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Species-specific TLR signaling—insight into human disease

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The selective utilization of IRAK kinases, which are thought to be recruited to MyD88 to form the 'Myddosome,' has been shown by Sun et al. to differ substantially in mouse and human cells. This finding has important implications for the development of therapeutics for inflammatory and autoimmune disorders associated with Toll Like Receptors (TLRs).

The myeloid differentiation primary response protein MyD88 (hereby referred to as MyD88) signaling pathway is central for the development of inflammation induced by nearly all Toll-like receptors (TLRs). However, our analysis of these mechanisms is often guided by murine models, which may or may not coincide functionally with the mechanisms in humans. Sun *et al.* performed a high-throughput RNA interference (RNAi)-based screen of human and mouse macrophage cell lines to identify species-specific differences in the relative importance of gene products that contribute to TLR signaling leading to *TNF* expression in humans or to NF-kB-driven and *Tnf* expression in mouse[1]. This work is a *tour de force* that resulted in the identification of a family of related proteins that differentially mediate TLR-dependent, MyD88-dependent signalling in mouse and human cells. The researchers initially selected 126 inflammatory genes for screening, including representative genes that encode receptor proximal signalling adaptors, and also signal transducers, negative regulators, and transcription factors. The greatest effect of RNAi-driven gene knockdown occurred when the levels of proximal signalling molecules were downregulated.

Sun *et al.* focused on species-specific differences in sensitivity to RNAi-driven silencing among the genes that encode IL-1 Receptor-Associated Kinases (IRAKs). IRAKs, a family of related proteins that have scaffolding and/or kinase activities, facilitate the formation of the Myddosome, a very large multi-component structure that mediates MyD88-dependent signaling. The principal findings of the study suggest that IRAK-2 and IRAK-4, but not IRAK-1, are required for MyD88-dependent, TLR-driven signaling in the mouse RAW 264 cell line, whereas IRAK-1 is the predominant IRAK in this pathway in human THP-1 macrophages, with knockdown of IRAK-4 and IRAK-2 having lesser effects on signal transduction.

The Myddosome is thought to assemble in a hierarchical order, with MyD88 being recruited initially to TLRs via interactions between two Toll–interleukin receptor resistance (TIR) domains [2, 3]. IRAK-4 is then recruited to MyD88 through death domain interactions,

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Competing interests

The authors declare no competing interests.

although it was earlier suggested that an intermediate domain within MyD88 was responsible for the interaction of this protein with IRAK-4[4]. Once docked onto MyD88, IRAK-4 enables the recruitment of IRAK-1 or IRAK2, via death domain interactions. Given the process by which the Myddosome is formed, the fact that IRAK-4 expression does not seem to be very important in human THP-1 cells, as revealed by the RNAi screen[1], is highly surprising. However, in subsequent experiments, the researchers did find that human macrophages require IRAK-4 expression, as peripheral blood mononuclear cells (PMBCs) from a patient with IRAK-4 deficiency had significantly decreased TLR4 signaling. These data are in agreement with previously published reports of multiple patients with mutations in *IRAK4*, the gene that encodes IRAK-4 [5–7].

Another point of divergence between human and mouse cells was shown by immunoprecipitation experiments in which murine MyD88 was seen to interact in a rapid and sustained manner with murine IRAK-4, whereas human MyD88–IRAK-4 interactions took longer to form and were more transient. This observation implies that a difference in the manner in which IRAK-4 initially interacts with MyD88 exists between mice and humans, with either the structure of MyD88 or that of IRAK-4 making this initial interaction weaker in humans. A rudimentary comparison of the amino acid sequences of mouse and human MyD88 using the Basic Local Alignment Search Tool (BLAST) showed that the greatest differences in sequence were in the death domains of the proteins.

These differences could explain the finding that the plasmid expression of murine IRAK-4 could not restore TLR signaling to human IRAK4-null cells and vice versa. A more extensive analysis of differences in the sequences of human and mouse MyD88 and IRAK4, confirmed by site-directed mutagenesis, would be required to investigate this hypothesis.

Studies from other research groups have revealed that the murine IRAK-4 possesses both catalytic and scaffolding activities [2]. Sun *et al.* also found that a mutant murine IRAK-4 in which kinase activity had been abolished could not reconstitute TLR signaling in murine IRAK4-null macrophages, whereas a human IRAK4, in which kinase activity had been similarly abolished, was able to mediate TLR signaling in human IRAK-4-deficient macrophages. This observation implies that TLR signalling in mouse cells requires the catalytic and scaffolding functions of IRAK4, whereas the TLR signaling and its effects on gene expression in human macrophages are normal for most TLR-induced genes in the absence of IRAK-4 kinase activity. This finding is somewhat perplexing, as patients with IRAK-4 deficiency often have mutations in the kinase domain of this protein [5]. Such mutations, and particularly those that generate stop codons within the kinase domain, might greatly reduce expression of, truncated IRAK-4 proteins in a patient’s cells. The reduced expression of these proteins could account for the failure of an appropriate response to TLR2 and TLR4 stimulation that occurs in patients with IRAK4 deficiency, and for the striking susceptibility of these patients to repeated infection with Gram-positive bacteria [5, 6]. However, other evidence suggests that IRAK4 proteins with mutations within the kinase domain can be functional. In a study in which two different constructs expressing IRAK4 catalytic mutations, which were found in a patient with a compound heterozygous genotype, were overexpressed in lipopolysaccharide (LPS)-responsive human cells, the mutated

IRAK4 proteins were detectable and behaved as dominant-negative inhibitors of MyD88-dependent signalling, presumably through their scaffolding regions [8].

The analysis by Sun *et al.* was further extended to investigating the role of IRAK-1 and IRAK-2 in mouse and human cells. As previously reported [9], *IRAK2*$^{-/-}$ murine macrophages were highly unresponsive to TLR2 ligands (P3C, PGN) or TLR7 ligand (R848) stimulation, as measured by diminished inflammatory gene induction compared with the WT macrophages, whereas the responses of *IRAK1*$^{-/-}$ murine macrophages to signalling induced by these TLRs were minimally affected. Conversely, IRAK-2 had a minimal role in human macrophages, whereas IRAK-1 was essential for TLR signalling.

Although this study presents us with many provocative findings, its experimental design imposes some limitations on the interpretation of the results. First, the effect of the RNAi knockdown experiments on induction of gene expression was only analysed with regards to the human and mouse genes encoding TNF. TNF is a widely used readout for TLR signal and an important inflammatory mediator, but many other such proteins exist. Unfortunately, the screen does not provide information about the behaviour of MyD88-independent TLR-responsive genes. Second, the TNF reporter gene was expressed from a lentiviral cassette inserted, presumably at random, into the genome. As such, the role of distal transcriptional enhancer elements and any differences in chromatin remodelling, which we now know are an important part of TLR responses, are not possible to analyse. A final limitation of the RNAi screening approach concerns its failure to identify roles for negative regulators of TLR responses. Silencing RNAs directed against several known negative regulators of these responses (IRAK-M, A20, CYLD, etc.) were included in the initial screen, but substantial enhancements of the TNF reporter were apparently not observed upon knockdown of these negative regulators. This lack of effect might result from the time points that were chosen for analysing the responses to RNAi in this screen, although this possibility would need to be investigated in further studies.

In conclusion, the work of Sun *et al.* suggests that potentially important caveats exist when translating structure–function relationships in TLR signaling from animal models to humans. The difficulties encountered in previous attempts to devise small-molecule inhibitors targeting IRAKs for the treatment of human inflammatory disorders [10] highlights the need to take these caveats into account.

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Biographies

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