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Ectosomes Released by Polymorphonuclear Neutrophils Induce a MerTK-dependent Anti-inflammatory Pathway in Macrophages*

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At the earliest stage of activation, human polymorphonuclear neutrophils release vesicles derived directly from the cell surface. These vesicles, called ectosomes (PMN-Ect), expose phosphatidylserine in the outer membrane leaflet. They inhibit the inflammatory response of human monocyte-derived macrophages and dendritic cells to zymosan A (ZymA) and LPS and induce TGF-β1 release, suggesting a reprogramming toward a tolerogenic phenotype. The receptors and signaling pathways involved have not yet been defined. Here, we demonstrate that PMN-Ect interfered with ZymA activation of macrophages via inhibition of NFκB p65 phosphorylation and NFκB translocation. The MerTK (Mer receptor tyrosine kinase) and PI3K/Akt pathways played a key role in this immunomodulatory effect as shown using specific MerTK-blocking antibodies and PI3K inhibitors LY294002 and wortmannin. As a result, PMN-Ect reduced the transcription of many proinflammatory genes in ZymA-activated macrophages. In sum, PMN-Ect interacted with the macrophages by activation of the MerTK pathway responsible for down-modulation of the proinflammatory signals generated by ZymA.

In general, intercellular communication occurs via membrane contact, soluble mediators (*e.g.* hormones, cytokines, chemokines), and small molecular mediators (*e.g.* nucleotides, bioactive lipids, nitric oxide ions) (1–3). However, recent evidence emphasizes the transfer of information by small vesicles released by one cell to its target cell (4, 5). In the literature, such vesicles go by various names, including microvesicles, nanovesicles, particles, and microparticles. Specific vesicles released from multivesicular bodies (preformed vesicles) from various cell types have been named exosomes and best described in the immune system, where they are capable of presenting specific antigens to T-lymphocytes (6, 7). Others, released at the time of cellular activation by budding directly from the cell surface, *i.e.* by ectocytosis, have been logically named ectosomes (8, 9).

Ectosomes are released by human polymorphonuclear neutrophils (PMN-Ect)[2] at the time of cellular activation. Interestingly, such ectosomes do not increase inflammatory processes; on the contrary, they were shown to down-modulate cellular activation of macrophages (10) and dendritic cells (DCs) (11). In human monocyte-derived macrophages (HMDMs), PMN-Ect could inhibit the release of TNFα and reduce the release of IL-8 and IL-10 induced by zymosan A (ZymA) and LPS (10). When immature monocyte-derived DCs were exposed simultaneously to PMN-Ect and LPS, their morphology was modified, their phagocytic activity and the expression of cell-surface markers (CD40, CD80, CD83, CD86, and HLA-DP, -DQ, and -DR) were reduced, cytokine release (IL-8, IL-10, IL-12, and TNFα) was inhibited, and their capacity to induce T-cell proliferation was impaired (11). These data suggested that ectosomes induce a tolerogenic phenotype in DCs, similar to what has been described for apoptotic cells (ACs).

So far, the mechanisms responsible for the biological effects of PMN-Ect remain speculative. The phosphatidylserine (PS) exposed in the outer membrane leaflet of PMN-Ect may be a major factor influencing macrophages and DCs, as shown for ACs and liposomes (12–14). Similar to ACs, PMN-Ect have been shown to induce the release of TGF-β1 by macrophages and DCs, which might be responsible for the down-regulation of their Toll-like receptor-mediated maturation (15–17).

Recent studies revealed that TAM (Tyro3/Axl/Mer) receptor tyrosine kinases bind PS and that antigen-presenting cell-produced TAM ligands are bridging these interactions in a calcium-dependent manner (*e.g.* GAS6 (growth arrest-specific 6) and Protein S) (18). TAM receptors, expressed in a large variety of cells, including macrophages and DCs, have been shown to have the capacity to down-modulate the inflammatory response (19, 20). *In vivo* and *in vitro* studies demonstrate that these receptors are required for AC clearance and homeostatic regulation of the immune system (21–23). Most of the analyses concerning ACs and TAM receptors have been carried out with MerTK (Mer receptor tyrosine kinase). Recently, ACs have been proposed to inhibit mouse DC activation via the induction of MerTK, which activates the PI3K/Akt pathway and inhibits NFκB (24).

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[2] The abbreviations used are: PMN-Ect, human polymorphonuclear neutrophil ectosome(s); DC, dendritic cell; HMDM, human monocyte-derived macrophage; ZymA, zymosan A; AC, apoptotic cell; PS, phosphatidylserine; Ab, antibody.

The transcription factor NFκB has a major role in regulating many aspects of cellular activity, in inflammation, cell proliferation, differentiation, and cell survival (25). The mammalian NFκB protein family consists of five proteins (p50, p52, p65 (RelA), c-Rel, and RelB) found in homodimers and heterodimers (25, 26). In the cytoplasm, NFκB is regulated by a class of inhibitor proteins called IκB proteins (27). Upstream activating signals cause the phosphorylation of IκB proteins and their ubiquitination and subsequent degradation, allowing NFκB dimers to enter the nucleus (25, 27, 28). Once translocated in the nucleus, NFκB undergoes post-translational modifications (such as phosphorylation of the p65 subunit at Ser^{276} and Ser^{536}) that affect its transcriptional activation (25). Whether PMN-Ect interfere with the NFκB signaling pathway is currently undetermined. Because PMN-Ect have immediate inhibitory effects on HMDMs and DCs (10, 11), we investigated whether PMN-Ect modify the NFκB signaling pathways of HMDMs and whether MerTK is involved.

TABLE 1
Primers for quantitative real-time RT-PCR

Primer	Sequences
GAPDH	
Forward	ATTGCCCTCAACGACCACT
Reverse	GCACAGGGTACTTTATTGATGG
IL-1β	
Forward	GGGCCTCAAGGAAAAGAATC
Reverse	AGCTGACTGTCCTGGCTGAT
IL-6	
Forward	CAGTTCCTGCAGAAAAAGGC
Reverse	ATCTGAGGTGCCCATGCTAC
IL-8	
Forward	ACATACTCCAAACCTTTCCACCC
Reverse	CAACCCTCTGCACCCAGTTTTC
IL-10	
Forward	CGAGATGCCTTCAGCAGAGTG
Reverse	GGGAAGAAATCGATGACAGC
IL-12	
Forward	CATAACTAATGGGAGTTGCCTGGC
Reverse	AACGGTTTGGAGGGACCTCG
TNFα	
Forward	GAGTGACAAGCCTGTAGCCCATGTTGTAGC
Reverse	GCAATGATCCCAAAGTAGACCTGCCCAGAC

EXPERIMENTAL PROCEDURES

Isolation, Culture, and Maturation of HMDMs—HMDMs were derived from monocytes isolated from fresh buffy coats. A buffy coat was diluted 1:1 (v/v) with Hanks' balanced saline solution (Invitrogen), layered over Histopaque-1077 (Sigma), and centrifuged for 30 min at 350 × *g*. Monocytes were recovered, washed, and cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin/streptomycin and 1% L-glutamine ($DMEM^+$) for 1 h at 37 °C. After incubation, non-adherent cells were removed by washing twice with prewarmed $DMEM^+$. The remaining adherent cells were then cultured in $DMEM^+$ supplemented with 10% normal human serum. The culture was maintained in 5% CO_2 at 37 °C, and the medium was replaced on days 4 and 7. On days 7–10, HMDMs were washed, and PMN-Ect and/or ZymA (5 μg/ml final concentration; Sigma) was added in fresh $DMEM^+$ without normal human serum (10).

Collection of PMN-Ect—To isolate PMNs, a fresh buffy coat was diluted 1:1 (v/v) with PBS/EDTA (2 mM), mixed with 0.25 volume of 4% dextran T500 (GE Healthcare), and left for 30 min for erythrocyte sedimentation. Leukocyte-rich supernatant was aspirated and centrifuged for 10 min at 200 × *g*. The pellet was resuspended in 9 ml of ultrapure water to lyse erythrocytes. Isotonicity was restored by the addition of 3 ml of KCl (0.6 M) and 40 ml of NaCl (0.15 M). Cells were then centrifuged for 10 min at 350 × *g* and resuspended in 20 ml of PBS/EDTA. This suspension was layered over 20 ml of Histopaque-1077 and centrifuged for 30 min at 350 × *g*. The PMN-rich pellet was recovered and washed twice with PBS/EDTA. All manipulations were performed at 4 °C, thus minimizing PMN activation (8–11).

For stimulation, pooled PMNs (1×10^7 cells/ml) from healthy blood donors were diluted 1:1 (v/v) in prewarmed (37 °C) RPMI 1640 medium (Invitrogen) with 1 μM formyl-methionyl-leucyl-phenylalanine and incubated for 20 min at 37 °C. PMNs were removed by centrifugation (4000 × *g* for 15 min at 4 °C), and PMN-Ect contained in the supernatant were concentrated with Centriprep centrifugal filter devices (MW 10,000 cut-off; Millipore) and stored in aliquots at −80 °C until used (8–11).

Detection of NFκB and Akt Activation—HMDMs were cultured in 96-well plates for 7 days. The phosphorylation levels of NFκB p65/RelA and Akt were quantified with CASE™ cellular activation of signaling ELISA (SABiosciences) according to the manufacturer's instructions. In some experiments, HMDMs were treated for 1 h at 37 °C with 20 μg/ml goat anti-MerTK antibody (Ab; AF891, R&D Systems), 20 μg/ml goat IgG Ab (R&D Systems), an isotype control, or the PI3K inhibitor LY294002 (50 μM; SABiosciences) or wortmannin (200 nM; Calbiochem) prior to PMN-Ect and/or ZymA incubation. All samples were measured in triplicates, and the results are given as an absorbance ratio of phosphorylated protein to total protein.

Western Blotting—Nuclear and cytoplasmic extracts were prepared from HMDMs using a nuclear extract kit (Active Motif, Rixensart, Belgium) according to manufacturer's instructions. Protein concentrations were quantified using a Bio-Rad *DC* protein assay kit. Proteins were separated by SDS-PAGE and transferred to Bio-Rad Trans-Blot transfer medium. The levels of NFκB p65 were measured in the nuclear extracts using a 1:500 dilution of an anti-N-terminal NFκB p65 Ab (Santa Cruz Biotechnology, Heidelberg, Germany). To monitor equal protein loading, blots were stripped and reprobed with a 1:2000 dilution of an anti-TATA-binding protein Ab (Abcam). Activation of Akt was assessed in the cytoplasmic extracts using a 1:1000 dilution of an anti-phospho-Ser^{473} Akt1 Ab (Millipore), and blots were then reprobed using a 1:200 dilution of antibodies against the non-phosphorylated form of Akt (Santa Cruz Biotechnology). The binding of HRP-labeled donkey anti-rabbit or sheep anti-mouse secondary Ab (GE Healthcare) or donkey anti-goat Ab (Santa Cruz Biotechnology) was analyzed using ECL detection reagents (GE Healthcare).

Fluorescence Microscopy—HMDMs were generated on 8-well culture slides (Falcon, BD Biosciences). After 7–10

days of culture, HMDMs were washed with DMEM$^+$ without normal human serum and incubated for the indicated times with or without ZymA in the absence or presence of PMN-Ect labeled with FITC-CD66b (GeneTex Inc.). Then, HMDMs were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. NFκB staining was performed by incubation with an anti-NFκB primary Ab (RelA; sc-109, Santa Cruz Biotechnology), followed by a Cy5-labeled donkey anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories). Labeled cells were mounted with VECTASHIELD containing DAPI (Vector Laboratories, Burlingame, CA). Analysis was performed on an Olympus BX61 microscope.

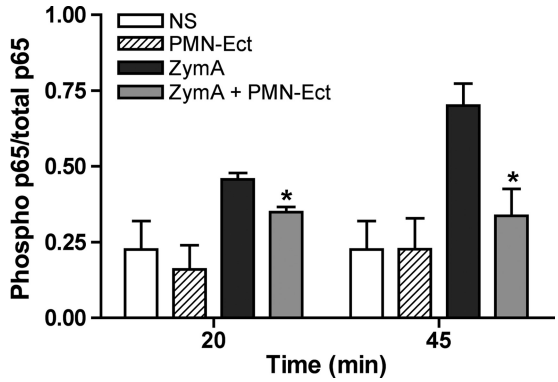

FIGURE 1. **Inhibition of NFκB p65 phosphorylation in ZymA-stimulated human macrophages by PMN-Ect.** HMDMs were incubated in medium alone (*NS*) or with PMN-Ect and in medium supplemented with ZymA in the presence or absence of PMN-Ect. At 20 and 45 min, the phosphorylation of NFκB p65 was analyzed. *, $p < 0.05$, ZymA + PMN-Ect *versus* ZymA. Results are the mean ± S.E. of three independent experiments.

Quantitative Real-time RT-PCR—On day 7, HMDMs were cultured for 45 min or 3 h alone or with PMN-Ect, ZymA, or ZymA and PMN-Ect. After 45 min or 3 h, total RNA was isolated with the NucleoSpin RNA/protein system (Macherey-Nagel, Düren, Germany) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. SYBR PCR was performed based on SYBR Green fluorescence (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA). Primers for GAPDH, IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα are shown in Table 1. The difference in the cycle threshold (ΔC_T) value was derived by subtracting the C_T value for GAPDH, which served as an internal control, from the C_T value for the transcript of interest. All reactions were run in duplicate using an ABI 7500 sequence detection system (Applied Biosystems). The mRNA expression levels of the transcripts were calculated relative to GAPDH from the ΔC_T values using the formula $2 - \Delta C_T$.

Quantitation of Cytokines by ELISA—HMDM supernatants were collected and spun at 500 × *g* for 10 min at 4 °C to remove cellular debris. The concentrations of various cytokines were measured using a human proinflammatory 7-Plex tissue

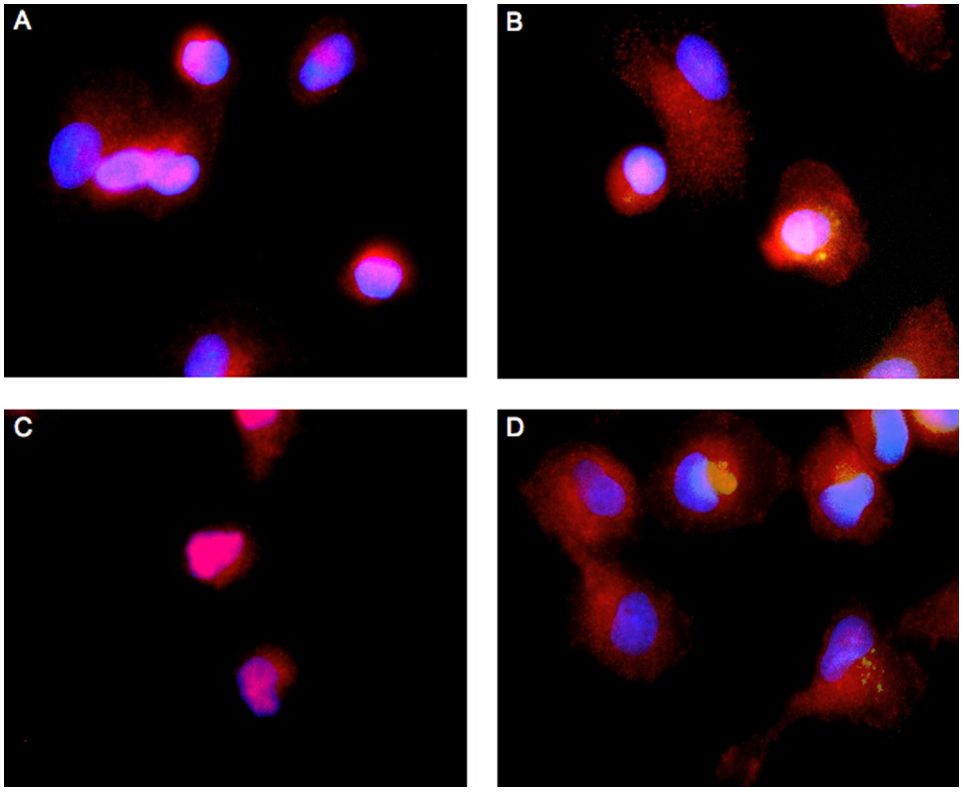

FIGURE 2. **Immunofluorescence microscopy of the inhibition of NFκB translocation in the nucleus by PMN-Ect in ZymA-stimulated human macrophages.** HMDMs were incubated in medium alone (*A*) or with PMN-Ect (*B*), with ZymA (*C*), or with ZymA + PMN-Ect (*D*) for 20 min. Cells were then fixed, stained with anti-NFκB primary Ab (RelA; sc-109) followed by Cy5-labeled donkey anti-rabbit secondary Ab (*red*), and analyzed by immunofluorescence microscopy. In *B* and *D*, *green dots* correspond to FITC-CD66b-prelabeled PMN-Ect that are present in the cytoplasm. The imaging medium was VECTASHIELD fluorescence mounting medium containing DAPI, and the analysis was performed on an Olympus BX61 microscope.

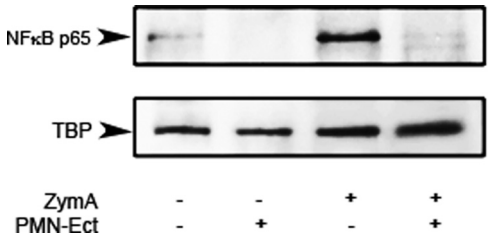

FIGURE 3. **Inhibition of NFκB p65 translocation in the nucleus in ZymA-stimulated human macrophages by PMN-Ect.** HMDMs were incubated in medium alone or with PMN-Ect and in medium supplemented with ZymA in the presence or absence PMN-Ect for 45 min. NFκB p65 was detected in nuclear extracts via Western blotting using the anti-N-terminal NFκB p65 Ab. The blots were stripped and reprobed with anti-TATA-binding protein (*TBP*) Ab to monitor loading. The blots are representative of three independent experiments.

culture kit (MSD, Gaithersburg, MD) according to the manufacturer's instructions. All samples were measured in duplicates.

Statistical Analysis—Data sets were tested for normality. Parametric analysis (two-tailed paired Student's *t* test) for normally distributed data and non-parametric analysis (Wilcoxon matched-pairs test) for non-normally distributed data were performed using GraphPad Prism software. Data are expressed as the mean ± S.E. A p value <0.05 was considered statistically significant.

RESULTS

PMN-Ect Inhibit NFκB Activation in HMDMs—As NFκB is the key transcription factor controlling major proinflammatory genes, we were interested in investigating whether NFκB signaling is involved in HMDM down-modulation upon encountering PMN-Ect. The NFκB p50/p65 dimer is typically sequestered in the cytoplasm by its interaction with IκB. Upon stimulation, IκB is phosphorylated, ubiquitinated, and degraded, allowing the NFκB dimer to enter the nucleus (25–28). Because phosphorylation of NFκB p65 at Ser^{536} is reported to enhance its transcriptional activation (25), we measured the phosphorylation of NFκB p65 in resting or zymosan-stimulated HMDMs in the absence or presence of PMN-Ect after 20 and 45 min (Fig. 1). PMN-Ect alone had no effect on NFκB p65 Ser^{536} phosphorylation. In contrast, ZymA alone induced a significant Ser^{536} phosphorylation of NFκB p65 compared with resting HMDMs. Strikingly, the effect of ZymA was significantly suppressed by PMN-Ect ($p < 0.05$ at both 20 and 45 min; $n = 3$). These results demonstrate that PMN-Ect have the capacity to interfere with NFκB p65 Ser^{536} phosphorylation at the time of HMDM stimulation, suggesting an inhibitory effect of PMN-Ect on NFκB transactivation.

To investigate whether this reduced phosphorylation was related to interference with the translocation of NFκB to the nucleus, we performed immunofluorescence microscopy. We first labeled PMN-Ect with the surface marker FITC-CD66b. We then incubated HMDMs with labeled PMN-Ect, ZymA, or both for 20 min and probed all samples with anti-NFκB Ab. We observed that, in resting HMDMs and in the presence of PMN-Ect alone, NFκB was mainly in the cytoplasm (Fig. 2, *A* and *B*). As expected, in ZymA-stimulated HMDMs, NFκB

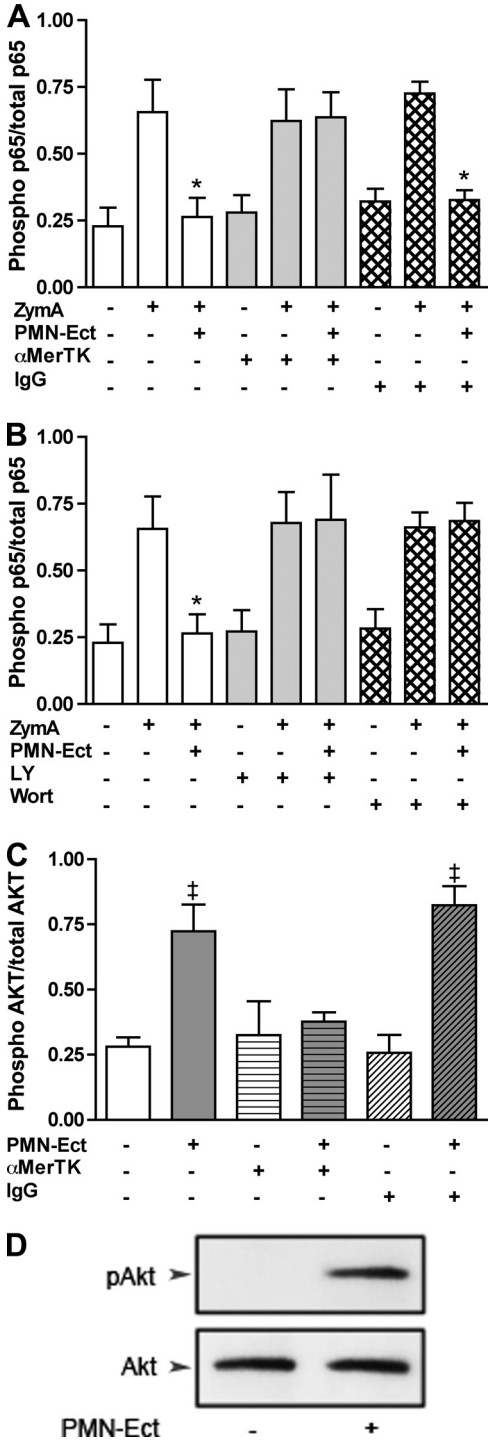

FIGURE 4. **Roles of MerTK and PI3K in PMN-Ect-induced inhibition of NFκB p65 in ZymA-stimulated human macrophages and effect of PMN-Ect on Akt phosphorylation in resting human macrophages.** HMDMs were pretreated for 1 h at 37 °C with 20 μg/ml goat anti-MerTK Ab or goat IgG Ab (*A* and *C*) or the PI3K inhibitor LY294002 (*LY*; 50 μM) or wortmannin (*Wort*; 200 nM) (*B*) prior to PMN-Ect and/or ZymA incubation. At 45 min, the phosphorylation of NFκB p65 (*A* and *B*) or Akt (*C*) was analyzed. All samples were measured in triplicates, and the results are given as an absorbance ratio of phosphorylated protein to total protein. *, $p < 0.05$, ZymA + PMN-Ect *versus* respective ZymA; ‡, $p < 0.05$, PMN-Ect *versus* respective macrophages alone. Results are the mean ± S.E. of at least three independent experiments. *D*, phosphorylation of Akt in cytoplasmic extracts was determined via Western blotting using an anti-phospho-Akt Ab (*pAkt*). The same blot was then reprobed for the non-phosphorylated Akt protein. The blots are representative of three independent experiments.

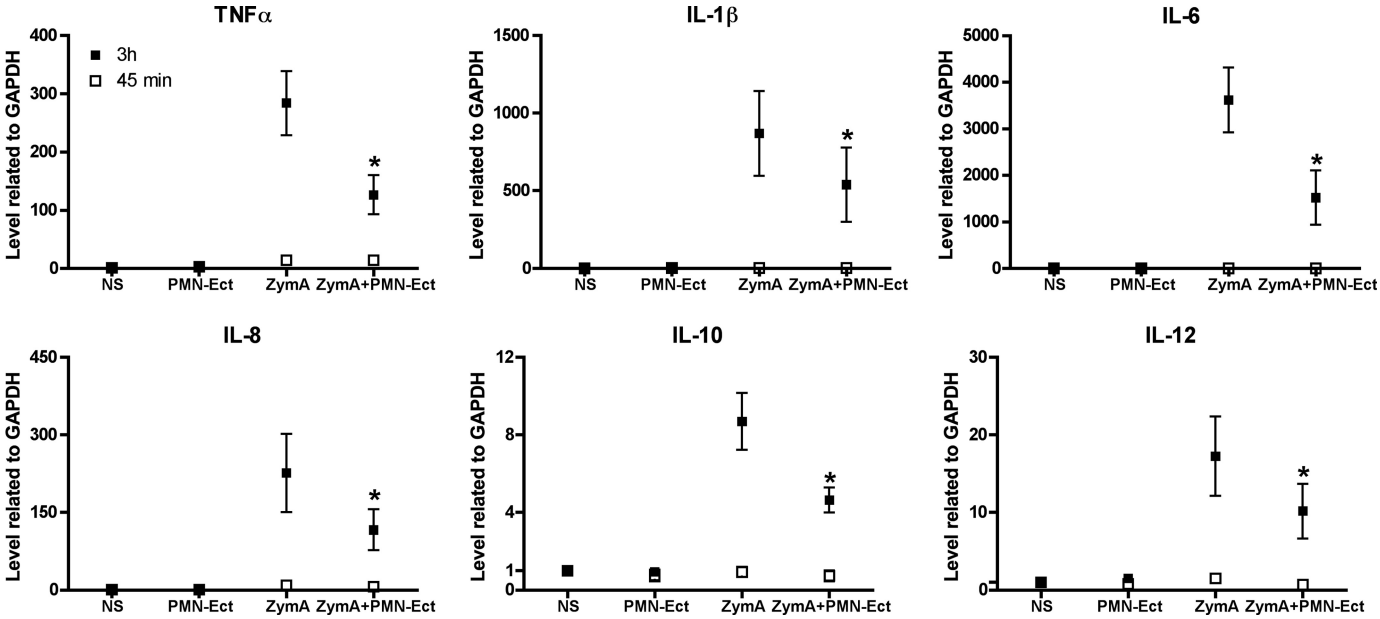

FIGURE 5. **Analysis of genes associated with inflammation and the NFκB signaling pathway by quantitative real-time RT-PCR.** HMDMs were incubated for 45 min or 3 h in medium alone (*NS*) or with PMN-Ect, ZymA, or ZymA + PMN-Ect. The differential expression of TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12 was measured by quantitative real-time RT-PCR. Results were normalized to non-stimulated (*NS*) = 1. *, $p < 0.05$, ZymA + PMN-Ect *versus* ZymA. Results represent the mean ± S.E. of at least three independent experiments measured in duplicates. □, results at 45 min; ■, results at 3 h.

was translocated to the nucleus (Fig. 2*C*). When co-incubated with ZymA and PMN-Ect, the translocation of NFκB to the nucleus was inhibited (Fig. 2*D*). We also determined the NFκB p65 levels in nuclear extracts of HMDMs alone or when they were incubated with PMN-Ect, ZymA, or both for 45 min by Western blot analysis (Fig. 3). NFκB p65 was poorly detected in resting cells, but not in the presence of PMN-Ect. Confirming our previous results, PMN-Ect abolished the ZymA-induced nuclear translocation of NFκB p65 (Fig. 3). Collectively, these observations confirmed that PMN-Ect inhibit the translocation of NFκB p65 to the nucleus and its subsequent transactivation.

PMN-Ect Induce MerTK- and PI3K/Akt-dependent Inhibition of NFκB—TAM receptors and, more specifically, MerTK are involved in the uptake of ACs by antigen-presenting cells and in the ensuing immunosuppression (18–22). Despite many structural differences, PMN-Ect share important biological properties with ACs, including the surface expression of PS, which might be bridged to MerTK (9–11). To determine whether PMN-Ect activate the downstream signaling via MerTK, we pretreated HMDMs with either anti-MerTK or control IgG Ab for 1 h and then incubated the cells with ZymA and/or PMN-Ect for 45 min and measured NFκB p65 phosphorylation. As shown in Fig. 4*A*, NFκB p65 phosphorylation was highly increased in non-treated and control IgG-treated HMDMs stimulated with ZymA, an effect that was significantly decreased by PMN-Ect. By itself, anti-MerTK Ab had no effect on the phosphorylation of NFκB in resting or ZymA-activated cells. However, the inhibitory activity of PMN-Ect on ZymA-activated cells was abolished with anti-MerTK Ab pretreatment, suggesting that MerTK is required for the biological activity of PMN-Ect.

In a recent study, AC inhibition of mouse DC activation by MerTK was mediated by the activation of the PI3K/Akt pathway, which was directly responsible for blocking NFκB signaling (24). Moreover, activation of PI3K/Akt pathway has been shown to negatively regulate NFκB in human monocytic cells (29). Thus, we investigated whether this pathway plays a role in the inhibitory effects of PMN-Ect. We pretreated HMDMs with the PI3K inhibitor LY294002 or wortmannin for 1 h and measured NFκB p65 phosphorylation after 45 min of incubation with ZymA and/or PMN-Ect. Resting or ZymA-stimulated HMDMs treated with LY294002 or wortmannin showed similar NFκB p65 phosphorylation compared with their non-treated counterparts. In contrast, LY294002 and wortmannin abolished the inhibitory effect of PMN-Ect on ZymA-stimulated HMDMs (Fig. 4*B*), demonstrating the involvement of PI3K in the inhibitory pathway induced by PMN-Ect.

To investigate whether PMN-Ect have the property to induce the Akt pathway even in the absence of ZymA, we measured Akt phosphorylation of HMDMs incubated with PMN-Ect for 45 min. Interestingly, Akt phosphorylation was highly increased in HMDMs incubated with PMN-Ect (Fig. 4, *C* and *D*). When HMDMs were treated with either anti-MerTK or control IgG Ab for 1 h, anti-MerTK Ab (but not IgG Ab) pretreatment significantly inhibited the Akt phosphorylation in HMDMs (Fig. 4*C*). As expected, pretreatment of HMDMs with LY294002 or wortmannin for 1 h blocked Akt phosphorylation (data not shown). Together, these results demonstrate that PMN-Ect have immediate inhibitory effects on ZymA-stimulated HMDMs by MerTK and the PI3K/Akt pathway-mediated inhibition of NFκB signaling.

Analysis of Gene Expression by Real-time RT-PCR—To further define the inhibitory activity of PMN-Ect in resting and ZymA-stimulated HMDMs, we investigated the early gene expression levels of six genes associated with inflammation and the NFκB signaling pathway: TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12. We measured the mRNA levels of these

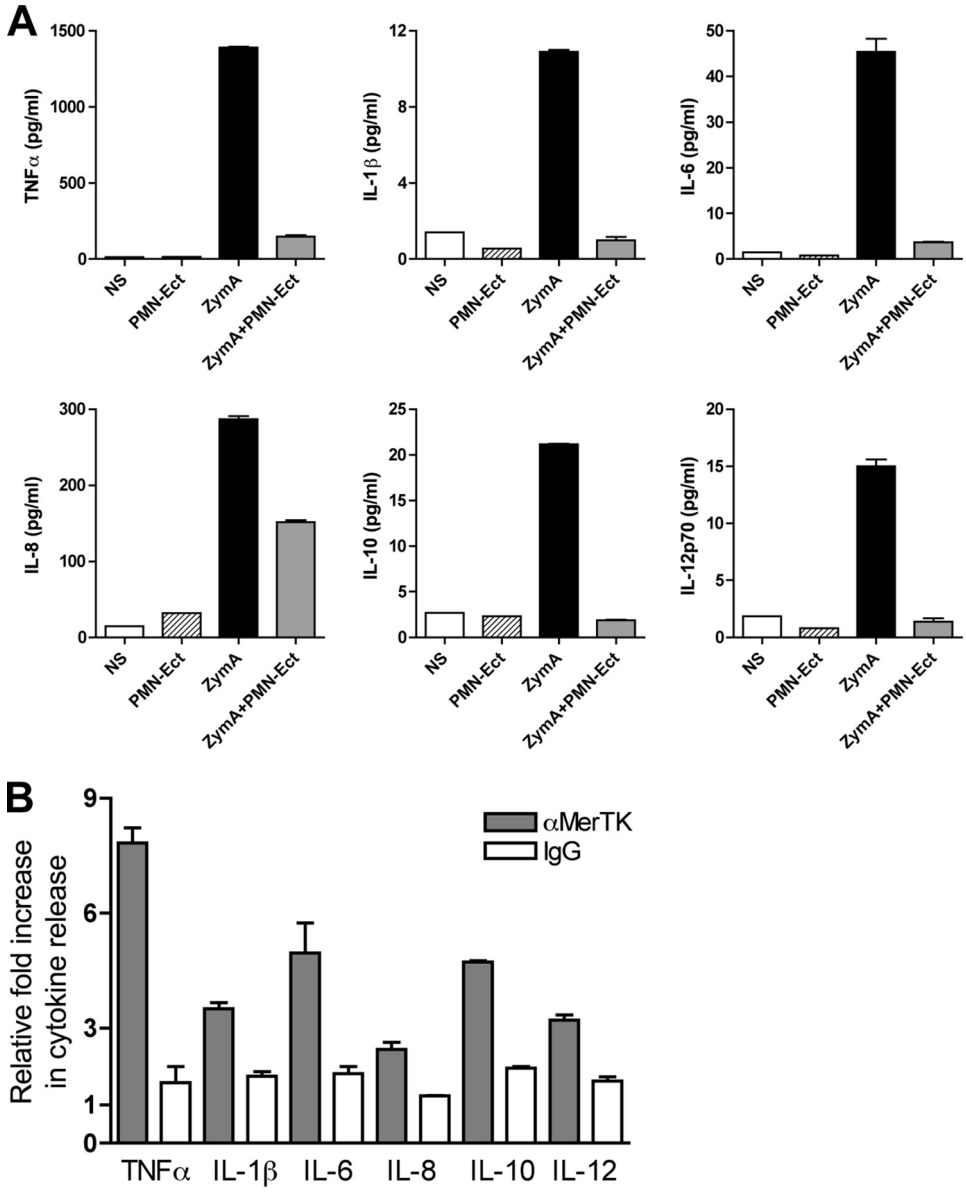

FIGURE 6. **Cytokine measurements in the supernatants of human macrophages.** *A*, HMDMs were incubated for 3 h in medium alone (*NS*) or with PMN-Ect, ZymA, or ZymA + PMN-Ect. The concentrations of TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12p70 were analyzed in the supernatants. The results shown are from one representative experiment ($n = 3$). *B*, HMDMs were pretreated for 1 h at 37 °C with 20 μg/ml goat anti-MerTK Ab or goat IgG Ab prior to ZymA and PMN-Ect incubation. The concentrations of TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12p70 were analyzed in the supernatants. The results are shown as relative -fold increase in cytokine release. The cytokine level of HMDMs incubated with ZymA + PMN-Ect = 1.

genes in HMDMs after 45 min or 3 h of incubation with or without ZymA in the absence or presence of PMN-Ect by quantitative real-time RT-PCR.

At 45 min, no changes in mRNA levels were detected whether cells were or were not stimulated with ZymA in the absence or presence of PMN-Ect. As expected, at 3 h, the mRNA levels of TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12 were highly increased when HMDMs were stimulated with ZymA (Fig. 5). Importantly, in the presence of PMN-Ect, the mRNA levels of these genes were significantly down-modulated: TNFα (level related to GAPDH), 283.7 ± 54.9 *versus* 126.3 ± 33.4 ($p = 0.011$); IL-1β, 869.1 ± 272.8 *versus* 538.8 ± 239.2 ($p = 0.046$); IL-6, 3616 ± 698.3 *versus* 1517 ± 582.9 ($p = 0.002$); IL-8, 226.3 ± 75.5 *versus* 116.6 ± 39.5 ($p = 0.046$); IL-10, 8.7 ± 1.5 *versus* 4.6 ± 0.6 ($p = 0.044$); and IL-12, 17.2 ± 5.1 *versus* 10.2 ± 3.5 ($p = 0.049$) (Fig. 5).

Measurement of Cytokines Released in the Supernatants—To confirm that the observations made by RT-PCR corresponded with the release of cytokines, we measured TNFα, IL-1β, IL-6, IL-10, IL-8, and IL-12p70 released in the supernatants. At 45 min, cytokine levels were under detection limits for all the conditions (data not shown). At 3 h, the levels of all cytokines remained unchanged when HMDMs were incubated with PMN-Ect compared with resting HMDMs (Fig. 6*A*). Compared with HMDMs alone, secretion of each of these cytokines was up-regulated when cells were stimulated with ZymA. However, co-incubation of HMDMs with PMN-Ect strongly down-modulated the release of TNFα, IL-1β, IL-6, IL-10, and IL-

12p70 and reduced the release of IL-8 (Fig. 6*A*). Similar results have been obtained previously (10), thus confirming that our RT-PCR results corresponded to protein synthesis and release.

Moreover, we measured the same cytokines in the supernatants of HMDMs pretreated with either anti-MerTK or IgG Ab for 1 h and then incubated with ZymA and/or PMN-Ect for 3 h. In the control experiments, anti-MerTK or IgG Ab pretreatment did not significantly modify the levels of cytokines released by HMDMs alone, in the presence of PMN-Ect, or stimulated with ZymA (data not shown). Of relevance was that the inhibitory effects of PMN-Ect on HMDMs stimulated with ZymA were significantly reduced by anti-MerTK Ab compared with the control IgG Ab, although the control IgG Ab had a slight nonspecific inhibitory activity (Fig. 6*B*). In sum, these results demonstrate that PMN-Ect down-modulate HMDMs through the MerTK-activated PI3K/Akt pathway, which inhibits NFκB transactivation.

DISCUSSION

Whereas the involvement of PMNs in enhancing inflammation by the release of multiple mediators and enzymes has been extensively studied, the mechanisms responsible for the control of this reaction are less well understood. That activated PMNs release ectosomes possessing anti-inflammatory and immunosuppressive properties toward macrophages and DCs is a new *in vitro* finding, which suggests that the very same cells known to be central in inflammation are involved in controlling it as well (10, 11). In this study, we identified essential signaling pathways involved in the inhibitory effect of PMN-Ect.

PMN-Ect inhibited the NFκB p65 translocation to the nucleus and its phosphorylation seen in ZymA-activated HMDMs. This NFκB blockade led to the down-regulation of the expression of major proinflammatory genes such as TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12 (Fig. 5) and logically to their down-modulation in protein release (Fig. 6*A*). Similar results were observed with DCs cultured with ACs (24); however, several studies on the down-modulation of macrophages by ACs have reported that NFκB activation was unaffected (30–32). Importantly, in some experimental setups testing for the effects of ACs or PS-expressing liposomes, macrophages or DCs had to be pretreated sometimes for several hours with ACs or PS liposomes to see an inhibitory effect on inflammatory stimuli (12, 24, 31–33). In this study, PMN-Ect were added at the same time with the stimulus, so the biological effects of PMN-Ect were observed immediately and were not dependent on a first phase of protein synthesis indispensable for these AC or PS liposome effects. Thus, our results emphasize that, despite similarities between PMN-Ect and ACs, there are also major differences in the pathways used to down-regulate inflammatory signals because PMN-Ect appear to have evident immediate effects.

MerTK is centrally involved in the inhibition of NFκB by PMN-Ect as demonstrated by using a blocking Ab. Recent *in vivo* and *in vitro* studies showed that MerTK is required for the "tolerogenic" clearance of ACs by macrophages and DCs (19, 21, 22, 34). PMN-Ect share PS exposure with ACs (9), and PS may play a key role in many of the immunomodulatory effects of both PMN-Ect and ACs. However, we do not know how the binding occurs, and in addition to PS, many other cell-surface components or soluble factors released by the target cells might be involved. For instance, GAS6 secreted by macrophages might be an efficient link to the PS exposed on PMN-Ect (18). Evidently, other possibilities will have to be explored, including the recently described Tim receptors (35), which might induce additional signaling pathways responsible for the modulation of the cellular response.

Our results showed that PMN-Ect induced inhibition of HMDMs by MerTK-mediated activation of the PI3K/Akt pathway, with a resulting blockade of NFκB (Fig. 4). The role of PI3K/Akt signaling was evidenced by using the specific inhibitors LY294002 and wortmannin. Consistent with our findings, activation of the PI3K/Akt pathway in LPS-stimulated human monocytes was shown to regulate negatively NFκB and to limit TNFα and tissue factor expression (29). Sen *et al.* (24) demonstrated similar results with AC-induced inhibition of mouse DCs. Importantly, PMN-Ect alone induced the phosphorylation of Akt (Fig. 4*C*). Thus, this inhibitory pathway was induced by PMN-Ect not only when HMDMs were activated by ZymA but also while they were resting, suggesting that PMN-Ect reprogram HMDMs immediately whether they are exposed to a specific stimulus or not.

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