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Toll-like receptors types 2 and 6 and the apoptotic process in human neutrophils

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Abstract

Introduction: Neutrophils (PMN) apoptosis plays an important role in limiting the last phase of inflammatory processes. It is unknown whether Toll-like receptor (TLR)2 acts independently or together with TLR6 in this process.
Materials and Methods: The aim of this study was to estimate the relationship between the expressions of TLR2 and TLR6 and the apoptosis of human neutrophils in physiological conditions. We investigated the influence of recombinant human interleukin (IL)-18 and N-formyl-metionyl-leucyl-phenylalanine (fMLP) on the relationships between these receptors and neutrophil apoptosis.
Results: Our results showed that after 4-h incubation, the percentage of apoptotic PMNs significantly increased compared with PMN counts before incubation. The stronger expression of TLR2 on the neutrophils suggests that this receptor contributes more significantly to the induction of PMN apoptosis than does TLR6. We also demonstrated an influence of recombinant human IL-18 (rhIL-18) on the expression of TLR6, whereas this effect was not observed in the expression of TLR2. We observed that both rhIL-18 and fMLP inhibited the apoptosis of PMNs and that rhIL-18 had a stronger effect than fMLP.
Conclusions: The obtained results suggest that not only TLR2, but also TLR6 plays an important role in the regulation of the apoptosis of PMNs. Changes in the expression of TLR6 and inhibition of apoptosis of PMNs by rhIL-18 seem to confirm the vital role this receptor and of rhIL-18 in regulating the survival of these cells. These data can be useful in developing methods to regulate PMN apoptosis in conditions associated with their excessive and unfavorable activation.

Key words: neutrophils (PMNs), TLR2, TLR6, apoptosis, IL-18, fMLP

Abbreviations: PMN – neutrophil, TLRs – Toll-like receptors, fMLP – N-formyl-metionyl-leucyl-phenylalanine.

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INTRODUCTION

Toll-like receptors (TLRs) play an important role in the recognition of pathogens. These type I transmembrane receptor family members are characterized by the presence of an extracellular leucine-rich repeat domain or one or two cystein-rich regions. The intracellular part of TLR contains a Toll/IL-1 region, homologous with the interleukin 1 receptor (IL-1R) or IL-18R, and is thus included in the IL-1 receptor family [22]. Data reported by Hayashi et al. [10] show the expression of all TLRs except for TLR3 on neutrophils (PMNs), although it is unclear whether the newly discovered TLR11 also occurs on the neutrophil surface [27]. Each of these receptors displays a variety of binding ligands. TLR2 and TLR4 are the best known. TLR2 is activated by the products of Gram-positive cell walls, lipoproteins, lipopolysaccharide (LPS), lipoteichoic acid (LTA), and yeast zymosan, while TLR4 binds mainly LPS and LTA [22]. Until now, no TLR6-specific ligands have been identified. However, TLR2 has been found to cooperate with TLR6 in response to zymosan. Hajjari et al. [9] demonstrated that phenol-soluble modulin-stimulated TLR2 activation on HEK 293 cells is enhanced in the presence of TLR6. Ozinsky et al. [23] observed that macrophage activation by Gram-positive bacteria and yeast molecules is caused by synergistic actions of TLR2 and TLR6, while TLR2 itself recognizes BLPs without TLR6. Signal transduction through TLRs induces the expression of many genes, including those regulating the production of pro-inflammatory cytokines, chemokines, inducible nitric oxide synthase, as well as co-stimulatory molecules which, apart from activating non-specific immune response cells, also trigger specific immune response mechanisms [22].

A number of reports indicate a significant role of TLR2 and TLR4 in the regulation of neutrophil apoptosis. The latest study by Sabroe et al. [25] revealed that TLR4 is an important factor that regulates PMN survival, while TLR2 inhibits, though to a lesser degree, the apoptotic process. The regulation of PMN apoptosis by TLR2 and TLR4 depends on factors that bind this receptor and occurs via pathways involving NF-κB, caspase 8, and caspase 3 activation. Zhang and Bliska [28] have shown that through TLR2 and TLR4 present on macrophages, bacterial lipoproteins (BLPs) or LPS induce the synthesis of factors that prolong the life of cells. However, evidence provided by Into et al. [13] indicates that TLR2 on BLP-stimulated lymphocytes, monocytes, and macrophages induces apoptosis via caspase activation. We found a correlation between TLR2 expression on recombinant human IL-15 (rhIL-15)--stimulated neutrophils and their apoptosis. The increased TLR2 expression on neutrophils was substantially enhanced due to rhIL-15 stimulation and the percentage of apoptotic cells was reduced compared with unstimulated PMNs [20]. There is, however, lack of evidence to confirm the involvement of TLR2 in apoptosis. It is unknown whether this receptor acts independently or together with TLR6. Moreover, no data are available on the role of TLR6 in the regulation of neutrophil apoptosis.

The aim of the current study was to determine the relationship between the expressions of TLR2 and TLR6 and the apoptosis of human neutrophils in physiological conditions. We evaluated the effect of rhIL-18 and N-formyl-metionyl-leucyl-phenylalanine (fMLP) on the relationship between these receptors and apoptosis.

MATERIALS AND METHODS

Cells

We examined 20 healthy donors aged 22–55 years (mean 38.5 years). PMNs were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient (1.115 g/ml). This method enables the simultaneous separation of two highly purified leukocyte fractions: mononuclear cells, containing 95% lymphocytes, and polymorphonuclear cells, containing 94%. The purity of the isolated PMNs and peripheral blood mononuclear cells was determined by May-Grünewald-Giemsa staining. The cells were suspended in culture medium (RPMI-1640, with 10% autologous serum, 10 U/ml penicillin, and 50 ng/ml streptomycin) to provide 5×10^6 cells/ml. The PMNs were cultured in 96-well plates (Falcon) for 4 h at 37°C in a humidified incubator with 5% CO_2 (NUAIRE™, USA). rhIL-18 (50 ng/ml; R&D Systems) and fMLP (50 ng/ml; Sigma--Aldrich, USA) stimulated PMN apoptosis, and the expressions of TLR2 and TLR6 in the PMN lysates were tested.

Apoptosis

Annexin V binding of neutrophils was performed using an apoptosis detection kit (APOTEST™-FITC, Nexins Research, Holland). Briefly, 10^6 cells/ml freshly isolated cells or cultured cells were labeled with FITC-conjugated annexin V and propidium iodide (PI) for 20 min at +4°C. The samples were analyzed by two--color flow cytometry using CellQuest Analysis software (Becton Dickinson, Epics Coulter, USA). Results were reported as the percentage of annexin V-positive cells, which reflects the relative proportion of apoptotic cells in the early and late stages of apoptosis. If a cell is undergoing necrosis, annexin V- and PI-positive cells would be expected, whereas the presence of FITC-annexin V-positive but PI-negative cells is indicative of apoptotic rather than necrotic cell death. At the same time, cells were marked with ethidium bromide and acridinium orange and counted by immunofluorescent microscopy.

Western blot

Cytoplasmic protein fractions of PMNs were analyzed for the presence of TLR2 and TLR6. The cells were lysed directly by sonication, and cytoplasmic proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA). The resolved proteins were transferred onto nitrocellulose (0.2 μm; Bio-Rad Laboratories, Hercules, CA, USA) and incubated with the primary anti-TLR2 and anti-TLR6 monoclonal antibodies (Alexis, Carlsbad, CA, USA). The membranes were incubated at room temperature with alkaline phosphatase anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). Immunoreactive protein bands were visualized by the BCIP/NBT Liquid substrate system (Sigma-Aldrich, USA), determined using Lab-Image 1 Gel software and estimated in arbitrary units.

Statistical analysis

Data were expressed as the mean ±SE. Differences between the groups of neutrophils were tested by Student's *t*-test, with $p<0.05$ considered the level of significance.

RESULTS

Neutrophil apoptosis

It was observed that 0.36% of the freshly isolated PMNs had features of early apoptosis (Annexin V binding cells), while 0.49% had features of late apoptosis/necrosis (Annexin V- and PI-binding cells). After a 4-h incubation the percentage of late-phase apoptotic PMNs increased significantly to 1.17%. Incubation of PMNs with rhIL-18 and fMLP reduced the percentage

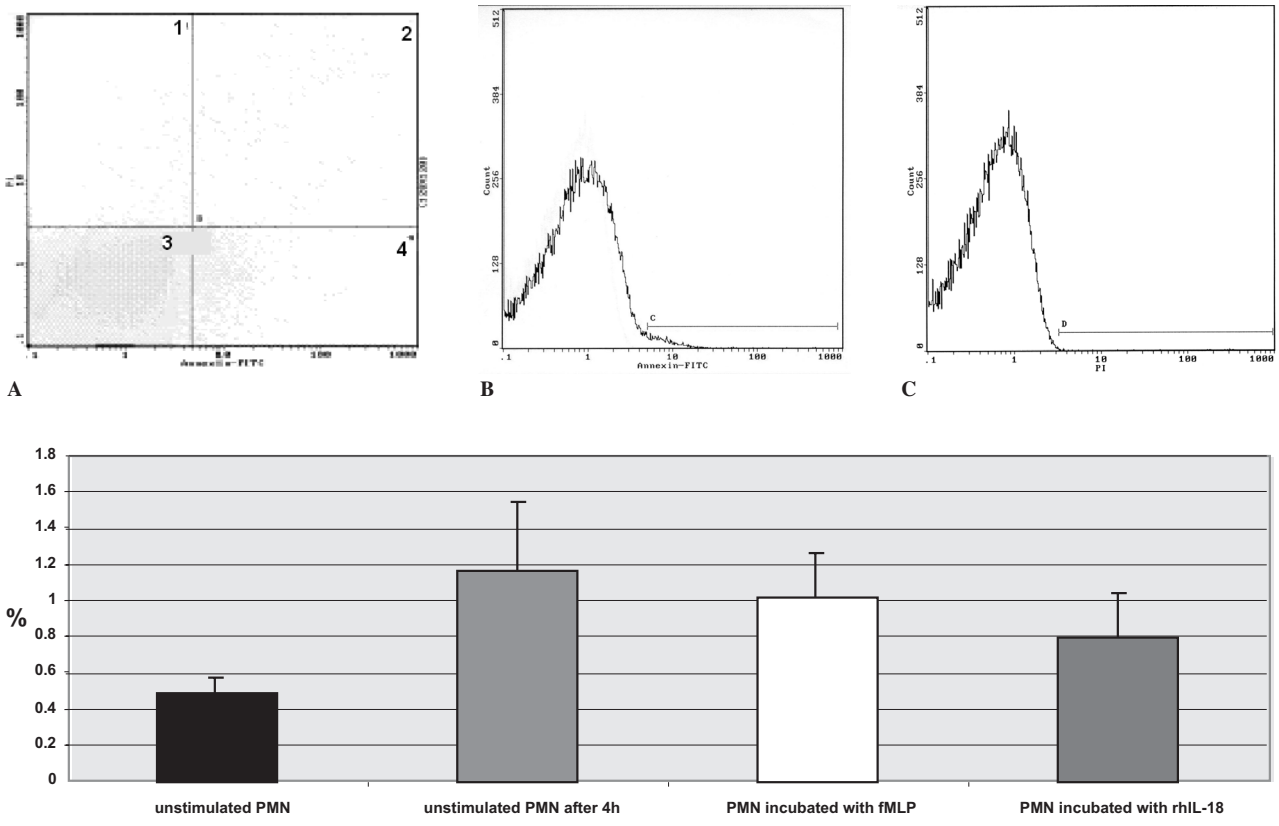

Fig. 1. One of the few experiments of apoptosis of PMN in flow cytometry. Flow cytometry expression of apoptosis PMN (**A**). These pictures show normal human PMN labeled with Annexin V-FITC (**B**) and propidium iodide (PI; **C**) from a normal volunteer. Numbers within quadrants represent the percentage of cells within each quadrant. Surviving cells (low in Annexin V and PI signal) appear in the lower left quadrant (number 3). Early apoptotic cells (high in Annexin V signal but low in PI signal) appear in the lower right quadrant (number 4). Late apoptotic cells (high in both Annexin V and PI signal) appear in the upper right quadrant (number 2). Necrotic cells (high in PI signal) appear in left upper quadrant (number 1). *Statistical difference between fresh isolated unstimulated PMN and unstimulated PMN after 4-h incubation.

of late-phase apoptotic cells to 0.8% (in the presence of rhIL-18) and to 1.025% (in the presence of fMLP) compared with unstimulated cells incubated under the same conditions (Fig. 1).

Neutrophil apoptosis was also assessed using immunofluorescence with acridine orange and ethidium bromide. After a 4-h incubation, the percentage of apoptotic PMNs increased significantly compared with that before incubation. Neutrophil incubation in the presence of rhIL-18 lowered the mean percentage of apoptotic cells compared with non-stimulated PMNs (Table 1).

Expressions of TLR2 and TLR6 in PMN lysates

The presence of intracellular proteins TLR2 and TLR6 was confirmed using specific monoclonal antibodies and Western blot. TLR2, a 90-kDa protein, was detected in these cells, its expression enhanced in fMLP-stimulated neutrophil lysates. However, its expression in the rhIL-18-stimulated cell lysates was comparable to that observed in unstimulated cells (Fig. 2). TLR6, a protein of approximately 92 kDa, was identified in PMN lysates. A comparison of the intensities of the bands corresponding to TLR2 and TLR6 revealed a weaker expression of TLR6 than of TLR2 in unstimulated PMNs. Moreover, neutrophil incubation with fMLP enhanced TLR2 expression in neutrophils, while rhIL-18 affected TLR6 expression (Fig. 2).

DISCUSSION

Effective neutrophil apoptosis plays a key role in the elimination and termination of the inflammatory

Table 1. Apoptosis of PMNs by the immunofluorescent agents

Unstimulated PMN	Unstimulated PMN after 4 h	PMN incubated with fMLP	PMN incubated with rhIL-18
4.857±3.6	10.85±3.4*	9.57±3.7	8.71±2.8

* Statistical difference between freshly isolated unstimulated PMN and unstimulated PMN after 4-h incubation.

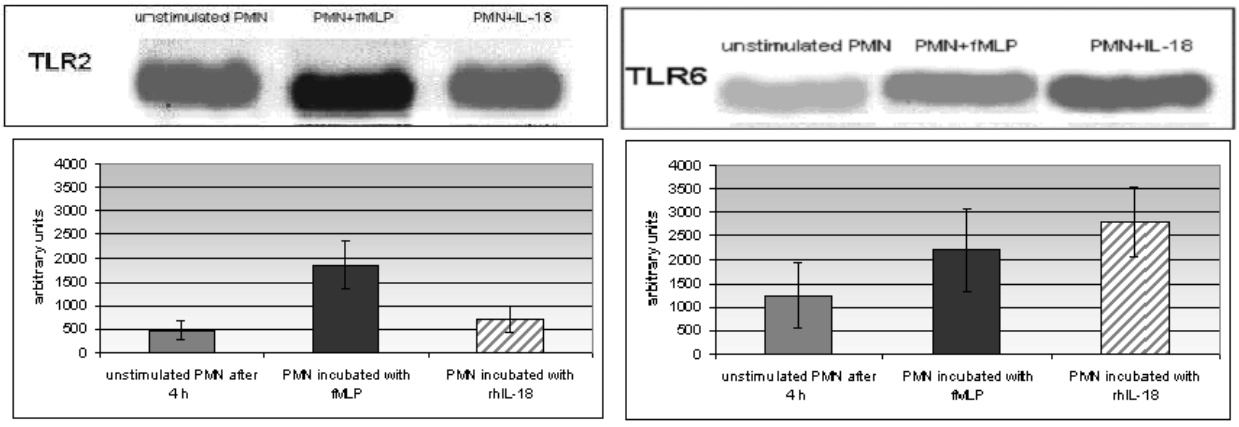

Fig. 2. Western blot analysis of TLR2 and TLR6 protein expression in human PMNs. Shown is the detection of TLR2 and TLR6 proteins from human PMNs; unstimulated PMN, fMLP stimulated PMN, rhIL-18 stimulated PMN. whole-cell lysates were obtained from fresh PMNs of healthy donors. One hundred micrograms of total protein from the cells were loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with 1:1000 dilution of monoclonal antibody anti-TLR2 and anti-TLR6. One representative Western blot of a few independent experiments is shown. The diagram below is shown statistical analysis of Western blot of TLR2 and TLR6 from a few independent experiments estimated by arbitrary units. * Statistical differences between fresh isolated unstimulated PMN and unstimulated PMN after 4-h incubation

process in which PMNs are involved. TLR2 and TLR6 present on the neutrophil surface may have a significant effect on apoptosis regulation. TLR2 appears to be a molecular link between microbial products, apoptosis, and host defense mechanisms, because the induction of cytokine production by microbial lipoproteins in human cells is mediated by TLR2 and other TLRs and because cell activation and apoptosis induced by bacterial products are signaled through human TLR2 [2]. Aleman et al. [1] revealed that apoptosis of *Mycobacterium tuberculosis*-activated PMNs proceeds through a TLR2-dependent pathway and involves the p38 mitogen-activated protein kinase pathway, which in turn would induce PMN activation and apoptosis. There is evidence that TLR-induced apoptosis modulates inflammation and immune activation during *M. tuberculosis* infection. Aliprantis et al. [2] observed that binding of TLR2 with BLPs in human THP-1 line monocytes is sufficient for apoptosis induction through MyD88 bound with FADD and the caspase 8 pathway. However, Into et al. [12] revealed that diacylated lipoproteins of *Mycoplasma fermentas* enhance apoptotic activity of the cell line HEK 293 along p38-, MyD88-, and FADD-dependent pathways not only through TLR2 mediation, but also with TLR6 involvement.

The latest reports by Francois et al. [7] found that TLR agonists, which delay apoptosis, also extend the functional life span of PMNs. They observed that agonists of TLR4 (LPS), TLR2 (peptidoglycan), TLR7/8 (R-848), and TLR9 (CpG-DNA) were equally effective in delaying spontaneous apoptosis of PMNs, while TLR1/2 (Pam_3CSK_4), TLR2/6 (macrophage-activating lipopeptide-2), TLR5 (flagellin), and TLR7 (loxoribine) were less effective or inactive [7]. Other authors also indicated that LPS completely prevented neutrophil apoptosis at early time-points (4 h) via a mechanism dependent on NF-κB and MAPK signaling cascades and, in contrast, Pam_3CSK_4-induced TLR2 signals showed less efficacy in preventing neutrophil apoptosis [24].

We observed relationships between the enhancement of neutrophil apoptosis and TLR2 and TLR6 expression in these cells. Four-hour incubation led to an increase in the percentage of apoptotic neutrophils and intensified the expressions of TLR2 and TLR6 on unstimulated cells. Stronger TLR2 expression on neutrophils indicates its more substantial contribution to apoptosis induction compared with TLR6.

It has been found that TLR expression may undergo a change when affected by a number of various factors, including cytokines. It has been noted, for instance, that TLR2 expression on neutrophils is enhanced under the effect of IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF), but reduced in the presence of tumor necrosis factor (TNF)-α [5, 15]. We observed that IL-15 is also responsible for the enhancement of TLR2 expression [20]. However, no reports are available on changes in the regulation of TLR6 expression, and this refers both to cytokines and other factors. The presence of IL-18-specific receptors on PMNs indicates that the cytokine can be exploited for the cell activity. Few studies have demonstrated the effect of IL-18 on neutrophil functions. It has been observed that this cytokine enhances the expression of adhesion molecules, migration capacity, and respiratory burst in these cells [6, 19]. Marino et al. [21] show that IL-18 differentially regulates apoptosis of liver endothelial cells mediated by the death-inducing factors, TNF and Fas. Yoon et al. [26] observed that transfection of the IL-18R selectively induced a slight enhancement of Fas via the up-regulation of intracellular reactive oxygen species and IL-18 in cervical carcinoma C33A cells, whereas there were no effects on the expression of p53, intercellular

adhesion molecules-1, and Fas ligand. We observed a modulatory effect of rhIL-18 on the increase in TLR6 expression. However, no such effect was observed on TLR2 expression in neutrophils.

No reports are available on the effect of fMLP on the expressions of neutrophil TLR2 and TLR6. Our study outcome shows that fMLP enhances them both. However, it has been found that fMLP affects TLR2 more than TLR6, which suggests that this factor is first of all a specific TLR2 stimulant. These observations appear particularly important, especially as literature data on the subject are lacking. Up to now, a number of studies have demonstrated that apoptosis undergoes cytokine regulation. It has been found that IL-1, IL-2, IL-4, IL-15, interferon γ, LPS, and GM-CSF inhibit cell apoptosis, while TNF-α, apart from IL-6, in high doses enhance the process in PMNs [8, 16, 18]. Our previous examinations have shown the ability of IL-15 to induce apoptosis of PMNs [20]. Results of the present study suggest that both rhIL-18 and fMLP inhibit PMN apoptosis, with rhIL-18 inducing a stronger effect than fMLP. Opinions concerning the effect of these two factors on apoptosis are contradictory. Leung et al. [19] observed no effect of IL-18, while Brach et al. [3] and Klein et al. [14] saw no effect of fMLP on neutrophil apoptosis. However, Colotta et al. [4], Hu et al. [11] and Lee et al. [16, 17] noted that fMLP inhibits neutrophil apoptosis despite high intracellular concentrations of Ca^{2+} ions, which disturb cell potential and induce apoptosis. Inhibition of neutrophil apoptosis by rhIL-18 or fMLP action prolongs the effective action of these cells, which may affect the conditions occurring with excessive amounts of these agents.

The current study was the first to indicate that not only TLR2, but also TLR6 could play a significant role in apoptosis regulation. The rhIL-18-induced changes in TLR6 expression and synergistic inhibition of apoptosis stimulated by this cytokine seem to confirm a significant role of this receptor and rhIL-18 in the regulation of neutrophil survival. These data can be useful in the evolution of methods to regulate PMN apoptosis in conditions associated with their excessive and unfavorable activation.

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