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Synergy in Toll-Like Receptors (TLRs)

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Commentary

Toll-like receptors (TLRs) were mostly studied for their function in innate immunity. These receptors identify distinct pathogen-associated molecular patterns from bacteria, viruses, fungi and parasites and initiate cascades of cellular events leading to the inflammatory response that fights the invading organisms [1].

The skeleton contains numerous blood vessels, tissue surfaces, and bone cells, all potential substrates for bacterial colonization. Bacterial diseases such as caries, periodontitis, periapical infection, osteomyelitis, septic arthritis and others severely affect the skeleton by causing accelerated bone loss [2]. The mechanism of these diseases includes activation of TLRs in immune cells by pathogen-derived molecules. It became apparent that functional TLRs are also expressed in the bone resorbing (osteoclasts [OCs]) and bone forming (osteoblasts [OBs]) cells [3-5]. Our studies are focused on OCs.

Communication between the immune and the skeletal systems is observed in normal physiological processes, but more obviously in autoimmune and other inflammatory diseases. This interrelationship prompted Arron et al. [6] to propose the term "osteoimmunology" to describe the interface between immunology and bone biology. We and others found that TLR ligands, in addition to their impact on bone indirectly *via* activation of TLRs in immune cells, are able to directly activate TLRs in bone cells, and thus modulate bone metabolism [3-5,7]. Focusing on the OC, the activation of TLRs in committed OC precursors (OCPs) results in increased OC differentiation and activity, and is probably a mechanism by which pathogen-induced bone loss occurs. On the other hand, activation of TLRs in early non-committed OCPs inhibits OC differentiation. This inhibition could serve as a mechanism for down-regulating excessive resorption and as a switch to promote the differentiation of common precursor cells into inflammatory cells [8].

TLRs Synergy in Osteoclast Differentiation

The organism is often challenged by more than one TLR ligand at a time (i.e. more than one type of organism or more than one type of TLR ligand in the same organism). Therefore, the impact of simultaneous challenges with different TLR ligands was studies. The focus of this commentary is our findings with simultaneous TLR ligands challenges of OC lineage cells. We examined modulation of both early non-committed OCPs and committed precursors. In the experimental settings commitment is obtained by treating the bone marrow-derived OCPs with the physiological OC differentiation factor, receptor activator of NF-κB ligand (RANKL) [9] before TLRs activation. As an example we analyzed the impact of poly (I:C) (mimicking viral single stranded RNA), lipopolysaccharide (LPS, derived from bacterial cell wall) and CpG-ODN (an oligonucleotide mimicking bacterial DNA), the ligands of TLR3, TLR4 and TLR9, respectively. In our analyses, in addition to examination of modulation of OC differentiation we also examined the modulation of c-Fos, a transcription factor with a key role in the induction of OC differentiation [10], as well as 2 cytokines that are also important for this process (IL-6 and TNF-α) [11,12]. A simultaneous activation of TLR4 and TLR9 and of TLR3 and TLR9 caused synergistic effects on OC differentiation; no synergy was observed with TLR4 and TLR9 simultaneous stimulation. The same pattern was obtained in the increase of c-Fos expression [13]. The overlap between the stimulation of OC differentiation and the increase in the cytokines expression was only partial. Simultaneous stimulation by the 3 TLR ligands did not show synergy in any of the parameters examined. Non-committed early precursors are bone marrow-derived OCPs without treatment with RANKL before TLRs activation. In addition to assaying the inhibition of OC differentiation we also measured the reduction in the expression of c-Fos and the increase of the anti-osteoclastogenic cytokine (IL-12) expression [14]. Activation of TLR4 and TLR9 and of TLR3 and TLR4 caused synergistic effects on OC differentiation; no synergy was observed with TLR3 and TLR9 simultaneous stimulation. The same pattern was obtained in the decrease of c-Fos expression and the increase in IL-12. As in the analyses of the committed precursors, also here simultaneous stimulation by the 3 TLR ligands did not show synergy in any of the parameters examined [13].

Increased expression of a TLR induced by ligands of other TLRs is a potential mechanism to mediate synergistic effects of these ligands. We indeed showed that TLR3 mRNA and protein levels were increased by TLRs 4 and 9 ligands. Similarly, TLR9 mRNA and protein levels were increased by TLRs 3 and 4 ligands. No significant increase was observed in TLR4 mRNA and protein levels with any of the ligands [13]. Up-regulation of TLRs in response to a variety of TLR ligands has been reported previously [15,16].

TLRs Synergy in Innate Immunity

As most aspects of TLRs, also their synergy was studied in immune cells more extensively than in bone cells. However, it is not the focus of this communication and will be dealt very briefly. TLR4 and TLR9 were shown to synergize in the production of TNF-α in mouse macrophages [17]. On the other hand [18] in dendritic cells (DCs) TLR4 and TLR9 did not show synergy in production of TNF-α, but synergistically induced IL-12p70 expression. These differences are in line with the specialized functions of macrophages and DCs; the macrophages in innate (TNF-α) and the DCs in adaptive (IL-12p70). Raman et al. [19] applied TLR synergy to enhance a parasite (Leishmania)-specific immune response. They used a well-established vaccine candidate, in conjunction with either, a TLR4 agonist, or a TLR9 agonist, or a combination of the two. Only mice treated with the vaccine plus the two TLR agonists were able to induce a strong

effective T cell response during disease and subsequently cured lesions and reduced parasite burden.

Concluding Remarks

What could be the significance of TLRs synergy?

In the case of the OCs, while the skeleton is affected at low doses of the TLR ligands also the switch between the OC-lineage and the inflammatory cells (both share the same precursor) occurs at low doses of the TLR ligands; thus the "war" against the pathogen starts shortly after invasion.

Regarding the immune system it was proposed that synergy between receptors for different microbial products would provide a safety mechanism, preventing inappropriate, potentially fatal reactions by reacting to low concentrations of ligands when more than a single ligand is present [20].

How does the processing of TLR inputs occur?

The TLR ligands interact with populations of cells. The resulting output could be derived from integration of the signals in the same cell or that each cell responds to a certain TLR ligand only. Kellogg et al. [21,22] examined this question for TLR2 and TLR4 ligands simultaneous stimulation. Independent stimulation of these ligands induced distinct NF-κB dynamic profiles of entry into the nucleus. Using automated microfluidic cell culture they found that single cells continued to show ligand-specific dynamic responses characteristic of TLR2 or TLR4 signaling rather than a mixed response. Thus, for simultaneous activation of TLR2 and TLR4 the mechanism, as termed by this group, is non-integrative processing. It remains to be shown how simultaneous signals from other TLR ligands combinations are processed.

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