Executive Summary:

Despite tremendous efforts to fight tuberculosis (TB), this disease remains a major health problem, particularly in low-income developing countries. TB causes 1.7 million deaths annually worldwide, and up to one third of the global population is estimated to carry latent Mycobacterium tuberculosis infection. The notion that some M. tuberculosis lineages, such as the W-Beijing genotype, are more virulent than others is supported by results obtained with animal and cellular models. Such variations between strains probably result from genetic diversity, which seems to be greater in mycobacteria than previously thought. Recent reports have shown that different clinical isolates of M. tuberculosis produce different proteins and lipids, according to their genetic lineage, and this probably affects mycobacterial virulence and the host immune response to infection. W-Beijing-specific expression of a phenolic glycolipid, for instance, has been associated with inhibition of the host immune response and the "hyperlethality" of this lineage. The inhibition of the host immune response by mycobacterial strains of the W-Beijing family has recently been confirmed in vivo. The W-Beijing lineage is one of the most successful M. tuberculosis families, in terms of morbidity and mortality, worldwide. These strains are currently attracting considerable attention worldwide, because they display important pathogenic features and are frequently associated with resistance to antimicrobial drugs, at least in some parts of the world. W-Beijing strains may be more able to acquire antimicrobial drug resistance than other strains, due to mutations in putative mutator genes and a higher level of adaptability. Strains of the W-Beijing lineage are responsible for 80 to 90% of TB cases in China, where TB incidence may exceed 50 per 100,000 in average (over 80 per 100,000 in the Shanghai area). The remarkable ability of these strains to spread locally in large clonal lineages probably reflects their high level of fitness/transmissibility and their adaptation to the infection of human populations. The predominance of the W-Beijing lineage probably results from genetic advantages, including unidentified virulence factors and the induction or modification of specific host cell responses not yet thoroughly investigated. The TB-VIR project aimed to unravel the links between differential host responses to M. tuberculosis infection and mycobacterial genetic diversity and virulence at the global genomic and post-genomic levels within the W-Beijing family, and between the W-Beijing family and other M. tuberculosis families, to improve our understanding of the epidemiological success of this particular lineage.

Important results have been obtained and published from the TB-VIR activity. This has helped increase our understanding of M. tuberculosis virulence and anti-mycobacterial immune response, in particular regarding the W-Beijing family of strains.

The genetic diversity within the W-Beijing family has been further elucidated using informative SNPs, and the role of transposable elements in the peculiar virulence of this family has been reported. Genotype-phenotype correlations could be established. In particular we showed that host cell response to W-Beijing sublineages is rather homogeneous and clearly impaired as compared to the immune response to other mycobacterial lineages. This has been shown using a combination of innovative approaches such as global gene expression profiling and protein survey. Candidate vaccine strains have also been isolated through TB-VIR activity, and are now tested in animal models.

In summary the TB-VIR project has been very successful not only to provide new knowledge on M. tuberculosis, and the W-Beijing lineage in particular, but also to generate new possible intervention tools (like vaccine candidates), and to promote euro-asian collaborations between laboratories in France, Germany and Spain, and laboratories in China and Korea.

Project Context and Objectives:

Despite tremendous efforts to fight tuberculosis (TB), this disease remains a major health problem, particularly in low-income developing countries. TB causes 1.7 million deaths annually worldwide, and up to one third of the global population is estimated to carry latent Mycobacterium tuberculosis infection (1). TB incidence varies from 7 per 100,000 in Northern Europe to up to 300 per 100,000 in parts of Africa. In Eastern Europe, the incidence of TB now exceeds 100 per 100,000, and the proportion of multidrug-resistant (MDR) M. tuberculosis strains is high (1). Furthermore, extensively drug-resistant (XDR) TB has emerged worldwide as a threat to public health and TB control, raising concerns about a possible future epidemic of virtually untreatable TB (2, 3). The frequency and distribution of XDR TB cases has been recently described by the American CDC, which determined that, during the 2000 to 2004 period, 20% of the 17,690 TB isolates were MDR and 2% were XDR (4). Population-based data on the drug susceptibility of M. tuberculosis isolates have also been obtained from the United States (for 1993-2004), Latvia (for 2000-2002), and South Korea (for 2004), where 4%, 19%, and 15% of MDR TB cases, respectively, were XDR.

Drug treatment has helped to decrease the incidence of TB in countries with effective public health systems, but vaccination is considered the most cost-effective intervention in most countries. However, the Mycobacterium bovis BCG vaccine currently in use, although effective against severe forms of TB in children, is of minimal and variable efficacy against the much more common and contagious pulmonary forms of TB in adults, particularly in TB-endemic areas, and therefore requires improvement (5).

Following M. tuberculosis infection, only 5 to 10% of immunocompetent individuals develop TB (6). Environmental factors probably play an important role, but it is increasingly thought that the virulence and load of the infecting strain, together with host genetic factors, such as polymorphisms in genes involved in anti-mycobacterial immunity, contribute to such differences between infected individuals.

On the host side, genetic variations in genes encoding key components of the immune system, such as the vitamin D receptor (7), monocyte chemoattractant protein (MCP)-1 (8), and the C-type lectin DC-SIGN (9), have been found to increase susceptibility to TB in certain parts of the world.

On the pathogen side, several reports have strongly suggested that the severity and clinical manifestations of TB depend on the immunogenicity and pathogenicity of the infecting strain of M. tuberculosis, these properties varying considerably between strains (10). A recent study on almost 700 clinical isolates collected from 1996 to 2000 in Arkansas (US) concluded that patients infected with M. tuberculosis W-Beijing strains were three times more likely to develop extrathoracic TB than patients infected with strains of other lineages (11). However, some other studies reported no such association between M. tuberculosis genotype and clinical features of TB (12-14).

The notion that some M. tuberculosis lineages, such as the W-Beijing genotype, are more virulent than others is supported by results obtained with animal and cellular models. For instance, the W-Beijing isolates HN878 and W4 induce more IFN-? and less Th1 cytokines in vitro and in vivo in mice than another non-W-Beijing isolate, CDC1551 (15-17), and are associated with lower survival rates in infected animals. Such variations between strains probably result from genetic diversity, which seems to be greater in mycobacteria than previously thought (18, 19). Recent reports have shown that different clinical isolates of M. tuberculosis produce different proteins (20) and lipids (21, 22), according to their genetic lineage, and this probably affects mycobacterial virulence and the host immune response to infection. W-Beijing-specific expression of a phenolic glycolipid, for instance, has been associated with inhibition of the host immune response and the "hyperlethality" of this lineage (21). The inhibition of the host immune response by mycobacterial strains of the W-Beijing family has recently been confirmed in vivo (23). The W-Beijing lineage is one of the most successful M. tuberculosis families, in terms of morbidity and mortality, worldwide (24).

On the basis of IS6110 typing and other genotyping methods, including spoligotyping, single nucleotide polymorphisms (SNPs), genotyping and large sequence polymorphism (LSP) analysis, M. tuberculosis strains of the W-Beijing family have been detected almost worldwide, in Asia, Russia, Europe, and North and South America (13), and new unexpected routes of transmission have been identified for these strains (25). This genotype was first identified in M. tuberculosis strains isolated in the Beijing area of China, hence its name (26).

These strains are currently attracting considerable attention worldwide, because they display important pathogenic features (29, 30) and are frequently associated with resistance to antimicrobial drugs, at least in some parts of the world (13, 31). W-Beijing strains may be more able to acquire antimicrobial drug resistance than other strains, due to mutations in putative mutator genes and a higher level of adaptability (Figure 2) (32). The W-Beijing genotype also contains a much larger number of IS6110 copies than other lineages (33). It has been demonstrated that IS6110 may increase the expression of neighboring virulence genes by generating new promoter sequences capable of driving their expression (34).

MDR-TB hot spots worldwide include China, Russia and India (35). The high frequency of drug-resistant and MDR strains of M. tuberculosis remains a serious problem for TB control in China (36, 37). Strains of the W-Beijing lineage are responsible for 80 to 90% of TB cases in China, where TB incidence may exceed 50 per 100,000 in average (over 80 per 100,000 in the Shanghai area). The remarkable ability of these strains to spread locally in large clonal lineages probably reflects their high level of fitness/transmissibility and their adaptation to the infection of human populations (28).

The predominance of the W-Beijing lineage probably results from genetic advantages, including unidentified virulence factors and the induction or modification of specific host cell responses not yet thoroughly investigated.

This project aimed to unravel the links between differential host responses to M. tuberculosis infection and mycobacterial genetic diversity and virulence at the global genomic and post-genomic levels within the W-Beijing family, and between the W-Beijing family and other M. tuberculosis families, to improve our understanding of the epidemiological success of this particular lineage.

The goals of the present project included:

1) Deciphering M. tuberculosis genetic diversity using highly discriminative technologies, and extracting strains representative of the W-Beijing subgenotypes, and of other genotypes. Genetic diversity of M. tuberculosis clinical isolates collected in the Shanghai area (China), where nearly 90% of the strains are expected to belong to the W-Beijing family, was investigated using spoligotyping, IS6110-RFLP profiling, MIRU-VNTR, RD-, and SNP-based genotyping. The Fudan University, in close collaboration with the Center for Disease Control (CDC) in Shanghai, has access to over 9,000 clinical isolates collected since 1999. At least 1,000 isolates were selected for highly discriminative genotyping. In particular, SNP-based genotyping, a new and very promising technique for the genotyping of mycobacteria and other pathogens, was carried out with the technical support of a SME, IntegraGen S.A. Diversity and evolution in the W-Beijing lineage have recently been correlated to SNPs in genes involved in DNA repair (32) or other genes (27, 33), and with LSPs or regions of difference (RD, (28)). SNPs provide a unique tool for phylogenetic studies, as they are less susceptible to the influence of selective pressure than other genetic markers, such as LSPs, and are unlikely to display the convergence sometimes observed with spoligotype or MIRU-VNTR markers. Selectively neutral SNPs should accumulate at a uniform rate and can thus be used to measure divergence. Here (WP2, see below), we aimed to make full use of the power of SNP-based phylogenies as a "gold standard" for assessing the accuracy of other DNA typing methods. This technique was compared with the most discriminative method currently in use, the MIRU-VNTR genotyping method (38), and with RD-based genotyping (28). In addition, the effect of IS6110 insertion on the expression of putative virulence genes was be investigated. Clinical isolates were clustered into lineages and sublineages and ~10 strains, representative of regional diversity, were selected for functional studies.

2) Understanding how host cells respond differentially to infection with M. tuberculosis W-Beijing subgenotypes and other genotypes. Comparative analysis of the changes in host cell gene expression following the infection of mouse and human macrophages with M. tuberculosis strains representative of the diversity of W-Beijing strains and with strains of other genotypes were realized. In particular, we carried out systematic analysis of changes in the host cell transcriptome in response to M. tuberculosis infection, using an approach combining the methods of experimental biology, bioinformatics and computational biology.

3) Identifying virulence genes in M. tuberculosis W-Beijing using functional genomics, and investigating mycobacterial virulome diversity in W-Beijing subgenotypes and in other genotypes. A genome-covering M. tuberculosis W-Beijing mutant library, generated by Partner 12 (IPAS), was screened by Partner 1 (CNRS) in host macrophages, using signature-tagged transposon mutagenesis (STM) and high-throughput confocal microscopy, a well established technique in the laboratory of Beneficiary 6 (IPK). Partner 6 (IPK) does not belong to an ICPC, but the participation of this partner in the project is invaluable, as high-throughput confocal microscopy will provide us with highly innovative and unique opportunities for identifying mycobacterial genes involved in macrophage parasitism by M. tuberculosis. This work allowed identifying virulence genes on a global, genome-wide level in a W-Beijing M. tuberculosis strain. Genetic diversity (SNPs, InDels etc.) in the genes identified here were subsequently investigated in representative members of the W-Beijing subgenotypes and of other genotypes.

4) Assessing differential host immune and pathological response to M. tuberculosis W-Beijing subgenotypes and other genotypes. Molecular and cell biology, biochemistry and immunology experiments were conducted in order to investigate the role of the host and mycobacterial genes identified by activities 1 to 3 in mycobacterial virulence and in the immune response to M. tuberculosis infection in vitro and in vivo. In particular, mycobacterial virulence and the immune response to infection with various representative strains were assessed in vitro in cultured macrophages, and in vivo in the mouse model of M. tuberculosis infection. A meta-analysis of the data generated through the activities described above made it possible to describe fine genotype-phenotype associations between M. tuberculosis genotypes and subgenotypes, and mycobacterial virulence and immunopathogenicity.

5) As we considered that a strong and integrated management of the above generated knowledge is an essential part of the success of this project, one WP (WP1) was dedicated to scientific and knowledge management.

References:

1. Frieden, T.R., T.R. Sterling, S.S. Munsiff, C.J. Watt, and C. Dye. 2003. Tuberculosis. Lancet 362:887-899.
2. Raviglione, M. 2006. XDR-TB: entering the post-antibiotic era? Int J Tuberc Lung Dis 10:1185-1187.
3. Raviglione, M.C., and I.M. Smith. 2007. XDR tuberculosis--implications for global public health. N Engl J Med 356:656-659.
4. 2006. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 55:301-305.
5. Kaufmann, S.H. 2006. Tuberculosis: back on the immunologists' agenda. Immunity 24:351-357.

6. Kaufmann, S.H. 2001. How can immunology contribute to the control of tuberculosis? Nat Rev Immunol 1:20-30.
7. Wilkinson, R.J., M. Llewelyn, Z. Toossi, P. Patel, G. Pasvol, A. Lalvani, D. Wright, M. Latif, and R.N. Davidson. 2000. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet 355:618-621.
8. Flores-Villanueva, P.O., J.A. Ruiz-Morales, C.H. Song, L.M. Flores, E.K. Jo, M. Montano, P.F. Barnes, M. Selman, and J. Granados. 2005. A functional promoter polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to pulmonary tuberculosis. J Exp Med 202:1649-1658.
9. Barreiro, L.B., O. Neyrolles, C.L. Babb, L. Tailleux, H. Quach, K. McElreavey, P.D. Helden, E.G. Hoal, B. Gicquel, and L. Quintana-Murci. 2006. Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. PLoS Med 3:e20.
10. Malik, A.N., and P. Godfrey-Faussett. 2005. Effects of genetic variability of Mycobacterium tuberculosis strains on the presentation of disease. Lancet Infect Dis 5:174-183.
11. Kong, Y., M.D. Cave, L. Zhang, B. Foxman, C.F. Marrs, J.H. Bates, and Z.H. Yang. 2007. Association between Mycobacterium tuberculosis Beijing/W Lineage Strain Infection and Extrathoracic Tuberculosis: Insights from Epidemiologic and Clinical Characterization of the Three Principal Genetic Groups of M. tuberculosis Clinical Isolates. J Clin Microbiol 45:409-414.
12. Borgdorff, M.W., H. Van Deutekom, P.E. De Haas, K. Kremer, and D. Van Soolingen. 2004. Mycobacterium tuberculosis, Beijing genotype strains not associated with radiological presentation of pulmonary tuberculosis. Tuberculosis (Edinb) 84:337-340.
13. Glynn, J.R., J. Whiteley, P.J. Bifani, K. Kremer, and D. van Soolingen. 2002. Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: a systematic review. Emerg Infect Dis 8:843-849.
14. van Crevel, R., R.H. Nelwan, W. de Lenne, Y. Veeraragu, A.G. van der Zanden, Z. Amin, J.W. van der Meer, and D. van Soolingen. 2001. Mycobacterium tuberculosis Beijing genotype strains associated with febrile response to treatment. Emerg Infect Dis 7:880-883.
15. Manca, C., L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J.M. Musser, C.E. Barry, 3rd, V.H. Freedman, and G. Kaplan. 2001. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. Proc Natl Acad Sci U S A 98:5752-5757.
16. Manca, C., M.B. Reed, S. Freeman, B. Mathema, B. Kreiswirth, C.E. Barry, 3rd, and G. Kaplan. 2004. Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. Infect Immun 72:5511-5514.
17. Manca, C., L. Tsenova, S. Freeman, A.K. Barczak, M. Tovey, P.J. Murray, C. Barry, and G. Kaplan. 2005. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res 25:694-701.
18. Fleischmann, R.D., D. Alland, J.A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J.F. Kolonay, W.C. Nelson, L.A. Umayam, M. Ermolaeva, S.L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, W.R. Jacobs Jr, Jr., J.C. Venter, and C.M. Fraser. 2002. Whole-genome comparison of Mycobacterium tuberculosis clinical and laboratory strains. J Bacteriol 184:5479-5490.

19. Gutacker, M.M., J.C. Smoot, C.A. Migliaccio, S.M. Ricklefs, S. Hua, D.V. Cousins, E.A. Graviss, E. Shashkina, B.N. Kreiswirth, and J.M. Musser. 2002. Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: resolution of genetic relationships among closely related microbial strains. Genetics 162:1533-1543.
20. Pheiffer, C., J.C. Betts, H.R. Flynn, P.T. Lukey, and P. van Helden. 2005. Protein expression by a Beijing strain differs from that of another clinical isolate and Mycobacterium tuberculosis H37Rv. Microbiology 151:1139-1150.
21. Reed, M.B., P. Domenech, C. Manca, H. Su, A.K. Barczak, B.N. Kreiswirth, G. Kaplan, and C.E. Barry, 3rd. 2004. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. Nature 431:84-87.
22. Reed, M.B., S. Gagneux, K. Deriemer, P.M. Small, and C.E. Barry, 3rd. 2007. The W/Beijing lineage of Mycobacterium tuberculosis overproduces triglycerides and is constitutively upregulated for the DosR dormancy regulon. J Bacteriol
23. Barczak, A.K., P. Domenech, H.I. Boshoff, M.B. Reed, C. Manca, G. Kaplan, and C.E. Barry, 3rd. 2005. In vivo phenotypic dominance in mouse mixed infections with Mycobacterium tuberculosis clinical isolates. J Infect Dis 192:600-606.
24. Bifani, P.J., B. Mathema, N.E. Kurepina, and B.N. Kreiswirth. 2002. Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains. Trends Microbiol 10:45-52.
25. Garcia de Viedma, D., F. Chaves, and J. Inigo. 2006. New route of importation of Mycobacterium tuberculosis Beijing genotype. Emerg Infect Dis 12:169-170.
26. van Soolingen, D., L. Qian, P.E. de Haas, J.T. Douglas, H. Traore, F. Portaels, H.Z. Qing, D. Enkhsaikan, P. Nymadawa, and J.D. van Embden. 1995. Predominance of a single genotype of Mycobacterium tuberculosis in countries of east Asia. J Clin Microbiol 33:3234-3238.
27. Filliol, I., A.S. Motiwala, M. Cavatore, W. Qi, M.H. Hazbon, M. Bobadilla del Valle, J. Fyfe, L. Garcia-Garcia, N. Rastogi, C. Sola, T. Zozio, M.I. Guerrero, C.I. Leon, J. Crabtree, S. Angiuoli, K.D. Eisenach, R. Durmaz, M.L. Joloba, A. Rendon, J. Sifuentes-Osornio, A. Ponce de Leon, M.D. Cave, R. Fleischmann, T.S. Whittam, and D. Alland. 2006. Global phylogeny of Mycobacterium tuberculosis based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J Bacteriol 188:759-772.
28. Gagneux, S., K. DeRiemer, T. Van, M. Kato-Maeda, B.C. de Jong, S. Narayanan, M. Nicol, S. Niemann, K. Kremer, M.C. Gutierrez, M. Hilty, P.C. Hopewell, and P.M. Small. 2006. Variable host-pathogen compatibility in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 103:2869-2873.
29. Lopez, B., D. Aguilar, H. Orozco, M. Burger, C. Espitia, V. Ritacco, L. Barrera, K. Kremer, R. Hernandez-Pando, K. Huygen, and D. van Soolingen. 2003. A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. Clin Exp Immunol 133:30-37.
30. Zhang, M., J. Gong, Z. Yang, B. Samten, M.D. Cave, and P.F. Barnes. 1999. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. J Infect Dis 179:1213-1217.
31. Cox, H.S., T. Kubica, D. Doshetov, Y. Kebede, S. Rusch-Gerdess, and S. Niemann. 2005. The Beijing genotype and drug resistant tuberculosis in the Aral Sea region of Central Asia. Respir Res 6:134.

32. Rad, M.E., P. Bifani, C. Martin, K. Kremer, S. Samper, J. Rauzier, B. Kreiswirth, J. Blazquez, M. Jouan, D. van Soolingen, and B. Gicquel. 2003. Mutations in putative mutator genes of Mycobacterium tuberculosis strains of the W-Beijing family. Emerg Infect Dis 9:838-845.
33. Gutacker, M.M., B. Mathema, H. Soini, E. Shashkina, B.N. Kreiswirth, E.A. Graviss, and J.M. Musser. 2006. Single-nucleotide polymorphism-based population genetic analysis of Mycobacterium tuberculosis strains from 4 geographic sites. J Infect Dis 193:121-128.
34. Soto, C.Y., M.C. Menendez, E. Perez, S. Samper, A.B. Gomez, M.J. Garcia, and C. Martin. 2004. IS6110 mediates increased transcription of the phoP virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. J Clin Microbiol 42:212-219.

Project Results:

At the end of the project, the partners of the TB-VIR consortium have achieved the following main scientific results:

- The phylogeny of M. tuberculosis (Mtb) Beijing strains has been constructed using polymorphisms in DNA replication, recombination and repair genes;
- The innate immune response to Mtb Beijing and other genotypes has been assessed;
- A high density mutant library in Mtb Beijing has been screened visually at the phagosome level using high throughput confocal microscopy;
- The role of IS6110 in Mtb Beijing has been deciphered;
- Novel techniques have been developed to study host cell promoter regions;
- Non-Beijing strains of Mtb in China have been studied;
- Mutations to study drug resistance in Mtb Beijing have been identified;
- Fluoroquinolone resistance in Mtb Beijing has been studied;
- The complete genome of several Mtb Beijing and non Beijing strains has been sequenced;
- The role of novel virulence genes involved in metal efflux has been studied in Mtb Beijing and non Beijing;
- The role of the NLRP3 inflammasome in immunity to Mtb Beijing and non Beijing has been studied.

Description of the main activities and achievements of the different workpackages (WP):

Work Package 2 - Evaluation of M. tuberculosis genetic diversity in clinical isolates from the Shanghai area, China

The main objectives of TB-VIR Work Package 2 were to:

- identify strains representative of the genetic diversity observed in the Shanghai area, where over 90% of the clinical isolates are expected to belong to the W-Beijing family;
- provide the scientific community with the know-how and proof of principle that SNP-genotyping can be successfully used to clusterize M. tuberculosis into genotypes and subgenotypes;
- sequence the IS6110 insertion sites in various representative strains within the W-Beijing genotype and among mycobacterial genotypes;
- assess the influence of IS6110 insertion on driving the expression of neighbouring genes.

The study of Beijing strains of the Shanghai area together with Beijing strains from other countries all over the world (partners 5, 9 and 12) allowed to propose a phylogenetic tree that confirm the successive acquisition of point mutations that can be used as markers to differentiate Beijing strains

(Mestre et al. PLoS One (2011) 6(1):e16020). The role of repetitive sequences in the evolution of Beijing strains was investigated by analysing IS6110 carrying regions (partner 4 and 12). They have been disseminated into the genomes either by transposition or homologous recombination (Alonso et al. Tuberculosis (2011) 91:117-26)

Thanks to the complete sequence of several Beijing strains genomes and their comparison with genomes of other family strains (partners 10 and 12) new single nucleotide polymorphisms (SNPs) were identified. They will be used for microevolution studies. For a more rapid and efficient typing of Beijing strains, the association of a limited number of SNPs and MIRU/VNTR loci was successfully tested in a collection of Beijing strains from the Shanghai area (partners 5, 9 and 12, manuscript in preparation).

The major goal of our work was to study the genetic diversity of the W-Beijing strains by SNP genotyping. To reach this goal, we sequenced the genomes of three Beijing strains using the Illumina Genome Analyzer GAII. Among these selected Beijing strains we included a strain namely GC1237. This pansusceptible strain is still responsible for an epidemic in the Gran Canaries Island. In addition we sequenced two other Beijing strains: one ancient and one modern Beijing strain selected by taking into account a previously published work based on the sequential accumulation of SNPs in 3R genes. For each strain, all generated Paired End (PE) reads have been analysed and aligned to the genome of the reference sequence H37Rv. This analysis led to the identification of 1211, 1287 and 1386 SNPs for the GC1237, R34-990172 and the W4 stain respectively.

Several specific SNPs to the GC1237 have been selected for microevolution purposes but after the withdrawal of IntegraGen (Partner 7) from the TB-VIR project we changed our strategy by retreiving all genome sequence data corresponding to 5 Beijing strains from different databases.

On one hand, in order to get more detailed insight into the genetic diversity of Beijing strains we included in our analyses the genome sequence data of 15 non-Beijing strains. Among these 15 strains, we included in our analysis the complete genome sequence data of M. bovis and the vaccine strain, the BCG strain. On the other hand, the genomes of two M. africanum strains were analysed. M. africanum is situated in an intermediate position between M. tuberculosis and M. bovis. The analysis of all these genomes led to the identification of 35,073 SNPs whose comparison yielded a set of 12,475 polymorphic sites.

These identified SNPs have been used to construct a phylogentic tree using the maximum likelihood. For The Beijing strains, we were able to identify by taking into account all identified SNPs, all specific ones for each strain and each Branch of the tree. These SNPs represent good candidates to perform genotyping test for microevolution purposes.

To identify the functions that have diversified to a larger extent in the MTBC complex, we analysed the distribution of SNPs in the genes using the Clusters of Orthologous Groups (COG) gene classification and the Tuberculist nomenclature. Overall, two thirds of the SNPs are non-synonymous (nsSNP) and one third are synonymous (sSNP). We therefore analysed all the intragenic SNPs in the 16 COG categories. When accounting for the relative size of COGs we found that genes playing a role in cell wall membrane and biogenesis are significantly enriched in SNPs ($p = 0.0015$). No other COG class significantly over-represents, or under-represents, SNP. The over-representation of SNP in the cell wall membrane is compatible with the previous observation that the most striking clusters of SNP occur in virulence associated functions.

To determine the prevalence and transmission potential of non-Beijing family strains in mainland China, we performed a population-based prospective study in three geographic areas. We collected 1004 M. tuberculosis clinical isolates from 988 culture positive pulmonary tuberculosis patients during December 1, 2006 to December 31, 2008. We selected the initial isolate from each patient for a total of 988 isolates that were analyzed in this study. Among the 988 clinical isolates, 304 (30.8%) had non-Beijing strains of M. tuberculosis. The percentage of non-Beijing strains varied in different geographic areas; 45.9% of the strains isolated from Sichuan were non-Beijing strains, while only 20.6% of the strains in Shandong Province were non-Beijing strains ($p < 0.0005$). The proportion of non-Beijing family strains in Sichuan Province was significantly higher than that in Shandong Province ($p < 0.0005$) and Shanghai ($p < 0.0005$). The proportion of non-Beijing strains in Shanghai was also significantly higher than that in Shandong Province ($p = 0.034$). Therefore, the prevalence of non-Beijing strains was different across the three study areas in mainland China. These differences may be due to factors such as human migration, transmission, or diversification and adaptation of the mycobacteria to different hosts.

Novel tools are urgently needed for the rapid, reliable detection of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis. To develop such tools, we need information about the frequency and distribution of the mycobacterial mutations and genotypes that are associated with phenotypic drug resistance. In a population-based study, we sequenced specific genes of M. tuberculosis that were associated with resistance to rifampin and isoniazid in 242 phenotypically MDR isolates and 50 phenotypically pan-susceptible isolates from tuberculosis (TB) cases in Shanghai, China. We estimated the sensitivity and specificity of the mutations using the results of conventional, culture-based phenotypic drug susceptibility testing as the standard. We detected mutations within the 81-bp core region of rpoB in 96.3% of phenotypically MDR isolates. Mutations in two structural genes (katG, inhA) and two regulatory regions (the promoter of mabA-inhA and the intergenic region of oxyR-ahpC) were found in 89.3% of the MDR isolates. In total, 88.0% (213/242) of the phenotypic MDR strains were confirmed by mutations in the sequenced regions. Mutations in embB306 were also considered a marker for MDR and significantly increased the sensitivity. Based on our findings, an approach that prospectively screens for mutations in 11 sites of the M. tuberculosis genome (rpoB531, 526, 516, 533, 513, katG315, inhA -15, ahpC -10, -6, -12, and embB306) could detect 86.8% of MDR strains in Shanghai.

This study lays the foundation for the development of a rapid, reliable molecular genetic test to detect MDR strains of M. tuberculosis in China.

The15- and 24-locus VNTR genotype methods have been used to study the molecular epidemiology in settings dominated by Beijing strains but the reliability of "clusters" defined by those methods has been questioned, as lots of clustered strains were found no epidemiology links. We combined SNP and VNTR to genotype Beijing strains in Shanghai and found SNP genotyping can both increase the discriminatory power of VNTR and exclude VNTR homoplasy which caused by convergent evolution.

In addition, other activities related to these tasks were performed:

1. To determine the prevalence and transmission potential of non-Beijing family strains in mainland China, we performed a population-based prospective study in three geographic areas. We collected 1004 M. tuberculosis clinical isolates from 988 culture positive pulmonary tuberculosis patients during December 1, 2006 to December 31, 2008. We selected the initial isolate from each patient for a total of 988 isolates that were analyzed in this study. Among the 988 clinical isolates, 304 (30.8%) had non-Beijing strains of M. tuberculosis. The percentage of non-Beijing strains varied in different geographic areas; 45.9% of the strains isolated from Sichuan were non-Beijing strains, while only 20.6% of the strains in Shandong Province were non-Beijing strains ($p < 0.0005$). The proportion of non-Beijing family strains in Sichuan Province was significantly higher than that in Shandong Province ($p < 0.0005$) and Shanghai ($p < 0.0005$). The proportion of non-Beijing strains in Shanghai was also significantly higher than that in Shandong Province ($p = 0.034$). Therefore, the prevalence of non-Beijing strains was different across the three study areas in mainland China. These differences may be due to factors such as human migration, transmission, or diversification and adaptation of the mycobacteria to different hosts.

2. Novel tools are urgently needed for the rapid, reliable detection of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis. To develop such tools, we need information about the frequency and distribution of the mycobacterial mutations and genotypes that are associated with phenotypic drug resistance. In a population-based study, we sequenced specific genes of M. tuberculosis that were associated with resistance to rifampin and isoniazid in 242 phenotypically MDR isolates and 50 phenotypically pan-susceptible isolates from tuberculosis (TB) cases in Shanghai, China. We estimated the sensitivity and specificity of the mutations using the results of conventional, culture-based phenotypic drug susceptibility testing as the standard. We detected mutations within the 81-bp core region of rpoB in 96.3% of phenotypically MDR isolates. Mutations in two structural genes (katG, inhA) and two regulatory regions (the promoter of mabA-inhA and the intergenic region of oxyR-ahpC) were found in 89.3% of the MDR isolates. In total, 88.0% (213/242) of the phenotypic MDR strains were confirmed by mutations in the sequenced regions. Mutations in embB306 were also considered a marker for MDR and significantly increased the sensitivity. Based on our findings, an approach that prospectively screens for mutations in 11 sites of the M. tuberculosis genome (rpoB531, 526, 516, 533, 513, katG315, inhA -15, ahpC -10, -6, -12, and embB306) could detect 86.8% of MDR strains in Shanghai.

This study lays the foundation for the development of a rapid, reliable molecular genetic test to detect MDR strains of M. tuberculosis in China.

The 15- and 24-locus VNTR genotype methods have been used to study the molecular epidemiology in settings dominated by Beijing strains but the reliability of "clusters" defined by those methods has been questioned, as lots of clustered strains were found no epidemiology links. We combined SNP and VNTR to genotype Beijing strains in Shanghai and found SNP genotyping can both increase the discriminatory power of VNTR and exclude VNTR homoplasy which caused by convergent evolution.

Nineteen IS6110 copies were localised for this strain. Sixteen of the nineteen copies present direct repeat sequences (DR) of 3 to 4 nucleotides in the flanking regions due to transposition events. The absence of DR in the other copies suggests rearrangments between IS6110 elements. One of these copies was localized in the hot spot between Rv0794 -Rv0797 in opposite orientation with respect to a copy in H37Rv. The other two locations correspond to the deleted regions of diference RD152 and RD207.

Nine copies were in the same orientation as the downstream gene and four of them could act as promoter according to the distance to the next gene. From these, we studied in depth the IS6110 located 31 bp of Rv 2179c. We investigated this location in other Beijing and non Beijing strains, and we observed that this insertion point was unique to GC 1237 strain.

By qRT-PCR we studied the expression of the genes preceded by IS6110 and we only found a clear increase of about 6 fold in the case of Rv2179 gene. We also studied the expression of this gene in macrophages and observed that the expression of this gene in GC1237 is higher than in H37Rv. The expression increases in intracelular conditions with respect to extracelular conditions.

We then studied the transcription of these genes in this region in GC1237 and H37Rv strains. The Rv2180c, Rv2179c and aroG gene are cotranscribed in H37Rv. In the case of GC1237, amplification products were obtained from 3'end of IS6110 to aroG gene suggesting that the three genes from the insertion sequence are cotranscribed in an operon.

We verified that IS6110 acts as a promoter by using the recombinant plasmids containing the promoterless gfp gene preceded by the region upstream Rv2179. Results showed that the fluorescence is about 5 fold higher when the IS is inserted indicating that the IS is acting as a promoter and the expression increased in intracellular conditions.

The promotor activity of IS6110 was then studied in different conditions and we observed upregulation during stationary but not under hypoxia conditions. In collaboration with Institut Pasteur of Korea (Partner 6), OPERA microscopy system was used to measure IS6110 promotor activity of different IS localizations. Using GFP as reporter gene, we have seen the strong promotor activity of the IS6110 located in the GC1237 strain upstream Rv2179c gene, confirming the data obtained by different techniques. Moreover, we studied the IS6110 upstream phoP gene, a known transcription factor associated with virulence. Again, a higher GFP intensity was measured in the cells infected with the construction including this IS6110.

In addition, we have studied the other 8 representative strains that present a variable pattern of MIRUS and RFLP. By IS6110 RFLP we observed that these strains contain 10 to 21 copies. We compared all locations from the nine strains, and there are 3 common points including the characteristic in the DR region and between dnaA and dnaN region and 2 other insertions were observed in 8 from the 9 strains. We studied the common location between dnaA and dnaN genes. In this location, IS6110 doesn't increase the expression of the downstream dnaN gene.

Work Package 3 - Study differential host cell gene expression modifications upon infection with various M. tuberculosis genotypes and subgenotypes representative of the W-Beijing and other mycobacterial families

The main objectives of TB-VIR Work Package 3 were to:

- identify gene expression changes after infection with different mycobacterial strains using DNA chip technologies;
- investigate the host-microbe crosstalk on the single gene level as a function of microbial diversity;
- identify distinct functional roles for the genes of each unit in host cell responses;
- identify transcription factors playing an important role in host responses and their potential target genes.

The TFBS assays performed in Period 1 pointed to the immunity- and development-related POU family TFs as putative regulators of genes differentially expressed in early phases of Mtb-infection in THP-1 cells. It was shown in period 2 that THP-1 transcriptomic responses are time-specific, regardless of sublineages of Beijing family strains. These conserved host responses are dramatically modulated between 4h and 18h. Genes exhibiting consistent changes within the strains and differential expression between 4h and 18h were selected through LIMMA-based methodology. Five genes including CXCL10, CXCL11, TNFSF10, IL15 and CCL8 were validated for consistent expression by qRT-PCR approach. TFBS enrichment analysis indicates that STATs, IFR-1, IRF-7 and Oct-1 are putative regulators responsible for transcriptional immune responses of THP-1 cells to Mtb infection.

Similarly to results obtained in human macrophages the overall transcriptome profiles between BMDM cells infected by 10 different Mtb strains were similar at each time point, irrespective of the strain phylogeny. Due to the highly similar/conserved transcriptional responses induced by H37Rv and the investigated members of the W-Beijing family a selection of differentially regulated genes was not feasible. Consequently, validation by RT-PCR of potential genes of interest was not performed.

During the second period of the project, WP3 aimed at deciphering the transcriptional signature of macrophages upon infection with representative W-Beijing Mtb strains. Based on the arrays data, the goal was to perform clustering of genes and identify host cell gene expression modifications induced by Mtb genotypes.

Bone marrow derived macrophages (BMDM) were infected with M. tuberculosis (Mtb) H37Rv (New York) as laboratory control strain and 9 different Mtb W-Beijing genotypes. Due to BSL3 capacity limitations experiments were carried out in 3 consecutive series, each series comprising the H37Rv control strain and 3 different Beijing genotypes. We generated two technical replicates by parallel sampling and performed two complete independent biological replicates at different days. Sample preparation for total RNA isolation took place at 3 different time points post infection (p.i.) (at 4h, 18h and 48h p.i.).

In total 190 different samples were hybridized and analyzed. For initial data analysis we compared all 41174 present microarray features of the whole genome microarray by divisive clustering.

The heat map of the differential expression shows that, independent of which strain was used, a similar pattern of expression was induced, i.e. there seems to be no difference between the induced regulation of expression between H37Rv and the Beijing strains.

In general it can be speculated that at the measured time points no significant and profound differences were identified between H37Rv and the investigated W-Beijing strains, although we do not exclude that other members of the Beijing family at other time points p.i. could show differences to H37Rv.

Due to the highly similar/conserved transcriptional responses induced by H37Rv and the investigated members of the W-Beijing family a selection of differentially regulated genes was not feasible. Consequently, validation by RT-PCR of potential genes of interest was not performed.

A follow up investigation was initiated by Partner 5 - FUDAN in human macrophages to explore differential transcriptional activity at early time-points between the lab strain in comparison with the Beijing isolates. Increasing sample size might provide significant power to the analysis. Partner P05 performed the infection and provided the cell RNA to Partner 8 for microarray assay. Differentiated THP-1 cells were infected with 12 representative M. tuberculosis clinical isolate from different sub lineages of W-Beijing family Mtb using MOI 5 for 4 hours. The cell RNAs and supernatants were collected at 0 hours, 4 hours, 18 hours and 48 hours. RNAs were extracted and sent to Partner 8 to perform global transcriptional analysis using microarray techniques.

As what displayed in the work of first 18 month period, host transcriptomic responses performed by Partner 8 were shown to be time-specific, regardless of sublineages of the pathogen. Also, these host-responsive genes are dramatically modulated between 4h and 18h. Accordingly, we speculated that the robustness of host responses might be attributed to the involvement of response-relevant transcriptional factors, and that these regulators could be predicted from the observed transcriptomic changes. In this regard, genes differentially expressed between 4h and 18h may represent the common signatures of Mtb-infected THP-1 cells. To such an end, a LIMMA-based methodology was applied to select genes exhibiting the consistent changes within the strains and differential expression between 4h and 18h. As validated in five randomly selected Mtb-infected samples by qRT-PCR approach, the CXCL10, CXCL11, TNFSF10, IL15 and CCL8 genes displayed consistent upregulation from 4h to 18h.

Differentially expressed genes identified can be further categorized into two groups: Cluster 1 for induction at 18h (or 48h) compared with at 4h, Cluster 2 for repression. Interestingly, most of differentially expressed genes (92%, 367/399) were induced. When enrichment analysis was applied to examine whether they shared functional or regulatory features, we only observed biological features associated with genes in Cluster1. On the contrary, no enrichment of biological relevance was observed in Cluster 2, which was thus excluded from further consideration in this study.

The most significant Gene Ontology (GO) terms are immunity-related responses. Consistent with this, the most significant transcription factor binding sites (TFBS, represented in the form of position weight matrixes [PWMs]) are immunity-related transcription factors or putative immunity-related regulators, including IFN-stimulated response element (ISRE), IRF-1, IRF-7 and Oct-1. Through extensive literature mining, we found that each of these predicted regulators was biologically relevant.

Collectively, transcription factors of STATs, IFR-1, IRF-7 and Oct-1 are putative regulators responsible for transcriptional immune responses of THP-1 cells to Mtb infection.

Work Package 4 - Screening of a genome-covering M. tuberculosis W-Beijing mutant library at the host cell level

The main objectives of TB-VIR Work Package 4 were to:

- infect murine macrophages with a 11,000-member genome-covering signature-tagged transposon mutagenesis library generated by Partner 12 (IPAS) in the W-Beijing strain GC1237;
- identify important mycobacterial virulence genes involved in host cell parasitism.

During the first 18 months of the project, we screened for the ten mutants over a 11,000 Mycobacterium tuberculosis mutant library that traffic most frequently into acidified compartments early after phagocytosis, suggesting that they had lost their ability to arrest phagosomal maturation. Molecular analysis of these mutants revealed mainly disruptions in genes involved in cell envelope biogenesis (fadD28), the ESX-1 secretion system (espL/Rv3880), molybdopterin biosynthesis (moaC1 and moaD1), LppM and periplasmic phosphate-binding pstS3 lipoproteins, as well as in genes from novel locus, Rv1503c-Rv1506c and Rv2295c. We went on studying the presence and the genetic diversity of these genes in M. tuberculosis strains from WP2. All these genes were present in the panel of M. tuberculosis Beijing strains sequenced in WP2. A number of non-synonymous (nsSNP) mutations was identified within the open reading frame of all genes. In particular, fadD28 has A to G nsSNP at position 1306 for four W/Beijing strains. In addition, a mutation in lppM encoded protein was identified in M. tuberculosis Beijing GC1237.

During the second period of the project, genotype/phenotype correlations were made for the Mtb virulence genes identified in WP04. In particular the residual virulence of the mutants was assessed in vitro in cultured macrophages, and in vivo in the mouse model of Mtb infection. The ability of the different Beijing strains to induce cytokines responses in cultured human phagocytes was assessed, and we could identify specific signatures of the Beijing family in terms of cytokine and chemokine secretion and host cell response to infection. Novel TFs identified in WP03 are still currently studied in the context of future projects.

Work Package 5 - Understanding genotype/phenotype relationships between M. tuberculosis genetic diversity and differential virulence and immune responses in vitro and in vivo

The main objectives of TB-VIR Work Package 5 were to identify:

- key genotype-phenotype relationships between M. tuberculosis genotypes and subgenotypes, in particular among the W-Beijing family;
- host cell responses at the transcriptome level;
- ability to overcome phagosome maturation and to parasitize host macrophages;

- host immune response to infection in vitro and in vivo and mycobacterial immuno-pathogenicity.

In the context of WP05, bone marrow derived macrophages (BMDM) were infected with M. tuberculosis (Mtb) H37Rv (laboratory control strain) and 9 different MtbW-Beijing genotypes. Due to BSL3 capacity limitations experiments were carried out in 3 consecutive series, each series comprising the H37Rv control strain and 3 different Beijing genotypes. We generated 2 technical replicates by parallel sampling and carried out 2 complete independent biological replicates at different days. Cell-free supernatants from MTB-infected BMDM were collected at 4, 18 and 48 h p.i.. In separate experiments BMDM were primed with IFN-? prior MTB infection. These experiments were carried out as above, except for we performed triplicates and collected cell-free supernatants at at single time-point, namely 24h p.i.

We measured up to 32 targets using the bead based assays (BioRad and Millipore) and detected 21 cytokines/chemokines. We could not identify a significantly different secretion pattern for most of the cytokines. For several W-Beijing members we determined a trend to induce more abundant release of IL-6 (eg. HM903, Cam22). However, this tendency did not hold true for other measured pro-inflammatory cytokines, like TNF-? or IL-1?. Moreover, the measurements at 24h p.i. did not recapitulate the previously mentioned observations. Classically activated BMDM responded in a divergent way in terms of release of several cytokines and chemokines. CXCL-10 levels were not significantly different between the tested bacterial strains. A similar tendency was reported for TNF-?, while IL-6 seemed to be less induced by several W-Beijing members at 24h p.i. These apparent discrepancies between kinetics measurements and the 24h measurement might be explained by the fact that BMDM present receptors for several of the quantified cytokines/chemokines and target-recepor binding results in reduced concentration of the target soluble mediator in the cell culture supernatant. Moreover, infection is a dynamic process and autocrine sensing of secreted cytokine might result in changes in density of the cell surface receptors and synthesis of decoy receptors, which may bind the mediator as well. As expected, activation of the BMDM with IFN-? resulted in increased levels of several cytokines compared with resting BMDM. Interestingly, IFN-? was able to restore the secretion of cytokine (TNF-?) for some Mtb strains while this propensity was not observed for IL-6.

Nitric oxide levels were quantified using the Griess reagent. As expected, IFN-? priming resulted in release of NO in culture supernatants. Abundance of the antimicrobial molecule was independent of the mycobacterial strain used to infect the BMDM.

To investigate whether the W-Beijing strains have a growth advantage in macrophages we infected resting and IFN-? primed BMDM with H37Rv and the Beijing strains and followed bacterial replication using 3H-Uracil incorporation. The radioisotope labeled base is integrated in bacterial RNA and thus upon replication and metabolic activity the retained radioactive levels increased. Firstly, we

incubated the lab strain and the W-Beijing strains in macrophage culture media and add the radionuclide labeled nucleic base. We observed that H37Rv, but also several of the tested Beijing strains grew well, showing a daily replication (GC1237, HNH5, N4, 990172), while other strains were defective in multiplication using macrophage media as substrate.

Overall, our results showed a conserved pattern of response of the lab Mtb strains and the W-Beijing members that were tested. The behavior recorded for isolated strains in a particular assay could not be extended to the whole subfamily or family. Thus, based on the tools and results described above, a significant difference between H37Rv and the clinical W-Beijing strains was not observed and cannot be stated.

Finally, apoptosis is a physiological way of cell death by which multicellular organisms control homeostasis, cell transformation and intracellular infection whith important implication in the host immune response. This process is triggered during the course of infections with intracelullar facultative pathogens, including M. tuberculosis. There have been proposed differences between Beijing and non Beijing strains in terms of apoptosis induction on host cells. Concretely, Beiijing strains have been shown to induce more necrosis and less apoptosis than non Beijing strains, however these studies are incomplete since they compare few strains. Thus, we used the nine Beijing reference strains to elucidate if this feature is intrinsic to Beijing family.

Using different cell death markers to discern between apoptosis and necrosis, we found that both Beijing and non Beijing strains clearly induced apoptosis on macrophages, and, although some of the Beijing strains were statistically less pro-apoptotic, no global differences between both groups were measured.

Intracellular cytokine expression of proinflamatory IL-6 was studied in mouse MH-S macrophages infected with the panel of 9 Beijing strains and with not Beijing strains. We found that none of the 9 Beijing strains studied induced IL-6 expression, while all the non-Beijing strains did it.

The ten M. tuberculosis GC1237 mutants identified in WP4 were further studied. Bone-marrow derived macrophages (BMDM) were inoculated with the various mutants and their growth was compared to that of wild type and of their respective complemented strains. BMDM were infected at different multiplicity of infection (MOI) ranging from 1 to 5 and intracellular bacteria were counted using colony-forming unit enumeration method at day 0, 3 and 5 post-infection. pstS3, lppM, moaD1Tn mutants were degraded within the macrophages, Rv1503c, Rv1506c, moaC1, Rv3880c Tn mutants survived intracellularly, in contrast Rv2295c, fadD28 and ppe54 Tn mutants were able to actively multiply within the host cells similarly to the wild type strain.

The cytokines release by BMDM that had been inoculated with the mutants was profiled at 24 hours using the Raybiotech Cytokine 32-Array and compared with that of their corresponding complemented strains and wild type. As expected M. tuberculosis GC1237 induced an increased secretion of IL-6 and GSCF compared to non-infected macrophages. Surprisingly, the Rv1506c Tn mutant was able to further augment the secretion of IL-6. Overall no specific pattern of cytokines expression using this technique was found among the different mutants.

We went on to study the phenotype of the mutants in the mouse model in vivo. All mutants but not fadD28 and Rv3880c for which data had been priory published by other groups, were inoculated to Balb/C mice via the intranasal route and compared to the wild type GC1237 strain and complemented strain. Groups of 5 animals were then sacrificed at three weeks and at six weeks and CFU were enumerated. All eight mutants were attenuated by displaying at least one log less CFU in the lungs compared to wild type control. The wild type phenotype could be restored for Rv1503c, Rv1506c, ppe54 and Rv2295 Tn mutants. Concerning lppM Tn mutant, our hypothesis for explaining the lack of phenotype restoration is that complementation was performed with plasmid encoding M. tuberculosis H37Rv LppM which has a mutation compared to that of GC1237 protein. With regards to the others, moaC1, moaD1, pstS3 complemented Tn mutants, there is an issue of bacterial growth fitness in vitro on agar plates.

In addition, the immune response generated by these mutants was investigated. Splenic T-cells from infected mice were analysed for CD3, CD4, CD8, CD44, CD45RB, CD62L, CD11b, CD11c, F4-80 and CD80 population. No significant variation had been found compared to wild type. Altogether these data showed that the mutants that are impaired in phagosome maturation arrest in vitro have all growth defects in vivo.

In collaboration with Partner 6 IPK, Partner 5 University of Fudan has used OPERA microscopy system to perform a comparative study of the trafficking of the different mutants in H37Rv (H37Rv ?phoP, H37Rv ?phoP pSO5k and H37Rv ?phoP pSO7k) and GC1237 (GC1237 ?phoP, GC1237?phoP pSO5k and GC1237 ?phoP pSO7k) inside macrophages and to analyse the influence of IS6110 as a promoter of phop gene (construction pSO7). We used DAPI, lysotraker-green and CypHer5 dyes. As a negative control we used non-infected cells were used, and as a positive heat-killed mutants.

After 2h of infection, nearly 100% of the cells were found infected and preliminary results show that the fraction of bacteria co-localizing with Lysotrake-positive staining are less than 10% in all the mutants and more than 60% of killed-bacteria were found to co-localize with acidified compartments. We did not observe any significant difference between H37Rv and the GC1237 constructions.

Work Package 1: Management and coordination of the Consortium

The main objectives of TB-VIR Work Package 1 were to:

- set up an effective management framework for the TB-VIR consortium;
- ensure the usual and contractual administrative tasks (financial, reporting, organization of (annual, Council, strategic) meetings;
- manage safety issues, legal, ethical and intellectual commercial / property-related issues, and gender equality promotion;
- coordinate the dissemination of knowledge (communication activities) inside and outside the network.

The TB-VIR coordination and management WP took care of the usual and contractual administrative tasks (financial, reporting, organization of consortium meetings, management of Intellectual Property, etc.), The project management team also focused on monitoring complex aspects related to the network's specific properties, notably some changes that emerged in the TB-VIR consortium and workprogramme, the organisation of access to the key deliverables (Biobase and Databases, compound library, microarray data) beyond the end of the EC contract, maintenance of the information hub (website), as well as identifying solutions (notably funding) for maintaining the momentum, added-value of cooperation and collections obtained by the TB-VIR network thanks to the EC support.

The management team acted as the interface between TB-VIR partners and European Commission, was in regular contact with EC Scientific Officer, and relayed problems and questions to the European Commission as soon as they occurred. The team was involved in the organization of a variety of meetings, promotional materials (leaflet, poster, newsletter, etc.), and the maintenance of the project's website.

Potential Impact:

a. Potential impact and exploitation of results

** Impact on the development of novel TB control strategies*

TB remains a major threat to mankind and cannot be conquered without an effective vaccination strategy. No completely effective vaccination strategy has yet been developed. M. tuberculosis is one of the most effective human pathogens, and one-third of the world's population is infected. The global incidence of TB increases by 2% annually. The vast majority of cases occur in developing countries, especially in adults between 15 and 45 years of age, the most economically active segment of the population. An additional major threat is posed by the increasing incidence of MDR strains of M. tuberculosis. Indeed, in several Eastern European countries, more than 14% of primary TB cases are MDR. This situation necessitates the development of new types of intervention and improvement of the follow-up of TB patients in these countries. This project, aimed at increasing our understanding of the links between differential host responses and M. tuberculosis genetic diversity has clearly contributed to the development of such new interventions. In particular, vaccine candidates have been isolated though TB-VIR activity. A provisional patent application entitled "Method for producing a vaccine for the treatment and/or prevention of Tuberculosis" has been filed by Partners 1 CNRS and 6 IPK (Brodin P., Neyrolles O. et coll.), on Oct. 9th, 2010. These vaccine candidates are currently under pre-clinical studies and will help provide novel intervention tools to combat TB.

** Impact on pathogen research and pathogen-related diseases*

The proposed work has identified the genes differentially expressed or regulated in response to various clinical isolates of M. tuberculosis with an emphasis on genes involved in the immune response. In addition, M. tuberculosis genes required for macrophage parasitism have been identified. Participation of beneficiary 6 (IPK) has been invaluable to identify mycobacterial genes involved in intracellular trafficking. WP4 as a whole has been highly complementary, and non-redundant, with the activity of the EC-funded project TB-MACS to which beneficiary 12 participated. The findings of this study have greatly increased our understanding of M. tuberculosis/host interactions and have been an invaluable reference for the TB research community. The results generated by this project have been disseminated to others in the form of high-impact publications and conference presentations. The datasets produced in this study have been a valuable reference for TB research worldwide. All datasets generated have been made publicly available via publications.

** Impact through innovation*

The proposed project was highly innovative because it made use of new developments in functional genomic technology to investigate M. tuberculosis pathogenesis. This project brought together technologies from the fields of microarray analysis, functional genomics and transcriptomics, cell biology, including high throughput confocal microscopy, bioinformatics in an overall pioneering

strategy that could be applied to the investigation of other intracellular pathogens. We therefore anticipate that the technologies used and the know-how obtained in this project will be used by researchers working on other pathogens. In addition, the application of high-throughput screens as a means of connecting genotypes and complex phenotypes will be of considerable interest to researchers in a wide range of general fields in functional genomics and studies of infection. In addition, the use of SNP genotyping to analyse M. tuberculosis W-Beijing population structure now provides the international community working on TB and other infectious diseases invaluable information for further studies on pathogen population structure and genotype-phenotype relationships.

** Impact through training and networking*

Training the next generation of scientist has been an important achievement in TB-VIR. Numerous exchanges of personnel have occured. For instance Dr O. Neyrolles and Mrs F. Levillain (Partner 1) have made two visits at the Shanghai CDC and Ruijin hopsital for over a week each time to start collaborative work and train local students involved in the project. Pr B. Gicquel, together with Dr Q. Gao and Dr J. Mei has organized a training workshop dedicated to international students on the topic of mycobacterial detection, evolution and drug resistance (see http://www.moleculartb.org/index.html).

The TB-VIR project has also allowed promoting and strengthening the links and scientific collaborations between European and Asian laboratories. This will help develop novel research project in the near future.

b. Main dissemination activities

As illustrated by the long list of TB-VIR-supported publications in high impact journals, the project contributed significantly to the development of our knowledge base concerning M. tuberculosis virulence and anti-mycobacterial immunity, in particular regarding the W-Beijing lineage, that is of particular concern. These achievements clearly confirmed and extended the leading role of the European laboratories in mycobacterial research and the development of novel intervention tools.

In addition to the scientific publications, principal investigators from the TB-VIR consortium presented their work at a large number of national and international meetings, thus adding to the visibility of the consortium and its main sponsor. Furthermore, fruitful collaborations with our Asian research partners were established, which are continuing beyond the end date of the TB-VIR project and may be a basis for future joint grant applications. Likewise, many of the European partners are continuing the collaborations that were initiated under TB-VIR, either to complete ongoing studies or to initiate research in new directions for which funding will be sought from the EC or other funding agencies.

The TB-VIR project resulted into 10 scientific publications in high impact journals, 2 articles currently under submission, to the participation of 13 conferences and symposiums to give oral presentations, and to 2 symposiums where posters were presented which described the project, its objectives and results.

A strong methodology has been set up to ensure the dissemination of the projects results. After verification that all possible steps have been taken for patent submission, when appropriate, the data have been made available outside TB-VIR through meetings and various media supports (publications in scientific journals or general public newspapers, Internet, etc). To give maximum visibility to our results, the TB-VIR consortium has developed the following tools to support its communication strategy.

- *Tools and ways of dissemination*

- Adapted communication tools: the TB-VIR public website, presentation brochure and poster. The TB-VIR public website (www.tb-vir.org) was presenting the consortium, the context of the project, its objectives and monitored the progress of the project. In addition, sections about the documentation, as well as for news and announcements, were proposed on the site. A brochure and a poster presenting the project were also issued to support the dissemination activities to a wider public.
- Workshops and conference presentations: When appropriate, the participants presented the project and related results to scientific and clinical conferences and workshops in Europe and outside Europe [such as the Fifth EDCTP Forum, the World Health Summit 2010, the 18th federation meeting of the Korean Basic Medical Scientists, the 20th European Congress of Clinical Microbiology and Infectious Disease, etc]. Attendance to these meetings gave the partners the opportunity to present the work of the consortium and develop interest of other experts but also develop contact with industrial partners.
- Publications in peer-reviewed and/or Open Access journals: Scientific results of the project were disseminated via publications in peer-reviewed and Open Access journals and in abstracts of national and international conferences. The TB-VIR project led to the publication of 9 articles in renowned journals, 2 additional articles are under submission.
- Outreach the general public: In addition to our public website, a press release was written for the kick off meeting and disseminated at national level to newspapers and to Public Relations offices of the participant's institutions, but also at European level through the commissions' communication services. When possible, peer-reviewed publications were adapted to publication in popular press to increase the visibility and transparency to a large public, beyond the close community. This has been for instance the case following the publication of the article "High content phenotypic cell-based visual screen identifies Mycobacterium tuberculosis acyltrehalose-containing glycolipids involved in phagosome remodeling" plublished in PLoS Pathogens in Oct. 2010.

- TB-VIR scientific publications

1. Wang C, Peyron P, Mestre O, Kaplan G, van Soolingen D, et al. 2010 Innate Immune Response to Mycobacterium tuberculosis Beijing and Other Genotypes. PLoS ONE 5(10): e13594. doi:10.1371/journal.pone.0013594
2. Mestre O, Luo T, Dos Vultos T, Kremer K, Murray A, et al. 2011 Phylogeny of Mycobacterium tuberculosis Beijing Strains Constructed from Polymorphisms in Genes Involved in DNA Replication, Recombination and Repair. PLoS ONE 6(1): e16020. doi:10.1371/journal.pone.0016020
3. Brodin P, Poquet Y, Levillain F, Peguillet I, Larrouy-Maumus G, et al. 2010 High Content Phenotypic Cell-Based Visual Screen Identifies Mycobacterium tuberculosis Acyltrehalose-Containing Glycolipids Involved in Phagosome Remodeling. PLoS Pathog 6(9): e1001100. doi:10.1371/journal.ppat.1001100
4. Alonso H, Aguilo J.I., Samper S., Caminero J.A., Campos-Herrero M.I., Gicquel B., Brosch R., Martín C., Otal I. Deciphering the role of IS6110 in a highly transmissible Mycobacterium ttuberculosis Beijing strain, GC1237. Tuberculosis 91 (2011) 117e126
5. Beisiegel,M. Mollenkopf H.J., Hahnke K., Koch M., Dietrich I., Reece S.T. and Kaufmann S.H.E. Combination of host susceptibility and Mycobacterium tuberculosis virulence define gene expression profile in the host. Eur. J. Immunol. 2009. 39: 3369-3384 DOI 10.1002/eji.200939615
6. Fang H., Yang Y., Li C., Fu F., Yang Z., Jin G., Wang K., Zhang J., Jin Y. Transcriptome Analysis of Early Organogenesis in Human Embryos. Developmental Cell 19, 174-184, July 20, 2010
7. Li X., Xu P., Shen X., Qi L., DeRiemer K., Mei J., Gao Q. Non-Beijing Strains of Mycobacterium tuberculosis in China. JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2011, p. 392-395
8. Luo T., Zhao M., Li X., Xu P., Gui X., Pickerill S., DeRiemer K., Mei J., Gao Q. Selection of Mutations To Detect Multidrug-Resistant Mycobacterium tuberculosis Strains in Shanghai, China. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2010, p. 1075-1081, Vol 54, No. 3
9. Wang K., Wang P., Shi J., Zhu X., He M., Jia X., Yang X., Qiu F., Jin W., Qian M., Fang H., Mi J., Yang X., Xiao H., Minden M., Du Y., Chen Z., Zhang J. PML/RAR? Targets Promoter Regions Containing PU.1 Consensus and RARE Half Sites in Acute Promyelocytic Leukemia Cancer Cell 17, 186-197, February 17, 2010
10. Xu P., Li X., Zhao M., Gui X., DeRiemer K., Gagneux S., Mei J., Gao Q. Prevalence of Fluoroquinolone Resistance among Tuberculosis Patients in Shanghai, China. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2009, p. 3170-3172
11. Wu K., Fang H., Dong D., Jin W. Levillain F., Mei J., Gicquel B., Wang K., Gao Q., Neyrolles O., Zhang J. Common Host Transcriptional Responses to Sublineages of Mycobacterium tuberculosis W-Beijing Strains and the Underlying Transcriptional Regulators. Submitted.
12. Dorhoi et al. Activation of the NLRP3 inflammasome by M. tuberculosis is uncoupled from susceptibility to active disease. Submitted.

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