TAILORED IMMUNOTHERAPIES IN 3D MODELS OF FOLLICULAR LYMPHOMA (TAIFOL)

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1. Project summary

Our study has focused on follicular lymphoma (FL), the second most frequent non-Hodgkin Lymphoma (NHL) and consider indolent. FL typically responds to standard chemoimmunotherapy regimens. However, despite its "indolent" nature, FL is very heterogeneous, with some patients showing long-lasting remission, while others relapse in the first two years. Thus, in this project we have analyzed the genetic alterations and immune profile in FFPE-derived biopsies in a series of FL patients at diagnosis, homogeneously treated with immunochemotherapy regimen, and followed-up for more than 11 years. By comparing patients who experienced relapse with those who did not, we have found a signature of 25 up-regulated genes in the relapse group. Among those genes, using multiplex immunofluorescence we validated the over-expression of CD70 in tumor B cells, which highly correlates with inferior progression free survival. Moreover, a fraction of T cells from the tumor microenvironment also expresses CD70, and higher levels were detected in both CD4+ and CD8+ T cells in the relapse group. CD27, the ligand of CD70, was also increased in the B cell population in patients who eventually relapse and in T follicular helper cells, while it was downregulated in CD8+ and CD4+ T helper non-follicular cells. To investigate the role of CD70 in FL pathogenesis we generated two CD70 Knock-out (KO) cell lines and FL patient derived cells using CRISPR/Cas9 technique. By co-culturing healthy donors' T cells with CD70+ or CD70- FL cells, we observed CD70+ tumor cells promote CD70 expression in T cells. In primary samples, we have demonstrated that CD70-KO B cells exhibit reduced response to proliferative stimuli. Finally, to advance towards personalized therapies for this high-risk FL patients, we have generated a dual CD19-CD70 CAR-T combining an approved academic product from the Hospital Clinic de Barcelona (CD19-CAR-T, ARI-0001) and a truncated CD27 protein-CAR-T

2. Results

FL-lymph node transcriptomic profile unravels differences at diagnosis in patients that relapse

We analyzed the transcriptome of 730 immune-related genes within the Nanostring® nCounter PanCancer Immune Profile panel in a FL patients cohort from a single-center (Hospital Clinic) treated with immunochemotherapy (mostly, R-CHOP). In our analysis,

we compared patients that did not relapse (n=20) with an extensive follow-up of more than ten years, or patients that eventually relapsed during the follow-up (n=12).We found 31 genes differentially expressed and 25 of them were upregulated in the relapse group, while only 6 were increased in the non-relapse group. Thus, we could demonstrate patients with different clinical responses to immunochemotherapy exhibit a different transcriptomic profile.

A gene-set enrichment analysis (GSEA) using genes upregulated in each condition showed the relapse group had an over-representation of genes related to B-cell proliferation, B and T-cell activation, cytokine regulation, extracellular matrix and cell adhesion, as well as different pro-oncogenic pathways (PI3K/Akt, IL6/JAK-STAT, IL4), among others. We also confirmed a decrease in BCR pathway in non-relapse patients, accompanied by interferon I signaling, T cytotoxicity pathway and Fcγ mediated phagocytosis, which could indicate a higher rituximab benefit that prolongs response to anti-CD20 treatment. Overall, we assessed patients that relapse or do not relapse have a previous differential immune profile at diagnosis.

CD70 is upregulated in relapse group and is associated to a lower mutational burden

Deciphering a differential immune profile between patients that relapse or not may be useful, not only to improve the FL prognosis, but also to better understand the biology of the disease and design new therapies for patients who repeatedly relapse, worsening their prognosis. Among the genes upregulated in patients who relapse, we further focus on CD70, as it has been defined as an oncogenic factor in many cancers, including B-cell lymphoma and it is expressed in the cell membrane, making it easily targetable. Interestingly, CD70 is not only increased in the Relapse but it is also associated with shorter progression-free survival (PFS) time, closely to significance ($p = 0.067$).

We then investigated whether CD70 expression was correlated with other genes. Among the 730 genes included in the Nanostring® Immune Profile panel, CD70 significantly correlates with 30 genes, and inversely with only 5 genes. Notably, some of the most represented genes that directly correlate with *CD70* are cytokines and chemokines, such as Th17/Treg chemoattractant CCL20, CCL22 a relevant cytokine in FL-TME, and IRF4 related to FL transformation into DLBCL.

The mutational profile from the patients used in our series has also been published by Mozas et al. Intriguingly, we found a tendency of a lower mutational burden in patients with higher CD70 RNA levels. In patients harboring less than 8 mutations, CD70 counts were significantly higher than in patients with at least 8 mutations. When analyzing individual mutations, we found significant differences in CD70 expression in CARD11, CIITA and TNFSRSF14, also known as HVEM. In all of them, patients with alterations in these genes have lower CD70 levels.

CD70 and CD27 protein expression is increased in FL cells from relapse patients

To validate our results at protein level, we performed multiplex immunofluorescence in FFPE biopsies from FL-LN patients of our cohort with available We confirmed CD70 is up-regulated in tumor cells from patients who eventually relapse. This data led us to anticipate an over-activation of the CD27/CD70 axis. Even though we did not find differences in CD27 RNA levels, we found an increase CD27 protein expression in the Relapse group and correlated with a shorter PFS.

CD70 is also expressed in T cells from FL-TME, and display higher levels in relapse patients

Furthermore, given the fact CD70 role has also been highlighted in FL-TME, we used mIF technique to decipher CD70 expression among T cells. To obtain a full and accurate picture of CD70 and CD27 expression across the different subtypes of T cells, we built a six-color panel including CD4, CD8, FOXP3, CXCR5, CD27 and CD70. As expected, CD4+ cells are more abundant in FL-LN than CD8+ cells. Remarkably, a higher protein expression of CD70 in both CD4 and CD8 populations has been observed in the relapse group. Within CD4+ cells, TFH, Treg and T helper non-follicular cells in relapse patients have higher expression of CD70, being TFH the population with a more pronounced rise. In the same line, $CD70^{high}$ expression in all T-cell populations were related to a lower PFS except for TFR.

To complete our analysis, we described CD27 expression in the different T-cell populations across FL-LN. CD27 is known to be a costimulatory molecule and a naive T-cell marker, which is lost after antigen contact. As done for CD70, we analyzed CD27 expression in the different CD4+ subpopulations. While TFH cells in Relapse group have higher CD27 protein levels and $CD27^{high}$ patients have a remarkably shorter PFS, its

expression is diminished in Th non-follicular cells and patients with $CD27^{low}$ have a worse survival. Overall, we could demonstrate an up-regulation of CD70 in both B and T cells, while CD27 is also increased in B cells but has a heterogeneous pattern in T cells.

CD70 expression in B cells induce higher levels of CD70 in T cells

We analyzed the correlation scores between CD70+ in B cells with CD70 or CD27 in the different populations analyzed. The percentage of CD70+ cells within tumor population significantly correlates with CD70+ in CD8+ cells and CD4+ cells. To understand this mutual regulation of the CD70-CD27 axis in B and T cells, we generated by CRISPR/Cas9 technology two FL-derived cell lines CD70-KO, WSU-FSCCL and SC-1, which are highly positive for CD70. After co-culturing T cells from healthy donors with CD70+ or CD70- FL cell lines, we analyzed CD27 and CD70 levels in T cells.

Remarkably, when they are co-cultured with a CD70+ cell line, the expression of CD70 in both CD4+ and CD8+ T cells is highly up-regulated, as it has been observed in patient samples. On the contrary, when the FL cell do not express CD70, this increase does not occur, indicating a CD70-specific effect. Regarding CD27, after the activation, T cells maintained a high percentage of positivity. This expression was dampened when T cells were cultured with CD70+ FL cells but not with CD70- FL cells. Overall, we could recapitulate the increase of CD70 and decrease of CD27 expression observed by multiplex immunofluorescence in CD8+ and CD4+ cells

CD70 expression is related to B cell proliferation in primary FL samples

CRISPR/Cas9 was also applied to primary FL samples. When FL cells were stimulated co-culturing with YK6 (cell line derived from follicular dendritic cells) expressing CD40L and IL21 (YK6-CD40L-IL21) and supplemented with IL-4 and IL-15 cytokines, we observed that CD70+ cells had a higher percentage of cells in S phase, indicative of a higher proliferative index. Thus, this experimental validation supports an oncogenic role of CD70 in FL biology and reinforces its therapeutic intervention.

A truncated ligand-based CAR-T targeting CD70 has a high anti-lymphoma efficacy and may be combined with CD19 CAR-T to obtain a novel dual CAR-T

Anti-CD19 chimeric antigen receptor (CAR) T cells have revolutionized the treatment of B-cell lymphoma. Nevertheless, a fraction of patients does not have durable responses

and a fraction of them experience CD19 loss-of-antigen relapse. As we demonstrated that CD70 is highly expressed in tumor cells in patients that relapse, we hypothesized CD70 could represent a promising target for CAR-T therapy in follicular lymphoma, especially in the context of a dual CD19-CD70 CAR-T.

In our approach, we have designed a ligand-based CAR-T using its natural ligand, CD27. Cytotoxicity experiments were performed using the single CAR-Ts CD27 and ARI-0001 (CD19-CAR-T). Using artificial presenting cells K562 modified to express CD19 and CD70, we demonstrated that CD27-CAR-T showed a similar efficacy than ARI-0001. In long-term assays co-culturing CD19+CD70+ FL cell lines with lower CAR-T numbers, we demonstrated that CD27 CART was as efficacious as ARI-0001 in the presence of both antigens. Overall, we considered that this novel CD27 CART will be further use generate a dual CD19-CD70 CAR-T. The co-transduction and further preclinical analysis is in progress and is financed through an international grant from the Follicular Lymphoma Foundation

3. Relevance for future

Thanks to the project financed by La Marató, we have been able to identify a gene signature that can predict at the time of diagnosis which patients might suffer a relapse. Within this signature, we have identified CD70 as a new relapse biomarker and a new therapeutic target in FL. In this sense and thanks to the advanced therapies program of the Hospital Clínic-IDIBAPS, we have developed a new CD19-CD70 dual CART that may improve the survival of these patients with the highest risk. Moreover, this new therapeutic product could be applied to other aggressive B-NHL, where CD19-CART offers shorter remissions, including Diffuse Large B cell Lymphoma and Mantle cell lymphoma. Thus, once preclinical studies will be finalized, an application to the AEMPS will be done, proposing a CD19-CD70 CAR-T clinical trial including these different types of B-NHL.

4. Bibliography

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