UNIVERSITY OF CALIFORNIA SAN DIEGO

Understanding the role of NFAT2 transcription factors in T-cell Exhaustion

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The Thesis of Payal Ramchandani is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

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University of California San Diego

2020

DEDICATION

Foremost, I'd like to dedicate my thesis to my parents and my brother. Thank you for your constant love, support and encouragement.

I would also like to dedicate this to Jigar. Thank you for being my rock through the busiest and the hardest times and always pushing me to do my best.

Lastly, I would like to dedicate this to Dr. Hogan. Thank you for teaching me everything fundamental to shaping me into the researcher I am today. This would not have been possible without your help and guidance.

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LIST OF ABBREVATIONS

NFAT	Nuclear Factor of Activated T-cells
IL2	Interleukin 2
DBD	DNA Binding Domain
NHR	NFAT Homology Region
TAD	Transactivation Domain
TCR	T-cell Receptor
CD	Cluster of Differentiation
PLCγ	Phospholipase C - gamma
PIP_2	Phosphatidylinositol 4,5-bisphosphate
IP_3	Inositol 1,4,5-trisphosphate
DAG	Diacylglycerol
ER	Endoplasmic Reticulum
CRAC	Calcium release-activated channels
AP-1	Activator Protein 1
IFNγ	Interferon Gamma
IL10	Interleukin 10
IRES	Internal ribosome entry site
GFP	Green Fluorescent Protein
WT	Wildtype
CA	Constitutively Active

PD1	Programmed cell death protein 1
LAG3	Lymphocyte-activation gene 3
TIM3	T-cell immunoglobulin and mucin-domain containing-3
PMA	Phorbol 12-myristate 13-acetate
ATAC-Seq	Assay for Transposase-Accessible Chromatin using Sequencing

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Results are being prepared for submission for publication of the material. Ramchandani, Payal; Mognol, Giuliana P; González-Avalos, Edahí; Rao, Anjana; Hogan, Patrick G.The thesis author will be a co-author of this material.

ABSTRACT OF THE THESIS

Understanding the role of NFAT2 transcription factors in T-cell Exhaustion

by

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Master of Science in Biology

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Professor Anjana Rao, Chair
Professor James Kadonaga, Co-chair

The transcription factor Nuclear Factor of Activated T-cells (NFAT) plays a key role in determining the fate of a T-cell and is an important target for immune modulation. NFAT drives different programs of gene expression depending on availability of its partner proteins. For instance, in the presence of Activator Protein 1 (AP-1), it drives an effector immune response however, in the absence of AP-1, it drives the exhaustion program. T-cell exhaustion is a state of dysfunction caused due to persistent antigen exposure that occurs in cancer and chronic infections that hinders the ability of T-cell to respond to the antigen.

In this study we examine the role of different isoforms of NFAT2 in T-cell exhaustion by overexpressing constitutively active NFAT2 isoforms in $CD8^+$ T-cells. We show that contrary to what is believed, the short NFAT2 isoform (NFAT2αA) is not able to drive the exhaustion program by itself, as it does not impair $CD8^+$ T-cells ability to produce IL2. Additionally, we show that the early steps of the T-cell exhaustion program elicited by NFAT proteins, assessed as differences in protein expression and $CD8^+$ T cell function, do not require chromatin rearrangement. Our study provides a valuable insight into the role of NFAT2 proteins in the T-cell exhaustion program.

INTRODUCTION

NFAT Family of Transcription Factors

Nuclear Factor of Activated T-cells (NFAT) is a family of transcription factors that consists of five major proteins – NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5. NFAT1 and NFAT2 were first identified as a part of an inducible nuclear protein complex at the IL2 promoter in activated T-Cells [1, 2, 31, 32]. Although their role is most studied in lymphocytes, NFAT proteins are also found in other cells and play an essential role in the nervous system, hematopoietic stem cells, skeletal muscles, heart, kidney, and other important organs [2, 10].

Four of these proteins – NFAT1, NFAT2, NFAT3 and NFAT4 – are regulated by calcium signaling and have two conserved regions: the DNA-Binding Domain (DBD) and the NFAT Homology Region (NHR). The DBD is the most conserved region in the four NFAT proteins and allows the NFAT proteins to bind to the same DNA core sequence, (A/T)GGAAA (Figure 1). It is also known as the Rel Homology Domain (RHD) because it is structurally related to the DNA-Binding domain of the Rel/ NFκB factors [3]. The NHR is moderately conserved and contains serine residues that are phosphorylated in resting conditions, which restricts the NFAT proteins in the cytoplasm of the cell (Figure 1). It also contains the binding sites for calcineurin and NFAT kinases, which regulate the translocation of these proteins to and from the nucleus [2, 5, 33, 34] .

Knockouts of individual NFAT proteins in mice cause mild alterations in their immune function. This suggests that other NFAT proteins may compensate for the protein knockout, which demonstrates a certain level of redundant function across the NFAT proteins. This may be explained by their conserved DBD and NHR regions,

which allow NFAT proteins to bind to the same DNA sequence and interact with some of the same proteins.

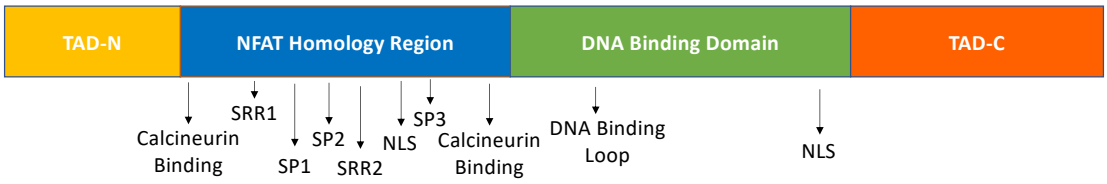

Figure 1: Schematic structure of NFAT family of transcription factors. Calcium-regulated NFAT proteins consist of an NFAT Homology Region, a DNA Binding Domain and Transactivation domains on the N and the C termini (TAD-N and TAD-C). The NHR contains the calcineurin docking sites and the serine rich motifs (SRR1, SP1, SP2, SRR2 and SP3) that are phosphorylated to keep the protein in the cytoplasm. The TAD-N and TAD-C are the least conserved in different NFAT proteins and their isoforms and contain transactivation/ repressor domains.

However, there are cases where the proteins and the isoforms of these proteins have distinct, or even opposite, functions. For example, while NFAT1 inhibits cell proliferation and induces apoptosis, NFAT2 is known to induce cell proliferation and inhibit T-cell apoptosis [6]. The variability in their functions could be explained either by the differences in their expression based on the cell-type or by their ability to partner with different proteins through their variable N and C termini. The N and the C termini are highly variable in the different NFAT proteins and their isoforms, and they contain trans-activation/ repression domains, which allow the NFAT proteins to interact with different partner proteins.

NFAT2 Isoforms

The murine NFAT2 gene has eleven exons and its expression is controlled by two promoters, P1 (upstream of exon 1) and P2 (upstream of exon 2), and two polyadenylation sites, pA1 (downstream of exon 9) and pA2 (downstream of exon 11) (Figure 2) [7, 8, 9]. Alternative promoter usage and alternative exon splicing of the NFAT2 gene can result in six different isoforms. Transcripts using P1, start at exon 1 and splice to exon 3, generating what are known as the α isoforms, whereas transcripts using P2 start at exon 2 and generate the β isoforms. The C terminus of the protein varies based on the polyAsite used. Transcripts using pA1 end at exon 9 and are known as the A or the short isoforms, whereas transcripts using at pA2 end at exon 11 and can generate either the B isoforms (uses partial exon 10 and splices to exon 11) or the C isoforms (uses complete exon 10).

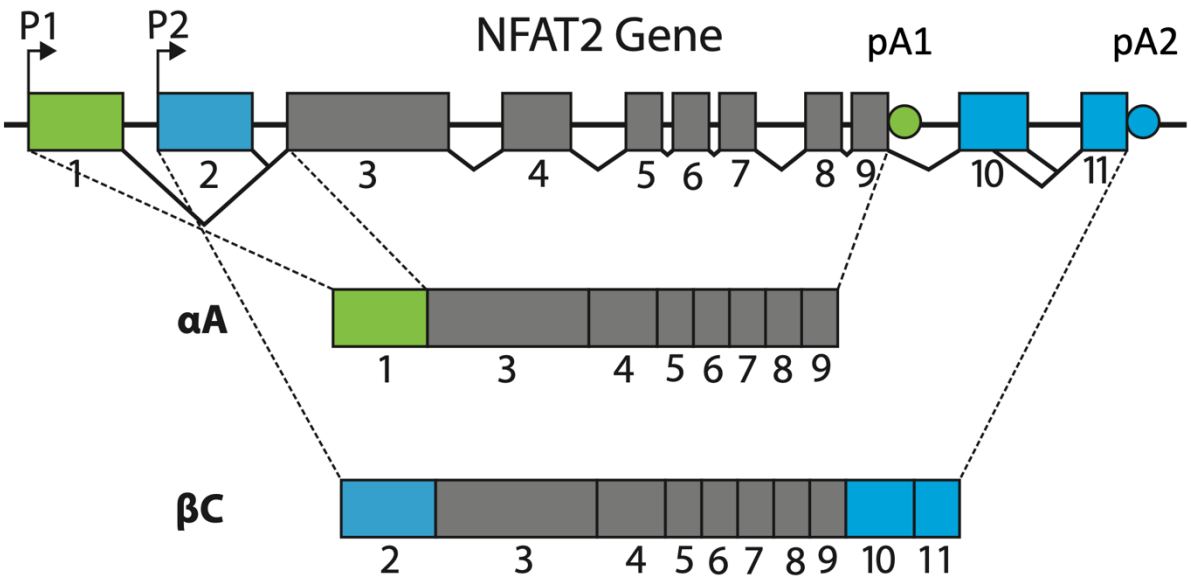

Figure 2: NFAT2 Gene Schematic and Different Isoforms. The NFAT2 gene has two different promoters and two different polyA-sites giving rise to multiple different isoforms. Two isoforms that differ in their N and C termini, and that are used in this study, are shown.

In resting T-cells, transcription starts at P2 and nearly always terminates at pA2, generating the βB (contains partial exon 10) or the βC (contains complete exon 10) isoform. Upon T-cell activation, the NFAT-inducible P1 promoter gains control and pA1 gets stronger, predominantly generating the short NFAT2αA isoform [9, 18, 28].

Figure 3: Schematic of NFAT proteins showing conserved TAD motifs. As in Figure 1, the TAD-N (yellow), NHR (blue), DBD (green) and TAD-C (orange) of the NFAT proteins are shown. Conserved motifs in TAD-N and TAD-C are shown as black, dark grey, and light grey squares.

The region between exon 3 and 9 is conserved amongst all of the NFAT2 isoforms and contains the DBD and the NHR. The isoforms only differ in the TAD-N and TAD-C regions, which contain trans-activation/ repression domains. While the N and C termini also vary considerably between different NFAT proteins, they have a few recognizable conserved motifs. As observed in figure 3, NFAT1 and NFAT2βC contain the conserved motifs in their TAD-N and TAD-C regions. However, those motifs are absent from NFAT2αA [2, 9, 20]. Thus, NFAT2αA is unique as it differs from NFAT2βC and NFAT1 in its structure and inducibility, making it an interesting isoform to study.

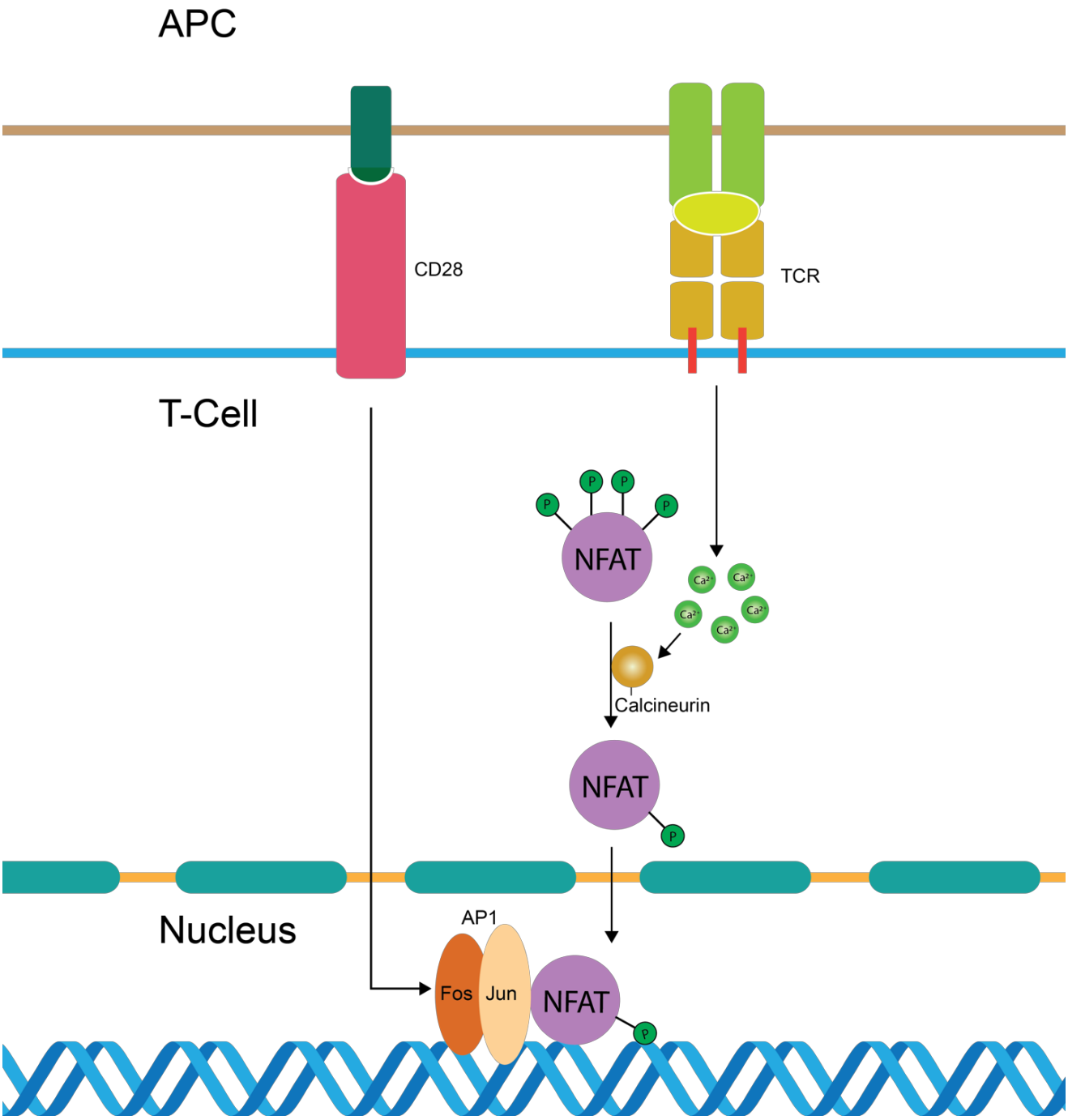

Figure 4: Mechanism of T-cell Activation. TCR recognition of an antigen triggers signaling that leads to an increase in intracellular calcium levels activating calcineurin, which dephosphorylates NFAT, causing it to translocate to the nucleus. Simultaneously the co-stimulatory signal through the CD28 molecules leads to activation of Fos and Jun proteins. Together these proteins turn on the T-cell activation program.

NFAT Regulation in T-Cells

The three major NFAT proteins in T-cells - NFAT1, NFAT2 and NFAT4 – are activated as a result of calcium signaling. TCR priming leads to PLCγ activation which hydrolyses PIP_2 to IP_3 and DAG. IP_3 binds to its receptor in the ER membrane, inducing an increase in cytoplasmic calcium as a result of release of stored Ca^{2+} from the ER. Loss of Ca^{2+} from the ER also triggers opening of Ca^{2+}-selective CRAC channels in the plasma membrane to maintain the increase in cytoplasmic Ca^{2+} levels. The calcium binds to calmodulin and activates calcineurin, a phosphatase, which dephosphorylates the serine motifs in the NHR of NFAT (Figure 4) [10, 11]. This induces a conformational change of the NFAT protein and exposes its nuclear localization signal, leading to its translocation to the nucleus. Continued elevation of cytoplasmic Ca^{2+} during signaling keeps NFAT dephosphorylated and in the nucleus [10].

In T-cell activation, the signaling through the TCR triggers an increase of intracellular Ca^{2+}, activating NFAT proteins. Simultaneously, the cells are receiving costimulatory signals through the CD28 molecules that lead to efficient activation of Fos and Jun proteins that form the AP-1 complex. Together with AP-1, the NFAT proteins elicit transcription of various effector genes such as IL2, IFNγ and GM-CSF, producing an effective immune response [2].

In case of chronic viral infections or cancer, there is persistent TCR stimulation in absence of adequate co-stimulation, causing NFAT activation that is not balanced by AP-1 activation. In the absence or relative deficiency of AP-1 proteins, NFAT, either by itself or with unknown partner proteins, turns on a completely different transcriptional program known as T-cell exhaustion [12, 19].

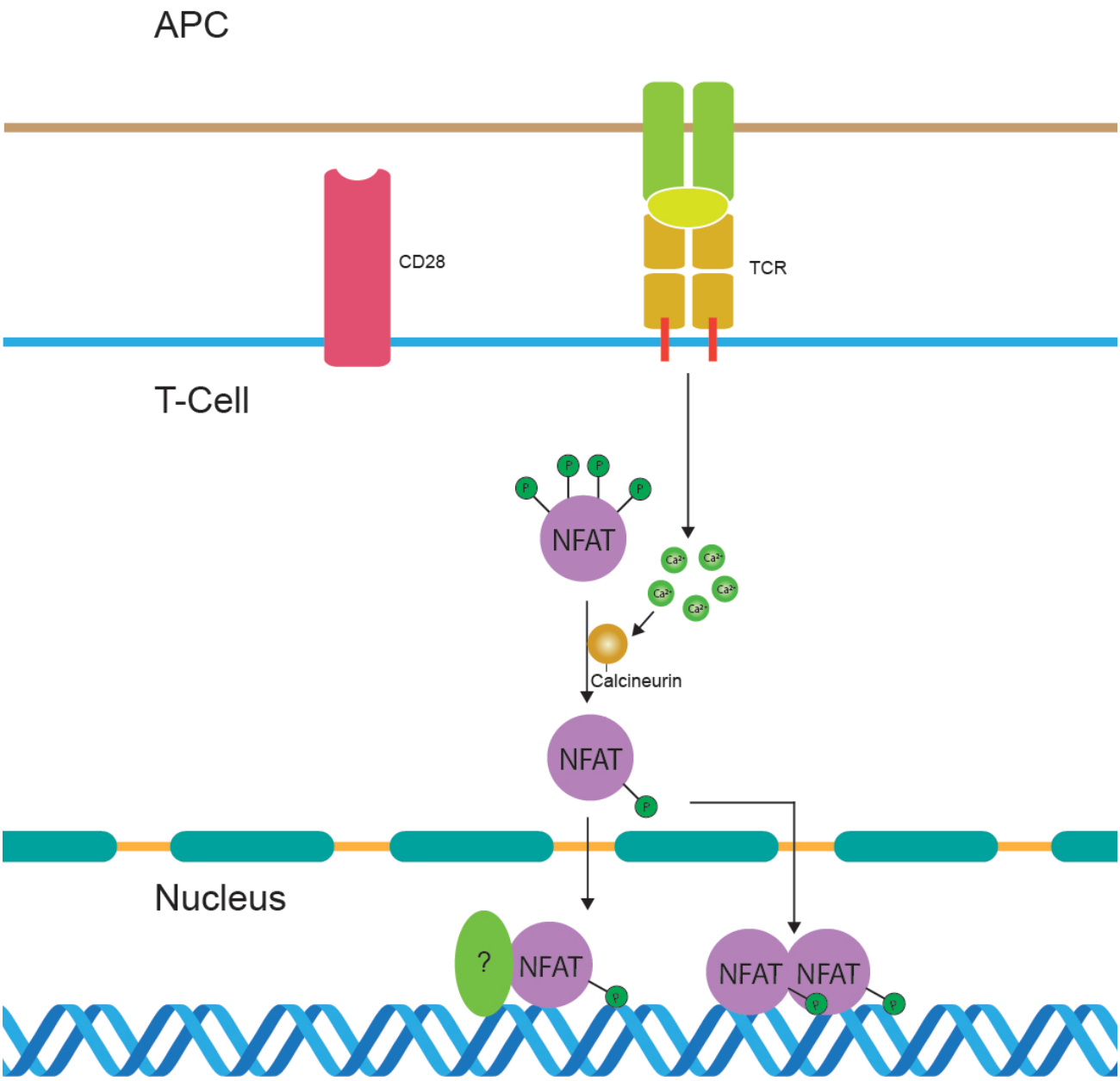

Figure 5: Mechanism of T-cell exhaustion. In absence of co-stimulation, or with diminished co-stimulation, TCR signaling leads to activation of NFAT without the presence of adequate levels of Fos and Jun proteins. In these conditions, NFAT by itself or with unknown partner proteins turns on the epigenetic program of T-cell exhaustion.

T-Cell exhaustion

T-cell exhaustion is a state of dysfunction that results in loss of the ability to proliferate and perform effector functions in response to antigen, and loss of the ability to form memory T cells. It is characterized by sustained expression of inhibitory receptors, loss of cytokine production and altered metabolism and transcriptional program [12, 13, 14, 15, 17].

Our lab has previously shown that introducing a mutant form of NFAT1, CA-RIT-NFAT1, into $CD8^+$ T-cells initiates a program of gene expression characteristic of T-cell exhaustion [12]. CA-RIT-NFAT1 was engineered to be constitutively active (CA), by mutating the serine residues that are usually phosphorylated to alanine to ensure that the protein is always in the nucleus and able to bind its target sites in DNA. Additionally, it has the RIT mutations (R468, I469 and T535) that render it to be defective in interaction with AP-1 proteins, the normal partners of NFAT in the T-cell activation program, shifting the balance of transcription toward the exhaustion program [29].

However, separate experiments with NFAT2- and NFAT4- deficient $CD8^+$ T-cells have indicated that NFAT1 alone is not sufficient for efficient expression of all exhaustion related genes. Furthermore, the cells transduced with CA-RIT-NFAT had elevated levels of *NFAT2* mRNA [12]. These results suggest that either NFAT1 and NFAT2 work together in driving the exhaustion program or CA-RIT-NFAT1 is simply upregulating NFAT2 which predominantly drives the exhaustion program. A similar upregulation of *NFAT2* mRNA was observed in exhausted tumor-infiltrating lymphocytes (TILs) and in exhausted T-cells isolated from mice with chronic LCMV infection [21, 22]. As a result of elevated *NFAT2* mRNA in exhausted cells and findings for NFAT2

knockout cells, other groups have proposed that NFAT2, specifically the upregulated isoform NFAT2αA, is the dominant NFAT family member controlling exhaustion [21].

However, the role of NFAT2 in driving the exhaustion program has not been studied directly. Our study aims to understand the role of NFAT2 in T-cell exhaustion and determine whether the different isoforms have distinct roles in the exhaustion program.

RESULTS

We generated engineered versions of the NFAT2 proteins – CA-NFAT2αA, CA-RLT-NFAT2αA, CA-NFAT2βA, CA-RLT-NFAT2βA, CA-NFAT2βC and CA-RLT-NFAT2βC. All the proteins are constitutively active (CA) as the serine residues in the NHR have been mutated to alanine to ensure that the proteins are always in the nucleus and able to bind DNA [29]. Some constructs were mutated further (RLT) to prevent the interaction of the expressed proteins with AP-1 (R485A/ L486A and T552G in case of CA-RLT-NFAT2αA and R471A/ L472A and T538G in case of CA-RLT-NFAT2βA and CA-RLT-NFAT2βC) [19, 30]. These engineered proteins are used to simulate the conditions the T-cell experiences in chronic viral infections or cancer, where the constant TCR signaling results in NFAT always being in the nucleus without AP-1 partner proteins.

The experimental scheme is adopted from a previous study by Martinez et al that studied the role of NFAT1 in T-cell exhaustion [12]. They used similarly engineered NFAT1 protein – CA-RIT-NFAT1 – and expressed it *in vitro* to study the effects on characteristics of exhaustion such as sustained inhibitory receptors and diminished cytokine production. Additionally, they demonstrated that CA-RIT-NFAT1-transduced cells adoptively transferred on day 6 have impaired function *in vivo* in viral and tumor clearance assays. Hence, the experiments in this study were replicated under the same conditions and timeline.

NFAT1 and NFAT2 deficient T-cells transduced with constitutively active NFAT proteins display characteristics of exhaustion

NFAT2 mRNA is elevated in $CD8^+$ T-cells transduced with CA-RIT-NFAT [12]. Therefore, to evaluate the role of NFAT2 in exhaustion, we first used cells deficient for NFAT1 and NFAT2 to study the effects of the proteins in isolation from the effects of endogenous NFAT proteins. $CD8^+$ T-cells were isolated from spleens of $NFAT1^{-/-}$ $NFAT2^{fl/fl}$ CD4 Cre mice that have a complete knockout of NFAT1 and a conditional knockout of NFAT2 in T-cells, the latter because complete knockout of NFAT2 is embryonic lethal. The cells were stimulated with anti-CD3 and anti-CD28 and transduced with an IRES-GFP retrovirus expressing one of the proteins – Empty (control), WT-NFAT1 (negative control), CA-NFAT1 (positive control), CA-RIT-NFAT1(positive control), CA-NFAT2αA, CA-RLT-NFAT2αA, CA-NFAT2βA or CA-RLT-NFAT2βA. Then, the cells were cultured in T-cell medium with 10U/mL IL2 and on day 5 the cells were either left unstimulated or re-stimulated with 10 nM PMA and 1 μM ionomycin for 6 hours to assess expression of extracellular markers and cytokines. Cells transduced with constitutively active NFAT1 and NFAT2 express higher amounts of inhibitory receptors compared to cells transduced with WT-NFAT1 and empty vectors. While the PD1 and LAG3 expression are considerably higher in constitutively active NFAT-cells, TIM3 expression is only slightly increased as observed by the slight right shift in the histograms (Figure 6B).

For cytokine production, a fraction of cells transduced with empty vector produced IL2, likely because of the endogenous NFAT4 in the cells (Figure 6C). As expected, a larger fraction of cells transduced with WT-NFAT1 expressed IL2 after re-

stimulation than in the case of cells transduced with empty vector, as NFAT1 plays a pivotal role in IL2 production [1, 2, 31, 32]. Fewer cells transduced with CA-NFAT1 and CA-RIT-NFAT1 expressed IL2 compared to cells transduced with empty vector, showing the hypo-responsiveness, as previously reported by Martinez et al [12]. In the initial experiments, similar observations were made for cells expressing CA-NFAT2αA, CA-RLT-NFAT2αA and CA-RLT-NFAT2βA, while CA-NFAT2βA had no effect on IL2 production compared to empty-vector transduced cells (Figure 6C).

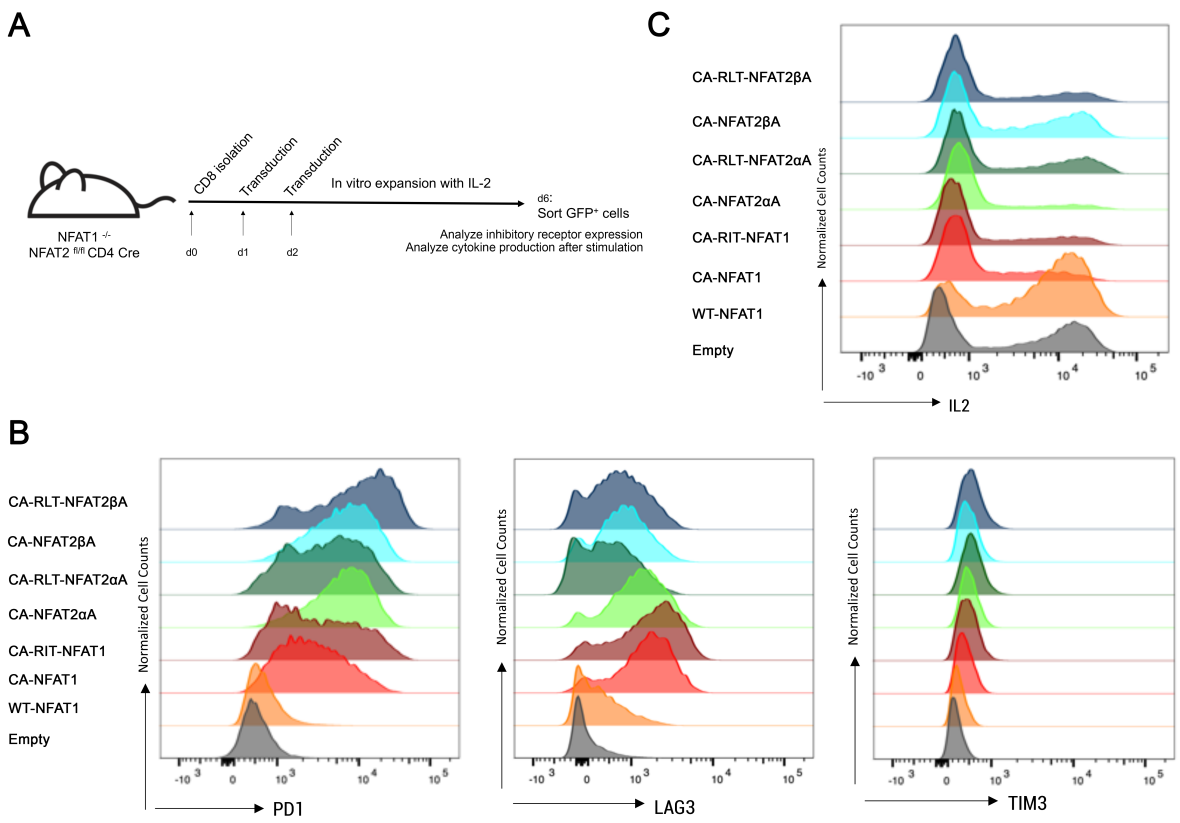

Figure 6: NFAT1- and NFAT2-deficient CD8⁺ T-cells overexpressing constitutively active forms of NFAT proteins showed increased expression of markers associated with exhaustion and reduced IL2 production. (A) Experimental design for assessing extracellular markers and cytokines of CD8⁺ T-cells transduced with various NFAT constructs. (B) Representative histograms of PD1 (left), LAG3 (middle) and TIM3 (right) expression in NFAT1 and NFAT2 deficient CD8+ T-cells transduced with different NFAT constructs on d5. All samples gated on live GFP +ve cells. (C) Histogram of IL2 production by NFAT1 and NFAT2 deficient CD8+ T-cells transduced with different NFAT constructs after 6-hour re-stimulation with 1μM ionomycin and 10 nM PMA on d5. All samples gated on live GFP +ve cells.

NFAT1- and NFAT2-deficient T cells display impaired development and an increased amount of an innate-like CD8$^+$ population

However, the trends for IL2 production by cells transduced with the different NFAT constructs were not consistent from experiment to experiment (Figure 7A). For example, in some cases cells transduced with CA-NFAT1 and CA-RIT-NFAT1 were producing as much IL2 as the cells transduced with empty vector, implying that CA-NFAT1 and CA-RIT-NFAT1 did not make the cells hyporesponsive. Additionally, the DNA accessibility profile of cells transduced with empty vector and cells transduced with CA-RIT-NFAT1 looked identical, contradicting previously published data (Figure 7B) [16]. Since the previous ATAC-seq data were obtained from WT-cells transduced with empty vector or CA-RIT-NFAT1, we wondered if the difference observed was because of deficiency of NFAT1 and NFAT2.

To investigate the difference between our cells versus WT cells, we performed ATAC-seq for naïve cells that were deficient for NFAT1 and NFAT2 and compared the observed pattern of chromatin accessibility to existing data from WT naïve cells and WT effector CD8$^+$ T-cells. We compared the top 3000 accessible peaks of WT naïve cells [16] to our data for NFAT1- and NFAT2-deficient naïve cells. Additionally, we compared the top 3000 peaks of WT effector cells [16] to the data for NFAT1- and NFAT2-deficient naïve cells. To our surprise, the chromatin accessibility signature of NFAT1- and NFAT2-deficient naïve cells had more resemblance to WT effector cells than to WT naïve cells (Figure 7C). Consulting the literature, we learned that spleens from NFAT2 $^{fl/fl}$ CD4-Cre mice have a major subset of 'innate-like' CD8+ T-cells that have expanded

in the thymus because of increased amounts of IL4 [23] and that might contribute to our unexpected results.

To confirm that this 'innate-like' subset of cells was present in our $CD8^+$ T-cell population, we used flow cytometry to determine expression of CD8α and CD8β molecules: Conventional $CD8^+$ T-cells express both CD8α and CD8β but innate-like $CD8^+$ T-cells do not express CD8β. $CD8^+$ T-cells were negatively isolated from spleens of WT and $NFAT1^{-/-}$ $NFAT2^{fl/fl}$ CD4 Cre mice and stained for CD8α and CD8β. While the cells obtained from WT mice expressed both CD8 molecules, splenocytes from the animals deficient for NFAT1 and NFAT2 in T cells comprised a mixture of populations – cells expressing both CD8α and CD8β (conventional CD8+ cells), cells expressing only CD8α (suspected innate-like cells) and cells expressing neither of the CD8+ molecules, demonstrating the presence of the innate-like CD8+ T-cells (Figure 7D).

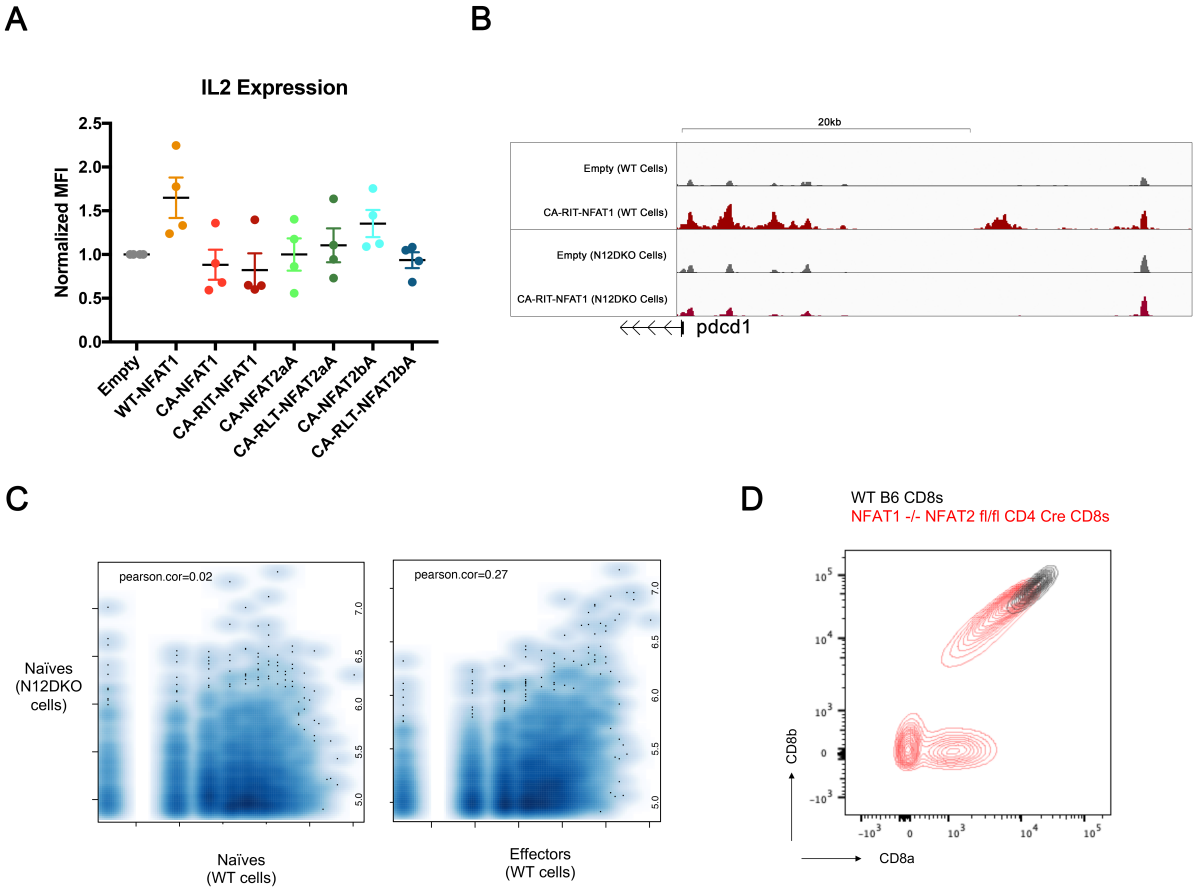

Figure 7: NFAT1 and NFAT2 double knockout T-cells have an increased population of innate-like CD8⁺ T-cells. (A) Normalized mean fluorescence intensities of 4 experiments measuring IL2 expression evoked by PMA + ionomycin, in NFAT1- and NFAT2-deficient CD8+ T-cells that had been transduced with different NFAT constructs. Normalized mean ± SEM for 4 independent experiments are shown, with the values for empty vector transduced cells set to 1. (B) Genome browser view illustrating a striking difference at the *Pdcd1* locus between previously published data in WT CD8⁺ T-cells transduced with empty or CA-RIT-NFAT1 vectors (top 2 rows) [16] and our data for NFAT1 and NFAT2 knockout CD8⁺ T-cells transduced with empty or CA-RIT-NFAT1 vectors (bottom 2 rows). (C) Scatterplot comparing top 3000 accessible peaks in WT naïve cells (left) and WT effector cells (right) to NFAT1 and NFAT2 deficient naïve cells. The graph shows the Pearson correlation between the cells compared. (D) Contour plot showing CD8α and CD8β expression for cells isolated from WT (black) or NFAT1$^{-/-}$ NFAT2 $^{fl/fl}$ CD4 Cre (red) mice. (Analysis in B and C by Edahi Gonzalez Avalos)

WT CD8$^+$ T-cells transduced with constitutively active NFAT proteins show phenotypic and functional characteristics of exhaustion

Since NFAT1- and NFAT2-deficient T cells were found to have developmental impairments, we turned to assessing the impact of different NFAT constructs in WT-cells. CD8$^+$ T-cells were isolated from spleens of WT B6 mice and stimulated with anti-CD3 and anti-CD28. A day later, the cells were infected with IRES-GFP retrovirus expressing one of the vectors – Empty (negative control), CA-NFAT1 (positive control), CA-RIT-NFAT1 (positive control), CA-NFAT2αA, CA-RLT-NFAT2αA, CA-NFAT2βC or CA-RLT-NFAT2βC and cultured in T-cell media with 10U/mL IL2 to generate memory-like CD8$^+$ T-cells. On day 6, cells were analyzed for the expression of inhibitory receptors such as PD1, LAG3 and TIM3. Compared to cells transduced with empty vector, cells expressing the constitutively active NFAT proteins tend to express higher amounts of PD1 and LAG3 proteins (Figure 8B and 8C). Perhaps due to scatter in the data and the correction for multiple testing, the upregulation of PD1 scores as statistically significant only for CA-NFAT2βC and CA-RLT-NFAT2βC. (Figure 8C). However, the percent of cells expressing PD1 is significantly higher in all constitutively active NFAT proteins except CA-RIT-NFAT (Figure S1A). LAG3 expression and percent of cells expressing LAG3 are preferentially increased by CA-NFAT1 and CA-RIT-NFAT1 (Figure 8C and S1A). TIM3 expression is not impacted much by the NFAT constructs, consistent with the previous finding that the effect of CA-RIT-NFAT1 on TIM3 is typically small.

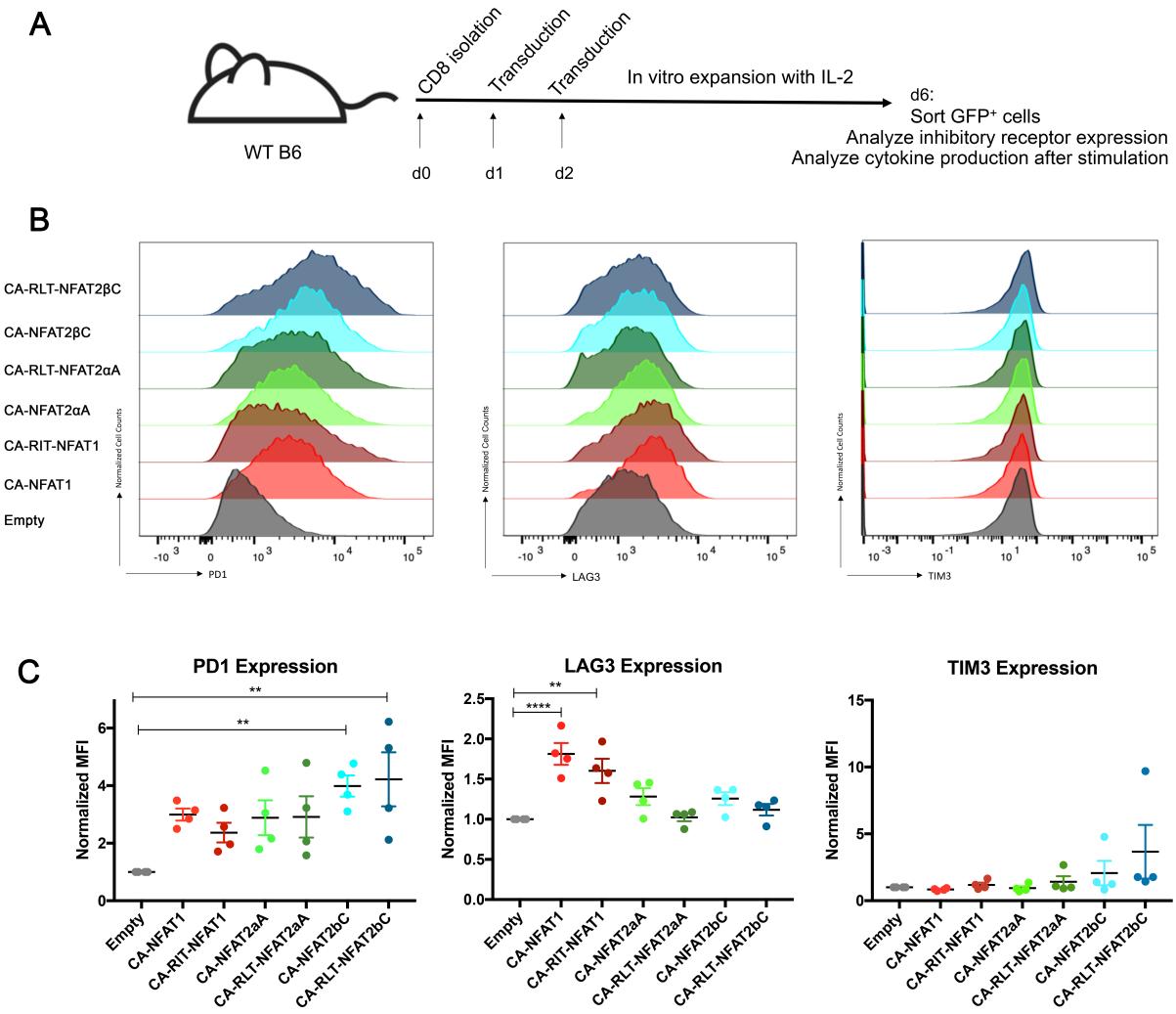

Figure 8: Overexpression of constitutively active forms of NFAT proteins in WT CD8⁺ T-cells results in increased expression of markers associated with exhaustion. (A) Experimental design for assessing extracellular markers and cytokines of CD8⁺ T-cells transduced with various NFAT constructs. (B) Representative histograms of PD1 (left), LAG3 (middle) and TIM3 (right) expression in WT CD8⁺ T-cells transduced with different NFAT constructs and examined without restimulation on d6. All samples gated on live GFP+ve cells. (C) Normalized mean fluorescence intensities of 4 experiments for PD1 (left), LAG3 (middle) and TIM3 (right) expression in WT CD8⁺ T-cells transduced with different NFAT constructs. Normalized mean ± SEM for 4 independent experiments are shown, with the values for empty vector transduced cells set to 1; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Transduced $CD8^+$ T-cells were further analyzed for cytokine production in response to restimulation, to determine whether constitutively active NFAT proteins have the effect of damping down effector functions. The cells were re-stimulated on d6 with 10 nM PMA and 1 μM ionomycin (to mimic signaling through the T-cell receptor and costimulatory molecule) and IL2 and IFNγ production was analyzed by flow cytometry. In this assay, there was a clear difference between sets of cells. Cells transduced with CA-NFAT1, CA-RIT-NFAT1, CA-NFAT2βC and CA-RLT-NFAT2βC exhibited significantly reduced IL2 production, whereas cells transduced with CA-NFATαA and CA-RLT-NFAT2αA showed no impact on IL2 production (Figure 9A and 9B). This observation suggests that the short isoform of NFAT2 (NFATαA) is doing something different than NFAT1 and NFAT2βC. This could be because of difference in expression of the NFAT proteins however we know that there is sufficient NFAT2αA (CA/RLT) in the cells to turn on expression of PD1 to the same extent as with NFAT1 (CA/RIT) and NFAT2βC (CA/RLT). Additionally, a preliminary check by western blot indicated equal expression of all the constitutively active NFAT proteins. The relative expression levels will be further judged by western blot because all the proteins carry the same epitope tag.

Additionally, CA-NFAT2βC and CA-RLT-NFAT2βC had a tendency to reduce IFNγ MFI and the percentage of cells producing IFNγ slightly, although the changes did not reach statistical significance (Figure 9A, 9B and S1B). Loss of the ability to make IFNγ upon stimulation occurs only at late stages of exhaustion *in vivo* [13].

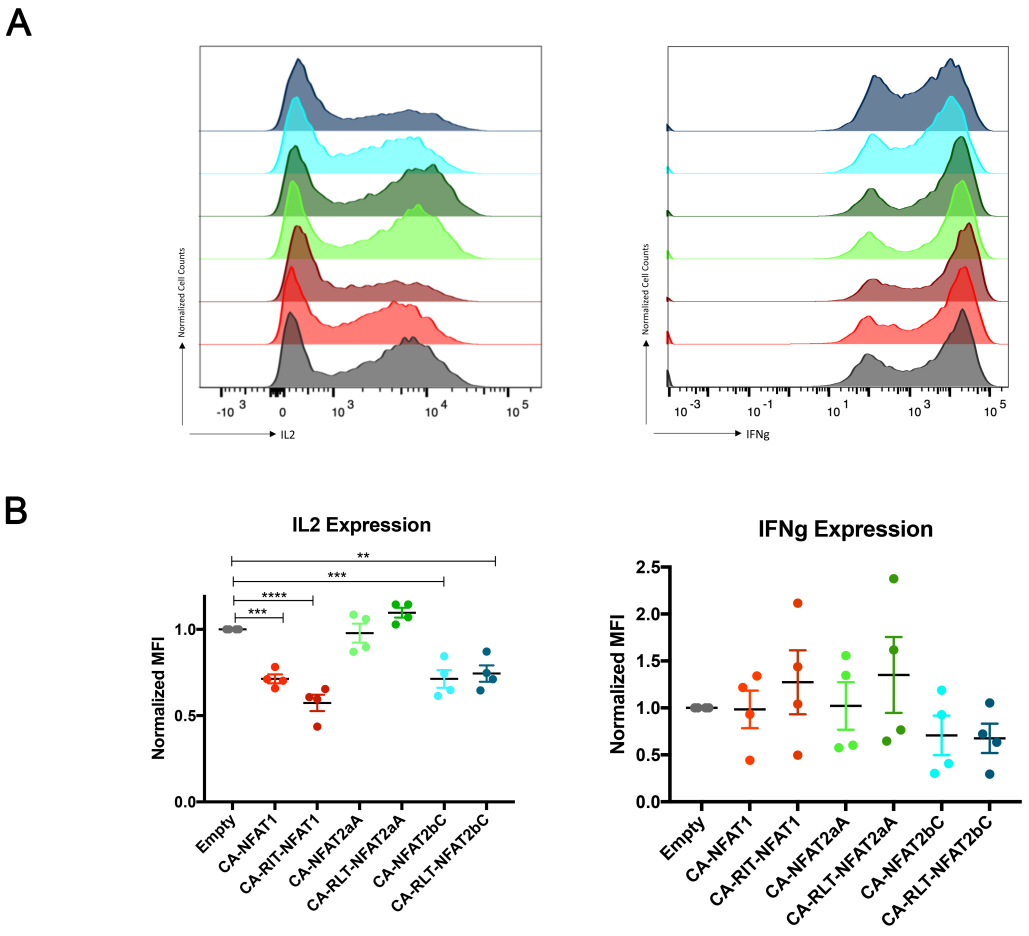

Figure 9: Effect of overexpression of constitutively active forms of NFAT proteins on evoked cytokine production in WT B6 CD8⁺ cells. (A) Representative histograms of IL2 (left) and IFNγ (right) production by WT CD8⁺ T-cells that had been transduced with different NFAT constructs, after 6 hour restimulation with 1μM ionomycin and 10 nM PMA on d6. All samples gated on live GFP +ve cells. (B) Normalized mean fluorescence intensities of 4 experiments for IL2 (left) and IFNγ (right) expression in WT CD8+ T-cells transduced with different NFAT constructs. Normalized mean $\pm$ SEM for 4 independent experiments are shown, with the values for empty vector transduced cells set to 1; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Changes observed in protein expression are not reflected in chromatin accessibility changes

To determine if the changes observed in protein expression are due to the NFAT proteins differentially opening or closing the chromatin, we performed ATAC-Seq. Total $CD8^+$ cells freshly isolated from spleen or cells transduced with Empty vector or with different constitutively active NFAT proteins were lysed, the transposition reaction was performed, and libraries were prepared to assess chromatin accessibility.

We observed that chromatin accessibility changes extensively as a result of stimulation with anti-CD3 and anti-CD28 and expansion *in vitro*. Cells transduced with Empty vector, expanded, and recovered on day 6 (Empty, in Figure 10A and 10B) showed 35,148 regions with increased accessibility compared to $CD8^+$ T-cells freshly isolated from the spleen (Total, in Figure 10A and 10B). However, cells expressing constitutively active NFAT proteins showed only very subtle further changes in chromatin accessibility as compared to Empty vector transduced cells (Figure 10C (left)). In particular, the changes observed in protein expression of PD1, LAG3 and IL2 amongst the NFAT transduced samples are not reflected as changes in chromatin accessibility (Figure S2A, S2B and S2B). For instance, CA-NFAT1, CA-RIT-NFAT1, CA-NFAT2βC and CA-RLT-NFAT2βC expressing cells produce significantly less IL2 compared to cells expressing CA-NFATαA and CA-RLT-NFAT2αA, however no qualitative change is observed in peaks in the *IL2* locus between different samples (Figure S2B).

Contrary to our expectations, the overall results suggest that chromatin accessibility does not change substantially due to the expression of different NFAT

proteins at this early time point (Figure 10C) and that the differences in protein expression are not a result of opening or closing of chromatin by the NFAT proteins. A caveat regarding PD1 and LAG3 proteins is that their elevated levels on day 6 might be due to changes in chromatin on prior days that have returned to the baseline of empty vector-transduced cells by day 6. This possibility will have to be tested by comparing chromatin accessibility in NFAT-transduced and empty vector-transduced T cells at earlier times in the experiment. However, the general conclusion is not altered by this caveat, since IL2 induction was measured here on day 6, and previous RNA-seq data documenting gene expression driven by CA-RIT-NFAT1 [12] were also obtained under the same experimental conditions on day 6.

Results are being prepared for submission for publication of the material. Ramchandani, Payal; Mognol, Giuliana P; González-Avalos, Edahí; Rao, Anjana; Hogan, Patrick G.The thesis author will be a co-author of this material.

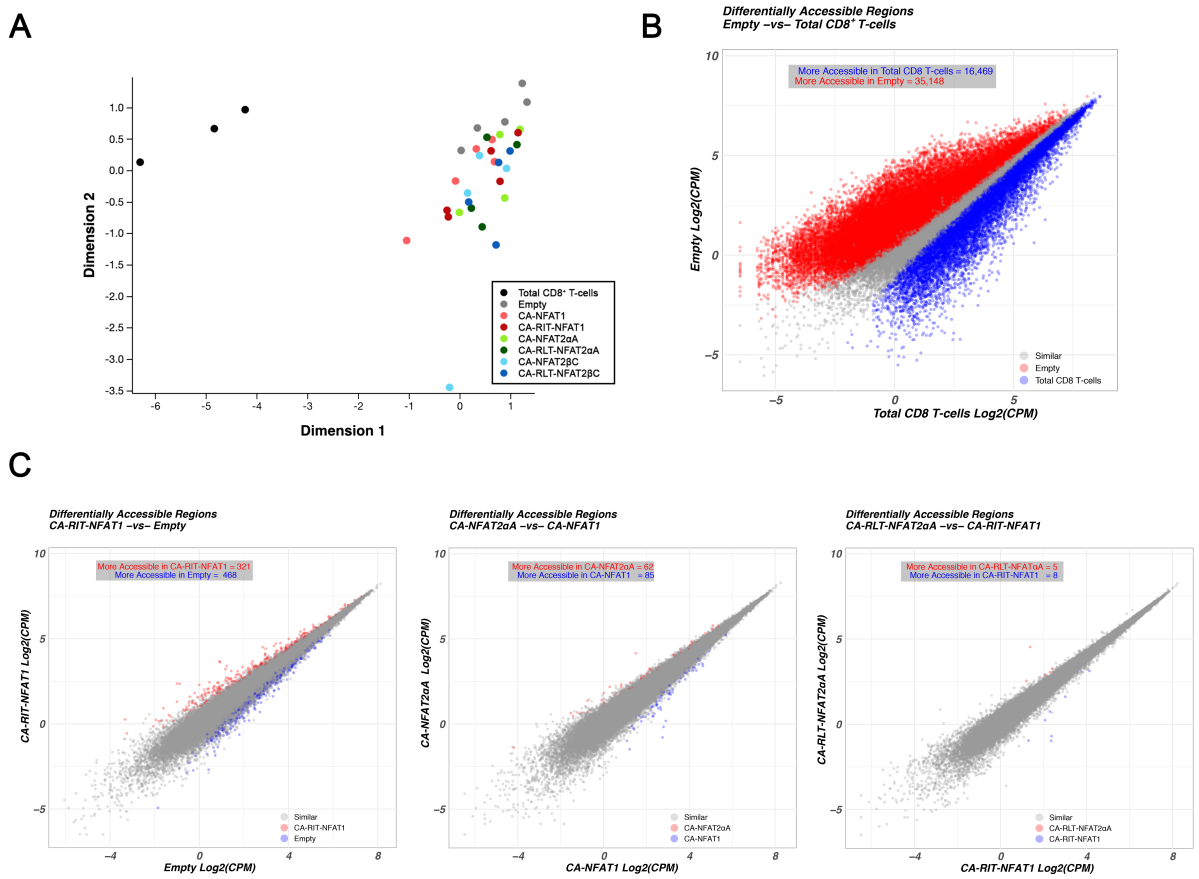

Figure 10: Stimulation and expansion of CD8⁺ T cells *in vitro* causes major changes in chromatin accessibility, but the expression of constitutively active NFAT proteins does not elicit additional dramatic changes. (A) Principal component analysis of peak accessibility in Total CD8⁺ T-cells directly isolated from spleen (black) and in cells transduced with Empty vector (grey), CA-NFAT1 (light red), CA-RIT-NFAT1 (dark red), CA-NFAT2αA (light green), CA-RLT-NFAT2αA (dark green), CA-NFAT2βC (light blue) and CA-RLT-NFAT2βC (dark blue). (B) Scatterplot comparing the averaged ATAC-Seq signal of individual regions in CD8⁺ T-cells transduced with Empty vector to Total CD8⁺ T-cells at the time of isolation from spleen in the same experiments. Each dot represents a peak. Regions more accessible in Empty-vector expressing cells than Total CD8⁺ T-cells are highlighted in red. Adjusted p value < 0.05 (C) Scatterplots of mean ATAC-Seq signal of individual regions, comparing cells expressing CA-RIT-NFAT1 to cells expressing Empty vector (left), cells expressing CA-NFAT2αA to cells expressing CA-NFAT1 (middle) and cells expressing CA-RLT-NFAT2αA to cells expressing CA-RIT-NFAT1 (right). Analysis in A, B and C is done by Edahi Gonzalez Avalos.

SUPPLEMENTARY RESULTS

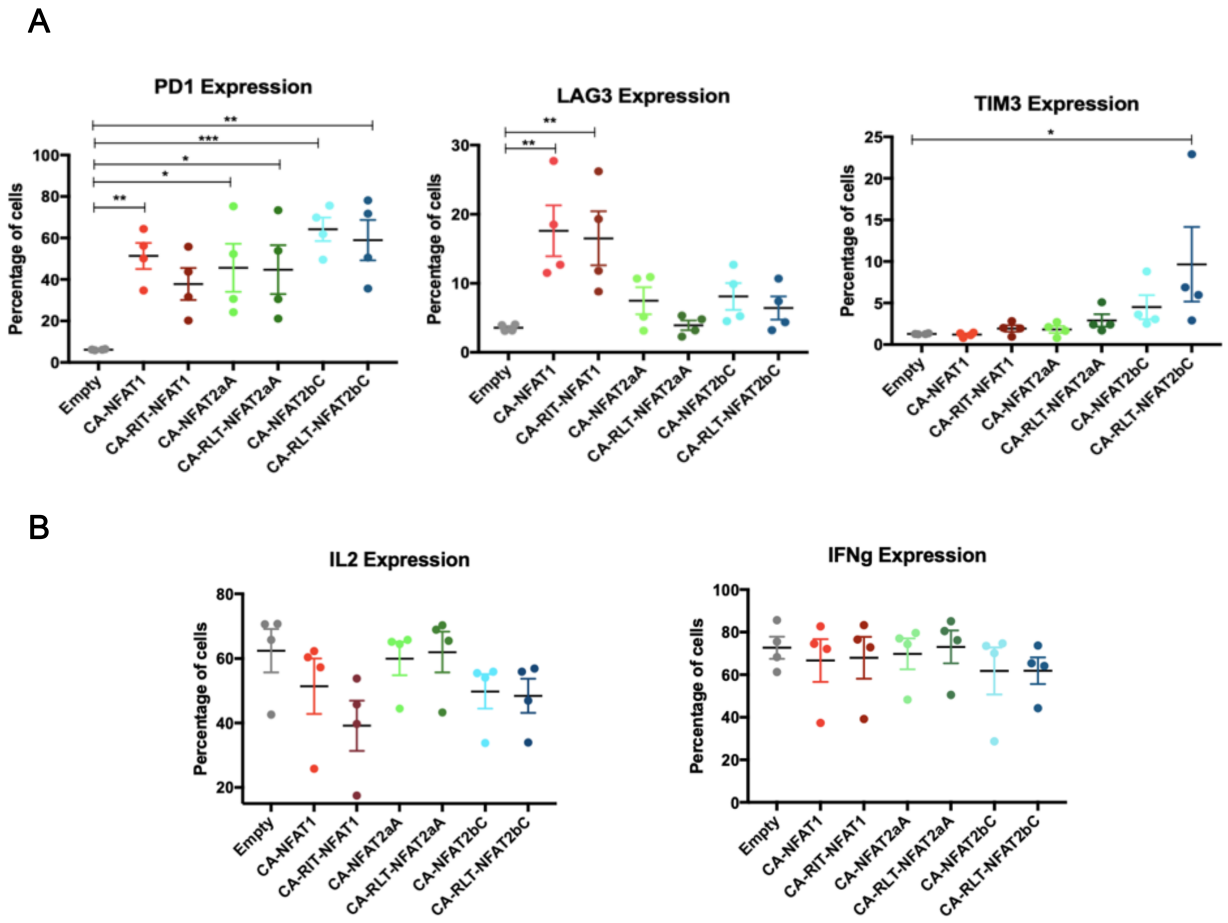

Figure S1: Effect of overexpression of constitutively active forms of NFAT proteins on percent of cells expressing inhibitory receptors and cytokines. (A) Percentage of cells expressing PD1 (left), LAG3 (middle) and TIM3 (right) when transduced with various constructs. Mean ± SEM for 4 independent experiments are shown, with the values for empty vector transduced cells set to 1; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (B) Percentage of cells expressing IL2(left) and IFNγ (right) when transduced with various constructs. Mean ± SEM for 4 independent experiments are shown.

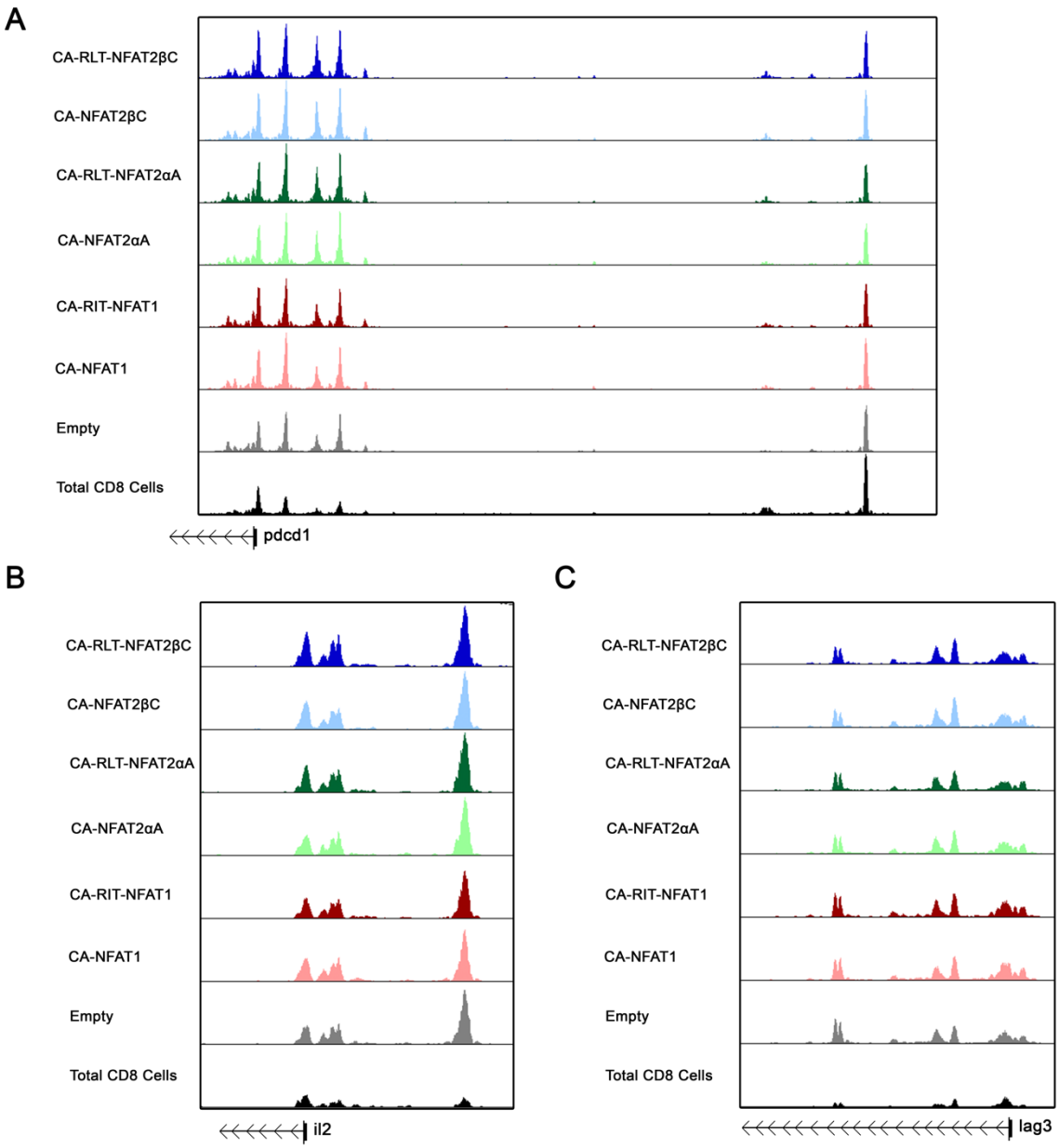

Figure S2: Lack of effect of overexpression of constitutively active forms of NFAT proteins on chromatin accessibility. Genome browser views of ATAC-Seq signal at the *Pdcd1* (A), *Il2* (B) and *Lag3* (C) loci, showing mean data of all replicates for Total CD8$^+$ cells from spleen (black) or cells transduced with empty vector or different NFAT expression vectors. Analysis done by Edahi Gonzalez Avalos (A, B and C).

DISCUSSION

In this study, we demonstrate that NFAT2 proteins can participate directly in the transcriptional program of T-cell exhaustion. We generated mutant NFAT2 proteins that were constitutively active (CA) and in some cases had additional mutations to prevent its interaction with AP-1 partner proteins (RLT). The CA-NFAT2 and CA-RLT-NFAT2 proteins *in vitro* simulate the conditions during a chronic viral infection or cancer as the NFAT proteins are always in the nucleus in absence of AP-1 complex, their partners during T-cell activation. These constructs were retrovirally transduced into $CD8^+$ T-cells to study the effects of these proteins on the exhaustion program in isolation from the activation program. The cells were then analyzed for characteristics of exhaustion.

NFAT1/NFAT2 double knockout cells seemed a logical choice for the analysis and were used in the initial experiments. However, while expression of constitutively active NFAT proteins in these cells did elicit characteristic markers of the exhaustion program, namely the upregulation of inhibitory receptors, the cells also exhibited troubling developmental differences as compared to WT $CD8^+$ T-cells. Not only was there an increased population of innate-like cells in the spleens of the $NFAT1^{-/-}$ $NFAT2^{fl/fl}$ CD4 Cre mice, but also the naïve conventional $CD8^+$ cells isolated from the $NFAT1^{-/-}$ $NFAT2^{fl/fl}$ CD4 Cre mice had a chromatin accessibility profile resembling that of WT effector T-cells (Figure 6C). Additionally, the cells transduced with CA-RIT-NFAT, our positive control [12], did not always exhibit a reduction in IL2 production when stimulated, compared to empty-vector control cells (Figure 6A). The increased amounts of innate-like cells can be chalked up to the presence of elevated levels of IL4 in the thymus during T-cell development in the absence of NFAT2 as observed by Pachulec

and colleagues [23]. However, it also possible that the altered thymic environment is affecting the development of conventional $CD8^+$ T-cells, and that this contributes to the activated phenotype observed.

Furthermore, we know that NFAT proteins are involved in T-cell development and therefore the absence of NFAT1 and NFAT2 might also have a cell-intrinsic impact on the development of $CD8^+$ T cells. Therefore, an acute knockout of NFAT2 post development would be a better system to study how transduced NFAT2 proteins control the exhaustion program in the absence of any contribution from endogenous NFAT2. NFAT2 $^{fl/fl}$ CD4 Cre-ERT2 or NFAT1$^{-/-}$ NFAT2 $^{fl/fl}$ CD4 Cre-ERT2 mice would be ideal as the NFAT2 knockout can be controlled by a tamoxifen-induced Cre. Therefore, the T-cells would develop in the presence of normal amounts of NFAT2 and could be isolated after they have developed normally and treated with Tamoxifen *in vitro* to activate Cre and knock out NFAT2.

While the NFAT2 $^{fl/fl}$ CD4 Cre-ERT2 were being bred, a time-consuming process, we took the alternative approach of transducing the NFAT constructs into WT $CD8^+$ T cells. All the constitutively active NFAT proteins increased expression of PD1 and LAG3, only NFAT2, specifically, the long isoform (βC), increased PD1 expression significantly and LAG3 expression was only significantly affected by NFAT1. TIM3 expression does not seem to change much because of the NFAT constructs but it has been shown previously that TIM3 is only poorly expressed *in vitro*. There is no striking difference between the proteins that are just constitutively active and the proteins that are constitutively active and unable to interact with AP-1. This could be because Fos and Jun are only transiently activated at the outset of *in vitro* expansion, so that even

though the NFAT proteins with just CA mutations can in principle interact with AP-1, post d2 *in vitro* there is insufficient Fos and Jun to form appreciable amounts of the AP-1 complex with the CA-NFAT proteins.

Although there is no dramatic difference in the NFAT2 isoforms in terms of elevated inhibitory receptor expression, NFAT2 isoforms have a differential impact on IL2 production. While CA-NFAT2βC and CA-RLT-NFAT2βC significantly reduce IL2 expression in response to restimulation (just like CA-NFAT1 and CA-RIT-NFAT1), CA-NFAT2αA and CA-RLT-NFAT2αA have no impact on IL2 production. This suggests that the short isoform of NFAT2 (αA) is doing something different and incomplete compared to NFAT1 and NFAT2βC, calling into question the belief that the short isoform of NFAT2 by itself is primarily driving the exhaustion program.

The difference in the NFAT2 isoforms might be explained by the differences in their N and C termini. As mentioned earlier, NFAT2αA lacks the transactivation/ repressor domain present in the C terminus of NFAT2C isoforms and NFAT1 and also lacks the acidic activation domain present in the N terminus NFAT1 and NFAT2β isoforms. It is likely that through these conserved motifs in their TAD-N and TAD-C, NFAT1 and NFAT2βC can interact with partner proteins that NFAT2αA cannot interact with. NFAT1 and NFAT2βC and these partner proteins could then bind to a distinct set of sites in DNA, promoting expression of a distinct subset of genes, and leading to distinct biological effects. This hypothesis could be tested directly by probing the NFAT binding sites with ChIP-seq, CUT&RUN, or CUT&Tag analyses.

This information on NFAT binding sites, along with ATAC-Seq data, could further be examined for enrichment of consensus binding motifs, to identify the transcription

factors that may be binding to these sites and possibly contributing as NFAT partner proteins in exhaustion.

One way that the different NFAT proteins could exert distinct effects is by differentially altering chromatin. We performed ATAC-Seq to determine changes in chromatin accessibility in CD8+ T-cells expressing the different NFAT2 proteins and compare with cells expressing CA-RIT-NFAT1 and empty vector to determine if there are certain regions of chromatin that open up uniquely under the influence of constitutively active NFAT2αA or constitutively active NFAT2βC and NFAT1. Substantial changes in chromatin accessibility changes occur in cells stimulated *in vitro* and transduced with empty vector and expanded under our conditions, compared to $CD8^+$ T cells isolated directly from spleen. These changes are the *in vitro* counterpart of the changes known to occur in the transition from resting to memory cells *in vivo*. Surprisingly, there is little to no change in chromatin accessibility in cells transduced with the NFAT constructs compared to the cells transduced with empty vector. This result contrasts with the finding in Scott-Browne *et al* [16] that CA-RIT-NFAT1 expression caused major changes in accessibility compared to the vector control. The most likely explanation is that Scott-Browne *et al* prepared the ATAC-seq samples on day 4, when the constitutively active NFAT proteins and residual effects of the earlier anti-CD3/anti-CD28 stimulation may act in concert. Our findings also contrast with the pronounced chromatin changes observed in later stages of exhaustion *in vivo* [15,16,27], despite the fact that cells prepared according protocol *in vitro* are functionally exhausted by day 6 [12], supporting the notion that early changes on the path to exhaustion need not involve irreversible changes in chromatin.

Since the observed changes in protein expression elicited by the NFAT proteins, for example the increases in PD1 and LAG3 expression, are not reflected as changes chromatin accessibility, these early changes in protein expression are likely a result of binding of NFAT and perhaps other transcription factors induced directly or indirectly by NFAT to DNA sites that are already accessible.

Since the comparisons in this project so far have been based on assessing only a few protein markers, our interpretation relies on the fact that we replicated the exact conditions of [12], where RNA-seq showed substantial CA-RIT-NFAT1-dependent changes in gene transcription at day 6. The conclusions will need to be buttressed in the future with RNA-seq data from our own RNA samples, which have been saved for analysis. The RNA-Seq data, in turn, will shed light on the more global picture of the effect of NFAT1 and the distinct NFAT2 isoforms, by showing all the genes affected and give more information about the extent to which the NFAT isoforms are driving a similar or different transcriptional program.

The NFAT-dependent program of exhaustion has significant relevance for the clinic as current therapies such as immune-checkpoint inhibitors target proteins that are downstream of NFAT signaling. Our data along with the RNA-Seq and ChIP-Seq data will provide more insight into molecules that can be targeted for future immune therapies. Additionally, this information can also be used to design converse therapies for autoimmune disorders where T cells are hyperactive, and it would be beneficial to induce a partial T cell exhaustion.

MATERIALS AND METHODS

Mice. All experiments were performed in compliance with the LJI Institutional Animal Care and Use Committee guidelines. Wildtype (WT) mice (C57BL/6J) were obtained from Jackson Laboratories. NFAT1$^{-/-}$ NFAT2 $^{fl/fl}$ CD4 Cre mice were generated by crossing NFAT1$^{-/-}$and NFAT2 $^{fl/fl}$ CD4 Cre mice (obtained by crossing NFAT2 $^{fl/fl}$ with CD4 Cre mice). Exon 3 of NFAT2 gene was floxed so all isoforms would be deleted. All mice were bred and maintained in the animal facility at La Jolla Institute for Immunology (LJI).

Retroviral Constructs: Murine NFAT1 constructs (WT-NFAT1, CA-NFAT1 and CA-RIT-NFAT1) were available in the lab. Murine NFAT2αA was obtained from Dr. Bruno Robbs (National Cancer Institute, Brazil) (UniProtKB: B5B2N5_Mouse) and the NFAT2βA was obtained from Addgene (plasmid #11102) (UniProtKB: Q9DBQ6_Mouse). The constructs had serine to alanine mutations in the regulatory domain of the proteins to ensure the NFAT2s are constitutively active (CA) (Okamura et al. 2000). The constructs were then cloned into an MSCV-based vector that expresses GFP from an IRES sequence as a marker of transduced cells. The CA-NFAT2αA was further engineered using site-directed mutagenesis to generate the replacements R485A/ L486A and T552G (CA-RLT-NFAT2αA) and CA-NFAT2βA was engineered to generate replacements R471A/ L472A and T538G (CA-RLT-NFAT2βA) in order to disrupt the interaction of the proteins with AP-1 complex.

The C terminal sequence of NFAT2βC (UniProtKB: B5B2N7_Mouse) was synthesized by Genscript and cloned into the vector containing the CA-NFAT2βA vector with and without the RLT mutations.

Sequence of C-terminal for the βC isoform instead of the last 59nt in βA isoform(nt):

TTCCAATTATAAAGACAGAACCCACGGACGACTTTGAGCCAGCTCTGACCTGTGGA
CCAATGAGCCAGGGGATTAGTCCTCTGCCGAGGCCTTACTACAGCCAACAGCTCA
CCATGCCTCCCGACCCCGGCTCCTGCCTCGTGGCTGGCTTCGCCCCCTGCTCCCA
GAGGAACACGCTGATGCCCACGCCTCCCAACGCAAGCCCGAAGCTCCACGACCTT
TCCTCTCCTGCCTACACCAAGGGCCTCACCAACCCGGGCCACAGTGGTCACCTTG
GACTTCAGCCACCCGCTTCGGAGGCCCCCACCATGCAGGAAGTGCCGAGACCCA
TGGCCATCCAACCCAACTCGCCTGAGCAGCCCCCATCCGCCAGGCTACAGCCGC
AGGTGAGTCCACATCTGAACAGTAGCTGTCCCCTTGGTCGCCGACAAGTGCTCTG
TCCCAACAGCCCCTCTTCTCCACTTCCATCTGCTGCCCAAGAGCCAGCCTGCTTAC
AGTCCTCAGCCCTCCCTCCTGACATGGGCCACCGGCAGCCACAACCGCAGAAGG
TTCAAAGAAATGAATCTCCAGCCGTATTGCCAGAGGTGTGTGAGGACAGTGGCCA
TAACTTGGCCCCTATTCCTGTAGTGATCAAGCAAGAGCCTGAGGAATTGGACCAGT
TGTACTTGGATGATGTAAATGAGATCATACGTAACGACCTCTCCAGCACGATCCCC
CACTCCTAA

Isolation, Culture and Restimulation of CD8+ T cells. Naïve (CD62L high and CD69low or CD62L high and CD44 low) or total CD8+ T cells were harvested from spleen and lymph nodes of 6 – 8 weeks old mice. The cells were purified by negative

selection (EasySep kit – Stem Cell Technologies) from RBC lysed cell suspension. The cells were cultured in T cell media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 1X penicillin-streptomycin, 1X non-essential amino acids, 1mM sodium pyruvate, 10mM HEPES pH 7.2, and 50μM 2-mercaptoethanol). The cells were plated at 10^6 cells/mL in 12-well plate and stimulated with plate-bound antibodies by first incubating 1μg/mL anti-CD3 (clone 2C11) and 1μg/mL anti-CD28 (clone 37.51) in plates coated with 50μg/mL goat anti-hamster IgG (MP Biomedicals). On day 2 the cells were removed from the antibody coated plate and re-cultured at 10^5 cells/mL in T cell medium with 10U/mL IL-2 (NIH). Every day the cell concentration was adjusted to 10^5 cells/mL in T cell medium with 10U/mL IL-2. On day 6 the cells were either left untreated (unstimulated) or re-stimulated with 1μM ionomycin (Sigma) and 10nM PMA (Sigma) for 6 hours. For cytokine production analysis, 1μg/mL brefeldin A was added 3 hours after stimulation.

Retroviral transduction. Viral supernatants were generated by transfection of PlatE cells according to the TransIT®-LT1 Transfection Reagent (Mirus Bio LLC) using pCL-Eco packaging vector. $CD8^+$ T cells were transduced twice, on day 1 and day 2, in a 12-well plate using 3mL of 0.45μM filtered viral titer and 8 μg/mL polybrene per well. Cells were centrifuged at 2000RPM for 1.5 hours at 30 °C in a prewarmed centrifuge and then incubated at 37 °C for 3-4 hours on day 1 and 1 hour on day 2.

Surface marker staining. Cells were stained for viability with 1:1000 final concentration of Fixable Viability Dye eFluor 780 (ThermoFisher) and for cell surface receptors with

1:300 final concentration of antibodies in FACS buffer (PBS + 1% FBS and 2mM EDTA) for 15 minutes. Antibodies used were anti-CD8α (clone 53-6.7), anti-CD8β (clone YTS156.7.7), anti-PD1 (clone 29F.1A12), anti-LAG3 (clone C9B7W) and anti-TIM3 (clone B8.2C12).

The cells were then fixed with 4% PFA (Affymetrix - ThermoFisher) for 10 minutes and analyzed for expression of extracellular inhibitory receptors.

Cytokine staining. Fixed cells were permeabilized with 0.5% Saponin in PBS for 5 to 10 minutes and then stained for cytokines with 1:100 final concentration of antibodies in complete permeabilization buffer (PBS + 2% FBS and 0.5% Saponin) for 45 minutes. Antibodies used were anti-IL2 (clone JES6-5H4) and anti-IFNγ (clone XMG1.2). The cells were washed twice with complete permeabilization buffer and analyzed for cytokine expression.

Flow Cytometry analysis. All flow cytometry analysis was performed using LSRFortessa (BD Biosciences) or the LSR-II (BD Biosciences). Flow cytometry data was analyzed using FlowJo v.10 (Tree Star, Inc).

ATAC-seq sample and library preparation. ATAC-seq samples were prepared as described by Corces and colleagues [24]. Live transduced $CD8^+$ cells (GFP +ve cells) were sorted in FBS and lysed. Transposition reaction was performed using Nextera Tn5 enzyme (Illumina) and was purified using MinElute kit (Qiagen) prior to PCR

amplification (KAPA Biosystems) with 9 – 11 cycles using Nextera barcode primers. Libraries were purified using MinElute kit (Qiagen).

ATAC-seq analysis. Analysis was done by Edahi Gonzalez Avalos. Reads were mapped to mouse genome (mm10) using a two-step mapping strategy. First, bowtie (v1.0.0) was used to map the untrimmed reads with parameters "-p 4 -m 1 --best --strata -X 2000 --tryhard -S --fr -- chunkmbs 2048". The unmapped reads were processed with Trim Galore! using parameters "--paired --nextera --length 37 --stringency 3 --three_prime_clip_R1 1 - -three_prime_clip_R2 1" and the filtered reads were mapped again. Both the mapping files were merged and processed to remove duplicated and mitochondrial reads. The filtered mapping results were further processed to identify nucleosome-free DNA fragments less than 100nt in length with a custom perl script. These sub-nucleosomal fragments were used to call peaks summits for each sequenced sample using MACS2. Peaks from individual samples were merged into a global set of peaks, used as region of interest for the Differential Accessibility analysis; the Tn5 footprint (9bp where the Tn5 makes the insertion) was used as counts for the analysis.

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