Genes & Diseases (2023) 10, 344–347

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RAPID COMMUNICATION

MyD88 in macrophages protects against colitis via inhibiting the activation of NLRP3 inflammasome in epithelial cells

The signal adaptor myeloid differentiation primary response 88 (MyD88) of Toll-like receptor (TLR) signaling is universally expressed in immune cells and non-immune cells, and myeloid cells play a significant role in modulating colitis. Macrophages are myeloid lineage cells which is important for maintaining intestinal homeostasis in inflammation.[1] And macrophages can recognize invading pathogens through pattern recognition receptors (PRRs), such as TLRs and the nucleotide oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3), and quickly infiltrate the injured site leading to inflammation.[2] MyD88 expression in myeloid cells can rescue the intestinal injury induced by dextran sulfate sodium (DSS) in murine models. However, the mechanism whereby MyD88 works in myeloid cells and influence the progress of the disease remain elusive. In this study, we used DSS induced colitis mouse model and found Lysm-cre-MyD88$^{fl/fl}$ (MyD88$^{\Delta M\Phi}$) mice were more susceptible to colitis. The deficiency of *MyD88* leads to up-regulation of S100A8, which activate NLRP3 inflammasome and their associated pyroptosis in intestinal epithelial cells in a RAGE dependent manner. Thus, MyD88 signaling in macrophages, which is necessary to maintain intestinal homeostasis, crucially prevents the development of colitis. Our results may shed new lights on potential targets that can be applied in colitis therapy.

MyD88 expression in macrophages is upregulated in murine model of colitis. C57BL/6 mice were orally administrated with 2.5% DSS for colitis induction (Fig. 1A). Inflammation and tissue damage were observed in colitis tissues (Fig. 1B). The number of F4/80^{+} macrophages and MyD88 expression is increased significantly in colitis tissues compared with that from untreated mice. Double immunofluorescence staining also revealed that MyD88 was highly expressed in F4/80^{+} macrophages in colitis tissues (Fig. 1C). Furthermore, protein levels of MyD88 and CD68 were both upregulated in colitis tissues compared with the control group detected by Western blot (Fig. 1D).

We then crossed mice carrying the loxP-flanked *MyD88* allele with Lysm-cre mice to achieve specific *MyD88* ablation in myeloid cells. The MyD88$^{\Delta M\Phi}$ mice, which were viable and fertile, were identified by PCR (Fig. S1A). Their littermate single-transgenic mice were used as control (MyD88$^{fl/fl}$) mice. And there were no differences in H&E staining and inflammatory cell infiltration in colon tissues of drinking water treated MyD88$^{\Delta M\Phi}$ mice and MyD88$^{fl/fl}$ mice (Fig. S1B–G).

Then, our results demonstrated that MyD88$^{\Delta M\Phi}$ mice were more susceptible to DSS induction. On day 8 and 10, the weight loss and disease score in MyD88$^{\Delta M\Phi}$ mice were much higher than those in MyD88$^{fl/fl}$ mice (Fig. 1E; Fig. S2A). Consequently, myeloid-specific *MyD88* knockout significantly increased the mortality of mice after DSS induction (Fig. S2B). In addition, the colon length in MyD88$^{\Delta M\Phi}$ mice was shorter than that in MyD88$^{fl/fl}$ mice on day 10 (Fig. 1F). Consistently, MyD88$^{\Delta M\Phi}$ mice exhibited much severer colonic damage and more inflammatory cell infiltration compared to those in MyD88$^{fl/fl}$ mice on day 10 (Fig. S2C–E).

Furthermore, we performed protein-coding mRNA-seq analysis of colon tissues from MyD88$^{\Delta M\Phi}$ mice and MyD88$^{fl/fl}$ mice after colitis induction. A total of 4236 differentially expressed genes (DEGs) were identified, including 3352 up-regulated and 884 down-regulated genes (Fig. S3A; padj <0.01). Among these up-regulated genes, the upregulation of *S100A8* is most significant in DSS-treated MyD88$^{\Delta M\Phi}$ mice compared with that in MyD88$^{fl/fl}$ mice (Fig. 1G, H). We also analyzed the public GEO data set GSE75214, and found that *S100A8* expression was dramatically upregulated in the colon tissues of patients with ulcerative colitis and Crohn's disease compared with healthy individuals (Fig. 1I).

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.04.021

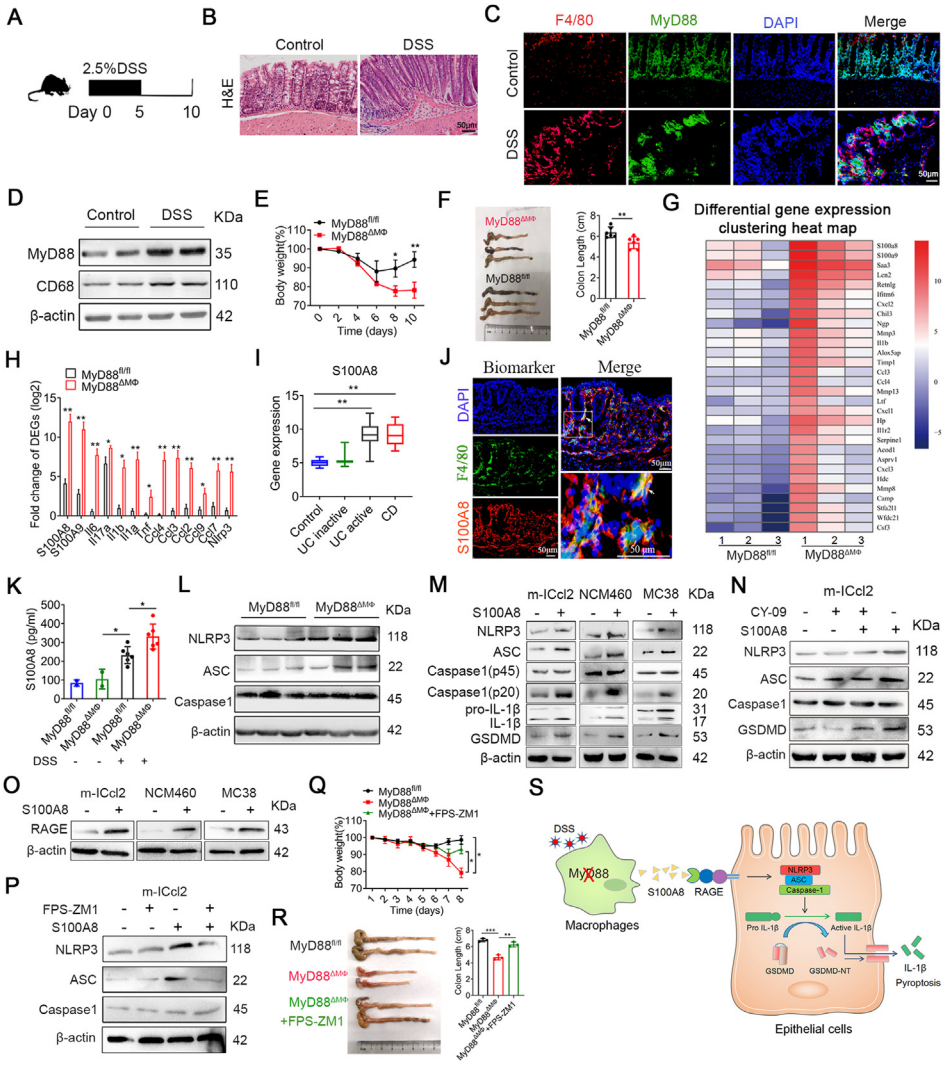

Figure 1 Deficiency of *MyD88* in macrophages leads to up-regulation of S100A8, which activate NLRP3 inflammasome and their associated pyroptosis in intestinal epithelial cells in a RAGE dependent manner. **(A)** Schematic representation of the DSS-induced colitis model. Groups of C57BL/6 mice were administered water or 2.5% DSS for 5 days to induce acute colitis, followed by a 5-day recovery period on normal drinking water ($n = 6$ per group). **(B)** H&E staining of colon specimens (scale bars: 50 μm). **(C)** Representative double-staining and statistical analysis of F4/80 (red) and MyD88 (green) in colon tissues (scale bar: 50 μm). **(D)** The expression levels of MyD88 and CD68 proteins in colitis tissues were determined by Western blot analysis. MyD88 were normalized to β-actin. **(E)** Body weight changes were monitored during the procedure of colitis. **(F)** Representative photographs of colon specimens from $MyD88^{fl/fl}$ and $MyD88^{\Delta M\Phi}$ mice on day 10 following DSS treatment. Colon lengths were measured on day 10. **(G)** Heatmap view of gene expression of the most significant up-regulated genes. **(H)** Analysis of fold change of DEGs. **(I)** The expression of S100A8 in colon tissues from ulcerative colitis (UC) and Crohn's disease (CD) patients in the colon dataset GSE75214. Control: $n = 11$; UC inactive: $n = 23$; UC: $n = 74$; CD: $n = 8$. **(J)** Double immunofluorescence staining for S100A8 (red) and F4/80 (green) in colitis sections (scale bars: 50 μm). Arrowhead indicate the double-staining cells. **(K)** The secretory protein levels of S100A8 in DSS treated macrophages were measured by ELISA. **(L)** The expression levels of NLRP3 inflammasome-associated assembly protein in colitis tissues were determined by Western blot analysis ($n = 3$). **(M)** 500 ng/ml S100A8 treated mouse intestinal epithelial cell line m-ICcl2, human colon epithelial cell line NCM460 and mouse colon cancer cell MC38 for 24 h; the expression levels of NLRP3 inflammasome-associated protein and its related pyroptosis markers were detected by Western blot analysis. **(N)** After pretreatment of m-ICcl2 cells with or without CY-09 (NLRP3 inhibitor) for 2 h, 500 ng/ml S100A8 recombinant protein was incubated for 24 h, and the expression of GSDMD was detected by Western blot analysis. **(O)** Intestinal epithelial cell lines m-ICcl2 cells, NCM460 cells and MC38 cells were stimulated with 500 ng/ml S100A8 recombinant protein for 24 h, and RAGE protein level was determined by Western blot analysis. RAGE was normalized to β-actin. **(P)** m-ICcl2 cells were pretreated with or without FPS-ZM1 (RAGE inhibitor), and then treated with S100A8 for 24 h, and the expression levels of NLRP3 inflammasome-related proteins were detected by Western blot analysis. NLRP3 and ASC were normalized to β-actin. **(Q, R)** Groups of $MyD88^{fl/fl}$ and $MyD88^{\Delta M\Phi}$ mice ($n = 3$ per group) were treated with DSS for colitis and were injected intraperitoneally with FPS-ZM1 (1 mg/kg) or PBS as control every day, starting from day 0. The body weight change (Q) and representative photographs and length of the colon (R) from 3 group mice on day 8 are shown. **(S)** Schematic of MyD88 signaling in macrophages in DSS-induced colitis model. DEGs, differentially expressed genes. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Furthermore, as shown in Figure S3B, the number of S100A8^{+} cells in colon tissues of MyD88$^{\Delta M\Phi}$ mice significantly increased in colitis tissues. Double immunofluorescence staining further revealed that S100A8 was highly expressed in most of F4/80^{+} cells and only a small number of Gr1^{+} cells in the colon tissues, demonstrating that S100A8 is mainly expressed in macrophages (Fig. 1J; Fig. S3C). Moreover, mouse primary macrophages were successfully isolated and the absence of MyD88 in myeloid cells was validated by double immunofluorescence staining in the MyD88$^{\Delta M\Phi}$ mice (Fig. S3D). Consistently, MyD88 significantly attenuated the RNA and protein levels of S100A8 in DSS-induced macrophage (Fig. S3E; Fig. 1K). These results indicate that macrophage-derived S100A8 may play important roles in the process of colitis.

NLRP3 was upregulated in colitis tissues of MyD88$^{\Delta M\Phi}$ mice based on RNA sequencing analysis (Fig. 1H). The number of E-cadherin and NLRP3 double-positive cells in MyD88$^{\Delta M\Phi}$ colon tissues significantly increased compared with that in littermate controls in colitis (Fig. S4A). In addition, NLRP3 and ASC, which are involved in the assembly of inflammasomes, were significantly up-regulated in colon tissues from MyD88$^{\Delta M\Phi}$ mice (Fig. 1L). Emerging evidences indicate that caspase 1 can proteolytically cleave the precursors of the inflammatory cytokines, such as interleukin-1β (IL-1β) and gasdermin D (GSDMD), into mature and active forms, and ultimately initiates cell pyroptosis.[3] Similar results were observed that the expression of NLRP3, ASC and caspases 1p20, IL1β, GSDMD in intestinal epithelial cells was significantly upregulated in response to S100A8 stimulation (Fig. 1M). It is also showed that a number of giant vesicles appeared in the perinuclear region in NCM460 cells stimulated with S100A8 (Fig. S4B). Moreover, the results of immunofluorescence staining demonstrated that S100A8 stimulation significantly enhanced the activation of GSDMD suggesting the occurrence of pyroptosis in NCM460 cells (Fig. S4C). To further investigate whether GSDMD in epithelial cells was associated with NLRP3 inflammasome activation, epithelial cells were pretreated with NLRP3 inhibitor and then treated with S100A8 recombinant protein for 24 h. It was found that NLRP3 inhibitor CY-09 specifically inhibited NLRP3 protein expression, and reduced GSDMD activation induced by S100A8 (Fig. 1N; Fig. S4D). The results indicated that activation of NLRP3 inflammasome was necessary for S100A8-induced pyroptosis in intestinal epithelial cells.

Then we examined whether S100A8-induced NLRP3 inflammasome and pyroptosis in intestinal epithelial cells depend on RAGE. The expression levels of RAGE are very low in m-ICcl2 and MC38 cells cultured without S100A8 protein, but they are clearly enhanced upon S100A8 stimulation (Fig. 1O). And NLRP3 and ASC could not be activated by S100A8 stimulation after RAGE-specific inhibitor FPS-ZM1 treatment (Fig. 1P; Fig. S5A, B). Then, we targeted S100A8 by RAGE inhibitor to examine the therapeutic value of S100A8 in mouse colitis model. We found that FPS-ZM1 significantly alleviated the disease severity in MyD88$^{\Delta M\Phi}$ mice, according to the body weight, disease activity and colon length (Fig. 1Q, R; Fig. S5C). Furthermore, evidence from H&E staining revealed that the colon of FPS-ZM1 group had mild inflammation compared with that of MyD88$^{\Delta M\Phi}$ group (Fig. S5D). Results from double immunofluorescence staining for E-cad and NLRP3 showed that NLRP3 activation in epithelial cells was significantly inhibited by FPS-ZM1 (Fig. S5E). Thus, these data indicated that targeting S100A8 by RAGE inhibitors could be an efficient treatment for colitis.

TLR signaling pathway plays an important role via MyD88 in protecting from colitis. *MyD88*-deficient mice develop severe intestinal inflammation in DSS-induced colitis.[4] As demonstrated by evidence from murine models, myeloid cells are involved in colitis regulation. Especially, MyD88 expression in myeloid cells is sufficient to rescue the intestinal injury in colitis.[5] From this study, our research provides a better understanding for the specific role of MyD88 in macrophage in intestinal bowel diseases. The schematic diagram of the present study is summarized in Figure 1S. The core factors MyD88/TLR, S100A8, RAGE, and NLRP3 might be several promising potential therapeutic targets on different levels for colitis prevention and targeted therapy.

Author contributions

Shi Liu and Jinhua Zhang designed the research. Shi Liu, Yanmin Wu, Haiqiang Chen, Shuang Ge conducted experiments. Shi Liu, Yanmin Wu, Haiqiang Chen, Qi Yuan, and Jinhua Zhang performed data analysis. Lishu Zhang and Lingling Hou provided advices for experiments and data analysis. Shi Liu, Tian Tian and Jinhua Zhang wrote the manuscript.

Conflict of interests

The authors declare no conflicts of interests.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81972689 and 81772497).

Acknowledgements

We thank Dr. Zhengquan Yu in China Agricultural University for generously providing us with murine intestinal epithelial cell line m-ICcl2.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.04.021.

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9 March 2022
Available online 18 May 2022

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