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Starch nanoparticles improve curcumin-induced production of anti-inflammatory cytokines in intestinal epithelial cells

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ABSTRACT

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, is a long-term condition resulting from self-sustained intestinal inflammation. Curcumin (Cur), a powerful, naturally occurring antioxidant and anti-inflammatory polyphenol, has been investigated as a therapeutic for IBD, but its poor stability and low bioavailability limits its efficacy. We investigated the use of crosslinked starch nanocarrier (NPL) on the intracellular delivery and the anti-inflammatory efficiency of curcumin. Caco-2 epithelial cells were stimulated with TNFα for 24 h and the anti-inflammatory effects of NPL/Cur formulations were evaluated at the early stages of inflammation (4 h) or later, when fully established (24 h). NPL allowed the intracellular delivery of curcumin, which was enhanced in inflammatory cells, due to a modification of the endocytosis pathways. NPL/Cur decreased the secretion of pro-inflammatory cytokines IL-1β, IL-6 and IL-8 while increasing the anti-inflammatory cytokine IL-10. Finally, the inflammation-related opening of the tight junctions better allowed NPL/Cur to cross the epithelium by paracellular transport. This was confirmed by *ex vivo* analysis where NPL/Cur, administered to colonic explants from chemically-induced acute colitis mouse model, delivered curcumin deeper in the epithelium. To conclude, NPL/Cur formulation emphasizes the anti-inflammatory effects of curcumin and could constitute a therapeutic alternative in the management of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a term commonly used to refer to Crohn's disease and ulcerative colitis, both characterized by a self-perpetuating inflammation of the intestinal epithelium. The incidence and prevalence of IBD are increasing (Hou et al., 2013), and the disease usually develops in patients between 25 and 30 years old (Siegel et al., 2017), with diagnosis according to precise clinical, endoscopic, radiologic and histological criteria.

Since the etiology of IBD remains unknown, these pathologies are generally experienced as chronic conditions, and medication has focused on resolving the inflammation state. During flare-ups, the first-line medical treatments target inflammation using non-steroidal anti-inflammatory drugs such as mesalazine (5-aminosalicylated acid – 5-ASA). Specifically, 5-ASA decreases inflammation by blocking cyclo-oxygenase (COX) activity and by inhibiting the production of the major pro-inflammatory mediators (IL-1β, IL-6, IL-8 and TNFα) (Na and Moon, 2019). Other conventional treatments involve the use of immunosuppressant and biological agents (*e.g.* anti-TNF-monoclonal antibodies) such as infliximab or adalimumab, which block inflammation mediators/promotors involved in the disease (Na and Moon, 2019).

However, despite a large therapeutic library, medication generally remains insufficient to completely cure the inflammation. Moreover, these drugs are expensive and exhibit severe side effects such as anemia, depression and insomnia, but also opportunistic bacterial infections, solid tumors and lymphomas (Shu et al., 2019).

To limit the side effects of current therapeutics and improve the compliance of patients, new therapeutic strategies need to be developed, including the use of natural products with anti-inflammatory properties (Bribi et al., 2016; Davatgaran-Taghipour et al., 2017; Mozaffari et al., 2014). Numerous studies have highlighted the natural anti-inflammatory and antioxidant activities of phytochemicals such as phenolic compounds and flavonoids. They have been shown *in vitro* to modulate the production of inflammatory mediators such as TNFα, IL-1β, IL-10, IL-6, inducible nitric oxide (NO) synthase (iNOS), prostaglandinE2 (PGE- 2) and cyclooxygenase 2 (COX2) (Aggarwal et al., 2013).

Curcumin (Cur; 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-

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heptadiene-3, 5-Dione) is a hydrophobic polyphenol, the principal curcuminoid of turmeric, traditionally used as a Indian spice in south Asian cooking, and is known to have numerous biological functions (antioxidant, anti-microbial, anti-parasitic, anti-cancer and anti-inflammatory effects) (Kunnumakkara et al., 2017). These are mainly explained by the ability of curcumin to modulate NF-kB, AP-1, c-Jun, Jak-STAT pathways (Aggarwal et al., 2003; Anand et al., 2008). Curcumin has been widely studied as a treatment for IBD and other diseases: clinical trials in humans demonstrated the safety and tolerability of curcumin (Epstein et al., 2010) and its efficacy as a chemo-protective agent (Shehzad et al., 2010) and an effective anti-inflammatory drug against ulcerative colitis (Hanai et al., 2006). However, several limitations were also observed in its clinical use after oral administration, mainly a low solubility in biological fluids resulting in a poor bioavailability, and a rapid metabolization resulting in limited plasma levels, much below its required therapeutic concentration (Pan et al., 1999; Sasaki et al., 2011).

An effective approach to treat IBD is the specific targeting of inflamed areas, in order to reduce systemic side effects and improve therapeutic efficacy, as local drug delivery results in higher drug concentrations in affected tissues, reduces histological degrees of inflammation, and enables better control of the disease (Grimpen and Pavli, 2010; Strojny et al., 2016).

In this regard, significant research has been conducted on new delivery systems that better target the gut and improve the protection of anti-inflammatory drugs against the digestive environment. The use of carriers provides many advantages, in particular to overcome the various biological barriers that limit access of the drug to the targets, and to improve the oral bioavailability of hydrophobic molecules such as curcumin (Grimpen and Pavli, 2010; Strojny et al., 2016). Moreover, the usefulness of curcumin associated with nanoparticles (NPs) has been reported in some pathologies such as skin disorders, cancer or wound healing (Kane et al., 2021; Vollono et al., 2019; Zhou et al., 2021).

Finally, NPs, depending on their composition, surface charge and physico-chemical properties, can passively accumulate at the sites of inflammation, taking advantage of the increased intestinal permeability (Tolstanova et al., 2012), which could enhance NPs uptake by infiltrating cells, and minimize systemic side effects (Lamprecht, 2010; Torchilin, 2014). In addition, due to their size, NPs can also penetrate deeply into the target tissue, which could be beneficial in particular for treatment of Crohn's disease.

In this sense, liposomal formulations, polymeric micelles or polymeric NPs are the most used vectors (Karthikeyan et al., 2021). Recently, liposomal formulations composed of lecithin and cholesterol were associated to Curcumin (5% drug loading and 90% encapsulation efficiency) to treat lung carcinoma. The formulations inhibited nuclear factor-kappaB (NF-κB) pathway and downregulated relevant inflammatory factors as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Shi et al., 2012). In another study, Xie X et al. produced biodegradable Curcumin-loaded PLGA (poly[lactic-*co*-glycolic acid]) NPs (5.75% drug loading and 91.96% encapsulation efficiency). The results indicated that PLGA NPs improved the oral bioavailability of Curcumin at 5.6-fold and had a longer half-life compared to free Curcumin. The authors thought that the improved oral bioavailability was associated with increased water solubility, higher release rate, inhibition of P-glycoprotein-mediated efflux (Betbeder et al., 2015) and increased residence time in the intestinal cavity (Xie et al., 2011). Noteworthy, these examples highlighted moderate drug loading which did not exceed 20% (Chen et al., 2020).

In this study, we attempted to develop a new nanoparticulated formulation that could efficiently help curcumin increasing its solubility and anti-inflammatory properties, as an alternative to current symptomatic treatments in IBD. We previously developed starch nanoparticles (hereafter called NPL) able to deliver proteins and biomolecules within airway epithelial cells (Dombu and Betbeder, 2013). They are composed of crosslinked and positively-charged maltodextrin (α1–4 D-Glucose polymer) with an inner core of anionic phospholipid (dipalmitoyl phosphatidylglycerol). NPL actively enter cells by endocytosis (Dombu et al., 2012), and due to their porous structure and lipophilic core, they are capable to be loaded with and to deliver hydrophobic molecules (Kroubi et al., 2010). Based on these findings, we hypothesized that NPL could deliver curcumin inside the epithelial cells in order to limit intestinal inflammation.

Here, in the context of IBD, we developed a formulation composed of NPL loaded with curcumin (NPL/Cur) to evaluate whether the anti-inflammatory activity of the curcumin is improved thanks to a better cellular delivery by NPL. We set up complementary models of "early" (4 h) and "late" (24 h) treatments of intestinal inflammation, to evaluate NPL/Cur anti-inflammatory properties against both a starting and an established inflammation. We first examined the ability of the NPL/Cur to deliver curcumin in the cells, for both models. The production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and the anti-inflammatory cytokine IL-10 were analyzed in cells treated by NPL/Cur by enzyme linked immune sorbent assay (ELISA) tests. The transepithelial passage of NPL and curcumin were determined using transepithelial electrical resistance (TEER) and Lucifer Yellow (LY) assay. Finally, delivery of curcumin was performed *ex vivo* on murine colonic samples from healthy *versus* chemically-induced colitis.

2. Materials and methods

2.1. NPL synthesis, labeling and curcumin association

2.1.1. Synthesis of NPL

The porous and cationic starch nanoparticles NPL were synthesized according to the previously described methods (Paillard et al., 2010). The maltodextrin (Roquette, France) was dissolved in a 2 N sodium hydroxide solution with magnetic stirring at room temperature, then epichlorohydrin and glycidyl trimethyl ammonium chloride (Sigma-Aldrich, France) were added, leading to the formation of a cationic hydrogel. The gel was then neutralized with acetic acid and crushed by a high-pressure homogenizer (LM20–30 microfluidizer, Microfluidics, France). The nanoparticles obtained (NP^+) were purified from oligosaccharides, low-molecular weight reagents and salts, by tangential flow ultra-filtration (Akta Flux6, GE Healthcare, France) using a 300 kDa cut-off hollow fiber. Finally, 70% (w:w) of dipalmitoyl-phosphatidylglycerol (DPPG, Lipoid, Germany) was incorporated into NP^+ at 80 °C for 2 h and filtered through 0.2 μm filter to obtain NPL nanoparticles.

2.1.2. Characterization of NPL and NPL/Cur formulations

The hydrodynamic diameter (*Z*-average) and the polydispersity index (PDI) of the nanoparticles and formulations was measured by dynamic light scattering (DLS, Zetasizer nanoZS, Malvern UK) at 25 °C, using 1 mg/mL NPL in an aqueous solution of 23 mM NaCl.

The zeta potential of the nanoparticles and formulations was measured by electrophoretic light scattering (ELS) at 25 °C, using NPL at 1 mg/mL in ultrapure water.

2.1.3. NPL labeling

Labeling the lipid part of NPL was performed by mixing NPL with a 1 mg/mL ethanolic solution of 1, 1″-dioctadecyl-3, 3, 3″, 3″-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonatesalt (DiD, 1 mg/mL) at 0.7% *w*/w overnight at room temperature. The resulting NPL_{DiD} was purified by exclusion chromatography (PD-10 column Sephadex ™ G-25, Sigma-Aldrich, France).

2.2. Formulation of Curcumin with NPL (NPL/Cur)

A 1 mg/mL curcumin (purity: 97%, lot n° KXGOG, Tokyo Chemical Industry, UK, Ltd. ®) solution in ethanol was mixed with NPL in a mass ratio of 1:1 under stirring during 2 h at room temperature. The resulting NPL/Cur were filtered through a 0.2 μm filter and characterized as

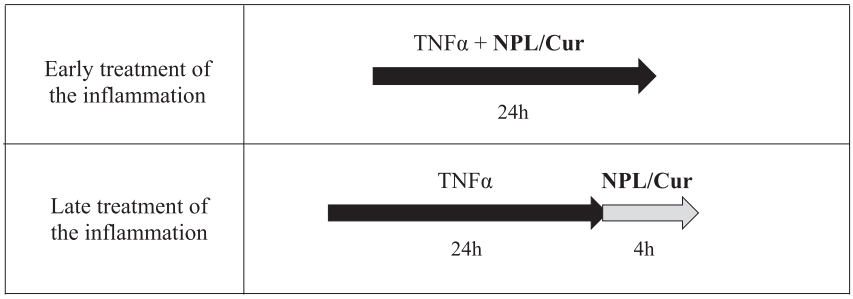

Fig. 1. Protocols developed as models for early and late treatments of the starting and established TNFα-induced inflammation.

Table 1
Characterization and encapsulation efficiency of free (NPL) and curcumin loaded (NPL/Cur) nanoparticles. The hydrodynamic diameter (*Z*-average, nm) and the polydispersity index (PDI) of nanoparticles were measured by dynamic light scattering while the zeta potential (mV) was measured by electrophoretic mobility. The encapsulation efficiency (EE) of curcumin into NPL was determined by separative filtration and expressed as a percentage of association over free curcumin. Analyses were measured in triplicate, and values represent the mean ± SD.

	Hydrodynamic diameter (nm)	PDI	Zeta-potential (mV)	EE (%)
NPL	65 ± 11	0.219	+37 ± 8	–
NPL/Cur	69 ± 14	0.204	+35 ± 6	80

described in Section 2.1.2.

The free curcumin was separated by filtration using a 30 kDa membrane (NanoSep, Pall, France) at 10,000 g for 5 min at room temperature. The amount of curcumin in the filtrate was quantified by fluorimetry using the ex: 485 nm/em: 538 nm wavelengths, over a curcumin standard curve ranging from 0.1 mg to 1 mg. The encapsulation efficiency (EE) of Cur in NPL was calculated as follows:

$$(\mathbf{m}_{cur}\ \mathbf{total} - \mathbf{m}_{cur}\mathbf{filtrate}/\mathbf{m}_{cur}\ \mathbf{total}) \times 100 = \%\boldsymbol{EE\ of\ Cur\ loaded\ in\ NPL}$$

2.3. Cell culture and treatments

The human intestinal epithelial cell line Caco-2 (ATCC #HTB-37) was maintained in DMEM supplemented with 20% heat-inactivated, fetal calf serum, 100 U/mL Penicillin, 100 mg/mL streptomycin and 1% L-glutamine at 37 °C in a humidified, 5% CO_2 atmosphere. Cells were seeded at a density of 1.0×10^5 cells per well or 5.0×10^4 for, respectively, 6-well plates (9.5 cm^2) and 12-well plates (3.5 cm^2) until confluence.

To study the treatment of the starting inflammation (early treatment), cells were treated with 10 ng/mL TNFα for 24 h, along with 8 μg/mL NPL/Cur, to limit the progression of the inflammation.

To study the treatment of the established inflammation (late treatment), cells were treated with 10 ng/mL TNFα for 24 h to induce inflammation. Then the medium was renewed, and 8 μg/mL NPL/Cur was added for 4 h.

The procedure is summarized on Fig. 1.

2.4. Characterization of NPL/Cur uptake by intestinal epithelial cells

The uptake of NPL and curcumin were evaluated using flow cytometry as previously described (Le et al., 2018).

Cells were washed twice with PBS and treated with 8 μg/mL of NPL_{DiD}, Cur or NPL_{DiD}/Cur either for 4 h or for 24 h, in both early and late treatment conditions following the Fig. 1. Cells were then washed again with PBS, harvested using trypsin and suspended in PBS for flow cytometry analysis.

To determine the uptake pathway of NPL/Cur, the cells were pre-incubated for 15 min at 37 °C with different endocytosis inhibitors: nystatin (20 μg/mL), filipin (10 μg/mL), chlorpromazine (CPZ, 10 μg/mL), phenylarsine oxide (PAO, 1 μg/mL), amiloride (23 μg/mL). Then 8 μg/mL NPL_{DiD}/Cur were added for 30 min before collecting the cells using trypsin and measuring nanoparticle endocytosis by flow cytometry (CYAN ADP Analyzer, Beckman Coulter). In this way, triplicate analyses were performed on 5000 cells.

2.5. Influence of NPL/Cur on cytokine secretion

Cells were incubated for 4 h or for 24 h with 8 μg/mL of NPL, Cur or NPL/Cur following the early and late treatment protocols in Fig. 1. The secretion of IL-1β, IL-6, IL-8 and IL-10 were measured by ELISA in each supernatant. Dexamethasone (Dex) was used at 0.1 mM as an anti-inflammatory positive control. The detection ranges of the ELISA kits (Invitrogen, France) were between 2 and 300 pg/mL.

2.6. Transcytosis of NPL/Cur on Caco-2 cells

Transcytosis of NPL/Cur was evaluated as previously described (Bernocchi et al., 2016). Caco-2 cells were seeded on Transwell® filters (3 μm porosity Transwell® filters, BD Bioscience, France) at a density of 1×10^5 cells/Transwell® (0.9 cm^2), in order to reproduce an intestinal epithelial barrier. Medium was changed every other day until confluence, which was checked by TEER measurement with an epithelial Volt/Ω meter (EVOM2, World Precision Instrument, USA, equipped with an STX2 electrode). The cells were pre-incubated with Hank's Balanced Salt Solution (HBSS, Life Technologies, France) during 30 min at 37 °C before measuring the permeability. As a positive control for the tight junction (TJ) opening, 50 μg/mL of a low molecular weight chitosan (CS; Sigma-Aldrich, France, 5 mg/mL at pH 6.5) was used, and a 50 μg/mL solution of lucifer yellow (Sigma-Aldrich, France) was added as control for paracellular and transcellular transports.

Cell monolayers were treated with 8 μg/mL of NPL_{DiD}, NPL/Cur, or Cur. The TEER was checked after 30 min, 1 h, 2 h, 3 h, 4 h and 24 h. The samples from the apical side and basolateral side were collected separately and the fluorescence was measured with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific, France) using the following filters: NPL_{DiD} λ_{ex}:633 nm/ λ_{em}:670 nm; Cur λ_{ex}:485 nm/ λ_{em}:538 nm.

2.7. Ex vivo uptake of NPL/Cur by colonic cells from Balb/c mice

Colonic segments of 1 cm from Balb/c mice, aged between 6 and 8 weeks, were collected. Tissues came from unused organs from animal experiments performed in accordance with the guidelines for animal experimentation (EU Directive 2010/63/EU), with a protocol approved by the local ethics review board (Nord-Pas-de-Calais CEEA N°75, Lille, France). Healthy mice were compared with mice suffering from Dextran Sulfate Sodium (DSS)-induced colitis. The inflammation of the colon was assessed by morphological observations (colon retraction and bowel

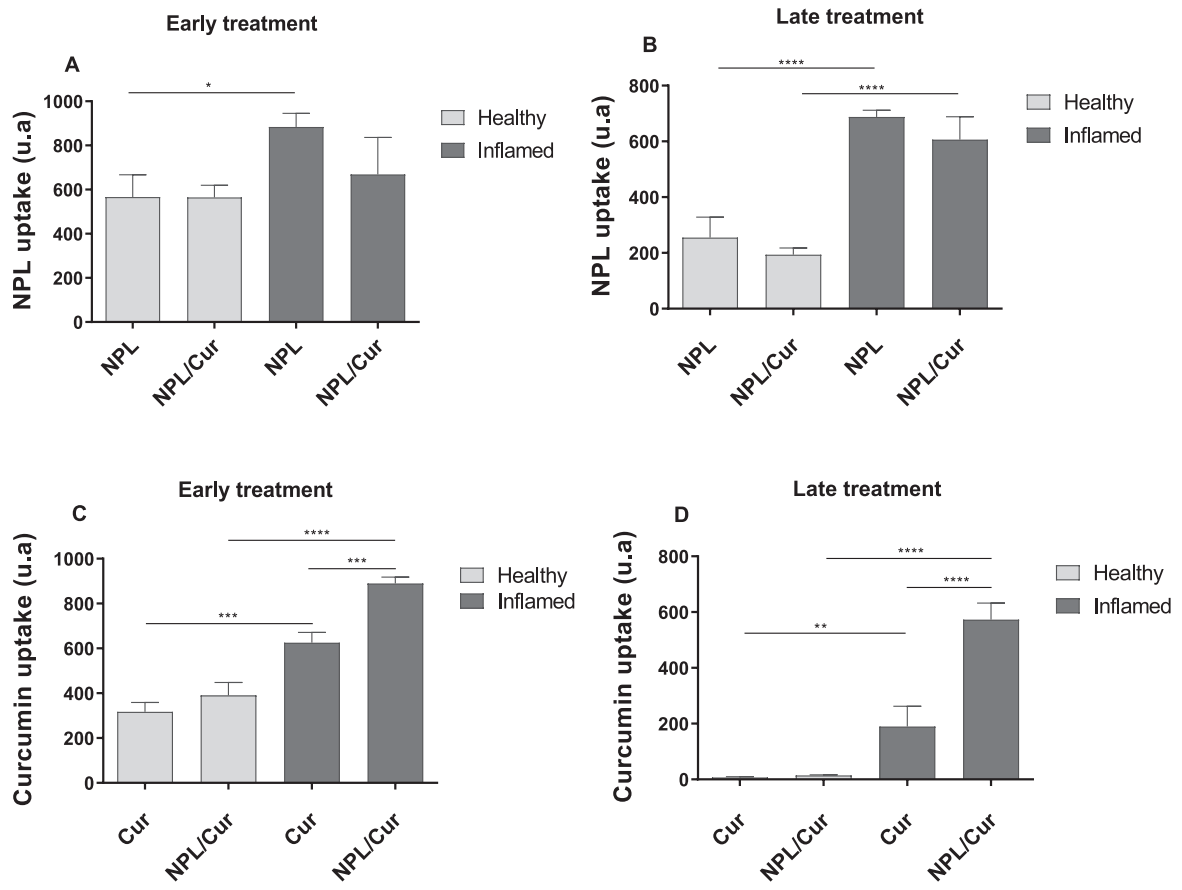

Fig. 2. Endocytosis of NPL and curcumin. The intracellular amount of NPL in non-inflamed ("healthy", light gray) or inflamed (dark gray) cells was analyzed by flow cytometry in the early 4 h (A) *versus* late 24 h (B) treatment conditions, using 8 µg/mL NPL or NPL/Cur. In a similar manner, the intracellular amount of curcumin, free- or NPL-associated, in the early (C) *versus* late (D) treatment conditions was measured. The analyses were performed in triplicate and values represent the mean ± SD of the mean fluorescence intensities based on a minimum of 5000 gated events. The significance of the statistical test is indicated as follow: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Table 2
Endocytosis inhibitors used in this study. The final concentration and the targeted endocytosis pathways are indicated.

Inhibitors Final	Concentration	Endocytosis pathway
Nystatin	20 µg/mL	Caveolae
Filipin	10 µg/mL	Caveolae
Chlorpromazine (CPZ)	10 µg/mL	Clathrin
Phenylarsine oxide (PAO)	1 µg/mL	Clathrin
Amiloride	23 µg/mL	Macropinocytosis

wall thickening) and clinical signs (loss weight, bloody stools, and diarrhea).

Segments were incubated for 4 h at 37 °C in HBSS buffer, and intraluminal instillations of Cur or NPL/Cur (8 µg/mL) were performed with a 32G needle. Tissues were then twice washed with PBS, fixed for 30 min with 4% paraformaldehyde at 4 °C, and frozen in Optimal cutting temperature (OCT) matrix (Sakura® Finetek, USA), after which 15 µm slices were obtained using a cryostat (Leica® CM3050 S). Nuclei were stained with Hoechst 33342 (5 µg/mL), and mounted with Fluoroshield™ (Merck Millipore, France), allowing visualization on a Zeiss LSM710 confocal microscope (Zeiss, France).

2.8. Statistical analysis

The results are represented as the mean ± standard deviation of at least 3 independent experiments and the analysis of variance was done using suitable ANOVA tests for each experiment.

3. Results

3.1. Characterization of the curcumin-nanoparticle formulation (NPL/Cur)

NPL are composed of a crosslinked maltodextrin shell with a core of phospholipids (DPPG). The NPL had an average hydrodynamic diameter of 65 nm and their PDI was 0.219. Moreover, they had a surface charge of +37 mV, which is important for their cell uptake and colloidal stability. The NPL/Cur formulation was made by adding equal amount of Curcumin (1 mg/mL) to NPL (1 mg/mL) leading to a 1:1 mass ratio formulation. The incorporation of curcumin by the NPL did not alter their size. The unaltered zeta potential of the NPL/Cur relative to NPL confirmed that the curcumin was associated with the lipid core of NPL, and not merely bound to their surface.

The efficiency of the curcumin encapsulation by the NPL, quantified by fluorimetry, was 80% (Table 1), and no curcumin release was measured in solution for at least 2 weeks of storage time. We thereby demonstrated that the curcumin was correctly associated with the lipid core of the NPL.

3.2. NPL/Cur uptake by caco-2 cells

To reproduce inflammatory conditions *in vitro*, cells were stimulated with 10 ng/mL of TNFα for 24 h at 37 °C.

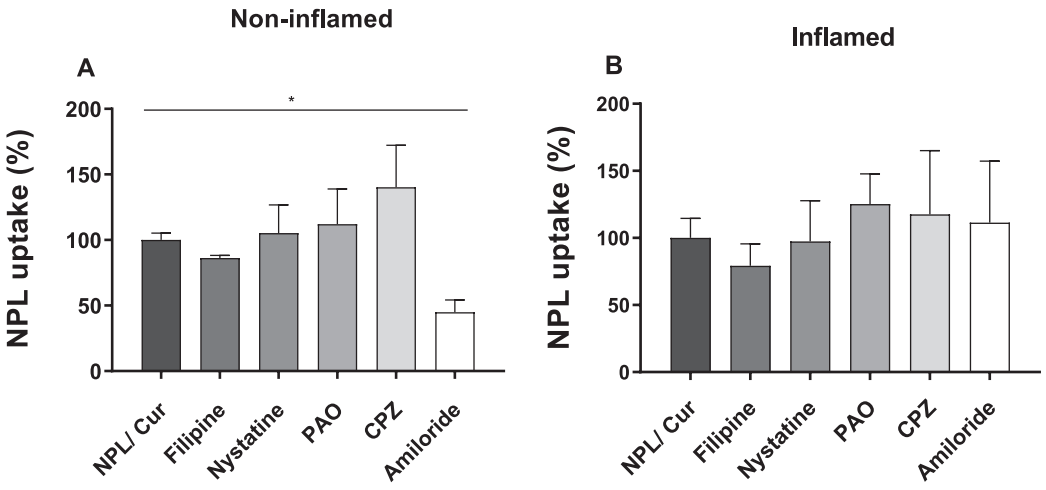

Fig. 3. Mechanisms of endocytosis of NPL/Cur in Caco-2 cells. Untreated, non-inflamed (A), or 24 h TNFα-induced inflamed (B) cells were treated with endocytosis pathway inhibitors for 15 min then 8 µg/mL NPL_{DiD}/Cur were added to cells for 30 min at 37 °C. Cells were then washed and immediately analyzed by flow cytometry. Data represent the mean fluorescence intensity +/− SD ($n = 3$) and cells not treated with inhibitors were set to 100% uptake. The significance of the statistical test is indicated as follow: * $p < 0.05$.

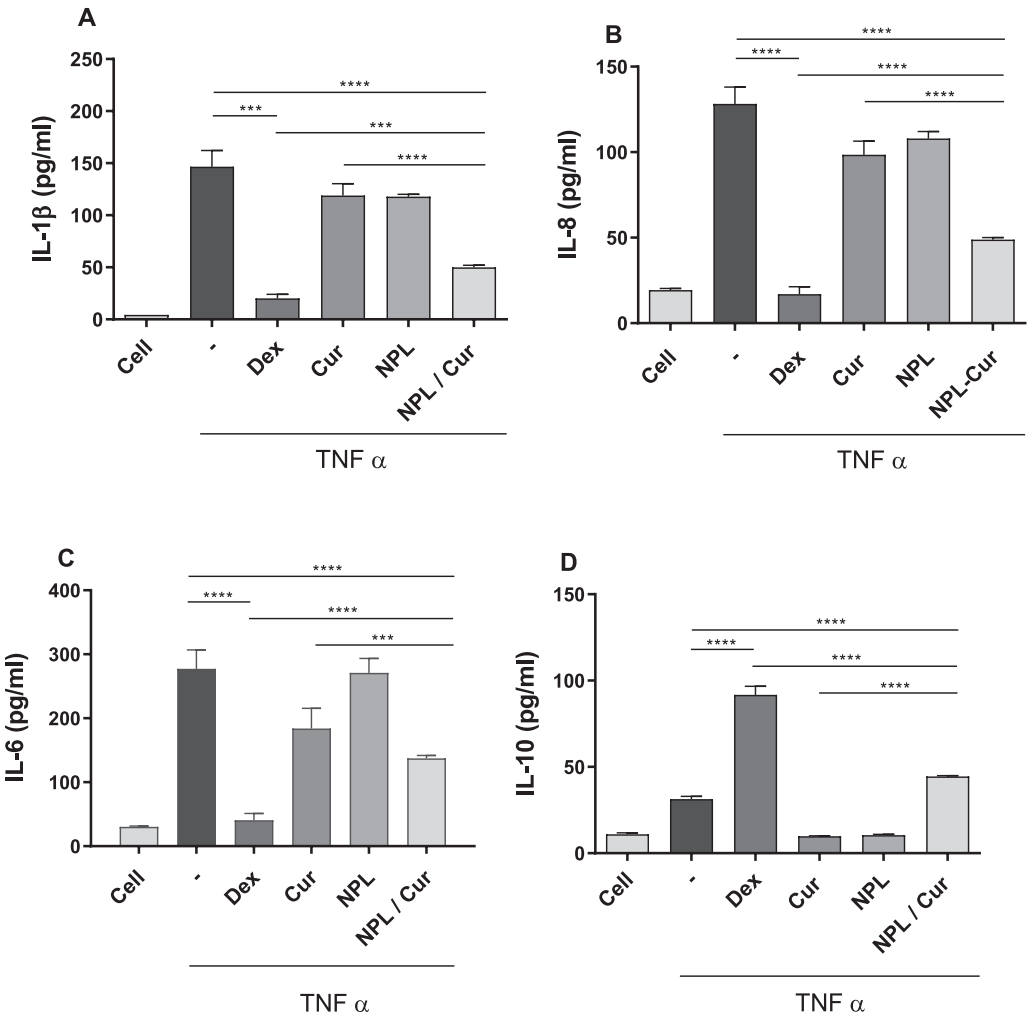

Fig. 4. Cytokine production in early treatment of inflamed Caco-2 cells. Cells were concomitantly stimulated with 10 ng/mL of TNFα and treated with either 8 µg/mL of NPL, Curcumin (Cur) or NPL/Cur during 24 h. The concentration of IL-1β (A), IL-8 (B), IL-6 (C) and IL-10 (D) cytokines in the supernatant was measured by ELISA. Values are expressed in pg/mL and represent the mean ± SD of triplicate analyses. The significance of the ANOVA statistical test is indicated as follow: *** $p < 0.001$; **** $p < 0.0001$.

Treatments with NPL, Cur or NPL/Cur were performed following two schemes: either during the presence of TNFα, to limit the progression of the inflammatory state (early treatment), or for 4 h after the TNFα-induced inflammation, to resolve an established inflammation (late treatment).

We first examined the influence of the inflammatory state on the endocytosis of NPL alone. In cells with an early treatment, a 1.56-fold increase of NPL uptake was observed compared to the healthy conditions (Fig. 2A). There was nevertheless an observable difference in cells with a late treatment, with a 2.7-fold increase of NPL uptake between healthy and inflamed conditions (Fig. 2B). The association of curcumin into NPL did not change this increase (x3.1).

Thereafter, the efficiency of curcumin delivery was examined. In the early treatment conditions, a significant increase of the NPL/Cur

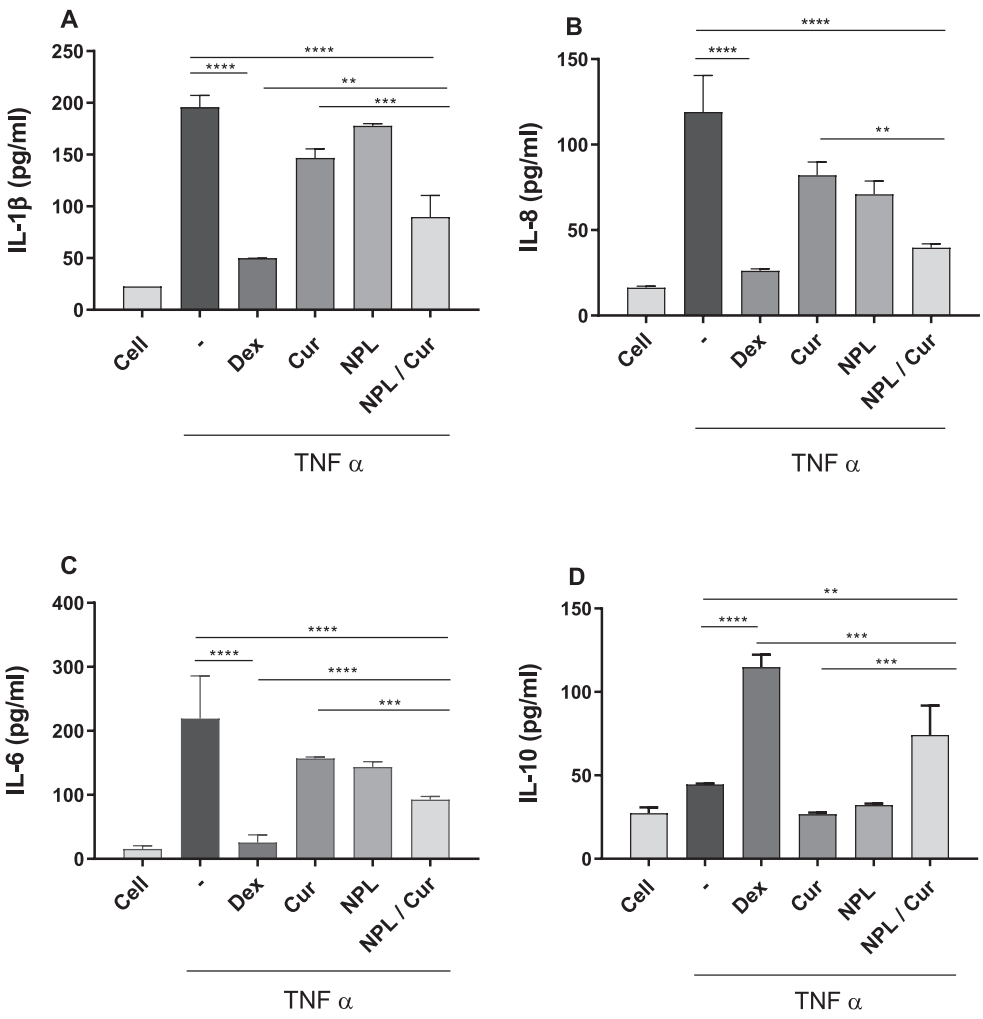

Fig. 5. Cytokine production in a late treatment condition of Caco-2 cells. Cells were pre-treated with 10 ng/mL of TNFα during 24 h to induce the production of inflammatory cytokines, then cells were treated for 4 h with either 8 µg/mL of NPL, Curcumin (Cur) or NPL/Cur. The concentration of IL-1β (A), IL-8 (B), IL-6 (C) and IL-10 (D) cytokines in the supernatant was measured by ELISA. Values are expressed in pg/ml and represent the mean ± SD of triplicate analyses. The significance of the ANOVA statistical test is indicated as follow: ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

delivery was observable between healthy and inflamed conditions (x2.8, Fig. 2C), as for the NPL endocytosis. In inflamed cells, a significant 1.4-fold increase of curcumin delivery was observed in presence of NPL. Concerning late-treated cells, an 81-fold increase of curcumin uptake was observed between free curcumin in healthy cells, and NPL/Cur in inflamed cells (Fig. 2D). Moreover, NPL increased the curcumin delivery by 3.6 in inflamed cells. As expected, the longer the incubation time, the higher the delivery. This showed that both the NPL nanocarrier and the inflammatory state synergistically increased the curcumin uptake.

These results suggest that in an inflammatory context such as IBD, the NPL nanocarrier could significantly increase curcumin delivery into the epithelial intestinal cells.

3.3. Endocytosis mechanism of NPL/Cur

Since the inflammatory state modified the uptake of NPL and curcumin (Fig. 2), we wondered whether the endocytosis pathways were redefined between these conditions. Cells were treated with known inhibitors of specific endocytosis pathways (Table 2), and the NPL/Cur uptake was assessed. In the healthy, non-inflamed cells, only the use of amiloride led to a significant inhibition of the NPL/Cur endocytosis, suggesting that macropinocytosis was the main pathway involved (Fig. 3A). However, in inflamed cells, none of these inhibitors impaired the uptake of the NPL/Cur (Fig. 3B). Thus, the TNFα-induced inflammation led to an uptake independent of the clathrin/caveola and macropinocytosis pathways.

3.4. Anti-inflammatory effect of NPL/Cur

To assess the efficiency of the NPL/Cur formulation on inflammation, the secretions of pro-inflammatory (IL-1β, IL-8 and IL-6) and anti-inflammatory (IL-10) cytokines were quantified by ELISA, on the Caco-2 cell line stimulated by TNFα according to the inflammation protocols depicted in Fig. 1.

Without any treatment, pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were highly produced, while the addition of dexamethasone (Dex), an anti-inflammatory glucocorticoid, totally inhibited this induction, as expected, and regardless of the early or late treatment protocol. Moreover, TNFα failed to induce IL-10 anti-inflammatory cytokine, contrary to Dex (Figs. 4 & 5). Noteworthy among these data was the observation that in healthy, non-inflamed cells, NPL, curcumin or NPL/Cur neither induced nor inhibited the production of cytokines (Supporting Fig. S1).

In the early treatment scenario, cells were concomitantly stimulated with TNFα and treated with NPL, curcumin or NPL/Cur for 24 h (Fig. 4). Production of IL-1β (Fig. 4A), IL-8 (Fig. 4B) and IL-6 (Fig. 4C) did not decrease in presence of free curcumin nor NPL whereas NPL/Cur had a significant effect. Moreover, only NPL/Cur was able to induce IL-10 secretion (Fig. 4D).

In the late treatment scenario, after 24 h of TNFα stimulation, cells were treated with NPL, curcumin or NPL/Cur for 4 h. As previously, IL-1β (Fig. 5A), IL-8 (Fig. 5B) and IL-6 (Fig. 5C) secretions only decreased in presence of NPL/Cur. Interestingly, NPL/Cur was as efficient as the Dex control in inhibiting the secretion of IL-8. Concerning the anti-inflammatory IL-10, while free curcumin and NPL alone had no effect,

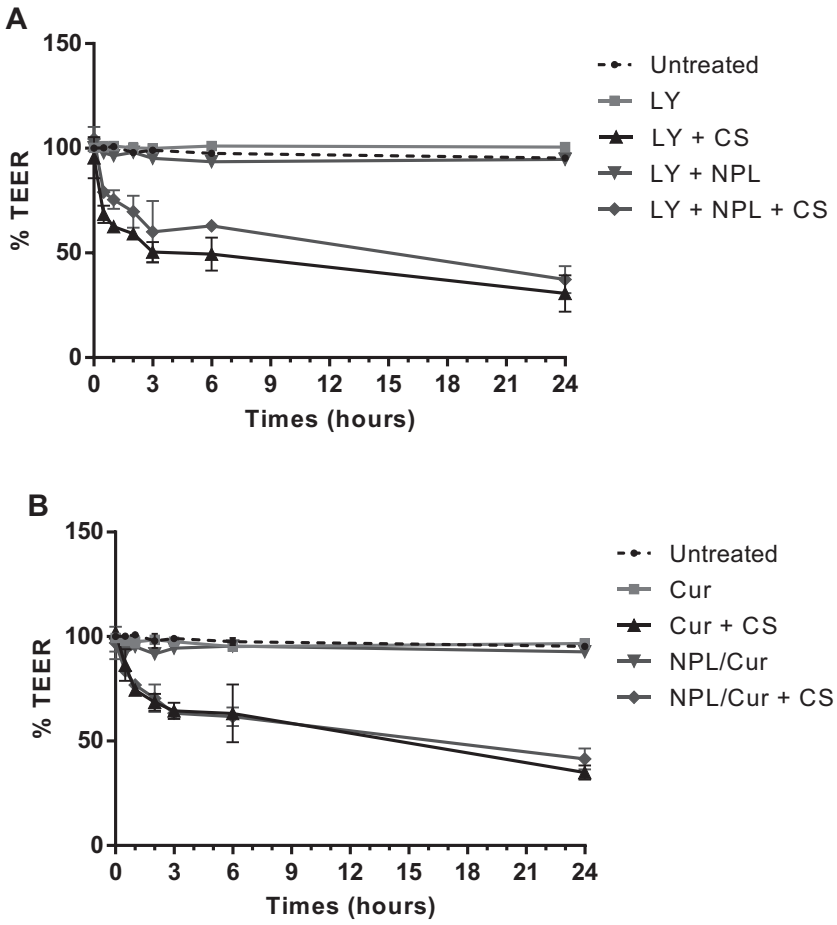

Fig. 6. Evaluation of tight junction opening in caco-2 cells at confluence. Cells were treated or not with chitosan (CS) (0.05% *w/v*) to loosen the epithelial junctions, and the influence of 8 µg/mL of NPL (A), curcumin or NPL/Cur (B) on the trans-epithelial electrical resistance (TEER) was evaluated. T0 is set to 100% and values represent the mean ± SD of triplicate analyses expressed as a percentage of the initial TEER value. Lucifer Yellow (LY) was used as a passive tracker of the transepithelial passage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NPL/Cur significantly increased its production (Fig. 5D).

3.5. Transcytosis and paracellular passage of NPL/Cur across Caco-2 epithelial barrier

Inflammation is a multicellular process involving epithelial, sub-epithelial, endothelial and circulating cells. To determine whether the NPL/Cur can cross an intestinal epithelium and resolve sub-epithelial inflammation, a Caco-2 model of epithelial barrier was established and the ability of NPL to deliver curcumin across the epithelial barrier was evaluated.

The Caco-2 cells were cultured on Transwell® filters until the cells reached confluence with a TEER value in the range of 350–450 $\Omega.cm^{-2}$. CS was used as positive control for the opening of the tight junctions (TJ), a mechanism mediated by integrin relaxation (Hsu et al., 2012; Hsu et al., 2013). Lucifer Yellow (LY) was used as a tracker of the transcytosis and paracellular passage. Treatment with CS exhibited a TEER reduction of 60% confirming tight junction opening. The LY and NPL had no effect on the TEER value, on either untreated or chitosan-treated epithelial barrier (Fig. 6A), neither did free curcumin or NPL-associated curcumin (Fig. 6B). This confirmed that this model was suitable for studying the trans-epithelial passage of curcumin.

To analyze the permeability of the epithelial barrier, the transport of LY across the intestinal epithelial barrier was assessed in parallel to the TEER measurements (Fig. 7). Without CS, the transcytosis of LY increased for 24 h (x2.68 between 0.5 h and 24 h), and as expected, a higher permeation was observed when the tight junctions were opened (x3.96 between 0.5 h and 24 h). The NPL did not modify the permeation of LY in presence or in absence of CS (Fig. 7A) which was consistent with TEER data (Fig. 6A).

We then examined the permeation of NPL across the epithelial barrier. Without CS, the transcytosis of NPL increased for 24 h (x3.58 between 0.5 h and 24 h), as depicted by the progressive increase of NPL amount in the basal compartment. CS increased this permeation (x7.61 between 0.5 h and 24 h). After 24 h, CS allowed a significant higher dose of NPL to cross the barrier, by a ratio of x2.9 (Fig. 7B). We noticed that the Curcumin did not alter the behavior of the NPL after 24 h of CS treatment (x2.6).

Finally, we analyzed if the permeation of curcumin was modified when associated to the NPL. Without CS, no difference of curcumin transcytosis was observed whether free or associated to NPL. However, CS increased both curcumin and NPL/Cur permeations after 1 h, with a higher and faster permeation of NPL-associated Cur than the free molecule. This was particularly obvious after 4 h and 6 h where a 2 -fold and a 1.3- fold increases were respectively observed between these conditions (Fig. 7C). This indicated that the NPL/Cur increased curcumin delivery across a loosened epithelial barrier.

3.6. Ex vivo study of NPL/Cur uptake by colonic cells of Balb/c mice

Since NPL were shown to effectively deliver Cur across an epithelial barrier with loosened tight junctions (Fig. 7), we examined the capacity of NPL/Cur to be taken up by colonic cells in DSS-induced colitis mice. Colonic explants were treated with free curcumin or NPL/Cur, and the presence of curcumin was analyzed by confocal microscopy (Fig. 8). DSS induced severe inflammation of the colon, confirmed by a thickened wall gut, a shorter colon, and weight loss (data not shown). Free curcumin slightly diffused across healthy epithelium but its association with the nanocarrier in the NPL/Cur allowed a deeper delivery. Moreover, NPL delivered Cur even better in DSS-damaged epithelium,

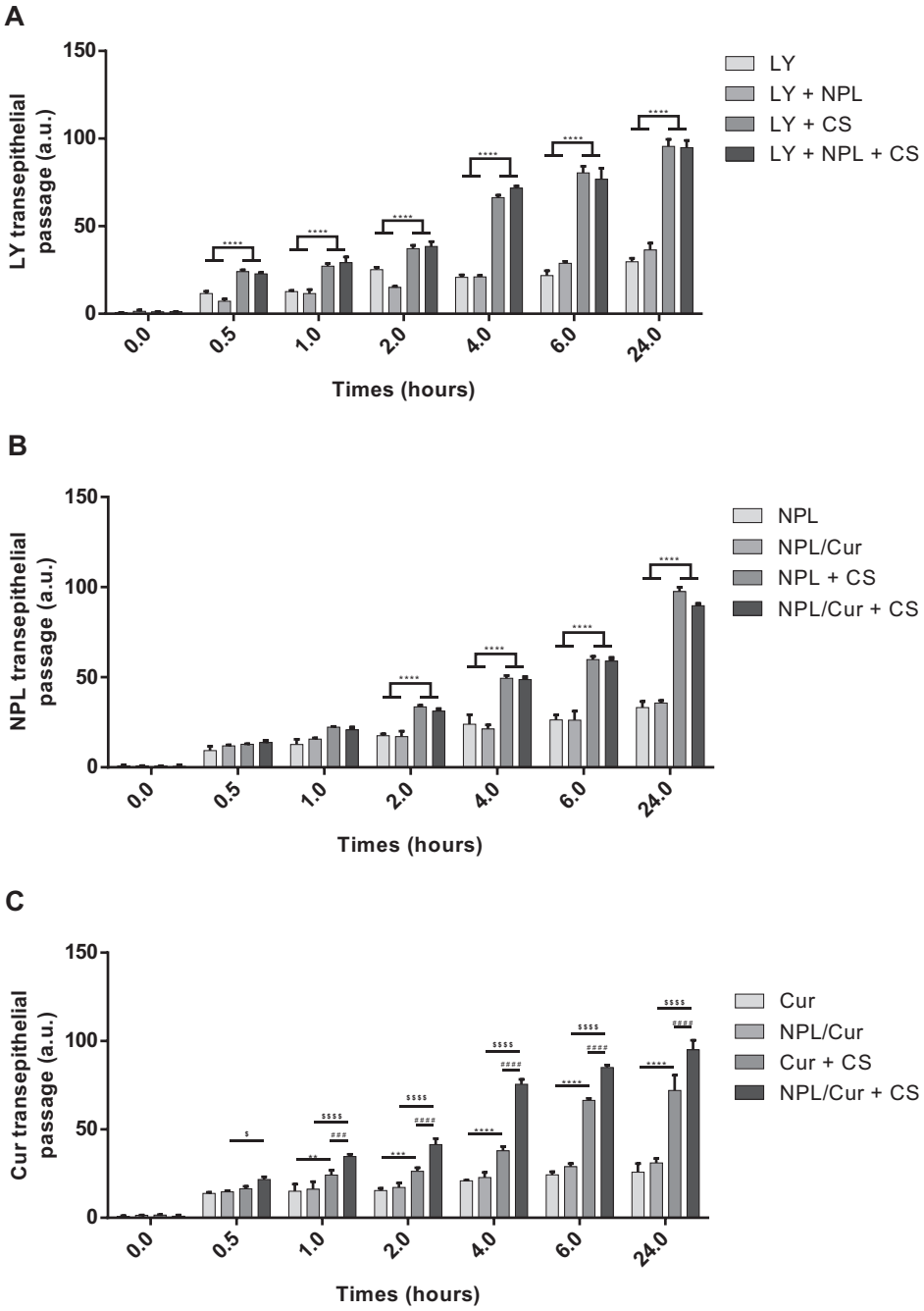

Fig. 7. Evaluation of the trans-epithelial passage of NPL/Cur in Caco-2 at confluence. A) Passage of the tracker Lucifer Yellow (LY): cells were treated with LY and influence of NPL in presence or absence of chitosan (CS) was shown. B) Passage of the NPL labelled with DiD: cells were treated with 8 µg/ mL of NPL_{DiD} or NPL_{DiD}/Cur in presence or not of CS. C) Passage of free or NPL-associated curcumin: cells were treated with 8 µg/mL of curcumin or NPL/Cur, in the presence or not of CS. Results are expressed as the means ± SD of triplicate measurements. Different symbols are used for clarity of the statistical ANOVA significance in C. The number of *, # or $ symbols represent the *p* value: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consistent with the *in vitro* permeation results (Fig. 7). NPL increased the curcumin bioavailability in the gut wall and thus could be considered as a good nanocarrier candidate to deliver curcumin to epithelial cells of an inflammatory gut.

4. Discussion

Curcumin exerts pleiotropic activities such as antioxidant, anti-carcinogenic and anti-infectious effects, all based upon the regulation of a large number of cellular signaling pathways (Calabrese et al., 2008). However, its poor solubility in biological fluid and its high rate of metabolization limit its potential clinical use. Nanomedicine as drug carrier and delivery system could overcome these limitations (Kumar et al., 2012). In the context of IBD promising results were already obtained with 5-ASA, corticosteroids, immunosuppressants or siRNA loaded in various NPs (Nunes et al., 2019; Taghipour et al., 2018). However, the clinical use of this strategy is still under evaluation.

In this study, we investigated the ability of the NPL nanocarrier, a crosslinked maltodextrin shell with a phospholipid core (Dombu et al., 2012), to deliver curcumin directly into inflamed intestinal epithelial cells and the anti-inflammatory effects of the NPL/Cur formulations.

In an inflammatory context, the electrical charge of the intestinal epithelial surface is perturbed, and the overall charge at the cells' surface becomes mainly cationic (Zhang et al., 2015). From this starting point, much research has focused on the use of anionic or neutral nanoparticles, aiming to increase their adhesion and epithelial residence time. Thus, it is important to state that, while the surface charge of the NPL is cationic, they behave as unique nanocarriers thanks to their anionic lipid core. Moreover, mucus production is increased in IBD, requiring nanoparticles that can penetrate and diffuse through the

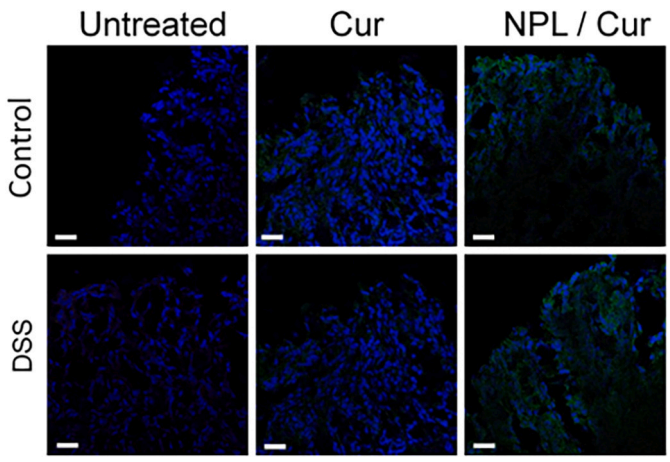

Fig. 8. Uptake of NPL/Cur into colonic explant in untreated *versus* DSS-induced colitis mice. Curcumin or NPL/Cur (8 μg/mL) were incubated for 4 h in the lumen of colonic explants and curcumin was visualized by confocal microscopy. Nuclei were stained with Hoechst 33342 (blue) while intrinsic curcumin fluorescence is shown in green. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mucus, which is the case for NPL (Murgia et al., 2018). Hence, the NPL combine three potential advantages for drug delivery in the context of IBD: (i) NPL are muco-penetrating nanocarriers, thanks to their size bellow 100 nm (here 65 nm, Table 1) and their phospholipid core (Fasquelle et al., 2020); (ii) they quickly interact and penetrate cells, due to their highly cationic surface charge (here +35 mV) (Le et al., 2018); (iii) they were proved to associate and deliver hydrophobic drugs (Kroubi et al., 2010). They can also protect a drug from oxidation, as shown for the anti-parasitic diminazene (Kroubi et al., 2010), which is a particular advantage when using curcumin (Betbeder et al., 2015). Thus, NPL are good candidates for curcumin cell delivery compared to other systems that only focus on increasing the drugs residence time in the epithelium's surface (Kumar et al., 2012).

The association of curcumin in NPL did not modify either the size or the zeta potential of the nanocarrier (Table 1), indicating an association between the curcumin and the phospholipids in the NPL core, as already described for diminazene and proteins (Bernocchi et al., 2016; Kroubi et al., 2010). Moreover, as a highly lipophilic molecule, hydrophobic interactions associated curcumin to the lipid core of the NPL; this latter being stably incorporated to the shell as previously demonstrated (Paillard et al., 2010), and explaining that no curcumin release is observed.

In the case of IBD, the inflammation state appears progressively, is continuous and self-sustaining (Luther and Dave, 2020). Rapid and efficient drug delivery would therefore be of interest to break this vicious circle. However, mimicking IBD *in vitro* is not possible and only specific features of inflammation can be reproduced. Here, we stimulated Caco-2 cells with TNFα to induce an inflammatory state, validated by measuring the secretion of pro-inflammatory cytokines IL-1β, IL-6 and IL-8 (Figs. 4 and 5). Treatments with anti-inflammatory compounds can either counteract or resolve the inflammation. In this regard, two protocols were examined, using NPL/Cur either in the early (during the TNFα-induced inflammation), or in the late (after the TNFα stimulation) steps of inflammation. In inflamed cells, we observed an increase of the NPL endocytosis leading to a better delivery of curcumin, compared with non-inflamed cells (Fig. 2). Under non-inflamed conditions macropinocytosis was the preferred endocytosis pathway for NPL (Fig. 3), while it was clathrin-, caveolae- and macropinocytosis-independent in inflamed conditions (Fig. 3), indicating a modification of the endocytosis mechanisms. We could hypothesize that nanoparticles use the flotillin pathway as an alternative, since its expression is abundant in inflamed enterocytes (Gauss et al., 2013).

Inflammatory cytokines and COX-2 are essential mediators of inflammation, and are known to be upregulated in IBD (Aggarwal et al., 2013; Singer et al., 1998). For instance, the excessive generation of IL-1β increases intestinal permeability, promoting activation of dendritic cells and macrophages (Al-Sadi and Ma, 2007; Al-Sadi et al., 2012; Coccia et al., 2012) and leading to acute and chronic inflammation (Halle et al., 2008; Larsen et al., 2009). Phytochemicals with anti-inflammatory and antioxidant activities have thus been studied in the management of IBD, as they can modulate various inflammatory mediators such as IL-1β, IL-6, IL-10, TNFα, PGE-2, iNOS, and COX-2 (Davatgaran-Taghipour et al., 2017; Zhang et al., 2016). We thus focused our study of the anti-inflammatory effects of curcumin, either free or delivered by the NPL, on the secretion of IL-1β, IL-6, IL-8 and IL-10 (Aggarwal et al., 2003). While neither curcumin alone nor NPL/Cur could reduce the secretion of the pro-inflammatory cytokines, NPL/Cur efficiently inhibited their expression (Figs. 4 and 5). On the contrary, IL-10 secretion was solely upregulated by NPL/Cur, despite the TNFα-induced inflammation. This proved that the NPL nanocarrier plays a key role in boosting the anti-inflammatory effects of curcumin. The mechanisms probably rely on the curcumin encapsulation which increases its solubility in biological fluids (Jurenka, 2009), and on the delivery of curcumin in cells (Le et al., 2019), (Fig. 2).

During a flare up of IBD inflammation, the epithelial barrier is damaged which induces disruption, or even the rupture, of the TJ. Here, we used chitosan, a cationic polysaccharide, to open the TJ (Chen et al., 2011). The initial aperture of epithelial TJ is thinner than 1 nm whereas chitosan can enlarge it to 150 nm, sufficient to allow the passage nanoparticles ≤50 nm across a Caco-2 cell monolayer (Lin et al., 2007). Here, the TJ opening allowed the passage of curcumin, NPL and NPL/Cur, (Figs. 6 and 7). However, this effect might be cell-type dependent as shown previously in airway epithelial cells (Bernocchi et al., 2016).

To better assess the NPL/Cur delivery in an inflamed intestine, an *ex vivo* murine colonic explant from a DSS-induced inflammation model was used. After 4 h of treatment, Cur was detected within the intestinal wall, not merely in the superficial layers of epithelial cells (Fig. 8). This supported the previous observation that NPL can cross a loosened intestinal epithelium (Fig. 7). This suggests that in IBD, NPL/Cur could also reach the subepithelial tissue and deliver curcumin to epithelial-associated immune cells, themselves implicated in the duration of the inflammation (eg. macrophages or dendritic cells).

5. Conclusion

Despite promising experimental evidence, clinical trials involving curcumin have failed to generate convincing results. Indeed, its therapeutic use could be either as a dietary supplement or a preventive treatment, or in association with other treatments (Iqbal et al., 2018). Owing to its low bioavailability and chemical instability when taken in free form, curcumin is an ideal candidate when loaded in delivery systems such as nanoparticles (Betbeder et al., 2015). Our results describe an easily scalable NPL/Cur nanoparticulated formulation. By encapsulating curcumin, NPL increased the intracellular drug delivery. This led to a boosted anti-inflammatory effect, evidenced by the downregulation of pro-inflammatory cytokines and the up-regulation of anti-inflammatory IL-10. This suggests that an adequate nanoparticulated formulation can actually help curcumin to be used as an efficient anti-inflammatory drug. NPL/Cur formulation described here needs to be further investigated to elucidate the exact mechanisms of the NPL-dependent, anti-inflammatory effects of curcumin, and to establish its efficiency *in vivo* on IBD induced animal models.

CRediT authorship contribution statement

Norhane Salah: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Laurent**

Dubuquoy: Funding acquisition, Writing – original draft. **Rodolphe Carpentier:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Didier Betbeder:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

D.B. is C.E.O and C.S·O of Vaxinano SAS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpx.2022.100114.

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