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ELEVENTH ANNUAL MEETING

RHOA G17V mutant potentiates CD28-induced NFAT transcriptional activity by modulating p300 activity: a step further in the understanding of follicular helper T-cell lymphomas

D. Vallois[1], M. Juilland[2], K. Ioannidou[1], F. Lemonnier[3], E. Missiaglia[1], B. Bisig[1], M. Thome[2] and L. de Leval[1]

1. Institute of Pathology, Department of Laboratory Medicine and Pathology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland
2. Department of Immunobiology, University of Lausanne, Lausanne, Switzerland
3. Unité Hémopathies Lymphoïdes, Hôpitaux Universitaires Henri Mondor, Assistance Publique des Hôpitaux de Paris, Créteil, France

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INTRODUCTION

The RHOA G17V mutation is highly recurrent in follicular helper T-cell (TFH) lymphoma of the angioimmunoblastic type (AITL) (60-70% of cases) and frequently associated with mutations in other T-cell receptor signaling related genes, including *CD28*.

AIM

We sought to elucidate how RHOA G17V (dominant negative ATPase activity) and CD28 (activating) variants may work in concert to sustain T-cell activation.

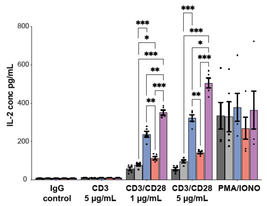
METHOD

- We generated stable Jurkat T-cell lines expressing either empty control vectors or the wild type (wt) or G17V mutant form of RHOA in combination with either the wt or T195P variant form of CD28.

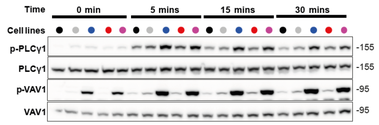


- We sought for RHOA G17V binding partners performing co-immunoprecipitation experiments, using Jurkat cells expressing empty vector or different RHOA forms (wt, G17V and K18N), followed by mass spectrometry analysis.
- p300 histone acetyltransferase activity was measured by quantifying the relative amount of the p300 specific H3K18ac and H3K27ac marks by:
  - Chromatin Immnuoprecipitation followed by qPCR in cells (ChIP-qPCR)
  - Multispectral immunofluorescence (IF) staining, scanning, image acquisition and analysis in cells and patients
- We stained AITL biopsies from four patients with RHOA G17V alteration to corroborate our observations about p300 activity

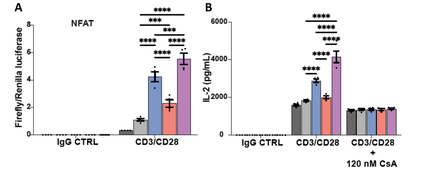
RESULTS



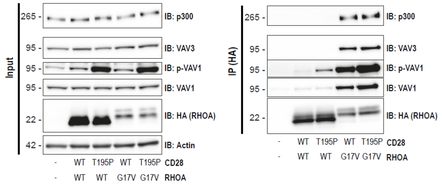
1. CD28 and RHOA G17V variants synergize in promoting IL-2 secretion and *IL-2* transcription. *In vitro* secretion of IL-2. 200 000 Jurkat cells per well, transduced with the lentiviruses indicated by the color code, were stimulated as indicated (phorbol myristate acetate (PMA; 20 ng/mL) and ionomycin (IONO; 1 µM)). 24h later, supernatants were collected and IL-2 was measured by ELISA. Data are represented as mean+/- SEM from 5 independent experiments realized in quadruplicates. *IL-2* transcription (not shown) shows the same pattern in time course experiment (0h-10h)



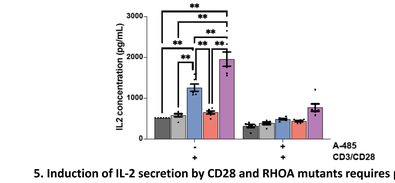
2. CD28 T195P but not RHOA G17V induces an increased phosphorylation of TCR signaling key molecules unpon CD3/CD28 co-stimulation. Representative Western blot of PLCγ1 and VAV1 phosphorylation



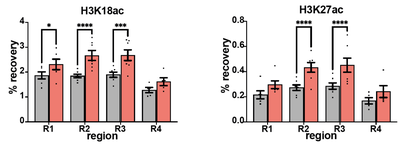
3. RHOA G17V and CD28 T195P synergize in promoting NFAT and AP1 transcriptional activity upon CD3/CD28 stimulation. (A) Luciferase reporter assays monitoring the activities of NFAT (AP1 not shown) upon co-stimulation of cell lines either with IgG control or anti-CD3, anti-CD28 and crosslinker at 2 µg/mL during 5 h and 30 min. **(B)** *In vitro* secretion of IL-2 upon costimulation with anti-CD3, anti-CD28 and crosslinker at 2 µg/mL with or without a small dose of cyclosporin A (120 nM) during 24 h.



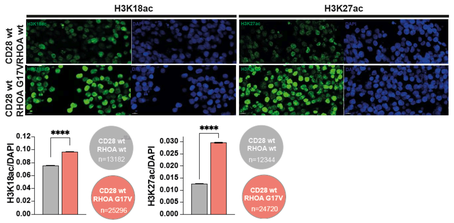
4. RHOA G17V interacts specifically with EP300 Representative Western blot analysis of lysates (left panel) and anti-HA immunoprecipitates of ectopically expressed HA-tagged RHOA (right panel) from the indicated cell lines. Cells were co-stimulated with anti-CD3, anti-CD28 and crosslinker at 2 µg/mL during 5 min.



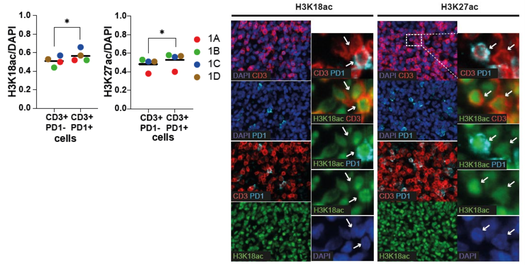
5. Induction of IL-2 secretion by CD28 and RHOA mutants requires p300 acetyltransferase-activity. *In vitro* secretion of IL-2 upon stimulation with anti-CD3, anti-CD28 and crosslinker at 2 µg/mL during 24 h with or without the specific p300 acetyltransferase inhibitor A-485 at 5 µM. Cells were pre-treated with 5 µM of A-485 during 48h. Data are represented as mean +/- SEM from 6 independent experiments realized in quadruplicates. Significant differences in activation activity were determined using 2-way analysis of variance (ANOVA) with tukey's multiple comparison test.



6. The *IL-2* promoter carries p300-specific marks of histone modification. Cells were CD3/CD28 co-stimulated at 2 µg/mL during 4 h. Chomatin Immunoprecipitation (ChIP) was performed using IgG control, anti-H3K4me3, H3K9ac, H3K18ac **(A)** and H3K27ac **(B)** antibodies. The relative enrichment of four regions (R1(-677; -567); R2(-226; -133); R3(+2; +100) and R4(+291; +386)) of the *IL-2* promoter was assessed by qPCR. R2 and R3 regions are considered as proximal *IL-2* promoter. Data are represented as mean +/- SEM from 7 independent experiments.

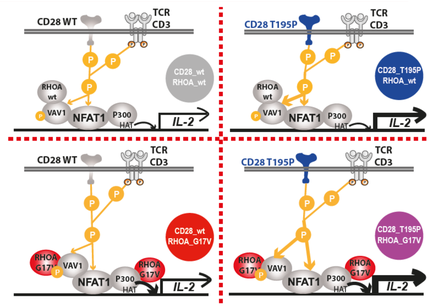


7. p300-specific histones' marks changes are genome wide. *Upper panel.* Cells were stained for H3K18ac (rabbit polyclonal ab1191, 1/800) or H3K27ac (rabbit polyclonal ab4729, 1/300) in combination with DAPI. *Lower panel.* We quantified and calculated H3K18ac/DAPI and H3K27ac/DAPI ratios of the mean intensities values for each cell. Data are represented as mean +/- SEM with the number of cells analyzed indicated in the figure.



8. TFH cells from Angioimmunoblastic T cells Lymphoma patients carrying RHOA G17V show increased H3K18ac and H3K27ac marks. 4 µm thick slides from four (1A, 1B, 1C and 1D) AITL patients were stained for DAPI, CD3, PD1 and either H3K18ac (left panel) or H3K27c (right panel). Mean intensity ratios of H3K18ac/DAPI and H3K27ac/DAPI were compared between thousands of CD3+PD1+ (TFH) and CD3+PD1- (reactive) T cells for each patient. The means of each patient are plotted and compared.

CONCLUSIONS



- The combination of CD28 T195P and RHOA G17V variants promote IL-2 secretion and *IL-2* transcription upon CD3/CD28 co-stimulation in a synergistic manner.
- This cooperation did not manifest at the level of phosphorylation of key TCR signaling molecules (which was induced by the CD28 mutant alone), but involved an increase in NFAT and AP1 transcriptional activities.
- RHOA G17V binds specifically the histone acetyltransferase p300
- Chromatin immunoprecipitations and immunofluorescence staining revealed an increase of p300-specific H3K18ac and H3K27ac marks at the *IL-2* promoter and at the whole genome level, respectively, in cells harboring the RHOA G17V mutant.
- Histological staining of tumor samples from four AITL patients carrying RHOA G17V variant corroborated these latest findings

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CONTACT INFORMATION

david.vallois@chuv.ch and laurence.deleval@chuv.ch