

# A potential role of toll-like receptors, IFN- $\gamma$ and the phosphatidylinositol 3-kinase pathway in the pathogenesis of acquired mediastinal lymphatic malformation

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## ABSTRACT

Sarcoidosis is a multisystem disorder with non-caseating granulomas in various organs. The etiology of sarcoid granuloma formation is not clear and likely an antigen-induced process. We came across a previously treated sarcoidosis patient who presented with worsening dyspnea on exertion for several months and several days of difficulty swallowing. On Chest CT imaging, large posterior mediastinal mass was found that subsequently diagnosed as macrocystic lymphatic malformation after surgical resection. Pathophysiology of development of acquired lymphatic malformations in a sarcoidosis patient is currently not clear. We hypothesize there might be a complex interplay of Toll-like receptors, IFN- $\gamma$  and the phosphatidylinositol 3-kinase pathway in the pathogenesis.

## Introduction

Sarcoidosis is a multisystem granulomatous disease, primarily affecting the lungs. The process of granulomas formation first starts within the mediastinal lymph nodes (LN), followed by the lungs. The etiology of sarcoid granuloma formation is thought to be an antigen-induced process [1] but the sequence of immunological events is unclear. Close interactions between macrophages, peri-lymphatic dendritic cells (DCs) and antigen producing cells (AEC)-II initiate the process of granuloma formation, synchronized by pro-inflammatory mediators including cytokines and chemokines [1]. In the majority of sarcoidosis patients, granulomas resolve spontaneously within a few years without any treatment. However, in several patients granuloma formation is an evolving process leading to chronic or end-stage disease [2]. Though some studies have suggested impaired immunosuppressive function of regulatory T (Treg) cells in granuloma persistence, the exact immunologic response is unclear [3].

Lymphatic malformations (LMs) are congenital vascular anomalies, arising from the embryonal remnant of lymphatic system that remains after birth. However, acquired LM can develop in areas of chronic lymphatic obstruction due to chronic inflammation, surgery or radiation later in life. Although development of acquired LMs in chronic infection and/or inflammation has been described, the pathophysiology

is uncertain [4]. It is also unknown why LM does not develop in all patients with chronic infection and/or inflammation.

## The hypothesis

We propose a hypothesis that development of acquired LM in persistent sarcoid granulomas may be the result of increased PTEN activity from the complex interplay of IFN- $\gamma$  Toll-like receptors (TLRs) and the phosphatidylinositol 3-kinase (PI3K) pathway.

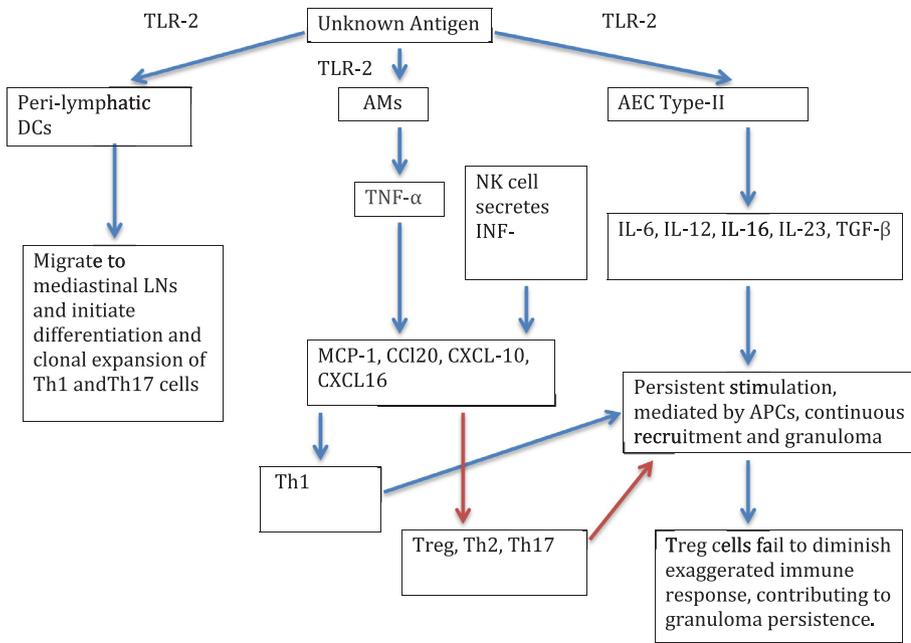
## Evaluation of the hypothesis

### Current understanding of persistent granuloma formation in sarcoidosis

Persistent granuloma formation in sarcoidosis appears to entail crosstalk and complex interplay of several signaling pathways leading to cascades of events resulting in the production of proinflammatory mediators [interleukins (IL-6, IL-12, IL-18, IL-23) and tumor growth factors (TGF- $\beta$ )] by macrophages, peri-lymphatic DCs, and AEC-II [1]. An outline of the pathophysiology of persistent granuloma formation in sarcoidosis is shown in Fig. 1. In this model an unknown microbial antigen simultaneously activates peri-lymphatic DCs, AMs and AEC-II. This process is initiated by TLR-2 ligands. TLRs are transmembrane

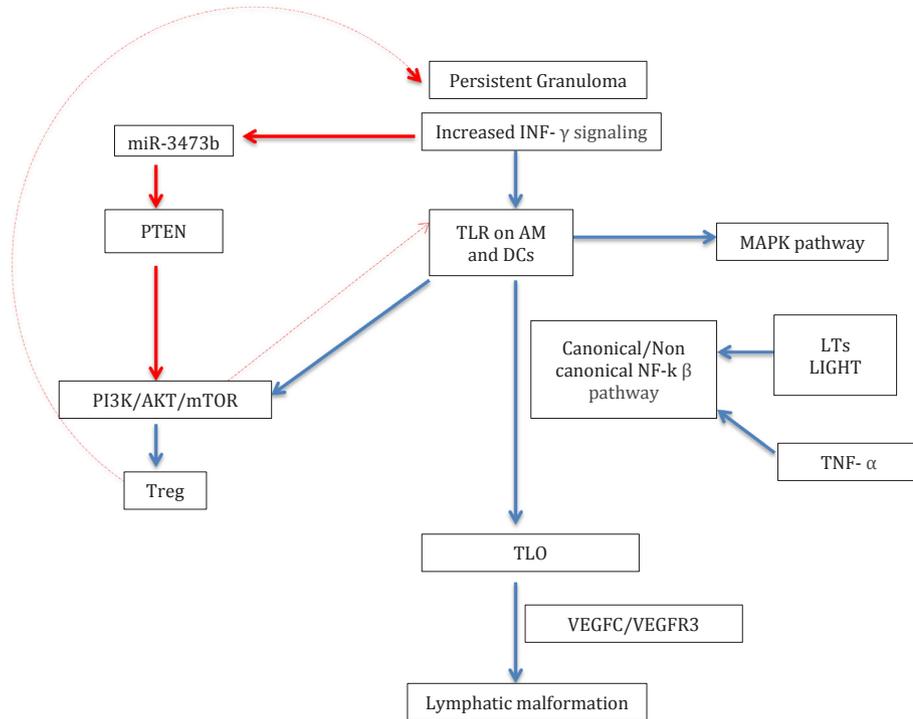
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**Fig. 1.** A schematic model for the role of IFN- $\gamma$  and TLR in persistent sarcoid granulomas. An unknown microbial antigen simultaneously activates peri-lymphatic DCs (dendritic cells), AMs (alveolar macrophages) and AEC-II (alveolar epithelial cells type-II). This process is initiated by TLR-2 (toll-like receptor-2) ligands. The interstitial DCs pick-up the alleged antigen and migrate to the mediastinal LNs (lymph nodes) and initiate differentiation and clonal expansion of Th (CD4 T helper) 1 and 17 cells. Simultaneously, AMs (alveolar macrophages) produce TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ), which further stimulates production of chemokine ligands (MCP-1, CCL20, CXCL10 and CXCL16) under stimulation of both TNF- $\alpha$  and NK (natural-killer) cells derived IFN- $\gamma$  (interferon- $\gamma$ ) and thereby sustains inflammation, maintain Th1 responses and inhibit differentiation of Th17, Th2 and Treg (T regulatory) cells. Th1 produce cytokines such as IL-6, IL-12, IL-18, IL-23, and TGF- $\beta$  in response to AEC (alveolar epithelial cell) type-II activation by TLR-2. Persistent stimulation, mediated by APCs (antigen presenting cells), leads to continuous cellular recruitment to the site of inflammation, which leads to granuloma formation. In many patients, granulomas persist for years likely due to chronically reduced differentiation of Treg cells by

increased IFN- $\gamma$  signaling. Blue arrow: positive regulation in chronic inflammation. Red arrow: negative regulation in chronic inflammation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** A schematic model for the role of lymphotoxins, TLR and PI3K pathways in acquired lymphatic malformation. The finding of persistent granuloma indirectly suggests increased IFN- $\gamma$  signaling which in turn leads to increased ligation of TLR (toll-like receptor) on AMs (alveolar macrophages) and peri-lymphatic DCs (dendritic cells). Increased TLR ligation leads to activation of the MAPK pathway, PI3K/AKT/mTOR pathway and canonical or non-canonical NF- $\kappa$ B pathway. Simultaneously, LTs (lymphotoxins) and LIGHT (LT-related inducible ligand) and TNF- $\alpha$  lead to production of VEGF-C (vascular endothelial growth factor C) in the stromal cells of TLOs (tertiary lymphoid organs) via activation of the canonical or non-canonical NF- $\kappa$ B pathway, which in turn leads to formation of LM (lymphatic malformation) via VEGF-C/VEGFR3. Note that increased PI3K/AKT/mTOR signaling serves to negatively regulate TLR signaling (dotted red arrow) thus resulting in down regulation of the NF- $\kappa$ B pathway. Also, PTEN (phosphatase and tensin homolog) negatively regulate PI3K signaling. In the case of chronic down regulation of PI3K signaling by increased activity of phosphatase and tensin homolog (PTEN), chronic upregulation of TLR signaling may lead to development of acquired LM via the NF- $\kappa$ B pathway. Moreover, chronic down regulation of PI3K signaling also results in reduced levels of Treg cells.

Blue arrow: positive regulation in chronic inflammation. Red arrow: negative regulation in chronic inflammation. Dotted red line suggestive of usual negative regulation in a healthy individual. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pattern recognition receptors which recognize microbial ligands and use several mechanisms to induce transcription of pro-inflammatory genes and type I interferon (IFN- $\alpha$ , IFN- $\beta$ ) in AMs and DCs, signals which serve to alert the host to the presence of infection and promote immune activation and pathogen clearance [5]. These mechanisms involve nuclear factor kappa-B (NF- $\kappa$ B) and mitogen activated protein kinase (MAPK) pathways [5]. In addition, TLR ligation also leads to activation of the PI3K/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway [6]. Whereas the NF- $\kappa$ B

and MAPK pathways are pro-inflammatory, PI3K signaling serves to negatively regulate TLR signaling in macrophages and DCs [6].

The interstitial DCs pick-up the alleged antigen and migrate to the mediastinal LNs and initiate differentiation and clonal expansion of Th (CD4 T helper) 1 and 17 cells. Simultaneously, AMs produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which further stimulates production of chemokine ligands including MCP-1, CCL20, CXCL10 and CXCL16 under stimulation of both TNF- $\alpha$  and IFN- $\gamma$  derived from natural killer (NK) cells. Priming by IFNs sensitizes cells, resulting in an exaggerated

response of IFNs and TLRs to extracellular inflammatory stimuli [7]. IFN- $\gamma$  also primes macrophage activation by increasing phosphatase and tensin homolog (PTEN) via miR-3473b down regulation [8]. Increased production of IFN- $\gamma$  via stimulation by tumor necrosis factor (TNF- $\alpha$ ) and natural-killer (NK) cells activates AMs via the JAK/STAT (Janus kinase/signal transducer and activators of transcription) pathway to produce chemokine ligands (CCL20, CXCL10, CXCL16 and MCP-1) [9]. By generating these inflammatory factors, IFN- $\gamma$  signaling sustains inflammation, maintains CD4+ T helper 1 (Th1) responses and inhibits differentiation of Treg cells, CD4+ T helper 2 (Th2) and Th17 cells [10]. Th1 cells produce cytokines such as IL-6, IL-12, IL-18, IL-23, and TGF- $\beta$  in response to AEC-II activation by TLR-2.

Despite these amplification steps, TLR and IFN- $\gamma$  signaling are generally short lived as cells also activate precise complementary inhibitory mechanisms at the plasma membrane, cytosolic and nucleus levels to stop over-production of inflammatory cytokines as they are toxic to the host and can cause pathological inflammation [6]. Given that IFN- $\gamma$  signaling inhibits differentiation of regulatory T (Treg) cells, granuloma persistence and integrity is indirectly suggestive of chronic exposure to IFN- $\gamma$  in sarcoidosis. Persistent stimulation mediated by antigen presenting cells (APCs) leads to continuous cellular recruitment to the site of inflammation and granuloma formation. In many patients, granulomas persist for years likely due to chronically reduced differentiation of Treg cells by a sustained increase in IFN- $\gamma$  signaling.

Fig. 2 outlines a proposed mechanism by which persistent granuloma formation may result in LM. A central theme of this hypothesis is that persistent granuloma formation indirectly suggests the presence of increased IFN- $\gamma$  signaling which in turn leads to increased ligation of TLRs on AMs and peri-lymphatic DCs.

There is evidence of heterogeneity of the lymphatic microvasculature in pulmonary sarcoid granulomas [11]. Lymphocytes are aggregated in a highly organized manner, resembling normal secondary lymphoid organs (e.g. lymph nodes) but these are actually inflammation-related tertiary lymphoid organs (TLOs). There is a slow process of new lymphatic vessel formation during the development of TLOs because these vessels provide routes for recruiting lymphocytes and release of local edema [12,13]. Lymphotoxins (LTs) and LT-related inducible ligand (LIGHT) are cytokines of the tumor necrosis factor (TNF) superfamily [14]. Previous studies have reported that LTs and LIGHT promote the formation of high endothelial venules and lymphatic vessels in TLOs by mediating the LT-related activation of stromal cells to produce vascular endothelial growth factors, VEGF-C and/or VEGF-A, through activation of either a classic (canonical) or an alternative (non-canonical) NF- $\kappa$ B pathway [15,16]. However, it is unclear if lymphotoxins are involved in the progression of lymphatic malformation in sarcoidosis. Also, it is still unknown if the classic or alternative NF- $\kappa$ B pathway is activated in LMs. Additionally, TNF- $\alpha$  is reported to activate endothelial cells directly as well as via the NF- $\kappa$ B pathway and promote their proliferation and migration, leading to new lymphatic vessel formation via the VEGF-C/VEGFR (receptor) – 3 signaling pathway [17]. Hence in persistent sarcoid granulomas in TLOs, chronic exposure to TLR may lead to progression of LM via activation of NF- $\kappa$ B pathways.

It is well known that IFN- $\gamma$  priming results in high TLR activators, which produce inflammatory cytokines through MAPK and NF- $\kappa$ B pathways, but PI3K signaling serves to negatively regulate TLR signaling resulting in down regulation of the NF- $\kappa$ B pathway. In the case of chronic down regulation of PI3K signaling as may occur by a mutation or by increased activity of phosphatase and tensin homolog (PTEN), chronic upregulation of TLR signaling will result, which may lead to production of VEGF-C and/or VEGF-A via the NF- $\kappa$ B pathway and hence development of acquired LM. The development of LM in our patient at the site of previously noted mediastinal LNs, which may actually have been TLOs, suggest that the patient might have developed impaired regulation of PI3K signaling.

PIK3CA is the gene associated with the catalytic PI3K subunit p110 $\alpha$ , the somatic mutation of which has been implicated in the

pathogenesis of LM [18]. Somatic mosaicism mutations of PIK3CA, when they occur in the cells of mesodermal origin during the 6th–7th weeks of gestation, are associated with several congenital overgrowth syndromes and LM, but when somatic mutations of PIK3CA occur in the epithelial cells of adults likely from second hit mutation, they are associated with the pathogenesis of several solid tumors (ovarian, breast, uterus, gastric and colorectal cancers) [19]. It seems unlikely that our patient's LM developed due to a PIK3CA mutation, given that it is not a congenital malformation and she had no history of any solid tumor. Instead we propose that a more likely mechanism to account for PI3K pathway down regulation is increased activity of PTEN. PTEN is a tumor suppressor gene, which negatively regulates the PI3K/Akt/mTOR signaling pathway [20]. As mentioned above, IFN- $\gamma$  priming results in increased PTEN and hence in this patient with chronic elevation of IFN- $\gamma$  secondary to sarcoidosis it would be expected that she has increased PTEN and therefore PI3K pathway downregulation. It has also been suggested that inhibition of PI3K in vivo can result in fewer Treg cells with reduced suppressive capacity [21]. As noted above impaired immunosuppressive function of Treg cells has been suggested to contribute to persistence of granulomas, further suggesting that our patient likely has inhibition of the PI3K pathway. While this mechanism may account for our patient's development of LM it does not provide an explanation for why LM does not develop in all patients of sarcoidosis with persistent granulomas. It is possible that this variability might be due to some alteration in one or more of these signaling pathways perhaps due to use of medications, which are often used such as immunosuppressants and various analgesics. While our patient was previously on prednisone she had not been taking it during the two years prior to this presentation.

### Consequences of the hypothesis and discussion

Currently immunosuppression has a central role in the management of acute symptoms of sarcoidosis. However, several case reports/series have suggested promising role of molecular targeted therapy in case of transition from acute to chronic disease, refractory or recurrent disease, which likely results from dysregulation of molecular pathways leading to persistent granulomas. For example some reports have described a beneficial role of therapy targeting Th1 (which are maintained by IFN- $\gamma$ ) by anti CD-20 therapy [22,23]. It is known that anti IFN- $\gamma$  therapy has shown promising role in treating several Th1 associated autoimmune diseases [24], so given its potential role in refractory sarcoidosis and acquired LM, anti-IFN- $\gamma$  based therapy may be worthy of further investigation in these settings.

In patients with extensive mediastinal LM impinging on the airways, initial temporary tracheostomy followed by surgical excision (similar to our case) may be the only therapeutic option. In cases with few or no symptoms or recurrence, non-surgical treatment such as targeted therapy should be considered in the light of potential postoperative complications. Currently, sirolimus (mTOR inhibitor) is the most commonly used targeted therapy for complex congenital LM [25], though in acquired LM its role is uncertain as the mTOR pathway may already be suppressed by increased PTEN activity [20]. The role of anti IFN- $\gamma$  therapy in acquired LM remains to be elucidated in future studies.

In summary, we suggest that the persistence of granulomas in the majority of sarcoidosis patients is likely due to suppressed activity of Treg cells, which may result from persistently increased IFN- $\gamma$  and downregulation of PI3K signaling. Downregulation of PI3K signaling leading to increase TLR activated lymphotoxins via NF- $\kappa$ B pathway may result in progression of LM in tertiary lymphoid organs. As we did not measure Treg cells in peripheral blood nor performed genetic testing of resected tissue for PTEN, we do not have direct supportive evidences for our hypothesis, a main limitation of our study. If such a mechanism is operative however it is not clear why all the patients with sarcoidosis and persistent granulomas do not develop LM. Further study is needed to further explore this potential mechanism as well as the role

of anti IFN- $\gamma$  therapy in persistent granulomas and LM in sarcoidosis.

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### Ethics approval

Our institution does not require ethical approval for reporting individual cases.

### Informed consent

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mehy.2019.109287>.

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