Toll-like Receptor-2 Expression Is Upregulated in Antigen-Presenting Cells from Patients with Psoriatic Arthritis: A Pathogenic Role for Innate Immunity?

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ABSTRACT. ***Objective.*** To study Toll-like receptor-2 (TLR-2) and TLR-4 expression in antigen-presenting cells from patients with psoriatic arthritis (PsA).

Methods. We measured expression of TLR-2 and TLR-4 in monocyte-derived dendritic cells from patients with PsA and with rheumatoid arthritis (RA), and in healthy controls. Dendritic cells were obtained from freshly isolated monocytes, stimulated with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) after 6 days in culture. To obtain mature dendritic cells, lipopolysaccharide stimulation and 2 additional days in culture were necessary. The expression of TLR-2, TLR-4, HLA-DR, and CD86 was studied at baseline, at 6 days, and at 8 days by flow cytometry. To establish the functional properties of TLR expression we studied the following cytokines in cell supernatants: tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, and GM-CSF. TLR-2 expression was confirmed by Western blot analysis.

Results. Ten PsA patients with active disease and 8 healthy controls were studied, along with 4 patients with RA. TLR-2 expression was increased in immature dendritic cells from patients with PsA. Monocytes and mature dendritic cells did not show statistically significant differences. No difference was observed in the expression of TLR-4 in any cell type. The supernatant expression of cytokines showed a Th1 pattern, mostly with increased expression of TNF-α, IFN-γ, and IL-2. Western blot analysis confirmed the increased expression of TLR-2.

Conclusion. Upregulation of TLR-2 expression provides support for a role of the innate immune system in the pathogenesis of PsA. (First Release Dec 15 2006; J Rheumatol 2007;34:374–9)

Key Indexing Terms:

TOLL-LIKE RECEPTORS | BACTERIA | PATHOGENESIS
DENDRITIC CELLS | RHEUMATOID ARTHRITIS | INNATE IMMUNITY

The pathogenesis of psoriasis and its related arthritis (PsA) is complex and is thought to be the result of the interplay of environmental, genetic, and immunological factors[1-3]. There is a growing body of evidence linking PsA with gram-positive bacteria, especially Streptococcus. It has long been recognized that the onset of guttate psoriasis is often preceded by throat infection with B-hemolytic streptococci in up to 40% of cases, and that this association decreased significantly with the discovery of penicillin[4-6]. Evidence of elevated levels of antibodies to streptococcal components (antideoxyribonuclease-B exotoxin) has been described in patients with PsA, and γ/δ-T cells from patients with PsA respond to streptococcal antigen[7]. The use of highly sensitive techniques has led to the detection of the V2 regions of 16S ribosomal RNA (rRNA) from 3 streptococcal species in the peripheral blood and synovial fluid of patients with PsA[8]. In addition, gram-positive bacteria, particularly *Staphylococcus aureus*, is highly prevalent in psoriatic skin plaques[9].

On the other hand, considerable evidence has accumulated linking psoriasis and PsA to a defect in cell-mediated immunity[10-12]. These disorders are characterized by a Th1 cell response, and evidence supporting a pivotal role for T cells in their pathogenesis is strong. In contrast, the role of the innate immune system in the pathogenesis of these disorders has not been clearly defined. This is of special interest considering that the innate immune system is immediately activated after infection. Toll-like receptors (TLR) are an essential part of the innate immune system involved in the response to microbial pathogens[13]. TLR recognize endogenous [heat shock proteins (HSP), fibronectin, and hyaluronic acid] and exogenous (bacterial, viral, and fungal) ligands and activate inflammatory cells via the nuclear factor-κB pathway[14,15]. TLR share the Toll/interleukin 1 (IL-1) homology domain, and 11 different human TLR proteins have been characterized[16]. TLR-4 has been the better characterized and studied and it recognizes lipopolysaccharides (LPS) from gram-negative bacteria,

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while peptidoglycan and lipoproteins from gram-positive bacteria are the main ligands for TLR-2[17,18]. TLR are expressed on different cell types including keratinocytes, uterine and urinary bladder cells, and synoviocytes, and on antigen-presenting cells (APC), such as monocytes and dendritic cells. TLR microbial recognition represents the link between the innate and the acquired immune systems by inducing the maturation of dendritic cells and directing the T cell-helper responses through a series of cytokine production mechanisms (Th1/Th2)[19]. Dendritic cells are professional APC and key actors in this bridge between our 2 immune systems[20].

We investigated the role of TLR in the pathogenesis of PsA. We studied the expression of TLR-2 and TLR-4 in APC from patients with active PsA.

MATERIALS AND METHODS

Patients. Consecutive patients with PsA attending the Louisiana State University Health Sciences Center Rheumatology Clinic were enrolled. Patients were diagnosed according to the Wright and Moll criteria[21]. Cutaneous disease was confirmed by skin biopsy by a dermato-pathologist. All patients were in the active phase of the disease. After all patients had given informed consent, we obtained 100 ml of fresh blood in sodium heparinized tubes. An additional serum sample was collected. All patients underwent a complete medical examination, including measurement of C-reactive protein and sedimentation rate. The Psoriasis Area and Severity Index (PASI) score was determined in these patients. Given the similarities between rheumatoid arthritis (RA) and PsA and also due to the confirmed findings of the expression of TLR in both serum and synovium of patients with RA[22], we studied TLR-2 expression in 4 patients with RA as well. Patients with RA were diagnosed according to the American College of Rheumatology criteria. Expression of TLR-2 and TLR-4 was also studied in 8 healthy controls. Demographic and clinical characteristics are summarized in Table 1.

Monocyte-derived dendritic cell study. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia, Biotech, Uppsala, Sweden). Monocytes were separated by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Begish Gladbach, Germany). The resulting population was ≥ 85% pure. Monocytes were adhered overnight and cultured for 6 days at 37°C in a humidified atmosphere containing 5% CO_2 and supplemented with RPMI L-glutamine, penicillin, streptomycin, human serum, and HEPES, and in the presence of IL-4 (50 U/ml) and granulocyte macrophage-colony stimulating factor (GM-CSF; 50 ng/ml) for 6 days to generate immature dendritic cells. Then dendritic cells were stimulated with LPS (30 μg/ml) for another 2 days. Viability was above 96% in all experiments as determined by trypan blue dye exclusion.

Table 1. Demographic and clinical characteristics of subjects.

Variable	PsA, n = 10	RA, n = 4	Controls, n = 8
Sex F/M	8/2	3/1	2/6
Mean age, yrs	39.4	52	42.5
Disease duration, yrs	6.1	10	NA
Treatment			NA
No treatment	0	0	6
PDN/MTX	7/10	2/4	NA
TNF-α blockers	2/10	0	NA
Abatacept		2/4	NA
PASI score	7.1	NA	NA
No. of swollen joints	4	6	NA
Tender joints	3	4	NA

NA: not applicable; PDN: prednisone; MTX: methotrexate; PASI: Psoriasis Area and Severity Index.

Flow cytometry analysis (FACScan). Monocytes and dendritic cells were studied using FACScan at Days 0, 6, and 8 (Beckton Dickinson FACScan). Cells were labeled using fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) conjugated antibodies against CD14, CD11c, CD86, HLA-DR, and TLR-2 and TLR-4 (e-Bioscience, San Diego, CA, USA).

Additionally, we determined the expression of different cytokines including IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, and TNF-α in the supernatant of the cells at Day 5 in culture using the Bio-Plex Human CytokineTh1/Th2 Panel (Bio-Rad, Hercules, CA, USA).

To confirm the expression of TLR-2 we performed Western blot analysis using TLR-2 antibody (e-Bioscience).

Statistical analysis. Mann-Whitney test or unpaired t test was employed. A p value < 0.05 was considered statistically significant and a 95% confidence interval was expressed. Statistical analysis was performed using GraphPad Prism, version 3.0.

RESULTS

Ten patients with PsA (8 women, 2 men, mean age 39.4 yrs) with active disease and 8 healthy controls (2 women, 6 men, mean age 42.75 yrs) were studied, along with 4 patients with RA (3 women and 1 man). The mean disease duration was 6.1 years for PsA patients. Mean PASI score was 7.1. Regarding treatment, 8 patients with PsA were receiving a combination of methotrexate (MTX) and prednisone (PDN) and the remaining 2 were receiving TNF-α blockers. Two patients with RA were receiving a combination of PDN and MTX, and 2 patients were receiving abatacept.

TLR-2 and TLR-4 expression in different APC. It was observed that CD14+ monocytes were more numerous in patients with PsA than in controls. By Day 5, 80% of the monocytes became immature dendritic (iDC) cells, as shown by the increased expression of CD11c+ and the low expression of CD14+ markers, and also by the decreased expression of HLA-DR compared with baseline (Figure 1). No statistically significant differences in the expression of TLR-2 and TLR-4 in freshly isolated monocytes from PsA and healthy controls (p = 0.7019; 95% CI –21.72 to 31.26) were observed. After 6 days in culture, TLR-2 was significantly upregulated in PsA patients compared to controls (p = 0.0223; 95% CI –43.79 to –4.06; Figure 2). However, no differences of TLR-4 expression were observed between PsA and controls (p = 0.0979; 95% CI –89.98 to 9.097). Interestingly, no statistical differences in the expression of TLR-2 (p = 0.3763; 95% CI –46.22 to 18.93) or TLR-4 (p = 0.930; 95% CI –32.68 to 35.38) were observed in mature dendritic cells from PsA patients and controls after 48 hours in culture with LPS. The results from TLR-2 and TLR-4 expression in different dendritic cell subsets are summarized in Table 2. There were no statistically significant differences in the expression of TLR-2 in iDC from patients with RA, compared to controls (p = 1.0000; sum of ranks 16,39) or PsA patients (p = 0.5167; sum of ranks 42,13; data not shown). There was, however, a statis-

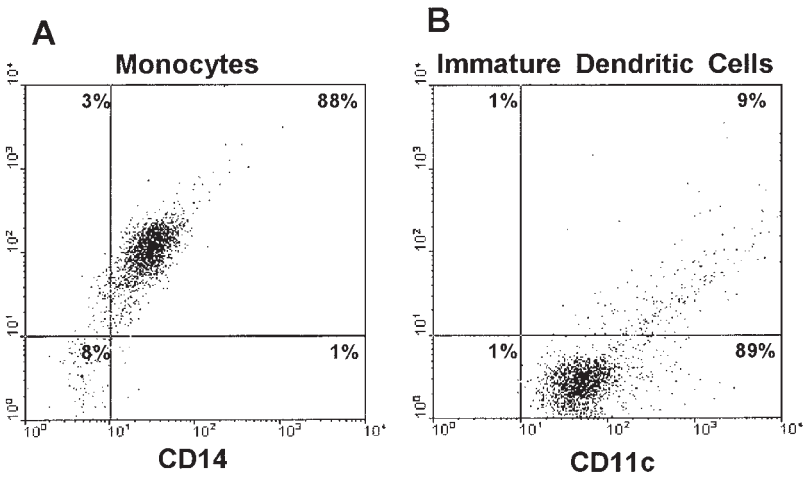

Figure 1. A. Flow cytometry analysis showing CD14+ monocytes and high expression of HLA-DR. B. Flow cytometry showing CD11c+ immature dendritic cells and decreased expression of HLA-DR compared to monocytes.

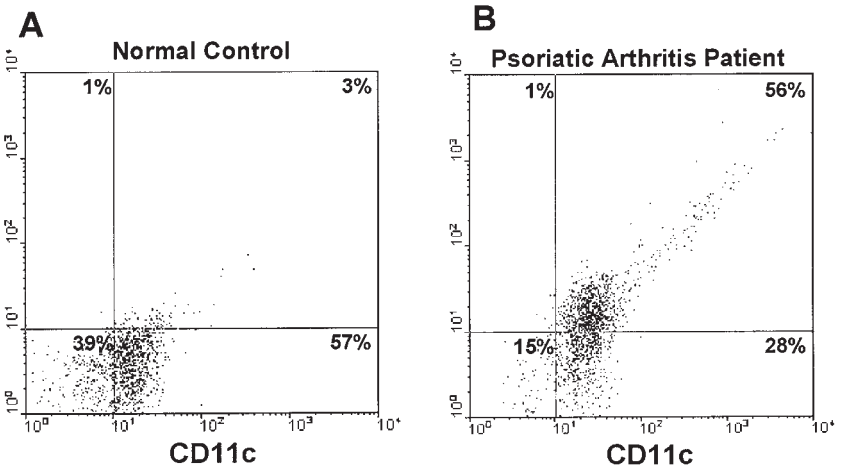

Figure 2. A. Flow cytometry showing TLR-2 expression in CD11c+ immature dendritic cells from controls. B. Flow cytometry showing TLR-2 expression in CD11c+ immature dendritic cells from PsA patients.

Table 2. Toll-like receptor 2 (TLR-2) expression on monocytes and dendritic cell subsets of PsA patients and controls.

Cell type	Surface Marker	PsA vs Controls, p (95% CI)
Monocytes	TLR-2	0.7019 (–21.72 to 31.26)
Immature dendritic cells	TLR-2	0.0223 (–43.79 to –4.06)*
Mature dendritic cells	TLR-2	0.3763 (–46.22 to 18.93)

* Statistically significant.

tically significant difference in the expression of TLR-2 in monocytes from RA patients compared to controls (p = 0.0180; 95% CI –11.69 to 93.43).

Cytokine panel in the supernatants of immature dendritic cells. The supernatant expression of cytokines is summarized in Figure 3. Briefly, TNF-α expression showed a trend (p = 0.06; 95% CI –57.49 to 2.241) when compared with healthy controls.

Expression of IFN-γ was increased in the patients with PsA compared to controls (p = 0.0351; 95% CI –2.57 to –13.15). IL-2 was elevated in the patients with PsA compared with RA patients (p = 0.0357; sum of ranks 30,6). We found no differences for the remainder of the cytokines including IL-5, IL-10, IL-12, IL-13, and GM-CSF.

Western blot analysis confirmed increased expression of TLR-2 in patients with PsA compared to controls (Figure 4).

DISCUSSION

A potential pathogenic role for Toll-like receptors is emerging in a variety of inflammatory and autoimmune conditions such as RA, systemic lupus erythematosus, asthma, sepsis, acne,

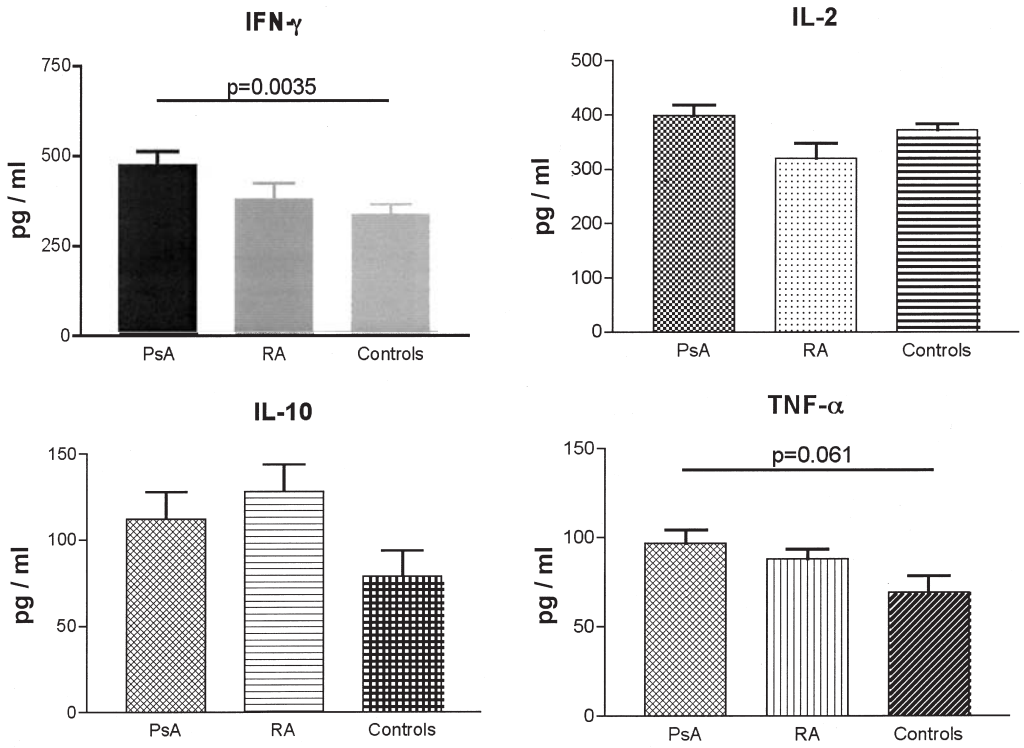

Figure 3. Th1/Th2 cytokine levels in the supernatants of monocytes/dendritic cells of PsA patients and controls.

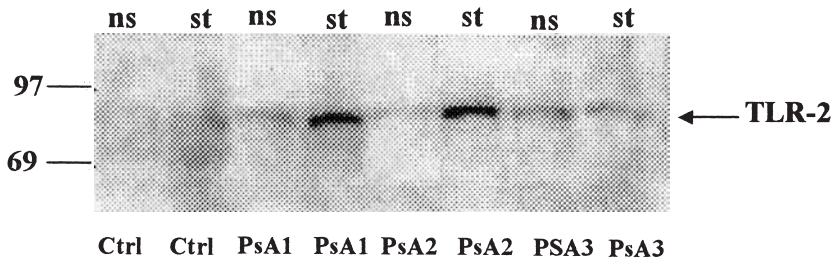

Figure 4. Western blot analysis confirming the expression of TLR-2 in patients with PsA and controls. ns: nonstimulated; st: stimulated; TLR-2: Toll-like receptor-2.

and reactive arthritis[23-27]. Studies in psoriasis have shown that epidermal keratinocytes in normal skin constitutively expressed TLR-1, TLR-2, and TLR-5, while TLR-3 and TLR-4 were barely detectable. In contrast, in lesional epidermis from patients with psoriasis, TLR-2 was more highly expressed on the keratinocytes of the upper epidermis than on the basal layer, while TLR-5 was downregulated in basal keratinocytes compared with corresponding nonlesional psoriatic epidermis[28]. Curry, *et al* expanded these observations and also described TLR-2 expression primarily by dermal dendritic cells and TLR-4 expression by epidermal dendritic cells and dermal dendritic cells[29]. Both studies showed the presence of distinctive patterns of innate immune-related receptors expressed by specific subsets of cells in normal and psoriatic skin, although the relevance of the findings remains to be defined.

Studies of this kind in PsA are scarce, although recent evidence suggests a role for TLR in the pathogenesis of reactive arthritis in animal models, as well as in patients with spondyloarthropathy[30,31]. A potential role for innate immunity-mediated inflammation in spondyloarthropathy is supported by the findings of DeRycke, *et al* describing increased TLR-4 expression in peripheral blood monocytes and inflamed synovium[32]. Notably, expression of TLR on mononuclear cells and synoviocytes was downregulated following treatment with TNF-α inhibitors. This was not observed in our 2 patients with PsA receiving TNF-α therapy. Raffeiner, *et al* have recently confirmed these observations[33].

The finding of upregulated expression of TLR-2 and not TLR-4 in PsA dendritic cells is in agreement with the report of TLR-2 upregulation in dermal dendritic cells in psoriatic skin, and TLR-2 expression has been shown to play a role in

the pathogenesis of zymosan-induced arthritis in mice[34]. A potential mechanism for this upregulation of TLR-2 in dendritic cells could be the presence of exogenous TLR ligands such as bacterial DNA and/or peptidoglycans described in PsA. Similar mechanisms have been described in RA[35]. Additionally, high levels of antipeptidoglycan antibodies have been described in patients with PsA[36]. A role for endogenous ligands such as HSP cannot be entirely ruled out at this time. Autoantibodies to HSP 60 are found in a sizable proportion (20%) of patients with PsA[37]. Extensive work has shown that HSP such as HSP 60, HSP 70, HSP 90, and GP 96 may activate the innate immune system leading to production of proinflammatory cytokines by the monocyte-macrophage system, and activation and maturation of dendritic cells via the TLR-2 and TLR-4 signal transduction pathways[38-40].

The pattern of cytokine production found in our patients with PsA reveals a Th1 pattern including IL-2, IFN-γ, TNF-α, IL-1ß, and IL-10. The cytokine pattern present in the supernatants of iDC in the PsA group compared with RA patients and healthy controls also exhibited a Th1 response with an increased production of TNF-α, IFN-γ, and IL-12. The cytokine assay that we used did not include IL-6, which has been shown to be an important contributor to the development of TLR-4-mediated acute arthritis in mice[30]. We have previously shown using an *in vitro* fibroblast culture assay that IL-6 is the most prominent cytokine produced by PsA fibroblasts[1]. This pattern (Th1) of cytokine production correlates directly with the TLR-2 expression we observed. Whether TLR-2 upregulation in patients with PsA is due to recognition of gram-positive bacteria ligands or to endogenous HSP products of joint and/or skin inflammation remains to be defined.

Our findings support a role for the innate immunity system in the pathogenesis of PsA. Further studies are needed to clarify its exact pathogenic role.

REFERENCES

1. Espinoza LR, van Solingen R, Cuellar ML, Angulo J. Insights into the pathogenesis of psoriatic arthritis. Am J Med Sci 1998;316:271-6.
2. Korendowych E, McHugh N. Genetic factors in psoriatic arthritis. Curr Rheumatol Rep 2005;7:319-24.
3. Ritchlin CT. Pathogenesis of psoriatic arthritis. Curr Opin Rheumatol 2005;17:406-12.
4. Rasmussen JE. The relationship between infection with group A beta hemolytic streptococci and the development of psoriasis. Pediatr Infect Dis J 2000;19:153-4.
5. Komine M, Tamaki K. An open trial of oral macrolide treatment for psoriasis vulgaris. J Dermatol 2000;27:508-12.
6. Vasey FB, Deitz C, Fenske NA, Germain BF, Espinoza LR. Possible involvement of group A streptococci in the pathogenesis of psoriatic arthritis. J Rheumatol 1982;9:719-22.
7. Grinlinton FM, Skinner MA, Birchall NM, Tan PL. Gamma delta + T cells from patients with psoriatic and rheumatoid arthritis respond to streptococcal antigen. J Rheumatol 1993;20:982-7.
8. Wang Q, Vasey FB, Mahfood JP, et al. V2 regions of the 16S ribosomal RNA used as a molecular marker for the species identification of Streptococci in peripheral blood and synovial fluid from patients with psoriatic arthritis. Arthritis Rheum 1999;42:2055-9.
9. Aly R, Maibach HE, Mandel A. Bacterial flora in psoriasis. Br J Dermatol 1976;95:603-6.
10. Espinoza LR, Gaylord SW, Vasey FB, Osterland CK. Cell-mediated immunity in psoriatic arthritis. J Rheumatol 1980;7:218-24.
11. Gladman DD, Keystone EC, Schacter RK. Aberrations in T-cell subpopulations in patients with psoriasis and psoriatic arthritis. J Invest Dermatol 1983;80:286-90.
12. Curran SA, Fitzgerald OM, Costello PJ, et al. Nucleotide sequencing of psoriatic arthritis tissue before and during methotrexate administration reveals a complex inflammatory T cell infiltrate with very few clones exhibiting features that suggest they drive the inflammatory process by recognizing autoantigens. J Immunol 2004;172:1935-44.
13. Sigal LH. Basic science for the clinician 27: Toll-like receptors and nucleotide oligomerization domains. J Clin Rheumatol 2005;11:176-9.
14. Beg AA. Endogenous ligands of Toll-like-receptors: implications for regulating inflammatory and immune responses. Trends Immunol 2002;23:509-12.
15. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-7.
16. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1-14.
17. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cells precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194:863-9.
18. Krutzik SR, Tan B, Li H, et al. TRL activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. Nat Med 2005;11:653-60.
19. Lanzavecchia A, Sallusto F. The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. Curr Opin Immunol 2001;13:291-8.
20. Kaisho T, Akira S. Dendritic cell function in Toll-like receptor and MyD88-knockout mice. Trends Immunol 2001;22:78-83.
21. Moll JMH, Wright V. Psoriatic arthritis. Semin Arthritis Rheum 1973;3:55-78.
22. Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? II. T cell-independent mechanisms from beginning to end. Arthritis Rheum 2002;46:298-308.
23. Iwahashi M, Yamamura M, Aita T, et al. Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. Arthritis Rheum 2004;50:1457-67.
24. Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. J Exp Med 2005;18:321-31.
25. Racila DM, Kline JN. Perspectives in asthma: molecular use of microbial products in asthma prevention and treatment. J Allergy Clin Immunol 2005;116:1202-5.
26. Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis 2005;15:S421-6.
27. Zhang X, Glogauer M, Zhu F, Kim TH, Chiu B, Inman RD. Innate immunity and arthritis: neutrophil Rac and Toll-like receptor 4 expression define outcomes in infection-triggered arthritis. Arthritis Rheum 2005;52:1297-304.
28. Baker BS, Ovigne JM, Powles AV, Corcoran S, Fry L. Normal keratinocytes express Toll-like receptors (TLRs) 1,2 and 5: modulation of TLR expression in chronic plaque psoriasis. Br J Dermatol 2003;148:670-9.
29. Curry JL, Qin JZ, Bonish B, et al. Innate immune-related receptors in normal and psoriatic skin. Arch Pathol Lab Med 2003;127:178-86.

30. Kyo F, Futani H, Matsui K, et al. Endogenous interleukin-6, but not tumor necrosis factor alpha, contributes to the development of toll-like receptor 4/myeloid differentiation factor 88-mediated acute arthritis in mice. Arthritis Rheum 2005;52:2530-40.
31. Jugeau S, Tenaud I, Knol AC, et al. Induction of toll-like receptors by Propionibacterium acnes. Br J Dermatol 2005;153:1105-13.
32. DeRycke L, Vandooren B, Kruithof E, DeKeyser F, Veys EM, Baeten D. Tumor necrosis factor alpha blockade treatment down-modulates the increased systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondyloarthropathy. Arthritis Rheum 2005;52:2146-58.
33. Raffeiner B, Dejaco C, Duftner C, et el. Between adaptive and innate immunity: TLR4-mediated perforin production by CD28 null T-helper cells in ankylosing spondylitis. Arthritis Res Ther 2005;7:R1412-20.
34. Frasnelli ME, Tarussio D, Chobaz-Peclat V, Busso N, So A. TLR2 modulates inflammation in zymosan-induced arthritis in mice. Arthritis Res Ther 2005; R370-9.
35. Roelofs MF, Joosten LAB, Abdollahi-Roodsaz S, et al. The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and co-stimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis Rheum 2005;52:2313-22.
36. Rahman MU, Ahmed S, Schumacher HR, Zeiger AR. High levels of antipeptidoglycan antibodies in psoriatic and other seronegative arthritides. J Rheumatol 1990;17:621-5.
37. Jarjour WN, Jeffries BD, Davis JS, Welch WJ, Mimura T, Winfield JB. Autoantibodies to human stress proteins. A survey of various rheumatic and other inflammatory diseases. Arthritis Rheum 1991;34:1133-8.
38. Vabulas RM, Ahmad-Nejad P, daCosta C, et al. Endocytosed HSP60s use toll-like receptor 2 (TLT2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem 2001;276:31332-9.
39. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. HSP70 as endogenous stimulus of the Toll-interleukin-1 receptor signal pathway. J Biol Chem 2002;277:15107-12.
40. Tsan MF, Gao BC. Heat shock protein and innate immunity. Cell Mol Immunol 2004;1:274-9.