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REVIEW

Tuberculosis vaccine research in China

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It is now privately acknowledged that there may be little if any perceptible impact of the national Bacille Calmette–Guerin (BCG) vaccination program on disease prevalence, despite the extensive coverage of the newborn infant population and likely benefit in the early years of life. A better preventive vaccine than BCG is now being sought by Chinese researchers. Urgency has been added to the control problem by the emergence of multidrug-resistant tuberculosis (TB). Furthermore, expensive second-line drugs seem unlikely to be made available by the government to treat drug-resistant cases, so attention in addition has turned to the potential of immunotherapy as an adjunct to chemotherapy. Research trends are summarized here.
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INTRODUCTION

There has been a striking upsurge in tuberculosis (TB) vaccine research in China in recent years. More than 80 papers reporting such research have been published since 2004 (Figure 1), many in English Language Journals. This has been driven and funded largely by Chinese Government agencies, since the scale of the TB problem and the difficulties in implementing fully effective control measures were recognized.[1–3] There seemingly has been little commercial interest. In considering the published output, we can note that there are at least two additional restricting factors limiting the progress of the field in China: few research groups have access to facilities for safely working with animals infected with a dangerous pathogenic organism such as the tubercle bacillus; infrastructure for undertaking vaccine clinical trials to international standards is sparse. In consequence, the universal problem of how to select among the promising approaches and candidate vaccines is particularly acute in China. Nevertheless, the basic research problems are being tackled with some enthusiasm and creativity, and the new 5-year program to improve TB control that was announced by the Chinese Ministry of Health on 1 April 2009 will have an impact. The antigens investigated to date and the locations of research groups are listed in Tables 1 and 2, respectively.

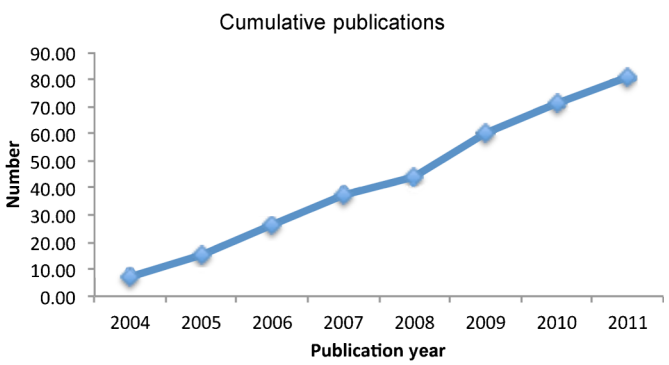

Figure 1 The number of TB vaccine research publications by Chinese Research Groups, 2004–2011.

TB IMMUNITY

A substantial body of work has gone into characterizing the immune responses to be found in TB infection and following vaccination. The twin purposes of identifying candidate antigens for use in vaccines and diagnostic tests and of defining immunological markers for latent infection and disease states drive these studies. These studies have not always been followed up with tests of protective or therapeutic efficacy in animal infection models.

The increased immunogenicity of mouse dendritic cells transfected with antigen heat shock protein 65 (hsp65) has been tested in Wuhan[4] and in Hong Kong, and differences have been found in the polarizing effect of intracellular *Mycobacterium bovis* Bacille Calmette–Guerin (BCG) vaccine bacteria on antigen presentation by human adult and cord blood dendritic cells.[5] Various forms of recombinant BCG expressing early secreted antigen 6 of *Mycobacterium tuberculosis* (ESAT6),[6] or antigen 85B (Ag85B) and antigen Rv3425,[7] or Ag85B and ESAT6 with tumor-necrosis factor-α,[8] or ESAT6 and human granulocyte macrophage-colony stimulating factor (GM-CSF)[9] have shown enhanced immunogenicity. Recombinant *M. smegmatis* expressing *M. tuberculosis* culture filtrate protein 10 (CFP10)/ESAT6 fusion protein was found stimulatory for macrophage inducible nitric oxide synthetase.[10] Recombinant *M. smegmatis* and BCG strains have been made that can express cloned antigens at a range of different levels under the control of modified *FurA* gene promoters.[11] Other BCG recombinants expressing ESAT6,[12] ESAT6/interleukin 2 (IL-2)[13] or Ag85B/ESAT6 fusions[14] have been made.

A range of known antigens has been tested for immunogenicity as mixtures, fusion proteins and peptides. These include chimeric

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Table 1 TB antigens tested.

Antigens	References
ESAT6	6, 9, 10, 12–15, 19, 25, 29, 30, 32, 35, 36, 39, 41–45, 50, 52–56, 64, 67, 68, 71, 80, 82
85B	7, 14, 15, 27–29, 31, 32, 48, 51, 53–56, 59–61. 63–65, 67, 68, 70, 72, 78, 84
MPT64	17, 33, 34, 45, 48, 51, 54, 55, 59–61, 65, 66, 72, 78, 84
Ag85A	32, 37–41, 50, 67, 80, 82, 83
hsp65	4, 32, 46, 69, 76, 85
CFP10	10, 19, 25, 30, 32, 71
Mtb8.4	23, 31, 48, 49, 51, 62
MPT83	56, 59–61, 72
hsp16.3 (hspX)	16, 47, 49, 71
38-kDa	25, 31, 33
MPT63	54, 55
BCG	5, 11
M. vaccae	73, 74
rec. *M. smegmatis*	75
MTB48	25
MPT70	65
TB10.4	65
CFP21	66
hsp70	77
Rv1009	18
Rv1987	26
Rv2450	20
Rv3425	7, 22
Rv3772	21
Rv3807c	26
Rv3887c	26
MPB64	68
M. bovis RD-1 to RD-16	24

Ag85B/ESAT6 with adjuvants monophosphoryl lipid A (MPL) and trehalose 6,6'-dimycolate,[15] hsp16.3 with dimethyl-dioctadecyl-ammonium bromide/MPL (DDA/MPL) adjuvant,[16] fusion protein M. tuberculosis protein 64 (MPT64)–ESAT6,[17] resuscitation-promoting factor B (Rv1009),[18] or CFP10, ESAT6 or RpfE (Rv2450) with nitrocellulose,[19,20] or Rv3772[21] and Rv3425[22] with incomplete Freund's adjuvant; or Ag85B/MPT64$_{190–198}$/Mtb8.4 plus a novel adjuvant of DDA with BCG extract.[23] In-silico analysis of putative MHC Class 1-restricted epitopes present in antigens encoded within the region of difference 1 (RD-1) to RD-16 regions of *M. bovis* genome has revealed potential high-affinity HLA binders[24] and profiles of human humoral responses to 38-kDa, MTB48, CFP10/ESAT6 antigens have been defined.[25] Screening of human immune sera against an expression library of *M. tuberculosis* open reading frames revealed three novel antigens among the top 20 most strongly recognized: Rv1987, Rv3807c and Rv3887c.[26]

More than a dozen studies have used DNA vaccination as a means of delivering antigens in immunogenicity tests. Many have shown Th1-biased immunogenicity without additional adjuvanting, for example with Ag85B,[27,28] Ag85B/ESAT6 fusion,[29] ESAT6/CFP10 fusion,[30] Mtb8.4/38-kDa/Ag85B fusion[31] and epitopes from ESAT6, Ag85A, CFP10 and Ag85B inserted within hsp65.[32] Targeting the expressed product for degradation via the ubiquitin pathway has been used to enhance MHC Class 1 presentation of epitopes from MPT64 and 38-kDa,[33] MPT64,[34] and ESAT6.[35,36] Liposome associated Ag85A DNA vaccine was shown to be immunogenic with oral delivery.[37] Enhanced responses to encoded antigens have been obtained by additionally encoding cytokines, such as interleukin 21 (IL-21), with Ag85A[38] or Ag85A/ESAT6,[39] and GM-CSF with Ag85A.[40] Inclusion of DNA

Table 2 The leading cities for TB vaccine research.

Main author affiliation	References
Beijing	
National Lab of Protein Engineering & Plant Genetic Engineering, Peking University	54–56, 59–61, 65, 72, 84
Institute for Tuberculosis Research, Second Hospital Affiliated to 309 General Hospital of the PLA	12, 25, 28, 80, 83
National Institute for Control of Pharmaceutical & Biological Products	71
Chengdu	
West China Center of Medical Science, Sichuan University School of Basic Medicine	9, 10, 52
Sichuan University, Chengdu	41
Chinese Cochrane Center, Chinese Evidence Based Medicine Center, West China Hospital, Sichuan University	73, 74
Chongqing	
Clinical Immunology Department, Chongqing University of Medical Sciences	13
Department of Pathobiology, Chongqing Medical University	75
Department of Microbiology, Chongqing University of Medical Sciences	57, 58
Department of Microbiology and Immunology, Chongqing University of Medical Sciences	78, 79
Haikou	
Department of Laboratory Medicine, Affiliated Xinhua Hospital of Hainan Medical College	31, 62, 63
Harbin	
Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science	68
Hong Kong	
Department of Pediatrics & Adolescent Medicine, Faculty of Medicine, University of Hong Kong	5
Jinan	
Institute of Basic Medicine, Shandong Academy of Medical Sciences	27
Lanzhou	
Institute of Pathogenic Biology, Lanzhou University	23
School of Basic Medical Sciences, Lanzhou University	48
Luzhou	
Department of Infectious Disease, Affiliated Hospital of Luzhou Medical College	77
Nanjing	
School of Basic Medical Sciences, South East University	38–40
Shanghai	
Institute of Genetics, Fudan University	7, 15, 21, 22, 24, 51
Ship Environmental Health Division, Institute of Naval Medicine Research	33, 42
College of Basic Medical Sciences, Second Military Medical University	33–36
Vaccine Research Laboratory, Shanghai H&G Biotechnology Co. Inc.	43, 44
Shanghai Public Health Clinical Center, Fudan University	85
Shenyang	
Department of Immunology, China Medical University	37
Wuhan	
Department of Immunology, Medical School of Wuhan University	4, 32, 53, 70
Department of Pathogen Biology, Huazhong University of Science & Technology, Tongji Medical College	50, 66, 67
Center for Proteomics Research, College of Life Science & Technology, Huazhong Agricultural University	26
Xi'An	
Fourth Military Medical University	6, 16–20, 45–47, 64, 69, 76
Research Center of Laboratory Animals, Fourth Military Medical University	30

expressing IL-12 enhanced prime/boost responses to BCG and to plasmids expressing Ag85A and ESAT6.[41] ESAT6 DNA priming and protein boosting has also been shown to give enhanced Th1 responses.[42–44]

TB PROPHYLAXIS

Many antigen preparations have been tested for their capacity to protect against challenge infection with virulent *M. tuberculosis*. Subcutaneous ESAT6/CFP10,[19] or ESAT6/MPT64[17,45] fusion proteins on nitrocellulose protected mice against H37Rv challenge, but not as effectively as BCG. hsp65/IL-2 fusion protein was found to elicit better protection than hsp65 given with DDA/MPL adjuvant and protection was equivalent to that produced by BCG.[46] Similarly, either hspX, a dormancy associated antigen, or synthetic epitope 91–104 gave protection equivalent to BCG when given with DDA/MPL.[47] Ag85B/$MPT64_{190–198}$/Mtb8.4 fusion protein with DDA adjuvant boosted protection against challenge in BCG primed mice.[48] A similar fusion protein in which Mtb8.4 was replaced by hspX gave a similar boost, but boosting with a mixture of the two fusion proteins was even better.[49] A fusion protein of ESAT6 and Ag85A also significantly boosted protection against H37Rv in mice.[50]

Recombinant BCG expressing the Ag85B/$MPT64_{190–198}$/Mtb8.4 fusion protein gave slightly better protection to mice against H37Rv challenge than parent BCG or BCG expressing rAg85B alone.[51] Recombinant BCG expressing a fusion protein of human interleukin (hIL)-12p70 and ESAT6 showed increased immunogenicity but less protective effect.[52] Recombinant *Salmonella typhimurium* that both expressed ESAT6/Ag85B fusion protein and delivered it as a DNA vaccine when given orogastrically gave protection similar to subcutaneous BCG and the combination was superior to either vaccine alone.[53]

Protection by DNA vaccination has been tested by many groups. The earliest reports indicated protection in mice superior to BCG when a divalent construct expressing both Ag85B and MPT64,[54] or a mixture of plasmids expressing Ag85B, MPT64, MPT63 and ESAT6[54,55] was used. The protection given by a mixture of three plasmids expressing MPT83, Ag85B and ESAT6 was enhanced by including DDA adjuvant[56] and an encoded fusion protein of Ag85B and MPT64 was superior to the separately encoded antigens.[57,58] Encapsulation in poly(lactide-co-glycolide) microspheres with DDA enhanced the protective efficacy in mice of DNA-encoding Ag85B/MPT64/MPT83 fusion antigen,[59] and strikingly the DNA mixed with DDA was superior to BCG in protecting cattle against challenge.[60] Inclusion of a plasmid expressing IL-2 improved protection by this plasmid[61] or by plasmid expressing Mtb8.4.[62] Plasmids expressing Ag85B,[28,63] Ag85B/ESAT6[29,64] or fusion proteins Mtb8.4/38-kDa/Ag85B[31] have given protection similar to BCG in mouse model challenge infections. A mixture of plasmids encoding Ag85B, MPT64, MPT70 and TB10.4 boosted protection by BCG,[65] as did a plasmid expressing a CFP21/MPT64 fusion protein.[66] Ag85B or Ag85A were superior to ESAT6 when compared separately for protection as DNA vaccines.[67] DNA expressing a fusion of MPB64/Ag85B/ESAT6 was superior to a mixture of plasmids expressing the separate antigens and gave protection equivalent to BCG.[68] The protective effect of DNA expressing hsp65 against BCG challenge was enhanced by incorporating epitopes of ESAT6, Ag85A/B and CFP10 within the hsp65 backbone.[32] The protective effect of hsp65 DNA against H37Rv challenge was increased by expression as a fusion with hIL-2, but did not surpass that of BCG.[69] Expression of Ag85B fused to bovine herpes virus 1 VP22 protein, which facilitates dissemination of antigen to adjacent cells, resulted in protection against H37Rv challenge in mice that was better than protection by BCG.[70] Few studies have been conducted with animals other than mice: a mixture of Ag85B, hspX and CFP10/ESAT6 fusion together with CpG and AlOH as adjuvant was immunogenic in mice but gave little protection against challenge with H37Rv in guinea pigs;[71] in contrast, combined DNA vaccines encoding antigens Ag85B, MPT64 and MPT83 given with DDA appeared to be better than BCG in protecting cattle.[72]

TB THERAPY

Interest in therapeutic vaccination has been sustained by clinical studies of a commercial Chinese product, M. vaccae extract. Recent meta-analyses of published data concluded that this product gave significant benefit in preventing TB in people at high risk (13 studies),[73] but there was only a minor benefit from treating new TB cases (54 studies).[74] In a unique and contrasting approach, a recombinant *M. smegmatis* delivering DNA expressing human granulysin and murine IL-12 was recently found to be therapeutic against H37Rv infection.[75]

Most research into therapeutic vaccines for TB has focused on the use of naked DNA vaccination. hsp65 DNA had a significant therapeutic effect against H37Rv in mice and the fusion hsp65/IL-2 was significantly better.[76] Treatment of infected mice with a DNA vaccine expressing a fusion protein of mycobacterial hsp70 and leukocyte cluster of differentiation antigen 80 substantially reduced acid-fast bacteria and pathology in liver and spleen, whereas BCG had no effect.[77] Although treatment with DNA expressing Ag85B was therapeutic, treatment with DNA expressing MPT64 was not, and the mixture was less effective than the Ag85B vaccine on its own,[78] and inclusion of DNA expressing IL-12 gave a slight additional benefit.[79] Studies of treatment of multidrug-resistant TB in mice have given encouraging results. Mice infected with a clinical isolate resistant to isoniazid and rifampicin responded well to treatment with the drugs plus DNA expressing chimeric Ag85A/ESAT6,[80] and appeared to respond better to treatment with the drugs plus DNA expressing Ag85A than to the DNA vaccine alone;[81] Ag85A DNA alone was at least as effective as Ag85A plus rifampicin in treating mice infected with a strain resistant to rifampicin and isoniazid, Ag85A/ESAT6 DNA was less effective[82] and Ag85A DNA worked in combination with pyrazinamide.[83] An immunogenic mixture of DNA vaccines expressing Ag85B, MPT64 and MPT83 has been found to work as a potent adjunct to isoniazid plus pyrazinamide therapy in mice[84] and to be therapeutic in cattle infected with *M. bovis*, reducing both pathology and bacterial numbers; a mixture expressing Ag85B, MPT64 and hsp65 was similarly effective.[72] Inclusion of immunostimulatory nucleotide motifs in the gene transcript enhanced the therapeutic efficacy of a plasmid expressing hsp65 when tested against H37Rv in mice.[85]

CONCLUSIONS

It is evident in considering this body of recent TB vaccine research in China that much of it has been tactical in nature, establishing credentials both nationally and internationally, and building new research bases. Additionally, there are creative and insightful studies of particular relevance to the needs of China. Researchers are now able to exploit cutting edge technologies in designing new TB vaccines and are increasingly able to test the vaccines in relevant animal models of infection. The evidence that DNA vaccines can provide effective therapy for TB in cattle[72] may be a significant pointer to the future. The potential benefits of adding immunotherapies/therapeutic vaccines to TB chemotherapy have been recognized in China, but both preventive and therapeutic approaches against human TB await development of clinical trial capacity for their proper assessment.

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