Genomic editing, mediated by CRISPR/Cas9, of *PDCD1*, *CTLA4* and *LAG3 loci* in human cells

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INTRODUCTION AND AIM

Cancer is a high prevalence disease, which justifies the studies in this area aiming to rising the rate of cure. Current treatments are generally aggressive and lead to unwanted side effects. 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 approaches, such as 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 the 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 their ligands mediated inhibition.

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 vectors and electroporated into human peripheral blood mononuclear cells (PBMC) and HEK293FT cell line. 72 hours later, HEK293T DNA was isolated by *Genomic DNA Extraction Kit Bioneer*. A PCR was designed to amplify each target *locus*. The PCR products were clone into 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, in PBMCs. Our data shown an editing rate of: 30-47% in HEK293T cells and 1-2,75% in PBMCs. Through *in silico* evaluation, we previewed the appearance of several stop codons in the edited sequences. We evaluated the effective response of edited cells against $CD19^+$, $MHCII^+$, $PD\text{-}L1^+$ and $CD86^+$ cells (Nalm-6) by lysis assay, and their possible enrichment among the T cell population. We propose here a system to knockdown a pathway 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. Our data demonstrated that all of gRNAs tested worked well in HEK293FT cells and with poor efficiency in PBMC.

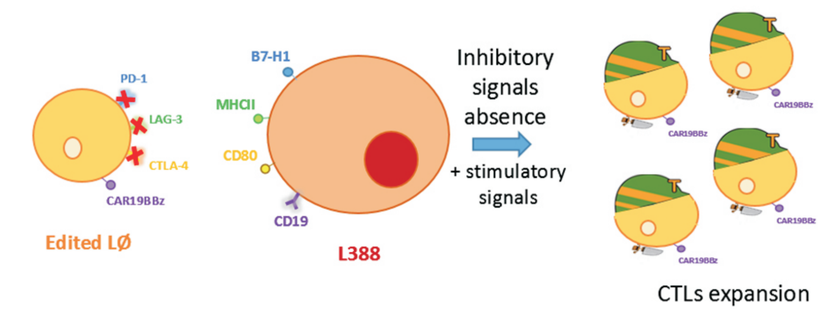


Fig. 1. Effects of CRISPR-mediated editing of human Lymphocyte. Representation of expected CRISPR edition effects, against tumor cells, in lymphocytes expressing CAR. In the absence of inhibitory signals through CRISPR editing PD-1, CTLA-4 and LAG-3 and presence of CAR stimulatory signal, we could improve the T lymphocyte effector potential against tumor cells.

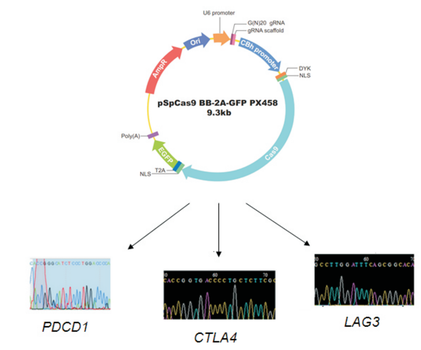


Fig. 2. Cloning confirmation of targets gRNAs in CRISPR vectors. *PDCD1, CTLA4* and *LAG3* gRNAs cloned in CRISPR plasmids and confirmed by sequencing.

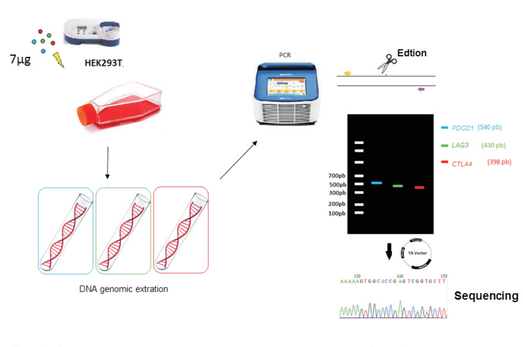


Fig. 3. Sample processing and indels analysis scheme. Experimental scheme of human cells (HEK293T and PBMCs) gRNAs electroporation, and subsequent indels analysis by amplifying the cloned products and indels evaluation by sequencing .

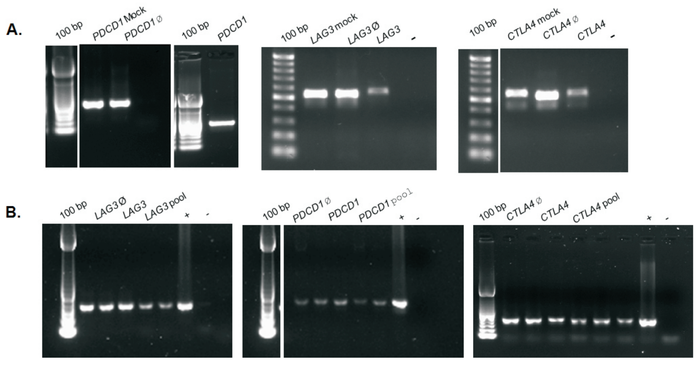
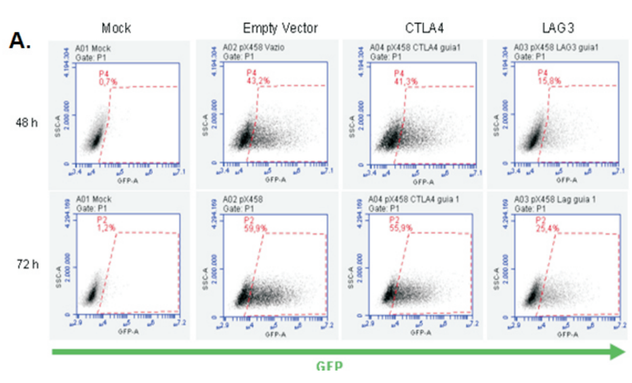


Fig. 4. Electrophoresis analysis. 1% Agarose gel of genomic PCRs of each *locus*. **(A)** HEK293T cell line **(B)** PBMC. Those PCR products were cloned and sequenced to evaluate the editions.



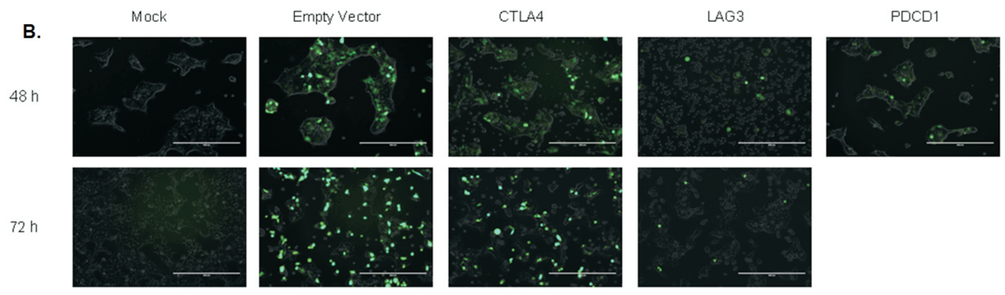


Fig. 5. GFP and viability analysis of HEK293FT CRISPR electroporation. **(A)** Flow cytometry analysis of HEK293T cell line with CRISPR vectors to evaluate GFP fluorescence (48 and 72 h after electroporation) in Mock, LAG3, CTLA4 and empty vector conditions. **(B)** Fluorescent microscopy of HEK293T cells to evaluate GFP fluorescence (48 and 72 h after electroporation) in Mock, Empty vector, CTLA4 and LAG3 conditions and 48 h to PDCD1 condition. **(C)** Plots quantification of flow cytometry of GFP+ cells.

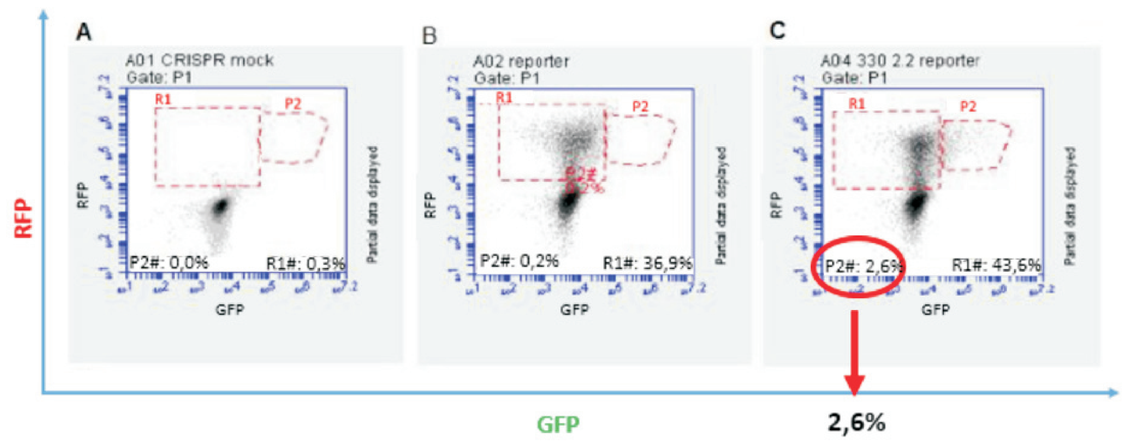


Fig. 6. (a) schematic representation of the Reporter system . When the PD-1 CRISPR specific construction recognizes and cleaves the reporter PD-1 linker, eventual editions will allow the GFP frame regeneration in one third of all editions. It occurs because the GFP truncated is missing one nucleotide and each indel could repair it adding or deleting nucleotides.**(b)** Possible editions represented by flow cytometry plot. **Edition eficiency (%) = [Edition (%)/ Transfection eficiency (%)] x 3x100; Edition eficiency = [2,6/46,2] x 3x100 ≈16,8 %.**

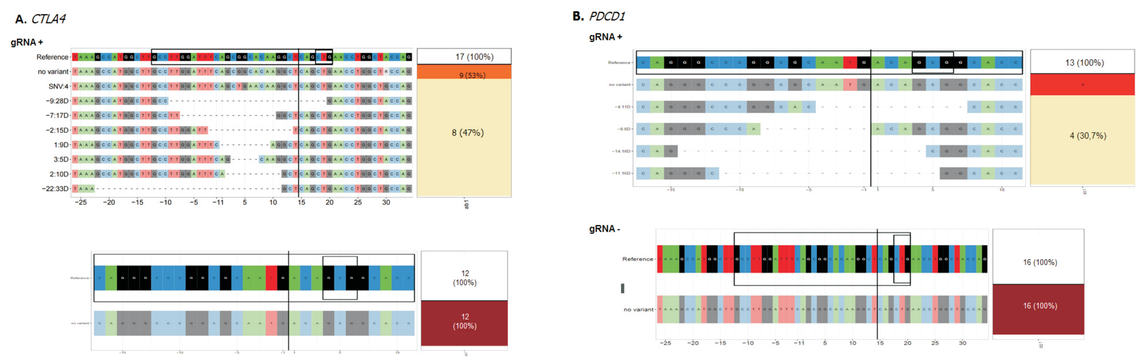


Fig. 7. HEK293FT sequencing indels analysis. *CTLA4* **(A)** *PDCD1* **(B)** and *LAG3* **(C)** *locus* editing 72 h after electroporation with (upper graphic) or without (bottom graphic) gRNA in HEK293T cell line. Colors are representing each different nucleotide. The black box in the reference sequence is highlighting the gRNA sequence and the line crossing the graphic shows the possible CRISPR region cleavages target. The right side of the image shows the editing percentage

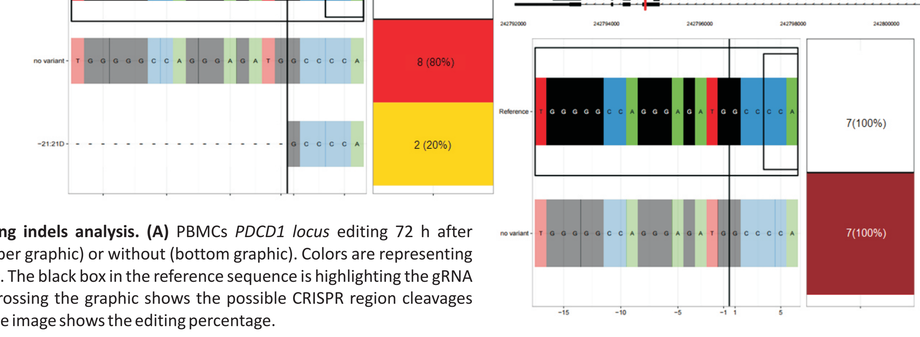


Fig. 8. PBMCs sequencing indels analysis. (A) PBMCs *PDCD1 locus* editing 72 h after electroporation with (upper graphic) or without (bottom graphic). Colors are representing each different nucleotide. The black box in the reference sequence is highlighting the gRNA sequence and the line crossing the graphic shows the possible CRISPR region cleavages target. The right side of the image shows the editing percentage.

CONCLUSION

We propose here a system to knockdown a pathway largely used by tumors to inactivate the immune response. Beyond HEK293F cell line, we tested the effects of PD-1, CTLA-4 and LAG-3 inactivation by CRISPR system in T Lymphocytes. We have validate CRISPRs for gene editing human LAG-3, CTLA-4 and PD-1 in HEK293T cells and are currently validating this approach in primary T lymphocytes.

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

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