Monitoring host response to *Mycobacterium Tuberculosis* infection

Abstract

Mycobacterium tuberculosis (Mtb) is a widespread pathogenic bacterial species which causes 1.5 million people death every year in the world (1-3). Moreover, *M. tuberculosis* infected about one third of world population, 5-10% of which developed acute clinical disease (4). To find a cure for this pathogen we need to understand how Mtb infects human beings and how the host environment responds to Mtb infection. Through lipid metabolism, Mtb can manipulate host gene expression to benefit its own sustention. Among those lipids, Trehalose 6,6'-dimycolate (TDM) is a cell wall glycolipid is proved to induce host response which is most similar to Mtb infection (6, 7). Therefore, we built reporter cell lines that can sense TDM induction, enabling us to monitor the progression by examing the GFP and luciferase expression.

Introduction

Mycobacterium tuberculosis (Mtb) is a widespread pathogenic bacterial species that killed 1.5 million people and infected approximately one third of world population (1, 2). Especially, TB disease mainly distributes in developing countries in Asia and Africa where about 80% of the population has been test positively infected (1). Additionally, people with compromised immune system, such as HIV patients, are much easier infected by TB and develop active diseases (3). However, the features of TB itself make it difficult to develop a rapid, convenient method to diagnose TB. *M. tuberculosis* divides every 16-20 hours, much longer than other bacteria do which usually divide in less than an hour (5). The bacterium has a hydrophobic cell wall which makes it more difficult to stain the cells (3). Besides, TB is extremely difficult to control since it can easily transmit through aerosol droplets from coughing,

sneezing, speaking and spitting (3). The infectious bacteria in the aerosol droplets may be inhaled by non-infected persons, and phagocytosed by macrophages (8), which induces an inflammatory response and attracts mononuclear cells to accumulate around the infected macrophages (8). Eventually, peripheral cells synthesize and secrete fibrous cuff to isolate the complex, which is called granuloma. Once the granuloma is formed, it is almost impossible for drugs to penetrate. At the late stage of TB infection, the structural granuloma corrupts (8) and releases thousands of infectious bacteria in the air, which continue similar infection cycles on other persons.

The lipid metabolism is proved vital for Mtb infection. Intracellular Mtb inside infected macrophage will synthesize and secrete cell wall lipid, which will accumulate in numerous small vesicles and then be exocytosed into the extracellular matrix (9). The vesicles can invade into uninfected macrophages and lead to the dysfunction of theses uninfected macrophage, which are induced to become foamy macrophage now. In the later stage of infection, foamy macrophages will die because of inflammation and release their necrotic debris inside the enclosed granuloma (9). As this debris accumulates, the granuloma will eventually corrupt and release the live Mtb inside, developing an active disease (9). The mechanism of Mtb infection related to lipid metabolism has been fully demonstrated, however, epigenetically, how the host responds to these lipids induction during infection, including different genes expression level changes and the order of these changes, are still unknown. Therefore, it is of great importance to monitor the progress of Mtb infection, in order to understand the mechanism of Mtb infection, based on which we can develop a cure against Mtb. Previously a microarray had been conducted in Dr. Russel's lab (Fig1), looking at the genes up-regulation and down-regulation (10). 11 genes (Table 1) are highly

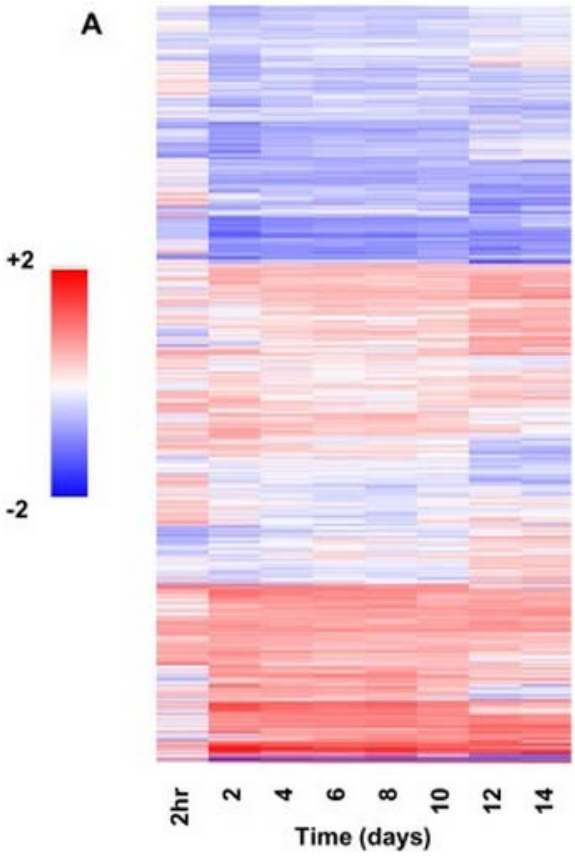

Fig 1 Microarray for gene expression of infected macrophage

up-regulated (we focus on genes that are up-regulated in the work), allowing us to build reporter cell lines to monitor the progression of Mtb infection. Therefore, we developed reporter cells lines that can sense the TDM induction and express GFP and luciferase, allowing us to keep track of the infection progress. Then we tested the GFP expression in vitro and luciferase expression in vivo, to see how efficient our reporter cell lines can sense the TDM induction.

Gene	Functional group	Gene	Functional group	Gene	Functional group
Angiotensin cleavage enzyme	Activation/Stress markers with antimicrobial activity	Arginase II	Alternative activation markers	Adipo-philin	Lipid metabolism
		Transferrin			
Apolipoprotin L		MMP2	Extracellular Matrix Degradation	Prosa-posin C	
Lipocalin 2		MMP9			
FAU		MMP10			

Table 1 Genes up-regulated during Mtb infection in infected macrophage

Results

1. Building reporter cell lines

We have a table of 11 genes (Table1) that are up-regulated during TB infection, kindly provided by Prof. Russell based on a microarray experiment. We located the genes' sequence in the gene bank (http://dbtss.hgc.jp/) and extracted the DNA sequence of their promoters.

Using modern molecular biology we successfully inserted different promoters into one specific vector with GFP and luciferase gene (Fig 2). The inserted promoters of the 11 genes can be induced by TDM, triggering the expression of GFP and luciferase.

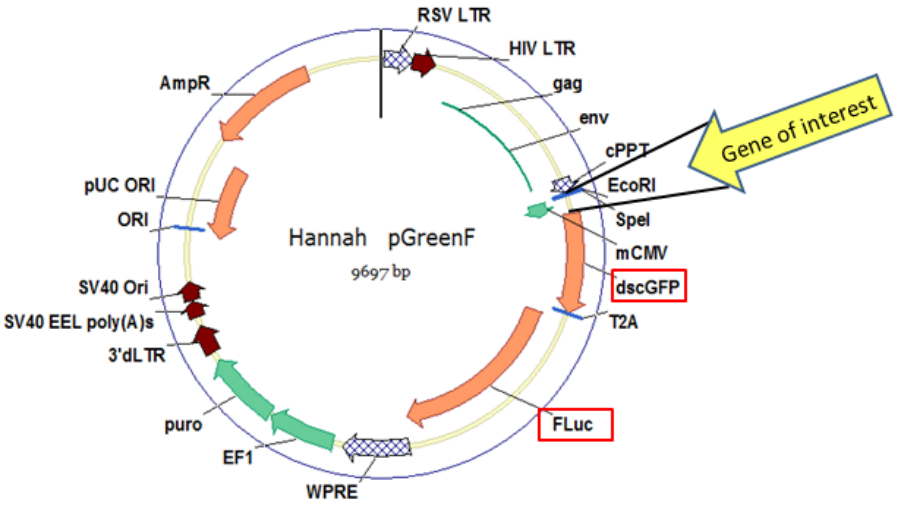

Fig 2 Gene map of vector pGreenFire. Gene inserted site is indicated with yellow arrow. Important genes include GFP and luciferase are highlighted in red boxes.

After successfully making these constructs, we transfected these plasmids with virus generating vectors to 293T cell lines to generate virus containing our plasmids. By collecting these virus media after transfection 48h, we infected our target cell line raw macrophage, allowing integration of our plasmids into its genome. We cannot see green fluorescence without induction since the promoters we inserted need to be induced by TDM. We introduce a positive control test our infection efficiency. pCMV is another vector with CMV self-driven promoter, which is our positive control. We can see green fluorescence in macrophage cell after infection.

Then we used puromycin to screen these cell lines and cultured them from single colonies. Totally we have 11 cell lines. Each cell lines have 8 single colonies cell lines in average. (Table 2)

Reporter	Clonies(picked up/ growed up)	Gene	Clonies(picked up/ growed up)	Gene	Clonies(picked up/ growed up)
Angiotensin cleavage enzyme (Ace)	12/14	Arginase II (Arg)	12/16	Adipophilin (Adi)	11/12
		Transferrin (Trf)	12/16		
Apolipoprotin L (apoe)	12/12	MMP2	12/16	Prosaposin C (ProC)	7/12
Lipocalin 2 (LCN2)	8/12	MMP9	9/12		
FAU	12/14	MMP10	12/13		

Table 2 Single colonies picked up and grow up for different reporter cell lines

2. In vitro test of reporter cell lines

We used conditional media and LPS to screen these cell lines to get the most “active” reporters. Conditional media contain a lot of cytokines which are induced by TDM. Therefore, we used conditional media and LPS to induce GFP and luciferase expression in our cell lines. Observing GFP under fluorescence microscope confirmed that our cell lines’ promoter can be triggered by conditional media and LPS (Fig 3 and Fig 4).

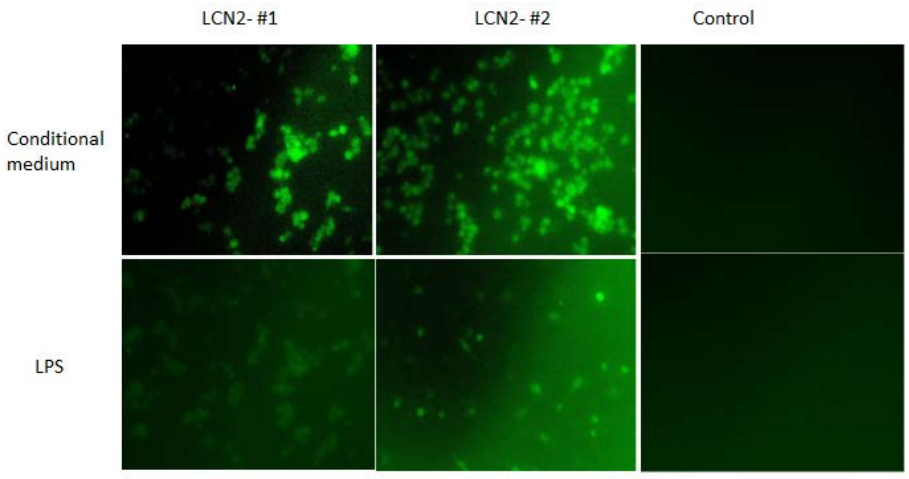

Fig 3 Reporter cell line LCN2 induced by conditional media and LPS shows green fluorescence, which is not showing in the control.

So the reporter cell line LCN2 is induced to express GFP by both condictional media and LPS. Same is MMP9, whose 7 single colony cell lines are all express GFP. While the other cell lines like Adipocalin, Apoliprotin L and Prosaposin C did not show high green fluorescence expression.

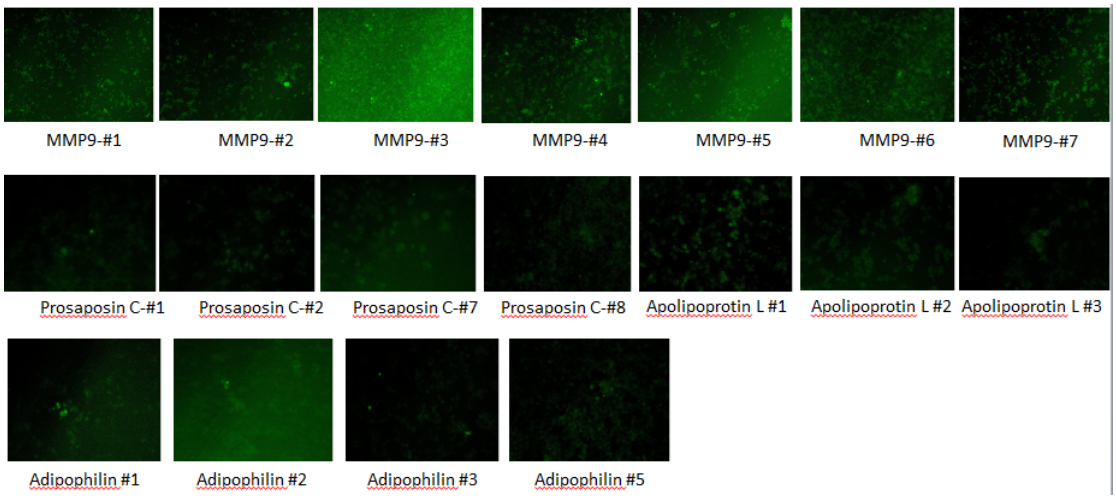

Fig 4 Four reporter cell lines induced by conditional media and LPS. Pictures for LPS induction and control are not shown.

We tried to quantify our result by testing the luminescence by adding our cell lysate to luciferase substrate (Fig 5).

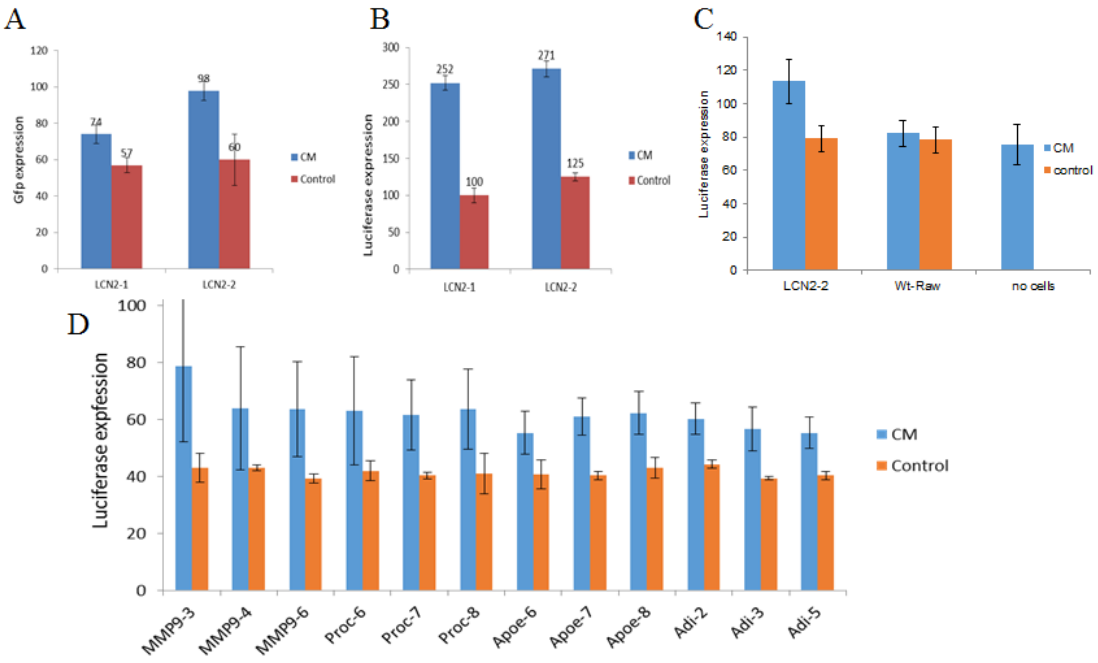

Fig 5 Quantitative fluorescence and luminescence signal generated by different cell lines induced by conditional media.

It turned out that there is always some background signal (Fig 5C). Then we figure out that is the from the lysis buffer we used to lyse the cells. Figure 5D shows some different cell lines' results for luciferase expression. Although we can see notable difference between experiment group and control group with and without conditional media induction, the value of the luminescence is far lower than normal value which range from 10^3 to 10^4. We speculated that these in vitro tests cannot mimic the real inflammatory response of cell lines to TB infection inside the granuloma, which makes it important to move our experiment into in vivo test.

3. In vivo test of reporter cell line

We employed the TDM model developed by Dr. Russell (See method). After injecting matrigel containing our cell lines and TDM coated beads for different time point, we inject luciferase substrate directly in the matrigel and test the luminescence signal (Fig 6). We kept these mice to get time points data, which can tell us different cell lines'

activity at different time points.

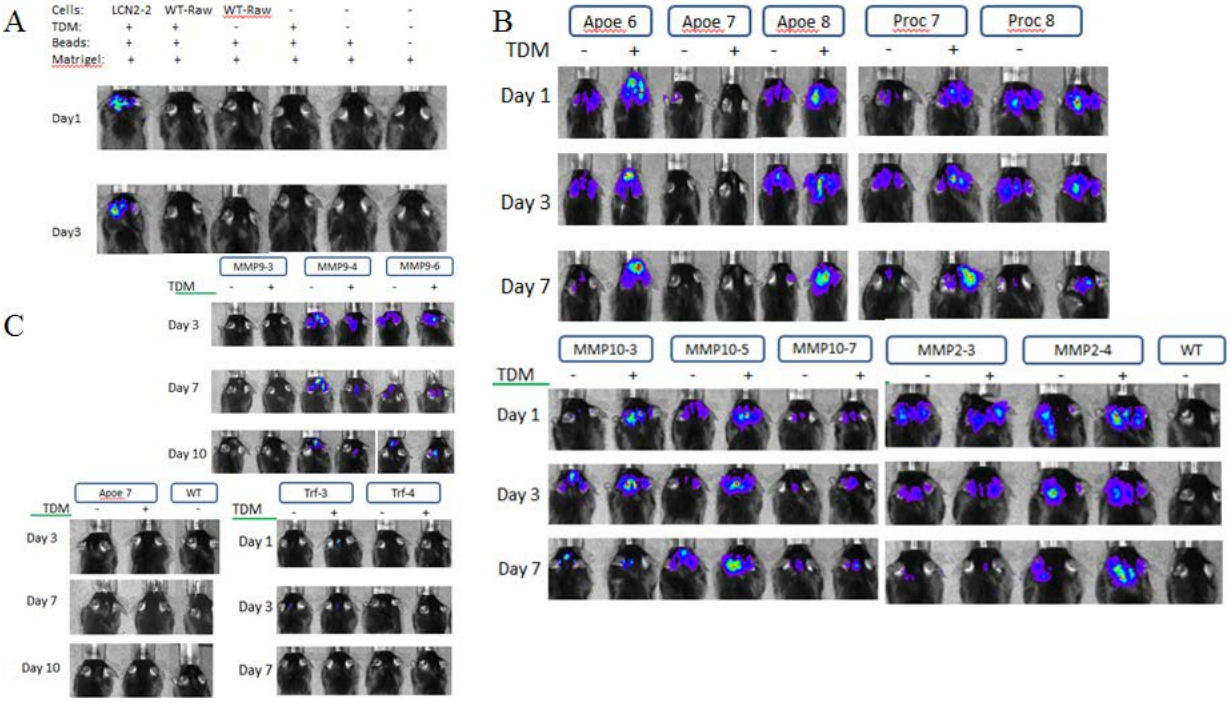

Fig 6 Luminescence signal in living mice indicates the luciferase expression level of different cell lines, induced by TDM. Data for Arginase and Adipophilin are not shown.

First of all we need to be sure that the TDM and the beads will not induce background signals. In Fig 6A, we proved that neither the TDM nor the beads will generate luminescence signals. Besides, the macrophage without our inserted genes cannot generate background signal, either. After we proved the effectiveness of this TDM model, we use it to test our single colonies cell lines. We mixed our reporter cell lines within this bead mitrigel, with or without TDM, and injected into the mice's scuff. In day 1, 3, 7 and 10, we injected luciferase substrate into the same spot, and tested the luminescence signal in a IVIS machine. Fig 6B shows some cells lines are expressing luciferase in day1, 3 and 7 including apoe-6, -8, and proC-7, -8 and so on. But some cell lines did not show luciferase expression (Fig 6C), like apoe-7, MMP9s and Transferrin-3, -4. Note that apoe-7 show different behavior with apoe-6 and apoe-8, meaning it is of importance to build the single colonies since different cells, even from the same lineage, may show different gene expression ability.

Discussion

In summary, we built reporter cell lines that will sense TDM induction and test them in vitro with GFP microscope and luciferase expression, and monitor the luminescence in living mice using the TDM model. We totally screened and characterized 17 clones in TDM mouse models. We have macrophage reporter lines from single colonies for LCN2, Apolipoprotein, Prosaposin C, MMP2 and MMP10

that work in the TDM mouse model.

Interestingly, the apoe and proc cell lines did not get express strong green fluorescence signal in the vitro test, which have high luciferase expression in the vivo test. The MMP9, in the other hand, express GFP in vitro relatively strong, compared to its luciferase expression in vivo. Since both GFP and luciferase are driven by the same promoters inserted, both of them should have similar expression level. However, our results show inconsistence to the in vitro and in vivo experiment. One explaination is that the difference of in vitro and in vivo tests can lead to the different behaviors of the same cell lines. This illustrates the complexity in living organism which has so many factors correlated that might not be understood by the behavior of cells in vitro, indicating that there may be no correlation between the macrophage reporters' activities in vitro and in vivo.

In the future, we are going to test our reporter cell lines in the TB model, using lived TB bacteria in the matrigel, which can mimic the TB infection in human better than the TDM model.

Method

Making plasmid constructs. After we get the 11 promoters' sequence from database, we designed primers and used PCR techniques to get DNA of our interest. Table 1 showed all the primers we used for PCR. Using restriction enzymes EcoRI and XbaI or ClaI and SpeI to digest our empty pGreenFire vector and PCR products, we were able to insert our promoter DNA into the plasmid.

Transfection and infection. We used LT1 transfection to introduce our own constructs with two lenti-virus helper vectors, pVSVG, the envelop vector and pds.217, the packing vector into 293T cell line. After 48h incubation, we harvested the media containing virus and added it into the raw macrophage. After 48h, we checked the fluorescence expression of positive control to rule out experimental bias.

Puromycin screening and making single colonies. We used 2ug/ml puromycin to screen our macrophage cell lines. After the cell lines stopped dying and regained growing, we counted the cell number and place 200 single cells into a 10cm-diameter disc. 4 or 5 days are needed for these single cells to grow into a colony which can be seen in naked eyes. Then we used cloning rings to pick up single colonies into 96-well plate.

In vitro test. We used 2ug/ml LPS to induce fluorescence of our cell lines. Conditional media is made using the TDM model. After injection of matrigel with TDM coated beads into mice scuff for 4 days, we scarified the mice and took the matrigel out and place it into phenol red-free DMEM media. After 7 days incubation, we collected and filtered the media, which is our conditional media. Quantitative test of luminescence is conducted in Dr. Dan Luo's lab. We cultured our reporter cell lines in 96-well plate, and added conditional media after 2 days. 2 more days later we removed the media and added lysis buffer. Then we mixed the supernatant with same volume of luciferase substrate and read the signal one by one.

In vivo test. We employed the TDM mouse model, developed by Prof. Russel (11). After mixing our reporter cell lines with beads and Matrigel, with or without TDM, we injected the mixture into the scuff of mice. On day 1, 3, 7, we injected luciferase substrate into the same spot to test the luminescence signal in the IVIS Lumina XR.

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