Study of Cytokine release from Macrophages and Myeloid Dendritic Cells in response to Lipopolysaccharide stimulation

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***Abstract*—Many members of the nucleotide-binding domain leucine-rich repeats (NLRs) play critical roles in pathogen recognition and inflammation. However, our understanding of the implications of most NLRs in the innate immune response remains limited. In particular, the characterisation of NLRP10, the only NLR lacking the putative ligand-binding leucine-rich-repeat domain, continues to be fragmentary. In this study, we analyzed cytokine secretion from lipopolysaccharide activated myeloid dendritic cells and macrophages at varying time points between wild type and knockout (with NLRP10 gene inactivated) mice. Our data revealed that NLRP10 functions as a negative regulator in IRF3-induced expression of IFN-β, but is not involved in early (MyD)88-dependent activation of NF-κB and late phase NF-κB activation controlled by TRIF-dependent signaling pathway. Taken together, our data revealed a novel role of NLRP10 in the innate immune response and provides a basis for drug development and future research targeting NLRP10.**

I. INTRODUCTION

The innate immune system is a sophisticated system that is able to sense signals of "danger", through both pathogenic and host-derived pattern-recognition signals[1]. One of the four major pattern recognition receptor (PRR) families is the nucleotide-binding domain leucine-rich repeats (NLRs). Some members of the NLR family are capable of forming a multi-protein complex, termed inflammasome[2]. The NLRs are composed of 3 subgroups (NOD, IPAF and NLRP). In particular, the NLRP family in human comprises 14 members with similar structure that are located in two clusters on chromosomes 11 and 19[3]. To date, over 20 human NLRs have been identified. However, besides NLRP1 and NLRP3, little is known about the role of other NLRPs in immune response towards pathogens or their potential activity in sensing danger signals. NLRP10 is the only NLR lacking the characteristic leucine-rich repeat domain, and has been thought to be a negative regulator of inflammation and apoptosis[4]. Characterizing the roles of these pattern recognition receptors have proved extremely important in furthering our understanding of various diseases that plague Mankind. With this knowledge, potential applications for cures could be developed. For example, it has been established that NLRP3 mutations are responsible for a continuum of auto inflammatory syndromes ranging from familial cold urticarial and Muckle-Wells syndrome to neonatal onset multisystem inflammatory disease[5].

In this study, we aim to compare the response of mouse macrophages and myeloid dendritic cells (DCs) to lipopolysaccharide (LPS) stimulation through the evaluation of cytokine release at varying time points. The comparison of cytokine release between cells from two types of mice, wild type (WT) and knockout (with NLRP10 gene inactivated) will allow us to gain a greater insight into the role of NLRP10 in the immune response - as a pro-inflammatory or anti-inflammatory agent. The other aim is to find out the molecular mechanisms in which NLRP10 is involved in. LPS, a component of the outer membrane of Gram-negative bacteria, binds to Toll-like receptor 4 (TLR4) on the surface of DCs and macrophages, triggering a few signalling pathways. It is of particular interest that we study these signalling pathways as they have been postulated to be associated with the pathogenesis of various inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease[6]. Within a susceptible individual, aberrant or dysfunctional TLR signaling may impair commensal-mucosal homeostasis, thus contributing to amplification and perpetuation of tissue injury and consequently leading to chronic inflammation in IBD[7]. Therefore, understanding which molecular pathway of TLR4 signalling is NLRP10 involved in could provide more clinical applications.

II. HYPOTHESIS

A. NLRP10 is postulated to have anti-inflammatory properties

This would be evidenced by higher levels of IFN-β (cytokines involved in mounting an early immune response) production after LPS stimulation in KO cells compared to WT cells.

B. NLRP10 interferes with IFN-β activation of NF-κB

Because NF-κB controls many genes involved in inflammation, it is also found to be chronically active in many inflammatory diseases. Many natural products (including anti-

oxidants) that have been promoted to have anti-cancer and anti-inflammatory activity have also been shown to inhibit NF-κB. Since it is postulated that NLRP10 inhibits NF-κB activation, this would result in reduced TNFα release in WT cells compared to KO cells. TNFα is a target gene of NF-κB.

III. Materials and Methods

Mice

12-week old C57Black6 and NLRP10KO from founder 30 were used for these experiments (IACUC #120777). All experiments were performed according to guidelines of NACLAR and AVA.

Culture for Bone Marrow Derived Dendritic Cells (BMDCs)

Culture medium used throughout was IMDM, consisting of 10% Fetal bovine serum (FBS), 10% Granulocyte macrophage colony-stimulating factor (GM-CSF), 1% 1-glutamine and 1% Penicillin Streptomycin

Culture for Bone Marrow Derived Macrophages (BMDMs)

Culture medium used throughout was IMDM, consisting of 10% Fetal bovine serum (FBS), 10% L929, 1% 1-glutamine and 1% Penicillin Streptomycin

Generation of BMDCs and BMDMs

Femurs and tibias from both WT and KO mice were collected after cutting the joints at the level of the hips and knees. Collected bones were moved into fresh and cold PBS/saline solution. A syringe filled with fresh PBS was then inserted inside the epiphyses and flushed inside the bone marrow cavity. The flush was repeated 3-4 times and cells collected in the petri dish were filtered with a strainer in a falcon tube. Cells were resuspended in 5ml of medium and counted (50 million live cells per bone). In order to differentiate BM cells to DCs, 7-8 million BM cells (including red blood cells) were plated in 100mm suspension petri dish in 10ml GM-CSF medium for 7 days, checking differentiation status using Fluorescence-activated Cell Sorting (FACS) with CD11c specific antibodies. MHC class II was also added to the analysis for better identification of DCs. In order to differentiate BM cells into macrophages, same procedure was repeated but in L929 medium.

LPS stimulation

DCs from WT and KO mice were plated in a 96-well plate. 55µl of WT DCs from original solution was added to 1945µl of IMDM to give a final concentration of 750,000 cells/ml. 24.4µl of KO DCs from original solution was added to 1976µl of IMDM to give the same concentration. 100µl of solution was pipetted into each well. DCs were then activated by LPS at a concentration of 100ng/ml. 100µl of LPS solution was added to each well to give a final volume of 200µl, except for the control (untreated with LPS), whereby medium was added instead of LPS. At time points of 1 hour, 2 hours, 3 hours and 4 hours respectively, cells were centrifuged at 1300rpm for 3 minutes and supernatant was collected and stored at -20°C until assayed. 130µl of TRIzol® Reagent was then added to RNA in each well for isolation and stored at -20°C as well. Steps were repeated exactly for macrophages onto a separate 96-well plate.

Acid guanidinium thiocyanate-phenol-chloroform extraction

26µl of chloroform (20% of the volume of TRIzol® sample) was added to each well and the mixture was spun in a microcentrifuge at 4°C, 15,000rpm for 15 minutes. This resulted in phase separation into an upper aqueous phase and a lower organic phase (mainly phenol). The nucleic acids partitioned into the aqueous phase and the top supernatant (transparent part) was immediately removed and transferred into a fresh 1.5ml tube (RNAase free). In order to recover the nucleic acids from aqueous phase by precipitation, equal volume of room temperature 70% ethanol was added, mixed and transferred immediately to RNAeasy (Qiagen) mini spin column, spinning at 10,000rpm for 1 minute at room temperature. 700µl of Buffer RW1 was added onto mini column, centrifuged at 10,000rpm for 1 minute at 25°C and flow-through discarded. 500µl of Buffer RPE mixed with ethanol was then added onto mini column, centrifuged at maximum speed of 15,000rpm for 1 minute at 25°C and flow-through discarded. 500µl of Buffer RPE mixed with ethanol was again added and centrifuged under the same conditions as before but for 2 minutes. To eliminate any chance of Buffer RPE carrying over, RNAeasy columns were centrifuged again at 15,000rpm for 1 minute at 25°C. RNAeasy column transferred to 1.5ml RNAasi free-tube and 40µl of DEPC water was added. The tubes were then centrifuged again at 10,000rpm for 1 minute.

Reverse Transcription Polymerase Chain Reaction

The following recipe was crafted (kit is from Applied Biosystems, Cat# 4368813): 2.0µl 10x RT Buffer, 0.8µl 25x dNTP (100mM), 2.0µl 10x RT Random Primers, 1.0µl MultiScribe Reverse Transcriptase, 1.0µl RNase Inhibitor and 13.2µl of RNA sample. The RT-PCR reaction is as follows: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C from then on.

Quantitative Real-time Polymerase Chain Reaction

RT-PCR product (cDNA) was then diluted to a concentration of 5ng/8.8µl. 8.8µl of sample was added to each well already containing 10µl 2x master mixture, 0.6µl primer FW IFN-β 5′TCAGAATGATGGTGGTTGC3′ and 0.6µl primer RV IFN-β 5′GACCTTTCAAATGCAGTAGATTCA3′. Plate was spun at 1000rpm briefly and kept at 4°C in dark until running program on real time PCR machine.

Enzyme-linked Immunosorbent Assay

Buffers: Coating buffer (0.1M Bicarbonate buffer, pH 9.6), PBS (100mM phosphate buffer, pH 7.4, 8.0g NaCl, 1.16g Na2HPO4, 0.2g KH2PO4, 0.2g KCl, add deionised water to 1L; pH to 7.4), Blocking buffer (PBS buffer with pH 7.4, 2% BSA), Wash buffer (PBS buffer with 0.05% Tween-20), Stop solution (1.0M H2SO4)

A capture antibody was first diluted in coating buffer at a final concentration of 2µl/ml and then 50µl was added to each well of the microtiter plate. The antibody coated plate was covered with Parafin and then incubated in the cold room

overnight. The next day, the plate was emptied and the unoccupied sites were blocked with 150µl of blocking buffer for 2 hours at room temperature. The plate was emptied and washed once with wash buffer. The antigen solution was first diluted in blocking buffer and then added to the plate in a volume of 50µl per well. The plate was incubated at room temperature for 3 hours. The plate was emptied again and washed thrice with wash buffer. The detection antibody against antigen was diluted in blocking buffer at a final concentration of 0.5µg/ml and then 50µl was added to each well and incubated at room temperature for 1 hour. The plate was emptied again and washed thrice with wash buffer. 50µl of diluted avidin-HRP solution (1:1000) was added with blocking buffer to each well, and incubated at room temperature for 30 minutes. Plate was washed 4 times with wash buffer. 50µl of TMB substrate solution was then added and incubated in the dark for 15 minutes. The reaction was stopped by adding 50µl of stop solution to each well. Positive wells should turn from blue to yellow.

IV. Results and Discussion

We generated DCs and macrophages by culturing bone marrow from wild type and knockout mice. We stimulated these cells with a bacterial endotoxin, LPS, for different periods of time (0hr untreated, 1hr, 2hr, 3hr and 4hr). RNA was then extracted from these cells, reversed transcribed to cDNA and real-time PCR was performed for detection of IFN-β as previously described. IFN-β is an example of a Type I interferon that plays an important role in the innate immune response by upregulating and downregulating a number of genes.

Fig. 1 shows that IFN-β mRNA levels in KO DCs increased significantly from the time it was stimulated with LPS and continued to increase until 2hrs post-stimulation. IFN-β mRNA levels were similar between WT and KO mice at early time points (0-1 hours) but it started to decrease in WT mice from 1 hour onwards. IFN-β expression in KO mice was also almost 2-fold higher than in WT mice at their respective peaks of 2 hours and 1 hour. Virtually identical results were obtained using macrophages as shown in Fig. 2, with IFN-β mRNA levels in KO mice increasing until a later time point of 2 hours as compared to WT mice. These results suggest that NLRP10 functions in inhibiting the production of IFN-β, substantiating the claims that NLRP10 is an anti-inflammatory NLR.

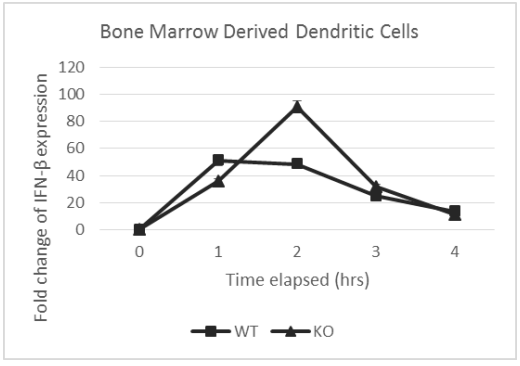

Figure 1. Line graph depicting fold change of IFN-β mRNA expression in BMDCs following LPS stimulation. IFN-β mRNA levels were detected using RT-PCR on cDNA.

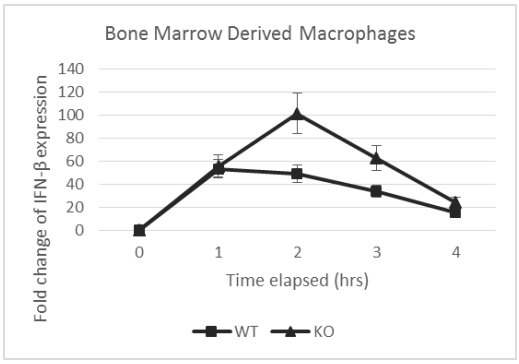

Figure 2. Line graph depicting fold change of IFN-β mRNA expression in BMDMs following LPS stimulation. IFN-β mRNA levels were detected using RT-PCR on cDNA.

LPS binds to TLR4 on the cell membrane, activating myeloid differentiation factor (MyD)88-dependent and (MyD)88-independent TLR4 signalling via different adaptor proteins, as shown in Fig. 3. The first pro-inflammatory cytokine under investigation is IFN-β, and it is produced through the MyD88-independent pathway. In unstimulated cells, interferon regulatory factor 3 (IRF3) is primarily present in the cytoplasm in an inactive form. However, stimulation with TLR4 ligands causes TBK1- and IKKi-mediated phosphorylation of the C-terminal regions of IRF3. This allows IRF3 to form a homodimer, translocate into the nucleus and bind its target sequences, such as IFN-stimulated response element (ISRE). In the nucleus, IRF3 forms a multiprotein complex called an enhanceosome, which binds the promoter–enhancer region of the IFN-β gene[8]. IFN-β production is induced transiently, peaking at around 2 hours, before being repressed progressively. This explains the trend of both IFN-β mRNA levels observed in WT and KO DCs and macrophages, accounting for the dramatic increase in mRNA levels immediately post-stimulation and the subsequent decline after a prolonged period of time. However, the production of IFN-β peaked and started to decline earlier for cells with NLRP10, suggesting that inflammation was reduced or regulated to a greater extent. This reaffirms the hypothesis that NLRP10 does aid in anti-inflammation post-stimulation.

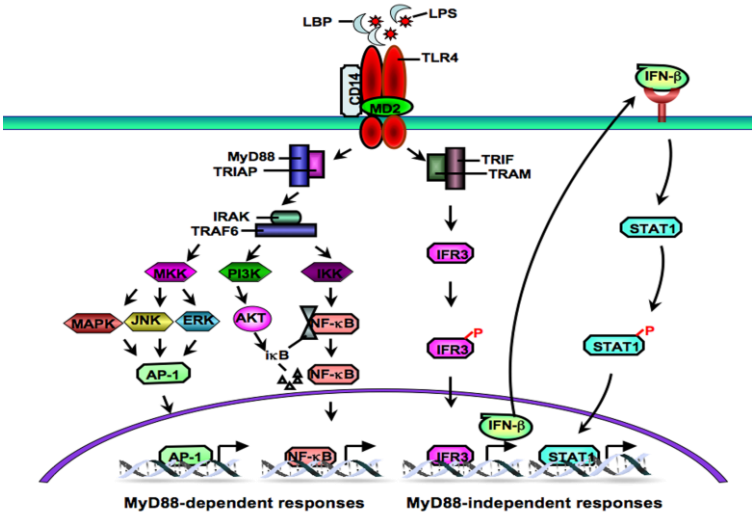

Figure 3. Schematic overview of Toll-like receptor 4 signalling pathway. LPS binds to TLR4 on the cell membrane, activating myeloid differentiation factor (MyD)88-dependent and (MyD)88-independent TLR4 signalling via different adaptor proteins.

Preliminary experiments involving β-carboline derivatives, which have been shown to inhibit IRF3, resulted in similar cytokine release in KO cells (treated with β-carboline) compared to untreated WT cells (untreated). This suggests that NLRP10 similarly plays a role in inhibiting IRF3 during production of IFN-β via MyD88- independent responses.

In order to investigate if NLRP10 interferes with IFN-β activation of NF-κB, we must first understand the process of activation in TLR4 signaling. MyD88 is composed of a TIR domain and a death domain. Upon TLR activation, through its death domain, MyD88 interacts with the death domains of members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases, including IRAK1, IRAK2, IRAK4 and IRAK-M[9]. IRAK4 is initially activated, which in turn phosphorylates and activates IRAK1. After IRAK4 and IRAK1 have been sequentially phosphorylated, they dissociate from MyD88 and interact with TRAF6. TRAF6 is a RING domain E3 ubiquitin ligase, and together with E2, Ubc13 and Uev1A, it promotes Lys63-linked polyubiquitination of target proteins, including TRAF6 itself and NEMO[10]. Lys63-linked ubiquitination is involved in protein interactions, activation of signaling pathways and subcellular localization. Ubiquitinated NEMO and TRAF6 subsequently activates a distinct pathway involving the IKK complex. IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein[11]. TNFα is a target gene of NF-kB activation[12].

In TRIF-deficient cells, early activation of NF-κB in response to LPS is normal. However, MyD88 and TRIF double deficiency results in a complete loss of NF-κB activation in response to LPS, which indicates that the late phase NF-κB activation is controlled by the TRIF-dependent pathway[12,13]. Collectively, these results suggest both MyD88- and TRIF-dependent pathways are required for induction of NF-κB in TLR4 signalling, unlike in signalling via other TLRs in which the activation of either MyD88- or TRIF-dependent pathways is sufficient for cytokine induction. IFN-β induces NF-κB activation through a "canonical" pathway of IκBα proteolysis mediated by PI3K/Akt and a "noncanonical" pathway of p100 processing mediated by NIK/TRAF[13]. Therefore, more IFN-β production would subsequently induce more TNFα production.

To test if NF-kB activation was affected in the absence of NLRP10, we determined the concentrations of TNFα, a target gene of NF-κB, using TNFα-specific ELISA on the supernatants collected earlier.

The mean absorbance for each set of triplicate standards, controls and samples were calculated. The estimation of TNFα concentration was then obtained based on the standard curve, as shown in Fig. 4. The standard curve was prepared by making serial dilutions of known concentrations of TNFα. To determine unknown TNFα concentrations in a sample, the mean absorbance value's corresponding cytokine concentration was read off from the standard curve.

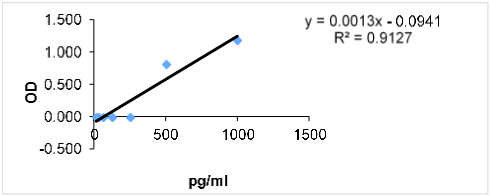

Figure 4. ELISA standard curve from TNFα

There was an upward trend in the concentrations of TNFα from the beginning to 4 hours. Since IFN-β mRNA levels were substantially higher for KO mice than WT mice in both DCs and macrophages, it should be expected that the amount of TNFα would also be greater. Figs. 5 and 6 show that TNFα is indeed higher in KO DCs and macrophages at all time points. This includes the 1 hour time point, even though IFN-β mRNA levels were marginally higher in WT than KO. IFN-β mRNA levels corresponded to relative TNFα concentrations in WT and KO mice, suggesting that NLRP10 is not involved in early (MyD)88-dependent activation of NF-κB and late phase NF-κB activation controlled by TRIF-dependent signalling pathway. Untreated WT and KO DCs and macrophages produced similar amounts of TNFα. This indicates that NLRP10 functions as a negative regulator in IRF3-induced expression of IFN-β, as difference in TNFα concentrations between WT and KO DCs and macrophages is only observed in the presence of LPS.

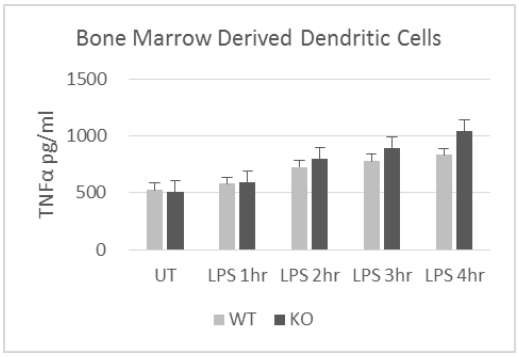

Figure 5. Bar graph depicting TNFα concentrations in pg/ml released by BMDCs stimulated with LPS for different time periods.

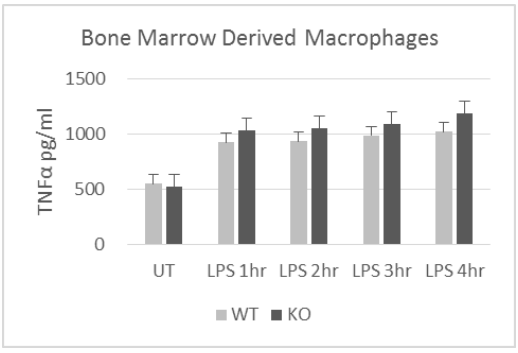

Figure 6. Bar graph depicting TNFα concentrations in pg/ml released by BMDMs stimulated with LPS for different time periods.

Stimulation of WT and KO cells with other types of TLR agonists results in similar concentration levels of TNFα at all time points, suggesting that NLRP10 is involved in LPS signalling pathways (TLR4). These data evidently indicates that NLRP10 is irrelevant in affecting IFN-β activation of signal transducers and transcription activators or factors, in particular NF-κB activation (triggered by Toll pathway).

Comparing TNFα concentrations between DCs and macrophages, it is also clear that significantly more TNFα was produced by macrophages. This is unsurprising given the fact that macrophages are the predominant source of TNFα production in an immune response.

V. Conclusion

The results obtained suggest that it is time to re-examine views about the role of NLRP10 in an immune response. Currently, there are two sets of conflicting hypotheses regarding NLRP10 – whether they induce pro-inflammatory or anti-inflammatory responses[14]. From this experiment, it is clear that NLRP10 is a negative regulator of IFN-β cytokine secretion, which is a key player in mounting an early immune response. NLRP10 deficient DCs and macrophages had increased levels of IFN-β mRNA and subsequently increased concentrations of TNFα production. Therefore, these data seem to affirm the hypothesis that NLRP10 functions in anti-inflammatory through the negative regulation of IRF3-induced expression of IFN-β.

With this knowledge of the role of NLRP10 in the innate immune response, it will form the basis for future research involving therapeutic applications. For example, enhanced production of type I IFN is associated with the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus. Pathways suppressing this synthesis will be important drug targets for treating autoimmune diseases[15]. Stimulatory ligands of NLRP10 may also be useful adjuvants to treat allergies and tumours, fostering new anti-inflammatory therapeutic approaches[16].

Toll-like receptor 4 has been shown to be important for the long-term side-effects of opioid analgesic drugs. Various μ-opioid receptor ligands have been tested and found to also possess action as agonists or antagonists of TLR4, with opioid agonists such as morphine being TLR4 agonists, while opioid antagonists such as naloxone were found to be TLR4 antagonists[17]. Activation of TLR4 leads to downstream release of inflammatory modulators such as TNFα. Constant low-level release of these modulators is thought to reduce the efficacy of opioid drug treatment with time, be involved in both the development of tolerance to opioid analgesic drugs, and in the emergence of side-effects such as hyperalgesia and allodynia that can become a problem following extended use of opioid drugs[18]. Since NLRP10 has shown promise as a negative regulator of IRF3-induced expression of IFN-β, it can be the subject of further studies to improve the efficacy of these opioid analgesic drugs.

VI. Acknowledgments

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VII. References

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