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Distinct Functions for Type I and Type II Interferon in Cancer Immunoediting

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DISTINCT FUNCTIONS FOR TYPE I AND TYPE II INTERFERON IN CANCER IMMUNOEDITING

by

Mark Stephen Diamond

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ABSTRACT OF THE DISSERTATION

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The idea that a functionally intact immune system can protect against cancer development forms the basis of the long-postulated and once controversial concept of cancer immunosurveillance. A substantial body of evidence, however, now exists defining a role for immunity in extrinsic tumor suppression – work that broadened our understanding of the tumor-immune interface and led to the cancer immunoediting hypothesis. The interferons, both type I (IFNα/β) and type II IFN (IFNγ), are critical mediators of cancer immunoediting, yet their respective roles in promoting anti-tumor immune responses remain unclear. Herein, we have examined the actions of IFNα/β and IFNγ during tumor rejection, providing evidence for distinct functions on the host as well as the tumor.

We have established that host hematopoietic cells represent important targets of IFNα/β's actions, however these cytokines can have potent stimulatory effects on both innate and adaptive immune cells. Using bone marrow chimeras, we demonstrated that IFNα/β sensitivity within innate immune cells, but not T or B lymphocytes, was essential

for the priming of tumor-specific T cells and the generation of protective immunity. Whereas NK cells were not required for IFNα/β-dependent tumor rejection, $CD8\alpha^+$ dendritic cells were critical, and the direct actions of type I IFN on $CD8\alpha^+$ DCs enhanced antigen cross-presentation.

When we instead examined the requirements for IFNγ during tumor rejection, we observed an important function for both hematopoietic and nonhematopoietic host cell sensitivity, as well as a more prolonged duration of action. Selective reconstitution and RNAi knockdown of IFNGR1 also corroborated the importance of tumor cell responsiveness to IFNγ, but not IFNα/β.

As exogenous type I IFN has shown clinical efficacy in cancer therapy, we performed similar studies using a model of IFNβ immunotherapy. We observed that local production of IFNβ could induce either reversible tumor equilibrium (at higher doses) or elimination (at lower doses). The effects of high-dose IFNβ were independent of adaptive immunity and required hematopoietic and nonhematopoietic IFNα/β responsiveness; in contrast, tumor elimination with low-dose IFNβ required adaptive immunity and responsiveness only in hematopoietic cells. Collectively, these studies add to our understanding of the protective functions of the interferons during anti-tumor immunity.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ADCC	antibody-dependent cellular cytotoxicity
APC	antigen-presenting cell
CPE	cytopathic effect
DC	dendritic cell
E14.5	embryonic day 14.5
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FLC	fetal liver cell
GAS	gamma-activated site
GFP	green fluorescent protein
HBSS	Hank's buffered saline solution
HSC	hematopoietic stem cell
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IFNα	interferon alpha
IFNα/β	type I interferon
IFNAR1	interferon alpha/beta receptor chain 1
IFNAR2	interferon alpha/beta receptor chain 2
IFNβ	interferon beta
IFNγ	interferon gamma
IFNGR1	interferon gamma receptor chain 1
IFNGR2	interferon gamma receptor chain 2
IL	interleukin
IRF	interferon regulatory factor
ISG	interferon stimulated gene

ISRE	interferon stimulated response element
JAK	janus kinase
LC	langerhans cell
LPS	lipopolysaccharide
mAb	monoclonal antibody
MCA	3′-methylcholanthrene
MDSC	myeloid-derived suppressor cell
MEF	mouse embryonic fibroblasts
MHC	major histocompatability complex
NK	natural killer
NKT	natural killer T
NO	nitric oxide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pDC	plasmacytoid dendritic cell
PRR	pattern recognition receptor
RAG	recombinase activating gene
RNAi	RNA interference
s.c.	subcutaneous
SA-APC	streptavidin-allophycocyanin
SA-PE	streptavidin-phycoerythrin
SCID	severe combined immunodeficiency
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TLR	Toll-like receptor
Treg	T regulatory cell
WT	wild type

CHAPTER 1

Introduction

THE EVOLUTION OF CANCER IMMUNOSURVEILLANCE

With the elucidation of molecular pathways normally regulating cell growth and differentiation, and their obligatory subversion by cancerous cells, the recognition of a common set of 'intrinsic' tumor suppressor mechanisms has emerged (1, 2). The idea that the immune system could also provide a barrier to cancer development by serving in an 'extrinsic' tumor suppressor capacity to eliminate or control neoplastic cells, is a notion that has long been postulated yet only more recently demonstrated experimentally. Originally proposed nearly a century ago by Paul Erlich, this view was more formally articulated some fifty years later by Macfarlane Burnet and Lewis Thomas (3). Prompted by the then recent demonstration that specific immune recognition of tumors could in fact occur, Burnet and Thomas postulated that the immune system may function to suppress the natural development of tumors – a theory that became known as 'cancer immunosurveillance' (4). Subsequent efforts to test this hypothesis using animal models, however, yielded little support for 'cancer immunosurveillance,' and by 1980 this idea had been largely abandoned (3).

In retrospect, early attempts to study possible immune control of tumor formation were limited by the experimental tools available at the time, and only more recently was this hypothesis revisited and rigorously investigated using molecularly-defined models of immunodeficiency (3). Indeed, a growing body of work over the past fifteen years has prompted a revival of the original idea of cancer immunosurveillance; yet, recent data

have also added new layers of complexity to the initial premise, leading to the formulation of the 'cancer immunoediting' hypothesis (3, 5, 6).

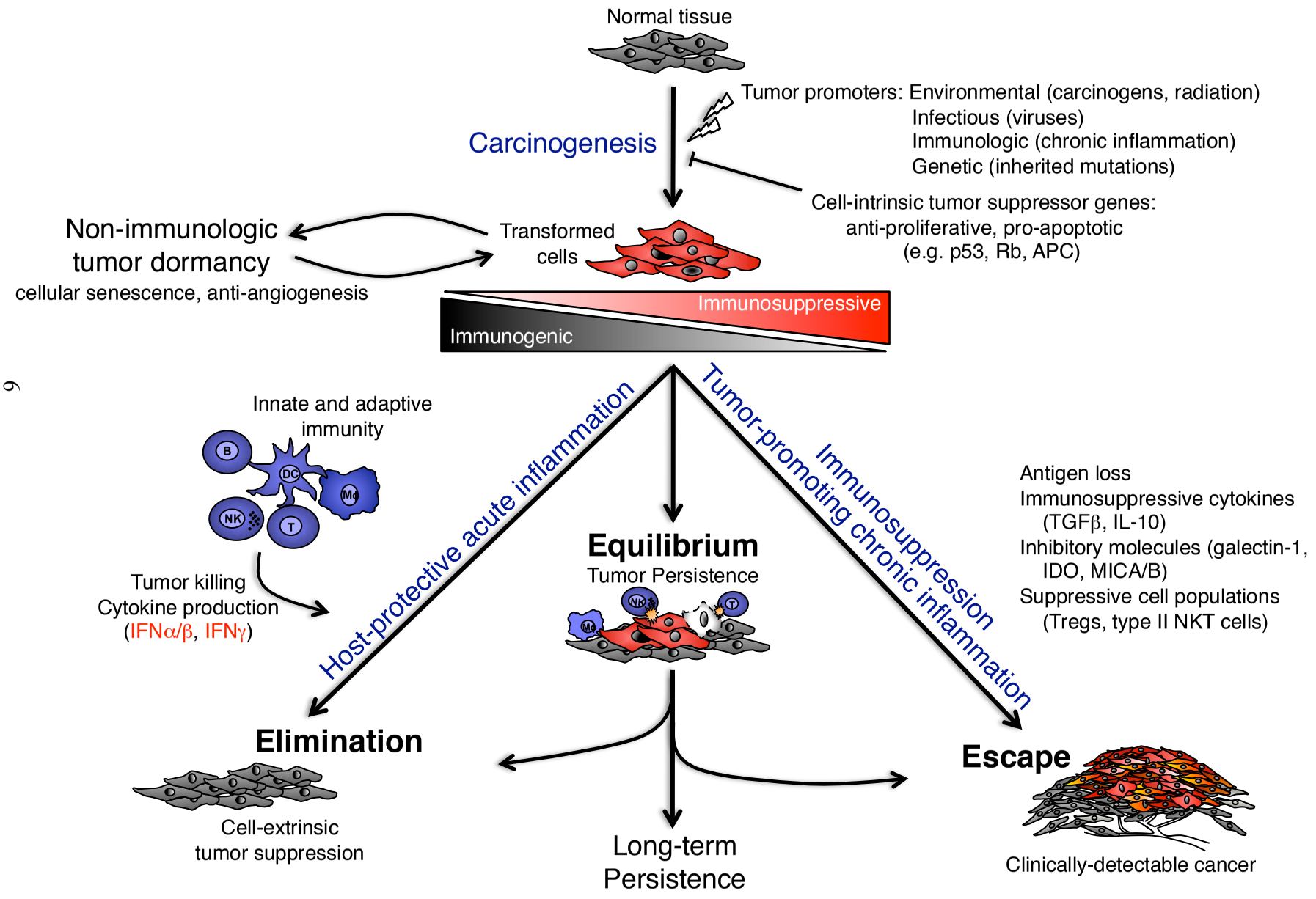
THE CANCER IMMUNOEDITING HYPOTHESIS

The concept of 'cancer immunoediting' emphasizes the tumor-sculpting as well as the protective activity of the immune system, and describes host-tumor interactions in the framework of three phases: elimination, equilibrium, and escape (7, 8) (Figure 1). The elimination phase, which encompasses the original immunosurveillance idea, involves the successful recognition and eradication of a developing tumor. A failure of these immune mechanisms may lead to the equilibrium or escape phases. During the equilibrium phase, tumor growth and immune destruction enter into a dynamic balance wherein an increasingly heterogeneous tumor is subjected to the sculpting forces of immunity. This process may then result in the emergence of a clinically apparent tumor which has escaped the selective pressure of the immune system.

Work from numerous laboratories has implicated a number of different cellular components and immune effector molecules in host protection against tumor development, including IFNγ, perforin, αβ T cells, γδ T cells, NKT cells, NK cells, IL-12, TRAIL, NKG2D, and IFNα/β (3, 9-11). Yet, while some of the important effectors are known, the processes leading to the initiation and progression (or failure) of a naturally occurring anti-tumor immune response remain incompletely understood.

Evidence suggesting a dynamic relationship between the immune system and developing tumors emerged from studies comparing tumors arising in different immunologic environments. Specifically, tumor transplantation experiments demonstrated that methylcholanthrene (MCA)-induced fibrosarcomas arising in immunodeficient RAG2$^{-/-}$ mice were, as a group, more immunogenic than MCA-induced tumors from WT mice (5). Whereas both sets of tumors grew progressively with similar kinetics in RAG2$^{-/-}$ mice, when transplanted into syngeneic immunocompetent hosts, 8/20 RAG2$^{-/-}$-derived tumors (termed RAG2$^{-/-}$ regressors) were rejected, in contrast to 0/17 WT-derived tumors. Therefore, the immunogenicity of these tumors was shaped by the immune environment in which they developed. In addition, the increased immunogenicity of tumors developing in other immunodeficient hosts – including nude, SCID, TCR $J_{\alpha}281^{-/-}$, perforin$^{-/-}$, and IFNAR1$^{-/-}$ mice – has been demonstrated (12-16). These observations thus prompted a refinement of the original cancer immunosurveillance theory into the current concept of cancer immunoediting.

Figure 1. Cancer immunoediting: a unifying principle describing the spectrum of tumor-immune system interactions. The progression of normal cells into clinically apparent cancers is facilitated by oncogenic stimuli and inhibited by both cell-intrinsic and cell-extrinsic tumor suppressor mechanisms. The cancer immunoediting process, which encompasses the tumor-protective and/or tumor-promoting activities of host immunity, is represented by the three proposed phases – elimination, equilibrium, and escape.


Normal tissue
Carcinogenesis
Tumor promoters: Environmental (carcinogens, radiation)
Infectious (viruses)
Immunologic (chronic inflammation)
Genetic (inherited mutations)
Cell-intrinsic tumor suppressor genes:
anti-proliferative, pro-apoptotic
(e.g. p53, Rb, APC)
Non-immunologic
tumor dormancy
cellular senescence, anti-angiogenesis
Transformed
cells
Immunosuppressive
Immunogenic
Innate and adaptive
immunity
B
DC
Mϕ
NK
T
Tumor killing
Cytokine production
(IFNα/β, IFNγ)
Host-protective acute inflammation
Tumor-promoting chronic inflammation
Equilibrium
Tumor Persistence
Antigen loss
Immunosuppressive cytokines
(TGFβ, IL-10)
Inhibitory molecules (galectin-1,
IDO, MICA/B)
Suppressive cell populations
(Tregs, type II NKT cells)
Elimination
Escape
Cell-extrinsic
tumor suppression
Long-term
Persistence
Clinically-detectable cancer

THE INTERERONS IN CANCER IMMUNOEDITING

Strong experimental evidence exists in support of a protective cancer immunosurveillance system, and the IFNs have emerged as critical components. In fact, early studies on the role of IFNγ in anti-tumor immunity were largely responsible for triggering a resurgence in the previously discarded theory of cancer immunosurveillance. Some of the key data implicating the IFNs in the cancer immunoediting process will now be reviewed.

Evidence for IFNγ-dependent Cancer Immunoediting

The initial findings supporting a role for endogenous IFNγ in anti-tumor immunity emerged from studies of the Meth A fibrosarcoma, an MCA-induced tumor derived from BALB/c mice. Although this tumor grows progressively when transplanted into syngeneic WT mice, its rejection can be induced upon intraperitoneal injection of a sublethal dose of bacterial lipopolysaccharide (LPS). Using a neutralizing mAb specific for IFNγ, it was discovered that blockade of endogenous IFNγ prevented the rejection of Meth A cells following LPS injection (17). Additional experiments revealed the central importance of IFNγ's actions on the tumor during this process, since abrogation of IFNγ sensitivity in Meth A cells – via overexpression of a dominant-negative IFNGR1 construct (IFNGR1.ΔIC) – also blocked LPS-mediated rejection. Importantly, it was also observed that (i) Meth A cells grew more rapidly in mice treated with anti-IFNγ mAb than in untreated mice, and (ii) IFNγ-unresponsive Meth A.IFNGR1.ΔIC cells displayed

more aggressive growth in unmanipulated WT mice than the IFNγ-responsive parental tumor. These studies therefore indicated that endogenous IFNγ, in the absence of an external LPS stimuli, had anti-tumor activity mediated at least in part through its actions directly on the tumor.

These results using transplantable tumor models prompted an examination of the role of IFNγ during the development of primary carcinogen-induced and spontaneous tumors. Such studies demonstrated that IFNγ-insensitive IFNGR1$^{-/-}$ or STAT1$^{-/-}$ mice on a 129 background developed MCA-induced tumors at a higher incidence and with a shorter latency than WT 129 control mice (18). In addition, when bred onto a p53-null genetic background, IFNγ-insensitive p53$^{-/-}$xIFNGR1$^{-/-}$ and p53$^{-/-}$xSTAT1$^{-/-}$ mice developed spontaneous tumors more rapidly than IFNγ-sensitive p53$^{-/-}$ control mice. Moreover, the spectrum of tumors discovered in the IFNγ-unresponsive doubly deficient mice was considerably more diverse, including a variety of non-lymphoid tumors, compared to the overwhelming development of lymphoid cancers in control mice. Additional work using gene-targeted mice on a C57Bl/6 strain that instead lack the IFNγ gene has also demonstrated an increased incidence of both carcinogen-induced and spontaneous tumors (15, 19).

In a subsequent study, the incidence of MCA-induced tumor formation in IFNGR1$^{-/-}$ and STAT1$^{-/-}$ mice was also compared with those in lymphocyte deficient RAG2$^{-/-}$ mice and in RAG2$^{-/-}$xSTAT1$^{-/-}$ mice lacking both lymphocytes and IFN responsiveness (5). Compared to genetically-matched WT control mice, each of these immunodeficient strains developed nearly three times the number of tumors following

carcinogen treatment. Based on these results, it was postulated that the mechanisms of tumor protection afforded by IFNγ and lymphocytes were largely overlapping. Taken together, the aforementioned studies – on both 129 and B6 genetic backgrounds, and using models of transplantable, carcinogen-induced, and spontaneous tumor development – have conclusively shown that IFNγ is an integral component of the protective cancer immunosurveillance system.

IFNγ's Actions on the Tumor

Initial studies using Meth A and MCA-207 revealed the importance of IFNγ's actions on the tumor cell (17), and the significance of this mechanism has been strengthened by a large amount of additional evidence. Analysis of MCA sarcomas from IFNGR1$^{-/-}$ mice showed them to be highly tumorigenic in WT mice (18). Yet, when their ability to respond to IFNγ was restored by introduction of the missing IFNGR1 receptor component, these tumors were now rejected by WT mice in a lymphocyte-dependent manner. If instead, components of the antigen processing and presentation pathway such as TAP1 and H-2D^{b} (known to be modulated by IFNγ) were constitutively expressed in IFNγ-insensitive tumor cells, they were also now rejected (5) (A.T. Bruce and R.D. Schreiber, unpublished data). These findings therefore indicate that the enhancement of tumor cell immunogenicity is an important downstream mediator of IFNγ's actions on the tumor. More recently, additional evidence was obtained from studies utilizing RAG2$^{-/-}$ regressor tumors, which are highly immunogenic and are rejected in WT mice. When RAG2$^{-/-}$ regressor cells (normally IFNγ responsive) were rendered insensitive to IFNγ

through overexpression of the dominant-negative IFNGR1.ΔIC construct, these cells now grew progressively in WT hosts (G.P. Dunn, C.M. Koebel, and R.D. Schreiber, unpublished data).

A logical extension of the prominent role for IFNγ's actions on the tumor is that some tumor cells may develop IFNγ insensitivity as a mechanism of immune escape. Indeed, one analysis of a panel of human lung adenocarcinoma cell lines revealed that 4/17 contained identifiable defects in the IFNγ signaling pathway which caused these cells to be IFNγ unresponsive (18). Similarly, other downstream components affecting tumor immunogenicity, such as MHC class I, TAP1, and β_2-microglobulin, have been found to be lost in a variety of tumors (20). Insight into the mechanism of IFN insensitivity was provided by a recent study showing that Jak1 expression in the LNCaP human prostate cancer cell line was repressed by epigenetic mechanisms (21).

IFNγ's Actions on the Host

Recent studies in our laboratory aimed at defining the host requirements for rejection of immunogenic RAG2$^{-/-}$ regressor tumors have also demonstrated a role for IFNγ's actions on host cells (G.P. Dunn, C.M. Koebel, and R.D. Schreiber, unpublished data). When a panel of 129 RAG2$^{-/-}$-derived sarcomas normally rejected in immunocompetent hosts were injected into syngeneic IFNGR1$^{-/-}$ mice, many were found to grow progressively. Since analyses of these same tumors using the IFNGR1.ΔIC construct or RNAi (see Chapter 4) to inhibit IFNγ sensitivity also showed the tumor cell to be an essential target, we could now conclude that both the tumor and the host were

relevant sites of IFNγ's actions during tumor rejection. These data are also consistent with prior studies indicating a role for IFNγ responsiveness at the level of the host, though this work relied on adoptive transfer or immunization and rechallenge tumor models.

In one such report, a $CD4^+$ T cell-mediated adoptive transfer model was employed involving transfer of activated tumor-specific $CD4^+$ T cells into SCID mice challenged with 6132A-PRO tumor cells (22). Rejection of this tumor challenge was found to require IFNγ since it could be blocked by treatment with anti-IFNγ mAb. Yet, tumor cell sensitivity was not required, prompting the conclusion that indirect effects of IFNγ on host cells, rather than on the tumor, was the key determinant of rejection. Although this work highlights the potential importance of host IFNγ responsiveness during tumor rejection, the observation that IFNγ's actions on the tumor were not required likely reflect the particular model used. Indeed, in our own work we have noted that rejection can also occur independent of tumor cell IFNγ sensitivity if mice are previously immunized with or reject the IFNγ-sensitive tumor cell counterpart (A.T. Bruce and R.D. Schreiber, unpublished data). This observation may merely reflect a lower MHC class I threshold for reactivation of previously activated or memory T cells compared to naïve T cells.

Another study using an immunization and rechallenge model primarily mediated by $CD4^+$ T cells also pointed to a prominent role for host cell IFNγ responsiveness (23). In this model, mice previously immunized with irradiated Mc51.9 tumor cells rejected a second challenge with live cells two weeks later, and this rejection was found to require

host cell IFNγ sensitivity. Interestingly, in this model IFNγ's actions on the host were only required during the effector phase, but not during priming of tumor-specific T cells. This conclusion was based on adoptive transfer experiments showing that splenocytes from immunized WT or IFNGR1$^{-/-}$ mice could provide equal protection when transferred into WT recipients but had no effect in IFNGR1$^{-/-}$ recipients. Subsequent experiments using reciprocal bone marrow chimeras with limited IFNGR1 expression then showed that sensitivity to IFNγ within nonhematopoietic host cells was necessary and sufficient for tumor protection in this model. Given that T cell responses and tumor infiltration by immune cells appeared to be comparable in WT and IFNGR1$^{-/-}$ hosts, the authors propose that angiostatic effects of IFNγ acting on host stromal cells are required for tumor inhibition. In support of this idea, immunostaining of tumor tissue appears to show reduced infiltration of CD31^{+} endothelial cells in WT mice compared to IFNGR1$^{-/-}$ mice.

In a later study by the same group, these observations were also extended to tumor models requiring CD8^{+} T cells for rejection (24). Again, however, rejection relied wholly on prior immunization with irradiated tumor cells. Nevertheless, a correlation between tumor rejection and decreased angiogenesis was observed. In addition, experiments using IFNγ-deficient or perforin-deficient mice indicated that IFNγ production but not perforin-mediated killing was responsible for the inhibition of tumor growth. Although the two previously described studies support the idea that anti-angiogenesis may be central to the effects of IFNγ on the host, it will be important to test this hypothesis in models of naturally-occurring immune responses to rejectable tumors in naïve mice.

Additional mechanisms for IFNγ's actions on the host have also been suggested, including its well-established function in promoting Th1 cell development as well as a more recently proposed role in inhibiting the activity of $CD4^+CD25^+$ T regulatory cells (Treg). Using $STAT1^{-/-}$ mice, it was shown that rejection of an immunogenic P815 tumor variant was impaired in the absence of host IFN responsiveness, though effects of IFNγ versus IFNα/β could not be distinguished (25). Nevertheless, this defect was found to correlate with a lack of T cell cytotoxic activity. In another study, it was demonstrated that the actions of IFNγ could modulate the generation/activation of Foxp3-expressing $CD4^+CD25^+$ Tregs following immunization against SEREX-defined self-antigens (26). Whereas immunization with these self-antigens has been shown to promote the generation and activity of Tregs and lead to enhanced tumor development, the presence of IFNγ effectively prevented this effect. Thus, early production of IFNγ may provide a key regulatory mechanism controlling the balance between suppressive $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Th cells promoting CTL responses. While the source of IFNγ was suggested to be $CD8^+$ T cells, it remains unclear if Tregs were the direct targets or if an indirect mechanism was involved.

Evidence for IFNα/β-dependent Cancer Immunoediting

Early Studies on Exogenously Delivered IFNα/β

Although type I IFN was originally identified due to its potent antiviral activity, the observation that it also had substantial growth inhibitory effects incited early investigations into its use against cancer (27). Several early studies indeed demonstrated

the efficacy of exogenous type I IFN treatment in murine tumor models, and these effects were presumed to be due to IFNα/β's direct actions on tumor cells. In Balb/c and C57Bl/6 mice injected with the RC19 and EL4 tumors, enhanced survival times were noted in mice treated with relatively crude interferon preparations (28, 29). These observations were later extended to the murine leukemia L1210 tumor cell line (30). The anti-tumor effects of exogenous IFNα/β, however, were also observed in mice injected with an IFNα/β-insensitive subline of L1210 (30, 31), suggesting that the activity of type I IFNs on host cells was perhaps more critical in mediating its effects. Subsequent experiments with IFNα/β-insensitive or IFNα/β-sensitive clones of Friend leukemia cells supported this finding as the effects of exogenous IFNα/β treatment in prolonging survival or inhibiting visceral metastases was equivalent regardless of tumor cell IFN responsiveness (32-34). More recent studies using the AGS-1 melanoma cell line, derived from a STAT1-deficient mouse, also showed that tumor cell IFNα/β responsiveness was not required for the antitumor effects of exogenously administered IFNα; in contrast, the effects of IFNα in this model were abrogated in $STAT1^{-/-}$ mice, even upon challenge with STAT1-reconstituted AGS-1 or WT-derived melanoma lines (35) Collectively, the findings from several murine models of tumor immunotherapy underscore the importance of host-dependent mechanisms in the efficacy of exogenous IFNα/β. In addition to exogenous treatment, various studies have demonstrated the immunotherapeutic effects of ectopically expressing IFNα/β in otherwise poorly immunogenic tumors cells (36, 37).

The administration of IFNα/β has also shown some efficacy in the clinical treatment of human cancer patients, though effectiveness has been demonstrated only in certain tumor types. The first observation of a therapeutic effect for recombinant IFNα was in the treatment of hairy cell leukemia, and it was approved by the FDA for this use in 1986 (27, 38). Since then, subsequent studies have shown beneficial effects for the treatment of over 14 types of human cancers including both hematological malignancies (e.g. hairy cell leukemia and chronic myeloid leukemia) and solid tumors (e.g. melanoma, renal cell carcinoma, and Kaposi's sarcoma) (39). Despite the fact that IFNα is now the most frequently used cytokine in patients, little is known about the mechanisms underlying its clinical efficacy (37). It is also unclear why some tumors are responsive to IFNα/β treatment yet others do not seem to be affected.

Critical Function for Endogenous IFNα/β

In addition to the therapeutic effects of exogenous IFNα/β, studies using murine tumor models have also demonstrated a role for endogenously produced IFNα/β during anti-tumor immune responses. Initial studies used polyclonal antiserum to murine IFNα/β to show that neutralization of the type I IFNs enhanced the growth of xenogeneic tumors in athymic nude mice (40). In subsequent studies, decreased survival times were noted in mice treated with anti-IFNα/β polyclonal antiserum and challenged with several syngeneic tumors, including the IFNα/β-insensitive Friend leukemia cell line (41). These findings thus suggested a role for endogenous IFNα/β in controlling the growth of transplanted tumors, while also indicating that tumor cell IFNα/β responsiveness was not

required for its effects. More recent studies have utilized gene-targeted IFNAR1$^{-/-}$ mice to demonstrate the enhanced growth of transplanted tumors in the absence of host IFNα/β responsiveness (42) – yet these studies were limited by the use of progressively growing tumors and an allogeneic transplantation system.

Recent studies from our laboratory have more definitively established a role for endogenous IFNα/β in immune-mediated protection against tumor development (16). Using a panel of highly immunogenic tumors derived from RAG2$^{-/-}$ mice, we demonstrated that global blockade of IFNα/β signaling by treatment with a novel anti-IFNAR1 mAb abrogated tumor rejection in immunocompetent WT mice. In addition, treatment of IFNα/β-unresponsive IFNAR1$^{-/-}$ mice with MCA yielded a higher incidence of primary tumors compared to WT controls at two different doses of carcinogen. Similar to RAG2$^{-/-}$-derived sarcomas, analysis of IFNAR1$^{-/-}$-derived tumors by retransplanting them into immunocompetent mice revealed an unedited phenotype – that is, as a group, these tumors were more immunogenic than comparable tumors originating in WT hosts. Specifically, although IFNAR1$^{-/-}$-derived tumors all grew progressively in RAG2$^{-/-}$ hosts, 4/11 were uniformly rejected in WT mice (designated IFNAR1$^{-/-}$ regressors), while one additional tumor showed an intermediate phenotype. WT-derived tumors, in contrast, grew progressively in both RAG2$^{-/-}$ and WT recipients. These data therefore indicate that type I IFNs are important components of the cancer immunosurveillance network.

IFNα/β's Actions on the Host

In subsequent studies, the critical targets of IFNα/β's actions were explored. Several lines of evidence suggested that tumor cell IFNα/β responsiveness was not essential in mediating its anti-tumor activity. First, the fact that IFNAR1$^{-/-}$ regressor tumors, which are totally unresponsive to the type I IFNs, can be rejected in WT mice affirms that the activity of these cytokines on the tumor are indeed dispensable for rejection. Additionally, experiments with the GAR4 tumor, an MCA-induced tumor derived from a IFNAR1$^{-/-}$ x IFNGR1$^{-/-}$ mouse, revealed that selective reconstitution with IFNGR1, but not with IFNAR1, converted this tumor from a progressor to a regressor. This result was corroborated by two other observations: (i) the reconstitution of IFNAR1 progressor tumors by enforced expression of IFNAR1 had no effect on their growth in WT mice, and (ii) an analysis of one IFNAR1$^{-/-}$ regressor demonstrated that abrogation of IFNγ signaling, through overexpression of a dominant-negative IFNGR1 construct, prevented its rejection (which normally occurred in the parental tumor independent of IFNα/β responsiveness). Thus, tumor responses to IFNγ, but not IFNα/β, appeared to be important for the rejection of this tumor. Taken together, these findings point to a critical role for IFNα/β on cells of the host.

In order to examine the significance of host IFNα/β responsiveness for anti-tumor immunity, the growth of several RAG2$^{-/-}$ regressor tumors in globally-insensitive IFNAR1$^{-/-}$ mice was assessed. Progressive growth of these tumors demonstrated that host type I IFN responses were indeed required for tumor rejection. Subsequently, bone marrow chimeras were used to further define the functionally relevant host cells as

derived from the hematopoietic or nonhematopoietic compartment. Lethally irradiated RAG2$^{-/-}$ or IFNAR1$^{-/-}$ mice were reconstituted with wild type bone marrow, generating two types of chimeras: WT→RAG2$^{-/-}$ (IFNα/β-responsive in both hematopoietic and nonhematopoietic cells) and WT→IFNAR1$^{-/-}$ (IFNα/β-responsive only in hematopoietic cells). The successful rejection of two RAG2$^{-/-}$ regressor tumors in both sets of chimeras – yet progressive growth in IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ control chimeras – indicated that IFNα/β responsiveness in the host hematopoietic compartment was sufficient for rejection. Conversely, the failure of tumor elimination by IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras (IFNα/β-responsive only in nonhematopoietic cells) showed that responsiveness in the hematopoietic compartment is also required for anti-tumor immunity. These data therefore demonstrate that the key mediators of IFNα/β's actions reside at the level of the host, and specifically host hematopoietic cells.

A more recent study using C57Bl/6 strain mice also confirmed the increased incidence of MCA-induced sarcomas in the absence of IFNα/β responsiveness (both IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ mice) (43), thus corroborating the importance of this cytokine family for tumor protection on a second genetic background. In this study, IFNα/β-unresponsive mice were also shown to be more susceptible to the growth of RMA-S tumor cells which are normally controlled in WT mice via the actions of NK cells. In this model, the early actions of IFNα/β were critical as anti-IFNAR1 mAb blockade at day 3 following RMA-S challenge of WT mice had no effect on tumor control. The temporal requirements for IFNα/β thus corresponded to those of NK cells, as NK depletion at day 3 also had no effect on tumor rejection. Since NK cell cytotoxic activity can be strongly

enhanced by type I IFN, it is likely that NK cells represented the relevant targets of IFNα/β, though this was not directly tested. Nevertheless, these data suggest that modulation of NK cell function may be an important component of IFNα/β's anti-tumor function.

Cellular Targets of the IFNs in Cancer Immunoediting

The previously described studies have established fundamental roles for IFNγ and IFNα/β in the cancer immunoediting process. Their respective cellular targets, however, do not completely overlap. Whereas type I IFN's actions on host hematopoietic cells were found to be essential for tumor rejection, IFNγ responsiveness within both the tumor and the host was required. Given the range of biologic activity ascribed to the IFNs, it is unclear which cellular components within the host are the functionally important responders. Prior to a more thorough discussion of the proposed anti-tumor functions of the IFNs, some of their relevant biology will be reviewed.

THE BIOLOGY OF THE INTERFERONS

Initially described over fifty years ago based on their potent antiviral activity (44, 45), interferons are now recognized as a heterogeneous family of cytokines with important immunoregulatory and antiproliferative, as well as antiviral, effects. The interferons are grouped into type I (IFNα/β), type II (IFNγ), and type III (IFNλ)

interferon subfamilies according to gene structure and sequence homology, biochemical and functional differences, and the usage of distinct cellular receptors. The type I IFN family is composed of a large array of related cytokines which can be produced by most cell types in response to infectious stimuli. In contrast, type II IFN consists of a single member, IFNγ, produced by specialized immune cells including activated T cells, NK cells, and NKT cells. Given its strong immunostimulatory activity and prominent role in T_H1 responses, IFNγ has long been a focal point in the study of immune regulation (46). More recently, a number of critical functions for type I IFNs in modulating immune responses have emerged; extending the scope of this cytokine family beyond the initial containment of viral spread, and establishing IFNα/β as a key link between innate and adaptive immunity (47). The recently described IFNλ family of type III IFNs includes IFNλ1, IFNλ2, and IFNλ3 (or IL-28A, IL-28B, and IL-29, respectively) (48, 49). While these cytokines display antiviral activity and share sequence similarities with the type I IFNs, they have a different chromosomal location and gene structure and signal through a distinct receptor complex consisting of IFNλR1 and CRF2-4. Given similarities in downstream signaling pathways and biologic effects mediated by the IFNλ's and IFNα/β (50, 51), it remains unclear whether the type III IFNs have unique functional properties and they will not be discussed further.

Type I Interferon

The type I IFN family is comprised of many subclasses, including the IFNα subtypes, IFNβ, IFNω, IFNκ, IFNε, IFNδ, IFNτ, and limitin. Several of these are

exclusive to a particular species, as IFNω is functional in humans but not in mice, limitin has been described only in the mouse, and IFNδ and IFNτ are confined to pigs and ruminants, respectively (52). In addition, relatively little is known about IFNκ, which is expressed in keratinocytes, and IFNε, which is found in the placenta and reproductive organs and may function during pregnancy (53). There is only a single IFNβ, but both the human and murine genomes contain numerous IFNα genes, encoding 13 functional proteins (54). Members of the IFNα subclass share 75-80% amino acid homology, whereas IFNβ is approximately 30% homologous to the IFNα's (39). The mouse type I IFN locus also includes an as of yet undefined number of limitin genes (55). Limitin, which is constitutively expressed in T lymphocytes and bronchial epithelial cells, shares several functional characteristics with other type I IFNs but lacks myelosuppressive activity (56). All of the type I IFNs function as monomers, act through a common IFNα/β receptor, and share certain biological properties such as the inhibition of viral replication.

The existence of so many different type I IFNs, and the particular abundance of IFNα subtypes, is intriguing considering the lack of such apparent redundancy with other known cytokines. This may reflect the tremendous importance of this cytokine family, such that the presence of many functionally equivalent molecules would be advantageous. It is also possible that this redundancy allows for the differential regulation of subtypes by various cells. Alternately, each of the type I IFNs, including the IFNα subtypes, may have distinct though partially overlapping functions. While these possibilities are not mutually exclusive, their relevance is poorly understood at

present. There is some evidence, however, for the presence of unique activity among the various type I IFNs (52, 57). For example, studies comparing the antiviral, antiproliferative, and NK cell stimulatory activities of different human IFNα subtypes have revealed unique activity profiles (58). Furthermore, no clear correlation among the activities was evident, indicating that differences were not attributable to the affinities of receptor binding (52). The preferential induction of certain genes by IFNβ has also been demonstrated (59, 60), which is consistent with differences in its engagement of the IFNα/β receptor (61). Thus, the natural existence of various subtypes may reflect in vivo functional differences not yet recognized.

The generation of mice lacking components of the IFNα/β receptor or alternately the use of receptor blocking antibodies, has permitted the in vivo study of biologic responses in the absence of signaling by all type I IFN subtypes. Consistent with their initial description as antiviral molecules, mice lacking IFNα/β sensitivity display more severe phenotypes upon infection with a number of different viruses (62-64). Evidence in humans also supports a role for IFNα/β in antiviral immunity, specifically, the identification of human patients with genetic deficiencies in the STAT1 or Tyk2 molecules, or the UNC-93B protein (involved in viral-induced IFNα/β production) (65). The observed deficits in antiviral immunity in the absence of IFNα/β can be attributed to multiple mechanisms involving both cell-intrinsic and non-cell autonomous effects (66). The direct antiviral actions of type I IFN, mediated by autocrine and paracrine signaling by cells responding to infection, includes the inhibition of viral replication, through induction of antiviral genes such as PKR, 2′,5′-OAS, RNase L, and Mx, as well as the

pro-apoptotic effects of IFNα/β on infected cells. More indirect immunoregulatory effects promote the generation of cellular immune responses through enhancement of NK, DC, and T cell function, via mechanisms discussed in more detail later. As previously outlined, these immunomodulatory activities are also critical outside the realm of viral infection, such as during naturally-occurring responses to tumors. Yet, the stimuli which might lead to IFNα/β production in this context remain unclear. Interestingly, the function of type I IFN during infection with non-viral pathogens (e.g. bacteria, protozoa, and helminths) appears more complex (67). In some cases, type I IFN may have host protective effects, yet in other cases such as during Listeria infection, mice lacking IFNα/β responsiveness are actually more resistant to infection (68-71).

Type II Interferon

In contrast to IFNα/β, type II IFN consists of a single member, IFNγ, which binds to a distinct cellular receptor and is encoded by a separate chromosomal locus. The critical importance of IFNγ in promoting immune responses to viral and intracellular bacterial infections, as well as to tumors, is corroborated by the phenotypes of mice and human patients with defects in IFNγ production or deficiencies in the necessary signaling components. Generally induced by immune and inflammatory stimuli, IFNγ has a prominent role in promoting the generation of innate and adaptive immune responses through its ability to upregulate antigen processing and presentation, induce macrophage activation, promote the production of proinflammatory cytokines, enhance the

development of CD4+ T cells into Th1 cells, and increase lymphocyte recruitment through the induction of chemokines (72, 73).

Interferon Signaling

The type I IFNs mediate their effects by binding to a specific cell surface receptor which is generally expressed on all cell types. The IFNα/β receptor is comprised of two subunits, IFNAR1 and IFNAR2, which are constitutively associated with members of the Janus kinase (JAK) family, Tyk2 and Jak1, respectively. Ligand-induced dimerization of the receptor subunits leads to the activation of associated Janus kinases by auto- and trans-phosphorylation. Upon activation, these kinases phosphorylate a critical tyrosine residue within the IFNAR1 intracellular domain, allowing the recruitment of STAT2 (which is pre-associated with STAT1 on IFNAR2) via its SH2 domain (53, 61). Phosphorylation of STAT2 is followed by recruitment and phosphorylation of STAT1 and the release of STAT1/STAT2 heterodimers. The phosphorylated heterodimer enters the nucleus by an unknown mechanism, where it associates with IRF-9 (p48) to form the heterotrimeric interferon stimulated gene factor 3 (ISGF3) complex. ISGF3 then initiates gene transcription by binding to regulatory regions containing IFN-stimulated response elements (ISREs) (74, 75). In addition to ISGF3, STAT1 homodimers and other STAT complexes can also form, although activation of certain STATs appears limited to particular cell types (76). Initiation of a number of ancillary pathways by receptor-associated JAKs has also been described, including the p38 MAPK and PI3K cascades

(76). Type I IFN stimulation leads to the modulation of many genes, yet the biochemical function of most of these remain unknown.

Signaling by IFNγ is also transduced via a JAK-STAT pathway upon engagement of a ubiquitous cell surface receptor composed of two subunits, IFNGR1 and IFNGR2. These receptor chains are constitutively associated with inactive forms of Jak1 and Jak2, which become activated following IFNγ binding and receptor oligomerization. The activated JAK kinases phosphorylate a key tyrosine residue within the cytoplasmic domain of IFNGR1, leading to binding and phosphorylation of STAT1. Upon dissociation from the receptor, reciprocal homodimers of phosphorylated STAT1 translocate to the nucleus, where they initiate transcription by binding to DNA regulatory elements known as gamma-interferon activation sites, or GAS elements (77, 78). In addition to tyrosine phosphorylation, serine phosphorylation of STAT1 is required for full transcriptional activity. Similar to IFNα/β signaling, ancillary pathways involving additional downstream molecules have been described for IFNγ signaling (76). Evidence has also emerged for IFNγ signaling independent of STAT1 (79, 80), though the majority of IFNγ-induced responses require STAT1, as well as Jak1 and Jak2 (6).

IFNα/β Production

As most cell types are capable of producing type I IFNs in response to viral and bacterial stimuli, there are many possible cellular sources of IFNα/β in vivo. In addition, at least two pathways contribute to IFNα/β production, one involving membrane receptors of the Toll-like receptor (TLR) family which are expressed on specialized cell

types, and the other employing more ubiquitously-expressed cytoplasmic receptors. Both classes of pattern recognition receptors (PRRs) recognize conserved microbial components and are therefore critical for the initial detection of invading pathogens. Signaling through these receptors leads to the production of proinflammatory cytokines/chemokines and the induction of costimulatory molecules, thus promoting the generation of both innate and adaptive immune responses. Multiple TLRs including TLR3, TLR4, TLR7, TLR8, and TLR9, as well as the cytoplasmic proteins retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation associated gene-5 (MDA-5) can mediate type I IFN production in response to infectious stimuli (66, 81). Yet, the possible induction of IFNα/β in response to other stimuli, such as the growth of immunogenic tumors, is poorly understood.

TLR-dependent IFNα/β production can occur in response to pathogen-derived components including extracellular dsRNA, LPS, viral ssRNA, and unmethylated CpG DNA, which activate TLR3, TLR4, TLR7 (or TLR8 in humans), and TLR9, respectively (82). The TLRs, which are expressed primarily on dendritic cells (DCs) and macrophages, display distinct patterns of subcellular localization and utilize different adaptor molecules to transduce their signals. Stimulation of TLR7 and TLR9 proceeds through the adaptor protein MyD88, whereas IFNα/β production mediated by TLR3 and TLR4 is MyD88-independent – instead utilizing the adaptor molecule TRIF, though TLR4 shows an additional requirement for the adaptor TRAM (81). TRIF, which can directly associate with the kinases TBK1 and IKKε (83), leads to activation of the interferon regulatory factor (IRF) family member IRF3 and IFNα/β transcription.

The cytosolic receptors RIG-I and MDA-5 provide a more universal and TLR-independent pathway to type I IFN induction. Although the two receptors detect different viral RNA structures, they both utilize the adaptor protein MAVS to induce activation of IRF3 and lead to IFNβ production. The phenotypes of RIG-I and MDA-5 deficient mice demonstrate that these two systems are functionally non-redundant, each being required for detection of particular viruses. The recent discovery of a cytoplasmic DNA receptor provides an additional pathway for activation of IRF3 and type I IFN production (84).

The production of type I IFNs is transcriptionally regulated, and some of the relevant transcription factors mediating its induction have been identified. As alluded to earlier, members of the IRF family, including IRF3 and IRF7, have important roles in IFNα/β induction. While IRF3 is constitutively expressed, most cells do not express or only weakly express IRF7, which is upregulated by IFNα/β-mediated ISGF3 activation (85). A classical positive feedback mechanism for full induction of the type I IFNs has thus been described – whereby initial activation of IRF3 leads to production of IFNβ and IFNα4, which signal through the IFNα/β receptor in an autocrine/paracrine manner to upregulate IRF7 (86, 87). Subsequent activation of IRF7 then mediates the expression of the majority of IFNα subtypes. Both IRF3 and IRF7 are activated by phosphorylation of carboxy-terminal serine residues by virus-activated kinases, including the IκB kinase-related kinases IKKε and TANK-binding kinase 1 (TBK1) (83, 88). Upon activation, IRF3 translocates into the nucleus where it associates with the coactivator CBP/p300 to drive IFNβ transcription (85). Induction of the IFNβ gene, whose promoter is well

characterized, depends on the cooperative binding of several transcription factors in addition to IRF3 – specifically, NF-κB and ATF-2/c-Jun (81).

Although all cells bearing the appropriate PRRs can respond to infectious stimuli and produce IFNα/β, a recently described subset of dendritic cells – the plasmacytoid dendritic cells (pDCs) – are particularly potent producers of IFNα/β during viral infections (89, 90). These cells are found in the T cell areas of secondary lymphoid organs and their recruitment from the blood is enhanced under inflammatory conditions (91). pDCs secrete high levels of IFNα/β upon stimulation of TLR7 and TLR9 present in endosomal compartments. This signaling pathway is mediated through the formation of a MyD88-TRAF6-IRF7 complex, leading to IRF7 activation and gene transcription (92). The transcription factor IRF7 is constitutively expressed at high levels in pDCs compared to other cell types and shows a preferential ability to activate IFNα promoters (91). This may explain the particularly robust production of IFNα in pDCs (though IFNβ is also produced). Yet, recent studies have also implicated the regulation of endosomal trafficking, as preferential retention of an IFN-inducing TLR9 ligand was apparent in pDCs but not conventional DCs (93). The MyD88-IRF7 pathway appears to be the major conduit to type I IFN production in pDCs since these cells do not express TLR3 or TLR4 and IFNα/β production by pDCs is abrogated in $MyD88^{-/-}$ as well as $IRF7^{-/-}$ animals (94, 95).

IFNγ Production

In contrast to the rather ubiquitous expression potential of type I IFN, IFNγ production is mediated by a relatively small subset of cell types – including NK, NKT, αβ T cells, and γδ T cells – generally in response to activation rather than viral infection. Additional studies also demonstrate IFNγ production by APCs such as macrophages and DCs (96-98), yet the functional significance of these cells as sources of IFNγ in vivo is unclear at present. NK cell IFNγ production in response to engagement of NK activating receptors is an important early source, whereas production by $CD4^+$ Th1 cells and $CD8^+$ T cells upon TCR stimulation are major sources of IFNγ during the adaptive phase of the immune response. An alternative pathway involving IL-12 and IL-18 can also lead to IFNγ production (6). In the context of anti-tumor immunity, prior studies in the literature support a role for γδ T cells as important early producers of IFNγ during responses to transplantable tumor challenge and during primary MCA-induced carcinogenesis (99). Other recent studies, however, have contested this conclusion, suggesting that innate immune cells rather than T cells are the relevant producers of IFNγ during anti-tumor responses (100). The recently described subset of interferon-producing killer dendritic cells (IKDC) has also been suggested to be an important source of IFNγ during immune surveillance of tumors (101).

POTENTIAL FUNCTIONS DURING CANCER IMMUNOSURVEILLANCE

Type I IFN

Despite fervent study using animal models and many years of clinical experience with its use, the critical functions mediating IFNα/β's anti-tumor effects remain poorly understood. The type I IFNs may exert their activity through both direct effects on the tumor and indirect immunostimulatory activity on various immune components. Indeed, IFNα/β has been shown to promote the differentiation and function of DCs, enhance the cytotoxicity of NK cells, boost the activation and survival of T cells, and enhance B cell function (37) (Figure 2). The diversity of IFNα/β's functions also raises the possibility that different mechanisms are operative depending on the tumor phenotype or the experimental model involved. Thus, whereas several studies have demonstrated that tumor cell responsiveness is dispensable for IFNα/β's effects (16, 32, 33), its antiproliferative and pro-apoptotic properties may also be important under certain circumstances.

Effects on Tumor and Stromal Cells

The antiproliferative and pro-apoptotic activity of the type I IFNs, properties shared with IFNγ, are potentially important mechanisms for the suppression of tumor growth and the containment of infection. While IFNα/β can inhibit the growth of both normal and transformed cells in vitro, different cell lines often show varying degrees of sensitivity. This activity has been linked to a prolongation of the G1 phase of the cell cycle and a reduction in the rate of entry into S phase (102). Specific cell-cycle

components affected include c-myc, pRB, cyclin D3, and cdc25A (61). The type I IFNs are also capable of inducing apoptosis under certain conditions, through the classic mechanisms involving caspase activation, increases in mitochondrial membrane permeability, and the release of cytochrome c (103). Caspase-8 activation appears to be an early event after the treatment of sensitive cells with IFNα, but the initiating events are unclear (104). Importantly for antiviral defense, IFNα/β can sensitize cells to inducers of apoptosis such as dsRNA and influenza virus that act through the FADD/caspase-8 pathway (105). Recent work identifying p53 as an IFNα/β-induced gene also suggests that these cytokines can sensitize cells to p53-mediated apoptosis in response to DNA damage (106).

Revealing a previously unrecognized link between IFNα/β and p53, Takaoka et al. demonstrated that type I IFN treatment of MEFs led to the upregulation but not activation of p53 protein (106). In addition to enhancing p53-dependent apoptosis upon exposure to DNA-damaging agents, IFNα/β treatment also mediated the suppression of oncogene-induced cellular transformation. This was demonstrated in transformation assays using MEFs expressing Ha-Ras and the HPV E6 protein, which is known to target p53 by ubiquitin-mediated degradation. While the significance of this mechanism in vivo is unknown, this data raises the possibility that IFNα/β modulates intrinsic tumor suppressor mechanisms to inhibit cellular transformation – through both an enhancement of the p53 pathway and antiproliferative effects which may be p53-independent. As mutational inactivation and loss of heterozygosity are frequently observed in human cancers (107), the effects of p53 induction within established tumors might be minimal.

Thus, at later stages the anti-tumor activity of IFNα/β may depend more on its immunostimulatory functions. Nevertheless, the role of type I IFNs in suppressing primary tumor formation via intrinsic versus extrinsic tumor suppressor mechanisms requires further study.

In addition to its antiproliferative and pro-apoptotic activity, other direct effects on the tumor and surrounding stroma are possible. For example, the upregulation of MHC class I molecules on tumor cells by both type I and type II IFNs could significantly enhance recognition by tumor-specific CTL. The IFNs can also inhibit angiogenesis within the tumor by reducing the production of pro-angiogenic factors like bFGF, while also stimulating the production of angiostatic chemokines of the non-ELR CXC family (i.e. IP-10, Mig, and I-TAC) (108, 109). The induction of chemokines such as IP-10 may also be important in promoting the recruitment of lymphocytes and monocytes to sites of inflammation (81).

Immunomodulatory Functions

The enhancement of NK cell cytotoxicity was one of the earliest recognized effects of the type I IFNs on innate immune cells. During the course of LCMV infection, the induction of early cytotoxic cells was found to correlate with the kinetics of type I IFN production in the spleen (110). Indeed, NK killing of sensitive targets in vitro is increased by treatment with IFNα/β, and antibody neutralization studies have demonstrated that IFNα/β is responsible for the augmentation of NK cell cytotoxicity in vivo following MCMV infection (111, 112). This increased cytotoxicity against virally-

infected cells may be partially mediated through the upregulation of TRAIL, which contains an ISRE within its promoter (113). Type I IFNs are also involved in viral-induced NK cell proliferation through the induction of IL-15 (114). Similarly, the homeostasis of peripheral NK cells (as well as CD8 memory T cells and NKT cells) is modulated by IFNα/β through the production of IL-15, which promotes survival possibly through maintenance of the anti-apoptotic protein Bcl-2 (115). Thus, type I IFNs may have a prominent role in supporting the function of NK cells, which are involved in host protective mechanisms against both infection and tumor formation (116).

The effects of IFNα/β in promoting DC maturation have been more recently elucidated. With a central role in the initiation and control of immune responses, DCs perform a number of critical functions. These cells are capable of acquiring and processing antigen, homing to the secondary lymphoid organs, and leading to the activation (or tolerization) of T and B lymphocytes. The outcome of this interaction is largely determined by levels of co-stimulatory molecules and cytokine production, which are induced by infection-associated stimuli including IFNα/β (47, 117). Thus, type I IFNs can have profound effects on T and B cell activation through their modulation of DC function.

Several studies have demonstrated that IFNα/β treatment of immature DCs in vitro can induce their phenotypic maturation, with upregulation of MHC class I and II, CD80, CD86, and CD40 (118, 119). The maturation of splenic DCs after in vivo exposure to IFNα/β was also shown, and this phenotype correlated with an increased ability to stimulate T cell proliferation in vitro (120). In vivo models have also been used

to demonstrate IFNα/β's actions in enhancing adaptive immune priming. Antigen-specific CD4 T cell and antibody responses to a poorly immunogenic soluble protein were augmented by exogenous treatment with IFNα/β or poly(I:C), an effect abrogated in IFNAR1$^{-/-}$ mice (121). Furthermore, adoptive transfer studies were used to show that the effect of type I IFNs on DCs alone was sufficient to mediate this enhanced antibody production and isotype switching. These experiments involved the injection of antigen-pulsed IFNAR1$^{-/-}$ or wild type DCs along with IFNα/β into IFNAR1$^{-/-}$ mice (such that only the DCs were IFNα/β responsive). Additional studies have also confirmed the strong adjuvant properties of IFNα when co-injected with a soluble antigen (119). Similarly, a role for virally-induced type I IFNs in promoting the cross-priming of CD8 T cells has recently been demonstrated (122). Thus, IFNα/β can act as a potent maturation factor for DCs, thereby enhancing the stimulation of T and B cell responses and effectively linking the innate and adaptive immune systems (123).

The actions of IFNα/β on adaptive immune cells are mediated by both direct and indirect effects. As previously discussed, the type I IFNs can enhance T cell activation through the upregulation of MHC and costimulatory molecules on APCs. Additionally, a role for IFNα/β in the induction or maintenance of CD8 memory T cells has been demonstrated (124). Although type I IFNs inhibit T cell proliferation in vitro, IFNα/β or IFNα/β-inducing agents lead to the selective proliferation in vivo of memory-phenotype (CD44^{hi}) CD8 T cells – through TCR-independent mechanisms involving IFNα/β-induced secretion of IL-15 by APCs (125, 126). In addition to these indirect effects, the enhancement of activated CD4 and CD8 T cell survival due to IFNα/β's direct actions on

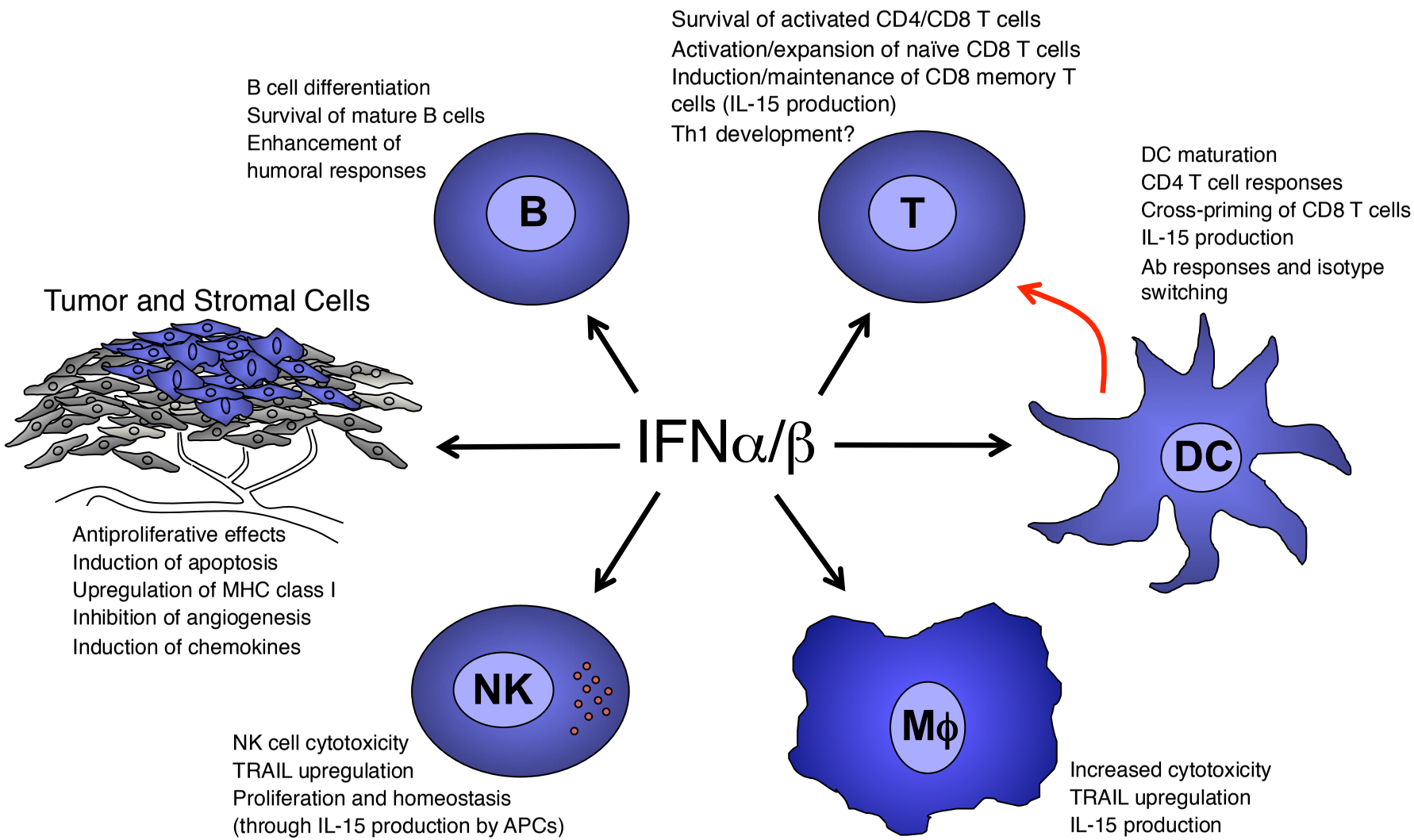
T cells has also been shown (127). These studies demonstrated that IFNα/β treatment, like IL-2, augmented the survival of previously activated CD4 and CD8 T cells in vitro, though treatment with other cytokines including IFNγ had no effect. Further experiments involving the co-culture of activated T cells derived from normal or IFNAR1-deficient mice indicated that such effects were dependent on T cell IFNα/β responsiveness, suggesting that indirect effects by IL-15 were not involved (127). In contrast to IL-15, it was also found that IFNα/β did not upregulate the anti-apoptotic proteins Bcl-2 or Bcl-XL in T cells, although other studies using activated human CD4 T cells noted increases in Bcl-2 and decreases in Bax upon IFNα treatment (128). It has therefore been postulated that IFNα/β induced by inflammatory stimuli may enhance immune responses through its role in preventing the apoptosis of activated T cells.

Recent work has also demonstrated the ability of IFNα/β to augment the activation of naïve CD8 T cells, aside from its enhancement of APC stimulatory activity. Using a model system involving the stimulation of naïve TCR-transgenic CD8 T cells with artificial APCs (microspheres bearing MHC class I/peptide Ag and the B7-1 ligand), it was shown that naïve T cell activation requires an additional or "third signal" provided by inflammatory cytokines for full clonal expansion and the development of effector function (129, 130). For naïve CD8 T cell activation, IL-12 was capable of providing this signal, and more recently the ability of IFNα/β to perform this function through its direct actions on the T cell was established (131). A role for IFNα/β in the activation of naïve CD8 T cells was also noted in prior studies, wherein IFNα/β-dependent CXCR3-mediated signals were found to be important (132). In addition to T cell activation, a

function for the type I IFNs in enhancing IFNγ production by CD8 T cells during LCMV infection was observed (133). The involvement of IFNα/β in promoting Th1 development has also been proposed (134), yet this remains controversial and may entail species-specific differences between mouse and human T cells (135, 136). Interestingly, while IFNα/β appears to augment T cell activation in most cases, recent studies have indicated that in the context of Listeria monocytogenes infection, IFNα/β signaling can actually be detrimental by enhancing lymphocyte apoptosis caused by bacterial pore-forming toxins such as listeriolysin O (68-70).

The modulation of B cell function by IFNα/β has also been described, yet the role of B cells during antitumor immune responses is unclear. Through its effects on DCs, IFNα/β can lead to the enhancement of humoral responses and isotype switching (121). In addition, IFNα/β produced by pDCs in response to viral infection was shown to promote the differentiation of CD40-activated B cells in vitro, which further differentiated into antibody-secreting plasma cells in the presence of IL-6 (137). Finally, although IFNα/β can exert inhibitory effects on B cell development, the type I IFNs have antiapoptotic effects on mature B cells, perhaps through their upregulation of the B cell survival factors BlyS and APRIL (53).

Figure 2. Potential functions of type I IFN in cancer immunoediting. The type I IFNs are potent immunomodulators which shape host immunity through direct actions on both innate and adaptive immune cells. In this schematic diagram, some of the immunostimulatory functions ascribed to IFNα/β are outlined. Given the ubiquitous expression of IFNα/β receptors and their pleiotropic effects, defining the critical cellular targets of type I IFN will be important for understanding their role in promoting anti-tumor immunity.


Survival of activated CD4/CD8 T cells
Activation/expansion of naïve CD8 T cells
Induction/maintenance of CD8 memory T cells (IL-15 production)
Th1 development?
B cell differentiation
Survival of mature B cells
Enhancement of humoral responses
B
T
DC maturation
CD4 T cell responses
Cross-priming of CD8 T cells
IL-15 production
Ab responses and isotype switching
Tumor and Stromal Cells
IFNα/β
DC
Antiproliferative effects
Induction of apoptosis
Upregulation of MHC class I
Inhibition of angiogenesis
Induction of chemokines
NK
Mϕ
NK cell cytotoxicity
TRAIL upregulation
Proliferation and homeostasis
(through IL-15 production by APCs)
Increased cytotoxicity
TRAIL upregulation
IL-15 production

Type II IFN

The potential anti-tumor functions of IFNγ, many of which are shared with type I IFN, also include both direct effects on tumor and stromal cells as well as a plethora of immunostimulatory activities. In contrast to IFNα/β, the importance of IFNγ's actions on the tumor cell has been extensively documented and some of the relevant mechanisms are apparent.

Effects on Tumor and Stromal Cells

As described earlier, IFNγ and IFNα/β share a number of common biologic activities including their capacity to exert anti-proliferative, pro-apoptotic, and angiostatic effects. IFNγ can exert growth inhibitory effects on a wide variety of tumor cells, through its induction of the cyclin dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$. In addition, sensitization of cells to apoptosis can be enhanced by IFNγ-induced caspase-1, PKR, cathepsin D, and surface Fas and Fas ligand (73). Upregulation of MHC class I molecules and other components of the antigen processing and presentation pathway (such as TAP1 and immunoproteasome subunits LMP2, MECL-1, and LMP7) are important effects for the enhancing immune recognition and promoting the immune response. IFNγ also has a prominent function in facilitating leukocyte-endothelial cell interactions through upregulation of cell adhesion molecules such as ICAM-1 and VCAM-1, as well as by inducing endothelial cell production of chemokines including MCP-1, fractalkine, IP-10, Mig, and I-TAC (138). As previously discussed, induction of

angiostatic chemokines as well as the inhibition of VEGF and basic FGF production within the tumor environment are key anti-angiogenic mechanisms.

Immunomodulatory Functions

The macrophage activating properties of IFNγ have been extensively characterized and are vital for both antimicrobial immunity as well as the induction of non-specific killing of tumor cell targets. Activation of macrophages by IFNγ leads to production of reactive oxygen intermediates, nitric oxide, enhancement of MHC class II and costimulatory molecule expression, as well as the secretion of proinflammatory cytokines such as TNFα and IL-12 (78). Adaptive immune responses are also promoted through direct effects on T and B cells, leading to enhanced $CD4^+$ Th1 development (and reciprocal inhibition of Th2 development) as well as B cell isotype switching (73). As previously discussed, inhibition of Treg development or function is another potentially important mechanism for promoting protective adaptive immune responses.

GOALS OF THIS STUDY

In the experiments presented in this thesis, we have addressed issues relevant both to our understanding of the roles of endogenous IFNα/β and IFNγ in the cancer immunoediting process and to the use of exogenous type I IFN for cancer immunotherapy. In the first study, we extended prior work demonstrating the importance

of the IFNs in anti-tumor immunity, while examining various parameters of IFN-dependent tumor rejection including the temporal requirements for their actions, contributions to tumor-specific adaptive immune priming, and role of host-derived IFNβ. The second study focused on defining the essential host cell targets of type I IFN during the rejection of immunogenic sarcomas. In the third study, we subsequently used similar approaches to localize the relevant host targets of IFNγ during tumor rejection, as well as confirm the importance of its direct actions on the tumor cell. Finally, we turned our attention to the anti-tumor activity of exogenous IFNα/β using a model of type I IFN immunotherapy. Through a more complete understanding of the protective effects of the IFNs during tumor development, as well as identification of critical immune pathways to augment therapeutically, such investigation will hopefully translate into more sophisticated approaches to cytokine-based cancer immunotherapy.

CHAPTER 2

Interferon-Dependent Tumor Rejection: Exploring the Parameters of the Anti-Tumor Immune Response

INTRODUCTION

Recent studies have begun to establish the key molecular and cellular components of host immunity involved in protective cancer immunosurveillance and immunoediting (8, 10). Both type I (IFNα/β) and type II (IFNγ) interferon were found to play critical roles in this process, and some of the relevant functions of these cytokines have begun to emerge. Mice deficient in either IFNα/β or IFNγ responsiveness display an increased susceptibility to both primary and transplantable tumor formation. Yet, further studies dissecting the important sites of IFN action have noted prominent differences. Whereas IFNγ's effects on the tumor cell were absolutely essential, a role for tumor cell IFNα/β sensitivity was not observed (5, 16-18). On the other hand, the actions of both IFNγ and IFNα/β on the host were found to be imperative for immune-mediated tumor rejection (16, 22, 23) (G.P. Dunn and R.D. Schreiber, unpublished data). Thus, while the cellular targets of the IFNs do not completely overlap, they both exert critical effects on the host in promoting anti-tumor immunity. Elucidation of the relevant mechanisms involved is central to our understanding of the cancer immunoediting process.

In addition to the functions mediated by type I IFN during this process, little is known about the specific contributions of individual members of this cytokine family. The type I IFNs include multiple IFNα subtypes, a single IFNβ, and several other poorly characterized species, all of which activate a common IFNα/β receptor ubiquitously expressed on all cells. Despite acting through a common receptor, there is some evidence for differential signaling among the IFNα/β subtypes, and individual species reportedly

possess different activity profiles in vitro (with regard to their antiviral, anti-proliferative, and NK cell stimulatory properties). Whether IFNα/β subtypes perform distinct biologic functions in vivo, however, remains unknown. Alternatively, the numerous type I IFN subtypes may be functionally redundant in vivo, yet certain subtypes could be more important due to their preferential induction during anti-tumor responses or non-redundant roles in the regulation/augmentation of IFNα/β production.

In this study, we have investigated several aspects of the IFN-dependent response to immunogenic tumors. Using antibody-mediated blockade at different time points, we established the temporal requirements for the actions of IFNα/β and IFNγ during tumor rejection. Analysis of tumor-specific T cell priming revealed a prominent defect in the absence of host sensitivity to the IFNs, while tumor mixing experiments provide evidence for specificity in the mechanisms of tumor elimination. We also assessed the specific contribution of IFNβ during tumor rejection using tumor transplantation studies in IFNβ-deficient mice.

MATERIALS AND METHODS

Mice. Inbred 129/SvEv and C57Bl/6 mice were purchased from Taconic Farms, and 129/SvPas mice were from Charles River Laboratories. 129 IFNAR1$^{-/-}$, IFNGR1$^{-/-}$, and RAG2$^{-/-}$ mice, as well as B6 RAG2$^{-/-}$ mice, were bred in our specific pathogen-free animal facility. B6 IFNAR1$^{-/-}$ mice (previously backcrossed by speed congenics to >99% B6 by Tony French and Wayne Yokoyama) were obtained and bred in our animal facility. STAT1^{Y701F} mutant mice were generated in our laboratory and maintained on a 129/SvEv background (K.S. Lai, J.M. White, and R.D. Schreiber, unpublished data). Homozygous mutant mice derived from the A10-C4 and A10-C8 ES cell clones were used with similar results. IFNβ$^{-/-}$ mice (139), originally on a mixed B6x129 genetic background, were backcrossed in our laboratory by speed congenics to >99% pure 129/SvEv or C57Bl/6 backgrounds.

Tumor cells. RAG2$^{-/-}$ regressor fibrosarcoma cell lines were previously generated by subcutaneous 3′-methylcholanthrene (MCA) injection of 129 or B6 strain RAG2$^{-/-}$ mice as described (5, 140), and are designated regressors since they are rejected when transplanted into syngeneic immunocompetent hosts. IFNAR1$^{-/-}$-derived progressor (d97m915) and regressor (d93m1244) fibrosarcomas have been described (16). Progressor tumors, which grow progressively when transplanted into syngeneic immunocompetent mice, were generated by MCA injection of WT 129 SvEv (F244) or WT B6 (9609, 9614) mice. GAR4.GR1 is a subclone of the GAR4 MCA-induced

fibrosarcoma (derived from a 129 IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ mouse) in which IFNγ-responsiveness has been restored by retroviral transduction of IFNGR1 (16).

Tumor transplantation experiments. Tumor cells were thawed from frozen stocks and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamate, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol (R-10 medium). After expansion for several passages, cells were harvested by incubation in 0.05% trypsin, washed once with R-10 medium, and washed three times with sterile, endotoxin-free PBS. Prior to the last wash, cells were counted using a hemacytometer and cell viability was assessed by trypan blue exclusion (injected cells were >90% viable). Cells were injected subcutaneously in a volume of 0.15 ml PBS into the shaved flanks of mice. Tumor size was measured on the indicated days and is presented as the average of two perpendicular diameters. When calculating percent tumor growth, mice with tumors larger than 6x6 mm in diameter at the end of the observation period were counted as positive.

Antibody Treatment. For IFNα/β blockade, mice were injected i.p. with a single 2.5 mg dose of the anti-IFNAR1 mAb MAR1-5A3 (64) or isotype control mAb GIR-208 (141) at day -1 or as indicated. For IFNγ neutralization, 750 μg of the anti-IFNγ mAb H22 (142) or isotype control anti-GST mAb PIP (143) was injected i.p. followed by a 250 μg dose every 7 days. Broad immunodepletion was achieved by i.p. administration of a mixture of the anti-CD4 mAb GK1.5 (144), anti-CD8 mAb YTS-169.4 (145), and

anti-IFNγ mAb H22. For this regimen, an initial dose of 750 μg of each mAb or of the control mAb PIP was followed by 250 μg of each every 7 days as described previously (140).

Ex vivo tumor-specific CTL killing assay. Spleens were harvested from H31m1 or d38m2 tumor-bearing WT, IFNAR1$^{-/-}$, or IFNGR1$^{-/-}$ mice 20 days after tumor implantation and cell suspensions were obtained by homogenization using frosted glass slides. $4x10^7$ splenocytes were cultured with $2x10^6$ IFNγ-treated, irradiated (10,000 rads) H31m1 or d38m2 tumor cells. 5 days later, the cells were harvested and used as CTL effector cells in a cytotoxicity assay. To generate target cells, H31m1, d38m2, or 1773 tumor cells were treated with 100 U/ml IFNγ for 48 hours before use. 10^6 tumor cells were radioactively labeled with 25 μCi of $Na_2{}^{51}CrO_4$ (PerkinElmer) for 90 minutes at 37ºC. The labeled target cells were washed three times and seeded at 10,000 cells/well in 96-well round-bottom plates. The effector and target cells were cocultured at indicated effector/target cell ratios for 4 hours at 37ºC in 5% CO_2. For blocking assays, 10 μg/ml of α-CD8 (YTS-169.4), α-CD4 (GK1.5), or control immunoglobulin (PIP mAb specific for glutathione S-transferase) were added to the cell culture of effector and target cells. Radioactivity was determined in the supernatants. Percent specific killing was defined as (experimental condition cpm - spontaneous cpm)/(maximal (detergent) cpm - spontaneous cpm) x 100. Data points were obtained in duplicate. All experiments were done at least twice.

IFNγ production assay. $2x10^7$ splenocytes obtained from H31m1 tumor-bearing WT or IFNAR1$^{-/-}$ mice 20 days after implantation were cultured with $2x10^6$ IFNγ-treated, irradiated (10,000 rads) H31m1 tumor cells. 5 days later, the cells were harvested and used as CTL effector cells in an IFNγ production assay. For target cells, H31m1 or 1773 tumor cells treated with 100 U/ml IFNγ for 48 hours were seeded at 10,000 cells/well in 96-well round-bottom plates. The effector and target cells were cocultured at indicated effector/target cell ratios overnight at 37ºC in 5% CO_2. Production of IFNγ in the supernatants was measured by using a mouse IFNγ ELISA kit (eBioscience).

Ex vivo analysis of tumors and immune infiltrate. Tumors were excised from euthanized mice, physically disaggregated by mincing with razor blades, then enzymatically disaggregated by digestion with 1 mg/ml collagenase IA (Sigma-Aldrich) in 10 ml HBSS medium for 1.5-2 hrs at room temperature with occasional mixing. Cell suspensions were washed once with R-10 medium, RBCs were lysed with Hybrimax RBC lysing buffer (Sigma-Aldrich), and cells were filtered through a 40 μm strainer to remove aggregates and debris. Cells were incubated with purified anti-CD16/CD32 mAb (2.4G2) (BD Biosciences) to prevent non-specific antibody binding to Fc receptors, then stained with FITC-conjugated anti-CD45.2 (BioLegend), and biotinylated anti-IFNAR1 mAb (MAR1-5A3) (64) followed by SA-APC (BioLegend). Immediately prior to flow cytometry, propidium iodide (PI) was added.

RESULTS

Immunogenic RAG2$^{-/-}$ Regressor Tumors of Different Genetic Backgrounds Display Interferon-Dependent Rejection

To extend our prior observations that endogenously produced IFNα/β and IFNγ play critical roles in the rejection of immunogenic 129 strain RAG2$^{-/-}$ regressor tumors, we examined the IFN requirements of a cohort of RAG2$^{-/-}$ regressor tumors derived from C57Bl/6 mice recently generated in the laboratory (C.M. Koebel, J.D. Bui, and R.D. Schreiber, unpublished data). These highly immunogenic tumors are termed "RAG2$^{-/-}$ regressors" since they are rejected when transplanted into syngeneic WT mice, but grow progressively in immunodeficient RAG2$^{-/-}$ hosts. Of the original group of eight 129 RAG2$^{-/-}$ regressors screened, four tumors (H31m1, d38m2, d42m1, and F510) were found to require host type I IFN responsiveness, as they failed to be rejected when transplanted into IFNAR1$^{-/-}$ mice (16) (also see Figure 1). Additionally, after screening several regressor tumors from a new bank of MCA-induced sarcomas derived from completely pure 129 SvEv RAG2$^{-/-}$ mice (C.M. Koebel and R.D. Schreiber, unpublished data), we observed that the 1773 tumor also required host cell sensitivity to IFNα/β for its rejection. In agreement with these results, when three representative B6 strain RAG2$^{-/-}$ regressors were transplanted into IFNAR1$^{-/-}$ mice, all three exhibited IFNα/β-dependent rejection, though 1969 and 7835 more strongly than the 6494 tumor (Figure 1).

When the same three B6 regressor tumors were transplanted into WT mice treated with the IFNγ-specific neutralizing monoclonal antibody (mAb) H22 (142), two out of the three (1969 and 6494, but not 7835) were found to require IFNγ signaling for their rejection (Figure 2). This finding is also consistent with data from the original 129 RAG2$^{-/-}$ regressors, of which 5/8 (H31m1, d42m1, d38m2, F510, and F515) required IFNγ for their rejection (C.M. Koebel, G.P. Dunn and R.D. Schreiber, unpublished data; also see Figure 2). Subsequent studies on this group of IFNγ-dependent 129 regressors identified both tumor cells and host cells as obligate sites of IFNγ's actions. For example, rejection of 4/5 tumors (H31m1, d42m1, d38m2, and F510) was impaired in IFNGR1$^{-/-}$ hosts, while overexpression of a dominant-negative IFNGR1 construct abrogated rejection of the same four tumors in WT hosts (G.P. Dunn, C.M. Koebel, and R.D. Schreiber, unpublished data). Oddly, the F515 tumor grows progressively with anti-IFNγ mAb treatment, yet neither grows in IFNGR1$^{-/-}$ mice nor grows when rendered IFNγ-insensitive via IFNGR1.ΔIC overexpression. Thus, for rejection of F515, IFNγ sensitivity in either the tumor or the host may be sufficient. A similar set of experiments to dissect the relevant sites of IFNγ's actions for rejection of the B6 regressor tumors is currently ongoing, and it has already been observed that tumor cell IFNγ responsiveness is required for 1969 rejection (S.H. Lee and R.D. Schreiber, unpublished data).

In establishing the IFN-dependence of B6 strain RAG2$^{-/-}$ regressor tumors, these data corroborate – on a second genetic background – the importance of endogenous IFNα/β and IFNγ for the rejection of immunogenic transplantable tumors. The current analysis, however, has also uncovered an intriguing point of divergence from previously

examined regressor sarcomas; specifically, the observation that rejection of 7835 tumor cells requires IFNα/β's actions on the host, but not the actions of IFNγ on the tumor or the host. This suggests that type I IFN performs a unique function on host cells during the lymphocyte-dependent rejection of 7835 tumor cells. In contrast, of the four original 129 regressors that required host cell sensitivity to IFNα/β for rejection, the same four also required host cell responsiveness to IFNγ. One additional tumor (F515) was IFNγ-dependent, but IFNα/β-independent, yet the functionally relevant targets of IFNγ during its rejection remain unclear. Nevertheless, the identification of tumors that specifically require type I but not type II IFN, or vice versa, provides preliminary evidence that the interferons may perform distinct functions, not only at the level of the tumor cell – which has already been established – but also at the level of the host, a possibility that we have investigated throughout this study.

Figure 1. Rejection of immunogenic 129 and B6 strain RAG2$^{-/-}$ regressor tumors requires host sensitivity to type I IFN. Groups of WT, IFNAR1$^{-/-}$, and RAG2$^{-/-}$ mice were injected s.c. with 1x10^{6} RAG2$^{-/-}$ regressor tumor cells as indicated and growth was monitored. Data represent mean tumor diameter ± s.e.m.

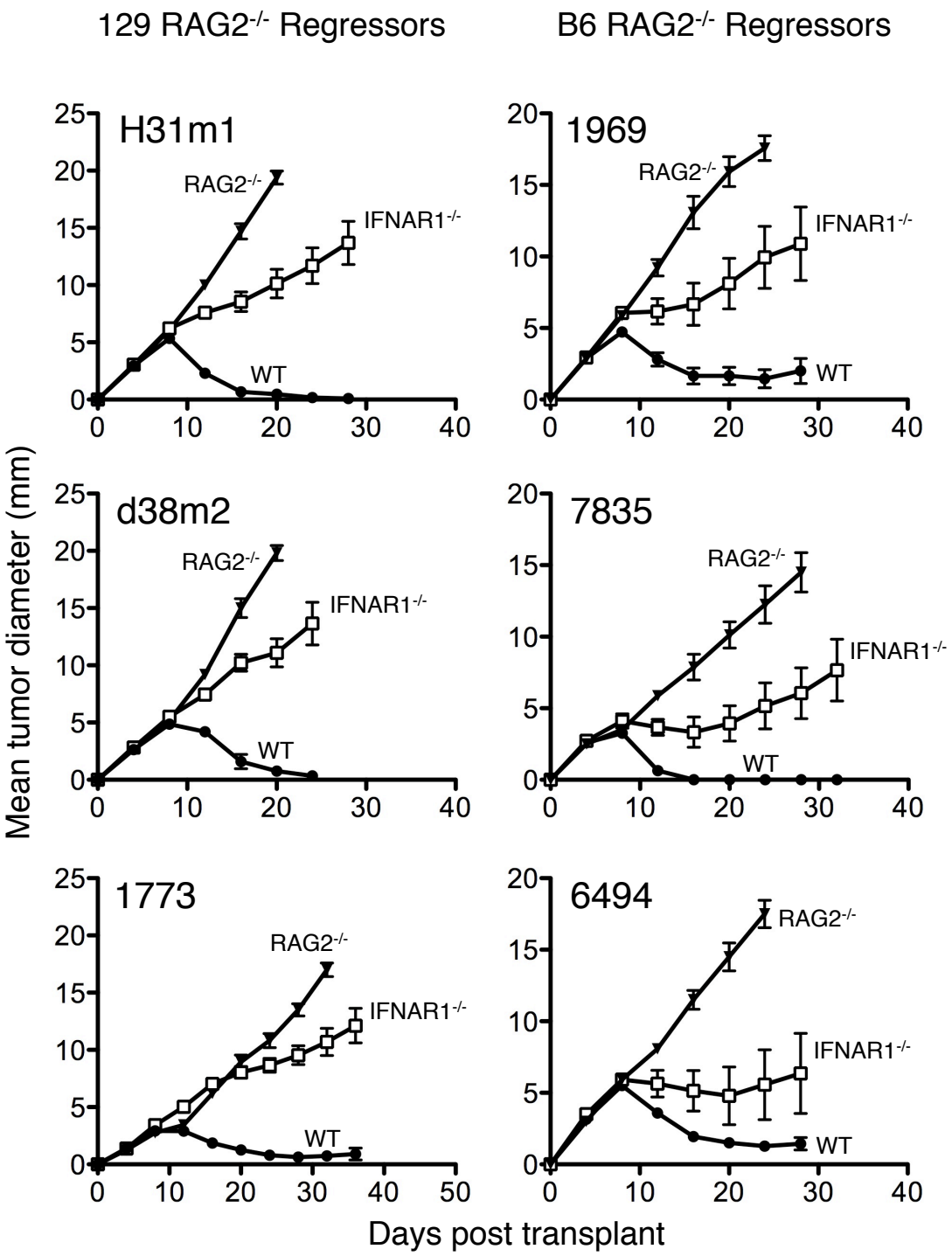
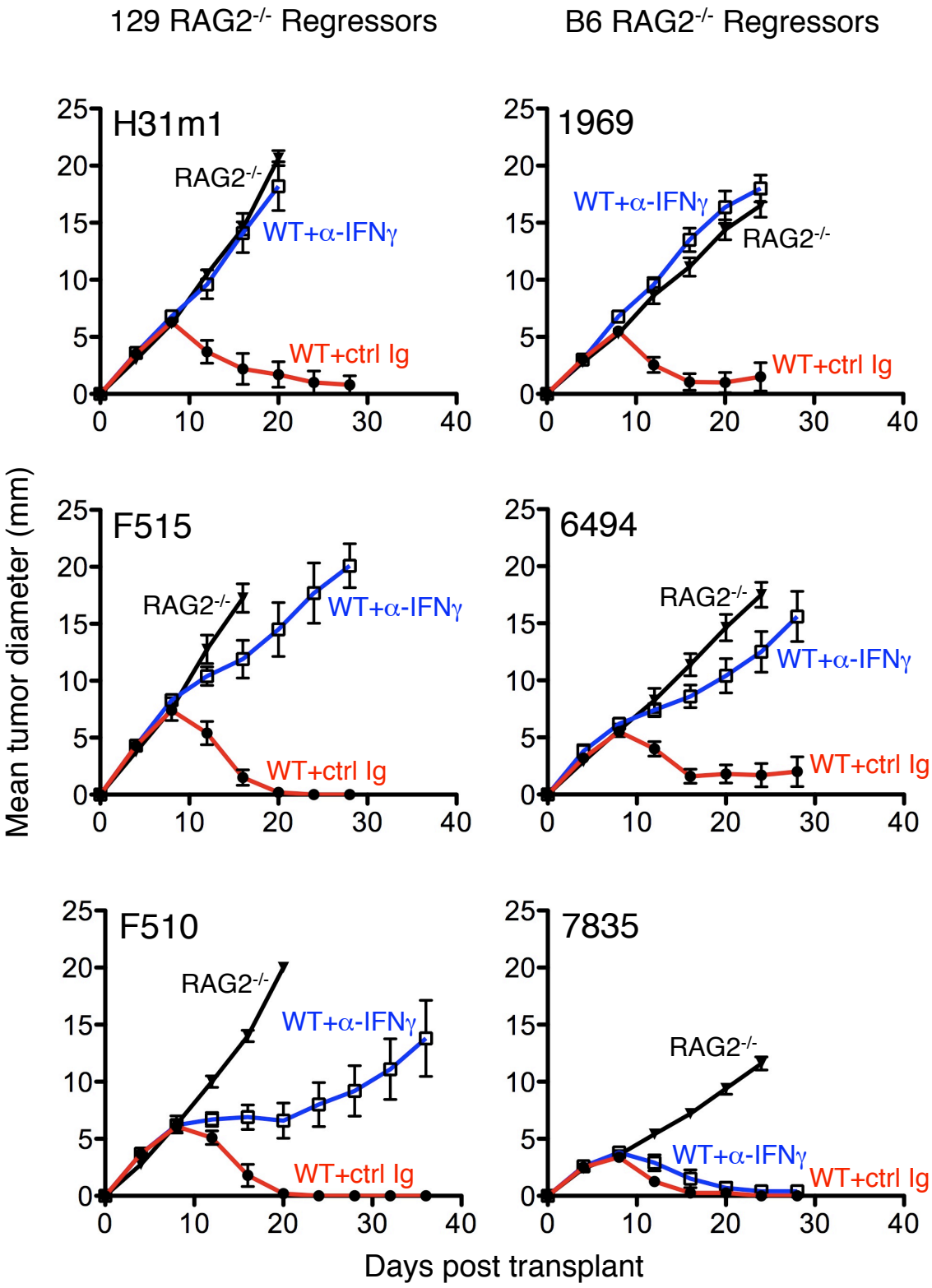

129 RAG2-/- Regressors
B6 RAG2-/- Regressors
H31m1
1969
d38m2
7835
1773
6494
RAG2-/-
IFNAR1-/-
WT
Mean tumor diameter (mm)
Days post transplant

Figure 2. Antibody-mediated blockade of endogenous IFNγ signaling abrogates rejection of 129 and B6 strain RAG2$^{-/-}$ regressor tumors. Groups of RAG2$^{-/-}$ mice or WT mice treated with either anti-IFNγ mAb H22 or isotype control mAb PIP were transplanted with 1x10^{6} tumor cells as indicated and growth was monitored. Mean tumor diameter ± s.e.m. over time is plotted.


129 RAG2-/- Regressors
B6 RAG2-/- Regressors
H31m1
1969
F515
6494
F510
7835
RAG2-/-
WT+α-IFNγ
WT+ctrl Ig
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

Enhanced Tumor Growth in the Absence of Host STAT1 Signaling

We postulated that if endogenous IFNα/β and IFNγ did indeed perform distinct functions on host cells, then RAG2$^{-/-}$ regressor tumor cells may exhibit more aggressive growth in mice unresponsive to both cytokines. For these studies we utilized gene-targeted mice engineered to express mutant STAT1 (Y→F mutation at position 701) recently generated in the laboratory (K.S. Lai, J.M. White, and R.D. Schreiber, unpublished data). STAT1 phosphorylation at tyrosine 701 is required for signal transduction as the Y701F mutant STAT1 displays no nuclear translocation, DNA binding activity, or IFN-mediated gene induction when introduced into STAT1-deficient U3A cells (146). As expected, cells isolated from homozygous STAT1^{Y701F} mutant mice were unresponsive to IFN treatment when examined in a variety of in vitro biologic assays (K.S. Lai, K.C.F. Sheehan, C. Arthur, and R.D. Schreiber, unpublished data).

To examine the growth of a transplantable tumor, H31m1 cells (which require for their rejection both IFNα/β and IFNγ at the level of the host) were injected into groups of WT, RAG2$^{-/-}$, and STAT1^{Y701F} mice (Figure 3A). Whereas this tumor challenge was rejected in WT hosts, the progressive growth of H31m1 tumor cells was almost identical in RAG2$^{-/-}$ and STAT1^{Y701F} mice. The H31m1 tumor would indeed be expected to grow in STAT1^{Y701F} mutant mice, yet the phenotype observed was more severe than that seen in either IFNAR1$^{-/-}$ or IFNGR1$^{-/-}$ mice, as summarized in Figure 3B from multiple independent experiments with each genotype. While H31m1 tumor cells grew with rapid kinetics in 100% of STAT1^{Y701} mice inoculated, growth in IFNAR1$^{-/-}$ or IFNGR1$^{-/-}$ mice

generally exhibits slower kinetics and is observed in a fraction of mice (ranging from 60-90% of mice between experiments) (see Figure 1 and Chapter 4, Figure 8).

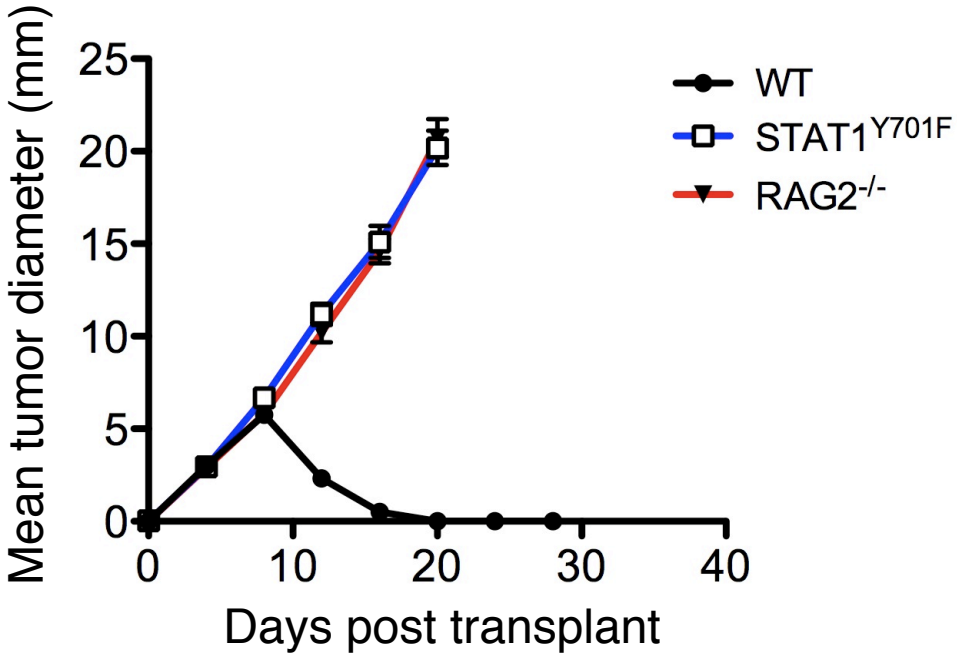
The additive effect of host deficiencies in both IFNα/β and IFNγ responsiveness on the growth of transplanted H31m1 cells provides additional indirect support for the notion that the IFNs exert non-redundant functions on the host. Experiments using IFNAR1$^{-/-}$xIFNGR1$^{-/-}$ doubly deficient mice, however, would be required to rule out any IFN-independent effects of the STAT1^{Y701F} mutation, though prior studies with STAT1$^{-/-}$ mice generated in the laboratory failed to show deficits in response to signaling via other cytokine ligands known to activate STAT1 – including EGF, GH and IL-10 (147). The differences we observed on the growth of transplanted tumor cells were not detected in previous work comparing the incidence of tumor development following MCA treatment of IFNGR1$^{-/-}$ mice compared to STAT1$^{-/-}$ mice (5, 18). This disparity may reflect differences in the respective models employed (i.e. tumor transplantation versus the multistep process of primary MCA carcinogenesis).

Interestingly, mice heterozygous for the STAT1^{Y701F} mutation showed an intermediate phenotype upon H31m1 challenge, with progressive tumor growth exhibiting slower kinetics and occurring in only 5/7 mice (data not shown). Additional studies in the laboratory have also documented intermediate responses in STAT1^{Y701F} heterozygous cells upon in vitro IFN treatment (K.S. Lai, K.C.F. Sheehan, C. Arthur, and R.D. Schreiber, unpublished data). These data are consistent with the reported ability of the STAT1^{Y701F} mutant protein to act in a dominant negative fashion when overexpressed in cells containing wild type STAT1 or cotransfected with a wild type STAT1 construct

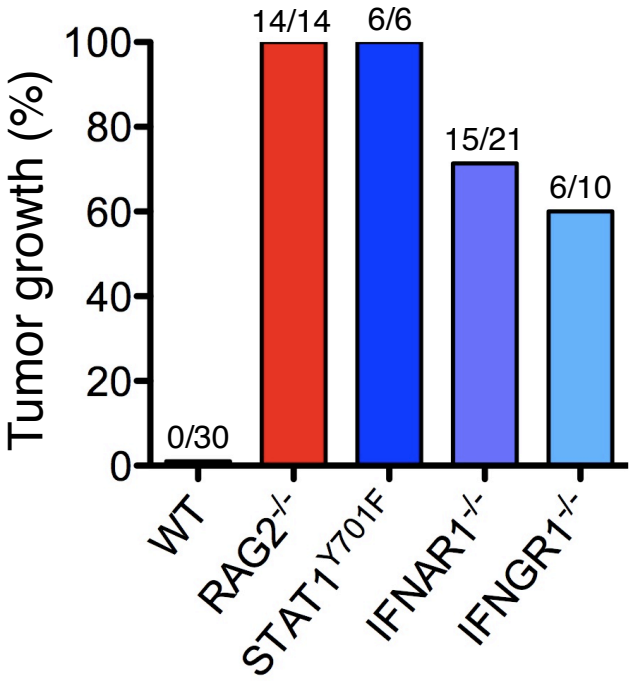
(148, 149); yet, they also demonstrate that such an effect is not merely an artifact of an overexpression system as it is also observed both in vitro and in vivo in cells expressing normal and mutant STAT1 from its endogenous promoter. The possibility that mutant $STAT1^{Y701F}$ protein could occupy phosphorylated receptor docking sites (thereby impeding the binding of wild type STAT1) or the recent demonstration that unphosphorylated STAT1 exists predominantly as a dimer in the cytoplasm (150) may provide an explanation for these findings.

Figure 3. Enhanced growth of H31m1 tumor cells in the absence of host responsiveness to both IFNα/β and IFNγ. (A) WT, RAG2$^{-/-}$, and STAT1^{Y701F} homozygous mutant mice were injected s.c. with 1x10^{6} H31m1 tumor cells. (B) Percent of WT, RAG2$^{-/-}$, STAT1^{Y701F}, IFNAR1$^{-/-}$, and IFNGR1$^{-/-}$ mice with progressively growing tumors following H31m1 injection. Mice with tumors >6x6 mm in diameter at the end of the observation period were considered tumor-positive. Cumulative results from 2-3 independent experiments are shown.

A


Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
Days post transplant
WT
STAT1Y701F
RAG2-/-

B


Tumor growth (%)
100
80
60
40
20
0
0/30
14/14
6/6
15/21
6/10
WT
RAG2-/-
STAT1Y701F
IFNAR1-/-
IFNGR1-/-

More Prolonged Requirement for the Actions of IFNγ Compared to IFNα/β During the Anti-Tumor Immune Response

Given the importance of both type I and type II IFN in the rejection of immunogenic transplantable tumors, we wanted to dissect the precise temporal requirements for their actions during the generation and execution of the immune response. In this set of experiments, type I IFN blockade was achieved by treatment of WT mice with anti-IFNAR1 mAb MAR1-5A3 (64), and IFNγ blockade via treatment with anti-IFNγ mAb H22. By initiating antibody treatment at different times following the inoculation of mice with H31m1 tumor cells, we were able to determine when the actions of the IFNs were no longer required for successful tumor rejection. As a control, we also treated mice with a cocktail of mAbs to deplete $CD4^+$ and $CD8^+$ cells and neutralize IFNγ (anti-CD4/CD8/IFNγ), in order to achieve a broad immunodepletion at different times and thus to better define the duration of immune function necessary for complete tumor rejection.

As summarized in Figures 4-5, anti-IFNAR1 mAb treatment of WT mice prior to H31m1 injection led to tumor outgrowth in 80% (12/15) of mice; whereas blockade at day 4 or day 6 (relative to tumor challenge at day 0) blocked rejection in a substantial, though slightly reduced fraction of mice – 53% (8/15) or 57% (8/14), respectively. In contrast, IFNα/β blockade beginning at day 8 or day 10 resulted in outgrowth in only 15% (3/20) or 13% (2/15) of mice, which was similar to that observed in mice treated with the same regimen of isotype control mAb (Figure 5 and data not shown). In

comparison, the actions of IFNγ were required for a more prolonged time period, as anti-IFNγ mAb treatment beginning as late as day 8 or day 10 was still able to abrogate rejection in a substantial number of animals, 55% (11/20) or 40% (6/15), respectively. This effect eventually diminished by day 12 or day 14. With anti-CD4/CD8/IFNγ mAb treatment, however, tumor recovery and outgrowth still occurred in a significant fraction of mice (50%, 4/8) even when the immune response was impeded as late as day 14.

These data indicate that the requirement for type I IFN is lost between day 6 and day 8, thus underscoring the importance of IFNα/β's actions during the early stages of an anti-tumor response. The finding that tumor outgrowth can still occur upon relatively late abrogation of immune function by anti-CD4/CD8/IFNγ mAb treatment, also suggests that type I IFN is not essential throughout the duration of immune effector function. This is in contrast to the more prolonged temporal requirement for IFNγ signaling during H31m1 rejection, perhaps signifying a more prominent role during the effector phase of the response due to its actions directly on the tumor cell in enhancing tumor immunogenicity and facilitating immune killing. An important role for tumor cell IFNγ sensitivity in H31m1 rejection has, in fact, been demonstrated both through overexpression of a dominant-negative IFNGR1 construct (G.P. Dunn, C.M. Koebel, and R.D. Schreiber, unpublished data) and by RNAi-mediated knockdown of IFNGR1 expression (see Chapter 4). This point may underlie another interesting observation emerging from these studies; specifically, the apparent discrepancy between the rapid and uniform growth of H31m1 in WT mice treated with anti-IFNγ mAb as compared to its less robust growth in IFNGR1$^{-/-}$ hosts. Antibody treatment would be expected to block IFNγ signaling in both

tumor and host cells, while the defect in IFNGR1$^{-/-}$ lies only in the cells of the host. Interestingly, given the hypothesis that IFNα/β's actions on the tumor cell is of little significance during the rejection of RAG2$^{-/-}$ regressors, the growth of H31m1 in WT mice treated with anti-IFNAR1 is more comparable to that observed in IFNAR1$^{-/-}$ mice.

Figure 4. More prolonged temporal requirement for IFNγ's actions than those of IFNα/β during tumor rejection. WT mice challenged with $1x10^6$ H31m1 tumor cells were treated beginning on the indicated day with anti-IFNAR1 mAb, anti-IFNγ mAb, or a mixture of anti-CD4/CD8/IFNγ mAb's and tumor growth was monitiored. Mice with tumors >6x6 mm at the conclusion of the observation period were considered tumor-positive. Control mice injected with H31m1 and treated similarly with isotype control mAb were included in each experiment, and data shown represent percent tumor growth above the control group. Data from 2-4 independent experiments were pooled.

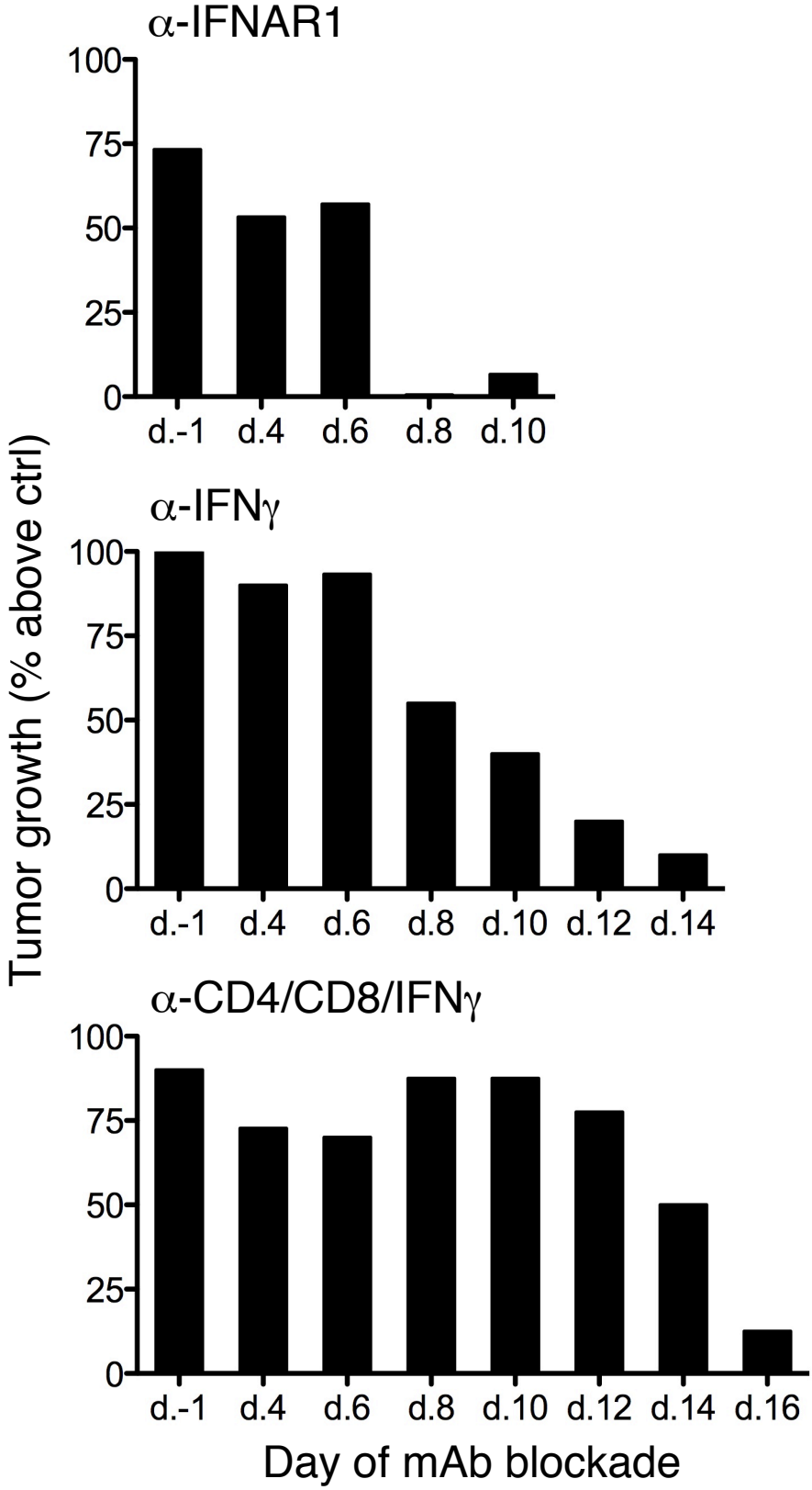
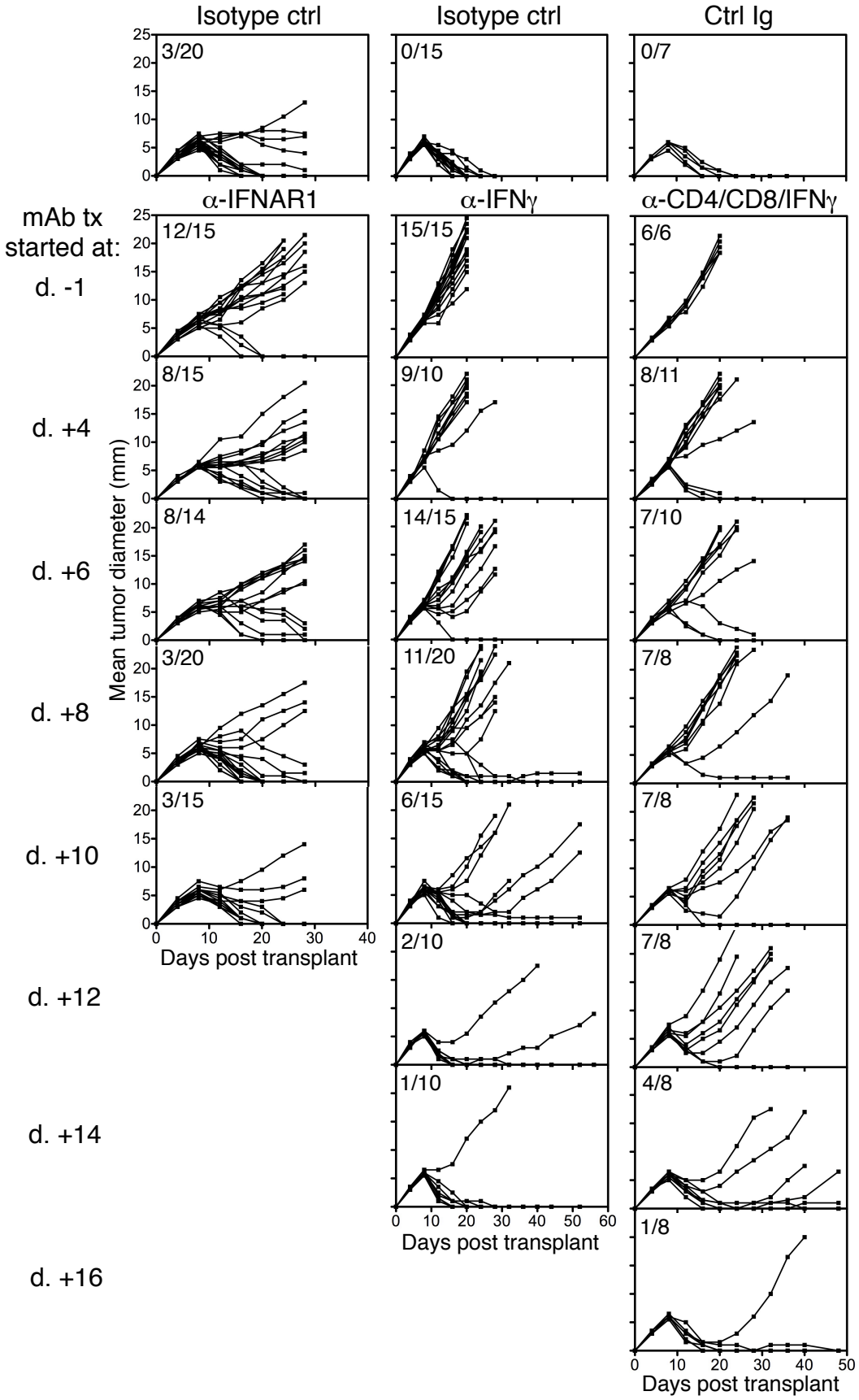

α-IFNAR1
100
75
50
25
0
d.-1 d.4 d.6 d.8 d.10
α-IFNγ
100
75
50
25
0
d.-1 d.4 d.6 d.8 d.10 d.12 d.14
α-CD4/CD8/IFNγ
100
75
50
25
0
d.-1 d.4 d.6 d.8 d.10 d.12 d.14 d.16
Tumor growth (% above ctrl)
Day of mAb blockade

Figure 5. Kinetics of H31m1 tumor growth following antibody-mediated IFN blockade at different times. Growth curves of H31m1 tumor cells in WT mice treated with anti-IFNAR1, anti-IFNγ, or anti-CD4/CD8/IFNγ mAb's beginning on different days after tumor injection are shown (as summarized in Figure 4). Similar treatments with the respective isotype control mAb were performed for all time points, though only one representative graph for each isotype control is shown. Lines represent individual mice and the fraction of tumor-positive mice is indicated. The higher background levels observed with GIR-208 mAb treatment compared to the other isotype controls is unexplained, though perhaps due to the higher mAb dose administered (2.5 mg single injection).


Isotype ctrl
Isotype ctrl
Ctrl Ig
α-IFNAR1
α-IFNγ
α-CD4/CD8/IFNγ
mAb tx started at:
d. -1
d. +4
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d. +8
d. +10
d. +12
d. +14
d. +16
3/20
0/15
0/7
12/15
15/15
6/6
8/15
9/10
8/11
8/14
14/15
7/10
3/20
11/20
7/8
3/15
6/15
7/8
2/10
7/8
1/10
4/8
1/8
Mean tumor diameter (mm)
Days post transplant
Days post transplant
Days post transplant

Impaired Generation of Tumor-Specific T Cells in IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ Mice

To examine the mechanism underlying the defect in anti-tumor immunity in the absence of host responsiveness to the IFNs, we looked specifically at the priming of tumor-specific T cells in IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice following tumor challenge. In collaboration with Hirokazu Matsushita in the laboratory, we utilized an ex vivo restimulation protocol to assay for the presence of tumor-specific T cells in the spleen. At a given time point following tumor inoculation, splenocytes were isolated and cocultured in vitro with irradiated IFNγ-treated tumor cells. Five days later, splenocytes were used as CTL effectors in a cytotoxicity assay with ^{51}Cr-labeled, IFNγ-treated tumor cells as targets at the indicated E:T ratios. In each case, killing of radiolabeled irrelevant tumor cells (to which the mouse should be naïve) was also assayed to ensure specificity of tumor cell killing. This protocol was used previously to demonstrate a lack of T cell priming in CD8α^{+} DC-deficient p21SNFT$^{-/-}$ mice following H31m1 tumor challenge (151). In addition, we have demonstrated that (i) tumor cell killing is CD8-dependent, since blocking mAb's to CD8 but not CD4 were able to inhibit killing (data not shown, see Chapter 3, Figure 12), and (ii) in vitro restimulation of naïve splenocytes yields no cytotoxic activity (see Chapter 3, Figure 11).

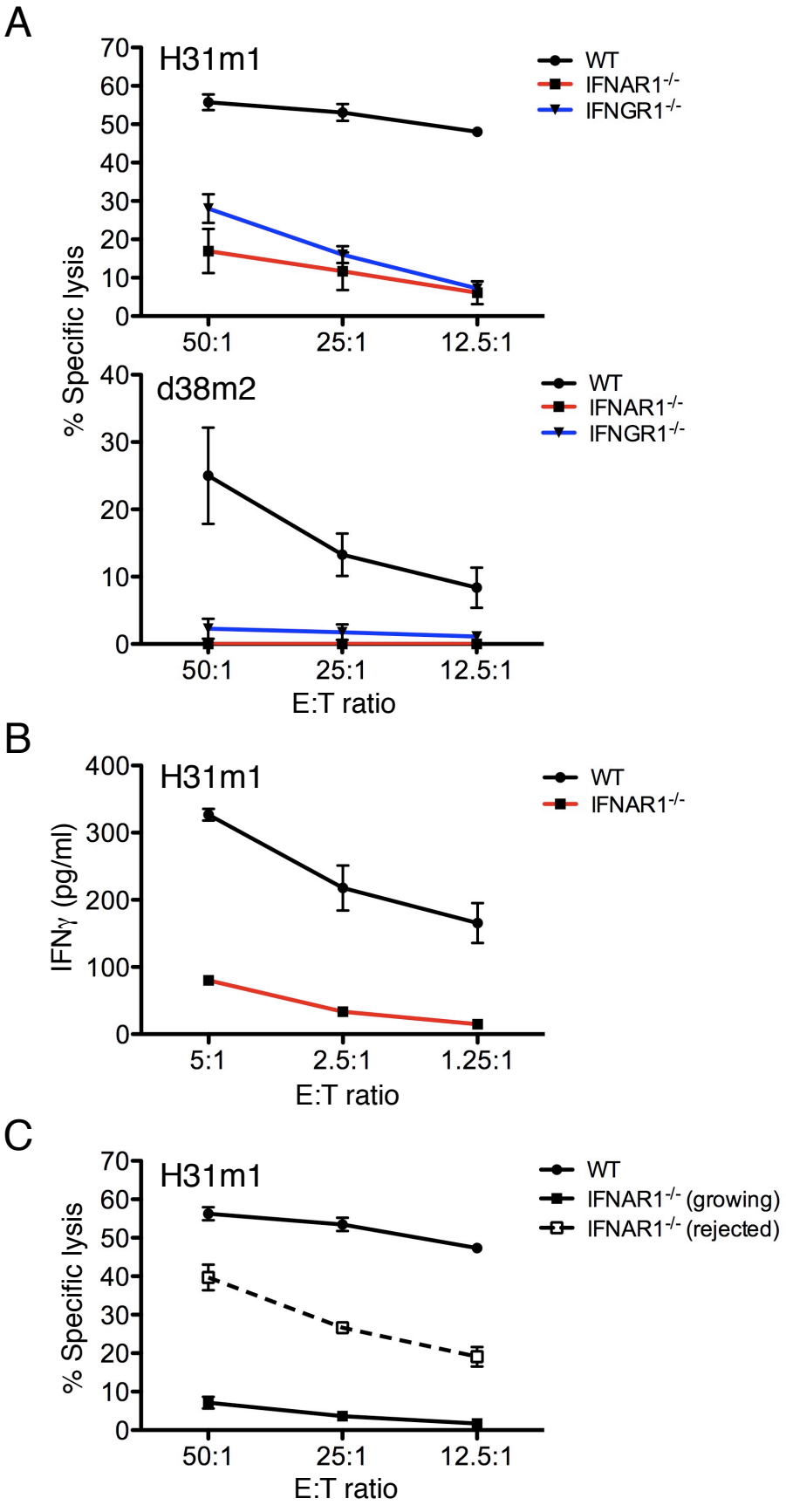
Using this experimental protocol, we assayed splenocytes from WT, IFNAR1$^{-/-}$, and IFNGR1$^{-/-}$ mice at day 20 following injection of H31m1 or d38m2 tumor cells. Although robust killing of the respective tumor targets was detected with splenocytes from WT mice, very little cytotoxic activity was observed with splenocytes from

IFNAR1$^{-/-}$ or IFNGR1$^{-/-}$ mice (Figure 6A). In addition to cytotoxicity, splenocytes from H31m1 tumor-challenged IFNAR1$^{-/-}$ mice showed impaired IFNγ production compared to WT mice when cocultured with tumor targets (Figure 6B). While poor tumor-specific T cell priming is not unexpected given the inability of IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice to reject these immunogenic tumors, these data suggest a defect relatively early in the anti-tumor response, as opposed to more downstream deficits in effector cell trafficking to the tumor or tumor cell killing.

As described previously, a minority of IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice challenged with RAG2$^{-/-}$ regressor tumor cells exhibit complete (though sometimes delayed) rejection of the tumor inoculum. To ask whether tumor-specific T cell generation correlates with the success of the anti-tumor response in IFNAR1$^{-/-}$ mice, we performed similar experiments on WT mice, IFNAR1$^{-/-}$ bearing progressively growing tumors, and IFNAR1$^{-/-}$ mice which had rejected the tumor by day 20 (Figure 6C). Whereas H31m1 tumor-bearing IFNAR1$^{-/-}$ mice again showed poor cytotoxic activity, IFNAR1$^{-/-}$ hosts that had rejected the tumor challenge displayed considerably better tumor cell killing – though levels were still intermediate compared to WT mice. While T cell priming in IFNAR1$^{-/-}$ hosts may be less efficient in all cases, these findings indicate that rarely the generation of tumor-specific T cells can surpass the threshold needed for successful tumor rejection. Though we have not specifically looked at IFNGR1$^{-/-}$ mice that have rejected a tumor challenge, it is likely that a similar finding would be observed given the importance of effector T cells for rejection. The IFN-independent compensatory pathways which can drive the generation of tumor-specific T cells (though perhaps less

efficiently) are unclear at present and will require further study. Interestingly, it has been suggested that either IL-12 or IFNα/β can function as "signal 3" (in addition to antigen and costimulation), necessary for full clonal expansion and acquisition of effector function by $CD8^+$ T cells (131, 152). In addition, during viral infection with LCMV, IL-12 was found to provide an alternative pathway to the generation of IFNγ-producing $CD8^+$ T cells in $IFNAR1^{-/-}$ mice, even though the T cell response was normally independent of IL-12 in WT hosts (133).

Figure 6. Lack of tumor-specific T cell priming in IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice. (A) Splenocytes from WT, IFNAR1$^{-/-}$, or IFNGR1$^{-/-}$ mice were isolated 20 days following tumor challenge, restimulated in vitro, and used as effector CTL in a cytotoxicity assay with radiolabeled tumor targets. Percent specific lysis at the indicated E:T ratio is plotted for n=2-4 animals/group assayed in duplicate and data is representative of multiple independent experiments. (B) IFNγ production by ELISA after overnight coculture of splenocytes and tumor cells. Data from one of two independent experiments with similar results are shown. (C) Cytotoxicity assay using splenocytes from WT mice, IFNAR1$^{-/-}$ with growing tumors, or IFNAR1$^{-/-}$ mice that had rejected H31m1 tumor cells. Data are from two independent experiments with n=2-4 mice/group assayed in duplicate.


A
H31m1
WT
IFNAR1-/-
IFNGR1-/-
% Specific lysis
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
d38m2
WT
IFNAR1-/-
IFNGR1-/-
40
30
20
10
0
50:1
25:1
12.5:1
E:T ratio
B
H31m1
WT
IFNAR1-/-
IFNγ (pg/ml)
400
300
200
100
0
5:1
2.5:1
1.25:1
E:T ratio
C
H31m1
WT
IFNAR1-/- (growing)
IFNAR1-/- (rejected)
% Specific lysis
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
E:T ratio

Immunogenicity of RAG2$^{-/-}$ Regressor Tumors is Cell Intrinsic, Suggesting Specificity in Immune-Mediated Tumor Elimination

Given the strong IFN-dependence of RAG2$^{-/-}$ regressor tumor rejection, one might hypothesize that the differences in immunogenicity between a regressor and a progressor sarcoma are related to a differential ability to induce interferon production in the host, either through mechanisms of innate immune recognition or by the elaboration of endogenous danger signals. If this were indeed the case, then coinjection of regressor tumor cells along with progressor cells may promote the initiation of an immune response and lead to rejection of a normally tumorigenic dose of progressor cells. To test this hypothesis we used mixtures of progressor and regressor tumor cells differing in their surface expression of IFNAR1, providing a neutral marker (since tumor cell IFNα/β sensitivity does not seem to affect tumor immunogenicity) with which to track the two different cell types.

When a 50:50 mixture of RAG2$^{-/-}$ regressor cells (H31m1 or d38m2) and IFNAR1$^{-/-}$-derived progressor cells (d97m915) were injected into mice, progressive tumor growth was still observed in both RAG2$^{-/-}$ and WT hosts (Figure 7). The converse mixture – that is, an IFNAR1$^{-/-}$-derived regressor (d93m1244) and a WT progressor (F244) – also yielded similar results. The addition of immunogenic regressor tumor cells was therefore unable to induce rejection of a tumorigenic inoculum of progressor cells, arguing against a divergence only in their ability to stimulate IFN production or induce early innate immune activation. An alternate possibility, however, is that mechanisms of

immune suppression mediated by progressor cells are dominant, and may thus prevent the elimination of immunogenic regressor cells within the mixture. To examine this possibility, we harvested late stage tumors (at day 24) from RAG2$^{-/-}$ and WT mice and analyzed the tumor cell composition by IFNAR1 staining. Interestingly, whereas the tumors from RAG2$^{-/-}$ mice contained a mixture of regressor and progressor cells (even somewhat skewed toward the regressor), those from WT mice consisted of tumors cells almost uniformly derived from the progressor cell component (Figure 8 and data not shown). A similar result was obtained with both d38m2/d97m915 and H31m1/d97m915 tumor cell mixtures, though we did not test different ratios of mixing.

These observations indicate that the immunogenicity of RAG2$^{-/-}$ regressor tumors is cell intrinsic, while also providing an example of lymphocyte-dependent immunoediting in a tumor transplantation setting. The inherent immunogenicity of regressor cells compared to progressor cells may be related to differences in the efficiency of immune priming (perhaps due to the spectrum of antigens they possess) or to a selective resistance against immune killing or other effector mechanisms (perhaps through expression of inhibitory ligands or antigen-specific suppression mediated by Treg cells). The capacity of the immune system to specifically eliminate the regressor cells within the mixture also argues against a mechanism of RAG2$^{-/-}$ regressor tumor rejection involving immune targeting of the tumor stroma or tumor-associated endothelium as postulated by others (153-155), since this would presumably affect the growth of progressor cells as well. In addition, it implies a mechanism of specific killing, consistent also with the requirement for T and B lymphocytes. While tumor cell killing

by T cells is perhaps the most likely explanation, NK cells and macrophages may be afforded the necessary specificity by tumor-specific antibody bound to the tumor targets (eliciting ADCC) or perhaps other mechanisms.

Figure 7. Coinjection of a progressor tumor with immunogenic IFNα/β- and IFNγ-dependent regressor tumor cells does not elicit rejection. Groups of WT and $RAG2^{-/-}$ mice were injected with 50:50 mixtures of the regressor and progressor tumor cells as indicated injected at a dose of $2x10^6$ cells/mouse ($1x10^6$ cells of each tumor). Tumor size was measured over time and is plotted as mean tumor diameter ±s.e.m. Data is representative of two independent experiments with n=3-4 ($RAG2^{-/-}$) or n=5-7 (WT) mice/group.

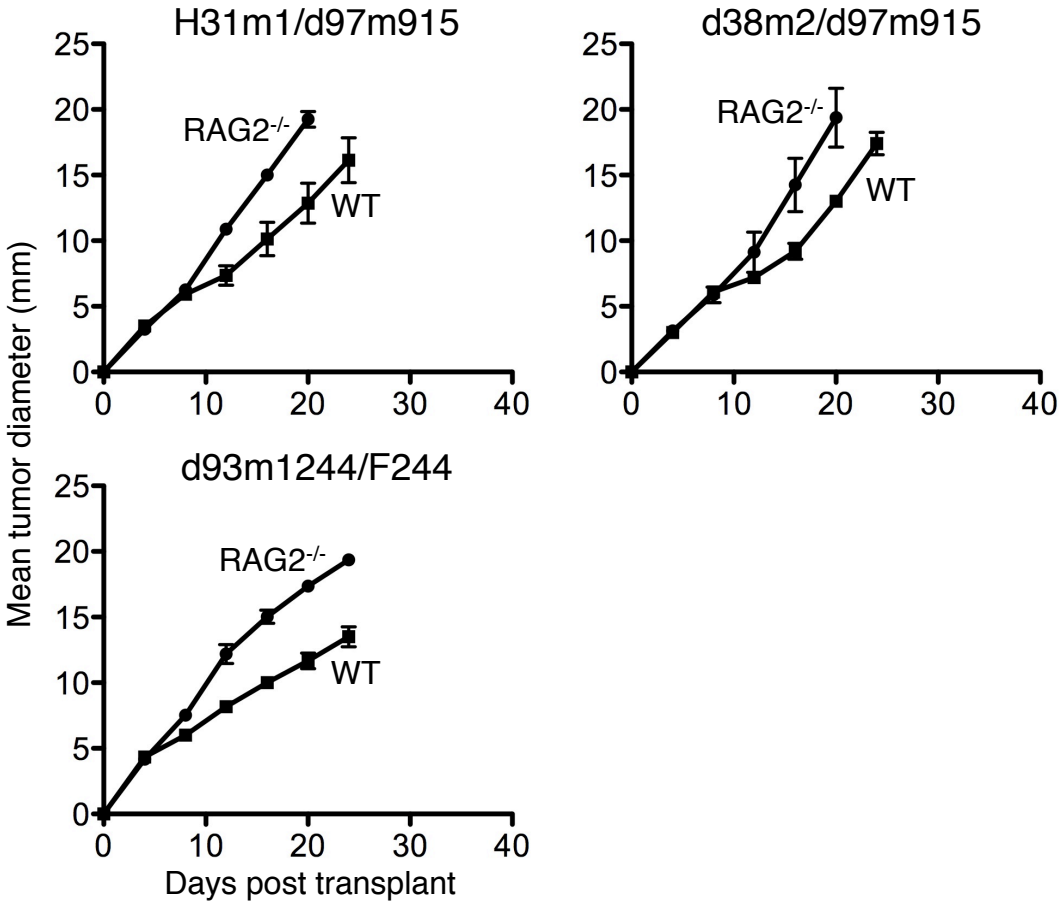
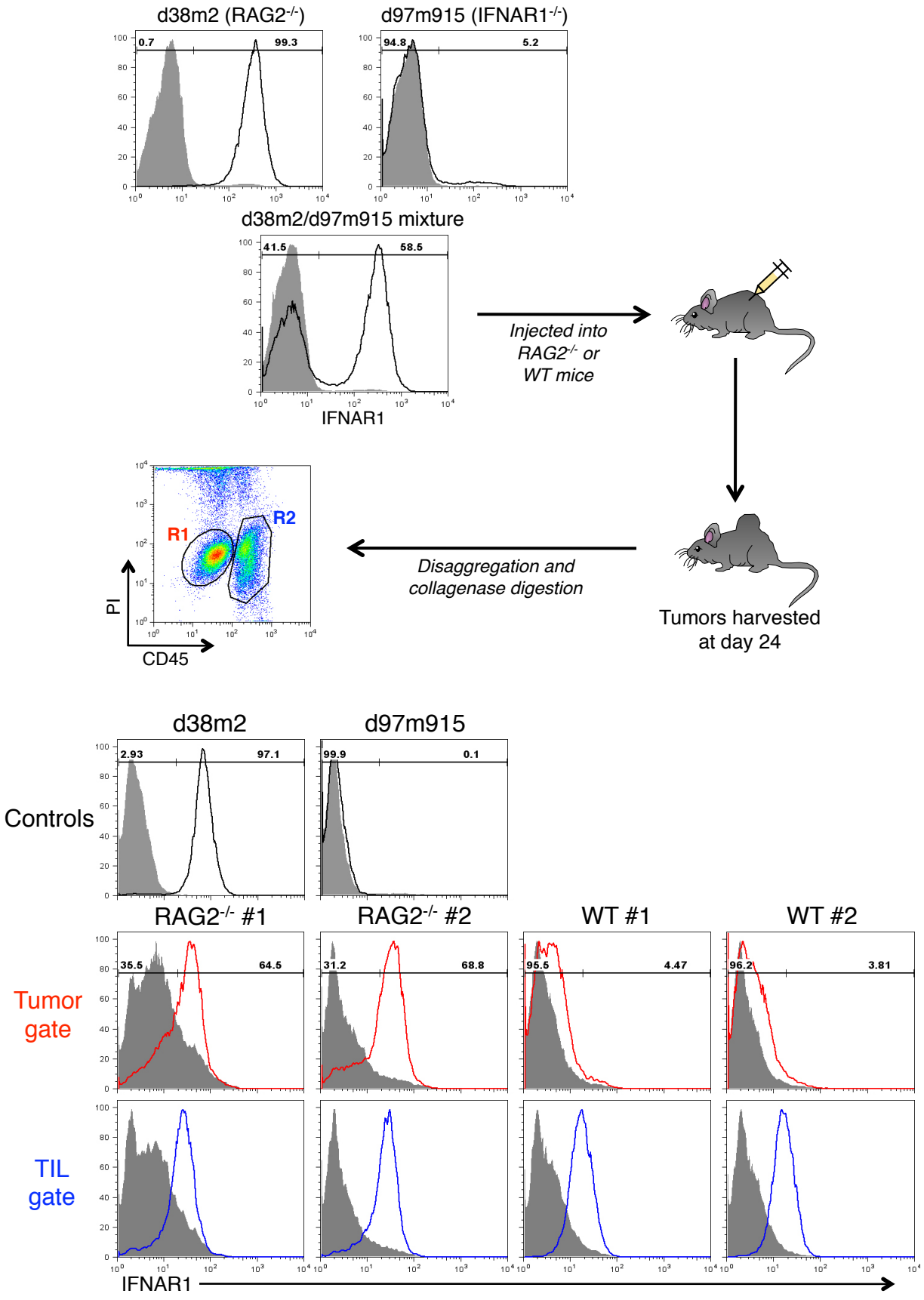

H31m1/d97m915
d38m2/d97m915
d93m1244/F244
RAG2-/-
WT
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

Figure 8. Immunogenicity of RAG2$^{-/-}$ regressor tumors is cell intrinsic. A 50:50 mixture of regressor (d38m2) and progressor (d97m915) tumor cells, distinguishable by differential expression of IFNAR1, were injected into RAG2$^{-/-}$ or WT mice. At day 24 post-injection, tumors were harvested, disaggregated, and stained for CD45 and IFNAR1 expression. FACS plots show IFNAR1 staining of cells within the tumor (CD45-PI-) or leukocyte (CD45+PI-) gates from two representative RAG2$^{-/-}$ and WT mice. Similar results were also observed when mixtures of H31m1 and d97m915 were analyzed ex vivo at day 24 (data not shown).


d38m2 (RAG2-/-)
d97m915 (IFNAR1-/-)
0.7
99.3
94.8
5.2
d38m2/d97m915 mixture
41.5
58.5
IFNAR1
Injected into RAG2-/- or WT mice
Tumors harvested at day 24
Disaggregation and collagenase digestion
R1
R2
PI
CD45
d38m2
d97m915
Controls
2.93
97.1
99.9
0.1
RAG2-/- #1
RAG2-/- #2
WT #1
WT #2
Tumor gate
TIL gate
35.5
64.5
31.2
68.8
95.5
4.47
96.2
3.81
IFNAR1

IFNβ is Not Essential for the Rejection of Immunogenic RAG2$^{-/-}$ Regressor Tumors

A critical role for type I IFN's actions on the host during tumor rejection have been well documented, yet little is known about the specific contributions of individual members of this cytokine family. In order to test whether IFNβ performs a unique function and is therefore required for the rejection of immunogenic transplantable tumors, we employed gene-targeted mice with a genetic deficiency in IFNβ due to insertion of the lacZ gene (139). Since these mice had been maintained on a mixed B6x129 genetic background, we backcrossed them onto the 129 SvEv strain using a speed congenics approach, thus allowing for tumor transplantation studies with our previously characterized regressor sarcomas. As shown in Figure 9, when three representative regressor tumors (H31m1, d38m2, and GAR4.GR1) were injected into IFNβ$^{-/-}$ mice, we observed no significant defect in the anti-tumor response – a very minimal difference perhaps following challenge with H31m1 (growth in 1/14 IFNβ$^{-/-}$ mice vs. 0/13 WT mice) or GAR4.GR1 (2/11 IFNβ$^{-/-}$ vs. 0/10 WT). In contrast, all three of these tumors grew progressively in IFNAR1$^{-/-}$ hosts as well as RAG2$^{-/-}$ control mice. Uniform rejection in IFNβ$^{-/-}$ mice was also observed when mice were challenged with two additional IFNα/β-dependent regressor tumors, F510 and 1773 (data not shown). Taken together, these data demonstrate that type I IFN-dependent rejection can occur normally in the absence of IFNβ, indicating either no role for IFNβ or potential redundancy in the functions of IFNβ and the relatively large number of other IFNα/β subtypes.

To ensure that tumor rejection in IFNβ$^{-/-}$ mice was still dependent on IFNα/β signaling, we treated IFNβ$^{-/-}$ mice with either anti-IFNAR1 or isotype control mAb prior to transplantation of H31m1 tumor cells. Whereas control mAb treatment had no effect on tumor rejection, IFNα/β receptor blockade abrogated rejection in the majority (9/12) of IFNβ$^{-/-}$ mice (Figure 10), comparable to the effect of anti-IFNAR1 treatment of WT mice. Although we also attempted to neutralize IFNα using several pan-IFNα-specific mAbs, treatment of WT or IFNβ$^{-/-}$ mice had no effect on tumor rejection (data not shown). Efforts to develop IFNα-specific mAb's of higher affinity and broader coverage against the different IFNα subtypes are currently ongoing in the laboratory.

The above experiments were performed using IFNβ$^{-/-}$ mice of approximately 95% genetic purity to the 129 SvEv strain (by microsatellite analysis), yet these results were subsequently confirmed with completely backcrossed 129 SvEv IFNβ$^{-/-}$ mice. Nevertheless, one could still argue in either case that very minor genetic differences remain which might contribute to an allogeneic immune response to the transplanted tumor cells. For this reason, we wanted to address the possible contribution of an allogeneic response to tumor rejection in IFNβ$^{-/-}$ mice, as well as the potential role of type I IFN in allogeneic tumor rejection. We thus tested the growth of a WT 129 SvEv strain progressor tumor (F244) in IFNβ$^{-/-}$ mice and observed no difference in its growth kinetics as compared to growth in WT 129 SvEv mice, despite the fact that F244 tumor cells were rejected in B6 strain WT mice and B6 IFNAR1$^{-/-}$ mice (Figure 11). This suggests the small contribution of B6 alleles to the background of IFNβ$^{-/-}$ mice is insufficient to cause an alloresponse, while also indicating that host sensitivity to IFNα/β

is not required for the rejection of allogeneic tumor cells. Therefore, we find it unlikely that an allogeneic immune response directed at minor antigens is contributing to tumor rejection in IFNβ$^{-/-}$ mice given that (i) a 129 SvEv WT progressor tumor grows in IFNβ$^{-/-}$ mice but not B6 strain WT or IFNAR1$^{-/-}$ mice, and (ii) type I IFN blockade abrogates rejection in IFNβ$^{-/-}$ mice, whereas allogeneic tumor rejection does not require IFNα/β responsiveness in the host (see also Figure 12).

Figure 9. Host-derived IFNβ is not required for the rejection of immunogenic tumors. (A) H31m1, d38m2, and GAR4.GR1 regressor tumor cells were injected at a dose of $1x10^6$ cells/mouse into groups of WT, $RAG2^{-/-}$, $IFNAR1^{-/-}$, and $IFN\beta^{-/-}$ mice. Mean tumor diameter ± s.e.m. over time is plotted and the percent tumor growth for each group is summarized in (B).

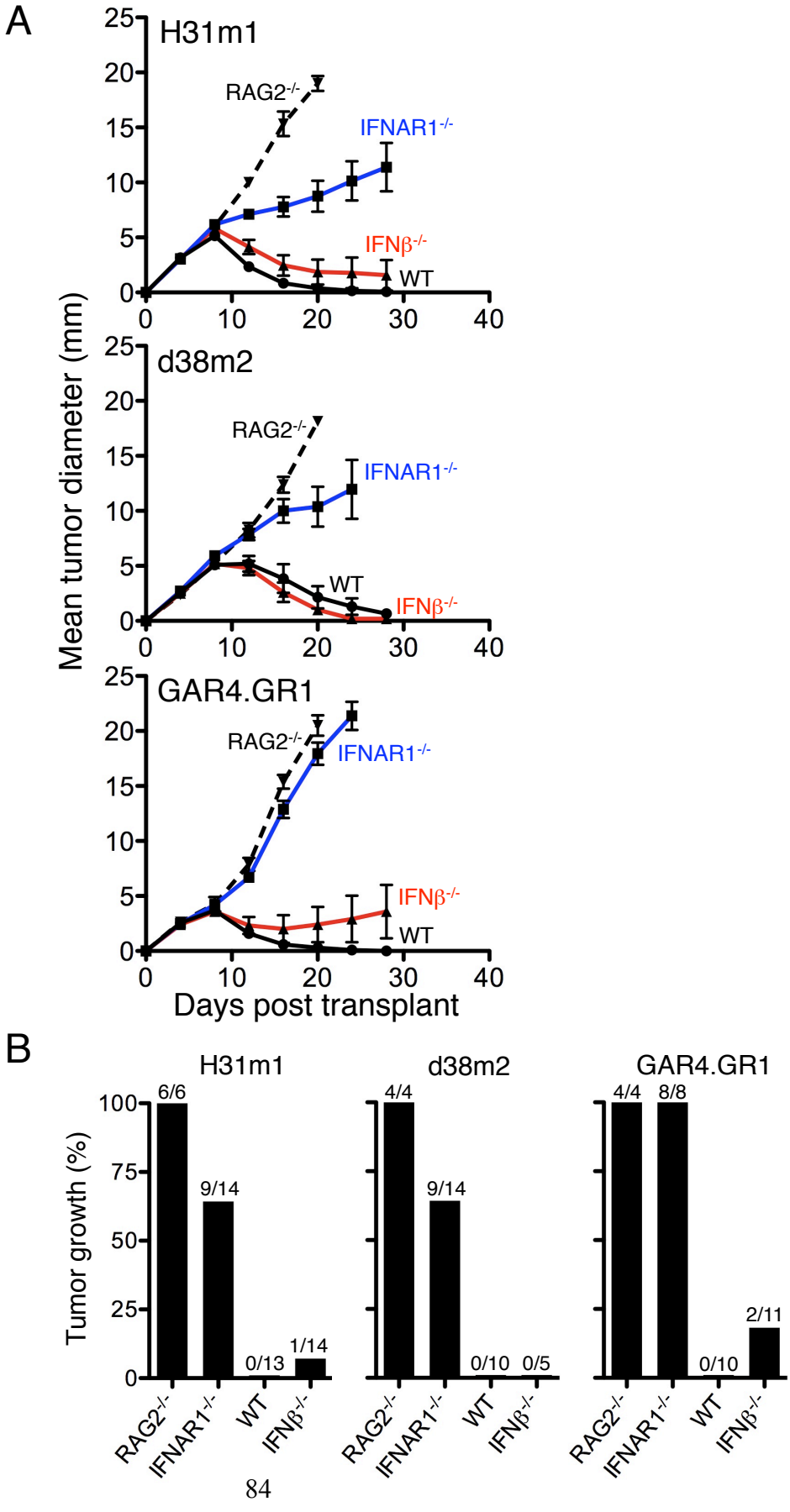
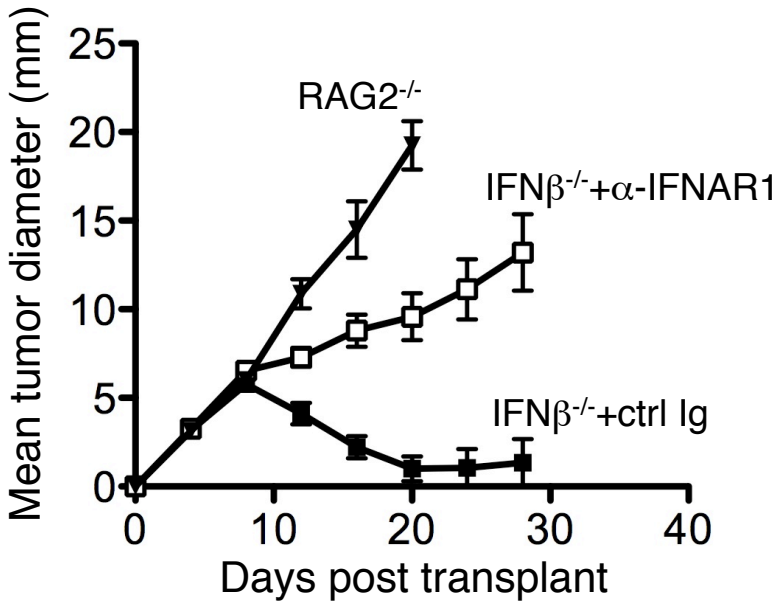

A
H31m1
RAG2-/-
IFNAR1-/-
IFNβ-/-
WT
d38m2
GAR4.GR1
Mean tumor diameter (mm)
Days post transplant
B
Tumor growth (%)
6/6
9/14
0/13
1/14
4/4
9/14
0/10
0/5
4/4
8/8
0/10
2/11
RAG2-/-
IFNAR1-/-
WT
IFNβ-/-

Figure 10. Tumor rejection in IFNβ$^{-/-}$ mice is still dependent on IFNα/β signaling. (A) Groups of IFNb-/- mice were treated with anti-IFNAR1 mAb MAR1-5A3 or isotype control GIR-208 mAb and transplanted with 1x10^{6} H31m1 tumor cells. Tumor growth over time for mAb treated and RAG2$^{-/-}$ control mice is shown as mean tumor diameter ± s.e.m. Percent tumor growth per experimental group is summarized in (B) from multiple independent experiments.

A


Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
Days post transplant
RAG2-/-
IFNβ-/-+α-IFNAR1
IFNβ-/-+ctrl Ig

B

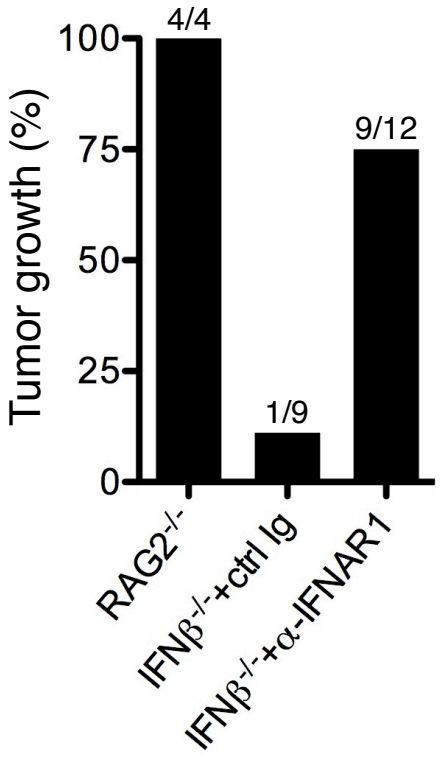
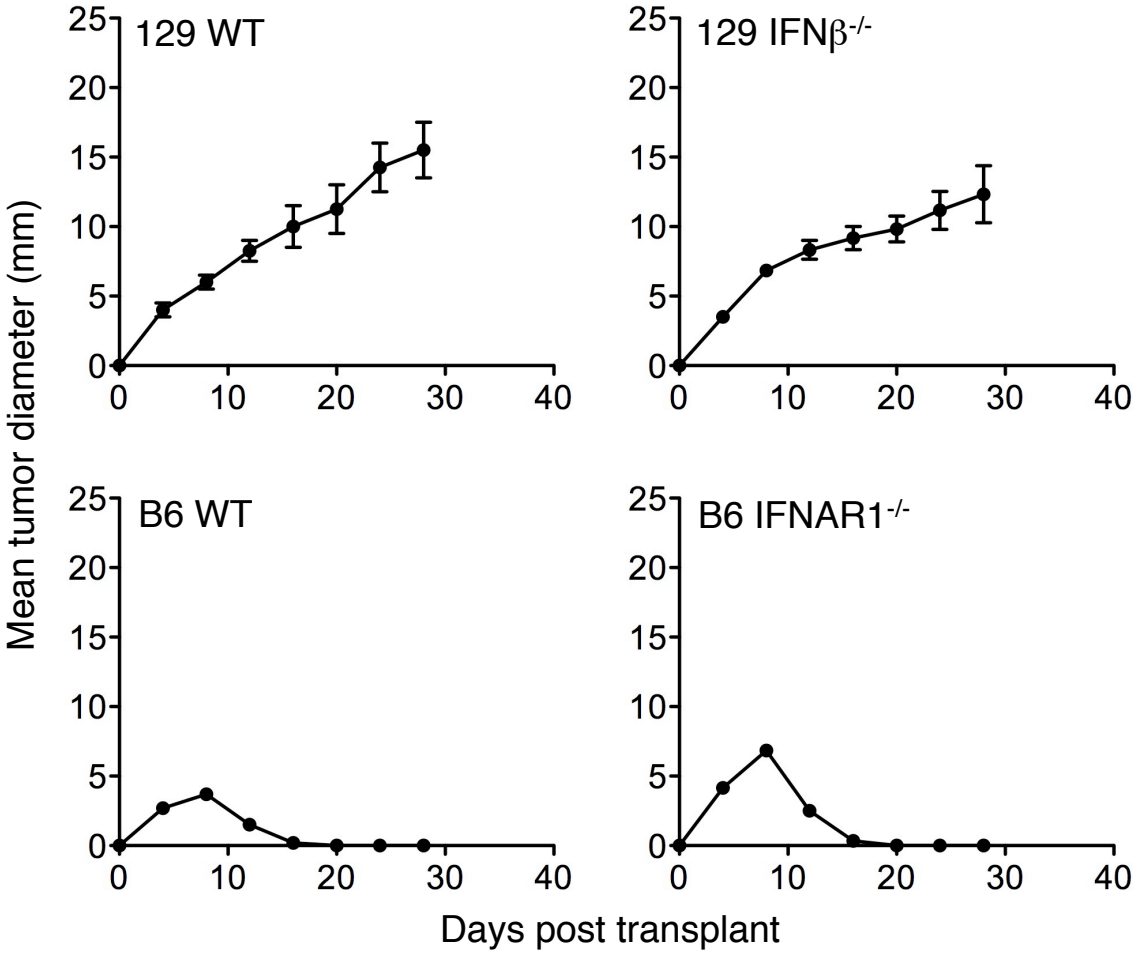

Tumor growth (%)
100
75
50
25
0
4/4
1/9
9/12
RAG2-/-
IFNβ-/-+ctrl Ig
IFNβ-/-+α-IFNAR1

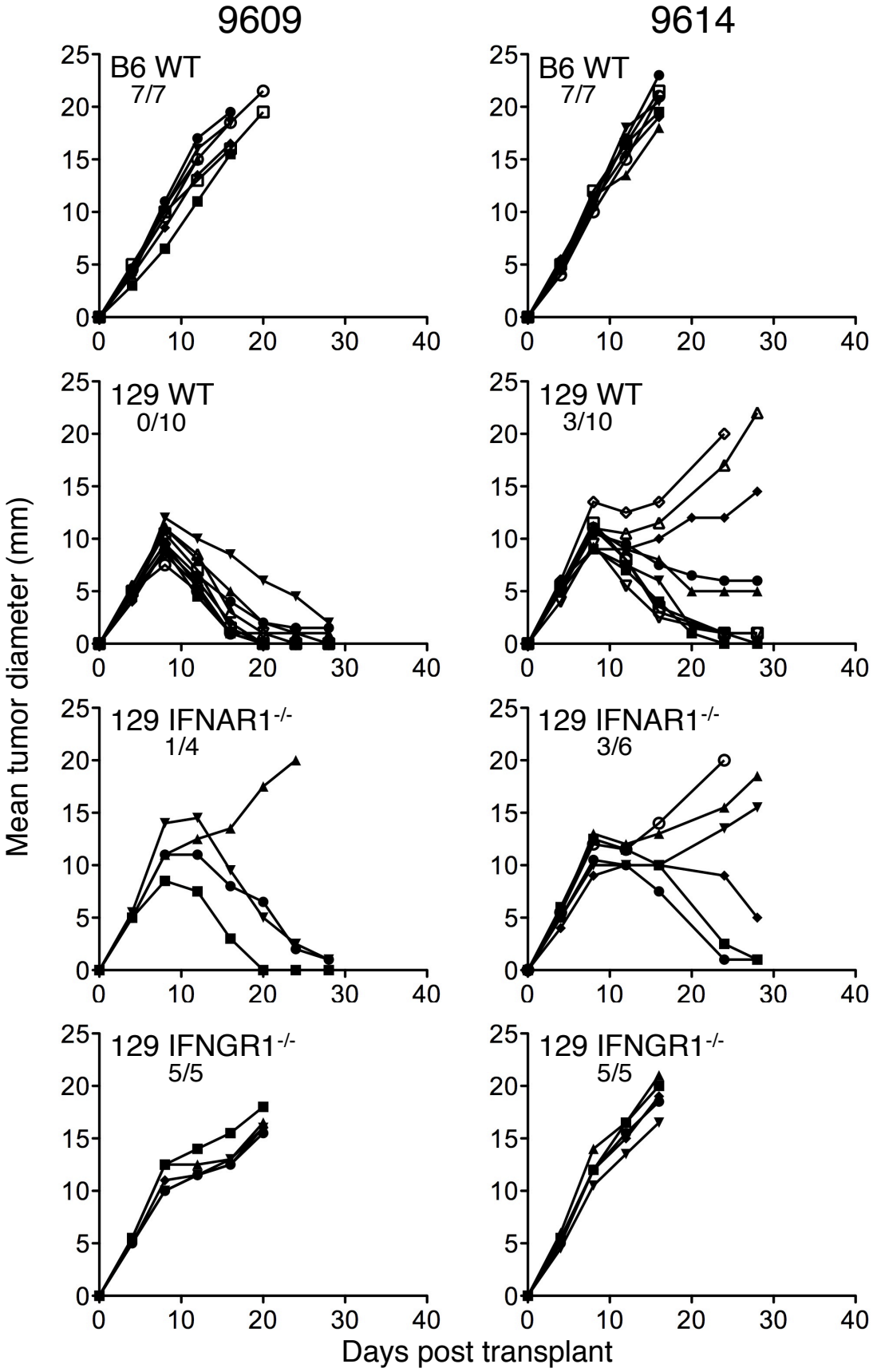
Figure 11. Absence of allogeneic tumor rejection in IFNβ$^{-/-}$ mice. 129 strain WT and IFNβ$^{-/-}$ mice as well as B6 WT and B6 IFNAR1$^{-/-}$ mice were challenged with 1x10^{6} F244 (129/SvEv-derived) progressor tumor cells and tumor growth was monitored over time. Data represent the mean tumor diameter ± s.e.m. of n=3-5 mice/group.


129 WT
129 IFNβ-/-
B6 WT
B6 IFNAR1-/-
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40

Host Cell Sensitivity to IFNγ But Not IFNα/β is Required for Rejection of an Allogeneic Tumor Challenge

Given the observation that the 129 SvEv strain WT progressor tumor F244 was rejected when transplanted into both WT and IFNAR1$^{-/-}$ mice on a C57Bl/6 background, we decided to investigate more fully the role of the IFNs in the rejection of an allogeneic tumor challenge. For these experiments, we employed two different B6 strain WT progressors, 9609 and 9614, which exhibit extremely aggressive growth when transplanted into syngeneic B6 WT hosts (Figure 12). In contrast, when transplanted into WT 129 SvEv (or 129 SvPas) strain mice, these tumors grew quite large initially (reaching approximately 10mm in average diameter by day 8), before being rapidly rejected in the majority of mice. Surprisingly, transplantation into 129 strain IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice revealed that the allogeneic rejection of these two tumors was largely independent of host IFNα/β responsiveness, yet almost completely abrogated in the absence of host IFNγ sensitivity. The reasons for the differential requirement of IFNα/β and IFNγ during allogeneic tumor rejection will require further study. Nevertheless, these findings highlight potentially interesting differences in the mechanism of allogeneic tumor rejection versus rejection of syngeneic, though still highly immunogenic, RAG2$^{-/-}$ regressor sarcomas.

Figure 12. Allogeneic tumor rejection requires host responsiveness to IFNγ but not IFNα/β. B6 strain progressor tumor cells 9609 and 9614 were injected at a dose of $1x10^6$ cells/mouse into groups of B6 WT, 129 WT, 129 IFNAR1$^{-/-}$, and 129 IFNGR1$^{-/-}$ mice. Each line represents an individual mouse and the fraction of mice with progressively growing tumors is indicated. Allogeneic rejection of 9609 and 9614 was observed in both 129/SvEv and 129/SvPas WT mice.


9609
9614
B6 WT
7/7
B6 WT
7/7
129 WT
0/10
129 WT
3/10
129 IFNAR1-/-
1/4
129 IFNAR1-/-
3/6
129 IFNGR1-/-
5/5
129 IFNGR1-/-
5/5
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

DISCUSSION

In order to further understand the immunostimulatory mechanisms promoted by the IFNs during anti-tumor responses, we examined various aspects of the host response to immunogenic tumors and the role of the IFNs in these processes. Using a new cohort of RAG2$^{-/-}$-derived regressor tumors from C57Bl/6 strain mice, we generalized original observations made with 129 tumors by showing that rejection of B6 regressors also required IFNγ signaling as well as host responsiveness to IFNα/β. To determine when during the immune response the respective IFNs were acting, we performed antibody blockade experiments and demonstrated that whereas the early actions of IFNα/β were sufficient for tumor rejection, the temporal requirement for IFNγ's actions was more prolonged. We further showed that in the absence of host sensitivity to either IFNα/β or IFNγ, generation of tumor-specific T cells with effector activity is severely impaired, suggesting a central role in promoting the initiation of adaptive responses to tumors. Using mixtures of immunogenic and non-immunogenic tumor cells, we then confirmed that specific adaptive immune killing was responsible for rejection, since regressor tumor cells were selectively eliminated within tumor cell mixtures. Finally, because the type I IFN family consists of a large number of individual subtypes which might possess non-redundant functions in vivo, we examined whether IFNβ was essential during the rejection of transplantable tumors. IFNβ-deficient mice, however, showed no defect in their ability to reject immunogenic 129 regressor tumors, suggesting potential redundancy or a more prominent role for the IFNα subtypes.

Several pieces of data gleaned from these initial studies provide preliminary evidence that the IFNs might be performing distinct functions on the host during tumor rejection. In addition to their differing temporal requirements, analysis of STAT1^{Y701F} mutant mice showed that growth of transplantable tumor cells was more aggressive in mice lacking responsiveness to both IFNα/β and IFNγ than in mice lacking sensitivity to one or the other. In addition, the identification of individual tumors that require only the actions of IFNα/β but not IFNγ (or vice versa) for their rejection, also implies their unique function. Although mechanisms contributing to allogeneic tumor rejection may be quite different from those involved in the rejection of immunogenic syngeneic tumors, we also observed that allogeneic rejection of aggressive progressor tumors required host sensitivity to IFNγ but not IFNα/β.

CHAPTER 3

IFNα/β's Actions on Innate Immune Cells are Critical for Tumor Rejection

INTRODUCTION

Although the anti-tumor properties of exogenously administered type I interferon (IFNα/β) have long been recognized, only more recently has a critical function for endogenous IFNα/β in tumor protection emerged. The type I IFNs were initially identified based on their antiviral properties, and consist of a large number of related cytokines all acting through a common cell surface receptor. It soon became apparent, however, that in addition to antiviral activity, these molecules had potent antiproliferative and proapoptotic effects on cells, and could inhibit the growth of a variety of cancer cells in vitro (27). Indeed, early studies demonstrated increased survival times of tumor bearing mice when treated with relatively crude, viral-induced interferon preparations (28-30). While initially assumed that the observed effects were due to direct actions of IFNα/β on the tumor, subsequent experiments established the in vivo efficacy of exogenous IFNα/β treatment even against IFN-insensitive cancer cells (30, 32, 33). A more recent study using the AGS-1 melanoma cell line (derived from a $STAT1^{-/-}$ mouse) showed that improved survival with IFNα treatment required IFN responsiveness within host cells, but not the tumor (35). Thus, collective results from a number of different model systems emphasize the importance of host-dependent mechanisms for the protective effects of exogenous IFNα/β.

The first study indicating a role for endogenous IFNα/β in inhibiting tumor growth used polyclonal antiserum against murine IFNα/β, demonstrating enhanced growth of xenogeneic tumor cell lines in nude mice (40). Additional work showed

decreased survival times in mice treated with anti-IFNα/β polyclonal antiserum when injected with several syngeneic tumors (41). Once again, this phenotype was observed when either IFN-sensitive or IFN-insensitive Friend leukemia cells were used. In a more recent report, the progressive growth of tumors was found to be enhanced in IFNα/β-insensitive IFNAR1$^{-/-}$ mice (42) – yet these studies were limited by an allogeneic transplantation system and the use of tumor cell lines that also grew progressively in WT mice.

Recent work from our laboratory has definitively established endogenous type I IFN as an important component of the host protective cancer immunosurveillance network using models of both primary and transplantable tumor formation (16). Utilizing a panel of highly immunogenic regressor tumors, we found that treatment of immunocompetent WT mice with a novel blocking mAb against IFNAR1 abrogated tumor rejection. In addition, mice deficient in IFNα/β responsiveness (IFNAR1$^{-/-}$ mice) were more susceptible than WT controls to the development of primary carcinogen-induced sarcomas. As previously demonstrated for tumors derived from other immunodeficient mice, IFNAR1$^{-/-}$-derived tumors were as a group more immunogenic (thus displaying an unedited phenotype) than tumors arising in WT mice.

To investigate the relevant targets of type I IFN, immunogenic IFNα/β-responsive tumors were transplanted into IFNAR1$^{-/-}$ hosts, revealing a requirement for IFNα/β's actions on the host during tumor rejection. In addition, selective reconstitution of IFN responsiveness in the GAR4 tumor (derived from an IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ mouse) determined that tumor cell sensitivity to IFNγ, but not IFNα/β could mediate tumor

rejection (see also Chapter 4). These data, along with the observation that IFNAR1$^{-/-}$-derived tumors could still be rejected in WT hosts, argued against an important role for IFNα/β acting on the tumor cell, similar to findings with exogenous IFNα/β treatment. Additional studies using bone marrow chimeric mice further localized the essential IFNα/β-responsive host cells to the hematopoietic compartment. This work therefore highlights the critical immunomodulatory functions of IFNα/β acting on host hematopoietic cells. The physiologic relevance of this anti-tumor mechanism is also supported by recent correlative data in human patients treated with high-dose adjuvant IFNα for metastatic melanoma (156). In this study, manifestations of autoimmunity detected in IFNα-treated patients were associated with significantly improved relapse-free and overall survival.

The key role of type I IFN in initiating and enhancing immune responses is being increasingly recognized through studies using a variety of experimental systems (47, 53). Yet, IFNα/β has been shown to mediate an array of potent immunoregulatory effects on cells of both the innate and adaptive immune system. One of the earliest described immunostimulatory effects of type I IFN is its profound enhancement of NK cell cytotoxic activity following IFNα/β induction in response to viral infection (110, 111). Type I IFN also has prominent effects in promoting the differentiation and maturation of DCs, inducing macrophage activation, and eliciting IL-15 production – thereby promoting T and B cell responses (67, 121, 122, 126). In addition to its effects on innate immune cells, IFNα/β can augment adaptive immunity through direct actions on T and B lymphocytes, including its enhancement of T cell activation and expansion (131, 157-

159), the survival of activated T cells (127), and B cell antibody production and class switching (160, 161).

Given the array of immunomodulatory activities ascribed to the type I IFNs, it is unclear at present which functions represent the relevant mediators of its anti-tumor activity. In this study, we have therefore investigated the host cell targets of IFNα/β required for the rejection of immunogenic tumors. We demonstrate that the actions of endogenous IFNα/β on cells of the innate immune compartment are essential, as selective reconstitution of bone marrow chimeric mice with IFNα/β sensitivity only in innate immune cells restored tumor rejection. To investigate the mechanisms involved, we subsequently show that depletion of NK cells had no effect on IFNα/β-dependent tumor rejection, whereas IFNα/β-responsive innate cells promoted the generation of tumor-specific CTL which were required for rejection. Additional studies using p21SNFT$^{-/-}$ mice with a selective deficiency in CD8α^{+} DCs, revealed an obligate function for this DC subset in tumor-specific CTL priming and tumor rejection. Taken together with previous data establishing the importance of early IFNα/β action (see Chapter 2), these findings underscore the critical role of type I IFN on innate immune cells for the generation of protective adaptive responses to immunogenic tumors.

MATERIALS AND METHODS

Mice. Inbred 129/SvEv, C57Bl/6, and 129xB6 F1 mice were purchased from Taconic Farms, and 129/SvPas mice were from Charles River Laboratories. 129 IFNAR1$^{-/-}$, 129 RAG2$^{-/-}$, and B6 RAG2$^{-/-}$ were bred in our specific pathogen-free animal facility. p21SNFT$^{-/-}$ mice generated and maintained on a pure 129/SvEv background have been described (151). OT-I transgenic mice on a RAG1$^{-/-}$ background were obtained through the NIAID Exchange Program, NIH (C57BL6-Tg(OT-I)-RAG1^{tm1Mom} 004175) (162, 163). C57Bl/6 strain MHC class I-deficient $K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice (164) were a gift from H. Virgin and T. Hansen (Washington University School of Medicine, St. Louis, Missouri).

Generation of bone marrow chimeras. 5-FU treated adult bone marrow cells or E14.5 fetal liver cells were used as donor HSCs for reconstitution of lethally-irradiated recipients. To isolate 5-FU treated bone marrow, donor mice were treated i.p. with 150 mg/kg 5-FU (American Pharmaceutical Partners) 4-5 days prior to harvest and elution of bone marrow cells. For harvest of E14.5 FLCs, embryos were extracted 14 days following implantation, livers were removed, and FLCs were isolated by homogenization through a metal mesh strainer with a 6 cc syringe plunger. Following RBC lysis by incubation in Hybrimax RBC lysing buffer (Sigma-Aldrich), cells were filtered through a 40 μm cell strainer, washed 2 times with sterile endofree PBS, and counted. Cells were resuspended in PBS for injection of $5x10^6$ (FLCs) or $1x10^6$ (bone marrow) cells per

mouse in a volume of 0.2 ml using a 0.5 cc 29 gauge insulin syringe. Recipient mice, lethally irradiated with a single dose of 9.5 Gy several hours prior, were anesthetized by i.p. avertin and HSCs were infused i.v. via retro-orbital injection. Animals were generally maintained on TMS water for 4 weeks following irradiation and reconstitution, and tumor transplantation experiments were performed at least 10-12 weeks post-reconstitution.

Flow cytometry. Single cell suspensions were isolated, incubated with purified anti-CD16/CD32 mAb (2.4G2) (BD Biosciences) to prevent non-specific antibody binding to Fc receptors, then stained with the indicated antibodies prior to data collection on a BD FACSCalibur (BD Biosciences) and data analysis using FlowJo software (Tree Star). The following were purchased from BioLegend: anti-CD3-FITC (145-2C11), anti-CD4-PE (RMA4-5), anti-CD8-APC (53-6.7), anti-DX5-PE (DX5), anti-B220-FITC (RA3-6B2), anti-CD11b-PE (M1/70), anti-CD11b-PerCP-Cy5.5 (M1/70), anti-NK1.1-FITC (PK136), anti-CD24-FITC (M1/69), and SA-APC. Anti-CD11c-PE (HL3) and anti-CD8α-PerCP-Cy5.5 (53-6.7) were from BD Biosciences, anti-F4/80-FITC (BM8) and anti-NKp46-PE (29A1.4) were from eBioscience, and anti-IFNAR1-biotin (MAR1-5A3) was described previously (64). Immediately prior to analysis, propidium iodide (PI) was added to assess cell viability.

Tumor cells. $RAG2^{-/-}$ regressor fibrosarcomas were generated by s.c. MCA injection of 129 or B6 strain $RAG2^{-/-}$ mice as previously described (5, 140). The WT progressor

tumor 1877 was generated by MCA treatment of WT 129/SvEv mice. RMA-S is an MHC class I-deficient mutant cell line derived from the Rauscher virus-induced lymphoma RBL-5 of B6 origin (165).

Tumor transplantation experiments. Tumor cells were thawed from frozen stocks and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamate, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol (R-10 medium). After expansion for several passages, cells were harvested by incubation in 0.05% trypsin, washed once with R-10 medium, and washed three times with sterile, endotoxin-free PBS. Prior to the last wash, cells were counted using a hemacytometer and cell viability was assessed by trypan blue exclusion (injected cells were >90% viable). Cells were injected subcutaneously in a volume of 0.15 ml PBS into the shaved flanks of mice. Tumor size was measured on the indicated days and is presented as the average of two perpendicular diameters. When calculating percent tumor growth, mice with tumors larger than 6x6 mm in diameter at the end of the observation period were counted as positive.

Antibody treatment. For depletion of $CD4^+$ and/or $CD8^+$ cells, mice were treated by i.p. injection with anti-CD4 mAb (GK1.5), anti-CD8 mAb (YTS-169.4), a mixture of both, or control mAb (PIP) at an initial dose of 750 μg followed by 250 μg every 7 days. NK cell depletion was achieved in B6 mice by i.p. injection of 200 μg anti-NK1.1 mAb (PK136) (BioLegend) on days -2, 0, and +2, then 100 μg every 5 days thereafter. Anti-asialoGM1

antibody (Wako Chemicals) was resuspended in 4 ml sterile endofree PBS and 100 μl (diluted to 0.5 ml in PBS) was administered by i.p. injection on days -2, 0, +2, and +7, then every 7 days thereafter. Control mice received i.p. injections of an equal volume of PBS. In all cases, effective cell depletion was confirmed by flow cytometry or functional assay.

Ex vivo tumor-specific CTL killing assay. Spleens were harvested from H31m1 tumor-bearing WT, IFNAR1$^{-/-}$, or bone marrow chimeric mice 20 days after tumor implantation and cell suspensions were obtained by homogenization using frosted glass slides. $4x10^7$ splenocytes were cultured with $2x10^6$ IFNγ-treated, irradiated (10,000 rads) H31m1 tumor cells. 5 days later, the cells were harvested and used as CTL effector cells in a cytotoxicity assay. To generate target cells, H31m1 or 1773 tumor cells were treated with 100 U/ml IFNγ for 48 hours before use. 10^6 tumor cells were radioactively labeled with 25 μCi of $Na_2{}^{51}CrO_4$ (PerkinElmer) for 90 minutes at 37ºC. The labeled target cells were washed three times and seeded at 10,000 cells/well in 96-well round-bottom plates. The effector and target cells were cocultured at indicated effector/target cell ratios for 4 hours at 37ºC in 5% CO_2. For blocking assays, 10 μg/ml of α-CD8 (YTS-169.4), α-CD4 (GK1.5), or control immunoglobulin (α-GST mAb PIP) were added to the cell culture of effector and target cells. Radioactivity was determined in the supernatants. Percent specific killing was defined as (experimental condition cpm - spontaneous cpm)/(maximal (detergent) cpm - spontaneous cpm) x 100. Data points were obtained in duplicate. All experiments were done at least twice.

NK cell cytotoxicity assay. Splenocytes were isolated from mice treated by i.p. injection of 300 μg polyI:C 24 hours prior and were used as effector cells with YAC-1 cells as tumor targets. 10^6 YAC-1 cells were radioactively labeled by incubating with 25 μCi of $Na_2{}^{51}CrO_4$ (PerkinElmer) for 90 minutes at 37ºC. The labeled target cells were washed three times and seeded at 5,000 per well in 96-well round-bottom plates. Splenocytes were added at the indicated E:T ratios. After 4 hours of culture, radioactivity was determined in the supernatants. Percent specific killing was defined as (experimental condition cpm – spontaneous cpm)/(maximal (detergent) cpm – spontaneous cpm) x 100. Data points were obtained in duplicate. All experiments were done at least twice.

DC adoptive transfer. WT or p21SNFT$^{-/-}$ donor mice were treated i.p. with 10 μg of Flt3 ligand (FL) Fc for 3 consecutive days. 10 days after initiation of FL Fc treatment, CD11c$^+$ splenocytes were positively selected by MACS (Miltenyi Biotec) (>90% purity). A fraction of enriched cells were stained for CD11c, CD8α, and B220 and analyzed by flow cytometry. Whole tumor cell lysates were prepared by harvesting H31m1 cells, washing them 3 times with sterile endofree PBS, then performing 5 quick-freeze/thaw cycles. Isolated CD11c$^+$ donor cells were cultured in the presence of tumor lysate (DC/tumor cell ratio: 2/1) and 1 μg/ml LPS (Sigma-Alrich) ex vivo for 4 hours. Cells were then washed 3 times with HBSS before transfer. p21SNFT$^{-/-}$ mice received 9x10^6 CD11c$^+$ cells both i.v. and s.c. in the right flank. Following DC transfer, mice were immediately challenged with 1x10^6 H31m1 tumor cells.

Antigen cross-presentation assay. Dendritic cell cross-presentation of antigen to $CD8^+$ OT-I T cells was assessed as previously described (151). Briefly, spleens from naïve WT and $IFNAR1^{-/-}$ mice were digested with collagenase B (Roche) and DNase I (Sigma-Aldrich), and cellular subpopulations were isolated by MACS purification (Miltenyi Biotec). Total $CD11c^+$ DCs were obtained by negative selection using B220, Thy1.2, and DX5 microbeads followed by positive selection with CD11c microbeads. $CD8\alpha^+$ DCs were recovered by B220, Thy1.2, DX5, and CD4 negative selection, followed by CD8α positive selection. $CD4^+$ DCs were isolated by B220, Thy1.2, DX5, and CD8α negative selection, followed by CD4 positive selection. In all cases, purity of the population of interest was >97%. Splenocytes from $K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice were prepared in serum-free medium, loaded with 10 mg/ml ovalbumin (Calbiochem) by osmotic shock, and irradiated (13.5 Gy) as described (151). OT-I T cells were purified from OT-I/$RAG1^{-/-}$ mice by CD11c and DX5 negative selection followed by positive selection with CD8α microbeads (purity >99%). T cells were fluorescently labeled by incubation with 1 μM CFSE (Sigma-Aldrich) for 9 minutes at 25°C at a density of $2x10^7$ cells/ml. For the assay, $5x10^4$ purified DCs were incubated with $5x10^4$ CFSE-labeled OT-I T cells in the presence of varying numbers of irradiated, ovalbumin-loaded $K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ splenocytes. After 3 days, cells were stained with anti-CD8α-APC and CFSE dilution was measured by flow cytometry. For exogenous IFNα treatment, recombinant murine IFNα5 (a gift from D. Fremont, Washington University School of Medicine, St. Louis,

Missouri) was added at 1,000 U/ml, whereas IFNα/β blockade was achieved by incubation with 5 μg/ml IFNAR1-specific mAb MAR1-5A3.

RESULTS

Host Cell Sensitivity to Type I IFN in the Hematopoietic Compartment is Both Necessary and Sufficient for Tumor Rejection

To confirm and extend our previous findings, we investigated the requirements for IFNα/β sensitivity within hematopoietic versus nonhematopoietic host cells during the rejection of several additional RAG2$^{-/-}$ regressor tumors. Bone marrow chimeric mice were generated by reconstituting lethally-irradiated recipient mice with donor hematopoietic stem cells (HSCs) from either 5-fluorouracil (5-FU)-treated adult bone marrow or fetal liver cells (FLCs) isolated from embryonic day 14.5 (E14.5) fetuses. Although our initial studies utilized 5-FU-treated bone marrow as a source of HSCs, the majority employed FLCs due to their ease of isolation as well as their enhanced hematopoietic reconstitution potential compared to adult bone marrow cells (166, 167). In order to verify the successful reconstitution of recipient mice using this protocol, we transplanted different doses of unfractionated FLCs from WT embryos into irradiated RAG2$^{-/-}$ mice and monitored both survival and repopulation of hematopoietic-derived cell lineages. While injection of 10^7 or 10^6 FLCs yielded recovery of immune cell subsets to WT levels, mice given lower doses of cells either failed to survive lethal irradiation or demonstrated reduced spleen cellularity and cell percentages (Figure 1). Thus, in subsequent experiments a dose of $5x10^6$ FLCs/mouse was typically used.

Mice with type I IFN responsiveness specifically in the hematopoietic compartment were produced by reconstituting IFNAR1$^{-/-}$ recipients with WT FLCs (WT→IFNAR1$^{-/-}$ chimeras), whereas mice with IFNα/β sensitivity only in nonhematopoietic cells were generated by reconstitution of RAG2$^{-/-}$ recipients with IFNAR1$^{-/-}$ FLCs (IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras). The expected phenotypes of these mice, along with WT→WT and IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ control chimeras, were confirmed by analysis of IFNAR1 expression on splenocyte subsets by FACS staining (Figure 2). We consistently observed that all immune cell lineages were entirely donor HSC-derived, with the exception of a small minority of the T cell population which remained recipient-derived. Thus, approximately 10-20% of T cells in WT→IFNAR1$^{-/-}$ chimeras were IFNAR1-deficient and probably represent long-lived radioresistant memory T cells. The use of RAG2$^{-/-}$ mice as recipients for IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras eliminated this potential caveat, and the entire T cell compartment lacked IFNAR1 expression. In addition to the expected IFNα/β receptor status, all of the bone marrow chimeras displayed normal cellularity and immune cell percentages in the spleen, as well as exhibiting normal splenic architecture (data not shown) – thus providing evidence for the normal hematopoietic reconstitution of these mice.

To assess the requirements for type I IFN sensitivity during tumor rejection, we transplanted the RAG2$^{-/-}$ regressor tumors H31m1 and d38m2 into groups of control and bone marrow chimeric mice (Figure 3). As previously described, these immunogenic tumors are rejected when transplanted into naïve syngeneic WT mice, but grow progressively in immunodeficient RAG2$^{-/-}$ or IFNAR1$^{-/-}$ hosts. Similar phenotypes were

also observed in control bone marrow chimeras, as H31m1 and d38m2 tumor cells were rejected in WT→WT chimeras but grew progressively in IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ chimeras. Importantly, IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras (IFNα/β-responsive only in nonhematopoietic cells) were unable to reject this tumor challenge; yet, WT→IFNAR1$^{-/-}$ chimeras (IFNα/β-responsive only in hematopoietic cells) displayed no defect in tumor rejection. Taken together, these results demonstrate that IFNα/β sensitivity within host hematopoietic cells is both necessary and sufficient for rejection of the H31m1 and d38m2 RAG2$^{-/-}$ regressor sarcomas.

Figure 1. Titration of unfractionated FLCs for hematopoietic reconstitution of lethally-irradiated recipients. WT FLCs at the indicated dose were transplanted i.v. into irradiated RAG2$^{-/-}$ recipients and mice were monitored for survival (A) and hematopoietic reconstitution at 12 weeks post-transplantation (B and C). Spleens from control and bone marrow chimeric mice were analyzed for cellularity and immune subset composition by FACS.

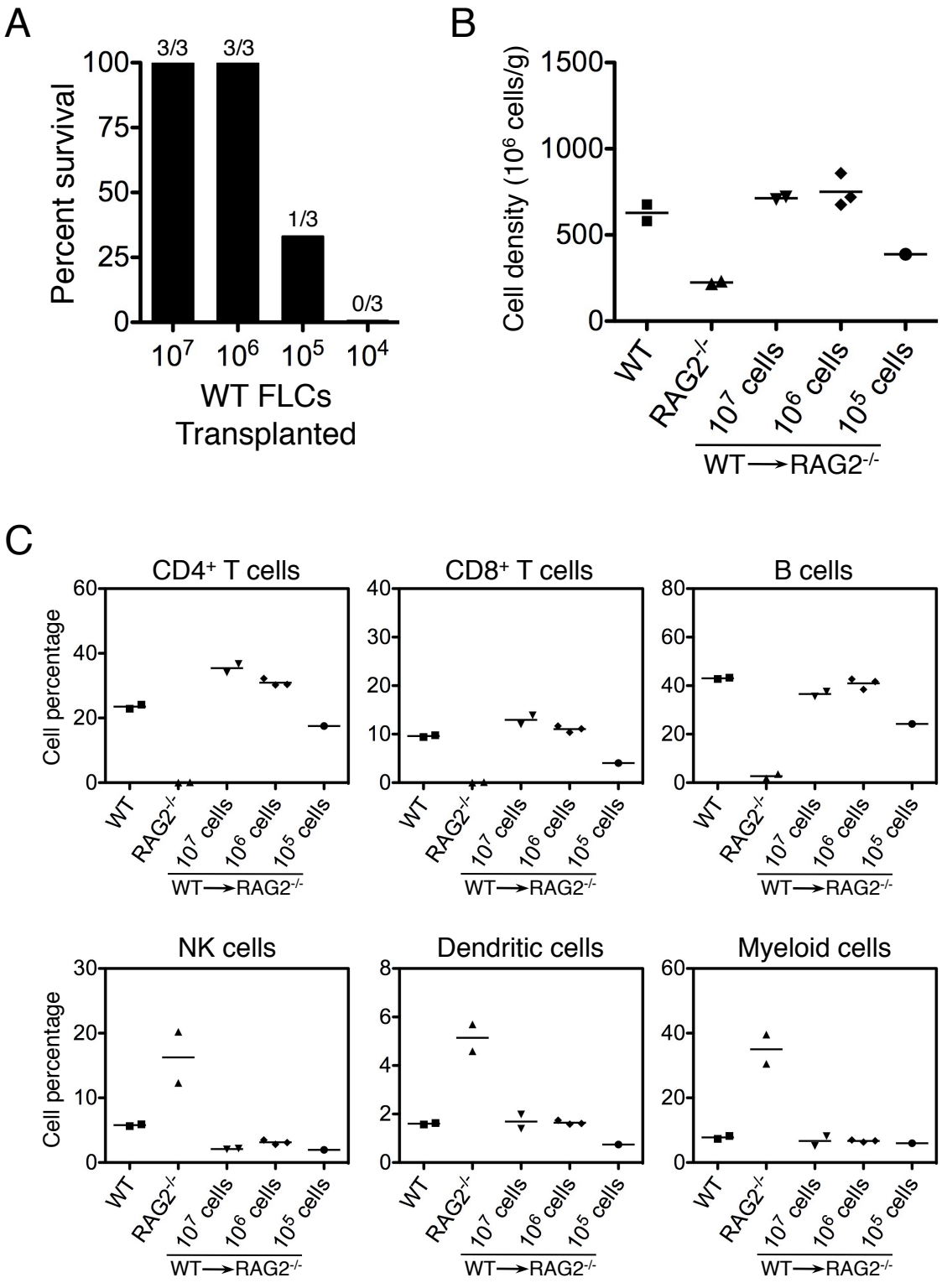

A
Percent survival
100
75
50
25
0
3/3
3/3
1/3
0/3
10^7 10^6 10^5 10^4
WT FLCs Transplanted
B
Cell density (10^6 cells/g)
1500
1000
500
0
WT
RAG2-/-
10^7 cells
10^6 cells
10^5 cells
WT→RAG2-/-
C
CD4+ T cells
CD8+ T cells
B cells
NK cells
Dendritic cells
Myeloid cells
Cell percentage
WT
RAG2-/-
10^7 cells
10^6 cells
10^5 cells
WT→RAG2-/-

Figure 2. Generation of bone marrow chimeras with selective IFNα/β sensitivity in the hematopoietic or nonhematopoietic compartment. (A) Experimental scheme for generating chimeras with selective IFNα/β responsiveness. (B) Immune compartment from a representative cohort of bone marrow chimeras was analyzed for expression of IFNAR1 by FACS. Data represent the percentage of $IFNAR1^{+}$ splenocytes within each cellular subset.

A

Group	Donor HSCs	Irradiated Recipient Mice	IFNα/β Sensitivity	
			Hematopoietic	Nonhematopoietic
Control	WT	WT	+	+
Control	IFNAR1$^{-/-}$	IFNAR1$^{-/-}$	-	-
Experimental	IFNAR1$^{-/-}$	RAG2$^{-/-}$	-	+
Experimental	WT	IFNAR1$^{-/-}$	+	-

B

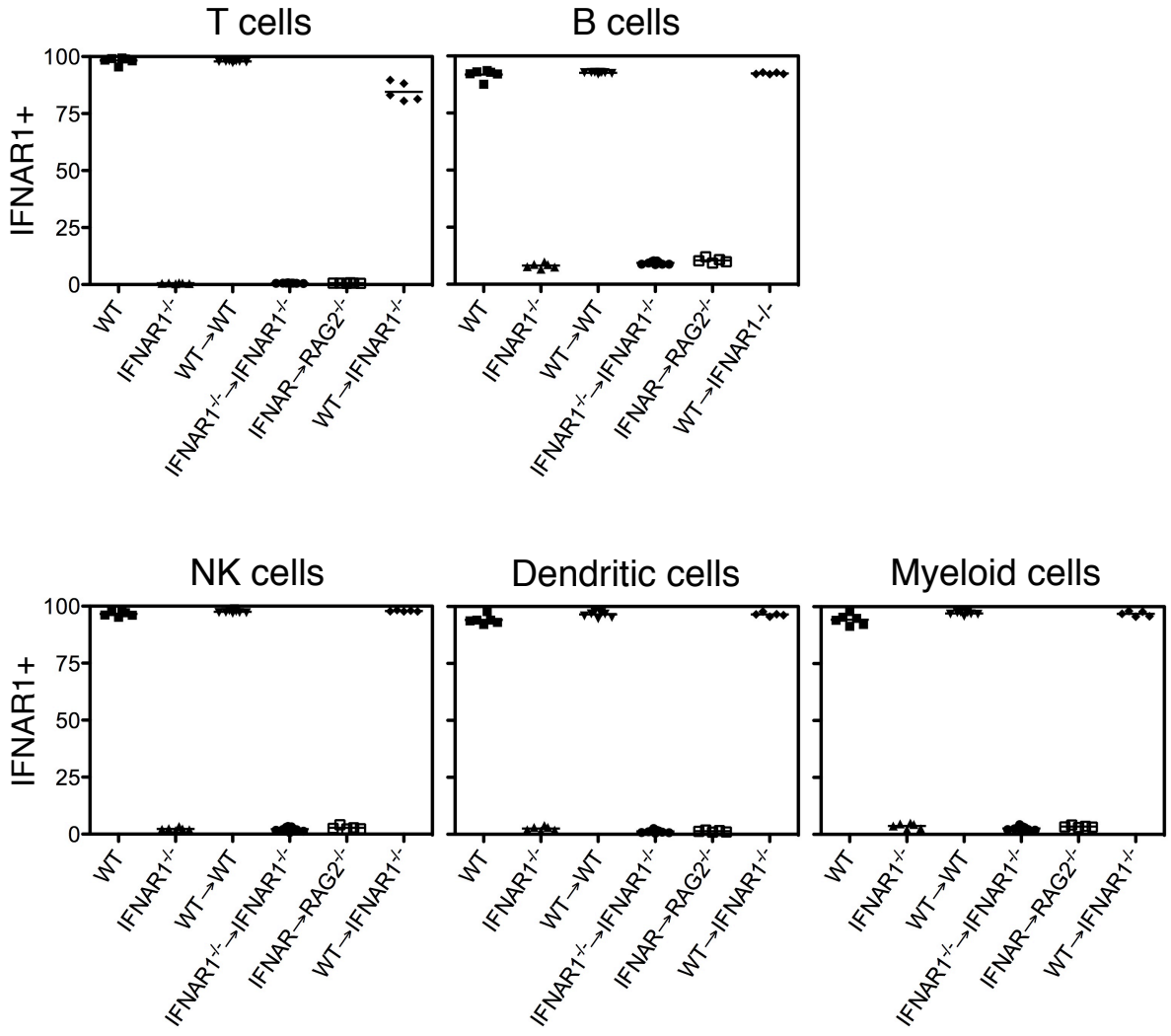
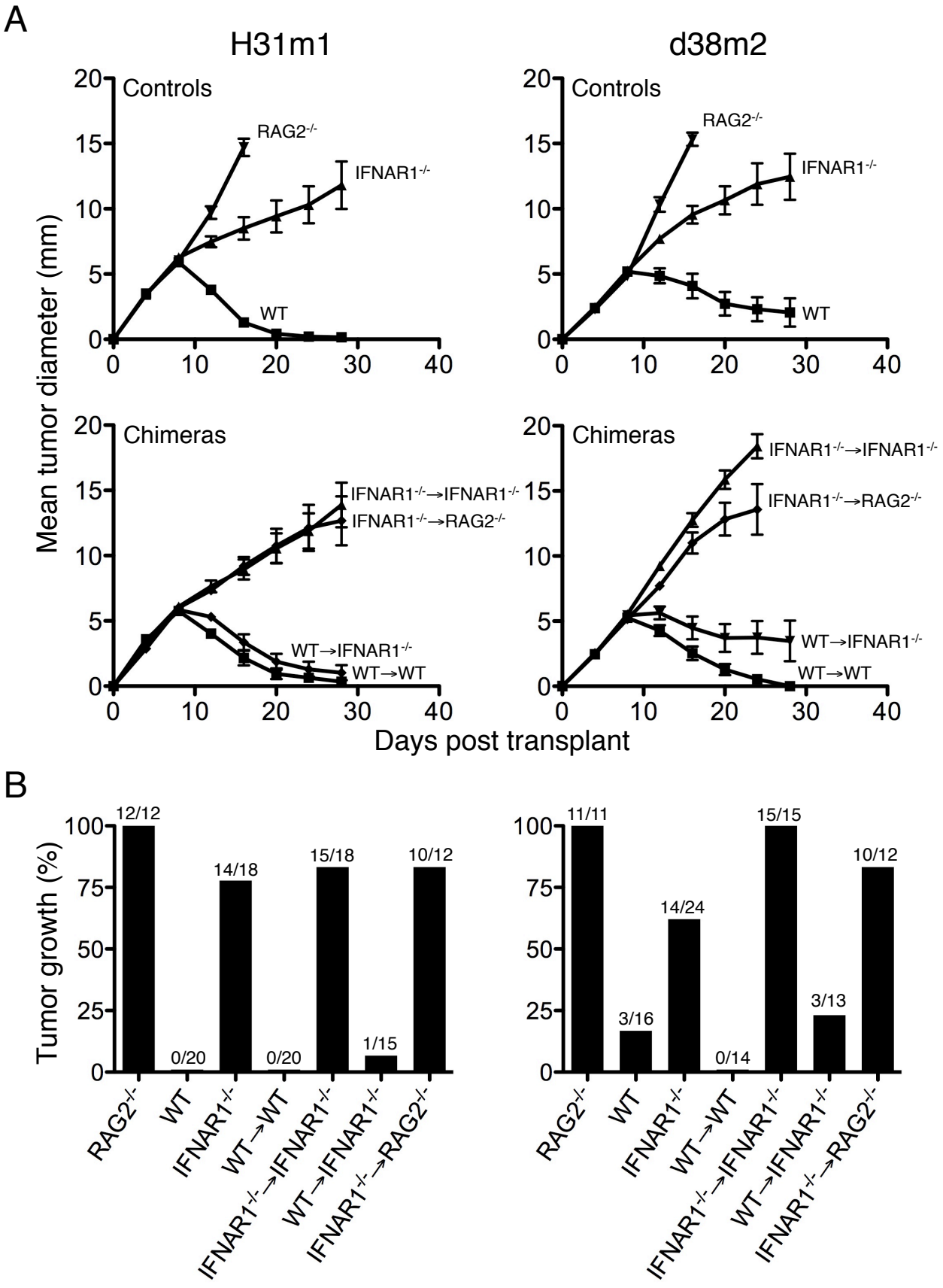


Figure 3. Host hematopoietic IFNα/β responsiveness is both necessary and sufficient for rejection of immunogenic tumors. Groups of control and bone marrow chimeric mice were injected with $1x10^6$ H31m1 or d38m2 tumor cells and tumor growth was monitored over time (A) for group sizes as indicated in (B). Results from 2-3 independent experiments with each tumor is shown.


A
H31m1
d38m2
Controls
RAG2-/-
IFNAR1-/-
WT
Chimeras
IFNAR1-/-→IFNAR1-/-
IFNAR1-/-→RAG2-/-
WT→IFNAR1-/-
WT→WT
Mean tumor diameter (mm)
Days post transplant
B
Tumor growth (%)
12/12
0/20
14/18
0/20
15/18
1/15
10/12
11/11
3/16
14/24
0/14
15/15
3/13
10/12
RAG2-/-
WT
IFNAR1-/-
WT→WT
IFNAR1-/-→IFNAR1-/-
WT→IFNAR1-/-
IFNAR1-/-→RAG2-/-

Generation of IFNAR1$^{-/-}$ Mixed Bone Marrow Chimeras with Partial IFNα/β Sensitivity Within the Hematopoietic Compartment

Having established the key function of IFNα/β on host hematopoietic cells during tumor rejection, we wanted to investigate more specifically the relevant cellular targets mediating its protective effects. After all, the hematopoietic compartment includes the entire immune system – a collection of diverse cell types, all of which can respond to and are potentially modulated by type I IFN. To determine the relative contribution of IFNα/β's actions on innate versus adaptive immune cells, we generated a series of mixed bone marrow chimeras as outlined in Figure 4, which take advantage of the selective deficiency in T and B lymphocyte development in mice lacking the RAG2 gene. For example, hematopoietic reconstitution of recipient mice using a mixture of RAG2$^{-/-}$ and IFNAR1$^{-/-}$ HSCs would yield an adaptive immune compartment (T and B cells) comprised entirely of IFNAR1$^{-/-}$ cells, whereas innate immune cells (including NK cells, macrophages, DCs, and granulocytes) would consist of a mixture of IFNAR1-sufficient (RAG2$^{-/-}$ HSC-derived) and IFNAR1-deficient (IFNAR1$^{-/-}$ HSC-derived) cells. In order to skew the composition of the innate immune compartment toward IFNα/β-responsive cells, we would simply use a greater ratio of RAG2$^{-/-}$:IFNAR1$^{-/-}$ HSCs. The resulting chimera would thus have IFNα/β sensitivity in innate but not adaptive immune cells. The converse condition – normal IFNα/β responsiveness in the adaptive but not innate immune compartment – could be similarly achieved by reconstitution using an unequal mixture of RAG2$^{-/-}$IFNAR1$^{-/-}$ and WT HSCs.

The prediction would be that in the absence of a competitive developmental advantage by one of two genetically distinct HSCs infused into an irradiated recipient, the mature cellular output of bone marrow-derived lineages would reflect the input ratio of the HSC mixture. Several studies using congenic bone marrow cells have, in fact, shown this to be the case (168, 169), suggesting that unequal mixing of HSCs could indeed be exploited to generate the desired chimeras. In order to test this approach and to arrive at an optimal ratio for donor stem cell mixing, we reconstituted lethally-irradiated $RAG2^{-/-}$ mice with five different ratios of $RAG2^{-/-}$:$IFNAR1^{-/-}$ HSCs (90:10, 80:20, 70:30, 50:50, and 10:90). At 10-12 weeks post-transplantation, the composition of various immune cell lineages in the resulting chimeras was assessed by IFNAR1 staining of splenocytes and peripheral blood cells (Figure 5 and data not shown). At all of the different ratios, T and B cells were found to be uniformly IFNAR1-negative as expected. In contrast, the composition of innate immune cells (e.g. NK cells, DCs, and myeloid cells) closely mirrored the input ratio, ranging from largely IFNAR1-positive at the 90:10 ratio to largely IFNAR1-negative at the 10:90 ratio. Although a careful analysis of absolute cell numbers was not performed, the cellular percentages of T and B cells at the 90:10 ($RAG2^{-/-}$:$IFNAR1^{-/-}$) ratio appeared notably diminished, whereas normal percentages were observed at the remaining ratios (data not shown). Subsequent studies have therefore used the 80:20 ratio of mixing to ensure full adaptive immune reconstitution by the minority component of the mixture.

Using this protocol we have specifically reconstituted $IFNAR1^{-/-}$ mice with either innate IFNα/β sensitivity ($RAG2^{-/-}$+$IFNAR1^{-/-}$→$IFNAR1^{-/-}$ mixed chimeras, hereafter

referred to as "innate" chimeras) or adaptive IFNα/β sensitivity ($RAG2^{-/-}IFNAR1^{-/-}$ +WT→$IFNAR1^{-/-}$, "adaptive" chimeras). As controls, reconstitution of both innate and adaptive compartments ($RAG2^{-/-}$+WT→$IFNAR1^{-/-}$, "innate+adaptive") or neither compartment ($IFNAR1^{-/-}$→$IFNAR1^{-/-}$, "neither") was achieved. IFNAR1 staining on different immune cell subsets from the spleens of a representative cohort of $IFNAR1^{-/-}$ mixed chimeras is shown in Figure 6. Normal type I IFN responsiveness of reconstituted cells was also confirmed by pSTAT1 staining following IFNα treatment (data not shown). In addition, a recent study examining the role of type I IFN during *Listeria monocytogenes* infection has similarly reported the generation of the "innate"-type $IFNAR1^{-/-}$ mixed chimeras by using a 5:1 ratio of $RAG2^{-/-}$ and $IFNAR1^{-/-}$ 5-FU-treated bone marrow cells to reconstitute lethally-irradiated $IFNAR1^{-/-}$ mice (71).

Figure 4. Strategy for the generation of IFNAR1$^{-/-}$ mixed bone marrow chimeras with IFNα/β responsiveness in either innate or adaptive immune cells. Shown is the experimental scheme and protocol for the generation of mixed chimeras by hematopoietic reconstitution using mixtures of E14.5 FLCs.

Group	Donor HSCs (4:1 ratio)	Recipient Mice	Immune IFNα/β Sensitivity		Compartment Reconstituted
			Innate	Adaptive	
Control	RAG2$^{-/-}$ + WT	IFNAR1$^{-/-}$	+	+	Innate + Adaptive
Control	IFNAR1$^{-/-}$	IFNAR1$^{-/-}$	-	-	Neither
Experimental	RAG2$^{-/-}$ + IFNAR1$^{-/-}$	IFNAR1$^{-/-}$	+	-	Innate
Experimental	RAG2$^{-/-}$IFNAR1$^{-/-}$ + WT	IFNAR1$^{-/-}$	-	+	Adaptive

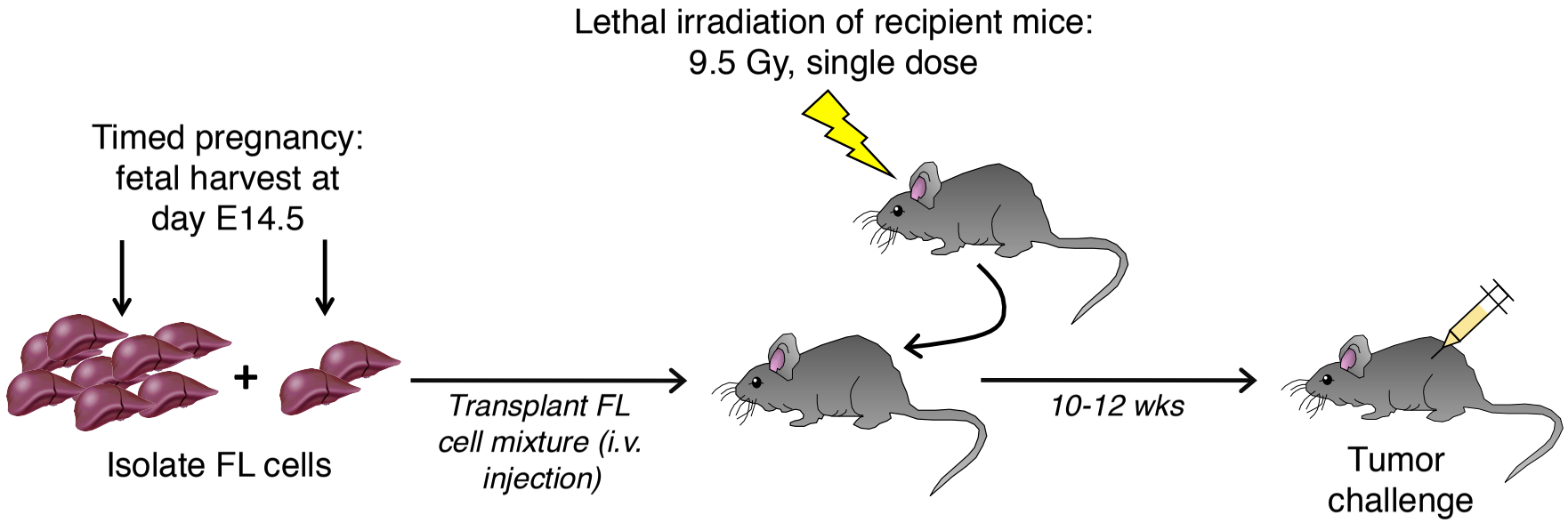


Figure 5. Mixing ratio of RAG2$^{-/-}$ to IFNAR1$^{-/-}$ HSCs determines the composition of type I IFN responsive and unresponsive cells within the innate immune compartment. Lethally-irradiated RAG2$^{-/-}$ mice were injected i.v. with mixtures of RAG2$^{-/-}$ and IFNAR1$^{-/-}$ HSCs at the indicated ratios (1x10^{6} total cells). At 10-12 weeks post-transplantation, splenocytes were analyzed for expression of IFNAR1 within various immune cell compartments. Similar results were obtained using either 5-FU treated adult bone marrow or FLCs as donor HSCs and the data was pooled, representing n=2-8 mice at each ratio.

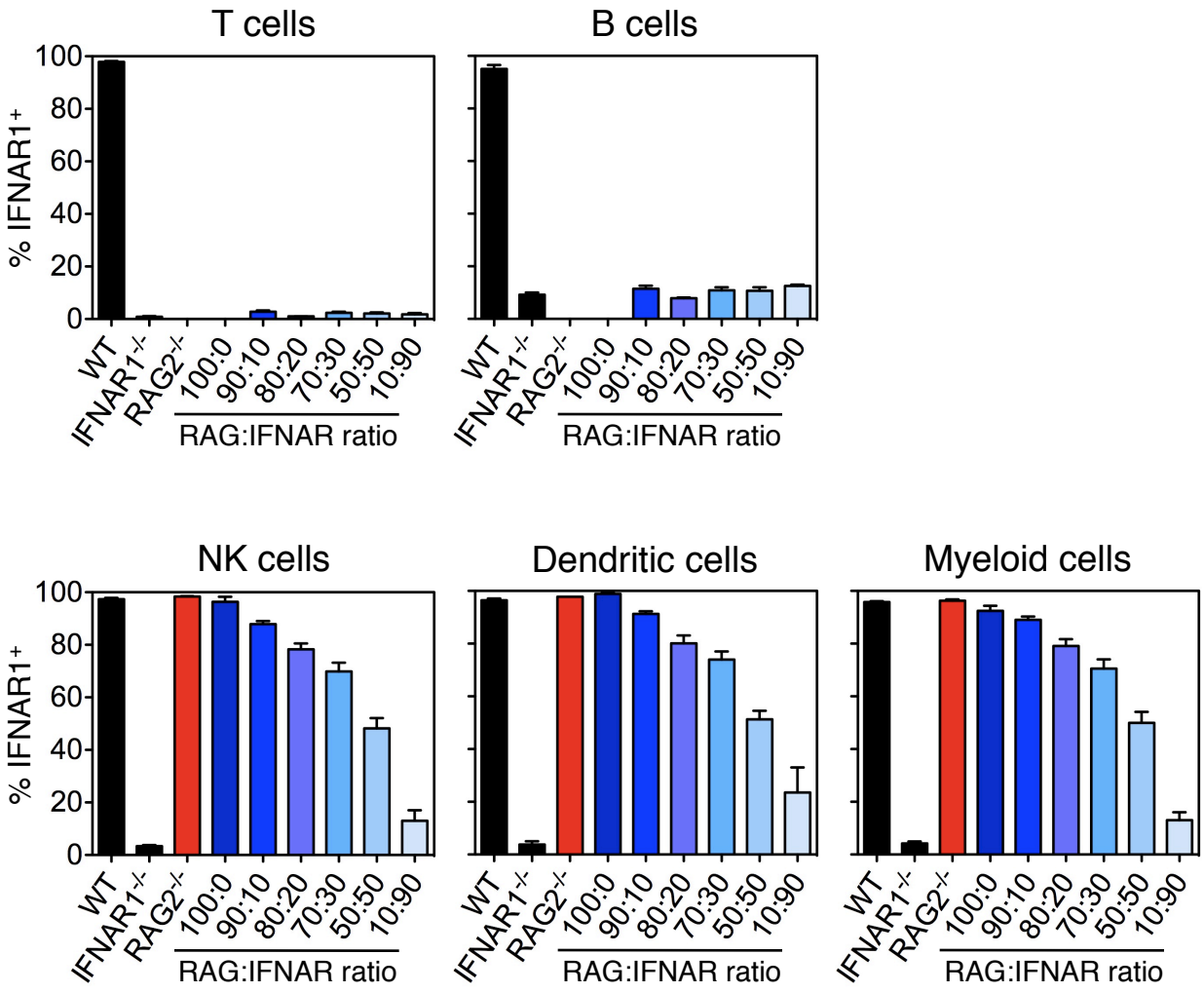
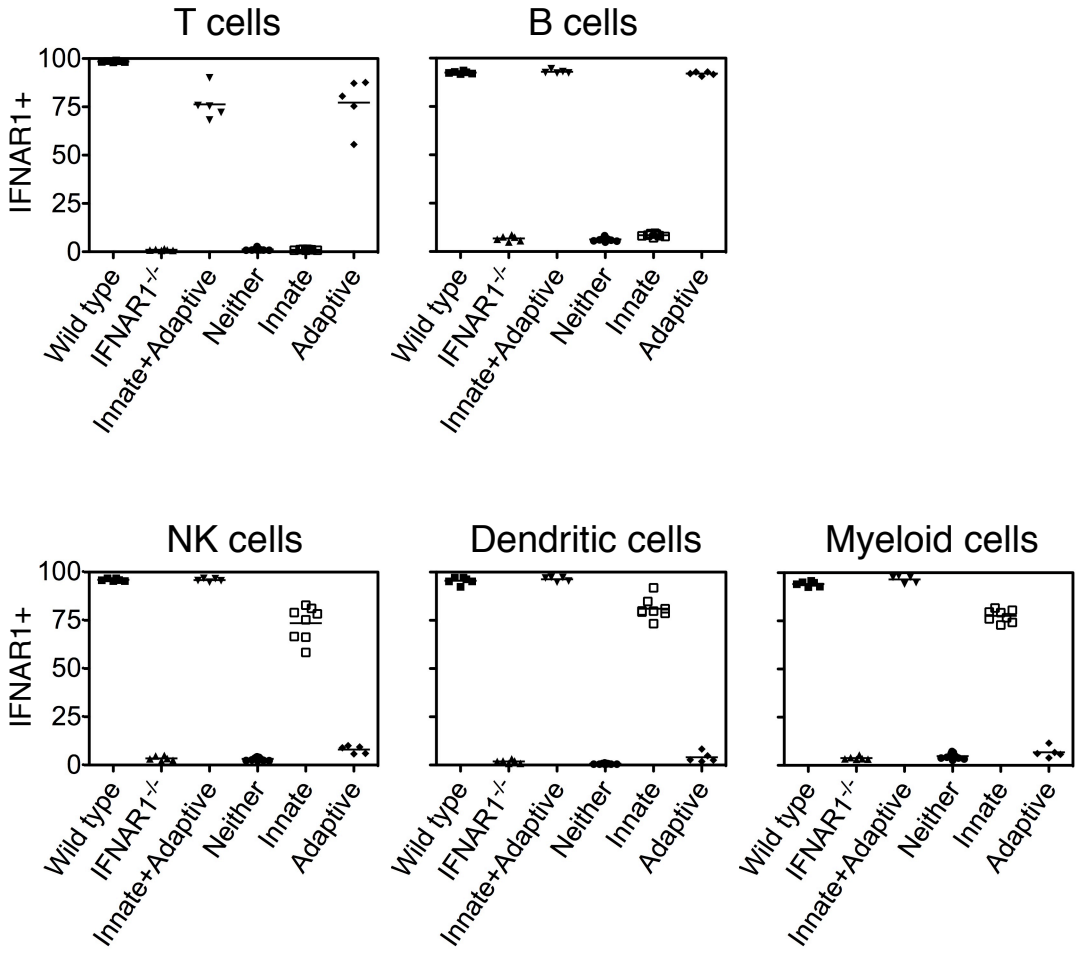

T cells
B cells
NK cells
Dendritic cells
Myeloid cells
% IFNAR1+
100
80
60
40
20
0
WT
IFNAR1-/-
RAG2-/-
100:0
90:10
80:20
70:30
50:50
10:90
RAG:IFNAR ratio

Figure 6. Validation of selective IFNAR1 expression within innate or adaptive immune cells in IFNAR1$^{-/-}$ mixed bone marrow chimeras. Splenocytes were isolated from a representative cohort of mixed chimeras at 12 weeks post-transplantation and IFNAR1 staining was analyzed by FACS. Shown are the percentages of IFNAR1^{+} cells within the indicated immune cell subsets.


T cells
B cells
NK cells
Dendritic cells
Myeloid cells
IFNAR1+
100
75
50
25
0
Wild type
IFNAR1-/-
Innate+Adaptive
Neither
Innate
Adaptive

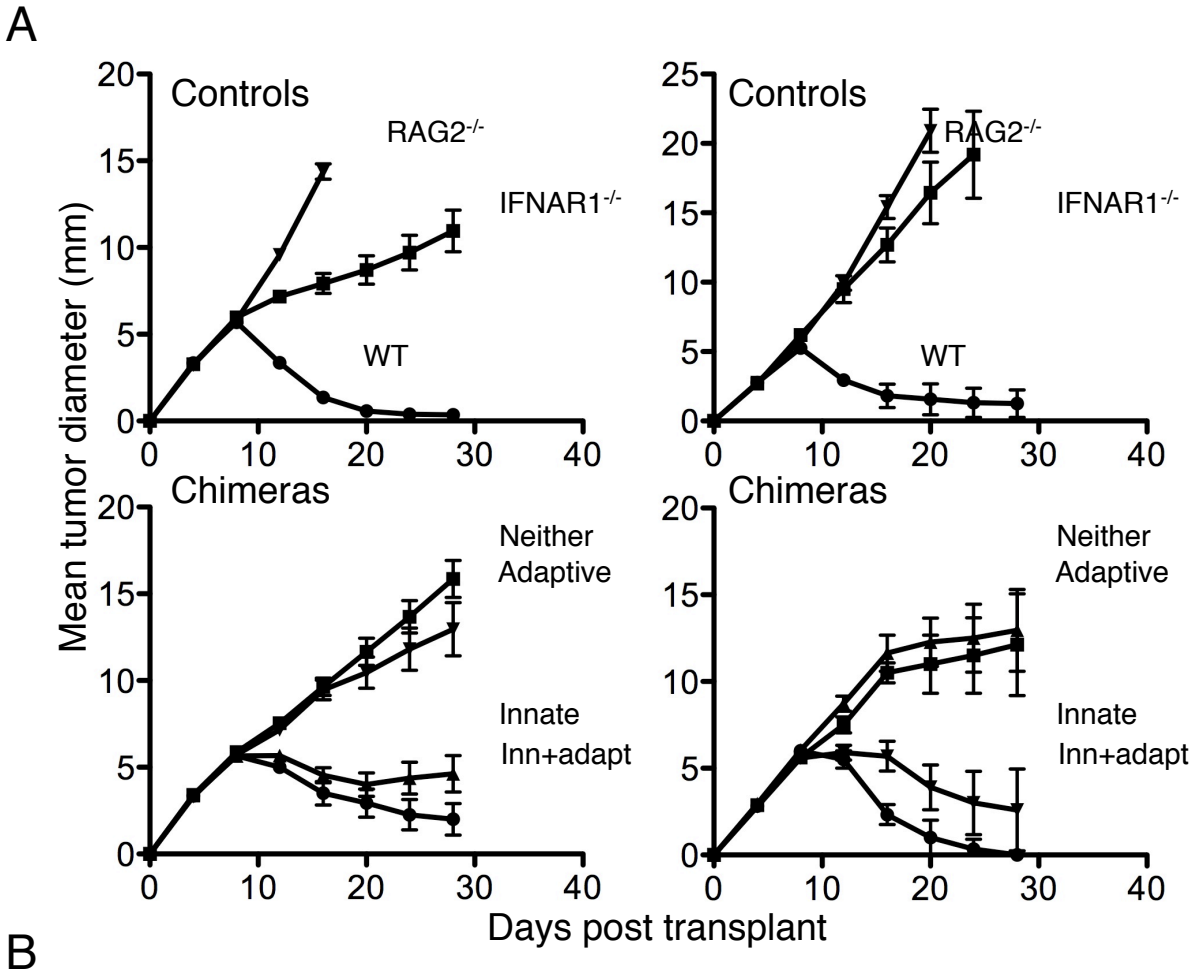
IFNα/β Sensitivity in Innate Immune Cells is Critical for Tumor Rejection

To assess the role of IFNα/β's actions on innate versus adaptive immune cells, we injected H31m1 RAG2$^{-/-}$ regressor tumor cells into groups of control and IFNAR1$^{-/-}$ mixed chimeric mice (Figure 7). While this tumor challenge is rejected in WT hosts, it grows progressively in RAG2$^{-/-}$ mice and in the majority of IFNAR1$^{-/-}$ mice. Consistent with our prior results, "innate+adaptive" IFNAR1$^{-/-}$ chimeras (IFNα/β-responsive within the entire hematopoietic compartment) were also able to reject H31m1 tumor cells, while "neither" IFNAR1$^{-/-}$ chimeras (globally IFNα/β-unresponsive) showed a considerable defect in anti-tumor immunity. Importantly, "adaptive" chimeras (IFNα/β-responsive only in adaptive immune cells) could not reject this tumor challenge, while the majority of "innate" IFNAR1$^{-/-}$ chimeras (IFNα/β-responsive only in innate immune cells) displayed normal rejection of H31m1.

Although there was not a statistically significant difference between the percent tumor growth in "innate+adaptive" as compared to "innate" IFNAR1$^{-/-}$ chimeras (2/17 versus 7/25, p=0.27), it is not clear whether complete recovery of anti-tumor immunity was achieved in "innate" IFNAR1$^{-/-}$ chimeras. There are two possibilities that might account for a potential difference, though at present we cannot distinguish between the two; either (i) there exists a minor role for T and/or B lymphocyte IFNα/β sensitivity, or (ii) the minor fraction of IFNα/β-unresponsive innate immune cells present in "innate" IFNAR1$^{-/-}$ chimeras (due to the 4:1 RAG2$^{-/-}$:IFNAR1$^{-/-}$ HSC mixing ratio) have an inhibitory effect. In either case, the previously described findings support the conclusion

that the major, and perhaps only, functionally relevant role of type I IFN on host cells during tumor rejection is mediated by the innate immune compartment.

Figure 7. Rejection of immunogenic tumor cells requires IFNα/β's actions on cells of the innate immune compartment. $1x10^6$ H31m1 tumor cells were injected into groups of control and $IFNAR1^{-/-}$ mixed bone marrow chimeric mice and tumor growth was monitored. The mean tumor diameter ± s.e.m. over time is shown in (A) and percent tumor growth is summarized in (B) for the indicated group sizes. Data represent the cumulative results of at least 3 independent experiments.


A
Controls
RAG2-/-
IFNAR1-/-
WT
Chimeras
Neither
Adaptive
Innate
Inn+adapt
Mean tumor diameter (mm)
Days post transplant
B
Tumor growth (%)
25/25
1/44
37/59
2/15
23/25
7/25
12/13
6/6
1/8
7/8
0/3
3/4
1/5
9/11
RAG2-/-
WT
IFNAR1-/-
Inn+Adapt
Neither
Innate
Adaptive
WT→WT

Demonstration of the Normal Functional Reconstitution of IFNAR1$^{-/-}$ Mixed Bone Marrow Chimeras

In order to address the possibility that tumor growth in IFNAR1$^{-/-}$ mixed chimeras was due to residual immune dysfunction or incomplete hematopoietic reconstitution, we utilized the F515 RAG2$^{-/-}$ regressor tumor, which requires lymphocytes and IFNγ, but not host IFNα/β sensitivity, for its rejection. As shown in Figure 8, F515 was rejected when transplanted into WT mice, WT mice treated with control mAb, and IFNAR1$^{-/-}$ mice, but grew progressively in RAG2$^{-/-}$ mice and WT mice treated with anti-IFNγ mAb. Similar to IFNAR1$^{-/-}$ control mice, this tumor challenge was rejected in IFNAR1$^{-/-}$ mixed chimeras of each type, confirming the functional reconstitution of the immune compartment in these mice. In addition, we also assessed spleen cellularity and confirmed the normal representation of various immune cell subsets within the spleens of mixed chimeras as another measure of hematopoietic reconstitution (Figure 9). Finally, to rule out the presence of a hyperactive immunological state in these reconstituted mice, we challenged groups of IFNAR1$^{-/-}$ mixed chimeras and control mice with the 1877 WT progressor tumor, which grew similarly in all of the mice (Figure 10).

Figure 8. Functional immune reconstitution in IFNAR1$^{-/-}$ mixed bone marrow chimeras. The indicated groups of control, mAb-treated, and mixed bone marrow chimeric mice were injected with 1x10^{6} F515 RAG2$^{-/-}$ regressor tumor cells. As shown, rejection of this tumor is independent of host IFNα/β sensitivity but requires IFNγ signaling. Results represent cumulative data from 2 independent experiments with n=7-10 mice/group.

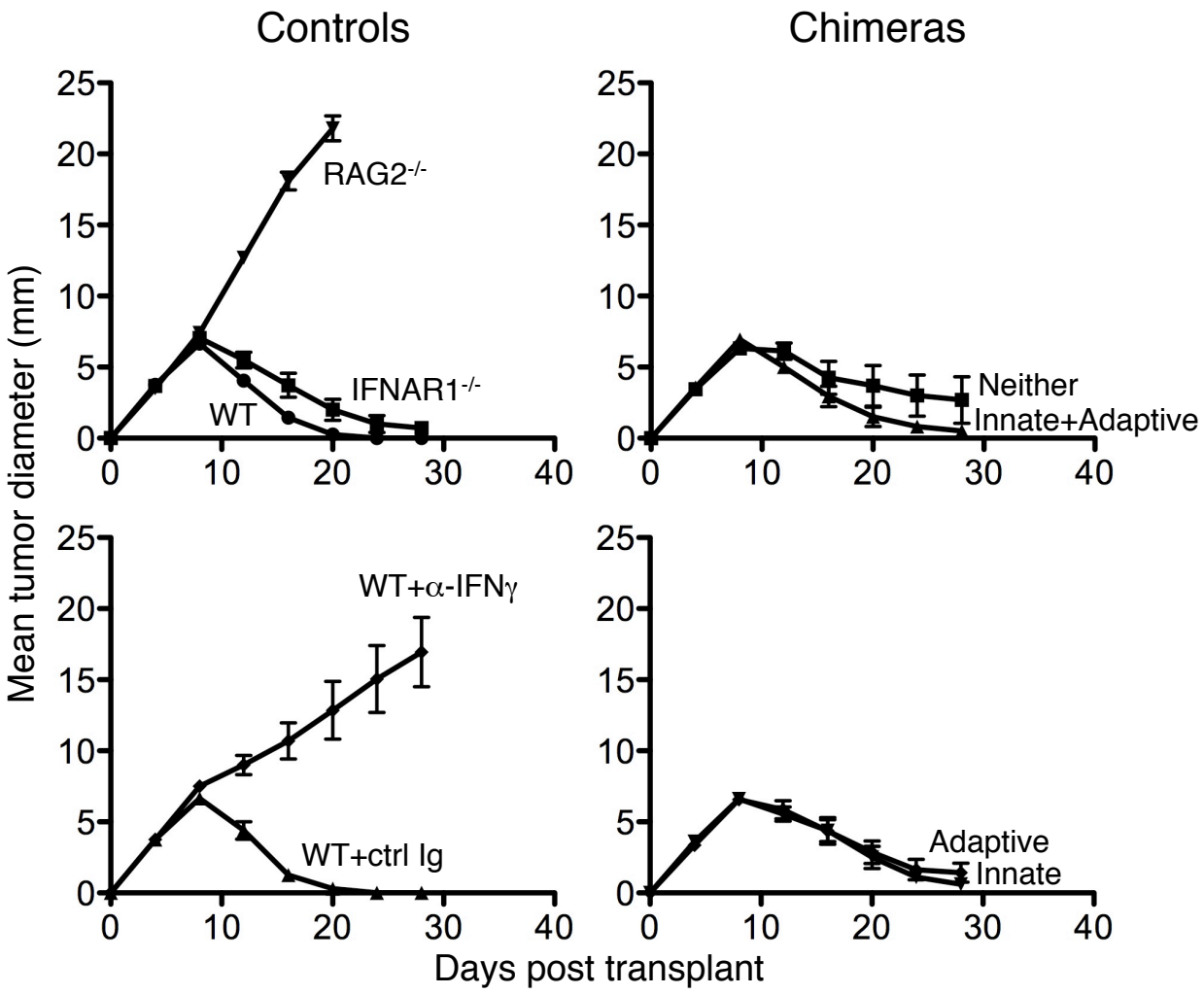
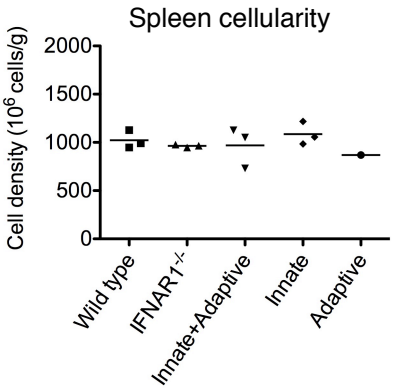

Controls
Chimeras
Mean tumor diameter (mm)
Days post transplant
RAG2-/-
IFNAR1-/-
WT
Neither
Innate+Adaptive
WT+α-IFNγ
WT+ctrl Ig
Adaptive
Innate
0
5
10
15
20
25
0
10
20
30
40

Figure 9. Normal spleen cellularity and immune composition in IFNAR1$^{-/-}$ mixed bone marrow chimeras. Spleens were harvested from mixed chimeras of each type at 12 weeks post-reconstitution, and both cellularity (A) and immune subset composition (B) were analyzed.

A


Spleen cellularity
Cell density (10^6 cells/g)
2000
1500
1000
500
0
Wild type
IFNAR1-/-
Innate+Adaptive
Innate
Adaptive

B

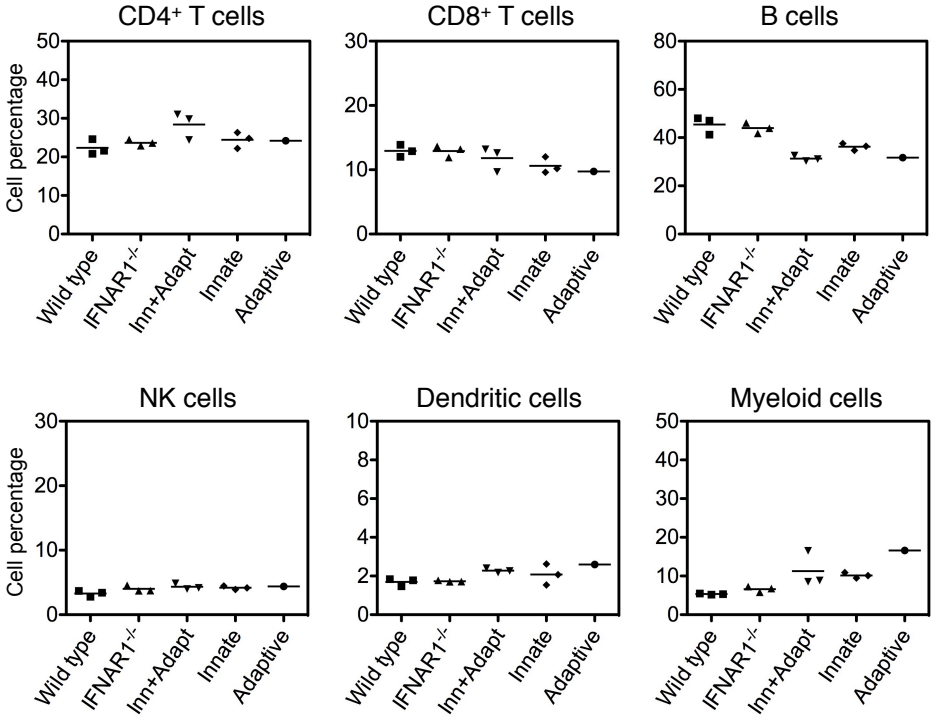
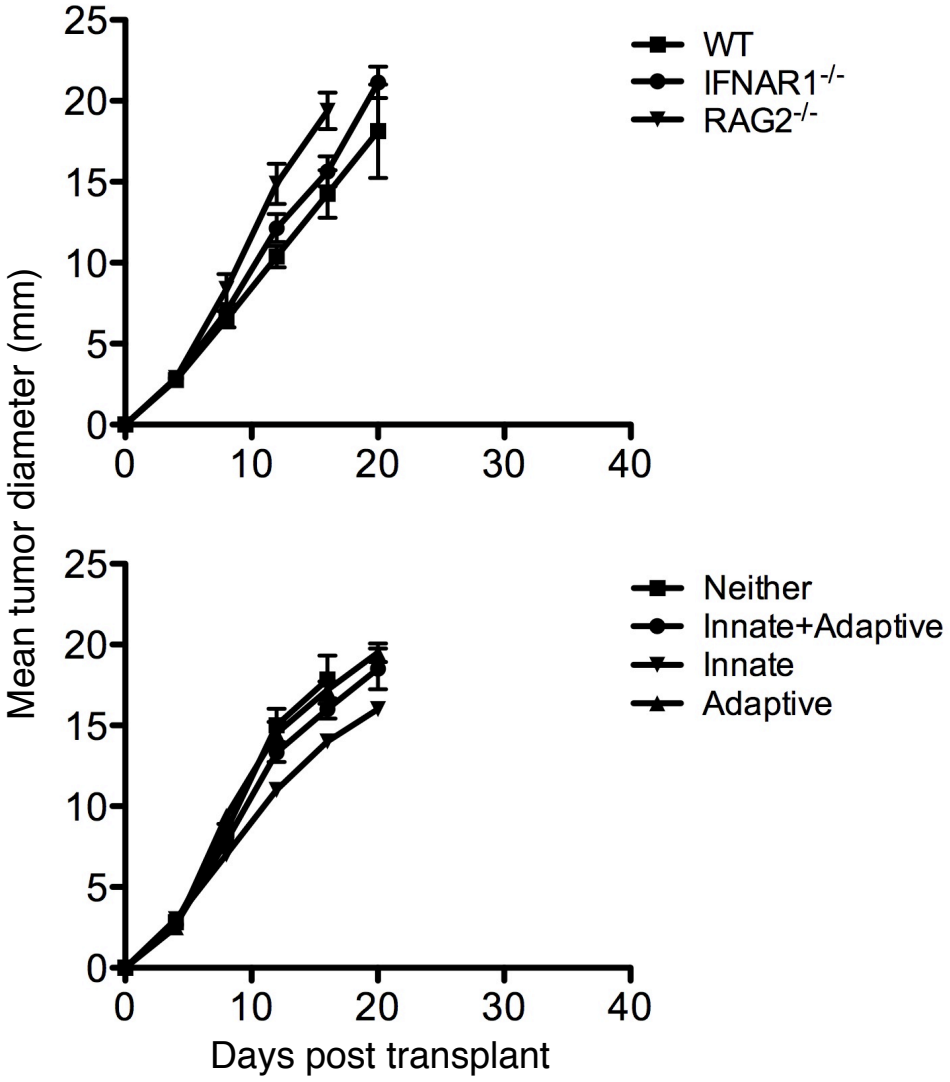

CD4+ T cells
CD8+ T cells
B cells
NK cells
Dendritic cells
Myeloid cells
Cell percentage
Wild type
IFNAR1-/-
Inn+Adapt
Innate
Adaptive

Figure 10. Absence of hyperactive immunological state in IFNAR1$^{-/-}$ mixed chimeras. Groups of control and mixed chimeric mice were injected with 1x10^{6} 1877 progressor tumor cells. Mean tumor diameter ± s.e.m. over time are shown.


25
20
15
10
5
0
0
10
20
30
40
Mean tumor diameter (mm)
WT
IFNAR1-/-
RAG2-/-
Neither
Innate+Adaptive
Innate
Adaptive
Days post transplant

Innate Immune Type I IFN Responsiveness Promotes the Generation of Tumor-Specific T Cells

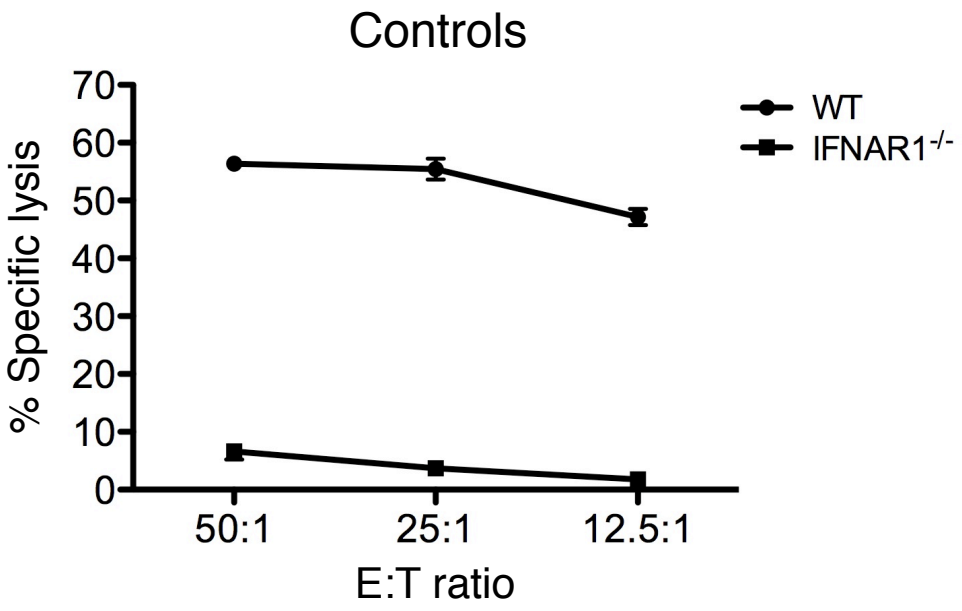
We have previously demonstrated that the anti-tumor defect in IFNAR1$^{-/-}$ hosts correlates with the absence of detectable tumor-specific T cells in the spleen after ex vivo restimulation (see Chapter 2, Figure 6). A similar type of analysis was thus performed with IFNAR1$^{-/-}$ mixed bone marrow chimeras to determine whether the presence of IFNα/β-responsive innate immune cells was sufficient to restore the generation of tumor-specific T cells to wild type levels. At day +20 relative to H31m1 tumor challenge, spleens from representative control and chimeric mice were harvested, splenocytes were restimulated once in vitro with irradiated tumor cells, and the cells were then used as effectors in a standard cytotoxicity assay with radio-labeled tumor (Figure 11). As observed in prior experiments, splenocytes from WT mice displayed robust tumor cell killing, whereas those from IFNAR1$^{-/-}$ mice did not. Similar results were also seen with control WT→WT and IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ chimeras. Reconstitution of IFNα/β sensitivity within hematopoietic cells (in "innate+adaptive" chimeras) restored tumor cell killing to levels comparable to WT→WT chimeras – further illustrating the importance of IFNα/β's actions on hematopoietic cells for the development of an anti-tumor immune response. Within the hematopoietic compartment, selective reconstitution of innate immune IFNα/β sensitivity (in "innate" chimeras) was able to recapitulate the normal generation of H31m1-specific T cells. In contrast, "adaptive" chimeras – containing

IFNα/β-responsive cells exclusively in the adaptive immune compartment – showed no recovery of tumor-specific T cell priming.

To assess whether tumor cell cytotoxicity was attributable to $CD8^+$ T cell killing, we added blocking mAb's specific for either CD4 or CD8 to the culture of effector and target cells. As shown in Figure 12, addition of a blocking anti-CD8 antibody, but not anti-CD4 antibody, inhibited the cytotoxic activity of "innate" chimera splenocytes against H31m1 tumor cells. This result also demonstrates that the defect in tumor cell killing observed in $IFNAR1^{-/-}$ mice is, in fact, due to the lack of T cell priming rather than inherent deficits in the effector function of $IFNAR1^{-/-}$ $CD8^+$ T cells, since T cells in "innate" chimeras are exclusively IFNAR1-deficient.

While IFNα/β's actions on innate immune cells can promote tumor-specific T cell generation, these data do not formally establish this effect as the mechanism responsible for tumor rejection in "innate" $IFNAR1^{-/-}$ chimeras. To directly address this question, we used a mixture of monoclonal antibodies that deplete $CD4^+$ and $CD8^+$ cells to ask whether T cells were indeed required for tumor rejection. As depicted in Figure 13, treatment of WT mice with the combination of anti-CD4/CD8 mAb's blocked the rejection of H31m1 tumor cells in 5/6 mice, while treatment with control mAb had no effect. Similarly, H31m1 tumor cells grew progressively in 5/5 "innate" $IFNAR1^{-/-}$ chimeras treated with anti-CD4/CD8 mAb's, yet in only 2/6 "innate" chimeras treated with control mAb. Taken together, these data underscore the important function of innate immune IFNα/β sensitivity for the generation of tumor-specific T cells, while confirming the essential role of these effector cells during tumor rejection.

Figure 11. Innate immune IFNα/β sensitivity restores the generation of tumor-specific T cells. Splenocytes were isolated from control and IFNAR1$^{-/-}$ mixed chimeric mice injected 20 days prior with 1x10^{6} H31m1 tumor cells. After a single in vitro restimulation, cells were used as CTL effectors in a 4 hr-cytotoxicity assay with radiolabeled H31m1 tumor cells. Percent specific lysis at the indicated E:T ratio is plotted for n=3-5 mice per group assayed in duplicate from 2-3 independent experiments. In each case, cytotoxicity against the irrelevant RAG2$^{-/-}$ regressor tumor 1773 was assayed to confirm specificity of killing (data not shown). Background levels of cytotoxicity obtained with naïve splenocytes following in vitro stimulation and tumor coculture are shown.


Controls
% Specific lysis
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
E:T ratio
WT
IFNAR1-/-

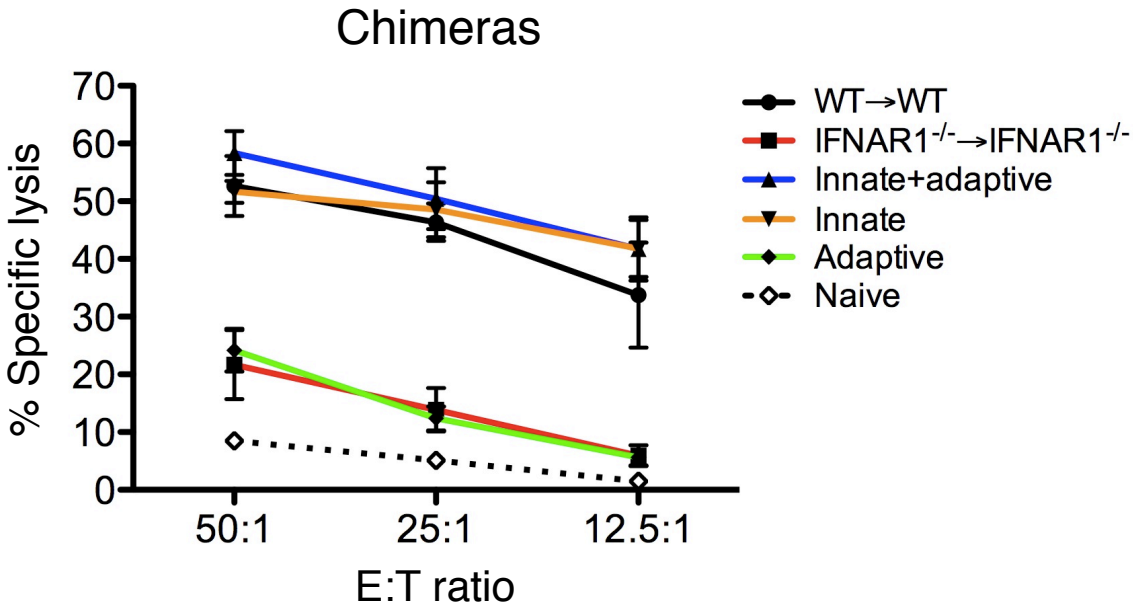

Chimeras
% Specific lysis
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
E:T ratio
WT→WT
IFNAR1-/-→IFNAR1-/-
Innate+adaptive
Innate
Adaptive
Naive

Figure 12. $CD8^+$ T cells are responsible for enhanced tumor-specific cytotoxicity elicited by IFNα/β-responsive innate immune cells. Splenocytes from an $IFNAR1^{-/-}$ mixed chimera with selective IFNα/β sensitivity in innate immune cells were isolated at day 20 post-transplant with $1x10^6$ H31m1 tumor cells. Ex vivo cytotoxicity was assayed in the presence of blocking anti-CD4 (GK1.5), anti-CD8 (YTS-169.4), or control (PIP) mAb. Percent specific lysis at the indicated E:T ratios are shown. Similar results were obtained using splenocytes from WT mice following H31m1 tumor challenge (data not shown).

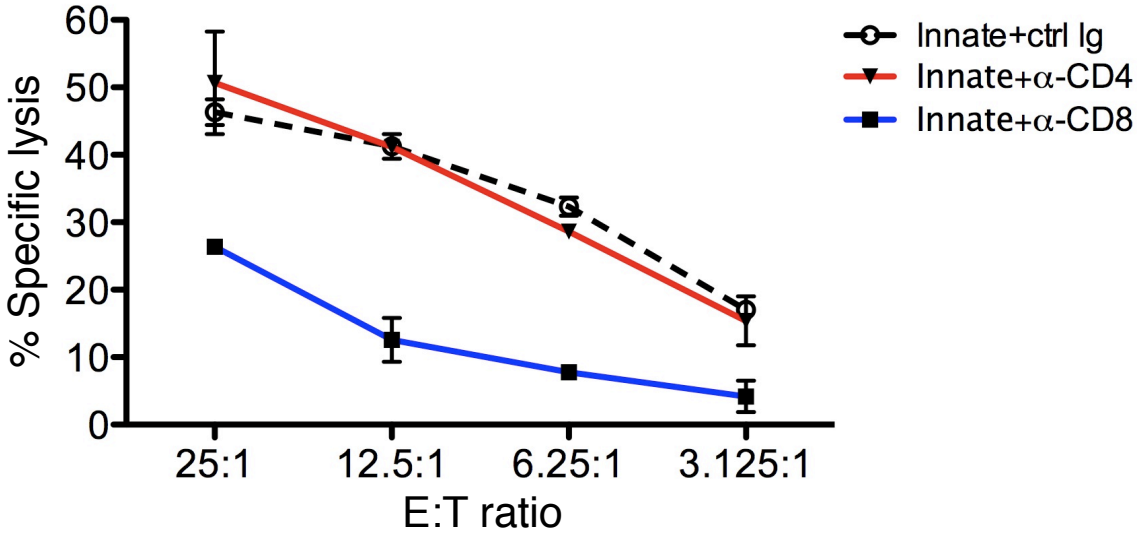
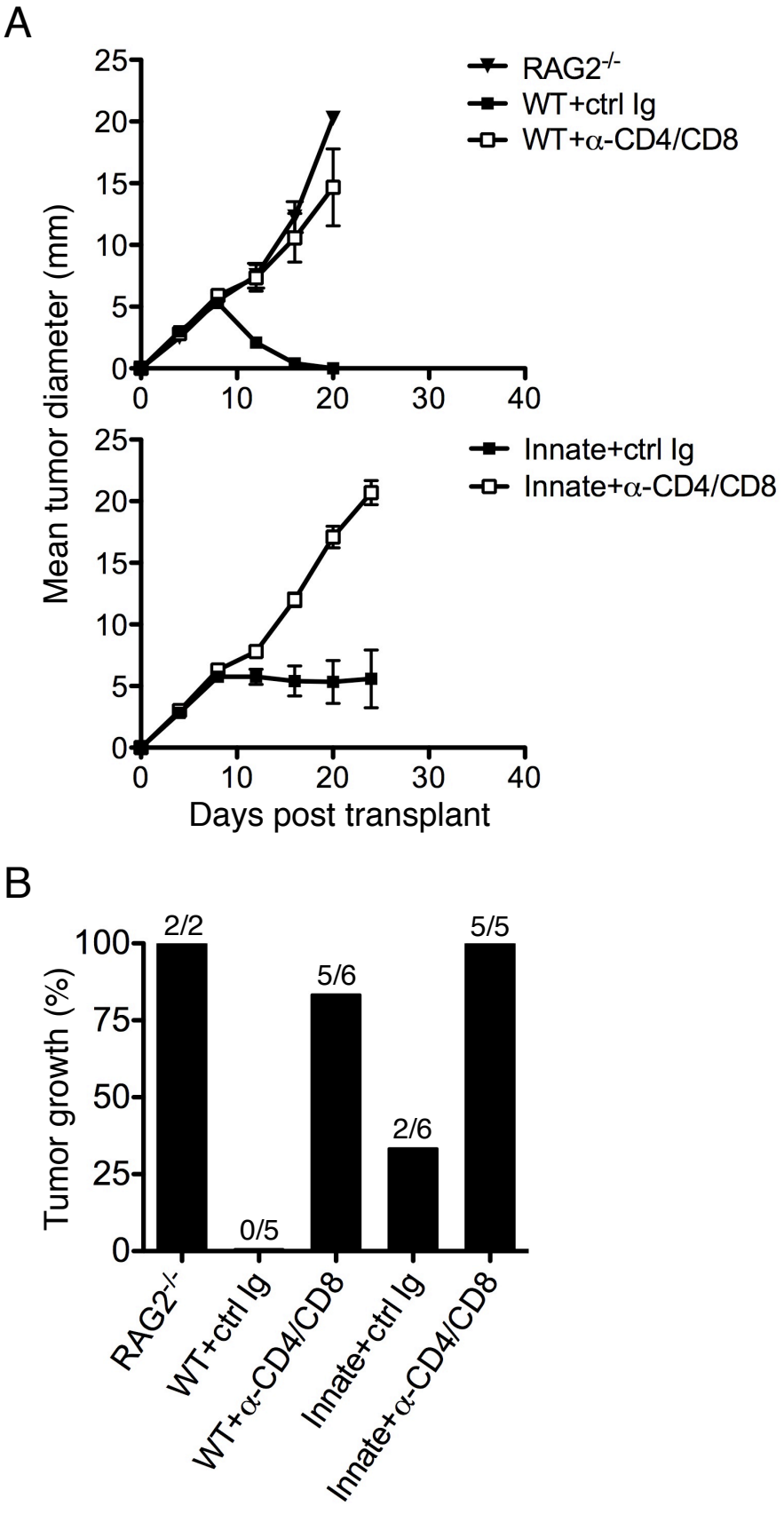

60
50
40
30
20
10
0
% Specific lysis
25:1
12.5:1
6.25:1
3.125:1
E:T ratio
Innate+ctrl Ig
Innate+α-CD4
Innate+α-CD8

Figure 13. T cells are required for tumor rejection in innate immune IFNα/β-responsive mixed bone marrow chimeras. (A) $1x10^6$ H31m1 tumor cells were injected into $RAG2^{-/-}$ controls or groups of WT mice and innate $IFNAR1^{-/-}$ mixed chimeras treated with either control PIP mAb or anti-CD4/CD8 mAb's. Mean tumor diameter ± s.e.m. over time is shown from 2 independent experiments. Percent tumor growth for the respective groups is indicated in (B).


A
RAG2-/-
WT+ctrl Ig
WT+α-CD4/CD8
Innate+ctrl Ig
Innate+α-CD4/CD8
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40
B
Tumor growth (%)
0
25
50
75
100
2/2
0/5
5/6
2/6
5/5
RAG2-/-
WT+ctrl Ig
WT+α-CD4/CD8
Innate+ctrl Ig
Innate+α-CD4/CD8

NK Cells are Not Required for IFNα/β-Dependent Tumor Rejection

Having established the importance of IFNα/β's actions on cells of the innate immune compartment, we sought to identify the functionally relevant responding cells within this compartment mediating its anti-tumor effects. As NK cells are one population of innate immune cells that (i) have a host protective role in some models of primary and transplantable tumorigenesis (116, 170), and (ii) display enhanced cytotoxic activity in response to type I IFN (171), we investigated the role of NK cells in the IFNα/β-dependent rejection of immunogenic fibrosarcomas. The finding that T cells play an obligate function in the rejection of $RAG2^{-/-}$ regressor tumors suggests that NK-mediated cytotoxicity is not the sole effector mechanism responsible for tumor elimination; however, early tumor cell recognition and killing, and/or the production of pro-inflammatory cytokines, may be imperative for the induction of an effective anti-tumor immune response.

For these experiments we utilized MCA-induced $RAG2^{-/-}$ regressor sarcomas on a C57Bl/6 background, which have recently been generated in the laboratory, in order to allow for NK cell depletion in B6 strain mice via treatment with the anti-NK1.1 mAb PK136 (172). We confirmed by FACS analysis the effective depletion of both NK and NKT cells in the spleen following anti-NK1.1 mAb treatment (Figure 14 and data not shown). A standard assay for NK cell function involving ex vivo killing of ^{51}Cr-labeled YAC-1 target cells following polyI:C injection, also showed complete abrogation of NK killing upon anti-NK1.1 mAb treatment (Figure 15A). In addition, the in vivo control of

a low dose challenge with MHC class I-deficient RMA-S tumor cells was abolished by anti-NK1.1 but not anti-CD4/CD8 mAb treatment (Figure 15B), as previously reported (173).

Despite the complete elimination of NK cell function using this treatment protocol, we observed no effect on the rejection of three representative B6 RAG2$^{-/-}$ regressor tumors (1969, 7835, and 6494), as shown in Figure 16. Similar to 129 SvEv strain RAG2$^{-/-}$ regressors, the immune-mediated rejection of these B6 sarcomas required IFNα/β responsiveness at the level of the host (see Chapter 2). Taken together, these data therefore indicate that the actions of endogenous type I IFN on NK cells are not absolutely required to mediate the protective effects of this cytokine family.

Figure 14. Effective NK cell depletion by anti-NK1.1 PK136 mAb treatment. WT B6 mice were treated with PBS or PK136 mAb by i.p. injection as indicated in Materials and Methods. Splenocytes were analyzed at day +2 of the treatment regimen by FACS analysis using the NK cell markers DX5 and NKp46. Splenocytes were gated on CD3- cells, and the percentages of $DX5^{+}NKp46^{+}$ cells are indicated. Similar depletion was also observed at day +6 (data not shown).

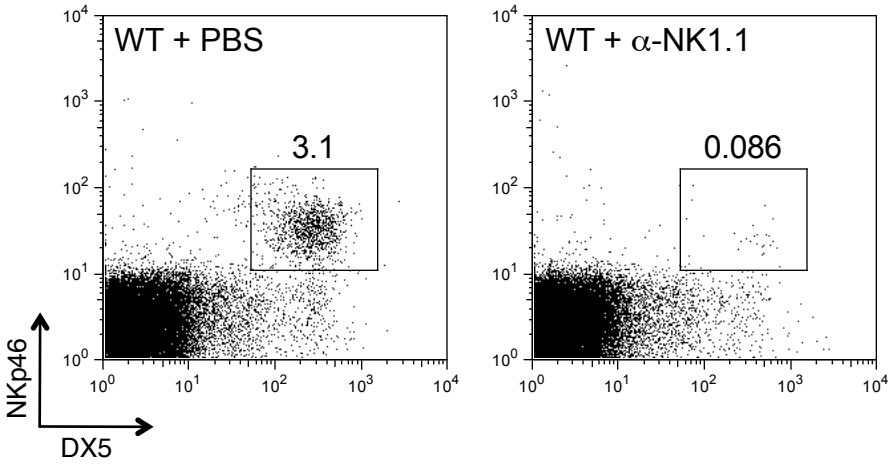
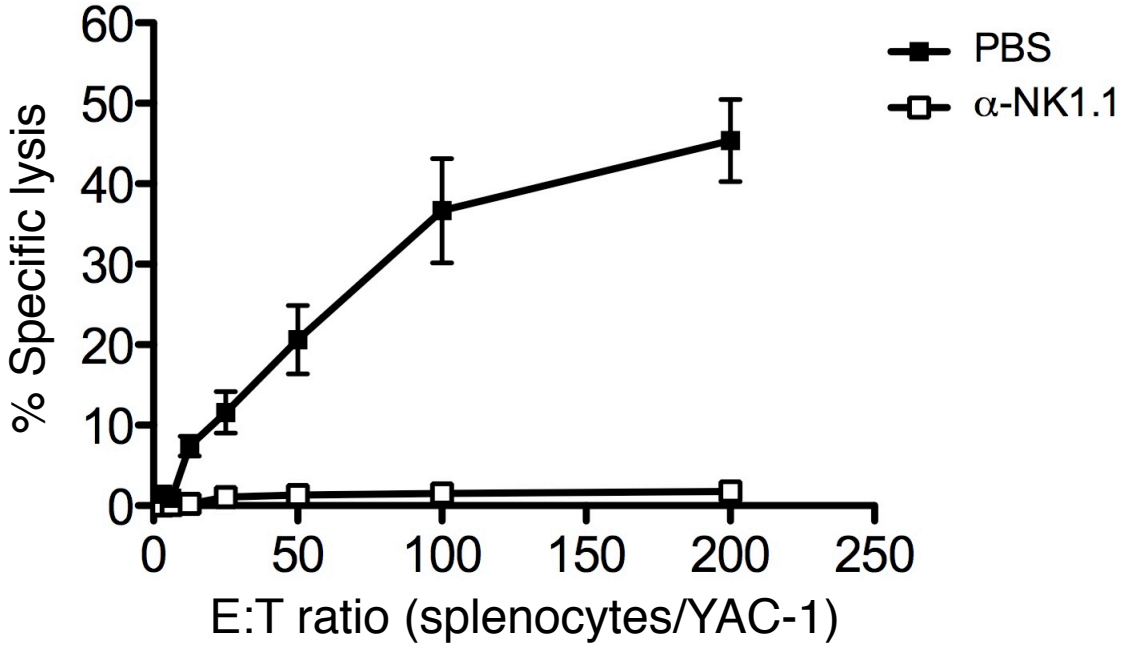

Gated on CD3- splenocytes
WT + PBS
3.1
WT + α-NK1.1
0.086
NKp46
DX5

Figure 15. Abrogation of ex vivo NK killing activity and in vivo NK cell function with anti-NK1.1 mAb PK136 treatment. (A) Splenocytes from WT B6 mice treated with either PBS or anti-NK1.1 mAb PK136 were isolated at day +4 of the treatment regimen, following i.p. injection of 300 μg polyI:C 24 hours prior. Cells were used as effectors in a standard 4 hr cytotoxicity assay with NK-sensitive YAC-1 targets. Percent specific lysis at the indicated E:T ratios is shown for n=4 mice/group assayed in duplicate from two independent experiments. (B) PBS or PK136 mAb-treated mice were injected s.c with a low-dose challenge of $1x10^5$ RMA-S cells and tumor growth was monitored. Mean tumor diameters ± s.e.m. for 3 mice/group are shown for one of two independent experiments with similar results.

A


% Specific lysis
60
50
40
30
20
10
0
0
50
100
150
200
250
E:T ratio (splenocytes/YAC-1)
PBS
α-NK1.1

B

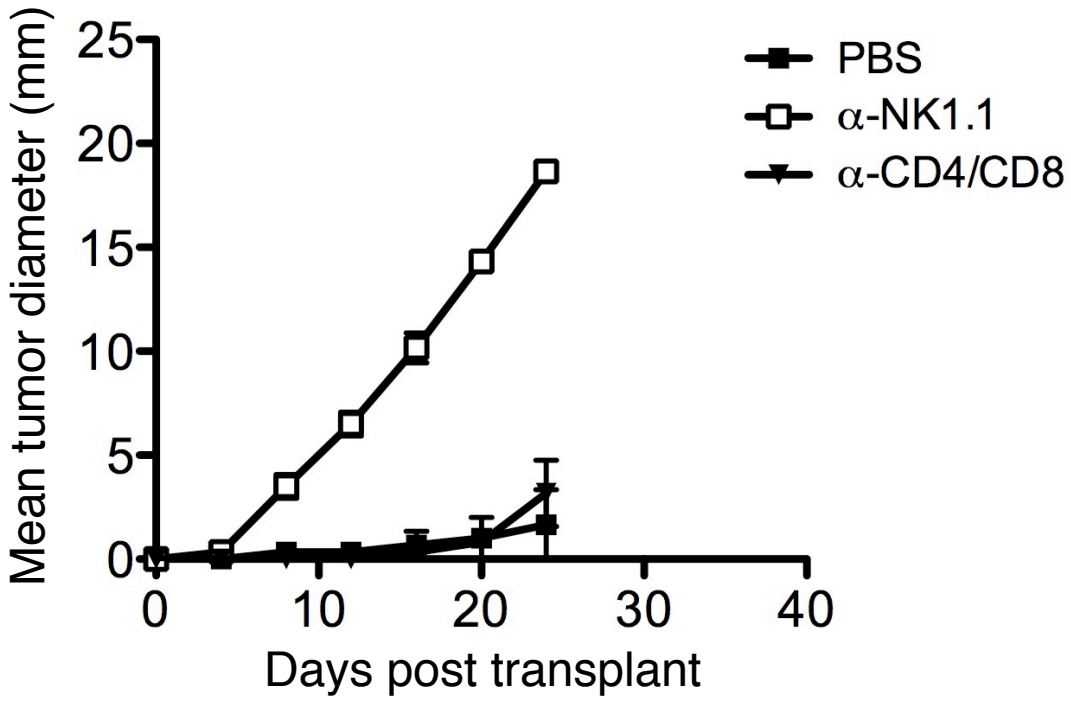
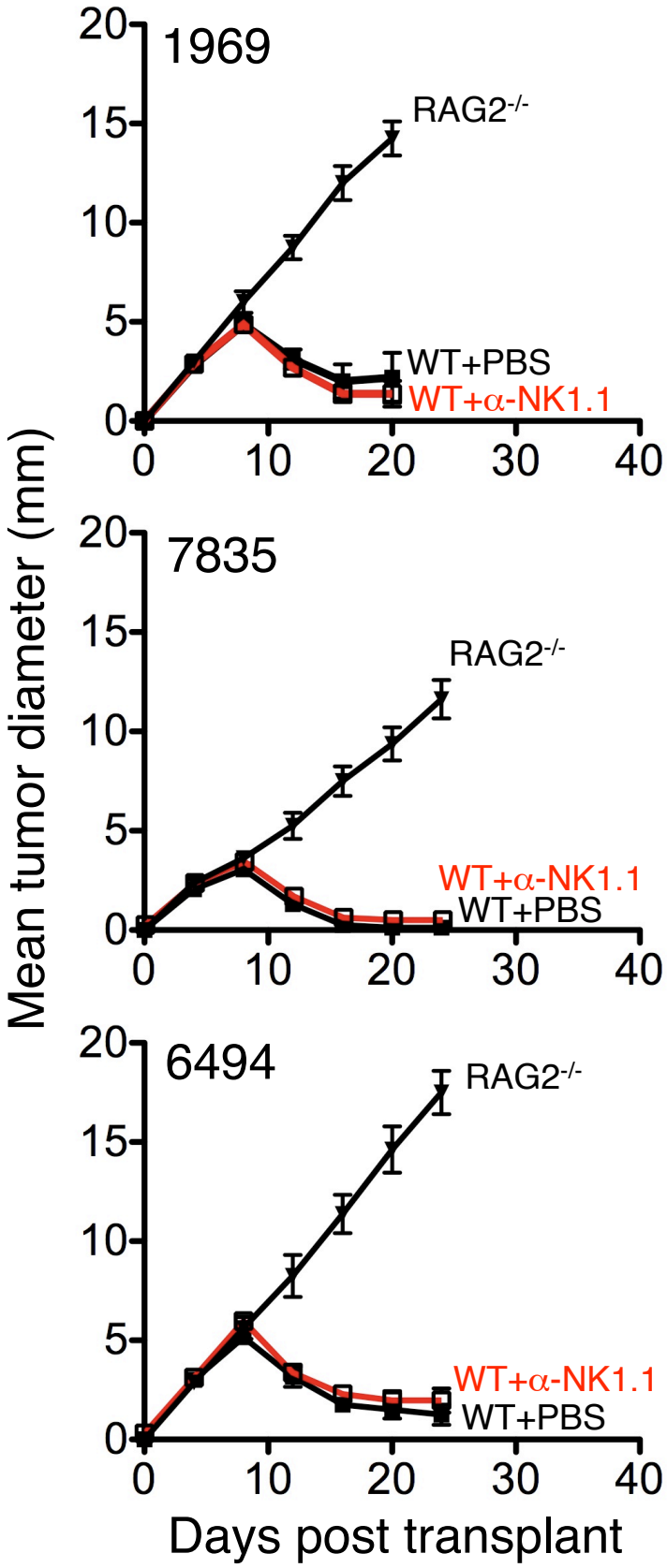

Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
Days post transplant
PBS
α-NK1.1
α-CD4/CD8

Figure 16. NK cells are not required for rejection of immunogenic sarcomas. Groups of $RAG2^{-/-}$ controls or WT mice treated with either PBS or anti-NK1.1 mAb PK136 were challenged with $1x10^6$ 1969, 7835, or 6494 B6 $RAG2^{-/-}$ regressor tumor cells. Data represent pooled results from two independent experiments with n=6-8 (WT) or n=4 ($RAG2^{-/-}$) mice.


1969
RAG2-/-
WT+PBS
WT+α-NK1.1
7835
RAG2-/-
WT+α-NK1.1
WT+PBS
6494
RAG2-/-
WT+α-NK1.1
WT+PBS
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
0
10
20
30
40

NK Cell-Independent Effects of Anti-asGM1 Antibody Treatment Inhibits Anti-Tumor Immunity

During the course of the prior studies, we observed that treatment of mice with polyclonal antiserum against the surface glycolipid asialo-GM1 (anti-asGM1 Ab) was able to inhibit the rejection of two 129 regressor tumors, H31m1 and d38m2, in a proportion of mice (Figure 17). This antibody is frequently used in non-NK1.1 expressing mouse strains to deplete NK cells; however, depletion is not specific since asialo-GM1 is also expressed on activated macrophages and on subsets of naïve and activated T cells. In fact, it has been reported that asialo-GM1 is induced on the majority of virus-specific $CD4^+$ and $CD8^+$ T cells following acute viral infection, and that these cells are largely depleted by in vivo administration of anti-asGM1 Ab (174, 175). In our studies, we found that anti-asGM1 Ab treatment depleted NK cells but not NKT cells (Figure 18) as previously reported (176), and that NK cell function is effectively eliminated when assessed using both in vitro and in vivo assays (Figure 19). In addition, percentages of $CD4^+$ and particularly $CD8^+$ T cells were reduced in the spleens of naïve 129 and B6 strain mice following anti-asGM1 Ab treatment (Figure 18 and data not shown). Though we did not observe depletion of $F4/80^+CD11b^+$ macrophages within the spleen, macrophage expression of asialo-GM1 at high levels is typical seen only upon activation (177).

Given the possibility that NK cell-independent effects of anti-asGM1 Ab might be responsible for its inhibition of anti-tumor immunity, we tested this hypothesis using the

three B6 regressor tumors previously shown to be rejected in mice lacking NK cells (see Figure 16). When transplanted into WT mice treated with anti-asGM1 Ab, two of the three tumors (1969 and 6494, but not 7835) now grew progressively. As these same tumors were rejected in WT mice treated with anti-NK1.1 mAb, the effects of anti-asGM1 Ab treatment must be mediated by depletion or blockade of a non-NK cell population. Although the depletion of effector T cells may be solely responsible, it is intriguing to speculate that the elimination of activated macrophages might be involved. Future studies using clodronate liposomes or CD11b-DTR mice to achieve macrophage depletion will be required to clarify their function during tumor rejection.

In addition to potential effects on macrophages, recent studies have suggested that a population of innate-like central memory $CD8^+$ T cells is readily depleted by anti-asGM1 Ab treatment of naïve mice (178). This subset of $asGM1^+CD8^+$ T cells (which also express CD44, CD122, CD62L, and CCR7) were potent producers of early IFNγ upon anti-CD3 stimulation both in vitro and in vivo compared to the $asGM1^-CD8^+$ central memory T cell population. In our experiments, we indeed noted significant depletion of a $CD3^+CD122^+$ T cell population with anti-asGM1 Ab treatment (data not shown). Recent studies in our laboratory have shown that most memory phenotype $CD8^+CD44^+$ T cells also express high levels of the chemokine receptor CXCR3 and can be selectively depleted in vivo using the anti-CXCR3 mAb CXCR3-173 (179) (R. Uppaluri, J.J. Brotman, and R.D. Schreiber, unpublished data). Depletion of this subset, however, had no effect on the rejection of several tumors which grew progressively with

anti-asGM1 Ab treatment (R. Uppaluri, J.J. Brotman, and R.D. Schreiber, unpublished data), suggesting that these cells are not the relevant targets of anti-asGM1 Ab.

Figure 17. Anti-asGM1 Ab treatment inhibits $RAG2^{-/-}$ regressor tumor rejection via NK cell-independent effects. $1x10^6$ cells of the indicated 129 or B6 strain regressor tumors were injected into groups of $RAG2^{-/-}$ mice, WT mice treated with PBS, or WT mice treated with anti-asGM1 as indicated in Materials and Methods.

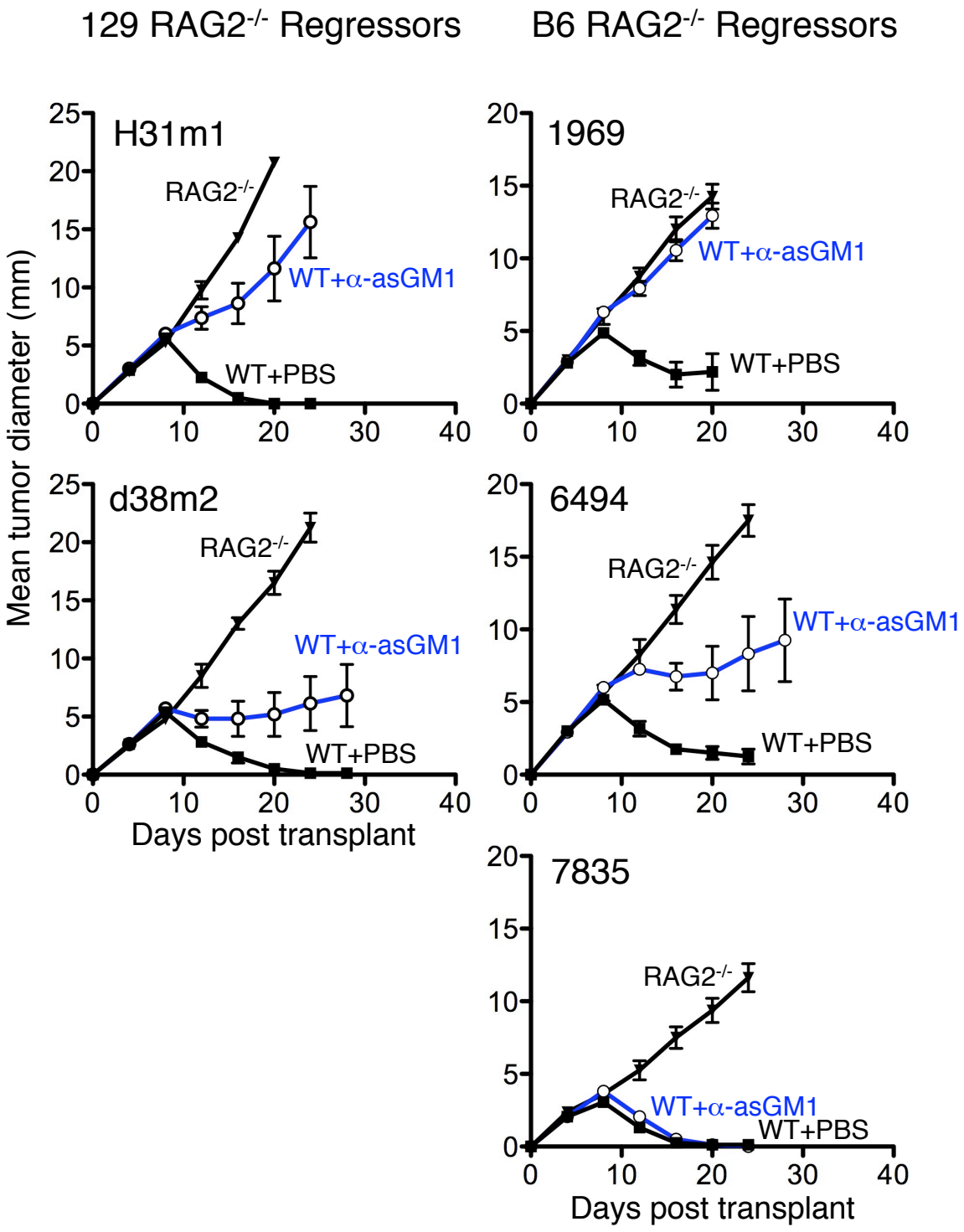

129 RAG2-/- Regressors
B6 RAG2-/- Regressors
H31m1
1969
d38m2
6494
7835
RAG2-/-
WT+α-asGM1
WT+PBS
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

Figure 18. Depletion of NK cells and T cell subsets in mice treated with anti-asGM1. Splenocytes were isolated at day +4 from naïve B6x129 F1 mice treated with either PBS or anti-asGM1 and immune cell subsets were examined by FACS. Similar NK cell and T cell, but not NKT cell, depletion was also observed in B6 strain mice (data not shown).

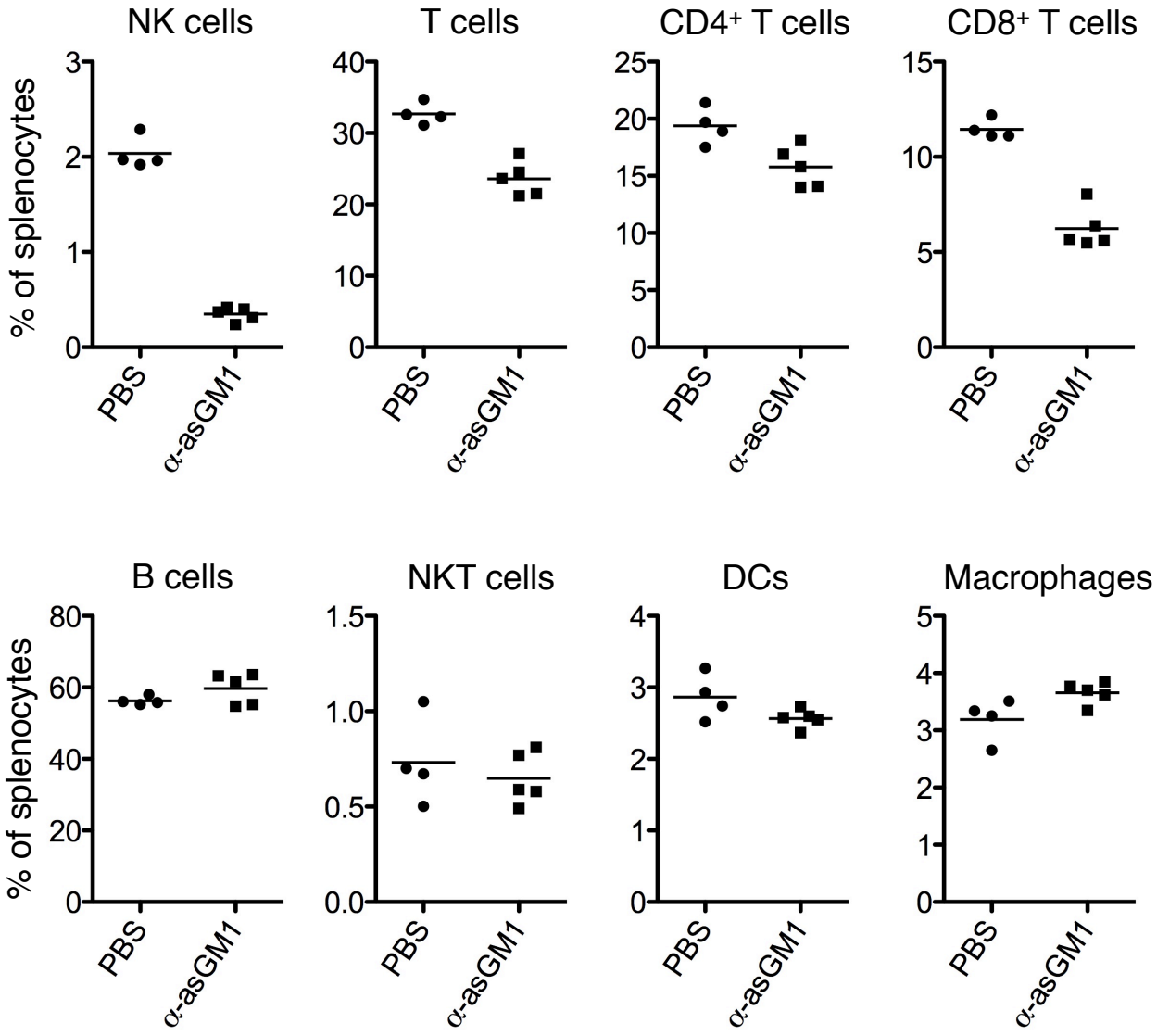
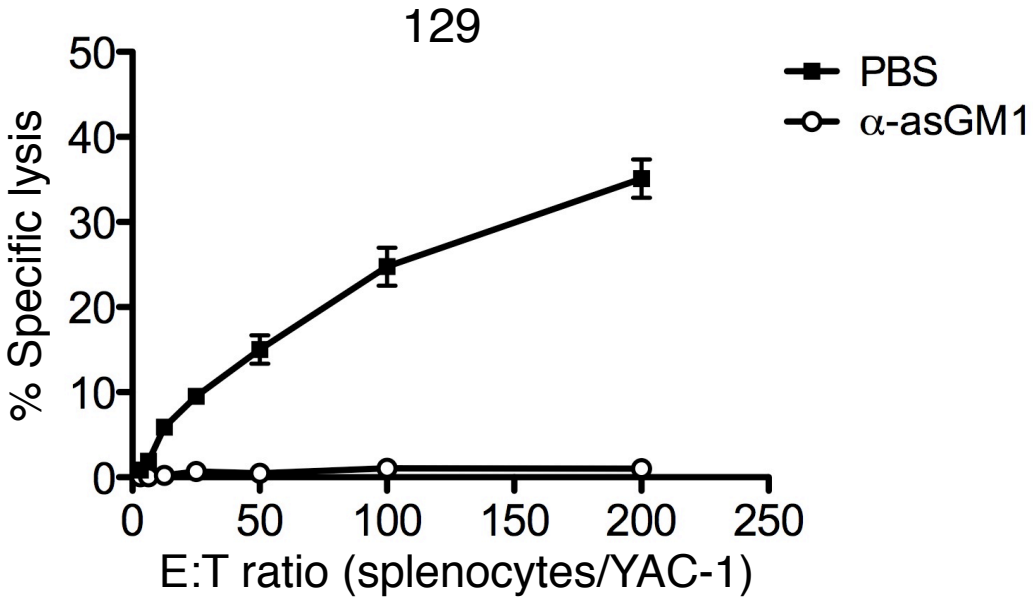
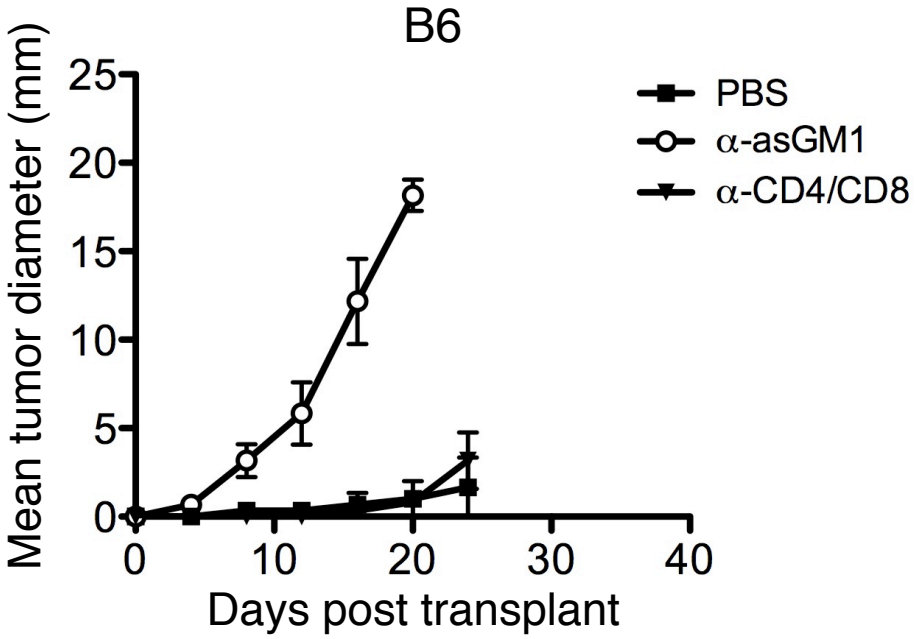

NK cells
T cells
CD4+ T cells
CD8+ T cells
B cells
NKT cells
DCs
Macrophages
% of splenocytes
% of splenocytes
PBS
α-asGM1

Figure 19. Elimination of NK cell function in 129 and B6 mice treated with anti-asGM1 Ab. (A) 129/SvEv mice were treated with either PBS or anti-asGM1 Ab and splenocytes were isolated at day +4, following injection of 300 μg polyI:C 24 hrs prior. NK killing assays were performed using YAC-1 target cells, and percent specific lysis is plotted for n=3-4 mice/group from two independent experiments. (B) B6 mice treated with anti-asGM1 were also injected with a low-dose RMA-S challenge ($1x10^5$ cells) and growth was monitored.

A


129
% Specific lysis
E:T ratio (splenocytes/YAC-1)
PBS
α-asGM1
0
10
20
30
40
50
0
50
100
150
200
250

B


B6
Mean tumor diameter (mm)
Days post transplant
PBS
α-asGM1
α-CD4/CD8
0
5
10
15
20
25
0
10
20
30
40

Cross-Presentation by CD8α$^+$ Dendritic Cells is Required for Tumor-Specific CTL Priming and Tumor Rejection

As our previous findings have (i) established an important role for innate immune IFNα/β sensitivity during tumor-specific T cell priming and tumor rejection, and (ii) ruled out a prominent function for NK cells during this process, we turned our attention toward other innate immune cell populations – including professional APCs such as DCs and macrophages which are particularly adept at initiating adaptive T cell responses. As a first step toward examining the specific function of IFNα/β on these cell populations during tumor rejection, we wanted to ask more broadly whether the presence of these cell types was absolutely essential for rejection of transplantable tumors. Unfortunately, there are few ways to achieve selective and long-term depletion of DCs and macrophages in vivo.

Gene-targeted mice lacking the AP-1 family member p21SNFT (also known as Batf3) were recently developed by Kai Hildner, Ken Murphy, and colleagues (151). Analyses of genome-wide expression profiles across an array of tissues and immune cell subsets had identified this gene as almost exclusively expressed in conventional DCs (cDCs), though low expression in monocytes was also observed. Interestingly, p21SNFT$^{-/-}$ mice were found to selectively lack the CD8α$^+$ cDC subset, while normal representation of other DC subsets and hematopoietic lineages was maintained. Detailed examination of tissue resident DCs within the skin and lung, however, also revealed the selective absence of a population of recently described migratory Langerin$^+$ DCs

(DEC205$^+$CD103$^+$Langerin$^+$) found in the dermis as well as lung and liver (180-182). This population is distinct from previously described dermal DCs (which are Langerin$^-$ and CD11b^{high}) and although they do not express CD8α, they share phenotypic markers (CD103$^+$, DEC205$^+$, and CD11b$^{lo/-}$) and functional similarities with the CD8α$^+$ cDC subset, including responsiveness to TLR3 ligands and the ability to cross-present antigen, though their functional significance in this regard is still unclear. The absence of Langerin$^+$ dermal DCs in p21SNFT$^{-/-}$ mice suggests that this population may be developmentally related to CD8α$^+$ DCs, yet further studies will be required to investigate this possibility.

When challenged with West Nile virus (WNV), p21SNFT$^{-/-}$ exhibited normal antibody and CD4$^+$ T cell responses, which are required for protective immunity, and hence they showed no increased susceptibility to infection (151). Examination of CD8$^+$ T cell responses, however, revealed a significant reduction in the generation of WNV-specific CD8$^+$ T cells. T cell adoptive transfer experiments demonstrated that this deficit was associated with the absence of CD8α$^+$ DCs rather than a cell intrinsic defect in CD8$^+$ T cell function. Additional studies also confirmed normal cell-intrinsic responses in CD4$^+$ and CD8$^+$ T cells as well as NK cells, supporting the conclusion that deficient DC function is responsible for the phenotype of p21SNFT$^{-/-}$ mice.

To assess the role of cross-presentation by CD8α$^+$ DCs in the rejection of immunogenic tumors, we challenged p21SNFT$^{-/-}$ mice with three representative syngeneic RAG2$^{-/-}$ regressor tumors. Whereas all three tumor lines (H31m1, 1773, and d38m2) were rejected in WT mice, these tumors grew rapidly in p21SNFT$^{-/-}$ hosts with

kinetics comparable to those observed in RAG2$^{-/-}$ controls (Figure 20). In addition, ex vivo analysis of tumor-specific CD8^{+} T cell cytotoxicity revealed a complete lack of tumor reactivity, and we confirmed that both CD8^{+} and CD4^{+} cells were required for rejection of H31m1 (Figure 22A-B). Interestingly, analysis of TIL populations within the tumors of WT and p21SNFT$^{-/-}$ mice at day 11 following H31m1 injection showed a substantial decrease in CD8^{+} T cell infiltration, but no change in CD4^{+} T cell abundance (data not shown). While p21SNFT$^{-/-}$ mice exhibit a striking defect in tumor rejection, dose titrations of H31m1 tumor cells showed that there is not an absolute deficiency in mounting adaptive responses, since a fraction of mice injected with $1x10^{5}$ or $1x10^{4}$ tumor cells could reject this challenge, in contrast to RAG2$^{-/-}$ mice (Figure 21). Moreover, those p21SNFT$^{-/-}$ mice that had rejected low-dose H31m1 challenge manifested some H31m1-specific CTL killing activity ex vivo, though perhaps only a partial response (Figure 22C). This result suggests that either cross-presentation of tumor-derived antigens by other APC populations can occur with lesser efficiency, or that there may be a small contribution of direct priming (directly by the tumor cells), which has been observed in other model systems (183).

Although adoptive transfer of DC populations is difficult due to their limited life span and the need to recapitulate proper trafficking in vivo, we tested whether transfer of WT DCs into p21SNFT$^{-/-}$ mice prior to tumor challenge could provide some recovery of anti-tumor function. For this purpose, WT and p21SNFT$^{-/-}$ donor mice were injected with a regimen of fms-like tyrosine kinase 3 (flt3) ligand-Fc to induce in vivo DC expansion. CD11c^{+} cells from the spleen were then positively selected by MACS,

incubated with whole tumor lysate in the presence of LPS, and injected into p21SNFT$^{-/-}$ recipients via both s.c. and i.v. routes immediately prior to tumor challenge. As shown in Figure 23, transfer of p21SNFT$^{-/-}$ DCs had no effect on H31m1 tumor growth in p21SNFT$^{-/-}$ mice, yet transfer of WT DCs induced a significant slowing of growth in a fraction of p21SNFT$^{-/-}$ recipients. This observation suggests that transferred WT DCs, but not p21SNFT$^{-/-}$ DCs (which lack the CD8α^{+} subset as shown in Figure 23B) can restore some anti-tumor immunity, though it would be interesting to assess levels of H31m1-specific T cell priming by ex vivo cytotoxicity assay. Collectively, these data demonstrate a prominent role for cross-presentation via the CD8α^{+} DC subset during the generation of protective anti-tumor responses.

Figure 20. Lack of tumor rejection in CD8α^+ DC-deficient p21SNFT$^{-/-}$ mice. Groups of WT, p21SNFT$^{-/-}$, and RAG2$^{-/-}$ mice on a 129/SvEv background were injected s.c. with 1x10^6 H31m1, 1773, or d38m2 fibrosarcoma cells and tumor growth was measured over time. Data are presented as mean tumor diameter ± s.e.m. of n=10 (H31m1) or n=3-5 (1773 and d38m2) mice/group.

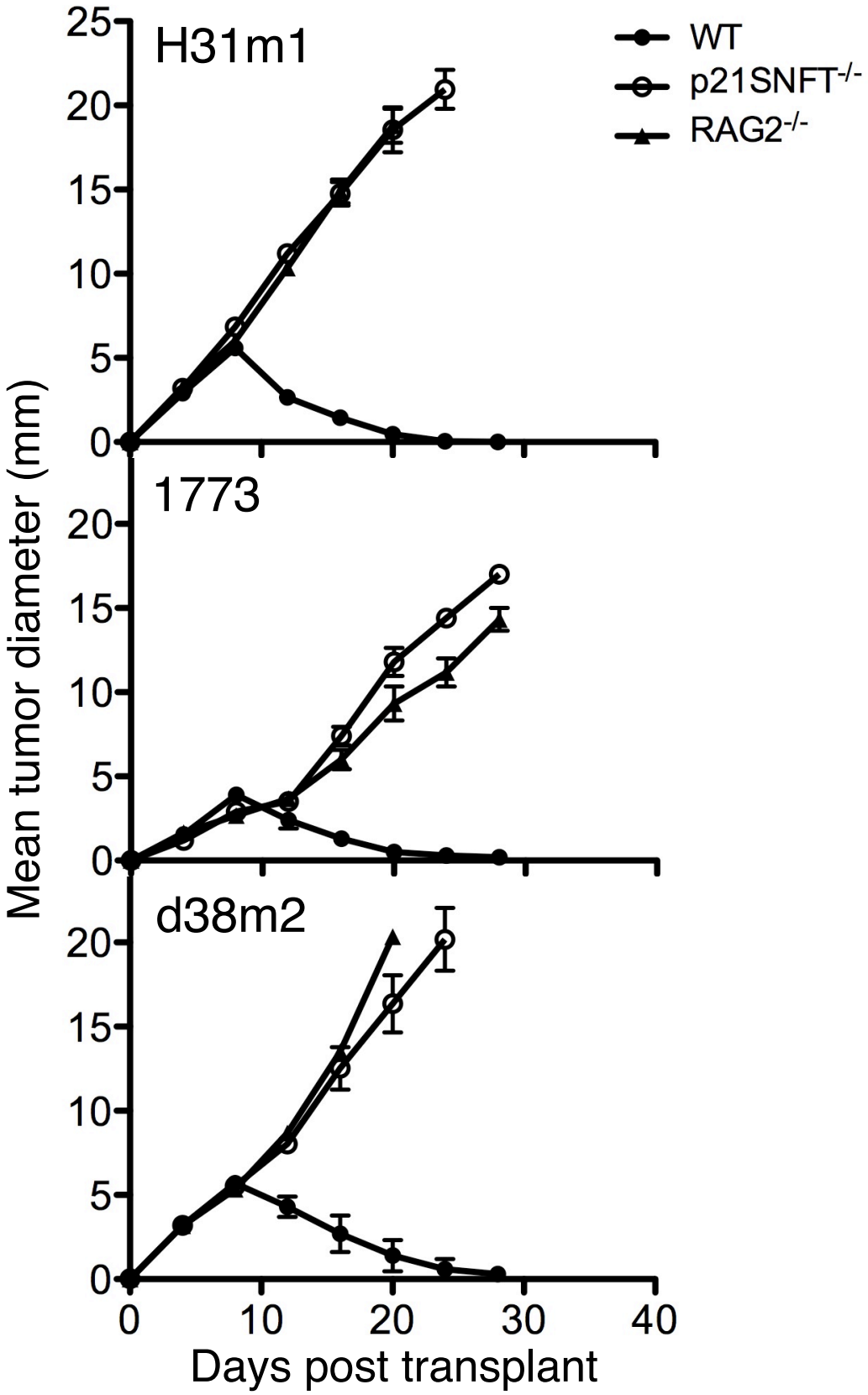

H31m1
1773
d38m2
WT
p21SNFT-/-
RAG2-/-
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

Figure 21. Titration of H31m1 tumor cells in RAG2$^{-/-}$ and p21SNFT$^{-/-}$ mice. Groups of RAG2$^{-/-}$ and p21SNFT$^{-/-}$ mice were challenged with 10^6, 10^5, or 10^4 H31m1 tumor cells and growth was monitored. Each line represents an individual mouse.

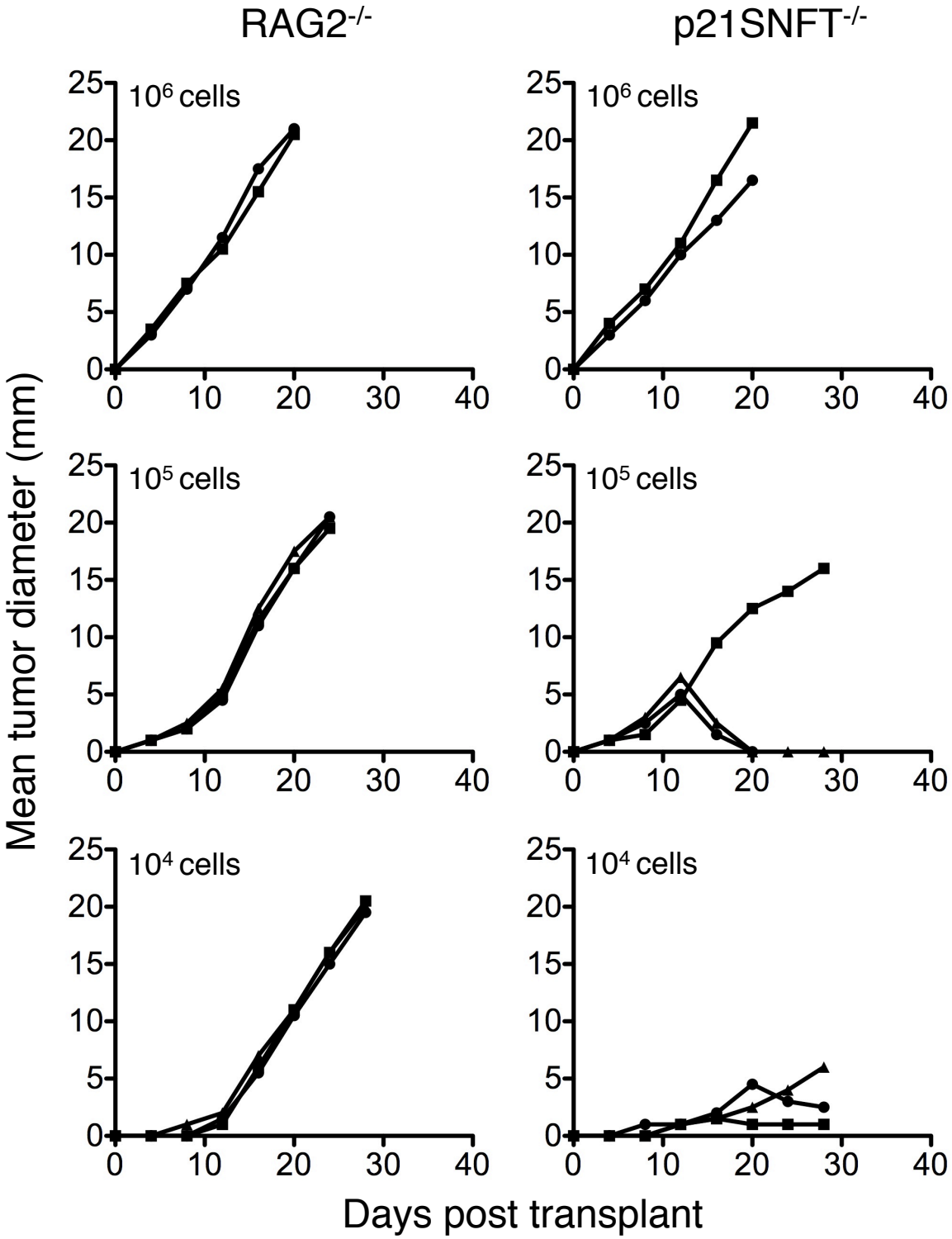

RAG2-/-
p21SNFT-/-
10^6 cells
10^5 cells
10^4 cells
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40

Figure 22. Lack of tumor-specific CTL response in p21SNFT$^{-/-}$ mice. (A) WT mice treated with either anti-CD4, anti-CD8, anti-CD4/CD8, or control mAb's as indicated were challenged with 1x10^6 H31m1 tumor cells. Data represent mean tumor diameter ± s.e.m. of n=4 mice/group. (B) WT and p21SNFT$^{-/-}$ mice were injected with 1x10^6 H31m1 tumor cells and splenocytes were isolated at day 9. Cells were cocultured with IFNγ-pretreated, irradiated H31m1 tumor cells and after 5 days, cells were used as CTL effectors in a 4 hr cytotoxicity assay with ^{51}Cr-labeled H31m1 or 1773 tumor cells as targets. (C) Splenocytes from p21SNFT$^{-/-}$ mice that had rejected a low-dose (1x10^5) H31m1 challenge were treated as in (B).

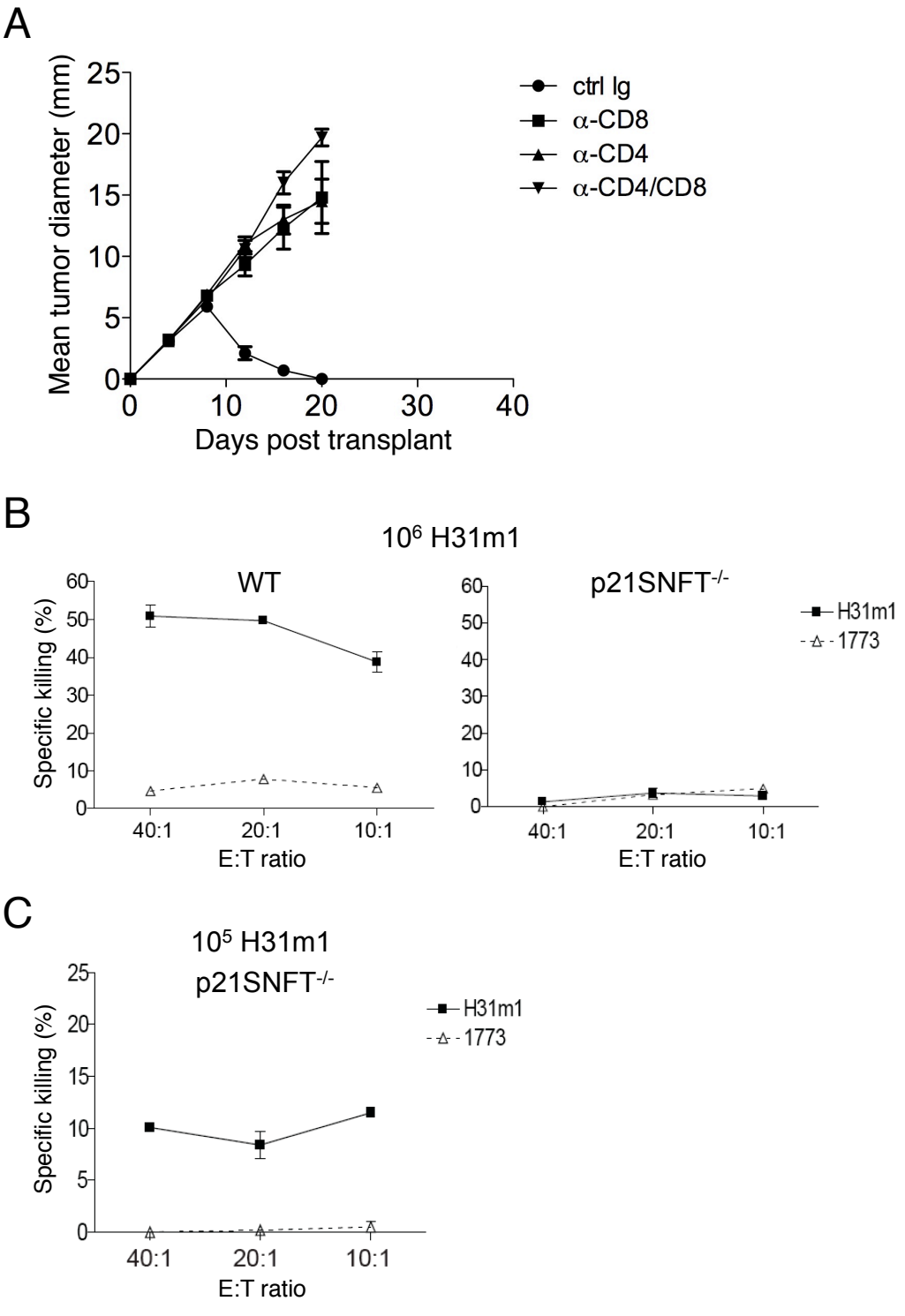
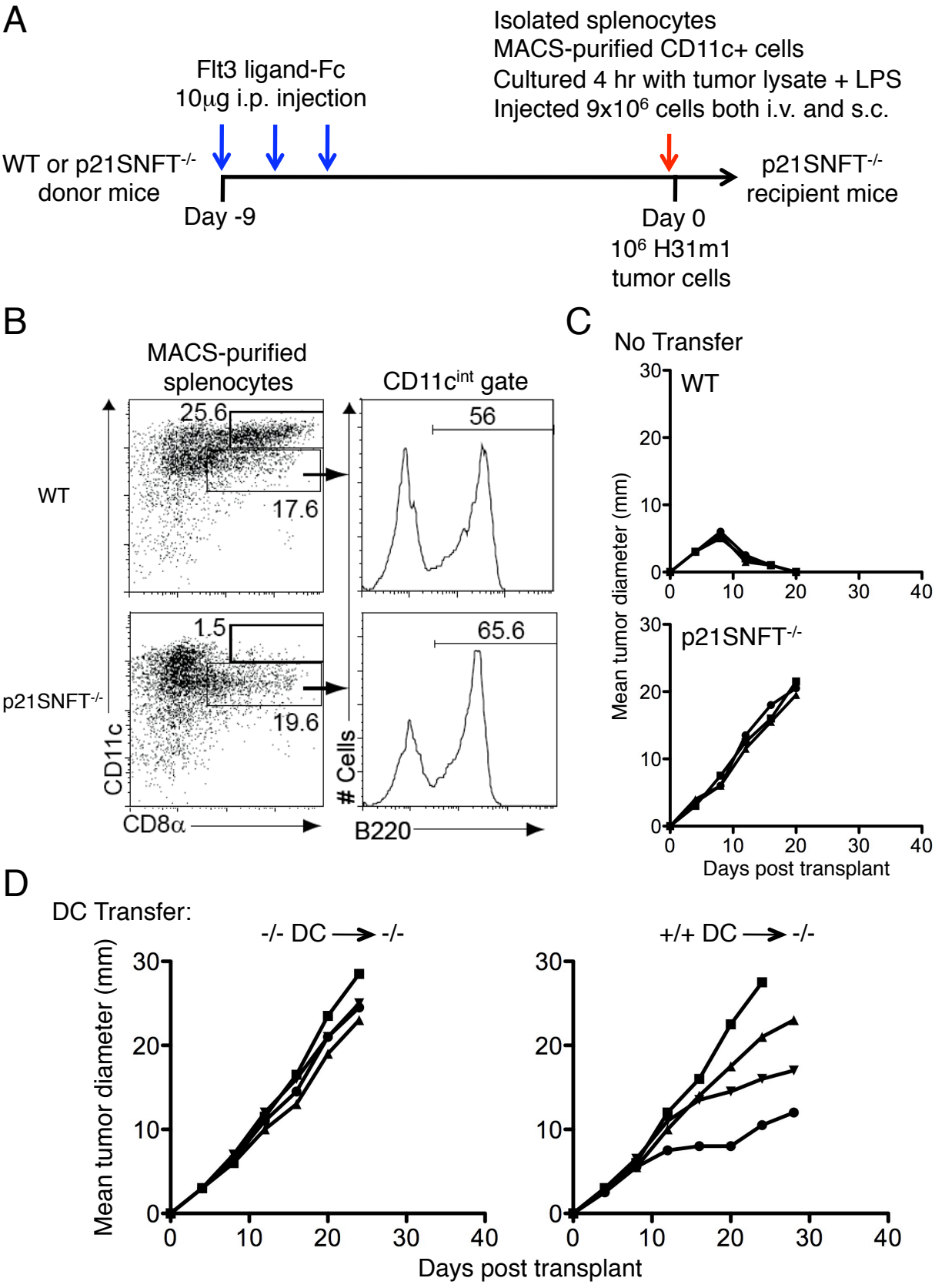

A
Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
Days post transplant
ctrl Ig
α-CD8
α-CD4
α-CD4/CD8
B
10^6 H31m1
WT
p21SNFT-/-
Specific killing (%)
60
50
40
30
20
10
0
40:1
20:1
10:1
E:T ratio
H31m1
1773
C
10^5 H31m1
p21SNFT-/-
Specific killing (%)
25
20
15
10
5
0
40:1
20:1
10:1
E:T ratio
H31m1
1773

Figure 23. Partial reconstitution of anti-tumor immunity by adoptive transfer of WT DC into p21SNFT$^{-/-}$ mice. (A) Protocol for adoptive transfer of WT or p21SNFT$^{-/-}$ DCs. Briefly, donor mice were treated i.p. with flt3 ligand-Fc for three consecutive days. After another 7 days, CD11c^{+} splenocytes were positively selected by MACS (purity>90%), cultured with H31m1 whole tumor lysates in the presence of LPS for 4 hrs ex vivo, and transferred into p21SNFT$^{-/-}$ recipients both i.v. and s.c. (9x10^{6} cells each). (B) CD11c^{+} MACS-purified splenocytes from WT and p21SNFT$^{-/-}$ mice were analyzed by FACS using the indicated markers to assess enrichment of CD8α^{+}CD11c^{hi} cDCs and CD11c^{int}B220^{+} pDCs. (C) Control WT and p21SNFT$^{-/-}$ mice receiving no cells were injected with 1x10^{6} H31m1 tumor cells. (D) p21SNFT$^{-/-}$ receiving either p21SNFT$^{-/-}$ or WT CD11c^{+} cells as indicated (n=4/group) were challenged with 1x10^{6} H31m1 cells. Each line represents an individual mouse.


A
Isolated splenocytes
MACS-purified CD11c+ cells
Cultured 4 hr with tumor lysate + LPS
Injected 9x10^6 cells both i.v. and s.c.
Flt3 ligand-Fc
10μg i.p. injection
WT or p21SNFT-/-
donor mice
Day -9
Day 0
10^6 H31m1
tumor cells
p21SNFT-/-
recipient mice
B
MACS-purified
splenocytes
CD11c^int gate
WT
p21SNFT-/-
25.6
17.6
1.5
19.6
56
65.6
CD11c
CD8α
Cells
B220
C
No Transfer
WT
p21SNFT-/-
Mean tumor diameter (mm)
Days post transplant
D
DC Transfer:
-/- DC → -/-
+/+ DC → -/-
Mean tumor diameter (mm)
Days post transplant

CD8α$^+$ DCs are Important Sites of IFNα/β's Actions

While the preceding experiments reveal an obligate function for CD8α$^+$ DCs during tumor rejection, they do not address whether these cells are the relevant type I IFN responder cells. A large amount of both in vitro and in vivo data show that IFNα/β can have potent effects in inducing functional maturation of DCs (including upregulation of MHC class I and II, CD40, CD80, CD86), which also correlates with their enhanced ability to cross-present antigen to naïve CD8$^+$ T cells (184). In addition, IFNα/β's actions specifically on DCs were found to promote the generation of antibody responses and class switching, though these experiments involved injection of exogenous IFNα (121). The effects of IFNα/β specifically on CD8α$^+$ DCs, however, has not been investigated, and whether these mechanisms are operative during anti-tumor responses is unclear.

We therefore hypothesized that DCs, and specifically the CD8α$^+$ cDC, may be a critical innate immune target of type I IFN during tumor rejection. We first confirmed the presence of normal numbers of DC subsets, including CD8α$^+$ cDCs, in the spleen and lymph nodes of IFNAR1$^{-/-}$ mice (Figure 24), as well as the ability of IFNAR1$^{-/-}$ DCs to expand normally in vivo in response to fms-like tyrosine kinase 3 (flt3) ligand-Fc treatment (data not shown). In collaboration with Mona Mashayekhi and Ken Murphy, we then examined the function of type I IFN during cross-presentation in vitro by culturing splenic DCs isolated from WT or IFNAR1$^{-/-}$ mice with irradiated ovalbumin-loaded MHC class I-deficient cells and OT-I T cells. Total CD11c$^+$ cells purified from

WT mice were more effective than IFNAR1$^{-/-}$-derived cells in inducing the proliferation of OT-I T cells, though this defect could be overcome at very high doses of antigen (Figure 25A). Additionally, cross-presentation by WT CD11c^{+} cells was enhanced by treatment with exogenous IFNα and inhibited by antibody-mediated IFNα/β blockade (Figure 25B). When WT and IFNAR1$^{-/-}$ DCs were further purified into CD8α^{+} and CD4^{+} subsets, the CD8α^{+} DC was confirmed to be the critical cross-presenting cell in this assay, and a more significant deficit was observed in the capacity of IFNAR1$^{-/-}$ CD8α^{+} DCs to activate OT-I T cells (Figure 25C). These findings reveal a critical function for type I IFN acting on CD8α^{+} DCs during cross-presentation of antigen to CD8^{+} T cells.

To address the role of IFNα/β's actions on DCs in vivo during tumor rejection, we pursued both adoptive transfer and mixed bone marrow chimera approaches. In collaboration with Hirokazu Matsushita in the laboratory, we adoptively transferred CD11c^{+} cells isolated from the spleens of naïve WT or IFNAR1$^{-/-}$ mice into IFNAR1$^{-/-}$ recipients challenged with GAR4.GR1 tumor cells. The IFNAR1-deficient GAR4.GR1 tumor line was used to ensure that potential priming of an anti-IFNAR1 immune response by transfer of WT cells into IFNAR1$^{-/-}$ mice could not contribute to tumor cell killing. Although transfer of WT CD11c^{+} cells but not IFNAR1$^{-/-}$-derived cells could induce a delay in tumor growth and a corresponding increase in tumor-specific CTL priming (data not shown), this effect was variable, perhaps due to the technical difficulty of such a transfer and the unknown half-life and trafficking properties of the transferred cells. In addition, since transferred CD11c^{+} cells contained a mixture of CD8α^{+}, CD4^{+}, and CD8α^{-}CD4^{-} cDC subsets as well as pDCs, future studies utilizing adoptive transfer of

purified CD8α$^+$ cDCs will be required to specifically examine their role during the anti-tumor response.

Mixed bone marrow chimeras with HSCs from CD8α$^+$ DC-deficient p21SNFT$^{-/-}$ mice were also generated in order to investigate the function of type I IFN on this DC subset. Mice with IFNα/β-unresponsive CD8α$^+$ DCs were produced by reconstituting lethally-irradiated IFNAR1$^{-/-}$ recipients with a 4:1 mixture of p21SNFT$^{-/-}$ and RAG2$^{-/-}$ IFNAR1$^{-/-}$ FLCs (p21SNFT$^{-/-}$+RAG2$^{-/-}$IFNAR1$^{-/-}$→IFNAR1$^{-/-}$). Control chimeras with IFNα/β-responsive CD8α$^+$ DCs were similarly generated by reconstituting IFNAR1$^{-/-}$ mice with unequal mixtures of p21SNFT$^{-/-}$ and RAG2$^{-/-}$ FLCs (p21SNFT$^{-/-}$+RAG2$^{-/-}$ →IFNAR1$^{-/-}$). Finally, chimeras lacking CD8α$^+$ DCs were made by reconstituting IFNAR1$^{-/-}$ mice with p21SNFT$^{-/-}$ FLCs alone (p21SNFT$^{-/-}$→IFNAR1$^{-/-}$). The phenotypes of these chimeras were confirmed by FACS analysis of splenocytes, which showed the expected hematopoietic reconstitution of all non-DC lineages (data not shown). Within the DC compartment, the percentage of CD8α$^+$ DCs in p21SNFT$^{-/-}$+RAG2$^{-/-}$IFNAR1$^{-/-}$ →IFNAR1$^{-/-}$ and p21SNFT$^{-/-}$+RAG2$^{-/-}$→IFNAR1$^{-/-}$ mixed chimeras was approximately half of that in unmanipulated control WT and IFNAR1$^{-/-}$ mice (data not shown). Interestingly, analyses of p21SNFT$^{+/-}$ heterozygotes also showed a reduction in CD8α$^+$ DC percentages (151), suggesting a haploinsufficiency of this gene as well as a possible cell-extrinsic effect related to occupation of a required developmental niche.

Despite a reduction in CD8α$^+$ DC percentages, analyses of IFNAR1 expression in splenocytes from p21SNFT$^{-/-}$+RAG2$^{-/-}$IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ mixed chimeras revealed that CD8α$^+$ DCs were IFNAR1-deficient whereas other DC subsets were predominantly

IFNAR1-sufficient (data not shown). Conversely, all DC subsets in p21SNFT$^{-/-}$+RAG2$^{-/-}$ →IFNAR1$^{-/-}$ chimeras were IFNAR1-sufficient. Preliminary tumor transplantation experiments – in which control and chimeric mice were challenged with immunogenic RAG2$^{-/-}$ regressor tumor cells – yielded results that were inconsistent, though suggestive of a role for IFNα/β sensitivity in CD8α^+ DCs. Additional experiments will be required to clarify these data and to further define the in vivo function of type I IFN acting on CD8α^+ DCs during tumor rejection.

Figure 24. Normal percentages of DC subsets in IFNAR1$^{-/-}$ mice. Cells were isolated from the spleens and lymph nodes of WT, IFNAR1$^{-/-}$, and p21SNFT$^{-/-}$ mice by collagenase digestion and DC subsets were analyzed by flow cytometry. (A) Relative number (shown as a percentage of live cells) of CD11c^{hi} conventional DCs (cDC) and CD11c^{int}B220^{+} plasmacytoid DCs (pDC) are shown. Data represent the average ± s.e.m. of spleens from 4 WT, 4 IFNAR1$^{-/-}$ mice, and 2 p21SNFT$^{-/-}$ mice, or values from pooled inguinal LNs. (B) Splenocytes and LN cells were gated on CD11c^{hi} cDCs and analyzed for CD8α, CD4, CD11b, and CD24 expression. FACS plots from a representative sample of WT, IFNAR1$^{-/-}$, and p21SNFT$^{-/-}$ splenocytes, or pooled LN cells are shown. The percent of cells in the indicated gates are noted.

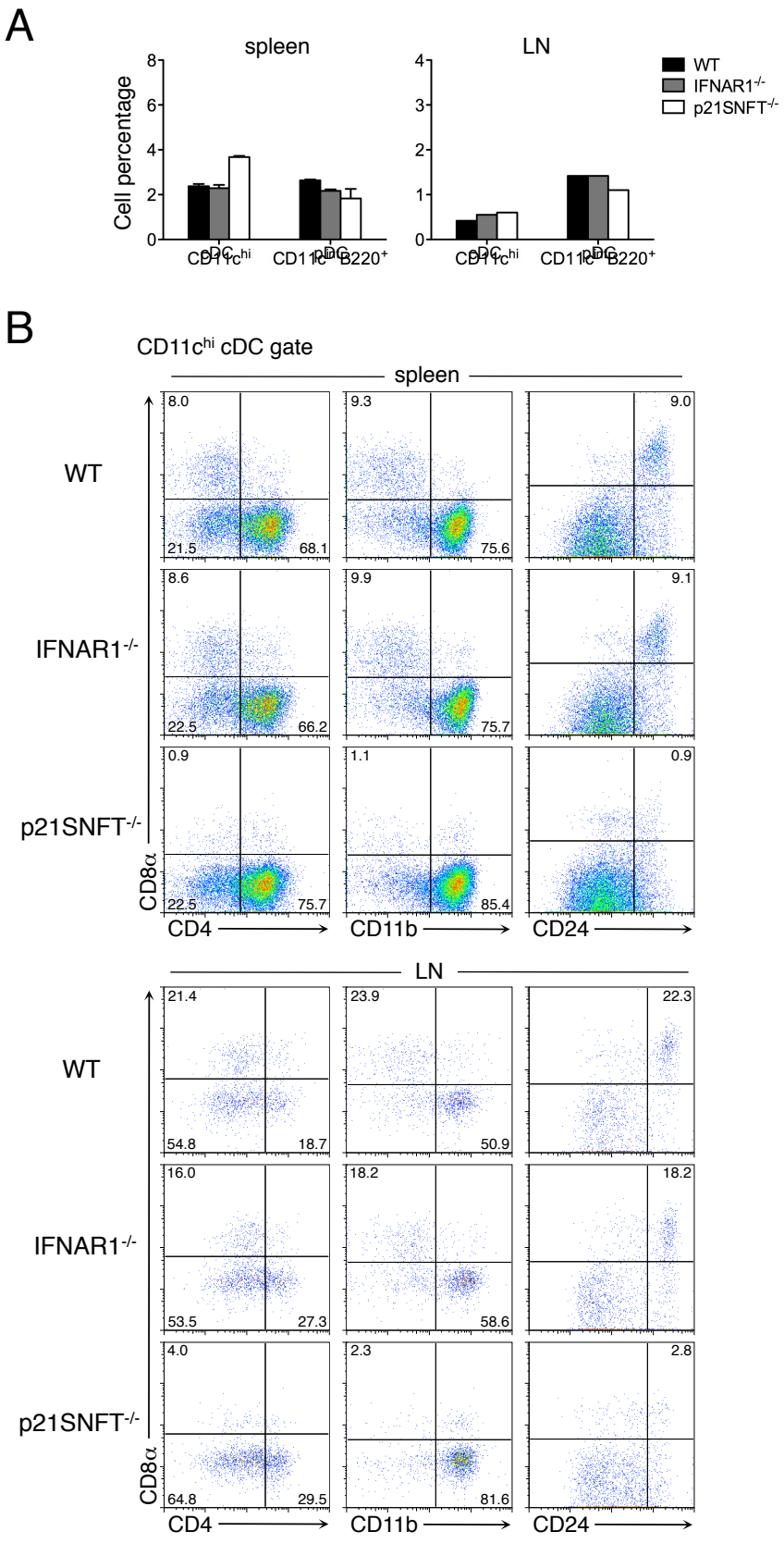
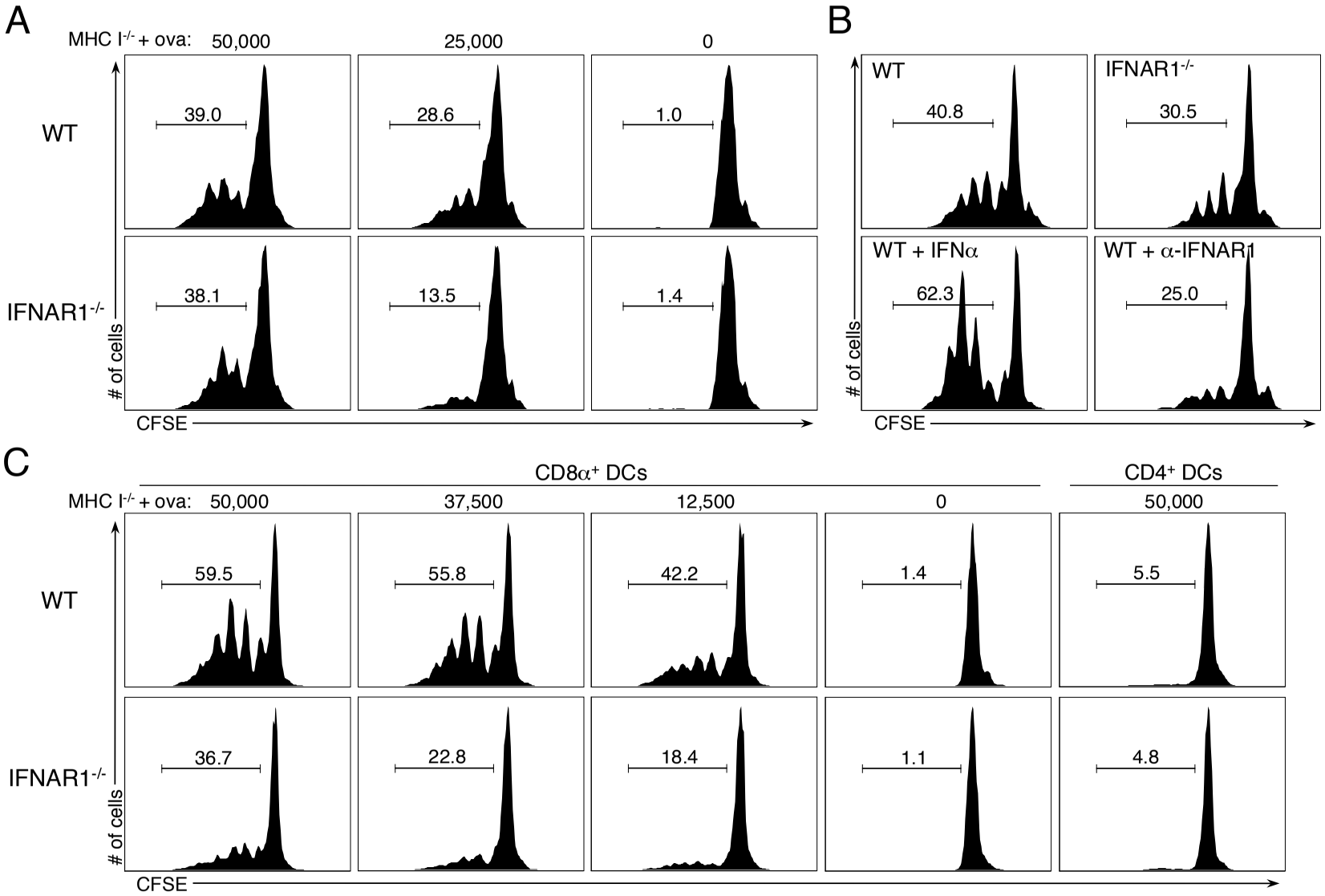

A
spleen
LN
WT
IFNAR1-/-
p21SNFT-/-
Cell percentage
CD11chi cDC
pDC
CD11c- B220+
B
CD11chi cDC gate
spleen
LN
WT
IFNAR1-/-
p21SNFT-/-
CD8α
CD4
CD11b
CD24
8.0
21.5
68.1
9.3
75.6
9.0
8.6
22.5
66.2
9.9
75.7
9.1
0.9
22.5
75.7
1.1
85.4
0.9
21.4
54.8
18.7
23.9
50.9
22.3
16.0
53.5
27.3
18.2
58.6
18.2
4.0
64.8
29.5
2.3
81.6
2.8

Figure 25. IFNα/β sensitivity in $CD8\alpha^+$ DCs enhances cross-presentation of antigen. (A) $CD11c^+$ cells isolated from the spleens of WT or $IFNAR1^{-/-}$ mice were cocultured with the indicated number of irradiated, ovalbumin-loaded MHC class $I^{-/-}$ splenocytes and CFSE-labeled OT-I T cells. After a 3-day incubation, proliferation of OT-I T cells was determined by CSFE dilution. Histograms represent CFSE levels in the $CD8^+$ T cell population, with the percentage of cells in the indicated gate noted. (B) WT and $IFNAR1^{-/-}$ $CD11c^+$ cells or WT $CD11c^+$ cells incubated with exogenous IFNα (1,000 U/ml) or IFNAR1-specific mAb MAR1-5A3 (5 μg/ml) were treated as in (A) at a dose of 25,000 MHC class $I^{-/-}$ splenocytes. (C) Purified $CD8\alpha^+$ and $CD4^+$ DC subsets isolated from WT or $IFNAR1^{-/-}$ mice were treated as in (A) with the indicated number of ovalbumin-loaded MHC class $I^{-/-}$ splenocytes. Data represent one of at least two independent experiments with similar results.


A
MHC I-/- + ova:
50,000
25,000
0
WT
IFNAR1-/-
39.0
28.6
1.0
38.1
13.5
1.4
# of cells
CFSE
B
WT
IFNAR1-/-
40.8
30.5
WT + IFNα
WT + α-IFNAR1
62.3
25.0
# of cells
CFSE
C
CD8α+ DCs
CD4+ DCs
MHC I-/- + ova:
50,000
37,500
12,500
0
50,000
WT
IFNAR1-/-
59.5
55.8
42.2
1.4
5.5
36.7
22.8
18.4
1.1
4.8
# of cells
CFSE

DISCUSSION

In this study, we have examined the host cell requirements for IFNα/β sensitivity during the rejection of immunogenic tumors. We first confirmed prior data using two additional RAG2$^{-/-}$ regressor tumors, demonstrating that IFNα/β's actions on host hematopoietic cells were necessary and sufficient for rejection of H31m1 and d38m2 tumor cells. As type I IFN can exert potent immunomodulatory effects on both innate and adaptive immune cells, mixed bone marrow chimeras with selective IFNα/β responsiveness were generated to assess the relative importance of IFNα/β's actions. These studies demonstrated that innate immune cells were the essential population requiring IFNα/β responsiveness for rejection.

We had previously shown that IFNAR1$^{-/-}$ mice exhibit a defect in the priming of tumor specific CTL (see Chapter 2). Examination of IFNAR1$^{-/-}$ mixed bone marrow chimeras revealed that IFNα/β-responsive innate immune cells were indeed sufficient to restore CTL priming to WT levels. In addition, mAb depletion studies confirmed that T cells were required for tumor rejection in these chimeras. To assess the role of NK cells in IFNα/β-dependent rejection, we utilized B6 strain RAG2$^{-/-}$ regressor tumors and achieved NK depletion via treatment with anti-NK1.1 mAb. Despite complete abrogation of NK cell function in mAb-treated mice, we observed no effect on the rejection of three B6 regressor tumors. Finally, tumor transplantation studies in p21SNFT$^{-/-}$ mice – which have a selective deficiency in the development of CD8α^{+} DCs

– showed that cross-presentation by this DC subset was required for the rejection of immunogenic tumors.

In a recent study, type I IFN was shown to have an important role in promoting NK cell-dependent control of RMA-S tumor growth (43). Antibody blockade experiments also showed that the temporal requirement for IFNα/β's actions corresponded to the requirements for NK function, as IFNα/β blockade or NK depletion at day 3 had no effect on RMA-S rejection. Although type I IFN can strongly augment NK cell cytotoxicity, we do not believe these cells represent an important innate immune target of IFNα/β in our model. Whereas anti-NK1.1 mAb treatment abrogated control of a low-dose RMA-S challenge in our studies, this treatment regimen had no adverse effect on the efficacy or kinetics of B6 regressor rejection. Nevertheless, since NK cells have been shown to be critical for protection against primary MCA tumorigenesis, experiments with mice having NK cell-specific conditional ablation of IFNAR1 will be required to clarify a possible role for type I IFN on NK cells during primary tumorigenesis.

The finding that $CD8\alpha^+$ DCs were required for the rejection of immunogenic tumors supports the notion that cross-presentation is the critical mechanism leading to CTL priming. Although some studies have similarly suggested a requirement for presentation of tumor antigens by bone marrow-derived cells (185), others have argued that direct priming can be a more efficient or merely redundant mechanism (183, 186). To investigate the role of IFNα/β's actions specifically on $CD8\alpha^+$ DCs, we employed an in vitro cross-presentation assay, demonstrating that type I IFN sensitivity within this DC subset promotes antigen cross-presentation and activation of naïve $CD8^+$ T cells. Work is

ongoing to test this potential mechanism during tumor rejection in vivo using adoptive transfer and conditional knockout approaches.

CHAPTER 4

More Extensive Requirement for IFNγ Sensitivity during Tumor Rejection

INTRODUCTION

There is strong experimental support for the existence of a cancer immunosurveillance system, and the IFNs have emerged as fundamentally important components. Some of the first studies inciting renewed interest in the immune-mediated surveillance of cancer focused on the role of IFNγ, demonstrating that tumor cell responsiveness to this cytokine was a significant factor influencing in vivo growth (17, 18). Subsequently, IFNγ unresponsive mice were shown to have an enhanced susceptibility to tumor development in various models of carcinogen-induced, spontaneous, and transplantable tumor formation (3, 11). Work to define the critical functions of IFNγ during this process has established the tumor cell as an important target. When IFNγ responsiveness was restored to various genetically deficient tumor cell lines, these progressively growing tumor cells were now rejected in immunocompetent hosts (5, 16, 18). Moreover, enforced expression of individual components of the antigen processing and presentation pathway in IFNγ-insensitive cells had a similar effect (5). Thus, enhancement of tumor cell immunogenicity represents one relevant downstream pathway induced by IFNγ's actions on the tumor.

Recent studies performed in our laboratory, also highlight a prominent role for host IFNγ sensitivity, since RAG2$^{-/-}$ regressors tumors exhibit progressive growth when injected into IFNGR1$^{-/-}$ mice (G.P. Dunn and R.D. Schreiber, unpublished data). Other groups have also reported that IFNγ's actions on the host were critical, though these studies predominantly used immunization and rechallenge tumor models or adoptive T

cell transfer (22-24). In one such study, it was suggested that nonhematopoietic host cells represented the critical IFNγ targets, and a mechanism involving IFNγ-mediated angiostasis was proposed (23). Since this model was entirely dependent on prior immunization and involved only delayed tumor growth rather than rejection, it will be important to test this hypothesis in models of naturally-occurring immune responses to rejectable tumors in naïve mice.

In this study we provide additional support for the important role of tumor cell IFNγ responsiveness, through experiments involving selective reconstitution of a genetically-deficient cell line as well as RNAi-mediated knockdown of IFNGR1 in a normally responsive regressor tumor. In addition, we examined the relevant targets of IFNγ's actions on the host, using reciprocal bone marrow chimeras to assess the role of hematopoietic versus nonhematopoietic cells. In contrast to our previous findings with IFNα/β, we demonstrate an essential function for IFNγ acting on both hematopoietic and nonhematopoietic host cells.

MATERIALS AND METHODS

Mice. Inbred 129/SvEv and 129/SvPas mice were purchased from Taconic Farms and Charles River Laboratories, respectively. 129 RAG2$^{-/-}$, IFNAR1$^{-/-}$, and IFNGR1$^{-/-}$ mice were bred in our specific pathogen-free animal facility.

Tumor cells. 129 RAG2$^{-/-}$ regressor fibrosarcomas were previously generated by s.c. MCA injection of RAG2$^{-/-}$ mice as described (5). 129 WT progressor tumors 1877 and F244 were induced by MCA treatment of WT mice. The GAR4 tumor cell line is an MCA-induced sarcoma derived from a IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ mouse, and has been reconstituted with either the missing IFNγ receptor component (GAR4.GR1 cells) or the missing IFNα/β receptor component (GAR4.AR1 cells) as previously described (16).

Tumor transplantation experiments. Tumor cells were thawed from frozen stocks and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamate, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol (R-10 medium). After expansion for several passages, cells were harvested by incubation in 0.05% trypsin, washed once with R-10 medium, and washed three times with sterile, endotoxin-free PBS. Prior to the last wash, cells were counted using a hemacytometer and cell viability was assessed by trypan blue exclusion (injected cells were >90% viable). Cells were injected subcutaneously in a volume of 0.15 ml PBS into the shaved flanks of mice. Tumor size was measured on the

indicated days and is presented as the average of two perpendicular diameters. When calculating percent tumor growth, mice with tumors larger than 6x6 mm in diameter at the end of the observation period were counted as positive.

Flow cytometry. Single cell suspensions were isolated, incubated with purified anti-CD16/CD32 mAb (2.4G2) (BD Biosciences) to prevent non-specific antibody binding to Fc receptors, then stained with the indicated antibodies prior to data collection on a BD FACSCalibur (BD Biosciences) and data analysis using FlowJo software (Tree Star). The following were purchased from BioLegend: anti-CD3-FITC (145-2C11), anti-DX5-PE (DX5), anti-B220-FITC (RA3-6B2), anti-CD11b-PE (M1/70), and SA-APC. Anti-CD11c-PE (HL3), anti-IFNGR1-biotin (GR20), anti-H-2D^b-PE (KH95), and anti-H-2K^b (AF6-88.5) were from BD Biosciences, and anti-IFNAR1-biotin (MAR1-5A3) was described previously (64). Immediately prior to analysis, propidium iodide (PI) was added to assess cell viability.

Generation of bone marrow chimeras. E14.5 fetal liver cells were used to reconstitute lethally-irradiated recipients. Embryos were extracted 14 days following implantation, livers were removed, and FLCs were isolated by homogenization through a metal mesh strainer with a 6 cc syringe plunger. Following RBC lysis by incubation in Hybrimax RBC lysing buffer (Sigma-Aldrich), cells were filtered through a 40 μm cell strainer, washed 2 times with sterile endofree PBS, and counted. Cells were resuspended in PBS for injection of 5x10^6 FLCs per mouse in a volume of 0.2 ml using a 0.5 cc 29 gauge

insulin syringe. Recipient mice, lethally irradiated with a single dose of 9.5 Gy several hours prior, were anesthetized by i.p. avertin and FLCs were infused i.v. via retro-orbital injection. Animals were generally maintained on TMS water for 4 weeks following irradiation and reconstitution, and tumor transplantation experiments were performed at least 10-12 weeks post-reconstitution.

Ex vivo analysis of tumors and immune infiltrate. Tumors were excised from euthanized control and bone marrow chimeric mice, physically disaggregated by mincing with razor blades, then enzymatically disaggregated by digestion with 1 mg/ml collagenase IA (Sigma-Aldrich) in 10 ml HBSS medium for 1-1.5 hours in a 37°C water bath with occasional mixing. Cell suspensions were washed once with R-10 medium, RBCs were lysed with Hybrimax RBC lysing buffer (Sigma-Aldrich), and cells were filtered through a 40 μm strainer to remove aggregates and debris. Cells were incubated with purified anti-CD16/CD32 mAb (2.4G2) (BD Biosciences) to prevent non-specific antibody binding to Fc receptors, then stained with FITC-conjugated anti-panCD45 (clone 30-F11) (BioLegend), PE-conjugated anti-CD31 (BioLegend), and biotinylated anti-IFNGR1 (BD Biosciences) followed by SA-APC (BioLegend). Immediately prior to flow cytometry, propidium iodide (PI) was added and cells were filtered again to prevent clogging.

RESULTS

Differential Requirements for IFNγ and IFNα/β Sensitivity during Immune-Mediated Rejection of the GAR4 Tumor

In this set of experiments, we have investigated the requirements for IFN sensitivity in both tumor cells and host cells using the MCA-induced sarcoma GAR4, which was derived from an IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ doubly deficient mouse. Although GAR developed in an immunodeficient environment (thus being unedited), this tumor grows progressively when transplanted into immunocompetent WT mice. As previous data had demonstrated the importance of IFNγ's actions on tumor cells for tumor rejection (17, 18), Allen Bruce in the laboratory decided to selectively restore IFNγ or IFNα/β responsiveness to GAR4 through retroviral transduction of the missing IFNGR1 or IFNAR1 receptor component. We screened clones of each type and isolated representative clones which displayed normal levels of the appropriate IFN receptor as well as normal biologic responsiveness to cytokine treatment (MHC class I upregulation and LPS+IFN-induced NO production) (Figures 1-2, (16) and data not shown).

To test the effect of selective reconstitution of IFN sensitivity on the in vivo growth of GAR4, we injected groups of WT and RAG2$^{-/-}$ mice with GAR4, GAR4.AR1, or GAR4.GR1 tumor cells (Figure 3). All three of these tumor cell lines grew progressively with similar kinetics in RAG2$^{-/-}$ hosts. Yet, whereas GAR4 and GAR4.AR1 also grew in WT mice, GAR4.GR1 tumor cells were rejected – indicating

that IFNγ sensitivity, but not IFNα/β sensitivity, was essential for GAR4 rejection by a functional immune system. We also injected GAR4.GR1 tumor cells, as well as GAR4 and GAR4.AR1 as controls, into IFNGR1$^{-/-}$ and IFNAR1$^{-/-}$ hosts to assess the function of host cell IFN sensitivity for rejection of reconstituted GAR4 (Figure 3). Although rejected in WT mice, GAR4.GR1 now grew progressively (similar to GAR4 and GAR4.AR1) in the absence of host cell responsiveness to IFNα/β or IFNγ. Taken together, these studies highlight the essential function of IFNγ on the tumor cell but both IFNγ and IFNα/β on host cells during the rejection of immunogenic tumors.

In the course of the above experiments, we noted that the growth of GAR4.GR1 was often more aggressive in IFNAR1$^{-/-}$ hosts as compared to IFNGR1$^{-/-}$ mice. To examine the relative dependency on host IFNα/β versus IFNγ sensitivity, we performed dose titration experiments in which groups of IFN receptor deficient or control mice were injected with decreasing doses of GAR4.GR1 tumor cells ranging from $3x10^6$ to $0.1x10^6$ cells/mouse (Figure 4). At the highest tumor dose, GAR4.GR1 cells grew with similar kinetics in both IFNAR1$^{-/-}$ and IFNGR1$^{-/-}$ mice (and also grew in WT mice, though with slower kinetics). At intermediate doses of tumor, however, slightly increased growth kinetics were observed in IFNAR1$^{-/-}$ compared to IFNGR1$^{-/-}$ mice, including growth in only 3/4 IFNGR1$^{-/-}$ mice at the $0.5x10^6$ cell dose but 4/4 IFNAR1$^{-/-}$ mice at the same dose. This difference was even more pronounced upon injection of $0.1x10^6$ cells/mouse, in which case the tumor grew in 0/3 IFNGR1$^{-/-}$ mice but 4/4 IFNAR1$^{-/-}$ mice.

To investigate the possibility that an immune response against the transduced IFNGR1 receptor component (foreign to IFNGR1$^{-/-}$ mice) might explain this differential

phenotype, we tested the growth of GAR4 cells transduced with a dominant-negative IFNGR1 receptor component lacking the cytoplasmic signaling domain (IFNGR1.ΔIC) in WT and IFNGR1$^{-/-}$ mice. As shown in Figure 5A, GAR4.GR1ΔIC cells contained highly overexpressed levels of IFNGR1 (at least a log above WT levels), yet still grew similarly in both WT and IFNGR1$^{-/-}$ mice (Figure 5B). This result not only confirms the importance of IFNγ's actions on GAR4 cells for their rejection in WT mice (since GAR4.GR1ΔIC but not GAR4.GR1 grows progressively), but also indicates the lack of an alloresponse against the IFNGR1 protein in IFNGR1$^{-/-}$ mice. These data therefore demonstrate that for rejection of the GAR4.GR1 tumor, host cell sensitivity to IFNα/β is more critical than host responsiveness to IFNγ. Similar to findings that the rejection of certain regressor tumors require IFNα/β but not IFNγ (or vice versa), this observation adds to the notion that endogenous type I and type II IFN are performing distinct functions on the host during the anti-tumor immune response.

Figure 1. Selective reconstitution of IFNγ or IFNα/β sensitivity in the GAR4 tumor. Parental GAR4 cells, IFNγ receptor-reconstituted GAR4.GR1, and IFNα/β receptor-reconstituted GAR4.AR1 cells were analyzed by FACS for expression of IFNGR1, IFNAR1, and MHC class I molecule H-2D^b as indicated. Isotype control Ab staining is shown in the thin black lines. For IFNγ and IFNα treatment, cells were cultured for 48 hrs with 1000 U/ml of the indicated cytokine prior to H-2D^b staining. A representative clone of GAR4.GR1 and GAR4.AR1 cells is shown.

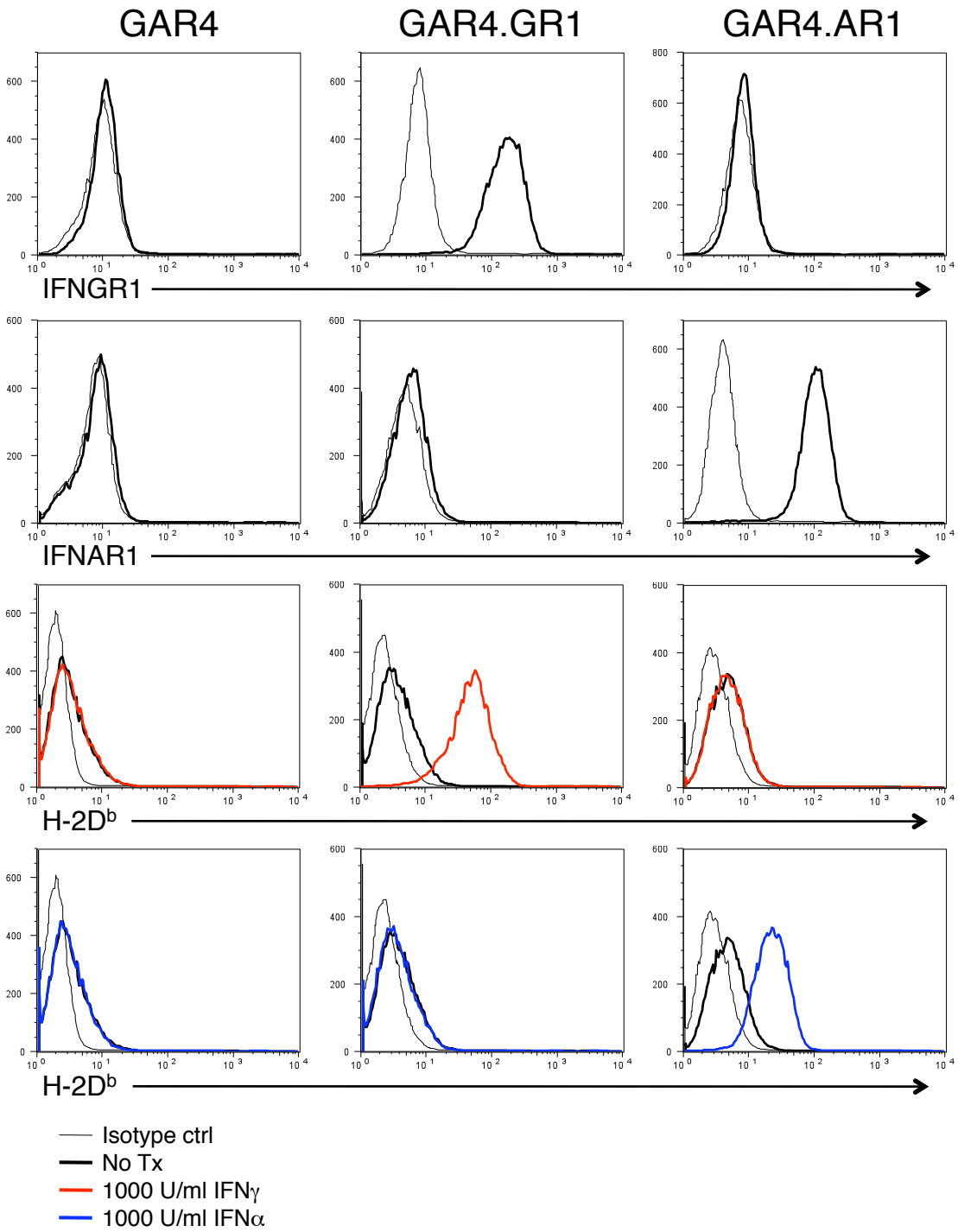

GAR4
GAR4.GR1
GAR4.AR1
IFNGR1
IFNAR1
H-2D^b
H-2D^b
Isotype ctrl
No Tx
1000 U/ml IFNγ
1000 U/ml IFNα

Figure 2. Appropriate cytokine responsiveness of reconstituted GAR4 clones. GAR4, GAR4.GR1, and GAR4.AR1 cells were treated with LPS plus either IFNγ or IFNα at the indicated doses for 48 hours and levels of NO production were assessed. One representative experiment of several with similar results is shown.

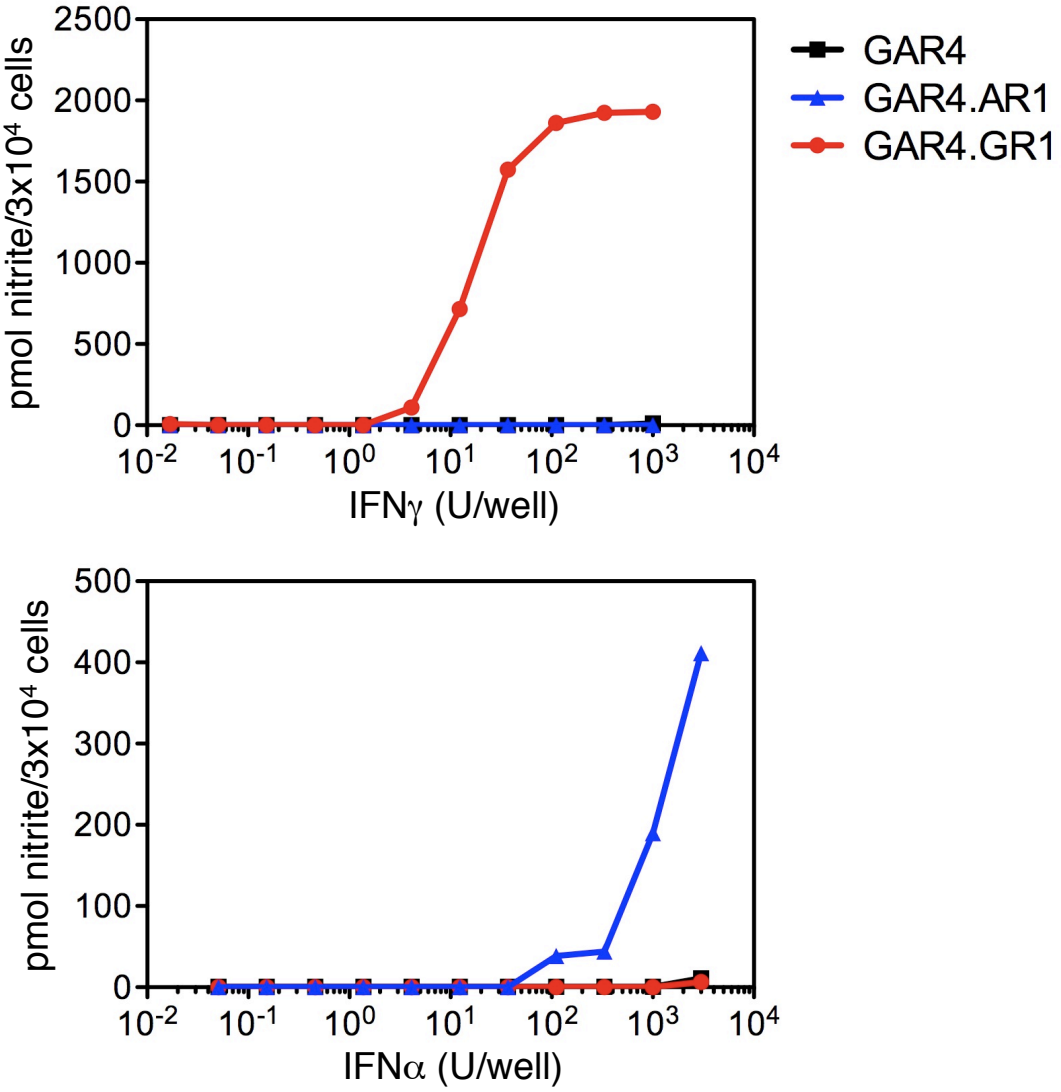

2500
2000
1500
1000
500
0
pmol nitrite/3x10^4 cells
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
IFNγ (U/well)
GAR4
GAR4.AR1
GAR4.GR1
500
400
300
200
100
0
pmol nitrite/3x10^4 cells
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
IFNα (U/well)

Figure 3. Tumor cell sensitivity to IFNγ but not IFNα/β is critical for GAR4 rejection, whereas host cell responsiveness to both IFNγ and IFNα/β is required. $1x10^6$ GAR4, GAR4.GR1, or GAR4.AR1 tumor cells were injected s.c. into groups of WT, $RAG2^{-/-}$, $IFNGR1^{-/-}$ and $IFNAR1^{-/-}$ mice and tumor growth was measured over time. Data from at least two independent experiments with n=5-11 mice/group is shown.

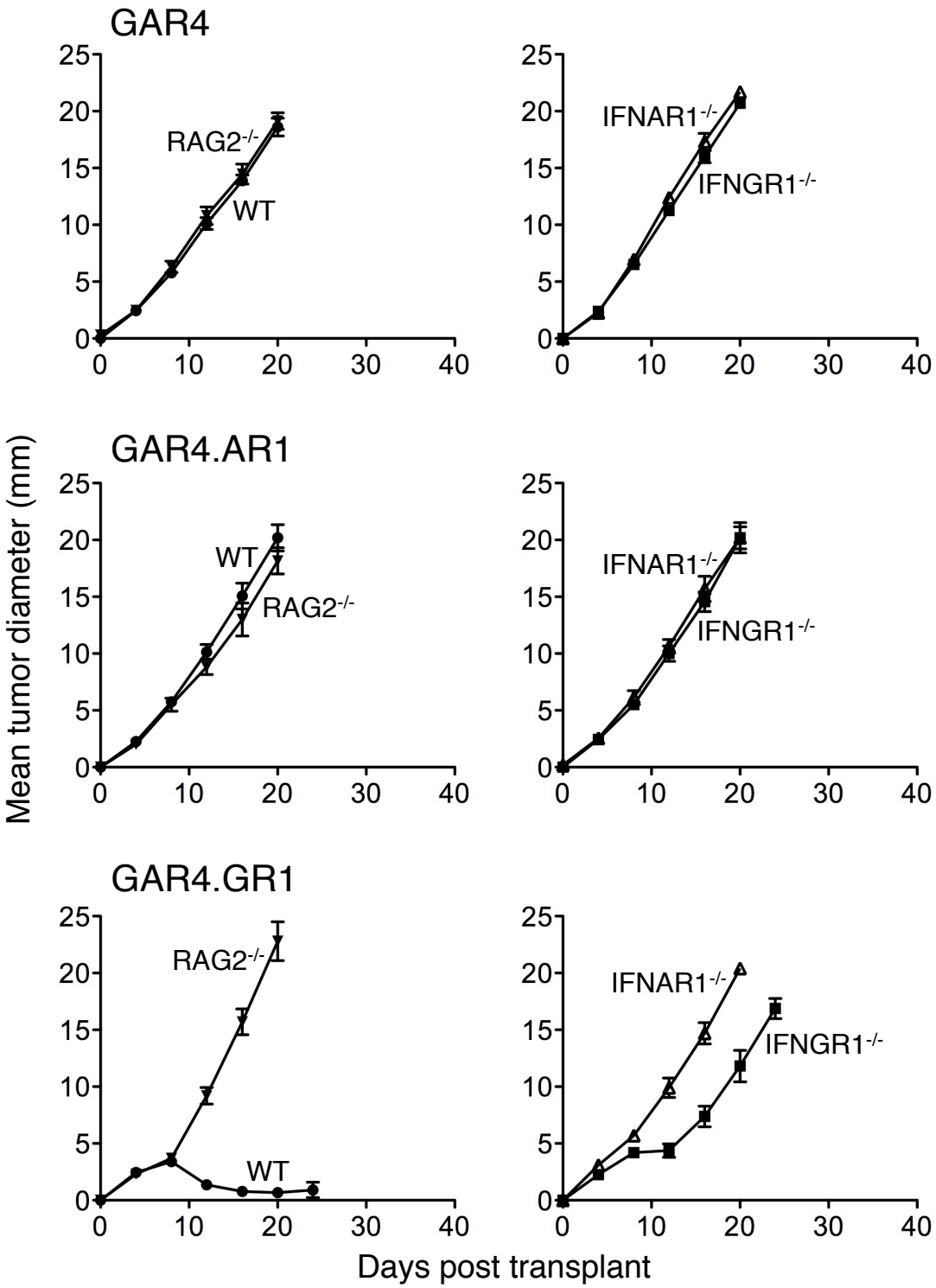

GAR4
RAG2-/-
WT
IFNAR1-/-
IFNGR1-/-
GAR4.AR1
WT
RAG2-/-
IFNAR1-/-
IFNGR1-/-
GAR4.GR1
RAG2-/-
WT
IFNAR1-/-
IFNGR1-/-
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40

Figure 4. Differential importance of host cell sensitivity to IFNγ and IFNα/β for rejection of reconstituted GAR4.GR1 tumor cells. A dose titration of GAR4.GR1 tumor cells was performed in which groups of WT, $RAG2^{-/-}$, $IFNGR1^{-/-}$, and $IFNAR1^{-/-}$ mice were injected with $3x10^6$, $1x10^6$, $0.5x10^6$, or $0.1x10^6$ GAR4.GR1 cells. Data are presented as mean tumor diameter ± s.e.m. of n=3-4 mice/group.

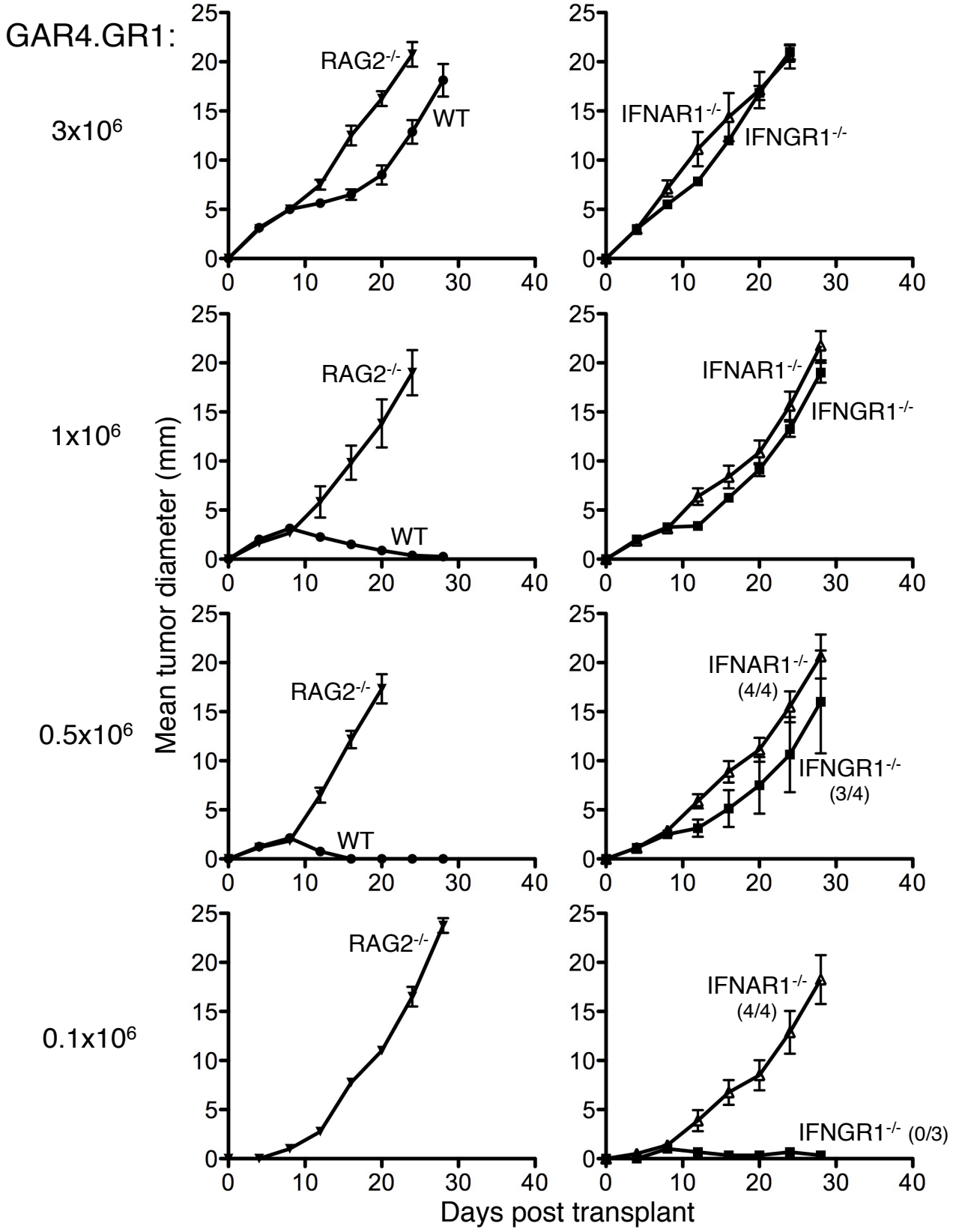
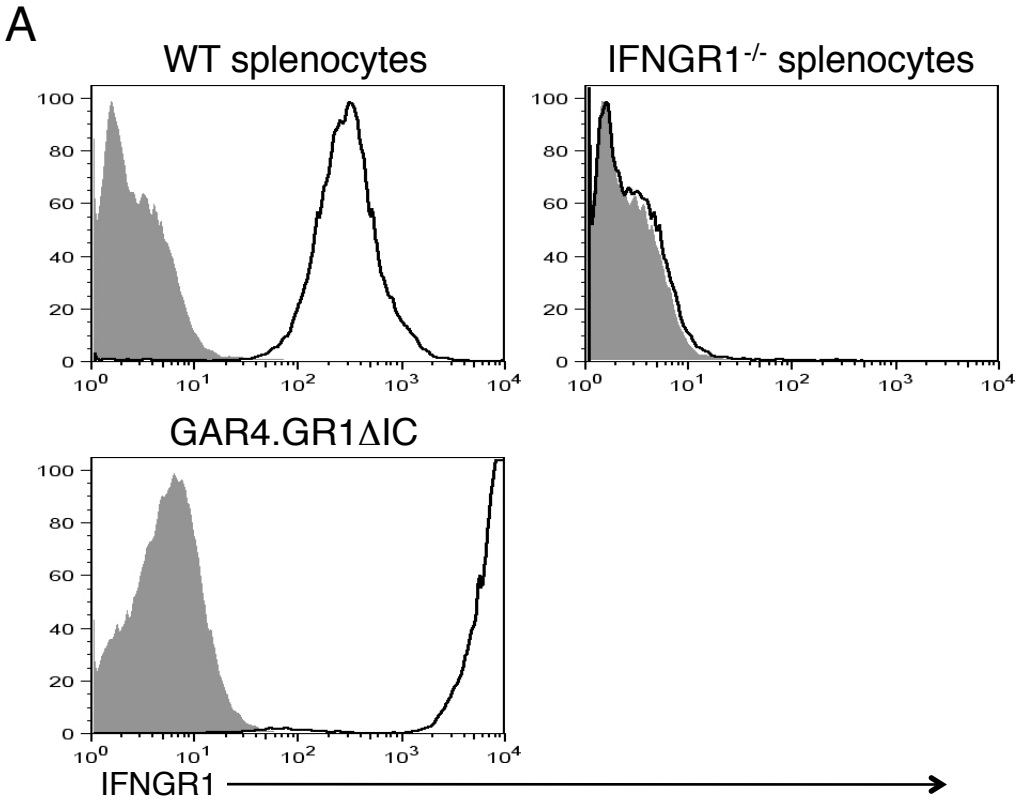

GAR4.GR1:
3x10^6
1x10^6
0.5x10^6
0.1x10^6
RAG2-/-
WT
IFNAR1-/-
IFNGR1-/-
(4/4)
(3/4)
(0/3)
Mean tumor diameter (mm)
Days post transplant

Figure 5. An alloresponse against IFNGR1 is not responsible for less robust growth of reconstituted GAR4.GR1 tumor cells in IFNGR1$^{-/-}$ mice. (A) IFNGR1 levels by FACS are shown for GAR4.GR1ΔIC tumor cells, which overexpress the nonfunctional IFNGR1ΔIC receptor chain, or splenocytes from WT and IFNGR1$^{-/-}$ mice as controls. (B) 1x10^{6} GAR4.GR1ΔIC tumor cells were injected into groups of WT and IFNGR1$^{-/-}$ mice (n=5 mice/group) and tumor growth was monitored over time.


A
WT splenocytes
IFNGR1-/- splenocytes
GAR4.GR1ΔIC
IFNGR1
B
GAR4.GR1ΔIC
IFNGR1-/-
WT
Mean tumor diameter (mm)
Days post transplant

RNAi-Mediated Knockdown of IFNGR1 in a RAG2$^{-/-}$ Regressor Sarcoma Abrogates Tumor Rejection

Prior work in the laboratory evaluated the importance of tumor cell IFNγ sensitivity for the rejection of RAG2$^{-/-}$ regressor sarcomas by overexpression of a dominant-negative IFNGR1.ΔIC construct, rendering these tumors IFNγ-unresponsive (G.P. Dunn, C.M. Koebel, and R.D. Schreiber, unpublished data). These studies showed progressive growth of 4/5 IFNγ-dependent 129 regressor tumors (H31m1, d42m1, d38m2, and F510) after paralysis of IFNγ signaling. Yet, the possibility remained that profound overexpression of IFNGR1.ΔIC (which can presumably still bind ligand) on the tumor cells could be binding and sequestering IFNγ within the tumor microenvironment, effectively inhibiting its actions on infiltrating host cells as well. In order to address this potential caveat, we took a second approach to abrogate IFNγ signaling within tumor cells – knockdown of IFNGR1 expression by RNAi.

We transduced H31m1 cells with a retroviral vector driving expression of IFNGR1-specific shRNA molecules, and following several rounds of transduction and FACS sorting, we isolated bulk cell lines with significantly reduced IFNGR1 expression. Sequential transduction and sorting were necessary as the retroviral construct contained no selectable marker, given concerns that any foreign protein might serve as a rejection antigen in vivo. Nevertheless, empty vector transduced cells treated similarly and sorted for the lowest IFNGR1 expressors yielded no reduction in receptor levels, signifying that IFNGR1 knockdown was mediated by the introduced shRNA and not due to naturally

arising variants present in the original population. After single cell cloning of the respective bulk lines, we screened clones for IFNGR1 expression levels and biologic responsiveness to IFNγ treatment by MHC class I upregulation. Shown in Figure 6 are representative empty vector and IFNGR1 shRNA transduced clones as well as the parental H31m1 tumor line. A number of clones were isolated expressing 10-15% of parental levels of surface IFNGR1 by FACS. These cells, however, showed no change in surface expression of several control genes, including IFNAR1, H-2K^{b}, H-2D^{b}, and CD1d (Figure 6 and data not shown). When treated with high doses of IFNγ in vitro, IFNGR1 shRNA transduced cells displayed greatly reduced, but not completely absent, biologic responses to IFNγ – as a minority of cells could upregulate MHC class I, though still not to levels seen in parental H31m1 cells. Further analysis has determined that IFNGR1 knockdown was stable for extended periods of time after both in vitro and in vivo passage (data not shown).

To assess the effect of IFNGR1 knockdown on in vivo growth of H31m1 cells, we injected several clones of each type into groups of WT and RAG2$^{-/-}$ mice. As shown for representative clones in Figure 7, both H31m1 parental and empty vector transduced cells were rejected in WT mice but grew progressively in RAG2$^{-/-}$ mice. In contrast, IFNGR1 shRNA transduced cells grew progressively in both RAG2$^{-/-}$ and WT hosts. These data therefore corroborate prior findings and confirm the importance of tumor cell IFNγ responsiveness for RAG2$^{-/-}$ regressor rejection using an additional experimental approach. These studies also provide proof of principle suggesting the utility of this

strategy for RNAi knockdown of other molecules in order to assess their contribution to tumor cell immunogenicity in vivo.

Figure 6. RNAi-induced knockdown of IFNGR1 expression and inhibition of IFNγ responsiveness in H31m1 tumor cells. Parental, empty vector-transduced, and IFNGR1 shRNA-transduced H31m1 tumor cells were analyzed by FACS for expression of IFNGR1, IFNAR1, and H-2K^{b}. IFN-induced upregulation of MHC class I was assessed following 3 day treatment with IFNγ (10 and 1000 U/ml; in red) or IFNα (3000 U/ml; in blue).

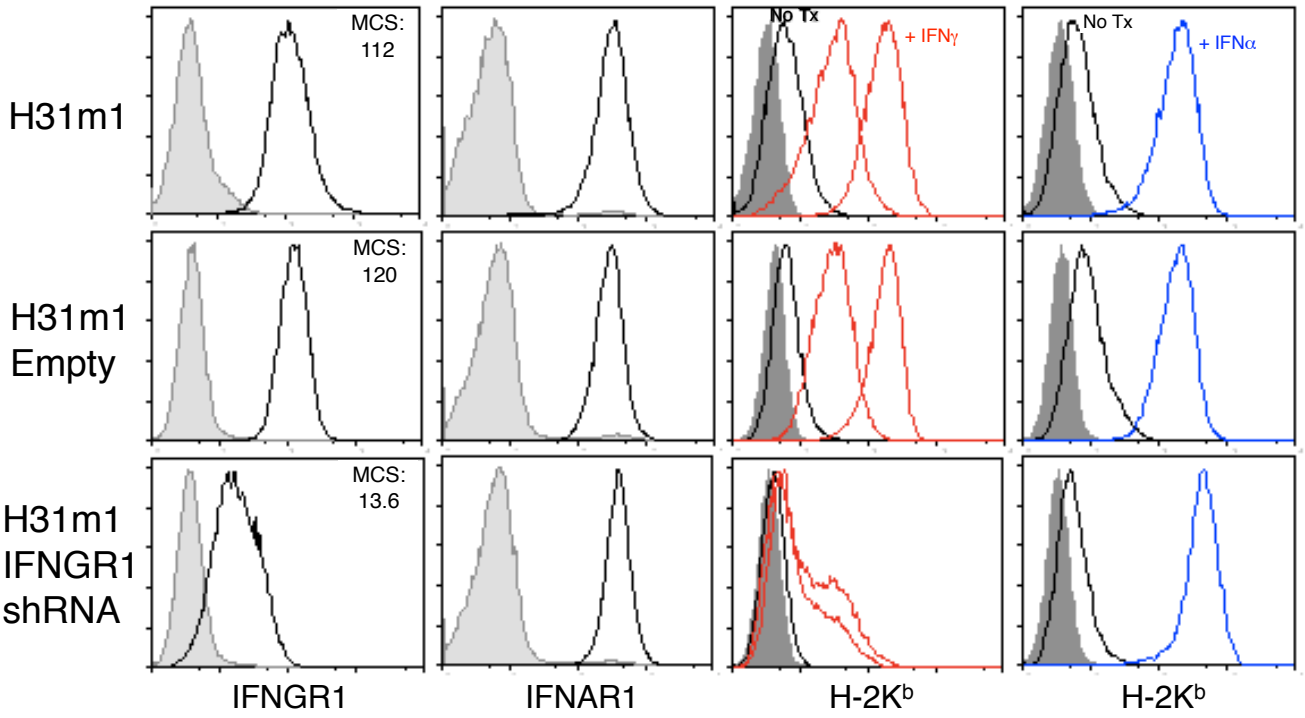
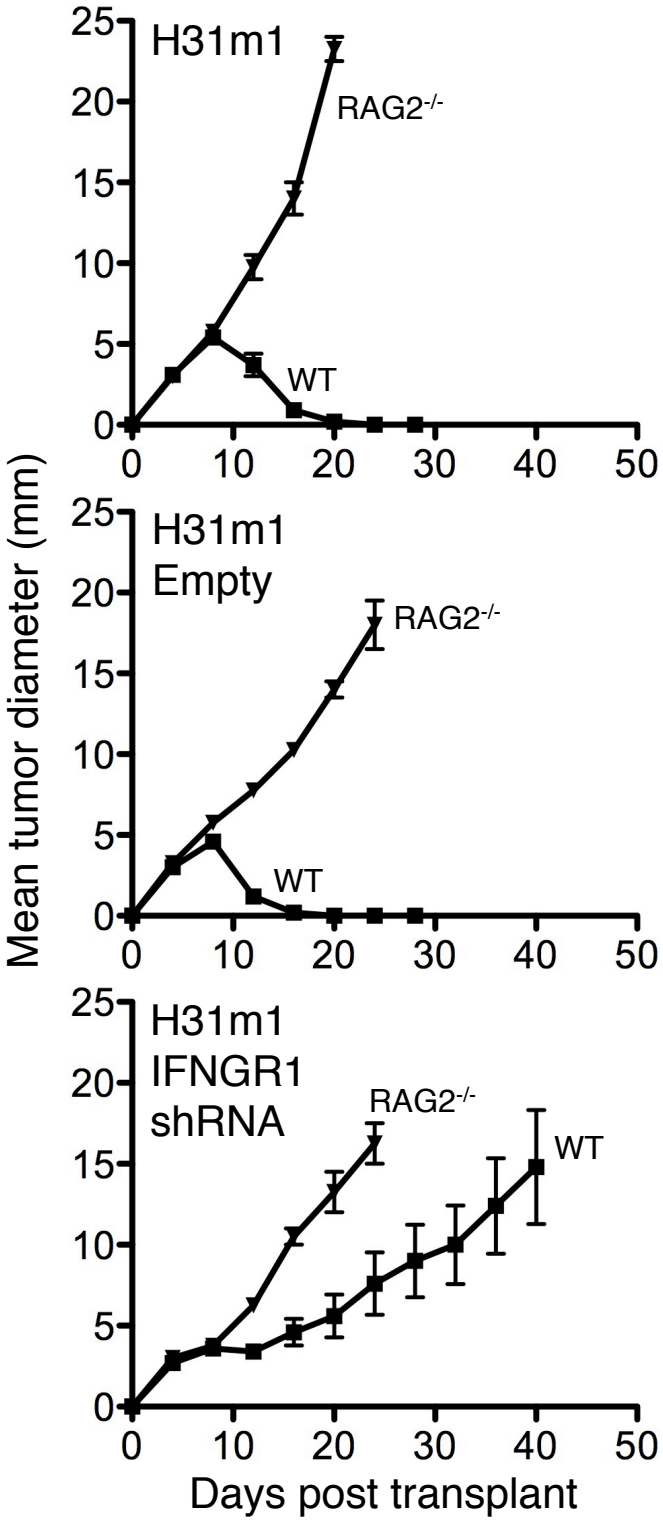

H31m1
H31m1
Empty
H31m1
IFNGR1
shRNA
MCS:
112
MCS:
120
MCS:
13.6
No Tx
+ IFNγ
No Tx
+ IFNα
IFNGR1
IFNAR1
H-2Kb
H-2Kb

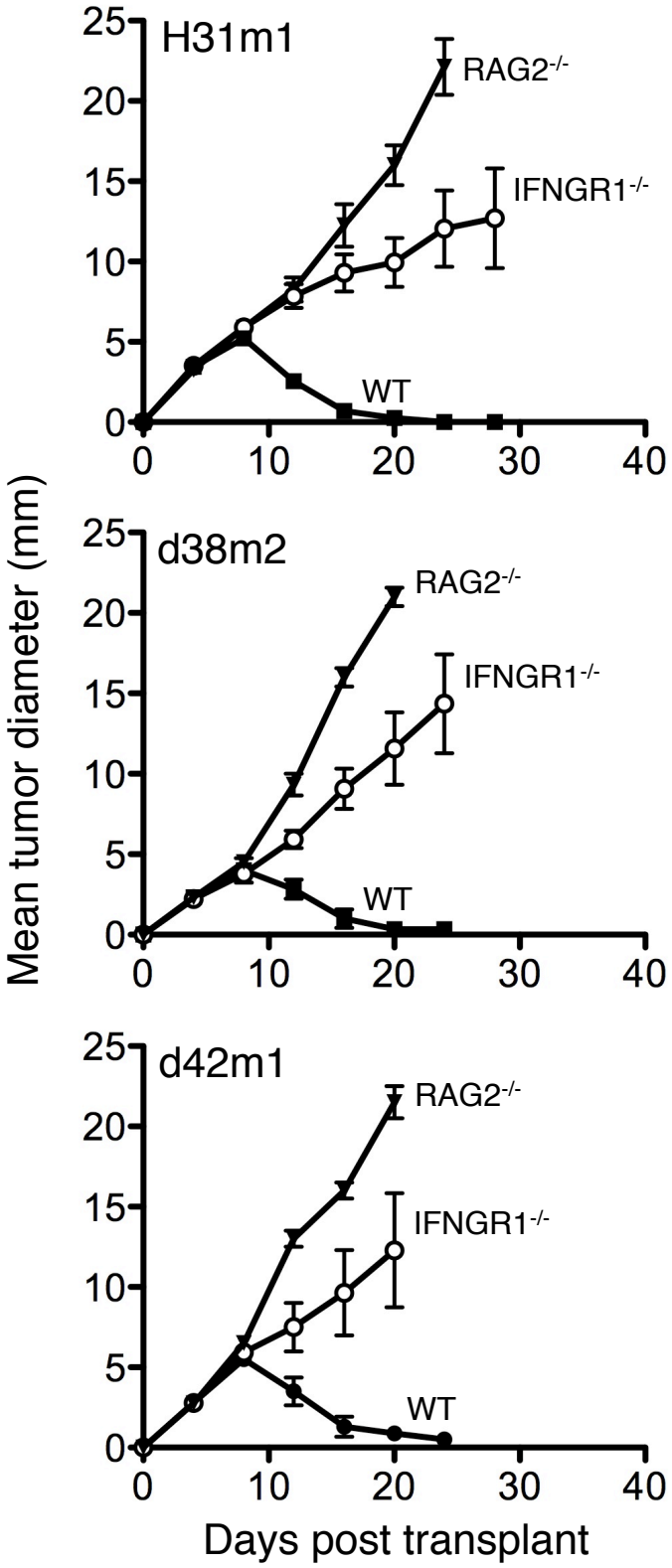
Figure 7. IFNGR1 knockdown in H31m1 tumor cells prevents their rejection. WT and RAG2$^{-/-}$ mice were injected with 1x10^{6} parental, empty vector-transduced, or IFNGR1 shRNA-transduced H31m1 tumors cells. Growth was measured over time and is presented as mean tumor diameter ± s.e.m. of n=5 (WT) or n=2 (RAG2$^{-/-}$) mice for each tumor.


H31m1
RAG2-/-
WT
H31m1
Empty
RAG2-/-
WT
H31m1
IFNGR1
shRNA
RAG2-/-
WT
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40
50

IFNγ Responsiveness in Host Cells is also Required for Tumor Rejection

In addition to the well-documented function for IFNγ signaling directly on the tumor cell, recent data in the laboratory has also revealed a role for IFNγ sensitivity of host cells for the rejection of immunogenic RAG2$^{-/-}$ regressor sarcomas (G.P. Dunn, and R.D. Schreiber, unpublished data). We confirmed these results by injecting groups of WT, RAG2$^{-/-}$, and IFNGR1$^{-/-}$ mice with several 129 regressor tumors (H31m1, d38m2, and d42m1) as shown in Figure 8. These tumors were rejected in WT mice, but grew progressively in RAG2$^{-/-}$ controls and in the majority of IFNGR1$^{-/-}$ mice injected. We have also shown that IFNGR1$^{-/-}$ animals, similar to IFNAR1$^{-/-}$ mice, displayed a defect in the generation of tumor-specific T cells (see Chapter 2, Figure 6). These 129 regressor tumors – requiring IFNγ's actions on host cells – were utilized in subsequent studies to investigate the functionally important host cell targets of IFNγ during the anti-tumor immune response.

Figure 8. Requirement for host sensitivity to IFNγ for rejection of immunogenic RAG2$^{-/-}$ regressor tumors. Groups of WT, IFNGR1$^{-/-}$, and RAG2$^{-/-}$ mice were injected with 1x10^{6} H31m1, d38m2, or d42m1 tumor cells and growth was measured over time.


H31m1
RAG2-/-
IFNGR1-/-
WT
d38m2
RAG2-/-
IFNGR1-/-
WT
d42m1
RAG2-/-
IFNGR1-/-
WT
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40

Host Sensitivity to IFNγ in Both Hematopoietic and Nonhematopoietic Cells is Critical for Tumor Rejection

Given the importance of IFNγ responsiveness at the level of the host for tumor rejection, we used bone marrow chimeras to localize the functionally relevant effects of IFNγ to either hematopoietic or nonhematopoietic cells. We employed a similar approach and protocol as used previously (see Chapter 3), in order to generate chimeric mice with selective IFNγ sensitivity only in hematopoietic cells (WT→IFNGR1$^{-/-}$), nonhematopoietic cells (IFNGR1$^{-/-}$→RAG2$^{-/-}$), or as controls, in both compartments (WT→WT) or neither compartment (IFNGR1$^{-/-}$→IFNGR1$^{-/-}$) (Figure 9A). The expected phenotypes of these mice were confirmed via IFNGR1 staining of splenocytes (Figure 9B). In addition, normal hematopoietic reconstitution was verified by examining the cell density and immune composition of the spleen (Figure 10).

Groups of control mice and bone marrow chimeras were then challenged with the IFNγ-dependent RAG2$^{-/-}$ regressor sarcomas d38m2 and H31m1, and tumor growth was monitored over time. As depicted in Figure 11, d38m2 tumor cells showed progressive growth in IFNGR1$^{-/-}$ and RAG2$^{-/-}$ control mice but were rejected in the presence of a wild type immune compartment. Similarly, d38m2 cells were rejected in WT→WT bone marrow chimeras, yet grew in 73% (24/33) of IFNGR1$^{-/-}$→IFNGR1$^{-/-}$ mice. When mice could only respond to IFNγ in hematopoietic cells (WT→IFNGR1$^{-/-}$ chimeras), we observed progressive growth in 42% (14/33) of mice; whereas selective IFNγ responsiveness in only nonhematopoietic cells (IFNGR1$^{-/-}$→RAG2$^{-/-}$ chimeras) yielded

growth in 50% (16/32) of mice. These findings reveal a requirement for both hematopoietic and nonhematopoietic IFNγ sensitivity for rejection of d38m2 tumor cells. Yet, given the partial phenotypes in WT→IFNGR1$^{-/-}$ and IFNGR1$^{-/-}$→RAG2$^{-/-}$ chimeras compared to globally unresponsive IFNGR1$^{-/-}$→IFNGR1$^{-/-}$ mice, sensitivity in either compartment alone appears to afford some tumor protection.

When cohorts of mice were instead challenged with H31m1 tumor cells, we again noted a prominent requirement for nonhematopoietic IFNγ responsiveness, with growth in 44% (7/16) of WT→IFNGR1$^{-/-}$ chimeras compared to 8% (1/12) of WT→WT and 69% (11/16) of IFNGR1$^{-/-}$→IFNGR1$^{-/-}$ controls (Figure 12). Similar to findings with d38m2, the defect in the absence of nonhematopoietic IFNγ sensitivity appeared partial compared to globally unresponsive chimeras. Although H31m1 cells did not grow in IFNGR1$^{-/-}$→RAG2$^{-/-}$ chimeras, IFNγ's actions on hematopoietic cells still seemed to be important as tumor rejection in these mice was considerably delayed compared to WT→WT controls. Taken together, data with both the d38m2 and H31m1 RAG2$^{-/-}$ regressor sarcomas support a role for IFNγ acting on both hematopoietic and nonhematopoietic cells of the host. We have therefore demonstrated with two different immunogenic tumors that the requirements for host cell sensitivity to IFNγ and IFNα/β are, in fact, distinct. While hematopoietic cells – and specifically, the innate immune compartment – are the essential targets of IFNα/β (see Chapter 3), sensitivity to IFNγ within both hematopoietic and nonhematopoietic cells is critical.

Figure 9. Generation and validation of bone marrow chimeras with hematopoietic or nonhematopoietic IFNγ sensitivity. (A) Strategy for producing chimeras with differential host cell IFNγ responsiveness. (B) Splenocytes from a representative cohort of chimeric and control mice were analyzed for IFNGR1 expression by FACS. Shown are the percent IFNGR1^{+} cells within the indicated gate for n=7-12 chimeras or n=4-5 controls.

A

Group	Donor HSCs	Irradiated Recipient Mice	IFNγ Sensitivity	
			Hematopoietic	Nonhematopoietic
Control	WT	WT	+	+
Control	IFNGR1$^{-/-}$	IFNGR1$^{-/-}$	-	-
Experimental	IFNGR1$^{-/-}$	RAG2$^{-/-}$	-	+
Experimental	WT	IFNGR1$^{-/-}$	+	-

B

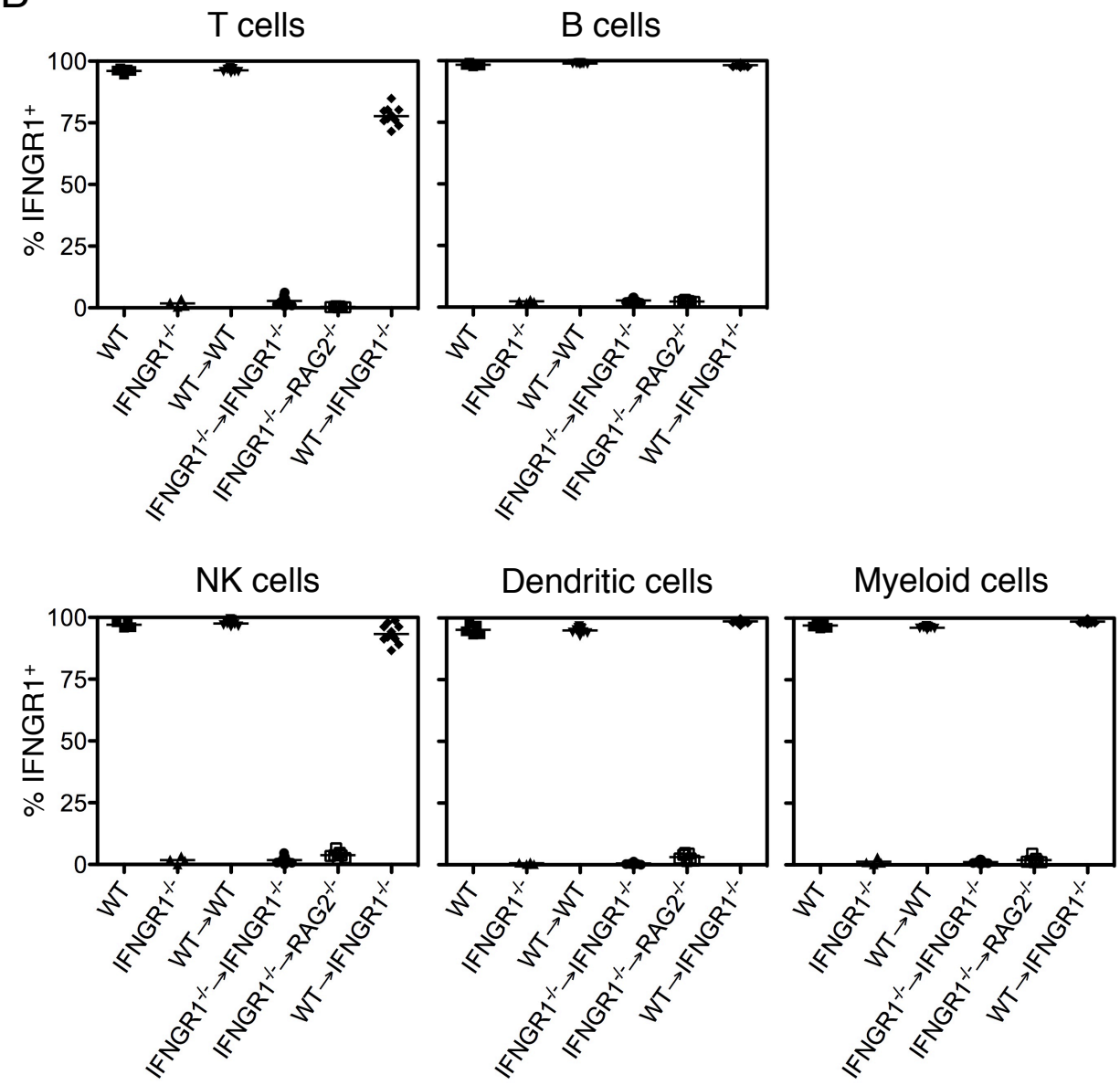


Figure 10. Reconstitution of the hematopoietic compartment in IFNGR1$^{-/-}$ bone marrow chimeric mice. Splenocytes were harvested from representative cohorts of control and IFNGR1$^{-/-}$ chimeric mice and analyzed for cellularity and immune composition. (A) Cell density of the spleen (calculated as total cell number/wt of tissue) is shown for 3 control and chimeric mice of each type. (B) Cellular percentages of the indicated immune cell subsets were determined by flow cytometry for control and chimeric mice of each type. Mean values (expressed as a percentage of total splenocytes) ± s.e.m. for 3 mice/group are shown, and cell populations were defined as follows (after gating for PI^{-} live cells): CD4^{+} T cells (CD3^{+}CD4^{+}), CD8^{+} T cells (CD3^{+}CD8^{+}), B cells (B220^{+}), NK cells (DX5^{+}CD3^{-}), dendritic cells (CD11c^{hi}), and myeloid cells (CD11b^{+}).

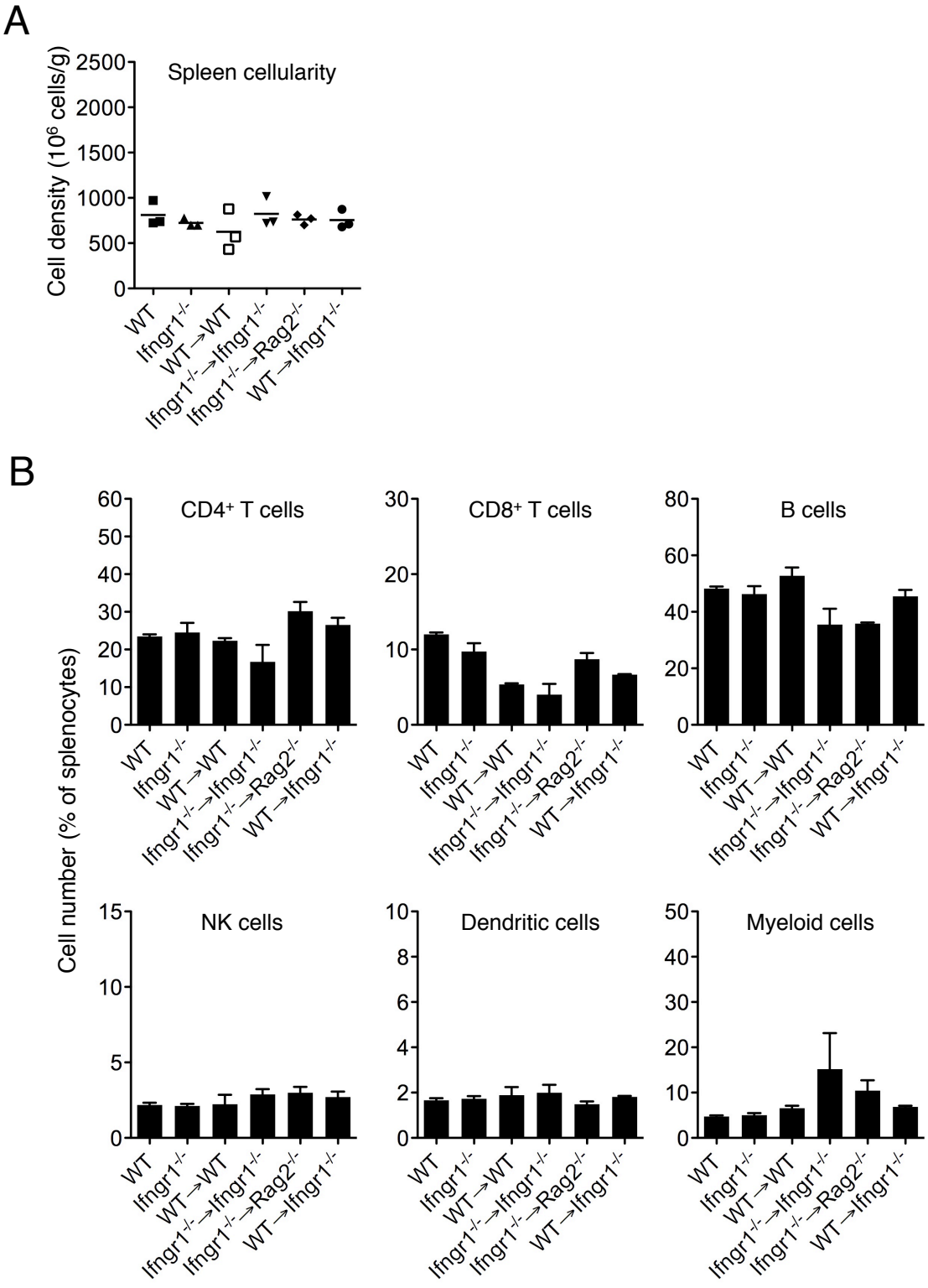

A
Spleen cellularity
Cell density (10^6 cells/g)
2500
2000
1500
1000
500
0
WT
Ifngr1-/-
WT→WT
Ifngr1-/-→Ifngr1-/-
Ifngr1-/-→Rag2-/-
WT→Ifngr1-/-
B
CD4+ T cells
CD8+ T cells
B cells
NK cells
Dendritic cells
Myeloid cells
Cell number (% of splenocytes)

Figure 11. Both hematopoietic and nonhematopoietic IFNγ responsiveness is critical for rejection of d38m2 tumor cells. (A) Groups of control and chimeric mice were injected s.c. with $1x10^6$ d38m2 $RAG2^{-/-}$ regressor cells and tumor size was measured over time. Percent of mice exhibiting progressive tumor growth is plotted in (B). Data represent cumulative results from five independent experiments with the indicated group sizes.

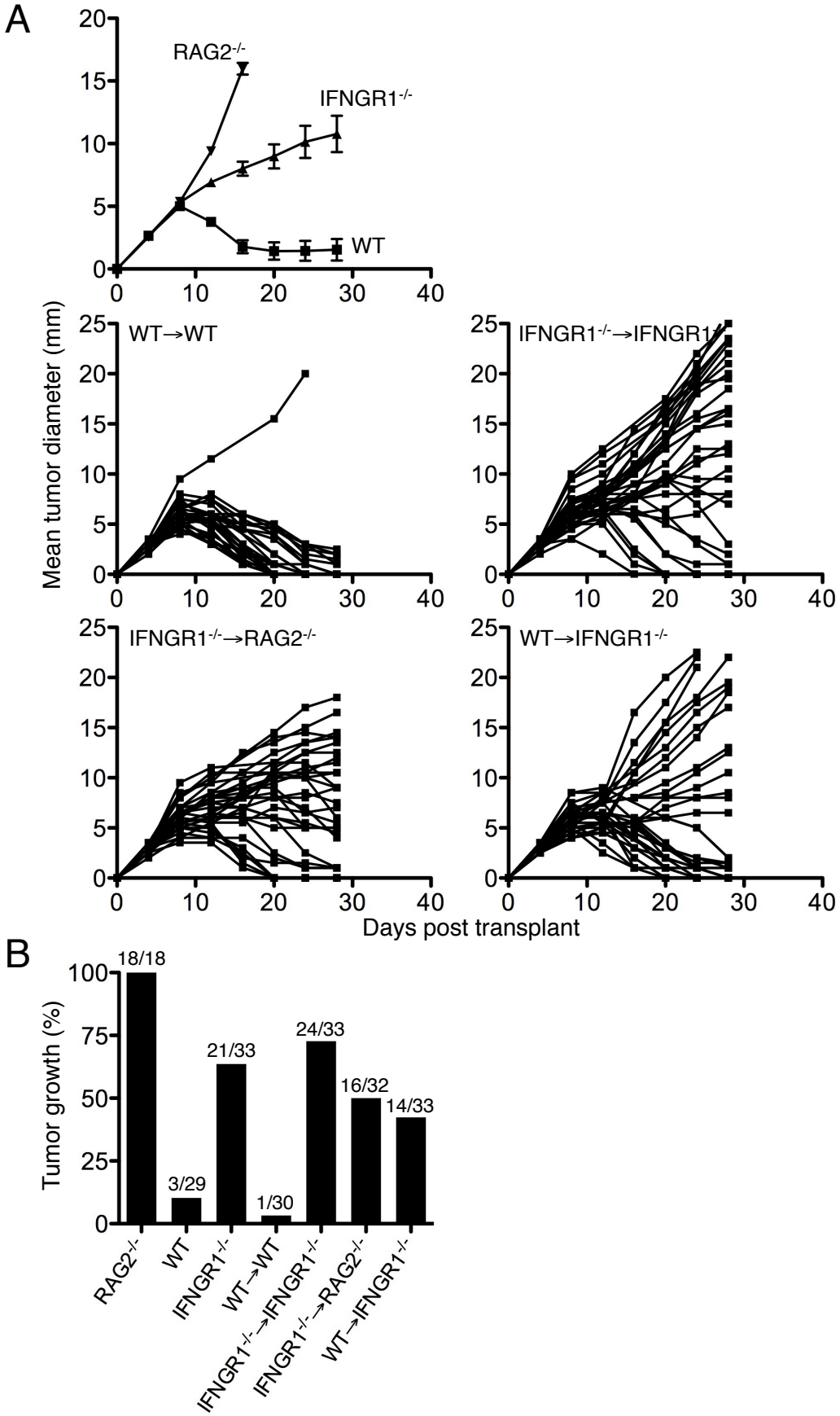
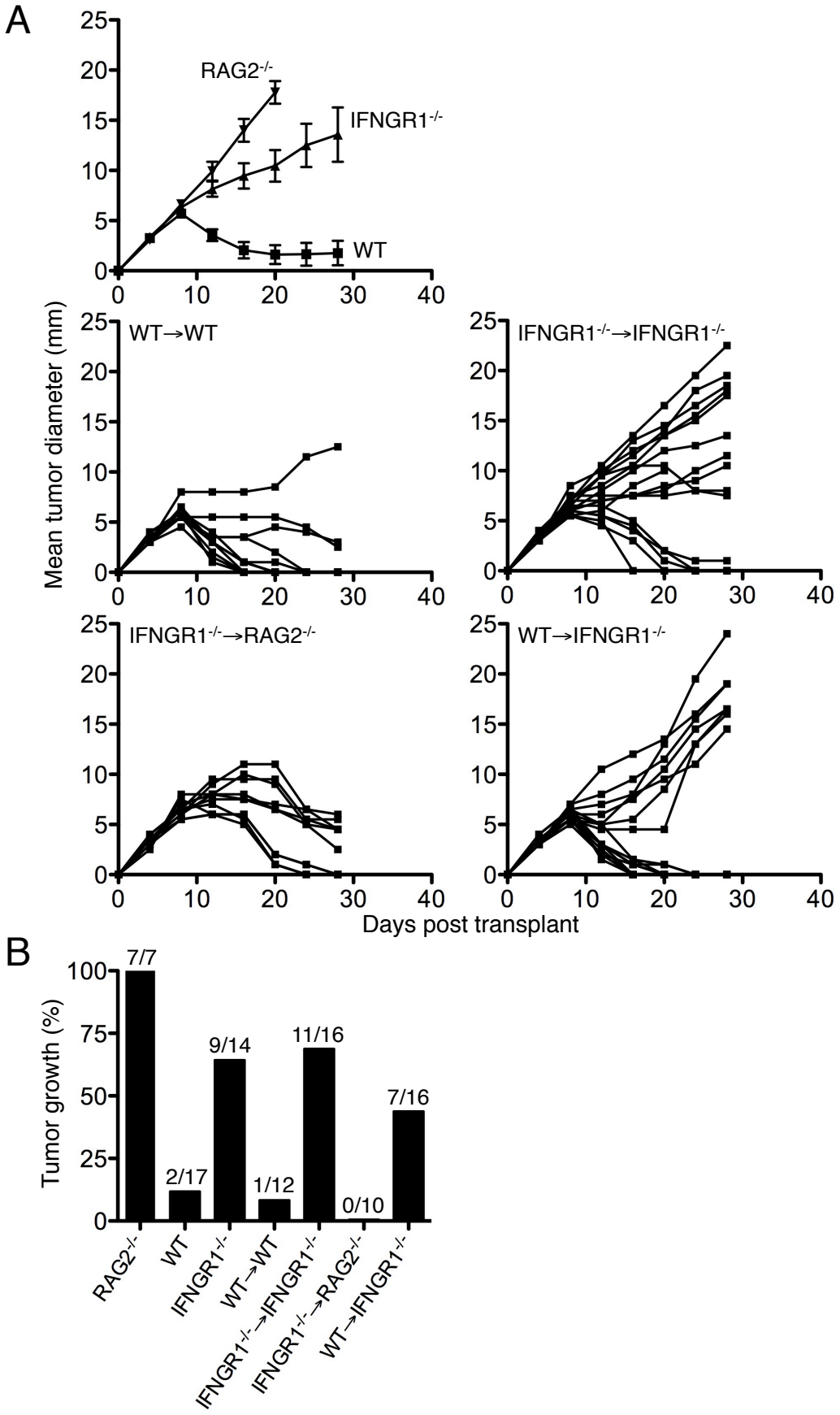

A
RAG2-/-
IFNGR1-/-
WT
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-
Mean tumor diameter (mm)
Days post transplant
B
Tumor growth (%)
18/18
3/29
21/33
1/30
24/33
16/32
14/33
RAG2-/-
WT
IFNGR1-/-
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-

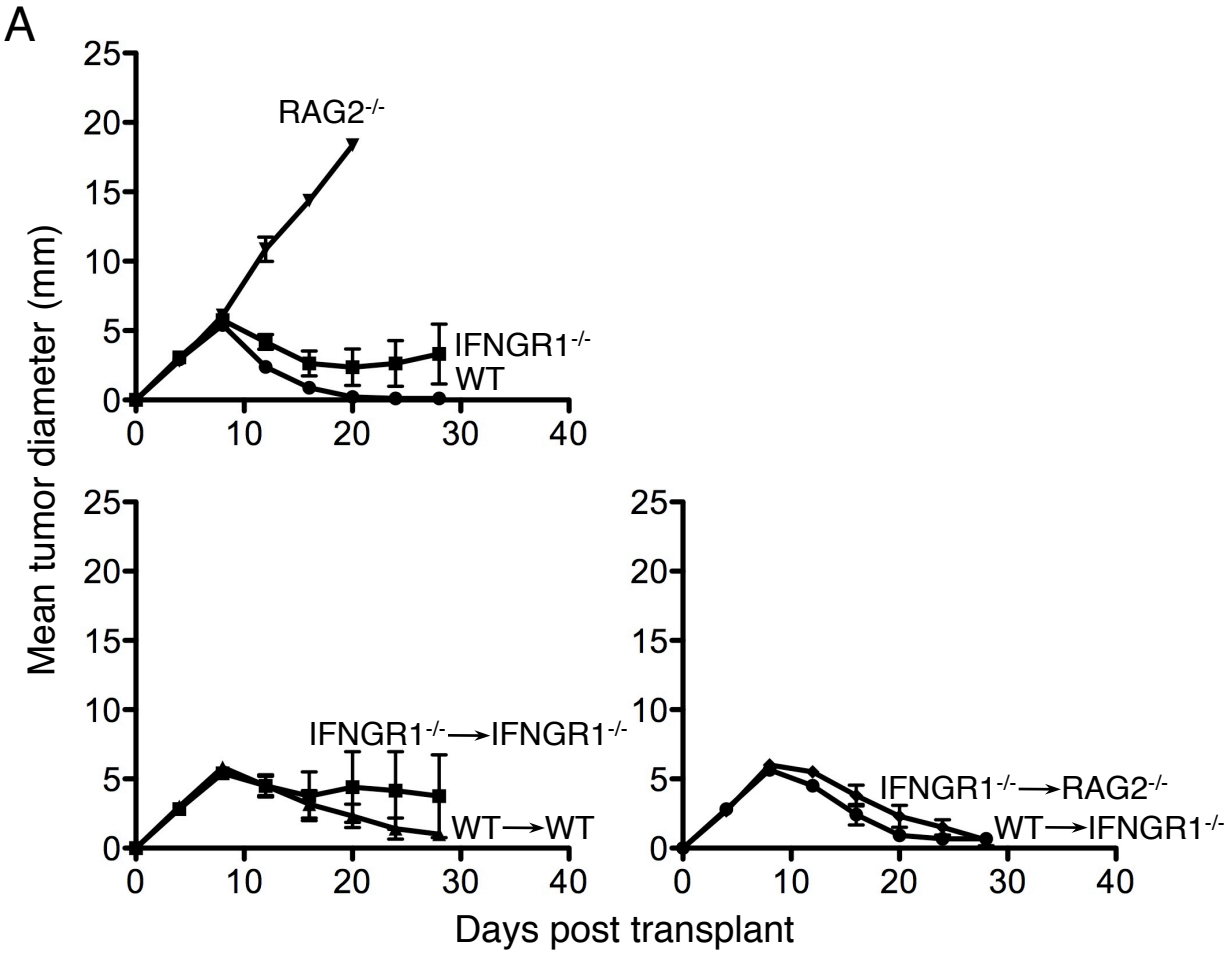
Figure 12. More important role for nonhematopoietic than hematopoietic IFNγ sensitivity for H31m1 rejection. (A) Control and bone marrow chimeric mice were injected with $1x10^6$ H31m1 tumor cells and growth was monitored over time. Data from three independent experiments are shown with group sizes as indicated in (B).


A
RAG2-/-
IFNGR1-/-
WT
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-
Mean tumor diameter (mm)
Days post transplant
B
Tumor growth (%)
7/7
2/17
9/14
1/12
11/16
0/10
7/16
RAG2-/-
WT
IFNGR1-/-
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-

Demonstration of the Normal Functional Reconstitution of IFNGR1$^{-/-}$ Bone Marrow Chimeric Mice

To confirm that the immune compartment in IFNGR1$^{-/-}$ bone marrow chimeras has been functionally reconstituted, we challenged control and chimeric mice with the F535 RAG2$^{-/-}$ regressor, a tumor rejected in WT mice in a lymphocyte-dependent but largely IFNγ-independent fashion (C.M. Koebel and R.D. Schreiber, unpublished data). As shown in Figure 13, F535 tumor cells were rejected in WT controls and in the majority of IFNGR1$^{-/-}$ mice transplanted, yet grew progressively in RAG2$^{-/-}$ hosts. When injected into IFNGR1$^{-/-}$ bone marrow chimeras of each type, the F535 tumor was also uniformly rejected, with the exception of a minor proportion (1/6) of IFNGR1$^{-/-}$ →IFNGR1$^{-/-}$ chimeras, comparable to the phenotype in IFNGR1$^{-/-}$ controls (2/11). We have additionally examined the growth of two representative WT progressor tumors, 1877 and F244, to rule out the presence of a hyperactive immunological state in the IFNGR1$^{-/-}$ chimeras (Figure 14). As expected, both tumors grew progressively with similar kinetics in control and chimeric mice.

Figure 13. Demonstration of functional immune reconstitution in IFNGR1$^{-/-}$ bone marrow chimeras. (A) Groups of WT, IFNGR1$^{-/-}$, and RAG2$^{-/-}$ control mice as well as bone marrow chimeric mice of each type were injected with 1x10^{6} F535 tumor cells. As shown, rejection of this tumor is independent of host IFNγ responsiveness. Data from two independent experiments with group sizes as indicated in (B) are shown.


A
Mean tumor diameter (mm)
RAG2-/-
IFNGR1-/-
WT
IFNGR1-/-→IFNGR1-/-
WT→WT
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-
Days post transplant

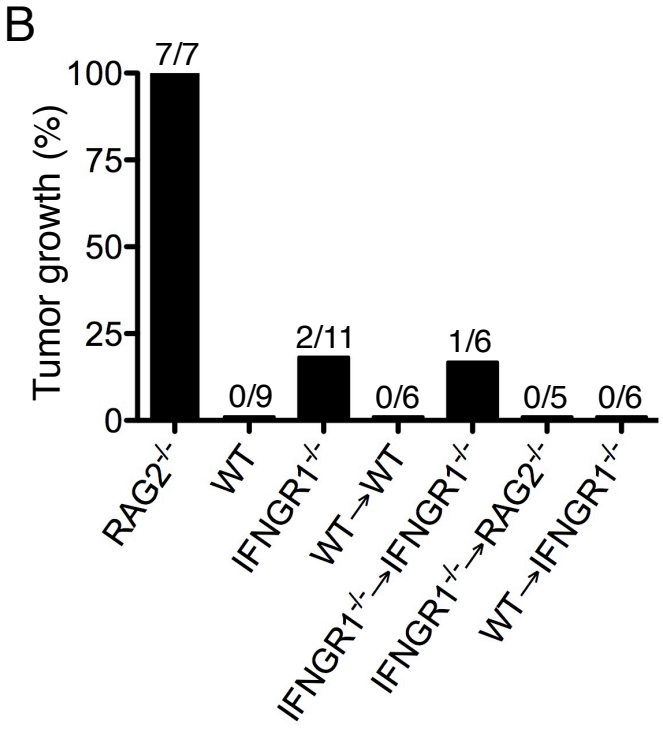
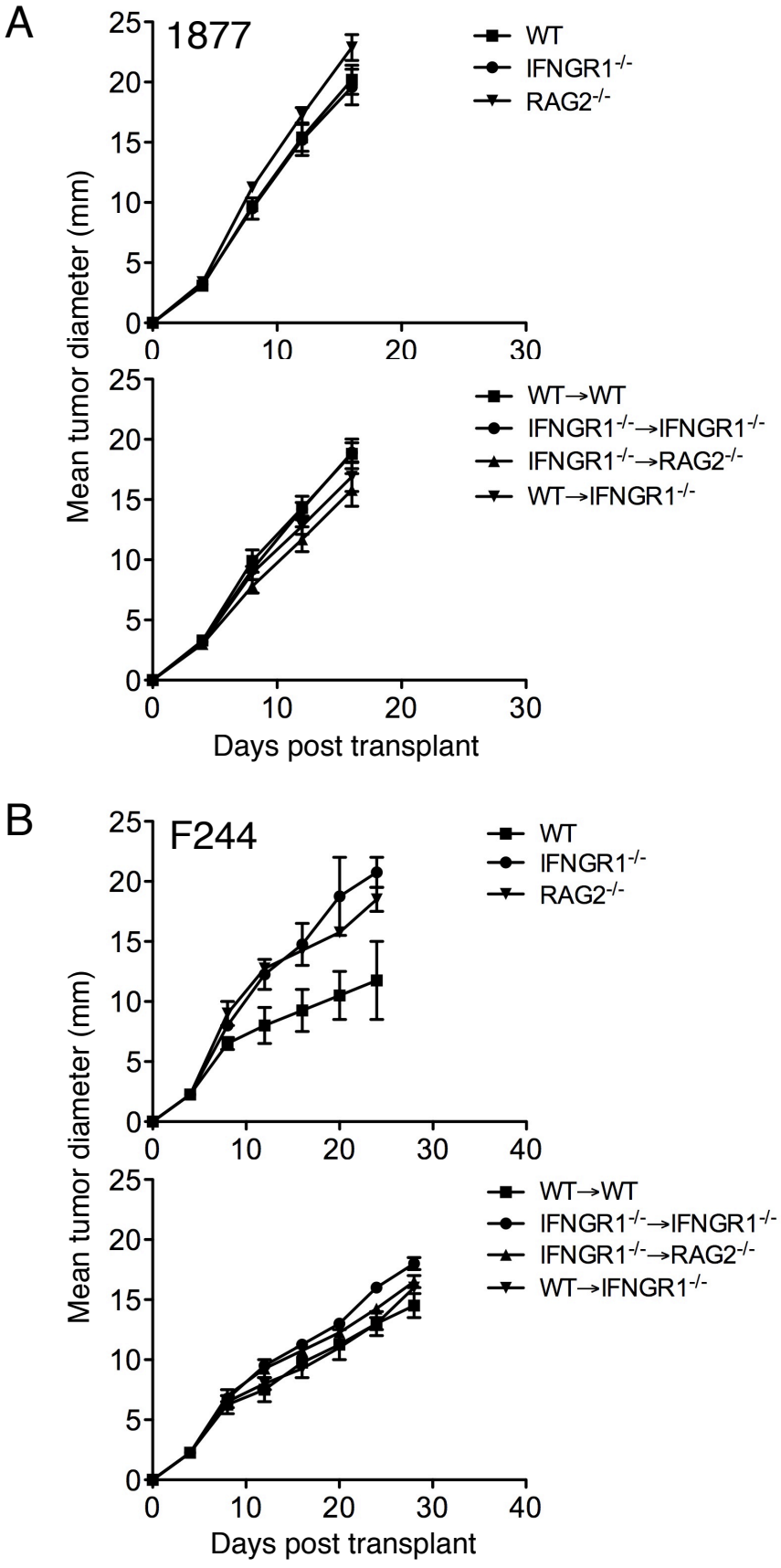

B
Tumor growth (%)
7/7
0/9
2/11
0/6
1/6
0/5
0/6
RAG2-/-
WT
IFNGR1-/-
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-

Figure 14. No evidence for immune hyper-reactivity in IFNGR1$^{-/-}$ bone marrow chimeras. Control and bone marrow chimeric mice were injected with either 1877 (A) or F244 (B) WT progressor tumor cells at a dose of $1x10^6$ cells/mouse. Mean tumor diameter ± s.e.m. over time is shown.


A
1877
WT
IFNGR1-/-
RAG2-/-
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-
Mean tumor diameter (mm)
Days post transplant
B
F244
WT
IFNGR1-/-
RAG2-/-
WT→WT
IFNGR1-/-→IFNGR1-/-
IFNGR1-/-→RAG2-/-
WT→IFNGR1-/-
Mean tumor diameter (mm)
Days post transplant

Tumor-Associated Endothelium in IFNGR1$^{-/-}$ Bone Marrow Chimeras is Derived from Nonhematopoietic Host Cells Whereas TILs are Donor Bone Marrow Derived

The origin of endothelial cells comprising the blood vessels which infiltrate and sustain a growing tumor has become increasingly controversial. In the adult, neovascularization was traditionally thought to arise through the process of angiogenesis – that is, via outgrowth from existing vessels through endothelial cell proliferation and migration. Some recent studies, however, have questioned this view, providing evidence for an alternate process known as vasculogenesis – involving the recruitment and differentiation of circulating bone marrow-derived endothelial precursors. Although the identity of endothelial precursor cells remains unclear due to the lack of definitive markers, a number of studies report the incorporation of bone marrow-derived cells into newly formed endothelium (187-192). Using different model systems including tumor growth, myocardial and hindlimb ischemia, and cutaneous wounding, genetically-marked cells arising from the bone marrow could be detected within the endothelium. In most cases the bone marrow contribution was rather small, yet it has been suggested that progenitor cells might be more prominent during the early stages of neovascularization but are then diluted out by later expansion of existing endothelium (190).

Additional recent data, however, refute these claims, finding that bone marrow-derived precursors do not contribute to newly formed endothelium in the adult and are not, in fact, required for tumor growth (193-198). In one such study, Purhonen et al examined vessel formation in models of both transplantable and spontaneous tumor

growth, yet failed to detect cells of bone marrow origin within the endothelium (198). Nevertheless, all of the aforementioned studies find abundant bone marrow-derived cells directly adjacent to and underlying the endothelial cell layer. Furthermore, there is increasing evidence that such cells – determined to be mostly leukocytes, but also pericytes and fibroblasts – play an important accessory role in promoting the formation of new vasculature (197, 199). While the precise contribution of bone marrow-derived cells remains contentious, it is likely that their role during neovascularization is tissue- and model-dependent.

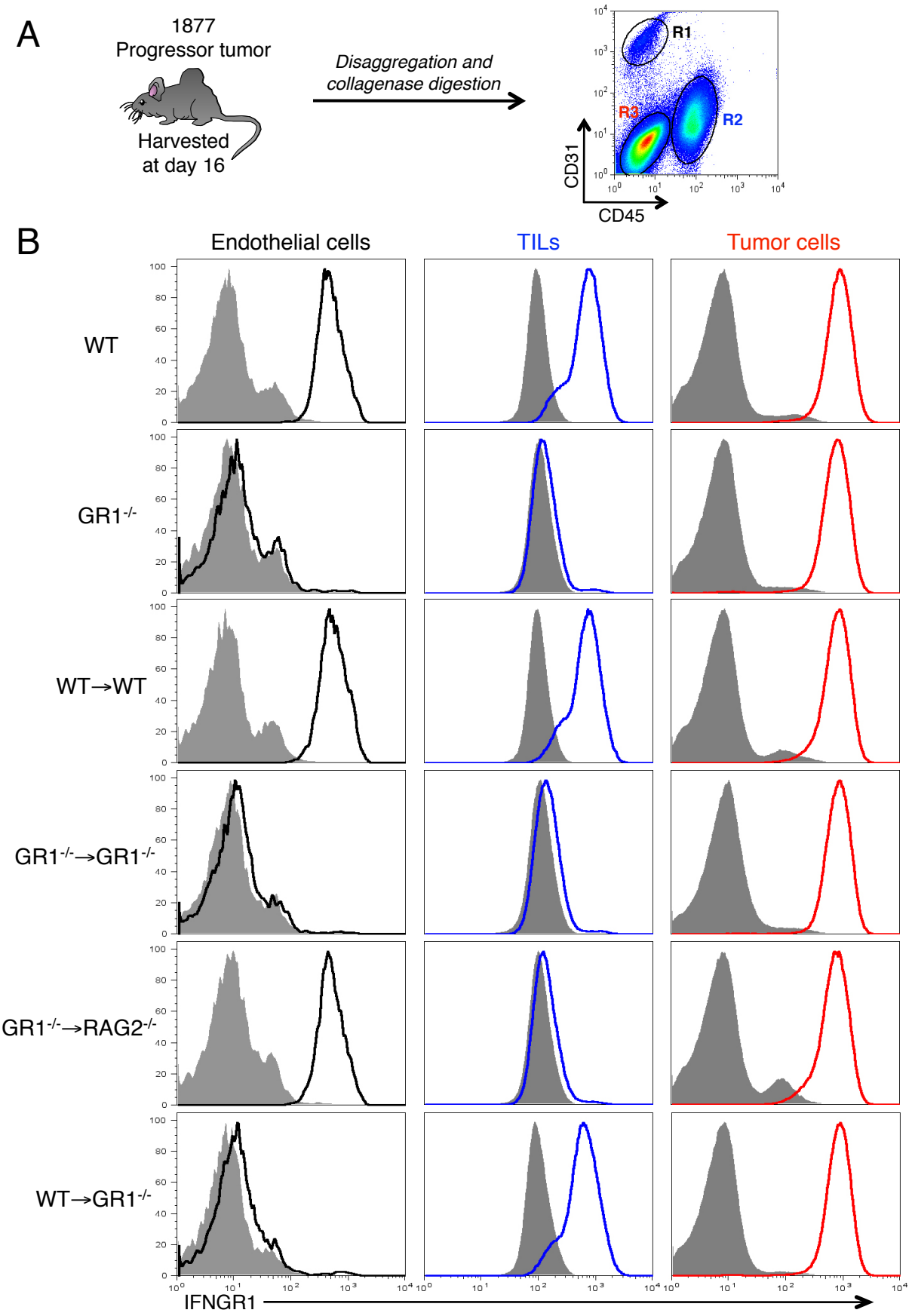
Given our findings that nonhematopoietic as well as hematopoietic IFNγ sensitivity is important for tumor rejection, we wanted to ask whether tumor-associated endothelium in IFNGR1$^{-/-}$ chimeras was recipient-derived or donor bone marrow-derived. We injected groups of control and chimeric mice with the WT progressor tumor 1877, then harvested tumors at day 16 for analysis. Following mechanical and enzymatic disaggregation, cells isolated from the tumor tissue were stained with antibodies specific for the endothelial cell marker CD31 (PECAM-1), the hematopoietic lineage marker CD45, and the IFNγ receptor subunit IFNGR1, allowing us to assess the origins of the endothelium as well as tumor-infiltrating leukocytes (TILs) within the tumor (Figure 15).

When we gated on tumor cells (CD45$^-$CD31$^-$), which comprised the major cellular population within the mixture, we detected similar IFNGR1-positive staining in all of the samples – thus providing an internal control for IFNGR1 staining. Consistent with our previous analyses of splenocytes from IFNGR1$^{-/-}$ chimeras (see Figure 9), when we instead gated on TILs (CD45$^+$CD31$^-$) we found these cells to be strictly donor bone

marrow derived. For example, tumors isolated from IFNGR1$^{-/-}$→RAG2$^{-/-}$ chimeras contained IFNGR1-negative TILs, while the TILs from tumor-bearing WT→IFNGR1$^{-/-}$ mice were IFNGR1-positive. In contrast, endothelial cells within the tumor (CD31^{+}CD45^{-}) were uniformly derived form the nonhematopoietic compartment, thus being IFNGR1-positive in IFNGR1$^{-/-}$→RAG2$^{-/-}$ mice and IFNGR1-negative in WT→IFNGR1$^{-/-}$ mice. Although it could be argued that any bone marrow contribution might be diluted out by day 16 of tumor growth, studies documenting this phenomenon can still detect some contribution in late tumors (190). Since we find no evidence of bone marrow derived cells, it is likely that in this model of tumor growth, infiltrating vessels are derived from existing endothelium.

In order to look more closely at different subpopulations of infiltrating leukocytes within the tumor, we also costained the TILs with CD45 and markers for myeloid cells (CD11b), dendritic cells (CD11c), or T lymphocytes (CD3). These analyses showed that all TIL subpopulations were donor bone marrow-derived (data not shown), ruling out the possibility that tissue resident macrophages or DCs persist following bone marrow transplantation and comprise a significant proportion of the tumor infiltrate. This possibility would perhaps also be unlikely given the large number of leukocytes within the tumor that are presumably recruited from the blood or differentiate from blood-borne precursor cells.

Figure 15. Tumor-associated endothelium in IFNGR1$^{-/-}$ bone marrow chimeras is derived from nonhematopoietic host cells. Tumors from control and chimeric mice were harvested 16 days after injection of 1x10^{6} 1877 tumor cells. Following disaggregation, cell suspensions were stained with antibodies for CD45, CD31, and IFNGR1. Tumors were analyzed by gating on endothelial cells (CD31^{+}CD45^{-}), tumor-infiltrating leukocytes (CD45^{+}CD31^{-}), or tumor cells (CD45^{-}CD31^{-}) as shown in (A). IFNGR1 expression levels were then examined on individual cell populations from control and bone marrow chimeric mice (B). IFNGR1 positivity on the cells of the tumor served as an internal control for staining.


A
1877
Progressor tumor
Harvested
at day 16
Disaggregation and
collagenase digestion
R1
R2
R3
CD31
CD45
B
Endothelial cells
TILs
Tumor cells
WT
GR1-/-
WT→WT
GR1-/-→GR1-/-
GR1-/-→RAG2-/-
WT→GR1-/-
IFNGR1

DISCUSSION

In this study, we have explored the functionally relevant sites of IFNγ's actions during anti-tumor immune responses. Initial experiments provided additional support for the conclusion that the tumor cell represents a critical target of IFNγ. Through selective reconstitution of GAR4 tumor cells (derived from an IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ mouse) with either the missing IFNγ receptor component (IFNGR1) or IFNα/β receptor component (IFNAR1), we found that rejection of this tumor required tumor cell IFNγ but not IFNα/β sensitivity. Yet, when we instead examined the host cell IFN requirements, both IFNγ and IFNα/β responsiveness were essential for its rejection. In another set of experiments, we took the converse approach using H31m1 cells, which are normally IFNγ-responsive and are rejected when injected into WT mice. RNAi-mediated knockdown of IFNGR1 expression in these cells showed that sensitivity to IFNγ was indeed required for its rejection, corroborating results obtained using a dominant-negative IFNGR1ΔIC construct.

Since prior work in the laboratory has also demonstrated a requirement for IFNγ's actions on host cells for rejection of RAG2$^{-/-}$ regressor tumors, we used bone marrow chimeras with selective IFNγ sensitivity to ask whether hematopoietic cells or nonhematopoietic cells represented the essential responders. Analyses of two independent regressor tumors revealed that host responsiveness to IFNγ in both hematopoietic and nonhematopoietic cells was important for tumor rejection. When injected with d38m2 tumor cells, chimeras lacking either hematopoietic or

nonhematopoietic IFNγ sensitivity showed a similar defect in anti-tumor immunity. Yet, this phenotype was not quite as severe as that observed in chimeras with complete IFNγ insensitivity, suggesting that IFNγ's actions on cells within either compartment can provide some tumor protection. Experiments with the H31m1 tumor also showed a similar defect, though again partial, in chimeras lacking nonhematopoietic IFNγ responsiveness. In chimeras selectively lacking hematopoietic responsiveness, we observed only significantly delayed rejection.

Taken together, our results demonstrate an essential function for IFNγ's actions on both hematopoietic and nonhematopoietic cells during tumor rejection. Analyses from the previous chapter using the same two regressor tumors established that IFNα/β responsiveness in hematopoietic cells was required, yet showed no role for nonhematopoietic IFNα/β sensitivity. Thus, although these tumors require sensitivity to both IFNγ and IFNα/β within the host for their rejection, the respective IFNs are acting on distinct tissue compartments.

In contrast to our results, a previous study suggested that IFNγ sensitivity in nonhematopoietic cells was necessary and sufficient for anti-tumor immunity (23). This work, however, relied on an immunization/rechallenge model in which mice were first immunized with irradiated tumor cells two weeks prior to challenge with live tumor. In addition, this tumor model depended primarily on $CD4^+$ T cells, and only a delay in growth rather than complete rejection was observed. Since our regressor tumor model employs naïve mice and both $CD4^+$ and $CD8^+$ T cells are required for rejection, differences in our respective results may be related to the models employed. Nevertheless

it will be important to address this question using additional experimental approaches, including the use of conditional knockout mice to dissect with more specificity the individual cell types responsible for tumor protection. It will also be essential to perform primary tumor induction studies in addition to tumor transplantation approaches.

CHAPTER 5

Local Production of IFNβ Induces Tumor Equilibrium or Elimination via Tissue-Selective Actions on the Host

INTRODUCTION

A large body of work – beginning as early as the 1960's – has demonstrated the ability of IFNα/β to inhibit tumor growth and improve survival when administered to tumor-bearing animals (11, 37, 200). IFNα administration has also shown some clinical efficacy in the treatment of several types of human cancer, and has been approved by the US Food and Drug Administration (FDA) for this use since 1986 (27, 38, 201). Nevertheless, surprisingly little is known regarding the relevant mechanisms of action, as well as the reasons why only some tumor types and a subset of patients seem to benefit from treatment.

In this study, we have turned our attention toward the protective anti-tumor functions of *exogenous*, as opposed to *endogenous*, type I IFN. While it is possible that exogenous IFNα/β merely augments the same pathway involved in tumor protection by endogenous type I IFN, it is equally plausible that distinct host effector mechanisms are invoked in the setting of high dose administration. Clearly, a better understanding of the mode of action would facilitate the development of more effective regimens or improved combination therapies.

Despite the ability of IFNα/β to inhibit tumor cell growth, promote apoptosis, and enhance immune recognition through upregulation of MHC class I molecules, it has become increasingly apparent that the efficacy of exogenous IFNα/β treatment is mediated largely by its effects on the host rather than the tumor (200). For example, it was shown that IFN-insensitive tumor cell clones were still responsive to exogenous

IFNα/β treatment (30, 32, 33), whereas the absence of host sensitivity to type I IFN abrogated the effect (35). Given the array of immunomodulatory activities ascribed to the type I IFNs – including the enhancement of NK and macrophage cytotoxicity, inhibition of angiogenesis, activation of DCs, and augmentation of T and B cell function – identification of the key mechanisms evoked by exogenous IFNα/β has been challenging.

A large number of studies have investigated potential mechanisms involved, primarily using model systems in which tumor cells were engineered to overexpress individual IFNα/β subtypes. Conclusions from this work were varied, however, as some reports suggested a prominent role for protective T cell responses, while others implicated anti-angiogenic effects or innate immune killing by NK cells and macrophages (200, 202, 203). Yet, since all of the cells of the host could potentially respond to IFNα/β in these models, distinguishing between effects on various cell populations was not possible.

In order to investigate the critical host cell mediators, we have utilized a model in which IFNα/β-unresponsive tumor cells (normally non-immunogenic and resistant to immunologic control) were engineered to constitutively express IFNβ. Given prior data indicating the importance of local type I IFN delivery and the obligate role for host IFNα/β sensitivity, the use of IFNAR1$^{-/-}$-derived tumor cells ensured that all effects were mediated by the host, while tumor cell secretion permitted localized IFNβ delivery. The use of bone marrow chimeras with selective IFNα/β responsiveness then allowed us to assess the effects on various host cell compartments.

MATERIALS AND METHODS

Tumor cells. IFNAR1$^{-/-}$-derived progressor tumors d103m503 and d97m915 and WT-derived progressor F244 were induced by MCA treatment as described (5, 16). GFP- and IFNβ-expressing tumor cell lines were generated by retroviral transduction. The IFNγ-unresponsive H31m1.IFNGR1ΔIC tumor was generated previously by overexpression of a dominant-negative IFNGR1 construct.

Flow cytometry. Tumor cells were stained directly, while splenocytes were first incubated with purified anti-CD16/CD32 mAb (2.4G2) (BD Biosciences) to prevent non-specific antibody binding to Fc receptors. Data was collected using a BD FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). Anti-H-2K^{b}-PE (AF6-88.5) and anti-CD11c-PE (HL3) were purchased from BD Biosciences; anti-DX5-PE (DX5), anti-CD11b-PE (M1/70), and SA-APC were from BioLegend; and anti-IFNAR1-biotin (MAR1-5A3) has been described (64). For MHC class I upregulation assays, cells were treated with the indicated dose of recombinant murine IFNγ (Genentech).

Mice. Inbred 129/SvPas mice were purchased from Charles River Laboratories, while 129 IFNAR1$^{-/-}$ and RAG2$^{-/-}$ mice were bred in our specific pathogen-free animal facility. RAG2$^{-/-}$IFNAR1$^{-/-}$ mice were generated previously in our laboratory by intercrossing RAG2$^{-/-}$ and IFNAR1$^{-/-}$ mice on a 129 background.

Tumor transplantation experiments. Tumor cells were thawed from frozen stocks and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamate, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol (R-10 medium). After expansion for several passages, cells were harvested by incubation in 0.05% trypsin, washed once with R-10 medium, and washed three times with sterile, endotoxin-free PBS. Prior to the last wash, cells were counted using a hemacytometer and cell viability was assessed by trypan blue exclusion (injected cells were >90% viable). Cells were injected subcutaneously in a volume of 0.15 ml PBS into the shaved flanks of mice. Tumor size was measured on the indicated days and is presented as the average of two perpendicular diameters. When calculating percent tumor growth, mice with tumors larger than 6x6 mm in diameter at the end of the observation period were counted as positive.

Antibody treatment. Anti-asialoGM1 antibody (Wako Chemicals) was resuspended in 4 ml sterile endofree PBS and 100 μl (diluted to 0.5 ml in PBS) was administered by i.p. injection on days -2, 0, +2, and +7, then every 7 days thereafter. Control mice received i.p. injections of an equal volume of PBS. Effective cell depletion was confirmed by flow cytometry and functional assays. For neutralization of IFNγ, mice were injected i.p. with 750 μg of anti-IFNγ mAb H22 or isotype control mAb PIP on day -1 and 250 μg every 7 days. IFNα/β blockade was induced by treatment at the indicated day with 2.5 mg anti-IFNAR1 mAb MAR1-5A3, followed weekly with a 0.5 mg i.p. injection.

Generation of bone marrow chimeras. E14.5 fetal liver cells were used to reconstitute lethally-irradiated recipients. Embryos were extracted 14 days following implantation, livers were removed, and FLCs were isolated by homogenization through a metal mesh strainer with a 6 cc syringe plunger. Following RBC lysis by incubation in Hybrimax RBC lysing buffer (Sigma-Aldrich), cells were filtered through a 40 μm cell strainer, washed 2 times with sterile endofree PBS, and counted. Cells were resuspended in PBS for injection of $5x10^6$ FLCs per mouse in a volume of 0.2 ml using a 0.5 cc 29 gauge insulin syringe. Recipient mice, lethally irradiated with a single dose of 9.5 Gy several hours prior, were anesthetized by i.p. avertin and FLCs were infused i.v. via retro-orbital injection. Animals were generally maintained on TMS water for 4 weeks following irradiation and reconstitution, and tumor transplantation experiments were performed at least 10-12 weeks post-reconstitution.

RESULTS

Type I IFN Sensitivity in Host Cells is Essential for the Control of IFNβ-Secreting Progressor Sarcomas

To establish a model of type I IFN cancer immunotherapy, we engineered three different progressor fibrosarcomas to constitutively express murine IFNβ, or GFP as a control. We utilized two MCA-induced tumors originally derived from IFNAR1$^{-/-}$ mice (d103m503 and d97m915), as well as one derived from a WT mouse (F244), because this would allow us to distinguish between potential effects of IFNβ on host cells versus autocrine effects on the tumor. After retroviral-mediated transduction, we confirmed expression of the introduced gene by FACS analysis for GFP levels or by testing cell culture supernatants for the presence of bioactive IFN via CPE assay or MHC class I upregulation (Figure 1). We also examined levels of MHC class I on the bulk transduced cell lines themselves (Figure 2). As expected, GFP- and IFNβ-expressing d103m503 and d97m915 lines showed comparable basal H-2K^{b} levels, whereas levels on F244.IFNβ were considerable elevated compared to F244.GFP. All of the cell lines, however, were able to equally upregulate MHC class I in response to IFNγ treatment. While growing these cells in culture, we further noted that growth of F244.IFNβ was measurably slower compared to its GFP-expressing counterpart, yet growth rates of the IFNAR1-deficient lines were comparable (data not shown).

When we tested the growth of these cells in vivo, all three of the GFP-expressing cell lines grew progressively in WT mice with kinetics similar to growth of the parental tumor (Figure 3). In contrast, the IFNβ-secreting tumor cells initially appeared to regress when injected into WT mice, then formed a persistent mass which remained stable in size. Control of these IFNβ-secreting tumors, however, was dependent on host sensitivity to IFNα/β since both GFP- and IFNβ-expressing cells grew progressively in IFNAR1$^{-/-}$ mice. The cells of the host – but not the tumor – must therefore be the relevant targets of IFNβ in this model, given that (i) IFNα/β-unresponsive d103m503.IFNβ and d97m915.IFNβ tumor cells could be controlled in WT, but not IFNAR1$^{-/-}$ mice, and (ii) IFNα/β-responsive F244.IFNβ cells still grew progressively in IFNAR1$^{-/-}$ mice. We have therefore used this model to dissect the important host cell populations responding to IFNβ within the tumor microenvironment and contributing to tumor control.

Figure 1. Generation of GFP- or IFNβ-expressing tumor cells by retroviral transduction and confirmation of bioactive IFNβ secretion. (A) GFP levels by FACS of the IFNAR1$^{-/-}$ progressor tumors d103m503 and d97m915 or the WT progressor F244 were assessed following retroviral transduction with either RV.GFP or RV.IFNβ constructs. (B) IFN secretion was confirmed by testing cell culture supernatants in a standard CPE assay for protection of L929 cells from viral lysis. (C) Supernatants from the indicated cell lines were also tested for their ability to mediate MHC class I upregulation in H31m1 cells. Shown are H-2K^{b} levels on H31m1 following incubation with the indicated supernatant for 3 days.

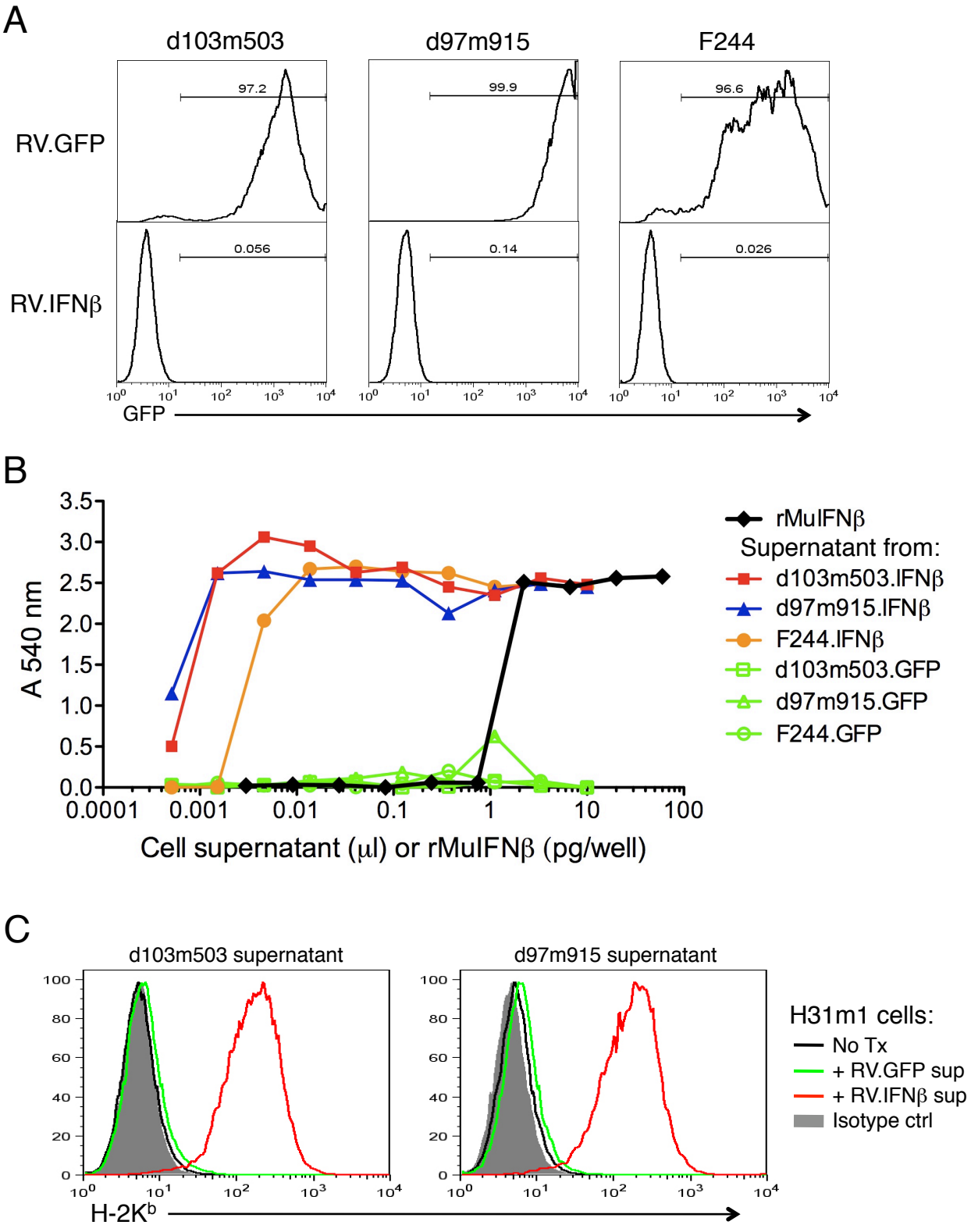

A
d103m503
d97m915
F244
RV.GFP
RV.IFNβ
97.2
99.9
96.6
0.056
0.14
0.026
GFP
B
A 540 nm
Cell supernatant (μl) or rMuIFNβ (pg/well)
rMuIFNβ
Supernatant from:
d103m503.IFNβ
d97m915.IFNβ
F244.IFNβ
d103m503.GFP
d97m915.GFP
F244.GFP
C
d103m503 supernatant
d97m915 supernatant
H-2K^b
H31m1 cells:
No Tx
+ RV.GFP sup
+ RV.IFNβ sup
Isotype ctrl

Figure 2. Basal and IFNγ-induced levels of MHC class I on IFNβ-secreting tumor cells. Expression levels of H-K^b on GFP- and IFNβ-expressing tumor cell lines were determined by FACS staining. Basal levels are shown in black whereas IFNγ-induced levels (1000 U/ml for 2 days) are in red. Irrelevant isotype ctrl Ab staining is solid gray.

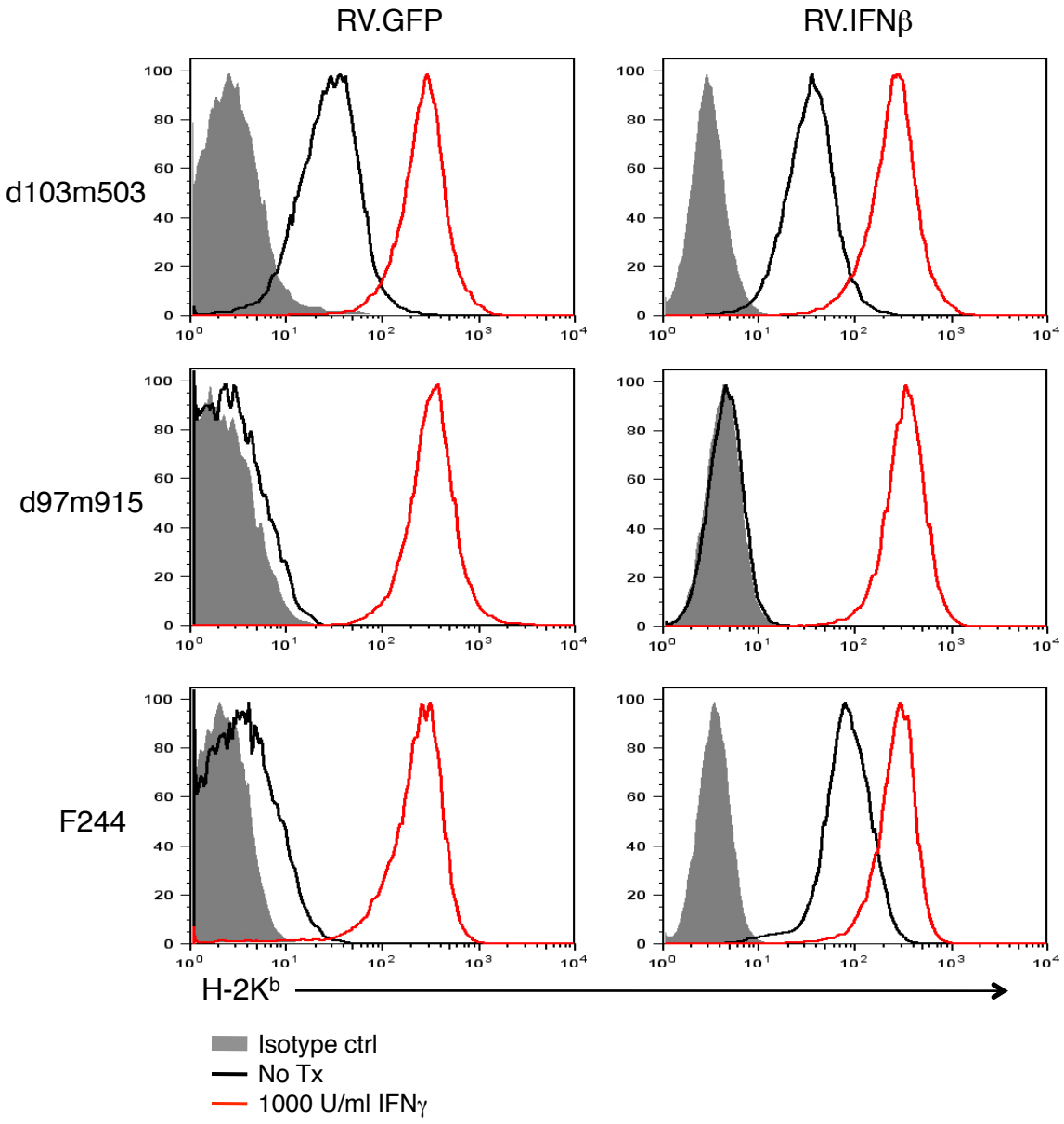
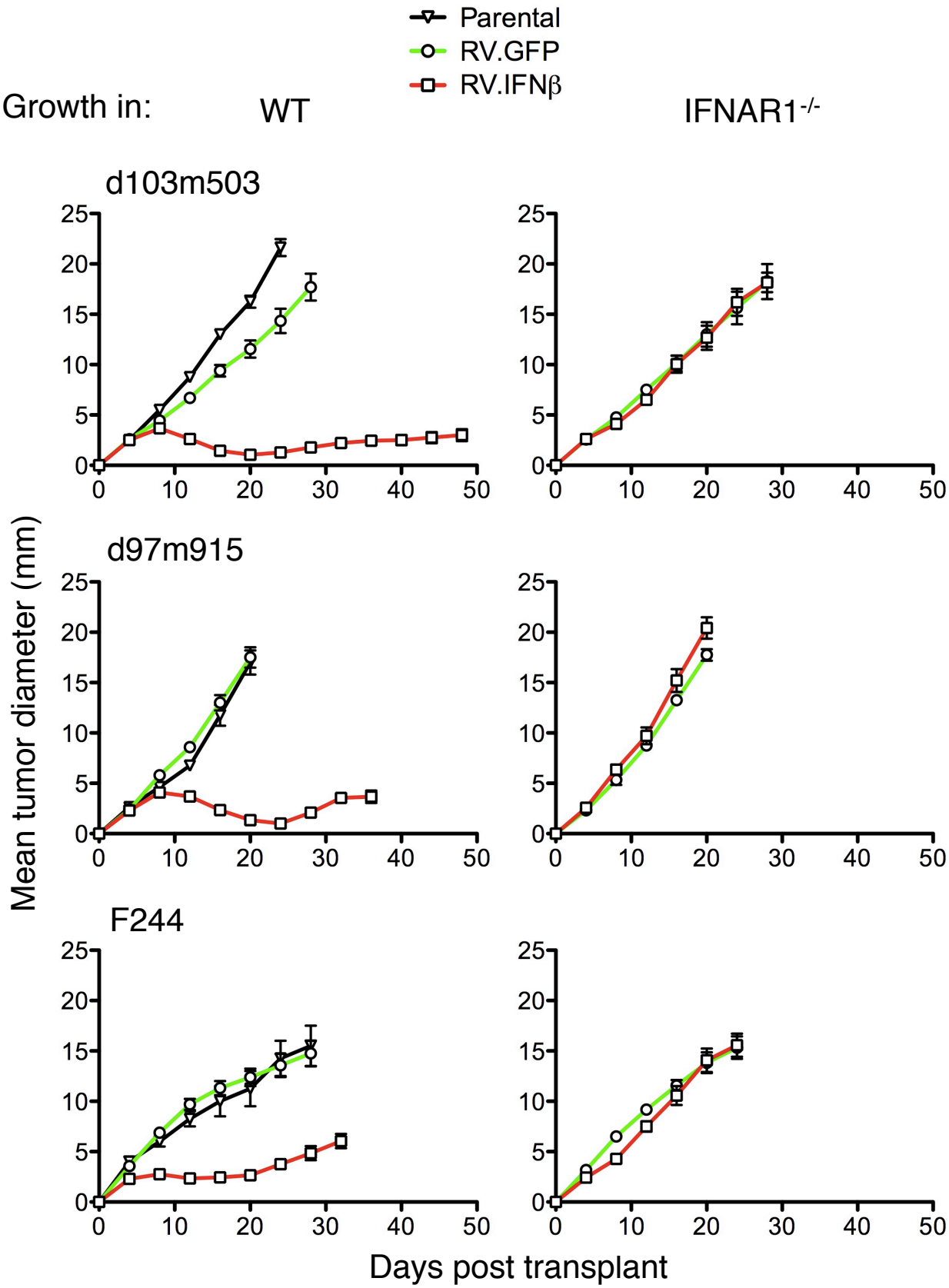

RV.GFP
RV.IFNβ
d103m503
d97m915
F244
H-2K^b
Isotype ctrl
No Tx
1000 U/ml IFNγ

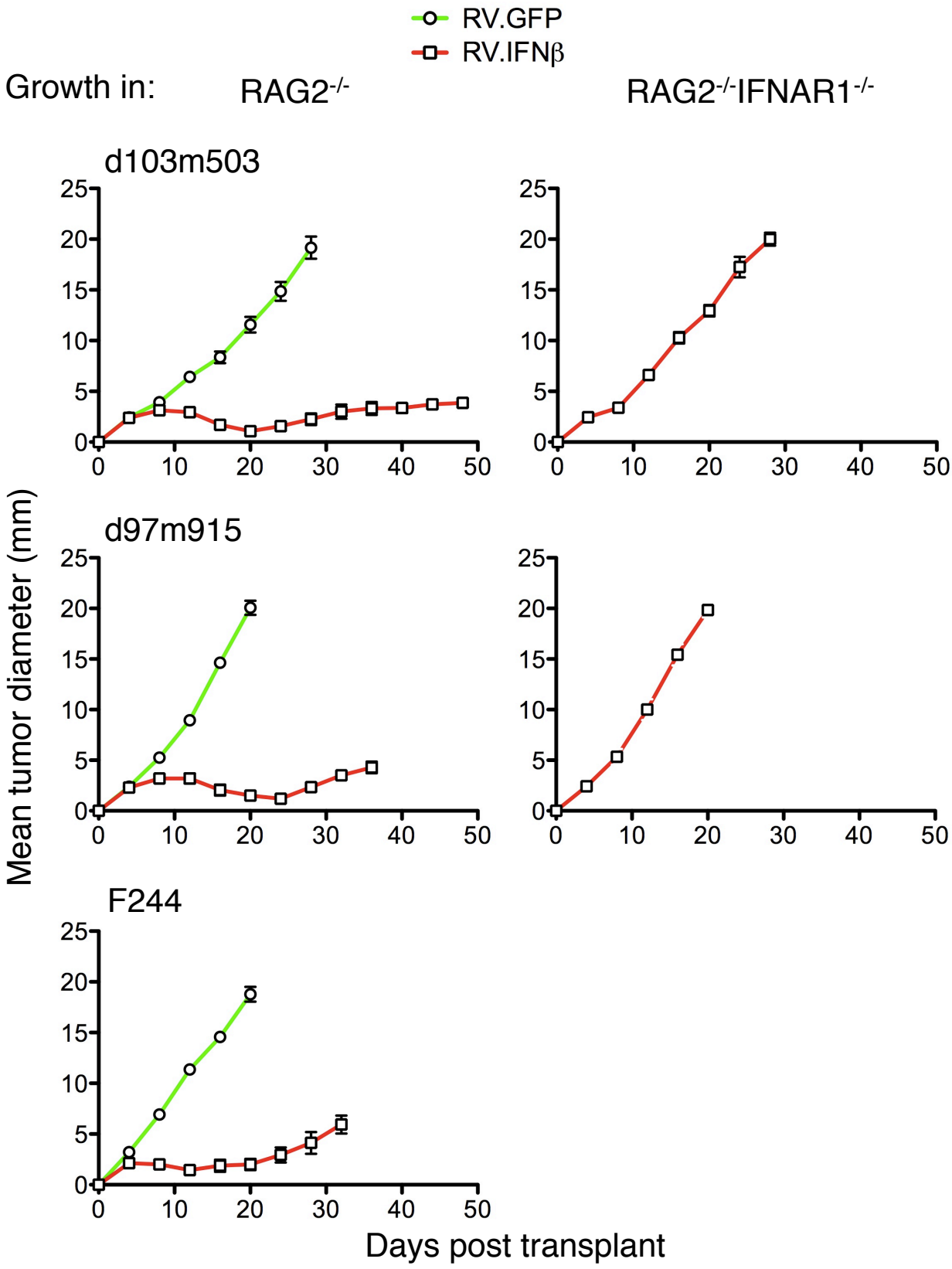
Figure 3. Host sensitivity to IFNα/β is required for control of IFNβ-producing tumors. Parental, GFP-expressing, or IFNβ-expressing d103m503, d97m915, and F244 tumor cell lines were injected at a dose of $1x10^6$ cells/mouse into groups of WT and $IFNAR1^{-/-}$ mice as indicated. Tumor size was measured over time and is depicted as the mean tumor diameter ± s.e.m. of n=6-10 mice/group from two independent experiments.


Parental
RV.GFP
RV.IFNβ
Growth in:
WT
IFNAR1-/-
d103m503
d97m915
F244
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
30
40
50

Effective Control of IFNβ-Secreting Tumors in the Absence of Adaptive Immunity

Having established the importance of host cell IFNα/β responsiveness for tumor control, we assessed whether adaptive immunity was required by injecting GFP- and IFNβ-expressing tumor cells into $RAG2^{-/-}$ or $RAG2^{-/-}IFNAR1^{-/-}$ mice (Figure 4). While both GFP- and IFNβ-expressing tumors again grew progressively in IFNα/β-unresponsive $RAG2^{-/-}IFNAR1^{-/-}$ mice, only the GFP-expressing cells displayed uncontrolled growth in $RAG2^{-/-}$ hosts. Control of IFNβ-secreting tumor cells was therefore maintained in the absence of T and B lymphocytes, suggesting the involvement of innate immunity or nonhematopoietic cells. Indeed, the growth of both IFNα/β-unresponsive and IFNα/β-responsive tumor cells in $RAG2^{-/-}IFNAR1^{-/-}$ mice demonstrates that IFNβ's actions on host cells were still required. Given the exclusive function of host cell IFNα/β sensitivity for tumor control, we used only $IFNAR1^{-/-}$-derived tumors in subsequent experiments to ensure that all effects were mediated by host cells.

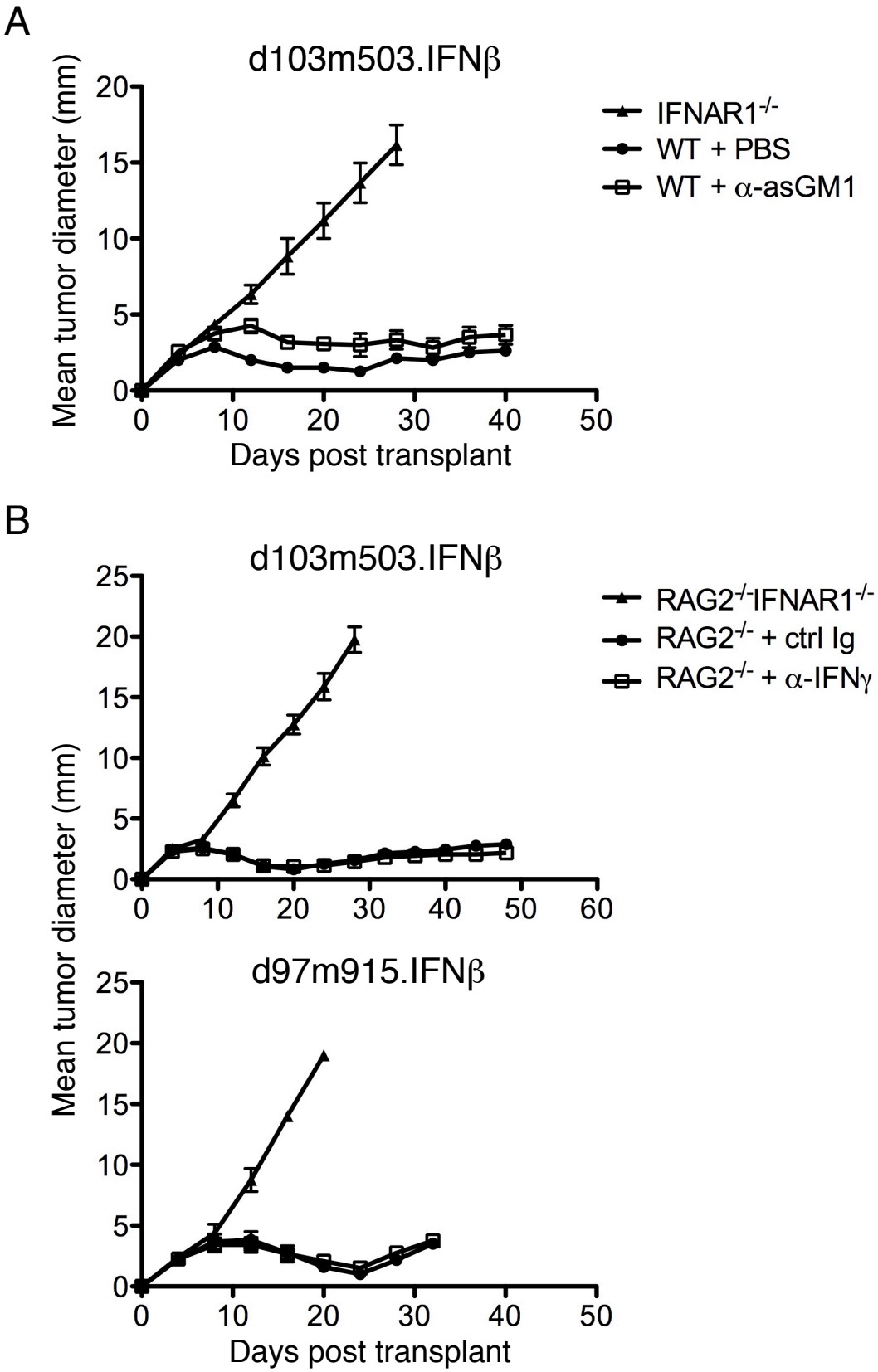
Figure 4. Tumor control mediated by IFNβ's actions on the host in the absence of adaptive immunity. $1x10^6$ GFP- or IFNβ-expressing d103m503, d97m915, and F244 tumor cell lines were injected into groups of $RAG2^{-/-}$ and $RAG2^{-/-}IFNAR1^{-/-}$ mice and growth was monitored.


RV.GFP
RV.IFNβ
Growth in:
RAG2-/-
RAG2-/-IFNAR1-/-
d103m503
d97m915
F244
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40
50

NK Cells and IFNγ Signaling are Not Required for Tumor Control Mediated by IFNβ Overexpression

Since control of IFNβ-expressing tumor cells was observed to be independent of adaptive immunity, we asked whether NK cells, which are potently activated by type I IFN, were required for tumor control. We injected d103m503.IFNβ tumor cells into WT mice treated with either PBS or anti-asGM1 polyclonal antibody, as well as IFNAR1$^{-/-}$ control mice. In prior studies, we have demonstrated that our anti-asGM1 treatment protocol depleted NK cell numbers and completely abrogated NK function in both ex vivo and in vivo assays, though depletion was not specific to NK cells (see Chapter 3). Nevertheless, anti-asGM1 treatment had no effect on tumor control, as we observed only a slight increase in tumor size and tumors still remained stable over time (Figure 5A). As IFNγ signaling also has important stimulatory functions on innate immune cells such as macrophages, we investigated the role of IFNγ during tumor control using IFNγ-specific neutralizing antibody. Again, no effect was detected upon injection of d103m503.IFNβ or d97m915.IFNβ tumor cells into RAG2$^{-/-}$ mice treated with anti-IFNγ mAb as compared to isotype control mAb treatment (Figure 5B).

Figure 5. NK cells and IFNγ signaling are dispensable for control of IFNβ-producing tumor cells. (A) d103m503.IFNβ tumor cells were injected at a dose of $1x10^6$ cells/mouse into groups of $IFNAR1^{-/-}$ control mice or WT mice treated with either PBS or anti-asGM1 Ab. Data from two independent experiments with n=4-6 mice/group are shown. (B) $1x10^6$ d103m503.IFNβ or d97m915.IFNβ tumor cells were injected into $RAG2^{-/-}IFNAR1^{-/-}$ controls or $RAG2^{-/-}$ mice treated with anti-IFNγ mAb H22 or isotype control mAb PIP. Tumor was monitored over time and is presented as mean tumor diameter ± s.e.m. of n=6-9 mice/group from two independent experiments.


A
d103m503.IFNβ
Mean tumor diameter (mm)
Days post transplant
IFNAR1-/-
WT + PBS
WT + α-asGM1
B
d103m503.IFNβ
RAG2-/-IFNAR1-/-
RAG2-/- + ctrl Ig
RAG2-/- + α-IFNγ
d97m915.IFNβ
Mean tumor diameter (mm)
Days post transplant

Induction of a Reversible State of Equilibrium Mediated by IFNβ Within the Tumor Microenvironment

Although IFNβ-expressing tumor cells initially appeared to regress, both WT and RAG2$^{-/-}$ mice eventually developed a persistent lesion at the site of tumor injection. Masses were stable for prolonged periods of time, though some lesions eventually assumed an inflammatory appearance with contracture and scar tissue formation in surrounding areas. Histologically, lesions were characterized by the presence of residual fibrosarcoma cells as well as abundant inflammatory infiltrate and areas of necrosis (data not shown). Over time, some mice also began to show signs of IFN toxicity, which was eventually lethal. Necropsy revealed alterations in hematopoiesis (i.e. anemia, thrombocytopenia, and hypocellularity of the bone marrow), hemorrhage in several organs, and microvesicular fatty degeneration of the liver – all potential findings of chronic IFNβ exposure. In addition, sera of mice bearing persistent lesions contained detectable IFN by CPE assay, and such activity could be inhibited by mAb's specific for IFNAR1 or IFNβ, but not IFNα or IFNγ (Figure 6). These observations suggest that residual tumor cells remain and thus cause pathology due to continual IFNβ production.

To investigate whether IFNβ-secreting tumors cells persisted within the stable masses, we treated RAG2$^{-/-}$ mice bearing d103m503.IFNβ tumor lesions with either anti-IFNAR1 MAR1-5A3 mAb or isotype control GIR-208 mAb beginning at day 65 (relative to tumor challenge). As expected, control mAb treatment had no effect on the persistent lesions and mice eventually succumbed to IFN-mediated toxicity (Figure 7A). In mice

treated with anti-IFNAR1 mAb, however, progressively growing tumors emerged from the lesions in 10/10 mice. The fact that anti-IFNAR1 mAb treated mice were rescued from lethality due to chronic IFN exposure also confirms this as the presumed cause of death in untreated animals. Similar results were obtained when RAG2$^{-/-}$ mice were treated with anti-IFNAR1 beginning at day 100, as tumors arose in 6/8 mice (Figure 7B). In this case, treatment was unable to rescue lethality in two mice which died shortly after the initiation of antibody blockade.

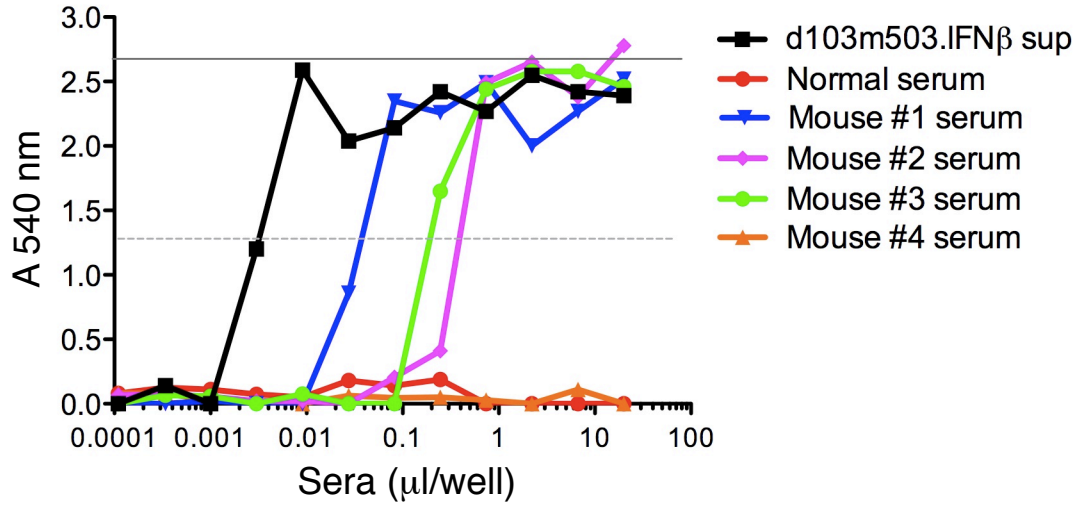
Treatment of WT mice bearing stable masses with anti-IFNAR1 mAb also yielded progressively growing tumors, though less frequently. As shown in Figure 8, masses in WT mice treated with control mAb at day 65 remained stable in size, whereas tumors progressed in anti-IFNAR1 mAb treated mice, ultimately leading to outgrowth in 5/8 mice. In several of the remaining mice, initial increases in tumor size were followed by eventual elimination of the mass, resolution of its inflammatory external appearance, and prolonged survival of the mouse (Figure 8 and data not shown). This observation is potentially explained by findings (described in a subsequent section) suggesting that under conditions of lower IFNβ levels an adaptive immune response can lead to tumor elimination. Such an effect would thus not be observed in RAG2$^{-/-}$ mice lacking lymphocytes.

In order to qualitatively examine the tumors that emerged following anti-IFNAR1 mAb treatment, we harvested tumors from RAG2$^{-/-}$ or WT mice following day 65 mAb treatment and generated cell lines. The tumorigenic potential of the harvested tumor cells was then assessed by transplantation into IFNAR1$^{-/-}$ and RAG2$^{-/-}$ mice. In all cases the

harvested tumor cells showed progressive growth in IFNAR1$^{-/-}$ mice but not in RAG2$^{-/-}$ hosts, suggesting that the cells continued to secrete IFNβ (Figure 9). Taken together, these data demonstrate that in both WT and RAG2$^{-/-}$ mice, poorly immunogenic progressor sarcomas can be maintained in a protracted state of equilibrium due to local production of type I IFN and its actions on host cells.

Figure 6. Detectable IFNβ in the sera of mice bearing stable masses. Sera from mice bearing stable d103m503.IFNβ tumor masses (beyond day 60 post-transplant) was tested for the presence of measurable IFN using a standard CPE assay (A). One positive serum sample was then assayed in the presence of anti-IFNAR1 (MAR1-5A3), anti-IFNβ (MIB-5E9), anti-panIFNα (TIF-C35), or anti-IFNγ (H22) mAb's as shown in (B).

A


3.0
2.5
2.0
1.5
1.0
0.5
0.0
A 540 nm
0.0001
0.001
0.01
0.1
1
10
100
Sera (µl/well)
d103m503.IFNβ sup
Normal serum
Mouse #1 serum
Mouse #2 serum
Mouse #3 serum
Mouse #4 serum

B

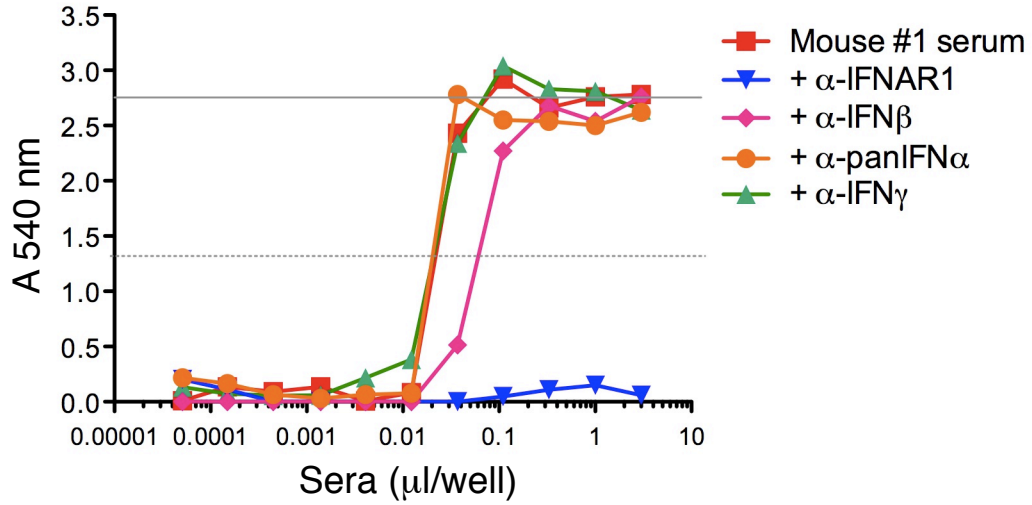

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A 540 nm
0.00001
0.0001
0.001
0.01
0.1
1
10
Sera (µl/well)
Mouse #1 serum
+ α-IFNAR1
+ α-IFNβ
+ α-panIFNα
+ α-IFNγ

Figure 7. IFNα/β receptor blockade at day 65 or day 100 reverses IFNβ-induced tumor equilibrium in RAG2$^{-/-}$ mice. (A) RAG2$^{-/-}$ mice bearing stable masses following injection of d103m503.IFNβ tumor cells were treated beginning at day 65 with either anti-IFNAR1 mAb MAR1-5A3 (n=10) or isotype control mAb GIR-208 (n=8) and tumor size was monitored over time. (B) RAG2$^{-/-}$ mice with stable masses were treated with anti-IFNAR1 mAb beginning instead at day 100 (n=8). Each line represents an individual mouse. A significant fraction of mice treated with control mAb eventually died due to chronic IFN exposure.

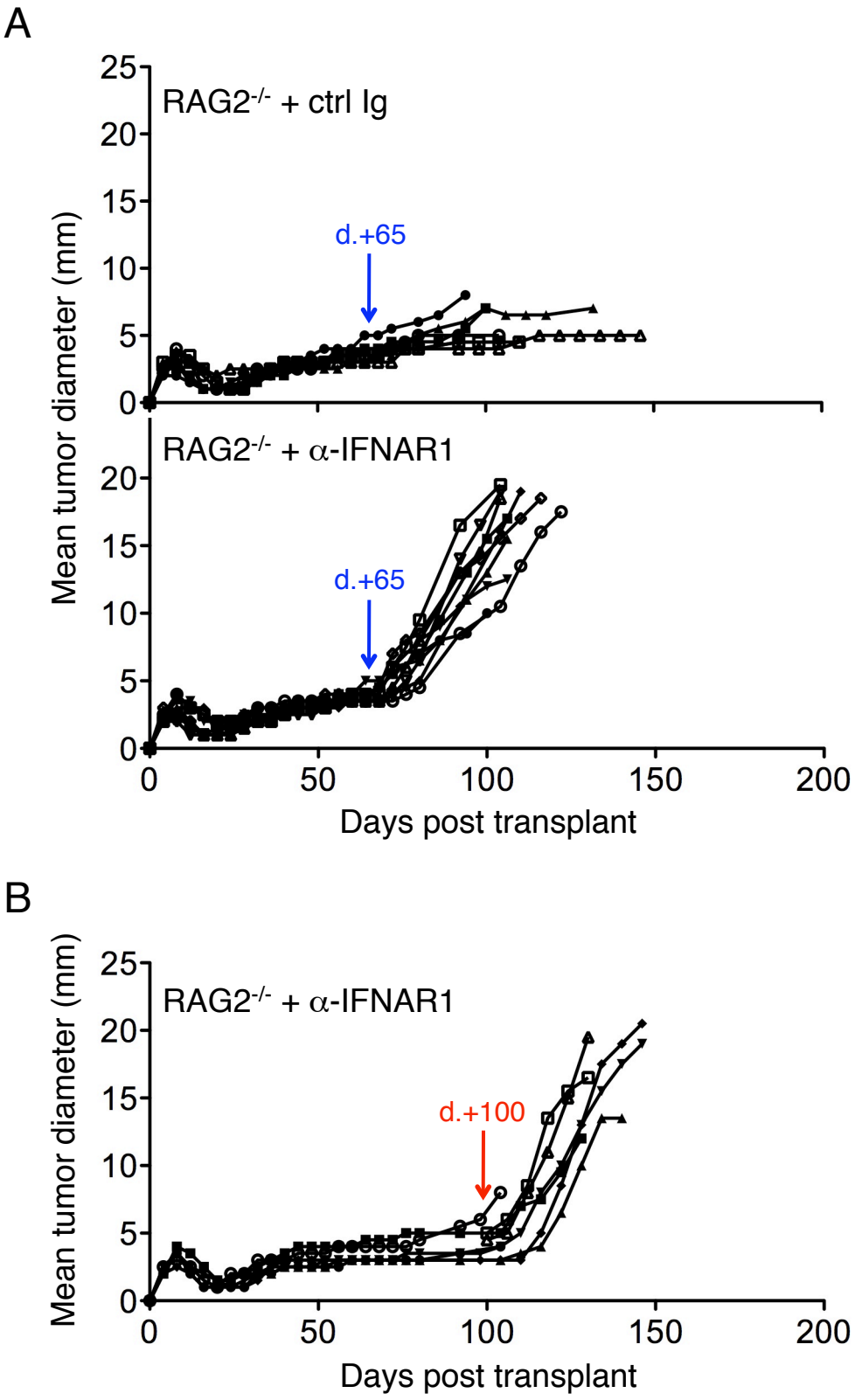

A
RAG2-/- + ctrl Ig
d.+65
RAG2-/- + α-IFNAR1
d.+65
Mean tumor diameter (mm)
Days post transplant
B
RAG2-/- + α-IFNAR1
d.+100
Mean tumor diameter (mm)
Days post transplant

Figure 8. Disruption of IFNβ-mediated equilibrium state in WT mice by IFNα/β receptor blockade at day 65. WT mice with stable masses following d103m503.IFNβ injection were treated beginning at day 65 with either anti-IFNAR1 mAb MAR1-5A3 (n=8) or isotype control mAb GIR-208 (n=6) and tumor growth was monitored.

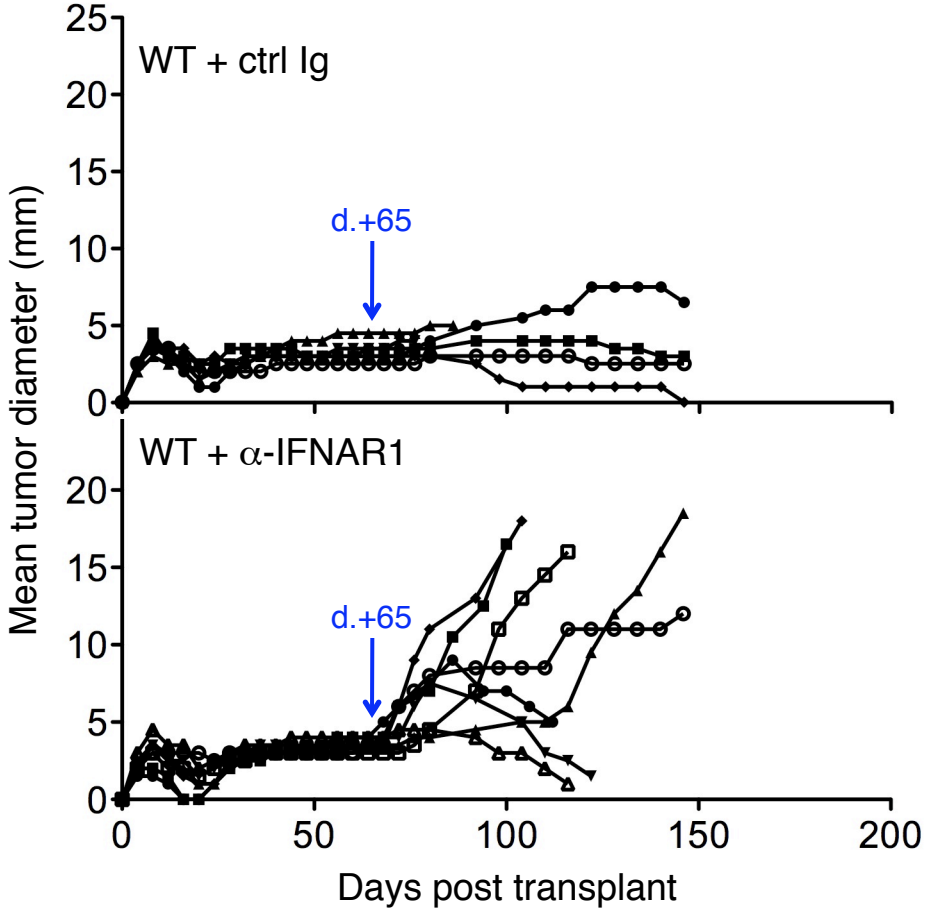
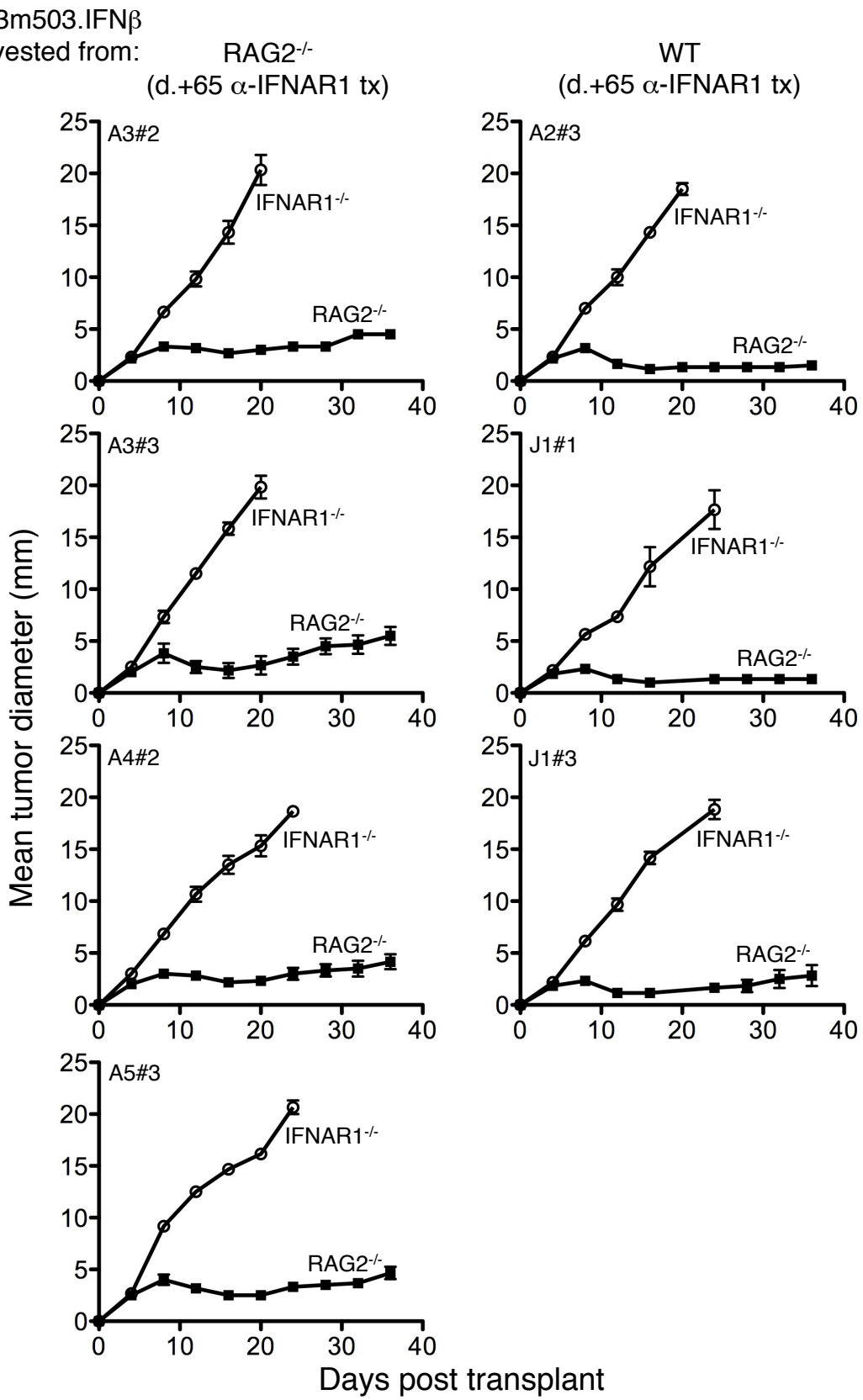

WT + ctrl Ig
d.+65
WT + α-IFNAR1
d.+65
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
50
100
150
200

Figure 9. Tumors harvested from RAG2$^{-/-}$ and WT mice following disrupted equilibrium are tumorigenic in IFNAR1$^{-/-}$ mice and still secrete IFNβ. Tumor cell lines established following anti-IFNAR1 mAb treatment were retransplanted into groups of IFNAR1$^{-/-}$ and RAG2$^{-/-}$ mice (n=3/group) and tumor growth was measured over time. The growth of 4 tumors originally harvested from RAG2$^{-/-}$ mice and 3 tumors harvested from WT mice is shown.


d103m503.IFNβ
harvested from:
RAG2-/-
(d.+65 α-IFNAR1 tx)
WT
(d.+65 α-IFNAR1 tx)
A3#2
A2#3
A3#3
J1#1
A4#2
J1#3
A5#3
IFNAR1-/-
RAG2-/-
Mean tumor diameter (mm)
Days post transplant

Critical Role for Both Hematopoietic and Nonhematopoietic Host Cell IFNα/β Sensitivity During Control of IFNβ-Secreting Tumors

We next wanted to delineate the role of hematopoietic versus nonhematopoietic type I IFN sensitivity for control of IFNβ-expressing tumors. Bone marrow chimeras were therefore generated as previously described (see Chapter 3), and we challenged control and chimeric mice with IFNβ-secreting d103m503 and d97m915 tumor cells (Figure 10). Similar to IFNAR1$^{-/-}$ and WT controls, these tumors displayed progressive growth in IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ chimeras but formed small stable masses in WT→WT chimeras. When injected into WT→IFNAR1$^{-/-}$ chimeras (IFNα/β-responsive only in hematopoietic cells), we again observed progressive tumor growth, indicating that IFNβ's actions on the host hematopoietic compartment is not sufficient for tumor control. Conversely, chimeras with IFNα/β sensitivity only in nonhematopoietic cells (IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras) also failed to control tumor growth, yet exhibited a slightly different phenotype. In these mice, tumors grew initially then began to level off upon reaching average diameters around 10mm, and generally remained stable in size until mice eventually manifested symptoms of IFN toxicity. We also noted differences in the gross appearance of tumors, since they did not protrude to the same degree (as seen in the absence of nonhematopoietic IFNα/β sensitivity) while becoming increasing necrotic toward their centers (Figure 11). These findings reveal that while IFNβ's actions on nonhematopoietic cells provide some inhibition to tumor growth, its activity on both hematopoietic and nonhematopoietic cells are required for full control.

Given prior studies showing no role for adaptive immune cells during tumor control, we performed similar experiments to dissect the requirements for host cell IFNα/β sensitivity in the absence of adaptive immunity. For this purpose, we generated two sets of bone marrow chimeras, both of which lacked T and B lymphocytes (Figure 12A). In the first group, lethally irradiated $RAG2^{-/-}IFNAR1^{-/-}$ mice were reconstituted with $RAG2^{-/-}$ FLCs, yielding mice responsive to IFNα/β in the innate immune compartment but not in nonhematopoietic tissues. In the converse type of chimera, lethally irradiated $RAG2^{-/-}$ mice were reconstituted with $RAG2^{-/-}IFNAR1^{-/-}$ FLCs, producing mice with type I IFN responsiveness in the nonhematopoietic compartment but not in innate immune cells. Analysis of splenocytes for IFNAR1 expression confirmed the expected phenotypes of these chimeras and also showed normal spleen cellularity (Figure 12B-C), suggesting adequate hematopoietic reconstitution. When transplanted with IFNβ-expressing tumor cells, we observed very similar phenotypes as seen with $IFNAR1^{-/-}$ chimeras (Figure 13). These data therefore demonstrate that cells of both the innate immune and nonhematopoietic host compartments constitute the critical targets for control of IFNβ-expressing tumor cells in vivo.

Figure 10. Requirement for both hematopoietic and nonhematopoietic host IFNα/β sensitivity for control of IFNβ-producing tumors. Groups of control IFNAR1$^{-/-}$ and WT mice as well as bone marrow chimeric mice with selective IFNα/β sensitivity were injected with 1x10^{6} d103m503.IFNβ (A) or d97m915.IFNβ (B) tumor cells and growth was monitored over time. Data represent n=4-5 mice/group from two independent experiments.

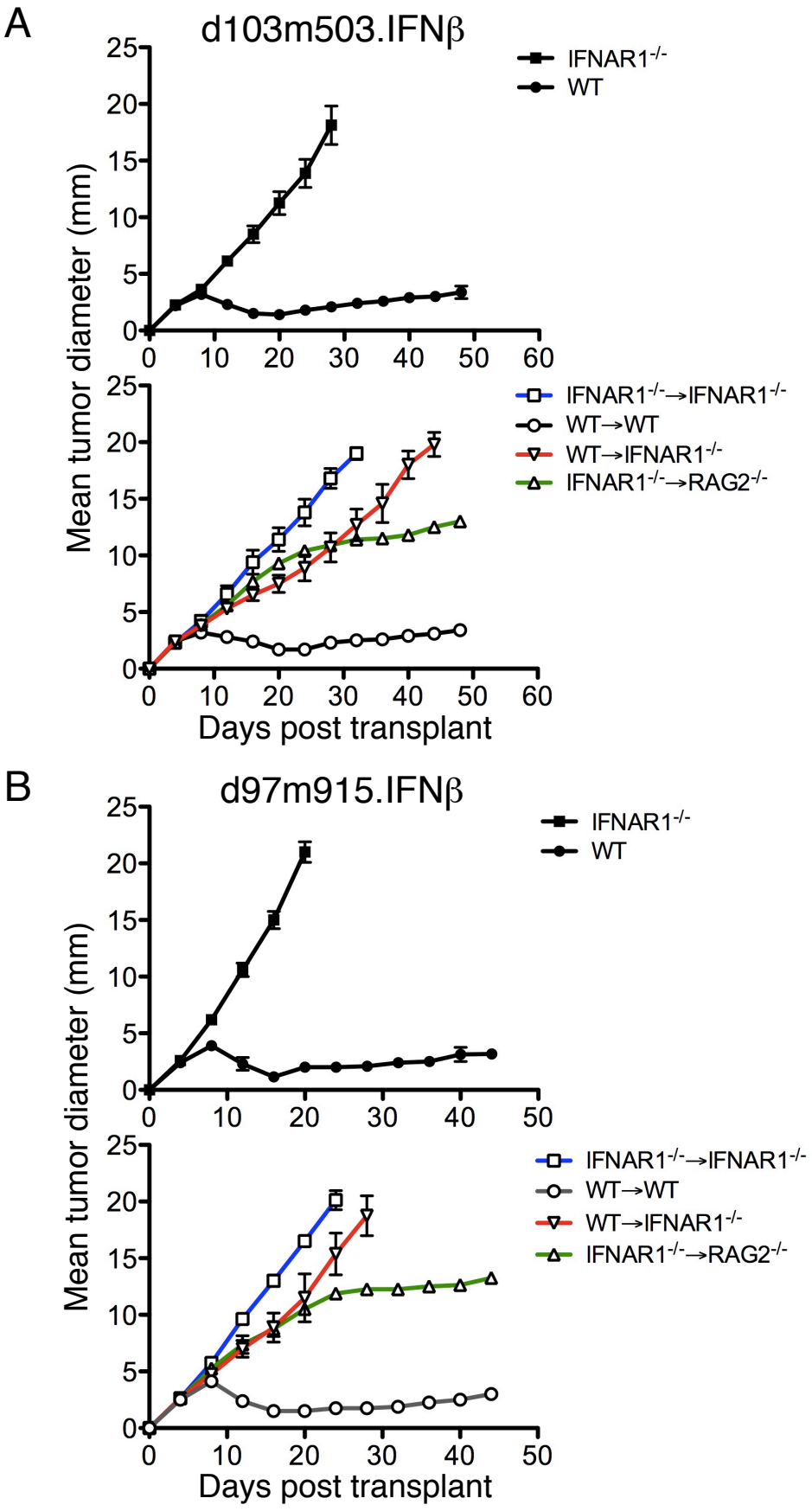
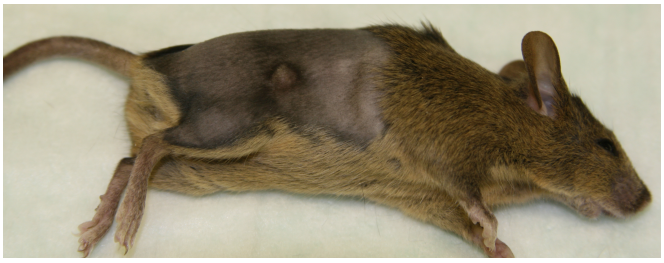
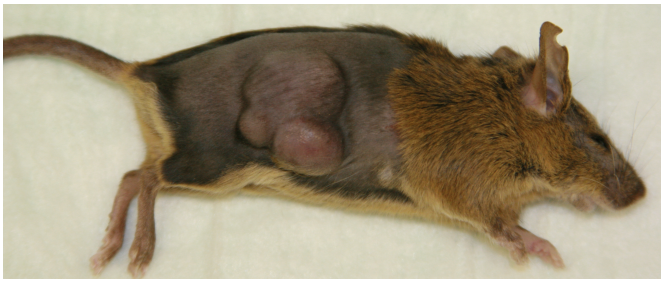

A
d103m503.IFNβ
IFNAR1-/-
WT
Mean tumor diameter (mm)
IFNAR1-/-→IFNAR1-/-
WT→WT
WT→IFNAR1-/-
IFNAR1-/-→RAG2-/-
Days post transplant
B
d97m915.IFNβ
IFNAR1-/-
WT
Mean tumor diameter (mm)
IFNAR1-/-→IFNAR1-/-
WT→WT
WT→IFNAR1-/-
IFNAR1-/-→RAG2-/-
Days post transplant

Figure 11. Differences in the gross appearance of d103m503.IFNβ tumors in the presence or absence of nonhematopoietic IFNα/β sensitivity. External appearance of tumors is shown for representative WT, IFNAR1$^{-/-}$, WT→IFNAR1$^{-/-}$, and IFNAR1$^{-/-}$ →RAG2$^{-/-}$ mice at the indicated day following injection of 1x10^{6} d103m503.IFNβ tumor cells. In the absence of nonhematopoietic IFNα/β responsiveness (WT→IFNAR1$^{-/-}$ mice), tumors grew progressively and protruded as a well-demarcated mass as observed in IFNAR1$^{-/-}$ controls. In the presence of hematopoietic IFNα/β sensitivity (IFNAR1$^{-/-}$ →RAG2$^{-/-}$ mice), tumors grew in a deeper location with less depth and became increasingly necrotic at the center, while also eventually stabilizing in size. Growth phenotypes of WT→WT and IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ control chimeras recapitulated that seen in WT and IFNAR1$^{-/-}$ mice, respectively (data not shown).

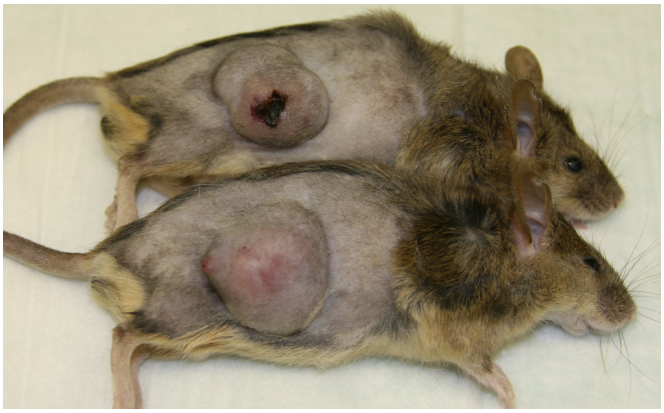
WT
d.75



IFNAR1$^{-/-}$
d.30



WT$\rightarrow$IFNAR1$^{-/-}$
d.40



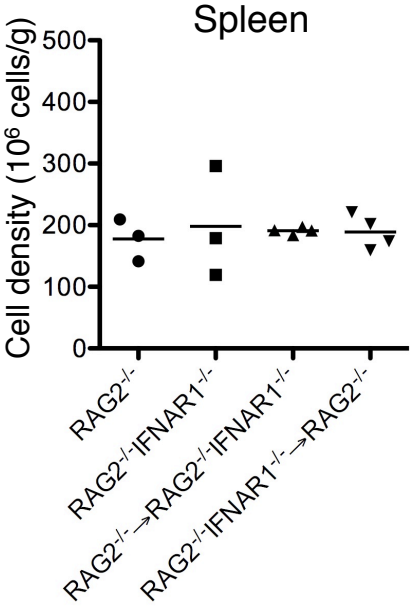
IFNAR1$^{-/-}\rightarrow$RAG2$^{-/-}$
d.75

Figure 12. Generation of bone marrow chimeras with selective IFNα/β responsiveness in nonhematopoietic or innate immune cells. (A) Strategy for producing chimeras with either innate immune or nonhematopoietic IFNα/β sensitivity. (B) Spleen cellularity from a representative cohort of bone marrow chimeras was assessed. (C) IFNAR1 expression levels on several immune cell subsets from the spleens of chimeric mice. Shown is the percent $IFNAR1^{+}$ cells within the indicated gate for n=3-4 mice/group.

A

Donor HSCs	Irradiated Recipient Mice	IFNα/β Sensitivity	
		Innate	Nonhematopoietic
$RAG2^{-/-}$	$RAG2^{-/-}IFNAR1^{-/-}$	+	-
$RAG2^{-/-}IFNAR1^{-/-}$	$RAG2^{-/-}$	-	+

B



C

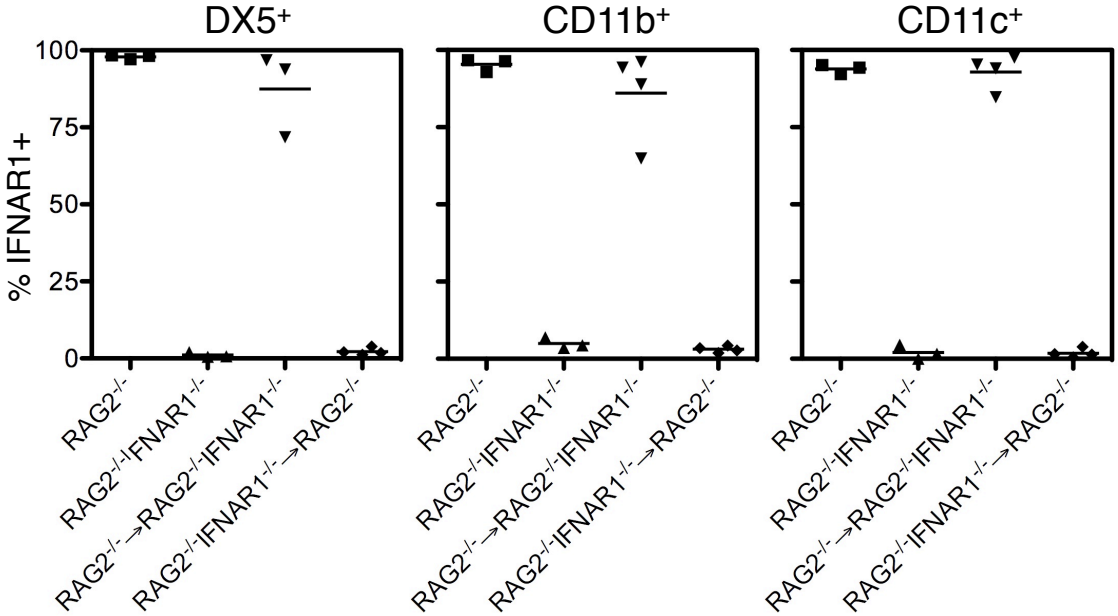
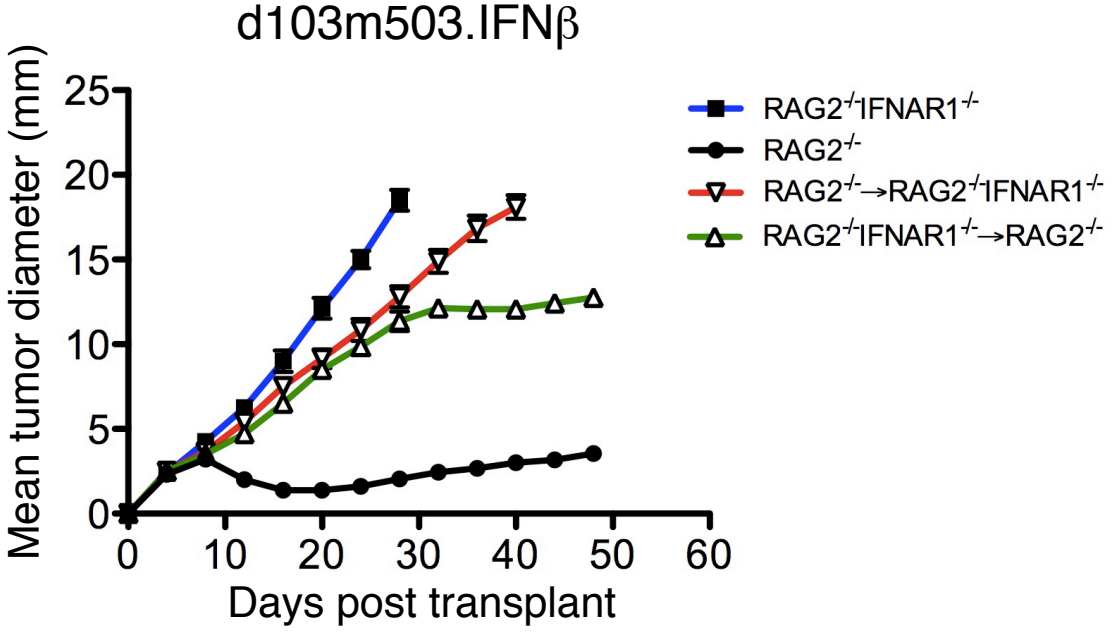
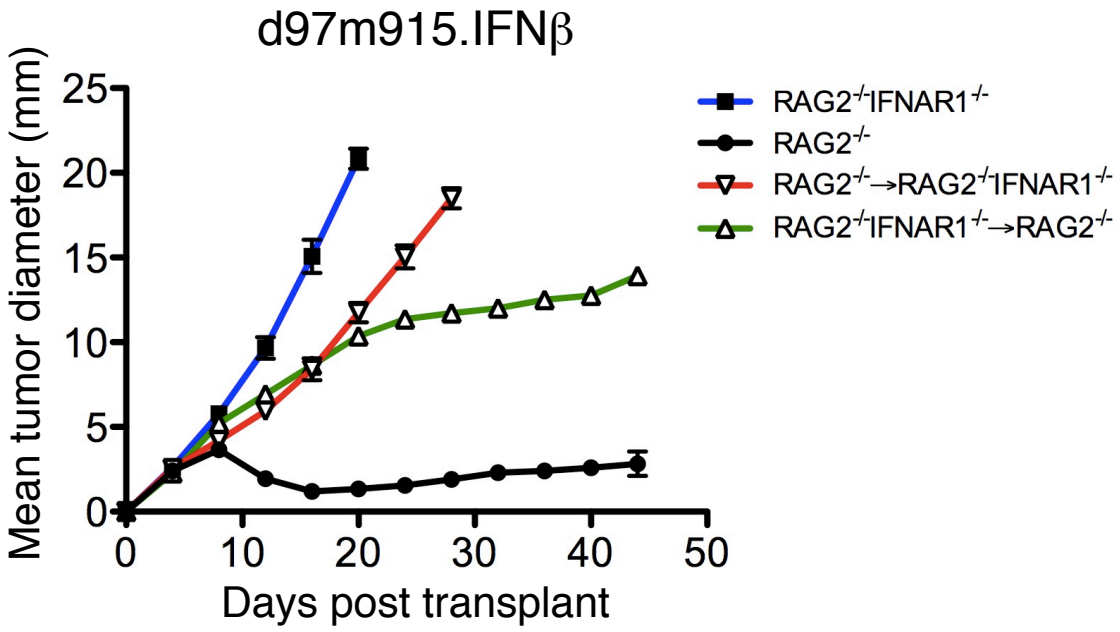


Figure 13. Important role for IFNβ's actions on both innate immune and nonhematopoietic cells. Cohorts of $RAG2^{-/-}IFNAR1^{-/-}$ and $RAG2^{-/-}$ control mice or $RAG2^{-/-}\rightarrow RAG2^{-/-}IFNAR1^{-/-}$ and $RAG2^{-/-}IFNAR1^{-/-}\rightarrow RAG2^{-/-}$ bone marrow chimeras were injected with $1x10^6$ d103m503.IFNβ (A) or d97m915.IFNβ (B) tumor cells and growth was monitored. Plotted are the mean tumor diameters ± s.e.m. for n=7-10 mice/group from two independent experiments.

A


d103m503.IFNβ
Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
50
60
Days post transplant
RAG2-/-IFNAR1-/-
RAG2-/-
RAG2-/-→RAG2-/-IFNAR1-/-
RAG2-/-IFNAR1-/-→RAG2-/-

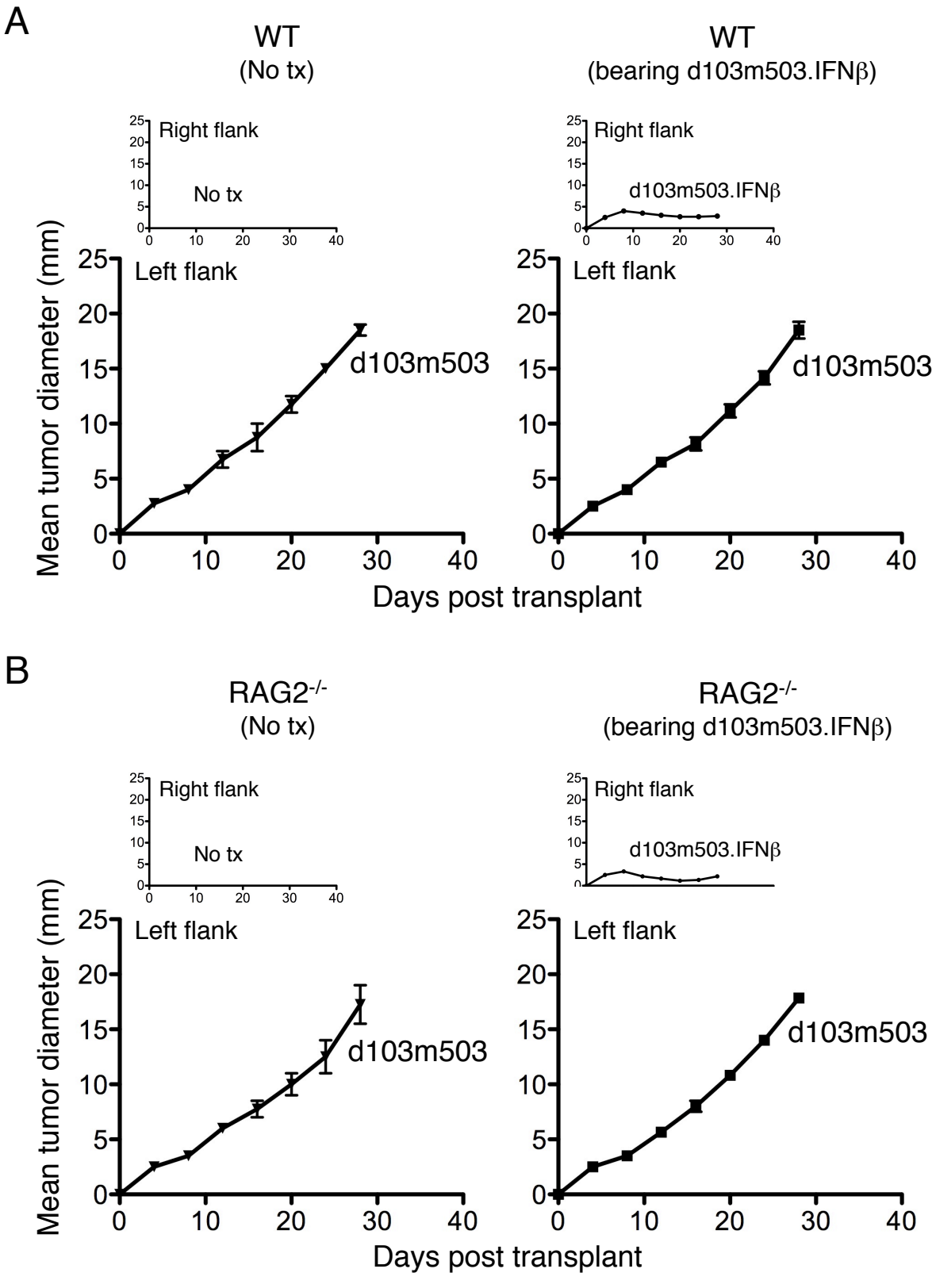
B


d97m915.IFNβ
Mean tumor diameter (mm)
25
20
15
10
5
0
0
10
20
30
40
50
Days post transplant
RAG2-/-IFNAR1-/-
RAG2-/-
RAG2-/-→RAG2-/-IFNAR1-/-
RAG2-/-IFNAR1-/-→RAG2-/-

Local IFNβ Production is Required for Tumor Control

To test whether IFNβ-producing tumor cells might provide protection against growth of the parental tumor at a remote site, we challenged WT and RAG2$^{-/-}$ mice with IFNβ-producing d103m503.IFNβ cells on one flank and parental d103m503 cells on the opposite flank. As shown in Figure 14, we found no evidence for the induction of concomitant immunity, as growth of the parental d103m503 tumor was identical in both uninjected and d103m503.IFNβ-bearing hosts. In addition, a similar result was observed when mice bearing stable masses (at day 65 following d103m503.IFNβ injection) were injected with parental tumor cells on the opposite flank (data not shown). Since IFNβ-producing tumor cells do, in fact, provide protection when mixed and coinjected with parental tumor cells (discussed in the next section), these findings suggest that local but not systemic delivery of IFNβ is efficacious in this model – a notion also supported by a very recent study utilizing local delivery of IFNα (204). In light of experiments outlined in the next section, which demonstrate lymphocyte-dependent protection at lower levels of IFNβ production, it would be interesting to repeat these experiments using an inoculum of low-expressing tumor cells. Nevertheless, the current results provide support for the idea that high-expressing tumors do not elicit an effective adaptive immune response.

Figure 14. Lack of concomitant immunity in mice bearing IFNβ-producing tumor cells. Groups of either untreated or d103m503.IFNβ-injected WT (A) or RAG2$^{-/-}$ (B) mice were injected on the opposite flank with 1x10^{6} parental d103m503 tumor cells. Shown is the growth of IFNβ-producing (above) and parental d103m503 (below) tumor cells over time (both were injected on the same day). Results represent the mean tumor diameter ± s.e.m. for n=2-3 mice/group. Similar data was also obtained when WT or RAG2$^{-/-}$ mice at day 65 following d103m503.IFNβ injection were challenged with parental d103m503 tumor cells (data not shown).


A
WT
(No tx)
WT
(bearing d103m503.IFNβ)
Right flank
No tx
Left flank
d103m503
Right flank
d103m503.IFNβ
Left flank
d103m503
Mean tumor diameter (mm)
Days post transplant
B
RAG2-/-
(No tx)
RAG2-/-
(bearing d103m503.IFNβ)
Right flank
No tx
Left flank
d103m503
Right flank
d103m503.IFNβ
Left flank
d103m503
Mean tumor diameter (mm)
Days post transplant

Dose-Dependent Effects of IFNβ Ranging from Long-term Tumor Persistence to Lymphocyte-Dependent Tumor Elimination Without Toxicity

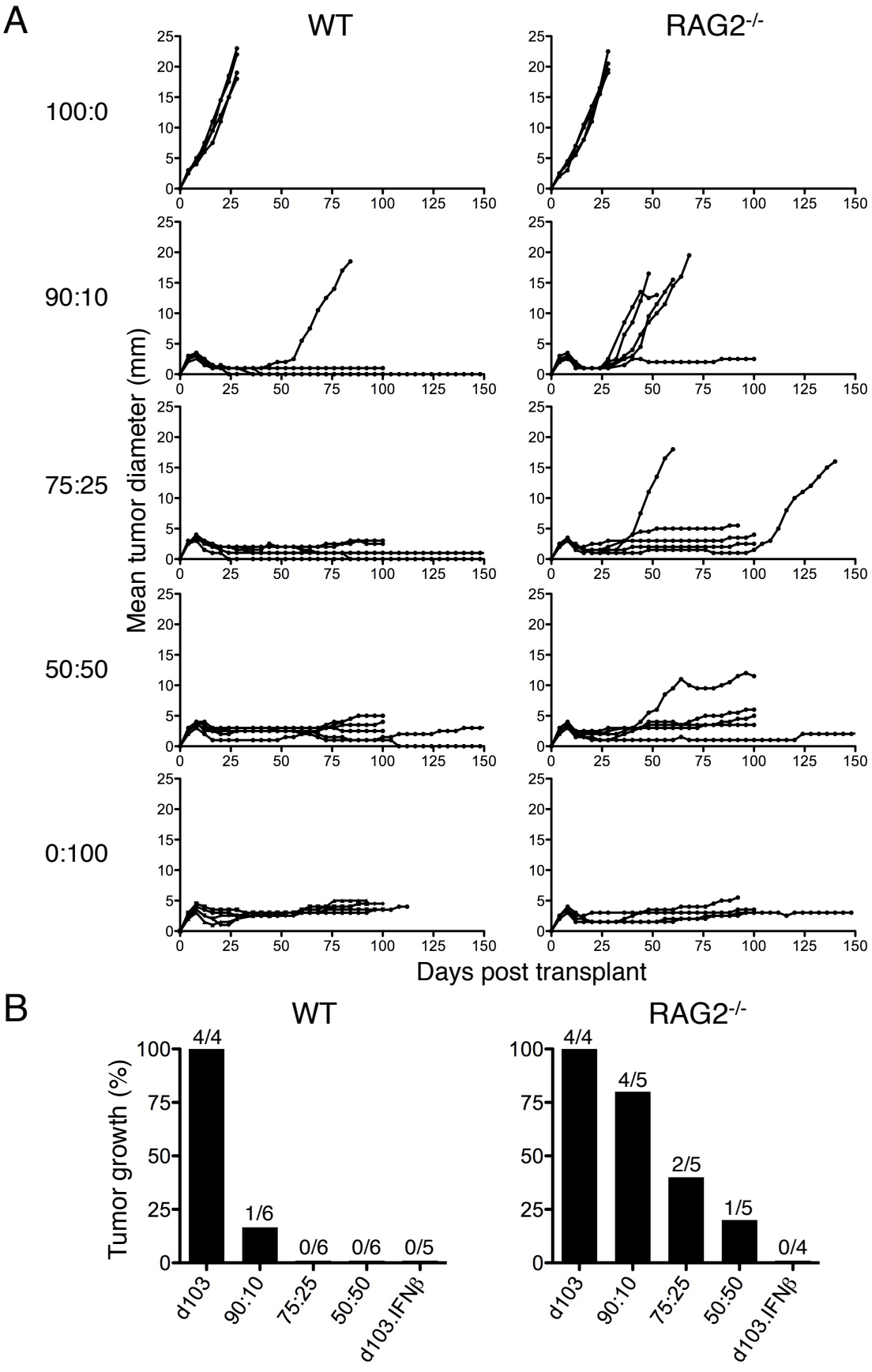
We have previously observed that production of high levels of IFNβ within the tumor microenvironment can mediate a reversible state of prolonged tumor persistence, yet also eventually results in significant toxicity due to chronic IFN exposure. In subsequent experiments, we have therefore assessed whether production of lower levels of IFNβ within the tumor can also provide protection without toxicity. To obtain tumor cell mixtures with varying levels of IFNβ production, we have mixed parental d103m503 and IFNβ-expressing d103m503.IFNβ cells at different ratios prior to injection into WT or $RAG2^{-/-}$ hosts. As plotted in Figure 15, we again observed progressive growth of d103m503 cells while d103m503.IFNβ cells formed stable masses in both WT and $RAG2^{-/-}$ mice. Similarly, 50:50 tumor cell mixtures also developed into persistent lesions in both WT and $RAG2^{-/-}$ recipients, though growth was seen in 1/5 $RAG2^{-/-}$ mice. This trend continued at the 75:25 cell ratio, since we found that most WT mice exhibited stable masses (complete elimination was seen in 2/6 mice), whereas all $RAG2^{-/-}$ mice developed stable masses and progressive growth was eventually observed in 2/5 mice. With low-dose IFNβ (at the 90:10 ratio), this difference was most dramatic, as 4/6 WT mice completely eliminated the tumor mass (1/6 showed progressive growth, and 1/6 developed a very small lesion) while progressive growth was observed in 4/5 $RAG2^{-/-}$ mice after an initial period of tumor control.

The differential tumor control in WT and RAG2$^{-/-}$ mice at low doses of IFNβ reveals an important function for lymphocytes in this setting. Interestingly, RAG2$^{-/-}$ mice were still capable of restricting tumor growth initially, though outgrowth eventually occurred around day 30 or beyond. This is in contrast to robust growth of the 90:10 mixture in IFNAR1$^{-/-}$ hosts (see Figure 17 in this chapter), suggesting that innate immune mechanisms can mediate initial control yet adaptive immune function is ultimately required for long-term tumor control as well as tumor elimination. At higher doses, however, tumor cells persist and lymphocytes are not, in fact, required for control of stable masses. In addition to successful tumor elimination in WT mice challenged with low-dose IFNβ-expressing tumor cells, we also found that mice did not manifest signs of IFN toxicity. Whereas no toxicity was evident in WT mice treated with the 90:10 tumor cell mixture, over half of WT mice injected with d103m503.IFNβ in this experiment eventually died due to chronic IFN exposure (Figure 15 and data not shown).

These findings may provide an explanation for the previously observed difference in tumor outgrowth between RAG2$^{-/-}$ and WT mice (each bearing stable masses after d103m503.IFNβ injection) upon anti-IFNAR1 mAb treatment at day 65 (see Figures 7-8). Whereas outgrowth occurred in all RAG2$^{-/-}$ mice, it was seen less frequently in WT mice. Moreover, a few tumors in WT mice initially grew and were then completely eliminated. Perhaps partial blockade of IFNβ's actions thus allowed for the generation or increased efficacy of an adaptive immune response, eventually leading to tumor elimination. This hypothesis would imply that high levels of IFNβ within the tumor are, in fact, inhibitory toward the adaptive immune response – a notion supported by the

observation that high-expressing tumors persist as stable lesions, whereas low-expressing tumors are completely eliminated.

Figure 15. Elimination of tumors expressing lower levels of IFNβ is lymphocyte dependent. (A) Groups of WT and RAG2$^{-/-}$ mice were injected with mixtures of parental d103m503 and d103m503.IFNβ tumor cells (1x10^{6} total cells/mouse) and tumor growth was monitored over time. Ratios indicate composition of d103m503:d103m503.IFNβ cells. Each line represents an individual mouse. (B) Summary of percent tumor growth for each group (n=4-6 mice/group) is plotted. Data from two independent experiments with similar results are shown.

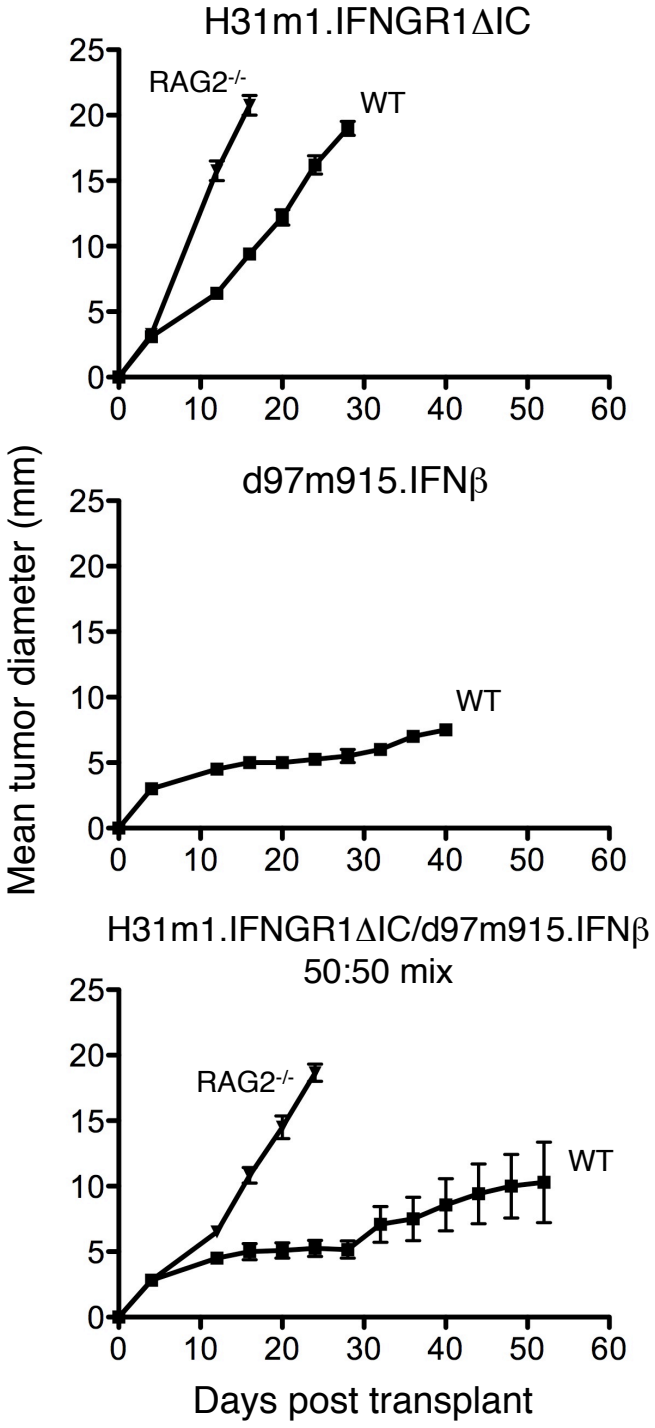

A
WT
RAG2-/-
100:0
90:10
75:25
50:50
0:100
Mean tumor diameter (mm)
Days post transplant
B
WT
RAG2-/-
Tumor growth (%)
4/4
1/6
0/6
0/6
0/5
4/4
4/5
2/5
1/5
0/4
d103
90:10
75:25
50:50
d103.IFNβ

Growth Inhibition of IFNγ-Unresponsive Tumor Cells via Local IFNβ Production is Lymphocyte Dependent

Although we have shown that coinjection of IFNβ-producing tumor cells with the parental progressor tumor is protective, we wanted to test whether local IFNβ production could also inhibit the growth of antigenically unrelated and IFNγ-unresponsive progressor tumor cells. We therefore coinjected a 50:50 mixture of d103m503.IFNβ cells and H31m1.IFNGR1ΔIC cells (which overexpress a dominant-negative form of the IFNγ receptor component IFNGR1 and hence are IFNγ-insensitive). When injected individually, H31m1.IFNGR1ΔIC cells formed rapidly growing tumors in both $RAG2^{-/-}$ and WT hosts, whereas d97m915.IFNβ tumor growth reaches a plateau around 5x5 mm and remains stable (Figure 16). In contrast, growth of the 50:50 tumor cell mixture was rapid in $RAG2^{-/-}$ mice but significantly inhibited in WT mice, though in a fraction of mice tumors eventually grew out. The actions of lymphocytes are therefore important in mediating delayed growth of this tumor mixture. Since the H31m1.IFNGR1ΔIC tumor is still responsive to IFNα/β, it is possible that tumor cell apoptosis mediated by local IFNβ is contributing to the development of an adaptive immune response – though rapid growth in $RAG2^{-/-}$ mice rules out direct anti-proliferative or pro-apoptotic effects as the primary cause of the delay. Alternately, local production of IFNβ may be inducing MHC class I upregulation on IFNγ-unresponsive H31m1 cells, thus rescuing the potential defect in adaptive immune recognition. It would be interesting to assess whether lower levels of local IFNβ production are more effective in mediating complete rejection of

H31m1.IFNGR1ΔIC cells as we observed with the parental d103m503 tumor.

Figure 16. Inhibition of IFNγ-unresponsive tumor cell growth by local production of IFNβ is mediated by lymphocytes. Groups of $RAG2^{-/-}$ or WT mice were injected with $1x10^6$ IFNγ-insensitive H31m1.IFNGR1ΔIC cells, IFNβ-producing d97m915.IFNβ cells, or a 50:50 mixture ($2x10^6$ total cells) of both cell lines and tumor growth was monitored over time. Data are presented as mean tumor diameter ± s.e.m. of n=2-3 ($RAG2^{-/-}$) or n=5-6 (WT) mice per group.


H31m1.IFNGR1ΔIC
RAG2-/-
WT
d97m915.IFNβ
WT
H31m1.IFNGR1ΔIC/d97m915.IFNβ
50:50 mix
RAG2-/-
WT
Mean tumor diameter (mm)
Days post transplant
0
5
10
15
20
25
0
10
20
30
40
50
60

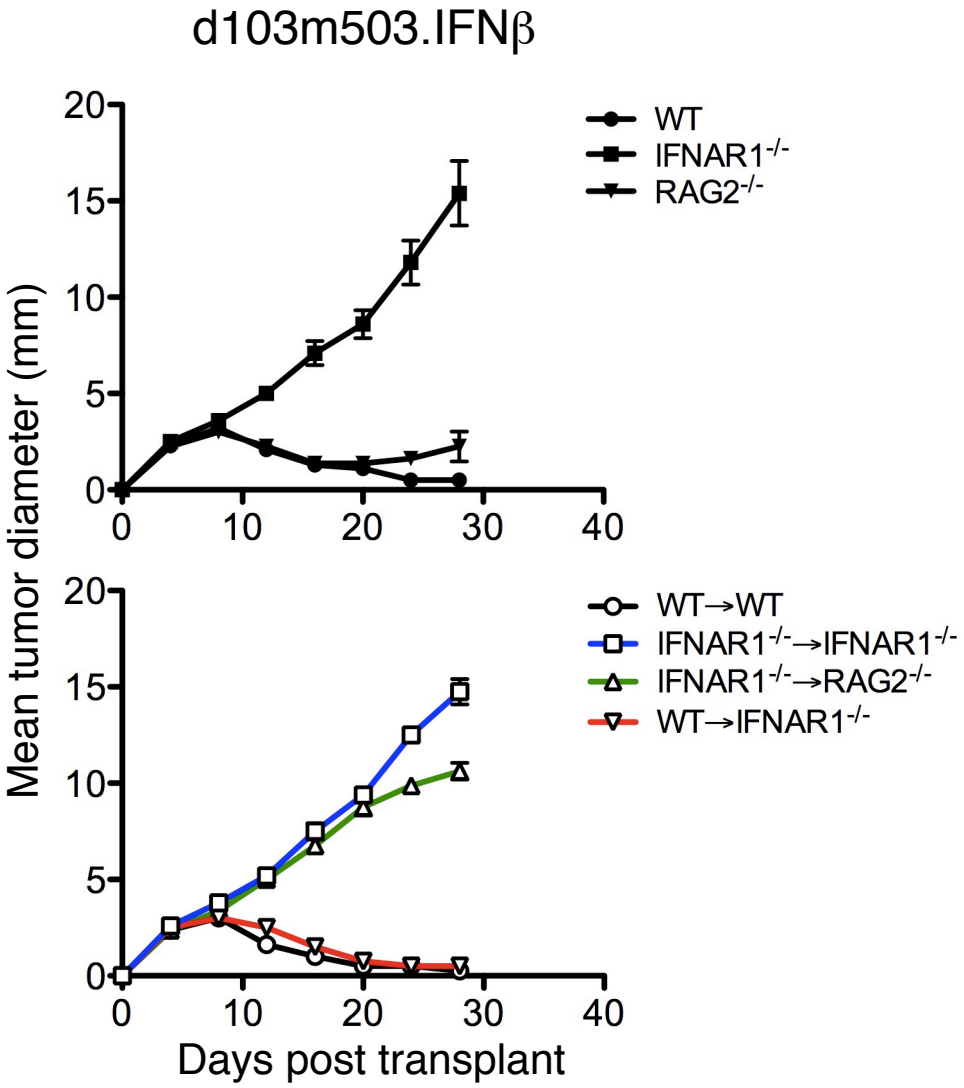
Tumor Rejection via Low-Dose IFNβ Reveals Tissue-Selective Requirements for Host Cell IFNα/β Sensitivity

Since the elimination of tumors with low-dose IFNβ production was found to require the actions of adaptive immunity, we postulated that the critical host cell targets of IFNβ may in this case be distinct from those with high-dose IFNβ. To test this hypothesis, we again utilized bone marrow chimeras with selective type I IFN responsiveness in hematopoietic or nonhematopoietic cells. Groups of control and chimeric mice were challenged with a 90:10 ratio of parental to IFNβ-expressing d103m503 tumor cells and growth was monitored over time (Figure 17). As in control mice, this tumor challenge was rejected in WT→WT chimeras but showed unrestrained growth in IFNAR1$^{-/-}$→IFNAR1$^{-/-}$ chimeras. Unexpectedly, IFNβ's actions on hematopoietic cells were now sufficient for tumor elimination, as WT→IFNAR1$^{-/-}$ chimeras exhibited an identical phenotype as WT→WT controls. In contrast, tumors in IFNAR1$^{-/-}$→RAG2$^{-/-}$ chimeras progressed initially then eventually leveled off, as seen with high-dose IFNβ. Nonhematopoietic cells could thus provide some protection against unrestrained tumor expansion, yet it is IFNβ's actions on hematopoietic cells (and the actions of lymphocytes as determined previously) that ultimately mediate tumor elimination.

With the injection of d103m503 tumor cells expressing higher levels of IFNβ, the outcome of tumor challenge in WT→IFNAR1$^{-/-}$ chimeras was dramatically different (see Figure 10A). Progressive growth in this setting indicates that high doses of IFNβ are, in

fact, inhibitory toward the anti-tumor actions of host hematopoietic cells. Taken together, these data reveal that (i) levels of type I IFN within the tumor environment represent an important parameter determining the efficacy of IFNα/β immunotherapy, and (ii) successful anti-tumor responses are mediated by exogenous IFNα/β's actions on host hematopoietic cells and require the actions of lymphocytes for complete tumor elimination.

Figure 17. Host hematopoietic type I IFN sensitivity is both necessary and sufficient for elimination of low dose IFNβ-producing tumors. Groups of WT, IFNAR1$^{-/-}$, and RAG2$^{-/-}$ control mice or bone marrow chimeric mice with selective IFNα/β responsiveness were challenged with 1x10^{6} d103m503.IFNβ tumor cells and growth was measured over time. Results represent mean tumor diameter ± s.e.m. of at least 4 mice/group.


d103m503.IFNβ
Mean tumor diameter (mm)
Days post transplant
WT
IFNAR1-/-
RAG2-/-
WT→WT
IFNAR1-/-→IFNAR1-/-
IFNAR1-/-→RAG2-/-
WT→IFNAR1-/-
0
5
10
15
20
0
10
20
30
40

DISCUSSION

Given the therapeutic efficacy of exogenously administered IFNα for the treatment of several types of human cancer, we wanted to explore whether the actions of IFNα/β in promoting anti-tumor immunity in a therapeutic setting was similar to that of endogenously produced type I IFN during a naturally occurring anti-tumor response. Prior work from several groups using mouse models of immunotherapy suggests that exogenous IFNα/β functions predominantly in a host stimulatory capacity, rather than through anti-proliferative or pro-apoptotic effects directly on the tumor. We therefore utilized MCA-induced progressor tumors derived from IFNAR1$^{-/-}$ mice, to ensure that all effects of IFNα/β treatment are due to actions on host cells. Although the parental tumor lines are poorly immunogenic and resistant to immune control, growth of the IFNβ-producing tumors could be controlled when injected into WT and RAG2$^{-/-}$, but not IFNAR1$^{-/-}$ hosts.

While tumors did not grow progressively, stable masses were observed to persist for long periods of time until a fraction of mice eventually died from IFN-mediated toxicity. To assess whether IFNβ-producing tumor cells persisted within these stable masses, we treated mice either at day 65 or day 100 with anti-IFNAR1 mAb. Such treatment could effectively interrupt IFNβ-mediated tumor equilibrium, leading to tumor outgrowth in both RAG2$^{-/-}$ and WT mice. In addition, harvest and retransplant of the resulting tumors showed that they still produced IFNβ and could be controlled in RAG2$^{-/-}$ mice, yet were highly tumorigenic in IFNAR1$^{-/-}$ hosts. Finally, experiments using bone

marrow chimeras demonstrated that IFNα/β sensitivity in both the nonhematopoietic and innate immune compartments was required for tumor control, though adaptive immunity was dispensable.

Since mice exhibiting stable masses eventually manifested significant toxicity due to chronic IFNβ exposure, we examined whether tumor cells expressing lower levels of IFNβ could be controlled without inducing toxicity. In this case, we observed that tumors were effectively eliminated with no remaining lesions (and no toxicity) in the majority of WT mice. Yet, adaptive immunity was found to be required, as low-dose IFNβ-producing tumor cells eventually grew progressively in $RAG2^{-/-}$ mice following a brief initial period of tumor control. The enhanced efficacy with low-dose IFNβ and the requirement for adaptive immunity, suggested that IFNβ at high levels within the tumor environment was, in fact, inhibitory toward the generation of a protective adaptive response and tumors therefore persisted. When we explored the cellular targets required for elimination of low-dose IFNβ-producing tumors, we found that in contrast to earlier observations, hematopoietic IFNα/β sensitivity was necessary and sufficient for tumor elimination. These data therefore reveal an interesting difference in the cellular mediators and ultimate outcome depending on levels of exogenous IFNβ within the tumor microenvironment. At high levels, IFNβ can inhibit tumor growth through actions on both innate immune and nonhematopoietic cells, but is inhibitory toward the generation of fully protective adaptive immune responses, and tumors persisted in a state of prolonged and reversible equilibrium. Alternatively, IFNβ at lower doses could

mediate complete tumor elimination through its actions on hematopoietic cells, but this outcome now required adaptive immunity.

CHAPTER 6

Summary and Future Directions

SUMMARY

Building on previous work identifying the type I (IFNα/β) and type II (IFNγ) interferons as key effector molecules in immunologic protection against tumor development, the overarching goal of this thesis was to further understand the critical host stimulatory functions mediated by these cytokines. Whereas prior studies have shown that tumor cell sensitivity to IFNγ, but not IFNα/β, is important, responsiveness within the host to both IFNα/β and IFNγ was found to be essential (11, 16). We reasoned that further characterization of the IFN-dependent anti-tumor response – and more specifically, the elucidation of the functionally relevant host cell targets of IFNα/β and IFNγ – would lead us closer to understanding the crucial immunomodulatory functions of the IFNs during this process.

In the first study, we took a descriptive approach to further examine the anti-tumor immune response to immunogenic tumors and its dependence on the IFNs. Using a new cohort of $RAG2^{-/-}$-derived regressor tumors from C57Bl/6 strain mice, we generalized original observations made with 129 tumors by showing that rejection of B6 regressors also required IFNγ signaling as well as host responsiveness to IFNα/β. To determine when during the immune response the respective IFNs were acting, we performed antibody blockade experiments and demonstrated that whereas the early actions of IFNα/β were sufficient for tumor rejection, the temporal requirement for IFNγ's actions was more prolonged. We further showed that in the absence of host sensitivity to either IFNα/β or IFNγ, generation of tumor-specific T cells with effector

activity is severely impaired, suggesting a central role in promoting the initiation of adaptive responses to tumors. Using mixtures of immunogenic and non-immunogenic tumor cells, we then confirmed that specific adaptive immune killing was responsible for rejection, since regressor tumor cells were selectively eliminated within tumor cell mixtures. Finally, because the type I IFN family consists of a large number of individual subtypes which might possess non-redundant functions in vivo, we examined whether IFNβ was essential during the rejection of transplantable tumors. IFNβ-deficient mice, however, showed no defect in their ability to reject immunogenic 129 regressor tumors, suggesting potential redundancy or a more prominent role for the IFNα subtypes.

Several pieces of data gleaned from these initial studies provide preliminary evidence that the IFNs might be performing distinct functions on the host during tumor rejection. In addition to their differing temporal requirements, analysis of $STAT1^{Y701F}$ mutant mice showed that growth of transplantable tumor cells was more aggressive in mice lacking responsiveness to both IFNα/β and IFNγ than in mice lacking sensitivity to one or the other. In addition, the identification of individual tumors that require only the actions of IFNα/β but not IFNγ (or vice versa) for their rejection, also implies their unique function. Although mechanisms contributing to allogeneic tumor rejection may be quite different from those involved in the rejection of immunogenic syngeneic tumors, we also observed that allogeneic rejection of aggressive progressor tumors required host sensitivity to IFNγ but not IFNα/β.

In the second study of this thesis, we have investigated the requirements for host cell sensitivity to IFNα/β during tumor rejection. Initial work confirmed and extended

prior studies demonstrating the importance of IFNα/β's actions on hematopoietic cells of the host. We now show that for rejection of two additional immunogenic RAG2$^{-/-}$ regressor tumors, H31m1 and d38m2, IFNα/β responsiveness in host hematopoietic cells is both necessary and sufficient for rejection. The hematopoietic compartment, however, includes all immune cells, and type I IFN can exert potent immunomodulatory functions on an array of cells. Therefore, we generated mixed bone marrow chimeras to specifically reconstitute IFNα/β sensitivity within either innate or adaptive immune cells. Using these chimeras, we found that cells of the innate immune compartment were the obligate targets of endogenous IFNα/β for rejection of H31m1 and d38m2 tumor cells. As a control, we verified the hematopoietic reconstitution in these chimeras, demonstrating normal rejection of F515 tumors cells, which require IFNγ signaling and adaptive immunity, but not IFNα/β responsiveness, for their rejection. Finally, we demonstrated that selective type I IFN responsiveness in innate immune cells was able to restore the defect in tumor-specific CTL priming, also confirming that T cells were, in fact, required for tumor rejection in innate IFNα/β-responsive chimeras.

Since type I IFN is a strong activator of NK cell function that has been shown to be important for NK cell-mediated anti-tumor responses (43), we asked whether NK cells were critical effectors during the rejection of immunogenic transplantable sarcomas. For these experiments, we utilized regressor tumors derived from C57Bl/6 strain RAG2$^{-/-}$ mice, allowing for depletion of NK cells by injection of anti-NK1.1 mAb PK136. When we examined three different B6 regressor tumors that required host sensitivity to IFNα/β for their rejection, we found that NK cell depletion had no effect their rejection. In

contrast, when we examined the role of CD8α^+ dendritic cells during tumor rejection, we observed a strong requirement for this immune subset (151). Using an in vitro cross-presentation assay, we furthermore showed that the direct actions of type I IFN on CD8α^+ DCs enhanced cross-priming of naïve CD8$^+$ T cells. Studies to ascertain whether CD8α^+ DCs are also essential direct targets of type I IFN during tumor rejection in vivo are currently ongoing.

In the third study, we examined the key cellular targets of IFNγ during the rejection of immunogenic tumors. Initial experiments provided additional evidence for the importance of tumor cell IFNγ sensitivity. Analysis of the GAR4 tumor (derived from IFNGR1$^{-/-}$xIFNAR1$^{-/-}$ mice) demonstrated that selective reconstitution of IFNγ responsiveness, but not IFNα/β sensitivity, could mediate rejection. When host cell IFN requirements were examined, however, responsiveness to both IFNγ and IFNα/β were required. In the course of these experiments, we noticed that rejection of the reconstituted GAR4.GR1 tumor showed a stronger requirement for host IFNα/β than IFNγ sensitivity, providing an additional hint that they might have non-overlapping functions on the host. Finally, to confirm the essential role of IFNγ's actions on the tumor, we also used RNAi to show that IFNGR1 knockdown in a regressor tumor normally responsive to IFNγ could now abrogate tumor rejection.

Since IFNγ sensitivity at the level of the host is also required for the rejection of RAG2$^{-/-}$ regressor tumors, we used bone marrow chimeras to examine the nature of this requirement. In contrast to type I IFN, we found that host responsiveness to IFNγ in both hematopoietic and nonhematopoietic cells was essential for tumor rejection. When

injected with d38m2 tumor cells, chimeras lacking either hematopoietic or nonhematopoietic IFNγ sensitivity showed a similar defect in anti-tumor immunity. This phenotype, however, was not quite as severe as that observed in chimeras with complete IFNγ insensitivity, suggesting that IFNγ's actions on cells within either compartment can provide some tumor protection. When we instead examined the requirements for H31m1 rejection, we found a similar defect – though again partial – in chimeras lacking nonhematopoietic IFNγ responsiveness. In this case, a more significant function was found for nonhematopoietic compared to hematopoietic IFNγ sensitivity, though chimeras selectively lacking hematopoietic responsiveness showed significantly delayed rejection. Taken together, these data demonstrate critical functions for IFNγ acting on both hematopoietic and nonhematopoietic host cells, but also suggest that these anti-tumor mechanisms may be independent of one another. Indeed, host responsiveness to IFNγ in one compartment or the other still afforded some tumor protection.

Importantly, we have shown that the constellation of requirements for IFNγ's actions on the host is, in fact, distinct from that of IFNα/β. Whereas immune-mediated rejection of H31m1 and d38m2 tumor cells required the actions of IFNα/β solely on hematopoietic cells, an essential role for IFNγ's actions on both hematopoietic and nonhematopoietic cells was found for these same tumors. This observation therefore establishes that IFNα/β and IFNγ must perform unique functions at the level of the host, in addition to their disparate importance at the level of the tumor.

In the final study of this thesis, we focused on the role of *exogenous* as opposed to *endogenous* IFN in mediating tumor protection. It has long been recognized that the

administration of exogenous type I IFN – either through injection of recombinant protein, in vivo delivery by viral vectors, or its overexpression in tumor cells – can inhibit tumor growth or induce fully protective anti-tumor responses through its actions on cell of the host (200). Relatively little is known, however, concerning the relevant cellular targets eliciting these effects. To address this issue, we have used a model of IFNα/β immunotherapy consisting of IFNβ-producing fibrosarcoma cells derived from IFNAR1$^{-/-}$ mice, thus ensuring that observed effects are due solely to actions on the host rather than the tumor. Although the parental tumor lines are poorly immunogenic and resistant to immune control, growth of the IFNβ-producing tumors could be controlled when injected into WT and RAG2$^{-/-}$, but not IFNAR1$^{-/-}$ hosts.

While tumors did not grow progressively, stable masses were observed to persist for long periods of time until a fraction of mice eventually died from IFN-mediated toxicity. To assess whether IFNβ-producing tumor cells persisted within these stable masses, we treated mice either at day 65 or day 100 with anti-IFNAR1 mAb. Such treatment could effectively interrupt IFNβ-mediated tumor equilibrium, leading to tumor outgrowth in both RAG2$^{-/-}$ and WT mice. In addition, harvest and retransplant of the resulting tumors showed that they still produced IFNβ and could be controlled in RAG2$^{-/-}$ mice, yet were highly tumorigenic in IFNAR1$^{-/-}$ hosts. Finally, experiments using bone marrow chimeras demonstrated that IFNα/β sensitivity in both the nonhematopoietic and innate immune compartments was required for tumor control, though adaptive immunity was dispensable.

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FUTURE DIRECTIONS

Identifying the critical targets of endogenous type I and type II IFN

Using mixed bone marrow chimeric mice, our prior studies have established that the relevant cellular targets of IFNα/β during tumor rejection reside in the innate immune compartment. Whereas NK cells were ruled out as obligate innate immune effectors during IFNα/β-dependent rejection, $CD8\alpha^+$ DCs were found to be absolutely required for successful anti-tumor immune responses. Furthermore, the direct actions of IFNα/β on $CD8\alpha^+$ DCs enhanced cross-presentation of cell-associated antigen to naïve $CD8^+$ T cells in vitro. Taken together, the evidence demonstrating that (i) IFNα/β promotes tumor-specific CTL priming, (ii) IFNα/β acts on innate immune cells to mediate its anti-tumor effects, (iii) $CD8\alpha^+$ DCs are absolutely required for CTL priming and tumor rejection in vivo, and (iv) IFNα/β acts directly on $CD8\alpha^+$ DCs to promote CTL priming in vitro, collectively supports a host protective function involving direct actions of type I IFN on $CD8\alpha^+$ cDCs. However, studies to more directly assess the role of IFNα/β on $CD8\alpha^+$ DCs in vivo will be instructive.

Although technically challenging, ex vivo and adoptive transfer approaches may be used to address this question. Since IFNα/β is a potent activator of DCs, ex vivo analysis of the activation status of DC subsets from WT and $IFNAR1^{-/-}$ mice at different time points following tumor challenge could be pursued. In addition, the ability of isolated DC subsets to prime T cells could be assayed by incubation with tumor-specific

T cell clones in vitro, using T cell proliferation or IFNγ production as a readout. This approach was employed in a recent study demonstrating the key role of skin-derived migratory $CD103^+$ DCs in $CD8^+$ T cell priming during herpes simplex virus infection (205). Since $p21SNFT^{-/-}$ mice were also found to lack the closely related subset of tissue-resident $CD103^+$ cDCs in addition to lymphoid resident $CD8\alpha^+$ cDCs (151, 206), comparable ex vivo experiments in the tumor system with tumor antigen-specific T cell clones (or naïve $CD8^+$ T cells from tumor antigen-specific transgenic mice) could clarify the respective functions of these DC subsets during cross-priming. Similarly, a possible tumor antigen transport function of Langerhans cells migrating from the skin to the dLN could be investigated using one of several animal models of Langerhans cell deficiency (207).

As discussed in Chapter 3, adoptive transfer of total $CD11c^+$ cells isolated from WT or $IFNAR1^{-/-}$ mice into tumor challenged $IFNAR1^{-/-}$ hosts yielded encouraging though inconsistent results. Recapitulating the normal function of adoptively transferred DCs is a significant obstacle to such approaches, especially given the limited half-life and unknown migratory and stimulatory properties of transferred cells; yet, preliminary studies have shown that tumor-specific CTL priming and anti-tumor responses can be augmented by transfer of WT $CD11c^+$ cells. Thus, additional studies – perhaps involving transfer of FACS-purified $CD8\alpha^+$ cDC populations instead of total $CD11c^+$ cells – are warranted. As a complementary approach, we also generated mixed bone marrow chimeric mice with $p21SNFT^{-/-}$ HSCs as described in Chapter 3, and additional work using this strategy may be helpful. Finally, conditional knockout strategies using

IFNAR1-flox mice, which were recently generated by Kalinke and colleagues (208), will be valuable for examining the effects of tissue-specific IFNAR1 ablation in conjunction with the appropriate cre-expressing strains (Table 1). We are currently in the process of backcrossing IFNAR1-flox mice onto a pure C57Bl/6 genetic background in preparation for their use in both tumor transplantation and primary MCA induction studies.

In addition to further clarifying the in vivo functions of type I IFN during tumor rejection, the mechanism involved in IFNα/β's enhancement of cross-presentation by $CD8\alpha^+$ cDCs is an area worthy of future investigation. The effects of type I IFN on $CD8\alpha^+$ cDC function at various levels can be envisioned, including modulation of antigen capture or processing, peptide shuttling and MHC loading, MHC class I and/or costimulatory molecule expression, cellular migration or survival, or the induction of secondary cytokines/chemokines. While current understanding of the cell biology of cross-presentation is limited, some data indicate that heightened or altered antigen processing, rather than better antigen capture, underlies the ability of the $CD8\alpha^+$ cDC to efficiently cross-present antigen (209). In vitro studies, including analyses of antigen processing and presentation as well as unbiased approaches such as global gene expression profiling, can be used to compare the relevant phenotypes of WT and $IFNAR1^{-/-}$ $CD8\alpha^+$ DCs, or of WT $CD8\alpha^+$ DCs in the presence or absence of exogenous type I IFN.

Interestingly, a recent study suggested that steady-state production of low levels of IFNβ promotes antigen presentation by cDCs to both $CD8^+$ and $CD4^+$ T cells via upregulation of heat shock protein 70, which boosts formation of MHC-peptide

complexes (210). Additional mechanisms must be involved, however, since baseline antigen presentation (in the presence of low-level IFNβ) induces cross-tolerance in the absence of DC activation triggered by inflammatory signals such as enhanced IFNα/β production (209). In fact, the presence of other inflammatory stimuli which may collaborate with type I IFN to activate $CD8\alpha^+$ cDCs is suggested by detection of residual low-level priming in the absence of IFNα/β signaling, as well as the somewhat more robust tumor growth in $p21SNFT^{-/-}$ mice (lacking $CD8\alpha^+$ cDCs) compared to $IFNAR1^{-/-}$ mice (containing normal numbers of IFNα/β-unresponsive $CD8\alpha^+$ cDCs). The involvement of other inflammatory stimuli and their interrelation with type I IFN therefore also remains to be investigated.

In our studies to evaluate the host cell targets of IFNα/β, we also demonstrated that type I IFN acts in a manner distinct from IFNγ, which also performs essential functions on the host during tumor rejection. Whereas hematopoietic-derived innate immune cells were obligate targets of IFNα/β's actions, IFNγ sensitivity within both hematopoietic and nonhematopoietic cells was critical. Additional work will be necessary to further delineate the relevant host cell targets of IFNγ. Interestingly though, our results suggested that several collaborating mechanisms may be involved – since mice with IFNγ sensitivity in either the hematopoietic or nonhematopoietic compartment alone appeared to have an intermediate phenotype compared to globally unresponsive mice. Future studies utilizing mixed bone marrow chimeras could assess the relative contribution within the hematopoietic compartment of IFNγ's actions on innate versus adaptive immune cells. A more targeted look, however, will require the use of

conditional knockout mice to abrogate IFNγ responsiveness in specific cell types. The generation of IFNGR1-flox mice will thus provide a critical reagent for examination of IFNGR1 ablation in defined cellular populations by intercrossing with tissue-specific cre mice (Table 1). Finally, since all of the experiments performed up to this point have relied on tumor transplantation approaches, it will be important to test tissue-specific knockout mice in primary MCA tumor induction studies as well as tumor transplantation.

Table 1. Cre-expressing mice useful for conditional ablation of IFN responsiveness. Intercrossing IFNAR1-flox and IFNGR1-flox mice with the indicated tissue-specific cre mice to generate hosts with cell type specific deletion of the respective IFN receptor will provide critical reagents for future studies. Both tumor transplantation and primary MCA induction studies using such mice will further clarify the important host cell targets of IFNα/β and IFNγ in cancer immunoediting.

Tissue-Specific Cre Mice	Cellular Compartment(s)
Vav-cre	Hematopoietic cells, endothelial cells
Lck-cre * *	T, B lymphocytes
CD4-cre	T lymphocytes, $CD4^+$ DCs
CD8-cre *	T lymphocytes, $CD8^+$ DCs
NKp46-cre	NK cells
CD11c-cre *	DCs, NK cells
LysM-cre *	Macrophages, granulocytes
Langerin-cre	Langerhans cells
VE-cadherin-creER^{T2} *	Endothelial cells *(inducible)*
Fibroblast-specific protein-1 (FSP1)-cre *	Fibroblasts

Priorities for: *IFNAR1-flox *IFNGR1-flox

Unique functions for IFNα/β subtypes?

Our studies using IFNβ$^{-/-}$ mice have suggested that IFNβ is not essential during the rejection of transplantable 129 strain regressor tumors. Since we have recently completed the backcrossing of this mouse to the C57Bl/6 strain, additional experiments using B6 RAG2$^{-/-}$ regressor tumors will be important to further test this conclusion. Moreover, use of both 129 and/or B6 strain IFNβ$^{-/-}$ mice in primary MCA tumorigenesis experiments would clarify the role of IFNβ during primary tumor formation. These approaches could also be supplemented by the generation and use of blocking mAb's specific for IFNβ, as this reagent would additionally allow for temporally-controlled blockade of IFNβ's actions.

Examination of the role of IFNα subtypes is more challenging given the large number of IFNα's and the difficulties in eliminating all subtypes or individual species. The number of IFNα genes would make genetic targeting of this locus difficult, while slight differences in structure could preclude the generation of an antibody reacting against all subtypes. Given the presumed importance of IFNα4 as an initially induced species critical for augmenting production of the remaining IFNα subtypes, specific targeting of this molecule (either through IFNα4-specific mAb's or gene-targeted mice) may be worthwhile. Alternatively, generation of a panel of mAb's each capable of neutralizing individual IFNα subtypes would allow for elimination of all IFNα's using a mAb cocktail. Such reagents will be critical for specific elimination of IFNα subtypes, but not IFNβ, in order to rigorously test whether the IFNα subtypes, or a subset thereof, are the critical mediators or merely functionally redundant with IFNβ.

What cells and molecular pathways mediate IFN production?

The observation that IFNα/β and IFNγ must act on distinct cellular targets during tumor rejection could reflect unique functions of these cytokines, or alternately could be a product of their differential sites of production. At present, however, we have very limited information as to where, when, and by whom the interferons are being produced during naturally-occurring anti-tumor responses. Although all cells are capable of making IFNα/β, the production of IFNγ has traditionally been ascribed to a comparatively smaller subset of cell types (which includes NK, NKT, αβ T cells, and γδ T cells). Some, however, have suggested that IFNγ can also be produced by APCs (96-98). Prior studies in the literature support a role for γδ T cells as important early producers of IFNγ during responses to transplantable tumor challenge and during primary MCA-induced carcinogenesis (99). Yet, other studies have indicated that innate immune rather than T cell IFNγ production is important for anti-tumor immunity (100). While this question has not been addressed for IFNα/β production in a tumor system, a recent study using IFNα6-GFP knockin mice indicated that alveolar macrophages were the major type I IFN producers during viral infection of the lung (211).

As an initial approach to study this question, we could examine levels of expression of IFNα/β and IFNγ by qRT-PCR in total RNA isolated from the tumor, draining lymph node, and spleens of mice at different days following tumor challenge. If this crude analysis is successful in localizing cytokine production, more specific approaches could then be used to specifically identify the relevant producing cells.

Sorting cells by FACS or MACS bead purification prior to RNA isolation would allow for examination of individual populations, and this strategy has been used previously to identify the primary producers of IFNβ in the context of DNase II-deficiency (212). Intracellular staining has also been reported for the detection of IFNα production (213). Additional approaches might involve the use of immunohistochemistry or in situ hybridization on tissue sections. As previously alluded to, gene-targeted reporter mice have also been employed for the detection of IFN production, hence these approaches could also be used.

Initial information regarding levels of IFN induction and relevant cellular producers during anti-tumor immunity will facilitate downstream studies into the molecular pathways leading to its induction. Since TCR stimulation or the actions of cytokines can lead to IFNγ production, the relevant stimuli mediating its induction are perhaps easier to surmise. For type I IFN, however, the major stimuli characterized to date are of microbial origin. It is therefore unclear what the physiologically important inducers in the setting of a growing tumor might be, although TLR-mediated recognition of endogenous danger signals such as heat shock proteins, HMGB-1, uric acid, and calreticulin have been suggested (214, 215). A number of different molecular pathways leading to type I IFN production have been described, including those transduced through the TLRs, RIG I-like helicases (RLHs), NOD-like receptors (NLRs), and lectin receptors, as well as a recently described cytoplasmic DNA receptor . Gene-targeted knockout mice or loss-of-function mutants are available for many of these receptors and/or their signaling components, in addition to some molecules (e.g. IRF-3, IRF-7, and IKKε)

common to multiple pathways. Use of such mice may provide initial information about the relevant stimuli associated with immunogenic tumors.

Therapeutic targets of exogenous type I IFN

Some of the same reagents and experimental approaches described earlier could also be used to identify the functionally relevant host cell targets of exogenous IFNα/β. Previous experiments with bone marrow chimeras and a model of IFNβ immunotherapy have suggested an important role for hematopoietic IFNα/β responsiveness during tumor elimination under conditions of low-level IFNβ production within the tumor. Conditional IFNAR1 knockout mice would therefore be useful to investigate individual cell populations within the hematopoietic compartment. one could use this approach to investigate the role of IFNβ's direct actions on T cells, B cells, DCs, macrophages, and NK cells. Alternately, mixed bone marrow chimeras could be generated to examine the relative importance of innate versus adaptive IFNα/β sensitivity.

Additional studies might also be instructive in determining the mechanism of tumor control and elimination. Whereas high-level IFNβ secretion led to tumor persistence and eventual toxicity due to chronic IFN exposure, low-level IFNβ mediated tumor elimination with no observable toxicity. Our studies in RAG2$^{-/-}$ mice showed that this effect is lymphocyte dependent, but we have not yet specifically examined the generation of tumor-reactive T cells in WT mice. It would be interesting to compare levels of T cell priming in mice challenged with tumor cells expressing high-dose versus low-dose IFNβ. This could be done by rechallenging mice with parental tumor cells on

the opposite flank or by ex vivo T cell killing assay using parental tumor cells as targets. Whereas a lack of tumor-specific CTL priming with high-dose IFNβ may suggest defects in innate immune function or T cell clonal expansion, the detection of equal levels of tumor-reactive T cells would instead indicate a defect in T cell effector function, perhaps due to high local concentrations of IFNβ within the tumor environment potentially inducing apoptosis or rendering effector cells anergic. These approaches could therefore provide further insight into the inhibition of adaptive immunity by high level IFNβ production within the tumor microenvironment.

An alternate hypothesis is that B cell function rather than T cell function is the critical mediator within the adaptive immune compartment contributing to tumor elimination. Such a possibility perhaps though tumor-specific antibody eliciting ADCC by NK cells and macrophages. This hypothesis is easily tested, however, by tumor transplantation into B cell-deficient mice. In additional to these functional assays, further characterization of the immune infiltrate by histology or FACS analysis might be useful, in addition to an examination of angiogenesis by tissue staining with endothelial cell markers.

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