Is targeting dysregulation in apoptosis splice variants in *Mycobacterium tuberculosis* (MTB) host interactions and splicing factors resulting in immune evasion by MTB strategies a possibility?

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Abstract

Mycobacterium tuberculosis (*Mtb*), is one of the foremost organisms causing mortality in humans, and has been for most of human history. When faced with an infection the human immune system is ordinarily very competent in killing both extracellular and intracellular bacilli. However, *Mtb* is able to evade the host immune system and is even able to establish a persistent infectious reservoir by "hiding" in the immune cells of the host. While the mechanisms by which the bacteria accomplishes this are not fully understood, it is known that the bacterium can subvert cellular processes in cells such as macrophages that prevent the lysis of the bacteria or the cell undergoing apoptosis. They are also able to interfere with immune cell signalling. One of the greatest effects that *Mtb* has is too alter the transcriptome of the macrophage. An easy way for the bacterium to accomplish this is to alter the alternative splicing patterns of the host. This can lead to a large change in the population of different protein isoforms, some of which have very different functions when compared to the original protein. At the same time the long history of *Mtb* infecting humans have led to specific immune reactions that occur in the host immune system in order to fight the infection. Many of these specific reactions involve new isoforms of host defence proteins. In this way the human host can use alternate splicing to create new isoforms of immune- related proteins that are more effective in defending against *Mtb*.

1. Introduction

Tuberculosis (TB) is a contagious, infectious disease, caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*). The bacterium is thought to have evolved 150 million years ago. The earliest records of TB in humans was found in 3300-year-old written records from India and 2300-year-old written records from China. TB was known to the ancient Greeks as Phitisus [1]and there are at least two probable references to TB mentioned in the Bible [2]. Archaeologists have identified skeletal abnormalities associated with TB in ancient Egyptian and Peruvian mummies [1]. The World Health Organization estimates that two billion individuals around the world are affected by *Mtb*, creating a massive inactive *Mtb*

reservoir, resulting in the reduction in the prospect of ever completely eradicating *Mtb* from the human population [3].

Tuberculosis (TB) remains a major health consideration around the world, with about nine million new cases and around two million deaths occurring every year [3,4]. Over 95% of new TB cases and death take place in developing nations, while most elevated morbidities are observed in Asia and Africa. This is further complicated by the ability of *Mtb* to resist the activity of the immune system, specifically macrophages, leading to persistent infections. The incidence rates of tuberculosis (frequency rates per 100,000 populace) by nation is presented in Fig. 1 [4].

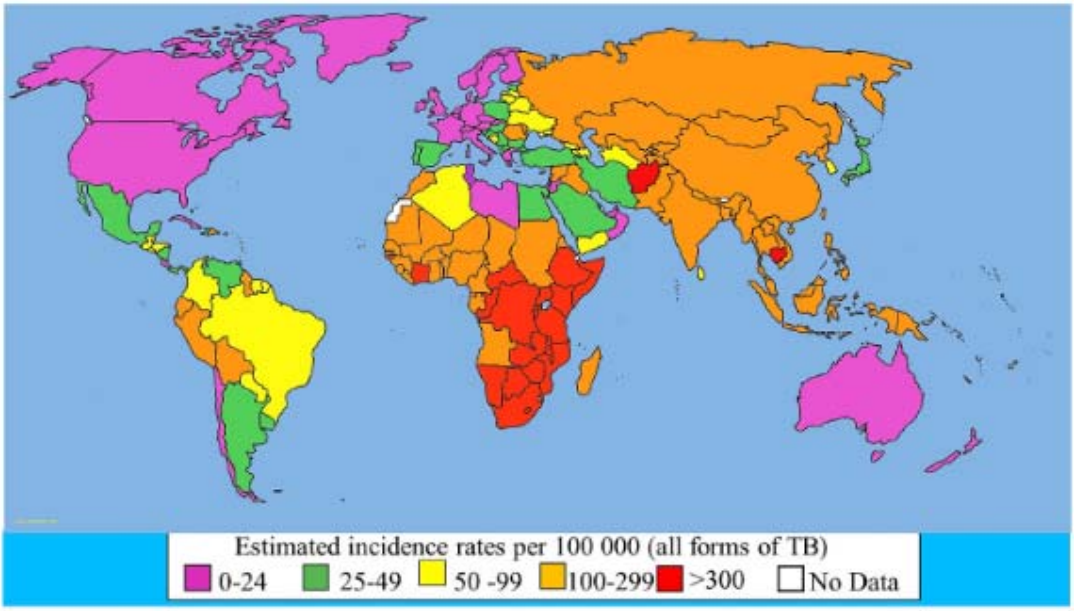

Fig. 1. **Incidence rates of tuberculosis**. The map depicts the incidence rate of *Mtb* worldwide as number of cases per 100,000 populace by nation. The highest rates occur in Africa (especially Sub-Saharan Africa) and Asia [3].

The highest incidence rates are found in Sub-Saharan Africa, where HIV infection has led to a massive increase in the frequency of TB infections in the past quarter century. Nations with particularly high incidence rates include Botswana, South Africa, Swaziland, and Lesotho. These countries have incidence rates of tuberculosis, which are >500/100,000. This is approximately 150-fold more than that seen in the USA. The nations of the Western Pacific and South Asia possess significantly lower frequency ratios. India as well as China simultaneously report approximately 40% of worldwide events of tuberculosis [4].

TB was once treated using cod liver oil, gold, bed rest, arsenic, herbs and clean atmosphere and sunshine [5]. However, none of these treatments were truly effective, with patients still eventually succumbing to the disease. The first effective treatment was streptomycin, developed in the year 1943. Following this a sequence of remarkable medications to treat TB were developed during the period between ‘‘1940s–1960s”, known as the golden age of antibiotics. The use of these medications produced a drop in TB incidence across the world. Yet, by 1982 infection rates increased as a result of the new epidemic of multidrug resistance that arose in the bacterium [6].

Mtb has developed evasion strategies against the host defence mechanisms [7]. Besides its capacity to resist the host's immune system, permitting its broad expansion, strains of *Mtb* have developed resistance to most antimicrobial agents. These strains progressively become much more resistant to the current medications. Strains that have been resistant to one anti-TB agent acquire further resistance to generate strains which are MDR (multidrug resistant), XDR (extensively drug resistant) as well as the TDR (totally drug resistant) [8]. Infections caused by TDR strains are virtually incurable using the present day TB drugs.

During infection, the *Mtb* bacteria exist in heterogeneous populations in varying environments in the host with the actively-replicating organisms that are susceptible to most antibiotics being found in macrophages and the persisters and dormant bacteria, which are drug tolerant/resistant, in the granuloma [9]. Furthermore, bacterial heterogeneity may be as a result of different host immune status due to underlying conditions such as HIV and co-morbidities [10]. Consequently, current alternatives for TB chemotherapy are extremely limited.

In order to develop effective intervention strategies against *Mtb*, an understanding of the underlying mechanisms defining bacterial and host interactions is required. Recently, few studies have demonstrated the usefulness of alternative splicing of RNA in providing insight into immune response against *Mtb*, demonstrating alterations in cellular physiology during *Mtb* infection [7,10].

2. Alternate splicing

Alternative splicing occurs post transcriptionally and leads to the generation of multiple transcripts from a single parent mRNA. These transcripts are then translated into multiple protein isoforms [11]. The control of alternative splicing is accomplished through competing spliceosomes, splice sites and splicing elements recognised by splicing factors [11]. These splicing factors are expressed differentially in different tissues at deferent developmental stages, allowing for variation of isoforms [12]. These isoforms may differ in function, sometimes acting antagonistically to the original protein [13]. Up to 94% of all human proteins are alternatively spliced [14]. Both *Cis*-and *Trans*-elements control alternative splicing. The *Trans*-elements include RNA-binding proteins and *Cis*-elements such as Serine/Arginine-rich (SR) proteins and the heterogeneous nuclear ribo-nucleoproteins (hnRNP) (Reviewed in Refs. [11,15]. Alternate splicing can be carried out in multiple ways. These include exon skipping, intron retention, through the use of alternate splice sites involving the 5′ and 3’ exons being shortened due to internal splice sites and through the use of mutually exclusive exons [16].

3. The immune response to TB

Initially the mycobacterium is recognised by macrophages and dendritic cells using toll-like receptors (TLRs). The mycobacteria are engulfed by phagocytic immune cells, upon activation of toll-like receptors TLR 2, TLR 9, and other pathogen-recognition receptors (including c-type lectin receptors and mannose receptors) [17,18]. Activated macrophages stimulate generation of Interleukin-12 (IL-12) in addition to the cell signalling cytokine Tumour necrosis factor alpha (TNF-α). IL-12 induces the two helper T-cell-mediated adaptive immune reactions that initiates cluster of differentiation (CD4) formation [19]. Activated macrophages release interferon-gamma (IFN-γ) which starts the autophagic

response, wherein the macrophage phagosome (containing the bacterium) fuses with the lysosome, [17,20].

Mycobacteria have the capacity to retard the start of T-cell-mediated immune defence, typically as a result of the failure of dendritic cells to emigrate from infected lungs toward territorial lymph hubs [21]. The immune response to *Mtb* is controlled by the action of the neutrophillss, NK cells (Natural killer cells), B lymphocytes, and T lymphocytes. The antigens are exhibited via MHC (major histocompatibility complex) class II molecules by dendritic cells (DCs) and macrophages on the exterior of the cells where a complex is formed between the MHC II molecule, the CD4 cell surface receptor and the Tyrosine kinase receptor. This results in T cell activation [21].

Mycobacterium tuberculosis is able to survive and replicate in human macrophages, evading host immunity. *M. tuberculosis* effectively evades lysosomal degradation by altering signalling within the macrophage. Phagosomes containing Mycobacterium lack the molecular signals that target them to the endosomal lysosome. In particular they lack the Two-Site Recognition of Phosphatidylinositol 3-Phosphate (two PI3P) Early Endosome Antigen 1 binding proteins (EEA1), Ras-related protein 7 (Rab7), HGS (hepatocyte growth factor-regulated tyrosine kinase substrate), the vacuolar H^+-ATPase (V-ATPase) and lysosome-associated membrane glycoprotein 1 (LAMP1) [22]. In this way *Mtb* takes advantage of proteins and lipid molecules to capture phagosome development in the initial phases [23].

In order to achieve these change, the bacterium has to manipulate the gene expression of the host. One of the simplest and most effective methods of accomplishing this is to influence the alternate splicing mechanisms of the host cell. Despite the fact that Mtb infection leads to changes in the alternate splicing of many genes, there are no changes in the splicing of genes controlling pre-mRNA splicing, such as Serine And Arginine Rich Splicing Factor 2 (SRSF2), Serine And Arginine Rich Splicing Factor 3 (SRSF3), Serine And Arginine Rich Splicing Factor 4 (SRp75) as well as Human splicing factor SF3a [7]. However, as we will discuss in section 4, the splicing factor PTB-associated splicing factor (PSF) is downregulated following Mtb infection [24]. Similarly, although the bacterium does not affect splicing of serine and arginine-rich (SR) proteins it does affect the expression levels of these proteins. In this way the bacterium can affect large changes to splicing in the macrophage [7].

4. Alternate splicing in the macrophages caused by *Mtb*

Mycobacterium tuberculosis possesses the ability to neutralize the anti-bacterial actions of the macrophages, and this enables the bacteria to establish a chronic surviving presence within a patient [25,26]. The infection of human macrophages by *Mtb* leads to massive alterations in splicing within the macrophage [10]. The significance of these alterations is hinted at through the change in the domains present in protein kinases following *Mtb* infection. The changes in the protein isoforms and the different functional domains present in these proteins is due to changes in alternate splicing [10]. Many of these splicing changes affect proteins that play a critical role in dictating the response of the macrophage to infection [27,28]. The physiology of the macrophage, which include antimicrobial effector mechanisms, is severely altered following infection by *Mtb* [27]. In particular these changes interfere with the ability of the macrophage to kill the bacterium [27]. The RAB8B protein is essential for the proper operation of the macrophage. Following infection with Mtb there is an alteration in alternative splicing, which results in a non-functioning transcript that codes

for an inactive protein [27]. The *Mtb* strains JAL2287 H37Ra and H37Rv all led to a high number of splicing changes in macrophages following infection [10].

Apoptosis is an important antimicrobial mechanism in cells, leading to reduction in pathogen viability, triggering death of infected macrophage [7]. One of the methods used by *Mtb* to evade the hosts immune system, is to inhibit macrophage apoptosis. Bacteria containing the Rv3659c gene mutant in the bacterial genome leads to an impaired ability of the bacterium to inhibit apoptosis in macrophages. This gene codes for a protein which is secreted into the cytoplasm of the macrophages. It blocks the extrinsic apoptosis pathway by cleaving the Polypyrimidine tract-binding protein-associated splicing factor (PSF). PSF regulates apoptosis by affective pre-mRNA splicing of Caspase-8 mRNA. Downregulation or cleavage of PSF leads to a decrease in the accessibility of caspase 8, leading to a decrease in apoptosis [24].

IL-4 is another important regulator of immune response. Interleukin 4 is involved in activating B-cell and T-cell proliferation. It also initiates the differentiation of B cells into plasma cells and igE. It up-regulates MHC class II production. It also stimulates apoptosis in certain cells. It also promotes the switch of macrophages into M2 cells involved in wound healing and inflammation. The expression of interlukin 4 is increased in cells infected with *Mtb* and IL-4 is involved in the transformation of a latent TB infection (LTBI) into an active dynamic infection. Studies have shown that alternative splicing of IL-4 takes place under the influence of *Mtb*. The mRNA is alternatively spliced to give rise to multiple isoforms. One of these isoforms lacks the second exon, known as IL-4delta2. This isoform is also known as VIL-4 or Il-4r. This isoform is thought to play an antagonistic role towards IL-4 stimulated T cell proliferation [29] and is expressed at higher levels during severe TB-induced lung injury [30,31]. Il-4 promotes cell proliferation in contrast to the cell death promoted by VIl-4 [32]. Both isoforms are expressed at high levels in THP-1 monocytes that are infected with *Mtb* [7]. The increase in IL_4 expression is thought to be detrimental to surviving *Mtb* infection [32]. However, increased expression of the antagonistic truncated isoform may inhibit the IL-4 induced cell death and tissue destruction. Allowing both infected macrophages and the bacterium within to evade the immune system. Therefore, the ability of the immune system to effectively defend again *Mtb* infection may depend on the ratio between il-4: IL-4δ2. In addition to this the ability of splice variant IL-4δ2 to act against IL-4 implies that recombinant IL-4δ2 may be used as a novel new therapy [[32], [33]]. Expression of the truncated isoform also lead to an increased + expression of CD23 [34].

Both macrophages and DCs (dendritic cells) contain TLRs on the surface of their cell membranes or on the surface of the of the endocytic vesicle membranes within these cells. The Toll-like receptor 4 TLR4) is involved in the identification of *Mycobacterium tuberculosis.* TLR4 is also involved in the initiation of NF-kB intracellular signalling [[35], [36], [37]]. Exons II and III are alternatively spliced in human TLR4 to give rise to four isoforms Fig. 2 [38]. In a THP-1 cell line, derived from an acute monocytic leukemia patient, infected with *Mtb*, there was a lower expression of TLR-4 and three of these isoforms [[38], [39]]. The functional role of TlR4 and any of its isoforms in *Mtb* infection are not known [7]. These changes in splicing frequently result in isoforms with less Leucine-rich repeats, which are involved in protein–protein interactions.

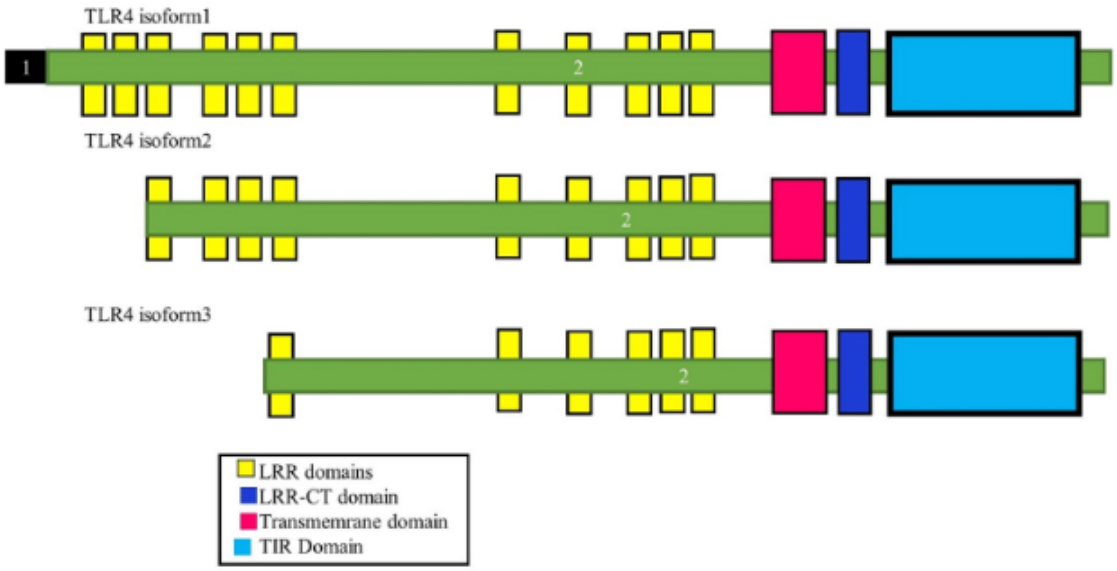

Fig. 2. Alternate splicing of TLR4. Splicing events result in the loss of exon 1 and alternate splicing of exon 2. Some of these isoforms can potentially act as inhibitory forms against the full-length form. Bacterial infection in patients with Cystic fibrosis generally lowers the expression of these inhibitory isoforms [38].

5. Alternative splicing in the host in response to *Mtb* infection

5.1. IL12Rβ1ΔTM

The IL12Rβ is a human gene essential for host defence against *Mtb* infection. The gene codes for a transmembrane receptor that is involved in the development of T-helper (Th)1 cells. Other names for this gene include NK cell stimulatory factor (NKSF2) and Cytotoxin lymphocyte maturation factor (CLMF). The mouse homologue, *il12rb1* is alternatively spliced in white blood cells, to give rise to a second isoform known as IL12Rβ1ΔTM. Unlike the original protein this protein lacks a transmembrane region and is secreted from transfected cells [40]. Splicing leads to IL12Rβ1ΔTM being equivalent to the interlukin 12 receptor subunit beta as well as a signal peptide and cytotoxic binding region also found in the full length protein is spliced from the mRNA [41,42]. The main difference is due to the removal of the C-terminus, coding for the transmembrane region Fig. 3. This also restricts the protein being localised within the endoplasmic reticulum (ER) within the cell [42]. It was observed that the expression of this isoform is induced by *Mtb* infection and mice lacking this isoform show increased susceptibility to *Mtb* infection [40]. The circulating nature of the protein allows it to defend the mouse against *Mtb* infection in extra-pulmonary organs [40,43]. In lungs infected with *Mtb*, the expression of IL12Rβ1ΔTM is initiated in the dendritic cells. DCs are an important part of the host defence against *M. tuberculosis* and are responsible for relocating *Mtb* to the mediastinal Lymph Node (MLN) [[44], [45], [46]]. DCs express higher levels of IL-12 family members during *Mtb* infection [47] and are essential to produce an effective T cell response [47]. The circulating nature of IL-12Rβ1ΔTM would therefore allow for enhanced DC activation [42] [48].

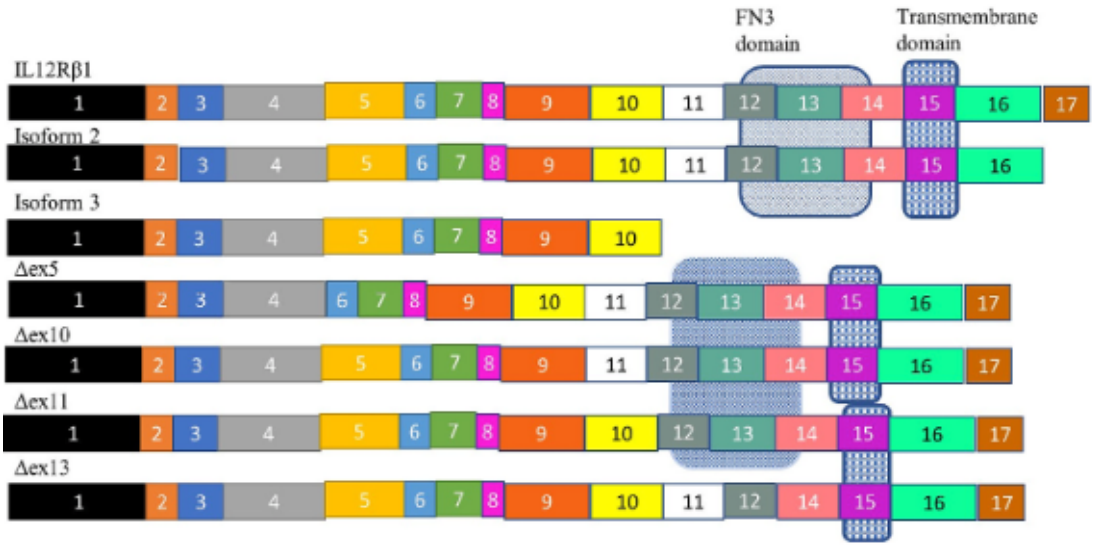

Fig. 3. **IL-12 Rβ1** splice variants. The splice variant isoform 3 is up-regulated in *Mtb* infections. This isoform lacks the transmembrane domain and the mouse version of this isoform has been shown to be secreted from transfected cells.

5.2. Il-7 and il-7r

In order to mount a successful immune response, immune cells have to communicate with each other. Cytokines such as interleukin-7 are secreted in response to macrophage infection with *Mtb*. This cytokine is able to stimulate the development of antigen-presenting cells and guarantees T-cell persistence. Overexpression of IL-7 leads to a decrease in *Mtb* load [49]. Both IL-7 and its receptor IL-7r play a crucial role in the differentiation of immune cells. Both were found to be alternatively spliced. IL-7 contains 6 exons with the isoform lacking exon 5 acting antagonistically to other IL-7 isoforms Fig. 4. The IL-7 receptor has splice variants that do not have a transmembrane region (sIL-7r) and acts as a competitive inhibitor if IL-7 binding to its receptor [50]. In a non-human primates (NHPs) tuberculosis vaccine model it was found that the expression of most IL-7 isoforms was higher in lung tissue. The exception being the short δ5 isoform. This increased expression leads to increased survival. At the same time the dominant form of IL-7r found in these lung cells is the shorter sIl-7R [50]. In addition to the increased expression of all 6 IL-7 isoforms, *Mtb* challenge lead to the increased expression of both Interferon and IL-17 and decreased expression of transforming growth factor-b (TGF-b). The splicing of IL-7 and its receptor was also found to be tissue specific [50].

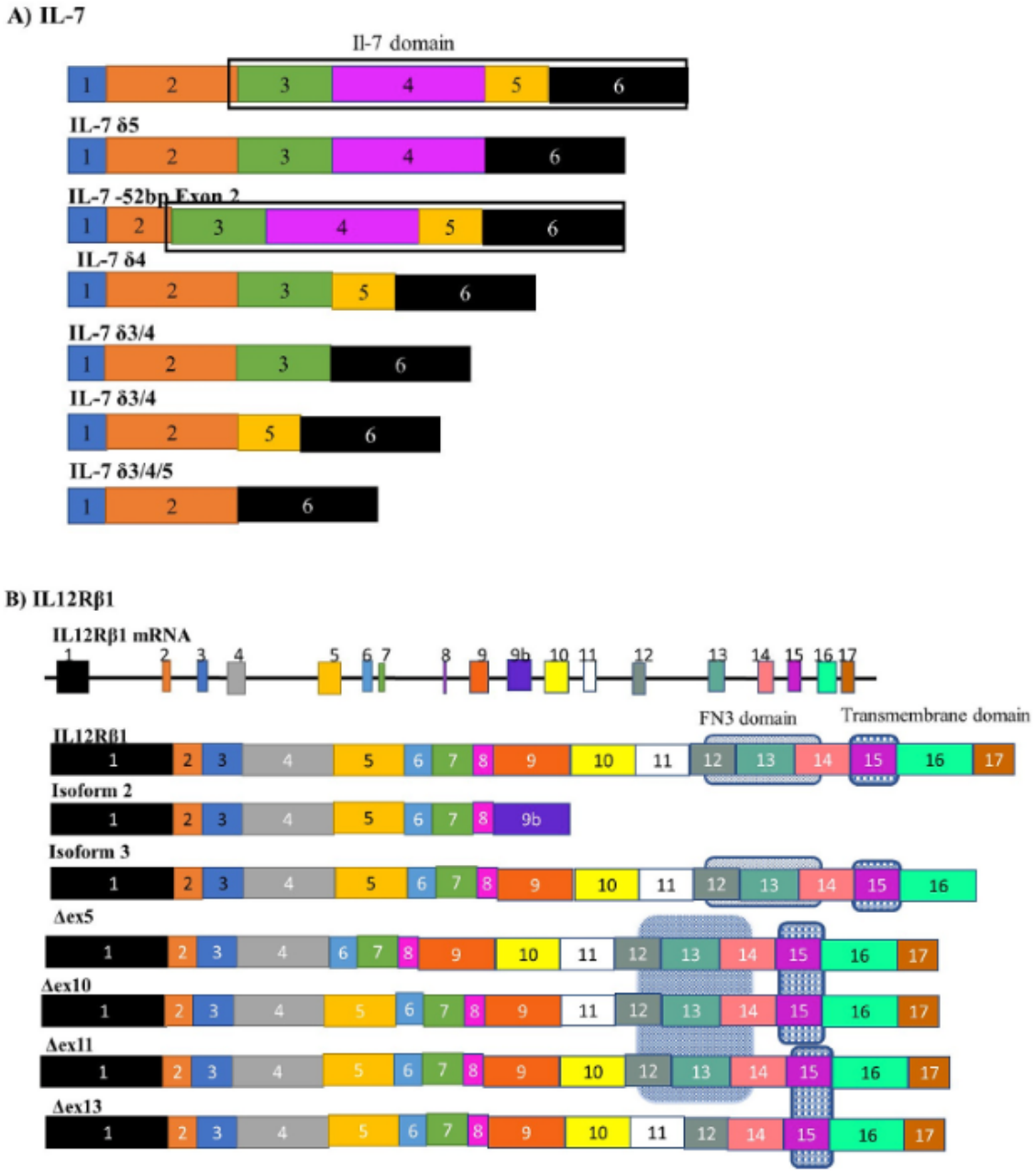

Fig. 4. **Alternate splicing of Il-7 and its receptor.** In panel A, the full length IL-7 protein is coded for by 6 exons. Two isoforms have been identified while others are predicted. The two identified isoforms include one in which exon 5 is absent and another in which exon 2 is truncated. The isoform lacking exon 5 is a competitive inhibitor of the full length protein and is downregulated following *Mtb* infection. Panel B represents isoforms of the human IL12Rβ.1 isoform 4 of the IL-7 receptor lacks a proper transmembrane region and is able to block ligand binding to the full length receptors.

As previously mentioned IL-57 initiates the expression of IL-17 A, which in the murine model seems to increase immunity against *Mtb* [51].The decrease in the expression of Transforming growth factor beta 1 (TGF-b) generation, associated with the increase in the expression of Interleukin 17A, following *Mtb* infection seems to be associated with granuloma production This granuloma production is independent of interferon gamma (IFN-g) activity [51]. Therefore the expression of Il-7 and IL-7r isoforms that act antagonistically

to the normal function of these proteins would also result in a decrease in the expression of IL-17A and a decrease in the ability of the host to resist *Mtb* infection [50].

6. Conclusion

Mycobacterium tuberculosis has been infecting and killing humans for most of their entire recorded history. This has led to the bacterium becoming perfectly adapted to their host and developing means to evade the hosts immune system. At the same time the host has developed ways in which they can respond to the bacterium. For both the host and the bacterium changes to the hosts gene expression is the main way to successfully evade the immune system or kill the pathogen. The most rapid and possibly easiest way to change gene expression is through changes to alternative splicing of mRNA. These isoforms can allow for the bacterium to evade the immune system through alternate splicing can allow for the bacteria to select isoforms that behave antagonistically towards the hosts immune genes, it would seem that the easiest way to accomplish this is by manipulating the levels of certain splicing factors. At the same time the host can increase the expression of certain isoforms which can more effectively target or direct the immune system against the mycobacterium. Understanding the basis of these splicing events may allow for the development of drugs that target certain isoforms. These new novel drugs would help to alleviate the current epidemic of strains of *Mtb* that are resistant to all of our current drug treatments.

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