

## Paired Immunoglobulin-Like Receptor B (PIR-B) Negatively Regulates Macrophage Activation in Experimental Colitis

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**BACKGROUND & AIMS:** Innate and adaptive immune responses are regulated by cross talk 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*<sup>-/-</sup> and wild-type (WT) mice to dextran sodium sulfate (DSS)-induced colitis. We assessed proinflammatory cytokine release and mitogen-activated protein kinase (MAPK) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation in *Pirb*<sup>-/-</sup> and WT macrophages following *Escherichia 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 homologues (immunoglobulin-like transcript [ILT]-2 and ILT-3) in colon biopsy samples from healthy individuals (controls) and patients with IBD. **RESULTS:** *Pirb*<sup>-/-</sup> mice had increased susceptibility to DSS-induced colitis. In vitro analysis showed increased production of proinflammatory cytokines (interleukin-6, interleukin-1 $\beta$ , and tumor necrosis factor  $\alpha$ ) and activation of MAPK and NF- $\kappa$ B in *Pirb*<sup>-/-</sup> macrophages following bacterial activation. Adoptive transfer of bone marrow-derived *Pirb*<sup>-/-</sup> macrophages into WT mice was sufficient to increase disease susceptibility. ILT-2 and ILT-3 were expressed on CD68<sup>+</sup> and CD68<sup>-</sup> mononuclear cells and intestinal epithelium in colon biopsy samples from patients and controls. **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.

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis (UC), are chronic relapsing inflammatory disorders and a substantial cause of mor-

bidity and colon cancer development.<sup>1</sup> Although UC and Crohn's disease are recognized to be CD4<sup>+</sup> T cell-dependent diseases, recent advances in our understanding of the role of commensal bacteria and pattern recognition receptors (eg, Toll-like receptor [TLR]-4 and caspase recruitment domain 15 [CARD15] polymorphisms) in the pathogenesis of IBD indicate a key role for innate immunity in colonic inflammation.<sup>2</sup> Consistent with this, activated macrophages are a prominent constituent of the inflammatory infiltrate in Crohn's disease and UC.<sup>3-5</sup> Macrophage depletion prevented disease onset in the *Il10*<sup>-/-</sup> spontaneous model of colitis.<sup>6</sup> Furthermore, macrophage colony-stimulating factor-deficient (op/op) mice, which are not able to develop mature macrophages, or wild-type (WT) mice administered neutralizing anti-colony-stimulating factor 1 antibody show decreased susceptibility to dextran sodium sulfate (DSS)-induced colitis.<sup>7,8</sup>

Recent experimental evidence suggests that inhibitory and activating immunoglobulin (Ig)-like receptors provide counterregulatory signals that balance immune responses to extrinsic stimuli.<sup>9</sup> Paired Ig-like receptor (PIR)-A (activating) and PIR-B (inhibitory) are orthologues of the human Ig-like transcript (ILT)/leukocyte Ig-like receptor (LIR) family of receptors,<sup>9</sup> which are expressed predominantly by myeloid cells in a pairwise fashion.<sup>10,11</sup> PIR-B possesses several immunoreceptor tyrosine-based inhibitory motifs (ITIM) within its cytoplasmic domain. The ITIM domains bind and activate intracellular phosphatases, including Src-homology 2 (SH2) domain-containing tyrosine phosphatase (SHP)-1 and

*Abbreviations used in this paper:* DSS, dextran sodium sulfate; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; Ig, immunoglobulin; IL, interleukin; ILT, immunoglobulin-like transcript; ITIM, immunoreceptor tyrosine-based inhibitory motif; JNK, c-Jun-N-terminal kinase; LIR, leukocyte immunoglobulin-like receptor; LP, lamina propria; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PIR, paired immunoglobulin-like receptor; SHP, Src-homology 2 (SH2) domain-containing tyrosine phosphatase; TLR, Toll-like receptor; TNF, tumor necrosis factor; WT, wild type.

SHP-2, which inhibit activating-type receptor-mediated signaling.<sup>12</sup> Recent data suggest an intricate link between PIR-B and tumor necrosis factor (TNF)- $\alpha$  and bacterial infections.<sup>13,14</sup> Nevertheless, the contribution of PIR-B to negative regulation of macrophage-mediated innate intestinal immune responses remains undefined.

Herein, we show that loss of *Pirb* expression on macrophages is sufficient enough to increase susceptibility to DSS-induced colitis. We show that direct activation of *Pirb*<sup>-/-</sup> macrophages by *Escherichia coli* leads to exaggerated mitogen-activated protein kinase (MAPK) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation as well as proinflammatory cytokine production. Finally, we show expression of PIR-B human homologues ILT-2 and ILT-3 in colonic biopsy specimens of healthy controls and pediatric patients with UC. 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*<sup>-/-</sup> mice (backcrossed >F9 to C57BL/6) were kindly provided by Dr Hiromi Kubagawa (University of Alabama, Birmingham, AL).<sup>15</sup> C57BL/6 WT mice were generated in-house (originally obtained from Taconic Laboratories, Hudson, NY). In all experiments, age-, weight-, and sex-matched mice were housed under specific pathogen-free conditions according to Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee-approved protocols.

### DSS-Induced Colonic Injury

DSS (ICN Biomedical Inc, Solon, OH; average mol wt, 41 kilodaltons) was supplied in the drinking water as a 2.5% (wt/vol) solution. Assessment of DSS-induced colonic injury was performed as described.<sup>16</sup>

### Punch Biopsies

The colons were flushed with phosphate-buffered saline and opened along a longitudinal axis. Punch biopsy specimens (3 mm<sup>2</sup>) were incubated for 24 hours in RPMI supplemented with 10% fetal calf serum and antibiotics. Supernatants were collected and assessed for cytokine expression.

### Macrophage Activation

Bone marrow-derived macrophages as well as peritoneal (resident and thioglycolate-elicited) macrophages were obtained as described.<sup>17</sup> The cells ( $2 \times 10^5$ ) were seeded onto 48-well plates and cultured overnight (37°C, 5% CO<sub>2</sub>). The next day, cells were treated with heat-inactivated pathogenic *Escherichia coli* (American Type Culture Collection #10799) for 24 hours and supernatants were assessed for cytokine production by enzyme-linked immunosorbent assay.

### Cytokine Determination

Cytokines were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Lower detection limits for interleukin (IL)-1 $\beta$ , IL-6, and TNF- $\alpha$  were 15.6, 15.6, and 32.25 pg/mL, respectively. In some experiments, cytokine/chemokine levels were determined by using a mouse multiplex kit (Millipore, Billerica, MA) according to the manufacturer's instructions.

### Flow Cytometry

Flow cytometry was performed on bone marrow-derived macrophages or enzymatically digested colon lamina propria (LP) cells as described in the supplementary materials.

### PhosphoFlow

Total peritoneal cells (resting or thioglycolate-elicited) were stimulated with heat-inactivated pathogenic *E coli* (American Type Culture Collection no. 10799) for the indicated time points (0–4 hours), and PhosphoFlow analysis was performed as previously described.<sup>18</sup> The mean fluorescent intensity for each intracellular signaling molecule and transcription factor in WT and *Pirb*<sup>-/-</sup> macrophages at time point 0 minutes was measured to identify basal phosphorylation levels. After confirmation of no significant differences in basal mean fluorescent intensity levels between groups for each molecule, the mean fluorescent intensity value for each time point was normalized to baseline and expressed as fold change over baseline.<sup>18</sup>

### 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 2 mmol/L orthovanadate (30 minutes, on ice). The cell lysate was precleared using either rat or goat IgG (8  $\mu$ g/mL) followed by protein A/G sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-PIR-A/B (6C1)<sup>10</sup> or anti-PIR-B (p91; Santa Cruz Biotechnology) (8  $\mu$ g/mL) was covalently attached to protein G Dynabeads (Invitrogen, Oslo, Norway) using DS<sup>3</sup> reagent (Pierce) and added to the precleared lysate (overnight, 4°C). This limits unspecific binding, including detection of heavy and light Ig chains. The immunoprecipitated complex was eluted from the protein G beads using NuPage LDS Buffer (Invitrogen, Carlsbad, CA) and analyzed by Western blot.<sup>19</sup>

### Bone Marrow-Derived Macrophage Transfer Experiments

Bone marrow-derived macrophages were generated as described.<sup>17</sup> The bone marrow-derived macro-

phages were detached using 10 mmol/L EDTA in phosphate-buffered saline, washed 3 times with saline, and intravenously injected ( $8 \times 10^6$  cells per mouse) into WT or *Pirb*<sup>-/-</sup> mice on day -1 and +1 of DSS treatment. Disease progression was monitored as described previously.<sup>16</sup>

### Patient Samples

Colonic biopsy samples were taken from patients with UC and healthy controls (aged 5–18 years). Colon biopsy specimens were obtained from the most proximal endoscopically affected segment and from the ascending colon in healthy controls. Patients with UC were diagnosed using established criteria as we have previously described.<sup>16</sup> Healthy controls were individuals who were evaluated for IBD and found to have normal diagnostic imaging as well as endoscopy and histologic appearance following upper endoscopy and colonoscopy. Samples from patients with UC were obtained at diagnosis, before therapy. The human studies were approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board.

### Immunofluorescence Microscopy

Immunofluorescence analysis was performed as described in the supplementary materials.

### Statistical Analysis

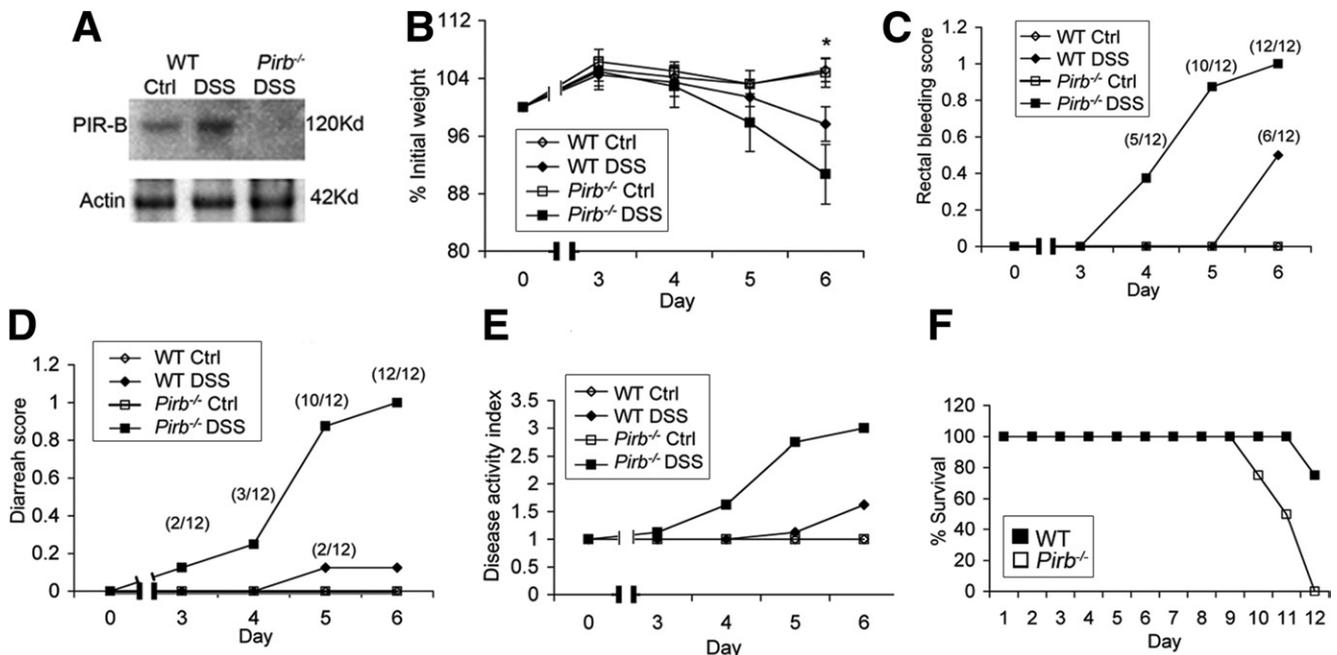
Data were analyzed by analysis of variance followed by Tukey post hoc test or by Student *t* test using

GraphPad Prism 4 (San Diego, CA) as indicated. Data are presented as mean  $\pm$  SEM; values of *P* < .05 are considered statistically significant.

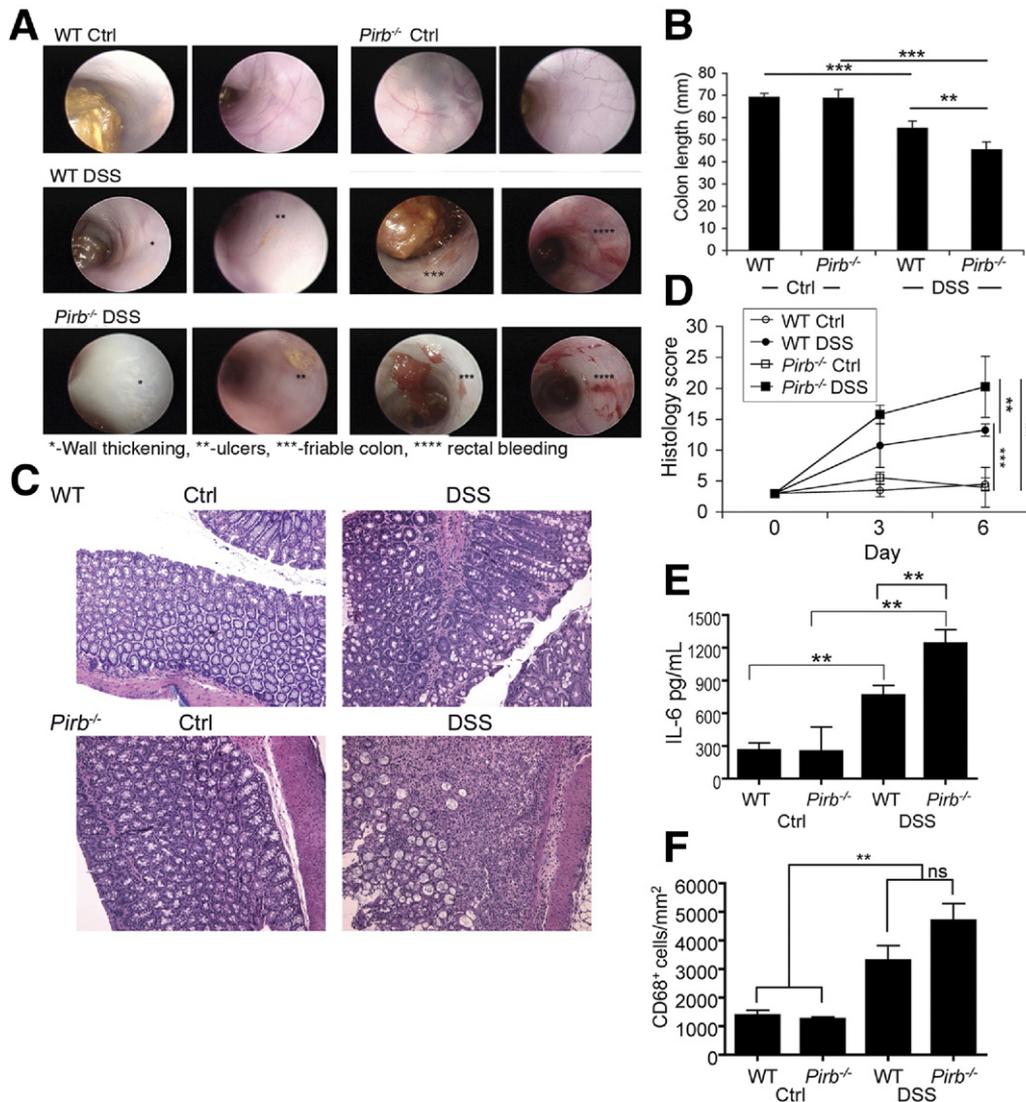
## Results

### Increased Susceptibility of *Pirb*<sup>-/-</sup> Mice to Experimental Colitis

Western blot analysis showed 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 shown by the absence of PIR-B expression in the colons of *Pirb*<sup>-/-</sup> mice. To delineate the role of PIR-B in innate intestinal inflammation,<sup>20</sup> *Pirb*<sup>-/-</sup> mice were subjected to DSS-induced colitis. Administration of DSS (2.5%) to WT and *Pirb*<sup>-/-</sup> mice induced colitis (Figure 1). Compared with DSS-treated WT mice, *Pirb*<sup>-/-</sup> mice displayed rapid onset and significantly increased weight loss (Figure 1B), rectal bleeding, and diarrhea (Figure 1C and D), resulting in increased disease activity at day 6 (Figure 1E). Increased susceptibility to DSS-induced colitis in *Pirb*<sup>-/-</sup> 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*<sup>-/-</sup> mice on day 6 revealed increased luminal thickening, severe intraluminal bleeding, and substantially increased ulcerations (Figure 2A). Consistent with increased intestinal disease, *Pirb*<sup>-/-</sup> mice displayed marked shortening



**Figure 1.** Increased susceptibility of *Pirb*<sup>-/-</sup> mice to experimental colitis. (A) Expression of PIR-B in whole colon lysates of WT and *Pirb*<sup>-/-</sup> mice. (B) Weight loss, (C) rectal bleeding, (D) diarrhea, (E) overall disease activity, and (F) survival rates in WT and *Pirb*<sup>-/-</sup> mice exposed to 2.5% DSS. In C and D, the numbers in parentheses represent the number of individual mice. Data are from a representative experiment of *n* = 4 (6–12 mice per group); in A, each lane represents a single lysate from an individual mouse. In B, \**P* < .05.



**Figure 2.** Increased histopathology in DSS-treated *Pirb*<sup>-/-</sup> mice. (A) Representative photographs of colonoscopy, (B) colon length, (C) H&E-stained colon sections, and (D) quantification of histology score (days 3 and 6) in WT and *Pirb*<sup>-/-</sup> mice following DSS or control treatment (Ctrl). (E) IL-6 levels in culture supernatants from distal colon punch biopsy specimens and (F) quantification of CD68<sup>+</sup> cells in the colon of control and DSS-treated WT and *Pirb*<sup>-/-</sup> mice. Data are from a representative experiment of n = 4 (6–12 mice per group). \*\*P < .01, \*\*\*P < .001. ns, not significant.

of the colon compared with DSS-treated WT mice (Figure 2B and Supplementary Figure 1).

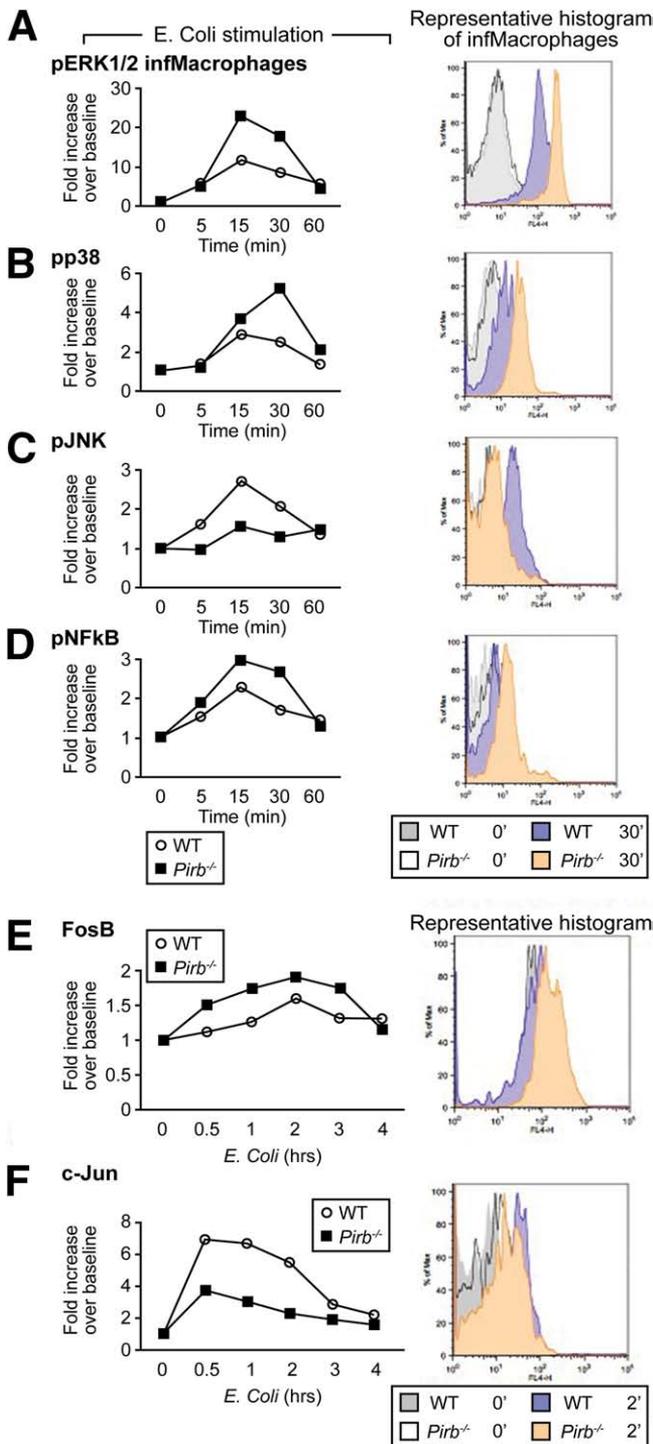
**Increased Histopathology in DSS-Treated *Pirb*<sup>-/-</sup> Mice**

Histologic analysis of colon sections obtained from DSS-treated *Pirb*<sup>-/-</sup> and WT mice revealed substantially increased edema, inflammation, and ulcerations of the epithelial layer of *Pirb*<sup>-/-</sup> mice (Figure 2C). Quantitative assessment showed substantially increased histopathology that was observed as early as 6 days of DSS exposure (Figure 2D, P < .01 comparing DSS-treated WT and *Pirb*<sup>-/-</sup> mice). Consistent with the increased histopathology in *Pirb*<sup>-/-</sup> mice, ex vivo colon punch biopsy cultures of DSS-treated *Pirb*<sup>-/-</sup> mice displayed increased IL-6 (Figure 2E) and IL-1β levels (data not shown) com-

pared with DSS-treated WT mice. Because macrophages are a primary source for IL-6 and IL-1β, we examined whether increased susceptibility to DSS treatment in *Pirb*<sup>-/-</sup> mice correlated with macrophage recruitment. Surprisingly, DSS treatment induced a comparable macrophage accumulation in the colon of both WT and *Pirb*<sup>-/-</sup> mice (Figure 2F).

**Regulation of PIR-B Expression on Macrophages**

To begin to delineate the molecular basis of increased susceptibility to DSS-induced colitis that was associated with PIR-B deficiency, we examined the cellular source for PIR-B using the 6C1 antibody that recognizes both PIR-A and PIR-B.<sup>10</sup> We showed expression of PIR-A and/or PIR-B on colonic LP macrophages, B cells,



**Figure 3.** Regulation of *E coli*-induced MAPK, NF- $\kappa$ B, and transcription factor activation by PIR-B. (A–F) Macrophages were stimulated for the indicated time points with heat-inactivated *E coli* (1:10). Quantification of fold increase in mean fluorescent intensity for (A) pERK1/2, (B) pp38, (C) pJNK, (D) pNF- $\kappa$ B, (E) FosB, and (F) c-Jun levels in WT and *Pirb*<sup>-/-</sup> inflammatory (inf) macrophages stimulated with heat-inactivated *E coli* (1:10). A representative histogram plot of inflammatory macrophages (A–F, histograms on the right) at the 30-minute (30') (A–D) and 2-minute (E and F) time point is shown. Data are representative of  $n = 5$  (inflammatory macrophages). Black squares and white circles indicate *Pirb*<sup>-/-</sup> and WT macrophages, respectively.

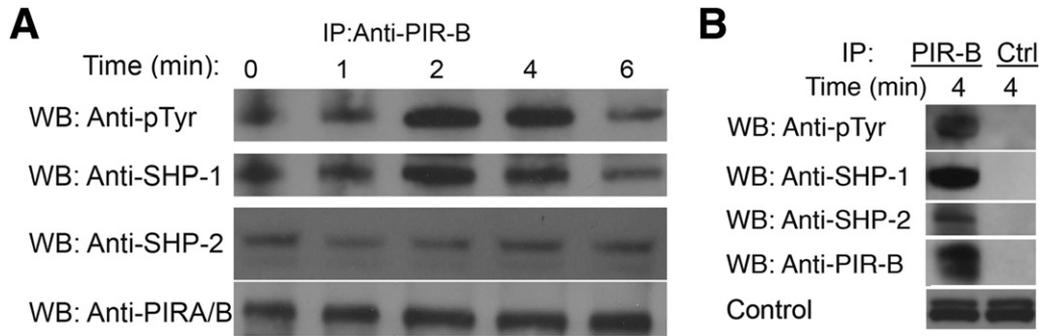
and neutrophils but not T cells (Supplementary Figure 2A–C). Following DSS treatment, PIR-B expression was significantly up-regulated on macrophages (Supplementary Figure 2D, pink histogram). As observed by analysis of DSS-treated *Pirb*<sup>-/-</sup> mice, PIR-A expression was also increased, albeit to a lesser extent (Supplementary Figure 2D, turquoise histogram). To delineate what components of innate immunity regulate PIR-B expression on macrophages, bone marrow-derived macrophages were stimulated with lipopolysaccharide (LPS), TNF- $\alpha$ , and IL-1 $\beta$ . LPS and TNF- $\alpha$  (but not IL-1 $\beta$ ) caused a significant, dose-dependent up-regulation in PIR-A/B expression (Supplementary Figure 3). These data suggest that innate stimuli and proinflammatory 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*<sup>-/-</sup> mice (Figure 2E), we hypothesized that PIR-B negatively regulates macrophage activation. To assess this hypothesis, proinflammatory cytokine production by macrophages from WT and *Pirb*<sup>-/-</sup> 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 a purified LP macrophage population, we used purified resident (ie, non-activated) and thioglycolate-elicited (ie, inflammatory) peritoneal macrophages. *E coli* stimulation of peritoneal resident and inflammatory WT and *Pirb*<sup>-/-</sup> macrophages induced IL-1 $\beta$ , IL-6, and TNF- $\alpha$  production (Supplementary Figure 4 and results not shown). The level of IL-6 and TNF- $\alpha$  production by *Pirb*<sup>-/-</sup> peritoneal resident macrophages was only modestly increased compared with WT (Supplementary Figure 4A and B). No difference was observed in IL-1 $\beta$  production (data not shown). In contrast, inflammatory *Pirb*<sup>-/-</sup> macrophages displayed substantially increased secretion of IL-6, TNF- $\alpha$ , and IL-1 $\beta$  compared with WT (Supplementary Figure 4C–E). Western blot analysis revealed that expression of PIR-A was equivalent between WT and *Pirb*<sup>-/-</sup> macrophages, indicating that increased macrophage activity was not due to increased expression of PIR-A (Supplementary Figure 5). Thus, PIR-B is a negative regulator of *E coli*-induced proinflammatory cytokine production in macrophages.

### *PIR-B Regulates MAPK and NF-κB Phosphorylation*

Bacterial-induced proinflammatory cytokine production is primarily mediated by MAPK and NF- $\kappa$ B-mediated pathways.<sup>21</sup> PIR-B binds bacteria and regulates *Staphylococcus aureus*-induced macrophage-derived cyto-



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

kinase production.<sup>14</sup> However, the signaling pathways involved in PIR-B negative regulation of macrophage activation are yet unknown. To assess this, we stimulated peritoneal resident and thioglycolate-induced cells with *E coli* for the indicated time points and assessed macrophage (CD11b<sup>+</sup>/F4/80<sup>+</sup>/FSC<sup>high</sup>) MAPK and NF- $\kappa$ B activation by PhosphoFlow analysis (Figure 3 and Supplementary Figure 6). Resident *Pirb*<sup>-/-</sup> and WT peritoneal macrophages displayed a minor but comparable increase in extracellular signal-regulated kinase (ERK)1/2 and p38 in response to *E coli* stimulation (Supplementary Figure 7). In contrast, inflammatory *Pirb*<sup>-/-</sup> and WT macrophages showed substantially increased phosphorylated levels of these kinases (Figure 3A and B). Notably, the level of activation of *Pirb*<sup>-/-</sup> macrophages was greater than that observed of WT (Figure 3). Unexpectedly, both resident and inflammatory *Pirb*<sup>-/-</sup> macrophages displayed a defect in c-Jun-N-terminal kinase (JNK) phosphorylation and only a minor increase in phosphorylation of NF- $\kappa$ B (Supplementary Figure 7C and D and Figure 3C and D). Importantly, the changes in mean fluorescent intensity were not due to differences in baseline phosphorylation changes because these were similar between WT and *Pirb*<sup>-/-</sup> 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-Jun.<sup>22</sup> We examined whether the altered phosphorylation pattern that was observed in *E coli*-stimulated *Pirb*<sup>-/-</sup> inflammatory macrophages correlates with changes in early-induced transcription factor expression. Indeed, *Pirb*<sup>-/-</sup> inflammatory macrophages demonstrated substantially increased FosB activation that was apparent as early as 30

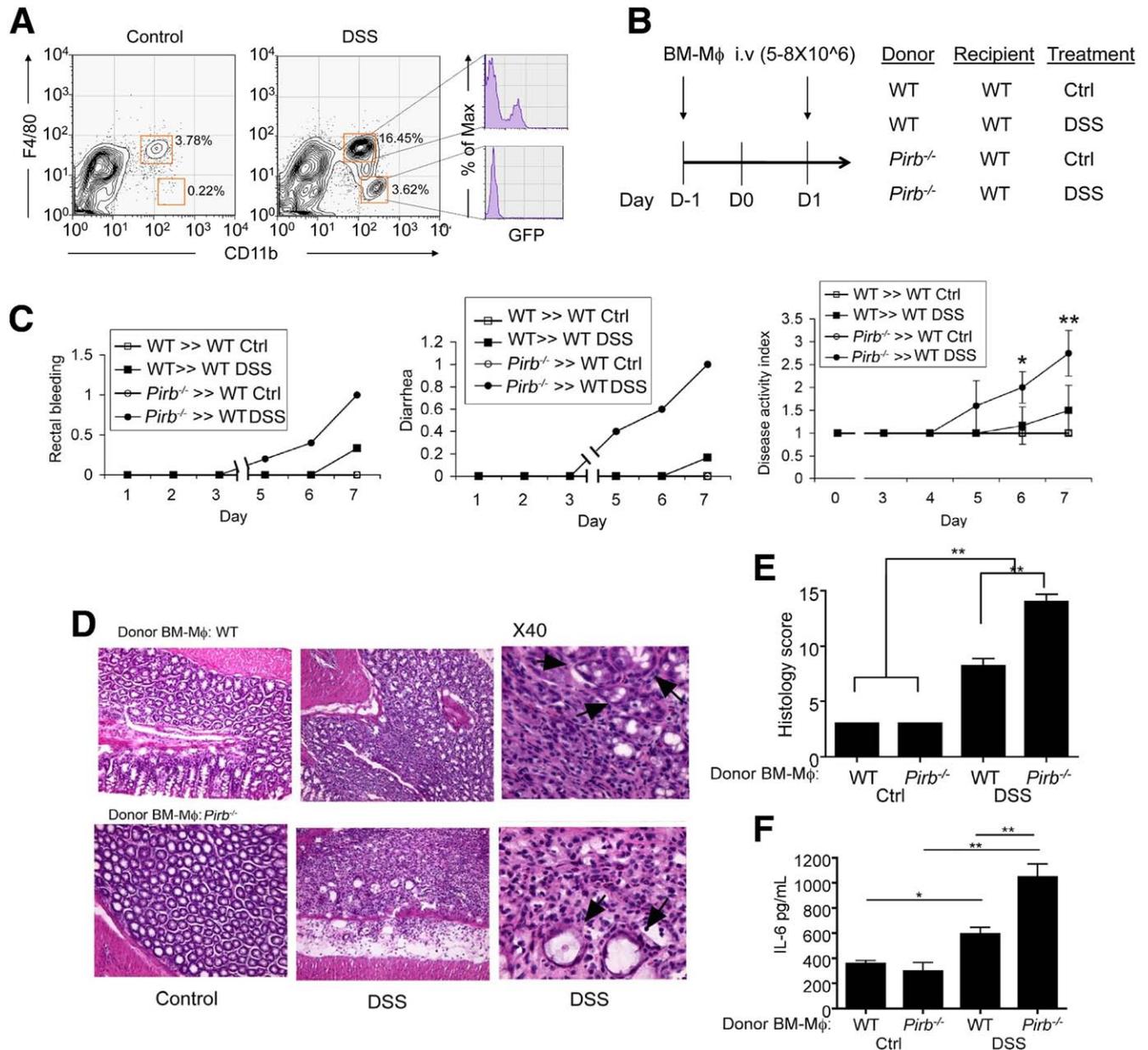
minutes and maximal at 2 hours after *E coli* stimulation (Figure 3E). These changes were independent of changes in c-Fos, which were comparable between inflammatory WT and *Pirb*<sup>-/-</sup> macrophages (Supplementary Figure 7E). Consistent with our observation that JNK phosphorylation is significantly impaired in *Pirb*<sup>-/-</sup> macrophages (Figure 3C), we observed a significant decrease in *E coli*-induced c-Jun expression in *Pirb*<sup>-/-</sup> macrophages (Figure 3F).

#### Assessment of SHP-1 and SHP-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 SHP-2.<sup>12,23,24</sup> 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 by increased association with SHP-1, but not SHP-2 (Figure 4A, middle panels). As a loading control, the membrane was probed with anti-PIR-A/B (Figure 4A, lower panel). These interactions were specific to PIR-B, because Ig control pull-down revealed no association with SHP-1 or SHP-2 (Figure 4B). Taken together, these data show a link between PIR-B-mediated suppression of ERK1/2-, p38-, and NF- $\kappa$ B-mediated pathways and SHP-1 recruitment and activation in macrophages.

#### Engraftment of *Pirb*<sup>-/-</sup> 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 increase susceptibility to DSS-induced colitis. To test this hypothesis, bone marrow-derived *Pirb*<sup>-/-</sup> macrophages were adoptively transferred into WT recipients and susceptibility to DSS-induced colitis was examined. Because PIR-B is expressed on other myeloid cells that have been implicated in the pathogen-



**Figure 5.** Assessment of DSS-induced colitis following adoptive transfer of *Pirb*<sup>-/-</sup> macrophages. (A) Flow cytometry analysis of colon from WT recipient mice engrafted with bone marrow–derived eGFP<sup>+</sup> macrophages following control treatment (*left panel*) or 6 days following treatment with 2.5% DSS (*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*<sup>-/-</sup> macrophages and exposed to DSS. (D) A representative microphotograph and (E) quantification of the histologic score of control and DSS-treated colons WT mice receiving adoptively transferred WT or *Pirb*<sup>-/-</sup> macrophages. (F) IL-6 levels in the culture supernatant of distal colon punch biopsy specimens from control and DSS-treated mice WT mice receiving adoptively transferred WT or *Pirb*<sup>-/-</sup> macrophages. Data are from a representative experiment of  $n = 2$  (6–8 mice per group). \* $P < .05$ ; \*\* $P < .01$ . (D) Original magnification 40 $\times$ . Black arrows indicate DSS-induced intestinal epithelial cell shedding.

esis of IBD (eg, neutrophils and dendritic cells) and there are inherent disease susceptibility complications associated with irradiation,<sup>10,11,25</sup> we developed a bone marrow–derived macrophage transfer model whereby WT and *Pirb*<sup>-/-</sup> bone marrow–derived macrophages were adoptively transferred into WT mice (Figure 5A and B). To confirm that WT and *Pirb*<sup>-/-</sup> donor macrophage populations were comparable and did not contain contaminating myeloid cells, we

examined the bone marrow–derived macrophage population by flow cytometry (Supplementary Figure 8). Indeed, the cell population was negative for B cell markers (B220<sup>-</sup>, IgM<sup>-</sup>), neutrophil markers (Ly6G<sup>-</sup>/Ly6C<sup>+</sup>), and dendritic cell markers (CD11c<sup>-</sup>). To confirm engraftment of the donor macrophages, syngeneic enhanced green fluorescent protein (eGFP<sup>+</sup>) bone marrow–derived macrophages were transferred into WT recipients. DSS treatment in-

duced a marked increase in LP macrophages (from ~3%–4% to 16%–17%; Figure 5A). Gating on macrophages (CD11b<sup>+</sup>/F480<sup>+</sup>/FSC<sup>high</sup>) revealed 2 populations: eGFP<sup>+</sup> cells corresponding to the population of adoptively transferred bone marrow–derived macrophages (Figure 5A, upper histogram) and the eGFP<sup>−</sup> infiltrating recipient macrophages (Figure 5A, lower histogram). Importantly, infiltrating neutrophils (CD11b<sup>high</sup>/F480<sup>−</sup>) did not express any eGFP (Figure 5A, lower histogram).

Adoptive transfer of WT bone marrow–derived macrophages into control-treated WT (WT<sub>mac</sub> → WT<sub>mice</sub>) mice resulted in no change in colonic phenotype (Figure 5B). Following DSS treatment, WT<sub>mac</sub> → WT<sub>mice</sub> displayed rectal bleeding, diarrhea, and increased disease activity index by day 6. Importantly, the disease severity was not significantly different from DSS-treated WT mice that were not engrafted with bone marrow–derived macrophages (compare Figure 1B–D and Figure 5C). Remarkably, WT mice that received *Pirb*<sup>−/−</sup> macrophages (*Pirb*<sup>−/−</sup><sub>mac</sub> → WT<sub>mice</sub>) and were 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<sub>mac</sub> → WT<sub>mice</sub> (Figure 5C and Supplementary Figure 9). Furthermore, histologic examination revealed increased epithelial ulceration (Figure 5D), edema, and overall histology score (Figure 5E) in *Pirb*<sup>−/−</sup><sub>mac</sub> → WT<sub>mice</sub> when compared with WT<sub>mac</sub> → WT<sub>mice</sub>. Notably, the susceptibility and severity of disease in *Pirb*<sup>−/−</sup><sub>mac</sub> → WT<sub>mice</sub> were similar to what was observed in DSS-treated *Pirb*<sup>−/−</sup><sub>mice</sub> in comparison to WT (Figure 1).

Increased susceptibility to DSS-induced disease was associated with increased proinflammatory cytokine production. Moreover, ex vivo colon punch biopsy specimens from DSS-treated *Pirb*<sup>−/−</sup><sub>mac</sub> → 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 (granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor) and IL-17 (Supplementary Figure 10). Collectively, these studies showed that loss of PIR-B expression on macrophages was sufficient to render WT mice susceptible to DSS-induced colitis. Notably, colonic interferon gamma levels were undetectable in the supernatants (data not shown).

#### **Engraftment of WT Macrophages Into *Pirb*<sup>−/−</sup> 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*<sup>−/−</sup> mice will have a protective effect. Thus, bone marrow–derived macrophages from WT mice were adoptively transferred into *Pirb*<sup>−/−</sup> mice, and disease susceptibility was assessed. Adoptive transfer of WT bone marrow–derived macrophages into *Pirb*<sup>−/−</sup> mice resulted in no change in colonic phenotype. By day 6, DSS-treated

*Pirb*<sup>−/−</sup> mice that received WT macrophages (WT<sub>mac</sub> → *Pirb*<sup>−/−</sup><sub>mice</sub>) displayed substantially decreased weight loss (Figure 6A; day 6) and exhibited decreased disease activity and histopathology when compared with DSS-treated *Pirb*<sup>−/−</sup> mice (Figure 6B–E and Supplementary Figure 11). Interestingly, assessment of IL-6 levels in ex vivo colonic punch biopsy specimens showed no statistically significant difference between DSS-treated *Pirb*<sup>−/−</sup> mice and WT<sub>mac</sub> → *Pirb*<sup>−/−</sup><sub>mice</sub> (Figure 6F).

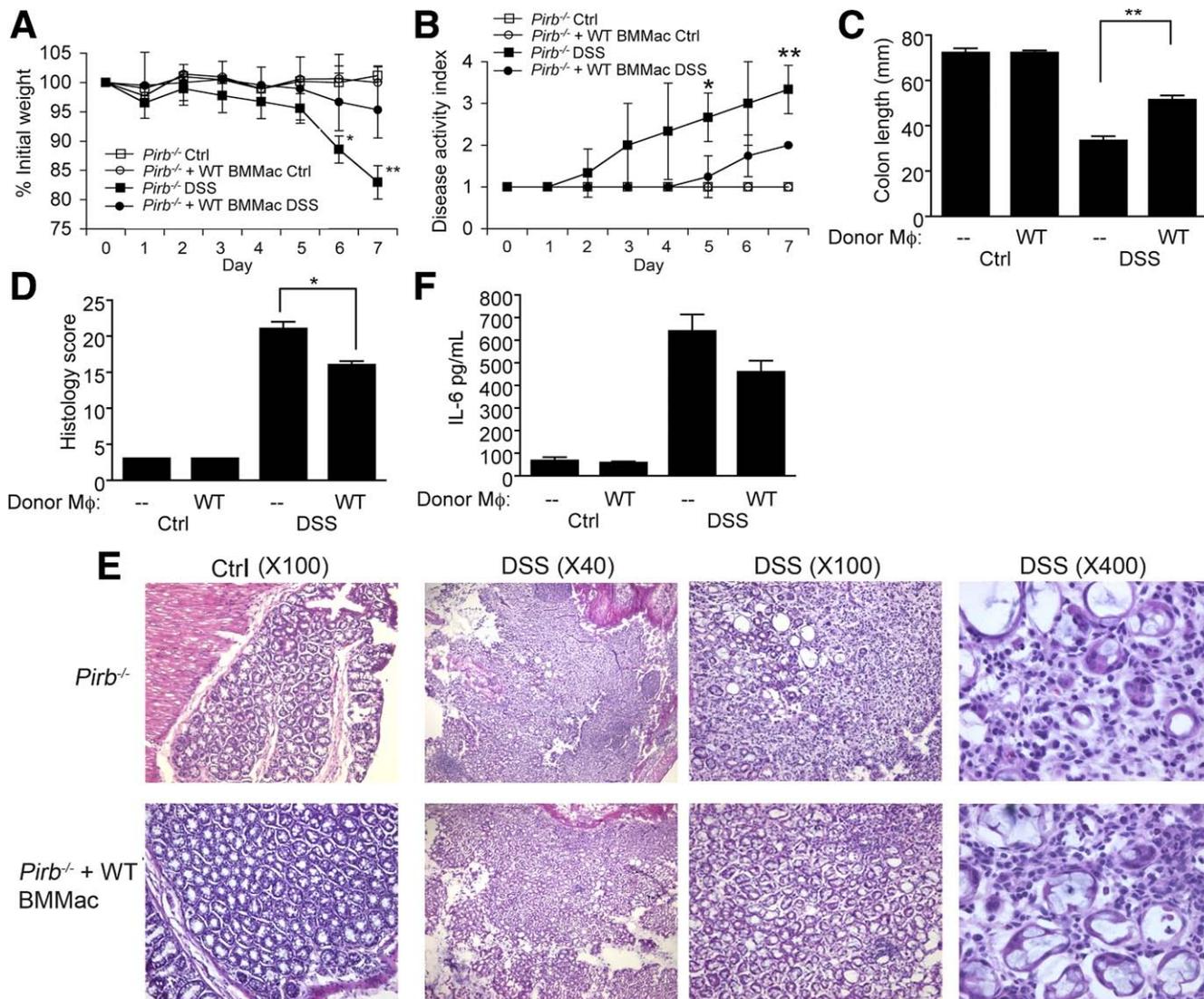
#### **Expression of Human ILTs/LIRs in IBD**

To gain insight into a possible role of PIR-B human orthologues in IBD, we assessed the expression of 2 inhibitory ILT/LIR family members in the colonic mucosa of healthy controls and patients with UC, namely ILT-2/LIR-1 (CD85J) and ILT-3/LIR-5 (CD85K).<sup>26,27</sup> Compared with an isotype control, we showed that CD85J is primarily expressed by mononuclear cells within the LP of the colon from healthy controls (Figure 7A and E). Notably, we observed CD85J expression on CD68<sup>+</sup> and CD68<sup>−</sup> cells (Figure 7A). In contrast, 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<sup>+</sup> cells within the LP of colonic biopsy samples from pediatric patients with UC, the CD85J and CD85K cellular specificity was similar to that observed in controls (Figure 7). These data show 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*<sup>−/−</sup> mice have increased susceptibility to DSS-induced colitis. The increase in disease severity was linked with elevated macrophage-associated proinflammatory cytokine production. In vitro analysis of *E coli*–stimulated bone marrow- and thioglycolate-derived macrophages revealed a link among 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 show expression of PIR-B human orthologues ILT-2 and ILT-3 on mononuclear cells within the LP, including CD68<sup>+</sup> macrophages and intestinal epithelial cells.

We show 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, dendritic cells, and B cells.<sup>28</sup> Notably, PIR-B nega-

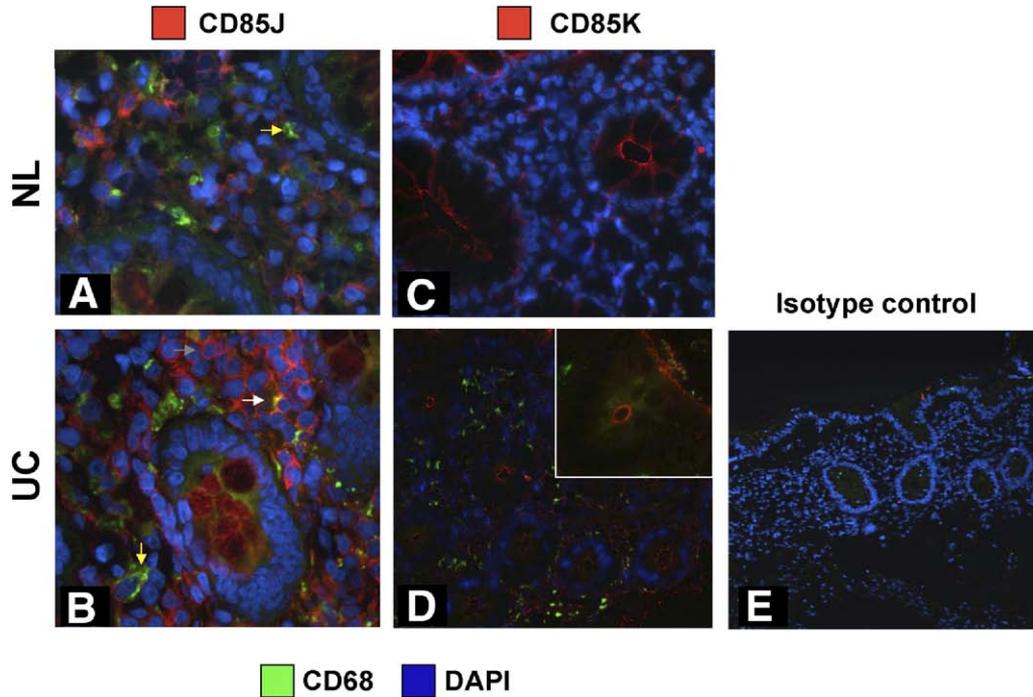


**Figure 6.** Assessment of DSS-induced colitis following adoptive transfer of WT bone marrow derived-macrophages (BMMac) into *Pirb*<sup>-/-</sup> mice. (A) Weight loss, (B) disease activity index, and (C) colon length in *Pirb*<sup>-/-</sup> mice receiving adoptively transferred WT macrophages and exposed to DSS. (D) Quantification of the histologic score and (E) representative photomicrographs of control and DSS-treated colons. (F) IL-6 levels in the culture supernatant of distal colon punch biopsy specimens from control and DSS-treated mice. Data are from a representative experiment of n = 2 (6–8 mice per group). \**P* < .05; \*\**P* < .01 vs *Pirb*<sup>-/-</sup> ctrl. (E) Original magnification 40×.

tively regulates neutrophil and eosinophil function.<sup>19,29</sup> Given that these cell types have a role in the pathophysiology of DSS-induced colitis,<sup>16,30,31</sup> we cannot rule out that hyperactivated neutrophils and eosinophils may contribute in part to the increased disease severity in *Pirb*<sup>-/-</sup> mice. However, our adoptive transfer experiments show that loss of PIR-B expression and of negative regulation of macrophage activation are sufficient to enhance susceptibility to DSS-induced colitis. Interestingly, transfer of WT bone marrow-derived macrophages into *Pirb*<sup>-/-</sup> mice delayed disease progression and had a protective effect. There are 2 possible explanations for these observations. First, WT bone marrow-derived macrophages could be displacing *Pirb*<sup>-/-</sup> macrophages in the colon and thus reducing the proinflammatory environment and disease pathol-

ogy alternatively, WT bone marrow-derived macrophages in *Pirb*<sup>-/-</sup> mice possess anti-inflammatory activity and negatively regulate *Pirb*<sup>-/-</sup> macrophage activation and suppress DSS-induced colitis. Previous studies have shown that myeloid-specific STAT3-deficient mice develop spontaneous enterocolitis.<sup>32</sup> Susceptibility to spontaneous enterocolitis was linked to a loss of anti-inflammatory IL-10/Stat3 signaling in macrophages.<sup>32</sup> 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 delineated.<sup>27</sup> Initial studies suggested that major histocompatibility complex class I (H-2) molecules are ligands for PIR-B; however, recent studies indicate interaction with *S aureus*



**Figure 7.** Cellular expression of ILT-2/LIR-1 (CD85J) and ILT-3/LIR-5 (CD85K) in pediatric UC. Immunofluorescence labeling for (A and C) CD68 and ILT-2/LIR-1 (CD85J) and (B and D) CD85K in colonic biopsy samples from healthy controls and pediatric patients with UC. Anti-CD85J and anti-CD85K-Alexa488 (green); anti-CD68/TRITC (red) nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (E) Isotype control stained colonic biopsy specimen. Results representative of 3 cases are shown. Original magnification 100 $\times$ ; inset 200 $\times$ . White arrow indicates CD68<sup>+</sup>CD85J cell CD85J, and yellow arrow indicates CD68<sup>+</sup>CD85J-cells.

(gram positive) and *E coli* (gram negative).<sup>9,14</sup> We show that stimulation of WT and *Pirb*<sup>-/-</sup> macrophages (resident or inflammatory) with *E coli*-derived LPS induced an equivalent increase in IL-6 and IL-1 $\beta$  production and MAPK/NF- $\kappa$ B phosphorylation (data not shown), whereas activation of *Pirb*<sup>-/-</sup> 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 inhibit TLR-mediated activation in response to bacterial stimulation. Consistent with this concept, TLR-2 agonist PAM<sub>3</sub>CSK<sub>4</sub> stimulation of WT and *Pirb*<sup>-/-</sup> 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- $\alpha$  production.<sup>14</sup> Although further investigation is required to define the bacterial cell wall components that bind and/or activate PIR-B, recent studies suggest that PIR-B may possess scavenger receptor-like binding activity toward bacteria.

TLR ligands and proinflammatory cytokines including IL-1 $\beta$  and TNF- $\alpha$  are believed to activate macrophage MAPK (p38, ERK1/2, and JNK) and NF- $\kappa$ B pathways, promoting gene expression and cytokine production, leading to cytokine-mediated inflammation and IBD.<sup>33</sup> Consistent with this, both the MAPKs (p38, JNK, and ERK1/2) and NF- $\kappa$ B are significantly activated in the inflamed colonic mucosa of patients with IBD.<sup>34</sup> Our

mechanistic analysis revealed that PIR-B negatively regulates bacterial-induced macrophage activation of MAPK, primarily ERK1/2 and p38, and to a lesser extent NF- $\kappa$ B phosphorylation. Pharmacologic blockade of MAPK activation, specifically p38, improved disease activity and histology disease score in a DSS-model of colitis.<sup>35</sup> Unexpectedly, we observed decreased JNK phosphorylation in *Pirb*<sup>-/-</sup> macrophages as well as decreased levels of the JNK target, c-Jun. Previous studies have shown that *Pirb*<sup>-/-</sup> myeloid dendritic cells are of immature phenotype and are unable to up-regulate major histocompatibility complex II molecules, a process that is dependent on JNK and c-Jun stimulation.<sup>15</sup> Notably, the observed elevation in IL-6, TNF- $\alpha$ , and IL-1 $\beta$  levels in *Pirb*<sup>-/-</sup> 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 $\kappa$ B and JNK; enhances ERK activation) in activated macrophages.<sup>36</sup> 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 following activation has been associated with the recruitment of SHP-1 and

SHP-2.<sup>12,23,24</sup> We show that stimulation of macrophages with *E coli*-induced tyrosine phosphorylation of PIR-B and recruitment of the phosphatase SHP-1 but not SHP-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) activation.<sup>37</sup> 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) inhibitor.<sup>38</sup> Furthermore, SHP-1 dephosphorylates vascular endothelial growth factor-induced ERK phosphorylation in endothelial cells.<sup>39</sup> In contrast to PIR-B, SIRP-1 $\alpha$ , another immunoreceptor tyrosine-based inhibitory motif-bearing receptor, inhibits LPS/TLR-4-mediated signaling primarily through sequestering SHP-2 but not SHP-1,<sup>40</sup> suggesting that different inhibitory receptors may utilize divergent intracellular phosphatases to elicit their inhibitory effect.

A limitation of these in vitro macrophage analyses is that all studies were conducted on bone marrow-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 show PIR-B expression on intestinal macrophages and show that in vivo transfer of bone marrow-derived macrophages leads to elevated cytokine production and exacerbates DSS-induced colitis, suggesting that PIR-B negatively regulates intestinal macrophage activation.

We have shown the expression of PIR-B human orthologues ILT-2/CD85J and ILT-3/CD85K in the colonic mucosa of healthy controls and patients with UC. ILT-2/CD85J was primarily expressed by CD68<sup>+</sup> and CD68<sup>-</sup> mononuclear cells within the LP, 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 nonhematopoietic cell function. The gastrointestinal tract is a tightly regulated immune organ that possesses suppressive immune mechanisms that prevent uncontrolled proinflammatory reactions toward 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 nonresponsiveness. We did not observe differences in ILT-2/LIR-1 or ILT-3/LIR-5 expression in the colonic mucosa of healthy controls and patients with UC. Consistent with this observation, levels of LIRs (ILT-1, ILT-4, and ILT-5) on monocytes from patients with rheumatoid arthritis were comparable to those of sex- and age-matched control subjects.<sup>41</sup> The mechanism of LIR mod-

ulation of inflammation remains unclear; however, it is postulated that LIRs regulate the threshold for activation of inflammatory cells and determine the severity of inflammation<sup>42</sup> via the relative balance of activating or inhibitory LIRs 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 gastrointestinal homeostasis in humans, 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

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: [10.1053/j.gastro.2010.04.006](https://doi.org/10.1053/j.gastro.2010.04.006).

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