Innate Immune Responses in the *Alternaria*-Dendritic Cell Interaction

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ABSTRACT

Exposure to spores and hyphae of *A*lternaria *alternata*, an airborne ubiquitous fungus, is clinically associated with allergic airway disorders including allergic rhinitis, asthma, and chronic rhinosinusitis. Dendritic cells are known as the type of antigen presenting cells most often associated with allergic inflammation. In this study, we used mouse bone marrow-derived dendritic cells (BMDCs) as a model to study the ability of *A. alternata* spores and different components of the spore cell wall to stimulate innate immune responses. We found that BMDCs were highly sensitive to *A. alternata* spores, chitin and the major allergen Alt a 1. Following stimulation with these molecules, the expression of MHC II and other co-stimulators, like CD40, CD86, and OX40L, were highly up regulated. In order to determine how different cell wall components affect the T cells, we conducted co-culture experiments of BMDCs and lymphocytes in this study. Both spores and Alt a1 did not induce IL-4 in mixed lymphocytes reactions. Interestingly, we found that Alt a 1 induced the switching of the CD4+ T cell population to the Th17 type, with a major increase in IL-17A secretion. This study reveals that *A. alternata* components may balance the innate immune responses between Th2 and Th17 pathways, and/or contributes to the development and exacerbation of more serve neutrophilic forms of asthma.

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GENERAL ABSTRACT

*A*lternaria *alternata*, is an airborne ubiquitous fungus, which is associated with allergic airway disorders including allergic rhinitis, asthma, and chronic rhinosinusitis. While dendritic cells, which are known as the classical antigen presenting cells, play an important function in antigen recognition at the early stage of immune response and then pass the signal to other immune cells. In this study, we used mouse bone marrow-derived dendritic cells (BMDCs) as a model to study the ability of *A. alternata* spores and different components of the spore cell wall to stimulate innate immune responses. As the results in this study, we found that BMDCs were highly sensitive to *A. alternata* spores and their cell wall components. Following stimulation with these molecules, the expression of major histocompatibility complex (MHC) molecules and other co-stimulators, were highly up regulated. In order to determine how different cell wall components affect other immune cells through BMDCs, we conducted co-culture experiments of BMDCs and different T cells population in this study. Both spores and cell wall component did not induce IL-4, a featured Th2 cytokine, in those co-culture studies. Interestingly, we found that cell wall component induced the switching of the T helper cell population to the Th17 type. This study reveals that *A. alternata* components may balance the innate immune responses between Th2 and Th17 pathways.

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Chapter I

Introduction and Overview of Research

Background

1. Allergic Inflammation

Allergic disorders are increasingly prevalent in the developed world and include allergic rhinitis (also known as hay fever), atopic dermatitis (also known as eczema), allergic (or atopic) asthma and food allergies[1]. Inflammation is produced in sensitized subjects (allergen-specific IgE) after re-exposure to a specific allergen. A single allergen exposure in sensitized patients produces an acute reaction, which is known as an early-phase reaction or a type I immediate hypersensitivity reaction/response. In many subjects, this is followed by a late-phase reaction. With persistent or repetitive exposure to allergen in sensitized patients, chronic allergic inflammation develops, often with associated tissue alterations such as airway remodeling[2].

In 1989, the idea that exposure to certain infections may decrease the risk of allergy was proposed as the “hygiene hypothesis”. This theory was extensively explained by immunologists and has become an important framework for the study of inflammatory disorders. In short, the hygiene hypothesis states that a lack of early childhood exposure to infectious agents (especially Th1-driving microbes like bacteria and viruses), symbiotic microorganisms (such as the gut flora or probiotics), and parasites increase susceptibility to allergic diseases by suppressing the natural development of the immune system[3]. This hypothesis has received the greatest attention in explaining the increase in asthma prevalence in recent decades.

Asthma is an immunological disease that has increased dramatically in prevalence over the past two decades. In industrialized countries, the incidence of asthma has nearly doubled since 1980. Meanwhile, in the United States and other industrialized countries, one in five to ten individuals may be affected in certain geographical regions. This disease is thought to be caused by a combination of genetic predisposition and environmental factors, such as allergen and pollution exposure and infections[3]. There are at least a dozen polymorphic genes found to be associated with the development of and exacerbation of asthma, controlling IgE, predominantly T helper type 2(Th2) cytokine and chemokine production as well as airway function and airway remodeling[4].The underlying reasons for the development of Th2 biased immune response associated with allergic inflammation including the over production of cytokines such as interleukin 4 (IL-4), IL-5, IL-13, still remains unclear[4].

Dendritic cells, known as the most efficient antigen presenting cells, are also a key player in the allergic inflammation process. Despite the known functions of DCs in primary immune responses and allergic sensitization, it is less clear whether airway DCs are also required for the presentation of allergens to resting memory Th2 and/or effector Th2 cells during a secondary immune response[5]. Suzuki et al (2009) demonstrated that bone marrow-derived DCs in which CD40 expression was silenced by a small interfering RNA approach could be used to both prevent and suppress established disease in antigen-specific manner in murine model of allergic rhinitis[6-7]. Hence, we can try to use modified DCs to suppress inflammation in our research or as a target cell for the development of novel therapeutics.

2. *Alternaria alternata*

Asthma can develop and may be exacerbated in response to a multitude of allergens and pathogens, including respiratory viruses (especially human rhinovirus [HRV]), pollen, indoor fungi (related to house mold/dampness), house dust mite, outdoor fungi and air pollutants (such as ozone and nitrogen dioxide)[8]. Among those factors, fungi constitute one of the most abundant and important immunogens. *A. alternata* is a clinically relevant allergen source that is associated with severe and fatal asthma exacerbations. With > 80% of asthmatic in the United States having sensitization to one or more fungi, of these individuals, 75% have reactivity to *A alternata*[9]. Exposure to *A. alternata* is characterized by a predominant Th2 response[9]. Mice treated with *A. alternata* develop an allergic airway response characterized by increase in lung expression of the Th2 cytokines IL-4 and IL-13, and high levels of serum IgE[9]. Other studies showed that *A. alternata* can also induce the production of factors associated with Th17 responses from epithelial cells[10]. In our proposed studies we will measure the expression level of several Th17-associated cytokines, such as IL-17A, in response to *A. alternata* stimulation as described later.

Fungi are ubiquitous and the species and numbers of fungi present in the air that we breathe depend on regional variation in vegetation, temperature, humidity and seasonal change[11]. All of those fluctuating factors may make studies difficult. However, the reason why outdoor fungi can trigger asthma exacerbations is mainly due to their allergenic proteins, other pro-inflammatory components such as chitin, and their small size (often 1-20 um in diameter), which provide a prerequisite for them to travel deep in the airways, causing inflammation and allergic responses. At present, a total of 17

allergenic proteins are known as allergens of *A. alternata* and listed in allergen database platforms (http://www.allergen.org and http://www.allergome.org/)[12-13].

Alt a 1 is an *Alternaria* allergen predominantly located in the cell wall of airborne spores that can enter the respiratory tract and induce allergic inflammation in patients affected by mold sensitization[13]. Approximately 80-100% of the patients who are sensitized to *A. alternata* have Alt a 1-specific IgE and thus can be considered a major, species-specific allergen[14]. Hence, Alt a 1 is considered to be a marker of primary sensitization to *A. alternata*. Natural Alt a 1 is a ~30kDa hetero-dimer that dissociates into 16.4 and 15.3kDa subunits under reducing conditions[13]. Research suggests that Alt a 1 possesses a highly stable dimeric structure presenting epitopes with proper orientation for IgE cross-linking compared with other allergens[13]. Kurup et al (2003) stated that there are two linear epitopes identified in the Alt a 1 protein sequence (K41-P50 and Y54-K63) showing reactivity with serum IgE from an *Alternaria* allergic patient[14]. In a recent study, recombinant Alt a 1 has been produced by molecular cloning and expression in *E. coli* and *Pichia pastoris* and retains its IgE reactivity[13]. Clinical research can use this recombinant allergen to improve diagnosis of mold allergy.

We already know the importance of Alt a 1 from what is described above. However, the true mechanism of the function of this major allergen remains unknown. Some hypotheses were raised by several labs. Mitakakis et al (2001) states that Alt a 1 might be involved in spore germination since it was found to be remarkably higher in germinating spores[14]. Cramer and Lawrence (2004) showed that the *A. brassicicola* Alt a 1 homolog was found to be highly expressed during the infection process of the model flowering plant *Arabidopsis thaliana* and may serve as a fungal virulence factor[15]. In

addition, Gomez-Casado et al (2014) demonstrated that Alt a 1 was found to induce the expression of and bind to plant defense proteins belonging to the PR5-TLP family and interact with it as a competitive inhibitor[16].

Alt a 1 is a canonical maker for *A. alternata* allergy. Some research showed that high levels of Alt a 1 are detected in the atmosphere even when spores are absent[13]. While the presence of Alt a 1 may be due to other fungi of the *Pleosporaceae* family that also express homologs, spores from these fungi were absent in the aerobiological analyses[17]. Although the sample size in this study is limited to some extent, this result may tell us that we need to investigate the Alt a 1 allergen in greater detail[17].

In former studies in our lab, Alternaria spores and recombinant Alt a 1 protein have been found to potently stimulate innate immune responses in different human and murine cell lines (Babiceanu et al., 2013; Hayes et al., 2018 unpublished). As described later, I plan to use spores and Alt a 1 to treat dendritic cells and measure cytokines and chemokines.

3. Chitin

Chitin, a potential allergy-promoting pathogen-associated molecular pattern (PAMP), is a linear polymer composed of N-acetylglucosamine residues which are linked by β-(1,4)-glycosidic bonds[18]. Chitin is the second most abundant polysaccharide in nature, which cannot be synthetized by mammals (**Figure 1-1**). However, mammals are potential hosts for chitin-containing protozoa, fungi, arthropods, and nematodes. Thus, chitin is considered as a possible target for recognition by the mammalian immune system. Since chitin is ubiquitous, exposure of the immune system to chitin is extremely

high. Former studies state that chitin was shown to be sensed primarily at host-pathogen interfaces, such as the gut or lungs, where it is active and cells of both innate and adaptive immunity are recruited and induce Th2 responses characteristic of allergies[18-19].

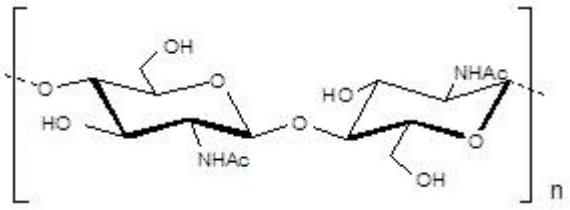

Figure. 1-1 Chemical structure of chitin. [20]

It has been shown that chitin has complex and size-dependent effects on immune responses. Large size chitin polymers (such as those of chitin containing pathogens) are biologically inert, while the small fragments of chitin have been shown to effectively induce immune response, which include recruitment of eosinophils, macrophages and IL-4/IL-13 expressing T helper 2 lymphocytes[21]. Also, the activation of IL-10 regulatory cytokine production was observed at the same time, which may play an anti-inflammatory role[22].

Moreover, chitin is a well-established PAMP that induces immune responses and during fungal, insect and nematode infections in plants[23]. Hence, we assume that there may be a similar mechanism in mammalian immune system. In order to explore the function of chitin, intranasal or intraperitoneal administration of chitin (varying in size, degree of acetylation and purity) to mice has been applied as a routine approach[22].

Chitin has three different crystalline allomorphs namely theα-chitin (the most prevalent form), β-chitin, and γ-chitin (found in mushrooms)[22]. Structural chitin present in microorganisms is thought to be degraded by host chitinases, such as acidic

mammalian chitinases. Smaller degraded molecules are thought to be sensed by mammalian receptors like FIBCD1, NKRP1 and RegIIIc, which are highly expressed enterocytes[22]. The immune recognition of chitin also involves pattern recognition receptors. Toll-like receptor (TLR) 2, Dectin-1 (β-glucan receptor mediates T helper 17 development and recruitment of neutrophils), and mannose receptors are all thought to participate in chitin signaling (**Figure 1-2**)[22]. Although a couple of signaling pathways have been proposed, the bona fide receptor for chitin recognition is not yet clear.

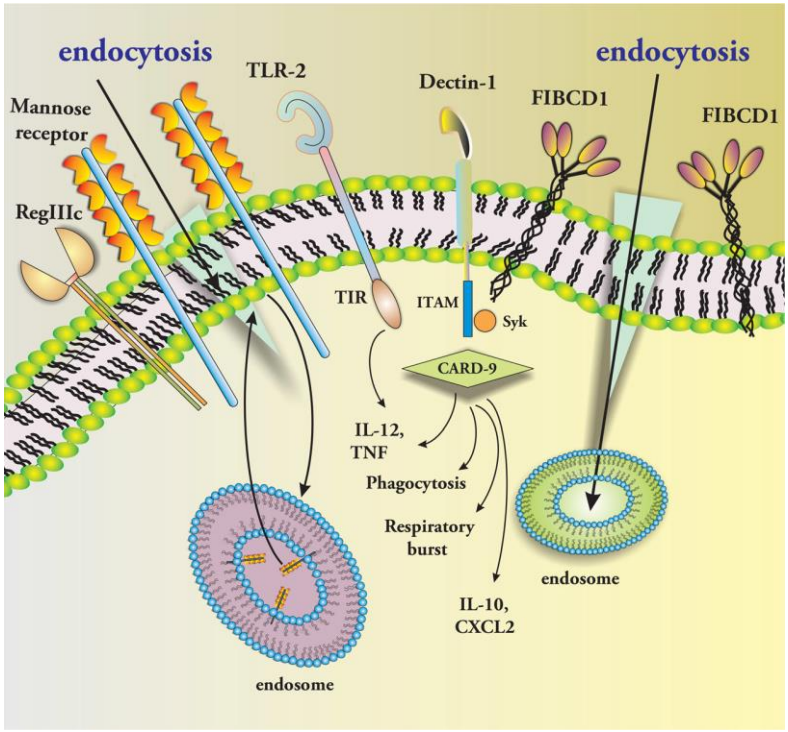

Figure. 1-2 Chitin signaling receptors from various superfamilies sense chitin fragments through different pathways and activate signaling. FIBCD1 binds chitin and directs acetylated components for degradation in the cytoplasmic endosomes through endocytosis. Dectin-1 through its signaling induces the respiratory burst and phagocytosis. TLR-2 signaling mediated by TIR domain results in inducing IL-12 and TNF production similar to Dectin-1. Mannose receptors participate in endocytosis of chitinous materials along with formation of endosomes. Mannose receptors in a pH-dependent manner dissociate from ligands and recycle back to the plasma membrane[22]

4. LysM

LysM (lysin-motif) domains found in proteins are a family of carbohydrate-binding modules with a length of approximately 40-50 amino acids and bind to N-acetylglucosamine (GlcNAc)-containing glycans, such as chitin, chitin-like compounds and peptidoglycan[24-25]. LysM-domain containing proteins (LysMs) have a βααβ structure in which the two β-strands form an antiparallel β-sheet. The abbreviation LysM is derived from the original name lysin motif, because they were first found in bacteriophage proteins. Then, LysM domains were also found in bacterial and eukaryotic proteins, and recently it has been established that they exist in all Kingdoms of life[25-27].

In reference to the putative chitin signaling pathways described above, **Figure 1-2**[22], we observe an ITAM motif in the intracellular part of Dectin-1. The ITAM motif is an acronym for Immune-receptor Tyrosine-based Activation Motif. It has been shown that signal transduction by the T cell and B cell antigen receptors and by receptors for a variety of immunoglobulins' Fc region is strictly dependent on a receptor subunit cytoplasmic ITAM motif[28]. ITAM motifs possess the consensus sequence YxxI/Lx(6–12)YxxI/L[29]. Receptor engagement is followed by a rapid and transient phosphorylation of tyrosine residues within their ITAMs, thereby creating temporary binding site(s) for Src homology 2 (SH2)-containing signaling molecules, such as SHP1/SHP2 and Syk, operating downstream of the activated receptor. On the other hand, the immune system may also employ negative regulatory receptors, immunoreceptor tyrosine-based inhibition motif (ITIM), with a consensus sequence of S/I/V/LxYxxI/V/L[29]. Ligand engagement by inhibitory receptors results in ITIM phosphorylation by Src and

recruitment of phosphotyrosine phosphatases (PTP), such as SHP-1 and SHP-2, or SHIP. This process can play an opposite role with ITAM-containing receptors[29].

Former and current research in our lab suggests that some LysM proteins in mammals (mouse and human), such as LysMD1, LysMD2, LysMD3 and LysMD4 may be receptors of and are involved in chitin signaling pathways. Mammalian LysM-containing proteins may recognize chitin by themselves or form a complex to initiate downstream signaling. We have already shown using microscopy, biochemical approaches, and gene specific siRNAs that LysMD3 is a transmembrane protein localized in the plasma membrane of epithelial and macrophage cells, which can activate the expression of pro-inflammation cytokines, such as IL-8 and IL-6 after stimulation by chitin, fungal spores, and peptidoglycan-like molecules (LPS and PAM3CSK4). Interestingly, similar immunological results were obtained for LysMD2. However, it is important to point out that unlike LysMD3, LysMD2 does not have a predicted membrane-spanning domain and is thought to be localized in the cytoplasm. Interestingly, we noticed that there is one half of a canonical ITAM domain in LysMD3(**Figure 1-4**), while there are two half ITAM domains in LysMD1(**Figure 1-3**). Interestingly, an ITIM motif was observed in LysMD4(**Figure 1-5**), which may function specifically as a negative regulator of inflammation. Indeed, experiments in our lab showed that knockdown of LysMD4 in human airway epithelial cells using gene specific siRNAs resulted in increased levels of cytokine and chemokine secretion (He et al., 2018, unpublished).

```
      10        20        30        40        50
MASPSRQPPP GGSGLLQGSR ARSYGSLVQS ACSPVRERRL EHQLEPGDTL
      60        70        80        90       100
AGLALKYGVT MEQIKRANRL YTNDSIFLKK TLYIPILTEP RDLFNGLDSE
     110       120       130       140       150
EEKDGEEKVH PSNSEVWPHS TERKKQETGA GRANGEVLPT PGQETPTPIH
     160       170       180       190       200
DLSASDFLKK LDSQISLSKK AAAQKLKKGE NGVPGEDAGL HLSSPWMQQR
     210       220
AVLGPVPLTR TSRTRTLRDQ EDEIFKL
```

Figure 1-3 Amino acid sequence of LysMD1. Highlighted parts are two half ITAM domains.

```
           10        20        30        40        50
MAGRHQNRSF PLPGVQSSGQ VHAFGNCSDS DILEEDAEVY ELRSRGKEKV
      60        70        80        90       100
RRSTSRDRLD DIIVLTKDIQ EGDTLNAIAL QYCCTVADIK RVNNLISDQD
     110       120       130       140       150
FFALRSIKIP VKKFSSLTET LCPPKGRQTS RHSSVQYSSE QQEILPANDS
     160       170       180       190       200
LAYSDSAGSF LKEVDRDIEQ IVKCTDNKRE NLNEVVSALT AQQMRFEPDN
     210       220       230       240       250
KNTQRKDPYY GADWGIGWWT AVVIMLIVGI ITPVFYLLYY EILAKVDVSH
     260       270       280       290       300
HSTVDSSHLH SKITPPSQQR EMENGIVPTK GIHFSQQDDH KLYSQDSQSP

AAQQET
```

Figure 1-4 Amino acid sequence of LysMD3. Highlighted part is half ITAM domain.

```
      10        20        30        40        50
MRHEELLTKT FQGPAVVCGT PTSHVYMFKN GSGDSGDSSE EESHRVVLRP
      60        70        80        90       100
RGKERHKSGV HQPPQAGAGD VVLLQRELAQ EDSLNKLALQ YGCKVADIKK
     110       120       130       140       150
VNNFIREQDL YALKSVKIPV RNHGILMETH KELKPLLSPS SETTVTVELP
     160       170       180       190       200
EADRAGAGTG AQAGQLMGFF KGIDQDIERA VQSEIFLHES YCMDTSHQPL
     210       220       230       240       250
LPAPPKTPMD GADCGIQWWN AVFIMLLIGI VLPVFYLVYF KIQASGETPN
     260       270       280       290
SLNTTVIPNG SMAMGTVPGQ APRLAVAVPA VTSADSQFSQ TTQAGS
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Figure 1-5 Amino acid sequence of LysMD4. Highlighted part is one ITIM domain.

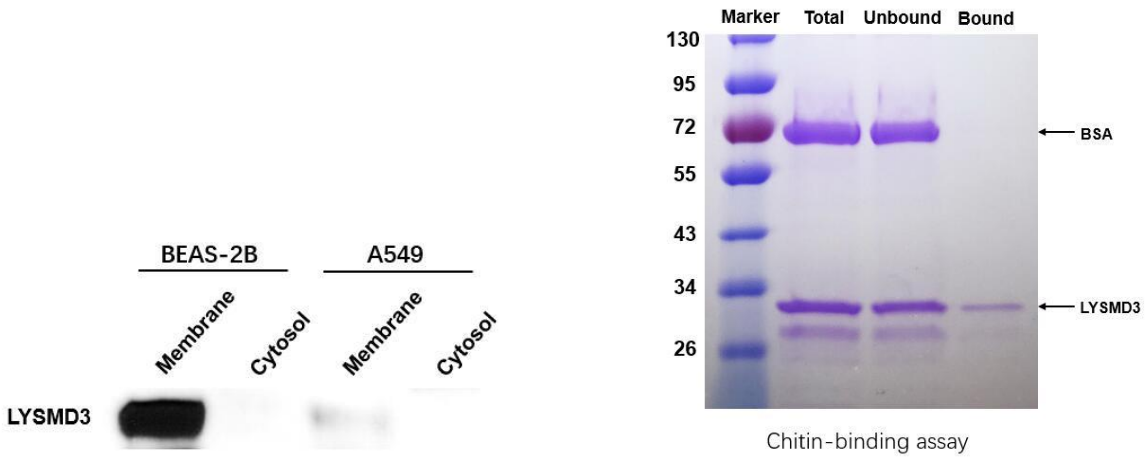


Figure.1-6 Cytosolic and membrane fractions expressed at the surface of pulmonary epithelial cells (left), LysMD3 is a chitin-binding protein (right). Work performed by Xin He, Lawrence lab.

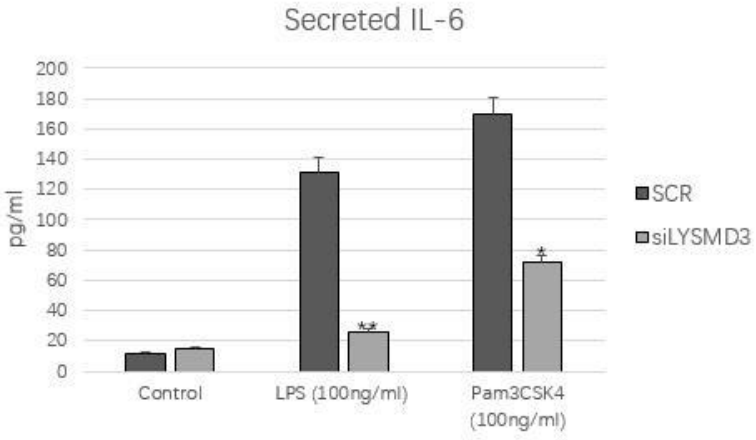


Figure 1-7. Silencing of LysMD3 transcripts results in significantly reduced pro-inflammatory cytokines secretion in human bronchial epithelial cells after stimulation with TLR ligands/Chitin. Work performed by Xin He, Mengyao Luo in Lawrence lab.

ELISA was conducted to measure the secreted IL-6 and IL-8 in different cell lines after treatment with different antigenic ligands. We found that after we use a siRNA transfection method to knockdown LYSMD3 and then treat cells, the secreted IL-6 and IL-8 decreases substantially compared to the scrambled control siRNAs.

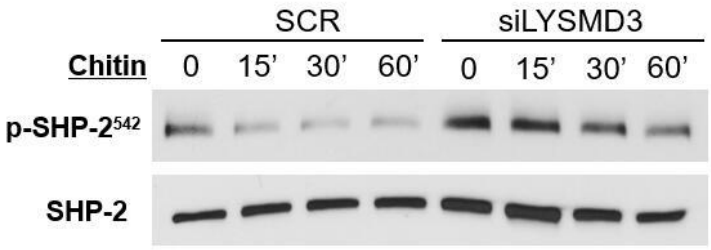


Figure 1-8 LYSMD3 knockdown affects phosphorylation of SHP-2 in Beas-2B cells. Work performed by Xin He, Mengyao Luo in Lawrence lab.

In addition, western blotting was conducted to determine if there is a correlation between SHP-2 and LysMD3. We found that following knockdown of LysMD3 with siRNAs resulted in enhanced phosphorylation of SHP-2. Now we would like to characterize the LysMD3 is a transmembrane protein, which can bind chitin as demonstrated in the Lawrence lab previously (**Figures 1-8**). For this project we now would like to characterize the response of dendritic cells to fungal spores and cell wall molecules such as chitin and the allergen Alt a 1.

Proposed Research

Hypothesis: *Alternaria and associated molecules induce immune responses in dendritic cells that are dependent upon pattern recognition receptors and downstream signaling components.*

Aim 1- Elucidate and characterize how Alt a 1, *Alternaria* spores and chitin stimulate dendritic cells harvested from C57BL/6 mice bone marrow, IRAK-1 KO and LysMD3 KO mice bone marrow.

As described previously, dendritic cells (DCs) are key immune cells that orchestrate the innate and adaptive immunity by playing a central role in antigen presentation, initiation and regulation of immune responses. In previous experiments in our lab, Alt a 1, Alternaria spores and Alternaria extract treated BALB/c mice suggest an obvious Th2 inflammatory response in vivo (**Figure 1-9**).

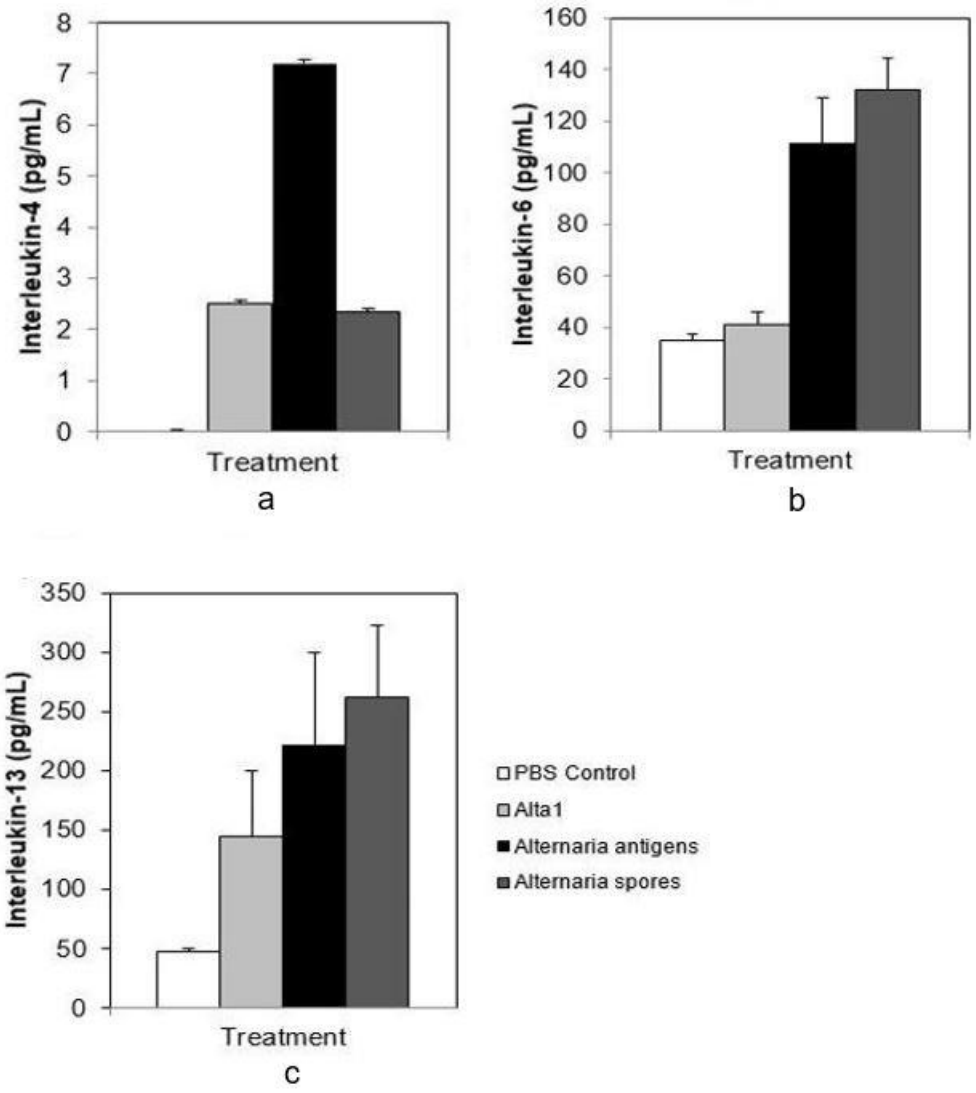


Figure 1-9. **Female BALB/c mice were anaesthetized by isoflurane and then intranasally challenged every other day over 14 days with equal volume of PBS(control), Alt a1(10ug), *Alternaria* antigens(50ug), or *Alternaria* spores (5x10000) Mice were euthanized 24h after the last challenge and lungs were immediately washed with 1mL of PBS.** Cell-free BALF was used for ELISA quantification of cytokines. (**a**) IL-4 (**b**) IL-6 (**c**) IL-13, N=4/group. Preliminary data. Work performed by Amanda Cornin-Rumore, Lawrence Lab.

However, the role of DCs in response to Alternaria spores or associated molecules like Alt a 1 or chitin has not been explored. Previous research in our lab has shown that Alt a 1 signaling in lung epithelial cells is dependent upon PRRs and associated

adaptors (TLR4, TLR2, MyD88, TIRAP, and IRAK-1). We will use TLR4 (and other pathway members) specific siRNAs, inhibitors, or blocking antibodies to investigate the role of these components in Alt a 1-induced immune responses specifically in DCs. Interestingly, previous experiments showed that there is a large increase of levels of IL-6 in wild type mouse BMDCs after stimulation with Alternaria spores or associated molecules like Alt a 1 or chitin (**Figure 1-10**).

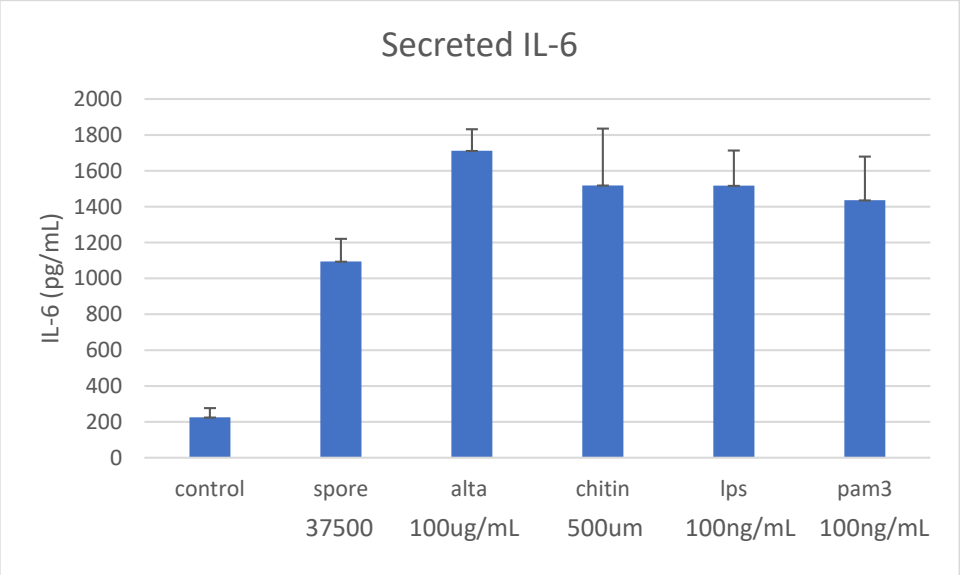


Figure.1-10 Increase of IL-6 secretion in wild type C57BL/6 BMDC after stimulation with *Alternaria* spores or associated molecules like Alt a 1 or chitin.

Methods and experimental plan

1) Generation of DCs (BMDCs) from mouse bone marrow cultures supplemented with Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF).

   A 6-day procedure for generating BMDCs from whole murine bone marrow precursors by Lutz et al (1999)[30] will be used for my experiments. Bone marrow will be harvested first. GM-CSF will be used to induce the differentiation of DCs. We

expect to obtain approximately 10^7 dendritic cells in 5-6 days. Immature BMDCs are obtained on day 6 of the culture in the presence of GM-CSF, which is more likely to play a role in antigen capture. These cells are characterized by high endocytic activity and low T-cell activation potential. Treatment of immature BMDCs on day 6 of culture with a maturation stimulus such as LPS (100 ng/ml) for 24h results in the maturation. Mature DCs may allow antigen presentation and T-cell activation[31].

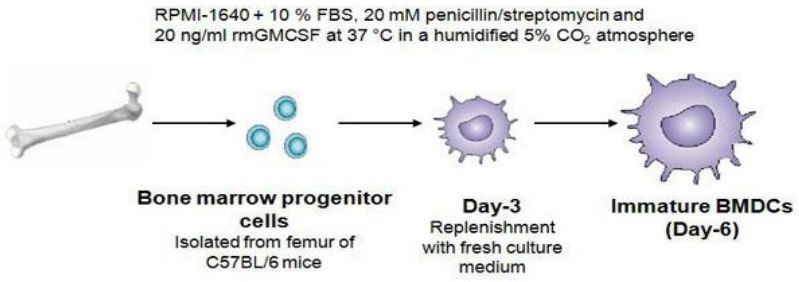


Figure. 1-11 Bone marrow harvest for DCs[31]

2) We will first collect the immature DCs. Surface expression of CD11c (a murine DC-specific marker) will be analyzed in BMDCs by flow cytometry.
3) Use *Alternaria* spores (5,000-100,000 spores), Alt a 1 (1 ug-100 ug/ml), and chitin (500um) to stimulate BMDCs.
4) After 4h, 8h, 12h, 24h we will measure IL-6, IL-8, IL-4, IL-5, IL-13, IL-25, IL-33 by ELISA. An initial screen with a cytokine array (Ray Biotech, Atlanta, GA) may also be used to detect which chemokines and cytokines are expressed following treatments.
5) Perform DC-T cell co-culture experiments and characterize the expression of markers, such as MHCII, some co-stimulators (CD40, CD80, CD252) and cytokine secretion, such as IL-4, RANTES, IL-17A.

Chapter II

Innate Immune Responses in the Alternaria-Dendritic Cell Interaction

Abstract

Exposure to spores and hyphae of *A*lternaria *alternata*, an airborne ubiquitous fungus, is clinically associated with allergic airway disorders including allergic rhinitis, asthma, and chronic rhinosinusitis. Dendritic cells are known as the type of antigen presenting cells most often associated with allergic inflammation. In this study, we used mouse bone marrow-derived dendritic cells (BMDCs) as a model to study the ability of *A. alternata* spores and different components of the spore cell wall to stimulate innate immune responses. We found that BMDCs were highly sensitive to *A. alternata* spores, chitin and the major Alternaria allergen Alt a 1. For example, following stimulation with these molecules, the expression of MHC II and other co-stimulators, like CD40, CD86, and OX40L, were highly up regulated. In order to determine how different cell wall components affect the T cell population, we conducted co-culture experiments of mouse BMDCs and lymphocytes in this study. Both spores and Alt a1 did not induce IL-4 in mixed lymphocytes reactions. Interestingly, we found that Alt a 1 induced the switching of the CD4+ T cell population to the Th17 type, with a major increase in IL-17A secretion. Although we haven't completely explained the signaling pathway by which Alternaria components induce innate immune response, this study reveals that *A. alternata* components may balance the innate immune responses between Th2 and

Th17 pathways, and/or contributes to the development and exacerbation of more serve neutrophilic forms of asthma.

Introduction

Allergic disorders are increasingly prevalent in the developed world and include allergic rhinitis, atopic dermatitis, allergic asthma, chronic rhinosinusitis, and food allergies[32]. Previous studies in our lab have been focused on the ubiquitous airborne fungus, *Alternaria alternata*. Sensitization to *A. alternata* is clinically associated with allergic rhinitis and moderate to severe atopic asthma. In former studies in our lab, Alternaria spores and recombinant Alt a 1 protein have been found to potently stimulate innate immune responses in different human and murine cell lines (Babiceanu et al., 2013; Hayes et al., 2018 unpublished). In this study, bone marrow-derived dendritic cells (BMDCs) were used as a model to study the effects of Alt a1 and other fungal cell wall components on the innate immune response. Dendritic cells (DCs) are thought to be the key players in bridging the innate and adaptive immune response to *A. alternata* by specifically activating naïve CD4+ T-cells and triggering their differentiation into disparate lineages of effector cells.

Virtually all T-helper subsets have been described to play a role during fungal infection, with the Th1 response being crucial for fungal clearance[37]. However, morbidity and mortality of inflammation can also be partly attributed to detrimental immune responses resulting from adaptive immune activation. Th2 responses benefit fungal persistence, and are the foundation of allergic forms of fungal invasion[37]. The Th17

response has two sides, although crucial for granulocyte recruitment, it can be involved in detrimental immunopathology. Regulatory T-cells, the endogenous regulators of inflammatory responses, play a key role in controlling detrimental inflammatory responses during infection[37].

Interleukin-17 (IL-17) is a pro-inflammatory cytokine produced by adaptive CD4+ T helper cells and innate lymphocytes. IL-17 activates signaling through the IL-17 receptor, which induces other pro-inflammatory cytokines, antimicrobial peptides and neutrophil chemokines that are important for antifungal activity[38]. The importance of IL-17 in protective antifungal immunity is evident both in mice and humans. Hematopoietic cells such as phagocytes (neutrophils and monocytes/macrophages), adaptive Th17 cells, natural (n) Th17 cells, dendritic cells (DC), non-major histocompatibility complex (non-MHC) restricted T-cells subsets such as γδ T-cell, as well as non-hematopoietic cells including mucosal epithelial cells participate in antifungal immunity[38]. The IL-17 family consists of six cytokines (IL-17A~ IL-17F) and five receptors (IL-17RA~IL-17RE). IL-17A (IL-17) and IL-17F forms homo- and heterodimers, and signals through a receptor complex (IL-17R) consisting of IL-17RA and IL-17RC subunits[38]. The IL-17A/IL-17R signaling axis has been studied most extensively in the context of candidiasis.

IL-17 is involved as a key regulator of anti-fungal immunity through induction of a signature gene profile including pro-inflammatory cytokines, antimicrobial peptides and chemokines. In addition to Th17 cells there are important innate cellular sources of IL-17, termed “Type 17” cells. Type 17 cells are dependent on IL-23 and RORt expression

for function and IL-17 production. Type 17 subsets include Th17, natural killer T (NKT), lymphoid tissue inducer and group 3 innate lymphoid cells (ILCs)[38].

In this study we aimed to characterize the immune response of DCs to Alternaria spores and molecules by profiling cytokines, chemokines, and the expression of various cell surface markers. In addition, we characterized T cell maturation in a series of DC-T cell co-culture experiments with a particular emphasis on Th2, Th1, and Th17 associated cytokines.

Materials and Methods

Mice

C57BL/6J(B6) and BALB/c mice were kindly provided by Dr. Liwu Li, Laboratory of Innate Immunity and Inflammation, Virginia Polytechnic Institute and State University. All mice were female and 8-10 weeks in age. The procedures and handling of mice were reviewed and approved by Institutional Animal Care and Use Committee (IACUC), Virginia Tech.

Fungal culture and Alt a1

A. alternata (ATCC #66981) was used as wild-type strain in spore experiments. Fungi were propagated on potato-dextrose -agar (PDA) (0.4% potato starch, 2% dextrose, 1.5% agar) and were grown at 25°C in the dark. Spores were harvested into sterile water from a 7-day old PDA plate. Suspensions were standardized to equal concentrations of 1 x 10^6 spores/mL after counting on a hemacytometer and then immediately added to

BMDCs culture plates at a ratio of 1/2=spore/cell. Alt a 1 (1.1mg/ml) was kindly provided by INDOOR Biotechnologies Inc.(Charlottesville, VA).

Isolation of mouse bone marrow derived dendritic cells (BMDCs)

BMDCs were harvested from C57BL/6J(B6) mouse bone marrow using an established protocol with minor modification. In brief, on day 1, mouse bone marrow was obtained from both femurs and tibias, followed by flushing the contents of marrow with 10 mL of RPMI 1640 media (Sigma Aldrich) using a 1-ml insulin syringe with a 29G × ½ needle. The contents were collected into a sterile 15 mL centrifuge tube. The cell suspension was centrifuged at 400 *g* for 6mins. Afterwards, 5 mL ACK buffer were added for erythrocyte lysis. After 5 mins at room temperature, 5 mL FBS were added to stop the lysis reaction. The cell suspension was transferred into a new sterile centrifuge tube with a 70-um filter. The suspensions were then centrifuged at 400 *g* for 5 mins. The cell pellets were re-suspended in culture medium (RPMI-1640 + 10% FBS + 20 mM penicillin/streptomycin+10 ng/mL murine GM-CSF) to achieve a final cell density of 2 × 10^6 cells/mL. Mouse recombinant GM-CSF was purchased from R&D SYSTEMS. Cells were plated into 6-well tissue culture plates and cultured. On day 3, the culture medium was discarded and 3mL RPMI-1640 medium was added to each well. The plates were gently shaken 30 times. The plates were washed two more times as above. Subsequently, 5 mL fresh complete culture medium with GM-CSF was added to each well and cells were cultured at 37 ℃ 5% CO_2. On day 5, the same procedures were conducted as those on day 3. On day 6, immature BMDCs were harvested in suspension medium. The cell suspension was centrifuged and the cell pellet was re-

suspended in completed culture medium without GM-CSF. The purity of CD11c+ DCs was 85% verified by flow cytometry.

BMDCs were stimulated with wide type *A. alternata* spores in a ratio of DC/Spore=2/1, Alt a1 protein, Chitin DP6 (Elicityl SA, Grenobyl, France) for 24 or 48h. For some experiments, Toll-like receptor 4 (TLR4) blocking antibody or LPS-RS (both from Invivogen, Madison, WI) were added 3 h ahead to block the TLR4 signaling pathway.

Cytokine Array

Mouse Cytokine Array Panel A kit (R&D systems, Minneapolis, MN) was used in this study. Culture supernatants from control and activated BMDCs were used as samples. 2mL Array Buffer 6 were added into each well of a specific 4-well multi-dish. Four membranes were put to each well and incubated for one hour on a rocking platform shaker. Next, 0.5 mL Array Buffer 4 was added to each sample. The final volume was adjusted to 1.5mL with Array Buffer 6. Then, 15uL reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail was added to each sample. Samples were mixed and incubated at room temperature for one hour. Array Buffer 6 was discarded from the multi-dish. Sample mixtures were added into each well and incubated with membranes overnight at 4 ℃ on a rocking platform shaker. Membranes were washed with washing buffer for 10mins and for a total of three washes. 2mL Streptavidin-HRP was then added into each well. Membranes were incubated with Streptavidin-HRP for half hour at room temperature. Membranes were then washed three times as before. 1mL Chemi Reagent Mix was evenly pipetted onto each membrane. Multiple exposures for 10s-60s were taken on Biorad Gel Doc system (BioRad, Hercules, CA) to optimize results.

Mixed Lymphocytes Reaction

96-well U shape tissue culture plates were coated with CD3 antibody overnight at 4℃. Splenocytes were harvested from BALB/c mouse. First, mouse spleens were mashed into with RPMI-1640 (10%FBS). Cells were filtered (70um pore size) into a 15mL new sterile centrifuge tube. The cell suspension was centrifuged at 400*g* for 5mins. The cell pellets were re-suspended with 1.5mL ACK lysis buffer at room temperature for 5mins. 1.5mL FBS were added to stop lysis reaction. The cell suspension was centrifuged at 400g for 5mins. The cell pellets were re-suspended into lymphocyte growth medium (RPMI 1640+10%FBS+20 mM penicillin/streptomycin+10 mM HEPES+ 50 mM 2-ME). Lymphocytes were seeded into 96-well plate at 1×10^6 cells/mL, 100uL/well. DC/T ratio=1/5, BMDCs were seeded at 5×10^6 cells/mL,100uL/well. The final volume of medium was 200uL. BMDCs and lymphocytes were co-cultured until for 3 days.

CFSE Proliferation Assay

Splenocytes were isolated as above. For the proliferation assay, prior to co-culture of lymphocytes with activated BMDCs, lymphocytes were stained with CFSE dye (BD Biosciences). First, 90ul DMSO was added to 50ug/uL CFSE stock solution to prepare 10mM CFSE solution. Lymphocytes were diluted to a density less than 20×10^6 cells/mL with PBS. 1ul 10 mM CFSE was mixed into 3 mL of lymphocyte suspension. Tube was incubated at room temperature for 8mins. 3mL warm FBS was added to the cell suspension and incubated at 37℃ for 10mins. Direct lighting should be avoided during the whole processes. The cell suspension was centrifuged at 400g for 5mins at 4℃. The

cell pellets were washed with RPMI 1640 medium containing 10%FBS four times. The cell pellets were re-suspended into complete lymphocyte growth medium.

Flow cytometry

For intracellular staining, BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). After co-culture for 3 days, the cell suspension was centrifuged in 96-well U shape plates at 400xg for 5mins. Supernatants were collected for ELISA. 4uL BD GolgiStop Protein Transport Inhibitor (BD Biosciences) was added for every 6mL of cell culture medium and mixed thoroughly. The cell pellets were re-suspended into single suspensions with 100uL mixture medium. The cell suspensions were incubated at 37℃, 5%CO_2 for 4 hours.

The cell suspension was centrifuged at 400xg for 5mins and medium containing inhibitor was discarded. The cell suspension was then incubated with 1ug Fc Block/10^6 cells in 100ul HANKS staining buffer for 15mins at 4℃. Anti-CD4, anti-CD8 and anti-ST2 antibodies (Biolegend Inc, CA) were added to each well and incubated with the cells for 30mins at 4℃. Fixation/permeabilization solution were added and incubated for 20mins at 4℃ to fix and permeabilize cells. 1× BD Perm/Wash buffer was used to wash cell pellets. Then, anti-IL4 and anti-IFN-α antibodies (Biolegend Inc, CA) were used to stain intracellular cytokines., Flow cytometry (FACS Calibur, BD Biosciences) was then performed to analyze the change of surface markers and intracellular cytokines.

Purification of CD4+ T cells

MojoSort™ Mouse CD4+ T Cell Isolation Kit was used to isolate CD4+ T cells (Biolegend Inc., CA). Splenocytes were harvested from 6-8 weeks old female BALB/c mice. 1.5 mL ACK buffer was added to lyse erythrocytes. The cell suspension was

centrifuged and re-suspended with 4ml MojoSort™ buffer in a 5mL (12 × 75 mm) polypropylene tube and kept on ice. The cell suspension was filtered with a 70um cell strainer and was centrifuge at 300xg for 5 minutes. The cell pellets were re-suspended with an appropriate volume of MojoSort™ Buffer. The cell suspension was counted and adjusted to 1 x 10^8 cells/mL. 100uL of cell suspension (10^7 cells) was added into a new tube. 10ul of Biotin-Antibody Cocktail was added, which contains Biotin anti-CD8a, CD11b, CD11c, CD19, CD24, CD45R/B220, CD49b, CD105, I-A/I-E (MHC II), TER-119/Erythroid, TCR-γδ. The cell suspension and antibody cocktail solution were mixed well and incubated on ice for 15mins. 10uL of Streptavidin Nanobeads were added, mixed well and incubated on ice for 15mins. 2.5ml of MojoSort™ buffer was then added. The tube with cells, antibody cocktail and nanobeads was inserted into the magnet and left for 5mins. Liquid containing the target CD4+ T cells was collected into a sterile new tube. The yield of CD4+ T cells using this protocol was approximately10% (10^6 CD4+ T cells from 10^7 splenocytes). The basic protocol is shown below.

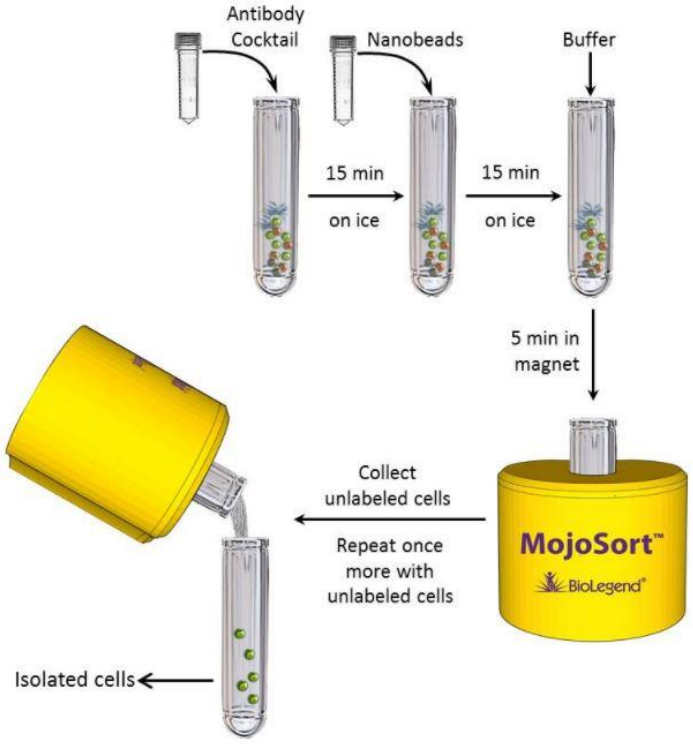


Figure 2-1. Protocol for CD4+ T cell isolation with MojoSort™ isolation kit (Biolegend, Inc, CA).

Antibodies & Antagonist

LEAF™ Purified anti-mouse CD3 Antibody [Clone: 17A2] and LEAF™ Purified anti-mouse CD28 Antibody [Clone: 37.51] were used to activate T cell proliferation. LEAF™ Purified anti-mouse TLR4 (CD284)/MD2 Complex Antibody [Clone: MTS510] was used to block TLR4 signaling pathway on BMDCs. LEAF™ Purified Rat IgG2a, κ Isotype Ctrl Antibody was set as a control group. (Biolegend Inc, CA)

For surface marker staining and intracellular cytokine staining, we choose Alexa Fluor® 647 anti-mouse CD11c antibody to check the purity of BMDCs. We also use Alexa Fluor® 488 anti-mouse I-A/I-E Antibody, PE/Cy7 anti-mouse CD40, APC/Cy7

anti-mouse CD86, PE anti-mouse CD80 and PE anti-mouse CD252 (OX40L) to measure the change of MHCII and co-stimulators on BMDCs. For lymphocyte and CD4 T cells, we choose Alexa Fluor® 488 anti-mouse CD4, Alexa Fluor® 647 anti-mouse IL-4, PE anti-mouse IFN-γR β chain, and PE/Cy7 anti-mouse IL-33Rα (IL1RL1, ST2) to measure the change of intracellular IL-4, intracellular IFN-γ, surface CD4 and surface ST2. All of these above-mentioned antibodies were purchased from Biolegend Inc, CA. LPS-RS, a TLR4 antagonist, were bought from InvivoGen, CA.

ELISA

The concentrations of IL-4, RANTES, TNF-α cell in cell culture supernatants were measured by ELISA (R&D SYSTEMS). The concentrations of IL-6, MCP-1, and IL-17A in the supernatants were measured by ELISA (Biolegend, Inc).

Statistical analysis

Data are presented as the mean with SD for the numbers of experiments indicated. The statistical significances of the differences between various treatment groups were assessed with ANOVA test or T-test; $p < 0.05$ was considered significant.

Results

A. alternata cell wall components stimulate innate immunity in BMDCs

Previous studies demonstrated that *A. alternata* cell wall components induce Th2-associated cytokines in vivo. We first investigated the ability of Alternaria to induce innate immunity in BMDCs. We stimulated BMDCs harvested from C57BL/6 mice with wild type *A alternata spores,* recombinant Alt a1 allergen and purified chitin oligomers (DP6) and measured secreted IL-6 using ELISA. LPS was used as a positive control. As shown in **Figure 2-2**, BMDCs stimulated with *A. alternata* spores, Alt a1, chitin DP6, or LPS, the secretion of IL-6 was highly induced, with Alt a1 exhibiting the most significant stimulating effect at the concentration tested. This indicated that *A. alternata* spores and cell wall components can trigger innate immune responses in BMDCs.

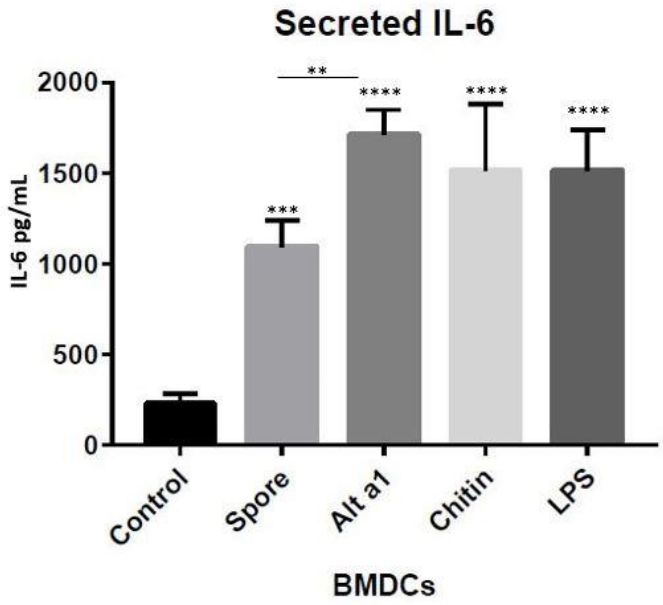


Figure 2-2. IL-6 induction by different *A. alternata* cell wall components and LPS. Bone marrow monocytes were isolated from 6-8 weeks old C57BL/6 mice and grown in RPMI + FBS + 1% Pen Strep+10ng/ml GM-CSF in 6-well plates for 6 days to harvest immature BMDCs. 7.5×10^4 BMDCs were stimulated with *A. alternata* wide type spore (spore/cell=1/2), Alt a1 (100ug/ml), chitin DP6 (500uM) and LPS (100ng/ml) for 24 h. Supernatants were collected and IL-6 concentration was assayed using a Biolegend mouse IL-6 kit. Data are represented as mean(SD). Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

In order to determine whether *A. alternata* spores and cell wall components can induce other cytokines and chemokines in BMDCs besides IL-6, we conducted a cytokine array profiling experiment (**Figure.2-3**). We found that *A. alternata* spores, chitin DP6, and Alt a1 induced many cytokines and chemokines in BMDCs (**Figure 2-3a, b, c**). IL-6, MIP-1α, MIP-1β, MIP-2, RANTES and TNF-α were among the most highly induced cytokines and chemokines. For experiments with spores, we chose a ratio of spore/DC=1/2. We observed spores undergoing active germination during the 24 h of stimulation.

As shown in **Figure.2-3 c**, RANTES was significantly induced by all three stimuli, MIP-1α and MIP-1β, also known as CCL3 and CCL4, respectively, were also significantly induced. Another induced chemokine in this study, MIP-2, is one of the CXC class of chemokines and is also called as chemokine CXC ligand (CXCL2). It was found that TNF-α and GM-CSF were also highly induced. The high production of these two cytokines are a known feature of Th17 cell phenotype. We measured the inducible expression of these two cytokines in BMDCs, which may lead to Th17 pathway in our later co-culture experiment. Details will be described in following context.

In summary, the cytokine array assay revealed that *A. alternata* spores and associated molecules (chitin and Alt a 1) induced BMDCs to secrete IL-6, RANTES, MIP-1αl MIP-1β, MIP-2, TNF-α, and GM-CSF.

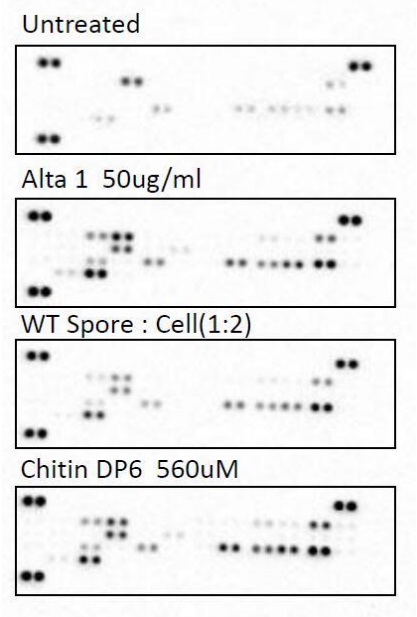


Figure 2-3a

Figure 2-3. Mouse cytokine array assay. Bone marrow monocytes were isolated from 6-8 week-old C57BL/6 mice and grown in RPMI + FBS + 1% Pen Strep+10ng/ml GM-CSF in 6-well plates for 6 days to harvest immature BMDCs. 7.5×10^4 BMDCs were stimulated with *A. alternata* wide type spore (spore/cell=1/2), Alt a1 (100ug/ml), chitin DP6 (500uM), or LPS (100ng/ml) for 24 h. Supernatants were collected and assayed using a R&D SYSTEMS Mouse Cytokine Array Panel A Kit. Data are represented as mean pixel density. (**a**) Four membranes corresponding to exposure to different stimulations. **(b**) A table showing the coordination of cytokine spots corresponding to (a). **(c)** Estimated mean pixel density of cytokine spots corresponding to those on membrane in (a).

PS	PS																					PS	PS
BLC	BLC	C5/C5a	C5/C5a	G-CSF	G-CSF	GM-CSF	GM-CSF	I-309	I-309	Eotaxin	Eotaxin	sICAM-1	sICAM-1	INF-γ	INF-γ	IL-1α	IL-1α	IL-1β	IL-1β	IL-1rα	IL-1rα	IL-2	IL-2
IL-3	IL-3	IL-4	IL-4	IL-5	IL-5	IL-6	IL-6	IL-7	IL-7	IL-10	IL-10	IL-13	IL-13	IL-12p70	IL-12p70	IL-16	IL-16	IL-17	IL-17	IL-23	IL-23	IL-27	IL-27
IP-10	IP-10	I-TAC	I-TAC	KC	KC	M-CSF	M-CSF	JE	JE	MCP-5	MCP-5	MIG	MIG	MIP-1α	MIP-1α	MIP-1β	MIP-1β	MIP-2	MIP-2	RANTES	RANTES	SDF-1	SDF-1
TARC	TARC	TIMP-1	TIMP-1	TNF-α	TNF-α	TREM-1	TREM-1																
PS	PS																					NS	NS

Figure 2-3b

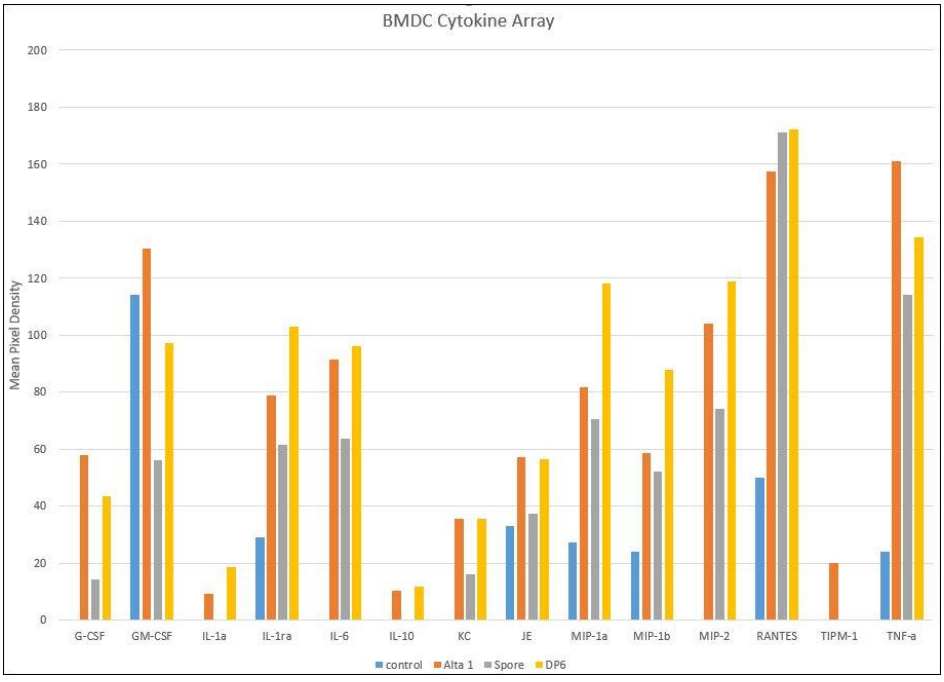


Figure 2-3c

Mouse BMDCs stimulated with *A. alternata* spores underwent maturation, shown by increased expression of the T-cell stimulatory molecules CD40 and CD86. Hence we conducted flow cytometry to determine expression patterns of BMCD surface markers (MHCII, CD40, CD80 and CD252) following stimulation with spores (**Figure 2-4**). The expression of MHCII decreased 38%, the expression of CD40 increased 85%, the expression of CD80 increased 88% and the expression of CD86 increased 81%. Interestingly, expression of CD252 (OX40L) increased 5 fold. MHC II levels were decreased in spore treated BMDCs compared to controls. Other co-stimulators, (CD40, CD80, CD86) were all increased in spores treated BMDCs compared to controls.

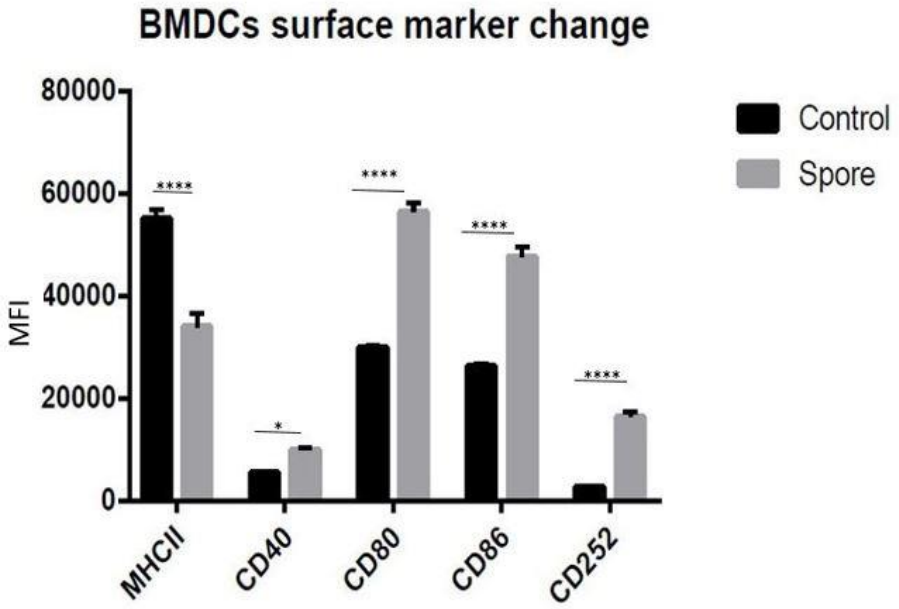


Figure 2-4. BMDCs surface marker expression after being stimulated by *A. alternata* wild type spore. Bone marrow monocytes were isolated from 6-8 week-old C57BL/6 mice and grown in RPMI + FBS + 1% Pen Strep+10ng/ml GM-CSF in 6-well plates for 6 days to harvest immature BMDCs. 7.5×10^4 BMDCs were stimulated with *A. alternata* wild type spore (spore/cell=1/2) for 24h. Activated BMDCs were stained with Alexa Fluor® 647 anti-mouse CD11c antibody, Alexa Fluor® 488 anti-mouse I-A/I-E antibody, PE/Cy7 anti-mouse CD40, APC/Cy7 anti-mouse CD86, PE anti-mouse CD80 and PE anti-mouse CD252 (OX40L) antibody. Flow cytometry was applied to measure relative surface maker expression. Data are represented as mean(SD). Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

Alt a 1 induced immune responses in BMDCs may be partially dependent upon TLR4

Previous experiments in our lab indicated that Alt a 1 induced innate immunity in lung epithelial cells is at least partially dependent upon TLR4 (Hayes et al., 2018, unpublished). In order to demonstrate that TLR4 plays an important role in Alt a1 induced immunity in BMDCs, LPS-RS was used in our study to block TLR4 pathway.

LPS from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) is a potent antagonist of LPS. Specifically, 20 ug/mL of LPS-RS was added to immature BMDCs in 6-well plates. After 3h incubation, 10 ng/mL LPS or 25 ug/mL Alt a1 was added to different wells. After 24h, expression levels of MHCII, CD40, CD80 were examined using flow cytometry. OX40L was measured after a 48h incubation. Following stimulation with Alt a 1, surface markers in cells pre-treated with LPS-RS were lower compared to controls indicating that TLR4 may be associated with Alt a1 induced innate immune response in BMDCs (**Figure.2-5**). Supernatants were also analyzed using ELISA (IL-4, RANTES, MCP-1, IL-6 and IFN-α. Consistent with the results of the cytokine array in **Figure 2-3**, increased levels of IL-6, RANTES, and IFN-α were detected (data not shown). However, no increase in IL-4 was detected.

Next we conducted experiments using anti-mouse TLR4 (CD284)/MD2 Complex Antibody [Clone: MTS510] to block TLR4 in BMDCs (**Figure 2-6**). Results of these experiments indicated that the TLR4 blocking antibody was not effective at reducing Alt a1 induced immune responses in BMDCs. Further optimization of antibody concentrations or choice of vendor may be necessary to yield tangible results.

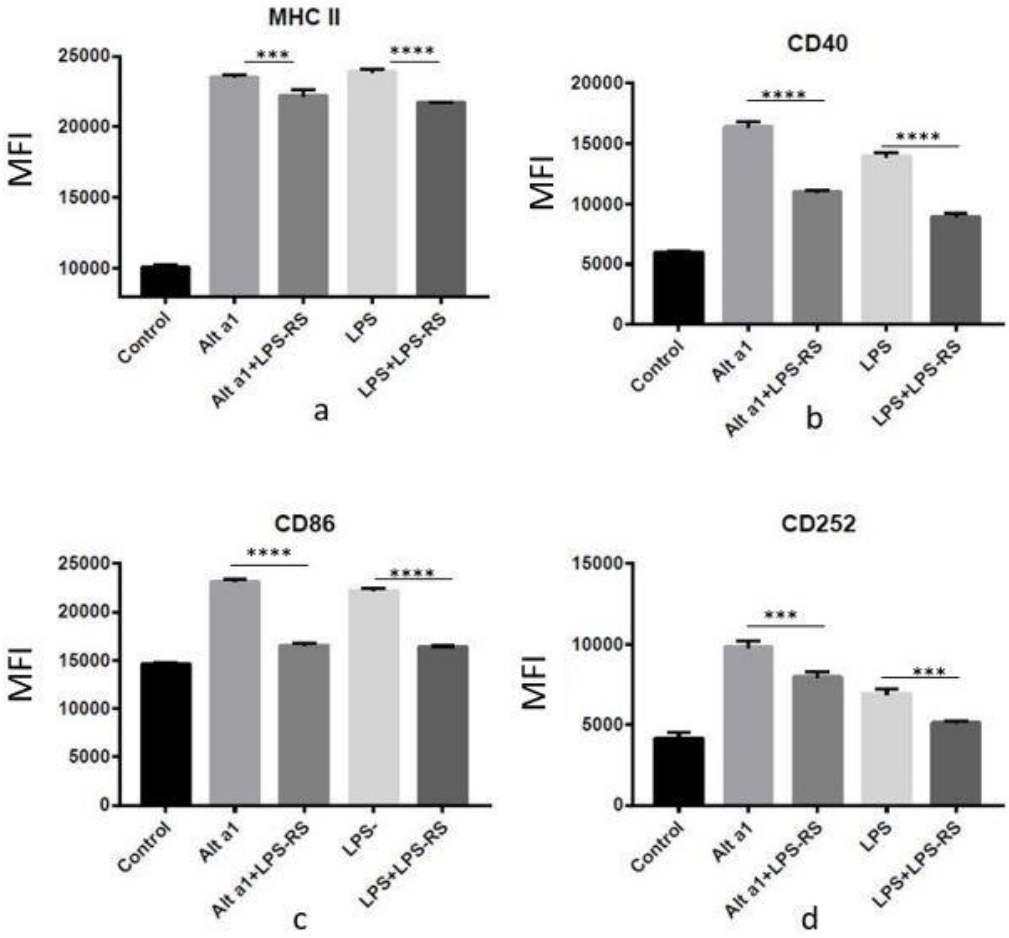


Figure 2-5. BMDCs surface marker expression after being stimulated by Alt a1, LPS and LPS-RS. Bone marrow monocytes were isolated from 6-8 week-old C57BL/6 mice and grown in RPMI + FBS + 1% Pen Strep+10ng/ml GM-CSF in 6-well plates for 6 days to harvest immature BMDCs. 7.5×10^4 BMDCs blocked with LPS-RS (20ug/ml) for 3h. Then Alt a1(25ug/ml) or LPS(10ng/ml) were added2. After 24h, activated BMDCs were stained with Alexa Fluor® 647 anti-mouse CD11c antibody, Alexa Fluor® 488 anti-mouse I-A/I-E antibody, PE/Cy7 anti-mouse CD40, APC/Cy7 anti-mouse CD86, PE anti-mouse CD80 antibody. PE anti-mouse CD252 (OX40L) antibody was stained after 48h. Flow cytometry was applied. (**a**) the expression of MHC II, (**b**) the expression of CD40, (**c**) the expression of CD86, (**d**) the expression of CD252. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

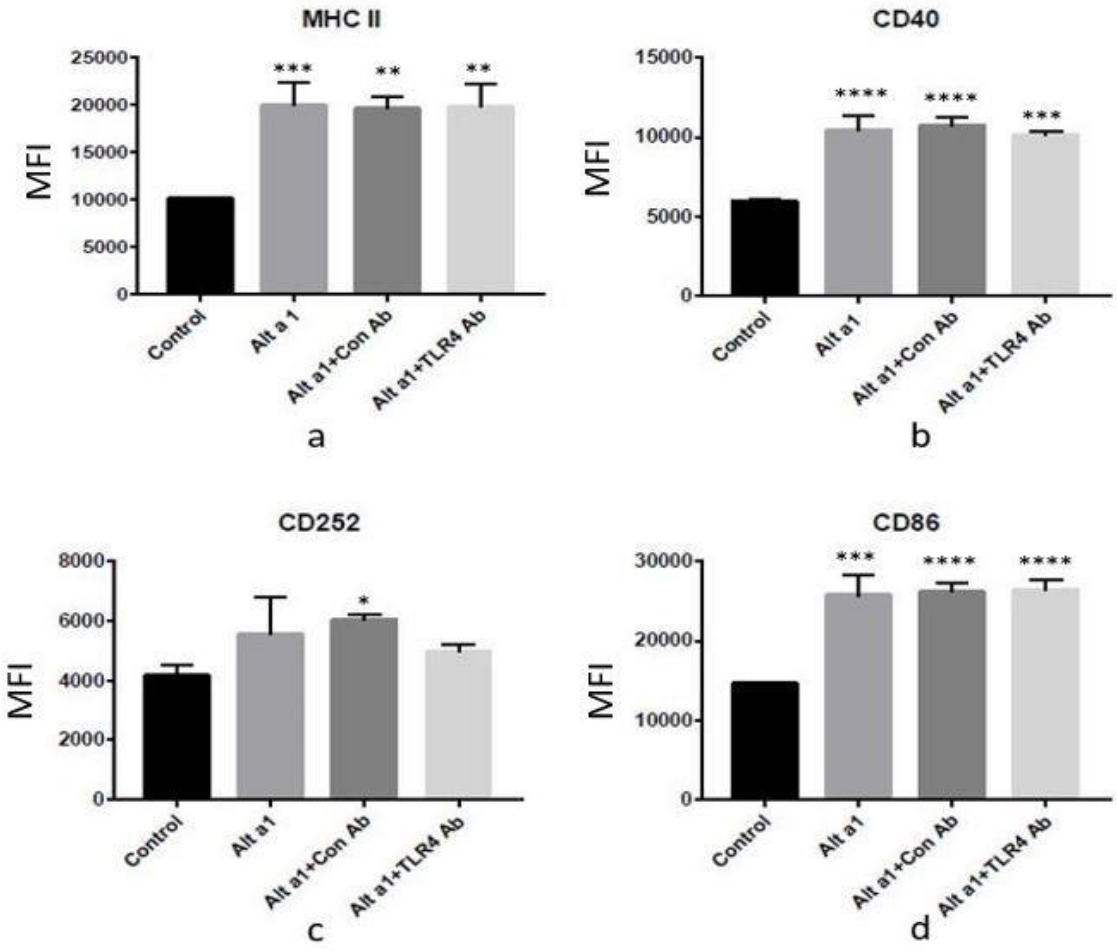


Figure 2-6. BMDCs surface marker expression after stimulated by Alt a1 with TLR4 Antibody or Control Antibody. Bone marrow monocytes were isolated from 6-8 weeks old C57BL/6 mice and grown in RPMI + FBS + 1% Pen Strep+10ng/ml GM-CSF in 6-well plates for 6 days to harvest immature BMDCs. 7.5×10^4 BMDCs blocked with TLR4 antibody(15ug/ml) or Control Antibody (15ug/ml) for 3h. Then Alt a1(25ug/ml) were added. After 24h, activated BMDCs were stained with Alexa Fluor® 647 anti-mouse CD11c antibody, Alexa Fluor® 488 anti-mouse I-A/I-E antibody, PE/Cy7 anti-mouse CD40, APC/Cy7 anti-mouse CD86, PE anti-mouse CD80 antibody. PE anti-mouse CD252 (OX40L) antibody was stained after 48h. Flow cytometry was applied. (**a**) the expression of MHC II, (**b**) the expression of CD40, (**c**) the expression of CD86, (**d**) the expression of CD252. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

Co-culture condition optimization

Cytokine array and surface marker results indicated that BMDCs respond to stimulation with spores and cell wall products. The ability of DCs to induce the proliferation of T cells in a mixed lymphocyte reaction (MLR) assay is commonly used for the evaluation of their function. To determine the mechanisms involved in DC-induced T cell activation in a primary MLR assay, we stimulated BMDCs with *A. alternata* spores. The MLR assays were carried out in round bottomed 96-well plates to ensure efficient DC/T cell contact[40]. Proliferation of different subsets of T cells was measured by CFSE staining.

We first optimized the co-culture conditions. We investigated three different co-culture ratios, DC/T=1/2, DC/T=1/5, DC/T=1/10, to study which ratio is optimal for T cell proliferation (Figure 2-7). A ration of DC/T=1/5 was found to be optimal for our experiments.

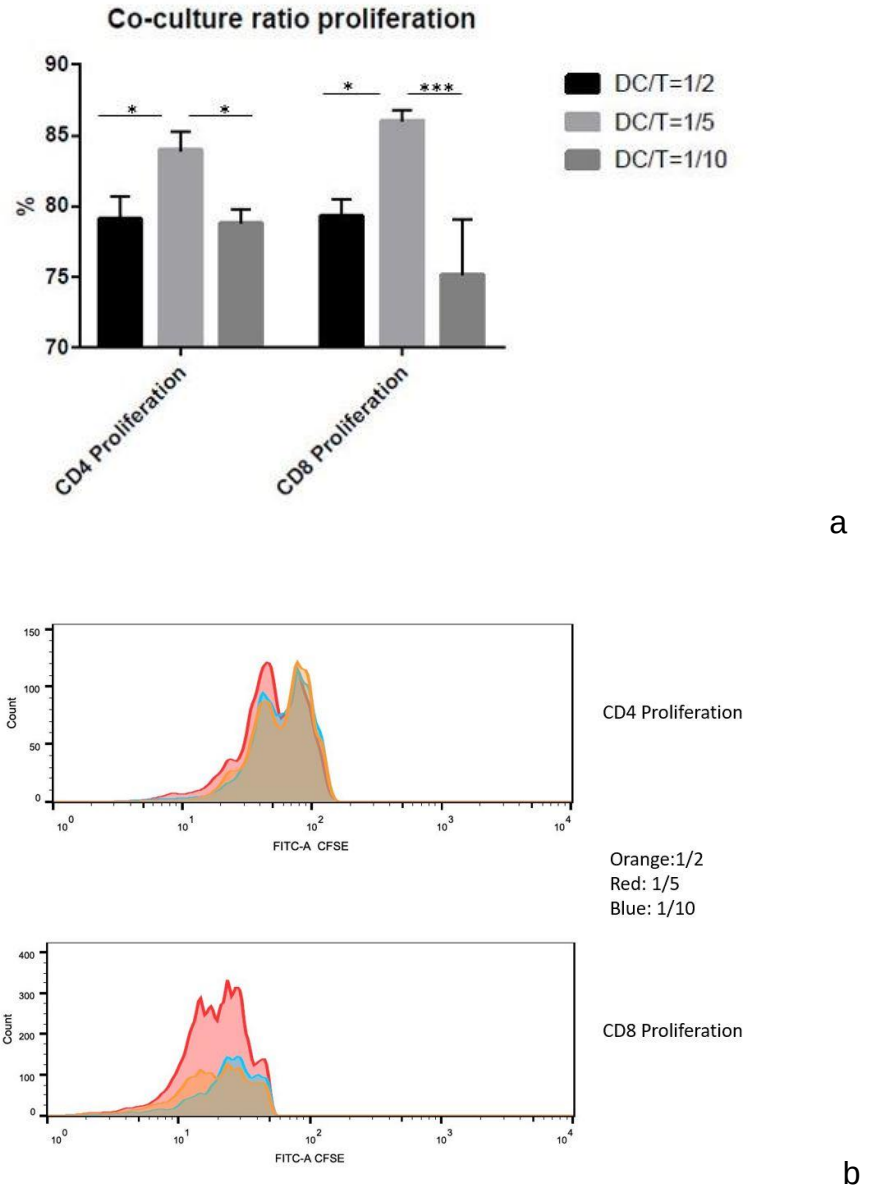
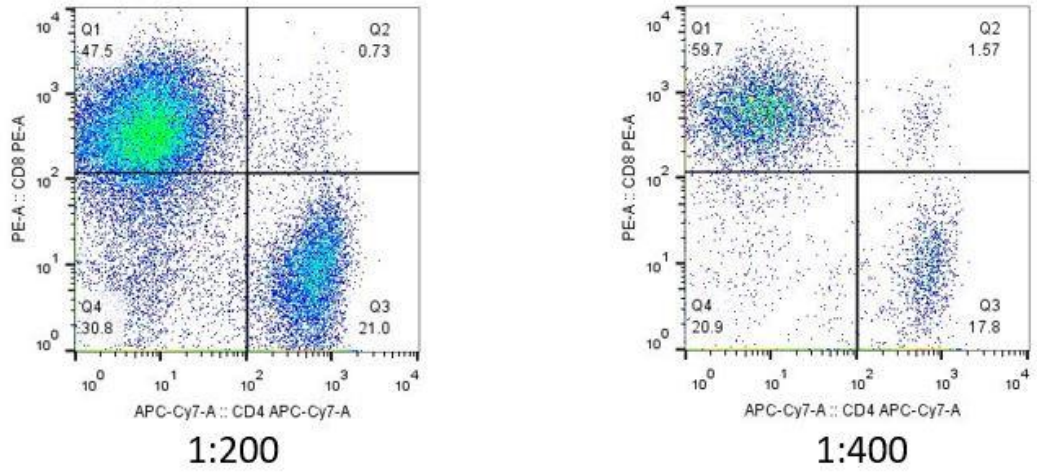
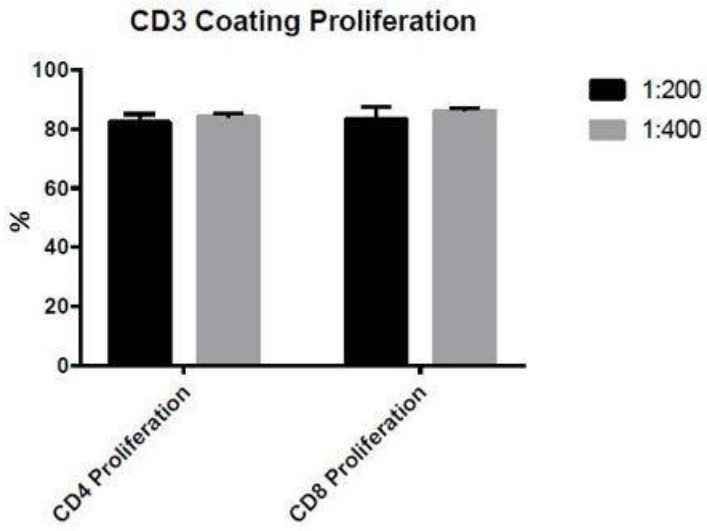


Figure 7. Co-culture ratio optimization. BMDCs from 6-8 weeks old female C57/BL6 mice were stimulated with *A. alternata* wide type spore (spore/DC=1/2) for 24h. Different amount of splenocytes from 6-8 weeks old female BALB/c mice were seed on U-shaped 96 well plate, which was coated with CD3 antibody overnight. Activated BMDCs were added. Co-culture DC and Splenocytes for 3 days. Splenocytes were stained with CFSE before co-culture. CD4 subset T cell proliferation and CD8 subset T cell proliferation was measured by flow cytometry. (**a**) Proliferation percentage of CD4 subset T cells and CD8 subset T cells. (**b**) Histogram of CFSE. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins[41]. In our experiments, we used CD3 antibody to activate T cells. For this step, we checked the effect of CD3 antibody coating concentration on T cell proliferation. Cells cultured in a non-coated plate died on day 3. Compared with other two coated plates, there is a smaller cell pellet at the bottom of each well (data was not shown). As shown in **Figure. 2-8 a**, for the coated plates, the number of viable T cells increased proportional to the CD3 antibody coating concentration. However, when we checked the results of CFSE labled proliferation, there was not much difference between 1/200 and 1/400 (**Figure. 2-8 b**). Hence, for the future experiments, we decided to use 1/400 diluted CD3 antibody to coat plates.



a



b

Figure 2-8. CD3 antibody coating concentration optimization. BMDCs from 6-8 weeks old female C57/BL6 mice were stimulated with *A. alternata* wide type spore (spore/DC=1/2) for 24h. Round bottom 96 well plate was coated with different concentration of CD3 antibody overnight at 4℃. Splenocytes from 6-8 weeks old female BALB/c mice were labeled by CFSE and seeded on 96 well plate. Activated BMDCs were added (DC/T=1/5). Co-culture DC and Splenocytes for 3 days. CD4 subset T cell proliferation and CD8 subset T cell proliferation was measured by flow cytometry. (**a**) Density plot of CD4 antibody and CD8 antibody. (**b**) Proliferation percentage of CD4 subset T cells and CD8 subset T cells. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

Co-culture of spore stimulated BMDCs with splenocytes.

After we optimized conditions, we conducted a co-culture study using *A. alternata* spores to stimultate BMDCs. Specifically, activated BMDCs were co-cultured with splenocytes for three days.

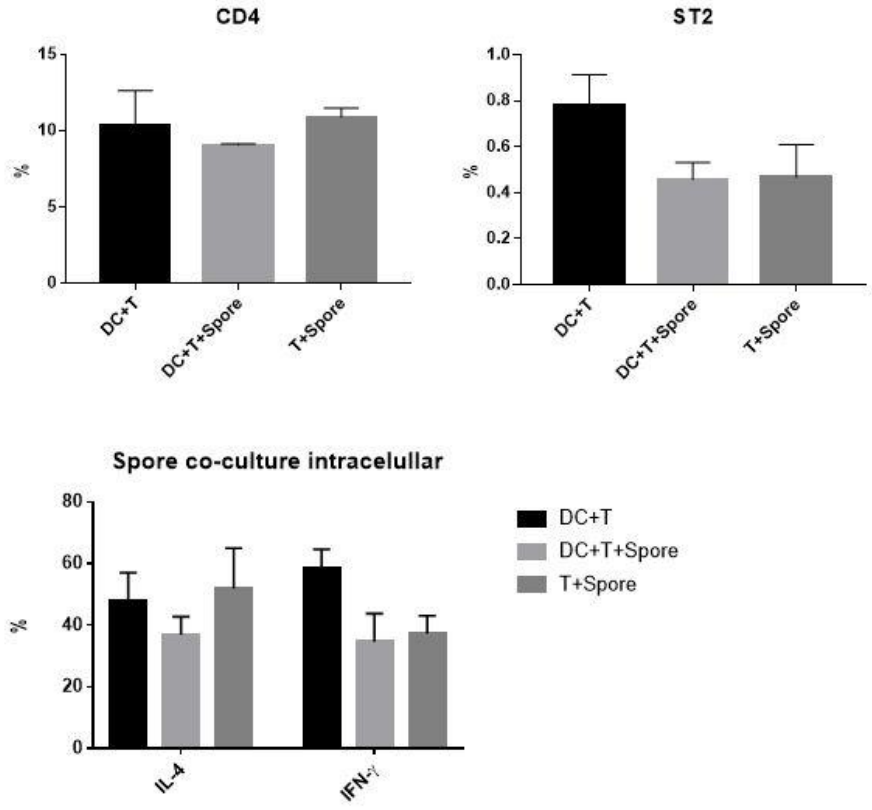


Figure 2-9. Spores treatment co-culture experiment. BMDCs from 6-8 weeks old female C57/BL6 mice were stimulated with *A. alternata* wide type spore (spore/DC=1/2) for 24h. Round bottom 96 well plate was coated with 1/400 diluted CD3 antibody overnight at 4℃. Splenocytes from 6-8 weeks old female BALB/c mice were seeded on 96 well plate. Activated BMDCs were added (DC/T=1/5). Co-culture DC and Splenocytes for 3 days. Flow cytometry was applied to measure the expression of CD4, ST2, intracellular IL-4 and IFN-a. (**a**)expression of surface CD4, (**b**)expression of surface ST2, (**c**)expression of intracellular IL-4 and INF-γ. DC+T stands for BMDCs co-cultured with splenocytes. DC+T+Spore means BMDCs activated by spore co-cultured with splenocytes. T+Spore stands for splenocytes co-cultured with spore. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

Before seeding DCs with lymphocytes, DCs were washed with complete RPMI-1640 medium to remove GM-CSF and *A. alternata* spores. Since the size of spores are quite close to the size of BMDCs, it was difficult to completely remove all spores. That's why we set a control group containing lymphocytes and spores without BMDCs. Also, in this experiment, we tried to answer a question, whether *A.alternata* spores could induce T cells switching to Th2 pathway. Thus we measured IL-4 and the IL-33 cell surface receptor ST2. We found no significant difference in terms of CD4+ expression level among these three groups (**Figure 2-9a**). Interestingly, the experimental group with spores, DCs and lymphocyte exhibited the lowest ST2 level (**Figure 2-9b**). One possible reason is that B cells in splenocytes may play an antigen presenting function in the co-culture process. Since the spores cannot be removed completely, they may continuously stimulate B cells. We also found no significant difference between each group, except the expression of INF-γ for two co-cultured groups with or without spores (**Figure 2-9c**).

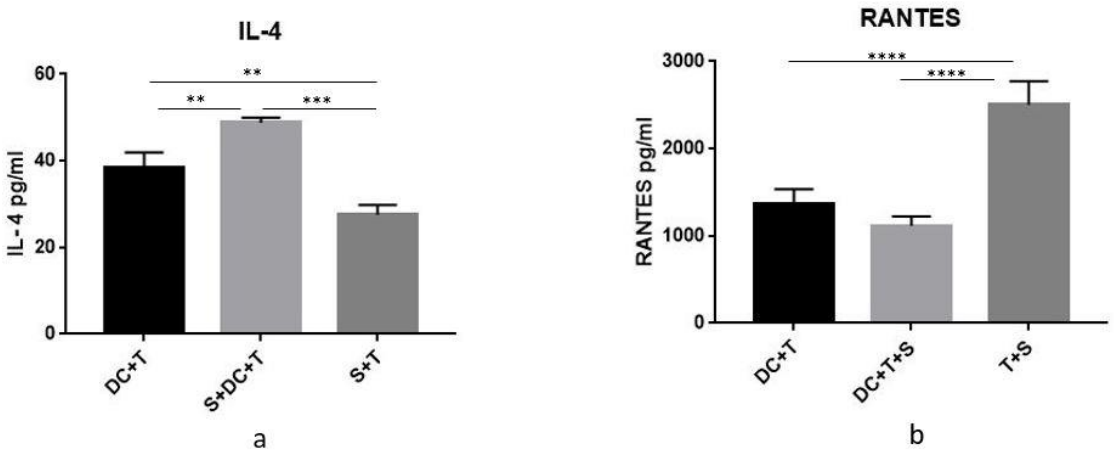


Figure 10. ELISA of supernatants collected form spores treatment co-culture experiment. BMDCs from 6-8 weeks old female C57/BL6 mice were stimulated with *A. alternata* wide type spore (spore/DC=1/2) for 24h. Round bottom 96 well plate was coated with 1/400 diluted CD3 antibody overnight at 4℃. splenocytes from 6-8 weeks old female BALB/c mice were seeded on 96 well plate. Activated BMDCs were added (DC/T=1/5). Co-culture DC and splenocytes for 3 days. ELISA for IL-4 and RANTES were applied with collected supernatants. (a)IL-4 tested in supernatants, (b)RANTES tested in supernatants. DC+T stands for BMDCs co-cultured with splenocytes. DC+T+Spore means BMDCs activated by spore co-cultured with splenocytes. T+Spore stands for splenocytes co-cultured with spore. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

From **Figure. 2-10 a**, we can see that the highest IL-4 was tested with spore stimulated co-culture medium, which indicates that BMDCs activated by *A. alternata* wide type spores pass the signal to T cells and induce the expression of IL-4. This results combined with **Figure 2-4**, five times CD252 was induced by spore, indicates that *A. alternata* wide type spores may trigger Th2 pathway in innate immune response. However, in **Figure. 2-10 b**, the group secreted highest level of RANTES is splenocytes cultured with spore only, which shows that spore may stimulate splenocytes, such as B cells, NK cells and different subsets of T cells to secreted RANTES. Still, it is hard to

explain why the activated BMDCs and splenocytes co-cultured group got the lowest level of RANTES, which need to be explored.

Alt a1 stimulates CD4+ T cells to Th17

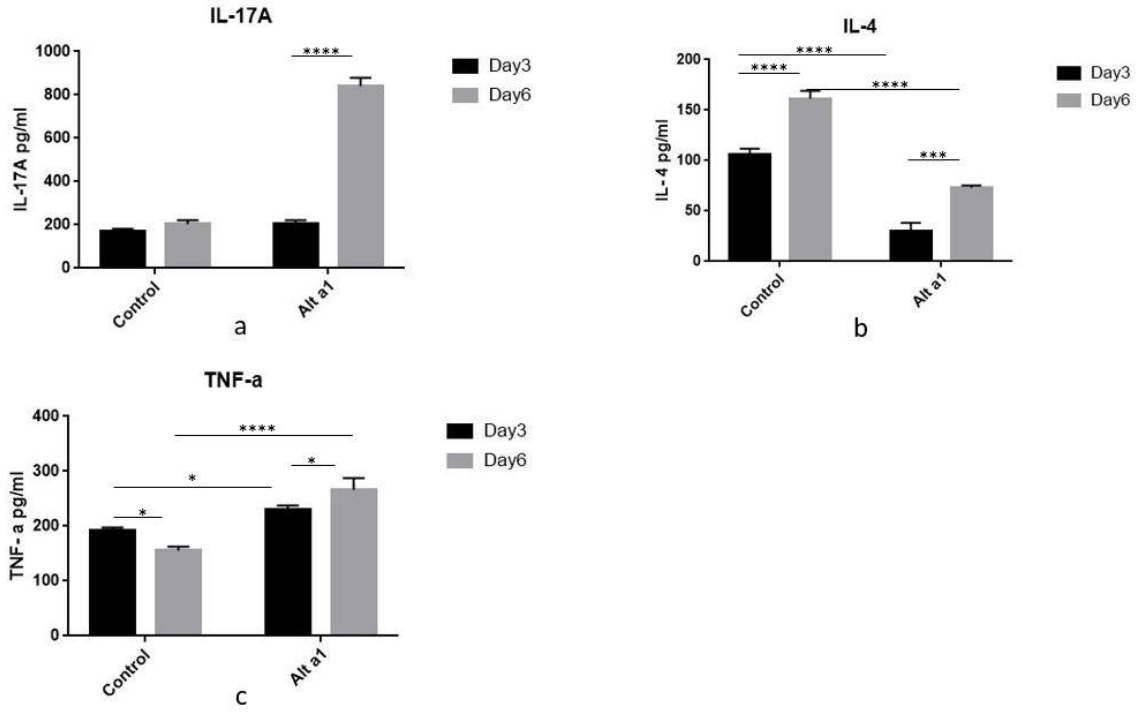


Figure 2-11. Alt a1 stimulated BMDCs co-cultured with isolated CD4 cells. BMDCs from 6-8 weeks old female C57/BL6 mice were stimulated with Alt a1 (25ug/ml) for 24h. Round bottom 96 well plate was coated with 1/400 diluted CD3 antibody overnight at 4℃. Isolated CD4 T cells from 6-8 weeks old female BALB/c mice were seeded on 96 well plate. Activated BMDCs were added (DC/T=1/5). Soluble CD3 antibody and CD28 antibody were supplemented on day 3. Supernatants were collected on day 3 and day 6 to apply ELISA. (**a**)secreted IL-17A tested with supernatant, (**b**)secreted IL-4 tested with supernatant, (**c**)secreted TNF-α tested with supernatant. Data are represented as mean(SD). ANOVA was performed and adjusted. Significantly different: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

CD4+ T cells were isolated with MojoSort^{TM} CD4 T cell isolation kit purchased from BioLegend. Inc. Activated BMDCs were co-cultured with purified CD4+ T cells for 3 or 6 days. Supernatants were collected and subject to ELISA assay. As shown in **Figure. 2-11 a**, on day 3, there was not much difference between the control group and Alt a1 stimulated group in terms of the IL-17A level. However, we detected a statistically significant increase of IL-17A on day 6, which may indicate that CD4+ T cells switched to Th17 after 6 days of co-culture. Meanwhile, we detected an increased level of secreted IL-4 on day 6, compared to that on day 3 (**Figure.2-11 b**). Interestingly, Alt a1 inhibited IL-4 expression on both day 3 and day 6, which is consistent to the results in the spore stimulation experiment we mentioned before. Also, from **Figure.2-11 c,** Alt a1 stimulated the secretion of TNF-α.

Discussion

In this study, we characterized the ability of *A. alternata* spores, Alt a1 and chitin to induce immune-related responses in BMDCs. We initially investigated IL-6 since it has been shown to play an important role in the pro-inflammatory state of chronic obstructive pulmonary disease (COPD), asthma, allergy, heart disease, rheumatoid arthritis and other lung injury diseases [44]. IL-6 is also a key cytokine that is consistently associated with fugal-induced airway injury[44]. In our studies, IL-6 was strongly induced in BMDCs following all challenges.

Based on results of cytokine array analyses, we selected RANTES, CCL3, CCL4 and TNF-α as target cytokines and chemokines in later studies. When RANTES (for 'regulated upon activation normal T cell expressed and secreted') was first identified, it appeared to be a typical chemokine in that was able to recruit leukocytes to sites of inflammation[45]. Later studies show that increased RANTES expression has been associated with a wide range of inflammatory disorders and pathologies, including allogeneic transplant rejection, atherosclerosis, arthritis, atopic dermatitis, inflammatory airway disorders such as asthma, delayed-type hypersensitivity reactions, glomerulonephritis, endometriosis, some neurological disorders (such as Alzheimer's disease) and certain malignancies. In all of these pathologies, RANTES is thought to act by promoting leukocyte infiltration to sites of inflammation[45]. Another interesting finding is that CCL3, CCL4 and RANTES were shown to be the most potent members of CC chemokine family released by CD8+ T cells that were able to suppress the replication of non-syncitium-inducing (NSI) HIV-1 strains in vitro[45].

We also observed induction of MIP-2 and TNF-α in our studies. MIP-2 affects neutrophil recruitment by binding to its specific receptors, CXCR1 and CXCR2[46]. Necrosis factor alpha (TNF-α), is an inflammatory cytokine produced during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis[47]. The role of MIP-2 and TNF-α has not been explored in *Alternaria*-associated inflammation and may warrant further investigation in vivo.

Another interesting finding is the large increase of OX40L expression on BMDCs after stimulation. Davinder Kaur et.sl reported on 2011, OX40L is directly mediated by thymic stromal lymphopoietin (TSLP), which is produced by epithelial cells, mast cells, airway smooth muscle, and dendritic cells, which are all related to the Th2 response[39].

TSLP activates immature CD11c dendritic cells to express OX40L, and these cells then become mature dendritic cells, which migrate to the draining lymph nodes. There they activate the differentiation of naive CD4 T cells by binding to the OX40 receptor, where they become inflammatory cells producing IL-4, IL-5, IL-13, tumor necrosis factor- a (TNF- α), and little or no IL-10[39]. Form the co-culture study, we did detect small amount of IL-4 and a high level of TNF-αwith the co-culture medium. Hence, in the future, we may try to measure the secreted TSLP from BMDCs stimulated medium and the co-culture medium, which may provide more evidence to support our conclusion.

Former studies in our lab showed that TLR2 and TLR4 may play an important role in Alt a1 recognition and downstream signaling. Thus, we chose to use TLR4 blocking antibody and LPS-RS, a TLR4 antagonist, to block the signaling pathway. LPS-RS was an effective antagonist in our study. Even though our hypothesis is not strongly supported by the TLR4 antibody experiment in this study, there are some explanations

that may have caused this result. There are only a few mouse TLR4 antibodies used to neutralize signaling pathway currently available. More experiments should be conducted with different antibodies at a range of concentrations in order to further optimize results.

The most interesting finding in this study is that the induced IL-17A expression gives us a new direction for future experiments. Fairly recently, in addition to Th1 and Th2 cells, a third subset of IL-17-producing effector T helper cells, called Th17 cells, have been discovered and characterized[40]. As INF-γ initiates the differentiation of Th1 cells and IL-4 triggers the differentiation of Th2 cells, TGF-β plays a similar role in the differentiation of Th17[40]. In 2006, three independent studies showed that a combination of TGF-β and IL-6 is required to induce IL-17 in naïve T cells. Travis et al. (2007) reported that DCs, though not primary producers of TGF-β, are essential to raising the level of active TGF-β in the local environment[41]. Meanwhile, Li et al (2007) reported that T cells themselves maybe the source of TGF-β[42]. In our study, **Figure 2-2** already shows that there is a high induction of IL-6 secretion with *Alternaria* stimulation. We can assume that the stimulated BMDCs may induce T cells to express more TGF-β to initiate the differentiation of Th17. Hence, in the future we can measure the secretion of TGF-β in the co-culture study to verify this hypothesis.

On the other hand, in order to further prove that there is a switch from Th1/Th2 subsets to Th17 subset, we need to measure additional Th17 related cytokines and chemokines. Besides IL-17A, Th17 cells also express a large amount of IL-17F, IL-21 and IL-22[43]. Similar to IL-2's role in Th1 differentiation, IL-21 produced by Th17 cells also acts in a positive feedback loop, amplifying the precursor frequency of Th17 cells[44]. IL-17A, IL-17F and IL-22 have broad effects on many cell types and induce the

production of pro-inflammatory cytokines and chemokines to attract neutrophils[45]. On the other hand, IL-17A and IL-17F are also produced by a variety of cells, including γδ T cells, NK cells, neutrophils and eosinophils[46]. What we can do in the future is co-culture isolated CD4+ T cells with stimulated BMDCs, then measure the expression of IL-17F, IL-21 and IL-22. In this scenario, there are only purified naïve CD4+ T cells, we can exclude other source of those Th17 related cytokines. Another interesting study we can perform is co-culturing neutrophils with activated T cells and characterize immune responses.

As we discussed above, assuming the stimulated BMDCs induce T cells to differentiate primarily into theTh17 subset, it is important to determine the function and pathological role of this subset in the context of allergic inflammation. Many studies have found that Th17 cells are important effector cells in host defense against certain pathogens such as *Candida albicans* and specific extracellular bacteria[47]. The broad response to Th17-related cytokines might be the basis for the prominent capability of Th17 cells to induce tissue inflammation and autoimmunity[48]. Interestingly, IL-17-producing CD4+ T cells contain a large fraction of cells reactive against fungal antigens, which suggest that Th17 cells may be preferentially induced in response to fungal infection and may play a meaningful role in orchestrating host defense against certain fungi[49]. It is possible that *A. alternata* may also be one of the fungi that induces Th17 related responses.

The ultimate goal of our study is to explore how *A. alternata* relates to human allergic diseases. Evidence suggests that Th17 cells are important in rheumatoid arthritis[50], multiple sclerosis[51], severe asthma[52] and some bacterial and fungal infections. Since

sensitivity to *A. alternata* has long been clinically associated with development and exacerbation of severe forms of asthma[12, 13], it begs the question does Th17 play an important role in this phenotype?

In conclusion this study is the first to report the immune-stimulatory effects of live *Alternaria* spores and Alt a 1 on BMDCs. MHCII and some co-stimulators, such as CD40, CD80, CD252, are highly induced with *Alternaria* stimulation. Alt a 1 induced immune responses in BMDCs may be partially dependent upon TLR4, which needs to be further confirmed in future optimized experiments. Probably the most striking result from these studies is the high level induction of IL-17A by Alt a 1 and may be another important direction we can explore in more detail in the future.

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