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_JmmunoHorizons_ 2019, 3 (8) 402-411
doi: [https://doi.org/10.4049/immunohorizons.1900043](https://doi.org/10.4049/immunohorizons.1900043)
http://www.immunohorizons.org/content/3/8/402

This information is current as of June 2, 2021.

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ISSN 2573-7732.
An Accord of Nuclear Receptor Expression in CD4+ T Cells in Rheumatoid Arthritis

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ABSTRACT

Chronically activated CD4+ T cells drive uncontrolled inflammation, leading to tissue damage in various autoimmune disorders, such as rheumatoid arthritis (RA). Investigation of the molecular mechanisms involved in RA and recent analysis of transcriptomic profiles has implicated members of the nuclear receptor (NR) superfamily in RA. NRs are required for the development, differentiation, and effector function of CD4+ T cells; therefore, it is thought that NRs are important in shaping the CD4+ T cell repertoire and associated inflammation in RA. Despite their relevance, the full potential of the NR superfamily in RA, either as biomarkers or disease targets, has not been harnessed. To gain insight on the NR members that are closely associated with RA disease activity, we generated an expression atlas for the NR superfamily in CD4+ T cells isolated either in a steady state or over the course of collagen-induced arthritis mouse model of RA. We observed discrete expression patterns among the NR superfamily during the disease stages. NRs that instigate anti-inflammatory programs underwent major downregulation during disease onset; however, during the fully developed disease stage we noticed that NRs that induce proinflammatory programs had reduced transcript levels. These animal findings corroborated well with the expression patterns of NRs in clinical samples obtained from RA patients. Furthermore, we observed that targeting NRs using synthetic ligands alleviates the progression of collagen-induced arthritis. Overall, our data demonstrates the potential of the NR superfamily as novel therapeutic targets for the treatment of autoimmune disorders. ImmunoHorizons, 2019, 3: 402–411.

Received for publication June 6, 2019. Accepted for publication August 7, 2019.

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This work was supported by Department of Biotechnology-India Project GAP0162 and the Council of Scientific and Industrial Research 12th Plan Network Project Bugs to Drugs, Infectious Disease (BS0221 and BS02210) (to P.G.). The Institute of Microbial Technology, a constituent laboratory of the Council of Scientific and Industrial Research, provided facilities and financial support. The funders had no role in the study design, data collection, and interpretation, or the decision to submit the work for publication.

A.S., S.M., and P.G. designed the research, A.S., S.M., E.B., R.K., and R.N. performed the experiments, A.S., S.M., R.G., N.K., and P.G. analyzed the data, A.S., S.M., and P.G. wrote the manuscript.

Abbreviations used in this article: CIA, collagen-induced arthritis; CII, type II collagen; CII-IgG, CII-specific IgG; Ct, cycle threshold; GMCH, Government Medical College and Hospital; IMTECH, Institute of Microbial Technology; NR, nuclear receptor; RA, rheumatoid arthritis.

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease associated with uncontrolled inflammation, generally characterized by synovial hyperplasia and irremediable damage to the joints (1). The prognosis of RA is challenging because little is known about the factors that initiate or sustain this autoimmune disease. The current model of RA incorporates signaling from both innate and acquired immune responses, but their relative contribution to the disease pathogenesis remains undetermined. Although the role of CD4+ T cells in RA initiation has not been clearly established, there is ample evidence to substantiate that they are crucial in the pathophysiology of the disease (2–4).

The involvement of CD4+ T cells in RA was deduced from earlier findings that demonstrated that the MHC class II HLA-DRB1 locus is a major genetic risk factor among individuals with RA. This gene locus was later recognized as influencing Ag presentation by inducing a repertoire of autoreactive CD4+ T cells (5–7). Recent genetic studies have also identified non-HLA alleles as susceptibility factors for RA; for example, polymorphism in genes encoding protein tyrosine phosphatase nonreceptor type 22 (PTPN22) (8), cluster of differentiation 28 (CD28) (9), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (9, 10), which control T cell activation show RA predisposition. Moreover, CD4+ T cells are among the most abundant immune cells present in the RA synovium (11), and cytokines secreted by activated T cells are associated with synovial inflammation (12, 13). This inflammatory environment created by CD4+ T cells directs not only the activation of other immune cells but also cells of the synovial tissue, such as activated synovial fibroblasts, to release effector molecules that further escalate destruction of joints (14).

Comprehensive transcriptome analysis has previously been attempted to identify transcriptional changes and signaling pathways that are central to CD4+ T cell activation in RA (15, 16); however, often such studies have been carried out on individuals with active RA and as a result preclude the possibility of describing genes that might be involved in the initial stages of disease progression. Consequently, it is extremely important to identify the changing gene pool in CD4+ T cells during initial and active disease stages to identify biomarkers as well as molecules that can be targeted in active disease, respectively.

Nuclear receptors (NRs) are a superfamily of transcriptional regulators whose differential expression pattern and transcriptional activity alterations have been reported in various autoimmune diseases (17, 18). It is becoming evident that NRs modulate the key features of RA pathogenesis (19, 20). Because of the involvement of NRs in the development and effector functions of CD4+ T cells (21–23), an imbalance in their homeostatic activity is likely to have a role in the development of arthritis. This idea is supported by research showing that members of a NR subfamily, the retinoic acid–related orphan receptor family (Ror/Nr1f), are required for the differentiation of Th17 cells that are a subset of CD4+ T cells critical in RA development (24, 25). Interestingly, synthetic ligands developed to direct the transcriptional activity of NRs have been used extensively to tease out the role of NRs in autoimmune diseases, including in RA. For example, administrating rosiglitazone, THR0921, or pioglitazone, all of which are synthetic ligands for peroxisome proliferator-activated receptor γ (Pparg/Nr1c3), attenuates the progression of RA (26–28). Similarly, ligands for retinoic acid receptors (Rar/Nr1b), glucocorticoid receptor (Gr/Nr3c1), and vitamin D receptor (Vdr/Nr1il) alleviate the chronic inflammation and tissue destruction associated with RA (29–31). However, to our knowledge there has not been a single study taking a comprehensive approach to systematically analyze all members of the NR superfamily in RA development. Thus, in this study we carried out an in-depth transcriptional analysis of NRs in a splenic CD4+ T cell population both at the induction and during fully developed RA. We also observed that pharmacological targeting of NRs alleviates RA symptoms; therefore, these findings highlight new potential remedies for the treatment of RA.

MATERIALS AND METHODS

Human ethics statement

The project was approved by the Ethics Committee of the Government Medical College and Hospital (GMCH), Sector 32, Chandigarh 160030, India (GMCH/TA-1 (19)/2013/14449), and the Ethics and Biosafety Committee of the Institute of Microbial Technology (IMTECH), Sector 39A, Chandigarh, India. The project was approved to Dr. P. Gupta (IMTECH) and collaborator Dr. R. Gupta (Department of Orthopedics, GMCH). The study was conducted strictly in accordance with the ethical guidelines for biomedical research on human subjects by the Central Ethics Committee on Human Research, Indian Council of Medical Research, New Delhi, and those as contained in the Declaration of Helsinki. Each subject was provided with written information about the study under the title “Information to the Patient,” and written consent on the consent form (in the language familiar to them) was obtained from each subject prior to his/her induction into the study.

RA patients belonging to different age groups and sex were included in the study group if they fulfilled the American College of Rheumatology/European League Against Rheumatism criteria of RA (32). Patients with any concurrent and overlapping chronic medical conditions likely to be associated with similar symptoms were excluded. Individuals that underwent arthroscopy for noninflammatory and non-RA disorders of the joint acted as healthy controls. Synovial fluid was obtained from human subjects and mononuclear cells isolated from it were used for expression analysis.

Mice

DBA/1 mice were bred and maintained under specific pathogen-free conditions in the animal facility at the IMTECH, Chandigarh, India. All animal procedures were conducted in accordance with protocols approved by the Institutional Animal Ethics Committee of the IMTECH and were in compliance with the guidelines from the National Regulatory Guideline issued by the Committee for the Purpose of Supervision of Experiments on Animals.
(No.55/1999/CPCSEA), Ministry of Environment and Forest, Government of India.

**In vivo experiments**

For the induction of collagen-induced arthritis (CIA), DBA/1 mice were immunized with 100 μg of Bovine type II collagen (CII; MD Biosciences) emulsified in CFA by injecting intradermally at the base of the tail on day 0. In some animals, a booster of 100 μg CII was given on day 21 after the priming, where CII was emulsified in IFA. Mice were inspected at regular intervals to ascertain the presence of arthritis. To evaluate the intensity of arthritis, a clinical scoring system of 0–4 points for each paw was used: 0, no sign of inflammation; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema; 4, extreme erythema and swelling. The clinical index was constructed by adding the scores from all four limbs for each animal. The drugs, CD437 (10 mg/kg), Cytosporone B (15 mg/kg), or CGP 52608 (10 mg/kg), were administered i.p. three times per week starting from day 21 of immunization as indicated. For histopathological analysis, the paws, ankles, and joints were stained with H&E.

**Isolation of CD4+ T cells**

The spleen was removed aseptically from mice and a single-cell suspension of splenocytes was prepared. These cells were then treated with RBC lysis buffer, so as to lyse the RBCs, and washed with PBS. This was followed by isolation of CD4+ T cells using a Mouse CD4 T Lymphocyte Enrichment Set (BD Biosciences) according to the manufacturer’s protocol.

**PCR array**

Total RNA was extracted from isolated CD4+ T cells using an RNeasy Mini Kit (Qiagen) and reverse transcribed with an RT^2 First Strand Kit (Qiagen), according to the manufacturer’s protocol. The abundance of 49 murine NRs in each sample was then determined by a customized RT^2 Profiler PCR Array (SA Bioscience) using the RT^2 SYBR Green ROX qPCR Master Mix, according to the manufacturer’s protocol. The analysis was performed as described previously (33); briefly, quality control for the arrays was performed using the RT^2 Profiler PCR Array Data Analysis version 3.5 software followed by calculation of the relative fold change using the 2^-ΔΔCt method, where Ct is cycle threshold.

**Quantitative real-time PCR**

Total RNA was extracted using TRIzol and reverse transcribed to cDNA using the Verso cDNA kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Gene expression was examined by quantitative real-time PCR using the SYBR Green method with the DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific). Expression of target genes was calculated relative to β-actin.

**ELISA**

Serum of the CIA mice was collected 42 d after the first immunization. ELISA was used to measure the levels of total CII-specific IgG (CII-IgG) and its subclasses, CII-IgG1, CII-IgG2a, and CII-IgG2b. Briefly, wells of the plate were coated overnight with bovine CII (5 μg/ml) at 4°C and blocked using 50 mM TBS (pH 8) containing 1% BSA. The diluted serum samples were then added and incubated overnight at 4°C, followed by addition of biotinylated anti-mouse Abs for 1 h at room temperature. The plates were subsequently incubated with avidin-HRP solution for 30 min at room temperature and developed with tetramethylbenzidine (TMB). The reaction was terminated by adding stop solution to each well, and absorbance was then measured at 450 nm. Pooled serum collected from wild type mice that had the highest arthritis scores were used for the standard curve.

**Statistical analysis**

The statistical significance of the data were calculated using either the unpaired two-tailed Student t test or one-way ANOVA followed by post hoc Tukey test, using GraphPad Prism version 7 and SigmaPlot. Statistical differences with a p value <0.05 were considered significant. Data reported are mean and error bars are SD.

**RESULTS**

**Expression analysis of NRs in CD4+ T cells from CIA mice**

RA pathophysiology is caused by a failure to induce T cell regulatory function as well as expansion of autoreactive, hyperactive CD4+ T cells (7, 34). Therapeutic interventions to manage RA currently result in broad immunosuppression rather than specifically targeting the activated CD4+ T cells. Thus, it is highly relevant to identify molecules that can be used to specifically target CD4+ T cells. NRs are druggable molecules, and they have been implicated in T cell function and differentiation in recent research. Consequently, in this study we set out to identify NRs that are expressed in CD4+ T cells. To address this question, we first isolated total CD4+ T cells from the spleen of normal DBA/1 mice and subjected them to PCR array analysis to examine the expression profile of the NR superfamily. We found that at steady state, T cells expressed 31 out of 49 murine NRs, with 17 being expressed at moderate levels and 10 expressed at high levels (Fig. 1A). In addition to the well-known receptors for T cells (Nr3c1, Nr1h2, Nr4a1, Nr4a2, Nr4a3, and Rora), we also observed high levels of thyroid hormone receptor α (Thra/Nr1a1), retinoid X receptor β (Rxrb/Nr2b2), Nr1d2, and Nr2c2. Collectively, these data revealed expression of NRs that were not previously known to be in T cells, providing compelling evidence to demand future detailed studies to unravel the mechanisms explaining functional role of NRs in T cell biology.

https://doi.org/10.4049/immunohorizons.1900043
Next, we adopted the CIA mice model of RA to identify NRs that might be regulating T cell functions during autoimmune responses. To explore this, we isolated splenic CD4+ T cells at the onset and fully developed stage of CIA and performed expression analysis for NRs (Fig. 1B, 1C). PCR analysis revealed that at 21 d postimmunization (disease onset), compared with unimmunized mice, $\text{Nr4a1}$, $\text{Nr4a2}$, $\text{Nr4a3}$, $\text{Pparg}$, and $\text{Rora}$ were downregulated, whereas $\text{Nr0b2}$ was upregulated in CD4+ T cells (Fig. 2). Of note, as shown in Fig. 1C, we observed further changes in the expression of several NRs 45 d postimmunization (the fully developed disease state). Overall, we observed several changes in the expression of NRs during RA progression.

**The level of NRs is indicative of arthritis outcome**

To further elucidate the role of NRs in the pathogenesis of arthritis, we followed a systematic approach to carefully analyze the data. We focused on NRs that had significantly altered expression (at least 2-fold expression change) in CIA mice compared with control mice. Intriguingly, we found that anti- and proinflammatory NRs both showed interesting expression patterns during the course of the disease (Fig. 2). During disease onset (21 d postimmunization), the expression of NRs considered to have anti-inflammatory functions (e.g., members of the Nr4a subfamily) were majorly downregulated; however, they displayed an evident rescue in their levels at peak incidence of arthritis (45 d postimmunization) (Fig. 2A–C). In contrast, the expression of NRs with illustrious proinflammatory functions...
roles, such as Nr1d1, Nr1d2, and Rorc, did not change much on day 21; however, on day 45 (after which the mice start recovering from the disease), they underwent a significant reduction in their expression (Fig. 2D–F). These observations demonstrate that during the onset of disease the major anti-inflammatory networks are shut off, whereas during the later stages of disease the anti-inflammatory networks become proactive and the proinflammatory pathways are negatively regulated. This led us to hypothesize that these NRs might be acting as critical genetic regulators of T cell homeostasis, deciding the outcome of the disease.

Expression of NRs in RA patients reiterates their expression observed in the CIA-RA model

Based on our findings, we speculated that changes observed in NR expression in the CIA mouse model of RA may also be detected in RA patients. To address this possibility, we obtained synovial samples from either control subjects or patients with active RA. In agreement with earlier reports (35, 36) we found increased expression of Nr4a2 in RA patients compared with control subjects (Fig. 3C). We also observed a significant reduction of Nr0b2 and Nr4a1 expression in RA patients compared with control subjects (Fig. 3A, 3B). A similar reduction in the expression levels of Rora was also observed in the RA group (Fig. 3D). Notably, we did not see any significant changes in the expression levels of Nr4a3 and Pparg in the analyzed samples (data not shown). Collectively, these findings suggest that NR expression changes for Nr4a1, Rora, and Nr0b2 noticed in RA patients corroborate the NR expression profile observed in the CIA mouse model of RA.

Targeting NRs alleviates the severity of CIA-induced RA

Pharmacological targeting of NRs using synthetic ligands has shown efficacy in preclinical models of infectious disease, such as
Mycobacterium tuberculosis, and autoimmune diseases, such as type 2 diabetes (37–40). Hence, we sought to determine whether targeting the key NRs that emerged from our screen would alleviate RA progression. To address this, we used three synthetic ligands, CD437, Cytosporone B, and CGP 52608, to selectively target Nr0b2, Nr4a1, and Rora, respectively. We observed that administration of these ligands starting from 21 d postimmunization significantly reduced the severity of CIA-induced RA in our CIA mouse model (Fig. 4A). Intriguingly, combined treatment (simultaneously targeting Nr0b2, Nr4a1, and Rora) was more effective than when each of these ligands was given alone (Fig. 4A). This finding was further supported by histological examination of the paws and joints of the mice. In vehicle-treated (control) mice their joints had inflammation with significant damage to the joint capsule. Conversely, animals treated individually with CD437, Cytosporone B, and CGP 52608 had significantly reduced infiltration of immune cells in their joints. Furthermore, mice receiving CD437, Cytosporone B, and CGP 52608 together displayed superior protection resulting in an almost normal synovium (Fig. 4B). A similar reduction in inflammation was also observed in and around the digits and elbow joints (Fig. 4C). Together, our data demonstrate that CD437, Cytosporone B, and CGP 52608 can reduce clinical symptoms and joint pathological condition in CIA mice.

The effects of CD437, Cytosporone B, and CGP 52608 on anti-CII Abs and inflammatory cytokines in CIA mice

We next examined the effects of CD437, Cytosporone B, and CGP 52608 on RA pathogenesis by measuring the level of CII-specific Abs in mice serum. We observed a significant reduction in the levels of total anti–CII-IgG and its subclasses, IgG1, IgG2a, and IgG2b, when mice were treated with CD437, Cytosporone B, or CGP 52608 (Fig. 5A–D). Additionally, combined treatment of these ligands was found to be more effective than when the animals received any of these ligands alone (Fig. 5A–D).

Furthermore, evidence suggests that the progression of RA results in the production of proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-17. To assess if CD437, Cytosporone B, or CGP 52608 can influence these cytokines in CIA mice, we examined the levels of cytokines in joint extracts and serum from CIA mice treated with CD437, Cytosporone B, or CGP 52608 separately or together. Interestingly, mice that received CD437, Cytosporone B, or CGP 52608 separately or together all showed a significant reduction in the levels of IFN-γ, TNF-α, and IL-17.
The emergence of self-reactive T cells results in the development of autoimmune diseases. RA is an autoimmune condition associated with the appearance of detrimental CD4+ T cells, which trigger a systemic inflammatory response (2). Earlier studies investigating the molecular framework of RA have recognized the role of transcription factors belonging to the NR superfamily in the pathophysiology of RA (9, 41). This has led to a generalized association between CD4+ T cells, NRs, and RA. A few NRs have been suggested to have a role in the development and differentiation of CD4+ T cells (21, 23), and this has been proposed as a possible mechanism for how these molecules direct the pathogenesis of RA. Nevertheless, not all members of the NR superfamily have been studied in the field of RA pathophysiology. Consequently, we aimed to determine the complete representation of the NR superfamily at different stages of RA progression, to provide valuable information for capitalizing on the therapeutic potential of NRs in RA. Hence, we performed comprehensive expression profiling of the NR superfamily in CD4+ T cells isolated from the spleen of healthy control mice or mice at different stages of RA development. The NR targets obtained from these mice studies were then validated in synovial samples obtained from RA patients. Additionally, we found that therapeutic targeting of these candidate NRs eased the severity of CIA-induced RA, thus suggesting that exogenous interventions targeting NRs’ activity can mitigate the severity of autoimmune diseases.

In this study, we observed that at steady state, 31 of 49 murine NRs were expressed in CD4+ T cells. Other than solely confirming the presence of well-documented members of NR superfamily, such as Pparg, Nr4a1’s, and Gr, our study identified the expression of many other molecules, such as Nr1d1 and Nr2c2, that were previously not known to be expressed in CD4+ T cells. Further analysis of the transcript levels revealed that many of these receptors had altered expression during RA development. Indeed, similar to work previously reported by Brenner et al. (41), we found a correlation between the functional (anti-versus proinflammatory) nature of NRs and the stage of arthritis. Receptors with anti-inflammatory properties, such as the Nr4a subfamily, were majorly downregulated during the induction phase of RA but came back to normal levels in mice with fully developed CIA. In contrast, levels of NRs with proinflammatory properties, such as Nr1d1, Nr1d2, Nr1h3, and Rorc, remained unchanged at the onset of RA but showed downregulation during the peak incidence of the disease. One plausible reason for this correlation could be that during the induction phase of arthritis it is necessary for the anti-inflammatory pathways to be downregulated to induce disease progression. In the CIA mouse model, the animal goes into a recovery phase after the peak incidence of RA, and this recovery phase could be marked by the prevalence of anti-inflammatory genes over proinflammatory genes.

In a healthy host, the immune system establishes a balance between pathogenic and protective Th cell subsets. During autoimmune diseases this balance is disturbed and the equilibrium shifts toward an increase in pathogenic Th cell subsets. Interestingly, RA was classically considered a Th1 driven disease, but this premise cannot explain the full spectrum of clinical manifestations. Subsequent findings lead to the discovery of Th17 in arthritic patients, which highlighted the potential role of these cells in RA disease activity. Th17 cells secrete various effector molecules, such as IL-17A, IL-17F, IL-22, IL-26, and GM-CSF, which all contribute to RA pathology. Supporting these observations, genetic mouse models showed that IL-17-deficient mice are resistant to CIA (42). The loss of IL-17 also prevents the spontaneous development of arthritis in IL-1R antagonist (IL-1Ra)–deficient mice (43). Additionally, Rorc has been shown to

FIGURE 5. Treatment with CD437, Cytosporone B, and CGP S2608 reduces CII-specific IgG levels.

The serum levels of CII-specific (A) IgG, (B) IgG1, (C) IgG2a, and (D) IgG2b Abs in CIA mice treated with CD437, Cytosporone B, and CGP S2608 separately or together. The Abs were measured by ELISA on day 42 postimmunization. Data display the mean and SD, with 10 mice in each group. *p < 0.05 (one-way ANOVA).

(Fig. 6A–C). Notably, in mice receiving treatment of CD437, Cytosporone B, and CGP S2608 separately or together, we also observed elevation of IL-10, a dominant suppressive cytokine in CIA (Fig. 6D). Furthermore, we analyzed cytokine production in cells isolated from the spleen of mice treated with CD437, Cytosporone B, or CGP S2608, separately or in combination and obtained similar results (Fig. 7). Overall, these results suggest that selective targeting of Nr0b2, Nr4a1, and Rora curtails the severity of RA.

DISCUSSION

The serum levels of CII-specific (A) IgG, (B) IgG1, (C) IgG2a, and (D) IgG2b Abs in CIA mice treated with CD437, Cytosporone B, and CGP S2608 separately or together. The Abs were measured by ELISA on day 42 postimmunization. Data display the mean and SD, with 10 mice in each group. *p < 0.05 (one-way ANOVA).
FIGURE 6. Administration of CD437, Cytosporone B, and CGP 52608 inhibits the inflammatory response in CIA mice. Joint protein extracts and serum were collected from mice treated with CD437, Cytosporone B, and CGP 52608, separately or together, on day 42 postimmunization, and the levels of (A) IFN-γ, (B) TNF-α, (C) IL-17, and (D) IL-10 were measured by ELISA. Data display the mean and SD, with 10 mice in each group. *p < 0.05 (one-way ANOVA).
regulate the development of Th17 cells, and inhibiting the transcriptional activity of Rorc limits the differentiation of Th17 cells (44). These studies led us to speculate that the expression pattern of NRs in arthritis might be modulating the disease outcome by regulating the Th cellular pool. Many members of NRs are ligand-activated transcription factors that mediate the effect of hormones, metabolites, vitamins, and xenobiotics. This feature of NRs makes them an ideal target for drug interventions to treat various metabolic, autoimmune, and infectious diseases. Synthetic NRs makes them an ideal target for drug interventions to treat various metabolic, autoimmune, and infectious diseases. Synthetic ligands targeting various NRs have been successfully tested in various animal models of arthritis. For example, a reduction in the severity of arthritis has been demonstrated upon administration of fenofibrate, a ligand that binds to Ppara (Nr1c1), and pioglitazone, a ligand that binds to Pparg. Similarly, administration of a ligand that targets the liver X receptor (Lxr/Nr1h3) significantly reduces the progression of CIA (20, 45). In this study, we found that specific targeting of Nr0b2, Nr4a1, and Rora with CD437, Cytosporone B, or CGP 52608, respectively, either separately or together, alleviates the progression of RA. Whether these ligands have any effect on maintaining the Th cell subset homeostasis is currently unknown and has yet to be identified; future work into this will be very valuable.

Overall, our findings suggest that NRs undergo dynamic expression changes in CD4⁺ T cells over the course of RA progression. Furthermore, we demonstrated that targeted interventions against Nr0b2, Nr4a1, and Rora alleviate the progression of RA in the CIA mice model. Thus, our work has provided new avenues for potential RA therapies.

DISCLOSURES

The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank Dr. B. N. Datta for the histopathology. We thank the IMTECH, a constituent laboratory of the Council of Scientific and Industrial Research, for facilities and financial support.

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