The role of the immune system in neurofibromatosis type 1-associated nervous system tumors

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Practice points

- Neurofibromatosis type 1 (NF1) patients carry mutations in the *NF1* gene and are at increased risk for tumors in the PNS and CNS.
- The product of the *NF1* gene, neurofibromin, downregulates RAS signaling and many cell types in NF1 patients show a hyperactive RAS phenotype.
- Mutation of *NF1* causes changes in cytokine levels, mast cells, macrophages, microglia, T cells and B cells, suggesting that immune system activities are altered.
- Mouse models of NF1-associated tumors have helped to clarify the causal relationship between immune cell alterations and tumorigenesis.
- The role of mast cells, microglia and cytokines in helping to drive tumor progression is providing therapeutic opportunities in molecularly targeted therapies and immunotherapy.

With the recent development of new anticancer therapies targeting the immune system, it is important to understand which immune cell types and cytokines play critical roles in suppressing or promoting tumorigenesis. The role of mast cells in promoting neurofibroma growth in neurofibromatosis type 1 (NF1) patients was hypothesized decades ago. More recent experiments in mouse models have demonstrated the causal role of mast cells in neurofibroma development and of microglia in optic pathway glioma development. We review here what is known about the role of *NF1* mutation in immune cell function and the role of immune cells in promoting tumorigenesis in NF1. We also review the therapies targeting immune cell pathways and their promise in NF1 tumors.

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Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disease in which patients are at risk for tumors in the PNS and CNS (Figure 1). NF1 patients carry mutations in the *NF1* gene encoding the protein neurofibromin, a RasGAP protein that acts to downregulate active Ras signaling. NF1 affects one in 3500 people and can be inherited or occur sporadically. Although NF1 can affect many different organ systems, effects on the nervous system are a defining feature. In the peripheral nervous system, NF1 is characterized by different Schwann cell tumors, particularly dermal neurofibromas and plexiform neurofibromas (PNF) that can progress to malignant peripheral nerve sheath tumors (MPNST). In the CNS, NF1 children are at an increased risk for optic

KEYWORDS

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pathway gliomas (OPG) and adults with NF1 are at risk for astrocytoma and glioblastoma, all tumors of glial cells.

Neurofibromas are complex tumors involving many cell types and occur in almost all NF1 patients. The peripheral nerve fiber normally consists of Schwann cells, perineurial cells, fibroblasts, and endothelial cells and all of these cells can be found embedded in neurofibromas. The initiating tumor cell has been shown to be *NF1*-null Schwann cells [1,2] in which the second wild-type copy of *NF1* has been mutated or deleted; however, *NF1* heterozygous stromal cells are also critical to tumorigenesis. Neurofibromas are divided into groups based on clinical features. Dermal (or cutaneous) neurofibromas occur on the surface of the skin in more than 90% of NF1 patients and can be particularly disfiguring. Subcutaneous neurofibromas occur below the epidermal layer. PNFs are associated with major nerves and can occur in both superficial and deep tissues. These PNFs occur in up to 50% of NF1 patients and are highly invasive, often spreading along the entire nerve branch [3,4]. Although benign, they can have serious morbidity or mortality for patients depending on

the tumor location. Standard of care for PNF is surgery, although surgery may not be feasible, due to the location of the tumor. New therapeutic options, particularly for patients for whom surgery is not an option, are desperately needed, despite an extensive history of clinical trials in NF1 [5].

PNFs can transform to MPNST, an aggressive sarcoma. The lifetime risk for MPNST in patients with NF1 is 8–16% [6,7]. Recent studies have shown that the transformation from PNF to MPNST can go through an atypical neurofibroma stage that frequently has mutation of *CDKN2A*, encoding p16^{INK4A} [8]. Once MPNSTs have formed, *TP53*, encoding p53 [9], and/or *SUZ12*, encoding a subunit of the PRC2 complex, are found [10,11]. Fifty percent of MPNSTs can also occur sporadically in the absence of a preexisting PNF or NF1. NF1-associated and sporadic MPNST do not significantly differ in chromosomal alterations [12], gene expression profiles [13,14] or RAS pathway phosphoprotein expression [15,16], although there is some indication that *BRAF* mutations occur in sporadic MPNST, but not NF1-associated MPNST [17,18]. NF1-associated MPNSTs have poorer prognosis

Figure 1. Development of nervous system tumors in NF1 patients.

GBM: Glioblastoma multiforme; MPNST: Malignant peripheral nerve sheath tumors; OPG: Optic pathway gliomas; PNF: Plexiform neurofibromas.

than sporadic MPNSTs and this may be due to the young age of onset and/or large volume at time of diagnosis, given that NF1-associated MPNSTs form within an existing PNF that confounds diagnosis [19–22]. The current standard of care for MPNST is surgery and chemotherapy [23], such as doxorubicin, etoposide and ifosfomide, as well as radiation therapy in some cases [20], but these treatments are seldom curative, except in some cases of complete surgical resection, and better targeted therapies are being actively sought [5].

OPG affect 15–20% of children with NF1, typically under 7 years of age. They form along optic nerves, chiasm or postchiasmal optic tracks. Children with OPG can present with vision defects, although roughly half of OPG are asymptomatic and never require intervention [24]. These tumors can stop growing as children get older, making it challenging to decide between intervention and ‘wait-and-see’ approaches in young children with vision deficits. Due to their location and indolent growth behavior, they are rarely surgically removed or biopsied, so less is known about their molecular biology. As in other NF1 tumors, OPG are cellularly complex, with hyperproliferative astrocytes, microglia, CD31⁺ endothelial cells, small clusters of progenitor cells and retinal ganglionic cells identified by immunohistochemistry [25]. Chemotherapy is the current standard treatment for OPG in cases where the patient’s vision is impaired [26]; however, new treatments are being tested [5]. Radiation therapy is avoided in NF1 patients, due to the risk of developing radiation-induced moyamoya lesions or secondary tumors in the brain.

Anaplastic astrocytoma and glioblastoma multiforme (GBM) are deadly malignant primary brain tumors that occur rarely in NF1 patients, although there is some evidence that the incidence in NF1 patients is greater than in the general population [27]. *NF1* is commonly mutated in sporadic GBM [28] and can also be lost through increased proteosomal degradation of neurofibromin [29]. The prognosis for anaplastic astrocytoma and GBM is very poor, and chemotherapy has thus far provided little improvement despite many ongoing clinical trials. The current standard of care for anaplastic astrocytoma and GBM is surgery with radiation therapy and chemotherapy that includes temozolomide.

The aim of this paper is to summarize what is known about alterations to the immune system in NF1 patients and how the immune system plays

a role in tumors of the nervous system in NF1, particularly as it relates to candidate therapeutics.

Method

To identify literature for this review, PubMed [30] was searched using the terms Neurofibromatosis type 1; immunotherapy of cancer; tumor microenvironment; T lymphocytes; mast cell and inflammation; immunology of CNS; as well as combinations between diseases terms (neurofibromatosis; plexiform neurofibroma; malignant peripheral nerve sheath tumor or MPNST; optic pathway glioma; astrocytoma; glioblastoma) and mouse model or clinical trial or immune system terms (lymphocyte; mast cell; microglia; macrophage; IFN; TNF; SCF; RANTES) or drug terms once they were implicated by other searches (ketotifen; imatinib; sunitinib; sorafenib; sulindac; pexidartinib; bevacizumab; GC-1008; fresolimumab; ipilimumab; nivolumab; pembrolizumab; tocilizumab; oncolytic virus). The NIH clinical trials database [31] was searched with the above disease terms and drug terms to search for unpublished, ongoing clinical trials.

Immune surveillance by peripheral immune cells in the CNS

OPG, anaplastic astrocytomas and glioblastoma in NF1 patients occur within the immune privileged CNS. The blood–brain barrier (BBB) protects the brain by regulating the passage of macromolecules and immune cells between the periphery and the CNS and by blocking pathogens from entering the brain. The cerebrospinal fluid (CSF) surrounds the brain and is important for the draining of CNS antigens into the deep cervical lymph nodes, via the meningeal lymphatics and the cribriform plate [32]. The permeability of the BBB and access by immune cells is regulated by cytokines. TGF- β is normally expressed in the brain and inhibits cell entry into the brain by blocking production of chemoattractants by astrocytes in the BBB and downregulating adhesion molecules on the surface of BBB endothelial cells that immune cells use to cross the BBB. TGF- β also inhibits proliferation of T cells in the brain under homeostatic conditions [33]. Conversely, TNF- α and IFN- γ enhance the adhesion properties of the BBB endothelial cells. TNF- α is not present in normal, healthy brain, but can be induced by inflammation, either in the brain or systemically. It increases the permeability of the BBB,

allowing increased passage of immune cells into the brain and increased release of brain antigens into the periphery [33].

• Microglia/macrophages

Brain-resident microglia are established prior to birth and perform many homeostatic and repair functions in the brain. Pathological changes in the brain cause microglia to upregulate proteins for antigen presentation. Microglia can both present antigens to T cells and induce an inflammation response to pathogens in the brains [34]. Macrophages and perivascular cells can also cross the BBB to enter the CNS and present antigens to T cells. These cells concentrate in the subarachnoid space where they activate incoming T cells from the periphery [35]. Both microglia and macrophages are found in astrocytic gliomas with levels of infiltration corresponding to tumor grade, suggesting a tumor supportive role in brain tumors [36–39].

• Lymphocytes

Because the normal, healthy brain has low levels of adhesion molecules on the BBB endothelial cells, T cells are relatively inhibited from entering the brain parenchyma, and so the surveillance of the brain by T cells is relatively low compared with the density of T cells in peripheral organs [33]. T cells that have been recently stimulated in the periphery cross the BBB, highlighting the importance of the draining CNS antigens in the deep cervical lymph nodes where they can be encountered by T cells in the periphery [33–35]. Cytotoxic T cells are present in brain tumors [40,41] and the survival of GBM patients was found to be correlated to increased numbers of effector T cells within the tumor, suggesting a tumor inhibitory role of cytotoxic T cells in GBM [42]. Cytotoxic T cells in the GBM microenvironment are suppressed by regulatory T cells and myeloid derived suppressor cells (MDSCs) [43–46].

Immunopathology of NF1-associated tumors: evidence in humans for the role of the immune system in NF1 tumors

• Myeloid malignancies

The increased incidence of myeloid disorders in NF1 patients, including myeloid dysplastic syndrome, juvenile myelomonocytic leukemia and acute myelogenous leukemia highlights the dysfunction of myeloid immune cells in NF1. The *NF1* protein product, neurofibromin, plays a role

in cytokine signaling [47] and *NF1*-mutant leukemic myeloid cells are hypersensitive to granulocyte-macrophage colony stimulating factor (GM-CSF) making them hyperproliferative [48]. Bone marrow of NF1 children with juvenile myelomonocytic leukemia show loss of the wild-type allele of *NF1*, supporting neurofibromin's role as a tumor suppressor in myeloid malignancies [49,50].

• Cytokine aberrations

NF1 patients have significantly higher serum concentrations of the inflammatory cytokines IFN- γ , TNF- α , IL-6 [51], stem cell factor (SCF) and midkine [52] compared with healthy individuals, suggesting that NF1 causes a hyper-inflammatory state that may contribute to tumorigenesis. Analysis of intracellular cytokine staining in stimulated lymphocytes has shown that the increase in TNF- α and IFN- γ in NF1 patients is specific to CD8⁺ lymphocytes, and IFN- γ increase is found in the CD8⁺/CD57⁺ effector T-cell subset [53]. This study also found increased expression of IL-2 in CD8⁺/CD57⁺ effector T cells of NF1 patients. Comparison of NF1 patients with and without MPNST has found upregulation of IGFBP1 and RANTES in patients with MPNST [51]. While some of these biomarkers and cytokines have been shown, or are expected, to act directly on tumor cells to drive proliferation [52], it is likely that they also act on immune cells to promote activation and chemotaxis and, as described above, may alter the permeability of the BBB. Alternatively, they may be markers of excessively activated immune cells in NF1 patients. Additional research is needed to better understand the complex interplay of these factors with tumor cell growth, angiogenesis and activation of immune functions.

• Tumorigenesis & immune suppression

Case reports on NF1 patients who have undergone organ transplant raise the possibility that immune suppression promotes tumorigenesis in NF1 patients. The development of post lung transplant lymphoproliferative disorder and MPNST was observed in an NF1 patient without any clinical evidence of sarcoma [54]. An astrocytoma was reported in another NF1 patient following a heart transplant [55], raising the possibility that normal immune function suppresses malignancies, but in the context of immune suppression after transplantation malignant tumors can develop. While these case reports do not conclusively provide a link

between immunosuppression and tumorigenesis, they highlight the need for further research into the role of immunosurveillance in inhibiting tumorigenesis in NF1 patients.

• Infiltrating immune cells

Infiltrating immune cells from the myeloid lineage are characteristic of many tumors associated with NF1. Mast cells have long been recognized as a major component of PNFs [56]. Immature mast cells are found in early stage neurofibroma and mature mast cells are found in late stage neurofibroma, and there is some evidence that mast cell stabilizers may block neurofibroma formation [57]. One study of NF1 patients found elevated serum IgE in a subset of patients examined. PNF patients had a higher incidence of elevated IgE compared with other NF1 patients [58], suggesting alteration in the B-cell compartment and a potential interaction with mast cells [59]. Macrophages have also been found at substantial levels in PNFs and MPNSTs, with higher levels in MPNST [60]. Optic gliomas show accumulation of microglia, a macrophage-type cell of the brain [61,62], as well as altered microglia morphology [63]. Studies in mouse models (see below) have now established causal relationships between infiltrating myeloid cells and nervous system tumorigenesis in NF1.

Mouse models for NF1-associated tumors

The NF1-associated nervous system tumors have all been successfully modeled in genetically engineered mice that has allowed the role of immune system cells in tumorigenesis to be better elucidated. Mouse models of PNF, MPNST, OPG and astrocytoma/GBM have all been generated by driving loss of the mouse *Nf1* gene in particular cell types or in combination with other tumor suppressors or oncogenes.

• Plexiform neurofibromas

PNFs have been modeled in mice using the Cre-lox system of targeted gene mutation, as *Nf1*^{-/-} mutant mice are embryonic lethal and *Nf1*^{-/+} mutant mice develop cancers late in life [64]. Loss of *Nf1* in *Krox20*-expressing (*Krox20*⁺) cells on a *Nf1*^{-/+} genetic background leads to the development of PNF [65]. In this case, heterozygosity in the stroma for *Nf1* mutation was critical for formation of PNFs and for mast cell infiltration into the tumors [66]. In a separate model, biallelic loss of *Nf1* in *Dhh*-expressing (*Dhh*⁺) cells on a wild-type genetic background gives rise to PNF that

similarly show infiltration of mast cells [67]. The difference in the dependence of PNF formation on *Nf1*^{-/+} stroma in these two models has been attributed to the progenitor cell compartments in which *Nf1* is mutated in each model.

• Malignant peripheral nerve sheath tumors

MPNSTs have been modeled in mice in a variety of different ways by breeding mutant mice together or by injection of viral vectors. The different MPNST models take advantage of spontaneous loss of heterozygosity of tumor suppressor genes, including *Nf1*, as mice age and/or by targeted loss of tumor suppressor genes in specific cell types using either the Cre-lox system or injection of lentiviral vectors expressing shRNAs. While mice mutants for *Nf1* alone do not develop MPNST, additional mutations in the tumor suppressors p53, PTEN, and p16^{INK4A}/p19^{ARF} can drive development of MPNST. Compound heterozygotes for *Nf1* and *Trp53* (encoding the p53 protein in mice) mutations develop high-grade MPNST through spontaneous loss of the wild-type copies of *Nf1* and *Trp53* [68–70]. In addition to spontaneous loss of *Nf1* and *Trp53*, loss of expression can be targeted using the Cre-lox system or expression of shRNAs against *Nf1* or *Trp53* using lentiviral vectors. Models in which a lentivirus-expressing shRNA against *Trp53* is injected into the sciatic nerve of mice with Cre-induced loss of *Nf1* in *Gfap*-expressing (*Gfap*⁺) or *Postn*-expressing (*Postn*⁺) cells develop low-grade MPNST, with mast cell infiltration of tumors [71]. Targeted loss of both *Nf1* and *Pten* in *Dhh*⁺ cells leads to high-grade MPNST with mast cell infiltration [72]. Targeted loss of *Nf1* and *Cdkn2a* encoding p16^{INK4A}/p19^{ARF} by injection of adenovirus expressing Cre into sciatic nerves leads to high-grade MPNST with mast cell infiltration [73]. The majority of reported MPNST genetically engineered models recapitulate the mast cell infiltration seen in human tumors.

• Optic pathway gliomas

OPGs have been successfully modeled in mice by targeted loss of *Nf1* in *Gfap*⁺ cells on a heterozygous *Nf1* genetic background [74]. *Nf1*^{-/+} stroma has been shown to be important for OPG development, and *Nf1*^{-/+} microglia promote OPG formation [25,75]. This mouse model of OPG recapitulates the microglia infiltration seen in human OPG [61] as well as changes in microglia morphology [63].

• Astrocytoma/glioblastoma

Many of the techniques used to model MPNST in mice have also been used to model astrocytoma and GBM. Compound heterozygotes for *Nf1* and *Trp53* on the C57BL/6J background develop astrocytoma and GBM with spontaneous loss of the wild-type alleles [76]. In these mice the tumor stroma is heterozygous for both *Nf1* and *Trp53*. Mice with targeted loss of *Nf1* in GFAP⁺ cells on a *Trp53* null background develop a range of astrocytoma and GBM [77]. Targeted loss of *Nf1*, *Pten* and *Trp53* on a *Trp53* heterozygous background develop GBM with high penetrance [78]. Although there are no published characterizations of immune cell infiltrates in *de novo* tumors in these models, an intracranial injection model of a cell line derived from the *Nf1/Trp53* compound heterozygote model shows increased infiltrations of activated microglia, monocytes/macrophages and lymphocytes in immunocompetent mice [79].

The effect of NF1 haploinsufficiency on immune cells

Experimental data from model systems have established the role for *NF1* mutation in phenotypes and functions of immune cells. This suggests that the tumor pathology of patients with germline mutation in *NF1* involves complex interactions between tumor cells and aberrant immune cells. Effects on immune cells are both developmental and functional, likely related to aberrant RAS signaling due to lower levels of neurofibromin.

• Hematopoietic progenitor cells

Hematopoietic progenitor cells isolated from *Nf1* null mouse fetal livers form more colonies and are hyper-responsive to growth factors in colony forming assays when compared with wild-type progenitors [80,81]. Effects were observed in granulocyte/macrophage progenitors, as well as primitive stem/progenitor cells. Significantly higher numbers of primitive stem/progenitor cells were found in *Nf1* null fetal livers and *Nf1* null fetal progenitor cells were better able to repopulate mouse bone marrow after irradiation of mouse hosts compared with wild-type progenitor cells. The engraftment of *Nf1* null progenitor cells is dependent on the growth factor GM-CSF in the microenvironment, such that postengraftment myeloid cell numbers are much lower in *Gmcsf*^{-/-} mice engrafted with *Nf1*^{-/-}; *Gmcsf*^{-/-} progenitors compared with wild-type mice engrafted with *Nf1*^{-/-}; *Gmcsf*^{+/+} progenitor cells [82]. This dependence of the *Nf1* mutant

effect on the growth factor GM-CSF is consistent with hyperactivation of RAS signaling downstream of the GM-CSF receptor through loss of *Nf1*. Targeted loss of *Nf1* in newborn mouse pups gives rise to spontaneous myeloid proliferative disease and higher numbers of leukocytes, monocytes and neutrophils [83].

• T cells

A direct role for T cells in NF1 nervous system tumorigenesis has not yet been established, but given the advances in T-cell-based immunotherapy it is important to understand the changes in T cells caused by *NF1* mutation. NF1 patients have lower levels of circulating lymphocytes overall. The levels of chronically activated CD8⁺/CD57⁺ T cells and CD8⁺/CD27⁺ effector T cells goes up in NF1 patients with low PNF tumor volume compared with NF1 patients with no PNFs, and then decreases with increasing tumor burden [53], suggesting that as tumor burden increases the T-cell response becomes muted. In *Nf1*^{-/-} mice, the thymus and spleen are hypercellular, with subtle increases in immature and mature T-cell subsets [84]. Bone marrow transplant of *Nf1* null cells into immune compromised mice results in increased thymic cellularity. Targeted loss of *Nf1* in maturing T cells did not lead to increased thymic cellularity, so this effect may be due to interactions between different cell types in the thymus [85]. In culture, both *Nf1*^{-/-} and *Nf1*^{+/-} thymocytes as well as mature splenic T cells proliferate less after IL-2 and CD3 stimulation than wild-type [84,86], whereas unstimulated proliferation of *Nf1*^{+/-} thymocytes is increased compared with wild-type. Loss of *Nf1* in maturing T cells leads to inhibition of positive selection in the context of MHC class I restricted T-cell receptor transgenic mice and fewer naive T cells in the spleen [85]. These data suggest that the effect of *Nf1* mutation on T cells is very specific to different stages of T-cell maturation and stimulation, and may depend on *Nf1* mutation in other cells of the immune compartment. Because some of the effects on T cells can be seen in *Nf1*^{+/-} cells, these data have implications for NF1 patients with germline mutations in *NF1*.

• B cells

Similar to T cells, a direct role of B cells in NF1 nervous system tumorigenesis has not been established, but B cells play a role in activating T cells and mast cells and therefore may

indirectly affect NF1 nervous system tumors. Neurofibromin has been found to relocate in B cells upon activation of surface Ig [87], suggesting a role in Ig signaling and the possibility that mutation of *NF1* impairs B-cell activation. Comparison of *Nf1*^{-/+} B cells and wild-type B cells showed subtle gene expression changes with most expressions differences being less than 1.5-fold [88]. Mouse and human cross-species comparison of *Nf1*^{-/+} versus wild-type B-cell gene expression found increases in cell cycle and DNA repair genes with inhibition of genes involved in the immune response. *Nf1*^{-/-} B cells proliferate well in response to IL-4 and antibodies to CD40, but do not proliferate as well as *Nf1*^{-/+} B cells in response to stimulation by antibodies to IgM [86]. Signaling downstream of IgM was found to be impaired in *Nf1*^{-/-} B cells.

• Mast cells

Nf1^{-/+} mice have slightly higher levels of mast cells and haploinsufficiency for *Nf1* rescues loss of mast cells in c-Kit mutant mice [89]. Mast cells from *Nf1*^{-/+} mice form more colonies in culture, suggesting that the role of *Nf1* haploinsufficiency is cell-autonomous. Increased proliferation of *Nf1*^{-/+} mast cells is dependent on RAS/MEK/RAC2/PAK1 signaling [90,91] consistent with hyperactivation of RAS signaling when neurofibromin levels are reduced. *Nf1*^{-/+} mast cells are also hypersensitive to migratory stimuli and degranulation signals secreted by Schwann cells [92,93].

• Microglia

Microglia from *Nf1*^{-/+} mice proliferate faster than wild-type microglia in primary cultures [61,75] and show twofold increased migration both in culture and in response to brain injury [75]. *Nf1*^{-/+} microglia also show higher expression of several transcripts including *Mgea5*, *Ptn*, *Jag1* [61], *Ptgs2*, *Il6*, *Nos2* and *Tgfa* [75]. In contrast to the relationship between mast cells and Schwann cells, *Nf1*^{-/+} microglia do not appear responsive to astrocyte cultured media, although only proliferation has been examined thus far [61], and other microglia functions may be affected in the interaction of astrocytes and *Nf1*^{-/+} microglia.

Immunomodulation of NF1-associated tumors: evidence in model systems for causal role of the immune system in NF1 tumors

The development of mouse models for NF1 tumors has led to rigorous experiments looking

at the effects of different immune cell compartments on tumorigenesis and whether haploinsufficiency for *Nf1* in the immune cell compartments accelerates tumor formation or growth. These experiments have also begun to elucidate which signaling molecules mediate the interactions between immune cells and tumors.

• Mast cells in PNF

Experiments in mouse models of neurofibromas comparing tumorigenesis on *Nf1* heterozygous versus wild-type genetic backgrounds suggest that *Nf1* heterozygosity in the tumor stroma, particularly in the bone marrow, is critical to drive tumorigenesis [65,66]. However, the dependence on *Nf1* heterozygous stroma may vary with the developmental stage at which the neurofibromas initiate, since tumors initiating through *Nf1* loss in *Krox20*⁺ cells require *Nf1* heterozygous bone marrow, whereas *Nf1* loss in *Dhhb*⁺ cells or in post-natal *Plp*⁺ cells drives neurofibromas on a wild-type stroma background [67,94]. Alternatively, differences in genetic background or pathogen status of the mice in these different experiments could explain the variation in the requirement for *Nf1* heterozygous stroma. The evidence that *Nf1* heterozygous stroma contributes to neurofibroma formation has led to the hypothesis that mast cells are a critical factor in promoting neurofibroma growth. Whether or not *Nf1*^{-/+} mast cells are necessary for neurofibroma formation, they likely promote tumorigenesis in NF1 patients. *Nf1*^{-/+} mast cells have enhanced functions that can contribute to tumorigenesis including increased migration and secretion of growth factors that modify fibroblast behavior, collagen secretion and remodeling of the tumor microenvironment [66,95].

• Microglia in optic pathway glioma

Similar to neurofibromas, OPG have shown mixed results on the requirement for *Nf1* heterozygous stroma. Loss of *Nf1* in astrocytes and glial progenitors gives rise to OPG whether or not the stroma is *Nf1* heterozygous [74]. When *Nf1* is lost in astrocytes specifically, and not in glial progenitors, *Nf1* heterozygosity is required in the genetic background for OPG to form [96]. Interestingly, injection of *Nf1* null astrocytes into the brains of *Nf1* heterozygous mice does not lead to tumors, suggesting the developmental timing and/or location is critical for tumors to form. Several studies have shown that *Nf1*^{-/+} microglia can promote OPG. *Nf1*^{-/+} microglia

secrete factors that promote growth of *Nf1* null astrocytes [61,63]. Inhibition of microglia, either pharmacologically [61,75] or genetically [97], inhibits the growth of OPG *in vivo*.

• Macrophages

The role of macrophages in NF1-associated nervous system tumors has not been thoroughly studied. A study using a c-KIT/FMS kinase inhibitor, Pexidartinib, in the *Dhh-Cre; Nf1^{flox/flox}* mouse model of PNF provides intriguing evidence for a different role for macrophages in early and late neurofibroma tumorigenesis [60]. Macrophage numbers are very high in plexiform tumors and numbers of macrophages go up with increased malignancy, from normal nerve to PNF to MPNST. Early inhibition of the c-KIT signaling pathway, important for mast cells, and the FMS kinase signaling pathway, important for macrophages, led to increased tumor growth [60]. Paradoxically, inhibition of these pathways once the tumors are established led to a subset of tumors shrinking, with the shrinkage correlating better to numbers of macrophages than to numbers of mast cells. The conflicting role of macrophages in tumor initiation and progression needs to be better understood before pharmacological intervention can be considered. Careful mouse genetic experiments are needed to dissect out the individual roles of macrophages and mast cells in the initiation and progression of PNF.

Implications of cytokine aberrations for immunomodulation in NF1

Although it has not been thoroughly studied in NF1, the high concentrations of cytokines found in the serum of NF1 patients may alter immune function in favor of immune suppression and tumorigenesis. IL-6, IFN- γ and TNF- α are upregulated in NF1 and all play immune suppressive roles. SCF is upregulated in NF1 patient serum and plays a tumor supporting role through its activity on mast cells. The chemokine RANTES/CCL5 is found at higher levels in the serum of MPNST patients and is overexpressed by optic pathway glioma associated microglia. The effect of RANTES on the immune system could be both immunosuppressive and immune activating.

• IL-6

IL-6 is produced by tumor-infiltrating myeloid cells [98]. It promotes cancer development and protects cancer cells from apoptosis. It is more highly expressed in *Nf1* heterozygous

microglia [75] and has been suggested to play a growth promoting role in OPG. It is upregulated in Schwann cells in which *Nf1* is knocked down [99] and promotes mast cell development and activation that may in turn favor PNF tumorigenesis. In combination with TGF- β it induces MDSCs to accumulate in the tumor microenvironment and upregulates their ability to suppress effector T cells [100], potentially blocking an antitumor immune response.

• IFN- γ

IFN- γ plays a crucial role in inducing Th1 immune response through CD4⁺ T cells, leading to CTL recruitment in the tumor microenvironment [101]. CD8⁺ T cells express IFN- γ within tumor tissue that upregulates the immune checkpoint proteins PD-L1 on tumor cells and PD-1 on CD8⁺ T cells, shutting down a cytotoxic antitumor T-cell response [101–103]. IFN- γ may also affect the immune response in the brain by increasing the expression of proteins on BBB endothelial cells necessary for T-cell transmigration into the brain [33].

• TNF- α

Mast cells secrete TNF- α and trigger the Th1 immune response [104]. TNF- α acts together with IFN- γ to upregulate expression of PD-L1 in tumor cells, blocking cytotoxic T-cell effects [101,103]. Chronic dysregulation of TNF- α synthesis in the tumor microenvironment activates fibroblasts and macrophages and leads to increased angiogenesis and tissue remodeling [105]. It promotes MDSC development in the bone marrow and recruitment to the tumor microenvironment, augmenting immunosuppression [106]. It can also increase permeability of the BBB, increasing the number of immune cells in the brain [33].

• SCF

SCF regulates mast cell differentiation, proliferation, degranulation and cytokine secretion [107]. As described above, many of the cytokines secreted by mast cells promote immunosuppression. Increased SCF release by *Nf1*-mutant Schwann cells activates the c-KIT receptor on *Nf1^{+/-}* mast cells leading to mast cell proliferation and their accumulation in the neurofibroma microenvironment.

• RANTES

RANTES is expressed by immune cells and promotes the migration of effector T cells, memory

T cells, neutrophils, monocytes, eosinophils, dendritic cells and macrophages. This could increase the level of immune cells in the tumor microenvironment for both antitumor effects and immunosuppressive effects. *RANTES* is part of a cytokine cluster on chromosome 17q11.2 that includes *MIP*, *CCL14*, and *CCL16*, all of which encode chemoattractants for immune effector cells. Because these genes are linked to the *NF1* gene, it will be interesting to compare the immune response to tumorigenesis in patients with large deletions on chromosome 17, compare to patients with mutations limited to the *NF1* gene. It is possible that *RANTES* plays different roles at different stages of tumorigenesis. As a macrophage chemoattractant, it may be tumor suppressive at early stages of tumorigenesis and tumor promoting at later stages, consistent with the effects of inhibiting macrophages at different times during tumor development [60].

Molecularly targeted therapy for NF1-associated tumors directed against the immune system

Several therapies expected to modulate immune cell function have been tested in patients or in animal models. These molecularly targeted therapies are predicted to inhibit immune cells in the tumor microenvironment, with or without inhibition of tumor cell growth, thereby blocking supportive effects of immune cells on the tumor.

• Ketotifen

Ketotifen is a mast cell stabilizer that is expected to block secretion of many of the cytokines that affect immune cell function in the tumor and promote tumor growth. Case reports on ketotifen show promising results for neurofibroma when used early as a prevention method [108]. An open-label double-blind clinical trial looking at pain and itching found favorable effects of continuous ketotifen administration [109]. Additional independent studies are needed to assess any potential benefit of ketotifen on PNF growth.

• Imatinib

Imatinib is a multityrosine kinase inhibitor, with activity against c-KIT, PDGFR and ABL. It has been predicted to inhibit PNF growth at several key points. The inhibition of c-KIT blocks mast cell proliferation and the inhibition of PDGFR blocks tumor cell proliferation, as well as angiogenesis and fibroblast proliferation. Imatinib was found to shrink PNFs, reduce

mast cell infiltration, and increase apoptosis in a mouse model. In a compassionate use case, imatinib treatment resulted in a 70% reduction in a PNF [66]. In a Phase II study of imatinib in NF1 patients with PNF, roughly a quarter of patients showed reduction in tumor volume, although all of the tumors that shrunk were very small when enrolled in the study [110].

• Sunitinib

Sunitinib is a multityrosine kinase inhibitor, with activity against VEGFR, PDGFR and c-KIT, and similar to imatinib is predicted to inhibit PNF growth by multiple mechanisms. In preclinical models, sunitinib reduced PNF number and volume in mice, and altered the cellular and biochemical properties of *Nf1* heterozygous mast cells and fibroblasts in culture [111]. Sunitinib is approved for use in renal cell carcinoma and gastrointestinal stromal tumors, however, a Phase II clinical trial (NCT01402817) of sunitinib in PNF patients has been suspended at the time of this writing, and so it remains to be seen whether sunitinib will show benefit in NF1 patients.

• Sorafenib

Sorafenib is a multikinase inhibitor with inhibition of VEGFR, PDGFR, RAF, and to a lesser extent c-KIT. It has been shown to inhibit tumor growth in a mouse plexiform model [112]. Unfortunately, it was not tolerated by pediatric patients in a Phase I study [113] and either no tumor response or acceleration of tumor progression was observed in studies on OPG (NCT01338857) [114], PNF (NCT00727233) [113] and MPNST (NCT00245102).

• Sulindac

Sulindac is a nonsteroidal anti-inflammatory drug that inhibits multiple biological functions through COX1/2-dependent and independent mechanisms [115,116]. Sulindac inhibits the bioactivity of IL6 [117], as well as inhibiting TGF- α and TGF- β and reduces the number of macrophages in the tumor microenvironment [118]. Sulindac has been shown to inhibit MPNST cells in culture by inducing apoptosis [119], and so could inhibit MPNSTs at multiple key pathways. It has not been tested in NF1 patients yet.

• Pexidartinib

As described above, pexidartinib inhibits FMS kinase, the receptor for M-CSF, and c-KIT, the

receptor for SCF and important in mast cell proliferation and migration. Treatment of mouse tumors after tumor formation was shown to shrink tumors [60]. Phase I clinical trials have recently been opened to test pexidartinib in PNF (NCT02390752) and in combination with the mTOR inhibitor sirolimus in MPNST (NCT02584647).

Potential immunotherapy for NF1-associated tumors

The goal of immunotherapy is to restore or augment antitumor immune responses by stimulating the immune system to eliminate cancer cells through immune-mediated cell killing processes. Immunotherapeutic approaches include cytokine therapies [120], tumor vaccines that elicit strong specific immune responses to tumor antigens [121], adoptive cell transfer of genetically engineered T cells expressing tumor-specific chimeric antigen receptors (CAR T cells) [122], monoclonal antibodies that target and block cell surface molecules on tumor cells [123], and approaches that block immunosuppressive molecules such as CTLA-4, PD-1 or Treg cells that dampen effective antitumor immune responses [124]. Aside from pegylated-interferon- α 2b (pegIFN- α 2b), most potential immunotherapies have not yet been tested in NF1-associated tumors. Several immunotherapies that have already shown promise in NF1 preclinical models or other tumor types may be useful in NF1.

• PegIFN- α 2b

IFN- α is secreted by fibroblasts and many types of lymphoid cells, and activates both the innate and adaptive immune response. It acts on NK cells and cytotoxic T cells to upregulate their cytotoxicity and survival [120,125]. It also can act directly on tumor cells to promote apoptosis and can block angiogenesis. Pegylation of IFNs extend their half-life in patient plasma and pegIFN- α 2b has been used in a clinical trial of PNFs [126]. In the Phase I clinical trial it was observed that just under 30% of patients had a 15–22% decrease in their tumor volume and many more had a decrease in volume of <10%. In addition, close to 70% of patients reported a decrease in pain symptoms. A Phase II trial of pegIFN- α 2b is ongoing (NCT00396019).

• Anti-TNF- α antibodies

Several humanized antibodies have been developed to target TNF- α for use in inflammatory

diseases, such as Crohn's disease [127] and rheumatoid arthritis [128], as well as cancer [129]. Inhibition of TNF- α is expected to inhibit mast cell activity, reduce the expression of immune checkpoint proteins on Treg cells and tumor cells, and inhibit MSDC activity, all of which could lead to increased immune response against NF1 tumors.

• Bevacizumab

Bevacizumab is a humanized anti-VEGF antibody. VEGF is a potent angiogenesis inducer. The therapeutic effect of bevacizumab is most often attributed to a normalization of tumor blood vessels that causes a decrease in tumor volume. In addition, VEGF contributes to recruitment of macrophages to tumors, is secreted by mast cells to inhibit differentiation of dendritic cells [130,131], thereby downregulating the antitumor immune response, and is a proliferation, survival and chemotactic factor for Schwann cells in the peripheral nervous system [132,133]. Bevacizumab has shown to delay tumor growth for a variety of tumors, including glioblastoma [134–136] and is showing promise for vestibular schwannomas associated with neurofibromatosis type 2 [137–139]. A trial of bevacizumab in combination with the mTOR inhibitor everolimus in MPNST is ongoing (NCT01661283). Bevacizumab has the potential to target tumor cells, tumor blood vessels and the immune response in NF1 tumors.

• Fresolimumab

Fresolimumab is a humanized anti-TGF- β antibody. TGF- β is secreted by *Nf1*^{-/-} mast cells and induces *Nf1*^{-/-} fibroblasts to proliferate and synthesize collagen, a major component of the stroma in PNFs [95]. The activation of *Nf1*^{-/-} fibroblasts occurs through hyperactivated RAS and c-ABL signaling downstream of TGF- β and inhibition of c-ABL with imatinib was shown to inhibit fibroblast proliferation and collagen production. In a mouse model of melanoma, TGF- β was shown to be required for tumorigenesis, and promotes migration of MDSCs into the tumor and the conversion of CD4⁺ naive T cells into Tregs, which play an important role in tumors escaping immune surveillance [140]. Inhibition of TGF- β has the potential to inhibit PNFs through both increasing immune surveillance and reducing stromal accumulation. Fresolimumab is under development for metastatic kidney cancer and malignant melanoma [141] and may show benefit in neurofibromas.

• Ipilimumab

Ipilimumab is an anti-CTLA4 antibody that has been approved to treat melanoma [142,143]. CTLA4 is expressed on tumor cells and antigen presenting cells and sends an immune suppressive signal to T effector cells. CTLA4 expression has not yet been evaluated in NF1 tumors, and is something to explore in the future.

• Nivolumab/pembrolizumab

Both nivolumab and pembrolizumab have been developed as anti-PD1 antibodies [144,145]. PD1 is expressed on T cells and mediates immune suppression. As with CTLA4, the upregulation of PD1 in NF1 tumors has not been thoroughly examined, however, many of the cytokines that regulate immune suppression, such as IFN- γ and TNF- α , are upregulated in NF1. A clinical trial of pembrolizumab in MPNST is opening (NCT02691026). Future studies may point to an important role for anti-PD1 antibodies in treatment of NF1 tumors.

• Tocilizumab

Tocilizumab is a humanized antibody against the IL-6 receptor and interferes with IL-6 signaling. It has been shown to downregulate the immunosuppressive function of MDSCs. Treatment with tocilizumab enhances T-cell mediated antitumor immune responses [100]. As more is learned about the role of IL-6 and immune surveillance by T cells in NF1, tocilizumab may be a promising therapy.

• Oncolytic viruses

Oncolytic viruses are being developed as therapy due to their ability to replicate in and selectively infect cancer cells. They kill cancer cells either by direct lytic activity of the virus life cycle or by activating the innate immune response against tumor cells. Studies in the *Nf1;Trp53* mutant astrocytoma/glioblastoma model show that tumor cells are susceptible to oncolytic myxoma viral infection and replication in culture, but are resistant when implanted into immune competent mice [146]. Infiltrating macrophages and monocytes, NK cells, and T cells were all found to play a role in inhibiting viral infection *in vivo*, suggesting that the innate immune system inhibits anticancer activity in this case. Oncolytic measles virus infects cells expressing CD46, and has been shown to infect MPNST cells selectively compared with normal Schwann cells. It inhibits tumor growth in an MPNST

xenograft model in immune compromised mice [147]. Oncolytic herpes simplex virus selectively infects cells with hyperactive RAS. It has been shown to infect MPNST cells in culture correlating to levels of RAS hyperactivity [148] and inhibit the growth of MPNST cells in immunocompromised mice [149] and immune competent mice [150]. A clinical trial of oncolytic herpes simplex virus therapy for MPNST has recently been opened (NCT00931931). Viral therapy, possibly in combination with therapies that suppress the innate immune response, may provide successful therapy for NF1 tumors in the future.

• Glioma-associated antigens vaccine

Vaccines for glioma associated antigens have been developed as a way to activate the immune system against gliomas [151]. A study using *Nf1;Trp53* mutant astrocytoma and glioblastoma cells demonstrated that the glioma antigen EphA2 is expressed in these cells and immunized mice generate cytotoxic T cells capable of killing the astrocytoma cells *in vitro* [152]. These vaccines are currently showing promise in pediatric low-grade glioma, but have not been examined specifically for brain tumors associated with NF1. Characterization of NF1 tumor specific antigens could provide another approach to therapy through tumor vaccines or T cells engineered to express chimeric antigen receptors.

Conclusion & future perspective

It is becoming clear that haploinsufficiency for *NF1* mutation has a profound effect on the immune system, but a clear understanding of how these alterations fit together to affect tumor progression or tumor suppression is still lacking. Further studies are needed to understand the potential rationale for immunotherapies in NF1 and how response to immunotherapies may or may not differ in NF1 patients compared with the general population. Research in mouse models has demonstrated that mast cells and neurofibroma tumor cells form positive feedback loops, each secreting factors that promote each others' proliferation. Less well understood is how these mast cells modulate T-cell recruitment and whether they are involved in activation or suppression of antitumor actions of T cells, dendritic cells or NK cells. Cytokine levels are altered in the serum of NF1 patients, but additional studies are needed to assess how these levels change during different stages of progression of different

NF1-associated tumors. If cytokine changes are predictive this could become a valuable biomarker for tumor development in NF1 patients.

There is currently great hope for the promise of immunotherapies in cancer, but given the complexity of immune system alterations in NF1, the role of molecularly targeted therapies should not be overlooked. Immunotherapies harness the body's own defenses to attack tumor cells, but therapies targeting signaling pathways may be needed either to combine with immunotherapies in eliminating tumor burden or to normalize the response of the immune system in NF1 patients by adjusting signaling pathways within the immune cells themselves. For example, a recent study has shown that the MEK inhibitor selumetinib can decrease circulating Treg cells, but increase expression of CTLA4 on Treg cells and increase the expression of PD-1 on Treg cells and CD8⁺ T cells, demonstrating the complex effects of altering signaling pathways in the immune system [153]. It is still unknown to what extent NF1-associated tumors mount immune suppressive defenses in the form of checkpoints such as CTLA4 or PD-L1. Depending on the extent to which the immune response is silenced, immune checkpoint therapies may provide exciting new

directions for NF1 tumor therapy. Learning how to combine different types of immunotherapy with different molecularly targeted therapies will first require a better understanding of the relationships between tumors and different aspects of the immune system in NF1 patients specifically.

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