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## Interleukin-1 in Monocyte Activation Phenotypes in Systemic Juvenile Idiopathic Arthritis: observations from a clinical trial of rilonacept, an interleukin-1 inhibitor

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### Abstract

Systemic juvenile idiopathic arthritis (sJIA) is a childhood rheumatic disease of unknown origin. Dysregulated innate immunity is implicated in disease pathology. We investigated if IL-1

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inhibition affects circulating cytokines and monocyte gene expression. CD14<sup>+</sup> monocytes from patients in the RAPPORT trial were analyzed by RT-PCR for expression of *IL1B* and transcription factors associated with monocyte activation. Serum IL-1ra decreased with treatment, and IL-18BP transiently increased. Serum levels of IL-1 $\beta$ , IL-6, IL-10 and IL-18 were unchanged. *IRF5* and *STAT6* were decreased, and *PPARG* was increased, independent of clinical response, and may represent a skew towards a PPARG-driven M2-like phenotype. *IL1B* expression was decreased in early clinical responders. A transient increase in *STAT1*, and a decrease in *SOCS1* preceded the reduction in *IL1B* in early clinical responders. Changes in IL1B/STAT1/SOCS1 could be associated with crosstalk between IL-1 and IFN pathways in sJIA. These transcriptional changes might be useful as drug response biomarkers.

## Keywords

Juvenile arthritis; Monocytes; Cytokine

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## 1. Introduction

Systemic juvenile idiopathic arthritis (sJIA) is a chronic rheumatic condition of childhood, with strong similarity to adult onset Still's disease; both are characterized by remitting fever, transient rash, and arthritis; sJIA etiology remains unknown [1]. Although the adaptive immune response may also contribute [2], several lines of evidence indicate that dysregulation of innate immunity, especially of the monocyte/macrophage lineage, plays a major role in sJIA pathology [1]. Activated monocytes constitute a significant component of the perivascular and synovial infiltrates observed during sJIA flares [3]. Several experimental studies of peripheral blood mononuclear cells show activation of innate immune cells, including upregulation of monocyte genes that cannot be explained solely by increase in monocyte number [4–8]. Exposure of monocytes from healthy donors to plasma or serum from sJIA patients induces expression of genes related to innate immunity and production of IL-1 $\beta$  and IL-6 [4, 9]. Additionally, the association of sJIA with macrophage activation syndrome (MAS) is a prominent, and potentially fatal, complication of the disease [10], although MAS also can occur in association with other conditions, including classical autoimmune diseases, such as SLE, and infections. Importantly, the exact nature of the immune dysfunction in sJIA is still unknown [1, 11].

The most compelling evidence for innate mediators, IL-1 and IL-6, as important drivers of the inflammation of sJIA, is the striking therapeutic responses to IL-1 or IL-6 blockade in patients [4, 12–16]. Treatment with anti-IL-1 medication is successful in treating a large percentage (close to 60%) of sJIA patients followed for up to 2 years [15, 17, 18]. It has been proposed that anti-IL-1 treatment may modify the course of the disease and reduce long term arthritis, if administered early in the disease [19–21].

We have observed a number of changes in circulating sJIA monocyte activation phenotype, with some features associated primarily with active disease [6, 7]. One aspect we have investigated is monocyte polarization. Polarization refers to distinct patterns of monocyte/macrophage activation, based on different types of stimulation. The extreme ends of the

polarization spectrum have been named M1 and M2 [22, 23]. The M1/M2 paradigm has its shortcomings, given the complexity of monocyte/macrophage activation [24, 25], but it is a useful framework to compare monocyte phenotype across different situations. The classically activated M1 is more proinflammatory, whereas the alternatively activated M2 is associated with resolution of inflammation and tissue repair [23, 26]. Despite the inflammatory nature of sJIA, we and others have found increased expression of genes and cell markers associated with M2-like response in peripheral blood monocytes, in addition to markers associated with M1-like response, suggesting these M2-associated markers may reflect a compensatory response to active inflammation in tissues [6, 8, 27]. In addition to the polarizing cytokines, monocytes and macrophages are highly sensitive to other cues in their microenvironments [9, 23]. Indeed, based on transcriptional analysis, striking heterogeneity is the hallmark of the programs expressed by these myeloid cells [28, 29].

Do the sJIA circulating monocytes contribute to IL-1-driven pathogenic activity in sJIA or do their phenotypes reflect the influence of elevated IL-1 on these cells? The RAPPORT trial (Randomized Placebo Phase Study of Rilonacept in the Treatment of Systemic Juvenile Idiopathic Arthritis) offered a unique opportunity to investigate changes in circulating monocyte phenotypes in the context of changes in *in vivo* IL-1 modulated by IL-1 blockade. RAPPORT was a randomized, placebo-controlled trial of rilonacept, an IL-1 “trap” [30]. Rilonacept is an Fc region of the human IgG1 fused to the extracellular domains of the 2 components of the IL-1 receptor, IL-1R1 and the IL-1 receptor accessory protein (IL-1RAcP). As such, it can bind IL-1 $\beta$ , IL-1 $\alpha$  and may also bind to the endogenous IL-1 inhibitor, IL-1ra. The RAPPORT clinical trial tested the short-term safety of rilonacept and its efficacy for articular and systemic manifestations of sJIA. The drug was well-tolerated and showed efficacy in treating active sJIA [30].

We hypothesized that IL-1 inhibition modifies the activation profile of circulating monocytes in sJIA. The activation profiles of monocytes/macrophages are shaped by transcription factors (TFs) that regulate downstream genes, determining the type of response manifest by the cell [28]. We examined a panel of key TFs associated with known monocyte activation profiles before and after treatment with rilonacept to determine the effect of IL-1 inhibition on these profiles. We also investigated whether specific changes in monocyte TF expression are associated with the clinical response to IL-1 blockade. We also investigated if circulating IL-1ra and other key cytokines previously implicated in sJIA [31] were altered by IL-1 inhibition.

## 2. Materials and Methods:

### 2.1 RAPPORT trial:

The Randomized Placebo Phase Study of Rilonacept in the Treatment of Systemic Juvenile Idiopathic (RAPPORT) was designed so that half the subjects were randomized to begin placebo for 4 weeks followed by active drug (“the placebo group”), while the other half received active drug without a placebo phase (“the rilonacept group”); the total duration of the trial was 24 weeks (6 months) [30]. Patients in the placebo group received placebo for 4 weeks and rilonacept for 20 weeks; patients in the rilonacept group received the drug for 24 weeks. A loading dose of the drug was administered at Day 0 (rilonacept) or Day 28

(Placebo), followed by weekly maintenance doses [30]. Patients continue to receive systemic corticosteroids during the trial. The trial enrolled 71 patients, below its planned enrollment of 100, with 57 completing the study. Inclusion/exclusion criteria and the baseline demographic and clinical characteristics of the study participants are described in Ilowite et al [30]. Time to response was the endpoint, based on the adapted American College of Rheumatology Pediatric 30 (ACR Pedi 30) criteria [32], together with absence of fever and tapering of the systemic steroids. Each subject was clinically evaluated every two weeks, starting from the beginning, for a total of 14 weeks, and then evaluated again at week 24. The median time to achieve the primary end point, defined by composite end point of an ACR Pedi 30 response, absence of fever and corticosteroid taper, was 4 weeks of treatment [30]. Based on this finding, for the purpose of this study we defined a patient as an early responder if the patient achieved ACR Pedi 30 (considered as a “Yes”) before 4 weeks or less of treatment, and continued to achieve ACR Pedi 30 or at least had more “Yes” at later visits (≥ 10 weeks in study) than earlier visits (<10 weeks in study). Patients were classified as late responders if it took more than 4 weeks of treatment to achieve ACR Pedi 30, or the patient did not achieve ACR Pedi 30 during the duration of the trial (24 weeks). We obtained samples from 35 patients (see below); 23 patients were classified as early responders and 12 as late responders.

## 2.2 Patients' samples:

De-identified, coded frozen PBMC samples from patients participating in the RAPPORT trial were received from the Tissue Repository Unit at Cincinnati Children's Hospital Medical Center (CCHMC, PI Susan Thompson). Total RNA isolated from CD14+ monocytes was received from the Baylor Institute for Immunology Research. Blood draws were performed at baseline (week 0), weeks 2, 4, 14 and 24 of the trial. PBMC samples or total RNA from a total of 35 individual patients were obtained; we did not obtain serial samples from all patients. Serum samples were obtained for ELISA assays from a total of 45 patients. Only samples from the RAPPORT study were analyzed, to avoid introduction of sample collection and processing bias. This study was approved by the Administrative Panels on Human Subjects Research from Stanford University.

## 2.3 ELISA for serum cytokines:

IL-1 receptor antagonist (IL-1ra) and IL-8 levels in serum were measured by kinetic sandwich ELISA (Peprotech, Rocky Hill, NJ, USA). Serum levels of IL-1 $\beta$ , IL-6 and IL-10 were measured using Cytometric Bead Array (CBA) (BD Biosciences, San Jose, CA). IL-18 serum levels were measured using Human IL-18 Platinum ELISA (Affymetrix/eBioscience/ThermoFisher Scientific); IL-18 Binding Protein (BP) was measured using IL-18BP ELISA from Abcam (Cambridge, MA).

## 2.4 Monocyte isolation:

Monocytes were isolated from frozen peripheral blood mononuclear cells (PBMCs) from RAPPORT subjects by positive selection with CD14 microbeads (Miltenyi Biotec, San Diego, CA, USA). Positive selection was chosen as the method of isolation that provided the highest yield and level of purity. Dead cells were removed prior to monocyte isolation using the Dead Cell Removal Kit (Miltenyi Biotec). Isolated monocytes were assessed for purity

using flow cytometry. The mean percentage of CD14<sup>+</sup> monocytes was 84% (range 60–99%) in post-sort samples.

## 2.5 Genes analyzed:

CD14<sup>+</sup> monocyte expression of the following genes was analyzed: Interleukin-1beta (*IL1B*) and the ATP Purinergic Receptor P2X7 (*P2RX7*), involved in IL-1 $\beta$  secretion [33]. Transcription factors (TF) and related genes: M1-associated TF: Interferon Regulatory factor (IRF) family 5 (*IRF5*); Signal transducers and activators of transcription (STAT) family 1 (*STAT1*) and its negative regulator suppressor of cytokine signaling 1 (*SOCS1*); M2 associated genes: *IRF4*, *STAT6*, Kruppel-Like Factor 4 (*KLF4*), and peroxisome proliferator-activated receptor gamma (*PPARG*). Primer sequences and brief description of genes analyzed are on Table 1. Housekeeping genes: eukaryotic translation elongation factor 1 alpha1 (*EEF1A1*), protein phosphatase 1, catalytic subunit, gamma isoform (*PPP1CC*) [27], and Ribosomal Protein L12 (*RPL12*). Primer sequences for all genes were obtained from primerdepot.nci.nih.gov/ and synthesized at Stanford University Pan Facility.

## 2.6 RT-PCR:

Total RNA from isolated monocytes was obtained using RNeasy Mini or Micro kit (Qiagen, Germantown, MD, USA) depending on monocyte number, and transcribed into cDNA using iScript Reverse Transcriptase (Bio-Rad, Hercules, CA, USA). cDNA (in duplicate) was amplified using SsoAdvanced™ SYBR® Green Supermix in a CFX384 qPCR machine (Bio-Rad). Relative amounts of transcripts for each gene were determined by the standard curve method, and adjusted for expression of housekeeping genes (combined expression of *PPP1CC*, *EEF1A1*, and *RPL12*). Each primer set was tested for assay efficiency, amplification of residual DNA and the profile of the melting curve. Additionally, cDNA from isolated monocytes from a single donor was used as a calibrator throughout the study.

## 2.7 Statistical analysis:

All statistical analyses were performed with IBM SPSS Statistics version 21. We obtained descriptive statistics from all data analyzed. We analyzed that data using a mixed effects regression model for repeated measures [34]. This approach allowed the missing data at some time points and accounted for partial pairing. To analyze if IL-1 inhibition, irrespective of clinical response, lead to change in any variable (cytokine or gene) tested, we analyzed for changes from baseline using time as a fixed effect. For analysis of effect of clinical response on the variables tested, we analyzed the early and late responder groups separately for changes during the duration of treatment. As this is an exploratory study, unpaired 2-group comparisons were also performed as described in the text, so as not to overlook any differences that may be small, but potentially biologically meaningful [35].

## 3. Results

### 3.1 Serum IL-1ra drops shortly after start of anti-IL-1 treatment

Levels of IL-1ra, the endogenous IL-1 receptor antagonist, positively correlate with IL-1 activity [36]. To assess whether treatment with rilonacept effectively reduces IL-1 activity, we measured the levels of circulating IL-1ra. In comparison to baseline, levels of IL-1ra

dropped significantly in the rilonacept group, 2 weeks after initiation of treatment, which was the earliest time point measured (Fig. 1A, Table 2). The level of IL-1ra stayed significantly below baseline levels in the rilonacept group throughout the 24 weeks (Fig. 1A, Table 2). For the placebo group, levels changed little during the placebo phase (weeks 0 to 4), decreasing significantly with treatment by weeks 14 and 24 of the trial (weeks 10 and 20 of the treatment) (Fig. 1B, Table 2). We also assessed circulating IL-1 $\beta$  levels, which were mostly close to the detection level of the assay (4 pg/ml), with no significant change during the trial (Table 2). These results are in line with the literature [37, 38] and our own data on an unrelated sJIA cohort (Macaubas et al, unpublished), which found that the levels are close to the assay detection levels, even during sJIA disease activity.

We also measured serum levels of IL-6, another cytokine involved in sJIA. We found no significant difference in circulating levels during the trial (Table 2). Another cytokine, IL-8, has been reported to respond to anti-IL-1 treatment [39], although in a short time frame. We did not observe a significant change in serum IL-8, suggesting that rilonacept treatment in sJIA does not alter this cytokine (Table 2). We also analyzed levels of IL-10, a regulatory cytokine often associated with suppression of immune response [40]; levels of serum IL-10 were unchanged throughout the trial (Table 2).

We also assessed levels of circulating IL-18 and IL-18 BP in a smaller set of samples (N=32). Elevated serum IL-18 is associated with sJIA [41], and treatment with IL-18BP, which binds IL-18 with high affinity and blocks its activity, is a potential treatment for sJIA [12]. Levels of serum IL-18 did not change with rilonacept treatment (Table 2). Levels of IL-18BP were increased at week 4 compared to baseline in the rilonacept group (Table 2); levels remains elevated in relation to baseline at week 14 but did not reach significance. By week 24, levels of IL-18BP were closer to baseline in the rilonacept group; no significant changes were observed in the placebo group (Table 2). It has been shown that IL-18 binding obscures measurement of IL-18BP [42], so the levels reported here are likely underestimated.

### 3.2 IL-1 inhibition decreases *IL1B* gene expression in CD14+ monocytes

We initially tested if IL-1 inhibition, irrespective of clinical response, leads to changes in gene expression in CD14+ monocytes. To perform this analysis samples were grouped based solely on duration (weeks) of treatment with rilonacept. The groups were weeks 0–4 (baseline), 10 (for placebo group) or 14 weeks (for the rilonacept group) and 20 (for placebo group) and 24 weeks (for the rilonacept group). There were no statistically significant differences at baseline between the placebo and rilonacept groups for any of the parameters tested ( $p > 0.05$ ).

We found that expression of *IL1B* in CD14+ monocytes was decreased significantly after 10–14 weeks and 20–24 weeks of rilonacept treatment (Fig. 2A), compared to baseline. We also measured expression of *P2RX7*, a purinergic receptor that recognizes ATP and mediates a range of functions, including inflammasome activation for IL-1 processing and secretion [33]. A significant decreased expression of *P2RX7* compared to baseline was observed at weeks 20–24 of treatment (Fig. 2B).

### 3.3 *IRF5* and *STAT6* expression decreases and *PPARG* increases following IL-1 inhibition

We chose to analyze a panel of TFs, based on their known roles in regulating monocyte/macrophage functional phenotype (Table 1). Monocyte expression of *IRF5*, a M1-related TF, was significantly decreased after 20–24 weeks of treatment (Fig. 2C). *STAT6* (a M2-related TF) expression by CD14<sup>+</sup> monocytes was significantly decreased after 10–14 and 20–24 weeks of treatment (Fig. 2D). The only TF that showed a statistically significant increase with riloncept treatment was *PPARG*. The elevation in *PPARG* expression achieved statistical significance after 20–24 weeks of treatment (Fig. 2E) in comparison to baseline.

### 3.4 Expression of other TFs analyzed did not change with IL-1 inhibition

We found that expression of the M1-related TF *STAT1* and its negative regulator *SOCS1* did not show significant changes from the baseline during the follow-up period (Fig. 3A, B). Similarly, the M2-related TFs *IRF4* and *KLF4* changed little from baseline throughout the treatment weeks (Fig. 3C, D)

### 3.5 Decreased expression of *IL1B* in CD14<sup>+</sup> monocytes in early treatment responders

We next compared gene expression changes in patients who achieved an early or late clinical response to treatment, defined as achieving ACR Pedi 30 before (early) or after (late) 4 weeks of treatment (See ‘Rapport trial’ under Material and Methods). There were no statistically significant differences between early responders and late responders at baseline for any of the genes analyzed. After 20–24 weeks of treatment, *IL1B* expression decreased significantly in CD14<sup>+</sup> monocytes of early responders, in comparison to baseline (Fig. 4A). In late responders, however, *IL1B* expression showed almost no change throughout the time that the samples were analyzed (Fig. 4A). Group to group comparison for week 20–24 time point show that *IL1B* expression was significantly lower in early responders in comparison to late responders ( $p < 0.05$ ). For *P2RX7* expression, both groups showed a tendency to decreased expression without reaching statistical significance (Fig. 4B).

### 3.6 Transient changes in *STAT1* and *SOCS1* expression in early treatment responders

Analyzing samples based on clinical response to treatment showed that *STAT1* was significantly increased in CD14<sup>+</sup> monocytes of early responders at weeks 10–14, in comparison to baseline; there was no change for late responders. Group to group comparisons showed that *STAT1* expression in early responders was significantly higher than in late responders at week 10–14 of treatment ( $p < 0.05$ , Fig. 4C). The increase in *STAT1* expression in early responders was transient, with values at week 20–24 closer to baseline. Concomitantly, *SOCS1*, the inhibitor of *STAT1*, was significantly lower in CD14<sup>+</sup> monocytes of early responders at the same time point as elevated *STAT1* (weeks 10–14 compared to baseline); no changes were observed for late responders (Fig. 4D). Using group to group comparisons, expression of *SOCS1* at weeks 10–14 was lower in early responder in comparison to late responders ( $p < 0.05$ ). At weeks 20–24, there were no statistical differences between early and late responders.

### 3.7 *IRF5*, *STAT6* and *PPARG* expression changes are independent of treatment response

Analysis of the samples based on treatment response showed a similar pattern for *IRF5* and *STAT6* expression in early and late responders, with a tendency toward decreased expression after 20–24 weeks (Fig. 5A, B). Similarly, for *PPARG* expression, there is a tendency for increased expression of *PPARG* in CD14+ monocytes in both early and late responders (Fig. 5C).

### 3.8 Response to treatment did not affect expression of *IRF4* or *KLF4*

Expression of the M2-related *IRF4* and *KLF4* in CD14+ monocytes did not show any noticeable changes associated with response to treatment (not shown)

## 4. Discussion

In this study, we find that systemic administration of the IL-1 trap riloncept is associated with changes in the expression patterns of IL-1 and key transcription factors related to monocyte activation in circulating monocytes. Some gene expression changes are associated with clinical response to the treatment, whereas other changes are related only to exposure to riloncept, arguing that distinct mechanisms may be involved in clinically related and clinically independent effects of IL-1 inhibition. As to some of its limitations, this study was performed with a limited number of samples, and changes between the early and late responders groups were statistically significant using only group to group comparisons. As such, further studies are necessary to confirm these observations.

We initially determined that IL-1 inhibition by riloncept is associated with a significant drop in circulating IL-1ra, detected at the earliest time point assessed (2 weeks). This finding fits with the known role of IL-1 in the induction of *IL-1RN* [43], indicating that inhibition of IL-1 by riloncept has a systemic effect on the IL-1 pathway. Effects of riloncept on other cytokine tested were less noticeable; specifically, there was no significant effect on circulating levels of IL-6. IL-1 inhibition by anti-IL-1 antibody canakinumab was shown to decrease levels of serum IL-6 rapidly [44]. The failure of riloncept to significantly decrease levels of serum IL-6 might be related to decreased efficiency of riloncept versus canakinumab, although no direct comparison between these drugs has been performed [45]. Similarly, no decrease in serum levels of IL-18 were observed with riloncept treatment, although a transient increase in serum IL-18BP was observed; the increase in IL-18BP may result in a temporary drop in active IL-18, even without changes in the total levels of IL-18. With canakinumab, IL-18 levels dropped significantly, although only after 8 weeks [44]. Elevated IL-18 levels have been shown to persist even during remission in sJIA [46]. Although IL-1ra levels dropped with riloncept treatment, indicating systemic effect on IL-1 pathway, other pro-inflammatory pathways involved in IL-6, IL-8 and IL-18 induction may persist during treatment with riloncept. For example, IL-18 has been shown to induce production of IL-6 and IL-8 independently of IL-1 $\beta$  [47]; the failure of riloncept to induce a significant drop of IL-18 levels may allow this cytokine to maintain production of IL-6 and IL-8, even when IL-1 pathways are inhibited. Riloncept may also potentially bind to IL-1ra, and this may, paradoxically, decrease or delay its efficacy. Another possibility is that

systemic induction of regulatory mechanisms, such as induction of IL-10, are not efficiently induced by rilonacept treatment.

We next examined if IL-1 inhibition by rilonacept affects expression of *IL1B* and transcription factors associated with monocyte activation in circulating CD14<sup>+</sup> monocytes. When we analyzed samples based solely on duration (weeks) of rilonacept treatment, we found that *IL1B* expression in CD14<sup>+</sup> monocytes is decreased compared to baseline following IL-1 blockade. This result agrees with the well-known property of IL-1 as an inducer of its own synthesis [48, 49]. Expression of *P2RX7*, which encodes a nucleotide receptor associated with inflammasome activation, IL-1 processing and release [33], also decreased, reaching significance after 20–24 weeks, arguing that blockade of IL-1 may also have moderate effect on *P2RX7* expression in CD14<sup>+</sup> monocytes. Other blood cells may be affected differently, as Quartier et al [15] found that expression of *P2RX7* was increased following treatment with anakinra (recombinant IL-1ra), in analysis of whole blood, perhaps due to changes in neutrophils [50].

Among the TF associated with monocyte activation, we observed that after IL-1 inhibition, the M1-related TF IRF5 was decreased. We have previously observed increased in both M1/M2-associated markers during sJIA flare [6]; IRF5 has been found to be important for control of inflammation in macrophages [51], and the reduction of IRF5 expression may be related to reduction in the pro-inflammatory monocyte phenotype with IL-1 inhibition. We also found that *STAT6* expression was decreased after rilonacept treatment. In the context of monocyte activation, STAT6 plays an essential role in M2 polarization mediated by IL-4 [26]. Given the previously observed mixed M1/M2-like skew of circulating sJIA monocytes [6], the decrease of *STAT6* expression may represent a reduction in the M2-like phenotype of circulating monocytes in sJIA, perhaps indicating diminishing levels of inflammation in tissues. However, expression of other tested TFs associated with M2 development were less affected by IL-1 inhibition. *KLF4* and IRF4 did not show a change in expression with IL-1 inhibition.

Another M2-related TF, PPARG [52], showed increased expression with IL-1 blockade. STAT6 has been shown to physically interact and amplify PPARG responses [53]. Nevertheless, blockade of IL-1 may play a direct role in regulating *PPARG* expression. In liver cells, treatment with IL-1, but not IL-6, reduces the mRNA expression of *PPARG* (and other PPARs) [54]. PPARG ligands also inhibit the secretion of IL-1 by stimulated monocytes, and induce production of IL-1ra [55]. Chen et al [56], in a study of monocytes from patients with atrial fibrillation, showed a significant negative correlation between serum levels of IL-1 and expression of *PPARG*. The inhibition of IL-1 may, directly or indirectly, contribute to increased expression of PPARG. Increased expression of *PPARG*, together with reduction in *IRF5* and *STAT6* expression, may skew monocytes in sJIA from a mixed M1/M2-driven to a PPARG-driven M2-like phenotype. Szanto et al [53] found that while the majority (85%) of genes induced by PPARG are STAT6-dependent, close to 30% of genes repressed by PPARG are STAT6 independent. PPARG activation has been shown to suppress IL-1 $\beta$  production [57]. These expression changes, if translated into active protein, could result in an increase of PPARG-repressive activity, independent of STAT6, following IL-1 blockade.

When we examined the transcription signatures in light of clinical responses to rilonacept (as determined by achieving ACR Pedi 30), we found that expression of *IL1B* was decreased significantly only in early responders after 20–24 weeks of treatment. At this point, the expression of *IL1B* was lower in early responders than late responders. Notably, the expression of *IL1B* in CD14+ from late responders was similar to baseline throughout the whole trial, although the number of late responders examined was small. This result indicates that, although rilonacept is generally associated with decreased *IL1B*, heterogeneity can be observed when clinical response is taken into account. In analysis of whole blood, the decrease in *IL1B* expression has also been observed to be more pronounced in sJIA responders to canakinumab, a human anti-IL-1 $\beta$  antibody [44]. Of note, *IL1B* mRNA expression is only one aspect of IL-1 $\beta$  biology. *IL1B* is translated into pro-IL-1 $\beta$ , which can be stored intracellularly; activation of IL-1 $\beta$  is dependent on inflammasome activation and cleavage by caspase-1, followed by secretion [58]. The significance of alterations in *IL1B* expression in sJIA monocytes following IL-1 inhibition needs to be confirmed by analysis of active protein.

We found that expression of *STAT1* was transiently increased (during week 10–14) in early responders in comparison to baseline and in comparison to late responders. Concomitantly, the expression of *SOCS1*, suppressor of cytokine signaling, was decreased in early responders and lower in the early responder group in comparison to late responders over the same time period. We have previously observed that STAT1 phosphorylation in response to IFN $\gamma$  and  $\alpha$  was decreased in monocytes from untreated sJIA patients with active disease in comparison to controls, indicating that response to IFN is dysregulated in sJIA monocytes [27]. Additionally, an animal model based on administration of Freund's complete adjuvant that reproduced several characteristic of sJIA, showed more robust and diverse sJIA-like symptoms in the absence of IFN $\gamma$  [59], indicating a contribution from dysregulation in the IFN $\gamma$  pathway in this model. We also found that IL-1 inhibition (with anakinra) or IL-6 inhibition (with tocilizumab) was associated with enhanced IFN $\gamma$ -induced STAT1 phosphorylation compared to controls [27]. Furthermore, it has been shown that blockade of IL-1 with anakinra revealed an IFN signature in whole blood from sJIA patients [15], and the levels of two IFN-inducible proteins (IP-10 and TRAIL) were elevated in serum following 6 months of anakinra treatment. These results suggest a relationship between IL-1 and IFN in sJIA. In other settings, reciprocal effects have been observed, including IFN inhibition of IL-1 activity [60, 61]. Work in host defense has indicated that production of IL-1 *in vivo* might be inhibited by IFN [62, 63], in a STAT1-dependent manner [62]. Guarda et al [61] showed that IFN signaling via STAT1 inhibits the activity of the NLRP1 and NLRP3 inflammasomes, consequently decreasing the production of mature IL-1 $\beta$  by caspase-1. In an animal model of tuberculosis, Mishra et al [64] found that the suppressive effect of IFN $\gamma$  on IL-1 production was mediated by effects of nitric oxide (NO) on NLRP3 inflammasome assembly.

The clinical response to IL-1 blockade may involve, directly or indirectly, normalization of IFN signaling in monocytes, either via increased expression of STAT1 and/or decreased expression of SOCS1. Of note, SOCS1 can be induced by IL-21 [65]; investigations of the level of IL-21 in sJIA might be informative. An increase in IFN activity may follow IL-1 inhibition, as suggested by the results described in [15]. Although the STAT1/SOCS1

changes were transient, maybe as a result of appearance of other regulatory mechanisms, the increase in IFN signaling and possible effects on the NLRP3 inflammasome in early responders might further reinforce the IL-1 inhibition, resulting in further decrease of IL-1 $\beta$  expression. Although Quartier et al, 2011 [15] did not find any changes in expression of genes associated with the NLRP3 inflammasome in whole blood of anakinra-treated sJIA patients, analysis of isolated monocytes may be informative. Effects of IL-1 inhibition in other important pathways, such as NF- $\kappa$ B, which is activated by IL-1 $\beta$  among other stimuli, may also occur. For example, there is evidence that IL-1 inhibition by anakinra may lead to decrease in nuclear translocation of NF- $\kappa$ B p65 [66].

The findings reported in this study refer only to gene expression. In addition to control of gene expression [67–69], multiple levels of post-transcriptional regulation exist, and it is unknown if IL-1 inhibition with riloncept also alters the rate and/or stability of the transcripts, or other processes that control gene expression, such as epigenetic regulation [70]. An analysis of circulating CD14<sup>+</sup> monocytes from RAPPORT subjects (Hay et al, unpublished) found that methylation changes in several genes could be detected when comparing monocytes at baseline with active disease to monocytes at week 24 with quiescent disease. Furthermore, it is unknown if the alterations in gene expression described here are reflected into translational changes of active proteins; additionally, post-translational modifications, such as phosphorylation, especially of STAT proteins, also influence molecule activity [71]. To understand the mechanism(s) of effects of IL-1 inhibition on sJIA monocytes, further studies analyzing protein levels and function will be needed.

Analysis of CD14<sup>+</sup> monocyte gene expression following IL-1 inhibition with riloncept shows that M1-associated IRF5 and M2-associated TF STAT6 are decreased, whereas *PPARG* expression increases, resulting in a monocyte phenotype of IRF5<sup>lo</sup> STAT6<sup>lo</sup> and PPARG<sup>hi</sup>, compared to baseline at active sJIA. The PPARG changes may be a direct effect of reduced IL-1. However, these changes are independent of therapeutic response, suggesting they may be necessary, but not sufficient, for clinical improvement. These changes may result in a persistent M2-like phenotype in circulating CD14<sup>+</sup> monocytes (and perhaps tissue monocytes) even during sJIA quiescence, under influence of PPARG rather than STAT6. Our results also show that reductions in *IL1B* transcripts correlate most robustly with achieving less active sJIA on riloncept. It will be of interest to determine whether diminished *IL1B* expression marks a distinct subset of blood monocytes compared to IRF5<sup>lo</sup>, STAT6<sup>lo</sup>, PPARG<sup>hi</sup> cells; single cell RNA sequencing will be required to answer this question. In addition to the association of reduced *IL1B* with clinical improvement, it is notable that approximately 30% of the early responders have higher baseline *IL1B* expression than the rest of all subjects studied. Thus, elevated initial monocyte *IL1B* transcripts may be a prognostic biomarker of responders to riloncept and to IL-1 inhibition more generally [44]. In a recent study, alleles associated with higher expression of *IL1RN* (gene coding for IL1-ra) were associated with failure to respond to anakinra [72]. Early reduction of monocyte *IL1B* expression in riloncept responders (and possibly reduction in monocyte P2XR7) strengthens the conclusion that IL-1 activity is a driver of sJIA in at least this subset of children, corroborating findings with other IL-1 inhibitors [15, 44]. An

important outstanding goal is to immunophenotype incomplete and non-responders to identify candidate alternative therapeutic targets for these patients.

The other monocyte transcriptional changes associated with an early response to riloncept (transient increase in STAT1 and decrease in SOCS-1 compared to late responders) suggest that a deficiency of interferon signaling may contribute to pathogenesis in IL-1 associated disease. Determining if the transcriptional changes are biomarkers of a therapeutic response to other medications that target IL-1 or to other interventions, such as IL-6 inhibition, will be useful.

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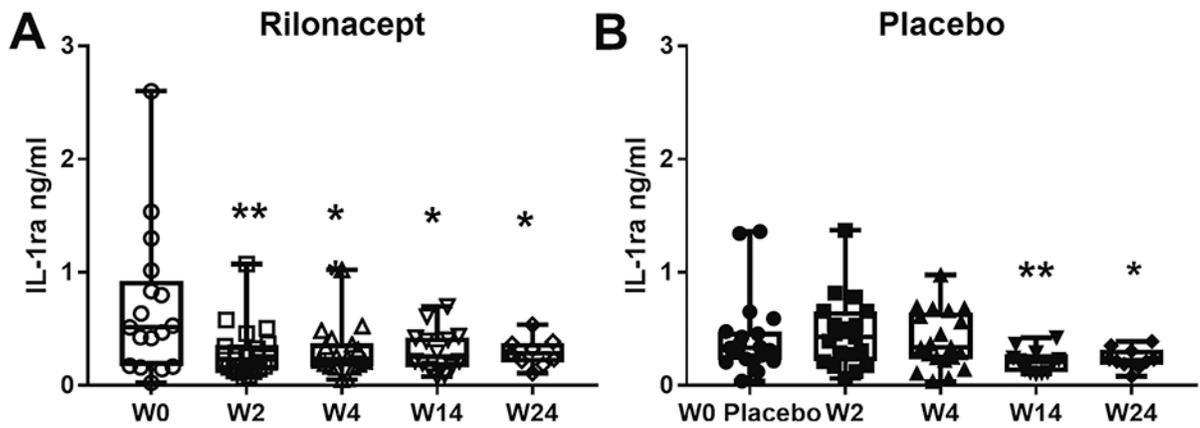
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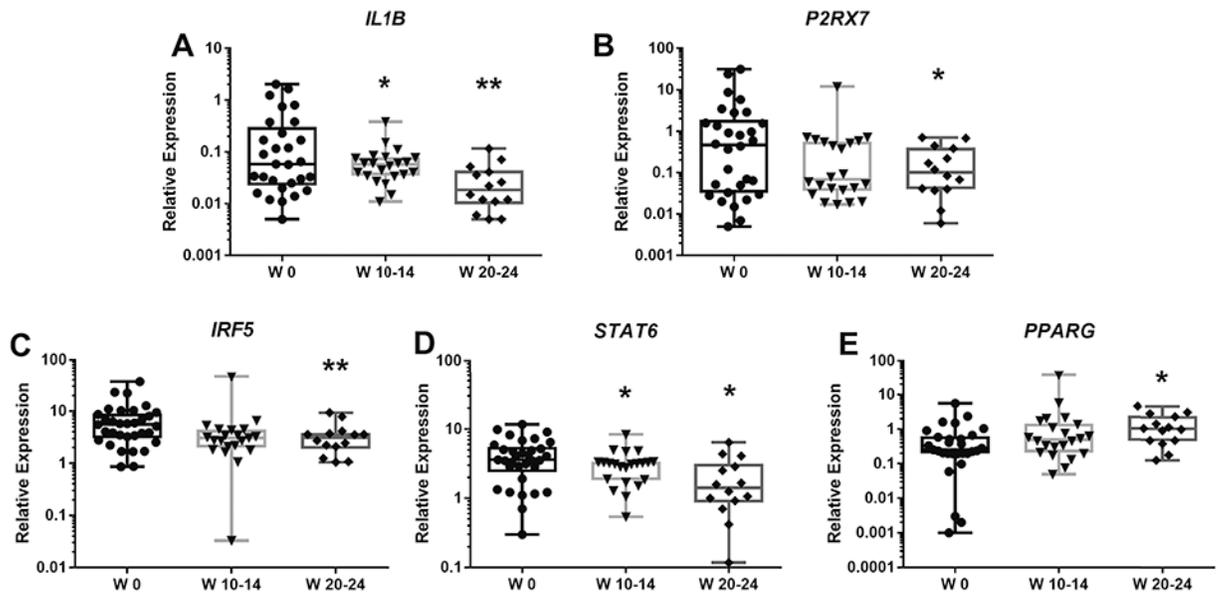
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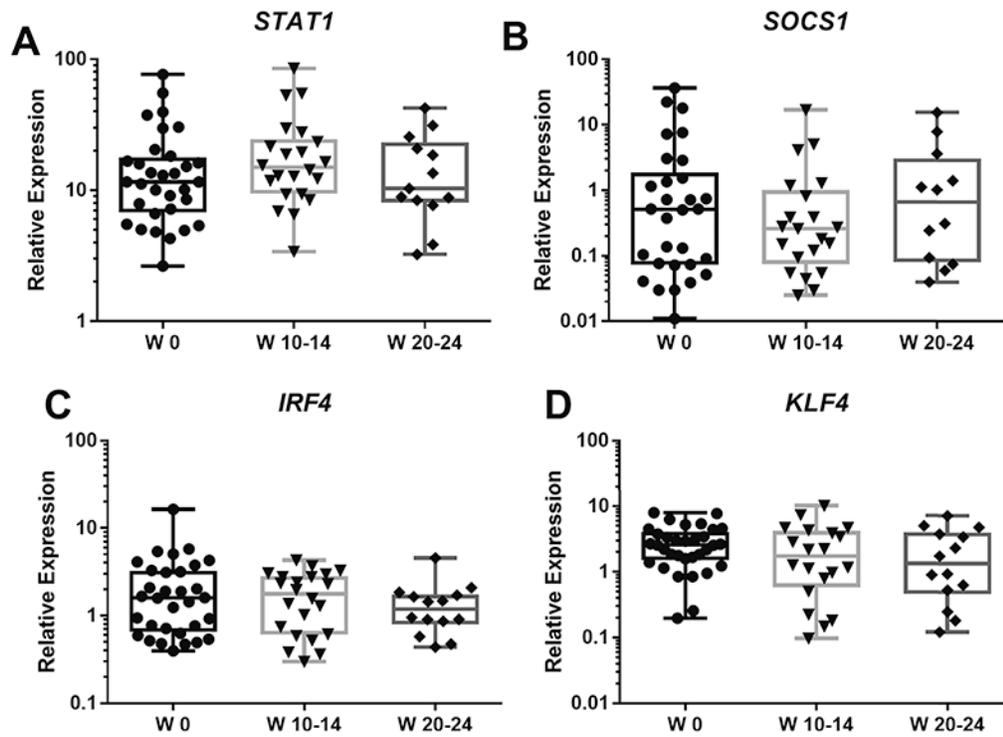
**Figure 1: Serum IL-1ra decreases after 2 weeks of rilonacept treatment.**

IL-1ra was detected in serum samples from patients in the RAPPORT trial as described in Material and Methods; (A), rilonacept group and (B), placebo group. Serum samples from a total of 45 patients were analyzed; samples were obtained at baseline, and week 2, 4, 14 and 24 of the trial; not all time points were available from all patients. Boxplots extend from the 25th to 75th percentiles, and the whiskers extend from the minimum to the maximum point; middle line represents the median. Statistical analysis used mixed effects regression model. P values associated with type III tests of fixed effects. One symbol,  $p < 0.05$ , two symbols,  $p < 0.01$  in relation to baseline.



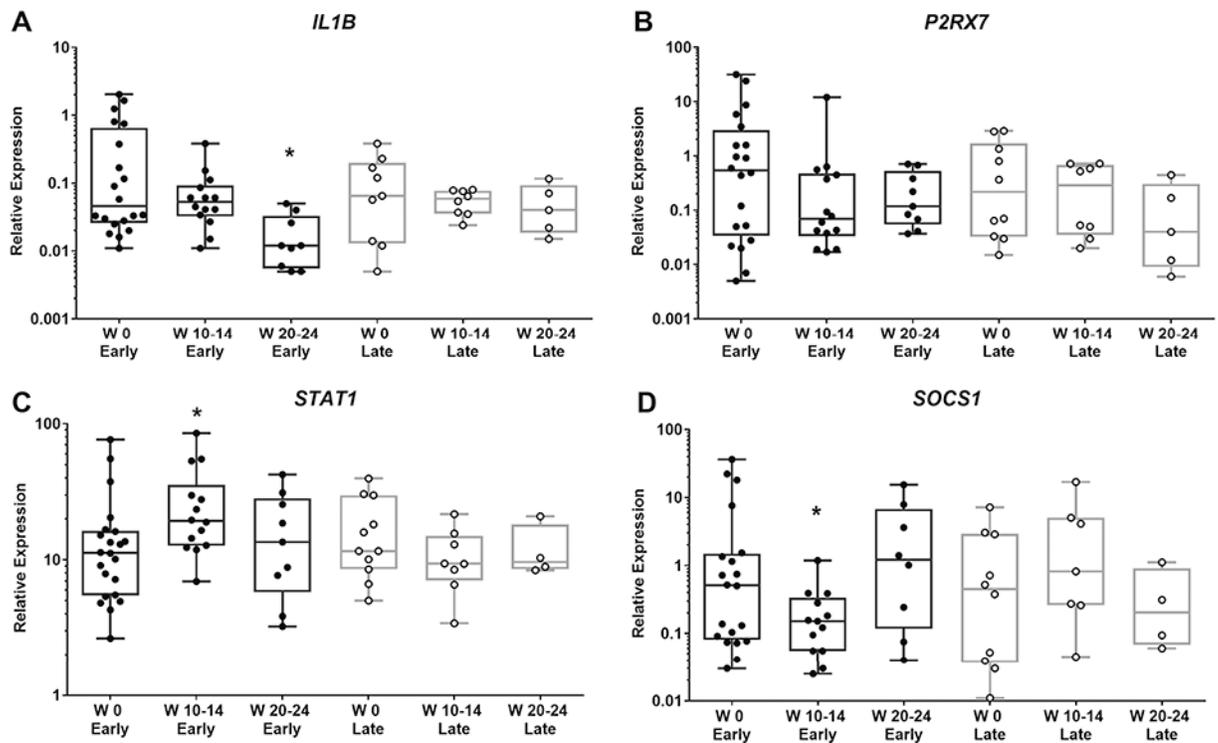
**Figure 2: Monocyte *IL1B*, *STAT6* and *IRF5* expression is reduced, and *PPARG* expression increased, after 20–24 weeks of rilonacept treatment.**

Total RNA was extracted from peripheral blood CD14<sup>+</sup> monocytes; gene expression was determined using RT-PCR, as described in Material and Methods. X-Axis indicates the number of weeks (W) of treatment with rilonacept. Boxplots extend from the 25th to 75th percentiles, and the whiskers extend from the minimum to the maximum point; middle lane represents the median. Y-axis shows log<sub>10</sub> scale. Statistical analysis used mixed effects regression model. P values associated with type III tests of fixed effects. One symbol,  $p < 0.05$ ; two symbols,  $p < 0.01$  in relation to baseline.



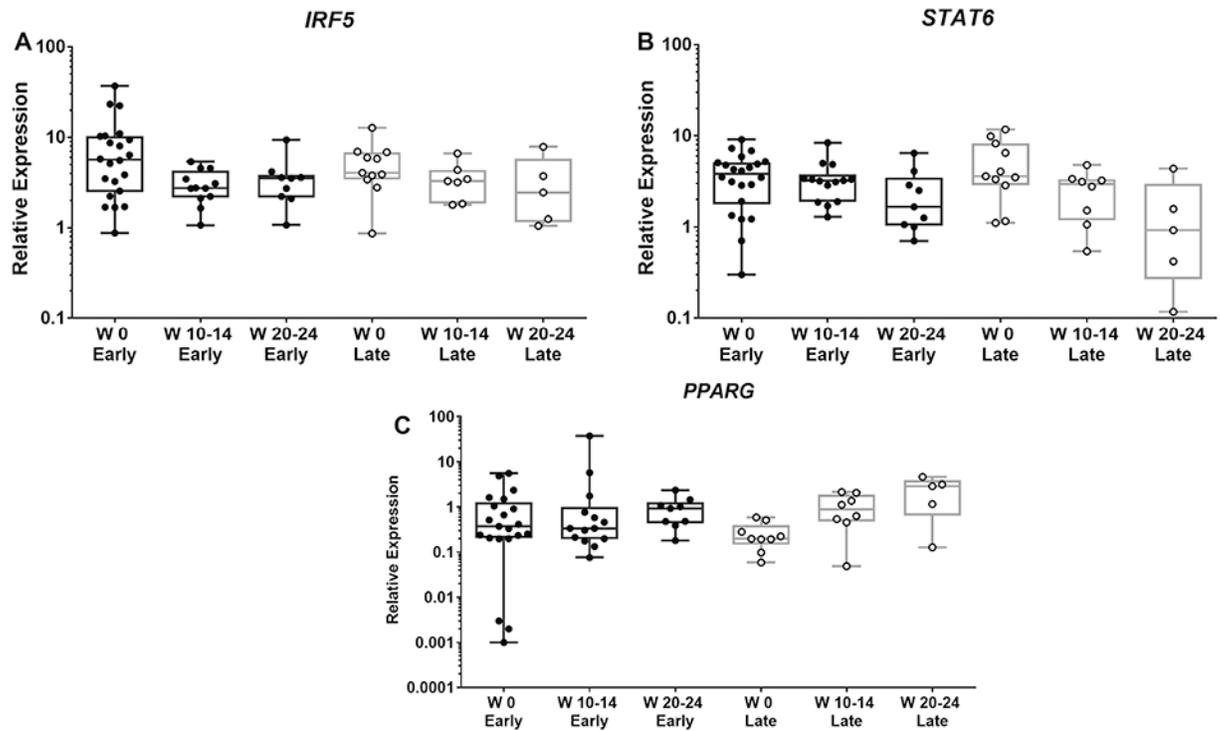
**Figure 3: Expression of *STAT1*, *SOCS1*, *KLF4* and *IRF4* does not change after riloncept treatment.**

Total RNA was extracted from peripheral blood CD14<sup>+</sup> monocytes; gene expression was determined using RT-PCR, as described in Material and Methods. X-Axis indicates the number of weeks (W) of treatment with riloncept. Boxplots extend from the 25th to 75th percentiles, and the whiskers extend from the minimum to the maximum point; middle line represents the median. Y-axis shows  $\log_{10}$  scale. Statistical analysis used mixed effects regression model. All comparisons  $p > 0.05$ , and deemed non-significant.



**Figure 4: *IL1B* expression is decreased in rilonacept early responders, while *STAT1* and *SOCS1* are transiently affected with rilonacept treatment.**

Total RNA was extracted from peripheral blood CD14<sup>+</sup> monocytes; gene expression was determined using RT-PCR, as described in Material and Methods. X-Axis indicates the number of weeks (W) of treatment with rilonacept. Early (responders): achieved ACR Pedi 30 before 4 weeks or less of treatment; Late (responders): more than 4 weeks of treatment to achieve ACR Pedi 30. Boxplots extend from the 25th to 75th percentiles, and the whiskers extend from the minimum to the maximum point; middle line represents the median. Y-axis shows log<sub>10</sub> scale. Statistical analysis used mixed effects regression model. P values associated with type III tests of fixed effects. One symbol, p < 0.05, in relation to baseline of specific responder group.



**Figure 5: *STAT6* and *PPARG* expression changes similarly in responders and nonresponders.** Total RNA was extracted from peripheral blood CD14<sup>+</sup> monocytes; gene expression was determined using RT-PCR, as described in Material and Methods. X-Axis indicates the number of weeks (W) of treatment with rilonacept. Early (responders): achieved ACR Pedi 30 before 4 weeks or less of treatment; Late (responders): more than 4 weeks of treatment to achieve ACR Pedi 30. Boxplots extend from the 25th to 75th percentiles, and the whiskers extend from the minimum to the maximum point; middle line represents the median. Y-axis shows log<sub>10</sub> scale. Statistical analysis used mixed effects regression model. All comparisons  $p > 0.05$ , and deemed non-significant.

**Table 1:**

Gene information and primer sequences.

Gene symbol	Function(s) in monocyte/macrophages	Right primer sequence	Left primer sequence
<i>IL1B</i>	Cytokine produced by activated monocytes/macrophages	AAG CCC TTG CTG TAG TGG TG	GAA GCT GAT GGC CCT AAA CA
<i>P2RX7</i>	Cell surface receptor for extracellular ATP; involved in IL-1 $\beta$ processing and release through activation of NLRP3 inflammasome	GCT TGT CAC TCA CCA GAG CA	CTC GGA TCC AGA GCA TGA AT
<i>M1-related genes</i>			
<i>IRF5</i>	Induces expression of IL-12 p40 subunit; suppresses IL-10 expression with GM-CSF	GAT GGA CTG GTT CAT GGC AG	CAG AGC TCA GCT TGG TCC C
<i>STAT1</i>	Induced by IFN $\gamma$ stimulation	TGA ATA TTC CCC GAC TGA GC	AGG AAG ACC CAA TCC AGA TGT
<i>SOCS1</i>	Inhibitor of STAT1 signaling	CAC ATG GTT CCA GGC AAG TA	CTA CCT GAG CTC CTT CCC CT
<i>M2-related genes</i>			
<i>IRF4</i>	Promotes expression of several M2-related genes in a chitin model (helminth derived), or in response to M-CSF; negative regulator of TLR signaling; increased expression with IL-4 stimulation	GAG TCA CCT GGA ATC TTG GC	CCT GCA AGC TCT TTG ACA CA
<i>KLF4</i>	Induced by IL-4 via STAT6; may inhibit expression of M1-induced genes	GTC AGT TCA TC TGA GCG GG	AGA GTT CCC ATC TCA AGG CA
<i>PPARG</i>	Induced by IL-4; PPARG activity amplified via STAT6	GAG AGATCC ACG GAG CTG AT	AGG CCA TTT TGT CAA ACG AG
<i>STAT6</i>	Activated by IL-4; essential role in M2-type polarization	ACT TTT TCT GGG GGC ATC TT	AGA AGA CAG CAG AGG GGT TG

Table 2:

Cytokine levels in serum of RAPPORT samples

Cytokine	Placebo Group					Rilonacept Group				
	Week 0	Week 2	Week 4	Week 14	Week 24	Week 0	Week 2	Week 4	Week 14	Week 24
IL-1ra	330 (222-477)	429 (210-657)	336 (228-647)	207 (114.8-276.8)*	222 (182-315.3)*	514 (171-925)	228 (137-357)*	231 (165.8-378.8)*	253.5 (160.5-424.5)*	285 (201-376)*
IL-1 $\beta$	5.5 (4.7-17.9)	5.2 (4.2-11.9)	7.1 (5-18.7)	5 (4.2-5.9)	12.4 (4.8-39.4)	5.2 (4.6-8.2)	6.9 (4.4-9.4)	9.1 (5.5-20.3)	5.3 (5.3-12.2)	6.3 (5-10)
IL-6	65.6 (23-359)	48.4 (24-75.3)	51.3 (26-178.9)	30.5 (22.6-103.7)	41.3 (26.1-120.1)	85.9 (31-150.8)	40.2 (24.2-121)	46.4 (24.9-100.2)	75.9 (27.2-84.4)	67.4 (33.7-118.7)
IL-8	72 (33-312)	165 (48-825)	376.5 (43.5-753)	141 (38.3-9229)	540 (72-477.2)	177 (58.5-1823)	111 (33-462.5)	120 (29.3-1092)	148.5 (26.3-514.5)	384 (120-7298)
IL-10	15.8 (12.4-24.3)	13.3 (11.8-16.9)	13.1 (12-18.9)	13.1 (11.6-14.7)	12.7 (11.9-15.4)	13.2 (10.9-17.4)	14.5 (13.1-18)	14 (12.5-17.9)	12.8 (11.9-16.7)	13.6 (13-15)
IL-18	849.5 (63.8-2446)	637.5 (92.5-3246)	970 (254.5-2749)	1599 (820.5-4947)	1001 (56.3-2295)	412 (41.5-8102)	1241 (177-2210)	2498 (66-7652)	2381 (1322-5919)	1789 (283-5640)
IL-18BP	58.5 (24.8-155.7)	56.9 (29.9-109.7)	63.6 (0.7-75.4)	64.6 (2-146.8)	40.7 (19.1-123.6)	31.2 (5.9-106.2)	60.8 (1-135.1)	165.9 (55.3-334.3)*	234.7 (59.7-778.2)	93.6 (0.4-205.4)

Values expressed as median and interquartile range; unit: pg/ml

Statistical analysis used mixed effects regression model. P values associated with type III tests of fixed effects. One symbol,

\* p&lt;0.05 in relation to baseline.