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Rv2820c of Beijing/W strains enhances *Mycobacterium tuberculosis* survival in human macrophages

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Abstract

The ability to survive in human macrophages is a hallmark of the virulence of *Mycobacterium tuberculosis*. Although the intracellular parasitism is apparent, the molecular determinants behind are still largely unknown. The truncated Rv2820c of the Beijing/W strains of *Mycobacterium tuberculosis* was previously shown to enhance the survival of *Mycobacterium smegmatis* in the human macrophages. The enhanced intracellular survival, however, was not observed in the recombinant harboring the intact Rv2820c of the non-Beijing/W strains. In the current investigation, the role of the truncated Rv2820c in *M. tuberculosis* was examined using a 'gain-of-function' manner. The truncated Rv2820c was transformed into non-Beijing/W strains of *M. tuberculosis* and the resulting recombinants were used to infect the monocytic cell line THP-1. The *ex vivo* infection showed that the non-Beijing/W *M. tuberculosis* recombinants survived significantly better than the vector controls after ten days of infection ($P < 0.05$; independent samples t-test, two-tailed). Similar levels of interleukin-6, interleukin-10, and tumor necrosis factor-alpha were secreted from the macrophages infected with those non-Beijing/W recombinants. This study showed that the Rv2820c of the Beijing/W strains is capable of enhancing the *M. tuberculosis* survival in the human macrophages, but is unlikely to evoke a different profile of cytokine secretion from the infected macrophages. It suggests that the truncated Rv2820c may be another Beijing/W-specific virulence determinant.

Keywords: *Mycobacterium tuberculosis*; Beijing/W strains; Rv2820c; THP-1 cells

Introduction

The phenotypic virulence of *M. tuberculosis* is not invariable among different strains. Particular strains of *M. tuberculosis*, such as the Beijing/W family, have proven to be more virulent using *ex vivo* [1,2] and *in vivo* models [3,4]. The Beijing/W family was shown to grow more rapidly inside human macrophages [1,2], induce a weaker cell mediated immunity [5] but a stronger humoral immunity [6], cause a higher and earlier mortality in mice [3,4,7], and increase the likelihood to cross blood brain barrier and cause disseminated infection in rabbits [8]. Unique virulent factors may be evolved and conserved among the descendants of the Beijing/W family.

The polyketide synthase-derived phenolic glycolipid (PGL) is one of these Beijing/W-specific virulence determinants [7]. The PGL is capable of suppressing the liberation of tumor necrosis factor-alpha (TNF-α), interlekin (IL)-6, and IL-12 from macrophage dose-dependently [7]. This PGL is commonly found in the Beijing/W strains but not in the non-Beijing/W strains, such as the H37Rv and the CDC1551 [7]. It is because a seven-base-pair frameshift deletion is present in the genome of the non-Beijing/W strains, which leads to the partition of the intact *pks15/1* gene into two separated genes *pks15* and *pks1* [9]. The polyketide synthase encoded by the *pks15/1* is essential for the production of PGL [9]. Inactivation of the *pks15/1* not only compromises the production of PGL but also attenuates the hypervirulent phenotype of the Beijing/W strains in mice [7].

The truncated Rv2820c is possibly another Beijing/W-specific virulence determinant [10]. The truncated Rv2820c enhances the survival of *Mycobacterium smegmatis* recombinant in human macrophages and causes earlier death of zebrafish after infection with *Mycobacterium marinum* recombinant [10]. The truncation in the Rv2820c is caused by the Beijing/W-defining deletion RD207 [11]. This deletion is commonly found among the Beijing/W strains [10,12]. Non-Beijing/W strains possess an intact Rv2820c, which does not enhance the mycobacterial virulence *ex vivo* and *in vivo* [10]. Although the truncated Rv2820c was demonstrated to enhance the survival of the surrogate host *M. smegmatis* in human macrophages, the role of this protein in *M. tuberculosis* is uncertain.

In the current investigation, the role of the truncated Rv2820c in *M. tuberculosis* was examined using a 'gain-of-function' manner. The truncated Rv2820c was transformed into non-Beijing/W strains of *M. tuberculosis*, which possess the intact Rv2820c only. To determine whether the truncated Rv2820c enhances the *M. tuberculosis* survival in human macrophages, the survivability of the non-Beijing/W recombinants inside the human monocytic cell line THP-1 was measured. The secretion of IL-6, IL-10, and TNF-α from the infected macrophages was also quantified to determine whether the truncated Rv2820c is involved with immunomodulation.

Materials and Methods

Bacterial strains and growth conditions

Two non-Beijing/W strains of *M. tuberculosis* were used in this

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study. They were the laboratory strain H37Rv (ATCC 27294) and a clinical isolate H83 from our previous study [13]. The H37Rv was purchased from the American type culture collection (ATCC). The non-Beijing/W genotype of both strains was verified using the mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) as described previously [14]. These two *M. tuberculosis* strains were grown in Middlebrook 7H9 broth supplemented with 10% oleic acid albumin dextrose catalase (OADC), 0.2% glycerol, and 0.05% Tween-80 or on Middlebrook 7H10 plates supplemented with 10% OADC and 0.5% glycerol. The broth culture was grown at 37 °C with shaking at 130 revolutions per minute (rpm) until mid-logarithmic phase. The agar plates were incubated at 37 °C until visible colonies formed. Hygromycin was included at 50 μg/ml when growing the recombinants harboring the expression vector pVV16 (Tuberculosis Vaccine Testing and Research Materials, Colorado State University).

THP-1 cells

The human monocytic cell line THP-1 (ATCC TIB-202) was purchased from the ATCC. They were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2. The THP-1 cells were induced with 20 nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate (Sigma Aldrich) at a density of 3 x 10^5 cells in each well of a 24-well plate for 24 hours to differentiate into macrophage-like phagocytic cells [10].

Construction of recombinant *M. tuberculosis* strains

The truncated Rv2820c of the Beijing/W strains were amplified using the *PfuTurbo Cx* hotstart DNA polymerase (Stratagene) and the primers Rv2820c'-F (5' TATAACATATGAACTCGCGGCTGTTTAGGTTCGAC) and Rv2820c'-R (5' TATAAAAGCTTTCAGATCAGAGAGTCTCCGGACTCACC). The restriction sites incorporated in the amplicon were exposed using the restriction endonucleases NdeI and HindIII (New England BioLabs) and were ligated to the expression vector pVV16 using the T4 DNA ligase (New England BioLabs). Correct insertion was confirmed using nucleotide sequencing and the sequencing primers Seq-Fwd (5' CGGTGAGTCGTAGGTCGGGACGG) and Seq-Rev (5' TGCCTGGCAGTCGATCGTACGCTAG). A total of 1 μg of the resulting plasmid was electroporated into the *M. tuberculosis* strains H37Rv and the H83 at 2.5 kV, 1 kΩ, and 25 μF. A naked pVV16 was also transformed as a vector control.

Ex vivo infection of THP-1 cells with *M. tuberculosis* recombinants

The differentiated THP-1 cells were infected with the mid-logarithmic recombinants of *M. tuberculosis* at a multiplicity of infection (MOI) of 1 at 37°C with 5% CO_2 for 24 hours. The mycobacterial suspension was passed through a 25-gauge needle (Terumo) ten times to disperse mycobacterial clumps prior to the inoculation [15]. The inoculum was enumerated using the plate counting method. To kill the extracellular mycobacteria, amikacin at 200 μg/ml was added to the cells and was incubated at 37°C with 5% CO_2 for 1 hour. This was considered as the day 0 of infection. The cells were lyzed with 0.1% SDS after 0, 3, 6, and 10 days of infection. The intracellular mycobacteria were also enumerated using the plate counting method.

Measurement of cytokine secretion

The differentiated THP-1 cells were infected with the mid-logarithmic recombinants of *M. tuberculosis* as described in the '*Ex vivo* infection of THP-1 cells with *M. tuberculosis* recombinants'. The culture supernatant was collected at 4, 24, 48, and 96 hours after infection. A mock control without the addition of mycobacteria was also included in parallel. The levels of IL-6, IL-10, and TNF-α in the culture supernatant were quantified using the OptEIA human ELISA kit II (Becton Dickinson).

Statistical analysis

To detect any significant differences in the survivability of *M. tuberculosis* recombinants inside the THP-1 cells, an independent samples t-test (two-tailed) was performed using the PASW (formerly SPSS) Statistics 17.0 program (International Business Machines).

Results

Rv2820c of Beijing/W strains enhances *M. tuberculosis* survival in human macrophages

The truncated Rv2820c of the Beijing/W strains was transformed into the two non-Beijing/W strains of *M. tuberculosis* (the clinical isolate H83 and the laboratory strain H37Rv) and their recombinants were used to infect the THP-1 cells. The *ex vivo* infection showed that the survivability of both non-Beijing/W strains was enhanced after acquiring the truncated Rv2820c of the Beijing/W strains (Figure 1) and was significantly different with the vector controls after ten days of infection (P_{H37Rv} = 0.036, P_{H83} = 0.047; independent samples t-test, two-tailed).

Rv2820c of Beijing/W strains did not induce a different cytokine secretion profile

The levels of IL-6, IL-10, and TNF-α secreted from the infected macrophages were quantified using the ELISA. The non-Beijing/W recombinants induced similar levels of IL-6, IL-10, and TNF-α from the infected macrophages (Figure 2). A different cytokine secretion profile was not observed for the non-Beijing/W strains after acquiring the truncated Rv2820c of the Beijing/W strains.

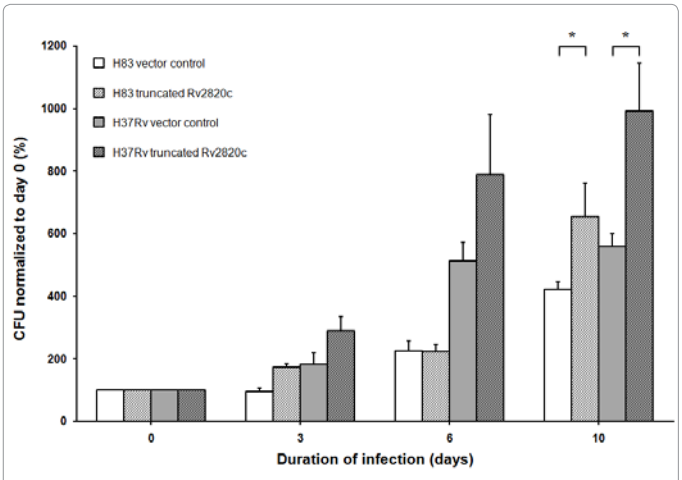

Figure 1: Survivability of *Mycobacterium tuberculosis* recombinants harboring truncated Rv2820c or vector alone inside THP-1 cells. The colony forming unit (CFU) of recombinants was normalized to the CFU on day 0. Mean percentage is shown while its error bars represents plus or minus standard error of the mean (±1 SEM) of three independent experiments performed in duplicate. * $P < 0.05$; independent samples t-test (two-tailed).

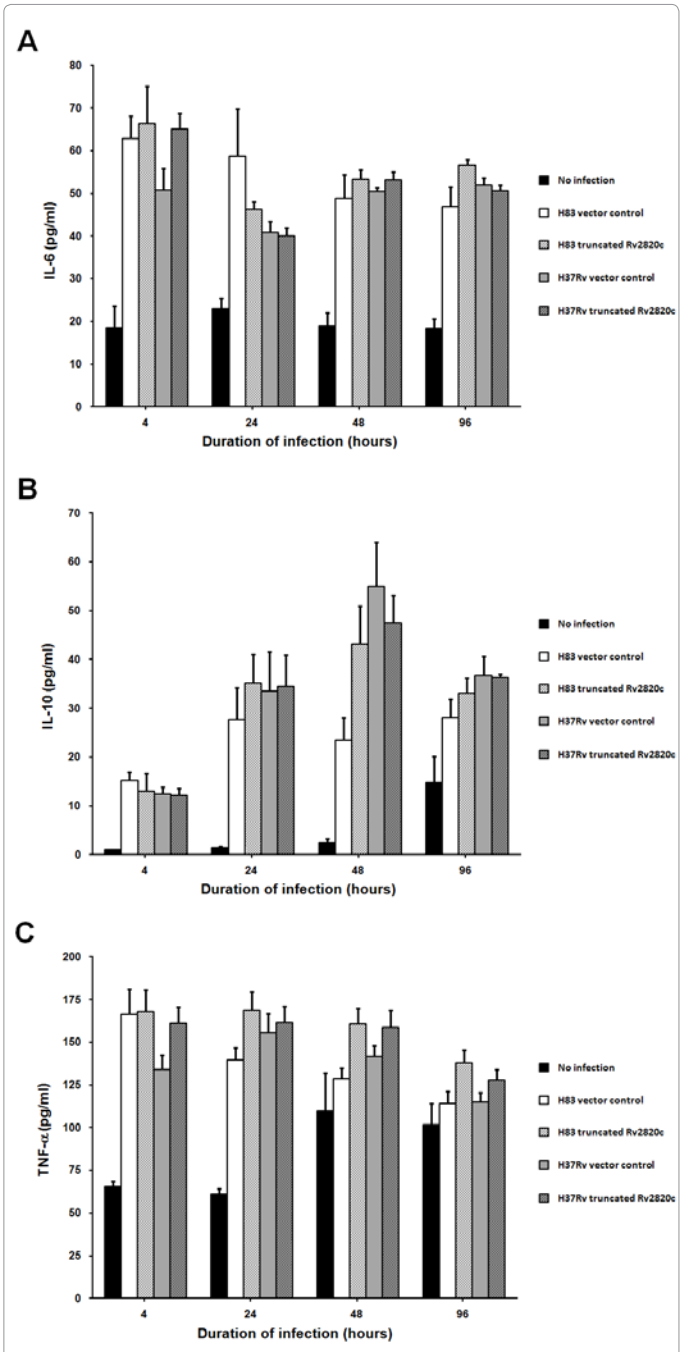

Figure 2: Secretion of interleukin-6 (A), interleukin-10 (B), and tumor necrosis factor-alpha (C) from THP-1 cells infected with *Mycobacterium tuberculosis* recombinants harboring truncated Rv2820c or vector alone. Mean concentration is shown while its error bars represents plus or minus standard error of the mean (±1 SEM) of three independent experiments performed in duplicate.

Discussion

The deletion RD207 is an early event in the evolution of Beijing/W strains [12]. It causes not only the characteristic pattern of spacer oligotyping (spoligotyping), which loses the spacers from 1 to 34 [16], but also the 3' truncation in the gene Rv2820c [11]. The truncated Rv2820c is capable of enhancing the survival of *M. smegmatis* in the human macrophages [10]. As the *M. smegmatis* is only a surrogate host, the results generated from this surrogate host may not be directly applicable to the *M. tuberculosis*. The current study examined the role of the truncated Rv2820c in *M. tuberculosis* using a 'gain-of-function' manner. The truncated Rv2820c was transformed into non-Beijing/W strains of *M. tuberculosis*, which possess the intact Rv2820c only. The intracellular survivability of non-Beijing/W recombinants, whether it was a clinical strain or a laboratory strain, was enhanced after acquiring the truncated Rv2820c. The current study confirmed the previous finding that the truncated Rv2820c of the Beijing/W strains enhances mycobacterial survival *ex vivo* [10].

A portion of the Beijing/W strains is capable of unbalancing the host immune response to a non-protective side [5,7,17]. They inhibit the production of pro-inflammatory cytokines, such as IL-6 and TNF-α [5,7], and simultaneously stimulate the production of anti-inflammatory cytokines, such as IL-10 [17]. Both TNF-α and IL-6 are essential for the proper formation of granuloma [18]. The TNF-α is critical for the initiation of granuloma formation, whereas the IL-6 is important for the maintenance of granuloma [18]. The anti-inflammatory cytokine IL-10 is originally used to calm the immune system down to avoid overreacting, but appears to be misused by the Beijing/W strains. To determine whether the truncated Rv2820c is involved with the immunomodulation, the levels of IL-6, IL-10, and TNF-α secreted from the infected macrophages were measured. A different cytokine secretion profile, however, was not observed in the macrophages infected with the non-Beijing/W recombinants.

In conclusion, the current study showed that the Rv2820c of Beijing/W strains is capable of enhancing the *M. tuberculosis* survival in the human macrophages, but is unlikely to evoke a different profile of cytokine secretion from the infected macrophages. It suggests that the truncated Rv2820c may be another Beijing/W-specific virulence determinant.

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