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DEVELOPMENT AND ANALYSIS OF NATURAL KILLER CELL-DEFICIENT MICE

by

Sungjin Kim

A dissertation submitted to the Graduate Faculty in
Biomedical Sciences in partial fulfillment for the degree of
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ABSTRACT

Development and Analysis of Natural Killer Cell-Deficient Mice

by

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Advisor: Dr. Wayne M. Yokoyama

Since natural killer (NK) cells were discovered two decades ago, it has not been possible to directly demonstrate the physiological role of NK cells in immune responses because of the lack of available animal model with a selective loss of NK cell activity. The present study describes transgenic (Tg) mice that express an inhibitory MHC class I receptor Ly-49A under control of the mouse granzyme A promoter. Splenocytes from the Tg mice fail to kill various tumor targets *in vitro* but this defect is not due to the action of Ly-49A as an inhibitory receptor. Instead, there is a remarkable decrease in the number of $CD3^-$ $NK1.1^+$ cells in peripheral organs. However, Tg mice possess a normal number of functional T, NK/T and B cells. Acute tumor rejection is impaired in Tg mice and can be reconstituted by the infusion of *scid* splenocytes. Taken together, these data demonstrate that Tg mice have a selective deficiency in the NK cell compartment. Studies of Tg mice also provide compelling evidence that NK cells are the major effector cells responsible for prevention of tumor metastasis, control of tumor outgrowth, acute rejection of β2m-deficient bone marrow (BM) grafts, and acute production of IFN-γ in response to bacterial endotoxin. In contrast to the peripheral organs, there is a significant increase in the number of $CD3^-$ $NK1.1^+$ cells in the BM of Tg mice, suggesting a defect in the maturation process. CD3$^-$ NK1.1$^+$ cells in Tg mice display a cell surface phenotype associated with immature NK cells. BM chimeric studies indicate that Tg mice have an intrinsic defect in the NK cell lineage with an intact microenvironment that can support normal NK cell maturation. Thus, this study also provides novel insights into the maturation process of NK cells.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AGM1	Asialo-GM1
B6	C57BL/6
B10.D2	C57BL/10.D2
β2m	β2-microglobulin
BM	Bone Marrow
BSA	Bovine Serum Albumin
CTL	Cytotoxic T Lymphocyte
Cy	Cy-Chrome
E:T	Effector-to-Target
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocynate
^{125}I-UdR	^{125}I-labeled 5-iodo-2-deoxyuridine
Ig	Immunoglobulin
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer Cell Inhibitory Receptor
LGL	Large Granular Lymphocyte
LPS	Lipopolysaccharide

MHC	Major Histocompatibility Complex
mAb	Monoclonal Antibody
NK	Natural Killer
NKC	Natural Killer Gene Complex
PE	Phycoerythrin
PI	Phosphatidylinositol
PLC	Phospholipase C
Poly-I:C	Polyinosinic-polycytidylic Acid
PTK	Protein Tyrosine Kinase
RBC	Red Blood Cell
SCID	Severe Combined Immunodeficient
TCR	T Cell Receptor
Tg	Transgenic
Th1	T Helper Type 1
TI	T Cell-independent
TNP	Trinitrophenyl
WT	Wild-type

INTRODUCTION

Natural killer (NK) cells constitute a population of lymphocytes that are able to kill, without prior sensitization or activation, a variety of target cells including tumor cells and cells infected with certain viruses, parasites or bacteria (1). The existence of NK cells was first discovered in the early 1970s by the observations that a subpopulation of lymphocytes from unimmunized humans as well as experimental animals have the natural capacity of killing certain tumor cells *in vitro* (2, 3). According to this natural killing activity, these lymphocytes were named natural killer cells. Based on the tumor killing activity *in vitro*, NK cells have long been speculated to play a role in tumor rejection *in vivo*. However, this role has not yet been established.

In addition to the cytolytic function, NK cells produce a broad spectrum of cytokines, including IFN-γ, TNF-α, and GM-CSF (4, 5). Several lines of indirect evidence have suggested that through these cytokines, NK cells may participate in a variety of immune responses, i.e., innate resistance and immunoregulation upon pathogen infections, humoral immune responses to T cell-independent (TI) antigens (Ag), and bacterial endotoxin-induced septic shock (6-8). In particular, NK cells produce IFN-γ during early stage of pathogen infection, which in turn induces activation of macrophages (9, 10). Importantly, this process appears to provide protection against infection before the development of the acquired immune responses mediated by T or B lymphocytes. In addition, IFN-γ, in conjunction with IL-12, appears to promote the differentiation of naive T cells into the T helper type (Th) 1 subset (11). Finally, it has been suggested that NK cells may play roles in regulation of hematopoiesis and acute rejection of MHC-disparate bone marrow (BM) grafts (12, 13).

Although the developmental pathways and stages of NK cells are poorly understood, it is believed that NK cells are derived from BM because NK cell activity is impaired in congenitally osteopetrotic mice or mice rendered osteopetrotic by treatment with the bone-seeking isotope ^{89}Sr or 17-beta estradiol (14, 15). Interestingly, however, these mice possess a normal number of peripheral NK cells that are capable of binding to target cells but are non-cytolytic (16, 17), indicating that an intact BM microenvironment is essential for the generation of mature NK cells. Thus, generation process of mature NK cells can be divided into two phases (18). In the first phase, lineage marker-negative NK cell progenitors develop into an immature form of NK cells; this process may not require an intact BM microenvironment. In the second phase, immature NK cells become mature, cytolytic NK cells and acquire the expression of various cell surface molecules; this process requires an intact BM microenvironment.

Unlike B and T cells, NK cells do not rearrange immunoglobulin (Ig) and T cell receptor (TCR) genes, and do not produce mature transcripts for these genes (19-21). Although mature B and T cells are absent, NK cells develop normally in severe combined immunodeficient (scid) mice and mice with targeted mutation in the RAG-1- or RAG-2-genes (22-25). Thus, NK cells do not require the V-D-J recombinase machinery for their development. Moreover, NK cells do not require a functional thymus for their development, as evidenced by the presence of normal NK cells in athymic nude mice that have no conventional T cells (26). However, consistent with the idea that NK cells belong to the lymphoid cell lineage, the development of NK cells is impaired in mice with a targeted mutation in the transcription factor Ikaros gene that is required for the development of all lymphoid lineages but not for that of erythroid and myeloid lineages (27). Similarly, NK cells are absent in Jak3 kinase- or IL-2Rγ subunit-deficient mice that have severe defects in general lymphopoiesis (28, 29).

Although NK cells constitute a distinctive lineage of lymphocytes, NK cells appear to be closely related to the T cell lineage in many aspects. Often, mice that have abnormalities in T cell lineage (i.e., IL-2Rβ subunit-, IRF-1-deficient, and CD3ε-transgenic mice) also display an impaired development of NK cells (30-32). In addition, it has been shown that there are bipotential T/NK progenitors in fetal thymus. Purified human $CD34^+$ $CD3^-$ $CD4^-$ $CD8^-$ or murine FcγRII/III^+ fetal thymocytes can give rise to both T and NK cells depending on the culture microenvironments (33, 34). Whereas these thymocytes differentiate into T cells under a thymic environment, they can differentiate into NK cells in other conditions. Moreover, NK cells and T cells utilize similar cytotoxic mechanisms for inducing target cell lysis. Both NK cells and cytotoxic T lymphocytes (CTLs) possess cytotoxic granules that contain perforin and a family of serine proteases, termed granzymes. Studies of mice with disrupted perforin or granzyme B genes indicate that both cell types kill target cells mainly by secreting these granular components (35, 36). A current granule exocytosis model postulates that these effector cells release components of granules upon recognition of target cells, perforin polymerizes to form pores on the target cell membrane, then granzymes enter through the pores and induce apoptosis and lysis of target cells (37, 38). Furthermore, like CTLs, NK cells can kill certain target cells via perforin-independent cytotoxic mechanisms, i.e., Fas-ligand-mediated cytotoxicity (39, 40).

However, in contrast to CTLs that generally require target cell expression of major histocompatibility complex (MHC) class I molecules for cytotoxic effector function, target cell killing by NK cells is non-MHC-restricted because NK cells kill syngeneic and allogeneic target cells at similar levels (1). Moreover, NK cells effectively kill target cells that lack the expression of MHC class I molecules (41, 42). Since NK cells do not express clonotypic antigen-specific receptors such as surface Ig and TCR, it is conceivable that NK cells utilize distinct cell surface receptors. Currently, however, there is no known unique NK cell-specific receptor that is responsible for recognition of target cells and triggering the cytolytic activity of NK cells.

Since NK cells were initially defined by their natural killing activity, there has been confusion over the identity and characteristics of NK cells with other cells types. Under certain circumstances, activated T cells or macrophages can mediate similar killing (43, 44). In general, NK cells display morphology of large granular lymphocyte (LGL) with a few azurophillic granules (45). Without reliable means to isolate enough number of pure NK cells, many early studies of NK cells were performed on cell preparations that were fractionated by physical properties (i.e., buoyant density) and chosen by the LGL morphology (46). However, the LGL morphology is not specific to NK cells and not all NK cells have this morphology (47). Thus, these studies sometimes generated artifactual information on the functional roles of NK cells due to the contamination of other cell types. In recent studies, to circumvent these problems, IL-2 activated NK cells or NK clones are being used. However, these cells often display characteristics different from resting NK cells *ex vivo.*

NK Cell Receptors

NK cells express various cell surface molecules that can function to activate NK cells (48). Among them, a low affinity IgG receptor (FcγRIII) CD16 is the best characterized receptor (49). CD16 binds the Fc portion of IgG and initiates transmembrane signaling. CD16 is expressed as a multimeric complex in association with disulfide-linked homodimers or heterodimers of the FcεRI-γ and TCR/CD3-ζ subunits (50-52). Crosslinking of CD16 appears to activate src-family protein tyrosine kinase (PTK) lck that induces the phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motifs of the FcεRI-γ and CD3-ζ subunits (53-55). Subsequently, there are a series of biochemical events including the activation of syk-family PTK ZAP-70, phospholipase C (PLC)-γ and phosphatidylinositol (PI) 3-kinase (56-58). The activated PLC-γ hydrolyzes PI into inositol-triphosphate and 1,2-diacylglycerol, which in turn lead to calcium mobilization and activation of protein kinase C (57, 59). Finally, these biochemical events result in activation of NK cell cytolytic function and production of various cytokines, including IFN-γ, TNF-α and GM-CSF (5, 60).

However, CD16 is not responsible for natural killing, because NK cells lacking this molecule display normal capacity of killing target cells (61).

The NKR-P1 molecule on rat and NK1.1 (MusNKR-P1C) on mouse NK cells are candidate activation receptors responsible for natural killing (62). These receptors are type II integral membrane proteins that belong to a family of highly related proteins with sequence homology to the C-type lectin superfamily. The genes encoding the NKR-P1 family receptors are located on a genetically linked locus, termed the NK gene complex (NKC) and display allelic polymorphism (63, 64). Crosslinking of these receptors with corresponding monoclonal antibody (mAb) can trigger a series of biochemical events similar to those seen during natural killing and induces granule exocytosis as well as cytokine production (65-68). Anti-NK1.1 mAb can induce NK cells to lyse otherwise resistant target cells that express Fc receptors through a phenomenon termed redirected lysis (69). More direct evidence for the role of NKR-P1 receptors in natural killing comes from a recent study with the rat RNK-16 NK cell line (70). This study shows that an NKR-P1 loss mutant of this cell line loses ability to kill certain tumor targets and transfection of the rat NKR-P1A gene into the mutant restores cytotoxicity against one of these targets. However, these receptors do not seem to account for all natural killing since NK cells from many strains of mice do not express these receptors (71, 72). Thus, it is likely that NK cells possess other receptors mediating natural killing. Importantly, NK1.1 is known as so far the most specific serological marker for murine NK cells in certain strains of mice (17, 73, 74), although it is also expressed on a subset of T cells, termed NK/T cells that share many functional and phenotypic characteristics with NK cells (67, 75, 76). Thus, NK cells in these mice are best characterized by the expression of NK1.1 and lack of TCR/CD3 (13, 19).

In the past years, a number of studies have demonstrated that there is an inverse relationship between MHC class I expression on target cells and their susceptibility to NK cell-mediated cytotoxicity (77). For example, certain MHC class I-negative cell lines generated by mutation are more susceptible than the parental cells to NK cell-mediated cytotoxicity (41, 42). Conversely, transfection of certain class I genes into susceptible MHC class I-negative cell lines confers protection from NK cell-mediated cytotoxicity (78). To account for these findings, it has been proposed that NK cells express inhibitory receptors that recognize MHC class I molecules on target cells and inhibit NK cell-mediated cytotoxicity.

The Ly-49A receptor expressed on a subset of murine NK cells is the first identified such receptor (79). In contrast to Ly-49A$^-$ IL-2-activated NK cells, Ly-49A$^+$ IL-2-activated NK cells are unable to kill target cells that express H-2 D^d MHC class I molecules. The presence of either anti-Ly-49A or anti-H-2 D^d mAb reconstitutes cytotoxicity, indicating that the engagement of Ly-49A with D^d class I molecules inhibits NK cell mediated cytotoxicity. Several lines of independent evidence also support the thesis that Ly-49A is an MHC class I-specific receptor (80, 81). In addition, the engagement of Ly-49A with D^d class I molecules inhibits cytokine production and granule exocytosis (68, 82). Despite opposing effects, Ly-49A shares many features with the NKR-P1 receptors. Ly-49A is a type II integral membrane that belongs to a family of highly related proteins with sequence homology to the C-type lectin superfamily (83, 84). The genes encoding the Ly-49 family receptors are located in the NKC and display allelic polymorphism (85). Similar to Ly-49A, other members of the Ly-49 family, including Ly-49C and Ly-49G2, inhibit cytotoxicity upon recognition of their respective class I ligands (86, 87).

An increasing number of inhibitory MHC class I receptors (i.e., p58 and NKB1) have been identified on human NK cells (88, 89). Interestingly, unlike the Ly-49 receptors, these human NK cell receptors are type I integral membrane proteins that have sequence homology to the Ig superfamily. These receptors are now termed killer cell inhibitory receptors (KIR) (90). Despite structural differences, both Ly-49 and KIR receptors appear to inhibit NK cell-mediated cytotoxicity through similar signal transduction pathway. Both types of inhibitory receptors have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Upon receptor engagement, the tyrosine residues in the ITIMs appear to be phosphorylated and recruit the cytoplasmic tyrosine phosphatase, SHP-1 (91-93). Presumably, this recruitment results in inhibition of NK cell-mediated cytotoxicity by dephosphorylating signal transduction molecules that are phosphorylated in the activation cascades.

Unlike NK1.1 that is expressed on all NK cells, Ly-49 receptors are expressed only subsets of NK cells (79, 94). For example, Ly-49A is expressed on 15-20% of NK cells. Interestingly, individual NK cells can express multiple Ly-49 receptors that have different specificity for class I molecules (95). Although the molecular mechanism underlying the regulation of Ly-49 expression is poorly understood, frequencies of NK cells expressing multiple Ly-49 receptors can be partly explained by a stochastic mechanism (96, 97). Host MHC class I molecules appear to play a role in shaping Ly-49 repertoire. Comparative studies of β2m-deficient mice that do not express class I molecules and normal mice with different MHC haplotypes demonstrate that the frequencies of NK cells expressing a given Ly-49 receptor or multiple receptors are influenced by host MHC class I molecules (98, 99). The cell surface expression of Ly-49 receptors is also modulated by interaction with MHC class I molecules, such that the expression level of Ly-49A is lower on NK cells in mice that express its ligand H-2 D^d class I molecules (100). These studies suggest that during their development, NK cells may undergo an "education process" to adapt to the host MHC environment (99, 101).

In Vivo Function of NK Cells

It has been particularly difficult to study the role of NK cells *in vivo* because there is no animal model in which NK cell activity is selectively deficient. In previous studies, various experimental protocols have been employed to investigate the role of NK cells in tumor rejection and other immune responses.

One protocol has been treatment of experimental animals with NK activity-inducing agents such as IFN-inducers, anti-coagulant agents, synthetic biological response modifiers, or bacterial adjuvants (102-106). Under these conditions, such treated animals exhibited enhanced anti-tumor activities as evidenced by enhanced acute clearance of injected tumor cells or decreased formation of experimental tumor metastasis. Alternatively, reduced anti-tumor or anti-metastatic activity was observed in animals when treated with split-dose radiation or immunosuppressive agents such as cyclophosphamide, urethane and 17β-estradiol (102, 103, 107). Correlated with increased or decreased host resistance against tumor cells, increased or decreased *in vitro* NK activity was observed when spleen or peripheral blood cells from such treated animals were tested for their killing ability against tumor targets. Although these studies established the correlation between the levels of in vitro NK activity and in vivo tumor rejection, the evidence that NK cells are responsible for such anti-tumor effects is only circumstantial due to possible non-specific effects of these treatments. Conversely, it has been shown that adoptive transfer of spleen cells, bone marrow cells or partially NK cell-enriched preparation from normal mice can restore the ability to acutely eliminate tumor cells or to prevent metastasis formation in immunosuppressed recipient animals (108-110). However, these studies did not exclude the effects mediated by other cell types, in particular T cells, possibly contaminated in adoptively transferred cell preparations.

Another protocol has been treatment of animals with antisera such as anti-asialo-GM1 (AGM1) and anti-NK1.2 to deplete NK cells *in vivo* (108, 111, 112), since these antisera can abolish *in vitro* NK activity of spleen cell preparations in the presence of complement. Such treatment led to markedly impaired acute tumor clearance or increased tumor metastasis formation (106, 108, 113), suggesting that cells reactive with these antisera may have anti-tumor activities. However, in addition to non-specific effects such as Fc receptor engagement on other cell types including macrophages and B cells, it is not clear whether the impaired acute tumor clearance and anti-metastatic activities resulted from selective depletion of NK cells, because these antisera are not specific for NK cells. Anti-AGM1 sera react with many other cell types (114, 115) and anti-NK1.2 sera react CTLs (116). Other studies showed that adoptive transfer of spleen cells could restore anti-tumor potential in antisera-treated or immunosuppressed animals and these effects were abolished if cell preparations were pretreated with complement and antisera (108, 109). However, again, these studies did not resolve the specificity issue.

In more recent studies, the anti-NK1.1 mAb has been widely used for *in vivo* depletion of NK cells because of its more defined and restricted reactivity with NK cells. In support of the role for NK cells in tumor rejection, anti-NK1.1-treated mice exhibited impaired anti-tumor potentials (74, 117). Importantly, however, the recent discovery of NK/T cells (76, 118) that express NK1.1 and are capable of killing tumor cells raised the possibility that NK/T cells are involved in tumor rejection. Indeed, most recent studies suggest that NK/T cells rather than NK cells may mediate *in vivo* anti-tumor effects (115, 119). Thus, despite numerous studies, the role of NK cells in tumor rejection has not yet been established. Taken together, none of the used protocols allow differentiating the contribution of NK cells from that of other cell types in tumor rejection.

Similar protocols have been used for studies of the role of NK cells in other immune responses. These studies have suggested that NK cells may act as a key player in host defense against pathogen infection by producing cytokines, particularly IFN-γ (6, 120). In addition, studies have suggested that NK cells may mediate acute rejection of allogeneic BM grafts (13). However, due to the non-specific effects as described above, it has not been possible to directly demonstrate the physiological role of NK cells in such immune responses.

The present study describes the generation and characterization of transgenic (Tg) mice that express Ly-49A receptor under control of the mouse granzyme A promoter. Initially, this study was intended to study the role of Ly-49A *in vivo* as an inhibitory MHC class I receptor and the possible effect of its expression on the formation of Ly-49 repertoire. Splenocytes from the Tg mice fail to kill various tumor targets *in vitro* but this defect is not due to the action of Ly-49A as an inhibitory receptor. Instead, there is a remarkable decrease in the number of peripheral $CD3^-$ $NK1.1^+$ cells in Tg mice. On the other hand, Tg mice possess a normal number of functional T, NK/T and B cells. Lung clearance experiments indicate that acute tumor rejection is impaired in the Tg mice. Tumor rejection can be reconstituted by the infusion of splenocytes from *scid* mice. This effect is abrogated by treatment with anti-NK1.1 mAb, indicating that the relevant cells are NK cells. Taken together, these data demonstrate that Tg mice have a selective deficiency in the NK cell compartment. Studies of Tg mice also provide compelling evidence that NK cells are the major effector cell population responsible for prevention of tumor metastasis, control of tumor outgrowth, acute rejection of β2m-deficient BM grafts, and acute production of IFN-γ upon injection with bacterial endotoxin. In contrast to the peripheral organs, there is a significant increase in the number of $CD3^-$ $NK1.1^+$ cells in the BM of Tg mice, suggesting a defect in the maturation process. Consistent with this, Tg $CD3^-$ $NK1.1^+$ cells display a cell surface phenotype associated with an immature form of NK cells. BM chimeric studies indicate that Tg mice have an intrinsic defect in the NK cell lineage but have an intact microenvironment that can support normal NK cell maturation. Thus, this study also provides novel insights into the maturation process of NK cells.

MATERIALS AND METHODS

Mice.

C57BL/6 (B6), C57BL/10.D2 (B10.D2), BALB/c, C57BL/6-*Hfh11*^{nu}, C57BL/6-*Prkdc*^{scid}/Sz, B6.SJL-*Ptprc*^{a}*Pep*^{b}/Boy (*Ly5.1*), C57BL/6-*Itgb2*$^{-/-}$, and C57BL/6-*β2m*$^{-/-}$ (β2m-deficient B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Cell Lines and Culture.

YAC-1 (Molony virus-transformed A/Sn-derived T cell lymphoma) was obtained from the American Type Culture Collection (ATCC: Rockville, MD). RMA-S is a Tap-2-deficient mutant originated from RBL-5 (Rauscher virus-induced C57BL/6-derived T lymphoma) and was provided by K. Karre (Karolinska Institute, Stockholm, Sweden). B16 (spontaneous C57BL/6-derived melanoma) was provided by W. Seaman (Veteran's Administration Medical Center, San Francisco, CA). Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 mM β-mercaptoethanol.

Antibodies and Reagents.

Hybridoma producing the following mAbs were obtained from ATCC; anti-NK1.1 (PK136), anti-ratκ (MAR 18.5), anti-CD3 (2C11), anti-Thy1.2 (30-H12) and anti-FcγRII/III (2.4G2). Anti-Ly-49A (JR9-318) was obtained from Dr. J. Roland (Pasteur Institute, Paris, France) and conjugated with fluorescein isothiocyanate (FITC) by standard methods (121). FITC-conjugated goat F(ab')$_2$ anti-mouse IgG and FITC-conjugated goat F(ab')$_2$ anti-hamster IgG were purchased from Caltag Laboratories (Burlingame, CA). The following mAbs were purchased from Pharmingen (San Diego, CA): anti-CD28 (37.51), phycoerythrin (PE)-conjugated anti-NK1.1 (PK136), FITC-, PE- or Cy-Chrome (Cy)-conjugated anti-CD3ε (145-2C11), PE-conjugated anti-CD4 (GK1.5), and FITC-conjugated anti-CD8 (53-6.7), anti-CD11a (2D7), anti-CD11b (M1/70), anti-CD11c (HL3), anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), anti-CD43 (S7), and anti-Gr-1 (RB6-8C5).

Human recombinant IL-2 was purchased from Chiron (Emeryville, CA). Polyinosinic-polycytidylic acid (poly-I:C), lipopolyssacharide (LPS; *E. coli* serotype 0127:B8, *Serratia marcescens*) and Percoll were purchased from Sigma (St. Louis, MO). Trinitrophenlyl (TNP)-Ficoll and TNP-bovine serum albumin (BSA) were purchased from Solid Phase Sciences (San Rafael, CA).

Generation of Transgenic Mice.

The murine granzyme A genomic DNA was used for the in vivo expression of Ly-49A. Since little is known about regulatory sequence regarding tissue specific expression of granzyme A, the entire genomic sequence (carrying 11 kb putative promoter and 7 kb structural gene with poly-A addition signal) (122, 123) was used as an expression cassette. The granzyme A-Ly-49A transgene was constructed by standard methods (124). To facilitate the expression of Ly-49A cDNA, the translation start codon of granzyme A gene was deleted by replacing 1032 bp Nae I-Sma I fragment (carrying putative promoter and start codon) with a truncated 1008 bp Nae I-Sma I fragment (carrying only putative promoter). The truncated fragment was prepared from PCR product that was amplified using primers: CCTGAAGCATGCTATCTCACGA, GTTCCCGGGCTCTCCACCCCAATCA.

Ly-49A cDNA was excised as 1.2 kb Xho I fragment from pA1.3 (83), filled with Klenow fragment, and inserted into the Sma I site of the modified granzyme A gene. The granzyme A-Ly-49A transgene was excised as 19 kb Sal I-Kpn I fragment and micro-injected into the pronucleus of a fertilized B6 egg by standard methods (125). Initial screening for founder mice carrying the transgene was performed by Southern blot analysis of tail DNA with the Ly-49A cDNA probe. To examine in vitro expression of the Ly-49A transgene, peripheral blood obtained from founder mice was cultured in RPMI 1640 media containing 10 % FCS and recombinant human IL-2 (1000U/ml) for 10 d. Cell surface expression was confirmed by flow cytometry analysis. After Tg lines were established from founder mice, Tg mice were screened by PCR analysis of tail DNA using transgene specific primers: ACCTCCATCTCCACCGGGCAGCA, CTGGCAGGTCTGTTTACATCCACTCC Heterozygous Tg mice and WT littermates were used in all experiments.

DNA Preparation from Tail.

For Southern blot analysis, tail (about 1 cm) was cut from 4 week old pups and incubated in digestion buffer I (50mM Tris, pH 7.5, 100 mM NaCl, 100mM EDTA and 1% SDS) containing 1 mg/ml proteinase K at 55°C overnight. Before DNA was precipitated with ethanol, the extract was treated with phenol twice then phenol/chloroform once. For PCR analysis, tail was incubated in digestion buffer II (50mM Tris, pH 7.5, 100 mM NaCl, 100mM EDTA, 0.01% gelatin, o.45% NP40 and 0.45% Tween20) containing 1 mg/ml proteinase K at 55°C overnight then directly used.

Cell Preparation and Flow Cytometry.

Under anesthesia, mice were exsanguinated by cardiac puncture then spleen, liver, lung and BM were collected. Single cell suspension from spleen was prepared by passage through sieves in HBSS (Gibco) containing 10% FCS and red blood cells (RBCs) were lysed with RBC lysis solution (0.14 M NH_4Cl and 0.017 M Tris, pH 7.2). BM cells were prepared from femur and tibia of the hindlimbs of mice by forcing RPMI 1640 medium through the bones using syringe and 20 gauge needle and RBCs were lysed with RBC lysis solution. Mononuclear cells from liver and lung were prepared as previously described (126). Peripheral blood was directly treated with RBC lysis solution. After RBC lysis, remaining cells in all preparations were washed with RPMI

1640 medium containing 10% FCS. For the phenotyping of marker expression, cells were incubated with anti-FcγRII/III antibody to block non-specific binding then stained with combinations of indicated fluorochrome-conjugated antibodies. Stained cells were analyzed using a FACScalibur (Becton Dickinson, Mountain View, CA). Dead cells were removed by prior centrifugation at 500x g for 20 min on Lympholyte-M or excluded from analysis by propium iodide staining. At least 3 mice per group were analyzed for each organ and representative data are shown.

Skin Allograft Rejection Assay and T Cell Proliferation Assay.

For skin allograft rejection assay, tail skin of B10.D2 was grafted onto dorsal surface of recipient as described (127). For T cell proliferation assay, 96 well flat bottom plate was incubated with 30 μl of anti-CD3 mAb at indicated concentrations at 4°C overnight then washed with PBS. Splenocytes were plated onto the plate at 2 x 10^5 cells/well and incubated. 2 d later, 0.5 μCi of [methyl-^{3}H] thymidine was added onto each well (Amersham) and incubated for additional 24 hr. Incorporated radioactivity was counted with a β counter.

ELISAs for Immunoglobulin Isotypes and Cytokines.

Basal levels of Ig isotypes were determined by clonotyping system (Southern Biotechnology Associates Inc., Birmingham, AL) following manufacture's instructions. For detection of TNP-specific Ig levels, Immulon2 microtiter plates (Dynex Technologies, Chantilly, VA) were incubated with 100 μg of TNP-BSA overnight at 4°C then washed with 0.3% Tween20-PBS. 2% BSA-PBS was added onto the plates, incubated at room temperature for 1 hr to block non-specific binding, and the plates were washed with 0.3% Tween20-PBS. Serum samples serially diluted in 2% BSA-PBS were added onto the plates, incubated at room temperature for 2 hr, and washed with 0.3% Tween20-PBS. Plate-bound Ig was detected with horseradish peroxidase-labeled isotype-specific antibodies in the clonotyping system followed by substrate reaction. Optical density was measured with a ELISA reader. Cytokines were measured using IL-4 and IFN-γ ELISA kits (Endogen, Woburn, MA).

In Vitro Cytotoxicity Assay.

NK cell activity was boosted by peritoneal injection of poly-I:C into experimental mice. 24 h later, splenocytes or BM cells were prepared as described above and natural killing was tested by standard ^{51}Cr-release assay as described previously (69). Briefly, tumor target cells (2 x 10^6 cells) were radiolabeled with 50 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) in RPMI 1640 medium containing 10% FCS. 90 min later, target cells were washed 3 times and added onto 96 well round-bottom plates at a constant number (10^4 cells/well). To achieve indicated effector-to-target (E:T) ratios, splenocytes or BM cells were plated onto at various cell densities. The plates were incubated for 4 hr and the radioactivity in the supernatants was counted with a gamma counter. Specific cytotoxicity was calculated according to the standard formula: % Specific lysis = 100 X (Experimental - Spontaneous)/(Total - Spontaneous) where Experimental represents the radioactivity from experimental wells, Spontaneous represents the radioactivity from wells with target cells alone, and Total represents the radioactivity from detergent (2% Triton-X100) lysed target cells.

In Vivo Tumor Rejection Assays.

The acute tumor clearance assay was performed as previously described (128) Target cells (8 x 10^6) were incubated with 50 μg of 5-fluoro-2-deoxyuridine (Sigma) in 2 ml volume of complete DMEM media. 15 min later at 37°C, 5 μCi of ^{125}I-labeled 5-iodo-2-deoxyuridine (^{125}I-UdR; Amersham, Arlington Heights, IL) was added and incubated for 2 hr. Cells were washed three times in PBS. Mice were injected intravenously through tail vein with 3 x 10^4 to 3 x 10^5 cells in 200 μl volume of PBS. 4 to 8 hr after injection, mice were sacrificed and the lungs were removed, rinsed with PBS then soaked in 70%

ethanol for 1 hr. Residual radioactivity in the lungs was counted with a gamma counter. The percentage of residual radioactivity was calculated as follows: % residual radioactivity = (residual radioactivity in the lungs/total injected radioactivity) x 100. For tumor metastasis assay, mice were injected intravenously through tail vein with 3 x 10^4 B16 melanoma cells in 200 μl volume of PBS. 14 d later, the lungs were removed and visible, black metastatic foci were counted. For long-term tumor outgrowth assay, mice were injected subcutaneously in the left flank with 1 x 10^2 to 1 x10^5 RMA-S cells in 100 μl volume of RPMI 1640. Mice were monitored for palpable tumors twice weekly for 8 weeks.

Bone Marrow Rejection Assay.

BM cells were prepared from β2m-deficient B6 donor mice as described but without RBC lysis. To count non-RBC cell number, an aliquot of BM preparation was treated with RBC lysis solution. Indicated recipient mice were injected intravenously through tail vein with 5 x 10^5 or 5 x 10^6 non-RBC BM cells after otherwise lethal γ-irradiation (9.5 Gy from a ^{137}Cs source) on day 0. 5 d after BM transplantation, the recipient mice were injected intravenously through tail vein with 2 μCi of ^{125}I-UdR then radioactivity in the spleens was counted with a gamma counter at day 6.

Production of Bone Marrow Chimeras.

BM cells were prepared from indicated donor mice as described above but without RBC lysis. NK and T cells were lysed by treatment with anti-NK1.1, anti-Thy1.2, and Low-Tox rabbit complement (Accurate Chemical & Scientific Corporation, Westbury, NY). Dead cells were removed by centrifugation on Lympholyte-M. Indicated recipient mice were injected intravenously through tail vein with 5 x 10^6 BM cells after otherwise lethal γ-irradiation (9.5 Gy) on day 0. Recipient mice were analyzed 8 to 12 weeks after BM transplantation.

RESULTS

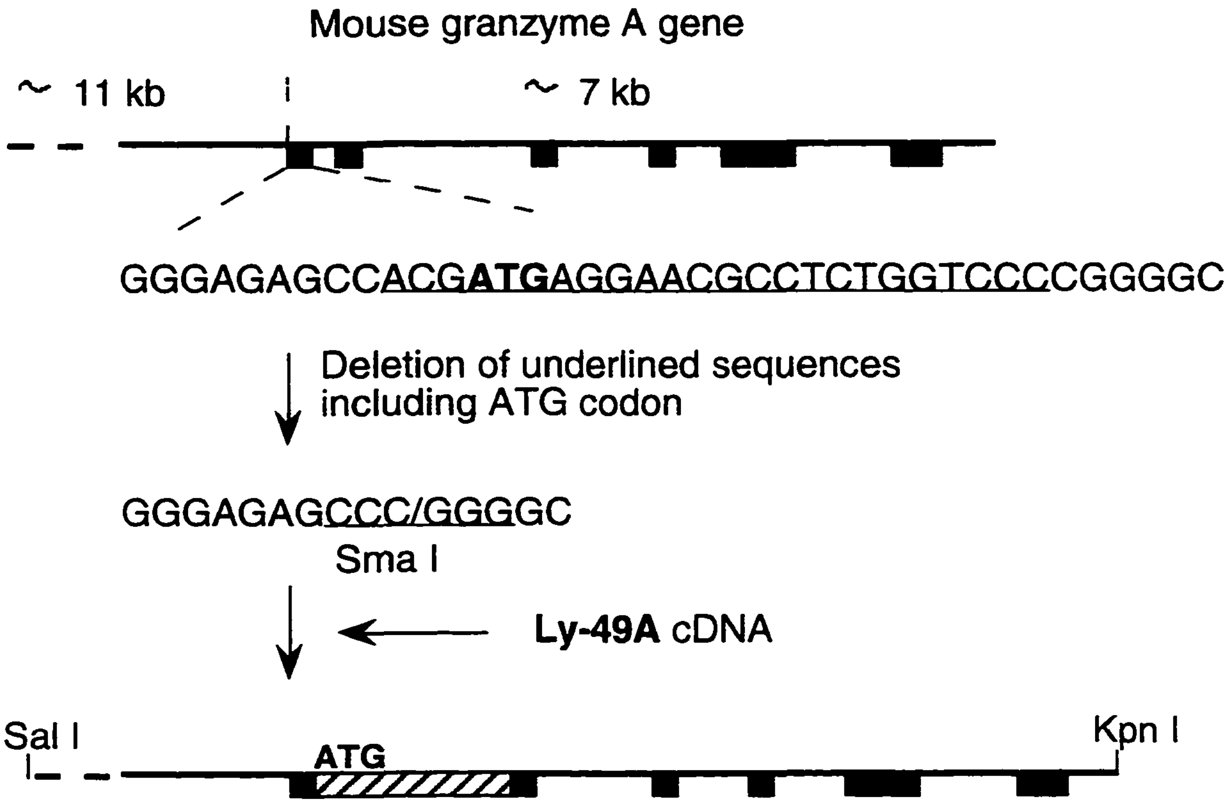
Generation of Ly-49A Transgenic Mice.

Murine granzyme A genomic DNA was used to facilitate in vivo expression of Ly-49A, since granzyme A expression is known to be restricted to cytotoxic T cells and NK cells. The Ly-49A cDNA was cloned into the exon 1 after removal of the granzyme A translation start codon (Figure 1). The granzyme A-Ly-49A transgene construct was excised and purified from the plasmid backbone and micro-injected into the pronucleus of fertilized eggs of B6 mice. To distinguish wild-type (WT) and Tg mice, 84 pups were screened by Southern blot analysis of tail DNA. Eight founder mice (founder #1 to 8) bearing the Ly-49A transgene were obtained and the transgene copy number ranged from 1 to 30: 1 to 5 copies (founder # 2, #3 and #4), 6 to 10 copies (founder #1, #6 and #8), 11 to 20 copies (founder #5), and 21 to 30 copies (founder #7). All founder mice were fertile and appeared normal.

Considering the capacity of IL-2 to support NK cell expansion *in vitro*, blood cells obtained from these founder mice or WT control mice were cultured in IL-2 and the expression of Ly-49A on the cell surface was examined by flow cytometric analysis using mAb specific for Ly-49A. Among IL-2-expanded cells derived from WT mice, a subpopulation (about 20%) expressed Ly-49A (data not shown), consistent with the known frequency of Ly-49A$^+$ cells among NK cells *in vivo*. The majority of cells derived from founder #3, 5, 7 and 8 expressed Ly-49A, indicating the expression of the Ly-49A transgene. However, cells derived from founder #1, 2, 4 and 6 displayed no sign of the Ly-49A transgene expression because their flow cytometric profiles were not different from those obtained from WT mice. To establish Tg mouse lines, founder mice were bred in the B6 background.

Figure 1. Production of Ly-49A Tg mice.

A partial map of the murine granzyme A gene is shown. After removal of the granzyme A translational initiation codon, the Ly-49A cDNA was inserted into exon 1. The 19 kb Sal I-Kpn I fragment was purified and injected into fertilized eggs of B6 mice by standard techniques to produce Tg mice. Exons of the granzyme A gene are displayed as filled boxes and the hatched rectangle represents the Ly-49A cDNA .


Mouse granzyme A gene
~ 11 kb
~ 7 kb
GGGAGAGCCACGATGAGGAACGCCTCTGGTCCCCGGGGC
Deletion of underlined sequences including ATG codon
GGGAGAGCCC/GGGGC
Sma I
Ly-49A cDNA
Sal I
ATG
Kpn I

The *in vivo* expression of the Ly-49A transgene was examined by flow cytometric analysis of unactivated spleen cells obtained from pups of each line. Since NK cells in B6 mice are currently characterized by the expression of NK1.1 and lack of CD3 expression, $CD3^-$ $NK1.1^+$ spleen cell populations were analyzed for their Ly-49A expression by staining with combinations of mAbs specific for Ly-49A, NK1.1 or CD3. Tg mice from line #7 displayed Ly-49A expression on most NK cells at intermediate levels, which were distinguishable from high levels of endogenous Ly-49A expression (Figure 2). However, Tg mice from lines #1, 2, 3, 4 and 6 displayed no sign of the Ly-49A transgene expression since only a subpopulation (about 20%) of NK cells expressed Ly-49A, similar to WT littermate control. Tg mice from lines #5 and 8 had an additional small population (5 to 10%) of NK cells with intermediate levels of Ly-49A expression (data not shown), indicating that only a minor population of NK cells in these mice expressed the Ly-49A transgene.

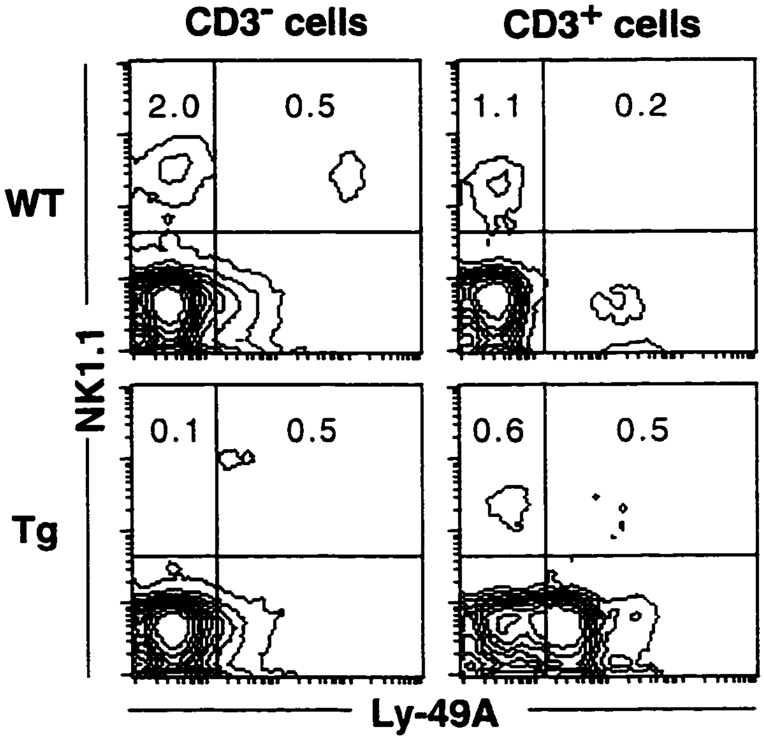
Spleen cells obtained from Tg mice from line #7 were further analyzed to examine the expression of the Ly-49A transgene on other cell populations. A substantial proportion (about 50%) of NK/T ($CD3^+$ $NK1.1^+$) and conventional T ($CD3^+$ $NK1.1^-$) expressed Ly-49A (Figure 2). There was no apparent difference in the Ly-49A staining profiles of non-NK and non-T ($CD3^-$ $NK1.1^-$) cells between WT littermates and Tg mice, indicating that the other cell populations such as B cells did not express the Ly-49A transgene. Thus, Tg mice and WT littermate mice derived from line #7 were used for further study.

Impaired *In Vitro* NK Cell Cytotoxicity in Transgenic Mice.

Interestingly, flow cytometric analysis of spleen cells showed that the percentage of NK cells was significantly lower in Tg mice, compared with WT mice (Figure 2). To evaluate NK cell cytotoxic activity, mice were injected with poly-I:C, an interferon inducer, which is known to augment NK cell cytotoxicity, and spleen cells were subjected to a standard ^{51}Cr-release assay against YAC-1 tumor cells, a prototype NK-sensitive target. Surprisingly, virtually no NK cell cytotoxicity was detected in spleen cells obtained from Tg mice, over a

Figure 2. Flow cytometric analysis of Ly-49A expression on spleen cells in Tg mice.

Single cell suspensions were prepared from the spleens and subjected to triple staining with Cy-conjugated anti-CD3, PE-conjugated anti-NK1.1 and FITC-conjugated anti-Ly-49A. Profiles for Ly-49A expression on cells gated on CD3-negative and CD3-positive populations are shown. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of WT and Tg mice are shown.


CD3⁻ cells
CD3⁺ cells
WT
Tg
NK1.1
Ly-49A
2.0
0.5
1.1
0.2
0.1
0.5
0.6
0.5

wide range of effector to target ratios, while WT spleen cells efficiently killed the target (Figure 3A).

This impaired cytotoxicity could not be attributed solely to the decrease in the number of NK cells in the spleen of Tg mice, because this number decrease was not usually greater than a factor of five. One possible explanation for this observation was that the engagement of the transgene-derived Ly-49A could prevent NK cells from killing of the target since YAC-1 cells expressed H-2 D^d MHC class I molecules, a known ligand for Ly-49A, at low but detectable levels. To test this possibility, further cytotoxicity assays were performed on syngeneic B6 mice-derived MHC class I-deficient RMA-S lymphoma and B16 melanoma targets. Interestingly, Tg spleen cells still failed to kill RMA-S to the expected levels (Figure 3B). Moreover, Tg spleen cells displayed no detectable killing of B16 (Figure 3C). These results suggest that in addition to decrease in NK cell number, there may be an qualitative difference in the NK cell compartment between WT and Tg mice.

Abnormal NK but Normal T, NK/T and B Cell Compartment in Transgenic Mice.

There were no significant differences in total spleen cell numbers between WT littermates ($52 \times 10^6 \pm 8 \times 10^6$, n=5) and Tg mice ($47 \times 10^6 \pm 5 \times 10^6$, n=5). On the basis of cell percentages determined by CD4, CD8 and sIg staining (Figure 4), Tg mice appeared to have a normal number of splenic $CD4^+$ T cells, $CD8^+$ T cells and B cells. In addition to the spleen, NK cells are known to be present in the peripheral blood, lung and liver at relatively high frequencies. To examine the distribution of NK cells, leukocytes were isolated from these organs and analyzed by flow cytometry. Similar to the observation in the spleen, there was a markedly reduced number of NK cells in each organ of Tg mice, compared with WT mice (Figure 5). There was no significant difference in the number of other cells between WT and Tg mice. In particular, Tg mice appeared to have a normal number of NK/T cells in the tested organs except for the spleen where the number of NK/T cells was slightly reduced (by

Figure 3. Impaired in vitro tumor cell killing activity of splenic NK cells in Tg mice.

WT (squares) and Tg (circles) mice were injected intraperitoneally with 150 µg of poly-I:C. 24 h later, the spleen cells were used in 4 hr ^{51}Cr-release cytotoxicity assays against YAC-1 (A), RMA-S (B) and B16 (C) target cells at varying effector:target (E:T) ratios as indicated. Results are expressed as the mean percent-specific lysis ± SD of triplicate wells.

Figure 4. Normal splenic T cell subsets and B cells in Tg mice.

Spleen cells were subjected to dual staining with PE-conjugated anti-CD3 and FITC-conjugated anti-Ig antibodies or PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 antibodies. The numbers represent the percentage of cells within each quadrant among all viable cells. Representative data from one of 3 WT and 3 Tg mice are shown.

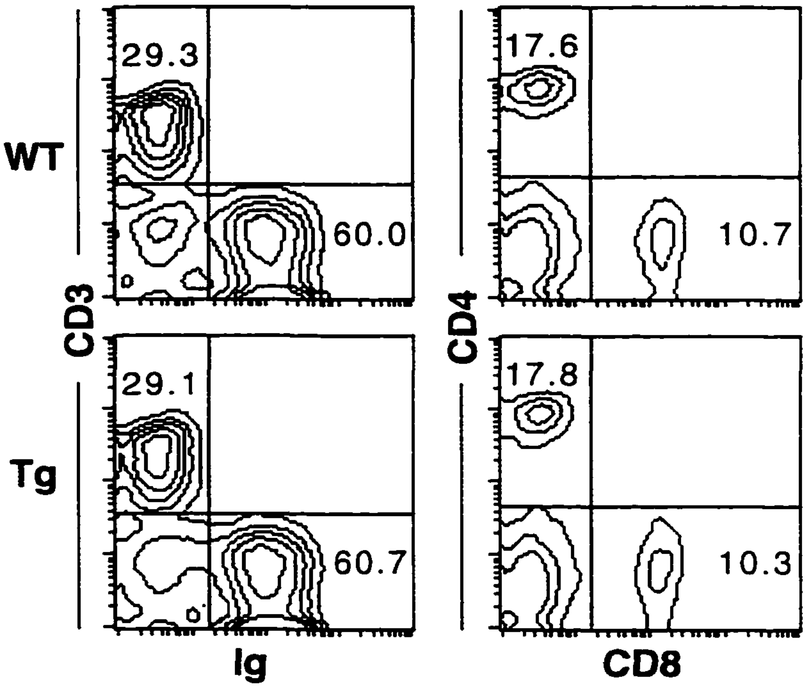
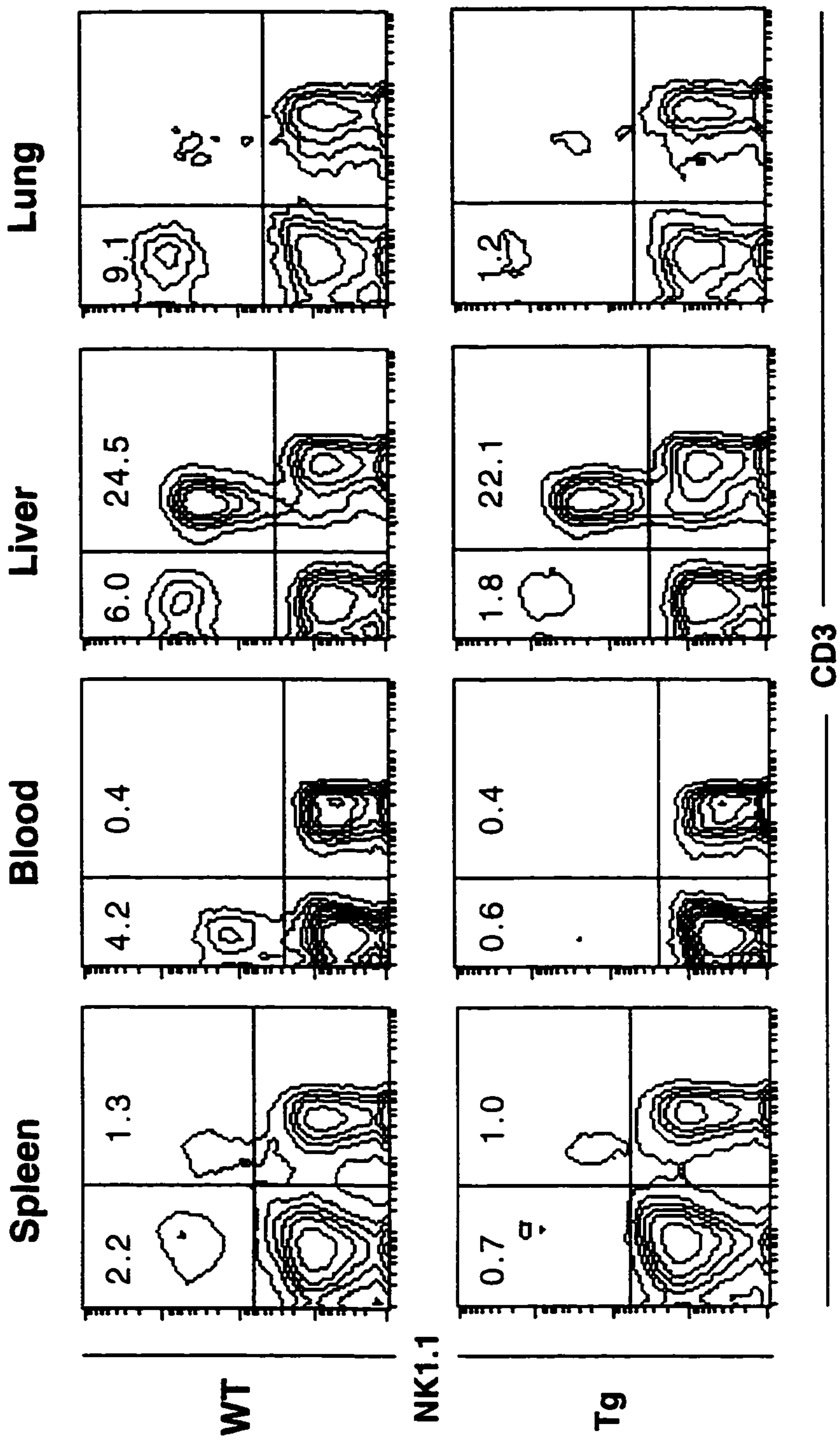

WT
Tg
CD3
Ig
CD4
CD8
29.3
60.0
29.1
60.7
17.6
10.7
17.8
10.3

Figure 5. Marked decrease of peripheral NK cells in Tg mice.

Single cell suspensions were prepared from indicated organs and subjected to dual staining with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 antibodies. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of 3 WT and 3 Tg mice are shown.


Spleen
Blood
Liver
Lung
WT
Tg
NK1.1
CD3
2.2
1.3
4.2
0.4
6.0
24.5
9.1
0.7
1.0
0.6
0.4
1.8
22.1
1.2

30%) (Figure 5). Moreover, Tg mice appeared to have a normal number of NK/T, CD4$^-$ CD8$^-$, CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$, and CD4$^+$ CD8$^+$ cells in the thymus (data not shown).

Given the known inhibitory effects of Ly-49A engagement on many effector functions (68, 79), it was interesting to test the possibility that Tg mice might be unable to reject skin grafts expressing ligands for Ly-49A, since a substantial population of T cells expressed Ly-49A (Figure 2). It was also reasoned that the expression of the Ly-49A transgene could be up-regulated if T cells are activated by recognition of allogeneic tissue because the transcription of granzyme A gene is known to be upregulated following activation of TCR and IL-2 receptor (129). However, tail skin grafts of B10.D2 mice expressing H-2 D^d MHC class I molecules were rejected by both WT and Tg mice with essentially identical kinetics (Figure 6A). It was not clear whether T cells without Ly-49A expression in Tg mice mediated skin graft rejection or whether inhibitory signals elicited by Ly-49A engagement was not able to inhibit T cell-mediated rejection of skin grafts. Though interesting, this issue was not further investigated. Nevertheless, the data suggest that T cell function is essentially normal in Tg mice. Consistent with this, when Tg and WT spleen cells were incubated with plastic-immobilized anti-CD3 mAb, essentially identical proliferative responses were observed (Figure 6B).

NK/T cells are known as the major producers of IL-4 and IFN-γ after intravenous administration of anti-CD3 mAb (130). To examine if NK/T cell function is normal, mice were injected intravenously with anti-CD3. Spleen cells from treated Tg mice produced slightly reduced (about 30%) but significant levels of IL-4 and IFN-γ as compared to spleen cells from treated WT (Figure 7). In contrast, no detectable levels of cytokines were produced by spleen cells isolated from non-treated mice. The degree of reduction in the cytokine production seemed to correspond to the decrease in the number of splenic NK/T cells in Tg mice (Figure 5), suggesting that NK/T cells in Tg mice are functional. Since Tg mice have a nearly normal number of NK/T cells in other organs, the NK/T cell compartment appeared to be normal in Tg mice.

Figure 6. Normal T cell functions in Tg mice.

(A) Rejection of allogeneic skin grafts. WT (n=6, squares) and Tg (n=8, circles) mice were grafted with C57BL/10.D2 skin. WT (n=4, diamonds) were grafted with B6 skin as control. Mice were monitored daily and when <20% of the graft remained, it was considered rejected. (B) Proliferative response of T cells. Spleen cells (2×10^5 cells/well) from WT (squares) and Tg (circles) mice were stimulated with anti-CD3 mAb immobilized at graded concentrations. 2 day later, cells were pulsed for 24 hr with [^{3}H]thymidine. Results are expressed as the mean cpm ± SD of triplicate wells.

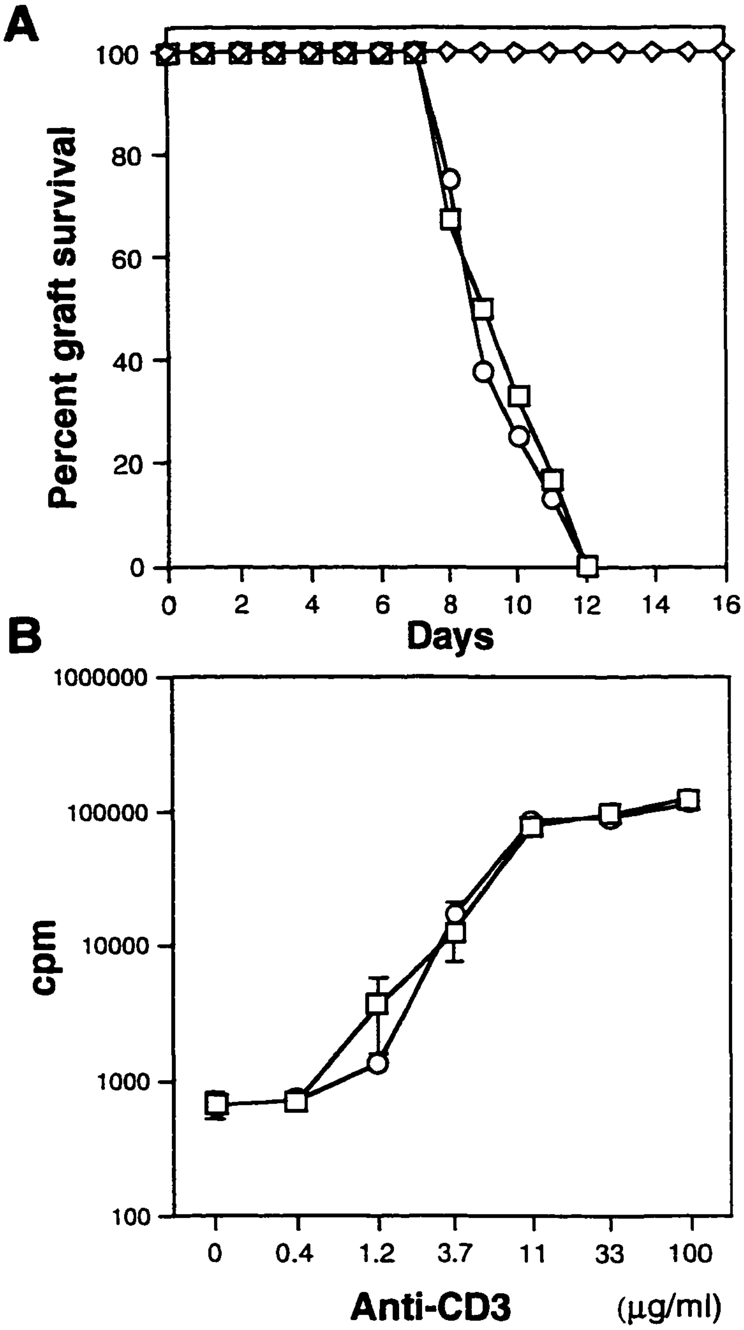
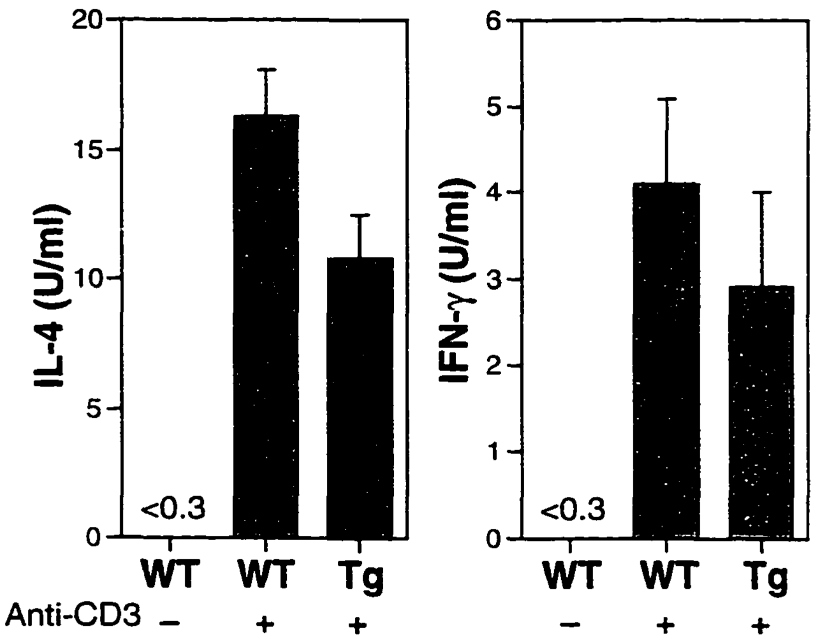

A
Percent graft survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Days
B
cpm
1000000
100000
10000
1000
100
0
0.4
1.2
3.7
11
33
100
Anti-CD3
(μg/ml)

Figure 7. Normal NK/T cell functions in Tg mice.

Mice were injected intravenously with PBS or 2 μg of anti-CD3 mAb. 90 min later, the spleen cells were harvested and incubated at 5×10^6 cells per ml for 2 hr. The supernatants were assayed for IL-4 and IFN-γ by ELISA. Results are expressed as the mean concentration ± SD from 3 mice per group.


IL-4 (U/ml)
20
15
10
5
0
<0.3
WT
WT
Tg
Anti-CD3
–
+
+
IFN-γ (U/ml)
6
5
4
3
2
1
0
<0.3
WT
WT
Tg
–
+
+

To examine if B cell function is normal, serum immunoglobulin levels were measured by ELISA. There was no significant difference in the levels of all tested isotypes (IgM, IgA, IgE, IgG1, IgG2a, IgG2b and IgG3) between WT and Tg mice (Figure 8). Previous *in vitro* studies have suggested that NK cells may play a role in the humoral immune response to TI Ag (7, 131). To test this possibility, mice were immunized with TNP-Ficoll, a prototypic type 2 TI Ag, and Ag-specific serum Ig levels were measured by ELISA. There was no apparent difference in IgM production between WT and Tg mice (Figure 9A). In addition, both WT and Tg mice produced essentially identical levels of IgG3 (Figure 9B), indicating that isotype switching occurs normally in Tg mice. Moreover, there was no apparent difference in the production of other isotypes between WT and Tg mice (data not shown). Similar results were observed in mice that were depleted of NK1.1$^+$ cells by administration of anti-NK1.1 mAb (data not shown). These results argue against the involvement of NK cells in the humoral immune response to type 2 TI Ag. Nevertheless, these data suggest that Tg mice have a normal B cell compartment. Collectively, despite of abnormalities in NK cell compartment, Tg mice appear to have normal T, NK/T and B cell compartments.

Impaired *In Vivo* Tumor Rejection in Transgenic Mice.

Numerous studies have suggested that NK cells may play a role in tumor rejection, based on correlative data from various experimental systems. However, due to the lack of animal model in which NK cell activity is selectively deficient, direct evidence is lacking. Taking advantage of the selective NK cell deficiency in Tg mice, the specific role of NK cells in tumor rejection was evaluated using 3 different tumor study models.

First, in order to evaluate the role of NK cells in eliminating tumor cells in short-term, WT control, anti-NK1.1-treated WT and Tg mice were injected intravenously with isotope-labeled tumor cells and residual radioactivity in the lungs was measured. When YAC-1 cells were injected into WT mice, the tumor cells were completely cleared within 4 hr, as evidenced by low levels of

Figure 8. Normal levels of serum Ig isotypes in Tg mice.

Sera from WT and Tg mice were assayed for Ig isotypes by ELISA using isotype-specific antibodies. Results are expressed as % control ± SD of three independent experiments. % control was calculated using a formula: % control = Ig levels of Tg mice/Ig levels of WT mice x 100.

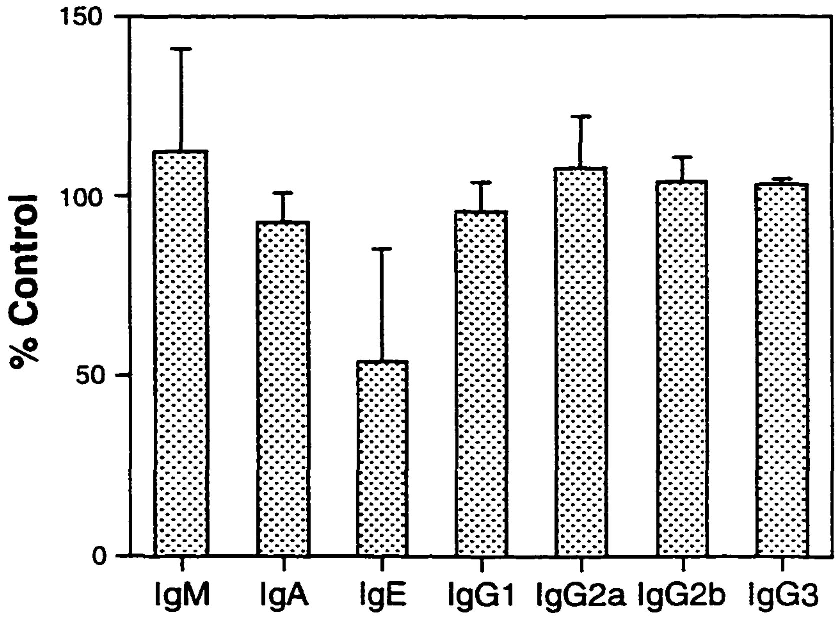
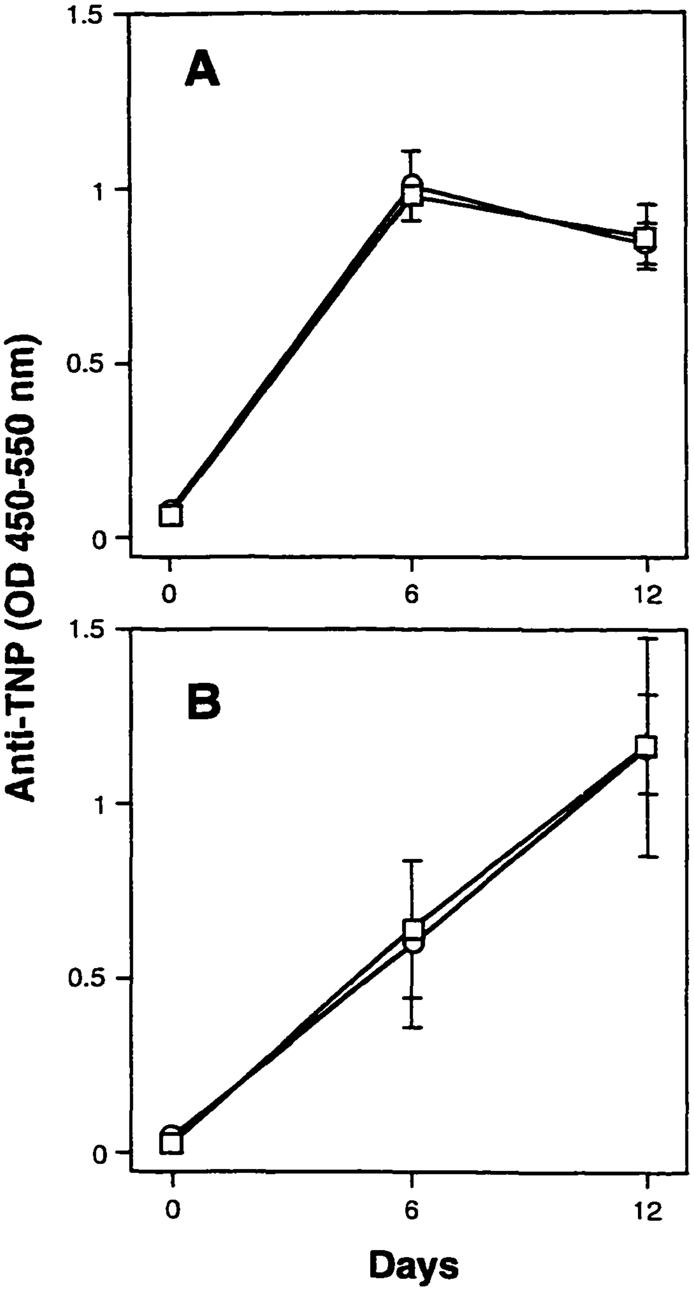

150
100
50
0
% Control
IgM
IgA
IgE
IgG1
IgG2a
IgG2b
IgG3

Figure 9. Normal humoral responses to type 2 TI Ag in Tg mice.

WT (squares) and Tg (circles) mice were immunized with intraperitoneal injection of 10 μg of TNP-Ficoll. Sera were diluted 500 fold and Ag-specific IgM (A) and IgG3 (B) titers were measured by ELISA on TNP-BSA-coated plates using isotype-specific antibodies. Results are expressed as the mean optical density ± SD from 3 mice per group.


1.5
A
1
0.5
0
0
6
12
1.5
B
1
0.5
0
0
6
12
Anti-TNP (OD 450-550 nm)
Days

residual radioactivity in the lungs (Figure 10A). In contrast, Tg mice retained high levels of residual radioactivity. Anti-NK1.1-treated mice that had no detectable NK and NK/T cells retained comparable levels of residual radioactivity. Similar results were observed with B16 tumor cells (Figure 10B).

Second, to evaluate the role of NK cells in tumor rejection in long-term, mice were injected intravenously with 3 x 10^4 B16 melanoma cells and visible lung or liver metastases were counted after 14 d. None of WT mice had more than 15 lung metastases (Figure 11). In contrast, all Tg mice had more than 200 metastases. Similarly, all anti-NK1.1-treated WT mice had more than 200 metastases. In addition, variable numbers (1 to 30) of liver metastases were found in Tg mice and anti-NK1.1-treated WT mice whereas none of WT mice had liver metastases. Interestingly, anti-NK1.1-treated WT mice generally had more liver metastases than Tg mice although this was not statistically significant.

Third, to evaluate the role of NK cells in tumor rejection in longer-term, mice were injected subcutaneously in the flank with variable doses of RMA-S cells and then monitored for palpable tumors. Once tumors were palpable, no signs of regression were observed. When 10^2 or 10^3 tumor cells were injected, most WT control mice were able to control tumor growth (Figure 12A and 12B). In contrast, none of anti-NK1.1-treated WT mice were able to control tumor growth under the same conditions. Likewise, most Tg mice were unable to control tumor growth. To achieve similar levels of tumor incidence in WT mice, 100 to 1000 fold more tumor cells were needed (Figure 12C). Collectively, these results demonstrate that NK cells are the major effector cell population mediating acute tumor clearance, prevention of tumor metastasis, and control of tumor outgrowth.

Figure 10. Impaired acute in vivo rejection of tumor cells in Tg mice.

Anti-NK1.1-treated WT mice, untreated WT and Tg mice were injected intravenously with ^{125}I-UdR-labeled 3x10^5 YAC-1 (A) and 1x10^5 B16 (B) tumor cells, respectively. The residual activity in the lungs was determined after 4 hr and 8 hr, respectively. Anti-NK1.1-treated mice were injected intraperitoneally with 200 μg of anti-NK1.1 mAb 2 day before tumor inoculation. Results are expressed as the mean percent residual radioactivity ± SD from 3 -5 mice per group.

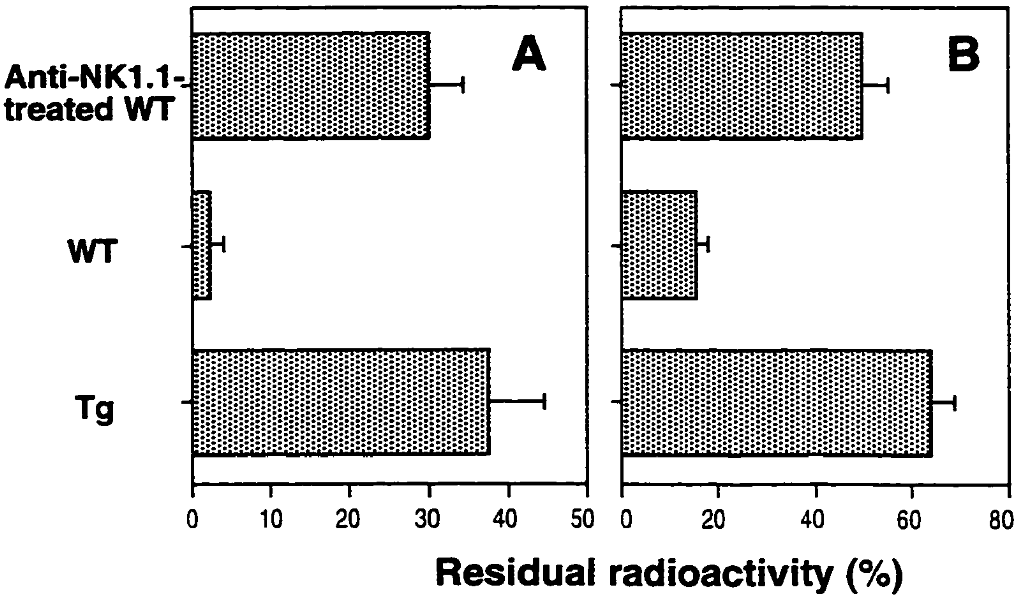

Anti-NK1.1-treated WT
WT
Tg
A
B
0
10
20
30
40
50
0
20
40
60
80
Residual radioactivity (%)

Figure 11. Increased formation of experimental lung metastasis in Tg mice. Anti-NK1.1-treated WT mice, untreated WT and Tg mice were injected intravenously with 3×10^4 B16 tumor cells on day 0. Macroscopic tumor metastases in the lungs were counted on day 14. Anti-NK1.1-treated mice were injected intraperitoneally with 200 μg of anti-NK1.1 mAb on days -2, +2 and +7. Results are expressed as the mean number ± SD from 4 mice per group.

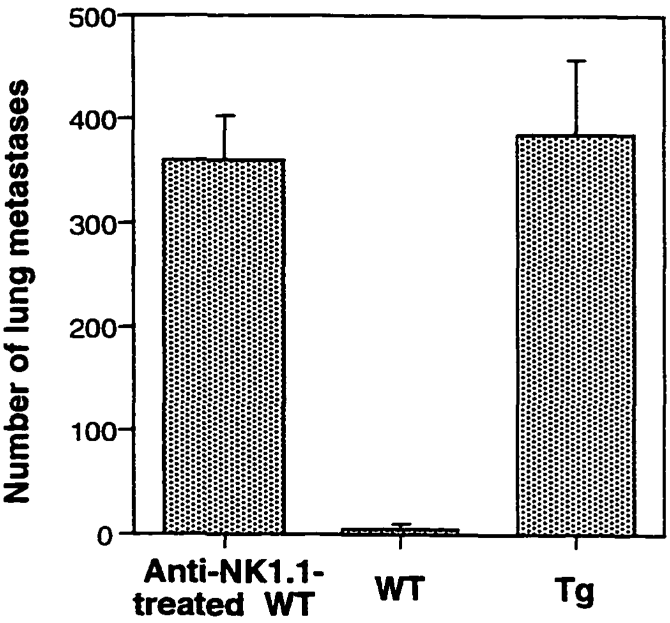
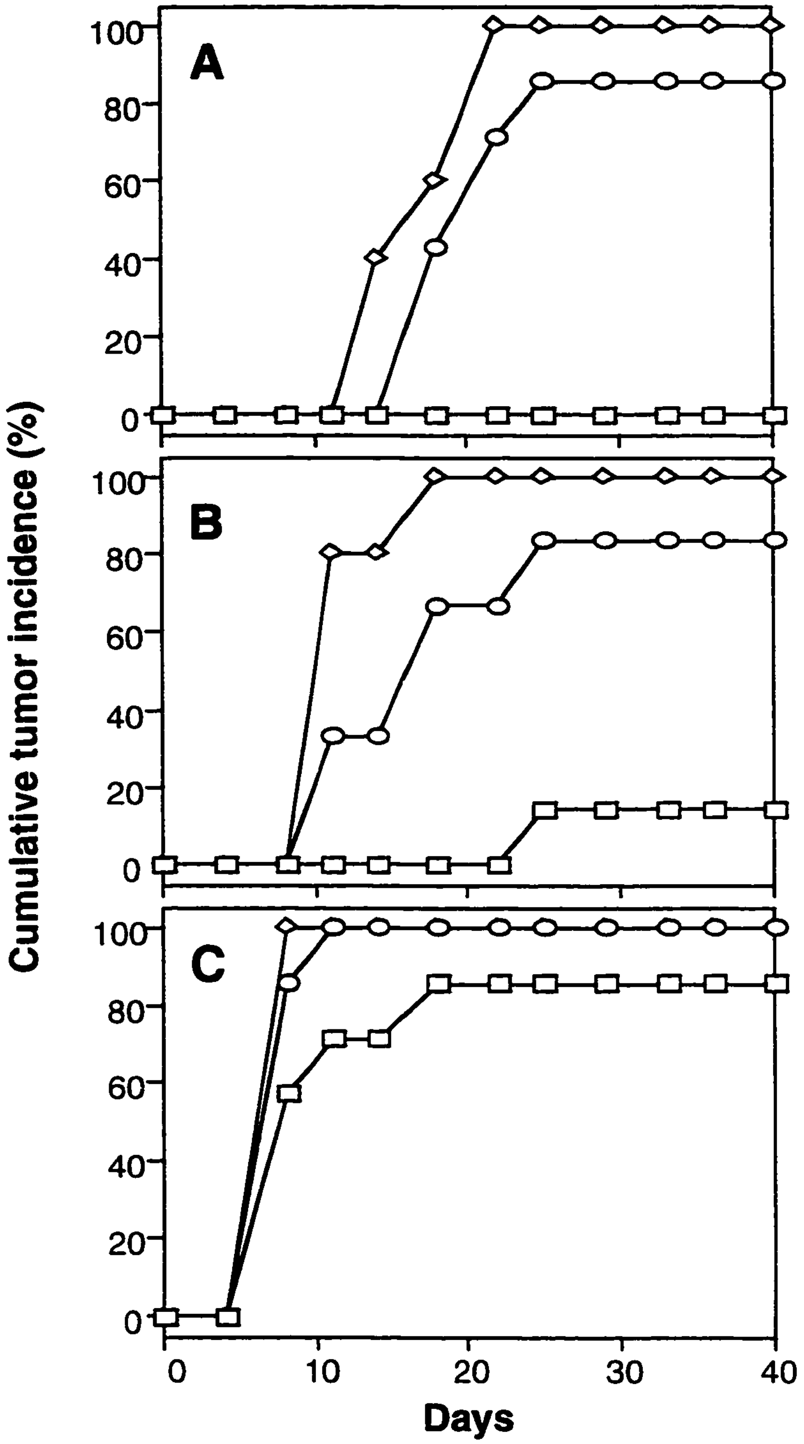

Number of lung metastases
500
400
300
200
100
0
Anti-NK1.1-treated WT
WT
Tg

Figure 12. Impaired control of long-term tumor growth in Tg mice.

10^2 (A), 10^3 (B), or 10^5 (C) RMA-S tumor cells were injected subcutaneously into the flanks of anti-NK1.1-treated WT mice (diamonds, 3-5 mice per group), untreated WT (squares, 7 mice per group) and Tg (circles, 6-7 mice per group), respectively. Mice were monitored for palpable tumors twice weekly. Anti-NK1.1-treated mice were injected intraperitoneally with 200 μg of anti-NK1.1 mAb on days -4, -2, +7, +14 and +21.


A
B
C
100
80
60
40
20
0
0
10
20
30
40
Days
Cumulative tumor incidence (%)

Reconstituted Acute Tumor Rejection by SCID Splenocytes in Transgenic Mice.

If an NK cell deficiency was the sole reason for the impaired tumor rejection, infusion of exogenous NK cells should reconstitute the tumor rejection in Tg mice. To test this possibility, mice were first infused with PBS,*scid* splenocytes (8 x 10^6 cells), or same number of NK cell-depleted *scid* splenocytes and subsequently injected with radiolabeled tumor cells. Similar to the previous observation obtained with other tumor cells, WT mice completely cleared RMA-S lymphoma cells, while this clearance was impaired in both anti-NK1.1-treated mice and Tg mice (Figure 13). The infusion of NK cell-containing *scid* splenocytes significantly restored tumor clearance in Tg mice. In contrast, the infusion of NK1.1^+ cell-depleted *scid* splenocytes into Tg mice failed to restore tumor clearance. Given that *scid* mice have no mature NK/T, T or B cells but have normal number of NK cells, these results provide further evidence that the functional defect in Tg mice is due to a deficiency in the NK cell compartment, not due to NK/T, T or B cell dysfunction.

Impaired Bone Marrow Graft Rejection in Transgenic Mice.

It has been known that NK1.1^+ cells mediate the rejection of β2-microglobulin (β2m)-deficient BM cell grafts in lethally irradiated β2m-sufficient mice (132). To evaluate the role of NK cells in this rejection, a series of BM transplantation experiments were performed. When 5 x 10^6 β2m-deficient BM cells were transplanted into lethally irradiated β2m-deficient mice, the recipient mice accepted marrow grafts as evidenced by the high levels of ^{125}I-UdR incorporation into spleen cells (Figure 14A). In contrast, β2m-sufficient recipient mice rejected marrow grafts. This rejection appeared to be mediated by NK1.1^+ cells because anti-NK1.1 mAb-treated, but not control mAb-treated, mice failed to reject marrow grafts, consistent with the previous report. Tg mice accepted marrow grafts at the levels comparable to those observed from β2m-deficient recipient mice and anti-NK1.1-treated recipient mice, indicating that

Figure 13. Reconstituted acute in vivo tumor elimination by *scid* splenocytes in Tg mice.

scid mice that had been injected with anti-NK1.1 or untreated on day -2 were injected with poly-I:C on day -1. 2 hr after the intravenous infusion of 8×10^6 of either untreated or anti-NK1.1-treated *scid* spleen cells, or PBS on day 0, the recipient mice were injected intravenously with ^{125}I-UdR-labeled 3×10^4 RMA-S cells. The residual activity in the lungs was determined after 6 hr. Anti-NK1.1-treated WT mice were injected intraperitoneally with 200 μg of anti-NK1.1 mAb on day -2. Results are expressed as the mean percent residual radioactivity ± SD from 3 -4 mice per group.

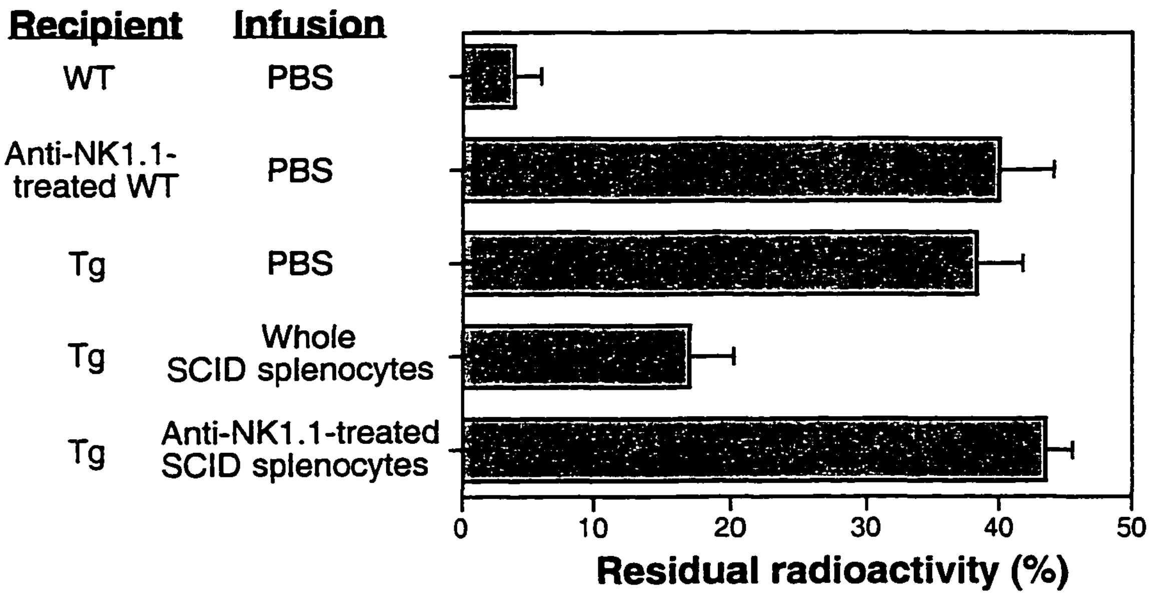
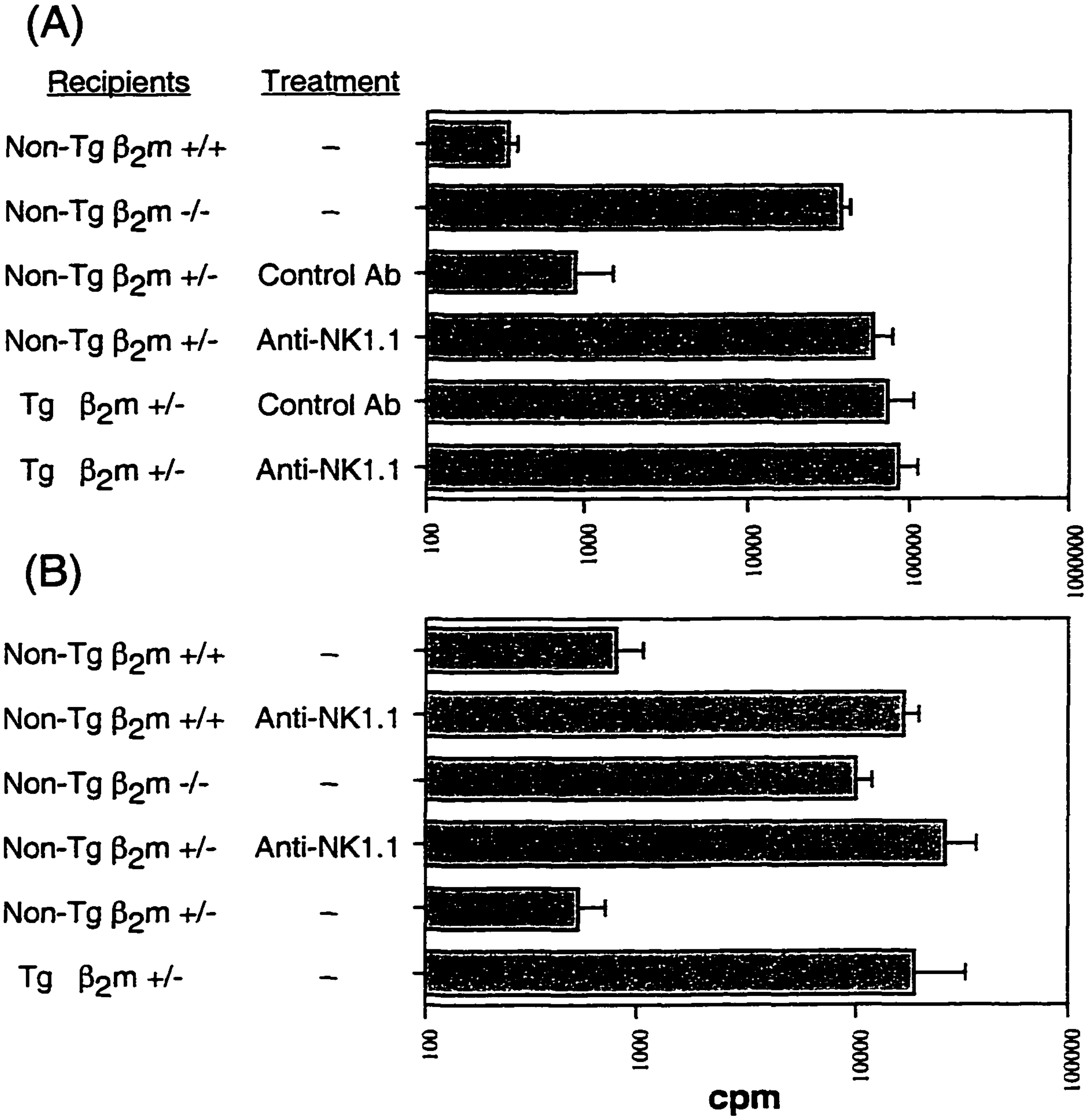

Recipient
Infusion
WT
PBS
Anti-NK1.1-treated WT
PBS
Tg
PBS
Tg
Whole SCID splenocytes
Tg
Anti-NK1.1-treated SCID splenocytes
0
10
20
30
40
50
Residual radioactivity (%)

Figure 14. Impaired rejection of β2m-deficient BM grafts in Tg.

Indicated recipient mice were injected intraperitoneally with anti-NK1.1 (200 μg), isotype-matched control antibody anti-rat κ (200 μg), or untreated on day -2. The recipients mice were injected intravenously with 5×10^6 (A) or 5×10^5 (B) β2m-deficient BM cells on day 0 and injected intravenously with 2 μCi of ^{125}I-UdR on day 5. Incorporated radioactivity in the spleens was determined on day 6. Results are expressed as the mean incorporated radioactivity ± SD from 4-6 mice per group.


(A)
Recipients
Treatment
Non-Tg β_2m +/+ –
Non-Tg β_2m -/- –
Non-Tg β_2m +/- Control Ab
Non-Tg β_2m +/- Anti-NK1.1
Tg β_2m +/- Control Ab
Tg β_2m +/- Anti-NK1.1
100
1000
10000
100000
1000000
(B)
Non-Tg β_2m +/+ –
Non-Tg β_2m +/+ Anti-NK1.1
Non-Tg β_2m -/- –
Non-Tg β_2m +/- Anti-NK1.1
Non-Tg β_2m +/- –
Tg β_2m +/- –
100
1000
10000
100000
cpm

the potential to reject marrow grafts was impaired in Tg mice. Moreover, anti-NK1.1 treatment did not significantly augment graft acceptance in Tg mice, consistent with a possibility that NK/T cells do not play a role in marrow graft rejection. However, it also seemed possible that Tg mice might still possess certain level of rejection potential but it was not sufficient to reject the transplanted dose of BM cells. To test these possibilities, another series of transplantation experiments were performed with a minimum dose (5×10^5) of BM cells. Again, Tg mice failed to reject marrow grafts, like β2m-deficient mice and anti-NK1.1-treated mice (Figure 14B), indicating that Tg mice are completely deficient in the rejection of β2m-deficient marrow grafts. Considering that Tg mice have normal NK/T cell compartment, these results suggest that NK cells are the major effector cells responsible for the acute rejection of β2m-deficient marrow grafts.

Impaired Acute IFN-γ Production by Transgenic Mice in Response to LPS.

Previously, $NK1.1^+$ or $AGM1^+$ cells were shown to mediate bacterial endotoxin LPS-induced generalized Shwartzman reaction, one of the experimental septic shock models (8). To test the possibility that NK cells are required for inducing the Shwartzman reaction, mice were injected intravenously with varying amounts (100 μg - 400 μg) of LPS 24 hr after priming injection of LPS into the footpad. As shown in Table 1, when challenged intravenously with 400 μg LPS, all WT control mice died following septic symptoms. Most Tg mice also died under this condition although there was a notable difference in survival time between WT and Tg mice; Most WT mice (5/6) died within 24 hr after the challenging injection, whereas most Tg mice (4/6) died between 24 - 48 hr. Similar results were observed with a lower dose (200 μg) of LPS injection. In contrast, none of anti-NK1.1-treated WT mice died.

To gain an insight into the role of NK cells upon bacterial infection, blood samples were collected from mice 7 hr after the intraperitoneal injection of

Table 1. Normal induction of the generalized Shwartzman reaction in Tg mice.

LPS (μg)		Anti-NK1.1-treated WT	WT	Tg
Priming[a]	Challenging	(dead/tested)		
5	100	0/4	1/9	0/5
5	200		3/6	4/6
5	300	0/4	6/6	5/6

a) Mice were primed with the footpad injection of 5 μg LPS (*Serratia marcescens*) 24 hr before the intravenous injection of varying amounts of LPS.

LPS and serum IFN-γ levels were determined by ELISA. Serum IFN-γ levels were significantly lower in Tg mice compared with WT control mice (Figure 15). Decreased IFN-γ levels were also observed in anti-NK1.1-treated WT mice. Collectively, these results suggest that whereas NK cells are not required for the Shwartzman reaction, NK cells are the major effector cells responsible for the acute production of IFN-γ in response to LPS.

Intrinsic Defect in NK Cell Development in Transgenic Mice.

In contrast to the peripheral organs, more than twice the number of NK cells were found in BM of Tg mice compared with WT mice (Figure 16), suggesting a defect in the maturation process of NK cells. To evaluate their cytotoxic activity, BM cells were isolated from poly-I:C-injected mice and subjected to ^{51}Cr-release assays. Like WT spleen cells, WT BM cells killed B16 target cells at certain levels, while Tg BM cells failed to kill this target (Figure 17A). In contrast, reflecting the presence of more NK cells, Tg BM cells killed RMA-S target at higher levels compared with WT BM cells, (Figure 17B). These data indicate that NK cells in Tg mice display target cell specificity different from that of NK cells in WT mice.

Based on the observation that Tg mice and anti-NK1.1-treated mice displayed similar levels of defects in tumor and marrow graft rejection, it was conceivable that the few peripheral NK cells in Tg mice might be an immature form of NK cells. To pursue this possibility, NK cells were analyzed for their cell surface expression of various marker molecules. Previously, it has been known that mature peripheral NK cells express the members of β2 integrin family, including LFA-1 (CD11a), Mac-1 (CD11b) and p150/95 (CD11c). Consistently, all or the majority of splenic NK cells in WT mice expressed CD11a, CD11b and CD11c (Figure 18). However, with the exception of CD11a, NK cells in Tg mice did not express detectable levels of CD11b and CD11c. In contrast, other cell populations in Tg mice normally expressed CD11b and CD11c, indicating the integrity of the β2 integrin genes. Similar expression patterns of the β2 integrins were also observed on NK cells in

Figure 15. Impaired in vivo IFN-γ production in response to LPS in Tg mice. Anti-NK1.1-treated WT, untreated WT and Tg mice were injected intraperitoneally with 20 μg of LPS (*E. coli* serotype 0127:B8). 7 hr later, blood samples were collected. Serum IFN-γ levels were determined by ELISA. Anti-NK1.1-treated mice were injected intraperitoneally with 200 μg of anti-NK1.1 mAb 2 day before LPS treatment. Results are expressed as the mean concentration ± SD from 5 mice per group.

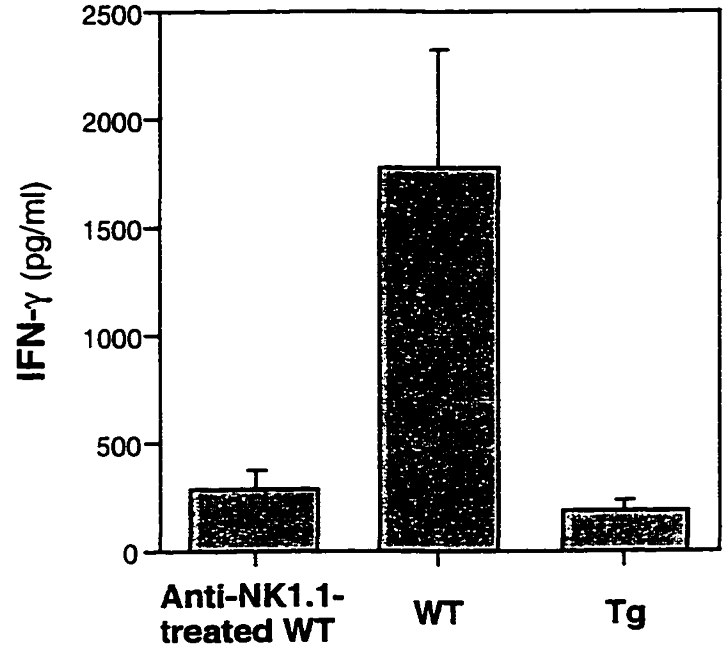

IFN-γ (pg/ml)
2500
2000
1500
1000
500
0
Anti-NK1.1-treated WT
WT
Tg

Figure 16. Marked increase of BM NK cells in Tg mice.

Single cell suspensions were prepared from BM and subjected to dual staining with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 antibodies. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of at least 5 WT and 5 Tg mice are shown.

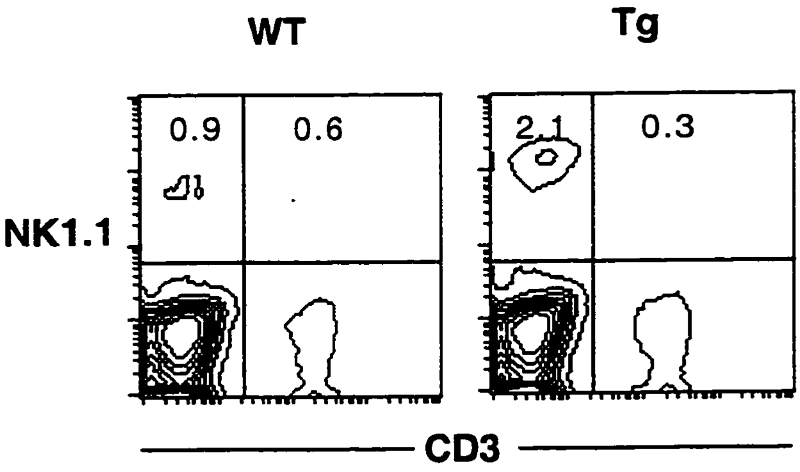

WT
Tg
0.9
0.6
2.1
0.3
NK1.1
CD3

Figure 17. Altered target cell specificity of BM NK cells in Tg mice.

WT (squares) and Tg (circles) mice were injected intraperitoneally with 150 μg of poly-I:C. 24 h later, BM cells from the treated mice or untreated WT (diamonds) and Tg (triangles) mice were used in 4 hr ^{51}Cr-release cytotoxicity assays against B16 (A) and RMA-S (B) target cells at varying effector:target (E:T) ratios as indicated. Results are expressed as the mean percent-specific lysis ± SD of triplicate wells.

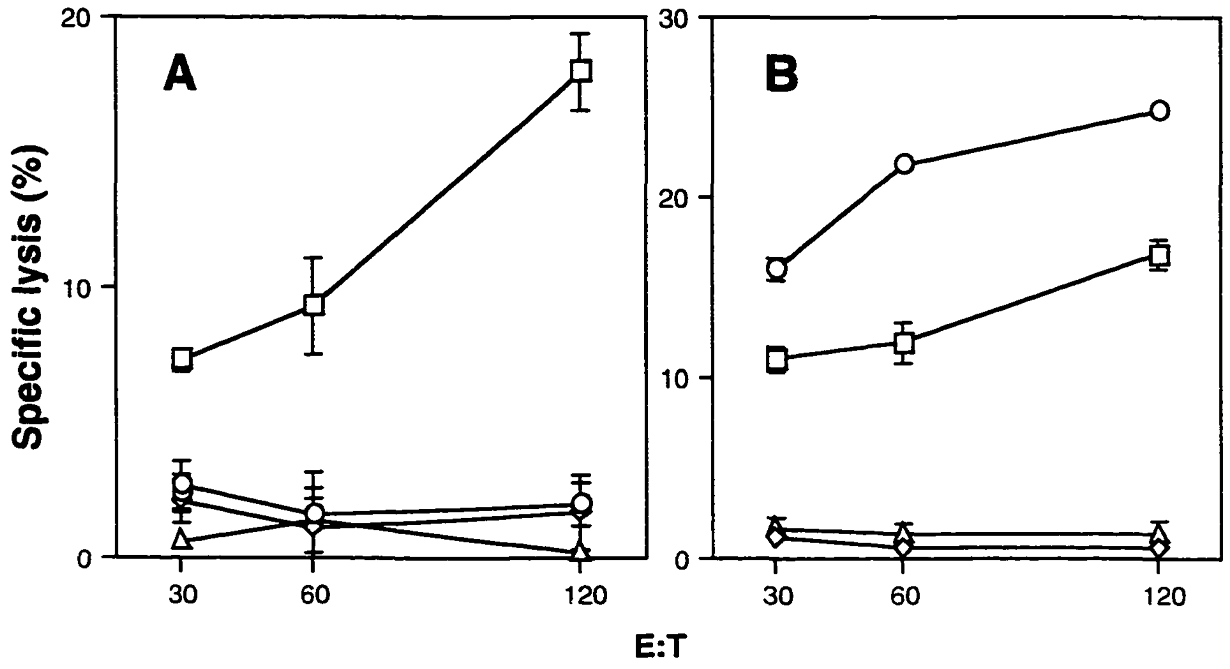
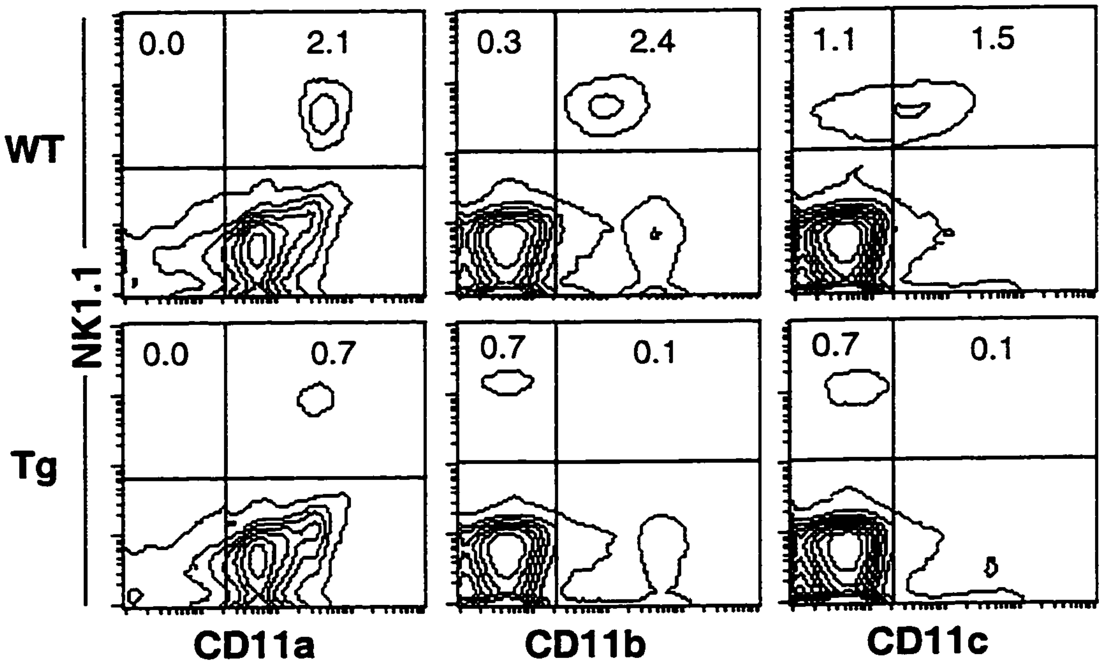

A
B
Specific lysis (%)
0
10
20
0
10
20
30
30
60
120
30
60
120
E:T

Figure 18. Absence of CD11b and CD11c expressing NK cells in Tg mice. Single cell suspensions were prepared from spleen and subjected to triple staining with Cy-conjugated anti-CD3, PE-conjugated anti-NK1.1 and FITC-conjugated antibody specific for CD11b or CD11c. Profiles for marker expression on cells gated on the CD3-negative population are shown. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of at least 5 WT and 5 Tg mice are shown.


WT
Tg
NK1.1
0.0
2.1
0.3
2.4
1.1
1.5
0.0
0.7
0.7
0.1
0.7
0.1
CD11a
CD11b
CD11c

other organs (data not shown). Most NK cells in WT mice displayed higher levels of CD11b and CD11c expression after in vitro stimulation with PMA (data not shown). However, the expression of these integrins was still undetectable on Tg NK cells even after this stimulation. Moreover, the frequency of Ly-49C$^+$ or Ly-49G2$^+$ NK cells was reduced in Tg mice (Figure 19). Furthermore, alterations in the expression of several other molecules (B220, CD43, Gr-1 and CD28) were observed on Tg NK cells (Figure 20). No significant proportion of NK cells in Tg mice expressed B220 and Gr-1, whereas a substantial proportion of WT NK cells expressed these markers. NK cells in Tg mice expressed lower levels of CD43 but expressed higher levels of NK1.1 and CD28, compared with WT NK cells. Taken together, the available data suggest that NK cells in Tg mice may represent a phenotypically and functionally immature form of NK cells.

The abnormal NK cell compartment in Tg mice may be due to an alteration of the BM microenvironment. Alternatively, Tg mice may have an intrinsic defect in the NK cell lineage. To address these possibilities, BM chimeric mice were constructed by transferring BM cells (5 x 10^6 cells) from normal congenic (Ly5.1) mice into Tg (Ly5.2) mice or vice versa. After 10 weeks, the proportions of donor-derived cells in spleens of chimeric mice were at least 80%, as determined by flow cytometric analysis using anti-Ly5.1 and anti-Ly5.2 mAbs (data not shown). Under this condition, transfer of BM cells from congenic mice into irradiated Tg mice resulted in the production of a normal number of NK cells (Figure 21). In contrast, transfer of BM cells from Tg mice into irradiated congenic mice resulted in the production of a reduced number of NK cells with abnormal marker expression, similar to Tg mice. Moreover, splenic natural killing of YAC-1 and RMA-S tumor targets was restored when Tg mice were reconstituted with normal bone marrow cells, while transfer of Tg bone marrow cells resulted in deficiency in natural killing of these targets (Figure 22). Thus, Tg mice appeared to have an intrinsic defect in the NK cell lineage rather than an alteration of host microenvironment.

Figure 19. Reduced frequencies of Ly-49C and Ly-49G2 expressing NK cells in Tg mice.

Single cell suspensions were prepared from spleen and subjected to triple staining with Cy-conjugated anti-CD3, PE-conjugated anti-NK1.1 and FITC-conjugated antibody specific for Ly-49C or Ly-49G2. Profiles for marker expression on cells gated on the CD3-negative cell population are shown. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of at least 5 WT and 5 Tg mice are shown.

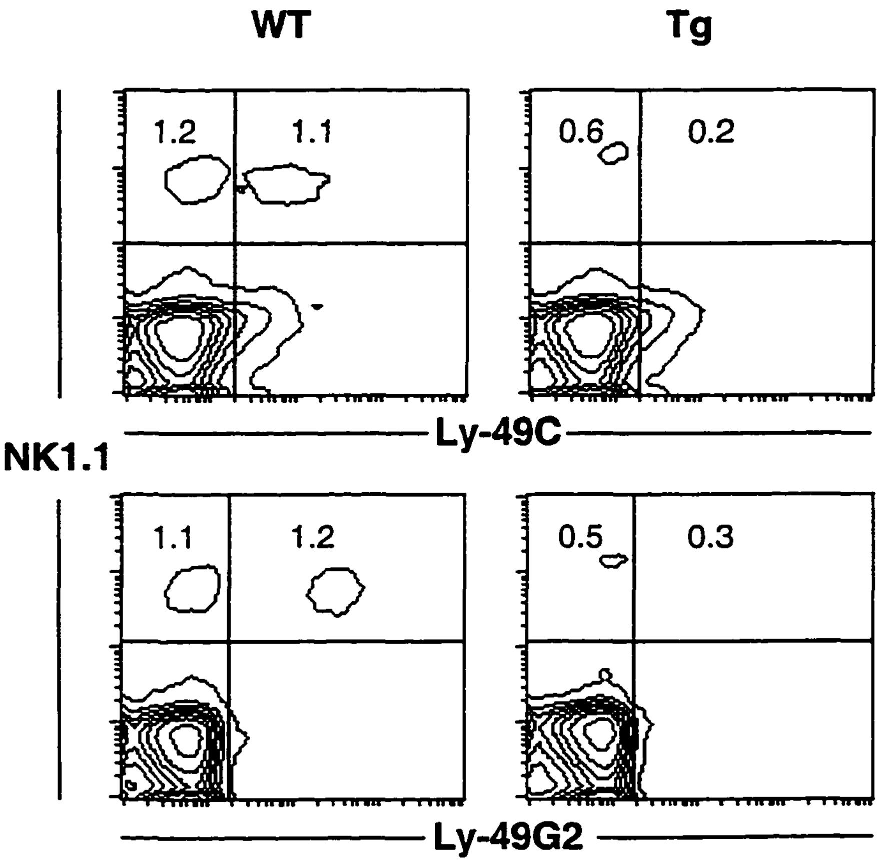

WT
Tg
1.2
1.1
0.6
0.2
NK1.1
Ly-49C
1.1
1.2
0.5
0.3
Ly-49G2

Figure 20. Abnormal marker expression on NK cells in Tg mice.

Single cell suspensions were prepared from spleen and subjected to triple staining with Cy-conjugated anti-CD3, PE-conjugated anti-NK1.1 and either FITC-conjugated antibody specific for B220, CD43, Gr-1. For the analysis of CD28, cells were stained with CD28 followed by FITC-conjugated anti-hamster IgG then stained with Cy-conjugated anti-CD3 and PE-conjugated anti-NK1.1. Profiles for marker expression on cells gated on the CD3-negative population are shown. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of at least 3 WT and 3 Tg mice are shown.

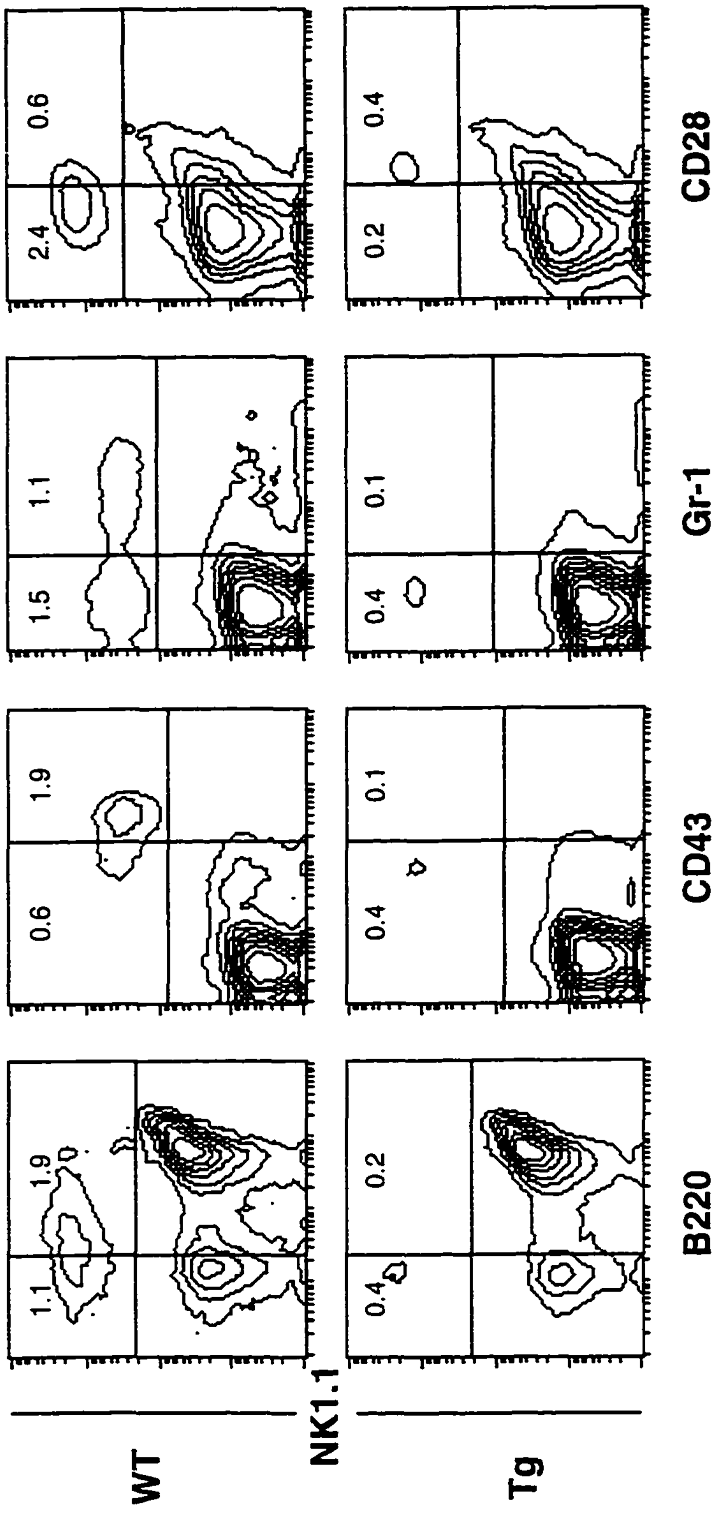

WT
Tg
NK1.1
B220
CD43
Gr-1
CD28
1.1
1.9
0.6
1.9
1.5
1.1
2.4
0.6
0.4
0.2
0.4
0.1
0.4
0.1
0.2
0.4

Figure 21. Intrinsic defect in the NK cell lineage in Tg mice.

BM cells from WT or Tg mice were transferred into irradiated B6-Ly5 congenic mice or vice versa as indicated. Ten weeks later, single cell suspensions were prepared from the spleens of chimeric mice and subjected to either dual staining with PE-conjugated anti-NK1.1 and FITC-conjugated CD3 antibodies (upper panel) or triple staining with Cy-conjugated anti-CD3, PE-conjugated anti-NK1.1 and FITC-conjugated CD11b antibodies (lower panel). Upper panel shows profiles for marker expression on whole spleen cells. Lower panel shows profiles for marker expression on cells gated on the CD3-negative population. More than 80% of the whole spleen cells were donor BM cell-derived in all chimeric mice. The numbers represent the percentage of cells within the quadrant among all viable cells. Representative data from one of three chimeric mice per group are shown.

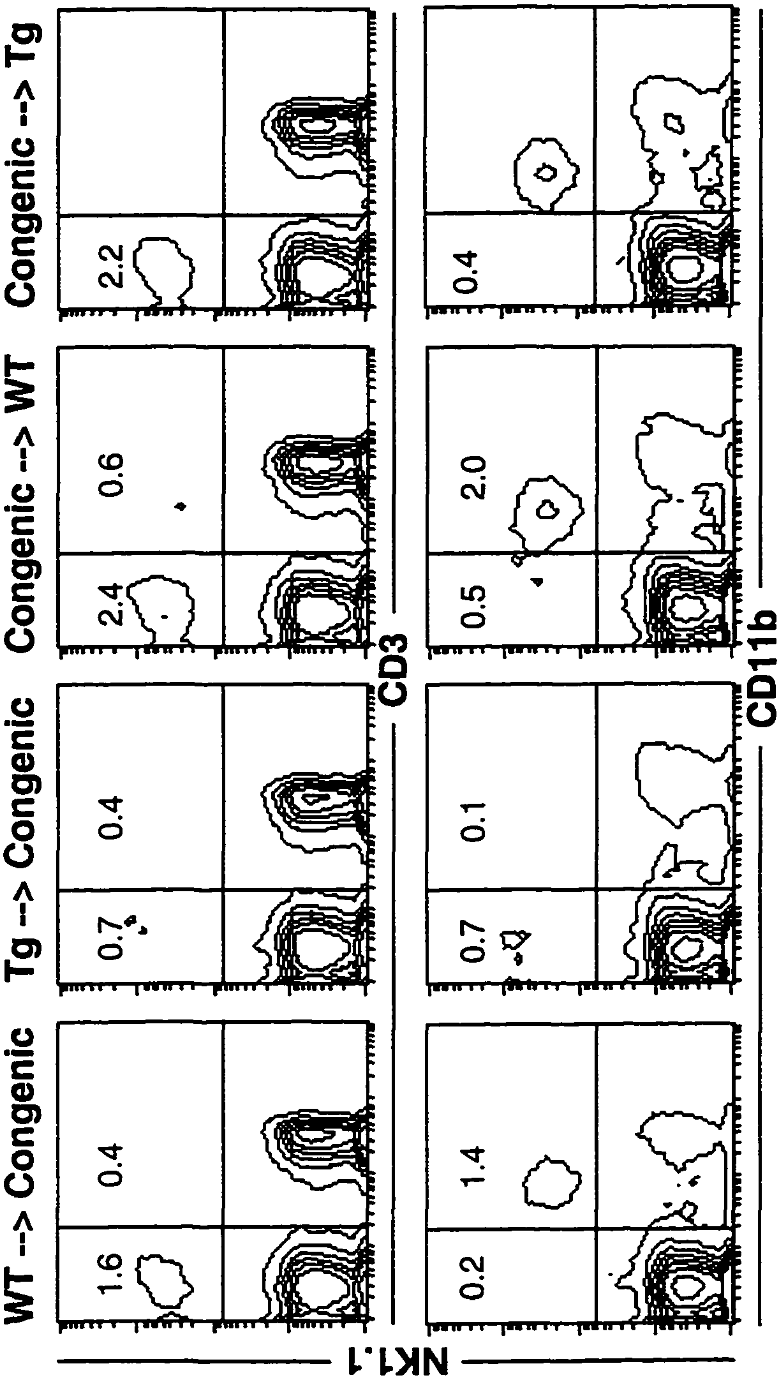
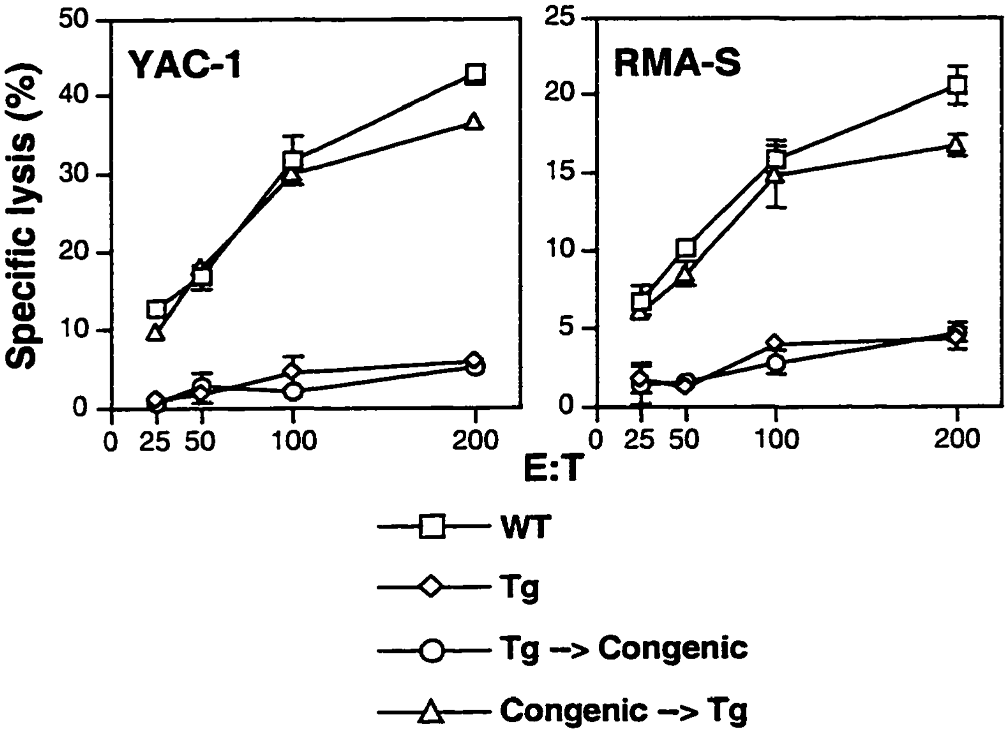

WT --> Congenic
Tg --> Congenic
Congenic --> WT
Congenic --> Tg
1.6
0.4
0.7
0.4
2.4
0.6
2.2
0.2
1.4
0.7
0.1
0.5
2.0
0.4
NK1.1
CD3
CD11b

Figure 22. Impaired in vitro tumor cell killing activity of Tg BM cell-derived splenic NK cells in congenic mice.

Indicated chimeric mice and WT and Tg control mice were injected intraperitoneally with 150 μg of poly-I:C. 24 h later, the spleen cells were used in 4 hr ^{51}Cr-release cytotoxicity assays against YAC-1 and RMA-S target cells at varying effector:target (E:T) ratios as indicated. Results are expressed as the mean percent-specific lysis ± SD of triplicate wells.


YAC-1
RMA-S
Specific lysis (%)
50
40
30
20
10
0
25
20
15
10
5
0
0
25
50
100
200
E:T
WT
Tg
Tg --> Congenic
Congenic --> Tg

DISCUSSION

Since the existence of NK cells was first reported two decades ago, numerous correlative studies have suggested that NK cells may mediate tumor rejection, acute marrow graft rejection and host defense against certain pathogen infection. However, it has not been possible to demonstrate that such effects are mediated exclusively by NK cells because of the lack of available animal model in which NK cell activity is selectively deficient. The present study describes the generation and characterization of Ly-49A Tg mice that have a selective deficiency in the NK cell compartment. In addition, this study provides evidences that 1) NK cells play essential roles in acute elimination of tumor cells, prevention of tumor metastasis formation and control of tumor outgrowth, 2) NK cells are the exclusive mediator of acute rejection of β2m-deficient marrow grafts, and 3) NK cells are the major effector cells responsible for the acute production of IFN-γ upon stimulation with bacterial endotoxin (LPS). Moreover, this study provides novel insights into the maturation process of NK cells.

Tg mice have a markedly reduced number of NK cells in the peripheral organs, such as spleen, peripheral blood, liver and lung, where NK cells are found at high frequencies in WT mice. Consistent with the decreased number of peripheral NK cells, splenic natural killing of a prototype NK-sensitive YAC-1 lymphoma, syngeneic MHC class I-deficient RMA-S lymphoma and B16 melanoma was dramatically decreased. On the other hand, other lymphocyte compartments appear to be normal. Tg mice possessed a normal number of CD4+ and CD8+ T cell subsets and B cells. Functional activities of T cells and B cells also appeared to be normal as manifested by normal skin graft rejection, normal proliferative response to mitogen and normal serum immunoglobulin levels. Moreover, the number of NK/T cells and their functional activities, such as IL-4 production, were essentially normal. Collectively, these data indicate that Tg mice have a selective deficiency in the NK cell compartment. Functional reconstitution study with *scid* splenocytes that are devoid of NK/T, T and B cells further supports this conclusion.

Tumor rejection studies using Tg mice provide compelling evidence that NK cells are the major effector cells mediating acute tumor clearance, anti-metastatic effect and long-term control of local tumor growth. Consistent with the impaired in vitro splenic natural killing of NK activity, Tg mice displayed a severely impaired potential to acutely eliminate 3 different types of tumor targets. Moreover, formation of B16 lung metastases was also dramatically increased in Tg mice compared with WT mice. Furthermore, as few as 10^2 RMA-S cells developed palpable tumors in most Tg mice, whereas about 100 to 1000 fold more tumor cells were needed to achieve similar levels of tumor incidence in WT mice. Finally, adoptive transfer of *scid* splenocytes restored acute tumor rejection potential in Tg mice and this effect was abrogated by treatment with anti-NK1.1. Taken together, these results formally establish the role of NK cells in tumor rejection.

The difficulties in defining role of NK cells in tumor rejection and other immune responses in previous studies largely stemmed from the fact that NK cells were initially described by their capacity of killing tumor cells rather than a molecular description. Thus, it was difficult to differentiate the effects of NK cells from those of other cell types, particularly T cells. Recent availability of mAb specific for NK1.1 that is known as the most specific serological marker for murine NK cells not only allowed better defined molecular description of NK cells but also provided a tool allowing study for more specific roles of NK cells *in vivo*.

Consistent with the role of $NK1.1^+$ cells in tumor rejection, administration of anti-NK1.1 led to impaired acute tumor rejection, increased formation of lung metastases and impaired control of tumor outgrowth as demonstrated in the present study and other studies (74, 117). Importantly, however, the contribution of NK cells in such effects was not known due to the existence of NK1.1-expressing NK/T cells that may have anti-tumor activity *in vivo* (119).

Comparative studies using Tg mice that are selectively NK cell-deficient and mice depleted of both NK cells and NK/T cells by administration of anti-NK1.1 allowed differentiation of the contribution of NK cells from that of NK/T cells in acute, intermediate and long-term tumor rejection. Acute tumor clearance appeared to be exclusively mediated by NK cells, because there was no significant difference in the retention levels of 3 different tumor cells between Tg mice and anti-NK1.1-treated mice.

In addition, NK cells appeared to be the major effector cells responsible for anti-metastatic effects against B16 melanoma, the most widely used tumor cells for experimental lung metastasis studies, because the number of lung metastases present in Tg mice was comparable to that present in anti-NK1.1-treated mice. Interestingly, however, Tg mice seemed to have a slightly smaller number of liver metastases than anti-NK1.1-treated mice. One interpretation for this observation is that the few residual NK cells in the liver of Tg mice may have anti-metastatic effect. However, this seems unlikely because residual NK cells in the lung did not have this effect. Alternatively, it is possible that this reduction is mediated by NK/T cells that are abundantly present in the liver of Tg mice. Along this line, a recent study suggests that NK/T cells may have anti-metastatic activity against certain tumors (115). Thus, NK and NK/T cells may mediate varying levels of anti-metastatic effects against different tumor cells.

Finally, NK cells appear to be the major effector cells mediating the control of local tumor growth, since despite the presence of essentially normal NK/T cell compartment, Tg mice failed to control tumor growth of as few as 10^2 RMA-S cells. The reason why Tg mice exhibited slightly lower levels of tumor incidence compared with anti-NK1.1-treated mice is not clear. This may be attributed to the effect of NK/T cells or the few peripheral NK cells present in Tg mice. Collectively, these data strongly suggest that NK but not NK/T cells are the major effector cells responsible for various modes of tumor rejection.

In addition to tumor rejection, it has been suggested that NK cells may mediate acute rejection of allogeneic BM grafts and hybrid resistance where F1 hybrid mice reject parental BM grafts (13). This notion originally came from the observation that failure of marrow graft rejection inversely correlated with the levels of NK activity in recipient animals (14, 133). It was also shown that administration of anti-AGM1 or anti-NK1.1 could abrogate marrow graft rejection (134, 135). More recently, however, this notion was challenged by the observation that spleen cells from normal mice when adoptively transferred into immunosuppressed mice could mediate both allogeneic resistance and hybrid resistance and these effects were mediated by cells expressing not only NK1.1 and AGM1 but also CD3 and αβTCR (136), a typical phenotype of NK/T cells. In addition, adoptively transferred T cell clones with NK-like activity could mediate similar effects (137, 138). Thus, it has been controversial whether marrow graft rejection is mediated by NK cells or NK/T cells. Along this line, although a recent study has demonstrated that $NK1.1^+$ cells in β2m-sufficient recipient mice mediate acute rejection of β2m-deficient otherwise syngeneic marrow grafts (132), it is not known whether NK or NK/T cells are responsible for this graft rejection.

BM transplantation experiments with Tg mice provide convincing evidence that NK cells are the major effector cells responsible for acute rejection of β2m-deficient marrow grafts. In contrast to β2m-sufficient WT mice that completely rejected β2m-deficient BM (5×10^6) grafts, β2m-sufficient Tg mice accepted the grafts at the levels comparable to those obtained from anti-NK1.1-treated recipient mice and β2m-deficient syngeneic recipient mice. The possibility that this graft acceptance is due to high dose of BM grafted cells that may overcome the rejection potential of Tg mice seems unlikely because Tg mice also failed to reject even minimum dose (5×10^5) of transplanted marrow cells. These results indicate that NK cells are the exclusive mediators of β2m-deficient marrow graft rejection, arguing against the involvement of NK/T cells in this graft rejection. However, the available data do not exclude the possibility that NK/T cells play a role in allogeneic resistance and hybrid resistance. This issue can be addressed by further comparative studies in allogeneic or hybrid resistance settings.

Another important function of NK cells is production of immunoregulatory cytokines, particularly IFN-γ. This cytokine is known as a key effector molecule mediating host resistance against numerous infectious pathogens such as *Salmonella typhimurium*, murine cytomegalovirus, *Listeria monocytogenes*, and *Toxoplasma gondii* (139-141). IFN-γ has been also suggested to promote the differentiation of naive T cells into the Th1 subset that mediates protective immunity in resistant hosts (11). Based on the observations that splenocytes from *scid* mice can efficiently produce IFN-γ *in vitro* in response to such pathogens or LPS (139, 142-144) and that administration of anti-AGM1 or anti-NK1.1 renders mice more susceptible to pathogen infection (145-147), it has been suggested that NK cells are a major source of IFN-γ during the early phase of pathogen infection. NK cell-mediated IFN-γ production is mainly induced by IL-12, a cytokine previously known as NK cell stimulatory factor (148, 149). Importantly, the recent findings (150, 151) that NK/T cells express higher levels of IL-12 receptor compared to NK cells and are the major producer of IFN-γ *in vivo* upon administration of IL-12 raises a question as to which cells are responsible for early IFN-γ production upon pathogen infection. In fact, none of previous studies differentiated the contribution of NK cells from that of NK/T cells in the early IFN-γ production.

The present study demonstrates that NK cells are the major effector cells responsible for acute IFN-γ production *in vivo* in response to LPS. Upon injection of LPS, serum IFN-γ levels in Tg mice were less than 20% compared with WT mice. The observation that Tg mice did not produce more IFN-γ than anti-NK1.1-treated mice suggests that NK/T cells may not produce significant amounts of IFN-γ during immediate early stage of infection.

On the other hand, IFN-γ, together with excessive amounts of other inflammatory cytokines, sometimes lead to pathological processes such as the generalized Shwartzman reaction (152). Consistent with the major contribution of NK cells in LPS-induced IFN-γ, a previous study has suggested that NK cells are essential components for this reaction (8). Thus, it was unexpected that Tg mice succumbed to this reaction, while anti-NK1.1-treated mice survived. These results suggest that NK cells are not required for the Shwartzman reaction. It is conceivable that NK/T cells may produce IFN-γ with slower kinetics. The observation that most Tg mice died slower than WT mice is consistent with this possibility.

Phenotypic analysis indicates that NK cells in Tg mice have abnormal expression of differentiation markers found on normal NK cells. For example, most or a substantial proportion of peripheral (presumably mature) NK cells in WT mice express CD11b, CD11c, B220 and surprisingly, Gr-1, a marker considered specific for neutrophils (153), but NK cells in Tg mice do not express these markers. In addition, smaller than normal percentages of NK cells in Tg mice express Ly-49 family receptors. On the other hand, NK cells in Tg mice express higher levels of CD28 and NK1.1 markers than WT NK cells. Although biological significance of these phenotypic characteristics remains to be established, these observations raise the possibility that the peripheral NK cells in Tg mice are not functionally normal. Indeed, most functional data are consistent with this notion. For example, splenic natural killing of YAC-1 and B16 tumor targets was undetectable even at extremely high E:T ratios where significant killing was expected by taking the NK cell number decrease into consideration. In addition, compared to the number decrease, Tg mice displayed more severely impaired NK cell activities with respects to tumor rejection and bone marrow graft rejection. These data indicate that NK cells in Tg mice are phenotypically and functionally different from WT NK cells. Thus, the functional defects observed in Tg mice may be caused by the combined effects of quantitative (NK cell number decrease) and qualitative (functional incompetence of residual NK cells) differences.

The NK cell number decrease in the periphery of Tg mice appears to be due to accumulation of NK cells in the BM. In contrast to the peripheral organs, more than twice the number of NK cells were present in the BM of Tg mice compared with WT mice. Together with the phenotypic and functional abnormalities of NK cells in Tg mice, this observation strongly suggests that the NK cell deficiency in Tg mice is associated with a developmental abnormality of NK cells. According to current understandings of NK cell ontogeny, the generation process of mature NK cells can be divided into two phases. In the first phase, lineage marker-negative NK cell progenitors develop into non-cytolytic NK1.1$^+$ NK cells. In the second phase, these NK1.1$^+$ NK cells mature into cytolytic NK cells that express many cell surface molecules, such as CD11b and Ly-49 receptors (18). It has been proposed that this later process requires an intact bone marrow microenvironment. By most criteria, it seems likely that the NK cell deficiency in Tg mice results from an abnormality in the differentiation process rather than the early developmental process.

Interestingly, NK cells in Tg mice share several phenotypic and functional characteristics with the previously reported NK cells present in the 17β-estradiol-treated osteopetrotic mice (154). These NK cells express NK1.1 and smaller than normal percentages of these cells express CD11b, B220 and Ly-49 receptors. In addition, these NK cells display an impaired cytotoxicity, such that they do not kill YAC-1 target as efficiently as normal NK cells, although their cytotoxicity against other targets are not known. Importantly, these NK cells can be induced to express CD11b and B220, and become capable of killing YAC-1 target in the presence of IL-15, a recently described cytokine that can support NK cell differentiation (155, 156). Based on these characteristics, the NK cells in 17β -estradiol-treated mice have been proposed as an immature form of NK cells whose differentiation process is arrested. Thus, together with these reports, the available data suggest that NK cells in Tg mice may represent a phenotypically and functionally immature form of NK cells.

Functional analysis of bone marrow NK cells from Tg mice treated with a poly-I:C inducer indicates that these NK cells have a limited target cell specificity compared with WT NK cells. Despite the presence of more NK cells, bone marrow cells from Tg mice had no detectable capacity to kill B16 tumor target, unlike bone marrow cells from WT mice that significantly killed this target. This observation is in agreement with the data obtained from the cytotoxic assays using spleen cells and provide an unequivocal evidence for a defect in the functional activities of NK cells in Tg mice. By contrast, compared with BM cells from WT mice, BM cells from Tg mice were able to kill RMA-S target at higher levels, reflecting the presence of more NK cells. This observation is surprising because it has been thought that immature NK cells are non-cytolytic. In fact, this concept was initially proposed based on limited cytotoxic experiments performed with a small panel of targets. Although the physiological relevance of these findings needs to be further elucidated, these data suggest that the inability of NK cells in Tg mice to kill other NK-susceptible targets is not due to the lack of cytolytic potential.

Consistent with this notion, a recent study has demonstrated that an immature form of human NK cells obtained from culture of lineage marker-negative umbilical cord blood cells possess cytotoxic granule-associated molecules such as perforin and serine esterase, despite inability to kill a prototype human NK cell target K562 (157). These immature NK cells express CD11a and NKR-P1A, a human homologue of the murine NK1.1 molecule, but do not express CD56, an adhesion molecule expressed on mature human NK cells although other marker expression is not known. Collectively, it is possible that mature NK cells possess many adhesion molecules (i.e., CD11b and CD11c in mice) and target cell recognizing activation receptors but some of these are expressed later than the acquisition of cytolytic potential. In this respect, identification of such receptor(s) responsible for B16 target recognition that are expressed on WT NK cells but not on NK cells in Tg mice will help to establish a molecular basis for the target cell specificity as well as maturation stage of NK cells.

On the other hand, it is interesting to speculate that candidate receptor(s) for RMA-S target recognition could be NK1.1 and/or CD28, because both receptors are expressed at high levels on NK cells in Tg mice and have been implicated as activation receptors that are involved in target cell recognition

(158, 159). Alternatively or additionally, it is possible that killing of RMA-S is mediated by granule exocytosis-independent cytotoxic mechanisms such as Fas-mediated mechanism. Consistent with this possibility, low but detectable levels of Fas antigen were expressed by RMA-S but not by B16 target (data not shown). However, whether NK cells in Tg mice can actually kill targets via this mechanism remains to be determined.

Another important finding with the immature human NK cells is that they are unable to produce IFN-γ, unlike mature NK cells. In accordance with this finding, NK cells in Tg mice appear to be unable to produce IFN-γ in response to bacterial endotoxin, because serum IFN-γ levels of Tg mice were not higher than those of anti-NK1.1-treated mice that had no detectable NK cells. Thus, this functional incompetence may be an important feature of immature NK cells. In addition to their role in host defense upon infection, NK cell-derived cytokines have been implicated as effector molecules in the regulation of hematopoiesis. Many in vitro studies have shown that when incubated with hematopoietic progenitor cells, NK cells produce cytokines such as TNF-α and IFN-γ and these cytokines have suppressive effects on the proliferation of hematopoietic progenitor cells (120, 160). In this respect, NK cell production of these cytokines may represent the molecular mechanism underlying acute rejection of BM allografts.

Consistent with this, recent studies using perforin, granzyme B or Fas gene-deficient mice have demonstrated that granule exocytosis dependent cytotoxic mechanisms are not required for bone marrow graft rejection (161, 162). It is possible that NK cells in Tg mice are not able to produce IFN-γ or TNF-α against transplanted bone marrow cells. The complete lack of bone marrow graft rejection in Tg mice may be explained by this possibility, in addition to the NK cell number decrease in the periphery. Nonetheless, comparative studies of NK cells in Tg mice and corresponding cell population in WT mice, in terms of cytokine production, cytotoxicity and cell surface marker expression, will help to formally establish the distinctive features of immature NK cells.

BM chimeric studies demonstrate that Tg mice have an intrinsic defect in the NK cell lineage. Transfer of bone marrow cells from Tg mice into irradiated recipient WT mice yields donor-derived splenic NK cells in the periphery at low levels and with abnormal marker expression as seen in Tg mice. By contrast, transfer of WT bone marrow cells into Tg mice results in a normal number of phenotypically mature NK cells in the periphery. Moreover, splenic natural killing of tumor targets was restored when WT bone marrow cells were transferred into irradiated Tg mice whereas transfer of bone marrow from Tg mice into WT mice resulted in a deficiency in natural killing. These data demonstrate that Tg mice possess intact microenvironment that can support normal NK cell development. This characteristic contrasts that recently reported in IRF-1-deficient mice that have alterations in microenvironments indirectly affecting development of NK cells as well as other cells (163, 164).

Molecular mechanisms underlying this intrinsic defect in the NK cell lineage remain to be thoroughly elucidated. Several possibilities may account for the abnormal NK cell development in Tg mice. First, based on the previous reports showing that engagement of Ly-49A with H-2 D^d MHC class I molecules can inhibit many NK cell-mediated functions (68, 165), it is possible that transgenic Ly-49A functions to interrupt normal NK cell development. As an attempt to address this possibility, Tg mice were crossed with β2m-deficient mice, then F1 hybrid Tg mice were backcrossed with β2m-deficient mice. Phenotypic analysis demonstrated that Tg mice in β2m-deficient background still had abnormal NK cell compartment (data not shown), similar to Tg mice. Although this observation seems to argue against the potential effect of Ly-49A engagement with MHC class I molecule, this experiment remains inconclusive because β2m-deficient mice are known to express class I molecules at low but significant levels (98, 166). Alternatively, Ly-49A may interact with as yet undefined ligands or carbohydrates as recently reported (167). Seemingly inconsistent with these ideas are the reported observations that NK cell development is not affected in two different Tg mice expressing Ly-49A under the control of MHC class I or CD2 promoter (168, 169).

However, it is possible that the temporal and spatial expression of the Ly-49A transgenic constructs are distinct, such that ectopic Ly-49A expression and its signaling function during a critical period of NK cell maturation process in Tg mice may result in interruption of this process. It is also possible that expressed Ly-49A competes for and sequesters a factor (e.g., SHP-1, a cytoplasmic phosphatase known to bind Ly-49A) (93) that may be critical for NK cell maturation. In this respect, expression of the Ly-49A transgene was detected on most NK cells in Tg mice at least day 7 after birth when endogenous expression of Ly-49A and other Ly-49 receptors was not normally detected (data not shown). Since other founder mice bearing the granzyme A-Ly-49A transgene construct do not express the Ly-49A transgene on a substantial proportion of NK cells, a correlation between NK cell-deficient phenotype and expression of transgenic Ly-49A can not be determined at present.

Second, it is possible that the granzyme A-Ly-49A transgene construct itself has an effect, such that high transgene copy number in Tg mice may titrate out a factor (e.g., transcription factor) critical for NK cell maturation. Seemingly inconsistent with this possibility is the observation that NK cell development is not affected in other founder mice obtained in the present study (data not shown). However, since these founders had fewer construct copy numbers, an effect due to differences in construct copy number can not be excluded. A recent study demonstrates that NK activity is partially reduced in mice bearing granzyme A-Diphtheria toxin transgene construct (122), but it is not clear whether this effect is due to the expressed toxin or the transgene construct.

Third, it is possible that transgene integration disrupts a gene or chromosomal locus whose product(s) is essential for normal NK cell development. Although this possibility seems unlikely because heterozygous Tg mice were used, a potential gene dosage effect can not be excluded. For example, IRF-1 or Jak3 gene-deficient heterozygote mice display certain abnormalities (28, 164). In this respect, it was possible that transgene integration might cause disruption of a gene encoding CD11b, CD11c or B220, since NK cells in Tg mice did not express these markers. However, the fact that other cells express these markers at normal levels and are present at normal frequencies in Tg mice excluded this possibility and indicates that abnormal phenotypes are specific to NK cells. In addition, CD18 (β subunit of β2 integrins)-deficient mice (170) had normal number of NK cells (data not shown) and CD45 (B220)-deficient mice were shown to have mature NK cells capable of killing YAC-1 (171). Thus, it seems unlikely that the lack of these marker expression resulted in the abnormality in the NK cell compartment in Tg mice. Nonetheless, marker analysis of Tg mice obtained by back-crossing each F1 hybrid Tg mice with β2m-deficient mice (β2m gene on chromosome 2), nude mice (nu gene on chromosome 11), BALB/c (2B4 on chromosome 1, NK1.1 on chromosome 6) or B10.D2 (H-2 class I Ag on chromosome 17) exclude the possibility that the transgene construct is integrated into these chromosomes (data not shown). Generation of more Tg mice using the granzyme A-Ly-49A construct or the construct without Ly-49A cDNA will help to resolve these issues.

As an in vivo model for studies of NK cell functions, Tg mice seem to be superior to other immunodeficient mice that are known to have defects in NK cell compartment. In earlier studies, *beige* mice were widely used as an NK-deficient animal model because of impaired NK activity (172-174). Although these studies provided important clues regarding many NK cell-associated functions, *beige* mice possess a mutation causing a defect in the protein sorting process that affects the granules and lysosomes of many different cell types (175, 176). Thus, the observations obtained in *beige* mice can not be interpreted as being specific NK cell-mediated effects. In addition, *beige* mice are not proper for studies of NK cell role in host defense against pathogens since NK cells in *beige* mice normally produce IFN-γ (177).

More recently, completely or partially impaired NK cell killing capacities have been documented in many gene-deficient mice such as perforin- (35), granzyme B- (36), Lag3- (178), IL-12- (179) and IL-18-deficient mice (180).

However, all of these mice, except for Lag3-deficient mice, have defects in other cell functions, particularly in T cell function. Although Lag3-deficient mice appear to have normal T cell function, like normal WT mice they are able to reject β2m-deficient marrow grafts, a function attributed to NK cells as shown in this study. Likewise, perforin- and granzyme B-deficient mice are capable of rejecting marrow grafts. Thus, these mice seem to have normal NK cell functions except for cytolytic function. IL-12- and IL-18-deficient mice display only partial reduction of NK cell cytolytic function and other NK cell functions are likely to be normal.

On the other hand, defects or abnormalities in NK cell compartment have been reported in many gene-deficient or transgenic mice such as Ikaros- (27), Jak3- (28), IL-2Rγ subunit- (29), IL-2Rβ subunit- (30), IRF-1-deficient (164), CD3ε- (32), FcεRIγ- (181), Diphtheria toxin- (122), IL-2Rβ subunit- (182) and TCR Vβ8.2- transgenic mice (119). However, all of these mice also have defects or abnormalities in other cell compartments, particularly in the T cell compartment. General lymphopoiesis is severely impaired in Ikaros-deficient and Jak3-deficient mice. There are developmental defects of whole or subsets of T cells in IL-2Rγ subunit-, IRF-1-deficient, CD3ε- and FcεRIγ-transgenic mice. Finally, Diphtheria toxin-, IL-2Rβ subunit- and TCR Vβ8.2-transgenic mice have an abnormal T cell compartment. Therefore, because of selective and profound defects in NK cell compartment, Tg should provide unique animal model for studies of the specific roles of NK cells in immune responses.

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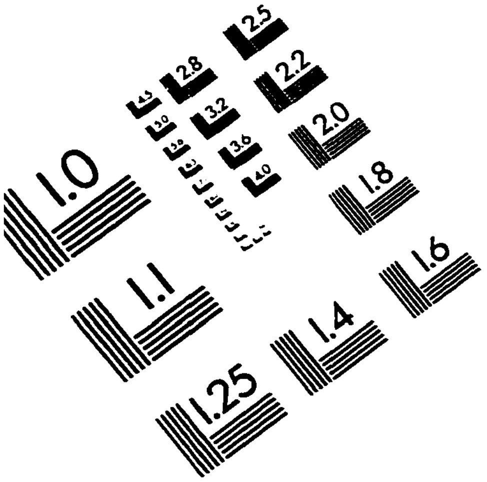
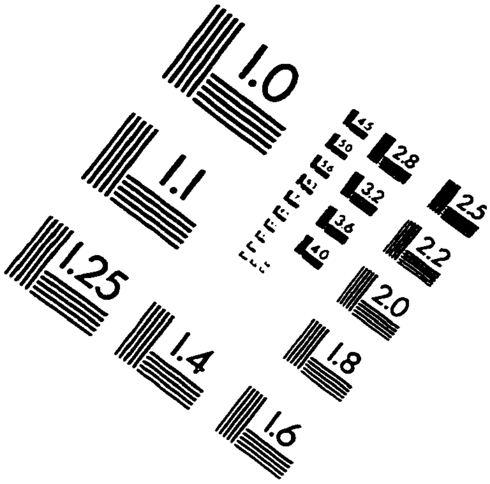
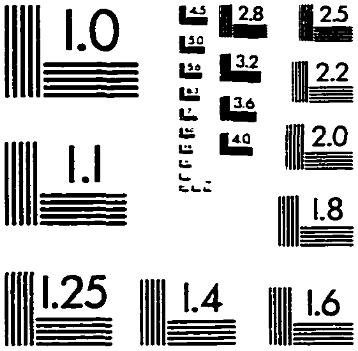
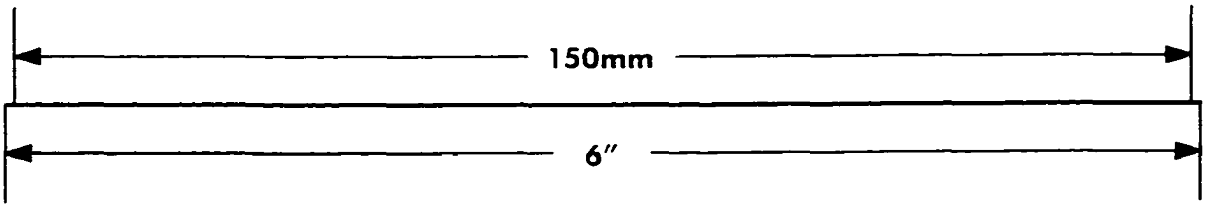
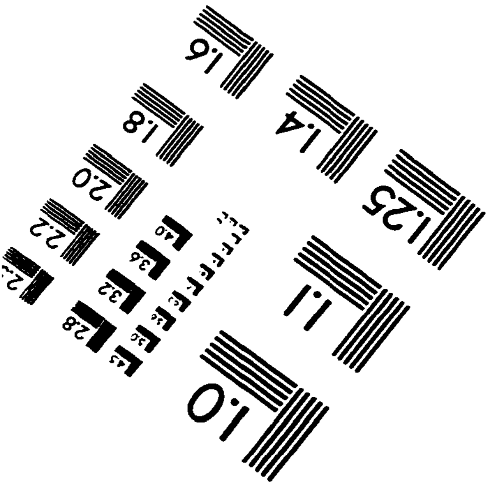
IMAGE EVALUATION
TEST TARGET (QA-3)

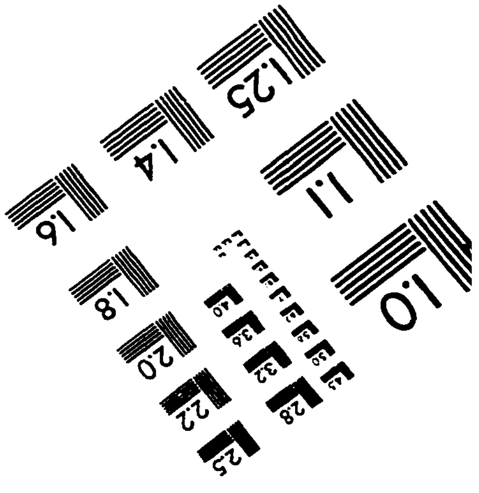












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