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TUNING THE ADAR1 RNA EDITING ENZYME TO BOOST TYPE I IFN AND NK CELL INNATE IMMUNE RESPONSES IN THE MODEL OF HPV-TRANSFORMED CELLS

Cristina Cerboni[1], Jean-Pierre Vartanian[2], Marcel Hollenstein[2]
1. IP Rome, 2. IP Paris

The hypothesis driving this project is that in high-risk (hr) HPV-transformed epithelia, the RNA editing enzyme ADAR1 could play a role in hrHPV-driven tumorigenesis, by its ability to dampen the activation of IFN-I pathways and innate immune responses. Therefore, we are investigating for the first time the interplay between ADAR1, IFN-I, and innate lymphocytes (i.e., NK cells and innate lymphoid cells, ILCs).

The project is divided in 3 work packages (WP), each one with a different leader.

In **WP1** (**leader: Jean-Pierre Vartanian**), the aim is to analyse the interaction partners of ADAR1 that could influence the degree of editing ***(task 1).*** We want to demonstrate that the burden of mutations following multiple rounds of ADAR1 induction during chronic inflammation will lead to tumorigenesis ***(task 2).*** Validation of the ADAR1 mutated phenotype will be performed in patients to correlate ADAR1 expression and editing activities with tumorigenesis ***(task 3).***
For ***task 1,*** we we are analyzing proteins interacting with p150 ADAR1 (full-length or isolated domains) in a yeast two-hybrid system. A cDNA library derived from the A549 cell line was introduced by transformation into a suitable strain of yeast and used to screen for ADAR1 interactors. We have now detected about 100 interactors to be sequenced.
In a parallel set of experiments, we developed a cell-based system for conditional expression of human ADAR1 to show that it edits by sequential mutational waves (***task 2).*** We obtained the constructs and HeLa cells will be now stably transduced with a doxycycline inducible GFP-ADAR1 and GFP-ADAR1* (catalytic mutant) lentiviral vector. Each ADAR1-expressing selected single cell will be expanded without induction and then subjected to 10 rounds of ADAR1 and ADAR1* induction and recovery. We also plan to make RNAseq analysis from cervical cancer (CC) cells, silenced or not for ADAR1, as well in fresh CC biopsies, in search for A-to-I mutations on ADAR1-edited sites ***(task 3).***

In **WP2** (**leader: Cristina Cerboni**), the aim is to explore how the IFN-I pathway can be manipulated through ADAR1 to promote inflammation and infiltration of innate lymphocytes in CC.
We are currently dissecting the *intrinsic effects* that ADAR1 silencing has on HPV-transformed CC cells (i.e., SiHa and CaSki cell lines), focusing on the IFN-I signaling pathway, pro-inflammatory cytokine production, and cell survival/proliferation of silenced

(KO) cells. Indeed, data collected so far clearly show that CC cell lines express boh ADAR1 isoforms (Fig. 1A), and that ADAR1 KO cells have a decreased proliferative capacity, particularly after treatment with IFN-beta (Fig. 1B). Innate immunity pathways are also activated in KO cells, with increased levels of IFN-alfa1, IFN-beta1, IFN-lamda1 and of other immune response genes (Fig. 1C), as well as of STAT1 and PKR phosphorylation (Thr446/Thr451) (Fig. 1D) (similar results for CaSki cells; not shown) ***(Task 1)***.
To correlate these findings with the dysregulation of NK cell effector functions against hrHPV-transformed cells (*extrinsic effects,* ***Task 2),*** supernatants (SNs) from KO SiHa cells were used to verify the release of pro-inflammatory cytokines/chemokines able to activate NK cells. Data show that NK cells incubated with such SNs increase their proliferation (Fig. 2A; similar data for CaSki cells, not shown), as well as their degranulation activity (CD107a+) against both K562 and the less sensitive target SiHa (Fig. 2B-C). These results suggest that ADAR1 silencing in tumor cells may (re)activate innate immune responses. We are currently aiming at identifying cytokines/chemokines modulated by ADAR1 inhibition and released in the supernatants, able to enhance NK cell activities.
For ***Task 3,*** by *ex vivo* and *in vivo* analysis, we are collecting and analyzing fresh biopsies and paraffin-embedded tissue sections of cervical cancers for the presence of innate lymphocytes (NK/ILCs) infiltrating the tumor. Preliminary results demonstrate the presence of a cervix-innate infiltrate, with the Lin-CD45+CD7+ cells including both NK and NCR+ILC3 (Fig. 3A). The correlation between ADAR1 expression and CD56+ (NCAM+) NK cells is under investigation also by IHC (Fig. 3B), with the aim of understanding an ADAR1-dependent reshaping of NK cell/ILC innate immune responses and/or impact on inflammation-driven immune responses. We also plan to extract RNA from the same biopsies and perform RNAseq and analysis of A-to-I mutations.
Finally, in relation to the development of scaffold-based 3D skin-like structures ***(Task 4)***, we are testing different compositions of matrices (with collagen, fibronectin and/or laminin), as well as a new approach based on organotypic cell cultures. These 3D cultures could be used to study a three-dimensional relationship between CC cells and innate leukocytes, as well as to validate aptamers developed by Dr. M. Hollenstein (see also WP3).

In **WP3** (**leader: Marcel Hollenstein**), the aim is to identify aptamers that specifically bind to hrHPV-transformed cells, and then convert them to a system capable of delivering an siRNA oligonucleotide capable of silencing the expression of ADAR1 and thus to restore IFN-I production. Ultimately, this would cause anti-tumor immunity and block of cell proliferation in hrHPV-transformed cells. In addition, since ADAR1 is overexpressed in various forms of cancer, we wish to exploit this feat to develop an alternative therapeutic approach yet based on aptamers. Indeed, we aim at harnessing the A-to-I editing property of endogenous ADAR1 to correct known cancer-related mutations (such as E542K/E545K on PIK3CA) by conjugating the afore-mentioned aptamers to AIMers, which are short,

chemically modified oligonucleotides that elicit A-to-I RNA editing (Monian P et al., Nature Biotechnol, 2022; Chen G et al., Biochemistry 2019). The aptamer part of these conjugates will deliver the AIMers specifically to CC cells and upon cellular internalization, the AIMers will make use of endogenous ADAR1 to specifically induce mutations in selected RNAs.

We are currently expressing the His-tagged protein ADAR1 to initiate SELEX against this protein, under typical conditions used in the laboratory (see e.g., Cheung YW et al., PNAS 2020) using standard DNA chemistry ***(Task 1)***. We have also prepared a degenerate library that will be used after purification of the protein ***(Task 1)***. Lastly, we have started to work on the synthesis of chemically modified RNA molecules ***(Task 2)***. Regarding AIMers, we have already synthesized various sugar-modified RNA oligonucleotides. Once ADAR1 protein will be expressedand purified, we will evaluate the editing capacity of the newly synthesized RNA oligonucleotides on a proof-of-principle RNA target sequence ***(Task 2 revised)***. Briefly, we will incubate target RNA with ADAR1 and the chemically modified oligonucleotides and evaluate RNA editing *in vitro* (see Stafforst et al., Angew Chem Int Ed Eng, 2012). If successful, the RNA oligonucleotides will be transfected into cancer cells and RNA editing will be evaluated *in vivo.*

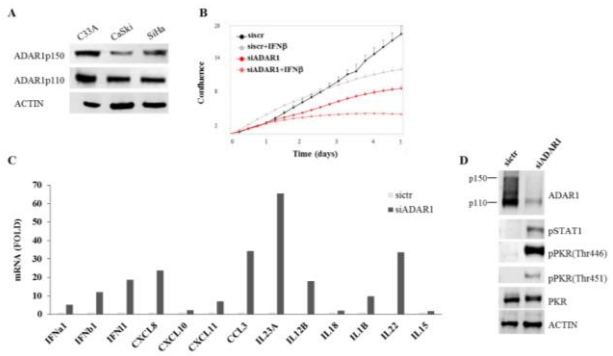


Figure 1. Analysis of ADAR1 expression in cervical cancer cell lines and impact on IFN-I pathway. A) Immunoblot analysis showing p110 and p150 ADAR1 expression in CC cell lines. **B)** Proliferation of siscr and siADAR1 (30 nM) SiHa cells, treated or not with IFNbeta (1000 IU/ml), was monitored over 5 days by the Incucyte® live-cell analysis system. **C)** qRT-PCR showing *IFNs* and ISGs expression in sictr and siADAR1 SiHa cells, 72 hrs following ADAR1 silencing. *GAPDH* was used as a control. **D)** Immunoblot showing increased STAT1 and PKR phosphorylation in siADAR1 SiHa cells compared to sictr, 72 hrs post-siRNA transfection.

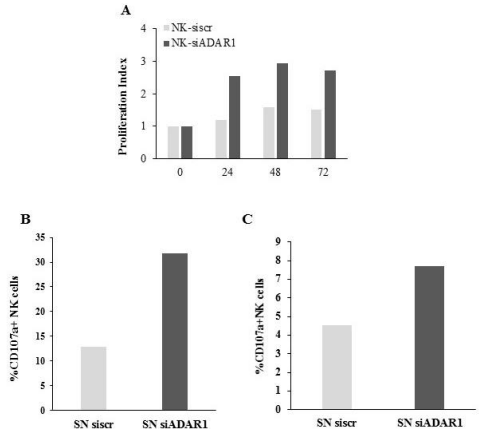
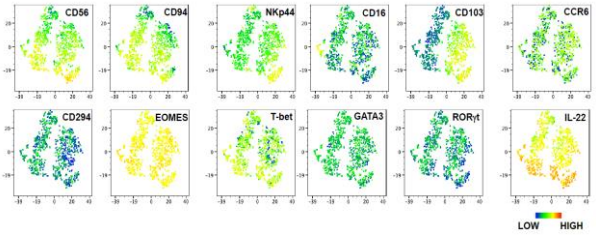


Figure 2. Impact of ADAR1 modification on NK cell phenotype. A) Conditioned supernatants (SNs) from sictr and siADAR1 SiHa cells were harvested and incubated for the indicated time points on purified NK cells, obtained from healthy donors. Proliferation was measured by Incucyte® live-cell analysis system and calculated by setting at 1 NK cells at day 0. **B**) NK cells were incubated O/N with conditioned SNs from sictr or siADAR1 SiHa cells and then used as effectors in a degranulation assay against K562 cells, or against SiHa cells (**C**), used as targets.



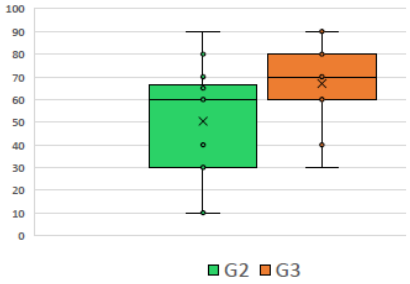


Figure 3. Human innate lymphocytes infiltrating neoplastic cervix. **A)** t-SNE plot of phenotypic markers on Lin-CD45+CD7+ innate lymphocyte infiltrate in a cervical cancer biopsy. Cells are coloured according to the expression level of the different markers. Results show the presence of NK cells (Lin-CD56+CD16+/-CD94+Eomes+T-bet+CD103-NKp44-IFNgamma+) and of NCR+ILC3 (Lin-CD56+Eomes+T-bet+CD103+NKp44+IL-22+). **B)** ADAR1 stained sections were divided into high (NCAM>5) and low NK numbers (NCAM<5) (NCAM=CD56) (data not shown). The graph shows that in tumors with low NK numbers (NCAM<5), more aggressive (G3) lesions have a higher cytoplasmic ADAR1 expression.

PUBLICATIONS

1. Tassinari V., **Cerboni C.,** Soriani A. Self or Non-Self? It Is also a Matter of RNA Recognition and Editing by ADAR1. Biology 2002. doi: 10.3390/biology11040568. IF: 5,168.
2. Girone G., Calati F., Lo Cigno I., Salvi V., Tassinari V., Schioppa T., Borgogna C., Lospinoso Severini L., Hiscott J., **Cerboni C**., Soriani A., Bosisio D., Gariglio M. The RIG-I Agonist M8 Triggers Cell Death and Natural Killer Cell Activation in Human Papillomavirus-Associated Cancer and Potentiates Cisplatin Cytotoxicity. Under second revision, Cancer Immunology Immunotherapy. IF: 6,630.

RESEARCH GROUP

Alessandra Soriani, Associate Professor
Marta Kaciulis, PhD student
Lorenzo Cuollo, PhD student
Stefano Petrai, PhD student

COLLABORATIONS

Prof. J.P. Vartanian, Pasteur Institute, Paris
Dr. M. Hollenstein, Pasteur Institute, Paris
Prof. H. Stabile, Sapienza University
Prof. M. Gariglio, University of Piemonte Orientale Medical School
Dr. I. Palaia, Sapienza University Umberto I Hospital
Dr. A. Pernazza, Sapienza University Umberto I Hospital