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Pharmacological repression of RORγ is therapeutic in the collagen-induced arthritis experimental model

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Abstract

Objective—The nuclear receptor RORγ (RAR-related orphan receptor gamma; T cell specific isoform is RORγt) is a key regulator of T_H17 cell differentiation controlling the production of the inflammatory cytokine IL17. Further it has been shown that LPS stimulation of monocytes leads to induction of RORγ. Previously we have shown that the potent and selective inverse agonist of RORγ, SR2211 was effective at suppressing IL17 production in EL4 cells. Further we demonstrate here that SR2211 treatment blocks proinflammatory cytokine expression in LPS stimulated RAW264.7 cells. Based on these findings SR2211 was administered to collagen-induced arthritis (CIA) mice to evaluate the ability of the compound to reduce joint inflammation.

Methods—Collagen was injected into the tail of DBA mice followed by a second boost inoculation 21 days later. Three days prior to the boost inoculation, SR2211 was administered into these mice twice daily for 15 days. Thymus, spleen and lymph node (DLN) were harvested and T_H17 cell differentiation and DLN stimulation were performed.

Results—Treatment of T_H17 cells with SR2211 suppressed the expression and production of inflammatory cytokines. Likewise, SR2211 reduced inflammatory cytokine production in LPS stimulated RAW264.7 cells. CIA mice administered SR2211 twice daily for 15 days exhibited statistically significant reduction in joint inflammation as compared to mice receiving only vehicle. Interestingly, systemic T_H1 cell activation was detected in SR2211 treated CIA mice as indicated by an increase in IFNγ.

Conclusions—These findings support targeting RORγ to therapeutically repress inflammatory T cell function and macrophage activation in rheumatoid arthritis. Compounds such as SR2211 have potential utility for the treatment of inflammatory disease.

Rheumatoid arthritis (RA) is an inflammatory disease that is characterized by extensive synovial hyperplasia, cartilage damage, bone erosion, and functional joint disability [1]. The inflammation in RA results from infiltration of inflammatory cells and the production of

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pro-inflammatory cytokines, prostaglandins and nitric oxide [2]. The cytokine TNFα has been shown to play a major role in the pathophysiology of RA and increased exposure to TNFα leads to degradation of cartilage and bone [3, 4]. The efficacy of anti-TNFα therapy in the treatment of RA is well documented and exemplified by clinical use of infliximab (Remicade), etanercept (Enbrel) and adalimumab (Humira). However, chronic administration of these anti-TNFα agents is directly associated with an increased risk of urinary tract and respiratory infections, and pneumonia. In addition to targeting TNFα, repression of other inflammatory cytokines such as IL1β [5], IL6 [6, 7], LTα1β2 [8], and IL17A [9] have shown efficacy in various animal models of arthritis. Targeted sequestration of IL17A, commonly referred to as IL17, using antibodies has gained significant momentum recently. The receptor for IL17 (IL17RA) was found to be overexpressed in peripheral whole blood of RA patients and the receptor was detected locally in synovium of the same patients [10, 11]. IL17 is an inflammatory cytokine produced by T_H17 cells and it has been shown that IL17 is present at sites of inflammatory arthritis and it synergizes the inflammatory response induced by other cytokines such as TNFα[12-14]. T_H17 cells differ from T_H1 and T_H2 lineages in that they develop under the influence of TGFβ, IL6, and IL1. Further, these cells have IL23 as a maturation factor and exclusively express the T cell specific isoform of RORγ, RORγt [15]. T_H17 cell differentiation and function in humans is associated with susceptibility to inflammatory bowel disease, rheumatoid arthritis, and psoriasis [16-18]. Recently, the therapeutic potential of anti-IL17 therapy was evaluated in a phase I study as adjunct therapy to patients taking oral disease-modifying anti-rheumatic drugs (DMARDs). As compared to placebo, patients given LY2439821, a potent anti-IL17 antibody, had reduced joint inflammation and erosion [19].

Members of the nuclear receptor (NR) superfamily are ligand-dependent transcription factors that regulate the expression of target genes that mediate a wide range of physiological processes from development, energy production and metabolism, to immunity. NRs are multi-domain DNA binding proteins that are activated by ligand binding to the receptors ligand-binding domain (LBD). Binding of ligand drives allosteric alterations in the receptors conformation dynamics facilitating the interaction and recruitment or the displacement of chromatin remodeling complexes [20]. The work presented here is focused on the NR1F subfamily containing the retinoic acid receptor-related orphan receptors or RORs. This subfamily contains three genes, RORα, RORβ, and RORγ and each of the RORs display significant sequence similarity and each gene generates several isoforms, differing only in their amino termini as a consequence of alternative promoter usage and exon splicing [21-24]. The RORs have been shown to bind to DNA as monomers on half-site elements with a 5'-A/T-rich extension [23, 25] and like most NRs, there are numerous DNA binding sites, known as response elements, for the NRF1 family within the promoter regions of a wide range of genes in a variety of tissues making it difficult to assign a precise role for each specific member of the family. However, studies have shown a clear role for RORα as a regulator of several metabolic pathways controlling the expression of genes such as Glucose-6-phosphatase (G6Pase) [26, 27]. Analysis of RORα-deficient *Stagger* mice clearly shows a role for this receptor in the development of the central nervous system [28, 29]. Interestingly, these animals display immune abnormalities associated with increased mRNA levels of IL1β, IL6 and TNFα while consuming a high-fat diet [30, 31]. In gain-of-

function studies with RORα it was demonstrated that this receptor negatively regulates the inflammatory response by interfering with the NF-κB signaling pathway [32].

The T cell specific RORγ isoform (RORγt) has been shown to be the key lineage-defining transcription factor to initiate the differentiation program of T_H17 cells [15]. Previously, we demonstrated that the synthetic LXR agonist T0901317, in addition to activation of LXR and to lesser degree FXR and PXR, binds to and represses the activity of both RORα and RORγ [33]. Therefore, to investigate the specific effects of repression of RORγ in T_H17 cell development independent of RORα, we applied retrosynthetic approaches to modify T0901317. This led to the development of the potent and specific RORγ inverse agonists SR2211 [34] and SR1555 [35], both which suppress T_H17 cell differentiation and block IL17 production. T_H17 cells produce IL17 and IL22 and they have been implicated in autoimmune diseases including rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [36, 37]. Importantly, mice lacking IL17 are resistant to developing experimental autoimmune encephalomyelitis or EAE, a model of multiple sclerosis, and to collagen-induced arthritis or CIA, a model of rheumatoid arthritis. As such, pharmacological inhibition of RORγ activity could offer a potential therapeutic approach for treatment of immune disorders including rheumatoid arthritis [38].

In addition to T_H17 cells, other cell types play major roles in inflammation. Macrophages are specialized differentiated mononuclear phagocytic cells that perform key roles in antimicrobial defense, autoimmunity, and inflammatory disease [39]. It has been shown that macrophages can produce a wide range of inflammatory cytokines including TNFα and IL17. Several studies have shown a role for RORs in regulating macrophage activation [40, 41]. Of relevance to the pathogenesis of RA are the effects of IL17 in driving osteoclastogenesis, leading to bone resorption [14, 42]. Prior reports have shown that neutralization of IL17 in mice decreases the severity of antigen-induced arthritis[43]. Further, the severity of collagen-induced arthritis was decreased in IL17-deficient mice and mice administered IL17 neutralizing antibodies [44]. Despite the complex etiology of RA, IL17 has been shown to be associated with the severity of RA [45, 46].

Previously we had shown that the potent and selective inverse agonist of RORγ SR2211 was effective at suppressing IL17 and IL23R gene expression in EL4 cells [44]. Here, we demonstrate that treatment of naïve T cells with SR2211 inhibits the differentiation towards T_H17 cells without blocking the differentiation towards T_H1 cells. In addition, compound treatment reduces the production of the inflammatory cytokines from T_H17 cells and macrophages. More importantly, we demonstrate that chronic systemic administration of SR2211 positively impacts the evolution of CIA in DBA mice.

MATERIALS AND METHODS

Mice

Wild-type DBA/1J mice were purchased from Taconic (Hudson, NY). EGFP-RORγt transgenic mice were purchased from Jackson laboratory (Bar Harbor, ME). Strain was B6.129P2(Cg)-$Rorc^{tm2Litt}$/J. We followed the genotyping protocol supplied by Jackson laboratory for generation of homozygote transgenic mice. All animals were handled and

housed in facilities in accordance with The Scripps Research Institute Institutional Animal Care and Use Committee.

Cell lines and T-cell differentiation *in vitro*

EL4 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics (penicillin and streptomycin; Invitrogen). The conditions for the different T-helper cell subsets were; 20μg/mL anti-IL4 (clone 30340, R&D systems) and 20μg/mL anti-IFNγ (clone H2, R&D systems) for T_H0 (neutral conditions); 20μg/mL anti-IL4, 20ng/mL IL12 (R&D systems) and 10ng/mL IFNγ (R&D systems) for T_H1 conditions; 20μg/mL anti-IFNγ, 20μg/mL anti-IL4, 1ng/mL TGFβ and 10ng/mL IL6 (R&D systems) for T_H17 conditions. All cultures were stimulated with 1μg/mL anti-CD3 (eBiosciences) and 1μg/mL anti-CD28 (eBiosciences). For naïve T cell differentiation, CD4+T cells were enriched using negative selection MACS kit (Millipore). Enriched CD4+ T cells were activated with 5μg/mL plate-bound anti-CD3 and 1μg/mL anti-CD28 in the presence of 20μg/mL anti-IFNγ, 20μg/mL anti-IL4, 1ng/mL TGFβ and 10ng/mL IL-6. Four to five days after activation, all cells were re-stimulated with 5ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma) and 500ng/mL ionomycin (Sigma) for 5h. The supernatant was harvested and the concentration of IL17 and IFNγ were measured using a Luminex assay kit (Invitrogen).

Quantitative real-time PCR analysis

RNA extraction and real time PCR analysis was performed as previously described [34]. IL1β and IL6 primer sets were as follows: IL1β, 5’-CAG GAT GAG GAC ATG AGC ACC-3’ (forward), 5’-CTC TGC AGA CTC AAA CTC CAC-3’ (reverse); IL6, 5’-GTA CTC CAG AAG ACC AGA GG-3’ (forward), 5’-TGC TGG TGA CAA CCA CGG CC-3’ (reverse); RORγ, 5’- ACC TCC ACT GCC AGC TGT GTG CTG TC-3’ (forward), 5’- TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3’ (reverse); TNFα, 5’- GAC GTG GAA GTG GCA GAA GAG -3’ (forward), 5’- TGC CAC AAG CAG GAA TGA GA -3’ (reverse).

Induction and clinical evaluation of CIA

CIA was induced in 8-12 week old female DBA/1J mice (Taconic) as previously described with some modifications [47-50]. Chicken type II collagen in an emulsion with complete Freund's adjuvant ((CFA); Hooke Laboratories) was used for initial immunization and chicken type II collagen in an emulsion with incomplete Freund's adjuvant ((IFA); Hooke Laboratories) was used for boosting. Collagen was delivered by intradermal injection at the base of the tail. Mice were observed daily for signs of joint inflammation and scored for clinical signs beginning on day 1 following administration of the boost. The scoring system for the severity of arthritic symptoms was the same as previously described [47]. Mice were scored daily on a scale of 0-4 for each paw: 0 = no clinical disease; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3 = entire paw inflamed and swollen; and 4 = severely inflamed and swollen paw or ankylosed paw. Ankylosed paws were determined by the inability of the animal to grip the wire top of the cage.

Compound synthesis and administration

SR2211 ($IC_{50:}$>0.32μM; 98% repression at 10μM) was synthesized as previously described [34] as was SR1555 ($IC_{50:}$>1.5μM; 59% repression at 10μM) [35]. Dexamethasone was purchased from Sigma–Aldrich. All test compounds were formulated in 15% cremophor EL at 2mg/ml unless otherwise specified. Mice were administered test compounds (dexamethasone, SR2211, SR1555) or vehicle only (15% cremophor EL) by intraperitoneal injection (ip) twice a day (BID). In the prevention paradigm test compounds were administered starting three days post initial collagen immunization. In the therapeutic paradigm, test compounds were administered starting three days prior to delivery of the immunization boost (day 18).

Histopathological examination

Mice were anaesthetized and sacrificed on day 32. Paws and knee joints were removed for histopathological examination after routine fixation, decalcification, and paraffin embedding of the tissue. Tissue sections were prepared and stained with haematoxylin and eosin (H&E). Adobe Photoshop CS3 software was used to count the number of stained cells in joints and the percentage of infiltrating cells was calculated setting the vehicle group to 100%.

Stimulation of monocytes

RAW264.7 cells (American Type Culture Collection) were pre-incubated with 5μM SR2211 or SR1555 for 18h then stimulated with 1μg/mL LPS (Escherichia coli 0127:B8, Sigma–Aldrich (St. Louis, MO)) for 6h.

Thymocyte, splenocyte and bone marrow cells isolation

Thymi and spleens were collected from SR2211 treated CIA mice, and a single-cell suspension was prepared in RPMI-1640 medium plus 10% FBS (both from GIBCO/BRL). RBCs were lysed by Tris-buffered ammonium chloride treatment, and viable nucleated cells (trypan blue dye-exclusion test) were counted in a hemocytometer. After RBC lysis, thymocytes and splenocytes were washed with cold staining buffer (PBS plus 2% FBS and 0.1% NaN3) and pelleted at 1-2 × 10^6 viable cells per tube. Cells were resuspended in 50μl of the same buffer plus 1~2μg of each antibody. After 30min incubation on ice, fluorescence was measured using a BD LSRII (BD Bioscience) instrument and data was analyzed using FlowJo software (Tree Star). For the analysis of all T cell subsets, B cells, NK cell, and macrophage, fluorophore-conjugated monoclonal antibodies were used; PE anti-IL17A (eBio17B7, eBioscience), A647 anti-IFNγ (XMG1.2, eBioscience), FITC anti-CD4 (GK1.5, eBioscience), PE anti-CD8 (SK1, eBioscience), FITC anti-B220 (RA3-6B2,eBioscience), PE anti-Mac1 (M1/70,eBioscience), PE anti-RORγ (AFKJS-9,eBioscience), Rat IgG2ακ isotype control PE (12-4321, eBioscience), A488 anti-TLR4 (UT41eBioscience), and APC anti-CD14 (Sa2-8,eBioscience). For isolation of bone marrow cells from EGFP-RORγt transgenic mice, 6-8 weeks female transgenic mice were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from adherent soft tissue. The bone marrow cells were filtered through a 70μm nylon mesh filter (BD, Falcon, USA). Cells were plated into 12-well plastic cell culture plates at a density of 10 × 10^6 cells per well in DMEM containing 15% fetal bovine serum (FBS; Invitrogen), 2mM L-glutamine (Gibco,

USA), 100μg/mL penicillin (Sigma) and 100μg/mL streptomycin (Sigma). Isolated bone marrow cells were stained with CD11b (PE anti mouse CD11b, M1/70 clone, e-bioscience) and sorted using FACS Aria (BD bioscience). Live CD11b cells were stimulated for 18h with 1μg/mL LPS (Escherichia coli 0127:B8, Sigma–Aldrich (St. Louis, MO)).

DLN cell culture and cytokine determinations

Draining lymph nodes (DLN) were isolated from vehicle-only and SR2211 treated CIA mice. Isolated DLN cells (5×10^5 per ml) were cultured for 24h in D-MEM supplemented with PSG (penicillin 100 μg/mL, streptomycin sulfate 100μg/mL and glutamine 2mM) and 10% FBS. IFNγ and IL17 production were measured in order to assess T_H1 and T_H17 cell activity, respectively. The production of IFNγ and IL17 in the DLN was monitored following stimulation with anti-CD3 (1.5μg/mL) and anti-CD28 (2μg/mL) monoclonal antibodies [49]. After 24h of *ex vivo* culture, cells were harvested and stained with anti-CD4, and intracellular staining was conducted using anti-IFNγ (XMG1.2,eBioscience) and anti-IL17 (eBio17B7, eBioscience). Flow cytometric analysis was performed on a BD LSRII (BD Biosciences) instrument and analyzed using FlowJo software (TreeStar).

Statistical analysis

The data were expressed as mean ± SEM (standard error of the mean). Statistical significance was evaluated using unpaired Student's t-test for the two groups. The results were considered significant as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

RESULTS

Inhibition of T_H17 cell development and IL17 secretion

RORγ has been shown to be essential for IL17 expression and the differentiation of T_H17 cells. Previously we demonstrated that SR2211 functions as a potent inverse agonist of RORγ with an $IC_{50:}$>0.3μM and affording 98% repression at 10μM [34]. The SR2211 scaffold was derived from the potent liver x receptor (LXR) agonist T091317 which we had shown previously to cross react as a repressor of RORα and RORγ [33]. Unlike T091317, SR2211 was found to be highly selective for RORγ. Using a cell based screen of SR2211 in a GAL4-NR chimeric co-transfection assay format demonstrated that SR2211 was devoid of RORα, LXR and FXR activity, but the compound repressed the activity at RORγ in a dose-dependent manner. This functional selectivity was confirmed in co-transfection assays with wild type full-length receptors and native promoter-reporter systems. Two additional screening paradigms were employed to examine the selectivity of SR2211. First, SR2211 was tested in an assay panel containing of all 48 human NRs and the compound showed selective repression of only RORγ. Second, SR2211 was screened against a panel of 40 GPCR's (G protein- coupled receptors), ion channels, and transporters and showed no off-target activity.

Treatment of stimulated-EL4 cells with SR2211 suppressed the expression of IL17 and IL23R mRNA in a dose-dependent manner (**Fig. 1a**). Next, we explored whether SR2211 could selectively block T_H17 cell differentiation. Enriched naïve CD4+T cells were cultured under T_H1 polarizing conditions (IL12 and IFNγ) or T_H17 polarizing conditions (low

concentration of TGFβ and IL6) in the presence of SR2211 or DMSO vehicle control for 3 days. The concentration of the cytokines IFNγ (a marker for T_H1 cells) and IL-17 (a marker for T_H17 cells) were measured using the Luminex system. As shown in **Fig. 1b**, SR2211 reduced the production of IL17 but not IFNγ suggesting the compound selectively inhibits T_H17 cell differentiation but not T_H1 cell differentiation.

Inhibition of pro-inflammatory cytokines in LPS stimulated RAW264.7 cells

Macrophages play a role in the immune defensive mechanism (innate immunity) and produce pro-inflammatory cytokines. Stimulation of macrophages with bacterial lipopolysaccharide (LPS) is TLR4-dependent and results in activation of NF-kB. Exposure of RAW264.7 cells to LPS (1μg/mL for 6h) resulted in a statistically significant increase ($p < 0.01$) in the expression of RORγ (**Fig. 2a and 2b**). Stimulation of these cells was confirmed by the observed increase in TLR4 and CD14 expression (data not shown). Furthermore, we confirmed the expression of RORγ in CD11b+ bone marrow cells from EGFP-RORγt transgenic mice and it was increased upon LPS stimulation (**Supplement Fig. 1**). Since proinflammatory cytokines play a major role in the pathogenesis of rheumatoid arthritis, the ability of SR2211 to suppress the expression of several cytokines was evaluated. As shown in **Fig. 2c**, treatment of LPS stimulated RAW264.7 cells with 5μM SR2211 repressed the expression of IL1β, IL6 and TNFα, cytokines that have been shown to be upregulated in RA patients [51-53]. Similar effects were observed following treatment with compound at lower concentrations. Interestingly, treatment of LPS stimulated RAW264.7 cells with SR1555, a structurally similar but less potent analog of SR2211, resulted in differential repression of pro-inflammatory cytokines as compared to SR2211 (see **supplemental Fig. 2**)

RORγ inverse agonist attenuates arthritis development in CIA mice

To investigate the therapeutic potential of inverse agonists of RORγ in the treatment of RA, we examined the efficacy of SR2211 and SR1555 in the collagen-induced arthritis (CIA) mouse model. Dexamethasone, a glucocorticoid receptor (GR) modulator with potent anti-inflammatory activity, was used as a control for the CIA model and was delivered to CIA mice at 0.25mg/kg twice a day (BID) by intraperitoneal injection three days post immunization. As shown in **Fig 3a**, dexamethasone completely prevented the development of clinical symptoms in the CIA mice. SR2211 was also administered to CIA mice at 20mg/kg BID in this disease prevention mode and similar to dexamethasone, SR2211 nearly completely blocked development of symptoms (data not shown). To determine if SR2211 would be efficacious in a therapeutic setting, compound was administered to CIA mice at three different dose levels (8, 16, or 20mg/kg BID) starting 18 days post immunization and three days prior to the collagen boost (CII/IFA). In these experiments the first signs of joint inflammation were typically observable at 19 days post immunization (two days prior to administration of the booster). In the animal group treated with SR2211 at 20mg/kg, clinical scores increased at a reduced rate during the first eight days (27 days post immunization) of drug treatment as compared to vehicle-only treated mice as shown in **Fig. 3a**. This was then followed by a rapid reduction in visible symptoms of joint inflammation and CIA scores decreased accordingly (**Fig. 3a**). In contrast, the vehicle-only treated group showed a steady and continuous increase in symptoms of joint inflammation and increased CIA scores until

the end of study (32 days). Thus, in this paradigm SR2211 administered at 20mg/kg resulted in a statistically significant ($p < 0.01$) reduction in the severity of disease symptoms in CIA mice monitored out to 32 days post immunization (**Fig. 3a**). The efficacy of SR2111 as determined by CIA clinical score, showed a clear dose-dependency with maximal efficacy observed at the highest dose (20mg/kg), modest efficacy at the intermediate dose (16mg/kg), and no efficacy observed at the lowest dose of 8mg/kg (**Fig. 3b**).

In addition to reducing CIA score, treatment of CIA mice with SR2211 at 20mg/kg resulted in reduced bone and cartilage erosion as compared to vehicle-only treated mice. As demonstrated in **Fig. 3c**, the severity of inflammation is visible in a representative paw of a vehicle-only treated mouse whereas inflammation in a representative paw from a SR2211 treated mouse is significantly reduced. Similar results were observed for all animals in both treatment groups and the reduction in inflammation was reflected in improved CIA clinical scores for the SR2211 treated group. Histopathological evaluation of paws revealed significant inflammatory cell infiltration in vehicle-only treated group. By contrast, paws from SR2211 treated mice had dramatically less inflammation.

CIA mice were also administered SR1555, a less potent analog of SR2211. Although the potency of SR1555 on RORγ is less than that of SR2211, the pharmacokinetics and plasma exposure is slightly better for SR1555 than that of SR2211 at similar doses. Consistent with reduced potency as a repressor of RORγ, SR1555 when administered at 20mg/kg twice a day afforded only modest efficacy as compared to the same dose of SR2211 (see **supplemental Fig. 3**).

IFNγ exerts both enhancing and suppressing influences on collagen-induced arthritis (CIA) disease progression in a temporal fashion and its expression and function varies with the degree of severity of symptoms [54]. To look at IFNγ stimulation we isolated the draining lymph nodes from SR2211 and vehicle-only treated CIA mice. The organs were subsequently stimulated *ex vivo* with CD3/CD28 for 24h. Interestingly, the expression of IFNγ was significantly increased ($p < 0.001$) in the SR2211 treated draining lymph nodes from the CIA mice as compared to those from vehicle-only treated mice (**Fig. 4**).

In the thymus, CD4+CD8+ double positive (DP) cells require expression of RORγt for survival [55]. Synthetic RORγ inverse agonists such as SR2211 might impact the T cell population if they reduce the survival of DP cells. Consistent with this mechanism, a significant reduction ($p < 0.001$) in total cell number as well as T cell subtype populations (CD4+CD8+ thymocytes, CD4+ CD8-T lymphocyte, and CD4-CD8+ T lymphocyte) were observed in thymus from SR2211 treated mice (Table 1). While a significant reduction ($p < 0.001$) of DP T lymphocytes was observed in SR2211 treated mice as compared with vehicle-only treated mice, the population of peripheral T cells (CD3+T lymphocyte) was unchanged (Table 1). Furthermore, the population of B220+ splenocytes, NK1.1 cells and Gr1+Mac1+ cells were also unchanged and similar with vehicle-only treated mice. The reduction of thymic T cell population is consistent with repression of RORγt and treatment of SR2211 appears to drive an immunosuppressive environment in the CIA mouse.

DISCUSSION

Previously we had shown efficacy of SR1001, a modestly potent dual RORα/RORγ inverse agonist, in the EAE murine model [38]. That study was the first to demonstrate the possibility of pharmacologically targeting RORγ for therapeutic intervention of an autoimmune disease. RORγ is essential for survival of CD4+CD8+ DP cells and for differentiation of T_H17 cells [15, 55, 56]. While both T_H17 cells, and macrophages as well, play important roles in host defense against bacterial and fungal infections, they have been linked to several autoimmune diseases, including psoriasis, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [57, 58]. T_H17 cells produce the inflammatory cytokine IL17, a protein that has been detected in RA associated synovial fluid. IL17 acts in synergy with IL1β and TNFα to drive the inflammatory environment in joints and other tissues [42, 59]. Here we demonstrate the link between inhibition of RORγ activity with efficacy in the CIA mouse, a model of rheumatoid arthritis, using a potent and selective RORγ inverse agonist (SR2211). In the studies presented here, SR2211 exhibited statistically significant reduction in joint inflammation along with improved clinical scores suggesting that pharmacological repression of RORγ activity alone is efficacious in the CIA model.

In addition to its ability to block differentiation of naïve T cells to T_H17 cells, SR2211 reduced the expression of inflammatory cytokines in activated macrophages. In comparison to SR2211, treatment of the same cells with a less potent but structurally related analog SR1555 showed a unique profile notably a decreased repressive effect on LPS-induced IL6 expression while maintaining the ability to suppress IL1β and TNFα expression. Not surprising, SR1555 was less efficacious in CIA mice than SR2211, consistent with both reduced ability to repress RORγ activity and the different profile in repression of inflammatory cytokines from macrophages. Previously we have shown using hydrogen/deuterium exchange mass spectrometry (HDX-MS) that structurally similar ligands can induce different conformational states of nuclear receptors. These unique ligand-dependent conformational states could drive differential recruitment of cofactors, which would likely result in different downstream pharmacological responses [34]. Studies are ongoing using chromatin immunoprecipitation (ChIP) looking at the unique promoter specific protein interactions that SR2211 and SR1555 drive upon to binding to RORγ.

Previously it has been shown that knocking out the IFNγ receptor in CIA exacerbates the disease symptoms [44, 60]. The protective role of IFNγ may stem from the ability of cytokines released from T_H1 and T_H2 cells to inhibit T_H17 cell maturation. In a recent study, activation of monocytes or macrophages resulted in the promotion of T_H1 polarization and IFNγ production which led to the suppression of T_H17 cell development in mice [61] and inhibition of RORγ and RORα expression [62]. In the studies presented here, repression of T_H17 cell differentiation also resulted in induction of IFNγ production in murine draining lymph nodes an observation that is consistent with the relationship of T_H17 cells to T_H1 cells.

In summary, the results presented here demonstrate that treatment of cells in culture or tissues *ex vivo* with SR2211 inhibits T_H17 cell differentiation, IL17 and IL23R expression,

reduces inflammatory cytokines expression in activated macrophages, and systemic activation of T_H1 cells as shown by the induction of IFNγ. More importantly, treatment of CIA mice with the potent and selective RORγ inverse agonist SR2211 afforded robust efficacy in reducing joint inflammation. Thus we were able to show that therapeutic strategies leading to reduction of T_H17 cells and inhibition of inflammatory cytokine expression in activated macrophages protects the severity of onset of autoimmune arthritis. As such, synthetic small molecules like SR2211 might be attractive starting point for the development of a novel therapeutic for the treatment of inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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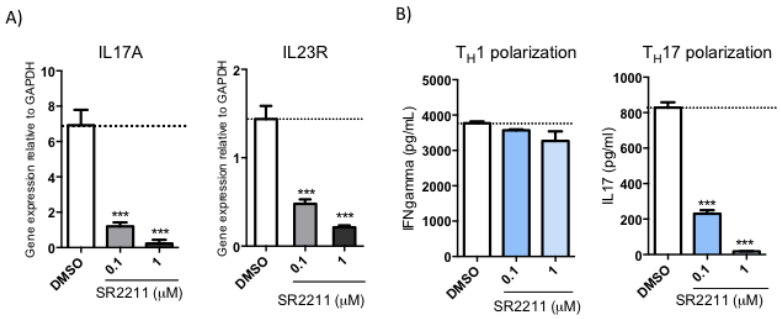

Fig. 1. The effect of SR2211 on IL17 gene expression and T_H17 cell differentiation

A) EL4 cells were pretreated with DMSO or SR2211 (0.1 or 1μM) for 20h followed by stimulation with PMA and ionomycin for 5 h. IL-17 and IL23R mRNA expression was quantitated and normalized to GAPDH. B) IFNγ or IL17 expression in splenocytes cultured under T_H1 polarizing conditions or T_H17 polarizing conditions with vehicle control (DMSO) or SR2211 (0.1μM or 1μM) for 3 days. Graphs represent the average concentration of IFNγ or IL17 cytokines (n=3). All error bars denote SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired *t*-test.

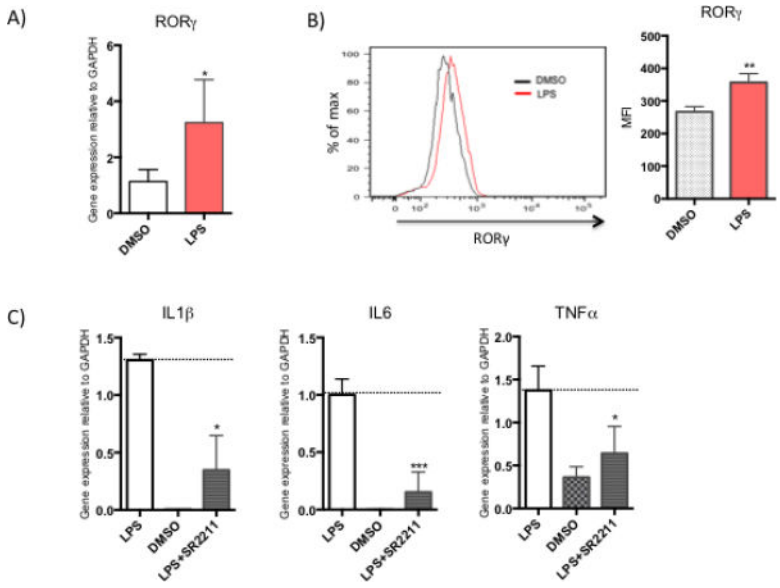

Fig. 2. The Induction of RORγ in LPS stimulated RAW264.7 cells and the inhibition of pro-inflammatory cytokines by SR2211

A) RAW264.7 cells were incubated with 1μg/mL LPS for 6h and measured the mRNA of mouse RORγ. B) The histogram represented the expression of intracellular protein of RORγ in RAW264.7 cells (n=3). Graph represent average of MFI (median fluorescence intensity). C) IL1β, IL6, and TNFα mRNA levels are measured by pre-incubation with 5μM SR2211 for 18 hours then added LPS (1μg/mL) with 5μM SR2211 for 6h. All error bars denote SEM. *$p < 0.05$, ***$p < 0.001$ by unpaired *t*-test.

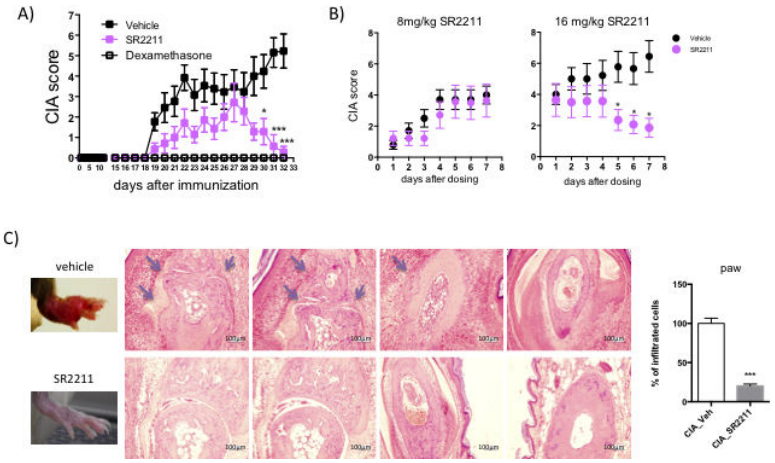

Fig 3. The therapeutic effect of SR2211 on arthritis onset

A) Treatment with SR2211 (20mg/kg) or dexamethasone (0.25mg/kg) suppressed the clinical severity of CIA [Vehicle (circle, n=13) or SR2211 (closed circle, n=7) or dexamethasone (red square, n=15)]. Clinical score was monitored every day after boosting during two weeks. B) Efficacy of SR2211 was dose-responsive, and C) Photograph inserts of a paw from one vehicle-only treated mouse and one from a mouse treated at 20mg/kg SR2211. Haematoxylin & eosion staining on paraffin sections of the joints on day 32 post immunization showing bone erosion (arrows) and infiltrated immune cells. The % infiltrated cells were counted on identical regions of swollen figures with vehicle group set to 100%. n= 3~4 mice/ group. All error bars denote SEM. $*p < 0.05$, $***p < 0.001$ by unpaired *t*-test.

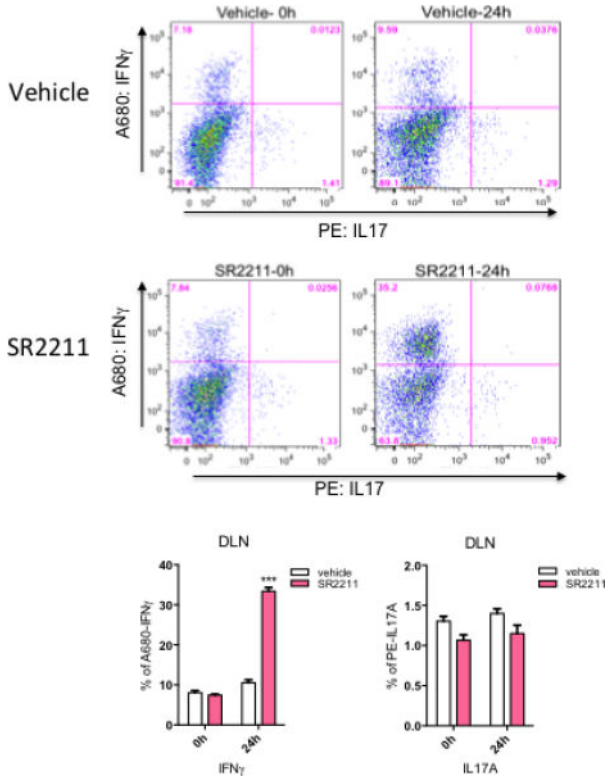

Fig. 4. Stimulation of systemic T_H1 cells by SR2211

Draining lymph nodes from Vehicle group or SR2211 treating group was isolated and activated with α-CD3 and α-CD28 mAb for 24h. IFNγ and IL-17 were measured by intracellular staining. Graphs represent the average percentage of IFNγ and IL17 expressing cells normalized to vehicle control (n=6). Error bars denote SEM. ***$p < 0.001$ by unpaired *t*-test.

Table 1

Immune cells profiles of total thymocytes and splenocytes from SR2211 treating CIA mice analyzed by flow cytometry.

Spleen	B220+[a]	CD3+[a]	Gr1+Mac1+[a]	NK1.1[a]	# of splenocytes[b] (×10[6])
Veh	51.5±2.12	24.5±0.70	11±1.41	16±1.41	214±22.62
SR2211	49±2.82	24±1.41	15±1.41	14±0	190±7.07

Thymus	CD4-CD8-[a]	CD4+CD8+[a]	CD4+CD8-[a]	CD4-CD8+ [a]	# of thymocytes[b] (×10[6])
Veh	13.5±2.12	74±1.41	9±1.41	3.5±0.70	161.5±4.94
SR2211	28±2.82**	22.5±3.53***	35.5±2.12***	9±141**	17.5±3.53***

CIA mice received SR2211 at 20mg/kg twice-a-day, i.p. or vehicle only. There were at least seven animals in each group.

[a] the percentage of positive population in each cells

[b] total cell number in each tissues

** $p < 0.01$

*** $p < 0.001$ by unpaired *t*-test.