Validation data for RAW-Dual™ KO-TLR4 cells

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Version 19L23-MM

RAW-Dual™ KO-TLR4 (IRF-Lucia/KI-[MIP-2]SEAP) cells were generated from RAW-Dual™ cells through stable gene knockout of Toll-like receptor 4 (TLR4). These cells derive from RAW 264.7 murine macrophages which express many pattern recognition receptors (PRRs) such as TLRs, the cytosolic DNA sensor (CDS) cGAS, and the cyclic dinucleotide (CDN) sensor STING. RAW-Dual™ and RAW-Dual™ KO-TLR4 stably express NF-κB and interferon regulatory factor (IRF) reporter genes encoding SEAP (secreted embryonic alkaline phosphatase) and Lucia luciferase, respectively. These cells were stimulated with various PRRs ligands, including TLR4 agonists such as the smooth (containing full-length O-antigenic polysaccharide chains) and rough (reduced or absent O-antigenic polysaccharide chains) lipopolysaccharides. Unlike their parental cell line, RAW-Dual™ KO-TLR4 do not respond to TLR4 agonists (Figures 1 & 2). A robust NF-κB activation to TLR2 and TLR7 agonists is observed (Figure 1), while CDNs and murine (but not human) type I interferon (IFN) induce IRF signaling (Figure 2). Interestingly, the response to 2'3'-cGAMP appears to be reduced, possibly due to overlapping gene networks, a phenomenon that appears to occur with other genes (Liu *et al.* 2018).

Ref: Liu Y. *et al.*, 2018. Innate responses to gene knockouts impact overlapping gene networks and vary with respect to resistance to viral infection. PNAS. 115(14):E3230-E7.

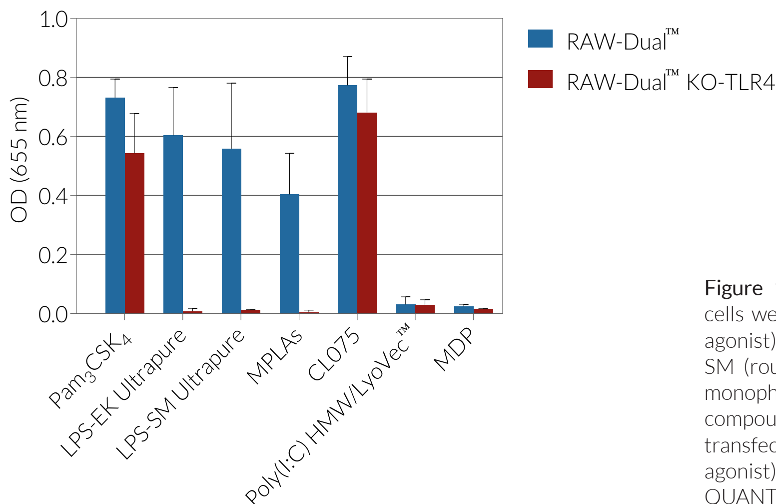

Figure 1: RAW-Dual™ KO-TLR4 cells and RAW-Dual™ (parental cell line) cells were incubated with 1 μg/ml Pam_3CSK_4 (triacylated lipopeptide; TLR2 agonist), LPS-EK Ultrapure (smooth LPS from *E. coli* K12; TLR4 agonist), LPS-SM (rough LPS from *S. minnesota* R595; TLR4 agonist), MPLAs (synthetic monophosphoryl lipid A; TLR4 agonist), 10 μg/ml CL075 (thiazoloquinoline compound; TLR7 agonist), Poly(I:C)/LyoVec™ (dsRNA complexed with transfection reagent; RIG-I agonist) or MDP (muramyldipeptide; NOD1 agonist). After overnight incubation, NF-κB activation was determined using QUANTI-Blue™ Solution, a SEAP detection reagent, and by reading the optical density (OD) at 655 nm.

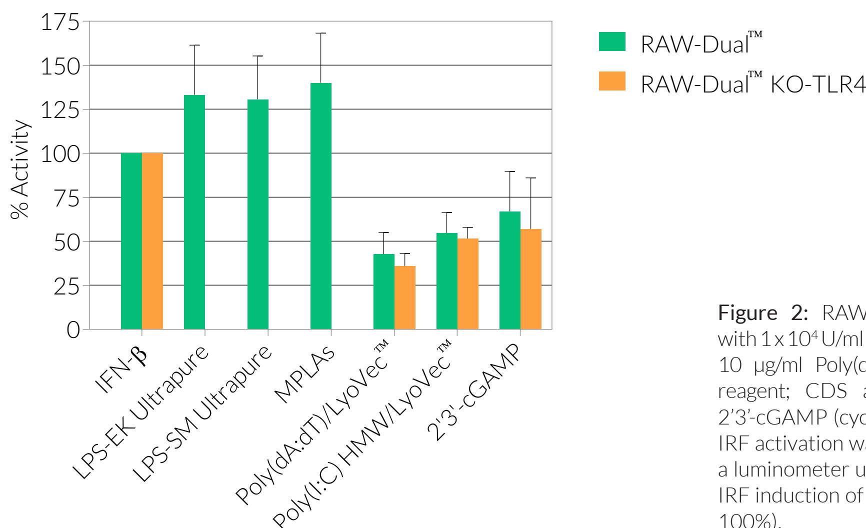

Figure 2: RAW-Dual™ KO-TLR4 and RAW-Dual™ cells were stimulated with 1 x 10^4 U/ml mIFN-β, 1 μg/ml LPS-EK Ultrapure, LPS-SM Ultrapure, MPLAs, 10 μg/ml Poly(dA:dT)/LyoVec™ (dsDNA naked complexed with transfection reagent; CDS agonist), Poly(I:C)/LyoVec™ (RIG-I agonist) or 10 μg/ml 2'3'-cGAMP (cyclic dinucleotide; STING agonist). After overnight incubation, IRF activation was determined by measuring the relative light units (RLUs) in a luminometer using QUANTI-Luc™, a Lucia luciferase detection reagent. The IRF induction of each ligand is expressed relative to that of mIFN-β (taken as 100%).