scientific reports

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Effects of surface-exposed sialic acid and LOS outer core on the ability of native *Neisseria meningitidis* outer membrane vesicles (nOMVs) to induce cytokine expression and pyroptotic pathways in THP-1-derived macrophages

Silvia Caterina Resta[1], Adelfia Talà[2], Antonio Baccante[3], Giovanni Saudino[3], Pasquale Petruccelli[3], Vito Di Cioccio[3], Cecilia Bucci[1] & Pietro Alifano[1]✉

Native outer membrane vesicles (nOMVs) are involved in meningococcal pathogenesis and are used for vaccine production. In this study, macrophages derived from the human monocytic cell line THP-1 were exposed to nOMVs from serogroup B *N. meningitidis* B1940 and derivative mutants B1940 *siaD*(+C), lacking the capsule, and B1940 *cps*, lacking both the capsule and the LOS outer core with sialic acid. Compared with THP-1 cells exposed to B1940 nOMVs, cells exposed to B1940 *cps* nOMVs showed significantly lower mRNA levels of genes encoding chemokines, interleukins, caspases, and gasdermin E (*DFNA5*). Furthermore, Western blot analysis showed a reduction in pro-IL-1β expression and activation of gasdermin E and caspase-4 in THP-1 macrophages treated with B1940 *cps* nOMVs compared to B1940 or B1940 *siaD*(+C) nOMVs. However, secreted pro-IL-1β and IL-1β were detected in the culture medium of THP-1 cells exposed to B1940 *siaD*(+C) nOMVs but not to B1940 or B1940 *cps* nOMVs. These findings provide genetic evidence that surface-exposed sialic acid and LOS outer core may contribute to the ability of meningococcal nOMVs to activate cytokine expression and pyroptotic pathways in THP-1-derived macrophages, providing new information to create safe nOMV-based vaccines.

Keywords *Neisseria meningitides*, Outer membrane vesicles, Lipo-oligosaccharide, Sialic acid, Inflammasome, Pyroptosis

Gram-negative bacteria spontaneously produce and release into the environment native outer membrane vesicles (nOMVs or blebs), nanoparticles with diameters between 50 and 200 nm, composed of a phospholipid bilayer containing outer membrane proteins, lipopolysaccharide/lipooligosaccharide (LPS/LOS), and a cargo of periplasmic proteins[1,2].

nOMVs play an important role in infection by *Neisseria meningitidis*, a bacterium that transiently colonizes the human nasopharynx and is occasionally responsible for meningitis and fulminant sepsis[3]. During invasive disease, *N. meningitidis* releases in the host large amounts of nOMVs. These vesicles are thought to play a role in the progression of infection as a source of LOS endotoxin[4,5], strongly contributing to the elevated endotoxin

[1]Department of Experimental Medicine, University of Salento, Campus Ecotekne-S.P. 6, 73100 Lecce, Italy. [2]Department of Biological and Environmental Sciences and Technologies, University of Salento, Campus Ecotekne-S.P. 6, 73100 Lecce, Italy. [3]Contraria Biotech, 53100 Siena, Italy. ✉email: pietro.alifano@unisalento.it

levels associated with fatal septic infection[6]. nOMVs have been detected in both cerebrospinal fluid and sera of patients with lethal meningococcal endotoxemia[4,7].

Furthermore, Neisserial nOMVs have been shown to interact with coinhibitory receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)[8] through opacity-associated (Opa) proteins, phase- and antigenically variable outer membrane proteins[9,10]. The interaction suppresses CD4+T cell function[8]. Moreover, more recently, evidence has been provided that *Neisseria gonorrhoeae* nOMVs target the major outer membrane porin PorB to mitochondria of macrophages, causing apoptotic cell death[11]. *N. meningitidis* PorB is also translocated to mitochondria, resulting, however, in protection of host cells from apoptosis[12–14], reflecting the different lifestyle of the gonococcus and meningococcus. Moreover, PorB is able to trigger Toll-like receptor 2 (TLR2) signaling[15,16].

nOMVs have been extensively studied and implemented as vaccines against serogroup B *N. meningitidis*[17], given the ineffectiveness of vaccines based on capsular polysaccharide for serogroup B meningococci[18]. Surface antigens presented in the context of natural nOMVs have been recently identified as vaccine candidates also for other bacteria[19,20]. For meningococci, a limitation is the large variability of surface antigens between strains due to antigenic variation. Consequently, the immune response elicited by OMV-based vaccines is strain-specific[17]. For instance, the MeNZB vaccine has been proven to be very effective in preventing disease caused by specific meningococcal strains[17]. This limitation has been overcome in vaccines currently in use, such as Bexsero (GSK)[21], which contains detergent-extracted outer membrane vesicles (dOMVs) from serogroup B meningococcal strain NZ98/54 to reduce toxicity associated with the meningococcal LOS, and three recombinant proteins conserved across *N. meningitidis*: Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp), and Neisseria Adhesion A (NadA)[22].

Chemical detoxification of meningococcal nOMVs by detergent treatment is a methodology currently used to create safe vaccines because of the reactogenicity determined by the extremely potent hexa-acylated LOS. Indeed, another limitation of nOMV-based vaccine manufacturing is their notable reactogenicity, which, if on the one hand guarantees the adjuvant action of the vaccine, on the other can cause even serious side effects. However, chemical detoxification of nOMVs does not allow antigens to be exposed in their native conformation and orientation, removes several protective antigens from the outer membrane, such as the loosely attached fHbp[23,24], and reduces the long-term stability of the vaccine[25]. Alternatively, nOMVs can be detoxified by genetic modifications[26,27], and can also be harvested from bacteria genetically modified to express heterologous proteins in order to elicit a broader antibody response[28]. These genetically detoxified and modified nOMVs (called mOMVs) represent the new frontier of OMV-based vaccines.

While the effects of LOS hexa/penta-acylation have been extensively studied in the past, the effects of surface-exposed sialic acids on OMVs reactivity have never been investigated. However, sialic acid plays a critical role in meningococcal virulence. Of the five meningococcal serogroups responsible for the epidemics, four (B, C, Y, and W-135) carry sialic acid in their capsular polysaccharides, which strongly contribute to resistance to phagocytosis[29].

Sialic acid is also found as a modification of the meningococcal LOS (instead of the terminal galactose residue) in serogroups with sialic acid-containing capsules[30]. LOS sialylation is required for resistance to complement-mediated killing via the alternative pathway[31–37]. Sialic acid-containing capsule and LOS sialylation are also important for meningococcal survival within infected cells[38], mediating the interaction of bacteria with host cell microtubules during cell infection[39], and protecting the bacteria against cationic antimicrobial peptides (CAMP)[38,40]. In a mouse model of meningococcal meningitis, surface-exposed sialic acids give bacteria a high tropism toward specific brain regions[41]. There is also evidence that sialylated LOS can lead to increased meningococcal susceptibility to phagocytic uptake through an interaction of the bacteria with sialic acid-binding immunoglobulin-like lectins 1 (Siglec-1 or sialoadhesin) and 5 (Siglec-5) expressed on the surface of macrophages[42]. Furthermore, it has been recently shown that the initial interaction (tethering) of sialylated meningococci with Siglec-5 is followed by a tighter interaction between NadA and Siglec-5 (or Siglec-14), leading to cell invasion[43]. Meningococcal LOS is also subject to phosphoethanolaminylation of lipid A, and there is evidence that reduced phosphoethanolaminylation and reduced sialylation would lessen the pathogenicity of the carrier isolates[35].

In this study, we have investigated the expression patterns of pro-inflammatory cytokines and caspases in human THP-1-derived macrophages exposed to *N. meningitidis* nOMVs derived from strains with or without sialic acid capsule or LOS sialylation / outer core extension. Chemokines with roles in immune cell recruitment and inflammation, pro-inflammatory interleukins involved in early and late responses, inflammatory caspases, inflammasomes, TLRs, as well as genes involved in nOMVs endocytosis and cytoskeletal dynamics were evaluated.

Results

Chemokine gene expression in THP-1 cells treated with Neisserial nOMVs

Transcript levels of genes coding for C–C motif chemokine ligand 2 (CCL2), C–C motif chemokine ligand 3 (CCL3), C–C motif chemokine ligand 5 (CCL5), C-X-C motif chemokine ligand 10 (CXCL10), intracellular adhesion molecule 1 (ICAM-1), and pro-platelet basic protein (PPBP) were determined after 24 h-exposure of differentiated THP-1 macrophages to nOMVs from wild-type and mutant *Neisseria* spp. strains by real-time RT-PCR using customized 96-well PCR plates (Fig. S1 and File S1). These chemokines were selected because they play important roles in immune cell recruitment. Specifically, CCL2 recruits monocytes[44,45], CCL3 is known to recruit T cells, B cells, and NK cells in addition to monocytes[46–50], CCL5 recruits various cell types and induces nuclear factor kappa-light-chain-enhancer of activated B cells 1 (NF-κB) signaling after binding to the CCR5 receptor[51], CXCL10 is important for recruiting Th1 cells[52,53] while PPBP attracts neutrophils to areas of inflammation[54].

Preliminarily, we evaluated the effect of nOMVs produced by wild-type strains of different *Neisseria* spp. to assess possible differences in inflammatory responses elicited by pathogenic and apathogenic *Neisseria* nOMVs. The results of this analysis demonstrated an increase (> fourfold) in the amount of *CCL2*, *CCL3*, *CXCL10,* and *ICAM-1* mRNA levels in THP-1 macrophages exposed to nOMVs from wild-type *Neisseria* spp. compared to unexposed cells (Fig. S2A). In particular, a significant increase of *CCL3* transcript levels was observed in nOMVs-exposed cells compared to untreated control (p value ranging from < 0.05 to < 0.01), and a significant increase of ICAM1 mRNA levels in cells exposed to B1940 nOMVs and *N. lactamica* nOMVs compared to unexposed cells (p value < 0.05) (Fig. S2A). The increase in mRNA levels of *CXCL10*, a chemokine gene induced by gamma interferon, was particularly accentuated, with values up to approximately one thousand times higher than those of basal levels in cells treated with nOMVs from serogroup B *N. meningitidis* B1940, and more than six hundred times higher with the other strains. Also notable was the increase in *CCL2* mRNA levels, with levels more than one hundred times higher than baseline in cells treated with nOMV from most wild-type strains (Fig. S2A). No significant differences in chemokines transcript levels were observed between cells exposed to pathogenic and apathogenic Neisserial nOMVs, nor between macrophages exposed to nOMV from the two *N. meningitidis* strains tested, although a slight non-significant reduction in *CXCL10* levels could be observed in cells exposed to nOMVs from strain 93/4286 compared to those exposed to nOMVs from strain B1940 (Fig. S2B).

The chemokine gene expression profile was then determined in THP-1 macrophages exposed to nOMVs from *N. meningitidis* B1940-derivative mutants B1940 *siaD*(+C), lacking the capsule, and B1940 *cps* lacking both the capsule and the LOS outer core with sialic acid (Fig. 1A, B). Primarily, we looked at the presence of capsular polysaccharides on the *N. meningitidis* strains and their nOMVs (Fig. S3A, B). Slot blot assay and immunofluorescence analysis confirmed the absence of the capsular polysaccharide in B1940 *cps* and B1940 *siaD*(+C) strains (Fig. S3A, B). Conversely, the capsular polysaccharide was detected both in the B1940 strain and in its nOMVs, indicating that these vesicles can carry polysaccharides from the capsule (Fig. S3A). The real-time RT-PCR did not reveal differences in *CCL2*, *CCL3*, *CCL5*, *CXCL10, ICAM-1* and *PPBP* mRNA levels between THP-1 macrophages exposed to nOMVs from B1940 and B1940 *siaD*(+C) applying the fourfold threshold (Fig. 1B). Nevertheless, mRNA levels of *CCL3* and *CXCL10* in macrophages treated with nOMVs derived from B1940 *cps* showed a tendency toward lower transcription levels which resulted significant with the ANOVA test (p values ranging from < 0.05 to < 0.01) compared to macrophages treated with nOMVs derived from the wild-type B1940 strain (Fig. 1B), suggesting a possible involvement of LOS sialylation/outer core extension in the activation of CCL3 and CXCL10 chemokines.

Interleukin gene expression in THP-1 cells treated with Neisserial nOMVs

Transcript levels of genes coding for the pro-inflammatory cytokines interleukin 18 (IL-18), interleukin 1 alpha (IL-1A), interleukin 1 beta (IL-1B), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 12 alpha (IL-12A), interleukin 23 alpha (IL-23A), NF-κB1, tumor necrosis factor alpha (TNF or TNF-α), and the regulatory, anti-inflammatory interleukin 10 (IL-10) were determined after 24 h-exposure of differentiated THP-1 macrophages to nOMVs from wild-type and mutant *Neisseria* spp. strains by real-time RT-PCR as described above (Fig. S2C, D).

Of note, IL-1β precursor is cleaved by caspase 1 to form mature IL-1β, a critical cytokine for the induction of the acute-phase response and fever[55]; IL-6 is a well-known acute-phase pro-inflammatory cytokine with a pleiotropic effect on inflammation, immune response, and hematopoiesis[56]; while IL-12 and IL-23, two cytokines belonging to the IL-12 family, play opposite roles in the polarization of the immune response toward T-helper type 1 (Th1) or T-helper type 17 (Th17) response, respectively[57]. IL-23-stimulated Th17 cells produce IL-17, a pro-inflammatory cytokine that enhances T cell priming and stimulates the production of other proinflammatory molecules such as IL-1, IL-6, TNF, NOS-2, and chemokines[57].

The results of real-time RT-PCR demonstrated an increase (> fourfold) in the amount of *IL-10*, *IL-1A*, *IL-1B, IL-23A, IL-6, IL-8,* and *NF-κB1* mRNA levels in THP-1 macrophages exposed to nOMVs from wild-type *Neisseria* spp. compared to unexposed cells (Fig. S2C). This increase was particularly pronounced for *IL-1A*, *IL-1B*, *IL-23A*, *IL-6*, *IL-8* mRNAs coding for pro-inflammatory cytokines and much smaller for *IL-10* mRNAs coding for the regulatory cytokine IL-10. In particular, the increase in mRNA levels of *IL-6* was particularly accentuated, with values up to approximately 2.5 thousand times higher than those of basal levels in cells treated with nOMVs from serogroup C *N. meningitidis* 93/4286, and more than one thousand times higher with the other strains (Fig. S2C). The cells exposed to *N. meningitidis* 93/4286 nOMVs also showed significantly higher levels of *IL-1A* mRNA levels, (p value < 0.01), compared to cells exposed to *N. meningitidis* B1940. Moreover, the mRNA for this interleukin was slightly but significantly increased in cells exposed to *N. cinerea* nOMVs compared to *N. meningitidis* B1940 nOMVs (p value < 0.05) (Fig. S2D). We also noted that mRNA levels of *IL-18*, encoding the potent pro-inflammatory cytokine IL-18, did not increase after nOMV stimulation. This finding may be due to the earlier and more transient pattern of expression of IL-18 in stimulated macrophages and/or to the up-regulation of the gene encoding IL-10, which exerts an inhibitory effect on IL-18 mRNA accumulation[58]. In fact, it has been shown that stimulation with 1 μg/mL of LPS is able to increase *IL-18* transcript levels in peripheral blood mononuclear cells (PBMCs) only in the first 6 h after stimulation[58].

The interleukin gene expression profile was then determined in THP-1 macrophages exposed to nOMVs from the *N. meningitidis* B1940 derivative mutants B1940 *siaD*(+C) and B1940 *cps* (Fig. 2A, B). No significant differences were observed when *IL-10*, *IL-1A*, and *IL-8* mRNA levels were compared in THP-1 macrophages exposed to nOMVs from B1940 and B1940 *siaD*(+C). In contrast, *IL-1B* (p value < 0.05), *IL-6* (p value < 0.05) and *IL-23* (p value < 0.001) mRNA levels were decreased in THP-1 macrophages exposed to nOMVs from B1940 *siaD*(+C) compared to nOMVs from B1940, indicating a detectable contribution of the sialic acid capsular polysaccharide on the induction of these interleukins (Fig. 2B). *IL-12A*, *IL-1B, IL-23A* and *IL-6,* mRNA levels in macrophages treated with nOMVs from B1940 *cps* were significantly lower than those in macrophages treated

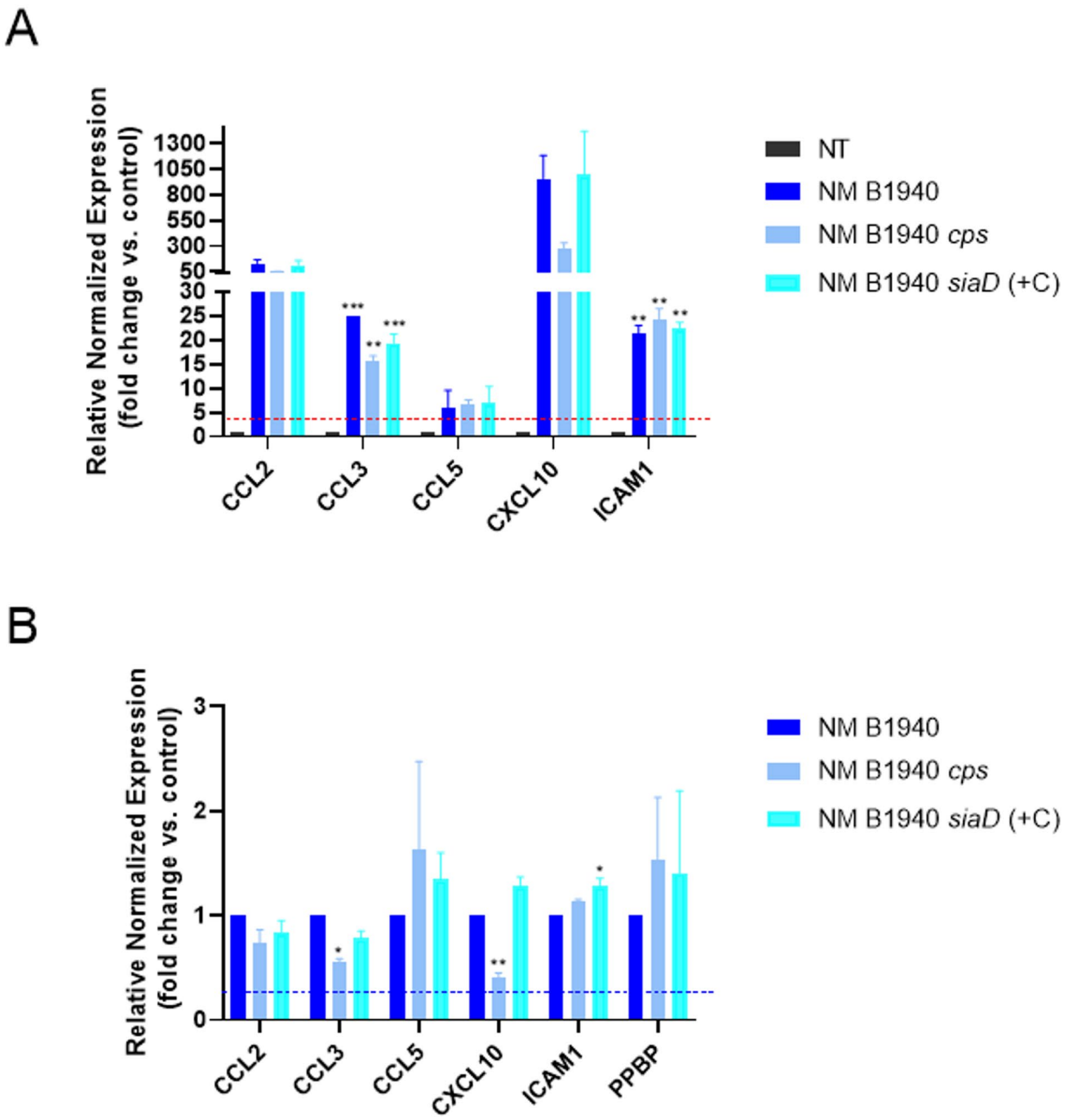

Fig. 1. Expression profile of genes coding for chemokines in THP-1 macrophages treated with *Neisseria* spp. nOMVs (10 µg/mL total protein). THP-1 macrophages were treated with nOMVs from serogroup B (NM B1940) meningococci or NM B1940 *siaD*(+C) or NM *cps* mutant strains. RT-qPCR normalized mRNA levels of genes coding for chemokines in nOMVs treated-THP-1 macrophages were compared either to the expression level of non-treated cells (NT) (**A**) or to expression levels of NM B1940 nOMVs treated macrophages (**B**). Red hashed lines indicate the cutoff of > fourfold applied for up-regulated genes. Values are the fold changes ± SEM of two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

with nOMVs from the wild-type strain B1940 (p values, ranging from < 0.05 to < 0.001, are shown in Fig. 2B). Of note, *IL-23A* mRNA transcript levels were significantly decreased to about the same extent in macrophages treated with nOMVs from B1940 *siaD*(+C) or B1940 *cps* suggesting an involvement of the sialic acid-containing capsule in the induction of IL-23, while levels of *IL-12A* (p value < 0.05), *IL-1B* (p value < 0.01) and *IL-6* ($p < 0.05$) mRNA were significantly lower in B1940 *cps* derived nOMVs treated cells compared to cells exposed to nOMVs derived from the encapsulated strain indicating an involvement of LOS outer core (Fig. 2B).

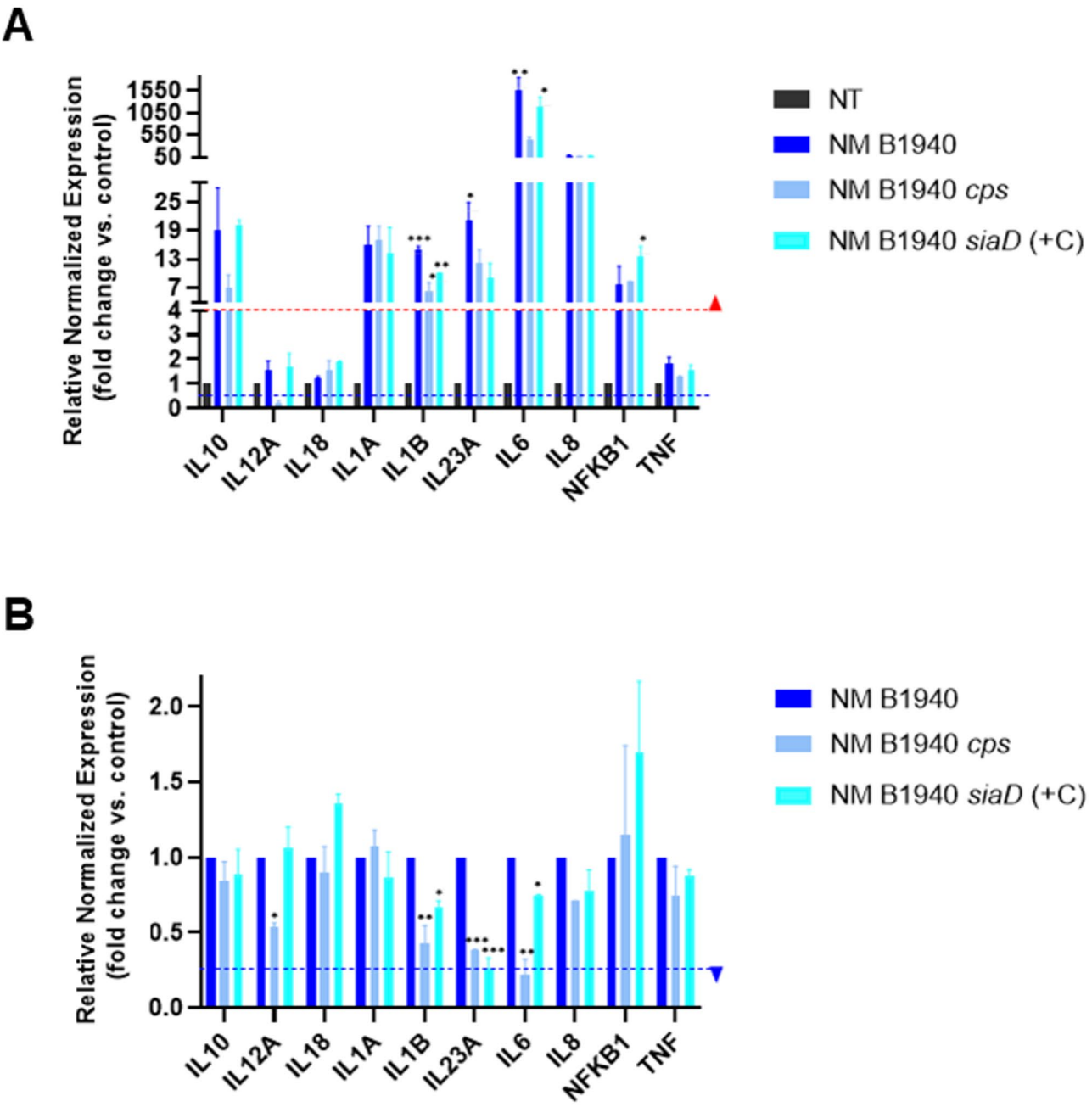

Fig. 2. Expression profile of genes coding for interleukins in THP-1 macrophages treated with *Neisseria* spp. nOMVs (10 µg/mL total protein). THP-1 macrophages were treated with nOMVs from serogroup B (NM B1940) meningococci or NM B1940 *siaD*(+C) or NM B1940 *cps* mutant strains. RT-qPCR normalized mRNA levels of genes coding for interleukins in nOMVs treated-THP-1 macrophages were compared either to the expression level of non-treated cells (NT) (**A**) or to expression levels of NM B1940 nOMVs treated macrophages (**B**). Red hashed lines indicated the cutoff of > fourfold applied for up-regulated genes. Blue hashed lines indicate the cutoff of < fourfold applied for down-regulated genes. Values are the fold changes ± SEM of two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Caspase gene expression in THP-1 cells treated with Neisserial nOMVs

Because pro-inflammatory interleukins such as IL-1β and IL-18 are transcriptionally induced during inflammasome priming, the first step of activation of the inflammasome and, consequently, caspase-1 activation[59], mRNA levels of genes coding for caspases were then evaluated using the same approach. In particular, we analyzed transcript levels of pro-apoptotic caspases, which are engaged in extrinsic (receptor-mediated) and intrinsic (mitochondrial) pathways of apoptosis, including the initiator caspase 8 (CASP8) and caspase 9 (CASP9), and the executor caspase 3 (CASP3) and caspase 7 (CASP7)[60]. We also examined mRNA levels of poly(ADP-ribose) polymerase-1 (*PARP1*), and inflammatory caspase 1 (CASP1), caspase 4 (CASP4),

and caspase 5 (CASP5), which are involved in cytokine maturation and pyroptosis[60]. CASP4 and CASP5 mediate the one-step non-canonical inflammasome pathway in human monocytes after LPS/LOS stimulation[61]. In particular, CASP4 is directly activated by cytosolic LPS/LOS released by internalized bacteria (Viganò et al., 2015), as shown in cell lines infected with *N. meningitidis*[62]. Moreover, nOMV-associated LPS/LOS can also activate the inflammatory response through cytosolic localization of LPS/LOS and non-canonical inflammasome activation[63]. The transcript levels of other genes coding for proteins involved in pyroptosis were also evaluated: gasdermin E (DFNA5), gasdermin D (GSDMD), NLR family pyrin domain containing 1 (NRLP1), NLR family pyrin domain containing 3 (NRLP3), and nucleotide-binding oligomerization domain 1 (NOD1).

We observed an increase (> fourfold) in the amount of mRNA coding for all inflammatory caspases examined (CASP1, CASP4, CASP5) and gasdermin E (DFNA5) in THP-1 macrophages exposed to nOMVs from wild-type *Neisseria* spp. compared to unexposed cells (Fig. S2E). In particular, the increases in mRNA levels of *CASP4* were significant with all *Neisseria* nOMV tested. The increase in *CASP5* transcript levels was particularly pronounced, with values up to approximately 30-fold higher than those of basal levels in cells treated with nOMVs from *N. cinerea*, and approximately 20-fold higher with the nOMVs from the other *Neisseria* spp. (Fig. S2E). Up-regulation of *CASP5* mRNA is consistent with the finding that *CASP5* mRNA levels are modulated by LPS and interferon-gamma[64]. Transcript levels of these genes were instead similar between cells exposed to nOMVs derived from the two *N. meningitidis* strains tested, as well as macrophages exposed to vesicles from pathogenic and non-pathogenic *Neisseria* spp. (Fig. S2F). However, even if below the threshold applied, a significant reduction in *CASP9* levels (p value < 0.01) was observed in cells treated with nOMVs from *N. meningitidis* 93/4286 compared to cells exposed to the vesicles derived from *N. meningitidis* B1940, together with a slight decrease in *NLRP1* levels in cells exposed to nOMVs from *N. meningitidis* 93/4286 or *N. cinerea* (p value < 0.05) compared to those exposed to B1940 nOMVs (Fig. S2F).

The caspase gene expression profile was then examined in THP-1 macrophages exposed to nOMVs from the *N. meningitidis* B1940 derivative mutants B1940 *siaD*(+C), and B1940 *cps* (Fig. 3A, B). Consistent with our observation of the effects of wild-type *Neisseria* nOMVs, treatment of THP-1 cells with either B1940 *cps* or B1940 *siaD*(+C) increased *CASP5, CASP4* and *CASP1* transcript levels compared to untreated cells (Fig. 3A). *DFNA5* and *NLRP1* mRNA levels in THP-1 macrophages treated with nOMVs from B1940 *cps* were significantly lower than those in macrophages treated with nOMVs from the wild-type strain B1940 (p values ranging from < 0.05 to < 0.01) (Fig. 3B). In contrast, it can be noted a tendency toward higher transcript levels of *CASP8* and *NOD1* in both B1940 *cps* and B1940 *siaD*(+C) nOMVs-treated macrophages compared to B1940 nOMVs-exposed cells, and a trend toward lower levels of *CASP1*, *CASP3* and *GSDMD* in cells treated with B1940 *cps* nOMVs compared to cells exposed to B1940 *siaD*(+C) nOMVs (Fig. 3B). This result was consistent with an involvement of LOS sialylation and/or LOS outer core (and, at much lesser extent, sialic acid encapsulation) in an induction of the inflammatory caspase gene expression.

Expression of TLRs and genes involved in the endocytic pathway in THP-1 cells treated with Neisserial nOMVs

We analyzed transcript levels of genes coding for Toll-like receptors (TLR2, TLR3, and TLR4) and proteins involved in the endocytic pathway (RAB5A, RAB7A), as nOMVs are internalized through endocytosis[65]. Furthermore, we analyzed transcript levels of genes involved in macrophage response to inflammation: superoxide dismutase 1 (SOD1), transforming growth factor beta 1 (TGFB1), and vascular endothelial growth factor A (VEGFA). Finally, the vinculin (VCL) transcript level was evaluated since nOMVs may influence macrophage cytoskeletal dynamics[66].

Using the same threshold (> fourfold for up-regulated genes, and < fourfold for down-regulated genes), we could observe a significant increase in the amount of mRNA coding for TLR3, and an increase in the amount of mRNA coding for VEGFA in THP-1 macrophages exposed to nOMVs from wild-type *Neisseria* spp. compared to unexposed cells. On the contrary, the transcript levels of vinculin (*VCL)* were significantly lower in cells exposed to *Neisseria* spp.-derived nOMVs compared to unexposed cells (Fig. S2G). No differences in transcript levels of these genes were observed in cells exposed to nOMVs from pathogenic or apathogenic *Neisseria* spp., nor between cells exposed to nOMVs from serogroup C or serogroup B meningococci (Fig. S2H). On the other hand, it could be noted a trend toward a lower transcript level of *SOD1* mRNA in *N. meningitidis* 93/4286 nOMVs-exposed macrophages compared to *N. meningitidis* B1940 nOMVs-treated ones (p value < 0.01) (Fig. S2H).

Analysis of THP-1 macrophages exposed to nOMV from B1940 *siaD*(+C) and B1940 *cps* (Fig. 4A and B) showed that the mRNA levels of *SOD1* (p value < 0.05), *TLR3* (p value < 0.01), and *TLR4* in THP-1 macrophages treated with nOMV from B1940 *cps* showed a tendency toward reduced transcription compared to macrophages treated with nOMV from the wild-type B1940 strain, while they did not change in macrophages treated with nOMV from B1940 *siaD*(+C) (Fig. 4B). However, *RAB7A* and *VEGFA* mRNA levels resulted slightly but significantly higher in cells exposed to B1940 *siaD*(+C) nOMVs compared to cells exposed to B1940 nOMVs (p values ranging from < 0.05 to < 0.01) although they did not reach the applied > fourfold threshold (Fig. 4B).

Western blot analysis of caspases, gasdermins, and cytokines involved in pyroptotic pathway in THP-1 cells treated with Neisserial nOMVs

Since in THP-1 cells exposed to Neisserial nOMVs, we found transcriptional activation of genes encoding inflammatory mediators, many of which are involved in pyroptosis pathways (Figs. S2E and 3A), and since nOMVs from some Gram-negative bacteria are sufficient to induce pyroptosis pathways[67], we evaluated by Western blot the activation of inflammatory caspases, gasdermins, pro-inflammatory and cell death mediators in THP-1 macrophages exposed to nOMVs.

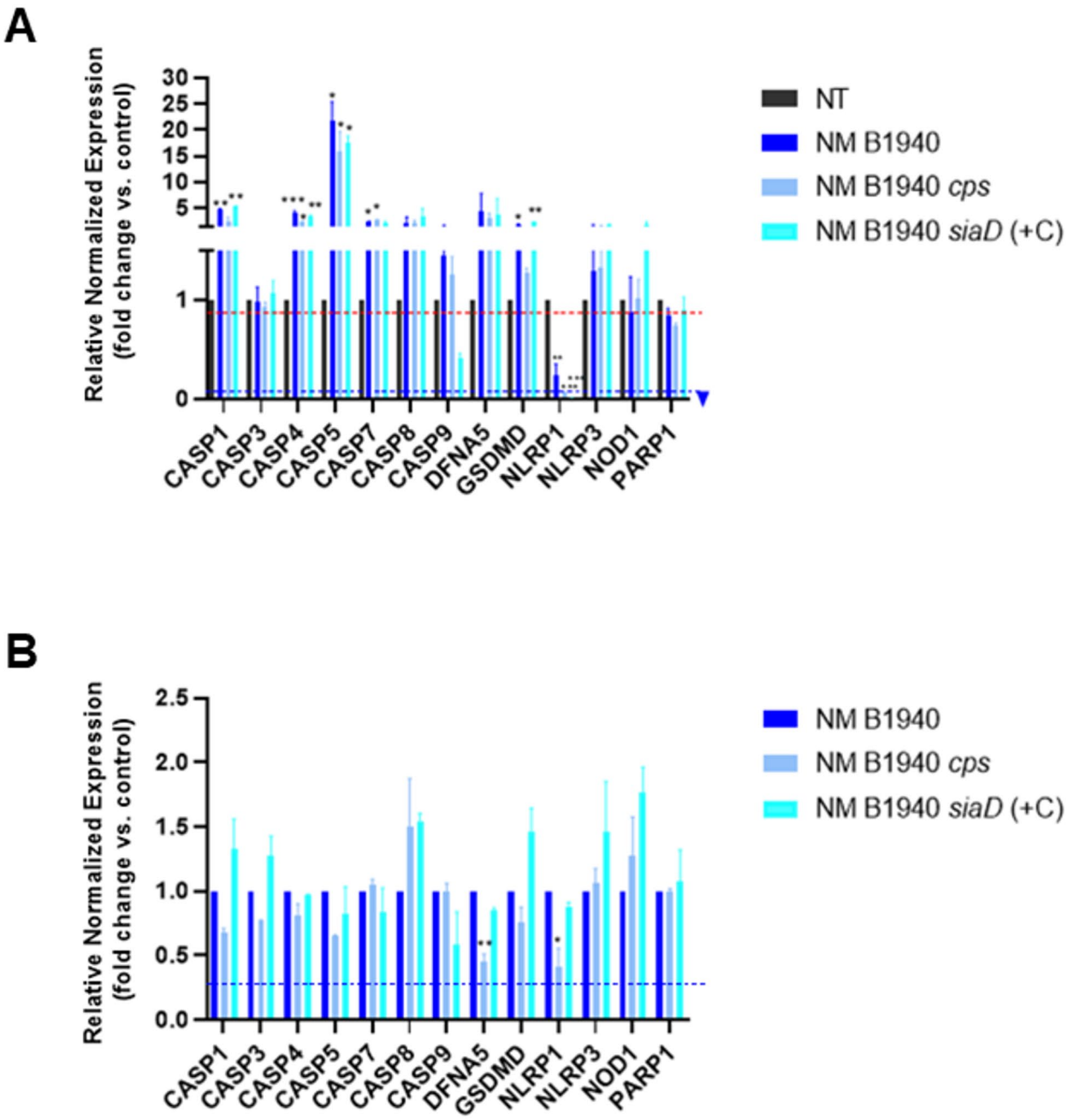

Fig. 3. Expression profile of genes involved in cell death pathways in THP-1 macrophages treated with *Neisseria* spp. nOMVs (10 µg/mL total protein). THP-1 macrophages were treated with nOMVs from serogroup B (NM B1940) meningococci or NM B1940 *siaD*(+C) or NM B1940 *cps* mutant strains. RT-qPCR normalized mRNA levels of genes involved in cell death pathways in nOMVs treated-THP-1 macrophages were compared either to the expression level of non-treated cells (NT) (**A**) or to expression levels of NM B1940 nOMVs treated macrophages (**B**). Red hashed lines indicated the cutoff of > fourfold applied for up-regulated genes. Blue hashed lines indicate the cutoff of < fourfold applied for down-regulated genes. Values are the fold changes ± SEM of two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Caspase-4 was activated upon exposure to *N. meningitidis* nOMVs (Fig. 5A, B). Important differences were observed when cells were treated with nOMVs from *N. meningitidis* B1940 and its derivative mutants. Caspase-4 activation was significantly lower in THP-1 macrophages exposed to B1940 *siaD* (+C) nOMVs and, in particular, to B1940 *cps* nOMVs, compared to the activation observed in cells exposed to B1940 nOMVs. (Fig. 5A, B). However, gasdermin D (GSDMD), cleaved by caspase-4 in the non-canonical pyroptosis pathway[61], remained inactive upon Neisserial nOMVs exposure (Figs. S4 and 5A). In fact, the active N-terminal GSDMD fragment at 31 kDa was not detected in any of the Neisserial nOMVs-exposed cells, probably because the low activation level of caspase-4 was not sufficient to activate this gasdermin.

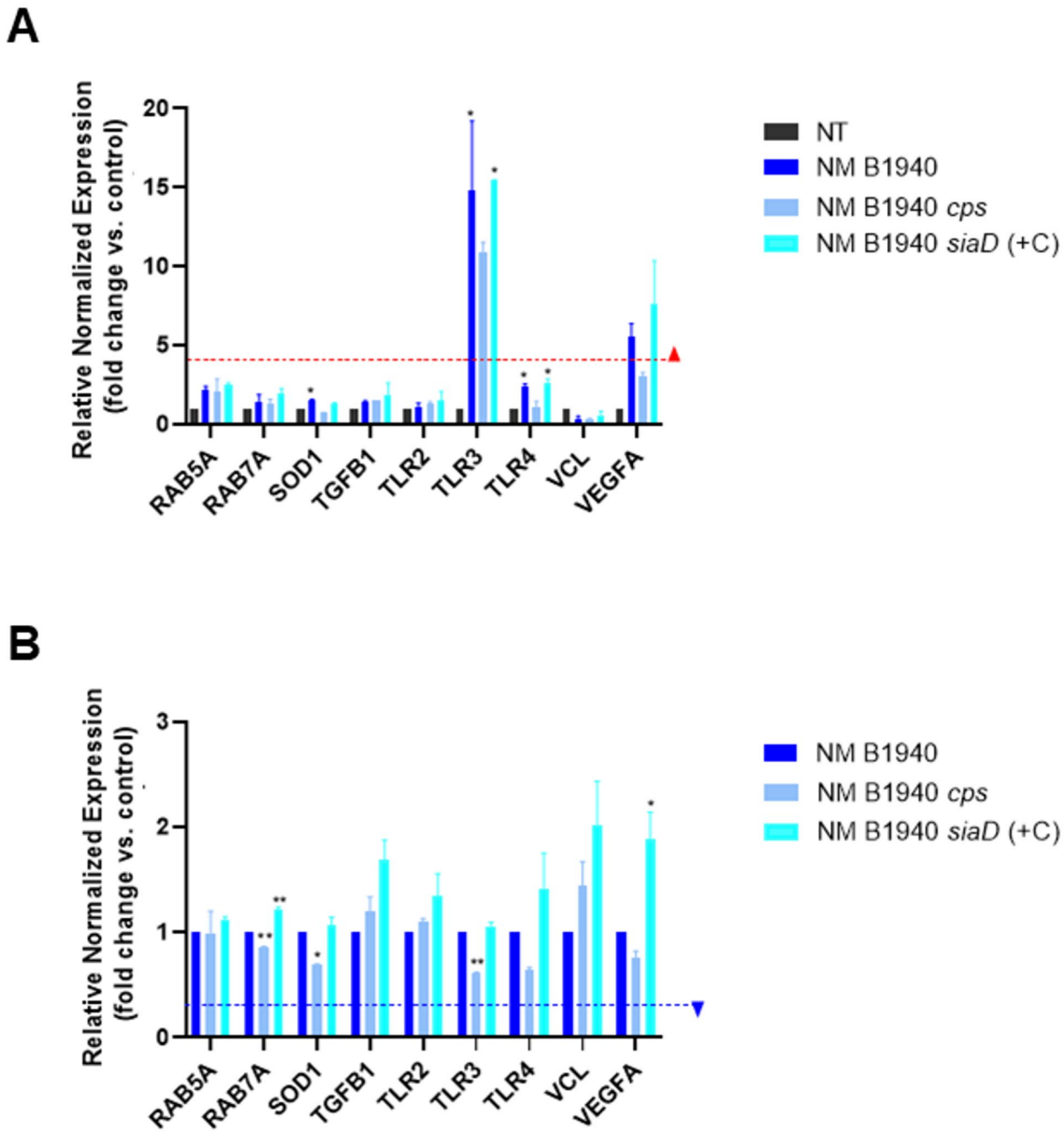

Fig. 4. Expression profile of genes coding for TLRs and genes involved in the endocytic pathway in THP-1 macrophages treated with *Neisseria* spp. nOMVs (10 µg/mL total protein). THP-1 macrophages were treated with nOMVs from serogroup B (NM B1940) meningococci or NM B1940 *siaD*(+C) or NM B1940 *cps* mutant strains. RT-qPCR normalized mRNA levels of genes coding for TLRs and genes involved in the endocytic pathway in nOMVs treated-THP-1 macrophages were compared either to the expression level of non-treated cells (NT) (**A**) or to expression levels of NM B1940 nOMVs-treated macrophages (**B**). Red hashed lines indicated the cutoff of > fourfold applied for up-regulated genes. Values are the fold changes ± SEM of two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Conversely, caspase-3 activation, which is involved in both apoptosis and pyroptosis pathways[68], was not detected by Western blot analysis upon nOMVs stimulation in THP-1 macrophages (Figs. S4 and 5A), in contrast to what was observed using the same antibody in other cell types infected with *N. meningitidis* B1940[62] and in THP-1 infected with *N. gonorrhoeae*[69]. However, the active N-terminal fragment of gasdermin E (GSDME), which can be produced by caspase-3 activity[68], was detected in THP-1 macrophages treated with nOMVs derived from all wild-type *Neisseria* strains (Fig. S4A–C). Of note, in THP-1 cells treated with nOMVs derived from B1940 *cps*, the pore-forming fragment was barely detectable (Fig. 5A, C). This result indicates that GSDME-mediated pyroptosis is strongly influenced by LOS outer core and/or sialylation.

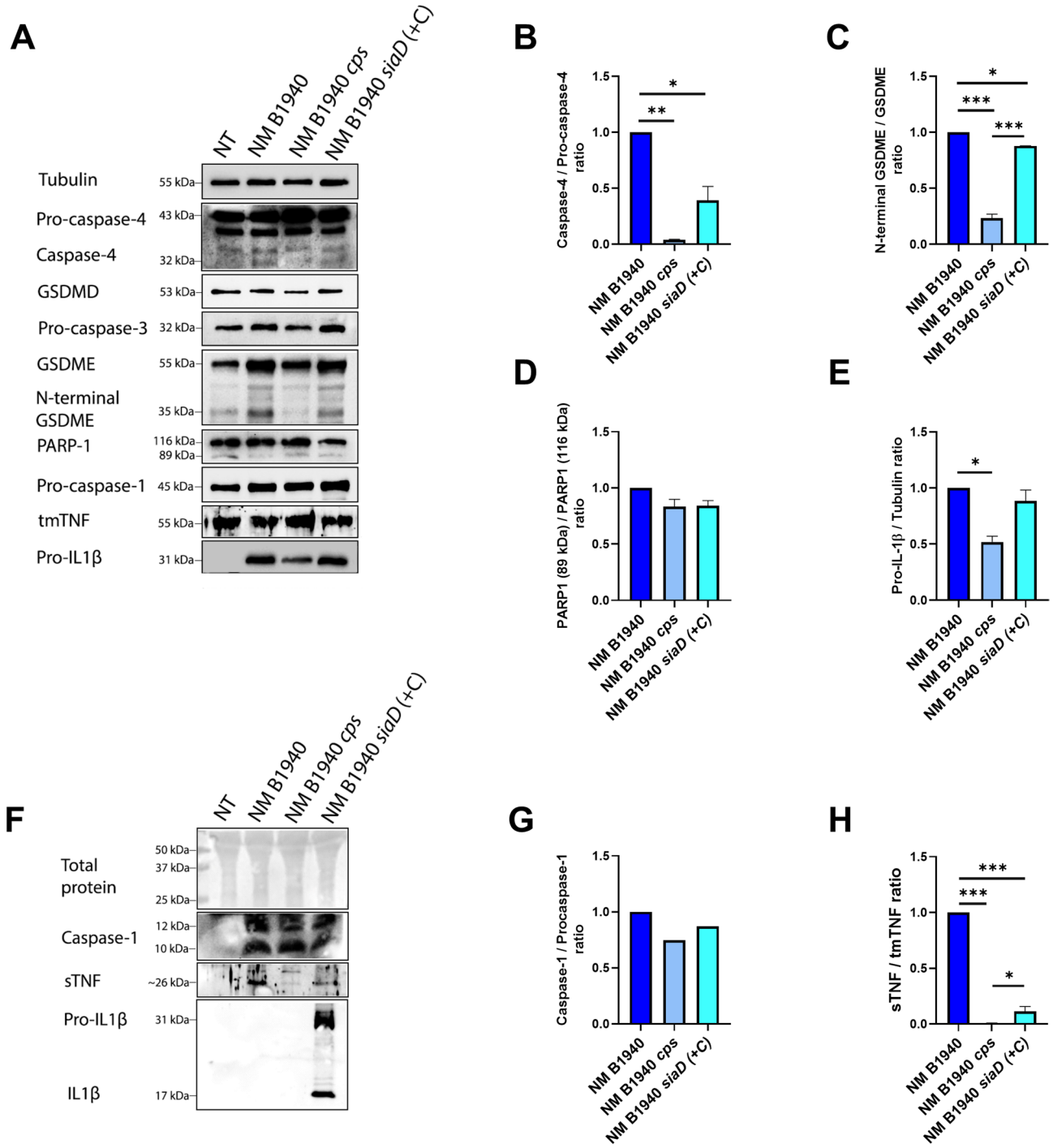

Fig. 5. Effect of capsule and LOS sialylation in nOMVs from *Neisseria meningitidis* on pyroptosis activation. THP-1 macrophages were treated for 24 h with nOMVs (10 µg/mL in protein content) derived from *N. meningitidis* B1940 strain, *N. meningitidis* unencapsulated strain B1940 *siaD* (+C) or *Neisseria meningitidis* unencapsulated, with LOS outer core truncation and lacking LOS sialylation (B1940 *cps*). Cell lysates (**A**) or proteins secreted in the cell culture medium (**F**) were analyzed by immunoblotting using antibodies against caspase-1, IL-1β, caspase-3, PARP1, gasdermin E (GSDME), TNF-α, and gasdermin D (GSDMD). Loading controls were performed using an antibody against tubulin for cell lysates and Ponceau staining of total proteins for secreted proteins. Imagines were quantified by densitometric analysis, and results were reported as fold changes of values obtained with THP-1 macrophages treated with *N. meningitidis* B1940 *cps* or B1940 *siaD*(+C) compared to macrophages treated with nOMVs from the B1940 wild-type strain (**B**–**E**, **G**–**H**). The levels of cleaved forms of caspase-1, PARP1, sTNF-α, caspase-4, and GSDME were normalized to the levels of the respective unprocessed forms, while the levels of pro-IL-1β were normalized to the levels of tubulin. Values are the means of two independent experiments ± SEM $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Original uncropped Western blots are shown in Figs. S6 and S7.

Given the activation of the GSDME-mediated pyroptosis pathway elicited by Neisserial nOMVs, we sought to determine whether other forms of cell death were involved. In particular, we analyzed the activation of apoptosis, a form of cell death normally inhibited by the meningococcus[14], and also wondered whether meningococcal nOMVs were able to inhibit this pathway. PARP1 activation, a hallmark of apoptosis[70], was significantly inhibited by all the wild-type *Neisseria* spp. nOMVs tested compared to untreated cells (Fig. S4A, D), without differences between pathogenic and non-pathogenic *Neisseria* nOMVs (Fig. S4A, E), suggesting that these vesicles retain the ability of bacteria to inhibit apoptosis by translocating PorB to mitochondria[12–14]. On the other hand, only a trend toward a slight reduction in the cleaved form of PARP1 was observed in THP-1 macrophages exposed to B1940 *siaD*(+C) or B1940 *cps* nOMVs compared to cells treated with B1940 nOMVs (Fig. 5A, D).

Western blot analysis did not reveal caspase-1 active forms in THP-1 macrophages in response to nOMVs treatment. However, THP-1 cells have been shown to secrete active caspase-1 when exposed to LPS[71], thus we searched for caspase-1 active forms in the cell culture media. Indeed, we found two caspase-1 forms released from the cells. In particular, we detected the mature active p10 subunit[71] and the p12 subunit, the latter reported as an alternative caspase-1 form unable to cleave pro-IL-1β[72] (Figs. S4H, I and 5F). We observed a significant reduction in p10 caspase-1 secretion when cells were exposed to nOMVs derived from *N. cinerea* compared to cells exposed to nOMVs derived from *N. meningitidis* 93/4286 strain (Fig. S4H, L). No effects of surface-exposed sialic acid or LOS outer core truncation were found. Indeed, levels of active p10 caspase-1 fragment were similar in macrophages treated with B1940 nOMVs, B1940 *siaD*(+C) nOMVs, and B1940 *cps* nOMVs (Fig. 5F, G).

Because the p10 form of caspase-1 that we detected in the culture medium of THP-1 cells treated with Neisserial nOMV is part of the active p20/p10 protease able to activate pro-IL-1β[73], we analyzed the expression and activation of this interleukin in THP-1 cells exposed to Neisserial nOMV. Pro-IL-1β was detected in THP-1 cells only after nOMVs exposure (Figs. S4A, F, G and 5A, E). Notably, we observed a reduction in pro-IL-1β expression in THP-1 macrophages treated with nOMVs derived from B1940 *cps* compared to THP-1 cells treated with nOMVs from either B1940 wild-type or B1940 *siaD*(+C) mutant (Fig. 5A, E), consistent with the changes observed in *IL1B* transcript levels (Fig. 2B), suggesting an involvement of LOS outer core and/or sialylation also in the induction of pro-IL-1β expression. However, when we looked for the active form of IL-1β, normally generated by active caspase-1 and secreted from the cell, Western blot analysis did reveal both pro-IL-1β and IL-1β in the culture medium when THP-1 cells exposed to the nOMVs of the unencapsulated strain B1940 *siaD*(+C) (Fig. 5F), but, unexpectedly, not in the culture medium of THP-1 cells exposed to the nOMVs of *N. meningitidis* B1940, B1940 *cps* (Fig. 5F) or the other *Neisseria* wild-type strains (Fig. S4H).

Finally, given the elevated levels of this cytokine reported in different inflammatory diseases[74–76], we evaluated the expression and secretion of TNF-α. Western blot detected tmTNF-α (transmembrane form) in cell lysates of THP-1 cells exposed to nOMVs as well as untreated control cells without a significant difference between the samples (Fig. 5A), consistent with RT-qPCR result (Figs. S2C and 2). The presence of tmTNF-α in untreated control cells could be a consequence of treatment of THP-1 monocytes with phorbol esters for their differentiation, as previously reported[77]. The soluble form sTNF-α was instead detected in the culture medium of THP-1 cells exposed to *N. meningitidis* nOMVs but not in untreated control cells, with differences between cells exposed to nOMVs from B1940, B1940 *siaD*(+C), or B1940 *cps* (Fig. 5F, H) indicating a general lower pro-inflammatory potential of nOMVs derived from mutated meningococci strains compared to nOMVs from B1940 wild-type, as also suggested by the analysis of GSDME and caspase-4 activation.

Discussion

There is evidence that nOMVs released in cerebrospinal fluid and sera of patients with invasive meningococcal disease strongly contribute to the elevated LOS endotoxin levels associated with fatal septic infection[4–7]. Understanding the molecular basis of nOMV reactogenicity is therefore important both to find new adjuvant therapies against meningococcal endotoxemia and to create safe nOMV-based vaccines.

The reactogenicity of Neisserial nOMVs is mainly determined by the potent hexa-acylated LOS[26,27,78]. It was previously found that the reactogenicity of *N. meningitidis* nOMVs was greatly attenuated with nOMVs containing a penta- (rather than hexa-) acylated form of LOS[26,27,78]. In this study, we found a contribution of sialic acid capsule and LOS sialylation/outer core in the ability of nOMVs to activate cytokine expression and pyroptotic pathways in THP-1-derived macrophages (Figs. 1, 2, 3, 4). To shed light on possible mechanisms, it is important to keep in mind that there are two main pathways through which the LPS/LOS associated with nOMVs can activate the inflammatory response.

The first pathway is activated extracellularly by CD14, which detects the LPS/LOS and initiates a stepwise transfer of LPS/LOS to MD-2 and TLR4[79], leading to the induction of the inflammatory response through TLR4-MyD88-NF-κB signaling[80]. Detection of LPS/LOS by CD14 also induces an inflammatory endocytosis pathway that is triggered by MD-2 binding to and dimerizing TLR4[81]. Evidence has been provided that LPS/LOS molecules passing through this stepwise transfer fall into a thermodynamic funnel, which enables the extraction of LPS/LOS from the bacterial outer membrane and transfer through the TLR4/MD2 receptor/coreceptor complex[79]. In this transfer cascade, the thermodynamic barrier, represented by the extraction of the LPS/LOS from the outer membrane, can be reduced by the interaction with LPS/LOS binding protein (LBP), which would determine an alteration of the ionic environment formed by divalent counterions that tightly bind the negatively charged headgroups of lipid A between them, destabilizing the outer membrane[79]. Penta- (vs. hexa-) acylation of LPS/LOS may affect the thermodynamic barrier of LPS/LOS extraction from nOMVs or the stepwise transfer of LPS/LOS from CD14 to MD-2 and TLR4. In fact, hypo-acylation and hypo-phosphorylation of LPS/LOS are immune evasion strategies of commensal or host-adapted bacteria to evade CD14 and TLR4 signaling[81]. The increased amount of mRNA coding for TLR3 (Figs. S2G and 4A) in THP-1 macrophages exposed to Neisserial nOMVs is consistent with the evidence that the expression of TLR3, a TLR that is activated by double-stranded RNA in the endosomal compartment, is up-regulated by LPS through TLR4-MyD88-NF-κB signaling[82]. Additionally, a

trend toward down-regulation of VCL mRNA following exposure of nOMVs (Fig. 4A) has never been described before and could be associated with the LPS-induced cytoskeletal rearrangements that are also essential for LPS signaling[66,83].

The second pathway through which nOMV-associated LPS/LOS can activate the inflammatory response is activated at the intracellular level by cytosolic localization of LPS/LOS and activation of inflammatory caspase-1 and caspases-4, and -5[63,67]. In particular, caspases-4 and -5 mediating the non-canonical inflammasome pathway are directly activated by cytosolic LPS/LOS released by internalized bacteria[61] as shown in cell lines infected with *N. meningitidis*[62,84]. These caspases cleave gasdermin D (GSDMD), leading to the release of N-terminal fragments of GSDMD that oligomerize to form membrane pores. This mechanism also triggers nucleotide-binding oligomerization domain–like receptor pyrin domain–containing-3 (NLRP3)–dependent caspase-1 activation by damage-associated molecular patterns (DAMPs), and, in turn, the proteolytic activation of IL-1β and IL-18 leading to pyroptosis[85]. Caspase-1 was indeed found activated following Neisserial nOMVs exposure (Figs. S4H and 5F), and the active forms were released from the cells, as already established in THP-1 cells infected with metapneumovirus or treated with LPS or ATP[71,86].

Notably, GSDME rather than GSDMD seems to mediate pyroptosis in THP-1 macrophages treated with nOMVs as previously observed in different cell lines infected with *N. meningitidis*[62]. GSDME can be cleaved and activated at the same site by caspase-3 or granzyme B, which is abundant in sera of patients with severe meningococcal disease[87]. However, caspase-3 was not activated by nOMVs treatment of THP-1 cells, contrary to previous observations in other cell lines infected with *N. meningitidis*[62]. Possible reasons for the lack of caspase-3 activation can be: (i) the need for a higher concentration of nOMVs or a prolonged incubation time to induce caspase-3 activation; (ii) the requirement for the presence of live bacteria rather than nOMVs in the cell; (iii) a different pyroptosis pathway activated in THP-1 macrophage cells compared to the cell lines previously infected with *N. meningitidis* B1940[62]. Granzyme B is synthesized as a pro-peptide and targeted to acidic secretory lysosomes[88], where the peptidase cathepsin C activates it[89], and it may be noted that in THP-1 macrophages infected with *N. gonorrhoeae,* cathepsin C is required for IL-1β secretion[90]. LPS/LOS induces granzyme B release into circulation in a TNF-α and IL-12 dependent manner[91]. Our data indicate that the LOS outer core and/or its sialylation have a great impact on these mechanisms. In fact, we observed a strong reduction of the active N-terminal GSMDE fragment levels as well as a reduction in secretion of sTNF-α in THP-1 macrophages treated with nOMVs from B1940 *cps* compared to THP-1 cells treated with nOMVs from wild-type bacteria (Fig. 5A, C, F, H). Furthermore, we observed a significant reduction of *IL-12A* transcript in THP-1 cells treated with B1940 *cps* nOMVs compared to cells treated with B1940 nOMVs (Fig. 2B). Moreover, a strong reduction in caspase-4 activation was observed in B1940 *siaD* (+C) nOMVs and particularly in B1940 *cps* nOMVs-treated cells (Fig. 5A, B). The use of a mutant defective in the LOS α-2,3-sialyltransferase enzyme encoded by *lst* gene will be important to test whether the effects observed with the nOMVs of B1940 *cps* are caused by the truncation of the LOS outer core or the lack of the terminal sialic acid.

In contrast to pyroptosis pathways, *N. meningitidis* inhibits apoptosis through the translocation of PorB to mitochondria[14]. PARP1 activation was inhibited in THP-1 cells exposed to Neisserial nOMVs (Figs. S2A, D, 5A, D), consistent with the evidence that PorB is found on nOMVs[11,92]. nOMVs from the commensal species *N. cinerea* and *N. lactamica* seem to share this inhibitory function with *N. meningitidis*, in contrast to *N. gonorrhoeae,* which induces apoptosis through its porin PorB[11].

An unexpected finding of this study is that LOS outer core and/or sialylation play important roles in the activation of pro-inflammatory cytokines and caspases in human THP-1-derived macrophages. The expression of most inflammatory markers was indeed significantly reduced when THP-1 cells were treated with nOMVs derived from nOMV B1940 *cps* compared to wild-type nOMV B1940. The expression of some inflammatory markers was also partly reduced with nOMVs derived from the unencapsulated B1940 *siaD*(+C), a mutant that does not express the sialic acid capsule but retains LOS outer core as well as LOS sialylation. The nOMVs, in fact, can retain a certain amount of capsular polysaccharide (Fig. S3), as has also been observed in nOMVs produced by *Vibrio vulnificus*[93], *Francisella turalensis*[94], as well as OMV from *Escherichia coli* engineered to express surface-exposed polysialic acid[95]. The mechanism by which LOS outer core and /or sialylation of LOS or surface-exposed sialic acid on nOMVs contribute to the activation of pro-inflammatory cytokines and caspases is unknown, since lipid A, which is not modified by sialylation, is primarily involved in the interaction between LOS, CD14, MD-2, and TLR4, which triggers TLR4-MyD88-NF-κB signaling. One possibility is that sialylation of LOS, which increases its negative charge, or a complete outer core extending from the lipid A moiety on the bacterial surface, may facilitate the extraction of LOS from nOMVs by CD14. Another possibility is that sialylation of LOS or surface-exposed sialic acid may increase the adherence of nOMVs to THP-1 macrophages and their internalization through sialic acid, such as Siglec-1, Siglec-5, Siglec-11, and/or Siglec-14, which are expressed on the surface of the human monocytic cell line THP-1[96,97], as observed with meningococci expressing sialylated LOS[42,43]. Finally, another possibility is that a complete LOS outer core and/or LOS sialylation, or surface-exposed sialic acid, may potentiate the inflammatory response triggered by the cytosolic LOS, facilitating the activation of caspase 4. A combination of the mechanisms described above is also possible. For example, the reduced activation of caspase-4 by nOMVs from the B1940 *cps* strain could be caused by the reduced uptake of these nOMVs by THP-1 cells through the Siglecs. The use of purified and well-characterized LOS from *N. meningitidis* B1940, B1940 *siaD*(+C) and B1940 *cps*, which was not used in the current study and represents a major limitation, will help validate the proposed influence of the LOS outer core and/or sialylation on immune induction in THP-1 cells as well as in other relevant cellular models, and to discriminate between these mechanisms.

As expected, TNF-α was detected in the culture medium of THP-1 cells exposed to *N. meningitidis* nOMVs but not in control cells not exposed to nOMVs (Fig. 5F, H), with differences between cells exposed to nOMVs from B1940, B1940 *siaD*(+C), and B1940 *cps*, as well as in the amounts of intracellular pro-IL-1β, caspase-4,

and GSDME (Fig. 5A–C, E). However, unexpectedly, we found that secreted pro-IL-1β and mature IL-1β were detected only in the culture medium of THP-1 cells exposed to nOMVs derived from the unencapsulated strain B1940 *siaD*(+C) but not from the other *Neisseria* strains (Fig. 5F). It can be also observed that caspase-1, which is known to process pro-IL-1β into mature IL-1β, was activated and secreted by THP-1 cells in response to nOMVs treatment without significant difference between cells exposed to nOMVs from B1940, B1940 *siaD*(+C) and B1940 *cps* (Fig. 5F, G). Therefore, we can hypothesize that the presence of the sialic acid capsule may inhibit a pathway in THP-1 cells leading to the secretion of pro-IL-1β, which can be processed to mature IL-1β by the extracellular caspase-1 after its secretion. Indeed, different mechanisms for pro-IL-1β and/ or IL-1β secretion have been described, such as secretory lysosome and secretory autophagy in response to intracellular Ca^{2+} increase[98]. This could explain the difference between cells treated with B1940 and B1940 *siaD*(+C) nOMVs, while the lack of pro-IL-1β secretion (and consequent maturation to IL-1β) by THP-1 cells treated with B1940 *cps* nOMVs could be due to poor immune induction of its LOS, as hypothesized above. The inhibitory mechanism exerted by nOMVs from encapsulated bacteria on pro-IL-1β secretion could be due to the shielding of LOS by capsular material, leading to a reduced activation of the pathway leading to pro-IL-1β secretion, possibly activated by LOS, or to an inhibition on this pathway mediated by binding of sialic acid capsule to receptors, dampening the activation of inflammatory cells such as Siglec-5 or Siglec-11[99]. In fact, sialic acid has been demonstrated to inhibit IL-1β maturation induced by LPS through Siglec-11, -7, and -9[100]. Additionally, an up-regulation of IL-1β has been accompanied by a down-regulation of TNF-α and IL-6 in human macrophages upon adaptation to LPS or lipid A stimulation[101]. TNF-α is a potent inducer of IL-6 in response to LPS exposure[102,103], and its levels often parallel those of IL-6 in inflammatory diseases[104–106] consistent with the observed pattern of TNF-α secretion and *IL-6* mRNA levels in our assays (Figs. 2, 5F, H). Thus, the interactions of sialic acid with Siglecs, a different LOS exposure, and a different internalization rate of nOMVs from B1940, B1940 *siaD*(+C), and B1940 *cps* strain may shape the inflammatory response in human macrophages, highlighting the importance of these structures for *N. meningitidis* pathogenesis and paving the way for the production of nOMVs that meet the needs in clinical practice.

Materials and methods

Bacterial strains and growth conditions

The origin, genotype, and phenotype of *N. meningitidis* serogroup B encapsulated strain B1940 and its derivatives, the unencapsulated B1940 *siaD*(+C) and B1940 *cps* mutants, have been described previously (Table S1). Specifically, *N. meningitidis* B1940 [B:NT:P1.3,6,15; LOS immunotype L3,7,9] is piliated and expresses Opa and Opc adhesins[33,107–109]. This strain shows immunoreactivity with the monoclonal antibodies SM1, 4B12, and B306 as reported previously[107]. Molecular typing of PorB variable loops, performed as described[110] by using the PubMLST database[111], revealed that B1940 harbors a class 2 *porB*, allele 599 [2–143] encoding peptide variable loops I.1 IV.10 V.19 VI.17 VII.1 VIII.2 (Table S1). The B1940 *siaD*(+C) mutant lacks the capsule due to frameshift mutations, a cytosine insertion in a polycytidine repeat located in the coding region of the polysialyltransferase gene (*siaD*)[112]. The B1940 *cps* mutant harbors a large deletion of the *cps* locus, including *galE,* and lacks both the capsule and the LOS outer core with sialic acid[107,109]. 93/4286 (aliases: L93/4286; NIBSC_2759; Z6417) is a *N. meningitidis* serogroup C reference strain belonging to the hypervirulent lineage ET-37, which was kindly provided by Novartis Vaccine and Diagnostics, Siena, Italy, and has been characterized previously[41,113–117]. Capsulation of meningococcal strains was previously checked by the latex slide agglutination test (Directigen *N. meningitidis* group B/*Escherichia coli* K1 and Directigen *N. meningitidis* groups A, C, Y, and W135 test kits; Becton, Dickinson and Company)[39]. Fine-typing, Multi-Locus Sequence Typing (MLST), and PorB typing data are provided in Table S1 as deduced from the PubMLST database. 93/4286 harbors a class 2 *porB*, allele 1 [2] encoding peptide variable loops I.1 IV.1 V.1 VI.1 VII.1 VIII.1. *Neisseria lactamica* NCTC 10617[118] and *Neisseria cinerea* ATCC 14685[119] are reference strains. Available data about these strains are provided in Table S1. All *Neisseria* spp. strains were cultured in Gonococcus (GC) broth or agar with 1% Polyvitox at 37 °C in a 5% CO_2 incubator[116,117].

Purification of nOMVs

All bacterial strains were streaked from frozen stock (− 80 °C) on GC agar base (OXOID), supplemented with 1% (v/v) Polyvitox (OXOID) and incubated overnight at 37 °C in a 5% CO_2 incubator. Then, some colonies were inoculated in flasks containing 250 mL of GC broth, whose composition (per liter) is as follows: 15 g proteose peptone, 0.5 g corn starch, 4 g K_2HPO_4, 1 g KH_2PO_4, 5 g NaCl, 1% (v/v) Polyvitox (OXOID). Bacterial cultures were grown in GC broth at 37 °C with shaking (150 rpm) until the late stationary phase. After incubation, whole bacterial cells were removed from cultures by centrifugation (20 min, 3000 × *g*, 4 °C) and by filtration through a 0.22 µm sterile filter. At the end of growth, 1 L of culture was collected in 20 × 50 mL Falcon tubes and centrifuged at 3000 × *g* for 20 min at + 4 °C. The biomass was discarded, and the supernatant containing nOMVs was filtered using a 0.22 µm filter (Sartorius Sartolab RF 150 PES) in order to reduce the bioburden. The obtained material was ultrafiltered by tangential flow filtration (TFF) on an ambr Crossflow® system from Sartorius (Sartorius Stedim Systems GmbH, Guxhagen, Germany), equipped with cassettes at a 300 kDa molecular weight cut-off (MWCO). The nOMVs were repeatedly ultrafiltered using Tris and PBS buffers to remove all the residual small contaminants. Total protein content in nOMVs preparation was quantified using the Lowry assay using the DC Protein Assay Kit II (Bio-Rad) following the manufacturer's instructions and reported as µg/mL. The samples were analyzed in triplicate for each dilution factor; the final result was extrapolated from at least 5 average values. The Quant-iT PicoGreen dsDNA assay kit (Life Technologies) was used to measure the double-stranded DNA content in nOMVs preparations, and an average of 4.7% ± 0.5 µg DNA/ mg of proteins was obtained.

Meningococcal capsule detection

Capsule expression on *N. meningitidis* B1940, B1940 *cps*, and B1940 *siaD*(+C) strains and their purified nOMVs was checked with the anti-Meningococcus group B monoclonal antibody (Remel, ZM51/30167501). Overnight-grown bacterial colonies were resuspended in PBS buffer and normalized according to their optical density ($O.D._{600\ nm}$) at 600 nm. The normalized suspensions, together with 90 µg in protein content of nOMVs resuspended in PBS, were subjected to a slot blot assay. A Hybrid Slot Manifold (BRL Life Technologies) was assembled with two Whatman 3MM sheets and a nitrocellulose membrane pre-equilibrated in PBS, and 150 µL of each sample was loaded onto the membrane through the apparatus connected to a vacuum pump. The membrane was blocked with 3% bovine serum albumin (BSA) in PBS-Tween 0.05% for 30 min and incubated overnight with the anti-meningococcal group B antibody diluted 1:100 in PBS-Tween 0.05%. After washing with PBS-Tween 0.05% the membrane was incubated with anti-mouse IgG (H + L)-HRP conjugated antibody (Bio-Rad), and signals were acquired with the ChemiDoc MP Imaging System (Bio-Rad). Additionally, *N. meningitidis* B1940 and B1940 *cps* strains were resuspended in PBS buffer, deposited onto polylysinated glass coverslips, and fixed with 3% paraformaldehyde (PFA) in PBS. Samples were incubated with the anti-meningococcal group B antibody diluted 1:10 in PBS for 1 h at room temperature after blocking with 1% BSA in PBS. Subsequently, samples were washed twice with PBS, incubated with anti-mouse Alexa Fluor 568 conjugated antibody for 20 min, and nucleic acids were stained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, coverslips were mounted onto slides with Mowiol mounting media and observed with a confocal laser-scanning microscope (ZEISS, LSM700) with a 63 × /1.40 NA oil immersion objective.

Cell culture conditions and treatment

In this study, THP-1 (ATCC TIB-202), a human monocytic cell line originally derived from an acute monocytic leukemia patient, was used. The cell line was obtained from ATCC and handled in accordance with biosafety standards and protocols. The THP-1 cell line is commercially available and was used in accordance with ethical standards; no human individuals were directly involved. This cell line is a common model to estimate the modulation of monocyte and macrophage activities[120] and was previously used to study the phenotype of meningococcal strains[38]. THP-1 cells were cultured in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/mL penicillin, and 10 µg/mL streptomycin (Euroclone) and incubated at 37 °C in a 5% CO_2 incubator. THP-1 monocytes were differentiated into macrophages with 2 nM of phorbol 12-myristate 13-acetate (PMA) for 48 h. The low dose was chosen to minimize unspecific activation, as previously reported[121–123], and THP-1 differentiation was initially based on the acquisition of a macrophage-like morphology, characterized by strong adhesion and spreading on the plate[124–126]. Differentiation of THP-1 was functionally confirmed based on their responsiveness to stimuli, such as the induction of TNF-α, IL-6, and IL-1β, consistent with a macrophage-like phenotype[125,127,128]. Subsequently, cells were treated with 10 µg/mL (total protein content) of nOMVs from the bacterial strains indicated for 24 h, conditions which were sufficient to induce an inflammatory response in THP-1 macrophages for *Legionella pneumophila*-derived vesicles[129]. Finally, cells were harvested, centrifuged at 500 × *g* for 5 min at 4 °C, and the pellet was immediately used for the subsequent RNA extraction.

RNA extraction and real-time RT-PCR

Total RNA was extracted from THP-1 macrophages after treatments with nOMVs using Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions. Total RNA quality and concentration were assessed by loading it onto an agarose gel and using the spectrophotometer NanoDrop (Thermo Scientific). Reverse transcription was conducted by iScript cDNA Synthesis kit (Bio-Rad), and the cDNA was used to perform a real-time PCR on a CFX96 System (Bio-Rad) using a customized 96-well PCR plate (H96 PrimePCR) (Bio-Rad) (Fig. S1). Each well of the PCR Plate contains primers for a selected gene or a control for genomic DNA contamination, RNA quality, reverse transcription (RT), and PCR efficiency. To provide biological relevance and robustness of the observed transcriptional changes, we applied a stringent cut-off of > fourfold change to define up- and down-regulated genes. This threshold was chosen to reduce the impact of biological variability and to focus on genes showing the most highly consistent and marked responses to nOMV exposure. Nevertheless, gene expression changes below this threshold were also discussed, if biologically relevant and/or consistent with other experimental observations or statistically significant according to ANOVA analysis, to better understand the effect of nOMVs.

Western blotting

THP-1 macrophages were treated with 10 µg/mL (total protein content) of *Neisseria* spp. nOMVs for 24 h. Subsequently, the cell media were collected, centrifuged at 1500 × *g* for 5 min at 4 °C, and filtered through a 0.22 µm filter to remove cell debris while cells were lysed in Laemmli buffer (100 mM Tris–HCl, pH 6.8 with 4% SDS, 20% glycerol, and 0.2% blue bromophenol). Cell lysates or cell media were subjected to SDS-PAGE as previously described[62]. The following antibodies were used: anti-caspase-1 (Abcam, ab179515), anti-caspase-4 (Abcam, ab238124), anti-PARP1 (Cell Signaling Technology, #9532), anti-caspase-3 (Cell Signaling Technology, #9665), anti-gasdermin E (Abcam, ab215191), anti-gasdermin D (Cell Signaling Technology, #39754), anti-TNF-α (Invitrogen, #AMC3012) and anti-IL1β (Invitrogen, #P420B). Anti-tubulin (Santa Cruz Biotechnology, sc-23948) was used as a loading control or as a negative control to exclude signals due to lysate cells when secreted proteins were analyzed. Images were acquired by the ChemiDoc MP Imaging System using Clarity and Clarity Max ECL Western Blotting Substrate (Bio-Rad) and analyzed by Image Lab software version 6.0.1 (Bio-Rad).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1 software. Statistical significance of comparisons was determined by one-way ANOVA with Dunnett's post-hoc test.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Received: 29 May 2025; Accepted: 25 November 2025
Published online: 01 December 2025

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Acknowledgements

We are grateful to professor M. Frosch (University of Würzburg) for gift of B1940, B1940 siaD(+C) and B1940 cps meningococcal strains

Author contributions

All authors met ICMJE criteria for authorship and contributed to the study conception and design. Material preparation, experiments and data collection were performed by SCR, AT, AB, GS, PP and VDC. Data analysis was performed by SCR, AT, AB, VDC, CB, and AP. Project administration and supervision were carried out by AT, AB, VDC, and PA. The first draft of the manuscript was written by PA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

Research reported in this study was partially supported by the following grants funded to PA: Grant Funded by the European Union—Next Generation EU PRIN 2022 PNRR (Project No. P2022LPT3R); Grant Funded by Italian Ministry of University and Research (MUR)—PRIN 2020 (Project No. 202089LLEH); Grant funded by Consorzio Interuniversitario Biotecnologie (DM 587, 08/08/2018; CIB N. 86/19). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-30502-7.

Correspondence and requests for materials should be addressed to P.A.