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Research paper

The truncated *Rv2820c* of *Mycobacterium tuberculosis* Beijing family augments intracellular survival of *M. smegmatis* by altering cytokine profile and inhibiting NO generation

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ABSTRACT

Genetic variations among genes of *Mycobacterium tuberculosis* may be associated with antigenic variation and immune evasion, which complicates the pathogenesis of *M. tuberculosis*. The hyper-virulent *M. tuberculosis* Beijing strains harbored several large sequence deletions, among which RD207 attributed to the deletion of CRISPR loci and several *Cas* genes. RD207 also gave rise to a truncated gene *Rv2820c-Bj* with 60% deletion in length at the 3′-end and a new 3′-end of five amino acid mutations. It has been reported that *Rv2820c-Bj* correlated with enhanced intracellular survival of *M. smegmatis* in macrophages when compared to its full-length counterpart *Rv2820c* in *M. tuberculosis*, however, the respective contribution of the truncation and the new 3′-end of Rv2820c-Bj to this enhancement was unclear. Here, by infecting THP-1 macrophages with Ms_Rv2820c-Bj, Ms_Rv2820c and MS_Rv2820c-Tr (expressing the truncated Rv2820c without five amino acid mutations at 3′-end), we found only Ms_Rv2820c-Bj was responsible for the enhancement of survival of *M. smegmatis* in macrophages. Furthermore, we detected that Ms_Rv2820c-Tr and Ms_Rv2820c-Bj induced similar cytokine profile and NO production after infection of macrophages, which was distinctly different from Ms_Rv2820c. However, Ms_Rv2820c-Bj evoked higher levels of interleukin-10 (IL-10) and lower levels of interleukin- 6 (IL-6), interleukin-1β (IL-1β) and interleukin-12 (IL-12) in infected THP-1 macrophages than Ms_Rv2820c-Tr. Accordingly, we concluded that the new 3′-end of Rv2820c-Bj was important to dampen host defense and enhance the intracellular survival of *M. smegmatis*.

1. Introduction

Tuberculosis remains second only to HIV/AIDS as the leading cause of infectious disease mortality worldwide, with estimated 10.4 million new instances and 1.4 million deaths in 2015(WHO, 2016). The causative agent of human tuberculosis, *Mycobacterium tuberculosis* (MTB), is currently classified into seven major lineages (lineage 1–7) on the basis of genomic diversity, including single nucleotide polymorphisms (SNPs) and large sequence polymorphisms (LSPs) (Coscolla and Gagneux, 2014; Dos et al., 2009; Filliol et al., 2006; Mostowy et al., 2002; Yimer et al., 2017). These genomic variations of MTB lineages display different phenotypic consequences ranging from virulence, immunogenicity to the emergence of drug resistance (Alland et al., 2007; Chen et al., 2014; Comas et al., 2010; Coscolla and Gagneux, 2014; Hershberg et al., 2008). Indeed, the complex interactions between MTB genomic diversity and pathogenesis exacerbate the control of tuberculosis. Improvement in the knowledge of the genetic basis of MTB pathogenesis will undeniably aid the development of vaccines and new therapy strategies for tuberculosis control.

Among the seven lineages of MTB, lineage 2, also known as Beijing family, is one of the most successful lineages and predominates in South and East Asia (Bifani et al., 2002; Liu et al., 2016). The important features of this lineage are hyper-virulent and capable of building up further drug resistance without impairing its ability to spread (Tsolaki et al., 2005). Much emphasis has been placed on investigating the association between genetic features and pathogenic characteristics of Beijing strains. There is increasing evidence that the accumulation of genetic diversity contributes to the pathogenicity of Beijing lineage. An

Abbreviations: RD207, region of difference 207; CRISPR, clustered regularly interspaced short palindromic repeats; Cas genes, CRISPR-Cas associated genes; iNOS, inducible nitric oxide synthase; NO, nitric oxide; IL-10, interleukin-10; IL-6, interleukin-6; IL-1β, interleukin-1β; IL-12, interleukin-12

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SNP within the gene directly upstream of dormancy survival regulator (DosR) creates an alternative internal transcriptional start site (TSS), which is associated with increased expression of the DosR regulon in Beijing strains (Rose et al., 2013). The strategy of DosR regulon overexpression and the occurrence of frameshift mutation in the gene encoding the DosT sensor kinase may aid the bacteria in the face of changing environmental conditions encountered in the host (Fallow et al., 2010; Reed et al., 2007). Large genomic deletions and mobile genetic elements, such as *IS6100*, are important sources of genomic variation. Missense mutations in repair genes (*Rv3908* and *mutT2*) of Beijing strains were supposed to benefit the rapid adaption to fresh environment since they might increase mutation rate (Liu et al., 2016). In lineage 2/Beijing strains, insertion of the *IS6110* into CRISPR region led to the Beijing specific deletion named RD207 (Bifani et al., 2002; Gagneux et al., 2006). The RD207 deletion includes two CRISPR loci and seven *Cas* genes (*Rv2814c-Rv2820c*), and these deleted segments belong to the CRISPR-Cas system of MTB responsible for adaptive immunity, potentially to confer resistance to invasive genetic elements (phage and plasmid DNA) (Garneau et al., 2010; He et al., 2012; Makarova et al., 2015). Until now, just one study uncovered that the deleted *Cas2* gene (*Rv2816c*) was deleterious to *M. smegmatis* survival (Huang et al., 2016). Therefore, the functional impact of polymorphisms in CRISPR-Cas system on mycobacterial phenotypes has not been fully determined.

Among the seven deleted *Cas* genes of RD207, *Rv2820c* is exceptional in lineage 2/Beijing strains due to the truncation of 119th to 303rd amino acids (Fig. 1A) (Lam et al., 2011). A previous study found that *Rv2820c-Bj* rather than full-length *Rv2820c* of the non-Beijing/W strain could enhance mycobacterial virulence ex vivo and in vivo mainly through promoting intracellular survival of *M. smegmatis* (Lam et al., 2011). And then it postulated that the deleted 60% segment in 3′-end of full-length Rv2820c might compromise intracellular survival, although the specific mechanism was unclear. Sequence analysis revealed that the truncation also gave Rv2820c-Bj a new 3′-end of five amino acid mutations. As genomic polymorphisms could alter the phenotypic virulence and functional effects of *M. tuberculosis* (Jiang et al., 2016; Reed et al., 2004; Shimono et al., 2003), the new 3′-end of Rv2820c-Bj may also contribute to the observed enhancement of survival of *M. smegmatis*. To further understand the respective contribution of the new 3′-end and the truncation of Rv2820c-Bj in mycobacterial virulence, we introduced Rv2820c-Tr as a control, which was a truncated variant of Rv2820c that had the same length as Rv2820c-Bj but a difference of five amino acids at 3′-end (Fig. 1B). We compared the functions of Ms_Rv2820c-Bj, Ms_Rv2820c-Tr and Ms_Rv2820c using cytokine and in vitro stresses assays to explore the relevance of the truncation and the new 3′-end of Rv2820c-Bj in the enhanced intracellular survival of *M. smegmatis*.

2. Materials and methods

2.1. Bacterial, cell line cultures

Genomic DNA of MTB H37Rv and Beijing lineage were kind gifts from Disease Control Central of Chengdu, China. *M. smegmatis* mc^{2}155 (ATCC) were grown in Middlebrook 7H9 broth (Difco, USA) supplemented with ADC (5 g bovine serum albumin (BSA), 2 g glucose and 0.85 g NaCl/l), 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80 at 37 °C with constant shaking. Bacterial cells were plated on Middlebrook 7H10 agar plates supplemented with ADC and were stored in 30% glycerol at −70 °C after enumeration. Antibiotics were added when necessary: kanamycin 50 mg/ml. Human macrophage cell line THP-1 macrophages (ATCC) were cultured in complete RPMI comprising RPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml) and maintained at 37 °C in a humidified incubator (5% CO2).

2.2. Construction of recombinant M. smegmatis strains

All target genes were amplified from MTB H37Rv and Beijing lineage genomic DNA using a specific set of forward and reverse primers listed in Table S1 for each gene that incorporated a 5′-end *Eco*R I and a 3′-end *Hin*d III site. The polymerase chain reaction (PCR) products were ligated into plasmid pMV261. These constructs plasmids (pMV261, pMV261_Rv2820c-Bj, pMV261_*Rv2820c*-Tr and pMV261_Rv2820c) were sequenced and electroporated into *M. smegmatis* mc^{2}155. The recombinant *M. smegmatis* strains were selected on MB 7H10 solid medium containing 50 μg/ml kanamycin (*Kan*). The positive recombinant strains harboring pMV261_Rv2820c, pMV261_Rv2820c-Tr and pMV261_Rv2820c-Bj three genes were confirmed by PCR amplification.

2.3. Quantitative real-time PCR (qPCR) analysis

For the qPCR analysis, total RNA was extracted using TRIzol reagent (Sigma, USA) from mid-log phase cultures of the four recombinant *M. smegmatis* strains and the infected THP-1 macrophages at each given time point, respectively. Results were carried out on a BIO-RAD CFX Connect qPCR system. For the detection of genes expression of four recombinant *M. smegmatis* strains, gene-specific primers are listed in Table S1B and the synthesis of cDNAs were performed using RevertAid Premium Reverse Transcriptase (Thermo Scientific) as per the manufacturer's instructions. qPCR was conducted using SYBR Green reagents, and the reactions conditions were as follows: 95 °C for 7 s, 57 °C for 10 s, and 72 °C for 15 s, repeated 45 times. The SigA gene in each recombinant *M. smegmatis* strain sample was used as reference gene.

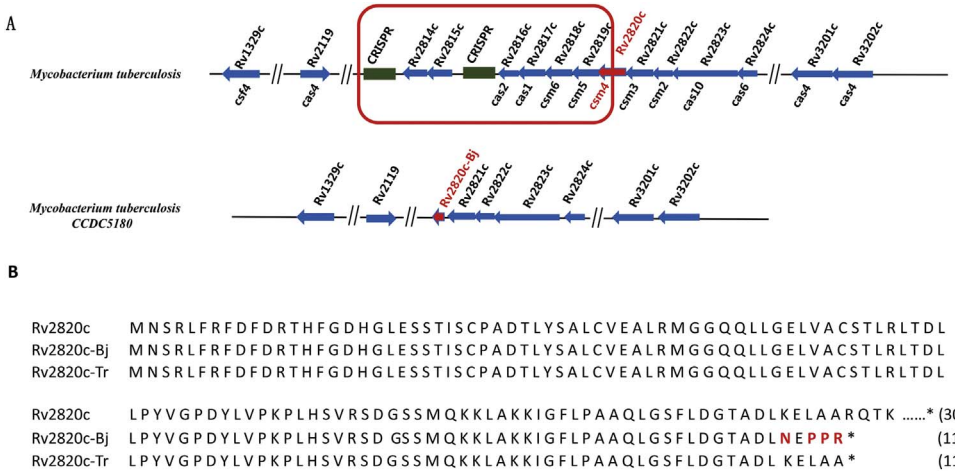

Fig. 1. Gene arrangement and amino acid sequence alignments. (A) Schematic representation of the gene organization in the region of *M. tuberculosis* H37Rv locus and orthologous regions of *M. tuberculosis* Beijing lineage. Arrows indicate the relative direction of transcription. The coloring scheme is as follows: the full-length *Rv2820c* in *M. tuberculosis* is red and the 3′truncated *Rv2820c*, namely, *Rv2820c-Bj* is red in *M. tuberculosis* CCDC5180. The contents of the red box represent RD207 in Beijing lineage. (B) Amino acid sequence alignment. Further amino acid sequence details of structural variation were revealed among Rv2820c, Rv2820c-Bj and Rv2820c-Tr. Highlights indicate the disagreement with each other. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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