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IL-12 inhibits glucocorticoid-induced T cell apoptosis by inducing GMEB1 and activating PI3K/Akt pathway

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ABSTRACT

Interleukin (IL)-12 is an important pro-inflammatory cytokine that has been shown to play a role in T cell survival, at least in part by activating the PI3K/Akt pathway. Glucocorticoid modulatory element binding protein (GMEB)1 and 2 are closely related proteins that modify the glucocorticoid receptor binding locus and thus modulate glucocorticoid-mediated gene induction effects, including apoptosis. GMEB1 associates with caspases and prevents apoptosis of cells in the nervous system. We have observed, in preliminary studies, that IL-12 up-regulates GMEB mRNA in human T cells, and postulated that this may contribute to the anti-apoptotic effect of IL-12 on T cells, in particular with regard to glucocorticoid induced apoptosis. Here, we confirm that IL-12 rescue of dexamethasone induced T cell apoptosis involves the PI3K/Akt pathway and that IL-12 induces GMEB1 and GMEB2. A siRNA knockdown of GMEB1 reverses the protective effect of IL-12 on dexamethasone induced T cell apoptosis. Thus, IL-12 protects T cells from glucocorticoid induced apoptosis via PI3K/Akt pathway and via induction of GMEB1, which is likely to reduce transactivation of the glucocorticoid receptor and induction of apoptotic genes. As glucocorticoid induced apoptosis occurs both in physiological and pathological/therapeutic situations, and IL-12 is actively involved in a variety of inflammatory and immune responses, the ability of IL-12 to inhibit steroid responses and increase T cell survival through GMEB1 has wide ranging implications. Manipulating GMEB may be used therapeutically to enhance the resistance or the sensitivity to steroids.

Introduction

Glucocorticoids (GC) are hormones essential for life, being involved in most homeostatic and metabolic functions and having a critical immunological role, where they are part of a feedback system that turns down inflammation. Synthetic GC are used as replacement therapy in endogenous GC deficiency states, and are important immunosuppressive and anti-inflammatory drugs used in inflammatory, autoimmune, and allergic diseases (Rhen and Cidlowski 2005). GC have pleiotropic effects in the immune system (Zen et al. 2011), including down-regulation of adhesion molecules, cytokine shift, regulation of cytokine signalling, suppressing matrix metalloproteinases, inducing regulatory T cells (Treg) and enhancing expression of the key transcriptional regulator of Treg cells, foxp3 (Karagiannidis et al. 2004; Braitch et al. 2008). It is also known that GC induce apoptosis in most nucleated cells of the immune system, such as thymocytes, monocytes, macrophages, and T and B lymphocytes as well as their malignant counterparts (Amsterdam et al. 2002; Herold et al. 2006; Opferman and Korsmeyer 2003). GC exerts their effects mainly through the interaction with the GC receptors (GR) and GR-mediated gene activation is an essential component of the apoptotic pathway (Reichardt et al. 1998). Although GC induced cell death does not directly proceed via one of the two classical apoptotic pathways, Bcl-2 proteins and caspases appear to be involved in this process (Herold et al. 2006; Tuckermann et al. 2005; Distelhorst 2002; Frankfurt and Rosen 2004).

Glucocorticoid modulatory element-binding protein 1 and 2 (GMEB1 and GMEB2), members of the family of KDWK proteins, form a heterodimer that binds DNA and modulates the transactivation function of GR and thus GC effects (Zeng et al. 2000). Furthermore, GMEB1 has been reported to be capable of binding to caspase-2, -8 and -9, and to inhibit caspase-mediated apoptosis (Nakagawa et al. 2008; Tsuruma et al. 2006, 2004).

Interleukin (IL)-12, a heterodimeric cytokine composed of the disulfide-linked p35 and p40 subunits, plays a key role in cell-mediated immune responses (Trinchieri 2003). IL-12 drives the

Abbreviations: FITC, fluorescein isothiocyanate; GC, glucocorticoids; GMEB, glucocorticoid modulatory element binding protein; GR, glucocorticoid receptor; IFN, interferon; IL, interleukin; Jak, Janus kinase; MS, multiple sclerosis; NK, natural killer; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; PI3K, phosphatidyl inositol-3 kinase; STAT, signal transductor and activator of transcription; Treg, regulatory T cells.

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differentiation of naïve CD4 T cells into Th 1 cells and enhances cytotoxic functions of CTLs and NK cells. IL-12 binds to the IL-12 receptor (IL-12R), which is expressed on T cells, NK cells, and dendritic cells. IL-12R is composed of two subunits, IL-12 β1 and β2. The IL-12 receptor signals through the Janus family of protein tyrosine kinase (Jaks), Jak2 and Tyk2 (Bacon et al. 1995) and activated Jaks phosphorylate STAT4; phosphorylated STAT4 then binds to the IL-12 responsive genes (e.g. IFN-γ) (Jacobson et al. 1995). IL-12 also activates the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway in T cells (Yoo et al. 2002). This pathway inhibits the apoptosis signal pathway and increases T cell survival. IL-12 has been shown to reduce T cell apoptosis (including CD8 and Th1 cells) in a number of settings (Radrizzani et al. 1995; Li et al. 2001; Fuss et al. 1999; Marth et al. 1999). However, while GC are known to inhibit Th1 responses through direct effects on IL-12 (Visser et al. 1998) or on its signalling (Franchimont et al. 2000; Fahey et al. 2006), the effects of IL-12 on GC induced T cell apoptosis are unknown.

In preliminary studies of IL-12 induced gene expression in T cells using microarrays, we detected an up-regulation of GMEB1 and GMEB2 along with other antiapoptotic genes (C. Constantinescu and L. Showe, unpublished data), and hypothesised that GMEB1 and GMEB2 may be involved in IL-12 rescue of GC-induced apoptosis in T cells.

In this study we investigate whether GC induced T cell apoptosis is affected by IL-12 and whether GMEB1 and GMEB2, as well as PI3K/Akt pathway, are involved in the IL-12 effects.

In previous studies, IL-7 was shown to inhibit dexamethasone-induced T cell apoptosis (Sade and Sarin 2003). In the current study we confirm this finding and use it as a positive control for rescue from GC-induced apoptosis by IL-12.

Materials and methods

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-Hypaque density gradient centrifugation (Sigma–Aldrich, Dorset, UK). PBMC were cultured in 24-well plates (10^6 cells/well) at 37 °C, 5% CO_2 containing RPMI-1640 supplemented with 10% fetal calf serum, 2 mM-glutamine, 20 mM HEPES, 100 μg/ml streptomycin, and 100 U/ml penicillin (Sigma–Aldrich, Dorset, UK).

For isolation of T-cells from fresh PBMC, PBMC were washed with media and stained with ECD conjugated anti-CD3 antibody (Beckman Coulter, Buckinghamshire, UK) for 20 min on ice. Stained cells were washed in PBA (phosphate-buffered saline, PBS, 0.5% bovine serum albumin and 1% sodium azide [Sigma–Aldrich]), and resuspended in complete culture medium. $CD3^+$ cells were collected using by an EPICS XL flow cytometer (Beckman Coulter, Buckinghamshire, UK).

When T cell blasts were used, PBMC were co-cultured with 10 μg/ml phytohemagglutinin (PHA; Sigma–Aldrich, Dorset, UK) for 72 h. Following PHA-induced proliferation, the cells were washed with media and stimulated with 100 U/ml IL-2 (R&D Systems, Minneapolis, MN) for 24 h. The cells were then allowed to rest for 24 h in serum-free media. Before stimulation, the cells were washed in culture medium.

Cell stimulation

All stimulations were performed with concentrations of stimuli established on preliminary experiments to be optimal. Isolated $CD3^+$ T cell from fresh PBMC were prepared at 10^6 cells/ml and stimulated with 100 ng/ml IL-12 (Peprotech EC, London, UK) for 24 h to study whether the expression of GMEB1 and GMEB2 in T cells were affected by IL-12 stimulation.

For the apoptosis assay, 500 nM dexamethasone was added in culture medium to induce apoptosis. The PBMC and PHA/IL-2-induced T cell blasts (10^6 cells/ml) were treated with/without 100 ng/ml IL-12 or 100 ng/ml IL-7 (Peprotech EC, London, UK) as a positive control for inhibition of dexamethasone induced apoptosis. We used a cell-permeable inhibitor of PI3K, LY294002 (25 mM) (Sigma–Aldrich Dorset, UK) to ascertain the role of PI3K in IL-12 mediated inhibition of dexamethasone induced apoptosis. Cells were incubated at 37 °C, 5% CO_2 for 24 h.

Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) was used to measure GMEB1 and GMEB2 mRNA abundance in IL-12 simulated T cells that had been isolated from fresh PBMC. Total RNA was extracted using a RNeasy mini kit (Qiagen, Surrey, UK) following the manufacturer's instructions. The quality and concentration of extracted RNA were measured by using Nanodrop 1000 (Thermo Scientific, Wilmington, USA). First-strand cDNA was synthesised from 500 ng total RNA using random primers and avian myeloblastosis virus reverse transcriptase using conditions as described by the manufacturer in a final volume of 25 μl. The synthesised cDNA was diluted 1 in 20 and 5 μl diluted cDNA was used for qRCR. Primers used (final concentration 300 nM) were as follows: for GMEB1, forward, 5′-TGG CCT AAT GGA CAC AGT C-3′; reverse, 5′-ATC CTG GCC TTG CTT CTT C-3′. For GMEB2, forward: 5′-CAA CAT CGT GCA GAA CTT CG-3′; reverse, 5′-ATG CTC ATC ACA CTG CTG CT-3′. As an internal control, the house-keeping gene β2-microglobulin (B2MG) was used: forward; 5′-CTC CGT GGG CTT AGC TGT G-3′; reverse, 5′-TTT GGA GTA CGC TGG ATA GCC T-3′. Real-time PCR was performed with Velocity™ SYBR® Green qPCR Master Mix (Stratagene, Amsterdam, Netherlands) in a final volume 25 μl. The reactions were carried out in triplicate on a MX4000® Multiplex Quantitative qPCR system (Stratagene, Amsterdam, Netherlands) under the following conditions: 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 50 s at 65 °C. The dissociation curve method was used to assess the specificity of the amplified products. Data were analyzed according to the standard curves methods using by Mx4000 system software v4.20. Results are presented as the normalized ratio against B2MG expression.

Measurement of apoptosis

Dexamethasone induced apoptosis was measured by utilizing the Annexin-PI assay kit (Beckman-Coulter, Buckinghamshire, UK). Cells stimulated with dexamethasone and other agents as mentioned above were washed with PBS. The cell pellet was resuspended in 100 μl annexin binding buffer and stained with 1 μl annexin V-FITC and 5 μl propidium iodide solution for 30 min on ice. After staining, 400 μl binding buffer was added in each samples. PHA/IL-2-induced T cell blasts were stained with ECD conjugated anti-CD3 (R&D system, Oxford, UK), for 30 min on ice. After surface staining, cells were washed with PBA (phosphate-buffered saline, 0.5% bovine serum albumin, 1% sodium azide; Sigma–Aldrich, Dorset, UK) and resuspended in 100 μl annexin binding buffer and 1 μl annexin V-FITC on ice for 30 min, subsequently 400 μl of binding buffer was added. Analysis was performed on an EPICS ALTRA flow cytometer (Beckman-Coulter, Buckinghamshire, UK).

siRNA

Human GMEB1 and GMEB2 siRNA (Ambion, Cambridgeshire, UK) were used to knock down the expression of each gene. siRNA sequences specific to human GMEB1 and GMEB2 were used as fol-