［課程―２］

審査の結果の要旨

氏名：陳 美寶

This study aims to examine the role of the lysosomal DNase, DNase II, in Toll-like receptor 9 (TLR9) DNA recognition and responses. The well-characterized TLR9 agonists, CpG-A and CpG-B were used in this study to activate TLR9. The two agonists have different properties in that CpG-A is a potent stimulator of type I interferon (IFN) production and can form nanoparticles inside the cell, but not for CpG-B. Due to the importance of TLR9 in host defence against virus/bacteria, and that CpG DNA is also widely used in vaccines as adjuvant, the role of DNase II in TLR9 responses as stimulated by CpG DNA was examined in this study. Using the *Dnase2a*$^{-/-}$*Ifnar1*$^{-/-}$ conventional double knockout mice (dKO) and the *Dnase2a*$^{flox/flox}$*Tie2-Cre* conditional knockout (KO) mice, the following results were obtained:

1. The requirement of DNase II in triggering TLR9-mediated responses was first examined. Bone marrow-derived conventional dendritic cells (BM-cDCs) obtained from dKO or conditional KO mice were stimulated with CpG-A or CpG-B, and cytokine production was measured by ELISA. The results showed that production of IL-12p40 and RANTES after CpG-A stimulation was significantly impaired, but the response to CpG-B was not affected. Similarly, type I IFN production by bone marrow-derived plasmacytoid dendritic cells (BM-pDCs) after CpG-A stimulation was also significantly impaired, with CpG-B responses being intact. These results suggested that in BM-cDCs and BM-pDCs, DNase II was required for TLR9 to respond to CpG-A.
2. To examine the underlying mechanism for the above observation, CpG DNA uptake in the BM-cDCs obtained from dKO mice was monitored by flow cytometry analysis. The amount of fluorescent-labeled CpG-A or CpG-B taken up into the cells after incubation was not different. This showed that CpG DNA uptake was not causing the difference in cytokine production.
3. Since DNase II may be involved in digesting CpG DNA after DNA internalization, the requirement of DNase II activity in TLR9 stimulation was investigated. Two histidine active sites DNase II mutants (H115A and H297A) were generated by site-directed PCR mutagenesis. The plasmid vector encoding the WT or mutant DNase II was transduced into the bone marrow stem cell-derived cDCs obtained from dKO mice. The reconstituted cDCs were stimulated with CpG-A and the production of IL-12p40 and RANTES was measured by ELISA. Only WT DNase II, but not the H115A or H297A DNase II mutant, was able to rescue cytokine production in the cells. This showed that the enzymatic activity of DNase II was required to confer the

TLR9 responsiveness to CpG-A.

4. The requirement of DNase II enzymatic activity indicated that DNase II may function to cleave or digest CpG-A before the DNA can activate TLR9. Purified WT, H115A or H297A DNase II protein was incubated with CpG-A or CpG-B in DNase II reaction buffer for 24 hours. The length of the CpG DNA after incubation was analyzed by Tris/Borate/EDTA (TBE) gel electrophoresis. Only CpG-A that was incubated with the WT DNase II protein revealed a band of shorter length. This was not observed when CpG-A was incubated with mutant DNase II, or when CpG-B was incubated with WT or mutant DNase II protein. Truncated CpG-A-like fragments composed of 9 or 11 nucleotides from the 5'or 3' end of the original full-length CpG-A sequence were synthesized. Running these truncated CpG-A DNA in parallel with the cleaved CpG-A by WT DNase II showed that the cleaved fragment was about 11 nucleotides in length. Among the 4 synthetic truncated CpG-A fragments (A5'9, A5'11, A3'9, A3'11), one of the fragments, the A3'11 CpG-A, was able to stimulate DNase II-deficient BM-cDCs to produce comparable amount of cytokine IL-12p40 as the control cells. This suggested the possibility that that the A3'11 fragment, which showed similar length as the cleaved product of CpG-A, may mimic the DNase II-cleaved product and stimulated TLR9 even in the absence of DNase II.

5. To confirm that DNase II was only required in CpG-A stimulation, but not in CpG-B or A3'11 fragment stimulation, subcellular localization of the endogenous DNase II was examined. Since monoclonal antibody against the murine DNase II was not available, it was generated in this study. Using the DNase II-specific monoclonal antibody, it was found that only CpG-A induced the recruitment of DNase II into LAMP2-positive lysosomal compartment in a time-dependent manner in BM-cDCs. When CpG-B or A3'11 fragment was added, no trafficking of DNase II to the LAMP2-positive lysosome was observed. The localization of fluorescent CpG DNA was also investigated. The results showed that only the full-length CpG-A was found to colocalize with the LAMP2 lysosomal marker. This suggested that DNase II and CpG-A was also found in the same compartment after stimulation. As DNase II has optimal activity in acidic environment, trafficking to the lysosome may facilitate cleavage of CpG-A for TLR9 activation.

The findings from this study demonstrated an unappreciated role of DNase II in cleaving TLR9 agonist essential for proinflammatory cytokine and IFN production. Such findings may contribute to the better understanding of TLR9-mediated DNA recognition, and implied a potential role of DNase II in vaccines that employ CpG-A DNA as adjuvant to enhance TLR9 responses. This work is worth for the conferral of a Ph.D. degree.