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TUBERCULOSIS

Novel M. tuberculosis specific IL-2 ELISpot assay discriminates adult patients with active or latent tuberculosis

INTRODUCTION

Tuberculosis (TB) is a highly contagious infectious disease caused by *Mycobacterium tuberculosis* (Mtb); is one of the oldest recorded human diseases and is still a major health problem worldwide (**Fig. 1**), with 1.2 million deaths among HIV-negative people and an additional 251,000 deaths from TB among HIV-positive people estimated in 2018 [1]. Although nearly 9 million new cases of active TB are still reported annually, the majority of infected individuals do not develop this form of the disease: 1.7 billion people in the world's population harbour a latent tuberculosis infection (LTBI). The LTBI is defined as a state of persistent immune response to Mtb in the absence of clinical, radiological and microbiological evidences [2]; people with LTBI are at risk of developing active TB disease during their lifetime [1].

The molecular epidemiological evidence suggests that the original infecting strain can lead to reactivation of TB up to 30 years after the initial infection [3]; this hypothesis is in keeping with previous reports that live and viable Mtb bacilli can be recovered from incidental TB lesions discovered post-mortem in individuals who died of other causes [4].

The preventive treatment of LTBI subjects is a key point to reduce TB at individual level, according to the WHO post 2015 End TB strategy. The early identification and treatment of patients with LTBI can reduce the burden of active TB, especially if the screening is performed on populations at high risk for progression to active disease, such as people with HIV infection or other immune deficiency, children aged under 5 years and household contacts with widespread exposure (all ages) to someone who has bacteriologically confirmed pulmonary TB [1, 5, 6].

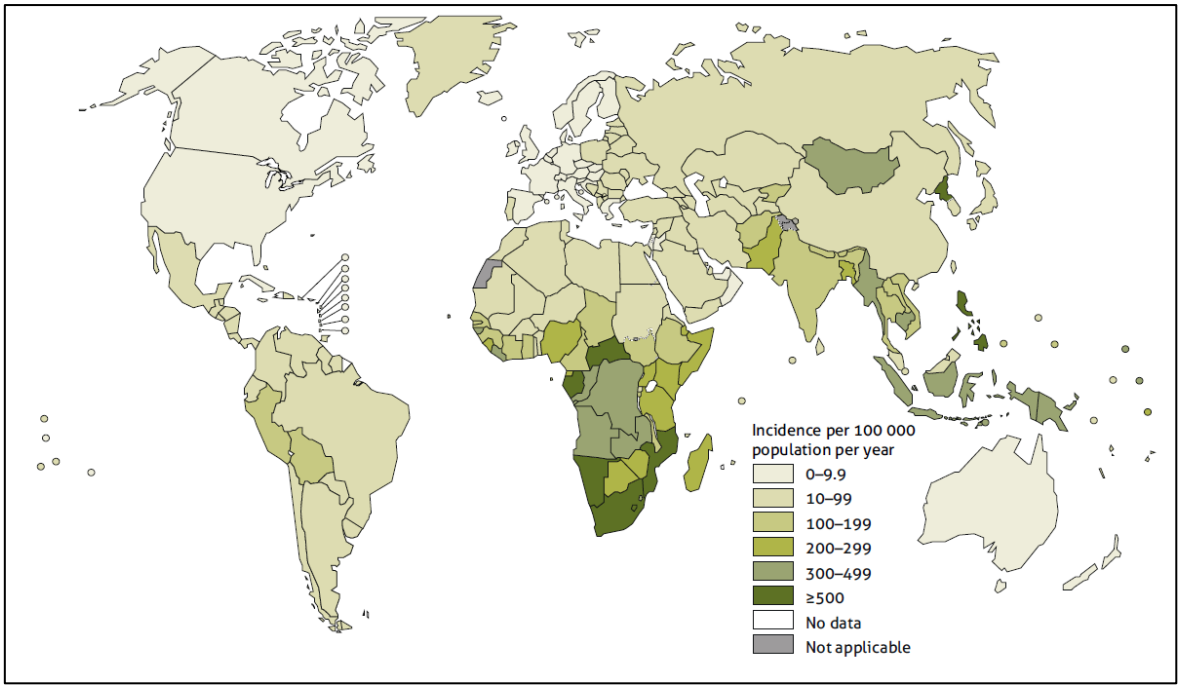

Figure 1. Estimated TB Incidence rate 2018 (Adapted from WHO global tuberculosis report 2019).

Aetiology and pathophysiology

The key concepts about microbiology and pathophysiology of TB became known slowly over the last 100 years. Mtb is an intracellular facultative bacillus first identified in 1882 by Robert Koch, who subsequently received the Nobel Prize in Physiology or Medicine for this discovery in 1905; the bacterium is also known as Koch's bacillus. Mtb and humans seem to have co-evolved during the period before the migration of humans out of Africa [7]. Mtb is a species of pathogenic bacteria in the family *Mycobacteriaceae*, Phylum *Actinobacteria*, order *Actinomycetales*. Mtb is part of a complex (MtbC) whose species are characterised by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. Assuming that they all are derived from a common ancestor, it is intriguing that some are exclusively human (*M. tuberculosis, M. africanum, M. canettii*) or rodent pathogens (*M. microti*), whereas others have a wide host spectrum (*M. bovis*) [8].

The bacillus requires oxygen to grow, it is highly aerobic and requires high levels of oxygen to duplicate, does not produce spores and is non-motile [9], his generation time is 15-20 hours and this is extremely slow compared to other bacteria.

Mtb has an unusual, waxy coating on its cell surface primarily due to the presence of mycolic acid and is likely responsible for its resistance to desiccation and is a key virulence factor. The Mtb's cell envelope differs substantially from the canonical cell wall structures of both gram-negative and gram-positive bacteria because in addition to the cell membrane and peptidoglycan layers, it contains mycolic acids and a vast array of lipids and glycolipids which confer extreme hydrophobicity to the outer surface of the organism. This coating makes the cells impervious to Gram staining and, as a result, Mtb can appear either Gram-negative or Gram-positive. Therefore, to identify Mtb with a microscope, Acid-fast stains such as Ziehl-Neelsen and the fluorescent stains such as auramine can be used [10]. Mtb cells are curved rod-shaped and are often seen wrapped together, due tothe presence of fatty acids in the cell wall that stick together; this appearance is referred to as cording, like strands of cord that make up a rope. Mtb can be grown in laboratory; commonly used media include liquids such as Middlebrook 7H9 or 7H12, egg-based solid media such as Lowenstein-Jensen, and solid agar-based such as Middlebrook 7H11 or 7H10 [9]. Visible colonies require several weeks to grow on agar plates.

Humans are the only known reservoirs of Mtb. It can be spread through air droplets generated by cough or sneeze of person with Mtb lung infection (Pulmonary tuberculosis) and inhaled by uninfected person [11]. The respiratory droplet nuclei are small enough in size (1–2 mm or less) to pass into the lower respiratory tract escaping the anatomical barriers of nasopharynx and upper respiratory tract. The inhaled bacilli are deposited in alveolar space and ingested by alveolar macrophages (AMs). Although AMs are the primary innate cells that the bacilli encounter, other various phagocytic cells recruited to infected lung including neutrophils, monocyte-derived macrophages and dendritic cells (DCs) also take part in the phagocytic process [12]. Phagocytosis of Mtb by AMs and DCs initiates a cascade of events involving the production of cytokines and chemokines, which stimulate the activation of phagocyte anti-microbial activities and recruit blood polymorphonuclear leukocytes (PMN) and additional mononuclear leukocytes into the tissue to the site of infection. The accumulation of mononuclear leukocytes around foci of infected cells leads to the formation of a macrophage-rich cell mass known as the granuloma. Macrophages serve as the major host cell niche for the growth and survival of Mtb. However, these cells are also responsible for activation of the protective immune responses, both innate and acquired, which are necessary to control or eliminate the infection. That outcome of primary infection is most often a latently infected healthy human host, in whom the bacilli are held in check by the host immune response and persist

in a sub-clinical (quiescent) form, the bacteria may become dormant or may persist at low numbers. This phase of LTBI is manifested only with a positive tuberculin skin test (TST) or positive interferon gamma release assay (IGRA), such individuals can develop active TB later in life with impairment in the immune system. In contrast, in a minority of infected individuals, the host immune response fails to control the growth of bacilli, and progressive granulomatous disease develops Primary TB, facilitating spread of the bacilli via infectious aerosols coughed out into the environment and inhaled by new hosts [12].

In the earliest events in the lung, there is impact of the initial environment encountered by the bacterium, this environment depends on the cells it first encounters, and these, in turn, may depend the genetics of the host.

Mtb is able to manipulate both the innate and acquired immune response of the host. Mtb expresses an extremely wide variety of virulence factors that counteract macrophage efforts in suppressing the pathogen. Among Mtb strategies we can include the inhibition of intracellular trafficking, the inhibition of autophagy, the acquisition of cytosol access, the induction of host cell death and the neutralization of toxic components as reactive oxygen species and toxic metals, Mtb lacks classical virulence factors such as toxins and its immune-escaping ability depends on the modulation of lipid metabolism, metal-transporter proteins, protease, proteins inhibiting the antimicrobial effectors of macrophages and many others [13]. In strong support of this concept, studies in zebrafish have demonstrated the ability of Mtb to manipulate the earliest recruitment of macrophages. This activity has been linked to the capacity of the bacterium to express lipids on its cell surface. Specifically, expression of the Mtb molecule phthiocerol dimycoceroserate (PDIM); limits innate recognition of Mtb, thereby reducing recruitment of Toll-like receptor (TLR)-activated macrophages; in addition to this subversion of the pathogen-recognition pathway, other lipids on the Mtb surface-the phenolic glycolipids (PGLs) - drive recruitment of permissive macrophages via a pathway that depends on the chemokine CCL2 and the chemokine receptor CCR2 [14].

Mtb infection can be influenced by autophagy, a process whereby components of the cytoplasm, including organelles and intracellular pathogens, are sequestered in an autophagosome and delivered to the lysosome for degradation. Activation of autophagy, by IFN-γ for example, results in phagosome maturation and an increase in its acidification and Mtb killing. However, in contrast to nonviable bacilli viable and virulent Mtb bacilli are able to prevent phagolysosomal fusion and persist in the phagosome, preventing acidification of the phagosomal compartment, thus adapting to the intracellular

environment of the macrophage and creating a niche for survival. Opsonisation of the bacilli prior to infection inhibits this blockade of phagolysosomal fusion. Macrophages can eliminate Mtb via different mechanisms if appropriately activated. Whilst IFN-γ is a key element in the containment of Mtb within macrophages, it is now widely recognized that performing this function requires the presence of vitamin D, because that has anti-inflammatory and bacteriostatic effect [15].

Infection of macrophages with Mtb can induce necrotic death, defined by cell lysis, which allows exit from macrophages and therefore cell-to-cell spread of the bacilli. Alternatively, infection can result in apoptotic death of the macrophages that maintain an intact plasma membrane and is associated with diminished pathogen viability and enhanced immunity [12].

Macrophages and DCs are key cells in the immune response to Mtb by presenting antigens to T cells in the context of both major histocompatibility complex (MHC) class I and II. Host immune response against Mtb is mediated by cellular immunity, in which cytokines and Th1 cells play a critical role: Th1cells activate macrophages to kill the bacteria by secreting IFN-γ and TNF-α. Furthermore, macrophages and DCs play an important role in cells recruitment at the site of infection by secreting the proinflammatory cytokines IL-1 and IL-6. DCs are prime antigen presenting cells (APCs) and they help in maximizing the recognition of antigens by T cells in the draining lymph nodes.

The migration of Mtb away from the lung occurs despite the generation of granulomatous responses and it occurs 7-9 days after initial infection; as a consequence, there is a delay of up to 18-20 days before antigen-specific T cells accumulate to sufficient numbers in the lung to stop bacterial growth. Even after vaccination, when a population of circulating memory T cells is present, this accumulation of antigen-specific T cells is accelerated by only five days, and Mtb still have 15 days in which to define the lung lesion before T cells arrive [16].

The acquired immunity to Mtb requires contributions by multiple T cell subsets: CD4+, CD8+, γδ T cells and CD1-restricted T cells. The initiation of the acquired response is preceded by the appearance of bacteria in the draining lymph nodes. Inside the lymph nodes, T lymphocytes undergo a process of activation and expansion of the specific populations for the mycobacterial (Mtb) antigens. However, at this point, the largest part is done, and the infection is now established. The acquired response is delayed relative to the initiation of infection. Cellular immune response can be evidenced 2-6 weeks after Mtb infection by the development of a delayed-type hypersensitivity response (DTH) to

intradermal injected tuberculin or purified protein derivative (PPD). Multiple mechanisms may explain the delay in the onset of T cell activation and functional effector T cell responses after primary or secondary infection with Mtb. Such mechanisms include Mtb inhibition of apoptosis of macrophages and neutrophils, as discussed above, which delays or inhibits the antigen-presenting capacity of DCs; Foxp3+ regulatory CD4+ T cells, which not only inhibit but also delay the arrival of effector T cells in the lung during early TB; and IL-10, which inhibits and delays production of the cytokines IFN-γ and IL-17 by CD4+ T cells in the lung. Many of these mechanisms also operate via effects on DC migration and function [12]. Once the acquired response has been initiated, T cells must migrate from the circulation into the parenchyma of the lung and then into the infected site, which is composed largely of macrophages and dendritic cells expressing a variety of effector functions [17]; this predominantly mononuclear environment is thought to be maintained by the expression of the protective cytokine IFN-γ.

CD4+ T cells play a dominant role in the protective response against Mtb. The Mtb reside within macrophage vacuoles; therefore, Mtb antigens are loaded on MHC class II and presented to CD4+ T cells through endocytic antigen presentation pathway. Upon activation, CD4+ T cells secret IFN-γ, IL-2 and TNF-α, which in turn activates macrophages. CD4+ cells are also important to enhancing the antigen presenting cell (APC) function through interaction of CD40-CD40L between CD4+ T cells and APC and this in turn can facilitate APC mediated induction of other T cells such as CD8+ T cells. The importance of IFN-γ production by CD4+ cells is particularly relevant at the early stages of Mtb infection as it is demonstrated that adequate IFN-γ levels can be obtained with 3 weeks of delay even in CD4+ - disrupted mice thanks to the compensation offered by other cell types like CD8+ [18]. Other roles played by CD4+ T cells include induction of apoptosis through perforin and granulysin, by Fas-Fas ligand interaction or TNF-α lytic pathways, help for B cells and CD8+ T cells, and production of other cytokines. CD8+ T lymphocytes have an activity against Mtb is conceivable considering that they recognize Mtb antigens through class I molecules of MHC, and produce IL-2, IFN-γ and TNF-α, which have a well-known role in controlling Mtb [19]. This direct cell-to-cell contact determines the apoptosis of the Mtb-infected cell especially macrophages depriving Mtb from its natural growth environment and at the same time reducing its viability by unknown mechanism [20]. On the other hand, CD8+ T cells produce IL-10 and TGF-β which favours the Mtb infection development.

Following the development of adaptive immunity, a complex and well-coordinated

mechanism is established between both immunity mechanisms, innate and adaptive, which seal the Mtb inside granulomas that isolate the bacteria from the rest of body [21]. This mechanism develops in at least 90% of the infected subjects and leads to LTBI. During LTBI, Mtb ceases to divide but doesn't die; thereby preventing eradication of the pathogen from the infected body, the subject is generally positive for the TST and for the IGRA.

The mechanisms inducing and maintaining the persistence of a pathogen in LTBI are still unclear.Wayne and Sohaskey refer to the latent physiological state as "non-replicating persistence" (NRP) when the bacilli evade the host immune response and survive inside the hostile macrophage environment, in a two step adaptation conditions: first in a stage of microaerophilic (NRP-1) and then anaerobic (NRP-2) persistence [22]. Since 1996, the Wayne model has been used in hundreds of studies, which greatly increased our knowledge about the Mtb dormancy. For example, Rustad et al. described expression of the 230 Mtb genes referred to as EHR (Enduring Hypoxic Response) genes: a rise of gene expression followed the induced DosR-mediated hypoxic response on days 4-7 of hypoxia. These genes include a surprising number of transcriptional regulators that could control the program of bacteriostasis [23].

The Mtb two-component system DosR-DosS (also called DevR-DevS) has received significant attention over the last decade as a key regulatory mechanism underlying Mtb dormancy. The histidine kinases DosS (DevS) and DosT phosphorylate and activate the cognate response regulator DosR in Mtb. Originally found to be induced by exposure of Mtb to progressive hypoxia, the dormancy survival regulator (DosR) regulon comprises 48 genes, including several believed to play an important role in Mtb adaptation to hypoxic stress, as well as energy acquisition from alternative carbon sources, such as fatty acid metabolism, and nitrate reductase genes. The DosR regulon acts shifting metabolism away from aerobic respiration and maintaining energy levels and redox balance [24].

The development of Mtb dormancy is a complex process, which radically remodels the genetic, metabolic, and functional systems of the mycobacterial cell; expression of certain genes changes the metabolism of bacterial cells. A number of studies have demonstrated that the transformation into dormant (nonreplicating) forms involves major cellular metabolic pathways. In general, characteristic of the metabolic shift is slowdown of all principal cellular processes, which results in the arrest of cell growth and division. Changes in the protein synthesis as a direct consequence of the changes in the transcriptome determine the further cell functioning. The whole proteome profiling studies have shown a downregulated expression of over 1500 proteins during Mtb dormancy. In addition, a

dormant Mtb synthesizes the proteins (including enzymes) that are not typically expressed during active replication [25]. As is known, the energy metabolism is changed towards a decline in the intracellular ATP level and an increase in the NADH/NAD ratio [24, 26]. Among the enzymes expressed during this dormancy is L-alanine dehydrogenase (Ala-DH), which converts pyruvate to alanine and glyoxylate to glycine concurrent with the oxidation of NADH to NAD; it is involved in the metabolic remodeling of Mtb through its possible interactions with both the glyoxylate and methylcitrate cycle.

The *ald* gene encodes Ala-DH, NAD (H)-dependent L-alanine dehydrogenase (EC 1.1.4.1; Ald) was identified as one of the major antigens present in culture filtrates of Mtb. The overexpression of the *ald* gene (Rv2780) is found under hypoxia and nutrient-starvation conditions. Upregulation of *ald* expression by alanine or under respiration-inhibitory conditions is mediated by AldR, a member of the Lrp/AsnC family of transcriptional regulators. Increased levels of this enzyme have been linked to the generation of alanine for peptidoglycan biosynthesis and maintain of cellular NADH/NAD+ homeostasis. The catabolic role of was showed to be required for mycobacterial utilization of alanine as sole nitrogen source and the adaptation of Mtb in anaerobic dormant stage in LTBI [27].

Ala-DH has important role in other bacteria that have specialized persistence programs, such as sporulation. In *Myxococcus xanthus*, an *ald* mutant showed delayed aggregation and reduced levels of sporulation. Ala-DH of Mtb is a unique enzyme involved in peptidoglycan biosynthesis since it only accepts L-alanine as substrate in contrast to Ala-DH from all other organisms studied, which also use serine as a substrate [28, 29]. In mycobacteria, it was identified as an enzyme absent from the vaccine strains of *M.* bovis BCG but present in virulent Mtb [30]. The *ald* gene of *M. bovis* contains a single nucleotide deletion and therefore lacks this enzyme. It was suggested that impairment of *M. bovis* BCG replication in humans due to the lack of a functional Ala-DH inhibited the development of protective immunity [31]. Ala-DH is present in other pathogenic mycobacteria such as *M. marinum* and *M. ulcerans.*

Griffin proposed that the main role of Ala-DH during hypoxia is to maintain the redox balance of Mtb. NAD is the principal oxidant in the cell and essential for many reactions, so NADH must be rapidly oxidised to prevent inhibition of important processes. The ratio between NAD and NADH is tightly regulated by redundant systems, and in Mtb is generally between 1:3 and 1:10. When oxygen levels decrease or aerobic respiration is blocked by NO the ratio of NAD to NADH shifts. This shift is also seen in Mtb in the lungs of mice, indicating that redox stress is present *in vivo*. In response to decreasing oxygen,

Mtb induces a set of dormancy genes that enable this obligate aerobe bacterium to adapt to and survive in low oxygen conditions. The *ald* transcription is upregulated in Mtb late in NRP-2 stage and then decreases as the cells enter the general shutdown characteristic of dormancy. Although transcription of *ald* decreases, the protein is stably maintained so that the cell will be ready to reactivate when oxygen levels increase. The late expression of *ald*, and a defect in regrowth of an *ald* mutant of Mtb suggested a role for Ala-DH during reaeration, infact, it is proposed that Ala-DH is involved in maintaining the optimal NADH/NAD ratio not only during dormancy, but also during reactivation when oxygen levels increase enough to support regrowth of Mtb [32]. Ala-DH catalyses the NADH-dependent conversion of pyruvate and ammonia to L-alanine and is involved in microbial carbon and nitrogen metabolism. The mycobacterial enzyme is secreted into the culture medium and is identical with the 40-kDa antigen that has been identified in culture filtrates of Mtb [33]. In solution, the enzyme forms a hexamer of six identical subunits (predicted molecular mass = 38,988 Da per subunit), as observed for most Ala-DH from other species. Agren et al. [27] demonstrated that three-dimensional structures of Ala-DH could alter the expression profile for adaptation to a state of latent infection. The authors also showed that the conformation and crystal structure of Ala-DH was changed from open to closed ternary forms in the phase of latent infection, which indicated the different host immune responses of LTBI. The structures reveal a conformational transition in the form of a 16° rotation upon binding of the dinucleotide, from an "open" to a "closed" conformation (**Fig. 2**). This conformational change ensures proper orientation of the substrates for hydride transfer to occur and excludes bulk water from the active site. The crystal structures and complementary site-directed mutagenesis studies support a mechanistic model where conformational changes are required to allow access of water and ammonia to the active site and where the active-site residues His96 and Asp270 participate in proton transfer steps during catalysis. The experimental studies have demonstrated that upon the binding of the coenzyme, Ala-DH undergoes a large-scale conformational transition from the open-apo to the closed-holo states, in which the substrate-binding domain (SBD) rotates by 16 toward the NAD-binding domain (NBD) [27]. The NBD includes a central seven-stranded β-sheet, as well as several surrounding α-helices; the SBD consists of an eight-stranded β-sheet and several α- helices. The crystal structure of the open-apo and closed-holo states of the protein have been determined by X-ray crystallography, which can be downloaded from the Protein Data Bank (PDB) with the access codes 2VOE and 2VHZ, respectively [34, 27].

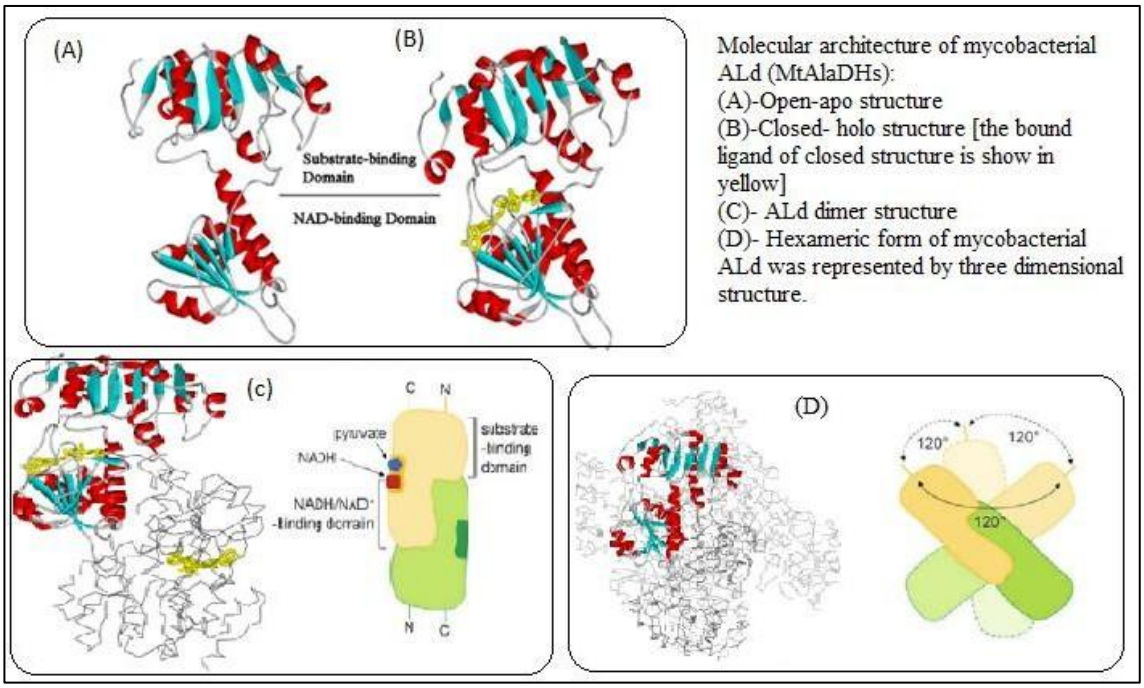

Figure 2. Molecular architecture of mycobacterial Alanine Dehydrogenase. Two forms of structures for Ala-DH including the dimer (Figure 2C) and the hexamer (Figure 2D) states. The hexameric molecule is made of three-fold non-crystallographic axes, and two monomers nearby form the fold axis. As shown in Figure 2A, each monomer of Ala-DH is composed of a substrate-binding domain (residues 1-128 and 309-371) and a NAD-binding domain (residues 129-308), which are connected by two α- helixes.

Tuberculosis diagnosis and guidelines

The TB disease is a leading cause of infectious disease morbidity and mortality worldwide, with many diagnostic uncertainties, and control relies on the identification and preventive treatment of individuals who are latently infected with Mtb, is important strategy in TB elimination. However, direct identification of LTBI is currently unavailable. Reactivation TB can be averted by preventive treatment. Currently available treatments have an efficacy ranging from 60% to 90% [35]. The potential benefit of treatment needs to be carefully balanced against the risk of drug-related adverse events. The presence of TB disease must be excluded before treatment for LTBI is initiated because failure to do so may result in inadequate treatment and development of drug resistance [36].

Targeted testing is an essential TB prevention and control strategy that is used to identify, evaluate, and treat persons who are at high risk for LTBI or at high risk for developing TB

disease once infected with Mtb.

The LTBI diagnosis is based on information gathered from the medical history, TST or Interferon-gamma release assays (IGRAs) result, chest radiograph (CXR), physical examination, and in certain circumstances, sputum examinations.

The lack of a gold standard for LTBI diagnosis has created uncertainty about test performance. The TST and IGRAs are current indirect methods for TB screening; neither IGRAs nor TST can distinguish active TB from LTBI [37, 38].

WHO recommends that either a TST or IGRA must be used to test for LTBI in both high- and low-TB-burden countries and that active TB disease be ruled out prior to prescribing preventive treatment, as per WHO guidelines [39]. There is no strong evidence that one LTBI test should be preferred over the other. The choice will depend on the availability, cost and the health infrastructure. The evidence reviewed and the recommendations in the 2018 consolidated guidelines on LTBI apply to the use of the TST and commercially available IGRAs (QuantiFERON®-TB Gold Plus and T-SPOT®.TB) [39, **Fig. 3**].

Active TB patients, in addition to TST positivity and a generally positive QFT-G-IT that can be negative in a certain number of cases, were detected through clinical, microbiological and radiological findings.

The diagnosis' confirmation required Mtb identification through fluorescence microscopy, polymerase chain reaction (PCR) as WHO-approved molecular test such as TB Xpert® MTB/RIF assay [39] or cultural assays upon 3 biological samples, such as sputum or bronchoalveolar lavage (BAL) for respiratory diseases, tissue biopsies, drainage liquid or needle aspirates for extrapulmonary localizations [40].

CXR, is an important tool for triaging and screening for pulmonary TB, and it is also useful to aid diagnosis when pulmonary TB cannot be confirmed bacteriologically. CXR can be used to select individuals for referral for bacteriological confirmation, and the role of radiology remains important when bacteriological tests cannot provide a clear answer.

The lack of a gold standard for LTBI diagnosis has created uncertainty about test performance. Whilst TST remains the most widely used, test performance is suboptimal in populations with a high prevalence of BCG vaccination and infection with environmental mycobacteria. There are two important causes of false-positive results: nontuberculous mycobacterium (NTM) infection and prior BCG vaccination, NTMs are not a clinically important cause of false-positive TST results, except in populations with a high prevalence of NTM sensitization and a very low prevalence of TB infection.

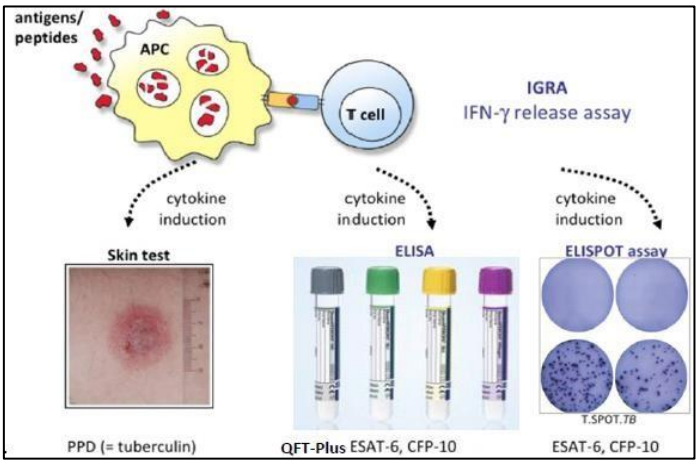

Figure 3. Diagnostic tests as TST or IGRA for active TB and LTBI. WHO Standard for diagnosing LTBI; recommended either TST or IGRA can be used to test for LTBI.

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The impact of BCG on TST specificity depends on when BCG is given and on how many doses are administered. If BCG is administered at birth (or during infancy) and not repeated, then its impact on TST specificity is minimal and can be ignored while interpreting the results. In contrast, if BCG is given after infancy (e.g., school entry) and/or given multiple times (i.e., booster shots), then TST specificity is compromised [41].

The TST is one of the few tests that have been continuously in use for about 100 years in clinical medicine. It attempts to measure a memory of cell-mediated immunity in the form of a DTH response to the most commonly used PPD of tuberculin. The TST, performed using the Mantoux technique [42], consists of the intradermal injection in 0.1ml of 5 tuberculin units (TU) of PPD-S or 2 TU PPD RT23 (these are considered equivalent); TST reactions in adults are measured by the diameter of induration, measured 48-72 h after antigen injection. Clinically, the TST reaction may already start a few hours after the injection of tuberculin PPD with a white or rose-coloured induration of the skin as a type-I or type-III immune reaction followed by the DTH reaction, which peaks after 48-72 h and may last for up to 1 month, depending on the quality and quantity of the initial reaction. Strong reactions may result in tissue necrosis. A positive TST reaction may lead to persistent discoloration of the skin. In interpreting a positive TST, it is important to consider much more than only the size of the induration (**Fig. 4**). Rather, the TST should be considered according to three dimensions: size of induration (for the current test as well as in relation to the induration on a previous test, if done), pretest probability of infection, and risk of disease if the person were truly infected. Menzies and colleagues developed a

simple, Web-based, interactive algorithm - the Online TST/IGRA Interpreter (version 3.0; www.tstin3d.com) - that incorporates all these dimensions [43] and also computes the risk of serious adverse events due to treatment.

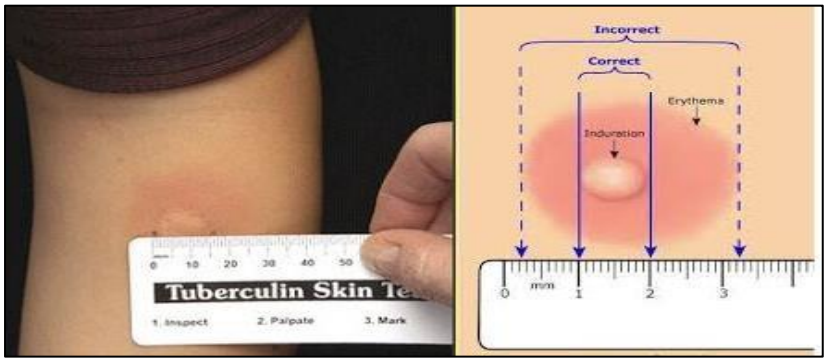

Figure 4. Tuberculin skin test (TST), performed using the Mantoux technique.

Based on the sensitivity and specificity of the PPD TST and the prevalence of TB in different groups three cut-points have been recommended for defining a positive tuberculin reaction: > 5 mm, > 10 mm, and > 15 mm of induration. For persons who are at highest risk for developing active TB if they are infected with Mtb (i.e., persons with HIV infection, who are receiving immunosuppressive therapy, who have had recent close contact with persons with infectious TB nor who have abnormal CXR consistent with prior TB), > 5 mm of induration is considered positive. For other persons with an increased probability of recent infection or with other clinical conditions that increase the risk for progression to active TB, > 10 mm of induration is considered positive. These include recent immigrants (i.e., within the last 5 yr) from high prevalence countries; injection drug users; residents and employees of high-risk congregate settings (including health care workers with exposure to TB); mycobacteriology laboratory personnel; persons with clinical conditions such as silicosis, diabetes mellitus, chronic renal failure, leukemias and lymphomas, carcinoma of the head or neck and lung, weight loss of > 10% ideal body weight, gastrectomy, and jejunoileal bypass; and children younger than 4 year of age or infants, children, and adolescents exposed to adults in high- risk categories. For persons at low risk for TB, for whom tuberculin testing is not generally indicated > 15 mm of induration is considered positive [44].

IGRAs are *in vitro* blood tests of cell-mediated immune response; they measure T-cell

release of IFN-gamma following stimulation by antigens specific to the MtbC, with the exception of BCG substrains. IGRAs measure interferon gamma release by memory T-cells after stimulation with Mtb-specific antigens absent in BCG and most NTMs and are, therefore, considered to be specific [42].

In 2001, QuantiFERON (QFT) became the first IGRA approved by the Food and Drug Administration (FDA) as an aid for diagnosing Mtb infection. This test used an enzyme-linked immunosorbent assay (ELISA) to measure the amount of IFN-γ released in response to PPD compared with controls, QFT specificity was less than that of TST despite the use of *M. avium* antigen as a control for nontuberculous mycobacterial sensitization and saline as a negative control [45]. QFT has not been available commercially since 2005. To improve specificity, new IGRAs were developed. These IGRAs assess response to synthetic overlapping peptides that represent specific Mtb proteins, such as early secretory antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10). These proteins are present in all Mtb and they stimulate measurable release of IFN-γ in most infected persons, but they are absent from BCG vaccine strains and from most NTM. Thus, as test antigens, these proteins offer improved test specificity compared with PPD. However, ESAT-6 and CFP-10 are present in *M. kansasii*, *M. szulgai*, and *M. marinum*, and sensitization to these organisms might contribute to the release of IFN-γ in response to these antigens and cause false-positive IGRA results. Because ESAT-6 and CFP-10 are recognized by fewer T lymphocytes and stimulate less IFN-γ release compared with PPD, a more sensitive ELISA than was used for QFT is required to measure IFN-γ concentrations and responses to ESAT 6 and CFP-10 [45].

In 2005, the QuantiFERON-TB Gold test (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia) became the second IGRA approved by FDA as an aid for diagnosing Mtb infection. It assesses the immunologic responsiveness of tested patients to ESAT-6 and CFP-10. In the QFT-G test, separate aliquots of fresh whole blood are incubated with controls and with two separate mixtures of peptides, one representing ESAT-6 and the other representing CFP-10. The amount of IFN-γ released in response to ESAT-6 or CFP-10 (i.e., the ESAT-6 response or the CFP-10 response) is calculated as the difference in IFN-γ concentration in plasma from blood stimulated with antigen minus the IFN-γ concentration in plasma from blood incubated with saline (i.e., Nil). For QFT-G, the TB Response is the higher of the ESAT-6 Response or the CFP-10 response. A stipulation for FDA approval was inclusion of interpretation criteria that addressed the potential for false-positive results accompanying high Nil values (i.e., >0.7 IU/ml) [45].

In October 2007, the Quantiferon Gold In Tube (QFT-G-IT) became the third IGRA approved by FDA. Their tubes contain test antigens that consist of a single mixture of 14 peptides representing the entire amino acid sequences of ESAT-6 and CFP-10 and part of the sequence of TB7.7 (Rv2654c), which is not an RD1 antigen**.** The two accompanying tubes serve as negative and positive controls: the negative-control tube contains heparin alone, and the positive-control tube contains heparin, dextrose, and phytohemaglutinin. Blood (1 ml) is collected into each of the three tubes, mixed with the reagents already in the tubes, and incubated for 16-24 hours. Plasma is separated, and the IFN-γ concentration in the plasma is determined using the same sensitive ELISA used for QFT-GT (**Fig. 5**).

To interpret QFT-G-IT as approved by the FDA, the TB response is calculated as the difference in IFN-γ concentration in plasma from blood stimulated with antigen (i.e., the single cocktail of peptides representing ESAT-6, CFP-10, and TB7.7) minus the IFN-γ concentration in plasma from blood incubated without antigen (i.e., Nil). The result is reported as quantification of IFN-γ in international units (IU) per millilitre [45].

As a particular advantage of *in vitro* testing, stimulation reactions with negative control and positive control (mitogen stimulus) are carried out in parallel to primarily evaluate test performance with respect to background signals or general T-cell responsiveness.

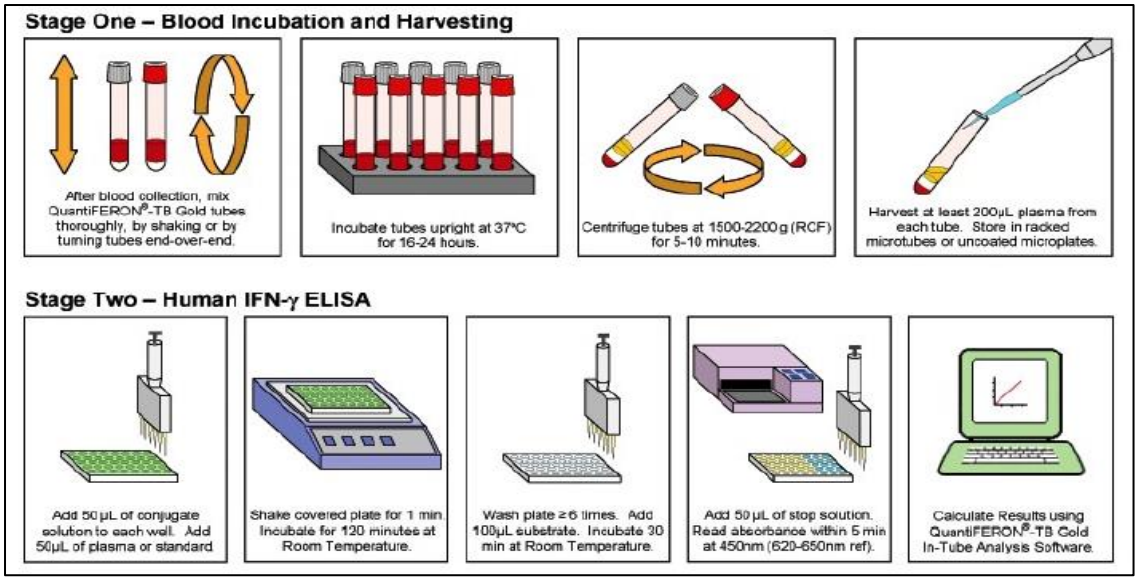

Figure 5. Overview of the stages of the QuantiFERON-TB GOLd® IN Tube assay.

In the setting of immunodeficiency, an impaired mitogen response may, in addition, be interpreted as a meaningful measure for the assessment of the overall extent of immunosuppression. Therefore, unlike skin testing, *in vitro* tests may be able to discriminate true negative responses from anergy.

T-SPOT.TB test (Oxford Immunotec, Oxford, UK) is a type of enzyme-linked immunospot assay (ELISpot), used for TB diagnosis, which belongs to the group of IGRAs, the test was approved by FDA on July 2008 [46, **Fig. 6**]. T-SPOT.TB test enumerates the response of effector T cells that have been sensitized to Mtb antigens. For this test peripheral blood mononuclear cells (PBMCs) are incubated with control materials and two mixtures of peptides, one representing the entire amino acid sequence of ESAT-6 and the other representing the entire amino acid sequence of CFP-10. The test uses an ELISpot to detect increases in the number of cells that secrete IFN-γ (represented as spots in each test well) after stimulation with antigen as compared to the media control (Nil). The result is reported as the number of IFN-γ -producing T cells (spot-forming cells, SFC). An individual is considered positive for Mtb infection if the spot counts in the TB antigen wells exceed a specific threshold relative to the negative-control wells [42].

The T-SPOT interpretation criteria approved by FDA for use in the USA differ from those used in other countries. Also, the majority of published studies evaluating T-Spot have used criteria that differ from those approved by FDA. The interpretation criteria for T-SPOT.TB approved by FDA included a borderline interpretation for a TB response equal to five, six, or seven [46].

Figure 6. T-SPOT.TB: an *in vitro* diagnostic test for detection of T cells specifically activated by Mtb antigens.

The last IGRA version, QuantiFERON-TB Gold Plus (QFT-P) (QIAGEN, Germantown, MD, USA), is 4th Generation QuantiFERON was approved by the USA. FDA on June 8, 2017. QFT-P employs two antigen tubes (TB1 and TB2) for diagnosis Mtb infection. Major differences between the QFT-G-IT and the QFT-P include the removal of TB7.7 peptides, the addition of a second antigen tube containing shorter peptides for ESAT-6 and CFP-10, aimed to elicit a response from CD8+ T-cells (**Fig. 7**). Currently, there is little data comparing the results of QFT-P and the QFT-G-IT based on blood collection. QFT-P is expected to be more sensitive than QFT-G-IT; however, early publications on the sensitivity of QFT-P has shown equal sensitivity compared to QFT-G-IT. Studies conducted in Japan, Italy, Germany, Belgium, and the Netherlands (low TB prevalence countries) found no significant differences between the sensitivity among bacteriologically and non- bacteriologically confirmed active TB patients and specificity among healthy subjects with low or no risk for TB between the third generation (QFT-G-IT) and fourth generation (QFT-P) assays. A study conducted among U.S. Health Care Workers (HCWs) found a positivity rate of 4% in the study population when using QFT-G-IT and 6% when using QFT-P with 96% agreement between the assays [47].

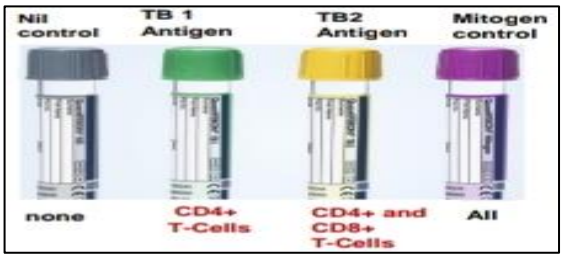

Figure 7. QuantiFERON-TB Gold Plus (QFT-P).

Treatment of Tuberculosis

Effective management requires prompt recognition using a combination of clinical, radiographic, microbiological, and histopathologic hallmarks and initiation of appropriate multidrug therapy. Effective medical therapy for TB has existed for more than half a century, yet TB remains among the most pressing public health issues of our day throughout the world but disproportionately afflicts low income nations. The challenges to effective solutions include lack of access to diagnosis and treatment, the frequent

coexistence of epidemics of TB and HIV, and the increasing prevalence of multidrug-resistant tuberculosis (MDR-TB).

Patients with active TB should be treated with multiple agents to achieve bacterial clearance, to reduce the risk of transmission, and to prevent the emergence of drug resistance. Antituberculosis (anti-TB) medicines or drugs are classified as first- and second- line drugs. First-line anti-TB drugs used to treat drug-susceptible TB (DST), include Isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (SM), (streptomycin is now considered a second-line TB medicine and used only as a substitute for amikacin when amikacin is not available or there is confirmed resistance to it). Second-line anti-TB medicine or drug is an agent reserved for the treatment of drug-resistant TB (DR-TB**)** include the Fluoroquinolones ((Ofloxacin (OFX), levofloxacin (LEV), moxifloxacin (MOX) and ciprofloxacin (CIP)), Injectable anti-TB drugs are the aminoglycosides (Kanamycin (KAN), amikacin (AMK) and capreomycin (CAP)). Less- effective second-line anti-TB drugs- Ethionamide (ETH)/Prothionamide (PTH), Cycloserine (CS)/Terizidone, P-aminosalicylic acid (PAS) [48].

For initial empiric treatment of TB, initial phase for 8 weeks, patient starts on a 4-drug regimen: isoniazid, rifampicin, pyrazinamide and either ethambutol or streptomycin. Once the TB isolate is known to be fully susceptible ethambutol or streptomycin, if it is used as fourth drug, can be discontinued. The first-line anti-TB agents are bactericidal and particularly effective against metabolically active organisms. Continuation phase for 16 weeks on 2 drugs, rifampicin and isoniazid. Effectively treating simplest forms of TB take between 6-9 months [48]. During drug treatment of human TB, susceptible organisms can be eradicated from sputum rapidly, usually within 2 months, but a continuation phase for months of treatment after cultures are negative is required to prevent re-emergence of the disease. Organisms that are still viable after months of treatment to which they are susceptible are termed persistors. Currently available TB drugs target mechanisms of cell growth and metabolism. Persistent organisms are metabolically less active, or differently active, making attacks on growth and metabolism less effective. It is unclear what signals initiate and maintain this less metabolically active state, but hypoxia and starvation within granulomas or phagocytic cells have been proposed. Rifampicin is somewhat active against persistors. This property may be what makes it critical to short course therapy. For a drug to kill persistors it will need to target some aspect of cell metabolism that remains vital in the less metabolically active state. Mtb organisms develop spontaneous resistance mutations that can interfere with a drug's mechanism of action, activation or entry into the cell.

Resistance emerges as a consequence of selective pressure caused by monotherapy. Treatment with multiple drugs to which the organism is susceptible will prevent this selection rates of drug resistance rise in the community, the potential for inadvertent monotherapy and subsequent emergence of more advanced drug resistance increases.

The patients with primary MDR-TB tend to exhibit a greater extent of lung cavitation and lower number of calcified nodules compared to patients with DST. A review also shed light on thicker cavity walls in MDR-TB lung lesions further to extensive tissue loss, the intense inflammatory processes unleashed by MDR-Mtb infection in the lungs [49]. Interestingly, patients with MDR-TB have also been shown to have slightly higher T-cell counts and IgM titres in serum compared to patients with DST [50].

TB strains with drug resistant (DR-TB) are more difficult to treat than DST, and threaten global progress towards the targets set by the End TB Strategy of the WHO. Globally in 2018, 51% of bacteriologically confirmed pulmonary TB cases were tested for rifampicin resistance, up from 41% in 2017 [1, 5]. The latest treatment outcome data show success rates of 85% for TB, 75% for HIV-associated TB, 56% for MDR/ Rifampicin resistance (RR-TB) and 39% for extensively drug-resistant (XDR-TB) [1]. MDR/RR-TB is harder to treat. In 2018, WHO released a Rapid Communication. Recommending the use of levofloxacin (or moxifloxacin), bedaquiline, linezolid, clofazimine and cycloserine (or terizidone) in longer MDR-TB regimens [39]. Second- line TB-drugs, three medicines are used for the treatment of DR-TB include levofloxacin or Moxifloxacin, Bedaquiline and linezolid are strongly recommended to use in a longer regimen, which is completed with other medicines ranked by a relative balance of benefits to harms. The proposed total duration of longer MDR-TB regimen is about 18-20 months, modified depending upon patient response. The standardised, short MDR-TB regimen maybe offered to eligible patients who agree to a briefer treatment (9-12 months). Injectable agents are no longer among the priority medicine when designing long MDR-TB regimens, with Kanamycin and capreomycin not recommended any more, and fully oral regimens should thus become the preferred option for most patients [39,48].

XDR-TB, is a form of TB which is resistant to at least four of the core anti-TB drugs. XDR-TB involves resistance to the two most powerful anti-TB drugs, isoniazid and rifampicin, also known as MDR-TB, in addition to resistance to any of the fluoroquinolones and to at least one of the three injectable second-line drugs. MDR/RR-TB and XDR-TB both take substantially longer to treat than ordinary, and require the use of second-line anti-TB drugs, which are more expensive and have more side-effects than the

first-line drugs used for drug-susceptible TB [39]. Treatment of XDR-TB or treatment-intolerant /non-responsive MDR-TB has historically been lengthy and complex; most XDR-TB patients currently take a combination of as many as eight antibiotics, some involving daily injections, for 18 months or longer [5]. In 2019, FDA approves the BPal regimen for treatment of highly drug resistance forms of tuberculosis [51], three-drug regimen consisting of bedaquiline, pretomanid and linezolid-collectively referred to as the BPal regimen-was studied in the pivotal Nix-TB trial across three sites in South Africa.

Even if all active cases of TB could be eliminated, the large pool of latently infected individuals would serve as a significant, reservoir for development of active cases for decades [35]. Preventive treatment which is offered to individuals who are considered to be at risk for TB disease in order to reduce that risk. Also referred to as LTBI treatment or preventive therapy. WHO guidelines for the management of LTBI are currently only available for people living with HIV and for children below 5 years of age who are household contacts of TB cases [39]. Isoniazid monotherapy for 6 months is recommended for treatment of LTBI in both adults and children in countries with high and low TB incidence, (strong recommendation, high-quality evidence. Existing recommendation) Rifampicin plus isoniazid daily for 3 months should be offered as an alternative to 6 months of isoniazid monotherapy as preventive treatment for children and adolescents aged < 15 years in countries with a high TB incidence, (strong recommendation, low-quality evidence. New recommendation). Rifapentine (RPT and isoniazid weekly for 3 months may be offered as an alternative to 6 months of isoniazid monotherapy as preventive treatment for both adults and children in countries with a high TB incidence. (Conditional recommendation, moderate-quality evidence. New recommendation). The following options are recommended for treatment of LTBI in countries with a low TB incidence as alternatives to 6 months of isoniazid monotherapy: 9 months of isoniazid, or a 3-month regimen of weekly rifapentine plus isoniazid, or 3-4 months of isoniazid plus rifampicin, or 3-4 months of rifampicin alone. (Strong recommendation, moderate–high-quality evidence. Existing recommendation). In settings with high TB incidence and transmission, adults and adolescents living with HIV who have an unknown or a positive TST and are unlikely to have active TB disease should receive at least 36 months of isoniazid preventive treatment (IPT), regardless of whether they are receiving antiretroviral therapy (ART). IPT should also be given irrespective of the degree of immunosuppression, history of previous TB treatment and pregnancy. (Conditional recommendation, low quality evidence. Existing recommendation) [39].

AIM OF THE STUDY

Accurate and rapid detection of either active and latent TB infection are critical for improving patient outcomes and decreasing TB transmission. Mycobacterial culture is generally considered the best available reference standard for TB diagnosis and is the first step in detecting drug resistance but it is a relatively complex and slow procedure.

Rapid testing for Mtb infection and treatment of LTBI in targeted populations is an important strategy in TB elimination, thus, discriminating LTBI from active TB may be challenging for novel screening tests because it is currently not possible; the TST and Mtb specific IGRAs are still the main screening tools used for the diagnosis of TB infection and, although the newer IGRAs show some improvements over TST [52-54], neither diagnostic test can differentiate between LTBI, active TB or past TB [42].

In view of this, this study was focused on improving TB diagnosis by developing a new blood test able to discriminate between active TB and LTBI according to the best cost/effectiveness ratio.

In 2012 our group showed, for the first time, that out of a number of Mtb antigens tested including Ala-DH, ESAT-6, CFP-10, PstS1, HSP-X and antigen-85B, only Ala-DH induced IL-2 production, measured by an ELISpot assay, that could clearly distinguish children with LTBI from those with active TB [55]. Thus, this study wants to confirm the role of Ala-DH as a diagnostic tool in IL-2 ELISpot in a population of adult subjects too.

MATERIALS AND METHODS

Patients and definition of study groups

This study was performed on peripheral blood samples collected from patients with confirmed active TB, from subjects diagnosed with LTBI and from healthy donors, who were consecutively enrolled during the period between September 2014 and August 2016 by the Careggi University Hospital, Florence, Italy. Subjects below 18, pregnant women, HIV/AIDS and all those people with any known immunocompromising condition (such as diabetes, haematological malignancies, end stage kidney disease and immunosuppressive therapy) were excluded from the study. None of the healthy patients had recent exposure to active pulmonary TB cases.

Following the approval by the ‘‘Area Vasta Centro, Regione Toscana, Ethical Committee ’’ (BIO 14.013), each patient, previously informed of the aim of the study, signed an informed consent.

All subjects were tested with TST (Sanofi Pasteur MSD SNC, Lyon, France) according to the Mantoux method [44] and with the IGRA test QFT-G-IT according to the manufacturer's instructions [45].

The subjects enrolled in the study were classified as active TB patients, LTBI patients or healthy people in accordance with the current guidelines [2, 40, 56].

Active TB patients, in addition to TST positivity and a generally positive QFT-G-IT that can be negative in a certain number of cases, were detected through clinical, microbiological and radiological findings. The diagnosis confirmation required Mtb identification through fluorescence microscopy, PCR or cultural assays upon biological samples, such as sputum or BAL for respiratory diseases, tissue biopsies, drainage liquid or needle aspirates for extrapulmonary localizations. In particular, all samples were tested with auramin-rhodamine fluorescence microscopic examination to detect acid-alcohol resistant bacilli (BAAR); PCR-based methods like Artus Mtb PCR Kit (Qiagen, Venlo, Netherlands) and GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA) were used to identify the *Mycobacterium* strain and to detect rifampicine resistance; then mycobacterial specific solid and liquid culture media were used for the isolation of the infectious agent.

LTBI was diagnosed by a positive test for Mtb infection in persons without history of BCG vaccination or by both TST and IGRA positivity in BCG vaccinated subjects, provided the exclusion of active TB by medical history, clinical, radiologic, and microbiologic evaluations [45, 57, 58].

A subject without BCG vaccination and discordant results between TST and QFT-G-IT was attributed to his study group based on the TST result according to the Mantoux method that is still the standard test for LTBI diagnosis; however, IGRAs are recommended to confirm LTBI diagnosis in TST positive BCG vaccinated individuals [2, 36, 52].

Patients with assessed TST and/or QFT-G- IT positivity and CXR positivity and/or in the presence of cough (N = 97) were tested for Mtb identification through fluorescence microscopy, PCR and cultural assays on biological samples. The Mtb identification tests were not performed in subjects with negative TST and negative QFT-G-IT.

Consequently, to the diagnosis, the 215 patients were classified as active TB cases (n = 73), LTBI cases (n = 88) and healthy individuals (n = 54). **(Table 1)** summarizes data from the 215 patients enrolled in the study.

	Healthy N (%)	**LTBI N (%)**	**Active TB N (%)**
	54 (25)	88 (41)	73 (34)
Age Median (IQR)	46 (32-63)	46 (35.5-65)	39 (31-61.5)
Immigrated	27 (50)	45 (51)	48 (66)
BCG vaccinated	21 (38.8)	38 (43.2)	32 (43.8)
TST (mm)			
<5	54 (100)	0 (0)	0 (0)
≥5 and <10	0 (0)	5 (5.8)	0(0)
≥10 and <15	0 (0)	13 (14.7)	8 (11)
≥15	0 (0)	70 (79.5)	65 (89)
QFT-G-IT			
Negative	50 (92.65)	5 (5.7)	4 (5.48)
Positive	3 (5.5)	80 (90.9)	68 (93.15)
Indeterminate	1 (1.85)	3 (3.4)	1 (1.37)
Fluorescence microscopy			
Positive	Not performed	0 (0)	42 (57.5)
Negative	Not performed	24 (100)	31 (42.5)
PCR- based tests			
Positive	Not performed	0 (0)	73 (100)
Negative	Not Performed	24 (100)	0 (0)
Mtb isolation from culture			
Positive	Not performed	0 (0)	73 (100)
Negative	Not performed	24 (100)	0 (0)

Table 1. Characteristics of the 215 subjects divided according to the diagnosis in the three study groups

Reagents

Lymphoprep was purchased from Fresenius Kabi Morge AS (Axis-Shield, Oslo, Norway), RPMI 1640 medium from Biochrome (Leonorenstr, Berlin), L-glutamine from Euroclone (Italy), betamercaptoethanol from Sigma-Aldrich (Darmstadt, Germany) Na-pyruvate and Non-essential aminoacids from Thermofisher scientific (Waltham, MA, USA), penicillin/streptomycin from PAN biotech (Aidenbach, Germany), fetal bovine serum (HyClone, GE Helathcare Life Science, Utah, USA).

LIOSpot® TB (LIONEX GmbH, Braunschweig, Germany) is an anti-human IL-2 ELISpot kit containing *M. tuberculosis* ESAT-6, CFP-10 and Ala-DH recombinant antigens, all produced at LIONEX to a purity of more than 98% and endotoxin free; Phytohaemagglutinin mitogen (PHA) was used as a positive control.

PBMCs isolation

PBMCs were isolated from blood samples of each enrolled patient, within 8 hours after venipuncture to ensure cell activity, by Ficoll-Hypaque density gradient centrifugation. Briefly, blood was layered on top of Lymphoprep, density gradient of 1.077 g/mL, and centrifuged at 800 x g for 25 minutes at room temperature (15-25°C) without brake. PBMCs layer was harvested and washed two times in PBS, pH 7.4. Cells were counted using the Burker chamber and then transferred into RPMI 1640 complete medium (supplemented with L-glutamine 1%, beta-mercaptoethanol 1%, Na-pyruvate 1%, non-essential aminoacids 1%, penicillin 50.000U and streptomycin 50 mg), with 5% fetal bovine serum to obtain a concentration of $2.5x10^6$ PBMCs/ml. Both freshly prepared and cryopreserved cells may be used in this assay.

Anti-human IL-2 ELISpot assay

The enzyme-linked immunospot (ELISpot) assay is a highly sensitive and specific method available for monitoring T and B cell response, permitting the *ex vivo* identification of cells actively secreting signalling proteins or antibodies after stimulation wit h appropriate stimulus *in vitro*. The T cell ELISpot assay has the lowest detection threshold among the assays viable for detection of T cell responses. With ELISpot assay it is possible to detect one specific cell out of 100 000 irrelevant cells, making this method much more sensitive

than traditional ELISA application because the secreted protein is captured directly on to the well of an ELISpot plat before having the chance to be diluted in the culture supernatant, degraded by proteases or captured by receptors on adjacent cells. The ELISpot assay is very similar to ELISA and is based on the same immunochemical 'sandwich' principle. The major difference is that an ELISpot is a combination of both an immunoassay and bioassay because living cells are cultured directly in the wells of the ELISpot plate. In this assay, cells are cultured on a surface coated with a specific capture antibody in the presence or absence of stimuli; the cytokines, secreted by the T cells after stimuli response, will be captured by the specific antibodies in each well. After an appropriate incubation time, cells are removed, and the secreted molecule is detected using a detection antibody in a similar procedure to that employed by the ELISA. The detection antibody is either biotinylated and followed by a streptavidin-enzyme conjugate or the antibody is directly conjugated to an enzyme. By using a substrate with a precipitating rather than a soluble product, the end result is visible as spots on the surface **(Fig. 8)**. A single cell forms a coloured 'footprint' (spot) on the bottom of the well representing its secretory activity. The frequency of spot forming cells can be quantified from the number of spots in the well and cell input. The term 'spot-forming cells or SFC' is used as a quantitative measure for number of cytokine or antibody secreting cells in the ELISpot assay. Each spot corresponds to an individual cytokine-secreting cell. ELISpot can be used to discriminate between subsets of activated T cells. For example, Th1 cells are characterized by their production of IFN-γ, IL-2, and TNF-α, whereas Th2 cells produce other cytokines such as IL-4, IL-5, and IL-13. Major advantages of ELISpot assay is easy performance, its potential for high throughput screening and no requirement for expensive instruments.

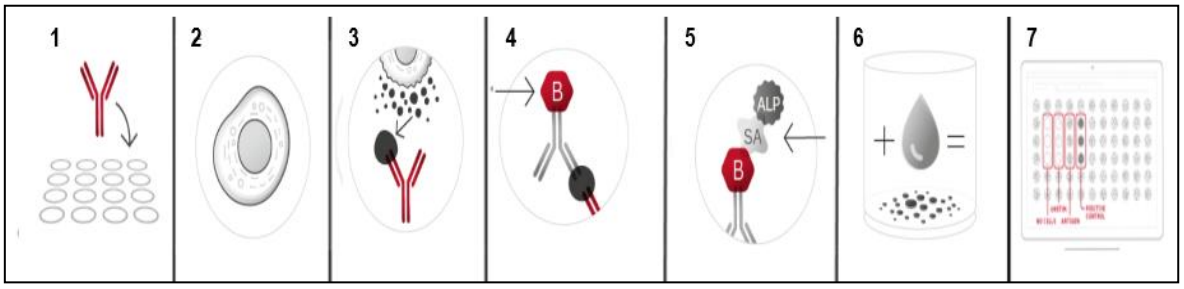

Figure 8. ELISpot step-by-step guide. The image shows the sequence of different steps to perform an ELISpot: 1) Antibody coating; 2) Cell incubation; 3) Cytokine capture; 4) Detection antibodies; 5) Streptavidin-enzyme conjugate; 6) Addition of substrate; 7) Analysis.

In our study was used the LIOSpot ®TB anti-human IL-2 ELISpot kit containing a 96-well plate coated with anti-human IL-2 for detection of IL-2 production by T cells stimulated with three Mtb antigens, like ESAT-6, CFP-10 (also used in IGRAs) and Ala-DH.
First we added the positive control (PHA) and the negative control (medium) as single determinations and the three different antigens ESAT-6, CFP-10 and Ala-DH in double determination (5µg/ml) (**Table 2**); then PBMCs from each patient were seeded in order to have $2.5x10^5$ cells per 0.1ml/well, working under sterile conditions.

A	Negative control:only medium
B	Ala-DH
C	Ala-DH
D	ESAT-6
E	ESAT-6
F	CFP-10
G	CFP-10
H	Positive control (mitogen): PHA

Table 2. Map of antigens used in IL-2 ELISpot plate.

The plate was incubated in a humidified incubator at 37°C, 5% CO2 for 16-24 hours: during incubation time the antigen will activates specific T cells to release IL-2 that will be captured by the antibody at the bottom of the well.
After the incubation time wells content was discarded and the plate was washed five times with a wash buffer (PBS 0.05% Tween-20) before adding the anti-human IL-2 biotinylated detection antibody to each well. After one hour of incubation, wash steps were repeated, and the conjugated horseradish peroxidase (HRP)-streptavidin solution was then added. The advantage of using HRP is its fast turnover rate (spots develop fast), The plate was incubated again for one hour at room temperature (RT) and, after being washed as already described, TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was dispensed into each well and incubated for ten minutes at RT in the dark, until spots were visible. The HRP substrate forms intense coloured spots but may be bleach in short period of time when stored in daylight. Therefore, plates should be protected from light and moisture

during storage at room temperature. Following the development of spots, the wells' content was discarded, and a stop solution was used. The plate was dried and the number of SFC (spot forming cells) was counted by an automated ELISpot reader using the AID ELISpot Software Version 3.2.3 (**Fig. 9**).

The SFC count in PHA positive control wells should be more then 50 or show saturation to 39 confirm cellular functionality and vitality. In the negative control it would be expected to have few or no spots; the number of SFC in the negative control is subtracted from the SFC mean value of the wells containing the ESAT-6, CFP-10 and Ala-DH antigens. Results were expressed as number of SFC per million of PBMCs.

Figure 9. ELISpot reader.

Statistical analysis

Descriptive statistics were used for the calculation of absolute frequencies and percentages for qualitative data, as well as for mean, median (IQR), and standard deviation of quantitative data. All distributions of ELISpot test results for healthy patients, LTBI subjects or active TB patients were compared using the Mann-Whitney inferential test or the Student t-test. $P < 0.05$ was considered statistically significant.

Test performance in terms of sensitivity (ability of the test to identify the true positive subjects) and specificity (ability of the test to identify the true negative subjects) was evaluated for each antigen by a ROC (Receiving Operating Characteristic) curve, the elective validation method of a quantitative diagnostic test in a population. The proportion of patients correctly diagnosed, that is the test accuracy, is proportional to the area under

the curve (AUC), which can assume values between 0.5 (50% accuracy) and 1 (100% accuracy). According to the classification proposed by Swets the test is not accurate for AUC = 0.5, the test is poorly accurate for 0.5 <AUC≤ 0.7, the test is moderately accurate for 0.7 <AUC≤ 0.9, the test is highly accurate for 0.9 <AUC< 1 and the test is perfect for AUC = 1 [59]. The ROC curve also allows to identify the best cut off value that maximizes the difference between true positive subjects and false positives ones; it is the best threshold value for anti-human IL-2 ELISpot result relative to each specific antigen (ESAT-6, CFP- 10 and Ala-DH) in order to discriminate between patients with active TB and subjects with LTBI.

To maximize both sensitivity and specificity, the Youden's index (= Sensitivity- [1-Specificity]) can be applied.

Finally, the correlation between the ELISpot diagnostic test for each antigen and the diagnosis performed on each patient using the current methods was calculated by Cohen's Kappa coefficient according to which there would be slight concordance for the value of k = 0.2, poor concordance for the value of K in the range 0.2-0.4, moderate concordance for k value in the range 0.4-0.6, substantial concordance for k value in the range 0.6-0.8, good concordance for k value in the range 0.8-1.

Statistical analysis was performed with SPSS for Windows software version 20.0.

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RESULTS

Two hundred and fifteen subjects were enrolled in this study: 73 patients with active TB, 88 with LTBI and 54 as uninfected controls. We performed LIOSpot® TB according to kit manual on PBMCs isolated from a blood sample of every participant by stimulation with Mtb antigens such as ESAT-6, CFP-10 and Ala-DH (**Fig. 10**) ; the T cell specific response to each antigen was evaluated in terms of IL-2 production (**Table 3**).

Mann-Whitney inferential test or Student t-test were used to compare ELISpot results for IL-2, as SFCs per million PBMCs, between healthy patients, LTBI subjects and active TB ones referring to each tested antigen. Comparing the results of infected and non-infected there were significant differences for all the antigens ($p \leq 0.0001$); similarly, comparing the results of patients in LTBI and the group of active TB patients there were significant differences for all three antigens too ($p \leq 0.0001$) **(Table 3).**

	Healthy (n=54)	**Latent TB (n=88)**	**Active TB (n=73)**	**P (Healthy vs infected)**	**P (Latent vs active disease)**
Ala-DH	0 (0;0)	7.5 (2.5;10)	280 (85;1120)	$p \leq 0.0001$	$p \leq 0.0001$
ESAT-6	2.5 (0;17.5)	180 (30;340)	392.5 (140;1300)	$p \leq 0.0001$	$p \leq 0.0001$
CFP-10	0 (0;15)	192.5 (77.5;660)	480 (325;1590)	$p \leq 0.0001$	$p \leq 0.0001$

Table 3. IL-2 based ELISpot test results for the 215 enrolled patients. IL-2 based ELISpot test results for the 215 enrolled patients, clustered according to the diagnosis. The values are expressed as mean and interquartile range of SFC per million PBMCs.

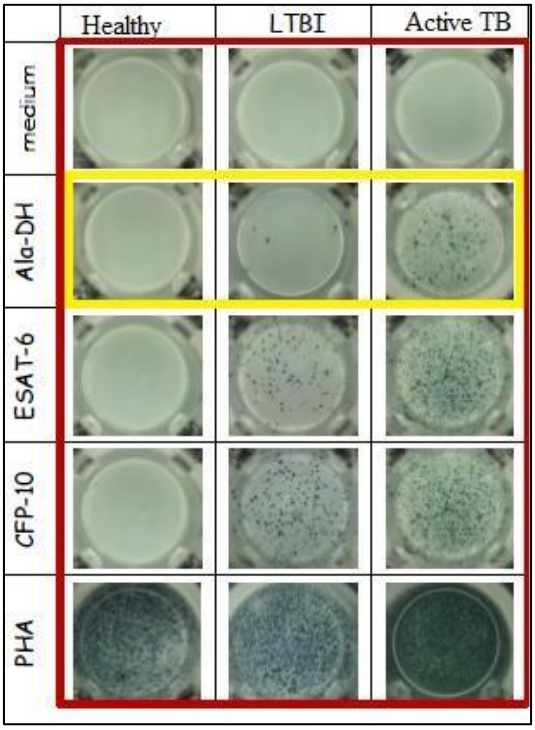

Figure 10. IL-2 ELISpot results. Example of test results as SFC for a healthy person, a LTBI subject and an active TB patient. Yellow highlighted the Ala-DH SFC.

ROC curve analysis was performed for each antigen in order to establish the best cut off of the ELISpot test for IL-2 in discriminating between LTBI and active TB. Considering the response to Ala-DH antigen, a best cut off value of 12.5 SFC per million PBMCs to have a 96% sensitivity and 100% specificity was established. The area under the ROC curve was 0.971 (IC 95%: 0.939-1), so the test was highly accurate in correctly diagnosing subjects with active disease and those with latent infection. For the ESAT-6 antigen, with a best cut off of 71.25 SFC per million PBMCs, a sensitivity of 86% and specificity of 36% was obtained. The area under the ROC curve was 0.713 (IC 95%: 0.632-0.794), the test is moderately accurate. Finally, the best cut off for CFP-10 was 231.25 SFC per million PBMCs, with AUC = 0.693 (IC 95%: 0.611-0.774); The test had a low accuracy, with sensitivity of 80% and specificity of 54% (**Fig. 11**)

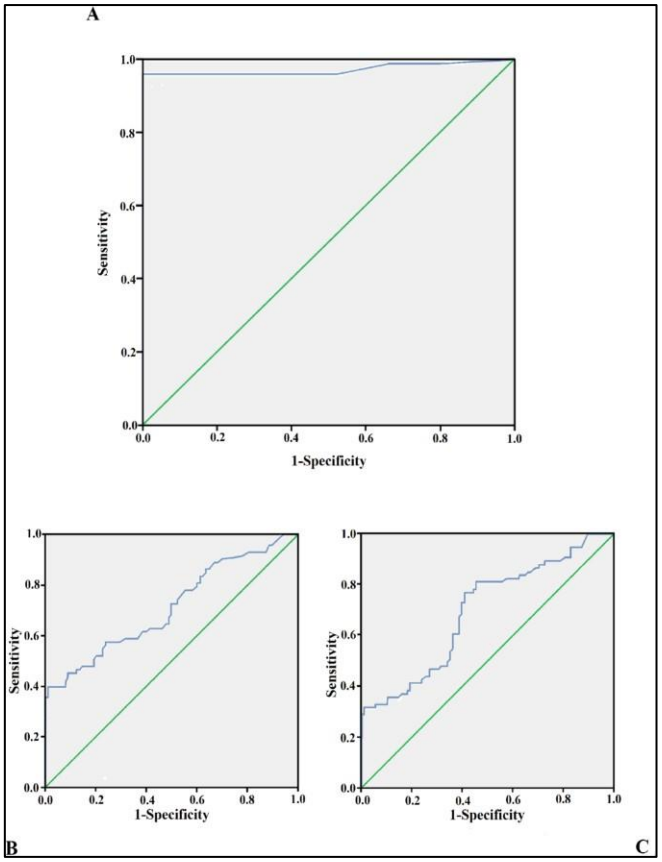

Figure 11. Receiver operator characteristic (ROC) curve. A ROC curve (blue line) plot of the LIOSpot® TB is shown, illustrating sensitivity and specificity for Ala-DH (A), ESAT-6 (B) and CFP- 10 (C) to discriminate subjects with active TB from LTBI ones. AUC is the area delimited by the ROC curve and the green reference line.

Once the best threshold value for the ELISpot IL-2 result was set for each specific antigen to discriminate between subjects with active TB and subjects with LTBI, the correlation between ELISpot and TST, that is still the standard test for Mtb infection diagnosis, was assessed for every antigen by computing The Cohen's Kappa coefficient and interpreting the data as described above.

The concordance between the ELISpot test for IL-2 and the TST test for all the results obtained in the 215 patients with Ala-DH, ESAT-6 and CFP-10 is: 92.56% (k = 0.82 very good concordance), 77.2% (k = 0.53, moderate concordance) and 63.7% (k = 0.35, poor concordance) respectively **(Table 4**).

TST vs ELISpot Ag	Concordance(%) Cohen's Kappa (k)
TST vs ELISpot Ala-DH	92,56%, k=0,82
TST vs ELISpot ESAT-6	77,2%, k=0,53
TST vs ELISpot CFP-10	63,7%, k=0,35

Table 4. Concordance between TST and IL-2 based ELISpot for each antigen in the study, for all the enrolled subjects.

The concordances between the different assays are shown in **(Table 5).** The five patients with indeterminate Quantiferon were excluded by this statistical analysis. ELISpot test for IL-2 and the QFT-G-IT test for all the results obtained in the 210 patients with Ala-DH is 57.1% (k = 0.25 poor concordance), with ESAT-6 is 79.5% (k = 0.57 moderate concordance) and with CFP-10 is 71% (k = 0.44, moderate concordance). These results are in agreement with results presented in this report since not all QFT-G-IT positive subjects were TB patients.

QFT-G-IT vs ELISpot Ag	Concordance(%) Cohen's Kappa (k)
QFT-G-IT vs ELISpot Ala-DH	57.1%, k=0.25
QFT-G-IT vs ELISpot ESAT-6	79.5%, k=0.57
QFT-G-IT vs ELISpot CFP-10	71.0%, k=0.44

Table 5. Concordance between QFT-G-IT and IL-2 based ELISpot for each antigen in the study, for all the 210 enrolled subjects.

DISCUSSION

Current TB diagnostics assays have limitations because they do not differentiate between LTBI and active TB. There is a great necessity for profiling biomarkers in addition to IFN-γ to improve TB diagnosis to facilitate quick and correct treatment implementation. This study aimed to identifying biomarkers of TB infection that are significantly different between active and latent status. During LTBI, Mtb lives in a non-replicating state, enclosed into the granuloma structure, as long as the host remains immunocompetent; in this period the bacillus still maintains the ability to reactivate and produce active disease when the host immune response is impaired [12, 60].

Studying gene expression profiles and proteomic analysis in both active and quiescent Mtb, a number of genes that are differently regulated during the latency phase have been found, in comparison to those expressed during active infection [27, 61], five proteins more abundant under low-oxygen conditions were identified from several lysates of Mtb: Rv0569, Rv2031c (HspX), Rv2623, Rv2626c, and Rv3841 (BfrB). In Mtb culture filtrates, two additional proteins, Rv0363c (Fba) and Rv2780 (Ald), were found in increased amounts under oxygen limitation **[**61]. Different antigens of latency are currently known, several of them were identified in the DosR-regulon [62-64]. Another gene, Rv2780, that encode Ala-DH, was found to be over- expressed during the Mtb dormancy phase, under nutrient starvation and lack of oxygen regimes [32, 61, 65].

Mtb Ala-DH catalyzes reversible conversion of pyruvate to alanine, and glyoxylate to glycine concurrent with the oxidation of NADH to NAD [66] to maintain the optimal NADH/ NAD ratio during anaerobiosis in preparation of eventual regrowth, and during the initial response during reoxygenation [32].

Thus, Mtb Ala-DH was thought to be a useful tool to discriminate between LTBI and active TB [67] and our previous study demonstrates that this antigen is able to stimulate IL-2 production in active TB but not in LTBI children; in that study we evaluated the performances of INF-γ and IL- 2 based ELISpot assay in children at risk for TB used an array of Mtb specific antigen (ESAT-6, CFP-10, TB 10.3, Ala-DH, Ag85B, PstS1,HspX (16 kDa) protein): the responses of 6 antigens in children with LTBI and active TBI were compared and their data revealed that IL-2 responses to Ala-DH antigen were significantly different between the children with active TB and the healthy control group but not between LTBI subjects and healthy ones [55].

Given these findings, in the present study we aimed to confirm our result in an adults cohort tested with the LIOSpot® TB kit, an ELISpot assay developed to detect IL-2 production by T cells stimulated with three Mtb antigens, like ESAT-6, CFP-10 (also used in IGRAs) and Ala-DH.

Cytokines play an important role in cell mediated immune responses to Mtb infection. The IFN-γ production by activated T cells has been widely considered to play a crucial role in protection against Mtb infection [12]. The choice of setting the LIOSpot® TB assay on IL-2 cytokine production is due to the fact that several studies have demonstrated that the IL-2 response to Mtb-specific antigens is significantly higher in active TB patients, suggesting that IL-2 could be a potential infection biomarker [68, 69].

Antigen-specific memory T-cells can be subdivided into effector memory T-cells (TEM) and central memory T-cells (TCM) [70]. TEM cells express receptors that enable them to migrate to the inflamed peripheral tissues and differentiate directly into effector cells. IL-2 is promoting T-cell replication and is essential for cellular immunity and granuloma formation. Although IFN-γ is predominantly produced by TEM, IL-2 is mostly produced by TCM, thus, it is related to ancient exposure [71, 72].

The secretion of IL-2 is secreted from cells also secreting IFN-gamma in both the early and the later stages of infection and when the antigen load has declined. Dual IFN-gamma /IL-2-secreting cells in active phase of TB can support their own expansion because IL-2 is a potent T cell growth factor. The presence of these cells inactive TB when the antigen load is high may therefore suggest their involvement in the initiation phase of the immune response. IL-2 in LTBI or when the antigen load is reduced or cleared may reflect its function in the termination of T cell responses. This proposed signalling function augments the growth and survival of regulatory T cells that control inflammatory responses [73].

A study conducted in Republic of Korea by Eun-Jeong Won et al. [67] measured 39 different cytokines in IGRA supernatants by Luminex assay: eight biomarkers (GM-CSF, IFN-γ, IL- 1RA, IL-2, IL-3, IL-13, IP-10, and MIP-1b) in the Mtb-infected group were significantly different from those of the healthy controls. The same study design was conducted among 19 active TB patients, 8 TB-negative individuals (controls) and 16 LTBI non-human immunodeficiency virus infected individuals in Nairobi, Kenya. Excess supernatants from the IGRA test were used to measure immune analytes using a luminex assay; the study suggested that IL-17F, MIP-3α, IL-13, IL-17A, IL-5, IL-9, IL-1β and IL-2, in addition to IFN-γ, could identify and uniquely discriminate between TB states [74] Despite the low sample size limitation, this study was able to identify putative biomarkers

of TB disease in Kenyans that warrant further investigation.

In another study conducted in China by Jing Wu et al. [75] the cytokine production by PBMCs after Mtb PPD stimulation was evaluated by luminex assay: the levels of IL-2, IL-10, IFN-γ, IP-10 and TNF-α were significantly higher in LTBI group than in active TB group, endorsing that cytokines such as IL-2 and IL-10 may serve as biomarkers for distinguishing healthy from infected patients and active TB from LTBI subjects too.

A further Chinese study setted a novel electrochemiluminescence (ECL)-sensing platform for the precise analysis of multiple LTBI markers IFN-γ and IL-2: the ECL intensity depended linearly on the content of IFN-γ and IL-2 and can be used to detect IFN-γ and IL-2 associated with LTBI [76].

Consistent with several other studies [68] is clear that the IL-2/IFN-γ ratio have the potential to be used as diagnostic biomarkers to distinguish between LTBI and active TB.

According to these findings, LIOSpot® TB was set as an anti-human IL-2 ELISpot assay able to detect IL-2 production after PBMCs stimulation with ESAT-6, CFP-10 and Ala-DH of Mtb.

In our study, all the healthy subjects showed no significant production of IL-2 (SFC per million of PBMCs) in response to T cell stimulation by the three different antigens: Ala-DH (0: 0-0), ESAT-6 (2.5: 0-17.5) and CFP-10 (0: 0-15). Furthermore, it is of note that none of the 21 BCG-vaccinated healthy subjects gave positive IL-2 responses to Ala-DH. The LIOSpot® TB detected a significant production of IL-2 after stimulation with ESAT-6 (392.5: 140-1300), CFP-10 (480: 325-1590) and with Ala-DH (280: 85-1120) in 73 patients group with active TB. Among people in the LTBI group, despite the smaller but substantial production of IL-2 in response to ESAT-6 and CFP-10 stimulation (180: 30-340 and 192.5: 77.5-660 SFC per million of PBMCs respectively) compared to active TB cases, Ala-DH induced a very low amount of IL-2 production by stimulated T cells (7.5: 2.5-10 SFC per million of PBMCs).

Even though comparing the LIOSpot® TB results for each tested antigen between uninfected and infected subjects and between people with LTBI and active TB, all the differences were significant ($p < 0.0001$), the ROC analysis demonstrated a high accuracy of the test only for Ala-DH: with a cut off value of 12.5 SFC per million PBMCs, the Ala-DH ROC curve conferred 96% sensitivity and 100% specificity to the test. Thus, the LIOSpot® TB test is highly accurate and is able to make a differential diagnosis between subjects with active TB and those with LTBI.

For the ESAT-6 antigen, with a best cut off value of 71.25 SFC per million PBMCs, a

sensitivity of 86% and specificity of 36% was obtained. Finally, the best cut off value for CFP-10 was 231.25 SFC per million PBMCs, with sensitivity of 80% and specificity of 54%.

Despite the low specificity, these thresholds confer to the LIOSpot® TB the ability to detect true tuberculosis infection. Limitation of our present study include has done in single-center study. Unclear if test would be practical in low-resource settings.

Overall, the present study demonstrates that the LIOSpot® TB test is a very useful diagnostic tool to discriminate between LTBI and active TBI. Accordingly has a diagnostic value for individuals with active TB. However, further prospective studies are needed to monitor the ELISpot assay of IL-2 responses to Ala-DH among patients exposed to TB infection in many center study.

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Systemic Lupus Erythematosus

Interleukin-17/Interleukin-21 and Interferon-γ producing T cells specific for β2 Glycoprotein I in atherosclerosis inflammation of systemic lupus erythematosus patients with antiphospolipid syndrome

INTRODUCTION

Systemic lupus erythematosus (SLE), commonly referred to as "lupus", is a multifactorial chronic and multisystem autoimmune disease of unknown causes with a highly heterogeneous pattern of clinical and serological manifestations, and a relapsing and remitting course. The pathogenesis most likely involves a complex interaction among gene susceptibility, hormonal influences and environmental factors resulting in an irreversible loss of immunologic self-tolerance. Autoantibodies (auto-Abs) production, immune complex (IC) deposition, complement activation and consequent dysregulation of the inflammatory response, leading to the induction and maintenance of the disease and resulting in damage and destruction of multiple organs. The pathogenic process is characterized by a sequence of events, which take a long time before ending into overt clinical manifestations [1, 2].

SLE can be severe and is associated with increased mortality from a variety of causes, including disease activity, in particular when vital organs or systems are involved, and treatment complications. Recent studies demonstrated that disparities according to age, sex, race/ethnicity, and socio-economic status are determinants that may affect the frequency and progression of SLE.

SLE is a predominately female disease and more than 90% of cases of SLE occur in women in childbearing age. However, 10-20% of patients have onset of disease in adolescence or younger named paediatric SLE (pSLE) [3]. On the other hand, Late-onset lupus, (i.e., after the age of 50 years) is reported to occur in 3-18% of patients typically showing a lower disease activity, including less incidence of nephritis, and less organ damage, but higher pulmonary manifestations. However, the overall outcome is poorer in patients with late-onset disease, probably because of increased comorbidity situations [4].

The incidence is higher in women during reproductive years; however, people of all ages, genders, and ancestral backgrounds are susceptible. The female-to-male incidence ratio varies with age as it approximates 1:1 during the first decade of life, followed by a sharp increase to 9:1 during the fourth decade and then a decline in subsequent years before increasing again during the seventh to the eighth decade [5]. Overall, the female-to-male ratio is 7-15:1 in adults and 3-5:1 in children [6]. Several studies support that the female predominance in SLE may result from a complex interaction among sex hormones, genetics and epigenetic factors, and gut microbiota [7]. The frequency of Klinefelter's syndrome (47, XXY), often subclinical, is increased in men with SLE by 14-fold compared with its prevalence in men without SLE. Prevalence of Klinefelter's syndrome in males with SLE has suggested a gene-dose effect at the X chromosome level. The presence of two X chromosomes increases the severity of SLE [8].

Notably, the time of disease onset, clinical manifestations, comorbidities, and the overall disease course differ considerably between male and female patients with SLE. Males tend to show more severe course of the disease with important clinical manifestation such as nephritis and serositis [9].

SLE, present across ethnicities, is more prevalent in non-Caucasians. While the prevalence in Europe and USA is higher in people of African descent, in USA the incidence of SLE in Afro-American females is >2.5 times higher than in white females with more sever disease and poorer clinical outcomes [10].

SLE is a worldwide disease, and in many countries, its prevalence approximates or even exceeds 80-100 per 100,000 adults. The estimate of the incidence and the prevalence of SLE, even within the same country or region, presented a high variability in various reports. Such a variation may be due to dissimilarities in the genetic make-up and environmental exposures of the population under study, although methodological differences are also critical. There are two well-conducted studies carried out by the Centres for Disease Control and Prevention (CDCP) in USA, that estimated the frequency of SLE in Michigan [11] and in Georgia [12] on the basis of data collected from 2002-2004, a period that reported very similar incidence (5.5 and 5.6 per 100,000 person-years) and prevalence (72.8 and 73.0 per 100,000) rates. Two other studies also provided estimates of the incidence and prevalence of SLE among residents of the New York (Manhattan) (4.6-6.2 per 100,000 person-years and 62.2-73.8 per 100,000 person-years, respectively) [13] and the San Francisco (California) (4.6 per 100,000 person-years and 84.8 per 100,000 person-years, respectively) [14] counties. Collectively, more

recent estimates of SLE frequency tend to be increased as compared with previous ones, at least in USA. Importantly, these data are generally considered as more accurate, because they originate from studies that were better designed using more strict case definitions, broader case-finding methodology and were corrected for possible case under-ascertainment. The prevalence of SLE among East Asians is higher than among Europeans [15].

Ethnicity has been identified as an important modifier of SLE outcome with higher mortality rates was found in Native and Afro-Americans than in Caucasians, Asian, and Hispanics. The study was based on data in CDCP database that were extracted from females death certificates issued between 2000 and 2015 in USA [16].

Furthermore, Tektonidou et al. [17] reported that during 2008-2016, the 5, 10, and 15 year survival rates in adult patients with SLE were 0.95, 0.89, and 0.82, in high-income countries, respectively, compared to 0.92, 0.85, and 0.79, in low/middle-income countries respectively.

Non-white race, and low socio-economic status are associated with more severe forms of the disease including increased rates of renal involvement. SLE is associated with a shorter life expectancy than in the general population. Standardized mortality ratios (SMR) of 2.4, comparable to diabetes, have been reported [18].

To date numerous SLE susceptibility genes have been identified. A sibling risk ratio of approximately 30 in SLE illustrates a strong genetic influence and the fact that observational studies have identified many families with multiple cases of SLE and other autoimmune conditions suggests the potential for shared genetic predisposition [19]. Studies showed that monozygotic twins have a ten-fold higher risk to develop SLE than dizygotic twins and a clear aggregation of SLE in families. This together with the findings in large-scale genome-wide association studies (GWAS) has allowed the recognition of >80 loci associated with SLE leads to the formation of key proteins involved in innate and adaptive immunity, each one contributing to a small increased risk and implicating a strong genetic background for SLE. More than 50% of lupus susceptibility genes are linked to the interferon (IFN) system [20]. In addition, gene loci involved in the NFkB signalling pathway, DNA degradation, apoptosis, phagocytosis, neutrophil, and monocyte/macrophage functions and signalling have been identified. The most pronounced association, however, was found for HLADR2/DR3, followed by BLK and PTPN22 involved in T and especially B cell activation and therefore adaptive immunity. Regulation of the gene expression appears to play a role in lupus pathogenesis. Each cell type might

have a specific and dynamic epigenetic profile. The interaction between the host and the presumed environmental factors resurfaces and suggests that they may directly interact at this level [21]. The certain miRNAs in SLE, miRNA (miR)-30a in B cells has been shown to decrease the expression of Lyn as central protein tyrosine kinase of B cell receptor signalling. The study arrived at the hypothesis that low levels of Lyn expression in SLE are due to an increased expression of miR-30a, which may facilitate B cell proliferation and antibodies (Abs) production [22].

Many sex hormones (including oestrogens, progesterone, androgens, and prolactin) affecting the susceptibility and development of SLE, may also play a role as triggers or protectors in SLE development. Studies in human populations and experimental models have indicated an elevated risk of SLE associated with the exposure to estrogens whereas progesterone and testosterone appear to play a protective role via counteraction of estrogens effects. Other sex hormones such as prolactin remains controversial.

The potential role of estrogens in autoimmune diseases has been extensively investigated, particularly for SLE. In genetically predisposed individuals, estrogens increase the risk of disease and have a pathogenic role in SLE. The pathogenic mechanisms may involve the production of type 1 (T1) IFN, survival of auto-reactive B cells and production of pathogenic immunoglobulin (Ig) G auto-Abs, and/or differentiation of $CD4^+$ Th cells.

Enhanced expression of T1 IFN-inducible genes has been detected in SLE, and this so-called “IFN signature” has been shown to play a critical role in the pathogenesis of SLE and is associated with active disease. T1 IFNs also increase estrogens signaling in immune cells by provoking the expression of ER-a, thereby contributing to SLE development and progression. Estrogens can also enhance the production of anti-double stranded DNA (anti-dsDNA) antibody and IgG or IgM by peripheral blood mononuclear cells (PBMCs) and serum, which enhances the disease severity causing flare-ups. Estrogens were also shown to exacerbate lupus disease severity via an ERa- independent mechanism along with other immune effects contributing to the lupus pathogenesis, including modulation of Toll-like receptor (TLR) pathways, dendritic cells (DCs) development, or E2-TWEAK signalling. These mechanisms might also contribute to the pathogenic mechanism of SLE, primarily affecting women [23].

Progesterone has been suggested to play an important protective role against SLE disease activity, counteracting the effects of estrogens. Progesterone has immuno-modulatory effects, it impairs IRF-7 activation in plasmacytoid dendritic cells (pDCs), which regulate the TLR-mediated decreased production of IFN-α, a major source of T1 IFN, through

depot medroxyprogesterone acetate (DMPA), thereby ameliorating the IFN signature and consequently SLE disease activity. Moreover, progesterone decreases the IgM to IgG class switch recombination via suppressing the transcription of activation induced cytidine deaminase (AICDA). High levels of progesterone will induce a T helper (Th) 2-type immunologic response and suppress a Th1-type one via inhibiting interleukine (IL)-12 signaling in SLE. Meanwhile, there is a decrease in mortality, glomerulonephritis and Th1-related auto-Abs production after DMPA treatment in NZB/NZW mice, which suggest that progesterone may have therapeutic benefit for SLE patients [24].

Androgens may play an important protective role in the pathogenic mechanisms contributing to the development of SLE. Similar to progesterone, androgens target key immune pathways that can protect against SLE by counteracting the effects of estrogens. In particular, androgens down- regulate the expression of Ifi202 gene, which in turn decreases the production of T1 IFN. Compared with estrogens, androgens may enhance checkpoints for the auto-reactivity of B cells. Androgens decrease the Th1 differentiation of $CD4^+$ T cells via inhibiting IL-12 signaling, which in turn mitigates $CD4^+$ responses in autoimmune disease. Androgens also appear to modulate the accumulation of $Gr1^+CD11b^+$ cells in male mice, $CD11b^+$ cells were shown to over-express DHT-regulated genes and colony-stimulating factor 3 receptor, which was identified to influence the development of lupus and rheumatoid arthritis (RA) [25].

Environmental factors, such as exposure to silicates, smoking and alcohol consumption, UV light, certain medications, vitamin D deficiency, viral infections, and estrogens are considered potential risk factors in the development of lupus. There is evidence for silica exposure [26] and a likely association for smoking and alcohol consumption [27] as contributors to SLE development. Vitamin D deficiency was found in most SLE patients [28]. As such, the role of vitamin D and SLE is complex. There is an association between vitamin D receptor single-nucleotide polymorphisms and a higher long-term cumulative damage in SLE has been reported [29]. Interestingly, vitamin status and CYP24A1 gene polymorphisms can have a combined role in the transition from incomplete to complete SLE, and this association is not confounded by the number of the American College of Rheumatology (ACR) classification criteria or exposure to sun at baseline [30].

The sun exposure, on the other hand, leads to a higher serum vitamin D concentration, that might be protective, but UV light exposure is also a risk factor for the induction of SLE and patient deterioration. UV light is known to aggravate pre-existing skin manifestation

and can cause severe lupus flares with a plausible pathogenic mechanism: UV light exposure induces increased apoptosis of keratinocytes, followed by a prolonged exposure of self-antigens (Ags) due to known clearance defects that subsequently causes precipitation of immune complexes (ICs) in the skin (‘lupus band’) resulting in an initial state of inflammation [31].

Bacterial and viral infections, such as EBV, CMV, parvovirus B19, human endogenous retroviruses and others, have been implicated in the development of SLE where these agents apparently ‘kick-start’ the immune activation and give rise to chronic inflammation. Molecular mimicry mechanisms involved in this ongoing autoimmunity process. Infections have been implicated in SLE for a long time; this is largely based on indirect associations. A number of studies have suggested a correlation of Epstein-Barr virus seroprevalence (anti-viral capsid antigen IgG) with SLE (OR 2.08) [32]. The Epstein Barr Virus Antigen 1 (EBNA-1) contains regions that are homologous to sequences of self-proteins such as Ro60 kDa and snRN. In SLE, McClain and colleagues could detect anti- EBNA-1 prior to the detection of anti-Ro. This suggests that EBV infection started with anti-EBNA-1 Abs production and due to similarities to Ro antigen, ‘switched’ to anti-Ro Abs production, probably after Ro60 kDa exposure due to apoptosis induced by UV light. This study demonstrated that anti-Ro Abs cross-react with EBNA-1 [33]. Bacterial infections contribute to SLE pathology through similar mechanisms. In SLE, endotoxins stimulation, such as lipopolysaccharides (LPS) of gram-negative bacteria displayed a substantial overlap demonstrating that they can in part mimic T1 IFN signature seen in the disease [34].

Various dietary factors have been proposed to play a role in SLE pathogenesis through epigenetic changes (e.g., affecting DNA methylation) and/or interplay with the gut microbiota [35].

The composition of gut microbiome is influenced by various factors such as genetics, sex hormones, diet, drinking water, and the use of antibiotics and probiotics. Hevia et al. [36] revealed lower levels of fecal Firmicutes/Bacteroidetes ratio in SLE patients than in healthy individuals. Importantly, intestinal dysbiosis has been linked to a disrupted balance between regulatory and pathogenic Th17 cells in SLE [37]. More recent data support the hypothesis of “leaky” gut epithelium in SLE, which leads to translocation of gut pathobionts, e.g. Enterococcus gallinarum, to the liver and lymph nodes, thus increasing the expansion of autoreactive T cells [38].

Psychosocial factors (including alexithymia, depression, anxiety, negative emotions,

perceived stress) affect the susceptibility and development of SLE. In recent years, depression and anxiety have also been suggested to play an important role in the activation of SLE, given their associations with proteinuria [39].

The clinical onset of SLE requires an interaction of genetic predisposition, environmental precipitants, immunological and hormonal factors. In such a permissive environment, along with proinflammatory stimuli such as T1 IFN and other cytokines, immune tolerance to self-Ags is lost [1]. Autoimmunity then follows driven by a complex interplay of defective clearance of apoptotic waste and ICs along with neutrophil extracellular traps (NETs), sensing of nucleic acids, disturbed T lymphocyte haemostasis, and disrupted IFN pathways [1].

The identification of strong T1 IFN signature in lupus recognized the pivotal role of the innate immune system [20]. SLE has a wide spectrum of disease expression, with aberrant host innate and adaptive immune responses against self-Ags which induce the production of auto-Abs and the deposition of ICs in tissues leading to the activation of complement, accumulation of neutrophils and monocytes, and self-reactive lymphocytes. SLE-associated auto-Abs and high serum IFN-α levels are two important heritable phenotypes in SLE which are thought to play a role in the disease pathogenesis. Breakdown of self-tolerance is critical in the development of SLE. T cells are defective and their aberration in lupus is complex [1, 40].

Regulatory T cells (Tregs) are important gatekeepers in preventing aberrant activation of self-reactive lymphocytes. Treg lymphocytes constitute a separate lineage of $CD4^+$ T cells which maintain the immunotolerance by a direct inhibitory effect on conventional effector T cells (Tconv) or by secretion of immunoregulatory cytokines. Tregs are induced from naïve $CD4^+$ T cells and IL-2 is critical for their differentiation and function. It is well known that IL-2 production by conventional Tconv is impaired in SLE. IL-2 gene is silenced through a transcriptional regulator, cyclic AMP response element modulator alpha, which is over- expressed by SLE Tconv cells. The absence of IL-2 probably favours the differentiation and the expansion of IFN-γ-producing Th1 cells and IL-17-producing Th17 cells, accumulating in organs such as the skin and the kidney [41]. Tregs which suppress Th cells and B cells, are impaired in SLE. Furthermore, an excess of double-negative T lymphocytes is observed and overall, an excess of autoreactive B cells, with B cell lymphopenia and hyperactivation of BLyS or BAFF pathways is detected. T cells stimulate B cell proliferation and are necessary for the secretion of high-affinity class-switched IgG

Abs, in a process called T lymphocyte help. T cells in SLE provide an excessive help to B cells for the Ab production and these auto-Abs are strongly associated with tissue damage in SLE [42, **Fig. 1**].

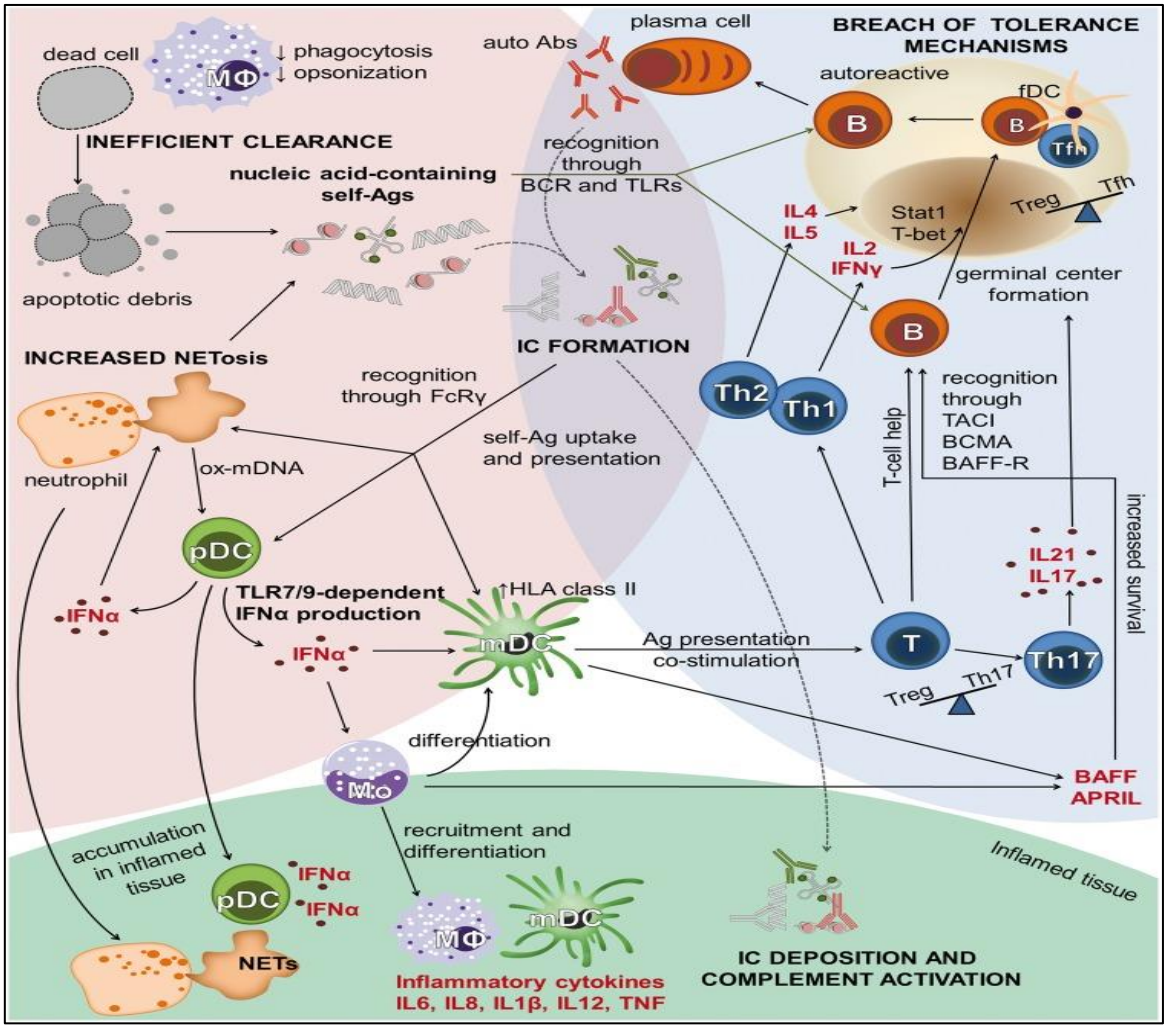

Figure 1. **Overview of immunological pathways leading to SLE.** The development of SLE occurs in three interconnected phases, illustrated by coloured back grounds. Loss of adaptive immune tolerance (blue) leads to an increase in autoreactive B cells. Signals from self-antigens, TLR ligands, BAFF/APRIL and T-cell-derived cytokines promote the formation of germinal centres and the production of autoantibodies. Innate immune defects leading to increased availability of self-antigens (pink) include increased NETosis, impaired clearance of apoptotic debris and reduced phagocytosis. Self-antigens form ICs with autoantibodies, enabling FcRg-mediated uptake and activation of several downstream pathways. Inflammation and tissue damage (green) is caused by mediators released by recruited inflammatory cells and IC-induced complement activation. Abs: antibodies; Ags: antigens; APRIL (CD256): a proliferation-inducing ligand; B: B cell; BAFF (CD257): B-cell-activating factor; BAFF-R: B-cell-activating factor receptor; BCMA: B-cell maturation antigen; BCR: B-cell antigen receptor; FcRg: Fc receptor-g; fDC: follicular dendritic cell; HLA class II: human leucocyte antigen class II; mDC:myeloid dendritic cell; M_: macrophage; Mo: monocyte; NET: neutrophil extracellular trap; ox-mDNA: oxidized mitochondrial DNA; pDC: plasmacytoid dendritic cell; Stat1: signal transducer and activator of transcription (a transcription factor); T: T cell; TACI (CD267): transmembrane activator, calcium modulator and cyclophilin ligand interactor; T-bet: a T-box transcription factor; Tfh: T follicular helper; TLR7/9: Toll-like receptors 7 and 9. (Zharkova2017).

There are other abnormalities in B and T cells including an abnormal T cell receptor complex, alterations on proteins that influence the T cell response to inflammation in various ways, such as mitogen activated protein kinase, decreased concentrations of blunting molecules such as Lyn (LCK/Yes-related novel tyrosine kinase, impaired signalling via the B cell inhibitory receptor FcgRIIB, and a faster response to a B cell proliferation stimulus such as proliferation-inducing ligand (APRIL) or B lymphocyte stimulator (BLyS) [22].

DCs play a key role in the production of T1 IFN and have also a role in the clearance and sensing of nucleic acids and ICs with known auto-Ags in lupus. The endogenous and external nucleic acids are a major antigenic stimulus in lupus. Auto-Abs targeting nucleic acid-bound Ags are one of the hallmarks of the disease. The major source of such Ags come from apoptosis and NETs [43]. Excess and impaired degradation of NETs are associated with lupus severity, lupus nephritis, anti-dsDNA Abs and complement consumption [44].

Apoptosis is pivotal in the pathogenesis of lupus. Apoptotic cells such as UV light exposed keratinocytes release blebs rich in auto-Ags including Ro, La, and RNP [45]. In *in vivo* photosensitivity for example, ICs with endogenous nucleic acids can activate pDCs via activation of TLR7 and 9 to produce conspicuous amounts of T1 IFN [46]. These particles are physiologically coated by molecules such as C1q and IgM to facilitate phagocytosis and clearance in an "immunologically silent" and anti-inflammatory environment as mediated by DCs [47].

Excessive non-cleared apoptotic waste is associated with inflammation and auto-Abs production. Dysregulated apoptosis and defective clearance of cellular debris increases auto-Ags exposure and tolerance breakdown. In SLE, apoptosis, particularly of T lymphocytes, is dysregulated in a Fas/Fas ligand (FasL)-dependent pathway in which FasL is hyperexpressed and correlates with SLE activity and auto-Abs concentrations.

Abnormalities in the innate immune system, including that of phagocytes and complement, are also linked to impaired recognition and clearance of apoptotic bodies. Subsequently, abnormal prolonged exposure of nuclear Ags that undergo multiple alterations creates neo-epitopes or uncovers hidden ones. The remaining apoptotic bodies then go through a process called secondary necrosis that leads to the release of even more nuclear material.

The presence of auto-reactive B and T cells in SLE, responsible for the aberrant production of a broad and heterogeneous group of auto-Abs, particularly dsDNA and small nuclear RNA-binding proteins. In 2004 Sherer et al. reported that one hundred sixteen auto-Abs

have been described in SLE patients [48].

In SLE, especially in the systemic form, auto-Abs directed to nuclear (ANAs), cytoplasmic, and cellular membrane Ags are considered the serological hallmark. ANAs consist of various types of auto-Abs characterized by different antigen specificities. These nuclear Ags include single strand (ssDNA) and double strand (dsDNA), histone proteins, nucleosome (histone-DNA complex), centromere proteins, extractable nuclear Ags (ENA), Smith antigen (Sm), Ro, La, ribonucleoprotein (RNP), etc. ANAs are present in about 95% of SLE patients with an active disease. In patients with prevalent cutaneous lesions, ANAs have been found positive in 75% of cases [49].

The presence of auto-Abs in SLE was envisaged when the lupus phenomenon was described by Hargraves et al. in 1948 [50] and then proven when it was understood that it was due to neutrophil phagocytosis of cell nuclei opsonised by auto-Abs.

In 1957, auto-Abs against DNA were identified [51] and in 1966 Tan and Kunkel found auto-Abs directed to Ags different from DNA and described the anti-Sm auto-Abs [52].

Common auto-Abs-mediated mechanisms of damage in SLE include immune complex mediate damage, cell surface binding and cytotoxicity, reactivity with auto-Ags expressed on apoptotic or activated cell surface, penetration into living cells, and binding to cross reactive extracellular molecules [42].

Auto-Abs to dsDNA are useful for the diagnosis of SLE, to monitor the disease activity, and correlate with renal and central nervous involvements. Anti-dsDNA auto-Abs have high specificity and are universally used as a diagnostic criterion for SLE (70–98% of patients are positive for such Abs) [49], and for monitoring the clinical course of the patient. Anti-dsDNA auto-Abs, in particular of the IgG isotype, have an important pathogenetic role in SLE. A clear-cut relationship exists, for example, between anti-dsDNA auto-Abs (R4A antibody) and disease activity in nephritis [53]. Moreover, anti-dsDNA auto-Abs also contribute to the end-stage lupus nephritis by directly binding exposed chromatine fragments in glomerular basement membrane [54]. On the other hand, IgM class anti-dsDNA auto-Abs seem to have a protective role for nephropathy [55]. Furthermore, De Giorgio et al. demonstrated that a subset of anti-DNA auto-Abs cross-reacts with N-methyl-D-aspartate receptors (NMDAR), and through an excitotoxic mechanism, could induce neuronal apoptosis. Anti-NMDAR auto-Abs are present in 40% of lupus patients and some reports have supported the correlation between such auto-Abs and the presence of neuropsychiatric lupus [56]. The anti-NMDAR auto-Abs also bind C1q; therefore, Franchin et al. hypothesized that this subset of anti-DNA Abs contributes to

lupus pathogenesis through direct targeting of C1q on glomeruli [57].

Anti-Sm auto-Abs are highly specific for SLE. Anti-nucleosome Abs are an excellent marker for SLE and good predictors of flares in quiescent lupus. Anti-Sm auto-Abs are a highly specific marker for SLE and Anti-Sm reactivity is not described in other diseases. Their sensitivity is however low. In fact, Anti-Sm Abs are detectable only in 20% of SLE white patients, but 30–40% in black and Asian people.

Anti-nucleosome auto-Abs are an excellent marker for SLE and good predictors of flares in quiescent lupus [58]. Anti-histone auto-Abs characterize drug-induced lupus, in about 96% of patients with SLE induced by procainamide [59] and 100% of patients with SLE induced by penicillamine, isoniazid [60], and methyldopa have anti-histone auto-Abs, while anti-SSA/Ro and anti-SSB/La Abs are associated with neonatal lupus erythematosus (NLE) and photosensitivity [61]. Anti-ribosomal P (Anti-RNP) Abs play a role in neuropsychiatric lupus, but their association with clinical manifestations is still unclear. Anti-C1q Abs amplify glomerular injury, and the elevation of their titers may predict renal flares [62]. Anti-RNP auto-Abs are a marker of Sharp's syndrome but can be found in SLE as well. Anti-proliferating cell nuclear antigen (PCNA) auto-Abs are present in 5–10% of SLE patients especially those with arthritis and hypocomplementemia [63].

Anti-phospholipid (aPL) Abs are a key feature in both Antiphospholipid syndrome (APS) and SLE [64]. They are found in all patients with APS and in 20–30% of patients with SLE [65], but only 1/3 of them develop clinical features of anti-PL syndrome [66]. Among SLE patients, auto-Abs including aPL Abs can be detected up to 10 years before diagnosis in 85% of SLE patients [67]. Auto-Abs lead to the formation of ICs that directly induce B cells to produce more Abs and enhance the TLR/IFN1 pathway, which also stimulates B cells to differentiate into plasma-blasts.

The diagnosis of SLE is made based on a combination of typical clinical manifestations and positive serologies. Given the wide heterogeneity of clinical manifestations, several sets of classification criteria have been developed over time for epidemiological and research purposes.

The ACR classification criteria, first published in 1971 and subsequently revised in 1982 and in 1997, has been the most widely used.

In 2012, the Systemic Lupus International Collaborating Clinics (SLICC) group published validated criteria that have higher sensitivity than the revised ACR criteria. They also have lower specificity, although not statistically significant, which might lead to the inclusion of more patients in clinical trials. Furthermore, the SLICC criteria showed some changes that

might improve their use in clinical practice. Some criteria (e.g. photosensitivity, malar rash) were reorganised in order to minimise overlaps, while others (e.g. neurological manifestations, arthritis) were redefined. Among the immunological criteria, ANA, anti-dsDNA, anti-Sm and anti-aPL auto-Abs are now considered different criteria, so each one can contribute to the classification of SLE. Low complement is a new criterion.

Using the SLICC classification, at least four criteria from a list of clinical and immunological features (**Table 1**), including at least one clinical criterion and one immunological criterion, should be present for a patient to be deemed to have SLE. However, biopsy-proven lupus nephritis (LN) together with ANA and anti-dsDNA may also be sufficient. The mandatory presence of clinical and immunological features is useful in order to avoid inclusion of patients without clinical manifestations or without positive Abs [68].

The 2018 European League Against Rhematism (EULAR) criteria, developed for research only, requires an ANA of 1:80 or higher, and then eight lupus manifestations (for a score of 10) [69]. Direct comparison of the 3 sets of criteria using the SLICC validation cohort showed that they perform similarly with a sensitivity and specificity of 89% and 90% for the EULAR, 83% and 96% for the ACR, and 97% and 84% for the SLICC criteria, respectively [70].

A systematic review and meta-analysis including 5236 patients and 1313 controls showed that the SLICC criteria have better sensitivity (94.6% vs. 89.6%) and similar specificity (95.5% vs. 98.1%) for adult SLE as compared to the 1997 ACR criteria [71].

SLE is highly heterogeneous clinical complex disease; it has variable presentation and an unpredictable course. It progresses in acute flares and periods of remission, although there are probably long periods of subclinical inflammatory activity. It can affect virtually every organ and system in the body, and during the flares more than one organ is usually affected. In mild forms, joints and skin are the main organs affected. In moderate forms, other organs are involved, but it is a severe disease, notably of the kidneys and heart, that can be life-threatening. Cardiac and cardiovascular system diseases are inter alia caused by atherosclerosis, vasculitis, and thromboembolic events. Many studies highlighted that SLE is associated with coronary heart disease and atherosclerosis [72-74]; an important prospective study has demonstrated that SLE patients have an accelerated progression of carotid plaque formations compared to non-lupus controls [75]. SLE patients have a reduced life expectancy mainly due to increased prevalence of cardiovascular diseases (CVD). Coronary artery disease is responsible for 30% of deaths in SLE [76].

1982 revised ACR criteria	SLICC classification system
Clinical criteria	**Clinical criteria**
Must meet 4 of 11 criteria -Malar rash -Discoid rash -Photosensitivity - Oral rash -Arthritis -Serositis: a) pleuritis; b) pericarditis -Renal disorder: a) proteinuria>0.5g/24h or 3+, persistently; b) cellular cast -Neurological disorder: a) seizures; b) psychosis (excluding other causes, eg drugs) -Haematological disorder: a) haemolytic anaemia with reticulocytosis; b) leukopenia or <4000/mm³ on two or more occasions); d) thrombocytopenia (<100,000/mm³) -Immunological disorders: a) positive lupus erythematous cell; b) raised anti-native DNA antibody binding; c) anti Sm antibody; d) false-positive serological test for syphilis, present for at least six months	Must meet 4 criteria (including 1 clinical and immunological) -Acute cutaneous lupus (1 or more of the following): a) malar rash; b) bullous lupus; c) maculopapular rash; d) photosensitive rash -Chronic cutaneous lupus (1 or more of the following): a) classic discoid (localised vs. generalised); b) hypertrophic lupus; c) lupus panniculitis; d) mucosal lupus; e) lupus erythematous tumidus; f) chilblanis lupus; g) discoid lupus/lichen planus overlap -Oral or nasal ulcers -Non scaring alopecia -Synovitis OR tendonitis: 2 or more joints; morning stiffness -Serositis: a) pleural; b) pericardial -Renal: a) urine protein-to-creatinine ratio with more than 500mg protein/24 h; b) red blood cells casts -Neurological disorders (1 or more of the following): a) seizures; b) psychosis; c) mononeuritis multiplex; d) myelitis; e) neuropathy; f) acute confusional state -Haematological disorder: a) haemolytic anaemia; b) leukopenia (<4000/mm³at least once), c) lymphopenia (<1000/mm³ at least once); d) thrombocytopenia (<100,000/mm³)
Immunological criteria	**Immunological criteria**
Abnormal titer of ANA	a) ANA above reference range; b) anti-dsDNA above reference range; c) anti-Sm; d) antiphospholipdantbody positivity; e) low complement; f) direct coombs (in absence of haemolytic anaemia)

Table 1. SLE classification criteria of ACR and SLICC (Santos, 2019).

Patients with SLE are at a higher risk of developing accelerated atherosclerosis. Multiple studies suggest that these patients have between a 9-fold and 50-fold increase in risk of developing CVD compared with non-SLE patients. These increases result from a combination of traditional risk factors, and dysfunctional immune and inflammatory mechanisms [77]. The data suggests that SLE is per se a strong independent risk factor for the development of CVE, comparable even to type I diabetes mellitus (DM) [78]. The relative risk of myocardial infarction is five to eight times greater than that of the general population, and more than 1/3 of SLE patients exhibit evidence of carotid plaques or coronary artery calcifications. Premature atherosclerosis has been recognized as a major comorbidity condition in SLE as evidenced by the increased carotid intima-media thickness (CIMT) score and prevalence of carotid plaques in several systematic reviews and meta-analyses [79].

Accelerated atherosclerosis in SLE

Accelerated atherosclerosis in SLE has been initially demonstrated in 1975 by Bulkley et al. [80] in a necroscopic study, that was further confirmed by Urowitz et al. [81]; they first reported an increased risk of CVD in SLE patients. This study outlined a bimodal curve of mortality for SLE, where early deaths (<1 year after diagnosis) were attributable to kidney disease and infections, while later deaths were associated with CVD [81].

Women with SLE in the 35-44 year old age group have an estimated 50-fold increased risk of myocardial infarction compared with age and sex-matched controls, as well as an increased incidence of subclinical atherosclerosis [82].

Atherosclerosis is a multifactorial disease for which a number of pathogenic mechanisms have been proposed. In addition to classical risk factors, in the last two decades the attention has focused on the inflammatory processes [83]. Increased atherosclerosis in SLE patients is thought to result from a combination of traditional risk factors together with the inflammatory burden and the immune dysregulation characterizing SLE (**Fig. 2**). Certain traditional CV risk factors have been found to be increased in patients with SLE, especially hypertension and a “lupus pattern” dyslipidaemia, SLE patients exhibit a proatherogenic lipid profile characterized by high levels of very low-density lipoprotein (VLDL) cholesterol, triglycerides, low levels of high-density lipoprotein (HDL) and high or unchanged low-density lipoprotein (LDL). Other factors such as smoking, DM, older age and hyperhomocysteinemia have all been linked to subclinical atherosclerosis and/or CV

events in these patients [84]. MTHFR 677 TT polymorphisms is the main genetic determinant for hyperhomocysteinemia and it has been shown to be an independent predictor for lupus related subclinical atherosclerosis [85].

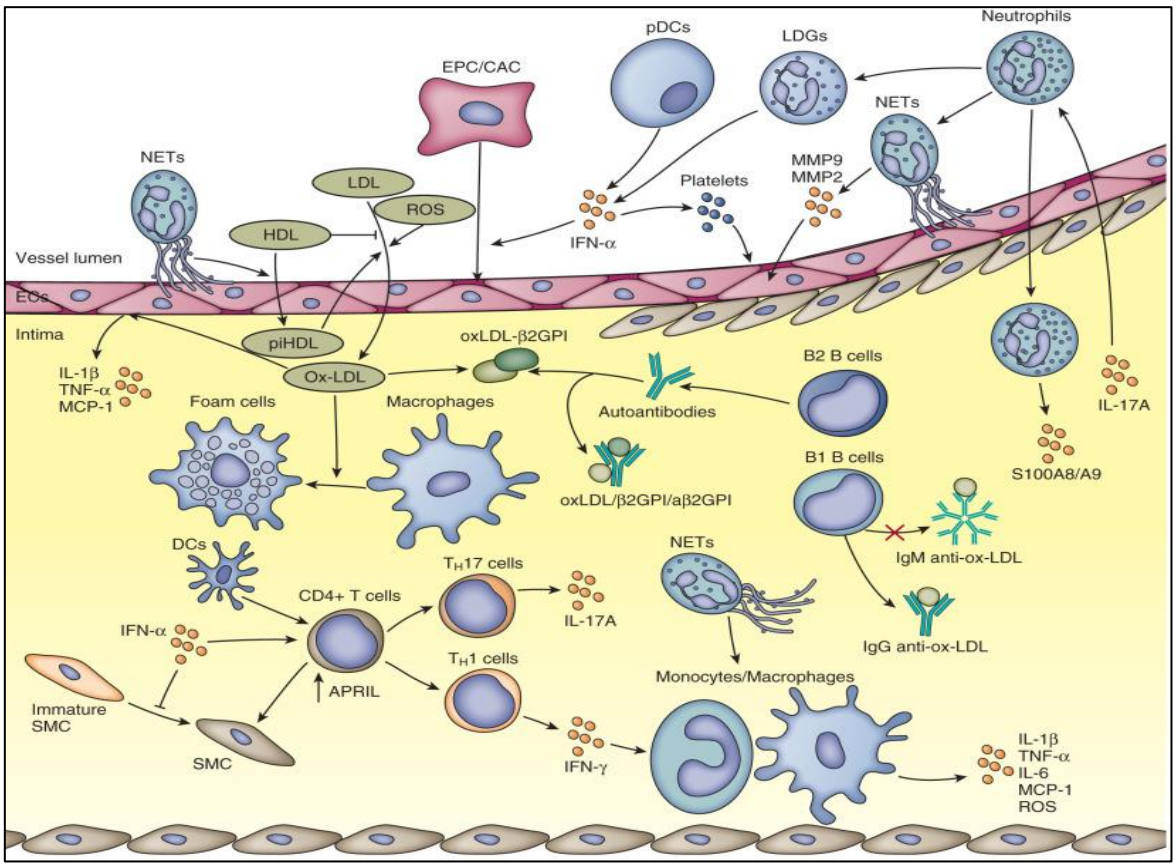

Figure 2. Mechanism of increased risk of cardiovascular disease in SLE. A number of pathogenic mechanisms contribute to the accelerated atherosclerosis and vascular injury in SLE. High-density lipoprotein (HDL), which protects low-density lipoproteins (LDLs) from oxidation by reactive oxygen species (ROS), becomes proinflammatory (piHDL). Ox-LDL activates endothelial cells (ECs) to produce proinflammatory cytokines, recruiting monocytes/macrophages to the lesions. Ox-LDL promotes differentiation of monocytes/macrophages into foam cells. Plasmacytoid dendritic cells (pDCs) and low-density granulocytes (LDGs) secrete type I interferons (IFNs), which impairs maturation of smooth muscle cell (SMC), promotes dysfunction of endothelial progenitor cell (EPC)/circulating angiogenic cell (CAC) differentiation into mature ECs, and induces platelet activation. Neutrophil extracellular traps (NETs) induce endothelial dysfunction and EC death and prime macrophages for interleukin (IL)-1β production. B1a B cells preferentially class switch to pathogenic IgG-producing B1a B cells. B2 B cells produce autoantibodies, such as anti–β2-GPI. Anti–β2-GPI forms complexes with ox-LDL/β2-GPI, further promoting foam cell formation and proinflammatory cytokine and chemokine expression. aβ2-GPI, Anti–β2-glycoprotein I antibodies; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloproteinase; ox-LDL, oxidized LDL; TNF, tumor necrosis factor. (Yudong Liu, 2019).

Atherosclerosis is a chronic inflammatory disease of the arterial wall, in which macrophages, T cells, and vascular smooth muscle cells (VSMCs) are important cellular components of the atherosclerotic lesion. Innate and adaptive immunoinflammatory mechanisms are involved in the formation of the early fatty streak, as reported, when the endothelium is activated and expresses chemokines and adhesion molecules, leading to monocyte/T lymphocyte recruitment and infiltration into the sub-endothelium. It also acts at the onset of adverse clinical vascular events when activated cells within the plaque secrete matrix metalloproteases (MMPs) degrading extracellular matrix proteins and weakening the fibrous cap, with consequent plaque rupture and thrombus formation [86]. Within the T cell population infiltrating the plaque, most cells are activated $CD4^+$ Th1 cells and Th17 cells expressing HLA-DR and the IL-2 receptor (IL-2R) [87]. DCs and B cells are also present in the plaque and in the adventitia and they play important roles in the disease process.

There are evidences indicating that autoimmunity can be detected within the atherosclerotic lesions [88]. Accordingly, self-phospholipids, such as oxidized low-density lipoproteins (oxLDL) and human heat shock proteins (hHSPs), drive T cell inflammation in atherosclerotic patients [89].

An early step in atherogenesis is the endothelial dysfunction, resulting in increased permeability to circulating LDL and recruitment of leukocytes. In SLE the integrity and repair mechanisms of endothelial cells (ECs) may be affected through direct binding of auto-Abs to ECs or deposition of circulating ICs [90]. The consequent endothelial damage promotes atherogenesis, which has been divided into three stages. The inflammatory process triggers the first stage, which consists in the expression of surface adhesion molecules. At the second stage, the adherent leukocytes migrate across the intima layer and penetrate the media layer [91]. The transmigration process is mediated by monocyte chemotactic protein-1 (MCP-1); in humans, elevated circulating levels of MCP-1 correlate to increased CIMT [92]. Tumor necrosis factor (TNF)-α, IL-1, and oxLDL have an important role in the first two stages of atherosclerosis, as they up-regulate adhesion molecules and MCP-1 [93]. Conversely, transforming growth factor-beta (TGF-β) suppresses smooth muscle (SMCs) and ECs proliferation. Decreased levels of TGF-β was associated with premature atherosclerosis in lupus-prone mice [94], supporting the hypothesis that the down-regulation of this cytokine can lead to arterial wall dysfunction with subsequent atherosclerosis development. Finally, in the last stage, macrophages foam

cells formation takes place along with replication of SMCs leading to plaque propagation and rupture [91]. This complex stage starts with the entrapment of LDL in the sub-endothelial space where they are exposed to reactive oxygen species (ROS) produced by nearby arterial wall cells and then converted to pro-inflammatory oxLDL. The exposure of ECs to oxLDL results in the production of more MCP-1, macrophage colony-stimulating factor (M-CSF) and GRO, which further contribute to monocyte binding, chemotaxis, and differentiation into macrophages [95]. In addition, when oxLDL are being exposed to macrophages, the inhibition of the phagocytosis of apoptotic cells takes place and the expression of the scavenger receptor CD36 is promoted, further increasing oxLDL phagocytosis [96], leading to foam cells formation around which atherosclerotic lesions are built. Elevated levels of circulating oxLDL have also been described in SLE patients, especially in those with a history of CVD, and in the general population affected by coronary heart disease. Monocytes and T cells infiltrate the margin of the plaque formed by foam cells and SMCs which encroach on the lumen of the vessel and ultimately lead to fibrosis, that renders the plaques fragile. The occlusion that results in MI can occur when one of these plaques ruptures takes place, or when platelets aggregate in the narrowed area of the artery.

In SLE, LDL display both greater susceptibility to oxidation and ability to increase oxidative stress levels [97]. Furthermore, a dysfunctional form of HDL (piHDL) that arises under inflammatory conditions, present in SLE patients, seems to lack the regular anti-inflammatory and antioxidant properties of HDL. As a result, it fails to efficiently participate in cholesterol efflux and enables the oxidation of LDL [98].

oxLDL, is highly immunogenic; unlike native LDL, they can also form complexes with β2-GPI, which are significantly elevated in SLE with or without APS [99]. The presence of IgG anti β2GPI increases oxLDL uptake by macrophages [100]. Both, elevated levels of circulating oxLDL and of auto-Abs against oxLDL, are more common in SLE patients with CVD, than in patients without CVD [101]. The increased levels of auto-Abs against epitopes of oxLDL has also been associated with maximum IMT scores and progression of atherosclerosis [102]. Once oxLDL become immunogenic promoting an immune humoral response against oxLDL resulting in B cell activation, Ab production and formation of ICs, all of which participate in the atherosclerotic process, in complex and distinct ways [103]. Nevertheless, the heterogeneity among Ig subclasses and the epitopes specificity and affinity might explain the diverse properties of oxLDL auto-Abs. In fact, only IgG are thought to promote atherogenesis, while IgM seem to protect atherosclerosis prone mice

from inflammatory oxidized moieties [104].

TNF-α also plays a role at the last stage of the disease as it suppresses lipoprotein lipase synthesis, thus inhibiting the metabolism of triglycerides and VLDL [105]. The protective mechanism of efflux of cellular cholesterol is thought to be impaired in SLE, as the responsible enzyme for this process, cholesterol 27-hydroxylase, has been shown to be decreased in cultured human monocytes and aortic ECs of SLE patients [106].

The relationship between lupus and atherosclerosis came with the identification of NETosis as a key pathophysiological element. NETosisis a type of cell death pathway that results from externalization of chromatin fibres decorated with granule-derived antimicrobial peptides and is one of the defense mechanisms of neutrophils against pathogens. NETs contain many proinflammatory antimicrobial molecules that induce ECs death and vascular dysfunction.

In SLE, there is a deficient clearing of these NETs, which increases the production and release of T1 IFN and further enhancing NETosis [107]. Impaired degradation of NETs is presumably due to the presence of Abs against deoxyribonuclease (DNase) I, the main degrader of NETs, or due to the presence of anti-NET Abs that protect NETs from degradation [108]. NETs also lead to increased inflammasome activation, by increasing the synthesis of activated IL-1β and IL-18, which induce a positive loop in NETs formation [109].

Low-density granulocytes (LDGs), a subtype of neutrophils prevalent in SLE, are particular prone to predispose to NETosis. LDG NETs are deleterious for the endothelium thus contributing to accelerated atherosclerosis in SLE, as they synthesize increased levels of pro-inflammatory cytokines, mainly IFN-α and disrupt the differentiation of endothelial progenitor cells (EPCs) to mature ECs. LDGs also display significant increases in mitochondrial ROS production. The oxidation of mitochondrial DNA (mtDNA), mediated by ROS, allows the migration of mtDNA to the cellular surface, thereby triggering potent pro-inflammatory and interferogenic responses [110]. Vitamin D regulates several important immune functions and its deficiency has been linked to premature atherosclerosis [111]. Vitamin D deficiency is common in patients with SLE and is associated with increased disease activity. Lower vitamin D is associated with increased aortic stiffness in patients with SLE. Increased disease activity may mediate the association between vitamin D deficiency and vascular stiffness [112].

T cells can be found in atherosclerotic plaques both in humans and mice, making up approximately 10% of the cells in the lesion [113]. Th cells form a main branch of the

inflammatory response in the pathogenesis of autoimmune diseases. T cells have a significant role in SLE pathogenesis. Overall, SLE T cells have been shown to be hyperactive, with a reduced threshold of activation [114]. Upon activation, these cells secrete inflammatory cytokines such as IFN-γ and IL-17. In addition, SLE T cells exhibit increased survival and are resistant to apoptosis [115]. These properties of SLE T cells contribute to disease pathogenesis and result in the activation of DCs and B cells [116].

There are different effects of each of T cells on atherosclerosis. The $CD4^+$ T cells can be classified into Th1, Th2 and Th17, and Treg cells based on the distinct cytokine repertoire. Each subset plays specific roles in atherosclerosis. The Ags (oxLDL, HSPs, phosphoryl choline, apoptotic bodies) may induce Th1, Th2, or both Th1 and Th2 pathogenic responses. The imbalance of Th subsets, including Th1, Th2, and Th17, is suggested to contribute to the development of SLE. Schulte et al. demonstrated that mice skewed more towards a Th1 phenotype dislpay increased atherosclerosis, while a bias towards a Th2 phenotype resulted in lower atherosclerosis [117].

The majority of T cells in atherosclerotic lesions, $CD4^+$ T cells with a Th1 phenotype are the primary T cell subset. The CD40/CD40L pathway is critical for Th1 differentiation. Th1cells are the main producers of IFN-γ, upon activation by plaque Ags. IFN-γ secretion leads to a further skewed Th1 response as it polarizes T cells toward the Th1 phenotype. The Th1 bias promotes atherosclerosis in both humans and mice [118].

A plethora of cytokines, including IFNs, has been shown to be important atherogenic regulators. Already in 1989, Hansson et al. [119] observed IFN-γ and IFN-γ–producing T cells to be abundant in human atherosclerotic plaques. IFNs are generally pro-atherosclerotic and have a complex role in different stages of atherosclerosis.

The pro-inflammatory cytokine IFN-γ is secreted by Th1. IFN-γ plays a role in cells recruitment to the atherosclerotic lesion. IFN-γ can subsequently activate macrophages resulting in the secretion of pro-inflammatory mediators such as IL-12 and IL-18. IFNs are also important regulators of macrophage migration toward the atherosclerotic plaque and under the influence of IFN-γ. Antigen presentation is enhanced by up-regulating both major histocompatibility complex class (MHC) I and class II molecules expression on activated macrophages and the induction of MHC II expression on mature DCs. This results in a second wave of T cell activation. IFN-γ is known to induce the expression of ECs adhesion molecules crucial for leukocyte recruitment to the plaque. Their expression can be further intensified by a cross talk between IFN-γ and lipopolysaccharide signalling

in atherosclerosis. IFN-γ also induces the expression of several chemokines, such as CXCL9, CXCL10, and CCL2, thereby further promoting cell recruitment toward the atherosclerotic plaque. Taken together these evidences demonstrate that IFN-γ can promote leukocyte recruitment to atherosclerotic lesions and promotes foam cell formation by increasing the expression of the scavenger receptor for phosphatidylserine and oxLDL. Lupus T cells synthesize significantly larger amounts of IFN-γ [120], one of the most important over-expressed cytokine in SLE, which up-regulates several pro-atherogenic processes such as the production of lipid mediators, platelet activating factors and eicosanoids, antigen presentation, and synthesis of TNF-α and IL-1 [121].

IFN-γ also inhibits collagen I and III production in VSMCs, destabilizing the plaque by reducing extracellular matrix production and inhibiting SMCs and ECs thereby increasing plaque vulnerability [122,123]. T1 IFN cytokine family is represented primarily by IFN-α and IFN-β, prototypical Th1 cytokine promotes, mainly produced by pDCs have also been found in atherosclerotic plaques. The activation of TLR7 and 9 is responsible for the up-regulation of IFN-α expression [124], which has been established as an atherogenic cytokine, and high levels of IFN-α production are induced by TLR 9-pDCs interaction. In subjects with SLE, pDCs induce the expansion of $CXCR3^+$ $CD4^+$ T cells and their migration from the bloodstream into the arterial wall, in which they may play pathogenic roles [125]. There is a chronic exposure to T1 IFN in SLE, which can promote and sustain chronic endothelial dysfunction, eventually leading to atherosclerosis. T1 IFNs may play a critical role in lupus CVD pathogenesis and the pro-atherogenic effect of IFN-α plays an important role in the development of premature atherosclerosis of SLE patient. The effect of IFN-α is supported by mice experiments, where a correlation among the depletion, dysfunction of EPCs and excessive T1 IFN levels was found [126]. An imbalance between ECs damage and repair develops as a result of alterations in EPCs and myelomonocyte circulating angiogenic cells (CACs) which are mediated by IFN-α. Furthermore, IFN-α is also responsible for plaque instability, as it inhibits the growth of SMCs, ECs, and the production of collagen trough mechanisms that are not fully understood. IFN-α also promotes atherosclerosis by various mechanisms by affecting adhesion and migration of leucocytes to plaques and by promoting plaque rupture [123].

Apoptosis is a common phenomenon in macrophage-rich areas of atherosclerotic lesions. Defective clearance of apoptotic cells, or efferocytosis, results in the formation of an inflammatory necrotic core, which makes plaques prone to rupture. Both T1 IFN and T2 IFN are implicated in the regulation of apoptosis.

Another T cell subset with a potential role in SLE-accelerated atherosclerosis is the Th17 subset. Th17 cells constitute a distinct lineage of Th cells characterized by the expression of transcription factor RAR-related orphan nuclear receptor (ROR) γt and by the production of pro-inflammatory cytokines [127]. IL-17 is known to play a role in both lupus and atherosclerosis.

Other Th17-related cytokines include interleukin IL-21, IL-22 and IL-6 as well as soluble CD40 ligand (sCD40L), which can be produced by Th17 cells and is involved in the development and differentiation of Th17 cells. Studies reported that serum levels of IL-21, IL-17A, IL-6, and sCD40L of SLE patients are all higher than the respective serum levels in healthy controls [128]. The levels of IL-17A are significantly increased and correlate with disease activity [129], tending to be higher in individuals with active nephritis [41].

There are complex interactions between Th17-related cytokines, it is reported that IL-21 can induce expansion of IL-17A-producing Th17 cells through initiating an alternative pathway [130]. However, other studies showed that IL-21 suppresses the production of IL-17 [131]. IL-17A can be produced by neutrophils when stimulated by IL-6 [132]. Moreover, IL-6 can enhance the cellular expression of IL-21R, IL-21, and activate STAT3, a main regulator of IL-21 and IL-6 signaling [133]. IL-6 is a key cytokine that inhibits Foxp3 expression during Treg cell differentiation [134]; increased production of IL-6 has been documented in SLE patients [134,135]. Thus, oxidative stress, which induces increased production of IL-6, may be the primary reason for the decrease in Treg cells in SLE patients. IL-6 is a potent cytokine produced by innate and adaptive cells. It stimulates B cell growth and B and T cell differentiation. The pro-atherogenic functions of CD40 ligation include augmented expression of MMPs, procoagulant tissue factor (TF), chemokines, and cytokines [136]. Th17 cells are present in atherosclerotic plaques of both mice and humans [137,138]. IL-17A also promotes plaque development in animal models of atherosclerosis [139,140]. The role of IL-17A in atherosclerosis development in SLE remains unknown, but LDLR knock-out mice receiving $CD4^{+}$T cells from lupus-prone mice develop accelerated atherosclerosis caused by an imbalance between IL-17 production and Tregs function. As the development of Th17 cells shares many similarities with Tregs' one and they are reciprocally regulated, the imbalance between these two cell subsets is a major immune deregulation occurring in SLE [141]. It has been suggested that a Tregs/Th17 imbalance could lead to a putative escape of autoreactive Th17 cells from Tregs control, thus facilitating autoimmunization. sCD40L, which is shed from activated platelets or T cells, is a marker of autoimmune diseases and platelet activation [142]. The

plasma concentration of sCD40L is increased in SLE patients, correlates with T cell activation, Th17/Treg cells balance and with the co-stimulatory pathways of humoral immunity via persistent chronic activation of B cells [143].

There is another subset of Th17 cells, named Th17/Th1 cells, that also secrete IFN-γ. Interestingly, Th17/Th1 and Th1 cells appeared more pathogenic than Th17 cells in an experimental mouse model. The ratio of Th17/Th1 appears to be dysregulated in SLE patients, with an increase in Th17 in SLE [144]. In addition, the ratio of Tregs/Th17 is reduced in SLE patients [145]. Given their potentially pathogenic role in atherosclerosis, the fact that Th17 cells are increased in SLE and that Tregs are reduced suggests that the dysregulation of these cell types may be a factor contributing to accelerated atherosclerosis in SLE. The significant activation of Th1 and Th17/Th1 cells in conjunction with a reduced Tregs population was observed in patients with ACS, suggesting a potential role of Th1, Th17/Th1 and Treg cells imbalance in the development of atherosclerotic plaque instability [146].

IL-21 can influence the immune response in many ways. This cytokine can be produced by follicular helper T (Tfh) cells, Th17 cells, or natural killer (NK) cells. IL-21 is an important pleiotropic cytokine, promotes Th17 immune responses acting as a key modulator of development, expansion, and differentiation of lymphocytes. It can also modulate the function of DCs, plasma cells, and NK cells, and provide instructional cues for maturation of B cells and germinal center formation [147]. IL-21 has an association with the increasing level of auto-Abs of IgG type, and the intracellular expression of granzyme B in B cells, leading to immune injury and apoptosis in SLE cases [148]. The genome-wide association studies on the IL-21 gene identified the association between polymorphisms in IL-21 gene and susceptibility to autoimmune diseases such as SLE, RA, and multiple sclerosis [149]. The Genetic studies that have accumulated over the past 10 years have supported that the IL-21 gene, located on chromosome 4q26–q27, has a role in the pathogenesis of SLE [150].

The role of B cells in lupus pathogenesis is more complex. In autoimmunity, B cells are hyperactivated and can produce pathogenic auto-Abs against nuclear elements. In addition to auto-Ab production, SLE B cell dysregulation can lead to T cell activation, DCs recruitment, induction of Th1 and Th17 cells and inhibition of Tregs, solidifying B cells as a major player in SLE pathogenesis [151]. Abs are produced by plasma cells, that represent the end point of B cell differentiation. There are two pathways for B cell

activation and antibody production: A T cell independent (TI) and T cell dependent (TD) one. Most studies indicate that TD antibody production is the main culprit [152]. In SLE, T cells display abnormal phenotypes, an altered activation threshold, and triggering of signalling pathways that lead to an increased activation and expression of co-stimulatory molecules such as CD40/CD40L [136]. T cell expression of CD40L is important in B cell differentiation, proliferation, Ig class switching, and antibody production via CD40 interactions on the B cell [143]. Increased CD40L/CD40 interactions lead to enhanced B cell activity and increased production of disease-promoting auto-Abs [153]. CD40L signalling is also important for atherosclerosis development, and inhibition of CD40L/CD40 interactions results in a more stable plaque phenotype. B cell subpopulations and distinct Abs have complex and opposing effects on atherosclerosis. B cells mainly contribute to spontaneous or TI antibody production of IgM or IgA class. B1 cells seem to bear an atheroprotective role through IgM antibody production which promotes reduction in necrotic core in atherosclerotic lesions. B2 cells are considered proatherogenic and appear to worsen atherosclerosis through the production of pathogenic IgG Abs against oxLDL epitopes and the promotion of an atherogenic cytokine profile [104]. SLE presents a suitable environment for LDL oxidation and creation of immunogenic oxLDL. This phenomenon coupled with aberrant B cell function in SLE may promote immune reaction skewed towards more prominent B2 cell and/or irregular B1 cell activation, as evidenced by the relative shortage of protective Abs in lupus patients resulting in an accelerated form of atherosclerosis development.

Accelerated atherosclerosis in SLE may be also related with the presence of aPL Abs, known to increase the risk of thrombosis in SLE through several mechanisms. aPL Abs interact with ECs and monocytes inducing a pro-inflammatory and pro-coagulant phenotype [154] and activating the complement cascade, that generates C5a which in turn activates neutrophils and the expression of TF responsible for the initiating of the extrinsic coagulation cascade [155]. Specific auto-Abs against phospholipids also induce the expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin [156].

Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by vascular thrombosis and/or pregnancy morbidity associated with the persistent presence of aPL Abs, which are found in all patients with APS and in 20–30% of patients with SLE [65], but only 1/3 of them develop APS clinical features, namely, venous thrombosis, arterial thrombosis, recurrent pregnancy loss, thrombocytopenia and haemolytic anaemia, livedo reticularis, and skin ulcers [157].

Among SLE patients with a positive lupus anticoagulant (LA), 50% will develop a venous thrombosis over a period of 20 years [158]. In 85% of SLE patients, auto-Abs including aPL ones can be detected up to 10 years before diagnosis in [159].

APS is considered a rare disease, with an estimated incidence of five cases per 100,000 people per year, that affects young adults of reproductive age, that adds significant morbidity to the affected individuals. In addition to physical sequelae, caused by thrombotic events, there is still considerable emotional repercussion from recurrent gestational losses.

aPL Abs is the most common cause of acquired hypercoagulability and were associated with accelerated atherosclerosis to explain cardiovascular manifestations of the syndrome.

Although APS and SLE differ in their clinical manifestations, there are significant overlaps in individual clinical features.

The syndrome is classified into primary (PAPS) when occurring in patient without known autoimmune disease or secondary (SAPS) associated with autoimmune diseases, particularly with SLE (SLE/APS).

The classification criteria for APS were developed in 1999 in Sapporo, and subsequently revised in 2006 at an international congress held in Sydney. At present, they comprise the requirement of at least one clinical criterion (thrombotic event or gestational morbidity) and at least one laboratory criterion (confirmed positive aPL Abs at two or more separate time points with a 12-week minimum interval), the laboratory criteria include three aPL Abs assays, one based on coagulation tests to reveal the presence of LA and two solid phase assays to detect IgG/IgM Abs targeting cardiolipin (CL)/β2GPI complexes or β2GPI alone (**Table 2**).

Two positive Ab measurements, separated by a minimum interval of 12 weeks, should be obtained to rule out the possibility of a transient positivity of aPL Abs induced by infections or drugs [160].

Clinical criteria	
Vascular thrombosis	One or more episodes of arterial, venous or small vessel thrombosis in any tissue or organ (confirmed by objective validated criteria (imaging study or histopathology)
Pregnancy complication	- One or more unexplained deaths of morphologically normal fetus≥10^{th} gestational week -One or more premature birth (≤34^{th} gestational week) of a morphologically neonate because of eclampsia, severe pre-eclampsia or placental insufficiency -Three or more unexplained consecutive spontaneous abortions ≤9^{th} gestational week (maternal anatomic and hormonal abnormalities and chromosomal abnormalities excluded)
Laboratory criteria	
Lupus anticoagulant present in plasma	Detected according to the guidelines of the international society of thrombosis and haemostasis (scientific subcommittee on lupus anticoagulant/phospholipid-dependent antibodies)
IgG and/or IgM anti-cardiolipin antibodies in serum or plasma	At medium/high titer (≥40 GPL or MPL or ≥99^{th} percentile) measured by standard ELISA
IgG and/ or IgM anti-β glycoprotein I antibodies in serum or plasma	Titer ≥99^{th} percentile measured by standard ELISA, according to recommended procedures

Table 2. Revised classification criteria for APS [Pavord, 2018]

According to the revised classification criteria, APS patients should be divided into four categories: category I includes patients with more than one positive test in any combination, while patients with a single positive test should be classified in category II (IIa if LA-positive, IIb if positive for Abs against CL (aCL), IIc if positive for anti-β2GPI Abs) [160].

Subjects positive for LA, high titers of aCL, and anti-β2GPI Abs (above the 99^{th} percentile) defined as "triple positives", more than any other aPL Abs profile, have high risks for thrombosis and pregnancy morbidity. The risk of recurrent thrombosis in triple-positive patients was around 30% over a 6-year follow-up period. Triple-positive aPL Abs patients usually have high titers of Abs to the major β2GPI epitope on domain I [161]. Thus, anti-domain I β2GPI auto-Abs, which frequently present in triple aPL-positive patients, confer LA activity, associate with the highest risk of thrombosis, predispose to both thrombosis and pregnancy loss [162].

The presence of these Abs is essential for the diagnosis and likely to play a pathogenic role

in various disease manifestations. The study of aPL Abs began in 1906 when Wasserman introduced his serological test for syphilis [163]. In 1941, the active component was found to be a phospholipid, which was called CL [164]. After the 1950s, it became clear that people with positive Wasserman-test did not necessarily have syphilis but that they may have had an autoimmune disorder instead, including SLE [165]. LA is the most specific test for the diagnosis of APS and should be performed according to the International Society for Thrombosis and Haemostasis; these are functional investigations and comprise three stages. aCL Abs is the most sensitive test for APS diagnosis. It is performed by a standardized enzyme immunoassay (ELISA) technique containing a source of β2GPI. Internationally approved positive controls are used so that this test has less variability and meets international standards. This test is not influenced by warfarin and heparins: aCL titers are divided into low (< 40 GPL or MPL), moderate (40 to 80 GPL or MPL) or high (> 80 GPL or MPL). Anti-β2GPI Abs are also used and detected by ELISA, and have recently been included in the classification criteria. There is still no standardization for this methodology [166].

aPL Abs recognize a number of anionic negatively charged phospholipids, including CL, LA, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). Neutrally charged auto-antigen targets include phosphatidyl ethanolamine, phosphatidyl choline, platelet activating factor and sphingomyelin. These Abs are usually detected with radioimmunoassay and ELISA. CL remains the most commonly used antigen for detecting aPL Abs by ELISA. aPL Abs bind to phospholipids and plasma or membrane proteins expressed in various cells (platelets, ECs, monocytes, fibroblasts and trophoblasts), producing a prothrombotic state. Many pathogenic Abs in APS do not target phospholipids themselves, but rather phospholipid-binding protein cofactors. In APS, the auto-Abs primarily recognize phospholipid-binding proteins, such as β2GPI and prothrombin. aPL Abs play a major pathogenic role in inducing clinical manifestations; however, there is growing evidence that inflammatory stimuli are pivotal for triggering thrombosis, while tissue distribution of β2GPI the major antigenic target as well as its post-transcriptional modifications or the fine epitope specificity of anti-β2GPI Abs may influence the type of clinical events or even their occurrence. Both APS and SLE are characterized by the production of high levels of IgG class-switched auto-Abs, consistent with a Th cell response. The primary target in both APS and SLE is β2GPI.The anti-β2GPI Abs are the most thrombogenic and may exert a pathogenetic potential either as a strong procoagulant factor in the cerebral circulation or by directly interacting with

neuronal tissue [167].

The mechanistic schema is that anti-β2GPI Abs potentiate thrombosis by engaging β2GPI protein that has been recruited to cell surfaces and thereby promoting cell activation. There are specific roles suggested for cell surface proteins such as annexin A2, apolipoprotein E receptor 2 (ApoER2), TLR2 and TLR4.

ApoER2 (also known as LDL receptor-related protein 8) is a receptor for β2GPI (and consequently β2GPI-dependent aPL Abs) on monocytes, ECs, and platelets. Indeed, in a 2011 study, Ramesh and colleagues demonstrated that ApoER2$^{-/-}$ mice are relatively resistant to thrombosis when confronted with aPL. More recently, it has been revealed that ApoER2 may play an important role in obstetric APS. Specifically, Ulrich and colleagues demonstrated enhanced placental trophoblast cell proliferation and migration *in vitro* when aPL Abs engage β2GPI/ApoER2 complexes on the trophoblast cell surface. Extending these studies to an *in vivo* model of aPL Abs-mediated pregnancy loss, they demonstrated protection in ApoER2$^{-/-}$ mice [168]. In another recent study, Mineo and colleagues developed a monoclonal Ab against β2GPI that prevents pathogenic aPL Abs binding, thereby protecting against aPL Abs-mediated cell activation. This Ab attenuated the association of β2GPI with ApoER2, thereby normalizing endothelial and trophoblast cell function *in vitro*, as well as preventing thrombosis and fetal loss *in vivo* [169]. Although further study is clearly needed, the intersection of aPL Abs, β2GPI, and ApoER2 warrants further investigation as a potential therapeutic target inpatients.

Among all SLE patients, approximately 40% of the aPL Abs-positive patients develop arterial and/or venous thrombosis, in comparison with 10%-20% of aPL Abs-negative patients ($p<0.001$) [170]. aPL Abs increase the risk of vascular events and death in SLE [171], but their role in the development of atherosclerosis in humans remains controversial. aPL Abs cross-react with Abs against oxLDL and may enhance its uptake by macrophages, which represents the initial step of atherosclerotic plaque formation. aPL Abs can also cross-react with HDL-complex (HDL-C) and apolipoprotein A-I (Apo A-I), a major constituent of the HDL-C, possibly reducing the antiatherogenic effects of HDL. Multiple molecules, essential in the haemostatic system, and acting as key players in thrombosis and atherosclerosis development, are altered in APS immune and vascular cells by the effect of aPL Abs, including TF, the vascular endothelial growth factor (VEGF) VEGF/Flt1 axis, several TLRs, annexins, protein disulfide isomerase. aPL Abs are a clinically important acquired risk factor for thrombosis and pregnancy loss and are thought to have a direct

prothrombotic effect *in vivo*. Data suggest that a major mechanism by which aPL Abs contribute to thrombophilia is the up regulation of TF on blood cells and vascular endothelium. TF is the physiological trigger of normal blood coagulation and thrombosis in many hypercoagulable conditions. TF was firstly described by Cuadrado et al. to be significantly elevated in monocytes from APS patients with a previous history of thrombosis and related to the presence of high tire of aPL Abs [172]. aPL Abs induce TF expression in monocytes in APS patients by activating simultaneously and independently, the phosphorylation of MEK1/ERK proteins, and the p38 MAP kinase-dependent nuclear translocation and activation of NF-kappaB/Rel proteins, with up-regulation of IL-6, IL-8 and induced nitric oxide synthase (iNOS). TF signalling activities are mainly mediated by the protease activators receptors (PARs), major mediators of thrombosis and haemostasis and inflammatory process. A study [173] demonstrated a correlation between PAR-2 expression level and IgG aPL titers. Previous reports indicated a close relationship between TF and VEGF, protein involved in normal vascular development, as well pathogenesis related to inflammation and cardiovascular disease [174]. VEGF can be considered a regulatory factor in aPL Abs-mediated monocyte activation and TF expression, contributing to the proinflammatory-prothrombotic status of APS.

TLR2, and TLR4 are membrane receptors known for their role in the activation of immune and ECs, pathogen recognition and cytokines production. TLR pathway activated in APS patients, in which PBMCs show a significant increase in the gene expression of TLR-2 and TLR-4 that mediate aPL Abs-induced vascular abnormalities.

aPL Abs can also activate platelets, promoting its aggregation, thus contributing to the development of thrombosis. There are other potentially significant antigenic targets for aPL Abs, which include prothrombin, tissue plasminogen activator (tPA), PS, plasmin, annexin 2, activated protein (APC), thrombin, antithrombin III (AT-III), and annexin V.

aPL Abs are associated to the development of atherosclerosis, and a direct correlation between serum levels of aCL and anti-β2GPI Abs and the occurrence of coronary syndrome myocardial infarction and stroke *in vivo* has been demonstrated [175].

A recent study found a strong association among higher aPL-IgG titers and development of thrombotic events, as well as with the presence of early atherosclerosis, as evidenced by an increased IMT [176]. Several studies confirmed the involvement of aPL Abs in the formation of the atheroma plaque *in vitro*, through the activation of ECs and leukocytes, and the induction of foam cell generation, by facilitating the adsorption of oxLDL by monocytes [177].

β2-glycoprotein I

β2GPI is major antigenic target of aPL Abs in both APS and SLE. It is a lipid-binding protein, 50 kDa β2 globulin involved in the regulation of blood coagulation [178] present in high levels, with largely unknown endogenous functions.

β2GPI possesses several properties that may bear relevance to progression of human atherosclerotic plaques; (1) it binds activated platelets and apoptotic cells upon exposure of inner-membrane PS; (2) it inhibits intrinsic blood coagulation pathway and ADP dependent platelet aggregation; (3) it serves as a key element in the activation of ECs induced by aPL Abs; and (4) it may cooperate in mediating the clearance of senescent cells and foreign particles from circulation [179].

β2GPI has good immunogenic properties, APS patient can produce Abs against several epitopes of the molecule. The presence of auto-Abs against β2GPI was described for the first time in 1990, when 3 different groups identified these Abs as an important subpopulation of auto-Abs in patients with APS [180-182].

The plasma glycoprotein β2GPI consists of 326 amino acids, arranged in 5 highly homologous complement control protein domains, designated domain I to V from the N-to the C-terminus. The first 4 domains consist of approximately 60 amino acids each, whereas the 5^{th} domain is larger due to a 6-residue insertion and a 19-residue C-terminal extension that constitute a phospholipid binding loop [183]. The domain V, which resembles a "hook", interacts with the PL in the cell bilayer; at the opposite end, domain I, is recognized by most clinically relevant Abs in APS [184].

Depending on the redox state of the extracellular milieu, domains I and V expose different epitope surfaces for antibody binding (**Fig. 3-4**). A tight interaction between domains I and V, which defines the circular form of β2GPI *in vivo*, shields various epitopes on domain I. The dissociation between the two domains gives rise to the linear, fishhook-like structure of β2GPI in which domain I epitopes are exposed [185]. Cysteine residues at positions 288 and 326 of domain V, which either remain as free thiols or form a disulphide bond, control the conversion between the two alternative *in vivo* conformations. In the plasma of healthy individuals, β2GPI occurs in the free thiol form, which folds into a ring configuration and blocks antibody access to the principal domain I epitopes [186].

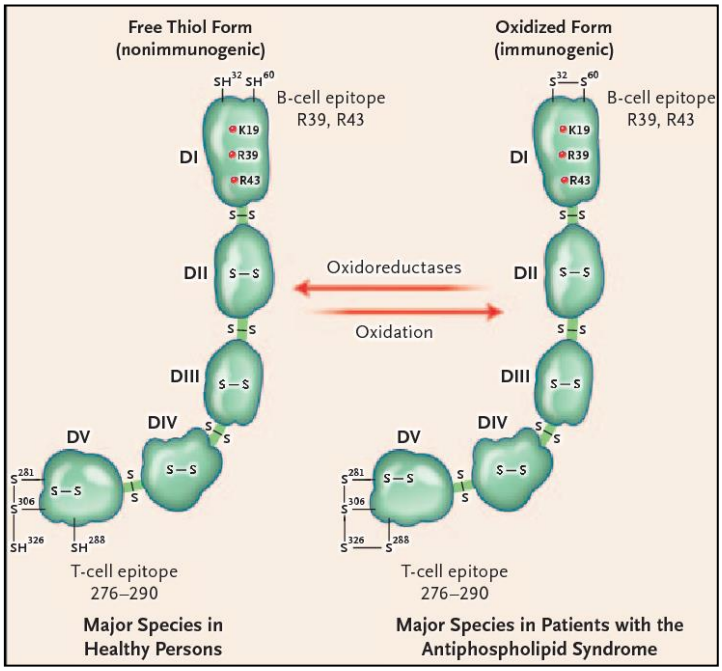

Figure 3. Schematic Representation of the Crystal Structure (Fishhook Configuration) of β2-Glycoprotein I (β2GPI) [Giannakopoulos, 2013].

In the plasma of healthy individuals, β2GPI occurs in the free thiol form, which folds into a ring configuration and blocks antibody access to the principal domain I epitopes [186].

Oxidative stress unfolds the ring conformation of β2GPI, exposing the normally shielded antigenic determinants of domain I, which are epitopes for pathogenic Abs binding [185]. This form inserts with domain V into the cell bilayer of anionic PL.

The presence of Abs alone does not cause thromboembolic manifestations, the current view is that a "second hit" is necessary to determine a thrombotic event [187].

In its circulating form, β2GPI is not recognized by pathologic anti-β2GPI Abs. Its recognition requires the conformational change that occurs either when it binds to negatively charged phospholipids exposed on a cell membrane, or because of oxidative stress [185]. Under these conditions, a cryptic epitope in the domain I of the molecule is exposed and recognized by these Abs. β2GPI is stabilized in this conformation, and the Abs-β2GPI complex can subsequently interact with several cellular surface receptors determining both vascular and obstetric events [187,188].

Raimondo et al. determined a strong positive correlation between IgG anti-domain I and

the proportion of oxidized β2GPI (oxβ2GPI), but not with IgM or IgA antidomain I [189]. This observation suggests that either anti-domain I IgG stabilizes the extended form of oxβ2GPI or that chronic inflammatory conditions lead to an abundance of oxβ2GPI that stimulates the production of anti-domain I IgG.

β2GPI is present in atherosclerotic plaques from carotid arteries [179]. There is a direct binding of β2GPI to ECs and the activation of inflammatory receptors on these cells.

The direct binding of β2GPI to ECs, a TLR4-mediated process, directly activates endothelia. Similarly, Laplante et al. [190] in a carotid artery injury model showed that anti-β2GPI activation of ECs is dependent on TLR4. The binding of β2GPI to TLR4 is enhanced by LPS and may reflect a possible scavenging of LPS. Current evidence indicates that autoimmunity can be detected within atherosclerotic lesions.

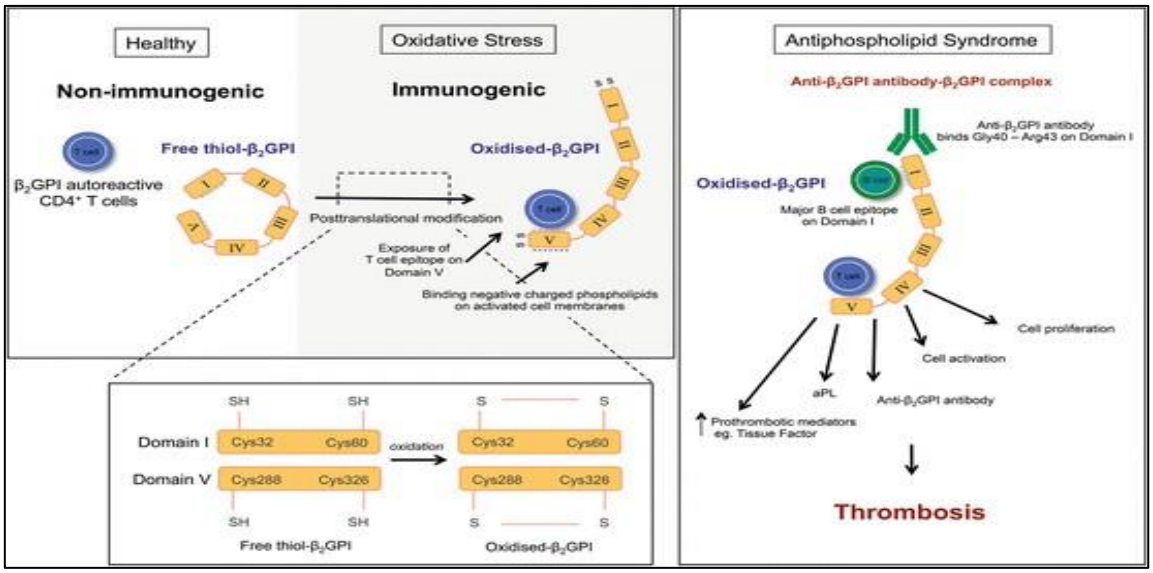

Figure 4. How oxidized β2GPI participates in the formation of thrombotic APS. During oxidative stress, free thiol β2GPI can undergo post-translational modification to form the immunogenic form, oxidized β2GPI after binding phospholipids. β2GPI autoreactive CD4+ T cells recognize newly exposed epitopes located on Domain V but not on free thiol β2GPI. A complex is formed between aβ2GPI, autoreactive CD4+ T cells and oxidized β2GPI triggering the production of aPL, specifically aβ2GPI, cell proliferation and the release of pro-inflammatory cytokines which are key events in the pathophysiology of thrombotic APS [P.G. de Groot, 2017].

A previous work of our group has examined the role of Th1 specific inflammatory responses to β2GPI in established atherosclerotic lesions of primary APS patients. Their work demonstrated that plaque-derived, β2GPI-specific $CD4^+$ T lymphocytes facilitate

perforin and Fas/Fas ligand-mediated cytotoxicity, pointing at a role for these auto-reactive T cells in plaque destabilization and potential athero-thrombotic events that are known to occur at a higher frequency in APS. They also demonstrated that β2GPI can induce proliferation and IFN-γ production by plaque-derived T cell clones. Furthermore, these T cells amplify monocyte responses, such as the production of TF and MMPs, which can be inhibited by an anti-IFN-γ antibody [191].

Pathogenic SLE immune response and the development of atherosclerotic plaques share some characteristics due to skewed T cell activation. aPL increase the risk of vascular events and death in SLE [171].

It has been hypothesized that the development of an anti-β2GPI-specific response in the target organ may contribute to atherothrombosis in SLE-APS patients. This hypothesis is largely based on the presence of β2GPI in human atherosclerotic plaques [179] and on the enhanced fatty streak formation in transgenic atherosclerosis-prone mice immunized with β2GPI [192,193]. Moreover, β2GPI-reactive T cells also promote early atherosclerosis in LDLR–deficient mice [194].

β2GPI is a target auto-Ag in both SLE and APS with good immune properties and seems to play a pathogenic role in atherosclerosis.

β2GPI is able to induce events leading to the stimulation of a specific T cell immune response in the atherosclerotic lesions of patients with SLE-APS or triple positive aPL Abs. The loss of local tolerance in atherosclerotic plaques and the increased plaque inflammatory process are important risk factors for clinical events.

The potential therapeutic strategies in SLE that target the immune system, and their impact on atherosclerosis and CVD: Novel immune-based therapies for CVD

CVD is an important complication of SLE, and effective preventive measures remain to be identified. The suggested novel target therapies are based on the pathogenic immune responses in SLE and the development of atherosclerotic plaques (**Fig. 5**), which share some similar characteristics, such as impaired efferocytosis and skewed T cell activation. Impaired efferocytosis, a common link between SLE and atherosclerosis, the loss of tolerance against LDL and other plaque Ags in atherosclerosis shares many characteristics with the loss of tolerance against self-Ags associated with disease development and organ damage in SLE. In particular, an impaired capacity to clear apoptotic cells and necrotic debris has been implicated as an important factor in both diseases [195].

The novel immune-based therapies for CVD are being developed and tested in clinical trial [196], it is likely that some of these may also show efficacy for the treatment of SLE. Novel immune-based therapies for CVD are being developed and may be effective in the prevention of CVD and in immunomodulation. Immunosuppressive drugs may reduce the progression of atherosclerosis and cardiovascular events, yet their exact mechanisms require further elucidation. To date monoclonal Abs like Belimumab have proven efficacy in the treatment of lupus nephritis [197] and several others monoclonal Abs like secukinumab, ixekizumab, and bimekizumab, which are IL-17A pathway blockers, and brodalumab an anti-17RA are approved for some immune-mediated inflammatory diseases [198].

In SLE Th17 cells produce IL-17, IL-21, and IL-22 and are involved in the development of inflammation in various organs. IL-17-producing cells play a crucial role in the disease pathogenesis and represent an attractive therapeutic target. Several IL-17A pathway blockers, there are preclinical and clinical studies that suggest a critical role of IL-23/IL-17 axis in SLE pathogenesis. Further studies, including a phase III study with ustekinumab, are needed to initiate the use of blockers of the IL23/IL-17 pathway in the treatment of SLE patients. Blocking IL-17 and/or Th17 cells might be beneficial in SLE but the effects in atherosclerosis and CVD are more uncertain and need to be carefully investigated [199].

Immunomodulation in atherosclerosis can basically have three targets: enhancing anti-inflammatory mechanisms, inhibiting cholesterol synthesis, and immunization against neo-Ags. In SLE there is an aberrant T cell signalling and there are many pathways involved in the disease and in atherosclerosis development. The possibility of using T1 IFN as a target

for future SLE therapies is currently under investigation. An anti-IFN-α monoclonal antibody was found to be safe, well tolerated, and effective and will be further investigated in clinical trials. Vaccination strategies leading to induction of anti-IFN-α Abs are also under development [200]. As T1 IFN also have a disease-promoting role in atherosclerosis, the blocking of these cytokines may be beneficial in CVD, both in SLE patients and in subjects without SLE.

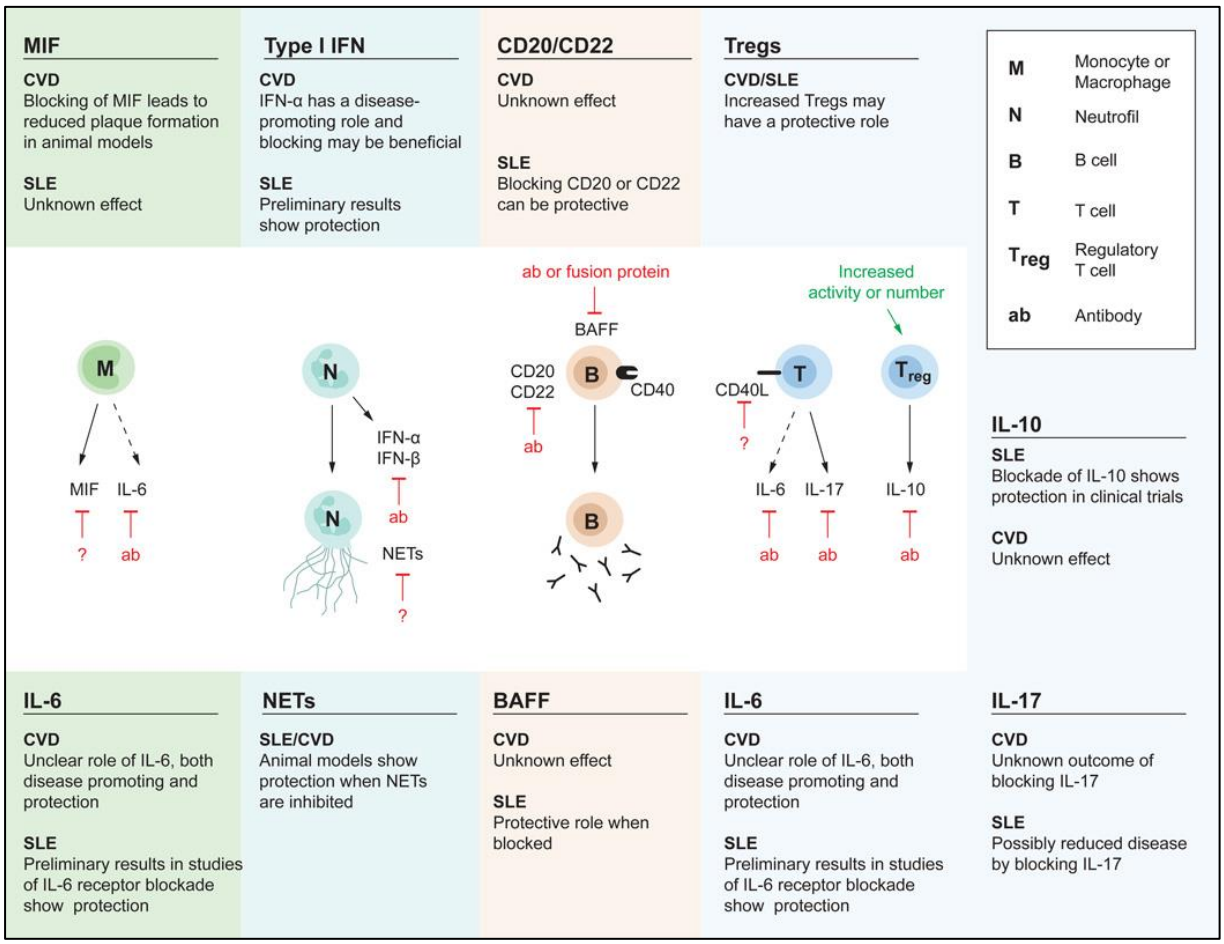

Figure 5. Potential future therapies targeting the immune system in systemic lupus erythematous (SLE) and cardiovascular disease (CVD) [Wigren, 2015].

AIM OF THE STUDY

This study was undertaken to evaluate the type of T cells responses elicited by β2GPI in established atherosclerotic lesions with SLE-APS patients. We investigated the cytokine production induced by β2GPI in activated T cells that infiltrate *in vivo* atherosclerotic lesions of SLE-APS patients. We examined the helper function of β2GPI-specific T cells for the TF production, as well as their cytolytic potential and their helper function for Abs production.

MATERIALS AND METHODS

Patients

Upon approval of the local Ethical Committee, 10 patients (10 females, mean age 51; range 42-56 years) with SLE-APS, 10 aPL Abs negative patients (10 females, mean age 51, range 43-55), 5 SLE aPL Abs positive patients (5 females, mean age 49, range 44-53), and 5 SLE aPL-negative patients (5 females, mean age 50, range 44-56), all affected by carotid atherosclerotic arteriopathy were included in the study. The carotid plaques were obtained by endarterectomy from each patient. The clinical informations of each patient are reported in (**Table 3-6**).

All patients studied (SLE- APS, SLE aPL Abs-positive, SLE aPL Abs-negative and aPL negative patients) were eligible for vascular surgery. SLE-APS patients were triple positive for aPL Abs, with high titters serum anti-β2GPI, aCL Abs and with positivity for LA. All SLE-APS patients in this study satisfied the Myiakis's criteria for APS, and they were on oral anticoagulation with vitamin K antagonists, then switched to low molecular weight heparin few days before surgery [160]. None of them displayed traditional risk factors for atherosclerosis and they were not receiving any anti-lipidemic drugs. All the SLE aPL-positive patients were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, anti-cardiolipin Abs or with positivity for LA. All SLE aPL-neg patients were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti-β2GPI, aCL Abs and with negativity for LA.

Patients	Age	Sex	Treatment	SLEDAI	Autoanti bodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of CD4 clones	No. of $CD4^+$ clones reactive to β2GPI
A	42	F	a+b	7	c+d+e	38	5	0	33	8
B	56	F	a+b	8	c+d+e+f	34	4	0	30	6
C	51	F	b	7	c+d	33	6	0	27	6
D	50	F	b	5	e	31	1	0	30	8
E	50	F	b	8	c+d+e	32	4	0	28	7
F	54	F	a+b	6	c+d+g	34	3	0	31	7
G	55	F	b	7	c+d+h	35	3	0	32	8
H	48	F	a+b	7	c+d+e	30	3	0	27	7
I	49	F	b	8	c+d+e+h	36	4	0	32	8
L	55	F	a+b	6	c+d	31	4	0	27	6

Table 3. **Clinical and lab information of the 10 SLE-APS patients.** All the 10 patients shown in this table were affected by SLE-APS and were triple positive for aPL, with high titers serum anti-β2GPI, aCL and with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

Patients	Age	Sex	Treatment	SLEDAI	Autoanti bodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of CD4 clones	No. of $CD4^+$ clones reactive to β2GPI
M	43	F	none	0	none	39	5	0	36	0
N	55	F	none	0	none	37	4	0	33	0
O	51	F	none	0	none	29	7	0	22	0
P	50	F	none	0	none	28	4	0	24	0
Q	50	F	none	0	none	36	4	0	32	0
R	54	F	none	0	none	31	3	0	28	0
S	55	F	none	0	none	38	5	0	33	0
T	48	F	none	0	none	26	3	0	23	0
U	49	F	none	0	none	34	5	0	27	0
V	55	F	none	0	none	32	2	0	30	0

Table 4. Clinical and lab information of the 10 aPL-neg patients. All the 10 patients shown in this table were affected by carotid atherosclerotic arteriopathy but not by SLE-APS and were triple negative for serum aPL, such as anti-β2GPI, aCL and with negativity for Lupus Anticoagulant, and they were all negative for any autoantibody. They were not treated with any steroids, nor other immune- suppressants.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of$CD8^+$ clones	No.of $CD8^+$ clones reactive to β2GPI	Total No. of CD4 clones	No. of $CD4^+$ clones reactive to β2GPI
Y	44	F	a+ b	6	c+d	32	4	0	28	4
YA	53	F	b	9	c+e	28	3	0	24	3
YB	52	F	b	8	c+d+e	25	5	0	20	5
YC	48	F	a+b	7	c+d	34	3	0	31	6
YD	49	F	a+ b	5	c+d+e+f	38	6	0	32	7

Table 5. Clinical and lab information of the 5 SLE aPL-pos patients. All the 5 patients shown in this table were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, aCL or with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of CD4 clones	No. of $CD4^+$ clones reactive to β2GPI
Z	52	F	b	7	c+d+e	29	5	0	26	0
ZA	49	F	a+b	9	c+e+g	34	4	0	28	0
ZB	51	F	a+b	8	c+e+h	31	7	0	24	0
ZC	44	F	b	6	c+h+g	39	6	0	33	0
ZD	56	F	b	7	c+e+h	33	8	0	25	0

Table 6. Clinical and lab information of the 5 SLE aPL-neg patients. All the 5 patients shown in this table were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti-β2GPI, aCL and with negativity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSBpositive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

Reagents

Human β2GPI was purified by perchloric acid treatment of pooled normal human sera obtained from blood donors followed by affinity purification on Heparin column (HiTrap Heparin HP, GE Healthcare, Milan, Italy) and by ion-exchange chromatography (Resource-S, GE Healthcare) [201].

We also ruled out the presence of contaminants by a limulus test. The human β2GPI used has been tested with a limulus test and resulted negative throughout the whole study. Human recombinant (hr)IL-2 and tetanus toxoid (TT) were provided by Novartis, Siena, Italy. Phytohaemagglutinin (PHA) was purchased from Life Technologies (Carlsbad, CA). Fluorochrome-conjugated human monoclonal antibodies (mAbs) anti-CD3, anti-CD4, anti-CD8, anti-IFN-γ and isotype-matched control mAb were purchased from BD Biosciences (San Jose, CA, USA).The fluorochrome conjugated anti-IL-17 mAb was obtained from eBioscience (San Diego, CA, USA). PMA, ionomycin and brefeldin A were purchased from BD Biosciences (San Jose, CA, USA).

Anti-phospholipid antibody detection

For the detection of aCL and aβ2GPI in patient sera, commercially available solid-phase ELISA employing purified human β2GPI in complex with CL and human β2GPI were used (Inova, Ca, USA).

Sera were considered positive when their concentration exceeded the cut-off of 10 U/mL for IgG and IgM. All samples were tested by the respective in-house assay as Described elsewhere [202].

The results of the two techniques were comparable. Analysis of LA was performed in accordance with the international recommendations as described [203].

Generation and characterization of T cell clones from atherosclerosis plaque' inflammatory infiltrates

Induction of T lymphocyte lines from atherosclerotic plaques in order to preferentially expand *in vivo*- activated T cells *in vitro*. A biopsy specimen of carotid plaques, obtained by endarterectomy, were investigated in both SLE-APS and in aPL negative patients under the same experimental conditions.

Plaque fragments were immediately cultured for 7 days with hrIL-2 (50 U/ml) in RPMI 1640 complete medium (supplemented with L-glutamine 1%, beta-mercaptoethanol 1%, Na-pyruvate 1%, non-essentialaminoacids 1%, penicillin 50.000U and streptomycin 50 mg), heat-inactivated human serum 3%, and fetal bovine serum (FBS)10%. After 7 days of culture the vital T lymphocyte found in medium were used to generate clones.

Specimens were then disrupted, and single T cells blasts were cloned under limiting dilution, (0.3 cells/well) in round-bottom microwell plates containing 10^5 irradiated PBMC (as feeder cells) in final volume in 0.2 ml of medium RPMI complete; added PHA (0.5% v/v), and hrIL-2 (30U/ml), After one week 0.1 ml of medium containing new feeder cells (irradiate PBMCs) (10^5/well) and hrIL-2 (30U/ml) was added to microculture [204,205].

This procedure was repeated at intervals weekly, after three weeks the originated clones were expanded and T cell blasts were used to assess their phenotype profile, T cell clones were screened by flow cytometry with fluorochrome conjugated anti-CD3, anti- CD4, anti-CD8 on a BD FACSCanto II (BD Bioscience), using the FACS Diva 6.1.3.software.

The repertoire of the TCR Vβ chain of β2GPI-specific Th clones was analysed with a panel of mAbs specific to the following: Vβ1, Vβ2, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7, Vβ8,Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2 and Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22,and Vβ23 (Beckman Coulter); Vβ6.7 (Gentaur) and Vβ3.1 (*In Vitro* Gen).

Isotype-matched nonspecific Ig were used as negative control. Vβ10, Vβ15, and Vβ19 T cell receptor typing were investigated by Clontech kit, according to the manufacturer's instructions.

T cell clones were then analyzed for their responsiveness to β2GPI by measuring [^{3}H] thymidine uptake after 60 h of co-culture with irradiated autologous PBMCs in the presence of medium, or β2GPI (10 nM). The mitogenic index (MI) was calculated as the ratio between mean values of counts per minute (cpm) obtained in stimulated cultures and those obtained in the presence of medium alone. MI >5 was considered as positive.

Assessment of T cell clones cytokine profile

To assess the cytokine production of β2GPI-specific T cell clones upon antigen stimulation, 5×10^5 T cell blasts of each clone were co-cultured for 48 h in 0.5 ml of serum-free medium with 5×10^5 irradiated autologous PBMCs in the absence or presence of β2GPI (10 nM). At the end of the culture period, duplicate samples of each supernatant were assayed for their IFN-γ, TNF-α, IL-4, IL-21 and IL- 17 (BioSource International, Camarillo, CA) production by ELISA. For further investigation, T cell blasts from each β2GPI-specific T cell clone were stimulated with medium or β2GPI (10 nM) in the presence of autologous APCs for 48 h in ELISpot microplates coated with anti-IFN-γ or anti-IL-17 antibody, respectively (eBioscience, Inc., San Diego, Ca, USA). At the end of culture period, the number of IFN-γ and IL-17 SFCs were counted by using an automated reader as described [206].

T helper assay to assess their ability to induce TF production and procoagulant activity (PCA) in autologus monocytes. T cell blasts (8×10^5 / ml) of β2GPI-specific T cell clones were co-cultured for 16 hrs with autologous monocytes (4×10^5 / ml) in the presence of serum-free medium or β2GPI (10 nM). At the end of the culture period, the amount of TF protein was quantitated by a specific ELISA (American Diagnostica, Greenwich, CT) in duplicate samples of supernatants obtained from cell suspensions after solubilization of membrane proteins with Triton X-100 and ultracentrifugation.

At the end of culture period, cell suspensions consisting of monocytes alone, or monocytes plus activated T cells were disrupted by repeated freezing and thawing followed by sonication.

Total cellular content of PCA was determined in a one-stage clotting assay and expressed in arbitrary units ($U/10^5$ monocytes) assigned by comparison with a standard curve derived from rabbit brain thromboplastin standard (Manchester Comparative Reagents, Manchester, UK) [206].

Our log-log plot was linear up to 200 seconds clotting time. Values less than 10 $U/10^5$ monocytes corresponded to clotting times ranging from 170 to 80 seconds. One thousand units corresponded to approximately 22 seconds clotting time.

PCA was characterized as factor VII-dependent procoagulant activity by evaluating its sensitivity to phospholipase C (Calbiochem, San Diego, CA), concanavalin A, and cysteine protease inhibitor (HgCI2), and by using factor VII- and factor X-deficient plasma samples [207].

T cell clones' helper assay to evaluate the induction of immunoglobulin (Ig) production by autologous B cell

T cell blasts of each clone were co-cultured at ratios of 0.2, 1, and 5 to 1 with autologous PBMCs in the absence or presence of β2GPI and, on day 10, culture supernatants were harvested and tested for the presence of IgM, IgG, and IgA by ELISA, Results represent the mean value (+/–SE) of Ig levels induced by T cell clones compared to the Ig spontaneous production in B cell cultures alone [208].

Statistical analysis

Statistical analyses were performed using Student's *t* test; data were considered significant if P values > 0.05.

RESULTS

Atherosclerotic lesions of SLE-APS patients and SLE aPL-positive patients harbour autoreactive β2GPI-specific $CD4^+$ T cell clones

Atherosclerotic plaque-infiltrating *in vivo* activated T cells were expanded *in vitro* in an hrIL-2 conditioned medium, subsequently cloned and studied for their phenotypic and functional profile. A total number of 297 $CD4^+$ and 37 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 10 SLE-APS patients. For each patient, $CD4^+$ and $CD8^+$ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium, or β2GPI. None of the $CD8^+$ T cell clones showed proliferation to β2GPI although they proliferated in response to mitogen stimulation (**Fig. 6**).

We have also investigated the amount of β2GPI-specific T cells present in the peripheral blood of SLE-APS patients and compared it with the one found in atheromas. The proportion of β2GPI- specific $CD4^+$ T cell clones generated from atherosclerotic plaques of SLEAPS patients was 24%, which is remarkably higher compared with the frequency of β2GPI-specific T cells found in the peripheral blood of the same patients (between 1:1900 and 1:3400). Each β2GPI-reactive $CD4^+$ T cell clone was stained by only one of the TCR-Vβ chain–specific monoclonal antibodies, showing a single peak of fluorescence intensity (**Fig. 7**). T cell blasts were used to assess their cytokine profile. The analysis was performed using FACS Canto II (BD). Dot plots expression of IFN-γ^+ and IL-17^+ on $CD4^+$ T cells of the 10 T cell lines obtained from the 10 SLE-APS patients (**Fig. 8**).

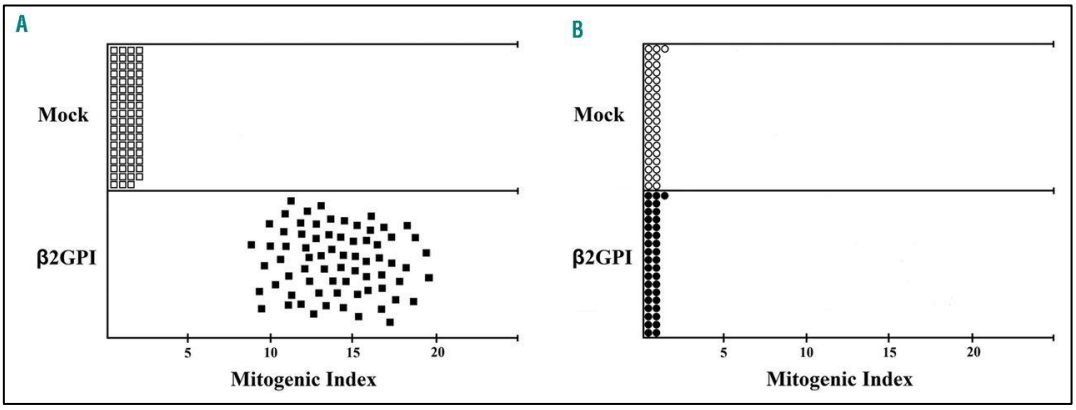

Figure 6. Antigen specificity of atherosclerotic plaque CD4$^+$ T and CD8$^+$ T cell clones obtained from SLE-APS patients. T cell clones were analysed for responsiveness to β2GPI (10 nM) (■), or medium (□) by measuring [^{3}H] Thymidine uptake after 60 h of co-culture with irradiated autologous PBMCs. 71 out of 297 CD4$^+$ T cell clones proliferated in response to β2GPI and are shown in panel **A**. None of the 37 CD8$^+$ T cell clone proliferated to β2GPI (panel **B**).

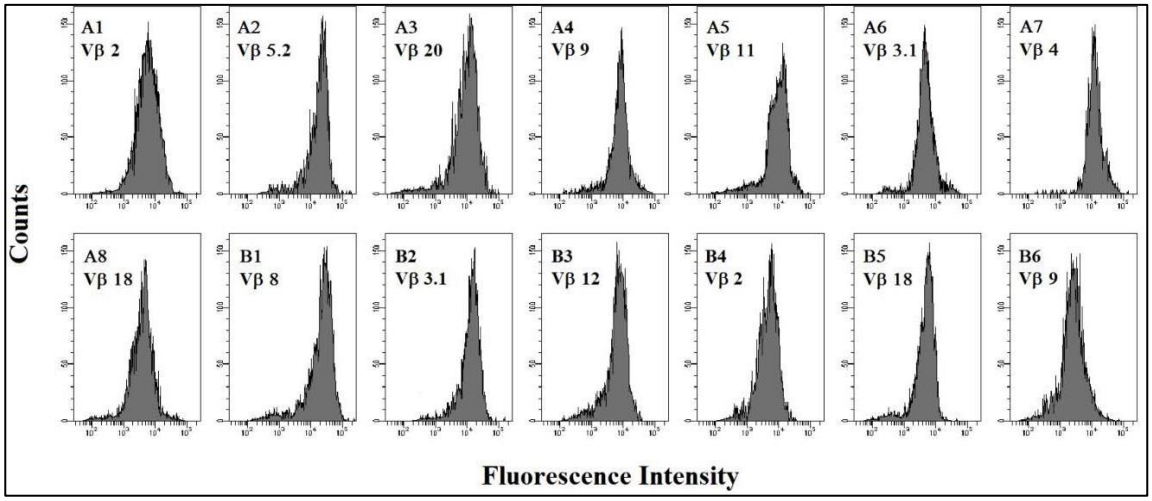

Figure 7. TCR Vβ chain repertoire of β2GPI-specific T cell clones derived from the atherosclerotic plaques of SLE-APS patients. All clones obtained from two representative patients, named "A" and "B", are represented in the figure.

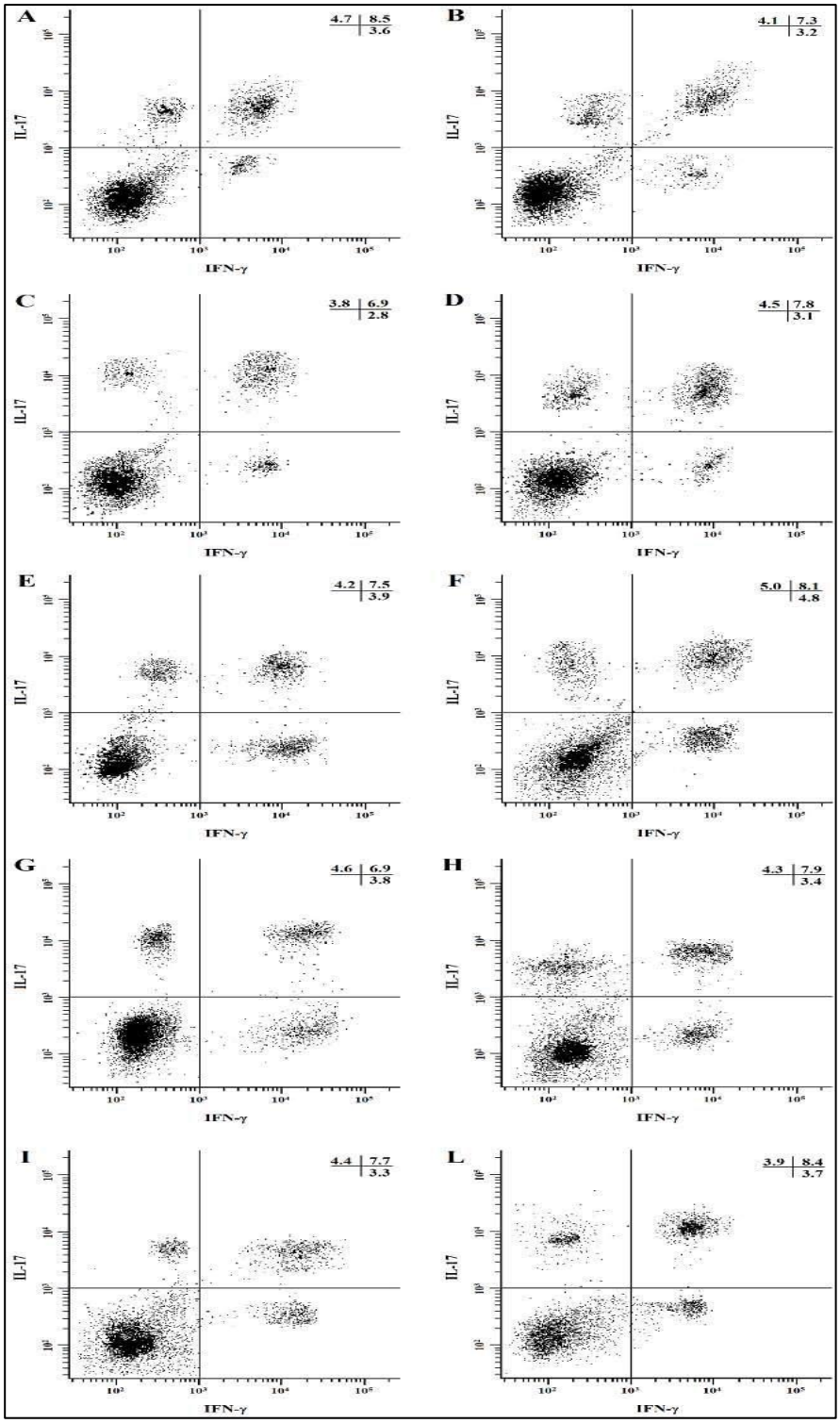

Figure 8. IFN-γ and IL-17 intra-cellular cytokine staining of plaque-infiltrating T cells of SLE -APS patients. Cells were stained for surface and intracellular markers with the following mAbs for flow cytometry: anti-CD4-PerCP, anti-IL-17-PE, and anti-IFN-γ-FITC (Becton Dickinson). Samples obtained from the atherosclerotic lesions of each of the SLE-APS patients were stimulated with PMA (25ng/ml) plus1μγ/ml ionomycinin the presence of brefeldin A (1μg/ml). The analysis was performed using FACS Canto II (BD), by the acquisition software FACS Diva 6.1.3. For each sample 5000 events were acquired. Dot plots expression of IFN-γ+ and IL-17+ on CD4+ T cells of the 10 T cell lines obtained from the 10 SLE-APS patients (named A, B, C, D, E, F, G, H, I, L) are shown.

Seventy-one (24%) of the 297 $CD4^+$ T cell clones generated from SLE-APS atherosclerotic plaque- infiltrating T cells proliferated significantly to β2GPI (**Fig. 6**). Each SLE-APS patient displayed a comparable percentage of $CD4^+$ T cell clones responsive to β2GPI (**Table 3**). On the other hand, a total number of 288 $CD4^+$ and 42 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of10 atherothrombotic patients, that were negative for aPL. For each patient, $CD4^+$ and $CD8^+$atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. None of the $CD4^+$ or $CD8^+$ T cell clones derived from the atherosclerotic lesions showed proliferation to β2GPI (**Table 4**). A total number of 135 $CD4^+$ and 21 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 5 SLE aPL-positive. For each patient, $CD4^+$ and $CD8^+$atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. 25 $CD4^+$ and no $CD8^+$ T cell clones derived from the atherosclerotic lesions of SLE aPL- positive patients showed proliferation to β2GPI (**Table 5**). A total number of 136 $CD4^+$ and 30 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 5 SLE aPL-negative. For each patient, $CD4^+$ and CD8+ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. None of the $CD4^+$ or $CD8^+$ T cell clones derived from the atherosclerotic lesions showed proliferation to β2GPI (**Table 6**).

All β2GPI-specific T cell clones, both those obtained from the atherosclerotic lesions of SLE-APS patients and those obtained from SLE aPL-positive patients, were stimulated with β2GPI and autologous APCs. Then, TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. In unstimulated cultures, level of TNF-α. IL-4, IFN-γ and IL-17 were consistently < 20pg/ml.

Upon antigen stimulation with β2GPI of the 71 β2GPI-specific T cell clones obtained from SLE- APS patients, 30 were polarized Th1 clones ($CD4^+$ T cell clones producing IFN-γ, but not IL-17 nor IL-4 were coded as Th1), 10 Th clones were Th17 ($CD4^+$ T cell clones producing IL-17, but not IFN-γ nor IL-4 were coded as Th17), 27 Th clones were Th17/Th1 ($CD4^+$ T cell clones producing IFN-γ, and IL-17, but not IL-4 were coded as Th17/Th1), and only 4 were able to produce IL-4 together with TNF-α (Th0 clones) (**Fig. 9**). Upon antigen stimulation with β2GPI of the 25 β2GPI-specific T cell clones obtained from SLE aPL positive patients, 10 were polarized Th1 clones, 6 Th clones were polarized Th17, 8 Th clones were Th17/Th1 and only 1 was Th0 (**Fig. 10**).

T cell blasts from each of the 71 β2GPI-reactive T cell clones obtained from atherosclerotic lesions of patients with SLE-APS were further screened by IFN-γ and IL-17 ELISpot in response to β2GPI (**Fig. 11**). Upon appropriate stimulation, IFN-γ SFCs were then counted by using an automated reader. After specific stimulation, 61/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IFN-γ. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels. IL-17 SFCs were then counted by using an automated reader. After specific stimulation 37/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IL-17. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels.
61 atherosclerotic derived atherosclerotic derived $CD4^+$ T cell clones produced IFN-γ, and thirty-seven produced IL-17 (**Fig. 12**). Interestingly, all IL-17-producing β2GPI-reactive T cell clones, produce IL-21 (mean ± SE, 3.3±0.5 ng/ml per 10^6 T cells) in response to antigen stimulation.

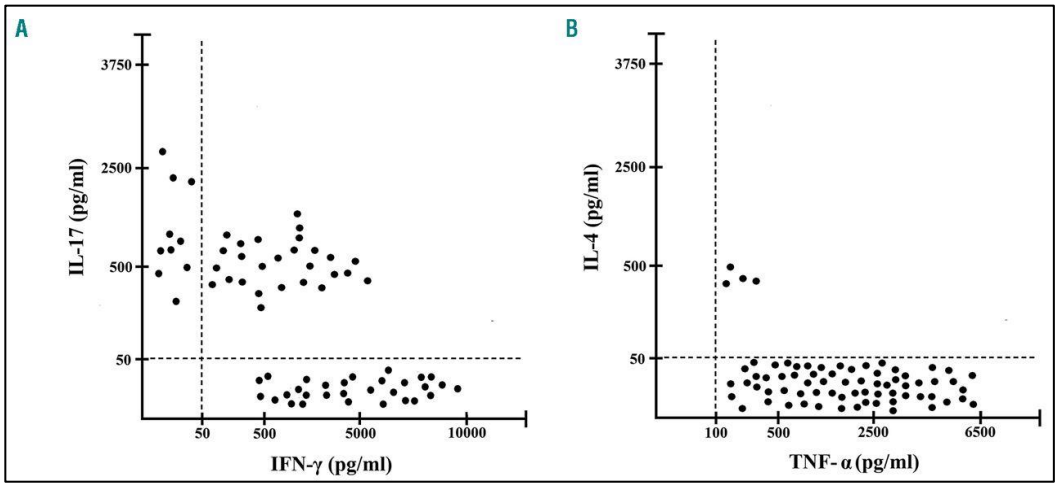

Figure 9. Cytokine profile of atherosclerotic plaque β2GPI-specific $CD4^+$ T cell clones obtained from SLE-APS patients. Th clones were tested for cytokine production (**A-B**). β2GPI-specificTh clones were stimulated with β2GPI and TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF-α, IL-4, IFN-γ and IL-17 were consistently < 20 pg/ml. $CD4^+$ T cell clones producing IFN-γ, but not IL-17 nor IL-4 were coded as Th1. $CD4^+$ T cell clones producing IL-17, but not IFN-γ nor IL-4 were coded asTh17. $CD4^+$ T cell clones producing IFN-γ, and IL- 17, but not IL-4 were coded as Th17/Th1. $CD4^+$T cell clones producing TNF-α and IL-4, but not IL-17 were coded asTh0.

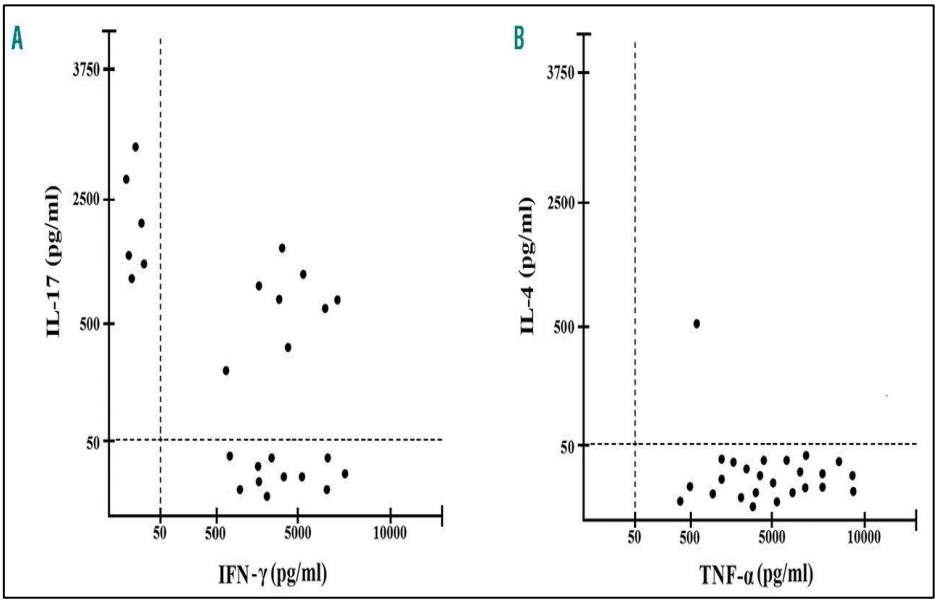

Figure 10. Cytokine profile of atherosclerotic plaque β2GPI-specific CD4$^+$ T cell clones obtained from SLE-aPL-positive patients. Th clones were tested for cytokine production (A-B). β2GPI-specific Th clones were stimulated with β2GPI and TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF-α, IL-4, IFN-γ and IL-17 were consistently < 20 pg/ml. CD4$^+$ T cell clones producing IFN-γ, but not IL-17 nor IL-4 were coded as Th1. CD4$^+$ T cell clones producing IL-17, but not IFN-γ nor IL-4 were coded as Th17. CD4$^+$T cell clones producing IFN-γ, and IL-17, but not IL-4 were coded asTh17/Th1. CD4$^+$ T cell clones producing TNF-α and IL-4, but not IL-17 were coded as Th0.

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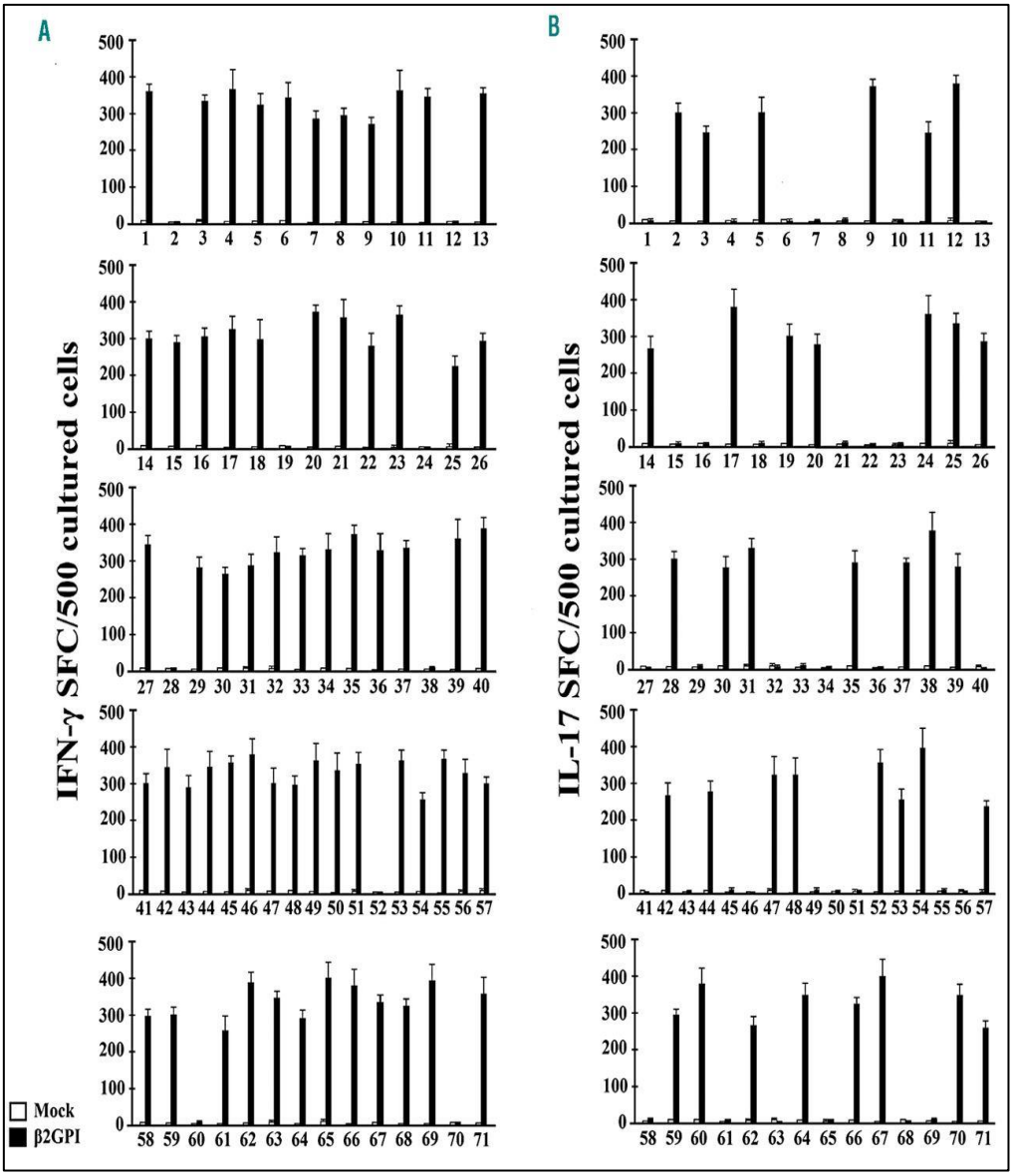

Figure 11. β2GPI driven IFN-γ and IL-17 secretion by β2GPI-specific atherosclerotic plaque derived Th clones from SLE-APS patients. Numbers of IFN-γ spot-forming cells (SFCs) after stimulation of atherosclerotic plaque derived T cell clones with medium alone, or β2GPI (**A**). T cell blasts from each clone were stimulated for 48 h with medium alone (□), or β2GPI (■), in the presence of irradiated autologous APCs in ELISpot microplates coated with anti-IFN-γ antibody. IFN-γ SFCs were then counted by using an automated reader. After specific stimulation, 61/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IFN-γ. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels.
Numbers of IL-17 spot-forming cells SFCs after stimulation of atherosclerotic plaque derived T cell clones with medium alone, or β2GPI (**B**). T cell blasts from each clone were stimulated for 48 h with medium alone (□), or β2GPI (■) in the presence of irradiated autologous APCs in ELISpot microplates coated with anti-IL-17 antibody. IL-17 SFCs were then counted by using an automated reader. After specific stimulation 37/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IL-17. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels.

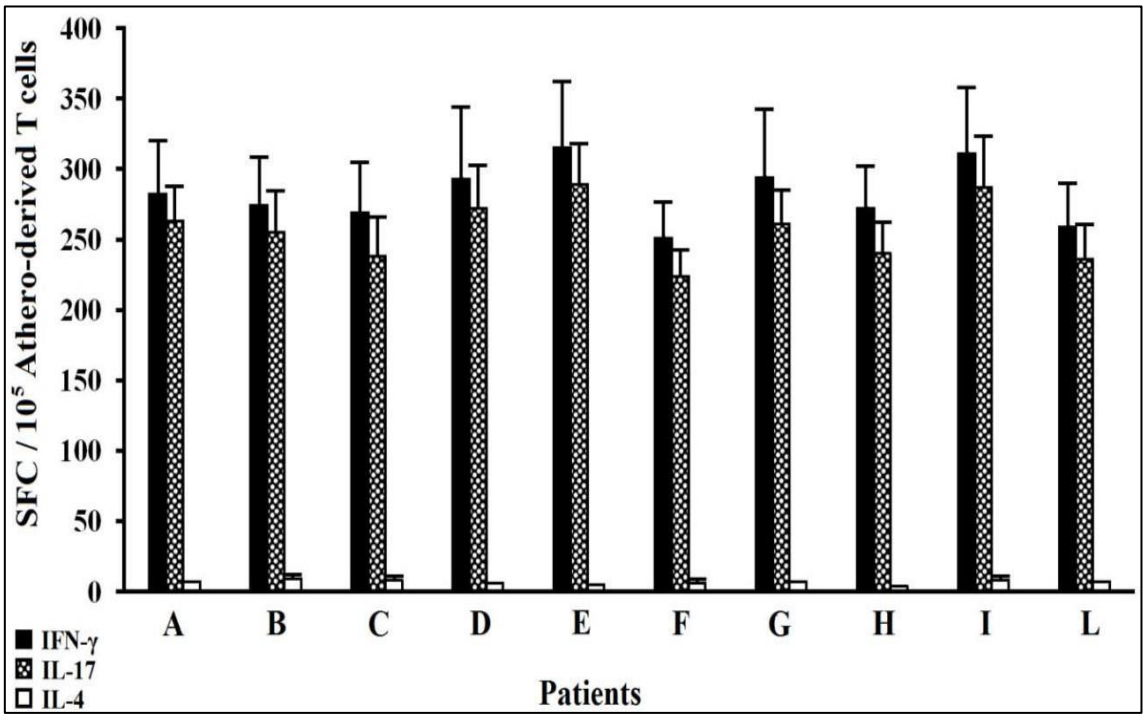

Figure 12. β2GPI driven IFN-γ and IL-17 secretion by β2GPI-specific atherosclerotic plaque derived T-cells from SLE-APS patients. Atherosclerotic plaque-derived T-cell lines were expanded from SLE-APS patients by addition of IL-2. At day 7, T-cell blasts from each line were stimulated for 48 h with ß2GPI or medium, in the presence of irradiated autologous APCs in ELISpot microplates coated with anti-IFN-γ anti-IL- 17, or anti-IL-4 antibodies. After specific stimulation with ß2GPI, a significant proportion of SLE- APS atherosclerotic plaque-derived Th cells produced IL-17 and IFN-γ, but not IL-4. Values are the mean ± SD number of SFCs per 10^5 cells over background levels.

β2GPI-specific atherosclerotic lesion-infiltrating T cells help monocyte TF production and PCA

Plaque rupture and consequent thrombosis are crucial complications of atherosclerosis. TF plays a key role in triggering atherothrombotic events being the primary activator of the coagulation cascade. We investigated whether atherosclerotic lesion-infiltrating β2GPI-specific T cells had the potential to express helper functions for TF production and PCA by autologous monocytes. Antigen-stimulated β2GPI-specific atherosclerotic lesion-derived T cell clones were co-cultured with autologous monocytes in the presence of medium or β2GPI. and levels of TF and PCA were measured by ELISA. Antigen stimulation resulted in the expression of substantial help for TF (**Fig. 13A**) production and PCA (**Fig. 13B**) by autologous monocytes.

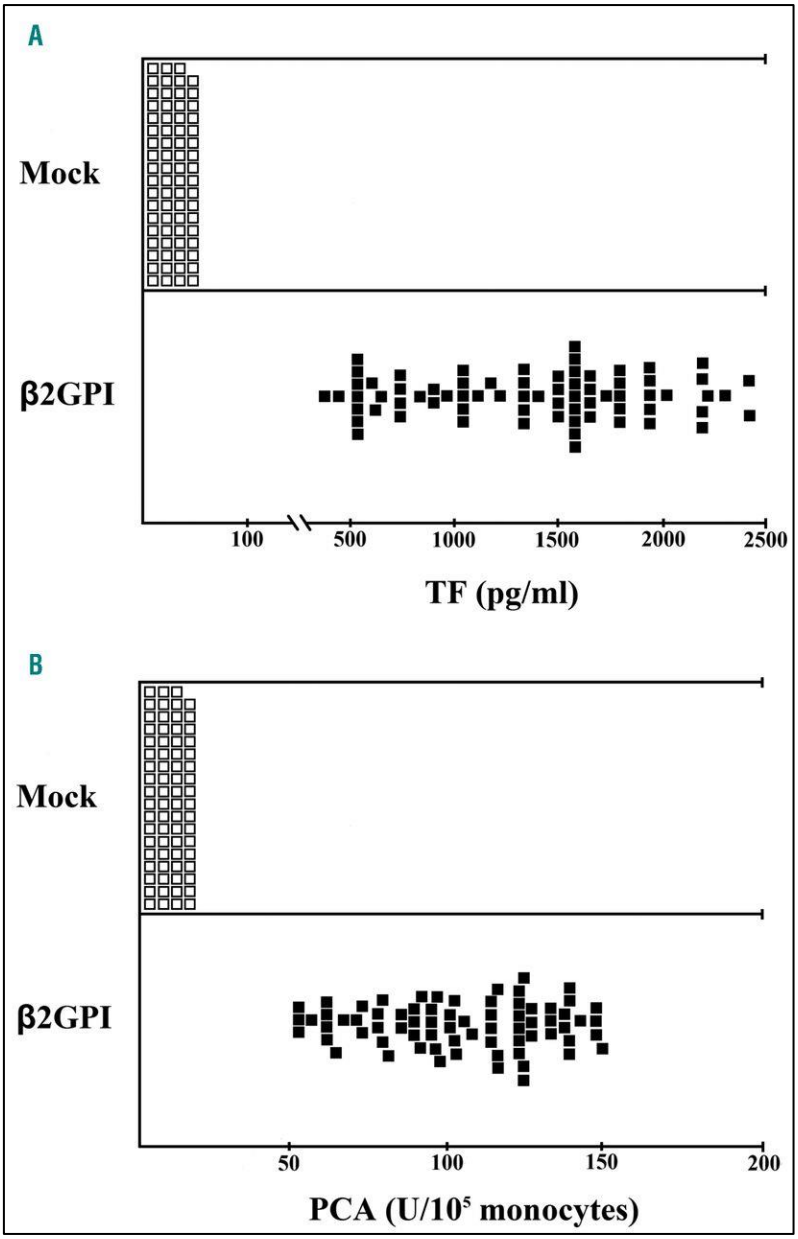

Figure 13. Induction of TF synthesis and PCA by atherosclerotic plaque β2GPI-specific T cells derived from SLE-APS patients. Atherosclerotic plaque β2GPI-specific T cells induce TF production and PCA by autologous monocytes. To assess their ability to induce TF production and PCA by autologous monocytes, β2GPI-specific Th clones were co-cultured with autologous monocytes in the presence of medium (□) or β2GPI (■) (**A**). TF production by monocytes was assessed by ELISA. The results shown represent TF levels induced by T cell clones over the TF production in cultures of monocytes alone. Atherosclerotic plaque-derived β2GPI-specific T cell induced PCA in autologous monocytes (**B**). β2GPI-specific Th clones were co-cultured with autologous monocytes in the presence of medium (□) or β2GPI (■). At the end of the culture period, cells were disrupted and total PCA was quantitated as reported in Materials and Methods. The results shown represent PCA induced by T cell clones in monocytes over the PCA in cultures of monocytes alone.

Atherosclerotic lesion-derived β2GPI-specific T cell clones express antigen-dependent help to autologous B cells for Ig production

T-B cell interaction is a multistep process resulting in B cell help depending on the functional commitment of the Th cells involved. So far, the ability of SLE-APS-derived β2GPI-specific T cell clones to provide B cell help for Ig synthesis was investigated. In the absence of the specific antigen, no increase in IgM, IgG, or IgA production above spontaneous levels measured in cultures containing B cells alone was observed. In the presence of β2GPI and at a T-to-B cell ratio of 0.2 to 1, all of the β2GPI-specific T cell clones provided substantial help for Ig production. At a 1-to-1 T/B cell ratio, β2GPI-dependent T cell help for IgM, IgG, and IgA production by B cells was remarkably higher (**Fig. 14**). However, at a 5-to-1 T/B cell ratio, co-culturing B cells with autologous β2GPI-specific T cell clones in the presence of β2GPI resulted in a much lower Ig synthesis.

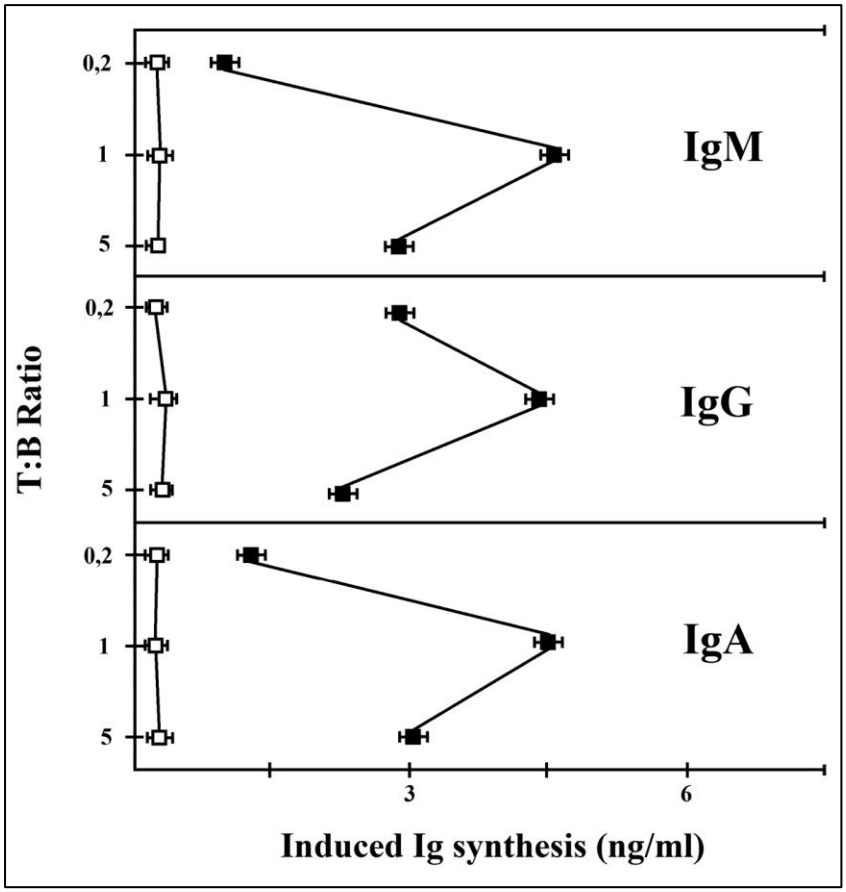

Figure 14. Helper function of atherosclerotic plaque β2GPI-specific T cells derived from SLE-APS patients. Autologous peripheral blood B cells (5 x 10^4) were co-cultured with β2GPI-specific T cell blasts at a T:B ratio of 0.2, 1, and 5 to 1 in the absence (□) or presence of β2GPI (■). After 10 days, culture supernatants were harvested and tested for the presence of IgM, IgG, and IgA by ELISA. Results represent the mean value (+/–SE) of Ig levels induced by T cell clones compared to the Ig spontaneous production in B cell cultures alone.

Atherosclerotic lesion-derived β2GPI-specific T cell clones display cytotoxic and proapoptotic activity.

The cytolytic potential of SLE-APS-derived atherosclerotic lesion-derived β2GPI-specific autoreactive T cell clones was assessed by using antigen-pulsed ^{51}Cr-labeled autologous EBV-B cells as targets. At an E:T ratio of 10:1, all Th1 and Th17/Th1 specific T cell clones were able to lyse β2GPI-presenting autologous EBV-B cells (range of specific ^{51}Cr release, 35–65%), whereas autologous EBV-B cells pulsed with control Ag and co-cultured with the same clones were not lysed (**Fig. 15A**). Likewise, 2 Th0 and all Th17 specific T cell clones were able to lyse their target (specific ^{51}Cr release, 50% and 25-45% respectively), while no lysis was observed when using autologous EBV-B cells pulsed with the control Ag.

Fas-FasL mediated apoptosis was assessed using Fas^{+} Jurkat cells as target. T cell blasts from each clone were co-cultured with ^{51}Cr-labeled Jurkat cells at an E: T ratio of 10, 5, and 2.5 to 1 for 18 h in the presence of PMA and ionomycin (**Fig. 15B**). Upon mitogen activation, 27 out of 30Th1, 24/27 Th17/Th1, 4/10 Th17, and 2 out of 4 Th0 clones were able to induce apoptosis in target cells (range of specific ^{51}Cr release, 25-61%).

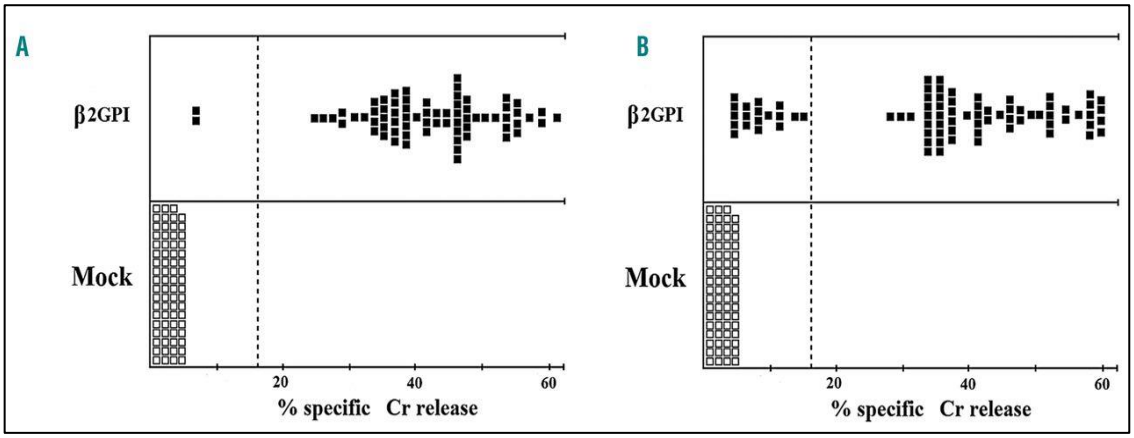

Figure 15. Cytotoxic and pro-apoptotic activity of β2GPI-specific atherosclerotic plaquederived CD4+ T cells derived from SLE-APS patients. (**A**) To assess their cytotoxicity, β2GPIspecific CD4+ T cell clones were co-cultured at different E:T ratios with ^{51}Cr-labeled autologous EBV-B cells pulsed with β2GPI (■) or medium alone (□). ^{51}Cr release was measured as index of specific target cell lysis.
(**B**) To assess their ability to induce apoptosis in target cells, β2GPIspecific CD4+ T cell clones stimulated with mitogen (■) or medium alone (□) were co-cultured with ^{51}Cr-labeled Fas+Jurkat cells, and ^{51}Cr release was measured as the index of apoptotic target cell death.

DISCUSSION

Several clinical studies and experimental models suggest a role for aPL in accelerating atherosclerotic plaque formation in SLE. On the other hand, there is growing evidence that aPL represent a risk factor for arterial thrombosis supporting their pathogenic role in cardiovascular events [64, 177, 209, 210]. Here in we report for the first time that a pro-inflammatory and procoagulant β2GPI-specific Th17, Th1 and Th17/Th1 infiltrate in human atherosclerotic lesions of patients with SLE-APS and may represent a key pathogenic atherothrombotic mechanism.

Many self-Ags, such as oxLDL, may theoretically be involved in SLE-APS atherosclerosis; oxLDL-specific peripheral blood-derived T cells displaying a Th1 profile were reported in APS patients [211]. However, there is no information on whether these cells are actively involved in atherosclerotic tissue lesions of SLE-APS patients. In addition, β2GPI was found to bind oxLDL [88] raising the issue whether or not the immune response is against oxLDL or β2GPI itself.

The relevance of the data presented in this study consists in the demonstration that all ten SLE-APS patients with clinically severe atherothrombosis harbored in their target tissues, such as atherosclerotic lesions, *in vivo*-activated $CD4^+$ T cells able to react to β2GPI. $CD4^+$ T cells specific for β2GPI were found also in the plaques of SLE aPL-positive patients but not in SLE aPL-negative patients nor in atherosclerotic patients without SLE. The results suggested that β2GPI drive inflammation in atherosclerotic plaques in SLE-APS and SLE aPL-positive patients, while in SLE aPL negative patients and in non SLE patients other Ags are involved in sustaining plaque inflammation. With the experimental procedure used in this study, the proportion of β2GPI-specific $CD4^+$ T cell clones generated from atherosclerotic plaques of atherothrombotic SLE-APS patients is remarkably higher than the frequency of β2GPI-specific T cells found in their peripheral blood.

In order to investigate plaque instability, we investigated fresh T cells coming from the atherosclerotic plaques of SLE-APS patients and we found that plaque-derived $CD4^+$ T cells specific produce IFN-γ and IL-17 in response to both β2GPI and to mitogen stimulation. Studying at clonal level the β2GPI-specific T cells found in the inflammatory atherosclerotic infiltrates of SLE-APS we found that 42% were polarized T helper 1 cells,

38% were Th17/Th1 cells, 15% were polarized Th17 cells, 5% were Th0 cells, and none of T cells were polarized Th2 cells. The lack of Th2 cells is an important risk factor in the genesis of atherosclerosis. T cells indeed play an important role in the genesis of atherosclerosis that has been defined a Th1-driven immunopathology [212, 213] and we have demonstrated that Th1 cells, producing high levels of IFN-γ, are crucial for the development of the disease [89, 191, 206]. Given that atherosclerosis can occur and progress even in IFN-γ- or IFN-γR–deficient mice, although with a lower lesion burden [214], other Th cells and factors are presumably involved in the genesis of the atheroma. A third subset of effector Th cells, namely Th17, has been discovered [215]. Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions [216, 217]. In the lymphocytic infiltrates of SLE-APS atherosclerotic plaques, we have found the presence of *in vivo*-activated plaque- infiltrating T cells able to produce IL-17 and IL-21 in response to β2GPI. Among the clonal progeny of T cells infiltrating the lesions, we demonstrated the presence of β2GPI -specific T cells able to secrete IL-17. A significant number (27%) of IL-17–producing T cells are also IFN-γ producers. This finding is in agreement with a previous report that demonstrated the concomitant production of IL-17 and IFN-γ by human coronary artery-infiltrating T cells in non SLE patients [138, 218, 219]. Plaque rupture and thrombosis are notable complications of atherosclerosis [206, 219]. The methodology used to obtain the plaque derived T cells encompasses a clonal expansion step, followed by limiting dilution to obtain single clones.

The β2GPI-reactive $CD4^+$ T cell clone found in atherosclerotic plaques were unique, based on the T-cell receptor-Vβ results obtained in the study. The β2GPI -specific T-cell clones revealed their ability not only to induce macrophage production of TF upon antigen stimulation but also were able to promote PCA.

Th17 cells were shown to play a key role in experimental mouse models of atherosclerosis; IL-17 is proatherogenic in experimental model of accelerated atherosclerosis in the presence of high fat diet (HFD) [220]. In fact, in IL-$17^{-/-}$ mice fed with HFD, the aortic lesion size and lipid composition as well as macrophage accumulation in the plaques were significantly diminished, and the progression of the process was remarkably reduced compared with WT mice. Furthermore IL-21 was produced by almost all Th1 and Th17/Th1 cells specific for β2GPI. IL-21 is actually up-regulated in patients with peripheral artery diseases [221]. Expression of IL-17 in human atherosclerotic lesions is

associated with increased inflammation and plaque vulnerability and increased Th17 cells [222]. An increased incidence of atherosclerosis associated with peripheral blood Th17 responses has been demonstrated in patients with SLE [223].

We have demonstrated that β2GPI was able to activate Th17 and Th1 responses in atherosclerotic lesions of SLE-APS patients. The relevance of Th17/Th1 cells in non SLE atherosclerosis patients have been demonstrated in other studies [224, 225], suggesting that Th1 and Th17 cells might plastically shift one into the other in different phases of the disease. It has been shown that Th17 cells might shift towards Th1 but not to Th2 via IL-12 receptor signalling [226].

Overall, our findings support the concept that a crucial component of atherosclerosis in SLE-APS is represented by T-cell–mediated immunity and that chronic Th response against β2GPI plays important role in the genesis of atheroma in SLE-APS patients [159].

Among β2GPI-specific IL-17–producing Th cells, the majority were polarized Th17 cells, whereas others were able to produce both IFN-γ and IL-17. Thus, it is possible to speculate that Th17 and Th1 cells co-migrate to the inflamed tissue and cooperate in the ongoing inflammatory process within the atherosclerotic lesion [87, 206, 216, 227].

In addition, upon appropriate Ag stimulation, the majority of atherosclerotic plaque-derived β2GPI- specific clones induced both perforin-mediated cytolysis and Fas/FasL-mediated apoptosis in target cells and were able to drive the up-regulation of TF production by monocytes within atherosclerotic plaques, thus further contributing to the thrombogenicity of lesions [138, 157, 219]. Our results demonstrate that β2GPI is a major factor able to drive Th17 and Th1 inflammatory process in SLE- APS atherosclerosis and suggest that Th17/Th1 cell pathway and β2GPI may represent important targets for the prevention and treatment of the disease.

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