



**HAL**  
open science

## Reduction of Pro-Inflammatory Markers in RAW264.7 Macrophages by Polyethylenimines

Emilie Frisch, Candice Dussouillez, Conor Mc Cartin, Juliette Blumberger, Chana Humbert, Luc Lebeau, Benoît Frisch, Béatrice Heurtault, Antoine Kichler, Sylvie Fournel

► **To cite this version:**

Emilie Frisch, Candice Dussouillez, Conor Mc Cartin, Juliette Blumberger, Chana Humbert, et al.. Reduction of Pro-Inflammatory Markers in RAW264.7 Macrophages by Polyethylenimines. *Macromolecular Bioscience*, 2024, 10.1002/mabi.202300492 . hal-04501929

**HAL Id: hal-04501929**

**<https://hal.science/hal-04501929>**

Submitted on 12 Mar 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Reduction of Pro-Inflammatory Markers in RAW264.7 Macrophages by Polyethylenimines

Emilie Frisch, Candice Dussouillez, Conor McCartin,\* Juliette Blumberger, Chana Humbert, Luc Lebeau, Benoît Frisch, Béatrice Heurtault, Antoine Kichler,\* and Sylvie Fournel\*

The physiological problem of chronic inflammation and its associated pathologies attract ongoing attention with regard to methods for their control. Current systemic pharmacological treatments present problematic side effects. Thus, the possibility of new anti-inflammatory compounds with differing mechanisms of action or biophysical properties is enticing. Cationic polymers, with their ability to act as carriers for other molecules or to form bio-compatible materials, present one such possibility. Although not well described, several polycations such as chitosan and polyarginine, have displayed anti-inflammatory properties. The present work shows the ubiquitous laboratory transfection reagent, polyethylenimine (PEI) and more specifically low molecular weight branched PEI (B-PEI) as also possessing such properties. Using a RAW264.7 murine cell line macrophage as an inflammation model, it is found the B-PEI 700 Da as being capable of reducing the production of several pro-inflammatory molecules induced by the endotoxin lipopolysaccharide. Although further studies are required for elucidation of its mechanisms, the revelation that such a common lab reagent may present these effects has wide-ranging implications, as well as an abundance of possibilities.

pathogen, with the goal being to eliminate this pathogen or repair the tissue. The response is characterized by the classical symptoms of heat, pain, swelling, and redness. The so-called “sentinel cells” residents within the tissue (mainly mast cells and resident macrophages) will respond to the damage or pathogen intrusion through the recognition of damage-associated molecular patterns (DAMPs) or microbe-associated molecular patterns (MAMPs) via pattern recognition receptors (PRRs).<sup>[1,2]</sup> The affected tissue will then undergo vasodilation of the surrounding blood vessels to allow the infiltration of plasma fluid and responding immune cells.<sup>[2]</sup> Macrophages are key in this response, acting to phagocytose any pathogens present, and to secrete pro-inflammatory signaling molecules such as nitric oxide (NO) (to directly damage pathogens and encourage vasodilation), pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6)) and chemokines to amplify and mediate the response to a resolution (and eventually repair of the tissue).<sup>[3,4]</sup> The aforementioned MAMPs which initiate this response are for example molecules commonly found on microorganisms such as

## 1. Introduction

Inflammation is part of the innate immune response which occurs either following damage to a tissue, or intrusion of a

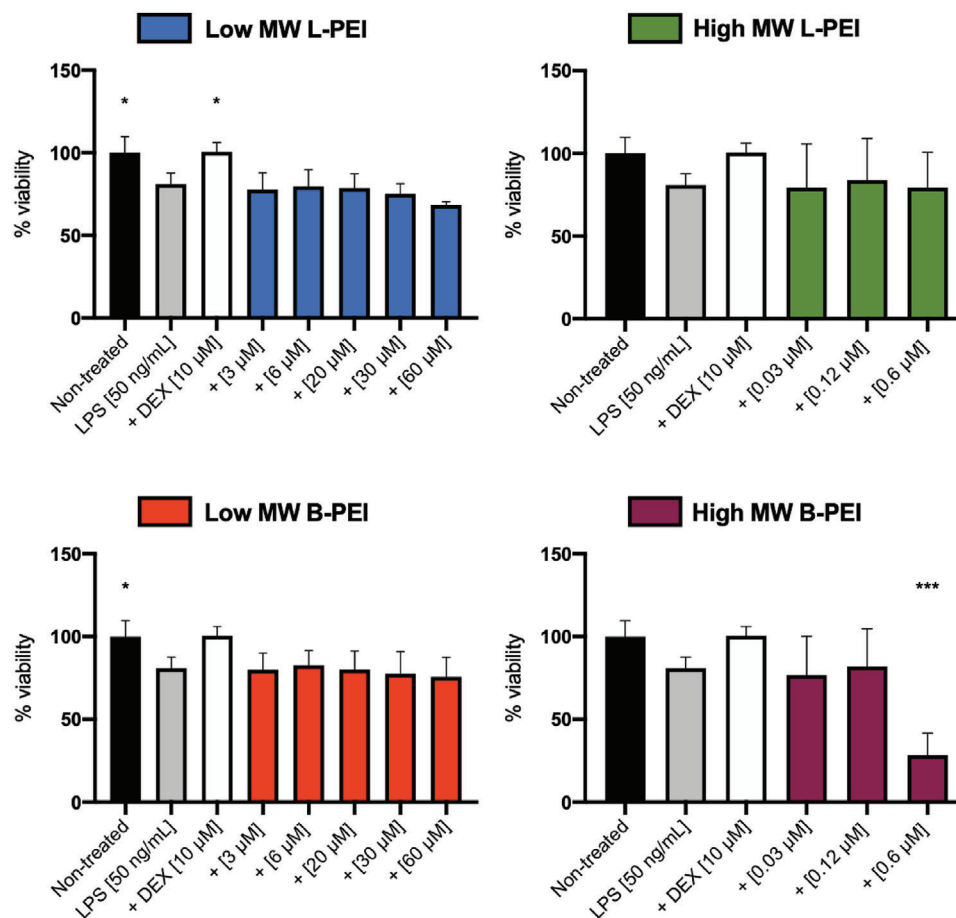
E. Frisch, C. Dussouillez, C. McCartin, J. Blumberger, C. Humbert, B. Frisch, B. Heurtault, A. Kichler, S. Fournel  
3Bio Team  
CAMB UMR7199  
Faculté de Pharmacie  
CNRS–Université de Strasbourg  
74 route du Rhin, Illkirch Cedex F-67401, France  
E-mail: [conormccartin@gmail.com](mailto:conormccartin@gmail.com); [kichler@unistra.fr](mailto:kichler@unistra.fr); [s.fournel@unistra.fr](mailto:s.fournel@unistra.fr)

E. Frisch, C. Dussouillez, B. Frisch, B. Heurtault, A. Kichler, S. Fournel  
Inserm UMR\_S 1121  
EMR 7003 CNRS  
Biomaterials and Bioengineering  
Centre de Recherche en Biomédecine de Strasbourg  
Université de Strasbourg  
1 rue Eugène Boeckel, Strasbourg F-67000, France  
L. Lebeau  
V-SAT Team  
Laboratoire de Conception et Application de Molécules Bioactives  
UMR 7199  
Faculté de Pharmacie  
CNRS-Université de Strasbourg  
Illkirch F-67401, France

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/mabi.202300492>

© 2024 The Authors. Macromolecular Bioscience published by Wiley-VCH GmbH. This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial](https://creativecommons.org/licenses/by-nc/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

DOI: 10.1002/mabi.202300492

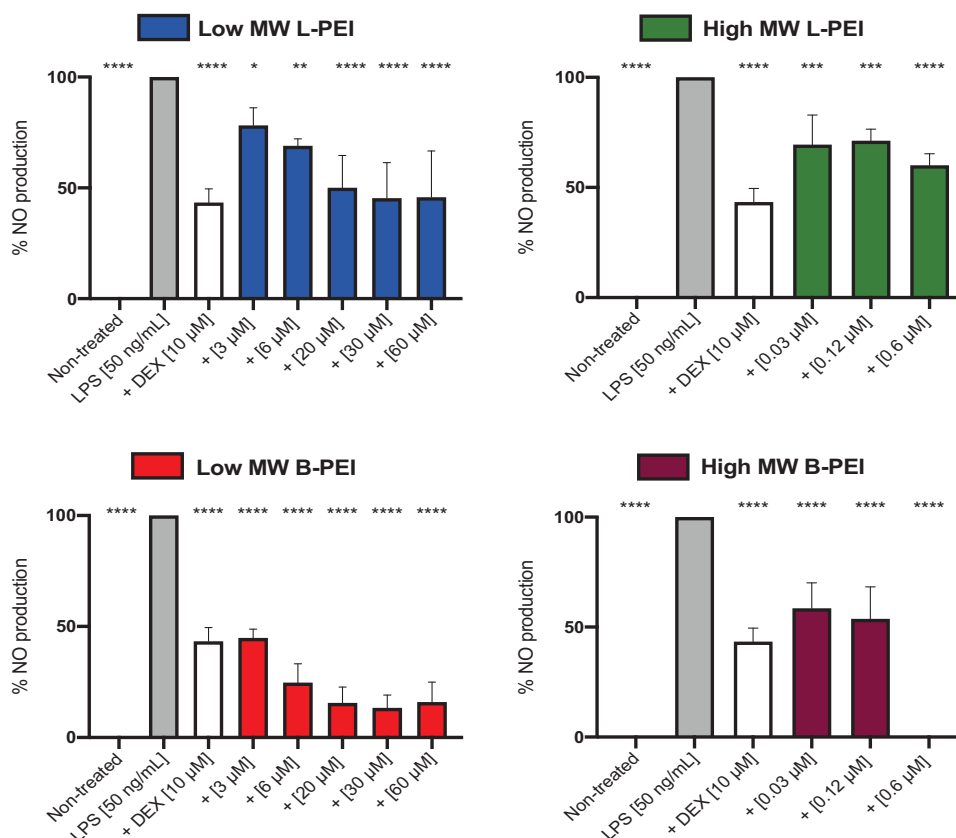


**Figure 1.** CellTiter Glo cytotoxicity assay of PEI treatments on LPS-stimulated RAW264.7. Results are expressed as the percentage of viable cells after 24 h treatment based on ATP quantification and expressed compared to non-treated condition (=100% viability). All conditions correspond to indicated treatments + LPS. Values represent the mean of  $n = 3$  independent experiments (each done in duplicates). Error bars represent one standard deviation (SD). The statistics represent a one-way ANOVA test in comparison to the LPS treated cells. Distribution normality was confirmed using a Shapiro–Wilk test. ns =  $P > 0.05$ . \* =  $P \leq 0.05$ , and \*\*\* =  $P \leq 0.001$ .

lipopolysaccharide (LPS) (an endotoxin found on the external membrane of Gram-negative bacteria),<sup>[5]</sup> or synthetic mimics such as Pam<sub>2</sub>CSK<sub>4</sub> (a synthetic analog of an endotoxin found on the external membrane of mycobacteria). LPS and Pam<sub>2</sub>CSK<sub>4</sub> are recognized by the PRRs Toll-like receptor 4 (TLR-4) and TLR2/6, respectively.<sup>[6,7]</sup> In addition to their chemical secretion-based signaling, macrophage activation may also be identified by cell surface markers such as CD86, major histocompatibility complex-II (MHC-II), and CD40.<sup>[4]</sup> While the acute phase of inflammation is important for proper tissue healing and repair, uncontrolled long-term inflammation has harmful effects on health causing a range of chronic inflammatory pathologies.<sup>[8,9]</sup> Chronic inflammation may have a range of causes, such as repeated exposure to an inflammatory inducer, defective biochemical signaling, or implantation of a biomedical device/biomaterial.<sup>[10,8,11]</sup> Anti-inflammatory compounds which may counteract this response are thus pharmacologically important and divided between several classes such as the non-steroidal anti-inflammatory drugs (NSAIDs) (e.g., piroxicam) or the steroidal class (e.g., the glucocorticoid dexamethasone).<sup>[12,13]</sup> Despite the recent develop-

ment of new strategies as the use of cytokine-targeted antibodies, current treatments are not free from side effects including susceptibility to infection or gastroduodenal toxicity.<sup>[14–16]</sup> Thus, the further development of novel anti-inflammatory compounds which may address these problems is of continued pharmaceutical interest.

One group of compounds showing such promise are cationic and anionic polymers, of which there are several examples in the literature that present anti-inflammatory effects. These include the naturally occurring chitosan and spermidine,<sup>[17,18]</sup> and more recently poly-arginine.<sup>[19]</sup> As for anionic polymers we may cite dendrimer glucosamine conjugates which showed a capacity to reduce the activation of LPS stimulated dendritic cells and macrophages *in vitro*.<sup>[20]</sup> This led us to investigate whether the very commonly used cationic transfection agent, polyethylenimine (PEI), would possess similar properties. We thus wished to test PEI of varying topology (linear vs branched) and size using LPS stimulated RAW264.7 murine macrophages as an inflammation model, to determine whether PEI could affect the induction of pro-inflammatory activation markers.



**Figure 2.** Reduction of LPS-induced NO production of RAW264.7 by PEI treatments. Results are expressed as percentage of NO production induced after 24 h LPS +/- PEIs treatment on RAW264.7 and compared to LPS control (=100% NO production) in corresponding experiments. Dexamethasone (DEX) at 10 μM was used as positive control. All conditions correspond to indicated treatments + LPS. Values represent the mean of  $n = 3$  independent experiments (each done in duplicates). Error bars represent one SD. The statistics represent a one-way ANOVA test against the LPS treated cells. Distribution normality was confirmed using a Shapiro–Wilk test. ns =  $P > 0.05$ , \* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ , and \*\*\*\* =  $P \leq 0.0001$ . Mean of RAW264.7 nitrite concentration in LPS condition = 29.15 μM.

## 2. Results and Discussion

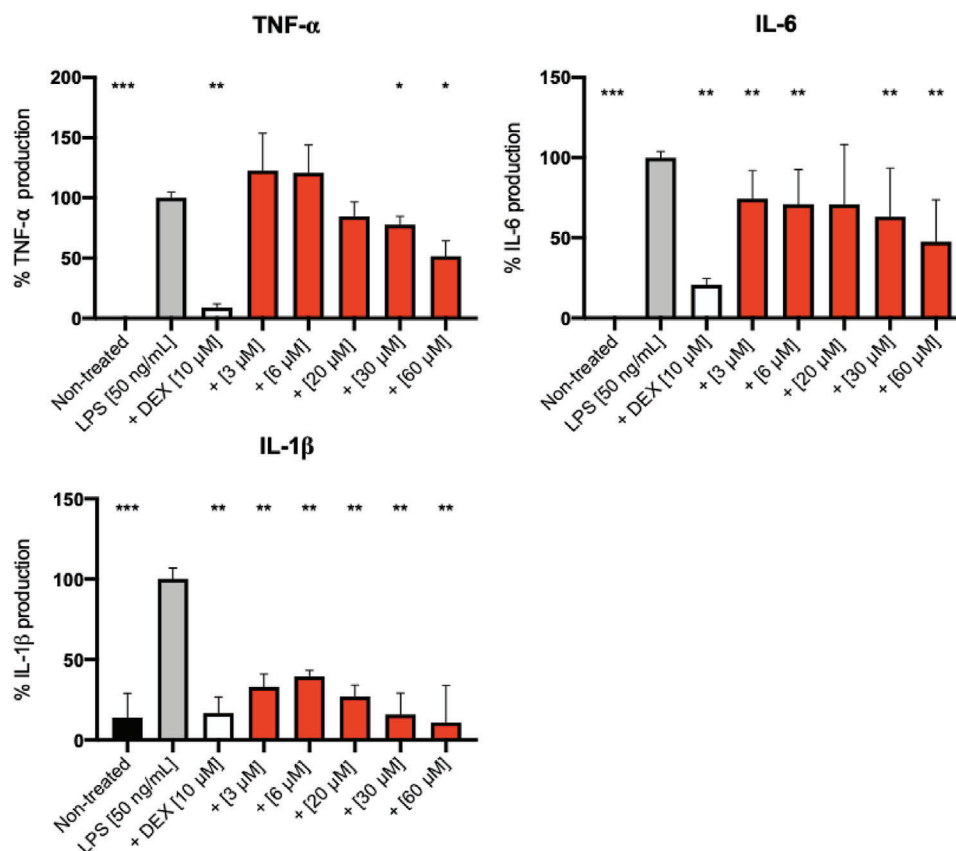
### 2.1. Toxicity of L-PEI and B-PEI of Varying Molecular Weight

In order to ensure that any reduction of pro-inflammatory markers was not due to a reduction in the number of cells, cell viability was measured using two different assays. RAW264.7 cells were treated with a range from 0.03 to 60 μM of polymer concentrations. The results obtained after a CellTiter-Glo 2.0 assay and the alamarBlue assay confirm that no cytotoxic effects were observed with both low molecular weight (MW) PEIs (branched polyethylenimine [B-PEI] 700 Da and linear polyethylenimine [L-PEI] 593 Da) at the tested concentrations (Figures S4 and S5, Supporting Information). By contrast, the two high MW PEIs (B-PEI 25 kDa and L-PEI 22 kDa), above 0.6 μM, severely affected the RAW264.7 viability (Figures S4 and S5, Supporting Information). When using 0.03, 0.12, and 0.6 μM the cell viability with the high MW PEIs remained high except for the 0.6 μM condition with the branched B-PEI 25 kDa. Moreover, to ensure that the toxicity observed was not related to LPS stimulation, we also tested the cytotoxicity of PEIs on RAW264.7 cells stimulated with LPS to induce an inflammatory phenotype. As shown in Figure 1 comparable results to those without LPS treatment were obtained. We there-

fore decided to continue our work using polymer concentrations from 3 to 60 μM for the low MW PEIs and from 0.03 to 0.6 μM for the high MW PEIs. Of note, dexamethasone which was used as anti-inflammatory control drug did not induce cell cytotoxicity at the concentration used (10 μM).

### 2.2. PEIs Reduce LPS-Induced Mouse Macrophage NO Production

The in vitro evaluation of anti-inflammatory properties is usually conducted using macrophages that can be activated by chemical compounds which mimic an inflammatory stimulus (LPS or Pam<sub>2</sub>CSK<sub>4</sub> in this study). The anti-inflammatory activity is then estimated by assessing the amount of various inflammation mediators produced by the cells, such as nitric oxide (NO) or pro-inflammatory cytokines. First, supernatants from RAW cells treated with LPS +/- PEIs were tested for nitrite (a stable product of NO), with the result showing a significant percentage reduction in the production of NO for all types of PEI tested (Figure 2). To note, it was the low MW B-PEI which induced the most efficiently an NO reduction. Importantly, the 700 Da B-PEI even reduced NO production to a level lower than that of dexamethasone



**Figure 3.** Reduction of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  production by B-PEI 700 Da. Results are expressed as percentage of TNF- $\alpha$ , IL-6, or IL-1 $\beta$  production induced by 24 h LPS stimulation in RAW264.7 and compared to LPS condition (=100% TNF- $\alpha$ , IL-6, or IL-1 $\beta$  production). All conditions correspond to indicated treatments + LPS. Values represent the mean of  $n = 3$  independent experiments (each done in duplicates) except for IL-1 $\beta$  where values are the mean of  $n = 2$  independent experiments. Error bars represent one SD. The statistics represent a Mann–Whitney test between each condition and the LPS alone. ns =  $P > 0.05$ , \* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , and \*\*\* =  $P \leq 0.001$ . Mean of RAW264.7 TNF- $\alpha$  concentration in LPS condition =  $9.19 \text{ ng mL}^{-1}$ ; mean of RAW264.7 IL-6 concentration in LPS condition =  $5.35 \text{ ng mL}^{-1}$ ; and mean of RAW264.7 IL-1 $\beta$  concentration in LPS condition =  $1.86 \text{ ng mL}^{-1}$ .

(DEX) which was used as a positive control. The total absence of NO production for B-PEI 25 kDa at  $0.6 \mu\text{M}$  could be explained by the fact that at this concentration the polymer induces important cell death (Figure 1 and Figure S4, Supporting Information).

To support the potential anti-inflammatory action and efficacy of the PEIs, additional experiments were conducted with Pam<sub>2</sub>CSK<sub>4</sub> as the inflammatory stimulus for RAW264.7 cells and the NSAID piroxicam as the positive control for NO reduction. The results showed a similar pattern as for the experiments done with LPS (Figure S6, Supporting Information), indicating the effect to be independent of the net charge of the inflammation-inducing molecule (net anionic for LPS and net cationic for Pam<sub>2</sub>CSK<sub>4</sub>).

As the PEIs can reduce both, LPS and Pam<sub>2</sub>CSK<sub>4</sub>-induced NO production, the mode of action of the polymers seems to be independent of the inflammatory activator and to the receptor used to induce macrophage activation. Indeed, while LPS is recognized by TLR4, Pam<sub>2</sub>CSK<sub>4</sub> is a TLR2/6 agonist.

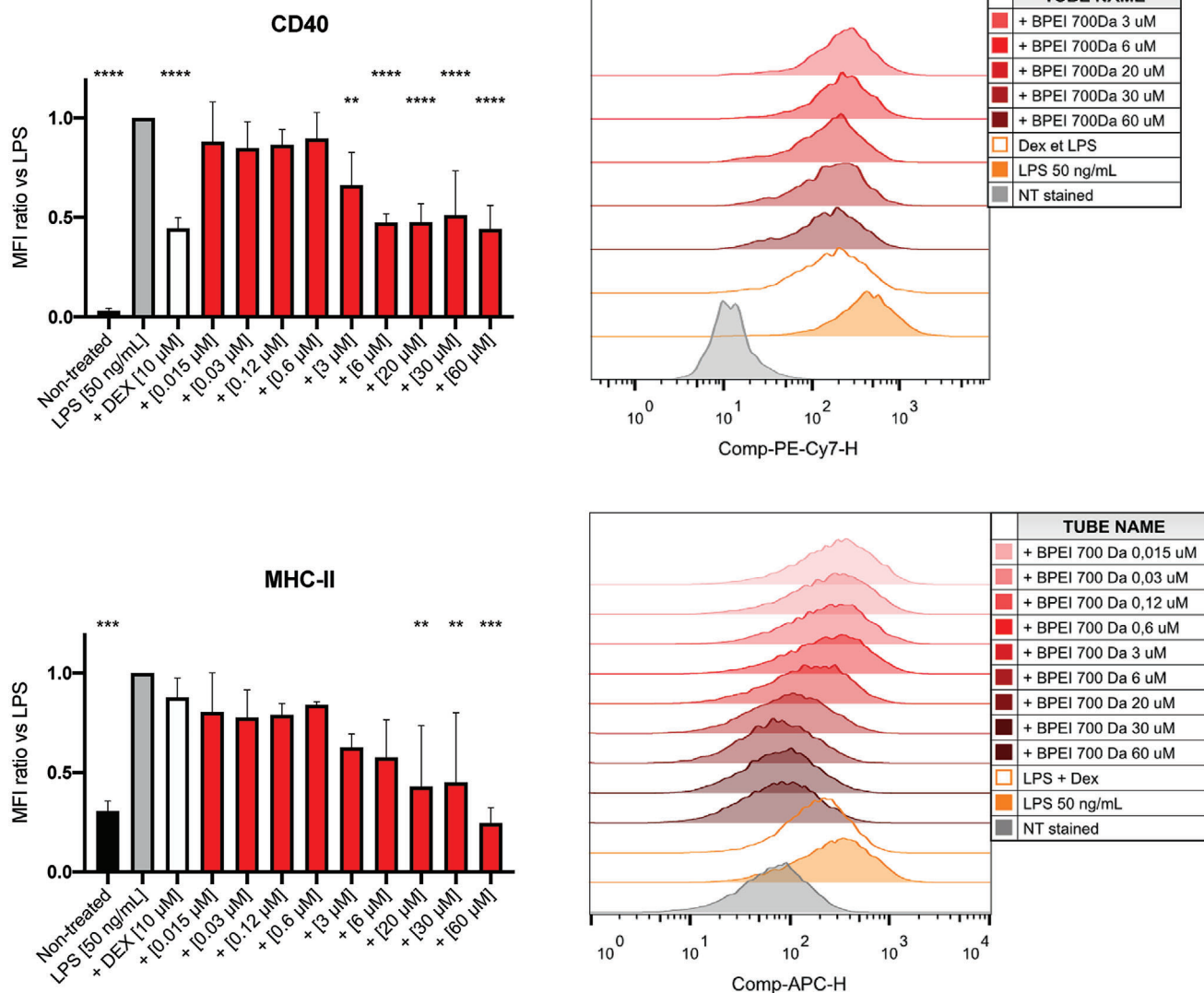
As the PEIs lowered NO production in LPS or Pam<sub>2</sub>CSK<sub>4</sub>-stimulated cells, the oxidative profile of LPS-stimulated cells after PEI treatment was characterized by using the mitochondrial reactive oxygen species (ROS) indicator MitoSOX Red. As shown in

Figure S7 (Supporting Information), none of the PEIs appeared to reduce superoxide production in LPS-stimulated cells.

### 2.3. Low Molecular Weight B-PEI Reduces LPS-Induced Mouse Macrophage TNF- $\alpha$ , IL-6, and IL-1 $\beta$ but not IL-12 Production

To confirm the observed capacity of the PEIs to reduce inflammatory macrophage activity, we wondered whether they could also reduce the secretion of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  or of the polarizing cytokine IL-12. In the same manner as before, the RAW264.7 cells were treated with LPS together with different concentrations of PEI for 24 h. An enzyme-linked immunoassay (ELISA) was then performed on the supernatants as described in the Experimental Section. With the most efficient polymer, namely the low MW B-PEI, the results showed a decrease in a dose-dependent manner of cytokine production, reaching a decrease of  $\geq 50\%$  with the highest concentration of polymer ( $60 \mu\text{M}$ ) for three of these cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  (Figure 3).

It should be noted that dexamethasone was however more efficient at lowering the cytokines production compared to B-PEI



**Figure 4.** Reduction of CD40 and MHC-II activation markers expression by B-PEI 700 Da. CD40 and MHC-II expression are expressed as a ratio of the geometric mean fluorescence intensity induced by LPS stimulation on RAW264.7 and compared to LPS control (=100% CD40 or MHC-II expression) in corresponding experiments (left). Histograms represent the percentage of CD40 and MHC-II expression in response to LPS stimulation on RAW264.7. They are one replicate specific but are representative of all independent triplicates (right). All conditions correspond to indicated treatments + LPS. Values represent the mean of  $n = 3$  independent experiments (each done in duplicates). Error bars represent one SD. The statistics represent a one-way ANOVA test being versus LPS-condition. Distribution normality was confirmed using a Shapiro–Wilk test. ns =  $P > 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ , and \*\*\*\* =  $P \leq 0.0001$ .

700 Da. Moreover, the other tested polymers were less efficient than the B-PEI 700 Da as shown in Figure S8 (Supporting Information).

All these results suggest that the branched topology, whether of low or high MW PEIs, seems to confer a higher efficiency to the polymers than the linear topology. Indeed, even if we exclude the results obtained using 0.6  $\mu\text{M}$  of B-PEI 25 kDa because of its high cytotoxicity, a greater effect was observed with 0.12  $\mu\text{M}$  of this polymer than with any concentration of L-PEI 22 kDa (Figure S8, Supporting Information). It is also worth mentioning that while all PEIs were able to reduce IL-6 and IL-1 $\beta$  production, it was not the case for TNF- $\alpha$  production since both low and high MW L-PEIs had no effect on the level of this cytokine.

Notably, Chen et al.<sup>[21]</sup> using peritoneal murine macrophages observed a reduction in LPS-induced TNF- $\alpha$  expression with a L-PEI of 25 kDa.<sup>[21]</sup> The discrepancy concerning the TNF- $\alpha$  inhibition may be explained by the fact these authors used primary macrophages and not cell lines as in our study but also a much higher amount of polymer (i.e., 50  $\mu\text{g mL}^{-1} = 2 \mu\text{M}$  polymer). Such concentrations induced important toxicities for the similarly sized 22 kDa L-PEI on the RAW cell line used in our study (>50% cell death when using 3  $\mu\text{M}$  of the polymer). As Chen et al. did not carry out any cytotoxicity assay for their treatment conditions, it cannot be ruled out that the observed pro-inflammatory cytokine induction is not due to a response to a toxic insult from the

cationic polymers, which could release endogenous activation signals.<sup>[22]</sup>

With regard to IL-12 production, no reduction was observed with the PEIs (Figure S9, Supporting Information). This is also in contrast to the study of Chen et al. which describes an induction of this cytokine for high MW L-PEI, leading the authors to assert a pro-inflammatory effect of PEI.<sup>[21]</sup> However as already mentioned above, the experiment settings in Chen's study differ from ours.

#### 2.4. Low Molecular Weight B-PEI Reduces LPS-Induced Mouse Macrophage Activation Markers CD40 and MHC-II

To further elaborate the observed effect of B-PEI 700 Da on the secretion of pro-inflammatory cytokines, we evaluated if low molecular weight B-PEI could similarly reduce the expression of the pro-inflammatory surface markers CD40 and MHC-II. This was carried out by co-treatment of the cells followed by flow cytometric quantification of markers expression. The results showed a diminution of CD40 expression by 50% starting from 6  $\mu\text{M}$  of B-PEI 700 Da in a similar level as the decrease induced by dexamethasone (Figure 4). Interestingly, a similar decrease in MHC-II expression was observed with >6  $\mu\text{M}$  B-PEI 700 Da while dexamethasone had no effect on MHC-II expression.

Below 3  $\mu\text{M}$ , the expression level of both markers returned to the level observed in LPS-treated cells. Concerning the three other PEIs, a reduction of CD40 expression was also observed for all the polymers, with the B-PEI 25 kDa being the most efficient one (Figure S10, Supporting Information). In contrast to CD40, only high MW PEIs appeared to slightly decrease MHC-II expression.

### 3. Conclusions and Perspectives

The observation that PEI, the decades-old, ubiquitous laboratory transfection reagent may have inherent anti-inflammatory properties is of great interest. Our results indeed convincingly show the effect of B-PEI 700 Da, and to a lesser extent PEI with higher MW or with a linear topology, in reducing several different pro-inflammatory markers including secreted and cell-surface based ones without significant cytotoxicity. This may have major implications for the results of immunological studies using PEI as a carrier for genetic (or other) materials. The present results also add further weight to the body of work showing the similar anti-inflammatory properties of other cationic polymers such as poly(amidoamine) dendrimers.<sup>[23]</sup> Such a property renders these compounds yet more interesting in the development of drug carriers, or especially biomaterials, where the battle against implant-induced inflammation is an important aspect of the domain's direction. Interestingly, the effect is maintained for low molecular weight versions of the PEI, which are considerably less toxic than the larger molecular weights which are classically used for transfection. The mechanism of this effect requires further investigation, although the ability to reduce pro-inflammatory markers induced via different agonists indicates that it is not a receptor-specific effect. In particular, it should be explored whether the effect is purely a block of pro-inflammatory activation, or whether

there is an induction of an anti-inflammatory cellular phenotype. To note, results from the literature suggest that PEIs do not directly block at the transcription level the expression of genes involved in inflammatory response pathways.<sup>[24]</sup> The maintenance (or not) of the characteristics observed in this study when incorporated into a material (such as a hydrogel) shall be important in determining the breadth of its biological applicability. The focus on PEI is often narrowed to its (highly important) use as a carrier of other compounds, however, clearly, work remains to be done on its own inherent interests.

### 4. Experimental Section

**Materials:** All tested PEI polymers 22 kDa L-PEI, 25 kDa B-PEI (#408 727, Sigma-Aldrich), 593 Da L-PEI (see synthesis below) and 700 Da B-PEI (#40871-9, Sigma-Aldrich), the inflammatory response inducers LPS (#tlrl-3pelps, InvivoGen), and synthetic diacylated lipopeptide Pam<sub>2</sub>CSK<sub>4</sub> (#tlrl-pms, InvivoGen) were stored as stock solutions in MilliQ water at  $-20^{\circ}\text{C}$ . The 22 kDa L-PEI was synthesized as described previously by Brisault et al., with slight modifications.<sup>[25,26]</sup> The anti-inflammatory positive control dexamethasone (#GP8543-56, Glentham Life Science) was stored as stock solution in absolute ethanol. Prior to use, stocks were heated and sonicated briefly at  $37^{\circ}\text{C}$  in a bath sonicator. Prior to the treatment of cells, stock compounds were diluted to desired concentrations in the culture medium of the cells being treated.

**Synthesis of the LPEI of 593 Da:** L-PEI was prepared by cationic ring-opening polymerization of 2-ethyl-2oxazoline, modifying experimental procedures reported in the literature<sup>[27,28]</sup> (Figure S1, Supporting Information). Briefly, *p*-toluenesulfonic acid methyl ester (37.6 g, 0.202 mol) was added as an initiator to ethyl oxazoline (200 g, 2.02 mol) in dry CH<sub>3</sub>CN under an argon atmosphere. The resulting mixture was refluxed under stirring for 20 h. It was then cooled to room temperature and quenched by the addition of water (8 mL) for 2 h. Volatiles were removed under reduced pressure and the polymerization mixture was dissolved in CHCl<sub>3</sub>, washed with saturated aqueous NaHCO<sub>3</sub>, brine and water. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and reduced under vacuum to yield  $\approx 220$  g of crude material. This material was suspended in HCl 6N (750 mL) and stirred under reflux for 10 h. Water was removed under reduced pressure, the residue was refluxed in MeOH (750 mL) for 2 h, and the mixture was cooled again to room temperature. The white precipitate was collected by filtration, washed with Et<sub>2</sub>O, and dried under vacuum to yield L-PEI in the hydrochloride form (155.1 g). The compound was characterized by NMR (Figure S2, Supporting Information) and ESI-ToF MS (Figure S3, Supporting Information). Calculated from the <sup>1</sup>H-NMR spectra, the average number of repeated units in the molecule (*n*) was found around 12.9. This was consistent with the ESI-ToF analysis which revealed a gaussian distribution of the polymer molecular weight centered at 592.6 Da, corresponding to the tridecamer (*n* = 13).

**Cell Culture:** The RAW264.7 (TIB-71, ATCC) murine macrophage cell line was kept in 10 cm<sup>2</sup> non-tissue culture-treated dish at  $37^{\circ}\text{C}$ , 5% CO<sub>2</sub>, and 80% humidity. Dulbecco's modified Eagle media—high glucose medium (#D0819-500ML, Sigma-Aldrich) was supplemented with 5% of fetal bovine serum (#10 270 106, Gibco) and streptomycin/penicillin (#P0781-100ML, Sigma-Aldrich) at 100 U mL<sup>-1</sup> and 100  $\mu\text{g}$  mL<sup>-1</sup>, respectively. Cell passage was carried out as follows: cells were first gently flushed and removed from the dish to be centrifuged at  $200 \times g$  for 4 min (room temperature—RT). The cell pellet was then resuspended in fresh culture medium and counted using the Countess II FL automated cell counter (Thermo Fisher Scientific) with Trypan Blue (#T8154-100ML, Sigma-Aldrich) at 1:1 v/v ratio to exclude dead cells from cell count. Cells were then seeded at  $5.10^6$  cells per dish for 3 days.

**Cell Treatment:** For 24-h treatments, cells were seeded at a density of  $3.10^4$  cells per well (in 100  $\mu\text{L}$ ) 1 day prior to treatment in 96-well plates (#236 105, Thermo Fisher). For treatment, the culture medium was first

removed and replaced with 100  $\mu\text{L}$  of culture medium per well containing the desired concentration of polymers +/- LPS (or Pam<sub>2</sub>CSK<sub>4</sub>).

**Cell Viability Assays:** To determine the amount of living cells remaining after LPS and PEI treatment, a CellTiter-Glo 2.0 cell viability assay (#9241, Promega) was conducted. This kit was based on cell metabolic activity by quantification of ATP released after cell lysis in the culture medium, which was directly proportional to the measured luminescence. According to manufacturer instructions, a volume of CellTiter-Glo reagent was added to each well of the experiment at a ratio of 1:1 v/v. The remaining culture medium was collected for other analyses. Following an agitation for 3 min to induce cell lysis and ATP release, the supernatants were then transferred into a white 96-well plate after 15 min of incubation (RT). Measurement of luminescence (expressed in RLU) was then conducted using a microplate reader (Safas Monaco SP2000, Genius 5801). Percentage of cell viability was defined by a percentage of the maximum viability (from the non-treated condition) and background luminescence was removed from the values from medium control alone.

To confirm the results obtained by the first cell viability assay, an AlamarBlue cell viability assay was done (#DAL1100, Invitrogen). AlamarBlue was based on a blue non-toxic and cell permeable compound called resazurin: the conversion of blue resazurin into red resorufin was due to cellular reduction performed by mitochondrial activity. The color change was quantifiable and, as with CellTiter-Glo assay, was directly related to cell viability. Based on the manufacturer instructions, the AlamarBlue reagent was added to the cells after removing the supernatants at a 1/10<sup>6</sup> dilution in culture media. After 3 to 4 h of incubation at 37 °C, the resulting absorbance was measured on a microplate reader (Safas Monaco SP2000, Genius 5801). The percentage of cell viability was expressed in the same way as for the CellTiter-Glo viability assay.

**Griess Assay:** To assess macrophage activation, the NO production was determined. A Griess assay was used to quantify stable breakdown product of NO radical, nitrite (NO<sub>2</sub><sup>-</sup>) in cellular supernatant. Following cell treatment as described above, 40  $\mu\text{L}$  of each supernatant were collected and transferred in a new 96-well plate (#655 001, Greiner Bio-One). To this volume were added 30  $\mu\text{L}$  of "S" solution (1% w/v p-aminobenzenesulfonamide, 30% acetic acid and 70% MilliQ water) and 30  $\mu\text{L}$  of "N" solution (0.1% w/v N-1-naphthylethylene diamine dihydrochloride, 60% acetic acid, and 40% MilliQ water). A standard curve was produced by cascade dilution of NaNO<sub>2</sub> from 100 to 1.56  $\mu\text{M}$ . Absorbance was measured using the same plate reader as for section 2.5 at 543 nm and nitrite concentrations were determined via interpolation of the standard curve.

**MitoSOX-Based Flow Cytometry:** To estimate the amount of ROS produced by LPS-stimulated cells after PEI treatments, a MitoSOX-based assay was performed. MitoSOX Red was used as an indicator of mitochondrial ROS and especially as a mitochondrial superoxide indicator, since its oxidation was only possible when superoxide was present. As the resulting oxidated product exhibits red fluorescence, an estimate of ROS production can be measured by flow cytometry. After 24 h of LPS stimulation and PEI treatments, cells were recovered with a 5 min incubation with trypsin-EDTA at 37 °C. Following a washing step with 50  $\mu\text{L}$  cold PBS per well, duplicate conditions were pooled in microfuge tubes (#MT-0105-R, Biotix) and centrifuged for 5 min at 350  $\times g$  (4 °C). Cell pellets were first stained with 1/1000<sup>e</sup> PBS-diluted LIVE/DEAD fixable near-IR dead cell stain kit (#L10119, Invitrogen) and incubated for 20 min at 4 °C. Cells were once again centrifuged for 5 min at 350  $\times g$  (4 °C) and washed a second time with 50  $\mu\text{L}$  cold PBS. Cells were then incubated in 1/5000<sup>e</sup> Hanks' balanced salt solution (HBSS, 14 025 092, Gibco)-diluted MitoSOX Red M36007, Thermo Scientific) for 10 min at 37 °C in the dark. Cells were finally re-suspended in HBSS after a last centrifugation (5 min, 350  $\times g$ ) before the acquisition on a BD FACS Canto. Data were analyzed using FlowJo 10.4 software.

**Enzyme-Linked Immunoassay:** To quantify pro-inflammatory cytokines TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-12p70 secretion in culture supernatants, a sandwich ELISA was performed. For TNF- $\alpha$ , the plate was coated with capture antibody after dilution at 1  $\mu\text{g mL}^{-1}$  (#12-7325-85, eBioscience), in a coating buffer (Na<sub>2</sub>CO<sub>3</sub> 15 mM, NaHCO<sub>3</sub> 35 mM, pH 9.6) followed by an incubation at 4 °C overnight. The plate was then washed thrice (PBS, 0.05% Tween 20 v/v) before the addition of 200  $\mu\text{L}$  per well of blocking buffer

(PBS, 0.05% Tween 20 v/v, 1% bovine serum albumin [BSA] w/v) for 1 h at 37 °C. The plate was again washed thrice as before. Cytokine standard range was made from 3.9  $\text{pg mL}^{-1}$  to 4  $\text{ng mL}^{-1}$  (#14-83211-63, eBioscience) and supernatants were then prepared and diluted in the appropriate culture medium. 50  $\mu\text{L}$  of standard and of each condition were added per well in the coated plates and incubated for 2 h at 37 °C. The plate was then washed thrice and the biotinylated detection antibody was added at 0.5  $\mu\text{g mL}^{-1}$  (#13-7326-85, eBioscience) in blocking buffer (50  $\mu\text{L}$  per well) for 1 h at 37 °C. The wells were once again washed thrice as before. Peroxidase-conjugated streptavidin (HRP Streptavidin) (#016-030-084, Jackson Immuno Research) was diluted at 1/5000 in blocking buffer (in 50  $\mu\text{L}$  per well) and added for 45 min at 37 °C. The wells were then washed twice with wash buffer (PBS, 0.05% Tween 20 v/v), and then twice with MilliQ water. Revelation was conducted by the addition of 75  $\mu\text{L}$  revelation solution (10% 3,3',5,5'-tetramethylbenzidine, 90% substrate buffer, 0.04% H<sub>2</sub>O<sub>2</sub> [Na<sub>2</sub>HPO<sub>4</sub> 70 mM, C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> 30 mM, pH5]) in each well and incubation at RT protected from light. The reaction was then stopped before apparent saturation by addition of 25  $\mu\text{L}$  HCl 1 M. Absorbance values were read at 450 nm using the microplate reader (SP2000, Safas Monaco).

The IL-6, IL-1 $\beta$ , and IL-12p70 quantification was carried out as indicated in the manufacturer's protocol (#DY-406, R&D systems, #432 601, Biolegend, #555 256, BD Biosciences). Briefly, the capture antibody was used at 2  $\mu\text{g mL}^{-1}$ , at a dilution of 200 times or 250 times, the biotinylated detection antibody at 0.75  $\mu\text{g mL}^{-1}$ , at a dilution of 200- or 250-times, with a standard curve made from 15.6  $\text{pg mL}^{-1}$  to 1  $\text{ng mL}^{-1}$  for IL-6 or from 31.3  $\text{pg mL}^{-1}$  to 2  $\text{ng mL}^{-1}$  for IL-1 $\beta$  and IL-12p70, respectively. Cytokine concentrations were determined via interpolation of the standard curves.

**CD40 and MHC-II Expression:** To evaluate the anti-inflammatory activity of PEI treatment on macrophage LPS-induced activation, the expression level of CD40 and of MHC-II (two activation markers whose expression increased under inflammatory conditions) was estimated by flow cytometry. Following RAW264.7 LPS induction and PEI treatments, cells were detached from plate with trypsin-EDTA for 5 min at 37 °C and each well was washed with 50  $\mu\text{L}$  of cold PBS. Duplicate conditions were pooled in microfuge tubes (#MT-0150-R, Biotix) and centrifuged at 350  $\times g$  for 5 min (4 °C). Cell pellets were re-suspended in 1/1000 PBS-diluted LIVE/DEAD fixable near-IR dead cell stain kit (a mortality marker, #L10119, Invitrogen) for 20 min (4 °C). Cells were once centrifuged as before and stained with 50  $\mu\text{L}$  of 1/100 PBS (0.5% BSA, 2 mM EDTA) diluted anti-CD40-PE Vio 770 (#130-116-112, Miltenyi Biotec) antibody, or anti-MHC-II-APC (#116 418, Biolegend). Cells were kept at 4 °C for 10 min and then centrifuged as before and re-suspended in 300  $\mu\text{L}$  in PBS (0.5% BSA, 2 mM EDTA). The cells were then analyzed for Live/Dead staining, CD40 or MHC-II expression using a BD FACS Canto. Fluorescence acquisitions were analyzed using FlowJo 10.4 software.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

## Acknowledgements

E.F., C.D., and C.M. contributed equally to this work. The authors would like to thank Stéphane Bellemin-Laponnaz for the synthesis of L-PEI 22 kDa. This work was supported by the Fondation ARC (Association pour la Recherche sur le Cancer project number ARCPJA32020060002306—Fournel Sylvie) and by the ITI Innovec (IdEx [ANR-10-IDEX-0002] and SFRI [ANR-20-SFRI-0012]).

## Conflict of Interest

The authors declare no conflict of interest.



## Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

## Keywords

anti-inflammatory properties, cationic polymer, immune modulation, lipopolysaccharide, macrophages, polyethylenimine

Received: October 27, 2023

Revised: January 31, 2024

Published online:

- [1] P. Italiani, D. Boraschi, *Front Immunol* **2014**, *5*, 514.
- [2] E. R. Sherwood, T. Toliver-Kinsky, *Best Pract. Res., Clin. Anaesthesiol.* **2004**, *18*, 385.
- [3] G. A. Duque, A. Descoteaux, *Front Immunol* **2014**, *5*, 491.
- [4] E. A. Ross, A. Devitt, J. R. Johnson, *Front Immunol* **2021**, *12*, 708186.
- [5] C. E. Bryant, D. R. Spring, M. Gangloff, N. J. Gay, *Nat. Rev. Microbiol.* **2010**, *8*, 8.
- [6] A. B. Schromm, J. Howe, A. J. Ulmer, K.-H. Wiesmüller, T. Seyberth, G. Jung, M. Rössle, M. H. J. Koch, T. Gutschmann, K. Brandenburg, *J. Biol. Chem.* **2007**, *282*, 11030.
- [7] K. Takeda, O. Takeuchi, S. Akira, *J Endotoxin Res* **2002**, *8*, 459.
- [8] M. Murakami, T. Hirano, *Front Immunol.* **2012**, *3*, 323.
- [9] E. Shacter, S. A. Weitzman, *Oncology* **2002**, *16*, 217.
- [10] J. Kzhyshkowska, A. Gudima, V. Riabov, C. Dollinger, P. Lavalle, N. E. Vrana, *J. Leukocyte Biol.* **2015**, *98*, 953.
- [11] M. Yanez, J. Blanchette, E. Jabbarzadeh, *Curr. Pharm. Des.* **2017**, *23*, 6347.
- [12] B. C. Bhavya, M. Haridas, in *Bioresources and Bioprocess in Biotechnology: Volume 2: Exploring Potential Biomolecules* (Eds: S. Sugathan, N. S. Pradeep, S. Abdulhameed), Springer Singapore, Singapore **2017**, pp. 235–1268.
- [13] H. S. Chiong, Y. K. Yong, Z. Ahmad, M. R. Sulaiman, Z. A. Zakaria, K. H. Yuen, M. N. Hakim, *Int J Nanomedicine* **2013**, *8*, 1245.
- [14] F. Atzeni, L. Gianturco, R. Talotta, V. Varisco, M. C. Ditto, M. Turiel, P. Sarzi-Puttini, *Immunotherapy* **2015**, *7*, 353.
- [15] L. Fugger, L. T. Jensen, J. Rossjohn, *Cell* **2020**, *181*, 63.
- [16] H. E. Vonkeman, M. A. F. J. van de Laar, *Semin. Arthritis Rheum.* **2010**, *39*, 294.
- [17] Y. H. Choi, H. Y. Park, *J Biomed Sci* **2012**, *19*, 31.
- [18] M. S. Kim, H. J. You, M. K. You, N. S. Kim, B. S. Shim, H. M. Kim, *Immunopharmacol Immunotoxicol* **2004**, *26*, 401.
- [19] V. Gribova, L. Petit, L. Kocgozlu, C. Seguin, S. Fournel, A. Kichler, N. E. Vrana, P. Lavalle, *Macromol. Biosci.* **2022**, *22*, e2200043.
- [20] S. Shaunak, S. Thomas, E. Gianasi, A. Godwin, E. Jones, I. Teo, K. Mireskandari, P. Luthert, R. Duncan, S. Patterson, P. Khaw, S. Brocchini, *Nat. Biotechnol.* **2004**, *22*, 977.
- [21] H. Chen, P. Li, Y. Yin, X. Cai, Z. Huang, J. Chen, L. Dong, J. Zhang, *Biomaterials* **2010**, *31*, 8172.
- [22] X. Zhang, D. Mosser, *J Pathol.* **2008**, *214*, 161.
- [23] A. S. Chauhan, P. V. Diwan, N. K. Jain, D. A. Tomalia, *Biomacromolecules* **2009**, *10*, 1195.
- [24] N. A. Raof, D. Rajamani, H.-C. Chu, A. Gurav, J. M. Johnson, F. W. LoGerfo, M. Bhasin, L. Pradhan-Nabzdyk, *BMC Genomics* **2016**, *17*, 20.
- [25] B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos, H. Cheradame, *Bioconj Chem.* **2003**, *14*, 581.
- [26] C. McCartin, J. Blumberger, C. Dussouillez, P. F. de Larrinoa, M. Dontenwill, C. Herold-Mende, P. Lavalle, B. Heurtault, S. Bellemin-Laponnaz, S. Fournel, A. Kichler, *J Funct Biomater* **2022**, *14*, 17.
- [27] R. Hoogenboom, R. M. Paulus, A. Pilotti, U. S. Schubert, *Macromol. Rapid Commun.* **2006**, *27*, 1556.
- [28] L. Tauhardt, K. Kempe, K. Knop, E. Altuntas, M. Jäger, S. Schubert, D. Fischer, U. S. Schubert, *Macromol. Chem. Phys.* **2011**, *212*, 1918.