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Glucocorticoid receptor *BcI* polymorphism associates with immunomodulatory response to stress hormone in human peripheral blood mononuclear cells

Lianbin Xiang* and Gailen D. Marshall Jr.

Laboratory of Behavioral Immunology Research, Division of Clinical Immunology and Allergy, Department of Medicine, University of Mississippi Medical Center

Lianbin Xiang: lxiang@umc.edu; Gailen D. Marshall: gmarshall@umc.edu

Summary

Glucocorticoid receptor (GR) variants have been found to be associated with stress-related disorders. Our previous *in vivo* study revealed that the CC allele of GR *BcI* single nucleotide polymorphism (SNP) was more common in the high stress group which had lower levels of both regulatory T cells (Treg) and Th1 cytokine. The current study was to investigate associations between GR *BcI* polymorphism and immunomodulatory response to stress hormone *in vitro* in human peripheral blood mononuclear cells (PBMC). Blood samples were collected from 18 normal volunteers including 9 subjects with *BcI* polymorphism GG allele and 9 with wild-type (WT) CC allele. PBMC were cultured with 10^{-8} M dexamethasone (DEX) which mimics the plasma cortisol level observed during periods of psychological stress for 24h and 11 days. Gene expressions of transcription factors, stress hormone and cytokine receptors were analyzed by real-time RT-PCR. FoxP3 mRNA was significantly altered in the *BcI* WT (decreased at 24h and increased at 11-day) but not in the GG allele. GR mRNA was up-regulated at 24h and down-regulated at 11-day in CC alleles ($p < 0.01$ and 0.05 , respectively), rather than in GG alleles. The expression of β -2 adrenergic receptor (β 2AR) was increased at 24h in both CC and GG alleles ($p < 0.01$ and $p < 0.001$), but decreased significantly at 11 days in only GG alleles. Expression of T-bet and GATA-3 was altered simultaneously in 24h culture with DEX from both groups. The *BcI* polymorphism of GR identifies different immunomodulatory responses to corticosteroids which may explain, at least in part, the variability in individual sensitivity to stressors.

Introduction

Stress is best thought of as a psychophysiological process, usually experienced as a negative emotional state. In response to psychological or physiological stress, a spectrum of autonomic, endocrine, and immunological responses are triggered, including hypothalamic-pituitary-adrenal (HPA) axis reactivity (Kumsta et al., 2007; DeRijk et al., 2008). Glucocorticoids (GCs) from the HPA axis and catecholamines (CT) from the sympathetic nervous system play important roles in response to stressors. Acute stress is experienced in response to an immediate perceived threat, either physical or psychological. The threat can be real or perceived. During an acute stress response, the hypothalamic-pituitary-adrenal (HPA) network and autonomic nervous system (ANS) are activated (Vellucci, 1997; Marshall, 2011). The host produces increased levels of both cortisol and adrenalin. Chronic stress results from a state of ongoing physiological arousal that can result from either a physical or psychological stimulus (or both). This may occur when the host experiences

*Lianbin Xiang, M.D., Laboratory of Behavioral Immunology Research, Division of Clinical Immunology & Allergy, Department of Medicine, University of Mississippi Medical Center, Tel: 601-815-3171, Fax: 601-815-4770, lxiang@umc.edu.

stressors with such frequency, persistence and/or intensity that the ANS and HPA cannot return to a homeostatic balance. Usually, acute stress can demonstrate an effect in a few minutes to a few hours or days, whereas chronic stress effects manifest within a few days to weeks or longer. In the present *in vitro* stress model, 24h exposure to DEX mimics an acute stress exposure and 11 days exposure mimics a chronic stress state.

We and others have previously demonstrated that GCs and CT can influence immunomodulation including changes in number of regulatory T cells (Treg), Th1/Th2 cytokine production, and costimulatory molecule mRNA expression (Ramirez et al., 1996; Agarwal & Marshall, 1998; Agarwal & Marshall, 2001). These immunoregulatory imbalances are associated with elevated cortisol *in vivo* and in the presence of GCs *in vitro*. Production of Th1 cytokine IFN- γ increased and Th2 cytokine IL-4 decreased in 24h culture (acute stress) with DEX. In contrast, 11-day exposure (chronic stress) to DEX decreased production of IFN- γ , increased production of IL-4 and induced a shift in the Th1/Th2 cytokine balance toward Th2 response in tetanus-stimulated PBMC (Agarwal & Marshall, 1998; Xiang & Marshall, 2011). Glucocorticoid receptors (GR), expressed in central nervous system and in peripheral tissue and blood cells, mediate the systemic effects of GCs, particularly those related to stress responsiveness (Svec, 1985; McEwen et al., 1986; de Kloet et al., 1991). The GR *BclI* single nuclear polymorphism (rs41423247), which had a very high allele frequency of approximately 37%, was associated with increased GC effects (de Kloet & Derijk, 2004; van Rossum & Lamberts, 2004) and changed corticosteroid-sensitivity as determined by several assays including skin vasoconstriction and the dexamethasone suppression test (Panarelli et al., 1998; Stevens et al., 2004). Wüst et al. showed strong genetic effects of the *BclI* polymorphism on HPA axis responses in which the *BclI* genotype GG was associated with a diminished cortisol response (Wüst et al., 2004). The *BclI* polymorphism had also been linked to high blood pressure, insulin sensitivity (Weaver et al., 1992), body mass index and abdominal fat distribution with GG-carriers showing increased abdominal visceral fat presumed to be an indicator of increased GC sensitivity (Bumann et al., 1997). In addition, the *BclI* GG genotype was associated with an increased susceptibility to develop major depression (van Rossum et al., 2006).

These findings document the functional relevance of the GR gene polymorphism. However, how the polymorphism may influence immunomodulatory responses to psychological stress is not defined. Our previous study showed that higher stress levels measured by Perceived Stress Scores (PSS) was inversely related to the level of regulatory T cells (Treg) in the group characterized by the CC allele of the *BclI* polymorphism (Xiang et al., 2008). To explore the immunomodulatory responses to stress hormone *in vitro* on *BclI* variant, we investigated how categorizing individuals by specific SNP would be associated with the effects of stress hormone DEX (a laboratory surrogate for cortisol) on the expression of transcription factors for Th1/Th2, cytokine receptors and stress hormone receptors including GR and β -2 adrenergic receptor (β 2AR) on PBMC. We hypothesized that GR *BclI* variant could have potential as a biomarker associated with changes in immunomodulatory response to stress hormones in our cultured PBMC model.

Materials and methods

Human subjects

One hundred thirty-four normal volunteers aged 18–50 were recruited in accordance with a UMMC Institutional Review Board approved protocol. All procedures were carried out with the adequate understanding and written consent of the participants. Genotyping assay for *BclI* SNP revealed that genotypes consisted of 14 GG homozygotes, 69 heterozygotes and 51 wild-type. Frequency of C allele is 63.8% and G allele is 36.2% which is similar to previous reports (van Rossum et al., 2003; Cuzzoni et al., 2012). This study focused on

comparison of two groups of homozygotes CC and GG for *BcII* SNP. We re-recruited volunteers from above subjects and only 9 of 14 GG alleles were available. To compare with the same number of GG alleles in the study, we re-recruited 9 of CC homozygotes that were matched for age, gender and race.

24h and 11-day cell cultures exposed to stress hormone

The PBMC culture system utilized in this study has been previously utilized as a model to study the effects of stress hormones on Th1/Th2 cytokine balance as previously described (Xiang & Marshall, 2011). DEX, at 10^{-8} M mimicking plasma cortisol levels during period of psychological stress (Kunz-Ebrecht et al., 2003; Agarwal et al., 2000), induces an *in vitro* shift in the Th1/Th2 cytokine balance of human PBMC toward a predominant Th2. Thus, 10^{-8} M DEX was used as the working concentration of corticosteroids (CS) in the present study. Briefly, 10^6 PBMC were stimulated with 1 μ g/ml purified tetanus toxoid, 10 IU/ml recombinant human IL-2 (rhIL-2) and 10^{-8} M DEX or media control containing RPMI1640, 10% pooled human AB serum and 2mM l-glutamine for up to 11 days at 37°C, 5% CO₂. On day 5, 0.5 ml of supernatant was removed from each well, replaced with fresh RPMI-C and 20 IU/ml rhIL-2 for expansion and incubated for an additional 6 days. Cell viability was consistently greater than 95% in 24h and 11-day cultures. After 24 h and 11 days, cells were harvested and RNA was extracted for further gene expression study.

Genomic DNA Preparation and *BcII* Genotyping assay

DNA was extracted using commercial Puregene DNA Isolation Kit (Centra Systems) and 15 ng of DNA was used in each genotyping assay. Genotyping of *BcII* polymorphism was performed by allelic discrimination real-time PCR using predesigned TaqMan probes (Applied Biosystems, Foster City, CA). The sequences of probes were 5'-FAM-TCTGCTGATCAATCT-3' and 5'-VIC-TCTGCTGATGAATCT-3' and primers were forward: GCACCATGTTGACACCA ATTCC and reverse: CAGGGTTCTTGCCATAAAGTAGACA. Control samples representing all possible genotypes and a negative control were included in each reaction. The results from TaqMan SNP assay were confirmed by PCR-RFLP analysis using the *BcII* restriction enzyme digestion and electrophoresis. The primers for RFLP analysis were forward: AAATTGAAGCTTAACAATTTTGGC-3' and reverse: GCAGTGAACAGTGACCAGACC (Fleury et al., 2003).

RNA preparation and real-time RT-PCR

RNA samples from cultured PBMC were prepared using RNAqueous RNA Isolation Kit. One microgram total RNA was treated with DNase and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using the Applied Biosystems 7300 Real-Time PCR System. The gene expression of the transcription factors including forkhead box P3 (FoxP3), T-bet, and GATA-3, stress hormone receptors GR and β 2AR, Th1 cytokine interferon- γ receptor (IFN- γ R), Th2 cytokine IL-4 receptor (IL-4R), and housekeeping gene 18s RNA was analyzed by relative qualitative RT-PCR. Pre-designed primer/probe sets were available from Applied Biosystems (specified in Table 1). The signal obtained from each gene primer/probe set was normalized to that of the unregulated housekeeping gene 18s rRNA.

Statistical Analysis

All data are presented in graph form as mean $\pm$ standard error (SEM). Comparison between two groups was done with paired student's T test. Statistical analyses were calculated with Prism (Version 4.0 Graphpad Software, San Diego, CA). Data from gene expression experiments were analyzed with the method for relative quantification RT-PCR as described

previously (Xiang & Marshall, 2011). The C_T values of each target gene were normalized by subtraction of the C_T value from 18s RNA (ΔC_T) generated from the identical reverse transcribed mRNA samples. For calculation of fold changes between control and experimental groups, ΔC_T values were converted using the $2^{-\Delta\Delta C_T}$ method (Vandesompele et al., 2002).

Results

DEX differentially alters stress hormone receptors expression in *Bcl* wild-type (CC) or variant (GG) in 24h and 11-day cultures

PBMC were cultured with or without 10^{-8} M DEX, harvested and analyzed after 24h and 11-days. Significant changes in GR mRNA were detected in CC group with increased expression at 24h and decreased at 11 days ($p < 0.01$ and 0.05 , respectively; Fig1A & B). There was no effect on PBMC from subjects in the GG allele group at either 24h or 11-day DEX cultures. $\beta 2AR$ expression was increased at 24h culture with DEX in both CC and GG alleles ($p < 0.01$ and 0.001 respectively; Fig1C & D), while significant decrease was only found in GG allele at 11-day culture ($p < 0.01$). The results revealed that $\beta 2AR$ was up-regulated with short term DEX exposure (acute stress model) for both alleles, whereas longer term DEX exposure (chronic stress model) down-regulated the expression only in GG allele.

FoxP3 mRNA expression in CC or GG alleles after short- versus long-term exposure to DEX

The FoxP3 mRNA level was decreased after 24h and increased after 11 day exposure to DEX in CC alleles. ($p < 0.01$, respectively; Fig 2A & B). There was no significant change of FoxP3 mRNA expression in GG allele at both 24h and 11-day cultures suggesting that PBMC from GG allele are more protected compared with CC alleles by FoxP3 gene expression under stress conditions.

DEX differentially alters IFN- γ R and IL4R expressions in CC or GG alleles

Th1 cytokine receptor IFN- γ R mRNA was significantly increased after 24h exposure to DEX in GG group ($p < 0.001$) and returned back to baseline after 11-day culture (Fig 3A & B). No significant changes were detected in CC group in either 24h or 11-day cultures. IL4R mRNA was increased in both CC and GG alleles from 24h cultures ($p < 0.05$ and 0.01 , respectively) but there were no DEX-mediated changes in IL-4R mRNA at 11 days in either group (Fig 3C & D).

Effects of DEX exposure on T-bet and GATA-3 in 24h and 11- day cultures

Gene expression of transcription factors T-bet and GATA-3 from short- and long-term cultures with DEX were analyzed. Both transcription factors were significantly decreased after 24h exposures to DEX in CC and GG alleles (Fig 4A & C). The alterations returned to baseline in both groups in 11-day culture.

Discussion

Changes in HPA axis reactivity in healthy family members of patients with affective disorders are documented, suggesting a genetic contribution through cortisol regulation (Ellenbogen et al., 2004; Holsboer et al., 1995). In patients with post-traumatic stress disorder, increased reactivity of the HPA axis and increased corticosteroid sensitivity has been demonstrated (Yehuda, 2002). Previous study revealed that the ER22/23EK and N363S polymorphisms in the GR gene can partly explain inter individual variation in sensitivity to GCs (Russcher et al., 2005). GR polymorphisms were associated with modulation of the

endocrine stress response and possibly represent one factor contributing to vulnerability for psychopathology (van Rossum et al., 2006; van West et al., 2006). Our study documents an association between different alleles of GR gene *BclI* polymorphism (e.g. CC and GG) and distinct immunological responses to *in vitro* stress hormone exposure of human PBMC.

In the *BclI* CC WT, GR mRNA was significantly increased at 24h exposure to DEX and decreased after 11 days. No such changes were observed in GG alleles. Variations in nucleotide sequence can result in amino acid changes if they are located in a codon. If in a noncoding region, such as the *BclI* polymorphism, they can influence gene regulation by effects on gene-splicing, transcription efficacy, promoter activity, translation efficacy or by introducing a stop codon (DeRijk et al., 2002). The current study was an attempt to investigate immune-altering effects of stress hormone DEX on PBMC based on *BclI* genotypes at the transcriptional level. Our results show that there was an effect of DEX on GR mRNA expression only in CC group in which it was up-regulated at 24 h and down-regulated after 11 days. However, when data from combining CC plus GG were analyzed, all differences was disappeared (data not shown) suggesting the polymorphism was associated with DEX-induced GR mRNA expression, even though it is located in a noncoding region. It is likely that the GG allele may be less sensitive to stressor, at least at 10^{-8} M DEX *in vitro* model which is a concentration mimicking the plasma cortisol concentration during periods of cognitive stress, on GR expression compared with WT. Therefore, the *BclI* SNP may be more protective in response to stressor compared to WT.

It has been reported by Rossum et al (van Rossum et al., 2003) that the *BclI* polymorphism in the GR gene is associated with GC sensitivity *in vivo* and the variant G-allele is associated with hypersensitivity to GC compared to WT C-allele. In their study, 0.25 and 1.0 mg DEX (pharmacological doses used in a clinical dexamethasone suppression tests *in vivo*) significantly decreased post-DEX cortisol levels in G-allele carriers. However, the concentration of DEX in our *in vitro* stress model was only 10^{-8} M which was much lower than pharmacological dose used *in vivo* (van Rossum et al., 2003). We speculate that different doses of DEX used in other studies might be a more likely cause for the effect differences reported by us and others. Future studies will be needed to search for differences between immune effects caused by varying concentrations of DEX and *BclI* polymorphism.

In anti-inflammatory processes, CS and CT can have either antagonistic or cooperative effects in anti-inflammatory processes (Johnson, 2004; Eickelberg et al., 1999; Straub et al., 2002). Previous studies of the immune impact of acute and chronic stress have demonstrated that cognitive stressors are strongly correlated with high levels of CS and CT. (Kirschbaum et al., 1993; Marshall et al., 1998). Both CS and CT systemically mediate a Th2 shift by up-regulating Th2-cytokine production and also by suppressing activities of antigen-presenting cells and Th1 cytokine synthesis (Elenkov & Chrousos, 2002). Our recent *in vitro* studies demonstrate that the combined effects of CS and CT (compared to either agent alone) significantly decreases the production of type-1 cytokines and simultaneously increases the expression of type-2 cytokines in PBMC cultures (Salicru et al., 2007). This suggests that each hormone has its own signal pathways. Variable cytokine responses due to their SNP-associated receptor variability could further explain between group differences. Therefore, in addition to GR mRNA expression, β 2AR mRNAs were measured as well in current study. DEX exposure significantly up-regulated the expression of β 2AR mRNA at 24h culture in both CC and GG alleles, whereas down-regulation was found only in GG alleles at 11-day culture. This suggested that, for β 2AR gene expression, the CC group may be more affected by acute stress, whereas the GG group may be more susceptible in both acute and chronic stress states. In addition, the increased β 2AR expression in short-term DEX culture is consistent with our *in vivo* study in which β 2AR mRNA from PBMC was significantly increased after laboratory stress tasks (Trier Social Stress Test) (Xiang et al., 2012). Thus,

the evidence from both of *in vitro* and *in vivo* studies further supports the concept that catecholamine signal pathway has more significant immunomodulatory effects after acute stress compared to HPA pathway which may be more prominent in chronic stress. Furthermore, because β 2AR is expressed only on Th1 cells, but not on Th2 cells (Sanders et al., 1997), increased β 2AR mRNA expression in 24h culture with DEX provides additional evidence that Th1 cells may be more affected by acute stress compared to Th2 cells.

Stress hormones can inhibit production of pro-inflammatory cytokines and stimulate the production of anti-inflammatory cytokines (Elenkov & Chrousos, 2002). Thus, active regulatory control of Th1/Th2 balance is a pivotal target in the host immune response to stress. In humans, FoxP3 is an intracellular transcription factor which, in CD3+ T cells, has regulatory capabilities and either originate from the thymus or can be induced in the periphery (Shevach, 2009). More than 95% of normal human peripheral blood FoxP3+ T cells are found in the CD4+ population. Therefore we chose to utilize FoxP3 mRNA expression as a surrogate for regulatory T cells (Treg) in current investigation. In our previous study, we found FoxP3 mRNA was decreased after 24h culture with DEX and returned back to control level by 11 days (Xiang & Marshall, 2011). We subsequently grouped subjects by *BcII* genotype in this study and found that FoxP3 mRNA was significantly decreased at 24h and increased at 11-day cultured with DEX only in CC WT group (Fig 2). There were no significant changes in GG SNP group at either 24h or 11-day culture. This finding suggests that the subjects with GG SNP may actually protect against stress hormone induced effects on FoxP3 expression compared to WT group. While the exact mechanism of DEX on FoxP3 expression remains to be established, it is unlikely that a substantial decrease in actual Treg numbers through apoptosis occurred *in vitro*, since most Treg cells typically do not proliferate (Wing & Sakaguchi, 2010). Rather, it seems more plausible that FoxP3 expression itself was modulated which would affect regulatory capability of this CD4+ subpopulation.

Immune deviation is primarily concerned with the balance between cytokines produced by Th1 cells (including IFN- γ and TNF- β) which promote cell-mediated immunity against intracellular pathogens vs. Th2 cells (including IL-4, IL-5, IL-9, and IL-13) which promote immunity against extracellular pathogens. In addition to regulating the metabolism of proteins, carbohydrates, and lipids, GCs have potent anti-inflammatory and immunosuppressive properties that can modulate gene expression involved in the priming of the innate and adaptive immune response. Their actions on the adaptive immune response include regulating the magnitude and balance between Th1 and Th2-mediated immune responses. It should be noted that the altered Th1/Th2 mRNA ratio after exposure to DEX in our previous studies replicated the shift noted when supernatant cytokine protein was measured in our original reports (Agarwal & Marshall, 1998; Agarwal & Marshall, 2001). Th1/Th2 cytokines modulate immune balance through binding their receptors. Thus, determining gene expression levels of cytokine receptors are very important in predicting changes in immunomodulation. This study showed that IFN- γ R mRNA increased only in GG allele at 24h culture with DEX and IL-4R was up-regulated in both alleles at 24h exposure to DEX suggesting GG allele may be more sensitive to acute stress effects on gene expression of Th1/Th2 cytokine receptors compared to CC allele.

To further understand the mechanisms of Th1/Th2 imbalance in response to stress hormones, we investigated the gene expression of transcription factors T-bet (TBX21) and GATA-3 which are important in T-cell proliferation and differentiation. T-bet acts as a key regulator of Th1 cell fate determination and directly activates IFN- γ and suppresses IL-4 (Szabo et al., 2000; Djuretic et al., 2007), whereas GATA-3 has a pivotal role in Th2 cell development and acts by activating IL-4 and suppressing IFN- γ (Zheng & Flavell, 1997; Chang & Aune, 2007). Both factors exert their roles at gene loci that encode Th1 and Th2

cytokines respectively. Previous observations from our group have shown that Th1 cytokine was increased after short term exposure to stress hormone (Agarwal & Marshall, 2001). However, the present study revealed that gene expression of T-bet and GATA-3 were repressed in both alleles after 24h culture with DEX and returned back to baseline after 11 days. It is likely that the regulatory role of T-bet on up-regulation of IFN- γ may have occurred earlier in the response to acute stress than our 24h measuring point. This notion is supported by our previous *in vivo* study which revealed that the production of IFN- γ was increased immediately after acute laboratory stressor and increased IFN- γ had returned to baseline by 2h after the completion of the stress task (Xiang et al., 2012). It is also possibility that other transcription factor pathways of T-cell differentiation may be more sensitive to stress hormone effects. These possibilities are being further investigated.

We conclude that the *BclI* polymorphism in the GR gene identifies different immunomodulatory responses to CS which may explain, at least in part, the variability in individual sensitivity to stressors.

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Fig 1. Effects of stress hormone on gene expression of stress hormone receptors GR and β 2AR on PBMC from subjects with CC or GG allele of *BclI* polymorphism (n=9 for each allele). Values are means $\pm$ SEM. Data are expressed as fold changes ($2^{-\Delta\Delta CT}$). The asterisk denotes a significant difference between groups indicated by lined demarcations (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig 2. FoxP3 mRNA expression from PBMC after 24h or 11 day incubation with 10⁻⁸ M DEX in CC or GG allele. Values are means ± SEM. The asterisk denotes a significant difference between groups indicated by lined demarcations (** *p* < 0.01).

Fig 3. Relative amounts of Th1 and Th2 cytokine receptors IFN γ R and IL-4R mRNA from PBMC were quantified by real-time PCR. Values are means $\pm$ SEM. The asterisk denotes a significant difference between groups indicated by lined demarcations (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig 4.

Gene expression of transcription factors T-bet and GATA-3 from PBMC cultured with DEX for 24h or 11 days. Values are means $\pm$ SEM. The asterisk denotes a significant difference between groups indicated by lined demarcations (** $p < 0.01$, *** $p < 0.001$).

Table 1

TaqMan Gene Expression Assay

Gene	Assay ID	RefSeq	bp
GR	Hs00230818	NM_000176	115
β 2AR	Hs00240532	NM_000024	65
FoxP3	Hs01085835	NM_014009	107
IFN- γ R	Hs00166223	NM_000416	110
IL-4R	Hs00166237	NM_000418	70
T-bet	Hs00894392	NM_013351	119
GATA3	Hs00231122	NM_1002295	80