

Expression of Toll-like Receptors and Their Signaling Pathways in Rheumatoid Synovitis

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ABSTRACT. Objective. Toll-like receptors (TLR) recognizing endogenous and exogenous danger signals could play a role in rheumatoid arthritis (RA). Our aim was to describe the presence, localization, and extent of expression of TLR and their adapters.

Methods. TLR 1, 2, 3, 4, 5, 6, and 9 receptors, and myeloid differentiation primary response protein 88, Toll/interleukin receptor (TIR) domain-containing adapter protein MyD88 adapter-like, and TIR domain-containing adapter-inducing interferon/TIR-containing adapter molecule-1 adapters were analyzed in RA (n = 10) and osteoarthritis (OA; n = 5) samples using real-time polymerase chain reaction (PCR). Their colocalization with cellular markers CD68, CD15, CD3, CD4, CD8, CD20, dendritic cell lysosomal-associated membrane protein (DC-LAMP), CD123, and 5B5 was analyzed in double immunofluorescence staining.

Results. In RA, β -actin standardized messenger RNA of TLR 2, 3, and 9 ($p < 0.001$) were particularly high. TLR 5 and 6 were also elevated ($p < 0.05$), but TLR 1 and 4 and adapters did not differ between RA and OA. In double-staining, TLR and adapters were strongly labeled in myeloid and plasmacytoid dendritic cells (DC), moderately in CD68+ type A lining cells/macrophages, and weakly to moderately in 5B5+ type B lining cells/fibroblasts. CD3+/CD4+ and CD3+/CD8+ T cells and CD20+ B cells in perivascular areas and in lymphoid follicles were moderately TLR- and weakly adapter-positive. In OA, TLR and adapters were weakly immunolabeled in vascular, lining, and inflammatory cells.

Conclusion. RA synovium showed abundant expression of TLR. RA synovitis tissue seems to be responsive to TLR ligands. DC, type A cells/macrophages, and type B cells/fibroblasts are, in that order from highest to lowest, equipped with TLR, suggesting a hierarchical responsiveness. In RA, danger-associated molecular patterns to TLR interactions may particularly drive DC to autoinflammatory and autoimmune cascades/synovitis. (J Rheumatol First Release Feb 15 2011; doi:10.3899/jrheum.100732)

Key Indexing Terms:

RHEUMATOID ARTHRITIS
SYNOVIAL MEMBRANE

TOLL-LIKE RECEPTORS
INNATE IMMUNITY

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Supported by the Grant-in-Aid for Scientific Research, the Ministry of Science and Culture (C2-17591546, C-21591929), Yamagata Health Support Society for the Promotion of Science 2006-2009, Japan; and the Sigrid Jusélius Foundation, Invalid Foundation, and the HUS evo program, Finland.

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Accepted for publication December 8, 2010.

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects multiple small joints simultaneously, causing a chronic and destructive synovitis.

In ordinary host defense, neutrophils and macrophages mediate innate host responses by recognizing microorganisms, followed by their phagocytosis and clearance. Further, macrophages and in particular dendritic cells (DC) can present pathogen antigens to initiate T cell-mediated adaptive immune responses¹, but can also activate autoreactive T cells by abnormal presentation of self-antigens.

Toll receptor was originally identified in *Drosophila melanogaster* as a receptor essential for the establishment of the dorsoventral pattern in developing embryos². In 1996, Lemaitre, *et al*, demonstrated that Toll-mutant flies were highly susceptible to fungal infections³. A mammalian Toll homologue was first reported in 1997 and named Toll-like receptor 4 (TLR 4)⁴. TLR belong to the family of pattern-recognition receptors. They are transmembrane proteins of inflammatory immigrant and resident stromal cells that share leucine-rich extracellular repeats and intracellular Toll/interleukin 1 receptor (TIR) domains. Thus, the innate

immune system recognizes conserved pathogen-associated molecular patterns (PAMP) through TLR. TLR 1, 2, 4, 5, and 6 are located on the cell surface. Bacterial lipoproteins are recognized by TLR 1, 2, and 6, lipopolysaccharides by TLR 4, flagellins by TLR 5, and fungal zymozans by TLR 2 and 6. TLR 3, 7, 8, and 9 are located in the endosomes. Double-stranded viral RNA is recognized by TLR 3, single-stranded RNA by TLR 7 and 8, and unmethylated CpG bacterial DNA by TLR 9^{5,6}. It was later found that TLR also bind endogenous alarmins⁷ such as monosodium urate crystals. Together with PAMP they form the danger signals or danger-associated molecular patterns (DAMP)⁸. DAMP stimulate innate inflammatory responses, but they also provide the danger or second signal to adaptive immune responses. For example, TLR 2 recognizes heat shock protein (Hsp) 60, 70, and gp96. TLR 4 recognizes HspB4 and HspB8, Hsp 60, 70, gp96, fibronectin 7EDA domain and oligosaccharides of hyaluronan, and TLR 9 recognizes chromatin-IgG complexes⁹. TLR engagement triggers intracellular adapter and signaling pathways. Adapter molecules include myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter protein (TIRAP), also known as MyD88-adapter-like, and TIR domain-containing adapter-inducing interferon (IFN)- β (TRIF), also known as TIR-containing adapter molecule (TICAM)-1¹⁰. The initial role of TLR is to function as sensors for danger signals and bind DAMP to initiate innate host responses^{3,4,11}. Ligand-TLR interactions lead to upregulation of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α)¹², IL-1 β ¹³, IL-6^{12,13,14}, and IFN- $\alpha/\beta/\gamma$ ^{12,14,15,16}, CC (or β) chemokines^{14,16,17}, and CXC (or α) chemokines^{14,16}. Ligand-TLR interaction also upregulates costimulatory molecules, such as intercellular adhesion molecule-1¹³, lymphocyte function-associated antigen-1, and lymphocyte function-associated antigen-3¹⁵. Costimulatory molecules are essential for the induction of pathogen-specific adaptive immune responses¹⁸. Thus, TLR link innate host responses to adaptive immunity^{19,20}. Some earlier reports have been published on the tissue distribution of some TLR in RA synovitis^{14,16,21,22}, but information on their cellular localization, levels, and adapter molecules is scanty. It was hypothesized that in RA synovitis, TLR in general and in different cellular subsets participate in pathomechanisms of RA. Therefore, we aimed to assess the eventual presence, cellular distribution, and quantity of some key TLR and their adapters in RA compared to osteoarthritis (OA).

MATERIALS AND METHODS

Patients and samples. Ten tissue samples of RA synovitis were obtained from synovectomy or joint replacement surgery. Eight patients were women and 2 were men, mean age 62.7 years (range 42–78 yrs). All samples were negative in bacterial and fungal cultures. All patients with RA fulfilled the American College of Rheumatology criteria. Ten patients with RA had been treated with the disease-modifying antirheumatic drugs methotrexate, salazosulfapyridine, bucillamine, low-dose glucocorticoids

(mean value 4.0 mg, range 0–7.5), and/or nonsteroidal antiinflammatory drugs. None of the patients had received any biologics. None had been treated with intraarticular corticosteroids within the previous 3 months. The mean value of rheumatoid factor was 104 IU/ml (range 8–350); C-reactive protein (CRP), 3.4 mg/l (range 0.2–11.8); and erythrocyte sedimentation rate (ESR), 52 mm/h (range 7–88) and 89 mm/2 h (range 17–148; Table 1).

As controls, 5 synovial membrane tissue samples were collected from patients with secondary OA caused by acetabular dysplasia who were undergoing primary total hip arthroplasty. Three of the patients were women and 2 were men (mean age 64 yrs, range 51–79; Table 2). All samples were negative in bacterial and fungal cultures. CRP and ESR levels were within the reference range. Half of each sample was snap-frozen in OCT (Lab-Tek Products, Miles Laboratories, Elkhart, IN, USA) for immunostaining and RNA extraction and stored at -80°C . The other half of the sample was fixed in 4% formalin in phosphate-buffered saline at 4°C for 24 h and embedded in paraffin.

All patients provided informed consent and the study protocol was approved by the Ethical Committee of Yamagata University School of Medicine.

Polymerase chain reaction (PCR). Total RNA was isolated from 100 μg frozen tissue with the Isogen kit (Nippongene, Tokyo, Japan). The quantity of total RNA was measured by spectrophotometry at 260 nm. After heating for denaturation at 65°C for 5 min, cDNA was produced by reverse transcription, using the Super ScriptTM III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

For quantitative real-time PCR, enzymatic amplification of the specific cDNA sequences was performed in a Light Cycler using Fast Start DNA Master SYBR Green I containing Fast Start Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The PCR was performed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at $60\text{--}62^{\circ}\text{C}$ for 5 s, and extension at 72°C for 10 s. PCR products were separated by 2% agarose gel electrophoresis.

Quantitative analysis was performed using Light Cycler Software (Roche), which detects real-time fluorescent signals that directly correlate to the concentration of the target cDNA, by comparing with the fluorescence of several dilutions of a standard. All messenger RNA levels were standardized using β -actin, which was diluted into different concentrations (10 pg/ml, 100 pg/ml, 1 ng/ml, and 10 ng/ml). The primers for TLR 1, 2, 3, 4, 5, 6, 9, MyD88, TIRAP/Mal, TRIF/TICAM-1, and β -actin were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA; Table 3).

Histopathological examinations. Six- μm -thick paraffin sections were stained with hematoxylin-eosin for histopathological analysis. Immunohistochemical analysis was performed using 6- μm -thick cryostat sections fixed in acetone for 5 min at 4°C . Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. Sections were treated serially with Block Ace (dilution 1:2; Vector Laboratories, Burlingame, CA, USA) for 1 h. Affinity-purified monoclonal mouse antihuman CD68 IgG1 (Dako Cytomation, Glostrup, Denmark) was used for macrophages, monoclonal mouse antihuman CD15 IgM (Dako) for granulocytes, monoclonal mouse antihuman 5B5 IgG1 (Dako) for fibroblasts, monoclonal mouse antihuman CD3 IgG1 (PS1; Nichirei, Tokyo, Japan) for mature T cells, monoclonal mouse antihuman CD4 IgG1 (Nichirei) for helper T cells, monoclonal mouse antihuman CD8₁ IgG (Nichirei) for cytotoxic T cells, monoclonal mouse antihuman dendritic cell lysosomal-associated membrane protein (DC-LAMP) IgG1 (104.G4; Immunotech, Marseille, France) for mature myeloid dendritic cells (mDC), and monoclonal mouse antihuman CD123 IgG1 (IL-3R, S-12; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for plasmacytoid DC (pDC). Antibodies against TLR and their adapters were all from Santa Cruz Biotechnology: rabbit antihuman TLR 1 IgG, rabbit antihuman TLR 2 IgG, rabbit antihuman TLR 3 IgG, rabbit antihuman TLR 4 IgG, rabbit antihuman TLR 5 IgG, rabbit antihuman TLR 6 IgG, rabbit antihuman TLR 9 IgG, goat antihuman MyD88 IgG, rabbit antihuman TIRAP IgG, and rabbit antihuman TRIF IgG. All were diluted 1:50–1:100 and treated with biotinylated antirabbit or anti-

Table 1. Profile of patients with rheumatoid arthritis.

Case	Age/Sex	Duration, yrs	DMARD	PSL, mg	RF, IU/ml	CRP, mg/dl	ESR, mm/h	ESR, mm/2h
1	70 M	3	MTX	7.5	60	2.1	38	71
2	40 F	4	MTX	5	44	6.9	47	77
3	67 F	30	MTX, BUC	5	350	2.0	88	127
4	59 M	3	SASP	0	53.2	2.74	48	85
5	60 F	20	SASP	5	88	1.5	90	148
6	78 F	14	SASP	0	89	11.75	60	120
7	69 F	13	GST	0	76	2.2	52	85
8	69 F	5	GST	7.5	256	1.0	39	66
9	42 F	16	GST	5	13	3.37	52	89
10	73 F	20	BUC	5	8	0.2	6.8	17
Averages	63	12.8		4.0	103.7	3.38	52.1	88.5

DMARD: disease-modifying antirheumatic drugs; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; MTX: methotrexate; SASP: salazosulfapyridine; BUC: bucillamine; GST: gold sodium isothiomalate; PSL: prednisolone.

Table 2. Profile of patients with osteoarthritis.

Case	Diagnosis	Age/Sex
1	SOA	67 F
2	SOA	56 F
3	SOA	51 M
4	SOA	79 M
5	SOA	67 F
Average		64

SOA: secondary osteoarthritis.

mouse IgG or IgM, as appropriate, and avidin-biotin peroxidase complex (Vector Laboratories), 30 min at room temperature each. The sites of peroxidase binding were visualized with a combination of H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (Wako Junyaku, Osaka, Japan). Sections were counterstained with hematoxylin. Between 2 steps, the sections were washed 3 times in 20 mM Tris-HCl buffered 150 mM NaCl, pH 7.5. The specificity of the staining was tested using normal mouse IgG of the same isotype (Sigma Chemical, St. Louis, MO, USA) or rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) at the same concentration or dilution as and instead of the specific primary antibodies. The average number of positive cells per 1 mm² in 3 random microscopic fields for each section was counted.

For immunofluorescence colocalization studies, Alexa Fluor 488-con-

Table 3. Sequence of oligonucleotide primers used for PCR and expected template size.

Target Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Expected Template Size, base pairs
TLR 1	CAG TGT CTG GTA CAC GCA TGG T	TTT CAA AAA CCG TGT CTG TTA AGA GA	107
TLR 2	GGC CAG CAA ATT ACC TGT GTG	AGG CGG ACA TCC TGA ACC T	69
TLR 3	CCA AGC CTT CAA CGA CTG AT	TTC CAG AGC CGT GCT AAG TT	205
TLR 4	TCC ATA AAA GCC GAA AGG TG	CTG AGC AGG GTC TTC TTC AC	195
TLR 5	TGC CTT GAA GCC TTC AGT TAT G	CCA ACC ACC ACC ATG ATG AG	75
TLR 6	GGA TAG CCA CTG CAA CAT CA	TTG GTT TTC ACG GGT AGG TC	185
TLR 9	GCT AGA CCT GTC CCG CAA TA	ACA CTT GGC TGT GGA TGT TG	196
MyD88	GAG CGT TTC GAT GCC TTC AT	CGG ATC ATC TCC TGC ACA AA	67
TIRAP/Mal	CCA GCC TTT CAC AGG AGA AG	ATA TTC GGG ATC TGG GGA AG	180
TRIF/TICAM-1	TGC CTT GAA GCC TTC AGT TAT G	CCA ACC ACC ACC ATG ATG AG	213
β-actin	TCA CCC ACA CTG TGC CCA TCT ACG A	CAG CGG AAC CGC TCA TTG CCA ATG G	295

PCR: polymerase chain reaction; TLR: Toll-like receptor; MyD88: myeloid differentiation primary response protein 88; TIRAP/Mal: Toll/interleukin receptor domain containing adapter protein MyD88 adapter-like; TRIF/TICAM: TIR domain-containing adapter-inducing interferon/TIR-containing adapter molecule-1.

jugated antirabbit or antimouse IgG or IgM antibodies and Alexa Fluor 546-conjugated anti-goat or antirabbit IgG antibodies (Molecular Probes Inc., Eugene, OR, USA) were applied according to the manufacturer's instructions. As a primary antibody, all these were applied at 4 µg/ml: affinity-purified monoclonal mouse antihuman CD68 IgG1 (Dako), monoclonal mouse antihuman CD15 IgM (Dako), monoclonal mouse antihuman 5B5 IgG1 (Dako), monoclonal mouse antihuman DC-LAMP IgG1 (104.G4; Immunotech), monoclonal mouse antihuman CD123 IgG1 (IL-3R, S-12; Santa Cruz Biotechnology), and the TLR and adapter IgG already mentioned. The images were observed under a fluorescence microscope (DMLB, DC200, DC viewer software, Leica Microsystems, Wetzlar, Germany).

Statistical analysis. The mean and SEM of each standardized mRNA was calculated. Kruskal-Wallis one-way ANOVA was used for the detection of differences between groups. If statistical significance was found, the Mann-Whitney U test was applied to determine which TLR mRNA expression level was significantly different between RA and OA samples. A value of $p < 0.05$ was regarded as statistically significant. Pearson's correlation coefficient (γ) was calculated for each standard curve to evaluate proper PCR performance with external standard.

RESULTS

Quantitative real-time PCR. Melting curve analysis showed that the peaks in curves represented the melting temperatures, with all TLR and β -actin showing only 1 peak, confirming accurate PCR performance without nonspecific products, except for TRIF/TICAM-1 in RA and TLR 6 in OA. Pearson's correlation coefficients (r) of the curves were < 0.995 , further confirming accurate PCR amplification. Quantification curves show the fluorescence data acquired once per cycle.

TLR 2, 3, and 9 were much higher in RA than in OA ($p < 0.001$ for all). TLR 5 and 6 followed the same pattern ($p < 0.05$ for both). In contrast, TLR 1 and 4 and none of the adapters differed between RA and OA (Table 4).

Histopathological examinations. In actively inflamed RA synovitis, CD68+ macrophage-like type A lining cells and macrophage infiltrates of the subsynovial stroma were notable (Figure 1A, 1B). Also abundant were 5B5+ fibroblast-like type B lining cells and fibroblasts of the sublining stroma (Figure 1C). CD15+ polymorphonuclear neutrophils

Target/ β -actin	RA	OA	p
TLR 1	0.73 \pm 1.17	0.045 \pm 0.028	0.54
TLR 2	1.87 \pm 3.21	0.015 \pm 0.0090	0.0007*
TLR 3	3.77 \pm 6.53	0.011 \pm 0.0078	0.0007*
TLR 4	0.55 \pm 0.96	0.045 \pm 0.038	0.74
TLR 5	2.83 \pm 4.64	0.017 \pm 0.0090	0.035*
TLR 6	3.15 \pm 6.08	0 \pm 0	0.035*
TLR 9	4.98 \pm 9.89	0.021 \pm 0.012	0.0001*
MyD88	0.26 \pm 0.37	0.027 \pm 0.018	0.33
TIRAP/Mal	1.77 \pm 3.12	0.074 \pm 0.045	0.91
TRIF/TICAM-1	0.083 \pm 0.083	0.028 \pm 0.024	0.90

* $p < 0.0001$. RA: rheumatoid arthritis; OA: osteoarthritis; TLR: Toll-like receptor; MyD88: myeloid differentiation primary response protein 88; TIRAP/Mal: Toll/interleukin receptor domain-containing adapter protein MyD88 adapter-like; TRIF/TICAM: TIR domain-containing adapter-inducing interferon/TIR-containing adapter molecule-1.

were occasionally observed in intravascular and perivascular areas. CD3+/CD4+ and CD3+/CD8+ T cells and CD20+ B cells were observed in the perivenular infiltrates and small and large lymphoid aggregates (Figure 1D).

For DC, mDC were frequently and pDC somewhat more sparsely observed in RA synovitis. Mature mDC, detected using DC-LAMP, were abundant and mainly observed in large and small lymphoid aggregates and around lymphoid follicles, but scarce in the sublining stroma and perivenular areas (Figure 1E). CD123+ IL-3R α immunoreactive pDC were scattered in perivenular infiltrates, small and large lymphoid aggregates, and around lymphoid follicles (Figure 1F). The negative control staining with nonimmune rabbit IgG proved the specificity of the staining.

All tissue samples from RA showed immunoreactivity to all TLR and their adapters examined (Figure 2A-F), except for TRIF/TICAM-1 (data not shown). Strong immunoreactivity of TLR studied was found in DC-like cells (shown for TLR 9 in Figure 2D). Otherwise, TLR 1, 2, 3, 4, 5, 6, and 9 were found in both the lining and sublining stroma, showing weak to moderate immunoreactivity (Figure 2A-F). In contrast, all OA samples showed weak immunoreactivity for TLR (TLR 1, 2, 3, 4, 5, 6, and 9) and their adapters (MyD88, TIRAP/Mal, and TRIF/TICAM-1) in endothelial and occasional lining and inflammatory cells (data not shown). Immunoreactive TLR 6 was not detected in OA samples. The negative control staining with nonimmune rabbit IgG proved the specificity of the staining.

Immunoreactivity disclosed cellular colocalization of strongly labeled TLR in DC-LAMP+ mDC and CD123+ pDC (green color, Figure 3A, 3B). TLR 1, 2, 3, 4, 5, 6, and 9 (red color, Figure 3A, 3B) were observed in the central cytoplasm of mDC, which were also strongly labeled for DC-LAMP (green color), with colocalization being detectable. In addition, colocalization of TLR 2, 4, and 9 was observed in CD123+ pDC identified in perivenular areas, large and small lymphoid aggregates, and around lymphoid follicles (data not shown). Similarly, moderately labeled TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were seen in CD68+ type A lining cells and infiltrating macrophages (Figure 3C-E), also showing MyD88, TIRAP/Mal, and TRIF/TICAM-1 (Figure 3E). TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were also found in 5B5+ type B lining cells and fibroblasts of the sublining stroma, with weak and/or at most moderate reactivity (Figure 3F, 3G). Thus, in RA, the strongest labeling of both TLR and their adapters was seen in DC, followed by synovial type A lining cells and infiltrating macrophages, and then by synovial type B lining cells and stromal fibroblasts, in that order. In addition, weakly to moderately immunoreactive TLR 1, 2, 3, 4, 5, 6, and 9 were seen in vascular endothelial cells. Moreover, colocalizations of TLR 1, 2, 3, 4, 5, 6, and 9 were to some extent found in CD3+/CD4+ and CD3+/CD8+ T cells and CD20+ B cells.

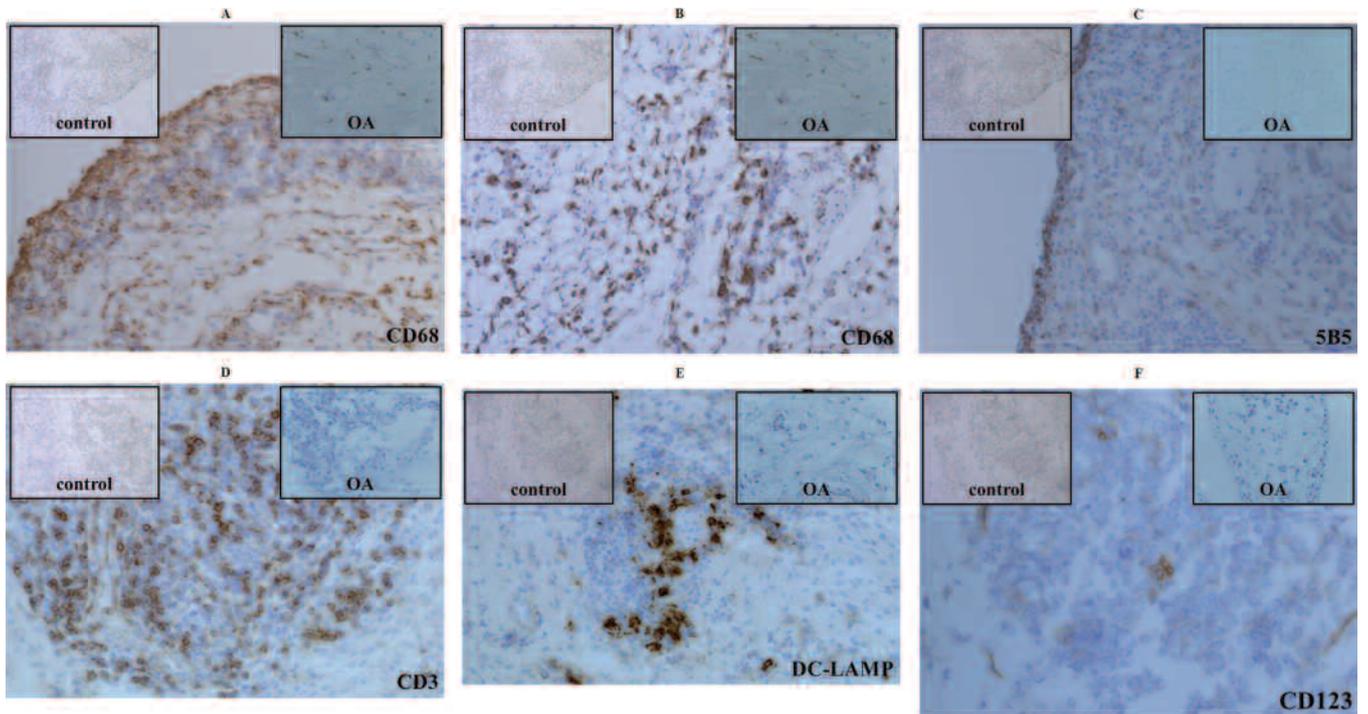


Figure 1. Immunohistochemistry of actively inflamed RA synovial tissues. A. Macrophage-like type A cells were seen in the lining layers with immunoreactivity of CD68. B. CD68+ macrophages of the sublining stroma were observed. C. 5D5+ fibroblast-like type B cells of the lining and fibroblasts in the sublining stroma. D. CD3, CD4, and CD8+ T cells and CD20+ B cells were observed. E. dendritic cell lysosomal-associated membrane protein (DC-LAMP) cells. F. CD123+ cells. Myeloid dendritic cells (DC), detected by DC-LAMP, were mainly observed in large and small lymphoid aggregations, but were scarce in the lining layer and perivascular area. Plasmacytoid DC, detected by CD123, were scattered in large and small lymphoid aggregations and the perivascular area. Upper right corner of each image shows the specific staining of osteoarthritis (OA) synovium. Upper left corner of each image shows the negative control staining with nonimmune rabbit IgG that proved the specificity of the staining.

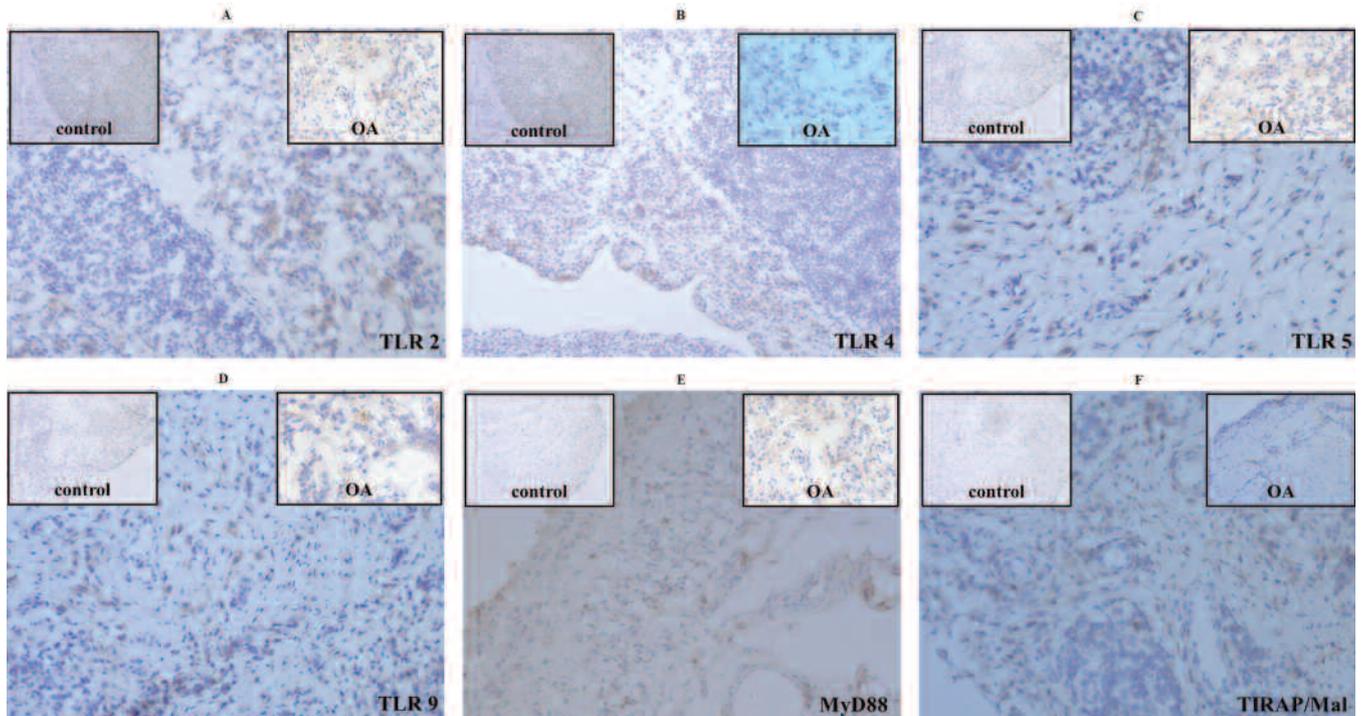


Figure 2. Immunohistochemistry of Toll-like receptors (TLR) and their adapter molecules in RA synovial tissues. A-D. TLR 2, 4, 5, and 9 were found in both the lining and sublining stroma with weak to moderate immunoreactivity. Marked immunoreactivity of TLR 9 was found in several dendritic-like cells (D). E, F. TLR adapter molecules were observed in lining and sublining stroma with weak immunoreactivity. Upper left corner of each image shows that the negative control staining with nonimmune rabbit IgG proved the specificity of the staining. Upper right corner of each image shows the negative control staining with nonimmune rabbit IgG that proved the specificity of the staining. OA: osteoarthritis; TIRAP: Toll/interleukin-1 receptor domain-containing adapter protein.

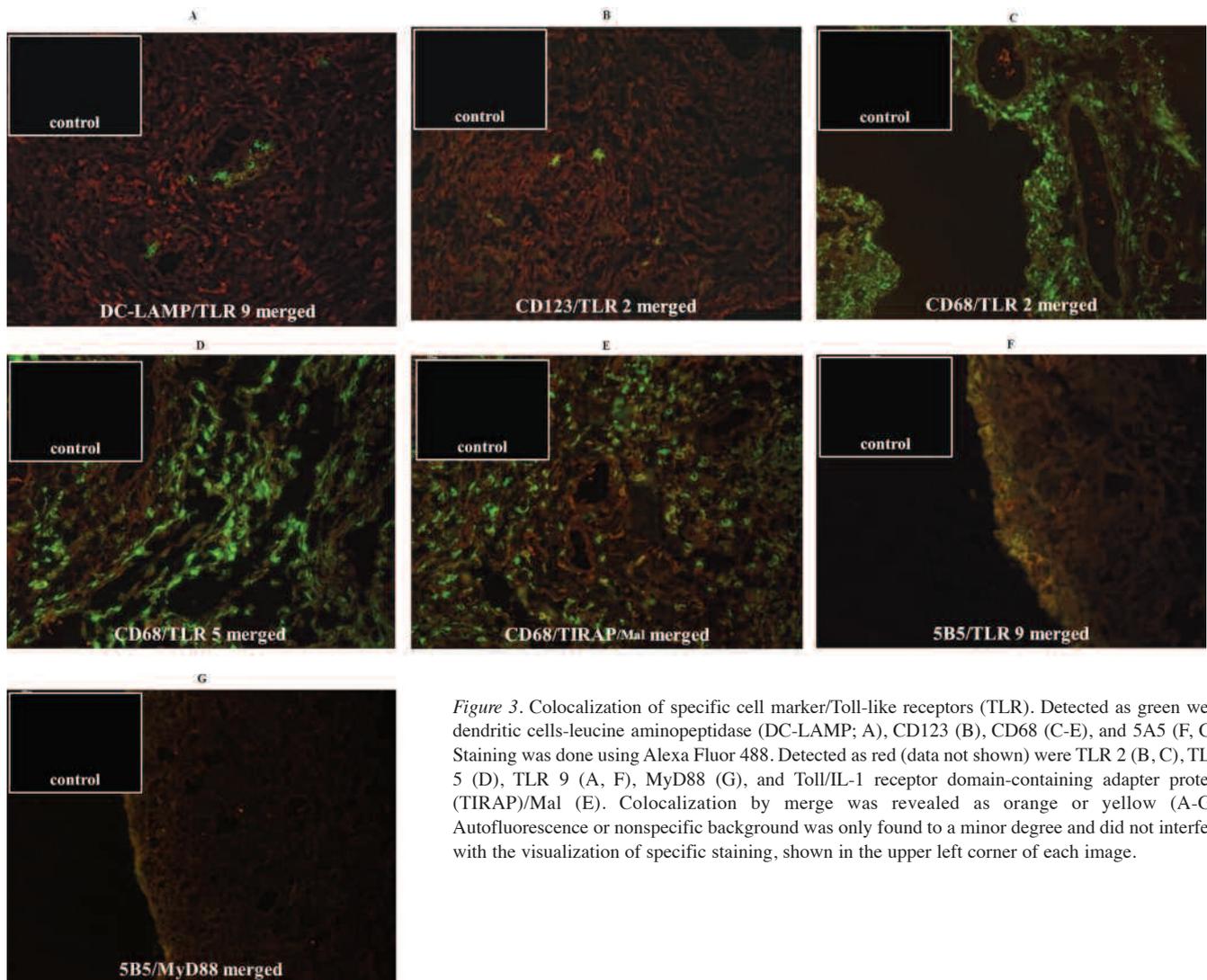


Figure 3. Colocalization of specific cell marker/Toll-like receptors (TLR). Detected as green were dendritic cells-leucine aminopeptidase (DC-LAMP; A), CD123 (B), CD68 (C-E), and 5A5 (F, G). Staining was done using Alexa Fluor 488. Detected as red (data not shown) were TLR 2 (B, C), TLR 5 (D), TLR 9 (A, F), MyD88 (G), and Toll/IL-1 receptor domain-containing adapter protein (TIRAP)/Mal (E). Colocalization by merge was revealed as orange or yellow (A-G). Autofluorescence or nonspecific background was only found to a minor degree and did not interfere with the visualization of specific staining, shown in the upper left corner of each image.

In the OA synovial membrane, large inflammatory cell infiltrates or lymphoid aggregates were not seen, but some thickening of synovial lining and infiltration of sublining stroma by mononuclear inflammatory cells was often observed. Some CD68+ macrophages and occasional CD15+ polymorphonuclear neutrophils were observed. Some CD3+/CD4+ and CD3+/CD8+ T cells and CD20+ B cells were observed in the lining and sublining stroma and in perivascular infiltrates. The negative control staining experiments confirmed the specificity of the staining (data not shown). Autofluorescence or nonspecific background was found to only a minor degree and did not interfere with the visualization of specific staining.

DISCUSSION

We demonstrate that in RA synovitis tissue, the β -actin standardized mRNA levels of TLR 2, TLR 3 and TLR 9 are high, those of TLR 5 and TLR 6 are elevated, but those of TLR 1 and TLR 4 do not differ between RA and OA. These

results suggest that RA synovitis tissue is particularly responsive to TLR 2, TLR 3, and TLR 9 stimulation. TLR 2 is stimulated by bacterial lipoproteins or fungal zymozan and TLR 9 is stimulated by bacterial unmethylated CpG. Although the bacterial components peptidoglycan and bacterial CpG-containing DNA have been identified in RA synovitis tissue and joint fluid^{23,24} as potential PAMP, it is more likely that TLR 2, TLR 3, and TLR 9 are stimulated by some endogenous alarmins. Alarmins are damage-associated molecular patterns released passively by diffusion from necrotic cells or secreted actively by stimulated cells to alarm the bystander cells to imminent or threatening damage. Together, PAMP and alarmins form DAMP^{7,8}. TLR 2 is stimulated by monosodium urate, an adjuvant necessary for immune responses and which causes gout upon excessive release^{25,26}. TLR 2 is also stimulated by high mobility group box 1, a DNA-binding nonhistone protein important for packaging and epigenetic regulation of genes, but which outside the cell functions as an alarmin and a

cytokine^{27,28,29}, binding to TLR 2 (and TLR 4 and receptor for advanced glycation product). TLR 3 is an endogenous sensor of tissue necrosis³⁰. TLR 9 binds chromatin-IgG complexes, but also mitochondrial DNA³¹ (Figure 4). Foci of necrotic cells are typically found in RA joints and were mentioned as one of the typical histopathological characteristics of RA synovitis in the 1958 revision of the then-American Rheumatism Association criteria for RA³². Because of the wide “pattern recognizing” specificity of TLR in general, it is likely that new endogenous ligands will be revealed for TLR 2, TLR 3, and TLR 9.

Colocalization of TLR 1, 2, 3, 4, 5, 6, and 9 were observed in DC-LAMP+ mDC. In addition, colocalization of TLR 2, 4, and 9 was observed in CD123+ pDC identified in perivascular areas, large and small lymphoid aggregates, and around lymphoid follicles. Moderately labeled TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were seen in CD68+ type A lining cells and infiltrating macrophages. TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were found in 5B5+ type B lining cells and fibroblasts of the sublining stroma, with weak and/or at most moderate reactivity. Thus, TLR-positive DC-LAMP+ and/or CD123+ DC were found in RA samples in this study. TLR 1, 2, 4, and 5 have been reported in immature mDC, but their expression levels decreased upon maturation of the mDC³³. However, TLR 3 was

expressed only in mature mDC³⁴. On the other hand, although peripheral blood pDC expressed only TLR 7 and 9, pre-pDC have been described to express TLR 1, 6, 7, 9, and 10, but not TLR 4. In contrast, mDC expressed TLR 1, 2, 3, 4, 5, 6, 8, and 10, but not TLR 7 and TLR 9^{25,35}. Therefore, our double-labeling findings suggest that both immature and mature mDC and pDC are found in RA synovitis tissue, but are rare in OA synovial membrane. In this respect, it is interesting that DAMP can, through TLR, activate resting DC so that they start to express costimulatory molecules and mature to professional antigen-presenting major histocompatibility complex (MHC) class II-rich cells³⁶. This local adjuvant effect might be important for the rheumatoid autoimmune processes.

Our study demonstrates the presence and cellular localization of TLR 1, 2, 4, 5, 6, and 9 receptor proteins in inflamed RA synovial tissues and adds new data to current reports. Expression of TLR 2 in RA synovial tissues was first reported by Seibl, *et al*²¹ and later confirmed by others^{12,37,38}. TLR 3^{14,38} and TLR 4^{12,37,38} have also been reported in RA. Fibroblasts isolated from RA synovial tissue expressed mRNA of TLR 1, 2, 3, 4, 5, and 6, but not those coding TLR 7, 8, 9, and 10³⁹. In our study, the cellular localization of different TLR was clarified using double-staining of TLR and cell-specific biomarkers. Previous reports were mainly focused on fibroblast-like synoviocytes^{21,37,39}, although some report on TLR in DC and CD16+ monocytes³⁸.

First, in our study the most intense TLR labeling was observed in mDC and pDC. Second, CD68+ lining cells and macrophages of sublining stroma were all positive for all the TLR examined and, in contrast to a previous report, also for TLR 2²¹. In addition, TLR adapters MyD88 and TIRAP/Mal were also strongly labeled in DC and moderately labeled in CD68+ macrophages. Weakly to modestly immunoreactive TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were found in type B lining cells and fibroblasts of sublining stroma. It seems that on a single-cell basis, DC particularly have the capacity to be engaged in DAMP-TLR signaling, followed by macrophage-like cells and then by mesenchymal stromal cells. The stimulating soluble and cell membrane-bound costimulatory molecules and MHC class II molecules might be important for the antigen-presenting and T cell-polarizing functions of DC/autoimmune responses. However, quantitatively dominant type A and B lining cells and stromal macrophages and fibroblasts may play a central role in local production of proinflammatory cytokines and tissue-destructive proteinases/autoinflammatory responses^{40,41}, to which T and B cells (according to current staining results) seem to contribute only slightly. TLR were weakly labeled in OA synovium, a condition that implies only mild or modest responsiveness to self-components and/or inflammatory products^{42,43,44,45,46} in OA compared to RA.

Tissue expression of TLR adapter molecules MyD88,

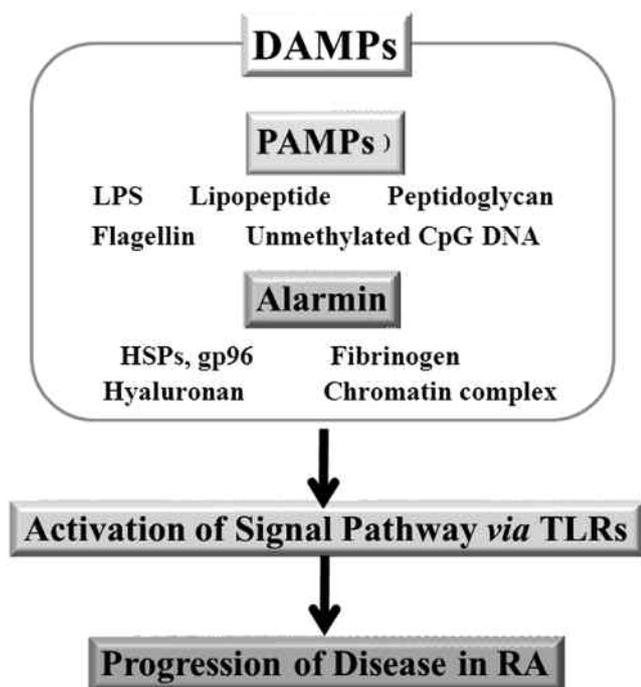


Figure 4. Possible scheme of disease progression by Toll-like receptors (TLR) and their ligands. RA synovial tissues can be exposed to exogenous pathogen-associated molecular patterns (PAMP) and endogenous alarmins. Exogenous PAMP such as viral and bacterial component and endogenous alarmins such as heat shock proteins, fibrinogen, and hyaluronic acid that exist around the microenvironment of joints can be recognized by TLR. PAMP and alarmins are also called danger-associated molecular patterns (DAMP). LPS: lipopolysaccharide; HSP: heat shock protein.

TIRAP/Mal, and TRIF was detected in all RA samples examined. MyD88 is the universal adapter for TLR⁴⁷, and the signaling pathway from MyD88 to the activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) is used by almost all TLR to control inflammatory responses (Figure 5). The activation of the MyD88-dependent pathway requires an additional adapter, TIRAP/Mal, in TLR 2 and TLR 4 signaling. Both MyD88 and TIRAP/Mal were found in RA and OA samples to a similar extent. TIRAP/Mal is not essential for MyD88-independent signaling, which instead requires TRIF/TICAM-1, an adapter for TLR 3 and TLR 4. TRIF is required for the development of tolerance to prevent overactivation of the host. The MyD88-independent pathway leads to activation of the transcription IFN regulatory factor-3 and induction of IFN- β and IFN signature^{6,48,49}. It was therefore interesting that although TRIF/TICAM-1 was detected in RA synovial tissues, its mRNA expression was weak (Table 4). None of the adapter molecules were significantly increased in RA synovitis, suggesting that the adapter protein assembly and activation, rather than synthesis *de novo*, is important in rapid TLR-mediated responses.

In contrast to this overall lack of difference in adapter molecule levels between RA and OA samples (analyzed using real-time PCR, which lumps together mRNA mole-

cules from all different cell subsets), double-staining experiments disclosed again some interesting cell subtype-specific findings. The strongest adapter molecule staining was found in mDC and pDC, while type A lining cells and infiltrative macrophages showed modest reactivity, and type B lining cells and stromal fibroblasts showed only weak to modest reactivity. This suggests again different potential responsiveness and reactivity of these cell subtypes to various DAMP, because strong TLR expression was paralleled by strong postreceptor expression of the adapters. It again seems that DC are particularly engaged in DAMP-TLR signaling, followed by macrophage-like cells. Stimulation of these cells by TLR enhances the NF- κ B signal transduction pathway, leading to increased production of inflammatory cytokines, which also determine the polarity of the T lymphocyte maturation along the Th1 (by IL-12) or Th17 (by IL-23) lineage⁵⁰, and of extracellular matrix-degrading proteinases^{12,13,14,16}. Thus, these immunopathological observations suggest that TLR, particularly TLR 2, TLR 3, and TLR 9, and the associated adapter proteins can contribute not only to autoinflammatory responses, but also to autoimmune responses by adaptive immunity in actively inflamed synovial tissues of RA^{40,41}.

Increased expression of TLR 2, TLR 3, and TLR 9 in general suggests a highly potent responsiveness of RA synovial tissue to various TLR 2, TLR 3, and TLR 9-binding DAMP, in particular endogenous alarmins, but possibly also some exogenous PAMP. Further, when this analysis was extended to cell subtypes, the strong expression of TLR 1, 2, 3, 4, 5, 6, and 9 in mDC and of TLR 2, 4, and TLR 9 in pDC, together with the associated adapters, suggests intense potential involvement of DC in adaptive autoimmune responses, although type A cells/macrophages and type B cells/fibroblasts may be mostly responsible for cytokine and proteinase production and autoinflammatory responses (Figures 6 and 7). Less intense inflammation and lower TLR mRNA and protein levels in OA suggest less potent responsiveness compared to RA. Similar expression of MyD88 and TIRAP/Mal adapters suggests competent downstream signal transduction pathways in both conditions and that the main difference between RA and OA lies in the general and cell subtype-specific levels of certain TLR rather than their adapters.

ACKNOWLEDGMENT

We sincerely thank Eiko Saito for her skillful technical support in immunohistochemistry.

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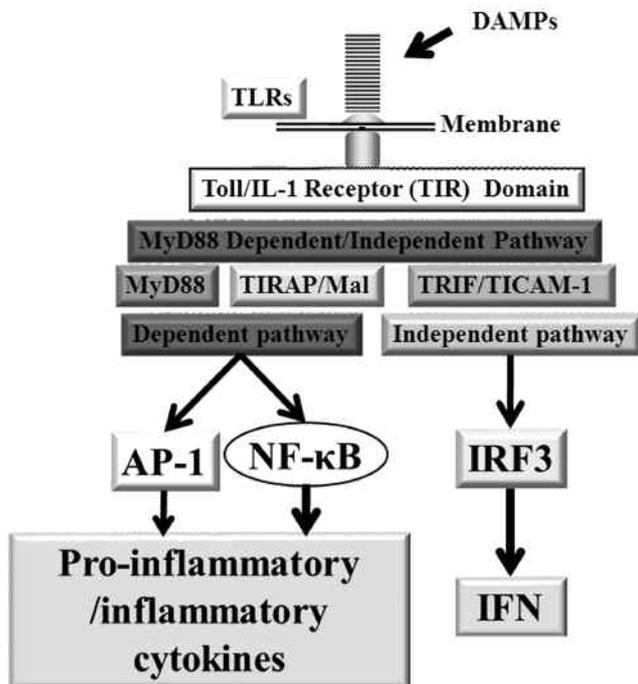


Figure 5. Two major pathways of Toll-like receptor (TLR)-related signals. The recognition of danger-associated molecular patterns (DAMP) can stimulate intracellular signal pathway through adapter molecules. By activation of activator protein-1 (AP-1) and NF- κ B, proinflammatory/inflammatory cytokines and extracellular matrix-degrading proteinases are possibly induced. Adaptive immune response is induced by type I interferon (IFN) production. TIRAP: TIR domain-containing adapter protein; TRIF: TIR domain-containing adapter-inducing IFN- β ; TICAM: TIR-containing adapter molecule; IRF: interferon regulatory factors.

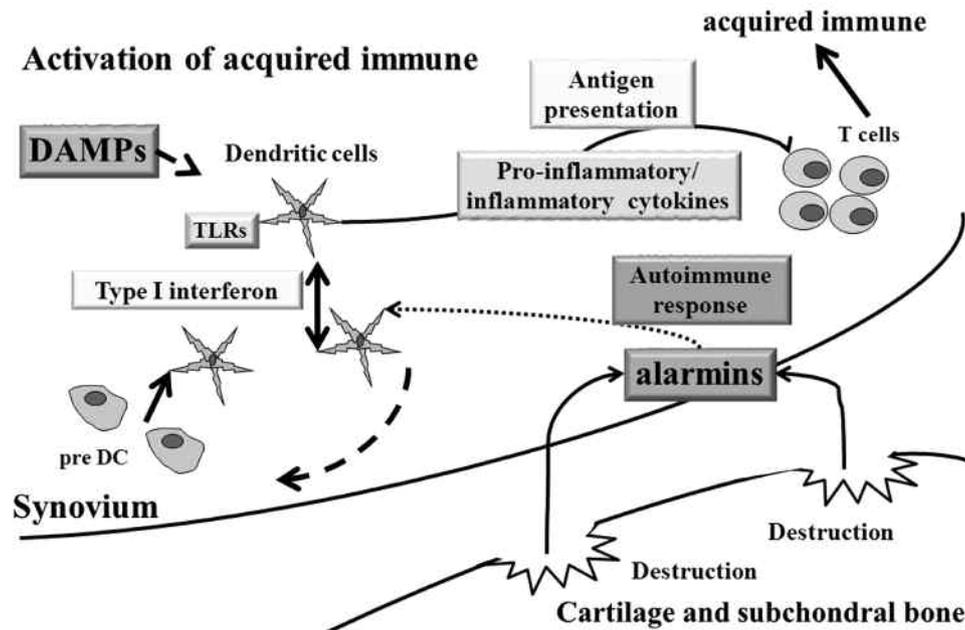


Figure 6. Pathologic role of Toll-like receptor (TLR) expressed in dendritic cells (DC). Danger-associated molecular patterns (DAMP) can through TLR activate resting DC so that they start to express costimulatory molecules and mature to antigen-presenting cells. This local adjuvant effect might be important for rheumatoid autoimmune processes. DC produce type I interferon (IFN) and proinflammatory/inflammatory cytokines by DAMP-TLR signaling. This type I IFN stimulates DC and pre-DC, leading to activation of DC and maturation of pre-DC. On the other hand, proinflammatory/inflammatory cytokines stimulate activation of acquired immunity. Damaged cartilage and subchondral bone may interact with DAMP to TLR expressed by DC. Autoimmune response may accelerate DAMP-TLR cycles.

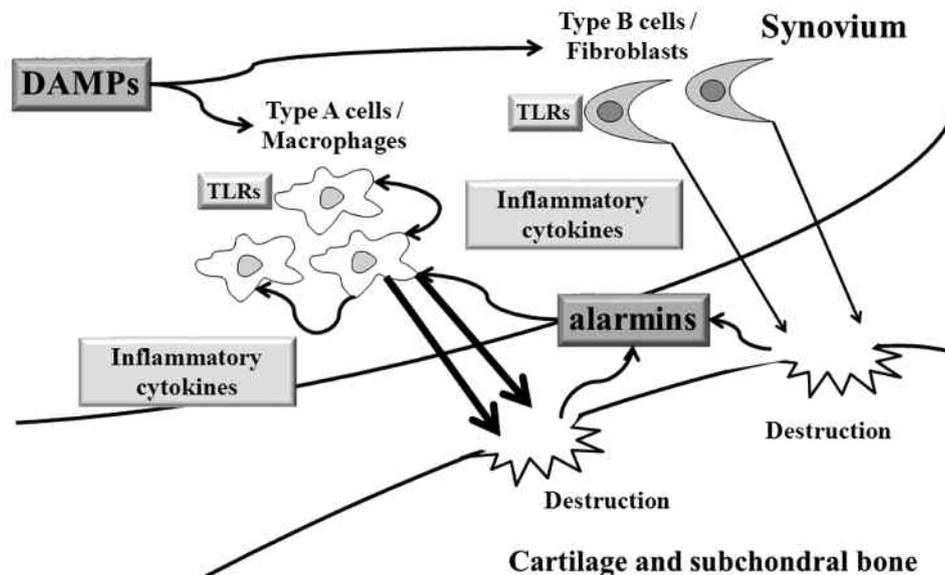


Figure 7. Pathologic role of Toll-like receptor (TLR) expressed in macrophage-like type A cells and fibroblast-like type B cells of the lining, and infiltrative macrophages and fibroblasts in the sublining stroma. Danger-associated molecular patterns (DAMP) also recognized by TLR expressed on type A cells/macrophages and type B cells/fibroblasts. These cells produce inflammatory cytokines by DAMP-TLR stimulation. These reactions activate each other and contribute to destruction of cartilage and subchondral bone. Debris of degraded cartilage and subchondral bone is recognized by TLR expressed on type A cells/macrophages and type B cells/fibroblasts. DAMP-TLR cycles between type A cells/macrophages and type B cells/fibroblasts may accelerate destruction of cartilage and subchondral bone.

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