PI: Francesca Granucci

Project title: **Role of calcineurin in affecting the tumor microenvironment and the response to immune checkpoint inhibitors**

Abstract

Immune checkpoint blockade (ICB) therapies are groundbreaking treatments that reactivate lymphocytes against cancer but, so far, only few patients have responded to this cure. This is probably due to differences in the microenvironments surrounding tumors cells. Thus, understanding how the tumor microenvironment forms and its composition is fundamental to predict the response to therapies and to develop new treatments. Calcineurin is a protein that promotes the development of several tumors but it is yet unclear how it works. We will study how calcineurin influences the composition of the tumor microenvironment by using different experimental models (mice, human cancer cells, and samples from patients). Our work will shed light on the process that leads to the reactivation of lymphocytes and will lay the basis for the discovery of new strategies that leverage our immune system against tumors.

Background, aims and significance of the proposed work

Calcineurin (CN) is a serine (Ser)/threonine (Thr) phosphatase that is ubiquitously expressed and is composed of a catalytic subunit A (CN) and a regulatory subunit B (CNB). CN is activated by calmodulin following an increase in intracellular calcium levels. CN interacts with substrates that have two major binding sites: the PxIxIT motif, which is considered the primary docking site, and the LxVP motif. Proteins containing one or both of these motives can be subdivided into regulators and effectors. Regulators are proteins that control CN activity by modulating its activation and subcellular localization or by recruiting effector molecules; effectors are transcription factors or kinases.

Clinical and experimental evidence has highlighted the role of CN in controlling cancer progression (1-5).

We have generated mouse cancer cell lines expressing the VIVIT peptide to inhibit the binding of CN to any substrates bearing the PxIxIT motif. Surprisingly, the VIVIT+ cells generated tumors characterized by a much stronger accumulation of $CD8^+$ T cells than the one observed in tumors induced by VIVIT- cells. Therefore, we concluded that the tumor-intrinsic inhibition of CN interaction with PxIxIT substrates affects the composition of the tumor microenvironment. Tumor infiltration by $CD8^+$ T cells is essential for the efficacy of immune-checkpoint blockade (ICB) therapies. Accordingly, we found that **the expression of the VIVIT peptide in tumor cells overcomes the resistance to ICB therapies** in mouse models.

The goal of the present project is to investigate the tumor-intrinsic role of CN in the modulation of tumor microenvironment.

The aims of this proposal are:

1) Comparing the tumor microenvironment of VIVIT+ and VIVIT- tumors with a particular focus on the characterization of $CD8^+$ T cells infiltrating VIVIT+ tumors.
2) Identifying the mechanism(s) through which tumor-intrinsic CN inhibition affects the tumor microenvironment.
3) Investigating the pathways regulated by CN in human tumors.

Experimental plan

Aim 1. Characterization of tumor microenvironments in VIVIT+ and VIVIT- tumors

Aim 1.1 Characterization of the immune infiltrate

We will inject in the subcutaneous space of syngeneic recipients VIVIT+ and empty vector, 4T1, CT26, and B16 cells, and we will assess the presence of immune cells in the tumor microenvironment by FACS analysis.

Absolute numbers and percentages of T, NK and B cells, macrophages, neutrophils/MDSCs, dendritic cells will be evaluated.

Aim 1.2 Characterization of CD8 T cells within the tumor microenvironment of VIVIT+ tumors

We have demonstrated that the $CD8^+$ T cells that accumulate in the microenvironment of VIVIT+ tumors express PD-1. Accordingly, we have seen that VIVIT+ tumors respond to anti-PD-1 treatments.

It has been shown that the $CD8^+$ T cells responsive to anti-PD-1 therapy are a subpopulation of progenitor exhausted tumor-infiltrating T cells. Progenitor exhausted CD8+ T cells are $Tcf1^+Tim3^-$ cells while terminally exhausted cells are $Tcf1^-Tim3^+$; the presence and relative frequency of these two populations will be investigated in VIVIT+ and VIVIT- tumors by cytofluorimetry to verify whether tumor-intrinsic CN inhibition favors the accumulation of $CD8^+$ T cells responsive to antibodies against PD-1. As the progenitor exhausted population has been shown to express a specific set of genes (Il7r, Xcl1, Tcf7, Slamf6), we will also assess the expression of this gene signature in $CD8^+$ T cells. The expression of CD103 will be also evaluated (see aim 2.1).

Finally, since tumors that efficiently respond to anti-PD-1 therapy have infiltrating $CD8^+$ T cells, the fine distribution of $CD8^+$ T cells in VIVIT+ tumors will be characterized by immunocytochemistry and compared with the one in VIVIT- tumors. Our preliminary results suggest that $CD8^+$ T cells infiltrate the VIVIT+ tumors, therefore we will complete this analysis including also the investigation of the interaction with specific immune cell types.

Aim 2 Identification of the mechanism(s) through which tumor-intrinsic CN inhibition affects the tumor microenvironment

Aim 2.1 TGF-β as a potential target molecule of the CN-PxIxIT axis to control the tumor microenvironment and the $CD8^+$ T cell tumor homing.

We have observed increased expression levels of TGF-β1 and 2 in VIVIT+ tumors compared to VIVIVT- tumors. TGF-β is required for the homing of $CD8^+$ T cells in the skin by inducing the expression of CD103, which interacts with the E-cadherin expressed by keratinocytes keeping the $CD8^+$ T cells in the skin. Since tumor cells often upregulate E-cadherin expression, it has been proposed that CD103 engagement may also be relevant in cancer. Moreover, $CD103^+CD8^+$ T cells are required to restrain melanoma growth and the expression of CD103 correlates with longer survival of melanoma patients.

On this basis, we hypothesize that the same process may occur also in VIVIT+ tumors, in which tumor-derived TGF-β may induce the expression of CD103 in $CD8^+$ T cells and favor their tumor homing.

However, TGF-β is also considered pro-tumorigenic, therefore, the assumption that TGF-β facilitates $CD8^+$ T cell tumor infiltration may seem paradoxical.

Recently, TGF-β was shown to inhibit the anti-tumor effect of the treatment with antibodies against PD-L1 in melanomas that were not infiltrated by $CD8^+$ T cells (which remained located in the external capsule). Nevertheless, the TGF-β response signature associated with stromal cells was present both in tumors that responded to anti-PD-L1 therapy and in non-responsive tumors. These data indicate that TGF-β per se does not explain the lack of responsiveness to ICB therapies. Interestingly, immune “desert” tumors, which are cancers that do not have CD8+ T cell infiltration either in the external capsule or in the tumoral mass, showed a very low TGF-β signature. This suggests that TGF-β may be required for $CD8^+$ T cell infiltration and that the site of TGF-β production may affect the localization of $CD8^+$ T cells.

To test the activity of TGF-β in our models, we will evaluate the presence of phosphorylated smad2/3 (which are phosphorylated downstream of the TGF-β receptor) by western blot in total tumor lysates and immunocytochemistry (which will also allow identifying the regions of the tumor with active TGF-β). We will perform double immunofluorescent staining of tumor sections to visualize phospho-smad2/3-positive $CD8^+$ T cells. We will then inhibit the TGF-β pathway by using inhibitors, such as SB-431542 (Cayman Chemical, cat. 13031) that we have already successfully used, and we will assess the composition of the tumor microenvironment after treatment. The treatment with TGF-β inhibitors will be performed at different time points after tumor transplantation.

Aim 2.2 Identification of the target genes of the tumor-intrinsic CN-PxIxIT axis affecting the tumor microenvironment

To identify additional CN targets involved in the generation of the tumor microenvironment, we will perform a comparative transcriptome analysis. Total RNA will be extracted from VIVIT+ 4T1 and B16 cells and the corresponding control cells to perform RNA-Seq experiments. The genes showing the highest changes in

expression levels and correlating with homing, recruitment or differentiation of $CD8^+$ T cells will be prioritized. Depending on the results of Aim 1.1, we will prioritize additional genes according to the immune cell populations affected by changes in the tumor microenvironment and related to the responsiveness to anti-PD-1 therapy. The transcriptome analysis will be performed in collaboration with Prof. S. Abrignani at the INGM institute, Milan, Italy.
The genes selected as potential targets of CN and its PxIxIT-substrates will be further studied: When possible, the tumors will be treated with soluble proteins and/or inhibitors to validate their role in tumor microenvironment formation; for those genes that do not encode soluble proteins or in case neither recombinant proteins nor inhibitors are available, we will validate their functions with molecular approaches. These genes will be knocked out (with CRISPR/Cas9) or knocked down (with shRNA) in 4T1, CT26, and B16 cells; the mutated tumor cell lines will be transplanted in vivo; and the tumor microenvironment will be analyzed. The responsiveness to anti-PD-1 therapy will also be evaluated.

Aim 3 Investigating the CN-regulated pathways in human tumors
To confirm the role of CN in human tumors, we will study the effect of the VIVIT peptide in human cancer cell lines, and we will investigate the potential association of CN nuclear translocation with the frequency of infiltrating $CD8^+$ T cells. A variety of human cancer cell lines will be infected either with the VIVIT vector or the empty vector. We will verify changes in the expression levels of TGF-β and the other genes identified in Aim 2 by real-time PCR or FACS analysis. We will also analyze tumor sections of Non-Small Cell Lung Cancer (NSCLC). NSCLC accounts for around 90% of all lung cancer cases and around 20% of patients positively respond to anti-ICB therapies. Tumor sections will be analyzed by immunofluorescence to identify phospho-smad 2/3+ $CD8^+$ T cells. The presence of $CD8^+$ T cells expressing phospho-smad 2/3 will be correlated with tumor infiltration or tumor exclusion.
CN activation may lead to CN nuclear translocation. Confocal microscopy will be used to detect CN in the nucleus of tumor cells in the tumor sections. We will correlate CN nuclear translocation with the presence of tumor infiltrating $CD8^+$ T cells.

Feasibility and financial support

Support from the Fondazione Regionale per la Ricerca Biomedica (Progetto IANG-CRC) and From AIRC (Progetto IG 2019Id23512)

Literature references

1. Peuker K, Muff S, et al. Epithelial calcineurin controls microbiota-dependent intestinal tumor development. 2016; Nature medicine. 22:506-15.
2. Quang CT, Leboucher S, et al. The CN/NFAT pathway is activated in diagnostic breast cancer cases and is essential to survival and metastasis of mammary cancer cells. 2015; Cell death & disease. 6:e1658.
3. Shoshan E, Braeuer RR, et al. NFAT1 Directly Regulates IL8 and MMP3 to Promote Melanoma Tumor Growth and Metastasis. 2016; Cancer research. 76:3145-55.
4. Tie X, Han S, Meng L, Wang Y, Wu A. NFAT1 is highly expressed in, and regulates the invasion of, glioblastoma multiforme cells. 2013; PloS one. 8:e66008.
5. Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, et al. NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence. 2016; Oncogene. 35:3282-92.

PI recent papers related to the topic

1. Mingozzi F, Spreafico R, Gorletta T, Soncini MC, Cigni C, Di Gioia M, Caccia M, Sironi L, Collini M, Rusconi M, Chirico G, Zanoni I and Granucci F Lymph node-resident NK cells become anti-tumor effectors upon prolonged contact with dendritic cells. EMBO Mol Med 2016 Sep 1;8(9):1039-51