Genomic editing, mediated by CRISPR/Cas9, of *PDCD1, CTLA4 e LAG3* loci in T lymphocytes expressing Chimeric Antigens Receptor (CARs)

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ABSTRACT

Introduction and Aim: The immune system plays an important role in tumor editing, being capable of eliminating this threat. The actual landscape of cancer treatment includes surgery, chemotherapy, radiotherapy and more recently immune-based approaches, such as the use of monoclonal antibodies, vaccines and adoptive immunotherapy. Different approaches to genetic modulate patients own immune response are being successfully used, such as CAR based treatment. However, occasionally tumor escapes. The Programmed Cell Death 1 (PD-1), Cytotoxic T-Lymphocyte-associated Protein 4 (CTLA-4) and Lymphocyte-activation gene 3 (LAG-3) pathways are constantly associated with cancer evasion from immune system. When associated with its ligands, PD-L1, CD80/86 and MHCII, respectively, these proteins inhibit T lymphocyte activation and proliferation, frequently blocking their effector function. Knockdown of PD-1, CTLA-4 and LAG-3 expression by genomic editing, may increase the anti-tumor functions of these cells. Recently, the Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR/Cas9) has emerged, as a new tool for site-specific genome editing. We propose here a CRISPR-based genetic engineering system to knockdown PD-1, CTLA-4 and LAG-3 expression in T-cells rendering these cells resistant to checkpoint inhibitors mediated inhibition. **Methods and Results:** We designed gRNAs using the Optimized CRISPR Design program at *crispr.mit.edu* targeting the *PDCD1*, *CTLA4* and *LAG3 loci*. DNA sequences for the gRNAs were cloned into the CRISPR plasmid vector and electroporated into human peripheral blood mononuclear cells (PBMC) and HEK293FT cell line. 24 hours later, DNA of CRISPR expressing cells was extracted by phenol-chloroform. A PCR was designed to amplify each target *locus*. The PCR products were cloned into the TA Cloning® Kit vector and the colonies were sequenced to validate the gRNAs. We are currently characterizing the functional edition of these *loci* by co-electroporating the CAR and the pool of gRNAs transcripts (*in vitro* transcription by MEGAshortscript™ kit), in PBMCs aiming tol evaluate the effective response of edited cells against CD19+, MHCII+, PD-L1+ and CD86+ cells by lysis assay, and their possible enrichment among the T cell population during in vitro lymphocyte expansion. **Conclusion:** We propose here a system to knockdown pathways largely used by tumors to inactivate the immune response. We will test the effects of PD-1, CTLA-4 and LAG-3 inactivation by CRISPR system in T Lymphocytes expressing a Chimeric Antigen Receptor.

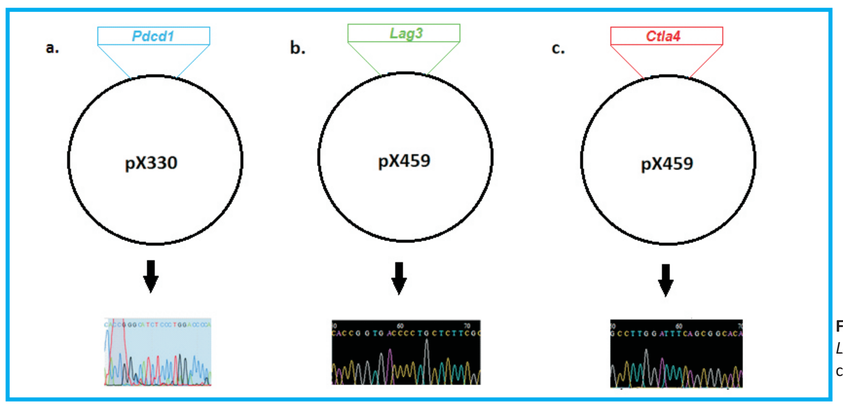

Fig. 1. Representation of *PDCD1, CTLA4* and *LAG3* gRNAs cloned in CRISPR plasmids and confirmation by sequencing.

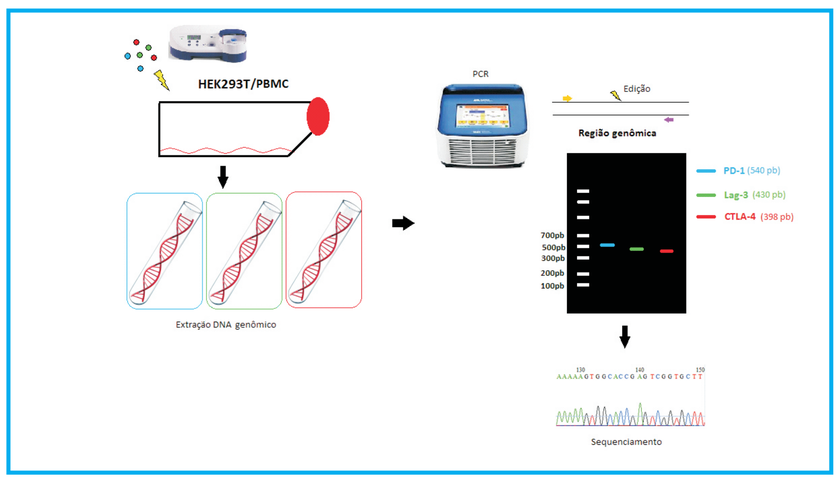

Fig. 2. Experimental scheme of gRNAs individual electroporation, in HEK293T cells and PBMCs, and subsequent indels analysis by PCR and sequencing .

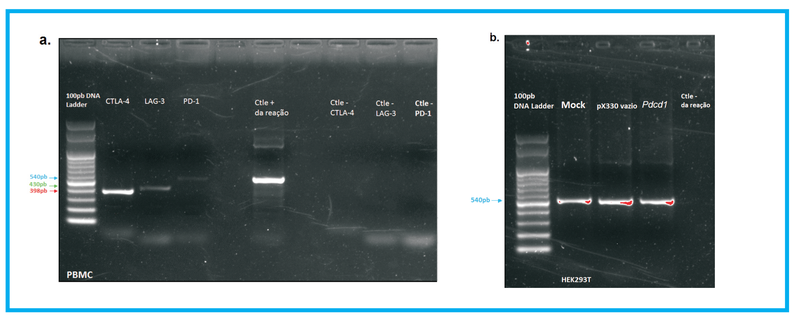

Fig. 3. 1.5% Agarose gel refers to amplification by PCR of genomic regions possibly edited of each gene. The expected sizes are represented in blue (*PDCD1*), green (*LAG3*) and red (*CTLA4*). (a) In PBMC for all genes and (b) in HEK293T to *PDCD1*.

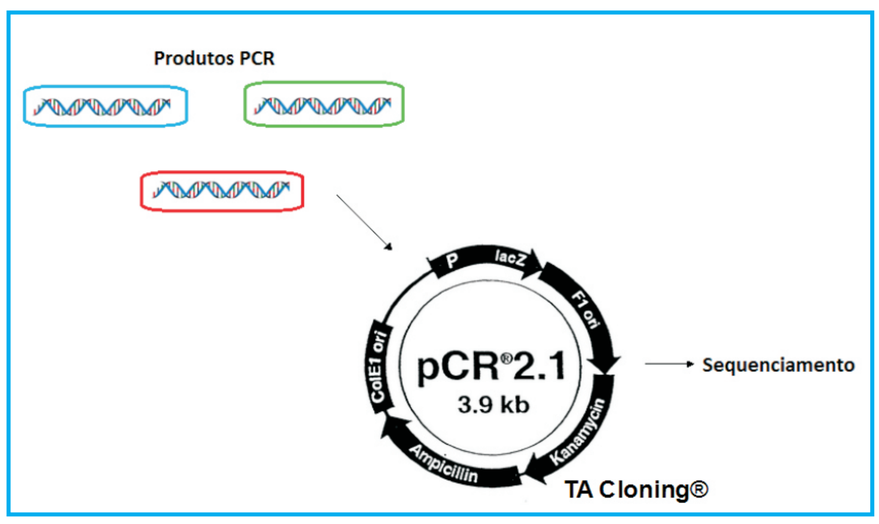

Fig. 4. Schematic representation of cloning amplified products of the three genes in the TA Cloning® system for sequencing and further indels analysis.

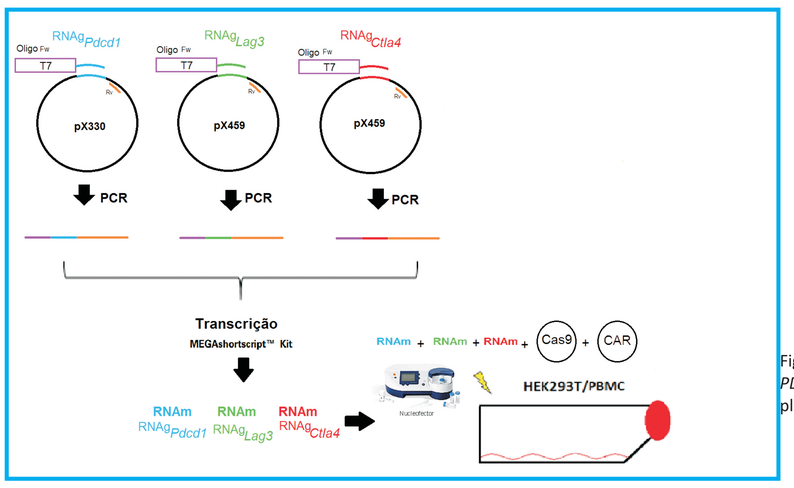

Fig. 5. Designed protocol for co-editing *LAG3* , *CTLA4* and *PDCD1* by co- electroporation of a RNAg pool and a plasmid adducing the Cas9 and CAR.

Test: FITC RNA + RFP (DNA - plasmid)

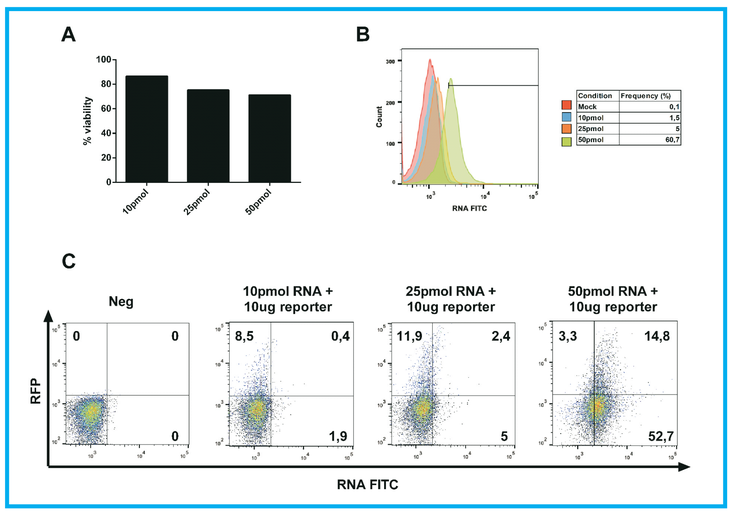

Fig. 6. Analysis one day after co- electroporation of a report plasmid (RFP) with a short FITC RNA showing high co-expression. A similar approach will be used to co-electroporation of Cas9 (plasmid) and RNAgs pool.

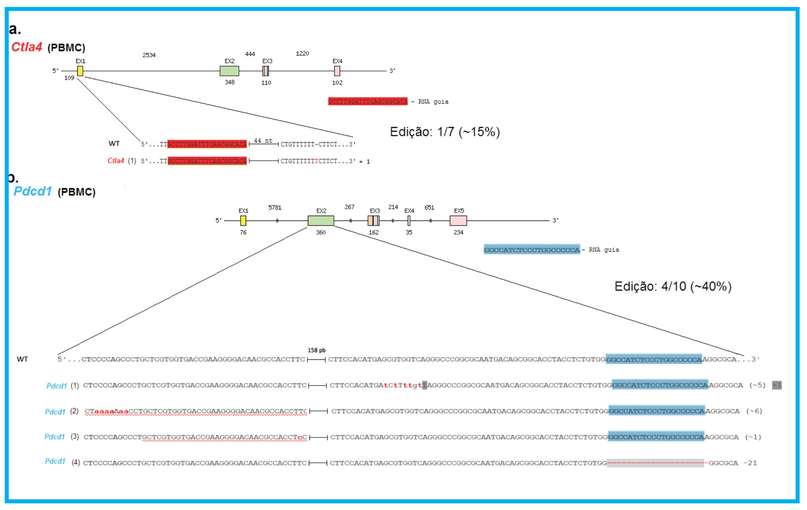

Fig. 7. *Locus* edition of *CTLA4* and *PDCD1*, one day after electroporation of plasmids containing the Cas9 and specific RNAg, in human mononuclear cells

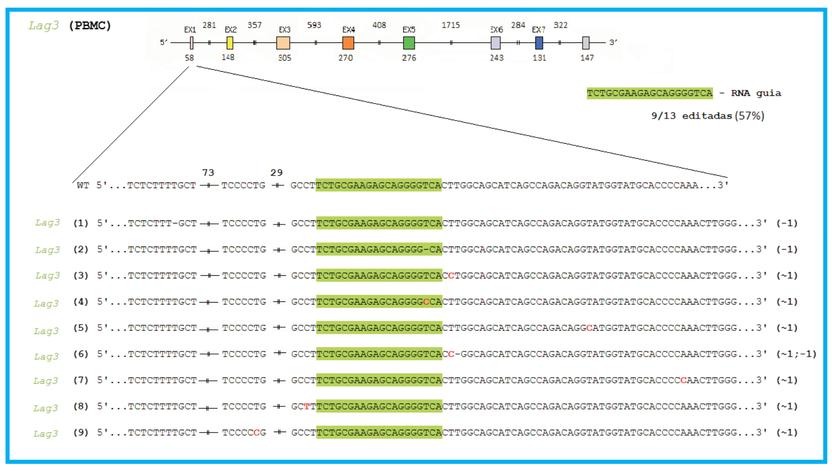

Fig. 8. *Locus* edition of *LAG3* one day after electroporation of plasmids containing the Cas9 and specific RNAg, in human mononuclear cells.

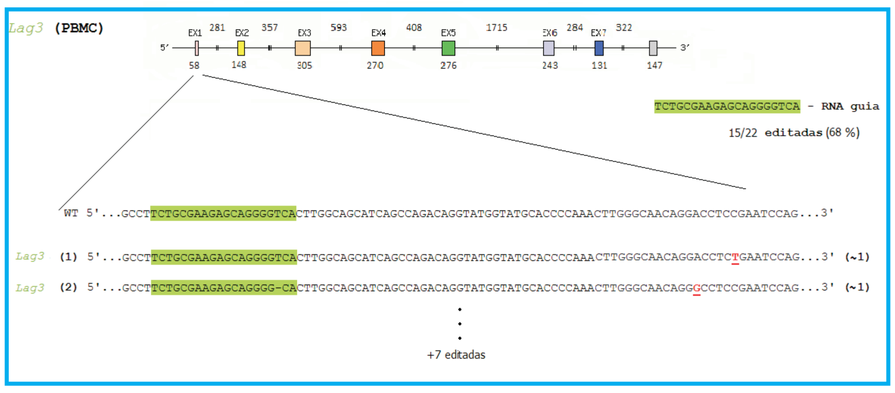

Fig. 9. *Locus* edition of *LAG3* CD3+/CD4+ one day after electroporation of plasmids containing the Cas9 and specific RNAg, in sorted human cells.

Table 1. Indel frequency of gRNAs transcripts.

Gene	*PDCD1*	*CTLA4*	*LAG3*
Edition % (Transcript)	52%	18%	45%

Table 2. Indel frequency of gRNAs in CRISPR plasmids .

Gene	*PDCD1*	*CTLA4*	*LAG3*
Edition % (Plasmid)	40%	15%	60%

Projeto Gráfico: Serviço de Edição e Informação Técnico-Científica / INCA

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