1 The mycotoxin Beauvericin exhibits immunostimulatory effects on **2 dendritic cells via activating the TLR4 signaling pathway**

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33

34 **Abstract**

35 Beauvericin (BEA), a mycotoxin of the enniatin family produced by various toxigenic fungi, has
36 been attributed multiple biological activities such as anti-cancer, anti-inflammatory, and
37 anti-microbial functions. However, effects of BEA on dendritic cells remain unknown so far.
38 Here, we identified effects of BEA on murine granulocyte–macrophage colony-stimulating
39 factor (GM-CSF)-cultured bone marrow derived dendritic cells (BMDCs) and the underlying
40 molecular mechanisms. BEA potently activates BMDCs as signified by elevated IL-12 and
41 CD86 expression. Multiplex immunoassays performed on myeloid differentiation primary
42 response 88 (MyD88) and toll/interleukin-1 receptor (TIR) domain containing adaptor inducing
43 interferon beta (TRIF) single or double deficient BMDCs indicate that BEA induces
44 inflammatory cytokine and chemokine production in a MyD88/TRIF dependent manner.
45 Furthermore, we found that BEA was not able to induce IL-12 or IFN β production in Toll-like
46 receptor 4 (*Tlr4*)-deficient BMDCs, whereas induction of these cytokines was not
47 compromised in *Tlr3/7/9* deficient BMDCs. This suggests that TLR4 might be the functional
48 target of BEA on BMDCs. Consistently, in luciferase reporter assays BEA stimulation
49 significantly promotes NF- κ B activation in mTLR4/CD14/MD2 overexpressing but not control
50 HEK-293 cells. RNA-sequencing analyses further confirmed that BEA induces transcriptional
51 changes associated with the TLR4 signaling pathway. Together, these results identify TLR4 as
52 a cellular BEA sensor and define BEA as a potent activator of BMDCs, implying that this
53 compound can be exploited as a promising candidate structure for vaccine adjuvants or
54 cancer immunotherapies.

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56 Introduction

57 Dendritic cells (DCs) represent a heterogeneous family of immune cells that link innate
 58 and adaptive immunity. They can be classified into two main subtypes: plasmacytoid DCs and
 59 conventional DCs, the latter being considered the most potent antigen presenting cells (1-3).
 60 DCs are key players in the immune responses, residing in peripheral organs in an immature
 61 state and acting as sentinels for a wide array of “danger” signals. These signals include
 62 danger-associated molecular patterns (DAMPs) or pathogen-associated microbial patterns
 63 (PAMPs), which are recognized by conserved pattern recognition receptors (PRRs) such as
 64 the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and cytoplasmic DNA receptors (4).
 65 Up to date, 10 (TLR1-10) and 12 (TLR1-9, 11-13) functional TLRs are identified in humans and
 66 mice, respectively and each is triggered by a distinct set of PAMPs. Based on the distinctive
 67 adaptors in these pathways, TLR signaling can be subdivided into two categories: signaling
 68 dependent on the MyD88-dependent pathway and signaling dependent on the
 69 TRIF-dependent pathway. Most TLRs interact intracellularly with MyD88, except TLR3 which
 70 transduces activating signals exclusively via TRIF. Effective TLR4 signaling depends on both
 71 adaptor molecules, TRIF and MyD88 (5).

72 Upon recognition of PAMPs, the TIR domain-containing adaptor proteins MyD88 and/or
 73 TRIF are recruited to the TLRs, and initiate signal transduction pathways that culminate in the
 74 activation of NF- κ B, interferon regulatory factors (IRFs), and mitogen-activated protein kinase
 75 (MAP) kinases to upregulate costimulatory molecules (CD40, CD80 and CD86), inflammatory
 76 cytokines (e.g. IL-12, IL-6, and TNF), chemokines (RANTES, IP-10, ENA78, etc.) and type I
 77 interferons (IFNs) that ultimately protect the host from microbial infection (6, 7). IL-12,
 78 composed of p35 and p40 subunits, is the critical factor for Th1 immune responses in the
 79 defense against bacterial and viral infection as well as cancer cells (8, 9).

80 Beauvericin is a cyclic hexadepsipeptide, belonging to the enniatin family and is produced by
 81 various fungi, such as *Beauveria bassiana* and *Fusarium spp.* (10, 11). As a mycotoxin, BEA is
 82 a very common contaminant of cereal and cereal based products (12, 13), but it is also found
 83 in other products such as nuts and coffee (14). Multiple and divers properties of BEA have

been reported such as enhancement of pesticide sensitivity (15), anti-bacterial activity against Gram-positive and Gram-negative bacteria (16), anti-viral activity against human immunodeficiency virus type-1 integrase (17), and cytotoxic activity against melanoma cells (18). BEA can also cause cell apoptosis by inducing reactive oxygen species (ROS) production (19). Moreover, BEA shows anti-inflammatory activity in macrophages by inhibiting the NF- κ B pathway (20). Although these various properties have been described for BEA, its impact on DCs has not been determined, yet. Here we found that Beauvericin shows immunostimulatory effects on BMDCs via activation of a TLR4 dependent signaling pathway.

Material and Methods

Mice

Wild type C57BL/6N and IL-12p40/GFP reporter mice (21) were used for GM-CSF culture of bone marrow cells. OT-II transgenic mice which express an OVA-specific, MHC class II-restricted TCR were used for T cell activation assays. Bone marrow from *Tlr3/7/9*^{-/-} mice and *Tlr4*^{-/-} mice were kindly provided by Prof. Carsten Kirschning. Bone marrow from *Myd88*^{-/-} and *Myd88*^{-/-} *Trif*^{-/-} mice was shared by Dr. Heike Weighardt and Prof. Ulrich Kalinke, respectively. No experiments on live animals were performed. Mice were euthanized by cervical dislocation before bone marrow was harvested. The euthanasia method used is in strict accordance with accepted norms of veterinary best practice. Animals were kept under specific pathogen-free conditions in the animal research facilities of the Universities of Düsseldorf, Essen-Duisburg, Bonn and the TWINCORE strictly according to German animal welfare guidelines.

GM-CSF cell cultures and stimulation conditions

2x10⁶ bone marrow cells were cultured in non-treated 10cm plates (Sarstedt) in 10 ml VLE DMEM (Biochrom) containing 10% heat-inactivated FCS (Sigma-Aldrich), 0.1% 2-mercaptoethanol (Thermo Fischer Scientific), and GM-CSF and kept for 9 days. 10 μ ml GM-CSF containing medium was added to the plates at day 3. On day 6 10 μ ml medium was carefully removed and centrifuged. The cell pellet was resuspended in 10 μ ml medium and added to the dish. On day 9 BMDCs were used for experiments.

111 For cytokine expression analyses, BMDCs were seeded (1×10^6 cells/well) on a 24-well plate
112 and were stimulated with BEA (purified by the lab of Prof. Rainer Kalscheuer, or purchased
113 from Cayman Chemicals), CpG 2216 (TIB MOLBIOL), LPS (*Escherichia coli* O127:B8, Sigma),
114 cGAMP (InvivoGen), R848 (Alexis Biochemicals), Poly I:C (InvivoGen), or Pam3csk4
115 (InvivoGen). After 24 hours, cell culture supernatants were collected for cytokine detection.

116 **Polymyxin B (PMB) neutralization assay**

117 BMDCs were seeded (1×10^6 cells/ml) on a 24-well plate and were stimulated with BEA
118 (purchased from Cayman Chemicals) or LPS (*Escherichia coli* O127:B8, Sigma) with or
119 without 100µg/ml PMB (InvivoGen). After 16 hours of incubation at 37°C, IL-12p40/GFP
120 expression was analyzed by flow cytometry. Alternatively, after 24 hours, cell culture
121 supernatants were collected and the IL-12p70 and IFNβ content was determined by ELISA
122 assays.

123 **Flow cytometry and cell sorting**

124 For cell surface staining, fluorochrome-conjugated monoclonal antibodies against mouse
125 CD3e (clone 145-2C11), CD19 (clone 1D3), CD4 (clone RM4-5), and MHC II (clone
126 N5/114.15.2) from BD Biosciences, and CD3e (clone 145-2C11), CD86 (clone GL-1) and
127 CD11c (clone N418) from BioLegend were used. For intracellular staining, cells were first
128 stained for surface markers and then fixed and permeabilized using Intracellular Fixation and
129 Permeabilization Buffer Set (eBioscience) before incubation with fluorochrome-conjugated
130 mAbs against mouse IFNγ (clone XMG1.2, BD Bioscience). Flow cytometry was performed on
131 LSRFortessa (BD Biosciences) or FACSCanto II (BD Biosciences) cytometers. The flow
132 cytometry data was analyzed using FlowJo V10.5. For RNA sequencing experiments live,
133 single, CD3⁺, CD19⁺, CD11c⁺ and MHCII^{high} BMDCs were FACS purified using FACS Aria III
134 (BD Biosciences).

135 **qRT-PCR**

136 RNA isolation was performed using Macherey-Nagel™ NucleoSpin™ RNA Plus kit

(Macherey-Nagel™). Complementary DNA synthesis was done by using the SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with 5x MESA Green (Eurogentec) on Bio-Rad CFX 96 Realtime PCR system. Primers used were as follows: *β-actin*: 5'-TGACAGGATGCAGAAGGAGA-3', 5'-CGCTCAGGAGGAGCAATG-3'; *IL-12p40*: 5'-ACAGCACCAGCTTCTTCATCAG-3', 5'-TCTTCAAAGGCTTCATCTGCAA-3'; *IFNβ*: 5'-CAGGCA ACCTTT AAG CATCA-3', 5'-CCTTTGACCTTTCAAATGCAG-3' *TNF*: 5'-TTGAGATCCATGCCGTTG-3', 5'-CTGTAGCCCACGTCGTAGC-3'; *IL-6*: 5'-CCAGGTAGCTATGGTACTCCAGAA-3', 5'-GCTACCAAACCTGGATATAATCAGGA-3'

RNA sequencing and analysis

RNA from sorted BMDCs was extracted using the Macherey-Nagel™ NucleoSpin™ RNA Plus kit (Macherey-Nagel™) and quantified using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). Quality was measured by capillary electrophoresis using the Fragment Analyzer and the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples in this study showed high quality RNA Quality Numbers (RQN > 9.4). Library preparation was performed according to the manufacturer's protocol using the 'VAHTS™ Stranded mRNA-Seq Library Prep Kit' for Illumina®. Briefly, 500 ng total RNA were used for mRNA capturing, fragmentation, the synthesis of cDNA, adapter ligation and library amplification. Bead purified libraries were normalized and finally sequenced on the NextSeq550 system (Illumina Inc. San Diego, USA) with a read setup of 1x76 bp. The bcl2fastq2 tool was used to convert the bcl files to fastq files.

The reads of all probes were adapter trimmed (Illumina TruSeq) and the clean reads were analyzed using FastQC software to identify potential issues with data quality. The clean reads were then mapped to the mouse reference genome (*Mus musculus*, GRCm39/mm39) using STAR software. The percentage of uniquely mapped reads were greater than 80%. The uniquely mapped reads to each gene were counted using featureCounts. In order to assess the sample quality, we performed the principal component analysis (PCA) and hierarchical clustering for all samples. No batch effect was detected. The differently expressed genes

(DEGs) ($|\log_2FC| \geq 1$, FDR < 0.05) between non-stimulation and BEA, LPS or BEA with LPS stimulation following the previously described methods (22) were identified using DESeq2 package. DEGs expression was visualized as clustered heat maps using pheatmap package. The functional enrichment analysis (KEGG pathways and GO terms) of DEGs was carried out using enrichR package. Gene Set Enrichment Analysis (GSEA, Version 4.0.3) was used to identify enriched functional gene sets based upon the definitions of the Molecular Signatures Database (23, 24). The included gene set collections were “C2 curated gene sets”, “C5 ontology gene sets” and “C7 immunologic signature gene sets”. An enrichment map of significantly enriched gene sets was produced via Cytoscape (Version 3.8.0) (25) and the GSEA Enrichment Map plugin (26). Since Cytoscape defines a FDR of 0.25 as significant, this value was used as a cut off for inclusion into the network. In the enrichment networks, nodes represent gene sets, while edges represent mutual overlap between gene sets. Genes with overlapping genes and functional annotations were clustered manually to highlight the functional results. These clusters were encircled and labeled with an encompassing terminology. To achieve a simplified and more precise figure all clusters with less than three signatures were discarded from the network.

Multiplex immunoassay

Cell culture supernatants were assessed for chemokine and cytokine concentrations. The ProcartaPlex Mouse Cytokine & Chemokine Panel 1A 36-plex (Invitrogen by Thermo Fisher Scientific) was used to measure the concentrations of IFN α , IFN γ , IL-12p70, IL-1 β , IL-2, TNF, GM-CSF, IL-18, IL-17A, IL-22, IL-23, IL-27, IL-9, IL-15/IL-15R, IL-13, IL-4, IL-5, IL-6, IL-10, Eotaxin (CCL11), IL-28, IL-3, LIF, IL-1 α , IL-31, GRO- α (CXCL1), MIP-1 α (CCL3), IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MIP-1 β (CCL4), MIP-2 (CXCL2), RANTES (CCL5), G-CSF, M-CSF, and ENA-78 (CXCL5) in cell culture supernatants, according to the manufacturer's instructions. Plates were read using the Bio-Plex 200 Systems (Bio-Rad, USA).

191 **ELISA**

192 Cell culture supernatants from BMDCs were analyzed by ELISA for IL-12p70 (R&D) and IFN β
193 (Invitrogen). Plates were read using a Tecan Sunrise microplate reader at 450 nm, and the
194 background was subtracted at 570 nm.

195 **Luciferase reporter assay**

196 HEK-293 cells stably expressing TLR4/MD2/CD14 were purchased from Invivogen. Cells were
197 seeded at 3.5×10^4 live cell/well in 96 well plates overnight and were transfected with
198 NF- κ B-luciferase reporter plasmid (50 ng/well) and Renilla plasmid (5 ng/well) with transfection
199 reagent jetPRIME (Polyplus-transfection Biotechnology) for 24 hours. Then cells were
200 stimulated with different concentrations of BEA (2.5 μ M, 5 μ M and 7.5 μ M) or LPS (1 μ g/ml) as
201 a positive control. After 24 hours of stimulation, the supernatant was discarded, and cells were
202 washed with PBS. 50 μ L of lysis buffer (Promega) was added and cells were lysed at room
203 temperature for 15 min on a shaker and luciferase activity was measured with the Dual-Glo
204 Luciferase Assay (Promega) in a Mithras LB 940 multimode microplate reader.

205 **T cell activation assay**

206 For BMDC / T cell coculture, BMDCs were treated with 2.5 μ M, 5 μ M, 7.5 μ M BEA for 24 hours
207 and then washed twice with PBS to remove residual BEA before use in subsequent culture.
208 Naive CD4 $^+$ T cells were purified from spleens of OT II mice by MACS (Miltenyi Biotec)
209 according to the manufacturer's protocol. Briefly, cells were Fc-blocked and incubated with
210 biotinylated anti-CD4 antibodies (BD Pharmingen). Subsequently, magnetic anti-biotin beads
211 (Miltenyi Biotec) were added and CD4 $^+$ T cells were positively selected by running cells along
212 a MACS magnet. CD4 $^+$ T cells were labeled with CellTrace Violet (Thermo Fisher Scientific)
213 and afterwards cultured with untreated or BEA treated BMDCs at a 10:1 ratio in 96 well round
214 bottom plates for 3 days and 5 days, respectively. Cell proliferation was measured at day 3 by
215 flow cytometry. At day 5, cell culture supernatants were collected and stored at -80°C. For
216 intracellular detection of IFN γ , Brefeldin A (BD Biosciences) was added to the cells in the last 6
217 hours before harvesting of the cells at day 5.

218 **Statistical analysis**

219 GraphPad Prism 9.0 software was used for data analysis. Data are represented as mean $\pm$
 220 SEM. For analyzing statistical significance between multiple groups, a one-way ANOVA with
 221 Dunnett's multiple comparisons test was used. For analyzing statistical significance for
 222 comparisons of more than two groups with two or more stimulations, two-way ANOVA with
 223 Sidak's multiple comparisons test was used, all p values < 0.05 were considered as
 224 statistically significant.

226 **Results**

227 **BEA activates BMDCs to increase inflammatory cytokine production and costimulatory** 228 **ligand expression.**

229 To study the effects of BEA on BMDCs, cells from IL-12p40/GFP reporter mice were treated
 230 with various concentrations of BEA in the presence or absence of suboptimal concentrations of
 231 LPS and CpG. BMDC activation was determined by IL-12p40/GFP and CD86 expression. BEA
 232 alone potently activated BMDCs leading to enhanced IL-12p40 and CD86 expression (Figure
 233 1A, B). As expected, BEA treatment can also enhance activation of LPS or CpG stimulated
 234 BMDCs leading to further increased IL-12p40 and CD86 expression (Figure 1A, B). In addition,
 235 we also detected significantly increased production of the inflammatory cytokines IL-12p40,
 236 IFN β , TNF and IL-6 in response to BEA stimulation by Real-time PCR with maximum levels
 237 reached at 6 hours post stimulation (Figure 1C). Taken together, BEA can upregulate IL-12 and
 238 other pro-inflammatory cytokines together with CD86 levels in BMDCs, indicating that BEA
 239 might be a potent BMDC activator.

240 **BEA promotes DC-mediated CD4⁺ T cell proliferation.**

241 Next, we aimed at investigating whether BEA could enhance the ability of BMDCs to induce T
 242 cell proliferation. BMDCs were cultured in the presence or absence of various concentrations
 243 of BEA for 24 hours and then cells were washed thoroughly as previous studies have shown

244 that BEA significantly inhibits T cell proliferation in TNBS-induced experimental colitis (27).
 245 Afterwards, untreated and treated BMDCs were co-cultured with OT II TCR transgenic naive
 246 CD4⁺ T cells for 3 days. While untreated BMDCs induced T cell proliferation without BEA
 247 stimulation to a certain level, T cell co-culture with BEA treated BMDCs led to increased
 248 numbers of T cell divisions (Figure 2A). Furthermore, we also analyzed intracellular IFN γ
 249 production of CD4⁺ T cells. While untreated and BEA treated BMDCs showed similar
 250 percentages of IFN γ producing CD4⁺ T cells (Figure 2B), significantly higher IFN γ levels were
 251 detected in supernatants of T cells that were co-cultured with BEA activated BMDCs for 5 days
 252 than with untreated BMDCs (Figure 2C). The increased amounts of IFN γ in the supernatant of
 253 T cells co-cultured with BEA treated BMDCs is due to increased T cell numbers of IFN γ
 254 producing T cells but not enhanced capacity for IFN γ production on a per cell basis. This
 255 suggests that BEA can enhance the ability of BMDCs to induce T cell proliferation, whereas it
 256 does not have an impact on differentiation or induction on cytokine production in individual
 257 cells. Taken together, BEA can induce BMDC-mediated CD4⁺ T cell proliferation.

258 **BEA mediated effects on BMDCs is not due to LPS contamination.**

259 The purity of BEA isolated from *Fusarium spp.* by the Institute of Pharmaceutical Biology and
 260 Biotechnology (Prof. Rainer Kalscheuer) and BEA purchased from Cayman Chemicals was
 261 above 95% as defined by HPLC-UV (data not shown). To further confirm this effect was not a
 262 result of endotoxin contamination, BMDCs derived from IL-12p40/GFP reporter mice were
 263 stimulated by indicated concentrations of BEA or LPS with or without PMB, which blocks the
 264 biological effects of LPS through binding to lipid A (28, 29). After 16 hours of stimulation,
 265 IL-12p40/GFP expression by BMDCs was analyzed by flow cytometry. PMB effectively
 266 blocked the LPS mediated activation of BMDCs resulting in undetectable IL-12p40 (Figure
 267 3A-B) and IL-12p70 levels (Figure 3C). However, amounts of IL-12p40 and IL-12p70 were
 268 comparable after BEA stimulation with or without additional PMB treatment (Figure 3A-C). This
 269 result demonstrated that production of IL-12 by BEA treated BMDCs is unlikely to result from
 270 any contamination of BEA with LPS.

271 **BEA induces BMDC cytokine production in a MyD88 and TRIF dependent way.**

272 MyD88 and TRIF are critical adaptors for TLR induced production of pro-inflammatory
 273 cytokines such as IL-12, TNF, IL-6 and IFN β by DCs (30, 31). Therefore, we aimed to
 274 investigate whether IL-12 and IFN β production by BEA stimulation are MyD88 or
 275 TRIF-dependent. To this end, BMDCs were generated from *Myd88*^{-/-} and *Myd88*^{-/-} *Trif*^{-/-} mice
 276 and stimulated with BEA, LPS or cGAMP, the latter serving as a positive,
 277 MyD88/TRIF-independent stimulation control. After 24 hours stimulation, cell supernatant was
 278 collected to assess IL-12 and IFN β production by ELISA. In this experiment, cGAMP can
 279 induce IFN β production in WT and *Myd88*^{-/-} or *Myd88*^{-/-} *Trif*^{-/-} BMDCs. As expected, neither
 280 *Myd88*^{-/-} nor *Myd88*^{-/-} *Trif*^{-/-} BMDCs released detectable amounts of IL-12 upon LPS stimulation.
 281 Production of IFN β was significantly diminished but still detectable in *Myd88*^{-/-} BMDCs,
 282 whereas it was undetectable in *Myd88*^{-/-} *Trif*^{-/-} BMDCs. Similarly, BEA did not induce IL-12p70
 283 production in either *Myd88*^{-/-} BMDCs or *Myd88*^{-/-} *Trif*^{-/-} BMDCs while IFN β production was
 284 significantly decreased in *Myd88*^{-/-} BMDCs and undetectable in *Myd88*^{-/-} *Trif*^{-/-} BMDCs (Figure
 285 4A). Furthermore, we determined production of other cytokines and chemokines by multiplex
 286 immunoassay. Production of the inflammatory cytokines TNF, IL-6, IL-1 β , IL-18, IL-27 and
 287 IL-10 (Figure 4B) and the chemokines GRO- α , MCP-3, ENA-78, MIP-1 β and RANTES (Figure
 288 4C) was significantly decreased in BEA simulated *Myd88*^{-/-} BMDCs and even lower in BEA
 289 simulated *Myd88*^{-/-} *Trif*^{-/-} BMDCs. However, production of IP-10 induced by LPS in *Myd88*^{-/-}
 290 BMDCs was similar to WT BMDCs, but was markedly decreased in LPS stimulated *Myd88*^{-/-}
 291 *Trif*^{-/-} BMDCs. Such findings are consistent with studies reporting that expression of IP-10 by
 292 LPS-stimulated bone-marrow-derived macrophages is mediated through a TRIF-dependent
 293 but MyD88-independent pathway (32). Interestingly, similar results were observed in BEA
 294 stimulated *Myd88*^{-/-} BMDCs and *Myd88*^{-/-} *Trif*^{-/-} BMDCs. Thus, the effects of BEA on BMDCs
 295 cytokine and chemokine expression profiles are mediated via activation of MyD88 and TRIF
 296 signaling pathways as similarly detected after LPS-stimulation.

297 **BEA activates BMDCs in a TLR4-dependent way.**

298 It has been shown that the TLR4 signaling pathway not only depends on the presence of the

299 MyD88 signal adaptor protein but also the TRIF signal adaptor protein (31). As we observed
300 that both, MyD88 and TRIF are involved in BEA induced BMDC cytokine production, we next
301 sought to determine whether BEA activates BMDCs in a TLR4 dependent manner. To this end,
302 we stimulated WT and *Tlr4*-deficient BMDCs with BEA in the presence or absence of CpG or
303 LPS for 24 hours. Measurement of IL-12p70 and IFN β in the supernatant by ELISA showed
304 that LPS and BEA did not induce IL-12p70 and IFN β production in *Tlr4*-deficient BMDCs
305 (Figure 5A, B). In contrast, CpG induced similar amounts of IL-12p70 and IFN β in both WT and
306 *Tlr4*-deficient BMDCs while BEA co-treatment with CpG failed to induce more cytokine
307 production as compared to *Tlr4*-deficient BMDCs stimulated with CpG alone, suggesting these
308 effects of BEA on BMDCs are TLR4 signaling dependent.

309 Furthermore, to investigate whether BEA can activate other TLR signaling pathways, WT and
310 BMDCs with a triple deficiency of TLR3, 7 and 9 were stimulated by BEA with or without CpG
311 (TLR9), R848 (TLR7) or Poly I:C (TLR3). Consistent with current knowledge, CpG and R848
312 can significantly induce IL-12p70 and IFN β production in WT, but not in *Tlr3/7/9*-deficient
313 BMDCs. In addition, we found that Poly I:C did not induce IL-12p70 production, which is
314 consistent with previous reports (30). In contrast, BEA induced similar amounts of IL-12p70
315 and IFN β in WT and *Tlr3/7/9* deficient BMDCs (Figure 5 C, D).

316 To further determine if BEA could directly activate TLR4-mediated signaling, we stimulated
317 HEK-293 cells stably expressing mTLR4/CD14/MD2 and transiently expressing the
318 NF- κ B-luciferase reporter and Renilla gene with various concentrations of BEA or LPS as a
319 positive control and measured NF- κ B activation. LPS treatment significantly induced NF- κ B
320 activation, which was similarly observed after BEA treatment (Figure 5E). Taken together, our
321 data indicate that BEA activates BMDCs via a TLR4 dependent signaling pathway.

322 **BEA induces transcriptional changes associated with TLR signaling and chemokine** 323 **signaling pathways.**

324 To define the underlying mechanisms by which BEA activates BMDCs, we used
325 whole-genome RNA sequencing (RNA-seq) to detect genome wide differences in gene
326 expression of BMDCs treated with or without BEA in an explorative study. MHC II^{high} CD11c⁺

BMDCs were sorted by flow cytometry followed by stimulation with BEA or LPS alone or BEA combined with LPS for 4 hours. Control samples were left untreated. PCA revealed that the four treatment groups cluster separately and that combined BEA with LPS treatment clusters in close proximity to that of BEA stimulation alone (Figure 6A). Similarly, heatmap and hierarchical clustering show that gene expression induced by BEA is different from LPS stimulation. Combined BEA and LPS stimulation induces a similar differential gene expression as BEA stimulation sharing differential regulation of endolysosome related gene expression (Lamp1, Lamp2, Lamtor3, CSTB, Vps35 and Mcoln1), cellular metabolism gene expression (HK3 and Fasn), mitochondrial gene expression (Polrmt, Slc25a29), autophagy gene expression (rptor) and transcription regulation (Zfp446, H4c3 and foxf2) (Figure 6B). KEGG pathway and GO analyses for BEA treated versus untreated BMDCs were enriched in those involved in “the innate TLR pathway”, “the MyD88 mediated pathway”, “the cytokine signaling pathway”, “the chemokine signaling pathway”, “response to lipopolysaccharide” and “regulation of interleukin-6 production”, amongst others, which further confirmed our Multiplex results (Figure 6C). Using Cytoscape to visualize molecular interaction networks we could show that BEA, LPS and BEA together with LPS similarly induced regulation of clusters related to DCs activation and cell cycle progression (Figure 6D and Supplemental Figure 1). In contrast, BEA led to additional clusters associated with cellular metabolism, T-cell activation, complement activation, type I IFN response, vaccine response, JAK-STAT signaling, ribosomes, translation, and autophagy/receptor recycling (Figure 6D). Moreover, BEA with LPS synergistically and additionally induced B cell antibody production networks and innate immune response (Supplemental Figure 1). Taken together, our results indicate that BEA activates BMDCs via a TLR4 dependent signaling pathway, but induces a gene expression profile different from LPS.

351

352 Discussion

BEA is a natural product found in various toxigenic fungi, for which several biological effects have been reported, such as cytotoxic, apoptotic, anti-cancer, anti-microbial,

insecticidal, and nematicidal activities (14). Moreover, BEA has been reported to exhibit anti-inflammatory activity in macrophages by inhibiting the NF- κ B pathway and in an experimental colitis model by inhibiting activated T cells (20, 27). However, little is known about the effect of BEA on DCs. In this study, we showed for the first time that BEA activates GM-CSF-cultured BMDCs, inducing inflammatory cytokines such as IL-12, IFN β , TNF, IL-6 together with CD86 expression in a MyD88 and TRIF-dependent way. Furthermore, BEA can enhance the ability of BMDCs to induce T cell proliferation, whereas it does not have an impact on differentiation or induction on cytokine production in individual cells. The purity of isolated and commercial BEA is above 95% and our PMB-blocking experiments also exclude any possibility of endotoxin contamination.

TLRs are crucial activating receptors on antigen presenting cells including macrophages and DCs. Upon recognition of PAMPs or DAMPs, they can induce a variety of cellular responses including production of inflammatory cytokines, chemokines, and type I IFNs. TLR signaling consists of at least two distinct pathways: a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the induction of IFN β (5, 33, 34). Signaling downstream of most of TLRs is MyD88-dependent, except for signaling downstream of TLR3, which is exclusively TRIF-dependent. TLR4 signals through both, the MyD88- and TRIF-dependent pathway to induce inflammatory cytokines, chemokines and type I IFNs production (30). To explore the mechanism by which BEA activates BMDCs, we analyzed inflammatory cytokine and chemokine production by BMDCs derived from *Myd88*^{-/-} or *Myd88*^{-/-} *Trif*^{-/-} mice after BEA stimulation. Production of cytokines and chemokines in response to BEA stimulation was strongly diminished in *Myd88*^{-/-} BMDCs and almost undetectable in *Myd88*^{-/-} *Trif*^{-/-} BMDCs. Thus, these results suggest BEA activates BMDCs using signaling pathways that are both MyD88- and TRIF-dependent. Thus, we reasoned that BEA activates BMDCs via activating the TLR4 signaling pathway. To test this, we analyzed the release of cytokines from *Tlr4*^{-/-} BMDCs. BEA significantly decreased IL-12p70 and IFN β production by *Tlr4*^{-/-} BMDCs. Consistently, Luciferase Reporter Assay shows that BEA significantly induced NF- κ B activation in HEK-293 cells stably expressing TLR4/MD2/CD14. Moreover, RNA sequencing and GO analyses showed that BEA-treated

384 BMDCs activate pathways related to TLR signaling, cytokines and inflammatory response,
 385 chemokine signaling, and IL-10 anti-inflammatory signaling, which were similarly activated in
 386 LPS-treated BMDCs. However, also marked differences exist between BEA-treated BMDCs
 387 and LPS-treated BMDCs. BEA-treated BMDCs show regulation of various signatures
 388 associated with cellular metabolism, T cell activation, complement activation, type I IFN
 389 response, vaccine response, JAK-STAT signaling, ribosomes, translation, and
 390 autophagy/receptor recycling, which was not found to the same extend in LPS-stimulated
 391 BMDCs. These differences could be attributed to the different affinity of TLR4 to BEA and LPS
 392 or by additional molecular targets of BEA within the cells. Of note, heat-killed conidia of
 393 *Aspergillus fumigatus* have been reported to activate TLR4 signaling to induce inflammatory
 394 cytokine production (35). However, which component of this fungi is responsible for TLR4
 395 activation was not elucidated. It is tempting to speculate that BEA or a derivative thereof
 396 produced by this fungus is responsible for this TLR4 stimulating activity, but this remains to be
 397 elucidated in future studies.

398 It has been reported that BEA shows cytotoxicity on human DCs derived from human
 399 umbilical cord blood CD14+ monocytes. Furthermore, BEA can affect LPS-induced DCs
 400 maturation by decreasing CCR7 expression and increasing IL-10 production (36), whereas,
 401 effects of BEA alone on human dendritic cell activation remain unknown. To determine
 402 whether BEA can activate human DCs is the aim of future studies. Furthermore, we found BEA
 403 pre-treated BMDCs could enhance T-cell proliferation, whereas no difference of T-cell
 404 proliferation was observed when BEA was present in the co-culture of BMDCs together with T
 405 cells (data not shown). This could be caused by direct inhibition of T-cell proliferation by BEA
 406 resulting in a neutralization of BMDC-mediated T-cell proliferation (27). Of course, further
 407 studies need to be done to verify effects of BEA *in vivo*. In addition, BEA has been reported to
 408 exhibit anti-inflammatory activity in macrophages by inhibiting the NF- κ B pathway (20). To
 409 determine why DCs and macrophages react differently to BEA is another task of future studies.
 410 Also, further studies need to be done to define the molecular mode of action of BEA-mediated
 411 TLR4 stimulation. Direct binding of BEA to TLR4 needs to be tested and if applicable the TLR4
 412 domains involved need to be identified. Alternatively, BEA-mediated TLR4 signalling could be

413 activated via the release or induction of endogenous proteins serving as ligands for TLR4 such
414 as Mrp8 (37), heat shock proteins (HSP60, 70, Gp96) (38) and high mobility group box 1
415 protein (HMGB1) (39).

416 Adjuvants are defined as molecules or formulations that enhance the efficacy of vaccines
417 without directly participating in the protective immunity. In recent decades, a variety of
418 preclinical and clinical studies have shown that purified TLR agonists could be exploited as
419 adjuvants to enhance adaptive responses during vaccination (40, 41). Monophosphoryl lipid A
420 (MPLA), a TLR4 agonist purified from *Salmonella minnesota* LPS has been used as adjuvant
421 in different vaccines against human papillomavirus (HPV) and hepatitis B virus (HBV)
422 infections (42). Moreover, MPLA is the only TLR4 agonist that has been clinically tested as an
423 adjuvant for cancer vaccines (43, 44). In our study, BEA potently activated DCs inducing a
424 range of inflammatory cytokines and chemokines in addition to MHC II upregulation. By means
425 of a cell directed delivery of BEA a specific activation of DCs could be achieved circumventing
426 its suppressive effects on T cell proliferation (27), thus suggesting that BEA can be a very
427 promising candidate of vaccine adjuvants and cancer immunotherapy. In addition, BEA has
428 been reported to neutralize the ATP-binding cassette (ABC) transporters, which contributes to
429 multi-drug resistance in human, nematodes and arthropods (15, 45). Therefore, combinational
430 therapy using BEA and other drugs can overcome multidrug resistance.

431 In summary, our data revealed a novel function of BEA on DCs in activating inflammatory
432 cytokine and chemokine production via activating the TLR4 signaling pathway. Our findings
433 suggest BEA can be exploited in the field of vaccine adjuvants and cancer immunotherapy.

434 **Author contributions**

435 S.S. conceived and supervised the study. X.Y. performed the experiments, analyzed the
436 results, X.Y., S.S., J.Q and M.U. wrote the manuscript. S.A. performed cell sorting and gave
437 suggestions to the project. V.S. performed qRT-PCR experiments. L.R. screened natural
438 products. C.K., H.W., P.L., U.K., J.S. and H.I. provided mice and isolated bone marrow. M.F.
439 and R.K. isolated BEA and performed purity analyses. C.A. performed the Multiplex
440 immunoassays. T.W and K.K performed RNA-seq experiments. X.Y., M.Z., J.D., S.B. and A.H.

441 analyzed the RNA-seq data.

442 **Declaration of interest**

443 The authors declare that the research was conducted in the absence of any commercial or
444 financial relationships that could be construed as a potential conflict of interest.

445 **Data availability statement**

446 RNA sequencing data in this study have been deposited in NCBI Gene Expression Omnibus
447 under the accession number GSE192689. Related website is:
448 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192689>.

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578

579

580 **Figure legend**

581 **Figure 1. Effects of BEA on BMDCs.**

582 **(A, B)** 5×10^5 BMDCs from IL-12p40/GFP reporter mice were stimulated with indicated
583 concentration of BEA with or without LPS (10 ng/ml) and CpG2216 (0.5 μ M) for 16 hours.
584 IL-12p40/GFP **(A)** and CD86 expression **(B)** by BMDCs were detected by flow cytometry. **(C)**
585 10^6 BMDCs were stimulated with 5 μ M BEA for the indicated time. IL-12p40, IFN β , TNF and
586 IL-6 were analyzed by Real time PCR. Data are shown as mean $\pm$ SEM. Results shown are
587 representative of two to three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not
588 significant.

589

590 **Figure 2. BEA-treated BMDCs enhance T cell proliferation.**

591 10^5 naïve $CD4^+$ T cells derived from OT II TCR transgenic mice were labeled with CellTrace
592 Violet and cultured with 10^4 untreated or BEA-treated BMDCs for 3 days. **(A)** T cell
593 proliferation was analyzed by flow cytometry based on CellTrace Violet dilution. **(B)** 10^5 naïve
594 OT II $CD4^+$ T cells were labeled with CellTrace Violet and cultured with 10^4 untreated or
595 BEA-treated BMDCs for 5 days. Percentage of IFN γ production by $CD4^+$ T cells was measured
596 by intracellular staining. **(C)** IFN γ production in the supernatant was detected by ELISA. Data
597 are shown as mean $\pm$ SEM. One representative experiment is shown out of two independent
598 experiments. ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

599

600 **Figure 3. Exclusion of BEA contamination with LPS.**

601 **(A, B)** 5×10^5 BMDCs derived from IL-12p40/GFP reporter mice were stimulated with 5 μ M BEA
602 or LPS (10 ng/ml) with or without PMB (100 ng/ml). After 16 hours of stimulation,
603 IL-12p40/GFP and CD86 expression of BMDCs were analyzed by flow cytometry. **(C)** 10^6
604 BMDCs derived from IL-12p40/GFP reporter mice were stimulated by 5 μ M BEA or LPS (10
605 ng/ml) with or without PMB (100 ng/ml). After 24 hours of stimulation, supernatants were
606 analyzed for IL-12p70 by ELISA. Results shown are representative of two independent
607 experiments. *** $p < 0.001$, ns: not significant.

608

609 **Figure 4. BEA promotes BMDC activation in a MyD88/TRIF dependent manner.**

610 10^6 BMDCs from WT, *Myd88*^{-/-} and *Myd88*^{-/-} *Trif*^{-/-} mice were stimulated with 5 μ M BEA, LPS
611 (10 ng/ml) and cGAMP (10 ng/ml) as control. After 24 hours of stimulation, supernatants were
612 analyzed for IL-12p70 and IFN β production by ELISA **(A)**, and inflammatory cytokine (TNF,
613 IL-6, IL-1 β , IL-27, IL-10) **(B)** and chemokine production (GRO- α , IP-10, MCP-3, ENA-78,
614 MIP-1 α and RANTES) **(C)** by Multiplex immunoassays. Results shown are representative of
615 two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

616

617 **Fig 5. BEA activates BMDCs via TLR4.**

618 **(A, B)** 10^6 BMDCs derived from WT and *Tlr4*^{-/-} mice were stimulated with 5 μ M BEA with or
619 without LPS (10 ng/ml) and CpG (0.5 μ M). After 24 hours of stimulation, supernatants were
620 analyzed for IL-12p70 and IFN β by ELISA. **(C, D)** 1×10^6 BMDCs derived from WT and

621 *Tlr3/7/9*^{-/-} mice were stimulated with 5 μ M BEA with or without LPS (10 ng/ml), CpG (0.5 μ M),
 622 R848 (1 μ g/ml), or Poly I:C (25 ng/ml). After 24 hours of stimulation, supernatants were
 623 analyzed for IL-12p70 and IFN β by ELISA. Results shown are representative of two
 624 independent experiments. **(E)** 3.5x10⁴ HEK-293 cells stably expressing mTLR4/CD14/MD2
 625 were transiently transfected with firefly luciferase NF- κ B reporter and Renilla plasmids. After
 626 24 hours, transfected HEK-293 were treated with indicated concentrations of BEA and LPS (1
 627 μ g/ml) as positive control and induction of NF- κ B was determined by luciferase activity.
 628 Results shown are representative of three independent experiments. *p<0.05, **p<0.01,
 629 ***p<0.001, ns: not significant.

630
 631 **Figure 6. BEA promotes transcriptional changes associated with chemokine and**
 632 **cytokine production and TLR signaling pathway activation but are distinct from LPS**
 633 **stimulation.**

634 MHC II^{high} CD11c⁺ BMDCs sorted by flow cytometry were followed by stimulation with BEA (5
 635 μ M) or LPS (1 μ g/ml) alone or BEA combined with LPS for 4 hours. **(A)** PCA of the
 636 quadruplicate biological replicates of each condition. **(B)** Heatmap showing expression profile
 637 for 4,015 genes that were found to be significantly regulated in at least one of the comparisons
 638 using untreated as baseline condition. **(C)** Enriched Reactome pathways (upper plot) and
 639 biological process (lower plot) in DEGs in BEA treated BMDCs compared with untreated
 640 BMDCs. **(D)** Cytoscape representation of significantly enriched signatures in BEA treated
 641 BMDCs compared with untreated BMDCs.

642
 643 **Supplemental Figure 1**

644 **(A)** Cytoscape representation of significantly enriched signatures in LPS treated BMDCs
 645 compared with untreated BMDCs. **(B)** Cytoscape representation of significantly enriched
 646 signatures in BEA with LPS treated BMDCs compared with untreated BMDCs.

Figure 1

A

B

C

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

C

D

Supplemental Figure 1