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Amplification loop of the inflammatory process is induced by $P2X_7R$ activation in intestinal epithelial cells in response to neutrophil transepithelial migration

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Cesaro A, Brest P, Hofman V, Hébuterne X, Wildman S, Ferrua B, Marchetti S, Doglio A, Vouret-Craviari V, Galland F, Naquet P, Mograbi B, Unwin R, Hofman P. Amplification loop of the inflammatory process is induced by $P2X_7R$ activation in intestinal epithelial cells in response to neutrophil transepithelial migration. *Am J Physiol Gastrointest Liver Physiol* 299: G32–G42, 2010. First published February 25, 2010; doi:10.1152/ajpgi.00282.2009.—Inflammatory bowel diseases (IBD) are characterized during their active phase by polymorphonuclear leukocyte (PMNL) transepithelial migration. The efflux of PMNL into the mucosa is associated with the production of proinflammatory cytokines and the release of ATP from damaged and necrotic cells. The expression and function of purinergic $P2X_7$ receptor ($P2X_7R$) in intestinal epithelial cells (IEC) and its potential role in the "cross talk" between IEC and PMNL have not been explored. The aims of the present study were *1*) to examine $P2X_7R$ expression in IEC (T84 cells) and in human intestinal biopsies; *2*) to detect any changes in $P2X_7R$ expression in T84 cells during PMNL transepithelial migration, and during the active and quiescent phases of IBD; and *3*) to test whether $P2X_7R$ stimulation in T84 monolayers can induce caspase-1 activation and IL-1β release by IEC. We found that a functional ATP-sensitive $P2X_7R$ is constitutively expressed at the apical surface of IEC T84 cells. PMNL transmigration regulates dynamically $P2X_7R$ expression and alters its distribution from the apical to basolateral surface of IEC during the early phase of PMNL transepithelial migration in vitro. $P2X_7R$ expression was weak in intestinal biopsies obtained during the active phase of IBD. We show that activation of epithelial $P2X_7R$ is mandatory for PMNL-induced caspase-1 activation and IL-1β release by IEC. Overall, these changes in $P2X_7R$ function may serve to tailor the intensity of the inflammatory response and to prevent IL-1β overproduction and inflammatory disease.

innate immunity; Crohn's disease

A LARGE EFFLUX OF POLYMORPHONUCLEAR leukocytes (PMNL) into the intestinal mucosa is a key histological feature of the active phase of inflammatory bowel diseases [IBD: Crohn's disease (CD) and ulcerative colitis (UC)] (11). Studying the mechanisms by which PMNL and intestinal epithelial cell (IEC) interact is a major challenge that should lead to the development of new therapeutic strategies for treatment of inflammation in IBD (28, 43). During acute inflammation, the migration of PMNL into the mucosa is tightly orchestrated by several chemoattractants, such as bacterial products (formyl peptides), host-derived chemokines (TNF-α, IL-1β, and IL-8) and immune activation products (complement fragments) (11, 28, 42).

Using an in vitro approach, we have shown that recruitment of activated PMNL into the epithelial barrier amplifies the local inflammatory response by causing the release of proinflammatory cytokines like TNF-α and IL-1β from IEC (5). However, little is known about the molecular events leading to release during PMNL-IEC "cross talk." Interestingly, among the different cytokines released by IEC during the inflammatory process, IL-1β is a key mediator of the host response to infection (17). After synthesis, the pro-IL-1β 31-kDa precursor is subsequently cleaved by interleukin-converting enzyme, also known as caspase 1, to produce the mature 17-kDa form (59, 62).

Cell death by apoptosis with release of ATP has been reported to stimulate the production and release of mature IL-1β from hematopoietic cells (32). ATP-stimulated K^+ efflux seems to be an important signal for the release of mature IL-1β (50). Ferrari et al. (23, 24) proposed that this effect is mediated by the nucleotide receptor $P2X_7$ ($P2X_7R$). Based on pharmacological profiling, two types of P2 receptors, P2X and P2Y, have been identified (7). Currently seven subtypes of P2XR and eight subtypes of P2YR are recognized (8). Among the P2XR family, $P2X_7R$ has been demonstrated to modulate production of several inflammatory mediators, including IL-1β, IL-18, TNF-α, and IL-6 (36). Activation of $P2X_7R$ by extracellular ATP is a key physiological inducer of rapid IL-1β release from lipopolysaccharide (LPS)-primed macrophages. Of particular importance, this rapid release response is not associated with immediate cell damage or cytolytic effects (49). Information concerning the intracellular signaling pathways activated downstream of $P2X_7R$ activation is currently limiting. Stimulation of $P2X_7R$ by ATP induces an increase in the intracellular Ca^{2+} concentration, cell membrane depolarization, and in most cases permeabilization of the cell membrane to large molecules (8, 10, 24). Pore formation associated with $P2X_7R$ activation requires the action of intracellular second messengers and the mitogen-activated protein kinases (MAPK) (18, 21).

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Other studies showed that activation of P2X$_7$R induced phosphorylation of p38 MAPK in hippocampal cells (48) and of MAPK (Erk1/2) in thymocytes (2). Finally, P2X$_7$R stimulation by ATP can also induce plasma membrane blebbing, which is dependent on Rho-associated kinases, although this action can be dissociated from ATP-induced release of IL-1β (61).

Purinergic signaling plays an important role along the gastrointestinal tract (9). However, most studies have focused on purinergic signaling in neurons in the submucosa and on infiltrating inflammatory cells in IBD (4, 9, 54). Previous in vitro studies have documented that 5′-AMP, which is released and broken down to adenosine during PMNL transepithelial migration, stimulates a P1 receptor (A2b) (38, 39, 56). When stimulated, this receptor induces epithelial cell Cl^- secretion (38, 39, 56). Moreover, extracellular ATP can also stimulate IEC Cl^- secretion through activation of epithelial P2X receptors (58). In this way, extracellular ATP released at sites of IEC damage and/or PMNL activation may activate the P2X$_7$R, which in turn triggers cytokine production. However, a large part of the ATP released by PMNL can also be rapidly metabolized to AMP by expression of CD39 (NTPDase1) on PMNL (12).

The presence of extracellular ATP may act as a "danger signal" for IEC, as in other cell types (33, 53). Previous studies have detected P2X$_7$R expression in the rat gastrointestinal epithelium (26) and on Caco-2, and HCT-8 cell lines (13). However, expression and regulation of P2X$_7$R on IEC, as well as its putative function in the cross talk between PMNL during the active phase of IBD and in the epithelium, have not been characterized.

The present study was designed to investigate the contribution of P2X$_7$R expressed by IEC and PMNL during PMNL recruitment and transepithelial migration. Among the many P2 receptors, we decided to focus on the role of P2X$_7$R for several reasons. Firstly, when examining the expression of the different P2 receptors on T84 cells, we found that P2X$_7$R was the most highly expressed P2 receptors in this cell line. Secondly, stimulation of PMNL can release both ATP and cathelicidin LL-37, two molecules that could activate P2X$_7$R in IEC (12, 23, 24, 45, 55). Thirdly, we showed previously that during PMNL transepithelial migration IEC release IL-1β after caspase 1 activation (5). We looked for a functional P2X$_7$R in the IEC line T84 and in IEC from intestinal human biopsies. Since both PMNL and epithelial cells can release extracellular ATP during inflammation, we tested whether P2X$_7$R activation could induce stimulation of caspase 1 and IL-1β release during PMNL transepithelial migration.

Our results show that the P2X$_7$R protein is only weakly expressed in intestinal biopsies from patients in the active phase of IBD when compared with biopsies of patients in the quiescent phase of IBD. We demonstrate that P2X$_7$R is expressed at the apical surface of T84 cells and, interestingly, that PMNL transepithelial migration was associated with significant downregulation of P2X$_7$R protein expression in the early phase, with P2X$_7$R redistribution from the apical to basolateral membrane of T84 cells. Our results provide evidence that epithelial P2X$_7$R is activated during PMNL transepithelial migration, inducing both caspase 1 activation and IL-1β release by IEC, which could lead to an amplified inflammatory loop. Importantly, we provide novel evidence that activation of epithelial P2X$_7$R is required to induce release of IL-1β by IEC.

MATERIALS AND METHODS

Reagents and antibodies. DMEM/HAM F-12 (Dulbecco-Vogt modified Eagle's medium), HBSS, ATP, 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP), oxidized ATP (oATP), *N*-formyl-L-methionyl-leucyl-L-phenylalanine (f-MLP), and LPS *Escherichia coli* O26:B6 were obtained from Sigma Aldrich (Paris, France). Trizol reagent, deoxyribonuclease I (amplification grade kit), and SuperScript first-strand synthesis system for RT-PCR were purchased from Invitrogen. The following custom Taqman specific primers for IL-1β were used: 5′-GACACATGGGATAACGAGGC and 3′-ACGCAGGACAGGTACAGATT (Applied Biosystems, Foster City, CA). The polyclonal anti-P2X$_7$R antibodies were purchased from Millipore (Cambridge, MA). Immunofluorescence P2X$_7$ antibodies were obtained from Prof. G. Burnstock (Autonomic Neuroscience Institute, UCL, London, UK; for epitope sequences, see Ref. 60). The P2Y$_4$ receptor antibody was purchased from Alomone Laboratories (Jerusalem, Israel). Noncommercially available P2X$_7$ antibodies were demonstrated previously to show subunit specificity (47). Controls for nonspecific binding of primary antibodies were performed by preincubating the immune sera overnight with the respective immunogenic peptide. We used the P2X$_7$R antagonist from Tocris Bioscience (A4308079; Bristol, UK). The antibodies used for immunoblotting against caspase 1 (polyclonal antibody, no. 2225), IL-1β (no. 2022), phospho-ERK (no. 9101), and the pan-ERK (no. 9102) were from Cell Signaling Technology (Danvers, MA). The caspase-1 antibodies used for immunofluorescence and immunohistochemistry were from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon (Hants, UK), respectively. The protease inhibitor cocktail (no. 11836145001) was purchased from Roche (Mannheim, Germany). The antibody to E-cadherin (mouse monoclonal) was purchased from BD Transduction Laboratories (Lexington, KY). The anti-myeloperoxidase monoclonal antibody was from Dako (Dakopatts, Coppenhagen, Denmark). LL-37 was from Peptide Specialty Laboratories (Heidelberg, Germany; purity >95%). In some experiments, cycloheximide (CHX) (Sigma) was used at 10 μg/ml for 1 h for inhibition of protein synthesis in T84 cells. In some experiments, Y-Vad-fmk (YVAD) (Alexis Biochemical, Villeurbanne, France) was used at 100 μM for 30 min for inhibition of caspase 1 in T84 cells and nigericin (Fermentek, Jerusalem, Israel) was used at 10 μM for 30 min for stimulation of caspase 1 in T84 cells. For immunofluorescence we used FITC or Texas red-conjugated secondary antibodies (anti-rabbit IgG) purchased from Dakopatts (Copenhagen, Denmark). Batimastat (BB94), a broad-spectrum metalloprotease inhibitor, was from British Biotech (Oxford, UK) and was used in some experiments at 10 μM (27).

Cell culture. T84 cells (ATCC, CCL-248, passages 54–90), a human colonic carcinoma cell line, were grown and maintained as confluent monolayers on collagen-coated permeable supports as previously reported (30). Monolayers were grown on 0.33-cm^2 or 4.25-cm^2 ring-supported polycarbonate filters (Thincert, Greiner Bio-One, Courtaboeuf, France).

P2X$_7$R knockdown. RNA interference against human P2X$_7$R was performed with 200 pmol of small interfering RNA (siRNA) (Ambion, Austin, TX) (sense strand, 5′-CUACACCACGAGAAACAUCtt-3′; antisense strand, 5′-GAUGUUUCUCGUGGUGUAGtt-3′) to knockdown P2X$_7$ receptors in T84 cells. Control T84 cells were transfected with an irrelevant scrambled siRNA (src hP2X$_7$) (Ambion) (sense strand, 5′-CAACACCACUAGAAGCAUCtt-3′; antisense strand, 5′-GAUGCUUCUAGUGGUGUUGtt-3′) according to the manufacturer's protocol. Briefly, 5×10^6 cells were plated on 10-cm culture dishes. Two hours after plating, cells were transfected by using lipofectamine RNAiMAX (Invitrogen 13778-075; Cergy

Pontoise, France) with siRNA for 48 h at 37°C. $P2X_7R$ knockdown in T84 cells was controlled by RT-PCR.

PMNL transepithelial migration assays. Human neutrophils were isolated from whole blood by a gelatin-sedimentation technique (37). Transepithelial migration of PMNL was performed as previously reported (30). The T84 monolayers were incubated for various times to allow for PMNL transepithelial migration (0 to 4 h). When indicated, T84 monolayers were incubated 30 min with oATP (irreversible $P2X_7R$ inhibitor, 400 μM) or with A4308079 (irreversible $P2X_7R$ inhibitor, 3 μM) before cell washing and the addition of PMNL for 4 h. As controls, cells were incubated with BzATP (chemical $P2X_7R$ agonist, 100 μM), LL-37 (endogenous $P2X_7R$ agonist, 5 μg/ml), and EGF (6 nM) for 30 min or left untreated. At the indicated times, the T84 cells were extensively washed with phosphate-buffered saline (PBS, Cambrex, Emerainville, France) and analyzed by quantitative real-time RT-PCR, immunofluorescence, and Western blotting to detect for the expression of $P2X_7R$, IL-1β, and caspase 1. In some experiments, the number of PMNL that transmigrated into the lower reservoirs was assayed in the presence or in the absence of the $P2X_7R$ antagonist A4308079 or oATP by quantification of the azurophil granule marker myeloperoxidase (30).

Western blot analysis. Western blot analysis of T84 cell was performed as previously described (35). T84 cells were probed overnight at 4°C with either anti-$P2X_7R$ (1:1,000), anti-caspase 1 (1:1,000), anti-IL-1β (1:1,000), or anti-phospho-Thr^{202}/Tyr^{204} ERK (1:2,000) antibodies. The primary polyclonal antibodies were revealed with a horseradish peroxidase conjugated-anti-rabbit antibody (1:10,000, Santa Cruz) and visualized with an Enhanced Chemiluminescence detection system (Perkin Elmer).

Measurements of the expression of mRNA of $P2X_7R$ and of other P2 receptors by real-time quantitative RT-PCR. Total RNA was extracted from T84 monolayers and digestive biopsy specimens with Trizol LS Reagent. RT-PCR amplification was carried out with the Applied Biosystems 7500 Fast RT-PCR kit, by using Taqman PCR Master Mix (No AmpErase UNG) and custom Taqman specific primers for $P2X_7R$ (Hs00175721_m1, Applied Biosystems Foster City, CA). We excluded the presence of residual PMNL by performing RT-PCR analysis of CD11b transcripts on T84 RNA extracts (PMNL marker; Hs00167304_m1, Applied Biosystems). In parallel, expression of other P2 receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) was examined in T84 cells by use of different primers. Primer sequences were taken from Lee et al. (34). The primers were synthesized by MWG-Biotech (Ebersberg, Germany).

IL-1β, TNF-α, and IL-8 production. The concentration of IL-1β, TNF-α, and IL-8 in the lower reservoir of migration chambers was assayed by an in-house ELISA performed in triplicate. The ELISA was carried out with monoclonal antibodies to IL-1β, TNF-α, and IL-8 and phosphatase-conjugated goat anti-IL-1β, TNF-α, and IL-8 polyclonal antibodies (25).

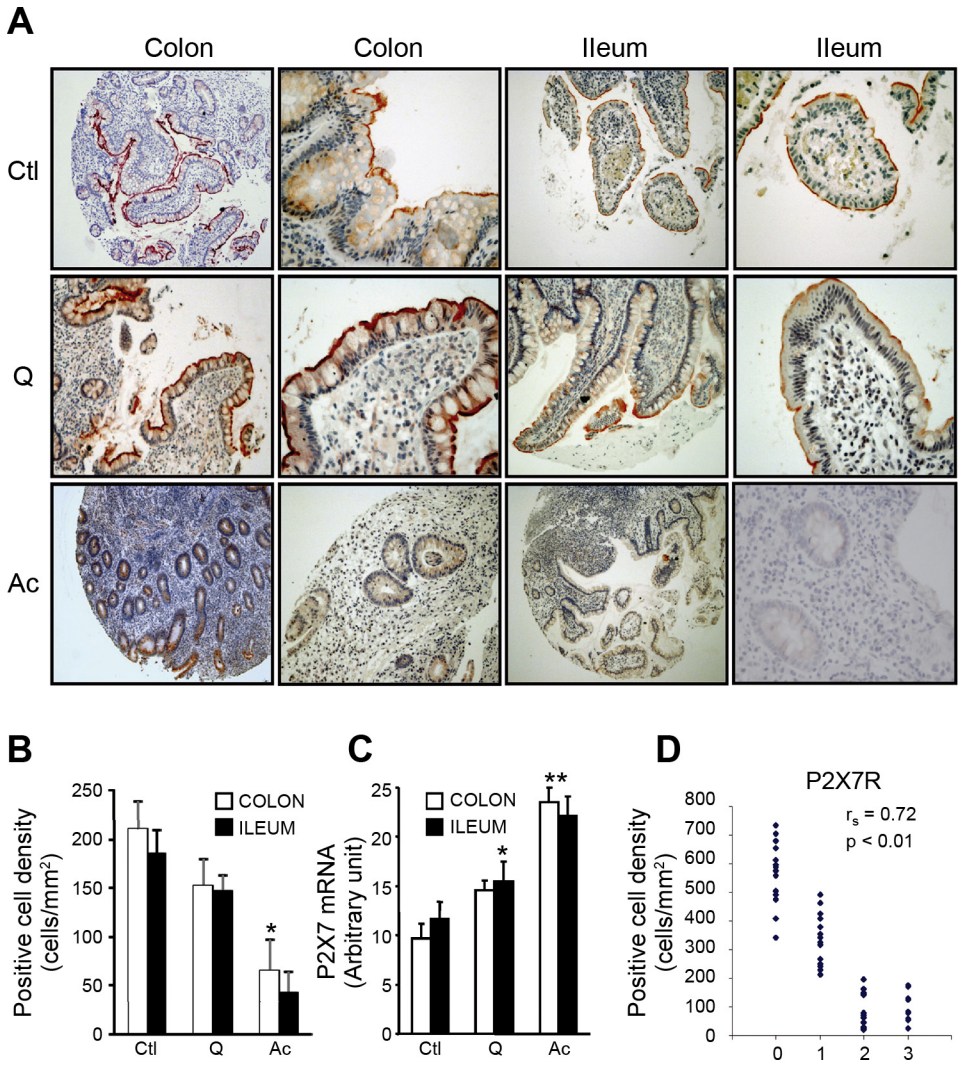

Fig. 1. The purinergic $P2X_7$ receptor ($P2X_7R$) protein level is strongly decreased whereas the RNA level is increased in inflamed intestinal epithelium from inflammatory bowel disease (IBD) patients. Expression correlates with the degree of polymorphonuclear leukocyte (PMNL) infiltration. *A*: immunohistochemical staining of tissue microarrays from colon and ileum biopsies of control subjects (Ctl), patients with quiescent (noninflammatory) IBD (Q), and patients with acute IBD (Ac). Each spot shows a representative tissue immunostained for $P2X_7R$ at a low original magnification (×100; *left*), and at a high original magnification (×400) (*right*). *B*: positive cell density for $P2X_7R$ in colon and ileum biopsies of control subjects, patients presenting with a quiescent noninflamed phase of IBD, and patients presenting with an acute inflamed phase of IBD, $^*P < 0.01$. *C*: level of $P2X_7R$ mRNA in colon and ileum biopsies of control subjects, patients with quiescent (noninflammatory) IBD, and in patients with acute IBD assessed by real-time RT-PCR ($^*P < 0.05$, $^{**}P < 0.01$). *D*: inverse relationship between $P2X_7R$ immunostaining and number of PMNL. Scores of PMNL according to myeloperoxidase staining: 0, none (control subjects); 1, mild (quiescent phase); 2, moderate; and 3, severe (acute phase).

P2X$_7$R, caspase 1, and E-cadherin localization by confocal fluorescence microscopy. T84 cell monolayers grown on permeable filters were fixed with 3.7% paraformaldehyde for 10 min at 4°C and permeabilized for 5 min at room temperature with Triton buffer (0.5% Triton, 2.5% goat serum, 1% bovine serum albumin, and 0.2% gelatine in PBS). Cells were incubated with anti-P2X$_7$R (5 μg/ml), anti-caspase 1 (1:100), or anti-E-cadherin (1:200) antibodies at 4°C for overnight. Cells were incubated with a FITC-conjugated anti-rabbit antibody (Molecular Probes, 1:2,000) for 1 h at room temperature. Pictures were taken with a ×63 magnification lens and a confocal microscope (Zeiss). The cells incubated with a control rabbit immunoglobulin G showed no staining.

Patients, biopsy specimens, and histology assessment. All patients were hospitalized in the Archet II Hospital (Nice, France) and gave signed agreement for this study. The protocol was approved by the local ethics committee of the University of Nice. The diagnosis of CD was made by established criteria of endoscopic analysis and from histology reports. The endoscopic index of severity was evaluated for each patient according to Mary and Modigliani (41).

For diagnosis, tissue microarray construction (TMA), and mRNA extraction, intestinal biopsies were obtained from macroscopically inflamed mucosa of the terminal ileum and of the colon in 65 CD patients (*group 1*) and in 37 UC patients (*group 2*), from macroscopically noninflamed mucosa of the terminal ileum and of the colon in 55 CD patients (*group 3*) and in 45 UC patients (*group 4*). In addition, biopsies were taken from the ileum and rectum of 40 control patients: individuals who had no significant pathological findings following endoscopic examination for changes in bowel habit, abdominal pain, or upper gastrointestinal bleeding or cancer surveillance.

TMA construction and immunohistochemistry. Representative intestinal biopsies performed for each individual for building TMAs were selected from hematoxylin and eosin-stained sections. These sections were examined by two pathologists (V. Hofman, P. Hofman) who were blinded to the experimental results. TMAs were set up as previously described (31).

Immunohistochemical methods were performed on serial 4-μm deparaffinized TMA sections. The sections were incubated with an anti-P2X$_7$R antibody (Chemicon) for 45 min. Sections were then

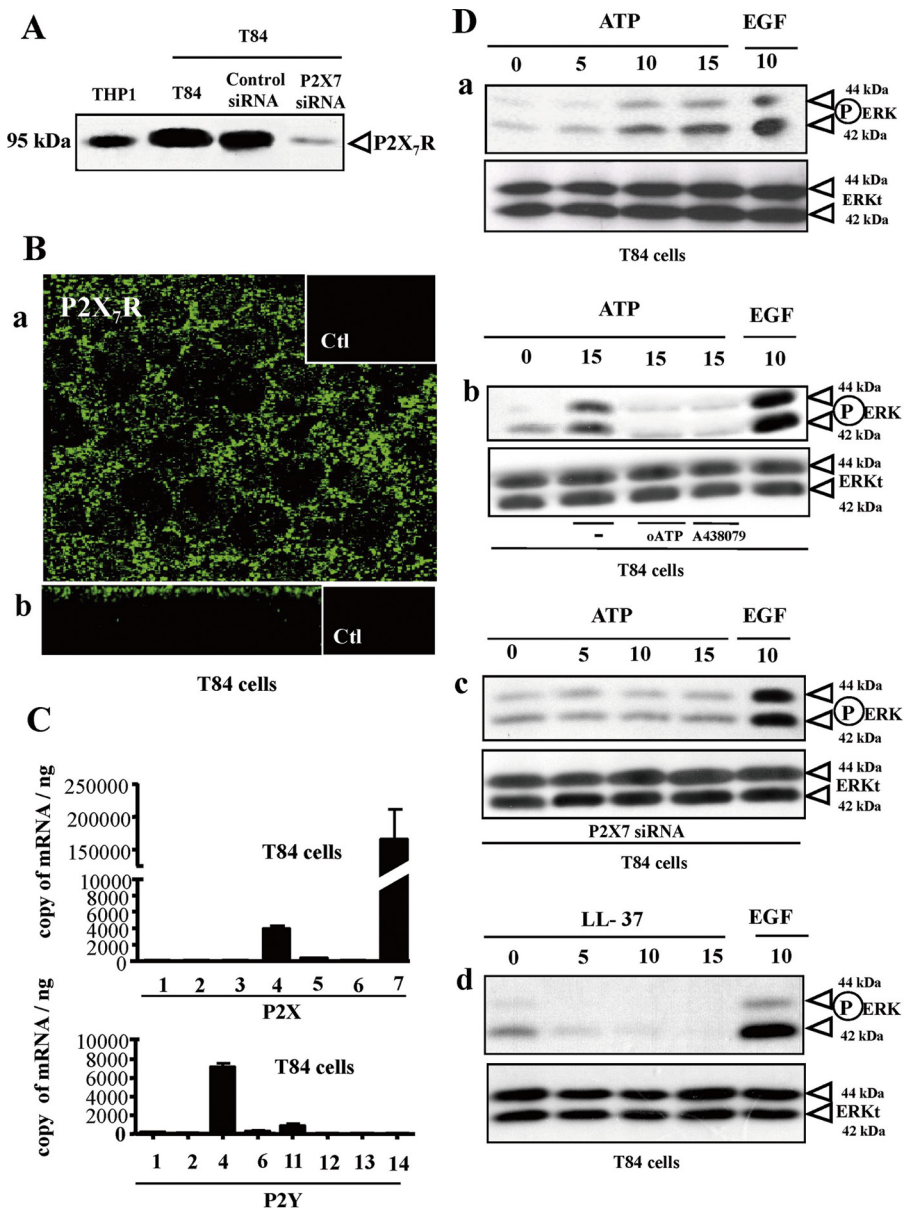

Fig. 2. Functional P2X$_7$R is expressed on T84 monolayers. *A*: P2X$_7$R expression in T84 and THP1 cells was determined by Western blot analysis. These cell lines express a major form of P2X$_7$R of 95 kDa. T84 cells invalidated for P2X$_7$ expression [P2X$_7$ small interfering RNA (siRNA)] showed a high decrease in the level of P2X$_7$R expression compared with T84 cells transfected with a nonspecific siRNA (control siRNA). *B*: P2X$_7$R localization on T84 monolayers was detected by confocal microscopy. P2X$_7$R is present on T84 cells at mainly the apical membrane. *Ba*: *x–z* axis (original magnification, ×400). *Bb*: *x–y* (original magnification, ×400). Ctl: control staining. *C*: levels of different P2XR and P2YR analyzed by quantitative PCR. *Da*: Western blots to phospho- (circled P) and total (suffix t) ERK of T84 stimulated with ATP (P2X$_7$R specific agonist; 100 μM; for 5–15 min). Positive control cells were stimulated with EGF (6 nM; 10 min). *b*: T84 cells preincubated with either oxidized ATP (oATP) or A438079 antibodies stimulated by ATP. Positive control cells were stimulated with EGF. *c*: P2X$_7$R transfected T84 cells stimulated with ATP. Positive controls were stimulated with EGF. *d*: T84 MAP kinases not stimulated by preincubation with LL-37 (50 μM; for 5–15 min).

incubated with peroxidase-labeled anti-mouse Igs (DAKO Envision System, DAKO, Carpinteria, CA) for 45 min. Slides were evaluated by two pathologists (V. Hofman, P. Hofman). For measurement of histological disease activity, a scoring system for histological abnormalities in CD mucosal biopsy specimens was used (13). Immunohistochemical results were scored by the method of quick score (29, 31).

Data analysis. Assays were compared by Student's *t*-test. Values are expressed as means ± SE of *n* number of experiments.

RESULTS

P2X7R expression is decreased in inflamed intestinal epithelium from IBD patients and is inversely correlated with the number of infiltrated PMNL. Immunostaining of P2X7R of the intestinal epithelium was continuous in the ileum and the colon (Fig. 1*A*). We observed weaker staining for P2X7R in intestinal epithelial biopsies with PMNL infiltrates compared with control biopsies (Fig. 1*A*). The faintness of the staining for epithelial P2X7R correlated inversely with the increase in the number of infiltrating PMNL. Although P2X7R expression in noninflamed colonic and ileal biopsies was not significantly different from controls, the trend was toward a slight decrease (Fig. 1*B*).

Figure 1*C* shows that in patients with acute active IBD, the P2X7R mRNA levels in the inflamed colon and ileum were increased compared with controls. In biopsies taken from IBD patients with quiescent disease, P2X7R mRNA levels were only slightly increased compared with controls (Fig. 1*C*). The activity of colitis (PMNL score) was graded from none (0), to mild (1), to moderate (2), and to severe (3), according to the myeloperoxidase staining. P2X7R staining showed an inverse correlation with PMNL infiltration (Fig. 1*D*). P2X7R protein expression was high and similar to controls in biopsies showing a low PMNL score for infiltration and was low in biopsies with a high PMNL score (Fig. 1*D*).

Functional P2X7R is expressed on T84 monolayers. Western blotting showed a 95-kDa band for T84 cells and for THP1 cells (positive control), which corresponds to the expected molecular weight of mature P2X7R (Fig. 2*A*). This was confirmed by confocal immunofluorescence microscopy, which showed strong expression of P2X7R in T84 monolayers, mainly at the apical membrane (Fig. 2*B*). T84 cells with P2X7R knockdown (siRNA-P2X7) showed a significant decrease in P2X7R expression (Fig. 2*A*). RT-PCR performed with primers for different P2X and P2Y receptors showed that P2X7R was the most highly expressed P2 receptor on the T84 cells used for this study (Fig. 2*C*). To verify the functionality of P2X7R expressed on T84 monolayers, we assessed its ability to activate MAPK (Erk1/2) (14). T84 cells were stimulated with one

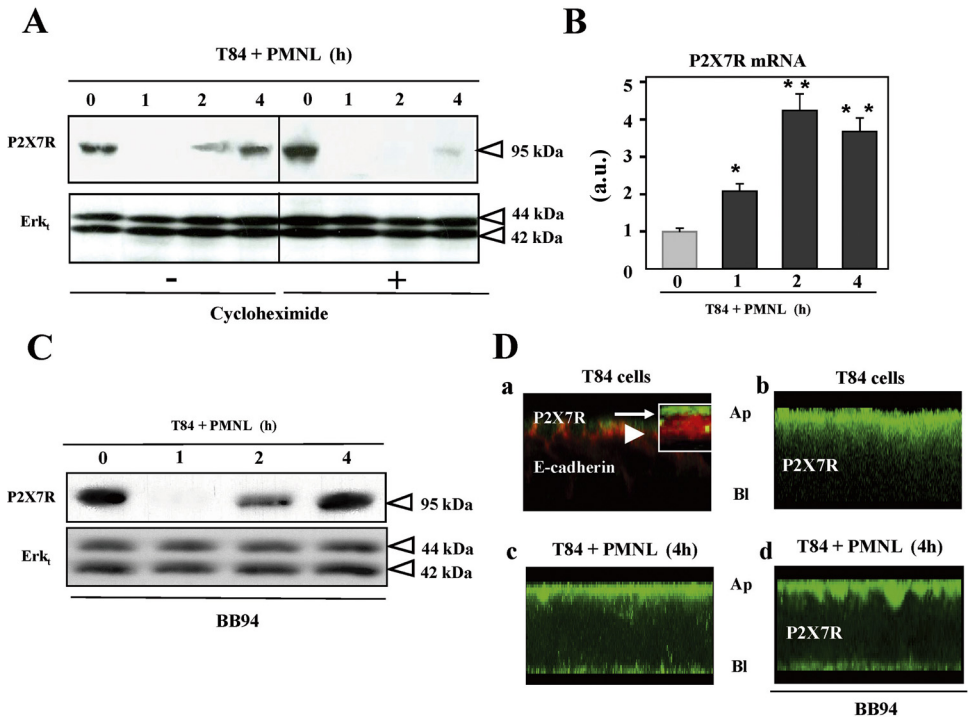

Fig. 3. PMNL transepithelial migration induces modulation of both intestinal epithelial cell (IEC) P2X7R protein and RNA levels. *A*: Western blotting of T84 cells using an anti-P2X7R antibody. During PMNL transepithelial migration, a decrease in P2X7R protein expression was observed at 1 h. After 2 h of PMNL migration the P2X7R protein level increased and was maintained for up to 4 h of PMNL migration (*left*). Preincubation of T84 monolayers with cycloheximide totally inhibited renewed expression of P2X7R at 2 and 4 h of PMNL transepithelial migration (*right*). One of 3 independent experiments is shown. *B*: total RNA was extracted from T84 cells and the mRNA level was measured by RT-PCR. An increase in the IEC P2X7R RNA level was noted after 1–4 h of migration, $*P < 0.05$; $**P < 0.01$. *C*: Western blotting to an anti-P2X7R antibody of T84 cell extracts obtained during PMNL transepithelial migration in the presence of the BB94 protease inhibitor. Similar levels of protein expression at 1, 2, and 4 h compared with the control time course in the absence of BB94 were noted. One of 4 independent experiments is shown. *Da*: detection of P2X7R (FITC) and E-cadherin (Texas red) on T84 monolayers shows apical expression of P2X7R (arrow) above the E-cadherin signal (arrow head). *b–d*: Expression of P2X7R on T84 monolayers before *b* and after *c*. *d*: PMNL transmigration detected by confocal microscopy. The P2X7R protein after 4 h of PMNL migration was distributed at the apical and the basolateral sides of T84 monolayers. P2X7R expression at the apical membrane was less intense after transepithelial migration (*c*) than in control monolayers (*b*). Similar P2X7R expression was observed in the absence (*c*) or in the presence (*d*) of the BB94 protease inhibitor after PMNL transepithelial migration. One of 3 independent experiments is shown.

of the three P2X$_7$R agonists: ATP (100 μm), BzATP (a nonhydrolyzable analog of ATP; 100 μM), or LL-37 (50 μM), and the activation of MAPK was analyzed by Western blotting with antibodies directed against the phosphorylated and active forms of ERK. As shown in Fig. 2*Da*, ATP induced a rapid and strong activation of both p42^{ERK2} and p44^{ERK1} in T84 cells at 10 and 15 min. MAPK activation was not observed in T84 preincubated with the oATP or A438079 antagonists (Fig. 2*Db*) and in P2X$_7$R siRNA-transfected T84 cells (Fig. 2*Dc*), incubated with ATP. Conversely, T84 MAP kinases were not stimulated in T84 cells when incubated with LL-37 for 5, 10 and 15 min (Fig. 2*Dd*).

PMNL transepithelial migration induces de novo P2X$_7$R mRNA expression and the P2X$_7$R protein is partially redistributed from the apical to basolateral surface in IEC. During PMNL transepithelial migration, P2X$_7$R protein expression significantly decreased at 1 h, before slowly recovering at 2 and 4 h of PMNL transmigration (Fig. 3*A*). However, the levels remained lower at 2 h compared with control monolayers. As shown in Fig. 3*B*, P2X$_7$R mRNA levels were significantly increased in IEC after 1–4 h of PMNL migration. As shown in Fig. 3*A*, preincubation of T84 cells with CHX inhibited induced expression of P2X$_7$R at 2 and 4 h of PMNL transepithelial migration, which allowed us to conclude that the increase in the P2X$_7$R protein observed in the absence of CHX during PMNL transmigration is preceded by renewed P2X$_7$R mRNA translation at 1 h. Interestingly, under the same conditions we were unable to detect any increase in the CD11b mRNA level, indicating little, if any, contamination of the T84 monolayers by PMNL (data not shown). Western blotting was performed to detect P2X$_7$R in T84 monolayers during PMNL transepithelial migration incubated in the presence of the BB94

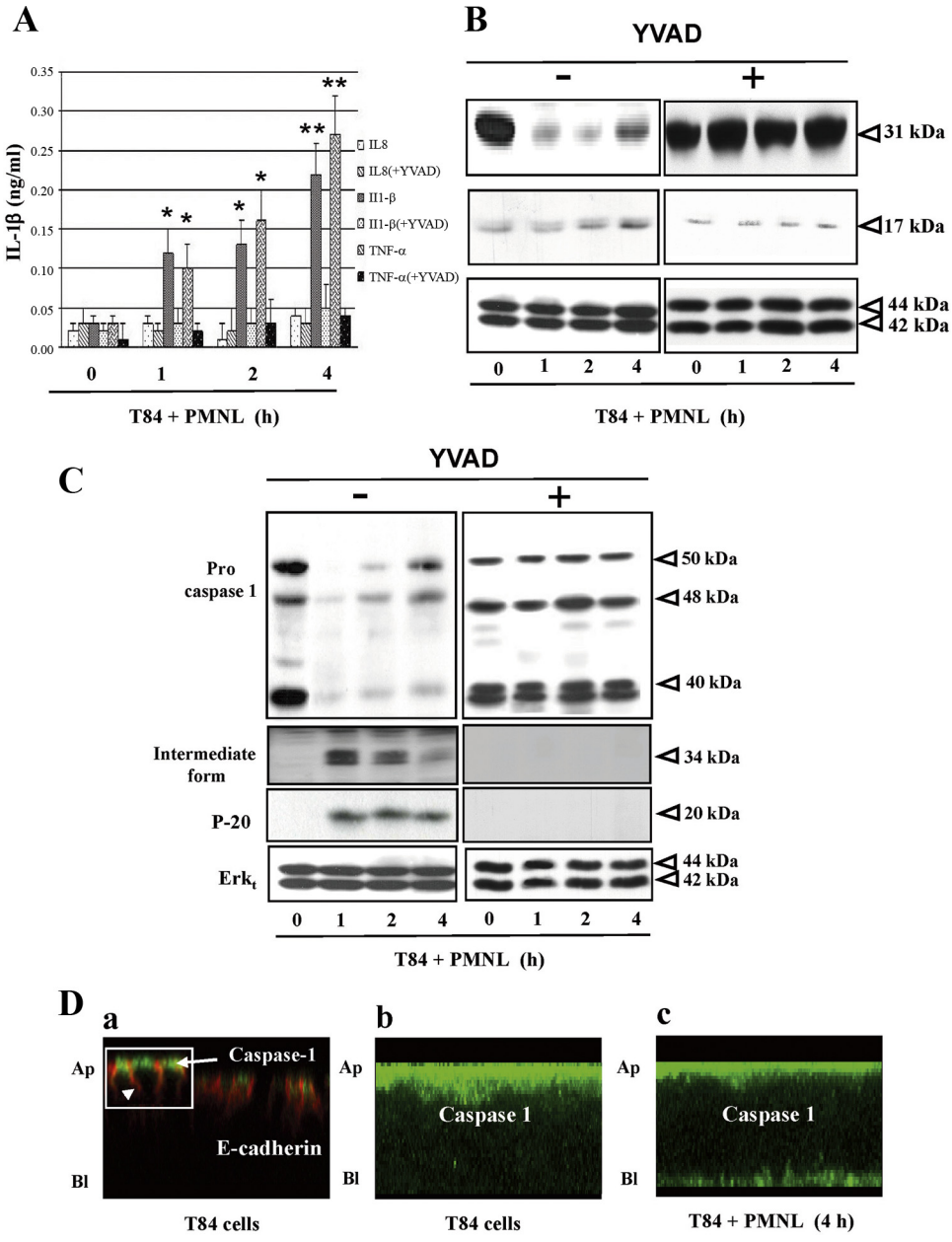

Fig. 4. PMNL transepithelial migration induced IL-1β release, pro-IL-1β cleavage, and caspase 1 activation in IEC. *A*: an increase in the secretion of IL-1β was seen during PMNL transepithelial migration. Secretion of TNF-α but not of IL-8 was increased during PMNL transepithelial migration. *$P < 0.05$; **$P < 0.01$. No effect on IL-β and TNF-α secretion was noted during PMNL transepithelial migration after preincubation of T84 cells with Y-Vad-fmk (YVAD; 100 μm, 30 min). *B* and *C*: a corresponding decrease in pro-IL-1β (*B*) and procaspase 1 (*C*) expression, indicating the presence of caspase 1 activation during PMNL transepithelial migration, was noted. No decrease in pro-IL-1β (*B*) and procaspase 1 (*C*) expression was noted after preincubation of T84 cells with YVAD. This was associated with membrane redistribution of P2X$_7$R in T84 cells, after 4 h of PMNL transepithelial migration, and a partial relocation of caspase 1 at the basolateral side, as observed by confocal microscopy (*D*). *Da*: caspase 1 was mainly located at the apical side (arrow) above the signal for E-cadherin (arrowhead). Distribution of caspase 1 in T84 cells before (*b*) and after (*c*) 4 h of PMNL transepithelial migration. One of 3 independent experiments is shown.

protease inhibitor. This inhibitor was added to the upper and lower reservoirs 30 min before the onset of migration and was maintained during the course of transepithelial migration. As shown in Fig. 3*C*, the signal obtained for $P2X_7R$ on T84 cells at 1, 2, and 4 h of PMNL transepithelial migration was not modified in the presence of BB94.

Analysis by confocal microscopy confirmed that $P2X_7R$ persists at the apical cell surface of T84 monolayers after 4 h of PMNL transmigration and was visible above the signal for E-cadherin (Fig. 3*D*). Partial redistribution of the $P2X_7R$ protein from the apical to basolateral membrane was also noted (Fig. 3*D*). The $P2X_7$ immunostaining observed on T84 cells after PMNL migration in the presence of BB94, added to the upper and lower migration chambers 30 min before the onset of migration, was similar to that in the absence of protease inhibitors (Fig. 3*D*).

PMNL transepithelial migration induced IL-1β release via caspase 1 activation and cleavage of pro IL-1β in IEC. As shown in Fig. 4*A*, an increase in the secretion of IL-1β was noted during PMNL transepithelial migration. In addition, we observed an increase in the secretion of TNF-α during PMNL transepithelial migration whereas the level of IL-8 was not modified during PMNL transepithelial migration (Fig. 4*A*). No increase in the secretion of IL-1β and TNF-α was noted during PMNL transepithelial migration performed after preincubation of T84 cells with YVAD (Fig. 4*A*). In parallel, a strong decrease in the pro-IL-1β level was observed by Western blotting for T84 cells at 1 and 2 h of PMNL transepithelial migration (Fig. 4*B*). As shown in Fig. 4*C*, there was a decrease in the level of the proform of caspase 1 (~40–50 kDa) at 1 and 2 h of PMNL transepithelial migration, indicating cleavage and activation of caspase 1. Using an antibody that recognizes both

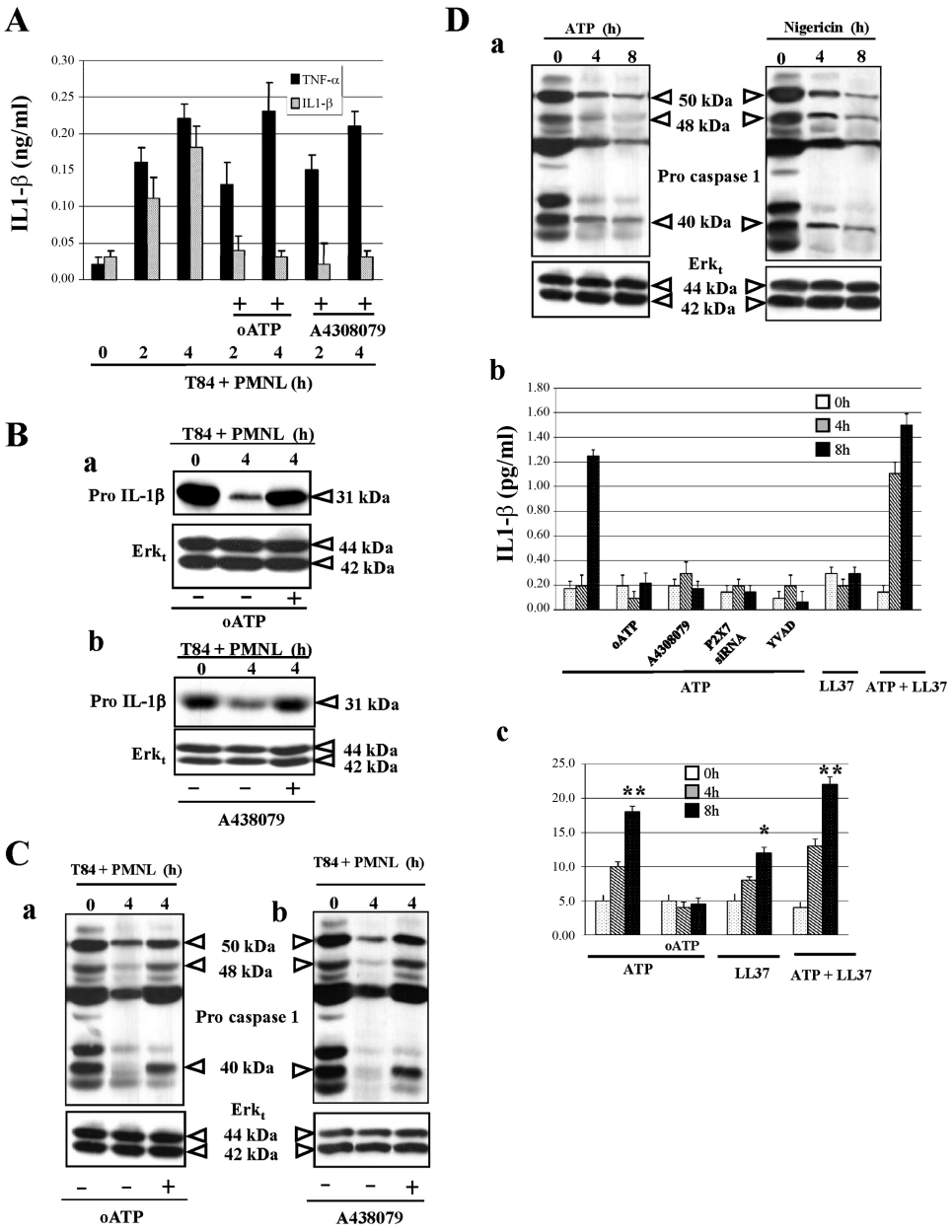

Fig. 5. PMNL transepithelial migration induced IL-1β release, pro-IL-1β cleavage, and caspase 1 activation in IEC through ATP-induced $P2X_7R$ stimulation. *A*: IL-1β secretion aftertk;1 pretreatment of T84 monolayers with oATP or A4308079 and subsequent PMNL transepithelial migration. Conversely, secretion of TNF-α still increased during PMNL transepithelial migration in T84 monolayers pretreated with oATP or A4308079. *B*: after oATP (*a*) or A430879 (*b*) pretreatment, followed by PMNL transepithelial migration, the level of pro-IL-1β in T84 monolayers was similar to that in control T84 monolayers. *C*: the level of the proform of caspase 1 in T84 monolayers was similar to control T84 monolayers. *Da*: the level of the proform of caspase 1 in T84 monolayers strongly decreased at 8 h of incubation with ATP and at 8 h after stimulation with nigericin (10 μM). *b*: T84 IL-1β secretion was noted when T84 monolayers were treated with ATP (150 μM) for 8 h. This latter secretion was not observed when T84 cells were preincubated with YVAD. Increased T84 IL-1β secretion was noted at 4 and 8 h when cells were treated with both ATP and LL-37. Secretion was not observed after preincubation of T84 monolayers with oATP or A43079 antibodies in T84 cells invalidated for $P2X_7R$ expression (siRNA-$P2X_7$). One of 3 independent experiments is shown. *c*: The IL-1β mRNA level increased at 4 and 8 h in T84 cells treated with ATP compared with controls and to T84 cells preincubated with oATP. An increase of IL-1β mRNA was observed at 4 and 8 h of incubation with LL-37, whereas a strong increase in the IL-1β mRNA was noted after 4 and 8 h of incubation with both LL-37 and ATP (*$P < 0.05$; **$P < 0.01$).

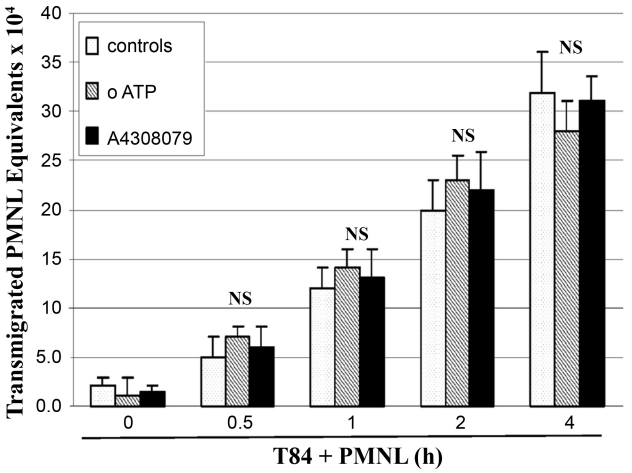

Fig. 6. P2X7R expression on PMNL and/or on T84 cells does not regulate the PMNL transepithelial rate of migration of PMNL when induced by *N*-formyl-L-methionyl-leucyl-L-phenylalanine (f-MLP). The rate of PMNL transmigration across T84 cell monolayers at different times in the presence (oATP and A4308079) or in the absence (controls) of P2X7R antagonists. An MPO assay indicates the total number of PMNL after migration when induced with f-MLP (10^{-7}). NS, nonsignificant. Results are means ± SE for 6 to 12 monolayers.

the pro- and mature forms of caspase 1 (Fig. 4*D*), we observed by confocal microscopy a basolateral localization of caspase 1 after 4 h of PMNL transepithelial migration, which was similar to the redistribution seen for $P2X_7R$. No decrease in pro-IL-1β (Fig. 4*B*) and procaspase 1 (Fig. 4*C*) expression was noted during the time course of PMNL transmigration performed after preincubation of T84 cells with YVAD.

Activation of intestinal epithelial $P2X_7R$ provides a costimulatory signal required, though not sufficient, for the production of IL-1β in response to PMNL transepithelial migration. Inhibition of the epithelial $P2X_7R$ activity by oATP pretreatment abrogated PMNL transmigration-induced effects on IL-1β secretion (Fig. 5*A*) and decreased pro-IL-1β levels (Fig. 5*B*), and procaspase 1 cleavage (Fig. 5*C*). Similar results were obtained with use of the A4308079 antagonist (Fig. 5, *A*-*C*). Inhibition of epithelial $P2X_7R$ activity by oATP or A4308079 antagonist pretreatment of T84 cells did not abrogate PMNL transmigration-induced effects on TNF-α secretion (Fig. 5*A*). Incubation of T84 cells with ATP (1 mM) for 4 and 8 h increased procaspase 1 cleavage (Fig. 5*Da*, *left*), whereas similar results were obtained after stimulation of T84 cells with nigericin (10 μM) (Fig. 5*Da*, *right*) Activation of the $P2X_7R$ by treatment of T84 cells with ATP (1 mM) induced IL-1β secretion by IEC only after incubation for a long-period time (8 h) (Fig. 5*Db)* whereas LL-37 (5 μg/ml) did not induce secretion at this time (Fig. 5*Db)*. Moreover, even after 8 h of incubation with ATP, T84 cells invalidated for $P2X_7R$ expression ($P2X_7$-siRNA) did not produce IL-1β secretion (Fig. 5*Db)*. Interestingly, in T84 cells treated with ATP after preincubation with YVAD, a strong decrease in IL-1β secretion was noted at 8 h (Fig. 5*Db)*. Moreover, IEC incubated with both LL-37 and ATP showed an increased in IL-1β secretion at 4 and 8 h of incubation (Fig. 5*Db)*. Additionally, Fig. 5*Dc* shows that the IL-1β mRNA level increased at 4 and 8 h in T84 cells treated with ATP compared with controls, and with T84 cells preincubated with oATP. An increase in IL-1β mRNA was observed at 4 and 8 h of incubation with LL-37, whereas a strong increase of IL-1β mRNA was noted after 4 and 8 h of incubation with both LL-37 and ATP (Fig. 5*Dc*).

$P2X_7R$ expressed either on PMNL and/or on T84 cells did not regulate the rate of PMNL transepithelial migration when induced by f-MLP. In response to f-MLP (10^{-7} M), the number of PMNL that transmigrated to the lower reservoirs increased from 0 to 4 h (2.0 ± 1.0, 5.1 ± 2.1, 12.2 ± 2.1, 20.5 ± 3.1, and 32.9 ± 4.0 × 10^4 PMNL cell equivalents per monolayer at 0, 0.5, 1, 2, and 4 h of transmigration, respectively) (Fig. 6). These numbers were similar in the absence and in the presence of P2X7R antagonists (oATP or A4308079), showing that $P2X_7R$ expression on PMNL and/or IEC alone does not regulate PMNL transepithelial migration (Fig. 6).

DISCUSSION

Over the last decade interest in the role of purinergic receptors in physiology and pathophysiology has experienced a revival (8–10, 36). Purinergic receptors are comprised of two groups, the P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) and P2 receptors, which are divided into two subcategories of P2X receptors ($P2X_{1-7}$) and P2Y receptors ($P2Y_{1-12}$) (10, 46, 47). In hematopoietic cells, activation of the caspase 1/IL-1β pathway is triggered by a member of the purinergic receptor family, $P2X_7R$ (57). This unique member of the P2X receptor group is a nucleotide-gated ion channel activated by extracellular ATP and is highly expressed on monocytes, macrophages, and lymphocytes (57). In these cell types, exposure to extracellular ATP leads to rapid release of IL-1β via activation of $P2X_7R$ (16, 20, 23, 36, 40). However, another endogenous ligand, the antimicrobial peptide LL-37, can also activate $P2X_7R$. LL-37 can be produced by several cell types, particularly PMNL and colonic epithelial cells (19). LL-37 is the major active cleavage product of the human cathelicidin hCAP18, which is upregulated under conditions of infection and inflammation (52, 63). In addition to its broad-spectrum antimicrobial activity and direct antiendotoxic effects, LL-37 possesses immunomodulatory functions (6). Moreover, LL-37 has been shown to induce

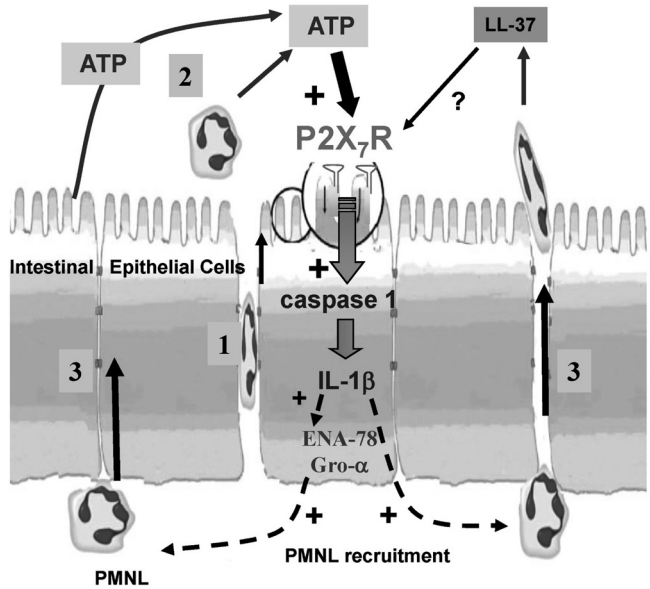

Fig. 7. Speculative mechanism by which PMNL interact with IEC in activating P2X7R and the effect on downstream molecules.

caspase 1 activation and secretion of mature IL-1β in LPS-primed monocytes through $P2X_7R$ activation (20). These studies indicate that, in addition to extracellular ATP, endogenous LL-37 may activate $P2X_7R$ on innate immune effector cells to modulate inflammatory cytokine release.

We show in the present study that T84 cells express functional $P2X_7R$, which following stimulation with extracellular ATP induce rapid and transient phosphorylation of the MAP kinase ERK1/2. $P2X_7R$ in these cells is expressed mainly at the apical cell membrane. We found that the $P2X_7R$ protein and mRNA levels are modified during PMNL transepithelial migration. The $P2X_7R$ protein level was initially decreased after 1 h of PMNL transmigration, an event that may reflect its internalization and degradation. After 2 and 4 h of PMNL transmigration, we saw an increase in the $P2X_7R$ mRNA level, which was associated with recovery of $P2X_7R$ protein expression. Correspondingly, in intestinal biopsies taken from patients with IBD, low expression of the $P2X_7R$ protein was observed in association with high mucosal infiltration of PMNL, whereas $P2X_7R$ expression in biopsy tissues from patients with quiescent disease had a low PMNL count that was similar to that of control tissues. In contrast, $P2X_7R$ mRNA levels were higher in biopsies from IBD patients with active disease compared with levels in samples from controls and patients with quiescent IBD. Thus these ex vivo findings are entirely consistent with findings in our T84/PMNL in vitro model.

The mechanisms by which PMNL transmigration down-regulate $P2X_7R$ protein expression but increase its mRNA levels remain unclear. One possibility is that internalization, degradation, recycling, and synthesis of activated $P2X_7R$ are modified. Indeed, it has already been reported that ATP stimulates the internalization and degradation of $P2X_7R$ in epithelial cells (22). Of particular interest, transient activation of $P2X_7R$ leads to a loss of mitochondrial membrane potential that does not lead to cell death unless receptor activation is prolonged (13). Therefore, we can speculate that the dynamic turnover of intestinal epithelial $P2X_7R$ induced by PMNL transmigration may help to protect IEC from excessive activation of $P2X_7R$ and thus from immediate cell death.

Even at a low level of expression, $P2X_7R$ may be sufficient to regulate the inflammatory response. We have provided evidence that IL-1β production by IEC during PMNL transepithelial migration is dependent on IEC $P2X_7R$ activation, since it was abolished when T84 monolayers were pretreated with the $P2X_7R$ antagonist oATP. We propose that release of extracellular ATP during PMNL transmigration may stimulate IEC $P2X_7R$ and activate pro-IL-1β. Moreover, the activation of IEC procaspase 1 during PMNL transepithelial migration leads to relocation of $P2X_7R$ to the basolateral side of the cell from which IL-1β is secreted.

We showed that activation of epithelial $P2X_7R$ by extracellular ATP or LL-37 alone may not, on its own, lead to IL-1β secretion. Epithelial $P2X_7R$ seems to be necessary, but not sufficient, to mediate the PMNL-induced IEC inflammatory response, as previously reported for LPS and ATP (20). Such sophisticated dual control may tailor the quality and direction of the inflammatory response and limit IL-1β overproduction during inflammation.

In summary, our results fit the following proposed model (Fig. 7): *1*) PMNL are initially attracted by various chemotactic stimuli in the intestinal mucosa, *2*) release of ATP in the extracellular space by activated PMNL and/or necrotic IEC activates the IEC $P2X_7R$; and *3*) stimulation of IEC $P2X_7R$ leads to activation of caspase 1 and secretion by the IEC of proinflammatory cytokines such as IL-1β. Even though IL-1β does not act directly as a powerful chemoattractant for PMNL, this molecule can induce production of other cytokines such as epithelial neutrophil activating peptide (ENA-78) and Gro-α, which by themselves can attract PMNL through the epithelial barrier (1). Moreover, secreted IL-1β can activate different matrix metalloproteinases that can degrade the lamina propria, thereby allowing efflux of PMNL from blood vessels (3). Our study highlights the critical role of epithelial $P2X_7R$ in initiating a positive amplification loop of PMNL recruitment into the intestinal mucosa during acute inflammation. Given this key function, we would expect that the expression and activity of the epithelial $P2X_7R$ is tightly regulated and that its dysregulation may contribute to the development of chronic IBD.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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