Expression of IGF-1, IL-27 and IL-35 Receptors in Adjuvant Induced Rheumatoid Arthritis Model

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ABSTRACT

Background: IGF-1 and certain other cytokines have been shown to exert inflammatory/anti-inflammatory roles in chronic joint diseases. **Objective:** To assess the effect of IGF-1, IL-27 and IL-35, their interaction and their receptor expression in a rheumatoid arthritis model. **Methods:** Freund's adjuvant-induced chronic joint inflammation was operated on 160 male rats. Animals were divided into histopathology and receptor expression groups, each composed of 10 subgroups including; control, vehicle, IGF-1, IL-27, IL-35, their antagonists, IGF-1+IL-27 antagonist and IGF-1+IL-35 antagonist. After two weeks, vehicle or agonist/antagonists were injected into the joint space every other day until day 28 where joint histopathology was performed. The expression of IGF-1, IL-27 and IL-35 receptors were assessed by western blot analysis. **Results:** IGF-1 did not show pro- or anti- inflammatory functions; endogenous IL-27 and IL-35, on the other hand, exerted inflammatory effects. IL-27 and IL-35 antagonists exerted the highest anti-inflammatory effects. The total inflammation scores were 0.55 ± 0.06, 4.63 ± 0.40, 3.63 ± 0.60, 2.50 ± 0.38 and 1.63 ± 0.40 regarding control, vehicle, IGF-1 Ant., IL-27 Ant. and IL-35Ant., respectively. IGF-1 receptor expression was reduced in chronic joint inflammation and all three antagonists augmented the IGF-1 receptor expression. IL-27 and IL-35 receptors were up-regulated by chronic joint inflammation. **Conclusion:** Overall, the results demonstrated the pro-inflammatory role of endogenous IL-27 and IL-35 along with the over expression of their receptors in chronic joint inflammation. IL-27 and IL-35 antagonists exerted the most anti-inflammatory effects and increased IGF-1 receptor expression. These two antagonists may be potential agents for new treatment strategies in chronic joint inflammatory diseases.

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Keywords: Chronic Joint Inflammation, Histopathology, IGF-1, IL-27, IL-35, Immunohistochemistry, Receptor Expression

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INTRODUCTION

Rheumatoid arthritis (RA) is a prevalent inflammatory arthritis affecting around 1% of the world's population (1). Its prevalence increases with age, approaching its highest rate in 40 to 70 years old (2). Considering the fact that RA entails heavy expenses and morbidity, it is important to assess the pathophysiology of the disease and its underlying mechanisms. Our previous works have revealed low oxygen tension in synovial fluid and its further reduction following joint inflammation despite the increase in joint blood flow (3,4). This condition stimulates the release of growth factors and pro-inflammatory cytokines, initiating processes that ultimately lead to joint destruction. The recently discovered IL-27 and IL-35 play an essential role in the differentiation of inflammatory cells (5,6). IL-27 obstructs the differentiation of Th-17 cells and may suppress diseases with auto-immune nature such as RA (6-8). On the contrary, Cao *et al.* (9) and Fearon *et al.* (10) have shown the inflammatory effects of IL-27 in arthritis induced by proteoglycans and adjuvants, respectively. The mice that lacked IL-27 receptors were also resistant to the progression of arthritis (11) further highlighting the inflammatory properties of IL-27. There is still uncertainty for the inflammatory and anti-inflammatory properties of IL-35. It seems that the diversities in the effects of IL-35 depend on the animal model of inflammation (12). The myriad studies on inflammatory bowel disease, collagen-induced arthritis (CIA), or other autoimmune diseases have proven the anti-inflammatory properties of IL-35 (5,13,14). In contrast, there exist studies indicating the pro-inflammatory properties of this cytokine. For instance, it is shown to act as an inflammatory mediator under various experimental conditions in Lyme arthritis, CIA and adjuvant induced arthritis (15,16,17). Furthermore, in a recent study, it was observed that IL-35 was up-regulated in the synovium of RA patients. These data imply the inflammatory properties of IL-35 in human and its potential role in the pathogenesis of RA (12). IGF-1 reduces the signaling of cytokines with pro-inflammatory properties through IL-10, NF-KB production or alleviation of the response to TNF-α (18,19). The reduction in the sensitivity to TNF-α has also been proposed as a mechanism for the elimination of diseases induced by pro-inflammatory cytokines (20). IGF-1 plays a role in the synthesis of extracellular matrix proteins of bones and cartilage, and the proliferation of the fibroblasts. Moreover, in contrast to IGF-1 binding proteins (IGFBPs), it either remains constant or decreases in RA disease (21).

Due to the controversies revolving around the function of IL-27 and IL-35 in joint inflammation, and the dearth of information regarding the interaction of such cytokines with IGF-1 in chronic joint inflammation (CJI), the present research was performed to analyze the role of IGF-1, IL-27, IL-35 and their antagonists in chronic joint inflammation. Further assessed was the influence of IL-27 and IL-35 on IGF-1 function in adjuvant induced CJI which is an experimental model that highly resembles the characteristics of human RA (22). As IGF-1 and cytokines exert their effects through their own receptors or interaction with other receptors, the effects of IGF-1, IL-27 and IL-35 on other receptor expressions in inflamed joints were also assessed as a probable mechanisms for their influence on CJI.

MATERIALS AND METHODS

Materials. Complete Freunds adjuvant (CFA) (Sigma, UK), Dry milk (GE Healthcare, USA), IGF-1 from rat (SRP-4121, Sigma, UK), IL-27 (SRP-4187, Sigma, UK), IL-35(10705-H02H, Sino biological, USA), IGF-1 receptor inhibitor (SC-204008, Santa Cruz, USA), IL-35 antagonist (SC-7925, Santa Cruz, USA), IL-27 antagonist (Mab 2109, R&D, USA), IGF-1 receptor antibody (SC-712, Santa Cruz, USA), IL-27 receptor antibody (SC-99082, Santa Cruz, USA), Protein G Sepharose (GE Healthcare, USA, Cat no 17-0618-01), SDS–PAGE 8, 10, 12% polyacrylamide gels), gp130 primary antibody (Anti-gp130, Alomone, ALR-023), secondary antibody (SC-2004, Santa Cruz, USA), and Sodium thiopental (Rotex Medica, Germany) were used.

Experimental Protocol. Experiments conformed to the national guidelines to conducting animal studies (Ethic committee code 93/222KA, Kerman University of Medical Sciences, Iran) and were performed on 160 male rats weighing 250-290 gr. Animals were housed at 23 ± 2˚C, 12-h dark/12-h light cycle, with access to water and food ad libitum. They were randomly divided into two main groups, according to histopathology (n=80) and receptor expression assessment (n=80). Each main group was subcategorized into 10 subgroups including: 1) Control (non-inflamed), 2) Vehicle (inflamed, intra-articular saline), and eight groups that were inflamed and received intra-articular, 3) IGF-1, 4) IL-35 , 5) IL-27, 6) IGF-1 antagonist, 7) IL-35 antagonist, 8) IL-27 antagonist, 9) IGF-1+IL-27 antagonist, and 10) IGF-1+IL-35 antagonist.

CJI was induced by injecting 0.2 ml CFA into the joint space of the right knee by inserting a 28 G needle through the mid-patellar tendon on day 0 (23,24) which is an experimental model of antigen-induced arthritis, mimicking human rheumatic arthritis (22). From day 14 (induction of chronic arthritis) to 28, 2 µg of agonist and/or 4 µg of antagonist, with a total volume of 0.2 ml, were injected into the joint space every other day. The doses were selected based on studies dealing with similar subjects using IGF-1 and IGF-1 (25), IL-35 and IL-35 (5) and IL-27 and IL-27 antagonists (7,11,26). The medio-lateral diameter of the knee was regularly measured as an inflammation indicator (27) using a caliper (abd, China). The frequency of measurement was once a day for the first week, and every other day for the next three weeks. The animals were weighed every other day over the first week and every three days for the next 21 days.

Histopathology Assessment. The animals were sacrificed under deep anesthesia (sodium thiopental, 50 mg/kg, ip) on day 28. Their right joints were removed from 1 cm above and 1 cm below the knee joint, cleaned from the muscles, and fixed in 10% formalin solution. So as to become decalcified and softened for sectioning, they were subsequently positioned in 10% nitric acid for at least 24 hours. Five 5 µm slices were prepared from paraffin blocks and following dehydration by alcohol, they were stained by hematoxylin and eosin. The severity of inflammation was graded based on the Kapila method (28) by a pathologist. The scoring system was:

A) Synovial hyperplasia: One to three cell layers = zero; 4 to 6 cell layers = 1; 7 cell layers or more = 2.

B) Villous Hyperplasia: Does not exist = Zero; low, scattered and short = 1; marked and long = 2; marked and abundant = 3.

C) Infiltration by mononuclear cells: Normal = zero; mild = 1; moderate = 2; severe = 3; marked cellular infiltration accompanied with lymphoid follicles = 4.

D) Pannus formation: Does not exist = zero; mild to moderate proliferation of synoviocytes and tissue invasion to the disc, cartilage, or bone = 1; moderate invasion = 2; severe invasion of mononuclear inflammatory cells to the disk, cartilage, or bone = 3. The sum of A, B, C, and D was considered as the total score with 12 being the maximum amount.

Western Blotting for IGF-1 and IL-27 Receptor Expression. On day 28, animals were anesthetized and sacrificed as stated in the histopathology assessment section. The synovial tissue around the joint space was removed quickly, immersed in liquid nitrogen and kept at -80°C until the day of experiment. Tissue samples were homogenized (Hielscher homogenizer, ultrasound technology, Germany) in ice cold lysis buffer with protease inhibitors. The homogenates were centrifuged (Eppendorf, Model 5810R, Germany) at 15000 ×g for 15 min at 4°C. Lysate (protein) concentration was determined through the use of Bradford method (Bio-Rad Laboratories, Munchen, Germany). An equal volume of 2×SDS sample buffer was added, and supernatant samples were resolved electrophoretically (Bio-Rad Company, USA) on a 12.5% SDS-PAGE gel and transferred to PVDF membranes. Following an overnight blocking at 4°C with 5% non-fat powdered milk in tris-buffered saline and Tween 20, the membranes were incubated for 3 h for IGF-1, 1.5 h for IL-27 and over night for IL-35 with a primary antibody (1:1000) at room temperature. The blots were washed 3 times in TBS-T and incubated with goat anti-rabbit IgG secondary antibody (1:10000) at room temperature for 1.5 h. All antibodies were diluted with blocking buffer. Enhanced chemiluminescence detection film was used in order to detect antibody-antigen complex. Image J software was used to measure the intensity of band densities. β-actin immuno-blotting was used as a control for loading. The density values of the receptor and β-actin band were obtained from gel analysis and band densitometry. These were expressed as receptor protein/β-actin ratio for each sample.

Immunohistochemistry (GP130 staining) for Assessing IL-35 Receptor Expression. As the antibody for IL-35 receptor has not yet been produced (commercially not available), we measured its receptor expression indirectly through gp130 subunit expression assessment using the IHC method. This was done because IL-35 receptor has two membrane subunits gp130 and IL-12Rβ2, and the gp130 subunit is the same as IL-27 receptor (29). As we assessed IL-27 receptor expression, we were able to analyze IL-35 receptor expression indirectly by measuring total gp130 and subtracting its relative IL-27 component from the total. 4 μm-sections were prepared from paraffin blocks and deparaffinized by three washes of xylene. The slides were consequently hydrated in 100%, 90%, 80% and 70% ethanol for 1 minute. Next, they were briefly dipped in water and fixed in microwave at 120ºC for 10 minutes. They were washed in phosphate buffer saline (PBS) (3 min), stranded in hydrogen peroxide (10 min) and exposed to anti gp130 primary antibody (60 min) at room temperature. Slides were then exposed to secondary antibody and washed by PBS. Prior to counterstaining with Hematoxylen Mayers, they were exposed to chromogen DAB. Finally, the slides were dehydrated in 70%, 90% and 100% ethanol, washed 3 times by xylene and mounted with Entellan. A pathologist blinded to the groups assessed the slides under a microscope and scored them semi-quantitatively for gp130 staining intensity through analyzing five different fields and using a four-point scale, where 0, 1, 2, 3, and 4 represented negative, weak , mild, moderate and strong staining, respectively (12). A lung slide was also stained as a positive control, since it has already been demonstrated that this tissue normally expresses high levels of gp130 (30).

Statistical Analysis. The data in the graphs and the table are presented as mean ± SEM. The scores of inflammatory indices and gp130 expression among the groups were compared using Kruskal-Wallis followed by Mann-Whitney tests. For weight, knee diameter and receptor expression data, the normal distribution was analyzed using Kolmogorov-Smirnov test. One–way ANOVA was used in order to compare the groups, followed by Tukey's post hoc test for pair-wise comparisons. The P values <0.05 were considered statistically significant.

RESULTS

Changes in Joint Diameter and Animal Weight.

Figure 1 illustrates the changes in joint diameter among the study groups throughout the 28 days of inflammation. Except for the control group, CFA injection increased the joint diameter of all groups, reaching its maximum on the third day (acute inflammation). Concerning joint diameter, there was no significant change in either group from day 15 to 28 (chronic inflammation). The animal weight increased from 260 ± 2.1 gr on day zero to 288 ± 2.3 gr on day 28 in the control group (10.7% increase). In the inflamed groups, this change ranged from a maximum 12.3% increase in the IL-27ant group to a minimum 0.3% increase in the IGF-1+IL-27ant group. There were no significant changes in weight either inside each group or among the ten groups (Due to space limitations, only the weight in three important time points are demonstrated in the Table) during the 28 days (Table 1).

Histopathology Findings.

Picture 1 demonstrates a sample histology section of each group. The total scores of the four histology indices, summarized in Figure 2, showed that IL-35 and IL-27 antagonists exerted strong anti-inflammatory effects (P<0.05). IGF-1 inhibited the anti-inflammatory effects of IL-27 and IL-35 antagonists (compare IGF-1+IL-27ant. and IGF-1+IL-35ant. groups with IL-27ant and IL-35ant. groups).

Table 1. The animal weights in grams (mean ± SEM) of different groups during the course of the experiment.

GROUP	Day 0	Day 15	Day 28	P value
Control	261 ± 14	275 ± 13	288 ± 12	0.37
Vehicle	270 ± 5	289 ± 6	296 ± 5	0.06
IGF-1	284 ± 5	294 ± 5	303 ± 5	0.20
IL-27	262 ± 9.8	273 ± 10.1	282 ± 10.6	0.22
IL-35	268 ±6	279 ± 6	288 ± 6	0.24
IGF-1ant	283 ±15	304 ± 18	295 ± 15	0.41
IL-27ant	284 ± 7.1	296 ± 7.1	319 ± 7.1	0.30
IL-35ant	257± 2	267 ± 5	263 ± 16	0.18
IGF + 27ant	259 ±3	269 ± 3	261 ± 14	0.90
IGF + 35ant	257 ±2	262 ± 2	269 ± 1	0.18

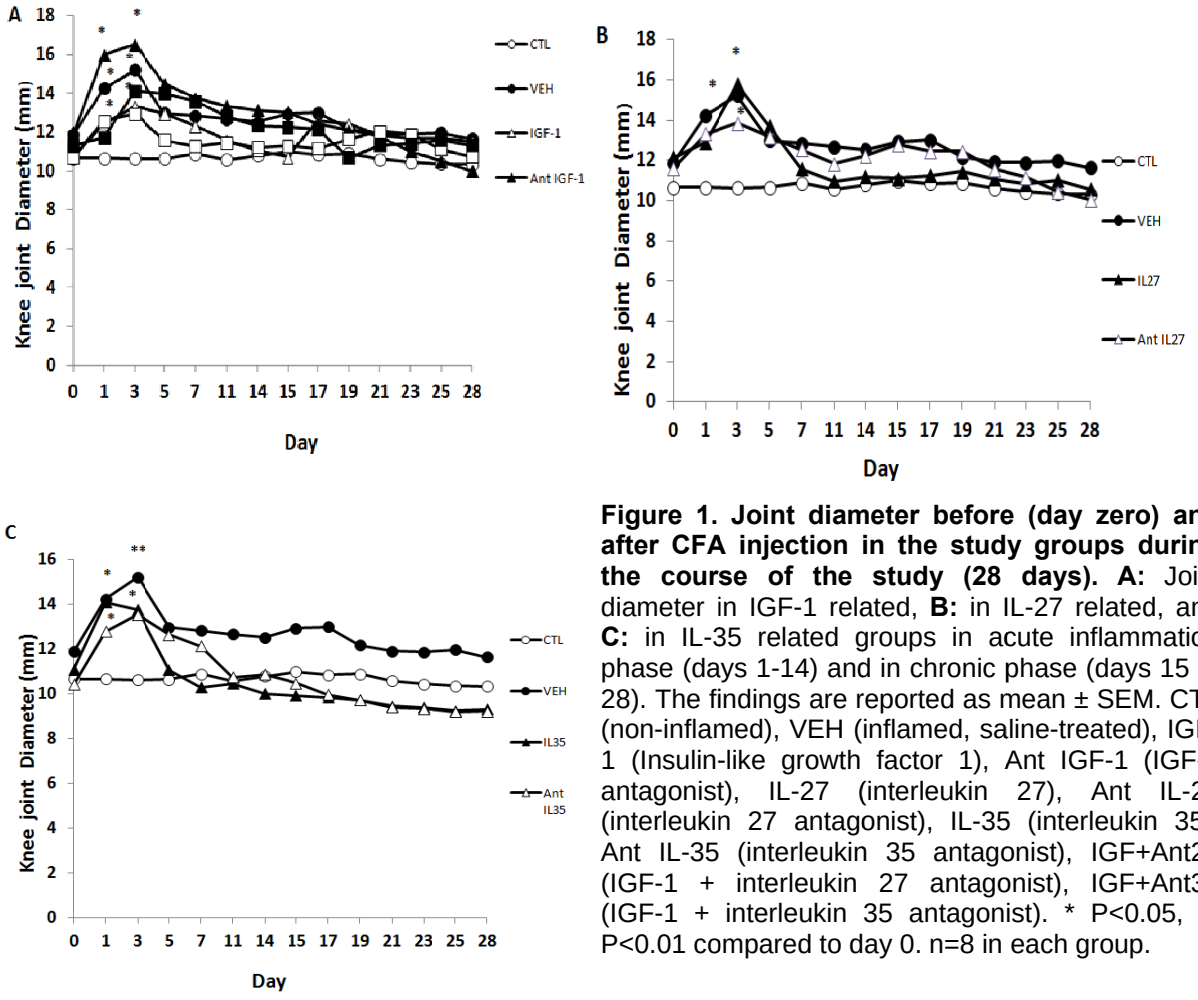


Figure 1. Joint diameter before (day zero) and after CFA injection in the study groups during the course of the study (28 days). A: Joint diameter in IGF-1 related, **B:** in IL-27 related, and **C:** in IL-35 related groups in acute inflammation phase (days 1-14) and in chronic phase (days 15 to 28). The findings are reported as mean ± SEM. CTL (non-inflamed), VEH (inflamed, saline-treated), IGF-1 (Insulin-like growth factor 1), Ant IGF-1 (IGF-1 antagonist), IL-27 (interleukin 27), Ant IL-27 (interleukin 27 antagonist), IL-35 (interleukin 35), Ant IL-35 (interleukin 35 antagonist), IGF+Ant27 (IGF-1 + interleukin 27 antagonist), IGF+Ant35 (IGF-1 + interleukin 35 antagonist). * P<0.05, ** P<0.01 compared to day 0. n=8 in each group.

Alterations in the Receptor Expression and the Effect of Agonists and Antagonists. Fig. 3 shows the receptor expression in the synovial tissue of different groups. As seen, IGF-1 receptors are significantly reduced by exogenous IGF-1 during chronic inflammation.

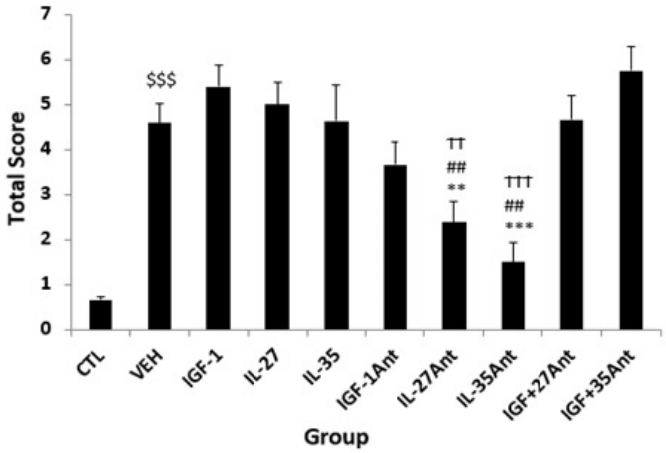


Figure 2. Histopathology total score (mean ± SEM) in the study groups (n=8 in each group). $ comparing with the control group.* comparing with the vehicle group, **#** Comparison of antagonist groups with their related agonist groups. **†** comparing with related combination group.

All three antagonists significantly increased IGF-1 receptors (Fig. 3A). IL-27 and IL-35 had no significant effect on IGF-1 receptors but the addition of IL-35ant. to IGF-1 reversed the suppressing effect of IGF-1 on its receptors. The IL-27 receptors were significantly increased during chronic inflammation; exogenous IL-27 reduced while IL-27 antagonist increased the IL-27 receptors significantly (Fig. 3B).

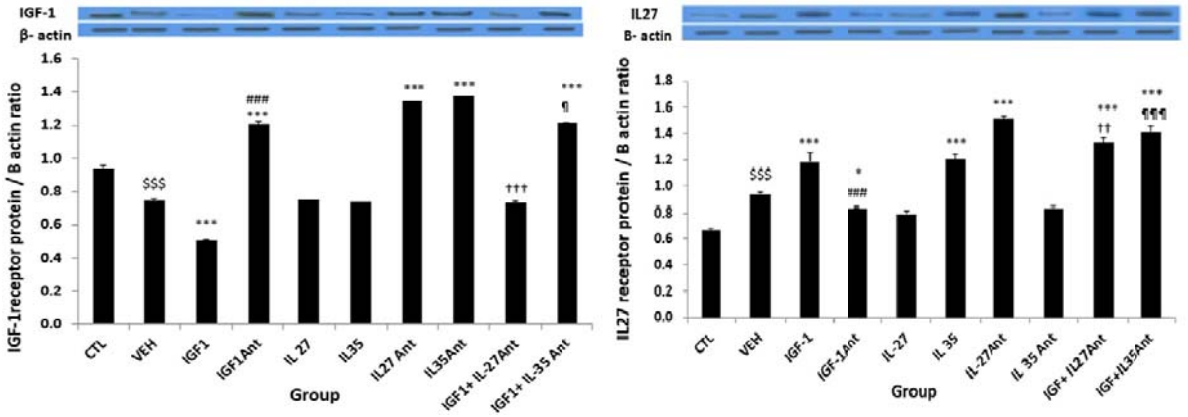


Figure 3. Alterations in receptor expression in the study groups (n=8 in each group). A: IGF-1 receptors, **B:** IL-27 receptors. Findings are reported as mean ± SEM. **$** comparing with control group,***** comparing with vehicle group, **#** comparing IGF-1ant group with IGF-1 group. **†** comparison with IL-27ant group, **¶** comparison with IL-35ant group.

Immunohistochemistry (IHC) Findings.

IHC was used to analyze the total expression of gp130 subunits of IL-35 and IL-27 receptors. A sample IHC section of each group is demonstrated in Picture 2. The strongest gp130 expression is seen in VEH, IL-27ant and IL-35ant groups (Picture 2B, E and H, respectively). Gp130 expression was mild to moderate in combination groups (I and J). IGF-1 did not affect gp130 expression and its antagonist reduced its expression (C and D). The expression of the gp130 component of IL-35 is represented in Fig. 4, and as observed, IL-35 receptors are over expressed by inflammation and exogenous IL-27. Exogenous IL-35 reduced and IL-35ant significantly increased the IL-35 receptor expression. IGF-1 inhibited the augmenting effect of IL-35ant on IL-35 receptor expression (compare IGF-1+IL-35ant group with IL-35ant group).

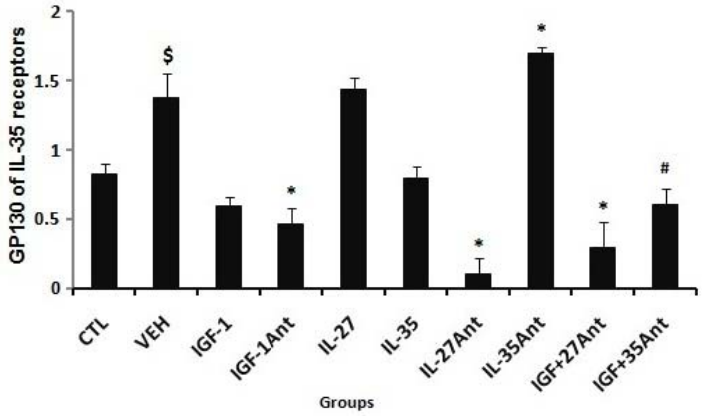
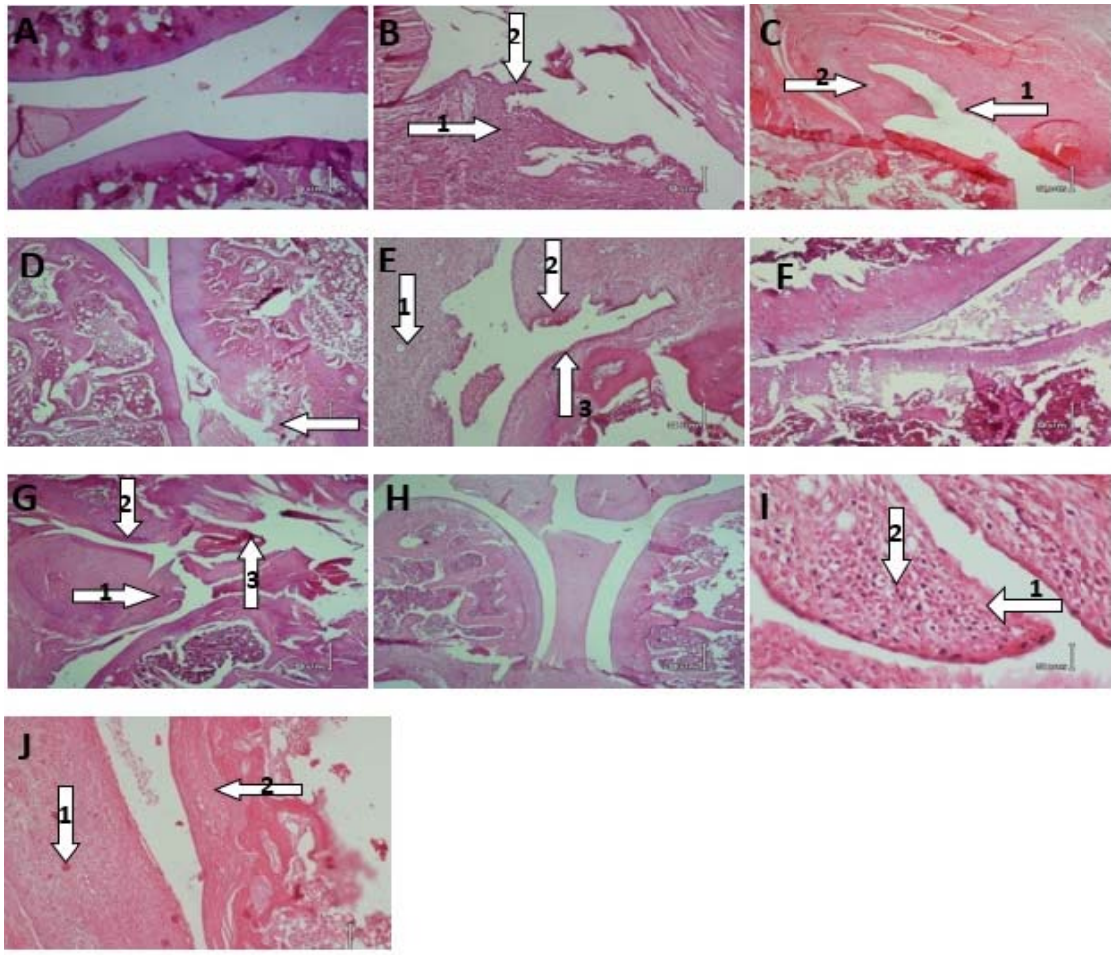


Figure 4. Alterations in gp130 subunit expression of IL-35 receptors in the study groups (n=8 in each group) assessed by immunohistochemistry. Findings are reported as mean ± SEM. **$**, P<0.05 comparing with control group,***** comparing with vehicle group, **#** comparing with IL-35ant group.

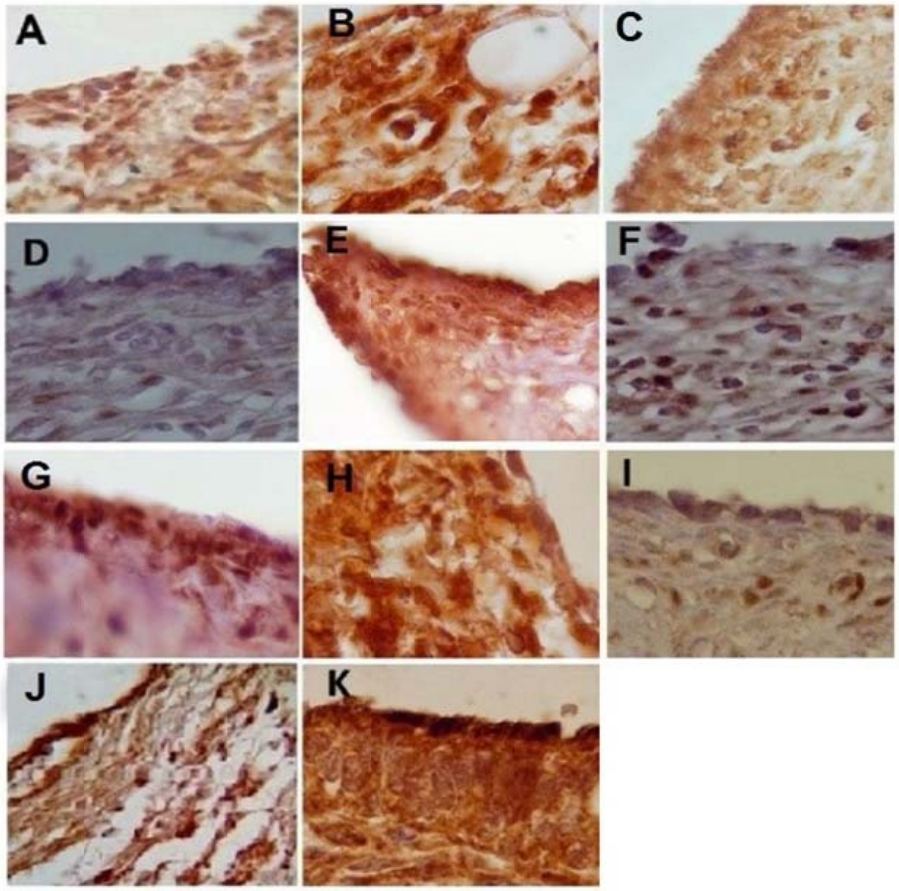
DISCUSSION

The main findings of the present study were that IL-27 and IL-35 antagonists significantly reduced inflammation indicating the inflammatory role of endogenous IL-27 and IL-35 in CJI. IGF-1 receptor expression was significantly reduced in chronic joint inflammation, and all three antagonists significantly increased the IGF-1 receptor expression. Joint diameter, as an index of inflammation, underwent the highest changes between days two and four (acute phase), reaching its peak on day 3 (Fig. 1). During the chronic phase of inflammation (days 15 to 28), the study groups showed no significant changes in joint diameter despite the progress of inflammation. This means that the degenerative changes at the tissue level have no external presentation. In the acute phase of inflammation, the joint diameter increased due to the increase in capillary permeability and leakage of fluid and proteins, leading to edema (31). The insignificant change in the weights of the animals (Table 1) proved that any change in the joint diameter is due to the inflammation process or study intervention, rather than the effect of animal growth over the 28 days of the study. No significant difference was observed between IGF-1 and IGF-1 antagonist groups with the vehicle regarding inflammation indices (Figure 2), implying that IGF-1 has no pro- or anti- inflammatory effects on this model of CJI. IL-27 and IL-35 antagonists significantly decreased inflammation indices; however, they lost this effect once employed in combination with IGF-1 (Fig. 2), meaning that exogenous IGF-1 had blocked the anti-inflammatory effects of IL-27 and IL-35 antagonists.



Picture 1. Microscopic sections of a knee joint in various groups on day 28 of chronic inflammation. A: the control joint with no sign of inflammation. **B**: vehicle group (saline-treated), chronic inflammation is observed on the surface of articular cartilages which includes congested vascular spaces and chronic inflammatory cells. Fibrosis and developing of inflammation into the internal surface of bone is presented in the lower part of the section (×10). Arrows, 1: infiltration of mononuclear cells, 2: villus hyperplasia. **C**: IGF-1 group; there is a dilated flattened villi which is attached to the internal surface of menisci, and granuloma are seen in its synovial lining. (×40). Arrows, 1: synovial hyperplasia 2: granuloma, **D**: IGF-1 antagonist group: synoviocytes are seen in one-row but in mucosal lining, fibrosis and proliferation of scattered fibro-histocytic cells are observed (×10). Arrow1: changes in panuss. **E:** IL-27 group: Deposition of fibrin materials in the joint and severe infiltration of mononuclear cells in mucosal lining on cartilaginous and non-cartilaginous surfaces is observed (×10). Arrows, 1: severe infiltration, 2: synovial and villus hyperplasia 3: synoviocytes hyperplasia. **F**: IL-27 antagonist group: damage to cartilage and inflammation in the joint is very mild, scattered and is almost normal (×100). **G**: IL-35 group: deposition of fibrin materials, fibrotic changes, and severe infiltration of mononuclear cells are seen in mucosal lining on cartilaginous and non-cartilaginous surfaces (×40). Arrows, 1: Severe infiltration, 2: synoviocytes hyperplasia, 3: villus hyperplasia. **H**: IL-35 antagonist group: There is no serious damage to the surface of cartilage and bone, except some fibrin deposition and mild fibrosis. On the non-cartilaginous surface of synovia, fewer inflammatory cells and mild fibrosis are seen (×10). **I:** IGF-1+IL-27Ant group: early villus and fibrosis changes on the cartilaginous surface and also fibrosis changes in mucosal lining of synovial membrane are seen (×40). Arrows, 1: early villus formation, 2: increase of mononuclear cells. **J**: IGF-1+IL-35Ant group: Pannus is formed on the cartilaginous surface which includes the proliferation of blood vessels and infiltration of lymphocytes and macrophages that contribute to destruction of cartilage and invading to bone surface under the cartilage (×100). Arrows, 1: inflammation changes with less severity 2: fibrosis formation.

It can therefore be concluded that 1) endogenous IL-27 and IL-35 have significant inflammatory effects on CJI; such effects are independent of IGF-1, as their antagonists inhibited inflammation and IGF-1 antagonist did not reduce their effects (fig. 2). 2) The inhibitory effect of exogenous IGF-1 on the anti-inflammatory effects of IL-27 and IL-35 antagonists is probably at the level of their receptors, because it was accompanied by the recovery of their receptors that had undergone changes during antagonism (compare combination groups with related antagonist groups in Figs 3B and 4). Certain studies have shown that the production of IGF-1 is suppressed in patients with RA and there is a reverse association between IGF-1 expression with the degree of inflammation (32,33). Based on a histologic assessment, the decrease in intra-articular IGF-1 found in patients with RA (34) was directly associated with synovial inflammation. The increase in the level of IGF-1 binding proteins (IGFBPs) may reduce the bioavailability of IGF-1 and reduce the response of cartilage to IGF-1 in osteoarthritis (OA) patients (35). When we look at IGF-1 receptor expression, expression was reduced during the process of inflammation (fig. 3A). IGF-1 antagonist increased the IGF-1 receptor expression while it had no significant effect on the inflammatory indices. IL-27 and IL-35 antagonists that increased the IGF-1 receptor expression (Fig. 3A) had the most anti-inflammatory effects. Accordingly, it might seem that there is a reverse association between the IGF-1 receptor expression and inflammation in the present model of RA. However the non-effectiveness of the endogenous/exogenous IGF-1 on inflammatory index found in the present study (fig. 2), despite the changes in its receptor expression, is not in line with the above conclusion. It seems that the changes in the level of IGF-1 receptors under such conditions are the result rather than the cause of inflammation. The reducing effect of IGF-1 on its own receptors and the augmenting effect of IGF-1 antagonist on IGF-1 receptor expression may be simply ascribed to the down-regulating effect of a ligand on its receptors. The mechanism by which the IL-27 and IL-35 antagonists increased the IGF-1 receptor expression is yet to be revealed. The results indicate that IL-27 led to joint damage (Picture 1E) and its antagonist significantly decreased the inflammation score (Figure 2). In accordance with the results of the present study, Cao and colleagues verified the inflammatory effects of IL-27 in proteoglycans-induced arthritis (9). In yet another study on patients with RA, an increase was observed in the serum level of IL-27 (27), highlighting the inflammatory activities of IL-27 in these patients. Goldberg *et al.* (2004) found that the mice which lacked IL-27 receptors were protected against the progression of arthritis (11). Conversely, certain studies have proven the suppressing effects of IL-27 on collagen- and osteocollagenesis-induced experimental arthritis (36). Moon and colleagues reported that IL-27 exerted anti-inflammatory effects via inducing regulatory T cells and obstructing Th17 differentiation (37). The differences in the method, time (acute vs. chronic phase) or duration of IL-27 administration may be the probable reasons for the discrepancy between the two groups of studies. In collagen-induced arthritis, for instance, the short-term administration of IL-27 at the onset of the disease significantly decreased the severity (26) or the progression (38) of the disease, yet it was ineffective in the stable phase. IL-27 administration during the onset of adjuvant-induced joint inflammation reduced, and if administered during a later phase, would have intensified the inflammation process (39,17).



Picture 2: IHC Micrographs of various groups (gp130 staining to analyze the IL-35 receptor expression) on day 28 after induction of arthritis (Magnification ×40 for all sections). A: the control joint with no sign of inflammation along with monolayer synovium (normal gp130 expression), **B:** vehicle group (inflamed, saline treated), abundant gp130 (brown colors) along with infiltration of inflammatory mononuclear cells. **C:** IGF-1 group: Bilayer synovium with almost normal degree of gp130 expression. **D:** IGF-1Ant group: low gp130 expression with macrophage cells relatively abundant. **E:** IL-27 group: Obvious gp130 with synovial layer proliferation and diffused number of lymphocytes and macrophages. **F:** IL-35 group: mild gp130 and mild to moderate inflammatory cells. **G:** IL-27Ant group: one or two synovial layers with dispersed inflammatory cells and moderate gp130 expression (mostly due to gp130 component of IL-27 receptors). **H:** IL-35Ant group: Strong gp130 expression with abundant fibroblast and mononuclear cells. **I:** IGF-1+IL27Ant group: Little gp130 expression is seen; Granuloma and fibrosis are mild and dispersed. **J:** IGF-1+IL-35Ant group; Moderate gp130 with low number of defending cells and mostly fibrosis in the field. **K:** Section of the lung as positive reference slide for gp130 staining.

The IL-27 receptor expression was increased during the process of inflammation (Fig. 3B, vehicle group). The Combination of IGF-1 with IL-27 antagonist or with IL-35 antagonist caused the highest inflammatory properties (Fig. 2) were accompanied by high levels of IL-27 receptor expression (Fig. 3B). Therefore, there may be a direct association between IL-27 receptor expression and chronic joint inflammation. Moreover, it seems that the level of IL-27 receptor expression is regulated by IGF-1. The evidence supporting this conclusion is that IGF-1 either alone or in combination with IL-27 antagonist increased the expression of IL-27 receptors (Fig. 3B). The

increment of IL-27 receptors by IL-27 antagonist may once again be due to the up-regulation of IL-27 receptors in the presence of their antagonist.

The results of the present study, as evident in Picture 1G, indicated the inflammatory role of IL-35 during chronic joint inflammation. IL-35 antagonist recognizably reduced the inflammation index (Fig. 2), thereby indicating the inflammatory role of endogenous IL-35. On the contrary, most of the previous studies have demonstrated the anti-inflammatory activity and inhibitory effect of this cytokine on the immune system. The suppression of Th17 cells (a pro-inflammatory cell) and the inhibitory effect of IL-35 on CIA are but a few examples (3,13). However, in agreement with our results, it has been shown that IL-35 increases the severity of Lyme arthritis (15). Thiolat ***et al.*** has recently reported that IL-35 intensifies the experimental RA (16) and Filkova and colleagues reported that IL-35 was up-regulated in the synovial tissue of RA patients suggesting the pro-inflammatory activity of this cytokine and its potential role in the pathogenesis of RA (12). In this regard, Senolt reported a reduction in the level of IL-35 during the treatment of RA and proposed IL-35 as a biomarker for the diagnosis and response to the treatment in these patients (40). It seems that similar to IL-27, the method, time and duration of IL-35 administration are determinants of the pro- or anti- inflammatory effect of this cytokine. In the present research, the changes in IL-35 receptor expression, indirectly assessed through measuring the expression of its gp130 subunit (Picture 2 and Fig. 4), imply that these receptors are significantly up-regulated by inflammation and by IL-35 antagonist. The anti-inflammatory effects of IL-27ant (Figure 2) is also accompanied by its strong reduction of IL-35 receptors (Figure 4). Thus, there may be a direct association between IL-35 receptor expression and chronic joint inflammation. The increment in IL-35 receptors by IL-35ant may once again be owing to the up-regulation of these receptors in the presence of their antagonist. It is probable that endogenous IL-35 has a regulatory (negative) role on IGF-1 receptor expression since the administration of IL-35ant. significantly increased IGF-1 receptor expression (Figure 3A). It also prevented the down-regulatory effect of IGF-1 on its receptors (compare IGF-1+IL-35ant with IGF-1 in fig. 3A).

There have been certain limitations to this study. It should be stated that all the aforementioned conclusions in this antigen-induced arthritis model were obtained from rats and may not be generalized to humans. Furthermore, the safety of IL-27 and IL-35 antagonists was not appraised in this study and requires an investigation into the animal models prior to considering their therapeutic effects in clinical studies.

In conclusion, the pro-inflammatory effects of endogenous IL-27 and IL-35 on CJI are accompanied by the overexpression of their receptors during the progress of inflammation. IL-27 and IL-35 antagonists increase IGF-1 receptor expression, have the most anti-inflammatory effect and are potential agents for the treatment of chronic joint inflammation. It is worth noting that the combination of these two antagonists would probably lead to better treatment outcomes.

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