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# NFAT5 induction by the tumor microenvironment enforces T cell exhaustion

Cropp Daniela

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**Daniela CROPP**

Pharmacienne diplômée de l'Université de Munich

**Jury**

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Lausanne, le 24 mars 2023

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Thierry Roger

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## Summary

Persistent exposure to antigen renders T cells exhausted. The molecular mechanisms regulating exhaustion are thought to be shared in infection and cancer, despite apparent differences in their microenvironments. We discovered that the NFAT family member NFAT5 was highly expressed in exhausted CD8 T cells during chronic infection and tumor development. NFAT5 deletion resulted in higher a accumulation of tumor-specific CD8 T cells that expressed lower levels of TOX and PD-1 and produced more cytokines, particularly among progenitor exhausted T cells. The observed changes in the phenotype of exhausted T cells upon NFAT5 KO led to improved tumor control. On the contrary, NFAT5 deletion did not affect chronic infection-induced CD8 T cell exhaustion, emphasizing a tumor-specific role of NFAT5 in CD8 T cells. Overexpressing NFAT5 in tumor-specific T cells resulted in a similar transcriptional profile as NFAT1 CA-RIT overexpressing T cells, along with a comparable delay of tumor control. Mechanistically we found that NFAT5 expression was triggered by NaCl- and KCl-induced hyperosmolarity *in vitro* and was TCR-driven *in vivo.* Finally, we discovered that hyperosmolarity was required for NFAT5 activity *in vitro,* consistent with the fact that NFAT5 was only active within the tumor microenvironment but not during chronic infection. Altogether, our data established that NFAT5 is a tumor-specific regulator of CD8 T cell exhaustion.

## Résumé

L'exposition persistante à l'antigène entraîne l'épuisement des lymphocytes T. On pense que les mécanismes moléculaires régulant l'épuisement sont partagés dans l'infection et le cancer, malgré des différences apparentes dans leurs micro-environnements. Nous avons découvert que le membre de la famille NFAT NFAT5 était fortement exprimé dans les cellules T CD8 épuisées au cours d'une infection chronique et du développement d'une tumeur. La délétion de NFAT5 a amélioré le contrôle des tumeurs en favorisant l'accumulation de cellules T CD8 spécifiques des tumeurs qui exprimaient des niveaux plus faibles de TOX et de PD-1 et produisaient plus de cytokines, en particulier parmi les cellules T épuisées progénitrices. À l'inverse, la délétion de NFAT5 n'a pas eu d'effet sur l'épuisement des cellules T CD8 induit par une infection chronique, ce qui souligne le rôle spécifique de NFAT5 dans les cellules T CD8. La surexpression de NFAT5 dans les cellules T spécifiques des tumeurs a entraîné un profil transcriptionnel similaire à celui des cellules T surexprimant NFAT1 CA-RIT et un retard similaire du contrôle de la tumeur. Sur le plan mécanique, nous avons constaté que l'expression de NFAT5 était déclenchée par l'hyperosmolarité induite par le NaCl et le KCl *in vitro* et qu'elle dépendait du TCR *in vivo*. Enfin, nous avons remarqué que l'hyperosmolarité était nécessaire à l'activité de NFAT5 *in vitro*, ce qui est cohérent avec le fait que NFAT5 n'était actif qu'au sein des tumeurs, mais pas pendant l'infection chronique. Dans l'ensemble, nos données ont établi que NFAT5 est un régulateur spécifique des tumeurs de l'épuisement des cellules T CD8.

# 1. Introduction

## 1. 1. The Immune System

The human body has three primary lines of defense to fight against pathogens. First, the physical barrier, composed of the skin and the mucosa, covering the lungs and gastrointestinal tract, preventing pathogens and toxins from entering our body. If a pathogen crosses this first barrier, for example, due to damage, the second line of defense, the innate immune system, reacts. The innate immune system is composed of cells and proteins which respond to patterns frequently found in pathogens. These Pathogen Associated Molecular Patterns (PAMPs) can be identified by pattern recognition receptors (PRRs) expressed by innate immune cells such as dendritic cells (DC), macrophages, monocytes, and mast cells. PRRs are specific for components found commonly in bacteria, fungi, and yeast, such as cell-wall carbohydrates, e.g., lipopolysaccharide (LPS) from gram-negative bacteria or unmethylated CpG DNA, allowing a broad and unspecific recognition of pathogens. Receptor-ligand engagement results in signals that initiate an immune response to eliminate or contain infectious agents[1]. Different signals initiate immunity in response to trauma, ischemia, and tissue damage. Damage-associated molecular pattern molecules (DAMPs) are a group of molecules, alarmins, released by cells upon stress or cell death, such as adenosine, ATP, and heat shock proteins upon others. As for PAMPs, innate immune cells harbor PRRs to recognize these danger signals in the absence or presence of pathogenic infection[2]. Our immune system has specific pathways for different pathogens, and each cell type reacts uniquely to the environmental stimuli followed by an infection. In the case of DCs, immature DCs will be stimulated to engulf and degrade the pathogen (phagocytosis). This process will trigger their migration to secondary lymphoid organs where mature DCs express pathogen-derived peptides, serving as antigens, on their cell surface through a major histocompatibility complex (MHC) molecule. Furthermore, mature DCs upregulate co-stimulatory molecules and cytokines, which provide, together with the presented peptide, the signals to activate T lymphocytes belonging to the adaptive immune system, the last line of defense[3, 4].

## 1. 2. Adaptive Immunity

The adaptive immune system comprises two types of lymphocytes, T and B cells. They build directed cellular (CD4 and CD8 T cell) and humoral (B cell) immunity. To do so, T and B cells harbor a receptor called T cell receptor (TCR) and B cell receptor (BCR), respectively, recognizing a specific antigen. Our body has just a few hundred T cells specific to an antigen. Naïve T cells circulate in the body and enter secondary lymphoid organs (SLO) such as lymph nodes and the spleen, facilitated by homing receptor expression such as CD62L, an L-selectin receptor. Once in the SLO, naïve T cells get activated by interacting with an antigen presenting cell (APC) presenting the cognate antigen on their surface. Through proper T cell activation, T cells differentiate and undergo clonal expansion to form a pool of short-lived effector cells (SLEC) with the ability to recognize the antigen and specifically kill the target cells, e.g., the pathogen or virus-infected cell. To do so, T cells leave the SLO where they were primed and travel to the site of infection. DCs express a site-specific surface signature allowing tissue-specific T cell imprinting leading to the T cell's specific tissue-homing receptors of the T cell[5].

CD4 and CD8 T cells have different functions in adaptive immunity. CD8 T cells are the primary cytotoxic cell type but rely on the help of CD4 T cells[6]. CD4 T cells recruit and help cells of the innate and adaptive immune system but also have direct effector functions. CD4 T cells engage with APCs to enhance their co-stimulatory function, e.g., through the CD40/CD40L interaction[7]. Furthermore, CD4 T cells produce chemokines important for the recruitment of T cells and produce cytokines such as Interleukin (IL)-2[8]. Depending on the context of the adaptive immune response, CD4 help can be more or less critical for cytotoxic functions of CD8 T cells and memory formation[9].

Activated T cells migrate to the site of infection, where they perform their killing capacities upon target cell encounter. Once the antigen is cleared, most antigen-specific T cells undergo apoptosis, marking the contraction phase. During activation, some T cells differentiate into memory precursor effector cells (MPEC) which will survive the contraction phase and form the immune system's memory repertoire[9]. The pool of MPEC cells comprises a small fraction of memory precursor cells which, upon further stimulation, yields SLEC and, in the absence of stimulation, differentiates to central memory cells[10]. Memory T cells ($T_{mem}$) can react faster to a secondary antigen encounter and build a more potent immune reaction. $T_{mem}$ can persist without antigen stimulation, and their maintenance relies on cytokines such as IL-7 and IL-15 and is regulated through the transcription factor T cell factor 1 (TCF-1).

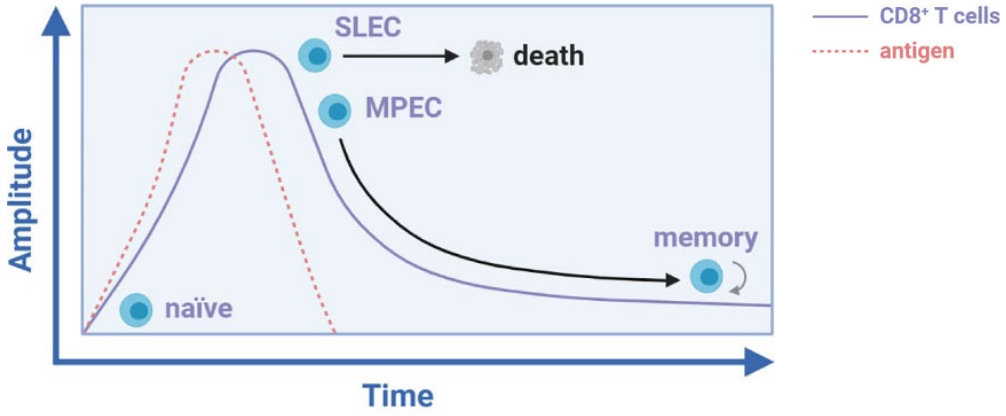


Figure 1: T cell response during acute infection: Naïve CD8 T cells become activated and form SLEC and MPECs. Upon antigen clearance, SLECs contract, and MPECs survive and differentiate into memory CD8 T cells. Adapted from Dolina et al. CD8+ T Cell Exhaustion in Cancer. Front. Immunol. (2021).

## 1. 2. 1 The three pathways underlying T cell activation

The adaptive immune response requires the activation and proliferation of antigen-specific T cell clones. Three signals are required to obtain fully differentiated T cells. The first signal is TCR triggering, the second is co-stimulation, and the third is cytokine stimulation.

### 1. 2. 1. 1. Signal one, TCR triggering

T cell activation requires the innate and adaptive immune system to communicate through the interaction of an APC and a T cell. This interaction of the two cell types is supported by forming a structure known as the immunologic synapse[11]. Multiple TCR and MHC-peptide complexes interact in the synapse, initiating intracellular signaling. The TCR is associated with a CD3 complex containing intracellular signaling domains to transmit the signal. Following the engagement of CD4 or CD8 with the MHC molecule, tyrosine kinases, such as the lymphocyte-specific protein tyrosine kinase (Lck), phosphorylate the tyrosine residues of the CD3 cytoplasmic chains, also known as immunoreceptor tyrosine-based activation motifs (ITAMs). Activated ITAMs lead to the recruitment of adaptor proteins such as Zeta-chain associated protein kinase (ZAP-70), followed by activation through Lck, ZAP-70 phosphorylates the scaffold protein, the linker of activated T cells (LAT). LAT is a key molecule since the formation of the LAT signalosome is crucial for the recruitment and activation of several downstream adaptor proteins. The following signaling cascades can be divided into three main pathways: The mitogen-activated protein kinase (MAPK), the nuclear factor-κB (NF-κB), and the calcium/calcineurin signaling pathway[12, 13].

One of the essential signaling proteins, phospholipase C γ (PLC-γ), catalyzes the production of second messengers by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG activates protein kinase C θ (PKCθ), leading to transcription factor NF-κB activation. IP3, in turn, is responsible for the release of Ca2+ by binding the IP3 receptor (IP3R) located on the endoplasmic reticulum (ER), the trigger for opening calcium-release-activated calcium (CRAC) channels in the plasma membrane. The increase in calcium concentration triggers calmodulin to activate the phosphatase calcineurin leading to the dephosphorylation of the Nuclear Factor of Activated T cells (NFAT)[14]. Dephosphorylated NFAT proteins translocate to the nucleus and induce the transcription of effector genes such as the growth factor IL-2. IL-2 is an autocrine and paracrine growth factor that enhances T cell proliferation and effector functions.

DAG induces the RAS-MAPK cascade through RAS guanyl nucleotide-releasing protein (RASGRP1), which is also induced by GRB2, leading to the Activator Protein 1 (AP-1) complex composed of c-Fos and c-Jun family members[12, 13].

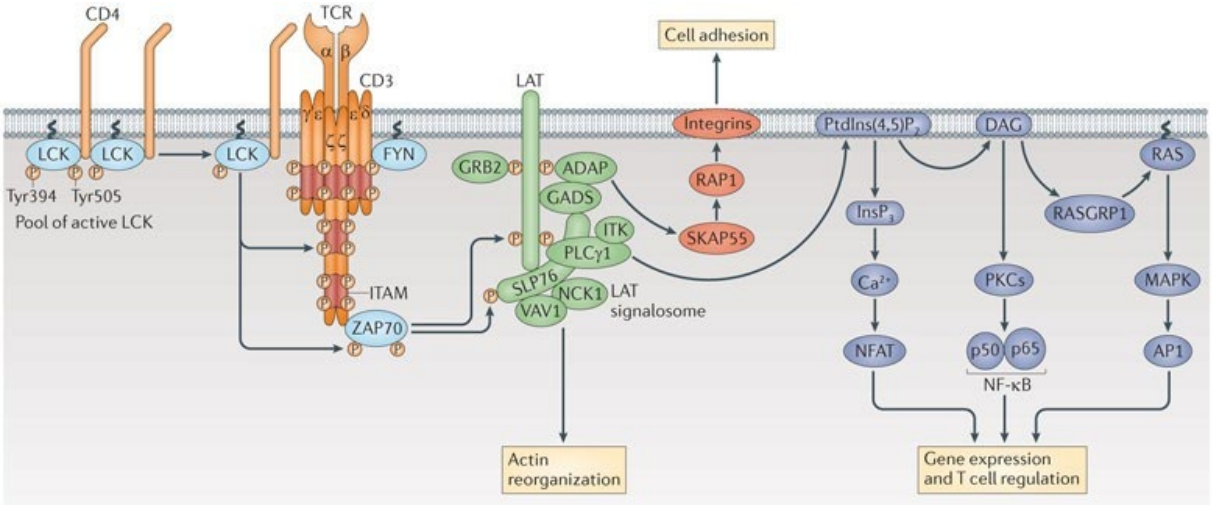


Figure 2.: T cell receptor signaling from Brownlie, R., Zamoyska, R. T cell receptor signalling networks: branched, diversified and bounded. Nat Rev Immunol (2013).

#### 1. 2. 1. 2. Signal two, co-stimulation

Stimulation of co-stimulatory receptors, such as CD28, serves as the second signal. Ligand-receptor interaction through the B7 family molecules CD80 and CD86, expressed by APCs, amplifies TCR signaling. CD28 stimulation leads to the recruitment of phosphatidylinositol-3-kinase (PI3K) to the phosphorylated cytoplasmic tail of CD28, which converts PIP2 to PIP3. AKT is recruited to PIP3, leading to AKT and PKCθ signaling inducing glycolysis through the mammalian target of rapamycin (mTOR), favoring NFAT nuclear localization through GSK3β

inhibition, and induction of NF-κB and AP-1[15]. T cells lacking CD28 can initiate T cell responses but are impaired in sustaining the response[16].

#### 1. 2. 1. 3. Signal three, cytokines

The third signal determining the fate of T cell activation is cytokines. During infections, inflammatory cytokines regulate effector functions and the survival of T cells. IL-12, produced by DCs, and IFNα/β[17] are the significant cytokines transmitting the third signal to CD8 T cells. Their signaling leads to T-bet upregulation through STAT4 and repression of TCF-1[18], leading to clonal expansion and the development of effector function. T cells lacking IFN receptor (IFN-1R) show an impaired clonal expansion[19], while IL-12 KO T cells are skewed towards the memory phenotype with weaker primary responses[20]. Depending on the infection, different cytokines enhance the effector functions of T cells[21].

### 1. 2. 2 CD4 differentiation

Naïve CD4 T cells differentiate into different T helper (Th) cell subsets depending on the polarizing signals they receive during activation. The main subsets of effector CD4 T cells include Th1, Th2, Th9, Th17, Th22, and regulatory T cells (Treg). Th1 cells are induced by IL-12, express the transcription factor T-bet, produce IFN-γ, and are essential for immunity against intracellular pathogens and tumors. Th2 cells are induced by IL-4, express the transcription factor Gata-3, produce IL-4, IL-6, IL-10, and TGF-β, and are a key player in immunity against helminth infections and allergies[22]. Th9 are induced by TGFβ and IL-4, express the transcription factors Foxo1 and PU.1, produce IL-9, and contribute immunity against extracellular pathogens and tumors. Th17 cells are induced by IL-23, express the transcription factor RORγt, produce IL-17, are essential in gut homeostasis, and are involved in autoimmunity. Finally, Th22, produce IL-22 in the absence of IL-17 and IFN-γ[23, 24, 25].

Tregs are immunosuppressive CD4 T cells producing TGF-β and IL-10 and are characterized by their expression of the transcription factor FoxP3[26]. Their role is to avoid autoimmune diseases by inhibiting immune cells through IL-2 consumption and CTLA-4 expression but also suppress anti-tumor immunity. Tregs have two different origins: Natural Tregs are thymus-derived through selection during thymic development by their moderate to high affinity to self-antigens. Induced Tregs (iTregs) have TCRs with a low or intermediate affinity for self-antigens and escaped negative selection in the thymus. Therefore, they derive from naïve CD4 T cells and are peripherally induced. iTregs can recognize self- and non-self-antigens and are essential in maintaining mucosal tolerance[24].

### 1. 2. 3 Characteristics of effector CD8 T cells

Activation of T cells leads to transcriptional and epigenetic changes accompanied by high metabolic demand. Naïve T cells are characterized by low nutrient uptake and low glycolysis and rely mainly on oxidative phosphorylation (OXPHOS). Activated T cells switch their energy source to glycolysis to synthesize the molecules necessary for clonal expansion and their effector T cell functions[27]. The central coordinator of anabolic metabolism is mTOR[28]. This metabolic change, coupled with increased glucose transporter expression (GLUT1), is linked to their activation through TCR and CD28 signaling via the PI3K-AKT pathway[29, 30].

Following activation, CD8 T cells acquire effector functions enabling them to kill infected or mutated cells in the body. The key features of effector T cells ($T_{eff}$) are their ability to produce perforins, granzymes, and cytokines IFN-γ, TNF-α, and IL-2. Furthermore, they express the Fas ligand (FasL) at their surface. IFN-γ triggers MHC-I upregulation by infected cells and MHC-II expression by APCs[31], which enhances antigen presentation to T cells, and promotes the polarization and activation of macrophages to pro-inflammatory M1 macrophages[32]. In DCs, IFN-γ signaling leads to their maturation and co-stimulatory molecule expression[33]. IL-2 serves as an essential survival and proliferation signal for lymphocytes. Furthermore, IL-2 signaling leads to STAT5-dependent upregulation of Eomes and thus effector differentiation[34, 35], while IL-2-dependent Blimp-1 expression serves as a negative feedback[36]. To kill target cells, perforins attack the membrane and form pores allowing granzymes to enter the cell and induce apoptosis of the target cell[37, 38]. Furthermore, FasL binding the Fas receptors and TNF-α and IFNγ binding, respectively, the TNF and IFN receptors on target cells induce apoptosis[39, 40].

Several transcription factors are essential for acquiring these features, such as the central transcription factors NFAT, NK-κB, and AP-1. While inhibitor of DNA binding 2 (Id2) is important for differentiation and maintenance[41], T-bet, Zeb2[42], Eomes, and Blimp1[43] promote the expression of perforins and granzymes, essential for the lytic function of $T_{eff}$.

## 1. 3. The role of the NFAT family in T cells

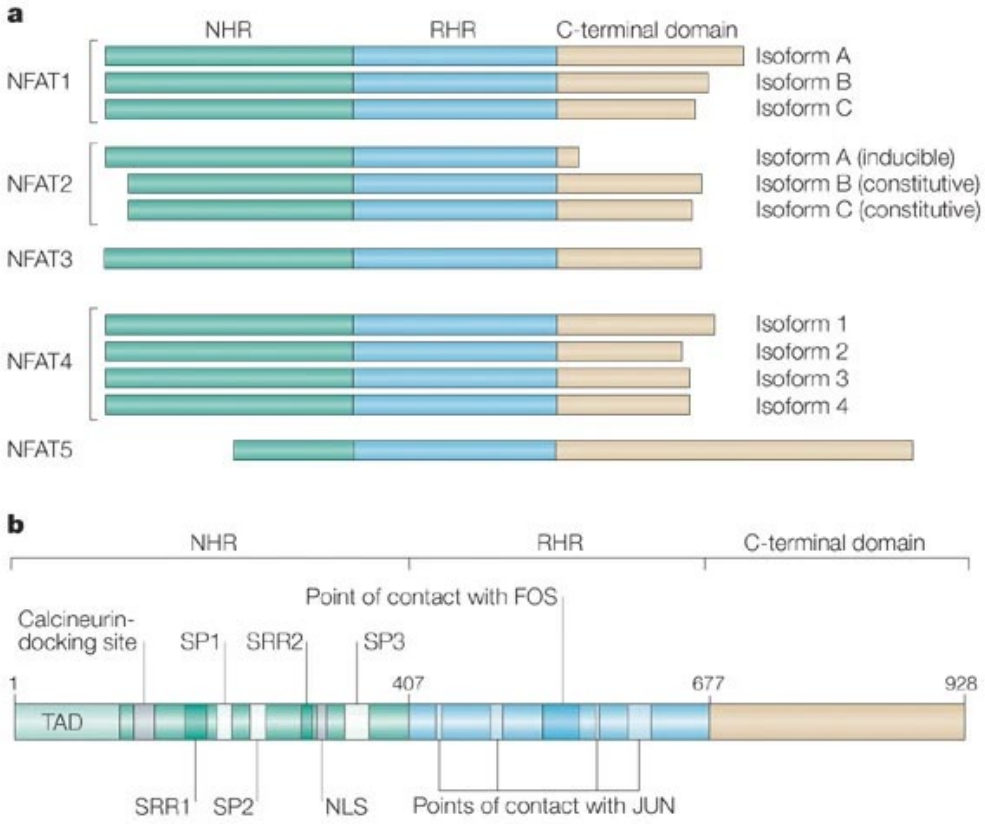


Figure 3.: The NFAT family of transcription factors. a) NFAT family members and isoforms with the three main domains: NFAT homology region (NHR), Rel-homology region (RHR), and C-terminal domain. b) NHR contains the transactivation domain (TAD) followed by a calcineurin docking site with respective dephosphorylation sites: SRR1, SRR2, SP1, SP2, and SP3. Dephosphorylation leads to exposure of the nuclear localization signal (NLS). The contact points for FOS and JUN interaction are located in the RHR. From Macian, F., NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol (2005).

The NFAT family comprises five members, NFAT1- NFAT5, and belongs to the Rel/NF-κB family of transcription factors. While NFAT3 is mainly expressed by non-immune cells and NFAT5 can be expressed by most cell types, NFAT1, NFAT2, and NFAT4 are expressed in immune cells and play a crucial role in their function and development. The NFAT family shares conserved regions, such as the REL homology domain containing the DNA binding domain (DBD), while the N-terminal domain is less conserved. Furthermore, several isoforms have been described due to alternative splicing and different promoters that differ in their N- and C-terminal domains[44].

The canonical pathway of NFAT activation depends on the receptor-ligand interaction through the TCR/BCR on T- and B-cells or the TLR on monocytes and mast cells. The initiated signaling cascade leads to the calcineurin-mediated dephosphorylation of several serine residues leading to a conformation change and the exposure of the nuclear localization signal (NLS)[45, 46]. The activation of NFAT downstream of the TCR is a crucial pathway in the generation of effector T cells, as described in the context of T cell activation above[47]. Furthermore, cytokines such as IL-2, IL-7, and IL-15 can regulate NFAT through an alternative pathway and induce NFAT binding to the CX3CR1 promoter[48]. NFAT1, NFAT2, and NFAT4 are co-expressed in T cells and have redundant roles linked to their shared DBD[49, 50], and demonstrated by the relatively small effect of a single KO of one of the three members compared to a double KO[51]. NFAT2A is the only isoform that is upregulated by NFAT members and can drive a self-sustaining feedback loop to maintain a high level of NFAT expression[52, 53]. NFAT plays distinct functions depending on the cell type, the stimuli, and the transcription partner. In Th1, Th2, and Th17 cells, NFAT controls the expression of the respective lineage cytokines IFN-γ, IL-4, and IL-17[54, 55, 56]. Furthermore, NFAT is responsible for the suppressive function of iTregs and can form complexes with FoxP3 leading to the expression of iTreg-associated genes such as *CD25* and *CTLA-4*[57].

The regulation by NFAT depends on its transcriptional partner[58], e.g., in Tregs, NFAT partners with FoxP3 to repress IL-2 expression[57]. In contrast, the binding to the IL-2 promoter during T cell activation relies on NFAT/AP-1 complexes[49]. Furthermore, it has been described that NFAT can form homodimers at specific palindrome sites. In $T_{eff}$, the glycolytic switch is supported by NFAT-dependent transcriptional regulators, such as IRF4, c-Myc, and HIF-1α[47, 59]. Overall, NFAT's role in T cell activation is crucial. NFAT1/2 KO mice, which show impaired cytokine production including IL-2, IL-4, IFNγ, and TNF-α, have a defect in controlling acute lymphocytic choriomeningitis virus (LCMV) infections and show an impaired memory formation[51].

Furthermore, NFAT is involved in alternative differentiation states such as anergy, tolerance, and exhaustion[60], which will be discussed in the next chapter.

## 1. 4. Alternative differentiation pathways of CD8 T cells

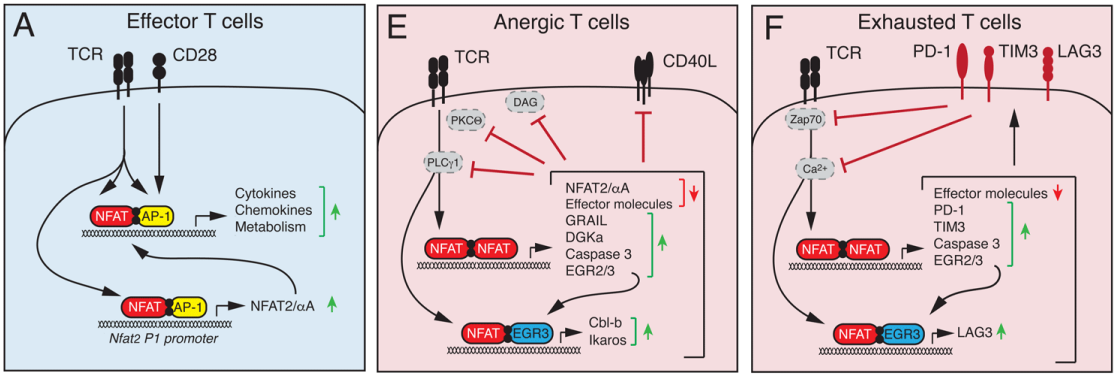


Figure 4.: NFAT partners and regulated genes in A) Effector T cells, E) Anergic T cells, and F) Exhausted T cells. From Vaeth M and Feske S. NFAT control of immune function: New Frontiers for an Abiding Trooper F1000Research (2018).

T cell activation under suboptimal conditions can lead to alternative differentiation programs such as anergy and exhaustion. T cell anergy describes T cells that encountered their specific antigen without co-stimulation. Anergic T cells are hyporesponsive, characterized by a low IL-2 production while remaining alive[61]. Without proper co-stimulation, NFAT does not form complexes with AP-1 but binds as a homodimer or a complex with EGR2 or EGR3 to a set of genes inducing a specific transcriptional program linked to anergy[62, 63]. Anergy-induced genes will further dampen T cell functions by degrading important signaling molecules downstream of the TCR and interfering with co-stimulatory pathways. This process is part of the peripheral tolerance to inactivate auto-reactive T cells and demonstrates the importance of the three signals for proper T cell activation.

## 1. 4. 1 T cell exhaustion

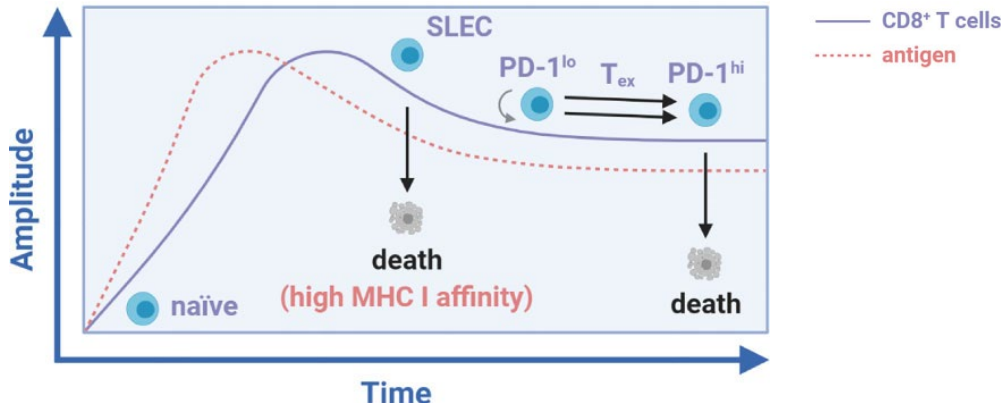


Figure 5.: T cell response during chronic infection: Initially gained effector functions are lost during the chronic phase of infection and with continuous antigen triggers exhausted T cells arise. Adapted from Dolina et al. CD8+ T Cell Exhaustion in Cancer. Front. Immunol. (2021).

T cell exhaustion is a dysfunctional state of CD8 and CD4 T cells characterized by a graduate loss of initially gained effector functions[64, 65]. Exhaustion is induced by chronic TCR stimulation in pathologies such as chronic infection, observed in HIV and HCV infections in humans, chronic LCMV infection in mice, and cancer[66, 67]. Exhausted T cells are characterized by their graduate loss of effector functions[68], limited proliferative potential[69], and sustained expression of multiple inhibitory receptors such as the programmed cell death protein 1 (PD-1), the T cell immunoglobulin domain, and mucin domain protein 3 (TIM3), and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) among others[70]. Inhibitory receptors impede the stimulatory signals from the TCR and co-stimulatory receptors[71]. APCs, tumor cells, fibroblasts, and endothelial cells express the PD-1 ligands, PD-L1 and PD-L2. Through receptor-ligand engagement, the tyrosine-protein phosphatases SHP-1 and SHP-2 are recruited to the cytoplasmic ITIM and ITSM domains. SHP-2 represses TCR signaling by inhibiting ZAP-70 phosphorylation, blocking PKCθ downstream of CD28 signaling, and dephosphorylation of PLCγ1 and ERK1/2, and PI3K/AKT/mTOR signaling through PI3K inhibition[72, 73, 74]. The inhibition of key signaling molecules downstream of the T cell activation cascade reduces cytokine production and cell proliferation and affects metabolism, such as inhibition of glycolysis. PD-1 inhibits peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), a critical metabolic regulator[75] controlling energy metabolism and mitochondrial biogenesis, resulting in altered glycolysis. Overall exhausted T cells show a different metabolic phenotype than $T_{eff}$, with decreased glycolytic activity and diminished mitochondrial fitness[76, 77].

Biophysiological T cell exhaustion is a brake for the immune system to protect the body from an overshooting immune response. Nevertheless, exhausted T cells maintain some effector functions and control chronic infections without causing immunopathology[78]. Moreover, the importance of exhausted T cells is also demonstrated by the increase in viral titers upon their depletion in an ongoing chronic infection[79].

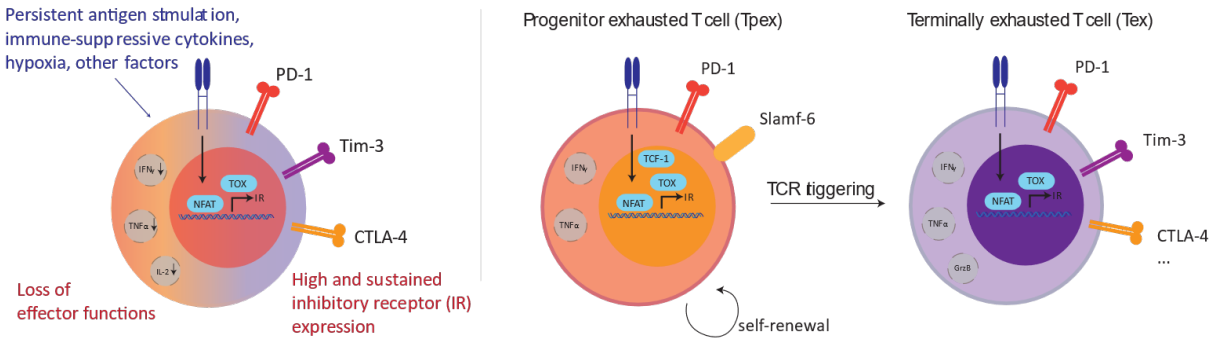


Figure 6.: Scheme of the characteristics of exhausted T cells (left), $T_{pex}$, and $T_{ex}$ (right). Created with Biorender.

Recent studies highlighted the existence of a progenitor T cell ($T_{pex}$, or memory-like T cells $T_{ML}$) pool with memory-like properties, which gives rise to terminally exhausted T cells ($T_{ex}$). $T_{pex}$ are characterized by their proliferative capacities while keeping a stem cell-like phenotype[80]. $T_{pex}$ depend on the expression of the transcription factor TCF-1 and can persist in chronic infections and cancer[81, 82]. The loss of the $T_{pex}$ population results in decreased viral/tumor control. With persistent antigen stimulation, $T_{pex}$ give rise to $T_{ex}$. $T_{ex}$, in contrast, downregulate TCF-1, which results in a reduced stemness capacity and short survival[83]. While PD-1 expression is common to $T_{pex}$ and $T_{ex}$, other inhibitory receptors such as Tim-3 and 2B4 are only upregulated on $T_{ex}$. $T_{ex}$ are terminally differentiated and have an increased capacity to produce cytokines and granzyme B. Strikingly PD-1 blockade treatment triggers a proliferative burst of $T_{pex}$ which can yield cytotoxic $T_{ex}$[82, 84]. Research has focused on understanding the complexity of the transition of $T_{pex}$ to $T_{ex}$, and it became clear that T cell exhaustion is not a single-defined cell population but more a developmental process with several transcription factors serving as checkpoints. Thus, exhausted T cells show a distinct transcriptional and epigenetic profile compared to effector T cells found in acute infections. We still need key information to understand the development and establishment of these stages and their complexity. Depending on the pathology and the unique microenvironment, we might face different transcription factors reacting to a specific combination of stimuli resulting in T cell exhaustion.

#### 1. 4. 1. 1. T cell exhaustion-associated transcription factors

Exhausted T cells have a distinct transcriptional profile from effector T cells, and several transcription factors have been associated with regulating exhaustion. As mentioned above, $T_{pex}$ rely on the expression of **TCF-1** for their maintenance. TCF-1 represses effector genes by promoting the expression of **EOMES**, **BCL-6**, and **c-MYB**[85, 86]. Furthermore, TCF-1 inhibits **Blimp-1**, which is generally involved in terminal differentiation, inhibitor receptor upregulation, and granzyme B production[43, 87]. Moreover, **ID3** is important for $T_{pex}$ survival by repressing cell death through the Fas-FasL axis[88]. While EOMES usually is essential for memory formation after a resolved infection[89], in exhausted T cells, its nuclear accumulation ratio to **T-bet** is higher, leading to inhibitory receptors upregulation. When nuclear T-bet is more abundant, it enforces normal effector differentiation[90].

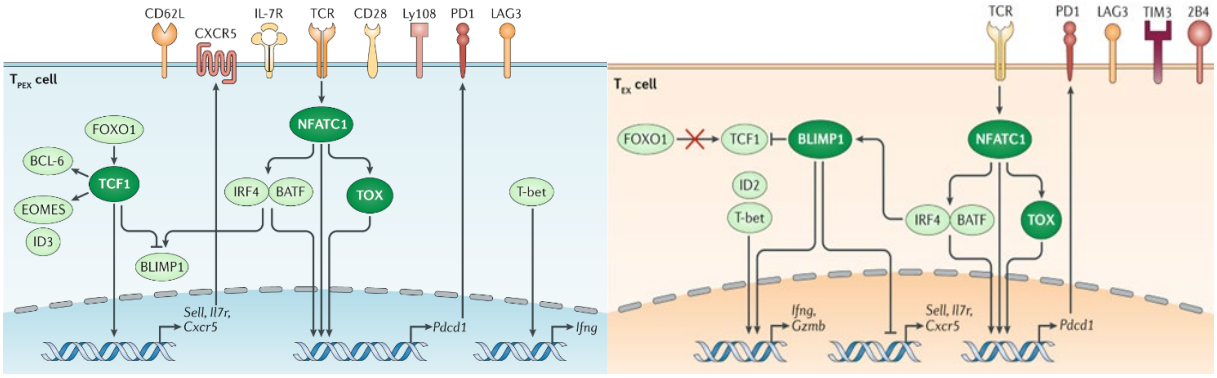


Figure 7.: Transcriptional regulation of $T_{pex}$ and $T_{ex}$ cells. Adapted from Kallies, A., Zehn, D. & Utzschneider, D.T. Precursor exhausted T cells: key to successful immunotherapy?. Nat Rev Immunol (2020).

The first work showing the mechanism linking **NFAT1** and exhaustion was done by Martinez et al. in 2015. An engineered, constitutively active (CA) version of NFAT1, unable to bind AP-1 due to an exchange of the amino acids necessary to the contact points with c-Fos and c-Jun (NFAT1 CA-RIT), controlled the transcription of a distinct set of target genes different to the genes that are controlled by classical NFAT-AP-1 complex. The monomeric binding of NFAT CA-RIT to the DNA upregulated exhaustion-associated proteins such as PD-1 and LAG3[91]. Interestingly, AP-1 expression was shown to be decreased in chronic LCMV infection, suggesting a shift to monomeric binding of NFAT[92]. NFAT is required for the development of exhaustion as an early transcriptional checkpoint. It drives the expression of BATF, IRF4, NR4A, and TOX[93, 94], while a positive feedback loop of BATF and IRF4 regulates NFAT.

The transcription factor thymocyte selection-associated high mobility group box protein, **TOX**, is the first transcription factor specifically associated with exhausted T cells, as classical $T_{eff}$ only express TOX transiently, and $T_{mem}$ express low levels of TOX[95]. TOX induction is Ca2+ sensitive and was shown to be regulated through the NFAT axis, where NFAT is necessary for TOX induction. Still, calcineurin signaling was dispensable for sustained TOX expression at later stages of exhaustion[96]. Interestingly, TOX does not bind DNA in a sequence-dependent manner but in a structure-dependent manner[97], driving inhibitory receptor expression while suppressing cytokines. The role of TOX in T cell exhaustion was demonstrated by the improved anti-tumor functions and ability to control LCMV infection of TOX KO CD8 T cells. At the same time, ectopic overexpression of TOX was sufficient to induce exhaustion *in vitro*[98]. Surprisingly, TOX KO CD8 T cells were found to decline in numbers at later time points in chronic LCMV infection, demonstrating the importance of TOX for the persistence of the antigen-specific CD8 T cell pool[98, 99]. This paradox outlines the role of transcription factors in different stages of T cell exhaustion development. TOX is essential for the formation and persistence of $T_{pex}$, which can self-renew and generate a sustainable pool of T cells. Thus, TOX induced exhaustion program serves for a more durable response with less immunopathology[93, 96, 98, 99, 100, 101, 102].

**IRF4** is expressed as the result of TCR stimulation partly through NFATs regulation and is involved in PD-1 upregulation and metabolic reprogramming towards reduced glycolysis and oxidative phosphorylation[103]. Together with **BATF**, IRF-4 promotes effector differentiation and drives the exhaustion program[94, 104].

Nuclear receptor subfamily 4 group A, **NR4A**, is another TCR-responsive transcription factor family composed of three members, NR4A1, NR4A2, and NR4A3. NR4A members are induced by NFAT and TOX, compete for the same binding sites, and have been proposed to bind the Pdcd1 locus while inhibiting proliferation and effector genes. A triple KO leads to enhanced anti-tumoral functions, decreased PD-1 and Tim-3 expression, and improved cytokine expression[105].

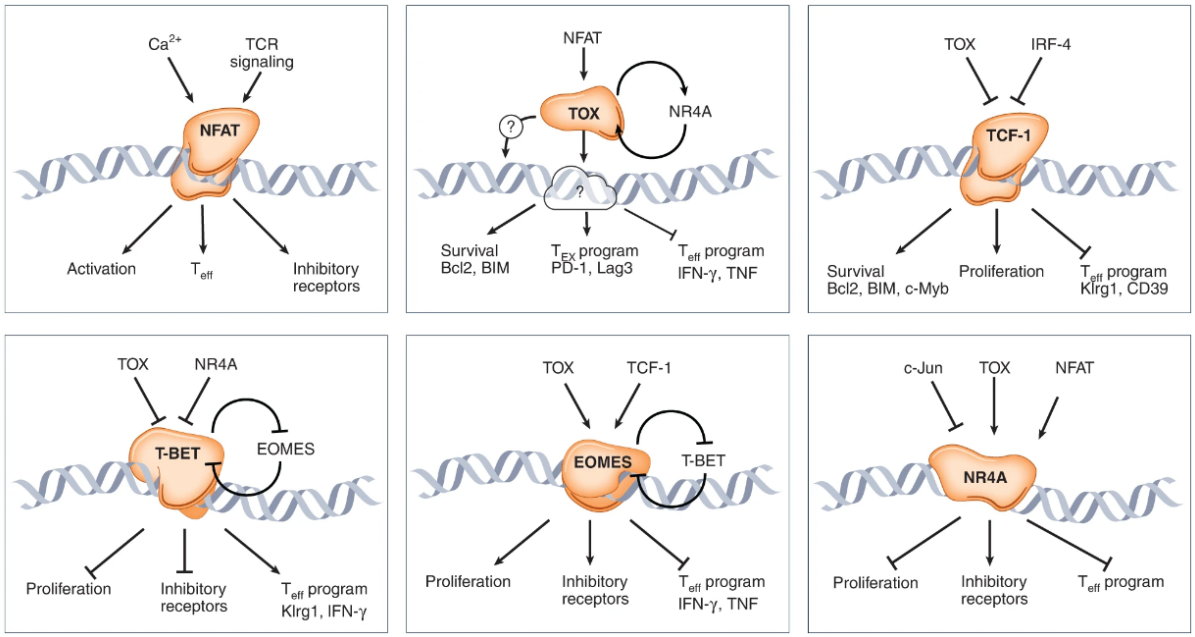


Figure 8.: Key transcription factors involved in the regulation of CD8 T cell exhaustion. Adapted from: Belk, J.A., Daniel, B. & Satpathy, A.T. Epigenetic regulation of T cell exhaustion. Nat Immunol (2022).

Exhaustion is imprinted in T cells. When isolated very early during chronic infection or cancer, exhausted T cells can differentiate into $T_{eff}$ and form $T_{mem}$. When isolated later, the acquired exhaustion state is not reversible[80]. This imprinting is mediated on the epigenetic level through DNA methylation and altered chromatin accessibility, where continuous TCR stimulation correlates with increased DNA methylation. PD-1 blockade did not alter the chromatin landscape of exhausted T cells underlying a stable change on the epigenetic level[106, 107].

## 1. 5. Tumor immunity

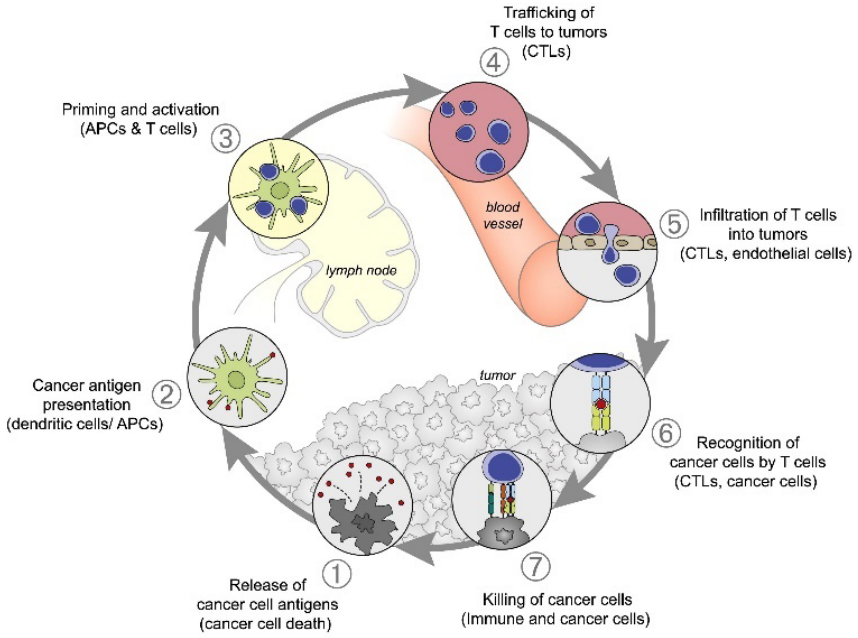


Figure 9.: The cancer immunity cycle. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. (2013)

Cancer is characterized by malignant uncontrolled cell proliferation leading to the invasion of the surrounding healthy tissue and is a leading cause of death worldwide (WHO). Cancer develops when genes that control how cells grow and divide mutate. Mutations can be inherited or occur during the lifespan due to damage to the DNA. Our body surveils cells for mutations and repairs or eliminates them, but this ability diminishes with aging. Nevertheless, not every DNA mutation leads to cancer, but specific genes are involved in cancer development once mutated. Among these are tumor suppressor genes, proto-oncogenes, and genes linked to DNA repair. During cancer development, cancer cells accumulate more mutations leading to the expression of mutated proteins, which can be presented as specific peptides by cancer cells. These peptides are called tumor-specific or neo-antigens and can be recognized by T cells as foreign antigens, allowing the immune system to detect and react to malignant cells. Tumor cells can also overexpress antigens expressed by non-malignant cells; these antigens are called tumor-associated antigens and are not recognized as foreign by the immune system. The last category of tumor-antigens are germ-line antigens, usually only expressed by germ cells and trophoblast tissues but not by somatic cells[108].

Tumor antigens are released during tumor cell death and are captured and processed by DCs, similarly to the mechanism found during pathogen-induced inflammation. In tumors, DCs get often activated through DAMPs. This stimulation is important for DC maturation and activation and improves tumor antigen processing and presentation[2].

Activated DCs migrate to the tumor-draining lymph node (TD-LN), presenting the antigen to antigen-specific T cells. Following priming, T cells leave the LN and travel through the bloodstream to the tumor site. Due to tumor-induced inflammation, vessels in the tumor's proximity display specific integrins and chemokines on their endothelium, allowing T cells to extravasate from the bloodstream. After infiltrating the tumor, T cells scan the cells for their cognate antigen, allowing the T cell to perform its cytotoxic action. Upon tumor cell killing, tumor-associated antigens are released and fuel the cycle and increase the efficiency of the response[109].

In theory, this cycle would lead to effective anti-tumor immunity, but in patients, this process is impaired at several stages. First, the released antigens are often poorly immunogenic since tumor cells also express self-antigens tolerated by the immune system. Furthermore, DCs activation might not be optimal depending on the DAMPs in the tumor microenvironment (TME)[2] and the inflammatory context. Insufficient DC priming leads to impaired T cell activation, induction of Tregs, and the generation of anergic T cells. Tumor cells can produce the Vascular Endothelial Growth Factor (VEGF), promoting the tumor's blood supply and inhibiting the endothelium's activation, leading to impaired T cell infiltration, excluding the T cells from the tumor site[110].

If T cells succeed in infiltrating the tumor, they still face several hurdles, described in the next chapter.

## 1. 6. Specificity of the tumor microenvironment

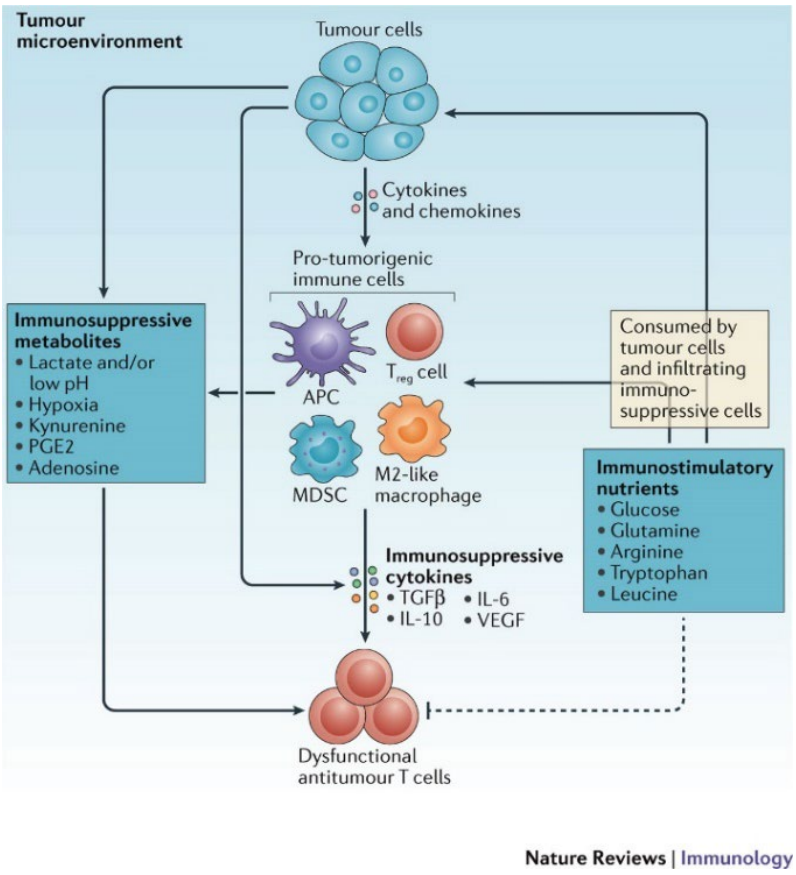


Figure 10.: Microenvironmental factors instructing T cell exhaustion. From Speiser, D., Ho, PC. & Verdeil, G. Regulatory circuits of T cell function in cancer. Nat Rev Immunol (2016).

The TME is composed not only of tumor cells but is a complex assembly of different immune and non-immune cells, such as cancer-associated fibroblasts (CAF) and endothelial cells, creating a tumor-specific environment. Often the TME is immunosuppressive, enabling the tumor cells to escape immune destruction. Tumor cells can attract pro-tumorigenic immune cells such as M2-like macrophages, Tregs, and monocyte-derived suppressor cells (MDSC). Together with tumor cells, pro-tumorigenic immune cells produce anti-inflammatory cytokines such as IL-10, IL-6, and TGF-β and compete for nutrients, amino acids, and oxygen with tumor-infiltrating lymphocytes (TILs)[111].

### 1. 6. 1 Metabolic challenges

The TME is poor in glucose, amino, and fatty acids and is enriched in cholesterol. Facing the TME, TILs must adapt to this harsh environment which leads to a switch of the metabolic, transcriptional, and epigenetic profile. Tumor cells have a high metabolic demand for glucose, amino acids, and fatty acids and rely mainly on aerobic glycolysis as their energy source. Even though aerobic glycolysis generates much lower ATP per mole glucose than classical oxidative phosphorylation, its higher kinetic allows tumor cells to consume more glucose to keep proliferating despite lacking nutrients[112]. Overexpression of lactate dehydrogenase (LDH), a glycolytic pathway enzyme converting reversibly pyruvate to lactate, further enables tumor cells to consume high amounts of glucose and, in turn, excess lactic acid production coupled with acidification of the TME[113]. The elevated lactic acid in the TME induces the expression of VEGF, promotes M2-like polarization[114], and skews plasmacytoid DCs towards tolerogenic functions and Treg promotion[115]. Low pH, through elevated lactate abundance, hinders TIL proliferation and effector functions[116]. Therefore, TILs must compete for glucose in the TME and adapt to environmental changes inhibiting their effector functions. Exhausted T cells have a repressed metabolism reflected in impaired glycolysis, while their mTOR activity is elevated compared to $T_{eff}$[117], reflecting their high metabolic demand. It has been shown that PD-1 interferes with glucose uptake, leading TILs to increase the production of mitochondrial reactive oxygen species (mROS), triggering NFAT signaling in a dose-dependent manner[118, 119]. The central mitochondrial dysfunctions in exhausted T cells include defects in the OXPHOS pathway, depolarization, and decreased mitochondrial mass and activity[120], rendering TILs metabolically inefficient and functionally exhausted.

### 1. 6. 2 Hypoxia

Hypoxia is a well-described environmental change in many tumors due to limited oxygen supply caused by abnormal vascularization and compromised blood flow primarily found within the tumor core. Oxygen levels range from 1-2% or even lower depending on the tumor type[121], and the localization, e.g., the tumor core, represents the most hypoxic niche. The physiologic levels vary from 5-10% in the human body. Hypoxia skews immune cells such as macrophages to a more pro-tumoral phenotype[122, 123], attracts Tregs[124], and on the other hand, increases inhibitory receptor expression on TILs[125]. Cells in the TME upregulate Hypoxia-inducible factor (HIF)-1α in response to hypoxia leading to a hypoxic stress response with

increased glycolysis[126, 127]. Expression of enzymes such as CD39[128] and CD73[129], responsible for generating immunosuppressive amounts of adenosine, are also hypoxia-induced. CD39 is also a marker of tumor reactivity[130] and terminal exhaustion of TILs[131]. Hypoxia further enhances glucose uptake by cancer cells, increases glycolysis, and exacerbates the acidification in the TME. The direct effect of hypoxia on CD8 T cells is complex. HIF-1α also regulates glycolysis in T cells, but hypoxia has been shown to have also adverse effects. Several studies have investigated the direct impact of hypoxia on CD8 T cells with different results. Depending on the study, hypoxic conditions were compared to normoxic cell culture conditions of 20% $O_2$, which are not physiological.

The KO of the central negative regulator of HIF-1α, VHL (von Hippel–Lindau) complex, improved CD8 T cell responses to chronic infection and tumors by enhanced cytokine functionality[132], suggesting a positive role of HIF-1α for T cells. *In vitro* activated CD8 T cells stimulated a second time in hypoxia are shifted towards terminal differentiation with higher expression of VEGF-A and effector molecules[133]. The combination of hypoxia and chronic TCR stimulation-induced T cell exhaustion i*n vitro* through Blimp-1 mediated repression of PGC-1α leading to increased mitochondrial ROS triggering NFAT nuclear localization and activity[117]. This effect was interestingly HIF-1α independent since HIF-1α KO T cells showed a similar Blimp-1 expression and dysfunction as HIF-1α expressing cells[117]. HIF-1α is also activated upon TCR stimulation through the PI3K/mTOR pathway and can also be induced through IL-6, independently of oxygen sensing[134]. Overall, hypoxia is a hallmark of many solid tumors and has pro-tumoral effects on immune infiltrates and tumor cells. In contrast, the direct impact on TILs is more complex and might have positive and negative effects depending on other stimuli in the TME.

### 1. 6. 3 Potassium

Due to the lack of nutrients, hypoxia, and rapidly dividing cells competing for limited local resources, cellular necrosis is often increased in tumors and is associated with poor prognosis for patients[135, 136]. Upon necrosis, intracellular content is released, altering the ion distribution of the TME, which challenges cells of the TME with a higher osmolarity. Potassium has been identified as the overabundant ion in the TME of patients and mouse models. High extracellular potassium leads to elevated intracellular potassium concentration in TILs, inhibiting PI3K–AKT–mTOR signaling, resulting in reduced effector functions[137].

Furthermore, T cells cultured in elevated potassium media hindered nutrient uptake and led to a phenotype of caloric restriction with increased autophagy and decreased histone acetylation at activation- but also exhaustion-associated gene loci, rendering T cells less differentiated. Finally, T cells cultured under high potassium conditions were better at controlling tumor growth due to their stem-like phenotype[138]. This study highlighted the importance of the ionic composition of the TME for T cell metabolism and functions.

### 1. 6. 4 Intra-tumoral niches and differences to chronic infection

Even though chronic infection and cancer-derived exhausted T cells share many characteristics, such as inhibitory receptor expression, defective effector capacities, and transcriptional, metabolic, and epigenetic profiles, the TME and the sides of chronic infections are distinct environments with different stimuli present or not for the T cells.

T cell activation depends on the quantity and quality of the antigen, TCR signal strength, and the context of priming, e.g., inflammatory versus non-inflammatory context[139]. T cells are primed through viral peptides in an inflammatory context during chronic infection, with mature APC delivering the three necessary signals. Therefore, T cells in chronic infections undergo an early effector phase for a few days before developing an exhausted phenotype in the long term. While in cancer, the antigen is not presented in the same inflammatory and acute context. Tumor-specific antigens are often poorly immunogenic self-antigens. Furthermore, APCs found in the TME, or the tumor-draining lymph node, are often activated under suboptimal conditions, e.g., non-inflammatory context. Therefore, poorly immunogenic antigens presented by insufficiently activated DCs, lead to poor costimulatory capacities reducing the quality of T cell activation. Especially early in tumor progression, anergic T cells can be found in the TME induced through the described mechanism. How these anergic tumor-specific T cells further develop still needs to be investigated. Still, it illustrates well that the T cell population in the TME is very heterogeneous, with different activation states depending on many factors, such as the time spent in the TME[140].

The cytokine composition found in tumors and chronic infection differs since immune cells' composition and polarization is different. The unique cytokine composition of tumors can trigger distinct regulators of exhaustion, such as the transcription factor c-Maf found highly expressed in TILs from melanoma patients and mouse melanoma models. Maf expression relies on TGFβ and IL-6 and induces the gene expression of *Il10* and *Bcl6* while repressing *Gzmb* and *Ifng* expression leading to the exhaustion of melanoma infiltrating CD8 T cells[141].

Within the TME, $T_{pex}$ are found in antigen-rich regions colocalized with antigen-presenting cells (MHC-II positive cells), often in proximity to lymphatic and blood vessels[142]. The number of DCs found in the TME correlates with $T_{pex}$ abundance, while $T_{ex}$ do not cluster in a specific niche but are dispersed in the TME. $T_{ex}$ do not recirculate in contrast to $T_{pex}$, preferentially recirculating to the TD-LNs and not to other SLO[143].

During chronic LCMV infection, exhausted subsets have their distinct niche within the spleen[82] as CD69+ $T_{pex}$ with low proliferative potential reside in the white pulp. Once they downregulate CD69 and start proliferating, they enter the red pulp and the bloodstream. Their progeny, transitory $T_{ex}$, express CX3CR1[144], are present in the blood and migrate back to the red pulp of the spleen during the differentiation to the terminally exhausted state[145]. Terminally exhausted T cells downregulate CX3CR1, are terminally differentiated, and can also be found in distant organs such as the liver.

Differential expression of chemokine receptors, such as CXCR5 by $T_{pex}$[82] and CX3CR1 by $T_{ex}$[144], might be involved in the localization/homing and contact with other cell types. These chemokine receptors have been better described in chronic infections where CXCR5 was expressed by $T_{pex}$ in SLO but was absent in $T_{pex}$ found in the bloodstream, secondary sites of infection, such as the liver.

Overall, these observations of different microenvironments pose the question if there are more regulators of exhaustion, such as c-MAF, which react to the unique composition of stimuli distinctly.

## 1. 7. NFAT5

The transcription factor NFAT5, also known as tonicity-responsive enhancer binding protein (TonEBP), was first described as an osmo-protective regulator responsible for the cellular adaptation to the persistent hypertonicity in the kidney medullary cells[146]. NFAT5 mRNA is ubiquitously expressed at a basal level, while tissue protein levels were only elevated in the thymus[147]. Unlike the other NFAT family members (NFAT1-4), NFAT5 can be activated by metabolic stress[148] and uniquely binds to the DNA as a homodimer with a lower disassociation rate than the NFAT1-AP-1 complex[149, 150]. The homodimer binding is mediated through one NFAT5 protein responsible for the contact with the consensus DNA site and the other NFAT5 protein binding the DNA backbone in a base-unspecific manner[151]. The difference in regulation and DNA binding is due to the structural properties of NFAT5. Interestingly, NFAT5 shares the common NFAT family-DNA core binding motif GGAA in its DNA binding motif (A/T)GGAAANN(C/T)N(C/T), which enables NFAT1/2 and NFAT5 the binding to similar promoter sites. Under hypertonic conditions, NFAT5 activated the 9xNFAT-Luc reporter, known to be induced by TCR stimulation by NFAT[152].

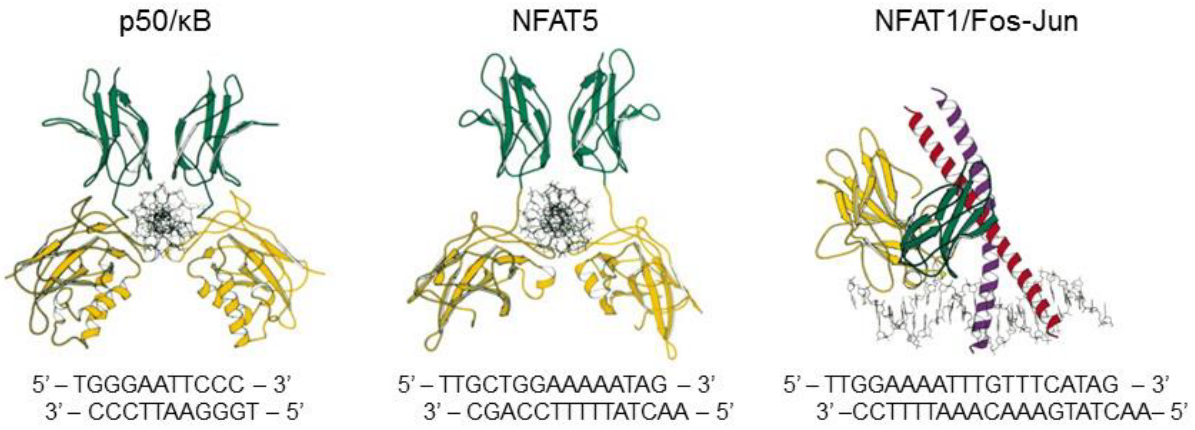


Figure 11.: Crystal structure of NF-κB, NFAT5, and NFAT/Fos–Jun DNA complexes with the DNA sequence of each complex shown below. Adapted from: Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. (2003).

Even though NFAT5 shares the Rel-homology domain with the other NFAT family members, it does not show other structural similarities, such as the 11 contact points for binding with c-Fos and c-Jun[146] and the calcineurin-dependent dephosphorylation sites. In contrast, it can be regulated by several phosphatases and kinases responsible for the de-/phosphorylation of the

different regulatory domains[153]. Three functional domains are part of the N-terminal half of NFAT5 and are responsible for nucleo-cytoplasmic trafficking. The auxiliary export domain (AED), nuclear export signal (NES), and nuclear localization signal (NLS), which contain several phosphorylation sites, determine the nuclear abundance of NFAT5[154]. The C terminal half has several transactivation domains (TAD), which upon phosphorylation, induce the activity of NFAT5.

Several NFAT5 activities are connected to diverse types of stress, including DNA damage, autoimmune signaling, inflammation, bacterial and viral infections, hypertonicity, and other metabolic stresses. Due to the complexity of its regulation, NFAT5 is able to respond to this wide range of stimuli and cellular stresses in different cell types.

## 1. 7. 1 NFAT5 regulation

Upon osmotic shock, NFAT5 recruitment to target genes takes as little as an hour to several hours, depending on the cell type. This mechanism relies on the translocation and activity of preexisting levels of NFAT5 to ensure rapid upregulation of NFAT5-responsive genes and, therefore, a quick adaptation to stress. *De novo* production of NFAT5 protein takes longer and plays an essential role in sustained cellular stresses. NFAT5 can be regulated at several levels: NFAT5 protein expression, mRNA stability and miRNA/lncRNA regulation, translocation, and finally, transcriptional activity can be modulated by distinct pathways.

#### 1. 7. 1. 1. NFAT5 expression

In macrophages, TLR stimulation leads to IKKβ-p65/NK-kB dependent NFAT5 upregulation, where p65/NF-κB was found to be recruited to the *Nfat5* promoter[155]. In combination with hypertonic stress, NFAT5 expression was p38α dependent[156]. The exact pathway to NFAT5 expression in T cells under hypertonic or isotonic conditions is yet to be fully defined. In reaction to high salt, the Brx- p38 MAPK pathways had been described to induce NFAT5 expression in lymphocytes[157]. Brx is an osmosensing scaffold protein localized near the plasma membrane. Brx is induced by changes in the cytoskeleton structure upon osmotic stress and can activate p38α MAPK through forming a complex with Rac1, a small G protein, JIP4, and kinases MKK3/6 which are responsible for the phosphorylation of p38α MAPK. How NFAT5 is induced downstream of activated p38α MAPK is not yet described. One of the possible

pathways could be p38 MAPK-dependent phosphorylation and activation of c-Fos, a component of AP-1. The promoter region of *Nfat5* contains two AP-1 binding sites and several interferon-sensitive responsive elements (ISRE) upstream of the transcription start site, which can be bound by interferon regulatory factors (IRFs)[157]. Thus, Nfat5 expression may be induced by p38 MAPK phosphorylation of transcription factors such as c-Fos and IRFs.

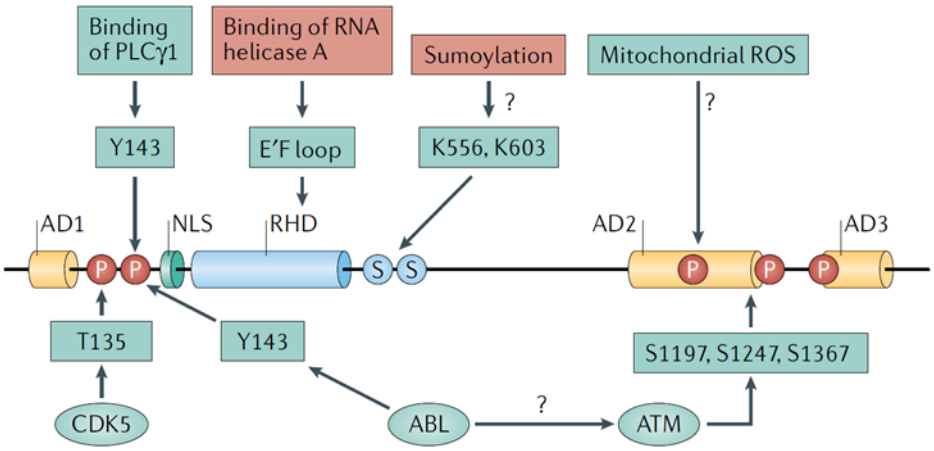


Figure 12.: NFAT5 regulation through post-translational modification of the protein, including phosphorylation and sumoylation. From: Choi, S.Y., Lee-Kwon, W. & Kwon, H.M. The evolving role of TonEBP as an immunometabolic stress protein. *Nat Rev Nephrol*. (2020)

#### 1. 7. 1. 2. NFAT5 regulation by kinases and phosphatases

Several kinases have been described to regulate the phosphorylation of NFAT5's threonine, serine, and tyrosine amino acid residues found in the transactivation domain (TAD). Upon these pathways, PKC-α contributes to the activation of NFAT5 through MAPK ERK[158], the Src kinase family-member Fyn[153, 154], the hypertonicity activated Cyclin-dependent kinase 5 (CDK5) which promotes nuclear localization[159], c-ABL promotes nuclear localization through phosphorylation of Y143 and promotes transactivation activity[160], through activating ataxia telangiectasia mutated (ATM). ATM, in turn, phosphorylates three serine residues in the transactivation domains of NFAT5[161]. While binding to phosphorylated Y143 of phospholipase Cγ1 (PLCγ1) simulates NFAT5 nuclear localization and transactivation activity[162], sumoylation of the lysine residues K556 and K603 suppresses it completely[163]. Glycogen synthase kinase 3β (GSK3β) negatively regulates the transcriptional activity of NFAT5. This regulation differs from the effect of GSK3β on NFAT1-4 proteins, where GSK3β-mediated phosphorylation of NFAT leads to nuclear export[164]. GSK-3β can be inhibited by PKA, AKT1, and PI3K, enhancing NFAT5 activity[165].

#### 1. 7. 1. 3. NFAT5 regulation by miRNA

NFAT5 is regulated by MicroRNAs (miRNAs) that repress translation by targeting the 3'UTR of *Nfat5* mRNA. Multiple miRNAs have been described to regulate *Nfat5* mRNA stability negatively, such as miR-200b and miR-717[166], while only one miRNA, miR-181a[167], stimulates *Nfat5* production. In response to hypertonicity, miRNAs are strongly downregulated[166].

### 1. 7. 2 NFAT5 and osmolarity

The average tissue osmolarity in mammals is 300 mOsm/kg $H_2O$ and is tightly regulated. The kidney is the central control element to adjust water excretion to sustain this physiologic level. Cells in the kidney medulla face extremely elevated osmolar levels, reaching up to 1200 mOsm/kg in humans. To protect cells from shrinking, osmotic stress leads as an immediate reaction to the efflux of water and the subsequent rise of the intracellular ion concentration, which can harm protein and DNA stability. Therefore, several ion transporters and enzymes are rapidly expressed, leading to the exchange of intracellular ions against organic osmolytes[168]. The rise in intracellular ionic strength induces *NFAT5* transcription with a peak at 10h post hypertonicity, stabilizes the mRNA, favors nuclear accumulation, and promotes its activity increasing overall active NFAT5 abundance[169].

Furthermore, hyperosmolarity promotes complete nuclear localization of NFAT5 since, in physiologic osmolarity, 50% of NFAT5 is located in the nucleus[149]. Mitochondrial ROS production is induced by hypertonicity and triggers NFAT5 transactivation activity, suggesting ROS-mediated transmission of the hypertonicity signal to NFAT5[170]. As a consequence of hypertonic stress, NFAT5 induces the transcription of genes containing a highly conserved sequence within their promoter region, known as tonicity responsive enhancer (TonE). Upon these targets are different transporter responsible for the exchange of ions and organic osmolytes, such as the Betaine Gaba Transporter 1 (BGT1), the Sodium/Myo-Inositol Co-Transporter (SMIT), the sodium chloride/Taurine Co-Transporter (TauT) and the enzyme Aldose Reductase (AR) which catalyzes the reduction of glucose to sorbitol. Finally, these mechanisms regulated by NFAT5 help the cell to equilibrate with the osmotic shock and survive in the harsh environment[154, 171].

### 1. 7. 3 NFAT5 and Hypoxia

Ischemia leads to hypoxia or anoxia of the affected tissue leading to ROS production by mitochondria. In rat kidneys and the brain, hypoxia-induced mitochondrial ROS production increased NFAT5 expression and transactivation activity[170]. Furthermore, NFAT5 is involved in ROS production following mitochondrial damage and can suppress the transcription of genes coding for essential proteins, e.g., components of ATP synthase, worsening mitochondrial dysfunction following kidney injury[13]. In the lung, NFAT5 has been proposed to limit ROS production, suppress OXPHOS-associated genes, and mitochondrial respiration in smooth muscle cells from pulmonary arteries in response to chronic hypoxia[172]. In macrophages, NFAT5 expression is triggered in response to hypoxia in inflammation, such as during rheumatoid arthritis[173], further exacerbating their inflammatory phenotype. Therefore depending on the cell type and pathology NFAT5 is differentially reacting to hypoxia and can have protective or destructive roles for the affected tissue.

## 1. 8. The role of NFAT5 in immune cells

NFAT5 regulates the response to osmotic stress in a cell type unspecific manner but has also been implicated in the regulation of different adaptations of immune cells to environmental stress and is associated with many other functions, such as the involvement in the immunoregulation of autoimmune diseases and chronic infection[174].

### 1. 8. 1 NFAT5 in macrophages

In macrophages, NFAT5 regulates diverse proinflammatory and antimicrobial functions. Upon TLR stimulation, NFAT5 mRNA and protein levels are progressively upregulated, which is controlled by NF-κB binding to the *Nfat5* promoter[155]. Furthermore, NFAT5 plays an important role in the pro-inflammatory polarization of macrophages (M1). Upon LPS stimulation of M1 macrophages, NFAT5 induces the upregulation of proinflammatory proteins such as TNFα, IL-6, cyclooxygenase 2 (COX2), and inducible nitric oxide synthase (iNOS)[175]. Thus, NFAT5 has been described to be involved in the antibacterial, antiviral, and antitumoral activity of macrophages and their ability to induce inflammatory T-helper responses[176]. The impaired control of parasitic infection by NFAT5 KO macrophages has further outlined its importance. NFAT5 induction upon hypertonic stress is p38/MAPK-dependent, further enhancing the antimicrobial responses of macrophages[177, 178].

### 1. 8. 2 NFAT5 in T cells

During T cell development in the thymus, NFAT5 is expressed in the transition from the double negative (DN) stage to the single CD4 or CD8 positive stages through the pre-TCR IKKβ dependent-signaling serving as a survival signal. Lck-Cre Nfat5$^{fl/fl}$ mice, where NFAT5 deletion occurs during the DN stage, show a defective transition from DN3 to DN4. This defect leads to the accumulation of DN3 thymocytes and decreased numbers of both double-positive and mature peripheral T cells[179]. Therefore, the generation of NFAT5 KO mice, where gene deletion only occurs at the double positive stage (CD4-Cre) with healthy T cell development and cellularity, was necessary to study T cells. NFAT5 KO T cells derived from CD4-Cre Nfat5$^{fl/fl}$ mice were studied for their alteration in T cell phenotype, function, and survival under hypertonic stress and isotonic conditions.

CD4-Cre Nfat5$^{fl/fl}$ mice show a slight bias towards effector memory CD8 T cells in their T cell compartment. When cultured *in vitro* under hypertonic conditions for three or six days, CD4 and CD8 NFAT5 KO T cells showed impaired homeostatic survival in response to IL-7 linked with a bias of NFAT5 KO cells towards an effector memory phenotype. Culture with concanavalin A (a mitogen) and IL-2 induced overall proliferation, especially in CD8 T cells. Additional hypertonicity reduced proliferation in WT CD8 T cells and abrogated expansion of NFAT5 KO CD4 and CD8 T cells in response to mitogen[180]. NFAT5 KO T cells face cell cycle arrest due to the role of NFAT5 in the cell cycle by inducing cyclins regulating G1 to G2/M progression[181].

Furthermore, CD4 and CD8 T cells depend on NFAT5 for CD24 expression during hypertonic stress combined with TCR triggering to sustain their proliferative capacity, which was abrogated in NFAT5 KO T cells *in vitro* and *in vivo*[180]. Reduced survival and proliferation of NFAT5 KO T cells were observed between 370mOsm/kg and 420mOsm/kg. These studies show the importance of NFAT5 for T cells to sustain expansion, cell cycle, and balance of naïve to memory phenotype in response to hypertonic stress.

*In vitro* culture of T cells under TCR triggering in isotonic conditions leads to upregulation of NFAT5, which has been described as calcineurin-dependent. At the same time, the induction of NFAT5 target genes in response to hypertonicity was not influenced by calcineurin blocking[149, 182]. Through TCR triggering and hyperosmolarity, NFAT5 promoted pro-inflammatory cytokines such as lymphotoxin β and TNF-α in Jurkat cells[149] and the Th17-like phenotype of CD4 T cells through RORγt induction[183].

*In vivo* activation of NFAT5 KO CD4 T cells via anti-CD3 injections leading to acute inflammation, showed a mild reduction of FOXP3+ cells and increased proportions of IFNγ+ and IL-17A+ cells. Treatment with Dextran Sodium Sulfate (DSS) led to exhibited exacerbated colitis coupled with IFNγ upregulation in NFAT5 KO mice[183]. In this study absence of NFAT5 led to autoimmunity showing that NFAT5 can act as a negative regulator of T cell activation.

Interestingly NFAT5 shows different regulation in response to TCR stimulation in isotonic or hypertonic conditions and depends on the cytokine milieu. In mature T cells, NFAT5 regulated pro-inflammatory gene expression in response to hypertonicity through the p38-NFAT5-SGK1 axis, such as *Il17*, *Tnf*, and *Il2*[183]. While in human Th17 cells, NFAT5 led to an anti-inflammatory switch by upregulating TGF-β and regulating FoxP3 and IL-17A under hypertonicity through its downstream kinase SGK1, but did not regulate the same pathway in isotonic conditions[184].

Thus, in CD4 T cells, NFAT5 can promote inflammatory and anti-inflammatory phenotypes depending on the stimuli present.

So far, research has focused on CD4 T cells for their alteration upon NFAT5 KO, and to date, no study has investigated the phenotype of NFAT5 KO CD8 T cells in different contexts. The unique induction profile of NFAT5, coupled with its role in immune cells, led us to study the role of NFAT5 in CD8 T cells in the context of anti-tumor activity, in detail we asked if NFAT5 has a role in CD8 T cell exhaustion.

# 2. Aims of the study

## 2. 1. Identify the kinetics of NFAT5 expression in TILs

The first question we wanted to address was whether and when NFAT5 is expressed in TILs. We, therefore, analyzed both the *Nfat5* RNA expression in mice and humans and the level of expression using an NFAT5-reporter mouse strain during a time-course experiment.

## 2. 2. Effect of NFAT5 overexpression on TILs

Since NFAT5 was highly expressed in TILs, we wondered if its expression regulated the anti-tumor function of TILs. To assess the effect of high NFAT5 expression on tumor-specific T cells, we overexpressed NFAT5 and NFAT Ca-RIT in a mastocytoma mouse model. We then compared the transcriptome by RNA-seq of NFAT5 and NFAT CA-RIT overexpressing TILs to control GFP-expressing ones.

## 2. 3. Effect of NFAT5 KO on TILs

NFAT5 overexpression dampened tumor control. By knocking out NFAT5 in CD8 T cells, we wanted to understand if this would rescue the impaired functions of TILs. We compared tumor control, phenotype, and transcriptome by RNA-seq of WT and NFAT5 KO TILs in a melanoma mouse model.

## 2. 4. Effect of NFAT5 KO on CD8 T cells during chronic infection

We aimed to translate the enhanced tumor control and effector functions observed by NFAT5 KO TILs in another pathology inducing exhaustion, chronic LCMV infection.

## 2. 5. Comparison of NFAT5 KO in the two pathologies

We matched the time course of chronic infection with our two tumor models to directly compare NFAT5 expression at several time points of exhaustion. Since we observed a differential NFAT expression in $T_{pex}$ and $T_{ex}$, we investigated the effect of NFAT5 KO on the two exhausted populations in chronic LCMV infection and the TME.

## 2. 6. NFAT5 induction and activity

To decipher the differences observed in chronic infection and tumors, we analyzed the induction profile of NFAT5 *in vitro* and *in vivo* using our NFAT5 reporter mouse model. Furthermore, we developed a luciferase reporter construct to study NFAT5 activity *in vitro*. To translate our findings *in vivo*, we generated a new reporter coupled to mCherry expression and analyzed NFAT5 activity in the TME and during chronic LCMV infection.

# 3. Results

Contribution to the manuscript “NFAT5 induction by the tumor microenvironment enforces CD8 T cell exhaustion” under revision at Nature Immunology:

This study has been performed in the laboratory of Dr. Grégory Verdeil in close collaboration with the lab of Prof. Werner Held for all the chronic LCMV-related experiments. Dr. Laure Tillé and I were equally involved in the conduction of the project in terms of planning, performing, analyzing experiments, and writing the article.

**I designed, performed, and analyzed the experiments for the following parts of the article:**

Figure 1 e, f, g, h, i, j

Figure 5 a, b, c, f, g

Figure 6 a, b, c, d, g, h, i, j, k, l, m

Extended Data Figure 1 a, c, d, e, f, g, h

Extended Data Figure 2 d

Extended Data Figure 3

Extended Data Figure 5 a, c, d

Extended Data Figure 6 b, c, f

**I analyzed the data for the following parts:**

Figure 4 c

Figure 5 d, e

Extended Data Figure 5 b

Extended Data Figure 6 a

## 3. 1. Manuscript

### NFAT5 induction by the tumor microenvironment enforces CD8 T cell exhaustion

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Author contributions: L.T, D.C, W.H, M.I, G.C and G.V conceived and designed the experiments. L.T, D.C, P.R, M.C, GB, M.M.L and G.V performed the experiments. J.L analyzed the scRNA-seq data and M.A, S.C and I.C performed bioinformatic analysis. C.L.R provided CD4-Cre NFAT5fl/fl mice. L.T, D.C and G.V prepared the figures and wrote the manuscript with input from all authors. D.E.S, W.H, C.L.R, S.C significantly reviewed the manuscript.

Running title: NFAT5 regulates T cell exhaustion in tumors

### 3. 1. 1 Abstract

Persistent exposure to antigen during chronic infection or cancer renders T cells dysfunctional. The molecular mechanisms regulating this state of exhaustion are thought to be common in infection and cancer, despite obvious differences in their microenvironments. We discovered that NFAT5, an NFAT family member lacking an AP-1 docking site, is highly expressed in exhausted T cells from murine and human tumors and is a central player selectively in tumor-induced exhaustion. While NFAT5 overexpression reduced tumor control, NFAT5 deletion improved tumor control by promoting the accumulation of tumor-specific CD8 T cells expressing less TOX and PD-1 and producing more cytokines among precursor exhausted cells. Conversely, NFAT5 had no effect on chronic infection-induced T cell exhaustion. While NFAT5 expression was induced by TCR triggering, its transcriptional activity required hyperosmolarity and was specific to the tumor microenvironment and not chronic infection. NFAT5 thus promotes CD8 T cell exhaustion in a tumor-selective fashion.

### 3. 1. 2 Introduction

CD8 T cells can actively recognize and eliminate tumor cells. However, CD8 tumor-infiltrating lymphocytes (TILs) are dysfunctional or exhausted. Exhausted CD8 T cells responding to chronic infection and cancer show high expression of multiple inhibitory receptors, reduced effector functions and are not able to efficiently control pathogens or tumors [1]. The exhaustion state is strongly related to the constant presence of antigen, resulting in continuous triggering of the TCR [2], but the composition of the local microenvironment further influences the gene expression of exhausted CD8 T cells [3].

Despite the tremendous progress in cancer immunotherapy during the past years [4], a large fraction of patients' cancers remain, or become, therapy resistant. Understanding the molecular mechanisms that regulate T cell exhaustion is a first step towards more efficient treatments. Recent studies highlighted the existence of a precursor exhausted T cell ($T_{pex}$) population with stem cell-like properties, which can further differentiate into terminally exhausted T cells ($T_{ex}$) that have cytolytic potential but are short lived. Several transcription factors (TF) such as TOX and NFAT play central roles in the establishment of T cell exhaustion, while others, such as TCF-1, maintain the stemness properties of $T_{pex}$ [1, 5, 6]. TOX drives the expression of inhibitory receptors and negatively regulates the production of inflammatory cytokines allowing T cell maintenance in the context of chronic antigen stimulation [7, 8]. Importantly, TOX is directly regulated by NFAT1 and NFAT2 [9, 10], which are activated by TCR signaling in calcineurin dependent fashion [11]. NFAT1 and NFAT2 are required for effective CD8 T cell differentiation into cytotoxic T cells by forming dimers with transcriptional partners such as AP-1 [12]. Indeed, the overexpression of a constitutively active version of NFAT1 unable to interact with AP-1 induces an exhausted phenotype in CD8 TILs [13]. As AP-1 expression in chronically stimulated T cells is reduced, exhaustion is partly induced by NFAT activation in the relative absence of AP-1 [13].

A previous transcriptomic analysis showed high expression of the NFAT family member NFAT5 in CD8 T cells from tumor-infiltrated lymph nodes (TILN) [14]. In contrast to the classical NFAT proteins (NFAT1 and NFAT2), NFAT5 lacks an AP-1 docking site and is not regulated by calcineurin [15]. Instead, NFAT5 is triggered by metabolic stress, such as hypertonicity, and regulates the transcription of proteins involved in the maintenance of an adequate osmotic balance in cell type-unspecific manner [16]. However, the activity of NFAT5 varies according to the cell type or the stimulus [17, 18]. Recent studies found that NFAT5

regulates inflammatory responses in macrophages [19] and CD4 T cells [20], but so far there is no report on a function of NFAT5 in peripheral CD8 T cells [17].

We found that NFAT5 was highly expressed in CD8 TILs from murine and human tumors. Overexpression of NFAT5 dampened CD8 T cell responses against tumor cells, while deletion of NFAT5 reduced T cell exhaustion and improved T cell functions and tumor control. Surprisingly, NFAT5 deletion in CD8 T cells during chronic LCMV infection had no effect on T cell exhaustion and virus control, emphasizing a tumor-specific role of NFAT5 in CD8 T cells. TCR triggering was the main inducer of NFAT5 expression and its transcriptional activity depended on hypertonicity, a feature specifically associated with the tumor microenvironment (TME). Therefore, our data identified NFAT5 as a tumor-specific regulator of CD8 T cell exhaustion.

## 3. 1. 3 Results

### 3. 1. 3. 1. NFAT5 is upregulated in tumor-infiltrating CD8 T cells

NFAT5 was previously found highly expressed in Melan-A-specific CD8 T cells obtained from metastasized lymph nodes [14]. To confirm the expression of NFAT5 in CD8 TILs, we took advantage of publicly available single cell RNA-seq (scRNA-seq) data from mouse B16 melanoma [21], mouse MC38 adenocarcinoma [22], human melanoma [23, 24] and human breast cancer [25]. The TILs were classified into naïve like, early activated, effector memory, $T_{pex}$ and $T_{ex}$ CD8 T cell subsets, using ProjecTILs [26]. NFAT5 was highly expressed in $T_{pex}$ and $T_{ex}$ compared to naïve like, early effector and effector memory TILs in all studies (Fig. 1a, Extended Data Fig. 1a). We used the same datasets to identify the most relevant TFs regulating T cell exhaustion by comparing their regulon activity (AUC score) in the different subsets. A regulon represents a gene set regulated by the same TF. The regulon activity of NFAT5 was upregulated in $T_{pex}$ and $T_{ex}$ and NFAT5 was one of the top eight TFs showing statistically significant differences comparing $T_{pex}$ and $T_{ex}$ to the other populations, together with Tbet, Runx2 and Bhlhe40 for upregulated regulons suggesting a regulatory role of NFAT5 in $T_{pex}$ and $T_{ex}$ (Fig. 1b). We further confirmed the upregulation of Nfat5 by quantitative PCR (qPCR) in CD8 T cells sorted from the spleen or tumors of B16 melanoma [27] tumor-bearing mice (Fig. 1c) and in human tumor-bearing mice (Fig. 1c) and in human infiltrating Melan-A-specific CD8 TILN cells, using circulating EBV-specific or naïve circulating CD8 T cells as controls (Fig. 1d).

To follow NFAT5 expression at the single cell level, we generated an NFAT5 reporter mouse strain, in which the stop codon in exon 14 of Nfat5 was replaced by a P2A-mCherry cassette (Extended Data Fig. 1b). Introducing the NFAT5-mCherry cassette had no adverse effect on the viability, breeding capacity, or thymic T cell development (Extended Data Fig. 1c-e). MCherry expression in CD8 T cells correlated with the level of Nfat5 mRNA (Extended Data Fig. 1f). We further confirmed that the introduction of the P2A-mCherry cassette did not alter NFAT5 expression by comparing the NFAT5 protein level in CD8 T cells from WT, NFAT5 KO and NFAT5mCherry mice (Extended Data Fig. 1g, h). Using this model, we showed that polyclonal CD8 TILs from B16 or MC38 tumors expressed significantly higher levels of NFAT5 compared to CD8 T cells from the tumor-draining lymph node (T-DLN) or the non-draining lymph node (N-DLN) on day 16 post tumor implantation (Fig. 1e-f). To define the kinetic of NFAT5 induction in CD8 TILs, we engrafted NFAT5mCherry mice with B16 expressing OVA (Fig. 1g). Seven days post engraftment, endogenous CD8 T cells in the tumor

and the LNs expressed similar levels of mCherry. At this time point (day 0) we transferred activated OT-I NFAT5mCherry CD8 T cells (OT-I cells), which express a TCR recognizing the OVA-derived SIINFEKL epitope, into tumor bearing mice. Three days after transfer, both endogenous and OT-I TILs showed an increased level of mCherry, which remained stable in OT-I cells but dropped in endogenous CD8 TILs at day 11, while tumor growth was transiently controlled, until day 11 (Fig. 1h). Once tumor growth resumed (after day 11), we observed a strong increase of mCherry expression in OT-I cells and to a lesser extent in endogenous CD8 TILs. In the absence of OT-I cell transfer, B16-OVA tumor growth was not controlled. In this situation, the level of mCherry also increased at day three and remained stable at day seven (Fig. 1i). The rapid growth of the tumors did not allow us to measure further time points in un-transferred mice. We followed mCherry expression in $T_{pex}$ and $T_{ex}$ of transferred OT-I cells by discriminating the two populations using SLAMF-6 and TIM-3, respectively [28]. Initially, the levels in $T_{pex}$ and $T_{ex}$ were similar but increased in $T_{pex}$ on day eleven, reaching a maximum on day 16 (Fig. 1j). Altogether, we found enhanced NFAT5 expression in $T_{pex}$ and $T_{ex}$ CD8 TILs both in human and murine tumors and increasing NFAT5 levels during tumor progression.

#### 3. 1. 3. 2. NFAT5 overexpression dampens CD8 T cell-mediated tumor control.

We next tested whether high NFAT5 levels impact the response of CD8 T cells against established tumors. We cloned naturally occurring NFAT5 isoforms that differ in the alternative splicing of the first or the last exons, into GFP-expressing retroviral vectors (Fig. S2a) and transduced TCR P1A-luc+ CD8 T cells (P1A cells), which express luciferase and a TCR recognizing the P1A epitope derived from P511 mastocytoma cells [29, 30]. Adoptive transfer of as few as $10^4$ transduced P1A cells was sufficient to induce regression of P511 mastocytoma in Rag1-/-B10D2 mice [31, 32]. Transfer of P1A cells overexpressing NFAT5 isoform A, which lacks the Nuclear Export Signal (NES) sequence[33] (Fig. 2a-b, Extended Data Fig. 2a), resulted in reduced tumor control compared to control eGFP-P1A cells (Fig. 2c). A similar reduction in tumor control was obtained with NFAT5 isoform D, which contains the NES, but previous studies have shown that the NES influence on the subcellular localization of NFAT5 is neglectable in hypertonic conditions such as the TME. An isoform lacking the DNA binding domain of NFAT5 restored tumor control (Extended Data Fig. 2) indicating that the transcriptional activity of NFAT5 was essential for hindering tumor control. Furthermore, overexpression of NFAT1 CA-RIT, a previously described constitutively active form of NFAT1 unable to bind AP-1, reduced tumor control to a similar extent as NFAT5 (Fig. 2c).

The presence of P1A cells in the tumor based on bioluminescence measurements was comparable throughout the experiment, suggesting that NFAT5 did not impair the infiltration of P1A cells into the tumor (Extended Data Fig. 2). To further characterize their phenotype, we sorted eGFP+ P1A TILs seven days after T cell transfer and performed RNA-seq analysis. Principal component (PC) analysis revealed that NFAT5 and NFAT1 CA-RIT-overexpressing P1A TILs clustered away from control eGFP-transduced P1A TILs (Fig. 2d). Most of the genes differentially expressed relative to control eGFP P1A TILs (n=35), were upregulated in both NFAT5 and NFAT1 CA-RIT-overexpressing CD8 TILs (Fig. 2e). Within the shared genes we found Dusp family phosphatases (2, 5 and 10) as well as Nr4a1 and Nr4a3, which have been associated with the regulation of T cell exhaustion [34]. Gene Ontology (GO) term analysis revealed similar transcriptomes of both NFAT5 and NFAT1 CA-RIT-overexpressing CD8 TILs, namely signatures associated with cell cycling (chromosome condensation, mitotic regulation), inhibition of MAPK signaling and cell differentiation. Altogether, NFAT5 overexpression in tumor-specific CD8 T cells reduced tumor control through the induction of a transcriptional program similar to that induced by an artificial NFAT1 construct that cannot associate with AP-1.

#### 3. 1. 3. 3. NFAT5 deletion in tumor-specific T cells improves tumor control

To assess whether NFAT5 deletion in tumor-specific CD8 T cells influenced the tumor response, we used CD4-Cre NFAT5flox/flox mice. The T cell-specific deletion of NFAT5 in these mice does not impair T cell development or alter the peripheral T cell compartment as already described [35]. We crossed the mice with the P14 TCR transgenic strain, in which all CD8 T cells recognize the LCMV-derived gp33 epitope (P14 cells). We transferred activated P14 CD4-Cre-/- NFAT5flox/flox (WT) or P14 CD4-Cre+/- NFAT5flox/flox (NFAT5 KO) CD8 T cells into mice bearing subcutaneous gp33-expressing B16 melanoma (B16-gp33) (Fig. 3a). Mice transferred with NFAT5 KO P14 cells developed tumors later and these remained smaller compared to mice transferred with WT P14 cells (Fig. 3b). Seven days after T cell transfer, the CD8 TIL compartments contained higher proportions of NFAT5 KO than of WT P14 cells. Strikingly, NFAT5 KO P14 TILs produced more IFN-γ, TNF-α and IL-2 upon ex-vivo stimulation and expressed less PD-1 than WT P14 TILs, while CD44 expression was comparable (Fig. 3c), indicating reduced exhaustion of NFAT5 KO P14 cells. To exclude the possibility that an altered TME contributed to the distinct phenotype, we co-transferred *in vitro* activated WT and NFAT5 KO P14 cells (Extended Data Fig. 3a) at a 1:1 ratio. On the day of

the transfer, both cell types showed comparable expression of CD25, CD44 and PD-1 (Extended Data Fig. 3b). Seven days later, the proportion of NFAT5 KO P14 TILs was increased and these expressed less PD-1 and TIM-3 compared to WT, confirming the results from the individual transfers and suggesting a cell intrinsic effect of NFAT5 KO on T cell exhaustion (Extended Data Fig. 3c). We next addressed the improved presence of NFAT5 KO P14 cells in the tumor. An increased migratory potential was unlikely as the expression of both CXCR3 and CXCR4 was decreased rather than increased on NFAT5 KO P14 cells. We did also not observe a difference in Ki67 staining suggesting no difference in their proliferative capacity. However, NFAT5 KO P14 TILs had a decreased fraction of Annexin V+ or active Caspase 3+ apoptotic cells compared to WT TILs, indicating an improved survival of NFAT5 KO P14 cells in the tumor (Extended data Fig 3d).

To decipher the molecular mechanism for the enhanced tumor control by NFAT5 KO P14 cells, we performed RNA sequencing of sorted P14 TILs seven days after transfer. PC analysis revealed that WT and NFAT5 KO P14 TILs clustered separately (Fig. 3d). We found that 458 genes were significantly upregulated and 833 genes significantly downregulated in NFAT5 KO compared to WT P14 TILs (Extended Data Table 1). By comparing the gene signatures to available scRNA-seq data from CD8 TILs [21-25], we found that the upregulated genes in NFAT5 KO P14 TILs were genes highly expressed in early activated CD8 TILs, while genes downregulated in NFAT5 KO P14 TILs were highly expressed in $T_{ex}$ and $T_{pex}$ (Fig. 3e). NFAT5 KO P14 cells overexpressed the cytotoxic molecule GzmA, activation-associated genes such as Tnfrsf4 (4-1BB), Tnfsfr18 (GITR) and Ccl5, genes expressed by T resident memory cells (Cd69, Cxcr6, Ccr8), memory T cells (Il7r) or associated with T cell differentiation (Rora) (Fig. 3f, Extended Data Table 1). Indeed several genes previously associated with CD8 T cell exhaustion were downregulated in NFAT5 KO P14 cells, including Cd244a (2B4), Entpd1 (CD39), Pdcd1 (PD-1), Ikzf2 (Helios) and Tox (Extended Data Table 1). Given the role of TOX in T cell exhaustion[7-9, 36], we assessed whether a reduced TOX expression accounted for the effect of NFAT5 KO. We compared our signature with the one obtained after Tox inactivation using GSEA [36]. We found that genes downregulated upon NFAT5 KO were significantly enriched among TOX-dependent genes and vice versa (Fig. 3g). To extend our findings to human T cells, we studied the correlation of NFAT5 expression with exhaustion-related genes in human melanoma CD8 TILs using scRNA-seq [37]. We calculated the Pearson correlation coefficients between NFAT5 and all detected genes in activated CD8 TILs expression. Consistently, we found that NFAT5 expression in human CD8 TILs

positively correlated with HAVCR2, PDCD1 and TOX expression. Conversely, NFAT5 expression negatively correlated with IL7R, GZMA and CD69 expression (Fig. 3h). The NFAT5 consensus binding sequence is similar to the one of NFAT1/2 illustrated by the JASPAR database (Extended Data Fig. 4a). Using UCSC Genome Browser, we identified potential NFAT5 binding sites in the promoter regions of Tox, Pdcd1 and Havcr2, i.e. in genes that are differentially expressed in NFAT5 KO P14 cells. Conversely, there was no site in the Tcf7 promoter, which expression was not altered in NFAT5 KO P14 cells (Extended Data Fig. 4b). Altogether, NFAT5 KO P14 TILs expressed less PD-1 and TOX and produced more inflammatory cytokines, resulting in a more efficient tumor control. Furthermore, we found that in both murine and human datasets, NFAT5 plays a key role in the regulation of exhaustion-associated genes.

#### 3. 1. 3. 4. NFAT5 does not alter the CD8 T cell response to chronic LCMV infection

T cell exhaustion is known to develop during chronic infection [38]. We therefore assessed whether NFAT5 regulated the CD8 T cell response to chronic viral infection. We adoptively transferred mice with naïve WT or NFAT5 KO P14 cells one day prior LCMV clone 13 (cl13) infection, which causes chronic infection (Fig. 4a). At day 28 post-infection, we found slightly higher proportions of NFAT5 KO P14 cells. WT and NFAT5 KO P14 cells produced comparable levels of effector cytokines (IFN-γ, TNF-α and IL-2) (Fig. 4c) and slightly overexpressed PD-1 and CD44. Finally, weight loss during infection, which is indicative of immunopathology, was comparable between mice receiving WT and NFAT5 KO P14 cells (Fig. 4b). Altogether, NFAT5 deficiency did not have a significant effect on the CD8 T cell response to chronic LCMV infection, suggesting a tumor-specific role of NFAT5 in T cell exhaustion.

#### 3. 1. 3. 5. NFAT5 is preferentially expressed in $T_{pex}$

To understand why NFAT5 inactivation did not restore CD8 T cell function during chronic infection while CD8 TILs function was strongly improved, we performed a more detailed analysis of NFAT5 expression in exhausted CD8 T cells. We used publicly available scRNA-seq data from murine CD8 T cells responding LCMV clone 13 infection to determine their Nfat5 expression[39]. We found that NFAT5 levels were higher in $T_{pex}$ compared to $T_{ex}$ and the other subtypes (Extended Data Figure 4c). Then, we compared our RNA-seq data from WT

vs NFAT5 KO P14 TILs to the same data set. We found that genes upregulated in NFAT5 KO P14 TILs were genes expressed in $T_{pex}$, $T_{ex}$ or Short-Lived Effector CD8 (SLEC) from P14 T cells in chronic LCMV infection (Extended Data Fig. 4d) suggesting a different role of NFAT5 during chronic infection than in the context of cancer. To directly compare NFAT5 levels in TILs and chronic infection, we took advantage of our NFAT5mCherry reporter mouse strain crossed to the P14 TCR transgenic mice (P14-NFAT5mCherry). Naive P14-NFAT5mCherry T cells were transferred into WT mice one day prior infection with LCMV clone 13 (LCMV cl13) or one day prior B16-gp33 melanoma inoculation, followed by vaccination with gp33 peptide + CpG seven days later (vacc.). Alternatively, P14-NFAT5mCherry T cells were preactivated *in vitro* and transferred into B16-gp33 melanoma-bearing mice (pre-act.). We then analyzed expression NFAT5 in 7, 14 and 21 days after antigen stimulation in the host. We observed that mCherry was upregulated within the TME at day seven (pre-act.) and day 14, while it remained expressed at low levels in chronic infection (Fig. 5a). At day 21, mCherry levels were further increased in the TME and also slightly elevated in chronic infection. In all conditions, $T_{pex}$ expressed more mCherry than $T_{ex}$ cells. Remarkably, mCherry in $T_{pex}$ from chronic infection was even higher than in the tumor models at days 14 and 21 (Fig. 5b). We did not measure significant mCherry expression in $T_{ex}$ from chronic infection or in P14 cells after acute infection with LCMV Armstrong (Extended Data Fig. 5a). In conclusion, NFAT5 was highly expressed in $T_{pex}$ cells in both tumors and chronic infection, while the expression in $T_{ex}$ cells was restricted to the TME, whereby levels increased as the diseases progressed.

#### 3. 1. 3. 6. NFAT5 inactivation strongly impacts $T_{pex}$ CD8 TILs

Considering the differential NFAT5 expression in $T_{pex}$ and $T_{ex}$ associated with chronic infection or tumors, we analyzed if NFAT5 KO had a different effect on these populations. In chronic infection, we did not observe significant changes in the proportions or absolute numbers of $T_{pex}$ and $T_{ex}$ (Fig. 5d). IFN-γ production, as well as the level of PD-1, were also similar (Fig. 5e). In contrast, we observed decreased proportion (but stable number) of NFAT5 KO $T_{pex}$ compared to WT, while both the proportion and number of NFAT5 KO $T_{ex}$ were increased seven days after T cell transfer in tumor bearing mice (Fig. 5f). Furthermore, NFAT5 KO $T_{pex}$ expressed less PD-1 and TOX but more IFN-γ as compared to WT, while NFAT5 KO $T_{ex}$ were less impacted by the NFAT5 deletion (Fig. 5g). Comparable results were obtained in a co-transfer setting (Extended Data Fig. 5). Thus, NFAT5 KO $T_{pex}$ displayed a less exhausted

phenotype and with more cytotoxic $T_{ex}$, resulting in an increased presence of tumor-specific CD8 T cells and improved tumor control.

#### 3. 1. 3. 7. NFAT5 expression is driven by TCR signaling in CD8 TILs

To explore the regulation of NFAT5 expression in the TME, we tested various stimuli known to be associated with the TME or described to regulate NFAT5 expression and/or activity. We determined NFAT5 expression in P14-NFAT5mCherry splenocytes cultured for 72h *in vitro*. TCR stimulation with gp33 peptide or anti-CD3 and anti-CD28 antibodies drastically increased mCherry levels. We further tested hyperosmotic and/or hypoxic conditions. The addition of NaCl or KCl to the cell culture medium lead to a dose-dependent (380mOsm vs 420mOsm) increase of mCherry levels. Strikingly, combining TCR triggering and hypertonic medium (with NaCl or KCl) led to maximal mCherry expression. On the other hand, hypoxic conditions (0,5% O2) did not induce mCherry expression alone or in combination with TCR triggering or high osmolarity (Fig. 6a).

To assess the importance of TCR triggering in NFAT5 induction in the TME, we co-transferred P14- and OT-I-NFAT5mCherry CD8 T cells into mice bearing a B16-gp33 and a B16-OVA tumor on opposite flanks (Fig. 6b). We observed strict a TCR dependent induction of mCherry expression in the two TME's. PD-1 upregulation followed the same pattern (Fig. 6c-d). Interestingly, OT-I T cells showed stronger induction of mCherry than P14 T cells. Thus NFAT upregulation correlated with the affinity of the TCR for its ligand (Kd OT-I/SIINFEKL = 5.9 µM [40] versus P14/gp33 = 3.5 µM [41]) (Fig. 6c, d). Therefore, TCR stimulation in the TME was necessary to induce NFAT5 expression. Since TCR stimulation leads to Ca2+/calcineurin-induced NFAT1 or NFAT2 activation [13], we wondered whether NFAT5 expression depended on the TCR-Ca2+/calcineurin-NFAT axis. The calcineurin inhibitor FK506 is widely used to block calcineurin targets such as NFAT1 and NFAT2. As in previous experiments, we transferred pre-activated P14-NFAT5mCherry into B16-gp33-bearing mice. Mice were treated with FK506 for either day 1 to day 3 (first phase) or day 4 to day 6 (second phase) post T cell transfer (Fig. 6e). The inhibition of calcineurin by FK506 partially decreased mCherry expression when mice were treated during the first phase, while it had no effect during the second phase (Fig. 6f). Calcineurin inhibition did not decrease NFAT5 expression in the T-DLN and N-DLN, showing a TME-specific effect (Extended Data Fig. 6a). Since NFAT5 expression is not dependent on Calcineurin[15] the data suggest that NFAT5 expression

depends in part on NFAT1/2, but other TCR induced signals or TME related signals are as well important in this regulation especially at later time points. To explore the contribution of NFAT1/2 versus NFAT5 in the regulation of exhaustion-associated genes, we similarly treated mice which were co-transferred NFAT5 KO or WT P14 cells. Mice were treated with FK506 from day 1 to day 3 or day 6 to day 9 post-adoptive cell transfer, and the expression of PD-1, TOX and TIM-3 were analyzed at day four and ten, respectively (Fig. 6g). FK506 treatment during the first phase, resulted in a 30 to 40% downregulation of PD-1, TOX and TIM-3 in WT P14 TILs. FK506 treatment during the second phase did not alter PD-1 expression, but reduced TIM-3 and TOX expression in WT P14 TILs. To address whether NFAT5 complemented NFAT1/2 in this process, we compared the effect of FK506 on NFAT5 KO P14 TILs. Early treatment reduced PD-1 expression in NFAT5 KO similarly to WT P14 TILs. Though TOX and TIM-3 were downregulated up to 20% more compared to WT P14 TILs.

After late treatment, NFAT5 KO P14 T cells showed stronger downregulation than WT P14 T cells for PD-1, TIM-3 and TOX. Thus, NFAT5-regulates TOX and TIM-3 already during the early phase but impacts PD-1 expression at later time points (Fig. 6h). The data suggest that NFAT5 is not sufficient to induce an exhausted phenotype but exacerbates NFAT1/2 induced exhaustion specifically within the TME.

It was previously established that the concentration of K+, but not Na+, is increased in the TME of melanoma compared to the serum or healthy tissue from both mouse and human [42]. We next addressed whether the ionic imbalance induced by K+ in the TME participated in the transcriptional regulation of NFAT5 as observed *in vitro*. We overexpressed KCNA3, a potassium channel enabling T cells to equalize intracellular potassium concentration, in activated P14-NFAT5mCherry CD8 T cells and transferred them into B16-gp33 tumor-bearing mice (Extended Data Fig. 4b). As a control we expressed a non-conducting KCNA3 mutant (KCNA3 mutant). At day seven post transfer, KCNA3-overexpressing P14 TILs showed a trend for decreased mCherry expression compared to the KCNA3 mutant control (Extended Data Fig. 4c).

#### 3. 1. 3. 8. NFAT5 transcriptional activity is specific to the TME

In addition to the level of expression, the capacity of NFAT5 to act as a TF is subject to further regulation [16, 43]. To test how different stimuli impacted the DNA binding capacity of NFAT5, we cloned two copies of a human tonicity-responsive enhancer (TonE) into a luciferase-expressing lentiviral vector and transduced Jurkat cells (Extended Data Fig. 6d). Culture of Jurkat cells, stably expressing the NFAT5 reporter construct, in the presence of NaCl or KCl induced luciferase activity in a dose-dependent manner. In contrast, TCR triggering, hypoxia, ROS inducers such as Butyl-Hydroperoxide or cytokine stimulation with TGF-β did not upregulate NFAT5 activity, suggesting a unique role for hypertonic stress to induce NFAT5 activity (Fig. 6i). To test whether NFAT5 activity is different between the TME and chronic infection *in vivo*, we generated another lentiviral reporter vector containing six mouse TonE repeated sequences upstream of mCherry expression (Extended Data Fig. 6e). We validated this construct *in vitro* using the T cell line PC60, and primary CD8 T cells to confirm the specificity for NFAT5. The expression of mCherry was increased when NaCl was added to the medium but was unchanged in the presence of Ionomycin which induces NFAT1/2 activation or FK506, which inhibits Calcineurin (Fig. 6j-k). Most importantly, mCherry was not induced in NFAT5 KO CD8 T cells (Fig. 6k). Finally, we co-transferred WT and NFAT5 KO P14 T cells expressing the NFAT5 activity reporter into B16-gp33 bearing mice. Seven days later, WT P14 TILs showed an increased activity of NFAT5 in the TME than in the T-DLN. NFAT5 activity was reduced in NFAT5 KO P14 T cells, demonstrating the specificity of the reporter *in vivo* and restricted NFAT5 activity in the TME. Further, we transferred transduced pre-activated WT P14 T cells into tumor bearing mice and mice which were pre-infected with LCMV clone 13. We found that NFAT5 was already active within the TME at day four, to a similar extend as observed in the co-transfer experiment on day seven. In contrast, NFAT5 activity was not observed in P14 T cells at day 14 or day 21 of chronic LCMV infection (Figure 6l, m).

Altogether, while TCR triggering was essential to drive NFAT5 expression *in vivo*, NFAT5 activity was only detected in the TME but not in chronic infection. Based on our *in vitro* analyses, the latter is likely explained by the osmolar changes specific to the TME.

### 3. 1. 4 Discussion

We showed that NFAT5, an unconventional member of the NFAT family, plays a crucial role in the regulation of tumor-induced T cell exhaustion. NFAT5 was expressed in CD8 TILs in various cancers (melanoma, adenocarcinoma, breast cancer) and in different species (mouse and human). In our study, overexpression of NFAT5 in tumor-specific CD8 T cells limited their anti-tumor response, while its inactivation strongly increased tumor control. Overexpression of different isoforms of NFAT5 containing or not the NES led to a similar effect, i.e. a reduced response toward the tumor. The nucleocytoplasmic trafficking of NFAT5 was originally characterized by the group of Pr. Ko[33]. An apparent lack of effect of the NES could be explained by its limited influence on NFAT5 subcellular localization in isotonic or mildly hypertonic conditions. NFAT5 has two export signals (NES and AED), and they have been found to be most effective in hypotonic conditions (250 mOsm/kg), not as much in isotonic medium (300 mOsm/kg). Since the TME is hypertonic, the NES will not be effective, explaining why there is no difference between the overexpression of isoform A and D. Furthermore, using this transfer model we have previously shown that CD8 T cells overexpressing exhaustion-associated genes are competed out, leading to a rapid enrichment of GFP- P1A cells that do not express the gene of interest [31, 32]. Therefore, even the slight, but significant, delay in tumor control was a good indicator of NFAT5-mediated impairment of CD8 TIL anti-tumor functions.

NFAT5 deletion had a different effect depending on the subtype of CD8 TILs. NFAT5 KO $T_{pex}$ showed increased production of cytokines and decreased levels of PD-1 and TOX, while their absolute number remained constant. In contrast, we found higher frequencies and absolute numbers of $T_{ex}$, but the effect of NFAT5 deletion on the function of these cells was limited. These effects correlated with the NFAT5 expression levels in $T_{pex}$ and $T_{ex}$ P14 TILs, with significantly higher NFAT5 expression in $T_{pex}$ compared to $T_{ex}$. RNA-seq analysis confirmed that NFAT5 KO P14 TILs expressed genes associated with early activation, with a signature that correlated with the one measured in TOX-KO CD8 TILs. We observed that the overexpression of NFAT5, or of a mutated form of NFAT1 unable to cooperate with AP-1 and involved in the regulation of exhaustion, has similar effects on the behavior and transcriptional program of tumor-specific T cells [13]. Interestingly, the Rel-like domain of NFAT5 binds the consensus DNA sequence for NFAT5 as a dimer, however, only one of the monomers does base-specific contacts while the other binds the backbone of the DNA in a base-nonspecific manner [44]. In contrast to NF-κB, NFAT5 does not recognize palindromic/symmetric DNA

sequences. This is supported by NFAT5 ChIP-seq experiments that identified the normal NFAT binding site in the fragments directly bound by NFAT5 but never in palindromic sites as for example the Ciita enhancer described by Buxadé et al.[45]. As the binding motif between NFAT1/2 and NFAT5 show strong similarities and that NFAT5 was shown to bind a NFAT1/2 reporter in mouse[46], NFAT5 could complement NFAT1/2 activity on enhancer/promoter regions from exhaustion related genes. Since TOX is a direct target of NFAT1 and 2 [9, 36], our data suggests that the phenotype observed by NFAT5 inactivation is mediated via the reduced level of TOX in $T_{pex}$. The TME inhibits NFAT activation through glucose deprivation or accumulation of lactic acid [47, 48]. This goes in parallel with an increased osmotic stress related to dead cell accumulation [42, 49]. We hypothesize that, at this stage, NFAT5 complements classical NFAT to enforce an NFAT-induced transcriptional program and thus stabilizes the expression of exhaustion-associated genes.

NFAT5 is primarily described to regulate osmolarity-regulated genes, which were not differentially expressed in our experiments. However, NFAT5 activity is not limited to the regulation of this panel of genes. In macrophages, expression of NFAT5 drives a pro-inflammatory phenotype, which further supports T cell-mediated tumor control [19]. Interestingly, while NFAT5 drives the expression of tonicity-responsive genes in macrophages cultured in a hypertonic environment, it drives IL-6 production when stimulated with LPS [50]. Therefore, NFAT5 regulates gene expression in a context- and cell type-dependent manner. Similarly, while NFAT5 drives the expression of tonicity-responsive genes in T cells cultured in a hypertonic environment [51], it was shown to negatively regulate IFN-γ production in CD4 T cells *in vitro* [20].

We demonstrated that the main inducer of expression of NFAT5 in T cells *in vivo* is TCR stimulation, at least in part through calcineurin activation, suggesting a regulation by classical members of the NFAT family. This regulation was more important during the first phase post T cell transfer (until day 3), while in a later phase (until day 6), calcineurin inhibition had a reduced effect on NFAT5 expression. A similar effect was observed for PD-1 expression, while the KO of NFAT5 increased the downregulation of TOX and TIM-3 observed after calcineurin inhibition. This observation enforces the role of NFAT5 in complementing NFAT1/2 activity.

Interestingly, the inactivation of NFAT5 in CD8 T cells in chronic infection with LCMV clone 13 did not improve viral control nor restore CD8 T cell functions. This suggests a tumor-specific role of NFAT5. Although reduced expression of NFAT5 in total virus-specific CD8 T

cells compared to CD8 TILs may account for part of this effect, the major difference between the two pathologies resides in the activity of NFAT5, that we could only detect in CD8 TILs but not in CD8 T cells from chronically infected mice. The massive death of tumor cells results in hypertonicity in the TME [42]. We showed that this factor slightly affected the regulation of NFAT5 expression within the tumor, but its main effect could be related to the activity of NFAT5 rather than its transcriptional regulation. Our study is in line with previous observations that increased osmolarity dampens T cell effector functions, unraveling another mechanism in place within the TME [42, 52].

Altogether, we uncovered a new central player in the regulation of T cell exhaustion, acting only within tumors, but not during chronic infection. This discovery is particularly important in the frame of adoptive cell therapy (ACT) where a patients' T cells are expanded before being transferred back into the patient. Acting on NFAT5, either genetically or using specific inhibitors, would favor a stronger T cell response against cancer, without decreasing the stemness capacity of transferred T cells.

### 3. 1. 5 Figure legends

**Fig. 1: NFAT5 is upregulated in tumor-infiltrating CD8 T cells.** a) NFAT5 mRNA expression levels in each indicated CD8 TIL subtype, as classified by ProjecTILs, across five different mouse and patient cohorts/studies (CF Material and methods). b) Heatmap showing the activity (AUC score) of the top 8 TFs with the greatest difference in regulon activity (either up or down regulation) when comparing terminal exhausted CD8 T cells ($T_{ex}$) and naïve-like CD8 T cells (Naive like) from tumor-infiltrating T lymphocytes (TILs dataset). c) Nfat5 mRNA levels from CD8 T cells homing B16-gp33 tumors or spleens. Ten mice pooled from two independent experiments. d) NFAT5 mRNA level in human naïve CD8 T cells, EBV (BMLF1280-288) tetramer+ and Melan-A26-35 (ELA) tetramer+ CD8 T cells, the latter from tumor-infiltrated lymph nodes (TILN). e) mCherry levels in non-draining lymph node (N-DLN), tumor-draining lymph node (T-DLN) and B16-gp33 tumors from NFAT5mCherry+/+ or NFAT5mCherry-/- CD8 T cells on day 16 post tumor injection. Four mice from one representative experiment out of two. f) mCherry levels in N-DLN, T-DLN and MC38 tumors from NFAT5mCherry+/+ or NFAT5mCherry-/- CD8 T cells on day 16 post tumor injection. Nine mice pooled from two independent experiments. g) Tumor growth measured in NFAT5mCherry+/+ mice transferred with OT-I-NFAT5mCherry+/+ CD8 T cells (day 7 post tumor injection) or no T cell transfer (w/o transfer). h) mCherry expression of OT-I-NFAT5mCherry+/+ (left) or endogenous- NFAT5mCherry+/+ (right) CD8 T cells from the N-DLN, T-DLN and tumor. Statistical comparison between CD8 T cells from the T-DLN and tumor. i) mCherry expression of NFAT5mCherry+/+ mice not receiving T cell transfer, tumor, T-DLN and N-DLN CD8 T cells. j) GMFI of mCherry of $T_{pex}$ and $T_{ex}$ at respective time points post-T cell transfer (left). Representative histograms of mCherry expression by OT1-NFAT5mCherry $T_{pex}$ and $T_{ex}$ at respective time points post-T cell transfer (right). g-j) Two pooled independent experiments with nine mice per condition. c) Paired student t-test. d) Mann-Whitney test. Mean. e-j) Two-way ANOVA. Mean with SD

**Fig. 2: NFAT5 overexpression dampens CD8 T cell tumor control.** a) Nfat5 mRNA levels in CD8 T cells transduced with a vector encoding for NFAT5 isoform A (NFAT5 A) (left) or control eGFP (right). b) Timeline of the experiment. Activated TCRP1A CD8 T cells were transduced and transferred into Rag1-/-B10D2 mice seven days post P511 tumor engraftment. CD8 T cells were sorted for RNA-sequencing on day 14. c) Tumor growth in mice transferred with TCRP1A CD8 T cells transduced with control eGFP, NFAT5 A, or NFAT1 CA-RIT. d) PC analysis of CD8 TILs transduced with control eGFP, NFAT5 A, or NFAT1 CA-RIT. e)

Venn diagram showing the number of genes upregulated in NFAT5 A transduced CD8 TILs, NFAT1 CA-RIT transduced TILs, or both. f) GO terms enriched in CD8 TILs with NFAT5 A (left), or NFAT1 CA-RIT (right). c) Two-way ANOVA. Error bars represent SEM. One representative experiment out of two with five mice per group.

**Fig. 3: NFAT5 deletion in tumor-specific T cells improves tumor control.** a) Timeline of the experiment: activated WT or NFAT5 KO P14 CD8 T cells were transferred into B16-gp33-bearing CD45.1.2 mice. CD8 TILs were analyzed seven days after transfer. b) Tumor growth of mice transferred with WT (grey) or NFAT5 KO (red) P14 CD8 T cells. In black is the control group without T cell transfer. c) WT or NFAT5 KO P14 CD8 TILs were analyzed by flow cytometry seven days after transfer. Bars represent the geometric mean. d) PC analysis of WT and NFAT5 KO P14 CD8 TILs. e) Violin plots showing the signatures (AUC) made of up-/down- regulated genes from NFAT5 KO CD8 TILs across tumor-infiltrating CD8 T cell subpopulations from a publicly available scRNA-seq dataset, including precursor exhausted ($T_{pex}$) and terminal exhausted ($T_{ex}$) cells. Dots on the plot represent the value of the signatures for each individual cell whereas the violins visualize the distribution of these values within each subpopulation. f) Heatmap displaying 1294 genes differentially expressed in WT versus NFAT5 KO P14 CD8 TILs, fold change of 1.5; adjusted p value <0.05). g) Genes differentially expressed in TOX KO CD8 T cells were compared to genes deferentially expressed in NFAT5 KO CD8 T cells using the GSEA analysis. h) Analysis of scRNA-seq data of human melanoma tumors: Pearson correlation coefficients were calculated between NFAT5 mRNA levels and mRNA levels of indicated genes. a-c) Seven mice per condition from one representative experiment out of three. Mann-Whitney test.

**Fig. 4: NFAT5 inactivation has no effect on CD8 T cell activity during chronic LCMV infection.** a) Timeline of the experiment. WT or NFAT5 KO P14 CD8 T cells were transferred into Vβ5 mice one day prior LCMV clone 13 infection. b) The body weight of infected mice was monitored over time and normalized to day 0. c) Flow cytometry analysis was performed 28 days after infection. Two pooled experiments. Mann-Whitney test.

**Fig. 5: NFAT5 inactivation strongly impacts $T_{pex}$ CD8 TILs.** a) mCherry levels of P14-NFAT5mCherry CD8 T cells from spleen or tumor at indicated conditions and time points. b) Paired mCherry GFMI of $T_{pex}$ and $T_{ex}$ at indicated conditions and time points. c) Representative histograms of mCherry expression by $T_{pex}$ or $T_{ex}$ P14-NFAT5mCherry compared to endogenous WT (dotted grey line) CD8 T cells at given time points. One representative

experiment out of two. d) Contour plots of Slamf6 and Tim-3 expression in WT (left) and NFAT5 KO (right) with the respective histograms comparing to endogenous CD8 T cells on day 28 post LCMV clone 13 (cl13) infection. Frequencies and numbers per spleen of $T_{pex}$ and $T_{ex}$ in NFAT5 KO and WT recipients are shown below. e) Immunophenotyping of $T_{pex}$ and $T_{ex}$ by their PD-1 expression and IFN-γ production. d-e) One representative experiment out of three. f) Contour plots of Slamf6 and Tim-3 expression in WT (left) and NFAT5 KO (right) with the respective histograms comparing to endogenous CD8 T cells on day 14 post tumor injection (day seven post T cell transfer). Frequencies and numbers per mm3 (tumor mass) of $T_{pex}$ and $T_{ex}$ in NFAT5 KO and WT recipients (below). g) Immunophenotyping of $T_{pex}$ and $T_{ex}$ by their PD-1 expression and IFN-γ production. Two independent experiments pooled out of three. a, b, d-g) Mann-Whitney test. c) Two ways ANOVA. Bars representing geometric mean.

**Fig. 6: NFAT5 expression is driven by TCR signaling in CD8 TILs and its activity is specific to the TME.** a) mCherry levels in NFAT5mCherry splenocytes ex-vivo, after three days in culture in the presence of IL-2 (naïve) or in combination with the indicated stimuli. b) Timeline of the experiment. Activated OT-I and P14 CD8 T cells were transferred into B16-OVA and B16-gp33-bearing mice. Flow cytometry analysis seven days after T cell transfer. c) Paired comparison of mCherry and PD-1 expression plotted as GMFI of indicated CD8 TILs within B16-OVA tumors. d) Paired comparison of mCherry expression plotted as GMFI of indicated CD8 TILs within B16-gp33 tumors. e) Timeline of the experiment. B16-gp33-bearing mice were treated with FK506 either the first three days after T cell transfer or 4-6 days after T cell transfer. CD8 TILs were analyzed on day four and day seven post T cell transfer. f) Fold change of mCherry between FK506 and DMSO-receiving mice at respective time points. Dotted line represents fold change equal to one. Two independent experiments pooled. Geometric mean with error bars representing SD. g) Timeline of the experiment. B16-gp33-bearing mice were treated with FK506 either the first three days or on days after co-transfer of WT and NFAT5 KO P14 T cells. CD8 TILs were analyzed on day 4 and day 10 post T cell transfer. h) Fold change of PD-1, TOX, Tim-3 and Tcf-1 expression between FK506- and DMSO treated mice at respective time points. Dotted line represents fold change equal to one. Two independent experiments pooled. Geometric mean with error bars representing SD. i) Luciferase activity measured from Jurkat-TonE reporter cells cultured for 24h with the indicated stimuli. j) PC60 cells were transduced with the TonE-mCherry construct and treated for 48h with the indicated stimuli. GMFI of mCherry of one out of three independent experiments. k) Primary NFAT5 KO or WT CD8 cells were transduced with the TonE-

mCherry construct and treated for 48h with the indicated stimuli. GMFI of mCherry of one out of three independent experiments. l) Frequency of mCherry+ $T_{pex}$ and $T_{ex}$ at indicated conditions and time points (left). Representative contour plots of mCherry expression of $T_{pex}$ and $T_{ex}$ at indicated conditions and time points (right). a-d, g) One representative experiment out of two. c-d) Paired student t-test. f) Mann-Whitney test.

### 3. 1. 6 Extended Data

**Extended Data Fig. 1:**

a) Fold change of Nfat5 expression level of $T_{pex}$ or $T_{ex}$ over indicated populations (left). Mean Nfat5 expression level of various CD8 T cell populations in indicated studies assessed by TILatlas (right) b) NFAT5 reporter mouse strain: The TAG stop codon in exon 14 of the mouse Nfat5 gene was replaced by CRISPR/Cas-mediated genome engineering with the P2A-mCherry cassette to create a knock-in NFAT5-P2A-mCherry reporter model in C57BL/6 mice. c-e) Spleen (c), lymph node (d) and thymus (e) from NFAT5mCherry-/-, NFAT5mCherry+/- and NFAT5mCherry+/+ mice were collected and analyzed for their immune compartment and thymic development (e) by flow cytometry. f) Relative expression (2-deltaCq) of mCherry and Nfat5 assessed by RT-PCR and normalized to β-2-microglobulin of NFAT5mCherry+/- or NFAT5mCherry+/+ CD8 T cells cultured for 72h under different hyperosmolar conditions ranging from 300mOsm/kg to 500mOsm/kg. g) Histogram showing mCherry expression of NFAT5mCherry+/+ CD8 T cells from the lymph node in comparison to a littermate control mouse (NFAT5mCherry-/-). h) Western blot showing the protein level of NFAT5flox/flox CD4-Cre-/- (WT), CD4-Cre+/- NFAT5flox/flox (NFAT5 KO), NFAT5mCherry +/- and NFAT5mCherry +/+ P14 CD8 T cells cultured in complete RPMI with 1uM gp33 peptide and 20U/ml rhIL-2 at 420mOsm/kg for 72 hours. β-actin serves as a housekeeping gene.

**Extended Data Fig. 2:**

a) The four murine isoforms of NFAT5 were aligned on the software Geneious using the entries from Genebank and Ensmbl. Domains described by Cheung et al. were aligned against mouse isoforms 203, whose length corresponds to human isoform C. NES: nuclear export signal, AD1: activation domain 1, AES: auxiliary export signal, DRL: DNA recognition loop, DD: dimerization domain, RHD: rel homology region, AD2: activation domain 2, AD3: activation domain 3. b) Gating for the sorting of NFAT5 A, NFAT1 CA-RIT or control eGFP transduced CD8 T cells. c) Individual tumor growth per mouse per group as described in Figure 2. d) Tumor growth comparing NFAT5 isoform A lacking the DNA binding domain (DBD) (left

panel) and NFAT5 isoform D (right panel) to the one of NFAT5 isoform A. e) Bioluminescence of the luciferase expressing TCRP1A CD8 T cells after injection of the mice with luciferin.

**Extended Data Fig. 3:**

a) Timeline of the experiment: activated WT (CD45.1) and NFAT5 KO (CD45.2) P14 CD8 T cells were transferred into B16-gp33-bearing CD45.1.2 mice. CD8 TILs were analyzed seven days after transfer. b) Overlay of the MFI of indicated proteins of WT and NFAT5 KO on the day of 1:1 transfer. c, d) Representative overlaid histograms of WT, NFAT5 KO P14 and endogenous CD8 TILs of indicated proteins (up) with paired comparison (below).

**Extended Data Fig. 4:**

a) Binding motif of NFAT5, NFAT1 and NFAT2 from the JASPAR database. b) Potential binding sites for the 3 TFs in the region located 3 to 5Kb before the transcription start site of the genes encoding for PD-1, TOX and TIM-3 but not for Tcf7, encoding TCF-1. c) Violin plot showing the Nfat5 expression level (number above) in LCMV infection (left) and fold change of Nfat5 expression level of $T_{pex}$ or $T_{ex}$ over indicated populations (right). d) Violin plots showing the signatures (AUC) made of up-/down-regulated genes from NFAT5 KO CD8 TILs across LCMV clone 13-specific CD8 T cell subpopulations from a publicly available scRNA-seq dataset, including precursor exhausted ($T_{pex}$) and terminal exhausted ($T_{ex}$) cells. Dots on the plot represent the value of the signatures for each individual cell whereas the violins visualize the distribution of these values within each subpopulation.

**Extended Data Fig. 5:**

a) mCherry levels of P14-NFAT5mCherry CD8 T cells from spleen or tumor at indicated conditions and time points. b) Representative histograms of mCherry expression of P14 CD8 T cells from acute or chronic (clone 13) LCMV infected mice at day seven or day 28 post infection. c) Frequency of WT and NFAT5 KO P14 among transferred cells and $T_{pex}$ and $T_{ex}$ among WT and NFAT5 KO P14 from the tumor seven days after transfer. d) GMFI of indicated protein expression of $T_{pex}$ and $T_{ex}$ among WT and NFAT5 KO P14 from the tumor seven days after transfer. Two independent experiments pooled. Paired students t-test.

**Extended Data Fig. 6:**

a) Fold change of mCherry calculated by DMSO receivers over FK506 receivers during indicated time periods in the T-DLN and N-DLN. b) Timeline of the experiment. B16-gp33 bearing mice were transferred with either KCNA3 or KCNA3 mutant overexpressing P14-NFAT5mCherry+/+ CD8 T cells ten days after tumor injection. c) mCherry expression plotted as fold change of P14 CD8 TILs over P14 CD8 T cells from N-DLN. d) Schematic representation of the luciferase NFAT5 activity reporter plasmid. Blue sequence represents the NFAT5 binding site (TonE). e) Schematic representation of the mCherry NFAT5 activity reporter plasmid. Blue sequence represents the NFAT5 binding site (TonE). f) Frequencies of mCherry+ WT and NFAT5 KO P14 CD8 T cells from the T-DLN or the tumor.

## 3. 1. 7 Materials and methods

### Patient material

To assess NFAT5 expression in human TILs by qPCR, we used amplified cDNA from Melan-A-specific CD8 TILs, EBV-specific and naïve (CD8+CD45+CCR7+CD27+CD28+) CD8 T cells isolated from healthy donor PBMCs, patient PBMCs and metastatic lymph nodes from stage III/IV metastatic melanoma patients (clinical study NCT00112229 14).

### Animals

CD45.1, CD45.1.2 and Rag1-/-B10D2 TCRP1A mice were bred in house. CD45.2 CD4-Cre NFAT5flox/flox mice were kindly provided by Prof. Cristina López-Rodríguez [51]. NFAT5-mCherry reporter mice were generated by Cyagen. The TAG stop codon in exon 14 of the mouse Nfat5 gene was replaced by CRISPR/Cas-mediated genome engineering with the P2A-mCherry cassette on a C57BL/6 background. CD45.2 CD4-Cre NFAT5flox/flox mice and NFAT5-mCherry reporter mice were crossed with P14 or OT-I TCR transgenic mice and kept on a C57BL/6 background. Mice were kept in an SPF animal facility. Experiments were approved by the veterinarian authorities and performed in compliance with the University of Lausanne internal regulations (authorization VD2943, VD3592). Tumor volume was calculated with the following formula: volume [mm3] = length [mm] x width [mm] x height [mm].

### Cell lines

Complete medium was composed of 10% heat-inactivated FBS (Gibco), penicillin/streptomycin 100 U/ml (Gibco), Hepes 10mM (Gibco), 1mM sodium pyruvate (Gibco) and 50µM 2-mercaptoethanol (Gibco). B16-gp33 [27] and B16-OVA were cultured in complete DMEM GlutaMAXTM-I with 100µg/ml G418 (Calbiochem). P511 [30], Jurkat and primary T cells were cultured in complete RPMI 1640 GlutaMAXTM-I. PlatinumE (PlatE) cells were cultured in complete DMEM with 10ug/ml Blasticidin (Invivogen) and 1ug/ml Puromycin (Invivogen). PC60 cells [53] were in RPMI 1640 GlutaMAXTM-I medium substituted with 10% heat-inactivated FBS (Gibco) and penicillin/streptomycin 100 U/ml (Gibco).

**Flow cytometry**

The following protocol was used for all tumor experiments: after the Fc receptor of the cells was blocked with anti-mouse CD16/32 (Biolegend, 101320), extracellular staining was performed in FACS buffer for 30 minutes. Dead cells were stained with Aqua Vivid (Invitrogen, L34966) in PBS for 15-20 minutes or by adding DAPI (Thermo Fischer Scientific, D3571) directly before flow cytometry analysis. After 20 minutes of fixation, intracellular staining was performed for 30 minutes. The Biolegend intracellular staining kit (421002) was used for cytokines and the FoxP3 staining kit (00-5523-00) was used for transcription factor staining. When cytokine levels were assessed, the cells were stimulated with their cognate peptide gp33 (10-6 M) and Golgistop (BD, 554724) for 5 hours. To detect active Caspase 3, cells were cultured for 4h at 37°C without growth factors, stained extracellular followed by intracellular staining with primary anti-Caspase 3 antibody for 1h at 4°C. The secondary antibody was added after washing and incubated for 15min. For Annexin V staining, cells were stained on the surface, followed by staining with Annexin V-APC Apoptosis Detection Kit (Biolegend), following the manufacturers protocol. A complete list of used antibodies can be found in in Extended Data Table 2.

**RNA extraction for sequencing / RT-qPCR**

Cells were centrifuged for 5 minutes at maximum speed and RNA was extracted with the RNeasy Plus Micro Kit (Qiagen) following the manufacturer's recommendations. For RNA sequencing, RNA quality was measured with a fragment analyzer. Reverse transcription was achieved using the High-capacity cDNA Reverse Transcription kit (Applied biosystems). For qPCR, KAPA SYBR® Fast qPCR Master Mix (2x) Kit (Sigma), was used. PCR amplification was performed in a 48 well plate (Illumina) on an Eco machine (Illumina). Primer pairs: β2M-F: AGACTGATACATACGCCTGCAG, β2M-R GCAGGTTCAAATGAATCTTCAG, Murine NFAT5-F: GGTACAGCCTGAAACCCAAC, Murine NFAT5-R TGCAACACCACTGGTTCATT, Human NFAT5-F: ATT GCA AAA CCA AGG GAA CA, Human NFAT5-R: TTG GAA TCA GGA TTT TCT TCG, mCherry-F: CCC ACA ACG AGG ACT ACA CC, mCherry-R: TTG TAC AGC TCG TCC ATG CC.

**Vectors**

NFAT1 CA-RIT (IRES-GFP) retroviral vector was obtained from Addgene (plasmid # 85181). MSCV-Kcna3-Thy1.1 (pMSCV-Thy1.1:F2A:mKcna3[NM_008418.2]) and non-conducting 'pore dead' construct MSGV-Kcna3-Thy1.1 (pMSCV-Thy1.1:F2A:mKcna3 W389F) (referred to as Kcna3 mutant) were generated by Vector builder.

Cloning of the four isoforms of NFAT5: The NFAT5 coding sequences were added after an enhanced GFP (eGFP) separated by the self-cleaving peptide P2A. The stop codon was removed and a FlagTag sequence was added at the end of the NFAT5 sequence. The four isoforms differ in the first and last exons. We first cloned the N- and C-termini of NFAT5 isoform A synthetized by Addgene into a pMSGV retroviral vector. We then inserted the core murine cDNA of NFAT5 obtained on the transOMIC platform to create the isoform A. We modified the first and last exon to achieve the three other complete isoforms by replacing the N- and C-termini sequences by newly synthetized sequences generated on GeneArt.

**Overexpression experiments**

TCRP1A CD8 T cells or P14-NFAT5mCherry cells isolated from the lymph nodes. Lymphocytes were activated with 1ug/ml of their respective peptides (P1A (LPYLGWLVF) or gp33 (KAVYNFATC)) and 20U/ml rhIL-2 (Proleukin Aldesleukin) one day before transduction. Viruses were produced in PlatE cells as previously described [54]. Briefly, transfection of the respective plasmids was done with lipofectamine 2000 (Life technologies) in DMEM. Viral supernatants were collected and filtered with 0.45µm filters (Sarstedt Ag & Co) and either used as crude supernatant or concentrated. Transduced CD8 T cells were injected i.v. (5x106) into tumor-bearing hosts one day post transduction. For NFAT5 overexpression, eGFP positive sorted cells were collected and injected i.v. into tumor-bearing mice (minimum 1x104 transduced cells per mouse). To follow CD8 T cell infiltration, mice were injected intraperitoneal with 3mg of luciferin (Biosynth, L-8220), anesthetized with 4% isoflurane and bioluminescence was captured with an IVIS LUMINA II machine. P511 tumors were cut into pieces and digested with 1mg/ml collagenase I and 100µg/ml DNAse (Sigma) for 30 minutes at 37°C. Tumors were passed through a 70µm cell strainer (Falcon). T cells from P511 were isolated with a Ficoll gradient (Fresenius Kabi Norge AS).

**T cell transfer experiments in tumor-bearing mice (B16-gp33 and B16-OVA)**

LN cell suspensions were cultured in complete RPMI + 1µg/ml gp33 peptide (KAVYNFATC) or 1µg/ml Ovalbumin peptide (SIINFEKL) + 20U/ml rhIL-2 (Proleukin Aldesleukin) for two days. CD8 T cells were counted and resuspended into PBS before i.v. injection (5x106 cells/mouse). On the day of the analysis, tumors were collected in complete RPMI and passed through 70µm cell strainers (Falcon). T cells from tumors were isolated with a 40/70 Percoll gradient (VWR 17-0891). When sorted for RNA sequencing, the cells were collected in RNA later (Invitrogen).

**T cell transfer in LCMV-infected mice**

Vβ5 mice were infected with 2x106 PFU one day after transfer of 1x103 P14 WT or NFAT5 KO P14 CD8 T cells. To stimulate cells for cytokine staining, cells were incubated 30 minutes with 1µM gp33 before addition of 5µg/ml of brefeldin A (Biolegend). The cells were then incubated 4 hours 30 minutes before staining. For TonE-mCherry NFAT5 reporter vector experiments, mice were infected with 2x106 PFU one day prior transfer of 5x104 transduced P14 WT cells.

**Jurkat TonE-luc NFAT5 reporter**

Jurkat-TonE was generated by lentiviral transduction of Jurkat cells with a plasmid encoding for luciferase under the control of 2x TonE repeated sequences from the promoter of mouse Akr1b3. 5x105 TonE-luc reporter-expressing Jurkat cells were plated in a 96-well plate and cultured for 24 hours with complete RPMI supplemented with the indicated (10ng PMA (Sigma-Aldrich) / 200ng ionomycin (Thermo Fisher), 10ug/mL CD3 (OKT3 - eBioscience)/CD28 (CD28.2 – eBioscience), ROS: 10uM Butyl-Hydroperoxide, 10ng/mL TGF-β (Roche), 0,5% O2 or NaCl / KCl as indicated). Luminescence was measured using the Bright-Glo™ Luciferase Assay System (Promega).

**TonE-mCherry NFAT5 reporter**

For viral production 1.25 x 106 293T cells were seeded in 2mL five hours before transfection in a 6 well plate. 293T cells were transfected with 4µg total DNA (divided as 0.666ug pVSVG-T2A-NovB2, 1.33µg of R874, and 2µg of 6x TonE-mCherry plasmid, using a mix of Lipofectamine 2000 (Invitrogen) and OptiMEM media (Invitrogen, Life Technologies, according to manufacturer's instructions). The cell culture medium was further supplemented with TNFα at 10ng/ml. The viral supernatant was harvested 48h post-transfection and was used

after filtration. P14 WT or P14 CD4-Cre NFAT5flox/flox CD8 T cells were obtained from the LN of respective mice. CD8 T cells were enriched by negative selection following the manufactures protocol (Stemcell) and plated at 0.4 106 cells /24-well with anti-CD3/28 antibodies and 20U/mL IL-2 24h pre transduction. Viral supernatants were supplemented with 20U/mL IL-2, Lentiboost at 1/2000 and 0.5mL were added to the CD8 T cells. Cells were spun for 60min at 1800RPM at 32°C and incubated for 24h-48h before T cell transfer or further stimulation. When PC60 cells were used, no prior activation or addition of IL-2 was needed.

**Statistical and bioinformatic analyses**

Statistical analyses were performed with Graphpad Prism version 9. Statistical tests are indicated in the legends. Comparisons of more than two groups and subsequent p-values were calculated by ANOVAs with corrections as needed and specified below the figures. A p-value of $<0.05$ was used as the threshold to define statistical significance.

**RNA-seq-Transcript quantification**

Transcript abundance quantification was performed with Salmon 0.14.1 [55] in quasi-mapping-based mode using the mouse reference transcriptome (assembly GRCm38.p2) obtained from ENSEMBLE [56]. Default parameters were used plus the --seqBias, --gcBias, --validateMappings, --fldMean 200 and –fldSD 30 parameters.

**Differential expression analyses**

Differential gene expression analyses were performed using DESeq2 1.24.0 [57]. Transcript-level abundances were summarized at the gene level using tximport 1.12.3 [58]. Genes with low read counts were filtered out (requiring genes to have a count of at least 10 in at least a number of samples equal to the smallest group size). Overall similarity between samples was assessed by first applying a regularized stabilizing transformation (rlog) to the gene-level count matrices using the rlog function, and then performing a principal components analysis (PCA) on the regularized matrix using the plotPCA function. Significant genes were identified using a false discovery rate (FDR) threshold of 5% and an absolute log2 fold change threshold of 1.5. The GO term analysis were performed with the EnrichR platform [59, 60] using the “GO Biological Process 2018” algorithm. Enrichment of the NFAT5 KO differentially expressed gene set in TOX up or downregulated genes was evaluated using the TOX expression data published in [36], available from the Gene Expression Omnibus (GEO) database under accession number GSE126973. NFAT5 KO differentially expressed genes were ranked based

on the log2 fold change (ranked list), and TOX signatures of up or down gene regulation were created based on TOX knockout differentially expressed genes with positive or negative log2 fold change. Enrichment scores and adjusted p-values were computed using the fgsea function and the ranked list and regulation signatures mentioned above, with the number of permutations set to 1,000. Enrichment plots were obtained using the plotEnrichment function.

**TIL atlas**

The TIL atlas dataset used in Fig.1a, b and Extended Data Fig.3, which includes 16,803 high-quality single-cell transcriptomes from 25 samples (B16 melanoma and MC38 colon adenocarcinoma tumors) from six different studies, has been collected, thoroughly analyzed and annotated by Andreatta and co-workers [26], and it is publicly available (https://doi.org/10.6084/m9.figshare.12478571).

**Regulon analysis of tumor-infiltrating T lymphocyte**

Regulons (gene sets regulated by the same transcription factor) and their activity were inferred and evaluated using the SCENIC pipeline (https://scenic.aertslab.org) [61], which can be described in three steps. Step 1) Infer gene regulatory network (GRN) using grnBoost2, which is a faster implementation of the original algorithm Genie3 [62]; scRNA-seq transcriptomics data is used as the input to infer causality from the expression levels of the transcription factors to the targets based on co-expression patterns. The importance of each transcription factor in the prediction of the target gene expression pattern is taken as an indication of a putative regulatory event. The aggregation of the top 50 targets per TF and the top 5 TF per target was used to define raw putative regulons. Step 2) Co-expression modules (raw putative regulons, i.e. sets of genes regulated by the same transcription factor) derived from the GRN generated in Step 1 are refined by pruning indirect targets by motif discovery analysis using cisTarget algorithm and a cis-regulatory motif database [63, 64]. We used mm9-500bp-upstream-7species.mc9nr.feather and mm9-tss-centered-10kb-7species.mc9nr.feather databases. The motif database includes a score for each pair motif-gene, so that a motif-gene ranking can be derived. A motif enrichment score is then calculated for the list of transcription factor selected targets by calculating the Area Under the recovery Curve (AUC) on the motif-gene ranking 1 using the RcisTarget R package (https://github.com/aertslab/RcisTarget). If a motif is enriched among the list of transcription factor targets, a regulon is derived including the target genes with a high motif-gene score. Step 3) evaluation of regulon activity of each individual cell using AUCell (https://github.com/aertslab/AUCell), which provides an AUC score for each

regulon; we discarded regulons with less than 5 constituent elements, as the estimation of the activity of small regulons is less reliable. In order to compare the regulon activity profile of CD8 exhausted T cells and CD8 naïve cells from the TILs dataset, we used the regulon activity (AUC score) matrix and performed, for each regulon, a Wilcoxon Rank Sum test implemented within the FindMarkers function of the SEURAT R package (version 4.0.3). Only regulons with an adjusted p-value for this test of 0.05 or less were considered as differentially active (Bonferroni correction).

**Evaluation of NFAT5 KO signature in tumor-infiltrating T lymphocytes**

Differentially expressed genes up (n=458) and down (n=832) regulated from the comparison NFAT5.KO Vs. WT were considered separately as two different signatures to be analyzed in the TILs dataset (NFAT5.KO.DEG.UP and NFAT5.KO.DEG.DOWN, respectively). The AUCell R package [61] was used to evaluate these signatures across tumor-infiltrating T lymphocyte subpopulations using the normalized gene expression matrix of this dataset.

**Western Blot**

NFAT5flox/flox CD4-Cre-/- (WT), CD4-Cre+/- (NFAT5 KO) NFAT5flox/flox, NFAT5mCherry +/- and NFAT5mCherry +/+ P14 CD8 T cells were cultured in complete RPMI with 1uM gp33 peptide and 20U/ml rhIL-2 (Proleukin Aldesleukin) for 72 hours. Whole cell extracts were isolated using RIPA lysis buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1% NP 40, 1% sodium deoxycholate, 0,1% SDS; Cell Signaling Technology) supplemented with DNase, 1x phosphatase inhibitor (PhosSTOP Roche) and 1x protease inhibitor (complete protease inhibitor cocktail Roche). Protein concentration was assessed by the Bradford Assay (Biorad Protein Assay Kit II) and 25ug of cell lysates from each condition were resolved by 8% SDS-PAGE with NuPAGE electrophoresis system (Invitrogen/Thermo Fisher Scientific). Proteins were then transferred to PVDF membrane (Invitrogen/Thermo Fisher Scientific) by Trans-Blot SD semidry transfer cell (Bio-Rad) at 18 V for 30 minutes. Membranes were blocked with 5% w/v nonfat dry milk (Sigma-Aldrich) and then incubated with the indicated primary antibodies at 4 °C overnight. The bands were visualized using Thermo Scientific™ Pierce™ ECL Western Blotting Substrate after incubation with horseradish peroxidase (HRP)-conjugated antibodies for 1 hour at room temperature. Primary antibodies: anti-NFAT5 antibody (Santa Cruz) and anti-beta-actin antibody (Invitrogen).

### 3. 1. 8 References

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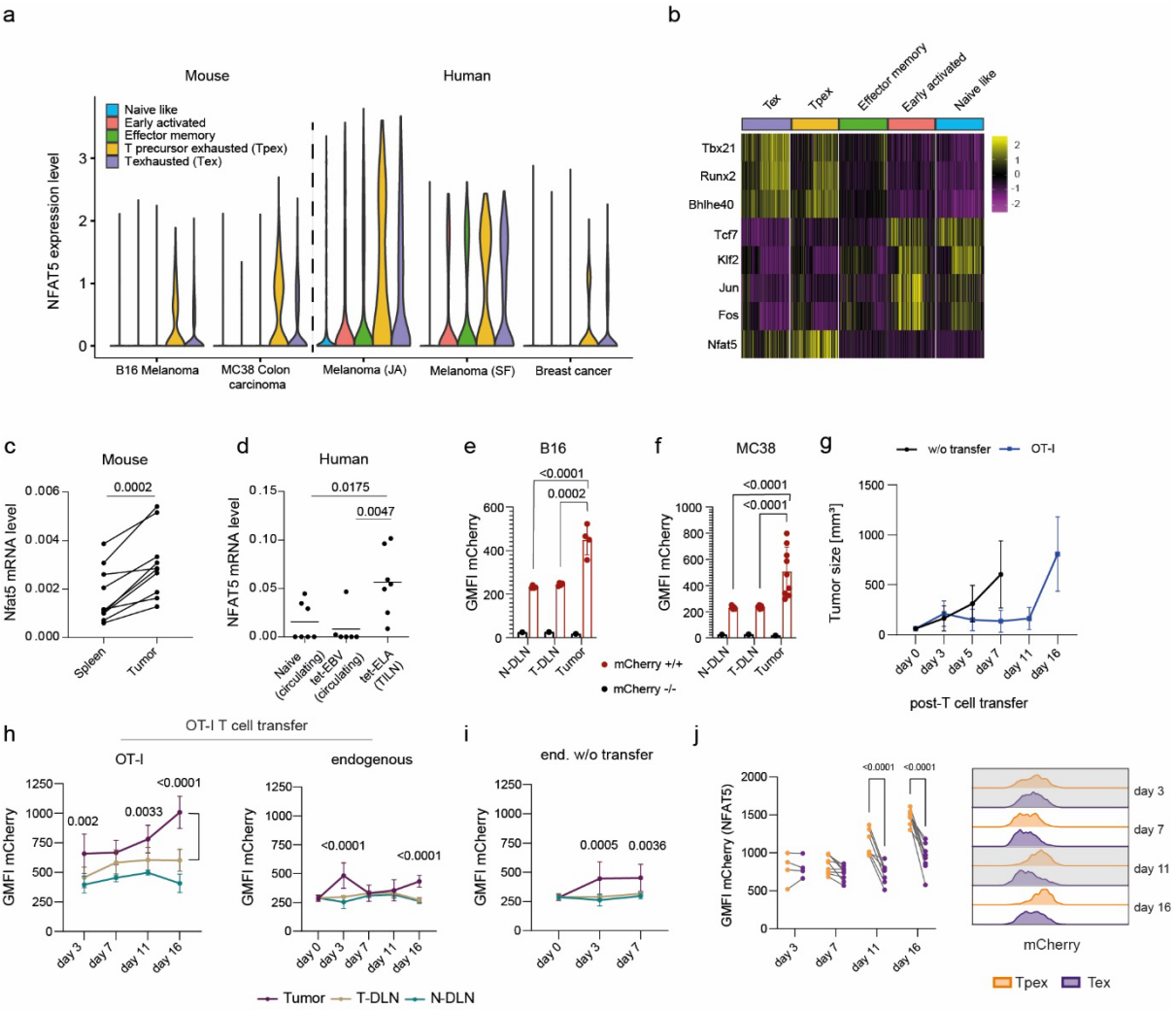
## 3. 1. 9 Figures

### 3. 1. 9. 1. Figure 1



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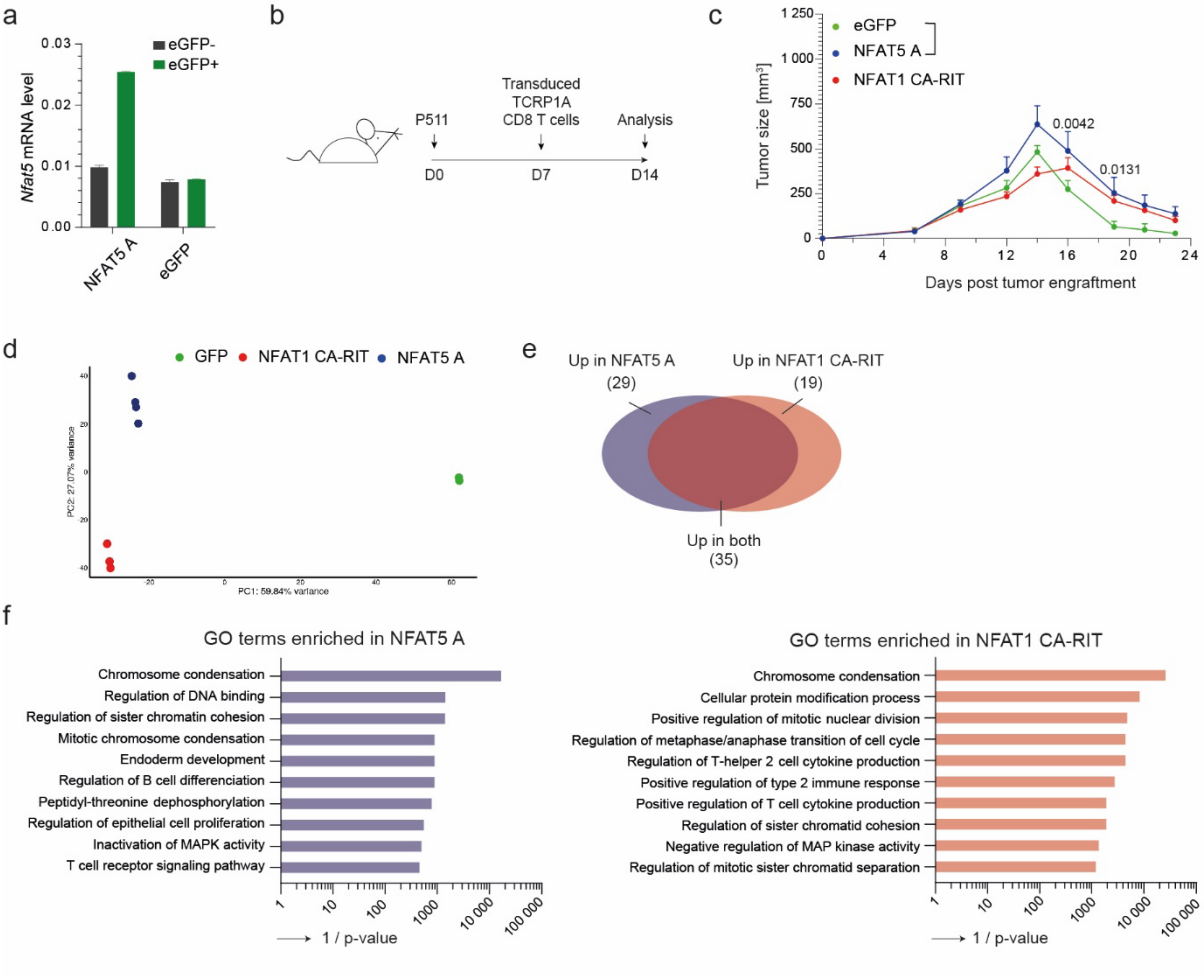

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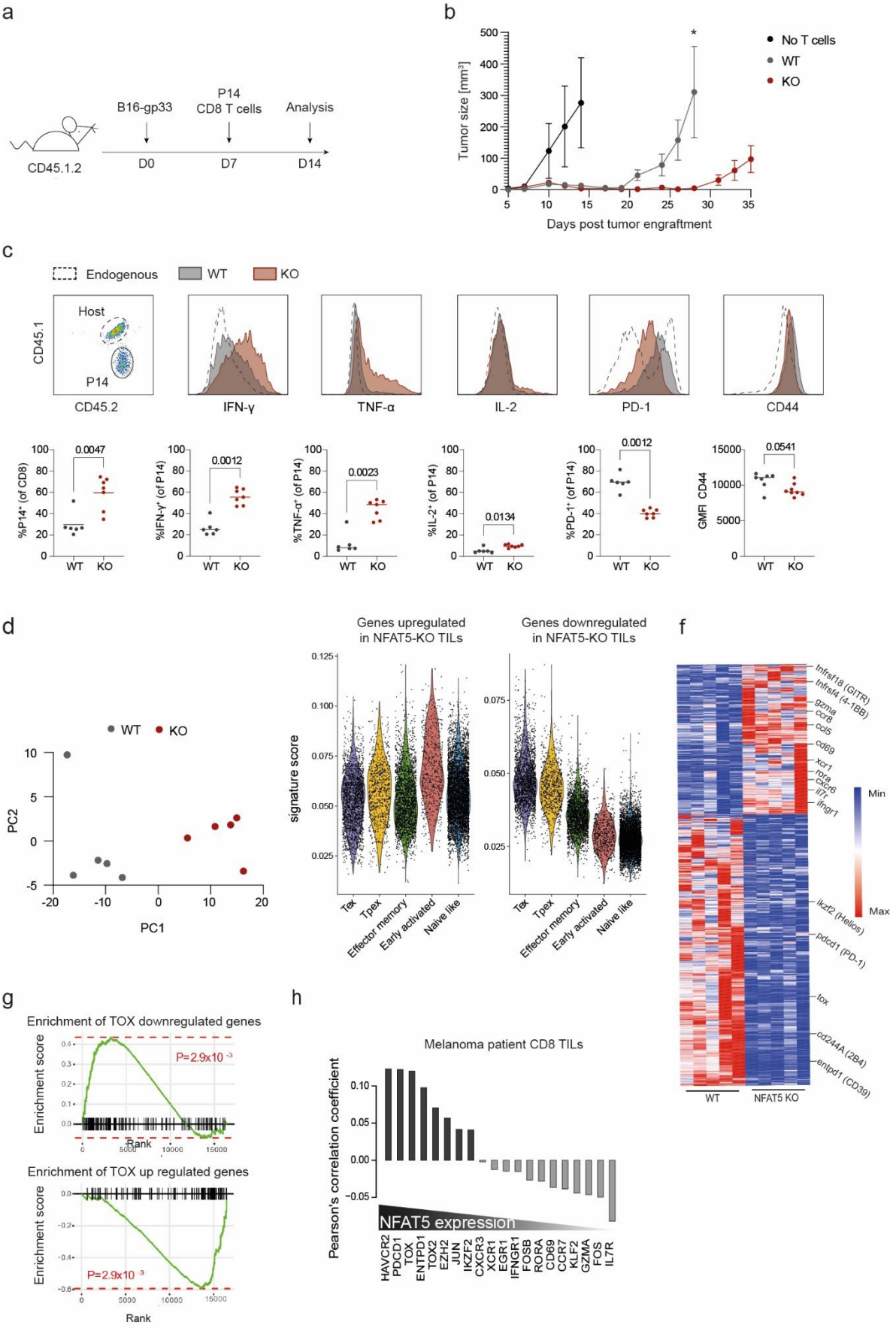

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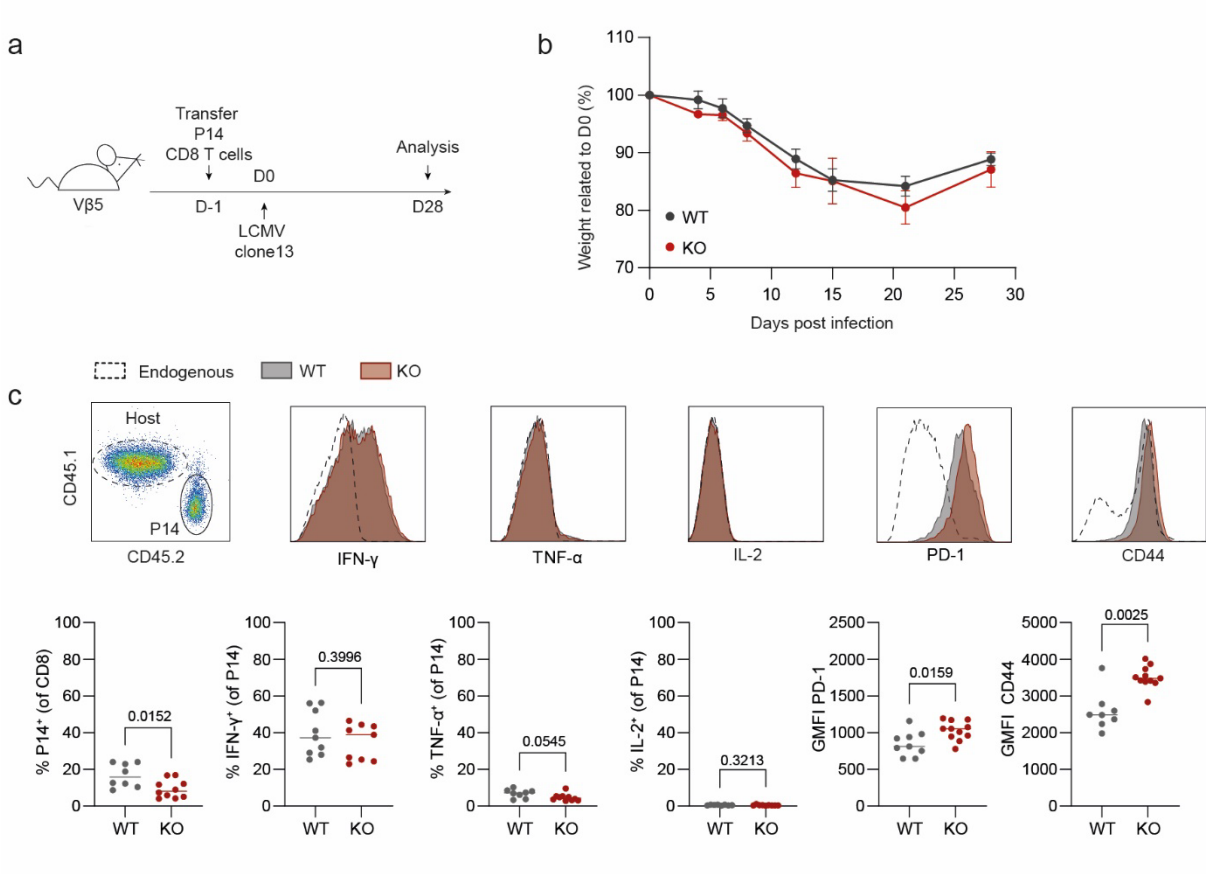
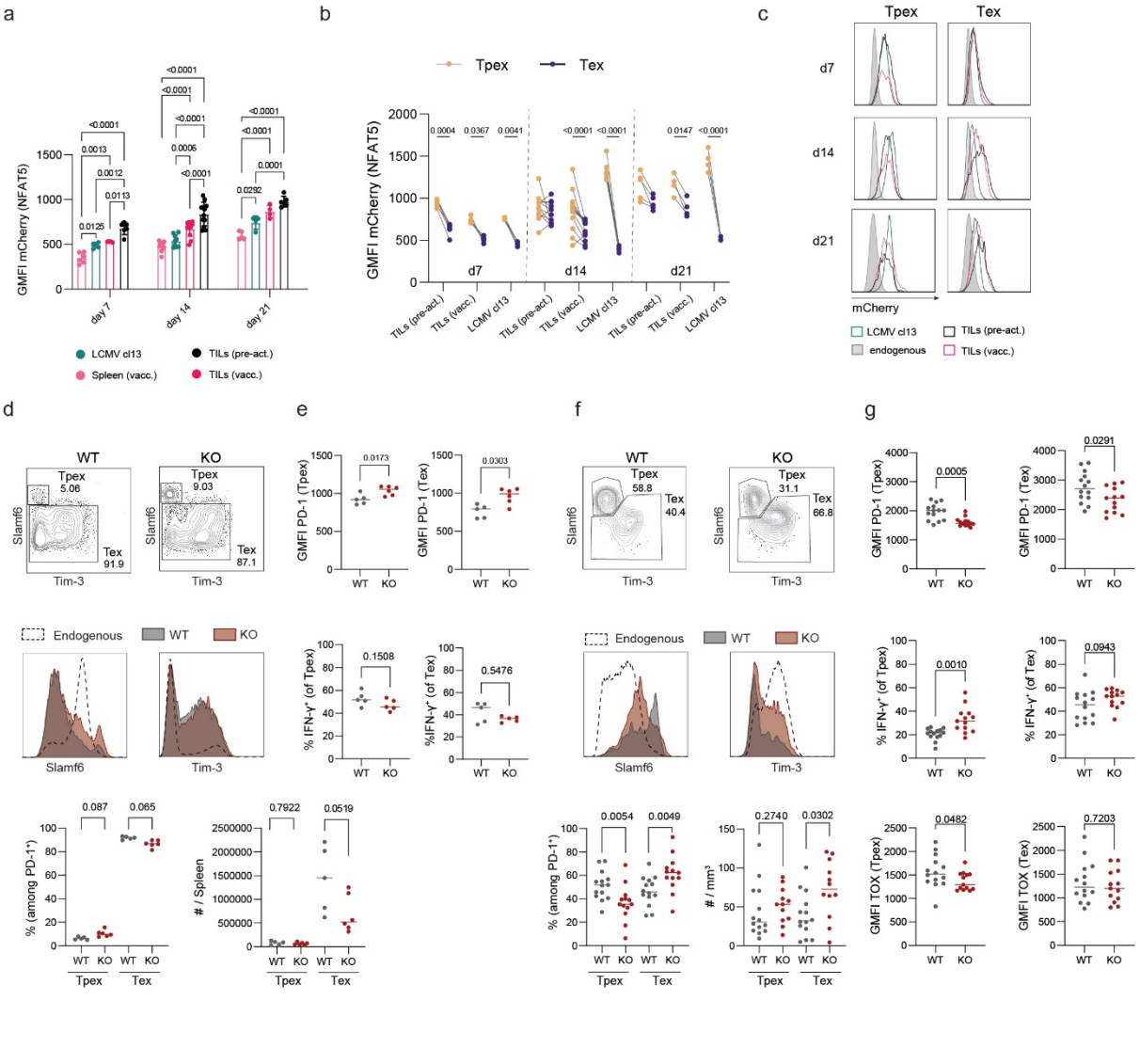


### 3. 1. 9. 5. Figure 5



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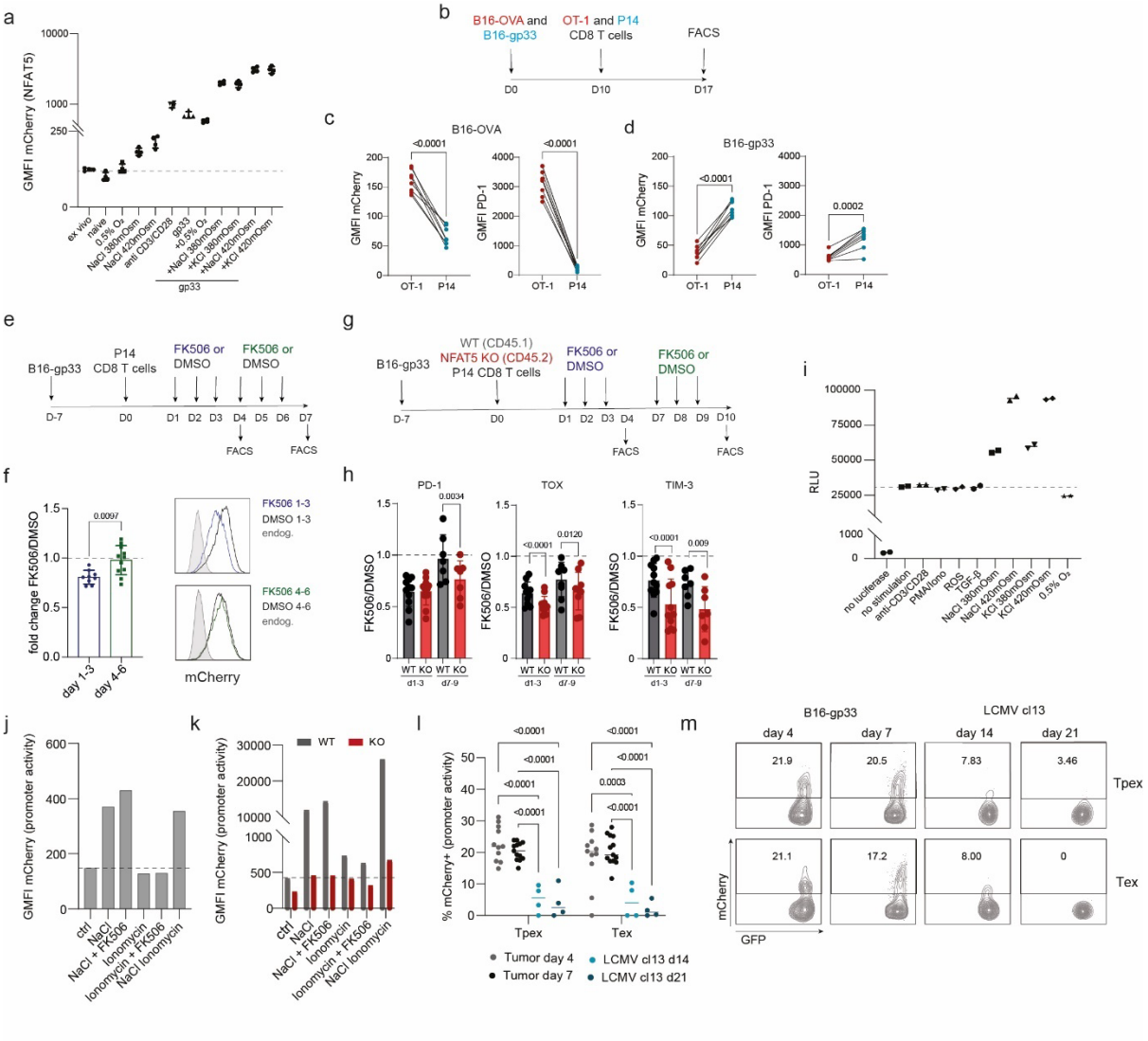
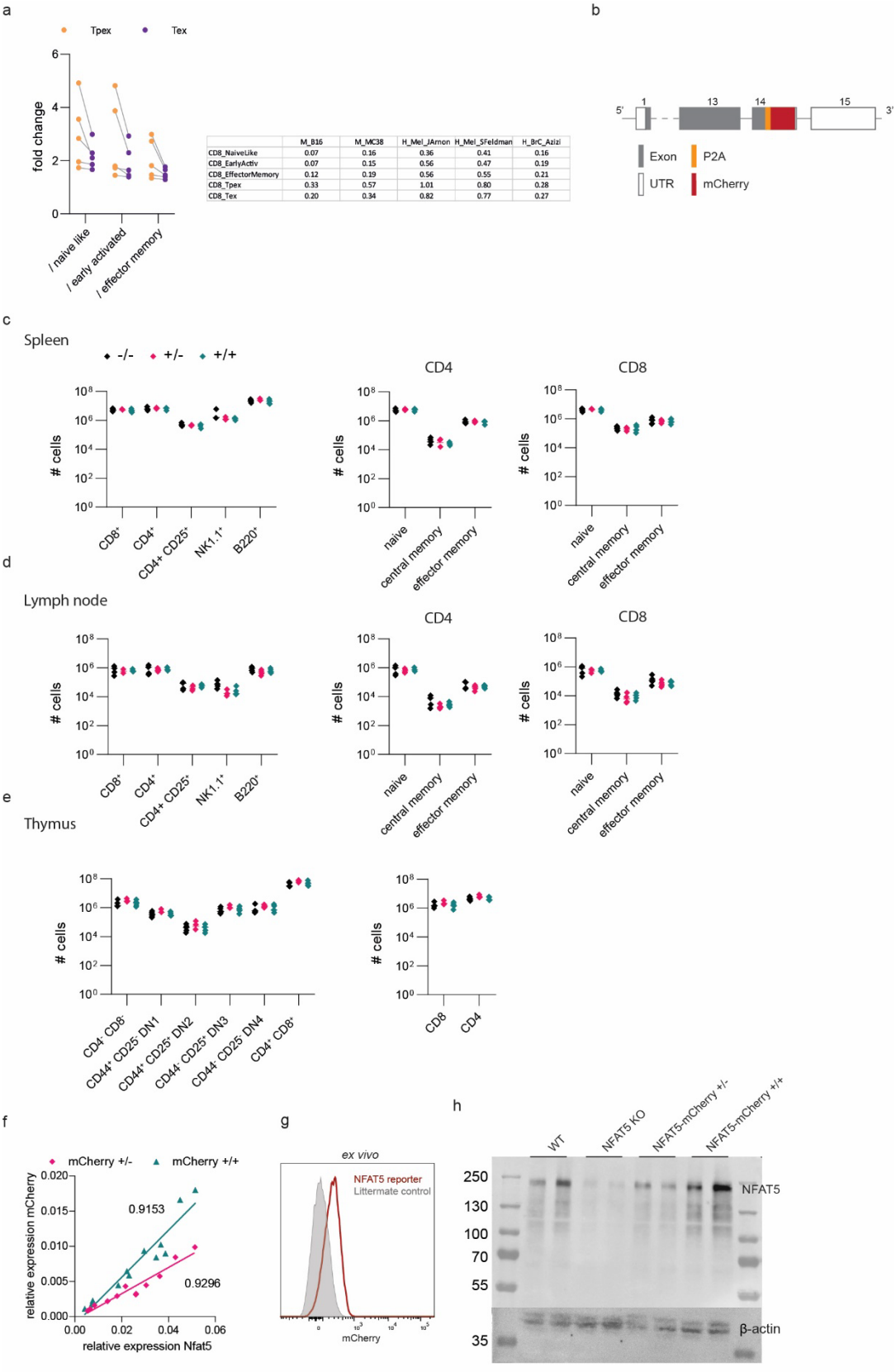


## 3. 1. 9. 7. Extended Data Figure 1



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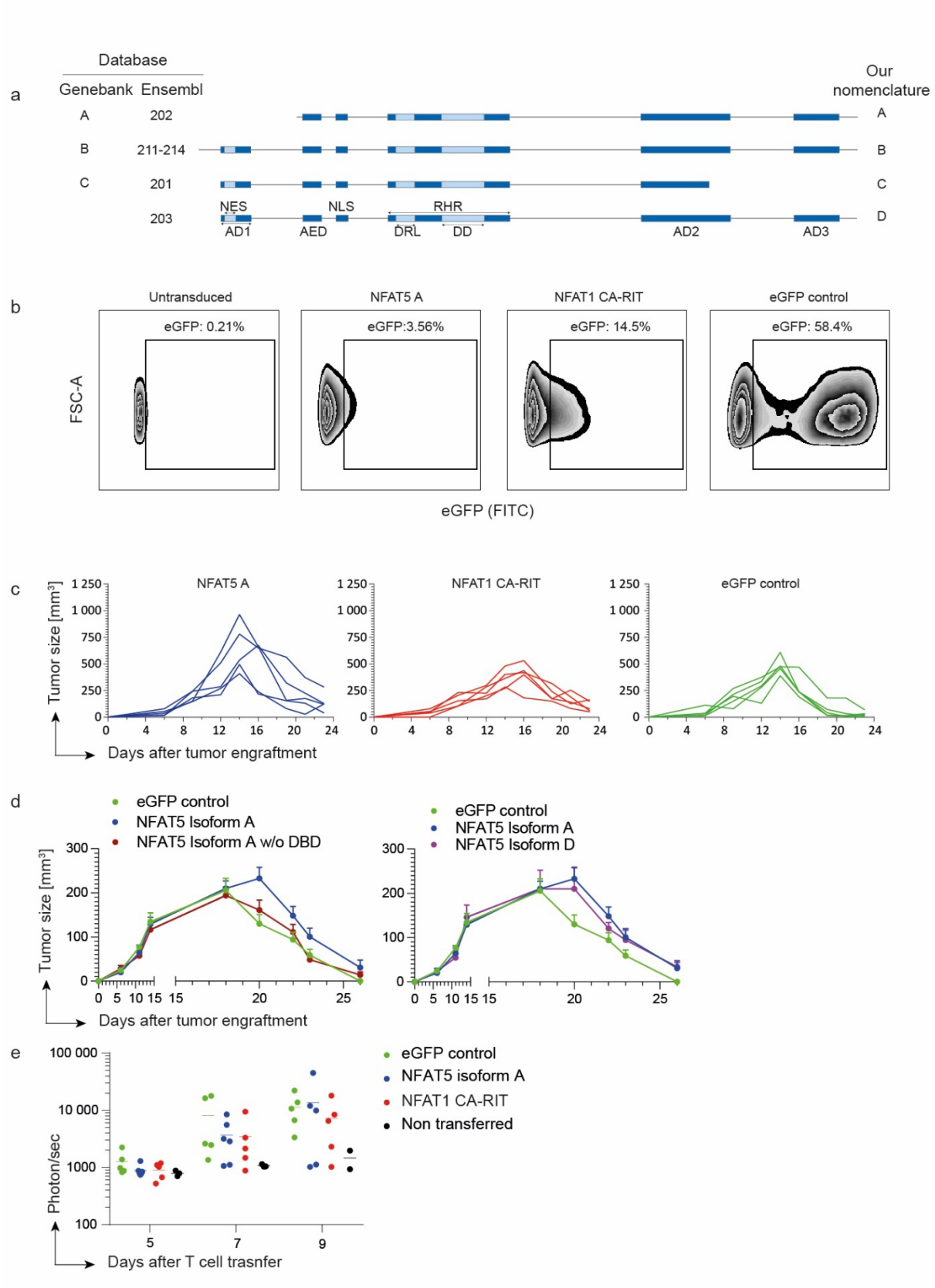

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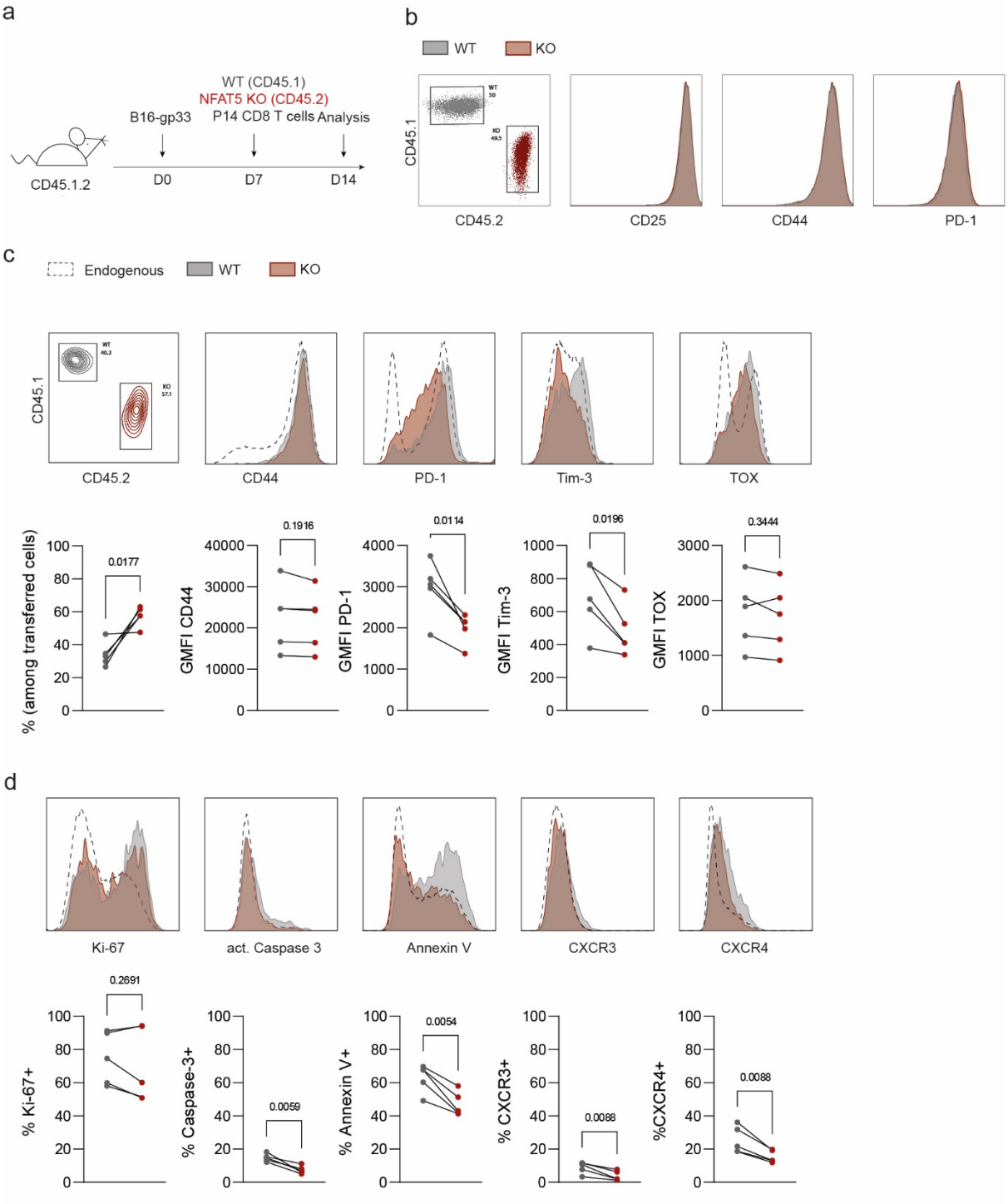
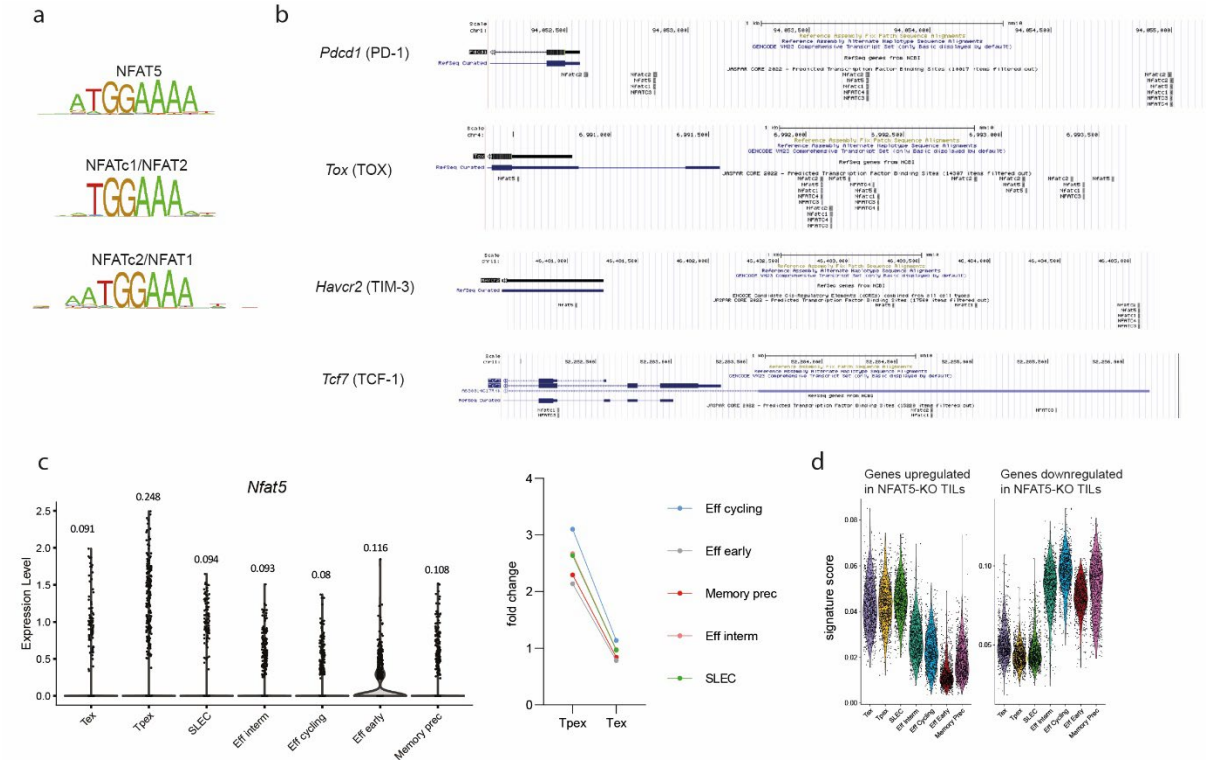


### 3. 1. 9. 10. Extended Data Figure 4



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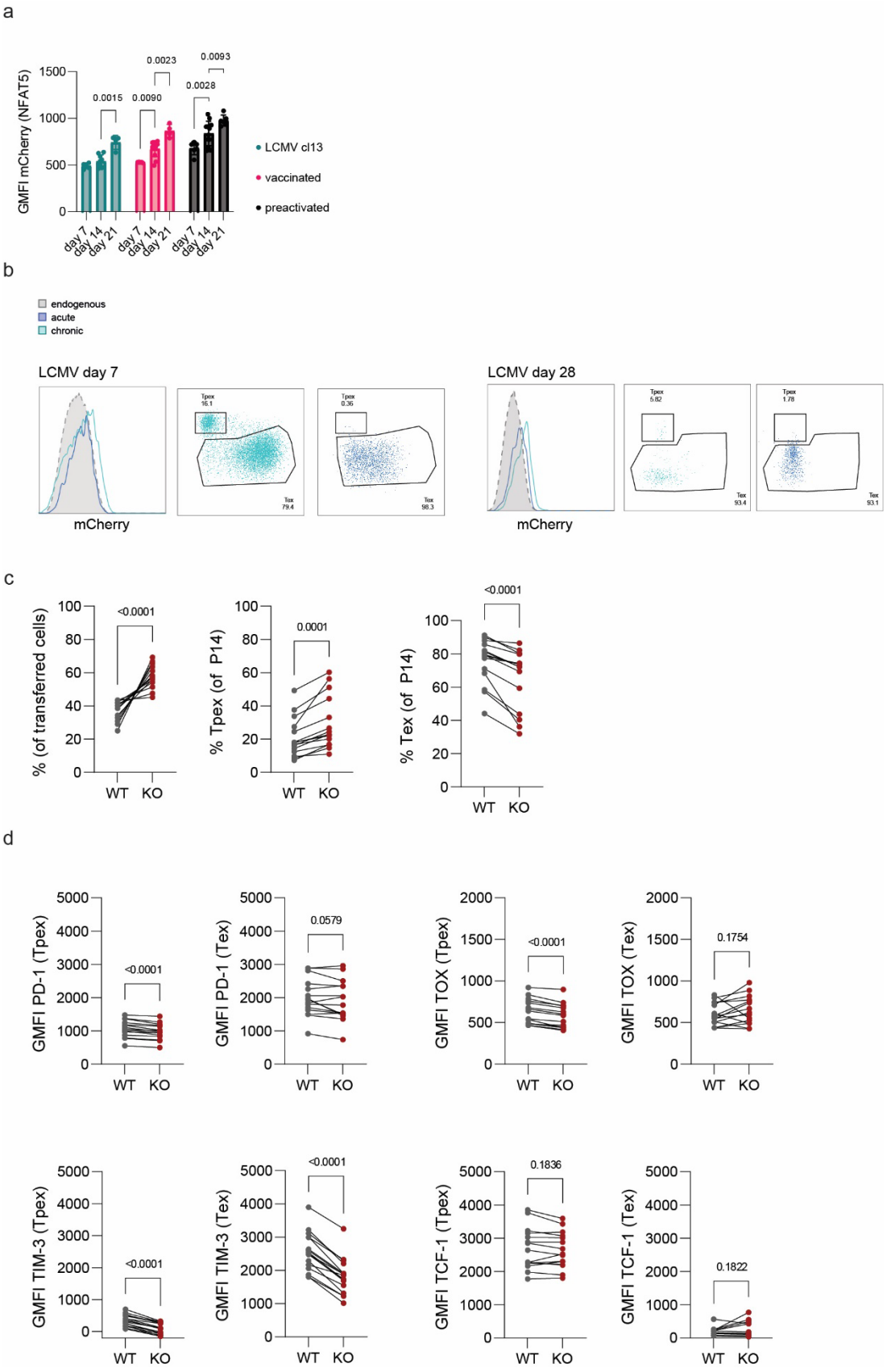
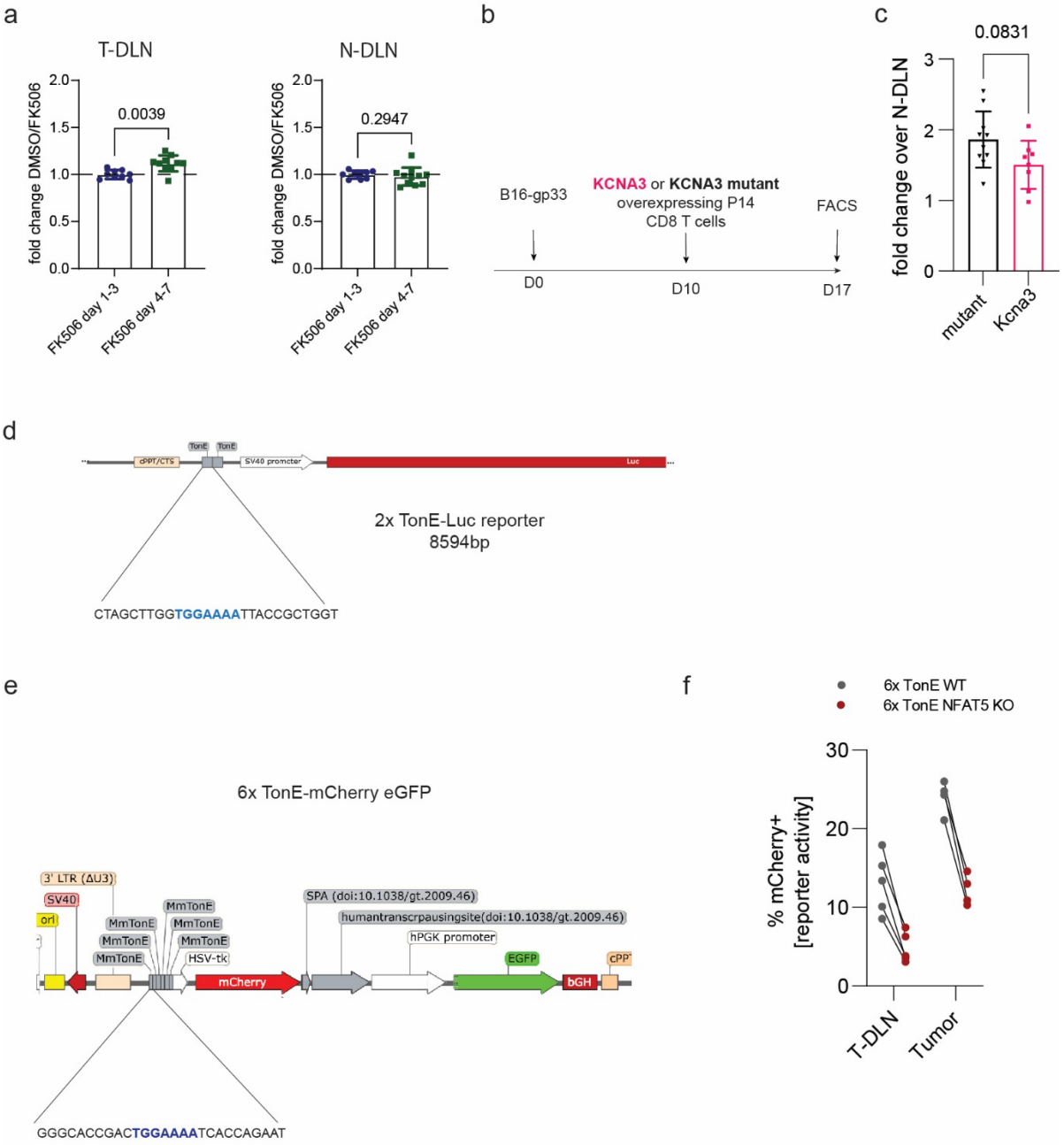


### 3. 1. 9. 12. Extended Data Figure 6



### 3. 1. 9. 13. Extended Data Table 1

List of antibodies used for flow cytometry

| Target | Flurochrome | Dilution | Clone | Host | Source | Lot no. |
|---|---|---|---|---|---|---|
| CD3 | BV650 | 50 | 17A2 | R | BioLegend | B282105 |
| | PE-Cy7 | 100 | 145-2C11 | H | eBio | 4304567 |
| CD4 | Pacific Blue (BV421) | 1000 | GK1.5 | R | LICR | 6.02.20 |
| CD8 | APC-Cy7 | 100 | 53.6.7 | R | eBio | 2011697 |
| | FITC | 200 | 53.6.7 | R | LICR | - |
| | APC | 100 | 53.6.7 | R | LICR | - |
| | BV786 | 100 | 53.6.7 | R | BioLegend | B285904 |
| | Alexa700 | 100 | 53.6.7 | R | LICR | 6.02.20 |
| | PE | 100 | 53.6.7 | R | eBio | 4300680 |
| CD11b | APC | 100 | M1/70 | R | LICR | - |
| CD11c | BV605 | 50 | N418 | H | BioLegend | B272371 |
| CD25 | Pacific Blue (BV421) | 1000 | PC61.5 | R | eBio | 1942891 |
| | BV711 | 100 | PC61.5 | R | eBio | 1946557 |
| CD44 | PE-Cy7 | 200 | IM781 | R | eBio | E07565-1635 |
| | FITC | 100 | IM781 | R | LICR | - |
| | APC | 100 | IM781 | R | LICR | - |
| | PerCP-Cy5.5 | 200 | IM781 | R | eBio | 1984139 |
| CD45.1 | BV421 | 100 | A20 | M | LICR | 3.06.20 |
| | BV650 | 100 | A20 | M | Biolegend | B279181 |
| CD45.2 | FITC | 100 | A20.1 | M | LICR | - |
| | PE | 100 | 104 | M | eBio | E02255-1630 |
| | PerCP-Cy5.5 | 100 | 104 | R | eBio | 2055168 |
| CD62L | PerCP-Cy5.5 | 1500 | Mel-14 | R | eBio | 1995503 |
| CD69 | Pacific Blue (BV421) | 100 | HI-F3 | M | eBio | B269770 |
| Thy1.1 (CD90.1) | APC | 100 | HIS51 | M | eBio | 1981294 |
| | FITC | 50 | H022.1.1 | R | LICR | - |
| B220 | APC-Cy7 | 1000 | RA3-6B2 | R | eBio | E10028-1635 |
| Dead cell marker (Aqua fluorescent reactive dye) | BV510 | 500 | - | - | invitrogen | L34966A |
| NK1.1 | FITC | 200 | PK136 | M | LICR | - |
| Tim-3 | BV786 | 100 | RMT3-23 | R | BioLegend | B295061 |
| TCR V alpha 2 | APC | 200 | 2142951 | M | invitrogen eBioscience | 17-5812-82 |
| PD1 | PE-Cy7 | 100 | RMPI-30 | R | BioLegend | B290858 |
| | BV711 | 200 | 29F.1A12 | M | BioLegend | B298663 |
| TOX | APC | 200 | TXRX10 | R | Invitrogen | 2246902 |
| LY108 | Biotin | 200 | 13G3-19D | M | Invitrogen | 2247484 |

**qPCR primers**

| | |
|---|---|
| β2M | F: AGACTGATACATACGCCTGCAG |
| | R GCAGGTTCAAATGAATCTTCAG |
| Murine NFAT5 | F: GGTACAGCCTGAAACCCAAC |
| | R TGCAACACCACTGGTTCATT |
| Human NFAT5 | F: ATT GCA AAA CCA AGG GAA CA |
| | R: TTG GAA TCA GGA TTT TCT TCG |
| mCherry | CCC ACA ACG AGG ACT ACA CC |
| | TTG TAC AGC TCG TCC ATG CC |

# 4. Discussion

Enhancing T cell functions is a novel and promising approach to improve cancer treatments and patient survival. Nevertheless, modern T cell-based therapy still faces limitations due to the suppressive TME causing T cell exhaustion. NFAT5 is a crucial regulator of the adaptation to environmental cell stress. In this study, we could show that NFAT5 is also a regulator of CD8 T cell exhaustion in a tumor-specific manner. NFAT5 KO T cells showed improved effector function and accumulation by rendering $T_{pex}$ more effector-like, resulting in better tumor control. In contrast, overexpression of NFAT5 resulted in dampened tumor control similar to the one observed by overexpressing NFAT1 CA-RIT. We wondered if NFAT5 was complementary to the known NFAT1-mediated T cell exhaustion by sustaining the long-term expression of exhaustion-associated genes.

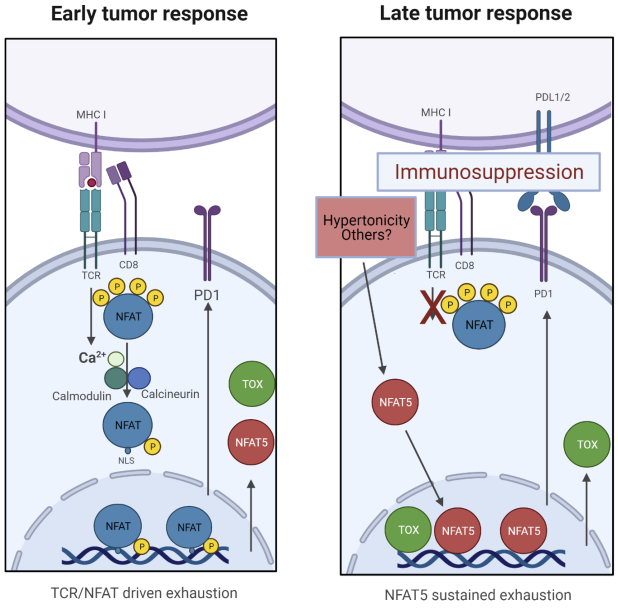



Figure 13.: NFAT and NFAT5 mediated exhaustion during early and late tumor response. Figure created by Dr. Verdeil using Biorender.

By inhibiting the phosphatase calcineurin, we showed that NFAT5 regulated independently of NFAT the expression of exhaustion-associated proteins such as PD-1 at later stages. In contrast, NFAT5 regulated TOX and Tim-3 expression throughout the tested period. These results demonstrate the importance of NFAT as an early regulator of exhaustion since FK506 treatment reduced the exhaustion phenotype early on, consistent with published data. Additionally, we could show that NFAT5 acts independently of NFAT on these hallmarks at different time points, thus proving the complementary role of the two family members.

We further found that calcineurin targets, such as NFAT, partly regulated NFAT5 since its expression was decreased when calcineurin was blocked at the early stages of tumor growth. No difference was observed between treated and untreated mice at later stages, suggesting that other triggers than calcineurin-dependent ones took over at this point. Possible secondary effects of FK506 treatment, such as nephrotoxicity affecting sodium and water homeostasis, are not responsible for this observation since mice received the same dose of FK506 at early and late time points. Therefore, the timing of the treatment is the only difference. In earlier studies, calcineurin has been proposed to regulate TCR-induced NFAT5 expression but not hypertonicity-induced expression *in vitro*[182]. It would be interesting to test the effect of FK506 at different doses and combinations on NFAT5 expression and compare the results to more NFAT-specific inhibitors, such as the NFAT-inhibitory peptide VIVIT. These experiments could further outline which combination of stimuli NFAT5 expression is calcineurin-dependent and if this relies on the reduced activity of NFAT or possibly other calcineurin targets.

The different NFAT5 KO phenotypes observed in CD8 T cells during chronic infection and cancer led us to investigate the induction and activation profile of NFAT5. Given that many stimuli have been described to act on NFAT5 upregulation, nuclear localization, and transactivation activity, we tested a set of triggers relevant to the TME. Hyperosmolarity and TCR triggering induced NFAT5 *in vitro,* while TCR stimulation was the dominant driver for NFAT5 expression *in vivo*. These findings matched the expression profile in CD8 T cells from chronic infection and the TME, where TCR triggering is equally present. Nevertheless, NFAT5, even though expressed in both pathologies, did not alter T cell function during chronic infection. We hypothesized that this effect might be due to different transcriptional activities of NFAT5. With an NFAT5 sensitive reporter construct, we showed that NFAT5 activity relied on hyperosmolarity and was independent of TCR stimulation *in vitro*. Finally, using this reporter construct, we found that NFAT5 was only active within the TME and not during chronic infection explaining the lack of improvement of NFAT5 KO T cells in the context of chronic infection where elevated osmolarity has not been described.

NFAT and NFAT5 have the same core DNA binding motif. Therefore, we validated the NFAT5 restricted specificity of the 6xTonE-reporter construct with the absence of signal in transduced NFAT5 KO CD8 T cells. Several reporter constructs have been used to study NFAT and NFAT5-specific transcriptional activities. Upon them, the 9xNFAT-Luc reporter was induced by PMA/ionomycin NFAT-dependently and showed NFAT5-dependent induction in response to hypertonicity. NFAT5-dependent reporter activity was reduced by FK506

depending on the cell type. T cells showed reduced reporter activity, while FK506 treatment did not impact Jurkat cells and macrophages[152]. We studied the effect of FK506 on primary CD8 T cells transduced with 6xTonE-reporter construct and cultured under either TCR stimulation or hypertonic conditions. FK506 treatment did not alter NFAT5-dependent reporter activity in any of the conditions, while it diminished the reporter activity of the 6xNFAT reporter tested in parallel (data not shown) as previously described. These results indicate that in CD8 T cells, NFAT5 recruitment and activity to the 6xTonE reporter was calcineurin independent.

## 4. 1. NFAT5 as part of the transcriptional network of T cell exhaustion

In this work, we have characterized the role of the transcription factor NFAT5 controlling a set of genes involved in tumor-induced T cell exhaustion. It is one of the first work showing a microenvironment-specific transcription factor regulating exhaustion.

Research has focused on understanding the development and possible interventions regarding T cell exhaustion. Interestingly, most discoveries share similarities between exhausted T cells found in chronic infections and those in tumors. A multi-omics study further revealed this phenomenon by comparing T cell exhaustion in cancer and infection[185]. By comparing CD8 T cells responding to acute or chronic infection and cancer from 12 different studies, they could dissect state-specific drivers of exhaustion using ATACseq and RNAseq. Upon identified drivers, *Nfat5* was shown to be specifically expressed by $T_{pex}$, in agreement with our findings of NFAT5 being highly expressed in $T_{pex}$ and KO influencing their phenotype over $T_{ex}$.

Nevertheless, the TME and an infected spleen are distinct microenvironments. Still, no study has been performed comparing the protein level, or the activity of transcription factors in chronic infection derived compared to tumor-induced exhausted T cells.

## 4. 2. Quality and limitations of the results

### 4. 2. 1 Role of hypertonicity on NFAT5 expression

NFAT5 is known to respond to hypertonic stress caused by high sodium chloride. In our study, NFAT5 reacted to hyperosmolarity caused by elevated NaCl or KCl in the culture medium, showing that NFAT5 sensed osmolarity independently of the cation causing it.

By blocking calcineurin with FK506, we found that NFAT5 expression was regulated through the calcineurin axis early after T cell transfer, but calcineurin was dispensable for NFAT5 expression at later stages. We hypothesized that TILs might face inhibited TCR signaling at later stages and are confronted with other TME stimuli, such as changes in the ion composition. As described, potassium is elevated in the tumor intestinal fluid (TIF) of patients and mouse models[137]. Potassium chloride-mediated elevation of the osmolarity of the cell culture media triggered both NFAT5 expression and activity. We, therefore, wanted to assess the role of high intracellular potassium on TILs *in vivo*. Overexpression of the voltage-gated channel Kcna3 has enabled T cells to equalize the high intracellular potassium concentration[137]. We hypothesized that Kcna3 overexpressing TILs would have a lower potassium concentration and, therefore, lower amounts of NFAT5. In our experiments, Kcna3 overexpressing TILs showed only a trend of decreased NFAT5 expression but not a significant decrease. This might be due to a not existing elevated intracellular potassium concentration of our TILs, which we will assess in the future using specific potassium probes such as Asante potassium green. As we have seen a dominant effect of TCR triggering on NFAT5 expression *in vivo*, it is also possible that the osmolarity in the TME has no impact on NFAT5 expression but instead uniquely influences the activity of NFAT5. Therefore, we would like to test the effect of potassium-elevated TIF on NFAT5 expression and activity *in vitro* and in combination with Kcna3 overexpressing T cells. If the activity is influenced in a potassium-dependent manner, we will test the effect of necrosis induction by VEGFR2 blocking on activity reporter expressing TILs *in vivo*.

### 4. 2. 2 NFAT5$_{mCherry}$ reporter mouse model

We generated NFAT5$_{mCherry}$ mice to investigate NFAT5 expression. These reporter mice allow the single-cell tracing of NFAT5 expression on the transcriptional level but do not give insight into the protein level since NFAT5 and mCherry are separated during the process of mRNA translation. We are currently engineering NFAT5-specific antibodies, which can be used for flow cytometry, western blot, and, hopefully, epigenetic studies, to overcome this limitation.

### 4. 2. 3 The role of NFAT5 in $T_{pex}$

Our study has outlined the higher expression of NFAT5 in $T_{pex}$ and the dominant effect of NFAT5 KO by rendering the phenotype of $T_{pex}$ more effector-like. Nevertheless, we need more key information to understand which role NFAT5 plays in $T_{pex}$. NFAT5 KO skewed $T_{pex}$ towards $T_{ex}$ differentiation while preserving the pool of $T_{pex}$ in numbers when NFAT5 KO and WT P14 cells were transferred to different hosts. We could hypothesize that NFAT5 hinders efficient $T_{ex}$ differentiation linked with reduced cytotoxicity leading to reduced tumor control. The bulk RNAseq performed on NFAT5 KO and WT TILs does not allow us to analyze subtype-specific effects. Therefore, we plan to perform scRNA-seq to compare the $T_{pex}$ and $T_{ex}$ signatures from the TME to those found in chronic LCMV infection.

### 4. 2. 4 Effect on apoptosis

NFAT5 is an important regulator for the adaptation to cellular stress, such as hyperosmolarity and hypoxia. Our experiments showed that NFAT5 KO TILs accumulated more than WT TILs in the TME. We investigated if increased accumulation was due to increased proliferation, infiltration, or survival. We found decreased apoptotic marker expression, such as active caspase-3 and Annexin V, of NFAT5 KO TILs suggesting their improved survival in the TME. *In vitro* culture under TCR triggering and hyperosmolarity decreased expansion of NFAT5 KO over WT CD8 T cells[180] (and data not shown). These data suggest a different reason than hypertonic stress for decreased apoptosis of NFAT5 KO TILs. Furthermore, HEK293 cells transfected with NFAT5 siRNA and exposed to 2.5% pO2 for 8 hours showed increased cleaved caspase-3 than control siRNA receiving HEK293 cells suggesting a protective function of NFAT5 to hypoxia-induced apoptosis[186]. On the other hand NFAT5 knockdown in HK-2 cells prevented hypoxia-induced apoptosis, suggesting a negative regulation of NFAT5 on cell survival[187]. Therefore the effect of hypoxia on NFAT5-mediated apoptosis is depending on the cell type and pathology.

NFAT has been shown to induce caspase-3 in anergic T cells[62], and NFAT-CARIT also increased caspase-3 expression in exhausted T cells[91]. Furthermore, elevated active caspase-3 can cleave NFAT and therefore is involved in regulating NFAT[188]. We do not know the osmolarity of the TIF in our experiments and plan to collect TIF with the described protocols and analyze the osmolarity with an osmometer. These results would also help to follow up on the activity of NFAT5 in the TME. Furthermore, we plan to assess apoptosis *in vitro* under different conditions, including hypoxia, in NFAT5 KO and WT CD8 T cells.

## 4. 3. Perspectives

### 4. 3. 1 NFAT5 regulated genes

To link the observed changes upon NFAT5 KO in TILs, such as reduced PD-1 and TOX expression in $T_{pex}$ and increased IFNγ, TNFα, and IL-2 expression, we plan to investigate which genes are directly controlled by NFAT5. NFAT5 KO CD8 T cells cultured for a short term *in vitro* did not show any phenotypical alteration compared to WT cells. This might be due to prominent NFAT activity at this stage. Therefore, we could not perform ChIP-seq since the required number of cells is above 1 million and not reachable with *ex vivo* sorted TILs. Our new strategy has two advantages. First, we plan to use Cleavage Under Targets and Release Using Nuclease (CUT&RUN) coupled with sequencing. CUT&RUN is an adapted protocol from ChIP that allows the use of cells in the native state (not cross-linked) and requires a lower cell number. CUT&RUN relies on Protein A-Protein G-Micrococcal Nuclease (pAG-MNase) mediated cleavage specific to the target-specific antibody binding region. With this strategy, anti-HA tag antibodies gave promising results for detecting transcription factor binding. Therefore, we will overexpress NFAT5-3xHA in NFAT5-KO CD8 T cells and detect its binding in an *in vitro* exhaustion model. As we identified NFAT5 as important for regulating exhausted related genes at later stages, we plan to chronically stimulate NFAT5-3xHA tag overexpressing NFAT5 KO T cells for ten days to induce exhaustion. To ensure the activity of NFAT5, we will combine chronic TCR triggering with elevated osmolarity of the cell culture media. Finally, we will compare conditions where NFAT was blocked for the last 48h by FK506 and conditions without FK506 blocking to understand the relevance of the NFAT/NFAT5 competition for the same binding sites at late stages. If the *in vitro* exhaustion protocol does not show any phenotype in NFAT5 KO CD8 T cells, we will set up a protocol using NFAT5-3xHA tag TILs sorted from tumors. In parallel, we are also cloning the TOX and PD-1 promoters in reporter constructs to test if their induction is NFAT5-dependent.

These experiments will demonstrate if NFAT5 binds to the promoters of genes downregulated upon NFAT5 KO, such as *Pdcd1*, *Tox*, and *Entpd1,* and if NFAT5 directly represses the expression of effector genes, such as *Ifng*, or if the phenotype relies on NFAT5-dependent TOX downregulation uniquely.

### 4. 3. 2 Local events driving NFAT5 expression and activity

We are intrigued by the NFAT5 expression profile of $T_{pex}$ and $T_{ex}$ in the TME and during chronic infection (Fig. 5b). The observed difference may be due to a specific niche in which the cells reside inside the tumor or spleen. Confocal microscopy will enable us to map NFAT5 expressing TILs and link local events in the TME to their appearance, e.g., necrosis or hypoxia. With this approach, we hope to understand if hypoxia can regulate NFAT5 expression in TILs *in vivo*. On the other hand, it will help us to understand the difference between $T_{ex}$ in the chronic infection model, which did not express NFAT5. Since $T_{ex}$ are more found in the circulation than in the spleen, they might not be exposed to TCR triggering as much as $T_{pex}$. Co-localization of NFAT5 expression in T cells and MHC expressing APC may give the answer.

### 4. 3. 3 Regulation of NFAT5

Still, there are open questions about how NFAT5 is differentially regulated during chronic infection and cancer and which signaling pathways are responsible for it. As suggested, confocal microscopy will give us an insight into the local events leading to NFAT5 expression. We performed scRNA-seq on sorted NFAT5 KO and WT P14 T cells from chronic infection. We plan to compare this data set to scRNA-seq of NFAT5 KO TILs to understand which regulators affecting NFAT5 activity and expression are differentially expressed in $T_{pex}$ and $T_{ex}$ in the two pathologies. To understand the difference in transcriptional activity, we plan to inhibit kinases involved in NFAT5 phosphorylation and compare their effect on our NFAT5 activity reporter construct *in vitro.* We hope to find candidates through the scRNA-seq data sets; otherwise, we will inhibit already described regulators. In this approach, we will also test a greater number of stimuli for their effect on NFAT5 activity, such as different cytokines, TIF, and co-culture with tumor cells. Since systemic use of inhibitors can have various effects, we plan to perform CRISPR/Cas9 mediated KO of promising regulators to investigate their impact on NFAT5 activity *in vivo*. For this approach, we would like to generate a new mouse strain with constitutive expression of the 6xTonE reporter construct and cross these mice to CD4-Cre Cas9 mice to perform the KO of NFAT5 regulators and compare the effect on NFAT5 activity in the TME.

Finally, we would like to investigate the downstream pathway leading to NFAT5 expression in our models, such as the TCR axis and response to hyperosmolarity. First, we want to broaden the stimuli tested *in vitro* on NFAT5 expression. Brx has been described to modulate NFAT5 expression upon osmotic stress in a p38 MAPK-dependent manner, but the exact mechanism

has yet to be discovered. Therefore, we plan to KO downstream targets of p38 MAPK and test the effect on NFAT5 expression. AP-1 and IRF family members are attractive candidates, as their binding sites in the *Nfat5* promoter were predicted[157].

### 4. 3. 4 Metabolism

NFAT5 has been described to be upregulated and control several metabolic stresses such as hyperlipidemia, hyperglycemia, hypoxia, and hypertonicity. NFAT5 is involved in ROS production following mitochondrial damage, and ROS in place induces NFAT5. NFAT5 can suppress the transcription of genes coding for essential proteins, e.g., components of ATP synthase, which could be involved in further worsening mitochondrial dysfunction in exhausted T cells[187]. In pancreatic ductal adenocarcinoma cells, NFAT5 regulated glycolysis by inducing its target gene phosphoglycerate kinase 1 (PGK-1), the first enzyme generating ATP in glycolysis[189]. In contrast to the positive regulation on glycolysis of NFAT5, it has been described to suppress the mTOR signaling pathway in intestinal cells[190].These studies indicate different regulatory role of NFAT5 on the metabolism of different cell types. Therefore we will assess the mitochondrial membrane potential and mitochondrial mass in NFAT5 KO TILs and compare them to WT TILs to understand if NFAT5 plays a role in the mitochondrial dysfunction seen in exhausted T cells.

## 4. 4. Significance of this work and future applications

Identifying a regulator of tumor-induced T cell exhaustion, which reacts to the specific microenvironment found in tumors, opens new strategies for treating cancer patients. Since NFAT1 is required for adequate T cell activation, targeting classical NFAT members will limit T cell effector functions. In contrast, NFAT5 is dispensable for T cell activation and effector function and could be targeted in CD8 T cells. Still, there are limitations since systemic NFAT5 inhibition would lead to many off-target effects, such as nephrotoxicity and defective pro-inflammatory function of macrophages and CD4 T cells. Therefore, NFAT5 inhibition directly in cellular therapy is the more promising approach. For example, during the adoptive cell therapy (ACT) protocol, patients' T cells are expanded *in vitro*, allowing a window for genetic engineering, such as KO of NFAT5, before transferring them back to the patients. With the same technique, CAR-T cells could be engineered to prevent their exhaustion in the TME. These approaches could be first tested *in vitro* by coculture of human T cells with human cancer cell lines under inhibition of NFAT5 with a small molecule inhibitor and be further carried out using CRISPR/Cas9 technology.

# 5. Appendix

## 5. 1. Contribution to articles

Article | 25 April 2022 | OPEN ACCESS TRANSPARENT PROCESS

### c-Maf enforces cytokine production and promotes memory-like responses in mouse and human type 2 innate lymphoid cells

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## Abstract

Group-2 innate lymphoid cells (ILC2s), which are involved in type 2 inflammatory diseases such as allergy, can exhibit immunological memory, but the basis of this ILC2 "trained immunity" has remained unclear. Here, we found that stimulation with IL-33/IL-25 or exposure to the allergen papain induces the expression of the transcription factor c-Maf in mouse ILC2s. Chronic papain exposure results in high production of IL-5 and IL-13 cytokines and lung eosinophil recruitment, effects that are blocked by c-Maf deletion in ILCs. Transcriptomic analysis revealed that knockdown of c-Maf in ILC2s suppresses expression of type 2 cytokine genes, as well as of genes linked to a memory-like phenotype. Consistently, c-Maf was found highly expressed in human adult ILC2s but absent in cord blood and required for cytokine production in isolated human ILC2s. Furthermore, c-Maf-deficient mouse or human ILC2s failed to exhibit strengthened ("trained") responses upon repeated challenge. Thus, the expression of c-Maf is indispensable for optimal type 2 cytokine production and proper memory-like responses in group-2 innate lymphoid cells.

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