Beauvericin Targets Toll Like Receptor 4 and Cathepsin B to Promote Dendritic Cell Activation

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Xiaoli Yang performed experiments, analyzed results, did literature research for experiment design, and wrote the manuscript (contribution: 40%). Shafaqat Ali performed cell sorting and gave suggestions to the project (contribution: 3%), Manman Zhao analyzed RNA seq data and created the PCA plot and figures of go terms and KEGG pathway (contribution: 3%). Lisa Richter screened natural products (1%). Vanessa Schäfer performed qRT-PCR experiments (contribution: 1%). Julian Schliehe-Diecks and Sanil Bhatia generated the cytoscape analysis (contribution: 1.5%), Rainer Kalscheuer and Marian Frank isolated NPs and measured the purity (contribution: 1.5%), Jing Qi and Markus Uhrberg contributed to writing the manuscript (contribution: 1%), Heike Weighardt, Ulrich Kalinke, Carsten Kirschning, Pia-Katharina Larsen, Jennifer Skerra and Heba Islam provided mice and isolated bone marrow (contribution: 2%), Thorsten Wachtmeister and Karl Köhrer performed RNA-seq experiments (contribution: 2%), Christina Alter performed the Multiplex immunoassays (contribution: 2%), Anfei Huang analyzed the RNA-seq data and created Heatmap figure (contribution: 2%) and Stefanie Scheu conceived and supervised the study and corrected the manuscript (contribution: 40%).

In this thesis, figures from the above publication have been included, modified, and marked as citations in the title part of each figure. Appropriate texts parts including abstract, material and methods, results, and discussion from the publication have also been copied, modified, and marked as citation.

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1. Abbreviations

A. fumigatus	*Aspergillus fumigatus*
ABC	Adenosine triphosphate binding cassette
ACT	Adoptive cell transfer
AD	Alzheimer's disease
AMR	Antimicrobial resistance
APCs	Antigen presenting cells
ASC	Adaptor apoptosis-associated speck-like protein
BCG	Bacillus calmette-guérin
BEA	Beauvericin
bHLH	Basic helix-loop-helix transcription factor
BMDCs	Bone marrow derived dendritic cells
BMDMs	Bone marrow-derived macrophages
C. albicans	*Candida albicans*
C. neoformans	*Cryptococcus neoformans*
CAG	Cycloastragenol
CAR-T cell	Chimeric antigen receptor T cell
cDC1	Conventional dendritic cells type 1
cDC2	Conventional dendritic cells type 2
CDP	Common dendritic cell progenitor
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
CNS	Central nervous system
CRD	Carbohydrate recognition domain
CSF	Cerebrospinal fluid
CTD	C-terminal domain
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTSB	Cathepsin B
Cytc	Cytochrome c
DAMP	Danger associated molecular pattern
DCs	Dendritic cells
DEGs	Differently expressed genes
DOX	Doxycycline

dsRNA	Double stranded RNA
ECM	Extracellular matrix
EFSA	European Food Safety Authority
ENN	Enniatin
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FLT3	FMS-related tyrosine kinase 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte–monocyte progenitors
GSEA	Gene Set Enrichment Analysis
GXM	glucuronoxylomannan
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1 protein
HPV	Human papillomavirus
HSC	Hematopoietic stem cells
HSP	Heat shock protein
IAV	Influenza A virus
iDCs	Immature dendritic cells
IFN	Interferons
IFNAR	IFNα receptor
IFNγ	Interferon-gamma
Ig	Immunoglobulin
iNOS	Nitric oxide synthase
IRF4	Interferon regulatory factor 4
IRF8	Interferon regulatory factor 8
ISG	Interferon-stimulated genes
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
LBP	Lipopolysaccharide binding protein
LCK	lymphocyte specific protein kinase
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LTβ	Lymphotoxin-β
mAb	Monoclonal antibody
MD2	Myeloid differentiation 2

MDA5	Melanoma differentiation-associated gene 5
MDP	Macrophage dendritic cell progenitor
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
MPLA	Monophosphoryl lipid A
MPP	Multipotent progenitor
Mrp8	Myeloid-related protein 8
MyD88	Myeloid differentiation primary response 88
NBD	Nucleotide-binding domain
NK cell	Natural killer cell
NLR	Nucleotide oligomerization domain (NOD)-like receptor
NLRP3	Nucleotide oligomerization domain (NOD)-leucine rich repeat and pyrin containing protein 3
NMR	Nuclear magnetic resonance
NPs	Natural products
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PCA	Principal component analysis
PD-1	Programmed cell death 1
pDCs	Plasmacytoid dendritic cells
PMB	Polymyxin B
PRRs	Pattern recognition receptor
PYD	Pyrin domain
PyMT	Polyoma Middle middle T
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene I
RLR	Retinoic acid-inducible gene I-like receptor
RNA-seq	RNA sequencing
S. scabiei	*Sarcoptes scabiei*
SAR	Structure-Activity activity Relationship relationship
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scRNA-seq	Single-cell RNA sequencing

ssRNA	Single stranded RNA
STAT	signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
T cells	T lymphocytes
T. gondii	*Toxoplasma gondii*
TAMs	Tumor-associated macrophages
TBI	Traumatic brain injury
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
TIM	T cell immunoglobulin and mucin domain
TIMP1/2	Tissue inhibitor of metalloproteinases 1 and 2
TIR	Toll/interleukin-1 receptor
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TNFR	TNF receptor
Treg	T regulatory cell
TRIF	Toll/interleukin-1 receptor (TIR) domain containing adaptor inducing interferon beta (TRIF)
UPEC	*Uropathogenic Escherichia coli*
WT	Wild type

2. Summary

BEA, a mycotoxin of the enniatin family produced by various toxigenic fungi, has been attributed multiple biological activities such as anti-cancer, anti-inflammatory, and anti-microbial functions. However, effects of BEA on DCs remain unknown so far. Here, we identified effects of BEA on murine granulocyte–macrophage colony-stimulating factor (GM-CSF)-cultured bone marrow derived dendritic cells (BMDCs) and the underlying molecular mechanisms. BEA potently activates BMDCs as signified by elevated IL-12 and CD86 expression. Multiplex immunoassays performed on myeloid differentiation primary response 88 (MyD88) and toll/interleukin-1 receptor (TIR) domain containing adaptor inducing interferon beta (TRIF) single or double deficient BMDCs indicate that BEA induces inflammatory cytokine and chemokine production in a MyD88/TRIF dependent manner. Furthermore, we found that BEA was not able to induce IL-12 or IFNβ production in Toll-like receptor 4 (*Tlr4*)-deficient BMDCs, whereas induction of these cytokines was not compromised in *Tlr3/7/9*-deficient BMDCs. This suggests that TLR4 might be the functional target of BEA on BMDCs. Consistently, in luciferase reporter assays BEA stimulation significantly promotes NF-κB activation in mTLR4/CD14/MD2 overexpressing but not control HEK-293 cells. RNA-sequencing analyses further confirmed that BEA induces transcriptional changes associated with the TLR4 signaling pathway. This paragraph is adapted from Yang *et al*. (2022).

In addition, by using an online *in silico* prediction tool, CTSB was predicted to be a target of BEA. This was confirmed by CTSB cell-based assays within human and mouse DCs. CTSB cell-free experiments further indicated that BEA can directly target human and mouse CTSB.

Together, these results identify TLR4 as a cellular BEA sensor and define BEA as a potent activator of BMDCs. Moreover, CTSB was identified as another direct target of BEA. These results imply that this compound can be exploited as a promising candidate structure for vaccine adjuvants or cancer immunotherapies.

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4. Introduction

Infectious diseases and cancer are major causes of morbidity and mortality worldwide, and there is an urgent need for new drugs to treat cancer and infections. Currently, immunotherapy has become an effective approach, and dendritic cell (DC)-based immunotherapy has been widely used in clinical trials. On the other hand natural products (NPs) are a rich source for drug discovery for cancer and infection. DCs, T cells, the crosstalk between T cells and DCs, and the lead NP Beauvericin (BEA) are the main topics of the thesis. In addition, Cathepsin B (CTSB) is described as a target of BEA.

4.1. Urgent need to identify new drugs for the treatment of tumors and infectious diseases

The data from the World Health Organization (WHO) shows that cancers and infections are among the top 10 causes of death in industrialized as well as in developing countries. In 2020, there were estimated 19.3 million people diagnosed with cancer and almost 10 million cancer deaths, which took up one in 6 deaths in the world (1). Infectious diseases have also been a big threat to the health of all living beings, including humans from ancient to modern times (2). In Europe, the number of infections and deaths caused by the most common multidrug-resistant bacteria was estimated above 670,000 and 33,000, respectively, in 2020 (3). Virus, bacteria, fungi, and protozoa are the major causes of infectious diseases (4). Treatments for cancer and infections are becoming more and more challenging due to the development of drug resistances against prevailing treatment approaches (5, 6).

In past decades, surgical resection, radiation therapy, and chemotherapy were commonly used for clinical treatments for cancer. Although chemotherapy and radiation can kill tumor cells, they can also damage healthy cells and tissues, leading to skin irritation, tiredness, and sickness. Besides, drug resistance in cancer chemotherapy continues to be the biggest challenge today (7) and this can lead to ineffectiveness of the drug treatment, resulting in 90% of cancer related deaths (8, 9). Approximately 50 new infectious agents have been identified in the last 40 years, including respiratory, central nervous system, and enteric infections, viral haemorrhagic fevers, hepatitides, systemic bacterial infections, and human retroviral and novel herpes virus infections (10). The situation is getting even worse with the

emergence of some new or unknown or even old infectious agents, which can affect the population globally. For example, the seasonal influenza virus was first recorded in 1580 and continues to cause epidemics globally each year due to the continuous evolution, resulting in around 500,000 deaths worldwide (11). Antimicrobial drugs are essential for the treatment of infectious diseases, and they are being more and more challenged due to antimicrobial resistance (AMR). Currently, AMR occurs due to abuse of antimicrobial drugs in humans and animals, making infections harder to treat and promoting mortality and morbidity (12). Antibiotic resistance in bacteria is especially urgent among the problems of AMR.

The mechanisms of drug resistance have been investigated by many researchers, and it was revealed that the mechanisms of drug resistance in cancer are very similar to those in infectious disease (13, 14). Such resistance mechanisms can be attributed into either intrinsic or acquired factors. Intrinsic resistance involves limiting uptake of drugs by lacking the respective target and activating drug efflux. For example, gram-negative bacteria with their LPS layer are naturally resistant to certain groups of large antimicrobial drugs, including β-lactams, quinilons, colistins and other antibiotics (13, 15). Tumor cells utilizing detoxifying enzymes, including cytochrome p450 and glutathione transferases, degrade therapeutic drugs and changes extracellular matrix (ECM) interactions to influence tumor environment mediated resistance (16). In contrast, acquired resistance involves the inactivation of the drug or modification of the drug target by mutation or post-translational alteration (17, 18). Various microorganisms can use β-lactamases to hydrolyse β-lactam antibiotics (19). Tumor cells can modulate anti-apoptotic gene expression and improve DNA damage repair capacity to improve their survival (16). Despite of the different molecular targets involved in the resistance mechanisms of cancer cells and microorganisms, the principles of underlying mechanism are quite similar, and this can support the idea of developing drugs that are effective against both.

Therefore, new treatment options urgently need to be developed to overcome the increase in deaths and the occurrence of resistant tumors and infections. Currently, immunotherapy has attracted higher interest in the scientific community and medicine fields. It works by reactivating immune responses that have been silenced by resistance mechanisms or suppressing the immune systems, and it has revolutionized

cancer treatment (20-22). Since natural products (NPs) have various structures, wide sources, less toxic side effects, and multi-targeted features, they have been good options for treatments for cancers and infectious diseases (23-26). In recent years, a variety of NPs has been used in immunotherapy (24). In the following section, NPs and immunotherapy are illustrated.

4.1.1. Natural products

NPs, also known as secondary metabolites, are molecules with low molecular weight. Compared to conventional synthetic molecules, NPs are characterized by diverse chemical structures and biological activities, and are a promising rich source of novel drugs (27). NPs have historically played a critical role in drug discovery for cancer and infectious diseases (28, 29). So far, approximately 60% of all newly discovered anticancer drugs are related to NPs, and 69% of all antibacterial agents are derived from NPs (30, 31). For instance, the antibiotics penicillin and gentamicin are derived from fungi are the most famous NP-derived drugs and have been used in clinics for the treatment of bacterial infections (32). Artemisinin isolated from *Artemisia annua* is a widely used drug for malarial infections (33), which was awarded a Nobel Prize in Physiology or Medicine to Youyou Tu in 2015. The nucleoside vidarabine, which originates from the sponge *Tethya crypta*, shows anticancer and anti-viral properties (34, 35). Overall, NPs exhibit promising efficacy in the treatment of inflammatory diseases, cancer, infections, and neurodegenerative diseases (36-39).

A variety of NPs have also been reported to show significant effects in the treatment against lymphoma, leukaemia, ovary, cervical cancer, and carcinoma in combination with cancer immunotherapy, including cancer vaccines, immune checkpoint inhibitors, and adoptive immune-cell therapy, and some of them are on clinical trials (24). For example, saponins can make pores on cell membranes, promoting antigens to access the cell interior and they have been widely used as immunological adjuvants of cancer vaccines designed for lymphoma, leukaemia, ovary, and carcinoma in clinical trials (40-45). Polysaccharides, characterized by their immune-stimulating ability in DCs, and showed improved efficacy in the adoptive treatment of cytokine-induced killer cells (CIKs) and can also be potential adjuvants for cancer vaccines (46, 47). Moreover, curcumin, which has been used in clinical trials for cervical cancer, can induce tumor-associated macrophages (TAMs) to acquire an M1 phenotype and suppress the M2 phenotype

(48). Since NPs have effective properties in cancer immunotherapy and treatments of infection, they will be the first option in the development of new drugs for treatments of infections and resistant tumors.

4.1.2. Immunotherapy

Immunotherapy has a long history, and it was first mentioned by William B. Coley in 1891, that he tried to utilize the power of the immune system to treat cancer. Coley injected patients with mixtures of live and inactivated bacteria, and durable complete remissions were observed in several types of malignancies. However, such kinds of treatments had to be given up due to the unknown mechanism of Coley's toxin and the risks of deliberately infecting cancer patients with pathogenic bacteria (49). More than a half century later, physicians and researchers used cytokines as cancer immunotherapy. Although clinical remissions were observed in some patients, significant adverse effects counterbalanced the therapy (50, 51). However, cytokines such as IL-2 and IFNα2b, have been approved by the Food and Drug Administration (FDA) for the treatments of renal cancer and malignant melanoma, respectively (52, 53). In the last few years, another revolution in cancer immunotherapy has been the discovery of T cell immune checkpoints. The most well-known examples of T-cell immune checkpoint molecules are the inhibitory molecules cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1). In physiological conditions, CTLA-4 and PD-1 are molecules expressed on T cells, and they can bind to B7 family proteins and PD-L1 on antigen presenting cells (APCs), respectively, resulting in suppression of T cell function (54). Combined blockade of PD-1 and CTLA-4 can improve the survival of more than 50% of patients as shown by three-phase clinical data (55), awarding of the 2018 Nobel Prize in Physiology or Medicine to Drs. Allison and Honjo.

Considering the critical roles of DCs in initiating immune responses and surveillance, DC-based immunotherapy has been used in clinical trials for various cancers (56). These trials can be classified into DC vaccines and other DC-related trials. DC vaccines involve DCs pulsed with proteins or lysates of autologous or allogeneic whole tumors or tumor cell lines have been used in treating numerous cancers (57-61). For instance, DCs pulsed with leukemic lysates from AML patients can induce immunological responses and increased autologous T cell ability to stimulate DCs (57). Moreover, DCs transduced with an adenoviral vector expressing the extracellular and transmembrane domains of HER2

(AdHER2) has been approved by FDA for HER2-expressing tumors (62). Other DC-related trials include the use of DCs in conjunction with various TLR agonist has been used to mature DCs for vaccination against various cancer treatment (63, 64). For example, a clinical-grade TLR4 agonist LPS has been used for vaccination in ovarian cancer patients and Bacillus Calmette-Guérin (BCG), a TLR2/4 agonist originating from *Mycobacterium bovis*14, is used to treat patients with early-stage bladder cancer (65).

DCs are already important targets for immunotherapy due to their critical roles in bridging innate adaptive immunity. Thus, Dendritic cells (DCs) are described in the following sections.

4.2. Dendritic cells

DCs function as sentinel cells in the immune system, sensing pathogens and tissue damage and subsequently migrating to lymph nodes to present antigens to naive T cells, playing crucial roles in innate and adaptive immune responses. DCs are derived from hematopoietic stem cells (HSCs), differentiate in the bone marrow, can recognize pathogens, and process and present antigens to naive T cells, inducing adaptive immune responses. At the same time, DC activation correlates with increased co-stimulatory molecule expression and multiple inflammatory cytokine productions. In the following section, the discovery and development of DCs and DC activation are introduced.

4.2.1. Discovery of dendritic cells

DCs were first described by German physician Paul Langerhans in 1868 as cells with a tree like morphology which were found in human skin. However, they were considered neurons since they were sharing morphology similarities (66). Following this, in 1973, Ralph Steinman found a unique adherent cell population by electron microscopy from mouse spleen, which was clearly distinct from macrophages. He noticed that these cells had a stellate morphology, were motile, contained less lysosomes, and he named such cells as DCs (67). Moreover, he reported that DCs express high levels of major histocompatibility complex (MHC) molecules, which were later confirmed to be the key factors for T cell priming (68). In 1978, through mixed lymphocyte reaction (MLR) assays, he found DCs were uniquely potent in inducing activation of naive T cells compared with other splenocytes. This led him

to hypothesize that DCs may provide other signals for T cells beyond MHC molecules, which were highly expressed on macrophages, as well (69). For these great findings, Steinman was awarded the Nobel Prize in Physiology or Medicine shared with Jules Hoffmann and Bruce Beutler in 2011. In 1992, Ken Shortman and colleagues discovered a DC subgroup in the spleen and thymus which highly expressing CD8α. These cells were later referred to as conventional dendritic cells type 1 (cDC1) (70). Eight years later, the same group reported another subgroup of DCs in the spleen and thymus which were highly expressing CD4. These cells were later referred to as conventional dendritic cells type 2 (cDC2) (71). Plasmacytoid dendritic cells (pDCs) were first depicted as a "lymphoblast" by Lennert and Remmele in 1958 according to their plasma cell-like morphology (72). In the following decades, they remained a mystery for immunologists and therefore they were called many different names such as "T-associated plasma cell" (73), "plasmacytoid T cell" (74, 75), "plasmacytoid T-zone cell" (76) and "plasmacytoid monocytes" (77) based on their localization and expression of surface molecules. In 1983, pDCs were termed "natural interferon (IFN) producing cells" or "NIPC" by Lars Rönnblomo (78). In 1999, it was determined that the NIPCs were identical to the plasmacytoid subset of dendritic cells (79), which are currently named pDCs.

4.2.2. Development of dendritic cells

DCs develop from HSCs in the bone marrow and undergo a multistep differentiation. In this process, a panel of regulatory elements are involved such as transcriptional factors and secreted and surface molecules such as cytokines or growth factors. In the following sections, stages of DC development and required signals are presented.

4.2.2.1. Stages of dendritic cell development

DCs, the most potent APCs, originate from progenitors in the bone marrow through hematopoiesis and the development process involves multiple cellular and molecular events. In mouse bone marrow, HSCs first give rise to multi-potent progenitors (MPPs) which can further differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs, one population of FMS-related tyrosine kinase 3 (FLT3) expressing cells, give rise to macrophage dendritic cell progenitors (MDPs) (80) and granulocyte–monocyte progenitors (GMPs). Developing from MDPs, common DC progenitors

(CDPs), which give rise to conventional DC progenitors (pre-cDCs) and pre-pDCs (45, 81). GMPs can further differentiate into granulocyte progenitors that further develop into mast cells, basophils, eosinophils, and neutrophils (82) and a common monocyte progenitor (cMoP) (83), which loses DC potential and generates only monocytes. CLPs can differentiate into T, B, and natural killer (NK) cells and other innate lymphoid cells. Pre-pDCs terminally develop to pDCs in the bone marrow and then localize to secondary lymphoid organs. In contrast, pre-cDCs migrate to both lymphoid and non-lymphoid organs and eventually differentiate into cDC1 and cDC2 (84-86). The differentiation stages are simplified summarized in Figure 1.

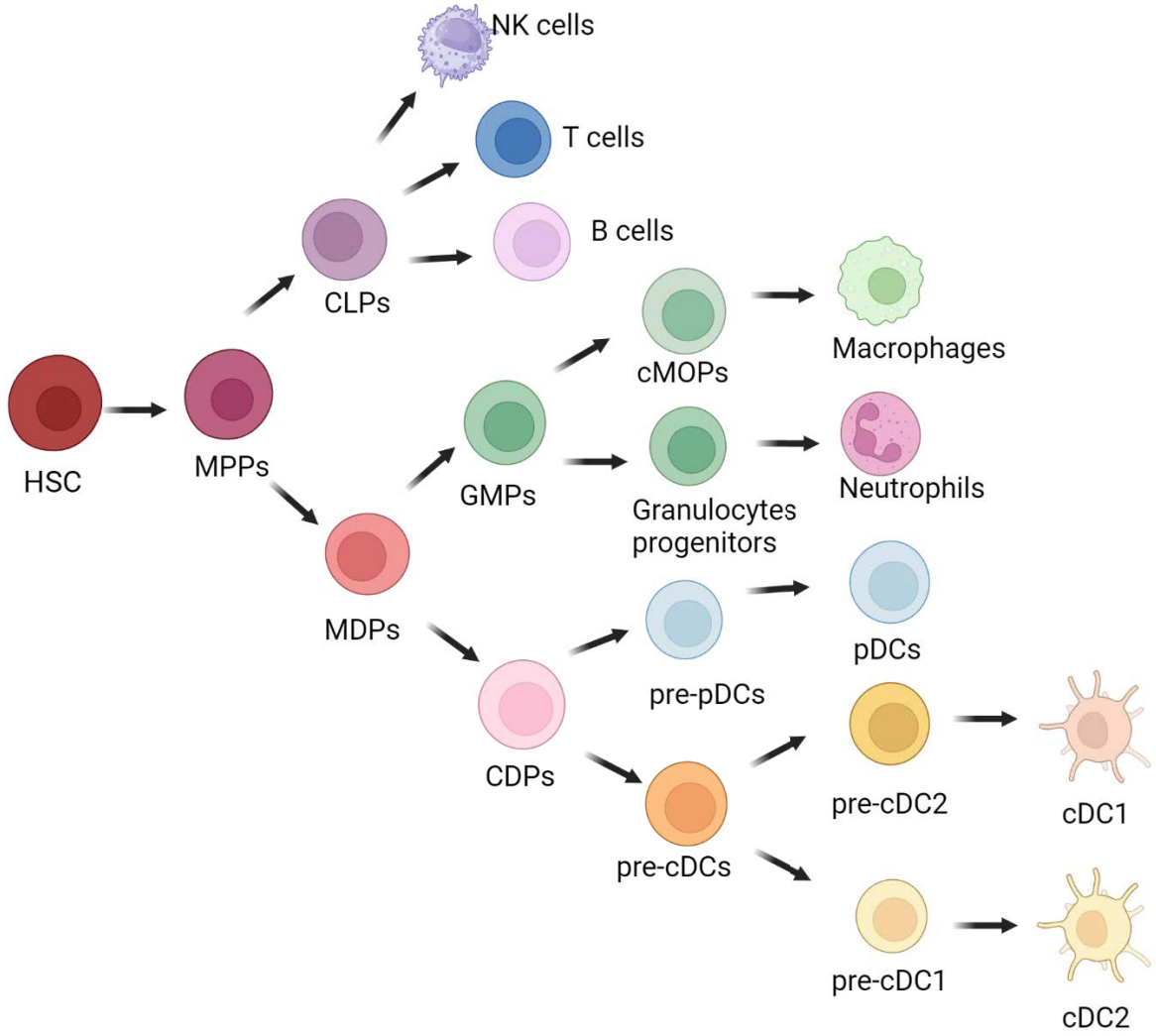

Figure 1. The development of DCs. This figure was generated by BioRender.com.

For many years, it was controversial whether pDCs are derived from a CLP or a CMP based on the initial classification of immune lineages (53, 87, 88). Previously, pDCs were commonly classified within the myeloid compartment (85). However, Shigematsu *et al*. revealed that pDCs can develop efficiently from both CMPs and CLPs. Consistently, Sathe *et al*. shows that CLPs can give rise to pDCs and the pDCs of both origins produced IFNα upon CpG oligonucleotides stimulation. Besides, Single-cell RNA sequencing (scRNA-seq) studies by Herman *et al*. revealed B cells and pDCs shared a common precursor cluster (89), which was further verified by Rodrigues *et al*. as pDCs originate from IL-7R$^+$ lymphoid progenitor cells (90). Additionally, Rodrigues *et al.* indicated that both origins of mature pDC subsets are able to produce Type I IFN and only myeloid-derived pDCs have the ability to process and present antigen effectively (90). Moreover, using scRNA-seq, mass cytometry and flow cytometry, Dress *et al.* showed that pDCs developed from Ly6D$^+$CD81$^+$ lymphoid progenitors, but not from myeloid progenitors (91). Conversely, DNA barcoding data by Feng *et al*. indicated that pDCs have a shared origin with cDCs in a common Flt3-driven developmental pathway and are clonally related to the cDC1 subset of cDCs (92).

4.2.2.2. Cytokines required for dendritic cell lineage

The differentiation, survival, and expansion of each hematopoietic lineage is regulated by different cytokines and growth factors. Here we summarize the main cytokines that control DC lineage commitment and differentiation in the BM and the maintenance of DC homeostasis in the periphery.

Flt3, a tyrosine kinase receptor, is specifically expressed on CD34$^+$ hematopoietic stem/progenitor cells (93, 94). Loss of Flt3L or its receptor leads to a remarkable decrease of both pDCs and cDCs in mouse spleen and lymph nodes. However, it has no impact on monocytes and granulocytes (95), indicating a key role of Flt3L in DCs development. In contrast, increased Flt3L levels induced by malaria infection promote DCs expansion *in vivo* (96). Furthermore, it has been reported that only bone marrow progenitors expressing Flt3 can give rise to DCs and overexpression of Flt3 in Flt3$^-$ haematopoietic progenitors can drive these cells towards differentiation into pDCs and cDCs (97-99). Accordingly, Flt3L is essential for the development of the DC lineage.

GM-CSF is a cytokine which promotes the proliferation of bone marrow cells and generates myeloid cell colonies (100, 101). In 1992, it was first shown that GM-CSF can induce DCs differentiation from mouse bone marrow cells *in vitro* (102). However, *in vivo*, the function of GM-CSF is dependent on the type of tissue. In mice deficient in GM-CSF or its receptor, cDCs in the spleen and lymph nodes differentiate normally (103). Interestingly, the numbers of cDCs in non-lymphoid tissues is significantly decreased in GM-CSF deficient animals due to apoptosis induced by increased mitochondria fission (104). Collectively, GM-CSF plays a critical role in the homeostasis of DCs in non-lymphoid tissues.

Lymphotoxin β (LTβ), a type II membrane protein of the TNF family, expressed in B cells regulates the proliferation of splenic DCs together with Flt3L (105, 106). LTβ can regulate proliferation of $CD11b^+$ spleen DCs as a heterotrimeric LTα1β2 complex. It has been shown that mice deficient in LTβR exhibit a decreased population of $CD11b^+$ DCs. Consistently, transgenic mice overexpressing LTα1β2 on B cells show an increased population of the $CD11b^+$ splenic DCs (107). This indicates the importance of LTβ for DC development and homeostasis.

4.2.3. Activation of dendritic cells

After completion of the development, DCs leave the bone marrow and reside in the peripheral lymphoid organs and non-lymphoid tissues such as skin and mucosal surfaces. Upon sensing of invading pathogens via pattern recognition receptors (PRRs), resident DCs from non-lymphoid tissues can become activated and migrate to the lymph node to activate naive T cells, thereby initiating adaptive immunity. Since PRRs are essential for DC activation, they are introduced in the following section.

4.2.3.1. Pattern recognition receptors

PRRs are a class of receptors that can directly sense pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). PRRs are essential elements in innate immunity and are mainly expressed by innate immune cells such as DCs, monocytes, and macrophages (108). Based on their localization, PRRs can be classified into membrane-bound PRRs including Toll like receptors (TLRs) and C-type lectin receptors (CLRs) and cytoplasmic PRRs including retinoic acid-

inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (109), which are presented in the following parts.

4.2.3.1.1. Toll like receptors

TLRs are membrane-bound proteins and were first described in the fruit fly by Jules Hoffmann in 1996 (110). TLRs are expressed in immune cells (e.g., DCs and macrophages) and non-immune cells, including fibroblasts and epithelial cells, playing essential roles in innate immunity. TLRs can recognize PAMPs or DAMPs in immune responses against antigens. Up to date, 12 functional TLRs have been identified in mice, including TLR1-9 and TLR11-13 (111). According to their localization, TLRs can be divided into two groups: cell surface TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, and intracellular TLRs, including TLR3, TLR7, TLR8, TLR9, TLR11, and TLR12. Although TLR4 is mainly found in the plasma membrane, it can also locate within the endosomal membrane (112). TLR2 can recognize bacterial lipoproteins and lipoteichoic acid, as well as fungal zymosan, by forming a heterodimer with TLR1 or TLR6. TLR5 can be stimulated by bacterial flagellin (113). However, TLR10 and TLR8 are not functional in the mouse (114, 115). TLR3 can sense double stranded RNA (dsRNA) like Poly I:C. TLR7 is mainly expressed in pDCs and can be activated by single stranded RNA (ssRNA) from viruses, synthetic poly(U) RNA, and certain small interfering RNAs. Similarly, human TLR8 can recognize fragments of ssRNA of viral and host origin. TLR9, by contrast, can be activated by unmethylated CpG-DNA from viruses and bacteria (116). TLR11, present in mice but not humans, recognizes flagellin from uropathogenic *Escherichia coli* (UPEC) and *Salmonella typhimurium*, and profilin of *Toxoplasma gondii* (117). Similarly, TLR12 can recognize profilin from *T. gondii* by forming either as a homodimer or a heterodimer with TLR11 (118). TLR13 recognizes conserved motif 23S rRNA of the large ribosomal subunit of bacteria (119). Upon recognition of PAMPs by members of the TLR family, TIR domain-containing adaptor proteins including MyD88 and TRIF are recruited, and subsequently downstream signal transduction is initiated to induce inflammatory cytokines (e.g., IL-12, IL-6, and TNF), chemokines (RANTES, IP-10, ENA78, etc.), and type I IFN expression. Based on the distinctive adaptors in these pathways, TLR signaling can be subdivided into two categories: the MyD88-dependent pathway and the TRIF-dependent pathway (111, 120). Most TLRs interact

intracellularly with MyD88, except TLR3, which transduces activating signals exclusively via TRIF. Effective TLR4 signaling depends on both adaptor molecules, TRIF and MyD88 (121).

TLR4 can be stimulated by lipopolysaccharide (LPS; endotoxin), a major component of the outer membrane of gram-negative bacteria. It has been reported that TLR4 recognizes lipid A, the active component of LPS by forming a complex with the secreted molecule myeloid differentiation 2 (MD-2) with the aid of LPS binding protein (LBP) and CD14 (122). Specifically, the lipid A of LPS first binds to LBP and then it was transferred to CD14, which can split LPS into monomeric molecules. The CD14-LPS complex facilitates TLR4 activation along with MD-2 of the TLR4 signaling pathway to form the TLR4/MD2/LPS complex (123). Subsequently, homodimerization of two MD2/TLR4/LPS complexes triggers two consecutive signaling pathways: the MyD88-dependent and TRIF-dependent signaling pathways to induce NF-κB, MAPK, and IRFs activation, resulting in the production of proinflammatory cytokines, chemokines, and type-I IFN production (124-126). The recognition of LPS by TLR4/MD2 is summarized in Figure 2.

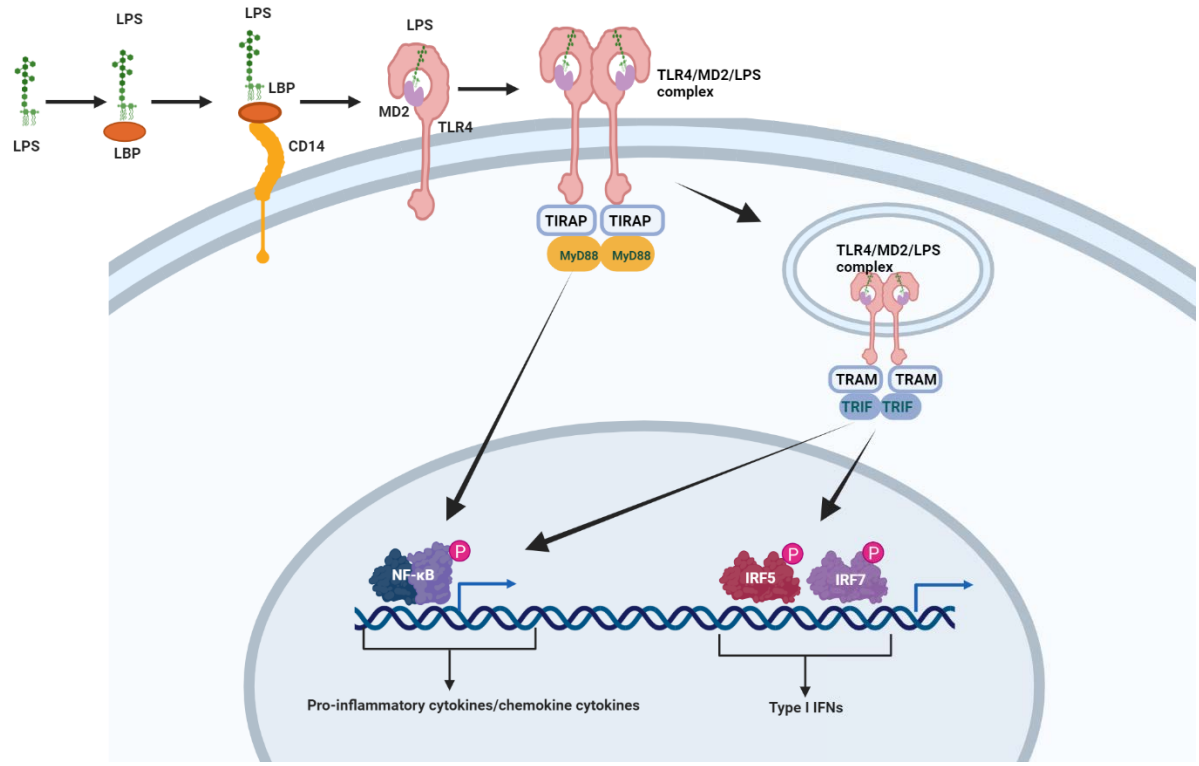

Figure 2. Simplified TLR4 signaling pathway in response to LPS stimulation. This figure was created by BioRender.com.

Apart from LPS, TLR4 can also recognize pathogens derived from respiratory syncytial virus and mouse mammary tumor virus (127, 128). In addition, TLR4 can also be activated by fungi including *Candida albicans* (*C. albicans*) O-linked mannans, Cryptococcus neoformans (*C. neoformans*) glucuronoxylomannan (GXM) (129), and *heat-killed conidia Aspergillus fumigatus* (*A. fumigatus)*. However, which component of *A. fumigatus* is responsible for TLR4 activation has not been elucidated (130). Additionally, TLR4 can also respond to endogenous proteins such as Heat shock proteins HSP60, HSP70, gp96, myeloid-related protein 8/14 (Mrp8/14) (131-134), and high mobility group box 1 protein (HMGB1) (135).

4.2.3.1.2. RIG-I-like receptors

RLRs are intracellular PRRs that act as cytoplasmic RNA sensors and can recognise virus dsRNA and induce type I IFN production, which is critical for antiviral immunity (136). The currently known RLR family members mainly consist of RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RIG-I was first discovered in acute promyelocytic leukemia cells induced by retinoic acid and is composed of three parts (137). The DexD/H helicase domain, showing ATPase and helicase activities and is a common part of the RLR family. Two CARDs are the N-terminal part, which is in charge of downstream signal transduction (138). A repressor domain (RD) and the C-terminal domain (CTD) form the C-terminal part. The RD can suppress activation of the receptor, and the CTD can recognize virus RNA. In the steady state, these three parts are folded and upon viral invasion the CTD recognizes viral RNA followed by a conformational change. RIG-I then uses its ATP hydrolase activity to expose and activate the CARD to recruit downstream signaling linker molecules (139). MDA5 has a similar structure but lacks the RD in its C-terminal domain. Although both RIG-I and MDA5 can recognize viral dsRNA, the lengths of the dsRNA they recognize are different. RIG-I can recognize short dsRNA below 1000 bp, whereas MDA5 can recognize long dsRNA above 1000 bp (140). Compared to other RLRs, LGP2 lacks a CARD domain (141, 142). LGP2 can regulate RIG-I-mediated recognition of viral dsRNA. Upon virus infection, LGP2 can interact with RIG-I and MDA5, promoting dsRNA recognition of RIG-I and MDA5 (143). As mentioned in the "Toll-like receptor" section, TLR3, TLR7, TLR8, and TLR9 expressed in the endosomes can respond to viral DNA

or RNA derived from virus or bacteria. In contrast, RLRs can directly recognize the virus in the cytosol. Therefore, RLRs are indispensable for antiviral immune responses (144).

4.2.3.1.3. NOD-like receptors

NLRs are intracellular PRRs that can recognize PAMPs and DAMPs in the cytosol. NLR family members NLRP1, NLRP2, NLRP3, NLRP6, NLRC4, and NLRP12 can act as sensors of the inflammasome complex, which is indispensable to innate immunity. NLRs are expressed in immune cells, including lymphocytes, macrophages, and DCs, as well as in non-immune cells (145). NLRs consist of three parts. The middle part is the central nucleotide-binding domain (NBD), also known as the NACHT domain, and it is a characteristic part of all NLR members. The NBD is responsible for the nucleic acid binding and oligomerization of NLRs. The C-terminal is composed of LRRs, which are responsible for ligand recognition. The N-terminal effector domain is either a CARD or pyrin domain (PYD), responsible for protein interaction (146, 147). NOD1 and NOD2 are the most well-known NLRs. NOD1 can recognize the conserved moieties of the Gram-negative bacterial cell wall (148). In addition, NOD2 can also respond to the ssRNA of the complete virus (149).

4.2.3.1.4. C-type lectin receptors

CLRs, the phagocytic PRRs, are essential to antifungal immunity. CLRs are mainly expressed on APCs, such as macrophages, DCs, and monocytes. CLRs are responsible for recognizing carbohydrates in fungal cell walls through the calcium-dependent carbohydrate recognition domain (CRD) (150, 151). Dectin-1 is a type II transmembrane protein composed of an intracellular signal transduction domain CTLD and an extracellular domain immunoreceptor tyrosine-based activation motif (ITAM). Dectin-1 can recognize diverse fungi (152), including yeast (153), *C. albicans*, and *Aspergillus* (154). Dectin-1, as a specific receptor of β-glucans (155), plays an important role in DCs. Specifically, the activation of Dectin-1 in DCs leads to the production of the inflammatory cytokines IL-6, TNF, and IL-12p40, which induce Th17 and Th1 cell polarization (156). DC-SIGN (also known as CD209), present on both macrophages and DCs, is a typical representative of CLRs. DC-SIGN recognizes fucose-based PAMPs through its CRD, leading to DC activation and IL-27 production (157).

4.2.3.2. Markers of DC activation

Upon recognition of PAMPs by PRRs, immature DCs convert into mature DCs and migrate to peripheral lymph nodes. Immature dendritic cells (iDCs) take up pathogens by phagocytosis or endocytosis, digest invading pathogens into small peptides, followed by their presentation on the cell surface through the formation of MHC/peptide complexes. Simultaneously, costimulatory molecules, MHC molecules, and cytokine production (e.g., IL-12, IFNβ, and TNF) are highly expressed, which leads to T-cell activation. In the following parts, costimulatory molecules, MHC molecules, and cytokine production (e.g., IL-12, IFNβ, and TNF) will be depicted. Mature DCs can eventually activate T cells via up-regulation of MHC and co-stimulatory molecule, and cytokines production and initiate adaptive immune response to clear the invading pathogens.

4.2.3.2.1. The MHC

The MHC is a group of genes that play a critical role in antigen presentation and immune recognition. The MHC family consists of several classes, with MHC class I (MHC-I) and MHC class II (MHC-II) directly involved in antigen presentation (158). MHC-I molecules are expressed on the surface of all nucleated cells and primarily present peptides, derived from endogenous proteins, such as viral proteins, to $CD8^+$ T cells (159). MHC-II molecules, on the other hand, are mainly expressed on APCs and present peptides from exogenous pathogens to $CD4^+$ T cells (160). Deficiencies in MHC molecules can lead to compromised innate and adaptive immunity, affecting the development and function of various immune cells. MHC-I deficiency can impair the function and development of NK cells and $CD8^+$ T cells (161-163), while MHC-II deficiency can result in incomplete development of $CD4^+$ T cells and a lack of antigen-specific antibody responses (164, 165). Therefore, MHC molecules play a critical role in both innate and adaptive immune systems.

4.2.3.2.2. Co-stimulatory molecules

Co-stimulatory molecules are proteins localized on the cell membrane that play a role in amplifying or counteracting the initial activating signals received by T cells through their T cell receptor (TCR) when interacting with the MHC/peptide complex. Co-stimulatory molecules represent a heterogenous group

of proteins, including members of the immunoglobulin (Ig) superfamily, the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily, and the T cell Ig and mucin (TIM) domain family (166). CD80 (B7.1) and CD86 (B7.2), members of the Ig family, are essential co-stimulatory molecules in T cell activation (167). CD80 and CD86 interact with CD28 on T cells to induce the production of interleukin-2 (IL-2), which promotes T cell expansion. CD40, another co-stimulatory molecule of the TNF receptor family, is also commonly used as a marker of DC activation. Interaction between CD40 and CD40L on activated T cells licenses DC to produce T cell stimulatory cytokines, such as IL-12 (168).

4.2.3.2.3. IL-12

IL-12, also known as IL-12p70, is a heterodimeric cytokine composed of the IL-12a (p35) and IL-12b (p40) subunits. IL-12 primarily acts on NK cells and T cells by binding to the IL-12 receptor, which consists of IL-12Rβ1 and IL-12Rβ2 subunits. Upon IL-12 binding, the receptor activates the janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, leading to the production of interferon-gamma (IFNγ) and promoting the differentiation of naive $CD4^+$ T cells into Th1 cells (169). IL-12, also considered as NK cell stimulating factor, has been shown to enhance NK cell-mediated cytotoxicity and stimulate the production of IFNγ by $CD8^+$ cytotoxic T lymphocytes (170, 171). Additionally, IL-12 can suppress the immunosuppressive function of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment (172).

Preclinical studies have demonstrated significant antitumor properties of IL-12 against various malignancies such as murine bladder cancer and sarcoma (173, 174), but early clinical studies using systemic administration of IL-12 have shown minimal effects with tolerated doses (175-177). Moreover, IL-12 produced by DCs has been found to increase the cytotoxicity of tumor-infiltrating T cells, making it an attractive candidate for immunotherapeutic approaches (178).

4.2.3.2.4. Type I IFNs

The type I IFN family consists of a single IFNβ and multiple IFNα subtypes (13 in humans and 11 in mice), as well as IFNε, IFNκ, and IFNω in most mammals (179). Type I IFNs are rapidly produced in

response to the recognition of pathogens such as viruses, bacteria, parasites, and fungi by PRRs. Although most cells in the body can produce type I IFN, pDCs have long been defined as professional type I IFN-producing cells due to their unique molecular adaptations for nucleic acid recognition and ability to produce high levels of type I IFN. However, recent findings show that the cell type responsible for type I IFN production depends on the specific infectious setting (180). Type I IFNs can bind to a common heterodimeric IFNα receptor (IFNAR), which consists of IFNAR1 and IFNAR2 subunits and is expressed on all nucleated cells of the body (181). Binding of type I IFN to IFNAR can activate the kinases JAK1 and TYK2, which in turn induce phosphorylation of STAT2 and STAT2 and promote the formation of STAT1–STAT2–IRF9 (IFN regulatory factor 9) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in the DNA to initiate the transcription of IFN-stimulated genes (ISGs) (179).

Type I IFNs have potent antiviral effects and are responsible for inducing ISGs that suppress viral replication in both human and murine cells (182). For example, it was shown *in vivo* that exogenous IFN can mediate resistance to influenza virus infections (183) and IFNAR1 deficient mice were susceptible to virus infection (179). They also play a crucial role in innate immunity by promoting the maturation of DCs, up-regulating MHC molecules and co-stimulatory molecules (184, 185), inducing chemokine receptor expression, and enhancing the response of $CD4^+$ T cells (186), $CD8^+$ T cells (187), and NK cells (188, 189).

Overall, mature DCs can activate T cells in the lymph node through up-regulations of MHC and co-stimulatory molecules, and cytokine production, thereby initiating an adaptive immune response to eliminate the invading pathogens. Therefore, T cells and the crosstalk between T cells and DCs are described in more detail in the following sections.

4.3. T cells

T cells are specialized lymphocytes that play a crucial role in adaptive immune responses. They are derived from CLPs in the bone marrow and undergo maturation in the thymus (190). T cell maturation involves multiple processes, including positive selection and negative selection. In brief, positive

selection induces thymocytes to differentiate into $CD4^+$ or $CD8^+$ single positive cells, and negative selection further eliminates thymocytes that carry TCR with a high affinity for self-peptide MHC complexes, which ensures that only those T cells that are self-tolerant can survive (191). During positive and negative selection, DCs play critical roles in promoting the survival of single-positive thymocytes and eliminating self-reactive T cells (192). The majority of immature T cells in the thymus differentiate into αβ T cells, while a small percentage become γδ T cells. Once mature, T cells with unique T cell receptors (TCRs) that are responsible for recognizing various antigens leave the thymus and enter the bloodstream (193).

4.3.1. The T cell receptor and CD3 chains

The TCR is a heterodimeric protein complex that plays a central role in T cell activation and antigen recognition. It was first discovered in 1982 and consists of highly variable α and β chains or γ and δ chains, which forms two distinct heterodimers: TCRα/TCRβ or TCRγ/TCRδ. The majority of mature T cells express TCRα and TCRβ isoforms referred to as αβ T cells, while a small percentage (0.5-5%) of T cells (γδ T cells) express TCRγ and TCRδ isoforms (194, 195). The TCR is a transmembrane protein with an extracellular domain responsible for recognizing peptides presented by MHC molecules on APCs or but also other nucleated cell surfaces, a transmembrane domain, and a cytoplasmic tail. The extracellular domain includes a variable immunoglobulin-like (V) domain and a constant immunoglobulin-like (C) domain that interacts with CD3 (196, 197). CD3 is a protein complex composed of four chains: CD3γ, CD3δ, and two CD3ε chains (198). These chains can form heterodimers CD3γε and CD3δε through non-covalent bonds, or homodimers CD3ζζ through disulfide bonds (199-201). The CD3 complex, along with the TCR, is involved in initiating TCR signaling (202).

4.3.2. T cell co-receptors

CD4 and CD8 are transmembrane glycoproteins that act as co-receptors of the TCR and are important markers for different subsets of T cells (203, 204). CD4 is mainly expressed on Th1, Th2, and Th17 helper cells, as well as regulatory T cells (Tregs). CD4 is a single-chain molecule with an extracellular section consisting of four domains (D1, D2, D3, and D4), a transmembrane section, and a cytoplasmic tail. CD4 binds to the β2 region of MHC-II molecules, enhancing T cell activation. CD4 has been shown

to increase the sensitivity of T cells to antigen recognition (205, 206), and blocking CD4 significantly reduces antigen recognition by T cells (207).

CD8, on the other hand, is primarily expressed on cytotoxic T lymphocytes (CTLs) (208). It exists as either a CD8αα homodimer on subsets of memory T cells, intraepithelial lymphocytes, NK cells, and DCs or as a CD8αβ heterodimer on MHC-I-restricted TCRαβ T cells (209). The α-chain of CD8 interacts with the α3 domain of MHC-I molecules to stabilize the interaction between the MHC/peptide complex and the TCR (210, 211). This interaction enhances the sensitivity of T cells to antigen recognition (212, 213). CD8 can also interact with the lymphocyte-specific protein kinase (LCK) via its cytoplasmic tail, initiating T cell activation.

4.4. Crosstalk between DCs and T cells

Upon recognition of invading pathogens by PRRs, immature DCs undergo maturation, leading to the up-regulation of MHC molecules, co-stimulatory molecules, and cytokine production, including IL-12 and type I IFN. Mature DCs can then present antigens to naive T cells, initiating an adaptive immune response against the pathogen or tumor. Naive T cell activation by DCs requires the following three signals (214).

Signal 1: involves the engagement of the T cell receptor (TCR) complex on $CD4^+$ or $CD8^+$ T cells with the antigenic MHC/peptide complex. This interaction is critical for antigen recognition and T cell activation (215-217). **Signal 2**: involves the binding of costimulatory molecules, such as B7 (CD80/CD86) on DCs, which binds to CD28 on T cells, CD40 binding to CD40L on T cells (218). This interaction provides a second signal that supports T cell activation and proliferation. **Signal 3**: Cytokines produced by DCs, such as type I IFNs, IL-12, IL-4, and IL-23, play a crucial role in directing and amplifying T cell polarization and proliferation. These cytokines influence T cell differentiation and can promote the development of specific effector functions and memory T cells (214, 219).

Signal 1 and signal 2 can initiate the proliferation of naive T cells. However, without signal 3, naive T cells are not able to develop specific effector functions or memory T cells (220). Signal 3, provided by DC-derived cytokines, is essential for the full development and differentiation of T cells. Specific

cytokines have distinct effects on T cell polarization. For example, IL-6 can induce naive $CD4^+$ T cell polarization into T follicular helper (Tfh) cells (221). IL-12 promotes IFNγ production by naive $CD4^+$ T cells and subsequently drives Th1 cell polarization (222). IL-4 drives naive $CD4^+$ T cell polarization into Th2 cells (223), while IL-23 enhances the proliferation of Th17 cells generated from naive $CD4^+$ T cells stimulated by a cytokine cocktail (TGF-β, IL-6, and IL-1α/β) (224). In addition, TGF-β together with IL-4 can induce Foxp3 expression in Treg cells (225). Moreover, IFNs and IL-12 promote the development of functional effector $CD8^+$ T cells by controlling chromatin remodelling (219), which is also involved in the process of Th1/Th2 cell differentiation (226).

Upon TCR recognition of the antigen-MHC complex, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 chains occurs via the Lck kinase bound to the cytoplasmic tail of the coreceptor CD4 and CD8 (191, 227). The phosphorylated ITAMs can then interact with the kinase ZAP-70 and induce its phosphorylation and activation. This cascade eventually activates nuclear transcription factors involved in T cell activation and differentiation. Lack of signal 2, which involves co-stimulatory molecule interactions, can induce anergy in $CD4^+$ T cells (228). Anergic T cells lack the capacity to proliferate or secrete IL-2 upon recognizing the MHC peptide complex, even in the presence of co-stimulatory molecule interaction (229). The cross-talk between DCs and CD4 T cell differentiation is summarized in Figure 3, which likely provides a visual representation of the complex processes involved in naive T cell activation and differentiation by DCs.

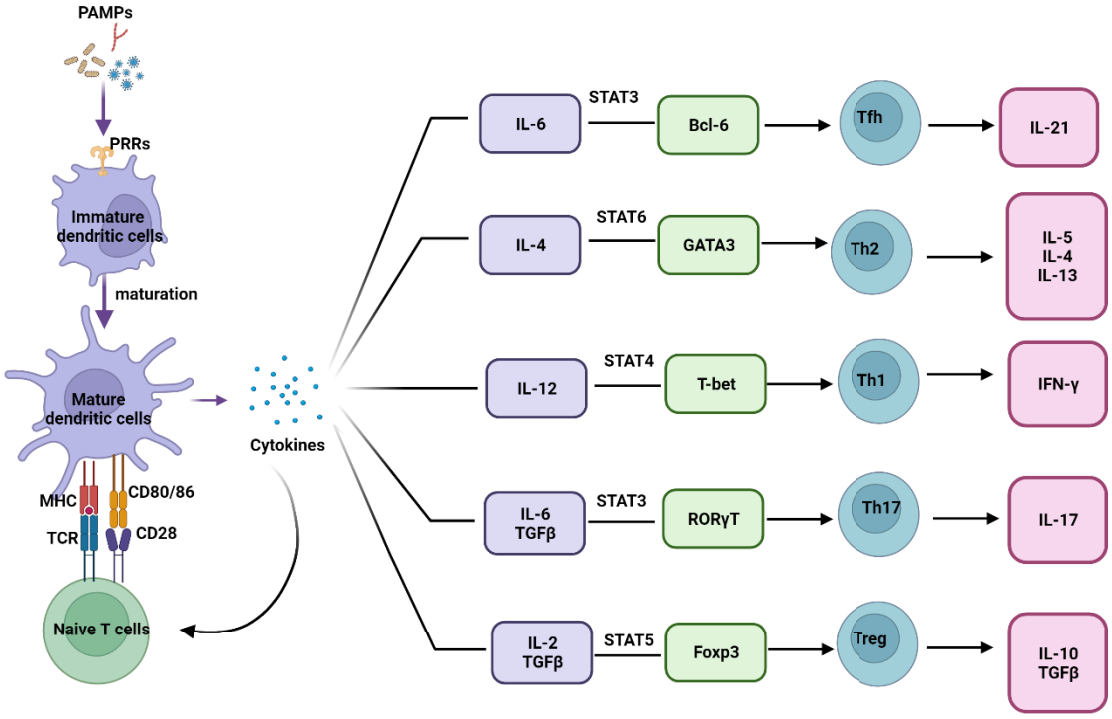

Figure 3. DC and T cell cross-talk. Figures were assembled with BioRender.com.

4.5. BEA

The mycotoxin BEA is a secondary metabolite produced by various toxic fungi, including *Beauveria bassiana* and *Fusarium spp*. Multiple biological functions of BEA have been reported, including cytotoxicity, anti-cancer, anti-virus, antimicrobial, immune-regulating, insecticidal, and pesticidal activity. In the following section, the origin and the occurrence of BEA and biological properties of BEA are introduced.

4.5.1. The origin and the occurrence of BEA

BEA is a cyclic hexadepsipeptide composed of three D-hydroxy-iso-valeryl and three N-methyl-phenylalanyl residues in an alternating sequence (230, 231) (as shown in Figure 4). This structure distinguishes BEA from other ENNs because of the N-methyl amino acid, which gives BEA distinct bioactivities compared to ENNs (232, 233). BEA was first identified from *Beauveria bassiana* by Hamill *et al*. in 1969 (230) and can be produced by various fungi, including several *Fusarium* species such as

F. anthophilum, F. bulbicola, *F. denticulatum,* and *F. phyllophilum* (234). BEA J has the same structure as BEA except for the hydroxyl group and it was also isolated from *Fusarium spp*.

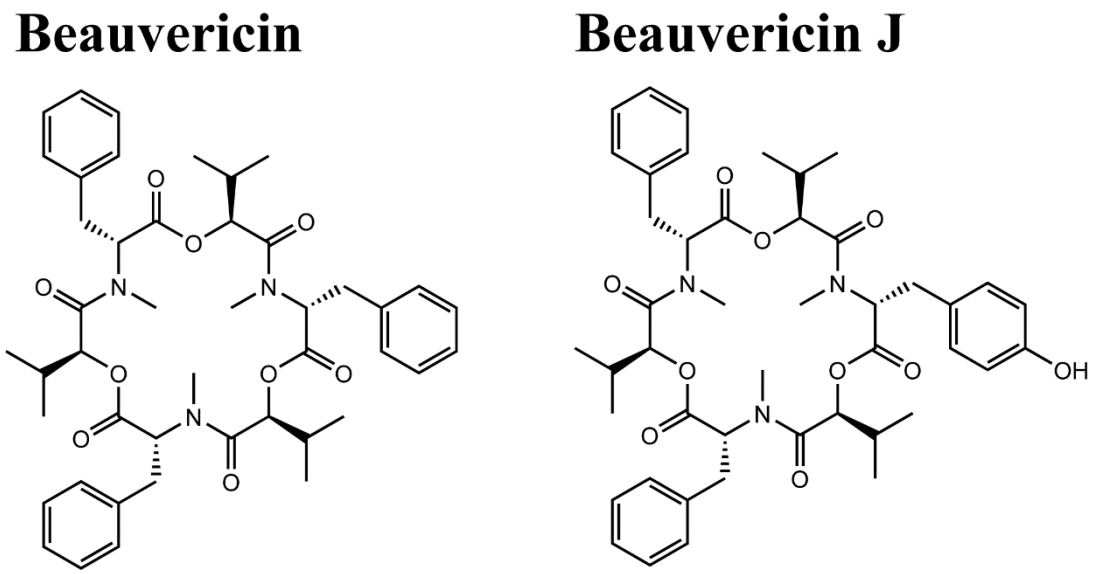

Figure 4. Structures of BEA and BEA J.

BEA is a common contaminant in food and feed products worldwide. It has been detected in cereal-based products, dried fruits, eggs, and infant formula (235-237). In Europe, BEA has been found in 54% of grains and cereal-based foods (237, 238). In feed and feed raw materials, BEA was detected in 98% of samples. High levels of BEA contamination were also observed in a rice-based infant cereal from Morocco (239). Furthermore, BEA contamination has been found in Chinese medicinal herbs, with an incidence of 20% (240). Besides, BEA was reported as the main mycotoxin in food and the contamination in the main markets of Abidjan, Bouake, and Korhogo, France and 91% of the rice, maize and peanut samples were contaminated with various mycotoxin including BEA (241, 242).

The high occurrence of BEA in food and feed products has led to studies investigating its potential impacts on human health. Chronic dietary exposure trials in poultry have shown that diets contaminated with BEA had no significant effects on performance parameters, biochemical blood parameters, or meat quality (243, 244). Similarly, dietary exposure of BEA to broilers and laying hens did not affect growth, feed intake, or egg production (237). The European Food Safety Authority (EFSA) also stated in 2014 that acute exposure to BEA is not a concern for human health (238). However, some studies have

indicated that BEA can penetrate through the skin and cross the blood-brain barrier in mice and humans, affecting the central nervous system (245, 246). Moreover, one recent paper reported that BEA can induce hepatotoxicity in pigs' precision-cut liver slices and HepG2 cells (247). The chronic exposure of humans to BEA is still unclear and requires further evaluation through in vivo toxicity assessments.

4.5.2. Bioactivity of BEA

The bioactivity of BEA has been studied already with regard to its anti-cancer, anti-virus, antimicrobial, immune-regulating, insecticidal, and pesticidal activity. In the following section, the bioactivity of BEA and the underlying mechanism are introduced.

4.5.2.1. Anti-cancer effects of BEA

Currently, anti-cancer effects of BEA are drawing more attention in the scientific community. It was well investigated for anti-cancer effects in vitro, and BEA exhibits cytotoxicity to a variety of human cancer cells, such as leukaemia cells (248, 249), brain cancer cells (250), breast cancer cells (250), hepatocellular carcinoma (251), colon carcinoma (252) and lung cancer cells (250, 253). Besides, BEA also shows anti-cancer effects by inhibiting the migration of metastatic cancer cells and the anti-angiogenic activity of tumor cells.

It has been indicated that BEA shows cytotoxicity via promoting extracellular Ca^{2+} influx into the cytosol (254), and a higher concentration of intracellular Ca^{2+} can increase the mitochondrial membrane potential, leading to the releases of cyt c and activates caspase-3 to induce cell apoptosis (249, 255). Moreover, BEA exhibits cytotoxicity via the cellular targets of mitochondria and the homeostasis of potassium ions (256). The balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins plays a crucial role in cancer cell apoptosis. This was indicated by Lin *et al.*, who showed that BEA induced the apoptosis of NSCLC A549 cells via up-regulating Bax, p-Bad and Bak and down-regulating phosphorylation of Bcl2 (253). Consistently, Kim *et al.* found that BEA can induce apoptosis of C6 glioma cells with dose-dependent activation of Caspase-3 and -9 and inhibition of mRNA expression of Bcl-2 (257). Ferrer *et al*. showed that BEA can reduce cell viability by increasing ROS production, leading to oxidative stress and malondialdehyde formation. Increased ROS production

induced by BEA treatment contributes to cell death through lipid peroxidation (258). Furthermore, BEA disrupts cell cycle distribution and checkpoint regulation, leading to cell cycle arrest and the prevention of mitosis (259, 260), which has been indicated that BEA predominantly arrests the cell cycle at the G2/M phase (261, 262). In addition, Kim *et al.* showed that BEA can interrupt actin polymerization and actin-actin interaction in C6 cells and induced cell membrane blebbing and apoptosis. Besides, BEA was found to directly interact with Src via C-terminal amino acids in C6 cells, suppressing the kinase activity of Src and regulating apoptosis through Src/STAT3 pathway. In addition, BEA can inhibit hepatocellular carcinoma (HCC) growth by inducing apoptosis via activating the PI3K/AKT pathway (251). These findings suggest that BEA can be a promising candidate for cancer therapy.

Moreover, BEA also shows anti-cancer effects by inhibiting the migration of metastatic cancer cells and anti-angiogenic activity of tumor cells. Wound healing assays showed that BEA can inhibit the migration of the metastatic prostate cancer (PC-3M) and breast cancer (MDA-MB-231) cells. In vitro, it was found that BEA starts to exhibit potent anti-angiogenic activity in HUVEC-2 cells and can complete disrupt of HUVEC-2 network formation (250). Apart from that, Yahagi *et al.* observed that BEA isolated from *Isaria sp*. inhibited the migration of human pancreatic cancer cell line PANC-1 (263).

Although the anti-cancer effects of BEA are well evaluated in vitro, in vivo effects were less studied. Heilos *et al.* conducted in vivo studies using allograft and xenograft mouse models and observed reduced tumor volumes and weights in BEA-treated mice without adverse effects. BEA was found to accumulate in tumor tissues, and treated mice exhibited increased necrotic areas within the tumor sections, suggesting its potential as a natural compound for anticancer therapy (252).

Overall, while the anti-cancer effects of BEA have shown promise in vitro and in preclinical models, further research is needed to better understand its mechanisms of action, optimize its efficacy, and evaluate its safety profile for potential clinical applications.

4.5.2.2. Anti-virus effects of BEA

Shin *et al*. investigated the anti-viral activity of BEA and other cyclic hexadepsipeptides against human immunodeficiency virus (HIV) integrase in vitro. Among the compounds tested, BEA exhibited the

most potent inhibitory activity on HIV-1 integrase. This suggests that the structural differences, particularly the N-methylation of BEA, may contribute to its higher efficacy as an inhibitor (233). These findings highlight the potential of BEA as a therapeutic agent against HIV.

Furthermore, computational analyses have indicated the potential anti-viral effects of BEA against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19. It was found that BEA can dock to pockets A and B of the main coronavirus protease as well as spike proteins. This suggests that BEA has the potential to interfere with viral replication and may serve as a promising therapy against coronaviruses, including SARS-CoV-2 (264).

It is important to note that these findings are based on in vitro and computational studies, and further research, including in vivo and clinical studies, is necessary to validate the anti-viral effects of BEA and its potential as a therapeutic agent for HIV and coronavirus infections.

4.5.2.3. Antimicrobial activity of BEA

BEA has been reported to show antibacterial characteristics against a variety of Gram-positive and Gram-negative bacteria that colonize or infect humans, animals, and plants (265-269). However, antibacterial mechanism of BEA is distinct from other antibiotics, which target the bacterial cell wall. It was shown that cell organelles or enzyme systems are the targets of BEA in bacteria (265, 270). According to the antibacterial activity of BEA against plant pathogens (266), BEA could be applied to control non-food crop diseases and drug-resistant bacteria (271). In addition to its extensive antibacterial activity, BEA was also reported to have effective antifungal activity. Specially, BEA was shown to exhibit synergistic antifungal activity in combination with ketoconazole or miconazole against *Candida albicans* (272, 273), whereas application of BEA and ketoconazole alone showed little to no effects. The mechanism of antifungal activity of BEA has been well studied (271, 274, 275), and the synergetic effect does not result from their pharmacokinetic interactions but from the blockage of ATP binding cassette (ABC) transporters as well as increased intracellular Ca^{2+} and ROS in *C. albicans* by BEA (271). Besides, BEA also shows higher efficacy against all developmental stages of the ectoparasite *Sarcoptes scabiei in vitro* (276), suggesting that BEA can be a promising candidate for the treatment of *S. scabiei*

infection. Collectively, the properties of anti-bacteria and anti-fungal resistance make BEA a potential strategy against bacterial, fungal, and mite infections.

4.5.2.4. Immune regulating properties of BEA

While the majority of research on BEA has focused on its anti-cancer, anti-microbial, apoptotic, and cytotoxic properties, there are few studies investigating its immuno-regulatory functions. NF-κB is a crucial transcription factor involved in innate and adaptive immune responses, regulating the expression of genes involved in immune and inflammatory processes, including cytokines such as TNF, IL-6, and IFNβ (277, 278). Yoo *et al.* reported that BEA exhibits anti-inflammatory activities in macrophages by regulating NF-κB (232). Specifically, BEA was found to inhibit the production of inducible nitric oxide synthase (iNOS) and IL-1β in LPS-treated RAW264.7 cells. They also demonstrated that BEA suppresses NF-κB activation by targeting Src and Syk downstream of MyD88 signaling. Similarly, strong inhibition of NF-κB signaling by BEA was observed in H4IIE cells (279).

Furthermore, Wu *et al*. showed that BEA possesses anti-inflammatory properties in a mouse model of colitis (280). Their study revealed that BEA attenuated trinitrobenzene sulfonic acid (TNBS)-induced colitis, resulting in reduced weight loss, diarrhea, and mortality, as well as decreased production of TNF and IFNγ in the serum. The anti-inflammatory effects of BEA were attributed to its suppression of T cell activation and proliferation by reducing the production of IL-2, TNF, and IFNγ. The authors also found that BEA targeted the PI3K/AKT signaling pathway to inhibit T cell activation and induce apoptosis in activated T cells. These findings suggest that BEA could be a novel therapeutic approach for the treatment of Crohn's disease (280). Recently, in vitro data by Xu *et al*. reported BEA can disrupt bovine mammary gland homeostasis by modulating expression of genes involved in innate immune function (281). However, this has only been observed in bovine and not in other mammals.

Overall, while the immune regulatory functions of BEA are less studied compared to its other properties, the existing research indicates its potential anti-inflammatory effects through modulation of NF-κB signaling and inhibition of T cell activation and cytokine production. Further investigations are needed

to fully understand the immuno-regulatory mechanisms of BEA and explore its potential therapeutic applications in immune-related diseases.

4.5.2.5. Insecticidal and pesticidal activity of BEA

The insecticidal properties of BEA were first discovered in 1969 when it was shown to have potent insecticidal activity against *Artemia salina*, a species of brine shrimp (230). Subsequent studies demonstrated its insecticidal activity against other insect species such as the blowfly *Calliphora erythrocephala*, the yellow fever mosquito *Aedes aegypti*, the polyphagous pests *Lygus spp*., the fall armyworm *Spodoptera frugiperda*, and the wheat aphid *Schizaphis graminum* (282-285). However, the insecticidal mechanism of BEA has been less investigated.

A study conducted by Khoury *et al.* was the first to demonstrate that BEA enhances insecticidal activity by neutralizing ABC transporters in arthropods (286). The researchers found that the combination treatment of BEA with pesticides effectively inhibited resistant populations of *Tetranychus urticae* compared to single pesticide treatments. Using in silico docking techniques, they also showed that BEA has a higher affinity for ABC transporter subfamilies than pesticides, suggesting its potential as a synergistic insecticidal agent. This could also be the mechanism of the antimicrobial activity of BEA against all developmental stages of *S. scabiei*.

BEA was also found to have significant nematicidal activity. Liu *et al*. reported that culture filtrates of *Beauveria bassiana* exhibited nematicidal activity against the northern root-knot nematode (*Meloidogyne hapla*), resulting in a reduction in nematode population densities, gall formation, and egg mass production (52). In addition, the nematicidal activities of BEA were also observed against the pine wood nematode *Bursaphelenchus xylophilus* and the free-living nematode *Caenorhabditis elegans* (287).

Furthermore, Steiniger *et al*. observed that octa-BEA showed effective antiparasitic activity against *Leishmania donovani* and *Trypanosoma cruzi* (288). However, the specific antiparasitic mechanism of BEA is still poorly understood and requires further investigation.

Overall, BEA exhibits diverse bioactivities, including insecticidal, nematicidal, and antiparasitic properties. The insecticidal mechanism of BEA has been associated with its ability to neutralize ABC

transporters, while the specific mechanisms underlying its nematicidal and antiparasitic activities require further investigation.

4.6. Cathepsin B

CTSB is a member of the lysosome cysteine protease family and plays an important role in intracellular proteolysis. In the following sections, structure, localization, function of CTSB, and CTSB-related disease were introduced.

4.6.1. Structure, localization, and function of CTSB

CTSB is a member of the lysosome cysteine protease family, which includes Cathepsins B, C, H, L, and S (289, 290). It is synthesized as a preproenzyme with 339 amino acids and undergoes post-translational glycosylation. In the Golgi apparatus, mature CTSB, consisting of a heavy chain and a light chain, is formed, with a molecular weight of approximately 38 kDa (291).

Structurally, cysteine proteases have two domains that fold to form a V-shaped active site cleft. CTSB, however, has a peptide occluding loop that overlays its active site, binding the C-terminus of the protein and inducing carboxypeptidase activity (292, 293). Most of the lysosomal cysteine proteases show endopeptidase activity, except for Cathepsin X and C, which show exopeptidase activity. In addition to endopeptidase activity, Cathepsin H also has aminopeptidase activity and CTSB has exopeptidase activity (292). This was confirmed in vivo by Sevenich *et al*., who showed that deficiency of either CTSB or Cathepsin L (also an endopeptidase) did not affect mouse survival. However, double-deficiency of CTSB and Cathepsin L resulted in lethality at four weeks of age, with symptoms of severe hypotrophy and motility defects (294). In a mammary cancer model, Vasiljeva *et al*. observed that loss of CTSB was compensated by up-regulation of Cathepsin X on the cell membrane, which is a carboxypeptidase (295).

The stability of the occluding loop in CTSB is controlled by pH and affects its enzymatic activity. CTSB exhibits exopeptidase activity at acidic pH, while at neutral/alkaline pH, it shows endopeptidase activity. Higher pH can disrupt the salt bridges between the occluding loop, leading to the exposure of the active site (293). As an exopeptidase, CTSB can remove two amino acids as a dipeptide from the C-terminal

site of a polypeptide substrate, and it is thus also referred to as a peptidyldipeptidase. As an endopeptidase, CTSB prefers to hydrolyze amino acids with large hydrophobic side chains in the P2 site, which is the active site for arginine and phenylalanine that is covered by an occluding loop under acidic pH conditions (293). Specific substrates of CTSB include Z-Arg-Arg-methylcoumarylamide and similar dibasic compounds.

CTSB is ubiquitously expressed in many tissues and cells (291). Its localization is mainly in lysosome or endosome compartments, where it is responsible for the degradation of intracellular and extracellular proteins (296). In these compartments, CTSB plays a crucial role in maintaining the balance of lysosome and autophagosome populations, as well as regulating the autophagic flux (297). However, under various stimulation conditions, CTSB can also be secreted into the cytosol due to lysosomal membrane permeabilization. In the cytosol, it functions as a proapoptotic mediator (298) or a cell death inducer (299). De Castro *et al*. demonstrated that the proteolytic activity of CTSB released into the cytosol can induce apoptosis by activating pro-apoptotic Bid and removing anti-apoptotic Bcl-xl. Additionally, CTSB indirectly accumulates Bax in cells by degrading a cysteine protease that can eliminate Bax, leading to cell fate determination towards apoptosis (300). In acute pancreatitis, the release of CTSB into the cytosol causes cell death, and low levels of CTSB activate apoptosis via an intrinsic pathway, while excessive CTSB levels shift the cell death pathway towards necrosis (299).

CTSB plays a critical role in both innate and adaptive immune responses. Inflammasomes are cytosolic multiprotein oligomers of the innate immune system, and among them, the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome has been extensively studied and implicated in various human inflammatory disorders (301). NLRP3 inflammasome activation leads to the activation of pro-caspase-1 and subsequent production of IL-1β and IL-18 (302), which are essential for innate and acquired immune responses (303, 304). CTSB has been shown to be indispensable for NLRP3 inflammasome activation through its interaction with NLRP3 (305). Deletion of CTSB partially or totally suppresses caspase-1 activation and IL-1β production in LPS-primed macrophages stimulated with different inflammasome activators. It has also been indicated that CTSB interacts with NLRP3 at the endoplasmic reticulum (ER) level (306). Additionally, CTSB is critical for the secretion of TNF, a

potent proinflammatory cytokine essential for initiating innate immune responses (307, 308). Inhibition of CTSB leads to the failed transportation of TNF-containing cargo vesicles to the cell membrane (307). Deng *et al*. demonstrated that inhibition of CTSB improves the antitumor immunity of CD8 T cells by suppressing MHC-I degradation. Cycloastragenol, derived from the flowering plant Astragalus membranaceus, used in Chinese traditional medicine, was found to effectively control tumor growth in mouse models, and the treatment promoted MHC-I binding in tumor cells. CTSB was identified as the target protein, and its inhibition suppressed CTSB-mediated MHC-I degradation, leading to improved antigen presentation of tumor cells (309). Furthermore, CTSB in APCs was shown by Gonzalez-Leal *et al*. to regulate mediators of the Th1 immune response during *Leishmania major* infection. CTSB-deficient BMDCs and bone marrow-derived macrophages (BMDMs) displayed increased expression of IL-12 following *Leishmania* infection, suggesting that CTSB-deficient BMDCs exhibit more pro-Th1 properties than their wild-type counterparts (310).

CTSB has also been implicated in the lifecycle of various viruses, including Ebola virus, influenza A virus, Nipah virus, Moloney murine leukemia virus, HIV virus, and feline coronavirus (311-317). In many cases, CTSB is involved in the proteolytic activation of viral membrane glycoproteins, facilitating viral release from endosomes into the cytoplasm and impacting viral entry (311, 312). The catalytic activity of CTSB is also reported to be necessary for optimal replication of Herpes simplex virus type I DNA (318). CTSB can participate in the proteolytic disassembly of the viral capsid of unenveloped reovirus in host endosomes (319). Additionally, CTSB cleaves capsid proteins of adeno-associated virus types 2 and 8 to initiate rapid capsid disassembly in the nucleus (320). Coleman *et al*. demonstrated that CTSB plays a key role in the optimal production of influenza A virus (IAV) by impacting the expression of IAV-PR8 HA protein (313). CTSB exhibits distinct properties compared to other Cathepsins, as it prefers an aromatic P2 residue and has a higher pH optimum (321). Furman *et al*. showed that murine noroviruses utilize CTSB to initiate the intrinsic apoptotic pathway, expanding the window of time for their replication (322). In a recent study by Padmanabhan *et al.*, a mathematical model predicted that CTSB could serve as a target against SARS-CoV-2 in combination with the serine protease TMPRSS2 (323).

These findings highlight the diverse roles of CTSB in various biological processes, including protein degradation, cell death regulation, immune response modulation, and viral lifecycle. Its involvement in these processes makes CTSB an important target for therapeutic interventions in diseases associated with dysregulated proteolysis, immune dysfunction, and viral infections.

4.6.2. CTSB related disease

Under normal physiological conditions, CTSB activity is well-controlled at multiple levels but plays an independent role in various oncogenic and pathological processes. Aberrant expression of the cysteine protease CTSB is observed in many different pathologies and oncogenic processes in humans, most notably Alzheimer's disease (AD) and cancer.

It has been shown that CTSB is highly expressed at the transcriptional and protein levels in several types of cancer, including prostate cancer (324, 325), gastric cancer (326), colon cancer (327), brain cancer (328), breast cancer (329), and lung cancer (330). The high expression of CTSB in these tumors has led to the hypothesis that this enzyme plays a causal role in tumor progression. Additionally, CTSB has been found to hydrolyze the inhibitors of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 & 2), resulting in elevated MMP activity and increased ECM degradation. This promotes the migration of endothelial cells and tumor invasion through the remodeled ECM, indicating its critical roles in tumor angiogenesis and metastasis (331-333). Numerous studies have verified this hypothesis, and Bengsch *et al*. showed that overexpression of CTSB in a mouse model of Polyoma Middle T (PyMT)-induced breast cancer promotes invasion of PyMT cancers by increasing proteolytic extracellular matrix protein degradation and enhancing cell invasion into adjacent tissue (334). Specifically, human CTSB was selectively overexpressed either in cancer cells or in macrophages in 3D co-cultures using a doxycycline (DOX)-inducible CTSB expression system. The increased invasiveness of tumor spheroids was observed in CTSB-overexpressing cancer cells. Consistently, ex vivo studies performed by Sevenich *et al*. indicated that transgenic expression of human CTSB promotes the progression and metastasis of PyMT-induced breast cancer in mice (335). In this study, double-transgenic animals were generated by crossing human CTSB transgenic mice with transgenic PyMT oncogene breast cancer mice, and CTSB overexpression did not alter tumor onset but promoted tumor

growth and increased the numbers of tumor-associated B cells, mast cells, as well as $CD31^+$ endothelial cells in the tumors. Furthermore, Gocheva *et al*. showed that loss of CTSB led to a decrease in tumor initiation, proliferation, angiogenesis, and invasion using a CTSB double transgenic mouse model for pancreatic islet cell carcinogenesis (336).

CTSB is one of the most abundant lysosomal Cathepsin proteases expressed in the human brain compared to other members of the lysosomal cysteine protease family, and it has been reported to be involved in various neurodegenerative diseases (337). It has been shown that CTSB is elevated in the serum of AD patients, which is also correlated with the cognitive status of AD patients (338). Moreover, numerous studies have indicated that up-regulation of CTSB is also observed in the plasma and cerebrospinal fluid (CSF) of patients with clinical traumatic brain injuries (TBI) and related conditions. Elevation of CTSB can lead to behavioral deficits and neuropathology in animal models of AD, TBI, and related brain disorders (339-341). Interestingly, loss of CTSB can improve behavioral deficits and ameliorate neuropathology in animal models of AD, TBI, and related disorders (339, 342-344). Similarly, CTSB inhibitors have also been indicated by numerous studies to improve cognitive dysfunctions and reduce neuropathology in AD, TBI, and TBI-related animal models (345-349). Neurodegeneration resulting from cell death and inflammation leads to deficits in cognition, memory, thinking, executive functions, and related behaviors. Mechanistically, CTSB in the cytosol, as a result of abnormal lysosome leakage, has been indicated to be an integral factor for cell death and inflammation in AD (350, 351), TBI (352, 353), and related brain disorders (354-356). Under normal conditions, CTSB exists in the lysosome; however, in patients with AD, TBI and related brain disorders, infection diseases and cancer, the lysosome membranes lose their integrity, leading to lysosomal leakage and redistribution of CTSB to the cytosol (357-360). CTSB released from disrupted lysosomes in the cytosol can initiate apoptosis by proteolysis of the Bcl-2 family members Bcl-xl, Bax, and Bid, inducing pro-apoptotic Bid and removing anti-apoptotic Bcl-xl. Furthermore, cytosolic CTSB indirectly accumulates Bax in the cells by degrading a cysteine protease that can degrade Bax (300). Moreover, cytosolic CTSB can induce the production of inflammatory IL-1β by activating the neuroinflammasome (361-365). It is known that the NLRP3 inflammasome in microglia is involved in AD and related neurodegenerative

diseases (361, 363), and CTSB can be induced by oligomeric Aβ and oxidative stress associated with AD and neurodegeneration to further mediate NLRP3 inflammasome activation. The activated NLRP3 inflammasome can convert pro-caspase-1 into the active caspase-1, which then induces the formation of mature IL-1β from pro-IL-1β via proteolytic cleavage (362, 366). Thus, CTSB can be a potential drug target for the therapeutic treatment of AD, TBI, and TBI-related brain disorders.

4.7. Aim of this thesis

Together, cancer and bacterial infections are the leading causes of death in the world. Treatments for cancer and infections are getting more and more challenging due to their resistance to currently established therapies. The increase in incidence rates and resistance causes an urgent need to find and develop new drugs especially for the most frequent disease groups of cancer and infections. Immunotherapies have become an alternative and promising therapeutic approach against cancer due to their characteristics of specificity and higher effectiveness, as well as less side effects. So far, DC-based immunotherapy has become a promising tool of cancer immunotherapy due to its activation of immune responses and their role in T-cell activation. NPs exhibit structural diversity and have played an important role in the history of drug development and several well-known NPs have been used in combinations of tumor immunotherapy. Currently, more than half of all newly discovered anticancer drugs and antibacterial agents are derived from NPs. However, only a small fraction of this pool has been evaluated and identified and the understanding of the underlying mechanisms induced by currently identified functional NPs for disease treatment is largely insufficient.

The aim of this study is to identify natural products which show immune activating effects on DCs. Here, the effects of compounds were tested on GM-CSF induced BMDCs derived from IL-12p40/GFP reporter mice in the absence or presence of sub-optimal of LPS or CPG. BMDCs activations were evaluated by the expression of IL-12p40/GFP and CD86. RNA sequencing (RNA-seq) will be performed to compare differential gene expression and to illustrate the underlying mechanism. For the promising NP, T cell activation will be analyzed for evaluating their immunologic properties. The functional targets will be predicted by using silicon prediction tools and confirmed firstly in BMDCs or

human iDCs-based assays. Subsequently, cell enzyme assays will be performed to see if it's a direct mode of interaction. The screening strategy on murine primary DCs and human cells provides more relevance to physiological conditions and simplifies the transition to in vivo experiments later on.

Taken together, this study aims to identify of new, promising, and reliable compounds that aid in the discovery of drugs against resistant cancers and infections.

5. Materials and methods

5.1. Mice

IL-12p40/GFP reporter mice (367) and wild type (WT) mice on a C57BL/6N background and *Tlr 3/7/9*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Myd88*$^{-/-}$*Trif*$^{/-}$ with respective WT controls on a C57BL/6J background were used for GM-CSF culture of bone marrow cells. OT-II transgenic mice on a C57BL/6N background which express an Ovalbumin (OVA)-specific, MHC-II-restricted T cell receptor (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. This chapter is adapted from Yang *et al*. (2022).

5.2. Reagents

5.2.1. Consumables and kits

Consumables and kits that have been used throughout this thesis are listed in Table 1.

Name	Supplier	Catalog number
non-treated 94x16 mm plate	Sarstedt	82.1473
VLE DMEM	Biochrom	P04-04515
FCS	Sigma-Aldrich	F7524
0.1% 2-mercaptoethanol	ThermoFisher Scientific	31350-010
non-treated 24 well plate	Sigma-Aldrich	3738
DPBS (1X), Dulbecco's phosphate buffered saline	Thermo Fisher Scientific	14190-094
2-mercaptoethanol (50 mM)	Thermo Fisher Scientific	31350-010
Erylysis buffer pH 7.2-7.4	Morphisto	12972-00500
LS columns	Miltenyi Biotec	130-042-401
Ovalbumin (323-339)	Sigma-Aldrich	O1641
Penicillin/Streptomycin	Biochrom GmbH	A2212
RPMI Medium 1640	Thermo Fisher Scientific	31870-025

UltraPure 0.5 M EDTA, pH 8.0	Invitrogen	15575-038
BEA	Cayman Chemicals,	11426
CpG	TIB MOLBIOL	CpG2216
LPS (Escherichia coli O127:B8)	Sigma	L3129-100MG
cGAMP	InvivoGen	tlrl-nacga23-5
R848	Alexis Biochemicals	ALX-420-038-M005
Poly I:C	InvivoGen	tlrl-pic-5
Pam3csk4	InvivoGen	tlrl-pms
Polymyxin B (PMB)	InvivoGen	tlrl-pmb
ProcartaPlex Mouse Cytokine & Chemokine Panel 1A 36-plex	Invitrogen by Thermo Fisher Scientific	EPX360-26092-901
magnetic anti-biotin beads	Miltenyi Biotec	130-090-485
Intracellular Fixation & Permeabilization Buffer Set	eBioscience	00-5523-00
CellTrace Violet	Invitrogen	65-0842-90
IL-2	Miltenyi Biotec,	130-12-333
Brefeldin A	Invitrogen	00-4506-51
Macherey-Nagel™ NucleoSpin™ RNA Plus kit	Macherey-Nagel™	740984.250
Qubit RNA HS Assay Kit	Thermo Fisher Scientific	Q32852
Total RNA Standard Sensitivity Assay	Agilent Technologies, Inc. Santa Clara, USA	DNF-471-0500
TLR4/MD2/CD14 HEK 293 cells	InvivoGen	293/mtlr4md2cd14
jetPRIME	Polyplus-transfection Biotechnology	114-15
Dual-Glo Luciferase Assay	Promega	E1980
NucleoSpin™ RNA Plus kit	Macherey-Nagel™	740984.250
SuperScript™ III Reverse Transcriptase	Invitrogen	18080-044
5x MESA Green	Eurogentec	RT-SY2X-06+WOULR
Mouse IL-12 p70 DuoSet ELISA	R&D	DY419
LEGEND MAX™ Mouse IFNβ ELISA Kit	Biolegend	439407

Mouse TNF-alpha DuoSet ELISA	R&D	DY410
Mouse IL-6 DuoSet ELISA	R&D	DY406
Mouse IFN-gamma DuoSet ELISA	R&D	DY485
DMSO	Sigma-Aldrich	D8418
Staurosporine	AdipoGen Life Sciences	AG-CN2-0022
MTT Cell Viability Assay	ThermoFisher	V-13154
Streptavidin	BD Biosciences	551419
BD CompBeads anti- Rat/Hamster	BD Biosciences	51-90-9000949
BD CompBeads negative control	BD Biosciences	51-90-9001291
Anti-biotin MicroBeads	Miltenyi Biotec	130-090-485
rhGM-CSF	ImmunoTools	11343125
rhIL-4	ImmunoTools	11340045
Recombinant human CTSB	R&D	953-CY
Recombinant mouse CTSB	R&D	965-CY
CTSB activity kit	Abcam	ab65300
Z-LR-AMC Fluorogenic Peptide Substrate	R&D	ES008
CA074	MedChemExpress	HY-103350

5.2.2. Antibodies

Antibodies that have been used for flow cytometry and MACS are listed in Table 2.

Name	Clone	Supplier	Catalog
Anti-mouse CD16/32 purified	93	Thermo Fisher Scientific	14-0161-85
Anti-mouse CD4	RM4-4	BD Biosciences	557443
Anti-mouse CD3e	145-2C11	BD Biosciences	551163
Anti-mouse CD3e	145-2C11	Biolegend	100353
Anti-mouse CD3e	145-2C11	BD Biosciences	553064
Anti-mouse CD19	1D3	BD Biosciences	553786
Anti-mouse CD4	RM4-5	Biolegend	100552
Anti-mouse CD4	RM4-5	BD Bioscience	553051
Anti-mouse MHC-II	M5/114.15.2	BD Biosciences	557000
Anti-mouse MHC-II	M5/114.15.2	eBioscience/Thermo	17-5321-81
Anti-mouse CD86	GL-1	BioLegend	105027
Anti-mouse CD86	GL-1	BioLegend	105014
Anti-mouse CD11c	N418	BioLegend	117324
Anti-mouse CD11c	N418	BioLegend	117317
Anti-mouse IFNγ	XMG1.2	BD Bioscience	554412
Anti-mouse IFNγ	XMG1.2	BioLegend	505836
Anti-mouse Foxp3	FJK-16S	eBioscience/Thermo,	12-5773-80
Anti-mouse IL-4	11B11	BioLegend	504119
Anti-mouse IL-17A	17B7	eBioscience/Thermo	51-7177-18
Anti-mouse CD4 Microbeads	L3T4	Miltenyi Biotec	130-117-043
Anti-human HLA-DR-BV605	L243	Biolegend	307640
Anti-human CD83	REA714	Miltenyi Biotec	130-110-561
Anti-human CD86	REA968	Miltenyi Biotec	130-128-567

5.3. Device

Devices that have been used in this thesis are listed in Table 3.

Name	**Supplier**
FACSCanto II cytometer	BD Biosciences
LSRFortessa cytometer	BD Biosciences
FACS Aria III cytometer	BD Biosciences
Bio-Plex 200 Systems	Bio-Rad, USA
NextSeq550 system	Illumina Inc. San Diego, USA
Mithras LB 940 Multimode Plate Reader	BERTHOLD TECHNOLOGIES
Bio-Rad CFX 96	Bio-Rad, USA
Sunrise™ microplate reader	Tecan
Infinite M200	Tecan

5.4. Cell culture

5.4.1. BMDC Culture and Stimulation Conditions

$2x10^6$ bone marrow cells were cultured in non-treated 94x16 mm dishes in 10 ml VLE DMEM containing 10% heat-inactivated FCS, 0.1% 2-mercaptoethanol, and GM-CSF and kept for 9 days. GM-CSF cultures were performed as previously described (368). 10 ml GM-CSF containing medium was added to the plates at day 3. On day 6, 10 ml medium was carefully removed and centrifuged. The cell pellet was resuspended in 10 ml medium and added to the dish. On day 9 BMDCs were used for experiments.

For cytokine expression analyses, BMDCs were seeded ($1x10^6$ cells/well) on a 24-well plate and were stimulated with BEA (purified by the lab of Prof. Rainer Kalscheuer, or commercial ones), CpG, LPS, cGAMP, R848, Poly I:C, or Pam3csk4. After 24 hours, cell culture supernatants were collected for cytokine detection. This chapter is adapted from Yang *et al*. (2022).

5.4.2. iDC Culture and Stimulation Conditions

iDCs were generated according to the protocol (369), namely, 1 x 10^6 THP1 cells were cultured in a T25 flask with 5ml RPMI including 10% heat-inactivated FCS, 0.1% 2-mercaptoethanol, 1% PenStrep, 1500 IU/ml rhGM-CSF and 1500 IU/ml rhIL-4 incubated at 37°C and 5% CO_2 for 5 days. At day 3, exchange the medium with 4 ml fresh medium including 1500 IU/ml rhGM-CSF and 1500 IU/ml rhIL-4. At day 5, cells are evaluated by FACS and subjected to further experiments.

For CTSB activity analyses, 1x10^6 iDCs were seeded on a 24-well plate and were stimulated with indicated concentration of BEA and CA074 (30 μM) for 24 hours. Afterwards, cells were lyzed with lysis buffer, processed for protein concentration measurement by BCA, and ready for CTSB assays.

5.5. Flow Cytometry and Cell Sorting

For cell surface staining, fluorochrome-conjugated monoclonal antibodies against mouse CD3e, CD19, CD4, MHC-II, Streptavidin, CD86 and CD11c were used. For intracellular staining, cells were first stained for surface markers and then fixed and permeabilized using Intracellular Fixation and Permeabilization Buffer Set before incubation with fluorochrome-conjugated mAbs against mouse IFNγ, Foxp3, IL-4, and IL-17A. Flow cytometry was performed on LSRFortessa or FACSCanto II cytometers. The flow cytometry data was analyzed using FlowJo. For RNA-seq experiments, live, single, $CD3^-$, $CD19^-$, $CD11c^+$ and MHC-II^{high} BMDCs were FACS purified using FACS Aria III. This text is adapted from Yang *et al*. (2022).

5.6. Polymyxin B (PMB) neutralization assay

BMDCs were seeded (1x10^6 cells/ml) on a 24-well plate and were stimulated with BEA or LPS with or without 100 μg/ml PMB. After 16 hours of incubation at 37°C, IL-12p40/GFP expression was analyzed by flow cytometry. Alternatively, after 24 hours, cell culture supernatants were collected and the IL-12p70 concentration was determined by ELISA assays. This text is adapted from Yang *et al*. (2022).

5.7. Multiplex immunoassay

Cell culture supernatants were assessed for chemokine and cytokine concentrations. The ProcartaPlex Mouse Cytokine & Chemokine Panel 1A 36-plex was used to measure the concentrations of IFNα, IFNγ, IL-12p70, IL-1β, 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-1α, IL-31, GRO-α (CXCL1), MIP-1α (CCL3), IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MIP-1β (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. This chapter is adapted from Yang *et al*. (2022).

5.8. T cell activation assay

For BMDC/T cell co-culture, BMDCs were treated with 2.5 μM, 5 μM, 7.5 μM BEA for 24 hours and then washed twice with PBS to remove residual BEA before use in subsequent culture. Naive CD4+ T cells were purified from spleens of OT-II mice by MACS according to the manufacturer's protocol. Briefly, OT-II splenocytes were Fc-blocked and incubated with biotinylated anti-CD4 antibodies followed by magnetic anti-biotin beads incubation. Alternatively, OT-II splenocytes were incubated with CD4 Microbeads. Subsequently, $CD4^+$ T cells were positively selected by running cells along a MACS magnet. For T cell proliferation, $CD4^+$ T cells were labeled with CellTrace Violet and afterwards cultured with untreated or BEA treated BMDCs at a 10:1 ratio in 96 well round bottom plates in the presence of 300nM OVA peptide (323-339) for 3 days. To detect impacts of BEA on T cell proliferation, $CD4^+$ T cells were labeled with CellTrace Violet followed by 2.5 μM, 5 μM, and 7.5 μM BEA stimulation in the presence of IL-2 for 3 days. For IFNγ production, $CD4^+$ T cells were cultured with untreated or BEA treated BMDCs at a 10:1 ratio in 96 well round bottom plates in the presence of 300 nM OVA peptide 323-339 for 5 days, cell culture supernatants were collected for IFNγ detection by ELISA. For the detection of intracellular IFNγ, Foxp3, IL-4 and IL-17A, Brefeldin A was added to the cells in the last 6 hours before harvesting of the cells at day 5. This chapter is adapted from Yang *et al*. (2022).

5.9. RNA sequencing and analysis

RNA from sorted BMDCs was extracted using the Macherey-Nagel™ NucleoSpin™ RNA Plus kit and quantified using the Qubit RNA HS Assay Kit. Quality was measured by capillary electrophoresis using the Fragment Analyzer and the 'Total RNA Standard Sensitivity Assay'. 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 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) (|log2FC| >= 1, FDR < 0.05) between non-stimulation and BEA, LPS or BEA with LPS stimulation following the previously described methods (370) 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 (371, 372). 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) (373) and the GSEA Enrichment Map plugin (374). 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. This chapter is adapted from Yang *et al*. (2022).

5.10. Luciferase reporter assay

HEK-293 cells stably expressing TLR4/MD2/CD14 were seeded at 3.5 x 10^4 live cell/well in 96 well plates overnight and were transfected with NF-κB-luciferase reporter plasmid (50 ng/well) and Renilla plasmid (5 ng/well) with transfection reagent jetPRIME for 24 hours. Then cells were stimulated with different concentrations of BEA (2.5 μM, 5 μM and 7.5 μM) or LPS (1 μg/ml) as a positive control. After 24 hours of stimulation, the supernatant was discarded, and cells were washed with PBS. 50 μL of lysis buffer was added and cells were lysed at room temperature for 15 min on a shaker and luciferase activity was measured with the Dual-Glo Luciferase Assay in a Mithras LB 940 multimode microplate reader. This chapter is adapted from Yang *et al.* (2022).

5.11. qRT-PCR

RNA isolation was performed using Macherey-Nagel™ NucleoSpin™ RNA Plus kit. Complementary DNA synthesis was done by using the SuperScript™ III Reverse Transcriptase according to the manufacturer's instructions. Real-time PCR was performed with 5x MESA Green 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'-CAGGCAACCTTTAAGCATCA-3', 5'-CCTTTGACCTTTCAAATGCAG-3' TNF: 5'-TTGAGATCCATGCCGTTG-3', 5'-CTGTAGCCCACGTCGTAGC-3'; IL-6: 5'-CCAGGTAGCTATGGTACTCCAGAA-3', 5'-GCTACCAAACTGGATATAATCAGGA-3'. This chapter is adapted from Yang *et al*. (2022).

5.12. ELISA

Cell culture supernatants were analyzed by ELISA for IL-12p70, IFNβ, TNF, IL-6, and IFNγ. Plates were read using a Tecan Sunrise microplate reader at 450 nm, and the background was subtracted at 570 nm. All experiments were performed according to the manufacturers' instructions. This chapter is adapted from Yang *et al*. (2022).

5.13. MTT assays

BMDCs were seeded at a density of $8x10^4$ cells/well in non-treated 96-well plates and then were stimulated with 2.5, 5 or 7.5 µM of BEA or with DMSO or staurosporine as controls for a final volume of 200 µl. After 16 hours of incubation at 37°C microplates were centrifuged to pellet the cells, medium was replaced with 100 µL of fresh medium and 10 µL of the 12 mM MTT stock solution was added to each well. Following an incubation for 4 hours at 37°C 100 µL of SDS-HCl solution was added to each well and mixed thoroughly followed by an additional incubation of 4 hours. After mixing each sample again absorption was measured at a wave length of 570 nm using a microplate reader (Tecan). This chapter is adapted from Yang *et al*. (2022).

5.14. Cell based CTSB activity

$1x10^6$ BMDCs from C57BL/6N mice or iDCs were stimulated with the indicated concentrations of BEA or CA074 for 16 hours in 12-well plates. After stimulation, the cells were washed with cold PBS and lysed with 50 µl of chilled cell lysis buffer on ice for 10-30 minutes. The supernatant of the cell lysates was then collected after centrifugation, followed by protein quantification by BCA. Equal amounts of protein were added to each well, followed by 50 µL of CB reaction buffer containing 100 µM CB substrate Ac-RR-AFC. The reaction was incubated at 37°C protected from light for 1 hour, and the output was measured on a fluorescence microplate reader at Ex/Em = 376/482 nm.

5.15. Cell free CTSB activity

Mouse or human recombinant CTSB was diluted to 10 µg/mL in activation buffer containing 25 mM MES, 5 mM DTT, pH 5.0 and incubated for 15 minutes at room temperature. The activated CTSB was then incubated with or without the indicated concentrations of BEA or CA074 in a black 96-well plate. After addition of the substrate, relative fluorescence intensity (RFU) was measured using a fluorescence microplate reader at excitation and emission wavelengths of 380 nm and 460 nm (top read), respectively, in kinetic mode for 1-2 hours.

5.16. Statistics analysis

GraphPad Prism 9.0 software was used for data analysis. Data are represented as mean ± SEM. For analyzing statistical significance between multiple groups, a one-way ANOVA with Dunnett's multiple comparisons test was used. For analyzing statistical significance for comparisons of more than two groups with two or more stimulations, two-way ANOVA with Sidak's multiple comparisons test was used, all p values < 0.05 were considered as statistically significant. This paragraph is adapted from Yang *et al*. (2022).

6. Results

6.1. Screening of natural products for DC activation

To identify functional NPs that play immune regulatory roles in BMDCs, bone marrow cells isolated from IL-12p40-GFP reporter mice were used for BMDC culture. We first determined the sub-optimal doses for LPS and CpG to activate BMDCs for the induction of IL-12p40. As shown in Figure 5A and 5B, BMDCs started to produce IL-12p40-GFP at 1.0 ng/ml of LPS and 0.5μM CpG stimulation and its production was concentration dependent.

To identify the promising NPs with immune modulating properties, screening assays were performed (as shown in Figure 5C). Briefly, IL-12p40-GFP BMDCs were stimulated either with NPs alone or with stimulations of 1ng/ml LPS or 0.5μM CpG. BMDC activation was assessed by the expression of IL-12p40-GFP by flow cytometry. As shown in Figure 5D, the expression of IL-12p40-GFP was markedly increased in P05E07-treated BMDCs compared with untreated control, or LPS, or CPG alone treated BMDCs. Taken together, P05E07 would be a functional candidate for DC activation.

P05E07 is a mycotoxin purified from *Fusarium* spp., and it is named BEA J (as shown in Figure 4). The biological and immunological roles of BEA J has not been investigated yet. However, its derivative BEA (as shown in Figure 4) has been well studied. BEA has been reported to exhibit anti-inflammatory effects in macrophages and TNBS-induced experimental colitis (232, 280). In addition, BEA has been shown to directly suppress T cell activation and proliferation in TNBS-induced experimental colitis (280). BEA showed anticancer activity by inducing apoptosis of small lung cancer cells (257) and potent antiviral activities against HIV integrase (233). However, the effects of BEA on DCs are still unclear.

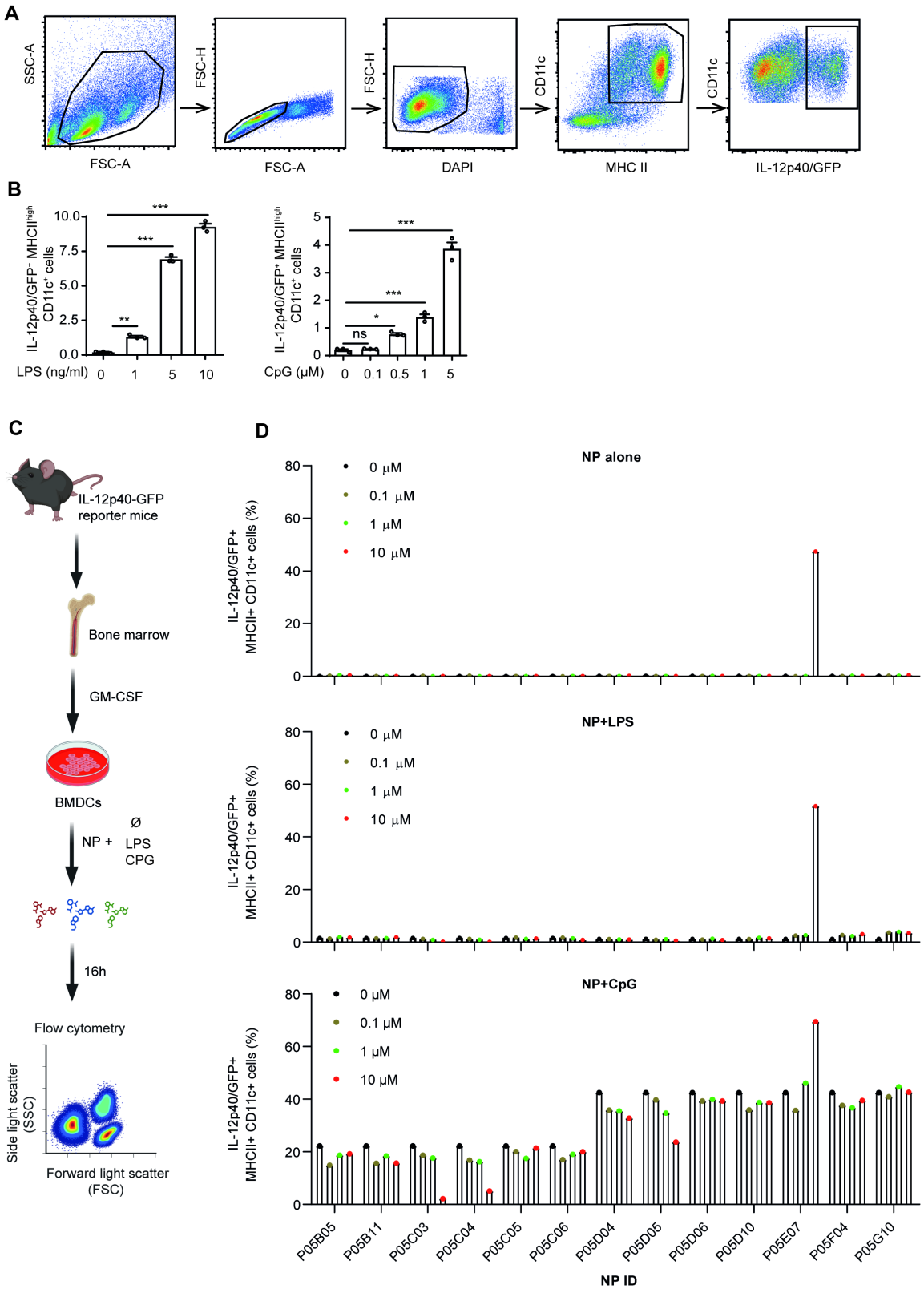

Figure 5. Screening of NPs for BMDC activation.

5x10^5 BMDCs from IL-12p40/GFP reporter mice were stimulated with indicated concentration of NPs with or without LPS (1 ng/ml) and CpG2216 (0.5 μM) for 16 hours. **(A)** The gating strategy is shown. **(B)** IL-12p40/GFP by BMDCs was detected by flow cytometry. Analysis of IL-12p40/GFP were gated on MHC-II^{high} CD11c$^+$ cells.

Results shown are representative of two independent experiments with cells from three mice per group (n=3). Data are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ns, not significant. **(C)** The NP screening workflow was created with BioRender.com. **(D)** 10^6 BMDCs were stimulated with different doses of NPs with or without LPS or CpG for the 16 hours. IL-12p40/GFP$^+$ were analyzed by flow cytometry.

6.2. Comparison of BEA and BEA J on BMDC activation

BEA J has the same structures as BEA except for the hydroxyl group. The similar structures prompt us to investigate whether they have a similar immune-activating role on BMDCs. The same different concentrations of BEA or BEA J were applied to BMDCs in the presence or absence of LPS or CPG stimulation. Flow cytometry analysis indicates that the expression of IL-12p40/GFP in BEA-treated BMDCs was slightly higher than that in BEA J-treated BMDCs, as shown in Figure 6A and 6B. Furthermore, both BEA and BEA J induced BMDC activation are in a dose dependent manner. Overall, both BEA and BEA J can activate BMDCs to induce IL-12p40 production and BEA has a slightly higher capacity. Therefore, we decided to use commercial BEA in the following experiments.

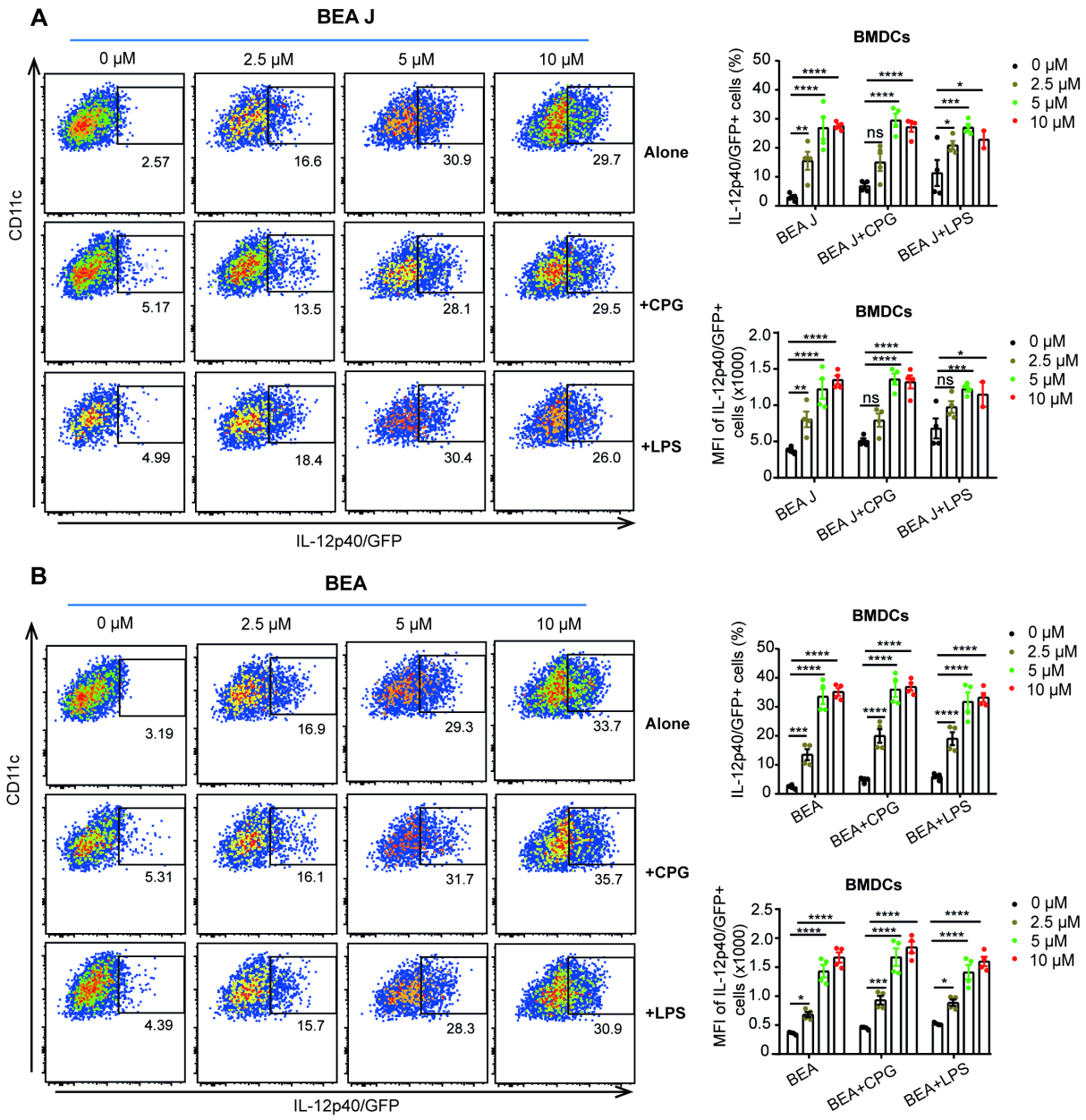

Figure 6. Comparable effects of BEA and BEA J on BMDC activation.

(A) 5x10^5 BMDCs from IL-12p40/GFP reporter mice were stimulated with the indicated concentration of BEA-J with or without LPS (1 ng/ml) and CpG2216 (0.5 mM) for 16 hours. IL-12p40/GFP expression by BMDCs was detected by flow cytometry. Analysis of IL-12p40/GFP was gated on MHC-II^{high} CD11c$^+$ cells. **(B)** 5x10^5 BMDCs from IL-12p40/GFP reporter mice were stimulated with the indicated concentration of BEA with or without LPS (1 ng/ml) and CpG2216 (0.5 mM) for 16 hours. IL-12p40/GFP expression by BMDCs was detected by flow cytometry. Analysis of IL-12p40/GFP was gated on MHC-II^{high} CD11c$^+$ cells. Results shown are representative of one independent experiment with cells from 4 mice per group (n=4). Data in B was presented as mean ± SEM and analyzed by two-way ANOVA with Dunnett's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, ns, not significant.

6.3. The expression of inflammatory cytokines and co-stimulatory ligand in BEA-stimulated BMDCs

To further confirm whether BEA can work as BMDC activator, similar experiments were performed as described above and the costimulatory molecule CD86 and IL-12p40 were analyzed. Consistently, BEA alone can potently activate BMDCs resulting in increased IL-12p40 and CD86 expression (Figure 7A, B). As expected, BEA treatment can also enhance the activation of LPS- or CpG-stimulated BMDCs, leading to further increased production of IL-12p40 and CD86 expression on BMDCs (Figure 7A, B). In addition, Real-time PCR shows that significant increased production of the inflammatory cytokines IL-12p40, IFNβ, TNF, and IL-6 can be observed in BEA-stimulated BMDCs at 6 hours post stimulation (Figure 7C). Taken together, BEA can induce the productions of IL-12 and other pro-inflammatory cytokines as well as CD86 expression in BMDCs, suggesting that BEA might be a potent BMDC activator. This paragraph is adapted from Yang *et al*. (2022).

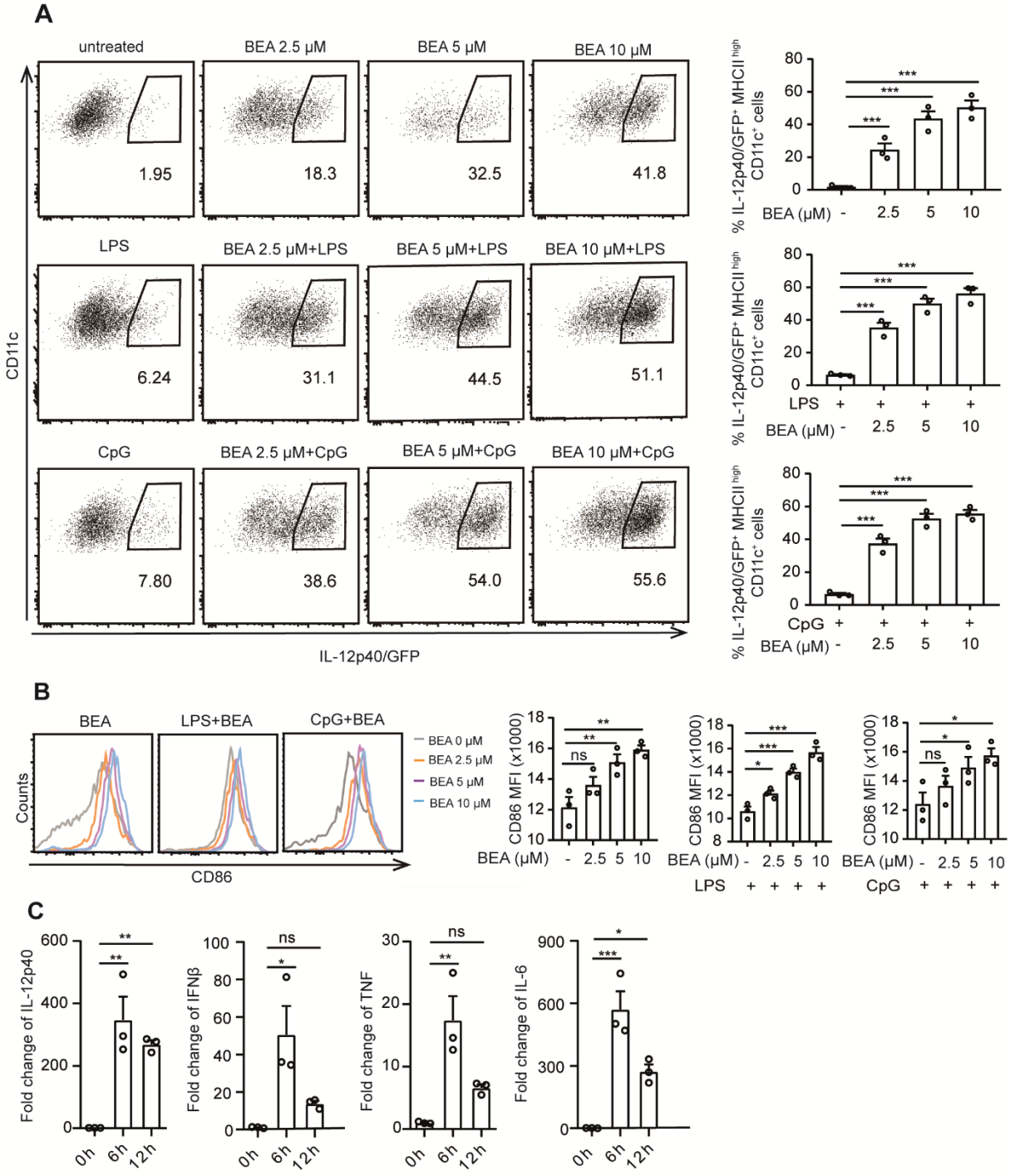

Figure 7. Effects of BEA on BMDC activation. This figure is adapted from Yang *et al.* (2022).

(A, B) 5x10^5 BMDCs from IL-12p40/GFP reporter mice were stimulated with indicated concentration of BEA with or without LPS (1 ng/ml) and CpG2216 (0.5 μM) for 16 hours. IL-12p40/GFP (**A**) and MFI of CD86 expression (**B**) by BMDCs were detected by flow cytometry. Analysis of IL-12p40/GFP and CD86 expression was gated on MHC-II^{high} CD11c$^+$ cells. Results shown are representative of three independent experiments using cells from three mice per group (n=3). **(C)** 10^6 BMDCs were stimulated with 5 mM BEA for the indicated time. IL-12p40, IFNβ, TNF and IL-6 were analyzed by Real time PCR. Results shown are representative of two

independent experiments using cells from three mice per group (n=3). Data in A, B and C are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ns, not significant.

6.4. Effects of BEA on DC-mediated CD4$^+$ T cell proliferation

We next sought to determine whether BEA could enhance the ability of BMDCs to induce T cell proliferation. BMDCs were cultured in the presence or absence of various concentrations of BEA for 24 hours and then the cells were washed thoroughly as previous studies have shown that BEA significantly inhibits T cell proliferation in TNBS-induced experimental colitis (280). Afterwards, the untreated and treated BMDCs were co-cultured with OT-II TCR transgenic naive CD4$^+$ T cells for 3 days. T cell co-culture with BEA treated BMDCs led to increased numbers of T cell divisions (Figure 8A). Furthermore, we also analyzed intracellular IFNγ production of CD4$^+$ T cells. While untreated and BEA treated BMDCs showed no significant changes in percentages of IFNγ producing CD4$^+$ T cells (Figure 8B), significantly higher IFNγ levels were detected in supernatants of T cells that were co-cultured with BEA activated BMDCs for 5 days than with untreated BMDCs (Figure 8C). Taken together, this suggests that BEA can enhance the ability of BMDCs to induce T cell proliferation. This paragraph is adapted from Yang *et al*. (2022).

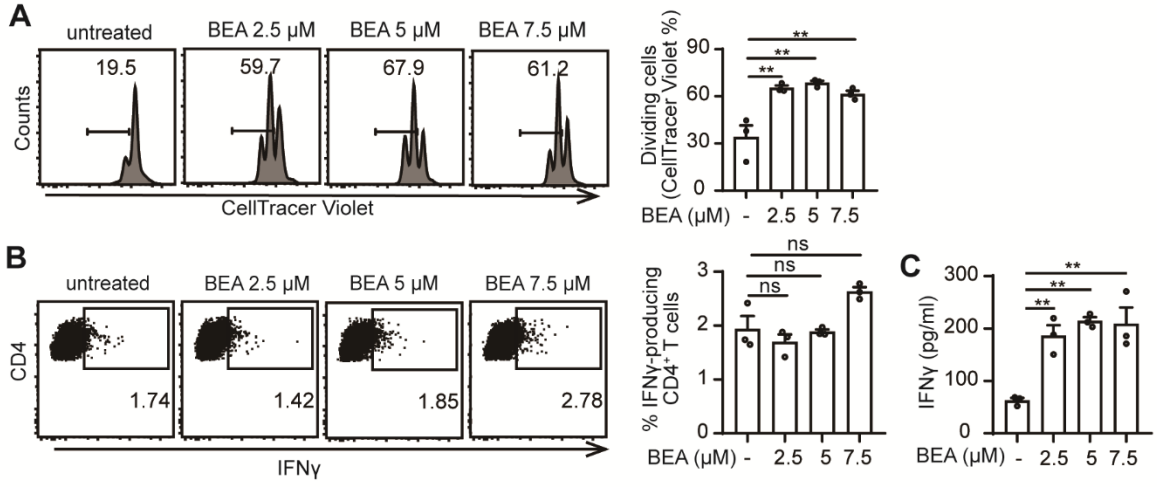

Figure 8. Effects of BEA on BMDC-mediated T cell proliferation. This figure is adapted from Yang *et al.* (2022).

10^5 naïve CD4$^+$ T cells derived from OT-II TCR transgenic mice were labelled with CellTrace Violet and cultured with 10^4 untreated or BEA-treated BMDCs in the presence of 300nM OVA peptide 323-339 for 3 days. **(A)** T cell

proliferation was analyzed by flow cytometry based on CellTrace Violet dilution. Results shown are representative of three independent experiments using cells from three mice per group (n=3). **(B, C)** 10^5 naïve OT-II $CD4^+$ T cells were cultured with 10^4 untreated or BEA-treated BMDCs in the presence of 300nM OVA peptide 323-339 for 5 days. **(B**) Percentage of IFNγ production by $CD4^+$ T cells was measured by intracellular staining. Results shown are representative of three independent experiments using cells from three mice per group (n=3). **(C)** IFNγ production in the supernatant was detected by ELISA. Results shown are representative of three independent experiments using cells from three mice per group (n=3). Data in A, B and C are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. **p<0.01, ns, not significant.

6.5. Effects of BEA on T cell differentiation and cytokine production

Furthermore, we also asked whether the effects we saw in the co-culture systems would be caused by the direct effects on CD4 T cells. To exclude this possibility, we cultured purified CD4 T cells in the presence of IL-2 and stimulated them with different doses of BEA. Flow cytometry data indicates that BEA even has a slight inhibitory role on T cell expansion (Figure 9A, B). Therefore, we could conclude that the enhanced T cell proliferation effects were not a T cell intrinsic effects by BEA.

IFNγ was undetectable in the supernatants when BMDCs were treated with BEA in the absence of T cells (Figure 9C, D). Thus, while BEA activated BMDCs do not by themselves exhibit a strong impact on Th1 cell differentiation under these culture conditions, the significantly increased amounts of IFNγ in the supernatant of T cells co-cultured with BEA treated BMDCs likely reflect the summary effect of a slightly increased IFNγ expression in a higher number of T cells due to the increased proliferation as compared to the co-culture with untreated BMDCs. Similarly, no relevant effect of BEA treatment was observed for the capacity of BMDCs to drive Treg, Th2, or Th17 differentiation (Figures 9E, F). Taken together, these data suggest that BEA can enhance the ability of DC induced T cell proliferation, while having a lesser impact on differentiation or induction of cytokine production in individual cells. This paragraph is adapted from Yang *et al*. (2022).

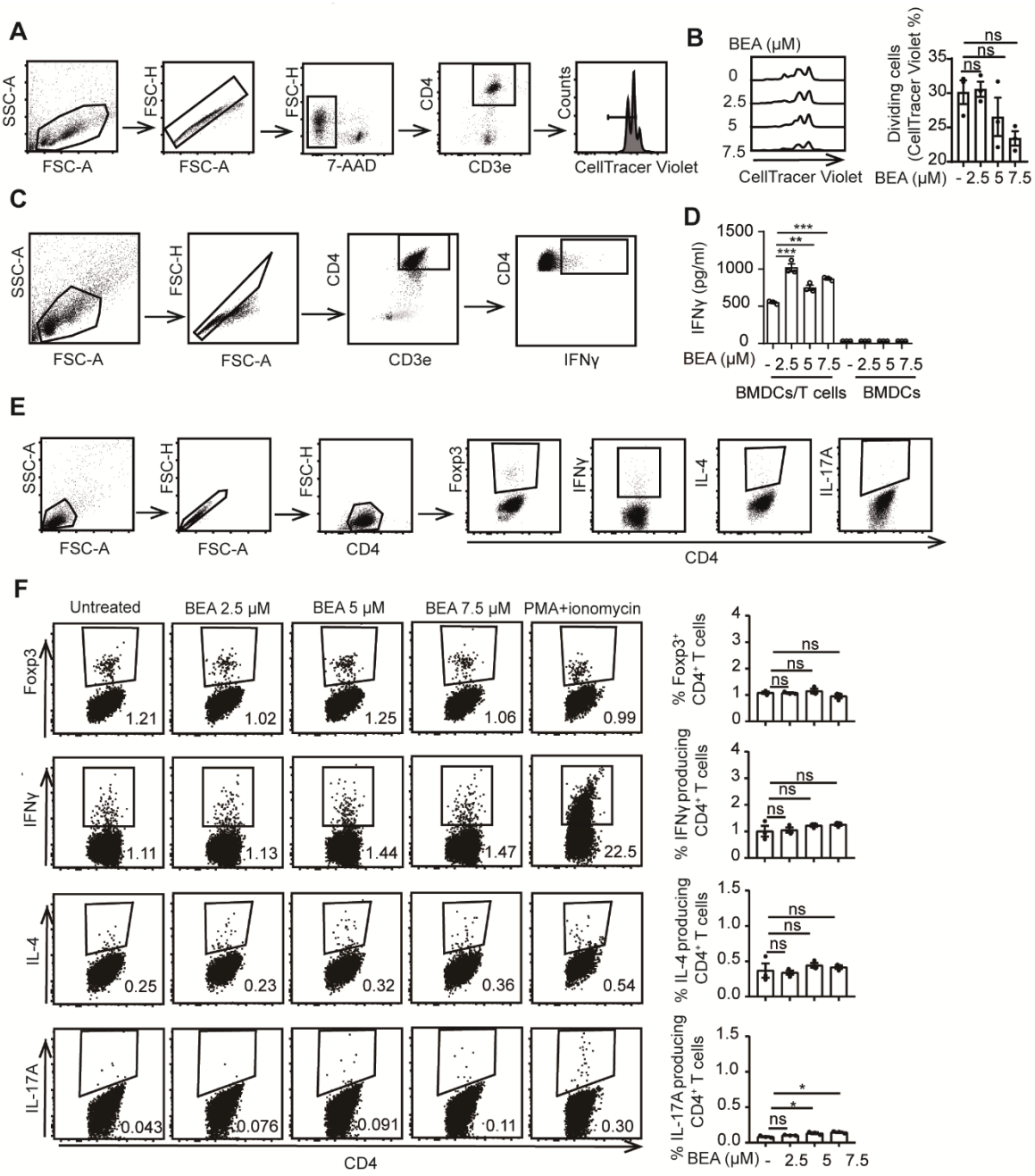

Figure 9. Effects of BEA-treated BMDCs or BEA on T cell proliferation and differentiation. This figure is adapted from Yang *et al.* (2022).

(A) Representative gating strategy for T cell proliferation. Arrows indicate that $CD4^+$ T cells were sequentially gated from single cells and live cells ($7\text{-}AAD^-$). Gated $CD4^+$ $CD3e^+$ T cells were analyzed for T cell proliferation. **(B)** Direct impact of BEA on T cell proliferation. 10^5 naïve OT-II $CD4^+$ T cells labelled with CellTracer Violet were stimulated with or without indicated concentration of BEA in the presence of IL-2. T cell proliferation was detected at day 3 by flow cytometry. Data shown are from one experiment using cells from three mice per group (n=3). Data are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test, ns, not significant. **(C)** Representative gating strategy for T cell IFNγ production. Arrows indicate that $CD4^+$ T cells were gated from single cells. Gated $CD4^+$ $CD3e^+$ T cells were analyzed for IFNγ production. **(D)** IFNγ

levels in the supernatants of BMDCs co-cultured T cells or BMDCs. 105 naïve OT-II $CD4^+$ T cells were cultured with 10^4 untreated or BEA-treated BMDCs for 5 days in the presence of OVA peptide. IFNγ production in the supernatant was detected by ELISA. Results shown are from one experiment using cells from three mice per group (n=3). Data are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. **p<0.01, ***p<0.001. **(E)** Representative gating strategy for combined analysis of T helper cell differentiation associated transcription factors and cytokines. Arrows indicate that $CD4^+$ T cells were gated from single cells. Gated $CD4^+$ T cells were analyzed for Foxp3 expression or production of IFNγ, IL-4, and IL-17A. **(F)** Expression of Foxp3, IFNγ, IL-4 and IL-17A in T cells co-cultured with untreated or BEA treated BMDCs. 10^5 naïve OT-II $CD4^+$ T cells were cultured with 10^4 untreated or BEA-treated BMDCs in the presence of OVA peptide. Expression of Foxp3, IFNγ, IL-4 and IL-17A were detected by flow cytometry. Data shown are from one experiment using cells from three mice per group (n=3). Data are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, ns, not significant.

6.6. Exclusion of LPS contamination with BEA

The purity of BEA isolated from *Fusarium* spp. by the Institute of Pharmaceutical Biology and Biotechnology (Prof. Rainer Kalscheuer) and BEA purchased from Cayman Chemicals was above 95% as defined by HPLC-UV. To further confirm this effect was not a result of endotoxin contamination, BMDCs derived from IL-12p40/GFP reporter mice were stimulated by indicated concentrations of BEA or LPS with or without PMB, which blocks the biological effects of LPS through binding to lipid A (375, 376). After 16 hours of stimulation, IL-12p40/GFP expression by BMDCs was analysed by flow cytometry. PMB effectively blocked the LPS mediated activation of BMDCs resulting in undetectable IL-12p40 (Figures 10A, B) and IL-12p70 levels (Figure 10C). However, amounts of IL-12p40 and IL-12p70 were comparable after BEA stimulation with or without additional PMB treatment (Figures 10A–C). This result demonstrated that the production of IL-12 by BEA treated BMDCs is unlikely to result from any contamination of BEA with LPS. This chapter is adapted from Yang *et al*. (2022).

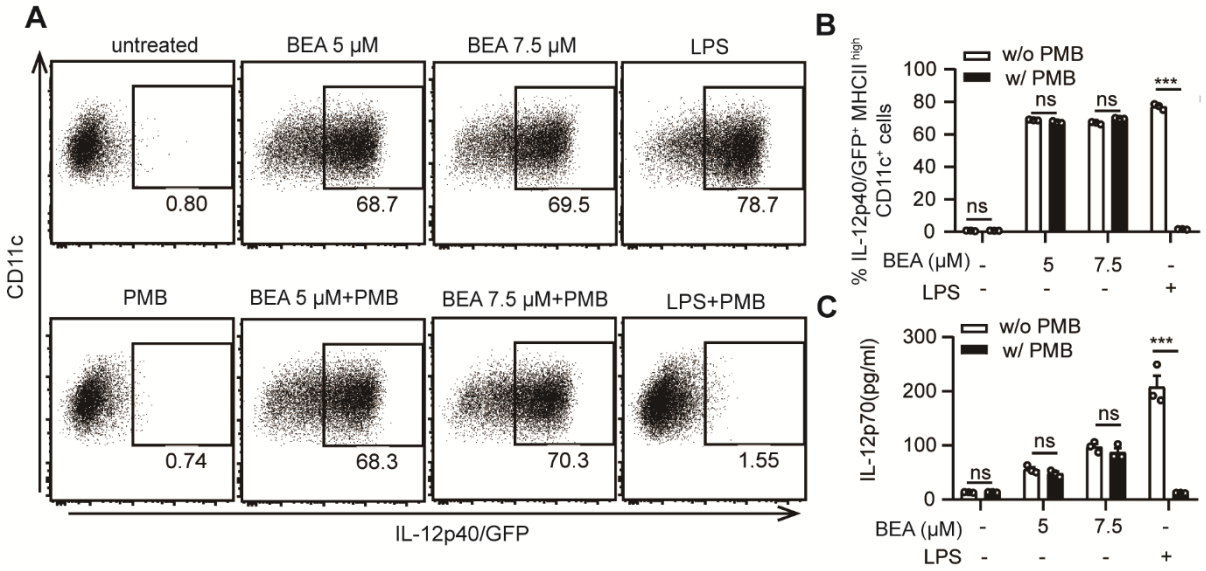

Figure 10. Exclusion of BEA contamination with LPS. This figure is adapted from Yang *et al.* (2022). **(A, B)** 5x10^5 BMDCs derived from IL-12p40/GFP reporter mice were stimulated with 5 µM, 7.5 µM BEA or LPS (10 ng/ml) with or without PMB (100 ng/ml). After 16 hours of stimulation, IL-12p40/GFP expression of BMDCs was analyzed by flow cytometry. Results shown are representative of two independent experiments using cells from three mice per group (n=3). **(C)** 10^6 BMDCs derived from IL-12p40/GFP reporter mice were stimulated by 5 µM, 7.5 µM BEA or LPS (1 mg/ml) with or without PMB (100 ng/ml). After 24 hours of stimulation, the supernatants were analyzed for IL-12p70 by ELISA. Results shown are representative of two independent experiments using cells from three mice per group (n=3). Data in B and C are presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's multiple comparisons test. ***p<0.001, ns, not significant.

6.7. Impacts of BEA on the MyD88 and TRIF signaling pathways

MyD88 and TRIF are critical adaptors for TLR induced production of pro-inflammatory cytokines such as IL-12, TNF, IL-6 and IFNβ by DCs (121, 377). Therefore, we aimed to investigate whether IL-12 and IFNβ production by BEA stimulation are MyD88 or TRIF-dependent. To this end, BMDCs were generated from *Myd88*$^{-/-}$ and *Myd88*$^{-/-}$ *Trif*$^{-/-}$ mice and stimulated with BEA, LPS or cGAMP, the latter serving as a positive, MyD88/TRIF-independent stimulation control. After 24 hours stimulation, cell supernatant was collected to assess IL-12 and IFNβ production by ELISA. In this experiment, cGAMP can induce IFNβ production in WT and *Myd88*$^{-/-}$ or *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs. As expected, neither *Myd88*$^{-/-}$ or *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs released detectable amounts of IL-12 upon LPS stimulation. Production of IFNβ was significantly diminished but still detectable in *Myd88*$^{-/-}$ BMDCs, whereas it was undetectable in *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs. Similarly, BEA did not induce IL-12p70 production in either

Myd88$^{-/-}$ BMDCs or *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs while IFNβ production was significantly decreased in *Myd88*$^{-/-}$ BMDCs and undetectable in *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs (Figure 11A). Furthermore, we determined production of other cytokines and chemokines by multiplex immunoassay. Production of the inflammatory cytokines TNF, IL-6, IL-1ß, IL-18, IL-27, and IL-10 (Figure 11B) and the chemokines GRO-α, MCP-3, ENA-78, MIP-1β and RANTES (Figure 11C) was significantly decreased in BEA stimulated *Myd88*-/- BMDCs and even lower in BEA simulated *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs. However, production of IP-10 induced by LPS in *Myd88*$^{-/-}$ BMDCs was similar to WT BMDCs but markedly decreased in LPS stimulated *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs. Such findings are consistent with studies reporting that expression of IP-10 by LPS stimulated BMDMs is mediated through a TRIF-dependent but MyD88-independent pathway (378). Interestingly, similar results were observed in BEA stimulated *Myd88*$^{-/-}$ BMDCs and *Myd88*$^{-/-}$ *Trif*$^{-/-}$ BMDCs. Thus, the effects of BEA on BMDCs cytokine and chemokine expression profiles are mediated via the activation of MyD88 and TRIF signaling pathways as similarly demonstrated after LPS-stimulation. This chapter is adapted from Yang *et al*. (2022).

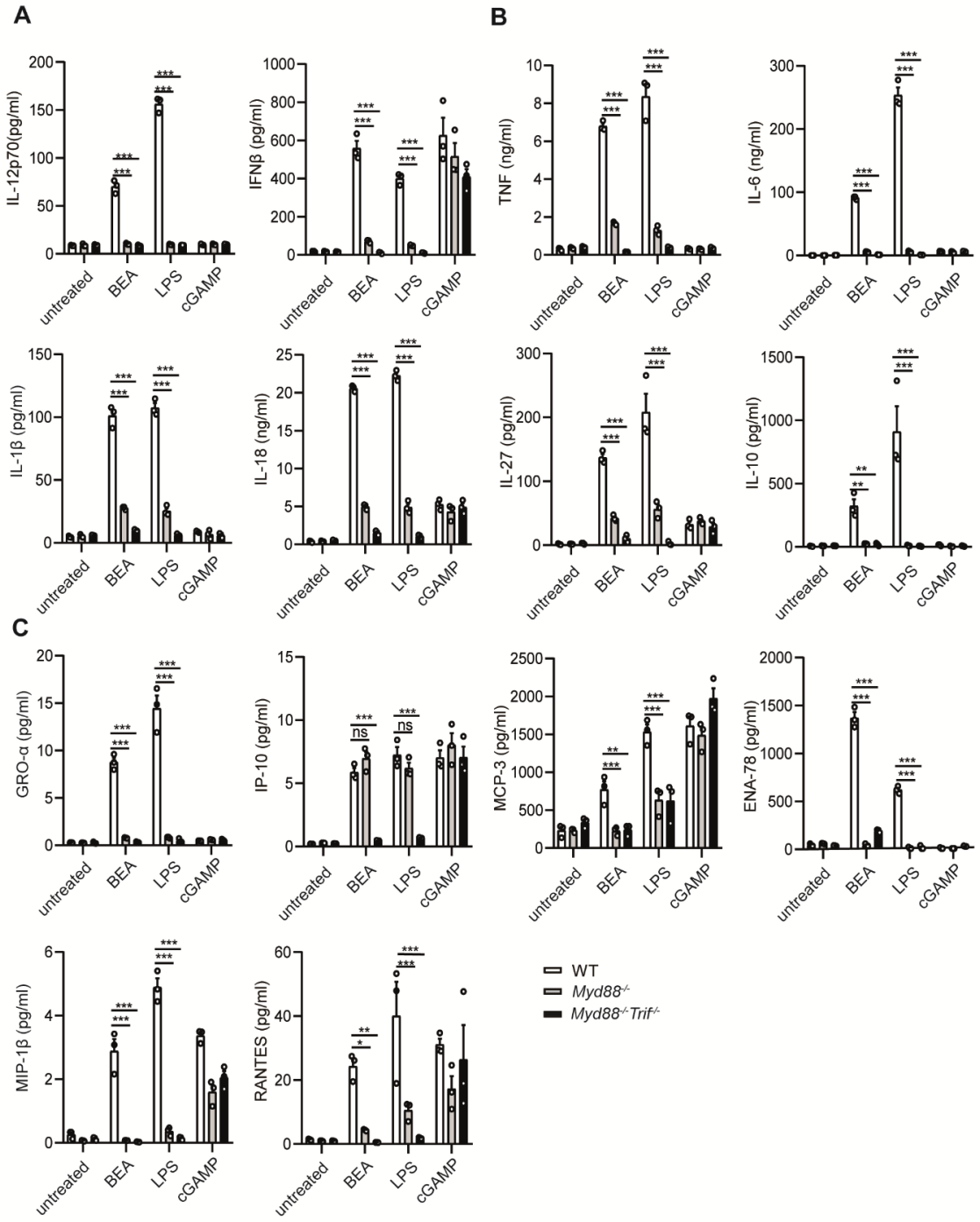

Figure 11. BEA promotes BMDC activation in a MyD88/TRIF dependent manner. This figure is adapted from Yang *et al.* (2022).

10^6 BMDCs from WT, *Myd88*$^{-/-}$ and *Myd88*$^{-/-}$ *Trif*$^{-/-}$ mice were stimulated with 5μM BEA, LPS (10 ng/ml) and cGAMP (10 ng/ml) as control. **(A)** After 24 hours of stimulation, supernatants were analyzed for IL-12p70 and IFNβ production by ELISA. **(B, C)** Production of inflammatory cytokines (TNF, IL-6, IL-1β, IL-27, IL-10) **(B)** and chemokines (GRO-alpha, IP-10, MCP-3, ENA-78, MIP-1a and RANTES) **(C)** by Multiplex immunoassays. Results shown are representative of two independent experiments using cells from three mice per group (n=3). Data in A, B and C are presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ns, not significant.

6.8. Impacts of BEA on TLR4 signaling pathways

It has been shown that the TLR4 signaling pathway not only depends on the presence of the MyD88 signal adaptor protein but also the TRIF signal adaptor protein (377). As we observed that both, MyD88 and TRIF are involved in BEA induced BMDC cytokine production, we next sought to determine whether BEA activates BMDCs in a TLR4 dependent manner. To this end, we stimulated WT and Tlr4-deficient BMDCs with BEA in the presence or absence of CpG or LPS for 24 hours. Measurement of IL-12p70 and IFNβ in the supernatant by ELISA showed that LPS and BEA did not induce IL-12p70 and IFNβ production in *Tlr4*-deficient BMDCs (Figures 12A, B). In contrast, CpG induced similar amounts of IL-12p70 and IFNβ in both WT and *Tlr4*-deficient BMDCs while BEA co-treatment with CpG failed to induce more cytokine production as compared to *Tlr4*-deficient BMDCs stimulated with CpG alone, suggesting these effects of BEA on BMDCs are TLR4 signaling dependent.

Furthermore, to investigate whether BEA can activate other TLR signaling pathways, WT and BMDCs with a triple deficiency of TLR3, 7 and 9 were stimulated by BEA with or without CpG (TLR9), R848 (TLR7) or Poly I:C (TLR3). Consistent with current knowledge, CpG and R848 can significantly induce IL-12p70 and IFNβ production in WT, but not in *Tlr3/7/9*-deficient BMDCs. In addition, we found that Poly I:C did not induce IL-12p70 production, which is consistent with previous reports (121). Moreover, we found a decreased LPS induced IL-12p70 production in *Tlr3/7/9*$^{-/-}$ cells as compared to WT cells, that can be explained by the convergent downstream signaling pathways shared by TLR4 and TLR3/7/9 which might be impacted by the triple deficiency in TLR3, 7, and 9 (379). Similarly, BEA induced slightly less amounts of IL-12p70 but comparable amounts of IFNβ in *Tlr3/7/9*-deficient BMDCs (Figures 12C, D). To further determine if BEA could directly activate TLR4-mediated signaling, we stimulated HEK-293 cells stably expressing mTLR4/CD14/MD2 and transiently expressing the NF-κB luciferase reporter and Renilla gene with various concentrations of BEA or LPS as a positive control and measured NF-κB activation. LPS treatment significantly induced NF-κB activation, which was similarly observed after BEA treatment (Figure 12E). Taken together, our data indicate that BEA activates BMDCs via a TLR4-dependent signaling pathway. This chapter is adapted from Yang *et al*. (2022).

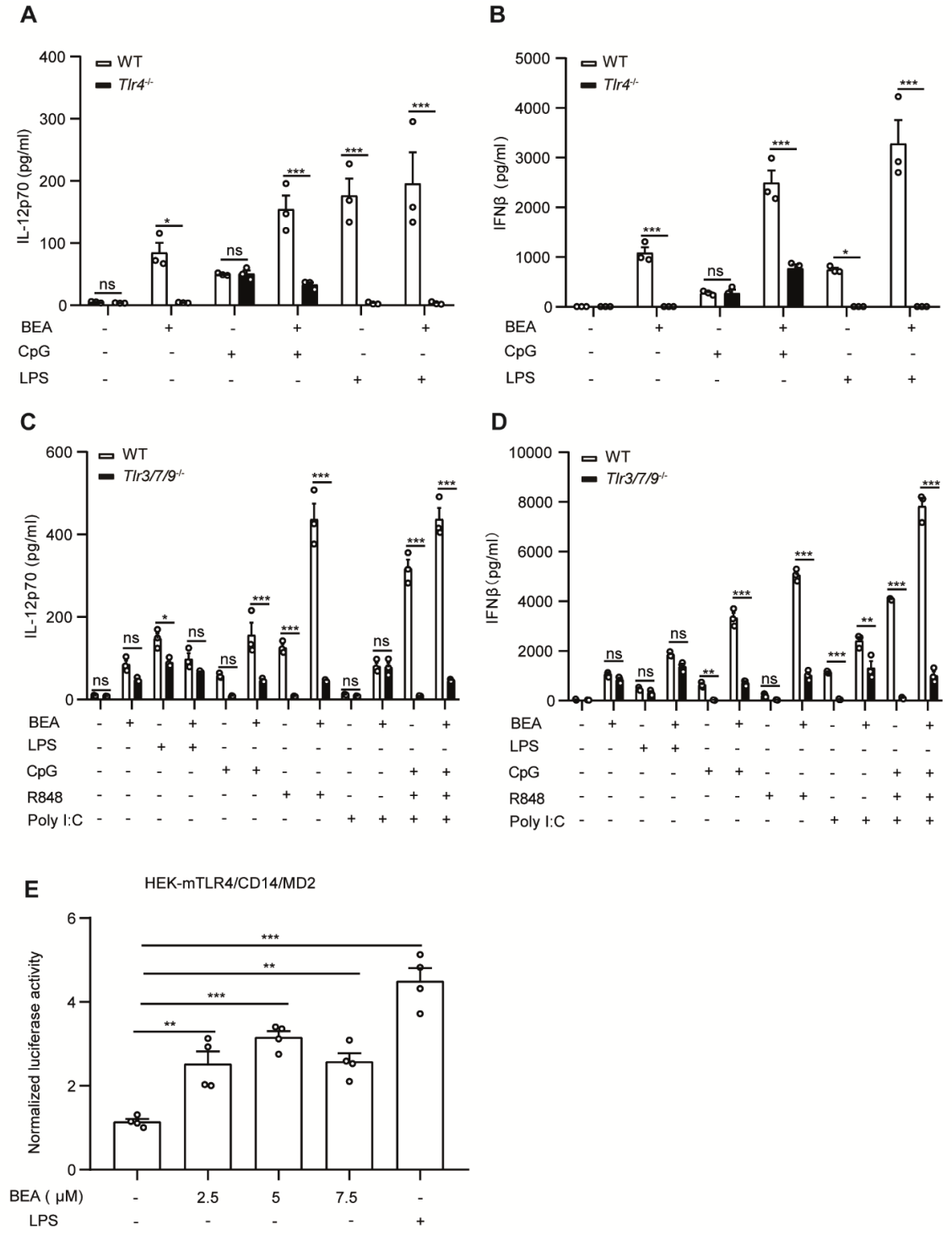

Figure 12. BEA activates BMDCs via TLR4. This figure is adapted from Yang *et al*. (2022).

(A, B) 10^6 BMDCs derived from WT and *Tlr4*$^{-/-}$ mice were stimulated with 5 μM BEA with or without LPS (10 ng/ml) and CpG (0.5 mM). After 24 hours of stimulation, supernatants were analyzed for IL-12p70 and IFNβ by ELISA. Results shown are representative of two independent experiments using cells from three mice per group (n=3). **(C, D)** 1x10^6 BMDCs derived from WT and *Tlr3/7/9*$^{-/-}$ mice were stimulated with 5 μM BEA with or without LPS (10 ng/ml), CpG (0.5 mM), R848 (1 mg/ml), or Poly I:C (25 ng/ml). After 24 hours of stimulation,

supernatants were analyzed for IL-12p70 and IFNβ by ELISA. Results shown are representative of two independent experiments using cells from three mice per group (n=3). Data in A, B, C and D are presented as mean ± SEM and analyzed by two-way ANOVA with Sidak's multiple comparisons test. **(E)** $3.5x10^4$ HEK-293 cells stably expressing mTLR4/CD14/MD2 were transiently transfected with firefly luciferase NF-κB reporter and Renilla plasmids. After 24 hours, transfected HEK-293 were treated with indicated concentrations of BEA and LPS (1 mg/ml) as positive control and induction of NF-κB was determined by luciferase activity. Results shown are representative of three independent experiments in quadruplicates (n=4). Data are presented as mean ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ns, not significant.

6.9. Effects of BEA on BMDMs

It has been reported that BEA show anti-inflammatory effects on LPS-stimulated RAW264.7 through inhibiting IL-1β production (232), however, the impacts of BEA alone on macrophages are undetermined. To this end, WT and *Tlr4*$^{-/-}$ BMDMs were generated and followed by different concentrations of BEA or LPS as a control. TNF and IL-6 in the supernatant were measured by ELISA. As shown in Figure 13, both TNF and IL-6 were induced in WT BMDMs, but the expression levels of TNF and IL-6 were very low and almost undetectable in *Tlr4*$^{-/-}$ BMDMs. These data suggest that BEA can also activate BMDMs in a TLR4 dependent way and a dose dependent manner. This paragraph is adapted from Yang *et al*. (2022).

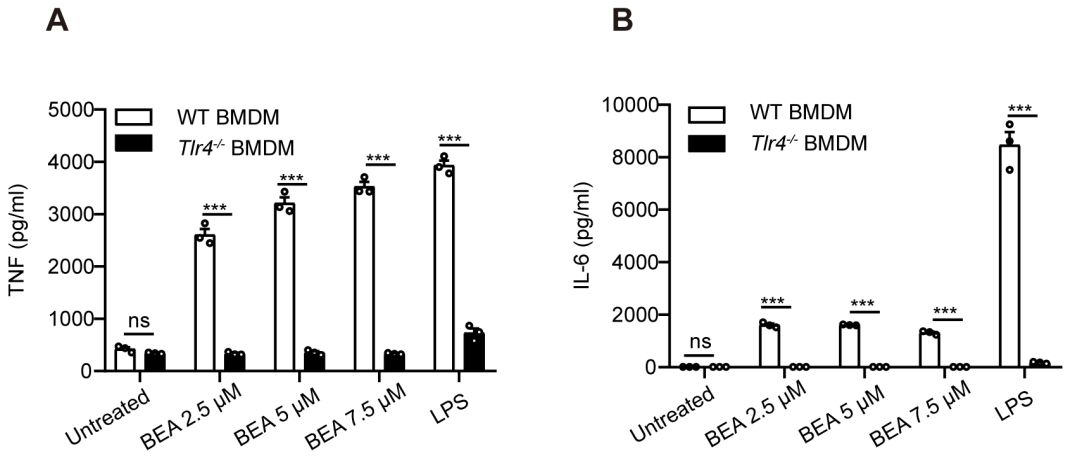

Figure 13. Effects of BEA on BMDMs. This figure is adapted from Yang *et al*. (2022).

$1x10^6$ BMDMs derived from WT and *Tlr4*$^{-/-}$ mice were stimulated with the indicated concentration of BEA or LPS (100 ng/ml) for 24 hours. TNF **(A)** and IL-6 **(B)** expression were analyzed by ELISA. Results shown are representative of two independent experiments using cells from three mice per group (n=3). Data in A and B are

presented as means ± SEM. Significance was analyzed by Two-way ANOVA with Sidak's multiple comparisons test. ***p<0.001, ns, no significance.

6.10. Analysis of gene expression profile in BEA-treated BMDCs

To define the underlying mechanisms by which BEA activates BMDCs, we used whole-genome RNA-seq to detect genome wide differences in gene 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 the combined BEA with LPS treatment clusters in close proximity to that of BEA stimulation alone (Figure 14A). Similarly, heatmap and hierarchical clustering show that gene expression induced by BEA is distinct from that induced by 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, CTSB, Vps35 and Mcoln1), cellular metabolism gene expression (HK3 and Fasn), mitochondrial gene expression (Polrmt, Slc25a29), autophagy gene expression (Rptor) and transcriptional regulation (Zfp446, H4c3 and Foxf2) (Figure 14B).

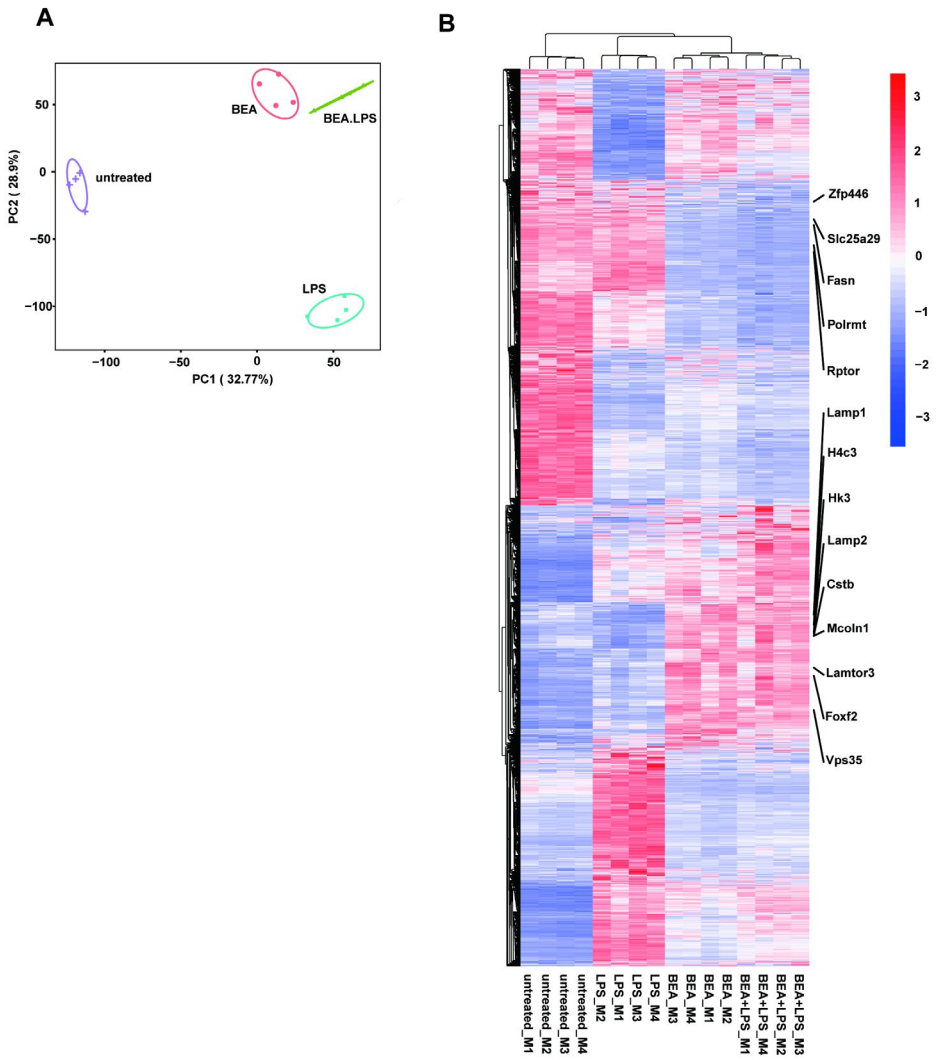

Figure 14. BEA induces transcriptional changes distinct from LPS stimulation. This figure is adapted from Yang *et al.* (2022).

MHC-II^{high} CD11c^{+} BMDCs sorted by flow cytometry were followed by stimulation with BEA (5 µM) or LPS (1 ng/ml) alone or BEA combined with LPS for 4 hours. Results shown are from one experiment containing 4 independent biological replicates (cells from 4 mice per group, n=4) for each condition. **(A)** PCA of the quadruplicate biological replicates. **(B)** Heatmap showing the expression profile for 4,015 genes that were found to be significantly regulated in at least one of the comparisons using untreated as baseline condition.

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 15A and 15B).

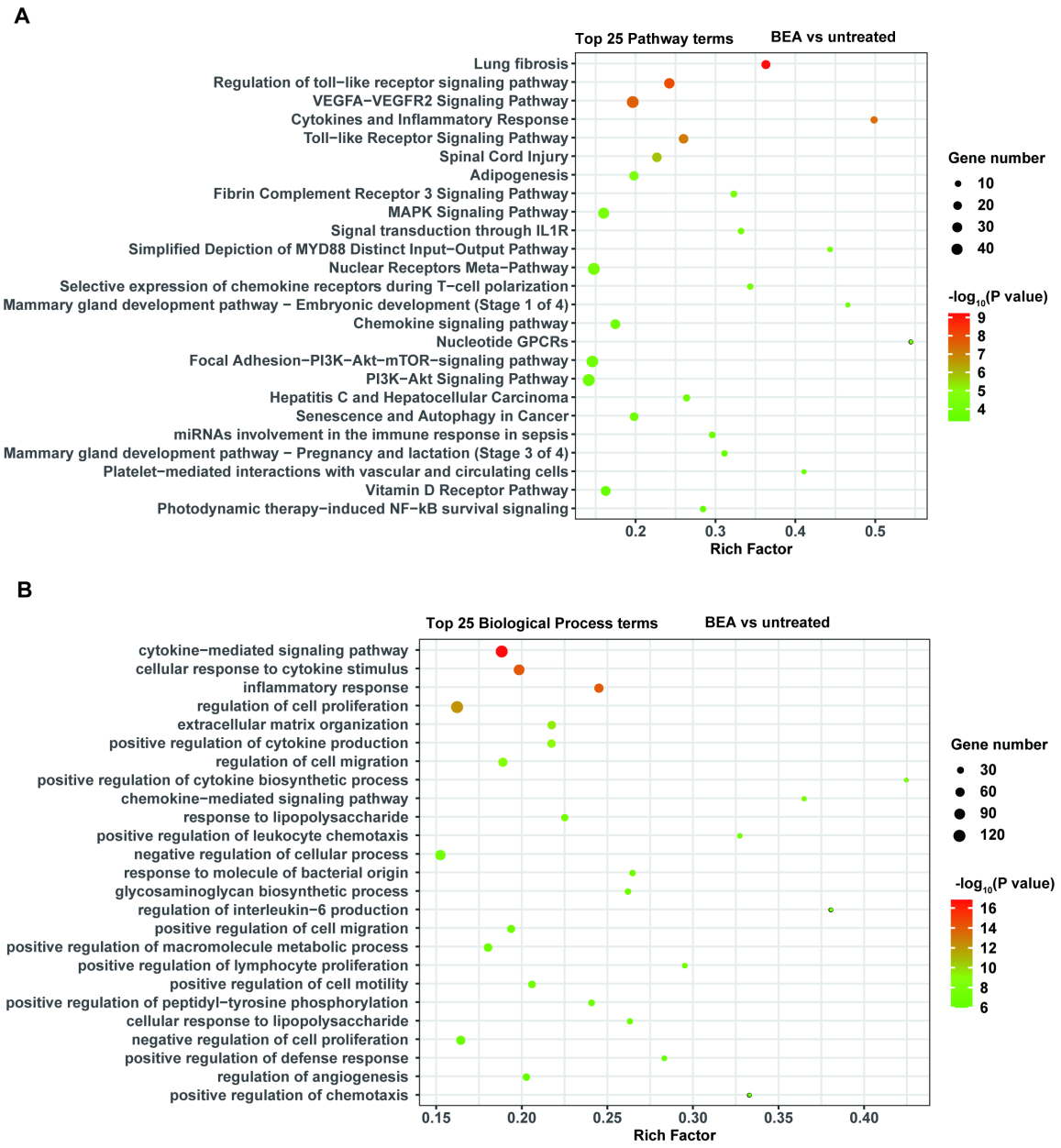

Figure 15. The transcriptional changes in BEA-treated BMDCs. This figure is adapted from Yang *et al.* (2022).

MHC-II^{high} CD11c^{+} BMDCs sorted by flow cytometry were followed by stimulation with BEA (5 µM) or LPS (1 ng/ml) alone or BEA combined with LPS for 4 hours. Results shown are from one experiment containing 4 independent biological replicates (cells from 4 mice per group, n=4) for each condition. **(A)** Enriched Reactome pathways and **(B)** biological process in DEGs in BEA treated BMDCs compared with untreated BMDCs.

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 DC activation and cell cycle progression (Figure 16A-16C). 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 16B). Taken together, our results indicate that BEA activates BMDCs via a TLR4 dependent signaling pathway, but induces a gene expression profile different from LPS. This chapter is adapted from Yang *et al*. (2022).

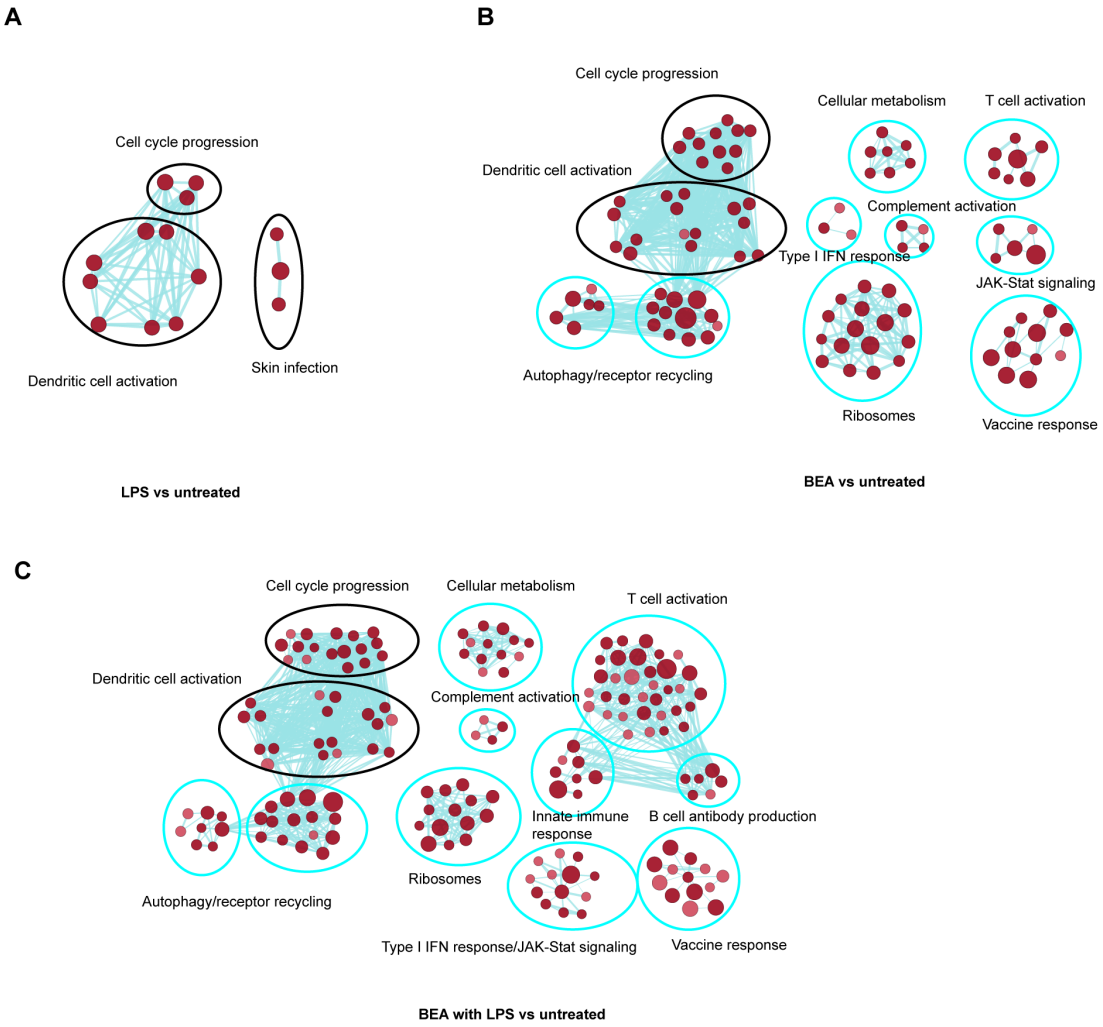

Figure 16. Cytoscape analysis of significantly enriched signatures in LPS (A) or BEA (B) or BEA together with LPS (C) treated-BMDCs. This figure is adapted from Yang *et al.* (2022).

MHC-II^{high} CD11c^{+} BMDCs sorted by flow cytometry were followed by stimulation with BEA (5 μM) or LPS (1 mg/ml) alone or BEA combined with LPS for 4 hours. Results shown are from one experiment with 4 independent biological replicates (cells from 4 mice per group, n=4) for each condition.

6.11. Target prediction of BEA

Although we have identified TLR4 as a target of BEA that activates BMDCs or BMDMs, we also wanted to explore whether there are any other functional targets with which BEA might interact. To address this question, we first used an online tool (ChemDIS: https://cwtung.kmu.edu.tw/chemdis), which can predict targets of a compound. As shown in Figure 17A, most of the cysteine-Cathepsin family proteins are predicted to be direct binding targets of BEA and CTSB was predicted to be in the top 5 targets of BEA. To further confirm that Cathepsin family proteins are the potential targets of BEA, another compound protein prediction tool was used (Super-PRED: https://prediction.charite.de/). Consistently, it shows that Cathepsin family proteins are the targets of BEA, Cathepsin D with the highest probability, and CTSB being on the 27th highest rank to be the target of BEA (Figure 17B). Interestingly, it has been reported that CTSB-deficient BMDCs exhibited enhanced IL-12 production in LPS-treated BMDCs (310), which highly matched our findings that BEA activates BMDCs via the TLR4 signaling pathway to produce IL-12 production. Suppression of CTSB by BEA may induce more IL-12 production. Taken together, with the online prediction tools, CTSB may be another target of BEA in DCs.

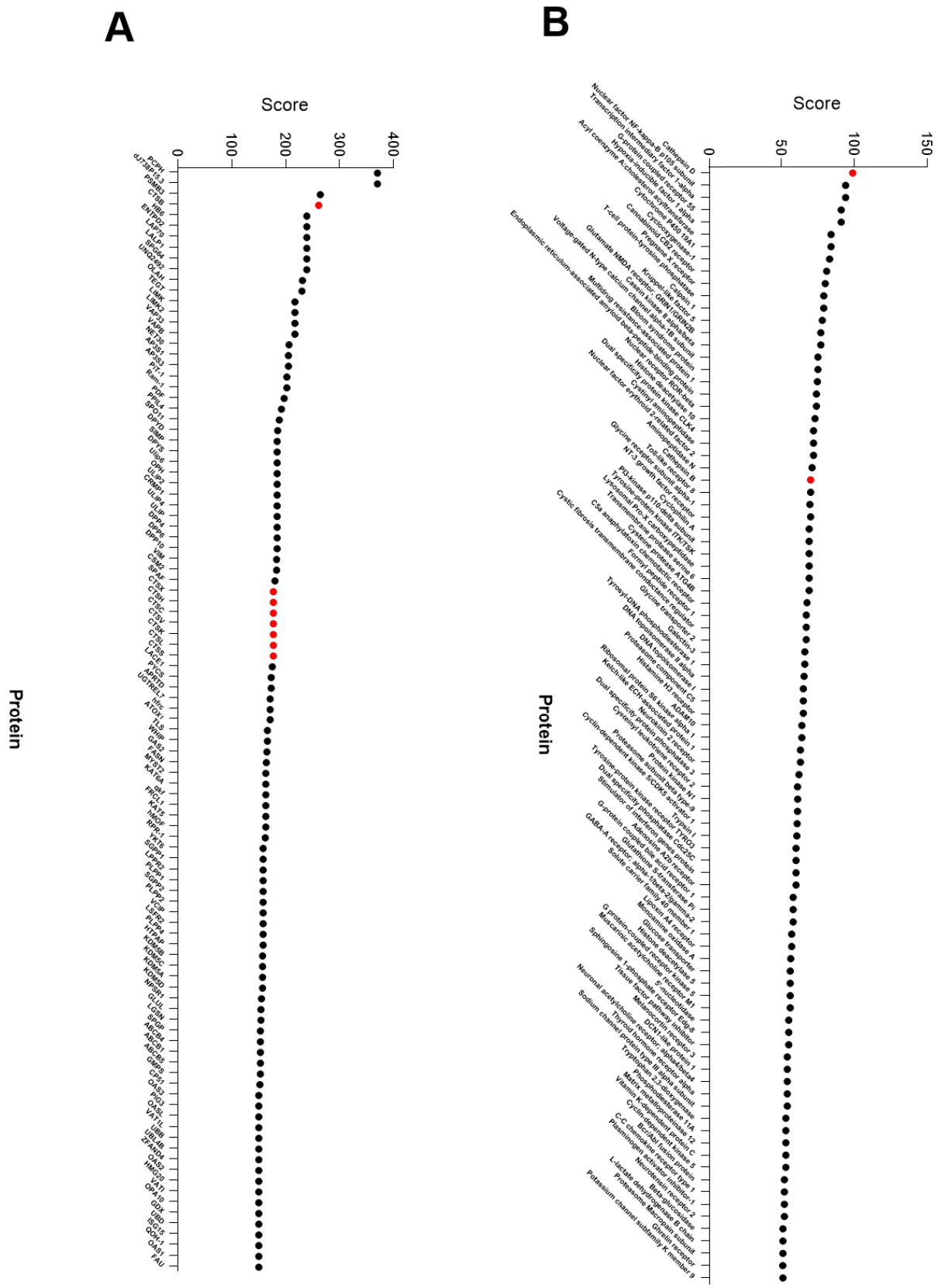

Figure 17. Prediction of BEA targets by digital databases.

(A) Prediction of BEA targets by ChemDIS: https://cwtung.kmu.edu.tw/chemdis. **(B)** Prediction of BEA targets by Super-PRED: https://prediction.charite.de/.

6.12. Direct suppression of mouse CTSB activity by BEA

As shown above, CTSB could be a potential target of BEA according to the digital analysis of two online target prediction tools. To verify the prediction result, BMDCs were stimulated with or without the indicated concentrations of BEA for 16 hours and CTSB enzyme activity was measured. It was shown that CTSB activity was suppressed with BEA stimulation at 2.5 μM, and the inhibition is in a dose-dependent manner, suggesting CTSB is a target of BEA in BMDCs (Figure 18A, B). However, it is still unclear whether BEA can directly bind to mouse CTSB and thus suppress its enzymatic activities. To address this concern, mouse recombinant CTSB protein was incubated with or without a different dose of BEA or a specific inhibitor, CA074 as a control, followed by the addition of substrate of CTSB to the reaction system and CTSB activity was measured (Figure 18C, D). As expected, CA074 can markedly reduce CTSB activity, and BEA also shows strong inhibition of mouse CTSB activity. Overall, BEA can directly suppress CTSB activity.

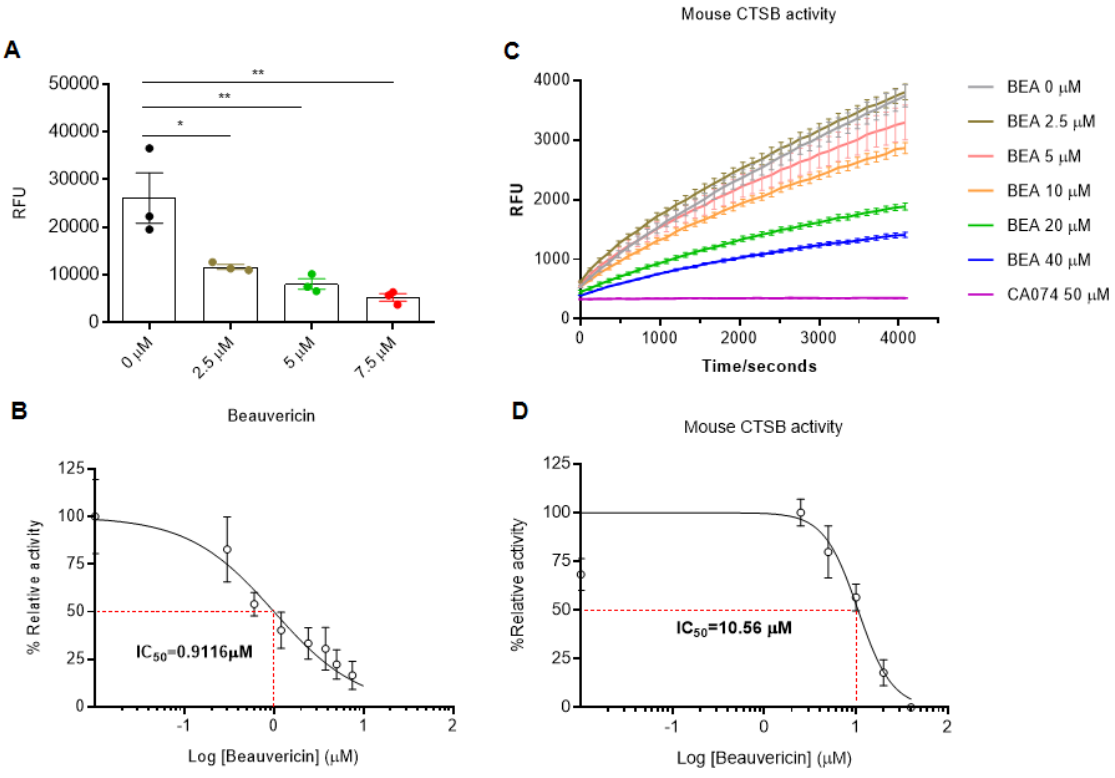

Figure 18. Direct inhibition of mouse CTSB by BEA.

(A, B) Mouse BMDCs were stimulated with indicated concentrations of BEA for 16 hours. **(A)** CTSB activity in BMDCs was indicated by RFU. **(B)** CTSB activity in BMDCs was indicated by IC_{50}. Representative data was

shown from three independent experiments (cells from three mice per group, n=3). Significance was analyzed by one way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ns, no significance **(C, D)** Recombinant mouse CTSB was incubated with indicated doses of BEA or CA074, and the substrate. **(C)** Mouse CTSB activity was indicated by RFU. **(D)** Mouse CTSB activity was indicated by IC_{50}. Representative data was shown from three independent experiments. Data are presented as means ± SEM.

6.13. Direct suppression of human CTSB activity by BEA

As described above, we conclude that BEA can directly target mouse CTSB. This led us to ask whether BEA can also inhibit human CTSB. To address this question, we first induced human monocyte-derived iDCs from THP-1 cells with the culture of human GM-CSF and IL-4 (Figure 19A). The efficiency of human DC induction was measured by flow cytometry (Figure 19B). iDCs can express increased levels of HLA-DR, CD86, and CD83 compared to undifferentiated THP1 cells. The iDCs were then subjected to various concentrations of BEA stimulation for 16 hours. Subsequently, cell lysates were collected for measurement of CTSB activity. As shown in Figure 19C, BEA can also suppress human CTSB activity and this inhibition is dose-dependent. Moreover, the IC_{50} of BEA on human CTSB activity is higher than that on mouse CTSB (Figure 19D). To further investigate whether BEA directly targets human CTSB, commercially available recombinant human CTSB was used for the activity assays. As shown in Figure 19E and 19F, human CTSB activity was highly suppressed by BEA in a dose dependent manner. Taken together, these data indicate that in addition to mouse CTSB BEA also directly targets human CTSB.

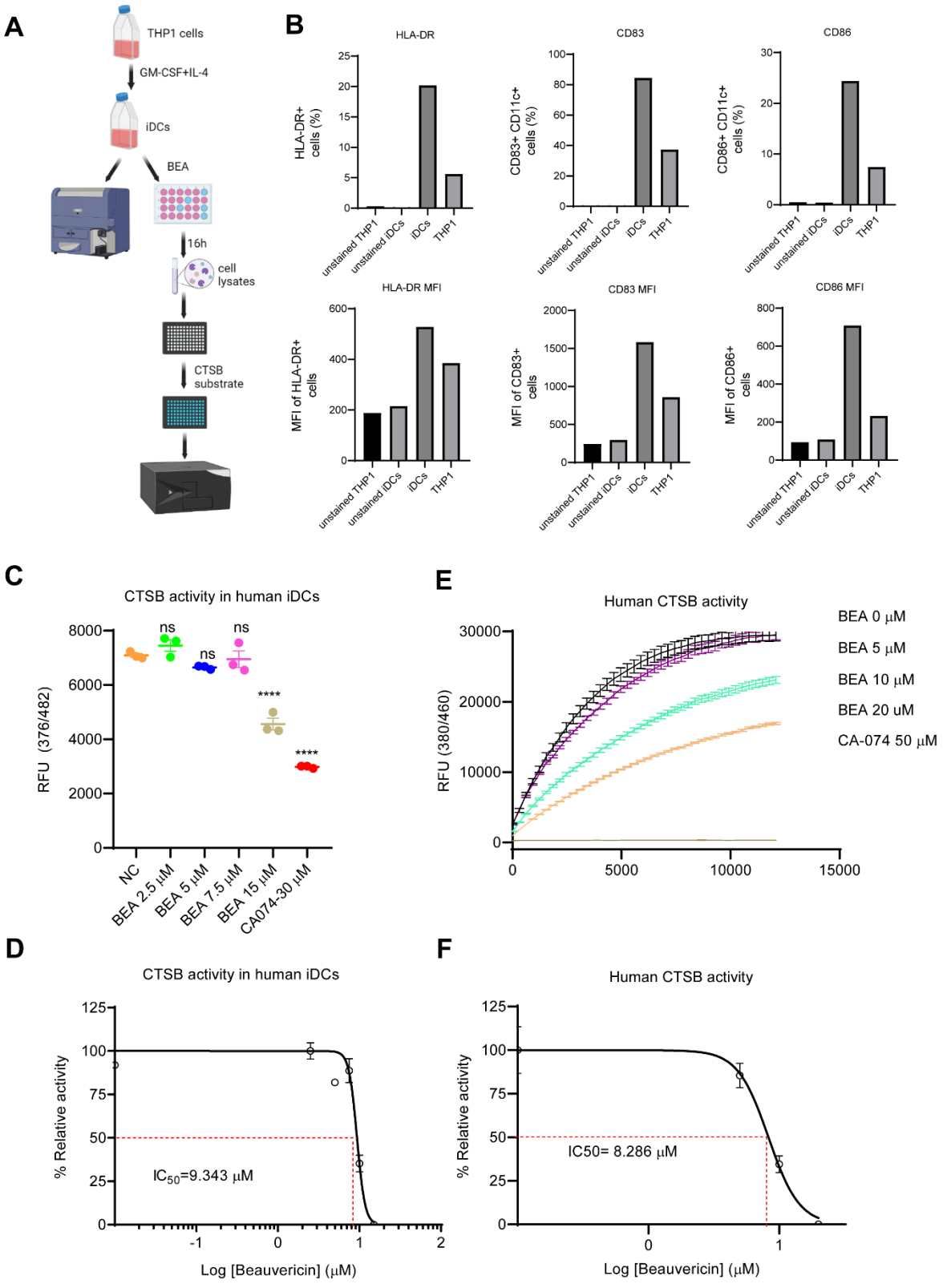

Figure 19. Direct inhibition of human CTSB by BEA.

(A) Workflow for the detection of CTSB activity in BEA-stimulated iDCs, created with BioRender.com. Briefly, human THP-1 cells were cultured with human GM-CSF and IL-4 for 5 days, cell culture medium was replaced

with fresh medium on day 3. On day 5, the induction of iDCs was first checked with the expression of surface molecules by flow cytometry. Then iDCs were used for detection of CTSB activity in iDCs with or without BEA stimulation. **(B)** The surface markers were detected by flow cytometry. Representative data was shown from two independent experiments. **(C, D)** Human iDCs were stimulated with the indicated concentrations of BEA for 16 hours. The cell lysates were used to measure the CTSB activities. **(C)** CTSB activity in iDCs with or without BEA stimulations was shown by RFU. **(D)** The IC_{50} of CTSB activity in cell lysates of iDCs with or without BEA stimulation was shown. Representative data was shown from three independent experiments. Data are presented as means ± SEM. Significance was analyzed by one way ANOVA with Dunnett's multiple comparisons test. ****$p<0.0001$, ns, no significance. **(E, F)** Recombinant human CTSB was incubated with indicated doses of BEA or CA074 and the substrate. **(E)** CTSB activities were shown by RFU **(F)** The IC_{50} of human CTSB activity was shown. Representative data was shown from two independent experiments. Data are presented as means ± SEM.

7. 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 (380). Moreover, BEA has been shown to exhibit anti-inflammatory activity in macrophages by inhibiting the NF-κB pathway and in an experimental colitis model by inhibiting activated T cells (232, 280). 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, IFNβ, 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. This paragraph is adapted from Yang *et al*. (2022).

TLRs are crucial activating receptors on APCs 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 IFNβ (111, 120, 381). 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 (121). 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 IFNβ production by Tlr4$^{-/-}$ BMDCs.

Consistently, Luciferase Reporter Assay shows that BEA significantly induced NF-κB activation in HEK-293 cells stably expressing TLR4/MD2/CD14. Moreover, RNA-seq and GO analyses showed that BEA-treated BMDCs activate pathways related to TLR signaling, cytokines and inflammatory response, chemokine signaling, and IL-10 anti-inflammatory signaling, which were similarly activated in LPS-treated BMDCs. However, also marked differences exist between BEA-treated BMDCs and LPS-treated BMDCs. BEA-treated BMDCs show regulation of various signatures associated with cellular metabolism, T cell activation, complement activation, type I IFN response, vaccine response, JAK-STAT signaling, ribosomes, translation, and autophagy/receptor recycling, which was not found to the same extent in LPS-stimulated BMDCs. These differences could be attributed to the different affinity of TLR4 to BEA and LPS or by additional molecular targets of BEA within the cells. For example, BEA was predicted to be a target of several Cathepsin family proteins, which is involved in producing immune modulators by the limited proteolysis processing. CTSB is essential for the production of interleukin-1β (IL-1β) (363, 382) and TNF (307) and Cathepsin K involves in TLR9 activation (383). Alternatively, BEA-mediated TLR4 signaling could be activated via the release or induction of endogenous proteins serving as ligands for TLR4 such as Mrp8 (384), HSP60, 70 and Gp96 (385) and HMGB1 (135). Of note, heat-killed conidia of *A. fumigatus* have been reported to activate TLR4 signaling to induce inflammatory cytokine production (130). However, which component of this fungi is responsible for TLR4 activation was not elucidated. It is tempting to speculate that BEA or a derivative thereof produced by this fungus is responsible for this TLR4 stimulating activity, but this remains to be elucidated in future studies. Besides, it was already known that mannans obtained from fungi such as *C. albicans* and *C. neoformans* were also able to induce innate immune activation through TLR4. Therefore, our data identified a novel fungal component capable of activating TLR4 signaling (386, 387). This paragraph is adapted from Yang *et al*. (2022).

It has been reported that BEA shows cytotoxicity on human DCs derived from human umbilical cord blood $CD14^+$ monocytes (388). However, in our study BEA showed significant cytotoxicity on BMDCs only at 7.5 μM while the relevant observations regarding the activating capacity of BEA on BMDCs are detectable at BEA concentrations (2.5μM, 5μM) exhibiting no cytotoxicity. Furthermore, BEA can

affect LPS induced DCs maturation by decreasing CCR7 expression and increasing IL-10 production (388), whereas effects of BEA alone on human DC activation remain unknown. To determine whether BEA can activate human DCs is the aim of future studies. Furthermore, we found BEA pre-treated BMDCs could enhance T-cell proliferation, whereas no difference of T cell proliferation was observed when BEA was present in the co-culture of BMDCs together with T cells (data not shown). This is in line with a direct inhibitory effect of BEA on T-cell proliferation that has been described before (280) and a similar tendency was also detectable in our study, which might result in a neutralization of BMDC-mediated T cell proliferation in our co-culture setup. This points to a cell type specific effects of BEA where in this case in T cells a positive stimulatory effect of BEA via the TLR4 signaling pathway, which is less dominant in T cells, is antagonized by so far unknown additional target molecules and more dominant inhibitory signaling pathways. Further studies are necessary to elucidate the underlying cell type specific molecular mechanisms. In addition, BEA has been reported to exhibit anti-inflammatory activity in macrophages by inhibiting the NF-κB pathway (232). However, when stimulating primary BMDMs with BEA we could observe an increase in TNF and IL-6 production which was also strictly dependent on the presence of TLR4. This discrepancy could be caused by different experimental systems: Yoo *et al*. used the RAW264.7 cell line (232), whereas we worked with primary BMDMs. Moreover, Yoo *et al*. did not analyze effects of BEA alone on RAW264.7 cells. Future studies will be needed to completely elucidate the underlying differences. TLR4 is a transmembrane protein of TLR family and it can't recognize LPS alone and has to work with several other proteins including MD2 and CD14 (389). Therefore, another interesting question to be addressed in the future is that if BEA can directly target the TLR4 receptor or if the TLR4/MD2/CD14 complex is required for BEA mediated activation of the TLR4 signaling pathway. Direct interaction between BEA and TLR4 or TLR4/MD-2 or TLR4/MD-2/CD14 complexes can be demonstrated in vitro by Nuclear Magnetic Resonance (NMR) spectroscopy. X-ray crystallography and Structure-Activity Relationship (SAR) can be used to analyse structure binding conformation between BEA and its specific targets. This paragraph is adapted from Yang *et al*. (2022).

Adjuvants are defined as molecules or formulations that enhance the efficacy of vaccines without directly participating in the protective immunity. In recent decades, a variety of preclinical and clinical studies have shown that purified TLR agonists could be exploited as adjuvants to enhance adaptive responses during vaccination (390, 391). Monophosphoryl lipid A (MPLA), a TLR4 agonist purified from *Salmonella minnesota,* has been used as an adjuvant in several vaccines against human papillomavirus (HPV) and hepatitis B virus (HBV) infections (392). Moreover, MPLA is the only TLR4 agonist that has been clinically tested as an adjuvant for cancer vaccines (393, 394). In our study, BEA potently activated DCs inducing a range of inflammatory cytokines and chemokines in addition to MHC-II up-regulation. Cell-directed delivery of BEA could achieve specific activation of DCs, bypassing its suppressive effects on T cell proliferation (280). Considering the cytotoxicity reported for human umbilical cord blood derived DCs, the suitability of BEA as a candidate of vaccine adjuvants and cancer immunotherapy needs to be carefully evaluated. Based on its lead structure, derivatives with higher specificity and efficacy of its stimulatory capacities could be developed. In addition, BEA has been reported to neutralize ABC transporters, which contribute to multidrug resistance in human, nematodes and arthropods (286, 395). Therefore, combinational therapy using BEA or an optimized derivative thereof together with other drugs could overcome multidrug resistance. This paragraph is adapted from Yang *et al*. (2022).

In addition, in this thesis, using the silico prediction websites, we found another target of BEA: CTSB. It is a cysteine protease that is primarily localized in lysosomes and can be released into the cytosol or extracellular space and plays critical roles in various physiological and pathological processes (396-398). The prediction results were first verified in BMDCs and it was shown that BEA starts to inhibit mouse CTSB activity in BMDCs at 2.5 μM after 16h stimulation with the IC_{50} being 0.9116 μM. Similar inhibition of human CTSB activity by BEA was observed in human iDCs generated from human monocyte THP-1 cells. Significant inhibition was observed at 7.5 μM with the IC_{50} being 9.343 μM. This roughly 10-fold difference in IC_{50} between mouse and human CTSB could be due to the different expression levels of CTSB in these two cell types. Furthermore, it has also been reported by Silva *et al.* that BEA isolated from *Fusarium proliferatum* of infected pineapple inhibits human CTSB activity with

IC_{50} of 6.8 ± 0.7 μM (399) resembling the range of our IC_{50} results for human cells. Conversely, Mohammed *et al.* reported that BEA has no inhibitory effects on cellular CTSB activity, as decreased CTSB activity was correlated with increased CTSB activity in the media, indicating that BEA can only induce extracellular CTSB secretion (400). Consistent with Silva's study, our cell-free data also showed that BEA can markedly inhibit mouse and human CTSB activity, with IC_{50} values of 8.286 μM and 10.56 μM, respectively. There are three main types of enzyme inhibition including competitive, non-competitive, and uncompetitive. In competitive inhibition, inhibitors can compete with the substrate to bind to the active site of enzyme (401). Non-competitive inhibition occurs when inhibitors bind to the non-active site of the enzyme and change enzyme activity via altering its conformation (401). In case of uncompetitive inhibition, the inhibitors bind only to the enzyme-substrate complex and form an inactive enzyme-substrate-inhibitor complex (401). Further enzymatic assays are needed to elucidate which kind of inhibition is induced by BEA. Also, X-ray crystallographic studies and molecular docking need to be performed to analyse the binding site of BEA to CTSB. Overexpression of CTSB was observed in many cancers including breast, cervical, bladder, colon, ovarian, bladder, lung, prostate, thyroid, and lung cancer (402). Moreover, BEA was shown to exert anticancer activity in vivo in an allograft mouse model (252). Given the high pharmacological relevance of CTSB in cancer, manipulating CTSB expression with its inhibitors could be applied to cancer therapy. However, most synthetic inhibitors of CTSB have peptidyl backbones with an electrophilic reactive group that forms either a reversible or an irreversible covalent bond with the active site cysteine. Thus, none of the existing CTSB inhibitors are used in clinical practice due to poor bioavailability, off-target side effects and high toxicity (403). In this study, we found BEA can not only activate BMDCs and BMDMs via targeting to TLR4 signaling pathway but also target to CTSB, suggesting that BEA can be a promising candidate for cancer therapy.

CTSB is a member of cysteine protease family including CTSB, C, F, H, K, L, O, S, V, X, and W. It was shown to be essential in intracellular proteolysis and also extracellular matrix remodelling of cellular proteolysis networks (398). It has been reported that CTSB deficiency can lead to increased MHC-II molecule expression and can induce IL-12 production by LPS-treated BMDCs (310). In this study, BEA targets TLR4 to activate BMDCs, inducing inflammatory cytokines production.

Considering that BEA also suppresses CTSB activity, we speculated that BEA might induce more IL-12 production after inhibition of CTSB activity or deletion of CTSB in BMDCs. However, we observed that IL-12 was slightly decreased in BEA-treated CTSB knockout BMDCs compared with WT controls (data not shown). This could result from compensatory effects of other Cathepsin family members, as copious reports on proteolysis pathways indicate that several protease can compensate for the loss of another. For example, genetic ablation of CTSB in a transgenic mouse model of mammary cancer can be compensated by redistribution of Cathepsin X to the membrane of these cells (295). Aggarwal *et al*. also showed that CTSB deficiency in a transgenic mouse model of pancreatic ductal adenocarcinoma delays progression of premalignant lesions and pancreatic carcinoma, while Cathepsin L is up-regulated in these tumors (404). Moreover, the slightly reduced production of IL-12 by CTSB-deficient BMDCs could be interpreted by the existence of other intracellular targets of BEA. For instance, BEA can suppress human Cathepsin V activity in cell-free assays (399) and BEA could also have some other targets in the cells as shown by the prediction results. In addition, it was indicated that CTSB can lead to cleavage of the calcium channel MCOLN1/TRPML1 and the inhibition of CTSB by BEA results in calcium efflux from the lysosomal lumen to the cytoplasm and induces the expression of lysosomal and autophagy-related proteins. Consequently, this leads to an increase in the size and number of populations of lysosomes and autophagosomes (297). Besides, it was shown by our RNA-seq data that BEA can regulate autophagy related genes such as *Rptor*. Therefore, we proposed that the inhibition of CTSB by BEA can regulate autophagy. However, no difference of autophagy-related gene (MAP1LC3B and SQSTM1) expression was observed in our experiments. This could result from autophagy inhibition by down regulation of NLRP3 inflammasome activation by CTSB. Specifically, in our work, BEA can significantly inhibit CTSB activity. CTSB was required for NLRP3 inflammasome activation in macrophages via interacting with NLRP3 (306), which can initiate autophagy via interacting with Beclin 1 (405-407). Inhibition of CTSB by BEA led to down-regulation of NLRP3 inflammasome activation, which subsequently down regulates autophagy. Therefore, changes of autophagy induction by BEA could be neutralized by the indirect down-regulation of autophagy led by inhibition of CTSB.

NPs are an important source of medicines and drug templates, playing crucial roles in a variety of diseases, including infections, cancer, and cardiovascular diseases (408). In the last two decades, approximately a third of all FDA-approved drugs were derived from NPs or their derivatives. Nature has been proven to be the most abundant source for drug discovery since it has many kinds of plants and microorganisms (409). Bioactive NPs have been isolated from plants (e.g. aspirin, galantamine, etc.), microbes (penicillins, streptomycin, etc.), and marine sponges (polyketides, alkaloids, etc.) (410, 411). Here, we defined that the fungal NP BEA can target TLR4 to induce DC activation. TLRs are important PRRs for DC activation and TLR-agonists have been used for cancer immunotherapy (108, 412, 413). For example, BCG, which originates from Mycobacterium bovis14, is used for treatments for patients with early stage bladder cancer (65). As a TLR4 agonist with anti-tumor properties and that also targets CTSB, which is overexpressed in various invasive and metastatic cancers (414), BEA shows potential efficacy in the treatment of cancer. Since all the data presented here are based on *in vitro* experiment and little is known about its anti-tumor effects in vivo, it is important to note all the well-known functions of BEA are also based on in vitro studies. Therefore, it is of major importance to evaluate the efficacy of BEA in tumor mouse models in vivo. Besides, various NP-derived TLR agonists have also been used as vaccines for virus infection (415, 416). For example, prophylactic administration of the TLR4 agonist fimbriae H protein derived from UPEC protects against lethal influenza A virus infection in a mouse model (417) and administration of another TLR4 agonist MPLA to mice prior to a lethal influenza infection can enhance both mucosal and systemic immune responses (418). Therefore, BEA might be a potential vaccine candidate for virus infections, but needs to be tested in specific vaccination mouse model. Apart from that, BEA shows anti-bacterial effects on some Gram-positive or Gram-negative bacteria by targeting cell organelles or enzyme systems and inhibits ABC transporters, which are responsible for drug resistance (419). Consequently, BEA might potentially show effects in the treatments of bacterial infection. Apart from that, BEA exhibits anti-virus properties on HIV by inhibiting HIV integrase (233) and on SARS-CoV-2 by binding to protease pockets and the spike glycoprotein (264). Therefore, potential efficacy might be observed in virus infection mouse models after BEA treatment. Most importantly, due to the cytotoxicity of BEA to many cell types (380), the

evaluation of in vivo toxicity needs to be determined before the analysis of effects on the therapy of tumors and infections and optimal dosing should be determined before analysis.

Although NPs have historically been a critical source for drugs discovery and medical use, sometimes the lead NPs have the drawback of undesirable toxicity, which prevents them from being direct therapeutic agents (420). However, structural optimization can overcome the disadvantages of toxicity to generate clinically useful structures. For example, the natural product β-lapachol, which exhibits significant anti-tumor activity, was later abandoned due to unacceptable toxicity (421). However, with structural modifications, the β-lapachone derivatives showed strong anticancer activity and was tested in clinical trials for advanced solid tumors (5). To better utilize the immune activating role of BEA in vivo, careful structure modification can be used to weaken its toxicity and keep its biological and immunological activities.

In summary, our data revealed a novel impact of BEA on DCs in activating inflammatory cytokine and chemokine production via activating the TLR4 signaling pathway. In addition, BEA can suppress human and mouse CTSB activity in both cell based and cell free assays. Our findings suggest that BEA can be exploited in the field of vaccine adjuvants and cancer immunotherapy.

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10. Declaration

I declare on oath that I have written the dissertation independently and without impermissible external assistance, in compliance with the “Principles for Ensuring Good Scientific Practice at the Heinrich Heine University Düsseldorf”.

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08/2018-08/2019: Research assistant; Institutes of Biology and Medical Sciences (IBMS), Soochow University, Suzhou, China

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Laboratory skills

- Cell Culture (primary & cell line): DC, T, BMDMs &BMDCs
- Animal experiments; i.v&i.p, etc.
- Multi-channel Flow cytometry
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- Histology& Imaging

Publications

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