Synopsis of Thesis

Thalidomide inhibits TLR induced Cytokine production by preventing the Cereblon mediated recruitment of Rabex5 to the early endosome

(サリドマイドの抗炎症作用は、IRF-3の活性化に必要はCereblon-Rabex-5複合体の形成阻害により発揮される)

The Teratogenic and therapeutic properties of Thalidomide are dependent on Cereblon. While the precise function of Cereblon remains unknown, the properties of Thalidomide suggest a potential role in immunity. In particular, Thalidomide is known to inhibit the TLR4 induced production of TNFα through an unknown mechanism. We therefore investigated the role of Cereblon in TLR4 induced Cytokine production.

Mouse peritoneal macrophages were treated with Pomalidomide, a potent derivative of Thalidomide, one hour before stimulation with LPS. Pomalidomide strongly inhibited the LPS induced production of TNFα and IL12, with a modest effect on IL6, at both the protein and mRNA level. Inhibition was not limited to a single Cytokine, suggesting that signaling pathways activated downstream of TLR4 are disrupted. Two distinct pathways are activated by TLR4, each dependent on a different adaptor protein, MYD88 and TRIF. In order to discern which pathway is affected, LPS responsive HEK293 cells were transfected with reporter constructs for NFκB, AP-1 and IRF3. While NFκB and AP-1 are predominantly MYD88 dependent, IRF3 is activated downstream of the TRIF pathway. Pomalidomide completely inhibited LPS induced IRF3 activation, with a modest effect on NFκB. This is consistent with the selective inhibition of the TRIF dependent pathway downstream of TLR4 .

In order to investigate the function of Cereblon, RAW264.7 cells were transfected with shRNA constructs and maintained under selection until a stable cell line was obtained. Cereblon deficient RAW264.7 cells produced significantly less TNFα, IL6 and IL12 in response to TLR4 activation. Interestingly, TNFα production induced by TLR3, which also signals through the TRIF adaptor, was not affected. With this exception, Cytokine production, in addition to the activation of IFN inducible genes, was broadly inhibited by Cereblon deficiency following stimulation with agonists for TLRs 2, 4, 7 and 9.

Following receptor ligation, TLR4 is ubiquitinated and targeted to the lysosomal pathway for degradation. The activation of IRF3 is known to be dependent on this intracellular trafficking process. We therefore investigated whether Cereblon is involved in the transport of the TLR4 receptor. RAW264.7 cells were transfected with a TLR4-GFP construct and the lysosomal compartment stained using Lysotracker®. In Cereblon deficient RAW264.7 cells, the TLR4 receptor failed to localise to the lysosome following stimulation with LPS. The same effect could be observed using Pomalidomide.

The lysosomal pathway is regulated by the Rab family of GTPases. We identified significant homology between Cereblon and Rabex5, a GDP/GTP exchange factor with activity specific to Rab5. Activation of Rab5, involved in the endosomal maturation process, requires the recruitment of Rabex5 to the early endosome. Using Co-Immunoprecipitation experiments, we demonstrated an interaction between Cereblon and Rabex5, which was disrupted by Thalidomide. Given that Thalidomide is known to inhibit the auto-ubiquitination of Cereblon and that Rabex5 contains a ubiquitin binding domain, it is likely that Rabex5 binds to ubiquitin chains conjugated to Cereblon, which are disrupted by Thalidomide.

In summary, we have identified a novel mechanism to explain Thalidomide's anti-inflammatory activity. By inhibiting Cereblon auto-ubiquitination, Thalidomide blocks the recruitment of Rabex5 to the early endosome, inhibiting the lysosomal pathway. In the context of TLR4 signalling, this prevents the activation of IRF3, resulting in reduced Inflammatory Cytokine and type 1 interferon production.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

Thalidomide is an immunomodulatory drug and used for several diseases including multiple myeloma. It also shows several adverse effects such as teratogenic potency. Therefore, it is very important to elucidate the molecular mechanisms by which Thalidomide functions. David Millrine has investigated the anti-inflammatory properties of Thalidomide, which remain poorly understood. Thalidomide derivatives selectively inhibited the MYD88-independent pathway downstream of a lipopolysaccharide (LPS) sensor, TLR4. Consequently, the activation of a transcription factor, IRF3, and the production of type-1 interferons, which are regulated by the MYD88-independent pathway, was inhibited by these compounds. Thalidomide has recently been shown to bind directly to Cereblon, part of a multi-subunit E3 ubiquitin ligase complex. Using the stable transfection of Cereblon targeting shRNA, David Millrine demonstrated that Cereblon is required for TLR4 induced signal transduction, including but not limited to the MYD88-independent pathway. To identify a precise function of Cereblon, amino acid sequences of Cereblon were compared to an online database leading to the identification of Rabex-5, a known regulator of endosomal trafficking, as sharing significant homology to Cereblon. Analysis by confocal microscopy revealed that, like Rabex-5, Cereblon localizes to Rab5 positive vesicles. Cereblon could physically associate with Rabex-5 and this association was inhibited by Thalidomide in a dose dependent manner. Rabex-5 knockdown inhibited inflammatory cytokine production *via* the MYD88 independent pathway, consistent with the observed effects of Thalidomide. Thus, David Millrine has, for the first time, demonstrated that Thalidomide inhibits inflammatory cytokine production *via* disruption of the Cereblon-Rabex-5 interaction. Although the molecular mechanisms by which cereblon and Rabex-5 function remains unclear, the findings are novel, very important and should shed light on the clarification of the positive and negative effects of Thalidomide.