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Circulating Muramyl Dipeptide Is Negatively Associated with Interleukin-10 in the Frail Elderly

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Abstract—Elevated levels of serum cytokines, a marker of immune activation and chronic inflammation, are commonly associated with age and are a significant risk factor for all-cause mortality in the elderly. This phenomenon is very similar to that exhibited by individuals with diseases of inflammatory etiology and chronic viral infections such as human immunodeficiency virus (HIV). Although the origin of chronically elevated cytokines with age is unknown, for chronic diseases and viral infections, a role for circulating bacterial products and other pattern recognition receptor (PRR) ligands has been suggested. Given this, we sought to examine whether the levels of circulating cytokines (tumour necrosis factor (TNF), interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12) in the advanced-age, frail elderly (n=135) correlated with plasma levels of lipopolysaccharide (LPS), muramyl dipeptide (MDP), 16S ribosomal DNA, total cell-free DNA and host-derived mitochondrial DNA. After adjusting for multiple testing, no associations between circulating products and donor age, sex or comorbidities were observed. However, a significant negative correlation between MDP and IL-10 was identified. Given the anti-inflammatory nature of IL-10, a negative relationship with a potent inflammatory agonist such as MDP is not surprising and suggests a potential role for circulating MDP in the propagation of age-related immune activation.

KEY WORDS: inflammaging; chronic immune activation; cytokines; lipopolysaccharide; cell-free DNA; mitochondrial DNA.

INTRODUCTION

A common feature of aging is an increase in the levels of cytokines in the blood. This phenomenon, commonly termed "inflammaging" [1], corresponds with the incidence of mortality and physical frailty in the elderly [2, 3], overactive immune responses to bacterial products [4, 5], as well as alterations in the frequency of important peripheral blood leukocytes, such as monocytes, T cells, and natural killer cells [6, 7]. Interestingly, it is very similar to the chronic immune activation exhibited during certain chronic infections and systemic diseases. For example, it is well known that human immunodeficiency virus (HIV)-positive individuals have elevated levels of serum cytokines such as interleukin (IL)-6, IL-10, and tumour necrosis factor (TNF) [8, 9] and often demonstrate an accelerated aging phenotype that includes early-onset cognitive impairment, cardiovascular disease and other age-associated conditions [10]. This is also true for chronic diseases such as cirrhosis [11] and for patients undergoing long-term peritoneal dialysis [12]. A factor these patients share is a rise in circulating bacterial products, namely endotoxin or lipopolysaccharide (LPS), which may be derived by the resident gastrointestinal microbiota via stress-induced leakage across the gut epithelium [12–15]. This has been suggested as a causal factor in chronic immune activation, since modifications to the gut microbiota by probiotic therapy

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lower the levels of circulating endotoxin as well as cytokines [16].

The cause of chronically elevated cytokines over the trajectory of aging is not known. It has been proposed, but not proven, that immune activation with age is caused by circulating bacterial products [6]. Others have suggested that host-derived damage-associated molecular patterns, such as nucleic acids, released into the circulation drive immune activation [17]. Herein, we tested the hypothesis that cytokine levels in the advanced-age, frail elderly, which we have previously shown to have elevated serum interferon-gamma (IFN-γ), TNF, IL-1β, IL-6 and IL-8 as compared to young controls [18], would correlate with circulating bacterial products (LPS, muramyl dipeptide (MDP) and 16S DNA), total cell-free DNA and host-derived mitochondrial DNA.

METHODS

Participants and Cytokine Quantification

Advanced-age, frail elderly participants (n=135, median age=88 years, range 68–99, male/female 21:114) were recruited from five local nursing homes in Hamilton, Ontario, between November 2010 and January 2011; all were at least vulnerable based on the Clinical Frailty Scale [19]. History of comorbidities was obtained from their medical charts. Individuals on immunosuppressive medication, chemotherapy or undergoing radiation therapy were excluded. A sample of 35 young adults (19–45 years old) was also recruited in the same time frame. These studies were approved by the McMaster Research Ethics Board, and written informed consent was obtained for all participants. Cytokines were quantified in cryopreserved plasma from heparinized venous blood using the Milliplex High Sensitivity Human Cytokine Kit (Millipore, MA, USA).

Bacterial Product and Nucleic Acid Detection in Human Plasma

For the detection of LPS and MDP in human plasma, a colourimetric reporter bioassay was generated allowing for the quantification of NF-κB activation by the pattern recognition receptors toll-like receptor (TLR)-4 and nucleotide-binding oligomerization domain 2 (NOD2), respectively, in response to the aforementioned bacterial products. Briefly, the MDP-responsive reporter line was generated by stable transfection of HEK293T cells with pNifty2-SEAP (Invivogen, CA, USA) and NOD2 (a generous gift from Dr. Jonathan Schertzer, McMaster University), while the LPS-responsive reporter line was generated by transiently transfecting the pNifty2-SEAP plasmid into a commercially available HEK293 cell line expressing TLR-4, MD2, and CD14 (Invivogen, CA, USA; a generous gift from Dr. Ali Ashkar, McMaster University). Cells were seeded at 4×10^3 per well in a 96-well plate in complete DMEM for 24 h. Media was removed prior to the addition of heat-inactivated plasma (30 μL, diluted 1:10) in HEK Blue Detection Media (Invivogen, CA, USA) to a final volume of 200 μL. Readings were performed at 630 nm, 24 h after stimulation, and background levels were subtracted from relative absorbance units. Standard curves constructed using different doses of purified LPS or MDP were used to calculate the relative amount of bacterial product in plasma. Only those samples that fell within the linear range of the standard curve (LPS, 5 to 500 ng/ml, r^2= 0.991; MDP, 0.05 to 5 μg/ml, r^2=0.997) were included for further analysis. In the frail elderly (n=135), for the measurements of LPS, the average and range were 68.0 (6.0–487)ng/ml, and 43 were excluded, and for MDP, the average and range were 1.51 (0.05–4.74)μg/ml, and 38 samples were excluded. In the sample of young adult controls (n=35), only six participants (0.063 (0.051–0.090)μg/ml) fell within the linear range of the standard curve; the remaining were below.

Total DNA was extracted from plasma (50 μL) using the QIAamp DNA mini kit (Qiagen, CA, USA). Cell-free DNA (cfDNA) was measured using the QuantiFluor dsDNA system (Promega, WI, USA) and a SpectraMax i3 plate reader (Molecular Devices, CA, USA). The average and range of cfDNA were 49.2 (1.9–216)ng/ml of plasma. Mitochondrial DNA (mtDNA) levels were estimated by measurement of mitochondrial NADH dehydrogenase (MT-ND1) by quantitative PCR using GoTaq qPCR Master Mix (Promega, WI, USA) and an ABI StepOnePlus (Applied Biosystems, CA, USA). Standard curves were prepared using purified mitochondrial DNA, and primer sequences are as follows: F- 5′ATACCCATGG CCAACCTCCT, R-5′GGGCCTTTGCGTAGTTGTAT. The average and range of mtDNA were 0.57 (0.01–6.83) ng/ml of plasma. Bacterial 16S DNA was also measured using quantitative PCR, amplifying the V3 region of the 16S rRNA. Standard curves were prepared using purified *Escherichia coli*-derived bacterial DNA. Bacterial 16S DNA levels were expressed as relative copy number.

Statistics

All statistical analyses were performed in R 2.11.1 (R Development Core Team, 2011). All multivariate analyses

were performed by linear regression on log-transformed circulating product values; log transformation was deemed necessary to approximate normality, according to Pearson's chi-square normality test. Pairwise comparisons were performed by Wilcoxon rank sum test and correlations using Spearman's rank correlation test. Experimental-wise significance threshold was determined by the Benjamini-Hochberg procedure for controlling false discovery rate.

RESULTS AND DISCUSSION

In a cohort of 135 elderly donors, we measured the levels of plasma IFN-γ, TNF, IL-1β, IL-6, IL-8, IL-10 and IL-12. Of all cytokines measured, 27, 17 and 35 % of donors did not have detectable levels of IFN-γ, IL-12 and IL-1β, respectively (Fig. 1a). Significant correlations between several cytokines were observed, in particular IL-6 and TNF (Spearman's rho=0.391, $p<0.0001$), IL-8 and TNF (rho=0.336, $p<0.0001$), and IL-12 and IFNγ (rho=0.346, $p<0.0001$) (Fig. 1b). The bacterial products lipopolysaccharide (LPS), muramyl dipeptide (MDP) and 16S ribosomal DNA, as well as total cell-free DNA (cfDNA) and host-derived mitochondrial DNA (mtDNA) were also measured in plasma (Fig. 1c). We hypothesized that these circulating molecules would correlate with plasma cytokines given their potency in stimulating immune activation via pattern recognition receptors TLR-4 (LPS), TLR-9 (16S DNA, cfDNA and mtDNA) and NOD2 (MDP). All

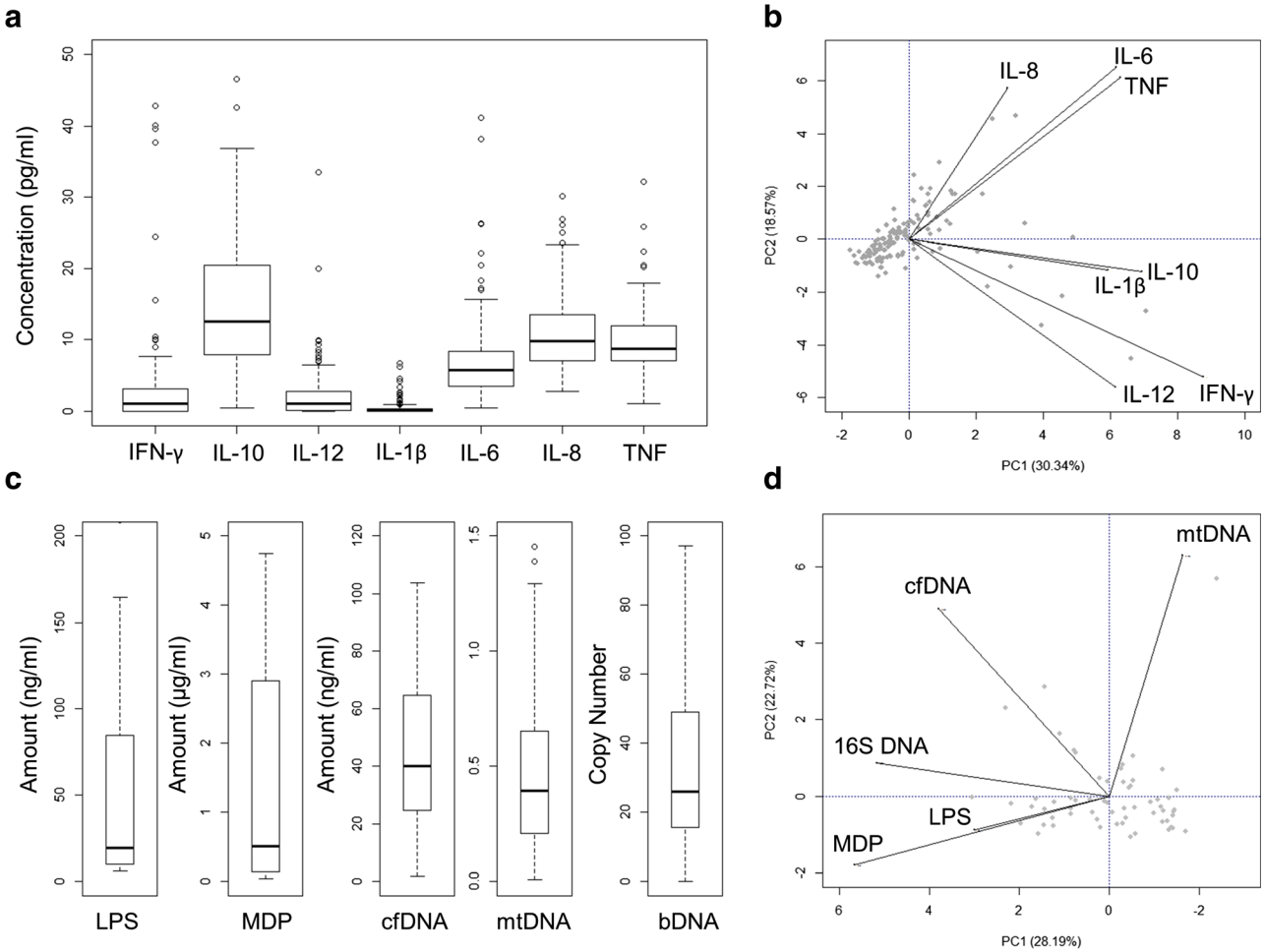

Fig. 1. Characteristics of cytokines, bacterial products and nucleic acids in the plasma of elderly donors. Plasma levels of **a** cytokines and **c** bacterial products and host-derived nucleic acids are presented. Principal component analysis of **b** cytokines and **d** bacterial products and host-derived nucleic acids indicates the degree of correlation between analytes, evident by the acuteness of the angle between vectors. *Axes* represent the percentage contribution of each principal component (PC) to the overall variation in the dataset and each *point* represents a study participant.

circulating bacterial products and nucleic acids were detectable in more than 75 % of donors, and none were associated with donor age, sex, smoking and frailty, or comorbidities such as anaemia, arrhythmia, asthma, congestive heart failure, chronic obstructive pulmonary disease, stroke, dementia or diabetes, after adjusting for multiple-testing correction. Unsurprisingly, levels of mitochondrial (rho=0.473, $p<0.0001$) and 16S DNA (rho=0.455, $p<0.0001$) correlate strongly with levels of total cfDNA, while MDP correlated with cfDNA (rho=0.294, $p=0.006$) and 16S DNA (rho=0.307, $p=0.003$) (Fig. 1d). Univariate linear regression analysis of plasma cytokines against the bacterial products and nucleic acids measured indicated that after adjusting for multiple-testing correction, MDP was significantly associated with IL-10 ($\beta=-2.98$, $p<0.001$); this association remained significant after testing using a multiple regression model, including all cytokines as independent variables ($\beta=-0.038$, experimental-wise $p=0.040$). Segregating donors into groups based on their measured levels of plasma MDP, it appears that although the relationship between MDP and IL-10 is not linear, those individuals that have greater than 0.5 μg/ml of MDP also have significantly less IL-10 than those lower than 0.5 μg/ml (Fig. 2).

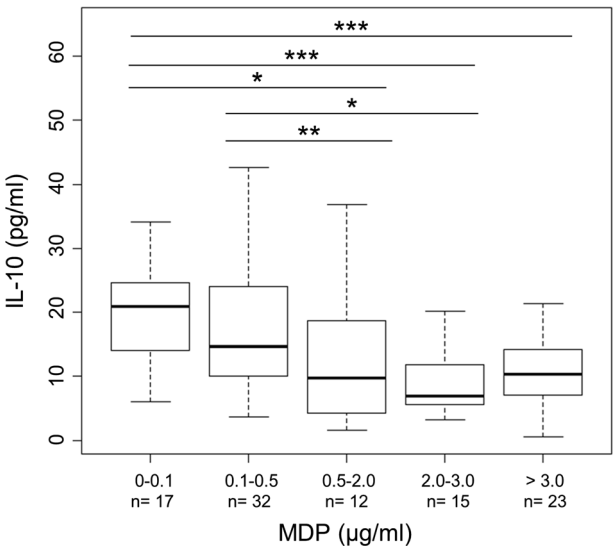

Fig. 2. Levels of circulating IL-10 are lowest in donors with the highest levels of muramyl dipeptide (MDP). IL-10 was measured by multiplexed bead ELISA, while MDP was measured using an *in vitro* bioassay in which MDP levels correlate with colourimetric absorbance following NF-κB activation. Significance was determined by Wilcoxon rank sum test and adjusted for multiple testing using the Benjamini-Hochberg procedure to control false discovery rate. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

These observations are not surprising, as it has been shown that signalling by MDP via NOD2 is tightly linked to the secretion of IL-10 by host cells [20]. In particular, pretreatment of peripheral blood mononuclear cells with MDP suppresses IL-10 production upon subsequent stimulation [21], and the induction of arthritis by MDP injection results in reduced serum IL-10 and IL-10 secretion by splenic leukocytes [22]. Thus, it is possible that the negative relationship between IL-10 and MDP we observe is due to the stimulation of host leukocytes by circulating MDP, resulting in a suppression of IL-10 secretion at basal conditions, thereby leading to less IL-10 in the blood.

While it is plausible that MDP is able to reduce levels of circulating IL-10, it begs the question of the source of MDP. Interestingly, it has been shown in patients suffering from irritable bowel syndrome that serum IL-10 negatively correlates with gut permeability; in other words, those with higher levels of IL-10 have superior gut barrier function [23]. This is important since increased permeability is thought to promote the translocation of luminal antigens, such as bacterial products, across the epithelial barrier thereby triggering low-grade inflammation [23]. These trends between IL-10 and gut permeability have also been observed in murine models of colitis, in which therapeutic intervention using IL-10 reduces gut permeability and inflammation [24], and complete loss of IL-10 worsens gut permeability and leads to subsequent endotoxemia [25]. Thus, we suggest that reductions in IL-10 may be related to the translocation of MDP derived from the luminal gut microflora into the circulation.

Given that our data was derived from the frail elderly, a population that is expected to have higher than average levels of chronic inflammation [3, 18], as well as altered immune/inflammatory signalling [26–28], it is difficult to surmise whether changes in the levels of IL-10 with age would contribute to the levels of MDP, or vice versa. Recent studies have shown that IL-10 does in fact decrease with age [1, 29], and preliminary analysis from our laboratory suggests that circulating MDP is elevated in the frail elderly as compared to young adults. In a sample of 35 young adults, only six participants had MDP levels high enough to reach the linear range of the standard curve. Of these, the average was 0.063 μg/ml, more than 20-fold lower than the frail elderly (1.51 μg/ml). Thus, although further study is required, we hypothesize that alterations in the levels of IL-10 and MDP with age are causally related and contribute to the inflammaging phenotype.

In summary, by comparing a comprehensive panel of circulating cytokines and bacterial products and nucleic acids, we report a novel, negative relationship between

IL-10 and MDP. This may be a causal relationship in which MDP derived from the gut suppresses the section of IL-10 by peripheral blood leukocytes, or it may be due to decreased gut barrier function in individuals with lower IL-10, which allows the release of MDP into the circulation. Such a phenomenon would be expected to contribute to age-related immune activation.

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