IL-6-PAD4 axis in the earliest phase of arthritis in knock-in gp130F759 mice, a model for rheumatoid arthritis

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ABSTRACT

Objective Animal models for human diseases are especially valuable for clarifying molecular mechanisms before or around the onset. As a model for rheumatoid arthritis (RA), we utilise knock-in mice gp130F759. They have a Y759F mutation in gp130, a common receptor subunit for interleukin 6 (IL-6) family cytokines. Definitive arthritis develops around 8 months old and the incidence reaches 100% around 1 year old. Careful examination in the clinical course revealed very subtle resistance in flexibility of joints at 5 months old. Therefore, pathophysiological changes in gp130F759 were examined to dissect molecular mechanisms for preclinical phase of RA.

Methods Severity of arthritis in gp130F759 was evaluated with a clinical score system and histological quantification. Serum cytokines, autoantibodies and C reactive protein (CRP) were measured. Changes in the synovium were analysed by real-time PCR, flow cytometry and immunohistochemistry.

Results Around 5 months old, various types of cytokines, rheumatoid factor (RF), anti-circular citrullinated peptide IgM and CRP increased in the sera of gp130F759. Enhancement of neovascularisation, synovial hyperplasia and fibrosis was observed. Also, increases in haematopoietic cells dominated by innate immune cells and gene expression of Il6 and Pad4 were detected in the joints. Il6 was expressed by non-haematopoietic synovial cells, whereas PAD4 protein was detected in the synovial neutrophils. Padi4 is induced in neutrophils in vitro by IL-6. Increases of phospho-STAT3 and PAD4 protein were detected in the synovium. Deletion of IL-6 in gp130F759 normalised the amount of PAD4 protein in the joints.

Conclusion The IL-6-PAD4 axis operates in the earliest phase of arthritis in gp130F759, implicating it in early RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease with 0.3%–1.5% morbidity. It is characterised by chronic, proliferative synovitis with bone erosion leading to joint destruction. Genetic factors (HLA-DRB1, PTPN22 and PADI4 genes, etc), as well as environmental factors (smoking and infection) are involved in breakdown of self-tolerance and activation of autoimmunity.

Critical findings in the understanding and treatment of RA are anti-citrullinated peptide autoantibodies (ACPAs) as specific for RA and effectiveness of anti-cytokine therapies. Development of biologics against TNFα or IL-6 has drastically changed the prognosis of RA and therapeutic strategies. Since serum ACPAs are detected much earlier than the onset of RA,1–3 anti-circular citrullinated peptide (CCP) antibody was included in the criteria for RA of The European League against Rheumatism/American College of Rheumatology (EULAR/ACR) 2010.4 This permit earlier therapeutic application of biologics and the possibility of complete cures for this disease. Recent investigation of RA has proposed a stepwise, progressive model of RA.5–8 But it is difficult to obtain information on the molecular events in the synovium during preclinical phases of RA. This limitation can be partly overcome by the study of animal models.
Citrullination of arginine is catalysed by peptidyl arginine deiminases (PADs), which results in generation of modified self-antigens and production of ACPA. PAD4 is one of the risk factors of RA identified by Genome Wide Association Study (GWAS). Single nucleotide polymorphisms (SNPs) of PAD4 is responsible for increased expression of PADI4, and deletion of Pad4 in mice ameliorated collagen-induced or anti-G6PI-induced arthritis. PAD4 mediates autoantibody production and inflammatory arthritis downstream of TNFα. However, molecular mechanisms for expression of PADI4 before the clinical onset of RA are poorly understood.

IL-6 is a multifunctional cytokine that regulates the development and functions of neuronal, haematological, inflammatory and immunological systems. Dysregulation of IL-6 is involved in various autoimmune diseases including RA. IL-6 receptor complex consists of an IL-6 receptor α chain and a signal transducing subunit gp130. Gp130 is a common receptor subunit for the IL-6 family cytokines, transducing signals through two independent pathways, YXXQ/STAT3 and Y759/SHP2 pathways. Gp130F759 knock-in mice have a mutation of tyrosine to phenylalanine at the 759th amino acid residue, and a defect in the negative regulatory pathway by SOCS3. In turn, this leads to prolonged activation of STAT3 by physiological ligands. In this regard, one amino acid mutation in a cytokine receptor can cause an autoimmune disease. The development of arthritis in gp130F759 is Rag2-dependent, but the Y759F mutation in non-haematopoietic cells is sufficient. Several pathophysiological mechanisms have been reported. Homeostatic proliferation of CD4+ T cells driven by IL-6/STAT3-dependent IL-7 production by non-haematopoietic cells is sufficient. IL-6-dependent acceleration of arthritis by transgene of HTLV-1 pX, triggering effects of polyclonal, antigen-non-specific Th17 accumulated in the joints, and a positive feedback loop of IL-6/IL-17A in chronic inflammation. From these studies, a four-step model was proposed that explains the mechanism for tissue specific autoimmune diseases by antigen-non-specific activation of the IL-6 amplifier. This intriguing model is based on the experiments utilising artificial manipulations, such as neonatal thymectomy, puncture or injection of chemokine to the joints, and over-expression of cytokines by plasmid injection. Arthritis of gp130F759 is characterised by late onset around 8 months old, equivalent to the predilection age of human RA, and gradually progresses. Therefore, we have exploited this model to learn that the IL-6-PAD4 axis is operative among early RA related changes.

MATERIALS AND METHODS

Mice
All mice in C57BL/6 background were maintained under specific pathogen free (SPF) condition in the animal facility in Kawasaki Medical School Central Research Institute. Gp130F759 is used as a notation for homotypic knock-in mice gp130F759/F759. In this paper, Gp130F759 was crossed with Il6 knockout mouse (Il6KO) to make double mutant Il6KO/gp130F759. The animal protocol for this experiment was approved by the Animal Care and Use Committee of Kawasaki Medical School.

Scoring of arthritis
The score system for arthritis from 0 to 4 points/a limb is based on the degree of restriction of joint flexibility (0, no restriction; 1, slight; 2, moderate; 3, severe), and +1 point for redness or swelling for a joint of each fore-limb and hind-limb (online supplementary figure 1). Two researchers who are not informed about genotype scored independently.

Cytokine measurement
Concentration of serum cytokines from gp130F759 (n=12) and wild type (WT) (n=8) were measured using Bio-Plex (Bio-rad, Hercules, California, USA). IL-6 and TNFα in culture supernatant of fibroblast-like synoviocytes were measured with ELISA kits for these cytokines (BioLegend, San Diego, California, USA) following manufacturers instruction.

Neutralising antibody for IL-6
Rat anti-IL-6 monoclonal antibodies (mAb) (IgG1; clone #MP5-20F3) and isotype matched control mAb were purchased from BioLegend and Zymed (San Francisco, California, USA), respectively.

Quantitative histology for arthritis
Ankle containing synovium was fixed in 10% neutral buffered formalin, decalcified with 10% formic acid and embedded in paraffin. Sliced tissue was stained with HE. For estimation of fibrosis, sliced tissue was stained with Sirius red F3BA (WALDECK, Münster, Germany). Using Image J software available on the web site, histological changes in preclinical to very early arthritis were quantitatively estimated using criteria as follows (figure 2B): For neovascularisation, the numbers of the transverse section typical for newly formed vasculature (black arrow) in the total region of synovium (light green lined) were counted. Hyperplasia was defined by synovial lining that has four cell-layers or more, and evaluated with a formula (the length of lining with hyperplasia (yellow line)/length of total synovial lining (blue line))×100 (%). For fibrosis, the ratio of fibrosis (fibrosis area positively stained with Sirius red/total area of synovium (light green lined))×100 (%) was calculated by Image J software.

Cell separation
Splenocytes were prepared by teasing the spleen with slide glasses. The synovium of the knee joint was dissected with scalpel, minced and digested with collagenase D (Roche Diagnostics, Mannheim, Germany) for 3 hours at 37°C in CO2 incubator. After washing and passing through mesh, synovial cells were used for flowcytometry. Synovial cells were separated to CD45+ haematopoietic and CD45- non-haematopoietic cells using rat
anti-mouse CD45 mAb (BD Biosciences) and magnetic beads coupled with sheep anti-rat IgG (H+L) (Thermo Fisher Scientific, Waltham, MA). Real-time PCR detected no transcripts for Cd45 in the cDNA prepared from CD45− fractions. Cultured for 3 days in MEMα containing 15% Fetal calf serum (FCS) and penicillin/streptomycin, CD45− fractions showed typical morphology for fibroblasts, indicating that synovial CD45− fractions are compatible with fibroblast-like synoviocytes.

Bone marrow neutrophils were purified with neutrophil separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) using MACS LS column. Purities of neutrophils (CD11b+Gr-1+) confirmed with FACSCanto II were usually more than 98%. Neutrophils were stimulated with various concentrations of human rIL-6 (2–100 ng/mL), E. coli 0111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA), or culture supernatant fluid of CD45− synovial cells for 6 hours. After each separation or incubation, the cells were collected into TRIzol (Thermo Fisher Scientific) and stored at −80°C until use.

Flow cytometry and typing of synovial cells
Splenocytes and synoviocytes were blocked with mixture of supernatant of anti-CD16/32 mAb (2.4G2) and 5% heat inactivated rat serum. Then the cells were stained with two sets of cocktail mAbs labelled with fluorescent dyes. Set 1 consists of FITC-anti-Ly-6G/Ly-6C (Gr-1) (RB6-8C5) and store at −80°C until use.

Figure 1 The clinical and histological changes of arthritis in gp130F759. (A) Incidence and severity of clinical arthritis of gp130F759 (n=13; seven of male and six of female) is shown. The severity score for each mouse, the sum of scores for four limbs, was determined as described in Methods section and online supplementary figure 1. Onset of clinical arthritis was certified by the score higher than two. Severity scores are expressed as the mean±SD. (B) Representative arthritic joint sections from the mice with distinct severity scores. Slices of the joints were stained with H&E. (C) Results of Sirius red staining of the slice specimens obtained from the same mice used in (B) are indicated. WT, wild type.
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Figure 2  The earliest pathological changes of arthritis in gp130F759 develop at 5 months of age. (A) Sera were collected from WT (n=5) and gp130F759 (n=6), and concentrations of cytokines were measured using Bio-Plex. Black bald line shows averages and open circle shows values for individual mice. *p<0.05. (B) Histological changes in the synovium of the hindlimb joint with score 0 from wild type (WT) (n=7) and gp130F759 (n=6) at 5–5.5 months old were estimated by degrees of neovascularisation (Nv), hyperplasia (Hp) and fibrosis (Fb) as described in Materials and methods section. The average and individual scores are indicated as horizontal bars and circles, respectively. *p<0.05. (C) Haematopoietic cell numbers in the synovium obtained by collagenase treatment and the spleen from WT (open bars) and gp130F759 (black bars) were analysed by flow cytometry. (D) Titres of CRP, RF-IgM, anti-circular citrullinated peptide (CCP)-IgM and anti-CCP-IgG antibodies in the sera of WT (open bars) and gp130F759 (black bars) were measured from 4 to 12 months old. *p<0.05. CRP, C reactive protein; Fb, fibrosis; Hp, hyperplasia; Nv, neovascularisation.

donkey anti-rabbit IgG (LI-COR, Lincoln, Nebraska, USA) with Odyssey CLx (LI-COR). Anti-pSTAT3 antibody was stripped with Western BLoT Stripping Buffer (TAKARA Bio, Shiga, Japan), and then the membrane was reprobed with rabbit anti-STAT3 antibody (Cell Signaling Technology).

Immunohistology
Ankle sections were made by Kawamoto’s film method using cryofilm type 2 (Section-Lab Co, Hiroshima, Japan). The sections were fixed with cold-acetone, blocked with Phosphate buffered saline (PBS) containing 5% donkey serum, 2% FCS and 0.09% NaN₃, and then incubated with rabbit anti-mouse PAD4 or anti-pSTAT3 with Can Get Signal immunostain buffer A (TOYOBO) for 2 hours at room temperature. After washed, secondary antibodies Cy3-labelled F(ab’)₂ donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) with Hoechst 33342 were incubated for 30 min at room temperature. For studying colocalisation, sections were first stained with rabbit anti-PAD4 antibody and Cy3-labelled F(ab’)₂ donkey anti-rabbit IgG (H+L). The sections were blocked with 5% rabbit serum, washed and then stained with biotinylated rabbit anti-pSTAT3 (Tyr705) monoclonal antibody (Cell Signaling Technology) and DyLight488-streptavidin (Vector Laboratories, Burlingame, California, USA). Sections were
mounted with VECTASHIELD (Vector Laboratories), scanned with LSM700 confocal microscope (ZEISS, Jena, Germany) and analysed with Zen (ZEISS).

For preparation of cytospin slides, synovial cells were centrifuged onto slide glass with Shandon Cytospin III (Thermo Fisher Scientific) and air-dried immediately.

Statistics
Serum concentrations of cytokines, autoantibodies and CRP, cell numbers, histological and biochemical quantification, and quantity of gene expression, were compared by the Mann-Whitney U test. A part of data was compared by the Kruskal-Wallis test and Dunn’s method was used as post-hoc testing. Data were analysed using GraphPad Prism8 (GraphPad Software Inc., San-Diego, CA).

RESULTS
Earliest pathological changes in gp130F759 mice develop at 5 months old
We re-evaluated the clinical course of arthritis in gp130F759 under SPF conditions using a scoring system described in Methods section and online supplementary figure 1. As reported previously,17 obvious restriction of joint flexibility appeared at 8 months old. This gradually increased in degree, leading to anklylosis. The incidence of clinical arthritis was 50% at 11 months old and reached 100% by 12 months old (figure 1A). There were no sex differences in the age of onset, progress and incidence of arthritis.

First, we examined the relationships between clinical scores and histological changes of ankle joint specimens stained with HE and Sirius red (figure 1B and 1C). Joint specimens in a group with sum of the scores 3 showed significant changes such as thickening of the synovial lining, and replacement of adipocytes by fibroblasts, and neovascularisation. At score 5, cellular infiltration increased. In mice with scores of 0, there were apparently no changes in gp130F759 compared with WT. These data indicate that our scoring system for clinical arthritis reflects histological changes of the joints.

To clarify the earliest changes in joints developing arthritis, we focused on the age around 5 months old, the earliest pathological changes in gp130F759 mice develop at 5 months old (figure 1D). Since expression of Padi4 gene was increased in joints of gp130F759 mice around 5 months old in more detail.

Padi4 gene expression was increased in the joints of gp130F759
Since serum anti-CCP antibody increased, we examined the expression of Padi family genes by real-time PCR. Expression levels of Padi4, but not Padi1, 2, 3 and 6, were significantly higher in the joints of gp130F759 than those of WT (figure 3A, online supplementary figure 4). Furthermore, expression of Il6 and Osm genes also increased in the joints (figure 3B). This strongly suggests that Padi4 gene expression is induced in the process of local inflammation mediated by neutrophils, which produce OSM. Among the chemokines known to attract neutrophils, expression of Cxcl1, Cxcl2 and Cxcl5 did not increase but that of Cxcl7 significantly increased by 54 times (figure 3B).

Since expression of Padi4 gene was increased in joints of gp130F759, we examined PAD4 protein levels and activation status of STAT3 by western blot analysis. As shown in figure 4A, increases of PAD4 protein and tyrosine-phosphorylation of STAT3 in gp130F759 were clearly demonstrated, suggesting the possible relationship between the local increase of Padi4 expression and abnormally enhanced activation of STAT3 in the joints of gp130F759. To confirm the spatial correlation of STAT3 activation and PAD4 production in the joint microenvironments, immunohistochemical analyses were performed using frozen sections of the joints of gp130F759 at 5 months old. Increased reactivities of anti-PAD4 antibody as well as those of anti-phospho-STAT3 antibody were detectably increased in gp130F759 (figure 4B). Furthermore, the reactivities of anti-phospho-STAT3 antibody, some of which showed nuclear localisation (inset), were increased in gp130F759.

These data clearly demonstrate that aberrant STAT3 activation and PAD4 production take place in the preclinical stage of arthritis in gp130F759. Importantly, very few cells, with frequency approximately two in 340 cells, showed colocalisation of reactivities of anti-phospho-STAT3 antibody and anti-PAD4 antibody (figure 4B, right inset), indicating that a relationship between gp130 signal and PAD4 production is observable in a single cell level.

Then, we investigated cells expressing Padi4 in the joints. In the synovia of gp130F759, Padi4 gene was highly expressed selectively by haematopoietic cells (figure 4C). Interestingly, increased expression of Il6 gene was observed in non-haematopoietic synovial cells in gp130F759. Actually, ELISA revealed increased production of IL-6 in culture supernatant fluid (CSF) of
CD45− synovial cells from gp130F759 compared with that of WT (figure 4D). Next, to characterise the haematopoietic cells expressing PAD4, synovial cells liberated with collagenase treatment were cytopun and stained with anti-PAD4 antibody. Characteristic nuclear morphology of PAD4+ cells indicated that they are neutrophil lineage (figure 4E photo and inset). Numbers of PAD4+ cells were higher in gp130F759 than WT mice (figure 4E graph).

To examine whether the increased expression of Padi4 depends on IL-6, neutrophils enriched from the bone marrow were stimulated with IL-6 (online supplementary figure 5). The expression levels of Padi4 in neutrophils from gp130F759 and WT similarly increased by IL-6 stimulation.

To confirm the interaction between non-haematopoietic synovial cells and neutrophils, we tested whether the CSF from non-haematopoietic synovial cells has activity to induce Padi4 expression in neutrophils. Stimulation with CSF of CD45− synovial cells from gp130F759 induced Padi4 gene expression in WT neutrophils (figure 4F). This Padi4-inducing activity was proved to be mediated mainly by IL-6, because preincubation with anti-IL-6 neutralising antibody but not control antibody inhibited Padi4 gene induction to the basal level. To confirm operation of IL-6 signal in a single cell level, WT neutrophils were incubated with CSF of CD45− synovial cells from gp130F759 for 30 min, cytopun, and stained with rabbit anti-phospho-STAT3 antibody with Cy3 labelled second antibody. Photos in figure 4G clearly indicate that the CSF strongly induced phosphorylation of STAT3, which is inhibited by pretreatment of CSF with anti-IL-6 neutralising antibody but not control IgG1. Thus, from these data, we conclude that Padi4 expression was induced in neutrophils by IL-6 produced from non-haematopoietic synovial cells in the joint microenvironment of gp130F759.

**Operation of the IL-6-PAD4 axis in preclinical arthritis**

To clarify the causative, in vivo roles for IL-6 in the increases of PAD4 and anti-CCP antibody, IL-6 was deleted by crossing gp130F759 with Il6KO. Complete deletion of Il6 gene in gp130F759 resulted in normalisation of splenomegaly (figure 5A,C), and serum levels of RF and anti-CCP antibodies (figure 5B). Importantly, PAD4 production (figure 5D) and synovial cell numbers (figure 5E) returned to almost normal levels. These data indicate that Padi4 induction in the synovium is IL-6 dependent and that operation of the IL-6-PAD4 axis in
Figure 4  PAD4+ cells in the synovium of gp130F759 are neutrophils. (A) Amounts of PAD4 protein and STAT3 phosphorylation in the wrist joints from wild type (WT) and gp130F759. Lysates of the joints from three mice of each genotype were pooled and subjected to western blotting. (B) Frozen sections of the synovium from WT and gp130F759 were stained with anti-PAD4 and pSTAT3 antibodies and Cy3-labelled F(ab')2, donkey anti-rabbit IgG (H+L). Representative pictures from four independent experiments are shown. Nuclei were stained with Hoechst 33342. Colocalisation was examined with anti-PAD4 and biotinylated pSTAT3 antibodies, which were visualised with Cy3-labelled anti-Rabbit IgG and Alexa488-streptavidin, respectively. A PAD4 producing cell whose STAT3 is phosphorylated (inset). The bars indicate 20 µm. (C) Gene expression levels of Padi4 and Il6 in haematopoietic (CD45+) or non-haematopoietic (CD45−) synovial cells which were separated with rat anti-mouse CD45 antibody and sheep anti-rat IgG magnetic beads. Summarised data from three independent experiments are shown. (D) IL-6 concentration in supernatant of primary culture of CD45− synovial cells from WT and gp130F759 at 5 months old (n=7). *p<0.05. (E) Morphology of synovial cells producing PAD4. The photos of representative cytospin specimens from WT and gp130F759 are shown. Synovial cells cytospun onto the slide glass were incubated with anti-PAD4 antibody (red) and Hoechst 33342 for nuclear staining (blue). In the graph, black bar shows average and the open circle shows individual value for each mouse. (F) IL-6 produced by CD45− synovial cells induced Padi4 expression in neutrophils. WT bone marrow neutrophils were stimulated for 6 hours with culture supernatant fluid (CSF) from gp130F759 pretreated with anti-IL-6 antibody or control IgG1. Then RNA from the neutrophils were prepared and transcription of Padi4 was estimated by real-time PCR using specific primers and SYBR green. Relative expression levels compared with Actb are shown. (G) IL-6 in the CSF from gp130F759 induced activation of STAT3 in neutrophils. WT bone marrow neutrophils were stimulated for 30 min with CSF from gp130F759 pretreated with anti-IL-6 antibody or control IgG1. The neutrophils were cyto-spun, air-dried and stained with anti-pSTAT3 antibody and Cy3-labelled donkey anti-rabbit IgG antibody. Pictures were taken with LSM700 confocal microscope. The bars indicate 10 µm.
vivo at an early preclinical phase of RA-like arthritis in gp130F759 (figure 6).

**DISCUSSION**

In this study, we focused on the natural course of RA-like arthritis in gp130F759, and especially on events during the preclinical phase. To understand kinetics of systemic immune or inflammatory responses, we first examined a set of serum cytokines with Bio-Plex. This revealed increases of multiple cytokines at 5 months old, much before the recognition of definitive, clinical arthritis at around 8 months old. In most of the cytokines, levels of increases were less than twofold, and not statistically significant. This is possibly because the initial age and progress of preclinical changes in each mouse are variable in the spontaneous arthritis in gp130F759, which is contrasted with other arthritis models induced by immunisation with type II collagen or antigen.

Since this time point, 5 months old, coincides with appearance of very faint resistance of joint flexibility much less than point 1 of the arthritis score, we extended the study around this age.

Increases of CRP, anti-CCP antibody and RF in the sera were detected at 5 months old, suggesting that as far as the laboratory data, gp130F759 around 5 months old is equivalent to fulfil the criteria of RA. Flow cytometrical analyses revealed increases of the haematopoietic cells competent in innate immunity. Although the clinical signs of arthritis were not obvious at 5 months old, quantitative histopathological analyses provided evidence for synovial hyperplasia and neovascularisation, the latter is also characteristic of early RA. Thus, it can be said that gp130F759 at 5 months old is a model for the preclinical to early phase of RA when minute pathological changes begin. Gene expression analyses revealed that at this time point, increases of *Mmp3* and *Il6* started in the joint, indicating that local inflammatory responses had already begun. Importantly, the expression of *Padi4* gene among members of *Padi* gene family was increased selectively in the joint. Increased production of PAD4 protein with increase of STAT3 phosphorylation was confirmed by biochemical as well as immunohistological measurements. The technique for magnetic cell separation revealed the role-sharing by non-haematopoietic cells expressing *Il6* and haematopoietic cells expressing *Padi4* in the synovial microenvironment. Cytological analyses revealed that PAD4 producing cells are neutrophils and stimulation of IL-6 induced *Padi4* gene expression in neutrophils. All of these data suggest that the IL-6-PAD4 axis participates in the preclinical phase of RA-like arthritis in gp130F759. We have verified the operation of the IL-6-PAD4 axis by four facts; increased production of IL-6 in CSF of CD45− synovial cells from gp130F759, induction of STAT3 phosphorylation and *Padi4* transcription in WT neutrophils stimulated with the CSF, and both inductions were inhibited by anti-IL-6 neutralising antibody.

In the promoter region of *Padi4*, presence of conserved binding sites for NF-κB, TATA-ETS-like, AP-1, Krüppel-like factor-6 (KLF-6) and SP1 are reported. Lack of typical STAT3 binding elements suggests that STAT3 indirectly

**Figure 5** IL-6-dependent changes in preclinical phase of arthritis in gp130F759. Multiple parameters of WT, *Il6KO* (abbreviated as KO), gp130F759 (*F759*) and *Il6KO/gp130F759* (*KO/F759*) at 5–6 months old (*n=4 each*) were examined. As systemic changes, (A) weight of the spleen, (B) serum titres of CRP, RF-IgM, and anti-CCP IgM are indicated at top. As a local changes in the joints, (C) *Il6* gene expression by real time PCR, (D) the relative amounts of PAD4 protein by western blot (the average ratio of band densities (PAD4/β-actin) of WT=1.0) and (E) synovial cell numbers are indicated at bottom. Statistical significance was estimated by the Kruskal-Wallis test and Dunn’s method. ND; not detectable. CCP, circular citrullinated peptide; CRP, C reactive protein.
Figure 6 The IL-6-PAD4 axis in the synovium of preclinical phase of rheumatoid arthritis (RA)-like arthritis in gp130F759. anti-citrullinated peptide autoantibodies (ACPAs) are detectable much earlier than onset of RA. Presence of ACPA indicates that tolerant self-antigens have been modified by citrullination and that the modified self-antigens become recognised as foreign by adaptive immunity. Thus, ACPA and anti-circular citrullinated peptide (CCP) antibody, which is adapted for clinical examination to detect ACPA as a whole, are useful biomarkers to predict development of RA. In other words, anti-CCP antibody can be used to define the preclinical phase of RA by the criteria: anti-CCP antibody positive and lack of clinical symptoms of arthritis. Since the natural course of RA is now appreciated as (1) preclinical RA, (2) early ‘clinically evident’ RA and (3) chronic ‘established’ RA,7 the molecular events and causal relationships between them in preclinical RA are gathering great attention. But the information of synovial microenvironment in preclinical RA is hardly obtainable from human. Using the mutant gp130 knock-in mice, gp130F759, we report here that gp130F759 around 5 months old could be an ideal model for the pathological phases spanning preclinical to early clinically evident RA. During this time window, the IL-6-PAD4 axis is generated by interaction of non-haematopoietic cells, most likely fibroblast-like synoviocytes, and neutrophils, representative of innate immunity, attracted by CXCL7. ERK, Extracellular Signal-regulated Kinase; MAPK, Mitogen-activated Protein Kinase; M.O.; months old.
the elevation of anti-CCP antibody was detected, systemic, low-level increases of typical inflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-17 already existed. Second, cytokines that are elevated in this phase showed no deviation to Th1 or Th2, that is, both Th1 and Th2 types were elevated. Third, an immune-suppressive cytokine, IL-10 also increased. None of the changes were specific to this animal model. On the contrary, the pre-clinical phase of RA. Especially, TNFα animal model. On the contrary, the pre-clinical phase also increased. None of the changes were specific to this animal model. As a STAT3-activating cytokine, IL-10 may have a unique role in early RA and dysregulation of IL-10 in RA synovial macrophages has been reported.

Another similarity to RA is induction of a chemokine CXCL7 attracting neutrophils, which was reported to be produced by synovial macrophages in early RA. Interestingly, our novel, infection-induced arthritis model using gp130F759 at 3 months old (unpublished observation by Yahagi et al.) revealed that systemic infection of Mycoplasma fermentans induced expression of Cxcl7 and accumulation of cells dominated by neutrophil in the synovium. This suggests intriguing possibility that aberrant cytokine signals and triggering by microorganisms utilise common pathway to initiate arthritis by induction of Cxcl7 and accumulation of neutrophils in gp130F759.

From all the data obtained in this paper, we propose a model for molecular events in the synovial microenvironments of pre-clinical phase of RA. Importantly, the IL-6-PAD4 axis operates to promote the transition from innate immunity to acquired immunity (figure 6). Thus, gp130F759 is useful to learn the molecular mechanisms for heterogenous pathways leading to a clinical entity RA, which shows variable clinical courses and responses to therapies.

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