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Gender differences in circulating levels of neutrophil extracellular traps in serum of multiple sclerosis patients

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ABSTRACT

Neutrophil extracellular traps (NETs) trap and kill pathogens very efficiently but also activate dendritic cells and prime T cells. Previously, we demonstrated that neutrophils are primed and circulating NETs are elevated in relapsing remitting multiple sclerosis (RRMS), a T cell-mediated autoimmune disease. Here, we demonstrate gender specific differences in circulating NETs but not in neutrophil priming in RRMS patients. Although the results from our systematic and in depth characterization of these patients argue against a major role of circulating NETs in this disease, they suggest that NETs may underlie gender-specific differences in MS pathogenesis.

1. Introduction

The main function of the innate immune system during infection is to eliminate the pathogen, and neutrophils are key players in innate immune responses. Women of reproductive age are more resistant to sepsis and subsequent morbidity and mortality than men (Schroder et al., 1998), and the incidence of sepsis in postmenopausal women increases to levels almost equal to those seen in age-matched men (Martin et al., 2003). Women also have a higher systemic neutrophil count compared with men (Bain and England, 1975a), and neutrophil counts correlate with estradiol levels during menstruation (Bain and England, 1975b) and pregnancy (Efrati et al., 1964) suggesting that sex hormones influence neutrophilia and overall resistance to sepsis most likely by delaying apoptosis in neutrophils (Molloy et al., 2003). In order to eliminate pathogens during infections, neutrophils are armed with a variety of weapons including engulfment and intracellular degradation of microbes (Hampton et al., 1998; Segal, 2005), release of oxygen species and granule proteins (Lehrer and Ganz, 1999) and release of extracellular chromatin fibers bound to granular, nuclear and cytoplasmic proteins called neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). NETs not only trap and kill pathogens very efficiently but also minimize collateral tissue damage by containing proteases to the DNA fibers and act as physical barriers preventing microbial spread. Despite the well documented importance of NETs as an effective antimicrobial first line defense mechanism, there is increasing evidence that NETs occur in various clinical settings in the absence of microbial infections and that they are probably also associated with pathophysiological conditions (Amulic et al., 2012; Logters et al., 2009). NETosis, the process of neutrophil cell death that leads to expulsion of NETs, can be triggered by different stimuli including pro-inflammatory cytokines such as IL-8 and TNFα phorbol 12-myristate 13-acetate (PMA) (Brinkmann et al., 2004; Fuchs et al., 2007), activated platelets (Clark et al., 2007) and endothelial cells (Gupta et al., 2010), and placental microparticles (Gupta et al., 2005). In addition to their bactericidal potential, NETs can also activate plasmacytoid- (pDCs) (Garcia-Romo et al., 2011; Lande et al., 2011) and myeloid (mDCs) (Sangaletti et al., 2012) dendritic cells and in consequence modulate inflammatory responses. The activation of DCs by NETs appears to play an important role in the pathogenesis of some autoimmune diseases such as psoriasis (Lande et al., 2007), systemic lupus erythematosus (SLE) (Barrat et al., 2005; Garcia-Romo et al., 2011; Lande et al., 2011; Means et al., 2005) small vessel vasculitis (Sangaletti et al., 2012) and type I diabetes (Diana et al., 2013). NET-activated pDCs produce large amounts of interferon that can lead to the maturation of myeloid DCs (mDCs) and exert an effect on T cell function. In SLE, pathogenic auto-antibodies have been associated with abnormal clearing of NETs and NETosis that results in abnormally high production and/or low degradation of NETs, which then leads to tissue damage and facilitated generation of large quantities of auto-antibodies creating a

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vicious cycle (Knight and Kaplan, 2012). In small vessel vasculitis, NETs can favor neutrophil proteins uploading into mDCs and the induction of anti-neutrophil cytoplasmic antibodies (ANCAs) (Sangaletti et al., 2012). Recently, we demonstrated that NETs are also able to directly prime T cells by reducing their activation threshold, which represents a novel link between innate and adaptive immune responses (Tillack et al., 2012).

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system with aspects of secondary neurodegeneration (Sospedra and Martin, 2005). There are two major forms of MS, relapsing-remitting (RR)-MS, which affects around 85%–90% of patients, and primary progressive (PP)-MS in around 10%–15%. Most RR-MS patients later develop secondary progressive (SP)-MS. It is not clear at present, which factors are responsible for the different courses. RRMS affects women about twice as often as men, while no gender differences are observed in PPMS. The activation of $CD4^+$ autoreactive T cells and their differentiation into a T-helper type 1 (Th1) phenotype are crucial events both in the initial phase and in the long-term evolution of RRMS. Damage of the central nervous system is however, most likely mediated by antibodies, complement, $CD8^+$ T cells, and factors produced by innate immune cells. Deregulation in other immune networks involving T-helper 2 (Th2) cells, regulatory $CD4^+$ T cells or NK cells is probably also involved. Compared to T cell- and antibody responses or to other innate immune cells such as DCs and monocytes, the role of neutrophils in MS has not been examined extensively. In a previous study (Naegele et al., 2012), we demonstrated that neutrophils in RRMS patients are more numerous and exhibit a primed state. Furthermore we found higher levels of NETs in serum from these patients. Gender specific differences were not addressed in this study. The chronic inflammatory environment in RRMS (Lund et al., 2004) probably underlies this inappropriate neutrophil priming, which may result in enhanced neutrophil activation during infection. The higher levels of NETs in serum might also be linked to the chronic inflammatory environment in MS, but may have been caused by other triggers such as infections, that have been associated with relapses in these patients (Granieri et al., 2001) or higher frequency of activated platelets (Sheremata et al., 2008) or endothelial cells (Minagar et al., 2001) both elevated in MS. Due to the central role of T cells in RRMS and the ability of NETs to modulate T cell immunity, an abnormally high level of NETs in this disease as consequence of neutrophil priming may play an important role in certain aspects of the pathogenesis and also explain some gender-specific differences.

Here, we have analyzed in detail a large cohort of MS patients including those with subforms other than RRMS, and we have found that only a subset of RRMS patients shows elevated circulating NETs. We also examined whether the higher levels of NETs as well as the primed state of neutrophils in these patients show sex differences, and our results indeed indicate that gender specific differences exist with respect to levels of circulating NETs but not in neutrophil priming. Further, we have performed a systematic and in depth characterization of these patients aimed to identify NET triggers as well as their putative role in MS pathogenesis. The results argue against a major role of circulating NETs in MS but suggest that NETs may underlie gender-specific differences in MS pathogenesis.

2. Material and methods

2.1. Patients

Patients were recruited from the INiMS outpatient clinic and day hospital at the University Medical Center Hamburg-Eppendorf. MS diagnosis was based on the revised McDonald criteria (Polman et al., 2005). Patients, who had not receive steroids for at least 4 weeks prior to enrolment or any immunomodulatory or immunosuppressive agent during the last 3 months, were considered untreated and included in the study. Patients were classified into the following MS subgroups: patients with a first demyelinating event suggestive of MS, termed clinically isolated syndrome (CIS, n = 126), RRMS (n = 168) and PPMS (n = 21). The CIS and RRMS groups included patients that were either neurologically stable for at least 30 days before sampling (remission state, CIS n = 76 and RRMS n = 99) or exhibited an acute episode of neurological worsening lasting for more than 24 h at the time of sampling and not having received steroids (relapse state, CIS n = 50 and RRMS n = 69). Patients did not show clinical signs or symptoms of acute infection. Controls included patients with inflammatory neurological disease other than MS (OIND, n = 5), patients with other non-inflammatory neurological diseases (OND, n = 12) and a cohort of healthy volunteers not suffering from any known infectious or inflammatory disorder (HC, n = 40). Table 1 summarizes the demographic characteristics of patients and controls. Supplementary Table 1 summarizes the diagnosis of patients with OIND and OND. This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg), and written informed consent was obtained from all patients and controls before blood was drawn.

2.2. Quantification of circulating NETs and dsDNA in serum samples

Detection of myeloperoxidase (MPO) associated with DNA was used to quantify circulating NETs. MPO-DNA complexes in serum samples were quantified as previously described (Kessenbrock et al., 2009). Briefly, 5 μg/ml of mouse anti-human myeloperoxidase (MPO)-specific capture antibody (AbD SeroTec, Duesseldorf, Germany) was coated to 96-well plates. After blocking with 1% BSA, serum samples were added together with a peroxidase-labeled anti-DNA monoclonal antibody (component 2 of the Cell Death ELISA kit, Roche). After 2 h of incubation, the peroxidase substrate was added according to the manufacturer's instructions. Absorbance was measured at 405 nm using a μQuant microplate reader (Bio-Tek, Winooski, VT, USA). Total dsDNA in serum was quantified using Picogreen dsDNA kit (Invitrogen, GIBCO, Paisley, UK) as previously described (Fuchs et al., 2007).

2.3. Neutrophil purification and in vitro stimulation

Neutrophils from RRMS patients and HC were isolated from freshly drawn peripheral blood using dextran-Ficoll, as described previously (Weiss et al., 1985). Briefly, erythrocytes were sedimented for 30 min in Hank's balanced salt solution (HBSS) (Invitrogen GIBCO, Paisley, UK) containing 3% pure dextran 200 (Serva, Heidelberg, Germany). The top phase was carefully layered onto 3 ml of LSM 1077 Ficoll solution (PAA Laboratories GmbH, Pasching, Austria) and centrifuged for 30 min at 2000 rpm without brakes. After centrifugation, the supernatant was discarded and the pellet washed. Remaining erythrocytes were lysed by adding 5 ml of ice-cold H_2O for 20 s. Neutrophil purity and viability was ≥97% and ≥95% respectively as assessed by expression of the neutrophil-specific marker CD16b and trypan blue exclusion as already described (Naegele et al., 2012).

Table 1
Demographic characteristics of MS patients and controls.

Group	n	F	M	F:M	Mean age ± SD
CIS	126	78	48	1.6	33.9 ± 8.8
• CIS remission	76	48	28	1.7	35 ± 9.4
• CIS relapse	50	30	20	1.5	32.3 ± 7.6
RRMS	168	106	62	1.7	36.5 ± 7.9
• RRMS remission	99	63	36	1.7	37.1 ± 7.5
• RRMS relapse	69	43	26	1.6	35.7 ± 8.5
PPMS	21	11	10	1.1	39.3 ± 6.6
OIND	5	3	2	1.5	47.8 ± 20.5
OND	10	5	5	1	45.3 ± 11
HC	40	20	20	1	32.2 ± 7.3