Do Changing Toll-like Receptor Profiles in Different Layers and Grades of Osteoarthritis Cartilage Reflect Disease Severity?

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ABSTRACT. ***Objective.*** Cartilage degeneration in osteoarthritis (OA) leads to release of potential danger signals. The aim of our study was to profile OA cartilage for the Toll-like receptor (TLR) danger signal receptors.

Methods. Osteochondral cylinders from total knee replacements were graded using OA Research Society International score and stained for proteoglycans, collagenase-cleaved type II collagen, and TLR 1–10, which were analyzed histomorphometrically.

Results. Grade 1 OA lesions contained 22%–55% TLR 1–9-positive cells in the surface zone, depending on the TLR type. In Grade 2 TLR, immunoreactivity was 60%–100% ($p < 0.01$) and it was even higher in Grades 3 and 4 ($p < 0.01$ vs Grade 1). TLR-positive cells in Grade 1 middle zone were low, 0–19.9%, but were 5.1%–32.7% in Grade 2 ($p < 0.01$) and 34%–83% in Grades 3–4 samples ($p < 0.001$). TLR values in Grade 5 were low (14.3%–28.7%; $p < 0.001$). In Grades 3–4 OA, cartilage matrix stained strongly for TLR. In Grade 1, COL2-3/4M was restricted to chondrocytes, but was increasingly seen in matrix upon progress of OA to Grade 4, and then declined.

Conclusion. Cells in the gliding surface zone are fully equipped with TLR in mild OA. Their proportion increases and extends to the middle or even the deep zone, reflecting OA progression. COL2A-3/4M staining suggests Endo180-mediated intake for intralysosomal degradation by cathepsins in Grade 1, but in higher grades this chondrocyte-mediated clearance fails and the matrix demonstrates extensive collagenase-induced damage. Detached and/or partially degraded matrix components can then act as endogenous danger signals (damage-associated molecular patterns or DAMP) and stimulate increasingly TLR-equipped chondrocytes to inflammation. At the peak inflammatory response, soluble TLR may exert negative feedback, explaining in part the low TLR levels in Grade 5 OA. (First Release March 15 2013; J Rheumatol 2013;40:695–702; doi:10.3899/jrheum.121159)

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Osteoarthritis (OA) is the most common joint disease of so-called diarthrodial or synovial joints[1]. This relates in part to the particular physico-mechanical properties of these joint structures. They carry weight and are subjected to trauma, shear stress, and wear and tear, in spite of tentative mixed-mode lubrication, consisting of hydrodynamic pressure film lubrication, and boundary (asperity, contact point) lubrication mediated by lubricin, hyaluronan, and other biolubricants[2]. This may be aggravated by the lack of nerves in cartilage so that even considerable traumatic and degenerative changes may go unnoticed, without pain. Second, cartilage does not have vascular or lymphatic vessels that could transport mesenchymal stem cells, nutrients, oxygen, and metabolites, which contributes to impairment of its inherent repair capacity. Instead, cartilage is supplied by diffusion, assisted by the pumping action of its own compression or by flexion of the elastic cartilage[3]. It is known that chondrocytes, which by volume form only 1%–5% of human cartilage, slowly remodel their pericellular matrix, with perhaps distant effects as well on intra-

and interterritorial areas[4]. The question remains how such remodeling activities are regulated.

Recent findings suggest that normal locomotion, age-related degenerative changes in OA, and traumatic injuries can all release cartilage matrix molecules from the extracellular matrix. Such detached and perhaps partially degraded and/or denatured molecules would form a feedback signal for the cells with a capacity to synthesize new intact molecules, to compensate for losses resulting from the dissolution, degradation, and denaturation caused by heavy use of the joints. If the articular cartilage damage is too extensive (i.e., pathological), given the lack of nociceptive nerves it may also be a signal of potential danger. According to current concepts, these matrix-derived molecules act as damage-associated molecular patterns (DAMP), which as endogenous danger signals can alarm the resident cells by stimulating them through pattern-recognizing danger receptors (PRR)[5]. One important set of these is formed by Toll-like receptors (TLR). Stimulation of chondrocytes by TLR leads to an inflammatory response, which often also represents the initial stage of tissue repair[6,7].

There are reports describing the TLR in cartilage and/or OA[8,9,10,11]. This is the first with systematic profiling of TLR in which the results are related to the severity of histological grading of the OA, using the Osteoarthritis Research Society International (OARSI) grading system[12], and also describes the location of these changes in the different zones of cartilage.

MATERIALS AND METHODS

OARSI grading and Alcian blue staining of OA tissue samples. Osteochondral cylinder samples were harvested from specimens of tibial plateaus obtained from total knee arthroplasty operations from knee joints of 9 female patients with primary OA. The tibial plateau was fixed to an in-house purpose-designed sample holder, using a hollow 9-mm bore attached to a power drill. Samples were collected from macroscopically different areas and graded in the laboratory using the OARSI grading[12].

Osteochondral samples were fixed in neutral buffered 10% formalin for 2 weeks and decalcified in 10% EDTA, pH 7.4, for 5 weeks at +4°C (until bones were pliable) before being dehydrated in ethanol series, cleared in xylene, and embedded in paraffin. Samples were cut using a Leica RM255 microtome (Leica) to 3 μm thickness on objective slides.

After deparaffinization in xylene and ethanol rehydration, OA tissue sections were stained with Safranin O, toluidine blue, and Alcian blue. The histopathological OARSI grading was carried out blindly by 3 independent researchers from coded samples using light microscopy[12]. Our samples contained no totally healthy grade 0 cartilage; grade 6 OA samples contain only subchondral bone, and therefore grade 6 samples lacking cartilage were not analyzed.

Immunohistochemical staining of different grades of OA cartilage tissue sections. Representative samples from all the different OARSI grades were used in immunohistochemistry analysis. After deparaffinization and rehydration, tissue sections were incubated for antigen retrieval in 10 mM citrate buffer, pH 6.0, for 1 hour at +70°C. After quenching of endogenous peroxidase in 0.3% H_2O_2 in deionized water for 25 min, sections were blocked with normal goat serum or normal horse serum (Vectorlabs) diluted in 0.1% bovine serum albumin-phosphate buffered saline (BSA-PBS) for 1 h. Sections were incubated as follows: (1) overnight at 4°C in 2 μg/ml rabbit anti-human TLR1 IgG (H-90), 1 μg/ml rabbit anti-human TLR2 IgG (H-175), 2 μg/ml rabbit anti-human TLR3 IgG, 1.3 μg/ml rabbit anti-human TLR4 IgG, 1.3 μg/ml rabbit anti-human TLR5 IgG, 1 μg/ml goat anti-human TLR6 IgG, 0.8 μg/ml goat anti-human TLR7 IgG, 1 μg/ml rabbit anti-human TLR8 IgG, 1 μg/ml rabbit anti-human TLR9 IgG (H-100), 1.6 μg/ml rabbit anti-human TLR10 IgG (all TLR from Santa Cruz Biotechnology), and 1:1000 mouse anti-human COL2A-3/4M IgG1 (Ibex); (2) then in biotinylated goat anti-rabbit or horse anti-mouse IgG (Vectorlabs); and (3) then in avidin-biotin-peroxidase complex. Color was developed using H_2O_2 and 3,3′-diaminobenzidine (Dako A/S) for 10 min, followed by counterstaining with Mayer hematoxylin for TLR (Merck) or with eosin for COL2A-3/4M for 1 min. After washes in deionized water the slides were dehydrated in ethanol series, cleared in xylene, and mounted. For negative staining controls, nonimmune IgG was used instead of and at the same concentration as the primary specific antibodies. All incubations were performed at 22°C, with PBS washes between the steps if not otherwise stated.

Stained samples were evaluated under a Leica DM6000 B/M microscope (Leica Microsystems) equipped with a motorized Leica XY-stage and connected to a Leica DFC 420 digital camera using Leica Application Suite version 3.8.0 software (Leica). The percentages of TLR-positive cells were evaluated from at least 5 high-magnification (200×) fields and separately in the surface, middle, and deep zone of the hyaline articular cartilage.

Statistical analysis. All values are provided as mean ± SD. For immunostaining data, statistical analysis was done using the Kruskal-Wallis 1-way variance test to compare differences in the proportion of TLR-positive cells in samples of different OA grades (from 1 to 5) and between different cartilage zones (surface, middle, and deep). Mann-Whitney U test was applied for pairwise comparison of groups. Wilcoxon signed-rank test was used for comparison of zonal distribution of TLR within the same OA sample. Nonparametric tests were applied because the data had ordinal levels of measurement. SPSS v18.0 for Windows was used for statistical analyses.

Our study was carried out with a permit from the local ethical committee (Dnro HUS 165/E6/03). Patients provided written informed consent to participate.

RESULTS

Alcian blue staining revealed progressive proteoglycan (glycosaminoglycan) depletion as OA advanced. Depletion was initially confined to the most superficial surface zone in grade 1 OA lesions (Figure 1), and reached its maximum in grades 3 and 4, which showed proteoglycan depletion in the whole surface zone and in part of the middle zone (Figure 1). In grade 5 lesions there was some Alcian blue staining in the superficial cartilage matrix, which was stronger than that in the superficial zone in grade 3 lesions, but still weaker than the much stronger staining in the pericellular and intraterritorial cartilage matrix in grade 5 lesions.

Immunostaining of collagenase-cleaved COL2A-3/4M neoepitope was already seen in chondrocytes in grade 1 OA lesions. With increasing OARSI grade of the OA, from grade 2 to 4, both the area and the intensity of the immunostaining of collagenase-cleaved COL2A-3/4M increased in the extracellular matrix (Figure 2B-2D). Extracellular matrix staining was at first mainly localized to the surface zone, with particularly intense staining of the regions with severe degenerative changes. Negative staining controls, with nonimmune mouse IgG_1 at the same concentration as the primary mouse anti-human COL2-3/4M-specific IgG_1, confirmed the specificity of staining (Figure 2F).

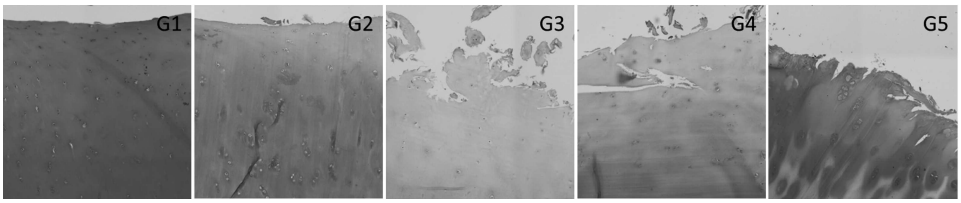

Figure 1. Alcian blue staining in relation to the grade of osteoarthritis lesions, graded according to the histological Osteoarthritis Research Society International (OARSI) grading system (Grade 1-Grade 5). Original magnification ×200.

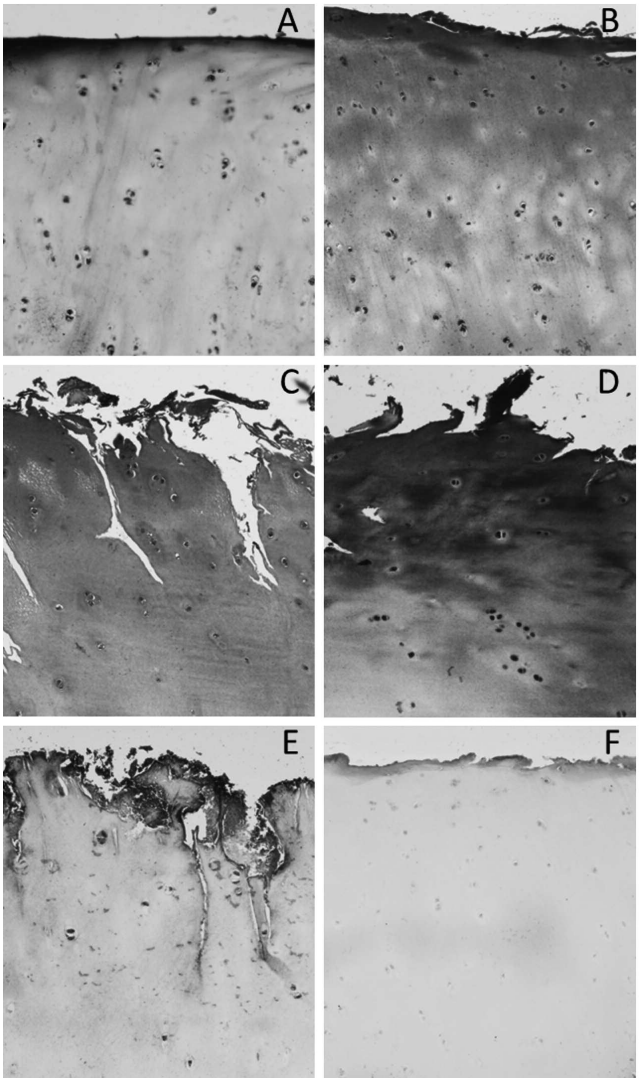

Figure 2. Immunostaining of COL2A-3/4M in relation to grade of osteoarthritis lesions, according to Osteoarthritis Research Society International (OARSI) grading system. A. Grade 1, COL2A-3/4M neoepitope is mainly seen in the chondrocytes. B. Grade 2, extracellular matrix is stained for COL2A-3/4M neoepitope. C. Grade 3, COL2A-3/4M neoepitope staining becomes visible in the chondron capsule, forming a halo-like staining patterns. D. Grade 4, COL2A-3/4M neoepitope staining is stronger and extends more deeply. E. Grade 5, COL2A-3/4M neoepitope staining is confined to the severely damaged "surface" zone. F. Negative staining control of grade 2 lesions confirms the specificity of the staining. Original magnification ×200.

Except for TLR10, all TLR were found in the human articular cartilage. In grade 1 OA lesions, all other TLR were seen mainly in some chondrocytes in the surface zone (Figure 3A). TLR3 results are shown in this example because its expression in relation to the OA grade and cartilage zone has not been published before. Staining was stronger in grade 2 lesions and was also seen in somewhat more deeply located chondrocytes and in the lamina splendens (Figure 3B). In grade 3 (Figure 3C) and grade 4 lesions (Figure 3D), fissures penetrated the cartilage and chondrocyte clusters stained for TLR, but the most prominent staining was now seen in the extracellular matrix, whereas in grade 5 lesions this TLR staining was almost extinguished (Figure 3E). Negative staining controls with nonimmune rabbit IgG used at the same concentration as the antibodies confirmed the specificity of staining (Figure 3F). Staining of all TLR samples is presented in Figure 4.

In grade 1 OA lesions, morphometric calculations and statistical analysis showed relatively low percentages (22%–55%) of TLR-positive cells in the surface zone, whereas the TLR-positive chondrocytes were very scarce in the middle zone. In grade 2 lesions the percentage of TLR-positive cells was higher than in grade 1 (60%–100%; $p < 0.01$) and positive cells were also more numerous in the middle zone (5.1%–32.7%; $p < 0.01$), with a further increase of the percentage of middle zone TLR-positive cells in moderate grade 3 (41%–83%; $p < 0.01$) and severe grade 4 lesions (34%–73%; $p < 0.01$). In very severe grade 5 lesions, most of the cartilage had been lost from the superficial and upper middle zone, relatively few chondrocytes stained for TLR, and there was practically no staining of extracellular matrix (Figure 5).

DISCUSSION

Toll was initially recognized in *Drosophila melanogaster* (the banana fly), which upon binding of *Aspergillus niger*-derived pathogen-associated molecular patterns (PAMP) triggers a simple protective, proinflammatory host response. Earlier during the ontogenesis, Toll binds to another molecular pattern, an endogenous molecule, *spätzle*, that helps to establish the dorsal-ventral polarity in the Drosophila embryo[13]. Similarly, the human Toll equivalents, TLR, can respond to exogenous PAMP but also to endogenous DAMP, suggesting a similar dual role.

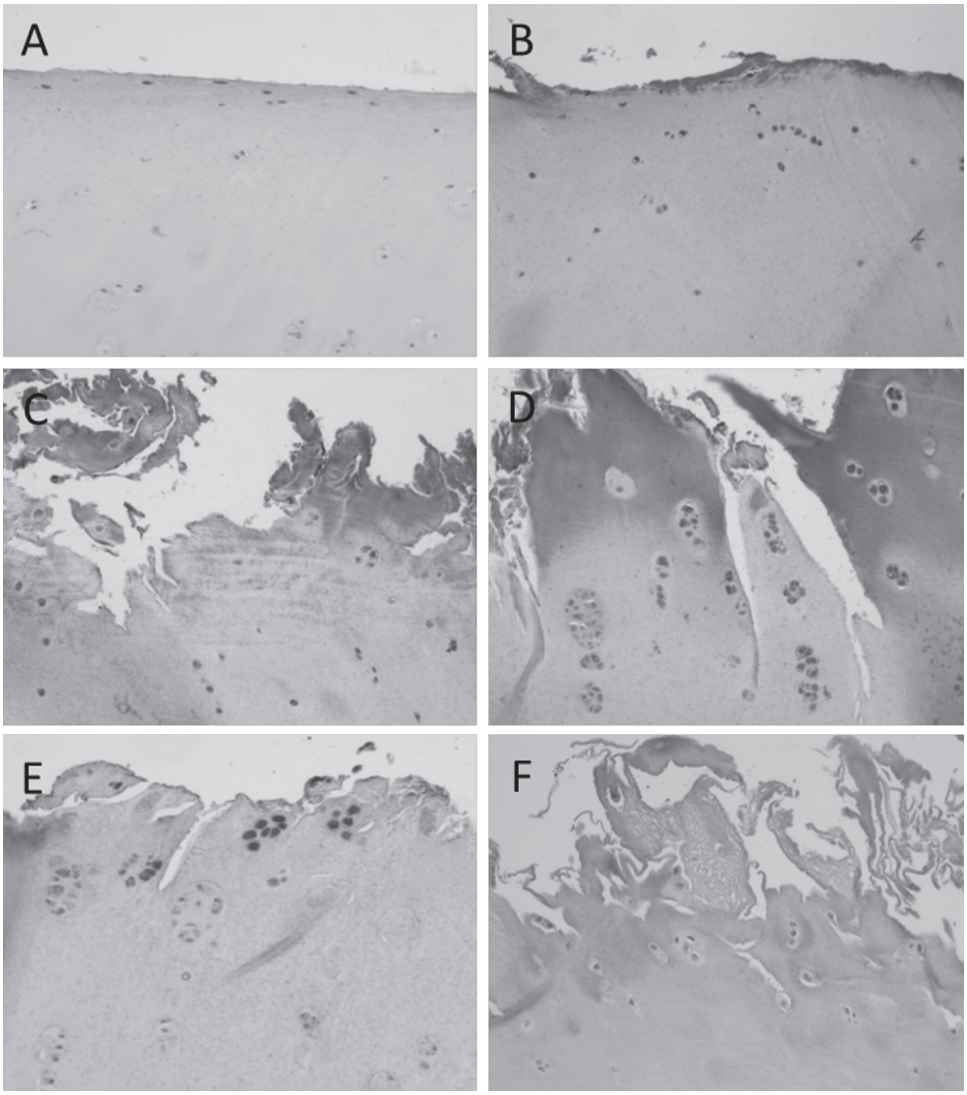

Figure 3. Immunostaining of Toll-like receptor 3 (TLR3) in relation to grade of osteoarthritis (OA) lesions, according to Osteoarthritis Research Society International (OARSI) grading system. A. Grade 1, TLR3 is seen mainly in chondrocytes of the surface zone. B. Grade 2, cellular TLR3 staining is stronger and extends more deeply, but can also be seen extracellularly in lamina splendens. C. Grade 3, TLR3-positive cells are seen in the surface and middle zones, but also in the superficial cartilage matrix. D. Grade 4, TLR3-positive cells now extend even deeper, and in particular the matrix staining is stronger and more extensive than in Grade 3. E. Grade 5, percentage of TLR3-positive chondrocytes is relatively low and matrix does not stain. F. Negative staining control of grade 3 lesions confirms the specificity of staining of both chondrocytes and extracellular matrix. It is emphasized that the extracellular matrix staining represents specifically soluble and/or solubilized TLR immunoreactivity, not nonspecific background; it is also different in different OARSI grade OA samples, further confirming its specificity. Original magnification ×100.

Although DAMP- and PAMP-mediated activation of TLR might culminate in common inflammatory responses through partial recruitment of the same molecular signaling machinery, it is known that their ligand recognition and activation effects are clearly distinct. Sometimes simultaneous stimulation by both a DAMP and a PAMP is necessary to activate the TLR, which has led to the proposal that DAMP can act as modulators of the early immune responses[14].

It is known that both PAMP and DAMP are recognized through leucine-rich repeat (LRR) motifs located in the extracellular or endosomal ligand-binding domains of TLR. However, to date there are no reports of the crystal structure of any endogenous ligand-TLR complexes, which leads to uncertainties over the recognition structure responsible for the binding of endogenous ligands. It has also become clear that DAMP require different coreceptors and accessory molecules than those used by PAMP[15].

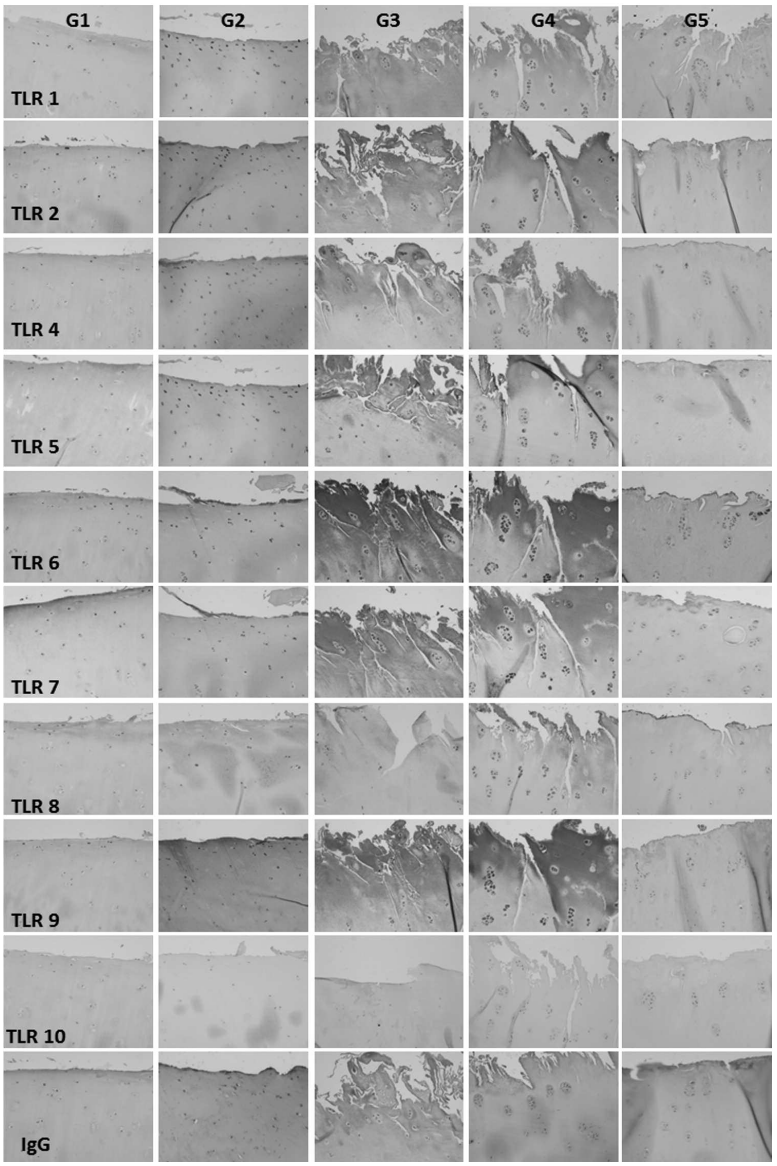

Figure 4. Immunostaining of Toll-like receptor 1 (TLR1) to TLR10 in relation to the grade of osteoarthritis (OA) lesions, according to the Osteoarthritis Research Society International (OARSI) system (Grade 1 to Grade 5). Negative staining controls of all grades confirmed the specificity of the staining. Original magnification ×100.

TLR agonists (ligands) induce TLR assembly into homo- or heterodimeric complexes. Dimerization of their cytoplasmic Toll-interleukin receptor (TIR) domains then triggers the downstream signaling through recruitment of specific adaptor molecules such as the myeloid differentiation factor 88 (MyD88), MyD88-adaptor like, TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule, and sterile alpha and HEAT-Armadillo motifs. These specific adaptor molecules are differently recruited by different TLR complexes induced by different TLR agonists. Such differential use of the TIR domain-binding adaptors provides specificity to individual TLR-mediated signaling pathways, which in part helps to prevent overactivation of the innate immunity and to balance the pro- or antiinflammatory responses.

In our study many of the human articular cartilage chondrocytes in the surface zone were found to be fully equipped with all TLR (except for TLR10, for which no ligand is known yet) in mild OA. This might relate to the wear and tear to which the superficial cells in the gliding zone (joint bearings) are subjected and where the surface fibrillation and focal loss of cation-staining (proteoglycan loss) are first seen.

The proportion of TLR-positive cells was significantly

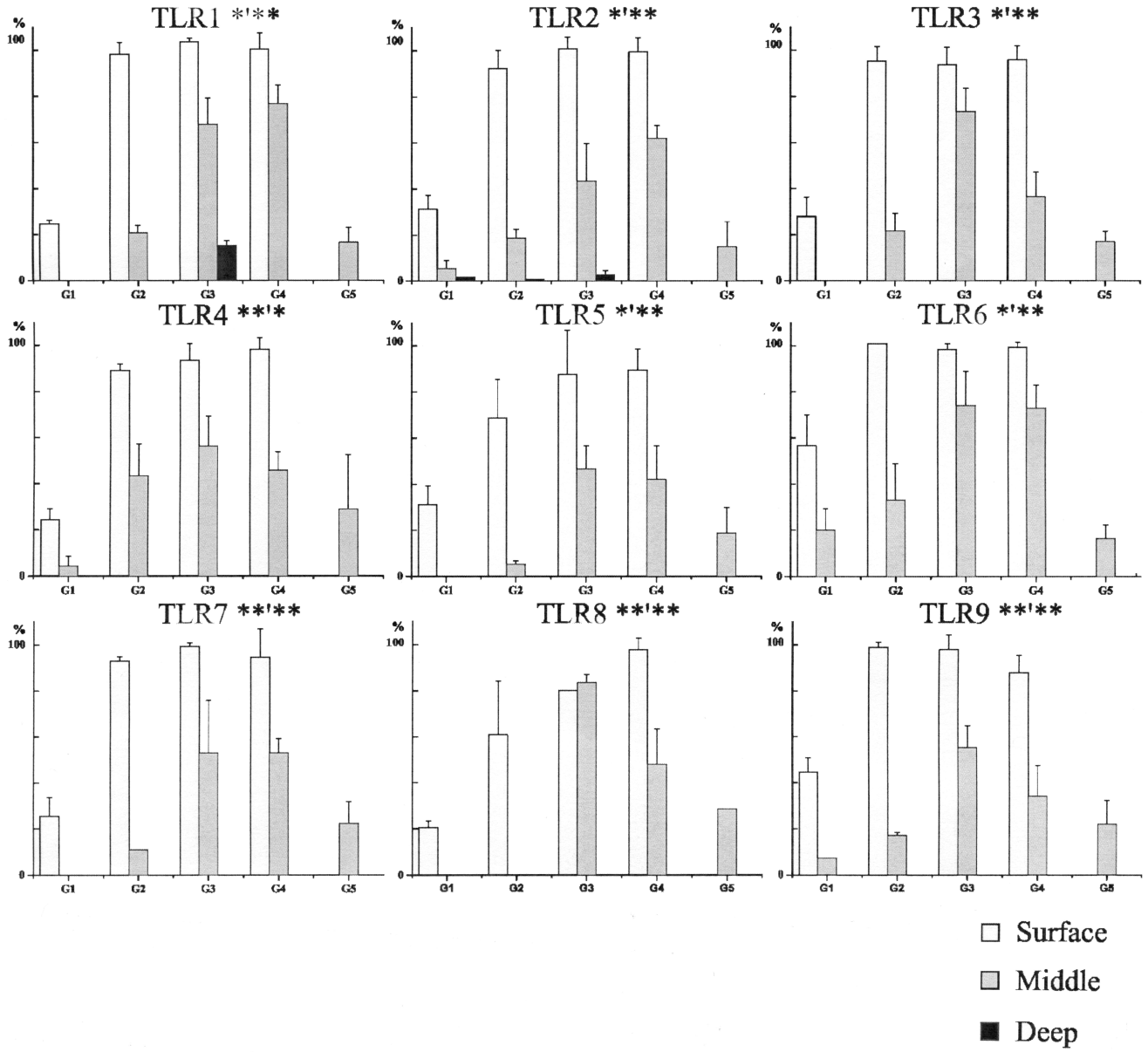

Figure 5. Histomorphometric calculations and statistical comparisons of all currently known human Toll-like receptors (TLR) are shown in relation to the grade of the osteoarthritis (OA) lesion and the cartilage zone. OA lesions were graded according to the Osteoarthritis Research Society International (OARSI) system. Cartilage zones evaluated were the surface (gliding, tangential), middle (transient), and deep (vertical) zones. TLR10 was not found in human OA cartilage. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate cartilage zone differences of TLR expression in different OA grades, separately for the surface zone (first asterisk) and for the middle zone (second asterisk). TLR1 was the only one that displayed statistically significant differences between different OARSI grades in the deep zone ($p < 0.01$).

increased in grade 2 OA lesions in both the superficial and middle zones of cartilage. This is in almost total congruence with the histological progression: matrix cracks pass through the surface zone, reaching down to the upper border of the middle zone, and proteoglycans are lost in the upper third of the cartilage thickness. The highest proportions of TLR were seen in grade 3–4 OA lesions, again totally in line with the deeper penetration signs of tissue degeneration and damage, e.g., deep vertical/branching fissures reaching into the upper border of the deep zone and proteoglycan loss down into the lower two-thirds of the cartilage thickness and finally in grade 4 matrix loss, and erosions extending through all cartilage zones.

These observations are in accord with other studies, which showed a similar pattern for TLR4[11] and are compatible with our working hypothesis: the areas of degenerative release of cartilage matrix-derived endogenous danger signals and the danger signal receptor-equipped cells match perfectly with each other. This would enable danger signal-TLR interactions to guide the chondrocyte responses

according to the severity and topology of the damage, e.g., through TLR1/2 heterodimers (Sillat, *et al*, in preparation).

In support of results described above, COL2A-3/4M immunostaining revealed clearly that in the surface zone of grade 2, 3, and 4 OA samples, the collagen network structure was compromised. This pattern of collagen degradation is in accord with studies showing increased cleavage of collagen type II together with matrix metalloproteinase (MMP)-type collagenases in the surface zone of OA cartilage1[16,17]. Interestingly, in addition to extracellular matrix, chondrocytes were also strongly stained, as previously shown for this COL2A-3/4M neoepitope and also for 2 additional cleavage-produced neoepitopes, Helix-II and CTX-II[18,19]. Such immunostaining can be explained by intake of collagenase-cleaved type II collagen for intracellular cathepsin-mediated degradation by endocytotic Endo180 receptor[20] located primarily on the chondrocyte surface[21]. This intracellular degradation mechanism was, from our results, already activated in grade 1 OA, which in grade 1 was enough to clear the surrounding pericellular matrix of COL2A-3/4M-positive collagen fragments. As the disease progresses and MMP-collagenase activity increases, this intracellular clearance mechanism becomes saturated and the pericellular and territorial matrix stains strongly for COL2A-3/4M. Nevertheless, at grade 5 there seems to be a break in the progression in proteoglycan depletion and COL2A-3/4M neoepitope staining. This has 3 possible explanations. First, in grade 5 lesions the proteoglycan-poor, weakly staining superficial zone and middle zone have been worn off. Second, it is possible that the chondrocyte-dependent and aggrecanase-mediated (ADAMTS4/5) lytic process is not as intense in the deep zone in grade 5 lesions (burnout lesions) as it is in the superficial zone in grade 3 lesions. Finally, it is possible that there is some attempt at fibrocartilagenous regeneration in these advanced lesions[12]. In grade 5 lesions the superficial zone contains some very lightly staining tissue islands, which together with the weakly staining surrounding cartilage matrix correlates quite well with COL2A-3/4M staining, which discloses MMP-type collagenase-mediated production of specific 3/4-1/4 ^{775}Gly-^{776}Ile cleavage sites.

Proteoglycan depletion and COL2A-3/4M seem to be concomitant[22], which may indicate a loss of the protective effect of aggrecan against collagen degradation[23]. Both aggrecanases and MMP-collagenases can be upregulated by activation of TLR[24,25], which might explain high TLR values in high-grade samples (except grade 5) characterized by both proteoglycan depletion and collagen degradation.

TLR profiling disclosed a somewhat surprising finding. TLR were also found deposited extracellularly in the peri-, intra-, and interterritorial matrix. Negative staining controls were in all instances strictly negative, confirming the specificity of the immunostaining. Further, this TLR immunostaining of the cartilage matrix was not uniform across the different OARSI grades, but varied and was dependent on the grade, and was most prominent in grades 3 and 4, which further confirms the specificity of the staining. This suggests that with progression of OA, TLR are increasingly produced in alternatively spliced, soluble form, are redirected to secretory pathways instead of cell surface, and/or are solubilized from the cell surfaces by TLR-sheddases[26,27,28]. Such soluble TLR might have multiple sources and could, according to our findings, derive from the articular cartilage, but also from the synovial membrane[29] and synovial fluid and cells[30,31]. Thus, to avoid overstimulation, chondrocytes seem to use the same negative feedback regulation that is used for many other receptors: after initial stimulation, the cell surface-associated TLR expression is diminished at the same time as the production of soluble "decoy" receptors is increased[32].

In very severe grade 5 OA lesions, much of the surface and some of the middle zone cartilage is worn away; chondrocytes form clusters but the rest of the cartilage matrix is hypocellular, and the normal composition and architecture are in deep disarray. It is possible that the extensive negative feedback in moderate to severe OA is enough to downregulate or turn off the chondrocyte-mediated inflammatory responses. Indeed, stimulation of TLR of chondrocytes increased production of proinflammatory interleukin 1β and tumor necrosis factor-α. From our results (data not shown), these cytokines upregulate TLR expression, thus forming a positive, self-amplifying cycle. Alternatively, chondrocytes in grade 5 lesions may be more engaged in a primitive attempt to synthesize extracellular cartilage matrix, because fibrocartilagenous tissue repair is a feature sometimes seen in grade 5[12].

It was also somewhat surprising that all the TLR expressed behaved qualitatively, albeit not quantitatively, in the same way. We show for the first time that the expression of all TLR, and not just TLR4[9], tend to be upregulated in parallel with greater severity of histopathologic changes as measured by the OARSI grading system. It should be considered that in contrast to the large diversity of, for example, T cell receptors (10^9 different clones), TLR are not conventional highly ligand-specific receptors. Instead, they recognize patterns, and because of their low number it is possible to provide even 1 cell, at least on demand, with all or almost all TLR (as found to be the case for chondrocytes) so that no PAMP or DAMP can escape the attention of the innate immune host defense. Our study shows conclusively that all TLR are actively expressed in OA cartilage and settles the debate about their expression in chondrocytes[8,9,10,11].

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