Syndecan4 On B Cell Regulates Generation Of Germinal Center In The Development Of Autoimmune Arthritis

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Introduction: Syndecan-4 (Syn4), a transmembrane heparan sulfate proteoglycan, is expressed on several types of cells. The functions of Syn4 are believed to be co-receptors or reservoirs for ECM proteins such as growth factors and chemokines through its heparan sulfate chains. Syn4 is also implicated as a critical mediator for inflammatory responses. Previous study showed that Syn4 was expressed on B cell lineage, suggesting that Syn4 is involved in B cell function (1). Rheumatoid Arthritis (RA) is a complex multisystem disease. Recent clinical study demonstrated that anti-CD20 B cell depletion therapy exerted therapeutic effect for the patients who were resistant for anti-TNF therapy (2). Moreover, some autoantibodies including rheumatoid factors and anti-collagen type II were increased in the serum of RA patients. These phenomena support that B cells possibly play critical roles in the pathogenesis of RA. Therefore, we hypothesized that Syn4 might have an important role for RA progression by regulating B cell function. The purpose of this study is to clarify the role of Syn4 in the development of murine RA model.

Methods: Animals
C57BL/6 mice were obtained from Japan SLC. Syn4 KO mice, backcrossed >10 times into the C57BL/6 background, were obtained from the Center for Animal Resources and Development (Kumamoto, Japan). All animal experiments were conducted in accordance with the guidelines of an institutional committee at Hokkaido University.

Induction of Collagen-Induced Arthritis (CIA)
Mice were intradermally immunized with 100 µg of bovine type II collagen (CII) (Chondrex), emulsified in CFA (Chondrex), three times every three weeks. The arthritic severity in each limb was graded on a 0-4 scale. In brief: 0 = no swelling, 1 = slight erythema, 2 = slight swelling, 3 = severe swelling and 4 = maximum swelling and deformity of limbs.

Flow cytometric analysis
The cell suspension from inguinal draining lymph node (iLN) was incubated with various combinations of antibodies as follows: (a) For follicular B cell staining; FITC-conjugated IgM, pacific blue-conjugated CD19 and APC/Cy7-conjugated CD21/CD35. (b) For germinal center (GC) B cell staining; FITC-conjugated GL7 and APC/Cy7-conjugated B220. (c) For follicular helper T cell staining; FITC-conjugated Gr-1, B220 and CD11b, Per CP/Cy5.5-conjugated CD4, PE/Cy7-conjugated PD-1 and biotin-conjugated CXCR5.

Histology
OCT frozen sections of iLN were stained immunohistochemically as previously described (3). Primary antibodies included biotin-conjugated rat anti-mouse B220 and biotin-conjugated Armenian hamster anti-mouse CD3. For immunofluorescence, primary antibodies included biotin-conjugated rat anti-mouse GL7 to detect GC and rabbit anti-mouse Syn4.

Measurement of Abs against CII
Serum levels of anti-mouse CII-specific antibodies were measured by ELISA seven days after 2nd immunization. Briefly, 96-well plates were coated with bovine CII (5 µg/ml) overnight at 4°C. After blocking with 1% BSA in PBS, diluted serum samples were added and incubated for two hours at room temperature. HRP-conjugated rat anti-mouse IgG antibodies were added at 1:10000 and incubated for one hour at room temperature. Absorbance was measured at 450 nm.

Statistical analysis
Statistical evaluation was performed based on the Student’s t test. For calculation of appearance ratio of arthritic joint, χ2 statistics were derived using the CHIDIST function of Microsoft Excel.

Results: Syn4-deficiency ameliorated the development of Collagen-Induced Arthritis (CIA) To investigate whether Syn4 plays a role in the development of RA, we used CIA model, which is commonly used to analyze both antigen recognition and effector phase of arthritis. In CIA model, Syn4 KO mice showed amelioration of RA symptoms and incidence (onset; WT mice: day 2 vs Syn4 KO mice: day 24, maximal average clinical score; WT mice: 5.5 vs Syn4 KO mice: 3 and incidence; WT mice: 50% vs Syn4 KO mice: 9%) (Fig. 1A), concomitantly with mild bone destruction and cartilage degeneration compared with WT mice (Fig. 1B).

Syn4 was highly expressed on B cells in the draining lymph node (dLN) To seek cellular sources of Syn4 in the dLN, we performed flow cytometric analysis of WT B cells. We found that B cell subsets, including follicular (FO) B cells and germinal center (GC) B cells, highly expressed Syn4 (Fig. 2A). Consistent with flow cytometric analysis, in immunohistochemistry, Syn4 was co-localized within B cell zone within the dLN (Fig. 2B).
Syn4 is a critical regulator for establishment of B cell follicle and germinal center

To confirm whether Syn4 is related to autoantibody production, we examined serum level of CII-specific antibodies. Syn4 KO mice showed significantly reduced levels of total IgG, IgG2a and IgG2b compared with WT mice after immunization (Fig. 3). Therefore we focused on the formation of GC in the dLN. In immunohistochemistry, primary follicles and GCs were well formed in WT mice after immunization (Fig. 4A upper panels). Interestingly, we detected that Syn4 KO mice failed to generate primary follicle and GC following immunization (Fig. 4A lower panels). To confirm these results, flow cytometric analysis showed that the numbers of B cell subsets (FO B cells and GC B cells) of Syn4 KO mice was significantly reduced, although the numbers of follicular helper T cells (Tfh) were comparable between both mice (Fig. 4B).

Discussion: In the present study, we exhibited that Syn4 was mainly expressed on B cell in the dLN and Syn4-deficiency induced a defect of GC formation and antibody production, resulting in amelioration of CIA progression. It is well known that GC critically contributes to antibody production. Recent studies also indicated that the interactions between Tfh and B cells are critical for GC formation and antibody production (4). These facts support that Syn4 on B cell regulates GC formation due to the intimately relation to T-B cell interaction. In addition, recent report indicated that the amount of serum CII-specific IgG is correlated to the clinical severity of CIA (5). Our data displayed that CII-specific antibody production (total IgG, IgG2a and IgG2b) in Syn4 KO mice was significantly reduced compared with that in WT mice. Therefore it was highly possible that attenuated clinical severity in Syn4 KO mice was due to the defect of GC formation and antibody production. In summary, our study revealed that Syn4 on B cells promotes the development of RA, by enhancing GC formation and autoantibody production.

Significance: Syn4 modulates the development of murine RA models through enhancing the generation of GC formation and antibody production.

Acknowledgments: nothing

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Figure 1  Syn4-deficiency ameliorates the development of both T and B cell-dependent arthritis. Comparison of clinical severity between WT and Syn4 KO mice in CIA model. (A) Disease score and incidence of arthritic mice in CIA model. Mice were immunized with bovine CII (100 μg), emulsified in CFA on day -21, day 0 and day 21. (This study was designated the day of 2nd immunization as day 0.) n= 20, 22, respectively. Significant differences between means are indicated: *, p < 0.05. (B) Histological evaluation of normal joints of WT mice and arthritic joints of both WT and Syn4 KO mice on day 44. Sections were stained with H&E or safranin-O.
Figure 2  B cells highly express Syn4. Analysis of Syn4 expression on B cell subsets in the dLN. (A) Seven days after 2nd immunization with CII, iLN cells of WT mice were stained for Syn4 expression on B cell subsets. (B) Representative histological staining to detect Syn4 expression in naïve iLN. Frozen sections were stained with anti-Syn4, anti-B220 Abs for B cell zone and anti-CD3 Abs for T cell zone.
Figure 3. Syn4-deficiency reduces production of CII-specific IgGs. Analysis of the effect of Syn4-deficiency on antibody production in the development of CIA. Serum levels of CII-specific IgGs were measured by ELISA seven days after 2nd immunization. Data were presented as the means ± SEM. Data are representative of three independent experiments with five mice per group. Significant differences between means are indicated: *, p < 0.05; **, p < 0.01.
Figure 4 Syn4 is critical for organization of B cell follicle and GC. Analysis of the impact of Syn4 deficiency on generation of GC in the dLN.

(A) Representative histological staining of the dLN in WT and Syn4 KO mice seven days after 3rd immunization with CII.

Arrowheads indicate GC.

(B) Seven days after 2nd immunization, number of B cell subsets and Th in the dLN was analyzed by Flow Cytometry. Data were presented as the means ± SEM. Data are representative of two independent experiments with five mice per group. Significant differences between means are indicated: *, p < 0.05; **, p < 0.01.