A Key Regulatory Role of the Transcription Factor NFATc2 in Bronchial Adenocarcinoma via CD8+ T Lymphocytes

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Abstract

The Ca2+-regulated calcineurin/nuclear factor of activated T cells (NFAT) cascade controls alternative pathways of T-cell activation and peripheral tolerance. Here, we describe reduction of NFATc2 mRNA expression in the lungs of patients with bronchial adenocarcinoma. In a murine model of bronchoalveolar adenocarcinoma, mice lacking NFATc2 developed more and larger solid tumors than wild-type littermates. The extent of central tumor necrosis was decreased in the tumors in NFATc2(−/−) mice, and this finding was associated with reduced tumor necrosis factor-α and interleukin-2 (IL-2) production by CD8+ T cells. Adoptive transfer of CD8+ T cells of NFATc2(−/−) mice induced transforming growth factor-β, in the airways of recipient mice, thus supporting CD4+ CD25+Foxp3+ glucocorticoid-induced tumor necrosis factor receptor (GITR)+ regulatory T (Treg) cell survival. Finally, engagement of GITR in NFATc2(−/−) mice induced IFN-γ levels in the airways, reversed the suppression by Treg cells, and co-stimulated effector CD4+CD25− (IL-2Rα+) and memory CD4+CD127− (IL-7Rα−) T cells, resulting in abrogation of carcinoma progression. Agonistic signaling through GITR, in the absence of NFATc2, thus emerges as a novel possible strategy for the treatment of human bronchial adenocarcinoma in the absence of NFATc2 by enhancing IL-2Rα+ effector and IL-7Rα− memory-expressing T cells. [Cancer Res 2009; 69(7):3069–76]

Introduction

There is substantial evidence that cancer patients harbor tumor-reactive T cells, although their number and/or activity is apparently insufficient to eradicate tumors (1). How such tumor-reactive T cells can be expanded and activated to turn against established tumors is a promising scientific endeavor in effective immunotherapy for cancer (2). Naturally occurring CD4+CD25+Foxp3+ regulatory T (Treg) cells maintain immunologic self-tolerance in the periphery but also inhibit immunosurveillance against autologous tumor cells (3–7).

Nuclear factor of activated T cells (NFAT) transcription factors were originally described as nuclear proteins that bind to and, thus, control the activity of the interleukin-2 (IL-2) promoter in T lymphocytes (8, 9). IL-2 and IL-7 have been used to activate T-cell responses in patients with tumors. The immunosuppressive agents cyclosporin A and FK506 target calcineurin and prevent the nuclear translocation and activation of NFAT factors, which hence emerge as critical activators of the immune response (10). We, thus, asked whether NFATc2 expression would be defective in the lungs of patients with lung adenocarcinomas.

Materials and Methods

Study population.

The study was approved by the local ethical review board (ethics committee of Rheinland-Pfalz). Lung cancer specimens derived from surplus tissues from the routine surgical pathology service were provided in an anonymous fashion from the tumor tissue bank of the program project (SFB432) supported by Deutsche Forschungsgemeinschaft. Ten control lung tissues were obtained from lung cancer patients. Tumor surgery for peripheral lung adenocarcinomas usually involves total lobectomy or even bilobectomy, which generates not only tumor tissue but mostly nonneoplastic lung tissue. During tissue cryoconservation, we aimed at selecting control tissue as far away from the tumor as possible. In addition, five control tissues from patients with pneumonia were analyzed. Pneumonia was confirmed histologically by the presence of (masses of) neutrophils in the airspaces in the absence of tumor cells.

In addition, 25 adenocarcinoma tumor specimens were investigated. In these patients, we selected the grading classification to establish a link between the tumor-directed immune system and a marker of tumor biology, as grading is linked to the degree of dedifferentiation, the speed of disease progression, and the putative response to antiproliferative cytotoxic treatment. Grading was done by expert histopathologic assessment of the tissue samples. In addition to grading, tumor staging is reported for all patients (Supplementary Fig. S1).

RNA extraction and reverse transcription–PCR.

Total cellular RNA was extracted from frozen tissue specimens using RNeasy Mini kit (Qiagen). The resulting RNA (5 μg) was primed with a dT18 oligonucleotide and reverse-transcribed with Superscript II (Invitrogen) according to the manufacturer’s instructions. Integrity of the obtained cDNA was tested by amplification of p53 transcripts in a 30-cycle PCR (sense 5′-CGT GAG CGC TTC GAG ATG TTC CG-3′, antisense 5′-CCT AAC CAG CTT CCC AAC ATG AG-3′; annealing temperature, 67°C). Only samples that had been tested for cDNA integrity by successful amplification of a 740-bp p53 product were subjected to expression analysis.

PCR primers were designed and used for specific amplification from first-strand cDNA stocks. To exclude false-positive PCR products due to contaminating genomic DNA in the RNA preparation or existence of pseudogenes, the individual gene-specific primer sets were designed to span exon/intron boundaries and quality-controlled by PCR reactions using either DNA or not reverse-transcribed RNA as template. For PCR analysis of individual gene transcripts, 0.5 μL first-strand cDNA were amplified with

Note: Supplementary data for this article are available at Cancer Research Online.
transcript-specific oligonucleotides (Operon), using 1 unit of Hot Star Taq DNA polymerase (Qiagen) in a 30-μL reaction mixture according to the manufacturer’s instructions. The primer sequences and the respective annealing temperatures are given for hypoxanthine phosphoribosyltransferase (HPRT; sense 5′-TGA CAC TGG CAA AAC AAT GCA-3′, antisense 5′-GGT CCT TTT CAC CAG CAA GCT-3′; annealing temperature 65°C), NFATc2 (sense 5′-ACA ACA TGA GGG CAA CCA TCG-3′, antisense 5′-GTC CAT CTG TGG TCT CTT CAG-3′; annealing temperature, 60°C), and glucocorticoid-induced tumor necrosis factor receptor (GTR, TNFSF18; sense 5′-TGG CTG TGG GCT CTT GAA ACC-3′, antisense 5′-GAG GCA CAG TCG ATA CTA GGC-3′; annealing temperature, 62°C).

Quantitative real-time reverse transcription–PCR (RT-PCR) analysis was performed using the ABI PRISM 3700 Sequence Detection System instrument and software (Applied Biosystems) with Quantitect SYBR Green PCR kit (Qiagen). Reactions were performed for 40 cycles in triplicates with specific primers (300 nmol/L each) with initial denaturation/activation for 15 min at 95°C, 30 s denaturation at 94°C, 30 s annealing, and 30 s amplification at 72°C. The relative expression level of specific transcripts was calculated with respect to the internal standard HPRT to normalize for variations in the quality of RNA and the amount of input cDNA. Expression levels were normalized to normal lung tissues using ΔΔCt calculation. In each experiment, a template-free negative control was included. The specificity of PCR reactions was confirmed by cloning and sequencing of amplification products from arbitrarily selected samples.

Mice. BALB/cj mice were obtained from Charles River Laboratories. NFATc2−/− mice were generously provided by Prof. L.H.Glimcher and were previously described (11). Animals were bred and maintained under specific pathogen-free conditions in our animal facility. Mice were treated according to institutional animal care and use guidelines.

Cell line. The cell line L1C2 is a murine (BALB/cj) bronchoalveolar carcinoma cell line obtained as a gift from Dr. Steven Dubinett (University of California-Los Angeles; ref. 12). The cell line is syngeneic to BALB/cj mice in L1C2 tumor models (13). Similar results were found in other syngeneic mouse tumor models in BALB/cj mice (i.e., mesothelioma cells; ref. 14). L1C2 lung adenocarcinoma cells were cultured in RPMI enriched with 10% FCS (Biofluids) and antibiotics, as previously described (12). This cell line was tested negative for Mycoplasma and other transmissible infectious agents. A total of 2 × 106 cells resuspended in 0.2 mL RPMI without supplements were later injected into the tail vein. At the indicated following days, lungs were removed, pooled in medium, and photographed under the stereomicroscope Stermi 2000-C and analyzed on AxioVision software (Carl Zeiss Vision GmbH).

Antibody treatment. On days 4 and 8, mice were i.v. injected with 100 μg of affinity-purified anti-GITR antibodies (DTA-1) in 200 μL stero- fundin. These antibodies are generous gifts from Prof. Shimon Sakaguchi and Prof. Edgar Schmitt. Anti-GITR antibodies were purified from ascites of severe combined immunodeficient mice by 40% ammonium sulfate precipitation twice and subsequently purified by protein G column (GE Healthcare). Purified rat IgG (Sigma-Aldrich) was used as a control antibody.

Two experiments were performed by enclosing four mice per group, thus resulting in eight independent values per group.

Collection and analysis of the bronchoalveolar lavage fluid. Collection and analysis of bronchoalveolar lavage fluid (BALF) of the right lung were performed, as described previously (15). Histologic analysis of tumor mass. For histologic analysis, lung samples were fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer sections were stained with standard H&E, and tumor load and necrosis for each lung were quantified by HAL using Photoshop-assisted image analysis (Adobe Systems, Inc., version 7.0), as previously described (16). For each lung, these sections were examined at 300-μm increments, and the slides with the maximum tumor involvement were quantified for final analysis.

Isolation, analysis, and reconstitution of total lung CD8+ T cells. Total lung cell suspension was obtained, as previously described (17). Alternatively, 7.5 × 10^3 purified CD8+ T cells, which were isolated at day 5 after L1C2 injection from BALB/cj and NFATc2−/− mice, were resuspended in 0.2 mL RPMI without supplements and i.p. reconstituted into 5-d tumor-bearing wild-type mice.

Fluorescence-activated cell sorting analysis. Total lung cells were stained with the fluorescence-activated cell sorting (FACS) antibodies and analyzed, as described above: antimouse CD4 (L3T4-FITC) antibody, antimouse CD8a (S3-6-7-APC) antibody, antimouse CD25 (PerCP-Cy5.5) antibody, control IgG2a (G155-178) and IgG2b (eB149/10H5; BD Bioscien-

ces) antibodies, antimouse GITR (FITC) antibody, rat anti-mouse CD127 (A7R34-FITC; eBioscience), and IgG (299Arm; eBioscience).

Intracellular Fos-3 protein analysis. Freshly isolated lung cells were permeabilized and fixed with Permfix solution (eBioscience) for 45 min at 4°C and washed with washing buffer containing 0.1 μg pg/μL saponin. After that, the cells were then incubated with APC-labeled antimouse Fos-3 for 30 min at 4°C in permeabilization fluid, washed twice with wash buffer, resuspended in 200 μL PBS, and then analyzed by FACS.

ELISA. Tumor necrosis factor-α (TNF-α), IL-2, and IL-10 levels were detected in cell supernatants using a specific sandwich ELISA set (TNF-α OptiEA standard range, 15.6–1,000 pg/mL; IL-2 OptiEA standard range, 62.5–4,000 pg/mL; IL-10 OptiEA standard range, 15.6–1,000 pg/mL; BD Bioscience). The IFN-γ-ELISA was performed in BALF, and cell super-
natants using a sandwich ELISA set (OptiEA standard range, 31.3–2,000 pg/mL; BD Pharmingen). Transforming growth factor-β1 (TGF-β1) ELISA has been performed from BALF using a sandwich ELISA, as previously described.

Statistical analysis. Differences were evaluated for significance (P < 0.05) by the Student’s two-tailed t test for independent events (Excel, PC). Data are given as mean ± SE.

Results

Defective transcription of the NFATc2 gene in the lung of human subjects affected by bronchial adenocarcinoma. In this study, we first asked whether NFATc2 expression is defective in the lungs of patients with bronchial adenocarcinoma. To address this question, we quantified using RT-PCR NFATc2 mRNA expression in lung samples of 25 patients with bronchial adenocarcinoma, 10 control lung samples, and 5 lung samples from patients with pneumonia (Fig. 1A, left). Expression of this NFATc2 mRNA transcript was significantly decreased in tissues derived from bronchial adenocarcinoma compared with control tissues and tissues showing histopathologic signs of pneumonia [single patient values are given in Fig. 1B (right) and Supplementary Fig. S1]. In these samples, CD3 mRNA expression was comparable between control and carcinomatous tissues (single patient values are given in Fig. 1C, right), whereas CD3 mRNA levels were significantly elevated in tissues with signs of pneumonia.

NFATc2-deficient mice display more aggressive growth of bronchial adenocarcinoma compared with wild-type littermates. We next analyzed the effects of NFATc2 deficiency in a murine model of bronchial adenocarcinoma induced by the injection of the bronchial adenocarcinoma cell line (Supplementary Fig. S2). On day 10 after tumor cell injection, some early small tumor nodules were found in NFATc2−/− mice, whereas no tumor colonies were seen in NFATc2+/+ mice (Fig. 2A, left). On day 21, the tumor nodules had grown into confluent masses, which were significantly larger in the NFATc2−/− mice, reaching mean values of almost 50% of the entire lung volume in NFATc2−/− mice compared with only ~10% in NFATc2+/+ mice (Fig. 2A, left and right). In agreement with the frequently observed preferred colonization of human metastatic disease in the lung periphery, we found the earliest metastatic tumor colonies on the pleural surface of L1C2-injected mice (Fig. 2A, right).
Despite this striking increase in total tumor load in NFATc2(−/−) mice, we found virtually no tumor necrosis in these mice (Fig. 2B, right). In contrast, the much smaller tumor nodules in the NFATc2(+/−) mice exhibited significant central necrosis, underscoring the striking survival advantage of tumor cells in NFATc2(−/−) mice (Fig. 2B).

Lack of correlation between IFN-γ release in the airways and tumor load in NFATc2(−/−) mice. We, thus, started to investigate the immunologic mechanisms responsible for the increased tumor load in the lung of NFATc2(−/−) mice. Accordingly, we analyzed levels of the cytokine IFN-γ, as previous findings suggested that this pleiotropic cytokine is produced by T lymphocytes and natural killer (NK) cells (18) and promotes antitumor responses (19). Surprisingly, as shown in Fig. 2C (left), no correlation between IFN-γ released in the BALF of NFATc2(−/−) and wild-type littermates bearing tumor and the tumor load was seen. In addition, no difference in IFN-γ production in the BALF between NFATc2(−/−) and wild-type mice was observed at comparable tumor load (Fig. 2C, right).

CD8+CD122+ cells have recently been described to suppress the proliferation of IFN-γ–producing CD8+ T cells (20, 21). As shown in Fig. 2D, mice lacking NFATc2 had significantly increased numbers of CD8+CD122+ T cells compared with wild-type littermates, and the number of these cells was even further expanded in the lung of NFATc2(−/−) mice bearing tumor. We, thus, reasoned that, in this case, CD8+CD122+ cells were responsible for the lack of correlation between IFN-γ release and tumor load in NFATc2(−/−) mice (Fig. 2D, right).

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**Fig. 1.** Decreased expression of the transcription factor NFATc2 in tumor specimens of patients with adenocarcinoma of the lung. RNA from lung tumor tissues classified in differentiation grades of adenocarcinoma G II (n = 7), G II-GIII (n = 7), and G III (n = 11), as well as nonneoplastic lung tissue from control patients (n = 10), and five samples from patients with pneumonia were isolated as described. A, the primers were selected in the coding region corresponding to the IPT (immunoglobulin-like, plexins, transcription factors), which is a conserved region downstream of the DNA-binding domain. B, NFATc2 mRNA expression in tumor tissues versus nonneoplastic control samples was evaluated by means of RT-PCR. C, no changes in CD3 mRNA expression were observed in neoplastic samples from patients with adenocarcinoma with the exception of the transition group GII-GIII, in which CD3 mRNA levels were significantly elevated compared with the nonneoplastic control samples (t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001). Single values and the corresponding tumor-node metastasis values are given in Supplementary Fig. S1.
lung tumor model, CD8+CD122+ T cells are increased but are apparently not capable to fully suppress IFN-γ production.

Defective IL-2 and TNF-α production by CD8+ T cells isolated from tumor-bearing lungs in NFATc2(-/-) mice. We next asked whether the decreased tumor necrosis would correlate with a decrease in TNF-α release in the airways of NFATc2(-/-) mice. As shown in Fig. 3A, wild-type mice revealed higher TNF-α levels in the airways compared with NFATc2(-/-) mice. Moreover, a rather constant amount of TNF-α was released in the BALF of NFATc2(-/-) mice as the tumor mass expanded (Fig. 3A).

Consistent with the decreased necrosis in the tumors of NFATc2(-/-) mice, we detected decreased TNF-α levels in the supernatants of lung CD8+ T cells isolated from tumor-bearing lungs of NFATc2(-/-) mice at day 21 compared with those isolated from the wild-type littermates (Fig. 3B). However, the lung CD8+ T cells from tumor-bearing NFATc2(-/-) mice expressed significantly increased levels of GITR (Fig. 3C). Along with a decrease in TNF-α, lung CD8+ T cells from NFATc2(-/-) mice released lower levels of IL-2 (Fig. 3D, left). By contrast, the immunosuppressive cytokine IL-10 was markedly increased in the supernatant of lung NFATc2(-/-) CD8+ T cells (Fig. 3D, right).

NFATc2 deficiency induced TGF-β1 and CD4+CD25+Foxp-3+ GITR+ suppressive cells in the airways in a mouse model of bronchial adenocarcinoma. To show a direct pathophysiologic link between the tolerogenic function of CD8+ T cells isolated from the NFATc2-deficient mice and advanced tumor growth, we adoptively transferred lung CD8+ T cells i.v. into wild-type and NFATc2(-/-) mice previously injected with L1C2 tumor cells (Fig. 4A). Wild-type CD8+ T cells could not induce TGF-β1, a Treg cell inducing cytokine, in the airways of NFATc2(-/-)-recipient mice and similarly CD8+ NFATc2(-/-) T cells could not induce TGF-β1 in the airways of wild-type littermates. TGF-β1 could only be induced by NFATc2-deficient CD8+ T cells in an environment also defective in this transcription factor (Fig. 4A).

We then asked the question whether the increased colonization of NFATc2(-/-) mouse lungs by L1C2 adenocarcinoma cells is directly linked to an increased suppressive function exerted by Treg cells in these mice (4, 22–24). CD4+CD25+ Foxp-3+Treg cells are a unique lineage of the T-cell population that inhibits the proliferation of defined immune cells, including CD4+ T cells and NK cells, as well as CD8+ T cells (25). By immunofluorescence analysis studies (FACS), CD4+CD25+Foxp-3+ T cells were found to be increased in lung tumors in NFATc2(-/-) mice when compared with wild-type controls (Fig. 4C). In parallel, TGF-β1 levels were up-regulated in the lung tumors of NFATc2(-/-) mice (Fig. 4B). Treg cells express GITR at higher levels than other T cells, although both Treg and non-Treg cells up-regulate its expression upon activation (26, 27). Interestingly, CD4+CD25+GITR+ T cells were significantly increased in the lung tumors of NFATc2(-/-) mice (Fig. 4D).

To rule out the possibility that the number of Treg cells would be dependent on the increased tumor load present in the lung of Fig. 2. NFATc2 protects mice from metastases of the lung adenocarcinoma. A, left, representatives of low-power histologic sections of the lungs of wild-type (top) and NFATc2(-/-) (bottom) mice are shown 10 d (left column) and 21 d (right column) after i.v. injection of the L1C2 tumor cell line. Columns, mean of three independent experiments (n = 5 mice per group); bars, SE. B, high-power histologic examination of the necrosis in the center of tumor nodules in NFATc2(-/-) mice (top) but virtually no necrosis in NFATc2(-/-) mice (bottom). C, IFN-γ release in the BALF and the tumor load measured in individual mice in both wild-type (open squares) and NFATc2(-/-) (solid squares) mice were correlated. Linear regression equation and correlation coefficient (R²) are given. Right, quantification of IFN-γ in BALF at day 21 after L1C2 i.v. injection (n = 5). D, increased number of CD8+CD122+ T cells in the lungs of NFATc2(-/-) mice before and 21 d after i.v. injection of L1C2 in a murine model of adenocarcinoma. Asterisks indicate statistically significant differences between animals of the two groups (t test: *, P < 0.05; **, P < 0.01; *** P < 0.001).
NFATc2(-/-) mice, we analyzed the equation correlating these two parameters. As shown in Supplementary Figs. S3 and S4, no increase in the number of Treg cells was seen in the lung of NFATc2(-/-) mice as the tumor developed, indicating that, in the absence of NFATc2, Treg preferentially develops in this tumor model. Taken together, these data suggest that the development of tumors in the lungs of NFATc2(-/-) mice is associated with increased number and activation of Treg cells in the lung via TGF-β1.

GITR is increased in the lungs of some patients affected by bronchial adenocarcinoma. T-cell stimulation by GITR attenuates Treg-mediated suppression and/or enhances tumor killing by CD4+ and CD8+ T cells, leading to the eradication of advanced neoplasms.
tumors in mice (28). For this reason, we looked at GITR expression in the samples of human carcinomas and control lungs analyzed in Fig. 1. Interestingly, we found that GITR expression was significantly up-regulated in the lungs of some samples from patients with grade G2 or G3 tumors. We also noted that patients with increased CD3 expression had also increased GITR levels (Figs. 1C, right and 5). We, hence, propose that, in the absence of NFATc2, anti-GITR therapy could be used to activate effective antitumor immune response when GITR is increased.

**Anti-GITR treatment ameliorated lung adenocarcinoma by inducing IL-2Rα⁺ and IL-7Rα⁺CD4⁺ T cells in the lung of NFATc2(−−/−) mice in a murine model of adenocarcinoma.** It has been previously shown that administration of an agonistic anti-GITR monoclonal antibody to neonatal mice can break self-tolerance and elicit autoimmune disease (27). We, thus, examined whether the immunostimulatory activity of agonistic anti-GITR antibodies would provoke effective antitumor immunity in mice lacking NFATc2 with advanced tumors.

We found that two consecutive i.v. injections of 100 μg each of anti-GITR antibodies (DTA-1) on days 4 and 8 after tumor inoculation resulted in a much reduced tumor load in the lungs of NFATc2-deficient mice (Fig. 6A, bottom). This finding was associated with a reduction of CD4⁺CD25⁺Foxp⁺ Treg cells (Fig. 6B) and with increased numbers of CD4⁺CD127⁺ (IL-7Rα⁺) effector T cells (Fig. 6C, left) in the airways of NFATc2(−−/−) mice, just as previously described for wild-type littersmates by Sakaguchi and colleagues (28). Moreover, we found that anti-GITR antibody treatment restored and increased IL-2 production by pulmonary CD8⁺ NFATc2(−−/−) T cells (Fig. 6C, right). The release of IFN-γ was also significantly increased in the airways of NFATc2(−−/−) mice compared with wild-type littersmates after anti-GITR treatment (Supplementary Fig. S5). However, no significant difference in IFN-γ production by CD8⁺ lung T cells was observed after anti-GITR antibody treatment in both wild-type and NFATc2(−−/−) groups (Supplementary Fig. S5). Finally, we noted the expansion of the CD4⁺CD127⁺ (IL-7Rα⁺) T cells after anti-GITR antibody treatment in the lung of NFATc2(−−/−) mice (Fig. 7; ref. 29). IL-7 is an important survival factor for CD4⁺ long-lived memory T cells that, together with T-cell receptor signals, regulates homeostasis of the CD4⁺ memory T-cell population in lymphopenic conditions and in the intact immune system (30).

**Discussion**

The current study reveals an unexpected role of the nuclear transcription factor NFATc2 in integrating the function of CD8⁺ and CD4⁺ T cells in a murine model of lung adenocarcinoma. The consequences of NFATc2 deficiency for the lung affected by adenocarcinoma comprise the local expansion of immunosuppressive CD4⁺CD25⁺Foxp⁺GITR⁺ T cells induced by CD8⁺ NFATc2(−−/−) cells via TGF-β1. This immunosuppression leads to increased tumor load and abrogates tumor central necrosis in NFATc2(−−/−) mice. These findings are in line with the documentation of defective TNF-α production by CD8⁺ T cells isolated from the NFATc2(−−/−) mice in a model of lung adenocarcinoma.

The clinical relevance of these findings is underlined by the defective expression of NFATc2 in samples of human bronchial adenocarcinoma compared with control tissue. It should be noted, however, that one cannot refer to these control specimens as “healthy” lung samples, as control tissue from lungs next to tumor tissue may harbor genetic abnormalities. In any case, lung tissue from patients with bronchial adenocarcinoma showed reduced levels of NFATc2 compared with such control tissue, as well as to lung samples derived from patients with pneumonia. Whether this defect is associated with a mutated NFATc2 gene and NFATc2 protein expression in T cells in these patients is currently not clear. One possibility is the presence of a mutated sequence in this gene, likely affecting the COOH terminal domain that has been recently described as enhancer of TNF-α expression (31). This possibility could be compatible with our findings showing defective TNF-α production by CD8⁺ T cells isolated from the NFATc2(−−/−) mice in a model of lung adenocarcinoma.

In subsequent studies, we found that TNF-α, rather than IFN-γ, levels in the BALF correlate inversely with tumor load in a model of lung adenocarcinoma. Furthermore, we have shown that TNF-α deficiency in CD8⁺ T cells from lungs of NFATc2(−−/−) mice is associated with a defect in IL-2 and, in turn, an increase in IL-10 production by these cells. In a recent report, in a murine model of allergy, we identified a subpopulation of CD8⁺ T cells, the CD8⁺CD122⁺ T-cell subpopulation, which is expanded in the absence of NFATc2 and selectively produces IL-10. We further showed in that report that the expansion of this CD8⁺CD122⁺ T-cell population leads to the down-regulation of IL-2 production by the CD8⁺NFATc2(−−/−) cells (21). Hence, NFATc2 expression in
CD8+CD122+ cells might lead to the expression of IL-2 and TNF-α, which in turn causes the suppression of IL-10. Consistent with these findings, we also found in our murine model of lung adenocarcinoma an increase of the CD8+CD122+ population within the lung CD8+NFATc2(C0/C0/C0) T-cell compartment in this murine model of adenocarcinoma. These data suggest that expressing NFATc2 in CD8+CD122+-(IL-2/IL-15Rβ chain+) cells could lead to increased IL-2 and decreased IL-10 production, thus eventually resulting in an inhibition of Treg cell functions. In support of this assumption, it has been recently reported that IL-2 expression is dependent on NFATc2 in memory but not in naive cells (32). Due to this altered cytokine milieu and the increased number of Treg cells, NFATc2-deficient mice have an increased colonization of the lung by exogenous L1C2 adenocarcinoma cells.

Depletion of Treg cells with anti-GITR antibodies at early time points after cancer cell injection in NFATc2(C0/C0/C0) mice abrogated the release of the tumor growth restoring CD8+ T-cell cytokine release, such as IL-2. Ko and colleagues described an increase in IFN-γ secreting T cells in the spleen of anti-GITR tumor-bearing mice (28). We looked at the lung and found increased IFN-γ levels in the BALF of anti-GITR antibody–treated NFATc2(C0/C0/C0) mice compared with wild-type littermates. Moreover, we measured IFN-γ in the supernatants of lung CD8+ T cells isolated from anti-GITR–treated and IgG-treated mice. No significant increase in IFN-γ release was observed in the supernatant of lung CD8+ T cells isolated from anti-GITR antibody–treated wild-type and NFATc2(C0/C0/C0) mice compared with the untreated groups. It is possible that the differences between our results and those described by Ko and colleagues in IFN-γ production after anti-GITR antibody treatment relate to the different tissues analyzed. Alternatively, the functional role of IFN-γ might depend on the

Fig. 6. In vivo injection of anti-GITR antibody abrogated lung adenocarcinoma in NFATc2(C0/C0/C0) mice by reducing CD4+CD25+Foxp3+ T cells and restoring IL-2 in CD8+ T cells. A, agonistic anti-GITR monoclonal antibody (mAb) given i.v. on days 4 and 8 after i.v. L1C2 cells decreased the lung metastatic area in NFATc2(C0/C0/C0) mice, as seen on H&E-stained sections. This treatment was accompanied by decreased numbers of CD4+CD25+Foxp3+ Treg cells (B), increased number of CD4+CD25+ effector T cells (C, left), and induced IL-2 production by lung CD8+ T cells (C, right). One representative experiment of two (n = 4 mice per group).

Fig. 7. Anti-GITR antibodies induced CD4+CD127+ memory T cells in NFATc2(C0/C0/C0) mice. Top, agonistic anti-GITR mAb given i.v. on days 4 and 8 after i.v. L1C2 cells induced CD4+CD127+ memory T cells at day 21 in NFATc2(C0/C0/C0), but not NFATc2(C0/C0/C0), mice. Bottom, representative FACS stainings. One representative experiment of two (n = 4 mice per group).
tumor model used, as Ko and colleagues used Meth A (BALB/c-derived fibrosarcoma cell line) compared with the L1C2 bronchial adenocarcinoma cell line used in the present study.

Depletion of Treg by anti-GITR treatment resulted in an expansion of the CD4+CD127+ (IL-7Rα) memory T cells and effector CD4+CD25+ (IL-2Rα) T cells, and this treatment exerted powerful antitumor activity (32–35). In this way, Treg cells can evade CD8+ T-cell control in the absence of Nfatc2. The clinical relevance of these findings is highlighted by the observation that Nfatc2 expression is suppressed in human lung adenocarcinoma. Moreover, the increased expression of GITR mRNA in the lung of some patients affected by bronchial adenocarcinoma suggests a possible intervention strategy in these patients by agonistic anti-GITR treatment.

The increase in effector CD4+CD25+ (IL-2Rα) cells and the decrease of CD4+CD25+Foxp3- T cells after anti-GITR treatment have been previously described by others (3), whereas documentation of an increase of CD4+CD127+ memory T cells is novel. The common γ-receptor chain of IL-2 and IL-7 is an important regulator of T-cell homeostasis (35). Although IL-2 is implicated in the acute phase of the T-cell response, IL-7 is important for memory T-cell survival. Moreover, it has recently been reported that IL-2 is a factor that regulates IL-7Rα expression and, consequently, memory T-cell homeostasis in vivo (35). Thus, inducing IL-2 production in the T-cell compartment results in the expansion of the memory cells that enhances a fast and tumor antigen–specific immune response.

In conclusion, cytokines released by CD8+ T cells are regulated by the expression of Nfatc2. The absence of Nfatc2 represses IL-2 and TNF-α production by CD8+ T cells and conversely induced the immunosuppressive cytokine IL-10. Due to this altered cytokine milieu and the resulting increased number of Treg cells, Nfatc2-deficient mice are more susceptible to pulmonary colonization by exogenous L1C2 adenocarcinoma cells. The GITR ligation in an environment deficient in Nfatc2 emerges as a novel possible strategy for the treatment of some human lung adenocarcinoma, because it induces the development of memory cells and augments the local immune response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prof. Jeffrey M. Drazen for critically reading the manuscript and Isabell Ernst and Francois Burri for their technical help.

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A Key Regulatory Role of the Transcription Factor NFATc2 in Bronchial Adenocarcinoma via CD8+ T Lymphocytes


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