL-27 are most likely impeded by the elevated amounts of sIL-27Rα, a natural antagonist, in MS patient's serum, similar to what has been reported for Crohn's disease patients.[53] There are no data available on the presence of sIL-27Rα in mouse models. Despite

promising results from mouse models, supporting IL-27 as novel therapeutic tool to block MS disease,[5,7–13] our work underscores that additional investigations are deemed essential to thoroughly characterise the IL-27/IL-27R axis in MS patients prior to attempt therapeutic interventions.

METHODS

Human participants

A written informed consent was obtained from MS patients and healthy controls (HC) in accordance with the local ethical committee, and studies were approved by the Centre Hospitalier de l'Université de Montréal ethical boards (BH 07.001 and HD 07.002). Patients with clinically relapse–remitting disease MS (RRMS) were recruited from the CHUM MS-Clinic. Each patient was diagnosed according to the revised 2017 McDonald criteria[76] and classified at the time of blood collection by a highly trained MS neurologist. Eighty-nine RRMS patients (31 men and 58 women) were enrolled in the study. As a result of the limited blood sample size, not all patients were analysed in all assays; number of patients for each assay is indicated in figure legends. MS patients had a mean age of 41 years (min 20 years, max 64 years), mean EDSS 1.5 (min 0, max 7) and mean disease duration of 10.8 years (min less than 1 year, max 40 years). HCs were age- and sex-matched with RRMS patients for each set of experiments. Patients undergoing lumbar puncture for diagnostic purpose and who were confirmed as non-MS patients were included in the other neurological disease (OND) group (examples of diagnosis include migraine, chronic fatigue, prolactinoma, ischaemic optic neuropathy, hypertensive leuco-encephalopathy, normal pressure hydrocephalus), were used as control for CSF assay and sex-matched with RRMS patients (17 women and 9 men).

Serum, cerebrospinal fluid and ELISA

Blood was collected in silica-coated tubes (Greiner bio-one, Monroe, NC, USA) and clotted for 30 min before centrifugation at 1800 *g* for 10 min, and then, sera were aliquoted and stored at −80°C until needed. Cerebrospinal fluid (CSF) samples were centrifuged at 420 *g* for 10 min, and supernatants were aliquoted and stored at −80°C until used. Sera and CSF were assessed for the levels of IL-27 using DuoSet ELISA Human IL-27 commercial kit (R&D Systems distributed by Cedarlane, Oakville, ON, Canada). Soluble IL-27Rα levels were measured as previously published.[53] IL-17A-F, GM-CSF and IFN-γ levels were assessed on culture supernatants from isolated $CD4^+$ and $CD8^+$ T cells using U-Plex immunoassay customised kit according to the manufacturer's instructions (Meso Scale Discovery, Rockville MD, USA).

Peripheral immune cell isolation and *in vitro* assays

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected in EDTA-coated tubes (BD Biosciences, Mississauga, ON, Canada) using Ficoll density gradient as previously described.[22,50,77] To determine IL-27-induced STAT signalling, PBMCs were incubated at 37°C in Iscove's Modified Dulbecco's Medium (Life Technologies Thermo Fisher Scientific, Burlington, ON, Canada) without serum for 1 h, then exposed to human recombinant IL-27 (R&D Systems) for 15 min and finally quickly put on ice for flow cytometry analysis as previously detailed.[22] Basal levels of STAT1 and STAT3 phosphorylation in CD4 and CD8 T cells from MS patients and HC were subtracted from levels after IL-27 stimulation.

To assess the impact of IL-27 on cytokine production, response to other cytokines (IL-12, IL-23) or surface marker expression by T cells, $CD4^+$ and $CD8^+$ T-cell subsets was isolated from PBMCs using anti-human CD4 or CD8 beads, respectively, according to manufacturer's protocol (Miltenyi Biotec, San Diego, CA, USA). Cell purity of each subset assessed by FACS was > 90%. $CD4^+$ and $CD8^+$ T cells were put in culture for 4 days in Iscove's Modified Dulbecco's Medium supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), sodium pyruvate (1 mM), non-essential amino acids (1× solution) and β-mercaptoethanol (50 μM). Cells were activated with plate-bound suboptimal doses of α-CD3 (clone OKT3, precoated for 24 h at 4°C, 0.6 μg mL^{-1} for $CD4^+$, 1.8 μg mL^{-1} for $CD8^+$) and soluble α-CD28 (0.6 μg mL^{-1}) (BD Biosciences) in the presence or absence of recombinant human IL-27 (100 ng mL^{-1}) (R&D Systems). To assess IL-12 and IL-23-induced STAT signalling, these αCD3/αCD28 pre-activated $CD4^+$ and $CD8^+$ T cells in the presence or not of IL-27 were harvested, incubated without serum for 1 h, subsequently exposed to human recombinant IL-12 (Invitrogen, Thermo Fisher Scientific, Burlington ON, Canada) for 30 min or IL-23 (R&D Systems) for 15 min and then quickly put on ice prior to flow cytometry analysis of STAT3 and STAT4.[22] To assess cytokine production, pre-activated $CD4^+$ and $CD8^+$ T cells were stimulated as previously described in the presence of phorbol 12-myristate 13-acetate (20 ng mL^{-1}) (Sigma-Aldrich, Oakville, ON, Canada), ionomycin (500 ng mL^{-1}) (Sigma-Aldrich), brefeldin A (5 μg mL^{-1}) (Sigma-Aldrich) and monensin (1 μM) (Sigma-Aldrich) for 5 h before flow cytometry analysis for cytokines.

Microarray

Naïve ($CD45RA^+CD45RO^-CCR7^+$) and central memory ($CD45RA^-CD45RO^+CCR7^+$) $CD4^+$ and $CD8^+$ T cells from three healthy donors and three untreated RRMS patients were first sorted on a BD FACSAria III (BD Biosciences, San Jose, CA, USA) and then stimulated for 3 days with immobilised αCD3 (naïve: 1.6 μg mL^{-1}, central memory: 0.32 μg mL^{-1}) and soluble αCD28 (1 μg mL^{-1}) in the presence or not of IL-27 (10 ng mL^{-1}). RNA samples were purified using RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen, Toronto, ON Canada). RNA quality was checked on RNA Nano chips using a 2100 bioanalyser (Agilent Technologies, Mississauga, ON, Canada). All samples had a RNA integrity number (RINs) > 8.3. Transcriptome-wide analysis of gene expression was performed by Genome Québec using the Clariom S HT array profiling more than 20 000 well-annotated genes. Differential expression

Table 2. List of antibodies used for flow cytometry

Targeted human antigen-fluorochrome	Clone	Source
CD3-Alexa Fluor® 700	UCHT1	BD Biosciences
CD4-BV786	SK23	BD Biosciences
CD8-Pacific Blue	RPA-T8	BD Biosciences
CD8a-Super Bright 702	RPA-T8	Invitrogen
CD14-APC-H7	M5E2	BD Biosciences
CD45RA-FITC	HI100	BD Biosciences
CD45RO-PerCP-Cy™5.5	UCHL1	BD Biosciences or Biolegend
CCR7-PE-Cy™7	G043H7	Biolegend
gp130-PE	28126	R&D
IL-27Rα	Polyclonal rabbit	LifeSpan Biosciences
PD-L1-biotin	MIH1	Invitrogen
IL-12Rβ1-APC	2.4E6	BD Biosciences
IL-17A-eFluor 660	64CAP17	eBioscience
IFN-γ-Alexa Fluor® 700	B27	BD Biosciences
GM-CSF-biotin	BVD2-21C11	BD Biosciences
STAT1p-PE	4a	BD Biosciences
STAT3p-Alexa Fluor® 647	4/p-STAT3	BD Biosciences
STAT4p-PE	38/p-STAT4	BD Biosciences
Total STAT1-Alexa Fluor® 647	1/STAT1	BD Biosciences
Total STAT3-PE	M5950	BD Biosciences
F(ab')2-Goat anti-Rabbit-Alexa Fluor® 647		Thermo Fisher Scientific
Streptavidin-BV605		BD Biosciences

analysis was done using the statistical software R (The R Foundation for Statistical Computing, c/o Institute for Statistics and Mathematics Vienna, Austria). Intensity files were processed using the oligo v1.19.0 and normalised with the RMA method. Differential expression was then assessed with the limma package v3.41.6 (Bioconductor open source software for bioinformatics). Genes were identified as significantly differentially expressed when the adjusted *P*-value ≤ 0.05 and the absolute fold change > 1.3. Microarray data have been deposited in the NCBI-GEO repository with the accession number GSE145334 (private access token for reviewers: gxglsmmyhxkbfeh). GSEA was performed with the fgsea package. The gene sets used can be found on the MSigDB website: http://www.gsea-msigdb.org/gsea/msigdb/index.jsp. Genes were ranked according to their *t*-statistic.

Flow cytometry

PBMCs and isolated T cells were stained for surface markers and/or intracellular cytokines as previously described.[22,50,77] Briefly, cells were blocked with normal mouse immunoglobulins (mIgG) (6 μg mL^{-1} per million of cells) (Invitrogen) during 15 min at 4°C prior to be incubated with fluorochrome-labelled antibodies targeting surface antigen (see list of antibodies in Table 2) for 30 min at 4°C. LIVE/DEAD Fixable Aqua Dead Cell Stain (Molecular Probes™, Thermo Fisher Scientific) was added together with the surface antibodies to exclude dead cells. To assess phosphorylation of STATs, PBMCs were first surface-stained, then fixed with 1.5% (w/v) paraformaldehyde and permeabilised during 25 min on ice with Perm Buffer III (BD Biosciences) according to the manufacturer's instructions prior to STAT labelling. Cells were acquired on a BD LSRII or a Fortessa flow cytometer (BD Biosciences) and analysed using FlowJo software (Treestar, Ashland, OR, USA). To ensure stringent single-cell gating, doublets were excluded using SSC and FSC height and width as recommended by the Flow Cytometry Network (www.thefcn.org); single events were first gated on the SSC-H vs. SSC-W and then on the FSC-H vs. FSC-W dot plots. Appropriate isotype controls were used in all steps. Staining specificity was confirmed using fluorescence minus one (all antibodies minus one). The change in median fluorescence intensity (MFI) was calculated by subtracting the fluorescence of the isotype from that of the stain.

Statistics

Data analysis was performed using the Prism 9.0 software (GraphPad, La Jolla, CA, USA). When data passed the D'Agostino & Pearson omnibus normality test, an unpaired or paired *t*-test or one-way ANOVA followed by Fisher's LSD test was performed. When data did not pass the normality test, the Mann–Whitney test, Friedman test or Kruskal–Wallis test was used. Values were considered statistically significant when probability (*P*) values were as follows: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) or $P \leq 0.0001$ (****). *P*-values between 0.05 and 0.1 are marked as #.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Marie-Laure Clénet: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. **Cyril Laurent:** Formal analysis; Investigation; Methodology. **Florent Lemaitre:** Data curation; Methodology. **Negar Farzam-kia:** Data curation; Methodology. **Olivier Tastet:** Formal analysis; Methodology. **Odile Devergne:** Methodology; Writing-review & editing. **Boaz Lahav:** Formal analysis; Investigation; Methodology. **Marc Girard:** Resources. **Pierre Duquette:** Resources. **Alexandre Prat:** Resources. **Catherine Larochelle:** Resources; Writing-review & editing. **Nathalie Arbour:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing-original draft; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.