Immunology of Tuberculosis
and Implications for the Diagnosis of Infection

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I. Overview of the Immune-Response

1. Infection

Patients first become infected by inhaling mycobacterial bacilli. At that point, the major host cells of the bacteria are the alveolar macrophages, located in the alveoli of the lungs. When the macrophages take up the mycobacteria, the bacilli begin to multiply. Eventually, the host cell will die, due to the overwhelming mycobacterial burden. Then, newly recruited macrophages would be attracted, take up the mycobacteria, become infected, and the cycle would continue. This would have been the natural course of the infection of macrophages, if people fail to develop efficient immune responses.

2. The Innate Immune-Response

However, fortunately, people have both innate and adaptive immune responses. The innate immune response begins during the first days of infection, and is guided by several features. The first is the release of molecules, which are capable of directly killing Mycobacterium tuberculosis. These include, for example, oxygen radicals, which are produced by the infected alveolar macrophages. It has been shown in-vitro that oxygen radicals have direct antimicrobial activity. Other antimicrobial peptides, such as LL-37, are also capable of directly killing the mycobacteria.

We believe that in some instances, this interaction alone can lead to the prevention of active infection. Thus, the innate immunity response will be able to clear the bacteria by itself. However, in most cases, it would not be sufficient.

3. The Adaptive Immune-Response

To attract help, the alveolar macrophages, which are activated by the mycobacteria infection, will release a group of molecules called chemokines, which include CCL-2, CCL-3, CCL-5, and Tumour Necrosis Factors (TNF). The task of these molecules is to recruit T-cells, which will then be attracted to the lymph node and activated.

C4+ and C8+ T-cells are antigen-specifically activated and initiate the adaptive phase of the immune-response. However, they must first be educated to perform the appropriate task; in this case, the secretion of Th1 cytokines and mediators that help the macrophages to kill M. tuberculosis. The education of the T-cells is performed by a second set of cytokines released by the alveolar macrophages, IL-12 and IL-18.

4. Deactivation

One of the great risks of tuberculosis is that the immune system overreacts and destroys lung tissue, thereby causing the clinical symptoms. In order to prevent this effect, the T-cells must
be deactivated once the major bacterial burden has been defeated. Thus, in order to prevent the immunopathology of tuberculosis, a set of immunosuppressive cytokines deactivate the immune-response.

II. Toll-Like Receptors

I would now like to focus on Toll-Like Receptors (TLRs), which are a set of receptors involved in the initial interaction between *M. tuberculosis* and the macrophages. TLRs belong to a large family of receptors. However, in the case of tuberculosis, the interaction is primarily conducted with two specific receptors, TLR2 and TLR4.

It has been shown that 19kDa lipoprotein, PIM, STF, and LAM all interact with TLR2, thereby initiating several signal transduction cascades, which lead to the activation of the macrophage. The interaction between *M. tuberculosis* and the macrophage triggers several biological functions, which are important in the protection against the disease. For example, the interaction with 19kDa lipoprotein induces the release of TNF and IL-1, thereby leading to the maturation of dendritic cells. The dendritic cells are then able to present mycobacterial antigens in the lymph node. The TLR interaction with macrophages also results in the modulation of antigen presenting molecules, and the release of IL12.

We were interested in finding out whether the interaction of TLR molecules with alveolar macrophages and dendritic cells would induce the killing of *M. tuberculosis*. We set up an experiment, where we infected alveolar macrophages with *M. tuberculosis*. Then, we stimulated the TLR2 axis, and examined its impact on the survival of mycobacteria. Our results indicate that the activation of TLR2/1, leads to a significant reduction in the number of colony forming units (CFUs) of *M. tuberculosis*.

Since dendritic cells are also an important host cell for *M. tuberculosis* in the lungs, we have conducted the same experiments using dendritic cells. We found that the activation of dendritic cells by the TLR2/1 ligand did not lead to a decrease in the number of CFUs of intracellular *M. tuberculosis*. We concluded that macrophages can perform functions that dendritic cells cannot.

III. The Role of the Vitamin D

We then investigated which molecule is responsible for killing *M. tuberculosis* in macrophages, but not in dendritic cells. In other words, we wanted to know the difference between TLR2/1 ligand activated macrophages and TLR2/1 ligand activated dendritic cells. We conducted a microarray analysis, and obtained a large list of molecules. Then, we tried to identify the molecules that are both regulated and have an impact on antibacterial activity. We have identified one such molecule, the Vitamin D receptor (VDR), which was up-regulated in the TLR2/1 ligand activated macrophages, but not in the dendritic cells.

We decided to focus on the vitamin D receptor for several reasons. First, studies have shown it to be important in mycobacterial immune responses. Second, there is a correlation between low 25(OH) vitamin D serum levels and the risk of developing tuberculosis. Third, vitamin D is expressed in alveolar lymphocytes in tuberculosis. Fourth, and most interestingly, Graham ROOK showed 20 years ago that vitamin D3 enhances the antimycobacterial activity of monocytes.
While vitamin D can be taken nutritionally, sunlight is required in order to transform it to the active form. It is the active form of vitamin D that possesses the biological functions that might protect against intracellular *M. tuberculosis*.

After combining data obtained from the literature with the results of the microarray analysis, we formulated the following hypothesis: The activation of the TLR pathway in human macrophages will increase the levels of intracellular vitamin D, and the expression of the vitamin D receptor. This would then induce the transcription of several genes.

To find out which genes were being transcribed, we tried to identify molecules that had a vitamin D receptor response element, and were involved in killing *M. tuberculosis*. One group of such molecules are the cathelicidins (LL-37). In addition, cathelicidin has many biological functions directed against bacteria, since it belongs to the cationic antimicrobial peptides (CAMPs) family.

We conducted an experiment to examine whether cathelicidin plays a role in fighting *M. tuberculosis*. We incubated extracellular *M. tuberculosis* with increasing concentrations of cathelicidin, and measured the metabolic activity of the mycobacteria. We discovered that cathelicidin is active against *M. tuberculosis*.

We then finalised our hypothesis: The TLR activation of macrophages leads to the up-regulation of the vitamin D dependent pathway. This leads to the increased expression of cathelicidin, which is capable of killing intracellular *M. tuberculosis*.

In order to prove this hypothesis, we first demonstrated that TLR2/1 ligands induce up-regulation of cathelicidin in human macrophages in a VDR-dependent manner. Then, the critical experiment was to examine whether this up-regulation would result in killing *M. tuberculosis*. For that purpose, we infected alveolar macrophages with virulent *M. tuberculosis*, and added a TLR 2/1 ligand. After 72 hours, we monitored the growth of the mycobacteria by measuring the number of CFUs.

As shown earlier, the activation of the TLR 2/1 pathway decreased the viability of *M. tuberculosis*. We repeated the experiment in the presence of an inhibitor of the vitamin D receptor. We discovered that the antibacterial activity was almost entirely inhibited. We were therefore able to confirm our hypothesis, that the killing of *M. tuberculosis* is mediated by vitamin D.

To summarise, the up-regulation of the vitamin D receptor by TLR 2/1 ligands increases the expression of cathelicidins, which then contribute to the killing of intracellular *M. tuberculosis*.

**IV. Vitamin D: An Historical Perspective**

1. **Sanatoriums**

The discovery of the role of vitamin D in fighting tuberculosis can help explain some historical observations. In the 19th century, Hermann BREHMER, a German biology student, suffered from tuberculosis. At the time, the agent of the disease was not known, and thus could not be treated. BREHMER decided to spend the presumably last days of his life in the Himalaya region. However, he was then unexpectedly cured from the disease. When he returned to Germany, he completed his PhD, in which he claimed that tuberculosis was a
curable disease. He then founded the first sanatorium in Silesia in 1854, since he believed the advantage of the Himalaya region was the fresh air. Until the 1930s, sanatoriums remained the method of choice for the management of tuberculosis patients.

Today, we would speculate that the vitamin D levels of sanatorium patients rose significantly when they travelled to high altitudes. Their concentration of antimicrobial peptides was increased, and contributed to the cure of tuberculosis. However, the problem with most sanatoriums was that the patients stayed mostly indoors.

2. UV-Therapy

Few people know that Robert KOCH was not the first scientist to receive the Nobel Prize for tuberculosis research. Two years earlier, in 1903, Danish scientist FINSEN received the Nobel Prize for discovering the use of UV-therapy in the treatment of tuberculosis of the skin (Lupus vulgaris). Again, we would suggest that the UV-exposure of patients has led to increased levels of vitamin D receptor response.

V. Clinical Trials Using Vitamin D

Several clinical trials have already examined whether vitamin D contributes to curing tuberculosis. A double blind, randomized, controlled trial was conducted by Robert WILKINSON. It was conducted in London, where people typically suffer from low vitamin D levels, due to low sun exposure. WILKINSON provided 192 healthy adult tuberculosis contacts with a single oral dose of 2.5 mg vitamin D.

Six weeks later, he drew blood from the participants. He discovered that the blood of the individuals who received vitamin D displayed an increased antmycobacterial activity in-vitro. This indicated that a single dose of vitamin D enhances immunity to mycobacteria. Since vitamin D is known to decrease the T-cell response, it was important to show that the release of the antigen-specific interferon-γ remained unaffected. It was indeed the case, and trials are already planned for testing the use of vitamin D as a treatment for MDR-TB.

VI. Cell-Mediated Immunity

I would now like to shift from the innate immune-response to the adaptive one. It is important to remember that innate immunity is not the major component in the protection against mycobacteria. Generally, cell mediated immunity against M. tuberculosis is based on CD4+ T-cells, whose major function is the production of interferon-γ. The function of interferon-γ is to activate macrophages infected with mycobacteria to produce nitrogen and oxygen radicals, which are capable of combating M. tuberculosis. The second important subset of T-cells are the CD8+ cytotoxic T-cells. They contain granules, which in turn contain several molecules that could take direct antimycrobial action against M. tuberculosis.

1. MHC Class II

I would now like to briefly describe how the antigens reach the cell surface to be recognised by antigen-specific T-cells. At first, the mycobacteria are taken up by macrophages. Then, the mycobacterial antigens are digested by defined mechanisms. Then, the lysosome, which now contains the mycobacterial antigens, fuses with a second compartment of antigen-presenting cells, the MHC class II containing compartments.
Following this fusion, the mycobacterial antigens and MHC class II molecules are located within the same compartment. The MHC class II molecules then transport the mycobacteria antigens to the cell surface. Finally, the T-cell receptor αβ+, CD4+ T cells recognise the antigens, and produce Th1 cytokines such as interferon-γ.

This process is probably the most important element in protection against *M. tuberculosis*. In fact, the reason HIV and TB occur together, is because this process is not functionnal in HIV patients.

2. **MHC Class I**

However, today, we know that CD8+ T-cells also play an important role in the immune-response against *M. tuberculosis*. This raises the question of how the MHC class I molecules, which are located in the cytoplasm, find the antigens. The answer is that some antigens escape from the phagolysosome and enter the cytoplasm, and thereby gain access to MHC class I molecules. This interaction then activates CD8+ cytotoxic T cells, leading to the reorientation of cytotoxic granules. The granules are then able to gain access to the infected cell, with the help of perforin. This, in turn, allows granzymes to enter the infected cell, leading to apoptosis of the host cells.

**VII. CD1**

I have so far overviewed MHC Class II and I. However, in 1995, a third group of antigen presenting cells that might play an important role in the immune response to *M. tuberculosis* was discovered. The group is called CD1, and I believe it is more important than was previously thought. Unlike MHC molecules, which present peptide antigens, CD1 molecules presents lipid antigens. The CD1 antigen presentation pathway is similar to that of MHC class II. There are two established groups of CD1 molecules, and a third is emerging.

1. **Group 1**

The CD1 group 1 molecules includes CD1 a, b, and c. They are non-polymorphic, and have two alleles, which is relevant for vaccination strategies. These molecules are expressed in B-cells and dendritic cells, but not in macrophages. They are present in humans and guinea pigs. Unfortunately, they are not present in mice, in which the majority of immunological research is performed. The antigen presented by this group is lipids, and there is some indication from in-vitro studies that they are protective. All the three molecules in the group are capable of presenting lipid antigens.

2. **Group 2**

Group 2 includes only one member, CD1 d, which is expressed in all antigen-presenting cells. Since it is present in both human and mice, extensive research has already been conducted on this molecule. The antigens are ceramides, which are present in marine sponges and in some bacteria. CD1 d has been shown to offer a certain amount of protection against *M. tuberculosis*, although it is not very impressive.

3. **Group 3**

In 2007, CD1 e has been discovered. It is very similar to the group 1 and 2 molecules on the molecular level. However, it has a completely different function, since it is involved in the
processing of lipids. Thus, it has an indirect effect on the antigen presentation of lipids, but not a direct one.

VIII. CD1 Importance in Vaccination

1. CD1 Advantages

CD1 is important in the context of vaccination for three reasons. First, CD4+ T-cells are not required, since both C8+ and CD4/CD8 negative T-cells respond to CD1 antigens. Thus, this antigen could be useful in the vaccination of HIV-positive patients, who do not have functional CD4+ T-cells. A second advantage is that CD1 is non-polymorphic. Finally, a major challenge in vaccinations is using the most efficient cell type to present the antigen. We know that the only cells capable of inducing primary immune responses are dendritic cells, not macrophages. By using lipid antigens, it is possible to ascertain that the antigen will be presented by dendritic cells.

2. Cytotoxic T-lymphocytes

In the late 1990’s, it was discovered that all the T-cells that respond to lipids have cytotoxic activity. It was then debated whether cytotoxicity is relevant for the protection against tuberculosis. At the time, most people believed that CTL activity leads to the lysis of the infected macrophage, which would then lead to the uptake and killing of bacilli by activated macrophages.

However, other scenarios are also possible. Let us consider a macrophage infected with mycobacteria, which is then killed. The macrophage could be lysed, and then the bacilli could spread throughout the immune system to different organs, and cause disease. Thus, theoretically, lysing the host cells could actually cause damage.

A second scenario is that the lysis could result in the release of \textit{M. tuberculosis}, which would then trigger freshly recruited monocytes to take up the mycobacteria.

The third, and optimal scenario, is that the interaction of cytotoxic T-cells with the macrophages infected with \textit{M. tuberculosis}, could directly result in killing the bacilli.

3. Role of CD8+

We thus wondered how our CD1-restricted cytotoxic CD8+ T-cells would interact with a macrophage infected with \textit{M. tuberculosis}. We therefore infected dendritic cells with \textit{M. tuberculosis}, and added CD1 restricted CD8+ T-cells. 72 hours later, we measured the number of CFUs that survived the attack, in-vitro. We discovered that the viability of intracellular mycobacteria decreased significantly in the presence of CD8+, but not in the presence of other CD1-restricted T-cell subsets, such as the double negative T-cells. Thus, we were able to demonstrate that CD8+ T-cells are capable of killing \textit{M. tuberculosis}.

4. Role of Granulysin

We then examined the molecules contained in the granules of C8+ T-cells. We wanted to know whether they could account for the antibacterial activity we have observed. None of the molecules were able to kill \textit{M. tuberculosis} except granulysin, which has already been shown to have antibacterial activity against various pathogens.
Granulysin is expressed in CD8+ cytotoxic T-cells, which recognise lipid antigens. It is expressed in patients that suffer from leprosy, which is a useful model for studying immune responses. When leprosy patients develop tuberculoid leprosy, it indicates that their immune-response is robust, and that they are thus able to contain the infection. However, as the cell-mediated immunity drops, some patients develop more severe forms of leprosy, such as lepromatous leprosy. We have obtained biopsies from the two types of leprosy patients, and then tested for granulysin expression.

The biopsies of patients who suffered from lepromatous leprosy, and therefore had no protection against the mycobacteria, did not contain granulysin. We were therefore able to infer indirectly that the expression of the antimicrobial peptide granulysin correlates with protection against mycobacterial disease. Unfortunately, since granulysin is not expressed in mice, further in vivo experiments cannot be conducted at the moment.

IX. Immunology Implications for Diagnostics

I would like to show how this immunological research guides the development of new diagnostic tools of tuberculosis. There are currently several ways to diagnose tuberculosis by immunological means.

1. Morotest

When using the Morotest, we place *M. tuberculosis* antigen on the skin of babies. If the baby has T-cells that are specific for mycobacteria, these T-cells would recognise the antigen presented by the dendritic cells in the skin.

2. The Tine and Heaf Tests

When using the Tine Test, tuberculin is injected into the skin. Only the patients who have had previous contact with mycobacteria would respond. In the British Heaf test, the antigen is put in the skin, and then punched into the subcutis.

3. Intracutaneous Tests

These tests are no longer used in many countries. The current state of the art are intracutaneous tests, such as the tuberculin skin test (TST). The advantage of these tests is that a defined amount of mycobacterial antigens are injected into the skin.

4. QuantiFERON

The use of Interferon-γ release assays (IGRA) tests is becoming more and more important. These tests currently include the QuantiFERON, the QuantiFERON-TB Gold, the QuantiFERON-TB Gold In tube, and the TB-Spot TB.

During the test, blood is drawn from the patient into a tube. The tube is coated with a mycobacterial antigen, specific for *M. tuberculosis*. These antigens will be presented to T-cells. Activated T-cells would then produce IFNγ. The test can be used as a measurement of T-cell specific responses, since no other type of cell in the blood is capable of producing IFNγ. Furthermore, merely patients who have encountered *M. tuberculosis* in the past would produce IFNγ. Others will not have any effector memory cells. Thus, their T-cells would not recognise the antigen, and no IFNγ would be produced.
a. Advantages of QuantiFERON

QuantiFERON is considered an extremely useful tool for several reasons. First, it provides high specificity to *M. tuberculosis*. This specificity is due to the fact that the antigens used are present in the RD-1 locus of *M. tuberculosis*. Thus, since they are not present in BCG, there is no risk of cross-reaction when testing vaccinated patients.

Second, it is more sensitive than TST in testing immuno-compromised patients, such as HIV patients. Third, it has a comparable sensitivity with TST for the diagnosis of active tuberculosis. Unfortunately, the sensitivity of both tests is moderate in this respect.

Fourth, it is easy to perform, since it requires drawing blood once. Finally, it has no booster effect. Typically when a TST is performed once a year, there is a risk that the repetitive exposure to the antigen might lead to a false positive result after a few years. However, in the case of QuantiFERON, this risk is eliminated, since the patient has no direct contact with the antigen.

b. Indications

The QuantiFERON can be used for several purposes. First, for the diagnosis of latent (and active) tuberculosis. Second, for screening contacts. Third, for screening of (often BCG-vaccinated) immigrants, whose TST tests is difficult to interpret. Fourth, for the monitoring of healthcare workers, in order to solve the problem of repetitive testing.

c. Limitations

Tuberculosis diagnosis tests still suffer from several weaknesses. First, neither TST nor QuantiFERON-Gold discriminate between latent and active tuberculosis. Second, both are not suitable for monitoring the course of disease. Patients that have been diagnosed with *M. tuberculosis* at any time point of their life, would obtain positive results in both tests. Finally, in the case of QuantiFERON, the amount of IFN-γ release does not correlate with the success therapy or the severity of the clinical response, since the effector memory T-cells are long-lasting. It is thus impossible to use the test for monitoring therapy.

X. Question and Answer Session

Participant

Do other toll-like receptors, such as TLR4 or TLR9 induce mechanisms that kill the bacteria, as TLR2 does?

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We have conducted experiments with TLR4, and have found no antibacterial activity. Other Toll-like receptors are not known to interact with *M. tuberculosis*, with the exception of TLR9. So far, it is not know whether TLR9 modulates the killing of the bacteria.