Deletion of diacylglycerol kinase alpha ameliorates chimeric antigen receptor T cell dysfunction by reducing receptor downmodulation

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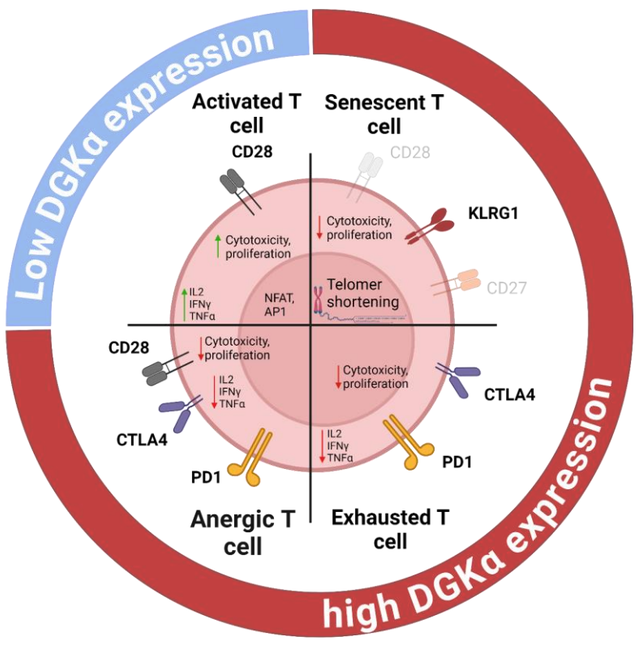
Rationale

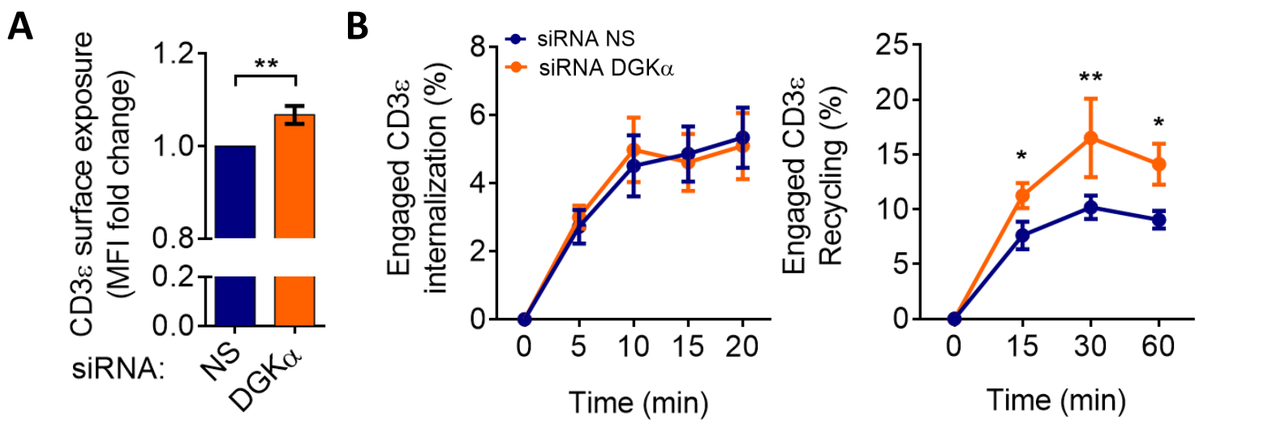


T cell senescence, exhaustion and anergy represent functionally similar, yet molecularly distinct, state of T cell dysfunction, that together contribute to the failure of long term Chimeric Antigen Receptor T-Cell (CAR-T) immunotherapy.

Diacylglycerol kinase alpha (DGKα), a well characterized negative regulator of TCR signaling, is upregulated in all the described conditions, suggesting that it may represent a promising target to revert the T dysfunctional phenotype. However, how DGKα affects T cell function has been only partially uncovered.

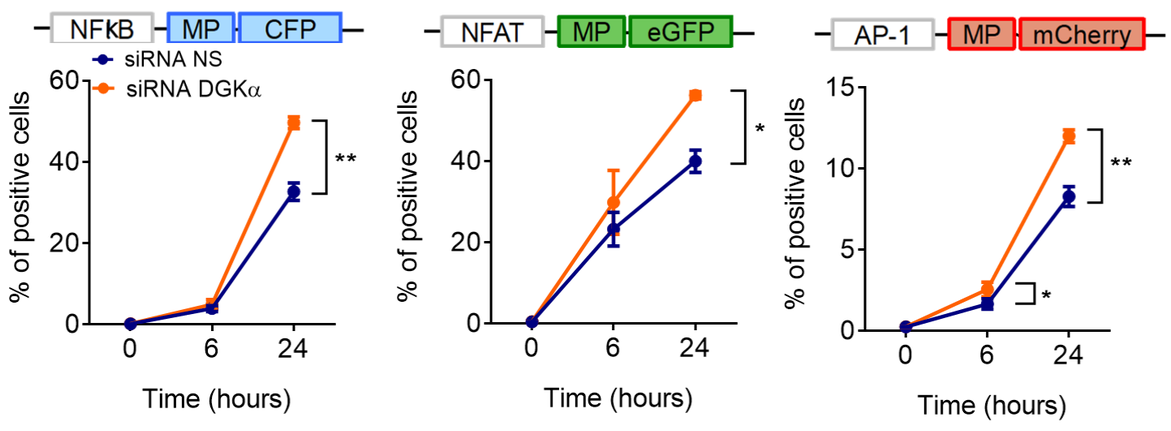
Image created with Biorender

DGKα controls TCR cell surface expression



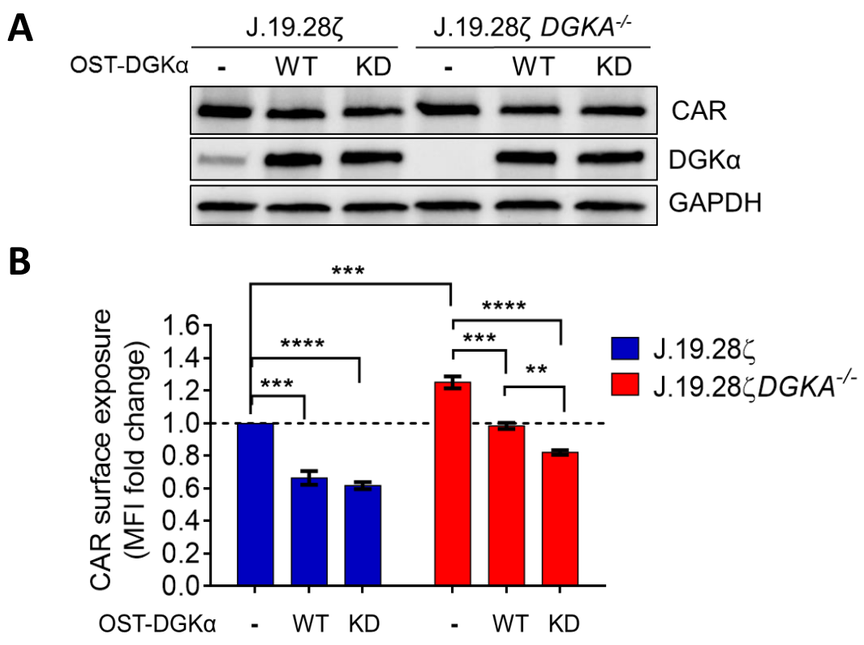
A) Activated human primary CD8⁺ T cells transfected with Non-Specific (NS) or DGKα siRNA were stained with saturating Alexa Fluor 488-labeled anti-CD3ε antibody (clone OKT3). Data are mean ± SEM of 22 ind exps, analyzed by FACS. Paired t test, two-tailed; **p < 0.01 **B) CD3ε internalization**; data are mean ± SEM of 7 ind exps, analyzed by FACS. Two-way ANOVA, Sidak's multiple comparison test: ns. **Stimulation-induced recycling of the TCR**. Graph shows the % of CD3ε recycling. Data are mean ± SEM of 4 ind exps. Two-way ANOVA, Sidak's multiple comparison test.:*p < 0.05; **p < 0.01.

DGKα controls TCR distal signaling



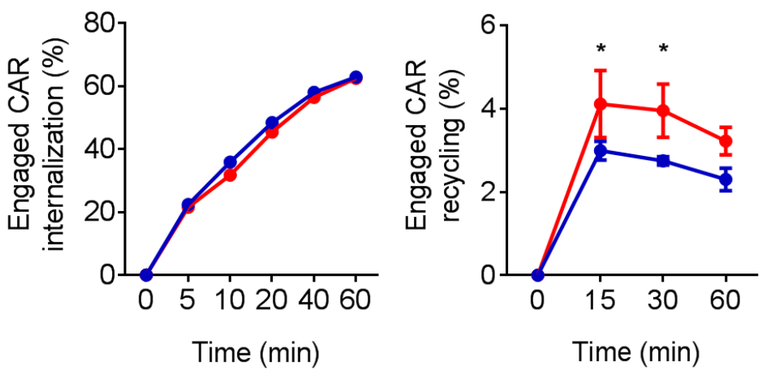
Jurkat triple parameter reporter (from Steinberg lab) were silenced for NS and DGKα and activated with plate-bound anti-CD3ε (clone UCHT1, 1 µg/ml) for the indicated time. NFκB, NFAT and AP-1 activity were detected simultaneously by flow cytometry analysis. Data are mean ± SEM of 3 technical replicates; Two-way ANOVA, Sidak's multiple comparisons test: * p < 0.05; **p < 0.01.

Strategy to investigate the role of DGKα in CAR trafficking



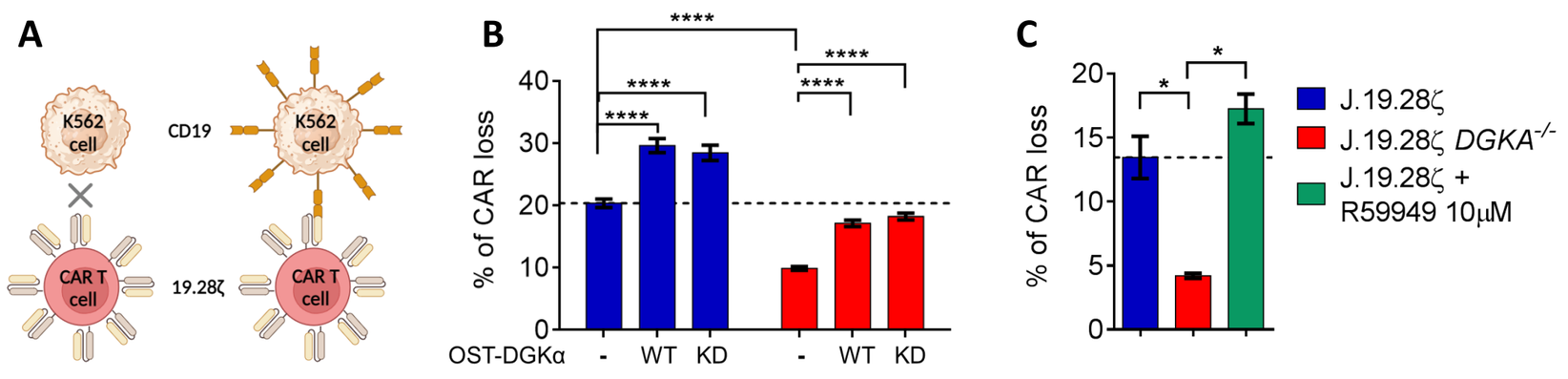
A) Representative blot of CAR and DGKα protein expression levels after transduction. **B)** J.19.28ζ and DGKα KO cell lines were stained with saturating APC-labeled anti-Myc antibody (clone 9B11) to measure CAR basal levels at plasma membrane; analazyed FACS analysis. Data are mean ±SEM of 3 independent experiments; Two-way ANOVA, Sidak's multiple comparison test: ** p < 0.01; *** p < 0.001; **** p < 0.0001

DGKα deletion promotes CAR recycling



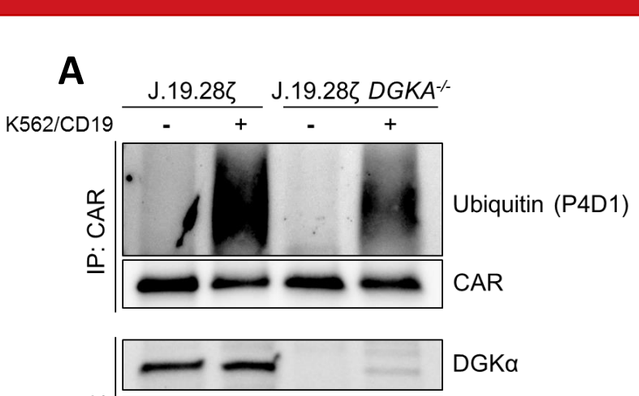
CAR internalization and recycling were followed by staining CAR-T cells with saturating concentration of APC-labeled anti-Myc (clone 9B11). Data are mean ± SEM of 3 independent exps; Two-way ANOVA, Sidak's multiple comparisons test: *p < 0.05).

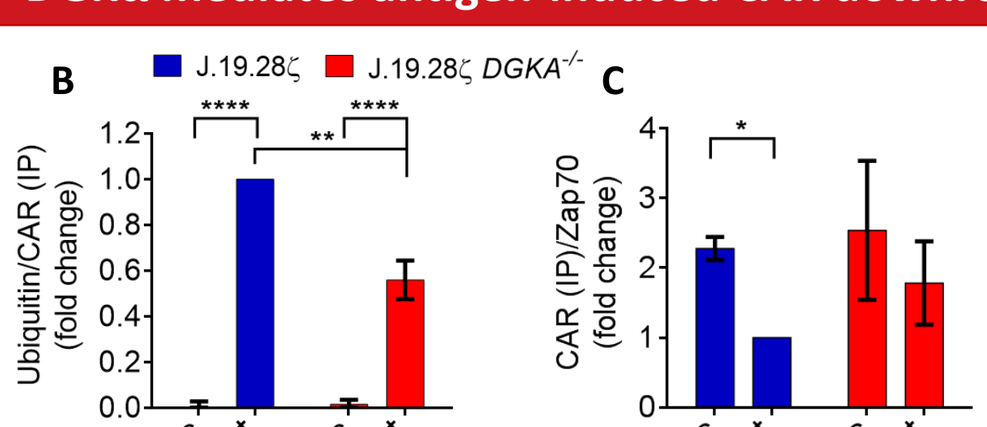
DGKα mediates antigen-induced CAR downregulation



A) Model for CAR-T cells stimulation. B) Quantification of CAR loss from cell surface upon 1 hour stimulation with target cells expressing CD19 (cognate antigen). Data are mean ±SEM of 4 independent experiments; Two-way ANOVA, Sidak's multiple comparison test: **** p < 0.0001. C) Same as described in B) but with a clone of J.19.28ζ DGKA-/-. J.19.28ζ are pre-treated for 30 min with R59949, an inhibitor of DGKα activity. Data are mean ±SEM of 2 independent experiments; One-way ANOVA, Tukey's multiple comparisons test: * p < 0.05.

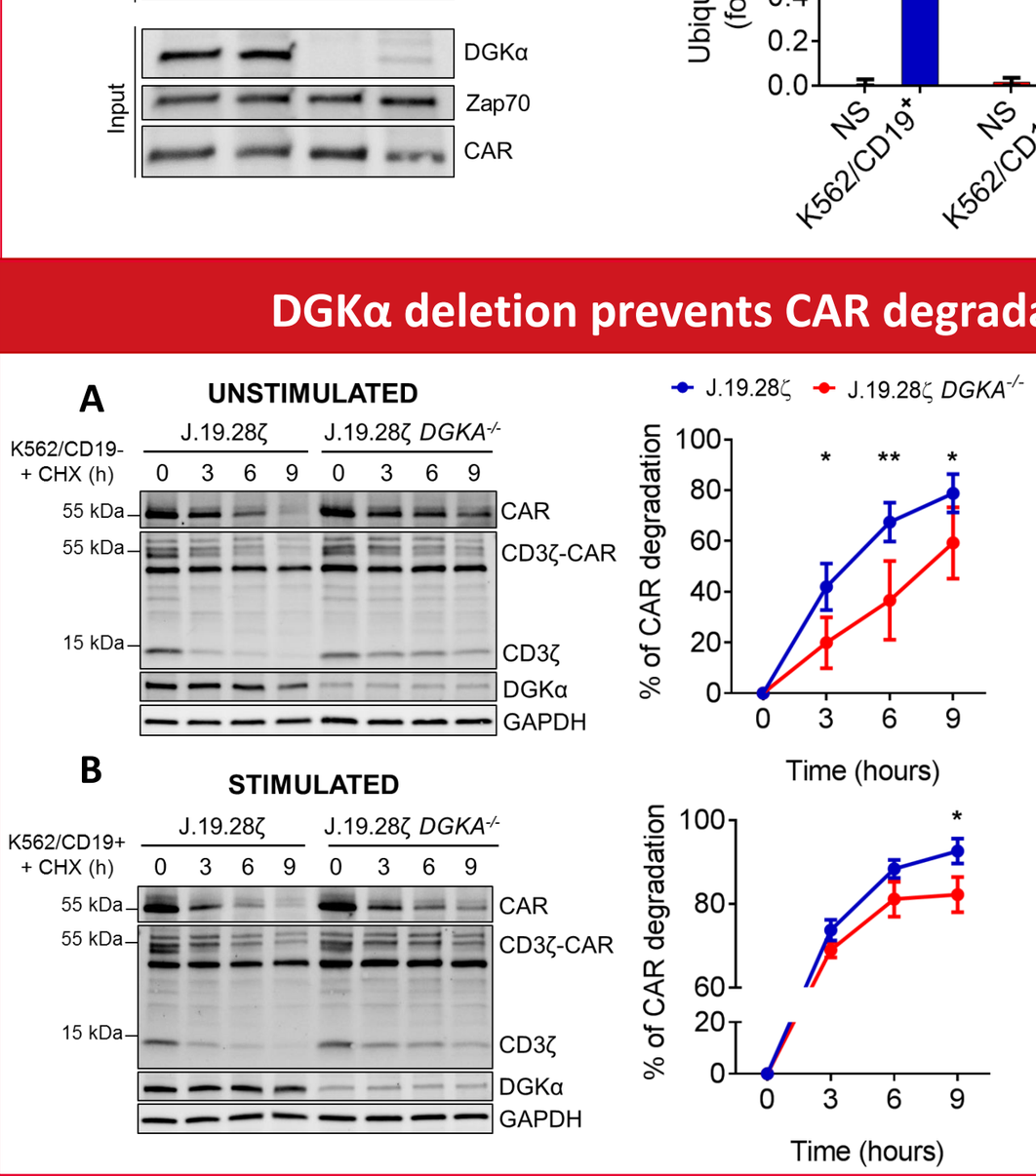
DGKα mediates antigen-induced CAR downregulation





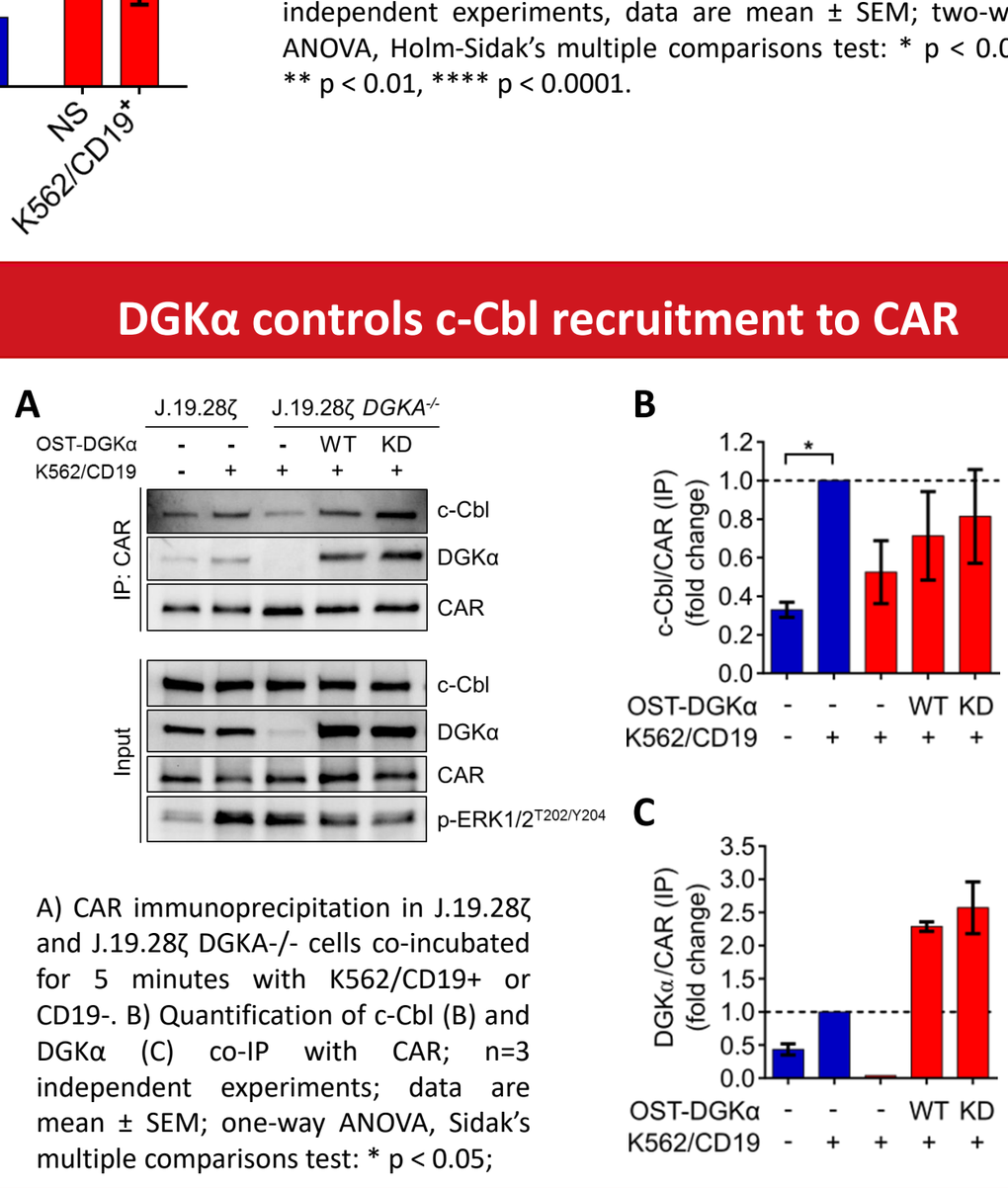
A) Representative blot of CAR immunoprecipitation in J.19.28ζ and J.19.28ζ *DGKA*$^{-/-}$ cells not stimulated (NS) or following engagement with K562/CD19+ cells. **B-C)** Quantification of immunoprecipitated ubiquitin normalized on immunoprecipitated CAR level for each condition (B) and quantification of immunoprecipitated CAR on Zap70 (C); n= 3 independent experiments, data are mean ± SEM; two-way ANOVA, Holm-Sidak's multiple comparisons test: * p < 0.05, ** p < 0.01, **** p < 0.0001.

DGKα deletion prevents CAR degradation



CAR expression was evaluated in J.19.28ζ and DGKα KO cells co-incubated with K562/CD19- (A) or with K562/CD19+ (B) plus CHX (100 µg/ml) for the indicated time. Representative Western Blot (left) and quantification of degraded CAR is shown (right). A small band was observed in DGKα KO due to the presence of K562 cells, physiologically expressing DGKα. Data are mean ± SEM of 3 independent experiments; Two-way ANOVA, Holm-Sidak's multiple comparisons test: * p < 0.05, ** p < 0.01.

DGKα controls c-Cbl recruitment to CAR



A) CAR immunoprecipitation in J.19.28ζ and J.19.28ζ DGKA-/- cells co-incubated for 5 minutes with K562/CD19+ or CD19-. B) Quantification of c-Cbl (B) and DGKα (C) co-IP with CAR; n=3 independent experiments; data are mean ± SEM; one-way ANOVA, Sidak's multiple comparisons test: * p < 0.05;

This work is supported by Ministero dell'Università e della Ricerca – Unione Europea – PNRR – Age-It (Spoke 2); AIRC; Ministero della Salute (RF) all to AG.

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