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Animal Model

Loss of the *Nf1* Tumor Suppressor Gene Decreases Fas Antigen Expression in Myeloid Cells

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Genetic loss of surface Fas antigen expression leads to reduced apoptosis of myeloid and lymphoid progenitor cells, and a propensity to develop autoimmunity and myeloid leukemia in mouse models. Oncogenic $p21^{ras}$ decreases surface Fas antigen expression and renders fibroblasts resistant to Fas mediated apoptosis. Neurofibromin, which is encoded by *NF1*, is a GTPase activating protein that negatively regulates $p21^{ras}$ activity. *NF1* loss leads to deregulation of $p21^{ras}$-effector pathways, which control myeloid cell survival. Heterozygous inactivation of *Nf1* increases mast cell numbers in *Nf1* +/− mice, and enhances mast cell survival in response to c-kit ligand (kit-L). Here, we show that *Nf1*-deficient mast cells have reduced surface Fas antigen expression in response to kit-L and are resistant to Fas ligand-mediated apoptosis. Using genetic intercrosses between *Nf1* +/− and class I_A-PI-3K-deficient mice, we demonstrate that hyperactivation of the $p21^{ras}$-class I_A PI-3K pathway is the mechanism for this phenotype. Finally, we demonstrate that mast cells from both Fas antigen-deficient mice and *Nf1* +/− mice are resistant to apoptosis following kit-L withdrawal *in vivo*. Thus, therapies designed to decrease $p21^{ras}$ activity and up-regulate Fas antigen expression may limit the pathological accumulation of myeloid cells in disease states where $p21^{ras}$ is hyperactivated. *(Am J Pathol 2004, 164:1471–1479)*

Mast cells are important mediators of host immune responses and are active cellular participants in allergic inflammation, neurodegenerative disorders, and some cancers.[1,2] Local cutaneous mast cell numbers increase during inflammatory processes, tissue repair, and certain tumor microenvironments.[1–5] The mechanisms involved in regulating mast cell numbers following these initiating events are not understood, but are likely complex and dependent on local paracrine or autocrine signals, which activate programmed cell death. Several *in vitro* studies suggest that Fas antigen (Apo-1/CD95) may directly or indirectly regulate the apoptotic program in mast cells.[6–8] Further, Fas antigen-deficient mice (*lpr/lpr*) have significantly increased numbers of myeloid precursors in both the bone marrow and spleen.[9] However, the role of Fas antigen signaling in regulating mast cell numbers *in vivo* in murine models of mast cell hyperplasia has not been investigated.

Mutations in the *NF1* tumor suppressor gene cause neurofibromatosis type 1, a pandemic autosomal dominant genetic disorder with an incidence of 1:3500. Neurofibromin, the protein encoded by *NF1,* functions as a GTPase activating protein (GAP) for $p21^{ras}$ by accelerating the hydrolysis of active $p21^{ras}$-GTP to inactive $p21^{ras}$-GDP.[10,11] Individuals with NF1 and genetically engineered mice harboring mutations at the *Nf1* locus have a number of myeloid cell abnormalities.[3,4,12–23] Specifically, NF1 patients develop neurofibromas,[24] which are infiltrated with a high density of degranulating mast cells, and recent genetic studies in mice suggest that *Nf1* +/− mast cells may play a central role in initiating tumorigenesis.[25–28] Further, mice transplanted with *Nf1* nullizygous fetal liver hematopoietic stem cells uniformly develop a myeloid leukemia that is highly reminiscent of the juvenile myelomonocytic leukemia observed in children with NF1.[19,20,23] Currently, the biochemical and cellular mechanisms underlying the expansion of both myeloid

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progenitors and mast cells in NF1 patients is poorly understood.

Fas signaling is tightly regulated by several mechanisms including expression of proteins that directly bind Fas or inhibit caspase activity and mitochondrial events.[29,30] Recent studies in non-myeloid cells demonstrate that Fas antigen expression is regulated by downstream $p21^{ras}$ signaling pathways.[31–33] Specifically, oncogenic $p21^{ras}$ signaling through the phosphoinositide-3-kinase (PI-3K) pathway has been reported to down-regulate Fas antigen expression in immortalized fibroblasts, which renders them resistant to Fas ligand-mediated apoptosis.[32] In support of these data, bone marrow-derived mast cells (BMMCs) derived from SH2-containing inositol 5-phosphatase (SHIP)-deficient mice, which have increased PI-3K activation, are insensitive to Fas ligand-mediated cell death.[34]

The murine c-kit receptor and its ligand, kit-L, are components of a signaling pathway that promote mast cell proliferation and survival.[3] We recently demonstrated that *Nf1* heterozygous BMMCs have increased $p21^{ras}$ activity and PI-3K activation both at baseline and in response to stimulation with kit-L.[3,4] As loss of neurofibromin leads to deregulated $p21^{ras}$ signaling and increased activation of the $p21^{ras}$-classI_A- PI-3K pathway, we tested whether *Nf1*-deficient BMMCs would have a reduction in Fas ligand-mediated apoptosis and Fas antigen expression. Here, using genetic intercrosses between *Nf1* +/− and class I_A-PI-3 kinase-deficient mice, we provide genetic and biochemical evidence to demonstrate that *Nf1*-deficient BMMCs have decreased Fas antigen expression and Fas-ligand mediated apoptosis via hyperactivation of the $p21^{ras}$-class I_A PI-3K signaling pathway. Further, we demonstrate that mast cells from both Fas antigen-deficient mice (*lpr/lpr*) and *Nf1* +/− mice are resistant to apoptosis following kit-L withdrawal *in vivo*.

Materials and Methods

Animals

Nf1 +/− mice were obtained from Dr. Tyler Jacks at the Massachusetts Institute of Technology (Cambridge, MA) in a C57BL/6.129 background and back-crossed for 13 generations into the C57BL/6J strain. *p85α* +/− mice were obtained in a C57BL/6J.129 background from Dr. Lewis Cantley at Harvard University (Boston, MA) and back-crossed for 10 generations into a C57BL/6J strain. *lpr/lpr* mice were obtained in a C57BL/6J background from the Jackson Laboratory (Bar Harbor, ME). Multiple FO founders were used to generate mast cells from embryonic day 13.5 fetal liver from the four F2 *Nf1* and *p85α* genotypes as outlined: FO: *Nf1 +/−; +/+* X *+/+; p85α +/−*. F1: *Nf1 +/−; p85α +/−* X *+/+; p85α +/−*. F2: *Nf1 +/−; +/+*, *+/+; p85α −/−*, *Nf1 +/−; p85α −/−*, *+/+; +/+*. The *Nf1* and *p85α* alleles were genotyped by polymerase chain reaction (PCR) as previously described.[4,35] These studies were conducted with a protocol approved by the Indiana University Laboratory Animal Research Center.

Mast Cell Cultures

Bone marrow-derived mast cells (BMMCs) or fetal liver mast cells (FLMCs) from embryonic day 13.5 fetal liver were established exactly as previously described.[3,4,36] BMMCs and FLMCs were cultured as previously described with minor modifications, and the homogeneity of BMMCs and FLMCs was determined by Giemsa staining.[3,4,37] Aliquots of cells were also stained alcian blue and safranin to confirm that they were mast cells. Furthermore, fluorescence activated cytometeric analysis (FACS) (Becton Dickinson, San Jose, CA) revealed similar forward and side light scatter characteristics and the same percentage of c-kit^+ expression in BMMCs and FLMCs for all murine experimental genotypes (data not shown). For assays examining the expression of cellular surface Fas antigen expression or Fas ligand-mediated cell death, 1×10^6 BMMCs or FLMCs were cultured in RPMI media containing 1% L-glutamine (BioWhittaker, Walkersville, MD) in the absence of both growth factors and serum for 16 hours. Mast cells were then cultured in RPMI media containing 10% fetal calf serum (Hyclone, Logan UT), 1% L-glutamine, 2% penicillin/streptomycin (1 mmol/L/ml) (BioWhittaker) and 100 ng/ml of recombinant murine kit-L (Peprotech, Rocky Hill, NJ) for 72 hours. Cells were then examined for expression of Fas antigen or assayed for Fas ligand-mediated apoptosis.

Flow Cytometric Analysis for Fas Antigen Expression

Surface Fas antigen expression was evaluated by fluoresence cytometry. Mast cells were stained for 30 minutes at 4°C with 1 μg/ml of FITC-conjugated anti-Fas monoclonal antibody (Jo-2) (Pharmingen, San Diego, CA) or an isotype control. Cells were washed three times with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and analyzed by FACS.

Assay for FasL-Induced Cell Death

Following 72 hours of kit-L stimulation (100 ng/ml) as described above, Fas ligand-mediated apoptosis of mast cells was induced by the addition of 100 ng/ml of recombinant human Fas ligand (Upstate Biotech, Waltham, MA). Apoptotic cells were identified by examining DNA fragmentation using the TUNEL method (Roche, Boulder, CO) as previously described.[38] Following 16 hours of incubation with recombinant human Fas ligand, TUNEL-positive cells were enumerated by fluorescence cytometry.

Generation of Recombinant Retroviral Plasmids

Previously developed recombinant retrovirus constructs were used in these studies.[37] The internal sequences of these constructs are under the transcriptional control of the myeloproliferative sarcoma retrovirus promoter. Constructs also contain a puromycin resistance gene, *pac*, which is under the transcriptional control of the phospho-

glycerate kinase (PGK) promoter. Three viruses were used in these experiments: a virus expressing the full-length *NF1* GTPase activating related domain (*NF1* GRD) and *pac* (MSCV-*NF1* GRD-pac); a virus expressing a GAP-inactive mutant of the *NF1* GRD that harbors a known human mutation in the arginine finger loop (R1276P)[39] and *pac* (MSCV-1276P *NF1* GRD-pac); and a virus expressing the selectable marker gene alone (MSCV-pac).[37]

Retroviral Transduction of BMMCs or FLMCs

The transduction protocol has been previously described and was used here with minor modifications.[37] Briefly, embryonic day 13.5 fetal liver cells recovered from genotyped livers or bone marrow mast cells were pre-stimulated for 48 hours in liquid cultures of RPMI containing 20% fetal bovine serum (Hyclone) supplemented with kit-L (100 ng/ml) and interleukin-6 (IL-6) (200 U/ml) (Peprotech). Cells were transduced on mitomycin C-treated E86 producer cells in the presence of kit-L, IL-6, and polybrene (5 μg/ml) for 48 hours. Transduced cells were then cultured under conditions to promote mast cell growth as described previously and as above.[37]

Detection of p21^{ras}-GTP Levels

BMMCs were deprived of serum and growth factors for 24 hours and stimulated with 10 ng/ml of kit-L for 5 minutes. p21^{ras} activation was subsequently determined using p21^{ras} activation assay kits (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol and as described previously.[4]

PI-3 Kinase and ERK Inhibition

BMMCs were preincubated with 5 μmol/L of LY294002 (Sigma), 50 μmol/L of PD98059 (Cell Signaling, Beverly, MA), or DMSO vehicle control for 2 hours. Following pre-incubation, 100 ng/ml of kit-L was added to cultures for up to 72 hours as described above. Following treatment with the inhibitors or vehicle alone, cells were analyzed for surface Fas antigen expression.

In Vivo *Cell Survival Assay*

Under light avertin anesthesia, micro-osmotic pumps (Alzet, Cuppertino, CA) containing kit-L were placed under the dorsal back skin of WT, *Nf1* +/−, or Fas antigen-deficient (*lpr*) mice. Osmotic pumps released a continuous infusion of kit-L at a rate of 20 μg/kg/day for 7 days. Mice were sacrificed and skin samples were recovered at the site of kit-L release at various times following pump depletion. Dorsal skin was stained with a drop of India ink at the point of exit of kit-L from the osmotic pump before pump removal. Three-cm sections of skin marked with India ink were removed, fixed in buffered formalin, and processed in paraffin-embedded sections. Specimens were stained with hematoxylin-eosin to assess routine histology and with Giemsa to identify mast cells. Cutaneous mast cells were quantitated in a blinded fashion by counting 2 mm^2 sections in proximity to the India ink stain. Mast cells undergoing apoptosis were detected using a DermaTACS *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD), which is based on DNA-end labeling using terminal deoxynucleotidyl transferase (TdT) and modified nucleotides. Detection of incorporated molecules is achieved using a chromogenic substrate with a horseradish peroxidase detection system. Reactions were performed without the labeling enzyme as a negative control.

Immunofluorescence

Skin sections were deparaffinized in xylene for 15 minutes then rehydrated in dilutions of ethanol (100%, 95%, 70%) and PBS for 5 minutes each. Sections were then blocked in 10% goat serum plus 3% BSA for 20 minutes and incubated with anti-Fas (Jo2) antibody at a dilution of 1:1000 or isotype control (1:1000) for 1 hour at room temperature. *In situ* immunofluorescence was assess using a Zeiss Axioskope 20 immunofluorescent microscope system (Upstate Biotech, Waltham, MA).

Results

Loss of Nf1 Results in Resistance to Fas Ligand-Mediated Apoptosis and a Reduction in Surface Fas Antigen Expression

Loss of *Nf1* increases p21^{ras} activity in *Nf1* +/− mast cells and *Nf1* −/− bone marrow cells.[4,19,20] To examine whether loss of *Nf1* renders mast cells resistant to Fas ligand-mediated apoptosis, WT, *Nf1* +/− and *Nf1* −/− BMMCs were cultured in kit-L (100 ng/ml) for 72 hours and then stimulated with recombinant Fas ligand (100 ng/ml) or vehicle control for 16 hours. Following culture, the percentage of cells undergoing apoptosis was determined by examining DNA fragmentation using the TUNEL method. *Nf1* +/− and *Nf1* −/− mast cell cultures contained fewer TUNEL-positive cells compared to WT cultures in an *Nf1* gene dose-dependent manner (Figure 1A). Importantly, mast cell cultures treated with vehicle alone did not show differences in TUNEL-positive cells in the three genotypes tested (Figure 1A). To ensure that differences in cell survival were not explained by alterations in c-kit receptor expression, we quantified surface expression of c-kit on WT, *Nf1* +/−, and *Nf1* −/− BMMCs. No differences in c-kit expression were observed between the three experimental genotypes (data not shown).

One potential explanation for resistance to Fas ligand-mediated apoptosis in *Nf1*-deficient mast cells is that surface Fas antigen expression is altered. Thus, we tested whether loss of neurofibromin decreases Fas antigen expression in response to kit-L. One $\times$ 10^6 WT, *Nf1*+/−, or *Nf1* −/− BMMCs were placed in RPMI serum-enriched cultures containing 100 ng/ml of kit-L for 72 hours. Following culture, cells were stained with an anti-Fas antibody and surface Fas antigen expression was

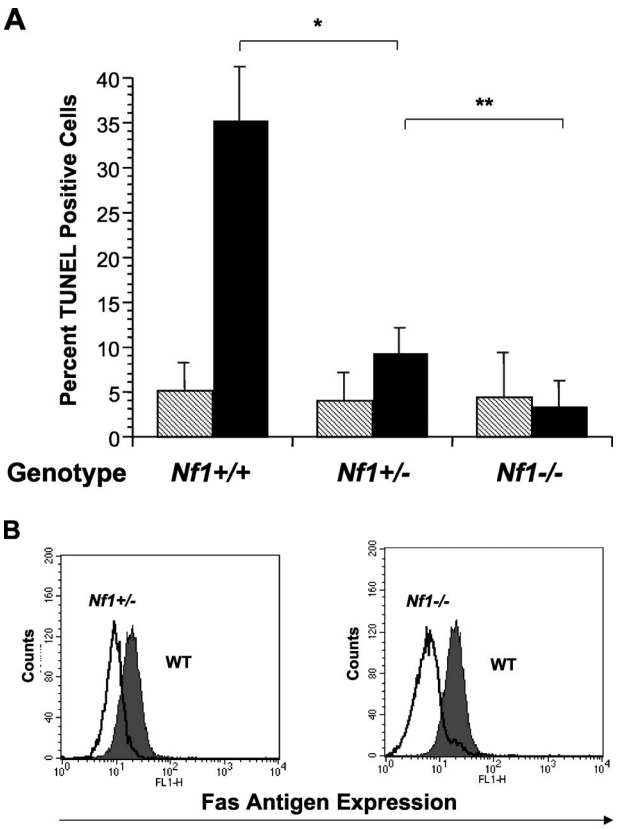


Figure 1. A–B: Neurofibromin-deficient mast cells are resistant to Fas ligand-mediated apoptosis and have reduced surface Fas antigen expression. **A:** WT, *Nf1 +/−*, or *Nf1 −/−* mast cells were cultured in serum-enriched medium and kit-L for 72 hours. Following culture, cells were stimulated with either Fas ligand (**black bars**) or vehicle (**hatched bars**). The percentage of cells undergoing apoptosis was determined by FACS analysis using the TUNEL method. Results represent the mean ± SEM of five independent experiments. * $P < 0.03$ for comparison of Fas ligand-treated *versus* vehicle-treated mast cell cultures within each experimental genotype by Student's paired *t*-test. ** $P < 0.05$ for comparison of Fas ligand-treated *Nf1 +/−* mast cells *versus* Fas ligand-treated *Nf1 −/−* mast cells by Student's paired *t*-test. **B:** WT, *Nf1 +/−*, or *Nf1 −/−* mast cells were cultured in serum-enriched medium and kit-L for 72 hours and analyzed for expression of surface Fas antigen following culture. The **dark profile** represents Fas antigen expression by WT mast cells and the **overlays** represent Fas antigen expression by *Nf1 +/−* and *Nf1 −/−* cells in the **left** and **right panels**, respectively. Data are representative of five other independent experiments with similar results.

determined by FACS analysis. *Nf1 +/−* and *Nf1 −/−* cells expressed diminished levels of Fas antigen compared to WT BMMCs (Figure 1B). However, no differences in surface Fas expression between the experimental genotypes were observed before stimulation with kit-L (data not shown). Thus, loss of neurofibromin imparts resistance to Fas ligand-mediated apoptosis in *Nf1*-deficient mast cells that is directly linked with reduced Fas antigen expression.

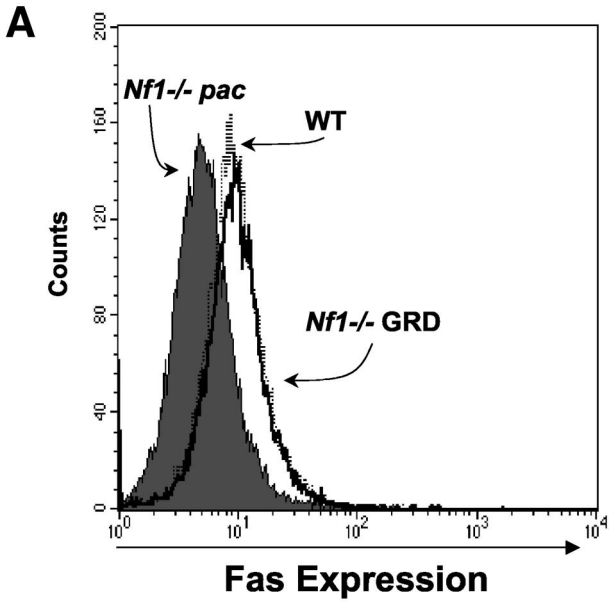
Expression of NF1 *GAP Related Domains into Nf1 −/− Mast Cells Increases Fas Antigen Expression, and Restores Fas Ligand-Mediated Apoptosis.*

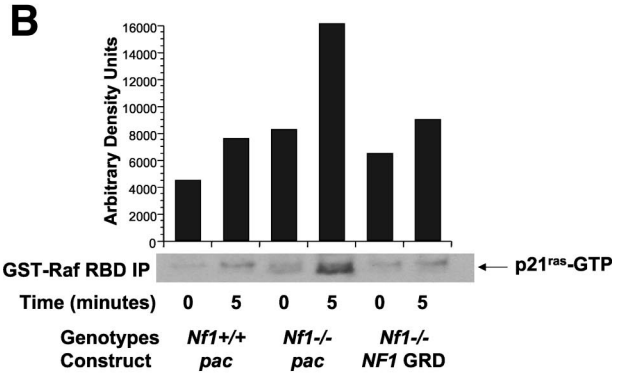
Expression of recombinant *NF1* GAP related domains (GRDs) in primary *Nf1*-deficient cells restores the activation of the $p21^{ras}$-Raf-Mek-ERK effector pathway to WT levels.[37] To determine whether reduced surface Fas antigen expression in *Nf1*-deficient cells is a function of increased $p21^{ras}$ activity, WT and *Nf1 −/−* mast cells were transduced with a recombinant retrovirus encoding full length *NF1* GRD and a selectable marker gene, *pac*. Following transduction and puromycin selection, mast cells were cultured in serum-enriched medium containing 100 ng/ml of kit-L for 72 hours, and surface Fas antigen expression was examined using fluorescence cytometry. In contrast to *Nf1 −/−* mast cells expressing *pac* alone, expression of *NF1* GRD in *Nf1 −/−* mast cells increased Fas antigen expression to WT levels (Figure 2A). A similar correction was observed when *NF1* GRD was expressed in *Nf1 +/−* mast cells. To test whether increased Fas antigen expression correlated with decreased $p21^{ras}$ activity in *Nf1 −/−* mast cells, we examined $p21^{ras}$-GTP levels in *NF1* GRD transduced cells. Expression of *NF1* GRD into *Nf1 −/−* mast cells, but not the control sequences (*pac*), reduced $p21^{ras}$ activity to WT levels (Figure 2B). However, expression of a known human mutant *NF1*GRD cDNA (R1276P), which contains a single point mutation that reduces NF1 GAP activity by 8000-fold,[39] neither restored Fas antigen expression nor increased Fas-induced apoptosis in *Nf1 −/−* mast cells (data not shown). Thus, increased $p21^{ras}$ activity is directly linked to reduced Fas antigen expression in *Nf1*-deficient cells.

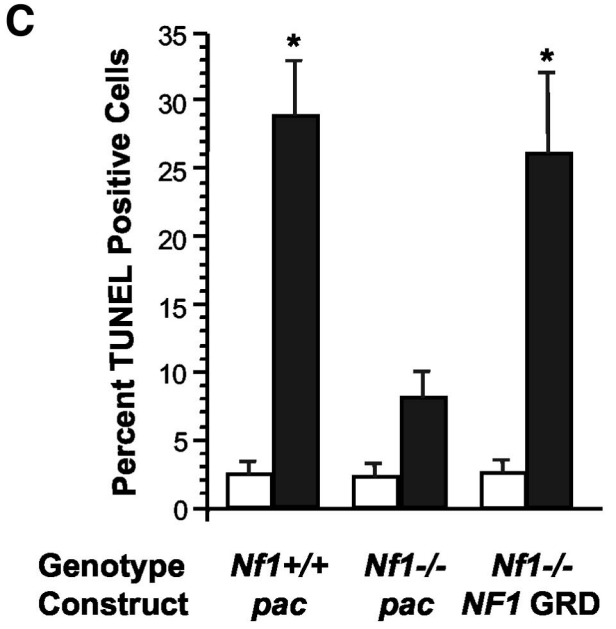
To test whether expression of *NF1* GRD sequences into *Nf1 −/−* cells would increase Fas ligand-mediated apoptosis to WT levels, *Nf1 −/−* BMMCs transduced with either the *NF1* GRD or the reporter construct sequences only (*pac*) and WT cells were cultured in kit-L (100 ng/ml) for 72 hours and then stimulated with recombinant Fas ligand (100 ng/ml) or vehicle control for 16 hours. Following culture, the percentage of cells undergoing apoptosis was determined by examining DNA fragmentation using the TUNEL method. Consistent with a restoration of surface Fas antigen expression, *Nf1 −/−* mast cells transduced with the *NF1* GRD were sensitive to Fas ligand-mediated apoptosis (Figure 2C).

Genetic Inactivation of Class I_A- PI-3K Activity in Nf1-Deficient Mast Cells Restores Fas Antigen Expression and Sensitizes Cells to Fas Ligand-Mediated Apoptosis

Given the biochemical link between $p21^{ras}$ activity and Fas antigen expression in neurofibromin-deficient cells, we next sought to identify which $p21^{ras}$ effector pathways regulate Fas antigen expression. Based on previous studies in immortalized fibroblasts[31] and the fact neurofibromin-deficient mast cells have increased PI-3 kinase and ERK activity in response to kit-L,[4] we initially tested whether pharmacological inhibition of the PI-3K or the $p21^{ras}$-Raf-Mek-ERK pathway would alter Fas antigen expression in WT, *Nf1 +/−*, or *Nf1 −/−* mast cells by using specific inhibitors of PI-3K (LY294002), and MEK (PD98059). BMMCs were pre-incubated with serum-enriched media and either LY294002 (5 μmol/L), PD98059 (50 μmol/L), or vehicle control for two hours, and kit-L







was then added to all cultures. Following 72 hours of culture, surface Fas antigen expression was analyzed by FACS. As was previously observed, Fas antigen expression was reduced in neurofibromin-deficient cells treated with vehicle control compared to WT cells (data not shown). While pretreatment with PD98059 did not restore surface Fas antigen expression in *Nf1*-deficient mast cells, preincubation of *Nf1* +/− and *Nf1* −/− mast cells with LY294002 significantly increased Fas antigen expression (data not shown). To more rigorously test whether class I_A PI-3 kinase alters surface Fas antigen expression in *Nf1* +/− mast cells, we intercrossed *Nf1* +/− mice with mice deficient in the p85α regulatory subunit of class I_A PI-3K (*p85*α−/−). While we were not able to generate *Nf1* −/−, *p85*α −/− mast cells secondary to the lethality of these embryos, we did establish *Nf1* +/−, *p85*α −/− mast cells along with the appropriate experimental controls from embryonic day 13.5 fetal liver to test our hypothesis. Consistent with our prior observations using similar culture conditions, *Nf1* +/− BMMCs expressed low levels of Fas antigen. However, *Nf1* +/−; *p85*α −/− BMMCs had Fas antigen expression comparable to WT controls (Figure 3A) and were sensitive to Fas ligand-mediated apoptosis (Figure 3B). Thus, these results show that increased Fas antigen expression and resistance to Fas ligand-mediated apoptosis in *Nf1* +/− mast cells is mediated by increased activation of the $p21^{ras}$-class I_A-PI-3K pathway.

Mast Cells from Nf1 +/− Mice Are Resistant to Apoptosis following kit Ligand Withdrawal and Do Not Express Fas Antigen after Stimulation with kit-L in Vivo

Injection of polyethylene glycol conjugated kit-L into the skin of WT mice or continuous infusion of kit-L via a

Figure 2. A–C: Expression of the *NF1*GRD in *Nf1* −/− mast cells increases surface Fas antigen expression and Fas ligand-mediated apoptosis. **A:** Cell surface Fas antigen expression in *Nf1* −/− mast cells transduced with a recombinant retrovirus expressing the *NF1*GRD. Following transduction with a recombinant retrovirus expressing either the *NF1*GRD or a puromycin-resistant gene (*pac*) and selection in puromycin, WT and *Nf1* −/− cells were cultured in serum-enriched media and kit-L for 72 hours and surface Fas antigen expression was measured by FACS. The **dark profile** depicts Fas antigen expression on *Nf1*-deficient cells transduced with the control virus and the **open overlay** represents Fas expression by cells transduced with *NF1* GRD sequences. The **dashed overlay** represents Fas expression by WT cells transduced with a retrovirus expressing *pac* only. Data are representative of five other independent experiments with similar results. **B:** $p21^{ras}$-GTP levels in *Nf1* −/− mast cells transduced with a recombinant retrovirus encoding either the *NF1*GRD or *pac* and WT mast cells transduced with a retrovirus encoding *pac* only. After transduction and selection in puromycin, mast cells were serum starved in the absence of growth factors for 24 hours and then stimulated with kit-L for 5 minutes. $p21^{ras}$-GTP levels were quantitated as described in Materials and Methods. Data are representative of five other independent experiments with similar results. **C:** Fas ligand-mediated apoptosis in *Nf1* −/− mast cells transduced with a recombinant retrovirus encoding the *NF1*GRD. Following transduction with a recombinant retrovirus encoding either the *NF1*GRD or a puromycin-resistant gene (*pac*) and selection in puromycin, WT and *Nf1* −/− cells were cultured in serum-enriched media and kit-L for 72 hours. Following culture, cells were stimulated with either Fas ligand (**black bars**) or vehicle (**white bars**). The percentage of cells undergoing apoptosis was determined by FACS analysis using the TUNEL method. Results represent the mean ± SEM of five independent experiments. * $P < 0.05$ for comparison of Fas ligand-treated and vehicle-treated mast cell cultures within each experimental genotype by Student's paired *t*-test.

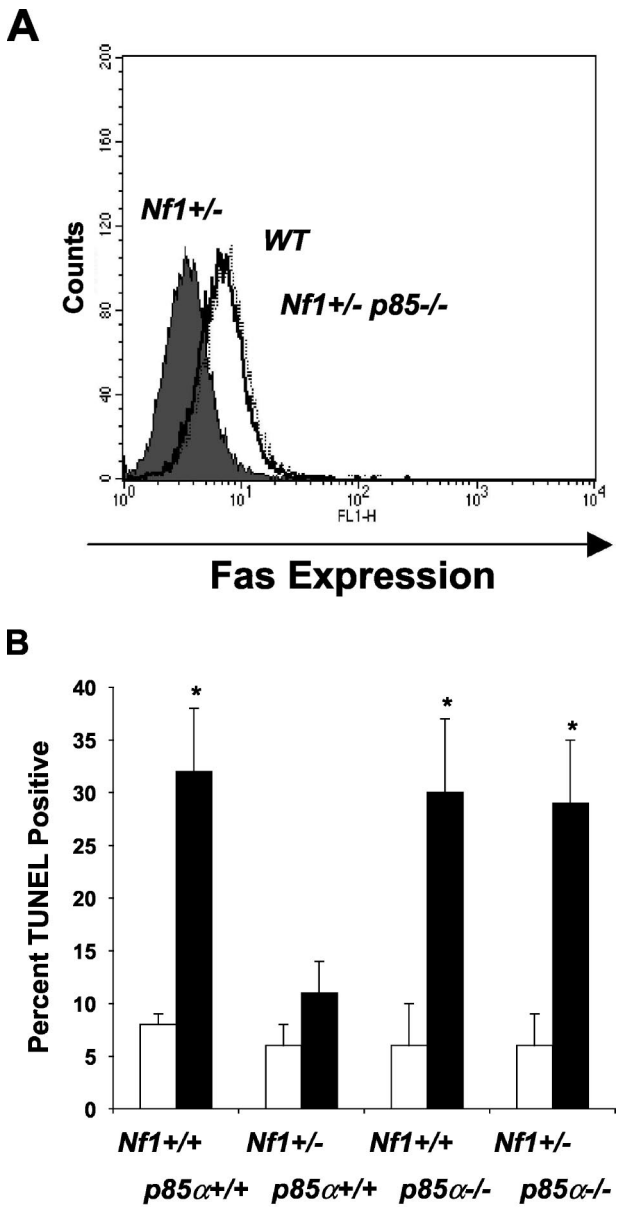


Figure 3. A–B: Genetic inhibition of class I_A PI-3 kinase increases surface Fas antigen expression and Fas ligand-mediated apoptosis in neurofibromin-deficient mast cells. **A–B:** Effect of genetic inactivation of *p85α* on Fas antigen expression and Fas ligand-mediated apoptosis in neurofibromin-deficient mast cells. FLMCs from the four F2 *Nf1* and *p85α* experimental genotypes were cultured for 72 hours in serum-enriched medium and kit-L. Following culture, an aliquot of cells was examined for cell surface Fas antigen expression and the remaining cells were stimulated with either Fas ligand or DMSO vehicle control for 16 hours. Following stimulation with Fas ligand, the percentage of apoptotic cells was examined by FACS using the TUNEL method. The **dark profile** in **B** depicts Fas expression on *Nf1* +/− mast cells, and the **open overlay** represents Fas expression on *Nf1* +/−; *p85α* −/−-deficient cells. The **dashed overlay** represents Fas expression on WT mast cells. Fas expression on WT and *p85α* −/− mast cells was not different (data not shown). In **C**, results represent the mean ± SEM of five independent experiments. * $P < 0.05$ for comparison of Fas ligand-treated (**dark bars**) and vehicle-treated mast cell cultures (**open bars**) within each experimental genotype by Student's paired *t*-test.

subcutaneous microsmotic pump results in mast cell accumulation at the site of injection that is associated with local mast cell proliferation.[4,40,41] To test whether heterozygous inactivation of *Nf1* would alter Fas antigen expression *in vivo* in response to kit-L, we administered kit-L to WT, *Nf1* +/−, and Fas antigen-deficient mice (*lpr* mice) via microsmotic pumps and examined Fas antigen expression by immunofluorescence. Micro-osmotic pumps were filled with a dose of kit-L to administer 20 μg/kg/day for 7 days and placed into the dorsal back skin. This dose of kit-L induces an approximate fivefold and sevenfold increase in the number of mast cells that accumulate in WT and *Nf1* +/− mice, respectively.[4] *In situ* immunofluorescence was performed on skin sections taken from mice of the various genotypes following 7 days of kit-L administration. Skin sections were stained with an FITC-conjugated anti-Fas antigen monoclonal antibody (Jo2) or an isotype control, and immunofluorescence on cutaneous mast cells of WT, *Nf1* +/−, and *lpr/lpr* mice was assessed. Figure 4 demonstrates that mast cells present in skin samples from WT mice express Fas antigen. However, Fas expression could not be detected in either *Nf1* +/− or *lpr/lpr* cutaneous mast cells (Figure 4). Mast cells in adjacent skin samples did not stain positive when immunofluorescence was performed with an isotype antibody control (data not shown). Thus, these studies verify that the biochemical mechanisms identified *in vitro* for decreased Fas antigen expression in neurofibromin-deficient mast cells are valid in an experimental model of mast cell stimulation with kit-L *in vivo*.

Finally, to genetically test the role of Fas antigen expression in regulating mast cells numbers following kit-L withdrawal *in vivo*, we administered kit-L to WT, *Nf1* +/−, and Fas antigen-deficient mice (*lpr* mice) via micro-osmotic pumps and examined cutaneous mast cell numbers following pump depletion. Withdrawal of kit-L after continuous administration to WT mice has previously been shown to rapidly reduce mast cell numbers by inducing mast cells apoptosis.[40] Micro-osmotic pumps were filled with a dose of kit-L to administer 20 μg/kg/day for 7 days and placed into the dorsal back skin. Pumps were removed 5 days following pump depletion and skin biopsies were taken from the site of pump insertion and stained with Giemsa to identify mast cells. Administration of kit-L induced a profound increase in mast cell numbers in mice of each genotype, as previously described[4] (data not shown). However, while the number of mast cells returned to baseline in WT mice 5 days after pump depletion, *Nf1* +/− mice retained elevated numbers of mast cells 5 days following pump depletion (Figure 5). Interestingly, *lpr/lpr* mice retained similar numbers of mast cells following pump depletion as *Nf1* +/− mice. Thus, decreased Fas antigen expression on *Nf1* +/− and *lpr/lpr* mast cells is associated with resistance to mast cell apoptosis following kit-L withdrawal *in vivo*.

Discussion

Individuals with NF1 and genetically engineered mice harboring mutations at the *Nf1* locus have a number of myeloid cell abnormalities.[3,4,12–23] Children with NF1 have a 500-fold increased incidence of developing myeloid leukemia associated with a loss of the normal NF1 allele.[13,14,18] Though *Nf1* −/− mice die *in utero* from complex cardiovascular abnormalities, adoptive transfer

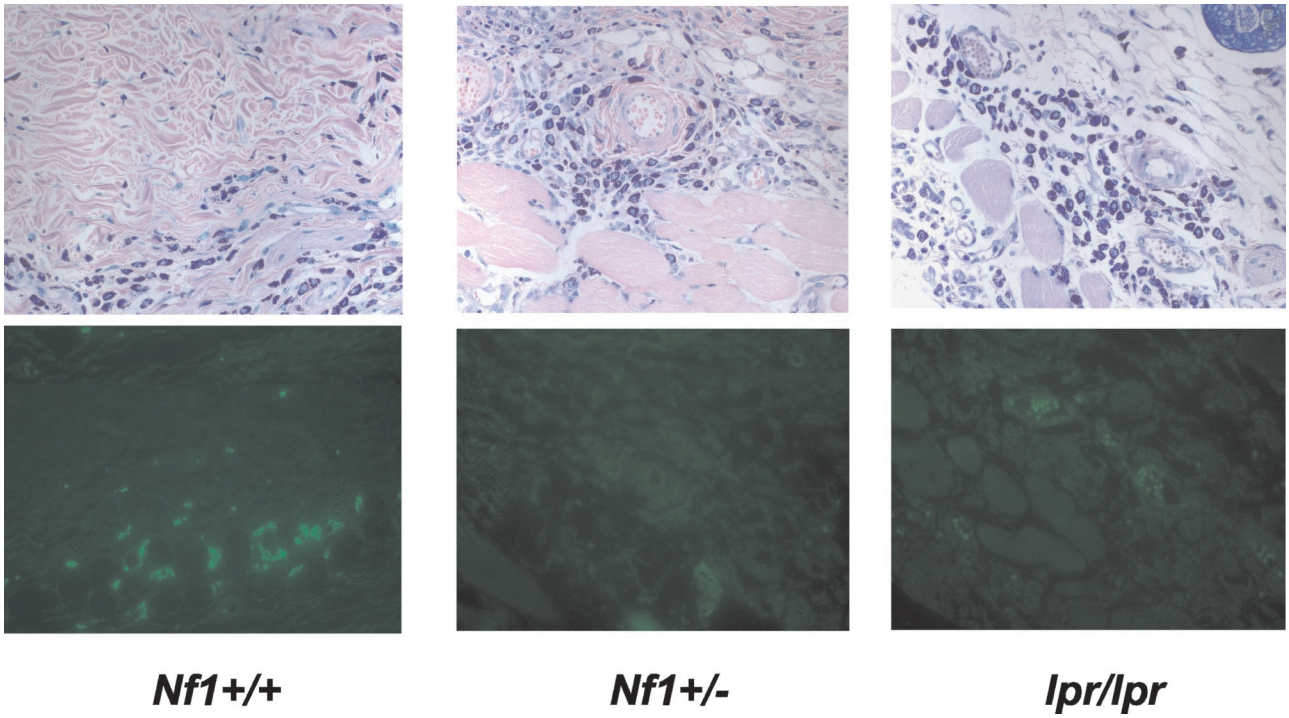


Figure 4. *In vivo* expression of Fas antigen in *Nf1*-deficient and Fas antigen-deficient (*lpr/lpr*) mice. Osmotic pumps were loaded with kit-L or PBS and placed in the subcutaneous mid-dorsum of WT, *Nf1* +/−, and *lpr/lpr* mice to locally administer 20 μg/kg/day of kit-L for 7 days. Skin sections were taken from mice at the point of pump insertion at the end of 7 days and stained with a FITC-conjugated antibody against Fas antigen. A representative skin section from each experimental group stained with either Giemsa to identify mast cells (**top panel**) or a FITC-conjugated antibody against Fas antigen to identify mast cells expressing Fas antigen (**bottom panel**) is shown. Data are representative of five other independent experiments with similar results.

of *Nf1* −/− fetal liver stem cells into WT recipients results in the development of a myeloid leukemia that is highly reminiscient of the human disease.[19,20,22,23] The development of neurofibromas, which are complex tumors composed of multiple cell types, is a hallmark of neurofibromatosis type 1.[24] Mast cells infiltrate plexiform and cutaneous neurofibromas in humans[28] as well as murine models of cutaneous tumors[25,42] where they secrete proteins that can remodel the extracellular matrix and initiate angiogenesis.[1,2,24,42] Consistent with these observations in NF1 patients, we have recently shown that *Nf1* +/− mast cells have increased proliferation[4] and migration[12], in response to kit-L and that *Nf1* +/− mice have increased numbers of cutaneous and peritoneal mast cells. Thus, identification of the biochemical mechanisms responsible for the expansion of *Nf1*-deficient myeloid cells is critical for understanding disease pathogenesis and the rational design of experimental therapeutics.

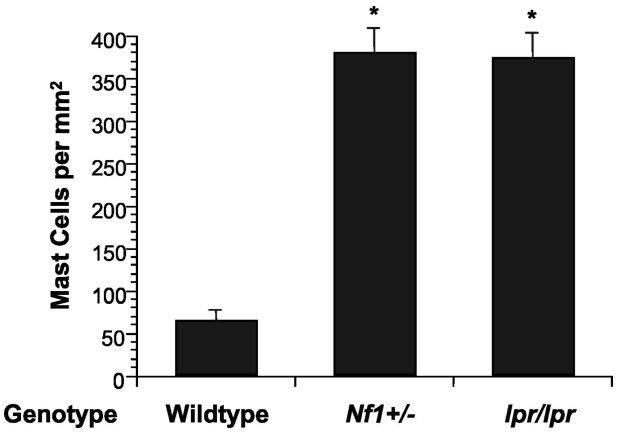


Figure 5. The effects of loss of *Nf1* or Fas antigen on accumulation of cutaneous mast cells in response to local administration and withdrawal of kit-L *in vivo*. **A:** Osmotic pumps were loaded with kit-L or PBS and placed in the subcutaneous mid-dorsum of WT, *Nf1* +/−, and *lpr/lpr* mice to locally administer 20 μg/kg/day of kit-L for 7 days. Skin sections were taken from mice 5 days after pump depletion and stained with Giemsa to identify mast cells. Cutaneous mast cells were quantitated by counting 2 mm^2 sections. Data represent the represent the mean ± SEM of five independent experiments. $^*P < 0.03$ for numbers of cutaneous mast cell in *Nf1* +/− and *lpr/lpr* mice compared to WT mice by Student's paired *t*-test.

Neurofibromin, the protein encoded by *NF1*, functions as a GAP for $p21^{ras}$ by accelerating the hydrolysis of active $p21^{ras}$-GTP to inactive $p21^{ras}$-GDP.[10,11,43] We and others have shown that neurofibromin-deficient myeloid progenitors and mast cells have increased $p21^{ras}$ activity in response to multiple hematopoietic cell growth factors, which is directly linked to increased proliferation.[4,19–21,37] While most studies have investigated which downstream $p21^{ras}$ effectors are altered in enhancing proliferation, the role of *NF1* in regulating myeloid cell survival is incompletely understood. These studies are important since growth factor regulation of both apoptosis and proliferation not only plays a critical role in normal hematopoiesis but can also contribute to leukemogenesis and tumor formation when one or more elements fail to function properly.

One of the most potent activators of programmed cell apoptosis in both hematopoietic and non-hematopoietic cell lineages is the death receptor, Fas antigen.[32] Interestingly, recent studies have shown that oncogenic

p21^{ras} inhibits the expression of Fas antigen and renders p21^{ras}-transformed cells resistant to Fas-mediated apoptosis.[31,32] The role of Fas signaling in regulating hematopoietic cell numbers has been described in both myeloid and lymphoid cell lineages.[9,44] *lpr/lpr* mice have increased numbers of both myeloid and lymphoid progenitors assayed in bone marrow and spleen compared to wild-type controls.[9] Moreover, Fas gene mutations have been implicated in development and progression of myelomas, lymphoid leukemias, and lymphomas.[45–47] Given that p21^{ras} activation has been linked to Fas antigen expression and that Fas signaling is important for regulating myeloid cell numbers *in vivo*, we designed studies to test whether Fas antigen expression was altered in neurofibromin-deficient mast cells.

Using genetic intercrosses between *Nf1 +/−* and class1$_A$-PI3-kinase-deficient mice, we provide several lines of evidence to demonstrate that *Nf1*-deficient mast cells have decreased Fas antigen expression and Fas-ligand-mediated apoptosis via hyperactivation of the p21^{ras}-class1$_A$-PI3-K pathway. These results are consistent with studies in *SHIP −/−* mast cells, which also have increased PI-3K-Akt activity and are insensitive to Fas-mediated apoptosis.[34] Expression of the *NF1* GRD in neurofibromin-deficient mast cells restores p21^{ras} activity to wild-type levels, increases Fas antigen expression and restores sensitivity to Fas ligand. Similar results were observed when pharmacological or genetic inhibition of PI-3K was tested in neurofibromin-deficient mast cells. Importantly, we have also identified a role for Fas ligand-mediated cell death in resolving mast cell numbers *in vivo*. The resolution kinetics of mast cells following kit-L-induced accumulation are similar in *Nf1 +/−* mice and *lpr/lpr* mice and cutaneous mast cells sampled from *Nf1 +/−* mice do not express detectable levels of Fas antigen *in vivo*. Finally, while the primary focus of these studies was to determine whether Fas antigen expression was altered in neurofibromin-deficient mast cells, we have initiated studies to test whether Fas antigen expression is decreased in myeloid progenitors harvested from mice transplanted with *Nf1 −/−* fetal liver hematopoietic stem cells. In preliminary studies, we have observed that Fas antigen expression is decreased in phenotypically defined populations of hematopoietic cells (*Sca*I$^+$ lin$^-$)[21] that are highly enriched for primitive and mature populations of myeloid hematopoietic progenitors (data not shown). Taken together, these results argue that reduced Fas antigen expression contributes at least in part to increased numbers of cutaneous mast cells in *Nf1 +/−* mice and demonstrate that alterations in p21^{ras} activity can alter Fas antigen expression in primary, non-transformed myeloid cells.

The development of myeloid leukemias and the contribution of mast cells to neurofibroma formation in NF1 patients likely involves increased proliferation and resistance to apoptosis of neurofibromin-deficient cells. Targeting the biochemical pathways responsible for both of these cellular phenotypes is an attractive strategy for treating NF1 patients. Recently, inhibition of oncogenic p21^{ras} by farnesyltransferase inhibitors has been shown to up-regulate Fas antigen expression under both basal growth conditions and in the presence of growth factors.[31] Currently, several different farnesyltransferase inhibitors have entered clinical testing in a number of disease states where p21^{ras} is hyperactivated, including NF1 patients with myeloid leukemia. In evaluating the clinical efficacy of these trials and in dissecting the pathogenic mechanisms for the accumulation of mast cells in neurofibromas, it will be important to test whether Fas antigen expression is altered in primary cells harvested from patient specimens. Given that increased PI3-kinase activation is responsible for the reduction in Fas antigen expression in neurofibromin-deficient cells, pharmacological inhibition of PI3-K may also decrease mast cell numbers within neurofibromas or potentially be useful in treating myeloid leukemias in NF1 patients. Finally, since NF1 patients have a wide variety of clinical manifestations including bone abnormalities, learning disorders, vascular abnormalities, and brain tumors, it will be interesting to test whether Fas signaling is altered in other neurofibromin-deficient cell lineages.

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