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## Epstein-Barr virus-encoded dUTPase enhances proinflammatory cytokine production by macrophages in contact with endothelial cells: Evidence for depression-induced atherosclerotic risk <sup>☆</sup>

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### Abstract

Increased levels of proinflammatory cytokines, TNF- $\alpha$  and IL-6, predict mortality and morbidity. In cardiovascular disease patients, they are observed in atherosclerotic lesions and serum. Factors behind the increased levels of these cytokines are multifaceted and may include latent herpesviruses, such as Epstein-Barr virus (EBV) that can be reactivated by stress. Previously, we showed that the EBV-encoded deoxyuridine triphosphate nucleotidohydrolase (dUTPase), a protein synthesized in the early phase of virus replication, can induce human monocytes/macrophages to produce TNF- $\alpha$  and IL-6. In this study, we modeled the interactions that take place between macrophages and endothelial cells *in vivo* using human umbilical vein endothelial cells (HUVEC). HUVEC were stimulated by soluble factors induced by EBV dUTPase-treated monocyte-derived macrophages (MDM) that resulted in the upregulation of VCAM-1 and ICAM-1. These changes were related to MDM production of TNF- $\alpha$  following the activation of NF- $\kappa$ B. In a previous study, chronically stressed dementia caregivers had elevations in plasma IL-6 levels, a risk for cardiovascular disease. We found a relationship between plasma IL-6 levels and neutralizing antibody titers to EBV dUTPase suggesting that one source of the plasma IL-6 observed in our previous study could be related to the effect of EBV-encoded dUTPase on macrophages. The results suggest that EBV-encoded dUTPase can enhance production of proinflammatory cytokines by monocytes/macrophages in contact with endothelial cells of blood vessels, and may play a role in cardiovascular pathology and chronic inflammation.

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**Keywords:** EBV; dUTPase; Proinflammatory cytokines; Endothelial cells; Monocytes/macrophages; Stress

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## 1. Introduction

Inflammation plays a central role in driving the evolution of atherosclerosis and coronary artery disease (Binder et al., 2002; Danesh et al., 1997; Rattazzi et al., 2005; Ridker et al., 2005a). Clinical investigations indicate that inflammation is an independent risk factor for the development of atherosclerosis and also interacts with established risk factors such as hypercholesterolemia (Binder et al., 2002; Ridker et al., 2005b; Steinberg, 2002). Evidence from model systems and patient studies have demonstrated increases in the concentration and activity of factors that drive inflammation and innate immunity, including the well described increase in C-reactive protein (CRP), as well as upstream factors, such as interleukin-1 beta (IL-1 $\beta$ ), IL-6, and TNF- $\alpha$  (Binder et al., 2002; Danesh et al., 1997; Rattazzi et al., 2005; Ridker et al., 2005a). These cytokines are increased in circulating plasma in patients with cardiovascular disease and are present within atherosclerotic lesions themselves (Rattazzi et al., 2005). In particular, TNF- $\alpha$  promotes the expression of pattern recognition receptors such as scavenger receptors and Toll-like receptors that facilitate the interaction between monocytes/macrophages and circulating lipid, thus leading to the formation of early atherosclerotic lesions (Rattazzi et al., 2005).

The factors that promote the activation of these inflammatory mechanisms are incompletely defined. Viruses, such as the latent herpes viruses Epstein-Barr virus (EBV) or cytomegalovirus (CMV), are a likely stimulus to inflammation, considering their persistence in the host and prevalence throughout the population; however, investigations have not provided consistent evidence that they play a significant role in atherogenesis (Danesh and Appleby, 1998; Horvath et al., 2000; Ibrahim et al., 2005). The failure to consistently link viruses with inflammation does not exclude the possibility that their proatherogenic role may be obscured by the fact that a significant percentage of adults are latently infected with EBV and CMV. Such apparent dormant states may exert proatherogenic effects through mechanisms that are yet to be defined (Glaser et al., 2006).

Depression is among the comorbid conditions that contribute to the evolution and progression of atherosclerosis and is, itself, influenced by proinflammatory pathways (Empana et al., 2005; Ferketich and Binkley, 2005a,b; Ferketich et al., 2000; Jones et al., 2003; Schulz et al., 2000). Depression can increase serum levels of cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and plasma proteins, such as CRP. Furthermore, depression enhances mortality in patients who have suffered a myocardial infarction or who have congestive heart failure (Rumsfeld et al., 2005). Depression may promote mortality by further activation of proinflammatory pathways in part through stress hormones, such as the catecholamines which have been shown to activate NF- $\kappa$ B (Bierhaus et al., 2003), thus accelerating the progression of cardiovascular disease. Accordingly, the

common proinflammatory pathways in atherosclerosis and depression imply a possible shared causal mechanism for the activation of these pathways.

Depression and psychological stressors can interrupt the steady-state expression of latent EBV and reactivate the virus (Glaser et al., 2005b, 1991; Kasl et al., 1979; Payne et al., 1999; Sarid et al., 2002). Moreover, reactivation of latent EBV may be abortive in that only some early viral proteins, e.g. viral-encoded enzymes, are synthesized (Glaser et al., 2005b, 1991). The EBV encodes for six enzymes which are involved in virus replication (Glaser and Kiecolt-Glaser, 2005a; Glaser et al., 2006). These viral enzymes are part of the early antigen (EA) complex and can be synthesized by the endogenous virus genome prior to, and independent of, virus DNA synthesis.

One of these viral proteins, the EBV-encoded dUTPase (Williams et al., 1985), can produce immune dysregulation *in vitro* and *in vivo*. For example, the EBV-encoded dUTPase induced unstimulated monocytes to upregulate the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-10 (Glaser et al., 2006). Accordingly, we are proposing that latent EBV plays a role in cardiovascular disease risk by the production of viral proteins during lytic and/or abortive replication following reactivation of the latent virus. The interaction of one or more EBV-encoded proteins (such as dUTPase) with monocytes/macrophages would contribute to the production of proinflammatory cytokines that would add to the process of chronic inflammation.

The interactions among aging, endothelial cells, macrophages, and EBV could have implications for chronically distressed older people. In a previous study, we found an age-related association between higher antibody titers to EBV EA and virus capsid antigen (VCA) in a group of healthy older subjects, average age of 72, compared to a younger group of students with an average age of 23 (Glaser et al., 1985). In a follow-up study of husbands and wives providing care for a spouse with dementia (average age 67), the caregivers had even higher levels of IgG antibody titers to EBV VCA than well matched controls of the same age (Kiecolt-Glaser et al., 1991). The differences in antibody titers to latent EBV are thought to reflect differences in virus-specific T-cell immunity (Glaser et al., 2005b). Higher antibody titers reflect a decrease in the control over the replication of the latent virus, which results in the synthesis of viral proteins and the immune response to the proteins. The end result is an increase in antibody titers to viral proteins (Glaser et al., 2005b). Caregivers' higher antibody titers to latent EBV (compared to noncaregiving controls) may be associated with the interaction between aging and chronic distress and their impact on the cellular immune response (Glaser et al., 1985; Kiecolt-Glaser et al., 1991). Moreover, distressed caregivers also showed four times the rate of increase in serum levels of IL-6 over a 6-year longitudinal study compared to well matched non-caregivers (Kiecolt-Glaser et al., 2003). Plasma IL-6 levels are a risk factor for cardiovascular disease (Papanicolaou et al., 1998), and dementia caregivers have a higher inci-

dence of cardiovascular disease than noncaregivers (Lee et al., 2003). Thus, the current study addressed the connection between EBV-encoded dUTPase stimulation of proinflammatory cytokines, and the consequent TNF- $\alpha$ -mediated induction of adhesion molecules, ICAM-1 and VCAM-1; in a setting that models the *in vivo* interactions between macrophages and endothelial cells.

## 2. Materials and methods

### 2.1. Endothelial cells and monocyte-derived macrophages (MDM)

Human umbilical vein endothelial cells (HUVEC) were isolated and propagated as previously described (Sedmak et al., 1990). Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (Histopaque, Sigma) density gradient centrifugation from buffy coats ( $n = 7$ ) purchased from the American Red Cross as previously described (Waldman et al., 1992). Because complete donor anonymity is a strict condition of this arrangement, no IRB human subjects protocol is required, as specified by the NIH and OSU IRB guidelines. To promote monocyte differentiation into the macrophage phenotype, PBMCs were suspended in RPMI 1640 medium (GIBCO) supplemented with 10% pooled human serum and incubated for 5–7 days in Teflon plates at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air, transferred to plastic tissue culture plates, and incubated 24 h prior to removal of non-adherent cells. Cells prepared in this manner routinely marked 90–95% positive for CD14 with undetectable levels of CD3<sup>+</sup> T cell contamination as determined by immunofluorescence flow cytometry.

### 2.2. Purification of EBV-encoded dUTPase

Detailed methods for the purification of the EBV-encoded dUTPase have been previously reported (Glaser et al., 2006; Williams et al., 1985). All EBV-encoded dUTPase preparations used were tested as described previously (Glaser et al., 2006) and were free of detectable levels of lipopolysaccharide (Rosenstein et al., 1999), peptidoglycan (SLP-HS), DNA or RNA. Protein concentration was determined with a Coomassie brilliant blue dye-binding assay (Bio-Rad Laboratories) using bovine serum albumin as the standard. The purified EBV-encoded dUTPase used in these studies was stored at 4 °C at stock concentrations of 0.5 and 1 mg per mL.

### 2.3. Assay of macrophage-mediated endothelial activation

To determine the impact of soluble factors induced by the EBV-encoded dUTPase-treated MDM upon proximal endothelia, MDM in 24-well culture plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air, with various concentrations of EBV-encoded dUTPase (0.125–15  $\mu$ g/mL) or culture medium alone (0.5 mL/well) and analyzed as described below. This concentration range was found to be optimum to induce proinflammatory cytokines in our previous study (Glaser et al., 2006).

Confluent HUVEC monolayers in 24-well culture plates were treated with MDM supernatants, culture medium alone, medium supplemented with 15  $\mu$ g/mL EBV-encoded dUTPase, or medium supplemented with 300 IU/mL human recombinant TNF- $\alpha$  (R&D Systems, Minneapolis, MN) as a positive control for adhesion molecule induction. Cells were incubated for 8 h (VCAM-1) or 24 h (ICAM-1), harvested by brief trypsin digestion, and assayed for ICAM-1 and VCAM-1 expression by immunofluorescence flow cytometry as previously described (Kristovich et al., 2004).

### 2.4. TNF- $\alpha$ and IL-6 quantification in MDM supernatants

All donors were presumed to be in generally good health since they qualified for donation by Red Cross standards; only HIV and hepatitis C serum negative blood samples were provided. Since donor anonymity

is part of the arrangement, donor age and gender were not known. Supernatants from EBV-encoded dUTPase-treated MDMs were analyzed for TNF- $\alpha$  and IL-6 production using the BD™ Human Inflammation Cytometric Bead Array kit (BD Biosciences, CA) according to the manufacturer's instructions. TNF- $\alpha$  was measured because it has been shown that this cytokine can modulate the expression of ICAM-1 and VCAM-1; IL-6 does not have the capacity to regulate the expression of these adhesion molecules, but it is an important cytokine in the pathophysiology of cardiovascular disease as previously discussed (Kiecolt-Glaser et al., 2003; Pober and Cotran, 1990). The lower limit of detection by this method for TNF- $\alpha$  is 3.7 pg/ml and 20 pg/ml for IL-6.

We have previously demonstrated EBV-encoded dUTPase-mediated induction of proinflammatory cytokines in freshly isolated PBMCs. Studies of CD14-depleted populations and of the adherent fraction of PBMCs implicated the monocytic fraction as a responsive component (Glaser et al., 2006). To determine whether differentiated macrophages are similarly responsive, MDM purified and differentiated from buffy coats obtained from seven individual blood donors were incubated for 24 h with various concentrations of EBV-encoded dUTPase (0.125–15  $\mu$ g/mL). These concentrations were chosen based upon the results of our previous study (Glaser et al., 2006). Culture supernatants were recovered for measurement of TNF- $\alpha$  and IL-6 content by flow cytometry.

### 2.5. NF- $\kappa$ B activation

Human PBMCs were isolated and subsequently treated with EBV-encoded dUTPase as described (Glaser et al., 2006). At indicated times, nuclear extracts were prepared (Guttridge et al., 1999) and electrophoretic mobility shift assay (EMSA) was performed probing for the DNA binding activity of NF- $\kappa$ B. Briefly, 2  $\mu$ g of nuclear extract were incubated with 1 mM phenylmethylsulfonyl fluoride and 1  $\mu$ g of poly(dI-dC)-poly(dI-dC) (Amersham Biosciences) for 10 min at room temperature. To this mixture  $2 \times 10^4$  cpm of a <sup>32</sup>P]-labeled oligonucleotide probe corresponding to the MHC promoter was added in a buffer consisting of 10 mM Tris-HCl, pH 7.7, 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Complexes were resolved on a non-denaturing 5% polyacrylamide gel, and then subsequently exposed on phosphorimaging screen (Kodak). For supershifts, antibodies raised against specific subunits of NF- $\kappa$ B, p65 (Rockland), p50 (NLS, Santa Cruz Biotechnology), and c-Rel (Santa Cruz Biotechnology) were preincubated with nuclear extracts for 10 min at room temperature before the addition of phenylmethylsulfonyl fluoride and poly(dI-dC)-poly(dI-dC).

To confirm that NF- $\kappa$ B contributed to cytokine production, dUTPase-treated PBMCs were incubated in the presence of NF- $\kappa$ B inhibitors, NBD and PS1145, which target the I $\kappa$ B kinase (IKK) responsible for NF- $\kappa$ B-controlled activity. PBMCs at 0.5 million cells/ml were pre-incubated with IKK inhibitors (NBD peptide, 100  $\mu$ M; or PS1145, 10  $\mu$ M) for 60 min followed by dUTPase treatment at 10  $\mu$ g/mL for an additional 1 h and 3 h. At this time, culture medium and cell pellets were collected for cytokine analyses and EMSA analysis, respectively, as described above.

### 2.6. Subjects

The subjects studied were part of a previous six-year longitudinal study on caregiving, stress, and health in older adults (Kiecolt-Glaser et al., 2003) in which serum levels of IL-6 were assessed (by ELISA) and depressive symptoms were assessed using the Beck Depression Index (Bonaccorso et al., 1998; Beck et al., 1988). The study was approved by our institutional review board and the subjects gave informed consent. The average age of the 34 men and 60 women chosen for this subsample was 72.18 (SD = 8.92) and 10 were non-white. The majority of older adults take some medication; in this sample the most common medications were aspirin and other over-the-counter analgesics ( $n = 27$ ), diuretics ( $n = 19$ ), estrogen ( $n = 13$ ), thyroid ( $n = 14$ ), calcium channel blockers ( $n = 11$ ),

and beta blockers ( $n = 10$ ). Health problems included arthritis ( $n = 61$ ), hypertension ( $n = 40$ ), prostate problems ( $n = 15$ ), digestive disorders ( $n = 13$ ), and oral health problems ( $n = 11$ ).

Levels of depression were positively correlated with plasma IL-6 levels (Kiecolt-Glaser et al., 2003). Serum neutralizing antibody titers to the EBV-encoded dUTPase were measured as previously described (Williams et al., 1985). Neutralizing antibody titers should be related to amount of EBV-encoded dUTPase being synthesized (Levitsky and Masucci, 2002).

### 2.7. Statistical analysis

To account for the correlations with the immune markers, mixed effects models were used to study the association between  $\log(\text{TNF-}\alpha)$  or  $\log(\text{IL-6})$  and  $\log(\text{dUTPase})$  concentrations. First, we tested the significance of a quadratic trend in (log-transformed) dose for each of the endpoints. Next, we performed all pairwise comparisons (using Tukey's adjustment) among the dose levels, using repeated measures analysis of variance (ANOVA).

For the study to confirm that NF- $\kappa$ B activation by EBV-encoded dUTPase was related to the induction of TNF- $\alpha$  and IL-6, two sample tests (with unequal variances) were used to compare TNF- $\alpha$  levels between groups with and without inhibitors. For the study involving antibody titers to the EBV-encoded dUTPase, linear regression analyses were performed to determine if there was an association between plasma IL-6 levels and antibody titers to the EBV-encoded dUTPase. Of the 94 subjects studied, 62 (66%) had no measurable level of antibodies against the EBV-encoded dUTPase. Values were determined for the remaining 32 subjects. An indicator variable that took on the value 0 for all patients with non-measurable and 1 for those patients with measurable dUTPase levels was created. We used this variable to stratify the population and compare IL-6 levels within the two groups. IL-6 levels were log transformed so as to better conform to the normality and equal variance assumption.

## 3. Results

### 3.1. EBV-encoded dUTPase induces TNF- $\alpha$ and IL-6 production by differentiated macrophages

Data generated by these experiments are presented in Fig. 1 as the mean  $\pm 1$  standard deviation (solid symbols and error bars) for  $\log \text{TNF-}\alpha$  among the seven donors at each concentration of dUTPase. Open symbols represent  $\log \text{TNF-}\alpha$  values for each individual donor. As evident in the figure, MDM produced TNF- $\alpha$  in response to EBV-encoded dUTPase treatment in a dose-dependent manner. Repeated measures analysis of variance supports the fact that mean  $\log(\text{TNF-}\alpha)$  levels differ significantly with different concentrations of dUTPase ( $p < 0.0001$ ) and multiple comparison methods suggest that the baseline levels using the two highest concentrations of dUTPase do not differ significantly from one another. For IL-6, the baseline level (0.05) differs significantly from all higher concentrations of dUTPase.  $\log(\text{IL-6})$  levels at the five highest concentrations of dUTPase do not differ significantly from one another suggesting that we had reached saturation.

### 3.2. EBV-encoded dUTPase induces macrophage-mediated endothelial inflammatory activation

To simulate the impact of soluble factors produced by EBV-encoded dUTPase-exposed-differentiated macrophages upon proximal vascular endothelium, HUVEC

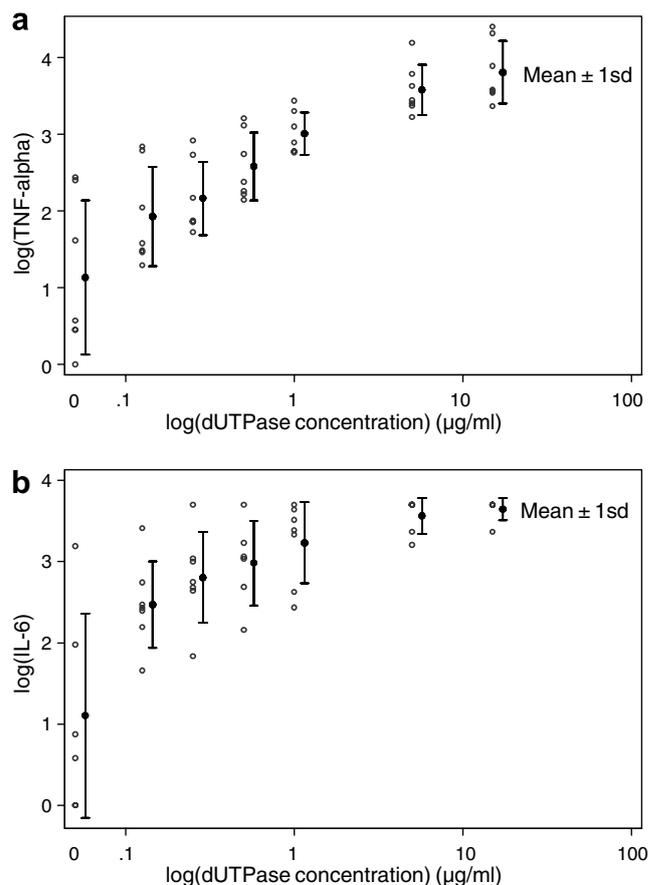


Fig. 1. (a) EBV-encoded dUTPase stimulates TNF- $\alpha$  production by human differentiated macrophages. Human macrophages isolated and differentiated from seven individual donors were treated for 24 h with various concentrations of EBV-encoded dUTPase. TNF- $\alpha$  content in culture supernatants was measured by flow cytometry using the BD™ Human Inflammation Cytometric Bead Array. Open symbols represent  $\log \text{TNF-}\alpha$  values for each individual donor. Closed symbols represent mean  $\log \text{TNF-}\alpha$  values among donors at each dUTPase concentration  $\pm 1$  standard deviation. (b) EBV-encoded dUTPase stimulates IL-6 production by human differentiated macrophages. The conditions were the same as described in (a).

were incubated with supernatants of MDM treated for 24 h with various concentrations of dUTPase (0.125–15  $\mu\text{g/mL}$ ). Controls included untreated HUVEC, HUVEC treated with 15  $\mu\text{g/mL}$  dUTPase, and HUVEC treated with 300 IU/mL human recombinant TNF- $\alpha$  as a positive control for adhesion molecule induction. We measured TNF- $\alpha$  and IL-6 levels in the supernatants. Following 8 h (VCAM-1) or 24 h (ICAM-1) of incubation, HUVEC were harvested and assayed for VCAM-1 or ICAM-1 expression by immunofluorescence flow cytometry. These incubation times were based upon numerous studies which demonstrate that endothelial cells treated with TNF- $\alpha$  express peak levels of VCAM-1 at 8 h and of ICAM-1 at 24 h (Poher and Cotran, 1990). Data generated by these experiments are presented in Fig. 2 as the mean  $\pm 1$  standard deviation (solid symbols and error bars) for  $\log$  endothelial ICAM-1 (2A) or VCAM-1 (2B) mean fluorescence intensity among the seven MDM donors

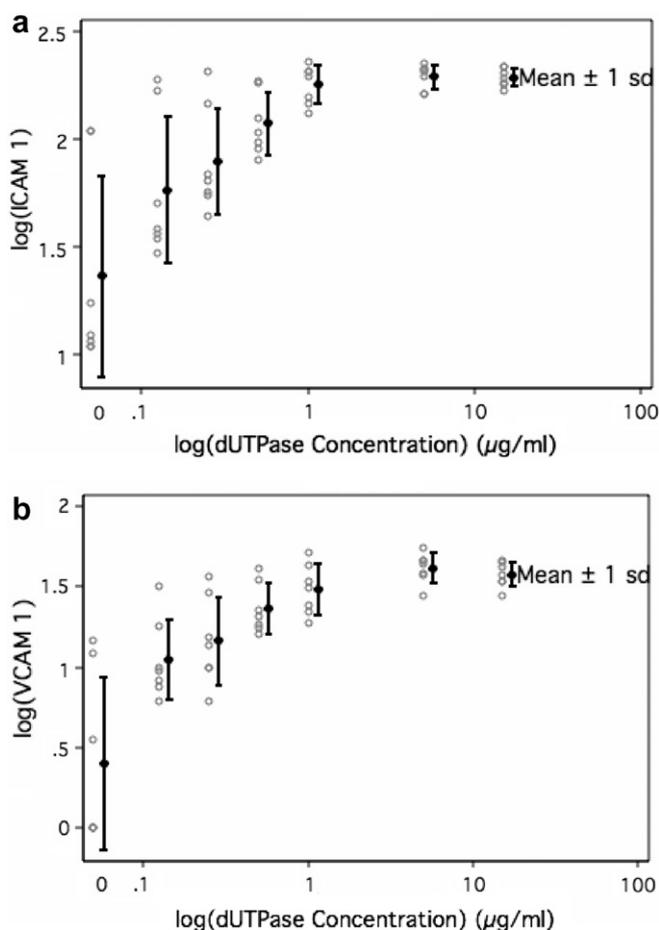


Fig. 2. (a and b) Soluble factors elaborated by EBV-encoded dUTPase-treated macrophages induce inflammation-associated adhesion molecule expression on human endothelial cells. Human umbilical vein endothelial cells were incubated for 8 h (VCAM-1) or 24 h (ICAM-1) with supernatants recovered from EBV dUTPase-treated macrophages, harvested and stained with FITC-conjugated monoclonal antibodies specific for ICAM-1 (a) or VCAM-1 (b), and analyzed by fluorescence flow cytometry. Open symbols represent log mean fluorescence intensity values for each individual donor. Closed symbols represent mean log mean fluorescence intensity values among donors at each dUTPase concentration  $\pm$  1 standard deviation.

at each concentration of dUTPase. Open symbols represent log mean fluorescence intensity values for each individual donor. As was true for TNF- $\alpha$  and IL-6 values shown in Fig. 1a and b, the magnitude of endothelial ICAM-1 and VCAM-1 induction by supernatants of EBV-encoded dUTPase-treated MDM varied among individual MDM donors, but exhibited a dose-response which plateaus at the higher levels of dUTPase. The association between each of these markers and log(dUTPase) is quadratic (in all cases  $p < 0.0011$ ), and in all cases the  $p$ -value for the repeated measures analysis of variance is significant at the  $p < 0.0001$  level. For ICAM-1, the baseline level (0.05) differs significantly from all but the 0.125 and 0.25 levels of dUTPase. Log(ICAM-1) levels at the highest concentrations of dUTPase does not differ significantly from either of the next two highest concentrations suggesting that we had reached saturation. For VCAM-1, the baseline

level (0.05) differs significantly from all higher levels of dUTPase. Log(VCAM-1) levels at the four highest concentrations of dUTPase do not differ significantly from one another again suggesting that we had reached saturation. The failure of direct dUTPase treatment of HUVEC to induce ICAM-1 or VCAM-1 verifies that endothelial adhesion molecules were induced by soluble factors produced by EBV-encoded dUTPase-treated MDM and not directly by residual dUTPase in MDM culture supernatants (data not shown).

### 3.3. EBV-encoded dUTPase activates NF- $\kappa$ B in macrophages

As a first step in elucidating mechanisms by which EBV-encoded dUTPase induces macrophage proinflammatory cytokine production, we measured NF- $\kappa$ B activation in dUTPase-treated PBMCs, since this signaling pathway is known to be a major positive regulator of a number of inflammatory responses, in particular the activation of TNF- $\alpha$  and IL-6 transcription. Furthermore, catecholamine stress hormones are elevated in depressed individuals and they can upregulate the production of proinflammatory cytokines through the activation of NF- $\kappa$ B. Results showed that NF- $\kappa$ B activity was induced at 1 h by EBV-encoded dUTPase treatment, which returned close to basal levels after 4 h of stimulation (Fig. 3). Furthermore, EMSA demonstrated that activation of NF- $\kappa$ B comprised primarily of p50/c-Rel heterodimers with a slight contribution from p50/p65 complexes (Fig. 4).

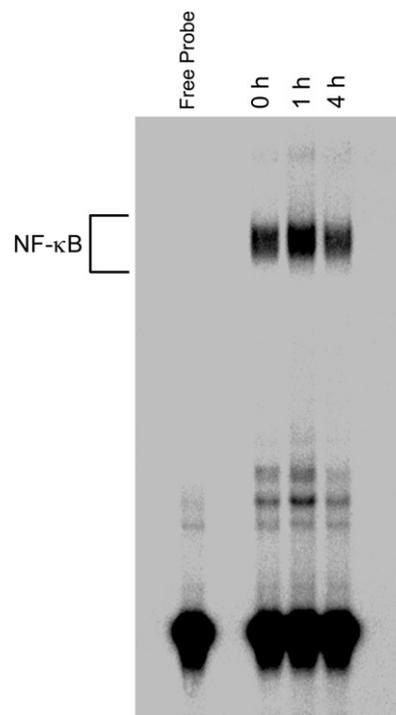


Fig. 3. NF- $\kappa$ B is activated by dUTPase treatment of macrophages. Cells were treated with EBV-encoded dUTPase and, at indicated times, nuclear extracts were prepared and EMSA analysis was performed.

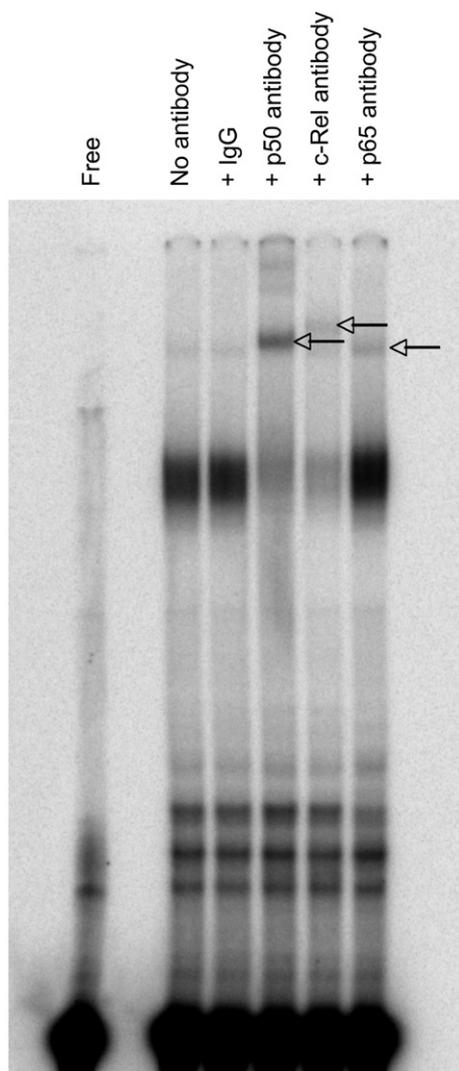


Fig. 4. dUTPase activates the p50/c-Rel complex of NF- $\kappa$ B. Nuclear extracts were pre-incubated with antibodies specific to NF- $\kappa$ B subunits and EMSA analysis was subsequently performed. Arrows indicate supershifted complexes.

To confirm that NF- $\kappa$ B contributed to cytokine production, dUTPase-treated PBMCs were incubated in the presence of NF- $\kappa$ B inhibitors, PS1145 and NBD, that target the IKK complex responsible for NF- $\kappa$ B controlled activity. In the presence of the inhibitors, TNF- $\alpha$  production was markedly reduced ( $p < 0.001$ ), which corresponded to a concomitant reduction of NF- $\kappa$ B DNA binding activity ( $p < 0.001$ ), and similar reductions were also observed for IL-6 (Fig. 5 and data not shown). Together, these data suggest that expression of inflammatory cytokines is mediated through dUTPase-induced activation of NF- $\kappa$ B transcriptional activity.

### 3.4. Antibody titers to the EBV-encoded dUTPase are related to plasma IL-6 levels

All subjects were part of our earlier longitudinal study on caregivers in which plasma IL-6 levels were measured

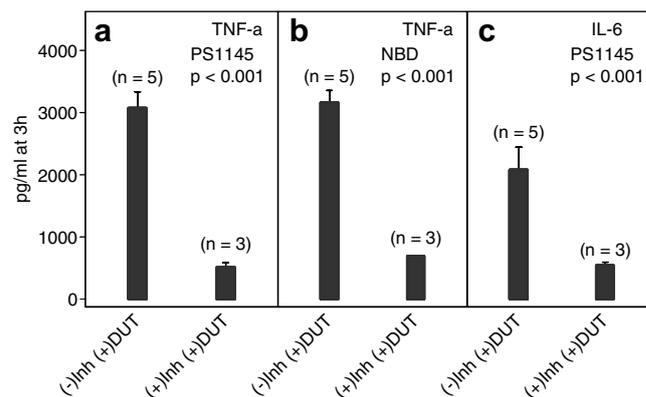


Fig. 5. dUTPase-induced secretion of TNF- $\alpha$  and IL-6 from PBMCs is NF- $\kappa$ B dependent. TNF- $\alpha$  (a and b) and IL-6 (c) released from dUTPase-treated PBMCs pre-incubated with and without NF- $\kappa$ B inhibitor compounds (Lynch et al., 1972), EBV dUTPase treatment at 10  $\mu$ g/mL, PS1145 (10  $\mu$ M) and NBD (100  $\mu$ M). Inh = inhibitor, DUT = dUTPase.

(Kiecolt-Glaser et al., 2003). Because circulating IL-6 levels can be a co-factor for depression and we have shown that the EBV-encoded dUTPase can upregulate the production of IL-6 in monocyte/macrophages, we assessed the association between plasma IL-6 levels and EBV dUTPase antibody titers. The subjects' plasma IL-6 values were below 15 pg/ml, with the exception of two participants whose values were 240.58 pg/ml and 831.49 pg/ml, or at least 90 SDs above the group mean; they were excluded from the sample. Both of these subjects were in the group with measurable EBV-encoded dUTPase neutralizing antibody titers; they were removed from the database so as to better conform to the assumptions of equal variance and normality of the IL-6 data within subgroups for the  $t$ -test, and because their extreme values could have reflected unreported health problems, e.g., acute illness that was either unknown to the subject or not reported. Subjects with measurable anti-EBV-encoded dUTPase neutralizing antibody levels had generally higher mean IL-6 levels (mean = .41, SEM = 0.06) than did the group whose anti-EBV-encoded dUTPase antibody levels were unmeasurable (mean = 0.34, SEM = 0.03), but the difference between the groups did not achieve statistical significance ( $p = 0.26$ ). While not statistically significant, the direction of the difference is suggestive. Fig. 6a presents a scatterplot of log(plasma IL-6 levels) on log(EBV dUTPase antibody titers) for only those patients with measurable levels of anti-EBV-encoded dUTPase antibody with the two outliers removed. The scatterplot demonstrates that, for those subjects with measurable EBV-encoded dUTPase antibody titers, IL-6 levels tend to increase as the antibody titers increase. This association is significant ( $p = 0.04$ ), with log(dUTPase) antibody titers explaining  $r^2 = 14\%$  of the variability in log(IL-6). After controlling for age, the association is still significant ( $p = 0.047$ ,  $r^2 = 14.5\%$ ).

Fig. 6b presents a scatterplot of BDI scores obtained in our previous study (Kiecolt-Glaser et al., 2003) on log EBV-encoded dUTPase antibody titers (only for those sub-

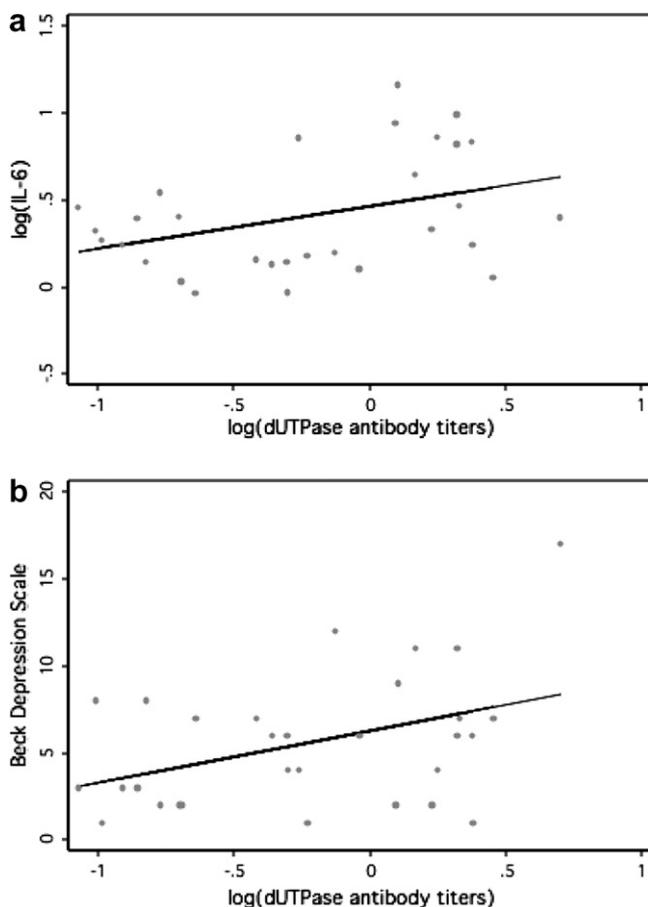


Fig. 6. Scatterplot and linear regression line of  $\ln(\text{IL-6})$  on  $\log(\text{dUTPase})$ . (a) Scatter plot of  $\log(\text{IL-6})$  vs.  $\log(\text{dUTPase})$  along with the linear regression model ( $\log(\text{IL-6}) = 0.464 + 0.243 \times \log(\text{dUTPase})$ ) based on  $n = 30$  subjects with no missing data and eliminating two subjects with  $\log(\text{IL-6}) > 3$ . This association was statistically significant ( $p = 0.039$ ). (b) Scatterplot and linear regression line of Beck depression scale on  $\log(\text{dUTPase})$ . (b) Scatter plot of the Beck depression scale vs.  $\log(\text{dUTPase})$  along with the linear regression model ( $\text{Beck} = 6.278 + 3.004 \times \log(\text{dUTPase})$ ) based on the same  $n = 30$  subjects as were used in (a). This association was statistically significant ( $p = 0.023$ ).

jects with measurable EBV-encoded dUTPase neutralizing antibody titers and eliminating the two subjects with extreme IL-6 levels). The relationship shown in the graph is significant ( $p = 0.0233$ ), with  $\log(\text{EBV dUTPase antibody titers})$  explaining  $r^2 = 17\%$  of the variability in depression scores.

This observed relationship between BDI scores and  $\log$  EBV-encoded dUTPase antibody titers remains significant in a linear model ( $p = 0.032$ ) even after controlling for  $\ln$  IL-6 levels. IL-6 levels were neither a confounder nor an effect modifier of the strong relationship between the depression scores and  $\log$  EBV dUTPase.

#### 4. Discussion

In this study the EBV-encoded dUTPase stimulated monocytes/macrophages to upregulate the expression of

TNF- $\alpha$  through a NF- $\kappa$ B mechanism and resulting in the upregulation of the surface expression of inflammation-associated endothelial adhesion molecules, VCAM-1 and ICAM-1; an upregulation of IL-6 was also observed. These data support the evidence that EBV induces an inflammatory cascade.

A recent study showing that EBV DNA can be detected (by PCR) in atherosclerosis plaques is consistent with the interpretation that EBV can lytically replicate in the monocytes/macrophages (Savard et al., 2000), which have become associated with the plaques (de Boer et al., 2006). EBV can induce macrophages to synthesize the macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ ), which can attract B- and T-lymphocytes to the site of inflammation (McCull et al., 1997); a recent study by Tugizov et al. found that EBV-infected B-cells can provide a source of EBV that can act as a source of the virus to infect monocytes (Tugizov et al., 2007). Furthermore, the deBoer et al. study showed that EBV-specific T-cells are generated at the site of the plaque as well, confirming that a local EBV-specific T-cell response can contribute to the inflammatory process presumably related to the EBV-infected macrophages (de Boer et al., 2006). This investigation provides a plausible basis for the initiation of pro-atherogenic inflammatory responses by latent viral infections, such as EBV.

Supporting the *in vitro* data, which provide a possible mechanism, distressed caregivers had significant elevations in plasma IL-6 levels (compared to noncaregivers), a known risk factor for cardiovascular disease. In accord with these data, other laboratories have documented caregivers' greater risk for cardiovascular disease (Lee et al., 2003; Schulz et al., 2001). The significant relationship between plasma IL-6 levels and neutralizing antibody titers to the EBV-encoded dUTPase in our subjects suggest that at least one source of plasma IL-6 could be related to the effect of the EBV-encoded dUTPase on monocyte/macrophage-induced inflammatory cytokines.

Although some subjects had antibody to the EBV-encoded dUTPase and some did not, all were latently infected with EBV as demonstrated by the presence of antibody to EBV VCA, consistent with previous reports (Glaser et al., 1985; Jones et al., 1988). Despite the fact that unique antibody patterns to several EBV-encoded early proteins in different groups of EBV-infected subjects have been demonstrated by our laboratory and others, the reason for these patterns is not known (Cheng et al., 1980; Glaser and Kiecolt-Glaser, 1998; Glaser et al., 2006, 2005b; Jones et al., 1988; Laichalk and Thorley-Lawson, 2005).

The data support the hypothesis that EBV-encoded proteins play a role in the pathogenesis of atherosclerosis and that distress-associated upregulation of the steady state expression of latent EBV could contribute to the increase in plasma IL-6 levels observed in our caregiver studies (Kiecolt-Glaser et al., 2003, 1991). The significant association of BDI depression scores with antibody titers to the

EBV-encoded dUTPase supports the relationships among depressive symptoms, virus reactivation, and the upregulation of proinflammatory cytokine synthesis. The data also suggest a possible mechanism by which viral protein(s) could participate in this process. These data provide the foundation for considering new strategies for the prevention and treatment of peripheral arteriosclerosis and coronary artery disease.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbi.2007.07.007](https://doi.org/10.1016/j.bbi.2007.07.007).

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