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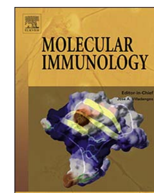

Full length article

Protective role of surface Toll-like receptor 9 expressing neutrophils in local inflammation during systemic inflammatory response syndrome in mice

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ABSTRACT

Clinically, systemic inflammatory response syndrome (SIRS) occurs after serious trauma or sepsis. In sepsis, neutrophils are the major effector cells responsible for eliminating pathogens. However, the role of neutrophils in development of SIRS, especially in local inflammatory area, is controversial. In this study, we established a SIRS mouse model characterized with cytokine-mediated lethal shock by intraperitoneal injection of oligodexynucleotides containing CpG motifs (CpG ODN) in D-galactosamine (D-GalN) sensitized mice based on our previous work and found that abundant neutrophils were rapidly recruited into the peritoneal cavity, where some neutrophils expressed surface TLR9 (sTLR9), defined as sTLR9$^+$ neutrophils. Along with the progression of SIRS, the expression of sTLR9 in sTLR9$^+$ neutrophils isolated from peritoneal lavage cells (PLCs) was declined in accompany with an increase in the level of the inflammatory cytokine TNFα and a decrease in the level of the anti-inflammatory cytokine IL-10 in Ly6G$^+$ PLCs. When using CCT ODN, an oligodeoxynucleotide with CCT repeats, which we have previously shown to be capable of rescuing mice from lethal shock, the expression of sTLR9 on neutrophils was significantly elevated. Adoptive therapy using early recruited neutrophil-rich PLCs containing sTLR9$^+$ neutrophils that express high levels of sTLR9, could rescue mice from SIRS. Overall, the data reveal that the early recruited sTLR9$^+$ neutrophils may, at least in the area of local inflammation, play a protective role during SIRS development and provide a method to rescue the patients with severe SIRS via the up-regulation of sTLR9 levels on the surface of neutrophils or via adoptive therapy with protective sub-populations of neutrophils.

1. Introduction

Systemic inflammatory response syndrome (SIRS), a major cause of morbidity and mortality worldwide, occurs after serious trauma or sepsis. It is characterized by shock and the compromised function of several organs (Calfee and Matthay, 2010). Neutrophils are the dominant participants in sepsis, forming the first line of defense against bacterial pathogens (Sadik et al., 2011). After entry into inflammatory tissue, a stimulus by the tissue causes neutrophils to become fully activated to release granule proteins and produce neutrophil extracellular traps, which have an enhanced pathogen-destructive capacity (Mayadas et al., 2014). In addition, neutrophils also contribute to the significant tissue damage that occurs as a result of acute and chronic disease processes. An imbalance between the pro-inflammatory response and anti-inflammatory response can result in host damage caused directly by extravagant inflammation, or indirectly through immune dysfunction (Buras et al., 2005). The massive recruitment of neutrophils, occurring during the sterile inflammation (McDonald and Kubes, 2011), may be detrimental due to their release of oxidants, proteases, and antimicrobial proteins, causing perpetual or nonresolving bystander injury (Chen and Nunez, 2010). However, up to now, few studies have examined the protective role of neutrophils in the development of SIRS.

SIRS caused by severe trauma has been attributed to a massive release of mitochondrial DNA (mtDNA) from the injured cells into the

Abbreviations: SIRS, systemic inflammatory response syndrome; D-GalN, D-galactosamine; CpG ODN, oligodexynucleotides containing CpG motifs; CCT ODN, oligodexynucleotides containing CCT motifs; mtDNA, mitochondrial DNA; PLCs, peritoneal lavage cells; sTLR9, surface Toll-like receptor 9; PMNs, polymorphonuclear cells; TNFα, tumor necrosis factor alpha; IL-10, interleukin-10; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns

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circulation (Zhang et al., 2010). As a result of its evolutionary history, the mtDNA shares many similarities with bacterial DNA, containing inflammatogenic unmethylated CpG motifs that are recognized by Toll-like receptor 9 (TLR9). It has been reported that CpG synthetic oligodexynucleotides (ODN)-induced TLR9 activation can induce an excessive cytokine-mediated lethal shock in D-galactosamine (D-GalN)-sensitized mice, during which massively released pro-inflammatory cytokines such as TNF-α and IL-12 were detected in the sera of mice (Hemmi et al., 2000; Sun et al., 2010; Wang et al., 2007). Conventionally, TLR9 is located in endosomal/lysosomal compartments and activated by unmethylated CpG motifs present in pathogen-derived DNA as well as in synthetic ODNs. Recently, TLR9 has been detected on the surface of human and mouse neutrophils. This type of TLR9, designated as surface TLR9 (sTLR9), can sense mtDNA and initiate signaling pathways that lead to acute inflammation (Lindau et al., 2013). The expression of sTLR9 on neutrophils can be upregulated in response to stimulation (Lindau et al., 2013). However, it remains elusive whether and how the sTLR9 expressing ($sTLR9^+$) neutrophils are involved in the development of SIRS.

Neutrophils have long been regarded as the main phagocytes, but recently have been found to produce a variety of chemokines or cytokines, in response to microenvironmental stimuli in a temporal and stimulus-dependent manner (Tecchio et al., 2014), constituting a key contribution to innate immunity by recruiting neutrophils, as well as, monocytes, macrophages, dendritic cells, natural killer cells, and T cell subsets (Tecchio and Cassatella, 2016). Among the cytokines, interleukin (IL)-10 is an anti-inflammatory cytokine that controls the degree and duration of inflammatory reactions. Neutrophils isolated from healthy donors do not express or secrete IL-10 (Tamassia et al., 2013). However, a population of IL-10-secreting neutrophils was found to play a regulatory role during various murine microbial infections *in vivo* (De Santo et al., 2010; Zhang et al., 2009). Neutrophils have been classified into distinct subsets on the basis of their morphology, cytokine production, surface antigens, and expressed TLRs. Moreover, neutrophils with distinct phenotypes show different susceptibilities to a specific pathogen (Pillay et al., 2012; Tsuda et al., 2004) and adjust to the stressor by changing their phenotypes in the course of inflammation (Kolaczkowska and Kubes, 2013).

In this study, we established a SIRS mouse model using CpG ODN to stimulate D-GalN-sensitized mice based on our previous work and found that neutrophils were massively and rapidly recruited into the peritoneal cavity, where some $sTLR9^+$ neutrophils were detected. Along with the progression of SIRS, the expression of sTLR9 in $sTLR9^+$ neutrophils in peritoneal lavage cells (PLCs) were declined in conjunction with an increase in the level of the inflammatory cytokine TNFα and a decrease in the level of the anti-inflammatory cytokine IL-10 in $Ly6G^+$ PLCs. When using life-saving CCT ODN, an ODN with CCT repeats, which we have previously shown to be capable of rescuing mice from SIRS-like lethal shock, the expression of sTLR9 on neutrophils was significantly elevated. Adoptive therapy with early recruited neutrophil-rich PLCs, containing a high ratio of sTLR9-high-expressing $sTLR9^+$ neutrophils, could rescue mice from the development of SIRS. Our observations could provide insights into the protective roles of neutrophils in the development of SIRS. Overall, the data may reveal an existence of a protective sub-population of neutrophils that are present during the early stages of SIRS development and suggest a way to up-regulate this population of the neutrophils for rescuing severe SIRS patients.

2. Materials and methods

2.1. Antibodies and reagents

APC rat anti-mouse Ly6G (560599) and PE rat anti-mouse CD19 (557399) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). FITC-conjugated active anti-TLR9 monoclonal antibody (26C593.2) was purchased from Abcam. CpG ODN (5′-TCCATGACGTTCCTGACGTT − 3′) and CCTODN (5′-CCTCCTCCT CCTCCTCCTCCTCCT-3′) were synthesized by Takara Co (Dalian, China). All ODNs were diluted in PBS and tested for endotoxin using the Limulusamebocyte lysate assay (Associates of Cape Cod, Inc. Falmouth, MA, USA). D-Galactosamine hydrochloride (D-galactosamine HCL, D-GalN) was purchased from DeBioChem (Nanjing, China). Trizol reagent (Noahcentury, NC0301) was purchased from Invitrogen (Carlsbad, CA, USA). A cDNA synthesis kit (Transgen Biotech, Beijing, China, I21021) and two-step SYBR green qPCR assays (Transgen Biotech, G31227) were also used.

2.2. Establishment of a SIRS mouse model

Eight-week-old female BALB/c mice (19 ± 1 g in weight) obtained from the Experimental Animal Center, Medical College of Norman Bethune, Jilin University, were given free access to standard rodent chow and water and were housed in pathogen-free cages. To establish a SIRS mouse model, mice were firstly injected intraperitoneally (i.p.) with 500 μl of D-GalN (0.01 g/g body weight) to make them become sensitive to immune stimulators; 1.5 h later, CpG ODN (10 μg/per mouse in 300 μl of PBS) was used to stimulate for triggering excessive SIRS-like syndrome in mice by i.p. injection. Mouse survival was recorded beginning after CpG ODN i.p. injection and monitored every 4 h. In the SIRS model mice pretreated with CCT ODN, the D-GalN-sensitized mice were injected i.p. with CCT ODN (50 μg/per mouse in 300 μl PBS) 0.5 h before CpG ODN injection. Experimental manipulation of the mice was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Science & Technology of Jilin Province.

2.3. Preparation of PLCs

To harvest PLCs from CpG ODN-induced SIRS model mice or PBS-injected control mice, the mice (n ≥ 3 per group) were euthanized at defined time points with 50 mg/kg pentobarbital sodium followed by the washing of peritoneal cavities using 6 ml ice-cold PBS per mouse. The peritoneal lavage fluid was centrifuged at 750*g* for 5 min at 4 °C to harvest PLCs. The PLCs were re-suspended in cold PBS for further use.

2.4. Cell counting

To count the number of polymorphonuclear cells (PMNs) in each peritoneal lavage sample, the samples were centrifuged at 750*g* for 5 min at 4 °C for fixing the PLCs on slides and then stained with hematoxylin-eosin followed by counting the cell numbers on hemocytometer (Beckman Coulter, Fullerton, CA) and taking images of the cells.

2.5. Cell culture and treatment

To observe the effect of CpG ODN and CCT ODN treatment on the expressions of sTLR9 in early recruited peritoneal neutrophils, the pooled PLCs were collected at 1 h after CpG ODN injection from the SIRS-like mice and maintained in RPMI 1640 supplemented with 10% (V/V) fetal bovine serum (GIBCO) and antibiotics (100 IU of penicillin/ml and 100 IU of streptomycin/ml). Cells were counted and then plated in 24-well cell culture plates (Costar, Cambridge, MA) at an approximate density of 7×10^5 cells/well. The PLCs were cocultured with CpG ODN (2 μg/ml), CCT ODN (2 μg/ml), or RPMI 1640 as a vehicle control for 1 h in a 5% CO2-humidified incubator at 37 °C. The PLCs were collected and stained with FITC-labeled anti-TLR9 mAb and APC-labeled anti-Ly6G mAb, followed by flow cytometry analysis to detect the expression of sTLR9 on $sTLR9^+Ly6G^+$ PLCs.

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