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Research Article
Alteration of Cellular and Humoral Immunity by the Blockage of *P2y11* Gene Attenuates on the Rheumatoid Arthritis

Hui Peng, Xiaoqiang Zhuang, Yu Bai, Xu Fang, Hui Huang and Shenglin Lu

Department of Orthopaedics, Affiliated National Hospital of Guangxi Medical University, Nanning, Guangxi, China

Abstract

Background and Objective: $P2Y_{11}$ is a sub type of purinergic receptors that altered the function of immune cells. Present study evaluates the beneficial effect of $P2Y_{11}$ receptor blockage on rheumatoid arthritis (RA). **Methodology:** Arthritis was induced by the intraplantar injecting the mixture of complete freund's adjuvant (CFA) and type II collagen in left hind paw. $P2Y_{11}$ receptor was blocked by $P2Y_{11}$ receptor antagonist (NF340). All the animals were separated in to five groups like control group, negative group (Rheumatoid arthritis group), NF340 0.3, 10 and 30 μM/day injected intrathecally for the period of 21 days. Development of RA was estimated by arthritic score, paw swelling and paw withdrawal latency. Pro-inflammatory cytokines in the synovial fluid, anti-type II collagen antibodies in serum and proliferation of T-cell was estimated in RA rats. **Results:** Data of the study reveals that treatment with NF340 attenuates the development of RA. There was significant ($p<0.01$) decrease in the pro-inflammatory cytokines in NF340 treated group compared to negative control group. Moreover treatment with NF340 significantly reduced anti-type II collagen antibodies and increase in the proliferation of T-cells compared to negative control group. **Conclusion:** Present investigation proves that gene expression of $P2Y_{11}$ receptor blocked by NF340 attenuates the RA by ameliorating the cellular and humoral immunity.

Key words: Arthritis, $P2Y_{11}$ receptor, NF340, immunity, inflammatory cytokines

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Corresponding Author: Shenglin Lu, Department of Orthopaedics, Affiliated National Hospital of Guangxi Medical University, Nanning, Guangxi, China Tel/Fax: 0086-0771-3117032

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rheumatoid arthritis is an auto-immune disorder characterized by destruction of bone and cartilage. All over the world around 1% of people are suffering from RA[1]. Chronic inflammation of joint develops the RA, due to infiltration of inflammatory cells and also the immune cells like neutrophils, macrophages, B and T-cells[2]. Literature reveals that the production of ROS and inflammatory cytokines enhanced due to infiltration of immune cells in the joint and which further destruct of synovial tissues and bone and causes inflammation of joint[3,4]. There are several therapies available for the management of RA such as disease modifying anti-rheumatic drugs, biological therapy and glucocorticoids[5]. However these drugs are costly and require the chronic use of it that causes many adverse effects. Thus a new therapeutic is required for the management of RA.

$P2Y_{11}$ receptor is present on the several immune cells such as lymphocytes, mast cells, granulocytes and dendritic cells[6]. Movement and differentiation of immune cells was reported into induce $P2Y_{11}$ receptor and it also modulate the function of cellular immune[7]. Literature reveals these receptors involved in myocardial infarction process for the inflammatory reaction[8]. Thus present study evaluates effect of $P2Y_{11}$ receptor antagonist against the rheumatoid arthritis.

MATERIALS AND METHODS

Chemicals: 4,4'-(Carbonylbis(imino-3,1-(4-methyl-phenylene)carbonylimino))bis(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340) was procured from Tocris Bioscience, UK. TNF-α, IFN-γ and IL-1β was procured from BioSource Europe, Belgium. Bovine serum albumin (BSA), Goat antimouse IgG, Freund's complete adjuvant (FCA), Bovine type II collagen procured from Sigma chemicals, USA.

Animals: Male albino wistar rats (150-180 g) were procured from Shanghai Medical College, Shanghai, China. Animals were stored under the standard condition as per the guideline. All the animals were kept for the period of one week for the acclimatization to laboratory condition with free access to normal standard chow diet and tap water. Protocol of this study is approved by institutional animal ethical committee of Affiliated National Hospital of Guangxi Medical University, China (NHGMU/IAEC/2017/08).

Induction of RA: Complete Freund's adjuvant (CFA) was mixed with an equal volume of type II collagen. Acetic acid (0.05 M, 1 mL) was used to dissolve 4 mg of type II collagen. Complete Freund's adjuvant (CFA) was injected by the intraplantar injection for the induction of arthritis in left hind paw. Mycobacterium tuberculosis killed by heat in 10 mg mL^{-1} concentration of sterile paraffin oil in adjuvant. After the administration of Freund's complete adjuvant plethysmometer was used for the estimation of paw volume of each animal on 0, 7, 14 and 21 days. Thirty wistar rats were separated in to 5 groups (n = 6) like control group, negative group (Rheumatoid arthritis group), NF340 0.3, 10 and 30 μM/day injected intrathecally for the period of 21 days.

Estimation of rheumatoid arthritis development: Rheumatoid arthritis development in all the animals were estimated by estimating thermal hyperalgesia, mechanical nociceptive threshold, arthritic score and paw volume on 0, 7, 14 and 21 days of protocol. Severity of arthritis was estimated on the five pointer scale. As 0 considered as no swelling, 1 for limited erythema and edema, 2 for erythema from the tarsal bone to ankle with slight edema, 3 for erythema from the tarsal bone to ankle associated with moderate edema, 4 for erythema of entire leg associated with edema.

Estimation of pro-inflammatory cytokines: ELISA method was used for the determination of concentration of inflammatory cytokines (TNF-α, IFN-γ and IL-1β) in synovial fluid. Level of inflammatory cytokines was estimated by using ELISA kit as per the instruction of manufacturer.

Estimation of anti-type II collagen antibodies: Blood was collected from the retro-orbital plexus of rats at 7th, 14th and 21st day of protocol. Saline solution was used for the dilution of serum. Type II collagen was coated to the microtiter plate and incubated at 4°C for the period of overnight. Later it was treated with 2% BSA in PBS (50 mL) at room temperature for the duration of 90 min for the blocking and then PBS buffer was used to wash the plate for the four times. Thereafter serum sample (50 μL) was seeded and later for the period of 1 h incubated at room temperature. Peroxidase-labeled rabbit antimouse IgG 50 μL (in 0.6 mol = L NaCl, 0.26 mol = L H_3PO_4 and 0.08N NaOH, pH 9.6) was seed to the plate carries sample and incubate it for the period of half an hour at room temperature. Sulphuric acid (0.8 N) was added to the plate to stop the reaction and at a wavelength of 493 nm plates were read by ELISA method.

T-cell proliferation assay: Spleen was isolated from all the animals and T-cells were separated out as per the previously

reported method[9]. Spleens were tested to yield a single cell suspension by incubating with 1 mL 0.9 % NH_4Cl for 1 min at 37°C, followed by adding RPMI, twice the volume of the incubation mixture. NH_4Cl traces were washed three times from the cells by washing and later B cell population was removed by incubating anti-mouse immunoglobulins (1:1000 PBS) coated plate with cells. Separation of unbounded cells was done carefully and fetal calf serum was used to resuspend the cell. Trypan blue exclusion was used check the viability of cells. Antigen-induced T-cell proliferation assay was applied for the cells. 2×10^5 cells were poured into each well of 96 wells microtiter plate with RPMI (250 mL). In presence and absence of several fractions of doses mitogen (1 and 2 µg) was used to stimulate the cells. Cell culture was incubated in CO_2 incubator at 37°C for the duration of 3 days. Further tritiated thymidine was added in the each well and incubation of culture was done for the period of 18 h. Nunc cell harvester was used to harvest the cells and scintillation fluid was added for the estimation thymidine incorporation by liquid scintillation counting.

Statistical analysis: All data were expressed as Mean±SD (n = 6). The statistical analysis was performed using one-way ANOVA. The *Post-hoc* comparison of means was carried out by Dunnett's *post hoc* test (Gradpad prism 6.1., CA, USA). The level of statistical significance was set at $p<0.05$.

RESULTS

Effect of $P2Y_{11}$ antagonist on paw swelling: Effect of $P2Y_{11}$ receptor antagonist on the paw swelling was estimated in RA rats on 0, 7th, 14th and 21st day of protocol was shown in Fig. 1. It was observed that paw swelling was significantly enhanced compared to control group on 7th, 14th and 21st day of protocol. However treatment with NF340 significantly reduces the paw swelling in RA rats compared to negative control group on 7th, 14th and 21st day of protocol in a dose dependent manner.

Effect of $P2Y_{11}$ receptor antagonist arthritic score and paw withdrawal latency: Figure 2 shows the effect of $P2Y_{11}$ receptor antagonist was observed on the development of RA in CAF induced arthritic rats. Arthritic score and paw withdrawal latency was estimated in RA rats on 0, 7th, 14th and 21st day of protocol in all the group of animals. There was significant increase in the arthritic score in all the group of CAF treated group compared to control group. However, arthritic score was found to be significantly enhanced and

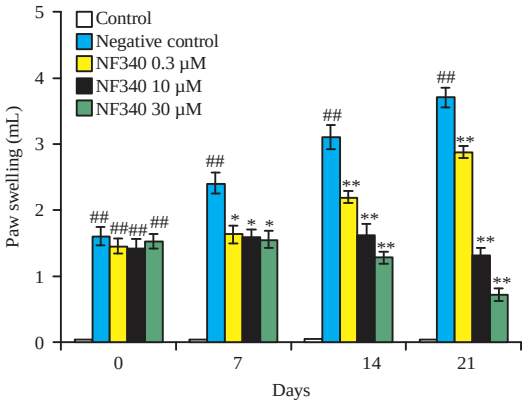

Fig. 1: Effect of $P2Y_{11}$ receptor antagonist on the paw swelling was estimated in RA rats on 0, 7th, 14th and 21st day of protocol

Data Mean±SD (n = 6), ##p<0.01 compared to control group, *p<0.05, **p<0.01 compared to negative control group

percentage of paw withdrawal latency significantly reduced continuously in negative control group compared to control group of rats. While NF340 treated group shows the significant reduction in the arthritic score and increase in the percentage of paw withdrawal latency on 7th, 14th and 21st day of protocol compared to negative control group. Effect of NF340 treatment on arthritic score and paw withdrawal latency was found in a dose dependent manner.

Effect of $P2Y_{11}$ receptor antagonist on pro-inflammatory cytokines: Effect of $P2Y_{11}$ receptor antagonist on pro- inflammatory cytokines such as IL-1β, TNF-α and INF-γ in the synovial fluid of arthritis rats was shown in Table 1. Level of pro-inflammatory cytokines was found to be significantly enhanced in the synovial fluid of negative control group compare to control group of rats. Treatment with NF340 significantly reduces the concentration of IL-1β, TNF-α and INF-γ in the synovial fluid of arthritis rats compared to negative control group.

Effect of $P2Y_{11}$ receptor antagonist on anti type II collagen antibody: Effect of $P2Y_{11}$ receptor antagonist on the anti type II collagen antibody was estimated in the serum of RA rats on 7th, 14th and 21st day of protocol was shown in Fig. 3. It was observed that level of anti type II collagen antibody (IgG) was found to be significantly enhanced in negative control group compared to control group of rats on 7th, 14th and 21st day of protocol. There was significant decrease in the level anti type II collagen antibody (IgG) found in NF340 treated group compared to control group of rats on 7th, 14th and 21st day of protocol in a dose dependent manner.

Table 1: Effect of $P2Y_{11}$ receptor antagonist on pro-inflammatory cytokines in the synovial fluid of arthritis rats

Groups	IL-1β (ng L^{-1})	TNF-α (ng L^{-1})	INF-γ (ng L^{-1})
Control	41.59±2.17	65.46±2.91	3.42±0.18
Negative control	194.20±9.23##	412.50±19.52##	8.27±0.42##
NF340 0.3 μM	163.60±11.46*	342.20±17.47*	6.38±0.28*
NF340 10 μM	109.20±10.19**	169.80±11.72**	5.14±0.24**
NF340 30 μM	70.35±3.91**	89.32±5.28**	3.94±0.21

Data Mean±SD (n = 6), ##p<0.01 compared to control group, *p<0.01, **p<0.01 compared to negative control group

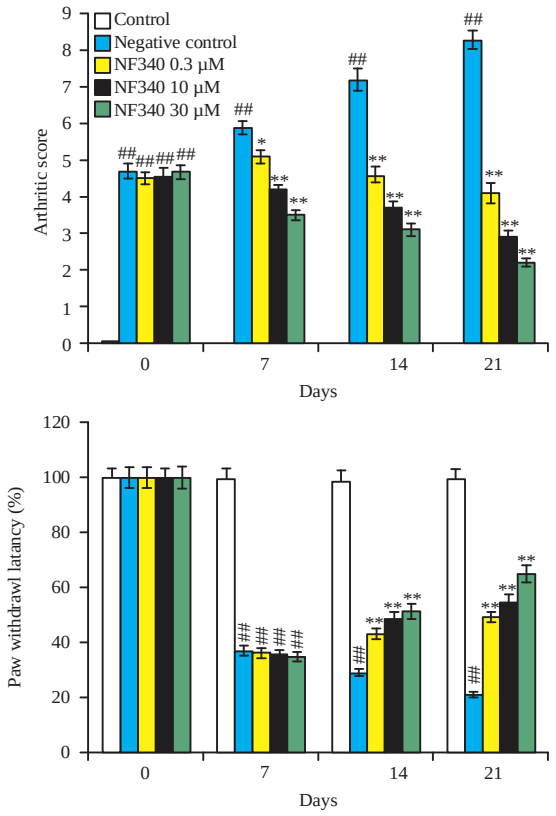

Fig. 2: Effect of $P2Y_{11}$ receptor antagonist on arthritic score and paw withdrawal latency was estimated in RA rats on 0, 7th, 14th and 21st day of protocol
Data Mean±SD (n = 6), ##p<0.01 compared to control group, *p<0.05, **p<0.01 compared to negative control group

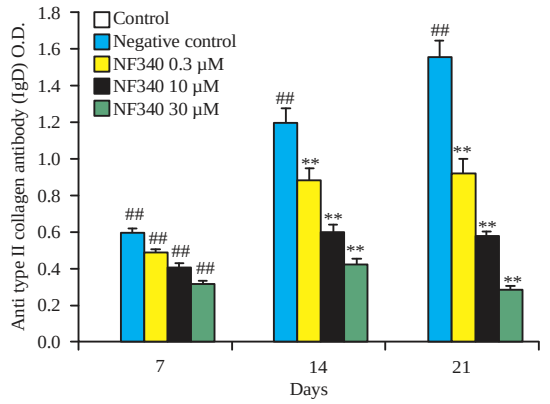

Fig. 3: Effect of $P2Y_{11}$ receptor antagonist on the anti type II collagen antibody was estimated in the serum of RA rats on 7th, 14th and 21st day of protocol
Data Mean±SD (n = 6), ##p<0.01 compared to control group, *p<0.05, **p<0.01 compared to negative control group

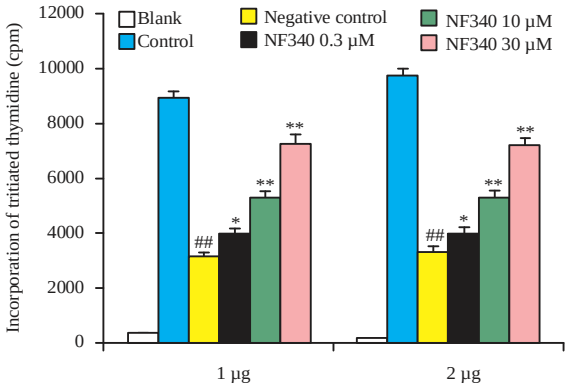

Fig. 4: Effect of $P2Y_{11}$ receptor antagonist on the proliferation of T-cells in RA rats
Data Mean±SD (n = 6), ##p<0.01 compared to control group, *p<0.05, **p<0.01 compared to negative control group

Effect of $P2Y_{11}$ receptor antagonist on the proliferation of T-cells: Figure 4 Shows the effect of $P2Y_{11}$ receptor antagonist on the proliferation of T-cells in RA rats. Proliferation of T-cell was estimated by determining the incorporation of tritiated thymidine. It was observed that the incorporation of tritiated thymidine significantly reduced in negative control group compared to control group of mitogen (1 and 2 μg) stimulated cells. However, treatment with NF340 significantly attenuated the incorporation of tritiated thymidine in mitogen (1 and 2 μg) stimulated cells of RA rats. Thus data of study reveals that treatment with NF340 restores the proliferation of T-cells in a dose dependent manner.

DISCUSSION

Present study evaluates the beneficial effect of $P2Y_{11}$ receptor antagonist against the RA rats. Moreover given study also postulate the mechanism of $P2Y_{11}$ receptor antagonist. $P2Y_{11}$ receptor antagonist (NF340) effect was estimated on the development of arthritis by determining the paw withdrawal latency and swelling and arthritis score on

7th, 14th and 21st day of protocol. Moreover level of pro-inflammatory cytokines, anti type II collagen antibody and proliferation of T-cell was estimated in the given report.

Data of the present report reveals that treatment with $P2Y_{11}$ receptor antagonist (NF340) attenuates the development of RA in CFA induced RA rats. In RA swelling of joints and the arthritis score parameters significantly enhanced and these parameters are responsible for the estimation of development of RA[10]. Literature reveals that drug used in the management of RA attenuates the developmental parameters of RA[11].

In RA concentration of pro-inflammatory cytokines enhanced which promotes the destruction of synovial tissue and thereby promotes the inflammation in the joints[12]. Result of given study reveals that treatment with NF340 attenuates the altered level of pro-inflammatory cytokines which was supported by previous reports[3]. Moreover immune cells play an important role in the development of RA as production of inflammatory cytokines is stimulated due to infiltration of immune cells[13]. Result of the study reveals that altered inflammatory cytokine level was attenuated by blocking $P2Y_{11}$ receptor in RA rats. Cellular and humoral immunity is responsible for the pathogenesis of RA[14]. Literature also reveals that immuno-suppressive agents have shown a proven effect in the management of RA[15,16]. Data of current report suggest that treatment with NF340 stimulates the proliferation of T-cells and also reduces the level of anti type II collagen antibody compared to negative control group.

CONCLUSION

Present investigation proves that gene expression of $P2Y_{11}$ receptor blocked by NF340 attenuates the RA by ameliorating the cellular and humoral immunity. Moreover alteration in the cellular and humoral immunity reduces the level of inflammatory cytokines in NF340 treated groups.

SIGNIFICANCE STATEMENT

Present study reports the role of $P2Y_{11}$ receptor blockage in the management of RA. This report also postulates that blockage of *P2y11* gene expression attenuates the altered cellular and humora, immunity in RA. Data of this study suggest that the blocking of $P2Y_{11}$ receptor could be a good target for the management of RA.

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