Interferon-regulated chemokine score associated with improvement in disease activity in refractory myositis patients treated with rituximab

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Abstract

Objective

The purpose of this study was to investigate whether serum interferon (IFN)-regulated chemokine and distinct cytokine response profiles are associated with clinical improvement in patients with refractory inflammatory myopathy treated with rituximab.

Methods

In a randomised, placebo-phase trial Rituximab in Myositis Trial (RIM), 200 refractory adult and paediatric myositis subjects received rituximab. Following rituximab, clinical response and disease activity were assessed. Serum samples and clinical data were collected at baseline and several time-points after rituximab treatment. Multiplexed sandwich immunoassays quantified serum levels of IFN-regulated chemokines and other pro-inflammatory cytokines. Composite IFN-regulated chemokine and Th1, Th2, Th17 and regulatory cytokine scores were computed.

Results

Baseline IFN-regulated chemokine, Th1, Th2, Th17 and regulatory cytokine scores correlated with baseline physician global VAS, whereas the baseline Th1, Th2 and Th17 cytokine scores correlated with baseline muscle VAS. We also found baseline IFN-regulated chemokine scores correlated with specific non-muscular targets such as baseline cutaneous (r=0.29; p=0.002) and pulmonary (r=0.18; p=0.02) VAS scores. Among all cytokine/chemokines examined, the baseline score of IFN-regulated chemokines demonstrated the best correlation with changes in muscle VAS at 8 (r=-0.19; p=0.01) and 16 weeks (r=-0.17; p=0.03) following rituximab and physician global VAS at 16 weeks (r=-0.16; p=0.04). In vitro experiments showed increased levels of IL-8 (p=0.04), MCP-1 (p=0.04), IL-6 (p=0.03), IL-1β (p=0.04), IL-13 (p=0.04), IL-10 (p=0.02), IL-2 (p=0.04) and IFN-γ (p=0.02) in supernatants of TLR-3 stimulated PBMCs from non-responder compared to patients responders to rituximab.

Conclusion

IFN-regulated chemokines before treatment is associated with improvement in disease activity measures in refractory myositis patients treated with rituximab.

Key words

refractory inflammatory myopathies, serum cytokine scores, rituximab, core set measures, toll-like receptors

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Introduction

Adult polymyositis (PM), dermatomyositis (DM) and juvenile DM are a heterogeneous group of idiopathic inflammatory myopathies affecting predominantly proximal skeletal muscles, although other organs may also be involved. Increasing evidence suggests that early aggressive management of idiopathic inflammatory myopathy (IIM) improves outcome, while a delay in treatment is associated with a longer time to reach remission and more complications (1). The optimal treatment for IIM remains to be determined, but the standard therapeutic approach includes corticosteroids and immunomodulatory agents. The effective strategy for the treatment of patients with IIM emphasises the early treatment with standard disease-modifying anti-rheumatic drugs (DMARDs) targeted to achieve a state of low or minimal disease activity, and the use of biologic DMARDs in patients with poor prognostic factors and/or inadequate response to standard DMARDs (2). Unfortunately, not all patients achieve long-lasting remission and many develop undesirable side effects.

Rituximab, the first chimeric mouse-human monoclonal antibody directed against the CD20 antigen on B-cells, has become a novel approach to treatment of refractory myositis (3-5). We have recently published the first prospective, randomised, double-blind, clinical trial (the Rituximab in Myositis [RIM] trial) assessing the efficacy of rituximab in the treatment of adults and children with refractory DM or PM (6). Although the RIM Trial did not meet its primary or secondary end-points, 83% of study patients achieved the definition of improvement (DOI), and individual core set measures (CSMs) improved in both rituximab-treated groups throughout the 44-week trial (6). Nevertheless, clinical response to rituximab varies widely between patients. Therefore, it would be highly desirable to identify biomarkers that allow early identification of myositis patients that could potentially respond better to rituximab treatment and ideally even predict non-responder status.

Increased expression of T helper (Th) 1 and Th17 cytokines in muscle tissue is a striking feature in PM and DM and correlates with active disease, implicating these cytokines in muscle fibre damage (7-9). Similarly, cytokine expression levels of IL-6, IL-8, and TNF-α and composite IFN-chemokine/cytokine scores are sensitive to change in disease activity in myositis, especially physician global and muscle VAS scores (10). Besides muscle disease, patients with myositis frequently present with a variety of extra-muscular clinical features involving skin, joints, lungs or other systemic involvement. Cutaneous and pulmonary manifestations may pose a significant challenge to treat. Moreover, clinical assessment of treatment response of these manifestations can also be difficult due to the imprecise nature of current disease activity measures. In this regard, we investigated whether serum interferon (IFN)-regulated chemokine and distinct cytokine response profiles are associated with clinical improvement in patients with refractory inflammatory myopathy treated with rituximab. The levels of 3 IFN-regulated chemokines, IP-10, I-TAC and MCP-1, were used to calculate an 'IFN-chemokine score'. However, these cytokines do not represent unique IFN-regulated chemokines but those that can be induced in response to type I IFN.

Toll-like receptor (TLR)-immune mediated responses result in the production of a variety of cytokines and chemokines such as IFN-α, IFN-γ, IFN-inducible IP-10, IL-12, IL-6 and TNF-α (11), which direct the adaptive immune response. Emerging evidence indicates that nuclear factor-κβ activation via both the TLR-MyD88-dependent and TLR-MyD88-independent pathways contributes to the proinflammatory milieu in IIMs (8, 12, 13). In the current study, we also examined whether the cytokine response by peripheral blood mononuclear cells in response to a subset of TLR ligands could discriminate between responders and non-responders to rituximab.

Subjects and methods

Subjects and study design

The RIM Trial enrolled 200 subjects with refractory adult (n=76) and juve-

nile DM (n=48) and adult PM (n=76). Details regarding study design, entry criteria and clinical assessments were described previously (6, 14). Briefly, refractory myositis was defined by the intolerance to or an inadequate response to glucocorticoids and at least one other immunosuppressive or immunomodulatory agents. Patients were randomised to either a 'rituximab early' (drug at weeks 0/1, placebo at weeks 8/9) or 'rituximab late' arm (placebo at week 0/1, drug at week 8/9), such that all patients eventually received active drug. Disease activity and peripheral blood samples were collected at baseline and several time-points after rituximab treatment. Baseline was defined as the CSMs and the serum cytokine/chemokine scores before (*i.e.* week 0 for the early arm and week 8 for the late arm) rituximab treatment. The 6 CSM (15) for this trial were: patient/parent global using a 10 cm visual analogue scale (VAS), physician's global assessment of disease activity also using a 10 cm VAS; health assessment questionnaire (HAQ) or childhood HAQ (C-HAQ); muscle enzymes level; global extramuscular disease activity (based on the investigator's composite assessment of disease activity on the constitutional, cutaneous, skeletal, gastrointestinal, pulmonary, and cardiac scales of the Myositis Disease Activity Assessment Tool [MDAAT]) (16); and manual muscle testing (MMT), assessed using the MMT-8 (17). This study was carried out in accordance with research protocols approved by Institutional Review Boards of the Mayo Clinic and the University of Pittsburgh and all patients or their legal guardians signed informed consent and/or assent.

Measurement of serum cytokines and chemokines

Serum was isolated from blood drawn into Serum-Separator Tubes (BD Vacutainer® Blood Collection Tube; Becton, Dickinson, USA) at baseline and 8, 16 and 24 weeks after the first dose of rituximab. A protease inhibitor (aprotinin; 1 ug/ml) was added to each sample, and aliquots were immediately frozen at -80°C. Multiplexed sandwich immunoassays (Meso Scale Discovery, Rockville, MD) were used to quantitate the serum levels of IFN regulated chemokines (monokine induced by gamma interferon [MIG/ CXCL9], macrophage inflammatory protein-1 α and β [MIP-1α /CCL3 and MIP-1β/ CCL4], monocye chemotactic protein-1 and 2 [MCP-1 and MCP-2], interferon gamma-induced protein 10 [IP-10/ CXCL10], interferon-inducible T-cell alpha chemoattractant/ chemokine [C-X-C motif] ligand 11 [I-TAC/CXCL11], TNF receptor type I [TNFR1], and interferon α and γ [IFNα, IFNγ]), and the serum levels of tumour necrosis factor α (TNF-α), interleukin-2 (IL-2), IL-1β, IL-4, IL-5 IL-6, IL-8, IL-10, IL12p70, IL-13, and IL-17. This panel of cytokines/chemokines was selected a priori (7-9, 18-21). Samples were run in duplicate and calibrated recombinant proteins were used to generate standard curves. A composite IFN-regulated score was generated based on serum levels of 3 IFN-regulated chemokines (IP-10, I-TAC and MCP-1), which exhibited the strongest correlations with disease activity in adult and juvenile DM in our previous report (20). To reduce the number of comparisons derived from all cytokine measurements, we grouped the cytokines into Th1-(IFN-γ, TNF-α, IL-2), Th2-(IL-4, IL-5, IL-10, IL-12p70, IL-13), Th17-(IL-6, IL-17, IL-1β) and regulatory cytokines (IL-10 and TNF-α); and cytokine scores were computed for each cytokine group. Our chosen cytokine scoring systems included individually validated markers by a number of reports in the literature (21-24). Herein, we included IP-10 within the composite IFN-regulated chemokine score as a marker of the effects of IFN and not to suggest it was specifically upregulated only due to IFN, since many cytokines and chemokines including IP-10 are a matrix of overlapping responses.

PBMCs isolation, cell culture and stimulation assay

For these set of experiments, whole blood samples from 21 responders and 13 non-responders were collected 4 weeks post-rituximab treatment into sodium heparin cell preparation tubes (BD Vacutainer® CPT™ , Franklin Lakes, NJ, USA), and then PBMCs separated in the tubes according to the manufacturer's recommendations. Patients were classified as responders if they achieved a sustained improvement of ≥20% in 3 of any 6 CSMs, with no more than 2 core set measures worsening by ≥25%. Non-responders did not achieve the DOI or worsened after achieving the DOI over 44-week trial.

Aliquots of purified PBMCs were cryopreserved and stored in liquid nitrogen until assayed. On the day of stimulation assay, the cryopreserved cells were thawed in a 37°C water bath until the cell suspension was almost completely melted. Each cell suspension was transferred to a 15-ml tube, and 10 ml of prewarmed (37ºC) complete RPMI (cRPMI) medium was slowly added. The tubes were centrifuged for 10 minutes at 1300 rpm in the Jouan centrifuge®, and the cells were washed and then re-suspend in 5 mL of cRPMI before counting. PBMC yield and viability were determined viability were determined with a hemocytometer and the trypan blue dye. The cell volume was adjusted to give a cell concentration of 8.0 x 105 cells/ml in cRPMI. PBMC were seeded into 48-well tissue culture plates (Falcon; BD Biosciences) at a density of 4.0 × 105 cells/well at 37°C in 5% CO2 for a minimum of 30 minutes before stimulation. The cells were then stimulated with or without the following TLR agonists: 10ug/mL TLR3 agonist poly (I:C) (InvivoGen, San Diego, CA, USA), 1ug/mL TLR4 agonist LPS (Sigma, St. Louis, MO, USA), 10ug/mL TLR9 agonist CpG oligodeoxynucleotide (InvivoGen, San Diego, CA, USA), 1ug/ml TLR 7/8 agonist (R848) (InvivoGen, San Diego, CA, USA) and 500u/mL recombinant human IFN-α (PBL Interferon Source, Piscataway, NJ, USA). The PBMCs were then incubated at 37ºC in 5% CO2 for 24 hours. The supernatants were subsequently collected and transferred to small vials. A protease inhibitor (aprotinin; 1 ug/ml) was added to each vial, and aliquots were immediately frozen at -80°C.

Statistical methods

Descriptive statistics (medians, percentages, etc.) were used to summarise

the data. Composite chemokine scores were determined by first calculating the 95th percentile of serum concentration levels for each chemokine and then setting concentration levels ≥95th percentile to the 95th percentile value to reduce extreme outliers as previously described (20). For each chemokine, the data were re-scaled to a maximum value of 1.0. For each subject, the re-scaled values were summed up, and the sum was adjusted to a 100-point scale. Paired comparisons between time points were performed using Wilcoxon signed-rank tests and correlations were performed using Spearman methods. Correlations between cytokine/chemokine scores and changes in disease activity measures were adjusted for baseline disease activity values. For each outcome, 5 comparisons were performed, so the Bonferroni correction indicating *p*-values of 0.05/5=0.01 should be considered statistically significant (25). Principal component analyses with varimax rotation were used to determine whether the cytokine/chemokine groupings defined *a priori* were optimal for these data. A likelihood ratio test was used to determine the number of principal components needed to adequately describe all the data.

Results

Baseline characteristics

Baseline cytokine and chemokine analysis, and clinical information were available for 177 of 200 patients from the RIM trial. At baseline, the clinical features and the values for the CSMs were similar between the early and late rituximab-treated groups, except for the muscle enzymes and muscle VAS which were higher in the early treatment group. The mean (SD) age of rituximab responders was 41.3 (±14.8) years, and in non-responders was 38.7 (±17.2) years. Most patients were Caucasian (70%) and female (73%), with a mean disease duration exceeding 5 years. There was no difference in demographic features between responders and non-responders. The values for the CSMs at baseline were similar between responders and non-responders, except for the baseline physician global assessment and muscle disease activity, which were statistically higher in the responders (both $p<0.05$).

There were marginally significant correlations between IFN-regulated chemokine and regulatory cytokine scores at baseline with disease duration at baseline (r=-0.14, $p=0.05$; r=-0.17, $p=0.02$, respectively), and between Th1 cytokine score at baseline and age (r=0.14, $p=0.04$). Notably, IFN-regulated chemokine score at baseline was positively correlated with aspartate aminotransferase (AST), aldolase (ALDL) and lactate dehydrogenase (LDH) serum levels at the start of treatment (r=0.18 [$p=0.01$], r=0.2 [$p=0.007$] and r=0.18 [$p=0.01$], respectively); while Th2 cytokine score at baseline was negatively correlated with creatine phosphokinase (CPK) levels (r=-0.16, $p=0.03$). However, there were no significant correlations between muscle strength scores and cytokine scores. The RIM trial showed no statistical difference in the time to achieving the DOI in the adult PM, adult DM, and juvenile DM subsets (6), and we found no significant differences in cytokine scores between the 3 disease subsets. Therefore, all subsequent analyses were conducted on a combination of adult PM, adult DM, and juvenile DM patients together.

Changes from baseline in cytokine and chemokine scores following treatment with rituximab

We examined the effect of rituximab treatment on a wide range of IFN-regulated chemokines, Th1, Th2, Th17 and regulatory cytokine scores. We chose these follow-up time points (8 and 16 weeks) since they were prospectively selected as part of the study protocol for full clinical assessments and collection of serum samples. No significant changes were detected in cytokine/chemokine scores from baseline to 8 weeks after the start of treatment (Table I). Next, we looked for changes in cytokine/chemokine scores from baseline to 16 weeks after treatment. Interestingly, increased levels of Th2 and Th17 cytokine scores were observed at 16 weeks after the start of treatment compared to baseline ($p=0.05$, $p=0.02$, respectively, Table I), whereas the levels of regulatory cytokine scores were reduced ($p<0.001$). There were no significant differences in baseline cytokine/chemokine scores between the three myositis subgroups (data not shown).

Ability of baseline cytokine scores to predict clinical improvement of disease activity core set measures following rituximab treatment

We first wanted to identify if baseline scores predict the rate of achieving DOI. The IFN-regulated chemokine score at baseline was associated with time to achieving the DOI (hazard ratio: 1.07 per 10 unit increase in IFN score; 95%CI: 1.00–1.15; $p=0.06$).

Next we examined the potential relationships between baseline cytokine/chemokine scores with the baseline disease activity CSMs. Clinical response was evaluated mainly using the

Table I. Distribution of cytokines from baseline to 8 and 16 weeks after treatment.

	Baseline	8 weeks after treatment	Paired Differences	*p*-value	16 weeks after treatment	Paired Differences	*p*-value
IFNCK	19.2 (1.4–100)	18.1 (1.6–100)	-0.1 (-76–55.6)	0.56	19.4 (2.1–94.5)	-0.2 (-82–35.3)	0.18
Th1	22.9 (3.8–93.3)	23.7 (5–100)	0.9 (-58.8–82.2)	0.59	23.3 (8.8–89.9)	-1.6 (-59.8–63.3)	0.073
Th2	10.7 (2.2–93.7)	10.9 (5.2–100)	0.4 (-76.9–92.2)	0.72	11.9 (3.7–84.3)	0.5 (-69.3–41.4)	**0.047**
Th17	24.3 (8.6–79.2)	27 (9.6–84.4)	2.3 (-36.8–57.1)	0.15	27.9 (8.6–100)	1.3 (-25.3–45.7)	**0.018**
Regulatory	23.5 (3.5–100)	24.8 (7.2–100)	-0.6 (-47.3–83.1)	0.41	21.3 (10–100)	-2.7 (-50.3–55.3)	**<0.001**

IFNCK: interferon-regulated chemokine. Values in the table are median (min, max).

physician global activity and muscle VAS; the selection of the physician global activity and muscle VAS was based on our previous study showing that these disease activity measures were the most sensitive to change when compared to the IFN-regulated genes and chemokines in DM (10, 20). Baseline IFN-regulated chemokine, Th1, Th2, Th17 and regulatory cytokine scores correlated with baseline physician global VAS ([r=0.20, p=0.01], [r=0.29, p<.001)], [r=0.16, p=0.03], [r=0.18, p=0.02] and [r=0.19, p=0.01], respectively). Similarly, the baseline Th1, Th2 and Th17 cytokine scores correlated with baseline muscle VAS ([r=0.22, p=0.003], [r=0.17, p=0.02] and [r=0.23, p=0.003], respectively) (Table II).

To determine if the baseline cytokine/chemokine scores correlated with changes in disease activity measures from baseline to 8 and 16 weeks post-rituximab, we performed a similar analysis. As shown in Table II, the baseline IFN-regulated chemokine score was better correlated with changes in physician global and muscle VAS among all cytokines examined. Specifically, subjects with higher baseline interferon-regulated chemokine scores had a significant improvement on physician global VAS (r=-0.16, p=0.04) at 16 weeks and in muscle VAS at both 8 and 16 weeks ([r=-0.19, p=0.01] and [r=-0.17, p=0.03], respectively) after rituximab treatment, whereas subjects with higher baseline Th1 and regulatory cytokine scores had better responses in muscle VAS at 8 weeks from the start of treatment ([r=-0.20, p=0.01] and [r=-0.22, p=0.005], respectively).

Table II. Spearman correlation of cytokine scores at baseline with the level of response for Physician Global VAS and Muscle VAS at 8 and 16 weeks after treatment.

At baseline	Value	Physician global VAS at baseline (n=177)	Muscle VAS at baseline (n=176)	Relative change in physician global VAS 8 weeks after treatment (n=174)	Relative change in physician global VAS 16 weeks after treatment (n=172)	Relative change in muscle global VAS 8 weeks after treatment (n=169)	Relative change in muscle global VAS 16 weeks after treatment (n=171)
IFNCK	r	**0.20**	0.08	-0.02	**-0.16**	**-0.19**	**-0.17**
	p	**0.010**	0.28	0.80	**0.036**	**0.013**	**0.027**
TH1	r	**0.29**	0.22	0.02	-0.03	**-0.20**	-0.15
	p	**<.001**	0.003	0.84	0.73	**0.010**	0.058
TH2	r	**0.16**	**0.17**	-0.002	0.08	-0.09	0.004
	p	**0.033**	**0.023**	0.98	0.30	0.24	0.96
TH17	r	**0.18**	**0.23**	0.07	-0.01	-0.05	-0.04
	p	**0.016**	**0.003**	0.37	0.91	0.55	0.56
Regulatory	r	**0.19**	0.13	-0.04	-0.03	**-0.22**	-0.11
	p	**0.012**	0.094	0.57	0.73	**0.005**	0.17

*Spearman correlation coefficients adjusted for baseline disease activity values, p-value

Table III. Spearman correlation of changes in cytokine scores from baseline to 8 weeks after the start of treatment with the changes in Physician global VAS and Muscle VAS.

Changes from baseline to 8 weeks later	Value	Relative change in physician global VAS 8 weeks after baseline (n=174)	Relative change in muscle VAS 8 weeks after baseline (n=169)	Relative change in physician global VAS 16 weeks after baseline (n=172)	Relative change in muscle VAS 16 weeks after baseline (n=167)
IFNCK	r	**0.17**	**0.19**	0.10	0.14
	p	**0.022**	**0.012**	0.21	0.077
TH1	r	-0.07	0.12	0.01	0.09
	p	0.38	0.14	0.92	0.22
TH2	r	-0.08	0.06	-0.10	-0.02
	p	0.27	0.46	0.18	0.78
TH17	r	-0.02	0.08	0.0005	0.08
	p	0.84	0.29	1.00	0.3
Regulatory	r	-0.001	**0.16**	0.04	0.10
	p	0.99	**0.04**	0.61	0.2

IFNCK: interferon-regulated chemokine. Values in the table are Spearman correlation coefficients and p-value.

Correlation between changes in cytokine/chemokine scores and changes in disease activity

We examined if changes in cytokine/chemokine scores at 8 and 16 weeks from the start of treatment correlated with changes in the myositis disease activity at 8 and 16 weeks. We found that changes in IFN-regulated chemokine score from baseline to 8 weeks significantly correlated with relative change in muscle VAS and physician global VAS 8 weeks from start of treatment ([r=0.19, p=0.01] and [r=0.17, p=0.02]). Changes in regulatory cytokine score from baseline to 8 weeks also correlated with relative change in muscle VAS from baseline to 8 weeks from start of treatment (r=0.16, p=0.04). The change in IFN-regulated chemokine score from baseline to 8 weeks also tended to correlate with the relative change from baseline to 16 weeks from the start of treatment in muscle VAS (r=0.14, p=0.07) (Table III).

Ability of baseline cytokine scores to predict clinical improvement of cutaneous and pulmonary disease activity

We also examined the correlation between baseline cytokine/chemokine scores and cutaneous (among DM and JDM patients only) and pulmonary (among all patients) disease activity measures. We found statistically significant correlations between baseline IFN-regulated chemokine and baseline

cutaneous and pulmonary VAS scores ([r=0.29, $p<0.002$], [r=0.18, $p=0.02$], respectively). Interestingly, baseline Th1 scores were positively correlated with relative change in cutaneous VAS from baseline to 8 after treatment (r= 0.21, $p=0.04$), indicating that patients with a high Th1 score at baseline were less likely to experience cutaneous improvement. In contrast, baseline Th17 score was negatively correlated with relative change in pulmonary VAS scores from baseline to 16 weeks after rituximab (r=-0.23, $p=0.04$), indicating patients with high Th17 scores at baseline were more likely to experience improvement in pulmonary VAS by 16 weeks after treatment.

Principle component analysis

Principal component analyses were used to determine if the cytokine/ chemokine groupings we defined *a priori* were optimal for these data. These analyses revealed 8 principal components, which explained 65% of the variability in the data. The first component was similar to our Th1 score (including loadings >0.5 for IL-5, IL-10, IL-12, IL13 and MIP-1α), the second and third were similar to our IFN-regulated chemokine score (including loadings >0.5 for IP-10, I-TAC, MCP-1, MIG and IP-10, I-TAC, MCP-2, respectively). The sixth component was similar to our Th17 score (including IL-4, IL-6 and IL-17). The other components did not correspond to any known biological pathways (IL-8 and IL-6, IL-2 and IL-5, IL-1β and IL4 and IFN-γ). As our *a priori* cytokine/chemokine groupings were similar to several of the principal components, additional analyses using these components were not performed.

Immune response profiles after TLR stimulation

Poly I: C-stimulated PBMCs from non-responder patients secreted significantly higher MCP-1 ($p=0.04$), IL-6 ($p=0.03$), IL-1β ($p=0.04$), IL-13 ($p=0.04$), IL-10 ($p=0.02$), IL-2 ($p=0.04$), IFN-γ ($p=0.02$) and IL-8 ($p=0.04$), compared to responder patients (Fig. 1). No significant differences were observed in the immune response profiles for LPS-, CpG-, R848- and IFN-α-stimulated PBMCs between non-responders and responders ($p>0.05$ for all comparisons).

Discussion

Cytokines and chemokines associated with innate and adaptive immune-pathways have emerged as important factors in the pathogenesis of inflammatory myopathies. Evidence suggests that an altered expression of cytokines and/or chemokines in peripheral blood and/ or muscle tissue of DM/PM patients may be particularly relevant in treatment-resistant disease. For example, expression of interleukin (IL)-18

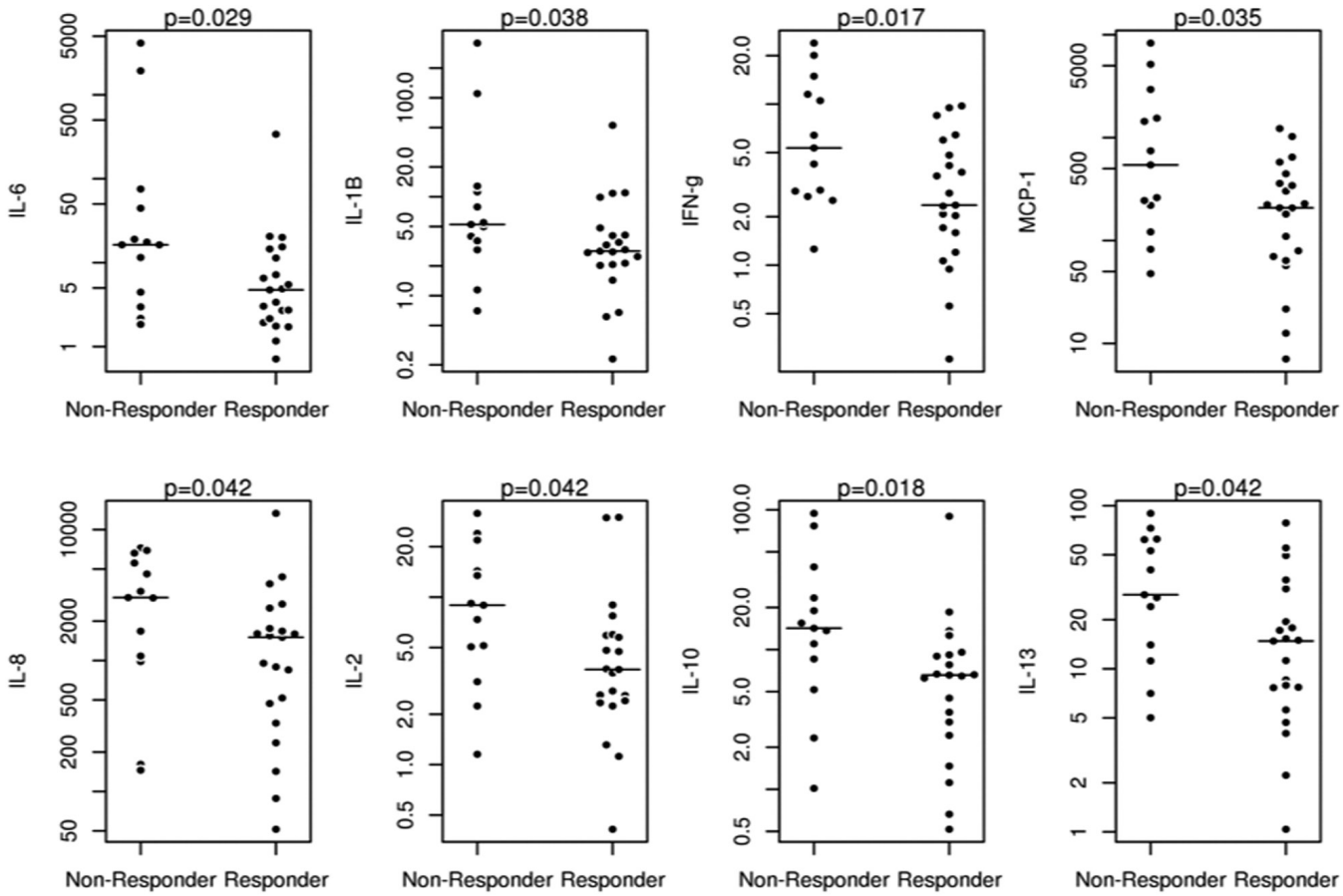

Fig. 1. Immune cytokine profiles of peripheral blood mononuclear cells *in vitro* TLR3 ligand stimulation in a set of refractory myositis patients by response status. Figures depict IL-6, IL-1β, IFN-g, MCP-1, IL-8, IL-2, IL-10 and IL-13 at 24 h following Poly I:C stimulation. TLR, toll-like receptors; IL, interleukin; IFN-γ, interferon gamma; MCP-1, monocyte chemotactic protein-1.

in muscle tissue from patients with inflammatory myopathies has been associated with treatment-resistant disease (26). IL-6 is another cytokine that has been associated with a more severe disease activity in DM patients (20). Interestingly, studies suggest that Th1- associated cytokines may mediate the immune response of corticosteroid-resistant interstitial lung disease in both PM and DM (27). In the earlier report of the RIM study (6), we demonstrated that nearly all patients successfully depleted their peripheral B cells at 4 weeks post-treatment with B cells beginning to return at ~32–36 weeks in both the early and late rituximab groups. There were no differences in the B cell counts between the responders and non-responders. Herein, we report the impact of B cell depletion on serum cytokine/chemokine profiles at two time points after rituximab treatment. Our data showed that the major effect of rituximab on cytokine profiles was seen 16 weeks after initiation of therapy, when B-cells were virtually absent in peripheral blood. Specifically, we found that the scores of serum Th2- and Th17-derived cytokines significantly increased, while the scores of regulatory cytokines (IL-10 and TNF-α) were decreased compared with baseline levels. Interestingly, IFN-regulated chemokine scores did not differ significantly from baseline at either of the two time points analysed. Like T cells, B cells are not homogenous with respect to their functions. The key role played by B cells in a range of autoimmune diseases has provided the rationale for B cell depletion therapy. B-cells can both amplify and suppress innate- and T-cell-immune responses through different mechanisms (28, 29). This suppressive or regulatory effect of B cells on effector T cells and other innate cells is mediated by the production of cytokines (30). For example, B cell-derived IL-10 can inhibit both Th1 and Th2 polarisation, antigen presentation, and the production of pro-inflammatory cytokines including TNF-α, IL-12p70, and IL-23 by myeloid cells (31). In contrast, in a model of experimental autoimmune encephalomyelitis (EAE), the absence of IL-10 production by B cells correlated with exacerbated production of pro-inflammatory Th1 and Th17 cytokines by autoreactive T cells and mice developed a persistent chronic disease, suggesting that recovery from EAE requires IL-10 production by B cells by modulating the pathogenic T cell response (32). The decrease of regulatory cytokine scores in our cohort of myositis patients treated with rituximab could, at least in part, be explained by a direct effect of depletion of cytokine-producing B-cells. Nevertheless, whether or not all changes in the cytokine profiles observed in our study are direct consequences of the B cell depletion or may be attributable to improvement of disease progression, remains to be investigated.

The aim of the present study was to investigate if particular profiles of serum chemokine and cytokines were associated with improvement in disease activity measures in patients with refractory myositis treated with rituximab. Herein, we examined a comprehensive panel of serum Th1-, Th2-, Th17-, regulatory-derived cytokines and interferon-regulated chemokines; most of these cytokines and chemokines have been linked to the pathogenesis of inflammatory myopathies as well as a more severe disease course (7-9, 18-20). We demonstrated that patients with higher baseline IFN-regulated chemokine scores had higher physician global, cutaneous and pulmonary VAS scores at the baseline visit. Taken together, these data confirm and extend our previous observations that type I IFN-regulated chemokines (IP-10, I-TAC, and MCP-1) and IL-6 levels are increased in the peripheral blood of patients with adult and juvenile DM, and show strong correlations with disease activity (20). In addition, we demonstrated that baseline scores of Th1, Th2 and Th17 cytokines correlated positively with disease activity as measured by the physician global and muscle VAS at baseline. Shen *et al*. reported that IL-17 and IL-23 (Th17-derived cytokines) are increased in the serum and the supernatant of stimulated PBMCs from patients with new-onset PM and DM compared to those with long-standing disease (9). Interestingly in our cohort of patients with primarily long-term and treatment-resistant disease, we found high baseline Th17 scores. A previous study from our group in patients with juvenile and adult DM showed stronger correlation between IFN-regulated chemokine score and physician global VAS (20) compared with the present study. The observed difference may be explained by the fact that the RIM study represented a cohort of patients with refractory myositis and long-standing disease; whereas the DM patients in our previous study had a lesser degree of baseline physician global VAS and most patients were newly diagnosed disease. Although the IMACs measures are useful for assessing extent of disease, it is thought that these measures often cannot discriminate between the effects of chronic muscle damage and active muscle inflammatory disease.

The RIM Trial demonstrated that rituximab is effective therapy for patients with treatment-resistant myositis (6). However, rituximab therapy did not benefit all patients. To support an 'individualised-medicine' approach to the treatment of IIM, the identification of biomarkers that predict a beneficial response to B cell depletion is necessary. In this study, the IFN-regulated chemokine score was the best predictor of muscle improvement (changes on muscle VAS) at 8 and 16 weeks, and physician global improvement at 16 weeks. Of note, among all myositis patients with higher Th1 cytokine scores had better responses in muscle VAS at 8 weeks. Similarly, DM subjects (among DM and JDM patients only) with a high Th1 score at baseline were less likely to experience cutaneous improvement at 8 weeks post-rituximab, whilst myositis subjects with a high baseline Th17 score were more likely to experience improvement in pulmonary VAS at 16 weeks. Collectively, these different patterns in clinical response in muscle/skin (Th1 profile) and pulmonary (Th17 profile) VAS scores concerning the cytokine profiles suggest that there may be different pathogenic pathways in myositis. In addition, Th1 and Th17 scores may also help in the assessment of disease activity in these features of extramuscular disease.

Earlier studies have analysed potential predictors to rituximab response in other autoimmune diseases. For example, the IFN type I signature appears to serve as a predictive biomarker for non-response to rituximab in rheumatoid arthritis (33). In a recent study, Roll *et al*. analysed the correlation of B cell subsets with response to rituximab in rheumatoid arthritis patients, noting that increased IgD^+, $CD27^+$ memory B cell subsets before therapy were the strongest predictors of non-response and relapse (34). We are currently analysing the B-cell phenotypes before and after rituximab treatment in the RIM cohort of patients to examine whether B cell subsets serve as useful biomarkers for rituximab response and whether B cell subsets could predict future relapses in refractory inflammatory myopathy (manuscript in preparation). In the present study, we did not address the question if the autoantibody profiles correlate to cytokine profiles; however, we have recently reported preliminary data showing that IFN-regulated chemokine score at baseline was higher in subjects with anti-syn, TIF1-γ and Mi-2 compared with other autoAb groups. Moreover, rituximab-treated myositis patients with high IFN-chemokine scores at baseline (pre-rituximab treatment) showed the greatest clinical improvement based on physician global and muscle VAS among patients with certain autoantibody groups such as Mi-2 (35).

Previous studies have demonstrated up-regulation of type I IFN-regulated transcripts and chemokines in peripheral blood, serum and muscle tissues of myositis patients (36-38). Niewold *et al*. (39) observed higher levels of serum IFN-α activity in juvenile DM patients who had untreated and newly diagnosed disease compared to those with long-standing disease (either 24 or 36 months after diagnosis), suggesting that IFN-α may be important in the early phase of disease. Importantly, the authors also observed a non-significant trend toward lower serum IFN-α activity in juvenile DM at 24 months post-diagnosis and a late increase in serum IFN activity at 36 weeks post-diagnosis in those who had discontinued treatment, potentially implicating that in addition to a role in DM pathogenesis, IFN signature may also predict treatment response.

Emerging evidence has suggested a key role for TLRs in the pathogenesis of myositis. TLR-3 and TLR-7 mRNAs and proteins were found highly expressed in affected muscle of PM and DM patients. In addition, cultured myoblasts expressing high levels of HLA class I antigens produced high levels of IL-6, a potent driver of Th17 differentiation, following TLR-3 stimulation with poly (I:C) (synthetic analogue of viral double stranded RNA) in the presence of necrotic myocytes (8). In the present study, we have analysed the *in vitro* cytokine response to various specific TLR agonists (TLR-3, -4, -7/8 and -9) in PBMCs collected 4 weeks after rituximab treatment, which coincided with the time of lowest peripheral blood B cell counts. We found that TLR-3-stimulated PBMCs from non-responders patients secreted significantly higher amount of individual cytokine/chemokine measures IL-6, IL-8, MCP-1, IL-1β, IL-13, IL-10, IL-2 and IFN-γ, compared to responder patients. Although the TLR-3 stimulation of PBMCs from patients with refractory myositis that did not respond to rituximab induced an increased production of cytokines/chemokines that are known to function in several distinct biological pathways, we and others have shown previously that these cytokine/chemokine signatures including the type I IFN-regulated chemokine MCP-1/CCL2, cytokines IL-6, IL-1β and IFN-γ and chemokine IL-8, reflect the cytokine production by cellular infiltrates seen in affected muscle and skin as well as in peripheral blood of juvenile and adult DM patients with active disease (10, 18, 20, 40, 41). The impact of TLR-activated B cells and TLR-activated DC on T cell-mediated inflammation in IIM is still unclear. The present study was initiated to examine the changes of cytokine/chemokine scores under condition of treatment with rituximab and their association with clinical improvement. Additionally, these results provide further evidence that TLR-3 signalling may be involved in the innate and adaptive inflammatory response in inflammatory myopathies, and it may play a crucial role in the unmitigated immune response in myositis patients. Therefore, additional studies are needed to fully elucidate the impact of rituximab treatment on TLR signalling and T-cell immunity.

Conclusion

In summary, in this large cohort of patients with refractory IIM treated with rituximab, our data demonstrated a moderate correlation between the cytokine/chemokine profiles before rituximab treatment, particularly the IFN-regulated chemokine score, with particular indicators of disease activity measures, which may prove useful in the assessment of clinical response to rituximab, and may help to identify patients who respond better to this therapy. Furthermore, these results provide additional evidence implicating IFN-regulated chemokines in the pathogenesis of IIM. Interestingly, it appears that B-cell depletion did not significantly alter the IFN pathway since there was no change in the IFN-regulated chemokine score at the follow-up treatment compared to baseline. Therefore, therapies targeting the IFN pathway in combination with rituximab may prove effective in treatment of resistant IIM disease in the future.

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