Supplementary Methods

Multiplex ELISA:

Multiplex ELISA was performed using using a BioPlex 200 System with HTF Multiplex Array System (Bio-Rad, Hurcules, CA). For mouse cytokines/chemokines in the colonic mucosa, a 20-plex kit (Life Technologies) was used, including IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, GM-CSF, FGF-basic, and VEGF. For human cytokine/chemokines secreted by colonic mucosal explant culture, a 10-plex was used, including IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-13, IL-17, MCP-1, MIG, MIP-1α.

The goal for the cytokine study is to determine specifically what immunological pathways have been affected when NLRP3 inflammasome is inhibited by glyburide. Therefore, the rationale for selection of cytokines measured include: 1) for mouse study, we chose the 20-plex multiplex kit that includes most of the major cytokines (TH1, Th2, Th17) and chemokines that are known to play pathogenic role in IBD; 2) for human study, in order to determined the relevance of the data obtained from mouse model of colitis to human IBD, we chose the 10-plex multiplex based the mouse data, of which we demonstrated these 10 cytokines/chemokines were suppressed by glyburide.

Supplement Table S1. Patient demographics

	CD	UC	CTRL (Healthy controls)
Patients	10	4	4
Sex (Male/Female)	5/5	2/2	2/2
Age (years)	39.2±11.5	41.4±9.2	47.4±8.4
Race (n)	White (9), African American (1)	White (4)	White (4)
Disease Duration (years)	9.5±5.1	11.2±6.2	-
Activity	Active colitis	Active colitis	-
Location	Colon	Colon	-
Medication	Biologics (7) Mesalamine (5) Corticosteroids (3) Immunomudulators (4)	Biologics (3) Mesalamine (4) Corticosteroids (2) Immunomudulators (3)	-

Supplement Table S2. PCR primers for evaluation of cytokine genes

Genes (mouse)	Sequences	Gene regions
IL-1β	Forward: 5'- TGCTGGTGTGTGACGTTCCCATT-3'	nt 406–428
	Reverse: 5'-GTCCGACAGCACGAGGCTTTTT-3'	nt485-464
Caspase-1	Forward: 5'- TGGTCTTGTGACTTGGAGGA-3'	nt 1278–1297
	Reverse: 5'-TGGCTTCTTATTGGCACGAT-3'	nt 1468-1449
IL-25	Forward: 5'-CAGCTGCTGCCCCAGCAAAGA-3'	nt 87-107
	Reverse: 5'-TGCTGTTGAGGGGGCCATCCT-3'	nt 229-209
IL-17F	Forward: 5'-TGAGGGAAGAAGCAGCCATTGGA-3'	nt 40–62
	Reverse: 5'-GGCTGTTTCACGGGTGCACTT-3'	nt 95-75
IFN-γ	Forward: 5'-GCTCTTCCTCATGGCTGTTT-3'	nt 141-160
	Reverse: 5'-GTCACCATCCTTTTGCCAGT-3'	nt 294-275
TNF-α	Forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3'	nt 401-425
	Reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3'	nt 575-553
IL-18	Forward: 5'-GTGGGGAGGGTTTGTGTTCCAGAA-3'	nt 744-767
	Reverse: 5'-CGAGGTCATCACAAGGCGCATGT-3'	nt 803-781
IL-22R	Forward: 5'-GGCCCGCTAGCACCTCTGACA-3'	nt 131-151
	Reverse: 5'-CAGTGTGGTTGCGGGTCTCCAT-3'	nt 268-247
GAPDH	Forward: 5'-CATGGCCTTCCGTGTTCCTA-3'	nt 756-775
	Reverse: 5'-CTGGTCCTCAGTGTAGCCCAA-3'	nt 906-886

Supplemental data -Figure S1

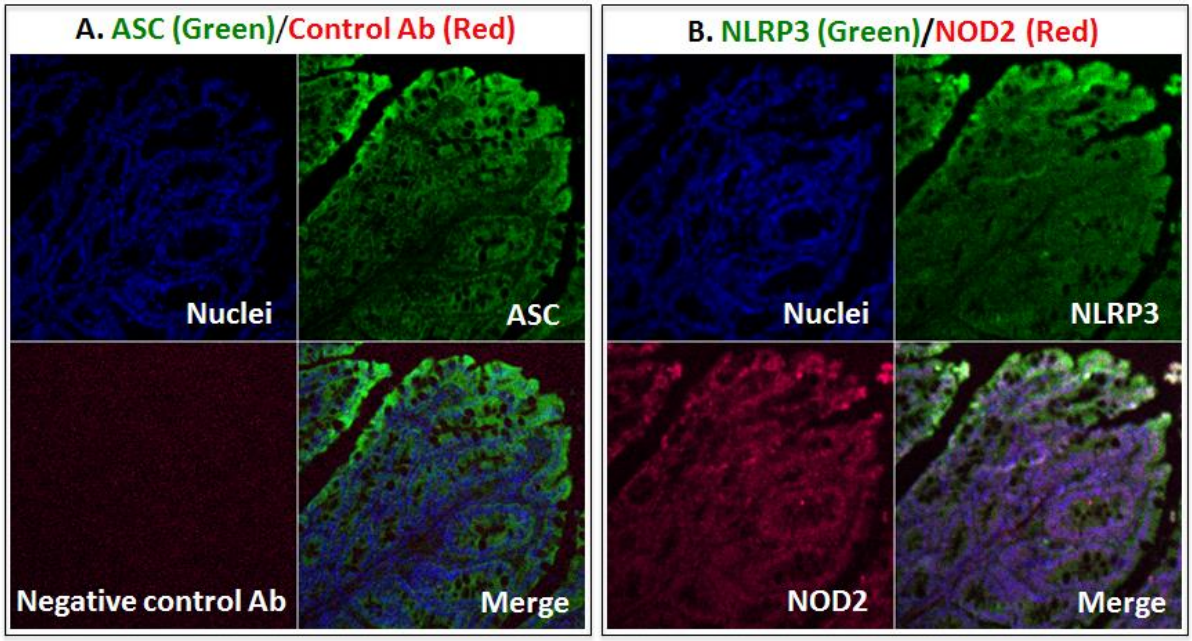


Figure S1

Expression of NOD2 protein in colonic mucosa of IL-10$^{-/-}$ mice. Experiments were performed as described in **Figure 4**. Immunofluorescent microscopy using consecutive sections of paraffin-embedded colon. Note: NLRP3 and ASC exhibit similar tissue distribution (strong luminal or sub-apical) as demonstrated in Figure 4B, while NOD2 expression is predominately intracellular. Representatives of at least three independent experiments were shown.

Supplemental data -Figure S2

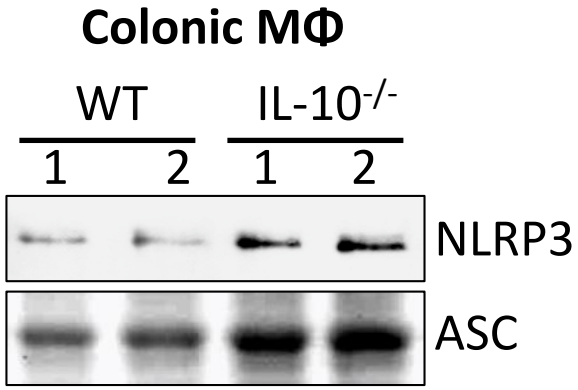


Figure S2

Comparison of NLRP3 expressions in colonic macrophages between WT and IL-10$^{-/-}$ mice. Like colonic mucosa (Figure 2), colonic macrophages also exhibit increased expression of NLRP3 and ASC inIL-10$^{-/-}$ mice compared to WT mice. Colonic macrophages were isolated using MACS® CD11b Microbeads (Miltenyi Biotec Inc, San Diego, CA). Proteins of colonic macrophages and CEC were analyzed by SDS-PAGE (loading: 65 µg protein per lane) and Western blot. Representatives of data from at least 5 mice in each experimental group were shown. Numbers 1 and 2 denote two independent mice in each group. Representatives of four independent experiments are shown.

Supplemental data -Figure S3

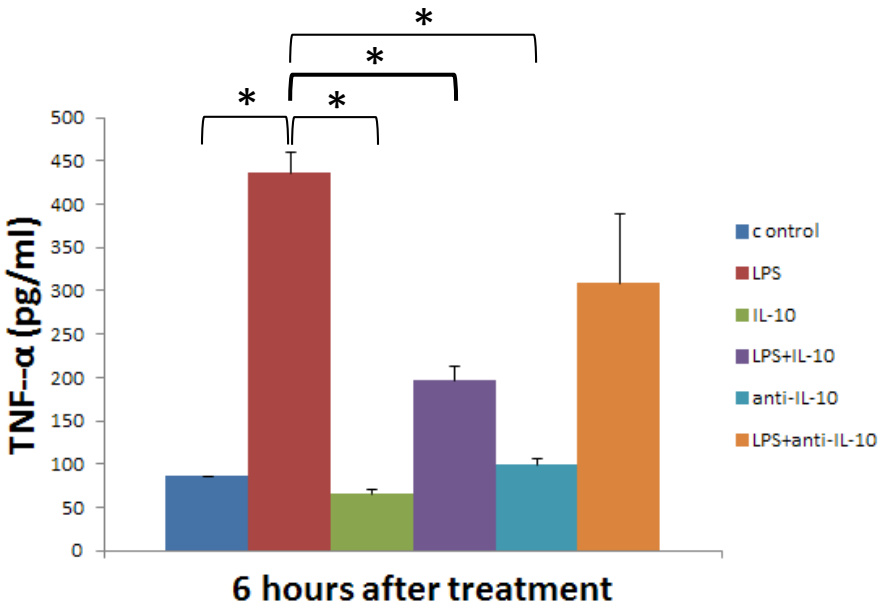


Figure S3

IL-10 alone effectively suppresses the LPS-induced TNF-α production by macrophage Raw264.7. Raw264.7 cells were incubated for 18 hours in the presence (or absence) of LPS (100 ng/ml), IL-10 (100 ng/ml), or both. Growth media were collected for the analysis of TNF-α level by ELISA (*P <0.05). Antibody neutralization of IL-10 using monoclonal anti-IL-10 antibody also appeared to reduce the LPS-induced TNF-α secretion, but did not reach statistic significance, suggesting that the Raw cells may express low level of TNF-α production.

Supplemental data -Figure S4

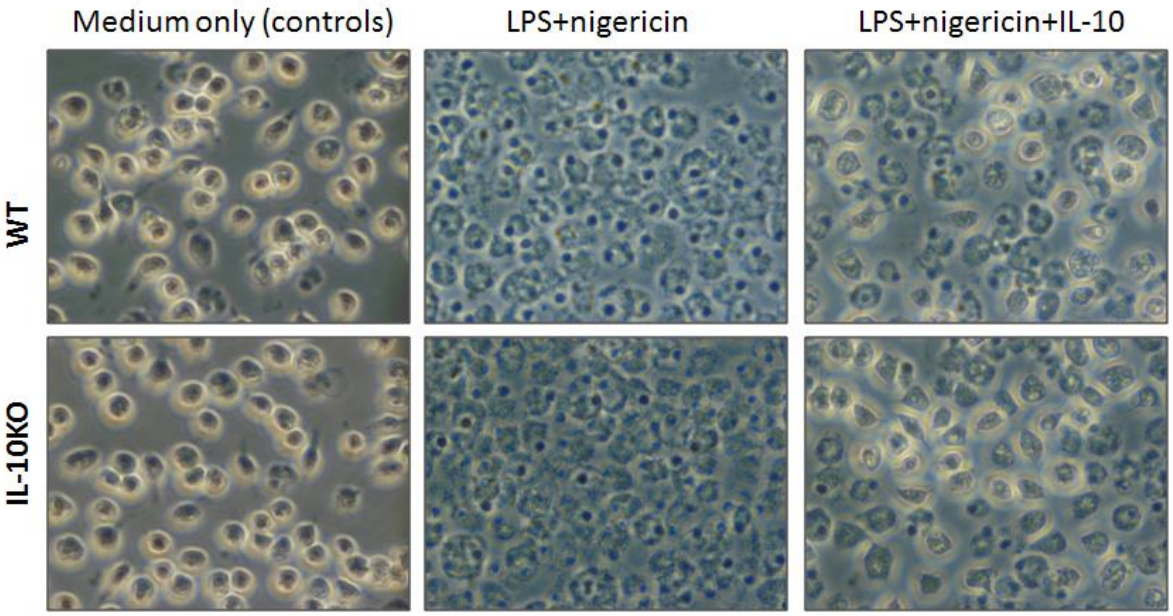


Figure S4

IL-10 alone can restore LPS + nigericin-induced morphological changes of macrophages. Mouse peritoneal macrophages from WT and IL-$10^{-/-}$ mice were incubated for 12 h with reagents as indicated (**see details in Figure 5**). Cells were photographed using Zeiss Axio Inverted Microscope (Zeiss, Thornwood, NY) equipped with an Olympus DP72 Digital Camera (Olympus America Inc., Center Valley, PA). Representatives of at least three independent experiments were shown.

Supplemental data -Figure S5

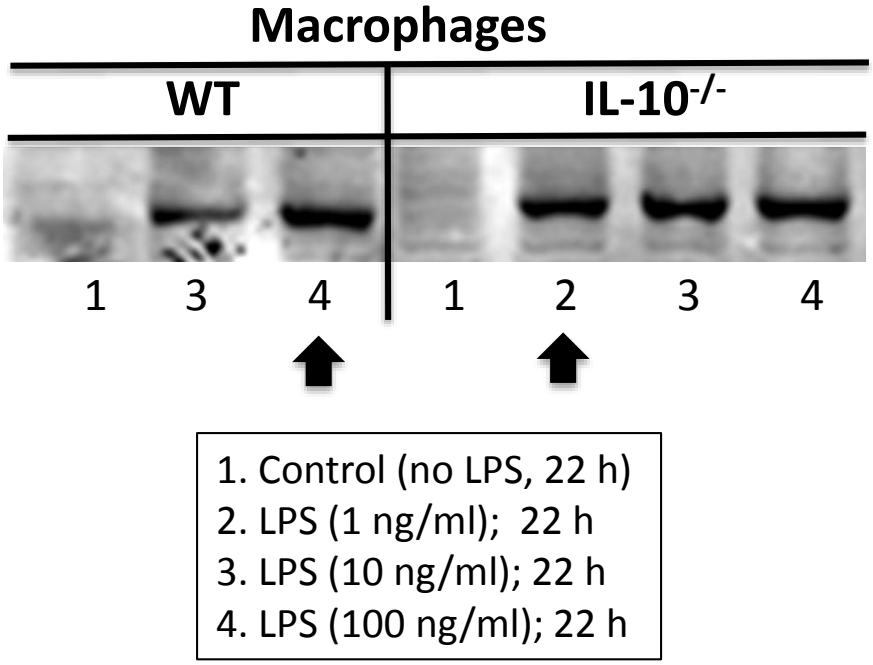
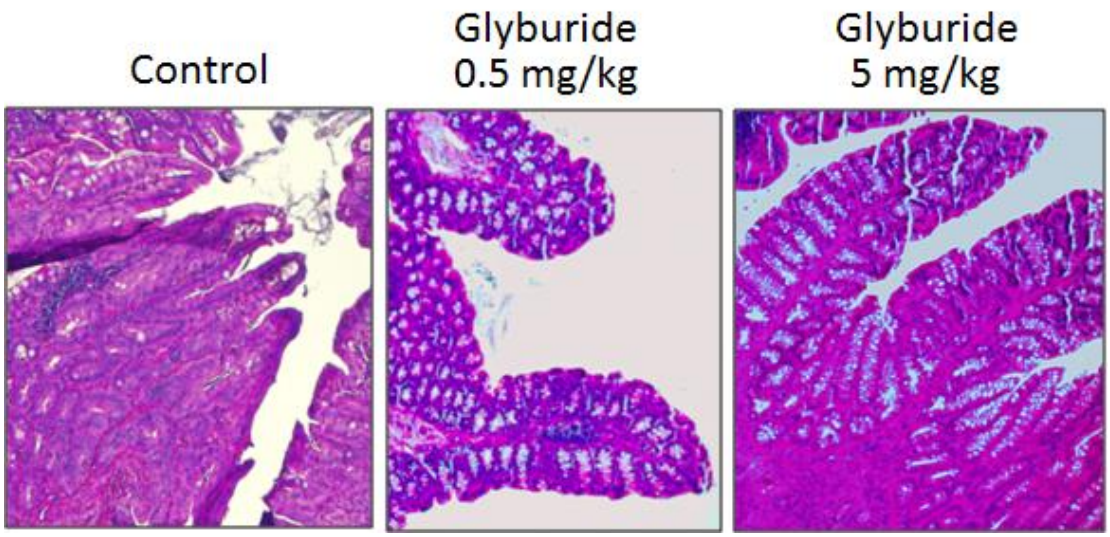


Figure S5

Enhanced sensitivity of NOD2 protein to stimulation of LPS of peritoneal macrophages from IL-10$^{-/-}$ mice, compared to that from WT mice. Isolated macrophages were incubated with or without (controls) LPS at the indicated concentrations for indicated times. Macrophages at each condition were collected and total Triton-X100- solubilized cellular proteins (45 μg per lane) were separated by SDS-PAGE and analyzed by Western blot with anti-NOD2 antibody. Note that NOD2 expression in IL-10$^{-/-}$ macrophages at 1 ng/ml of LPS was similar to that in WT mice at 100 ng/ml (see lanes marked by arrows)，indicating an approximately 100-fold difference in the sensitivity to LPS. Representative of at least three independent experiments was shown.

Supplemental data -Figure S6

A. H&E stain of colonic mucosa



B. Inhibition of IL-1β activation by glyburide

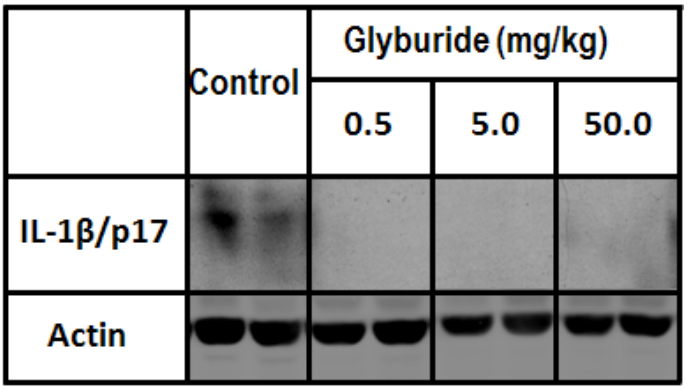


Figure S6

Inhibition of NLRP3 inflammasome with glyburide at lower concentration (0.5 or 5.0 mg/kg) resulted in alleviation of chronic colitis. IL-10$^{-/-}$ mice (6-month old) were intraperitoneally administrated daily with 0.5 or 5.0 mg/kg glyburide, for 3 consecutive days then every other day for 11 days (2 weeks total) as described in **Figure 6**. Glyburide group, n=15 (5 per subgroup); control group, n=10. (**A**) Representative of histological changes before (control) and after glyburide treatments. While the colonic mucosa of control mice had excessive hyperplasia, leukocyte infiltration, and loss of crypts and Goblet cells, glyburide treated mice exhibited a relative normal appearance. (**B**) Glyburide markedly suppressed the activation of IL-1β (activated IL-1β: IL-1β/p17) at all three concentrations of glyburide (0.5, 5, and 50 mg/kg). Mucosal extracts from control and glyburide treated groups were separated by SDS-PAGE and analyzed by Western blot with different antibodies as indicated. Actin was used as a loading control.

Supplemental data -Figure S7

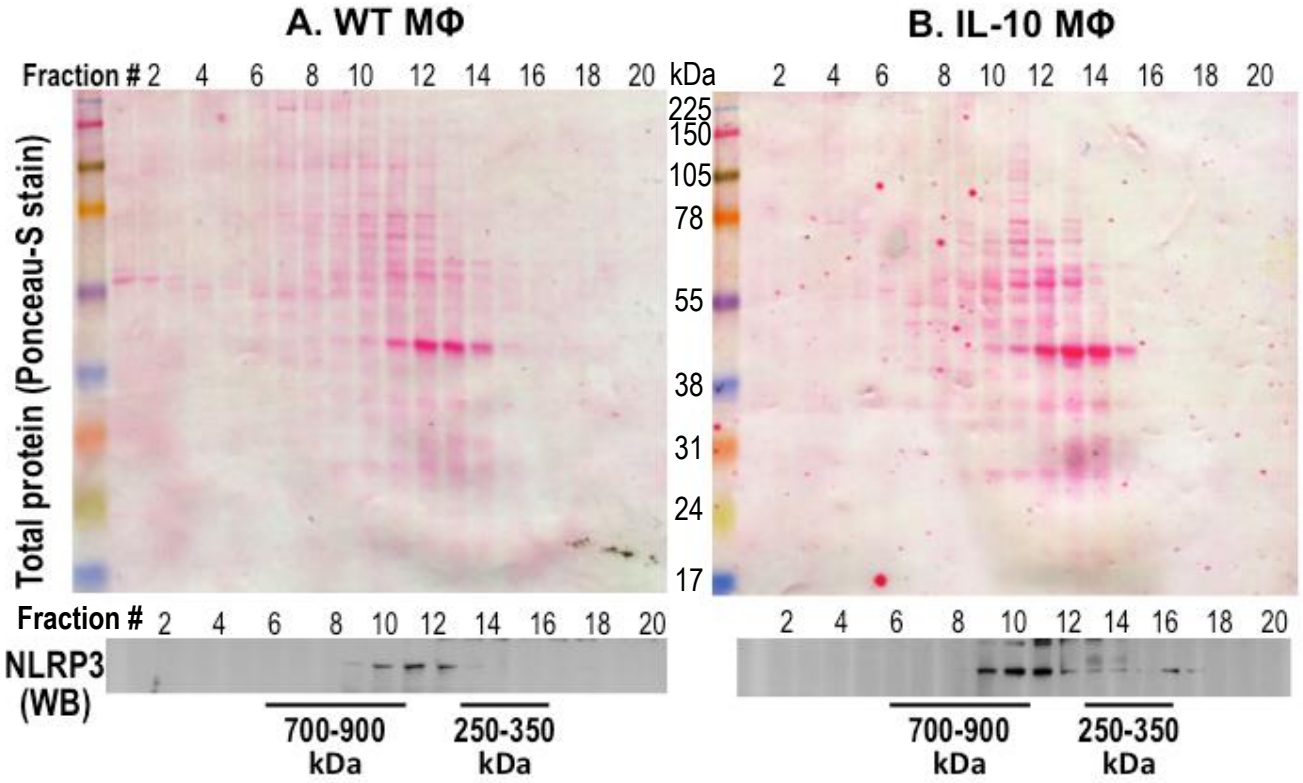


Figure S7

Large complexes of NLRP3 inflammasome in peritoneal macrophages isolated from WT and IL-10$^{-/-}$ mice (3-month old). Peritoneal macrophages were purified from mice as we described previously (see Ref 63 in the main article). Protein complexes were separated by sucrose density gradient and analyzed by SDS-PAGE and Western blot as described in Figure 3. (**A**) WT mice. (**B**) IL-10$^{-/-}$ mice. Ponceau-S staining (upper panels) shown the overall profiles of total proteins along the density gradients. NLRP3 (lower panel) was shown to be present in complexes ranging 400-750 kDa. V-ATPase, consisting of the full complex (around 600-900 kDa) and the V1 peripheral subcomplexes (around 250-350 kDa), was used as molecular weight markers to indicate the size of the protein complexes along the density gradient (See Figure 3A, bottom panels).

Supplemental data -Figure S8

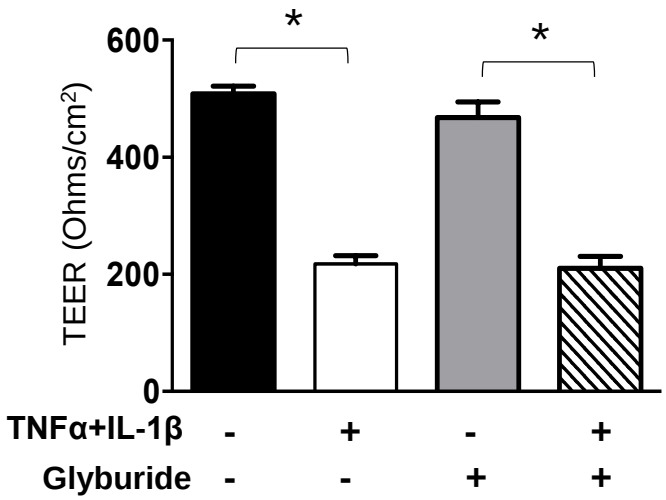


Figure S8

Inhibition of inflammosome 3 by glyburide exhibits no effect on the transepithelial electrical resistance (TEER) in colonic epithelial cell line Caco-2 cells. Caoc-2/BBE cells were cultured on CORNING Transwell inserts (0.4 μm pore size) in 6-well plates for 5 days post-confluence. Cells were then treated under 4 different experimental conditions, including controls (CTRL), with TNFα+IL-1β, with glyburide, and with TNFα+IL-1β + glyburide, as indicated, for 18 hours. TEER (Ohms/cm^2) was measured using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). Three consecutive measurements for electrical resistance of each filter insert was performed until similar values were recorded. A combination of TNFα and IL-1β was chosen to disrupt TEER because we found, through our preliminary pilot experiments, this combination led to the maximum reduction in TEER.

Supplemental data -Figure S9

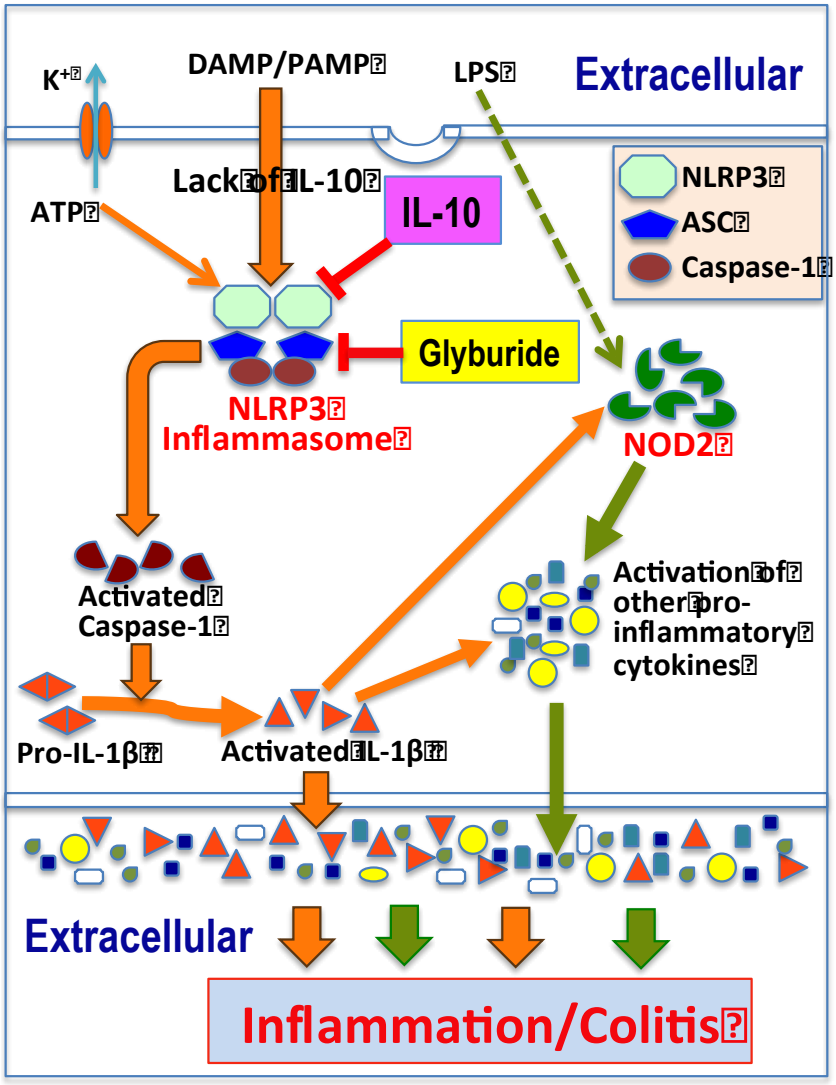


Figure S9

A working model to illustrate the involvement of the NLRP3 inflammasome and other factors in the pathogenesis of colitis in IL-10$^{-/-}$ mice. In the absence of IL-10, the NLRP3 inflammasome is not only spontaneously activated (Figure 5), but also upregulated and further activated by either microbial (DAMP, PAMP, etc) or cellular stress signals (changes in K^+ or ATP) or both. A cascades of down-stream events of NLRP3 inflammasome lead to the activation of IL-1β (Figure 2), followed by activation of other proinflammatory cytokines, such as INF-γ, TNF-α, IL-1α, IL-6, IL-17, IL-18, IL-25 (Figure 1E&F). The activation of the NLRP3 inflammasome is an early event of pathogenesis, occurring prior to the onset of colitis, and propagating continuously and progressively over the course of disease progression (indicated by the orange-color arrows) (Figure 2A). However, the expression of NOD2, which is hypersensitive to LPS in IL-10$^{-/-}$ mice (Figure S5), occurs at a later stage around the onset of disease, and such an activation persists throughout the late stage of disease development (Figure 2A), as indicated by green arrows. Early NLRP3-mediated activation of IL-1β may be directly involved in the upregulation of NOD2 through activated IL-1β during the late stages of inflammation. Hyper-sensitivity of NOD2 to microbial products such as LPS (Figure S5) in IL-10$^{-/-}$ mice may further accelerate the inflammation. The sustained secretion of multiple proinflammatory cytokines leads to colonic inflammation/colitis in IL-10$^{-/-}$ mice. IL-10 acts as a negative regulator of the NLRP3 inflammasome (Figure 5). Glyburide suppresses the activation of NLRP3 inflammasome (Figures 6, 7, 9, &S6).