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Exacerbated intestinal inflammation in $P2Y_6$ deficient mice is associated with Th17 activation

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ABSTRACT

Extracellular nucleotides are released as constitutive danger signals by various cell types and activate nucleotide (P2) receptors such as $P2Y_6$ receptor. $P2Y_6$ activation on monocytes induces the secretion of the chemokine CXCL8 which may propagate intestinal inflammation. Also, $P2Y_6$ expression is increased in infiltrating T cells of Crohn's disease patients. As inflammatory bowel disease (IBD) is associated with immune cell recruitment, we hypothesised that $P2Y_6$ would participate to the establishment of inflammation in this disease. To address this, we used $P2Y_6$ deficient (*P2ry6*$^{-/-}$) mice in the dextran sodium sulfate (DSS) murine model of IBD. In disagreement with our hypothesis, $P2Y_6$ deficient mice were more susceptible to inflammation induced by DSS than WT mice. DSS treated-*P2ry6*$^{-/-}$ mice showed increased histological damage and increased neutrophil and macrophage infiltration that correlated with increased mRNA levels of the chemokines KC and MCP-1. DSS treated-*P2ry6*$^{-/-}$ mice exhibited also higher levels of Th17/Th1 lymphocytes in their colon which correlated with increased levels of IFN-γ and IL-17A in the sera as well as increased mRNA levels of IFN-γ, IL-17A, IL-6, IL-23 and IL-1β in *P2ry6*$^{-/-}$ colons. This inflammation was also accompanied by a decreased cell proliferation and goblet cell number. Importantly, injection of anti-IL-17 intraperitoneally partially protected *P2ry6*$^{-/-}$ mice from DSS-induced colitis. Taken together, in the absence of $P2Y_6$, an exacerbated intestinal inflammation to DSS was observed which correlated with increased recruitment of Th17/Th1 lymphocytes. These data suggest a protective role of $P2Y_6$ expressed on leukocytes in intestinal inflammation.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic multifactorial disease known under two major phenotypes in humans: Crohn's disease (CD) and ulcerative colitis (UC). This pathology is characterized by inflammation-mediated damage to the intestinal mucosae where inflammatory immune cells are recruited which include T cells, macrophages and neutrophils. As a consequence, immune cell dysfunction results in the upregulation of synthesis and release of several proinflammatory mediators such as reactive oxygen metabolites, chemokines, and cytokines, which actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response in the intestine [1]. IBD has long been recognized to be associated with Th1 cell recruitment, which secretes the inflammatory cytokine IFN-γ. This cytokine plays an important role in upregulating antigen presentation and secretion of proinflammatory cytokines such as TNF-α and IL-1β. Emerging evidence suggest that Th17 also play a key pathogenic role in chronic inflammatory conditions, including IBD [2]. Genetic studies have shown a link between genetic susceptibility to Crohn disease and Th17 cytokine expression [3]. Interestingly, it was proposed that the IL-23/IL-17-mediated axis of inflammation plays an important role in T-cell-mediated diseases such as intestinal inflammation [4–6]. Increased level of IL-17A expression has also been detected in IBD [7–9]. IL-17 is a highly inflammatory cytokine that can induce the production of proinflammatory cytokines and chemokines responsible for the attraction of neutrophils and monocytes to the inflammatory sites [9,10]. Furthermore, due to their plasticity, Th17 can convert into Th17/Th1 cells and also produce IFN-γ [11,12].

During inflammation, in response to inflammatory aggressions, epithelial and immune cells release nucleotides such as adenosine-5′-triphosphate (ATP) and uridine-5′-triphosphate (UTP) along with their respective diphosphate derivatives (ADP and UDP) in the intestinal

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lumen s constitutive danger signals [13]. Once released, nucleotides contribute to the inflammatory response by interacting with nucleotide receptors at the cell surface which activate downstream signalling pathways. Indeed, $P2Y_{1,2,4,6,11-14}$ (G proteins coupled protein) and P2X1-7 (ligand-gated ion channels) receptors [14,15] modulate a variety of immune pathologies and pathways associated with inflammation and immune cell activation [15,16].

Emerging evidence suggests a role for $P2Y_6$ signalling in the regulation of immune functions related to inflammation [17,18]. An interesting observation about $P2Y_6$ is that its expression increases in infiltrating T cells of CD patients [19]. In monocytes, $P2Y_6$ receptor activation induces the production of the chemokine CCL2 [20]. We have also shown that $P2Y_6$ regulates neutrophil migration by controlling TLR2-induced CXCL8 release from human monocytes under inflammatory conditions, suggesting that its agonist UDP was released by these cells upon TLR stimulation [21]. CXCL8 is a powerful chemokine that attracts neutrophils and lymphocytes which is associated with the extended tissue damage observed in IBD patients [22]. In another study, Zhang et al. demonstrated that the activation of $P2Y_6$ receptor promotes host defence against bacterial infection via monocyte chemoattractant protein-1-mediated monocytes/macrophages recruitment which suggest that $P2Y_6$ could be a novel mediator in upregulating innate immune response against the invaded pathogens [23].

These data suggest that $P2Y_6$ receptor expressed on leukocytes might play a proinflammatory role in IBD. In opposite, we found in this study that $P2Y_6$ deficient mice had a dramatic susceptibility to DSS-induced colitis that was associated with an increased immune cell infiltration in the intestine, especially Th17/Th1 lymphocytes.

2. Material and methods

2.1. Reagents

Dextran Sulfate Sodium (DSS) was purchased from MP biomedicals (OH, USA). K_2HPO_4 was purchased from Acros organics (New Jersey, USA). KH_2PO_4 was obtained from EMD Millipore (Mississauga, ON, Canada). O-dianisidine hydrochloride, H_2O_2, phorbol 12-myristate 13-acetate (PMA), brefeldin A and ionomycin were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fetal Bovine Serum (FBS), HBSS and RPMI 1640 were obtained from Wisent (St-Bruno, Canada). Trizol and dispase were purchased from Invitrogen (Carlsbad, CA, USA). Cytofix/Cytoperm Kit Plus, anti-mouse CD16/CD32 antibody, anti–CD4-Alexa Fluor 647, anti–IL-17A–Alexa Fluor 488 and anti–IFN-γ–PE were purchased from BD Bioscience. F4/80 antibody was obtained from R&D Systems (Minneapolis, USA). Tgo DNA polymerase, Collagenase D, Syber Green and DNAaseI were obtained from Roche Diagnosis (Indianapolis, IN, USA) and antibodies to Muc2, Ki67 and CD3 were obtained from Abcam Inc. (Toronto, ON, Canada).

2.2. Animals

All experiments were conducted according to the guidelines of the Canadian Council on Animal Care (CCAC), and the protocols were approved by the Animal Care Committees of *Université Laval*.

P2yr6$^{-/-}$ mice obtained from B. Robaye (Université Libre de Bruxelles, Belgium) [18] were backcrossed 10 times with C57BL/6 mice from Charles River. A few backcrosses with WT females were performed to ascertain that the mitochondrial DNA is the same in mutant and control mice. Heterozygotes were then mated to obtained homozygote mice to generate a colony of *P2yr6*$^{-/-}$ mice. These deficient mice [18] behave similarly to WT mice in terms of growth, behaviour, and reproduction. No differences were observed with WT mice in absence of DSS in this work. *P2yr6*$^{-/-}$ mice as well as C57BL/6 mice were derived from specific-pathogen-free elite section and were bred in house. A few complementary experiments were performed with WT controls from Charles River (Pointe-Claire, QC, Canada) which responded similarly as the WT mice bred in house. Animals were maintained in a specific pathogen-free environment in a temperature-controlled room (21 °C) on a 12-hour/12-hour light and dark cycle and given unrestricted access to standard diet and tap water (or specified drinking solution). Mice were allowed to acclimate to these conditions for at least 7 days before experiments. Adult males (8–12 weeks) were used.

2.3. DSS induced acute-colitis

Colitis was induced by DSS 3% (*w/v*) (36,000–50,000 kDa), added to the drinking water for 7 days. Control mice received standard drinking water. Disease activity index (DAI) was evaluated daily by scoring percent of weight loss, intestinal bleeding (no blood, presence of blood), and stool consistency (normal stool, loose stool, or diarrhea) as previously described [24].

After 7 days of DSS treatment, the colon was collected, its length measured then divided in 3 parts: the proximal colon used to perform myeloperoxidase activity (MPO), the medial colon used for RNA extraction and the distal colon used for histological analysis.

2.4. Histological assessment of colitis

Colon specimens were fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C then embedded in paraffin. Five-micrometer tissue sections were stained with hematoxylin & eosin (H&E) then histology was observed and photographed with a BX51 Olympus microscope. Colonic sections were examined for evidence of colitis as previously published using 7 criteria: inflammatory cell infiltration, crypt density, muscle thickening, crypt hyperplasia, and goblet cell loss using a scale of 0–3 for each parameter, and the presence (1 point) or absence (0 point) of ulceration and also of crypt abscesses for a maximum of 17 points [24,25].

2.5. Myeloperoxidase activity assay

Neutrophil infiltration was evaluated by the quantification of the enzymatic activity of myeloperoxidase (MPO), which is most abundantly expressed in neutrophil granulocytes, as described previously [26]. Briefly, colon samples were homogenized in ice-cold potassium phosphate buffer (50 mM K_2HPO_4 and 50 mM KH_2PO_4, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The homogenates were centrifuged 20 min at 20,000 × *g*. The reaction was started by incubating the supernatant in 1 mg/ml o-dianisidine hydrochloride and 0.0005% (vol/vol) H_2O_2, then the change in absorbance at 450 nm was measured every 30 s over 3 min. MPO activity was expressed in units per milligram of tissue, where 1 unit corresponds to the activity required to degrade 1 μmol H_2O_2/min/ml at 24 °C [27].

2.6. Immunohistochemistry

Paraffine embedded tissue were used for immunohistochemistry which was performed as previously described [28]. Briefly, tissue sections collected 7 days after DSS treatment, were incubated at 4 °C for 18 h with the indicated primary antibodies then at 25 °C for 1 h with the appropriate biotinylated secondary antibody. Isotype-matched IgG was routinely included as a control. Proliferation was analysed using Ki67 antibody. The presence of Goblet cells was analysed with anti-Muc2 antibody which stained the mucus secreted by goblet cells. The infiltration of macrophages and lymphocytes were assessed with F4/80 and anti-CD3 antibodies, respectively. Percentage of stained areas from positive cells per microscopic field of colon was quantified by Image J software as detailed before [29]. Lymphocytes positive with CD3 antibody were counted on the 20× (objective) images of colon.

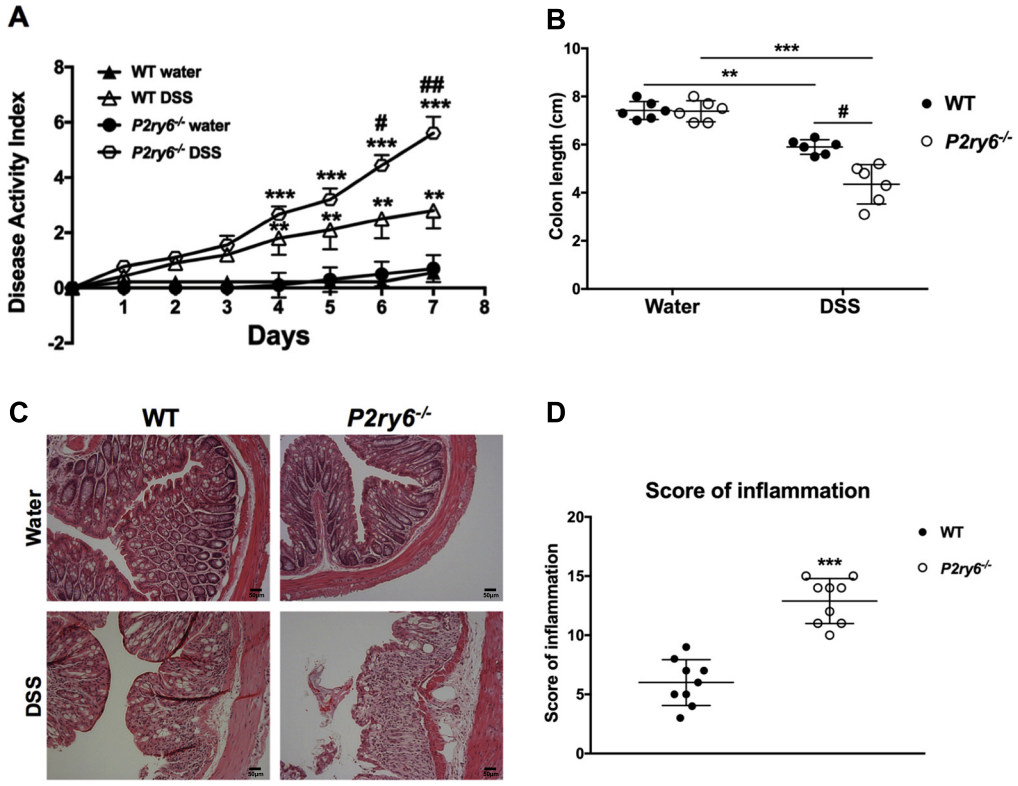

Fig. 1. *P2ry6*$^{-/-}$ mice are more susceptible to DSS-induced colitis. A. Disease activity index (DAI) of WT and *P2ry6*$^{-/-}$ mice during DSS treatment. $n = 20$. The data are shown as the mean ± S.E.M. B. Colon length from DSS-treated WT and *P2ry6*$^{-/-}$ mice at day 7 of acute DSS-induced colitis. $n = 6$. The data shows the individual data points and the mean ± S.E.M. A. B. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to non-treated mice. $^{\#}p < 0.05$, compared to DSS-treated WT. C. Representative histological photos of H&E-stained colon sections from the indicated groups at day 7 of acute DSS-induced colitis. Scale bar: 50 µm. D. The score of inflammation from the indicated groups at day 7 of DSS treatment. Individual data points are presented together with the mean ± S.E.M. of data quantified from 3 sections per colon for 3 mice.

2.7. Evaluation of lymphocyte subset infiltration

Cellular suspension including lymphocytes was isolated from mice colons as previously described [30–32]. Briefly, intraepithelial cells were prepared from longitudinally opened colons, washed to remove fecal content, and incubated with HBSS containing 1 mM EDTA, 1 mM DTT and 1% FBS for 30 min at 37 °C in a shaking water bath. After filtration through a 100 µm stainer, the cells were washed with RPMI 1640 containing 4% FBS, centrifuged at 780 × *g* for 20 min at 25 °C then resuspended in RPMI 1640 containing 10% FBS. For lamina propria cells, remaining tissue was treated with 1 mg/ml collagenase D, 0.5 mg/ml dispase, and 40 mg/ml DNaseI in RPMI 1640 containing 4% FBS for 3 h at 37 °C in a shaking water bath. The digested tissue was washed with HBSS supplemented with 5 mM EDTA then cells were collected and centrifuged as for the above collected fraction.

After isolation, cellular suspensions from both collected fractions were resuspended in the 40% fraction of a 40:80 Percoll gradient then overlaid on the 80% fraction. Percoll gradient was performed by centrifugation for 20 min at 2500 rpm. Lymphocytes were collected at the interphase of the Percoll gradient, washed then resuspended in FACS buffer to be activated with PMA (0.1 µg/ml) and ionomycin (1 µg/ml) for 6 h in presence of Brefeldin A (10 µg/ml) at 37 °C. Surface Fcγ receptors were blocked by anti-mouse CD16/CD32 for 15 min at 4 °C. Subsequently, cells were washed and first stained with anti-CD4-Alexa Fluor 647 or isotypic-Alexa Fluor 647 for 30 min at 4 °C. The cells were then washed, fixed and permeabilized with a CytoFix/CytoPerm kit for 10 min at 4 °C. Intracellular cytokine staining was performed with anti-IL-17A-Alexa Fluor 488 and anti-IFN-γ-PE for 30 min then expression of IL-17A and IFN-γ in CD4^{+} T cells was analysed using a BD FACSCalibur [33]. Cells stained with isotopic antibodies were used as a control.

2.8. mRNA expression of cytokines and chemokines in colon

Total RNA was isolated from mouse colon with Trizol then RNA was reverse transcribed to generate cDNA. Primers specific for cytokines and chemokines along with SYBR Green Supermix were used for quantitative real time PCR (qRT-PCR) using life technologies light cycler 7900. Standard curves were used to determine mRNA transcript copy number in individual reactions. Primer sequences for cytokine analysed were purchased from Qiagen. GAPDH and ACTB were used as reference genes to normalize results [34].

2.9. ELISA

The blood was collected by cardiac puncture then the serum was isolated by centrifugation at 3000 rpm for 10 min. The serum was then collected and frozen at −80 °C until determination of IL-17A and IFN-γ concentrations by ELISA kits following the manufacturers' instructions (R&D Systems).

2.10. Intraperitoneal injection of anti-mouse IL-17 antibody

The neutralizing rat anti-mouse IL-17A monoclonal antibody (mAb) and matched-purified rat IgG1 κ isotype control were purchased from BD Bioscience. One hundred micrograms of mAb was administered intraperitoneally 12 h after the beginning of DSS treatment. The administration of the same dose of mAb (blocking Ab or control isotype) was repeated three times at every 48 h until the end of the DSS treatment [35].

2.11. Statistical analysis

Statistical analysis was performed with Graphpad Prism 7 software. Results are expressed as mean ± standard error of the mean (S.E.M.). The statistical significance of differences between mean values was assessed by non-parametric test for DAI and two-way ANOVA followed by Bonferroni's correction for comparison of multiple groups with the control group using graph-prism software. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Increased inflammation in DSS-treated $P2Y_6$ deficient mice

To evaluate the role of $P2Y_6$ receptor in the development of acute colitis, WT and *P2ry6*$^{-/-}$ mice were exposed to 3% DSS in the drinking water. DSS treatment induced inflammation in both groups as measured by DAI when compared with control mice that received regular water. In opposite to what we were expecting, the DAI was dramatically increased in *P2ry6*$^{-/-}$ mice compared to WT mice from day 6 to the sacrifice of the mice on day 7 (Fig. 1A). The reduction in colon length, which reflects aggravated intestinal inflammation, was also significantly more pronounced in DSS-treated *P2ry6*$^{-/-}$ mice (Fig. 1B). The score of inflammation was also dramatically elevated in the histopathology analysis of DSS-treated *P2ry6*$^{-/-}$ colons (Fig. 1C, D).

3.2. Colonic crypts of DSS-treated *P2ry6*$^{-/-}$ mice showed a decreased proliferation and a decreased mucus production

Cell proliferation of colonic epithelial cells is known to be decreased by DSS treatment and this correlates with the exacerbation of inflammation [36]. DSS also destabilizes mucus secretion by goblet cells, of which Muc2 is the major structural component [37]. Dysregulation of biophysical mucus layer properties causes mucus barrier disruption and allows bacterial penetration and subsequent epithelial barrier disruption [38] which also lead to inflammation. Accordingly, we evaluated cell proliferation and mucus secretion in DSS-treated mice by immunohistochemistry.

Proliferation was quantified on colon transversal sections with an anti-Ki67 antibody (Fig. 2A, B). In basal state, WT and *P2ry6*$^{-/-}$ mice showed statistically comparable level of proliferation. When treated with DSS, both groups displayed a significant decrease in Ki67 positive cells which was more pronounced in *P2ry6*$^{-/-}$ intestine (10% ± 5% for WT mice vs 5% ± 3% for *P2ry6*$^{-/-}$ mice, $p < 0.05$).

As for Ki67, immunolabelling of Muc2 showed comparable level of staining in WT and *P2ry6*$^{-/-}$ sections of intestine before treatment and a decreased staining after DSS treatment which was significantly more pronounced in *P2ry6*$^{-/-}$ mice (9% ± 4% for WT mice vs 5.5% ± 2.5% for *P2ry6*$^{-/-}$ mice, $p < 0.05$). This suggests that there was a reduction of mucus production at the surface of *P2ry6*$^{-/-}$ intestinal epithelial cells after DSS treatment (Fig. 2C, D).

3.3. Increased number of infiltrated immune cells in the colon of *P2ry6*$^{-/-}$ mice

Because the deletion of $P2Y_6$ receptor resulted in aggravated intestinal inflammation in response to DSS, we questioned whether it correlated with a change in leukocyte subsets infiltration in the colon. Neutrophil infiltration was evaluated by the measurement of myeloperoxidase enzymatic activity into the colon 7 days after DSS treatment. *P2ry6*$^{-/-}$ intestines had significantly increased MPO activity when compared to control mice that received water, and also to WT mice treated with DSS (Fig. 3A).

Colon sections were stained with F4/80 and CD3 antibodies to evaluate the infiltration of macrophages and T lymphocytes, respectively. A marked increase in the number of macrophages and T lymphocytes was noted in DSS-treated *P2ry6*$^{-/-}$ mice, which was less prominent in DSS-treated WT intestines (Fig. 3B–E).

3.4. Cytokine and chemokine expression profile in DSS-treated *P2ry6*$^{-/-}$ mice

Having demonstrated an increased infiltration of neutrophils, macrophages and T lymphocytes (Fig. 3), we sought to determine the levels of cytokines and chemokines that are associated with these cell subsets. The results in Fig. 4 show increased mRNA levels of the chemokines KC and MCP-1 in DSS-treated knockout mice compared to WT ones. Both of these chemokines are chemoattractant for myeloid cells [10,39].

With regard to T lymphocytes, DSS-induced inflammation has been shown to be associated with Th17 and Th1 responses. We thus examined the cytokines associated with such phenotypes. We found that in comparison with WT mice, IL-17A and IFN-γ levels are increased in

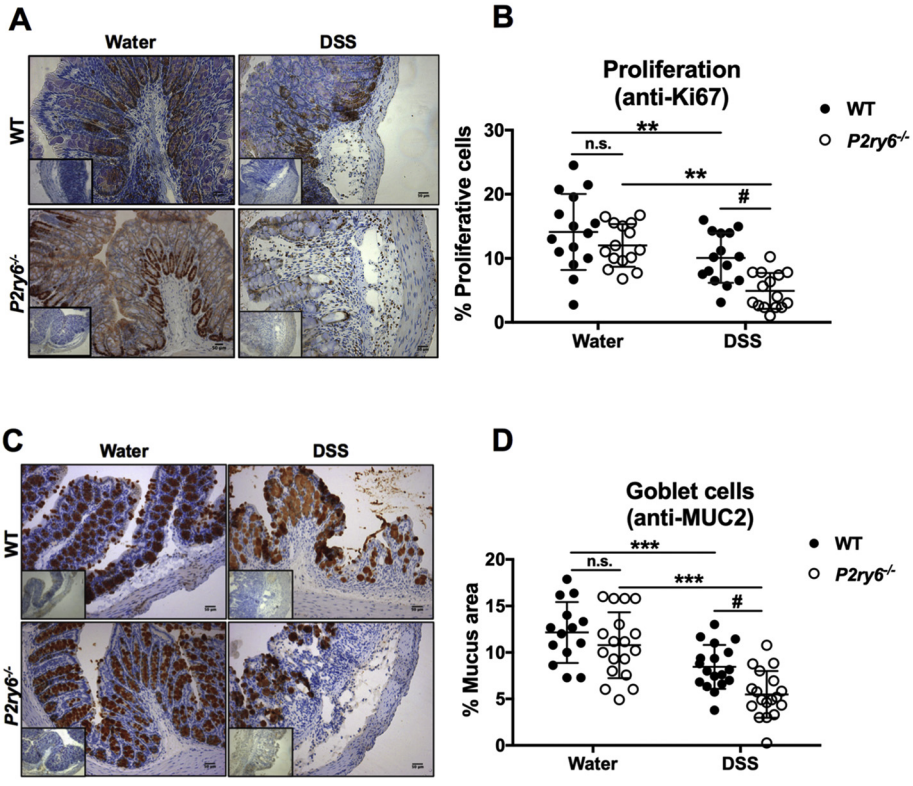

Fig. 2. DSS-treated *P2ry6*$^{-/-}$ mice show a decrease of proliferation and Goblet cell number in colonic crypts. A. Proliferation was analysed on paraffin colon sections using anti-Ki67 antibody. B. Quantification of labelled area corresponding to Ki67^{+} cells with Image J software. C. Staining of mucin-filled goblet cells with an anti-Muc2 antibody on paraffin colon sections from WT and *P2ry6*$^{-/-}$ mice after DSS treatment. D. Quantification of labelled area corresponding to Muc2^{+} cells with Image J software. B., D. Individual data points are shown together with the mean ± S.E.M. of data quantified from 3 sections per colon for 6 mice. Representative pictures are shown. Inset: IgG isotype control. Scale bar: 50 µm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to non-treated mice. $^{\#}p < 0.05$ compared to DSS-treated WT. n.s.: not significant.

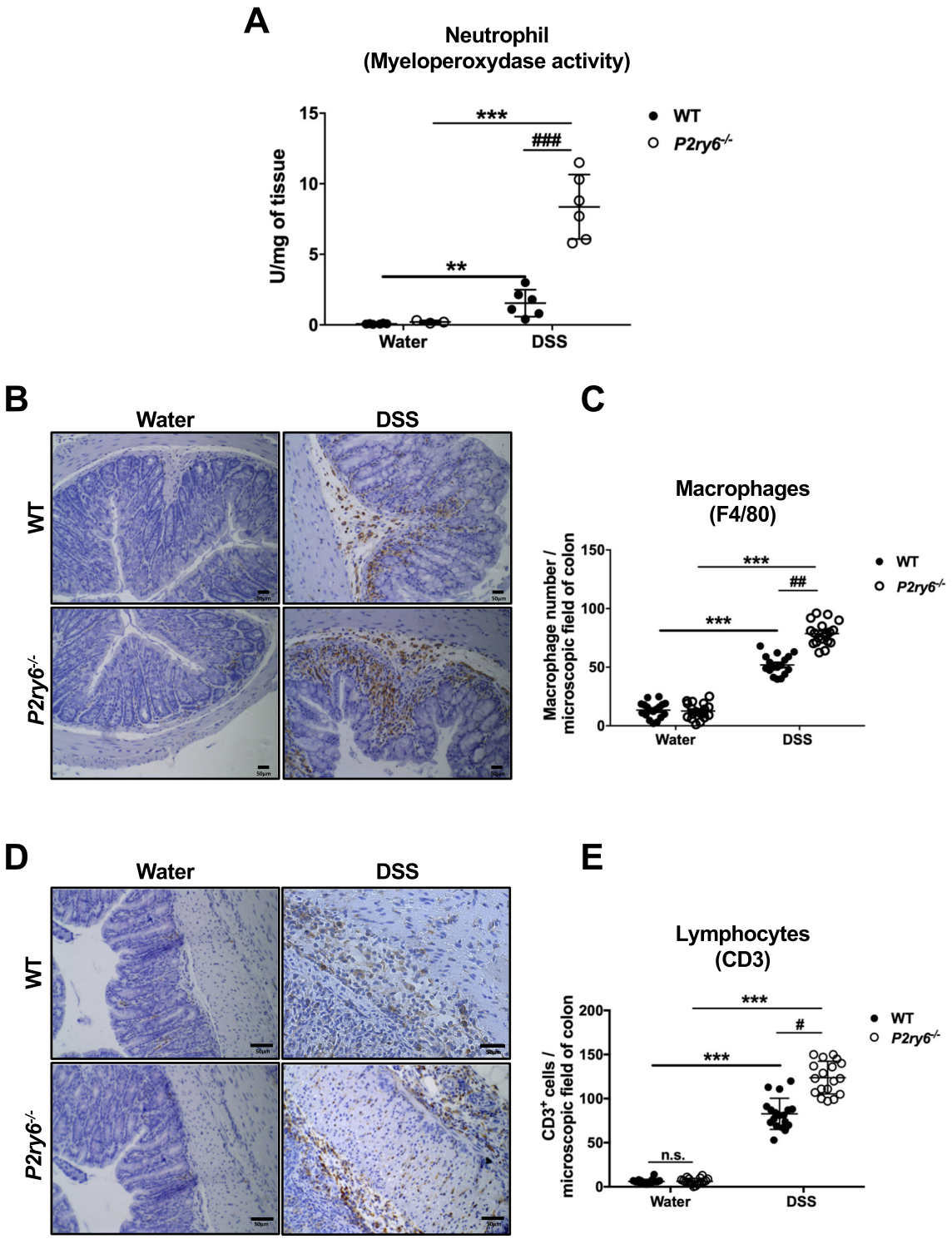

Fig. 3. Increased infiltration of immune cells in the colons of DSS-treated *P2ry6*$^{-/-}$ mice.
A. Measurement of myeloperoxidase activity (MPO) in the homogenate of the colon as an indicator of polymorphonuclear leucocytes (neutrophils). Individual data points are shown with the mean ± S.E.M. of the colons from 6 mice. B. Macrophage infiltration was analysed on paraffin colon sections using F4/80 antibody. Scale bar: 50 µm. C. Quantification by Image J software of macrophages infiltration evaluated by immunohistochemistry with F4/80. D. Lymphocyte infiltration was analysed on paraffin colon sections using CD3 antibody. Scale bar: 50 µm. E. Quantification of $CD3^+$ lymphocytes infiltration evaluated by immunohistochemistry. Individual data points with the mean ± S.E.M. of quantified cells from 3 sections per colon for 6 mice. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to non-treated mice. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared to DSS-treated WT.

DSS-treated knockout mice but the mRNA level of the Th1-differentiating cytokine IL-18 did not change (Fig. 4). Interestingly, there was a clear increase in cytokines involved in Th17 differentiation including IL-6, TGF-β1, IL-23 as well as IL-1β. In contrast, we found that the levels of TNF-α which can be produced by T cells and macrophages did not increase in DSS-treated wild type vs DSS-treated-knockout mice. Finally, the expression of MIP-2 increased similarly in DSS-treated mice of both genotypes.

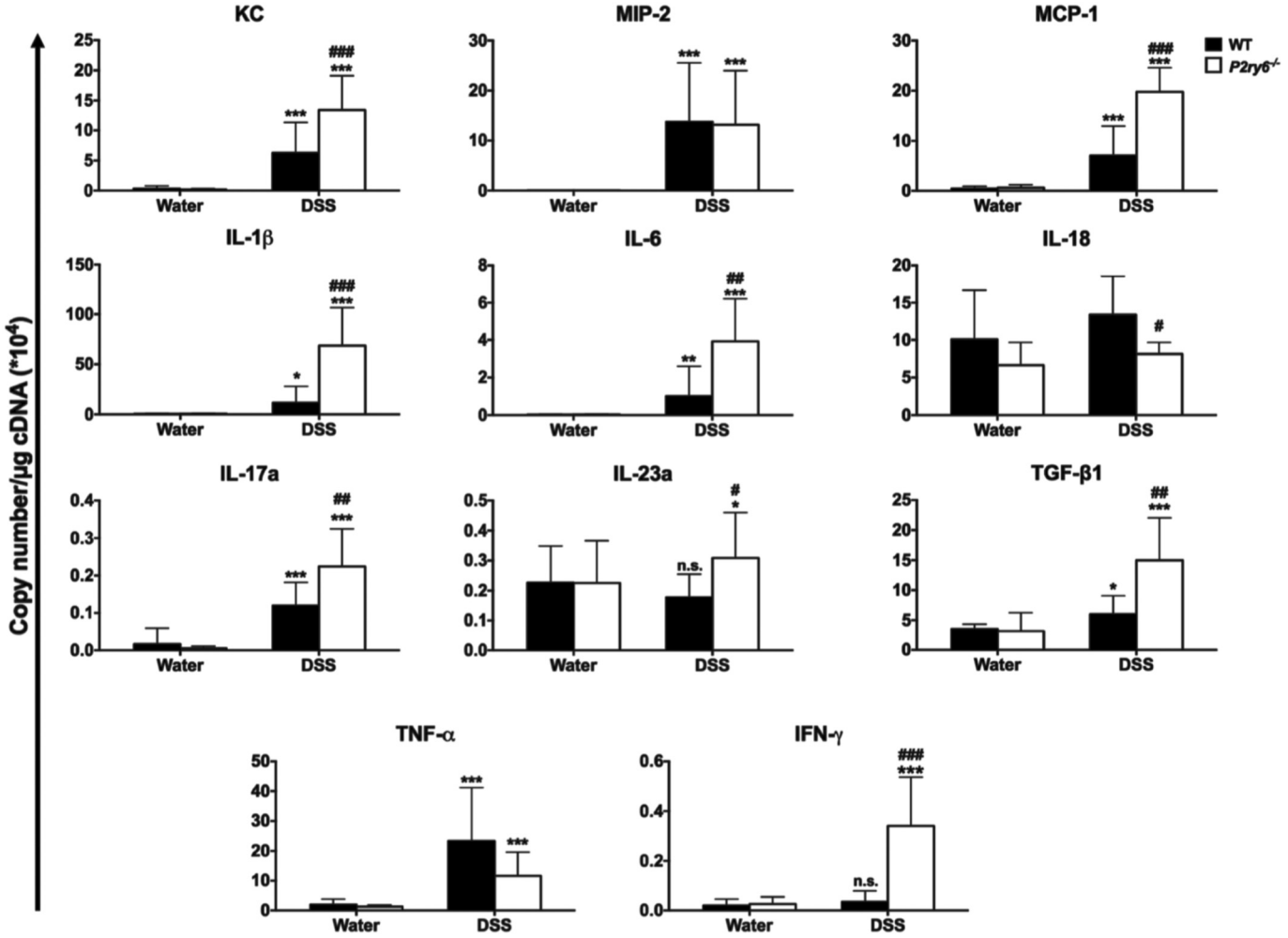

Fig. 4. Cytokine and chemokine expression profile in the colon of DSS-treated mice. The mRNA coding for chemokines and cytokines were quantified by qRT-PCR. Data were normalized with GAPDH mRNA level. Data shown are the mean ± S.E.M. of 3 pooled experiments of 4 mice each. $n = 12$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to non-treated mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to DSS-treated WT. n.s.: not significant.

3.5. Th17/Th1 cell accumulation in P2ry6$^{-/-}$ DSS-treated colon

Expansion and infiltration of effector Th1 and Th17 cells has been implicated in the pathogenesis of intestinal inflammation [1,30,40,41]. To assess the impact of the absence of $P2Y_6$ on the accumulation of Th1 and Th17 cells in the large intestine, cellular suspensions isolated from colon of WT and *P2ry6*$^{-/-}$ mice treated with DSS were stained intracellularly for IL-17A and IFN-γ on CD4^{+} cells. As shown in Fig. 5A, the total number of Th1 (CD4^{+} IFN-γ^{+} IL-17^{-}) and of Th17 (CD4^{+} IFN-γ^{-} IL-17^{+}) in DSS-treated mice was significantly higher in *P2ry6*$^{-/-}$ mice ($5.6 \pm 4.0 \times 10^4$ and $3.1 \pm 2.5 \times 10^4$ cells, respectively) in comparison to WT mice ($1.3 \pm 1.0 \times 10^4$ and $1.3 \pm 0.8 \times 10^4$ cells, respectively). Likewise, the total number of CD4^{+} T cells producing both IL-17 and IFN-γ was significantly higher in the *P2ry6*$^{-/-}$ colons ($177 \pm 80 \times 10^4$ cells) compared to WT mice ($60 \pm 40 \times 10^4$ cells). The Supplementary figure and Fig. 5B show a representative flow cytometry analysis of SSC/FSC gating strategy and Th1, Th17, and Th17/Th1 cells in WT and *P2ry6*$^{-/-}$ mice treated or not with DSS, respectively.

In agreement with these data, IFN-γ and IL-17A protein levels, which were barely detectable by ELISA in mice sera in absence of DSS (data not shown), increased at day 7 of DSS treatment in both WT and *P2ry6*$^{-/-}$ sera, and dramatically more in the sera of DSS-treated *P2ry6*$^{-/-}$ mice. IFN-γ protein level was increased by 13 folds in *P2ry6*$^{-/-}$ mice comparing to WT mice while IL-17A was increased by 18 folds in *P2ry6*$^{-/-}$ mice comparing to WT mice (Fig. 5C).

3.6. Blocking anti-IL-17 antibodies partially protects P2ry6$^{-/-}$ mice from DSS-induced colitis

IL-17 is a highly inflammatory cytokine involved in IBD and as seen in Fig. 5C its level increased in DSS-treated *P2ry6*$^{-/-}$. To investigate the role of IL-17 in DSS-induced colitis in *P2ry6*$^{-/-}$ mice, we have administrated an anti-mouse IL-17A mAb [35], or an isotype-matched control rat IgG1 κ, 4 times starting 12 h after the beginning of DSS treatment and followed by three injection at every 48 h. As shown in Fig. 6A, on days 6 and 7 after the initiation of colitis by DSS, the DAI was significantly lower in the anti-IL-17 mAb-treated mice than in the control rat IgG1 κ-treated mice. Accordingly, the length of the colon was significantly longer in the anti-IL-17 mAb-treated mice than in the control rat IgG1 κ-treated mice (Fig. 6B). DSS-colitis induced in *P2ry6*$^{-/-}$ mice injected with the isotype control was characterized by edema, infiltration of inflammatory cells into both the mucosa and submucosa, destruction of epithelial cells, and mucosal thickening (Fig. 6C). The observation of H&E photos of colons showed that the administration of anti-IL-17 blocking mAb reduced colitis damaged in *P2ry6*$^{-/-}$ mice (Fig. 6C) as seen with the score of inflammation (Fig. 6D).

As shown in Fig. 6E, the myeloperoxidase activity was significantly decreased in the colon of anti-IL-17A mAb-treated mice. Finally, mice

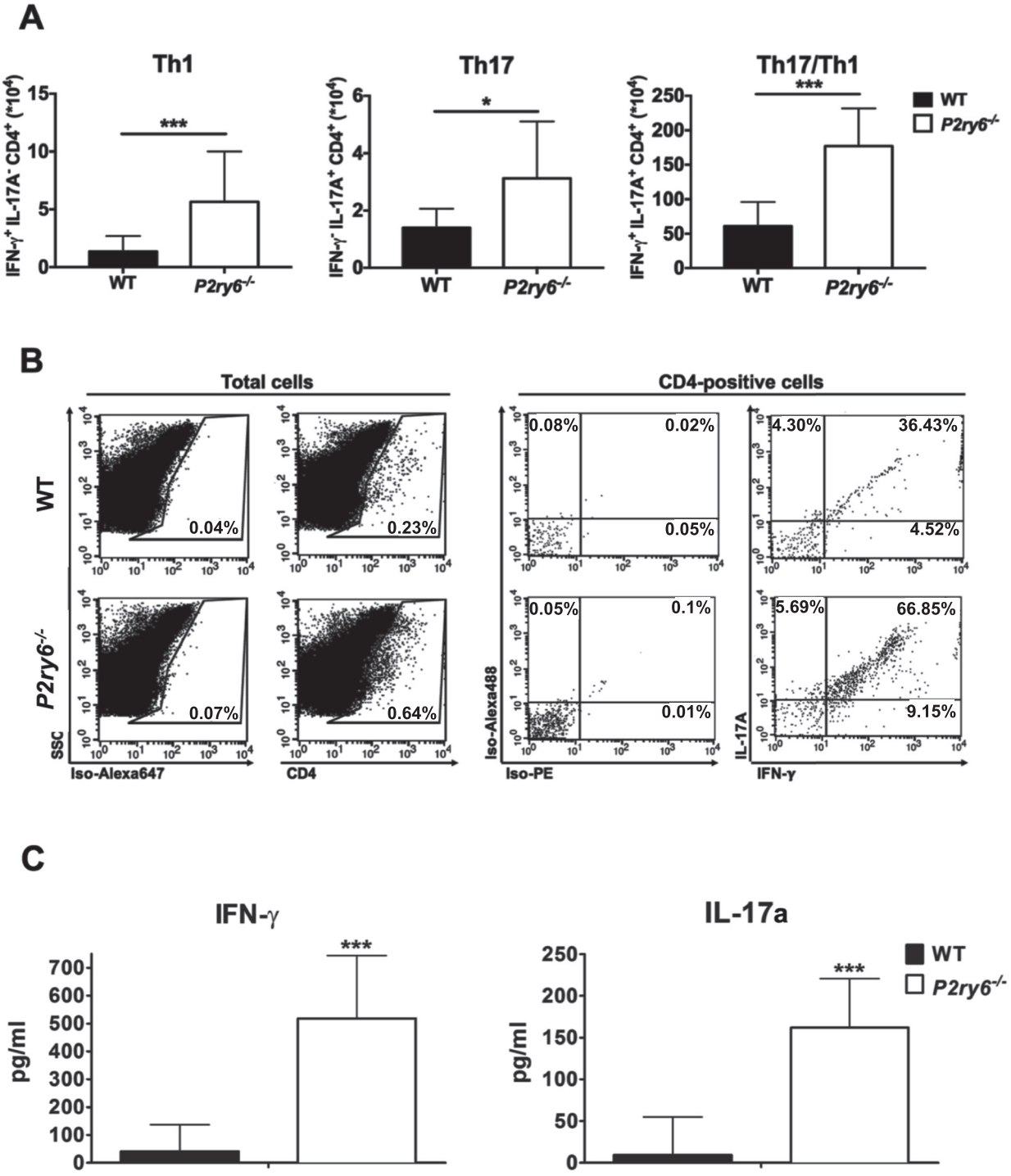

Fig. 5. Accumulation of Th1, Th17 and Th17/Th1 in the gut of DSS-treated *P2ry6*$^{-/-}$ mice. After 7 days of DSS treatment, cellular suspensions were prepared from colon, stimulated with PMA+ ionomycin in the presence of Brefeldin for 6 h and then stained with Alexa Fluor 647–, Alexa 488– and PE–conjugated isotypic (Iso) antibodies or with Alexa 647–anti–CD4, Alexa 488–anti–IL-17A, and PE–anti–IFN-γ mAbs as described in Material and methods. A. The cells expressing IFN-γ, IL-17A or both were analysed by intracellular staining using flow cytometry. Data are shown as mean ± S.E.M. from 3 pooled experiments of 4 mice each. $n = 12$. B. Representative flow cytometry profiles of total CD4$^+$ T cell population and IL-17A/IFN-γ positive cells gated on the CD4$^+$ T cell population from colon. C. Cytokines produced in the sera of DSS treated mice evaluated by ELISA. Each column represents the means ± SEM. $n = 8$. $^*p < 0.05$, $^{***}p < 0.001$.

treated with DSS in the presence of anti-IL-17A mAb showed a reduced mRNA expression in the colon of the chemokines KC and MCP-1 as well as of the cytokines TNF-α, IL-1β, IL-6, IL-17A, IL-18, IL-23a, TGF-β1 and of IFN-γ compared to mice which received the IgG isotype control (Fig. 6F).

4. Discussion

Several lines of evidences suggest that $P2Y_6$ receptor is proinflammatory as this receptor has been implicated in a number of proinflammatory effects in vitro and in vivo [42–44]. Regarding IBD, it was thought that the UDP/$P2Y_6$ signalling pathway is responsible of neutrophils recruitment through secretion of chemoattractant and by

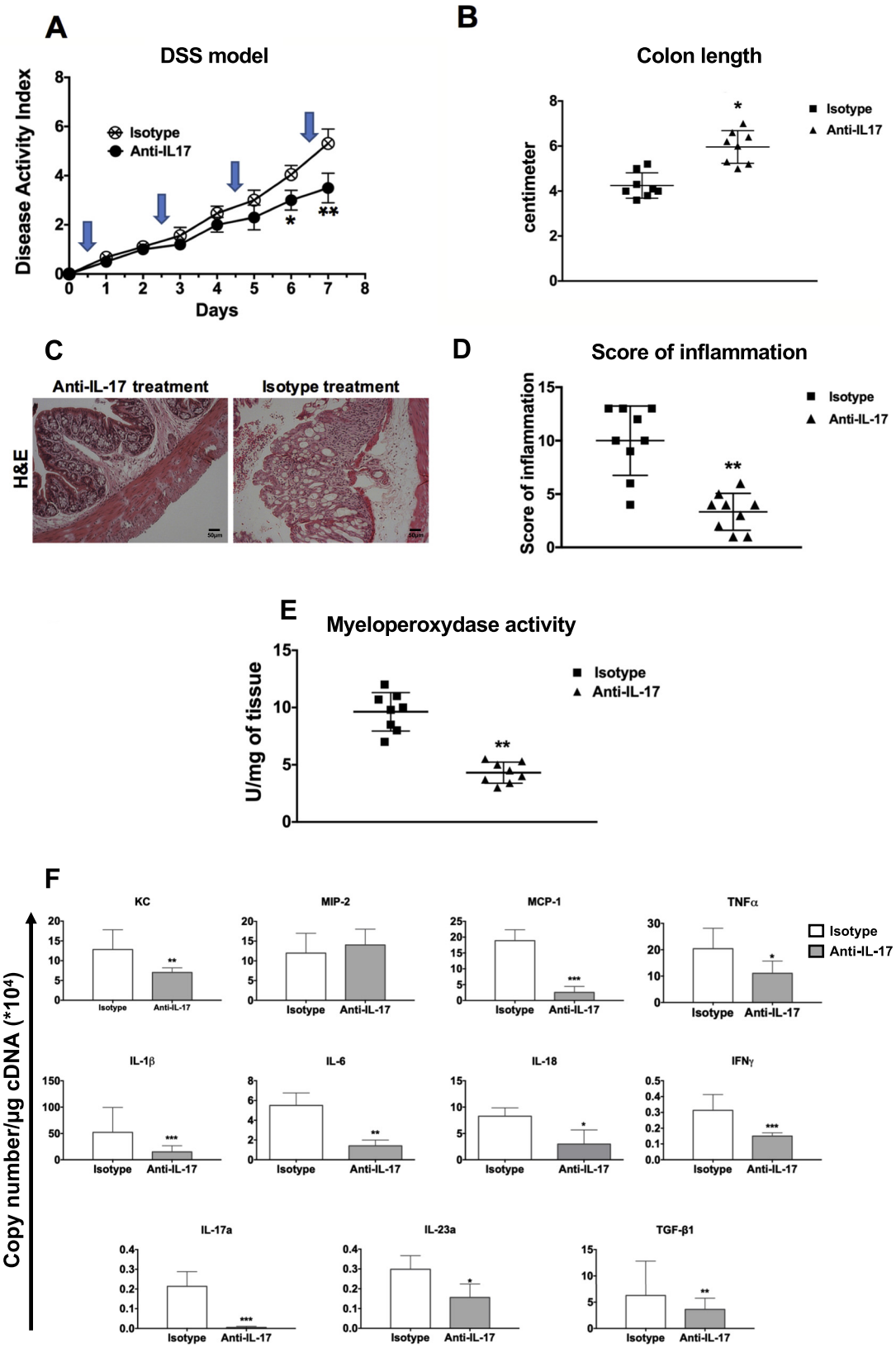

Fig. 6. IL-17 blocking antibodies partially protected *P2ry6*$^{-/-}$ mice from colitis induced by DSS. During the DSS treatment, *P2ry6*$^{-/-}$ mice were injected intraperitoneally with a blocking anti-IL-17A antibody (100 µg) or its isotype control every 48 h, as indicated by the arrows. A. DAI was recorded daily. B. Colon length at day 7 of acute DSS-induced colitis. C. Representative histological photos of H&E-stained colon sections from the indicated groups at day 7 of DSS treatment. Scale bar: 50 µm. D. The histopathological score of inflammation from the indicated groups at day 7 of DSS treatment. Individual data points are shown together with the mean ± S.E.M. of data quantified from 3 sections per colon for 3 mice. E. Measurement of myeloperoxidase (MPO) activity in the homogenate of the colon as an indicator of polymorphonuclear leucocytes (neutrophils). F. The mRNA expressed in the colon coding for chemokines and cytokines were quantified by qRT-PCR. Data were normalized with GAPDH mRNA level and are shown as the mean ± S.E.M., $n = 8$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to mice treated with the matched isotype control.

this way participated to the pathology [22]. In contrast, the present study shows that mice with full deletion of $P2Y_6$ receptor exhibit high susceptibility to DSS-induced colitis as seen with the elevated DAI, the decreased colon length, the severe lesions throughout the mucosa and the alteration of epithelial structure (Fig. 1). The exacerbated inflammation observed in $P2Y_6$ deficient mice treated with DSS was also associated with a loss of intestinal epithelial cell proliferation and a decreased mucus production. In addition, an increased infiltration of leukocytes was observed in the colon of *P2ry6*$^{-/-}$ mice treated with DSS, namely macrophages, neutrophils and Th1, Th17 and Th17/Th1 lymphocyte subsets. The accumulation of these immune cells in the *P2ry6*$^{-/-}$ colons correlated with the increased expression of several chemokines and proinflammatory cytokines as measured by qRT-PCR in colon homogenates of these mice. Taken together, these findings suggest a protective role of $P2Y_6$ receptor in the regulation of intestinal inflammation in the DSS model.

In the settings presented in this work, we noted a decrease of proliferation in both WT and *P2ry6*$^{-/-}$ colons treated with DSS which was more pronounced in *P2ry6*$^{-/-}$. These observations are in agreement with studies that showed that DSS reduces cell mitosis by arresting cell cycle at the G0 stage and also by inducing apoptosis [36].

P2ry6$^{-/-}$ mice also exhibited altered production of Muc2 which is produced by goblet cells. Muc2 is a highly glycosylated multimerized protein and the main structural mucus component that creates net-like sheets that stack upon each other after secretion forming a sieving mucus structure that is normally impenetrable to commensal colonic bacteria [38,45–47]. There is strong evidence that mucus layer disruption contributes to ulcerative colitis [48]. The results presented in this study are in agreement with these studies that propose that intestinal inflammation could be associated with alteration of mucus secretion by goblet cells [49,50]. It is therefore possible that $P2Y_6$ controls intestinal inflammation partly by regulating mucus production. Whether the reduction in Muc2 seen in the *P2ry6*$^{-/-}$ intestines represents a trigger of inflammation or a consequence of inflammation in these mice needs further investigation.

IBD is characterized by an increase number of immune cells including neutrophils and macrophages in the intestine. When activated, these cells release chemokines which recruit effector cells such as lymphocytes to the inflammatory site [51–53]. The results presented in this study show that the increased intestinal inflammation in $P2Y_6$-deficient mice was associated with a significant increase of macrophages, neutrophils as well as of Th1, Th17 and Th17/Th1 lymphocytes in colon. Although it was previously shown that $P2Y_6$ receptor contribute to leukocyte chemotaxis [23,54,55], we noted a significant increase in macrophages and neutrophils in the *P2ry6*$^{-/-}$ mice suggesting that other important mechanisms are in place.

In addition to immune cell infiltration, the expression of proinflammatory cytokines that contribute to the pathophysiology of IBD was shown to be increased in DSS-induced colitis [1]. Colons of *P2ry6*$^{-/-}$ mice treated with DSS showed an abnormal profile of RNA expression of chemokines and proinflammatory cytokines. In the colon, cytokines and chemokines are generally produced by cells from the immune system such as lymphocytes, macrophages and dendritic cells which exacerbate inflammation [39,56,57]. This suggests that $P2Y_6$ receptors expressed on these cells may be involved in these effects. Whether these cytokines are the cause or the consequence of the inflammation observed here is not known and would require further evaluation.

The full deletion of $P2Y_6$ resulted in the accumulation of neutrophils in colon which is in agreement with the results of chemokine RNA expression. Indeed, the intestine of DSS-treated *P2ry6*$^{-/-}$ mice exhibited increased expression of the chemoattractants KC and IL-1β. KC is a chemokine that attracts neutrophils. During acute inflammation, the migration of polymorphonuclear leukocytes into the mucosa is also tightly orchestrated by several other chemoattractants such as IL-1β released by hematopoietic cells [10,53,58]. This migration of inflammatory cells leads to deleterious effects such as tissue destruction via oxidative and proteolytic damage which most likely contribute to chronic and irreversible inflammation [52]. The expression of MCP-1, a chemokine responsible for macrophage infiltration, was also increased in the colon of *P2ry6*$^{-/-}$ mice which correlated with the increased macrophage infiltration observed by immunohistochemistry.

Interestingly, IL-1β was highly expressed in *P2ry6*$^{-/-}$ mice while WT mice expressed higher levels of IL-18 mRNA. These cytokines have also been reported to be markedly up-regulated in other proinflammatory conditions [1]. Colons of *P2ry6*$^{-/-}$ mice treated with DSS expressed higher levels of IL-23 than those of WT mice. IL-23, a proinflammatory cytokine that activates the same intracellular signalling as IL-1β, is produced by antigen presenting cells and is known to stimulate the production of IL-17 and IL-6 from T-cells [5,6,53,59,60], which were indeed elevated in the colons of DSS-treated *P2ry6*$^{-/-}$ mice. IL-1β, IL-23 and IL-6 are critical for the differentiation of $CD4^+$ into Th17 cells [60,61], which could explain the observed accumulation of Th17 in the large intestines.

P2ry6$^{-/-}$ mice showed also significant increase of Th1 cell infiltration which correlated with higher expression and secretion of IFN-γ after DSS treatment when compared to WT mice. Th1 subset participates in tissue damage during inflammation, and contributes to the observed exacerbation of intestinal inflammation [62].

In addition, Th17 cells orchestrate tissue inflammation by producing proinflammatory cytokines such as IL-17, which induces from various cell types, chemokines (CXCL-8, CXCL-1, and CXCL-10), cytokines (IL-1β, IL-6), and growth factors (GM-CSF and G-CSF) [63,64] that contribute to inflammation and PMN recruitment [65–67]. Th17 cells are predominant in Crohn's disease and crucial in mediating host defence against both resident bacteria and extracellular pathogens [9]. Thus, it is likely that Th17 cells could play an important role in the exacerbated inflammation observed in *P2ry6*$^{-/-}$ mice. In agreement, the colon of DSS-treated *P2ry6*$^{-/-}$ mice revealed higher expression and secretion of IL-17 than WT mice. At the end of the DSS-treatment the intestine of *P2ry6*$^{-/-}$ mice had 3 times more Th17/Th1 cells (Fig. 5A) and 18 folds more IL-17A in their sera than WT mice (Fig. 5C). In addition, the pathological signs of colitis were ameliorated by the administration of an anti-IL-17A neutralizing mAb in *P2ry6*$^{-/-}$ mice (Fig. 6). These data indicate that IL-17A promotes the initiation and progression of inflammation in *P2ry6*$^{-/-}$ mice in the acute DSS-colitis model. Hence, our study identified the $P2Y_6$ pathway as a negative regulator of Th17 cells.

Recent evidence indicates that the Th17/Th1 cells are the most pathogenic T cell subsets in various autoimmune diseases including rheumatoid arthritis, multiple sclerosis and IBD [11,12,68,69]. Our results showed that there was also an increased infiltration of the Th17/Th1 subset, suggesting that they could also be targeted by $P2Y_6$ as well.

Although the majority of studies so far suggest that blocking $P2Y_6$ receptor would reduce inflammation [70], there are also studies, as the one herein, that show that $P2Y_6$ activation can also induce pathways that can protect from inflammation. Bone marrow macrophages deficient for $P2Y_6$ receptor were more susceptible to infection by vesicular stomatitis virus while $P2Y_6$ activation by UDP protected from viral infection [71]. In other studies, the $P2Y_6$ natural ligand UDP induces phagocytosis in microglia in a $P2Y_6$-dependent process that may contribute to clearance of cellular debris following nerve injury [72,73]. The latter observation could explain part of the increased inflammation observed in *P2ry6*$^{-/-}$ mice as a reduction of phagocytosis by macrophage would prevent the clearance of infiltrating bacteria into the intestinal lamina propria that may in turn favour cell activation and inflammation. $P2Y_6$ receptor is the most highly nucleotide receptor expressed in macrophages [17,21]. In agreement with a protective role of $P2Y_6$ expressed on leukocytes, bone marrow transplantation studies showed a dramatic increase of inflammation in DSS-treated mice where $P2Y_6$ receptor was deleted only in hematopoietic cells (manuscript in preparation). These data correlate with those presented in this

manuscript with the full $P2Y_6$-deficient mice. Also in agreement with the actual study, Giannattasio et al. reported that conditional mutant mice with absence of $P2Y_6$ in T cells had increased T cell activation and increased pathogenicity in an allergic lung model [43].

5. Conclusion

The results presented in this paper show that the full deletion of $P2Y_6$ in mice exacerbates intestinal colitis induced by DSS through increase of immune cell infiltration especially Th17/Th1 subset as well as macrophages and neutrophils. These data correlated with an increased expression of proinflammatory cytokines in the intestines of *P2ry6*$^{-/-}$ mice. In addition, a neutralizing anti-IL-17 mAb reduced the exacerbated inflammation in DSS-treated *P2ry6*$^{-/-}$ mice. These results suggest that $P2Y_6$ has a protective role in the development of IBD at least partially through the negative regulation of Th17 cells.

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Author contributions

M.S. designed the study, performed all experiments, drafted the manuscript and analysed the data. J.P. was responsible of colony maintenance and provided help with mouse work and histopathology analysis. M.A.A. and F.A. provided support in cytometry experiments and analysis. B.R. provided $P2Y_6$ deficient mice. J.S. supervised the work. B.R., F.A. and J.S. also participated to the redaction of the manuscript.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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