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RESEARCH HIGHLIGHTS

FOXO1 or not FOXO1: that is the question

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Two groundbreaking articles in *Nature* by Evan W. Weber [1] and Philippe Darcy teams [2] revealed that overexpressing the transcription factor Forkhead Box O1 (FOXO1) boosts Chimeric Antigen Receptor-T (CAR-T) cell antitumor activity against various tumors, including solid ones. Paradoxically, we recently described that pharmacological inhibition of FOXO1 transcriptional activity by AS184856 treatment in resting T cells enables the generation of non-activated CAR-T cells that outperforms solid tumor eradication compared to *ex vivo*-activated CAR-T cells [3]. Our findings confirm the interest in using non-activated CAR-T cells, echoing two other studies that achieved more potent CAR-T cells by transducing resting T cells cultured with interleukin 7 (IL-7), with [4] or without [5] the addition of IL-15.

Although these results may seem contradictory at first, experimental evidence shows that this contradiction is only apparent and can be resolved by taking into account the initial status of the cells: activated versus resting T cells. Overexpressing FOXO1 in activated T cells leads to a similar phenotypic and functional differentiation state as inhibiting FOXO1 activity in resting T cells. Indeed, both strategies lead to significant changes in cell metabolism, specifically to an increase in mitochondrial activity [1, 2, 6]. Similarly, both of these apparently opposed processes also lead to an increase in cytotoxic functions. One of the proteins essential for cytotoxic activity, granzyme B, was described to be increased at the transcriptomic and protein level, either after inhibition of FOXO1 in resting T cells [3, 6] or after FOXO1 overexpression in activated T cells [2]. In both cases, granzyme B rise is associated with an in vivo tumor killing increase [1–3]. Finally, in both configurations, T cells show no exhaustion markers and differentiate into stem cell memory T (TSCM)-like cells [1–3], a T cell differentiation stage associated with a greater antitumor activity [7].

Taken together, these results suggest that the correlation between the level of FOXO1 transcriptional activity and the antitumor potential of CAR-T cells is not straightforward. FOXO1 maintains quiescence in unstimulated cells. In naïve T cells, TCR triggering (or cytokines) allows a rapid, yet prolonged nuclear exclusion of this transcription factor, downstream the PI3K/Akt pathway [8]. Since T cell activation leads to the shutdown of FOXO1 transcriptional activity [8], one would expect that FOXO1 overexpression in activated T cells would have no effect. Instead, the results from Weber's and Darcy's teams show that overexpression may maintain a small but sufficient amount of FOXO1 activity, responsible for the beneficial effects observed in CAR-T cells [1, 2]. Similarly, inhibition of FOXO1 activity in resting T cells is partial, since invalidation of the *FOXO1* gene cannot recapitulate the effects caused by pharmacological inhibition of FOXO1 activity [3]. This could result from the molecular mechanism

List of abbreviations: CAR-T, Chimeric Antigen Receptor-T; FOXO1, Forkhead Box O1; IL-7/15, interleukin 7/15; TSCM, Stem Cell Memory T cell.

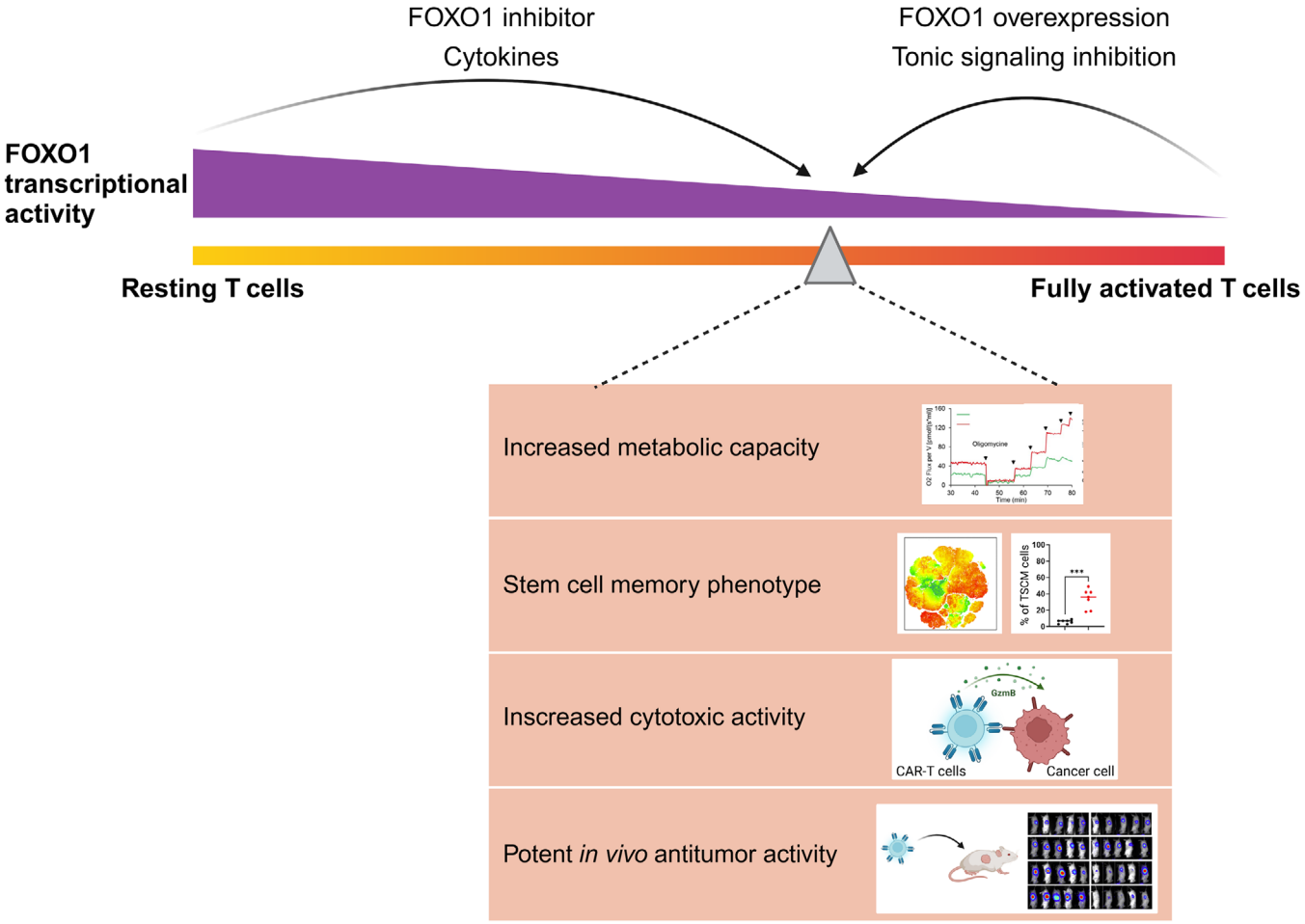

FIGURE 1 **FOXO1 is the key driver of CAR-T cell responses.** Inhibiting FOXO1, pharmacologically or by cytokine treatment, in resting T cells makes them permissive to lentiviral infection, allowing a CAR expression, and fully competent to destroy the tumor cells. Conversely, overexpressing FOXO1 or inhibiting tonic signaling in activated T cells reverse CAR-T cell exhaustion and boost antitumoral activity. Depending on the initial state of the treated T cells, both inhibition and overexpression of FOXO1 allow to reach an optimal activation state characterized by a high mitochondrial activity, a TSCM-like cell phenotype and a huge amount of Granzyme B and cytotoxic activity where FOXO1 partially exerts its transcriptional activity. Created with BioRender.com # PH27ELCT85. Abbreviations: CAR-T, Chimeric Antigen Receptor-T; FOXO1, Forkhead Box O1.

of FOXO1 inhibition by AS1842856. AS1842856 binds to FOXO1 to selectively inhibit its interactions with its consensus DNA motif and thus blocking its transcription factor activity [9]. As AS1842856 does not affect its expression, FOXO1 may still be capable of carrying out its other regulatory functions, such as chromatin remodeling [10] or interacting with its nuclear partners [11]. In this context, it will be interesting to investigate the epigenetic changes induced by both FOXO1 inhibition and overexpression respectively in resting and activated T cells.

Thus, both methods targeting FOXO1 cells allow to achieve an intermediate level of FOXO1 activity placing the T cells in a specific activation state. Previous results from Crystal Mackall's team emphasize the critical importance of precisely tuning CAR-T cell activation for optimal efficacy. They demonstrate that inhibiting antigen-independent tonic signaling from constitutive CAR expression increases CAR-T cell activity and reverses exhaustion, a cell phenotype associated with an opening in the chromatin of FOXO1 target regions [12]. This is in line with our model (Figure 1) which proposes that a significant increase in the antitumor activity of CAR-T cells can be achieved through fine-tuning the activation signal or directly regulating the activity of FOXO1.

Ultimately, all of the strategies described here to enhance CAR-T cell efficacy converge on a common message: FOXO1 is the key driver of CAR-T cell responses. Optimized CAR-T cells can be obtained by fine-tuning the level of FOXO1 activity, either by overexpressing FOXO1 in activated T cells or by inhibiting FOXO1 activity in resting T cells. The inhibition of FOXO1 activity in resting T cells can be obtained both pharmacologically or by cytokine

treatment leading to inhibition of FOXO1 activity via the PI3K/Akt signaling pathway. To establish a signature for highly effective CAR-T cells it is necessary to confront the two strategies with global molecular approaches. The regulon identified in the study by Weber's team [1], which encompasses a set of gene alterations suitable for CAR-T cell efficacy consecutive to FOXO1 overexpression, serves as a promising draft that could be refined through such comparisons. In conclusion, these two strategies are in fact not contradictory, they tell the same story.

AUTHOR CONTRIBUTIONS

Maude Marchais and Marianne Mangeney have co-built the concept and co-written the manuscript.

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Not applicable.

CONFLICT OF INTEREST STATEMENT

Marianne Mangeney and Maude Marchais are the inventors on a patent application related to a method to generate more efficient CAR-T cells (Application number EP22306289.4). Maude Marchais is employed by Viroxis.

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DATA AVAILABILITY STATEMENT

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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