Lipopolysaccharide-Induced M2 to M1 Macrophage Transformation for IL-12p70 Production Is Blocked by Candida albicans Mediated Up-Regulation of EBI3 Expression

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Abstract

Macrophages are heterogeneous cell populations that are present in all tissues. Macrophages can be divided into classically activated inflammatory macrophages (M1) and alternatively activated anti-inflammatory macrophages (M2). It has been generally accepted that M1 macrophages are polarised in an inflammatory environment to produce pro-inflammatory cytokines, whilst M2 macrophages are involved in anti-inflammation and aid tissue repair in wound healing. Bacterial endotoxin (lipopolysaccharide; LPS) is a potent factor in infection, which induces M1 macrophages resulting in higher levels of iNOS, TNFα and IL-12p70 which dictate inflammatory T cell responses. M2 macrophages can be transformed into M1 macrophages following LPS stimulation to promote inflammation. Candida albicans is a commensal fungal microorganism, which has been suggested to induce immune tolerance; however, the mechanism of C. albicans-induced immune tolerance has not been investigated in detail. IL-35 is a recently identified anti-inflammatory cytokine which is a heterodimeric protein consisting of the Epstein-Barr virus-induced gene 3 (EBI3) and IL-12p35. IL-35 shares the protein subunit p35, with IL-12p70. IL-12p70 is the most potent cytokine to induce Th1 responses during inflammation. In this study, we demonstrate that heat-killed C. albicans (HKC) strongly suppressed LPS-induced IL-12p70 production in M2 macrophages. Candida albicans induced a high level of EBI3 expression in M2 macrophages, which served as a mechanism for IL-12p70 suppression by competitive binding of the common protein subunit (p35) of IL-35 and IL-12p70. To demonstrate that EBI3 expression had the ability to block IL-12p70 production intracellularly, a Chinese Hamster Ovary (CHO) cell line with bicistronic expression of IL-12p40 and p35 was constructed, followed by ectopic over-expression of EBI3. The over-expression of EBI3 in the IL-12p70 producing cell line effectively suppressed IL-12p70 production. IL-35 secretion was also detected in the cell line, with suppressed IL-12p70 production by immune-precipitation Western blotting. However, this secretion was not evident in M2 macrophages following stimulation by HKC. This can be explained by the constitutive expression of IL-35 receptors (gp130 and IL-12Rβ2) in M2 macrophages for cytokine consumption. Our results have indicated that C. albicans can suppress host inflammatory responses in mucosal skin by suppressing LPS-induced IL-12p70 production. Lower IL-12p70 production may avoid an unnecessary Th1 response in order to retain immune tolerance, which may be one of the mechanisms by which C. albicans achieves a successful commensal lifestyle without having a detrimental effect on the host’s health.

Introduction

Macrophages are dispersed throughout the body and represent a highly heterogeneous cell population which demonstrate plasticity in their cell polarisation. Macrophages play an important role in immune surveillance [1,2], as they produce cytokines to alert host immune cells and can also be phenotypically transformed by a tissue environment. It has been widely accepted that at least two types of macrophages, with distinct phenotypes, can be found in the processes of infection and wound healing: classically activated macrophages (M1) and alternatively activated macrophages (M2) [3,4,5]. In an inflammatory environment, inflammatory T cells, such as Th1 and Th17, produce Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) which drives macrophage maturation. GM-CSF together with bacterial lipopolysaccharide (LPS) induce inflammatory macrophages (M1) from bone marrow-derived macrophages (BMDM) which exhibit high levels of iNOS, IL-12 and TNFα production [6]. In contrast, M2 macrophages can be generated in vitro by culturing BMDM with macrophage colony-stimulating factor (M-CSF) together with IL-4. M2 cells predominantly produce higher levels of arginase-1
and IL-10 [6,7]. Macrophages switch their phenotypes depending on the tissue environment [9,8,10] and this phenotypic plasticity is of benefit to both the host and pathogen, since inappropriate host responses can result in tissue damage which may lead to chronic wounds and pathogen invasion.

_Candida albicans_ is an opportunistic fungal pathogen, capable of causing life-threatening infections in immune incompetent individuals [11,12]. However, _C. albicans_ is found on moist mucosal surfaces in the majority of healthy humans, without causing disease and this commensal existence has been associated with host immune tolerance. _Candida albicans_ has two basic morphological growth forms, namely hyphae and yeast. It is often stated that hyphae on mucosal surfaces induce an immune response, whilst a predominant yeast presence is linked to a commensal existence on the mucosal surface and may be more potent at inducing immune tolerance. A fine balance between host immune responses to _C. albicans_ and bacterial LPS stimulation may play a key role in maintaining healthy mucosal surfaces; however, the detailed mechanism of _C. albicans_ inducing immune tolerance has not been extensively studied.

Macrophages are a major cell source for production of the IL-12 family of cytokines during infection and inflammation. IL-12 consists of two protein subunits, IL-12p40 and p35, forming a heterodimeric cytokine IL-12p70, which is an effective cytokine in driving Th1 responses. IL-35 shares the IL-12p35 subunit and is heterodimeric with EBI3. IL-35 is one of the anti-inflammatory cytokines that promotes regulatory T cells (Treg) to suppress immune response [13,14,15]. The macrophage phenotypes are largely dependent on cytokine production in the tissue environment. Switching a macrophage from an inflammatory cytokine producing cell to an anti-inflammatory producing cell may hold the key to induced non-immune responses or peripheral tolerance.

In this study, we found that LPS induced an M2 to M1 cell phenotype transformation, thereby inducing IL-12p70 production. Heat-killed _C. albicans_ (HKC) suppressed LPS-induced IL-12p70 production in a dose-dependent manner in M2 macrophages and this inhibition was associated with induced EB13 expression, a subunit of IL-35. Over-expression of EB13 in an IL-12p70 expression cell line reduced IL-12p70 production and detectable secretion of IL-35. This result demonstrated that _C. albicans_ induced anti-inflammatory cytokine IL-35 production in M2 macrophages and blocked LPS-induced M2 to M1 macrophage phenotype transformation. We postulate that this serves as the mechanism behind _C. albicans_-induced immune tolerance.

**Materials and Methods**

**M1 and M2 Macrophages Culture and Stimulation**

The protocols for animal handling were previously approved by our institutional Animal Ethics Committee according to UK Home Office guidance. Bone marrow cells were harvested from the femurs of male C57/Black mice and prepared accordingly for culture of bone marrow M1 (classical) and M2 (alternative) macrophages using 10 ng/ml recombinant GM-CSF (ImmunoTools) and M-CSF respectively [ImmunoTools] [9]. Briefly, bone marrow cells were cultured in RPMI 1640 containing 10% (w/v) foetal bovine serum (FBS); 100 U/ml penicillin/streptomycin (Invitrogen, Glasgow, UK) and either GM-CSF or M-CSF. The medium was changed every other day for 7 days. Harvested M1 and M2 macrophages were seeded at a density of 1×10^5 cells/well in a 24 well plate, then cultured with full medium for 24 h. To fully polarise M1 and M2 macrophages, GM-CSF derived M1 macrophages were stimulated with 10 ng/ml bacterial LPS and M2 macrophages were cultured in M-CSF with 10 ng/ml IL-4 (ImmunoTools). In certain experiments, macrophages were stimulated with increasing concentrations of heat-killed _C. albicans_ (HKC). The cell lysate was harvested at the indicated time points for RNA extraction for quantitative RT-qPCR. Cell lysates and culture supernatants were also harvested at the indicated time points for Western blotting and an enzyme-linked immunosorbent assay (ELISA). To block EB13 protein transportation, 3 μg/ml of Brefedlin A (eBiosciences Ltd, USA) was added to the cell cultures 1 h before cell harvesting.

**Preparation of Heat-killed _C. albicans_**

A well characterized clinical isolate of _C. albicans_ was used for the challenge studies [16]. This isolate had previously been identified based on traditional biochemical analysis as well as sequencing of rDNA gene sequences. The isolate was cultured overnight at 37°C in Yeast Nitrogen Base medium supplemented with 0.5% v/v glucose. The cells were harvested by centrifugation at 3000 g and washed three times with phosphate buffered saline (PBS) before being heated at 98°C for 10 min. Yeast viability was then assessed by culture on Sabouraud dextrose agar to confirm total cell death.

**RT-qPCR to Detect mRNA Expression in Mouse Macrophages**

To detect the expression of mouse IL-12p40, p35 and EB13 as well as iNOS and Arginase 1, total RNA was extracted from lysed cells using RNeasy (Qiagen) following the manufacturer’s instructions. The RNA yield and purity were measured using a spectrometer (nanodrop), and 1 μg of each RNA sample was used for reverse transcription of cDNA. The mRNA levels of mouse iNOS, Arginase-1, IL-12p35 and EB13 as well as IL-12Rβ2 and gp130 (IL-35 receptor chains) [17] and TLR4 were compared with the housekeeping genes β-actin or mHPRT. The following primer pairs were used: mouse iNOS sense, 5′-TGG CTC GGT TTT CCA CGG AGC AGA CCG A and anti-sense, 5′-GGA GCT GCT ACG ACA GGA AGG CAG CGG G. Mouse Arginase-1 sense, 5′-AGT GCC TGG TGT GGT GGC AGA GTT CCA and, anti-sense, 5′-GGG TGG ACC CTG CGG TGG CCA GAG ATG CT. Mouse IL-12p35 sense, 5′-CCC TTG CAT CTG GCG TCT ACA CTG CTG C and, anti-sense, 5′-AGG AGG GCA AGG GTG GCC AAA AAG AGG A. Mouse EB13 sense, 5′-GCC TCC TAG CCT TTG GTG CTG AGC GAA A and, anti-sense, 5′-AGA GAG AAG ATG TTC GGG AAG GGC CAG. Mouse IL-12Rβ2 sense, 5′-CAG GGA GCA TGA CGA AGT TTC CCC CAC A and, anti-sense, 5′-TTT GTG CCTT GGA GTT ACC CCC GAG GTG G. Mouse gp130 sense, 5′-CAG GAA GAC GCT ACC GTG CAT AAT GCG ACC C and, anti-sense, 5′-CTG TCG GAG CAG GGC CTT TGT TGA GGT A. Mouse TLR4 sense, 5′-GAC TGT CAT GGC ACT GTT CTT CTC C and, anti-sense, 5′-CAG GCA CTG TGC TGA TGT GTT CAC GGA C. Mouse β-Actin sense, 5′-TCT TCG CAG CTT CTC CTG TGG CCG TCC, and anti-sense, 5′-GTC TCTT CTT CTG ACC CTT CAC CAG CAC A. Mouse HPRT sense, 5′-TTG ATT GTT GAA GAT ATA ATT GAC ACT and, anti-sense, 5′-TTC CAG TCT TTT CAC TAA TGA CAC A.

PCR products were obtained using the following thermal cycles, 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 40 cycles. RT-qPCR results were visualised using a 1.5% (w/v) agarose gel containing ethidium bromide. To quantify the mRNA levels present in M1 and M2 macrophages, relative gene expression levels were determined using a SYBR Green qPCR kit (Sigma). All samples were run in triplicate. The CT value of each sample was acquired following a calculation of the 2^-ΔΔCt, and all data were expressed as a percentage of gene/β-actin (%).
lysate or 1 ml of cell culture supernatant was pre-treated with a housekeeping gene was used for equal loading control in all samples.

After this, 2–5 μl of anti-IL-12p35 antibody binding, 20 μl of pC3-p40-IRES-p35 plasmid DNA followed by selection of pC3-p40/p35 bicistronic expression, the cells were transfected with increasing concentrations of mouse EBI3 cDNAs were cloned via RT-PCR with cDNA extracted from LPS stimulated J774 cells as previously described [14]. The cDNAs containing the open reading frames (ORFs) of IL-12p40 and p35 were inserted into a pcDNA3.1A-IRES vector to give the plasmid pC3-p40-IRES-p35. To establish Chinese Hamster Ovary (CHO) clones with IL-12p40/p35 bicistronic expression, the cells were transfected with 1 μg of pC3-p40-IRES-p35 plasmid DNA followed by selection of G418 resistant clones over a 14-day culture period. IL-12p40/p35 expressing CHO clones were identified and expanded in culture.

Functional IL-12p70 production by the CHO cell clones was confirmed by stimulation of IFNγ production in purified CD4+ T cells from a mouse spleen. The EBI3 expression vector was constructed through insertion of EBI3 cDNA into pcDNA4/TTO-A. EBI3 expression in CHO cells was also demonstrated by Western blotting with antibodies against EBI3.

Detection of Memory Bound EBI3 on Macrophage Cell Surface by FACS Analysis

The Mouse RAW264.7 macrophage cell line was cultured in the M2 macrophage conditional medium (RPMI 1640 full medium containing 20 ng/ml of M-CSF and 20 ng/ml of IL-4) for 4 days. After this time, 5×10^6 cells per well of a 6-well plate were incubated overnight with or without 10^6 HKC/ml. The macrophages were then harvested and washed with cold PBS before staining with anti-mouse EBI3 antibody (eBioscience) or isotype control antibody, followed by PE-Texas red-labelled secondary antibody. Cell surface bound EBI3 was then detected by FACS analysis.

Statistical Analysis

Results were calculated as means ± standard deviations (SD). Statistical significance was determined using a one-way ANOVA and Tukey-Kramer or Bonferroni multiple comparisons post-tests to analyse differences between groups; P<0.05 was considered significant.

Results

Phenotypes of M1 and M2 Macrophages Derived from Mouse BMDM

To demonstrate the phenotypes of M1 and M2 macrophages generated in our culture conditions, iNOS Arginase-1 mRNA expression in M1 and M2 macrophages were examined by RT-qPCR. M1 macrophages cultured in GM-CSF/LPS expressed much higher levels of iNOS mRNA compared with M-CSF/IL-4 cultured M2 macrophages, whilst a higher level of arginase-1 expression was detected with M2 macrophages when compared with M1 macrophages (Figure 1A). Significantly higher levels of IL-12p70 and TNFα were also detected with M1 macrophages, but not with M2 macrophages (Figure 1B). This result indicated that successful polarisation of M1 and M2 from BMDM was achieved in the culture conditions used.
Candida albicans Suppresses LPS-induced IL-12p70 in M2 to M1 Phenotype Switching

Macrophages demonstrate plasticity in phenotype switching. Inflammatory M1 macrophages can be polarised into M2 macrophages within tissues during the resolving stage of infection and wound healing. M2 macrophages produced lower levels of inflammatory cytokines, but higher anti-inflammatory cytokines and growth factors following HKC stimulation (data not shown). To demonstrate plasticity of macrophage polarisation, fully polarised M1 and M2 macrophages were stimulated with 10 ng/ml bacterial LPS and the cell culture supernatants harvested 48 h after stimulation. In these experiments, IL-12p70 and TNFα production was determined via ELISA. It was evident that following LPS stimulation, a 20-fold higher IL-12p70 expression in M2 occurred compared with M1 macrophages. However, TNFα production only increased 2-fold in M2 macrophages, with no significant difference when compared with M1 macrophages (GM-CSF/LPS) (Figure 2A). Using qRT-PCR a 3-fold greater TLR4 mRNA expression in M2 macrophages was evident compared with that in M1 macrophages (Figure S1). An increased concentration of HKC resulted in dose-dependent suppression of LPS-induced IL-12p70 production by M2 macrophages, but the levels of TNFα production did not alter in these cells (Figure 2B). Since IL-12p70 is a critical pro-inflammatory cytokine, which...
dictates Th1 polarisation, suppressing IL-12p70 production induced by LPS, may serve as a critical mechanism of by which *C. albicans* induces immune tolerance.

*Candida albicans* Stimulates Increased Levels of EBI3 mRNA and Protein in M2 Macrophages

Previously we reported that HKC induced a higher level of EBI3 expression in human monocytes/THP-1 cells [13]. In this study, we have confirmed that HKC stimulation results in much higher EBI3 levels in mouse BMDM compared with LPS stimulation. In contrast, LPS and HKC stimulation resulted in comparable levels of IL-12p35 expression (Figure 3A). In macrophages, EBI3 mRNA was expressed at much higher levels than p35 mRNA, which was demonstrated by the percentage expression of genes of interest compared with the mouse housekeeping gene, adenine phosphoribosyltransferase (HPRT) (Fig. 3A). Moreover, HKC was found to induce dose-dependent EBI3 expression at both the mRNA and protein level in M2 macrophages in the presence of LPS stimulation (Fig. 3B). Although we have detected expression of both IL-35 protein subunits in macrophages after stimulation by HKC, immune precipitation Western blotting failed to detect IL-35 secretion in cell culture medium (data not shown). However this can be explained by detecting IL-35 receptors expression in M2 macrophages (Figure 3C). The consumption of IL-35 by its receptors on M2 macrophages was further evidenced by detection of cell membrane bound EBI3 by FACS staining with anti-EBI3 antibody (Figure 3D). However expression of the IL-35 receptor did not trigger cell signalling to inhibit IL-12p70 production, since adding recombinant IL-35 or IL-27 to M2 macrophage cell cultures did not alter LPS induced IL-12p70 production (Figure S2). Furthermore, HKC induced EBI3 expression plays a role in suppression of LPS induced IL-12p70 production. This was supported by results obtained following EBI3 ‘knock down’ in the mouse macrophage cell line (RAW264.7) which rescued HKC inhibition of LPS-induced IL-12p70 production (Figure S3). Competition between EBI3 and IL-12p40 for IL-12p35 coupling was further confirmed by the following experiments.

Over-expression of EBI3 in IL-12p70 Expressing CHO Cell Lines Suppresses IL-12p70 Production

To further investigate the impact of EBI3 expression on IL-12p70 production, IL-12p70 producing CHO cell lines were established by transfection to give bicistronic expression of IL-12p40 and p35. Two clones with comparable levels of IL-12p40 and IL-12p70 expression were used for testing IL-12p70 biological activity using purified mouse CD4+ T cells (Figure 4A). Cell culture supernatants collected from both clones were able to

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**Figure 2. Heat-killed *C. albicans* inhibits LPS-induced M2 to M1 shifting for IL-12p70 production.** A. M1 and M2 macrophages were stimulated with 10 ng/ml LPS for 48 h before harvesting the cell culture supernatant to detect IL-12p70 and TNFα expression via ELISA. B. M2 macrophages were stimulated with increasing concentrations of HKC plus 10 ng/ml LPS. IL-12p70 and TNFα production was quantified by ELISA. The results are representative of three experiments. **p<0.01.

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A

RT-qPCR

Western blot

mEBI3/HPRT (%)

mp35/HPRT (%)

Med LPS HKC Med LPS HKC

B

RT-qPCR

Western blot

EBI3/mhprt (%)

0 105 106 cell/ml HKC 10 10 10 ng/ml LPS

C

HKC stimulation

0 4 8 24 h

-ml-12Rβ2

-mgp130

-β-Actin

D

Macrophage cell population (%)

Isotype Ab Medium HKC

FL3-H
stimulate IFNγ production (Fig. 4B). We selected one of the two clones for transfection with the EBI3 over expression vector. Three representative clones with higher EBI3 expression levels were identified following the detection of IL-12p40 and IL-12p70 in the culture medium. IL-12p70 producing CHO cells with over-expression of EBI3 showed a large reduction in IL-12p70 production (Fig. 4C) which was associated with detectable levels of IL-35 secretion in the culture supernatant (Fig. 4D). This result demonstrated that intracellular over-expression of EBI3 effectively suppressed IL-12p40 and p35 dimersiation for IL-12p70 production. This effect provides an intrinsic mechanism of regulation for the IL-12 family of cytokines, which serves to balance immune responses.

Discussion

Host innate and adaptive immune cells orchestrate their development to control pathogen invasion and also limit tissue damage or promote tissue repair. Candida albicans is an opportunistic pathogen which is normally effectively controlled by the host immune system. One of damaging features of host immunity is its over-reaction to a pathogen. In a host-commensal C. albicans relationship, C. albicans is able to induce innate immune responses, but these are sufficiently mild so as not to trigger severe inflammation and tissue damage. The mechanism behind this fine balance of the host immune response has not been established in detail. Macrophages are one of the most important host innate immune cells and exist in all tissues including mucosal surfaces. Macrophages play a key role in sensing and controlling C. albicans and bacterial infections by phagocytosing the pathogen and releasing cytokines. Macrophages are heterogeneous in phenotype and exhibit plasticity in polarising to adapt to different tissue environments. Two types of macrophages have been identified in infection and tissue repair, namely M1 and M2 macrophages. M1 macrophages are usually associated with higher pro-inflammatory cytokine production, which initiates adaptive immune T cell responses and induces tissue inflammation by producing cytokines such as IL-12p70, IL-23 and TNFα [18,19,20,21,22,23]. This inflammatory response promotes host defences against C. albicans and bacterial invasion of the mucosal epithelium. However, prolonged inflammatory cytokine production induced by M1 macrophages may lead to persistent inflammation as a result of excessive Th1 and Th17 responses. Although large numbers of Th1 and Th17 cells migrating into inflamed tissue help enforce macrophage killing, they also cause tissue damage [24,25]. Resolving pathogen invasion and the initiation of tissue repair is associated with M2 macrophages through increased concentration of growth factors and release of anti-inflammatory cytokines into the tissues [26,27]. M2 macrophages also exhibit phenotypic and functional plasticity [8].

Bacterial products, such as LPS, are potent bioactive factors which result in the switch of macrophage phenotype from M2 to M1 [9]. The aim of this study was to elucidate how C. albicans could potentially impact on these key innate immune cells (macrophages) in terms of response and tolerance through use of an in vitro macrophage culture model. We used GM-CSF to drive BMDM to become M1 macrophages. The development of classical M1 macrophages occurs in a tissue environment containing high levels of GM-CSF and IFN-γ produced by Th1 and Th17 cells [5,9]. GM-CSF effectively induced IFN-β production in an autocrine manner, triggering STAT1-1 signaling, which has the same effect as IFN-γ [20]. It is generally accepted that GM-CSF plus LPS produces M1 macrophage phenotypes in vitro. M2 macrophages were produced by culturing bone marrow macrophages in a medium containing M-CSF and IL-4. M1 and M2 macrophages generated in this study showed typical M1 and M2 phenotypes. M1 macrophages produced higher levels of iNOS, TNFα and IL-12p70, while M2 macrophages demonstrated higher levels of arginase-1 and vascular endothelial growth factor (VEGF), but lower levels of iNOS, TNFα and IL-12p70. Although M2 macrophages expressed a higher level of EBI3 than M1 macrophages, HKC stimulation further enhanced EBI3 expression in M2 macrophages.

Bacterial products (e.g. LPS) can induce inflammation and promote M1 macrophage polarisation. However, LPS can also cause M2 macrophages involved in wound healing to reverse to the inflammatory M1 phenotype, with a higher level of iNOS expression and pro-inflammatory cytokines, such as IL-12p70 and TNFα production [9,29]. Although this M1 inflammatory macrophage effectively controls pathogen invasion, it can also impede the wound healing process and induce tissue damage [30,31]. The appropriate transformation of macrophages from M1 to M2 phenotype in the process of resolving infection towards wound healing, is crucial for limiting tissue inflammation and promoting tissue repair. Maintaining the M2 macrophage phenotype is key for retaining immune tolerance. In general, C. albicans stimulated lower responses in macrophages compared with those following LPS stimulation. We found that HKC was not able to stimulate iNOS, TNFα or IL-12 production in M2 macrophages, but was able to with M1 macrophages. In contrast, LPS stimulated iNOS, TNFα and IL-12 production in both M1 and M2 macrophages to a different degree. However, HKC effectively suppressed LPS-induced IL-12p70 production only in M2 macrophages. Candida albicans stimulated high EBI3 expression by M2 macrophages which blocked switching of LPS-induced M2 to M1 cell phenotype with IL-12p70 production. Production of the IL-35 heterodimer protein (EBI3/p35) as detected by immune precipitation, was associated with decreased IL-12p70 secretion by transfected CHO cells. However, we failed to detect IL-35 production by M2 macrophages, which may be explained by induced expression of IL-12Rβ2 and gp130, a receptor for IL-35 (p35/EBI3). IL-35 receptor expression in M2 macrophage may bind to IL-35 and thereby restrict its secretion; this is supported by the detection of both EBI3 and p35 expression in lysed M2 macrophages and detection of increased cell surface bound EBI3 by FACs staining (Fig. 3D).

To demonstrate an autocrine role of IL-35 on macrophages for LPS induced IL-12p70 production, we added increasing concentrations of either recombinant mouse IL-35 or IL-27 [14,32] into M2 macrophages cell cultures, followed by LPS stimulation. This
Figure 4. Over-expression of EBI3 blocks IL-12p70 production in CHO cells. **A.** Biscistronic IL-12p40 and p35 expression were compared before being introduced into a CHO cell by transfection. Two clones (C1 and C2) with comparable levels of IL-12p40 and IL-12p70 as detected by ELISA were selected. **B.** The biological activity of IL-12 was detected by inducing IFNγ production in C57/black mouse CD4+ T cells. **C.** The EBI3...
did not significantly alter the levels of IL-12p70 in M2 macrophages culture supernatant following HKC stimulation (Figure S3). We did not detect IL-27 production in M2 macrophage culture supernatant from EBI3 KO mice produced higher IL-12p70 in response to LPS stimulation, showing that EBI3 plays an intrinsic role in suppressing IL-12p70 production in mice (Figure 7 in USA patent file: Pat No. US2009/022498-A1). We have also shown in this study that knocking down EBI3 by esiRNA rescued the HKC suppression for LPS-induced IL-12p70 production in mouse macrophages (Figure S3).

These results demonstrate for the first time that C. albicans induces EBI3 expression in M2 macrophages and blocks LPS-induced M2 to M1 macrophage phenotype transformation. This may serve as one of the mechanisms utilised by C. albicans to induce immune tolerance.

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