Screening of proteins related to the immunological checkpoint Lymphocyte activation gene-3 (LAG-3) through the BioID method

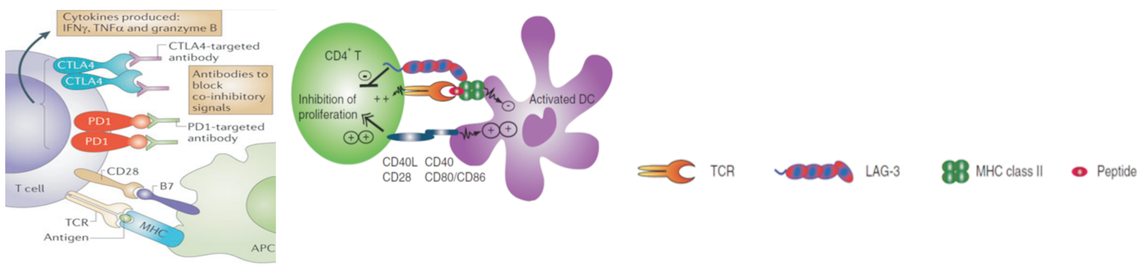
Priscila Rafaela Ribeiro (DO)[1], Marco Antônio Pretti[1], Leonardo Chicaybam,[1,2] Martin Hernan Bonamino[1,2].

1. Instituto Nacional de Câncer José Alencar Gomes da Silva, INCA.

2. Fundação Oswaldo Cruz, FIOCRUZ.

INTRODUCTION

Inhibitory receptors, such as PD-1, LAG-3, TIM-3 and CTLA-4 have gained attention as potential targets for immunotherapy, once the manipulation of the negative signals mediated by these receptors may provide new therapeutic approaches for infectious diseases, transplantation, autoimmune diseases or cancer. More recently, CD-4 like lymphocyte activation gene-3 (LAG-3) was described as a cell surface molecule that interacts with high affinity through its cytoplasmic domain with MHC class II molecules. The identification of molecules that interact with inhibitory receptors is a key step to better understand the funcions of these receptors. Once identified, such molecules can also become possible new pharmacologic targets. In order to identify interactions between proteins, a new method called BioID was developed. This method is based on the fusion of a protein of interest linked to a mutated biotin ligase (R118G) from Escherichia coli, wich is called BirA (CHOI-RHEE et al., 2004; Cronan, 2005; Roux et al, 2012.) This enzyme is able to biotinylate proteins associated with particular target protein. Once biotinylated, the proteins may be recovered by affinity (Kd = 10^{-14}) through beads conjugated to streptavidin and subsequently identified by mass spectrometry.



OBJECTIVE

The objective of this project is to conduct a screening of proteins that interact with LAG-3`s cytoplasmic domain by using the BioID method and identify the possible signaling pathways (*in silico* analysis) with which these proteins are involved, validate the presence of these proteins by western blot and / or flow cytometry.

METHODOLOGY

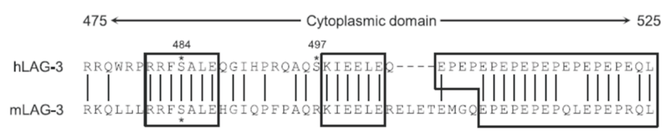
- Chimeric antigen receptors were built with extracellular domain scFv anti-CD20 (chimeric receptor antigen - CAR) containing the intracellular domain of Lag-3wild type, Lag-3 Kmut (mutation K => Non KIEELE) Lag3 EPdel (EP domain deleted) and Lag-3 Kmut EPdel (double mutant), all fused to the BirA Domain;

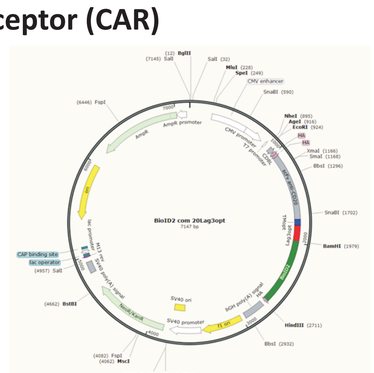
- CARs were expressed in in HEK293FT and CD4 + T lymphocyte Jurkat cell lines;

- Identification and Quantification of Proteins Associated to the inibithory receptor Lag-3 will be performed by mass spectrometry;

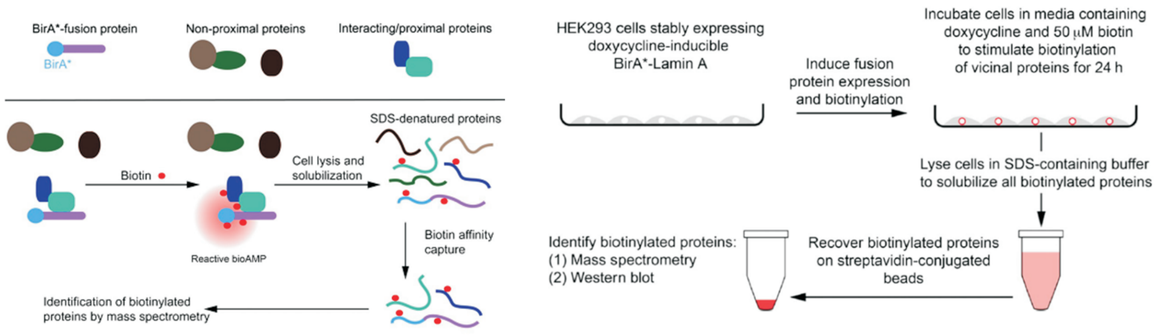
- The *in silico* analysis of possible downstream signaling pathways related to Lag3 will be performes using the Metacore platform.

Construction of the chimeric antigen receptor (CAR)





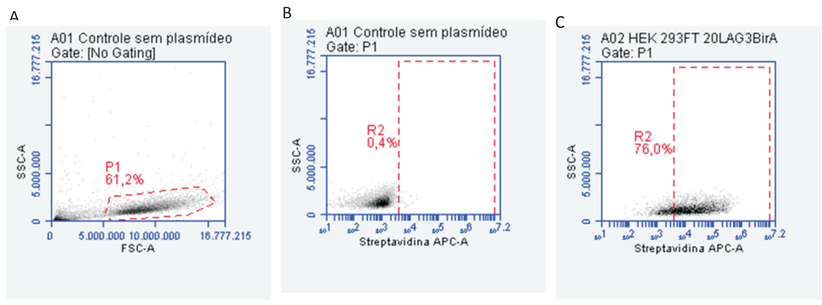
Identification and quantification of proteins by mass spectrometry



Roux et al., 2012

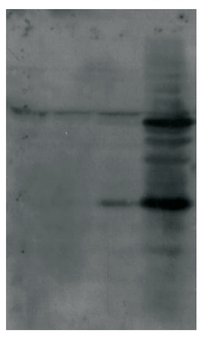
PRELIMINARY RESULTS

CAR 20LAG3BirA expression in HEK 293FT cells detected by flow cytometry



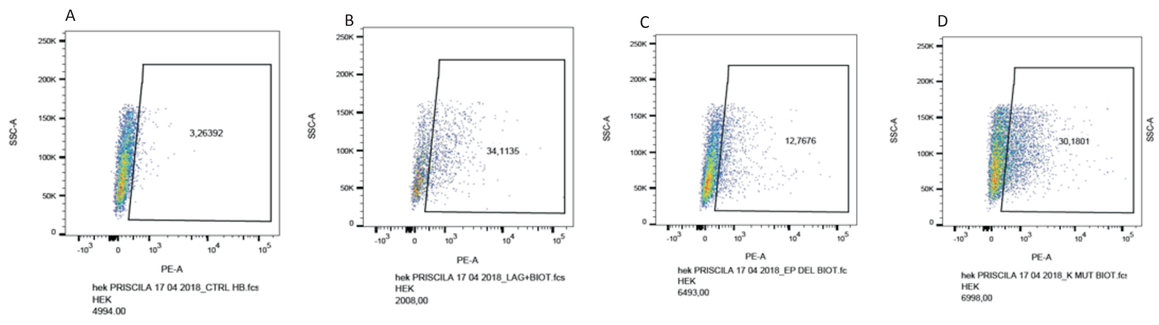
HEK 293FT cell line was electroporated with 10 μg of CAR 20Lag3 BirA and was incubated with primary antibody (anti-Fab 1:200) and secondary antibody (streptavidin APC 1:200) to detect the presence of the CAR. Cell viability and CAR expression were analyzed after 24h by flow citometry in two independent experiments Results represent one experiment.
A) Gate of viable cells B) Cells stained just with secondary antibody- negative control for Streptavidin/APC; C) Percentage of positive cells for SPV/APC, indicating expression of the CAR

Western Blot analysis of biotinylation



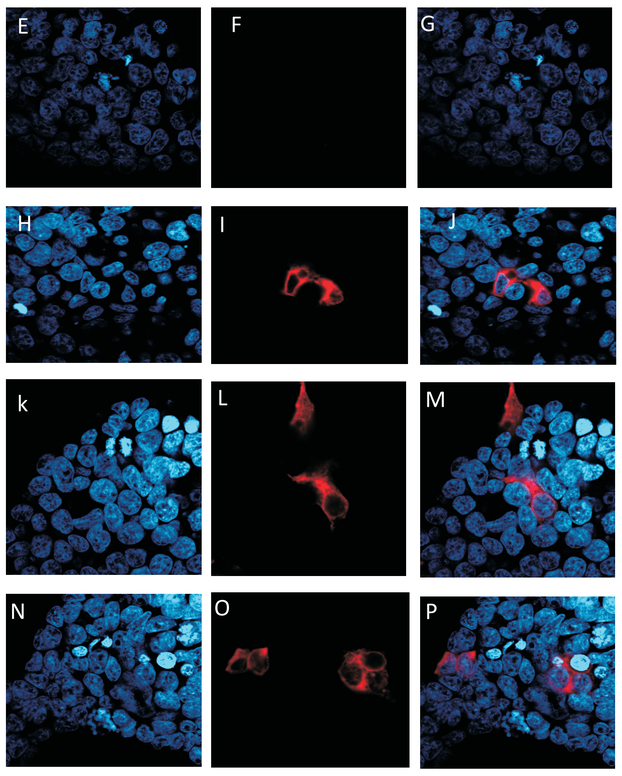
Western blot of HEK293 FT cells electroporated or not with CAR 20Lag3 BirA for biotinylation analysis. The cells were grown under addition of excess of biotin to the cell culture medium (50 M final concentration).
Following SDS-PAGE separation, non transfected and 20Lag3 BirA cells were probed with streptavidin-HRP. The extensive biotinylation of proteins in the BioID-20Lag3 BirA conditions can be observed.
(C=Control; CB=Control plus biotin-50μM; Lag=cells electropored with CAR 20Lag3BirA; LagB*= cells electropored with CAR 20Lag3 BirA plus biotin-50μM)

CARs expression pattern in HEK 293FT



HEK 293FT cell line was electroporated with 10 μg of CAR 20Lag3 BirA, Ep del and Kmut and both conditions were incubated with primary antibody (anti-HA 1:20) and secondary antibody anti F(ab)'2 PE (1:200) to detect the presence of the CAR. Cell viability and CAR expression were analyzed after 24h by flow citometry.
A) Cells not transfected- Control. B) Percentage of positive cells for anti- F(ab)'2 PE in LAG3BirA, C) in Ep delBirA and D) in Kmut BirA condition.

Immunofluorescence



For immunofluorescence, HEK 293FT cells were stained for DAPI (blue) and anti-HA antibody (1:30) followed by Alexa-Fluor 546 (1:250- red) and observed at confocal microscope.
All the pictures represents DAPI staining (blue) followed by the condition (Control, Lag3BirA, Epdel and Kmut) stained for HA antibody and Alexa Fluor 546 (red) and merge.
E-G: Control (not tranfected cells)
H-J: Lag3 BirA WT
K-M: Epdel
N-P: Kmut

PROSPECTS

- Proceed to the identification and quantification of proteins by mass spectrometry;
- Validate the presence of these proteins identified by flow cytometry and / or Western blot.
- Perform functional assays to validate the proteins

CNPq FAPERJ

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

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