

T helper 9 cells induced by plasmacytoid dendritic cells regulate interleukin-17 in multiple sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by persistent inflammation orchestrated by cluster of differentiation (CD) 4 T helper (Th) cells. In particular, Th1 and Th17 cells amplify, whereas T regulatory (Treg) cells moderate inflammation. The role of other Th subsets in MS is not clear. In the present study, we investigated the generation of different Th responses by human dendritic cells (DCs) in MS. We compared the production of several Th cytokines by naive CD4⁺ T-cells polarized with myeloid and plasmacytoid DCs (mDCs and pDCs) in healthy donors (HD) and relapsing–remitting (RR)-MS patients. We found that resiquimod-stimulated mDCs were able to activate Th17 differentiation, whereas pDCs induced interleukin (IL)-10-producing Th cells. Surprisingly, resiquimod-stimulated pDCs from MS patients also significantly induced the differentiation of Th9 cells, which produce IL-9 and are known to be involved in allergic diseases. We investigated the potential role of IL-9 in MS. We found that IL-9 activated signal transducer and activator of transcription (STAT) 1 and STAT5 phosphorylation and interfered with IL-17 and interferon (IFN) regulatory transcription factor (IRF)-4 expression in Th17-polarized cells. Moreover, in the cerebrospinal fluid (CSF) of 107 RR-MS patients, IL-9 inversely correlated with indexes of inflammatory activity, neurodegeneration and disability progression of MS. High levels of IL-9 were associated with the absence of IL-17 in the CSF of RR-MS patients. Our results demonstrate a Th9-inducing potential of pDCs in MS, suggesting an immunoregulatory role leading to attenuation of the exaggerated Th17 inflammatory response.

Key words: cerebrospinal fluid, dendritic cells, interleukin-9, interleukin-17, multiple sclerosis, T helper cells.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Autoreactive T-cells that are normally controlled by ‘the tolerance machinery’ generate autoimmune reactions against myelin, thus resulting in severe inflammation and CNS damage [1]. Cytokine-producing cluster of differentiation (CD) 4 T helper (Th) cells act as principal modulators of the effector T-cell immune response. Upon

activation by the innate immune system, distinct Th lineages are generated from naive CD4⁺ T-cells depending on the environmental signals present during activation [2]. Until now, two major cell subsets, Th1 and Th2, were described in the adaptive immune responses established to eradicate pathogens through the production of interferon (IFN)- γ or interleukin (IL)-4, -5 and -13 respectively [3,4]. In the past 10 years, three additional Th cell subtypes have been discovered and designated Th17, Th9 and Th22 cells, according to the signature cytokine secreted by

Abbreviations: ARR, annualized relapse rate; CD, cluster of differentiation; CNS, central nervous system; CSF, cerebrospinal fluid; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; HD, healthy donor(s); HIL9, high IL-9; i.v., intravenously; IFN, interferon; IL, interleukin; IL-9R, IL-9 receptor; IRF, interferon regulatory transcription factor; L-IL9, low IL-9; mDC, myeloid dendritic cell; MS, multiple sclerosis; MSSS, MS severity scale; MV, macular volume; NFL, neurofilament light chain; OCT, optical coherence tomography; OR, odds ratio; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; PI, progression index; RNFL, nerve fibre layer; ROR, retinoic acid-related orphan receptor; RR, relapsing–remitting; RT, reverse transcription; SE, standard error; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; Th, T helper; TLR, Toll-like receptor; TNF, tumour necrosis factor; Treg, T regulatory.

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each subset after activation (IL-17, IL-9 and IL-22 respectively) [5–7]. Moreover, dendritic cells (DCs) may also lead to the development of T regulatory (Treg) cells, which exert inhibition and neutralization of effector cells by several mechanisms, including release of IL-10 [8,9]. Treg cells are known to be protective in MS [10], whereas effector Th1 and Th17 cells contribute to tissue inflammation and exacerbation of autoimmune pathology [11,12]. Moreover, the presence of IFN- γ -producing Th1 and IL-17-producing Th17 cells in the cerebrospinal fluid (CSF) of MS patients firmly points to their involvement in human CNS inflammation [13,14].

The presence of Th22 cells in MS has also been previously described [15,16], whereas the differentiation and the role of Th9 cells in the disease have never been investigated.

The differentiation of Th subsets is mediated by activated DCs. There are two main subsets of peripheral blood DCs in humans: myeloid (mDC) and plasmacytoid (pDC), each expressing distinct sets of pattern-recognition receptors and responding to different microbial products, suggesting that they may activate different Th cell responses [17,18]. Thus, the response of DCs to activating signals and the activation of an appropriate Th response is a critical issue in autoimmune disorders, where the balance between inflammatory and regulatory responses is deregulated.

To date, little is known about the generation of Th cells in MS patients. In the present study, we identified a pDC-driven Th9 immunoregulatory pathway, which may contribute to decrease pathogenic Th17 inflammation and disease progression.

MATERIALS AND METHODS

MS subjects for blood and CSF collection

Patients with relapsing–remitting (RR)-MS according to established criteria [19] were enrolled in the study. Demographic and clinical data of RR-MS patients included in the study are described in Table 1 (for blood sampling) and Table 2 (for CSF sampling).

As controls, we used blood or CSF from age- and gender-matched individuals without inflammatory or degenerative diseases of the central or peripheral nervous system. These subjects were volunteers that underwent drawing (for blood) or patients who underwent lumbar puncture because of a clinical suspicion of acute peripheral neuropathy, meningitis or subarachnoidal haemorrhage, which were not confirmed (for CSF).

Approval by the ethics committee of the Policlinico Tor Vergata, Santa Lucia Foundation and San Camillo Hospital and written informed consent in accordance with the Declaration of Helsinki from all participants were obtained before study initiation.

After their admittance, patients underwent blood and CSF sampling, full neurological assessment and brain (and in selected case also spinal) MRI scan. All patients included in the blood study did not take immunomodulant or immunosuppressive compounds at least 2 months before recruitment (Table 1). All subjects included in the CSF study were not treated before CSF collection (Table 2).

Table 1 Demographic and clinical characteristics of MS subjects at the time of naive-DC co-culture

Parameter	Value
Number	26
Gender (male/female)	6/20
Age (years)	42 \pm 14
Disease duration (years)	13 \pm 12
EDSS	2 \pm 2.0
MRI (gadolinium+/-)	5/21

For the follow-up of the CSF study, all RR-MS patients started immunomodulatory treatment (IFN- β 1a, $n = 44$; IFN- β 1b, $n = 33$; glatiramer acetate, $n = 30$) after CSF collection. Mitoxantrone [12 mg/m² i.v. (intravenously) every 3 months with a lifetime maximum of 140 mg/m²], natalizumab (300 mg i.v. every 4 weeks) and fingolimod [0.5 mg p.o. (*per os*) every day] were considered as second-line treatments for patients who experienced at least two relapses during 1 year of therapy with other approved immunomodulatory agents. Demographic and clinical informations were derived from medical records. MS disease onset was defined as the first episode of focal neurological dysfunction indicative of MS. Disease duration was estimated as the number of years from onset to the last assessment of disability. Relapses were defined as the development of new or recurrent neurological symptoms not associated with fever or infection lasting for at least 24 h.

Purification of naive CD4⁺ T lymphocytes from adult blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (GE Healthcare) from 100 ml of whole blood of RR-MS patients or healthy donor (HD) volunteers, stained with the following antibodies: lineage (CD3, CD14, CD16, CD19)–PE/eCy7 (phycoerythrin/indotricarbocyanine) (Coulter), CD11c–FITC (Miltenyi Biotec), human leukocyte antigen (HLA)–DR–V450 (eBioscience), CD4–ECD (Coulter), CD27–APC (allophycocyanin) (eBioscience), CD45RA–phycoerythrin (PE) (BD). pDC (lineage⁻, CD4⁺, CD11c⁻), mDC (Lineage⁻, CD4⁺, CD11c⁺), CD4⁺ naive T-cells (lineage⁺, CD4^{high}, CD45RA^{high}, CD27⁺) were sorted simultaneously with a MoFlo high speed cell sorter (Coulter). Gating strategy is shown in Supplementary Figure S1(A). All sorted cells had a purity of over 96%, as shown by flow cytometry (Supplementary Figure S1B).

Cells purified from healthy blood donor volunteers were used as controls in the experiment of Figure 1; cells purified from buffy coats (Policlinico Tor Vergata) were used in all other experiments where comparison with fresh blood from MS patients was not required.

Th cell differentiation assay

Naive CD4⁺ T-cells were cultured in 96-well round-bottomed plates (Corning) at a density of 5×10^4 per well in X-VIVO 15 serum-free medium (Lonza) in the presence of Dynabeads CD3–

Table 2 Demographic and clinical characteristics of MS subjects at the time of CSF collection

	IL-9			P
	Total	High (>100 pg/ml)	Low (<100 pg/ml)	
Number	107	50	57	–
Gender (male/female)	42/65	19/31	23/34	0.84
Age (years)	32.0 ± 6.0	32.2 ± 6.1	31.8 ± 6.0	0.77
Disease duration (years)	4.8 ± 2.8	5.3 ± 2.9	4.4 ± 2.8	0.11
EDSS	1.6 ± 1.0	1.3 ± 0.9	2.0 ± 1.0	<0.05
MRI (gadolinium+/-)	44/63	21/29	23/34	1.0

CD28 T-cell expander (one bead per cell; Life Technologies) and indicated cytokines: IL-1 β (10 ng/ml), IL-6 (20 ng/ml), transforming growth factor (TGF)- β (1 ng/ml) and IL-23 (100 ng/ml) (Miltenyi) for Th17 differentiation, as previously described [20], TGF- β (5 ng/ml) and IL-4 (50 ng/ml; Miltenyi) for Th9 differentiation. In some experiments recombinant human IL-9 and IL-2 (Miltenyi) were used. After 5–6 days, cells were harvested, extensively washed and viability was determined by Trypan Blue exclusion. Then, 1×10^6 cells/ml were re-stimulated with Dynabeads CD3–CD28 T-cell expander (one bead per cell) for 24h [for cytokine analysis and reverse transcription (RT)-PCR].

Analysis of cytokine production

Cytokines in culture supernatants were measured by IL-17 ELISA (R&D Systems), IL-9 ELISA (eBioscience), IFN- γ , IL-13, IL-22 and IL-10 Flow Cytomix (eBioscience) according to the manufacturer's instructions.

Real-time quantitative RT-PCR

Total RNA was extracted using an RNeasy Microkit (Qiagen). A mixture containing random hexamers, Oligo (dT)₁₅ (Promega) and Super Script II Reverse Transcriptase (Life Technologies) were used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an LC480 (Roche) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Taqman Gene expression Master Mix (Life Technologies). The following probes were used (identified by Applied Biosystems assay identification number): IL-17A, retinoic acid-related orphan receptor (ROR) C, IFN regulatory transcription factor (IRF)-4, IL-9 receptor (IL-9R). For each sample, mRNA abundance was normalized to the amounts of ribosomal protein L-34.

Western blot analysis

For protein extraction, Th cells were resuspended in RIPA buffer (50 mM Tris/HCl, pH 8, 200 mM NaCl, 2 mM EDTA, 1 % NP-40, 0.5 % sodium deoxycholate, 0.05 % SDS, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 5 mM NaF and 1 mM PMSF freshly added). After 15 min on ice, cell lysates were centrifuged for 15 min at 15 000 g at 4 °C and the supernatants were collected and used for Western blot analyses. Cell extracts were diluted in Laemmli buffer and boiled for 5 min at 95 °C. Proteins were separated on SDS/PAGE (8 % gel) gels and transferred on to nitrocellulose membranes (Whatman, Sigma–Aldrich, GE Healthcare Life Science) using a wet blotting apparatus (Amersham Biosciences). Membranes were saturated for 1 h at room

temperature with 3 % non-fat dry milk in PBS, containing 0.1 % Tween 20. Membranes were incubated with the following antibodies overnight at 4 °C: rabbit polyclonal anti-human phospho signal transducer and activator of transcription (STAT) 1 (Cell Signaling Technology; 1:1000 dilution), rabbit polyclonal anti-human phospho-STAT3 (Cell Signaling Technology; 1:1000 dilution), mouse IgG1 anti-human phospho-STAT5 (BD Transduction Laboratories; 1:500 dilution), mouse anti-human actin (Abcam; 1:10000 dilution). All antibodies were diluted in 3 % non-fat dry milk in PBS, containing 0.1 % Tween 20. Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Cell Signaling Technology) were incubated with the membranes for 1 h at room temperature at a 1:2000 dilution in PBS containing 0.1 % Tween 20. Immunostained bands were detected using a chemiluminescence method (Pierce ECL Western Blotting Substrate, Thermo Scientific).

CSF analysis

CSF was centrifuged to eliminate cells and cellular debris and immediately stored at –80 °C until analysed. All samples were processed using identical standardized procedures using Bio-Plex Multiplex Cytokine Assay (Bio-Rad Laboratories), according to the manufacturer's instructions. Concentrations of IL-4, IL-9 and IL-17 were calculated according to a standard curve generated for each target and expressed as pg/ml. When the concentrations of the cytokines were below the detection threshold, they were assumed to be 0 pg/ml.

Neurofilament light chain (NfL) levels in CSF were assayed using a commercially available ELISA kit (UmanDiagnostics NF-light assay). The levels of NfL in CSF were determined by fitting data to a four-parameter standard curve using the GraphPad Prism Software Package.

Analysis of clinical parameters

The annualized relapse rate (ARR) was defined as the number of relapses per year. Disability was determined by a specially trained and certified examining neurologist using the expanded disability status scale (EDSS), a 10-point disease severity score derived from nine ratings for individual neurological domains [21]. The EDSS, evaluated every 6 months since diagnosis, was used in combination with disease duration to calculate two measures of disease severity, the progression index (PI) and the mean MS severity scale (MSSS). PI was defined as EDSS/disease duration [22]. The MSSS is an algorithm that relates EDSS scores to distribution of disability in patients with comparable disease

durations [23]. Four years of follow-up were available for each patient.

Optical coherence tomography

Medical history with respect to visual symptoms was taken from all MS subjects. Self-report and physician report were confirmed by record review. A subset of RR-MS patients ($n = 88$) without history of optic neuritis and ophthalmological disease underwent measurement of nerve fibre layer (RNFL) thickness and macular volume (MV) for both eyes using Stratus Optical Coherence Tomography (OCT) software version 4.0.2, Carl Zeiss Meditec, Inc. [22,24]. Briefly, for MV, retinal thickness was measured automatically as the distance between the vitreoretinal interface and the anterior boundary of the retinal pigment epithelium. Stratus OCT images were generated using the fast map scan protocol consisting of six radial scans spaced 30° apart, with each scan measuring 6 mm in length. Each image had a resolution of $10 \mu\text{m}$ axially and $20 \mu\text{m}$ transversally. All Stratus OCT images had signal strength of $6 \mu\text{m}$. RNFL thickness measurements were read from the automated measurements generated by the machine using the Fast RNFL analysis. For the study, scanning was performed after pharmacological dilation. Average RNFL thickness for 360° around the optic disc was recorded. Values were adjusted for age. One randomly chosen eye from each subject was included in the study. Testing was performed by trained technicians experienced in examination of patients for research studies and patients wore their habitual glasses or contact lenses for distance correction.

MRI

MRI examination (1.5 Tesla) consisted of dual-echo proton density, fast fluid-attenuated inversion recovery, T2-weighted spin-echo images and pre-contrast and post-contrast T1-weighted spin-echo images. All images were acquired in the axial orientation with 3-mm-thick contiguous slices. The presence of gadolinium (0.2 ml/kg i.v.)-enhancing lesions was evaluated by a neuroradiologist who was unaware of the patient's clinical details [25].

Statistical analysis

For pair-wise comparisons of different conditions from the same donors or different donors, we used a non-parametric two-tailed paired or unpaired Student's *t* test respectively. *P*-values of 0.05 or less were considered statistically significant. Differences among groups were compared by univariate analysis using Student's *t* test or Mann-Whitney test for continuous variables and Fisher's exact test for categorical variables. Survival curves were analysed using log-rank (Mantel-Cox) test. Correlation analysis was performed by calculating Pearson's coefficient. Logistic regression model was constructed for the disability as outcome. We estimated the degree of disability by means of the dichotomous EDSS (cut-off point of 4.0, at which restriction in ambulation starts to be appreciated).

As reported in the literature, the lower scale values (0–4.0) are influenced by impairments detected by the neurological exam of eight functional systems, whereas the values above 4.0 are mainly based on walking ability and values above 6.0 mainly on

patients' handicaps [21,26–28]. Acquisition of EDSS steps 4 or above is considered a sufficient milestone because, once reached, MS inevitably progresses [29]. In line with this, the degree of disability in MS is usually analysed by means of the dichotomous EDSS with a cut-off point of 4.0 [30–32].

Six variables (years of disease, age, gender, ARR, use of second-line treatments and CSF cytokine contents) were included as predictor variables. Coefficients with standard error (SE) and odds ratio (OR) with 95 % confidence interval are provided. Two-way ANOVAs were performed to analyse the main effects and interactions of two conditions on the dependent variables.

Data are presented as means \pm S.D. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Plasmacytoid dendritic cells from RR-MS patients induce Th9 polarization

Given the crucial role of DCs in polarizing Th responses, we hypothesized a differential role of DCs obtained from MS patients compared with DCs from HD in driving Th differentiation. To test this hypothesis, naive CD4^+ T-cells from HD and RR-MS patients were co-cultured with autologous pDCs or mDCs stimulated with resiquimod (R-848), an agonist for Toll-like receptor (TLR) 7 expressed by pDCs and for TLR8 expressed by mDCs (Table 1).

We analysed cytokine production by polarized Th cells after 6 days of co-culture. We found that IFN- γ and IL-13 were not induced after Th cell activation by either pDCs or mDCs in both HD and MS (Figures 1A and 1B). In contrast, in the same conditions, stimulated mDCs induced significant levels of IL-17 whereas pDC induced IL-10 by Th cells in both groups of individuals (Figures 1C and 1D).

We found that resiquimod-simulated pDCs also promoted Th22 differentiation in HD (Figure 1E) and surprisingly, activated pDCs from MS patients induced significant IL-9 production by Th cells (Figure 1F). The induction of IL-9 by pDCs from MS patients was found regardless of the phase of disease, indicating that blood pDCs are not influenced by the stage of inflammation (results not shown). We verified that pDCs from MS patients and HD expressed similar levels of TLR 7, indicating that differences in Th9 induction were not related to a different sensitivity to the stimulus (Supplementary Figure S2).

In order to investigate whether the higher Th9 polarization observed in MS patients compared with HD was related to a differential response of pDCs or rather to a specific predisposition of naive CD4^+ T-cells in MS patients, we cultured naive CD4^+ T-cells with Th9-polarizing cytokines in the presence of anti-CD3 and anti-CD28. IL-9 production in Th9 conditions was compared with control Th0, generated in the absence of polarizing cytokines. The results showed that naive CD4^+ T-cells cultured in the presence of IL-4 and TGF- β produced similar levels of IL-9 in HD and in MS patients (Supplementary Figure S3). These results indicated that the Th9 differentiation machinery in naive CD4^+ T-cells was not altered in MS patients, implying that the

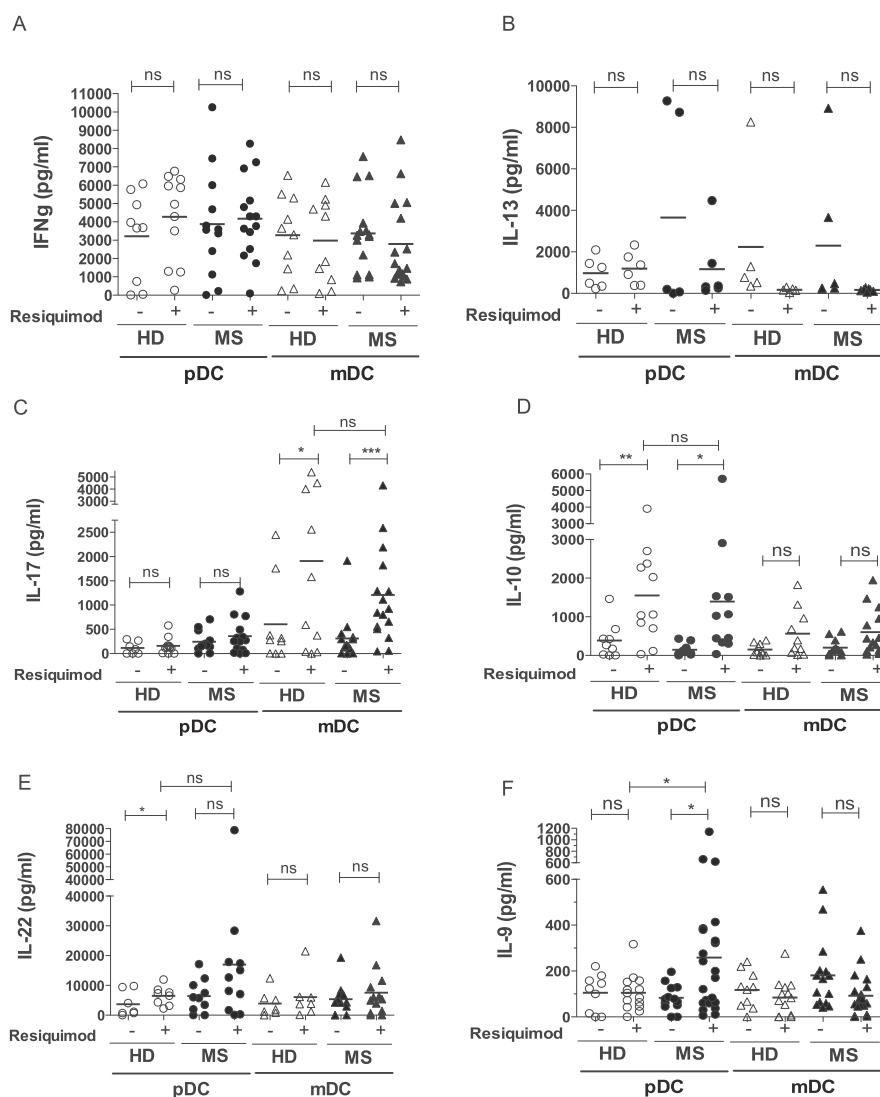


Figure 1 pDCs from RR-MS patients induce Th9 polarization
 pDCs and mDCs from HD and RR-MS patients were activated for 24 h with resiquimod and co-cultured with autologous naive CD4⁺ T-cells (ratio 1:1) in the presence of anti-CD3-CD28-coated beads for 6 days, then re-stimulated for 24 h. Protein expression of T-cell-derived cytokines IFN- γ (A), IL-13 (B), IL-17 (C), IL-10 (D), IL-22 (E) and IL-9 (F) was analysed by Flow Cytomix or by ELISA. A paired Student's *t* test was used to compare no cytokines and Th9 cocktail. An unpaired Student's *t* test was used to compare HD and RR-MS. **P* < 0.05, ***P* < 0.005.

differential Th9 polarization observed in MS was rather related to a peculiarity of pDCs.

IL-9 in the CSF of RR-MS subjects inversely correlates with disease severity

To investigate the role of IL-9 in MS, IL-9 levels were measured in the CSF of control subjects (*n* = 70, 27 males, aged 31.6 ± 6.1 years) and RR-MS patients (*n* = 107, 42 males, aged 32.0 ± 6.0 years). No significant difference was observed between these two groups (control: 132.3 ± 230.3 pg/ml; MS: 193.3 ± 244.6 pg/ml; *P* = 0.09). Furthermore, no significant dif-

ferences were found categorizing MS patients according to the absence (gadolinium-, *n* = 63; 180.6 ± 231.4 pg/ml) or the presence (gadolinium+, *n* = 44; 211.4 ± 264.1 pg/ml) of contrast-enhancing lesions at baseline MRI (*P* > 0.1), indicating that IL-9 levels in the CNS were not influenced by the acute stage of inflammation.

To further investigate the potential role of IL-9 on disease course, RR-MS patients were stratified according to IL-9 levels in the CSF in a high IL-9 (H-IL9, *n* = 50) and a low IL-9 group (L-IL9, *n* = 57), using a cut-off value of 100 pg/ml (Table 2), which was near to mean levels in controls and followed sample

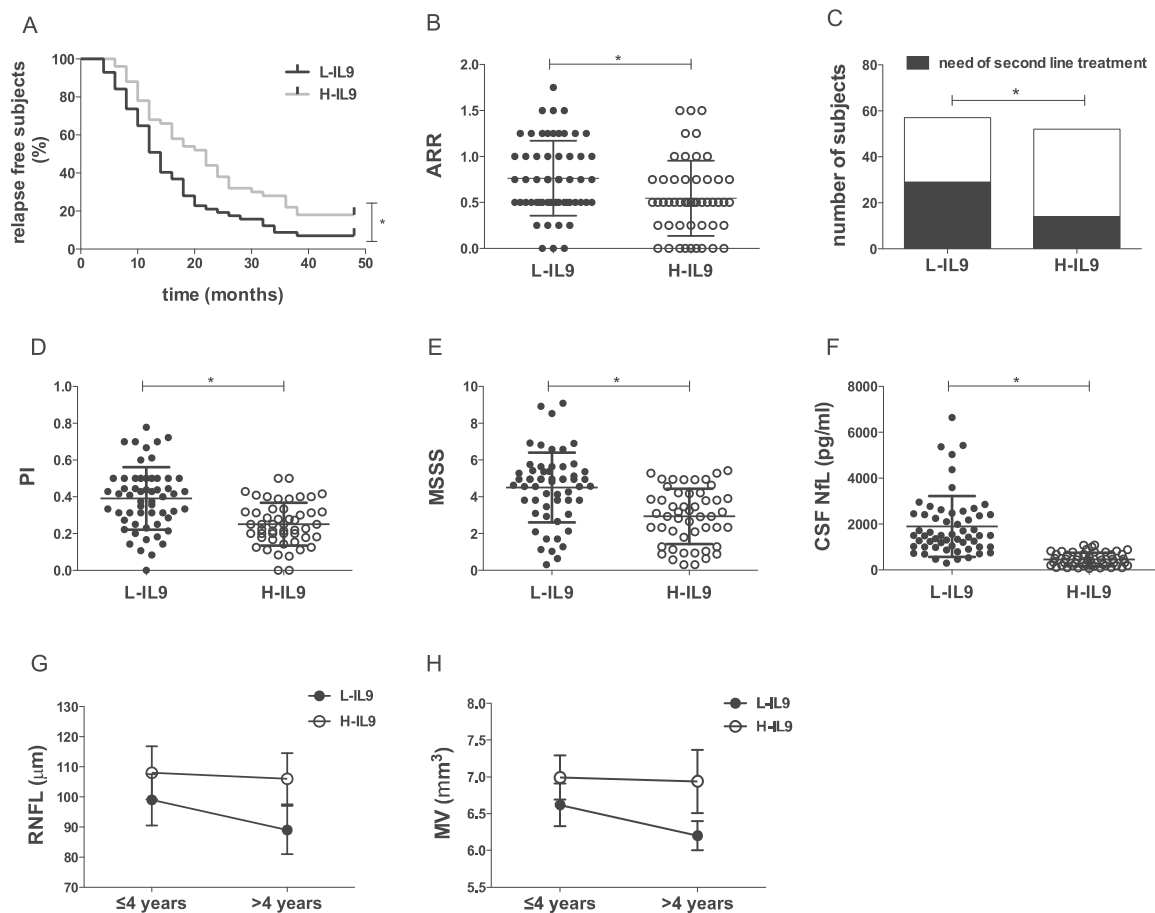


Figure 2 IL-9 in the CSF of RR-MS subjects inversely correlates with disease severity. Survival analysis for the time to first clinical relapse since diagnosis, among subjects with high or low levels of IL-9 in the CSF (100 pg/ml as cut-off value; **A**). Mean ARR (**B**), the number of subjects treated with second-line drugs (**C**) and the disability progression, measured as a PI (**D**) or MSSS (**E**), were reported in RR-MS subjects with L-IL9 and H-IL9 in the CSF (100 pg/ml as cut-off value). NfL, a soluble marker of neurodegeneration, was measured by ELISA (**F**) in CSF of RR-MS subjects with L-IL9 and H-IL9 (100 pg/ml as cut-off value). Interaction between IL-9 in CSF and disease duration was analysed based on OCT parameters, RNFL thickness (**G**) and MV (**H**). Data are represented as means \pm S.D. of 50 H-IL9 and 57 L-IL9 MS patients. * $P < 0.05$.

distribution for statistical analysis. Moreover, we fixed the cut-off level at 100 pg/ml to divide the 107 MS patients in two balanced groups, in order to have a similar number of patients in the H- and L-IL9 groups.

We found that the time to first clinical relapse was affected by the CSF levels of IL-9, being lower in the L-IL9 group, as assessed by survival curve analysis (16.9 ± 11.8 compared with 24.1 ± 14.2 months; $P = 0.007$; Figure 2A). Consistently, the mean ARR in the first 4 years since diagnosis and the number of subjects treated with second-line drugs were significantly lower among subjects with high CSF levels of IL-9 (H-IL9: 28% compared with L-IL9: 51%; $P < 0.05$ for each comparison; Figures 2B and 2C). These data show that high level of IL-9 in the CNS is associated with a less severe inflammatory disease activity in RR-MS.

Furthermore, baseline EDSS, PI and MSSS were significantly lower in the H-IL9 group ($P < 0.05$ for each comparison; Figures 2D and 2E), suggesting that increased levels of IL-9 within the CNS could contrast disability progression in RR-MS patients. Multivariate analysis confirmed the protective effect of IL-9 on disability progression besides its anti-inflammatory activity, predicting a lower risk to reach EDSS 4.0 in the H-IL9 group (coefficient: -1.43 , SE: 0.65, OR: 0.24, $P = 0.02$), at equal values of age, gender, disease duration, ARR and use of second-line treatments.

We then analysed the CSF contents for NfL, which is known to be a valid biomarker of axonal damage and neurodegeneration [33]. Our results showed lower levels of NfL in the H-IL9 group ($P < 0.05$; Figure 2F), suggesting a neuroprotective role for IL-9 in MS. The axonal and neuronal cell loss in MS has been

convincingly associated with reduced RNFL thickness and MV at the OCT [34–36]. Thus, we investigated the possible relationship between IL-9–CSF contents and OCT parameters in a subgroup of RR-MS patients with similar disease duration (disease duration ≤ 4 years: H-IL9 $n = 15$, L-IL9 $n = 24$; disease duration > 4 years: H-IL9 $n = 25$, L-IL9 $n = 24$). A significant effect of IL-9 CSF levels was revealed analysing both RNFL thickness ($F = 50.2$, $P < 0.0001$) and MV ($F = 63.7$, $P < 0.0001$), indicating a more severe damage of neuronal structures within the L-IL9 group. Disease duration slightly affected OCT parameters (RNFL thickness: $F = 10.7$, $P < 0.01$; MV: $F = 11.4$, $P < 0.01$) with a significant interaction with IL-9 contents (RNFL thickness: $F = 4.8$, $P < 0.05$; MV: $F = 7.1$, $P < 0.01$), confirming less severe neurodegenerative damage in subjects with H-IL9 levels in the CSF, a longer disease duration notwithstanding (Figures 2G and 2H).

Since IL-9 is a cytokine associated with Th2 cells, we assessed the specificity of Th9 in the protective effects on MS, replicating the analyses with CSF levels of IL-4, the major cytokine involved in Th2 responses. No association was found between CSF IL-4 levels and any index of disease severity. Comparing between a set of homogeneous patients (no differences in terms of demographic characteristics) with undetectable ($n = 66$) and detectable ($n = 41$) CSF levels of IL-4, we found that mean ARR and PI were not significantly different in the 4 years of follow-up ($P > 0.05$ for each parameter; Supplementary Figures S4A and S4B). In line with this observation, no significant differences in NfL contents were found between the two groups of patients ($P > 0.05$), ruling out the involvement of IL-4 in neuroprotective effects (Figure 3C). Of note, a lack of correlation was also found between IL-9 and IL-4 CSF levels ($P = 0.72$, $r = 0.03$), confirming an independent value of the two cytokines (Supplementary Figure S4D).

IL-9 activates STAT1, STAT5 and reduces IL-17 and IRF4 expression in Th17 cells

Previous data demonstrated an immunosuppressive function of IL-9 that indirectly inhibited the production of pro-inflammatory cytokines [37]. In order to investigate the role of IL-9 in modulating T-cell responses, we analysed the effect of IL-9 on different Th subsets expressing the IL-9R (Supplementary Figure S5). We found that, among all Th profiles, Th17 cells were the most responsive to IL-9 regarding the ability to produce cytokines. IL-9 reduced IL-17 production by Th17 cells and its inhibitory effect was dose-dependent (Figure 3A). Moreover, we found that IL-9 was able to down-regulate *IL17* mRNA, indicating that it exerts its inhibitory effect on IL-17 at the transcriptional level (Figure 3B). IL-9 had no effect on other cytokines produced by Th17 cells, such as TNF- α , IL-6, IFN- γ and IL-10 (Supplementary Figure S6).

In order to identify the mechanisms affected by IL-9 during Th17 polarization, we analysed the key transcription factor for the differentiation programme of Th17 cells, ROR γ t, encoded by the *RORC* gene [38]. We found a strong expression of *RORC* transcript in Th17 cells, but its expression was not modulated by IL-9 treatment (Figure 3C).

However, ROR γ t is not the unique transcription factor regulating IL-17 expression. IRF4 was reported to be also essential for Th17 cell differentiation [39]. In this context, we confirmed the induction of IRF4 in Th17 cells and we found that IL-9 significantly reduced *IRF4* transcript (Figure 3D), suggesting that IL-9 reduced the optimal expression of IL-17 by Th17 cells by inhibiting IRF4 expression.

It is known that upon binding to its cell-surface receptor IL-9 induces recruitment and cross-phosphorylation of Janus kinases (JAKs) 1 and 3 followed by activation of STAT1, STAT3 and STAT5. Consequently, STAT complexes translocate to the nucleus to drive transcription of IL-9-inducible genes [40]. Thus, we analysed the phosphorylation of STAT1, STAT3 and STAT5 on Th17 cells stimulated with IL-9. Interestingly, STAT3, known to be involved in Th17 polarization [41,42] was already phosphorylated on Th17 cells and no modulation was measured after stimulation with IL-9. In contrast, STAT1 and STAT5, which are known to inhibit Th17 polarization [43,44], were activated by IL-9 (Figure 3E) suggesting their potential role in IL-9-mediated modulation of the Th17 response.

IL-9–IL-17 interaction is associated to disability progression and neurodegeneration in MS

Given the inhibitory effect of IL-9 on IL-17 production, we investigated whether IL-9 could reduce IL-17 levels also *in vivo*. We analysed the CSF contents of the two cytokines in RR-MS patients. The proportion of subjects with undetectable levels of IL-17 was significantly higher in the H-IL9 group (90% compared with 58%, $P < 0.01$; Figure 4A).

A 2×2 ANOVA constructed with IL-9 and IL-17, as exploratory conditions, revealed a significant interaction between the two cytokines on both PI ($F = 4.2$, $P < 0.05$; Figure 4B) and NfL CSF contents ($F = 4.3$, $P < 0.05$; Figure 4C). In fact, among subjects of the L-IL9 group, the concomitant detection of IL-17 significantly increased indexes of disability progression (PI) and neurodegeneration (NfL levels).

Conversely, the presence of IL-17 in the CSF was not associated with increases in the ARR ($F = 1.1$, $P > 0.05$), without a significant interaction with IL-9 ($F = 0.35$, $P > 0.05$), ruling out the involvement of IL-17 in the effects of IL-9 on inflammatory activity. A significant effect on relapse rate was in fact observed only for IL-9 levels ($F = 4.3$, $P < 0.05$; Figure 4D), in line with primary univariate analysis.

Thus, the inverse correlation of IL-17 and IL-9 observed in the CSF of MS patients indicated that inhibition of IL-17 by IL-9 might occur also *in vivo*.

DISCUSSION

This is the first study comparing the polarizing ability of pDCs and mDCs derived from the same MS patient in response to the same TLR agonist in an autologous setting. We found that the two DC subsets activate different Th responses: resiquimod-treated pDCs induce IL-10, whereas resiquimod-treated mDCs drive IL-17 production. In MS, bacterial or viral infection can trigger the

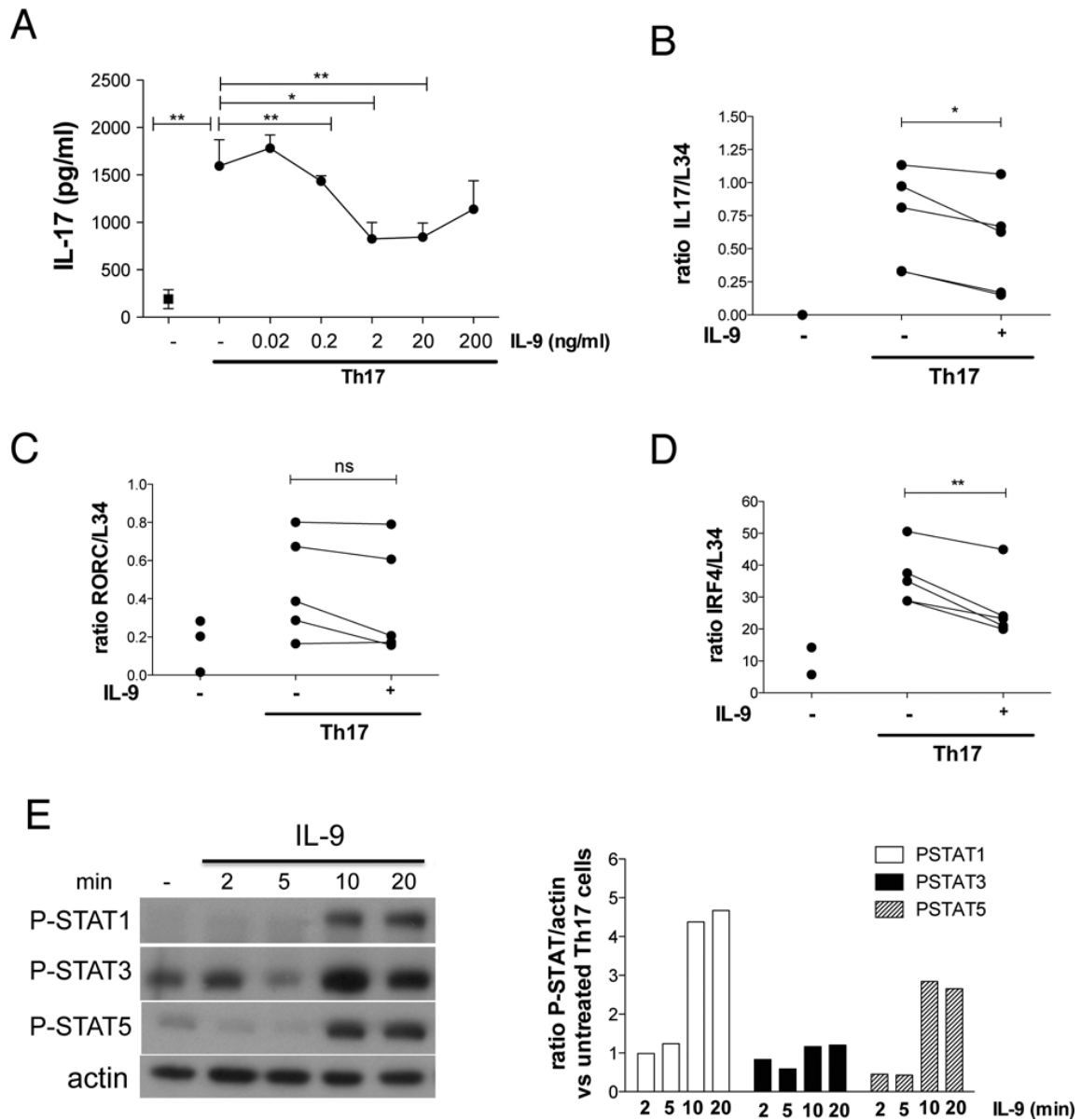


Figure 3 IL-9 activates STAT1 and STAT5 and reduces IL-17 and IRF-4 expression by Th17 cells
 Naive CD4⁺ T-cells were stimulated with anti-CD3 and anti-CD28 in the presence of Th17-polarizing cytokines for 5 days. At day 4, cells were washed and cultured in fresh medium. After 12 h, cells were treated or not with IL-9 for the last period of culture. IL-17 protein in the supernatants after 24 h of re-stimulation with anti-CD3-CD28 was analysed by ELISA. Data are represented as means \pm S.D. for seven donors (A); *IL17* (B), *RORC* (C) and *IRF4* (D) mRNA were analysed after 24 h of re-stimulation with anti-CD3-CD28 by RT-PCR; a paired Student's *t* test was used to compare sample conditions. **P* < 0.05, ***P* < 0.005. STAT1, STAT3 and STAT5 were analysed by Western blot (E) and results from a representative experiment are reported.

onset and/or relapses of the disease via mechanisms of molecular mimicry and via TLR signalling [10,45]. Our results suggest that the same trigger (TLR7/8 agonist) activates both pDCs and mDCs with consequent induction of opposite Th responses, Treg and Th17 respectively, which mediate the balance between

regulation and inflammation. Thus, the type of DC which encounters the stimulus is crucial for the generation of the appropriate Th response.

Our results are consistent with previous data showing that incubation of human PBMC with TLR7/8 ligands promotes the

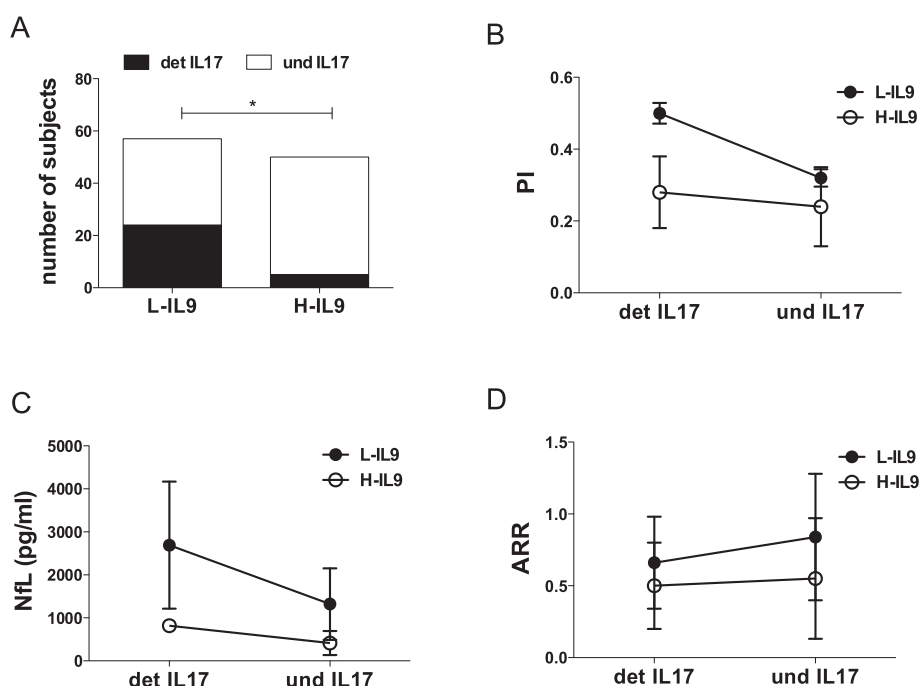


Figure 4 IL-9-IL-17 interaction is associated with disability progression and neurodegeneration in MS

The number of RR-MS subjects with detectable (det) or undetectable (und) IL-17 in CSF of patients with L-IL9 and H-IL9 in the CSF (100 pg/ml as cut-off value) was reported (A). Interaction between IL-9 and IL-17 CSF contents was performed by analysing PI (B), NfL levels (C) and ARR (D) in RR-MS subjects with different levels of cytokines in CSF. Data are represented as means \pm S.D. of 50 H-IL9 and 57 L-IL9 MS patients. * $P < 0.05$.

differentiation of Th17 cells from naive T-cells [46]. In the present study, we find that mDCs are responsible for Th17 induction. Divergent results are shown in another report, where human pDCs stimulated with TLR7 ligands promote Th17 differentiation from naive T-cells [47]. However, in those experiments allogeneic co-cultures were set up, in contrast with the autologous assays used in our study and this could explain the discrepancy. Nonetheless, in line with that paper we observed that stimulated pDCs enhance Th17 responses in memory CD4⁺ T-cells derived from HD (results not shown).

The differentiation of human Th17 cells has been studied using polyclonal activators and recombinant cytokines [20,48], activated monocytes [49,50], inflammatory DCs from ascites [51] and mDCs stimulated with resiquimod [52]. In line with others, we find that blood mDCs activated with resiquimod are capable of polarizing autologous naive CD4⁺ T-cells in Th17 cells, indicating that blood DCs are potential inducers of Th17 cells and that an appropriate environment may disclose their potentiality.

We show that resiquimod-stimulated pDCs are capable of inducing IL-10 producing cells. The induction of IL-10-producing Treg cells by pDCs has been demonstrated following activation with IL-3, CD40 ligand and TLR9 agonists [9,17,53]. This indicates that activation of IL-10 is a typical feature of pDCs, regardless of the nature of the activating stimulus.

We find that resiquimod-stimulated pDCs also promote Th22 differentiation in HD (Figure 1E) in accordance with previous

data demonstrating that oligodeoxynucleotides (CpG)-stimulated pDCs efficiently prime for Th22 polarization through the release of IL-6 and TNF- α [54]. Moreover, we observed for the first time that pDCs from MS patients induce the Th9 profile.

Given the well-described plasticity of pDCs [18], the modulation of new Th profiles by activated pDCs could be expected. Recently, it was described that mDC stimulated with thymic stromal lymphopoietin induce Th9 polarization in asthmatic patients [55]. However, the role of pDC in inducing the Th9 profile has never been reported.

Although the presence of pDCs in different autoimmune diseases, including systemic lupus erythematosus, psoriasis, Sjogren's syndrome, rheumatoid arthritis and MS has been observed [56–59], their protective or inflammatory role is not clear.

On the other hand, IL-9 is a cytokine implicated in human and murine asthma, anaphylaxis, resistance to nematode infection, antiviral immunity and tumorigenesis [60–69]. The role of IL-9 in autoimmunity has been investigated in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). However, these studies have generated contrasting results, which do not allow assigning a clear role for IL-9 in this model [37,70,71].

The role of IL-9 in human autoimmunity has been investigated in psoriasis, where it was shown that IL-9R is increased in lesional compared with healthy skin [72] and in lupus erythematosus, where IL-9 was reported to be highly expressed in the serum [73,74]. However, the lack of correlations with clinical

parameters does not permit to unequivocally define a specific role of IL-9 in human autoimmunity, yet.

Our analysis correlates IL-9 levels in the CSF of RR-MS patients with the course of the disease. A previous study in MS patients showed that IL-9 levels in the CSF were lower during clinical relapses and increased following prednisolone treatment, thus confirming the role of IL-9 in the maintenance of the remission phase in MS [75].

Consistent with our data, it has been demonstrated that mice lacking IL-9R exhibit an increase in Th17 cells during EAE, suggesting that IL-9 could have an inhibitory effect on Th17 cell differentiation *in vivo* [37]. However, in the same paper, *in vitro* experiments indirectly suggest that IL-9 enhances Th17 polarization [37]. Similarly, culture of human PBMC or of purified CD4⁺ T-cells with IL-9 determines an increase in IL-17 production [72].

The divergent effects of IL-9 on human cells could be due to a differential outcome of IL-9 when it interacts with mixed cellular populations present in whole PBMCs or in bulk CD4⁺ lymphocytes, compared with the highly purified CD4⁺ naive T-cells used in the present study.

The activation of STAT1 and STAT5 by IL-9 on Th17 cells indicates that the IL-9R on human Th17 cells is fully functional and suggests that the inhibitory effect of IL-9 on IL-17 production could be mediated by STAT1 or STAT5, which are already known to inhibit Th17 polarization [43,44]. Consistent with our data, the inhibition of IL-17 mediated by STAT5 is independent of ROR γ t. In fact, STAT5 directly represses IL-17 induction by binding to the *IL17* locus, where it removes accessible histone marks and displaces STAT3 occupancy [44]. Our data identify a new possible mechanism contributing to STAT5-mediated IL-17 inhibition, which involves repression of the transcription factor IRF-4.

Importantly, the interaction between IL-17 and IL-9 reveals a general mechanism regulating the Th17 response. In MS, activation of Th9 cells could be an important mechanism regulating the pathogenic inflammation generated by Th17 cells. Moreover, the production of IL-9 by Th17 cells [37] suggests an autocrine loop in which IL-9 produced by Th17 cells participates in controlling and reducing further amplification of the pathogenic and inflammatory activity of these cells.

Our results reveal that in MS pDCs are the first trigger for the activation of the immunoregulatory response of Th9 cells. Interestingly, we found that IL-9 inversely correlates with the progression of MS, probably due to an *in vivo* inhibition of IL-17. Thus, in the context of the discordant literature in the field, our findings highlight an anti-inflammatory role for IL-9 in MS. The present study adds another level of complexity to the intricate network of cells and cytokines involved in MS and opens perspectives on the possible involvement of Th9 cells in other autoimmune diseases and in the progressive form of MS. Th9 cells could have regulatory properties, ultimately contributing to protection from inflammation (such as production of anti-inflammatory cytokines) that go beyond their impact on Th17 cells.

Further studies aimed at characterizing human Th9 cells and at identifying the mechanisms responsible for their activation by pDCs could help discover new therapeutic targets to potentiate an immunoregulatory response in MS.

CLINICAL PERSPECTIVES

- We have identified a novel regulatory role for IL-9 in MS.
- Our results reveal that pDCs are the first trigger which induce IL-9 production and that this cytokine exerts anti-inflammatory properties. Specifically, we show that IL-9 activates STAT1 and STAT5, interferes with IRF4 transcription and reduces the production of the inflammatory cytokine IL-17. Moreover, IL-9 expressed in the CSF of MS patients inversely correlates with the severity of the disease and with the expression of IL-17 in the CSF.
- Thus, the mechanisms triggering IL-9 secretion and the ability of this cytokine to reduce IL-17 production could be therapeutically targeted and amplified to reduce the inflammatory response in MS.

AUTHOR CONTRIBUTION

Gabriella Ruocco performed the experiments and contributed to the experimental design. Silvia Rossi selected and recruited some MS patients, performed and supervised the analysis of clinical data and contributed to writing the paper. Caterina Motta, Giulia Macchiarulo and Francesca Barbieri performed the analysis of the clinical data. Marco De Bardi performed cell sorting experiments. Giovanna Borsellino and Luca Battistini contributed to experimental design and writing the paper. Annamaria Finardi performed some experiments. Maria Grasso, Serena Ruggieri, Claudio Gasperini and Diego Centonze selected and recruited some MS patients. Elisabetta Volpe supervised the study, designed the experiments and wrote the paper.

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REFERENCES

- 1 Gorman, J. (2009) Autoimmune T cell responses in the central nervous system. *Nat. Rev. Immunol.* **9**, 393–407 [CrossRef PubMed](#)
- 2 O'Shea, J.J. and Paul, W.E. (2010) Mechanisms underlying lineage commitment and plasticity of helper CD4⁺ T cells. *Science* **327**, 1098–1102 [CrossRef PubMed](#)
- 3 Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348–2357 [PubMed](#)

- 4 Coffman, R.L. (2006) Origins of the T(H)1-T(H)2 model: a personal perspective. *Nat. Immunol.* **7**, 539–541 [CrossRef PubMed](#)
- 5 Park, H., Li, Z., Yang, X.O., Chang, S.H., Nuriava, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q. and Dong, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* **6**, 1133–1141 [CrossRef PubMed](#)
- 6 Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M. and Weaver, C.T. (2005) Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–1132 [CrossRef PubMed](#)
- 7 Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorioso, T., Traidl-Hoffmann, C., Behrendt, H. et al. (2009) Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J. Clin. Invest.* **119**, 3573–3585 [PubMed](#)
- 8 O'Garra, A. and Vieira, P. (2007) T(H)1 cells control themselves by producing interleukin-10. *Nat. Rev. Immunol.* **7**, 425–428 [CrossRef PubMed](#)
- 9 Ito, T., Yang, M., Wang, Y.H., Lande, R., Gregorio, J., Perng, O.A., Qin, X.F., Liu, Y.J. and Gilliet, M. (2007) Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J. Exp. Med.* **204**, 105–115 [CrossRef PubMed](#)
- 10 Zhang, X., Koldzic, D.N., Izikson, L., Reddy, J., Nazareno, R.F., Sakaguchi, S., Kuchroo, V.K. and Weiner, H.L. (2004) IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25⁺CD4⁺ regulatory T cells. *Int. Immunol.* **16**, 249–256 [CrossRef PubMed](#)
- 11 Bettelli, E., Oukka, M. and Kuchroo, V.K. (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol.* **8**, 345–350 [CrossRef PubMed](#)
- 12 Panitch, H.S., Hirsch, R.L., Haley, A.S. and Johnson, K.P. (1987) Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet* **1**, 893–895 [CrossRef PubMed](#)
- 13 Giunti, D., Borsellino, G., Benelli, R., Marchese, M., Capello, E., Valle, M.T., Pedemonte, E., Noonan, D., Albini, A., Bernardi, G. et al. (2003) Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. *J. Leukoc. Biol.* **73**, 584–590 [CrossRef PubMed](#)
- 14 Brucklacher-Waldert, V., Stuermer, K., Kolster, M., Wolthausen, J. and Tolosa, E. (2009) Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* **132**, 3329–3341 [CrossRef PubMed](#)
- 15 Xu, W., Li, R., Dai, Y., Wu, A., Wang, H., Cheng, C., Qiu, W., Lu, Z., Zhong, X., Shu, Y., Kermod, A.G. and Hu, X. (2013) IL-22 secreting CD4⁺ T cells in the patients with neuromyelitis optica and multiple sclerosis. *J. Neuroimmunol.* **261**, 87–91 [CrossRef PubMed](#)
- 16 Rolla, S., Bardina, V., De Mercanti, S., Quaglino, P., Palma, R.D., Gned, D., Brusa, D., Durelli, L., Novelli, F. and Clerico, M. (2014) Th22 cells are expanded in multiple sclerosis and are resistant to IFN- β . *J. Leukoc. Biol.* **96**, 1155–1164 [CrossRef PubMed](#)
- 17 Risoan, M.C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R. and Liu, Y.J. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283**, 1183–1186 [CrossRef PubMed](#)
- 18 Liu, Y.J. (2005) IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* **23**, 275–306 [CrossRef PubMed](#)
- 19 Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L. et al. (2011) Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* **69**, 292–302 [CrossRef PubMed](#)
- 20 Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S., Hupé, P., Barillot, E. and Soumelis, V. (2008) A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat. Immunol.* **9**, 650–657 [CrossRef PubMed](#)
- 21 Kurtzke, J.F. (1983) Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33**, 1444–1452 [CrossRef PubMed](#)
- 22 Rossi, S., Studer, V., Motta, C., Germani, G., Macchiarulo, G., Buttari, F., Mancino, R., Castelli, M., De Chiara, V., Weiss, S. et al. (2014) Cerebrospinal fluid detection of interleukin-1 β in phase of remission predicts disease progression in multiple sclerosis. *J. Neuroinflammation* **11**, 32 [CrossRef PubMed](#)
- 23 Roxburgh, R.H., Seaman, S.R., Masterman, T., Hensiek, A.E., Sawcer, S.J., Vukusic, S., Achiti, I., Confavreux, C., Coustans, M., le Page, E. et al. (2005) Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology* **64**, 1144–1151 [CrossRef PubMed](#)
- 24 Rossi, S., Mancino, R., Bergami, A., Mori, F., Castelli, M., De Chiara, V., Studer, V., Mataluni, G., Sancesario, G., Parisi, V. et al. (2011) Potential role of IL-13 in neuroprotection and cortical excitability regulation in multiple sclerosis. *Mult. Scler.* **17**, 1301–1312 [CrossRef PubMed](#)
- 25 Rossi, S., Furlan, R., De Chiara, V., Motta, C., Studer, V., Mori, F., Musella, A., Bergami, A., Muzio, L., Bernardi, G. et al. (2012) Interleukin-1 β causes synaptic hyperexcitability in multiple sclerosis. *Ann. Neurol.* **71**, 76–83 [CrossRef PubMed](#)
- 26 Ebers, G.C., Heigenhauser, L., Daumer, M., Lederer, C. and Noseworthy, J.H. (2008) Disability as an outcome in MS clinical trials. *Neurology* **71**, 624–631 [CrossRef PubMed](#)
- 27 Meyer-Moock, S., Feng, Y.S., Maeurer, M., Dippel, F.W. and Kohlmann, T. (2014) Systematic literature review and validity evaluation of the expanded disability status scale (EDSS) and the multiple sclerosis functional composite (MSFC) in patients with multiple sclerosis. *BMC Neurol.* **14**, 58 [CrossRef PubMed](#)
- 28 Amato, M.P. and Ponziani, G. (1999) Quantification of impairment in MS: discussion of the scales in use. *Mult. Scler.* **5**, 216–219 [CrossRef PubMed](#)
- 29 Freedman, M.S. (2011) Improving long-term follow-up studies of immunomodulatory therapies. *Neurology* **76**, S35–38 [CrossRef PubMed](#)
- 30 Weinstock-Guttman, B., Galetta, S.L., Giovannoni, G., Havrdova, E., Hutchinson, M., Kappos, L., O'Connor, P.W., Phillips, J.T., Polman, C., Stuart, W.H. et al. (2012) Additional efficacy endpoints from pivotal natalizumab trials in relapsing-remitting MS. *J. Neurol.* **259**, 898–905 [CrossRef PubMed](#)
- 31 Paolicelli, D., D'Onghia, M., Pellegrini, F., Dizenzo, V., Iaffaldano, P., Lavolpe, V. and Trojano, M. (2013) The impact of neutralizing antibodies on the risk of disease worsening in interferon β -treated relapsing multiple sclerosis: a 5 year post-marketing study. *J. Neurol.* **260**, 1562–1568 [CrossRef PubMed](#)
- 32 Rossi, S., Studer, V., Moscatelli, A., Motta, C., Coghe, G., Fenu, G., Caillier, S., Buttari, F., Mori, F., Barbieri, F. et al. (2013) Opposite roles of NMDA receptors in relapsing and primary progressive multiple sclerosis. *PLoS One* **8**, e67357 [CrossRef PubMed](#)
- 33 Kuhle, J., Malmestrom, C., Axelsson, M., Plattner, K., Yaldizli, O., Derfuss, T., Giovannoni, G., Kappos, L. and Lycke, J. (2013) Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis. *Acta Neurol. Scand.* **128**, e33–e36 [CrossRef PubMed](#)
- 34 Frohman, E.M., Fujimoto, J.G., Frohman, T.C., Calabresi, P.A., Cutter, G. and Balcer, L.J. (2008) Optical coherence tomography: a window into the mechanisms of multiple sclerosis. *Nature clinical practice. Neurology* **4**, 664–675 [CrossRef PubMed](#)
- 35 Barkhof, F., Calabresi, P.A., Miller, D.H. and Reingold, S.C. (2009) Imaging outcomes for neuroprotection and repair in multiple sclerosis trials. *Nat. Rev. Neurol.* **5**, 256–266 [CrossRef PubMed](#)

G. Ruocco and others

- 36 Burkholder, B.M., Osborne, B., Loguidice, M.J., Bisker, E., Frohman, T.C., Conger, A., Ratchford, J.N., Warner, C., Markowitz, C.E., Jacobs, D.A. et al. (2009) Macular volume determined by optical coherence tomography as a measure of neuronal loss in multiple sclerosis. *Arch. Neurol.* **66**, 1366–1372 [PubMed](#)
- 37 Elyaman, W., Bradshaw, E.M., Uyttenhove, C., Dardalhon, V., Awasthi, A., Imitola, J., Bettelli, E., Oukka, M., van Snick, J., Renaud, J.C. et al. (2009) IL-9 induces differentiation of TH17 cells and enhances function of FoxP3⁺ natural regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 12885–12890 [CrossRef PubMed](#)
- 38 Ivanov, I.I., Zhou, L. and Littman, D.R. (2007) Transcriptional regulation of Th17 cell differentiation. *Semin. Immunol.* **19**, 409–417 [CrossRef PubMed](#)
- 39 Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E., Mak, T.W., Kamradt, T. and Lohoff, M. (2007) The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* **8**, 958–966 [CrossRef PubMed](#)
- 40 Knoops, L. and Renaud, J.C. (2004) IL-9 and its receptor: from signal transduction to tumorigenesis. *Growth Factors* **22**, 207–215 [CrossRef PubMed](#)
- 41 Chen, Z., Laurence, A., Kanno, Y., Pacher-Zavisin, M., Zhu, B.M., Tato, C., Yoshimura, A., Hennighausen, L. and O’Shea, J.J. (2006) Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 8137–8142 [CrossRef PubMed](#)
- 42 Wei, L., Laurence, A., Elias, K.M. and O’Shea, J.J. (2007) IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J. Biol. Chem.* **282**, 34605–34610 [CrossRef PubMed](#)
- 43 Laurence, A., Tato, C.M., Davidson, T.S., Kanno, Y., Chen, Z., Yao, Z., Blank, R.B., Meylan, F., Siegel, R., Hennighausen, L. et al. (2007) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* **26**, 371–381 [CrossRef PubMed](#)
- 44 Yang, X.P., Ghoreschi, K., Steward-Sharp, S.M., Rodriguez-Canales, J., Zhu, J., Grainger, J.R., Hirahara, K., Sun, H.W., Wei, L., Vahedi, G. et al. (2011) Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat. Immunol.* **12**, 247–254 [CrossRef PubMed](#)
- 45 Iwasaki, A. and Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**, 987–995 [CrossRef PubMed](#)
- 46 Kattah, M.G., Wong, M.T., Yocum, M.D. and Utz, P.J. (2008) Cytokines secreted in response to toll-like receptor ligand stimulation modulate differentiation of human Th17 cells. *Arthritis Rheum.* **58**, 1619–1629 [CrossRef PubMed](#)
- 47 Lombardi, V., Van Overtvelt, L., Horiot, S. and Moingeon, P. (2009) Human dendritic cells stimulated via TLR7 and/or TLR8 induce the sequential production of IL-10, IFN- γ , and IL-17A by naive CD4⁺ T cells. *J. Immunol.* **182**, 3372–3379 [CrossRef PubMed](#)
- 48 Manel, N., Unutmaz, D. and Littman, D. (2008) The differentiation of human T(H)-17 cells requires transforming growth factor- β and induction of the nuclear receptor ROR γ tt. *Nat. Immunol.* **9**, 641–649 [CrossRef PubMed](#)
- 49 Acosta-Rodriguez, E.V., Napolitani, G., Lanzavecchia, A. and Sallusto, F. (2007) Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* **8**, 942–949 [CrossRef PubMed](#)
- 50 Zielinski, C.E., Mele, F., Aschenbrenner, D., Jarrossay, D., Ronchi, F., Gattorno, M., Monticelli, S., Lanzavecchia, A. and Sallusto, F. (2012) Pathogen-induced human TH17 cells produce IFN- γ or IL-10 and are regulated by IL-1 β . *Nature* **484**, 514–518 [CrossRef PubMed](#)
- 51 Segura, E., Touzot, M., Bohineust, A., Cappuccio, A., Chiocchia, G., Hosmalin, A., Dalod, M., Soumelis, V. and Amigorena, S. (2013) Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* **38**, 336–348 [CrossRef PubMed](#)
- 52 Chiurchiu, V., Cencioni, M.T., Bisicchia, E., De Bardi, M., Gasperini, C., Borsellino, G., Centonze, D., Battistini, L. and Maccarrone, M. (2013) Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis. *Ann. Neurol.* **73**, 626–636 [CrossRef PubMed](#)
- 53 Gilliet, M. and Liu, Y.J. (2002) Human plasmacytoid-derived dendritic cells and the induction of T-regulatory cells. *Hum. Immunol.* **63**, 1149–1155 [CrossRef PubMed](#)
- 54 Duhon, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. and Sallusto, F. (2009) Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat. Immunol.* **10**, 857–863 [CrossRef PubMed](#)
- 55 Froidure, A., Shen, C., Gras, D., Van Snick, J., Chanez, P. and Pilette, C. (2014) Myeloid dendritic cells are primed in allergic asthma for thymic stromal lymphopoietin-mediated induction of Th2 and Th9 responses. *Allergy* **69**, 1068–1076 [CrossRef PubMed](#)
- 56 Ronnblom, L. and Alm, G.V. (2001) A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. *J. Exp. Med.* **194**, F59–F63 [CrossRef PubMed](#)
- 57 Nestle, F.O., Conrad, C., Tun-Kyi, A., Homey, B., Gombert, M., Boyman, O., Burg, G., Liu, Y.J. and Gilliet, M. (2005) Plasmacytoid dendritic cells initiate psoriasis through interferon-alpha production. *J. Exp. Med.* **202**, 135–143 [CrossRef PubMed](#)
- 58 Gottenberg, J.E., Cagnard, N., Lucchesi, C., Letourneur, F., Mistou, S., Lazure, T., Jacques, S., Ba, N., Ittah, M., Lepajolec, C. et al. (2006) Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren’s syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2770–2775 [CrossRef PubMed](#)
- 59 Lande, R., Gafa, V., Serafini, B., Giacomini, E., Visconti, A., Remoli, M.E., Severa, M., Parmentier, M., Ristori, G., Salveti, M. et al. (2008) Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta. *J. Neuroimmunol. Exp. Neurol.* **67**, 388–401 [CrossRef PubMed](#)
- 60 Dodd, J.S., Lum, E., Goulding, J., Muir, R., Van Snick, J. and Openshaw, P.J. (2009) IL-9 regulates pathology during primary and memory responses to respiratory syncytial virus infection. *J. Immunol.* **183**, 7006–7013 [CrossRef PubMed](#)
- 61 Fallon, P.G., Smith, P., Richardson, E.J., Jones, F.J., Faulkner, H.C., Van Snick, J., Renaud, J.C., Grecnis, R.K. and Dunne, D.W. (2000) Expression of interleukin-9 leads to Th2 cytokine-dominated responses and fatal enteropathy in mice with chronic *Schistosoma mansoni* infections. *Infect. Immun.* **68**, 6005–6011 [CrossRef PubMed](#)
- 62 Faulkner, H., Humphreys, N., Renaud, J.C., Van Snick, J. and Grecnis, R. (1997) Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur. J. Immunol.* **27**, 2536–2540 [CrossRef PubMed](#)
- 63 Faulkner, H., Renaud, J.C., Van Snick, J. and Grecnis, R.K. (1998) Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris*. *Infect. Immun.* **66**, 3832–3840 [PubMed](#)
- 64 Forbes, E.E., Groschwitz, K., Abonia, J.P., Brandt, E.B., Cohen, E., Blanchard, C., Ahrens, R., Seidu, L., McKenzie, A., Strait, R. et al. (2008) IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J. Exp. Med.* **205**, 897–913 [CrossRef PubMed](#)
- 65 Gessner, A., Blum, H. and Rollinghoff, M. (1993) Differential regulation of IL-9-expression after infection with *Leishmania major* in susceptible and resistant mice. *Immunobiology* **189**, 419–435 [CrossRef PubMed](#)

- 66 Knoop, L., Louahed, J., Van Snick, J. and Renaud, J.C. (2005) IL-9 promotes but is not necessary for systemic anaphylaxis. *J. Immunol.* **175**, 335–341 [CrossRef PubMed](#)
- 67 Lu, Y., Hong, S., Li, H., Park, J., Hong, B., Wang, L., Zheng, Y., Liu, Z., Xu, J., He, J. et al. (2012) Th9 cells promote antitumor immune responses in vivo. *J. Clin. Invest.* **122**, 4160–4171 [CrossRef PubMed](#)
- 68 Osterfeld, H., Ahrens, R., Strait, R., Finkelman, F.D., Renaud, J.C. and Hogan, S.P. (2010) Differential roles for the IL-9/IL-9 receptor alpha-chain pathway in systemic and oral antigen-induced anaphylaxis. *J. Allergy Clin. Immunol.* **125**, 469–476 [CrossRef PubMed](#)
- 69 Shimbara, A., Christodoulou, P., Soussi-Gounni, A., Olivenstein, R., Nakamura, Y., Levitt, R.C., Nicolaidis, N.C., Holroyd, K.J., Tsicopoulos, A., Lafitte, J.J. et al. (2000) IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J. Allergy Clin. Immunol.* **105**, 108–115 [CrossRef PubMed](#)
- 70 Nowak, E.C., Weaver, C.T., Turner, H., Begum-Haque, S., Becher, B., Schreiner, B., Coyle, A.J., Kasper, L.H. and Noelle, R.J. (2009) IL-9 as a mediator of Th17-driven inflammatory disease. *J. Exp. Med.* **206**, 1653–1660 [CrossRef PubMed](#)
- 71 Jager, A., Dardalhon, V., Sobel, R.A., Bettelli, E. and Kuchroo, V.K. (2009) Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J. Immunol.* **183**, 7169–7177 [CrossRef PubMed](#)
- 72 Singh, T.P., Schon, M.P., Wallbrecht, K., Gruber-Wackernagel, A., Wang, X.J. and Wolf, P. (2013) Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis. *PLoS One* **8**, e51752 [CrossRef PubMed](#)
- 73 Leng, R.X., Pan, H.F., Ye, D.Q. and Xu, Y. (2012) Potential roles of IL-9 in the pathogenesis of systemic lupus erythematosus. *Am. J. Clin. Exp. Immunol.* **1**, 28–32 [PubMed](#)
- 74 Ouyang, H., Shi, Y., Liu, Z., Feng, S., Li, L., Su, N., Lu, Y. and Kong, S. (2013) Increased interleukin9 and CD4⁺ IL-9⁺ T cells in patients with systemic lupus erythematosus. *Mol. Med. Rep.* **7**, 1031–1037 [PubMed](#)
- 75 Matsushita, T., Tateishi, T., Isobe, N., Yonekawa, T., Yamasaki, R., Matsuse, D., Murai, H. and Kira, J. (2013) Characteristic cerebrospinal fluid cytokine/chemokine profiles in neuromyelitis optica, relapsing remitting or primary progressive multiple sclerosis. *PLoS One* **8**, e61835 [CrossRef PubMed](#)

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