Published in final edited form as:

Gastroenterology. 2010 August ; 139(2): 530–541. doi:10.1053/j.gastro.2010.04.006.

Paired immunoglobulin-like Receptor B (PIR-B) Negatively Regulates Macrophage Activation in Experimental Colitis

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Abstract

Background & Aims—Innate and adaptive immune responses are regulated by crosstalk between activation and inhibitory signals. Dysregulation of the inhibitory signal can lead to aberrant chronic inflammatory diseases such as the inflammatory bowel diseases (IBD). Little is known about negative regulation of innate intestinal immune activation. We examined the role of the inhibitory receptor paired immunoglobulin-like receptor B (PIR-B) in the regulation of macrophage function in innate intestinal immunity.

Methods—We examined the susceptibility of *Pirb*^{-/-} and wild-type (WT) mice to dextran sodium sulfate (DSS)-induced colitis. We assessed proinflammatory cytokine release and MAPK and NFκB activation in *Pirb*^{-/-} and WT macrophages following *E. coli* stimulation. Macrophage transfer experiments were performed to define the role of PIR-B in the negative regulation of macrophage function in DSS-induced colitis. We also assessed expression of PIR-B human homologs (ILT-2 and ILT-3) in colon biopsy samples from healthy individuals (controls) and patients with IBD.

Results—*Pirb*^{-/-} mice had increased susceptibility to DSS-induced colitis. *In vitro* analysis demonstrated increased production of proinflammatory cytokines (IL-6, IL-1β and TNF-α) and activation of MAPK and NFκB in *Pirb*^{-/-} macrophages following bacterial activation. Adoptive transfer of bone marrow-derived *Pirb*^{-/-} macrophages into WT mice was sufficient to increase disease susceptibility. ILT-2 and ILT-3 were expressed on CD68⁺ and CD68⁻ mononuclear cells and intestinal epithelium in colon biopsy samples from patients and controls.

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Writing assistance: A.M designed and performed experiments, analyzed and interpreted data and wrote the manuscript, R.A. X. H., T.W., E.T.C and K.G performed laboratory experiments, A.W, designed and performed experiments, L.D. and K.S discussed experimental design and data analysis, M.E.R discussed experimental design, data analysis and wrote manuscript, S.P.H designed and performed experiments, analyzed and interpreted data and wrote the manuscript.

There is no conflict of interest to disclose for all authors.

Conclusions—PIR-B negatively regulates macrophage functions in response to pathogenic bacteria and chronic intestinal inflammatory responses. Inhibitory receptors such as PIR-B might be used as therapeutic targets for treatment of patients with IBD.

Keywords

Inhibitory receptors; macrophages; IBD

Introduction

The inflammatory bowel diseases (IBD), crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory disorders and a substantial cause for morbidity and colonic cancer development¹. Although UC and CD are recognized to be CD4⁺ T cell dependent diseases, recent advances in our understanding of the role of commensal bacteria and pattern recognition receptors (e.g toll-like receptor 4 (TLR-4) and caspase recruitment domain 15 [CARD15] polymorphisms) in IBD pathogenesis, indicate a key role for innate immunity in colonic inflammation². Consistent with this, activated macrophages are a prominent constituent of the inflammatory infiltrate in CD and UC³⁻⁵. Macrophage depletion prevented disease onset in the *Il10*^{-/-} spontaneous model of colitis⁶. Furthermore, M-CSF deficient (op/op) mice, which are not able to develop mature macrophages, or wild type (WT) mice administered neutralizing anti-CSF-1 antibody, demonstrate decreased susceptibility to DSS-induced colitis^{7, 8}.

Recent experimental evidence suggest that inhibitory and activating Ig-like receptors provide counter regulatory signals that balance immune responses to extrinsic stimuli⁹. One such family of receptors is the paired Ig-like receptor (PIR)-A (activating) and PIR-B (inhibitory). PIR-A and PIR-B are orthologues of the human immunoglobulin-like transcript (ILT)/ leukocyte Ig-like receptor (LIR) family of receptors⁹, which are expressed predominantly by myeloid cells in a pair-wise fashion^{10, 11}. PIR-B possesses several immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within its cytoplasmic domain. These domains bind and activate intracellular phosphatases including Src-homology 2 (SH2)-domain-containing tyrosine phosphatase 1 (SHP-1) and SHP-2 inhibit activating-type receptor-mediated signaling¹². Recent data suggest an intricate link between PIR-B and TNF α and bacterial infections^{13, 14}. Nevertheless, the contribution of PIR-B to negative regulation of macrophage-mediated innate intestinal immune responses remains undefined.

Herein, we demonstrate that *Pirb* gene deletion causes exaggerated DSS-induced colonic injury that was critically dependent on the expression of PIR-B on macrophages. We show that direct activation of *Pirb*^{-/-} macrophages by *E. coli* leads to exaggerated MAPK and NF κ B activation as well as proinflammatory cytokine production. Finally, we demonstrate expression of PIR-B human homologues ILT-2 and -3 in colonic biopsies of healthy controls and pediatric UC patients. Collectively, these studies emphasize a key role for PIR-B in the negative regulation of macrophage functions in innate intestinal immune reactions.

Materials and Methods

Mice

Male and female, 8- to 12-week-old *Pirb*^{-/-} mice (backcrossed >F9 to C57BL/6) were kindly provided by Dr. Hiromi Kubagawa (Univ. of Alabama)¹⁵. C57BL/6 WT mice were generated in house [originally obtained from Taconic Laboratories (Hudson, NY)]. In all experiments age-, weight and gender-matched mice were housed under specific pathogen-free conditions according to CCHMC IUACAC approved protocols.

DSS-induced colonic injury

DSS (ICN Biomedical Inc., USA, average molecular weight of 41kDA) was supplied in the drinking water as a 2.5% (w/v) solution. Assessment of DSS-induced colonic injury was performed as described ¹⁶.

Punch Biopsies

The colons were flushed with PBS and opened along a longitudinal axis. Three mm² punch biopsies were incubated for 24hrs in RPMI supplemented with 10% FCS and antibiotics. Supernatants were collected and assessed for cytokine expression.

Macrophage activation

Bone marrow (BM)-derived macrophages, peritoneal and thioglycolate-elicited macrophages were obtained as described ¹⁷. The cells (2×10^5) were seeded onto 48 well plates and cultured overnight (37°C, 5% CO₂). The next day, cells were treated with heat inactivated pathogenic *E. coli* (ATCC #10799) for 24hrs and supernatants were assessed for cytokine production by ELISA.

Cytokine determination

Cytokines were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Lower detection limits for IL-1 β , IL-6 and TNF- α were 15.6, 15.6 and 32.25 pg/ml, respectively. In some experiments cytokine/chemokine levels were determined by a mouse multiplex kit (Millipore, Billerica, MA) according to the manufacturer's instructions.

Flow cytometry

Flow cytometry was performed on BM-macrophages or enzymatically digested colon lamina propria cells as described in Supplementary materials.

PhosphoFlow

Total peritoneal cells (resting or thioglycolate-elicited) were stimulated with heat inactivated pathogenic *E. coli* (ATCC, #10799) for the indicated time points (0-4 hrs) and phosphoflow analysis was performed as previously described ¹⁸. The mean fluorescent intensity (MFI) for each intracellular signaling molecule and transcription factor in WT and *Pirb*^{-/-} macrophages at time point 0 minutes was measured (Figure 3 and Supplementary Figure 7 histograms) to identify basal phosphorylation levels. Following confirmation of no significant differences in basal MFI levels between groups for each molecule, MFI value for each time point was normalized to baseline and expressed as fold-change over baseline.

Immunoprecipitation

Following stimulation, thioglycolate-elicited [inflammatory] macrophages were lysed using M-PER lysis buffer (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) and 2mM orthovanadate (30 min, on ice). The cell lysate was precleared using either rat or goat IgG (8 μ g/ml) followed by protein A/G sepharose beads (Santa Cruz, CA). Anti-PIR-A/B (6C1) ¹⁰ or anti-PIR-B (p91, Santa Cruz) (8 μ g/ml) were covalently attached to protein G dynabeads using DS³ reagent (Pierce, Rockford, IL) and added to the precleared lysate (over night, 4°C). This limits unspecific binding including detection of heavy and light immunoglobulin chains. The immunoprecipitated complex was eluted from the protein G beads using NuPage LDS Buffer (Invitrogen, Carlsbad, CA) and analyzed by western blot ¹⁹.

BM macrophage transfer experiments

BM macrophages were generated as described¹⁷. The BM macrophages were detached using 10 mM EDTA in PBS, washed three times with saline and intravenously injected (8×10^6 cells per mouse) into WT or *Pirb*^{-/-} mice on day -1 and +1 of DSS-treatment. Disease progression was monitored as described above.

Patient samples

Colonic biopsy samples were taken from UC and normal healthy controls (5-18 years). Colon biopsies were obtained from the most proximal endoscopically affected segment, and from the ascending colon in healthy controls. UC patients were diagnosed using established criteria as we have previously described¹⁶. Healthy controls were individuals who were evaluated for IBD and found to have normal diagnostic imaging, endoscopy and histologic appearance following upper endoscopy and colonoscopy. Samples from UC patients were obtained at diagnosis, prior to therapy. The human studies were approved by the CCHMC IRB.

Immunofluorescence Microscopy—Immunofluorescence analysis was performed as described in Supplementary materials.

Statistical analysis

Data were analyzed by ANOVA followed by Tukey post hoc test or by students *t*-test using GraphPad Prism 4 (San Diego, CA) as indicated. Data are presented as mean $\pm$ SEM; values of *p* < 0.05 were considered statistically significant.

Results

Increased susceptibility of *Pirb*^{-/-} mice to experimental colitis

Western blot analysis demonstrated that PIR-B and PIR-A (data not shown) are expressed in colonic tissue at baseline (Figure 1A). Specificity of anti-PIR-B antibody was demonstrated by the absence of PIR-B expression in the colons of *Pirb*^{-/-} mice. To delineate the role of PIR-B in innate intestinal inflammation²⁰, *Pirb*^{-/-} mice were subjected to DSS-induced colitis. DSS (2.5%) administration to WT and *Pirb*^{-/-} mice induced colitis (Figure 1). Compared to DSS-treated WT mice, *Pirb*^{-/-} mice displayed rapid onset and significantly increased weight loss (Figure 1B), rectal bleeding and diarrhea (Figure 1C-D) resulting in increased in disease activity at day 6 (Figure 1E). Increased susceptibility to DSS-induced colitis in *Pirb*^{-/-} mice was associated with increased mortality with 100% mortality by day 12 (Figure 1F, n=12). In contrast, only 30% of the WT mice died by day 12 (n=12). Colonoscopic examination of DSS-treated *Pirb*^{-/-} mice on day 6 revealed increased luminal thickening, severe intra-luminal bleeding and substantially increased ulcerations (Figure 2A). Consistent with increased intestinal disease, *Pirb*^{-/-} mice displayed marked shortening of the colon in respect to DSS-treated WT mice (Figure 2B and supplemental Figure 1).

Increased histopathology in DSS-treated *Pirb*^{-/-} mice

Histological analysis of colon sections obtained from DSS-treated *Pirb*^{-/-} and WT mice revealed substantially increased edema, inflammation and ulcerations of the epithelial layer of *Pirb*^{-/-} mice (Figure 2C). Quantitative assessment demonstrated substantially increased histopathology that was observed as early as 6 days of DSS exposure (Figure 2D, *p* < 0.01 comparing DSS-treated WT and *Pirb*^{-/-} mice). Consistent with the increased histopathology in *Pirb*^{-/-} mice, ex-vivo colon punch biopsy cultures of DSS-treated *Pirb*^{-/-} mice displayed increased IL-6 (Figure 2E) and IL-1 β levels (data not shown) compared to DSS-treated WT. Since macrophages are a primary source for IL-6 and IL-1 β , we examined whether

increased susceptibility to DSS-treatment in *Pirb*^{-/-} mice correlated with macrophage recruitment. Surprisingly, DSS-treatment induced a comparable macrophage accumulation in the colon of both WT and *Pirb*^{-/-} mice (Figure 2F).

Regulation of PIR-B expression on macrophages

To begin to delineate the molecular basis of increased susceptibility to DSS-colitis that was associated with PIR-B deficiency, we examined the cellular source for PIR-B employing the 6C1 antibody that recognizes both PIR-A and -B¹⁰. We demonstrate expression of PIR-A and/or -B on colonic lamina propria (LP) macrophages, B cells, neutrophils, but not T cells (Supplemental Figure 2A-C). Following DSS treatment, PIR-B expression was significantly upregulated on macrophages (Supplemental Figure 2D-pink histogram). As observed by analysis of DSS-treated *Pirb*^{-/-} mice, PIR-A expression was also increased, albeit to a lesser extent (Supplemental Figure 2D, turquoise histogram). To delineate what components of innate immunity regulate PIR-B expression on macrophages, BM macrophages were stimulated with LPS, TNF- α and IL-1 β . LPS and TNF- α (but not IL-1 β) caused a significant dose-dependent upregulation in PIR-A/B expression (Supplemental Figure 3). These data suggest that innate stimuli and pro-inflammatory cytokines associated with DSS-induced colitis regulate the expression of PIR-B on macrophages.

PIR-B negatively regulates *E. coli*-induced macrophage activation

Following our demonstration of PIR-B expression on intestinal macrophages and elevated macrophage-associated proinflammatory cytokines in the colons of DSS-treated *Pirb*^{-/-} mice (Figure 2E), we hypothesized that PIR-B negatively regulates macrophage activation. To assess this hypothesis pro-inflammatory cytokine production by macrophages from WT and *Pirb*^{-/-} mice following stimulation with heat inactivated *E. coli* was examined. Due to the heterogeneity of PIR-B expression on multiple cell types and inability to obtain purified LP macrophage population, we used purified resident peritoneal and thioglycolate-elicited (i.e. inflammatory [Inf]) macrophages. *E. Coli* stimulation of resident peritoneal and inflammatory WT and *Pirb*^{-/-} macrophages induced IL-1 β , IL-6 and TNF- α production (Supplemental Figure 4 and results not shown). The level of IL-6 and TNF- α production by *Pirb*^{-/-} resident peritoneal macrophages was only modestly increased compared to WT (Supplemental Figure 4A-B). No difference was observed in IL-1 β production (data not shown). In contrast, inflammatory *Pirb*^{-/-} macrophages displayed substantially increased secretion of IL-6, TNF- α and IL-1 β compared to WT (Supplemental Figure 4C-E). Western blot analysis revealed that expression of PIR-A was equivalent between WT and *Pirb*^{-/-} macrophages indicating that increased macrophage activity was not due to increased expression of PIR-A (Supplemental Figure 5). Thus, PIR-B is a negative regulator of *E. coli*-induced proinflammatory cytokine production in macrophages.

PIR-B regulates MAPK and NF κ B phosphorylation

Bacterial-induced proinflammatory cytokine production is primarily mediated by MAPK and NF κ B-mediated pathways²¹. PIR-B binds bacteria and regulates *S. aureus*-induced macrophage-derived cytokine production¹⁴. However the signaling pathways involved in PIR-B negative regulation of macrophage activation are yet unknown. To assess this, we stimulated resident and thioglycolate-induced peritoneal cells with *E. coli* for the indicated time points and assessed macrophage (CD11b⁺/F4/80⁺/FSC^{high}) MAPK and NF κ B activation by Phosphoflow analysis (Figure 3 and see supplemental Figure 6). Resident *Pirb*^{-/-} and WT peritoneal macrophages displayed a minor, but comparable increase in ERK1/2 and p38 in response to *E. coli* stimulation (Supplemental Figure 7). In contrast, inflammatory *Pirb*^{-/-} and WT macrophages showed substantially increased phosphorylated levels of these kinases (Figure 3A-B). Notably, the level of activation of *Pirb*^{-/-} macrophages was greater than that observed of WT (Figure 3). Unexpectedly, both resident and

inflammatory *Pirb*^{-/-} macrophages displayed a defect in JNK phosphorylation and only a minor increase in phosphorylation of NFκB (Supplemental Figure 7C-D; Figure 3C-D). Importantly, the changes in MFI were not due to differences in baseline phosphorylation changes as these were similar between WT and *Pirb*^{-/-} macrophages (Figure 3, histograms). Thus, PIR-B negatively regulates *E. coli*-stimulated macrophage MAPK-activation and has significantly greater inhibitory activity in inflammatory macrophages than in resident macrophages.

Regulation of downstream transcription factors by PIR-B in response to *E.coli* stimulation

Activation of the MAPK pathway by innate immune components induces gene transcription via various transcription factors including c-Fos, FosB and c-Jun²². We examined whether the altered phosphorylation pattern that was observed in *E. coli* stimulated *Pirb*^{-/-} inflammatory macrophages correlates with changes in early-induced transcription factor expression. Indeed, *Pirb*^{-/-} inflammatory macrophages demonstrated substantially increased FosB activation, that was apparent as early as 30 minutes, and maximal at 2 hrs following *E. coli* stimulation (Figure 3E). These changes were independent of changes in c-Fos, which were comparable between Inflammatory WT and *Pirb*^{-/-} macrophages (Supplemental Figure 7E). Consistent with our observation that JNK phosphorylation is significantly impaired in *Pirb*^{-/-} macrophages (Figure 3C), we observed a significant decrease in *E. coli*-induced c-Jun expression in *Pirb*^{-/-} macrophages (Figure 3F).

Assessment of SHP-1 and -2 recruitment to PIR-B following *E. coli* stimulation

The inhibitory activity of PIR-B has been linked with the recruitment of SH2 containing phosphatases, SHP-1 and -2^{12, 23, 24}. We hypothesized that stimulation of macrophages with *E. coli* will result in PIR-B phosphorylation and subsequent phosphatase recruitment. Stimulation of inflammatory macrophages from WT mice induced a rapid and transient increase in PIR-B tyrosine phosphorylation (Figure 4A top panel). Increased tyrosine phosphorylation was accompanied with increased association with SHP-1 but not SHP-2 (Figure 4A middle panels). As a loading control, the membrane was probed anti-PIR-A/B (Figure 4A lower panel). These interactions were specific to PIR-B, as Ig control pull down revealed no association with SHP-1, or -2 (Figure 4B). Taken together, these data demonstrate a link between PIR-B mediated suppression of ERK1/2, p38 and NFκB mediated pathways and SHP-1 recruitment and activation in macrophages.

Engraftment of *Pirb*^{-/-} macrophages into WT mice is sufficient to increase WT mice susceptibility to DSS-induced colitis

We next hypothesized that loss of PIR-B on macrophages is sufficient to render increased susceptibility to DSS-induced colitis. To test this hypothesis, BM-derived *Pirb*^{-/-} macrophages were adoptively transferred into WT recipients and susceptibility to DSS-induced colitis was examined. Since PIR-B is expressed on other myeloid cells that have been implicated in IBD pathogenesis (e.g. neutrophils and dendritic cells) and there are inherent disease susceptibility complications associated with irradiation^{10, 11, 25}, we developed a BM-macrophage transfer model whereby WT and *Pirb*^{-/-} BM macrophages were adoptively transferred into WT mice (Figure 5A and B). To confirm that WT and *Pirb*^{-/-} donor macrophage populations were comparable and did not contain contaminating myeloid cells, we examined BM-macrophage population by flow cytometry (Supplemental Figure 8). Indeed, the cell population was negative for B cell markers (B220⁻, IgM⁻), neutrophil markers (Ly6G⁻/Ly6C⁺) and dendritic cell markers (CD11c⁻). To confirm engraftment of the donor macrophages, syngeneic eGFP⁺ BM-derived macrophages were transferred into WT recipients. DSS-treatment induced a marked increase in LP macrophages (from ~3-4% to 16-17%, Figure 5A). Gating on macrophages (CD11b⁺/F480⁺/FSC^{high}) revealed two populations; eGFP⁺ cells corresponding with the population of

adoptively transferred BM macrophages (Figure 5A- upper histogram) and the eGFP⁺ infiltrating recipient macrophages (Figure 5A- lower histogram). Importantly, infiltrating neutrophils (CD11b^{high}/F480⁺) did not express any eGFP (Figure 5A and lower histogram).

Adoptive transfer of WT BM-macrophages into control treated WT (WT_{mac}→WT_{mice}) mice resulted in no change in colonic phenotype (Figure 5B). Following DSS-treatment, WT_{mac}→WT_{mice} displayed rectal bleeding, diarrhea and increased disease activity index by day 6. Importantly, the disease severity was not significantly different to DSS-treated WT mice that were not engrafted with BM macrophages (Compare Figure 1B-D and Figure 5C). Remarkably, WT mice that received *Pirb*^{-/-} macrophages (*Pirb*^{-/-}_{mac}→WT_{mice}) and treated with DSS exhibited early onset of rectal bleeding and diarrhea by day 5 which resulted in increased disease activity and marked colon shortening in comparison to WT_{mac}→WT_{mice} (Figure 5C and Supplemental Figure 9). Furthermore, histological examination revealed increased epithelial ulceration (Figure 5D), edema and overall histology score (Figure 5E) in *Pirb*^{-/-}_{mac}→WT when compared to WT_{mac}→WT_{mice}. Notably, the susceptibility and severity of disease in *Pirb*^{-/-}_{mac}→WT mice were similar to what was observed in DSS treated *Pirb*^{-/-} mice in comparison to WT (Figure 1).

Increased susceptibility to DSS-induced disease was associated with increased pro-inflammatory cytokine production. Moreover, ex-vivo colon punch biopsies from DSS-treated *Pirb*^{-/-}_{mac}→WT mice showed a significant increase in IL-6 levels (Figure 5F). Furthermore, multiplex analysis of these supernatants revealed a substantial increase in macrophage related cytokines (G-CSF, GM-CSF and M-CSF) and IL-17 (Supplemental Figure 10). Collectively, these studies demonstrate that loss of PIR-B expression on macrophages is sufficient to render WT mice susceptible to DSS-induced colitis. Notably, colonic IFN- γ levels were undetectable in the supernatants (data not shown).

Engraftment of WT macrophages into *Pirb*^{-/-} mice protects from DSS-induced colitis

To substantiate our previous observations (Figures 1 and 5), we hypothesized that adoptive transfer of WT macrophages into *Pirb*^{-/-} mice will have a protective effect. Thus, BM-macrophages from WT mice were adoptively transferred into *Pirb*^{-/-} mice and disease susceptibility was assessed. Adoptive transfer of WT BM macrophages into *Pirb*^{-/-} mice resulted in no change in colonic phenotype. DSS-treatment of *Pirb*^{-/-} mice that received WT macrophages (WT_{mac}→*Pirb*^{-/-}_{mice}) displayed by day 6 substantially decreased weight loss (Figure 6A; day 6) and exhibited decreased disease activity and histopathology when compared to DSS-treated *Pirb*^{-/-} mice (Figure 6B-E and Supplemental Figure 11). Interestingly, assessment of IL-6 levels in ex vivo colonic punch biopsies showed no statistically difference between DSS-treated *Pirb*^{-/-} mice and between WT_{mac}→*Pirb*^{-/-} mice (Figure 6F).

Expression of human ILTs/LIRs in IBD

To gain insight into a possible role of PIR-B human orthologues in IBD, we assessed the expression of two inhibitory ILT/LIR family members in the colonic mucosa of normal healthy control and UC patients, namely ILT-2/LIR-1(CD85J) and ILT-3/LIR-5 (CD85K)^{26,27}. Compared to isotype control, we show that ILT-2/CD85J is primarily expressed by mononuclear cells within the lamina propria of the colon from normal healthy controls (Figure 7A and E). Notably, we observed ILT-2/CD85J expression on CD68⁺ and CD68⁻ cells (Figure 7A). In contrast, ILT-3/CD85K was not expressed on mononuclear cells within the LP, but rather expression was predominantly localized to the apical membrane or surface and crypt epithelium (Figure 7C). While we observed increased CD68⁺ cells within the LP of colonic biopsy samples of pediatric UC patients; the ILT-2/CD85J and ILT-3/CD85K cellular specificity was similar to that observed in controls (Figure 7). These data

demonstrate expression of PIR-B human homologues, ILT-2 and ILT-3 in the colon during intestinal inflammatory disease. Further these analyses indicate selective expression of PIR-B homologues on specific cell types within the intestine.

Discussion

Herein, we provide several lines of evidence for a key inhibitory role for PIR-B on macrophage function during DSS-induced colonic injury. We show that *Pirb*^{-/-} mice have increased susceptibility to DSS-induced colitis. The increase in disease severity was linked with elevated macrophage-associated pro-inflammatory cytokine production. *In vitro* analysis of *E. coli*-stimulated BM- and thioglycolate-derived macrophages revealed a link between PIR-B deficiency, elevated proinflammatory cytokine production and MAPK (particularly ERK1/2 and p38) and FosB activation. Adoptive transfer experiments confirmed a critical role for PIR-B in the negative regulation of macrophage function in DSS-induced colitis. Finally, and relevant to human disease, we demonstrate expression of PIR-B human orthologues, ILT-2 and -3 on mononuclear cells within the LP including CD68⁺ macrophages and intestinal epithelial cells.

We demonstrate that DSS exposure of mice deficient in *Pirb* leads to exaggerated cytokine production and intestinal disease. PIR-B is expressed on various cell populations including macrophages, neutrophils, mast cells, eosinophils, DC- and B-cells²⁸. Notably, PIR-B negatively regulates neutrophil and eosinophil function^{19, 29}. Given that these cell types have a role in the pathophysiology of DSS-induced colitis^{16, 30, 31}, we cannot rule out that hyper-activated neutrophils and eosinophils may contribute in part to the increase disease severity in *Pirb*^{-/-} mice. However, our adoptive transfer experiments demonstrate that loss of PIR-B expression and negative regulation of macrophage activation is sufficient to enhance susceptibility to DSS-induced colitis. Interestingly, transfer of WT BM-macrophages into *Pirb*^{-/-} mice delayed disease progression and had a protective effect. There are two possible explanations for these observations. First, WT BM-macrophages could be displacing *Pirb*^{-/-} macrophages in the colon and thus reducing the pro-inflammatory environment and disease pathology; alternatively, WT BM-macrophages in *Pirb*^{-/-} mice possess anti-inflammatory activity and negatively regulate *Pirb*^{-/-} macrophage activation and suppress DSS-induced colitis. Previous studies have demonstrated that myeloid-specific STAT-3 deficient mice develop spontaneous entero-colitis³². Susceptibility to spontaneous entero-colitis was linked with a loss of anti-inflammatory IL-10:Stat3 signaling in macrophages³². The involvement of altered IL-10:Stat3 signaling in exaggerated *Pirb* deficient macrophage proinflammatory cytokine production is currently under investigation.

The ligands of PIR-B are not yet fully delineated²⁷. Initial studies suggested that MHC class I (H-2) molecules are ligands for PIR-B; however, recent studies indicate interaction with *S. aureus* (Gram-positive) and *E. coli* (Gram-negative)^{9,14}. We show that stimulation of WT and *Pirb*^{-/-} macrophages (resident or inflammatory) with *E.coli*-derived LPS induced an equivalent increase in IL-6 and IL-1 β production and MAPK/NF κ B phosphorylation (data not shown). Whereas, activation of *Pirb*^{-/-} macrophages with *E. coli* increased proinflammatory cytokine production and MAPK activation. These studies indicate that PIR-B may not be directly activated by TLR-4 ligands such as LPS, but rather inhibits TLR-mediated activation in response to bacterial stimulation. Consistent with this concept, TLR-2 agonist PAM₃CSK₄ stimulation of WT and *Pirb*^{-/-} macrophages led to equivalent cytokine production, however activation with whole *S. aureus*, which exerts its proinflammatory effects primarily via TLR-2, led to exaggerated IL-6 and TNF- α production¹⁴. While further investigation is required to define the bacterial cell wall components that bind and/or activate PIR-B, recent studies suggest PIR-B may possess scavenger receptor-like binding activity towards bacteria.

TLR ligands and proinflammatory cytokines including IL-1 β and TNF α are thought to activate macrophage MAPK (p38, ERK1/2 and JNK) and NF κ B pathways, promoting gene expression and cytokine production, leading to cytokine-mediated inflammation and IBD³³. Consistent with this, both the MAPKs (p38, JNK and ERK1/2) and NF κ B are significantly activated in the inflamed colonic mucosa of IBD patients³⁴. Our mechanistic analysis revealed that PIR-B negatively regulates bacterial-induced macrophage activation of MAPK-, primarily ERK1/2- and p38- and to lesser extent the NF κ B-phosphorylation. Pharmacological blockade of MAPK activation, specifically p38, improved disease activity and histology disease score in a DSS-model of colitis³⁵. Unexpectedly, we observed that *Pirb*^{-/-} macrophages have a substantial defect in JNK phosphorylation and hallmark target of JNK, c-Jun. Previous studies have shown that *Pirb*^{-/-} myeloid dendritic cells are of immature phenotype and are unable to upregulate MHC-II molecules, a process that is dependent on JNK and c-Jun stimulation¹⁵. Notably, the observed elevated IL-6, TNF- α and IL-1 β in *Pirb*^{-/-} macrophages following bacterial stimulation occurred in the absence of heightened JNK:c-Jun activation. These data suggest that increased activation of p38 and ERK1/2 in the absence of PIR-B is sufficient to regulate increased macrophage activation. Similarly, PECAM-1, another inhibitory receptor has also been shown to have divergent inhibitory effects on downstream signaling intermediates (inhibits I κ B, and JNK; enhances ERK activation) in activated macrophages³⁶. The dependency of global attenuation of the JNK:c-Jun pathway or negative regulation of DSS-induced ERK1/2- and p38-activation by PIR-B on exacerbation of colitis is yet to be defined.

The inhibitory activity of PIR-B has been associated with the recruitment of SHP-1 and -2 following activation^{12, 23, 24}. We demonstrate that stimulation of macrophages with *E. coli*, induced tyrosine phosphorylation of PIR-B and recruitment of the phosphatases SHP-1 but not -2. These findings clearly indicate that PIR-B is activated upon ligation with *E. coli* and suggest that SHP-1 may be involved in the negative regulation of ERK1/2 and p38 by PIR-B. SHP-1 can directly and indirectly negatively regulate MAPK kinase (ERK and JNK) activation³⁷. Nitric oxide-induced dephosphorylation of ERK1/2 in rat vascular smooth muscle cells was associated with SHP-1 interaction and activation. Notably, ERK1/2 dephosphorylation was attenuated by a protein phosphatase 1 (SHP-1) inhibitor³⁸. Furthermore, SHP-1 dephosphorylates vascular endothelial growth factor-induced ERK phosphorylation in endothelial cells³⁹. In contrast to PIR-B, SIRP-1 α , another ITIM-bearing receptor, inhibits LPS/TLR-4-mediated signaling primarily through sequestering SHP-2 but not -1⁴⁰ suggesting that different inhibitory receptors may utilize divergent intracellular phosphatases to elicit their inhibitory effect.

A limitation of these in vitro macrophage analysis is that all studies were conducted on BM-derived or thioglycolate-elicited macrophages and not intestinal macrophages. We would have preferred to use intestinal macrophages, however purification of intestinal macrophages is technically challenging, often complicated by significant cellular contamination and insufficient cell yields for in vitro analyses. Consistent with our in vitro analyses, we demonstrate PIR-B expression on intestinal macrophages and show that in vivo transfer of BM-macrophages leads to elevated cytokine production and exacerbates DSS-induced colitis suggesting that PIR-B negatively regulates intestinal macrophage activation.

We demonstrated the expression of PIR-B human orthologues ILT-2/LIR-1 and ILT-3/LIR-5 in the colonic mucosa of normal healthy control and UC patients. ILT-2/CD85J was primarily expressed by CD68⁺ and CD68⁻ mononuclear cells within the lamina propria, whereas ILT-3/CD85K was localized to the crypt epithelium. The differential expression of PIR-B human orthologues suggests that these molecules may negatively regulate both hematopoietic and non-hematopoietic cell function. The gastrointestinal tract is a tightly regulated immune organ that possesses suppressive immune mechanisms that prevent

uncontrolled proinflammatory reactions towards enteric flora. We speculate that ILT-2/CD85J and ILT-3/LIR-5 may play a role in the maintenance of intestinal mononuclear cell and epithelial cell immune quiescence or non-responsiveness. We did not observe differences in ILT-2/LIR-1 or ILT-3/LIR-5 expression in the colonic mucosa of normal and UC patients. Consistent with this observation, levels of LIRs (ILT-1, ILT-4 and ILT-5) on monocytes from rheumatoid arthritis (RA) patients were comparable to that of sex- and aged-matched control subjects ⁴¹. The mechanism of LIRs modulation of inflammation remains unclear, however it is postulated that LIRs regulate the threshold for activation of inflammatory cells and determine the severity of inflammation ⁴² via the relative balance of activating or inhibitory LIR's expressed on a particular cell. Thus assessment of expression patterns of ILT/LIR family members in IBD will be important in defining their relative contribution to human gastrointestinal homeostasis including the exacerbation and contraction of the intestinal inflammatory response and mucosal recovery.

In summary, our results define a key role for PIR-B in the regulation of inflammatory macrophage activation during colonic inflammation predominantly by negative regulation of bacterial induced-ERK and -p38 activation. We confirm that the human homologues of PIR-B are expressed on both immune and epithelial cells in the inflamed and normal colon. These data highlight inhibitory receptors such as PIR-B as novel targets for suppression of macrophage functions in inflammatory settings such as IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Dr. Hiromi Kubagawa for providing the *Pirb*^{-/-} mice for this study and to Dr. Jochen Mattner for providing the heat inactivated *E. coli*.

Grant support: This work was supported by an American Heart Association research fellowship (A.M), NIH P01 HL-076383 (M.E.R.), R01 AI057803 (M.E.R.), the CURED and Buckeye Foundations (M.E.R), the Food Allergy Project (M.E.R), The Crohn's and Colitis Foundation of America Career Development Award (S.P.H) and NIH R01AI073553-01 (S.P.H).

Abbreviations

IBD	Inflammatory bowel disease
PIR-B	Paired immunoglobulin-like receptor B
DSS	Dextran sodium sulfate
ITIM	Immunoreceptor tyrosine based inhibitory motif
ILT	Ig-like transcript
LILR	leukocyte Ig-like receptor
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor kappa B
UC	ulcerative colitis
CD	Crohn's disease
TLR-4	Toll-like receptor 4
SHP	Src-homology 2 (SH2)-domain-containing tyrosine phosphatase

WT	Wild type
BM	Bone marrow
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
FACS	Fluorescent-activated cell sorting

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Figure 1. Increased susceptibility of *Pirb*^{-/-} mice to experimental colitis
 (A) Expression of PIR-B in whole colon lysates of WT and *Pirb*^{-/-} mice. (B) weight loss, (C) rectal bleeding, (D) diarrhea, (E) overall disease activity and (F) survival rates in WT and *Pirb*^{-/-} mice exposed to 2.5% DSS. In (C-D) the numbers in parentheses represent the number of individual mice. Data is a representative experiment of n=4 (6-12 mice per group); *-p<0.05. In (A) each lane represents a single lysate from an individual mouse.

Figure 2. Increased histopathology in DSS-treated *Pirb*^{-/-} mice

(A) Representative photographs of colonoscopy, (B) Colon length, (C) H&E stained colon sections and (D) quantification of histology score (day 3 and 6) in WT and *Pirb*^{-/-} mice following DSS or control treatment (Ctrl). (E) IL-6 levels in culture supernatants from distal colon punch biopsies and (F) quantification of CD68⁺ cells in the colon of control and DSS-treated WT and *Pirb*^{-/-} mice. Data is a representative experiment of n=4 (6-12 mice per group); (B) **-p<0.01, ***-p<0.001. (D); **-p<0.01. (E) **-p<0.01. (F). ns-non significant; **-p<0.01.

Figure 3. Regulation of *E. coli*-induced MAPK, NFκB and transcription factor activation by PIR-B

Macrophages were stimulated for the indicated time points with heat inactivated *E. coli* (1:10) (A-F). Quantification of fold increase in mean fluorescent intensity (MFI) for (A) pERK1/2, (B) pp38, (C) pJNK and (D) pNFκB, (E) FosB and (F) c-Jun levels in WT and *Pirb*^{-/-} inf Macrophages stimulated with heat inactivated *E. coli* (1:10). A representative histogram plot of inf macrophages (A-D-right histograms) at the 30 min (30') time point is shown. Data are representative of n=5 (inf Macrophages). Black squares and white circles indicate *Pirb*^{-/-} and WT macrophages, respectively.

Figure 4. Assessment of PIR-B:SHP-1 and -2 interactions following *E. coli* stimulation
 Western blot analysis of (A) PIR-B and (B) control-Ig immunoprecipitated cell lysates from WT thioglycolate-elicited macrophages for phosphotyrosine (pTyr), SHP-1, SHP-2 and PIR-B following *E. coli* stimulation.

Figure 5. Assessment of DSS-induced colitis following adoptive transfer of *Pirb*^{-/-} macrophages (A) Flow cytometry analysis of colon from WT recipient mice engrafted with BM-derived eGFP⁺ macrophages following control treatment (A-left panel) or six days following 2.5% DSS (A-right panel). LP cells were stained with CD11b-PE-Cy5 and F4/80-PE and analyzed for eGFP expression (A-histograms). (B) A schematic representation of the adoptive transfer strategy. (C) Rectal bleeding, diarrhea and disease activity index in WT mice receiving adoptively transferred WT or *Pirb*^{-/-} macrophages and exposed to DSS. (D) A representative microphotograph and (E) quantification of the histological score of control and DSS-treated colons. (F) IL-6 levels in the culture supernatant of distal colon punch biopsies from control and DSS-treated mice. Data is a representative experiment of n=2 (6-8 mice per group). ns-non significant; *-p<0.05; **-p<0.01, ***-p<0.001. (D) Magnification x40. Black arrows indicate DSS-induced intestinal epithelial cell shedding.

Figure 6. Assessment of DSS-induced colitis following adoptive transfer of WT macrophages into *Pirb*^{-/-} mice

(A) Weight loss, (B) disease activity index (C); and colon shortening in *Pirb*^{-/-} mice receiving adoptively transferred WT macrophages and exposed to DSS. (D) Quantification of the histological score and (E) representative photomicrographs of control and DSS-treated colons. (F) IL-6 levels in the culture supernatant of distal colon punch biopsies from control and DSS-treated mice. Data is a representative experiment of n=2 (6-8 mice per group). ns-non significant; *-p<0.05; **-p<0.01. (E) Magnification x40.

Figure 7. Cellular expression of ILT-2/LIR-1 and ILT-3/LIR-5 in pediatric UC

Immunofluorescence labeling for CD68 and ILT-2/LIR-1 (A and C), and ILT-3/LIR-5 (B and D), in colonic biopsy samples from normal and pediatric UC patients. Anti-ILT-2/LIR-1 and ILT-3/LIR-5-Alexa488, green; Anti-CD68-TRITC (red) nuclei were stained with DAPI, blue. (E). Isotype control stained colonic biopsy. Results representative of 3 cases are shown. Original magnification (x 100) inserts (x 200). White arrow indicates CD68⁺ ILT2⁺ cells; grey arrow indicates CD68⁻ ILT2⁺ cells and yellow arrow indicates CD68⁺ ILT2⁻ cells.