Study of the Role of NFAT5 in Tumor-Specific T Cells

Project

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Specific Aims

The tumor microenvironment creates a highly suppressive contexture that favors the recruitment of suppressive immune cells, such as regulatory T cells, and strongly inhibits common T cells. Cancer induces T cells that phenotypically and functionally differ from the "classically activated T cells" known to protect from disease. These cells are so-called "exhausted", i.e. poorly functional. The mechanism behind this dysfunctional state, causing T cells to be unable to eradicate tumors, is not fully understood and is one of the limiting factors for the success of cancer immunotherapies. Therefore, investigating the pathways leading to dysfunctional mechanisms upon repeated activation of tumor-specific T cells is an important approach to enhance the future treatment for patients. NFAT5 is a transcription factor that was shown by our research group to be overexpressed in tumor-infiltrated T cells. Furthermore, knocking out this gene in tumor-specific T cells led to an increased control of tumor growth. Our aims are:

1. to find out which are the mechanisms regulating NFAT5 expression in tumor-infiltrated T cells by taking advantage of a NFAT5-reporter mouse strain created by the group.
2. to functionally validate its role in tumor mouse models by studying the effect of its overexpression or knock out in tumor-specific CD8 T cells.
3. to uncover the molecular mechanisms regulated by NFAT5 to enforce T cell exhaustion.

Background and Significance

NFAT5 belongs to the family of the nuclear transcription factors of activated T cells (NFAT). It was first described as an osmosensitive transcription factor regulating the cellular response to hypertonicity, and was initially identified in the kidney medulla. Unlike the other members of the NFAT family, namely NFAT1-4, NFAT5 can be activated by osmotic stress and uniquely regulates cytokine gene expression through homodimerization. Further research has questioned the function of NFAT5 in the immune response and has associated NFAT5 with autoimmune diseases, chronic infection and tumor development. The aim of this project is to investigate the regulation of NFAT5 expression in tumor-specific T cells and furthermore to determine how NFAT5 promotes the exhausted state of CD8 T cells. Chronic T cell receptor (TCR) stimulation plays a key role in T cell exhaustion, but the cellular pathway linking both occurrences is not fully understood. The main known stimuli affecting NFAT5 expression, namely osmotic stress, hypoxia and TCR stimulation, indicate that NFAT5 plays a unique role as a transcription factor and is an interesting target to study T cell exhaustion in the tumor microenvironment. NFAT5 induction may be one of the main pathways leading to T cell exhaustion. Further experimental investigation is required.

Experimental Approach

1. Regulation of NFAT5 expression

This research approach will include characterizing the expression of the NFAT5 gene in tumor-infiltrating CD8 T cells to confirm which factors are involved in the signaling inducing NFAT5 expression. Three general stimuli regulating NFAT5 levels are known: osmotic stress, hypoxia and T cell receptor stimulation. Stimuli such as osmotic stress and hypoxia most likely depend on the condition of the surrounding tissue type, e.g. the kidney medulla, but can also be caused by pathogenic conditions such as chronic inflammation and tumors, as is the case for TCR stimulation.

So far, *in vitro* experiments have shown that osmotic stress increases the expression of NFAT5 in CD8 T cells. Furthermore, culturing T cells in 1% oxygen showed similar effects. To investigate the effect of chronic TCR stimulation, we will use anti-CD3 antibody-coated beads and examine whether NFAT5 is upregulated or not. The effect of the tumor microenvironment on NFAT5 expression is not well described. Therefore, CD8 T cells will be stimulated in the presence of tumor-conditioned medium and potential candidates will be determined by Meso-Scale Discovery (MSD) so that they can be blocked with specific antibodies.

In vivo investigation of the role of chronic TCR stimulation: for this approach, mice bearing B16 melanoma on each flank, one with the supplement of GP33 antigen, will be treated with pre-activated TCR transgenic P14 T cells. P14 T cells specifically recognize the GP33 antigen expressed only on one flank of the mouse. The tumor-infiltrating T cells will be sorted by flow cytometry in order to compare the levels of NFAT5 transcript in the two different tumor setups and to determine the effect of antigen TCR stimulation on the NFAT5 level. To confirm these results, a NFAT5 reporter mouse strain has been generated and will be used for flow cytometry studies.

1. NFAT5 function in tumor-specific CD8 T cells

We will validate the impact of NFAT5 and its role in T cell dysfunction by studying overexpression or knock out of NFAT5 in tumor-specific CD8 T cells in mouse tumor models.

Knock out: Previously, the effect of the inactivation of NFAT5 in tumor-specific CD8 T cells was studied in our lab by treating B16 melanoma-bearing mice with pre-activated tumor-specific T cells obtained from either NFAT5-KO animals or wildtype. Tumor-specific NFAT5-KO T cells expressed much lower levels of the inhibitory receptor PD-1 and displayed a higher capacity for controlling tumor growth coupled with higher production of IFNγ and IL-2 upon restimulation. To identify which genes are affected by NFAT5 knock down, we will perform RNAseq analysis with sorted tumor-specific WT or NFAT5-KO T cells isolated from the melanoma. In addition to the *in vivo* approach, we will test the same setup *in vitro* with WT T cells or NFAT5-KO T cells 3 days after stimulation with anti-CD3/CD28 antibodies or GP33 peptide. The comparison of the expression profiles from WT T cells or NFAT5-KO T cells will determine which genes are regulated by NFAT5.

Overexpression: To examine the effect of NFAT5 overexpression on T cell functions, we cloned the four known isoforms of NFAT5 into a retroviral vector also carrying the coding region of the

fluorescent protein eGFP. We added at the end of each isoforms a tag to be able to label the protein. We will overexpress these isoforms in tumor-specific CD8 T cells. A mutant inactivated form of NFAT5, unable to bind DNA, will be used as a control. We will monitor their impact on CD8 T cells and their function in the anti-tumor response. For example, we will follow the *in vivo* kinetics of T cell infiltration into the tumors using bioluminescence. Flow cytometric readouts for exhaustion-associated proteins (such as the inhibitory receptors PD-1 and CTLA-4), for the level of activation (CD25, CD44, CD62L, GzmB) and for the capacity of cytokine production will reveal differences between the overexpressed isoforms and the mock form with respect to the T cell functions. Furthermore, we will determine the transcriptional profile induced by the isoforms using RNA sequencing. To fully understand the effect of NFAT5 overexpression, the results will be analyzed with respect to the findings from our previously described P14 NFAT5-KO T cell experiments.

2. Epigenetic regulation by NFAT5 in tumor-specific CD8 T cells

To determine whether NFAT5 directly or indirectly regulates the genes identified in the previous aims, we will perform epigenetic studies. We will use chromatin immunoprecipitation followed by sequencing to analyze the genomic distribution of NFAT5 in CD8 T cells. NFAT5-transduced CD8 T cells will be stimulated with anti-CD3 and anti-CD28 for 24h. As controls, we will use our NFAT5-KO and WT T cells. The cells will be transduced with NFAT5 constructs or inactivated controls. After 6 days, cells will be either stimulated with PMA and ionomycin or rested. After chromatin immunoprecipitation, sequencing will be performed and analyzed by Sina Nassiri, a postdoctoral fellow in our group. Combining these findings with the transcriptomic data will reveal which genes are directly targeted by NFAT5.

In summary, our research approaches will support the hypothesis that NFAT5 sustains the expression of exhaustion-associated genes, and will help us develop strategies to modulate this profile. In a clinical approach, we are also developing a test to find NFAT5-inhibiting molecules. Promising compounds will be tested *in vivo* to determine the effect on NFAT5-regulated gene expression and anti-tumor response. With our objective, i.e. characterizing the role of NFAT5 during the anti-tumor response of CD8 T cells, we will provide novel insights into the negative regulation of T cell activation. These findings will support clinical applications and therefore give new impulses to develop more effective ways to selectively manipulate the immune system.