Thesis progression

Thesis title: Development of Diagnosis and Monitoring Approaches for NTM Infected Patients in The Northeast of Thailand

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1. Introduction

Non-tuberculous mycobacteria (NTM) referred to mycobacteria other than *Mycobacterium tuberculosis* complex and its related species, as well as *M. leprae* and *M. lepromatosis* (Haworth et al., 2017; Kham-Ngam et al., 2018). NTM were omnipresent in environment that are isolated from soil, water, domestic, plant, wild animal and other items (Chetchotisakd et al., 2007; Tortoli, 2009). They can cause opportunistic infection in human with underlying conditions (Griffith et al., 2007). NTM infection can generally classified into four types including pulmonary disease, lymphadenitis, skin-soft tissue-bone disease, and disseminated disease (Griffith et al., 2007; Tortoli, 2009).

Diagnosis of NTM infection is a challenging. The American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) have jointly released diagnostic guidelines for this purpose. These guidelines suggest that the diagnosis of NTM pulmonary disease (NTM-PD) should be based on several factors such as clinical symptoms related to the lungs, radiological evidence consistent with NTM disease, and positive microbiological cultures of expectorated sputum, bronchial wash, or lavage. Additionally, lung biopsy is also recommended with histopathological features indicative of mycobacterial infection such as granulomatous inflammation or acid fast bacilli (AFB), and a positive culture for NTM or biopsy. However, even the bacterial culture method is considered as a gold standard in laboratory diagnosis. But this method is known to be time-consuming, taking around 1-8 weeks for mycobacterial growths and lack of sensitivity in detecting disease-causing bacteria (Forbes et al., 2018). Several molecular techniques are employed for NTM identification to improve NTM diagnosis efficiency including matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, polymerase chain reaction (PCR), whole genome sequencing (WGS), next generation sequencing (NGS), multi-locus sequence typing (MLST), line probe assay (LiPA) are

available for detection of NTM (Alcaide et al., 2018). These molecular methods have a sensitivity ranging from 29-76%, and they still require culture isolates for accurate identification.

Many previous studies have investigated the serological diagnosis of NTM-PD patients. They reported that human plasma IgA against a component on the surface of NTM cells, glycopeptidolipid (GPL)-core, can be a useful diagnostic tool for patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD) (Kitada et al., 2008; Kitada et al., 2002). The diagnosis kit is now available commercially known as Capilia MAC Ab enzyme linked immunosorbent assay (ELISA). Diagnostic efficacies had been evaluated in Japan, Taiwan, South Korea, and the United States with sensitivity of the assay were ranging from 60-90% and specificity rates ranging from 91-100%, (Jeong et al., 2013; Kitada et al., 2008; Kitada et al., 2013; Kitada et al., 2002; Kitada et al., 2005; Shu et al., 2013). However, it should be noted that the prevalence of NTM infections may differ across the various regions that a slow growing mycobacteria (SGM) is common in Japan and outside Asia, but a rapidly growing mycobacteria (RGM) was reported to be commonly found in China and Southeast Asia (Hase et al., 2017). For instance, in the Northeast of Thailand, *M. abscessus*, one in the RGM group, is a predominant cause of NTM infections (Kham-Ngam et al., 2018).

According to the differences in bacterial distribution, diseases, and exposure factors in Thailand and other countries. Therefore, the first project in this study aims to evaluate the possibility of applying the Capilia MAC Ab ELISA in diagnosis of patients with NTM infection in Northeast of Thailand.

On the different aspect, some NTM infected patients presented with anti-interferon-**γ** autoantibodies (anti-IFN-**γ** auAb) causing disseminated NTM (dNTM). The dNTM is an infection at least two distinct organs and cause severe disease in humans (Chetchotisakd et al., 2007; Griffith et al., 2007; Sun et al., 2022; Yuan et al., 2021). The diagnosis of dNTM requires a combination of clinical, radiographic, microbiologic, and histopathologic features (Griffith et al., 2007; Musaddaq & Cleverley, 2020). Detection of neutralizing anti-IFN-**γ** auAb found in disseminated NTM by either indirect ELISA or inhibitory ELISA had been introduced. The inhibitory ELISA had a higher specificity of 100%, compared to the indirect ELISA which had a specificity of only 35% (Nithichanon et al., 2020). A low specificity of indirect ELISA based approached can be interfered with polyreactive antibodies (PAb) in plasma sample which are upregulated in people with abnormal immunity condition such as infections (Labombarde et al., 2022). Many previous study suggested that PAb and loss of central and peripheral self-tolerance potentially resulted to autoreactive and might developed into autoimmune disease such as Myasthenia gravis (MA), Rhumatoid artritis (RA), Systemic lupus erythematous (SLE) and Sjögren's Syndrome (SjS) (Lee et al., 2016; Sáez Moya et al., 2021; Samuels et al., 2005; Yurasov et al., 2005; Zhang et al., 2009)

The second research project will explore the effect of polyreactive antibody against the diagnosis prior development of a novel detection approach for neutralizing anti-IFN-γ autoantibody. Firstly, this study aims to observe the polyreactivity of plasma and peripheral mononuclear cells (PBMCs) samples including to identify neutralizing monoclonal antibody clone from dNTM patients with anti-IFN-γ auAb. Next, to reduce turnaround time, more cost effective of dNTM diagnosis, the development of rapid test to detect anti-IFN-γ auAb for dNTM diagnosis was interested. Furthermore, this study could potentially reveal some aspects of the disease mechanism that are still not well understood.

This thesis progression

According to previous results, the correlation between the polyreactive antibody marker antigen and the IFN-γ antigen was positive and statistically significant, suggesting that the polyreactive antibody might interfere with the detection of anti-IFN-γ antibodies using ELISA. Furthermore, upon re-evaluating our IFN-γ probes with Anti-mouse IgG beads, we observed a low signal from the conjugated dye. In response, we have ordered a new protein conjugating dye and will assess the probes for both quantity and quality using sandwich ELISA and flow cytometry. Following this evaluation, we will re-analyze the presence of anti-IFN-γ IgG+ memory B cells..

Previous results and discussion

The study involves the analysis of plasma samples obtained from 36 healthy controls (HC), 36 patients with disseminated non-tuberculous mycobacterial (dNTM) infection and positive anti-IFN-γ autoantibody (auAb) titers (Titer positive), 36 dNTM patients with negative anti-IFN-γ auAb titers (Titer negative), and 34 individuals with other bacterial systemic infections serving as control (Other infections). The observations indicated the presence of polyreactivity across all sample groups, as illustrated in Figure 1. Notably, a subset of samples from the Other infections group exhibited reactivity with a non-specific antigen (FBS). Further scrutiny of the positive results revealed a noteworthy pattern: the identical samples exhibited reactivity among different antigens within each sample group. Additional scrutiny of the

positive results revealed a noteworthy pattern: the identical samples exhibited reactivity among different antigens within each sample group.

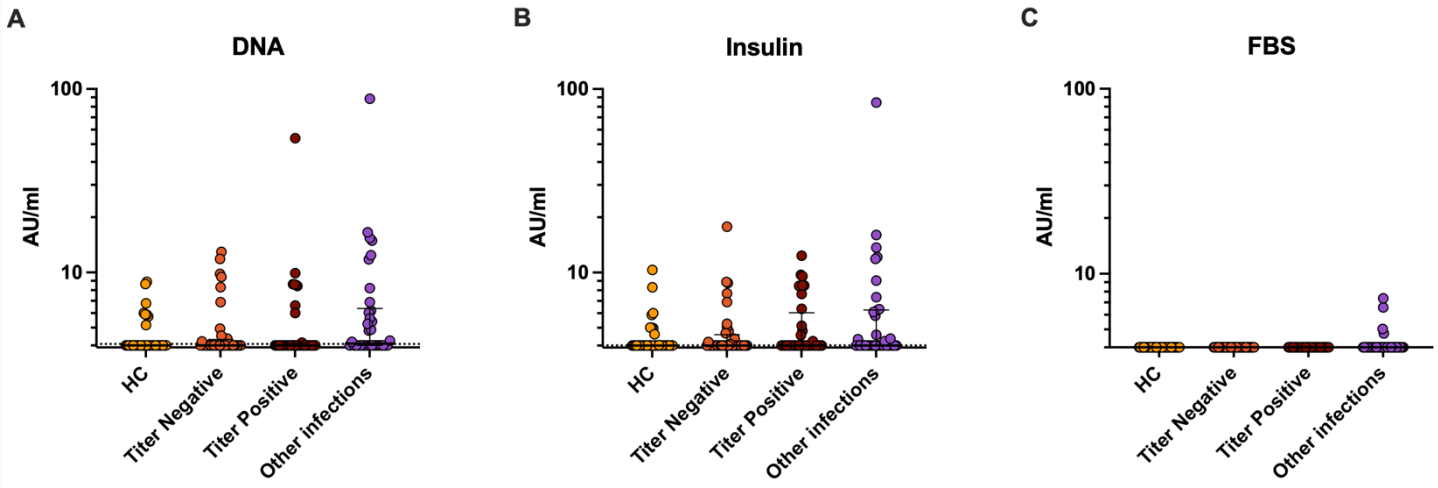

Figure 1. Polyreactive ELISA result. plasm sample from dNTM patient with anti-IFN-γ auAb (titer positive), dNTM patient without anti-IFN-γ auAb and healthy control against three different antigens. Comparison of anti-dsDNA IgG **(panel A),** comparison of anti-insulin IgG **(panel B)** and comparison of anti-FBS IgG **(panel C)** were plotted as dot plots with a line representing the median and interquartile range. Statistical differences among sample groups were tested using the Kruskal–Wallis test, and only significant P-values are shown in the figure.

Furthermore, our investigation revealed instances of false positive reactivity within the titer-negative group (Titer negative, including HC and Other infections) against the IFN-γ antigen, as illustrated in Figure 2A. Building upon the earlier observation that individual samples demonstrated reactivity across various antigens, we conducted a closer examination of the correlation between polyreactivity and false positivity against the IFN-γ antigen.

The results unveiled a significant correlation, with anti-DNA antibodies exhibiting a 75% correlation with false positive anti-IFN-γ results ($P < 0.0001$), (Figure 2B). Similarly, anti-insulin antibodies showed a notable correlation of 74% with false positive anti-IFN-γ results ($P < 0.0001$) (Figure 2C). These findings strongly suggest that the occurrence of false positivity in indirect ELISA may be attributed to the presence of polyreactive antibodies in plasma.

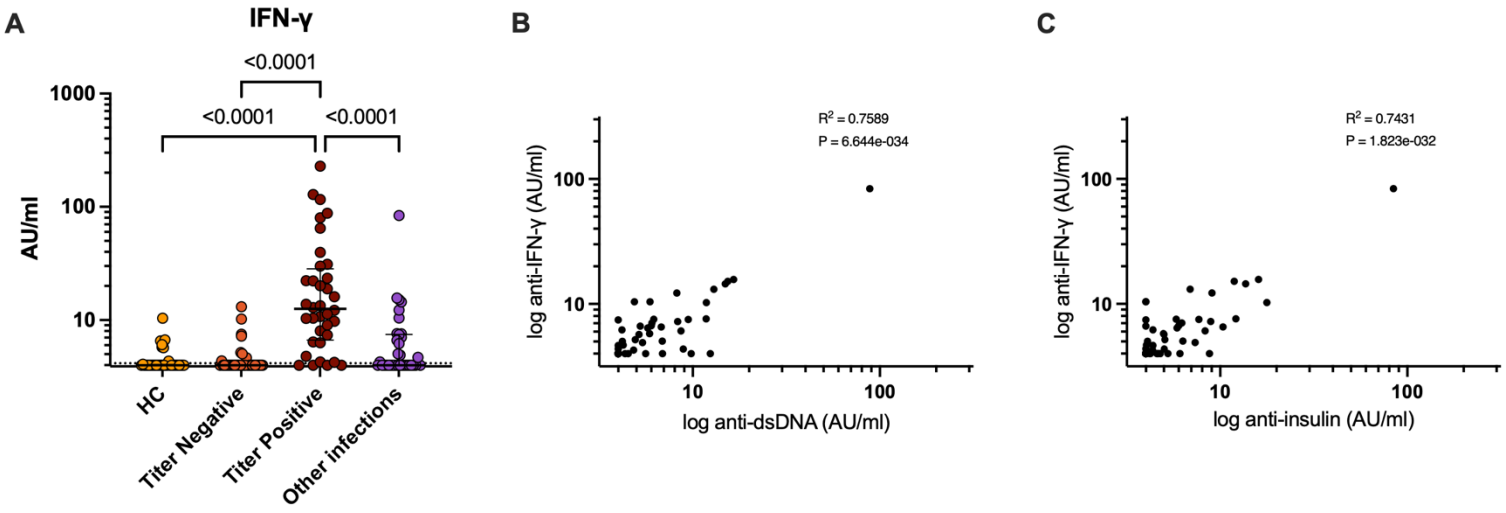

Figure 2. Indirect ELISA anti-IFN-**γ**, Comparison of anti-IFN-**γ** IgG **(panel A).** were plotted as dot plots with a line representing the median and interquartile range. Statistical differences among sample groups were tested using the Kruskal–Wallis test, and only significant P-values are shown in the figure. Correlation between anti-dsDNA and false positive anti-IFN-**γ** results **(panel B),** Correlation between anti-insulin and false positive anti-IFN-**γ** results **(panel C)** were plotted as dot plots with log AU/ml. Statistical correlation among each antigen reactivity were tested using Pearson correlation coefficient.

The assessment of the IFN-**γ** probe revealed a low intensity for both APC and PE tagged probes (Figure 3). This observation suggests a potential loss of the probe's properties after being tagged with the fluorescence dye, leading to reduced binding with antibodies. The commercial dye utilized in this study did not offer insights into the principles underlying the dye's attachment to the protein. In response to these challenges, we have taken the initiative to address this issue by ordering a new protein tagging dye specifically designed to bind to NH_2 groups.

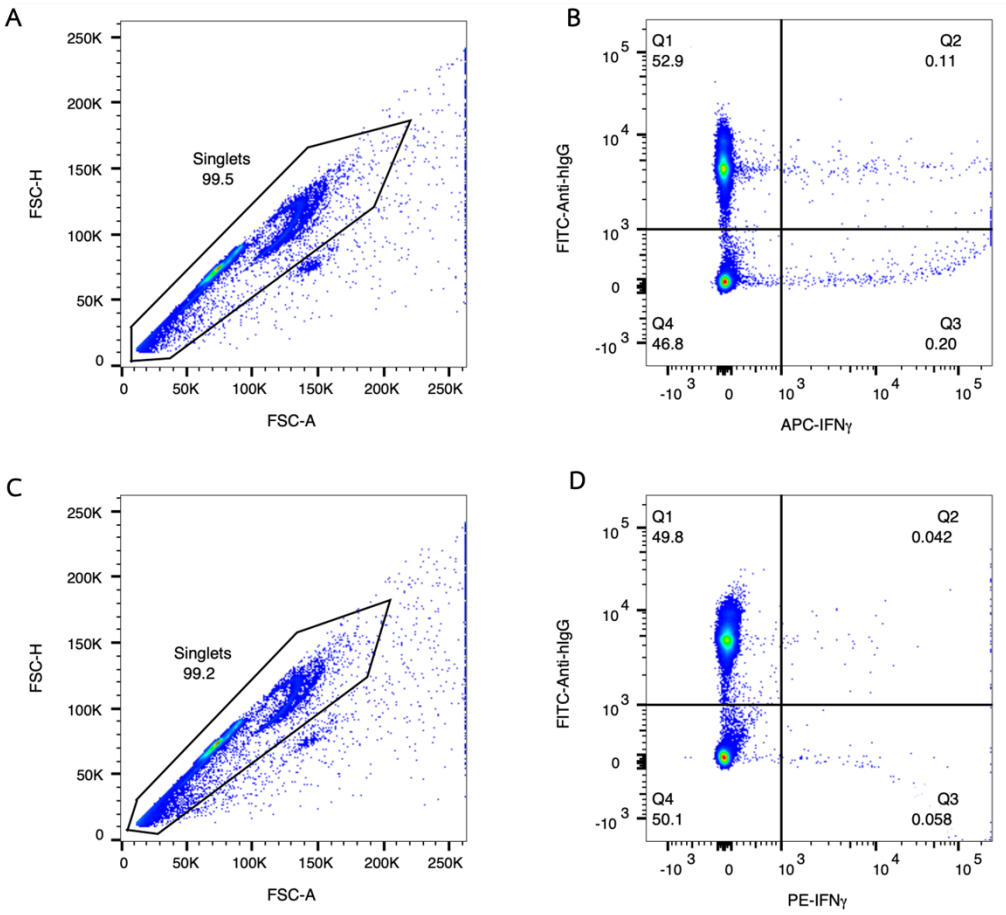

Figure 3. IFN-γ probe checking for surface staining anti-IFN-γ IgG+ memory B cell optimization results

Result and discussion

After conjugating a new IFNγ probe with a novel dye from Dojindo, Japan, following the manufacturer's labeling protocol, we assessed the quantity of the conjugated IFNγ. This was done to determine if the protein had lost its antibody-binding function or had become denatured. A sandwich ELISA was used to measure the concentration of the newly labeled IFNγ probe. The results indicated that the concentrations of both the PE-IFNγ and APC-IFNγ probes remained as expected (Figure 4A), suggesting that they were still intact and capable of binding to antibodies. After verifying that the PE-IFNγ and APC-IFNγ probes remained intact and capable of antibody binding, we evaluated the quality of the conjugated dye to determine if the newly labeled IFNγ probes exhibited strong fluorescence signals. Using flow cytometry, we analyzed the fluorescence intensity of the probes. The results demonstrated a strong signal from the IFNγ probe, with clear distinction between the positive and negative anti-

mouse IgG bead populations (Figure 4B). These results indicate that our new probe can effectively differentiate between anti-IFNγ antibody-positive and negative beads. Consequently, in the subsequent experiment, we used this probe as a marker for surface staining of anti-IFNγ IgG+ memory B cells from dNTM patients. This was done to investigate whether dNTM patients possess memory B cells that target IFNγ.

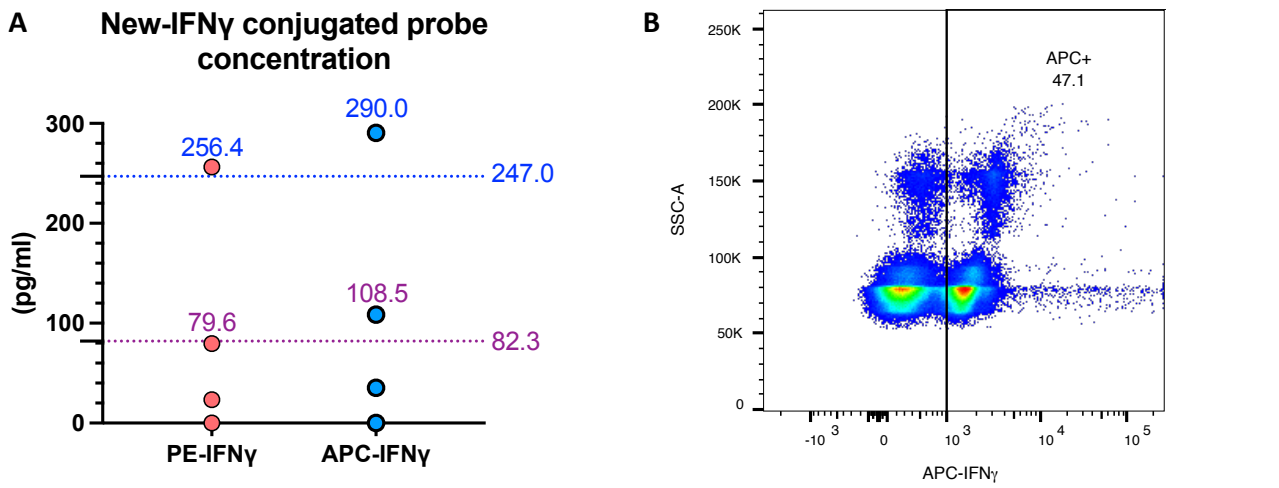

Figure 4. Probes investigate for quantity and quality. Sandwich ELISA were plotted as dot plots with a line representing the expected concentration, PE-IFNγ were plotted in pink dot and APC-IFNγ were plotted in blue dot **(panel A).** The intensity of the conjugated dye on the conjugated IFNγ **(panel B)**

Surface staining for anti-IFNγ IgG+ memory B cells from dNTM patient

An independent experiment was conducted to investigate the presence of anti-IFNγ IgG+ memory B cells in dNTM patients. The results from both experiments revealed that after gating for IgG+ memory B cells (Figure 5A), the dNTM patients' cells did not show double-positive binding with IFNγ compared to healthy controls (Figures 5B and 5C). This suggests that other types of antibody-secreting cells may play a more significant role in this autoimmune disease.

Previous studies on autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA), as well as research on autoreactive B cells, suggest that Toll-like receptor (TLR) signaling can drive autoreactive B cells into plasmablasts, which can develop into long-lived plasma cells in the bone marrow. TLR signaling can even induce

somatic hypermutation (SHM) extrafollicularly under T cell-independent conditions (Herlands et al., 2008; Hess et al., 2013; Hiepe et al., 2011; Wen et al., 2023). This might explain why IgG+ memory B cells against IFNγ were not detected in our study, as the B cells may not have followed the normal T cell-dependent pathway that drives activated B cells into memory B cells and subsequently into antibody-secreting cells (ASCs), such as plasmablasts or plasma cells.

Further research on plasma cells holds promise for better understanding the disease mechanism of anti-IFNγ autoantibodies (auAb) and for identifying new anti-IFNγ antibody clones. This could aid in the development of point-of-care testing (POCT) kits, improving the diagnosis of dNTM.

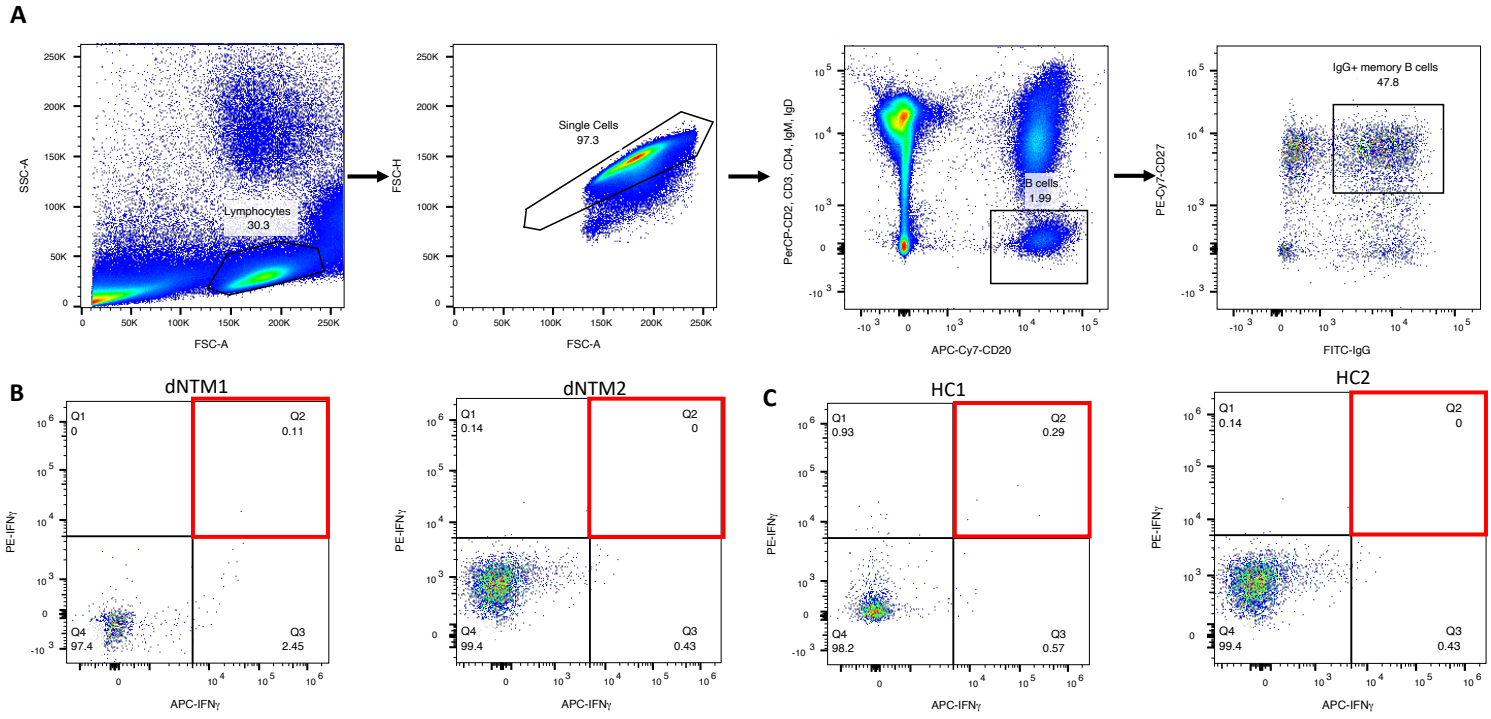

Figure 5. Surface staining for anti-IFNγ IgG+ memory B cell from dNTM patient. Gating strategy for IgG+ memory B cell from peripheral blood mononuclear cell (PBMC) from dNTM patient **(panel A).** The IgG+ memory B cell from dNTM patient binding to IFNγ probes **(panel B).** The IgG+ memory B cell from healthy control (HC) binding to IFNγ probes **(panel B)**

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