NFAT1 supports tumor-induced anergy of CD4+ T cells

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Precis: Results directly implicate CD4+ T cell anergy in immune escape and open the possibility of targeting a transcription factor known to induce T cell anergy as a general strategy to improve immunotherapy or immunochemotherapy for cancer treatment.

Abstract

Cancer cells express antigens that elicit T cell-mediated responses, but these responses are limited during malignant progression by the development of immunosuppressive mechanisms in the tumor microenvironment that drive immune escape. T cell hypo-responsiveness can be caused by clonal anergy or adaptive tolerance, but the pathophysiological roles of these processes in specific tumor contexts has yet to be understood. In CD4+ T cells, clonal anergy occurs when the T cell receptor is activated in the absence of a co-stimulatory signal. Here we report that the key T cell transcription factor NFAT mediates expression of anergy-associated genes in the context of cancer. Specifically, in a murine model of melanoma we found that cancer cells induced anergy in antigen-specific CD4+ T cell populations, resulting in defective production of several key effector cytokines. NFAT1 deficiency blunted the induction of anergy in tumor antigen-specific CD4+ T cells, enhancing anti-tumor responses. These investigations identified tumor-induced T cell hypo-responsiveness as a form of clonal anergy, and they supported an important role for CD4+ T cell anergy in driving immune escape. By illustrating the dependence of tumor-induced CD4+ T cell anergy on NFAT1, our findings open the possibility of targeting this transcription factor to improve the efficacy of cancer immunotherapy or immunochemotherapy.

Introduction

Successful responses of the immune system against tumor cells are frequently hindered by the presence of an immunosuppressive tumor microenvironment (1). A series of mechanisms prevent the recognition of tumor-associated antigens presented by tumor cells from activating a productive response able to clear transformed cells. Several processes have been described to contribute to the immune escape that allows tumor cells to block anti-tumor immunity including, among others, the down-regulation of antigen presentation by tumor cells and the expression of suppressor factors or the recruitment of cells with suppressor activity (1-5). One of the effects reported to occur in the tumor microenvironment is the induction of antigen-specific tolerance in CD4+ and CD8+ T cells (6, 7). Several mechanisms have been proposed to account for the anergic phenotype of tumor antigen-specific T cells. Ligation of inhibitory receptors and defective activation of antigen presenting cells, among others, appear to contribute to the establishment of a hypo-responsive state in tumor specific T cells (3, 8). Dendritic cells (DC) in tumor microenvironments appear to play a crucial role in the induction of anergy in CD4+ T cells (9). Several models of intrinsic T cell hyporesponsiveness have been proposed, each regulated by a specific set of molecular mechanisms that maintain T cells anergic (10, 11).The factors that determine which mechanisms are activated *in vivo* in a given context or how they may regulate specific processes is currently unknown. When stimulated through engagement of their antigen receptor in the absence of co-stimulatory signals, CD4+ T cells become anergic to subsequent re-stimulations. Their hypo-responsive state is characterized by decreased proliferative response and reduced cytokine production following re-stimulation even in the presence of co-stimulation. Clonal anergy in CD4+ T cells is established as a result of the activation of a program of gene expression that is dependent on the transcription factor NFAT. Proteins encoded by those genes are responsible for the maintenance of an unresponsive state. This is accomplished through the inhibition of signaling pathways downstream of the TCR and through direct repression of cytokine gene expression (12). Little is known however on the mechanisms

that regulate tumor-induced hypo-responsiveness in T helper cells. Here, using a B16 melanoma tumor model expressing the tumor surrogate antigen chicken albumin (OVA), we show that tumor antigen specific CD4+ T cells are rendered anergic *in vivo* through a mechanism that requires NFAT1 activity and involves the expression of anergy specific genes. Furthermore, by specifically targeting the regulation of anergy induction using NFAT1-deficient mice, our results support that tumor-induced CD4+ T cell anergy participates in the evasion of anti-tumor responses, as NFAT1-deficient T cells become resistant to tumor-induced anergy, delaying tumor appearance and slowing tumor growth.

Materials and Methods

Mice

C57Bl/6, B6.Pl-Thy1^{a}/CyJ, B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), B6.129S2-*Cd8a*^{tm1Mak}/J, BALB/c, and BALB/c-Tg(DO11.10)10Loh/J mice were purchased from Jackson Laboratories. *Nfat1*$^{-/-}$OT-II mice were generated by crossing B6.*Nfat1*$^{-/-}$ (13) with OT-II mice. *Nfat1*$^{-/-}$DO11.10 mice were generated by crossing BALB/c.*Nfat1*$^{-/-}$ with DO11.10 mice. Mice were maintained in pathogen-free conditions. All animal work was performed according to the guidelines of the Institutional Animal Care Committee at the Albert Einstein College of Medicine.

Tumor cell lines

The B16-F1 melanoma and EG7 thymoma cell lines were purchased from the American Type Culture Collection (ATCC). Cells received from the ATCC were not re-authenticated and were used within two months after receiving them. The B16-OVA cell line was a gift from EM Lord (University of Rochester Medical Center, NY). The lung carcinoma TC-1 cell line was a gift from TC Wu (Johns Hopkins University, MD). These cell lines were not genetically authenticated, but melanin production was assessed by microscopy and OVA expression confirmed by real time PCR. Cells were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 250IU Penicillin/Streptomycin, and 50μM β-mercaptoethanol. For tumor injections, $5x10^5$ cells in Hank's Balanced Salt Solution were injected subcutaneously into either the intrascapular or the lumbar regions. Where indicated mice were vaccinated with apoptotic cells prepared by irradiating TC-1 cells (10^7/ml in PBS) with 10,000 rads. Mice were injected intraperitoneally with 100 μl of irradiated cell suspension mixed with equal volumes of incomplete Freund's adjuvant. Vaccinations were performed once a week starting 2 weeks prior to tumor injections. Tumor growth was recorded by measuring perpendicular diameters and the volumes were calculated as $\pi/6 x 1.6 x (\textit{length x width})^{3/2}$.

Primary CD4+ T cell isolation and culture

CD4+ T cells were isolated using antiCD4-coupled magnetic beads (Invitrogen). To generate Th1 cells, CD4+ T cells were stimulated with plate bound anti-CD3ε (0.25μg/ml) and anti-CD28 (0.25μg/ml) antibodies (BD Biosciences), and differentiated with 10ng/ml of mouse IL-12 (eBioscience), 10μg/ml of blocking anti-mouse IL-4 antibody, and 10U/ml recombinant human IL-2 (Biological Resources Branch of the National Cancer Institute) for 6 days. Cells were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 50μM β-mercaptoethanol, essential vitamins, 550nM L-Arg, 240nM L-Asn, and 14nM folic acid.

Adoptive transfer and re-isolation of T cells

Nfat1$^{-/-}$ or *Nfat1*$^{+/+}$OT-II naïve CD4+ or *in vitro* differentiated OT-II Th1 cells were adoptively transferred ($5x10^6$ cells per mouse) into the tail veins of B6.PL-Thy1^a mice or CD8-deficient mice. Where indicated, adoptively transferred cells were re-isolated using a biotinylated anti-CD90.2 antibody and streptavidin coated magnetic beads (Invitrogen).

Isolation of tumor infiltrating T cells

Tumors were resected from mice and minced to 1-3mm fragments, then treated with 1mg/ml collagenase-D (Roche) and DNase-I (40U/ml, Sigma) in RPMI and incubated at 37 °C for 1 hour. Cells were then passed through a 40μm-mesh, washed and T cells isolated using antiCD4-coupled magnetic beads.

ELISA

$2.5\text{-}5x10^4$ CD4+ T cells were stimulated in 96 well plates with either anti-CD3ε/anti-CD28 antibodies or freshly isolated, T cell-depleted, $OVA_{323\text{-}339}$-loaded splenocytes added to responder T cells at 5:1 ratio. Supernatants were collected 12-24 hours after stimulation and IL-2, IL-4, IL-17 or IFNγ levels measured by sandwich ELISA.

5-Bromo-2-deoxy-uridine (BrdU) incorporation assay

2-5x10^4 CD4+ T cells were stimulated in 96 well plates with anti-CD3ε/anti-CD28 antibodies. After 48-72 hours of incubation, cells were pulsed with BrdU (Roche) for 8-10 hours, followed by fixation and detection according to the manufacturer's protocol.

Real Time PCR

RNA was isolated from cells using Trizol (Invitrogen) and cDNA was synthesized using Superscript-III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed with PowerSYBR (Applied Biosystems) using a StepOnePlus Real-Time-PCR system (Applied Biosystems). Expression of each gene was normalized to actin. The following primer sets were used: *Actin*: F-GTGACGTTGACATCCGTAAAGA; R-GCCGGACTCATCGTACTCC; *Grail*: F-ATGCAAGAGCTCAAAGCAGGAAGC; R-GTGCGCAGCTGAAGCTTTCCAATA; *Ikaros*: F-GCTGGCTCTCGGAGGAG; R-CGCACTTGTACACCTTCAGC; *Casp3*: F-ACGCGCACAAGCTAGAATTT; R-CTTTGCGTGGAAAGTGGAGT; *Egr2*: F-TCAGTGGTTTTATGCACCAGC; R-GAAGCTACTCGGATACGGGAG *Grg4* F-TCACTCAAGTTTGCCCACTG; R-CACAGCTAAGCACCGATGAG; *Itch*: F-GTGTGGAGTCACCAGACCCT; R-GCTTCTACTTGCAGCCCATC.

Statistical Analysis

Differences between groups were analyzed with Student's t test.

Results

Tumor-specific CD4+ T cells become hyporesponsive and produce less IL-2

To characterize tumor-induced hyporesponsiveness in antigen-specific CD4+ T cells, we used a B16 melanoma cell line that had been stably transfected to express OVA (B16-OVA) as a surrogate tumor antigen, and OT-II mice, whose T cells express a transgenic MHC class II-restricted TCR that recognizes $OVA_{323-339}$ peptide. We transferred naive wildtype or OT-II CD4+CD90.2+ T cells into congenic B6.PI-Thy1^{a}CD90.1+ mice challenged with B16-OVA cells injected subcutaneously in the intrascapular region. Seven days post-transfer CD4+CD90.2+ T cells were isolated from the tumor draining lymph nodes (DLN) and stimulated *ex vivo* with anti-CD3 and anti-CD28 antibodies. OT-II CD4+ T cells isolated from B16-OVA bearing mice produced significantly less IL-2 than non-TCR transgenic cells, suggesting that tumor antigen-specific T cells had become hyporesponsive, possibly through tumor-induced anergy (Fig. 1A). When B16-OVA growth kinetics was analyzed, we did not observe any significant differences between mice that received either of those two T cell populations, indicating that tumor-specific CD4+ T cells could not mount an efficient anti-tumor response (Fig. 1B). To rule out that this effect could be specific to B16 melanoma, similar experiments were performed using the more immunogenic EG7-OVA thymoma cell line. T cells isolated from DLN also showed decreased production of IL-2, which correlated with an inability of OT-II cells to control EG7-OVA growth (Suppl. Fig. 1A-B).

Studies have shown that Th1 skewing of CD4+ T cell populations is associated with anti-tumor immunity (14). We therefore determined whether tumor-specific Th1 cells could control tumor growth better than naïve cells. Experiments similar to those described above were carried out but adoptively transferring *in vitro* differentiated Th1 cells. Similar to what we had previously seen in naïve cells, OT-II Th1 cells transferred into mice bearing B16-OVA melanomas became hyporesponsive and produced less IL-2 than the transferred control non-TCR transgenic Th1

cells (Fig. 1C). Adoptive transfer of *in vitro* differentiated OT-II Th1 cells delayed slightly tumor appearance; however, it still allowed B16-OVA growth with similar kinetics as those measured in mice transferred with naive OT-II CD4+ T cells (Fig. 1D). To confirm that the decreased ability of the OT-II T cells to produce IL-2 following restimulation was a consequence of the recognition of a specific tumor antigen, we compared the behavior of OT-II CD4+ T cells in mice challenged with either B16-OVA or the parental B16 melanoma cell line. There was no significant difference in tumor growth between both tumors; however OT-II cells transferred into B16-OVA-bearing mice showed a significant decrease in IL-2 production compared to OT-II T cells isolated from mice challenged with B16 tumors, supporting a tumor-antigen specific induced hyporesponsiveness (Fig. 1E-F).

B16-OVA specific CD4+ T cells activate an anergy-associated program of gene expression characteristic of clonal anergy

To further validate our findings, we injected B16-OVA cells into the lumbar flanks of OT-II mice and compared responses of tumor-specific T cells that could have been presented with tumor antigen (isolated from inguinal DLN) and T cells that likely had no contact with melanoma antigens (isolated from distal cervical non-DLN). CD4+ T cells from DLN produced significantly lower amounts of IL-2, IFN-γ, and IL-17 compared to non-DLN (Fig. 2A-C). Ruling out these results could respond to Th2-skewing, IL-4 production was not detected at significant amounts in either location (Fig. 2D). In addition, CD4+ T cells isolated from DLN were also hypo-proliferative compared to T cells obtained from non-DLN (Fig. 2E).

Clonally anergic T cells activate a program of gene expression that leads generates proteins responsible for inhibiting TCR-mediated signaling and activation-induced cytokine expression (12). To determine if a similar mechanism was responsible for tumor-induced anergy, we assessed the expression of several anergy-associated genes in T cells isolated from DLN of B16-OVA bearing mice and compared it to T cells obtained from non-DLNs of the same mice.

Confirming our hypothesis, T cells from DLN upregulated expression of 6 out of 7 different genes we assayed, including the E3 ubiquitin ligases *Grail*, *Cblb* and *Itch*, the transcription factors *Egr2* and *Grg4*, and *Caspase3* (Fig. 2F).

Tumor infiltrating T cells isolated from tumors that had grown for 9 days in OT-II mice also produced much lower levels of IL-2 than cells isolated from non-DLN or spleen, and interestingly even less than cells from DLN (Fig. 3A and data not shown). Analysis of RNA extracted from the tumor infiltrating CD4+ T cells revealed also highly upregulated expression of *Grail* (Fig. 3B). We observed no significant differences in the percentages of CD25+CD4+ T cells or *Foxp3* expression between DLN and non-DLN or spleens (data not shown), ruling out the possibility that decreased CD4+ T cell responses in these mice might be due to increased regulatory T cells (Treg) recruitment.

B16-OVA specific *Nfat1*$^{-/-}$ T cells are resistant to tumor-induced anergy and show improved control of tumor growth

Tumor-induced T helper anergy may contribute to immune escape (6, 15), however its role in this process has not been quantitated. As *Nfat1*$^{-/-}$CD4+ T cells were resistant to soluble peptide-induced anergy *in vivo* (Suppl. Fig. 2), we determined if *Nfat1*$^{-/-}$ T cells could also be resistant to tumor-induced anergy. We generated *Nfat1*$^{-/-}$OT-II mice and compared T cell responses in these mice and *Nfat1*$^{+/+}$OT-II mice when challenged with B16-OVA. CD4+ T cells isolated from DLN and non-DLN of B16-OVA-challenged *Nfat1*$^{+/+}$ and *Nfat1*$^{-/-}$ OT-II mice were stimulated *ex vivo* with $OVA_{323-339}$ peptide-loaded splenocytes. As expected, CD4+ T cells from the DLN of OT-II mice produced significantly less IL-2, IFN-γ and IL-17 than cells isolated from non-DLN, indicating B16-OVA-induced anergy (Fig. 4A-C). However, CD4+ T cells from DLN of *Nfat1*$^{-/-}$OT-II mice failed to become anergic and produced similar (IL-2) or even higher (IFNγ) levels of Th1 cytokines than cells isolated from non-DLN (Fig. 4A-B). IL-17 production was,

however, also reduced in the DLN of *Nfat1*$^{-/-}$OT-II mice (Fig. 4C). Interestingly, there were also higher frequencies of tumor infiltrating CD4+ T cells in *Nfat1*$^{-/-}$ mice than in wildtype mice (0.81± 0.64% vs. 0.11±0.03%). As expected, *Grail* expression was upregulated in T cells from DLN in *Nfat1*$^{+/+}$OT-II mice, however, indicating resistance to tumor-induced anergy, no upregulation of *Grail* was detected in T cells from DLN of *Nfat1*$^{-/-}$OT-II mice (Fig. 4D).

Given that anergy-induction of Th1 responses was deficient in *Nfat1*$^{-/-}$ mice, we used this model to determine whether preventing B16-induced anergy would result in improved control of tumor growth. Excised tumors from *Nfat1*$^{-/-}$OT-II mice were significantly smaller in size and weight than melanomas formed in *Nfat1*$^{+/+}$OT-II (Fig 6A). Furthermore, tumor appearance was significantly delayed and tumor growth slowed in *Nfat1*$^{-/-}$ mice, with complete protection observed in one mouse (Fig. 5B). To further corroborate that control of B16 growth was Th1 cell-mediated, we adoptively transferred *in vitro* differentiated Th1 cells isolated from *Nfat1*$^{+/+}$OT-II or *Nfat1*$^{-/-}$OT-II mice into C57Bl/6 mice. Following adoptive transfer, mice were challenged with B16-OVA cells and tumor growth kinetics analyzed. As we had seen in *Nfat1*$^{-/-}$ OT-II mice, melanomas in mice that received *Nfat1*$^{-/-}$OT-II Th1 cells showed delayed onset and slower growth rate compared to mice receiving *Nfat1*$^{+/+}$OT-II Th1 cells (Fig. 5C). Additionally, we confirmed that the observed effect on tumor onset in *Nfat1*$^{-/-}$OT-II mice was caused by the presence of tumor-specific CD4+ T cells, as tumors implanted in non-transgenic *Nfat1*$^{-/-}$ mice behaved similarly than those injected in wildtype mice (Fig. 5D). The capacity of anergy-resistant NFAT1-deficient T cells to control tumor growth appeared to reside in their ability to regulate the activity of CD8+ T cells, as no significant differences in B16-OVA growth were detected when *Nfat1*$^{+/+}$ or *Nfat1*$^{-/-}$ CD4+ T cells were transferred into B16-OVA-bearing CD8-deficient host mice (Fig. 5E).

To exclude the possibility that the effects observed using B16-OVA could be specific for this cell line or the expression of OVA, we performed experiments using the lung cancer TC-1 cell

line. Mice were first vaccinated with apoptotic TC-1 cells to generate an anti-tumor specific T cell response. Accordingly, vaccinated mice controlled the growth of transferred TC-1 cells better than unvaccinated mice (Fig. 6A). Then we compared TC-1 growth in previously vaccinated wildtype and *Nfat1*$^{-/-}$ deficient C57Bl/6 mice. *Nfat1*$^{-/-}$ mice also showed significantly delayed growth of TC-1 tumors, supporting the role of NFAT1 in the establishment of tumor-induced tolerance (Fig. 6B). We obtained similar results when experiments were performed in mice challenged with the EG-7-OVA thymoma cell line (Suppl. Fig. 1).

Discussion

Transformed cells can express tumor-associated antigens that should be recognized as non-self by T cells and elicit effective anti-tumor responses. However, immunosuppressive mechanisms are engaged in the tumor microenvironment that account for the frequently inefficient anti-tumor T cell response (1, 4). Tumor-specific CD4+ T cells can be rendered hypo-responsive by both solid and hematologic cancers (6, 16). Using a B16-melanoma model, our data supports that tumor-specific T helper cells become anergic and show reduced ability to secrete several effector cytokines, including IL-2, IFNγ and IL-17. In addition, this hyporesponsive state bears similarity to the induction of clonal anergy as it is dependent on the transcription factor NFAT1, and leads to the up-regulation of clonal anergy associated genes.

Several models have been proposed to explain how T cells become anergic, including clonal anergy and adaptive tolerance. These processes respond to the inactivation of distinct signaling pathways and also differ in the requirement for continuous antigen presence (10-12). Little is known about the specific physiological contexts in which different forms of T cell inactivation may occur. Our results indicate that a hyporesponsive state similar to clonal anergy is induced by cancer cells in antigen specific CD4+ T cells, and therefore identify clonal anergy as a mechanism of tumor-induced hyporesponsiveness *in vivo*. Clonally anergic T cells not only secrete less cytokines and proliferate less when stimulated but they also upregulate the expression of anergy-associated genes (11, 17-24). Our studies show that B16-OVA antigen-specific CD4+ T cells isolated from tumor DLN upregulate the expression of several anergy associated genes, including *Grail*, *Itch, Cblb, Casp3*, *Grg4* and *Egr2*, supporting that T cells that have seen the tumor antigens become anergic.

It has been previously shown that DCs fail to properly activate in the tumor microenvironment and show decreased expression of B7 costimulatory ligands (3, 15, 25). Consequently, treatment with activating anti-CD40 antibodies preserved the functional

responsiveness and activation of tumor-specific T cells that would otherwise be tolerized by tumors (26). Interestingly, CD40L surface expression has been shown to be down-regulated by Grail in anergic T cells (27). This down-regulation may impair T cells' ability to further license DCs. Tolerogenic immature DCs would therefore anergize tumor-specific T cells, which in turn would fail to activate and induce maturation of those DCs. Under these circumstances, inactivated DCs would deliver signals that induce anergy in CD4+ T cells.

The role of Tregs in downregulating anti-tumor T cell responses has also been reported in several tumor models, and depletion of this population enhances tumor immunity and causes tumor rejection *in vivo* (28, 29). We did not detect increased Treg populations in tumor DLN, supporting the existence of an intrinsic mechanism of self-inactivation of tumor antigen-specific T cells. Myeloid derived suppressor cells (MDSCs) have been identified as another major mechanism involved in the inhibition of anti-tumor T cell activity (30). The suppressive mechanism of MDSCs requires cell-cell contact, as many of the suppressive mediators are short-lived (31). Although we do not rule out that melanoma infiltrating T cells and CD4+ T cells in DLNs may have experienced suppression by MDSCs, we performed our assays on isolated CD4+ T cells and stimulated them *in vitro* in rich medium. Therefore, it is unlikely that their hypo-responsiveness is solely due to the effect of MDSC populations.

The transcription factor NFAT1 is critical for the induction of T cell anergy. *Nfat1*$^{-/-}$ mice show T cell hyper-reactivity (13), and CD4+ T cells from these mice are resistant to anergy *in vitro* (11). We were able to confirm that NFAT1 was also required for the induction of anergy *in vivo* using the soluble peptide-induced model of anergy. This *in vivo* observation provided us with a tool to study the significance of CD4+ T cell anergy in the context of tumor tolerance. Functionally, CD4+ T cells isolated from the DLN of *Nfat1*$^{-/-}$ tumor-bearing mice did not show the anergic phenotype we observed in wildtype T cells, failing to up-regulate the expression of

anergy-associated genes and maintaining levels of IL-2 and IFNγ expression, which supports that tumor-induced Th1 cell anergy is also dependent on NFAT1 activity.

Our data indicates that anergy affects both Th1 and Th17 responses. Th1 cytokines, such as IFNγ and IL-2, are associated with cell-mediated immunity involving activation of CD8+ T cells in anti-tumor responses. (32-34). The function of Th17 cells has been characterized in the context of inflammation and autoimmunity, however their role in tumor immunity is still under much debate (35, 36). Some reports have shown that IL-17 may promote tumor development (37, 38); however evidence seems to also support an anti-tumor role of Th17 cells and their signature cytokine IL-17 (39, 40). We observed decreased IL-17 production in T cells from the DLN of mice bearing B16-OVA tumors. Interestingly, although *Nfat1*$^{-/-}$ OT-II CD4+ T cells from the DLN of mice harboring B16-OVA melanomas produced more IL-17 than wildtype controls, there was still a decrease in the production of this cytokine compared to T cells from non-DLNs, suggesting that *Nfat1*$^{-/-}$Th17 cells could still be anergized. Gene expression profiles of *in vitro* anergized Th17 cells, appeared to indicate, though, that this process may depend on the calcium-induced expression of anergy-associated genes (data not shown). Although additional experiments will be required to address this discrepancy, it is possible that the expression of those genes in Th17 cells may still be regulated by calcium/NFAT signaling, but that these cells may be less dependent on NFAT1 and able to express anergy genes through the activation of other NFAT family members.

Underscoring the importance of CD4+ T cell activity in orchestrating effective anti-tumor responses, our results show that mice harboring B16-OVA-specific TCR transgenic *Nfat1*$^{-/-}$ CD4+ T cells delay the onset of tumor appearance and decrease the rate of growth. These effects were not observed in *Nfat1*$^{-/-}$ mice with a wildtype TCR repertoire, suggesting that this was indeed an antigen and CD4+ T cell specific effect. It is likely that the delay in tumor growth was due to augmented CD8+ T cell responses due to increased CD4+ T cell activity, NFAT1-

deficient CD4+ T cells fail to delay B16 growth in mice that lack CD8+ T cells. It has been proposed that activation-induced non-responsiveness in CD8+ T cells, which occurs as a consequence of lack of adequate CD4+ T cell help, may be responsible for inefficient CD8+ responses against tumor cells (41). However, it is still possible that those CD4+ T cells might also mediate tumor destruction directly as indicated by previous reports (42, 43). While we cannot rule out that NFAT1-deficiency may also alter other aspects of the tumor microenvironment (44), our data indicates that anti-tumor responses directed towards limiting local development of B16 tumors at the primary site of injection in NFAT1-deficient mice are dependent on antigen-specific CD4+ T cells. Additionally, there is a possibility that NFAT1 deficiency may also influence the suppressive ability of Tregs (45), though *Nfat1*$^{-/-}$ Tregs appear to conserve their function *in vitro* (46).

Using *Nfat1*$^{-/-}$ CD4+ T cells has allowed us to quantitate the role of CD4 T cell anergy in tumor immunity. NFAT1-deficient T cells show normal activation-induced responses, likely due to compensation by NFAT2, as T cells that lack both transcription factors are characterized by profound defects in cytokine production following TCR engagement (13, 47). However, in response to tolerizing stimuli, NFAT1 is indispensable to form the transcriptional complexes that direct the expression of anergy-inducing genes (48). By using NFAT1-deficient mice or transferring NFAT1-deficient T cells, we have been able to specifically target clonal anergy induction without affecting other aspects of the regulation of T cell activation. We observed significant delays in tumor appearance and slowed rates of tumor growth in mice harboring tumor-specific anergy-resistant *Nfat1*$^{-/-}$CD4+ T cells, supporting that the establishment of tumor-induced NFAT-dependent anergy is an important mechanism of immune evasion. Our results support that anergy may occur early in the anti-tumor response, as has been suggested before (6). However, CD4+ T cells resistance to anergy may not be sufficient to prevent immune evasion as other dominant mechanisms of tolerance (e.g. MDSC or downregulation of tumor

antigen presentation) could still be able to further suppress anti-tumor responses and block T cell responses. It would be interesting to evaluate if combination of other immunotherapeutic approaches with anergy prevention may be able to further enhance anti-tumor responses.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: B16-OVA melanoma cells cause antigen-specific hyporesponsiveness in CD4+ T cells. B6.Pl-Thy1^a mice were challenged with 5x10^5 B16-OVA cells followed by adoptive transfer of 5x10^6 naïve CD4+ T cells from C57Bl/6 or OT-II mice (**A**) or *in vitro* differentiated OT-II Th1 cells (**C**). Transferred cells were re-isolated from DLN and stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28. IL-2 production was measured by ELISA. Results are presented as mean±SEM from 2-4 mice. *P<0.05. **B and D**. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM from 5-9 mice. **E**. B6.Pl-Thy1^a mice were challenged with 5x10^5 B16 or B16-OVA cells and adoptively transferred with 5x10^6 OT-II CD4+ T cells. Transferred cells were re-isolated and stimulated with anti-CD3 and anti-CD28. IL-2 was measured by ELISA. **F**. Tumor volumes were calculated from perpendicular diameters recorded daily. Results are presented as mean±SEM from 13-15 mice. **P<0.01.

Figure 2: B-16-OVA melanoma induces anergy in antigen specific CD4+ T cells in the tumor DLN. A-D. OT-II mice were challenged with 5x10^5 B16-OVA cells. Ten to twelve days post tumor challenge, CD4+ T cells were isolated from the DLN and distal non-DLN and stimulated with $OVA_{323-339}$ peptide-loaded splenocytes. IL-2, IFNγ, IL-17 and IL-4 production were measured by ELISA. Results are presented as mean+SEM from 3-5 mice. *P<0.05; **P<0.01. **E**. Cell proliferation was also measured in cells stimulated with anti-CD3 and anti-CD28 by BrdU incorporation. Results from are presented as mean±SEM from 4 independent experiments. **F**. CD4+ T cells isolated from DLN and non-DLN of the B16-OVA challenged OT-II mice (10^6 B16 cells per mouse) were lysed after isolation for extraction of RNA. cDNA was synthesized and levels of anergy-associated genes transcripts were measured by real-time PCR. Results, expressed as fold induction of the expression of anergy-associated genes in T

cells from DLN and non-DLN compared to T cells from control non-tumor bearing mice, are presented as mean±SEM from 3 to 4 independent experiments.

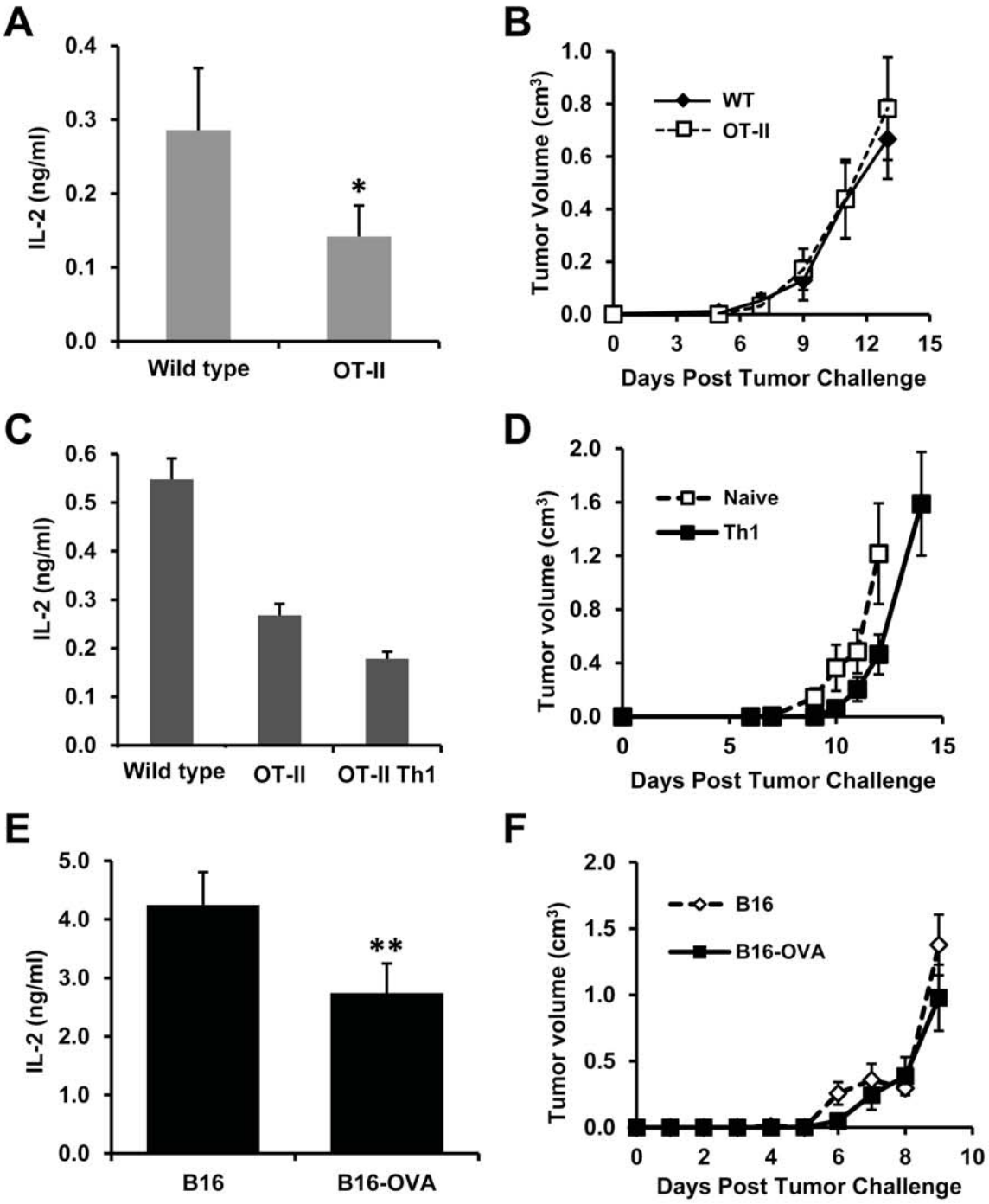
Figure 3. B-16-OVA melanoma induces anergy in tumor-infiltrating antigen specific CD4+ T cells. A. CD4+ T cells from DLN, non-DLN and tumor infiltrating lymphocytes (TIL) were isolated from B16-OVA tumor bearing ($5x10^5$ cells) OT-II mice and stimulated with anti-CD3 and anti-CD28 antibodies. IL-2 production was measured by ELISA and is presented as mean±SEM from 3 independent experiments. *P<0.05 (TIL vs. non-DLN). **B**. CD4+ TIL and T cells from spleens of B16-OVA bearing mice were isolated and immediately lysed for RNA extraction. *Grail* message levels were detected by real-time PCR. Results are presented as mean±SEM from 3 mice.

Figure 4. *Nfat1*$^{-/-}$ OT-II CD4+ T cells are resistant to tumor-induced anergy. A-C. CD4+ T cells were isolated from the tumor DLN and non-DLN of B16-OVA bearing *Nfat1*$^{+/+}$ or *Nfat1*$^{-/-}$ OT-II mice and stimulated with $OVA_{323-339}$ peptide-loaded splenocytes. IL-2, IFNγ and IL-17 production were measured by ELISA Results are presented as mean±SEM from 4-5 independent experiments. *P<0.05; **P<0.001. **D.** CD4+ T cells from the B16-OVA tumor DLN and non-DLN were isolated from *Nfat1*$^{+/+}$ and *Nfat1*$^{-/-}$/OT-II mice and immediately lysed for extraction of RNA. Transcript levels were measured by real-time PCR. Results are presented as mean±SEM from 3 independent experiments.

Figure 5. Anergy-resistant *Nfat1*$^{-/-}$ OT-II T cells delay tumor growth. A. $5x10^5$ B16-OVA cells were injected subcutaneously into TCR-transgenic *Nfat1*$^{+/+}$OT-II and *Nfat1*$^{-/-}$OT-II mice. 10 days

post-tumor challenge tumors were excised from *Nfat1*$^{+/+}$OT-II and *Nfat1*$^{-/-}$OT-II mice and weighed. Results are mean±SEM of 2 groups of 6 mice. *P<0.05. **B**. B16-OVA cells were injected subcutaneously into *Nfat1*$^{+/+}$OT-II and *Nfat1*$^{-/-}$OT-II mice. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM from 5-9 mice. *P<0.05; **P<0.01; ***P<0.001. **C**. C57Bl/6 mice were injected with 5x10^5 B16-OVA cells and then adoptively transferred with PBS or 5x10^6 *in vitro* differentiated *Nfat1*$^{+/+}$ or *Nfat1*$^{-/-}$ OT-II Th1 cells. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM from 3 groups of 5 mice. **D**. 5x10^5 B16-OVA cells were injected subcutaneously into *Nfat1*$^{+/+}$ or *Nfat1*$^{-/-}$ mice. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM from two groups of 5-6 mice. **E.** 5x10^5 B16-OVA cells were injected subcutaneously into the flanks B6.129S2-*Cd8a*^{tm1Mak}/J mice followed by intravenous transfer of 5x10^6 *Nfat1*$^{+/+}$ or *Nfat1*$^{-/-}$ OT-II T cells. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are representative of one of two independent experiments and are presented as mean±SEM of data obtained from 3 mice per condition.

Figure 6. Anergy-resistant *Nfat1*$^{-/-}$ T cells delay TC-1 tumor growth A. C57Bl/6 mice were vaccinated with by intraperitoneal injection of 10^6 apoptotic TC-1 cells (one injection per week for two weeks), followed by subcutaneous injection of 5x10^5 TC-1 cells. Control animals were injected with equal volumes of PBS prior to receiving the live tumor cells. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM of data obtained from 3 mice. **B.** B6.*Nfat1*$^{+/+}$ and B6.*Nfat1*$^{-/-}$ mice were vaccinated as above, followed by subcutaneous injection of 5x10^5 TC-1 cells. Tumor volumes were calculated from perpendicular diameters recorded every 1-2 days. Results are presented as mean±SEM of data obtained from four mice analyzed in two independent experiments.*P<0.05.



Abe et al. Figure 1

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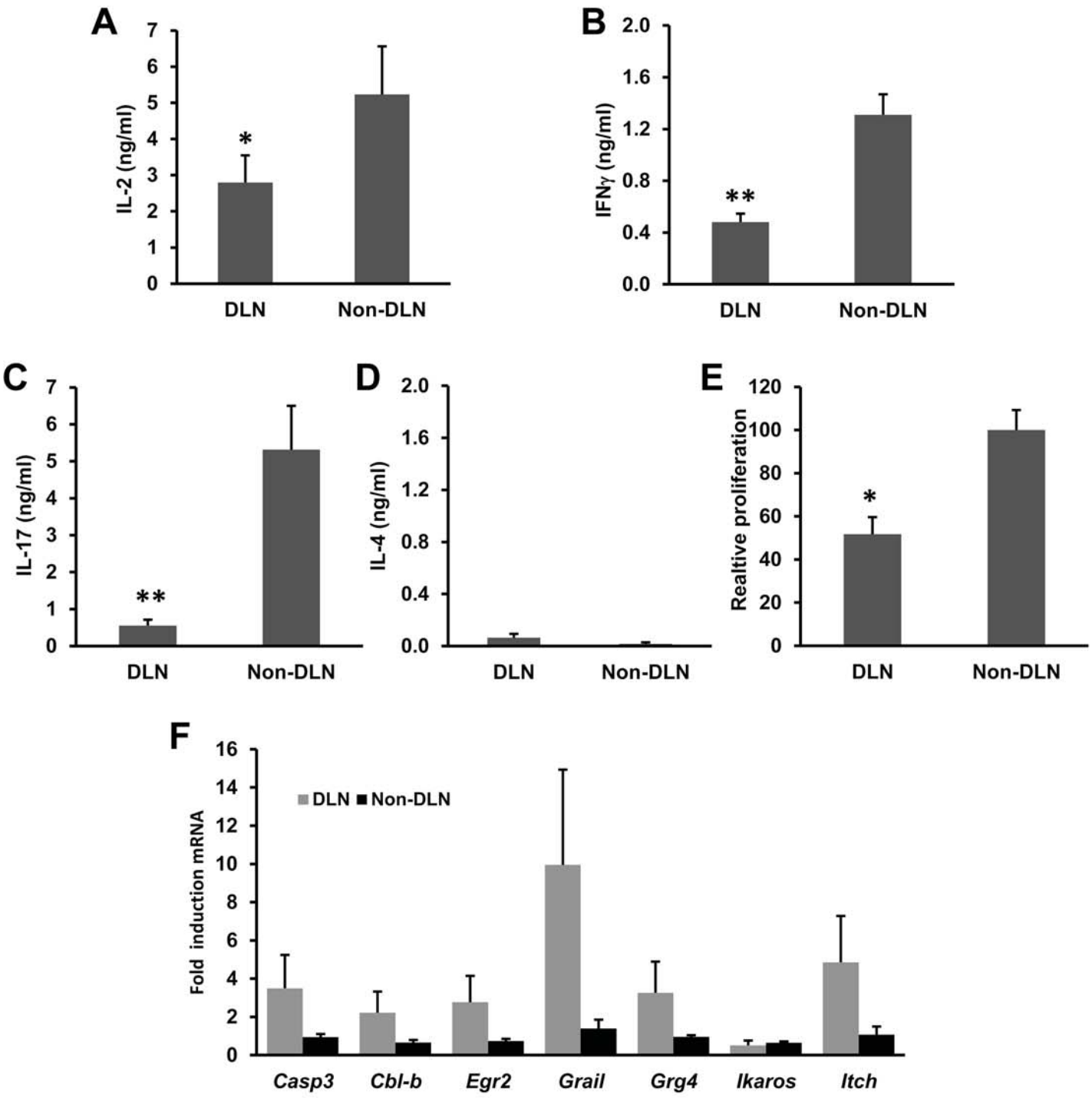
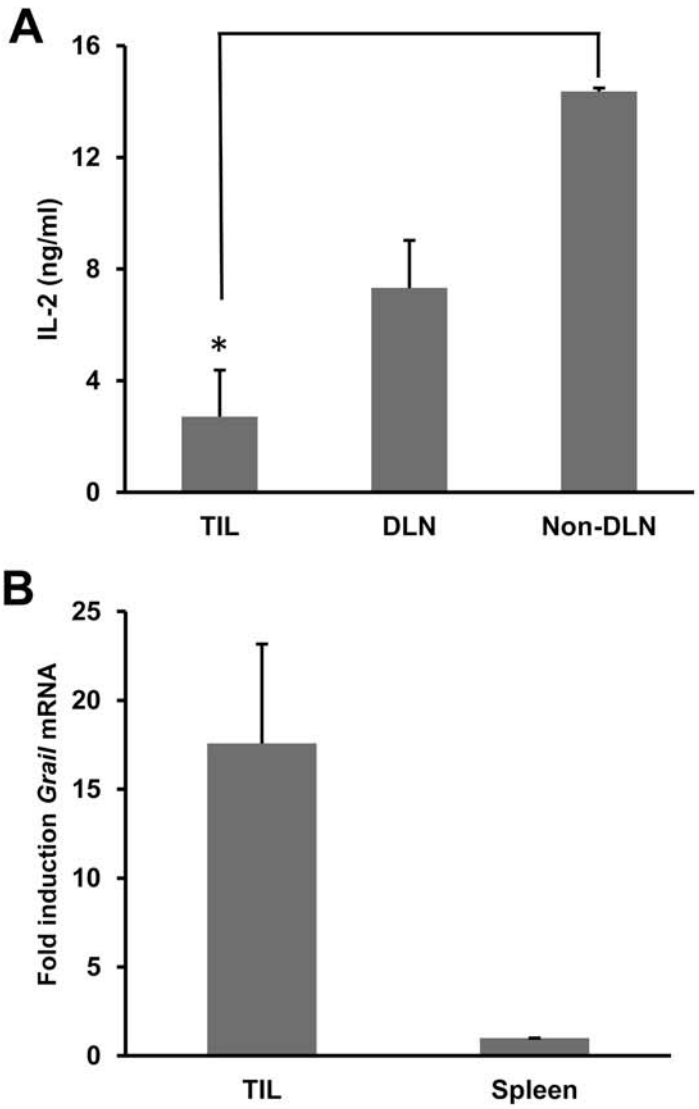


Abe et al. Figure 2



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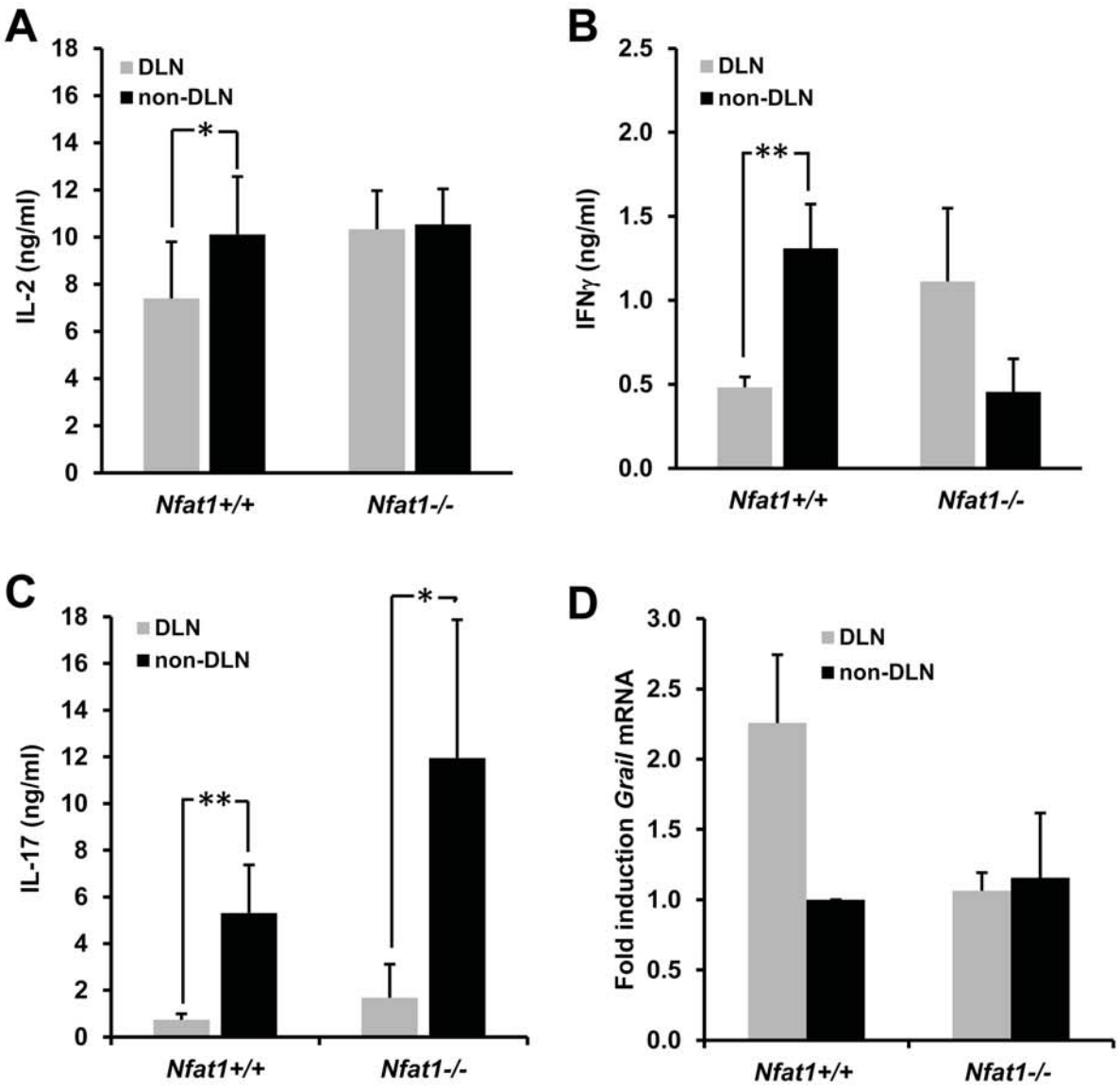

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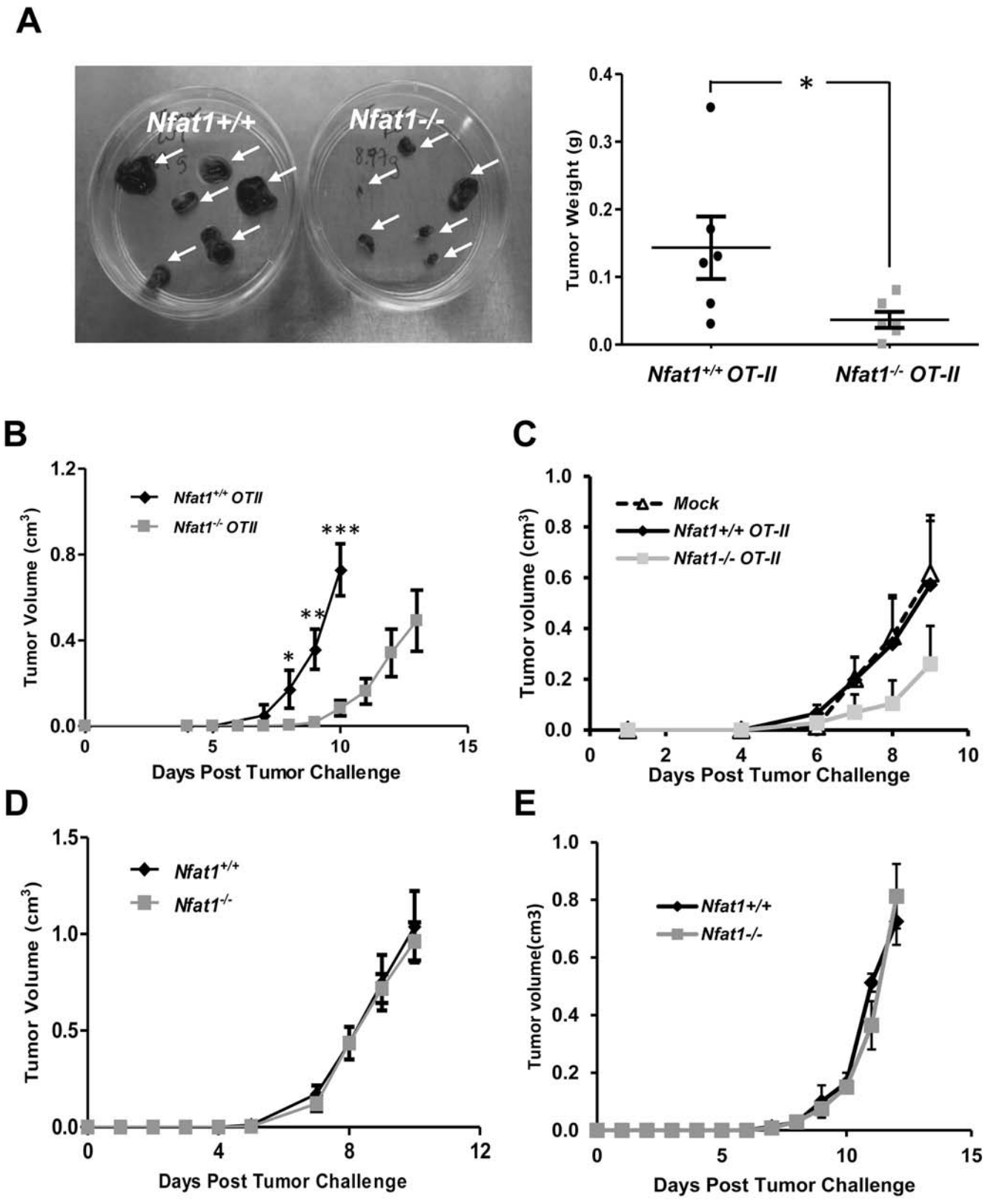

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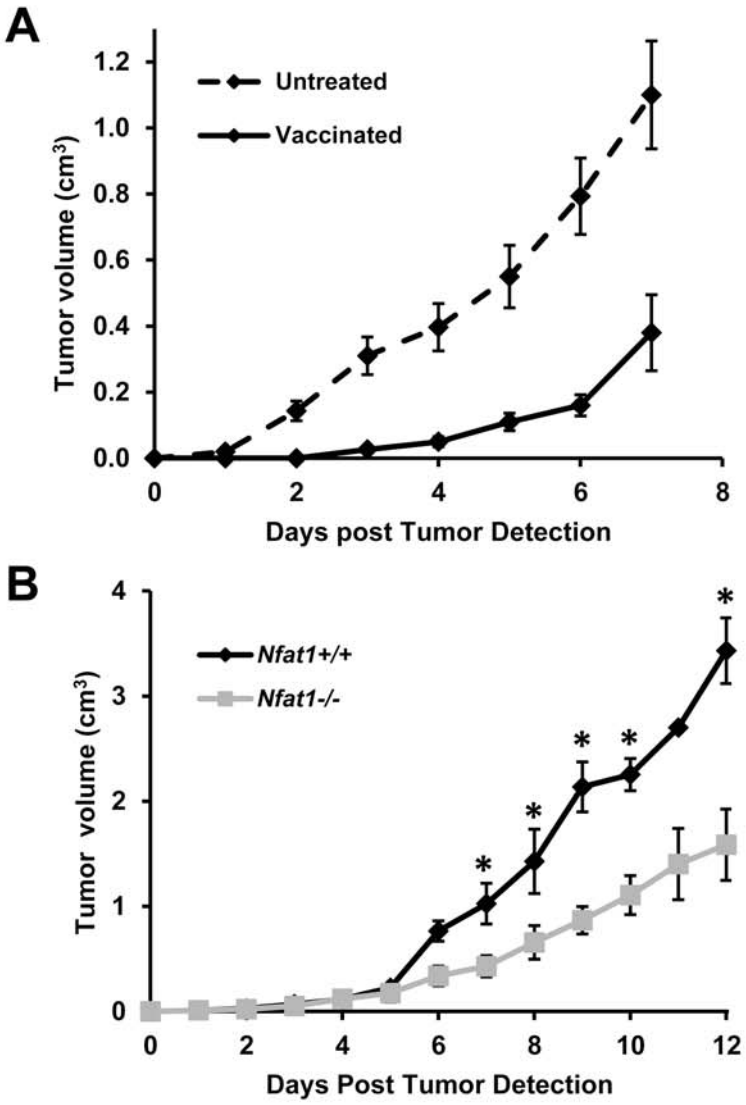

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NFAT1 supports tumor-induced anergy of CD4+ T cells

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