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Title PAF-R on activated T cells: role in the IL-23/Th17 pathway and relevance to multiple sclerosis

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Abstract

IL-23 is a potent stimulus for Th17 cells. These cells have a distinct developmental pathway from Th1 cells induced by IL-12 and are implicated in autoimmune and inflammatory disorders including multiple sclerosis (MS). TGF-beta, IL-6, and IL-1, the transcriptional regulator ROR-gamma-t (RORC) and IL-23 are implicated in Th17 development and maintenance. In human polyclonally activated T cells, IL-23 enhances IL-17 production. The aims of our study were: 1). To validate microarray results showing preferential expression of platelet activating factor receptor (PAF-R) on IL-23 stimulated activated T cells. 2). To determine whether PAF-R on activated T cells is functional, whether it is co-regulated with Th17-associated molecules, and whether it is implicated in Th17 function. 3). To determine PAF-R expression in MS. We show that PAF-R is expressed on activated T cells, and is inducible by IL-23 and IL-17, which in turn are induced by PAF binding to PAF-R. PAF-R is co-expressed with IL-17 and regulated similarly with Th17 markers IL-17A, IL-17F, IL-22 and RORC. PAF-R is upregulated on PBMC and T cells of MS patients, and levels correlate with IL-17 and with MS disability scores. Our results show that PAF-R on T cells is associated with the Th17 phenotype and function. Clinical Implications Targeting PAF-R may interfere with Th17 function and offer therapeutic intervention in Th17-associated conditions, including MS.

Keywords Platelet activating factor/receptor; interleukin-17; T-helper 17; T cells; multiple sclerosis

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Data will be made available on request

15 July 2020

To Prof Wilhelm Schwaeble

Editor in Chief, Immunobiology

On behalf of all the authors I hereby submit the revised paper, “PAF-R on activated T cells: role in the IL-23/Th17 pathway and relevance to multiple sclerosis” for consideration of publication in Immunobiology.

The revised version takes into account all the reviewers’ very helpful suggestions, which have improved the paper. The responses to reviewers are provided in a separate file.

Please address any correspondence related to the manuscript to me at the address given.

Many thanks for an excellent editorial and review process.

We look forward to hearing from you and thank you in advance.

Best wishes

Cris S Constantinescu

Professor of Neurology

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Journal: Immunobiology

Dear Professor Schwaeble,

Thank you very much for the editorial comments and to the reviewers for the careful and constructive review of our paper.

I am submitting the revised version, modified according to the helpful comments which I believe have improved the paper.

I am addressing the reviewers' comments point by point below.

Comments from the editors and reviewers:
-Reviewer 1

The study by Angela Midgley et al.shows evidences of a new pathway associated with Th17 cells. Various in vitro experiments show the mechanistic relationships between PAF and Th17. Moreover, they found a link between T cells from multiple sclerosis patients and the upregulation of this pathway.Although, the association between PAF and Th17 has been reported in two previous papers, they found a link with MS pathogenesis, which is an interesting and valuable finding.

The reviewer suggests to include the following information.

1. MS patient demography including type (RR, SP, PP),disease duration (if possible duration from the transition to SPMS), treatment.

Response: Thank you for this important suggestion. We have included Supplementary Table 2 with the demographic and clinical information on the patients.

2. Th17 cells are reported (for example, by the following review) to be heterogeneous with regard to pathonenecity, cytokine productions etc.

Bystrom, J., Clanchy, F. I., Taher, T. E., Al-Bogami, M., Ong, V. H., Abraham, D. J., ... & Mageed, R. A. (2019). Functional and phenotypic heterogeneity of Th17 cells in health and disease. European Journal of Clinical Investigation, 49(1), e13032.

The readers would want to know about where do Th17 cells with activated PAF-PAFR pathway positioned in the heterogeneous Th17 cells.

Response: Thank you for the valuable comment. We have included a discussion about the potential groups of Th17 cells the PAF-R+ T cells may belong to, and which they probably do not (Th1-17 and Th22). We also included additional references including that by Bystrom et al. above

-Reviewer 2

-

This is a very interesting paper addressing the expression of platelet activating factor (PAF) receptor on activated T cells, its co-expression with IL-17 and other associated Th17 markers, and its upregulation on PBMC and T cells of MS patients.
COMMENTS TO THE AUTHORS:

1) In the header (top right) of the manuscript a different surname appears which is not included in the list of authors. Please clarify and/or assign the correct first author credentials.

Response: Thank you for the observation. The first author's name has changed but it is the same person. The correct surname is Midgley.

2) The aims of the study are well stated in the sections of Abstract and Introduction, however it would be nice if the authors could also add a small paragraph in the beginning of Discussion addressing whether these aims were adequately reached

Response: Thank you for the good suggestion. The discussion has now been modified to comment on how the aims were achieved in this study. It also offered the opportunity to comment on some limitations of the study and thus address some of the further comments of the reviewer (e.g. the lack of flow cytometry data from MS patients).

3) All demographic and clinical details all the MS patients included in the study should be summarized in a supplementary table alongside with EDSS scores and other relevant values

Response: This is a very important suggestion. We have now included the information in the Supplementary Table 2.

4) In line 117 the authors state that PBMCs were collected and CD3+ cells were separated from a separate group of 8 patients, their details if they represent a different group, should also be provided in the table
5)

Response: The details of the second, smaller group are also given in Supplementary Table 2.

6) In line 120 the abbreviated (PBD) should be also written in full. Conversely, in line 233 only the abbreviated form should be provided. The authors can also offer a brief comment on the reason behind PBD/Ionomycin option instead of PMA/Ionomycin

Response: Thank you for the observation. The abbreviation is now explained when used the first time, and subsequently only the abbreviated form is used. One sentence mentioning PDB which had been left in error as it was referring to data not shown was removed. In our laboratory we found no major differences between the various phorbol esters. Our flow cytometry colleagues prefer PDB rather than PMA for flow cytometry experiments in particular those involving T cells, and prefer PMA for monocyte/macrophages; but I am not aware of strong scientific data to support these preferences.

7) In lines 125-128 the precise panels of stimulations for each experimental design should be provided alongside with the concentrations used, as written in figure legends. This will facilitate the reader to better understand each treatment and the rationale behind this (ie to induce Th17 cells etc)

Response: This information is provided more clearly now.

8) In line 129 a reference should be given for PFA-R antagonist CV3988

Response: The reference is provided

9) In line 138 the authors state that brefeldin A was added 2 hours following cytokine stimulation, and after 24h cells were fixed in 2% PFA. If I understand it correctly, the cells were left in brefeldin A for 22 hours. Brefeldin A is a toxic addition and rather not be left in cultured cells for more than 6-7 hours. The authors should provide a comment on whether they maintened high cellular viability in these specific assays.

Response: Thank you for the observation. This was likely an editing error. The stimulation was for 24 hours and the brefeldin was added for maximum 6 hours. The viability was checked routinely.

10) In line 147 representative illustrations of these preliminary data can be included as a supplementary figure, or an additional small panel on top of the flow cytometry plots (Figure 2, Panel E?). This is important for the reader in order to fully grasp the specificity and validation of the two step intracellular staining flow cytometric assay for PAB-R.

Response: Thank you for the suggestion; this would be ideal; however these figures are not available and the raw data related to the experiments is not feasible to retrieve, as the flow cytometer facility has been relocated and the flow cytometry expert and the first author have both left the University.

11) In line 274 the authors state that they used WEB2016 PAF-R antagonist. A reference should be provided and details of it should also be mentioned in methods section similarly to CV3988. Why the authors opted for this one in addition to CV3988?

Response: We provide a reference for the PAF-R antagonist. We performed some experiments in collaboration with the platelet laboratory, where WEB2086 is preferentially used and routinely available. We corrected the error (it is WEB2086 not 2016), and provide a reference showing that they have similar effects.

12) Figure numbers are not shown in the illustrations at the end of the manuscript. In some illustrations (for instance some flow cytometric plots) the letters indicating the order of the panels are missing alongside with key depictions in the flow cytometric plot axes (ie when the secondary isotype control is shown it should be labelled as human IgG-PE rather than PE or FITC only). In the same figure together with the flow cytometric plots showing IFNγ and IL-17 production, a positive control should also be provided showing high IFNγ and IL-17 production. This will facilitate evaluation

regarding the specificity and efficient detection of the flow cytometric staining.

Response: Thank you for the comments. We have numbered the figures and made the changes suggested. We acknowledge the lack of positive controls in this experiment as a limitation of the study.

13) The manuscript is lacking flow cytometric experiments and evaluation of PAF-R and IFNγ, IL-17 within PBMCs or separated cells from MS patients. I don`t fully grasp the reason why only RNA was extracted and measured from the PBMCs of MS patients.

Response: Thank you for the comments. We acknowledge the lack of flow cytometry data in MS as a limitation of the study. We set off the study to validate the microarrays in normal cells as we were intrigued to find PAF-R on T cells. As we showed its association with Th17 we thought it would be important to look at MS (previous microarrays found it up-regulated in MS brain), and used a convenience sample to test it in RNA from PBMC.

13) The authors state in line 67 that they identified platelet activating factor receptor (PAF-R), to be significantly upregulated by IL-23 but down-regulated by IL-12. In the supplementary table it is not entirely clear which are the 205 genes up-regulated by IL-23 and down-regulated by 8-fold, and which are the 126 genes showing the opposite regulation. Perhaps a different illustration (shaded boxes) or a line showing the separation would be extremely helpful. The indexes with values/ratios according to different stimuli are difficult to follow. In addition, platelet activation factor receptor (PAF-R) appears twice in the given table one as highlighted and one time exactly 24 rows below with other values and ratio indexes given. The authors should clarify on these aspects and also comment on the last entry of the table which is IFNγ and why it appears with similar index ratios to PAF-R. Are they similarly expressed?

Response: We apologise for submitting the table in error in the original submission. We are submitting the correct and verified table. The array used had two clones for some of the genes, and PAF-R was one of these. It shows some degree of internal consistency as the values go in the same directions, and have now been validated in this paper by quantitative PCR.

We have revised the legend of the table. We explain that the numbers to look for are in the last column, and have now colour-coded the genes up-regulated preferentially by either IL-23 (positive numbers) or IL-12 (negative numbers). In the text of the paper, we comment on IFN-gamma as indicating the plausibility of the arrays as it is significantly induced by IL-12. We also include a previous paper where we validated another gene induced by IL-12 in the same array results.

As the Wistar Institute has now changed the genomic platforms and the bioinfomatician on this study has left Wistar, we have decided against a separate publication of the microarray

data to Data in Brief. In addition, none of the methods are completely novel, and therefore we did not think that publishing them in MethodsX would bring novelty.
We thank the reviewers and you, the Editor, for the helpful and thorough review of the paper, and believe they have helped to considerably improve its quality.

We hope it is now suitable for publication in Immunobiology

I would be pleased to answer any questions or clarify any issues related to this paper.

With best wishes

Sincerely yours,

Cris S Constantinescu MD PhD FRCP

PAF-R on activated T cells: role in the IL-23/Th17 pathway and relevance to multiple sclerosis

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16 **Abstract**

17 IL-23 is a potent stimulus for Th17 cells. These cells have a distinct developmental
18 pathway from Th1 cells induced by IL-12 and are implicated in autoimmune and
19 inflammatory disorders including multiple sclerosis (MS). TGF-β, IL-6, and IL-1, the
20 transcriptional regulator RORγt (RORC) and IL-23 are implicated in Th17
21 development and maintenance. In human polyclonally activated T cells, IL-23
22 enhances IL-17 production.
23 The aims of our study were: 1). To validate microarray results showing preferential
24 expression of platelet activating factor receptor (PAF-R) on IL-23 stimulated T cells.
25 2). To determine whether PAF-R on activated T cells is functional, whether it is co-
26 regulated with Th17-associated molecules, and whether it is implicated in Th17
27 function. 3). To determine PAF-R expression in MS.
28 We show that PAF-R is expressed on activated T cells, and is inducible by IL-23 and
29 IL-17, which in turn are induced by PAF binding to PAF-R. PAF-R is co-expressed
30 with IL-17 and regulated similarly with Th17 markers IL-17A, IL-17F, IL-22 and
31 RORC. PAF-R is upregulated on PBMC and T cells of MS patients, and levels
32 correlate with IL-17 and with MS disability scores. Our results show that PAF-R on
33 T cells is associated with the Th17 phenotype and function.
34 **Clinical Implications** Targeting PAF-R may interfere with Th17 function and offer
35 therapeutic intervention in Th17-associated conditions, including MS.

36 **Abbreviations:** PAF, platelet activating factor, PAF-R, platelet activating factor
37 receptor, ROR, retinoic orphan receptor,

38

39 ## 1. Introduction

40 The role of Th1 cells, which produce interferon (IFN)-γ, Th17 cells (which produce
41 IL-17), and Th1-17 cells (which produce both IFN-γ and IL-17 and are likely
42 pathogenic) in multiple sclerosis (MS) in particular has been amply studied
43 (Constantinescu and Gran, 2014); (Edwards, et al., 2010). Several immunotherapeutic
44 agents for MS have been reported to reduce the levels of these cytokines (Balasa, et
45 al., 2017) (Montes Diaz, et al., 2018).

46 IL-12 and IL-23 are related proinflammatory cytokines involved in the development
47 and maintenance of Th1 cells and Th17 cells, respectively. IL-12 and IL-23 share the
48 p40 subunit which is covalently linked with a unique p35 or p19 subunit, respectively
49 (Teng, et al., 2015).

50 Although TGF-β, in a proinflammatory environment characterised by the presence of
51 IL-6, and possibly IL-1, along with the master regulatory transcription factor, retinoic
52 orphan receptor-γt (ROR-γt), or its human orthologue RORC, are required for Th17
53 development and differentiation from naïve T cells, the presence of IL-23 receptor is
54 required for Th17 terminal differentiation and effector functions (Bettelli, et al., 2006)
55 (O'Garra, et al., 2008).

56 IL-23 stimulates the production of large amounts of IL-17 by supporting and
57 expanding Th17 cells, a phenomenon observed both in the murine and the human
58 immune system (Gaffen, et al., 2014). In turn, IL-17 induces a wide range of
59 cytokines, chemokines and metalloproteinases, which contribute to inflammation and
60 tissue destruction (McGeachy, et al., 2019).

61 The ability of IL-23 to induce IL-17 production in human polyclonally activated T
62 cells and additional differential effects of IL-23 and IL-12 have been demonstrated
63 (Hoeve, et al., 2006). In this study, we investigated, using custom human

64 microarrays, transcripts differentially induced by IL-12 and IL-23 in polyclonally
65 activated human T cells (phytohemagglutinin-induced T cell blasts) These studies
66 identified 205 genes significantly (over 8-fold relative to IL-12) up-regulated by IL-
67 23 and 126 up-regulated over 8-fold relative to IL-23 by IL-12 (supplementary table
68 1). We identified platelet activating factor receptor (PAF-R), a G-protein coupled 7-
69 transmembrane domain receptor, to be significantly upregulated by IL-23 but down-
70 regulated by IL-12. The enzyme, cytosolic phospholipase A2α, which catalyses the
71 synthesis of three inflammatory mediators, including leukotrienes, prostaglandins and
72 PAF, was also upregulated by IL-23 and downregulated by IL-12, suggesting that the
73 PAF pathway is differentially regulated by these cytokines. We subsequently
74 confirmed these findings in activated, purified T cell populations. Platelet activating
75 factor (PAF), the ligand for PAF-R is a potent phospholipid inflammatory mediator
76 that is associated with diverse effects on a variety of cells (Kihara, 2019) (Honda, et
77 al., 2002).
78 PAF plays an important role in asthma and anaphylaxis, but also is elevated in the
79 peripheral blood and cerebrospinal fluid of patients with MS (Callea, et al., 1999), in
80 other human autoimmune diseases (Edwards and Constantinescu, 2009), and in the
81 central nervous system of mice with experimental autoimmune encephalomyelitis
82 (EAE) (Lock, et al., 2002), the most widely used model of MS (Constantinescu, et al.,
83 2011). PAF-R was also among the genes associated with allergies that were
84 discovered by microarray analysis in brains of patients with MS, suggesting
85 PAF/PAF-R may contribute to the MS pathology (Lock, et al., 2002) (Pedotti, et al.,
86 2003). This possibility was subsequently validated by the same group of investigators
87 in EAE, where a PAF-R antagonist suppressed disease (Pedotti, et al., 2003);. This
88 approach was successful in other EAE models as well (El Behi, et al., 2007); (Howat,

89 et al., 1989). Moreover, cPLA2a deficient mice are resistant to EAE and PAF-R
90 deficient mice develop less severe disease (Marusic, et al., 2005, Marusic, et al.,
91 2008). In addition, PAF has been involved in neurodegeneration in EAE and PAF-R
92 blockade is neuroprotective (Bellizzi, et al., 2016).
93 Our microarray findings that PAF-R is expressed on activated T cells (which until
94 now was not clearly known) and induced by IL-23 implicates PAF-R in the Th17
95 response and Th17 cell phenotype. This is also supported by recent findings showing
96 a role for PAF-R in Th17 responses.
97 Here, we provide evidence to validate our microarray results and confirm that PAF-R
98 is upregulated by IL-23 in part through IL-17, and show PAF-R to be a potential
99 marker of Th17 cells and the PAF-PAF-R pathway to be potentially important for the
100 development or maintenance of this pathogenic T cell population.
101

102 ## 2. Materials and Methods

103 ### 2.1. Cell preparation

104 Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by
105 gradient centrifugation with Histopaque 1077 (Sigma-Aldrich Dorset UK), prepared
106 at $1x10^6$ cells/ml in RPMI 1640 with 2mM glutamine, 20mM Hepes, 0.1mg/ml
107 penicillin and streptomycin and 10% fetal calf serum and cultured with 10µg/ml PHA
108 at 37°C and 5% CO_2 for 72 hours, then stimulated with 100U/ml IL-2 for 24h and
109 then rested 24h in serum free media.
110 CD3+, CD4+ and CD8+ T cell populations were separated immunomagnetically
111 using EasySep® (StemCell Technologies, UK).
112 PBMC were obtained as above from MS patients (20 females, 10 males, mean ±SD
113 age, 43.8 ± 8.5) and matched controls (11 females, 9 males; mean ±SD age, 42.1. ±

114 7.8). Ethical approval was obtained from the Nottingham Research Ethics Committee
115 2 (NS090102). Subjects gave informed consent. MS was scored clinically using the
116 Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). PAF-R and IL-17 mRNA
117 were measured by qRT-PCR (see below). In a separate group of 8 patients (6
118 females, 2 males, mean ±SD age, 43.1± 8.1) and 7 controls (4 females, 3 males, mean
119 age 38 years), CD3+ T cells were separated and PAF-R was measured in PBMC and
120 T cells. The proportion of PAF-R RNA from T cells was consistently 50-60%.
121
122 **2.2. Cell stimulation**
123 Human PHA/IL-2 T cell blasts or CD4+ cells stimulated with anti CD3/CD28, both at
124 $1x10^6$cells/ml, were either left untreated or treated as follows: incubated with IL-12
125 (100ng/ml); IL-23 (10ng/ml); IL-12 (100ng/ml) & anti-IFN-γ (2.5ng/ml); IL-23 &
126 anti-IL-17 (10 ng/ml); IFN-γ (2.5ng/ml); or IL-17 (0.5ng/ml) for 24h at 37°C and
127 5%CO_2. Rabbit and goat IgG were used as controls for anti-IFN-γ and anti-IL-17 ,
128 respectively.
129 In other experiments T cells were also incubated with TGF-β (50ng/ml) & IL-6
130 (20ng/ml), or TGF-β & IL-6 & IL-23 for 24h. The method was refined so optimum
131 cytokine concentrations were used. PAF-R was inhibited using antagonist CV3988
132 (Biomol) (10μM/ml) (Terashita, et al., 1983). In other experiments, the PAF-R
133 antagonist WEB2086 was used (Casals-Stenzel, et al., 1987). Both inhibitors are
134 reported to have similar effects (Hellewell and Williams, 1989). Cells were stimulated
135 with PAF (Sigma) (3.6 nM) or IL-23 for 24h. In some experiments, T cells were also
136 stimulated in solution with 1 μg/ml each of anti-CD3 and anti-CD28 antibodies
137 (Beckman Coulter, Paris Nord, Roissy, France) in the presence or absence of IL-12

138 (100ng/ml), IL-23 (10ng/ml), IL-12 (100ng/ml) & anti-IFN-γ (2.5ng/ml), IL-23 &
139 anti-IL-17, IFN-γ (2.5ng/ml) or IL-17 (0.5ng/ml) for 24h.
140
141 **2.3. Intracellular Staining**
142
143 Brefeldin A was added for the last 4-6h of a 24h incubation with cytokines. After the
144 24h, cells were fixed in 2% formaldehyde at room temperature for 5 min, washed by
145 centrifugation once in PBA (PBS, 0.5% bovine serum albumin and 1% sodium azide),
146 once in saponin buffer (PBA + 0.1% saponin) and once in 10% FCS in saponin buffer
147 at 300g for 5 min. The supernatant was poured off and the cells resuspended in the
148 residue, to which 10µl anti-PAF-R (mouse anti-human monoclonal IgG2a antibody,
149 Cayman Chemical) or isotyope control (Zymed) was added. Preliminary experiments
150 on human PBMC showing that the adsorption of the antibody with the immunizing
151 peptide LGFQDSKFHQ (Cayman Chemical) abolished the fluorescence on flow
152 cytometry proved the specificity of the antibody. Cells were incubated, washed by
153 centrifugation with saponin buffer, then incubated with 1µg of FITC-conjugated goat
154 anti mouse IgG (Zymed) for 30 min at room temperature. Staining with the directly
155 PE-conjugated primary anti-IFN-γ and anti-IL-17 mAb (eBioscience) was as above
156 except the second step (secondary antibody) was skipped. For double staining, cells
157 were then blocked with unlabelled mouse Ig (Zymed) before staining with directly
158 labeled mouse mAb. All cells were washed with 1ml saponin buffer and resuspended
159 in 0.5% formaldehyde for flow cytometry.
160
161 **2.4. Quantitative real time PCR and non-quantitative PCR**

162 qRT- PCR was used to assess PAFR, IL-17A and F, IL-22 and RORC mRNA
163 abundance in human T cells. RNA was extracted using RNeasy kit (Qiagen). First-
164 strand cDNA synthesis was initiated from 0.5 µg total RNA, using random hexamers
165 and AMV reverse transcriptase (Promega). Oligonucleotide primer sequences were as
166 follows: PAFR: forward 5'CCTCCTTAGCACCAACTGTGTC 3', reverse 5'
167 CAACCACTTCAGTGACCGTATCC 3'; β2microglobulin forward 5'
168 CTCCGTGGCCTTAGCTGTG 3', reverse 5' ATGTGTCTGGGTTTCATCCATC
169 3'; IL-17A forward 5' GCACAAACTCATCCATCCC 3', reverse 5'CATAGTGAA
170 GGCAGGAATCAC 3'; IL-17F forward 5'TGCACAAAGTAAGCCACCAG 3',
171 reverse GCTTGCCTTTCTGAGTG AGG 3'; RORC forward 5'
172 TGCCAACAACCACACAGTCT 3', reverse 5'GATGGAAAGCCAGTTCCAAA 3'.
173 IL-22 forward 5'CTCCTTCTCTTGGCCCTCTT 3', reverse
174 5'GTTCAGCACCTGCTTCATCA 3'. qRT-PCR was carried out as described (Fahey,
175 et al., 2006).

176 Non-quantitative PCR was also carried out on purified CD3+ cells as follows: PBMC
177 were isolated by gradient centrifugation with Histopaque 1077 (Sigma Aldrich,
178 Dorset, and U.K) and stimulated with PHA for 72h and recombinant human IL-2 for a
179 further 24 hours. The CD3+ human T cell enrichment kit (EasySep®) was utilized to
180 obtain a purified population of CD3+ T cells. One million T cells were plated out into
181 a 24 well plate and untreated or treated with 10ng/ml of recombinant human IL-23
182 (Peprotech), or 2ng phorbol dibutyrate (PDB) /1µM Ionomycin for 18 hours. The total
183 RNA was extracted from the T cells by employing an RNeasy miniprepkit (Qiagen
184 UK). DNase treatment of RNA samples was performed prior to RT-PCR (RQ1
185 RNase–free DNase, Promega, UK). The samples were analyzed by gel
186 electrophoresis.

187

188 **2.5. Measurement of intracellular calcium.**

189 Intracellular calcium was measured as previously described (Fox, et al., 2004) on a
190 Becton-Dickinson FACScan flow cytometer using Cellquest acquisition and analysis
191 software. T cell blasts ($5x10^{-6}$cells/ml) were loaded with the calcium fluorescent dye
192 Fluo-3AM (5µM fc) at 37ºC for 30 min in the presence of the anion channel blocker
193 probenecid (2.5mM fc) to prevent leakage of the probe from the cells. 50µl aliquots of
194 the T cells were diluted in 940µl of HEPES Tyrodes buffer containing 10µl of
195 calcium chloride to give a final concentration of 1mM calcium. 250µl of this
196 suspension was applied to the flow cytometer to measure baseline fluorescence at
197 time 0 sec. A further 480µl of the suspension was then added to 20µl of PAF (3.6nM
198 fc) and median fluorescence measurements were recorded at 5, 15, 30, 60 and 120s
199 following addition of PAF. Further tests were performed in which no PAF was added
200 and recordings were made at 5, 15, 30, 60 and 120s. Untreated cells and cells that had
201 been pre-treated with either IL-12 or IL-23 for 30 min were studied using this
202 procedure.

203

204 **2.6. ELISA**

205 ELISA was performed to measure IL-17 in supernatants of stimulated T cells
206 according to the manufacturer's instructions (R&D, Abingdon, UK).

207 **3. Results**

208 **3.1. PAF-R gene expression in T cells.**

209 In this study we validated our microarray analysis which had suggested that PAF-R is
210 expressed by a subgroup of T cells and it was upregulated by IL-23 and down
211 regulated by IL-12. PAF-R mRNA expression was assayed using quantitative real-

212 time PCR (qPCR) in PHA/IL-2-derived T cell blasts and isolated CD4+ T cells from
213 normal donors. Since IL-17 is a key cytokine induced by IL-23 we investigated the
214 role of IL-17 in the IL-23 mediated induction of PAF-R mRNA.
215 Both IL-23 (p=0.021, unpaired t-test) and IL-17 alone (p=0.04, unpaired t-test)
216 increased PAF-R mRNA expression in both T cell blasts (figure 1a) and purified
217 CD4+ T cells stimulated with anti-CD3/anti-CD28 (figure 1b) (p=0.001 for IL-23 and
218 for IL-17, unpaired t-test) compared to cells not exposed to these cytokines. In the
219 presence of a neutralizing IL-17 antibody (figure 1a and b), but not a control antibody
220 (not shown), the induction of PAF-R by IL-23 was reduced to nearly baseline levels.
221 Conversely, both IL-12 and IFN-γ decreased PAF-R mRNA expression (figure 1a &
222 b) in both sets of stimulated cells (T cell blasts: IL-12, p=0.021; IFN-γ, p=0.03,
223 unpaired t-test; CD4+ T cells: IL-12, p=0.001; IFN-γ, p=0.02, unpaired t-test). By
224 adding a neutralizing IFN-γ antibody, but not a control antibody (not shown), the
225 suppression induced by IL-12 was lost suggesting suppression is mediated by IFN-
226 γ (figure 1a & b).
227 Because the PAF-R gene consists of a single exon, we took the following measures to
228 rule out genomic DNA amplification by PCR: we used a RNA extraction kit that
229 removes genomic DNA; we detected no product in the condition without reverse
230 transcriptase; and we exposed the RNA to RNAse free DNAse without detecting
231 differences in PCR products (Figure 1c).
232

233 **3.2. PAF-R protein expression in T cells**

234 PAF-R expression was assessed at the protein level using flow cytometry. First we
235 found very low level of expression of PAF-R protein in resting total CD3+ and CD3+
236 CD8- T cells when performing flow cytometry on whole unstimulated PBMC with

237 gating on live lymphocytes. Positive fluorescence was also present in CD3- cells
238 within the lymphocyte population (largely B cells) (Figure 2a). The PAF-R protein
239 level increases significantly in cells activated with PBD (20 ng/ml) and ionomycin (1
240 μg/ml) overnight. (Figure 2a). We confirmed that PAF-R protein is also upregulated
241 by IL-23 and downregulated by IL-12 in T cell blasts (figure 2b). The increase in
242 fluorescence intensity compared to unstimulated cells ranged between 60 and 130%
243 for IL-23. The fluorescence intensity reduction induced by IL-12 was between 40 and
244 100% (n=5, p=0.04 and 0.03; two-tailed unpaired t-test). The effects of IL-23 and IL-
245 12 appear to be mediated, at least in part, through IL-17 and IFN-γ respectively. IL-
246 23 up-regulation of PAF-R protein was reduced in the presence of a neutralising anti-
247 IL-17 antibody (Figure 2c) but not a control antibody (not shown). IL-12 suppression
248 of PAF-R protein expression was reversed by adding a neutralizing IFN-γ antibody
249 (Figure 2d) but not a control antibody (not shown) (n=3, p>0.05 for IL-12 plus anti-
250 IFN-γ and IL-23 plus anti-IL-17 compared to unstimulated cells; unpaired t-test).
251 IFN-γ reduced the PAF-R protein by 43-120% (n=5, p=0.04), whereas IL-17
252 increased it by 50-100% (n=4, p=0.06). The results, expressed as percent change in
253 mean fluorescence intensity, are shown in Figure 2d. Combining IL-23 and IL-12
254 stimulation led to a slight (non-significant) reduction in the PAF-R level (n=3, p=0.1)
255 (Figure 2d).

256 **3.3. Functionality of PAF-R on T cells**

257 Although PAF-R is known to be expressed on a variety of cells, expression has not
258 been extensively investigated on T cells**.** Generally, PAF-R is not thought to be
259 expressed on resting T cells but our data indicated it could be up-regulated on
260 activated T cells. Like many GPCR, PAF-R increases intracellular calcium, and PAF-
261 R functional activity has been previously demonstrated in other cells by measuring

262 intracellular calcium concentration. We found that 30 or 60 second stimulation with
263 PAF 3.6 nM (figure 3a) or 7.2 nM (not shown) induced a significant increase in
264 intracellular calcium concentration in the T cell blasts but this was not observed in
265 cells exposed only to the PAF diluent and medium alone. Concentrations of PAF of
266 18 or 36 nM induced changes in size and granularity of the T cells within 120 seconds
267 as noted on flow cytometry, suggesting decreased viability. There were no
268 differences in the degrees of calcium increase in cells that had been briefly (30 min,
269 unlikely to significantly modify PAF-R expression) pre-exposed to IL-12, IL-23 or no
270 cytokine prior to stimulation with PAF 3.6 nM. (figure 3)
271 We have shown that IL-23 increases both PAF-R transcription and protein expression
272 and that this effect is in part dependent on IL-17 and that IL-17 alone can enhance
273 PAF-R expression. Investigating the time frame of these events using quantitative
274 realtime PCR (qRT-PCR) we found that PAF-R mRNA expression is greater than IL-
275 17 mRNA expression after 6h of stimulation with IL-23 (1.6 vs 1.1 fold increase
276 compared to unstimulated cells, respectively). However, by 12h IL-17 message levels
277 slightly exceed the PAF-R message levels (1.82 vs 1.78-fold induction) (Figure 4a).
278 We therefore hypothesised that PAF-R may not simply be induced in the development
279 of Th17 cells but that PAF/PAF-R pathway may influence the IL-17 expression. We
280 then examined the effect of PAF stimulation of T cell blasts on IL-17A production
281 using ELISA. We found that PAF treatment induces IL-17A protein production,
282 which can be blocked using PAF-R antagonist WEB2086 (Figure 4b), suggesting a
283 positive autocrine feedback loop inducing IL-17 expression and self-perpetuating
284 Th17-mediated inflammation. In support of this we show that the addition of the
285 synthetic competitive PAF-R antagonist, CV3988, also reduced the level of IL-17
286 induced by either IL-23 or PAF at both the mRNA (figure 4c) and protein level (data

287 not shown), indicating that both IL-23 and PAF induce IL-17 and can jointly
288 contribute to Th17 development.
289

290 **3.4. PAF-R is co-regulated with IL-17 and other Th17 associated**
291 **molecules**

292 We used intracellular staining for IL-17 or IFN-γ followed by flow cytometry to
293 determine whether PAF-R on T cell blasts co-expresses either or both of these
294 cytokines. We show that PAF-R is co-expressed on cells producing IL-17 but not
295 IFN-γ (Figure 5). A population of cells in these experiments done on T cell blasts
296 (>95% CD3+) appear to express PAF-R without coexpressing IL-17. We suspect
297 they may represent CD8+ expressing PAF-R as shown in our experiments using
298 PBD/ionomycin stimulation, and/or Th2 (PAF-R being involved in the allergic
299 response, (Kasperska-Zajac, et al., 2008)) or uncommitted activated T cells. Our
300 results, however, suggest that PAF-R expression may distinguish Th17 cells from
301 IFN-γ expressing Th1 cells.
302 We investigated the expression of PAF-R in human T cells after stimulation with a
303 combination of TGF-β, IL-6, and IL-23 as well as IL-23 and IL-12 alone. PAF-R
304 mRNA expression was compared to that of IL-22, IL-17 and RORC. (see figure 6).
305 PAF-R expression followed a very similar trend in expression when compared to the
306 other Th17 associated molecules, suggesting that PAF-R is co-regulated with other
307 Th17 markers and molecules required for their development. In this serum-containing
308 system, and using a mixed population of T cells including both naïve and memory
309 cells, significant induction of Th17 markers was similar using IL-23 compared with
310 TGF-β plus IL-6, or TGF-β, IL-6 and IL-23 in combination. Thus, compared to
311 unstimulated cells, induction of PAF-R, RORC, IL-17A, IL-17F, and IL-22 was

312 statistically significant ($p<0.05$, unpaired t-test, n=5 experiments). There were no
313 differences between the induction methods using IL-23, TGF-β plus IL-6 or TGF-β
314 plus IL-6 plus IL-23 ($p>0.05$ for all other Th17 markers) with the exception of IL-22,
315 which showed significantly less induction with IL-23 alone compared to the other
316 stimuli ($p<0.05$ for both TGF-β plus IL-6 and TGF-β plus IL-6 plus IL-23 compared
317 to IL-23 alone; unpaired t-test); and for IL-17F which showed less induction with
318 TGF-β plus IL-6 than with IL-23 alone (p=0.045, unpaired t-test). Although we
319 found significant induction of IL-17A, IL-17F, RORC and PAF-R in response to IL-
320 22 (data not shown) this response was modest compared to that achieved with IL-23.
321 IL-12 appeared to downregulate (PAF-R, RORC, IL-17A, IL-17F, $p<0.05$) or fail to
322 upregulate (IL-22, $p>0.05$) these molecules (figure 6).
323
324 **3.5. Expression of PAF-R in multiple sclerosis patients**
325 To determine the expression of IL-17 and PAF-R in peripheral blood of MS patients,
326 we extracted RNA from peripheral blood mononuclear cells from 30 patients with
327 relapsing MS and 20 age and sex matched controls. The demographic and clinical
328 characteristics of these patients are listed in supplementary table 2. IL-17 and PAF-R
329 mRNA expression was measured using real time PCR. MS patients have significantly
330 higher PAF-R and IL-17A mRNA expression compared to controls; (figure 7 a, b); p=
331 0.0001 and 0.02, respectively. PAF-R and IL-17A mRNA levels correlated well;
332 Pearson's r= 0.66; p= 0.023. Moreover, PAF-R mRNA correlated highly with MS
333 disability scores as measured by the Expanded Disability Status Scale (EDSS) score;
334 Pearson's r=0.61; p=0.0003 (figure 7 c).
335 To determine the proportion of the PBMC PAF-R mRNA that is of T cell origin, we
336 analysed a further group of 8 additional relapsing MS patients and 7 additional control

337 subjects. The PBMC from each donor were divided in two samples. PAF-R RNA was
338 measured from one sample (unfractionated PBMC) and from the other (CD3+ cells)
339 after CD3+ cells were magnetically separated. The average proportion of RNA
340 extracted from CD3+ cells represented 63% of the RNA extracted from total PBMC
341 in MS patients and 67% of the PBMC RNA of controls (Figure 7 d). In this smaller
342 group, the differences between PAF-R expression in MS and controls only showed a
343 trend toward higher levels in total PBMC and in CD3+ cells in patients (p=0.09 and
344 0.17, respectively, two-tailed unpaired t-test).
345 However, the results indicate that a substantial and comparable proportion of PAF-R
346 mRNA in both MS patients and controls is of T cell origin.
347
348 **4. Discussion**

349 The aims of this study were 1) to validate our previous gene expression profiling
350 results that identified PAF-R on activated T cells as a potential Th17 molecule; 2) to
351 determine its functionality and co-regulation with other Th17-associated molecules;
352 and 3) to explore its role in MS. We confirmed the microarray results by qPCR and
353 flow cytometry. Importantly, both the arrays and our T cell samples were validated in
354 several ways. Some genes on the arrays have 2 clones, including PAF-R. The
355 regulation by IL-12 and IL-23 is in the same direction, denoting internal consistency.
356 Also, the classical IL-12 target IFN-γ was shown to be up-regulated as expected
357 (Supplementary Table 1). In addition, we previously validated other array results, e.g.
358 the regulation of glucocorticoid modulatory element binding proteins (GMEB) and
359 went on to show its functional role in T cell survival (Kawabe, et al., 2012). We also
360 showed intracellular Ca increase indicating the functionality of PAF-R. We also
361 provided evidence supporting a potential role in MS, in that its mRNA level
362 expression is increased compared to controls and correlates with disability scores. A

363 limitation of our study is that we did not expand our flow cytometry studies in normal
364 cells into a more detailed examination of an MS cohort (although preliminary data,
365 not reported here, suggest an up-regulation at protein level in MS as well). We took
366 advantage of a collection of RNA samples from PBMC of untreated MS patients, in
367 part collected as part of a study of interferon responsiveness (Tanasescu, et al., 2017);
368 but without corresponding PBMC. Future studies investigating more specifically MS
369 and other autoimmune diseases are warranted. Another limitation of this study was
370 that, in the flow cytometry experiments showing co-expression of PAF-R and IL-17
371 and mutual exclusion of PAF-R and IFN-γ, we only used PHA/IL-2 stimulation but
372 did not use positive controls with strong inducers of these cytokines to show the
373 magnitude of cytokine induction.
374 Increasing evidence implicates Th17 cells in the pathogenesis of autoimmune
375 diseases. They produce chemokines, cytokines, metalloproteases and other
376 inflammatory compounds that compromise the blood brain barrier, relevant to MS.
377 In this study we provide evidence for PAF-R expression in activated human T cells.
378 Although not directly demonstrated (as most prior studies were on resting T cells),
379 induction of PAF-R on T cells by CD2 or CD3 stimulation has already been suggested
380 (Vivier, et al., 1990), and canine T cells were also shown to express functional PAF-R
381 that upregulate intracellular Ca after PAF stimulation (Calabresse, et al., 1992). A
382 previous study of human T cells that used unstimulated expression of HLA-DR as
383 activation marker did not show PAF-R on T cells, but PAF RNA was measured by
384 Northern blotting, a less sensitive method than the qPCR used here (Simon, et al.,
385 1994). Moreover, the similar parallel regulation of Th17-associated genes by IL-23,
386 IL-17, IL-12, and IFN-γ strongly suggests that our results are not an artefact. We used
387 strong stimuli (PHA/IL-2 followed by cytokines or PAF) which may explain our

388 higher yield of Th17 cells compared with other studies. In addition, our findings are
389 further validated by results on separated CD3+ and CD4+ cells simulated with anti-
390 CD3. Th17 cells show considerable phenotypic and functional heterogeneity
391 (Bystrom, et al., 2019). Our findings do not allow a definitive conclusion regarding
392 whether the PAF-R+ Th17 cells belong to a specific subpopulation of Th17 cells, but
393 in view of their associated cytokines and IL-23 responsiveness, they appear to be
394 classical Th17 cells, and do not appear to be Th22 cells. The absent co-expression of
395 IFN-γ indicates that PAF-R can distinguish them from the proinflammatory cells
396 concomitantly expressing Th1 and Th17 markers (Th1-17 cells) which have also been
397 associated with MS pathogenesis (Edwards, et al., 2010)
398
399 We show that PAF-R on T cells increases intracellular Ca in response to PAF
400 stimulation, and that PAF effects on T cells, including IL-17 induction, are blocked by
401 PAF-R antagonists. We also show PAF-R up-regulation by IL-23, and demonstrate
402 that its pattern of expression is similar to that of other Th17 associated molecules.
403 The PAF-R ligand, PAF, an inflammatory mediator with pleiotropic effects, appears
404 early in inflammation. Here, we show that it induces IL-17, and that PAF-R
405 expression is upregulated by IL-23 even before the upregulation of IL-17. Thus,
406 PAF/PAF-R interactions may be involved in the early events leading to Th17
407 differentiation and trigger a self-amplification process similar to the CCR6/CCL20
408 loop described previously for Th17 cells (Acosta-Rodriguez, et al., 2007).
409 Our results confirm two additional studies implicating PAF/PAF-R in Th17-mediated
410 responses (Singh, et al., 2011) (Drolet, et al., 2011). Both of these studies support our
411 findings; however our study is the first to focus on the role of T cells in this new role
412 of PAF/PAF-R pathway. We postulate that an inflammatory milieu containing PAF in

413 addition to TGF-β may skew the T cell development towards Th17 and away from
414 Treg commitment. This argument is strengthened by the fact that PAF itself induces
415 IL-6, a key element in the induction of Th17 cells and suppression of Treg cell
416 development (Hamel-Cote, et al., 2019).
417 Besides IL-23, IL-1 is a potent Th17 stimulus (Acosta-Rodriguez, et al., 2007).
418 Interestingly, IL-1 has also been shown to upregulate the PAF pathway (Lee, et al.,
419 2000), and PAF plays an important role in asthma, and possibly also in MS and other
420 autoimmune conditions. It is therefore plausible that PAF is an important member of
421 an inflammatory network that enhances and perpetuates inflammation with Th17
422 predominant pathogenesis.
423 PAF/PAF-R pathway may be implicated in the optimal functioning of this T cell
424 subset with crucial roles in MS, asthma, and other inflammatory disorders.
425 While it is still debatable whether the asthma and MS coexist more or less as
426 frequently as expected based on the figures of their independent life time prevalence,
427 the fact that they do coexist in a significant number of patients cannot be denied
428 (Edwards and Constantinescu, 2004) (Manouchehrinia, et al., 2015). Therapeutic
429 strategies that avoid exacerbation of one during treatment of the other should target
430 shared pathogenic pathways. Notably, PAF inhibition has shown some promise for
431 both MS and asthma (Brochet, et al., 1995) (Chu, et al., 2011). Thus, targeting
432 PAF/PAF-R pathway and, possibly through this, the Th17 pathway, may become a
433 justified and worthwhile therapeutic approach in the treatment of these conditions.

435 **Figure Legends**

436 **Figure 1. PAF-R gene expression in T cells.** Quantitative reverse transcriptase real-
437 time PCR was used to assess PAF-R abundance in human PHA/IL-2 T cell blasts (a)
438 and CD4+ cells stimulated with anti CD3/CD28 (b).Both sets of cells were either left
439 untreated (US) or incubated with IL-12 (100ng/ml), IL-23 (10ng/ml), IL-12
440 (100ng/ml) & anti-IFN-γ (2.5ng/ml), IL-23 & anti-IL-17, IFN-γ (2.5ng/ml) or IL-17
441 (0.5ng/ml) for 24h at 37°C and 5%CO_2. Rabbit and goat IgG were used as controls
442 for anti-IFN-γ and IL-17, respectively and did not show an effect. Differences were
443 as follows: a) means and SD are shown from 5 independent experiments. p=0.023 US
444 v IL-12; p=0.021 US v IL-23; p=0.03 US v IFN-γ; p= 0.04 US v IL-17. p values for
445 US v the other conditions were not significant; p=0.04 IL-12 v IL-12 v IL-12 + anti-
446 IFN-γ; p=0.03 IL-23 v IL-23 + anti-IL-17 . b) p=0.01 US v IL-12; p=0.01 US v IL-
447 23; p=0.03 US v IFN-g; p= 0.03 US v IL-17; p=0.01 IL-12 v IL-12 v IL-12 + anti-
448 IFN-γ; p=0.02 IL-23 v IL-23 + anti-IL-17. p values for US v the other conditions were
449 not significant (c) PCR confirmation of PAF-R expression on purified CD3+ T cells.
450 Agarose gel electrophoresis of PCR products as follows: m=molecular weight marker;
451 1: RNA from CD3+ cells stimulated with IL-23 (10 ng/ml) as described in materials
452 and methods; 2: RNA from CD3+ cells stimulated with PDB/ionomycin; 3: DNAse
453 treated RNA from CD3+ cells stimulated with PDB/ionomycin; 4: RNA from
454 unstimulated CD3+ cells; 5: DNAse treated RNA from unstimulated CD3+ cells. 6.
455 Lane loaded with the “no reverse transcriptase” negative control showing absence of
456 genomic DNA. Representative gel of 3 separate experiments.

457

458 **Figure 2. PAF-R protein expression in T cells**

459 PAF-R protein expression was measured using flow cytometry. (A). This left panel
460 shows unstimulated cells, gated on CD3+CD8- lymphocytes, stained using anti-PAFR
461 primary antibody with a FITC-labelled secondary antibody. Staining of PAFR on
462 unstimulated CD4+ lymphocytes is < 1%. The right panel shows cells have been
463 stimulated with PDB/ionomycin overnight, stained and gated as above which
464 significantly increases PAFR expression (6.17%). Representative scatterplot of 3
465 experiments; $p<0.01$ for unstimulated vs stimulated.
466 B) Histogram showing PAF-R expression on PHA/IL-2 T cell blasts stimulated with
467 IL-12 vs IL-23. Results are shown from one representative experiment out of 5.
468 ($p=0.029$ for US vs IL-23; $p=0.04$ for US vs IL-12).
469 C) Involvement of IL-17 and IFN-γ in the effects of IL-23 and IL-12, respectively;
470 and direct effects of IL-17 and IFN-γ on PAF-R protein expression. Representative
471 results of 3 experiments. P=0.04 for IL-17 and 0.045 for IFN-γ.
472 D). Graph depicting percent changes in the expression of PAF-R in all the above
473 stimulation conditions. Asterisk= $p<0.05$; half-asterisk= $p=0.05$.
474 **Figure 3. Functionality of PAF-R on T cells**
475 Stimulation of T cells by PAF induces intracellular calcium release**.** Intracellular free
476 calcium was measured using a FACScan flow cytometer after stimulation with 3.6
477 nM PAF (asterisk= $p<0.05$). Results are shown as means of 3 independent
478 experiments.
479 **Figure 4. PAF/PAF-R interaction increases IL-17 expression in T cells blasts**
480 (a) PAF increases IL-17 expression by T cell blasts as shown by intracellular staining
481 (b). The addition of a competitive PAF-R antagonist (CV3988) reduced the level of
482 IL-17 mRNA induced by both IL-23 and PAF.
483

484 **Figure 5. PAF-R is co-expressed with IL-17 but not with IFN-γ.** T cell blasts were
485 stained either alone with anti-IL-17 PE labelled antibody, anti-IFN-γ PE labelled
486 antibody, and anti-PAF-R FITC labelled antibody or double stained with anti-IL-17
487 and anti-PAF-R or anti-IFN-gamma and anti-PAF-R antibodies. Expression and co-
488 expression of the target proteins were measured using flow cytometry.

489

490 **Figure 6. PAF-R expression is regulated in the same way as other Th17**
491 **associated molecules.** PHA/IL-2 induced T cell blasts ($1x10^6$cells/ml) were either left
492 untreated or incubated with IL-12 (100ng/ml), IL-23 (10ng/ml), TGF-β (50ng/ml) &
493 IL-6 (20ng/ml) , or TGF-β (50ng/ml) & IL-6 (20ng/ml)& IL-23 (10ng/ml) for 24h at
494 37°C and 5%CO_2. RNA was extracted and PAF-R, IL-22, IL-17A&F and ROR γ T
495 mRNA expression was measured using real time PCR.

496

497 **Figure 7. Expression of PAF-R in multiple sclerosis patients.** RNA was extracted
498 from peripheral blood mononuclear cells isolated from MS patients and age and sex
499 matched controls. PAF-R (30 MS patients, 20 controls) (a) and IL-17A (12 MS
500 patients, 7 controls) (b) mRNA expression was measured using real time PCR. EDSS
501 scores were obtained during a standardised neurological examination at the time of
502 blood collection. Pearson's correlation coefficient was used to explore correlations
503 between PAF-R and EDSS (c).

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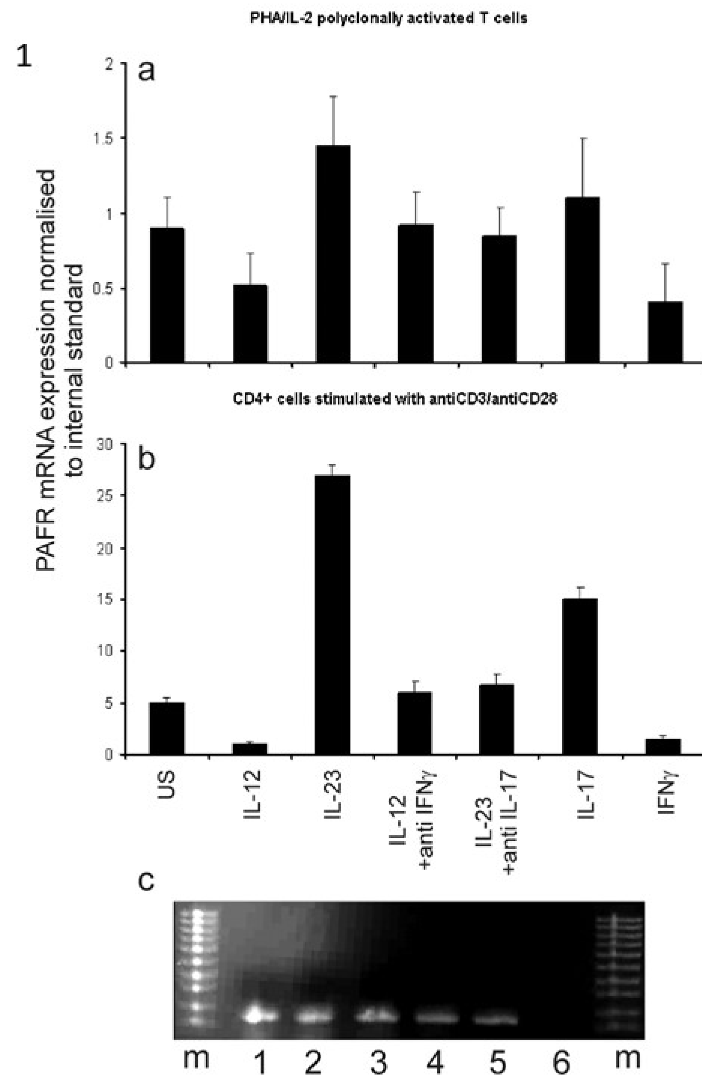
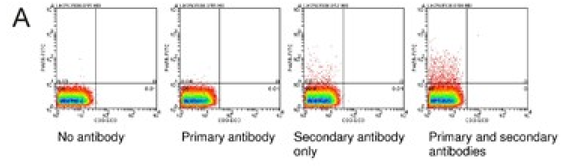
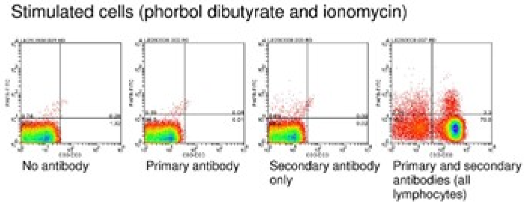
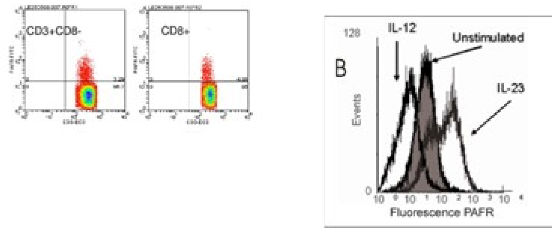


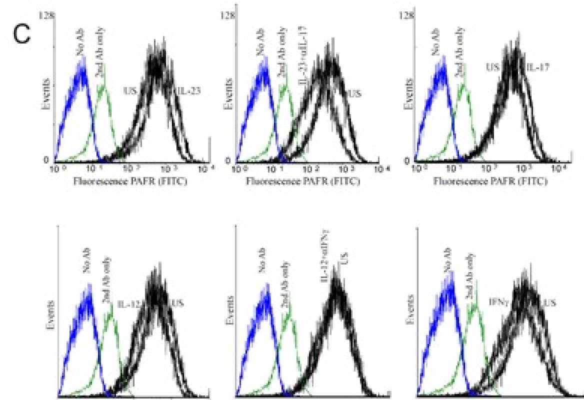
PHA/IL-2 polyclonally activated T cells
1
a
b
c
PAFR mRNA expression normalised to internal standard
CD4+ cells stimulated with antiCD3/antiCD28
US
IL-12
IL-23
IL-12 +anti IFNγ
IL-23 +anti IL-17
IL-17
IFNγ
m
1
2
3
4
5
6
m

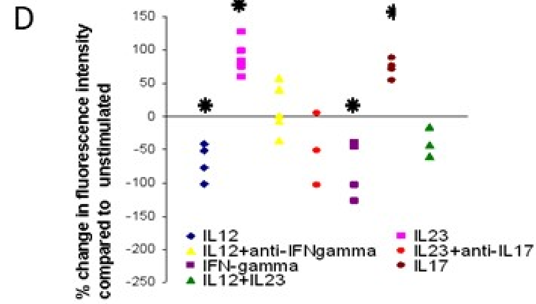
2











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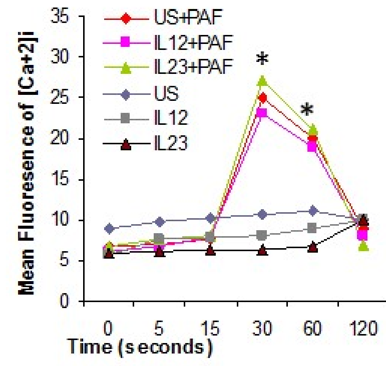

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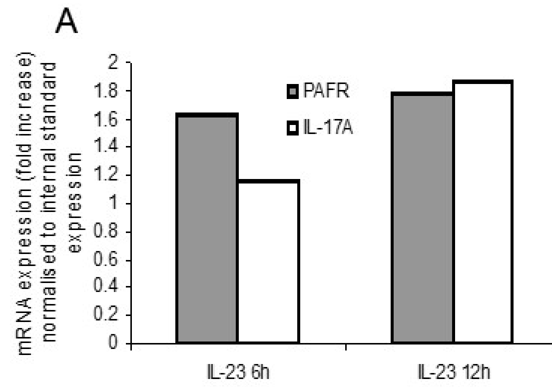
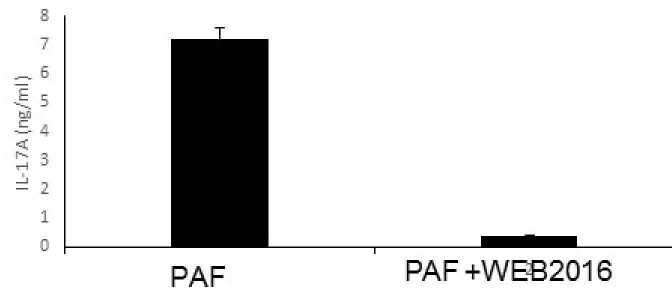
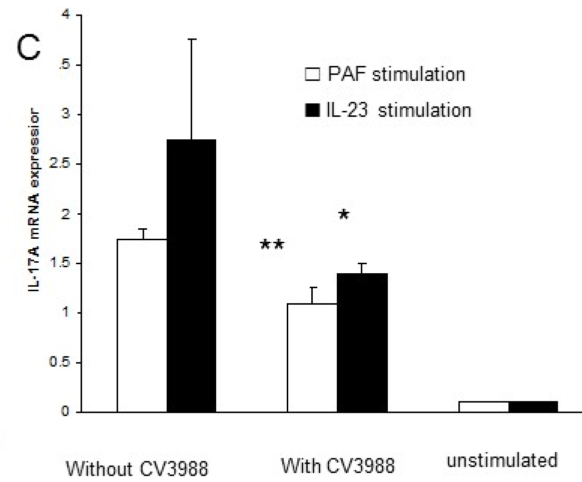
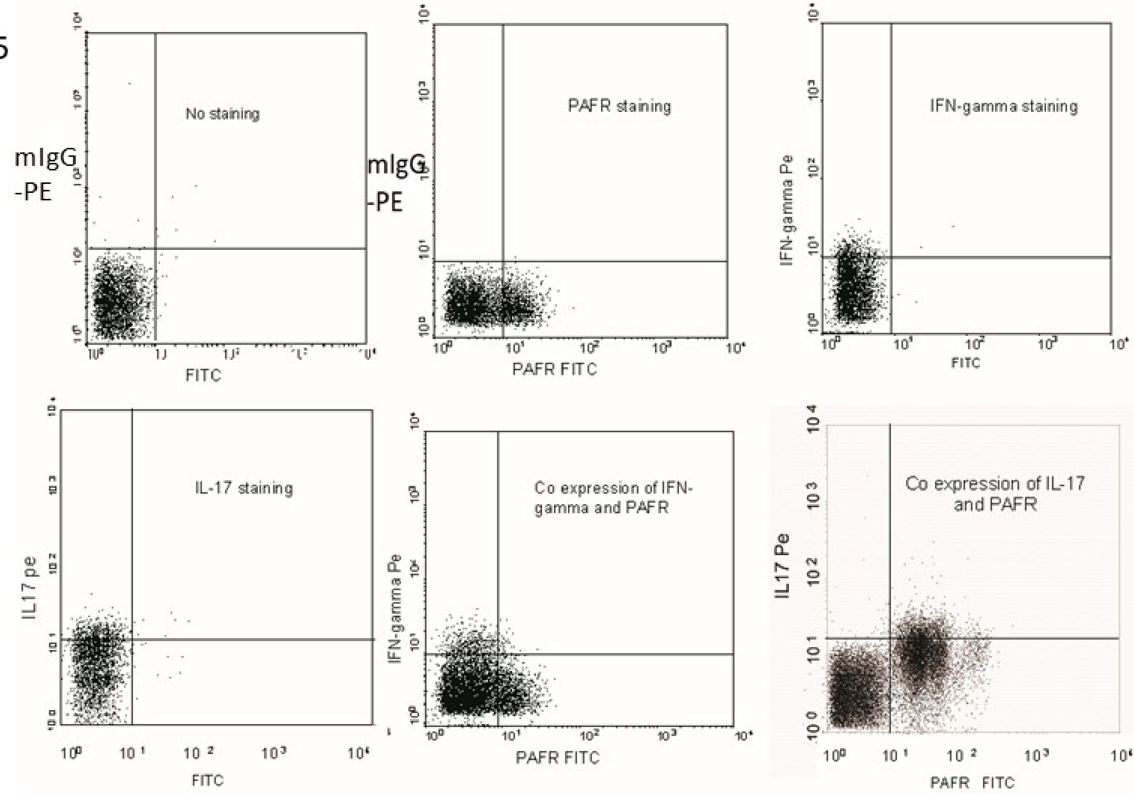


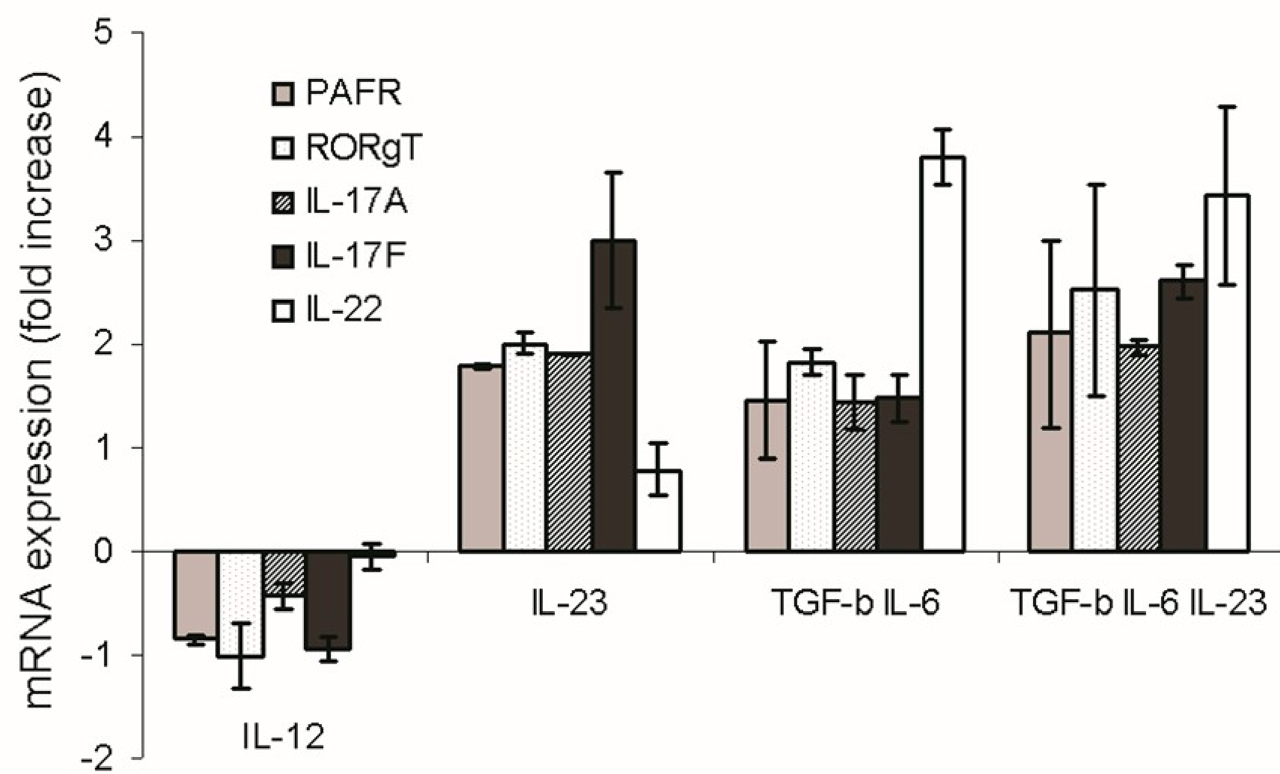






5
mIgG
-PE
No staining
FITC
mIgG
-PE
PAFR staining
PAFR FITC
IFN-gamma staining
IFN-gamma Pe
FITC
IL-17 staining
IL17 pe
FITC
Co expression of IFN-
gamma and PAFR
IFN-gamma Pe
PAFR FITC
Co expression of IL-17
and PAFR
IL17 Pe
PAFR FITC

6



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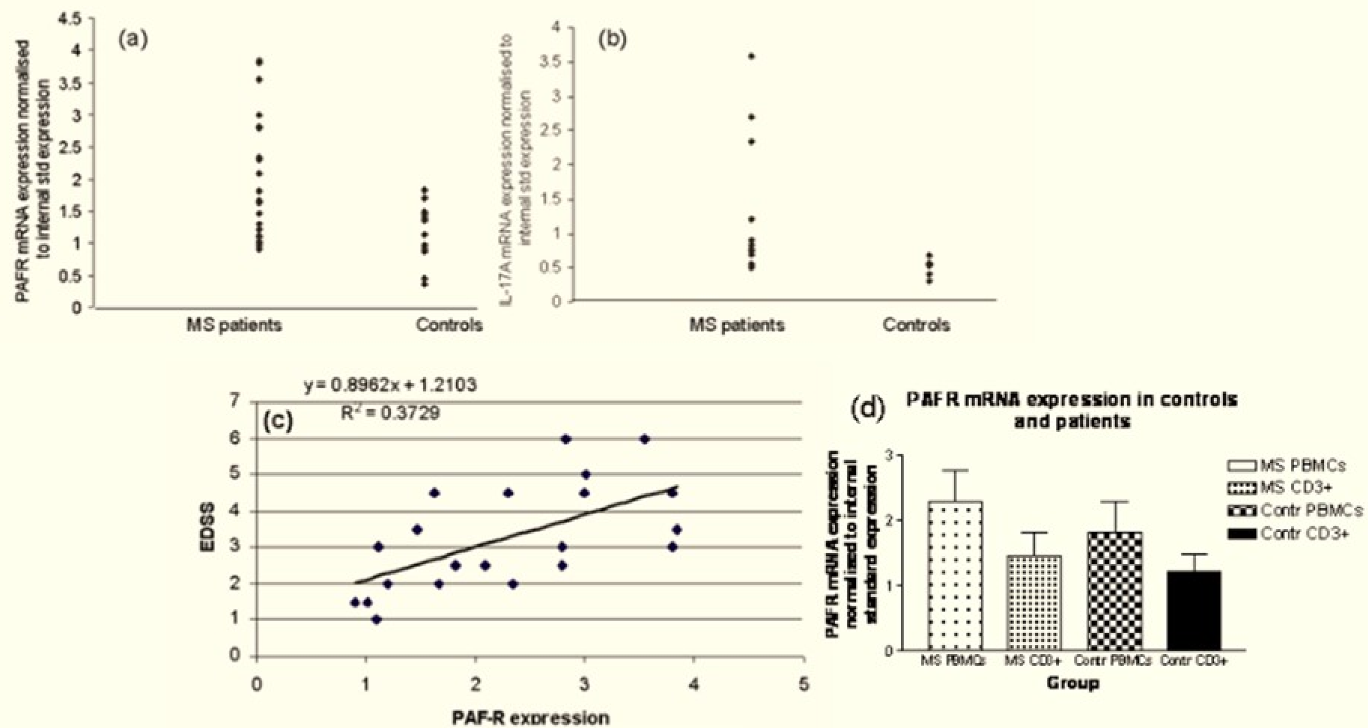

Midgley et al, **PAF-R on activated T cells: role in the IL-23/Th17 pathway and relevance to multiple sclerosis**

Supplementary Table 1

Gene Name	Symbol	IL12/PHA*	IL23/PHA**	IL23/IL12***
Deleted in bladder cancer 1	DBC1	-3.1	4.3	13.5
Sulfatase modifying factor 2	SUMF2	-6.2	2.0	12.2
TNF receptor-associated factor 5	TRAF5	-5.9	2.0	11.7
Mitogen-activated protein kinase kinase kinase 1	MAP3K1	-8.6	1.3	11.2
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	GALNTL2	-5.7	1.9	11.0
Prion protein 2 (dublet)	PRND	-6.3	1.7	10.9
Transmembrane protein 63A	TMEM63A	-3.2	3.4	10.8
Chloride channel CLIC-like 1	CLCC1	-6.2	1.7	10.7
DNA fragmentation factor, 45kDa, alpha polypeptide	DFFA	-7.5	1.4	10.7
Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	ELN	-3.9	2.7	10.7
Melanoma antigen family A, 5	MAGEA5	-7.1	1.5	10.7
ARS2 protein	ARS2	-6.8	1.6	10.5
platelet-activating factor receptor	PAF-R	-4.4	2.3	10.3
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	SEMA3F	-5.5	1.9	10.3
Actin binding LIM protein 1	ABLIM1	-4.9	2.1	10.2
Acyl-Coenzyme A dehydrogenase, short/branched chain	ACADSB	-7.1	1.4	10.2
Cholesterol 25-hydroxylase	CH25H	-4.5	2.2	10.0
Islet cell autoantigen 1, 69kDa	ICA1	-3.1	3.3	10.0
Zinc finger protein 193	ZNF193	-9.4	1.1	10.0
DnaJ (Hsp40) homolog, subfamily C, member 3	DNAJC3	-5.4	1.8	9.8
Lysophosphatidylglycerol acyltransferase 1	LPGAT1	-5.5	1.8	9.8
Mucin 5B, oligomeric mucus/gel-forming	MUC5B	-6.4	1.5	9.8
Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	-5.4	1.8	9.7
platelet-activating factor receptor	PAF-R	-5.4	1.8	9.6
Selectin E (endothelial adhesion molecule 1)	SELE	-5.1	1.9	9.6
SRY (sex determining region Y)-box 10	SOX10	-3.9	2.5	9.6
Syntaxin 5	STX5	-2.0	4.7	9.6
Vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	-2.2	4.3	9.6
Casein kinase 2, alpha prime polypeptide	CSNK2A2	-2.6	3.7	9.5
Coagulation factor II (thrombin)	F2	-4.9	1.9	9.5
Frizzled homolog 9 (Drosophila)	FZD9	-8.3	1.1	9.5
Protoporphyrinogen oxidase	PPOX	-4.6	2.1	9.5
Tyrosine hydroxylase	TH	-4.7	2.0	9.5

Zinc finger protein 544	ZNF544	-4.9	1.9	9.5
Chromosome 1 open reading frame 148	C1orf148	-3.8	2.5	9.4
GTF2I repeat domain containing 1	GTF2IRD1	-8.6	1.1	9.4
Homeobox C4	HOXC4	-6.6	1.4	9.4
Microtubule-associated protein 7	MAP7	-7.6	1.2	9.4
Rhomboid domain containing 3	RHBDD3	-2.9	3.2	9.4
Chromosome 16 open reading frame 70	C16orf70	-2.1	4.4	9.3
Leucine rich repeat containing 28	LRRC28	-3.3	2.9	9.3
Serine/threonine kinase 4	STK4	-5.1	1.8	9.3
Chromosome 15 open reading frame 38	C15orf38	-4.7	1.9	9.2
CD302 molecule	CD302	-7.6	1.2	9.2
Inositol polyphosphate-5-phosphatase F	INPP5F	-6.6	1.4	9.2
Josephin domain containing 1	JOSD1	-8.0	1.1	9.2
RAB, member of RAS oncogene family-like 2B	RABL2B	-6.0	1.5	9.2
PRKC, apoptosis, WT1, regulator	PAWR	-5.8	1.6	9.1
Disrupted in schizophrenia 1	DISC1	-6.5	1.4	9.1
Mitochondrial translational release factor 1	MTRF1	-1.6	5.8	9.1
Peroxisomal biogenesis factor 11B	PEX11B	-8.0	1.1	9.1
Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	-1.3	6.9	9.1
Trafficking protein, kinesin binding 1	TRAK1	-2.6	3.4	9.1
Zinc finger, CCHC domain containing 2	ZCCHC2	-1.8	5.0	9.1
Annexin A7	ANXA7	-7.9	1.1	9.0
Filamin C, gamma (actin binding protein 280)	FLNC	-2.4	3.8	9.0
General transcription factor IIIC, polypeptide 1, alpha 220kDa	GTF3C1	-3.7	2.4	9.0
ISL1 transcription factor, LIM/homeodomain, (islet-1)	ISL1	-3.0	3.0	9.0
Protein phosphatase, EF-hand calcium binding domain 1	PPEF1	-7.7	1.2	9.0
Small breast epithelial mucin	SBEM	-8.5	1.1	9.0
Solute carrier family 35, member E3	SLC35E3	-6.8	1.3	9.0
Contactin associated protein-like 2	CNTNAP2	-6.3	1.4	8.9
Cofactor required for Sp1 transcriptional activation, subunit 2, 150kDa	CRSP2	-3.8	2.3	8.9
Hypothetical protein LOC51057	LOC51057	-6.0	1.5	8.9
Phospholipase A2, group IVA (cytosolic, calcium-dependent)	PLA2G4A	-7.4	1.2	8.9
Chromosome 15 open reading frame 17	C15orf17	-6.2	1.4	8.8
Sterol-C4-methyl oxidase-like	SC4MOL	-7.9	1.1	8.8
Calmodulin regulated spectrin-associated protein 1-like 1	CAMSAP1L1	-5.4	1.6	8.7
Forkhead box M1	FOXM1	-6.3	1.4	8.7
Similar to Apolipoprotein C-III precursor (Apo-CIII)	LOC440838	-4.7	1.9	8.7
Methyltransferase like 2A	METTL2A	-5.0	1.8	8.7
PDZ and LIM domain 5	PDLIM5	-6.5	1.3	8.7
Rap guanine nucleotide exchange factor (GEF) 1	RAPGEF1	-5.3	1.7	8.7
Small nuclear RNA activating complex, polypeptide 4, 190kDa	SNAPC4	-3.1	2.8	8.7

Dishevelled, dsh homolog 2 (Drosophila)	DVL2	-5.4	1.6	8.6
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	NFKBIB	-6.2	1.4	8.6
Protein phosphatase 1J (PP2C domain containing)	PPM1J	-3.5	2.5	8.6
V-rel reticuloendotheliosis viral oncogene homolog (avian)	REL	-3.2	2.7	8.6
Tenascin C (hexabrachion)	TNC	-3.7	2.3	8.6
V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	YES1	-6.7	1.3	8.6
ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C2	ATP6V1C2	-6.6	1.3	8.5
Complement component 1, r subcomponent-like	C1RL	-8.3	1.0	8.5
Chromosome 3 open reading frame 63	C3orf63	-7.1	1.2	8.5
Choline kinase beta	CHKB	-6.8	1.2	8.5
Diacylglycerol kinase, delta 130kDa	DGKD	-2.3	3.8	8.5
Coagulation factor II (thrombin) receptor	F2R	-7.8	1.1	8.5
Similar to zinc finger protein 91	FLJ44894	-4.5	1.9	8.5
Prodynorphin	PDYN	-4.0	2.1	8.5
ATPase family, AAA domain containing 2	ATAD2	-7.0	1.2	8.4
Chromosome 10 open reading frame 79	C10orf79	-3.4	2.5	8.4
Cell growth regulator with ring finger domain 1	CGRRF1	-3.5	2.4	8.4
Cytochrome b5 type B (outer mitochondrial membrane)	CYB5B	-5.6	1.5	8.4
F-box and leucine-rich repeat protein 17	FBXL17	-8.5	-1.0	8.4
Progestin and adipoQ receptor family member VIII	PAQR8	-6.8	1.2	8.4
Peptidase D	PEPD	-7.2	1.2	8.4
Phosphoserine phosphatase	PSPH	-4.1	2.1	8.4
Src homology 2 domain containing adaptor protein B	SHB	-5.8	1.5	8.4
CART prepropeptide	CARTPT	-7.7	1.1	8.3
Cofactor required for Sp1 transcriptional activation, subunit 7, 70kDa	CRSP7	-7.2	1.2	8.3
Elastase 3B, pancreatic	ELA3B	-7.9	1.1	8.3
Poly (ADP-ribose) glycohydrolase	PARG	-8.6	-1.0	8.3
WAS protein homology region 2 domain containing 1	WHDC1	-7.8	1.1	8.3
Calcitonin receptor	CALCR	-3.5	2.4	8.2
Citron (rho-interacting, serine/threonine kinase 21)	CIT	-6.9	1.2	8.2
Eukaryotic translation initiation factor 2 alpha kinase 4	EIF2AK4	-6.6	1.2	8.2
Heparan sulfate proteoglycan 2	HSPG2	-7.1	1.1	8.2
Internexin neuronal intermediate filament protein, alpha	INA	-6.1	1.3	8.2
KIAA1407	KIAA1407	-6.2	1.3	8.2
RAB8B, member RAS oncogene family	RAB8B	-3.6	2.3	8.2
Reticulon 4 receptor	RTN4R	-7.9	1.0	8.2
Snail homolog 1 (Drosophila)	SNAI1	-8.0	1.0	8.2
CCR4-NOT transcription complex, subunit 8	CNOT8	-5.8	1.4	8.1

Coenzyme Q7 homolog, ubiquinone (yeast)	COQ7	-5.7	1.4	8.1
Cytochrome b-561	CYB561	-5.8	1.4	8.1
Desmoglein 3 (pemphigus vulgaris antigen)	DSG3	-9.5	-1.2	8.1
Integrin, beta 8	ITGB8	-2.0	4.0	8.1
Hypothetical protein LOC283588	LOC283588	-2.9	2.8	8.1
Major histocompatibility complex, class I-related	MR1	1.4	11.2	8.1
Phosphodiesterase 6B, cGMP-specific, rod, beta (congenital stationary night blindness 3, autosomal dominant)	PDE6B	-7.5	1.1	8.1
RAB38, member RAS oncogene family	RAB38	-5.1	1.6	8.1
A kinase (PRKA) anchor protein 4	AKAP4	-7.8	1.0	8.0
Calpain 7	CAPN7	-4.7	1.7	8.0
Caspase 7, apoptosis-related cysteine peptidase	CASP7	-7.6	1.0	8.0
CAMP responsive element binding protein 1	CREB1	-5.6	1.4	8.0
Embryonal Fyn-associated substrate	EFS	-6.4	1.2	8.0
Guanylate cyclase activator 2B (uroguanylin)	GUCA2B	-2.5	3.2	8.0
Keratin associated protein 10-11	KRTAP10-11	0.0	8.0	8.0
Mitogen-activated protein kinase kinase kinase 7	MAP3K7	-7.2	1.1	8.0
Zinc finger and BTB domain containing 32	ZBTB32	0.0	8.0	8.0
Chromosome 8 open reading frame 42	C8orf42	1.1	-7.5	-8.0
CD200 molecule	CD200	8.0	0.0	-8.0
Cyclin-dependent kinase 4	CDK4	2.4	-3.3	-8.0
F-box protein 4	FBXO4	8.0	0.0	-8.0
Opposite strand transcription unit to STAG3	GATS	8.0	0.0	-8.0
Jumonji domain containing 1C	JMJD1C	8.0	0.0	-8.0
Leucyl-tRNA synthetase 2, mitochondrial	LARS2	8.0	0.0	-8.0
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	MTHFD1L	5.1	-1.6	-8.0
N-myc downstream regulated gene 1	NDRG1	8.0	0.0	-8.0
Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	PKIA	3.9	-2.1	-8.0
Psoriasis susceptibility 1 candidate 2	PSORS1C2	8.0	0.0	-8.0
Poliovirus receptor	PVR	1.9	-4.3	-8.0
Splicing factor, arginine/serine-rich 2, interacting protein	SFRS2IP	1.8	-4.6	-8.0
Syntrophin, gamma 1	SNTG1	6.9	-1.2	-8.0
TATA element modulatory factor 1	TMF1	1.4	-5.9	-8.0
Zinc finger protein 236	ZNF236	8.0	0.0	-8.0
Cathepsin S	CTSS	5.8	-1.4	-8.1
Deleted in bladder cancer 1	DBC1	1.1	-7.2	-8.1
Leukotriene B4 receptor	LTB4R	1.9	-4.3	-8.1
Neurofilament, light polypeptide 68kDa	NEFL	-1.1	-8.6	-8.1
ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	1.5	-5.4	-8.2

ADAM metallopeptidase domain 6	ADAM6	2.1	-3.9	-8.2
Claudin 1	CLDN1	-1.1	-8.8	-8.2
Cullin 1	CUL1	2.0	-4.2	-8.2
Dynein, light chain, LC8-type 1	DYNLL1	4.5	-1.8	-8.2
Farnesyl-diphosphate farnesyltransferase 1	FDFT1	1.7	-4.8	-8.2
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	KRAS	1.1	-7.7	-8.2
Opioid binding protein/cell adhesion molecule-like	OPCML	3.2	-2.5	-8.2
Tribbles homolog 1 (Drosophila)	TRIB1	1.1	-7.6	-8.2
Chemokine (C-C motif) ligand 14	CCL14	2.6	-3.2	-8.3
Homeobox A9	HOXA9	3.9	-2.1	-8.3
Neurturin	NRTN	2.6	-3.2	-8.3
Cyclin A2	CCNA2	5.6	-1.5	-8.4
Cellular retinoic acid binding protein 2	CRABP2	3.2	-2.6	-8.4
Dyskeratosis congenita 1, dyskerin	DKC1	10.0	1.2	-8.4
Nucleosome assembly protein 1-like 4	NAP1L4	1.9	-4.4	-8.4
Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	1.4	-6.2	-8.4
Glutamyl-tRNA synthetase 2, mitochondrial (putative)	EARS2	1.6	-5.2	-8.5
Epidermal growth factor receptor pathway substrate 8	EPS8	7.3	-1.2	-8.5
Hypothetical protein LOC55565	LOC55565	1.3	-6.4	-8.5
Exportin 5	XPO5	3.0	-2.8	-8.5
Hypothetical gene supported by BC040598	LOC400960	1.7	-5.2	-8.6
Sperm associated antigen 6	SPAG6	2.6	-3.4	-8.6
BCL2-associated athanogene	BAG1	2.3	-3.8	-8.7
Glucocorticoid modulatory element binding protein 2	GMEB2	9.1	1.0	-8.7
Interleukin 18 (interferon-gamma-inducing factor)	IL18	4.4	-2.0	-8.7
Neural retina leucine zipper	NRL	1.2	-7.0	-8.7
GLE1 RNA export mediator-like (yeast)	GLE1L	1.3	-6.6	-8.8
Human interleukin 2	IL-2	3.2	-2.7	-8.8
Pleckstrin and Sec7 domain containing 2	PSD2	1.6	-5.4	-8.8
Zinc finger, CCHC domain containing 4	ZCCHC4	2.8	-3.1	-8.8
CDC42 effector protein (Rho GTPase binding) 2	CDC42EP2	2.6	-3.4	-8.9
CDC5 cell division cycle 5-like (S. pombe)	CDC5L	2.3	-3.8	-8.9
Deleted in liver cancer 1	DLC1	3.3	-2.7	-8.9
Glutamyl-tRNA synthetase 2, mitochondrial (putative)	EARS2	2.4	-3.7	-8.9
CXYorf1-related protein	FLJ00038	1.6	-5.6	-8.9
Keratin 27	KRT27	1.8	-4.9	-8.9
Leucine-rich repeat-containing G protein-coupled receptor 4	LGR4	6.0	-1.5	-8.9
Membrane associated guanylate kinase, WW and PDZ domain containing 2	MAGI2	-1.0	-9.0	-8.9
Solute carrier family 35 (UDP-glucuronic	SLC35D1	1.4	-6.2	-8.9

acid/UDP-N-acetylgalactosamine dual transporter), member D1				
Synaptotagmin XIII	SYT13	1.6	-5.5	-8.9
TNF receptor-associated factor 1	TRAF1	5.1	-1.8	-8.9
Adenosine deaminase, RNA-specific	ADAR	2.7	-3.3	-9.0
ATPase, Class II, type 9B	ATP9B	1.2	-7.7	-9.0
Calcium channel, voltage-dependent, alpha 2/delta 3 subunit	CACNA2D3	1.4	-6.5	-9.0
Cell cycle related kinase	CCRK	1.4	-6.3	-9.0
Kinesin family member 1B	KIF1B	1.8	-5.0	-9.0
Nucleolar and coiled-body phosphoprotein 1	NOLC1	4.9	-1.9	-9.0
Regulatory factor X, 4 (influences HLA class II expression)	RFX4	5.2	-1.7	-9.0
Cadherin 2, type 1, N-cadherin (neuronal)	CDH2	2.9	-3.1	-9.1
Sialidase 1 (lysosomal sialidase)	NEU1	2.9	-3.1	-9.1
Excision repair cross-complementing rodent repair deficiency, complementation group 6	ERCC6	0.0	-9.2	-9.2
Granulin	GRN	1.3	-7.0	-9.2
Human immunodeficiency virus type I enhancer binding protein 2	HIVEP2	3.7	-2.5	-9.2
Protein phosphatase 5, catalytic subunit	PPP5C	2.2	-4.2	-9.2
Actin, gamma 2, smooth muscle, enteric	ACTG2	1.5	-6.3	-9.3
Fibroblast growth factor (acidic) intracellular binding protein	FIBP	2.5	-3.7	-9.3
KIAA1166	KIAA1166	2.3	-4.1	-9.3
Phospholipase A2-activating protein	PLAA	5.9	-1.6	-9.3
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	3.0	-3.2	-9.4
Zinc finger protein 532	ZNF532	2.4	-3.8	-9.4
Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 6	EDG6	5.0	-1.9	-9.5
Nuclear receptor subfamily 4, group A, member 1	NR4A1	3.8	-2.5	-9.5
Thymidine kinase 2, mitochondrial	TK2	2.2	-4.3	-9.5
Early growth response 1	EGR1	3.4	-2.8	-9.6
Uroporphyrinogen decarboxylase	UROD	1.3	-7.3	-9.6
Upstream transcription factor 2, c-fos interacting	USF2	1.1	-8.6	-9.6
Synapsin III	SYN3	1.2	-8.2	-9.8
Interferon, gamma	IFNG	6.6	-1.5	-9.9
Steroid receptor RNA activator 1	SRA1	1.6	-6.3	-9.9
Transmembrane 7 superfamily member 3	TM7SF3	2.1	-4.7	-9.9
Importin 7	IPO7	2.6	-3.9	-10.2
Rabaptin, RAB GTPase binding effector protein 1	RABEP1	2.1	-5.0	-10.4
Glia maturation factor, beta	GMFB	1.8	-5.7	-10.5
Chromosome 14 open reading frame 138	C14orf138	4.1	-2.6	-10.6
ATP-binding cassette, sub-family C (CFTR/MRP), member 10	ABCC10	3.2	-3.3	-10.7

Forkhead box P1	FOXP1	2.6	-4.1	-10.8
Threonyl-tRNA synthetase	TARS	2.1	-5.4	-11.1
Phosphodiesterase 6D, cGMP-specific, rod, delta	PDE6D	4.4	-2.6	-11.3
Chondroitin sulfate proteoglycan 5 (neuroglycan C)	CSPG5	3.5	-3.3	-11.6
SEC24 related gene family, member D (S. cerevisiae)	SEC24D	3.9	-3.0	-11.6
Ethanolamine kinase 1	ETNK1	4.5	-2.6	-11.7
Amiloride-sensitive cation channel 2, neuronal	ACCN2	3.8	-3.2	-11.9
Tumor protein D52-like 2	TPD52L2	6.9	-1.8	-12.3
BCL2-like 2	BCL2L2	1.6	-7.8	-12.4
Excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	ERCC3	3.6	-3.4	-12.4
V-ski sarcoma viral oncogene homolog (avian)	SKI	3.2	-4.3	-13.7

The array studies were carried out using cDNA arrays carrying 19,200 clones prepared by the Wistar Genomics Core as previously described (Kari et al.). The gene names and symbols were derived from Source (https://source-search.princeton.edu)

Only the 236 annotated genes (of 330) with ratios greater that 8 fold up or down are listed. The data has been QLZ-transformed (quantile normalized, Log2-transformed, with subsequent column standardization by z-score transformation). The ratios in the last 3 columns are based on Normalized Median Densities (expression values) and are calculated by squaring the given values for the log2 ratios and can be considered as fold change. The array study was used as an exploratory tool to identify the genes that were most differentially expressed by IL-12 or IL-23 treatment for further study. The data are sorted by the last column.

*fold change for PHA blasts stimulated with IL-12; **fold change for PHA blasts stimulated with IL-23; *** (last column): ratio of fold induction by IL-23 to fold induction by IL-12. Positive values represent induction by IL-23 by more than 8 fold relative to IL-12 (highlighted in yellow in the last column); negative values represent induction by IL-12 by more than 8 fold relative to IL-23 (highlighted in blue in the last column).

Reference

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Supplementary Table 2. Clinical and demographic characteristics of MS patients who provided samples for this study

Pt no.	Age	Sex	MS duration	MS type (yrs SP)	EDSS	Expt Fig
1	28	M	2	RR	2	5 a-c
2	39	F	7	RR	2.5	5 a-c
3	48	F	11	RR	4.5	5 a-c
4	44	F	18	RR	2	5 a-c
5	38	M	8	SP (2)	6	5 a-c
6	46	F	10	RR	3	5 a-c
7	52	F	2	RR	3	5 a-c
8	53	F	10	RR	3.5	5 a-c
9	41	M	2	RR	1	5 a-c
10	40	M	3	RR		5 a-c
11	65	F	28	RR	1.5	5 a-c
12	46	F	2	RR		5 a-c
13	41	M	5	RR	3.5	5 a-c
14	54	M	2	SP		5 a-c
15	44	F	9	RR	4.5	5 a-c
16	39	F	21	SP (1)	5	5 a-c
17	49	F	9	RR	2.5	5 a-c
18	38	F	19	RR	2	5 a-c
19	35	F	29	RR (trans SP)		5 a-c
20	38	F	2	RR (trans SP)	4.5	5 a-c
21	41	F	4	RR	1.5	5 a-c
22	44	M	2	RR	2.5	5 a-c
23	35	M	1	RR	3	5 a-c
24	34	F	1			5 a-c
25	39	F	3	RR		5 a-c
26	55	F	21	SP (3)	6	5 a-c
27	32	M	7	RR	4.5	5 a-c
28	41	F	13	SP (4)		5 a-c
29	57	M	4	SP (5)		5 a-c
30	57	F	10	SP (3)		5 a-c
31	45	M	3	RR (trans SP)	6	5d
32	48	F	10	RR (trans SP)	4.5	5d
33	50	F	25	RR	2	5d
34	30	M	6	RR	2	5d
35	43	F	6	RR	3	5d
36	33	F	2	RR	2.5	5d

37	54	F	15	SP (3)	6	5d
38	42	F	7	RR	2.5	5d

Pt= patient; MS duration is in years from the onset of symptoms; RR=relapsing-remitting; SP=secondary progressive; RR (trans SP)= RR in transition to SP; number in brackets after SP represents number of years since transition to SP; EDSS= expanded disability status scale score (Kurtzke 1983); Expt Fig refers to the figure in the text depicting the experiment for which the sample of the patient was used.

Reference:

Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology*. 1983;33(11):1444-1452. doi:10.1212/wnl.33.11.1444

The authors report no conflict of interest related to this study.

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