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The Opposite Effects of DNA and Protein Components of *Listeria monocytogenes* and *Toxoplasma gondii* on Immunologic Characteristics of Dendritic Cells

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ABSTRACT

The innate immune system utilizes pattern recognition receptors (PRRs) to recognize microbes. Pathogens contain various molecules with diverse effects on immune response. In this study, we evaluated the effect of DNA and protein components derived from two intracellular microorganisms including *Listeria monocytogenes* (*L.monocytogenes*) and *Toxoplasma gondii* (*T. gondii*) on dendritic cells (DCs) activation and ensuing adaptive immune responses.

DNA and protein components of *L. monocytogenes* and *T. gondii* were prepared using relevant kits. DNA and protein components of these two pathogens were added to immature DCs (iDCs). Subsequently, co-stimulatory expression and cytokine production by DCs were measured. Finally, we evaluated the stimulatory capacity of mature DCs (mDCs) in DC-T cells co-culture.

The results showed that protein matured-DCs produced higher level of IL (Interleukin)-12p70. There was also a significant increase in Interferon-Gamma (IFN- γ) production and proliferative capacity in T cells co-cultured with protein matured-DCs. On the other hand, DNA matured-DCs produced significantly higher amounts of Transforming growth factor-beta (TGF- β).

Collectively, these results imply a regulatory nature for DNA and potent stimulatory character for protein components of these two intracellular microorganisms.

Keywords: Dendritic cells; Immunity; *Listeria monocytogenes*; *Toxoplasma gondii*.

INTRODUCTION

The mammalian immune system comprises innate and adaptive immunity, which work together to protect the host against different threats.^{1,2} The innate immune

system uses functionally diverse strategies to provide different forms of protection against pathogens.¹ The recognition of pathogens as non-self agents by mammalian cells is a fundamental step in mounting innate immune responses.³ Innate immune recognition

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relies on a limited number of germline-encoded receptors known as Pathogen Recognition Receptor (PRRs).^{1,4} PRRs provide a critical link between the innate and adaptive immune systems.⁵ These receptors evolved to recognize conserved products of microbial components termed pathogen-associated molecular patterns (PAMPs) which are produced by microbial pathogens, but not by the host.

PAMPs are molecular signatures typical of whole classes of microbes, and their recognition plays a key role in innate immunity.⁶ PAMPs engage the PRRs, thereby signal the presence of an infection, and subsequently induce innate immune responses. PAMPs such as lipopolysaccharides (LPS), double-stranded RNA (dsRNA) and flagellin are molecular structures, which are expressed by microbes but are not expressed by the human host.⁷ Activation of PRRs by PAMP ligation initiates an inflammatory cascade that attempts to clear the offending pathogen, prompting a specific adaptive immune response.³ On the other hand, there is growing evidence to propose that in addition to their well-characterized immune stimulatory properties, PRRs may also directly regulate the immune responses. This regulatory role of PRRs is important in two aspects. Firstly, these mechanisms enable pathogens to subvert the immune response and thereby generating conditions that ensure their survival for an extended period.^{8,9} Secondly, these strategies control the extent of immune-mediated pathology and so limit the collateral damage to self-tissues that can result from an exuberant immune response.^{9,10} Accordingly, this speculation arises that different components in pathogens play differential roles in immune responses.

During immune responses against pathogens, DCs as professional antigen presenting cells (APCs) play crucial roles in initiating immune responses against them.¹¹⁻¹³ In vivo, immature DCs (iDCs) reside in the periphery where they serve as sentinels for microbial pathogens. DCs, in their immature state, detect the presence of pathogens and subsequently uptake and process antigens.¹³ Interaction with microbes induces a critical maturation program during which the DCs modulate the expression of cell surface molecules and migrate to lymph nodes where potent interactions with T cells initiate the acquired immune response.¹⁴ In addition to their role in initiating immune responses, DCs play a crucial role in the immunoregulation. In vitro experiments have demonstrated that DCs with an

immature phenotype induce the differentiation of anergic/suppressive T cells.¹⁵ Therefore, DCs are central in the orchestration of the various forms of immunity and tolerance and are supposed to regulate immune responses.^{9,13} Their immunoregulatory role mainly relies on the ligation of certain receptors that initiate and modulate DC maturation resulting in the development of functionally different effector DC subsets.⁹ These receptors include PRR and it is clear that DCs constitutively express a range of PRRs specifically designed to bind PAMPs involved in pathogen recognition.^{16,17}

Immune responses against intracellular pathogens including *T.gondii* and *L. monocytogenes* are mainly instructed by DCs.^{2,18,19} It has been revealed that these pathogens are able to induce DC maturation and skew immune responses towards TH (T Helper)1 phenotype.^{20,21} In contrast, several studies have indicated that these intracellular pathogens are capable in inducing IL-10 production and thereby suppressing immune responses.^{22,23} Therefore, it has been supposed that these pathogens may have various PAMPs, which play different roles both in immune activation and in regulation. The aim of this study was to investigate the in vitro effects of different PAMPs on immune cells especially DCs for future use in DC-based immunotherapy. The effects of nucleic acid and protein components of these two intracellular pathogens on DCs maturation and ensuing adaptive immune responses were compared.

MATERIALS AND METHODS

Animals and Cell Lines

Six-to-eight-week-old female *BALB/c* mice were purchased from Pasteur Institute of Iran. Mice were handled according to the NIH 1978 Guide for the Care and Use of Laboratory Animals. *BALB/c*-derived fibrosarcoma (*WEHI164*) and colon carcinoma (*CT26*) cell lines were maintained in RPMI-1640 (Gibco, Grand Island, MA, USA) with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, MA, USA), antibiotics, and 2mM L-glutamine (Sigma-Germany).

Preparation of DNA and Protein Components

L. monocytogenes (ATCC 19115) was purchased from Iranian Research Organization for Science and Technology (IROST). Bacteria were grown to mid-

logarithmic phase, pelleted, washed, and suspended in phosphate buffered saline (PBS). *T. gondii* (RH strains) was obtained from Pasteur Institute of Iran. Tachyzoites of *T. gondii* were prepared by intraperitoneal (i.p) passages in female mice as previously described.²⁴ After 72h, tachyzoites were harvested from the peritoneal cavity and washed with PBS. Pellet of both microorganisms was sonicated in the presence of Complete, Mini Protease Inhibitor Cocktail Tablets (Roche). Subsequently, the lysates were dialyzed against PBS 10mM overnight and then lysates were passed through a 0.2- μ m pore filter. DNA and protein components of *L. monocytogenes* and *T. gondii* were isolated using QIAamp DNA mini kit (Qiagen Ltd., Germany) and Qproteome Bacterial Protein Prep Kit (Qiagen Ltd., Germany), respectively. The protein and DNA concentrations were measured by Bradford protein assay (Thermo Scientific) and by measuring the absorbance at 260nm in a spectrophotometer (Nanodrop; Thermo Scientific), respectively. DNA and protein components were stored at -70°C until use.

Generation of Bone Marrow-derived DC

Bone Marrow-derived Dendritic Cells (BMDCs) were generated as described by a modified protocol of Inaba et al.²⁵ Briefly, bone marrow cells were obtained from the femur and tibia of female BALB/c mice; 1×10^6 cells/ml were placed in 24-well plates in RPMI-1640 plus 10% FBS, 20 ng/ml GM-CSF (R&D systems, USA) and 10 ng/ml IL-4 (R&D systems, USA). After 3 days, fresh media was added. On day 5, 10 μ g protein components, 2 μ g DNA components or 10 μ g total lysate of two microorganisms were added to iDCs culture. In some wells 2 μ g/ml LPS (cat. no: L-6526 Sigma) was added as control. Non-adherent DCs were harvested on day 7 of the culture for further assessments.

Monoclonal Antibodies (mAbs) and Flow Cytometry

DCs were stained with FITC-conjugated mAbs against cell surface molecules including CD80, CD86, CD40, MHC-II and PE-conjugated mAbs against CD11c (BD Pharmingen, USA) and analyzed with FACS Analysis System (Becton-Dickinson). In all experiments, isotype controls were included using an appropriate mAb of the same Ig class or subclass.

Cytokine Measurement

DC and T cell cytokine production were detected respectively in supernatants of DCs culture (IL-12p70, TGF- β) and co-culture of DC and splenic cells (IL-10, IFN- γ). Supernatants were collected and kept frozen at -70°C until use. Cytokine concentration was measured by enzyme-linked immunosorbent assay (ELISA) Kit (BenderMed system, Austria) according to the manufacturer's instructions.

Assessment of Stimulatory Capacity of Mature DCs

To determine the capacity of DCs in inducing T cell proliferation, DCs as stimulators (S) and primed BALB/c lymphocytes as responders (R) were co-cultured. In this regard, priming of the responder cells was achieved by Subcutaneous (S.C) injection of tumor cell lysate 2 weeks before splenocyte isolation. DCs matured by DNA and protein components were γ -irradiated (3000 rad) in Shariati Hospital and added to lymphocytes at the 1:50 ratio (S:R). Cultures were established in triplicate in 96-well, round-bottom micro culture plates (Nunclon Intermed, Roskilde, Denmark) and maintained in RPMI-1640 for 96 h. Proliferation of lymphocytes was determined using colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. The BrdU ELISA was performed according to the manufacturer's instructions (Roche Applied Science, Indianapolis).

Statistical Analysis

The results are expressed as means \pm SEM. Statistical analysis was performed using non-parametric Mann-Whitney test. *P* value < 0.05 was considered significant. All calculations were performed using GraphPad Prism software 5 (GraphPad Software Inc).

RESULTS

Effects of Protein and DNA Components on the Expression of Maturation Markers

In order to determine the effects of DNA and protein components on co-stimulatory molecules and MHC-II expression, DCs were cultured in the presence of DNA and protein components. The expressions of MHC-II and co-stimulatory molecules were analyzed by flow cytometry. About 70% of DCs expressed mouse-specific DC marker CD11c (data not shown).

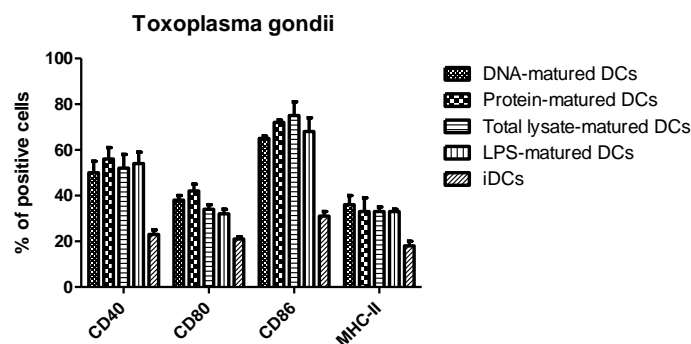
As shown in Figure 1, co-stimulatory molecules and MHC-II expression were not significantly different between the two groups. In addition, analysis of mean fluorescence intensity (MFI) showed similar expression levels of MHC-II CD80, CD86 and CD40 molecules (data not shown).

Protein-Matured DCs Produced Higher Level of IL-12p70

To compare the effects of DNA and protein components derived from intercellular microorganisms on IL-12p70 production by DCs, supernatants of

cultured DCs were collected and cytokine concentration was measured by ELISA method. Protein-matured DCs produced higher level of IL-12p70 compared to DNA and total lysate-matured DCs (Figure 2a). Moreover, Protein-matured DCs produced higher level IL-12p70 compared to LPS-matured DCs. The results also showed a significant increased production of TGF- β in the supernatant of DNA-matured DCs compared to protein-matured DCs and iDCs. The lowest concentration of this cytokine was found in supernatants of protein-matured DCs.

(a)



(b)

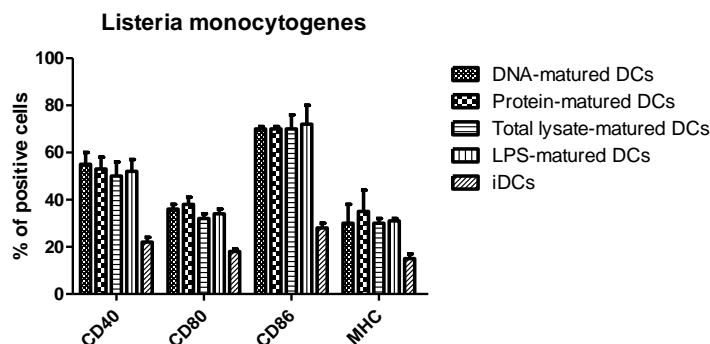


Figure 1. Phenotypic changes of BMDCs in response to different components of (a) *T. gondii* and (b) *L. Monocytogenes*. iDCs were exposed to DNA and protein components of these two pathogens. On day 7, iDCs and mDCs were stained with FITC-conjugated mAbs against cell surface molecules CD86, CD80, CD40, and MHC-II and then were analyzed by flow cytometry for the expression of DC maturation markers. Bars indicate means \pm SEM. Data are representative of at least 3 independent experiments. Isotype-identical antibodies served as negative controls.

Effects of Microbial Products on DCs Maturation

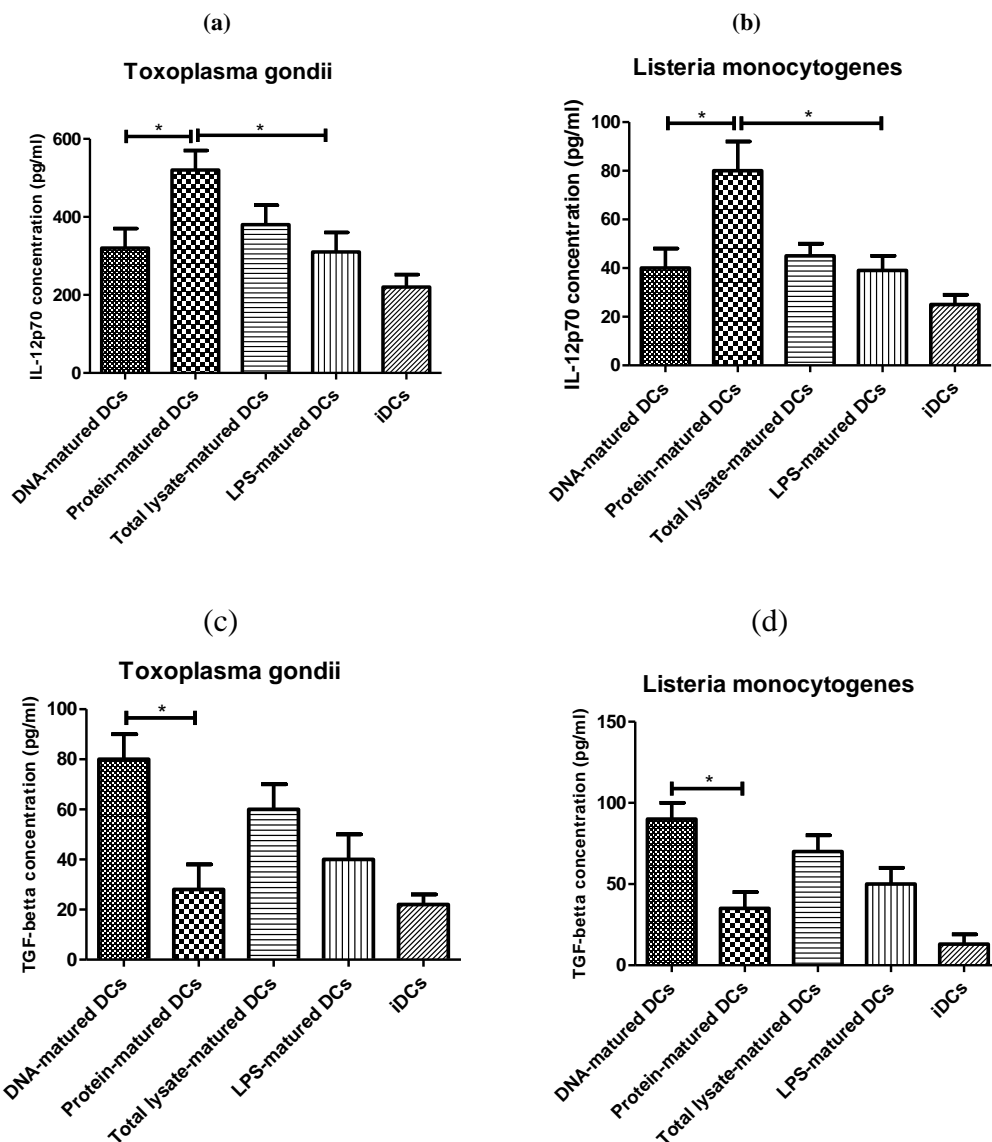


Figure 2. Cytokine production of DCs and T cells. a, b) iDCs were cultured in the presence of DNA and protein components of *T. gondii* and *L. monocytogenes*. On day 7, supernatants were collected and the concentration of IL-12 and TGF- β were determined. c, d) DCs and splenic cells were co-cultured and subsequently IFN- γ and IL-10 concentration were measured in the supernatants. Bars indicate means \pm SEM. Data are representative of at least 3 independent experiments. *, $P \leq 0.05$.

Protein-Matured DCs Drive T Cells toward Th1

To assess the capacity of DCs in inducing T cells toward Th1 or Th2, IL-10 and IFN- γ concentrations in co-culture supernatants of DCs and T cells were determined. As shown in figure 2b, *T. gondii*-derived protein efficiently matured DCs to drive T cells toward producing IFN- γ cells. Accordingly, minimum amount of IL-10 production was seen in protein-matured DCs. The IL-10 concentration in iDCs was significantly higher compared to the other groups.

Proliferation of T Cells Induced by Protein-Matured DCs

To compare the capacity of matured DCs to induce T cell proliferation, primed splenocytes and matured DCs were co-cultured for 96 h. The cell proliferation of the responders was determined by BrdU uptake using ELISA method. Compared with both LPS and DNA-matured DCs, protein-matured DCs significantly increased capacity to induce T cell proliferation (Figure 3).

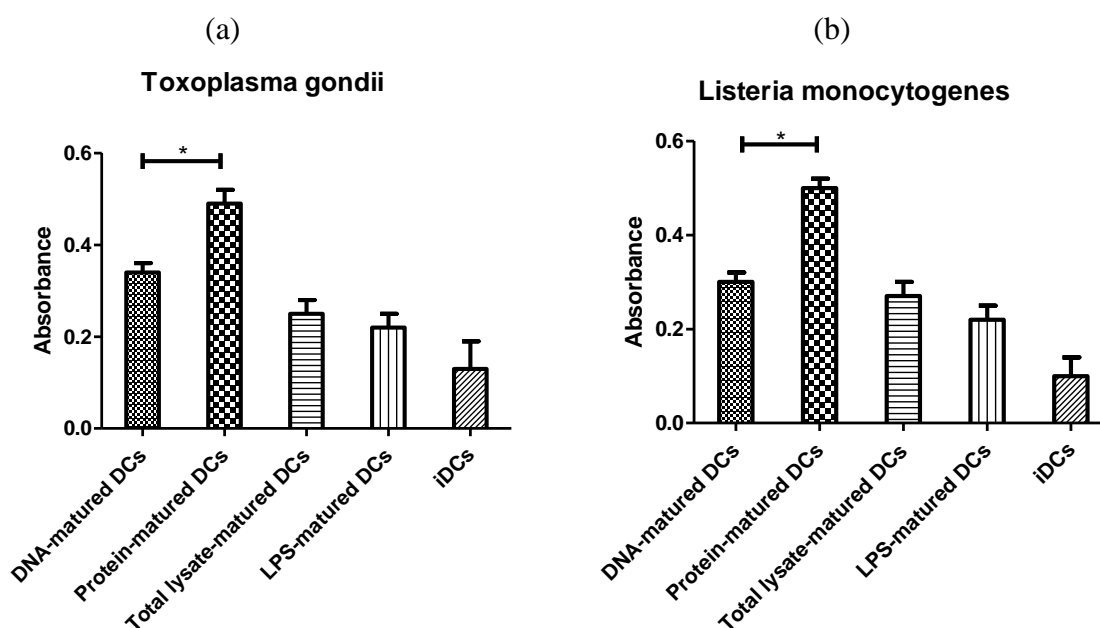


Figure 3. T cell proliferation induced by DCs matured in the presence of different components of (a) *T.gondii* and (b) *L.monocytogenes*. DCs matured by DNA and protein components were co-cultured with primed lymphocytes. After 5 days, the proliferation of lymphocyte was determined by BrdU ELISA method. Bars indicate means \pm SEM. Data are representative of at least 3 independent experiments. * $P \leq 0.05$.

DISCUSSION

DCs play a primary role in host defense in that they are the only APCs capable of activating naive lymphocytes, resulting in the initiation of protective immune responses against intracellular pathogens.^{9,13,26} Critical to this function is a program of maturation induced by microbial products that enhances the Ag-presenting and costimulatory functions of DCs. In the present study, we have demonstrated that microbial components have differential effects on DC maturation. The results showed that protein components of *T. gondii* and *L. monocytogenes* compared to DNA components are more potent in activating DCs and skewing immune response toward Th1. In contrast, a regulatory nature for DNA components was found. DNA matured-DCs skew immune response to Th2. Consistent with our study, another study showed that different microbial compounds polarize the maturation of human myeloid DCs into stably committed Th1 cell-promoting (DC1) or Th2 cell-promoting (DC2) effector DCs.²⁷ They demonstrated that protein extract derived from the helminth *Schistosoma mansoni* induced the development of DC2 cells while toxin from the

intracellular bacterium *Bordetella pertussis* induced the development of DC1s with enhanced IL-12 production.

Until now, several microbial products have been introduced which are able to mature DCs. In good agreement with this, numerous studies showed that proteins derived from different microorganisms efficiently mature and activate DCs.²⁸ One study showed that *Trypanosoma cruzi* Tc52-released protein induces human DC maturation.²⁹ They showed that Tc52-treated iDCs acquire CD83 and CD86 expression. Another study demonstrated that culture of iDCs with lipopeptides from *Mycobacterium tuberculosis* induced DC maturation via TLR-2³⁰. One study showed that *Listeria*-derived ActA (Actin A) is an effective adjuvant in cancer immunotherapy as it can stimulate APCs such as DCs.³¹ Several studies revealed that some protein components in *T. gondii* are able to activate DCs to produce IL-12. One study identified a profilin-like protein from the protozoan parasite *T. gondii* that generates a potent IL-12 response in murine DCs.³² These studies are consistent with the current study demonstrating that protein components derived from microorganisms are potent activators for DCs maturation and could be considered for DC-based

Effects of Microbial Products on DCs Maturation

vaccines in cancer immunotherapy.

In contrast, our study demonstrated that DNA components derived from *L. monocytogenes* and *T. gondii* inhibit DC maturation. Based on the achieved data, it can be speculated that DNA components derived from these two organisms have regulatory nature and can induce tolerogenic DCs. Until now, there was controversial data about the effects of nucleic acids on DC maturation and immune response activation. One study has demonstrated that bacterial DNA and CpG-ODN cause maturation and activation of DCs to bring about conversion of iDC into professional APCs.³³ However, there is in fact some evidence that DNA components inhibit immune system responses.³⁴⁻³⁶

One study also has demonstrated that CpG-ODNs can induce both stimulatory and regulatory responses. This study showed that CpG-ODNs induce selective Indoleamine 2,3-dioxygenase (IDO) up-regulation in splenic DCs, and these DCs can be considered as tolerogenic DCs.³⁷ Another study showed that CpG treated-DCs expand regulatory T cells following injection into tumor bearing mice.³⁸ The underlying mechanisms for suppressive effects of nucleic acids are not fully understood yet. DNA components are capable to increase IDO expression in DCs.³⁷ In addition, IL-10 production by macrophages probably is induced via TLR-mediated MyD88- or TRIF-dependent pathways, as well as via non-TLR signals.³⁹ Based on these achieved data, inhibitory properties for DNA components can be supposed. Therefore, it is thought that DNA components could be a candidate for producing tolerogenic DCs.

In conclusion, different PAMPs can have varying effects on APCs. The current findings suggest that protein and DNA components of microorganisms have differential impacts on professional APCs such as DCs. It has been proposed that three types of PAMPs including type 1, type 2 and regulatory can be found in pathogens that promote the development of naïve T cells into Th1, Th2 or regulatory T cells, respectively.⁹ It seems that DNA components can be further investigated for a good candidate to make DCs tolerogenic to attenuate immune responses in autoimmune diseases. Oppositely, protein components induce DC maturation and can be used to instruct immune responses against tumor antigens. The exact mechanisms involved in these effects of PAMPs on immune cells need to be clarified and explained by

further experiments.

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