The influence of rhIL-17 on the expression of Toll-like receptor-2 and neutrophil apoptosis – a preliminary study

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SUMMARY

The influence of rhIL-17 on the expression of Toll-like receptor-2 and neutrophil apoptosis – a preliminary study

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Effective elimination of neutrophils (PMNs) via the apoptotic pathway is an essential mechanism regulating the number of these cells in blood circulation. Disorders in neutrophil apoptosis are a likely cause of inflammatory states responsible for tissue damage. Numerous reports show that survival of PMNs depends on different molecules, including Toll-like receptor type 2 (TLR2). According to many data, its expression on neutrophils is regulated by various cytokines, such as IL-1, IL-2, IL-3, IL-6, GM-CSF, TNF-α. The last findings seem to confirm a significant role of TLR2 in the regulation of PMN apoptosis. TLR2 is a new "death receptor" that enhanced apoptosis machinery – TLR2 signals for apoptosis through MyD88 and then FADD and caspase-8.
The aim of the current study was to estimate the influence of rhIL-17 on neutrophil TLR2 expression and neutrophil apoptosis in physiological conditions.
***Results.** The findings indicate enhanced apoptosis and a simultaneous increase in TLR2 expression on the rhIL-17-stimulated PMNs.*
***Conclusions.** The study suggests a potential role of rhIL-17 in the regulation of PMN survival via a TLR2-dependent pathway.*

Key words: *PMNs, TLR2, rhIL-17, apoptosis*

STRESZCZENIE

Wpływ rhIL-17 na ekspresję receptora Toll-like-2 i apoptozę neutrofilów – badania wstępne

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Skuteczna eliminacja neutrofilów (PMNs) na drodze apoptozy jest podstawowym mechanizmem regulującym liczbę tych komórek w obiegu krwi. Zaburzenia apoptozy neutrofilów są prawdopodobną przyczyną stanów zapalnych odpowiedzialnych za uszkodzenia tkanek. Liczne raporty pokazują, że przeżycie PMNs zależy od różnych cząsteczek, w tym Toll-like receptora typu 2 (TLR2). Według wielu danych ich ekspresja na neutrofilach jest regulowana przez różne cytokiny, takie jak IL-1, IL-2, IL-3, IL-6, GM-CSF, TNF-α. Ostatnie badania wydają się potwierdzać istotną rolę TLR2 w regulacji PMN apoptozy. TLR2 jest nowym „receptorem śmierci", który zwiększa mechanizm apoptozy – sygnał TLR2 do apoptozy następuje za pośrednictwem MyD88 a następnie FADD i kaspazy-8.
***Celem obecnej pracy** była ocena wpływu rhIL-17 na ekspresję TLR2 na PMNs i apoptozę w warunkach fizjologicznych.*
***Wyniki.** Wyniki badań wskazują zwiększenie apoptozy i jednocześnie nasilenie ekspresji TLR2 na PMNs stymulowanych rhIL-17.*
***Wnioski.** Badanie sugeruje potencjalną rolę rhIL-17 w regulacji przeżycia PMN na drodze TLR2 zależnej.*

Słowa kluczowe: *PMNs, TLR2, rhIL-17, apoptoza*

Polymorphonuclear neutrophils (PMNs), like other cells of the human body, are subjected to the mechanisms regulating their number via the apoptotic pathway. The process of apoptosis is one of the mechanisms protecting the surrounding tissues against a deleterious action of reactive oxygen species (ROS) and enzymes that are released by the cells. Many cytokines are involved in neutrophil apoptosis, including TNF superfamily members: TNF-α, FasL, and TRAIL [19]. Some investigators have reported that the regulation of PMN apoptosis involves the apoptotic signaling pathway activated by Toll-like receptors (TLRs) [5, 18].

Toll-like receptors (TLRs) are known to recognize pathogen-related molecular patterns [4,13]. Among 13 human TLRs so far identified, TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 have been found to occur on the neutrophil surface [22].

TLR2 and TLR4 are the best known Toll-like receptors. It has been shown that TLR2 plays an important role in the induction of nonspecific response, as it recognizes lipoproteins that build cell membranes of such bacteria as *Borrelia burgdorferi*, *Treponema pallidum*, *Staphylococcus aureus*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, LPS, lipoteichoic acid (LTA), peptydoglicans (PGN) and yeast zymosan [12, 21]. Moreover, it has been observed that TLR2 activation is associated with the production of proinflammatory cytokines. Previously, we revealed a relationship between the increased TLR2 expression on neutrophil surface and elevated IL-6 – and inhibited IL-1β production by these cells isolated from blood of patients with Lyme disease [10].

It has been reported that TLR expression may undergo a change when affected by a number of various factors, including proinflammatory cytokines [11, 16, 17]. One of them is IL-17 which regulates many vital neutrophil functions e.g. their recruitment and release of elastase, mieloperoxydase (MPO) and metalloproteinase [7, 15, 20].

The aim of the current study was to estimate the influence of rhIL-17 on neutrophils (PMNs) TLR2 expression in confrontation with their apoptosis.

MATERIAL AND METHODS

The study group included 20 healthy volunteer blood donors, aged 22-55 years (mean 38.5 years). PMNs were isolated from peripheral blood using Gradisol G – 1.115g/ml according to *Zeman* et al. The suspension of granulocytes contained approximately 95% of neutrophils on average, which was assessed in specimens stained by the May-Grunwald-Giemsa method. Cell vitality evaluated by trypan blue dye was 98% on average. Cells ($5x10^6$ cells/ml) were suspended in culture medium (RPMI-1640, 10% patients' own serum, 10 U/ml penicillin, 50 ng/ml streptomycin) and incubated in microplates at a temperature of 37°C, in an incubator with a flow of 5% CO_2 (NUAIRE™) for 4 and 20 hours. Cells were stimulated using fMLP (N-formyl-methionyl-leucyl-phenyloalanine) (50 ng/ml) and rhIL-17 (50 ng/ml).

Apoptosis

Assessment of apoptosis was performed by the immunofluorescence method using acridine orange and ethidium bromide after 4 and 20 hours. Cells with features of apoptosis were evaluated using cell- and nucleus-based fluorescence (orange colour). Results were presented as the percentage of apoptotic cells.

Neutrophil apoptosis was also assessed by flow cytometry using Annexin and propidine iodine.

Western blot

After removal of the PMN culture supernatant, cell suspensions were rinsed with cooled PBS, suspended in TRIS-Cl pH 7.5 and then subjected to lysis by sonification. Protein was determined in cell homogenates by Lowry's method. The material obtained was suspended in Laemly's buffer. The cytoplasmic fraction of proteins underwent polyacrylamide gel electrophoresis (SDS-PAGE). The isolated proteins were transferred onto nitrocellulose (0.2 μm) and incubated with primary monoclonal antibodies anti-TLR2. Next, the membrane was incubated at room temperature with mouse anti-IgG antibodies labelled with alkaline phosphatase. Immunoreactive TLR2 protein bands were visualized using the BCIP/NBT liquid substrate system, determined using LabImage 1 Gel software and estimated by arbitrary units.

Statistics

Results concerning the percentage of apoptotic neutrophils were subjected to statistical analysis using the test for comparison of differences between two indices. However, the values of TLR2 expression showing normal distribution in the Kołmogorov-Smirnov test were analysed using the t-Student test. Calculations were performed by means of a statistical package Statistica 5.2 PL. The differences were considered statistically significant for $p<0.05$.

RESULTS

Neutrophil apoptosis assessed by flow cytometry and by immunofluorescence microscopy

Statistically significant differences were found between the percentage of apoptotic PMNs isolated directly from blood and those incubated for 4hr and for 20hr, as shown by flow cytometry and immunofluorescence with acridine orange and ethidium bromide. Prolonged incubation (up to 20 hr) increased the percentage of apoptotic PMNs as compared to the values obtained after 4hr incubation (tab. 1).

Moreover, after 4hr incubation with rhIL-17, the percentage of apoptotic cells increased significantly compared to the percentage of non-stimulated apoptotic cells assessed in the same period. The same results was observed after 20hr of incubation with rhIL-17 (fig. 1, tab. 1). Compared with rhIL-17-treated neutrophils, no significant effect on apoptosis was observed over the first 4hr when cells were stimulated with fMLP. In contrast, incubation of PMN with fMLP resulted in a statistically significant increase in the percentage of apoptotic neutrophils at 20hr.

Table 1. Percentage of apoptotic neutrophils

%	PMN directly after isolation	PMN after 4hr incubation			PMN after 20hr incubation		
		Non-stimulated PMN	fMLP – stimulated PMN	rhIL-17 stimulated PMN	Non-stimulated PMN	fMLP – stimulated PMN	rhIL-17 stimulated PMN
	6,5± 1,29	14,25*± 3,8	11,25 ± 3,7	26,25[a] ± 3,7	24,25*#± 4,03	19,75[b]± 6,2	39,75[a] ± 10,53

* – statistically significant differences between PMN directly after isolation and PMN after incubation
# – statistically significant differences between non-stimulated PMN and PMN after incubation
a – statistically significant differences between non-stimulated and rhIL-17 PMNs
b – statistically significant differences between PMN directly after isolation and fMLP-stimulated

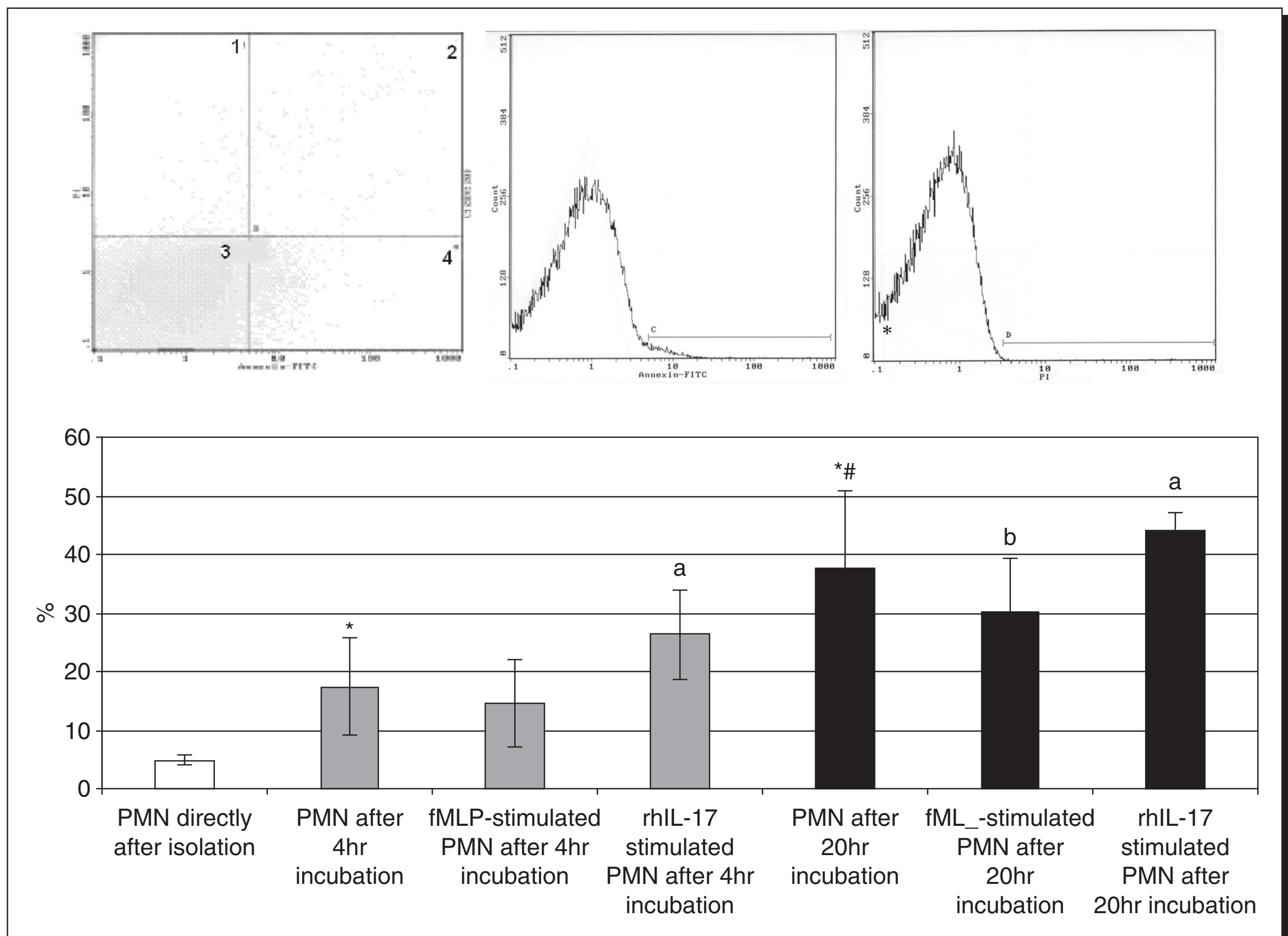

Fig. 1. An example of neutrophil apoptosis assessed by flow cytometry
Flow cytometry expression of apoptosis PMN (A). These diagrams show normal human PMN labeled with Annexin V-FITC (B) and propidium iodide (PI) (C) from a normal volunteer. Numbers within quadrants represent the percentage of cells 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). * – statistically significant differences between PMN directly after isolation and PMN after incubation, # – statistically significant differences between non-stimulated PMN and PMN after incubation, a – statistically significant differences between non-stimulated and rhIL-17 PMNs , b – statistically significant differences between PMN directly after isolation and fMLP-stimulated

The presence of intracellular protein TLR2 was confirmed by the Western blot method using specific monoclonal antibodies. TLR2, 90 kDa, was detected in unstimulated PMNs in 4hr and 20hr incubation. The expression was enhanced in the fMLP-stimulated neutrophil lysates and increased in the rhIL-17-stimulated cell lysates as compared to the non-stimulated cells (fig. 2).

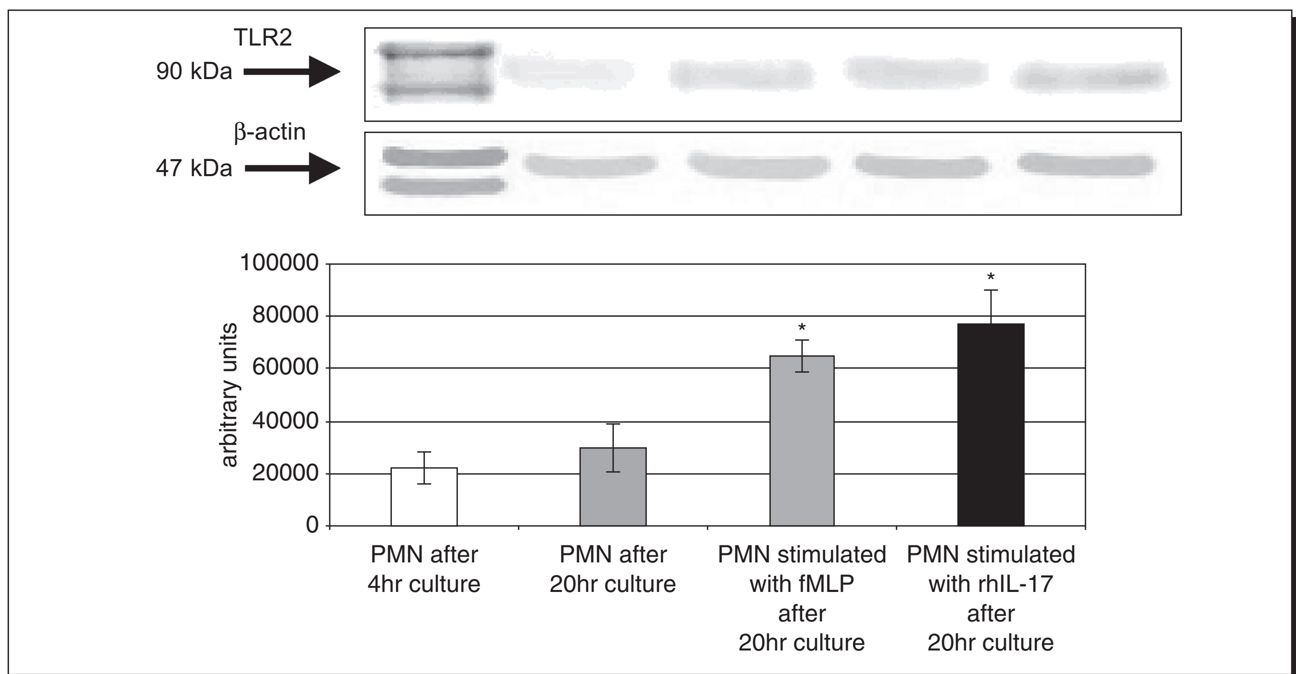

Fig. 2. Western blot analysis for TLR2 in PMN after 4hr and 20hr culture
* – statistically significant differences between non-stimulated and rhIL-17 stimulated cells after 20hr incubation

DISCUSSION

Our study shows that rhIL-17 increased of neutrophils apoptosis. We observed the similar effect to that reported by *Dragon* et al., suggesting that IL-17 enhances apoptosis of these cells in vitro. Moreover, they have noted that IL-17 affects PMN apoptosis and that it is associated with reduced anti-apoptotic Mcl-1 protein levels [3].

Simultaneusly, we found for the first time that rhIL-17 enhances TLR2 expression in human neutrophils. As revealed in our earlier study, rhIL-15 inhibits neutrophils apoptosis and at the same time increases TLR2 expression [14]. We observed a similar effect of rhIL-18 on PMN apoptosis [9]. However, in contrast to rhIL-15, we found no change in the expression of TLR2 on PMNs stimulated with rhIL-18.

TLR2 appears to be a molecular link between microbial products, apoptosis and host defense mechanisms, because cell activation, induction of cytokine production and apoptosis in human cells is mediated by TLR2 and induced by bacterial products [1]. *Into* et al. have observed that TLR2 on lymphocytes, monocytes and macrophages stimulated by BLPs induced apoptosis via activation of caspase pathway [8].

Also *Aliprantis* et al. have reported that apoptosis induction in the experimental cell line 293 occurred with the involvement of TLR2, called "death receptor" [1]. They indicated that BLPs in 293 cells induce apoptosis directly through TLR2. The TLR2 by binding with the adaptor protein MyD88 and then with FADD (death domain binding Fas and TRAIL) activates caspase-8 [17, 18, 22]. MyD88 contain sequences with homology to conventional DD (domain death). Interaction between the DD of MyD88 and FADD transmits the signals for NF-κB activation and apoptosis downstream of TLR2 activated by bacterial lipoproteins (BLPs) in both 293 cells and monocytes [1].

However, *Lotz* et al. have reported that PMN apoptosis is inhibited by lipoteichoic acid (LTA), i.e. a ligand for TLR2 [13]. Also *Francois* et al. observed the same effect when PMNs were stimulated by another agonist of TLR2, peptidoglycan (PGN), and noted that apoptosis inhibition by PGN was accompanied by elevated levels of the anti-apoptotic proteins Mcl-1 and A1 and reduced caspase-3 [5].

Recent studies provide evidence that cytokines regulate the expression of Toll-like receptor 2 (TLR2) on the surface of human neutrophils and process of neutrophils apoptosis. It has been found that GM-CSF decrease expression of TLR2 and PMN apoptosis [2, 4, 6]. However, *Muzio* et al. and *Kurt-Jones* et al. have obtained different results, demonstrating that G.

CONCLUSIONS

The changes in TLR2 expression found simultaneously with increased number of rhIL17-stimulated apoptotic neutrophils suggest that this cytokine may play a role apoptosis neutrophils by the TLR2-dependent mechanism. The above observations indicate a new aspects of biological activity of IL-17. Further studies could elucidate whether enhancement

of neutrophils apoptosis by IL-17 occurs via the receptor or mitochondrial pathway.

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