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ORIGINAL ARTICLE

NFAT1 transcription factor is central in the regulation of tissue microenvironment for tumor metastasis

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Abstract Members of the nuclear factor of activated T cell (NFAT) family of transcription factors were originally described in T lymphocytes but later shown to be expressed in several immune and non-immune cell types. NFAT proteins can modulate cellular transformation intrinsically, and NFAT-deficient (NFAT1−/−) mice are indeed more susceptible to transformation than wild-type counterparts. However, the contribution of an NFAT1−/− microenvironment to tumor progression has not been studied. We have addressed this question by inoculating NFAT1−/− mice with B16F10 melanoma cells intravenously, an established model of tumor homing and growth. Surprisingly, NFAT1−/− animals sustained less tumor growth in the lungs after melanoma inoculation than wild-type counterparts. Even though melanoma cells equally colonize NFAT1−/− and wild-type lungs, tumors do not progress in the absence of NFAT1 expression. A massive mononuclear perivascular infiltrate and reduced expression of TGF-β in the absence of NFAT1 suggested a role for tumor-infiltrating immune cells and the cytokine milieu. However, these processes are independent of an IL-4-induced regulatory tumor microenvironment, since lack of this cytokine does not alter the phenotype in NFAT1−/− animals. Bone marrow chimera experiments meant to differentiate the contributions of stromal and infiltrating cells to tumor progression demonstrated that NFAT1-induced susceptibility to pulmonary tumor growth depends on NFAT1-expressing parenchyma rather than on bone marrow–derived cells. These results suggest an important role for NFAT1 in radio-resistant tumor-associated parenchyma, which is independent of the anti-tumor immune response and Th1 versus Th2 cytokine milieu established by the cancer cells, but able to promote site-specific tumor growth.

Keywords NFAT · Cancer · Tumor microenvironment · Melanoma B16F10

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Introduction

Tumor progression and metastasis are complex phenomena that depend on the cross talk between the transformed cell and the tumor microenvironment. The relevance of this network is underscored by findings that disruption of the tumor microcirculation leads to death of tumor cells and that manipulation of tumor-infiltrating cells, like tumor-associated macrophages (TAM) or mast cells, can alter cancer vascularization and growth [1–4]. New insights into the molecular mechanisms contributing to tumor-promoting microenvironments will lead to a better understanding of the pathways exploited by cancer cells to facilitate its growth and may uncover new targets to be used in directed therapy.

The nuclear factor of activated T cell (NFAT) family of transcription factors is expressed by immune and non-immune cells. It is composed of four calcium-responsive members: NFAT1 (NFATc2/NFATp), NFAT2 (NFATc1/NFATc), NFAT3 (NFATc4) and NFAT4 (NFATc3/NFATx), which are activated by calcineurin-mediated

dephosphorylation in response to a sustained increase in intracellular calcium. NFAT1 and NFAT2 modulate cellular transformation, possibly by regulating expression of cell cycle–related proteins [5–8]. Whereas NFAT1 has tumor-suppressor activities in fibroblasts [9], NFAT2 acts as an oncogene in pancreatic and haematological cancers [9–15].

The role of NFAT signaling in the context of tumor microenvironment is less clear. Members of the family are expressed by endothelial, immune and stromal cells, all of which contribute to tumor homeostasis. The cytokine milieu of tumor-associated tissue can direct the differentiation of infiltrating cells toward tumor-static or tumor-promoting phenotypes. The Th1 cytokine IFN-γ promotes resistance against tumor growth [16–18]. The Th2 cytokine IL-4 has a more controversial role. Although it generally promotes immunomodulatory TAMs and impairs anti-tumor immunity, its stimulation of type 2 cytotoxic $CD8^+$ T cells (Tc2) can favor tumor killing [4, 19–21]. NFAT transcription factors participate on the cytokine balance by regulating the expression of IL-2, IL-4, IL-5, IL-13 and IFN-γ expression by lymphocytes and therefore have the potential to impact both pro- and anti-tumorigenic responses [22–24]. Analysis of single knockout mice deficient for NFAT1 showed that NFAT1-deficient (NFAT1−/−) T cells express greater amounts of Th2 cytokines upon in vitro stimulation; NFAT1−/− animals are more susceptible to *Leishmania major* infection and following antigen sensitization develop eosinophilia and an IL-4-dependent allergic phenotype [25–27].

Tumor-associated stromal and endothelial cells, tumor-infiltrating immune cells, and the cytokine profile of the tumor microenvironment are essential components to cancer progression and can be modulated by expression of NFAT family members. Here, we sought to address the impact of NFAT1 expression by the cancer cell extrinsic tumor microenvironment in tumor progression. We used NFAT1−/− mice as hosts of the well-established B16F10 melanoma tumor homing model and observed that B16F10 cells showed reduced growth in NFAT1−/− lung parenchyma despite similar ability to home to the lungs as in wild-type mice. This phenotype was independent of host IL-4 expression or bone marrow–derived cells, but may involve NFAT1-driven TGF-β expression. Our results demonstrate that NFAT1 expression is important for the tumor-promoting capacity of host radio-resistant cells.

Materials and methods

Mice and tumor cell line

NFAT1−/− and control wild-type (NFAT1+/+) mice were generated as described [28]. Mice deficient for NFAT1 and IL-4 were generated by crossing NFAT1−/− and IL-4−/− animals [22]. Eight to 12-week-old mice were used, following the "Principals of laboratory animal care" (NIH publication No. 85–23, revised in 1985). B16F10 cells were cultured in DMEM supplemented with 10% FCS, L-glutamine, streptomycin-penicillin, essential and non-essential amino acids, sodium pyruvate, vitamins, 2-mercaptoethanol (Invitrogen, Carlsbad, CA) at 37°C in 5% of CO_2.

B16F10 homing assay

At least three NFAT1+/+ and NFAT1−/− animals were inoculated per time point with 2×10^5 B16F10 melanoma cells intravenously. At indicated times, mice were euthanized and the organs of interest harvested for analysis.

^{51}Cr-B16F10 labeling and homing assay

In the culture medium, 2.5×10^6 melanoma cells/ml were labeled with 25 μCi of ^{51}Cr/ml at 37°C for 1 h. Cells were washed 3 times with cold PBS and inoculated intravenously in groups of three NFAT1+/+ and NFAT1−/− experimental mice. Designated groups were euthanized 5, 15, 30 min, 1, 2, 6 or 24 h after ^{51}Cr-B16F10 inoculation. Organs analyzed: blood, spleen, thymus, lymph nodes, liver, lungs, thyroid gland, skeletal muscle, bone, testis/ovary, kidney, brain, stomach, intestines, heart and pancreas. A sample of each organ was dissected, weighted and had its radiation measured in a gamma counter.

Histopathology

Organs were fixed for 24 h in buffered formol. Standard procedures for paraffin embedding followed. Thin slices of tissue (4 μm) were stained by hematoxilin and eosin.

Flow cytometry

Cells were stained with the following fluorochrome-labeled antibodies: anti-CD3, anti-B220/CD45R, anti-CD4 and anti-CD8 (BD-PharMingen, San Diego, CA) and analyzed on a FACScan using CellQuest software (BD Biosciences, San Jose, CA).

Western blot

Mononuclear splenocytes isolated in Ficoll gradient were lysed in resuspension buffer (40 mM Tris (pH 7.5), 60 mM NaPPi, 10 mM EDTA) containing 5% SDS. Total protein extracts were analyzed by electrophoresis on 6% SDS–PAGE and blotted with polyclonal antibody 67.1

anti-NFAT1 (kind gift from Dr. A. Rao, Harvard University, Boston, MA) as described [29].

Bone marrow transplant

Mice were fed acidic water (pH 3.0) for 15 days prior to full body irradiation with 10 Gy [30]. Irradiated mice received 2×10^6 bone marrow cells intravenously in PBS [31]. Two months later all groups were inoculated with 2×10^5 B16F10 melanoma intravenously and the number of tumor nodules in the lungs was counted 15 days later.

In vitro T cell activation and ELISA

Total lymph node cells were stimulated in vitro for 72 h with plate-bound anti-CD3 (1 μg/mL; clone 2C11; BD-Pharmingen) in non-polarizing condition [26]. After 24 h, IL-2 (20 U/mL; PeproTech, Rocky Hill, NJ) was added to the cultures. On day 3, cells were harvested, washed and rested for 48 h in the presence of IL-2 alone (20 U/mL). Cells were then harvested, washed and challenged for 48 h with plate-bound anti-CD3 (1 μg/mL). Cell-free supernatant was assessed for IFN-γ and IL-4 protein levels by ELISA, according to the manufacturer's protocol (BD-Pharmingen).

RNase protection assay

Total mesenteric lymph node cells were stimulated in vitro as described earlier. At the end of stimulation, 10^7 cells were collected and RNA was extracted by Trizol, following manufacturer's protocol (Invitrogen, Carlsbad, CA). RNase protection assay was performed according to manufacturer's protocol, using the probe kit mCK-1 or mCK-3 (Probe-Quant, BD-Pharmingen).

Statistical analysis

For single comparisons unpaired Student's t test was used. For multiple comparisons, an analysis of variance followed by the Student–Newman–Keuls was used. Differences with $P < 0.05$ were considered statistically significant.

Results

Reduction in growth of B16F10 melanoma in the lungs of NFAT1-deficient mice

Increasing evidence supports the role of type 1 immunity in suppressing tumorigenesis and tumor growth. IFN-γ is a hallmark of these responses and has been shown important in reducing the susceptibility to tumor development and progression [18]. Even though NFAT1−/− mice generally have a Th2 phenotype [26], NFAT1 is important for endothelial cells to respond to vascular endothelial growth factor (VEGF), leading to angiogenesis, an important step in tumor establishment [32]. To evaluate the susceptibility of NFAT1−/− animals to tumor growth, B16F10 melanoma cells were inoculated intravenously into cohorts of mice. Interestingly, NFAT1−/− mice showed reduced susceptibility to B16F10, with fewer tumor nodules growing in the lungs of these mice in comparison with NFAT1+/+ animals (Fig. 1). Whereas the number of melanoma nodules in the lungs of wild-type mice progressively grew to the extent that over 250 nodules were counted 15 days after tumor inoculation, NFAT1−/− hosts showed no more than 20 nodules at the same time point (Fig. 1a). Furthermore, the nodules observed in NFAT1−/− mice were smaller than those of wild-type counterparts, suggesting that besides colonization, NFAT1 expression by the host supports melanoma progression (Fig. 1b). It remained to be shown if this phenotype was due to NFAT1 deficiency in tumor parenchymal cells, potentially impairing homing of tumor cells or tumor-induced angiogenesis; if lack of NFAT1 expression by tumor-infiltrating immune cells could alter the tolerant state induced by growing cancer; or if both phenomena could be at play.

Homing of B16F10 cells is not altered by NFAT1 deficiency in host animals

Altered pulmonary capillary bed could influence tumor cell access to the lung parenchyma, impacting colonization of this organ and explaining our observations. Alternatively, an altered balance between members of the NFAT family in NFAT1−/− animals could influence the adhesion molecules expressed by endothelial cells in the lungs of these mice and impair cell migration. We therefore went on to evaluate whether B16F10 melanoma cells were homing differently in NFAT1+/+ and NFAT1−/− mice. B16F10 cells were labeled with ^{51}Cr, and cohorts of mice were euthanized different times after intravenous inoculation. A sample of the lungs and other organs from each mouse was dissected, weighted, and the relative radiation emitted relative to organ mass calculated. No significant differences between homing of labeled melanoma cells was observed in lungs of NFAT1+/+ and NFAT1−/− mice, demonstrating that the differences in tumor growth observed in these mice are not due to altered access of B16F10 cells to the lung tissue (Fig. 1c). Radioactivity was also detected in the liver and kidneys, but no significant differences between NFAT1+/+ and NFAT1−/− mice ensued (data not shown). These data demonstrate that B16F10 tumor cells are able to circulate equally well through relevant organs in NFAT1+/+ and NFAT1−/− mice, suggesting that events following tumor cell homing

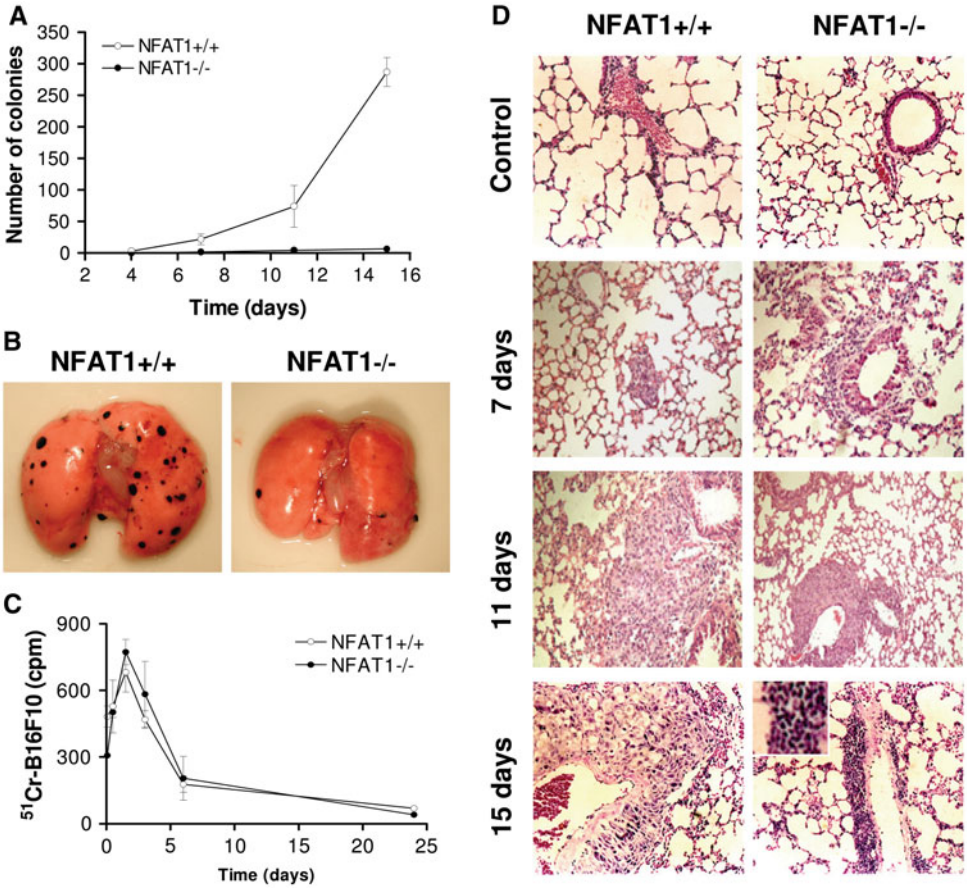

Fig. 1 NFAT1-deficiency decreases susceptibility to melanoma growth in the lungs. **a** Number of melanoma nodules in the lungs of NFAT1+/+ and NFAT1−/− mice after i.v. inoculation of 2×10^5 B16F10 melanoma cells. Results shown as mean ± SEM ($n = 3$), representative of three independent experiments. *$P < 0.05$. **b** Representative pictures of lungs from NFAT1+/+ and NFAT1−/− mice 15 days after melanoma inoculation ($n = 3$). **c** NFAT1+/+ and NFAT1−/− mice were injected i.v. with ^{51}Cr-labeled melanoma cells. At indicated times, 3 animals of each group were euthanized and a sample of the lungs was analyzed. Results are expressed as cpm/mg of tissue, and shown as mean ± SEM ($n = 3$). **d** Lungs of NFAT1+/+ and NFAT1−/− mice were dissected at the indicated times after B16F10 i.v. inoculation. Representative pictures of H & E-stained paraffin sections are shown ($n = 3$). Representative of at least three independent experiments

and not the homing itself is impairing melanoma growth in the absence of NFAT1.

Melanoma growth promotes accumulation of perivascular mononuclear infiltrates in NFAT−/− lungs

B16F10 melanoma cells display similar distribution upon intravenous inoculation in NFAT1+/+ and NFAT1−/− mice. The next steps in tumor establishment involve cell migration and growth in the tissue parenchyma. We investigated if fewer tumor cells were reaching the lung parenchyma in NFAT1−/− compared to wild-type animals by histopathological analysis of tumor-bearing lungs. Tumor masses could be observed already 7 days after inoculation of tumor cells in both NFAT1+/+ and NFAT1−/− mice (Fig. 1d). However, the progressive increase in tumor mass was slower in NFAT1−/−, and by 11 days after inoculation the melanoma nodules in NFAT1−/− lungs were smaller than those in wild-type counterparts. By 15 days after tumor inoculation NFAT1−/− lungs displayed massive perivascular infiltrates, what was not observed in NFAT+/+ tumor-bearing lungs (Fig. 1d). These results show that mononuclear cells are being recruited to the site of tumor growth in the absence of NFAT1 and, may be due to the cytokine milieu established in this site, may play a role in impairing the growth of melanoma in this tissue.

TGF-β expression by cells from tumor-bearing mice requires NFAT1 signaling

Cells of solid tumors promote the recruitment of diverse cell types, among them blood-born monocytes and

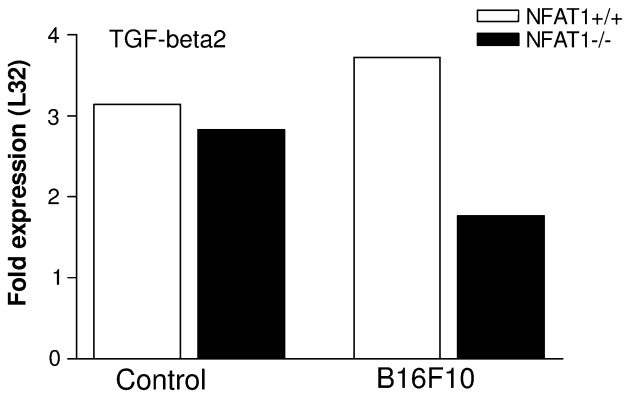

Fig. 2 Reduced expression of TGF-β by stimulated NFAT1−/− leukocytes from tumor-bearing hosts. Total lymph node cells were pooled from 3 tumor-bearing mice and activated in vitro by plate-bound anti-CD3. Cells were harvested 24 h after challenge and RNA was extracted for RPA analysis. Representative of two different experiments

lymphocytes, which once at the tumor microenvironment further differentiate into tumor-promoting and/or immunosuppressive cells, a crucial step in cancer progression [4]. Recent data demonstrated that B16F10 melanoma cells produce and secrete membrane fragments known as microvesicles, structures that induce the production of transforming growth factor (TGF-)β by macrophages [33]. At later stages of cellular transformation, TGF-β stimulates anchorage-independent cell growth, promoting tumor progression. We therefore investigated the expression of TGF-β by NFAT1−/− leukocytes. In vitro stimulated lymph node cells from melanoma-bearing NFAT1−/− mice express less TGF-β than wild-type counterparts (Fig. 2), suggesting that NFAT1 may be involved in the expression of this cytokine in the context of tumor growth. It remains to be shown if NFAT1 is also important for the expression of TGF-β by epithelial cells and fibroblasts, important components of the tumor microenvironment.

B16F10 growth in NFAT1−/− mice is independent of host IL-4

Cytokines and chemokines secreted by transformed cells compose the tumor microenvironment by recruiting blood-born and stromal cells, which may differentiate into tumor-promoting and/or immunosuppressive cells on site [4]. The Th2 cytokine IL-4 is of particular interest in this context since its expression is increased in the absence of NFAT1, and it has a controversial role in anti-tumor immunity. IL-4 skews monocyte differentiation toward trophic, immunoregulatory TAMs, and increased numbers of B16F10 pulmonary nodules in host mice are associated with increased levels of IL-4 [4, 19]. However, IL-4 is essential for Tc2 differentiation and activation of host anti-tumor immune responses [21]. We therefore tested if IL-4 expression was playing a role in our model by analyzing B16F10 homing and growth in mice that lack both IL-4 and NFAT1 (IL-4−/− × NFAT1−/−). Surprisingly, NFAT1−/− animals lacking IL-4 expression are more susceptible to B16F10 melanoma growth, displaying a higher number of tumor nodules in the lungs 15 days after melanoma inoculation when compared to IL-4-sufficient littermate controls (Fig. 3). However, NFAT1−/− mice had a lower number of melanoma nodules in the lungs than NFAT1-sufficient mice even in the absence of IL-4 (Fig. 3). NFAT1-dependent IL-4-skewed immune cell differentiation or NFAT1-dependent IL-4-induced changes in the pattern of adhesion molecule expression by endothelial cells are therefore not a critical step mediating susceptibility to B16F10 tumor growth.

NFAT1 expression by the lung parenchyma is responsible for increased susceptibility to B16F10

B16F10 melanoma cells have access and are able to initiate perivascular growth in both NFAT1+/+ and NFAT1−/− lungs. However, few tumor nodules progress in NFAT1−/− mice, where a mononuclear infiltrate is observed 16 days after inoculation. Furthermore, despite

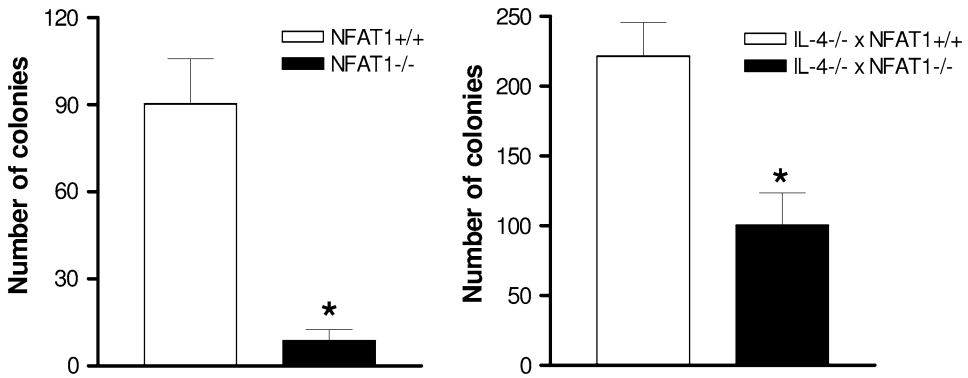

Fig. 3 IL-4 expression does not alter melanoma colonization of NFAT−/− lungs. Analysis of melanoma nodules in the lungs of NFAT1+/+, NFAT1−/−, IL-4−/− × NFAT1+/+ and IL-4−/− × NFAT1−/− mice 15 days after B16F10 i.v. inoculation. Results shown as mean ± SEM ($n = 3$). *$P < 0.05$. Representative of two independent experiments

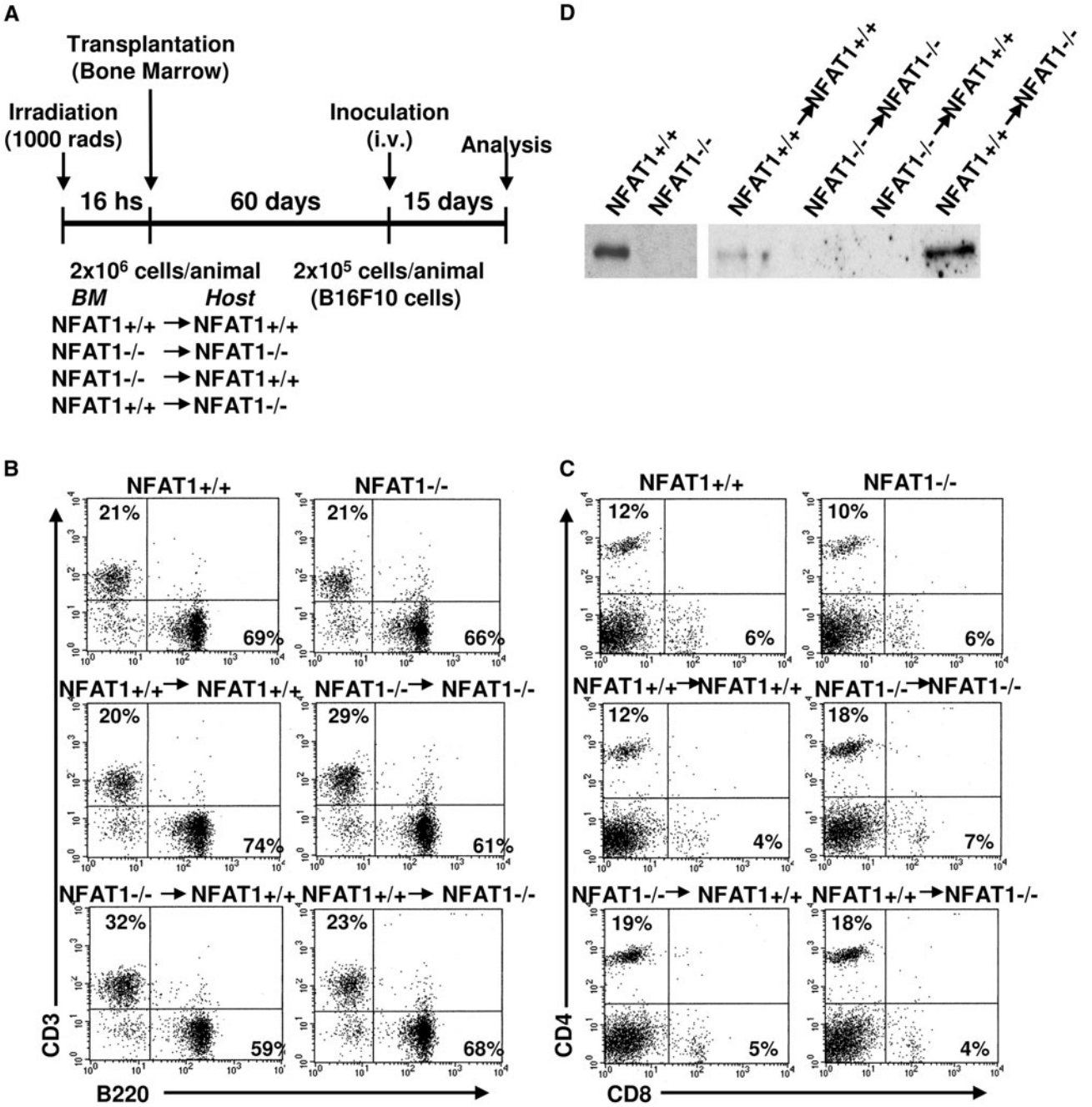

Fig. 4 BM chimera model for study of BM-derived versus parenchymal cells in tumor progression. **a** Experimental outline: irradiated NFAT1+/+ or NFAT1−/− mice received NFAT1+/+ or NFAT1−/− BM cells 16 h after full-body irradiation ($n = 4$). Eight weeks after BM transfer mice received 2×10^5 B16F10 cells i.v.. Fifteen days after tumor inoculation animals were euthanized. Representative plots with the percentage T and B cells (**b**) and of the $CD4^+$ and $CD8^+$ subsets of T cells (**c**) from BM-chimera lymph nodes 60 days after BM transfer, assayed by FACS ($n = 4$). **d** Western blot for NFAT1 of representative samples of mononuclear splenocytes isolated from NFAT1 chimeric mice at euthanasia ($n = 4$). Representative of two independent experiments

marked differences between IL-4 expression in NFAT1−/− and NFAT1+/+ mice, this cytokine does not mediate the phenomenon herein described since NFAT1−/− animals are less susceptible to melanoma growth in the lungs than NFAT1+/+, in the presence or absence of IL-4. To determine the contribution of NFAT1-expressing cells of parenchymal versus immune origin to the growth of melanoma, we transplanted NFAT1+/+ bone marrow (BM) into lethally, full body-irradiated NFAT1−/− mice and vice versa, following the time course depicted in Fig. 4a. Irradiated mice were completely depleted of mature lymphocytes and developed BM aplasia 7-10 days after

irradiation if not receiving BM transplant (data not shown). Irradiated host animals which received BM cells displayed normal percentages of B and T lymphocytes, as well as major T cell subsets 60 days after BM transfer, at which point mice received B16F10 melanoma cells intravenously (Fig. 4b, c). NFAT1 expression by mononuclear splenocytes tested by western blotting followed the genotype of the transferred BM, confirming the absence of residual host-derived cells in wild-type animals reconstituted with NFAT1−/− BM, what led us to infer that the protocol used successfully generated fully chimeric mice (Fig. 4d).

We went on to check the cytokine profile of control and chimeric mice in this setting, since IFN-γ and IL-4 have both been shown to alter the course of B16F10 growth in vivo [16, 17, 19]. Fifteen days after melanoma intravenous inoculation, when tumor nodules were quantified, we analyzed cytokine expression by in vitro activated T cells by ELISA and RNase protection assay (Fig. 5). As shown in Fig. 5, control mice (NFAT1+/+→ NFAT1+/+ and NFAT1−/− → NFAT1−/−) had the same pattern of IFN-γ and IL-4 production as not-manipulated wild-type and NFAT1−/− mice, respectively. Chimeric mice showed a different pattern, where NFAT1+/+ mice receiving NFAT1−/− BM showed increased IL-4 expression with maintenance of high IFN-γ production, whereas chimeric NFAT1−/− animals receiving NFAT1+/+ BM maintained high IL-4 expression while increasing IFN-γ production. Overall, independent of the combination assayed, chimeric mice displayed high expression of both IL-4 and IFN-γ.

Analysis of the number of melanoma nodules in the lungs of chimeric and control mice 15 days after

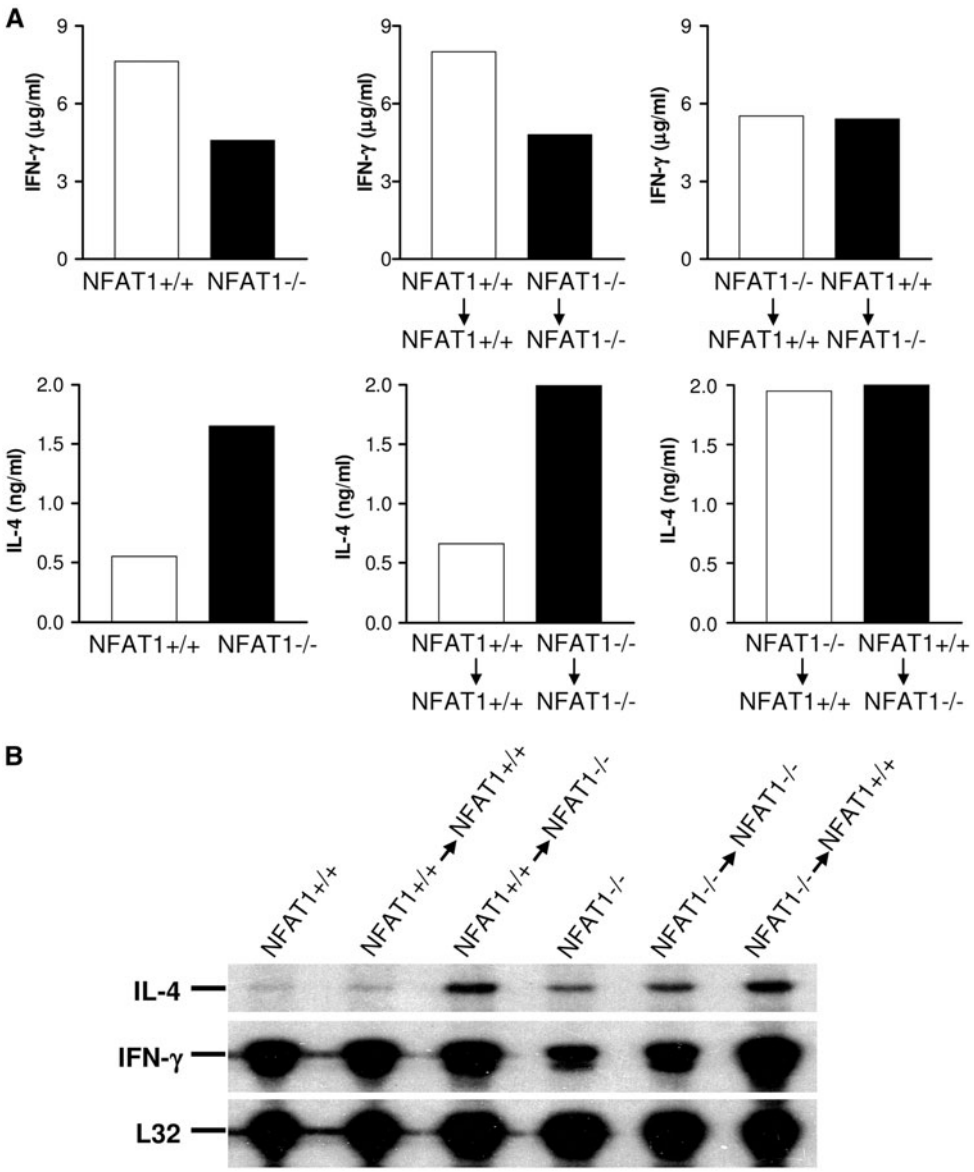

Fig. 5 Profile of cytokine expression by BM-chimera animals. **a** Total lymph node cells were pooled from 3 independent tumor-bearing mice and activated in vitro by plate-bound anti-CD3. IL-4 and IFN-γ were quantified by ELISA in cultures supernatants 48 h after challenge. **b** Samples were treated as depicted in (**a**), but cells were harvested 24 h after challenge and RNA was extracted for RPA analysis

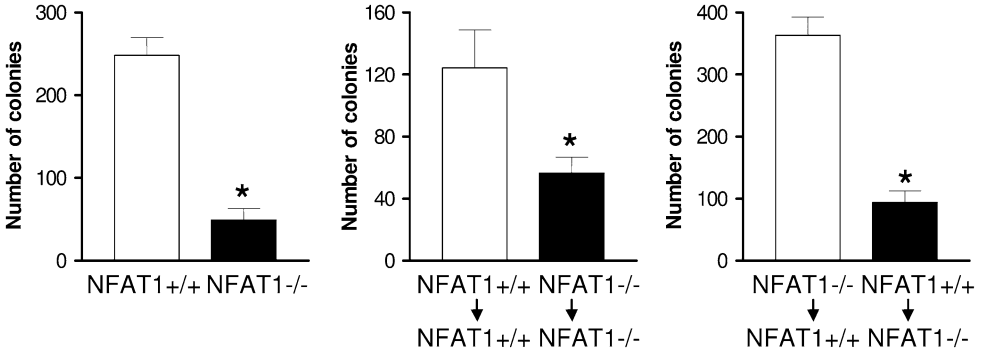

Fig. 6 Parenchyma and not BM-derived cells modulate melanoma growth in the absence of NFAT1. Analysis of melanoma nodules in the lungs of chimeric mice 15 days after B16F10 i.v. inoculation. Results shown as mean ± SEM ($n = 4$), $^*P < 0.05$. Representative of two independent experiments

inoculation revealed that lack of NFAT1 in the lung parenchyma, and not in BM-derived cells, was responsible for the decreased growth of B16F10 tumors (Fig. 6). Put together, our data demonstrate that NFAT1−/− lung parenchyma does not support the growth of highly aggressive melanoma cells as efficiently as wild-type counterparts. Independently of the genotype of tumor-infiltrating immune cells or the Th1 versus Th2 cytokine balance present in these mice, NFAT1 expression in tumor-associated radio-resistant tissue promotes the growth of cancer, leading to increased number and size of B16F10 nodules in the lungs of NFAT1+/+ mice.

Discussion

We have previously shown that NFAT1 is a tumor-suppressor gene, capable of subverting the transformed phenotype of neoplastic cells [9]. NFAT1−/− mice are indeed more susceptible to chemically induced carcinogenesis than wild-type littermate controls [9]. In the current study, we sought to address if, besides the demonstrated intrinsic tumor-suppressor role of NFAT1, this transcription factor could modulate the tumor microenvironment and alter tumor growth extrinsically. We demonstrated that NFAT1 expression is associated with an increased susceptibility to B16F10 melanoma growth in the lungs of host mice (Fig. 1a, b). This phenotype is not due to altered homing since tumor cells reach the lung tissue equally well in NFAT1−/− and wild-type mice, suggesting the NFAT1−/− lung's capillary bed is normally formed (Fig. 1c). The finding of a prominent perivascular mononuclear infiltrate suggested involvement of immune cells and the cytokine milieu (Fig. 1d). Indeed, NFAT1−/− lymph node cells express less TGF-β than wild-type counterparts (Fig. 2). However, despite being Th2-proned, with high expression of IL-4, the Th1 versus Th2 milieu showed little impact since IL-4−/− × NFAT1+/+ animals were still more susceptible to melanoma than IL-4−/− × NFAT1−/− mice (Fig. 3). We mapped this susceptibility to the lung parenchyma. Despite similar production of IL-4 and IFN-γ in NFAT1+/+ bearing NFAT1−/− BM and NFAT1−/− bearing NFAT1+/+ BM chimeric mice (Fig. 5), NFAT1−/− hosts had less melanoma tumor nodules in the lungs than NFAT1+/+ hosts (Fig. 6).

The relevance of tumor microenvironment for the establishment and growth of cancer cells is being increasingly acknowledged and exploited for therapy. It comprises the stromal cells sustaining the tumor, immune cells that in general differentiate toward tumor-promoting regulatory cells, and endothelial cells recruited during angiogenesis. NFAT1 expression has not been described in stromal cells, but it is present in innate and adaptive immune cells, epithelial and endothelial cells, making it a good candidate to be influencing the tumor microenvironment [11, 34].

Regulation of an anti-tumor immune response is essential for tumor growth. As postulated by Schreiber et al., to avoid elimination, tumor and the host immune response must reach equilibrium. However, the tumor mass will grow if the cancer cells evade host immunity, feed on growth factors and promote angiogenesis [35]. NFAT1−/− mice are immunocompetent but mount a Th2-biased immune response due to increased IL-4 expression [22, 24]. This characteristic could render NFAT1−/− animals more susceptible to B16F10 cells since IL-4 contributes to melanoma growth [16, 17, 19]. Nevertheless, our results indicate the opposite, with NFAT1−/− mice being more resistant to tumor growth than wild-type counterparts. In fact, IL-4 production has a protective role in our model since both IL-4−/− and IL-4−/− × NFAT1−/− mice are more susceptible to melanoma growth in the lungs than IL-4+/+ counterparts. Even though increased IL-4 expression in NFAT1−/− animals does not fully explain our results, this cytokine shows an important anti-tumor effect in NFAT1−/− mice given the important increase in susceptibility of these mice when IL-4 is absent (11 × increase in melanoma nodules in NFAT1−/− mice versus 2.5 × increase in NFAT1+/+ in IL-4−/− background),

mirrored in the reduced difference in tumor nodules between wild-type and NFAT1−/− mice in the IL-4-deficient background (45.3% vs. 9.6%, respectively). Furthermore, NFAT1 collaborates with the transcription factor FOXP3 in the regulation of suppression-associated genes in naturally occurring regulatory T cells (Tregs), suggesting that Treg function in NFAT1−/− mice could be altered [36].

Our data suggest, however, that the impact of NFAT1 deficiency in non-immune cells dictates the progression of B16F10 melanoma in vivo. B16F10 cells secrete membrane-derived microvesicles, which promote tumor growth and immune suppression by inducing TGF-β production by TAMs, and stimulating Treg development and function [33, 37]. We show reduced expression of TGF-β by NFAT1−/− lymph node cells. Even though it remains to be shown if the same is true for NFAT1-deficient endothelial and stromal cells, these results reconcile the increased susceptibility of NFAT1−/− mice to transformation, with the reduced establishment of transformed cells in their lung parenchyma, since TGF-β exposure leads to an arrest in cell cycle progression of primary endothelial cells while it promotes anchorage-independent growth of transformed cells [38]. These observations support our findings, which are in opposition to those of Maxeiner et al. (2009). Using a different mouse strain, they observed increased tumor load in NFAT1−/− mice inoculated with a cell line of lung adenocarcinoma and showed an increase in FOXP3$^+$ Tregs and TGF-β levels [39]. Increased Treg numbers do not correlate with inhibition of anti-tumor responses [40], and FOXP3 might not be a good marker in this system since in the absence of NFAT1 its capacity for inducing suppression-associated genes may be impaired [36]. However, similarly to us, effector cytokines did not play a role in their system [39]. It would be of interest to test if L1C2 lung adenocarcinoma secretes microvesicles and if Balb/c mice respond like C57Bl/6 animals to these structures.

Taken together, our results demonstrate a second role for NFAT1 in tumor progression. In addition to its cell-intrinsic tumor-suppressor role, we demonstrate a cell-extrinsic tumor-promoting role dependent on the host parenchyma and tumor microenvironment. NFAT1 expression, possibly in a TGF-β-dependent manner but independently of IL-4 and IFN-γ, can promote growth of transformed cells and is therefore a potential target for directed therapy of selected advanced malignancies.

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