Genetic and pharmacological inactivation of the purinergic P2RX7 receptor dampens inflammation but increases tumor incidence in a mouse model of colitis-associated cancer

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Abstract

Colitis-associated cancer (CAC) is a complication of inflammatory bowel disease (IBD). Binding of extracellular ATP to the purinergic receptor P2RX7 has emerged as a critical event in controlling intestinal inflammation, acting to limit elevation of pro-inflammatory mast cells and cytokines and promote survival of regulatory T cells (Treg) and enteric neurons. In this study, we investigated the effect of P2RX7 blockade in an established mouse model of CAC. Using genetic and pharmacological tools, we found unexpectedly that while P2RX7 mediated inflammatory responses it also acted at an early time to suppress CAC development. P2RX7 blockade enhanced proliferation of intestinal epithelial cells and protected them from apoptosis. The proliferative effects of P2RX7 blockade were associated with an increased production of TGF-ß1 that was sufficient to stimulate the proliferation of intestinal epithelial cells. Lastly, P2RX7 blockade also altered immune cell infiltration and promoted Treg accumulation within lesions of the digestive system. Taken together, our findings reveal an unexpected role for P2RX7 in preventing colitis-associated cancer, suggesting cautions in the use of P2RX7 inhibitors to treat IBD given the possibility of increasing risks CAC as a result.

Precis: Timely and provocative findings suggest cautions in the clinical development of P2RX7 antagonists to treat inflammatory bowel disease, highlighting a need for additional investigations to gain a more complete understanding of how P2RX7 may influence risks in the development of inflammation-associated colon cancer.
Introduction

Purinergic receptors form a family of adenosine (P1) and ATP (P2) receptors involved in a complex signaling network that affects various cellular functions such as cell proliferation, cell differentiation and cell death, but also exocrine and endocrine secretion, immune responses and inflammation (1). They are widely expressed throughout the digestive tract, where they are involved in the regulation of intestinal motility. Moreover, it was shown that this family of receptors regulated several neuronal and non-neuronal gut functions, visceral sensation, and the immune cell activity (2). Therefore, it is now widely accepted that purinergic receptors actively cooperate in the maintenance of gut homeostasis.

P2X receptors (P2RX) display a conserved topology with two transmembrane spanning regions, a large extracellular region sensitive to the ligand and intracellular amino- and carboxyl-termini that contain signaling binding motifs. Upon gating by eATP, P2RX7, also referred to as P2X7R, forms a poorly-selective channel leading to membrane depolarization, potassium efflux and calcium and sodium influx (3). P2RX7 triggering also induces the formation of larger membrane pores that allow the passage of molecules reaching up to 900 Da. This particular feature has been linked to the presence of a long cytoplasmic C-terminal tail and to the possible activation of a pore-forming molecular partner suggested to be pannexin-1 (4,5). Importantly, both P2RX7 activation and pannexin-1 mediate NLRP3 inflammasome assembly, caspase-1 (casp1) activation, and maturation of the pro-inflammatory cytokines IL1B (IL-1β) and IL18 (6).

At the level of the gastrointestinal tract, P2RX7 is expressed on immune and non-immune cells (7,8). Its low level of sensitivity to eATP limits its activation within inflammatory sites and in the tumor microenvironment where eATP concentrations are in the 100 micromolar range (9). We previously reported that P2RX7 is differentially expressed in the mucosa of...
patients with active and quiescent inflammatory bowel disease (IBD) and that its activation could be triggered in response to neutrophil trans-epithelial migration (8). These results, together with published data based on the use of P2RX7 antagonists or $P2rx7^{-/-}$ mice, support the notion that P2RX7 participates in the initiation as well as in the regulation of the inflammatory response notably via the processing and release of IL1B (10-12).

It is widely accepted that chronic inflammation can promote tumor development (13). In this scenario, the pro-inflammatory role of P2RX7 in immune and epithelial cells would be expected to favor carcinogenesis. However, P2RX7 also regulates cell proliferation and/or cell death (14-17). Moreover, adding to the complex interplay between inflammation and carcinogenesis, a study has revealed the role of the pro-inflammatory P2RX7/NLRP3/casp-1 cascade in the priming of dendritic cells and in the immunogenicity of dying tumor cells following chemotherapy, which favors the emergence of an endogenous anti-tumor immune response (18). Finally, P2RX7 appears to be over expressed in a variety of neoplastic disorders and tumors (19-21) and P2RX7 expression increases the tumorigenic properties of human embryonic kidney cells (16). Hence, based on the apparently contradictory evidence, the precise role of P2RX7 in vivo in the context of inflammation-associated carcinogenesis needs to be carefully addressed.

In this study, we investigated the role of P2RX7 in CAC and we showed, using $P2rx7^{-/-}$ mice as well as the P2RX7 specific pharmacological antagonists, that the P2RX7 activity suppressed tumor development. While deciphering the cellular and molecular events leading to this suppressive function, we demonstrated that P2RX7 inactivation leads to: 1) over production of TGFB1, 2) recruitment of Tregs within inflammatory lesions and 3) TGFB1-mediated proliferation of colonic epithelial cells.
Materials and Methods

Animal strains

This study was approved by the Institutional Care and Use Committee of the University of Nice-Sophia Antipolis. Animal protocols were approved by the committee for Research and Ethics of the PACA region (CIEPAL azur, #PEA 12-125) and followed the European directive 2010/63/UE. The generation of C57BL/6 mice harboring targeted disruption of the \textit{P2rx7} gene has been described previously (10). Control C57BL/6J mice were supplied by Harlan laboratories (Gannat, France).

Induction of colitis and inflammation-driven tumor formation

Acute inflammation was induced as followed: intraperitoneal (i.p.) injection of azoxymethane (AOM) at day 0 prior to 5\% DSS treatment from day 1 to day 5. CAC was induced by a single i.p. injection of AOM followed by 3 cycles of 3\% DSS in the drinking water. Each cycle lasted 5 days and was separated by 2 weeks, as illustrated in Figure 4A. We used the following semi-quantitative clinical score which was adapted from (22). Body weight loss was scored as follows: (0) no loss, (1) loss < 5\%, (2) loss < 10\%, (3) loss < 20\% and (4) loss > 20\%. The scores for stool consistency were measured as (0) normal, (1) loose stools, (2) watery diarrhea or (3) severe watery diarrhea. Rectal bleeding was scored as (0) no blood, (1) presence of petechia, (2) stools with a trace of blood or (3) bleeding. Inflammation score was performed by trained pathologists. Briefly, the severity of inflammation (none, mild, moderate, severe), extent of inflammation (none, mucosa, mucosa and submucosa, transmural), crypt damage (none, basal to 1/3, basal to 2/3, crypt
loss, crypts and epithelium loss), and percentage of tissue affected by inflammation (0, 25, 50, 75 and 100%) were scored.

**Macroscopic polyp analysis and histopathology**

Colons (caecum to rectum) were removed from animals and processed for histopathology as described in the Supplementary Materials and Methods.

**Serum, colon organ cultures and ELISA**

Blood was collected with a heparin treated needle from the tail vein and serum was obtained by centrifugation (15 min, 3000 rpm at 4°C). After resection, colon tissues were cultured overnight in DMEM media, supernatants were collected and production of IL1B, TGFB1, CXCL1 and CXCL2 assayed by an ELISA as described by manufacturers (see Supplementary Materials and Methods).

**Quantitative real time PCR and protein gel blotting**

Total RNA and protein were isolated from colonic tissues using TRI Reagent following manufacturer instructions and processed as described by manufacturers (see Supplementary Materials and Methods). We used the following antibodies: Phospho-STAT3 (#9131), STAT3 (#9139) and BCL2 (Bcl2, #50E3) antibodies were from Cell Signaling, anti-BCL2L1 (Bcl-XS/L, sc-634) and anti-BAX (Bax, sc-526) were from Santa Cruz and the anti-ACTB (Actin, clone AC40) antibody was from Sigma Aldrich.

**Immunohistochemical analyses of mouse colon tissues**
Resected mouse colon tissues were fixed in 10% formalin, paraffin embedded and processed for immunohistochemical analyses as described previously (8) and Supplementary Materials and Methods. We used the following antibodies: anti-PCNA (Epitomics), anti-cleaved-caspase-3 (Imgenex), anti-CD3 (Abcam), anti-Foxp3-biotin (ebioscience), anti-F4/80 (Abcam), and anti-Ly6G (Abcam).

**Treatment with the P2RX7 antagonists and depleting antibodies**

Competitive P2RX7 antagonists A438079 and A740003 and depleting antibodies were given i.p. during the DSS treatment as indicated in the Supplementary Materials and Methods.

**Statistical Analysis**

All data are represented as mean values and error bars represent SEM. The unpaired Mann and Whitney t test and ANOVA were used to evaluate the statistical significance between groups.
Results

Genetic and pharmacological inactivation of P2RX7 dampen DSS-induced colonic inflammation

The inflammatory environment generated by DSS is associated with the release of eATP. We reasoned that P2RX7 inactivation should impact the overall inflammatory response and we used the acute DSS colitis model to test this hypothesis. After AOM injection, WT and P2rx7−/− mice were fed a 5%-DSS solution during 5 days and then clean drinking water for 4 or 9 days. As expected, when considering that P2RX7 triggered inflammasome assembly and pro-inflammatory cytokine release, P2rx7−/− mice were less susceptible to this treatment (Figure 1A). P2rx7−/− mice survived better and lost less weight. Further, they showed milder signs of colitis with less diarrhea and less rectal bleeding. We also observed a less dramatic shortening of the colon length at necropsy. Such protection required the mutation of the two alleles since heterozygous P2rx7+/− mice showed the same phenotype as WT mice (Supplementary Figure 1). Interestingly, these results were reproduced on WT mice treated with competitive P2RX7 antagonists (Figure 1B and Supplementary Figure 2). Systemic blockade of P2RX7 with A438079 or A740003 improved the survival of the mice and reduced all the hallmarks of colitis. Indeed, 9 days after DSS treatment, the loss of body weight and the disease activity score decreased by around 25%, whereas the colon length increased, as observed with P2rx7−/− mice.

Histological analyses of colon tissues were performed to evaluate the degree of inflammation. In the non-treated condition, both WT and P2rx7−/− mice showed a normal colonic mucosa, with the crypts being straight, well defined, and sitting on the muscularis mucosa (Figure 2A). At the end of the DSS regimen (day 5), histological colonic transversal tissue sections of both WT and P2rx7−/− mice showed diffuse hemorrhagic walls with multiple
ulcerations, mucosal edema, transmural neutrophil infiltration, and the presence of large areas of erosion. Scoring for inflammation, which demonstrated no difference between WT and KO mice, nearly reached the maximum (which is scored as 14). By contrast, 4 days after the end of the DSS challenge (day 9), histological mid colon tissue sections of WT mice still showed a high level of inflammation (12 ± 0.7), whereas that of P2rx7/- mice showed signs of re-epithelialization, as indicated by better delineated crypts and milder inflammation of the mucosa (inflammatory index of 5.1 ± 1.5). Finally, most of the colonic mucosa of P2rx7/- mice presented a normal aspect 9 days after DSS treatment (day 14). By contrast, recovery of WT mice was partial with a mean inflammatory index of 9 ± 0.9 as compared to 2.2 ± 0.6 for KO mice. These differences were reproduced in WT mice treated with the P2RX7 antagonists (Figure 2B and Supplementary Figure 2). In particular, mice that received the competitive antagonist showed weak inflammatory infiltrates with no mucosal erosion and no epithelial cell defects within the crypts. Further, histological scoring of mid colon tissue sections of antagonist-treated mice demonstrated a very low inflammatory index, which was statistically significant as compared to the control group.

Taken together, these results demonstrate that both genetic and pharmacological inactivation of P2RX7 dampen DSS-induced colonic inflammation. Notably, a time course analysis demonstrated that mice deficient in P2RX7 activity recovered more rapidly from inflammatory lesions than WT mice.

P2rx7/- mice exhibits increased colonic epithelial cell proliferation and decreased apoptosis after AOM/DSS treatment

The prompt recovery of P2rx7/- mice to acute AOM/DSS challenge led us to evaluate whether P2RX7 regulated colonic mucosal epithelial cell proliferation. For that, colon tissue
sections were stained for proliferating cell nuclear antigen (PCNA). In non-treated WT and
\( P2rx7^{-/-} \) animals, quantification of PCNA-positive cells per 30-50 well-formed crypts showed 5
to 10% PCNA positive cells (Supplementary Figure 3A). Five days after acute AOM/DSS
treatment no well-defined crypts were visible (Figure 2B). Therefore we evaluated cell
proliferation at days 9 and 14. The proliferation indices of AOM/DSS-treated animals were
significantly higher in \( P2rx7^{-/-} \) as compared to WT mice (Figure 3A). Such an effect could
directly dependents on P2RX7 expressed by colonic epithelial cells (8) and (Supplementary
Figure 3B), or alternatively, depends on infiltrating immune cells. To address this point, we
evaluated whether this effect could be reproduced \textit{in vitro} on cultivated colonic epithelial
cells expressing P2RX7. Indeed, blockade of P2RX7 enhanced the \textit{in vitro} proliferation of the
T84 colonic epithelial cell line (Supplementary Figure 3C). The effect of P2RX7 blockade on
epithelial cell proliferation was further confirmed \textit{in vivo} since A438079 administration to
AOM/DSS-treated mice increased colonic epithelial cell proliferation (Supplementary Figure
3D). We next evaluated whether P2RX7 also regulates apoptosis of intestinal epithelial cells.
For this, inflammatory tissue lesions were stained with cleaved caspase-3 antibody. We
observed significantly less apoptotic cells in \( P2rx7^{-/-} \) mice at day 9 and their complete
absence at day 14 post AOM/DSS treatment (Figure 3B). We then examined the expression
of anti-apoptotic genes. No changes were observed in the expression of BAX. By contrast, a
significant increase in the expression of the genes encoding BCL2 and BCL2L1 (Bcl-xL) was
observed in the whole colon of \( P2rx7^{-/-} \) mice at day 14 after AOM/DSS administration (Figure
3C). Finally, the increase in epithelial cell proliferation correlated with dysplasia in the
colon mucosa of \( P2rx7^{-/-} \) mice. We consistently found abnormal crypts characterized by
misalignment of the epithelial nuclei and a partial or total loss of mucosal secretion (Figure
3D). \( P2rx7^{-/-} \) mice were particularly prone to develop dysplasia, as they were also present in
mice that received a short and low dose of DSS (Supplementary Figure 4) that induced no
signs of colitis and only a very low inflammatory index. Taken together, these results
demonstrate that the functional activity of the P2RX7 receptor is required to control the
level of intestinal epithelial cell proliferation and apoptosis in response to an inflammatory
insult and suggest that alteration in P2RX7 functionality may favor possibly tumor
progression.

P2RX7 mediates gastrointestinal tumorigenesis in the colitis-associated cancer model
To investigate the effect of P2RX7 on inflammation-induced colorectal cancer, AOM-
challenged mice received 3 cycles of DSS to mimic chronic colitis (Figure 4A). Both WT and
P2rx7−/− mice survived AOM-DSS treatment and showed comparable disease activity indices
during the first 10 weeks of treatment. However, P2rx7−/− mice displayed a higher disease
activity scores at the end of the protocol (Supplementary Figure 5). Unexpectedly, despite
their lower susceptibility to acute DSS-induced inflammation, P2rx7−/− mice developed
significantly higher numbers of macroscopic polyps (mean of 9 polyps per mouse) as
compared to WT mice (mean of 2 polyps per mouse). As expected for the CAC model,
tumors were located at the distal end of the colon in WT mice whereas they invaded up to
the medial part of the colon in P2rx7−/− mice (Figure 4A). In addition, the 4-fold increase in
tumor burden in P2rx7−/− mice was accompanied by a 2-fold increase in polyp size (Figure 4B).
Histological examination of colons from WT and P2RX7 deficient mice showed that 80% of
the KO mice developed colonic lesions ranging from hyperplasia and dysplasia to
adenocarcinoma (Supplementary Figure 6A). Adenocarcinoma was observed in more than
60% of KO mice whereas only 25% of the WT cohort displayed a carcinoma.
Further, we investigated the pharmacological effect of P2RX7 blockade in the CAC model using the A438079 competitive antagonist. Changes in body weight were followed during the entire protocol and the colonic tumor burden was monitored 10 weeks after AOM injection. As observed for WT and P2rx7−/− mice, antagonist-treated and non-treated mice displayed comparable body weight variations during the course of the treatment (Supplementary Figure 5, lower panel). However, A438079 treated mice harbored higher numbers of macroscopic polyps and tumors were significantly larger in size than in control mice (Figure 4C). Collectively these results demonstrate that alteration of the P2RX7 functionality enhanced tumor promotion and/or progression. Interestingly, tumor progression was also found to be controlled by P2RX7 in an unrelated tumor mouse model based on subcutaneous injection of Lewis Lung Carcinoma (LLC) cells. As shown in Supplementary Figure 6B, mice injected with LLC cells developed tumors within two weeks. Importantly, excised tumors from P2rx7−/− mice were bigger than tumors from WT mice confirming that P2RX7 behaved as a tumor suppressor in this tumorigenic mouse model.

**P2RX7 shapes the inflammatory microenvironment**

P2RX7 stimulation is known to trigger inflammasome assembly and casp1 activation and to favor the release of the processed mature form of IL1B, thereby initiating the inflammatory response (4,10). As the early level of inflammation can influence the quality and the quantity of recruited immune cells, we next characterized the inflammatory microenvironment before tumor onset. We first checked the capacity of colonic explants from acute DSS-treated mice to secrete IL1B. Following in vivo DSS treatment, IL1B was readily produced ex vivo in colon tissue cultures derived from WT mice (Figure 5A). By contrast, and not surprisingly, very little IL1B was produced by colonic explants from DSS-
treated P2rx7−/− mice. In agreement with their lowered inflammatory response, we also observed significantly lower activation of STAT3 in P2RX7 deficient mice at day 14 after DSS treatment as compared to WT control mice (Figure 5B).

We next evaluated how the lower P2RX7-dependent IL1B production influenced the composition of the inflammatory infiltrate in the colon of DSS-treated mice. For that, colon tissue sections from acute DSS-treated mice were stained for markers specific for T lymphocytes (CD3), Treg lymphocytes [FOXP3 (Foxp3)], macrophages [EMR1 (F4/80)] and neutrophils (LY6G). Colonic-lamina propria tissue sections from P2rx7−/− mice displayed statistically more CD3+ (9% versus 3%), FOXP3+ (15% versus 3%) and EMR1+ (F4/80) (16% versus 5%) cells as compared to WT animals (Figure 5C). By contrast, no difference between the two groups of mice was observed in regard to LY6G-positive cells. However, we noticed that colon tissue sections from both groups harbored a significantly high number of neutrophils (reaching 8 to 10% of total immune cells). This observation is particularly relevant to the field of IBD pathogenesis. Indeed, acute inflammatory lesions of IBD patients are characterized by a large influx of neutrophils within the intestinal mucosa (23). Keeping in mind the recent concept of the role of neutrophils in tumor biology (24), we wondered whether the P2RX7 activity participated in the polarization of tumor-associated neutrophils (TAN). To assess this question, we analyzed the expression of genes encoding chemokines that have been used to distinguish TAN from other tumor-associated myeloid cells (25). As illustrated in Figure 5D, the P2RX7 deficiency resulted in increased expression of the Ccl17 transcript and decreased expression of Cxcl10, a molecular profile that has been associated with genuine TAN (24,25).
TGFB1 is frequently associated with poor prognosis in patients with cancer (26). This immunoregulatory cytokine can be produced by immune as well as tumor cells and has been implicated in the polarization of various immune cell subsets but also in the establishment of a suppressive microenvironment that favors tumor growth (27). The accumulation of immunosuppressive cells within the inflammatory lesions of mutant mice could possibly be associated with the local production of TGFB1. Indeed, a 3-fold induction of TGFB1 production was observed in acute inflammatory of colon explant culture from P2rx7−/− versus WT mice (Figure 6A). These results were confirmed at later time points, since P2rx7−/− mice subjected to the chronic AOM/DSS treatment displayed an overexpression of Tgfb1 and Tgfb2 transcripts, and their cultured explanted colonic tissue showed increased secretion of the TGFB1 cytokine (Figure 6B).

Since TGFB1 is known to promote the suppressive activity of FOXP3+ Tregs, we further characterized the composition of the cellular infiltrate in colon tissue biopsies from chronic AOM/DSS-treated mice. As described before for the inflammatory colonic mucosa, a significant increase in the content of FOXP3+ cells was observed within tumors derived from P2rx7−/− mice (Figure 6C), whereas there was no difference in the numbers of macrophages (EMR1, F4/80+ cells) nor total T lymphocytes (CD3+ cells). Since TGFB1 has been proposed to control differentiation of TAN in mice (24), we next investigated the presence of neutrophil infiltration in their colonic mucosa. We found an enrichment in the proportion of LY6G+ cells in P2rx7−/− mice (Figure 6C), in agreement with the higher concentration of CXCL1 and CXCL2, two chemokines involved in neutrophil recruitment, in the serum of these animals (Supplementary Figure 7A). Interestingly, neutrophil enrichment appeared to be restricted to colonic tumor tissues as analysis of bone marrow and spleen cells did not show any difference in the composition of immune cells (Supplementary Figure 7B). Moreover, the
cytokine profile observed in tumor lesions of P2rx7−/− mice is compatible with the molecular profile associated with genuine TAN (Supplementary Figure 7C) (25).

In addition to its immunosuppressive function, TGFB1 has been reported to increase proliferation of colonic epithelial cells (28). Therefore, overproduction of TGFB1 in inflammatory and tumor lesions of P2rx7−/− mice may account for the increased proliferation of epithelial cells observed in this strain (Figure 3). To test this hypothesis, neutralizing anti-TGFB antibody was injected in AOM/DSS-treated P2rx7−/− mice. As shown in Figure 7A, body weight and colon length were not significantly different in mice treated with isotype-control or with the anti-TGFB antibodies. In contrast, the number of colonic epithelial cells positive for PCNA staining was reduced by 2-fold in mice that received the neutralizing anti-TGFB antibody (Figure 7B). Indeed, while 35 ± 4% of intestinal epithelial cells were PCNA+ in mice treated with the isotype control antibody, only 15 ± 2% of cells were detected to be positive in mice treated with the neutralizing antibody, a level comparable to what was observed in WT mice (see Figure 3).

Taken together, these results suggest that enhanced secretion of TGFB1 in P2RX7−/− favors the establishment of an immunosuppressive microenvironment and stimulates epithelial cell proliferation, thereby enhancing the development of colon cancer.
Discussion

P2RX7 is an ATP gated ion-channel that can induce multiple cellular responses. In the early 90s, studies demonstrated cell-permeabilizing and cytolytic effects of P2RX7 in response to high doses of eATP (29,30). A few years later, it appeared that stimulation of P2RX7 by low doses of eATP enhanced proliferation of human primary cells (31). This observation was further confirmed in vitro using heterologous expression systems (32) as well as in vivo using xenograft tumor models (16). P2RX7 appeared to be overexpressed in a variety of malignant tumors (reviewed in (33)). However, P2RX7-dependent signaling could also favor an anti-tumor effect in vivo. Indeed, P2RX7 participates in inflammasome assembly and may thereby alert the immune system through processing and liberation of the pro-inflammatory IL1B cytokine (6). It has been demonstrated that P2RX7 plays a crucial role in the immunogenicity of dying tumor cells in response to chemotherapy (a process named immunogenic cell death) through the activation of dendritic cells and the consequent promotion of an adaptive anti-tumor immune response that prevents tumor relapse (18). Of particular interest, in sporadic breast cancer treated with anthracyclines, patients bearing loss-of-function alleles of P2RX7 (rs3751143, NM_002562.4:c.1487A>C) are more prone to develop metastases than patients without mutation. Such results fuel the hypothesis that P2RX7 plays an important role in the anti-tumor response.

In this study, we conducted experiments in the context of acute and chronic colitis with the aim of examining the global in vivo contribution of P2XR7 signaling to inflammation and cancer progression. Using a mouse model of acute AOM/DSS-induced colonic inflammation, we found that P2rx7−/− mice displayed reduced signs of inflammation. This finding is in agreement with recent studies demonstrating that prophylactic systemic blockade of the P2RX7 activity prevented TNBS-induced colitis in rats (12) and that overexpression of P2RX7
in the intestinal mucosa was associated with the pathogenesis of CD (34). In the present study we found that P2RX7 inactivation using selective antagonists as well as genetic deletion of the P2rx7 gene attenuated the weight loss and the overall disease activity score (Figure 1). However, whereas the histological inflammatory score of DSS-treated animals was similar in both WT and P2rx7−/− mice at day 5 (i.e. at the end of DSS treatment), we paradoxically observed a faster re-epithelialization of the digestive mucosa of P2rx7−/− mice during the recovery phase (Figure 2).

Considering that the faster recovery of P2rx7−/− mice correlated with enhanced epithelial cell proliferation, reduced apoptosis and higher expression of BCL2L1 (Bcl-xL) and BCL2 survival factors (Figure 3), our results support the notion that P2RX7 plays a role in the control of epithelial cell proliferation and/or apoptosis in response to injury. We indeed confirmed in vivo in P2rx7−/− mice as well as in WT mice treated with P2RX7 pharmacological inhibitors, that P2RX7 inactivation increases epithelial cells proliferative index (Figure 3 and supplementary Figure 3D). Concordantly, we confirmed in vitro using the human T84 epithelial cell line derived from a colon cancer that pharmacological inhibition of P2RX7 significantly stimulates cell proliferation (Supplementary Figure 3C). This results support the notion that P2RX7 activity directly inhibit epithelial cell proliferation. Whether immune cells, that we found to be recruited at higher level in P2RX7-inactivated mice (i.e, FOXP3+ Tregs and LY6G+ neutrophils) also contribute to the control of epithelial cell proliferation need to be further address in future studies.

Our finding, which demonstrated that a lack of P2RX7 is linked to increased epithelial cell proliferation, may explain, at least partly, the increased tumor burden observed in chronic AOM/DSS treated P2rx7−/− mice (Figure 4). Another nonexclusive mechanistic explanation may reside downstream of IL1B signaling. As expected, little if any IL1B was detected from...
the colonic mucosa of $P2rx7^{-/-}$ mice at early time points following AOM/DSS treatment (Figure 5). This was associated with reduced STAT3 phosphorylation, which may indicate lower activation of immune cells and may favor the establishment of an immunosuppressive microenvironment prone to tumor development. However, down regulation of IL1B and STAT3 phosphorylation could not represent the sole mechanism as it has been reported that genetic inactivation of IL1BR or of STAT3 in the intestinal epithelial cell compartment ($\Delta ICE^{Stat3}$ mice) decreased tumor development (35,36).

Additionally, we found that FOXP3$^+$ Tregs, a cell population that has been associated with colon cancer progression (37), was increased in the colonic mucosa of acute AOM/DSS-treated $P2rx7^{-/-}$ mice (Figure 5C) in association with lower STAT3 phosphorylation (Figure 5B) and higher TGFB1 production (Figure 6). Interestingly, we previously demonstrated that P2RX7 activation at the surface of Tregs inhibited their suppressive function and induced cell death (38,39). Therefore, inactivation of P2RX7 should protect Tregs from the deleterious effect of endogenous P2RX7 ligands (i.e. eATP and NAD) and should promote their early accumulation in the damaged colonic mucosa, as observed in the present study (Figure 5C).

In addition, local production of TGFB may further enhance their suppressive activity and may even participates in the conversion of conventional CD4$^+$ T cells to induced Tregs (iTregs). Of note, several reports have linked TGFB1 to negative regulation of tumor-specific cytotoxic T lymphocyte responses (40-44). Therefore, up-regulation of TGFB production in the colonic mucosa of AOM/DSS-treated $P2rx7^{-/-}$ mice may also inhibit tumor-specific cytotoxic T cells activity and thereby further participates in tumor promotion. In addition to its immunosuppressive role, TGFB has been reported to enhance proliferation of epithelial cells (28). Concordantly, we show here that treatment of DSS-treated $P2rx7^{-/-}$ mice with an anti-TGFB antibody significantly reduced the proliferation of colonic epithelial cells (Figure 7B).
During the course of this study, we also observed the recruitment of LY6G+ neutrophils within tumor lesions of P2rx7−/− mice. These cells shared the characteristics of genuine TAN (Supplementary Figure 7), a population of cell that has been recently associated to tumor progression (24). Therefore we cannot exclude that TAN also participate in the reinforcement of the immunosuppressive environment in the colonic mucosa of AOM/DSS-treated P2rx7−/− mice and in the stimulation of tumor growth.

We demonstrate in our study that P2RX7 inactivation dampens inflammation but paradoxically enhances tumor susceptibility. Interestingly, we reproduced this finding in an independent tumor mouse model consisting of s.c. injection of LLC cells into C57BL/6 mice (Supplementary Figure 6B). Several polymorphisms have been described for the P2RX7 gene and their impact on the P2RX7 function, the mechanisms and relationship with diseases were characterized for a number of mutants (45-47). Surprisingly, none of these variants were found in a meta-analysis of IBD (CD and UC) that included a Genome-Wide Association study (48). However, this analysis highlighted the importance of TGFB signaling in IBD and reinforced the link with colorectal cancer. Further, P2RX7 was reported to be over expressed in chronic B lymphocytic leukemia (19), prostate cancer and adjacent tissue (21), papillary thyroid carcinoma (49) and gastric, colon, kidney and ovarian cancers (16). However, despite the large number of P2RX7 mutants and of P2RX7 variants, no attempts were made to characterize their functionality in tumor lesions, highlighting the need for additional studies to provide a better understanding of the role of P2RX7 in cancer onset.
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References


Figure legends

Figure 1: Genetic and pharmacological inactivation of P2RX7 decreases susceptibility to develop colitis

A. Survival (p≤0.01, Log-rack (Mantel-Cox) test), body weight loss, stool consistency, intestinal bleeding, and colon length from cohorts of 8 to 10 WT and P2rx7/- mice subjected to colitis. Data are presented as means ± SEM; * p≤ 0.05, ** p ≤ 0.01.

B. Effect of selective P2RX7 antagonist A438079 on survival (p≤0.01, Log-rack (Mantel-Cox) test), body weight loss, stool consistency, intestinal bleeding and colon length Data are presented as means ± SEM; * p≤ 0.05.

Figure 2: Mice deficient in the P2RX7 activity recovered more rapidly from inflammatory lesions than WT mice

A. WT and P2rx7/- were submitted to colitis. Colon were removed either at the end of the DSS treatment (day 5), 4 days after (day 9), or 9 days after (day 14) treatment. Tissue sections were stained with hematoxilin and eosin and analyzed microscopically (scale bar = 100μm). Histological scoring of mid colon tissue sections was calculated as indicated in the supplementary Materials and Methods. Data are presented as means ± SEM; * p≤ 0.05.

B. Non-treated and A438079-treated WT mice were submitted to colitis. Colon tissue sections were removed, stained with hematoxilin and eosin either at the end of the DSS treatment or 4 days after (day 9) treatment. Histological scoring of mid colon tissue sections is shown. Data are presented as means ± SEM; ** p≤ 0.01.

Figure 3: P2RX7-dependent signaling prevented early excessive colonic epithelial cell proliferation following AOM/DSS treatment
A. Cohorts of 8 to 10 WT and \( P2rx7^{−/−} \) mice were treated for colitis and epithelial cell proliferation was evaluated by PCNA-staining performed on colon tissue 4 and 9 days after DSS treatment (day 9 and day 14, respectively). Representative results are shown on the panels on the left and the number of proliferating cells (PCNA positive, arrows) per crypt was determined at day 9 and 14 (right panel). Arrows show crypts containing epithelial cells with PCNA nuclear staining. A higher magnification is shown in the inset.

B. Detection of the cleaved form of caspase-3 (see arrows). Representative pictures are shown on the left panel. Quantification of the number of apoptotic cells at day 9 and 14 is shown on the right panel. A higher magnification is shown in the insets.

C. Immunoblot analysis of BCL2, BCL2L1 (Bcl-XL) and BAX expression in the colon tissues of WT and \( P2rx7^{−/−} \) mice, treated for colitis, were performed at day 14. The total actin (ACTB) level was monitored as a control for equal protein loading.

D. Quantification of the number of low dysplasia in animals. Data, shown as arbitrary unit, are representative of two independent experiments (mean ± SEM). * \( p \leq 0.05 \), ** \( p \leq 0.01 \).

**Figure 4**: The P2RX7 activity impeded tumorigenesis in the colitis-associated cancer model

A. Schematic representation of CAC

B. CAC treated WT and \( P2rx7^{−/−} \) mice were euthanized and colons were resected and opened longitudinally to visualize angiogenic (arrows) and non-angiogenic polyps (star). Macroscopic polyps were then counted, sized and colon tissue sections were fixed in formalin and stained with hematoxilin/eosin for histological examination.

C. The CAC protocol was applied to cohorts of 10 none-treated and A438079-treated mice. Colons were resected and macroscopic polyps were counted and sized. Data are presented as means ± SEM; * \( p \leq 0.05 \), ** \( p \leq 0.01 \).
Figure 5: P2RX7 shapes the inflammatory microenvironment

A. Cohorts of 8 to 10 WT and P2rx7−/− mice were treated for colitis. Expression levels of Il1b mRNA and of the corresponding secreted protein (IL1B) were determined by RT-qPCR and ELISA.

B. Proteins were extracted from the mid to distal colon tissue sections of DSS-treated WT and P2rx7−/− mice. The activation of STAT3 was analyzed by immunoblotting using an anti-phospho-STAT3 antibody. The total actin (ACTB) level was monitored as a control for equal protein loading. Data are presented as means ± SEM; * p≤ 0.05.

C. Immunohistochemistry was performed on day 9 after DSS treatment. The quality of infiltrating cells was evaluated using antibodies directed against characteristic cell markers: anti-CD3 (T cells), anti-FOXP3 (Foxp3, Tregs), EMR1 (F4/80, macrophages), and anti-LY6G (Ly6G, neutrophils). Results are presented as means ± SEM; * p≤ 0.05, ** p ≤ 0.01.

D. The expression of Ccl17 and Cxcl10 (IP-10) was examined by RT-qPCR. Data are presented as means ± SEM; * p≤ 0.05.

Figure 6: Increased expression of TGFB1 in colon tumor biopsies from P2rx7−/− mice

A. Cohorts of 8 to 10 WT and P2rx7−/− mice were treated for colitis. Secretion of TGFB1 was assayed by ELISA on supernatants from organo-cultures. Data are expressed as mean ± SEM; * p≤ 0.05.

B. Tgfb1 and Tgfb2 transcripts were analyzed by RT-qPCR and the levels of secreted TGFB1 by ELISA from CAC-treated cohorts of 8 to 10 WT and P2rx7−/− mice. Data are expressed as mean ± SEM; * p≤ 0.05.
B The number of CD3-, FOXP3 (Foxp3)-, EMR1 (F4/80)- and LY6G (Ly6G)-positive cells was determined. Arrows highlight positive cells. Data are presented as means ± SEM; * p≤ 0.05. (Scale bar = 100 μm).

Figure 7: Treatment with anti-TGFB antibody inhibited colon epithelial cell proliferation in AOM/DSS treated $P2rx7^{-/-}$ mice.

Cohorts of 6 $P2rx7^{-/-}$ mice were injected with depleting antibodies and treated for colitis.

A. Body weight loss and colon length were analyzed as described in the legend of Figure 1. Data are presented as means ± SEM; Results are not statistically different.

B. Epithelial cell proliferation was evaluated by PCNA-staining performed on colon tissue sections 9 days after DSS treatment. Representative results are shown on the panels on the left. The number of proliferating cells is shown on the right panel. A higher magnification is shown on the insets. Data are presented as means ± SEM. ** p≤ 0.01.
Figure 1

A

Cumulative Survival

Delay (days)

WT

P2rx7-/-

Body weight scoring

DSS 8%

DSS 5%

Time (days)

WT

P2rx7-/-

Disease activity score

Colon length (% of non treated mice)

DSS 5%

WT

P2rx7-/-

B

Cumulative Survival

Delay (days)

NT

A438079

Body weight scoring

DSS 5%

5% DSS

INH

Time (days)

WT

P2rx7-/-

Disease activity score

Colon length (% of non treated mice)

5% DSS

INH

NT

A438079
Figure 2

A

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**Inflammatory Index**

Day 5: WT vs. P2rx7^−/−

Day 9: WT vs. A438079

Day 14: WT vs. P2rx7^−/−
Figure 4

A. Schematic diagram showing the experimental timeline with AOM (arrows) followed by DSS treatment on days 1, 5, 10, 25, 30, 45, and 50. The timeline also indicates Day 80 for animal harvest.

B. Images showing WT and P2rx7−/− mice with arrows indicating macroscopic polyps.

C. Bar graphs illustrating the average number of macroscopic polyps per mouse and polyp size for NT and A438079 groups. The histograms show the distribution of polyp sizes, with bars differentiated by size category (>/=2 mm and <2 mm).
Genetic and pharmacological inactivation of the purinergic P2RX7 receptor dampens inflammation but increases tumor incidence in a mouse model of colitis-associated cancer

Paul Hofman, Julien Cherfils-Vicini, Marie Bazin, et al.

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