Neurofibromin and IL-4 as Regulators of T Cell Development, Function, and Homeostasis

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
in The University of Michigan
2012

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Acknowledgements

There are so many people who have helped and supported me while I completed this dissertation. The following is my attempt to acknowledge and thank them all.

First, I thank my advisor, Phil King, for teaching me to approach research critically and to communicate my work clearly and concisely, for interesting, and often humorous conversation, for fostering a friendly and cooperative work environment, and for providing me with the opportunity to work on a dream project. In the same vein, I thank my current lab mates, Phil Lapinski, Beth Lubeck, and Melissa Meyer and honorary lab mate Natasha Del Cid, as well as past lab mate Tim Bauler, for assistance with research, help with the navigation of graduate school, and their invaluable friendship. I was proud to work with all of them in the King lab.

Next, I would like to thank all of my committee members for their careful attention to my research projects and for their helpful comments and suggestions. I would also like to thank various members of the Chang, Dunnick, Zhu, Chensue, Laouar, O'Riordan, Zhang, Fox, and Raghavan labs at the University of Michigan for their assistance with experimental design, implementation, and analysis. I would next like

to acknowledge the staff of University Laboratory Animal Management (ULAM), as well as all of the mice that were sacrificed for these experiments.

I would like to thank all of my friends, both near and far, for their continuous support through good times and bad. Special thanks go to the music ministry at Sacred Heart Parish in Dearborn, MI, for allowing me the creative outlet of making music with a most excellent and talented group of musicians. Thanks also go to the PhD Phorummers, who provided valuable advice and humor throughout my career as a graduate student, and to the ladies formerly of CAF, who have provided me with much-needed support and laughter.

Finally, I wish to thank my family, both immediate and extended. My parents have always encouraged me to follow my dreams, and have sacrificed much that I could do so. Their love and support have been invaluable and I am proud to be their daughter. My in-laws have welcomed me as one of their own, and I am very grateful for their love and understanding. My best friend has been like a sister to me for many years, and I am grateful for our rich friendship. Last but certainly not least, I thank my husband, Jeff, for being my staunchest supporter and for encouraging me to return to school. He has been a loving partner and friend and my greatest champion.

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List of Abbreviations

AICD: activation-induced cell death

CM-AVM: capillary malformation-arteriovenous malformation

CTL: cytotoxic T lymphocyte

DAG: diacylglycerol

DN: double negative

DP: double positive

Eomes: Eomesodermin

Floxed: flanked-by-loxP

IFN-γ: interferon-γ

IL: interleukin

IP3: inositol trisphosphate

JAK: Janus kinase

KO: knockout (of a protein)

MACS: magnetic-assisted cell sorting

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

n: number in sample

N.D.: not detectable

NF1: neurofibromin or neurofibromatosis type 1

NKT: natural killer T cell

PI(4,5)P_2: phosphotidylinositol-(4,5)-bisphosphate

RASA1: RAS p21 protein activator 1

RasGAP: Ras GTPase-activating protein

RasGEF: Ras guanine nucleotide-exchange factor

STAT: signal transducer and activator of transcription

T cell Het: T cell-specific NF1-heterozygous mouse

T cell KO: T cell-specific NF1-deficient mouse

T-ALL: T cell-acute lymphoblastic lymphoma/leukemia

TCR: T cell antigen receptor

Tg: transgenic

Th: helper T cell

γc: common γ-chain

Abstract

The regulation of intracellular signaling is critical to the ability of a T cell to develop, survive, and function in an immune response. In this work, we examined the effect of T cell-specific deletion of the Ras GTPase-activating protein (RasGAP) neurofibromin (NF1) on T cell development and function. We have determined that NF1 has a non-redundant role in T cell development in the thymus as well as in peripheral T cell homeostasis. NF1-deficient naïve CD8+ T cells express higher amounts of the effector cytokine interferon-γ (IFN-γ) and the transcription factor Eomesodermin, and this is likely the reason for the ability of T cell-specific neurofibromin-deficient mice to respond more efficiently to *Listeria monocytogenes* infection *in vivo*.

We have also determined that the cytokine IL-4 is an inducer of IFN-γ expression in wild-type CD8+ T cells. IL-4 directly induces transcription at the *ifng* locus, which is augmented by concomitant T cell antigen-receptor (TCR) stimulation, and is likely due to IL-4-mediated upregulation of the transcription factors Eomesodermin and T-bet. The effects of IL-4 on IFN-γ expression in CD8+ T cells are mediated by Ras-ERK and PI3K signaling, with dependence on STAT6 activation when the TCR is activated concomitantly with the IL-4 receptor.

Finally, we show that the induced systemic loss of both NF1 and the related RasGAP RASA1 in adult mice promotes the development of T cell lymphoma. Lymphoma cells were found to populate all hematopoietic and were $CD4^{+}CD8^{+}$ $CD25^{+}TCR^{-}$ cells of thymic origin. Tumor cells were also found to possess activating mutations in Notch1, a hallmark of both human and murine T cell acute lymphoblastic lymphoma/leukemia (T-ALL), in all but one instance of disease. Altogether, this work demonstrates the importance of regulation of signal transduction pathways in normal T cell function.

Chapter 1

Introduction

In the following work, I have sought a clear understanding of the molecular mechanisms that govern T cell development and function. Such knowledge that is acquired in basic research can be applied to translational and clinical research. Ultimately, elucidation of as-yet unknown causes behind phenotypically well-characterized human diseases and disorders and better treatments for such diseases may result from such research.

1.1 The small GTPase Ras

Ras signaling and function

The small GTPase Ras, a 21 kDa protein, is a key component of intracellular pathways in all cells that transmit signals from the extracellular matrix to the nucleus (2). The activation and subsequent tyrosine phosphorylation of a cell surface receptor, such as a cytokine, growth factor, or antigen receptor, initiates a signaling cascade that results in the exchange of Ras-bound GDP for cytosolic GTP. This exchange induces a conformational change in the tertiary protein structure of Ras and renders Ras active (5). Once active, Ras further transmits signals that promote cell growth, proliferation, and survival (6). Activated Ras transmits signals primarily through the

mitogen-activated protein kinase (MAPK) and phosphoinoside 3- kinase (PI3K) pathways (6). Ras-GTP binds Raf-1, a serine-threonine kinase that functions as a MAPK kinase kinase, which in turn phosphorylates the MAPK kinase MEK. MEK activates ERK1/2 by phosphorylation of tyrosine and threonine residues (7, 8). Upon activation, ERK phosphorylates and activates transcription factors including NF-AT, Elk, and c-fos, which translocate to the nucleus (8). Ras activates PI3K by acting directly on the p110 kDa subunit (9). Akt is activated downstream of PI3K and acts to promote cell growth and proliferation through mTOR and other cell cycle-related proteins as well as Bcl-2-mediated survival (10) (Fig. 1).

Ras isoforms

Ras is expressed in three isoforms: K-Ras, N-Ras and H-Ras, which are understood to localize to distinct locations within the cell and to signal differently through downstream effectors (11). K-Ras, for example, is reported to more strongly promote Raf activation and thus to promote ERK activation; H-Ras is reported to preferentially promote PI3K activation (12, 13). Studies of Ras isoform deletion and embryonic lethality in mice using null alleles showed that K-Ras was the only isoform required for embryonic development, although its activation must be tightly controlled, as expression of constitutively active K-Ras also results in embryonic lethality (14, 15). The importance of K-Ras is underscored by experiments in which double-deletion of H-Ras and N-Ras failed to result in embryonic lethality (14, 16,

17). N-Ras-deficient mice were found to have abnormal thymocyte development and attenuated T cell activation in response to viral infection (18).

Oncogenic Ras signaling

Approximately one-third of human tumors have been reported as consequent to dysregulated Ras signaling (19), due either to the loss of the ability of Ras to function as a GTPase or inability to bind to negative regulatory proteins due to Ras mutations (20). Given the role of Ras as a promoter of cell growth, proliferation and differentiation, it is understandable that unchecked Ras activity could lead to malignant transformation of a cell. Although Ras is expressed in three isoforms, K-Ras is the most commonly mutated isoform found in human tumors, occurring in approximately 20% of human tumors, or nearly two-thirds of all Ras-directed tumors (21). In hematopoietic cells, N-Ras mutations are most common (22), but K-Ras mutations are widely reported in T-cell acute lymphoblastic leukemia (T-ALL) (20, 23).

Regulation of Ras signaling

Regulation of Ras activity is important to maintain tissue homeostasis and cellular effector function as well as to prevent unchecked growth and survival. Ras is positively regulated by the Ras guanine-nucleotide exchange factors (RasGEFs) (24).

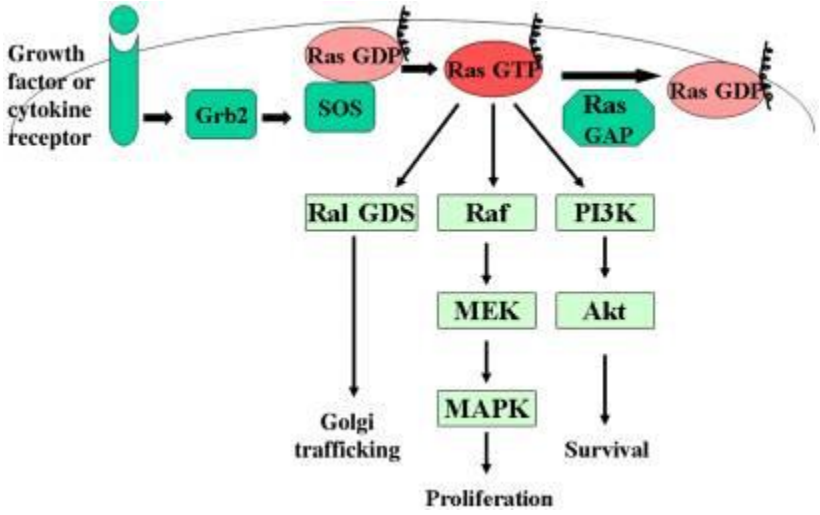

Figure 1. Ras signaling in the cell. (2)

Although several RasGEFs are known to be expressed in humans and mice, the best-studied RasGEF family member is SOS, which is highly conserved across species (2). Upon activation, SOS is recruited to the plasma membrane by the adapter protein Grb2, which docks to phosphorylated tyrosine residues on activated receptors or intermediate scaffolding proteins (2, 25). SOS is then able to interact with Ras and facilitate the release of bound GDP. Although RasGEFs do not directly promote the binding of GTP over GDP, the high ratio of free GTP to GDP in the cytosol increases the likelihood that GTP will fill the empty Ras binding pocket (24). Negative regulation of Ras activity is controlled by a family of catalytic proteins known as the Ras-GTPase activating proteins.

Ras GTPase-activating proteins

Within the cell, the Ras GTPase-activating proteins (RasGAPs) are responsible for the negative regulation of Ras activity. The RasGAP family is comprised of multiple members, all of which possess a catalytic GAP-related domain (26). The proteins are further categorized by the possession of other modular binding domains that facilitate protein-protein interactions as well as localization within the cell (5, 26-28). RasGAPs are differentially expressed in tissues and during developmental stages, which has presented challenges to loss-of-function studies involving null or conditional-null allele mutations *in vivo* due either to embryonic lethality or to redundancy of RasGAP function due to overlapping expression patterns (29-32).

RasGAPs exert their effects on Ras via the above-mentioned GAP domain. This domain permits the binding of RasGAP to GTP-bound Ras, at which point the RasGAP facilitates the cleavage of the γ-phosphate from the bound GTP, promoting its release as inorganic phosphate and returning Ras to its inactive GDP-bound state (33). In the two best-studied RasGAPs, RAS p21 protein activator 1 (RASA1) and neurofibromin (NF1), an arginine-finger motif was determined to act as a key catalytic residue within the GAP domain, where it acts to stabilize both the position of key residues on Ras itself and the charge distributions within the catalytic reaction (34, 35), as well as catalyze the hydrolysis of GTP to GDP and Pi (36).

Research into the requirements for individual RasGAPs within a cell is complicated by the understanding that the importance of individual RasGAPs may differ with the

activity levels of a cell. For example, NF1 is thought to act as a basal regulator of Ras due to its much higher binding affinity for Ras-GTP, while its fellow RasGAP RASA1, which has a lower RasGTP-binding affinity, is thought to regulate Ras in activated cells, when Ras-GTP levels are at a higher concentration within the cell (37, 38). RasGAPs are also believed to differentially regulate different Ras isoforms, which localize differently within the cell and thus affect signal transduction patterns (11, 26).

RASA1

RASA1 is a 120 kDa protein comprised of a number of modular binding domains as well as the catalytic GAP domain (39). In addition, RASA1 contains two SH2 domains, a SH3 domain, pleckstrin-homology (PH) domain, and a calcium-dependent lipid-binding (C2) domain (5, 26, 39). These domains support interactions with other signaling or scaffolding proteins, leading to the regulation of Ras activity and related downstream events (40, 41). RASA1 is also known to interact with p190 RhoGAP through its SH2 domains, thus impacting on pathways regulated by Rho GTPase, such as cell migration (42, 43).

In humans, a mutated allele of *Rasa1*, which codes for the protein RASA1, is known as a causative agent of the disorders capillary malformation-arteriovenous malformation (CM-AVM), an autosomal-dominant disorder characterized by defects in blood vasculature development (44). In mice, full deletion of RASA1 was found to induce embryonic lethality due to defects in cardiovascular development (29),

underscoring the critical role of RasGAPs across species. Induced systemic deletion of RASA1 in adult mice led to hyperplasia of the lymphatic vasculature (45). RASA1 inactivation-related abnormalities in the blood vasculature of adult mice were observed, although only in the context of tumor-related angiogenesis (46), an example that further underscores the differential requirements for RasGAPs in tissues.

Neurofibromin

Neurofibromin (NF1) is a 250 kDa protein comprised of the requisite GAP domain as well as a tandem Sec14 and PH-like domain, which is believed to bind to glycerophospholipids, indicating its potential importance in directing NF1 to plasma membranes (47, 48). Although NF1 is a large protein, no other functional domains have been identified to date, although the frequency of reported non-truncating mutations throughout the length of NF1 in humans with NF1-related disease indicates that other functional domains have yet to be identified (49).

Although comparatively little is known about the structure of NF1, it is well known in the context of human disease. Mutations in the gene *Nf1* are responsible for the disorder neurofibromatosis type 1, an autosomal-dominant disorder that affects approximately 1 in 3500 people (50, 51). Neurofibromatosis type one is classically accompanied by the development of peripheral nerve sheath tumors, or neurofibromas, which are frequently benign. Other presentations include the

appearance of café au lait macules on the skin, optic gliomas, cognitive deficiencies, and an increased susceptibility toward leukemias of the myeloid cell compartments (52-54). Similar phenotypes have been observed in mice with tissue-specific conditional deletions of NF1, indicating that the roles of NF1 are well-conserved between species.

Ras-related disorders in humans

A number of human disorders are related to dysregulation of Ras signaling. These 'RASopathies' can result from gain- or loss-of-function mutations in Ras itself as well as in proteins that regulate Ras and its downstream effectors (22). Human disorders that result from such mutations include Noonan, LEOPARD, Costello, and cardio-facio-cutaneous syndromes, all of which are characterized by developmental abnormalities in the musculoskeletal, cardiovascular and neural systems (55, 56). Some overlap of phenotype has been observed between Ras- and RasGAP-related disorders. In studies of interferon-inducible gene targeting, expression of constitutively active KRas was shown to induce the development of myeloproliferative disorder (57), similar to that which is observed in both NF1-deficient mice and humans with loss-of-function mutations in *nf1*.

1.2 Signal transduction pathways in T cells

TCR-mediated signaling

Interaction of the T cell antigen receptor (TCR) with its cognate antigen-MHC complex sets in motion a number of downstream signaling cascades (Fig. 2). In the

instance of such an interaction, the coreceptors CD4 and CD8 localize to the immunological synapse with the TCR. CD4 and CD8 interact with MHC class II and class I molecules, respectively, and in doing so draw the Src-family kinase Lck, which is associated with the cytoplasmic tails of CD4 and CD8, close to the TCR-associated CD3 complex. Lck, much of which is constitutively activated in thymocytes, is then able to phosphorylate the CD3ζ chains, permitting the binding and phosphorylation of ZAP-70 (58, 59). Activated ZAP-70 then phosphorylates multiple tyrosines on the membrane-tethered scaffolding protein LAT, which facilitates the branching of proximal TCR signaling into several distal signaling pathways through its role as a docking site for several SH2-domain-containing proteins (60).

LAT phosphorylation promotes activation of the small GTPase Ras in two ways. First, the protein PLC-γ binds to LAT, which facilitates the ability of PLC-γ to cleave phosphotidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) to diacylglycerol (DAG) and inositol trisphosphate (IP_3) (3, 61). DAG localizes to the plasma membrane, where it is able to attract and bind the RasGEF RasGRP1 (62). Second, the adaptor protein Grb2 also binds to LAT and in so doing is able to bind and activate the RasGEF Sos (61). The differential localization of these activated RasGEFs within the T cell is thought to contribute to differential Ras signaling in response to the strength of TCR interaction with the cognate antigen-MHC complex (62, 63). Following activation by RasGRP1 and Sos, Ras exerts its effects on T cell development, growth and differentiation primarily through the Raf-MEK-ERK and PI3K pathways (3, 64, 65).

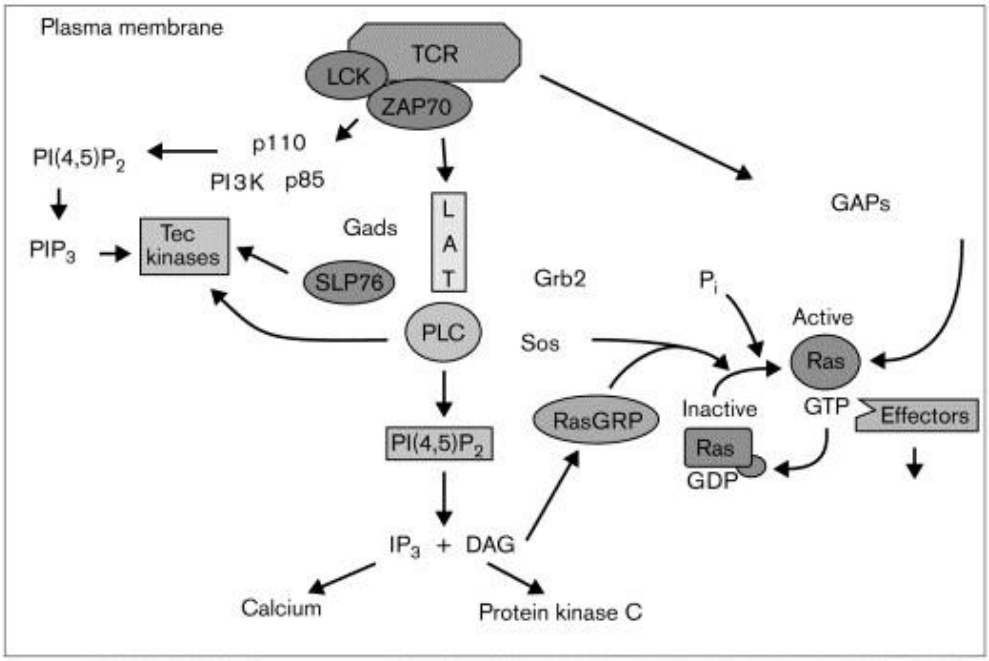

Figure 2. Activation of Ras downstream of the TCR. (3)

T cells also receive signal through costimulatory receptors. Concomitant signals through these receptors can provide 'signal 2' to induce full activation of T cells when 'signal 1' is received through the TCR. The requirement for a second signal prevents inappropriate activation of T cells, i.e. activation of T cells in response to self-antigen (66, 67). One of the best-studied coreceptors expressed on T cells is CD28. Its ligand is CD80/CD86, expressed on activated dendritic cells, and binding of CD28 to its ligand occurs when the TCR is in contact with its cognate antigen-MHC complex on the dendritic cell (68). Signal transduction through CD28 further enhances PI3K activation in T cells (69).

Common γ-chain cytokines in homeostasis and effector function

The common–γ-chain (γc) family designates cytokines that recognize a group of receptor complexes, all of which include the γc as one receptor subunit. IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 belong to the γc family, and all exert effects on T cells to promote development, survival, and effector function (70). All of these cytokines signal through the γc-associated transcription factors signal transducers and activators of transcription (STAT) 5a/b, which are activated by the receptor-associated Janus kinase 3 (JAK3) to the extent that deletion of STAT5 proteins in adult mice significantly impairs normal T cell development and function (71, 72). Individual effects of each γc cytokine are largely mediated by signal transduction events, both STAT-associated and otherwise, downstream of the other subunit or subunits that comprise the individual cytokine receptors (70, 73).

γc cytokines exert their affects on T cells in a number of ways. IL-7 is well-known as a peripheral survival factor for T cells (70, 74, 75). IL-15 is thought to promote homeostasis of central memory T cells, although it has also been shown to be required for the maintenance of naive $CD8^+$ T cell homeostasis (74, 76). IL-21 has been shown to promote the survival of naïve $CD8^+$ T cells in mice and to act with IL-7 and IL-15 to promote both naïve and memory $CD8^+$ T cell proliferation and effector functions *in vitro* (77). IL-4 is a prominent inducer in the differentiation of $CD4^+$ T cells to the type 2 helper T cell (Th) lineage and is a hallmark effector cytokine of Th2 cells (70). IL-9 is also considered to be an effector cytokine of the Th2 and Th lineage (70).

IL-2 is well known as a growth factor for T cells, although IL-2-deficient mice have been found to have dysregulated T cell proliferation accompanied by autoimmune disease (70, 78, 79). This is due to the role of IL-2 in the maintenance of peripheral tolerance and T cell homeostasis by promoting the survival of regulatory T cells as well as by regulating the apoptosis of effector T cells through activation-induced cell death (AICD) following the resolution of the immune response to infection (70, 79, 80). IL-2 is also known to promote the differentiation of $CD4^+$ T cells to the T helper cell type 2 (Th2) lineage (81).

Ras signaling in T cells

Ras activity has been shown to be a key mediator of signal transduction downstream of the TCR, with both developmental and functional consequences (82). Expression of constitutively active Ras in T cells permitted the reversal of induced anergy, while expression of active NRas resulted in the development of autoimmune lymphoproliferative syndrome (83, 84). Further, expression of a constitutively active form of ERK, to mimic continuous signaling through the Ras-ERK pathway, resulted in the loss of peripheral T cells (85). Although the factors involved in positive regulation of Ras in T cells are well understood, less is known about the role of RasGAPs in the negative regulation of Ras signaling in T cells. The RasGAP family members RASA1, NF1, and RASAL3 are known to be expressed in thymocytes and T cells, and this overlapping expression is likely to result in some redundancy of function. However, it is becoming clear that the individual RasGAPs have non-redundant roles in the T cell compartment. NF1 has been reported to regulate

thymocyte and T cell homeostasis and activation-induced proliferation (86). More recently, RASA1 has been reported to regulate thymocyte positive selection as well as peripheral T cell survival (32). However, much remains to be determined regarding the functions of these negative regulators of Ras signaling in T cells.

1.3. Thymocyte development

The primary organ for T cell development is the thymus. In the event of inability to develop a thymus, as seen in *nude* mice or humans with DiGeorge's syndrome, T cell production is inhibited and the host is severely immunocompromised (87, 88). The thymus is comprised of several lobes, each containing both cortical and medullary zones. Developing thymocytes progress from the cortex to the medulla, encountering thymic epithelial cells throughout the process.

Early thymocyte development

The thymic cortex is seeded by early thymic progenitors (ETPs), descendants of hematopoietic stem cells in the bone marrow (89) (Fig. 3). At this early stage, ETPs are also known as double-negative (DN) thymocytes, due to their lack of the T cell co-receptors CD4 and CD8, as well as lack of expression of the T cell-antigen receptor (TCR). DN thymocytes can be further analyzed to belong to four developmental stages: DN1, characterized by high expression of CD44; DN2, characterized by continued expression of CD44 as well as high expression of CD25/IL-2 receptor α (IL-2Rα); DN3, low expression of CD44 and continued high

expression of CD25; and DN4, low expression of both CD44 and CD25 (90). The downregulation of CD44 correlates not only with the downregulation of the early thymocyte marker c-kit, but also with the rearrangement of the TCRβ locus at the transition from DN2 to DN3 (90). The pre-TCRα is also expressed in DN3 and dimerizes with the newly expressed TCRβ. The resulting pre-TCR must be able to conduct signal to the nucleus via interaction with the CD3 complex (91, 92); failure to properly signal through the pre-TCR results in a developmental block at DN3 (93). Thymocytes that express functional TCRβ subunits are able to transition to DN4, characterized by downregulation of CD25 expression (94).

Thymocyte selection

Transition to the double-positive (DP) stage of thymocyte development is characterized by expression of CD4 and CD8. Following surface expression of the TCRαβ, DP thymocytes undergo the process of thymocyte selection, in which the newly formed TCR is tested for appropriate signal transduction. This process is critical for the production of a T cell repertoire that can respond to survival signals from MHC molecules bound to self-antigen peptides as well as foreign peptides within the context of self-MHC. It is also essential to prevent the development of autoimmune disorders, many of which are characterized by strongly self-reactive T cells. Under the widely accepted "strength of signal" model, DP thymocytes that respond to self-antigen are selected for further maturation (95). 'Positive selection' distinguishes thymocytes that can respond to self-antigen, as presented by cortical

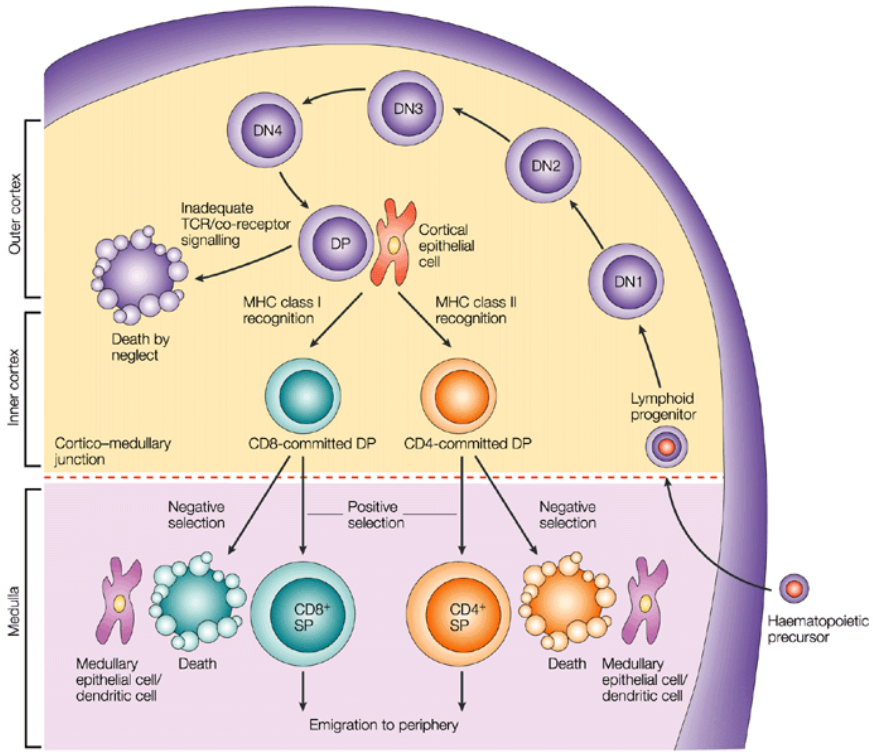

Figure 3. Thymocyte development. (4)

thymic epithelial cells, strongly enough to reach the minimum signal threshold for survival (96). The majority of DP thymocytes (approximately 90%) are unable to reach this minimum threshold and die by neglect (4). Of the remaining cells, those that respond very strongly to self-antigen, expressed by medullary thymic epithelial cells under the control of the transcription factor Aire, are deleted in a process called 'negative selection' (97). Surviving thymocytes respond to peptide in the context of either MHC class I or class II and downregulate surface expression of CD4 or CD8 accordingly (4).

Ras signaling in T cell development

The Ras signaling pathway is understood to play a critical role in thymic selection. Ras-ERK signaling is known to be required for the transition from the DN to DP stages of thymocyte development (98, 99). ERK1/2 phosphorylation downstream of Ras activation has been shown to have a role in the regulation of positive selection (99, 100). Recently, the protein Themis has been discovered to be critical for positive selection through its binding of Grb2, an adaptor protein that binds and activates the RasGEF Sos (101, 102). Expression of a constitutively active form of ERK2 in murine thymocytes resulted in preferential transition of DP thymocytes to the CD4 SP lineage (85). However, the mechanisms by which Ras is negatively regulated in thymocytes are still largely unclear.

1.4. T cells in the periphery

Peripheral survival and homeostasis of naïve T cells

Fully mature T cells, upon exit from the thymus, circulate through the bloodstream. Peripheral T cells circulate through the lymphatic vasculature and lymph nodes for two reasons: first, circulating T cells receive survival signals from stromal cells in the lymph nodes; second, in the event of an infection, circulation increases the likelihood that an antigen-specific T cell will encounter its cognate antigen in a draining lymph node. Maintenance of the peripheral T cell compartment, or homeostasis, is critical to the ability of a host to efficiently respond to pathogens and to prevent autoimmune reactions from highly self-reactive T cells that escaped negative selection.

Naïve T cells continuously receive survival signals during circulation. Efficient thymic selection should ensure that all peripheral T cells can respond to self-MHC at a basal level while deleting cells that respond strongly enough to increase the likelihood of autoimmune responses (103). Thus, T cells are able to continuously sample self-MHC complexes, allowing them to receive low-level TCR activation that promotes survival (62, 74, 103). Survival signals are also transmitted through the IL-7 receptor (IL-7R), expressed on resting T cells, which is activated in response to IL-7 that is produced by epithelial cells and fibroblasts (70). Beyond mechanisms that promote survival, T cells maintain peripheral homeostasis through carefully regulated homeostatic proliferation, in which T cells in lymphopenic hosts expand to fill an underpopulated niche. The mechanisms behind lymphopenia-induced proliferation are not entirely understood. IL-7 is believed to play a role, as IL-7- or IL-7Ra-deficient mice are chronically lymphopenic while overexpression of IL-7 can overcome intrinsic controls on the size of the T cell pool (74, 103, 104).

T cell activation and effector functions

Activated T cells comprise one arm of the host adaptive immune response against pathogens. In the event of pathogen infection, immature dendritic cells at the infection site mature into antigen presenting cells (APCs) as they take up antigenic material from the pathogen. APCs then travel to the draining lymph nodes, where they present antigen, costimulatory ligands, and effector cytokines to the circulating naïve and central memory T cells (105). T cells that encounter their cognate antigen-MHC complex on a fully activated APC are induced to enter cell cycle to allow for

clonal expansion of a pathogen-specific T cell population, and are influenced toward differentiation to effector subsets by signals received from APCs (106).

Helper T cells

$CD4^+$ effector T cells are also known as helper T (Th) cells, and 'help' or direct effector cells primarily through the expression of effector cytokines, which influence the nature and scope of the response to pathogens. Type 1 or Th1 cells are characterized by the expression of the master transcription factor T-bet and the production of the inflammatory cytokine IFN-γ, which promotes the immune response to intracellular pathogens (107). Th2 cells express the transcription factor GATA-3 and are characterized by expression of the cytokine IL-4. These cells promote immune responses against extracellular pathogens by facilitating antigen-specific B cell immunoglobulin class switching to produce opsonizing and neutralizing antibodies against such pathogens, as well as promoting the activation of the granulocytic eosinophils (108). Th17 cells express the transcription factor ROR-γt and are characterized by production of the cytokine IL-17 (109). These cells are involved in the immune response against extracellular bacterial and fungal infections at the mucosal surface (106). Both Th1 and Th17 cells have also been implicated in autoimmune pathology (106). Although each Th subset expresses characteristic transcription factors and cytokines, differentiation to these subsets is no longer believed to be terminal, and the issue of Th cell plasticity is of much interest (106, 110).

Cytotoxic T Lymphocytes

$CD8^+$ T cells also require encounters with their target antigen-MHC complex on APCs as well as costimulatory ligands and cytokines to become fully activated. However, $CD8^+$ T cells also require assistance from $CD4^+$ T cells in the form of enhancement of APC function, as well as from secreted cytokines such as IL-2 (111, 112). $CD8^+$ T cells enter cell cycle and begin to express the transcription factors T-bet and Eomesodermin (Eomes), which promote the expression of the effector cytokine IFN-γ (113). Interestingly, the differential expression of T-bet verses Eomes in CTLs has recently been shown to predict the capacity of a CTL to differentiate into a short-lived effector CTL versus a memory $CD8^+$ T cell, respectively (114-117). Cytotoxic proteins such as perforin and granzyme B are also expressed and are stored in lytic granules within the activated $CD8^+$ T cell (111). These granules are released to kill infected cells which bear the target antigen-MHC complex for the activated $CD8^+$ T cell (118). For this reason activated $CD8^+$ T cells are also known as cytotoxic T lymphocytes (CTLs). CTLs are critical in the adaptive immune response to viral infections as well as some intracellular bacterial pathogens (118).

Memory T cell differentiation and homeostasis

Following a successful adaptive immune response to a pathogen, the T cell compartment contracts to a fraction of its size at peak activation, in a process known as activation-induced cell death (AICD). Not only does this process allow for a return

to maintenance numbers of peripheral T cells, but it also reduces the number of clonal and potentially inflammatory effector T cells in the host (119). The cells that remain are those that have been targeted for differentiation to the memory T cell lineage. Maintenance of this memory T cell population is critical for the development of immune memory, in which the host is able to respond swiftly and effectively to a previously-encountered pathogen.

Memory T cells can be divided into two major subsets. Effector memory T cells mainly reside within tissues, allowing for swift activation upon a secondary antigen encounter. Central memory T cells reside within the peripheral lymphoid organs and are reactivated upon antigen challenge in a much more rapid manner than are naïve cells, allowing for a faster response against infections (120). The latter subset is the target of effective T cell-mediated vaccination, as these cells are long-lived in the host (120).

1.5 Scope of the thesis

In this thesis, I have outlined three research projects, each of which investigates the effects of perturbations of signal transduction pathways on T cell development and function. In the first section, I investigated how the deletion of the RasGAP NF1 in the T cell compartment affects thymocyte development and peripheral T cell homeostasis. In the second section, I reported on the role of IL-4 in the promotion of $CD8^+$ T cell effector function and provide an analysis of the signaling mechanisms by

which IL-4 exerts its effects on these cells. Finally, I described the first systemic concurrent deletion of the RasGAPs NF1 and RASA1 in adult mice, and demonstrate how this double deletion predisposes mice to the development of Notch1-mediated T cell lymphoma. This work has furthered our understanding of the regulation of Ras signaling in T cells as well as the molecular mechanisms that underlie CTL effector function.

Chapter 2

The RasGAP neurofibromin as a regulator of T cell development

2.1. Abstract

Negative regulation of Ras activation downstream of the T cell antigen receptor is poorly understood. The RasGAP neurofibromin (NF1), which facilitates the inactivation of Ras, was conditionally deleted from the T cell compartment in mice at an early stage of thymocyte development. Heterozygous or total loss of NF1 resulted in a partial block of thymocyte development at the double-negative 3 (DN3) stage, and a partial block in the transition from the DP to CD8 single positive stage is evident in TCR-transgenic thymocytes. Mice with heterozygous or total loss of NF1 had significantly fewer naïve peripheral T cells, particularly in the $CD8^+$ subset, indicating a non-redundant role for NF1 in T cell homeostasis. NF1-deficient mice were more efficient in mounting a response to *Listeria monocytogenes* , clearance of which requires the activation of $CD8^+$ T cells, in a five-day infection. Short-term *in vitro* stimulation of pan-T and $CD8^+$ T cells demonstrated an increase in per-cell expression of interferon-γ and Eomesodermin in the naïve $CD8^+$ T cell compartment in response to TCR stimulation. However, loss of NF1 did not affect Ras-ERK signaling in short-term *in vitro* cultures in either freshly isolated $CD4^+$ or $CD8^+$

T cells, nor was Ras-ERK signaling altered in previously activated T cell blasts. These findings suggest that the role of NF1 is non-redundant in thymocyte development and the maintenance of peripheral T cell homeostasis with moderate effects on T cell effector function.

2.2. Introduction

The small GTPase Ras is a key modulator of signal transduction downstream of many cell surface receptors, including the T cell antigen receptor (TCR). Once the TCR is activated through binding to its cognate antigen in complex with the MHC on an APC, a signaling cascade is triggered that leads to the activation of Ras (121). Ras in turn promotes signaling through the MEK-ERK and PI3K pathways, leading to the translocation of the transcription factors AP-1, NF-κB and NFAT to the nucleus and the transcription of key functional genes (3, 122-124). Ras signaling is critical not only for the development of T cells in the thymus, but also for the induction and maintenance of T cell effector functions in the periphery (125-127). The role of Ras in cell activation is significant in that its dysregulation is directly and positively associated with many types of tumors, including T cell-acute lymphoblastic leukemia (T-ALL) (128-130). Thus, it is important to understand the processes by which Ras is negatively regulated within the cell.

Ras is negatively regulated by a family of proteins collectively known as the Ras GTPase-activating proteins (RasGAPs) (5). RasGAPs share a common GTPase-activating protein (GAP) domain that facilitates the hydrolysis of the Ras-bound GTP to GDP and inorganic phosphate (33). The family members are distinguished from one another by the presence or absence of other modular protein domains, which assist in intracellular localization and scaffolding, as well as by tissue expression patterns (131). The high number of RasGAPs in the proteome yields the possibility of overlap of expression and function in a given cell type (27, 132). Although RasGAPs

are expressed in T cells, the specific functions of each are not yet well elucidated (3). Our lab has recently reported that mice bearing a T cell-specific deletion of the RasGAP family member RASA1 do not have grossly dysregulated T cell function, but that RASA1-deficiency does affect thymocyte development and peripheral T cell survival (32).

The RasGAP neurofibromin (NF1) is named for its causative role in the human disorder neurofibromatosis type 1 (NF1) (133, 134). NF1 is an autosomal-dominant disorder, and many manifestations of this disorder result from a heterozygous mutation of the *Nf1* gene (31, 135), as *Nf1* is haploinsufficient in many tissues (136-141). The disorder NF1 is classically characterized by the appearance of plexiform or dermal neurofibromas, café-au-lait spots and peripheral nerve sheath tumors that are malignant in a minority of cases (51, 142-145). Mutated forms of the protein NF1 have also been implicated in murine and human cardiovascular disease, neural development, cognitive abnormalities, cell cycle maintenance, bone and muscular development (30, 31, 138, 146-153), and are associated with a greater risk of myeloid leukemia development (52-54, 154-156).

NF1 is expressed in T cells, although a role for this protein in T cells has yet to be established. *Nf1* mutations have been reported in a small number of patients with T-cell acute lymphoblastic leukemia in the absence of clinical NF1 symptoms (157) and NF1 has been reported to modulate ERK activation downstream of N-Ras in a murine lymphoma model (158), indicating that NF1 may act as a tumor suppressor in the T

cell compartment. One previous report has indicated that although lymphocyte development appeared to be unaffected, RAG-deficient donor mice that had received NF1-deficent fetal liver cells showed proliferation of peripheral CD4+ T cells in response to TCR stimulation (159). Similar experiments also demonstrated great increases in overall thymocyte and peripheral T cell numbers as a result of NF1-deficiency, also accompanied by a reduced ability to proliferate in response to stimuli even with concurrent increases in Ras-MAPK signaling, suggesting that NF1 acts to regulate steady-state proliferation in the T cell compartment (86). Further, several cases have been reported in which human NF1 patients were consequently diagnosed with the T cell-mediated autoimmune disorder multiple sclerosis, at rates higher than were expected given the statistics for the individual disorders (160-163).

In these studies, we investigate the role of NF1 specifically in the T cell compartment through use of a Cre-LoxP conditional knockout mouse model (1). We report that NF1 is non-redundant and haploinsufficient in T cells with regards to thymocyte development and peripheral T cell homeostasis. We further report that homozygous inactivation of *nf1* in T cells enhances infection clearance in a murine model of *Listeria monocytogenes* infection. The enhanced response to *L. monocytogenes* is likely due to increased expression of interferon-γ (IFN-γ) and the transcription factor Eomesodermin in naïve NF1-deficient $CD8^+$ T cells, the population in which pathogen-specific cells would exist prior to infection. However, the mechanisms behind the increased effector function in naïve $CD8^+$ T cells remain unclear.

2.3. Materials and Methods

Mice

nf1$^{fl/fl}$ mice on a C57BL/6 x 129 Sv background were crossbred with *plckcre* Tg mice (Taconic) (164) to generate T cell-specific NF1-deficient (T cell KO) mice and Cre-negative littermate controls. *nf1*$^{fl/fl}$*-plckcre* mice were bred to wild-type C57BL/6 mice (Jackson Labs) to generate mice that were heterozygous for the floxed *nf1* allele in the T cell compartment, with (T cell Het) and without Cre. PCR genotyping for the floxed *nf1* allele was done using a set of primers that distinguish between the wild-type, floxed, and recombined floxed alleles, as previously described (1). T cell-specific NF1-deficient TCR-transgenic mice were generated by crossbreeding *nf1*$^{fl/fl}$*-plckcre* mice to HY TCR Tg mice (Taconic). All animals were maintained in specific pathogen-free conditions at the University of Michigan Medical School unless otherwise specified. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Peripheral T cell survival assay

Pan-T cells were isolated from pooled spleen and lymph node suspensions using a MACS pan-T cell negative selection kit (Miltenyi). Purity of cells was routinely established at > 90% T cells via flow cytometry. Cells were plated at a concentration of 10^6 cells/ml in RPMI 1640 containing 10% FBS and antibiotics (RPMI-FBS) at a final volume of 1 ml/well of a 24-well plate, with the addition or not of 5 ng/ml

recombinant murine IL-7 (R&D Systems). Cell viability was analyzed using flow cytometry after 72 hours in culture.

Activation-induced cell death assay

Pan-T cells were isolated as described above. T cells were stimulated at a concentration of 10^6 cells/ml in a total volume of 1 ml per well in a 24-well plate that had been pre-coated with CD3 mAb (1 μg/ml; eBioScience). CD28 mAb (1 μg/ml; eBioScience) and recombinant murine IL-2 (100 ng/ml; R&D Systems) were added to all wells. T cells were harvested after 72 hours, washed twice, and replated in RPMI-FBS or RPMI with or without IL-2. Cell viability was analyzed by flow cytometry after 48 additional hours in culture.

Stimulation of peripheral T cells

Pan-T cells or $CD8^+$ T cell populations were isolated as described above. T cells were stimulated at a concentration of 10^6 cells/ml RPMI-FBS in a volume of 200 μl/well of a 96-well U-bottomed plate. For CD3/CD28 mAb stimulation, wells were pre-coated with CD3 mAb, and soluble CD28 mAb was included in the culture medium. Neutralizing anti-IL-2 mAb (BD Pharmingen) was added to wells at 1 μg/ml. Recombinant murine IL-2 was added to wells at 100 ng/ml. Cells were stimulated for 48 hours unless otherwise indicated.

HY TCR-tg T cells were stimulated at a concentration of 10^6 cells/ml RPMI-FBS at a volume of 200 μl/well of a 96-well U-bottomed plate. Irradiated adherent splenocytes

were used as antigen-presenting cells at a concentration of 10^6 per well. Cells were stimulated with 1 or 10 μg/ml HY peptide (New England Peptide) or not, or with CD3 and CD28 mAb as described previously.

Helper T cell differentiation

$CD4^+$ T cells were isolated from pooled spleen and lymph node suspensions using a MACS CD4+ T cell negative selection kit (Miltenyi). $CD4^+$ T cells were initially cultured at a density of 10^6 cells per ml RPMI-FBS in 96-well plates that had been pre-coated with CD3 mAb. Control cells were plated in RPMI + 10% FBS alone. All initial skewing conditions received CD28 mAb at a final concentration of 1 μg/ml. For Th0 skewing, 100 ng/ml IL-2 was added to the culture medium. For Th1 skewing, IL-2 plus 10 ng/ml IL-12 (R&D Systems) and 10 μg/ml IL-4 neutralizing mAb (BD Pharmingen) were added to the culture medium. For Th2 skewing, IL-2 plus 10 ng/ml IL-4 (R&D Systems) and 10 μg/ml IFN-γ neutralizing mAb (BD Biosciences) were added to the culture medium. For Th17 skewing, IL-2 and neutralizing mAb against IFN-γ and IL-4 as well as TGF-β at 5 ng/ml, IL-6 at 10 ng/ml, and IL-23 at 10 ng/ml (R&D Systems) were added to the culture medium.

At 72 hours, cells in each well were transferred to a fresh well in a 48-well plate. Th0, Th1, and Th2 cells had 100 ng/ml IL-2 added to the culture medium for a final volume of 600 μl. Th17 cells had 50 ng/ml IL-2, 5 ng/ml each IL-6 and IL-23, and 5 ng/ml each of neutralizing IFN-γ and IL-4 mAb added to the culture medium for a final volume of 500 μl. Cells were incubated in these conditions for 48 hours.

Cells were restimulated at a concentration of 10^6 cells/ml in 96-well plates that had been pre-coated with anti-CD3. All conditions were plated in triplicate. Supernatants were removed from cultures 24 hours later and were analyzed by ELISA.

Generation of CD4+ and CD8+ T cell blasts

Freshly isolated $CD8^+$ or naïve $CD4^+$ T cells from NF1 KO and control littermate mice were cultured in RPMI-FBS in 24-well plates that had been previously coated with CD3 mAb. 1 μg/ml CD28 mAb and 100 ng/ml IL-2 were added to the culture medium. Cells were initially plated at a volume of 1 ml per well and a density of $2x10^6$ cells/ml. After 72 hours ($CD8^+$ T cells) or 96 hours (naïve $CD4^+$ T cells), cells from each well were transferred to wells of a fresh 6-well plate. RPMI-FBS plus 100 ng/ml IL-2 was added to each well to a final volume of 5 ml per well. After 48 hours, cells were harvested, washed 1X in fresh RPMI, and plated in fresh 6-well plates or T-75 flasks at a density of $1\text{-}1.5x10^6$/ml in RPMI+10% FCS. Cells were harvested the following day for use in functional assays.

Flow cytometry

Single-cell suspensions of freshly isolated thymocytes, spleens and lymph nodes were analyzed for cell surface expression of CD4, CD8 and CD44 by flow cytometry using fluorochrome-labeled mAb (BD Pharmingen). Thymocytes were further analyzed for surface expression of CD25 and T3.70 (HY TCR) using fluorochrome-labeled mAb (BD Pharmingen and eBioscience). For proliferation experiments, cells were labeled with CFSE prior to stimulation or not as indicated above. For survival and cell death

experiments, unfixed cells were labeled or not with fluorochrome-labeled Annexin-V and 7-AAD for 10 minutes prior to analysis. For intracellular staining, cells were treated with PMA and ionomycin (50 ng/ml and 1.5 μM respectively; Sigma Aldrich) for 5 h with addition of brefeldin A (1:1000 dilution of stock; BD Biosciences) for the last 4 h of culture. Cells were surface stained with mAb against CD4, CD8 and CD44 prior to fixation and permeabilization and staining for intracellular expression of IFN-γ, Eomes and T-bet (eBioscience except IFN-γ, which was purchased from BD Pharmingen). Cellular data were collected on a FACSCanto flow cytometer equipped with FACSDiva software (BD Biosciences).

ELISA

Concentrations of IFN-γ in pan-T or $CD8^+$ T cell culture supernatants were determined using a Duo-set ELISA kit (R&D Systems) according to manufacturer specifications. Concentrations of IFN-γ, IL-4 and IL-17 in $CD4^+$ Th culture supernatants were determined with the use of Duo-set ELISA kits (R&D Systems).

In-vitro signaling and immunoblotting

For TCR signaling experiments, $5x10^5$ to 10^6 $CD4^+$ or $CD8^+$ T cells were treated as follows: CD3/CD28-stimulated cells were resuspended in RPMI containing 5 μg/ml anti-mouse CD3 and CD28 mAbs (eBioscience) and incubated for 20 minutes on ice. Cells reserved for the "zero" timepoint were incubated in RPMI without antibodies for 20 minutes on ice. 10 μl of RPMI containing 50 μg/ml goat anti-Armenian hamster IgG (Jackson Immunolabs) was then added to all tubes to crosslink the bound

primary antibodies. All cells were incubated in a 37 C water bath for the indicated timepoints. Cells reserved for the "zero" timepoint were incubated at 37 C for 2 or 5 minutes. Cells were chilled on ice, pelleted briefly, and supernatants were removed and replaced with 1x SDS-PAGE sample buffer containing protease inhibitor cocktail (Roche) and sodium orthovanadate. All samples were boiled for at least 10 minutes prior to separation on 10% acrylamide/bis tris-glycine SDS-PAGE gels followed by transfer to PVDF membranes. Blots were probed with antibodies against phospho-ERK1/2, total ERK1/2, phospho-Akt, total Akt, phospho-p70S6K, and total p70S6K (Cell Signaling).

Listeria monocytogenes infection

nf1$^{fl/fl}$*-plckcre* and Cre-negative littermate controls were injected intraperitoneally with 5x10^5 *L. monocytogenes* CFU as previously described (165). Five days post-infection, spleens and livers were harvested and homogenized. Organ homogenates were plated via serial dilution on LB-agar and were incubated for 24 hours prior to counting formed colonies.

2.4. Results

Generation of T cell-specific NF1-deficient mice

In order to investigate the effects of NF1 loss in T cells, we needed to circumvent the issue of embryonic lethality that was reported to occur in mice that were homozygous for a null allele of *nf1* (30, 31). We accomplished this through the use of Cre-LoxP conditional knockout technology. Mice bearing a flanked-by-LoxP (floxed) allele of *nf1*, the gene that codes for the protein NF1, were previously generated (1). In the targeted allele, exons 31 and 32 of *nf1* are floxed (Fig. 2.1). Exon 31 had been chosen for deletion in the design of both *nf1* null alleles that previously led to embryonic lethality as it is a site of observed mutations in three human families affected by the disorder NF1 (30, 31, 166), and for this reason it was chosen for recombination in the floxed allele. The non-recombined floxed allele of *nf1* has been demonstrated to act indistinguishably from wild-type alleles, and the recombined floxed allele acts as a null allele, indicating that this is an excellent model with which to circumvent embryonic lethality and study gene and protein deletion in adult mice (1).

To recombine *nf1* specifically in the T cell compartment, $nf1^{fl/fl}$ mice were crossed with mice bearing the Cre transgene under the control of the proximal Lck promoter (*plckcre)* to generate $nf1^{fl/fl}$*-plckcre* (T cell KO) mice and Cre-negative littermate controls. The proximal Lck promoter induces expression exclusively in the T cell compartment at the DN3 stage of thymocyte development, allowing for gene inactivation prior to thymic selection and maturation events (164). Due to technical issues, rather than assessing protein deletion by western blotting, we chose to analyze

gene recombination using PCR as the recombined allele had been previously established to act as a null allele (1). As seen in Figure 4, expression of the *plckcre* transgene consistently results in complete recombination of the floxed *nf1* locus in both thymocytes and peripheral T cells. Low expression of the non-recombined floxed allele that is observed in peripheral T cell PCR samples is likely from residual B cells or myeloid cells that are present due to imperfect magnetic cell sorting, as thymocyte samples from the same mice show complete recombination of the floxed *nf1* allele (Fig. 4). We are confident that the experiments described herein are an analysis of full deletion of NF1 in the T cell compartment.

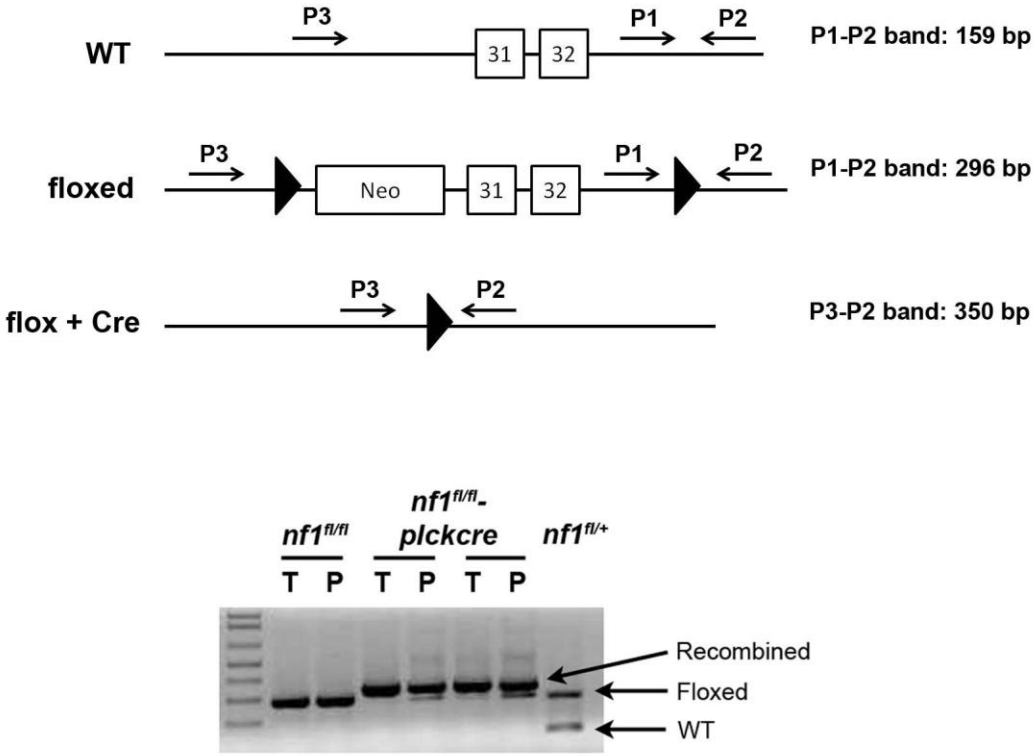

Figure 4. Genotyping of T cell-specific NF1-deficient mice.

Conditional deletion of NF1 was achieved using previously generated mice that bear flanked-by-loxP (floxed) alleles of *nf1*. Top, diagram showing the position of genotyping primers (P1, P2, P3) on the *nf1* locus. LoxP sites (filled triangles) surround exons 31 and 32, which were previously described to be essential to NF1 function (1). Bottom, T cell-specific deletion of NF1 was achieved via breeding of $nf1^{fl/fl}$ mice with *plckcre* transgenic mice. Recombination of the floxed *nf1* allele in thymocyte (T) and peripheral T cell (P) compartments for one control and two T cell KO mice were confirmed by PCR. Data are representative of multiple experiments.

Thymic cellularity in T cell-specific NF1-deficient mice

Upon generation of *nf1*$^{fl/fl}$*-plckcre* (T cell KO) mice and appropriate Cre-negative littermate controls, we first investigated cellularity within the thymus to determine the effects, if any, of NF1 loss on thymocyte development. ERK activation is well established as a dominant factor in thymic selection (63), so we thought it possible that perturbation of Ras-ERK signaling via deletion of NF1 could affect selection dynamics as well as thymocyte numbers and subset frequencies. Flow cytometric analysis of thymocytes from T cell KO and control mice demonstrated that gross thymocyte development is moderately affected by NF1 deficiency (Fig. 5A). T cell KO mice had significantly fewer CD4 and CD8 single positive (SP) thymocytes, both in frequency of total thymocytes as well as in absolute numbers. A slight but significant difference in DP numbers did not translate into a significant difference in the frequency of DP cells. Conversely, although DN cell numbers were not significantly different, T cell KO mice had a slight but significant increase in DN cell frequency. Analysis of the developmental subsets within DN cells revealed that the frequency of DN3 cells was significantly increased in NF1 KO mice, along with a corresponding decrease in the frequency of DN4 cells (Fig. 5B), indicating a potential block in the transition from DN3 to DN4. Analysis of absolute cell numbers within the DN population showed a significant increase in T cell KO mice in the DN3 subset (Fig. 5B).

We also investigated thymic cellularity in *nf1*$^{fl/+}$*-plckcre* (T cell Het) and littermate control mice. Mutation of a single allele of *nf1* has been shown to be causative in the

disorder NF1, and the potential effects of heterozygosity on thymocyte development had not been explored in detail (50, 51, 86). We found that T cell Het thymocytes showed slight but significant increases in both the frequency and total cell number in the DN thymic subset (Fig. 6A). Contrary to our observations in T cell KO mice (Fig. 6A), we observed no other significant changes in other thymocyte populations in T cell Het mice. Further analysis of the DN population indicated that the frequencies of DN2 and DN3 cells were significantly increased in NF1 het mice, along with a significant decrease in the frequency of T cell Het DN 4 cells (Fig. 6B). Analysis of DN cell numbers show that the absolute numbers of all DN cell populations are significantly increased in T cell Het mice (Fig. 6B). We note that the increases in DN3 cells are similar in mice with homozygous and heterozygous deletions of NF1, suggesting that NF1 is haploinsufficient with regards to thymocyte development.

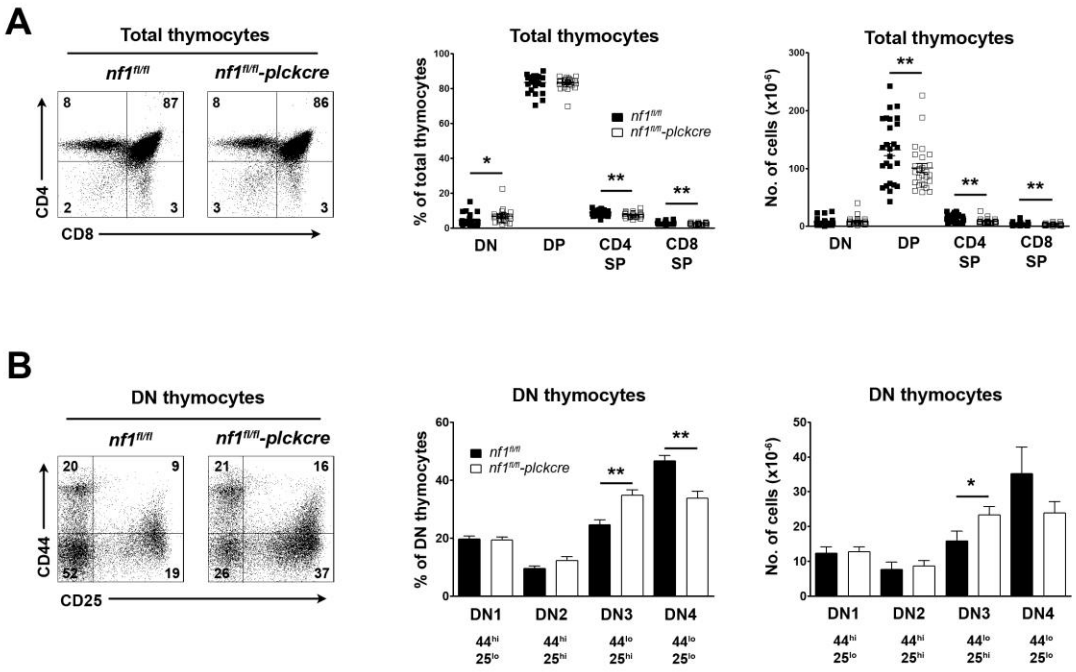

Figure 5. Thymic cellularity in T cell-specific NF1-deficient mice.

A, Thymocytes from T cell KO and control mice were analyzed by flow cytometry. Left, representative CD4 vs CD8 flow cytometry plots to demonstrate population analysis. Numbers in dot plot corners represent population frequency. Right, graphical analysis of thymic subset populations. B, Developmental subsets of DN thymocytes from T cell KO and control mice were analyzed by flow cytometry. Left, representative CD44 vs. CD25 flow cytometry plots. Right, graphical representation of DN subset frequencies. All graphs show the mean + SEM. n = 26 for control and T cell KO mice (A), n = 22 for control and T cell KO mice (B). Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$, **$p < 0.01$.

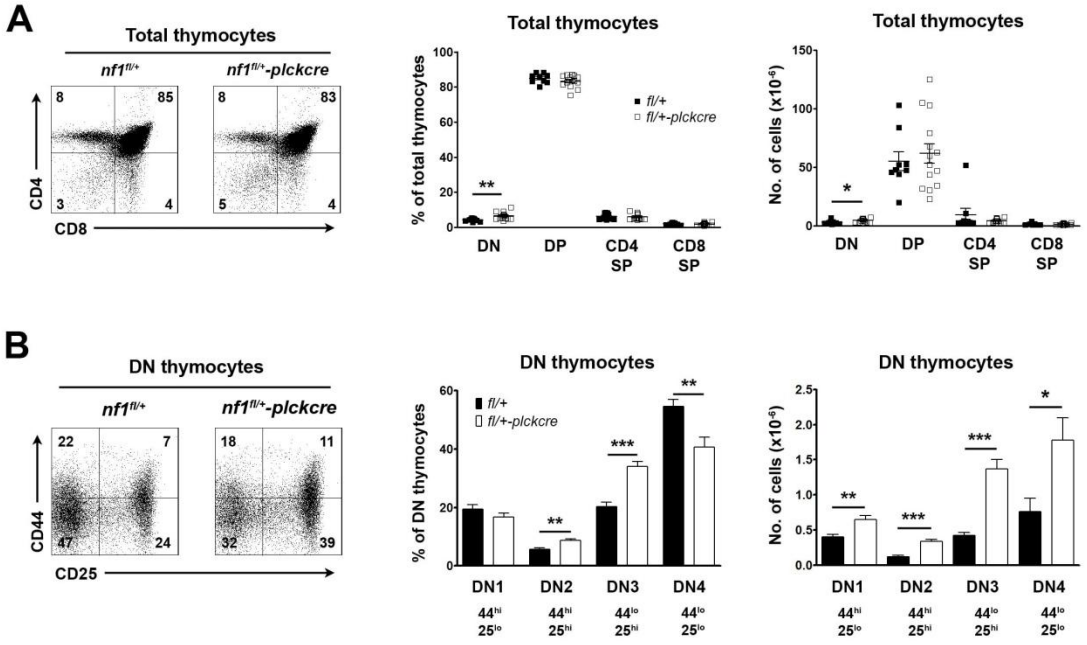

Figure 6. Thymic cellularity in T cell-specific NF1-heterozygous mice.

A, Thymocytes from T cell Het and control mice were analyzed by flow cytometry. Left, representative CD4 vs. CD8 flow cytometry dot plots to demonstrate population analysis. Numbers in dot plot corners represent population frequency. Right, graphical analysis of thymic subset populations. B, Developmental subsets of DN thymocytes from T cell Het and control mice were analyzed by flow cytometry. Left, representative CD44 vs. CD25 flow cytometry plots. Right, graphical representation of DN subset frequencies. All graphs show the mean + SEM. n = 9 for control mice and 14 for T cell Het mice (A), and n = 7 for control mice and 10 for T cell Het mice (B). Statistical significance was determined using the unpaired one-tailed Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Peripheral cellularity in T cell-specific NF1-deficient mice

Loss of NF1 in thymocyte precursor cells has been shown previously to result in increased numbers of peripheral T cells in murine adoptive transfer models (86). Unexpectedly, we observed modest but significant decreases in CD4 and CD8 SP cells in T cell KO mice (Fig. 5A). To determine if peripheral T cell populations were similarly affected by the loss of NF1, we investigated the cellularity of splenocytes and lymph node cells from T cell KO and littermate control mice. Flow cytometric analysis showed striking and statistically significant decreases in $CD4^+$ and $CD8^+$ T cell numbers and frequencies in both spleen and lymph node cell populations (Fig. 7A). We initially observed higher frequencies of $CD44^{hi}$ T cells in the peripheral $CD4^+$ and $CD8^+$ compartments in T cell KO mice (Fig. 7B). However, analysis of cell numbers within the $CD4^+$ and $CD8^+$ T cell subsets indicated to us that in fact, the numbers of $CD44^{hi}$ T cells were unchanged in NF1 KO mice. On the contrary, the increased frequencies of $CD44^{hi}$ cells in these mice were due to large, statistically significant decreases in $CD44^{lo}$ naïve T cells in both the $CD4^+$ and $CD8^+$ compartments (Fig. 7B). Analysis of T cell Het mice demonstrated a similar significant reduction in peripheral T cell numbers (Fig. 8A). We further observed that the reductions in T cell numbers in NF1 Het mice were due to significant decreases in $CD44^{lo}$ naïve T cells specifically, similar to what we had observed in T cell KO mice (Fig. 8B).

Diminished numbers of peripheral T cells in T cell KO mice, especially in the naïve cell compartments, could be attributed to a reduced ability of these cells to respond to

pro-survival signals in the periphery, and thus to reduced survival. IL-7 is well-known to be an essential cytokine for the promotion of naïve and memory T cell survival in the periphery (167). To determine if NF1-deficiency resulted in a reduced ability of cells to respond positively to IL-7, we cultured pan-T cells from T cell KO and control mice with IL-7 or not. After 72 hours, we assessed cell viability using flow cytometry. We observed no differences in pan-T cell survival between T cell KO and control cells (Fig. 9, left). Further analysis of $CD4^+$ and $CD8^+$ naïve and memory-like T cell subsets demonstrated that, again, there were no differences in IL-7-mediated survival between T cell KO and control T cells (Fig. 9, right).

We also wished to determine whether NF1-deficient T cells were more susceptible to activation-induced cell death (AICD). Following an adaptive immune response, the majority of active Ag-specific T cells undergo apoptosis upon the withdrawal of IL-2 (79). We considered the possibility that T cell KO T cells might respond abnormally to homeostatic conditions and thus might be more susceptible to AICD in resting conditions. Increased AICD might account for the reduced numbers of peripheral naïve T cells in T cell KO mice. To determine this, we cultured pan-T cells with CD3/CD28 mAb in the presence of IL-2 for 72 hours. We then replated the cells in medium with or without FBS, IL-2, or both, and analyzed cell viability after a further 48 hours of culture. We observed little difference in cell viability between T cell KO and control T cells in any of the conditions studied (Fig. 10). Thus, we conclude that NF1 is likely not a major factor in the contraction of the T cell-mediated adaptive immune response.

Peripheral T cells undergo proliferation to increase the number of T cells and maintain homeostasis (74). Ras signaling, Erk2 activation in particular, has been reported as an important regulator of $CD8^+$ T cell proliferation (64). However, the reduced numbers of peripheral naïve T cells in T cell KO mice indicated that perhaps homeostatic proliferation was defective. We assessed the proliferative capabilities of NF1 KO T cells *in vitro*. Analysis of CFSE-labeled pan-T cells from NF1 KO and control mice that had been stimulated with CD3/CD28 mAb for 72 hours revealed no apparent differences in $CD4^+$ or $CD8^+$ T cell proliferative responses between NF1 KO and control T cells (Fig. 11).

We expected that the effects of NF1, as a negative regulator of Ras, might be best observed in the cellular signaling pathways that are downstream of Ras activation. Ras activation is well-known to upregulate activation of the protein ERK, a member of the MAPK family (8). As Ras had been previously shown to be hyperactivated in T cells that had developed from NF1-deficient bone marrow (86), we expected that this may be true in T cells from our T cell-specific NF1-deficient mice. To investigate potential Ras-ERK signaling irregularities, we isolated $CD4^+$ or $CD8^+$ T cells from the spleens and lymph nodes of naïve mice and stimulated them in vitro for a short time course with anti-CD3 and anti-CD28, followed by lysis and Western blotting. However, we observed no increase in Ras-ERK activation in NF1-deficient T cells as measured by ERK phosphorylation (Fig. 12). We next analyzed activation of Akt in $CD8^+$ T cells, as Ras signaling can promote Akt phosphorylation by activation of PI3K (9). We also analyzed phosphorylation of the mTOR1 pathway component

p70S6 kinase, as mTOR is known to be activated downstream of Akt (10). Analysis of Akt phosphorylation in these cells as well as that of the mTOR pathway component p70S6K similarly failed to show evidence of increased Ras signaling in the absence of NF1 expression (Fig. 12). Thus, the mechanisms that underlie the reduction in peripheral naïve T cell numbers in T cell KO mice remain unclear.

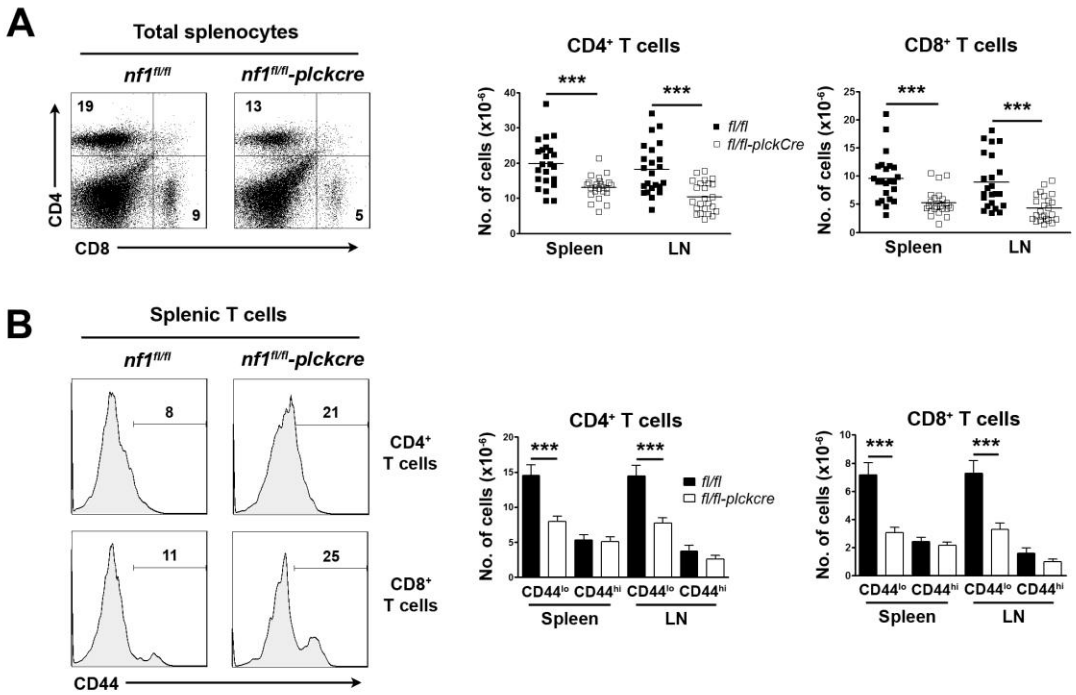

Figure 7. Peripheral cellularity in T cell-specific NF1-deficient mice.

A, peripheral T cell populations from T cell KO and control mice were analyzed by flow cytometry. Left, representative CD4 vs. CD8 dotplot to demonstrate gating. Numbers in dot plot corners represent population frequency. Right, graphical representation of CD4+ and CD8+ T cell populations in the spleen and lymph nodes. B, CD4+ and CD8+ T cells from T cell KO and control mice were analyzed by flow cytometry for the presence of the memory marker CD44. Left, representative histograms demonstrating the frequency of $CD44^{hi}$ cells in splenic T cells. Right, enumeration of $CD44^{lo}$ and $CD44^{hi}$ subsets within peripheral T cell populations. All graphs show the mean + SEM. n = at least 21 for control and 22 for NF1 KO mice. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. ***$p < 0.001$.

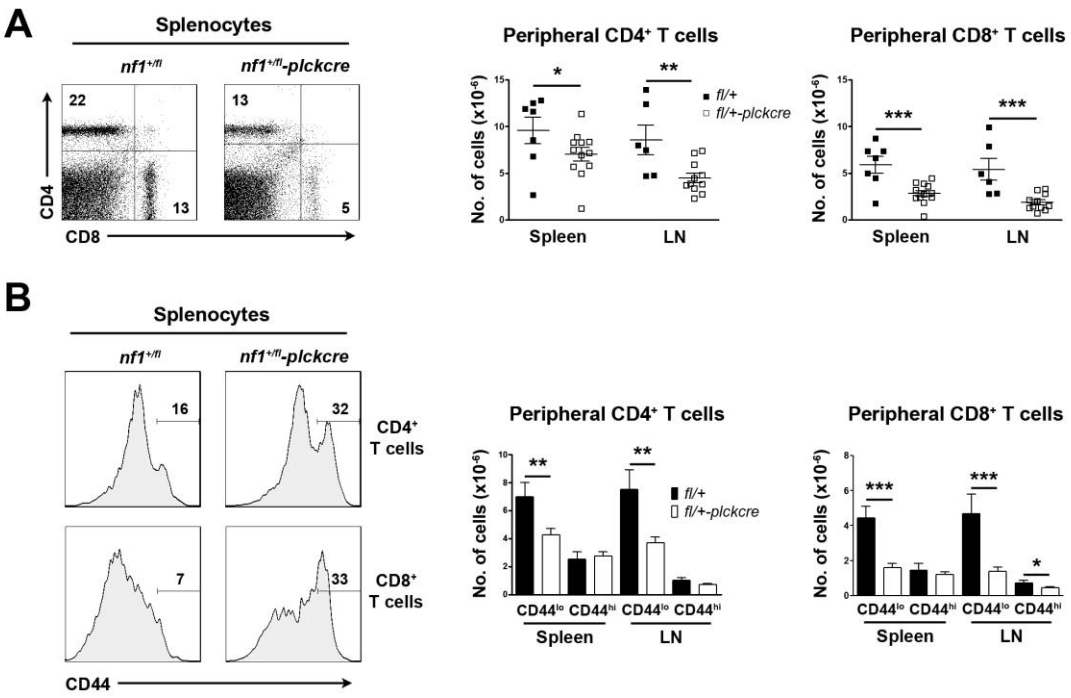

Figure 8. Peripheral cellularity in T cell-specific NF1-heterozygous mice.

A, peripheral T cell populations from NF1 Het and control mice were analyzed by flow cytometry. Left, representative CD4 vs. CD8 dotplot to demonstrate gating. Numbers in dot plot corners represent population frequency. Right, graphical representation of CD4+ and CD8+ T cell populations in the spleen and lymph nodes. B, CD4+ and CD8+ T cells from NF1 Het and control mice were analyzed by flow cytometry for the presence of the memory marker CD44. Left, representative histograms demonstrating the frequency of CD44hi cells in splenic T cells. Right, enumeration of CD44 lo and CD44hi subsets within peripheral T cell populations. All graphs show the mean + SEM. n = at least 6 for control and 10 for NF1 het mice. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

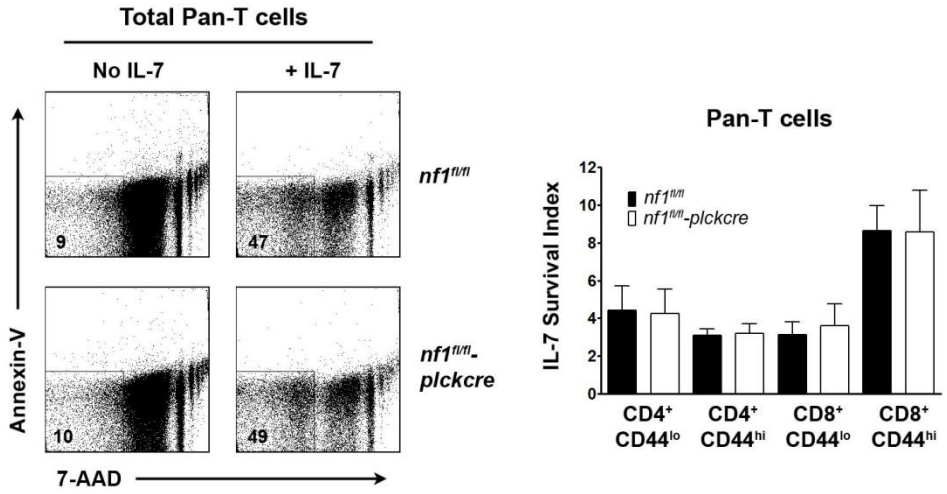

Figure 9. NF1-deficient peripheral T cells do not respond abnormally to IL-7.

Left, representative dot plots of total pan-T cells from T cell KO and control mice. Cells were treated with or without IL-7 for 72 hours prior to analysis of cell viability. Right, IL-7 survival index was determined as the ratio of Annexin-V/7-AAD double-negative T cells in IL-7-treated cells vs. untreated cells. Ratios were determined separately for each of the indicated peripheral T cell subsets. The graph shows the mean + SEM. n = 3 mice for each genotype.

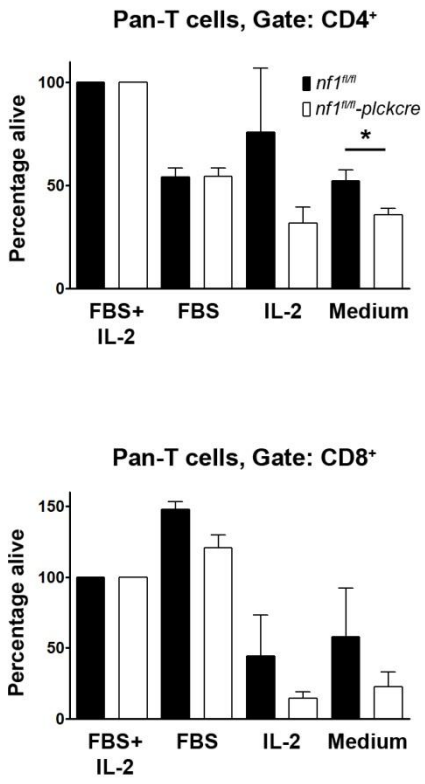

Figure 10. NF1-deficiency does not affect activation-induced T cell death (AICD) in CD8+ T cells.

Freshly isolated T cell KO and control pan-T cells were stimulated with CD3 and CD28 mAb and IL-2 for 72 hours prior to 48 hours of culture in the indicated conditions. Cell viability was assessed using Annexin-V and 7-7AAD staining as described in Figure 9. Cell viability is represented as a percentage of viable FCS+IL-2-treated cells. All graphs show the mean + SEM. n = at least 3 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. * $p < 0.05$.

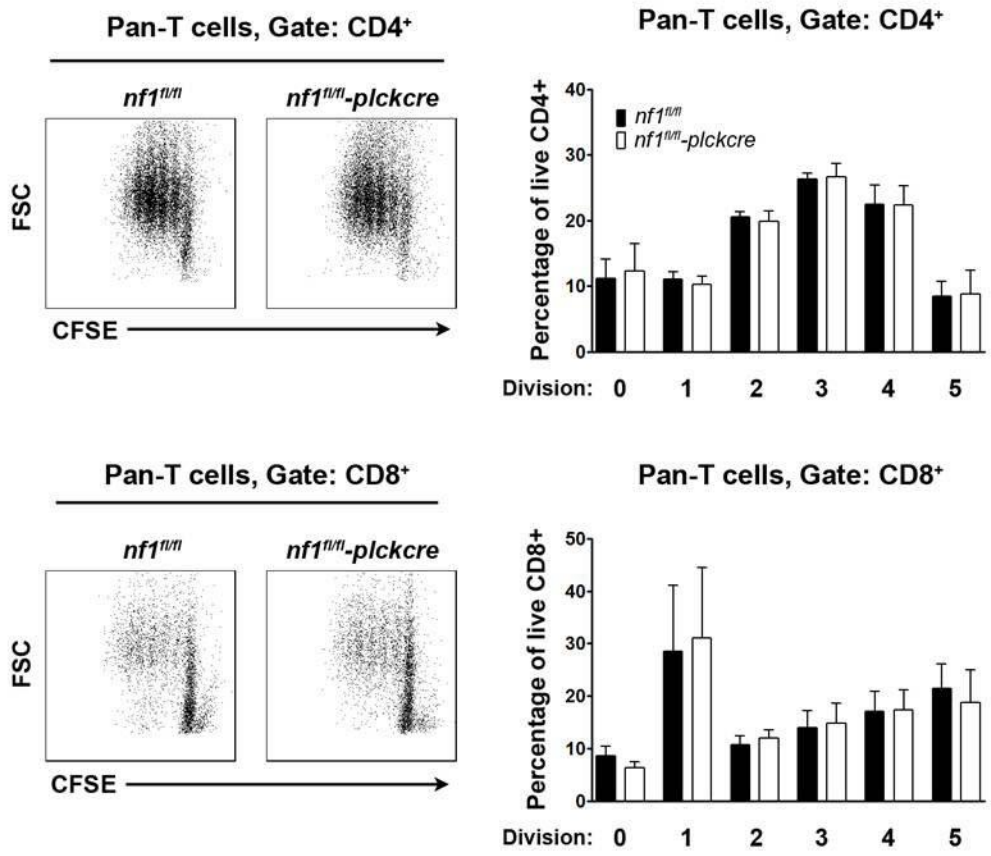

Figure 11. NF1-deficiency does not affect activation-induced T cell proliferation.

Freshly isolated T cell KO and control pan-T cells were labeled with CFSE prior to stimulation with CD3 and CD28 mAb. Cell division was analyzed by flow cytometry following 72 hours of culture. Left, representative CFSE vs. forward scatter dot plots of CD4+ and CD8+ T cells from T cell KO and control mice. Right, cell divisions represented as a percentage of total CD4+ and CD8+ T cells. All graphs show the mean + SEM. n = 4 mice per genotype.

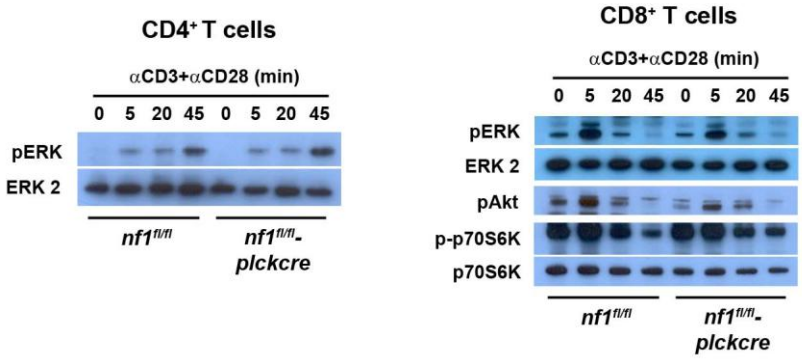

Figure 12. NF1-deficiency does not yield increased Ras signaling in freshly isolated T cells.

Freshly isolated T cell KO and control CD4+ or CD8+ T cells were stimulated with anti-CD3 and anti-CD28 in vitro for the indicated times. Cells were lysed and proteins were separated using SDS-PAGE. Phosphorylation of ERK, Akt, and p70S6K were detected via Western blotting of proteins that had been transferred to PVDF membrane. Data are representative of multiple experiments.

Analysis of CD8+ T cell effector function in NF1-deficient T cells

T cells are critical to the development of adaptive immunity against pathogens, to the extent that humans and mice with severe T cell deficiencies have a very high mortality rate early in life (87, 88). Thus, as our NF1-deficient mice possessed significantly reduced numbers of peripheral naïve T cells, we hypothesized that these mice might have a reduced ability to mount a T cell-mediated response against a pathogen, as pathogen-specific T cells would reside within the naïve compartment. We chose *Listeria monocytogenes* as it is an intracellular pathogen and the host must be able to induce activation of $CD8^+$ T cells to clear the infection fully and efficiently (168). We injected T cell KO and control mice intraperitoneally with *L. monocytogenes* and monitored the mice for five days to allow for initiation of an adaptive immune response. We then harvested the spleens and cultured spleen homogenates to determine the number of remaining *L. monocytogenes* colony-forming units (CFU). Unexpectedly, we determined that NF1 KO mice were better able to clear *L. monocytogenes* as determined by CFU counts (Fig. 13).

Although $CD8^+$ T cells are the primary effector cells in the host response to *L. monocytogenes*, $CD8^+$ T cells do require help from $CD4^+$ T cells, primarily in the form of IL-2, during the course of initial activation in the lymph nodes (112). We considered the possibility that increased cytokine production from NF1-deficient $CD4^+$ T cells could promote $CD8^+$ T cell activation and effector function and that we may be able to more easily observe such changes *in vitro.* We cultured freshly isolated pan-T cells from T cell KO and control mice with or without anti-CD3 and

anti-CD28 (CD3/CD28) for 48 hours. We analyzed the cells for the expression of IL-2 as well as the effector cytokine IFN-γ by intracellular cytokine staining and flow cytometry. We found that T cell KO $CD4^+$ T cells did not differ significantly from control cells in cytokine expression when stimulated with CD3/CD28 (Fig. 14). In CD3/CD28-stimulated T cell KO $CD8^+$ T cells, we noted a significant increase in the expression of both IL-2 and IFN-γ in the naïve cell compartment only (Fig. 14). Analysis of cytokine expression in T cell Het and control pan-T cell cultures demonstrated no increase in T cell Het $CD8^+$ IFN-γ expression (Fig. 15).

We next examined the expression of IFN-γ in stimulated $CD8^+$ T cell cultures. We stimulated $CD8^+$ T cells with CD3/CD28 alone or in the presence of IL-2, to mimic CD4+ T cell help, or IL-4, a $CD4^+$ T cell produced cytokine that shares a receptor subunit with IL-2. We then determined the frequency of IFN-γ-positive cells using intracellular cytokine staining and flow cytometry. We observed no significant changes between T cell KO and control cells in the frequencies of IFN-γ-positive cells in $CD44^{hi}$ $CD8^+$ T cells (Fig. 16A). However, we did find significantly increased frequencies of IFN-γ-positive cells in T cell KO $CD44^{lo}$ $CD8^+$ T cells that were stimulated with CD3/CD28 alone (Fig. 16A). We also analyzed levels of secreted IFN-γ in supernatants from the stimulated T cell KO and control $CD8^+$ T cell cultures that were described in Figure 16A. Using ELISA, we determined that T cell KO cells did secrete significantly higher levels of IFN-γ in response to all stimuli (Fig. 16B). Calculation of the ratio of T cell KO over control IFN-γ secretion demonstrated the greatest fold difference in cytokine secretion in the cells treated with only

CD3/CD28; addition of cytokine stimulation reduces the difference in cytokine secretion between T cell KO and control cells (Fig. 16B). A caveat is that the increased frequency of $CD44^{hi}$ T cells in T cell KO mice makes it impossible to determine whether the increased IFN-γ secretion is due to CD44hi cells or to increased activity by T cell KO naïve $CD8^{+}$ T cells (Fig. 16A).

The transcription factors Eomesodermin (Eomes) and T-bet are critical determinants in $CD8^{+}$ T cell effector and memory fate decisions (114, 116). We were curious whether increased expression of Eomes and T-bet could account for the increased expression of IFN-γ in naïve T cell KO $CD8^{+}$ cells (Fig. 16A). We used intracellular staining and flow cytometry to determine the frequency of expression of Eomes and T-bet in $CD8^{+}$ T cells that had been stimulated with CD3/CD28 in the presence or absence of IL-2 or IL-4. Similar to our observations with IFN-γ expression, we observed significant increases in Eomes expression following stimulation only in T cell KO naïve $CD8^{+}$ T cells, although we did note increased basal levels of Eomes expression in $CD44^{hi}$ T cell KO $CD8^{+}$ cells (Fig. 17). T-bet expression was significantly increased only in T cell KO $CD44^{lo}$ cells following stimulation (Fig. 18).

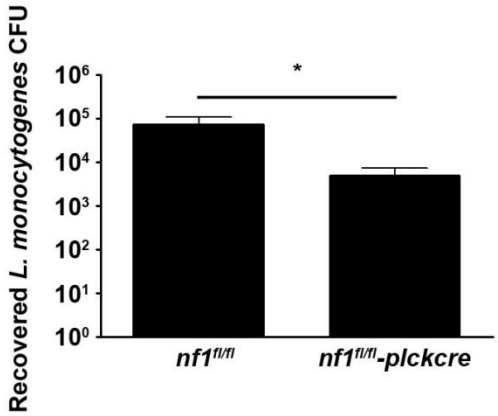

Figure 13. Response to *Listeria monocytogenes* in NF1-deficient mice.

NF1 KO and control mice were injected intraperitoneally with $5x10^4$ colony-forming units (CFU) of *L. monocytogenes*. Mice were sacrificed five days later, spleens were harvested, and spleen homogenates were incubated on LB-agar. CFU were counted 24 hours later. N = 5 for control and NF1 KO mice. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. $*p < 0.05$.

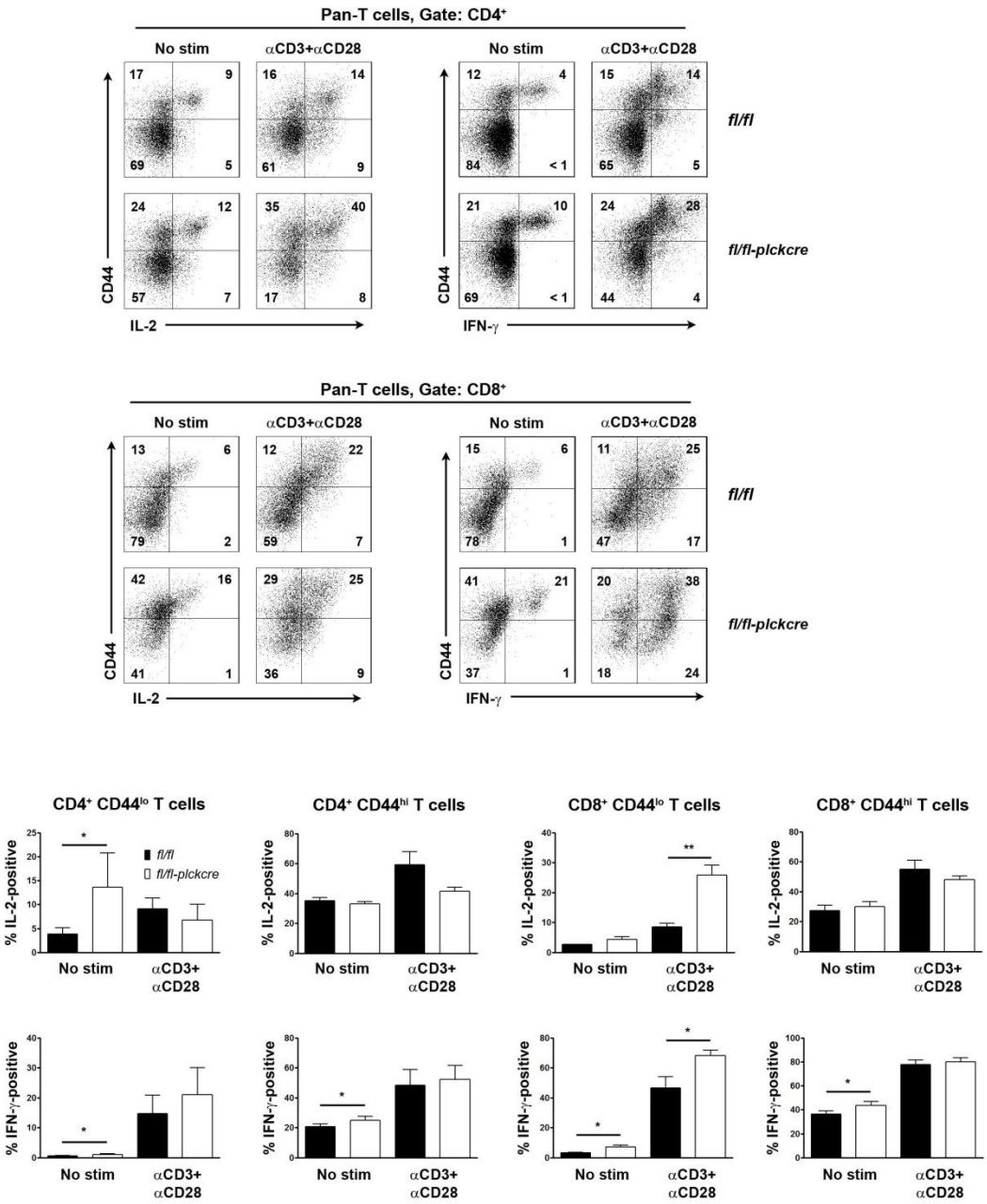

Figure 14. Analysis of NF1-deficient T cell cytokine production.

Freshly isolated T cell KO and control pan-T cells were cultured with CD3 and CD28 mAb or not for 48 hours prior to restimulation with PMA and calcium ionophores. IL-2 and IFN-γ cytokine production were determined using flow cytometry. Top, representative flow plots of T cell KO and control CD4+ and CD8+ T cells from pan-T cell cultures. Bottom, CD4+ and CD8+ T cell subsets represented as percent cytokine-positive cells of total $CD44^{lo}$ and $CD44^{hi}$ cells. All graphs show mean + SEM. n = at least three mice per genotype. Statistical significance was determined using the unpaired two-tailed Student's *t*-test. $*p < 0.05$, $**p < 0.01$.

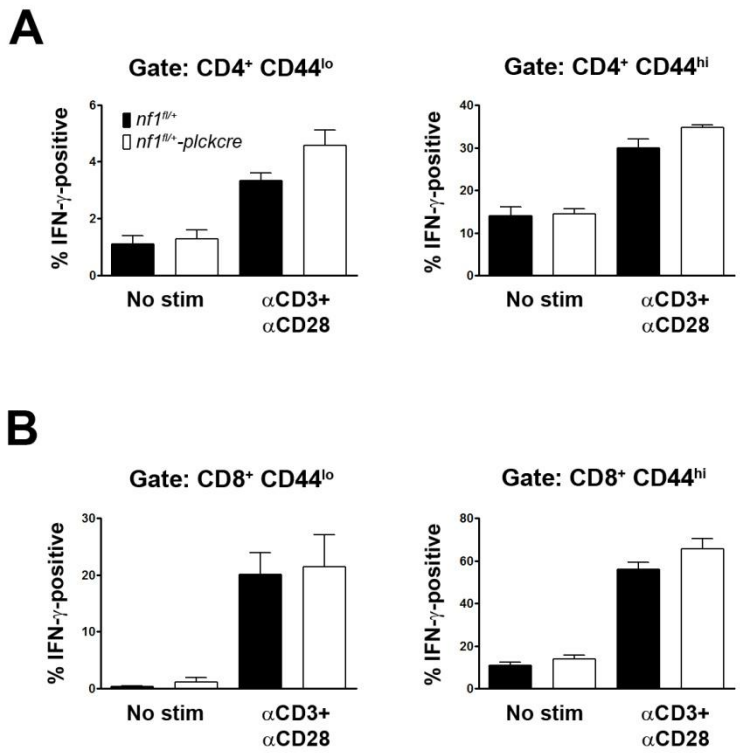

Figure 15. NF1-heterozygosity does not affect T cell effector function.

Pan-T cells from T cell KO and control mice were stimulated or not with CD3 and CD28 mAb for 48 hours. IFN-γ expression in CD4+ (A) and CD8+ T cells (B) was determined by flow cytometry. Graphs show the mean + SEM. n = 4 for each genotype.

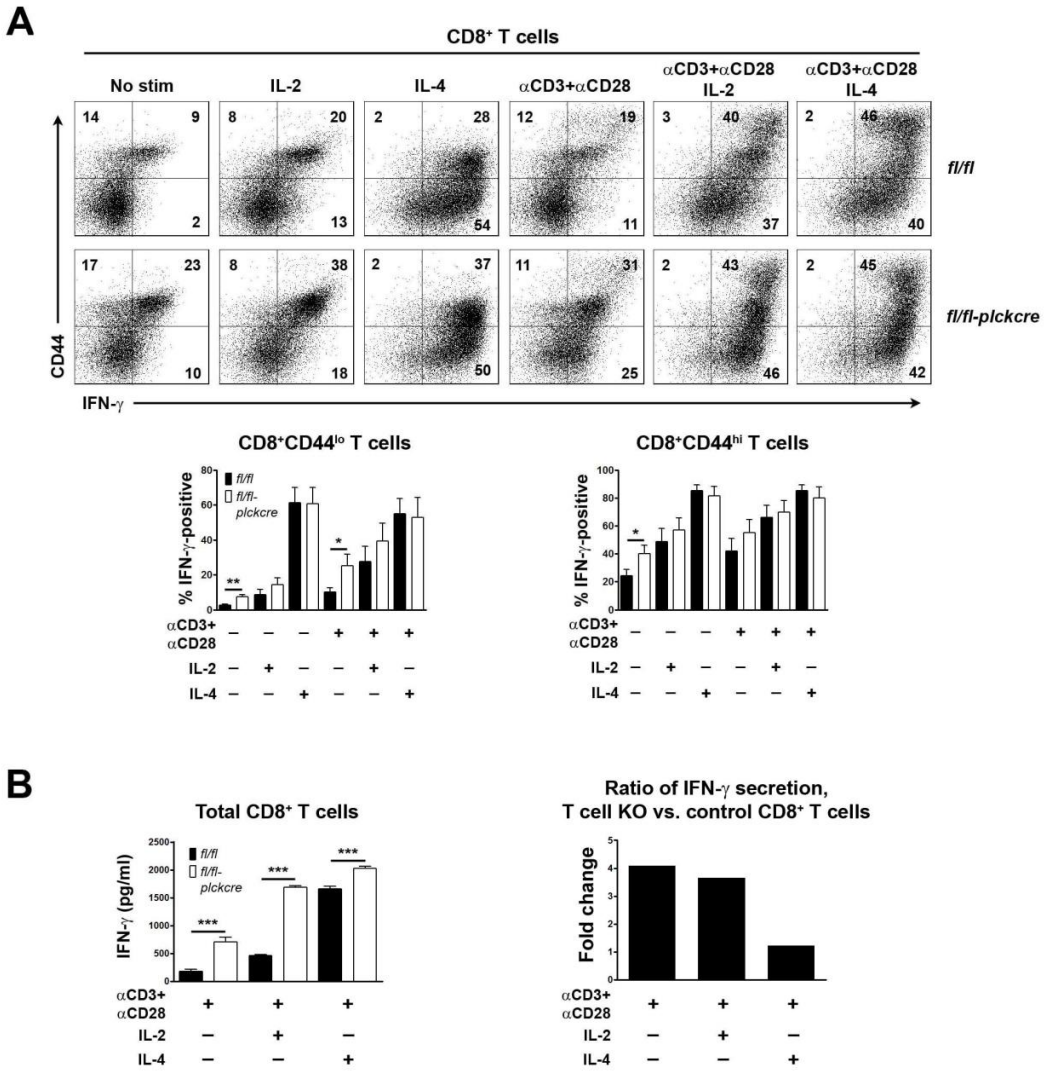

Figure 16. NF1-deficiency promotes naive CD8+ T cell effector function on a per-cell basis.

A, freshly isolated peripheral CD8+ T cells from T cell KO and control mice were stimulated for 48 hours with CD3 and CD28 mAb or not, in the presence or absence of rIL-2 or rIL-4, followed by restimulation with PMA and ionomycin. Cellular IFN-γ expression was determined by flow cytometry. Top, representative dotplots of CD44 vs. IFN-γ expression. Bottom, IFN-γ-positive cells expressed as a percentage of total CD44lo or CD44hi cells. B, CD8+ T cells were stimulated as in (A). Supernatants from these cultures were analyzed for IFN-γ secretion using ELISA. Left, comparison of IFN-γ secretion. Right, ratios of KO IFN-γ secretion to WT IFN-γ secretion. All graphs show mean + SEM. n = at least three mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

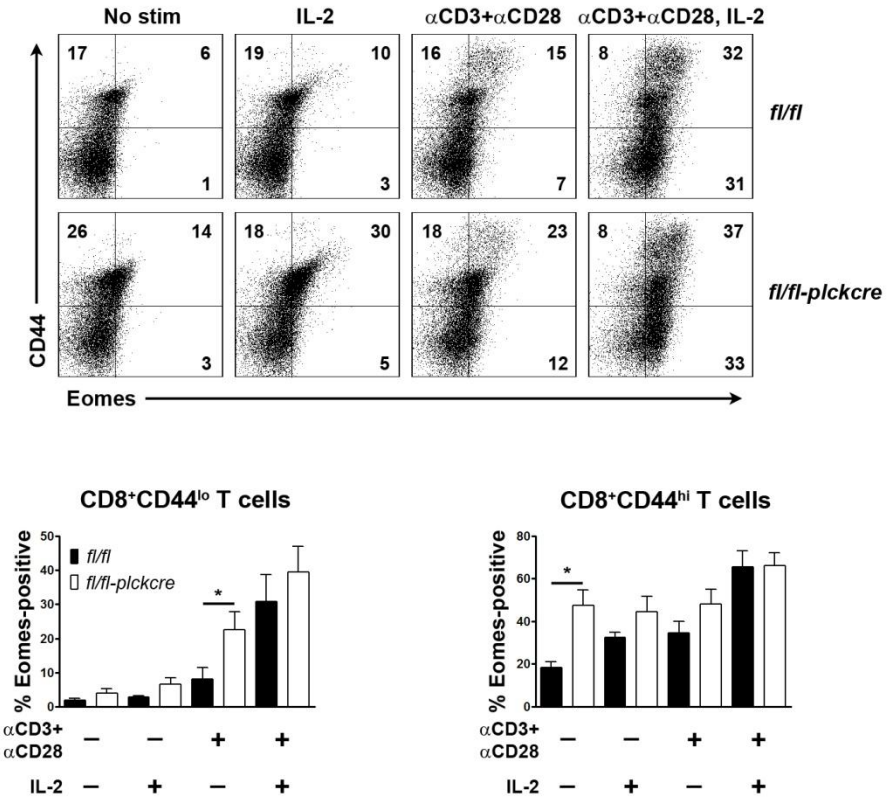

Figure 17. Expression of Eomesodermin in NF1-deficient CD8+ T cells.

Freshly isolated peripheral CD8+ T cells from T cell KO and control mice were stimulated for 48 hours with CD3 and CD28 mAb or not, in the presence or absence of rIL-2. Cells were restimulated with PMA and ionomycin prior to analysis of intracellular Eomes expression using flow cytometry. Top, representative dotplots of CD44 vs. Eomes expression. Bottom, Eomes-positive cells as a percent of total CD44lo or CD44hi cells. Graphs show the mean + SEM. n = 5 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$.

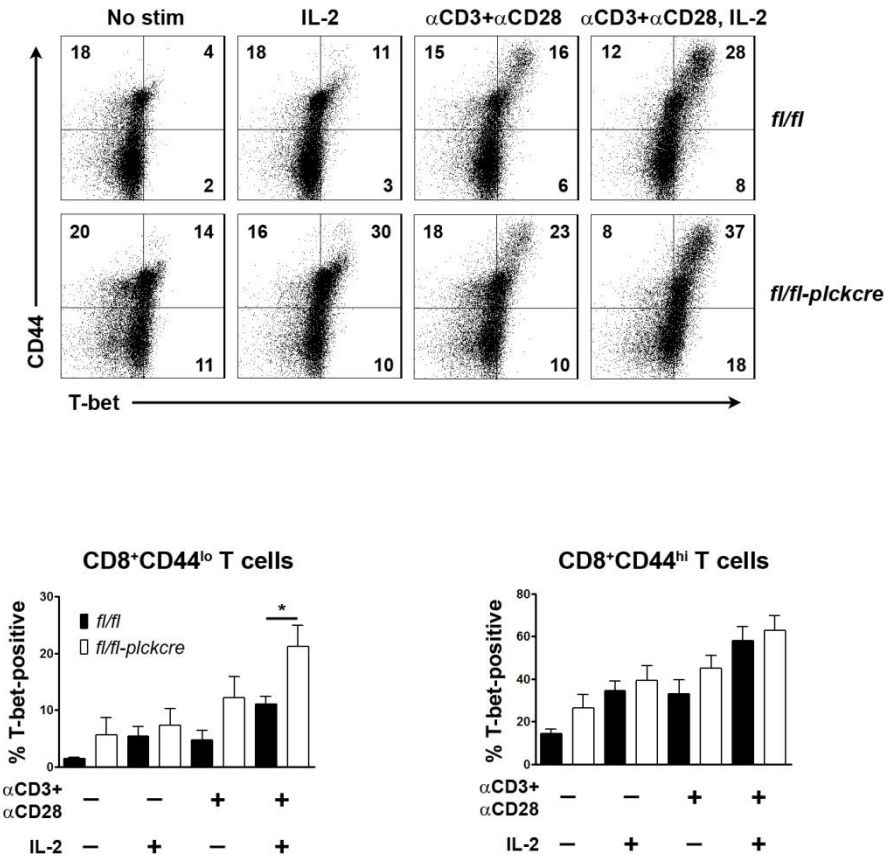

Figure 18. Expression of T-bet in NF1-deficient CD8+ T cells.

Freshly isolated peripheral CD8+ T cells from T cell KO and control mice were stimulated for 48 hours with CD3 and CD28 mAb or not, in the presence or absence of rIL-2. Cells were restimulated with PMA and ionomycin prior to analysis of intracellular T-bet expression using flow cytometry. Top, representative dotplots of CD44 vs. T-bet expression. Bottom, T-bet-positive cells as a percent of total CD44lo or CD44hi cells. Graphs show the mean+ SEM. n = 4 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. **p* < 0.05.

Helper-T cell differentiation in NF1-deficient $CD4^+$ T cells

Although $CD8^+$ T cells are the primary effector cells in the immune response against *L.* monocytogenes, $CD4^+$ T cells not only direct the course of the adaptive response to infections but also provide cytokine help that promotes the activation of $CD8^+$ T cells, primarily in the form of IL-2 (112). Thus we considered it important to determine if the loss of NF1 had any significant impact on the effector function of $CD4^+$ T cells. We cultured isolated $CD4^+$ T cells under helper-T cell skewing conditions for 120 hours prior to restimulation with CD3 mAb. 24 hours later, supernatants from these skewing cultures were analyzed by ELISA for the secretion of IFNγ, IL-4 and IL-7, cytokines known to be produced following Th1, Th2, and Th17 skewing, respectively. We noted that NF1 deficient T cells appeared to respond similarly to control T cells with regards the production of each cytokine under the conditions that would be expected to induce it (Fig,. 19A-C). Curiously, we also noted that NF1-deficient T cells expressed higher amounts of cytokines that would not be expected to be induced under the initial skewing conditions. Most notably, NF1-deficient T cells secreted significantly higher amounts of IFN-γ under conditions that would be expected to suppress IFN-γ expression and instead promote IL-4 or IL-17 expression (Fig. 19A). We further observed similar albeit lesser increases in IL-4 and IL-17 secretion under non-Th2 or non-Th17 conditions (Fig. 19B,C).

One caveat of the T helper cell skewing experiments is that NF1-deficient T cell populations have a higher frequency of $CD44^{hi}$ memory-like cells. Although the actual numbers of $CD44^{hi}$ cells are unchanged within the peripheral lymphoid organs,

the decreased numbers of $CD44^{lo}$ cells means that the resulting increase in $CD44^{hi}$ to $CD44^{lo}$ ratio must be taken into account. We noted that the increased frequency of $CD44^{hi}$ cells could be responsible for the increase in cytokine production observed in Figure 19A-C. The presence of an activated phenotype on these cells could also indicate that the cells had been previously committed to a Th lineage *in vivo*, which would account for the increased Th subset plasticity that we observed.

We did not previously observe differences in the strength or kinetics of Ras-ERK signaling in freshly isolated $CD4^+$ T cells (Fig. 12), but we wished to determine if perhaps differences in ERK phosphorylation, which may explain the abnormal cytokine secretion in Fig. 19A-C, would be evident in blasts generated from naïve CD4+ T cells. Western blotting of $CD4^+$ T cell blast lysates demonstrated no consistent increase in Ras-ERK signaling upon restimulation between T cell KO and control cells (Fig. 19D). We conclude that the Th plasticity that we observed in NF1-deficient $CD4^+$ T cells is the result of *in vivo* activation and subset differentiation.

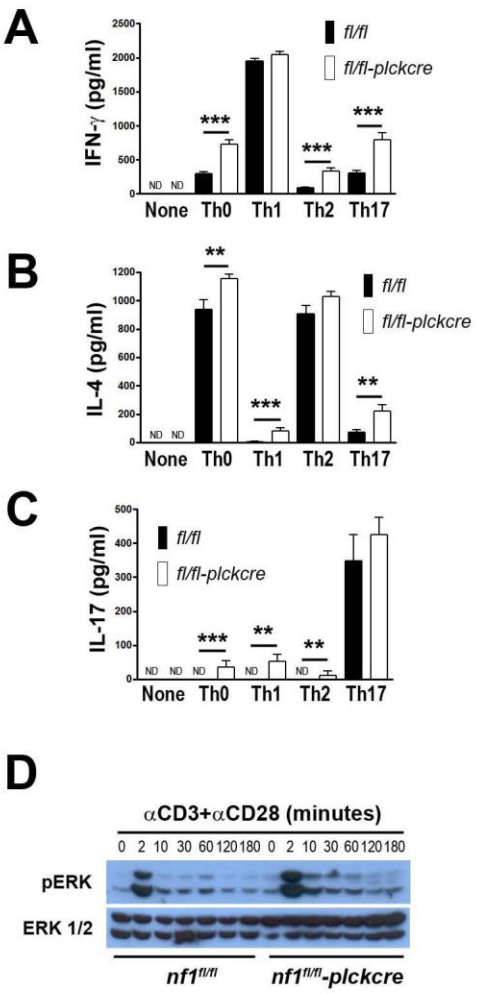

Figure 19. Plasticity in differentiated helper-T cells.

Freshly isolated CD4+ T cells from T cell KO and control mice were stimulated for 5 days under the indicated helper-T cell skewing conditions or not, followed by restimulation with CD3 mAb for 24 hours. Supernatants from restimulation cultures were analyzed by ELISA for IFN-γ (A), IL-4 (B) or IL-17 (C). D, CD4+ T cell blasts were analyzed by western blotting following CD3+CD28 mAb stimulation. Graphs show the mean + SEM. n = 6 for both genotypes (A-C). Data are representative of multiple experiments (D). Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

NF1-deficiency affects TCR-transgenic thymocyte development

We observed earlier that NF1-deficiency moderately affects thymocyte subset populations but has a considerable effect on peripheral T cell populations, although we could not ascertain the reasons behind the observed peripheral naïve T cell losses seen in NF1-deficient mice (Fig. 5-9, 11,12). However, it is possible that we are unable to adequately observe abnormal thymocyte events within a polyclonal T cell population. Abnormal thymic selection of a clonal population might not be observable within the polyclonal thymus, although it could have considerable impacts on peripheral T cell maintenance and function. The use of TCR-transgenic mice allows for the study of a clonal T cell population during development in the thymus as well as of peripheral homeostasis and effector function.

We chose to investigate cellularity within NF1-deficient mice that are transgenic for the HY TCR (HY TCR-tg), which recognizes a peptide from the male HY antigen in the context of MHC class 1 receptor H-2D^{b} (169). This peptide is recognized as self-antigen in male mice, leading to the deletion of all HY TCR-expressing T cells at the thymic negative selection checkpoint. In female mice, this transgenic TCR permits positive selection of HY TCR-positive thymocytes on thymic APCs that express H-2Db. As such, in female mice, all mature HY TCR-positive T cells should express CD8^{+}. This TCR-transgenic mouse model allows for the analysis of positive thymic selection in a clonal thymocyte population, as well as the analysis of peripheral survival of a clonal T cell population and antigen-specific CD8^{+} T cell effector function.

Thymic cellularity analysis of T cell KO and control HY TCR-tg female mice first revealed that a significantly lower percentage of thymocytes expressed the HY TCR in T cell KO mice as compared to littermate controls (Fig. 20A). We concluded that the reduced frequency of T cell KO HY TCR-positive thymocytes was due to decreased positive selection. To better address the possibility of defective positive selection in T cell KO mice, we looked at developmental subsets within the HY TCR-positive cells. Similarly to polyclonal thymocytes, HY TCR cells transition from the DN to DP to CD8 SP stage, and in a clonal population, disturbances in this progression may be more readily apparent. In analysis of CD4 and CD8 expression on HY TCR-positive thymocytes, we observed that NF1-deficient thymocytes appeared to be partially blocked at the transition from DP to CD8 SP. Analysis of the CD8 SP to DP ratio revealed that, although it is approximately 3:1 in control thymocytes, the ratio is reduced to 1:1 in NF1-deficent animals (Fig. 20B). This indicates that perhaps NF1-deficiency results in a reduced ability to transition normally through thymic development and that the death of cells that fail positive selection could account for the reduced frequency of HY TCR-positive thymocytes.

We were interested to determine if the previously observed peripheral T cell homeostatic abnormalities that we observed in polyclonal T cell KO mice were present in the clonal TCR-transgenic peripheral T cell population or in the non-transgenic cells. Analysis of total T cell populations in the spleens of HY TCR-transgenic mice demonstrated that T cell KO HY^+ cells were present in similar frequencies and numbers as those observed in non-Cre mice (Fig. 21A). In the HY-

negative compartment, we did observe a modest but significant reduction in peripheral $CD4^+$ T cells in NF1-deficient mice, although this was not accompanied by the reduction in $CD8^+$ T cell frequency and numbers that we had observed in polyclonal T cell populations (Fig. 7, 8, 21B). Furthermore, in the HY-negative population, we failed to observe the losses in peripheral naïve T cells that we had observed in polyclonal T cell populations (Fig. 7, 8, 21B).

We also wished to address the issue of effector T cell function in a clonal population that had been stimulated with cognate antigen-MHC complex. The use of a specific peptide antigen allows for more physiologically relevant stimulation of T cells, and it is possible that responses to specific antigens would not be observed in a broadly-stimulated polyclonal setting. We stimulated peripheral $CD8^+$ T cells from T cell KO and littermate control HY TCR-tg mice with HY peptide-pulsed antigen-presenting cells for 48 hours to induce expression of effector proteins, along with CD3 and CD28 mAb as a control to assess the level of stimulation. Using flow cytometry, we determined that the expression of IFN-γ, Eomes and T-bet in HY TCR-positive cells was not significantly different in NF1-deficient $CD8^+$ T cell cultures in response to antigen stimulation (Fig. 22).

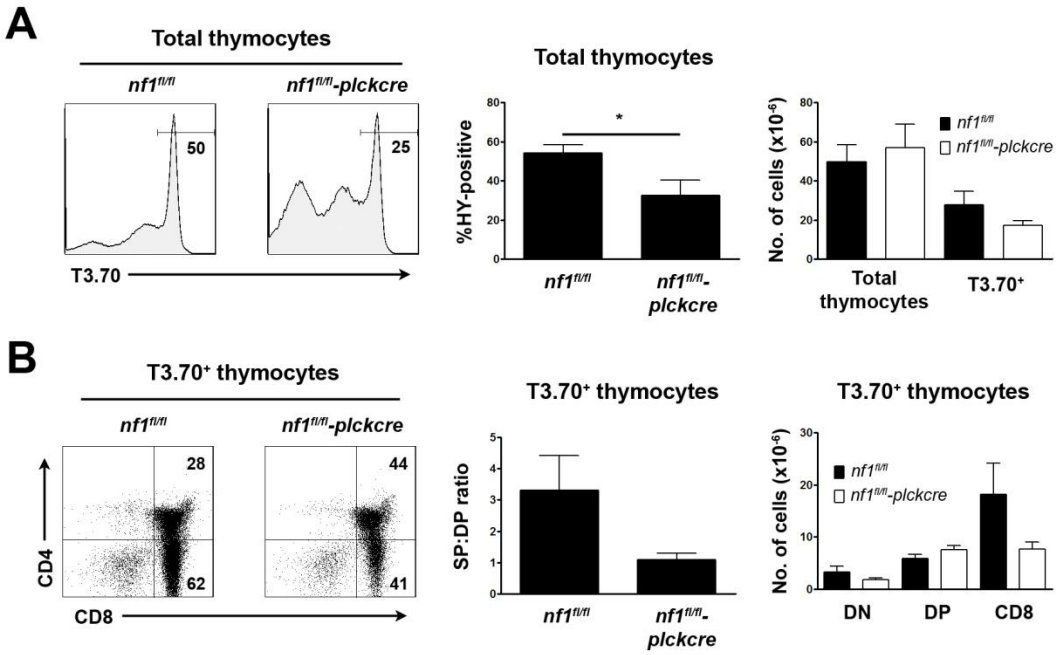

Figure 20. NF1-deficiency affects thymocyte development and cellularity in HY TCR-transgenic mice.

A, representative histograms showing HY TCR expression on thymocytes (left) and HY TCR-positive percentage of total thymocytes (right). B, representative dotplots showing CD4 and CD8 expression on HY TCR-positive thymocytes (left) and ratios of CD8 SP to DP cells (right). Graphs show the mean + SEM. n = 3 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. *$p < 0.05$.

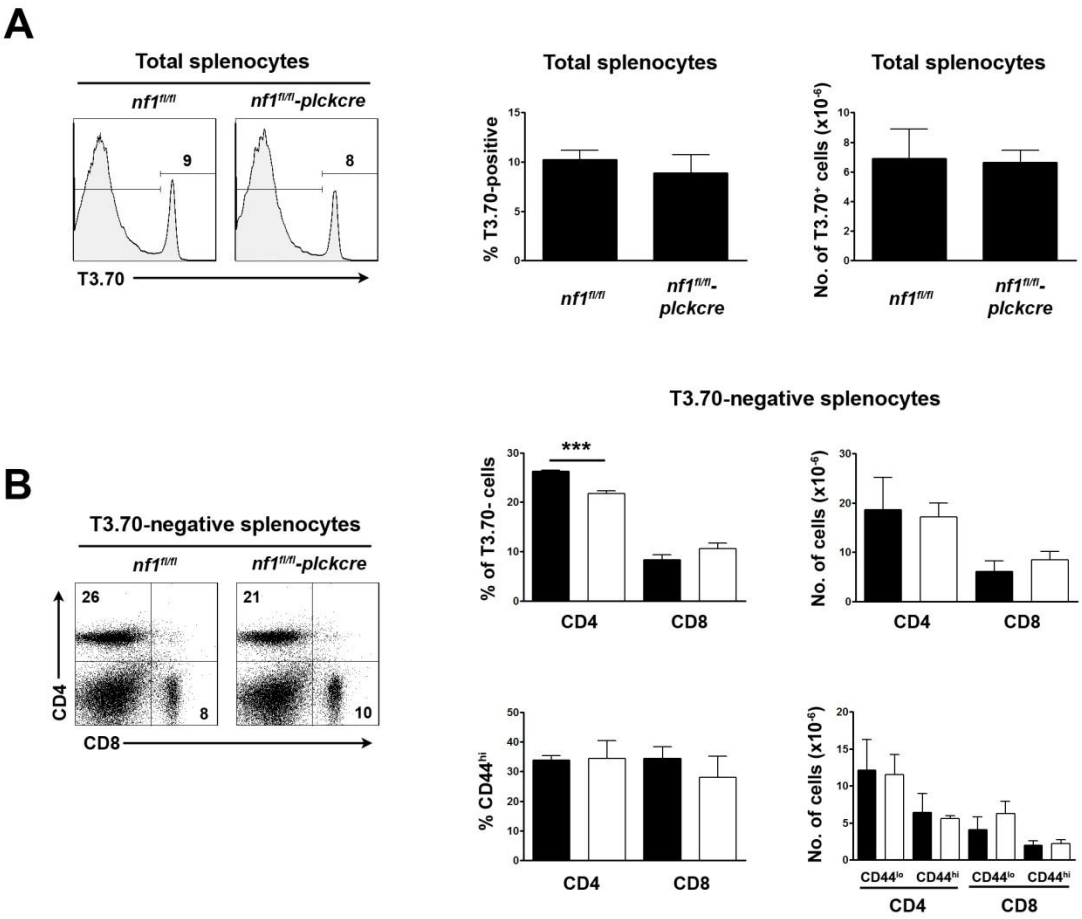

Figure 21. NF1-deficiency affects peripheral CD4+ T cell homeostasis in HY TCR-transgenic mice.

A, left, representative histograms showing HY TCR expression on T cell KO and control splenocytes. Right, percentage and absolute numbers of HY TCR-positive cells in spleens. B, left, representative dotplots showing CD4 and CD8 expression on HY-negative splenocytes. Right, top, percentage and number of HY-negative CD4+ and CD8+ cells. Bottom, percentage and number of HY-negative CD44hi T cells. Graphs show the mean + SEM. N = 3 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's *t*-test. ***$p < 0.001$.

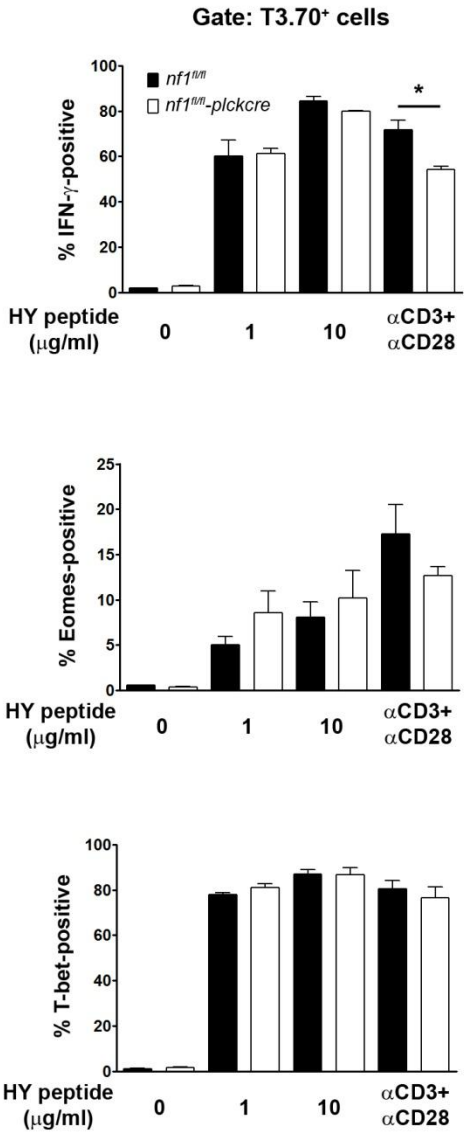

Figure 22. NF1-deficiency does not affect antigen-specific CD8+ T cell effector function in HY TCR-transgenic mice.

Purified CD8+ T cells were stimulated with the indicated concentrations of HY peptide or CD3 and CD28 mAb for 48 hours. IFN-γ (top), Eomes (middle) and T-bet (bottom) expression were measured in HY TCR+ CD8+ T cells using flow cytometry. N = 2 mice per genotype. Statistical significance was determined using the unpaired one-tailed Student's t-test. *$p < 0.05$.

2.5. Discussion

We have shown that NF1 acts in T cells during thymocyte development, as determined by disruptions in thymic developmental stages, most notably a partial block in the transition from DN3 to DN4. NF1 acts in thymocyte development in a haploinsufficient manner, as determined through investigation of mice with a heterozygous T cell-deficiency of NF1. NF1 may also affect positive selection of T cells as determined by an apparent partial block in the DP to CD8 SP transition that we observed in NF1-deficient HY TCR-transgenic mice. Further, T cell-specific NF1 deficiency impacts peripheral T cell homeostasis, as determined by our observations of losses in the naïve $CD4^+$ and $CD8^+$ T cell compartments in both NF1-deficient and NF1-heterozygous mice. However, NF1 appears to play a modest role in the regulation of T cell effector function, as we only observed moderate significant differences in effector cytokine or transcription factor expression in naïve $CD8^+$ T cells upon TCR stimulation. Increases in secreted cytokine that we observed in NF1-deficient T cell cultures are likely the result of higher frequencies of $CD44^{hi}$ effector/memory cells in NF1-deficient T cell populations, due to the partial loss of naïve T cells.

Disruptions in thymocyte development as a result of NF1 deletion were not particularly surprising. The role of Ras-MAPK signaling has been well established in thymocyte development. More recently, a related RasGAP, RASA1, was found to affect thymocyte positive selection and survival (32). NF1 may also affect positive selection in thymocytes, as observed in our studies with HY TCR-transgenic mice.

Curiously, in HY TCR-transgenic mice, NF1 appears to promote transition from DP to CD8 SP; RASA1 was reported to have the opposite effect on CD4 T cell positive selection on the AND TCR-transgenic background. NF1 and RASA1 apparently act at different stages of thymocyte development and on different developmental stages.

Loss of peripheral naïve T cells has been previously reported in mice with a T cell-specific deletion of RASA1, as well as in mice that express a constitutively active form of ERK2 (32, 85). For this reason, the homeostatic abnormalities that resulted in peripheral T cells losses in T cell KO and Het mice were not surprising. In RASA1-deficient T cells, this loss of naïve T cells was attributed to dysregulated signaling downstream of the Il-7 receptor, as IL-7 is a critical promoter of peripheral T cell survival. However, we did not observe a similar lack of survival response to IL-7 in NF1-deficient T cells. It is possible that abnormal responses to other survival factors are the cause of reduced naïve T cell numbers. It is also possible that the moderate effects of NF1-deficiency on thymocyte development may affect peripheral T cell homeostasis at a greater level. Continued experiments with TCR transgenic mice are expected to provide insight into the effects of NF1-deficiency on clonal T cell populations.

The finding that NF1-deficient mice were able to respond to *Listeria monocytogenes* infection more efficiently was interesting. Initially we hypothesized that NF1-deficient T cells were more likely to become activated in response to TCR-mediated Ras signal transduction, and that perhaps NF1-deficient T cells would mount a

stronger proliferative response. However, in vitro experiments with T cells from uninfected animals demonstrated that NF1-deficient T cells do not mount an abnormal proliferative response, nor could we detect changes in ERK phosphorylation, which we would expect downstream of dysregulated Ras activation. Future experiments in which T cells from *L. monocytogenes*-infected mice were analyzed for effector responses may shed light on this phenomenon.

The surprising findings in our *L. monocytogenes* experiment led us to wonder if NF1-deficient T cells would demonstrate significantly greater effector function in vitro. We hypothesized that differences in function would become apparent over a longer course of activation, although analysis of previously activated $CD4^+$ T cells failed to show a change in Ras-ERK signaling. In fact, we did observe increased effector cytokine secretion in longer-term cultures of both $CD4^+$ and $CD8^+$ T cells, although we conclude that this is due to a higher frequency of $CD44^{hi}$ memory T cells in the NF1-deficient cultures that results from the loss of naïve cells. Flow cytometry analysis showed an increase in the frequency of cells expressing IFN-γ and Eomes in T cell KO $CD8^+$ T cells in response to antibody-mediated TCR stimulation. However, we failed to observe increased an increase in the frequency of cytokine or transcription factor expression in a clonal $CD8^+$ T cell population in response to stimulation with cognate antigen-MHC complex. The HY peptide used in the clonal antigen stimulation experiments is not a particularly strong effector peptide; perhaps our observations in polyclonal T cell cultures and *in vivo* are due to increased strength of signal received through the TCR.

Our findings with regards to cell numbers and proliferative capabilities are in contradiction to an earlier report in which liver or bone marrow cells from fetal *nf1*-null homozygous mice were transplanted into RAG-deficient mice. In this model, the resulting NF1-deficient thymocytes and peripheral T cells were hyperproliferative, with a high constitutive level of Ras activation (86). This is in clear contrast to our observations that T cell-specific NF1 deficiency does not generally affect thymocyte numbers and that it negatively affects peripheral T cell numbers. A major caveat of the earlier report was that, although the recipient mice were deficient only in lymphocytes, NF1-deficiencies in hematopoietic cell populations have been widely reported to promote myeloproliferative disorders in mice and men (52, 54, 154, 170). NF1-deficient myeloid cells have also been reported to be hyperresponsive to cytokine stimulation, which may result in activation that would affect the T cell population indirectly. We believe that our model of T cell-specific deletion allows us to more accurately determine the effects of lineage-specific NF1 deficiency on thymocyte development and the maintenance of the T cell compartment.

We are also aware that, overall, the effects of NF1 on the T cell compartment are moderate. We believe that this is likely due to the expression of multiple RasGAPs in T cells. Some redundancy of function is likely to be present as a result. NF1-deficient T cells, although they possess somewhat different developmental abnormalities in the thymus, behave much as RASA1-deficient T cells in the periphery. However, we do not expect that NF1 and RASA1 are each simply able to compensate for the loss of

the other; in initial experiments with mice bearing T cell-specific deletions of NF1 and RASA1 concurrently, we did not observe greater abnormalities in thymocyte development or peripheral T cell homeostasis (data not shown). This indicates to us that likely another RasGAP is primarily responsible for the negative regulation of Ras in the T cell compartment. The RasGAP RASAL3 has been recently reported to be phosphorylated downstream of the TCR (171). Little is currently understood about RASAL3; it is known to be strongly expressed in the thymus and peripheral lymphoid organs (unpublished data) almost to the exclusion of expression in non-lymphoid tissue. Future investigation into the role of this protein in the T cell compartment will hopefully shed light on the mechanisms of negative regulation of Ras.

In conclusion, we believe that we have demonstrated a significant role for NF1 in thymocyte development. Further, we have demonstrated that NF1 affects peripheral T cell homeostasis although we have yet to determine the mechanism by which this occurs. Finally, we have shown that NF1 deficiency promotes the immune response to an intracellular bacterial pathogen and enhances naïve $CD8^+$ T cell effector responses, although the cellular mechanisms by which these occur are as yet undetermined. Future studies are expected to shed light on this phenomenon.

Chapter 3

IL-4 acts as a potent stimulator of IFN–γ expression in CD8+ T cells through STAT6-dependent and independent induction of Eomesodermin and T-bet

3.1. Abstract

$CD8^+$ T cell synthesis of IFN-γ is an important component of the $CD8^+$ T cell immune response. The cytokine IL-4 has been reported to promote $CD8^+$ T cell activity *in vivo*, although whether IL-4 acts directly on $CD8^+$ T cells or indirectly through action on another cell type is unclear. In short-term cultures of murine pan-T cells, we found that IL-4 was the principal cytokine responsible for driving IFN-γ synthesis by CD3/CD28-activated $CD8^+$ T cells. IL-4 was able to induce low levels of IFN-γ mRNA in $CD8^+$ T cells even in the absence of CD3/CD28 engagement, although concomitant CD3/CD28 stimulation was necessary for IFN-γ secretion. IL-4 induction of IFN-γ was explained by its ability to induce Eomesodermin and T-bet transcription factors whose expression was further increased by CD3/CD28. Expression of Eomesodermin, T-bet and IFN-γ induced by IL-4 was partially dependent upon activation of MAPK and PI3K but independent of the canonical IL-4-activated transcription factor, STAT6. In contrast, expression of IFN-γ induced by IL-4/CD3/CD28 stimulation showed additional dependency upon STAT6 which functions to increase expression of Eomesodermin specifically. These novel findings

point to a function for IL-4 as a direct regulator of IFN-γ expression in $CD8^+$ T cells and reveal the molecular mechanisms involved (172).

3.2. Introduction

$CD8^+$ T cells are the principal effector cells of the adaptive immune response directed toward intracellular pathogens. Initial activation of $CD8^+$ T cells results from TCR and costimulatory receptor recognition of MHC class I-antigenic peptide complexes and costimulatory ligands respectively that are both displayed upon the surface of professional APC. Once activated, effector $CD8^+$ T cells then target infected cells through TCR recognition of the same MHC class I-peptide complexes displayed on the target cell surface. Another important mechanism by which $CD8^+$ T cells control intracellular infections is through synthesis of IFN-γ that has antiviral and macrophage-activating properties.

In many, although not all types of infection, activation of $CD8^+$ T cells and development of memory $CD8^+$ T cells is additionally dependent upon signals provided by $CD4^+$ T cells or other immune cell types (173-177). One important $CD4^+$ T cell-derived factor that promotes $CD8^+$ T cell activation is the IL-2 cytokine (178). In addition, IL-4, classically studied as a regulator of $CD4^+$ Th2 cell differentiation and B cell immunoglobulin class switching and differentiation (179, 180), has also been implicated as a helper factor for $CD8^+$ T cell responses. In lymphochoriomeningitis virus and vaccinia virus infection models, mice that are double-deficient for IL-2 and IL-4 show a more severe impairment in an ability to generate specific CTL upon restimulation *ex vivo* compared to mice singly-deficient for IL-2 (181). In addition, in an influenza virus infection model, mice deficient in the IL-4R alpha receptor showed reduced CTL responses *ex vivo* (182). IL-4 has also

been shown to regulate $CD8^+$ T cell responses in parasitic infection models. In *Plasmodium yoelii* mouse malaria, *Leishmania Donovani* and *Schistosoma mansoni* infection models, generation of IFN-γ expressing $CD8^+$ T cells following *ex vivo* restimulation was profoundly reduced in IL-4-deficient animals (183-185). However, in each of these studies, it is unclear if IL-4 acts directly upon $CD8^+$ T cells to regulate IFN-γ synthesis or cytotoxic activity or indirectly through action upon another cell type. Moreover, IL-4 has been shown to be required for the generation of memory $CD8^+$ T cells which compared to naïve T cells synthesize much greater quantities of IFN-γ and cytotoxic effector molecules (186). Therefore, an apparent role for IL-4 in the induction of IFN-γ and cytoxicity as revealed upon *ex vivo* stimulation could reflect a role for this cytokine in induction of memory cell formation *in vivo* rather than its ability to directly regulate these responses *per se*.

In the current studies we show that IL-4 acts as a direct regulator of $CD8^+$ T cell function independent of its role as an inducer of $CD8^+$ T cell memory. The mechanism by which IL-4 induces expression of IFN-γ in $CD8^+$ T cells was investigated in detail. IL-4 induces expression of Eomesodermin (Eomes) and T-bet transcription factors that activate transcription of the *ifng* gene. Coupling of the IL-4R to both transcription factors in part depends upon IL-4-mediated activation of the intracellular signaling enzymes, MAPK and PI3K. In addition, in the presence of concomitant TCR and costimulatory receptor stimulation, an important role for IL-4-mediated STAT6 transcription factor activation in IFN-γ induction, specifically through induction of Eomes, was demonstrated.

3.3. Materials and methods

Mice

C57BL/6 x 129 Sv mice were bred in the animal facility at the University of Michigan Medical School. C57BL/6, IL-4-deficient and STAT6-deficient (both on a C57BL/6 background) and BALB/c mice were purchased from the Jackson Laboratory. All mice were 2-3 months old at the time of experiments. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Isolation and stimulation of peripheral T cells

Pan-T cells or $CD8^+$ T cell populations were prepared from pooled spleen and lymph node cell suspensions using MACS pan-T cell or $CD8^+$ T cell negative selection kits (Miltenyi) respectively according to manufacturer's instructions. For isolation of NKT cell-depleted pan-T cells and $CD44^{lo}$ $CD8^+$ T cell populations, NK1.1 and CD44 mAb (eBioScience) were used in conjunction with pan-T cell and $CD8^+$ T cell isolation kits respectively. Purity of negatively selected T cell populations was routinely determined by flow cytometric analysis.

T cells were stimulated in wells of 96 well U-bottomed plates in RPMI 1640 containing 10% FBS and antibiotics (2×10^5 cells/200 ml/well). For CD3/CD28 mAb stimulation, wells were pre-coated with CD3 mAb (1 μg/ml; eBioScience) and soluble CD28 mAb was included in the culture medium (1 μg/ml; eBioScience). Neutralizing anti-IL-2 and anti-IL-4 mAb (BD Pharmingen) were added to wells at 1

μg/ml. Recombinant murine IL-2 and IL-4 (R&D Systems) were added to wells at 100 and 10 ng/ml respectively. PD98059 and wortmannin inhibitors (Calbiochem) were added to wells at 50 and 1 nM respectively.

Flow cytometry

Cells were harvested from wells and analyzed for cell surface expression of CD4, CD8, CD44, CD49b (DX5) and intracellular expression of IFN-γ, Eomes and T-bet by flow cytometry using respective fluorochrome-labeled mAb (BD Pharmingen except CD49b, Eomes and T-bet mAb which were purchased from eBioScience). CD1d-α-GalCer tetramer used for detection of NKT cells was purchased from the NIH Tetramer Core Facility. For intracellular staining, harvested cells were treated with PMA and ionomycin (50 ng/ml and 1.5 μM respectively; Sigma Aldrich) for 5 h with addition of brefeldin A (1:1000 dilution of stock; BD Biosciences) for the last 4 h of culture. Cells were then surface stained prior to fixation and permeabilization and intracellular staining. Cellular data was collected on a FACSCanto flow cytometer equipped with FACSDiva software (BD Biosciences).

ELISA

Concentrations of IFN-γ in well supernatants were determined with the use of a Duo-set ELISA kit (R&D Systems). To assay Granzyme B secretion, stimulated T cells were harvested and restimulated in CD3 mAb coated wells as above. Granzyme B concentrations in well supernatants were then determined with the use of a Granzyme B Duo-set ELISA kit (R&D Systems).

Real time RT-PCR

RNA was isolated from stimulated CD8+ T cells using Trizol (Invitrogen) and reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Real time PCR was performed using established IFN-γ and β-actin Taqman primer/probe sets in a 7500 Fast Lightcycler (Applied Biosystems). Fold change in IFN-γ mRNA expression relative to unstimulated $CD8^+$ T cells was determined as described (187).

3.4. Results

A major role for IL-4 in induction of IFN-γ expression in $CD8^+$ T cells

As shown by intracellular staining, stimulation of pan-T cells with CD3 mAb (directed to the TCR complex) and mAb against the CD28 costimulatory receptor for 48 h *in vitro* results in $CD8^+$ T cell expression of IFN-γ (Fig. 23A). Substantial induction of IFN-γ is observed in both $CD44^{lo}$ naïve and $CD44^{hi}$ memory phenotype $CD8^+$ T cells in these cultures. In contrast, purified $CD8^+$ T cells synthesize little IFN-γ in response to the same stimulus, at least as detected by intracellular staining (Fig. 23A). Thus, other T cells provide help to $CD8^+$ T cells in order for them to synthesize IFN-γ.

IL-2 is considered an important helper factor for $CD8^+$ T cell responses (178). To address this, we included a neutralizing anti-IL-2 antibody in pan-T cell cultures. However, blockade of IL-2 only partially inhibited the ability of $CD8^+$ T cells to synthesize IFN-γ in pan-T cell cultures (Fig. 23B). Therefore, we examined the effect of blocking antibodies against other cytokines whose receptors share the same common gamma chain subunit with the IL-2R (70, 188). Of these, antibodies against IL-7, IL-9, IL-15 and IL-21 had no effect upon $CD8^+$ T cell IFN-γ synthesis (data not shown). In contrast, a neutralizing antibody against IL-4 profoundly blocked $CD8^+$ T cell IFN-γ expression, particularly in $CD44^{lo}$ $CD8^+$ T cells (Fig. 23B). Furthermore, combined blockade of IL-2 and IL-4 essentially abrogated the ability of both $CD44^{lo}$ and $CD44^{hi}$ $CD8^+$ T cells to synthesize IFN-γ (Fig. 23B).

Based on these findings, we next examined if addition of IL-4 to cultures of purified $CD8^+$ T cells could restore IFN-γ expression in response to CD3/CD28 mAb stimulation. In comparison with IL-2, IL-4 was found to be a potent inducer of IFN-γ expression in CD3/CD28 mAb-stimulated $CD8^+$ T cells, particularly in the $CD44^{lo}$ subset (Fig. 23C).

To confirm a predominant role for IL-4 in induction of $CD8^+$ T cell synthesis of IFN-γ, we examined IFN-γ expression in $CD8^+$ T cells from IL-4-deficient mice. Consistent with results of antibody blocking studies, $CD8^+$ T cells in CD3/CD28-stimulated pan-T cell cultures from IL-4-deficient mice synthesized reduced levels of IFN-γ in comparison to $CD8^+$ T cells from C57BL/6 control mice (Fig. 24). Again, the dependency upon IL-4 was most apparent in $CD44^{lo}$ $CD8^+$ T cells. A major role for IL-4 in the induction of IFN-γ synthesis in $CD44^{lo}$ $CD8^+$ T cells was also demonstrated in mouse strains other than C57BL/6 or 129 Sv, e.g. BALB/c (Fig. 25).

NKT cells are known to rapidly synthesize IL-4 in response to TCR engagement (189). Therefore, since NKT cells would be present in pan-T cell cultures, we asked if they were required for $CD8^+$ T cell synthesis of IFN-γ through provision of IL-4. For this purpose, we performed additional negative selection for the NK1.1 marker that is expressed upon the majority of mature NKT cells in the peripheral immune system of mice (Fig. 26)(190). As shown by staining with CD1d-α-GalCer tetramers and antibodies against the DX5 marker that is also expressed by NKT cells(190), NK1.1 negative selection was only partially effective at depleting NKT cells in C57BL/6 x

129 Sv mice. Furthermore, NK1.1 depletion did not affect $CD8^+$ T cell synthesis of IFN-γ in response to CD3/CD28 mAb stimulation in this strain. By contrast, the same NK1.1 negative selection procedure resulted in depletion of the vast majority of NKT cells in C57BL/6 mice, which could potentially be explained by differences in the level of expression of NK1.1 between the two strains. More importantly, in C57BL/6 mice, NKT cell depletion resulted in much reduced IFN-γ synthesis by $CD44^{lo}$ $CD8^+$ T cells following stimulation with CD3/CD28 mAb. In addition, the residual IFN-γ response of these cells was not inhibited with an anti-IL-4 mAb. These results, therefore, indicate that NKT cells are the principal source of IL-4 that drives $CD8^+$ T cell synthesis of IFN-γ at least in the $CD44^{lo}$ subset.

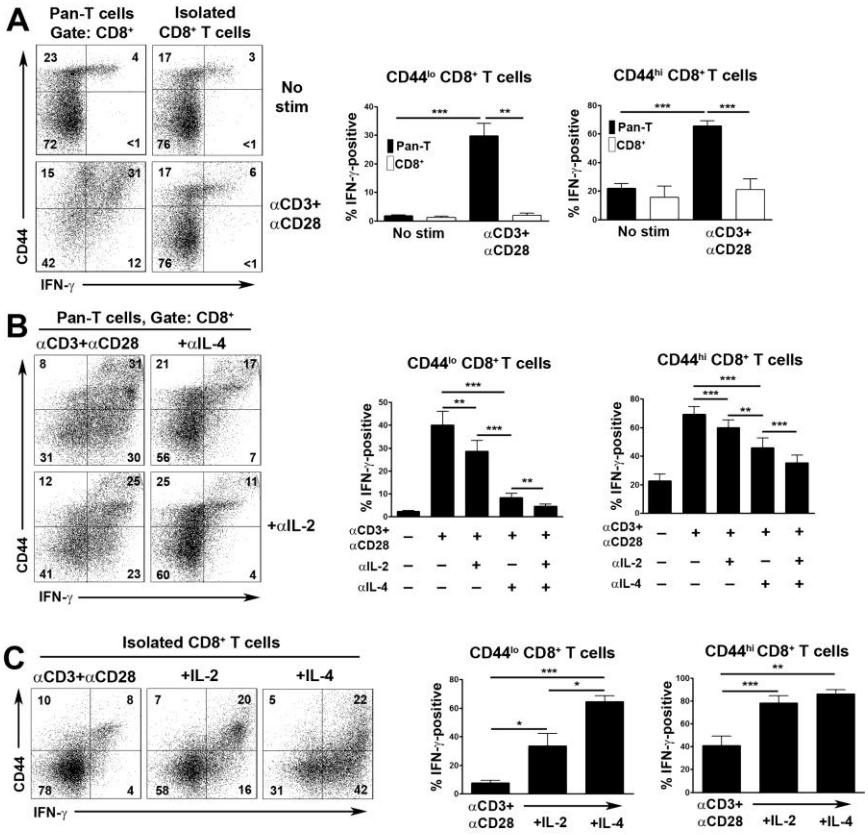

Figure 23. Role of IL-2 and IL-4 in induction of IFN-γ expression in CD8+ T cells.

Experiments were performed with T cells from littermate C57BL/6 x 129 Sv (A-C) or C57BL/6 (D) mice. A, Pan-T cells or purified CD8+ T cells were stimulated or not with CD3 and CD28 mAb. After 48 h, expression of IFN-γ upon $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells was determined by flow cytometry. At left are shown representative two color flow cytometry plots of CD44 versus IFN-γ staining. Numbers indicate percentage of CD8+ T cells in each quadrant. Bar charts at right show the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells determined in repeat experiments (n=5 mice). B, Pan-T cells were stimulated as in (A) in the presence or absence of neutralizing anti-IL-2 and/or anti-IL-4 antibodies. Representative flow cytometry plots of CD44 versus IFN-γ staining are shown at left. Bar charts show mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells under each stimulation condition determined in repeat experiments (n=5 mice). C, Purified CD8+ T cells were stimulated as in (A) in the presence or absence of recombinant IL-2 or IL-4. Representative flow cytometry plots of CD44 versus IFN-γ staining are shown at left. Bar graphs at right show the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells under the indicated stimulation conditions determined in repeat experiments (n=4 mice). Statistical significance was determined using the unpaired Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

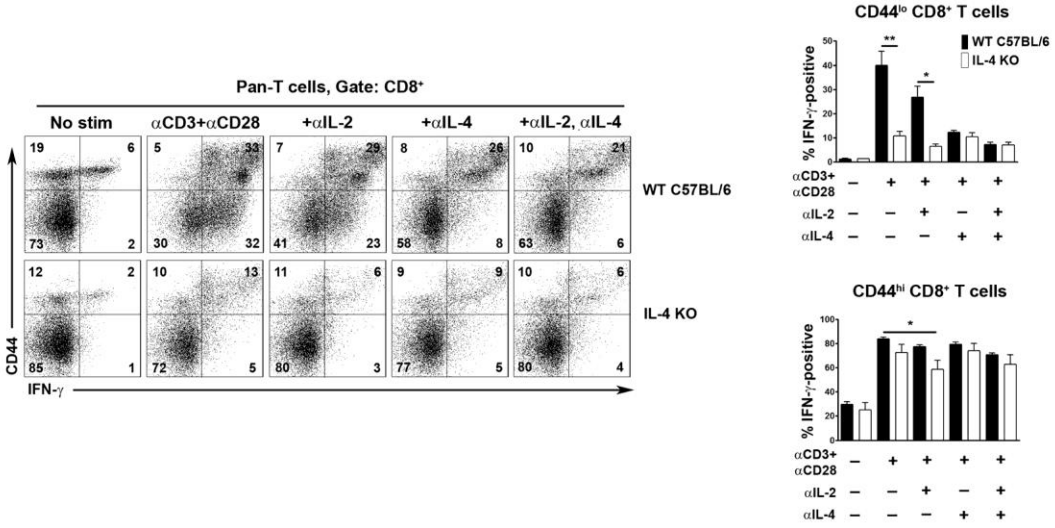

Figure 24. IFN-γ expression in IL-4-deficient T cells.

Pan-T cells from C57BL/6 IL-4-deficient (IL-4 KO) and wild type control C57BL/6 mice were stimulated or not with CD3 and CD28 mAb in the presence or absence of neutralizing anti-IL-2 and/or anti-IL-4 mAb. Expression of IFN-γ upon $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells was determined by flow cytometry after 48 h of culture. At top are shown representative flow cytometry plots of CD44 versus IFN-γ staining. Bar graphs show the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells for each mouse genotype and condition of stimulation as determined in repeat experiments (n=5 mice each genotype). Statistical significance was determined using the paired Student's t-test. *$p < 0.05$, **$p < 0.01$.

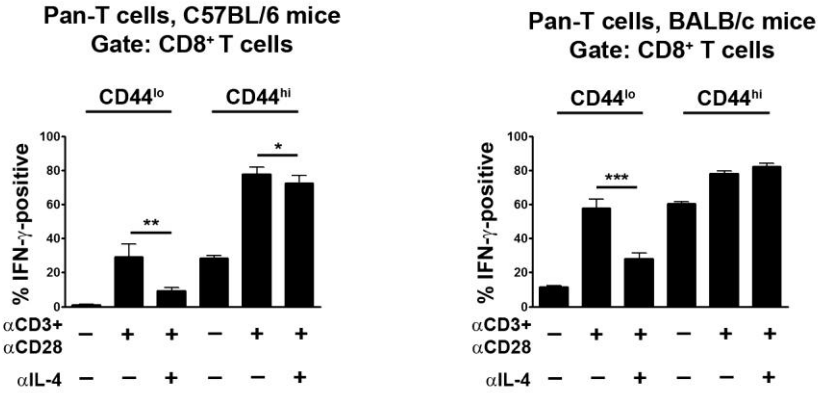

Figure 25. Role of IL-4 in CD8+ T cell synthesis of IFN-γ in BALB/c mice.

Pan-T cells from wild type C57BL/6 and BALB/c mice were stimulated or not with CD3 and CD28 mAb in the presence or absence of neutralizing anti-IL-4 mAb. After 48 h, expression of IFN-γ upon $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells was determined by flow cytometry. Bar graphs show the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells for each mouse genotype and condition of stimulation as determined in repeat experiments (n=5 mice each genotype). Statistical significance was determined using the paired Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

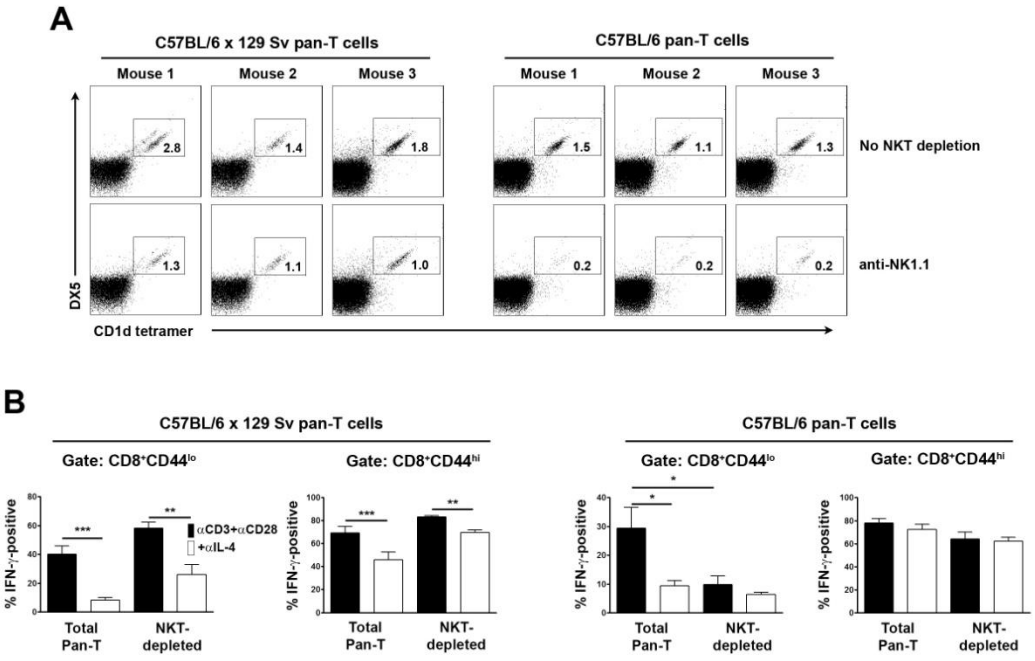

Figure 26. Cellular source of IL-4 for IFN-γ induction in CD8+ T cells.

A, Pan T cells were depleted or not of NKT cells by negative selection using an NK1.1 mAb. Shown are two-color plots of DX5 mAb (CD49b) and CD1d-α–GalCer tetramer staining from three mice of each genotype. B, Pan-T cells or NKT cell-depleted pan-T cells from wild type C57BL/6 x 129 Sv mice or C57BL/6 mice (as in A) were stimulated with CD3 and CD28 mAb in the presence or absence of a neutralizing anti-IL-4 mAb. After 48 h, expression of IFN-γ upon $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cell subsets was determined by flow cytometry. Bar graphs show the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells for each mouse genotype as determined in repeat experiments (n=5 mice each genotype). Statistical significance was determined using the two sample Student's t-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

IL-4 induces the expression of intracellular IFN-γ in $CD8^+$ T cells in the absence of TCR and costimulatory receptor engagement

As a negative control in experiments, we examined the influence of IL-4 alone upon $CD8^+$ T cell expression of IFN-γ. Surprisingly, as detected by intracellular staining, IL-4 alone induced similar levels of IFN-γ expression in $CD44^{lo}$ and $CD44^{hi}$ $CD8^+$ T cells to that observed when T cells were stimulated with IL-4 plus CD3 and CD28 mAb (Fig. 27A). However, as determined by ELISA of well supernatants, none of the IFN-γ induced by IL-4 in the absence of CD3/CD28 engagement was secreted from cells but instead remained intracellular (Fig. 27B). Thus, CD3/CD28 engagement is necessary for the secretion of IL-4-induced IFN-γ in $CD8^+$ T cells. With this knowledge, we considered that IL-4 plus CD3/CD28 engagement might in fact induce much higher levels of IFN-γ expression in $CD8^+$ T cells compared to IL-4 stimulation alone. However, the difference would not be detected by intracellular staining since concurrent CD3/CD28 stimulation would result in continual release of *de novo* synthesized IFN-γ whereas in IL-4 alone stimulated $CD8^+$ T cells the *de novo* synthesized IFN-γ would accumulate. To confirm this possibility, we examined IFN-γ mRNA levels in $CD8^+$ T cells by real time RT-PCR. As predicted, stimulation of $CD44^{lo}$ $CD8^+$ T cells with the combination of IL-4 plus CD3/CD28 mAb resulted in much higher levels of IFN-γ mRNA expression than stimulation with IL-4 alone (Fig. 27C). Thus, although IL-4 alone is able to induce IFN-γ expression in $CD8^+$ T cells, IL-4 is in fact a relatively weak inducer of IFN-γ in the absence of concurrent CD3/CD28 stimulation. In addition, stimulation of $CD8^+$ T cells with IL-4 alone does not result in IFN-γ secretion.

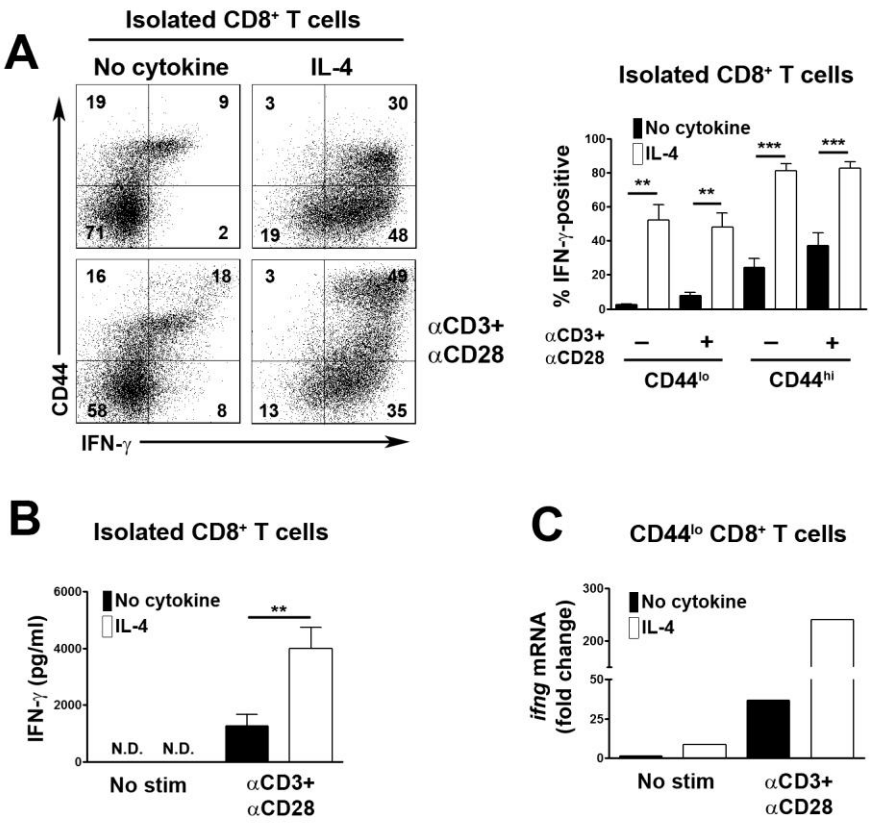

Figure 27. IL-4 induced synthesis of IFN-γ in CD8+ T cells with and without concurrent CD3/CD28 stimulation.

All experiments were performed with T cells from C57BL/6 x 129 Sv mice. A, Purified CD8+ T cells were stimulated or not with IL-4 and/or CD3 and CD28 mAb for 48 h. Expression of IFN-γ in $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells was determined by flow cytometry. At left are shown representative flow cytometric plots of CD44 vs IFN-γ staining. Bar graph at right shows the mean percentage of IFN-γ positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells under different stimulation conditions as determined in repeat experiments (n=7 mice). B, Purified CD8+ T cells were stimulated as in (A). Concentrations of IFN-γ in well supernatants were determined by ELISA. Shown is the mean concentration of IFN-γ + 1 SEM for each stimulation condition as determined repeat experiments (n=4 mice). N.D., not detectable. C, Purified CD8+ $CD44^{lo}$ T cells were stimulated or not with IL-4 and/or CD3 and CD28 mAb for 6 h. Relative expression levels of IFN-γ mRNA were determined by real time RT-PCR. Results are expressed as fold change in IFN-γ expression compared to non-stimulated T cells. Similar results were obtained in a repeat experiment. In (A) and (B) statistical significance was determined using the paired Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

IL-4 promotes the cytotoxic function of CD8+ T cells but is not required for CD8+ T cell proliferation

We further asked if IL-4 was necessary for the acquisition of $CD8^+$ T cell cytotoxic function *in vitro*. Efficiency of CTL priming was measured by expression of the lysosomal marker CD107a that is induced in $CD8^+$ T cells during the acquisition of cytotoxic function (191). In wild type mice, CD3/CD28 mAb stimulation of pan-T cells resulted in expression of CD107a in $CD44^{hi}$ $CD8^+$ T cells that was blocked by approximately 50 percent by anti-IL-2 or anti-IL-4 mAb and completely by both types of mAb when included in priming cultures (Fig. 28A). We also examined secretion of the cytotoxic effector molecule, Granzyme B, by ELISA. Blockade of either IL-2 or IL-4 during CD3/CD28 mAb-induced priming of wild type pan-T cells, or intrinsic IL-4 deficiency, was sufficient to almost completely block the synthesis and secretion of Granzyme B by $CD8^+$ T cells (Fig. 28B). Consistent with these results, in IL-4-deficient mice, induction of CD107a surface expression in $CD8^+$ T cells was substantially reduced (Fig. 28A). IL-4 also induced expression of CD107a in purified $CD44^{lo}$ and $CD44^{hi}$ $CD8^+$ T cells, albeit not to the levels observed with concurrent CD3 and CD28 stimulation (Fig. 28C).

We also asked if IL-4 played a role in the induction of $CD8^+$ T cell proliferation in CD3/CD28-stimulated pan-T cell cultures by monitoring $CD8^+$ T cell dilution of CFSE label by flow cytometry. In these experiments, blocking IL-2 and IL-4 mAb, when used either alone or together, did not affect the $CD8^+$ T cell proliferative response (Fig. 29).

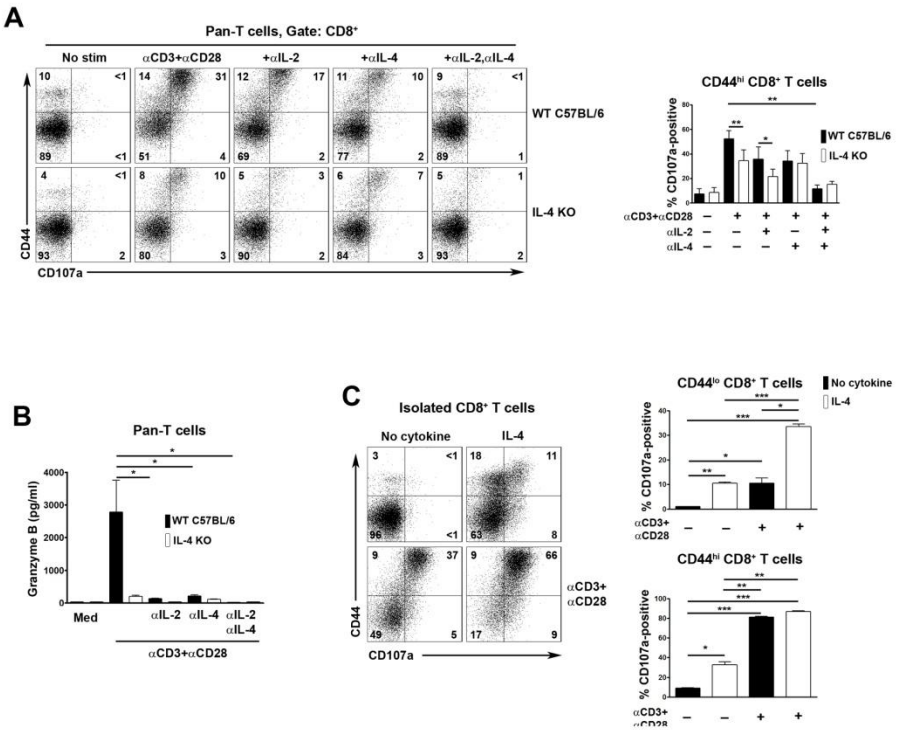

Figure 28. Role of IL-4 in induction of CD8+ T cell cytotoxic function.

A, Pan-T cells from IL-4-deficient and wild type control C57BL/6 mice were stimulated or not with anti-CD3 and CD28 mAb in the presence or absence of neutralizing anti-IL-2 and/or anti-IL-4 mAb. After 72 h, cells were restimulated in CD3 mAb coated (1 mg/well) wells (1 x 10^5 cells/well) in medium containing labeled CD107a mAb before staining for additional cell surface markers. Expression of cell surface CD107a upon CD8+ $CD44^{lo}$ and $CD44^{hi}$ T cell subsets was determined by flow cytometry. At left are shown representative flow cytometry plots of CD44 and CD107a staining. The bar graph at right shows the mean percentage of CD107a-positive cells + 1 SEM among CD8+ $CD44^{hi}$ cells under different conditions of stimulation in the two strains of mice as determined in repeat experiments (n=4 mice each genotype). B, Purified CD8+ T cells from C57BL/6 mice were stimulated or not with CD3 and CD28 mAb and/or IL-4 as in (A) and expression of CD44 and CD107a was determined by flow cytometry. At left are shown representative flow cytometry plots of CD44 and CD107a staining. Bar graphs at right show the mean percentage of CD107a-positive cells + 1 SEM for $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cells under different stimulation conditions as determined in repeat experiments (n=3 mice). C, Pan-T cells from IL-4-deficient mice and wild type control C57BL/6 mice were stimulated as in (A). After 4 h restimulation, concentrations of Granzyme B in well supernatants were determined by ELISA. Shown is the mean concentration of Granzyme B +1 SEM for the different stimulation conditions and mouse genotypes as determined in repeat experiments (n=4 mice each genotype). Statistical significance was determined using the paired Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

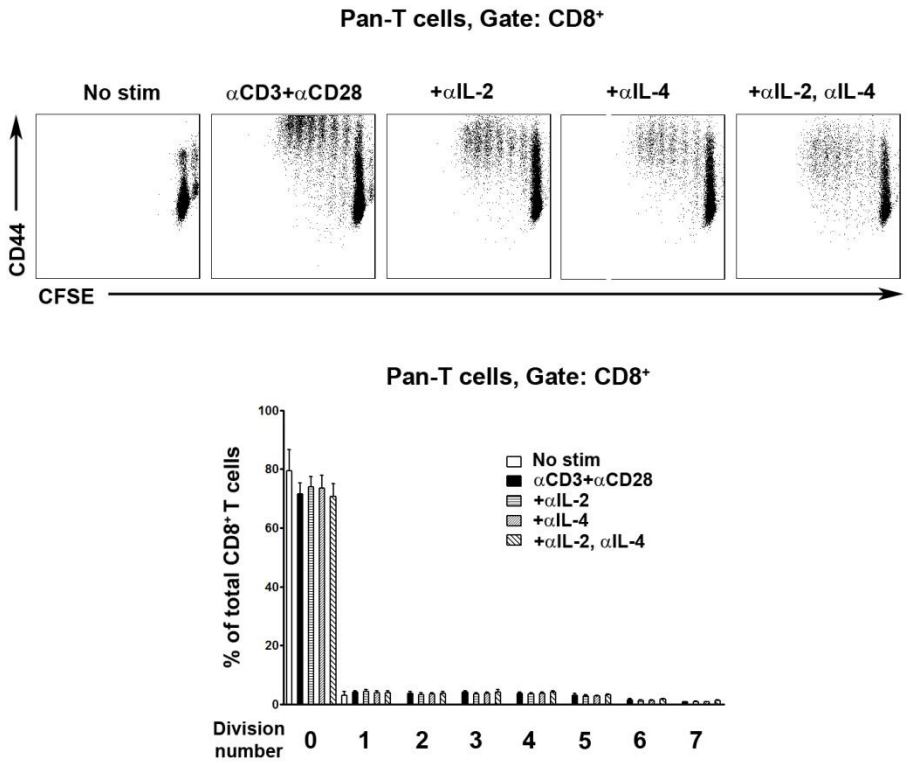

Figure 29. Role of IL-4 in CD8+ T cell proliferation *in vitro*.

CFSE-labeled pan-T cells from C57BL/6 mice were stimulated or not with anti-CD3 and CD28 mAb in the presence or absence of neutralizing anti-IL-2 and/or anti-IL-4 mAb. After 96 h, CFSE fluorescence of CD8+ T cells was determined by flow cytometry. At left are shown representative flow cytometry plots of CFSE fluorescence versus CD44 staining. The bar graph at right shows the mean percentage of live CD8+ T cells + 1 SEM that have undergone the indicated number of divisions under the different conditions of stimulation as determined in repeat experiments (n=4 mice). Statistical significance was determined using the paired Student's t-test.

IL-4 induces the expression of Eomes and T-bet transcription factors in $CD8^+$ T cells

Expression of IFN-γ in $CD8^+$ T cells is controlled by Eomes and T-bet transcription factors (115, 192). Therefore, we asked if IL-4 induced the expression of either transcription factor in $CD8^+$ T cells that would account for IFN-γ gene transcription. First, we examined if expression of Eomes and T-bet in $CD8^+$ T cells in CD3/CD28 mAb-stimulated pan-T cell cultures was dependent upon IL-4. As shown, CD3/CD28 mAb stimulation of pan-T cells resulted in an induction of Eomes and T-bet expression in CD8+ T cells (Fig. 30, 31). In $CD44^{lo}$ T cells, stronger induction of Eomes compared to T-bet was noted, whereas in $CD44^{hi}$ subpopulations these transcription factors were induced to a similar degree. Significantly, expression of both of these transcription factors in both $CD8^+$ T cell subpopulations was inhibited by a blocking anti-IL-4 mAb (Fig. 30, 31).

We next examined if IL-4 could induce expression of either transcription factor in purified $CD8^+$ T cells. IL-4 alone induced expression of Eomes and T-bet in $CD44^{lo}$ and $CD44^{hi}$ $CD8^+$ T cell subpopulations, although in $CD44^{lo}$ cells, Eomes was more strongly induced, consistent with findings with pan-T cells (Fig. 30A,B). Furthermore, the combination of IL-4 plus CD3/CD28 mAb resulted in increased expression of both transcription factors in $CD8^+$ T cells (Eomes in $CD44^{lo}$ and $CD44^{hi}$ cells and T-bet in $CD44^{hi}$ cells only), consistent with increased levels of IFN-γ mRNA (Fig. 27 C; Fig. 30B, 31B). We conclude that IL-4 drives IFN-γ expression in $CD8^+$ T cells through induction of the expression of Eomes and T-bet.

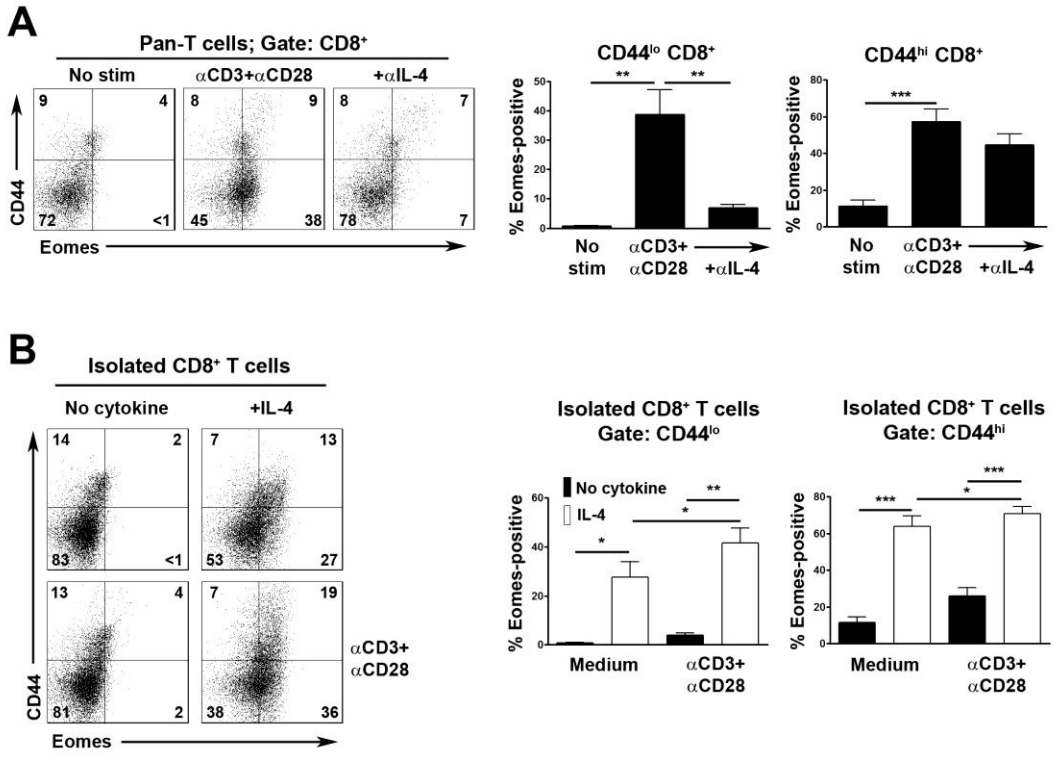

Figure 30. IL-4-mediated induction of Eomes and T-bet transcription factors in CD8+ T cells.

Pan-T cells (A) or purified CD8+ T cells (B) from C57BL/6 x 129 Sv mice were stimulated or not with CD3 and CD28 mAb in the presence or absence of a neutralizing IL-4 mAb (A) or with or without IL-4 or with IL-4 alone (B) as indicated. After 48 h, expression of CD44 and Eomes upon CD8+ T cells was determined by flow cytometry. At left are shown representative flow cytometry plots of CD44 versus transcription factor staining. Bar graphs at right shown the mean percentage of transcription factor positive cells + 1 SEM within $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cell populations under the different stimulation conditions as determined in repeat experiments. N = 5 for each genotype. Statistical significance was determined using the unpaired two-tailed Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

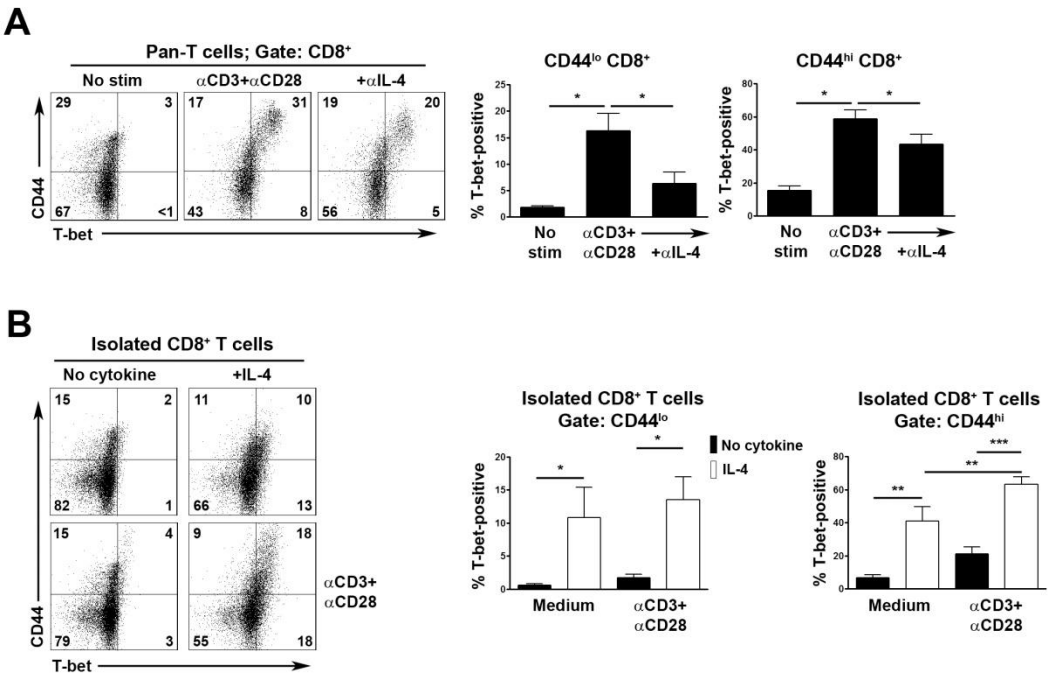

Figure 31. IL-4-mediated induction of T-bet transcription factor in CD8+ T cells.

Pan-T cells (A) or purified CD8+ T cells (B) from C57BL/6 x 129 Sv mice were stimulated or not with CD3 and CD28 mAb in the presence or absence of a neutralizing IL-4 mAb (A) or with or without IL-4 or with IL-4 alone (B) as indicated. After 48 h, expression of CD44 T-bet upon CD8+ T cells was determined by flow cytometry. At left are shown representative flow cytometry plots of CD44 versus transcription factor staining. Bar graphs at right shown the mean percentage of transcription factor positive cells + 1 SEM within $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cell populations under the different stimulation conditions as determined in repeat experiments. N = 4 for each genotype. Statistical significance was determined using the unpaired two-tailed Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

STAT6 promotes IFN-γ expression in $CD8^+$ T cells induced by the combination of IL-4 and CD3 plus CD28 mAb stimulation

IL-4 signal transduction proceeds through the JAK-STAT pathway (193, 194). Binding of IL-4 to the IL-4R results in activation of receptor-associated JAK protein tyrosine kinases, which phosphorylate the IL-4R leading to recruitment of the STAT6 transcription factor. JAK-mediated phosphorylation of STAT6 then promotes STAT6 dimerization and nuclear translocation and activation of gene transcription programs. Given the established role of STAT6 in the IL-4 signal transduction pathway, we examined if this transcription factor was necessary for IL-4 induced expression of IFN-γ. To this end, we examined the ability of IL-4 to induce expression of IFN-γ in purified $CD8^+$ T cells from STAT6-deficient mice. Surprisingly, when stimulated with IL-4 alone, loss of STAT6 did not impact upon the ability of $CD8^+$ T cells to synthesize IFN-γ (Fig. 32A). In contrast, loss of STAT6 did result in reduced levels of IFN-γ expression in $CD44^{lo}$ $CD8^+$ and $CD44^{hi}$ $CD8^+$ T cells following stimulation with the combination of IL-4 and CD3/CD28 mAb (Fig. 32A). Therefore, stimulation of $CD8^+$ T cells with IL-4 plus CD3/CD28 mAb appears to license STAT6 involvement in a signaling pathway that is associated with much increased levels of IFN-γ gene transcription (Fig. 27C).

We further examined if STAT6 was necessary for IL-4 induced expression of Eomes and T-bet in $CD8^+$ T cells. Loss of STAT6 did not affect expression of Eomes or T-bet in $CD8^+$ T cells that were stimulated with IL-4 alone (Fig. 32B and C). However, as with IFN-γ induction, loss of STAT6 did result in reduced levels of expression of

Eomes in $CD44^{lo}$ $CD8^{+}$ T cells following stimulation with IL-4 plus CD3 and CD28 mAb (Fig. 32B). Expression of T-bet was not affected by loss of STAT6 in $CD8^{+}$ T cells following stimulation with IL-4 plus CD3 and CD28 mAb (Fig. 32C). From these findings we conclude that STAT6 functions to increase IFN-γ expression in IL-4 plus CD3/CD28 mAb-stimulated $CD8^{+}$ T cells primarily through increasing the expression of Eomes.

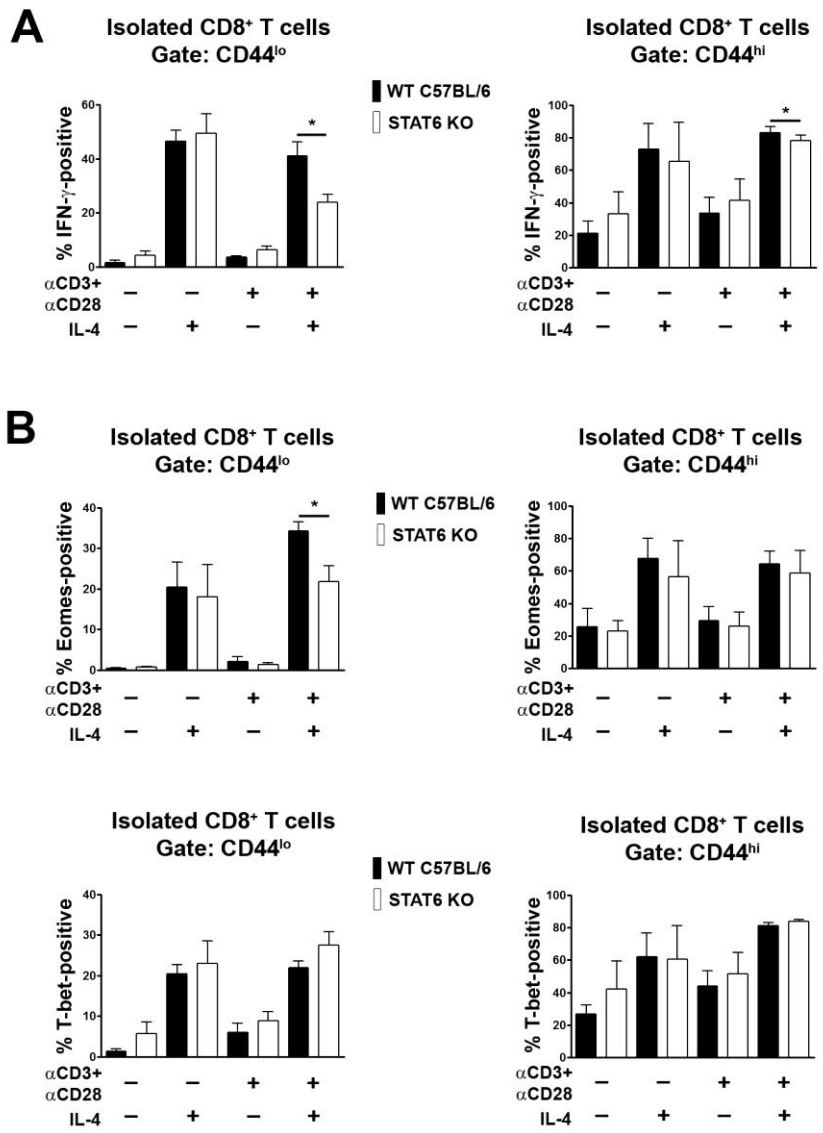

Figure 32. Role of STAT6 in IL-4-mediated induction of IFN-γ expression in CD8+ T cells.

Purified CD8+ T cells from STAT6-deficient (STAT6 KO) or wild type C57BL/6 control mice were stimulated or not with CD3 and CD28 mAb and/or IL-4. After 48 h, expression of IFN-γ (A), Eomes and T-bet (B) within $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cell populations was determined by flow cytometry. Bar graphs show the mean percentage of IFN-γ or transcription factor-positive cells + 1 SEM for each condition of stimulation and mouse strain as determined in repeat experiments (n= at least 3 mice each genotype). Statistical significance was determined using the paired Student's t-test. *$p <$ 0.05.

IL-4 induction of IFN-γ expression in $CD8^+$ T cells is partially dependent upon activation of MAPK and PI3K

JAK-mediated phosphorylation of the IL-4R also leads to recruitment of the insulin receptor substrate adapter proteins that couple the IL-4R to activation of MAPK and PI3K (193-195). Furthermore, MAPK and PI3K are activated upon TCR and CD28 engagement and are essential for a number of different types of T cell response (196, 197). Therefore, we asked if MAPK and PI3K activation are necessary for IL-4-induced $CD8^+$ T cell expression of IFN-γ. For this purpose, purified $CD8^+$ T cells were stimulated with IL-4 and/or CD3 and CD28 mAb in the presence or absence of the MAPK inhibitor, PD98059, or the PI3K inhibitor, wortmannin. PD98059 and wortmannin both partially inhibited $CD8^+$ T cell expression of IFN-γ induced by IL-4 alone and IL-4 plus CD3/CD28 mAb (Fig. 33A and B). Furthermore, this was associated with partial inhibition of expression of Eomes and T-bet for both types of induced response (Fig. 33C and D). Therefore, MAPK and PI3K promote IL-4 and IL-4 plus CD23/CD28-induced IFN-γ expression in $CD8^+$ T cells by increasing the expression of Eomes and T-bet.

We additionally examined the effect of combined MAPK and PI3K inhibition and STAT6 loss upon IL-4 plus CD3/CD28-induced IFN-γ secretion in $CD8^+$ T cells (Fig. 33E). Pair-wise combination of inhibitors and STAT6 loss resulted in further reduction of IFN-γ secretion compared to single inhibitors or STAT6 loss alone. Furthermore, combination of MAPK and PI3K inhibitors with STAT6 loss resulted in a complete blockade of the $CD8^+$ T cell IFN-γ response.

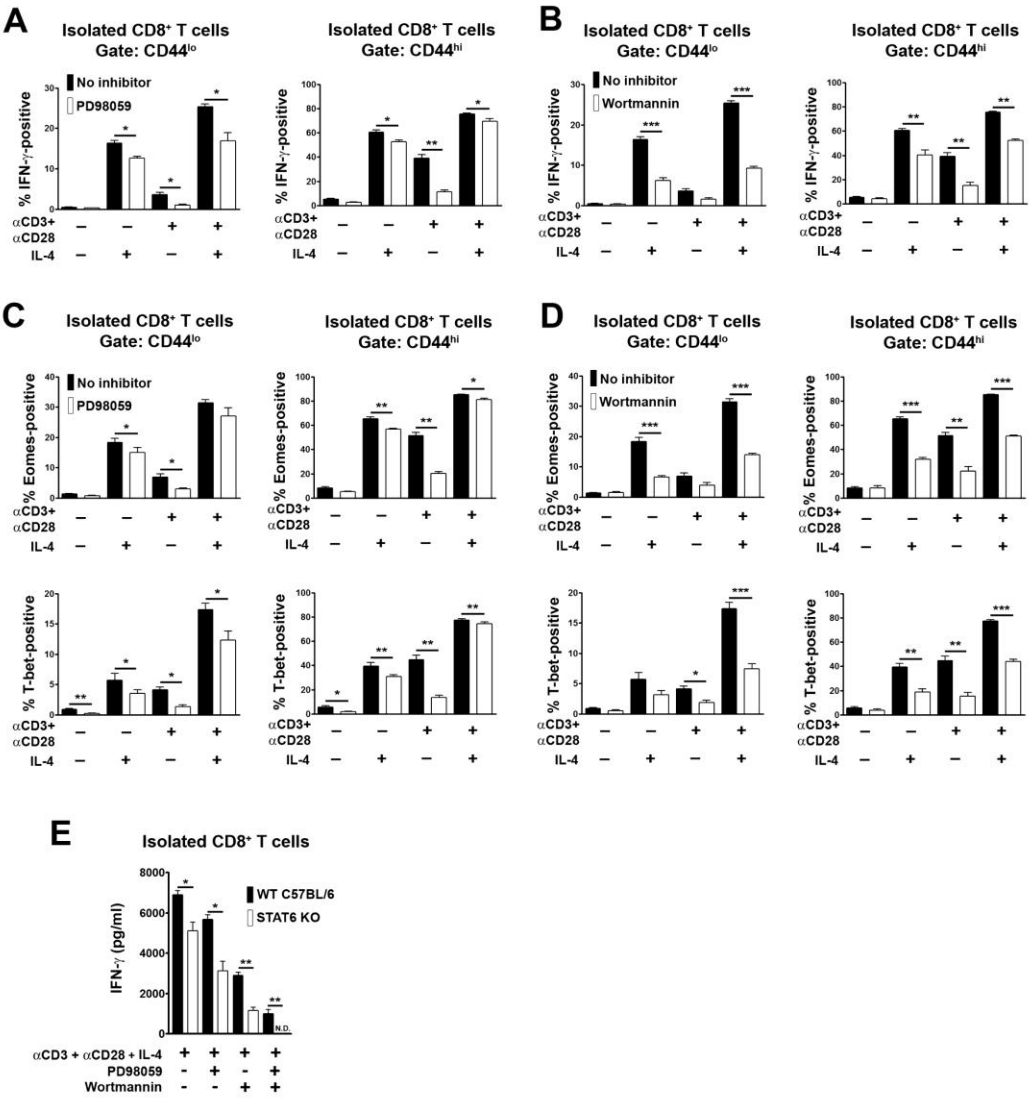

Figure 33. Roles of ERK and PI3K activation in IL-4-mediated induction of IFN-γ expression in CD8+ T cells.

Purified CD8+ T cells from C57BL/6 mice were stimulated or not with CD3 and CD28 mAb and/or IL-4 in the presence or absence of the ERK inhibitor PD98059 (A,C) or the PI3K inhibitor wortmannin (B,D). After 48 h, expression of IFN-γ (A,β), Eomes (C,D) and T-bet (C,D) within $CD44^{lo}$ and $CD44^{hi}$ CD8+ T cell populations was determined by flow cytometry. Bar graphs show the mean percentage of IFN-γ or transcription factor-positive cells + 1 SEM for each condition of stimulation as determined in repeat experiments (n= at least 3 mice each genotype). Statistical significance was determined using the paired Student's t-test. (E) Purified CD8+ T cells from STAT6-deficient or wild type C57BL/6 control mice were stimulated or not as in (A-D). After 48 h, the concentration of IFN-γ in well supernatants was determined by ELISA. Shown is the mean concentration of IFN-γ + 1 SEM for each condition of stimulation as determined in repeat experiments (n=3). Statistical significance was determined using the two sample Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.5. Discussion

We show here that IL-4 acts as a potent stimulator of IFN-γ synthesis and cytotoxic activity in $CD8^+$ T cells. These functions for IL-4 cannot be explained on the basis of any role for IL-4 in $CD8^+$ T cell memory development or proliferation. Importantly, therefore, findings expand upon previous studies and show that IL-4 activates $CD8^+$ T cell IFN-γ synthesis and cytotoxic effector function directly.

Regarding the mechanism by which IL-4 induces expression of IFN-γ in $CD8^+$ T cells, interestingly, some induction of IFN-γ was observed when $CD8^+$ T cells were stimulated with IL-4 alone in the absence of TCR and costimulatory receptor engagement. However, the IFN-γ synthesized upon IL-4 stimulation alone was not secreted from cells but was instead retained intracellularly. Therefore, IL-4 produced in the course of an immune response would not trigger IFN-γ secretion from bystander antigen non-specific $CD8^+$ T cells unless their TCR and costimulatory receptors were also engaged. If IL-4 alone-induced IFN-γ synthesis in $CD8^+$ T cells has a physiological purpose, one possibility is that it serves to amplify a TCR/CD28 plus IL-4 induced IFN-γ response of antigen specific $CD8^+$ T cells.

The role of TCR/CD28 engagement upon $CD8^+$ T cells in induction of IFN-γ secretion is not limited to an ability to stimulate release of IFN-γ. Thus, TCR and CD28 signals synergize with IL-4 signals to induce much higher levels of IFN-γ gene transcription. This is associated with increased expression of Eomes and T-bet transcription factors that are known activators of IFN-γ gene transcription in $CD8^+$ T

cells (192). Eomes and T-bet also induce the expression of genes that mediate CTL lysis of target cells (192). Therefore, an ability of IL-4 with and without TCR/CD28 engagement to induce both transcription factors in CD8+ T cells would also account for the role of this cytokine in the acquisition of cytotoxic effector function.

IL-4-alone and IL-4 plus TCR/CD28-induced $CD8^+$ T cell IFN-γ synthesis was partially dependent upon activation of MAPK and PI3K. This could be accounted for on the basis that both signaling intermediates are necessary for full induction of Eomes and T-bet expression. Exactly how MAPK and PI3K promote Eomes and T-bet expression in $CD8^+$ T cells is unknown at present. However, MAPK and PI3K participate in signaling pathways that culminate in the activation of transcription factors such as activator protein 1 and nuclear factor kappa-B that have the potential to activate transcription of the Eomes and T-bet genes directly.

STAT6 was not required for $CD8^+$ T cell IFN-γ synthesis induced by IL-4 alone but did promote IFN-γ synthesis in response to IL-4 plus TCR/CD28 engagement. However, the magnitude and kinetics of STAT6 activation in $CD8^+$ T cells as detected by phospho-STAT6 western blotting was comparable following IL-4 alone or IL-4 plus TCR/CD28 activation (data not shown). Therefore, participation of STAT6 specifically upon additional TCR/CD28 engagement cannot be explained by increased activation of this transcription factor. Instead, TCR/CD28 triggering must act to license STAT6 involvement in the IFN-γ response through an as yet undetermined mechanism. Whatever this mechanism, STAT6 appears to increase

IFN-γ synthesis through increased expression of Eomes but not T-bet. Potentially, this specificity could be accounted for by an ability of STAT6 to activate transcription of the Eomes gene directly.

The discovery that IL-4 promotes the expression of Eomes in naive $CD8^+$ T cells is exciting in light of recent findings that state the critical role of Eomes in the promotion and maintenance of memory $CD8^+$ T cell populations. Eomes-deficient CD8+ T cells have been reported as defective in establishing central memory cell populations *in vivo* (115). In addition, IL-4 is required for the development of a recently described population of innate $CD8^+$ T cells in mice with a memory-like phenotype and constitutive expression of Eomes (198). IL-4 has also been shown been shown to promote memory development in adaptive $CD8^+$ T cells *in vivo*, as well as their survival and proliferation (186, 199, 200). With regards to memory development, in the current studies we also found that blockade of IL-4 in CD3/28-mAb stimulated pan-T cell cultures or addition of IL-4 to CD3/CD28 mAb-stimulated purified $CD8^+$ T cells inhibited or promoted the generation of $CD44^{hi}$ memory phenotype $CD8^+$ T cells respectively (data not shown). Potentially, therefore, the role of IL-4 in $CD8^+$ T cell IFN-γ expression might be explained by increased generation of memory phenotype $CD8^+$ T cells and that memory $CD8^+$ T cells intrinsically have an increased propensity to synthesize this cytokine in comparison with naïve $CD8^+$ T cells. However, this possibility can be ruled out since IL-4 was shown to induce high levels of expression of IFN-γ in $CD44^{lo}$ naïve $CD8^+$ T cells. In addition, IL-4 was

generally shown to induce higher levels of IFN-γ expression in $CD44^{hi}$ memory $CD8^+$ T cells compared to memory $CD8^+$ T cells deprived of this cytokine.

In conclusion, we have identified IL-4 as a major direct inducer of IFN-γ expression in $CD8^+$ T cells and have dissected the mechanisms involved. In concert with the TCR and costimulatory receptors, IL-4 induces expression of Eomes and T-bet and *de novo* transcription of the IFN-γ gene. Induction of both transcription factors is partially dependent upon activation of MAPK and PI3K enzymes in $CD8^+$ T cells. However, only expression of Eomes is regulated by the STAT6 transcription factor. Altogether, these studies illuminate an important molecular mechanism involved in the control of $CD8^+$ T cell immunity.

Chapter 4

Synergistic interactions of the RasGAPs RASA1 and neurofibromin in the initiation of thymic lymphomas in mice

4.1. Abstract

The RasGAPs RASA1 and NF1 are negative regulators of Ras signaling in many cell types from embryonic development through the lifetime of the organism. Loss of NF1 alone or with one allele of RASA1 largely predisposes mice to myeloid leukemia, similar to that which is observed in previously reported deletion models of NF1. We report for the first time the concomitant induced systemic deletion of RASA1 and NF1 in mice. There is little evidence that double-deficient mice possess characteristics reported in induced systemic single-deletion mice. Instead, a substantial subset of mice develop thymic lymphoma that resembles reported T-cell acute lymphoblastic lymphoma, in which lymphoid organs are seeded by an immature $CD4^{+}CD8^{+}$ population that originates in the thymus. A majority of these tumors are positive for activating Notch1 mutations, a further characteristic of T- cell acute lymphoblastic lymphoma (T-ALL). We believe that these novel findings are important in the study of RasGAP function in adult animals as well as in the studies of overlapping and non-redundant function of multiple RasGAPs within a given cell type.

4.2. Introduction

Negative regulation of the Ras signaling pathway is essential to the maintenance of cell and tissue homeostasis. Although activation of the Ras GTPase is a central component of many cell surface receptor pathways that transmit signals to the nucleus that promote cellular survival and growth, unchecked Ras signaling leads to negative consequences. Ras is considered an oncogene, as its dysregulation is regarded as causative in many human tumors (201).

Ras activity is known to be negatively regulated by a family of proteins known as Ras GTPase-activating proteins (RasGAPs). These proteins share a catalytic GAP domain, which facilitates the cleavage of the γ-phosphate from Ras-bound GTP, thus rendering Ras inactive (36, 202, 203). As these proteins are critical to normal cell function, it is perhaps not surprising that there are often multiple RasGAPs expressed in a given cell type (132). This overlap of expression has presented many challenges in our understanding of the roles of each individual RasGAP as well as of potential synergistic and antagonistic interactions between RasGAP family members.

The RasGAP neurofibromin (NF1) has been quite well studied in both mouse and man. NF1 is named for the human disease neurofibromatosis type 1, which is considered an autosomal-dominant disorder (204). The phenotypic indications

of NF1 are caused by heterozygous loss-of-function or truncation mutations in the gene *Nf1*. Unchecked cellular growth is evident in the development of nerve sheath tumors, or neurofibromas, that are characteristic of neurofibromatosis type 1 (50, 142). Although the majority of these neurofibromas are benign, patients with NF1 are predisposed to malignant nerve sheath tumors as well as myeloid leukocyte tumors (52, 54, 142, 154, 155, 205). Neurofibromatosis type 1 is also characterized by dysregulated cardiovascular development as well as neural defects, possibly due to non-redundant Ras regulatory functions in these tissues (1, 30, 31, 146, 150).

Less well studied in human disease but also of interest is RASA1, a fellow member of the RasGAP family. RASA1 has been reported as the causative agent in the human disorder capillary malformation-arteriovenous malformation (CM-AVM), believed to be due to germline inheritance of one mutated *Rasa1* allele followed by the loss of heterozygosity in the affected tissues (44, 206-210). CM-AVM is characterized by port-wine skin lesions in which developmental abnormalities result in arterial vasculature that is connected directly to venous vasculature (206). A number of RASA1 mutation-associated CM-AVM patients are afflicted with the disorder Parkes-Weber syndrome, in which large skin lesions are accompanied by subcutaneous arterialized veins and hypertrophy of the affected tissues (44, 211). In a minority of cases, chylothorax and chylous ascites, the pooling of lymphatic fluid in the thoracic or peritoneal cavities, were observed (44).

The generation of appropriate mouse models with which to study NF1 and RASA1 deficiency has been challenging. Complete deletion of either RasGAP due to homozygous expression of null *nf1* or *rasa1* alleles has resulted in embryonic lethality (29-31). Recently, LoxP conditional knockout mouse models for *nf1* and *rasa1* have been reported. Systemic deletion of RASA1 in adult animals, by ubiquitously-expressed inducible Cre recombinase, has been shown to result in lymphatic vessel hyperplasia and leakage, culminating in the development of chylothorax and chylous ascites .This hyperplasia has been shown to be due to dysregulated Ras signaling downstream of the VEGFR3 on lymphatic endothelial cells (45). Systemic deletion of NF1 in adult mice and consequent myeloproliferative disorder has been described in one report (54); however, deletion of NF1 in specific tissues has shown that the loss of NF1 has similar effects in murine tissues to those observed in human tissues (1).

In this report, we attempt to describe and understand the interactions between RASA1 and NF1 in murine tissues by generating heterozygous or homozygous RASA1-deficiency concurrently with homozygous NF1 deletion. We demonstrate that our model of systemic deletion of NF1 in adult mice results in the development of myeloproliferative disorder, similar to previous observations of systemic deletion of NF1 in all murine tissues as well as NF1-related human myelodysplastic syndrome. We show that *rasa1* heterozygosity, previously reported to have no phenotypic effect in single-deletion mouse models, correlates with less predictable hematopoietic phenotypes when combined with NF1-deficiency. We further show that

homozygosity for both RASA1- and NF1-deficiency yields a further shift in hematopoietic disorders from myeloid leukemias, accompanied by a sharp increase in the development of thymic lymphoma, accompanied by activating Notch mutations.

4.3. Materials and methods

Mice

The generation of *rasa1*$^{fl/fl}$ and *nf1*$^{fl/fl}$ mice has been described previously (1, 212). Tamoxifen-inducible NF1-deficient, RASA1-NF1-double deficient, and RASA1-heterozygous-NF1-deficient mice were generated by crossing *rasa1*$^{fl/fl}$ and *nf1*$^{fl/fl}$ mice with *ubErt2Cre* transgenic mice, which express Cre recombinase fused to a mutated estrogen receptor under the control of the ubiquitin promoter (213, 214). Floxed alleles were deleted in all tissues by the administration of tamoxifen on two subsequent days as previously described (212). Mice were 2-3 months of age at the time of injection. All mice are on a mixed C57BL/6 and 129 Sv background. C57BL/6 mice were purchased from JAX. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Flow cytometry

Single-cell suspensions of spleens, lymph nodes, thymuses and bone marrow as well as whole blood were depleted of red blood cells. Leukocytes were analyzed for surface expression of B220, TCRβ, Gr-1, CD11b, Ter119, CD4, CD8, CD44, and CD25 via staining with fluorochrome-labeled mAbs (BD Biosciences) followed by analysis on a FACSCanto (BD Biosciences) with FlowJo software (Treestar).

Histology

Whole tissues were fixed in 10% formalin immediately following dissection. Five micrometer sections were stained with hematoxylin and eosin according to University of Michigan University Laboratory Animal Management Histology core protocols.

Western blotting

Thymocytes from *rasa1*$^{fl/fl}$*nf1*$^{fl/fl}$*-ubErt2Cre* mice, thymocytes and splenocytes from C57BL6 mice were lysed at a concentration of 2x10^{7} cells/ml prior to SDS-PAGE protein separation and western blotting. Normal and truncated Notch1 intracellular domain (NICD) was determined using a cleaved Notch-specific antibody (Cell Signaling).

4.4. Results

Induced NF1-deficient mice present with myeloid leukemia

As *nf1* mutations are associated with myelodysplastic syndromes in both mice and men, we wished to determine if our inducible NF1-deficient mice would present with a similar phenotype. Following the administration of tamoxifen, we observed enlarged spleens in both observed *nf1*$^{fl/fl}$*-ubErt2Cre* mice (Table 1). Flow cytometric analysis of the hematopoietic tissues of these animals revealed abnormally high frequencies of Gr-1^{lo}CD11b^{+} and Gr-1^{hi}CD11b^{+} cells in the spleens of both mice, with the highest frequencies observed in the older mouse (Fig. 34A). This corroborates earlier reports in which induced deletion of NF1 in adult mice resulted in overgrowth of myeloid cells in spleens (54). We also observed high frequencies of Gr-1^{+}CD11b^{+} granulocytes in the bone marrow of both animals, and increased frequencies of CD11b^{+}Gr-1^{lo} monocytes, especially in the older mouse (Fig. 34B). This also agrees with earlier reports of increased monocyte frequencies in induced NF1-deficient animals (54). We did not observe overt abnormalities in cardiovascular or neural function in either animal.

Time to death (d)	Presentation	Diagnosis
148	Enlarged spleen	Myeloid dysplasia (33%/50% GM in spleen/BM)
256	Enlarged spleen	Myeloid dysplasia (60%/75% GM in spleen/BM

Table 1. Analysis of *nf1*$^{fl/fl}$ *–ubErt2Cre* mice.

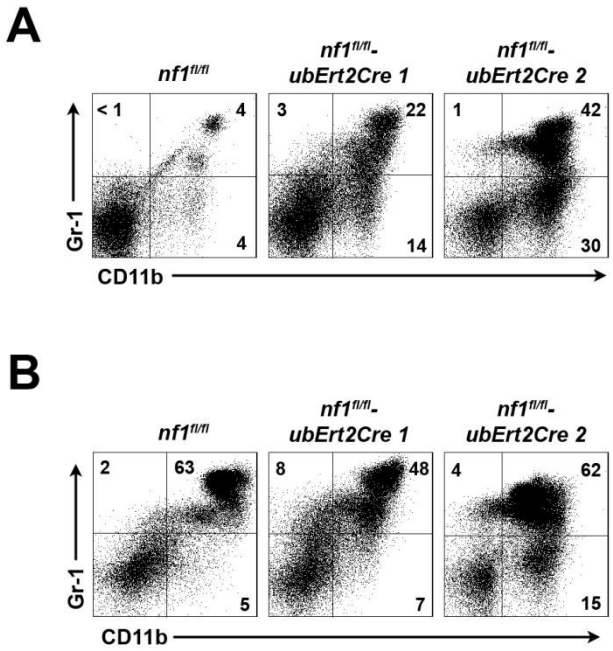

Figure 34. Myeloid leukemia in mice with induced systemic NF1-deficiency..

Shown are representative dot plots of live splenocytes (A) and bone marrow cells (B) from induced NF1-deficient and control mice that were analyzed for Gr-1 and CD11b expression. n = 2 mice.

Concurrent *rasa1* heterozygosity and *nf1* deletion yield mixed hematopoietic phenotypes

Rasa1 heterozygosity has not previously been shown to induce abnormal phenotypes ((29) and Lapinski, unpublished). To our knowledge, *rasa1* heterozygosity has not been investigated concurrently with NF1 deletion. We generated *rasa1*$^{fl/+}$*nf1*$^{fl/fl}$ – *ubErt2Cre* (RASA1 het) mice and induced deletion with tamoxifen. We expected development of myeloid leukemia, as we had observed in NF1-deficient mice, and in fact we observed this in 12 of 18 mice (Table 2).

In the remaining mice, we observed phenotypes that were more difficult to classify, ranging from erythoid cell dysplasia to thymic lymphoma (Table 2). Further analysis of a mouse with a mixed phenotype showed histiocyte infiltration into the liver and spleen (Fig. 35A). Flow cytometric analysis revealed that the ratios of B and T cells were abnormal in the spleen, lymph nodes, and thymus (Fig. 35B). Analysis of the non-lymphocyte population in these tissues demonstrated that the ratios of Gr-1^{hi}CD11b^{+} granulocytes to Gr-1^{lo}CD11b^{+} monocytes were also abnormal, with high frequencies of monocytes and few granulocytes (Fig. 35B)

Time to death (d)	Presentation	Diagnosis
157	Labored breathing, enlarged spleen	Myeloid dysplasia (60% GM in BM)
164	Hemangioma	Hemangioma
267	Labored breathing, growth on spleen	Mixed (70% GM in BM; CD8+TCR- thymoma)
279	Labored breathing	Erythroid dysplasia (elevated Ter119 levels)
419	Very enlarged spleen	Myeloid dysplasia (>60% GM in spleen, BM)
553	Labored breathing; Enlarged spleen, thymus, ascites	Erythroid dysplasia (elevated Ter119)
644	Aged, enlarged spleen	Myeloid dysplasia (60% GM in spleen)
68	Dermatitis	Myeloid dysplasia (34% GM in spleen)
138	Enlarged spleen, LN	Myeloid dysplasia (>70% GM in blood)
138	Enlarged spleen, LN	Myeloid dysplasia (>70% GM in blood)
516	Dermatitis	Myeloid dysplasia (76% GM in spleen)
160	Enlarged spleen	Myeloid dysplasia (40% GM in spleen, BM)
273	Very enlarged spleen	Thymic lymphoma (DP TCR-ve cells in spleen)
513	Aged	Myeloid dysplasia (35/74% GM in spleen, BM)
513	Aged	Myeloid dysplasia (57/90% GM in spleen, blood)
513	Aged	Mixed T cell/myeloid dysplasia
513	Aged	Myeloid dysplasia (50/83% GM in spleen, blood)
513	Aged	Myeloid dysplasia (45/67% GM in spleen, blood

Table 2. Analysis of *rasa1*$^{fl/+}$*nf1*$^{fl/fl}$ *–ubErt2Cre* mice.

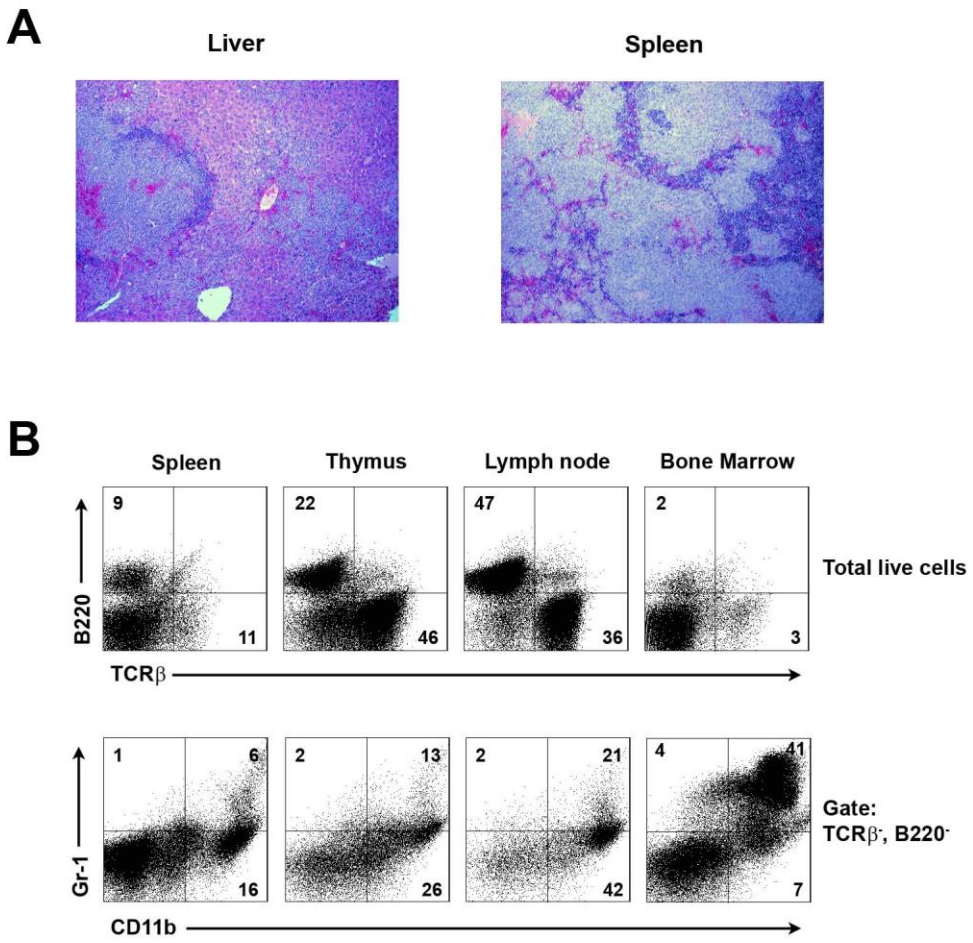

Figure 35. NF1-deficient RASA1 heterozygous mice develop mixed/myeloid dysplastic syndromes..

A, H&E sections demonstrating diffuse large-cell lymphoma, characterized by the infiltration of histiocytes. B, top, abnormal B and T cell distributions are observed in a mouse with a mixed dysplastic syndrome. Bottom, abnormal CD11b expression in non-lymphoid cells. Data are representative of several experiments.

RASA1-NF1 double-deficient mice are predisposed to lymphoma development

Next, we wished to investigate the effects of concomitant induced deletion of RASA1 and NF1 in adult animals. Few studies have been conducted on the interactions between these proteins. We report here the first instance of double-deletion in adult animals. Double deletion of RASA1 and NF1 has been shown previously to result in a more severe embryonic phenotype than does either single deletion, although all are embryonic lethal (29). We considered the possibility that we might observe mice with phenotypic characteristics of both single deletions.

Out of 14 mice observed, we categorized five with erythroid dysplasia, seven with non-myeloid dysplasia, and the remainder with various other disorders (Table 3). Curiously, only one mouse presented with chylothorax, the expected phenotype for a RASA1 single-deficient mouse. We have also observed that induced systemic single-deletion of RASA1 in adult mice does not result in malignancy (unpublished observation). Further, only one case of myeloproliferative disorder, a common presentation in NF1 single-deficient mice, was observed. Clearly, concomitant double deletion did not result in a compounding of single-deletion phenotypes.

The majority of mice showed abnormalities in a specific cell type. The mice that were classified as succumbing to erythroid dysplasia were so categorized due to abnormally high frequencies of $Ter119^{+}$ cells. It is possible that erythroid dysplasia is due to dysregulated K-Ras signaling, which has been reported previously to be the major regulator in erythroid progenitor development (215), although we note that the

observations could have been due to the age of the mice. We did not investigate these cells further.

Our initial observations of lymphoma in double-deficient mice were of grossly enlarged hematopoietic organs that resulted in abdominal swelling and/or labored breathing due to compression of the lungs and diaphragm (Fig. 36). H&E analysis of these organs showed massive infiltration of lymphocytes into the spleens and thymuses of double-deficient animals. We noted the complete loss of splenic red pulp as well as the loss of thymic architecture, as shown by a lack of demarcation between the cortex and medulla regions (Fig. 36).

Using flow cytometry, we investigated the cellularity of the enlarged hematopoietic organs in double-deficient mice. We observed that there were abnormally low frequencies of the Gr-1^{hi}CD$11b^{+}$ and Gr-1^{lo}CD$11b^{+}$ granulocyte and monocyte populations in the spleens and bone marrow of double-deficient mice, indicating that abnormal proliferation in the myeloid compartment was not responsible for the malignancies (Fig. 37A). Further analysis of Gr-1/CD11b double-negative cells indicated abnormally low expression of B220 and TCR β-chain on double-deficient cells, markers of mature B and T cells respectively, indicating the possibility of tumor cells with an immature phenotype (Fig. 37B). We noted that thymocytes from double-deficient animals presented with abnormal expression of TCRβ, indicating that further investigation of the thymic compartment was required.

Time to death (d)	Presentation	Diagnosis
86	Labored breathing; Very enlarged thymus	Thymic lymphoma (thymus, BM, spleen)
349	Enlarged spleen	Erythroid dysplasia
371	Enlarged spleen	Erythroid dysplasia
33	Enlarged heme organs	Thymic lymphoma (thymus, LN, spleen)
85	Enlarged spleen	Erythroid dysplasia
104	Enlarged heme organs	Thymic lymphoma
244	Labored breathing; Very enlarged thymus	Thymic lymphoma
432	Labored breathing; Enlarged spleen	Chylothorax and erythroid dysplasia
112	Labored breathing; Enlarged spleen	Myeloid dysplasia (high GM in spleen, LN)
160	Hemangioma, very enlarged spleen	Undetermined
294	Labored breathing; Very enlarged thymus	Thymic lymphoma (thymus, spleen, LN)
300	Anogenital tumor	Mixed hematopoietic dysplasia
460	Hemangioma	Undetermined
109	Labored breathing; Enlarged spleen	Erythroid dysplasia

Table 3. Analysis of *rasa1*$^{fl/fl}$ *nf1*$^{fl/fl}$ *–ubErt2Cre* mice.

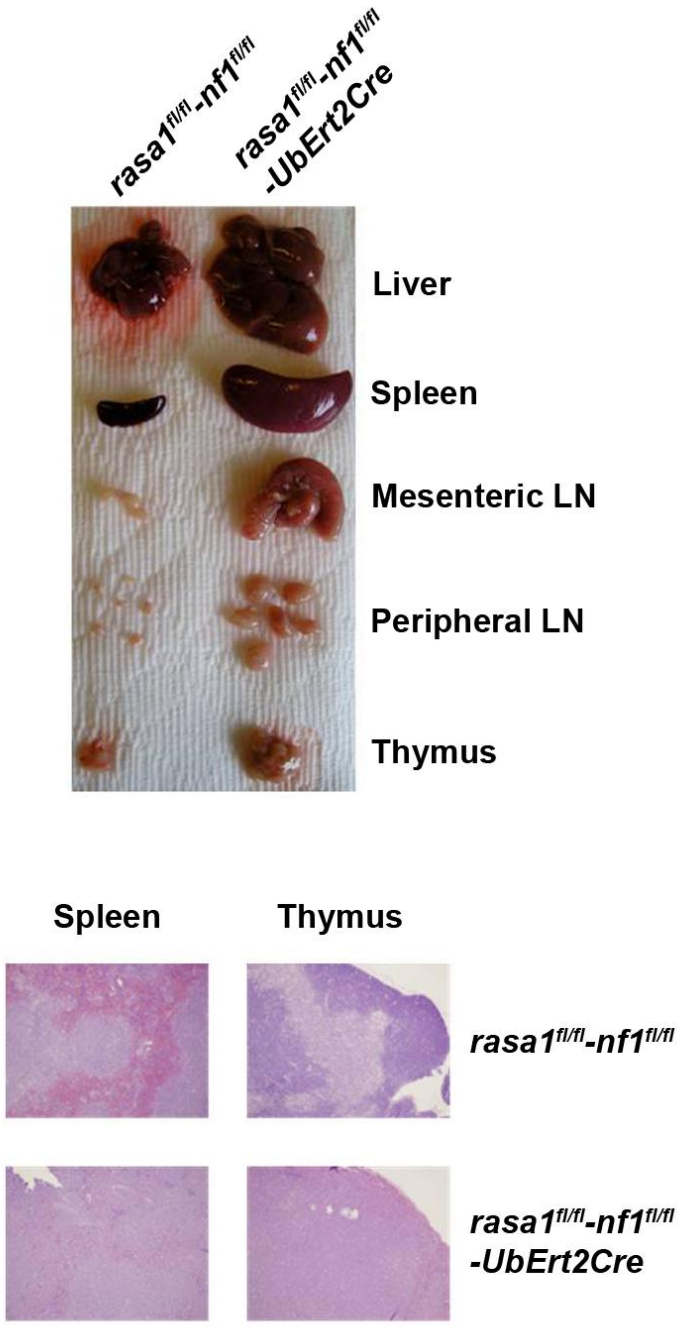

Figure 36. Lymphoma development in RASA1-NF1 double-deficient mice.

Top, hematopoietic organs that had been removed from a control and double-deficient mouse. Bottom, H&E-stained sections of spleen and thymus from the organs shown at top. Observe the loss of splenic red pulp and overall loss of thymic architecture in the double-deficient organs. Data are representative of multiple tumor-bearing mice.

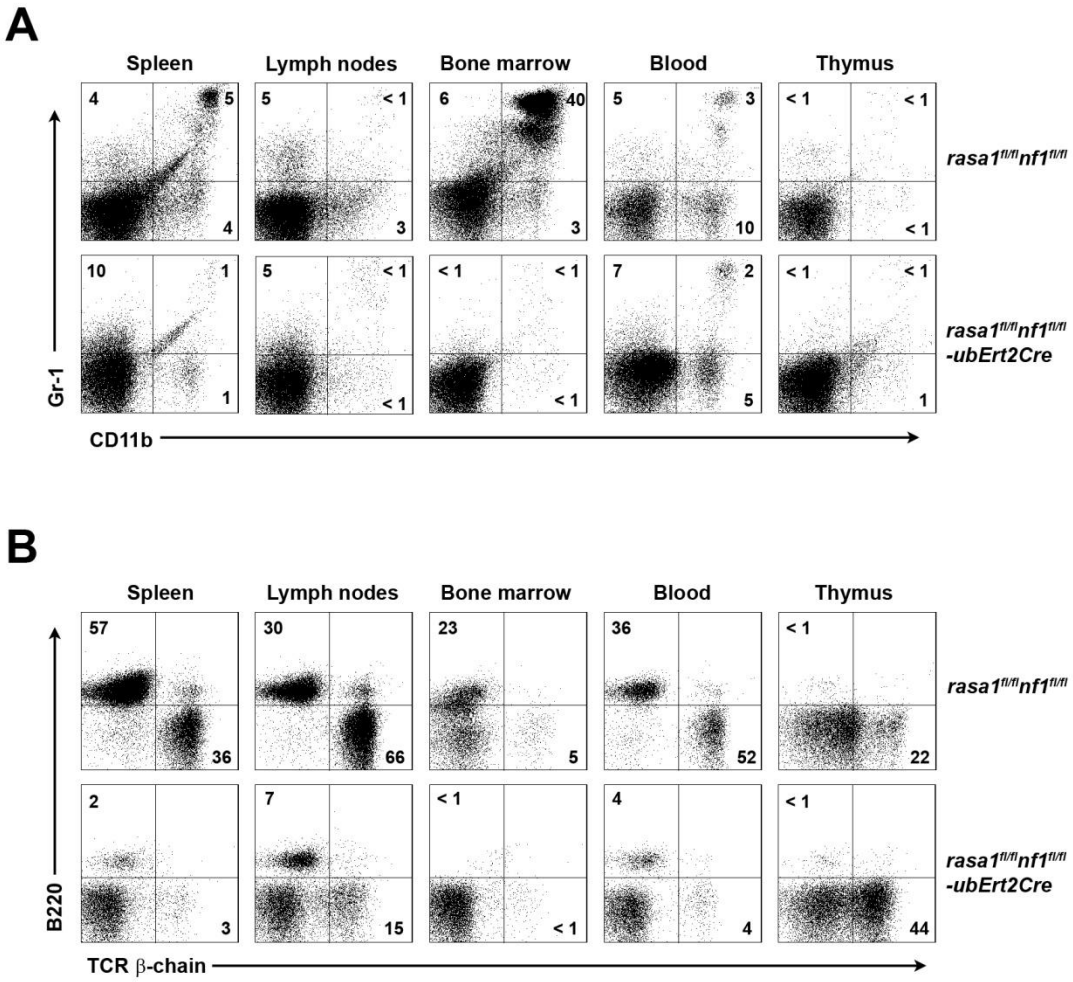

Figure 37. RASA1-NF1 double-deficient mice develop non-myeloid lymphomas.

Live cells from hematopoietic organs of double-deficient and control mice were analyzed by flow cytometry. A, Gr-1 and CD11b expression on live cells. B, B220 and TCR β-chain expression on double-negative cells from (A). Data are representative of multiple experiments.

Characterization of thymic lymphoma resulting from RASA1-NF1 double-deletion

We wished to characterize the malignancies we described in Figure 4.4. We were intrigued that, although we observed abnormally low frequencies of mature hematopoietic cell markers in the peripheral organs, we observed increased TCRβ expression in thymocytes (Fig. 37B). Analysis of hallmark thymocyte developmental subsets in double-deficient animals showed a large expansion of $CD8^+$ cells with varying expression of CD4, indicating the development of thymic lymphoma (Fig. 38A). We next wished to determine if the uncharacterized tumor cells observed in the periphery were in fact $CD4^+CD8^+$ cells of thymic origin. We analyzed $CD8^+$ cells from peripheral hematopoietic tissues for CD4 expression and found that nearly all $CD8^+$ cells in the periphery also expressed high levels of CD4 (Fig. 38A).

As we noted in Figure 37 that the tumor cells were largely TCR-negative, we further analyzed $CD8^+$ cells (which included CD4/CD8 double-positive cells) for TCRβ expression and found that all splenic tumor cells were TCR-negative, as were the majority of $CD8^+$ cells in lymph nodes and blood (Fig. 38B). $CD8^{hi}$ T-ALL thymocytes had been reported to express CD25 at high levels well past the DN3 stage of development, in which it is downregulated in normal thymocytes (20, 216). Using flow cytometry, we determined that nearly all $CD8^+$ cells in the hematopoietic tissues of double-deficient mice expressed high levels of CD25 (Fig. 38B). Thus, the lymphoma cells that we observed were characteristic of previously described murine T-cell acute lymphoblastic leukemia/lymphoma (T-ALL).

Activating Notch1 mutations have been reported in more than half of human and mouse T-ALL cases, and are believed to act either as the causative agent or as an accelerant of disease (217). In particular, up to 50% of murine T-ALL cases were found to possess truncating mutations in the Notch1 PEST domain, which normally facilitates protein degradation through ubiquitinylation (218). In mice, PEST domain mutations alone are not thought to be causative; however, murine T-ALL has been shown to present with RAG-mediated recombination events in *Notch1* that result in the loss of the extracellular Notch1 domain, leaving the active intracellular domain (ICD) largely unchecked (219). Of particular interest to our work are reports that activating Notch1 mutations were found to be causative in multiple studies of murine T-ALL associated with expression of constitutively active KRas (23, 218, 220, 221).

To further confirm that the concomitant loss of RASA1 and NF1 expression was driving the development of T-ALL, we investigated the incidence of Notch1 mutations in tumor cells isolated from double-deficient mice. Western blot analysis of thymocytes from the third thymic lymphoma case (Table 3) showed that the Notch1 (ICD) band, expected at 100 kDa, appeared at a lower molecular weight than did the control sample, suggesting a truncating mutation (Fig. 39A). The increased density of the Notch1 ICD band in the lymphoma cell sample indicates increased protein expression (Fig. 39A). Sequence analysis of DNA from thymic lymphomas demonstrated activating Notch mutations in six of seven cases (Fig. 39B). The majority of mice expressed both Type 1 mutations and PEST domain mutations, which prolong the activity of Notch ICD in the nucleus.

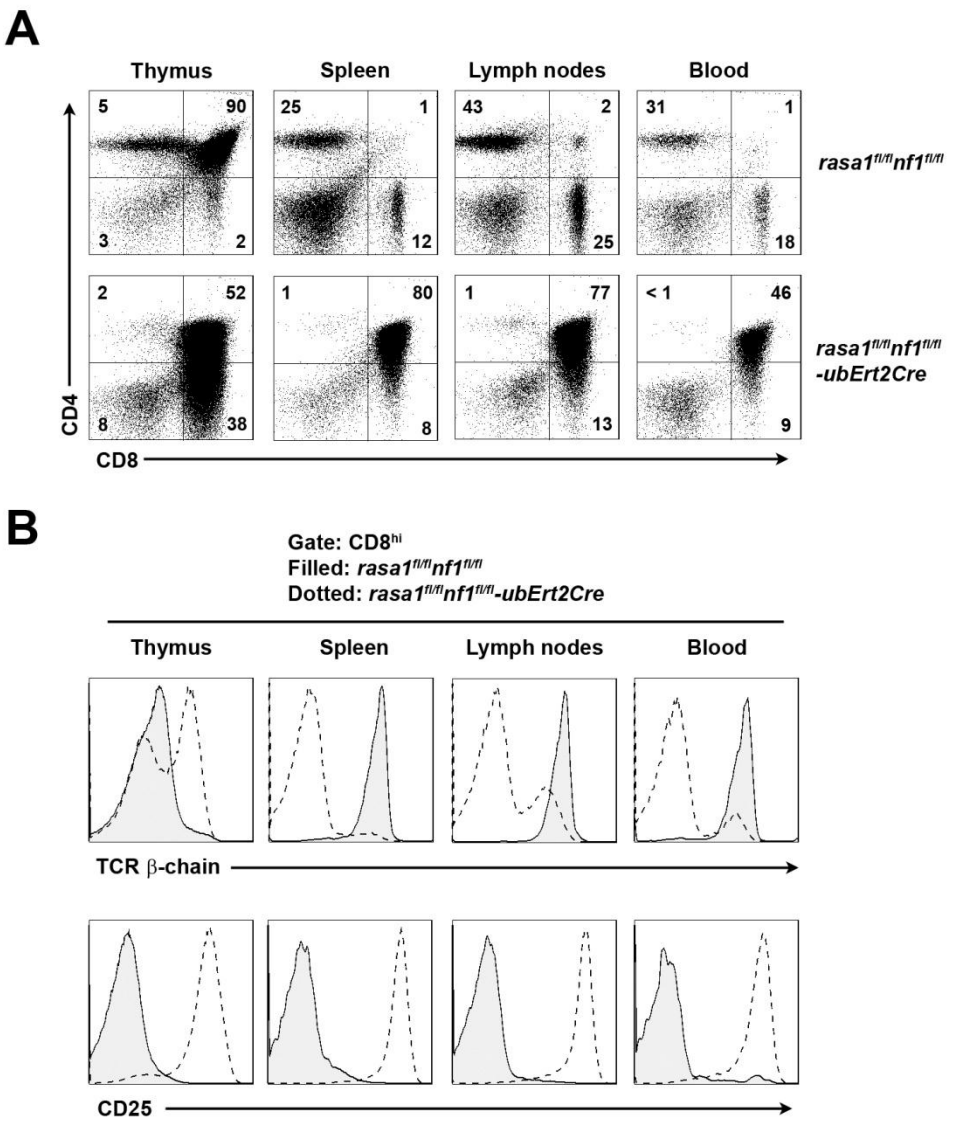

Figure 38. Double deficiency-associated lymphomas originate in the thymus.

A, thymocytes were analyzed for developmental subsets using flow cytometry. Shown are representative CD4 vs. CD8 dotplots of total thymocytes. B, CD8+ cells from hematopoietic tissues were analyzed for the expression of CD4, TCR β-chain, and CD25. Filled histograms represent control mice and dotted open histograms represent double-deficient mice. Data are representative of multiple experiments.

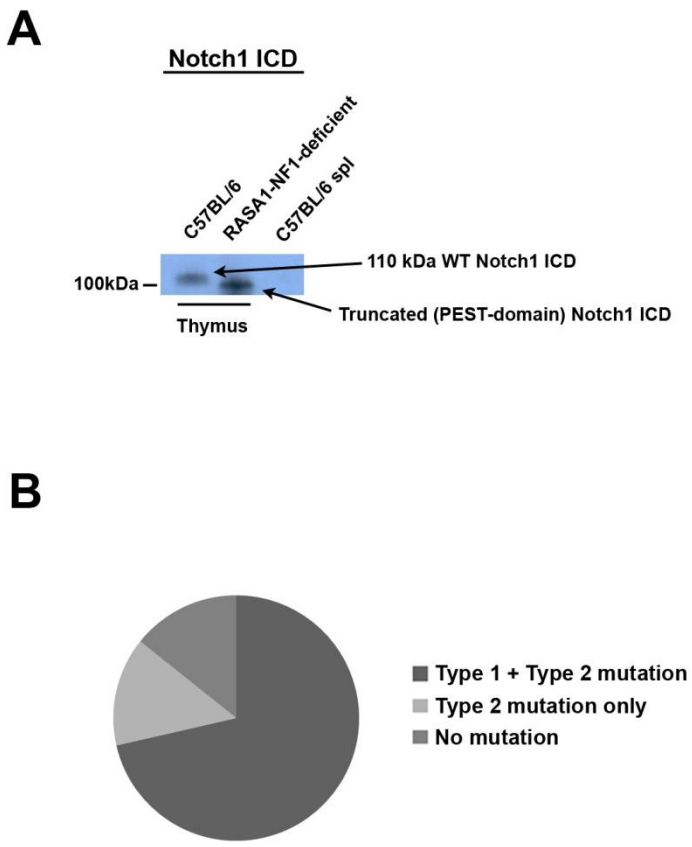

Figure 39. Activating Notch1 mutations in double-deficient thymic lymphomas.

A, C57BL/6 control thymocytes and thymic lymphoma cells were analyzed for the presence of Notch1 ICD truncation via western blotting. At right are C57BL/6 splenocytes, which do not express detectable Notch1 ICD. B, graphical representation of activating Notch1 mutations associated with T-ALL in double-deficient mice. N = 7 mice.

4.5. Discussion

We have demonstrated that induced systemic deletion of NF1 in adult mice results in the development of myeloid dysplasia, similar to that which has been previously reported in murine models of NF1 deletion. We have also reported that heterozygous loss of RASA1 concurrently with deletion of NF1 results in less predictable hematopoietic phenotypes including myeloid dysplasia but also abnormal expansion of lymphoid and erythroid populations. Furthermore, we have shown that concurrent systemic deletion of RASA1 and NF1 in adult mice does not result in the development of diseases that are associated with either single deletion, but instead results in the development of thymic lymphomas with nearly 50% penetrance.

Rasa1 has been shown previously to be haplosufficient in murine fetal development as well as somatic tissue function, although studies of the latter have been limited. For this reason, we were surprised that NF1-deficient mice with heterozygous expression of RASA1 did not simply recapitulate the NF1 single-deficient phenotype. Perhaps in the absence of one normal allele of *rasa1*, NF1 is able to substitute when Ras regulation is needed. Future studies will better illuminate the phenotypes that arise from heterozygous deletion of RASA1 concurrently with full deletion of NF1.

Curiously, in our double-deficient mice, we observed very few instances of disorders that were characteristic of either single-deficient mice. For example, although chylothorax was commonly observed in RASA1-deficient mice, we observed only one such instance in double-deficient mice. Similarly, although NF1-deficiency has

been strongly associated with myeloproliferative disorders in our mice as well as in previously reported models, we observed only one clear instance of myeloid leukemia in double deficient mice. The reasons for this are unclear. We considered the possibility that perhaps disorders such as thymic lymphoma appear so quickly in double-deficient mice that other disorders are not afforded time to develop, but we noted that not all mice develop thymic lymphoma and those that do not develop lymphoma may survive for more than a year, by which time all RASA1 single-deficient mice would have succumbed to chylothorax (45). The most likely explanation is that the simultaneous loss of two RasGAPs results in Ras-induced cellular senescence (222), although it is possible that effects resulting from each deletion are able to cancel out the effects of the other deletion. In future experiments, the interactions of these proteins to regulate hematopoietic cell development and homeostasis must be investigated further.

We further consider that the lack of apparent cardiovascular and neural defects in our induced double-deficient adult mice is quite different from what was observed in homozygous RasGAP null-allele embryos. We did not closely investigate mice for abnormal vasculature or neural tissues, but other than sporadic hemangioma, we observed nothing overt beyond hematopoietic disorders. This could be attributed to unforeseen differences in response to RasGAP deletion between mouse and man. More likely, however, the timing of deletion relative to the age of the mouse is essential. RASA1 and NF1 may be less important to the maintenance of the blood vasculature network in adults, although they are critical for its development *in utero*.

The development of thymic lymphoma in some double-deficient mice is of much potential interest. Interestingly, although NF1 mutations are normally associated with myeloid leukemias in human patients, NF1 inactivation has been reported in the literature as a contributing mutation to T-ALL (157, 223). Notch mutations have also been reported to be a common feature in human and murine T-ALL (23, 157, 217). We described earlier that 6 of 7 double deficiency-associated thymic lymphomas possessed activating Notch1 mutations, similar to a previous report that a majority of human T-ALL have associated Notch1 mutations (224). Type 1 extracellular domain deletion mutations and PEST domain mutations have been described in murine T-ALL associated with activating K-Ras mutations (20, 23), as have PEST domain mutations in human T-ALL (157, 224). We conclude that the acquisition of a Notch1 mutation is the determining factor in the development of T-ALL in our double-deficient mice, although the mechanisms by which this mutation is acquired and by which Notch1 signaling encourages the development of T-ALL have yet to be determined. We expect that this model may provide useful insight into the signaling events involved in Notch-mediated T-ALL.

We also note that, although data have yet to be published, we did not observe abnormalities indicative of oncogenic Ras activity in mice that were RASA1-NF1 double-deficient solely in the T cell compartment, due to transgenic expression of Cre under the control of a T cell-specific promoter. These mice had grossly normal thymocyte numbers and subset frequencies, as well as losses in peripheral T cell numbers similar to what we have previously observed in T cell-specific models of

RASA1 or NF1 single-deficiencies (unpublished observations). Our findings that a majority of mice with thymic lymphoma present with activating Notch1 mutations support the hypothesis that the timing of RASA1 and NF1 double-deletion, relative to T cell development, is critical. Further supporting this hypothesis is the understanding that regulation of Notch signaling is essential during early stages of T cell development. Loss of Notch1 signaling in thymic progenitors results in a loss of thymocytes and the expansion of B cell and myeloid populations (225-227). Conversely, overexpression of constitutively activated Notch1 in hematopoietic stem cells results in excessive T cell development (228). A better understanding of the relationship between the loss of these RasGAPs and the acquisition of Notch mutation is required.

In conclusion, we have presented the first studies of systemic NF1 deletion and RASA1-NF1 double-deletion in adult mice. We have shown that NF1 single-deficient mice succumb to myeloid leukemia, while the additional loss of one RASA1 allele unexpectedly results in more mixed hematopoietic abnormalities. We have reported that nearly half of double-deficient mice develop T cell lymphomas that are phenotypically similar to human and mouse T-ALL. Finally, we have shown that more than half of double-deficient mice with T-ALL present with activating Notch mutations that likely accelerate disease.

Chapter 5

Conclusion

The experiments described thus far have increased our understanding of the regulation of homeostasis in the T cell compartment in mice, from development to effector function to suppression of oncogenesis. Throughout each part of this work, we have investigated the effects of regulations on TCR-activated signal transduction pathways. We believe that this research underscores the complexity involved in understanding how deletion of a single regulatory protein affects the development and maintenance of the T cell compartment. We have also demonstrated the complex interactions between signaling pathways downstream of the TCR and cytokine receptors and the influence of these interactions on T cell effector function, as well as the importance of timing on the deletion of RasGAPs and their roles as tumor suppressors in the T cell compartment.

NF1 as a regulator of T cell development

Our studies of T cell-specific NF1 deletion at an early stage in thymocyte development led us to the conclusion that NF1 does have a moderate effect in the thymus. T cell-specific NF1 deletion, whether homozygous or heterozygous, results in a partial block in transition of cells through the DN stages of development. We found little evidence that other stages of thymocyte development were affected until we investigated clonal thymocytes in HY TCR-transgenic mice. Thymocytes in those mice appeared to have a partial block in the transition from the DP to CD8 SP developmental stages. We were unable to conduct studies of thymocyte development in mice that express a transgenic TCR that responds to peptide bound to MHC class II but expect that, in the future, such studies will yield further information on the role of NF1 in T cell development and $CD4^+$ T cell function.

Although we noted that NF1-deficiency negatively affects peripheral naïve T cell numbers, we were unable to determine the cause behind this phenomenon. NF1-deficient T cells did not appear to be defective in IL-7-dependent survival, AICD, or proliferation. It is possible that NF1-deficient T cells may be hyporesponsive to another peripheral survival signal *in vivo*, leading to death by neglect. It is also possible that NF1-deficiency leads to increased activation of Ras and downstream signaling pathways, resulting in deletion of highly reactive cells as a means to maintain tolerance and lymphopenia-induced proliferation of remaining cells (74).

We observed that NF1-deficient naïve $CD8^+$ T cells were more potent effectors on a

per-cell basis when stimulated *in vitro* with CD3/CD28 mAb. However, we did not observe improved effector function when the cells were stimulated concurrently with IL-2 or IL-4, nor did we observe changes in the per-cell effector responses in $CD4^+$ T cells. Upon cell activation, perhaps other RasGAPs are able to exert enough control over Ras signaling so as to maintain normal cell function in the CD4+ T cell compartment. It is also possible that our *in vitro* experiments are of limited physiological relevance. We did observe that T cell-specific NF1-deficient mice more efficiently responded to *L. monocytogenes* infection, so perhaps abnormalities in NF1-deficient T cells are more evident in an *in vivo* infection model. We note that pathogen-specific T cells would reside in the naïve cell compartments prior to infection, and that the increased effector capabilities observed in NF1-deficient naïve CD8+ T cells are likely responsible for the improved response to infection that we observed. However, we have not yet studied NF1-deficient T cells *ex vivo* after infection with *L. monocytogenes* to determine if numbers or frequencies of pathogen-specific T cells are significantly different, or if increased effector function was evident upon restimulation. We have also not studied memory T cell responses in NF1-deficient mice in response to pathogen immunization and challenge.

IL-4 as a promoter of CD8+ T cell effector function

At first glance, IL-4 might seem to be the odd man out in this story. In fact, the IL-4 experiments had their origins within our investigations into the role of NF1 in T cells. IL-4 caught our attention as, when used as a control for IL-2 in the *in vitro* activation

of $CD8^+$ T cells, it broadly stimulated cells in both the NF1-deficient and control cells to produce very high amounts of IFN-γ. Although IL-4 has been reported to promote $CD8^+$ T cell effector functions, little attention had been paid to the specifics of the effector functions or the cellular mechanisms that drove them.

We have shown that IL-4 is able to stimulate *ifng* gene expression *de novo* in $CD8^+$ T cells via the upregulation of the transcription factors Eomes and T-bet, which we believe to be exciting and novel information that sheds new light on the abilities of cytokines to induce a variety of responses in different cell types. IL-4 has been long understood as the master cytokine behind the type 2 T helper cell (Th2) immune response and has thus been studied more widely in those cells. Canonical knowledge holds that, in Th2 cells, IL-4 signaling downregulates and even blocks IFN-γ expression. As $CD8^+$ T cells that are differentiated under CTL type 2 skewing conditions also produce IL-4, it was expected that IL-4 induced similar gene activation and repression in $CD8^+$ T cells as in $CD4^+$ T cells. We have clearly demonstrated that this is not so, and we expect that future studies will demonstrate IL-4-driven IFN-γ expression is the primary effector in models of infection that require IL-4 to promote efficient CTL responses and pathogen clearance.

IL-4 had been reported in $CD4^+$ T cells to activate the PI3K pathway as well as to promote the activation of the ERK MAPK (229). IL-4 was similarly reported to activate those pathways in previously stimulated $CD8^+$ T cells (193). It made sense to target those pathways in our in our studies. We observed that both PI3K and ERK

activation are required for the full induction of IL-4-mediated IFN-γ expression. We are uncertain of all the signaling events involved: certainly ERK and PI3K can be activated by Ras, and it is likely that further investigation would show induction of Ras signaling. We are also unsure of the mechanisms linking PI3K and ERK activation in IL-4-stimulated cells to the upregulation of Eomes and T-bet. One possibility is the activation of the mTOR pathway downstream of PI3K. mTOR signaling has been reported to enhance T-bet expression and repress Eomes expression downstream of the IL-12 receptor in $CD8^+$ T cells (116). Although preliminary experiments with Rictor-deficient $CD8^+$ T cells, in which the mTOR2 pathway is inactivated, indicated that mTOR2 is most likely not a factor (data not shown), it is possible that the mTOR1 pathway may be modulated downstream of the IL-4 receptor.

Although we have conducted a thorough analysis of the direct effects of IL-4 on IFN-γ expression in $CD8^+$ T cells *in vitro*, we have yet to demonstrate the relevance of these findings *in vivo*. $CD8^+$ T cells isolated from IL-4-deficient mice following inoculation with *Schistosoma mansoni* eggs and immune challenge with *Schistosoma* egg antigen failed to show a significant change in IFN-γ expression upon restimulation *ex vivo*, although moderate reductions were observed in Eomes expression (unpublished data). It is possible that the pathogen model used was not appropriate for $CD8^+$ T cell studies, although it has been reported to induce Th2 differentiation *in vivo* (230). Further, isolated $CD8^+$ T cells from mice that had been previously infected with live schistosomes were found to express IFN-γ upon *ex vivo*

restimulation with antigen, although the mechanism behind this phenomenon was not explored (184). It is possible that IL-4-deficient mice are able to induce alternate responses *in vivo* to ensure appropriate immune responses against pathogens, thus masking the effects of IL-4 deficiency in our experiments.

RASA1 and NF1 as tumor suppressors in the T cell lineage

We are excited about the finding that concomitant systemic deletion of RASA1 and NF1 in adult animals can lead to the development of T-ALL, and that we observe Notch1 mutations in our murine T-ALL model at a similar frequency to that observed in human T-ALL (221). Although these data further our knowledge of RasGAP tumor suppressor activity in T cells as well as of factors that contribute to T-ALL initiation and severity, we are left with questions.

First, what factor (or factors) initiates T-ALL in these mice? With one exception, we have only observed T-ALL development in mice that were double-deficient for RASA1 and NF1, and of those, fewer than half developed T-ALL. Although RASA1 and NF1 are both known to regulate the activity of K-Ras, a known oncogene in T-ALL (20, 23), clearly the loss of both RasGAPs is not enough to favor T-ALL tumorigenesis. As noted, activating Notch1 mutations were found in all but one of the T-ALL cases observed in double-deficient mice. We suspect that the T cell tumors that we have observed are clonal in nature; perhaps these cells derive from a single

mutated bone marrow progenitor. It would be of great interest to look at other transcription factors and signaling pathways that are known to be active and non-redundant in thymocyte progenitor or early T cell development as perhaps one or more of these can also act as an oncogene. Constitutively activated Notch has been reported to bypass RAG-mediated recombination of the genes that code for the TCR, a crucial thymocyte developmental checkpoint, in RAG-deficient mice (231). Mutations in the DNA-binding transcriptional regulator Ikaros, which plays a non-redundant role in thymocyte development, have also been reported in murine T-ALL along with activating Notch mutations (232, 233).

Second, what mechanistic changes in signal transduction and regulation might account for the phenotypes observed in double-deficient mice, as opposed to those observed in single RasGAP-deficient mice? As noted in chapter 4, RASA1 is indispensable in the maintenance of lymphatic vasculature, while NF1 is essential as a tumor suppressor in the myeloid hematopoietic cell lineages; yet with few exceptions, we observe neither phenotype in double-deficient mice. A better understanding of the interactions between RasGAP proteins is required to explain this phenomenon.

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