Development of TB recombinant mycobacterial vaccine without antimicrobial resistance marker

Ana Paula Junqueira-Kipnis[1,3, 4], Eduarda Pereira Soares Dias[2,4], Patrícia Vaz França[2,4] Gabriel Costa[2,4], Guilherme Silva[2,4], André Kipnis[2,4]

[1] Laboratory of Immunopathology of the infectious diseases, Federal University of Goiás, *Brazil,* [2] Laboratory of molecular bacteriology, Federal University of Goiás, *Brazil.* [3]*Brazilian Network for Tuberculosis diseases.* [4]*Goiás Tuberculosis network. Tuberculosis*

Background

The Goiás Tuberculosis Research Network has been developing several vaccines for tuberculosis. Among the vaccines developed, vaccines using vehicles that contain the same infection mechanisms and that stimulate defense mechanisms like *Mycobacterium tuberculosis* infection are the most promising. The vaccine expressing the chimeric molecule containing immunodominant epitopes of *M. tuberculosis* antigens: Ag85c, MPT51, and the whole HspX molecule was previously shown. *M. smegmatis* was chosen as vector because of its ability to induce protection against Tuberculosis. These results can be seen in the introductory figures (Figure 1) published in PLoS One.

Considering aspects such as decreased lung inflammation and better reduction of bacterial load, it was decided to improve the mc²-CMX vaccine. Among the requirements for a live vector-based vaccine, there was a need to abolish the use of plasmids requiring the use of antibiotics. The strategy present here removes resistance genes that are located within *dif* regions, which allows natural excision by mycobacteria through cleavage with endogenous recombinases.

Objective:

To develop live attenuated mc²-based vaccines containing the genome-integrated CMX protein with no mark of antimicrobial resistance.

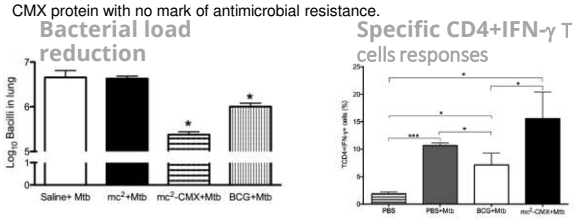
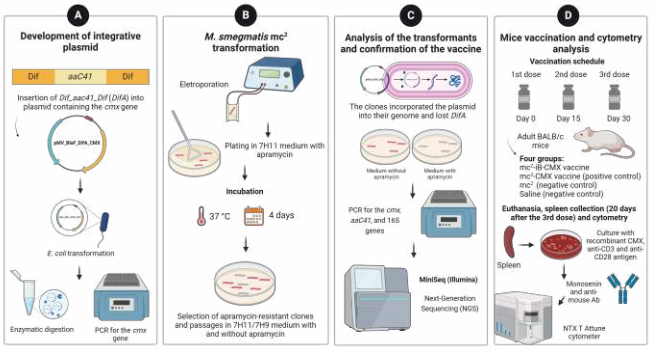


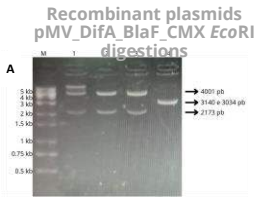
Figure 1. Reduction in the lung bacterial load in animals vaccinated with mc²-CMX and specific CD4+IFN-γ T-cell populations induction. 1. Groups of mice vaccinated with saline or PBS, mc², mc²-CMX, or BCG (A), and then vaccinated with saline, IKE, IKE-CMX, or BCG (B) were challenged with *Mtb* 45 days after immunization. Thirty days after infection, their lungs were collected and CFU counted. *p<0.05 when compared with the saline+*Mtb* group. **2.** Lung CD4⁺ T-cell response to *ex vivo* stimulation with CMX. CD4⁺ T cells selected with lymphocyte gating for IFN-γ were quantified Published at: Junqueira-Kipnis et al, PLoS One. 2013; 8(11): e78639. and Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 111(4), April 2016

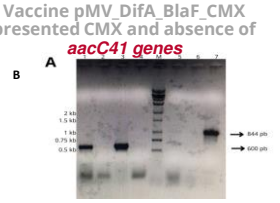
Methods



(A) Construction of integrative plasmids, in which the antibiotic resistance gene apramycin flanked by Dif sequences (DifA_aac41_DifA) was cloned in pMV306K_Hsp60_CMX and pMV306K_BlaF_CMX giving rise to pMVh_DifA_CMX and pMV_BlaF_DifA_CMX, respectively. (A) Confirmation of cloning was according to the enzymatic digestion and/or PCR profiles of the cmx gene. (B) *Mycobacterium smegmatis* mc² were transformed with the recombinant plasmids and seeded in 7H11/OADC medium with apramycin. Resistant clones were incubated in replicates by several passages in medium with and without apramycin to identify the clones that lost resistance to apramycin. (C) Clones with the expected phenotype (sensitive to apramycin) were analyzed to verify the loss of the aac41 gene and the permanence of the CMX gene by PCR and NGS MiniSeq sequencing (Illumina). (D) mice were vaccinated with saline, mc², or mc²-CMX or mc²iB – CMX vaccines with three doses 15 days apart. After 20 days of the last immunization, the animals were euthanized, and the spleens cells were cultured for 2 hours with recombinant CMX, anti-CD3 and anti-CD28 antigen. After this period, BD Golgi stop solution was added and the cultures were kept for 4 hours. Cells were labeled with anti-mouse antibodies CD4-FITIC, IL-17-PERCP, IFN-APC, TNF-PE-CY7 (BD Pharmingen) using Permfix/PermWash- BD Pharmingen protocol and evaluated using NTX T Attune cytometer. The results represent 300 thousand events.

Results





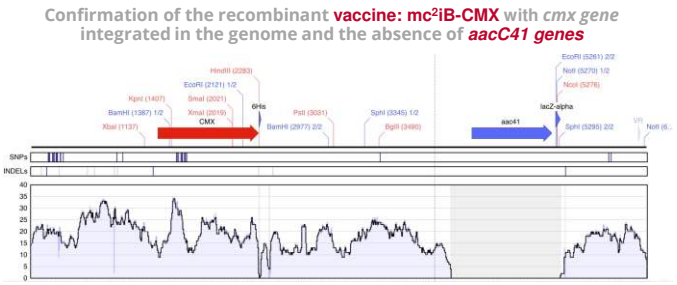


Figure 2. A. Agarose gel electrophoresis of clones 1, 2, 3 and 4 of pMV_DifA_BlaF_CMX digested with *Eco*RI. Clones 2 and 3 had lanes with sizes of 4001 bp and 2173 bp, and clone 4 had two bands of similar sizes (3140 bp and 3034 bp). Both profiles were expected according to the orientation of the insert. M- Molecular mass marker.
B. PCRs for the cmx and *aacC41 genes* of clones 2.5 and 10.7 of mc² transformed with pMV_BlaF_DifA_CMX. One clone (10.7) did not present the expected profile (lane 2). Lanes: 1 - clone CMX 2.5 mc2/pMV_BlaF_DifA_CMX; 2 - clone 10.17 mc2/pMV_BlaF_DifA_CMX; 3 - Positive control for CMX; 4 - Negative control for CMX; M - Molecular mass marker; 5 - PCR for apramycin gene from clone 2.5; 6 - PCR for apramycin gene from clone 10.7; 7 - Positive control for apramycin gene. Vaccine clone 2.5 had the expected profile. (B) Alignment of the NGS sequences of the mc² 2.5 clone with the plasmid pMV_BlaF_DifA_CMX proving the presence of the CMX gene (red arrow) and absence of the apramycin resistance gene (aac41, blue arrow), thus conforming the mc² iB-CMX vaccine.

Similar immunogenicity of mc²iB-CMX and mc²-CMX vaccinated animals

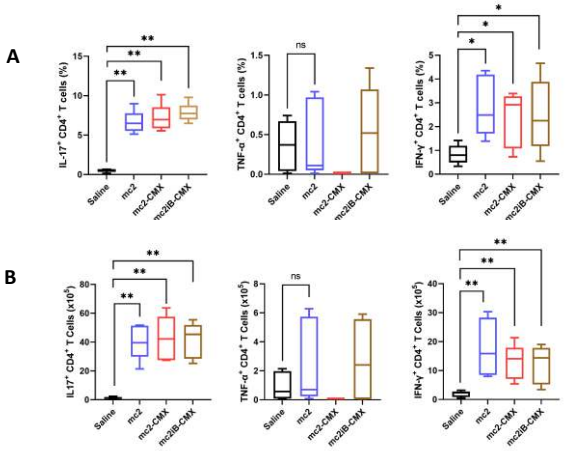


Figure 4. (A) Percentage of splenic CMX specific CD4+ T cells and (B) Total CD4⁺ T cells from mice vaccinated with saline, mc², or mc²-CMX. Adult BALB/c mice were vaccinated with three doses 15 days apart with the saline, mc², or mc²-CMX or mc²iB – CMX vaccines.

Conclusion

A live attenuated mc²-iB- CMX recombinant vaccine with no antimicrobial resistance marker was developed.

Acknowledgements

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