

**IMPACT OF BACTERIA ON THE PHENOTYPE, FUNCTIONS,
AND THERAPEUTIC ACTIVITIES OF INVARIANT
NKT CELLS IN MICE**

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NKT CELLS IN MICE**

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DEDICATION

To my loving wife and parents
for their love and support

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LIST OF ABBREVIATIONS

α -GalCer	alpha-galactosylceramide
ALT	alanine aminotransferase
APC	antigen presenting cell
β 2m	beta-2-microglobulin
B6	C57BL/6
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CFU	colony forming unit
CFSE	carboxyfluorescein succinimidyl ester
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
ConA	concanavalin A
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DGK	diacylglycerol kinases
EAE	experimental autoimmune encephalomyelitis
Egr	early growth response
ETP	early thymic progenitor
FACS	fluorescent-activated cell sorting
FITC	fluoresceine isothiocyanate

HSC	hematopoietic stem cell
GAP	GTP activating protein
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	granulocyte monocyte progenitor
GPI	glycophosphatidylinositol
GSC	germline stem cell
IFA	incomplete Freund's adjuvant
IFN	interferon
iGb3	isoglobotrihexosylceramide
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
iNKT	invariant natural killer T
KO	knockout
LCMV	lymphocytic choriomeningitis virus
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAPC	multipotent adult progenitor cell
MCA	methylcholanthrene
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis

MSC	mesenchymal stem cell
NFAT	nuclear factor of activated T cells
NK	natural killer
NKT	natural killer T
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PD-1	programmed death-1
PD-L1/2	programmed death-ligand 1/2
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PMA	phorbol 12-myristate 13-acetate
SCF	stem cell factor
SEB	staphylococcal enterotoxin B
SSEA-1	stage-specific antigen-1
TCR	T cell receptor
TGF	transforming growth factor
Th1	T helper type 1
Th2	T helper type 2
TLR	toll-like receptor
TNF	tumor necrosis factor

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Innate and adaptive immunity

The mammalian immune system is a composite that the innate and adaptive immune responses work together to protect the host from invading pathogens. The innate immune system is the first-line defense, and depends on humoral and cellular components including the complement system, neutrophils, macrophages, dendritic cells (DCs), mast cells, basophils, eosinophils, and natural killer (NK) cells. The innate immune system responds non-specifically to conserved molecular patterns present on pathogens and eliminates them through contact or phagocytosis. As the initial line of defense against pathogens, the innate immune response is immediate upon exposure to foreign pathogens. In addition to their direct role in elimination of pathogens, some of the cells involved in innate immunity also act as antigen presenting cells (APCs) that present specific antigens to T cells of the adaptive immune system. The adaptive immune system is maintained through the cooperation of humoral and cellular components as well. Specific antibodies against invading pathogens are produced by B cells, while direct killing of pathogens expressing specific antigens is mediated by cytotoxic CD8 T cells. CD4 T cells orchestrate the immune response of the adaptive immune system through secretion of various cytokines. The hallmark of the adaptive immune response is the pathogen- and antigen-specific response and the immunological memory characterized by

a delayed weaker primary response and more rapid and robust secondary response (1).

Natural killer T (iNKT) cells

NKT cells are a unique subset of T lineage cells co-expressing T cell receptors (TCRs) and NK lineage receptors. Although these cells express TCRs and share common developmental pathways with T cells, many functional characteristics of these cells categorize them as a component of the innate immune system. Most NKT cells express a semi-invariant TCR, V α 14-J α 18/V β 8,V β 7, or V β 2 in mouse (2-4), and V α 24-J α 18/V β 11 in human (5, 6). Consequently, these cells have a limited ligand repertoire, and they are referred to as invariant NKT cells (iNKT cells) or Type I NKT cells. The remaining NKT cells express non-invariant T cell receptors, and have different ligand specificities (7-11). These cells are referred as Type II NKT cells (12). Both types of NKT cells are specific for glycolipid antigens presented by the major histocompatibility (MHC) class I-related protein CD1d, in contrast with conventional T cells that recognize peptides presented by MHC class I or class II proteins. Although there is growing evidence for an important immune function of Type II NKT cells (13-16), the focus of this thesis work is on iNKT cells.

The phenotype of iNKT cells

The phenotype of iNKT cells shows interesting features. Several receptors commonly found on NK cells are also expressed on iNKT cells, most notably the C-type lectin NK1.1 (Nkrp1c or CD161) expressed in mouse strains such as C57BL/6. Engagement of this activation receptor has been shown to bias iNKT cells towards IFN- γ secretion (17). A significant subset of iNKT cells also expresses NKG2D (18, 19).

NKG2D is a highly conserved C-type lectin-like membrane glycoprotein expressed on most NK cells and on certain T cell subsets. NKG2D acts as an activation receptor for enhancing cytolytic activity and cytokine secretion, and it has been implicated in T and NK cell responses against viruses and tumors as well as autoimmunity (20-24). In addition, some iNKT cells, particularly thymic iNKT cells, express various Ly49 receptors, most of which are inhibitory receptors (25). iNKT cells also express CD94, a component of the inhibitory receptor CD94/NKG2A or the activating receptor CD94/NKG2C (25). Approximately 60% of iNKT cells also express CD4, which enhances TCR signaling in T cells (26-28), and there has been some evidence for distinct functions of CD4⁺ and CD4⁻ iNKT cells (29). Also, in comparison to conventional T cells, the expression level of TCR on iNKT cells is lower, and iNKT cells exhibit an activated phenotype with high expression level of CD69 and CD44, and low level of CD62L (30, 31).

iNKT cell responses are restricted by CD1d molecules

Reactivity of the semi-invariant TCR of iNKT cells is restricted by CD1d molecules. The mouse CD1d molecule is encoded by two genes, CD1d1 and CD1d2, on chromosome 3 (32-35). They exhibit close homology with the human CD1d gene found on chromosome 1 along with other CD1 family genes, CD1a, b, c, and e (36-40). CD1d molecules consist of a heterodimer of a glycosylated heavy chain and β 2-microglobulin (41). While related to MHC molecules, CD1d is structurally quite distinct with a binding pocket well-adapted to bind microbial and endogenous glycolipid antigens (42-44). CD1d is constitutively expressed on APCs such as DCs, macrophages, and B cells that mediate activation of iNKT cells in the periphery, and it is particularly abundant on marginal zone

B cells in the spleen (45-47). CD1d is also conspicuously present on cortical thymocytes, and it is required for development of iNKT cells during the selection process. In addition, high levels of CD1d are found on Kupffer cells, liver sinusoidal endothelial cells, and hepatocytes of liver where the frequency of iNKT cells reaches 30-50% of total T cells in mice (48). CD1d is expressed in the intestine (49) and is also upregulated on microglial cells during inflammation in the brain (50).

Glycolipid antigens for iNKT cells

All iNKT cells react with the glycolipid α -galactosylceramide (α -GalCer) presented by CD1d. This was the first iNKT cell ligand described, and was originally isolated from *Agelas mauritanus*, a marine sponge (51). α -GalCer is a glycosphingolipid, an uncommon antigen for T cells that usually recognize peptides, and its discovery lent strong support for the glycolipid reactivity of iNKT cells (52). Although the physiologic relevance of α -GalCer was doubted early on as the α -anomeric form of glycolipids is largely absent in mammals, this molecule was crucial in studying iNKT cells. α -GalCer bound on CD1d elicits extremely strong interaction with murine iNKT cell receptor with a Kd in the neighborhood of 100 nM (53, 54). The interaction is somewhat weaker with human iNKT cell receptor, but remains robust. Such conservation of antigen specificity of iNKT cells is also observed in rats and primates as well (Figure 1A).

More recently, it has become apparent that several microbial glycolipids can act as ligands for iNKT cells. In particular, iNKT cells react with the α -anomeric glycosphingolipid derived from the cell wall of *Sphingomonas* bacteria, a Gram-negative, LPS-negative α -proteobacterium ubiquitously present in marine and soil environment (55-57). These glycosphingolipids are strong stimulators of iNKT cells and seem to be

important in the host defense against *Sphingomonas*. Interestingly, *Agelas mauritanus* is colonized by *Sphingomonas*, and it is possible that α -GalCer may actually derive from this bacterium (58). Also, α -galactosyl-diacylglycerols from the spirochete *Borrelia burgdorferi* (59), the etiologic agent of Lyme disease that also lacks LPS, has been shown to activate iNKT cells and plays an important role in the clearance of infection (Figure 1B).

In addition to relevant roles of microbial iNKT cell ligands during infection, much attention has been given to identification of endogenous ligands, which were postulated to mediate autoreactivity of human iNKT cells exhibit to CD1d-expressing cells both in mice and in humans (60-62). It has been shown that activation of iNKT cells by certain toll-like receptor (TLR) ligands requires autoreactivity towards CD1d (63, 64). Autoreactivity is also thought to mediate iNKT cell development in the thymus during positive and negative selection of iNKT cells as well as their subsequent maturation in the periphery (65-67). Recent findings have demonstrated that the glycosphingolipid isoglobotrihexosylceramide (iGb3) can activate a majority of mouse and human iNKT cells, and Hexb deficient mice lacking lysosomal enzymatic activity to degrade a precursor lipid to iGb3 also lacked iNKT cells (68-70). However, CD1d tetramer loaded with iGb3 is unable to stain iNKT cells probably due to weak binding of this molecule to CD1d. Indeed, it appears a 100-fold higher concentration of iGb3 is required to stimulate comparable levels of activation of iNKT cells than α -GalCer. A recent detection of iGb3 in thymus has further lent support for the physiological relevance of this molecule (71), although its detection in the peripheral tissues such as spleen and liver remains to be demonstrated. A contradictory report also demonstrated that the deficiency in iGb3 synthase, a putative enzyme essential for iGb3 production, did not affect iNKT cell

ontogeny and function (72). Nevertheless, it is possible that an alternate synthesis pathway to iGb3 exists in vivo (Figure 1C).

iNKT cell response to glycolipid antigens

Although a subset of the T cell lineage, iNKT cells seem to play a pivotal function in bridging innate and adaptive immunity. As the identity of physiological ligand has remained elusive, the function of these cells has been studied employing α -GalCer and its derivatives. While iNKT cells are capable of cytotoxic activity through expression of perforins and granzymes as well as membrane bound tumor necrosis factor (TNF) family including Fas ligand, their primary immune response seems to involve cytokine secretion (73-76). The hallmark of iNKT cell activation is rapid secretion of a variety of cytokines such as interferon (IFN)- γ , interleukin (IL)-4, IL-2, IL-5, IL-10, IL-13, IL-21, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF- α , and TNF- β immediately following TCR engagement. Among these cytokines are T helper (Th) 1 cytokines such as IFN- γ that drives cellular immunity against viruses and other intracellular pathogens as well as cancer, and Th2 cytokines such as IL-4 drives humoral immunity to upregulate antibody production against extracellular organisms. This is in direct contrast with naïve conventional T cells that require prolonged primary stimulation prior to secretion of cytokines, which are also biased to either Th1 or Th2 cytokines, unlike iNKT cells that can secrete Th1 and Th2 cytokines simultaneously. iNKT cells initiate IFN- γ and IL-4 transcription during thymic development and abundant mRNA transcripts are detectable in naïve iNKT cells allowing rapid production of these cytokines (77). These cytokines secreted by activated iNKT cells amplify the immune response initiated by iNKT cells through transactivation of other immune cell types,

including DCs, NK cells, B cells, conventional T cells, and macrophages (78, 79). iNKT cells also upregulate CD40 ligand upon activation and crosslink CD40 on DCs inducing upregulation of CD40 and CD80/CD86, and secretion of IL-12 by DCs, which results in potentiation of the immune response that begins from DCs (80, 81). Maturation of DCs also reciprocally affects iNKT cell activation and cytokine production (82-86).

While iNKT cells can produce explosive amounts of Th1 and Th2 cytokines, the balance between Th1 vs. Th2 cytokines can be variable according to the glycolipid antigen employed to stimulate iNKT cells. α -GalCer shows equally potent secretion of Th1 and Th2 cytokines (79). The C-glycoside analogue of α -GalCer, α -C-GalCer exhibits Th1 bias (79, 87). However, glycolipids with shorter or less saturated lipid chains such as OCH exhibit a Th2 bias in cytokine production (79, 88-90). The mechanism by which different glycolipids induce variable cytokine secretion is unclear. One hypothesis is that the duration and strength of T cell receptor engagement by different glycolipids might explain differences in cytokine production (91), but TCR on and off rates determined by plasmon resonance or crystal structures of CD1d have shown minor differences among various glycolipids (43). Alternatively, it is possible that glycolipid trafficking and uptake might depend on lipid solubility and differences in lipid solubility owing to modifications of lipid chains that might result in increased or decreased uptake by APCs such as DCs that in turn secrete the Th1-inducing cytokine IL-12 (43, 92). Also, differences in tissue distribution of glycolipids might result in variable cytokine production as tissues may offer different cytokine milieu in which iNKT cells are activated.

iNKT cell response to α -GalCer

During the primary response of naïve conventional peptide-reactive T cells, a strong antigenic stimulation in conjunction with adequate costimulation is followed by a prolonged period of maturation of these naïve cells into mature effector cells, which can take several days. After clearance of the particular antigen, a population of memory T cells with the same antigen specificity emerges to mount a more rapid and effective resolution of the secondary challenge (1).

The response of lipid-reactive iNKT cells is quite distinct from that of conventional T cells. A detailed analysis of the *in vivo* response of murine iNKT cells to α -GalCer has been reported by our laboratory and others. The response of iNKT cells has been assessed using the CD1d-tetramer loaded with α -GalCer (93, 94), which specifically binds to the invariant TCR of iNKT cells. With appropriate fluorochromes linked to the tetramer, iNKT cells can be identified by flow cytometry. Consistent with the activated phenotype exhibited by naïve iNKT cells, these cells downregulate their TCR and NK1.1 immediately following the primary α -GalCer challenge (95-97), which renders these cells undetectable to staining by CD1d tetramer loaded with α -GalCer. As early as 24 hours after injection their TCR is re-expressed, but NK1.1 remains downregulated for several months. During this time, iNKT cells undergo an extensive *in vivo* expansion reaching maximal levels by day 3 with 10- to 15-fold increase in cellularity in spleen. *In vivo* expansion of iNKT cells is also observed in other organs such as lymph nodes, peripheral blood, liver and bone marrow, but no expansion is observed in thymus. Following the peak response, iNKT cells gradually decrease in number to levels slightly lower than before α -GalCer treatment.

The secondary response by iNKT cells following rechallenge with α -GalCer is

characterized by a hyporesponsive phenotype (25, 98, 99). For at least 1 month after the initial challenge, iNKT cells show significantly suppressed capacity to proliferate and secrete cytokines in response to rechallenge with α -GalCer. This decrease in cytokine production is associated with inability of iNKT cells to transactivate DC, B cells and NK cells. The hyporesponsive phenotype of iNKT cells induced by the initial α -GalCer challenge was shown to be cell autonomous indicating that these iNKT cells are in an anergic state. These anergic iNKT cells are unable to show anti-tumor activities in B16 melanoma metastasis model, and thus α -GalCer-induced anergy may limit the utility of iNKT cell-based therapies.

iNKT cells and cancer

iNKT cells may function in immune surveillance against cancer even in the absence of exogenous stimulation of iNKT cells by α -GalCer (100). In patients with many forms of cancer, such as myelodysplastic syndromes, iNKT cell function was found to be severely compromised. Also, studies of a fibrosarcoma carcinogenesis model induced by methylcholanthrene (MCA), a chemical carcinogen, revealed the protective effects of iNKT cells against cancer. This protection was found to be mediated by IL-12 and cytolytic activity of iNKT cells. A subsequent study has shown that the NKT-NK axis of activation was also critical for suppression of MCA-induced carcinogenesis (101). However, this tumor model is the only example providing evidence for physiological tumor surveillance by iNKT cells in the absence of exogenous stimulation of these cells.

α -GalCer was first identified while screening for the molecule responsible for anti-tumor activity against B16 melanoma in marine sponge, and as such, the role of iNKT cells in protection against cancer has been carefully studied. Results have shown

promising anti-tumor effects in various types of metastatic malignancy by α -GalCer and its analogs as well as DC pulsed with α -GalCer (102).

More importantly, a number of clinical studies on iNKT cell-based therapy employing α -GalCer or α -GalCer-pulsed DC are underway (103-107). A phase I study of α -GalCer carried out in the Netherlands has shown no significant dose-limiting side effects or toxicity, and α -GalCer was well tolerated by patients (106). However, no significant anti-tumor effect with clinical improvement was observed with this initial study. Because α -GalCer-pulsed DC exhibit more potent effects against B16 melanoma metastasis (108, 109), Nakayama and colleagues carried out a phase I clinical trial using α -GalCer-pulsed DC on advanced non-small cell lung cancer patients and have seen some clinical improvements (105). Nicol and colleagues have also reported some success during phase I clinical trial of α -GalCer-pulsed DC involving patients with metastatic malignancy (110).

iNKT cells and autoimmunity

Potent immunomodulatory function of iNKT cells has been exploited to impart protection against a number of autoimmune disorders including type I diabetes, experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease (111-125). Successful protection or amelioration against certain autoimmune disease models, in particular, Type I diabetes, EAE, and rheumatoid arthritis, have been observed employing α -GalCer and its derivatives to impart Th2 bias as pathogenic cells have been indicated to be Th1 biased cells destroying the tissue of interest. However, treatment efficacy depended on the dose, route and timing of administration as well as the number of injections and the strain of

mice used in the particular study. Such results reflect the complexity of iNKT cell functions in the modulation of the immune system. The delicate balance between Th1 and Th2 cytokines seems to be crucially regulated by iNKT cells, and further understanding of their normal functions as well as their responses to pharmacologic ligands is necessary to fully cultivate their therapeutic potential in treatment of autoimmune diseases.

iNKT cells and infection

Most of the known iNKT cell antigens have the α -anomeric form that is not found in mammals. Few exceptions include β -GalCer and iGb3 with relatively weak activity compared to α -anomeric ligands. It has therefore been speculated that these cells might have originally arisen in defense against foreign microbes. Because iNKT cells have the capacity to rapidly produce cytokines that can enhance immune responses by DCs, NK cells, and conventional T and B cells, these cells were thought to bridge and amplify the host immune response during early phases of the immune response. A whole body of literature now exists revealing the importance of these immunomodulatory cells during infection with bacteria, viruses, fungi and parasites (126). The role of iNKT cells during infection was assessed by using J α 18 deficient mice that specifically lack iNKT cells, or CD1d deficient mice that lack iNKT cells as well as non-invariant CD1d restricted T cells, or neutralizing antibody against CD1d. As iNKT cells function primarily as immunomodulators, outcome of infection was in some cases ameliorated in the absence of iNKT cell function. Moreover, iNKT cell contribution to host defense was sometimes variable according to the bacterial strain, route of administration, and strain of mice used. For instance, intranasal infection with the D4 strain of *Pseudomonas aeruginosa* exacerbated infection in CD1d deficient mice (127), whereas intratracheal

infection of PAO1 strain of *Pseudomonas aeruginosa* showed no significant difference in the severity of disease in $J\alpha 18$ deficient mice (128). Interestingly, bacteria that are known to express microbial glycolipid antigens for iNKT cells, *Sphingomonas* and *Borrelia burgdorferi*, have been found to depend on iNKT cell function for efficient clearance (55, 56, 59, 129, 130). More examples of the role for iNKT cells during bacterial infection are summarized in Table 1.

iNKT cell activation by microbes

Activation of conventional T cells requires recognition by the TCR of specific peptide antigen derived from microbial proteins. Activation of iNKT cells by microbes is unique in that even in the absence of direct recognition of cognate glycolipid antigen derived from microbes by the TCR, iNKT cells have been shown to be involved in the clearance of diverse species of microbes. This non-specific activation of iNKT cells, putting them in the category of the innate immune system, can be explained by two modes iNKT cell activation, the direct mechanism and the indirect mechanism.

The direct mechanism of iNKT cell activation relies on the presence of microbial glycolipid antigen that is presented by CD1d molecules on APC and directly engages the semi-invariant TCR (Figure 2A). Early studies have shown that glycosphosphatidylinositol anchor purified from *Plasmodium* and *Trypanosoma* species (131), lipophosphoglycan extract from *Leishmania donovani* (132), and phosphatidyl inositol tetramannosides enriched from *Mycobacterium* can activate a minor subset of iNKT cells (133), although some of these results remain controversial. Subsequently, variants of glycosphingolipids in *Sphingomonas capsulata* (55, 130, 134) and galactosyl diacylglycerol antigens from *Borrelia burgdorferi* (59) have been found to strongly

activate most iNKT cells.

However, the vast majority of pathogens are not considered to express microbial antigens specific for iNKT cells. These pathogens activate iNKT cells through an indirect mechanism of activation that does not rely on specific recognition of microbial glycolipid antigens and instead is mediated by activation of DCs by pathogen-associated molecular patterns (PAMPs), which in turn leads to non-specific activation of iNKT cells (Figure 2B). In response to LPS from *Salmonella typhimurium*, TLR signaling in DCs induces IL-12 secretion, which in conjunction with CD1d presentation of hypothetical endogenous ligand activates iNKT cells (55, 63). However, variations on this theme of iNKT cell activation have been noted. For instance, iNKT cell activation by DCs sensitized with *Schistosoma mansoni* eggs has been shown to dependent on endogenous antigen alone (135). In the case of *Escherichia coli* LPS, release of proinflammatory cytokines such as IL-12 and IL-18 by DCs is sufficient for iNKT cell activation, and autoreactive TCR engagement by endogenous ligand is not required (136).

T cell anergy

Anergy is defined as a tolerance mechanism involving the intrinsic functional inactivation of lymphocytes in response to antigen encounter (137). Anergy is often evoked, either in vivo or ex vivo, by the unbalanced stimulation of lymphocytes through antigen receptors, in the absence of co-stimulatory signals, by chronic antigen stimulation, or by stimulation with weak agonist antigens in the presence of full co-stimulation (137). The precise molecular and biochemical events responsible for the development and maintenance of the anergic state remain to be fully characterized, and might differ for the particular tolerance model studied (138). Studies with multiple anergy models have

demonstrated a critical role for the mobilization of intracellular free Ca^{2+} (139), resulting in activation of the Ca^{2+} -sensitive protein phosphatase calcineurin and the nuclear factor of activated T cells (NFAT) (140). NFAT (most notably NFAT1), activated in the absence of its transcriptional partner AP-1 (Fos/Jun), then enters the nucleus and induces the transcription of a variety of anergy-associated genes, including the early growth response gene 2 (Egr2) and Egr3 (141), the E3 ubiquitin ligases GRAIL, Cbl-b, and Itch (142, 143), and diacylglycerol kinases (DGK)- α and - ζ (144-146). Egr2 and Egr3 are transcription factors that are thought to be important for the induction of anergic factors, possibly including several of the E3 ubiquitin ligases (141). The anergy-associated ubiquitin ligases are thought to promote the monoubiquitination of a variety of receptors and signaling components (147, 148). It has been suggested that these events, together with the termination of diacylglycerol-dependent signaling mediated by activated DGKs, lead to uncoupling of the TCR from downstream signaling events, most notably Ras activation. As a consequence of these abnormalities in proximal signal transduction, defective IL-2 gene transcription is a common characteristic of T cell anergy (137). In many cases, the anergic phenotype can be reversed by withdrawal of the anergy-inducing stimulus, by exposure to signals (e.g., ionomycin plus phorbol myristate acetate) that bypass proximal TCR signaling events and/or by exposure to exogenous IL-2 (137).

Purpose of this thesis work

Past studies in our laboratory have found that, unlike conventional T cells that exhibit memory responses, iNKT cells undergo a long period of anergy following a single injection of α -GalCer, a potent synthetic ligand for iNKT cells. This result might have biological significance in that the primary response of iNKT cells is already extremely

strong with an explosive secretion of various cytokines and extensive transactivation of other immune cells. Furthermore, iNKT cells mount non-specific responses to multiple pathogens. Repeated responses of iNKT cells at high magnitude might do more harm than good as the tissue damage during infection is often a result of the excessive immune responses. In this light, limiting the subsequent activity of iNKT cells following the initial activation might be beneficial.

Recently, with identification of various microbial glycolipid antigens for iNKT cells, there is growing evidence that iNKT cells have important functions during host defense against pathogenic microbes in addition to their crucial roles in autoimmunity and tumor surveillance. In fact, iNKT cells have been found to play an important role during immune responses against pathogenic bacteria, viruses, fungi and parasites; and this iNKT cell activity during infection may be the original function for this relatively small subset of T cells when viewed from an evolutionary perspective. It has been found that these cells with a limited TCR repertoire can mount a response to a wide array of pathogens in the presence of specific microbial glycolipid antigens for iNKT cells, or even in the absence of a cognate antigen indirectly through combination of proinflammatory cytokines from APC in conjunction with presentation of endogenous autoantigens. Although the role of iNKT cells in defense against invading pathogens has been well documented, the impact of pathogens on iNKT cells remains incompletely understood.

In this context, I tested the hypothesis that bacteria induce long-term hyporesponsiveness of iNKT cells. This hypothesis was tested in two integrated Specific Aims. In Aim 1, I tried to determine whether bacteria induce long-term phenotypic changes in iNKT cells accompanied by induction of a hyporesponsive state, and how this

relates to iNKT cell function in disease models. The results from this aim are reported in Chapter II. In Aim 2, the mechanism of iNKT cell hyporesponsiveness induced by bacteria was explored, and the results are reported in Chapter III.

Significance of this thesis work

Since identification of iNKT cells, their immunomodulatory functions have attracted significant attention throughout the scientific community for their therapeutic potential. In the past several years, significant advances were made in the field of iNKT cell biology aided by development of important tools to study iNKT cells including CD1d deficient mice, generated in our own laboratory and others, CD1d tetramers, and other reagents, providing novel insights into the importance of this relatively small subset of T cells that have key immunomodulatory functions during various immune responses. A wide variety of glycolipid antigens for invariant TCRs of iNKT cells have been identified and the response of iNKT cells to these antigens has been carefully tested and documented. The therapeutic potential of iNKT cells has been explored and expansive amounts of research have been performed to delineate the role of iNKT cells against various types of cancer, autoimmune disorders, and infection. Recently, several clinical trials have been initiated, using α -GalCer or α -GalCer-pulsed DCs to treat cancer. During these clinical trials, it has become apparent that iNKT cell number and function has wide individual variability in humans, which likely is contributed by genetic factors as well as environmental factors. In this thesis work, I present evidence that infection by pathogenic bacteria, which is a regular occurrence in the human population, and more so in cancer patients or patients with autoimmune disorders who are often immunocompromised due to the disease itself or due to the treatment, impacts iNKT cell function with long-term

effects on therapeutic activity of these cells. This result might in part explain some of the difficulties encountered during clinical application of iNKT cell-based immunotherapy and an may provide insight into the design of clinical protocols to optimize efficacy of treatment.

Bacteria (strain)	Route of infection	Effect of NKT cell deficiency on infection			Mouse strain	Ref
		CD1d ^{-/-}	Jα18 ^{-/-}	Anti-CD1d Ab		
<i>Pseudomonas aeruginosa</i>						
D4	Intranasal	Exacerbated		Exacerbated	B6 or BALB/c	(127)
PAO1	Intratracheal		Not exacerbated		B6	(128)
<i>Sphingomonas</i> spp.						
<i>S. yanoikuyae</i>	Intraperitoneal	Exacerbated	Exacerbated		B6	(56)
<i>S. capsulata</i>	Intravenous	Exacerbated	Exacerbated		B6	(55)
<i>Mycobacterium tuberculosis</i>						
Erdman	Intravenous	Not exacerbated			B6 or BALB/c	(149, 150)
Kurono	Aerosol		Not exacerbated		BALB/c	(151)
H37Rv	Intravenous			Exacerbated	B6	(152)
<i>Mycobacterium bovis</i>						
Pasteur	Intravenous		CFU unchanged inflammation ↑		B6 or BALB/c	(153)
Tokyo 172	Intratracheal		Not exacerbated		B6	(154)
<i>Salmonella</i> spp.						
<i>S. choleraesuis</i>	Intraperitoneal		CFU unchanged Liver injury ↓		B6	(155)
<i>S. typhimurium</i>	Oral	Not exacerbated			B6	(156)
<i>Listeria monocytogenes</i>						
Unknown strain	Intravenous			Ameliorated	B6	(157)
10403S	Intravenous or oral	Exacerbated			B6/129	(158)
LO28	Oral		Not exacerbated		B6	(159)
<i>Chlamydia</i> spp.						
<i>C. muridarum</i>	Intranasal				BALB/c	(160, 161)
<i>C. pneumoniae</i>	Intranasal	Exacerbated	Exacerbated		B6 or BALB/c	(160)
Other						
<i>Borrelia burgdorferi</i> N40	Intradermal	Exacerbated			B6 or B6/129	(129)
<i>Ehrlichia muris</i>	Intraperitoneal	Exacerbated			B6	(55)
<i>S. pneumoniae</i> serotype 3	Intratracheal		Exacerbated		B6	(162)

Table 1. The role of NKT cells in host defense against bacterial infection. The table summarizes results from an extensive collection of articles on the role of NKT cells during bacterial infection. Various strains of bacteria were introduced into mice of the indicated strain, with NKT cells deficient or neutralized by disruption of CD1d or Ja18, or injection of blocking antibody against CD1d. CFU: colony forming unit.

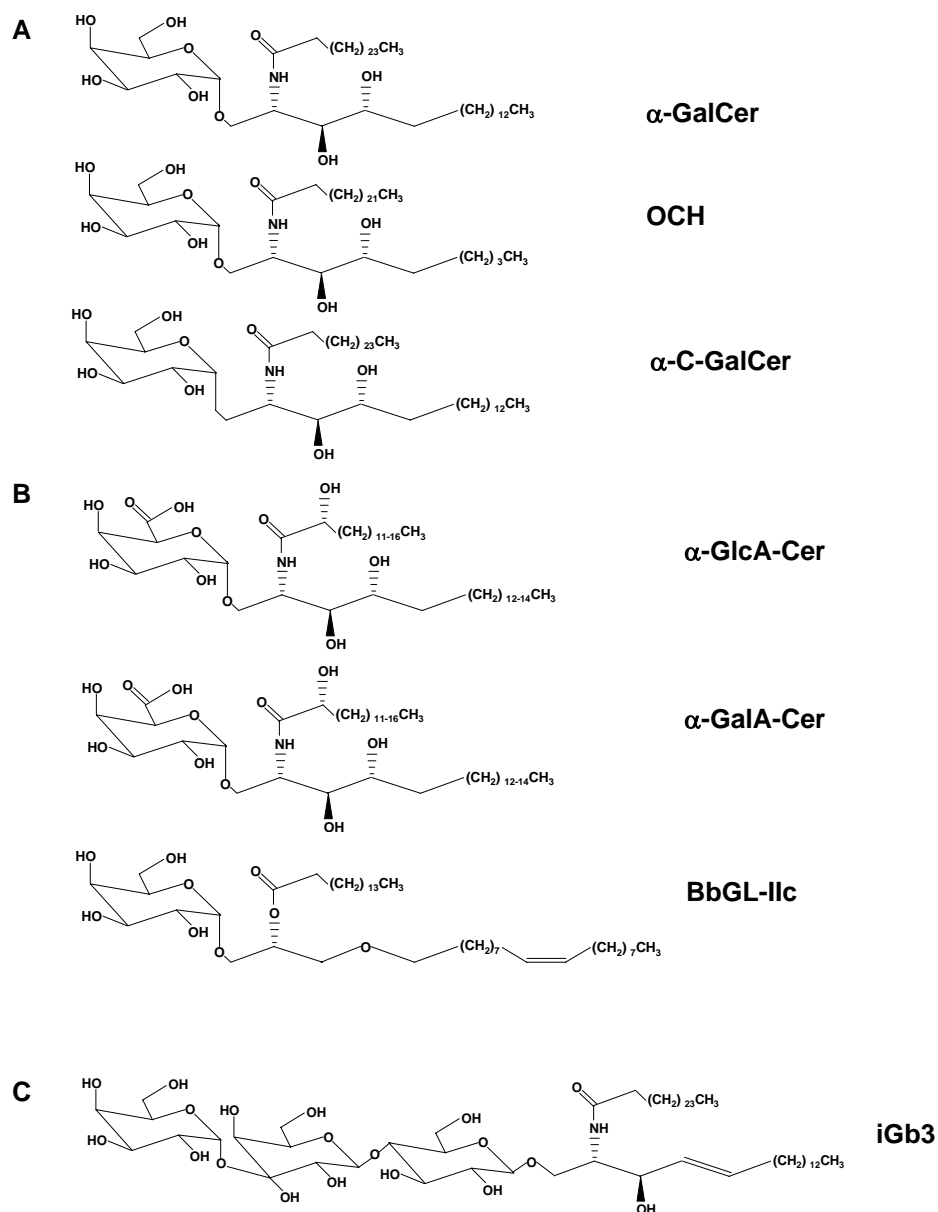


Figure 1. Glycolipid ligands for iNKT cells. **(A)** Synthetic glycolipid ligands for iNKT cells. α -GalCer is the synthetic derivative of a potent iNKT cell antigen purified from a marine sponge. OCH is a variant of α -GalCer with a shortened sphingosine chain. α -C-GalCer is a C-glycoside analogue of α -GalCer. **(B)** Microbial glycolipid ligands for iNKT cells. α -GlcA-Cer and α -GalA-Cer are microbial glycolipid ligands present in *Sphingomonas capsulata*. BbGL-IIc is a microbial glycolipid ligand in *Borrelia burgdorferi*. **(C)** Endogenous glycolipid ligand for iNKT cells. Isoglobotrihexosylceramide (iGb3) is an endogenous glycolipid antigen that appears to mediate autoreactivity of iNKT cells in the absence of an exogenous ligand.

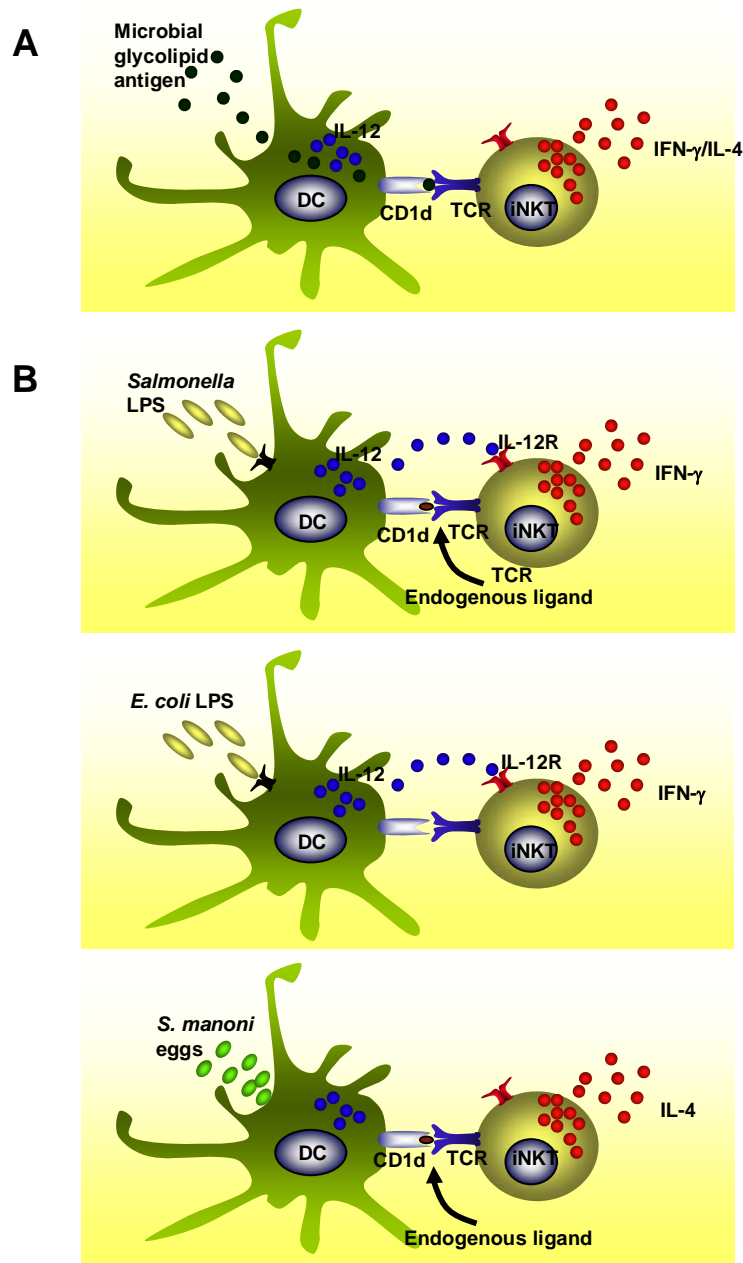


Figure 2. Activation of iNKT cells by microbes. (A) Direct activation of iNKT cells by microbial glycolipid ligands. Certain microbes such as *Sphingomonas capsulata* or *Borrelia burgdorferi* express microbial glycolipid ligands that can be presented by CD1d on APC and engage with TCR of iNKT cells. (B) Indirect activation of iNKT cells by microbial products. *Salmonella* LPS activates DCs which present the endogenous ligand and secrete IL-12 to activate iNKT cells to secrete IFN- γ . Activation of iNKT cells and the resultant secretion of IFN- γ by *E. coli* LPS is solely dependent on cytokine secretion by DCs such as IL-12 or IL-18. *S. mansoni* eggs sensitize DCs to present an endogenous ligand to iNKT cells, and this activation results in IL-4 secretion by iNKT cells.

CHAPTER II

INDUCTION OF INKT CELL HYPORESPONSIVENESS BY MULTIPLE BACTERIA

Abstract

Invariant natural killer T (iNKT) cells are innate-like lymphocytes that recognize glycolipid antigens in the context of the MHC class I-like antigen-presenting molecule CD1d. Our laboratory has previously demonstrated that in vivo activation of iNKT cells with the glycolipid α -galactosylceramide (α -GalCer) in mice results in the acquisition of a hyporesponsive or anergic phenotype by these cells. Because iNKT cells can become activated in the context of infectious agents, we have evaluated whether iNKT cell activation by microorganisms can influence subsequent responses of these cells to glycolipid antigen stimulation. We found that murine iNKT cells activated in vivo by multiple bacterial microorganisms became unresponsive to subsequent activation with α -GalCer. This hyporesponsive phenotype of iNKT cells was associated with changes in the surface phenotype of these cells, reduced severity of Concanavalin A-induced hepatitis, and alterations in the therapeutic activities of α -GalCer. These findings have important implications for the development of iNKT cell-based therapies.

Introduction

Conventional T cells respond to invading pathogens through recognition of specific antigenic peptides derived from the pathogens and presented by MHC Class I or Class II molecules on APCs. Conventional T cells are able to mount a response to a wide range of non-self peptides through a diverse TCR repertoire achieved through somatic recombination and the positive and negative selection process during development in the thymus. The primary response of naïve conventional T cells towards pathogen-derived peptides is characterized by a delayed and moderate response, which requires time to generate mature effector cells to adequately control infection. Once the pathogen is cleared however, a population of memory T cells emerges that can rapidly and effectively resolve subsequent infection by the same pathogen. Conventional T cells therefore exhibit classic features of the adaptive immune system (1).

Unlike conventional T cells, iNKT cells have a severely restricted TCR repertoire. These cells express a semi-invariant T cell receptor, V α 14-J α 18/V β 8, V β 7, or V β 2 in mouse (2-4), and V α 24-J α 18/V β 11 in human (5, 6). Interestingly, the TCRs of iNKT cells are reactive to glycolipid ligands. The most well-documented glycolipid ligand for iNKT cells is α -GalCer, a potent synthetic derivative of a marine sponge product that elicits a strong response by iNKT cells.

The iNKT cell response was originally studied using antibodies against TCR β and NK1.1 to identify iNKT cells. In vivo administration of anti-CD3, IL-12, or α -GalCer in mice resulted in rapid disappearance of these cells in all organs except thymus and bone marrow (163). It was initially proposed that in vivo administration of iNKT cell antigens resulted in activation-induced cell death of iNKT cells. However, apoptotic

disappearance of iNKT cells following activation was unable to explain extensive proliferation of iNKT cells observed in vitro (164, 165). This original hypothesis was revised when CD1d tetramers that can specifically stain for the semi-invariant TCR of iNKT cells became available (95-97). It was determined that the apparent loss of iNKT cells during early phases of iNKT cell responses to antigens was due to profound downregulation of NK1.1 and the TCR by iNKT cells rendering these cells undetectable by conventional methods of identification using anti-TCR β and anti-NK1.1 antibodies. Additional studies revealed that this TCR downregulation was transient and the cells could be detected as early as 24 hours following initial activation using CD1d tetramer staining once TCR levels returned to normal levels even though NK1.1 downregulation was sustained and persisted even 6 months after initial activation (95-97).

During the primary response naïve iNKT cells have been shown to undergo extensive in vivo expansion following α -GalCer treatment (95, 96). Maximal expansion was observed around 3 days after the treatment reaching 10 to 15 fold increase in the number of iNKT cells in spleen. Once iNKT cell numbers reached their peak, they gradually declined to untreated levels by 10-14 days and continued to decline over the period of several months. In summary, during the primary response of iNKT cells to in vivo α -GalCer stimulation, iNKT cells undergo transient downregulation of TCR followed by rapid clonal expansion and homeostatic contraction accompanied by downregulation of NK1.1.

Adaptive immunity is characterized by initially latent and weaker primary responses, and rapid and explosive memory responses, and T cells are an integral part of the adaptive immune system critical for clearance of foreign pathogens (1). Although iNKT cells are a subset of T cells, these cells do not show memory responses during the

recall response to α -GalCer, and instead exhibit an anergic response characterized by absence of clonal expansion and cytokine production. Studies from our laboratory and others provided a detailed documentation of the secondary response shown by iNKT cells following rechallenge with α -GalCer in mice previously treated with α -GalCer (25, 98, 99). When splenocytes were rechallenged ex vivo following different time points after a single dose of α -GalCer, iNKT cells no longer exhibited extensive proliferation and cytokine production in response to α -GalCer normally observed in naïve animals. This blockade in iNKT cell response was prolonged and was observed for at least 1 month. Among various cytokines normally produced by iNKT cells, blockade in IFN- γ was more pronounced than IL-4. Loss of cytokine secretion during secondary challenge was also accompanied by absence of transactivation of DC, B and NK cells. This suppressed iNKT cell activity during the recall response was associated with loss of anti-tumor activities of these cells in the B16 melanoma metastasis model, but interestingly the protective effect for EAE was retained. As a result, this anergic phenotype of iNKT cells induced by α -GalCer has been implied as a limiting factor for therapeutic application of iNKT cell-based therapies using α -GalCer and its analogues.

The role of iNKT cells during immune defense against microbial pathogens is well documented. Since Brenner and colleagues have postulated a model of physiological iNKT cell activation during infection dependent on autoreactive CD1d presented endogenous ligand and IL-12 during *Salmonella typhimurium* infection (63), it has become apparent that even with a limited repertoire of the semi-invariant T cell receptor, iNKT cells are able to be activated and respond to a broad spectrum of pathogens. Additionally, several specific microbial lipid antigens that bind to CD1d and activate iNKT cells have been identified in *Sphingomonas capsulata* (55, 130, 134) and *Borrelia*

burgdorferi (59).

During the primary response of iNKT cells to *S. typhimurium*, which is now thought to activate iNKT cells through toll-like receptor (TLR) ligands such as LPS and flagellin, a similar disappearance of iNKT cells around 3 to 5 days after infection was observed when these cells were identified based on the surface expression of TCR β and NK1.1 as was also observed with α -GalCer (166-168). Consistent with the α -GalCer studies, iNKT cells remained detectable during those time periods when studied with α -GalCer-loaded CD1d tetramer, and the initial result of iNKT cell disappearance was attributed to profound downregulation of NK1.1 (96). As results from studies with α -GalCer have shown that NK1.1 downregulation by iNKT cells coincided with long-term periods of suppressed iNKT cell function following initial activation by α -GalCer (25, 98, 99), bacteria may also induce iNKT cell hyporesponsiveness. Based on these previous studies, we hypothesized that iNKT cells can be activated by multiple bacterial organisms, and we evaluated a large panel of bacteria for their impact on the phenotype, functions, and therapeutic activities of iNKT cells.

Results

Mouse iNKT cells become activated in vivo by diverse bacterial species.

Prior studies have shown that iNKT cells can become activated in response to various infectious agents, either through direct recognition of microbial glycolipid antigen, or indirectly through cytokines secreted by DCs in conjunction with endogenous antigens expressed by activated DCs (126). We tested the capacity of a wide variety of bacteria, including the gram-positive organisms *Listeria monocytogenes* and *Staphylococcus aureus*, and the gram-negative organisms *Escherichia coli*, *Salmonella typhimurium*, and *Sphingomonas capsulata* to activate iNKT cells and to modulate the functions of these cells. As we were primarily interested in the long-term effects of bacterial microorganisms on iNKT cell functions, the choice of bacteria was not limited to known pathogens that depend on iNKT cells for their clearance. Apart from *L. monocytogenes* and *S. capsulata*, bacteria were heat-killed prior to challenge. Activation of iNKT cells was assessed by their prevalence and numbers and by their surface phenotype, such as expression of CD69, an early activation marker, and NK1.1, which becomes downregulated on activated iNKT cells (95, 96) and remains expressed at low levels on iNKT cells rendered anergic in α -GalCer-treated animals (25). Analyses were performed 24 hrs after i.v. injection of bacteria. Naïve mice and mice injected with 5 μ g α -GalCer were used as controls.

Consistent with prior studies (95-97), 24 hrs after α -GalCer injection, TCR downregulation rendered iNKT cells undetectable by tetramer staining (Figure 3). Minor decreases in iNKT cell numbers were observed in the spleens of mice injected with *L. monocytogenes* and *S. aureus*, and in livers of mice injected with *L. monocytogenes*, *S.*

aureus, *S. capsulata* and *S. typhimurium*. Differences in iNKT cell numbers in the liver reached statistical significance only after *L. monocytogenes* and *S. capsulata* injections.

Each of the bacterial organisms tested induced upregulation of CD69 on iNKT cells, suggesting activation of these cells. However, the extent of CD69 upregulation was variable, reflecting potential differences in the degree or kinetics of iNKT cell activation induced by distinct organisms. NK1.1 downregulation by spleen iNKT cells was observed for heat-killed *S. aureus*, *S. typhimurium*, and live *L. monocytogenes*, but was less evident for heat-killed *E. coli* and live *S. capsulata*. The changes observed in hepatic iNKT cells mirrored changes in splenic iNKT cells, except for downregulation of NK1.1, which was only evident for *S. aureus* and *S. typhimurium* (Figure 3).

Next, we examined the prevalence, cell number and surface phenotype of iNKT cells 3 weeks after injection of α -GalCer or bacteria. Consistent with prior studies (25), α -GalCer injection resulted in a modest decrease in the frequency of iNKT cells in the spleen and liver accompanied by sustained NK1.1 downregulation in spleen (Figure 4). Similar changes were observed in mice that received heat-killed *E. coli*, *S. aureus* or *S. typhimurium*. Notably, inoculation of live *L. monocytogenes* resulted in a substantial loss of iNKT cells and sustained downregulation of NK1.1. By contrast, *S. capsulata* did not induce sustained changes in the surface phenotype of iNKT cells.

In summary, all bacteria tested were able to induce early activation of iNKT cells, but the changes in surface phenotype of these cells induced by different bacteria were distinct, and were different from the phenotype of iNKT cells induced by α -GalCer.

Impact of bacteria-induced iNKT cell activation in vivo on the response of splenocytes to subsequent α -GalCer stimulation ex vivo

Prior studies have demonstrated that α -GalCer treatment of mice results in long-term suppression of subsequent iNKT cell responses to α -GalCer ex vivo and in vivo (25, 99, 169). Several of the bacteria tested activated and induced phenotypic alterations in iNKT cells that were characteristic of anergic iNKT cells induced in response to α -GalCer treatment (Figure 3, 4). Therefore, we treated mice with heat-killed or live bacteria and 3 weeks later we measured responses of splenocytes from these animals to stimulation with α -GalCer. Consistent with prior studies (25, 99, 169), splenocytes from α -GalCer-injected mice showed dampened proliferation and cytokine production as compared with naïve splenocytes (Figure 5). Interestingly, splenocytes from mice injected with heat-killed *E. coli*, *S. aureus* or *S. typhimurium*, or with live *L. monocytogenes* also showed significant defects in proliferation and cytokine production in response to subsequent ex vivo stimulation of iNKT cells with α -GalCer (Figure 5). For most of these bacteria there was a trend for a more profound defect in IL-4 than IFN- γ production by hyporesponsive iNKT cells, whereas iNKT cells rendered anergic by α -GalCer had a more profound defect in IFN- γ than IL-4 production (Figure 3 and (25)). In sharp contrast to the effect on iNKT cell responses, bacteria did not alter conventional T cell function (Figure 6 and 18A). Collectively, our findings suggest that bacteria can impair iNKT cell functions in vivo.

Kinetics of iNKT cell responses in mice treated with heat-killed E. coli or live L. monocytogenes

We selected two organisms, heat-killed *E. coli* and live *L. monocytogenes*, which showed the strongest effects on iNKT cell responses, to perform a detailed characterization of the kinetics of iNKT cell responses. We measured iNKT cell numbers,

expansion, surface phenotype and functions at different time points after treatment.

After treatment with heat-killed *E. coli* there was a modest decrease in total numbers of splenic iNKT cells over time (Figure 7A, B), but this did not reach statistical significance. The frequency of liver iNKT cells on the other hand dropped between 3 and 4 weeks, which was due to an influx of conventional T cells into the liver (data not shown), but the prevalence of iNKT cells returned to relatively normal levels around 6 weeks. NK1.1 surface levels became downregulated in the spleen and liver around 2-3 weeks, returned to normal levels in the liver by week 6, but remained suppressed in the spleen until week 6 (Figure 8). Analysis of iNKT cell responses revealed suppressed capacity of splenocytes to proliferate and produce IFN- γ and IL-4 upon in vitro stimulation with α -GalCer at 3 and 4 weeks after treatment with heat-killed *E. coli* (Figure 9A). In contrast with α -GalCer-injected controls, the blockade in IL-4 production induced by *E. coli* appeared to be more profound than that for IFN- γ production. Despite sustained NK1.1 downregulation on iNKT cells, splenocytes generated relatively normal responses to *E. coli* by week 6. To assess effects on iNKT cell proliferation and cytokine production more directly, we performed carboxyfluorescein succinimidyl ester (CFSE) dilution and intracellular staining experiments. Results demonstrated reduced capacity of iNKT cells from *E. coli*-treated animals to proliferate (Figure 9B) and to produce cytokines (Figure 9C) in response to α -GalCer stimulation ex vivo.

In contrast to heat-killed *E. coli* and α -GalCer, treatment of mice with live *L. monocytogenes* resulted in a dramatic reduction in iNKT cell frequency and numbers in both spleen and liver (Figure 10A, B). By week 4, numbers of iNKT cells had recovered in the liver but not spleen. The NK1.1 expression pattern following infection with *L. monocytogenes* closely mimicked that seen after α -GalCer treatment (Figure 11). NK1.1

downregulation was evident by day 1 and persisted until week 4. These alterations in iNKT cell numbers were accompanied by profound changes in the response of splenocytes to α -GalCer stimulation (Figure 12A). In addition, intracellular staining revealed reduced capacity of iNKT cells from *L. monocytogenes*-infected animals to produce cytokines in response to α -GalCer stimulation ex vivo (Figure 12B).

Bacteria induce long-term iNKT cell hyporesponsiveness in vivo

To determine whether bacteria can modulate iNKT cell responses in vivo, we injected mice with heat-killed *E. coli*, *S. aureus* or *S. typhimurium*, or with live *L. monocytogenes* and treated these animals at different time points thereafter with α -GalCer to observe iNKT cell expansion in vivo. Consistent with prior results (25, 98), α -GalCer injection (1 μ g/mouse, i.p.) into naïve mice induced dramatic iNKT cell expansion, whereas iNKT cells failed to expand in mice treated 3 weeks earlier with a single dose of α -GalCer (Figure 13A-H). In mice treated with each of the bacteria tested, α -GalCer failed to induce substantial iNKT cell expansion. This inhibition of iNKT cell expansion persisted for at least 3 weeks for heat-killed *S. aureus* (Figure 13E, F) and *S. typhimurium* (Figure 13G, H), and 4 weeks for heat-killed *E. coli* (Figure 13A, B) and live *L. monocytogenes* (Figure 13C, D). Additional data revealed that these iNKT cells were defective in inducing CD86 expression on B cells and DCs, as well as CD69 expression and IFN- γ production by NK cells (Figure 14). These findings indicate that bacteria can induce iNKT cell hyporesponsiveness in vivo.

To investigate whether heat-killing of bacteria influences their capacity to induce iNKT cell hyporesponsiveness, we compared the impact of heat-killed vs. live *E. coli* or *L. monocytogenes* on iNKT cell responses. Results showed that both heat-killed and live

bacteria induced iNKT cell hyporesponsiveness (Figure 15).

Impact of bacteria-induced iNKT cell hyporesponsiveness on ConA-induced hepatitis

To determine whether bacteria can influence iNKT cell-mediated effector functions in a disease setting, we evaluated iNKT cell function in a model of hepatitis induced by Concanavalin A (ConA). ConA-induced hepatitis is a well-characterized mouse model for human autoimmune hepatitis that is dependent on iNKT cell function (170). Consistent with prior studies (170), CD1d-deficient mice, compared with wild-type mice, showed significantly reduced liver damage following ConA injection (Figure 16A). Likewise, mice treated with heat-killed *E. coli* or *S. aureus*, or with live *L. monocytogenes*, as compared with naïve mice, experienced significantly less liver damage, as assessed by serum alanine aminotransferase (ALT) levels (Figure 16B, C). Interestingly, bacteria conferred better protection from ConA-induced liver injury than α -GalCer (Figure 16C).

Impact of E. coli-induced iNKT cell hyporesponsiveness on the therapeutic activities of α -GalCer

The immunomodulatory properties of iNKT cells have been exploited for the development of immunotherapy for cancer and for preventing autoimmunity (36, 171, 172). We therefore tested whether exposure to bacteria can influence the therapeutic activities of α -GalCer, using a model for lung metastasis induced by B16 melanoma cells and the EAE model of multiple sclerosis. Mice were treated with heat-killed *E. coli* or live *L. monocytogenes* and three weeks later these animals were injected with B16 cells for induction of tumor metastases or treated with myelin oligodendrocyte glycoprotein

(MOG)₃₅₋₅₅ peptide in complete Freund's adjuvant (CFA) for induction of EAE. Mice were then treated with a series of vehicle or α -GalCer injections. Results showed that, even in the absence of α -GalCer treatment, tumor burden in *E. coli*-treated animals, but not *L. monocytogenes*-treated animals, was substantially lower when compared with the tumor burden in naïve animals (Figure 17). This may be due to primed innate immune responses in *E. coli*-treated animals. However, α -GalCer was unable to enhance the clearance of B16 tumors from *E. coli*-treated mice, and instead slightly enhanced tumor burden in these animals (although differences were not statistically significant). Likewise α -GalCer was unable to promote tumor clearance in mice treated with *L. monocytogenes*. In contrast, however, iNKT cells in *E. coli*-treated animals retained their capacity to prevent the development of EAE (Figure 18A). A prior report similarly demonstrated the capability of α -GalCer-experienced iNKT cells to suppress EAE (25). This ability of hyporesponsive iNKT cells to provide protection against EAE might be due to increased secretion of IL-10 by DC in *E. coli*- or α -GalCer-treated animals compensating for the hyporesponsive iNKT cells (Figure 18B).

Discussion

Immune responses mediated by peptide-reactive, MHC-restricted T cells are characterized by a period of T cell activation, followed by proliferation and differentiation, elaboration of effector functions, a decline phase in which the pool of antigen-specific T cells contracts, and the development of immunological memory. In sharp contrast, little is known regarding the immune response mediated by glycolipid-reactive, CD1d-restricted iNKT cells. We and others have shown that treatment of mice with the potent iNKT cell agonist α -GalCer results in the rapid activation and proliferation of these cells, followed by homeostatic contraction of the iNKT cell population and acquisition of an anergic phenotype (25, 95, 99). Here, we have demonstrated that iNKT cells activated in response to multiple bacterial microorganisms acquire a similar hyporesponsive phenotype, which can significantly impact subsequent iNKT cell-mediated immune responses and the efficacy of iNKT cell-based immunotherapy.

We tested the impact of bacteria on the phenotype and functional responses of iNKT cells. While each of the bacteria tested, as evidenced by CD69 induction and NK1.1 downregulation (Figure 3 and data not shown), was able to activate iNKT cells, we observed long-term effects on iNKT cell function for *E. coli*, *S. aureus*, *S. typhimurium* and *L. monocytogenes* (Figure 5), but not *S. capsulata* (Figure 5), *E. faecalis* (data not shown) and *S. pyogenes* (data not shown). Whether bacteria were heat-killed or live did not impact the outcome on iNKT cell responses (Figure 15).

Each of the bacterial organisms investigated in this study likely has multiple mechanisms to induce the production of pro-inflammatory cytokines by APC and, thus,

to activate iNKT cells. As such, induction of iNKT cell hyporesponsiveness might be influenced by a variety of factors, including the mechanism and extent of iNKT cell activation. In this regard, we noticed that the capacity of bacteria to induce iNKT cell hyporesponsiveness correlated with sustained NK1.1 downregulation in the spleen (Figure 4A) and with transient iNKT cell depletion in the liver (Figure 4B), both of which are also observed after α -GalCer treatment (25, 98). In the case of *L. monocytogenes*, we also observed transient iNKT cell depletion in the spleen (Figure 10A, B), which likely reflects strong iNKT cell activation. A similar but more sustained depletion of iNKT cells has been observed in mice following an acute infection with lymphocytic choriomeningitis virus (LCMV) (173).

iNKT cell hyporesponsiveness induced by bacteria exhibited a number of similarities with iNKT cell anergy induced by α -GalCer (25). First, as already discussed, iNKT cell hyporesponsiveness correlated with sustained NK1.1 downregulation by splenic iNKT cells (Figure 4A). Second, iNKT cell hyporesponsiveness correlated with a transient decrease in liver iNKT cell numbers (Figure 4, 7A, B, 10A, B). Third, iNKT cell hyporesponsiveness was maintained for at least 4 weeks (Figure 5, 9, 12). These similarities between bacteria- and α -GalCer-induced iNKT cell hyporesponsiveness suggest similar mechanisms.

iNKT cell activation can have a number of detrimental effects in mice, including the induction of liver injury (174), abortions (175), and exacerbation of atherosclerosis (176) and allergic reactions (177). As such, it is likely that bacteria-induced iNKT cell hyporesponsiveness serves to avoid such deleterious outcomes of iNKT cell activation. In addition to inducing iNKT cell hyporesponsiveness, some pathogens, such as *L. monocytogenes* (the present study) and LCMV (173), induce significant iNKT cell

apoptosis, which likely represents an additional mechanism to avoid the deleterious effects of sustained iNKT cell activation. Our finding that treatment of mice with *E. coli*, *S. aureus*, or *L. monocytogenes* suppressed ConA-induced hepatitis (Figure 16) supports this hypothesis.

In humans, it has been well-documented that iNKT cell numbers and functions differ widely among individuals (178, 179). Our finding that many bacteria can induce iNKT cell hyporesponsiveness, together with the observation that certain pathogens can induce short-term (e.g., *L. monocytogenes*; this study) or long-term (e.g., LCMV (173)) depletion of the iNKT cell population, provides a potential explanation for this observation. A role for microbial pathogens in the variability of human iNKT cell numbers and functions is also consistent with the finding that iNKT cell numbers in humans are suppressed during certain chronic infections, including infections with HIV (164) and *Mycobacterium tuberculosis* (179).

iNKT cells are promising targets for immunotherapy of a variety of diseases, including cancer and autoimmunity (171, 172, 180, 181). Our studies revealed that bacteria-induced iNKT cell hyporesponsiveness impacts the efficacy of iNKT cell-based immunotherapies. We demonstrated that heat-killed *E. coli* and live *L. monocytogenes* abrogated the capacity of α -GalCer to protect mice against the development of B16 tumors, but we did not observe any effects of *E. coli* on the capacity of α -GalCer to protect mice against the induction of EAE (Figure 18A). These effects of bacteria on the therapeutic activities of α -GalCer are very similar to those mediated by α -GalCer-induced iNKT cell anergy (25). Although the precise mechanisms remain unclear, IFN- γ and iNKT-cell mediated transactivation of DCs and NK cells likely play important roles in the therapeutic effects of α -GalCer against B16 tumor cells (182), whereas IL-4, IL-10

and IFN- γ all have been implicated in the therapeutic efficacy of α -GalCer against EAE (116, 117, 119). Our finding that α -GalCer-activated, hyporesponsive iNKT cells are defective in transactivating DCs and NK cells (Figure 14) provides a potential explanation for loss of the beneficial effect of iNKT cells against B16 metastases. In the case of EAE, it is possible that the levels of cytokines produced by hyporesponsive iNKT cells are sufficient to promote the tolerogenic activities of these cells and prevent EAE disease. Indeed, we found that DCs from mice treated with α -GalCer three weeks after initial challenge with α -GalCer or heat-killed *E. coli* exhibited a profound increase in IL-10 secretion in response to in vitro stimulation with LPS or CpG (Figure 18B).

In conclusion, multiple bacteria have been shown to induce phenotypic and functional changes in iNKT cells rendering these cells hyporesponsive during the secondary response. These changes impacted the physiological function of iNKT cells in ConA-induced hepatitis model and also affected the therapeutic activities of these cells. These findings argue that infections and vaccination might limit the utility of α -GalCer therapy. In order to apply our findings to the development of novel strategies targeting iNKT-cell based therapies, it is crucial to gain mechanistic understanding of iNKT cell hyporesponsiveness induced by bacteria. In the next chapter, we tried to provide insight into the mechanism by which bacteria induce and maintain the hyporesponsive phenotype of iNKT cells.

Materials and Methods

Mice. Female C57BL/6 (B6) mice were purchased from the Jackson Laboratory. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. α -GalCer (KRN7000) was kindly provided from Kirin Brewery Co., Ltd. (Gunma, Japan) and was reconstituted in PBS containing 0.5% polysorbate-20 (Sigma-Aldrich). CD1d monomers were obtained from the National Institutes of Health. Fluorescently labeled tetrameric CD1d molecules loaded with α -GalCer (CD1d tetramers) were prepared as described previously (183). Anti-TCR- β -fluorescein isothiocyanate (FITC) and -allophycocyanin, anti-NK1.1-phycoerythrin (PE) and -allophycocyanin, anti-B220-peridinin chlorophyll protein (PerCP), anti-CD3-PerCP, anti-CD80-PE, anti-CD86-PE, anti-IL-4-allophycocyanin, anti-IFN- γ -FITC, anti-CD69-FITC, anti-CD11c-allophycocyanin, and streptavidin-PE-cyanide dye 5 were obtained from BD Biosciences-Pharmingen, complete and incomplete Freund's adjuvant from BD Biosciences-Pharmingen, CFSE from Invitrogen Corp., *Salmonella* LPS from Sigma, and CpG from Invivogen.

Treatment of mice with heat-killed or live bacteria. *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *S. pyogenes* (ATCC 19615) were obtained from Dr. Yi-Wei Tang (Vanderbilt Medical Center), *S. typhimurium* (χ 4550) was obtained from Dr. Roy Curtiss (Arizona State University, Tempe, AZ), and *L. monocytogenes* was obtained from Dr. Hao Shen (University of Pennsylvania School of

Medicine, Philadelphia, PA). Each of these organisms was grown on Brain Heart Infusion agar (BD Difco) plates and individual colonies were cultured overnight in Brain Heart Infusion broth, diluted in fresh broth and grown for 8 hr at 37°C to stationary phase, washed and resuspended in PBS. *S. capsulata* (ATCC 14666) was obtained from the ATCC and grown in Mueller-Hinton broth, washed and diluted in PBS buffer. Heat-killed bacteria were prepared by 2 hr exposure to 75°C except for *S. typhimurium*, which was incubated in boiling water for 45 min. Heat-killed bacteria were subsequently stored at -80°C. Heat-killed bacteria ($0.75-1 \times 10^9$ CFU in 200 μ l PBS) were intravenously injected into mice. Live bacteria were administered intravenously in 200 μ l PBS, at a dose of 5×10^4 colony forming unit (CFU) for *L. monocytogenes*, $1-2 \times 10^8$ CFU for *S. capsulata*, or 5×10^5 CFU for *E. coli*. Mice were sacrificed and analyzed at various time points after injection.

Flow cytometry. Single-cell suspensions of the spleen and liver were prepared and stained with fluorescently-labeled mAbs as described previously (79). In all experiments, dead cells were excluded from the analysis by electronic gating. The iNKT cell population was identified as B220⁻TCR- β ⁺tetramer⁺ cells. For intracellular cytokine staining, cells were permeabilized with Cytofix/Cytoperm reagents (BD Biosciences-Pharmingen) according to the manufacturer's protocol. For staining of DCs, Fc receptors were first blocked by addition of anti-CD16/32 antibodies (BD Biosciences-Pharmingen) and DCs were identified on the basis of high CD11c expression. Flow cytometry was performed using a FACSCalibur instrument with CellQuest software (BD Immunocytometry Systems) and the acquired data were analyzed using FlowJo software (Tree Star Inc.).

Measurement of in vivo and in vitro responses to α -GalCer. For evaluation of in vivo iNKT cell responses to α -GalCer, mice were injected i.p. with 1 μ g α -GalCer in 200 μ l PBS containing 0.025% polysorbate-20 (vehicle). At different time points, splenocytes and liver mononuclear cells were stained with fluorescently labeled mAbs and analyzed by flow cytometry. For evaluation of in vitro iNKT cell responses, splenocytes were plated in U-bottomed 96-well plates at 2×10^5 cells per well in RPMI medium containing 10% FCS (R-10) in the presence of titrated doses of α -GalCer or vehicle. For proliferation assays, 1 μ Ci of [3 H]thymidine (MP Biomedicals, Inc.) was added to the wells after 60 hrs of culture, and cells were cultured for an additional 12 hrs. Cells were then harvested, and uptake of radioactivity was measured in β -counter. For measurement of cytokine secretion in vitro, supernatants were harvested after 60 hrs of culture, and cytokine levels were measured by ELISA.

ELISA. A standard sandwich ELISA was performed to measure mouse IFN- γ , IL-4, IL-10, IL-12 and IL-2. IFN- γ - and IL-4-paired antibodies were obtained from R&D Systems Inc., and IL-10, IL-12 and IL-2-paired antibodies were obtained from BD Biosciences-Pharmingen. Cytokine standards were obtained from BD Biosciences-Pharmingen. For detection, streptavidin-HRP conjugate (Zymed Laboratories Inc.) was used, and the color was developed with the substrate 3,3',5,5'-tetramethylbenzidine (Dako Corp.) in the presence of H₂O₂.

CFSE dilution analysis. Total splenocytes or enriched iNKT cells were labeled with 1 μ M CFSE for 15 min at 37°C in PBS containing 5% FCS, and washed twice with R-10 medium. Labeled splenocytes (2×10^5 cells per well) were then stimulated with α -

GalCer (100 ng/ml) with or without addition of IL-2 (10 ng/ml) in the culture media. Cells were washed 3 times with R-10 medium and cultured for an additional 96 hrs in R-10 medium without α -GalCer. At the end of the culture, cells were harvested, stained with PE-labeled CD1d tetramer and anti-B220–PerCP, and analyzed by flow cytometry. Dead cells were excluded from the analysis by electronic gating. CFSE dilution analysis was performed on B220⁻tetramer⁺ iNKT cells.

Assessment of ConA-induced hepatitis. ConA (350 μ g in 200 μ l PBS) was injected intravenously into mice. Mice were sacrificed 24 hrs later and serum was collected and analyzed for alanine aminotransferase (ALT) levels using Prochem-V (Drew Scientific) according to the manufacturer's protocol.

Determination of lung metastases of B16 melanoma. B6 mice were injected i.v. with 3×10^5 syngeneic B16 melanoma cells suspended in PBS. Mice were treated with α -GalCer (5 μ g per injection) or vehicle at 0, 4, and 8 days. Fifteen days after challenge, mice were sacrificed, lungs were removed, and the number of metastatic nodules was counted as described (184).

Induction and evaluation of EAE. Mice were immunized s.c. with 200 μ g of MOG₃₅₋₅₅ peptide (Bio-Synthesis, Inc.) emulsified in CFA (BD Biosciences) on day 0 and in incomplete Freund's adjuvant (IFA) on day 7, as described (116). Mice also received 250 ng of pertussis toxin (Invitrogen Corp.) i.p. on days 0 and 2. Mice were treated with 5 μ g of α -GalCer or vehicle on days 0, 4, and 7 by i.p. injection. Clinical symptoms were monitored daily after the first immunization. The clinical score was graded as follows: 0,

no disease; 1, tail limpness; 2, hind-limb weakness; 3, hind-limb paralysis; 4, forelimb weakness; 5, quadriplegia; and 6, moribund. Mice were sacrificed at grade 6.

Statistical analysis. Statistical significance between two groups was determined by application of an unpaired 2-tailed Mann-Whitney U test. A *P* value less than 0.05 was considered significant. Statistical significance between multiple groups was determined by application of ANOVA followed by Bonferroni post-hoc test for samples determined to approximate normal distribution by Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefors *p* value. When samples were determined not to approximate normal distribution, Kruskal-Wallis followed by Dunns post-hoc test was used instead. A *P* value less than 0.05 was considered significant for the multiple comparison tests as well.

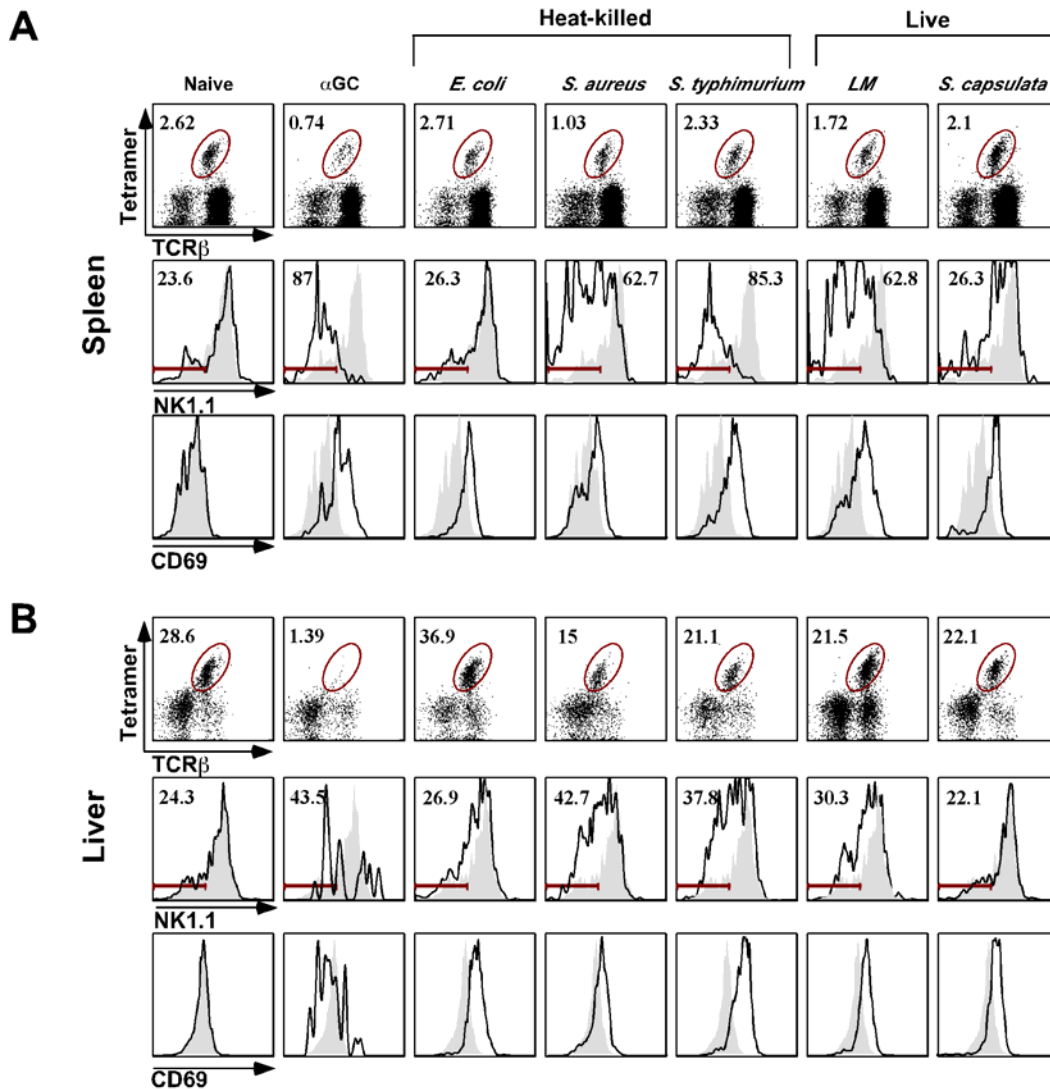


Figure 3. Multiple bacterial microorganisms activate murine iNKT cells. **(A)** and **(B)** The in vivo response of mice to treatment with heat-killed or live bacteria at day 1 in spleen (A) or liver (B). Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or with the indicated heat-killed or live bacteria (i.v.), sacrificed at day 1, and spleen or liver mononuclear cells were prepared and stained with anti-TCR β -FITC, anti-CD69-FITC, anti-NK1.1-PE, anti-B220-PerCP, and CD1d-tetramer-APC and analyzed by flow cytometry. Numbers indicate the percentage of TCR β ⁺tetramer⁺ cells among B220⁻ cells, or the percentage of NK1.1⁻ cells among iNKT cells. The shaded area represents the staining of naïve iNKT cells and the solid line represents the staining of iNKT cells from mice treated with α -GalCer or bacteria. Representative plots from 4-8 mice per group are shown.

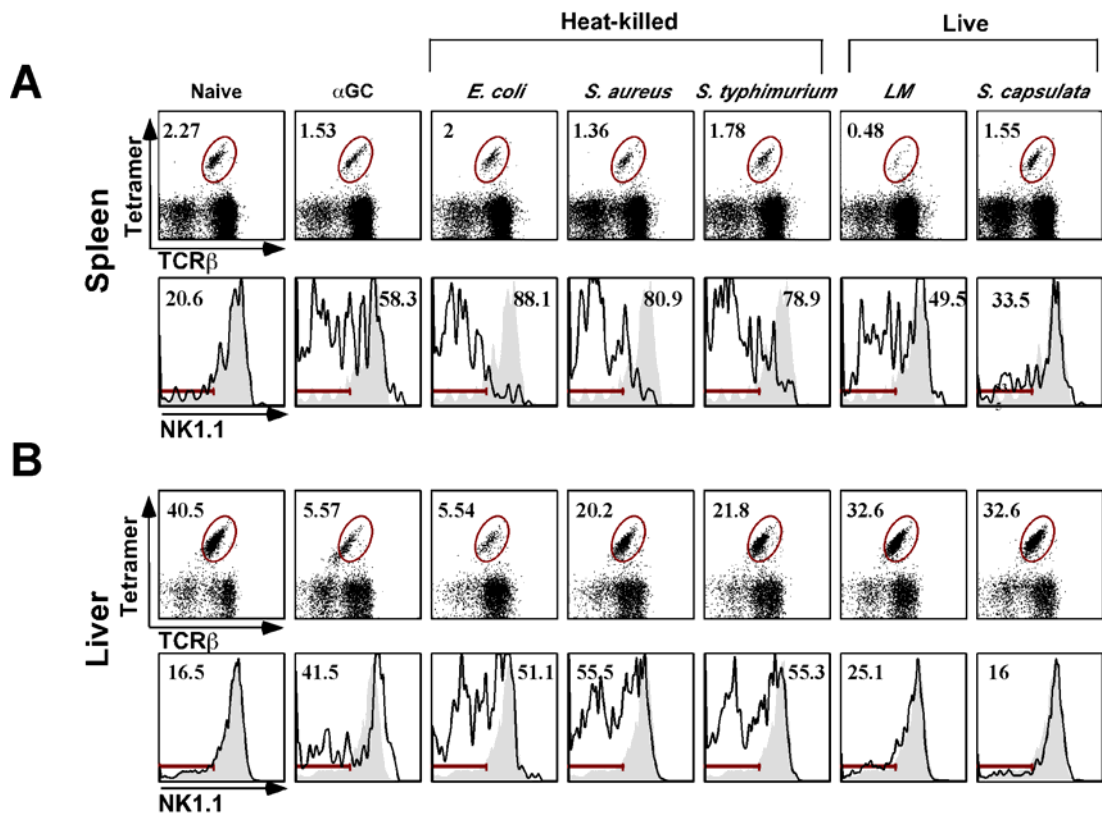


Figure 4. Some bacterial microorganisms induce sustained changes in the prevalence and surface phenotype of iNKT cells. (A) and (B) The in vivo response of mice to treatment with heat-killed or live bacteria at week 3 in spleen (A) or liver (B). Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or with the indicated heat-killed or live bacteria (i.v.), sacrificed at week 3, and spleen or liver mononuclear cells were prepared and stained with anti-TCR β -FITC, anti-CD69-FITC, anti-NK1.1-PE, anti-B220-PerCP, and CD1d-tetramer-APC and analyzed by flow cytometry. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells, or the percentage of NK1.1 $^-$ cells among iNKT cells. The shaded area represents the staining of naïve iNKT cells and the solid line represents the staining of iNKT cells from mice treated with α -GalCer or bacteria. Representative plots from 4-8 mice per group are shown.

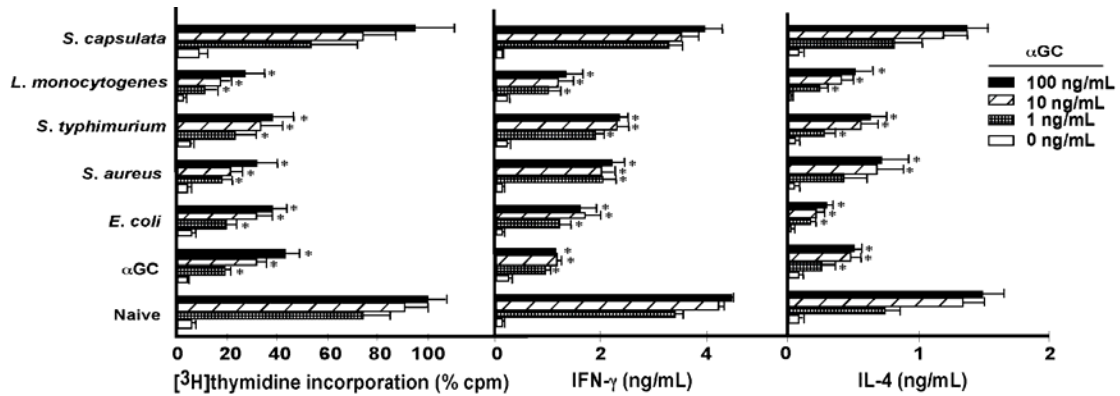


Figure 5. Some bacterial microorganisms induce suppressed response of splenocytes to α -GalCer rechallenge. Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or the indicated bacteria, sacrificed 3 weeks later, and splenocytes (2×10^5 per well) were cultured with graded doses of α -GalCer. After 3 days, proliferation was assessed by [3 H]thymidine incorporation and culture supernatants were evaluated for IL-4 and IFN- γ levels by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 6-11 mice per group, pooled from 3 experiments. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of α -GalCer.

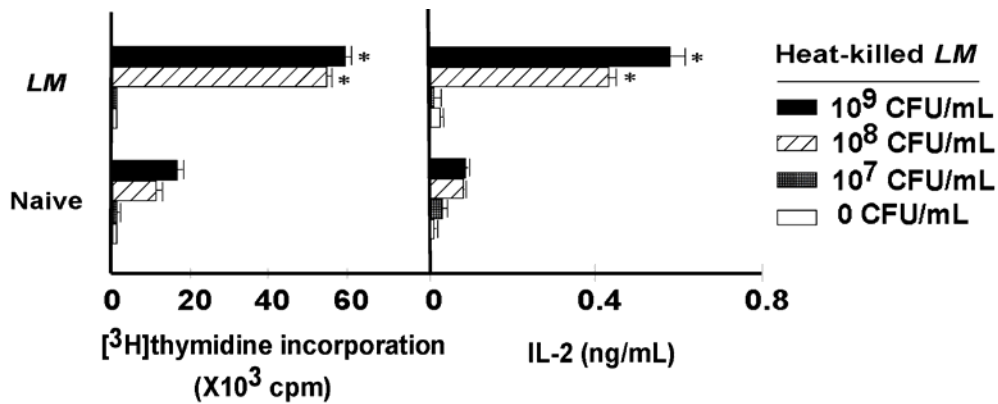


Figure 6. The memory response of conventional T cells following *L. monocytogenes* infection. Mice were infected with 5×10^4 CFU of *L. monocytogenes*, sacrificed 3 weeks later, and splenocytes (2×10^5 per well) were cultured with graded doses of heat-killed *L. monocytogenes*. After 3 days, proliferation was assessed by [³H]thymidine incorporation and culture supernatants were evaluated for IL-2 by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 4 mice. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of heat-killed *L. monocytogenes*.

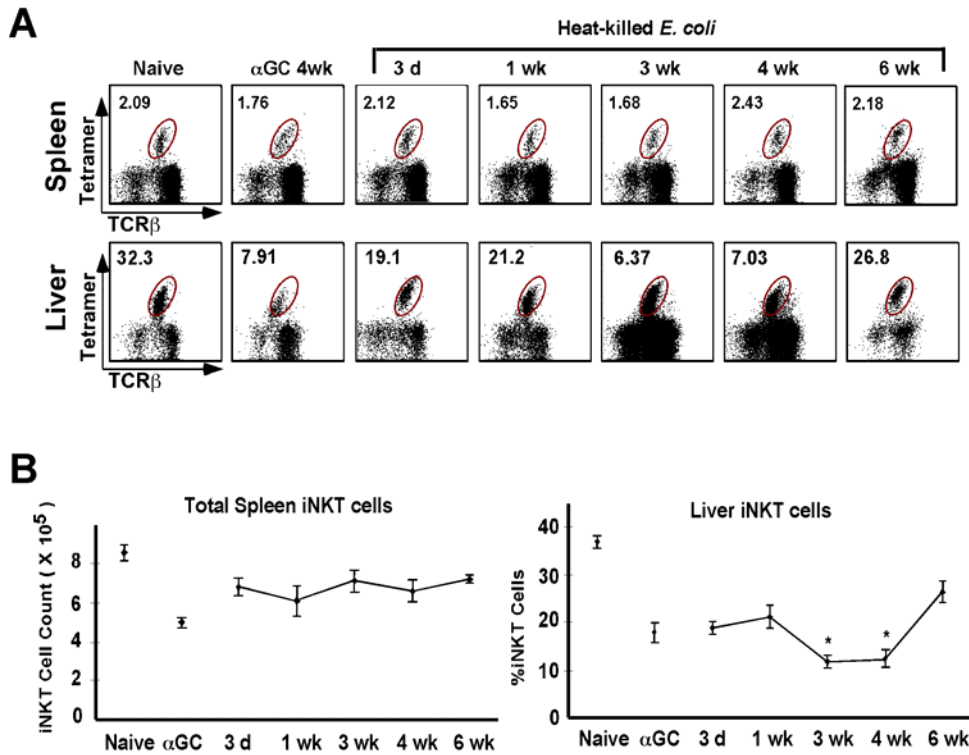


Figure 7. In vivo dynamics of the iNKT cell population in response to heat-killed *E. coli*. (A) Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or heat-killed *E. coli*, sacrificed at the indicated time points, and spleen and liver mononuclear cells were prepared and stained for the identification of iNKT cells with anti-TCR β -FITC, anti-NK1.1 PE, anti-B220-PerCP, and tetramer-APC. The percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells is shown. Representative plots from 5-10 mice per group are shown. (B) Graphical representation of the total spleen iNKT cell counts and the percentage of liver iNKT cells at the indicated time points, for a total of 5-10 mice per group, pooled from 2 separate experiments. *, $p < 0.05$ as compared with naïve animals.

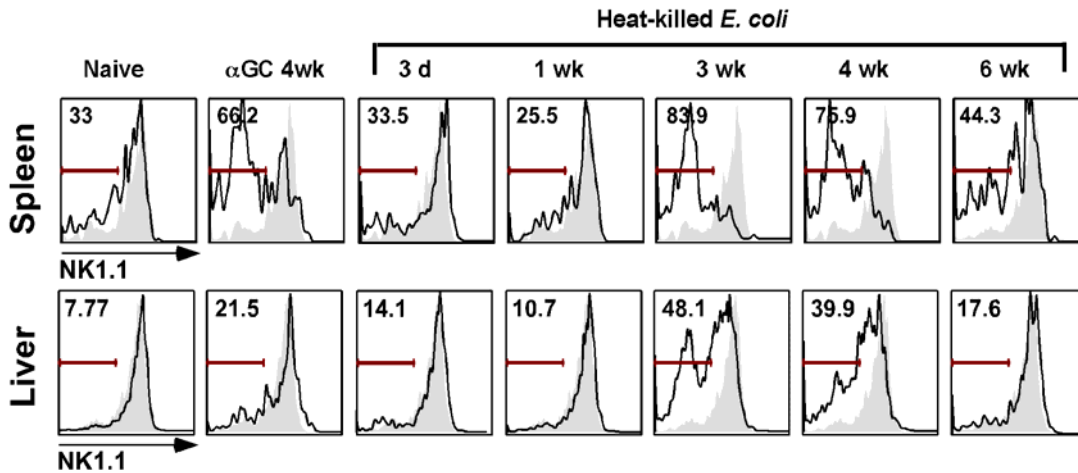
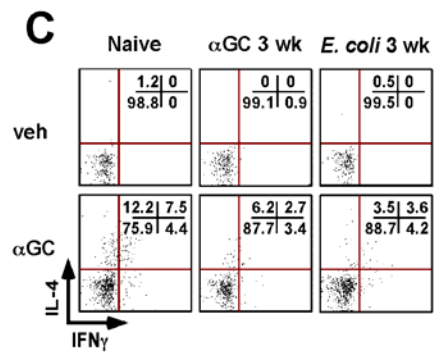
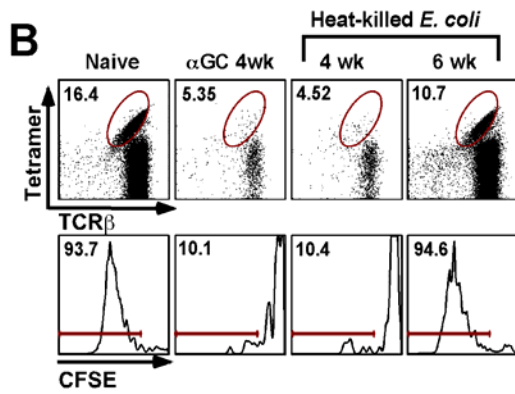
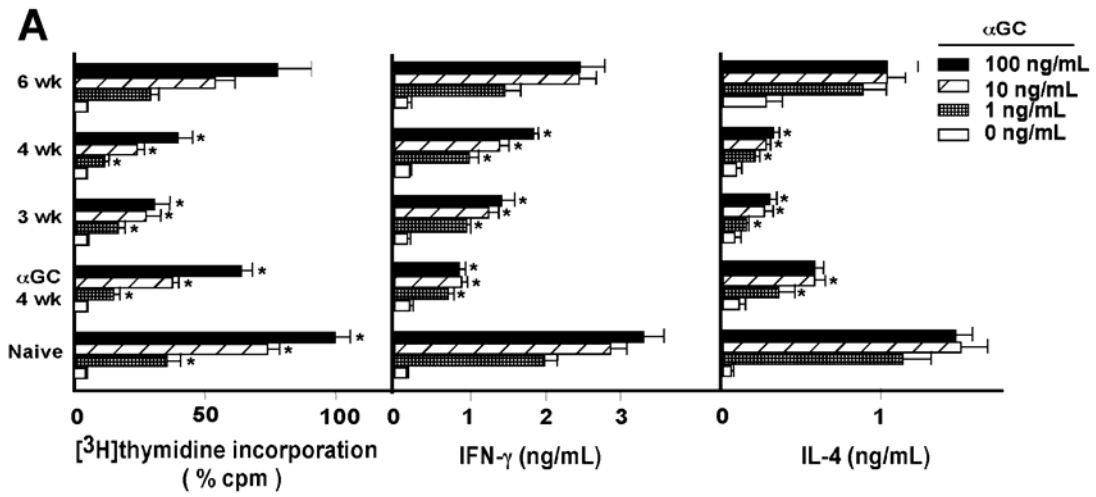


Figure 8. In vivo dynamics of NK1.1 expression by iNKT cells in response to heat-killed *E. coli*. Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or heat-killed *E. coli*, sacrificed at the indicated time points, and spleen and liver mononuclear cells were prepared and stained for the identification of iNKT cells with anti-TCR β -FITC, anti-NK1.1-PE, anti-B220-PerCP, and tetramer-APC. The percentage of NK1.1⁺ cells among iNKT cells is shown. The shaded area represents the NK1.1 staining of naïve iNKT cells, and the solid line represents the staining of iNKT cells from mice treated with α -GalCer or bacteria. Representative plots from 4-10 mice per group are shown.

Figure 9. Heat-killed *E. coli* induces hyporesponsiveness of iNKT cells to α -GalCer rechallenge ex vivo. **(A)** α -GalCer recall response of mice at the indicated time points after treatment with heat-killed *E. coli*. Mice were injected with α -GalCer or heat-killed *E. coli*, sacrificed 3, 4, or 6 weeks later, and splenocytes (2×10^5 per well) were cultured with graded doses of α -GalCer. After 3 days, proliferation was assessed by [3 H]thymidine incorporation and culture supernatants were evaluated for IL-4 and IFN- γ levels by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 6-9 mice, pooled from 2 experiments. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of α -GalCer. **(B)** Proliferative defect in iNKT cells from mice treated with heat-killed *E. coli*. Spleen cells from naïve mice or from mice injected 4 or 6 weeks earlier with α -GalCer or heat-killed *E. coli* were labeled with CFSE. Cells (2×10^5 per well) were then cultured with α -GalCer (100 ng/ml) for 24 hrs, then washed and cultured for an additional 96 hrs without α -GalCer. At the end of the culture period cells were harvested, stained with anti-TCR β -PE, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of the TCR β^+ tetramer $^+$ cells among B220 $^-$ cells. CFSE dilution was analyzed on B220 $^-$ TCR β^+ tetramer $^+$ cells. The data shown are representative of 3 separate experiments with 2 mice per group. **(C)** iNKT cell cytokine production. Spleen cells were prepared at the indicated time point and 2×10^5 cells were cultured for 6 hrs in plain medium (alone) or 100 ng/ml α -GalCer (α GC) in the presence of GolgiPlug. Cells were then harvested and surface-stained with tetramer-PE and anti-B220-PerCP, followed by intracellular staining with anti-IFN- γ -FITC and anti-IL-4-APC. Data are shown for B220 $^-$ tetramer $^+$ cells. Numbers indicate the percentage of cells within each quadrant. Results shown are representative of 4 independent experiments.



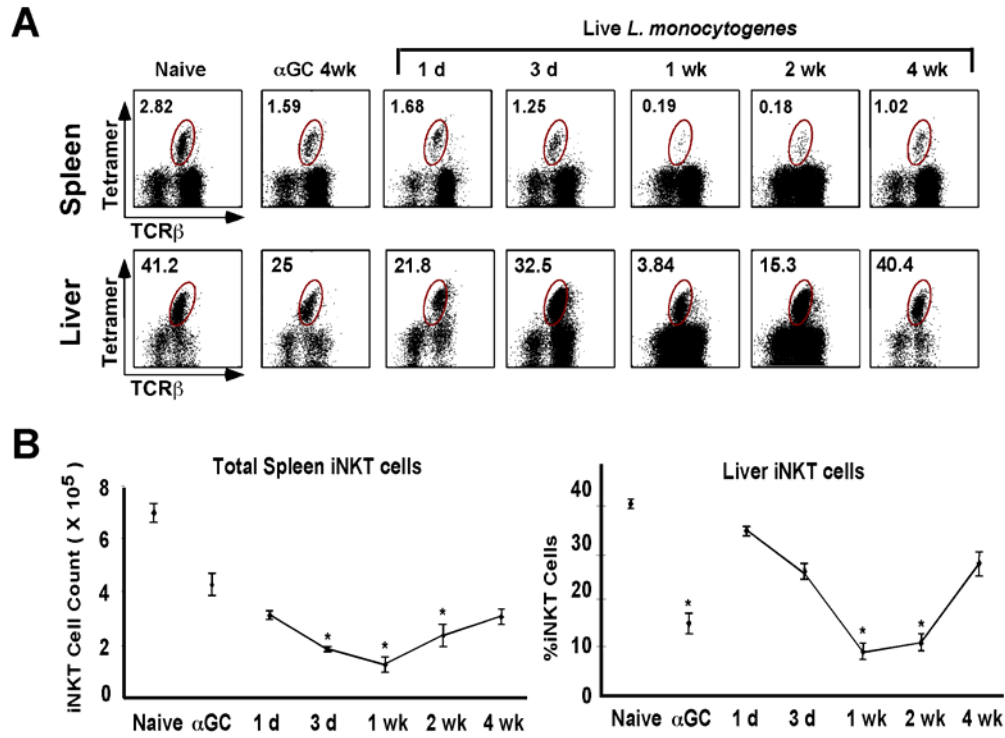


Figure 10. In vivo dynamics of the iNKT cell population in response to *L. monocytogenes* infection. (A) Mice were injected with α -GalCer ($5\mu\text{g}/\text{mouse}$, i.p.) or infected with *L. monocytogenes*, sacrificed at the indicated time points, and spleen and liver mononuclear cells were prepared and stained with anti-TCR β -FITC, anti-NK1.1 PE, anti-B220-PerCP, and tetramer-APC. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells. Representative plots from 4-7 mice per group are shown. (B) Graphical representation of the total spleen iNKT cell counts and the percentage of liver iNKT cells at the indicated time points, for a total of 4-7 mice per group, pooled from 2 separate experiments. *, $p < 0.05$ as compared with naïve animals.

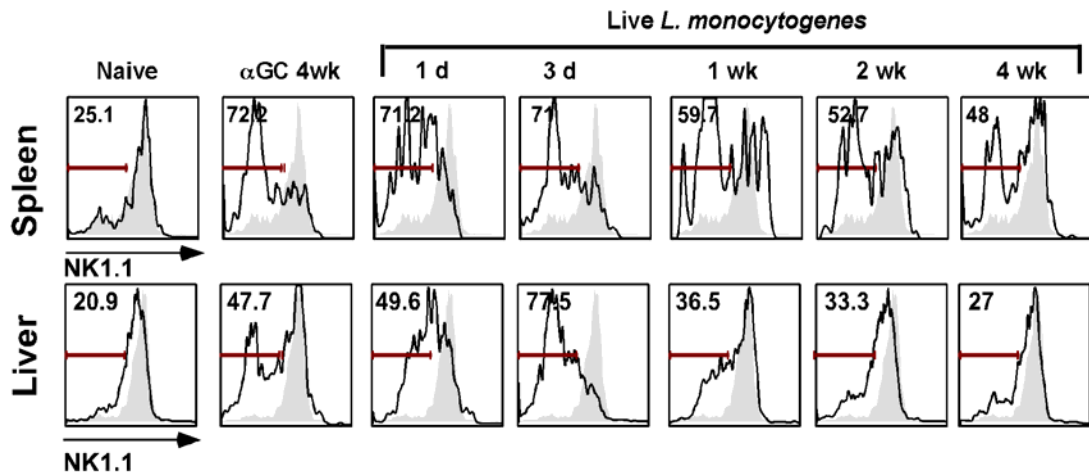


Figure 11. In vivo dynamics of NK1.1 expression by iNKT cells in response to live *L. monocytogenes*. Mice were injected with α -GalCer (5 μ g/mouse, i.p.), live *L. monocytogenes*, sacrificed at the indicated time points, and spleen and liver mononuclear cells were prepared and stained for the identification of iNKT cells with anti-TCR β -FITC, anti-NK1.1-PE, anti-B220-PerCP, and tetramer-APC. The percentage of NK1.1⁺ cells among iNKT cells is shown. The shaded area represents the NK1.1 staining of naïve iNKT cells, and the solid line represents the staining of iNKT cells from mice treated with α -GalCer or bacteria. Representative plots from 4-10 mice per group are shown.

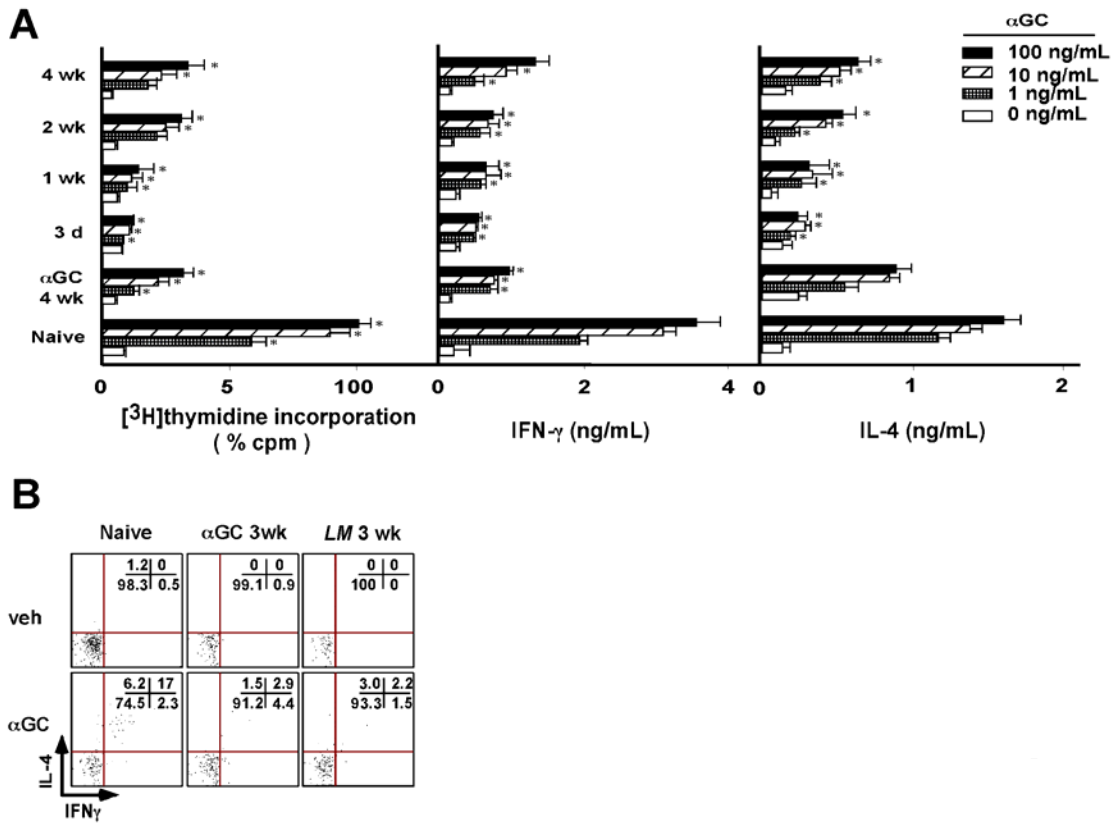


Figure 12. Live *L. monocytogenes* infection induces hyporesponsiveness of iNKT cells to α -GalCer rechallenge ex vivo. **(A)** The in vitro α -GalCer recall response of mice at the indicated time points after infection. Mice were infected with *L. monocytogenes*, sacrificed 3 days or 1, 2, or 4 weeks later, and splenocytes (2×10^5 per well) were cultured with graded doses of α -GalCer. After 3 days, proliferation was assessed by $[^3\text{H}]$ thymidine incorporation and culture supernatants were evaluated for IL-4 and IFN- γ levels by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 4-8 mice pooled from 2 separate experiments. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of α -GalCer. **(B)** iNKT cell cytokine production. Spleen cells were prepared at the indicated time point and 2×10^5 cells were cultured for 6 hrs in plain medium (alone) or 100 ng/ml α -GalCer (α GC) in the presence of GolgiPlug. Cells were then harvested and surface-stained with tetramer-PE and anti-B220-PerCP, followed by intracellular staining with anti-IFN- γ -FITC and anti-IL-4-APC. Data are shown for B220 tetramer $^+$ cells. Numbers indicate the percentage of cells within each quadrant. Results shown are representative of 2 independent experiments.

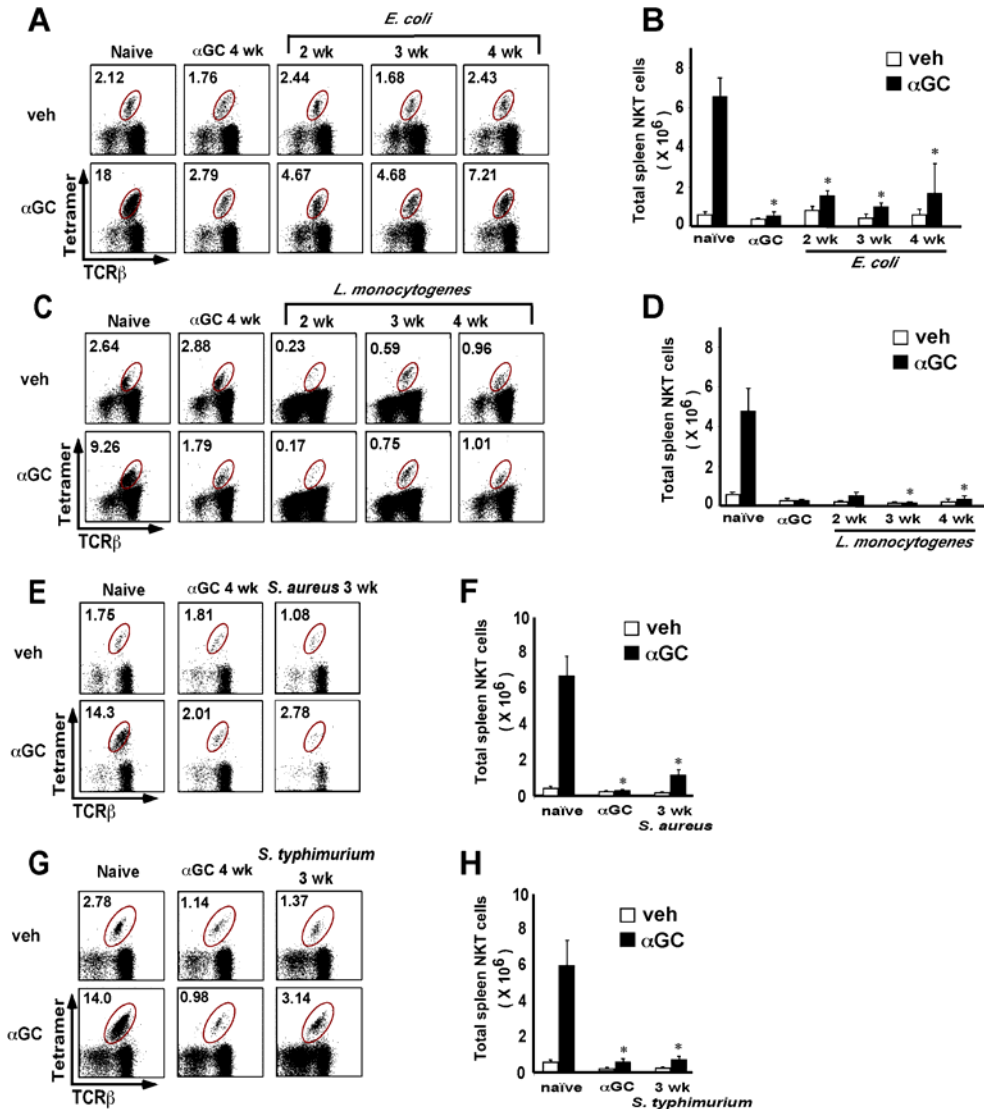


Figure 13. Bacteria can induce iNKT cell hyporesponsiveness to α -GalCer rechallenge in vivo. (A), (C), (E) and (G) At the indicated time points after injection with heat-killed *E. coli* (A), live *L. monocytogenes* (C), heat-killed *S. aureus* (E), or heat-killed *S. typhimurium* (G) mice were rechallenged in vivo with vehicle or α -GalCer (1 μ g/mouse, i.p.). Mice were sacrificed 3 days later and spleen cells were stained with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells for representative data from 5–7 mice per group in at least 2 separate experiments. (B), (D), (F) and (H) Graphical representation of the total spleen iNKT cells calculated from the experiments shown in (A), (C), (E), and (G) respectively. *, $p < 0.05$ as compared with naive mice rechallenged with α -GalCer.

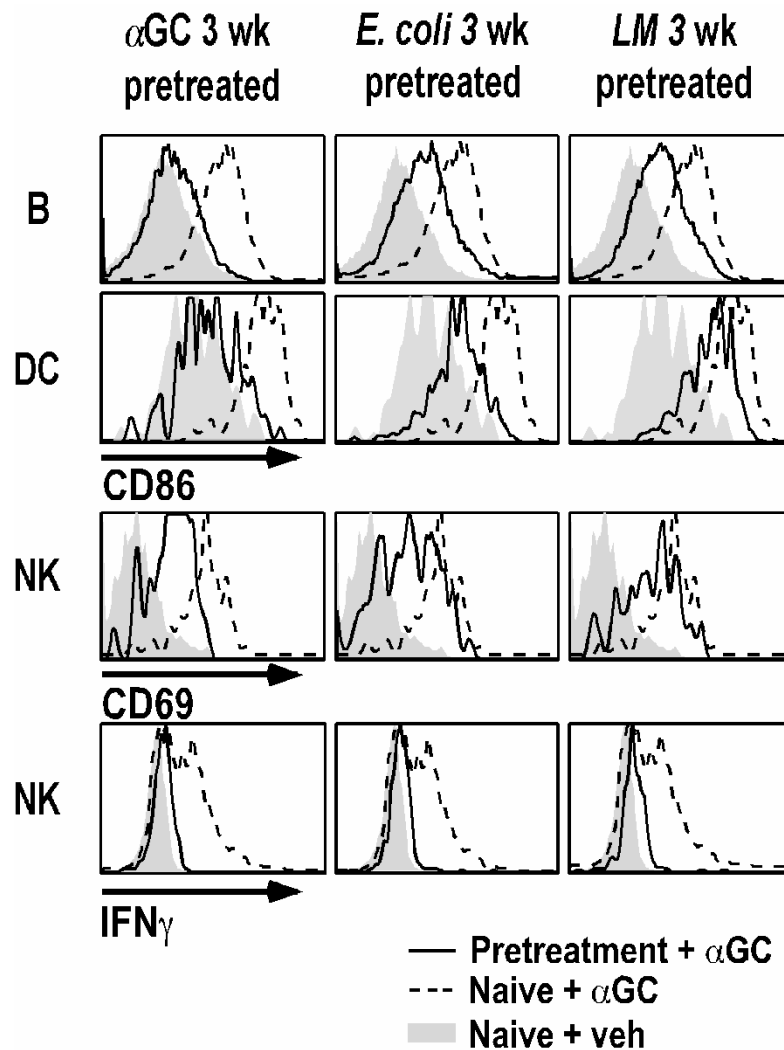


Figure 14. Hyporesponsive iNKT cells are defective in transactivating B cells, DCs and NK cells in vivo. Mice were injected with the indicated bacteria and rechallenged with α -GalCer (1 μ g/mouse, i.p) 3 weeks later. Mice were then sacrificed at the 24-hr time point and spleen mononuclear cells were stained with different combinations of anti-CD86-PE, anti-B220-PerCP, anti-CD11c-APC, anti-CD69 FITC, anti-NK1.1-APC, and anti-TCR β -PE. For IFN- γ staining on NK cells, mice were sacrificed 6 hrs following α -GalCer rechallenge and spleen mononuclear cells were cultured 2 hrs in the presence of GolgiPlug. Cells were then stained with anti-IFN- γ -FITC, anti-NK1.1-APC, and anti-TCR β -PE. Data shown are representative of 6 mice per group from 2 separate experiments.

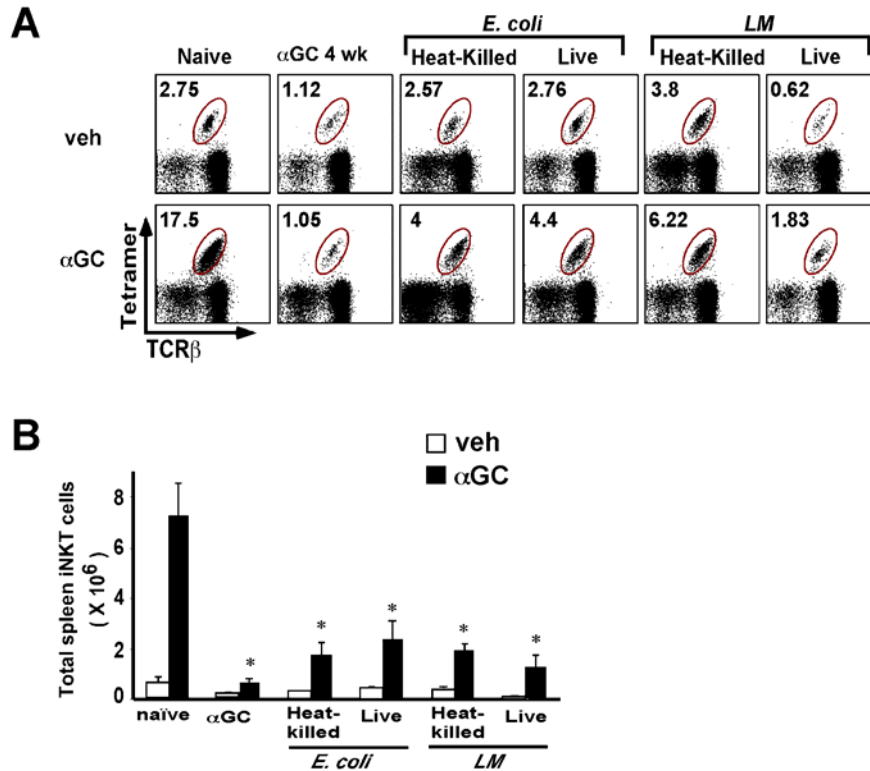


Figure 15. Both heat-killed and live bacteria induce iNKT cell hyporesponsiveness. **(A)** Mice were injected with heat-killed or live *E. coli* or *L. monocytogenes* and, 3 weeks later, rechallenged in vivo with vehicle or α -GalCer (1 μ g/mouse, i.p.). Mice were sacrificed 3 days later and spleen cells were stained with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells from representative plots of 5-6 mice per group from 2 experiments. **(B)** Graphical representation of the total spleen iNKT cells calculated from the experiments shown in (A). *, $p < 0.05$ as compared with naïve mice rechallenged with α -GalCer.

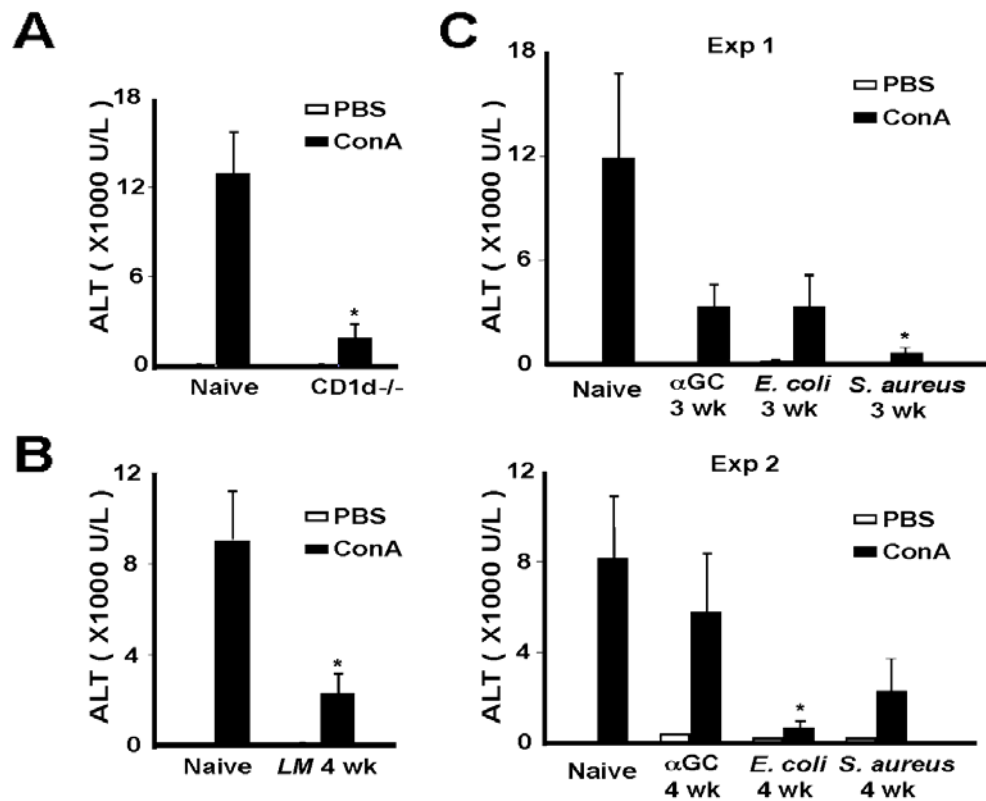


Figure 16. Impact of bacteria-induced iNKT cell hyporesponsiveness on ConA-induced hepatitis. Wild-type or CD1d-deficient mice were injected with α -GalCer (A), live *L. monocytogenes* (B), or heat-killed *E. coli* or *S. aureus* (C), and, 3-4 weeks later, mice were challenged with PBS or ConA (350 μ g/mouse in PBS). Mice were bled 24 hrs later and serum ALT levels were measured. Results represent the mean \pm SEM of 8 mice per group in ConA-treated groups or 2 mice per group in PBS-treated groups. *, $p < 0.05$ as compared with naïve mice treated with ConA.

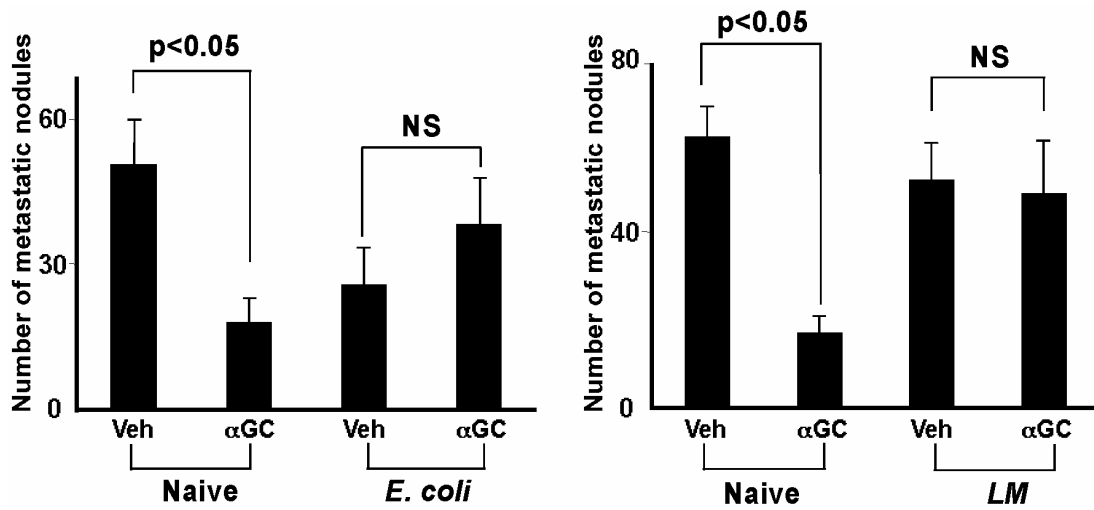


Figure 17. Impact of *E. coli*-induced iNKT cell hyporesponsiveness on the anti-tumor activities of α -GalCer against B16 tumor lung metastasis formation. B6 mice were left untreated or injected with bacteria and, 4 weeks later, mice were challenged i.v. with 3×10^5 syngeneic B16 melanoma cells and treated with α -GalCer (5 μ g/injection) or vehicle at 0, 4, and 8 days after tumor challenge. Mice were sacrificed after 15 days and the number of metastatic nodules in the lungs counted. Results shown are the average of 2 experiments with 4 mice in each group per experiment. *, $p < 0.05$; NS, not significant.

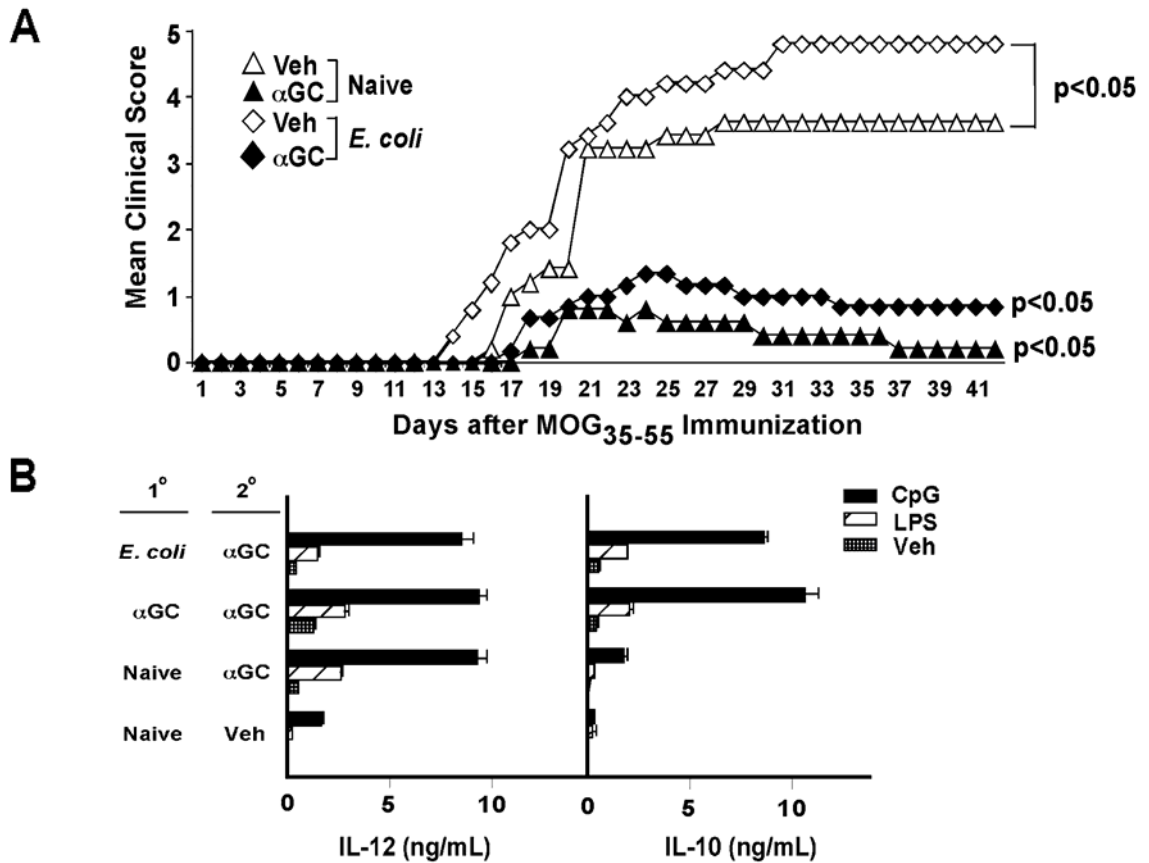


Figure 18. Impact of *E. coli*-induced iNKT cell hyporesponsiveness on the therapeutic activities of α -GalCer against EAE. (A) B6 mice were treated with heat-killed *E. coli* and, 3 weeks later, mice were immunized with MOG₃₅₋₅₅ peptide for induction of EAE, treated with α -GalCer (5 μ g/injection) or vehicle on days 0, 4, and 7, and followed for clinical signs of EAE. Results shown are one representative experiment of 2 with 5-6 mice in each group. (B) Development of tolerogenic DCs following α -GalCer or heat-killed *E. coli* treatment. Mice were injected with α -GalCer (5 μ g/mouse, i.p.) or heat-killed *E. coli* (indicated as 1°) and, 3 weeks later, rechallenged with vehicle or α -GalCer (5 μ g/mouse, i.p.) (indicated as 2°). Mice were sacrificed 24 hrs following α -GalCer rechallenge and DCs were MACS-purified from spleens. Purified DCs were then cultured for 48 hours in the presence of vehicle, 10 μ g/ml *Salmonella* LPS or 1 μ M CpG ODN. Supernatants were analyzed for IL-12 and IL-10 by sandwich ELISA. Data shown represents mean \pm SD from 3 wells per group.

CHAPTER III

THE MECHANISM OF INKT CELL HYPORESPONSIVENESS INDUCED BY BACTERIA

Abstract

Invariant natural killer T (iNKT) cells are innate-like lymphocytes that recognize glycolipid antigens in the context of the MHC class I-like antigen-presenting molecule CD1d. Unlike conventional T cells, iNKT cells can be activated directly through recognition of a cognate antigen such as α -GalCer as well as indirectly through cytokine and/or endogenous lipid antigen presentation by DC during bacterial infection. We have previously shown that multiple bacterial organisms can induce iNKT cell hyporesponsiveness, and therefore we have investigated the mechanism by which bacteria induce this hyporesponsive phenotype in iNKT cells. We have found that murine iNKT cells activated in vivo by bacterial LPS or flagellin, became unresponsive to subsequent activation with α -GalCer suggesting TLR ligands as causative agents of bacteria-induced hyporesponsiveness. Furthermore, while a distinct mechanism of activation resulted in a requirement for IL-12 in bacteria- but not in α -GalCer-induced energy, both share several common anergic phenotypes, implying similar underlying mechanisms of anergy. These findings provide insights into understanding iNKT cell function and may result in novel strategies for therapeutic application of iNKT cells.

Introduction

T cell anergy is defined as an extended period of functional inactivation and hyporesponsiveness of T cells following an antigenic stimulation. Functional inactivation may refer to combinations of suppressed cell division, differentiation/maturation, and/or cytokine production. Most importantly, this hyporesponsive state should be cell autonomous and distinct from bystander tolerance mediated by other immunoregulatory cells. Also, the period of the hyporesponsive state should last at least 24 hours and is distinguished from an apoptotic process characterized by caspase activation (185, 186).

T cell anergy falls into two main categories, clonal anergy and adaptive tolerance (137). Clonal anergy arises from incomplete T cell activation of previously activated T cells and usually is not associated with loss of effector functions. Adaptive tolerance, also termed *in vivo* anergy, often ensues from *in vivo* activation of naïve T cells in the absence of adequate costimulation or in the presence of strong coinhibition, for instance mediated by cytotoxic T lymphocyte antigen-4 (CTLA-4) (187).

Several features distinguish these two distinct forms of T cell anergy. While a block in IL-2 production and therefore proliferation is observed in both forms of anergy, only adaptive tolerance typically results in blockade of all cytokines with the exception of IL-10. Clonal anergy often retains effector functions. Also, unlike clonal anergy, adaptive tolerance also requires persistent presence of antigenic stimulation in order to maintain the anergic phenotype. In addition, in most adaptive tolerance models, the proliferative block cannot be reversed by exogenous IL-2 due to defective IL-2 receptor signaling which seems to involve CTLA-4 signaling (188).

Previous reports on α -GalCer-induced iNKT cell anergy provided important

perspectives for understanding the mechanism of iNKT cell hyporesponsiveness induced by bacteria with a number of shared characteristics observed in conventional T cell anergy (98, 189). The hyporesponsive phenotype exhibited by iNKT cells following initial α -GalCer injection was cell autonomous, and was not indirectly dependent on the activity of regulatory T cells, tolerogenic dendritic cells, or other cell types. Also, α -GalCer was able to induce anergy in thymectomized mice indicating peripheral rather than central tolerance mechanisms. With regard to roles for costimulation during anergy induction, α -GalCer pulsed B cells with low levels of costimulatory molecules but not α -GalCer pulsed DCs induced iNKT cell anergy. Additionally, α -GalCer was shown to induce iNKT cell anergy in IL-4, IL-10, or IFN- γ deficient mice excluding involvement of these cytokines during induction of iNKT cell anergy. Also, the surface phenotype of iNKT cells during anergy induced by α -GalCer correlated with sustained downregulation of NK1.1, but no change in Ly49 receptor expression was observed. α -GalCer-induced anergy was also rescued by phorbol myristate acetate and ionomycin, and the proliferative defect of anergic iNKT cells was corrected by the administration of exogenous IL-2.

Activation of iNKT cells by bacteria is however distinct from α -GalCer. As a potent specific antigen for semi-invariant TCR of iNKT cells, α -GalCer presented by CD1d on APCs activates iNKT cells by strong prolonged TCR engagement. In sharp contrast, despite extremely limited substrate specificity of their semi-invariant TCR, iNKT cells have been shown to be involved in the clearance of diverse species of microbes implying activation of iNKT cells in the absence of iNKT cell antigen. This is explained by two modes iNKT cell activation, the direct mechanism and the indirect mechanism. The direct mechanism of iNKT cell activation relies on the presence of

microbial glycolipid antigen that is presented by CD1d molecules on APC and directly engages the semi-invariant TCR. Glycosphingolipids in *Sphingomonas capsulata* (55, 130, 134) and galactosyl diacylglycerol antigens from *Borrelia burgdorferi* (59) are good examples of microbial glycolipid antigens strongly activating most iNKT cells. The indirect mechanism of activation does not rely on specific recognition of microbial glycolipid antigens. This non-specific activation is mediated by activated DCs in response to microbial products, most notably TLR ligands. For instance, TLR signaling in response to LPS from *S. typhimurium* results in IL-12 secretion by DCs, which in conjunction with endogenous ligands activates (55, 63). In the case of *E. coli* LPS, release of proinflammatory cytokines such as IL-12 and IL-18 by DCs without presentation of autoreactive antigen is sufficient for iNKT cell activation (136).

In this chapter, we have dissected the mechanism of bacteria-induced iNKT cell hyporesponsiveness, and we show that bacteria-induced and α -GalCer-induced iNKT cell anergy share a number of features, yet exhibit a number of striking differences as well. The findings from this research will provide novel insights into our understanding of iNKT cell biology and for developing improved iNKT cell-based therapies.

Results

Role for TLR ligands in bacteria-induced iNKT cell hyporesponsiveness

Bacteria that lack cognate iNKT cell antigens can activate iNKT cells in a manner that depends on the activation of DCs by TLR ligands (63, 126, 190). We therefore investigated whether TLR ligands can induce iNKT cell hyporesponsiveness. Our results showed that LPS and flagellin from *E. coli* and *Salmonella* were able to induce hyporesponsiveness in iNKT cells (Figure 19A-C). Interestingly, while a single dose of flagellin was sufficient to induce hyporesponsiveness, a repeated injection of LPS was required implying flagellin as a more potent inducer of hyporesponsiveness. Ex vivo assay of splenocytes prepared from mice previously treated with LPS or flagellin also showed relatively less iNKT cell impairment by LPS than flagellin (Figure 19C). Furthermore, DH5 α , a flagellin deficient strain of *E. coli*, while inducing relative hyporesponsiveness with respect to naïve animals, was much less efficient in the induction of iNKT cell hyporesponsiveness (Figure 20).

Role for IL-12 in bacteria-induced iNKT cell hyporesponsiveness

Because IL-12 has been implicated as a critical cytokine in the capacity of bacteria and bacterial products to activate iNKT cells (63, 126, 190), and because both LPS and flagellin induce IL-12 production by APCs (191), we investigated the role of this cytokine in bacteria-induced iNKT cell hyporesponsiveness using IL-12-deficient mice. Consistent with previous studies indicating an important role for IL-12 in the reciprocal interactions of iNKT cells and DCs (80), splenocytes from IL-12-deficient mice showed a suppressed response to α -GalCer as compared with wild-type

splenocytes, regardless of prior in vivo treatment. Results (Figure 21A, B) showed that the capacity of heat-killed *E. coli* to induce iNKT cell hyporesponsiveness required IL-12 expression, whereas induction of iNKT cell anergy mediated by α -GalCer was independent of IL-12 expression. Also, NK1.1 downregulation by iNKT cells consistently observed following bacteria treatment was absent in IL-12-deficient mice (Figure 22). Furthermore, we found an important role of IL-12 for iNKT cell hyporesponsiveness induced by LPS and flagellin (data not shown). These findings indicate a critical role of TLR ligands and IL-12 in the capacity of bacteria to induce iNKT cell hyporesponsiveness.

Costimulatory molecules are not required for bacteria-induced iNKT cell hyporesponsiveness

For bacteria-induced iNKT cell hyporesponsiveness, a strong stimulation of iNKT cells during the early response to bacteria might be required to impart the subsequent hyporesponsive phenotype. As costimulation amplifies the T cell response to TCR engagement in general, we investigated the role for CD86 in bacteria-induced iNKT cell hyporesponsiveness. Results showed that *E. coli* treatment in CD86 deficient mice induced long-term iNKT cell hyporesponsiveness in vivo (Figure 23) indicating costimulation is dispensable for bacteria to induce iNKT cell hyporesponsiveness.

Bacteria-induced iNKT cell hyporesponsiveness is thymus-independent

iNKT cell hyporesponsiveness induced by bacteria might involve central or peripheral tolerance mechanisms. We therefore tested whether iNKT cell hyporesponsiveness required an intact thymus. No differences were observed in the iNKT

cell response of euthymic vs. thymectomized animals pretreated with heat-killed *E. coli*, heat-killed *S. aureus*, or live *L. monocytogenes* (Figure 24). In addition, we observed a similar pattern of NK1.1 downregulation in bacteria-treated euthymic and athymic mice (Figure 25). Moreover, Ly49 upregulation observed in iNKT cells generated de novo during central tolerance was not observed following bacteria treatment (Figure 26). We therefore concluded that central tolerance mechanisms do not play a significant role in the induction of iNKT cell hyporesponsiveness by bacteria.

Bacteria-induced iNKT cell hyporesponsiveness is predominantly cell autonomous

iNKT cell tolerance induced by bacteria might be intrinsic to these cells or mediated by extrinsic factors such as tolerogenic APC. We tested this issue for mice treated with heat-killed *E. coli*. We cultured splenic DCs, purified from naïve or *E. coli*-treated mice, with liver iNKT cells purified from naïve or *E. coli*-treated animals, in the presence of α -GalCer. Results showed that iNKT cells derived from naïve mice proliferated and secreted cytokines at normal or slightly reduced levels in the presence of DCs derived from all mice (Figure 27A). In sharp contrast, liver iNKT cells from mice treated with heat-killed *E. coli* showed dampened proliferation and cytokine secretion in response to DCs from both naïve and *E. coli*-treated animals. To confirm these findings, we labeled splenic iNKT cells enriched from naïve or bacteria-treated animals with CFSE, cultured these cells in vitro with α -GalCer-loaded, splenic DCs enriched from naïve or bacteria-treated animals, and analyzed CFSE dilution among iNKT cells (Figure 27B). Results showed that iNKT cells enriched from naïve animals exhibited significant CFSE dilution, regardless of the source of DCs used for stimulation. Further, naïve DCs were unable to rescue hypoproliferation of iNKT cells purified from α -GalCer- or *E. coli*-

treated mice. These findings suggested that DC alterations have only a minor impact on the development of iNKT cell unresponsiveness.

To confirm these findings, we enriched DCs from naïve mice, loaded these cells *ex vivo* with α -GalCer, and injected the cells into naïve or bacteria-treated animals for evaluation of iNKT cell expansion in the spleen. Results showed that naïve DCs loaded with α -GalCer were unable to rescue the hyporesponsive phenotype of splenic iNKT cells from bacteria-treated animals (Figure 28). These findings indicate that iNKT cell hyporesponsiveness induced by heat-killed *E. coli* and live *L. monocytogenes* is not due to alterations in DC function and is most likely intrinsic to these cells.

PMA plus ionomycin, or α -GalCer and IL-2, can overcome bacteria-induced iNKT cell hyporesponsiveness

Next, we tested whether a combination of PMA and ionomycin, which bypasses proximal TCR signaling events, can overcome the hyporesponsive phenotype of iNKT cells induced by bacteria. Results showed that this treatment was able to overcome iNKT cell hyporesponsiveness induced by heat-killed *E. coli* and live *L. monocytogenes* (Figure 29). However, in most experiments, rescue of iNKT cell function in bacteria-treated animals was not as complete as in α -GalCer-treated animals.

We also investigated the role of IL-2 in iNKT cell hyporesponsiveness. Correlated with decreased proliferation by splenocytes from mice previously treated with α -GalCer, *E. coli*, *S. aureus* or *L. monocytogenes*, IL-2 secretion was observed to be decreased (Figure 30A). Next we tested whether exogenous administration of IL-2, which can overcome the proliferative defect of iNKT cells rendered anergic in response to α -GalCer treatment (25, 98), can rescue iNKT cell proliferation in mice treated three weeks earlier

with heat-killed *E. coli*. We found that IL-2 was able to restore proliferative function of hyporesponsive iNKT cells (Figure 30B), suggesting similarities between α -GalCer and bacteria-induced iNKT cell hyporesponsiveness.

Activating receptors are downregulated on hyporesponsive iNKT cells

Downregulation of NK1.1 has been shown to correlate with hyporesponsive phenotype of iNKT cells. We tested whether other activating receptors were also downregulated on hyporesponsive iNKT cells. We found that, in addition to NK1.1, NKG2D, an important activating receptor in NK cells and certain T cell subsets, was significantly reduced on α -GalCer- or bacteria-induced hyporesponsive iNKT cells (Figure 31). Interestingly, CD94, which is a component of both the activating receptor NKG2C/CD94 and the inhibitory receptor NKG2A/CD94, was also downregulated (Figure 31). These findings suggest activating receptor downregulation may contribute to the hyporesponsive phenotype of iNKT cells induced by bacteria.

Nitric oxide is not required for bacteria-induced iNKT cell hyporesponsiveness

A previous report has suggested a role for nitric oxide produced by CD11b⁺Gr-1⁺ myeloid cells in cancer-bearing animals in mediating iNKT cell hyporesponsiveness (192). We investigated whether similar mechanism might be active during bacterial infection to modulate iNKT cell function. Results showed that treatment with an iNOS inhibitor that interferes with nitric oxide production did not alter relative hyporesponsiveness of α -GalCer- or bacteria-pretreated animals compared to naïve animals (Figure 32). However, iNOS inhibitor treatment resulted in profound suppression of iNKT cell response in naïve animals suggesting that nitric oxide might be important

for the generation of effective iNKT cell responses.

Programmed Death-1 (PD-1) is upregulated on hyporesponsive iNKT cells induced by α -GalCer or bacteria

PD-1 has been implicated in T cell exhaustion or anergy during chronic infection (193-197). Prior studies have shown that PD-1 plays an important role in the induction of iNKT cell anergy by α -GalCer (Parekh et al, unpublished data). We investigated whether this molecule might also play a role in bacteria-induced iNKT cell hyporesponsiveness. Results have shown early upregulation of PD-1 on iNKT cells following bacteria treatment, although the levels were relatively lower than those seen after α -GalCer treatment (Figure 33A). Interestingly, this upregulation of PD-1 following *E. coli* treatment was absent in IL-12-deficient animals (Figure 33B), in which iNKT cell hyporesponsiveness is not induced by *E. coli*. These findings suggest a potential role for PD-1 in the induction of iNKT cell hyporesponsiveness by bacteria.

Discussion

In Chapter II, we tested the impact of bacteria on the phenotype and functional responses of iNKT cells and found that *E. coli*, *S. aureus*, *S. typhimurium* and *L. monocytogenes*, but not *S. capsulata*, *E. faecalis* and *S. pyogenes*, induced a hyporesponsive phenotype in iNKT cells characterized by downregulation of NK1.1 and functional unresponsiveness to subsequent challenge with α -GalCer which was used as surrogate antigen studying recall response. These divergent effects of distinct bacteria on iNKT cell function may be due, in part, to differences in the mechanisms by which iNKT cells become activated.

Although the precise mechanisms by which iNKT cells become activated in response to bacteria remain incompletely understood, two general mechanisms have been proposed (126, 190). Some bacteria, such as *Sphingomonas* and *Borrelia* species, contain glycolipids in their cell walls that can bind CD1d and directly activate iNKT cells. In this context, it was surprising that live *S. capsulata*, despite its capacity to activate iNKT cells, failed to induce iNKT cell hyporesponsiveness, even when used at a sublethal dose. Bacteria that lack cognate iNKT cell antigens can activate iNKT cells by stimulating the production of pro-inflammatory cytokines by activating TLRs on APC (63, 126). In this regard, we found that the TLR4 agonist LPS and the TLR5 agonist flagellin (Figure 19A-C, 20), but not the TLR9 agonist CpG, the TLR7 agonist imiquimod, the TLR2 agonist lipoteichoic acid, or the TLR3 agonist polyinosinic acid-polycytidylic acid (data not shown), induced iNKT cell hyporesponsiveness. Prior studies have indicated a key role of IL-12 for activation of iNKT cells by bacteria and bacterial LPS (55, 63, 126, 136). We confirmed that IL-12 plays an important role in activating iNKT cells in response to heat-

killed *E. coli* (data not shown), and studies with IL-12-deficient mice revealed a critical role of this cytokine for inducing iNKT cell hyporesponsiveness to heat-killed *E. coli* (Figure 21A, B, 33B).

These findings suggest that the mechanism by which certain bacteria are able to induce hyporesponsiveness of iNKT cells but not others likely depend on the specific combination of pathogen-associated molecular patterns (PAMPs) or TLR ligands present in distinct bacteria. Indeed, we have identified likely causative molecules responsible for iNKT cell hyporesponsiveness in *E. coli* and *S. typhimurium*, namely LPS and flagellin, while ruling out molecules that do not induce iNKT cell hyporesponsiveness. Moreover, consistent with whole bacteria-induced iNKT cell hyporesponsiveness, we found that LPS and flagellin also depend on IL-12 to induce iNKT cell hyporesponsiveness, reinforcing the idea that specific PAMPs in bacteria are responsible for iNKT cell hyporesponsiveness. Therefore, a differential array of PAMPs in distinct bacteria may result in different effects of these bacteria on iNKT cells.

iNKT cell hyporesponsiveness induced by bacteria shares a number of key mechanistic similarities with α -GalCer-induced anergy (78). First, although NK1.1 has been shown to be dispensable in the induction and maintenance of anergy, iNKT cell hyporesponsiveness is correlated with sustained NK1.1 downregulation by splenic iNKT cells following bacterial injection as well as α -GalCer injection. Indeed, NK1.1 downregulation was correlated with a hyporesponsive phenotype in experiments performed with thymectomized mice as well as IL-12-deficient mice. We also observed a similar downregulation of the activating NK cell receptor NKG2D and the CD94 subunit of the NKG2/CD94 family of NK cell receptors (Figure 31). These alterations in NK cell receptor expression might contribute to the hyporesponsive phenotype of iNKT cells.

Second, iNKT cell hyporesponsiveness was induced independently of a functional thymus (Figure 24), with absence of characteristic Ly49 expressing iNKT cells observed during central tolerance (Figure 26), implying peripheral rather than central tolerance mechanisms. Third, hyporesponsiveness was predominantly iNKT cell autonomous (Figure 27, 28). Fourth, iNKT cell hyporesponsiveness could be overcome by treatment of these cells with reagents (i.e., phorbol 12-myristate 13-acetate (PMA) + ionomycin) that can bypass early TCR signaling events (Figure 29). Fifth, hyporesponsive iNKT cells produced and secreted reduced levels of IL-2, and the proliferative capacity of these cells could be rescued by treatment with α -GalCer in the presence of IL-2 (Figure 30). Sixth, iNKT cell hyporesponsiveness was not rescued by iNOS inhibitor that prevents production of nitric oxide (Figure 32). Finally, iNKT cells upregulated PD-1 expression, a molecule that is responsible for certain forms of conventional T cell exhaustion or anergy, during early phases of iNKT cell activation both by bacteria and α -GalCer, and this upregulation was dependent on IL-12 for bacteria but not for α -GalCer (Figure 33). Collectively, these similarities between bacteria- and α -GalCer-induced iNKT cell hyporesponsiveness suggest similar mechanisms.

On the other hand, there was one striking difference in the capacity of bacteria and α -GalCer to induce iNKT cell hyporesponsiveness. *E. coli*-induced hyporesponsiveness of iNKT cells required IL-12 expression, but IL-12 was dispensable for α -GalCer-induced iNKT cell hyporesponsiveness (Figure 21A, B). Consistently, NK1.1 downregulation and PD-1 upregulation was absent in IL-12-deficient animals treated with bacteria but not α -GalCer (Figure 22, 33). Such difference may be attributed to distinct mechanisms of iNKT cell activation by α -GalCer and bacteria. Previous studies have indicated an important role for IL-12 in the reciprocal interactions of iNKT

cells and DCs (80), and accordingly splenocytes from IL-12-deficient mice showed a suppressed response to α -GalCer as compared with wild-type splenocytes. Regardless, as a potent ligand for the invariant TCR of iNKT cells, α -GalCer can activate these cells directly through TCR ligation and likely does not depend as heavily on IL-12 secretion by DC. On the other hand, a previous report showed that indirect activation of iNKT cells by *E. coli* LPS requires proinflammatory cytokine secretion such as IL-12 by DC while TCR ligation was dispensable. It should be noted that among various TLR ligands, LPS along with flagellin seems to be the causative agent for iNKT cell hyporesponsiveness by *E. coli*.

Interestingly, a recent study has shown that sulfatide, a ligand for a subset of CD1d-restricted NKT cells expressing diverse TCRs, can also induce hyporesponsiveness in iNKT cells (198). Unlike the anergy induced by α -GalCer, but similar to bacteria-induced hyporesponsiveness, anergy induced in iNKT cells by sulfatide required IL-12 expression (198). These findings suggest that multiple stimuli and pathways can result in iNKT cell hyporesponsiveness with overlapping but distinct mechanisms of induction and, possibly, maintenance.

Anergy or hyporesponsiveness in conventional T cells is categorized as clonal anergy or adaptive tolerance (137). Clonal anergy ensues following incomplete T cell activation of previously activated T cells resulting in growth arrest but usually with intact effector function. Adaptive tolerance is induced by T cell stimulation in the absence of adequate costimulation, or in the presence of strong coinhibition such as CTLA-4. This form of anergy cannot be reversed by exogenous IL-2 in most cases due to defects in IL-2R signaling (188), whereas clonal anergy is typically rescued by exogenous IL-2. From the observation that naïve iNKT cells show an intermediate activated T cell phenotype, as

exemplified by CD69 and CD44 expression (30, 31), and that TCR ligation may be weak or absent during initial activation by bacteria, bacteria-induced hyporesponsiveness exhibits features of clonal anergy. Furthermore, adaptive tolerance usually requires persistent presence of cognate antigen, which is unlikely to occur during bacteria-induced hyporesponsiveness. Indeed, there is no known microbial antigen for iNKT cell present in bacteria shown to induce iNKT cell hyporesponsiveness. However, prolonged presentation of endogenous ligand or persistence of TLR ligands responsible for iNKT cell hyporesponsiveness remains possible following bacterial infection. Indeed, reported upregulation of CD1d in the context of bacterial infection suggests such a possibility (156, 199, 200). Recovery of proliferation with exogenous IL-2 is also more typical of clonal anergy. On the other hand, severely compromised effector function such as IFN- γ and IL-4 production is often found with adaptive tolerance. Collectively, bacteria-induced hyporesponsiveness exhibits features of both categories of anergy and further investigation of the underlying molecular mechanisms is required to better understand this hyporesponsive phenotype induced by bacteria.

In summary, the mechanism of iNKT cell hyporesponsiveness induced by bacteria shares several common features with α -GalCer-induced iNKT cell anergy. Further understanding of the mechanism will provide insight into the development of novel strategies to manipulate iNKT cells for therapeutic applications.

Materials and Methods

Mice. Female C57BL/6 (B6) mice, thymectomized adult B6 mice, and IL-12p40-deficient mice were purchased from the Jackson Laboratory. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. In addition to reagents described in Chapter II, mouse recombinant IL-2 was obtained from BD Biosciences-Pharmingen, PMA and ionomycin from MP Biomedicals, Inc., complete and incomplete Freund's adjuvant from BD Biosciences-Pharmingen, N^G-monomethyl-L-arginine, lipoteichoic acid, *E. coli* LPS from Sigma, and imiquimod and *Salmonella* flagellin from Invivogen, and polyinosinic acid-polycytidylic acid from Amersham Pharmacia. *E. coli* flagellin was purified from cultures as described (201).

Treatment of mice with heat-killed or live bacteria. Bacteria were grown and prepared as described in Chapter II.

Flow cytometry. Flow cytometry was performed as described in Chapter II.

Measurement of in vivo and in vitro responses to α -GalCer. Evaluation of in vivo and in vitro iNKT cell responses to α -GalCer were performed as described in Chapter II.

ELISA. A standard sandwich ELISA was performed as described in Chapter II.

Isolation of splenic DCs. Spleens were cut into small pieces and digested with 0.2 mg/ml collagenase D (Roche Diagnostics Corp.) in FCS-free RPMI medium for 45 min. The digestion was terminated by addition of cold R-10 medium. Red blood cells were lysed using ACK lysing buffer (Lonza). DCs were enriched based on expression of the CD11c marker by magnetic sorting (Miltenyi Biotec) according to the manufacturer's protocol. Purity of enriched cell populations was 80–85% for DCs (data not shown). Purified DCs were pulsed for 3 hrs with 200 ng/ml α -GalCer at 37°C. Cells were then washed 3 times in R-10 medium to remove excess α -GalCer and injected i.v. into B6 mice (2×10^5 DCs per mouse). Mice were sacrificed 3 days later for analysis of iNKT cell function.

Enrichment of iNKT cells. For enrichment of liver iNKT cells, livers were perfused with cold PBS and then pressed through a 70- μ m cell strainer. Cells were suspended in 40 ml RPMI medium in a 50-ml conical tube and allowed to stand on ice for 45 min. The supernatant was then centrifuged, resuspended in cold 40% Percoll (GE Healthcare), and underlaid with 60% Percoll. Cells were centrifuged at 1,500 g for 20 min at 4°C. Mononuclear cells at the interphase of the 40% and 60% Percoll solutions were collected and washed twice with R-10 medium. Two rounds of panning, 2 hrs each, were then carried out to remove plastic-adherent APCs. The frequency of liver iNKT cells was then analyzed by flow cytometry in order to normalize numbers of iNKT cells in the subsequent culture with isolated splenic DCs. For splenic iNKT cells, single-cell suspensions of splenocytes were prepared and iNKT cells were enriched based on negative selection of B220-, CD11c-, CD62L-, Gr-1-, and CD11b-expressing cells by magnetic sorting (Miltenyi Biotec) according to the manufacturer's protocol. The enriched cells were then labeled with CFSE and cocultured with α -GalCer-loaded DCs

described above.

CFSE dilution analysis. Total splenocytes or enriched iNKT cells were labeled with 1 μ M CFSE for 15 min at 37°C in PBS containing 5% FCS, and washed twice with R-10 medium. Labeled splenocytes (2×10^5 cells per well) were then stimulated with α -GalCer (100 ng/ml) with or without addition of IL-2 (10 ng/ml) in the culture media, or stimulated with purified α -GalCer-loaded DCs for 24 hrs in R-10 medium. Cells were washed 3 times with R-10 medium and cultured for an additional 96 hrs in R-10 medium without α -GalCer. At the end of the culture, cells were harvested, stained with PE-labeled CD1d tetramer and anti-B220-PerCP, and analyzed by flow cytometry. Dead cells were excluded from the analysis by electronic gating. CFSE dilution analysis was performed on B220⁻tetramer⁺ iNKT cells.

Statistical analysis. Statistical analysis was performed as described in Chapter II.

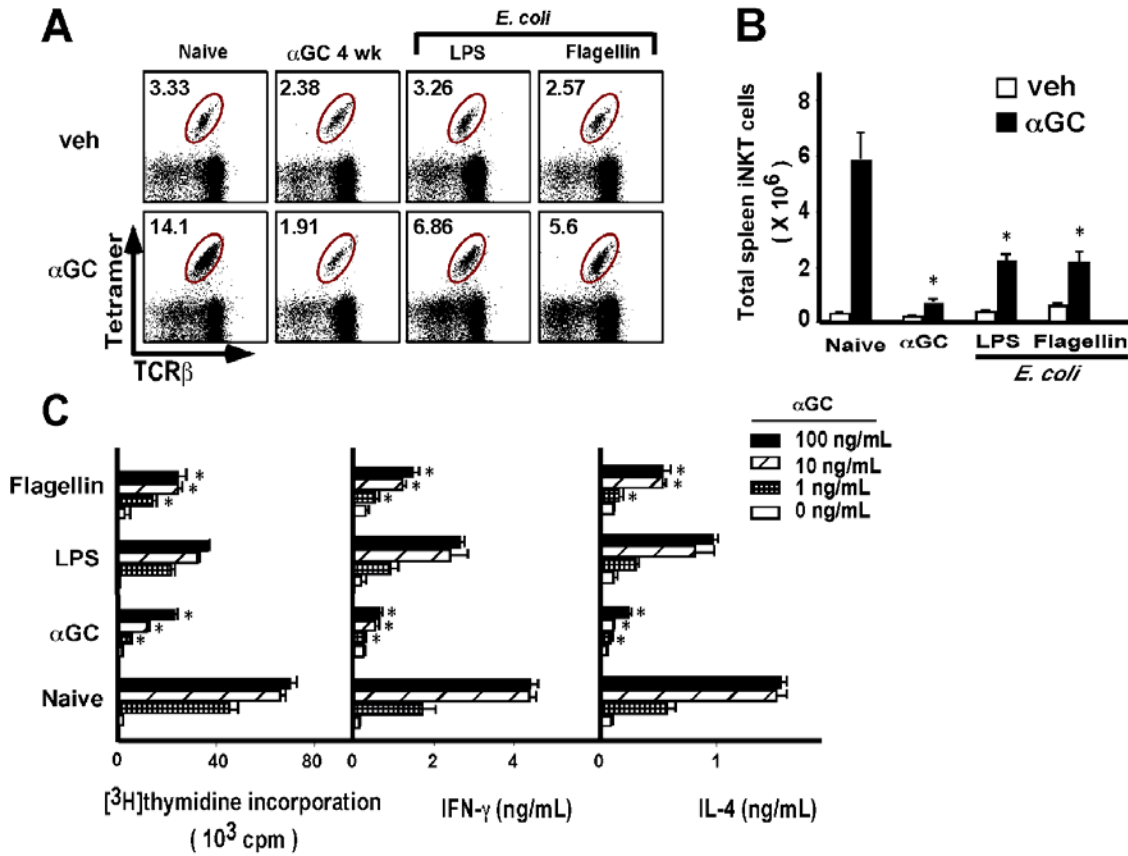


Figure 19. Role of bacterial TLR ligands in the induction of iNKT cell hyporesponsiveness. (A) Mice were injected with *E. coli* flagellin (20 μ g) or LPS (3 doses of 10 μ g every 3 days), and 3 weeks later mice were rechallenged in vivo with vehicle or α -GalCer (1 μ g/mouse, i.p.). Mice were then sacrificed 3 days later and spleen cells were stained with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCR β ⁺tetramer⁺ cells among B220⁻ cells for representative data of 6-9 mice per group from 2 experiments. (B) Graphical representation of the total spleen iNKT cells calculated from the experiments shown in (A). *, $p < 0.05$ as compared with naive mice rechallenged with α -GalCer. (C) The in vitro response of splenocytes to α -GalCer from mice treated 3 weeks earlier with α -GalCer, *E. coli* flagellin, or *E. coli* LPS. Mice were injected with α -GalCer or the indicated TLR ligands, sacrificed 3 weeks later, and splenocytes (2×10^5 per well) were cultured with graded doses of α -GalCer. After 3 days, proliferation was assessed by [³H]thymidine incorporation and culture supernatants were evaluated for IL-4 and IFN- γ levels by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 4 mice. *, $p < 0.05$ as compared with naive splenocytes.

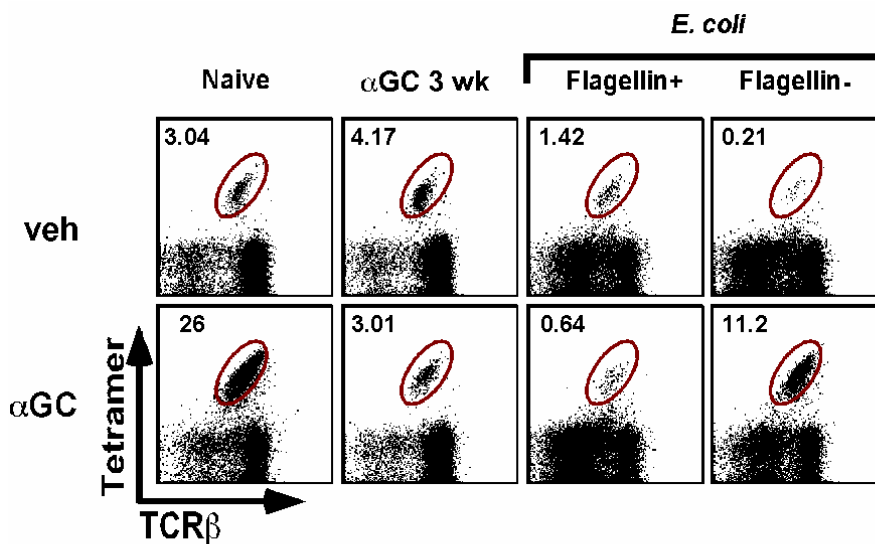


Figure 20. Role of flagellin in the induction of iNKT cell hyporesponsiveness. Mice were injected with flagellin⁺ *E. coli* or flagellin⁻ DH5α, and 3 weeks later mice were rechallenged in vivo with vehicle or α-GalCer (1 μg/mouse, i.p.). Mice were then sacrificed 3 days later and spleen cells were stained with anti-TCRβ-FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCRβ⁺tetramer⁺ cells among B220⁻ cells for representative data of 4-6 mice per group from 2 experiments.

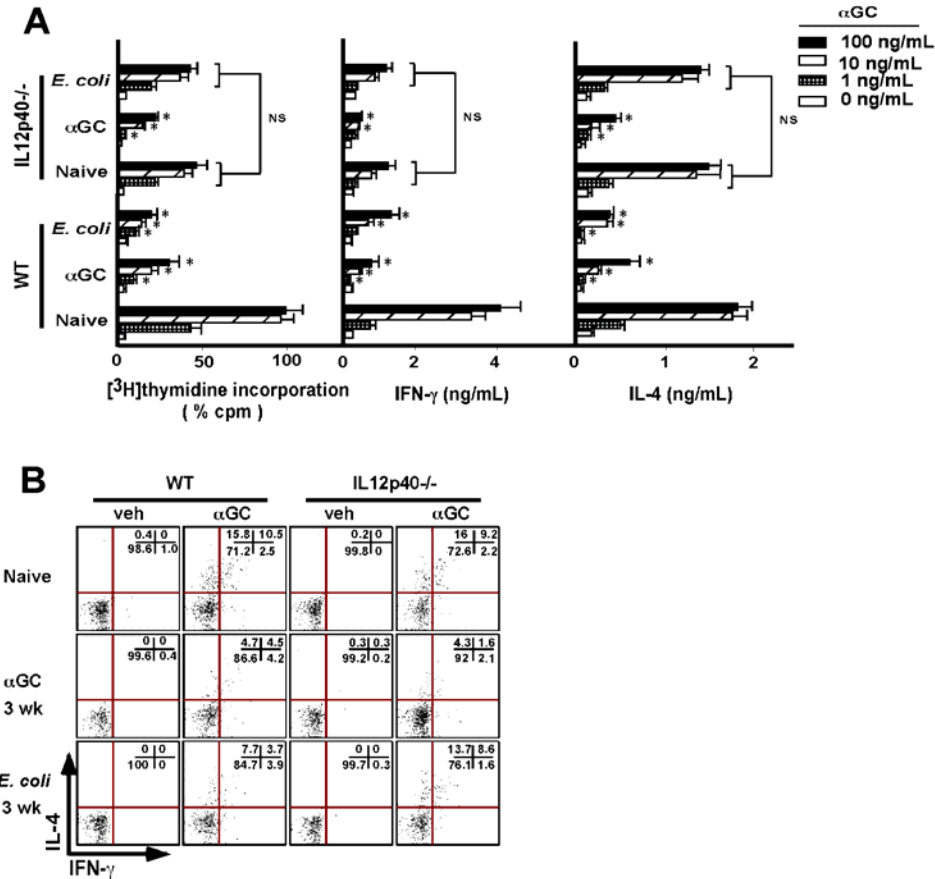


Figure 21. Role of IL-12 in the induction of iNKT cell hyporesponsiveness. **(A)** Wild type mice and IL-12p40-deficient mice were treated with α -GalCer or heat-killed *E. coli*. Three weeks later, mice were sacrificed and splenocytes (2×10^5 per well) were cultured with graded doses of α -GalCer, proliferation was assessed 3 days later by [3 H]thymidine incorporation and culture supernatants were evaluated for IL-4 and IFN- γ levels by ELISA. Proliferation and cytokine results represent the mean \pm SEM of 4-9 mice pooled from 2 separate experiments. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of α -GalCer. Wild type mice pretreated with α -GalCer or heat-killed *E. coli* was compared with wild type naïve mice whereas IL-12p40-deficient mice pretreated with α -GalCer or heat-killed *E. coli* were compared with IL-12p40-deficient naïve mice. NS, not significant. **(B)** Spleen cells were prepared at the indicated time points and 2×10^5 cells were cultured for 6 hrs in plain medium (alone) or 100 ng/ml α -GalCer (α GC) in the presence of GolgiPlug. Cells were then harvested and surface-stained with tetramer-PE and anti-B220-PerCP, followed by intracellular staining with anti-IFN- γ -FITC and anti-IL-4-APC. Data are shown for B220⁺tetramer⁺ cells. Numbers indicate the percentage of cells within each quadrant. Data shown are representative of 3-4 mice per group.

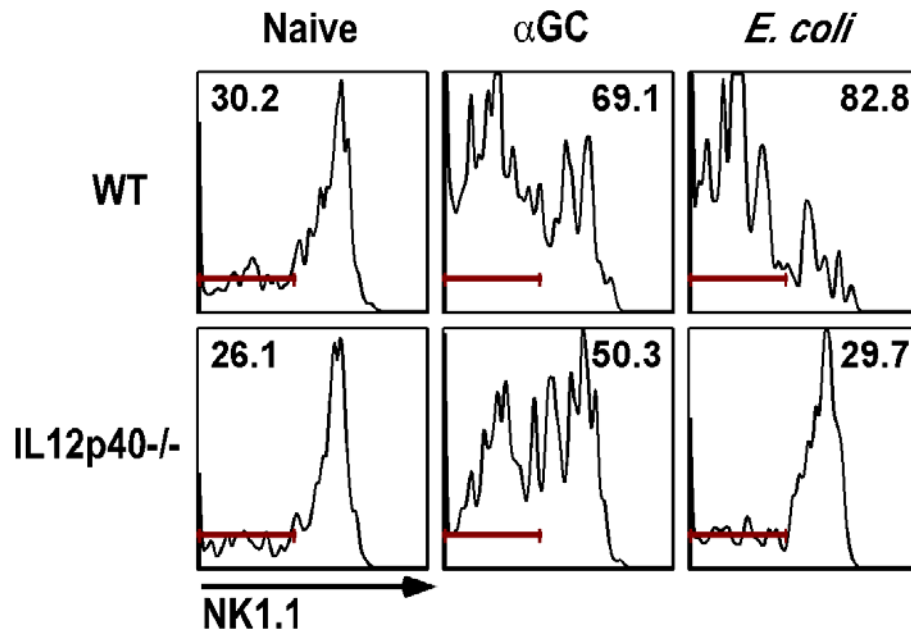


Figure 22. Role of IL-12 in bacteria-induced iNKT cell activation. Wild type mice and IL-12p40-deficient mice were treated with α -GalCer or heat-killed *E. coli* and at day 1, mice were sacrificed and splenocytes were stained for the identification of iNKT cells with anti-TCR β -FITC, anti-NK1.1-PE, anti-B220-PerCP, and tetramer-APC. The percentage of NK1.1⁺ cells among iNKT cells is shown. Data shown are representative of 4 mice per group.

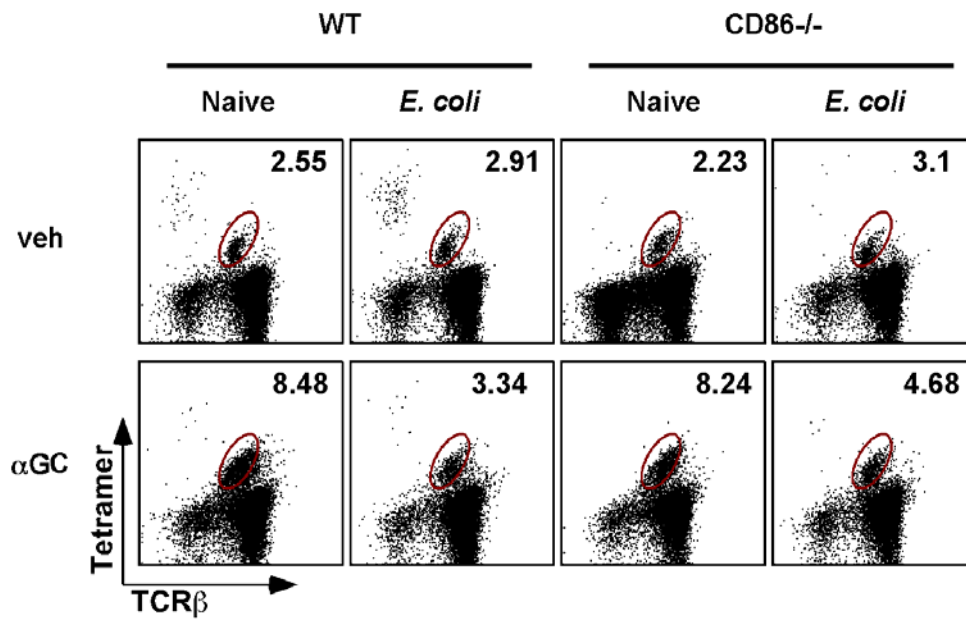


Figure 23. Costimulation is not required for the induction of iNKT cell hyporesponsiveness. Wild type mice and CD86-deficient mice were treated with heat-killed *E. coli* and 3 weeks later, mice were rechallenged with vehicle or α -GalCer (1 μ g/mouse, i.p) in vivo. Mice were sacrificed 3 days later and splenocytes were stained for the identification of iNKT cells with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC and analyzed by flow cytometry. Numbers indicate the percentage of TCR β ⁺tetramer⁺ cells among B220⁻ cells for representative data of 2 mice per group.

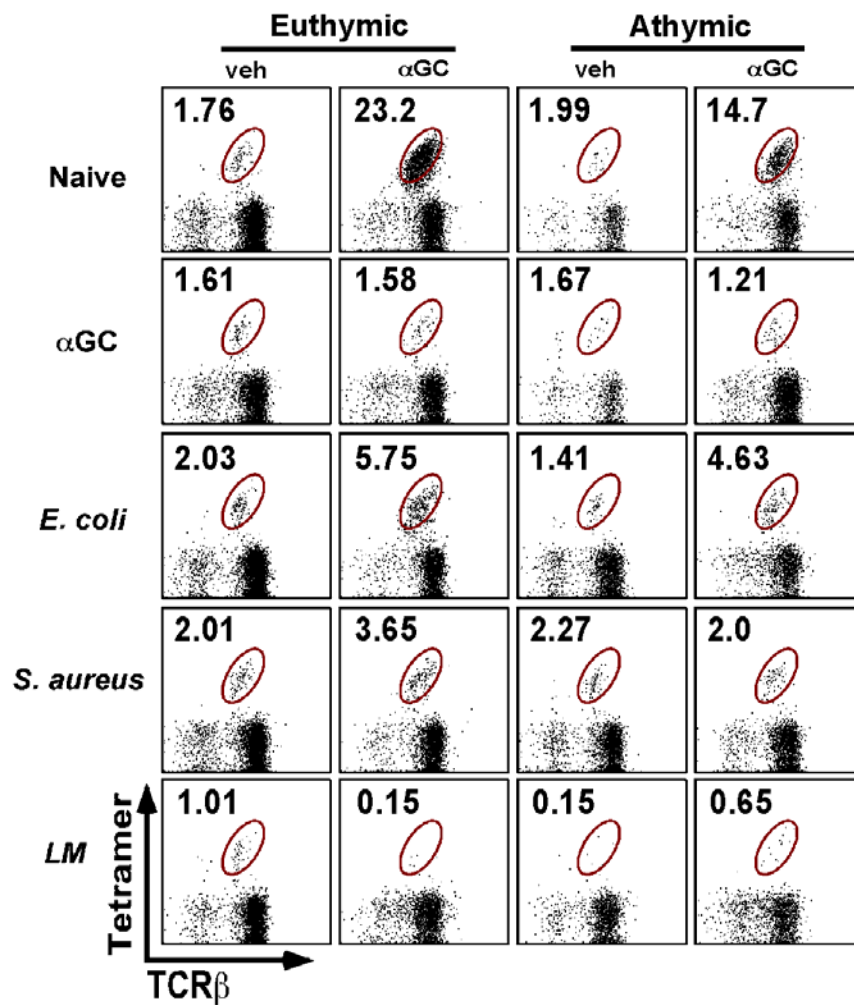


Figure 24. Bacteria-induced iNKT cell hyporesponsiveness is thymus-independent. Thymectomized and non-thymectomized B6 mice were treated with α -GalCer (5 μ g/mouse, i.p) or the indicated bacteria. Three weeks later, mice were rechallenged with vehicle or α -GalCer (1 μ g/mouse, i.p) in vivo. Mice were sacrificed at day 3 and spleen cells were stained with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells for representative data of 2 mice per group.

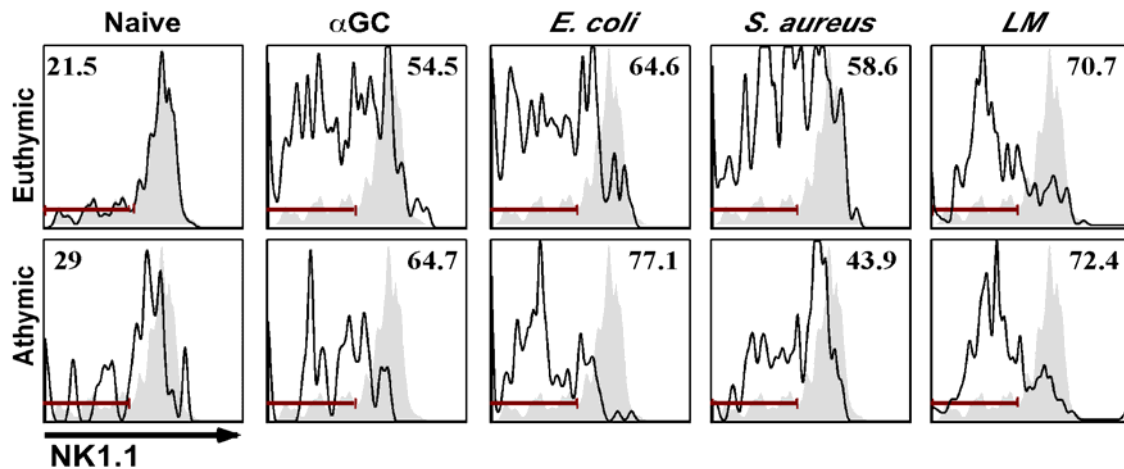


Figure 25. Expression of NK1.1 in euthymic and athymic mice treated with bacteria. Euthymic and athymic B6 mice were treated with α -GalCer (5 μ g/mouse, i.p.) or the indicated bacteria. Three weeks later, mice were sacrificed and spleen mononuclear cells were stained with anti-TCR β -FITC, anti-NK1.1-PE, anti-B220-PerCP, and tetramer-APC and analyzed by flow cytometry. Histograms were gated on B220⁻TCR β ⁺tetramer⁺ cells. Numbers indicate the percentage of NK1.1⁺ cells. Shaded area indicates NK1.1 staining on naïve animals. Data shown are representative of 2 mice per group.

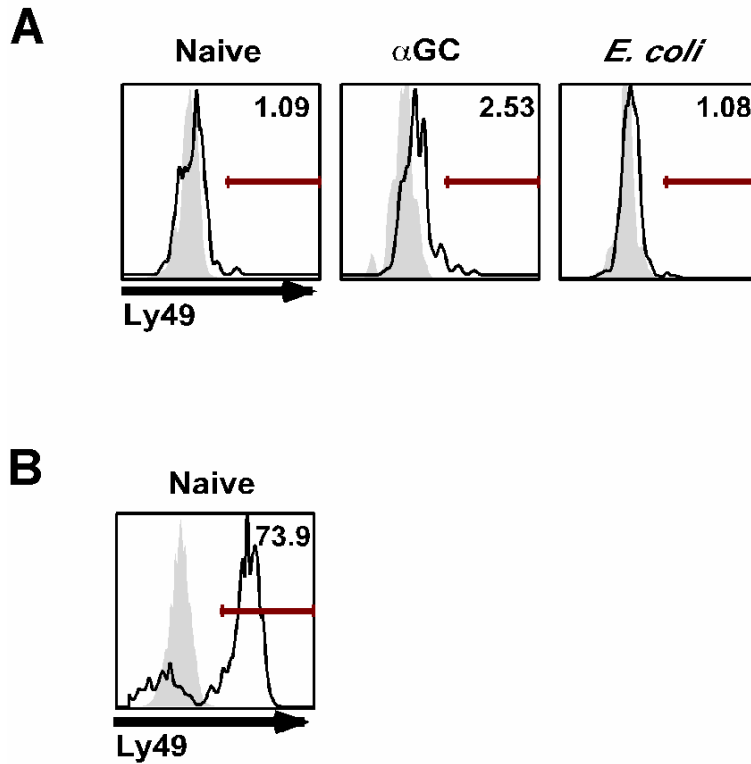


Figure 26. Surface expression of Ly49 on iNKT cells and NK cells. **(A)** Surface expression of Ly49 on iNKT cells. Mice were treated with α -GalCer or heat-killed *E. coli*, and spleen mononuclear cells were stained with anti-Ly49-FITC cocktail, anti-TCR β -PE, anti-B220-PerCP, and tetramer-APC. Histograms were gated on B220⁻TCR β ⁺tetramer⁺. Numbers indicate the percentage of Ly49⁺ cells. Shaded areas indicate isotype controls for Ly49 histograms. **(B)** Surface expression of Ly49 on NK cells. Naïve mice were sacrificed and spleen mononuclear cells were stained with anti-Ly49-FITC cocktail, anti-TCR β -PE, anti-B220-PerCP and anti-NK1.1-APC. Histograms were gated on B220⁻TCR β ⁻NK1.1⁺ cells. Numbers indicate the percentage of Ly49⁺ cells. Shaded area indicates the isotype control. Data are representative of 4-6 mice per group from 2 separate experiments.

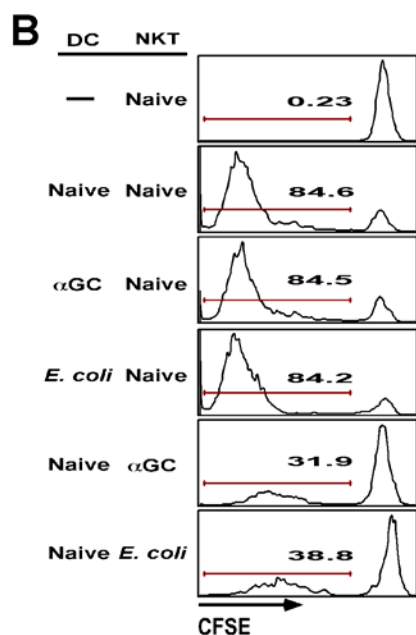
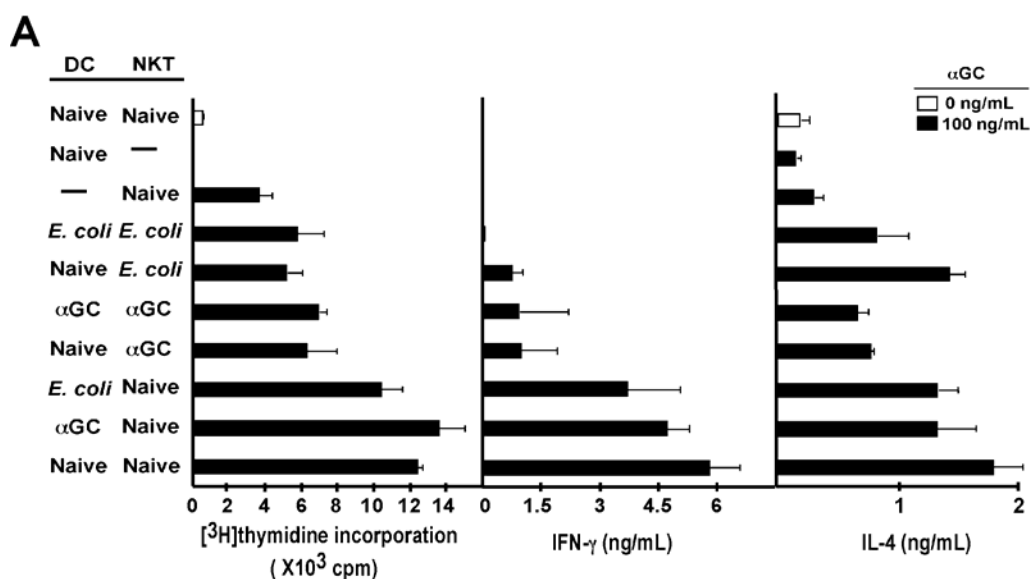


Figure 27. Bacteria-induced iNKT cell hyporesponsiveness is predominantly iNKT cell autonomous. (A) Mice were injected with α -GalCer or heat-killed *E. coli* and sacrificed at 3 weeks. DCs from the spleen and iNKT cells from the liver were then enriched as described in Methods. iNKT cells (1×10^5 per well) and DCs (5×10^4 per well) were then cultured in different combinations in the presence or absence of α -GalCer. Proliferation was assessed by [³H]thymidine incorporation, and IFN- γ and IL-4 levels in the supernatant were evaluated by ELISA. Data shown are the mean \pm SD of wells per group and representative of 2 independent experiments. (B) Mice were injected with α -GalCer or heat-killed *E. coli* and sacrificed at 3 weeks. DCs and iNKT cells were then MACS-purified as described in Methods. DCs (2×10^4 per well). were loaded with α -GalCer and cultured with splenic CFSE-labeled iNKT cells (1×10^5 per well). Cells were harvested 3 days later and stained with anti-B220-PerCP, tetramer-APC and analyzed by flow cytometry. Data shown are CFSE staining on iNKT cells. Numbers indicate the percentage of CFSE⁻ iNKT cells. Three mice per group were pooled for the experiment.

wells per group and representative of 2 independent experiments. (B) Mice were injected with α -GalCer or heat-killed *E. coli* and sacrificed at 3 weeks. DCs and iNKT cells were then MACS-purified as described in Methods. DCs (2×10^4 per well). were loaded with α -GalCer and cultured with splenic CFSE-labeled iNKT cells (1×10^5 per well). Cells were harvested 3 days later and stained with anti-B220-PerCP, tetramer-APC and analyzed by flow cytometry. Data shown are CFSE staining on iNKT cells. Numbers indicate the percentage of CFSE⁻ iNKT cells. Three mice per group were pooled for the experiment.

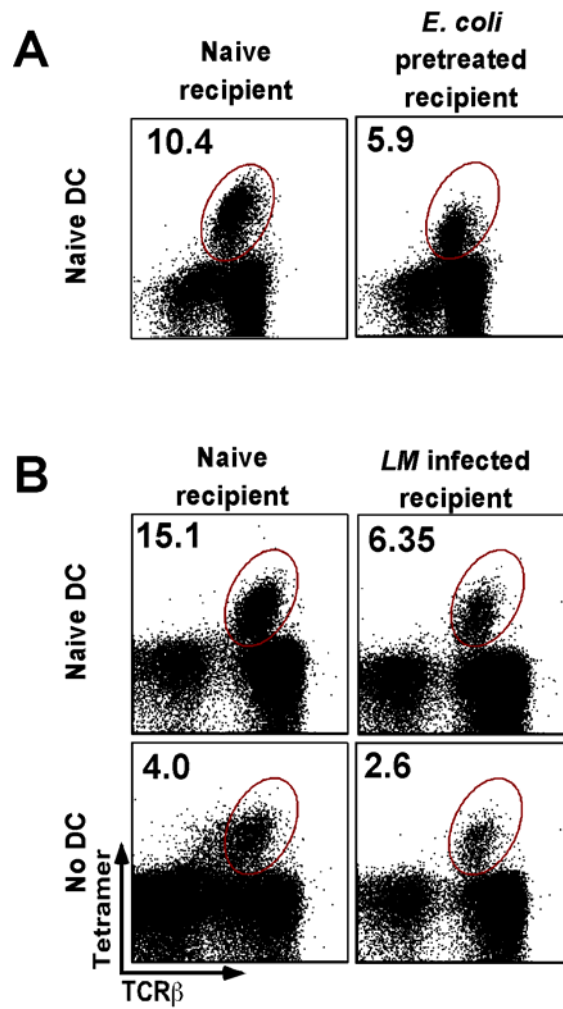


Figure 28. Bacteria-induced iNKT cell hyporesponsiveness is predominantly iNKT cell autonomous. Mice were left untreated or injected with heat-killed *E. coli* (A) or live *L. monocytogenes* (B). DCs were MACS-purified from naïve mice, pulsed with α -GalCer, washed and then injected i.v. (2×10^5 DCs per mouse) into naïve mice, or mice treated three weeks earlier with the indicated bacteria. As a control, mice were also treated without DC (no DC). Mice were sacrificed 3 days later, splenocytes were stained with anti-TCR β -FITC, anti-B220-PerCP, and tetramer-APC, and analyzed by flow cytometry. Numbers indicate the percentage of TCR β^+ tetramer $^+$ cells among B220 $^-$ cells. Data shown are representative of 2 mice per group.

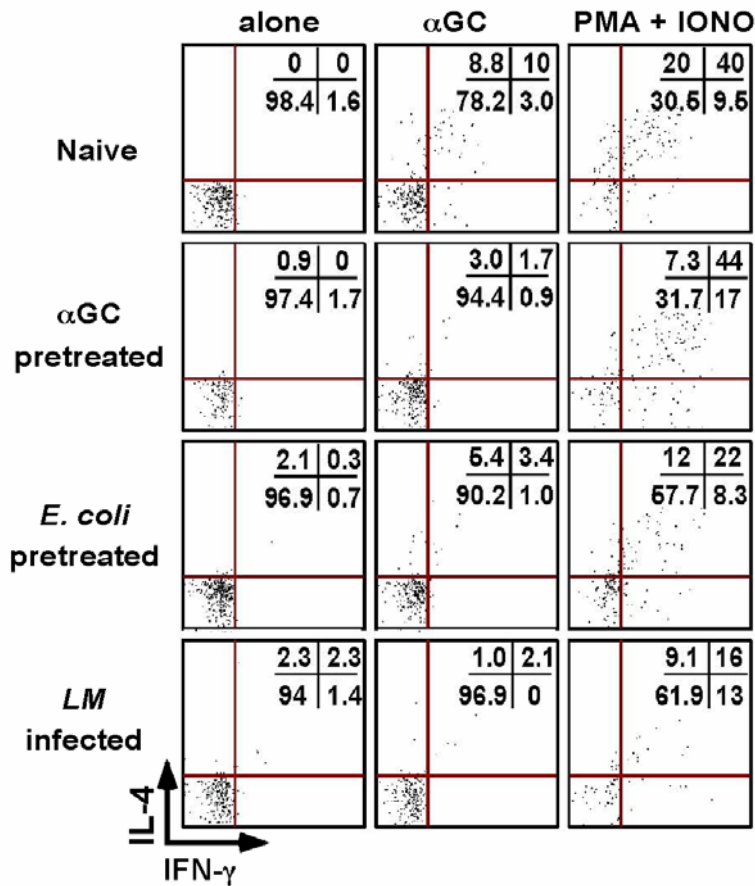


Figure 29. Bacteria-induced iNKT cell hyporesponsiveness can be overcome by treatment with PMA plus ionomycin. Spleen cells were prepared from mice treated 4 weeks earlier with α -GalCer or the indicated bacteria, cultured in vitro (2×10^5 per well) for 6 hrs in plain medium (alone), 100 ng/ml α -GalCer (α GC), or a combination of 10 ng/ml PMA and 1 μ M ionomycin (PMA + IONO), in the presence of GolgiPlug to allow intracellular accumulation of cytokines. Cells were then harvested and surface-stained with tetramer-PE and anti-B220-PerCP, followed by intracellular staining with anti-IFN- γ -FITC and anti-IL-4-APC. Data are shown for B220⁺tetramer⁺ cells. Numbers indicate the percentage of cells within each quadrant. Data shown are representative of 3 independent experiments with 2 mice in each group per experiment.

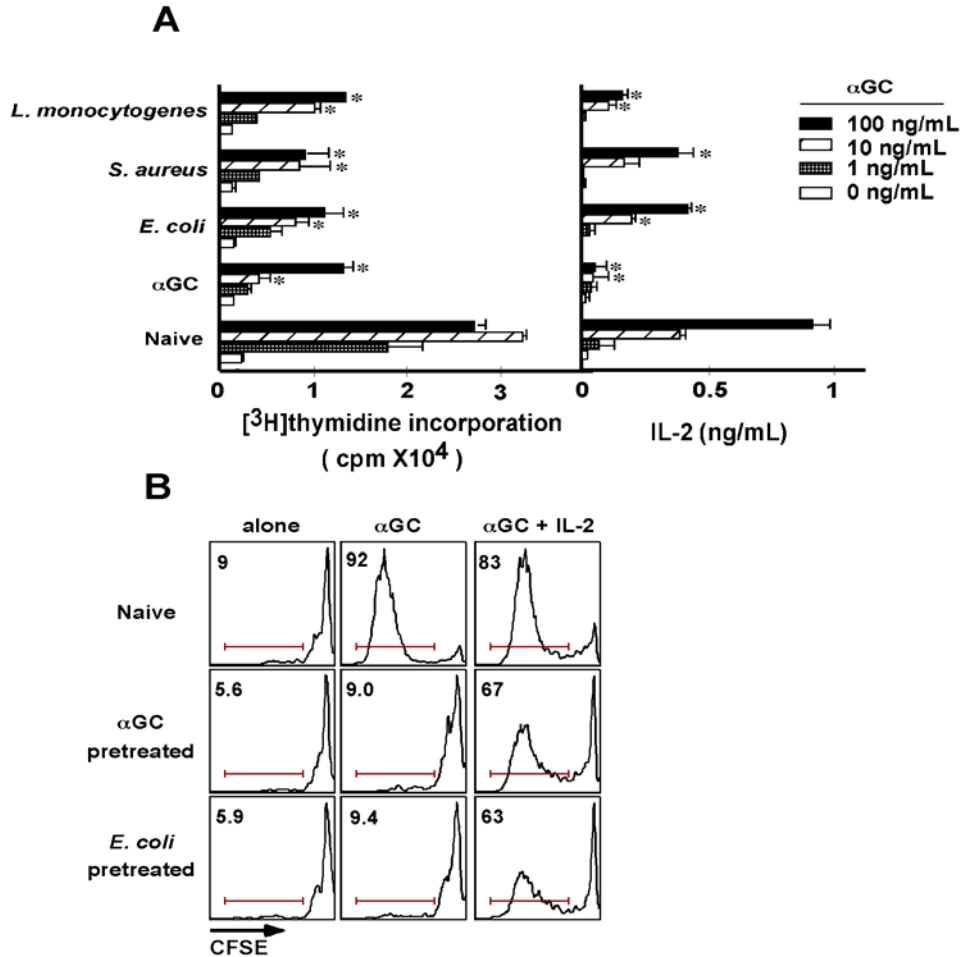


Figure 30. Bacteria-induced iNKT cell hyporesponsiveness correlates with reduced IL-2 secretion and can be overcome by treatment with α -GalCer plus IL-2. **(A)** Spleen cells were prepared from mice treated 4 weeks earlier with α -GalCer or the indicated bacteria, cultured in vitro (2×10^5 per well) with graded doses of α -GalCer. After 3 days, proliferation was assessed by [³H]thymidine incorporation and culture supernatants were evaluated for IL-2 levels by ELISA. Results represent the mean \pm SEM. *, $p < 0.05$ as compared with naïve splenocytes cultured with the same dose of α -GalCer. **(B)** IL-2 overcomes the proliferative defect of hyporesponsive iNKT cells in vitro. Spleen cells from naïve mice or from mice injected 1 month earlier with α -GalCer or heat-killed *E. coli* were labeled with CFSE. Cells (2×10^5 per well) were then cultured with α -GalCer (100 ng/ml) for 24 hrs in the presence or absence of IL-2 (10 ng/ml). Cells were then washed and cultured for an additional 96 hrs without α -GalCer in the presence or absence of IL-2. At the end of the culture period, cells were analyzed by flow cytometry. CFSE dilution was analyzed on B220⁺tetramer⁺ cells. Numbers indicate the percentage of CFSE⁻ cells among B220⁺tetramer⁺ cells. Representative data from 2 independent experiments are shown.

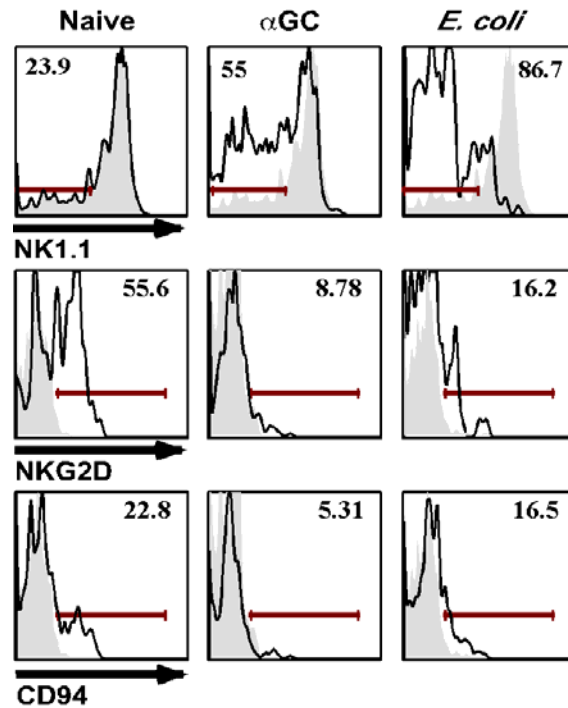


Figure 31. Surface expression of NK1.1, NKG2D and CD94 on iNKT cells. Mice were treated with α -GalCer or heat-killed *E. coli* and spleen mononuclear cells were stained with different combinations of anti-NK1.1, anti-NKG2D-biotin, anti-CD94-biotin, streptavidin-FITC, anti-TCR β -PE, anti-B220-PerCP, and tetramer-APC. Histograms were gated on B220⁻TCR β ⁺tetramer⁺. Shaded areas indicate isotype controls for NKG2D, CD94 and Ly49 histograms, and NK1.1 expression on naïve iNKT cells for NK1.1 histograms. Numbers indicate the percentage of NK1.1⁻, NKG2D⁺, or CD94⁺ cells among iNKT cells. Data are representative of 5-6 mice per group from 2 independent experiments.

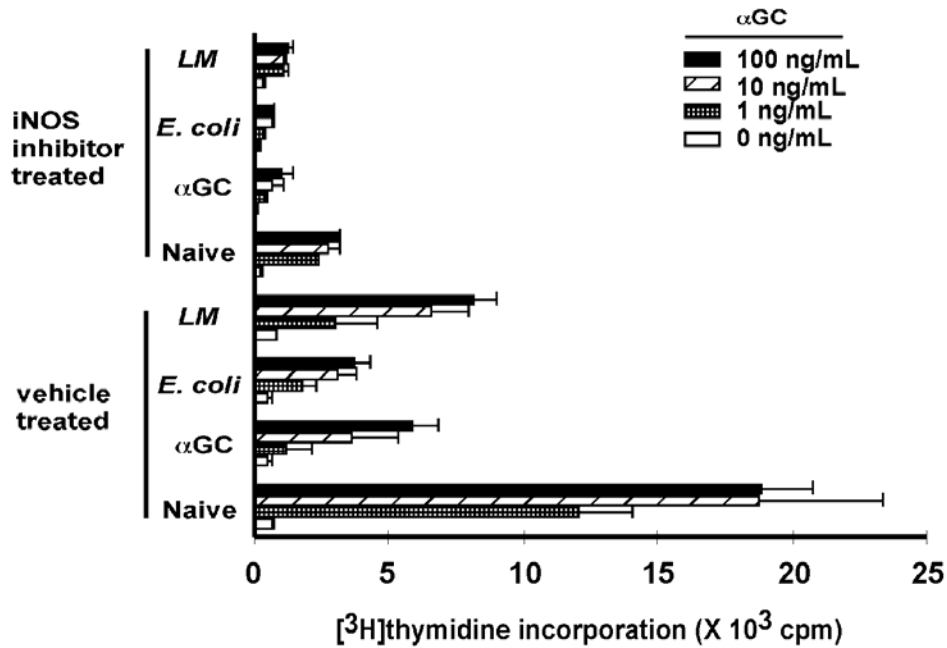


Figure 32. Nitric oxide does not contribute to the bacteria-induced hyporesponsive phenotype of iNKT cells. Spleen cells from naive mice or from mice injected 1 month earlier with α -GalCer, heat-killed *E. coli*, or live *L. monocytogenes* were cultured in vitro (2×10^5 per well) with graded doses of α -GalCer in the presence or absence of inducible nitric oxide synthase (iNOS) inhibitor, N^G-monomethyl-L-arginine. After 3 days, proliferation was assessed by [³H]thymidine incorporation. Proliferation results represent the mean \pm SEM.

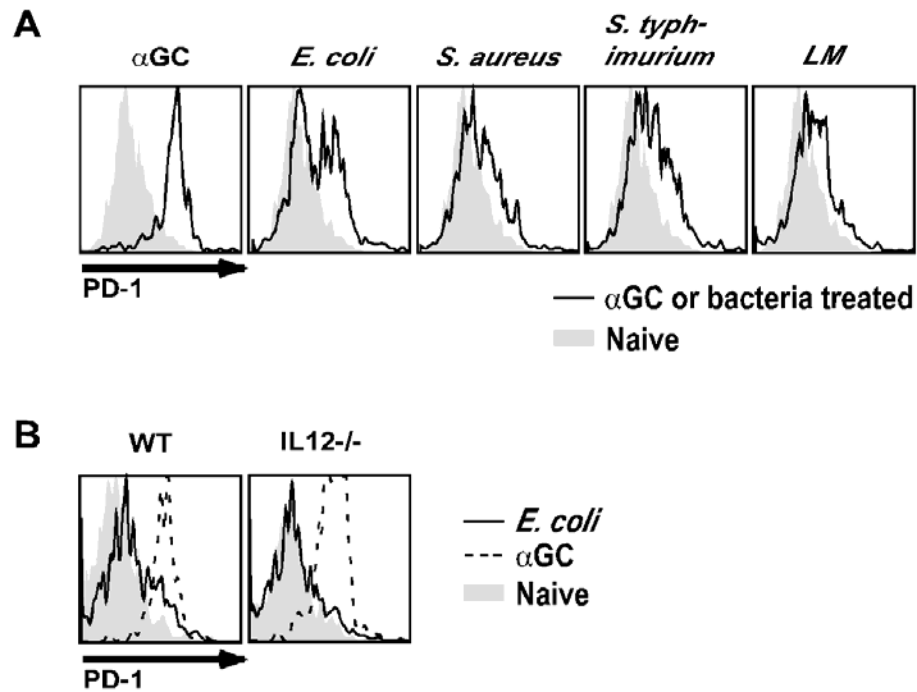


Figure 33. Expression of PD-1 in mice treated with bacteria. **(A)** Mice were treated with α -GalCer (5 μ g/mouse, i.p.) or the indicated bacteria and mice were sacrificed at day 1, and spleen mononuclear cells were stained with anti-TCR β -FITC, anti-PD-1-PE, anti-B220-PerCP, and tetramer-APC and analyzed by flow cytometry. Data shown are gated on B220⁻TCR β ⁺tetramer⁺ cells. The shaded area represents the PD-1 staining of naïve iNKT cells, and the solid line represents the staining of iNKT cells from mice treated with α -GalCer or bacteria. **(B)** Wild type or *IL12*^{-/-} mice were treated with α -GalCer (5 μ g/mouse, i.p.) or heat-killed *E. coli* and spleen mononuclear cells were stained as above for PD-1. The shaded area represents the PD-1 staining of naïve iNKT cells, dotted line α -GalCer, and the solid line heat-killed *E. coli*. Data shown are representative of 2-4 mice per group.

CHAPTER IV

CONCLUSIONS AND PERSPECTIVES

Natural killer T (NKT) cells are unique T lymphocytes that co-express T cell receptors (TCRs) and natural killer (NK) cell markers (36, 171, 172, 202-204). Most NKT cells, referred to as invariant NKT (iNKT) cells, express a semi-invariant TCR consisting of a $V\alpha 14$ - $J\alpha 18$ chain paired predominantly with a $V\beta 8.2$ chain in mice (94). Unlike conventional T cells, which recognize peptides presented by MHC class I or class II proteins, iNKT cells are specific for glycolipid antigens presented by the MHC class I-related protein CD1d. In response to TCR engagement, iNKT cells can rapidly produce a variety of cytokines and, hence, these cells can impart potent immunoregulatory properties. As such, iNKT cells can promote protective immune responses against infectious agents, suppress autoimmunity, promote natural tumor immunity and regulate allergic airway inflammation, atherosclerosis, colitis and contact hypersensitivity in mice.

The physiological antigens that are recognized by iNKT cells remain incompletely understood (205). All iNKT cells react with the glycosphingolipid α -galactosylceramide (α -GalCer), which was originally isolated from a marine sponge. More recently, it has been demonstrated that iNKT cells can react with α -anomeric glycosphingolipids derived from the cell wall of gram-negative *Sphingomonas* bacteria (55, 56, 130) and with α -galactosyl-diacylglycerols from the spirochete *Borrelia burgdorferi* (56), the etiologic agent of Lyme disease. However, the endogenous ligands

for iNKT cells remain to be fully characterized (206).

The immunomodulatory properties of iNKT cells have been exploited for the development of immunotherapies (36, 171, 172, 204, 207). In most of these studies, derivatives of α -GalCer have been employed. α -GalCer potently activates iNKT cells to secrete a mixture of T helper (Th) 1 and Th2 cytokines. iNKT cells activated in this manner transactivate a variety of other cell types, including antigen presenting cells (APCs), NK cells and conventional T and B cells (78). The potent immunostimulatory activities of α -GalCer on APCs, in particular dendritic cells (DCs), have been exploited for the development of vaccine adjuvants. In addition, repeated injection of α -GalCer can prevent development of tumor metastases and Th1-dominant autoimmunity in mice (36, 171, 172, 204, 207).

A thorough understanding of iNKT cell responses to various stimuli is important in order to develop effective iNKT cell-based adjuvants and immunotherapies for human disease. Prior studies have shown that a single injection of α -GalCer to mice results in rapid iNKT cell activation and cytokine production. This activation also results in transient downregulation of the invariant TCR and sustained downregulation of the NK cell marker NK1.1 on iNKT cells (95-97). In vivo-activated iNKT cells rapidly proliferate, leading to profound expansion of this cell population in multiple organs, which peaks around three days after α -GalCer treatment. This period of rapid iNKT cell expansion is followed by a contraction phase mediated by homeostatic mechanisms. Importantly, restimulation of these α -GalCer-experienced iNKT cells with α -GalCer results in a suppressed response because the iNKT cells acquire an anergic phenotype (25, 92, 98, 99). iNKT cell anergy induced in this manner is maintained for at least one month. Additional studies showed that iNKT cell anergy has a profound impact on iNKT

cell-mediated functions and the therapeutic properties of α -GalCer (25).

iNKT cell activation has been observed in the context of glycolipid antigens, cytokines, multiple microorganisms and inflammatory stimuli (36, 126, 171, 172, 208). In the case of certain microorganisms, such as *Sphingomonas* and *Borrelia*, iNKT cell activation involves specific, pathogen-derived glycolipid antigens. For many other microorganisms, however, there is no evidence for direct iNKT cell activation by microbial glycolipid antigens. Instead, many microorganisms might activate iNKT cells in a non-specific manner, by stimulating the production of pro-inflammatory cytokines by DCs that can activate iNKT cells by themselves, in concert with endogenous glycolipid antigens, or by inducing CD1d expression on APCs (55, 63, 190, 209). Whether iNKT cell activation by microorganisms influences subsequent responses of these cells to antigenic stimulation remains unclear.

In this dissertation, I report results demonstrating that bacteria have a profound impact on the functional status of iNKT cells with long-term effects on the therapeutic activities of these cells.

In chapter II, I tested the capacity of a wide variety of bacteria, including the gram-positive organisms *L. monocytogenes* and *S. aureus*, and the gram-negative organisms *E. coli*, *S. typhimurium*, and *S. capsulata* to activate iNKT cells and to modulate the functions of these cells. While activation of iNKT cells was observed immediately following infection by all the organisms, sustained long-term phenotypic changes characterized by NK1.1 downregulation, generally associated with iNKT cell hyporesponsiveness in studies with α -GalCer (25), were only observed with *E. coli*, *S. aureus*, *S. typhimurium*, and *L. monocytogenes*. Notably, *L. monocytogenes* induced

drastic loss of cellularity. Interestingly, *S. capsulata* did not induce sustained changes in the surface phenotype of iNKT cells.

In accordance with results from phenotypic analysis, ex vivo and in vivo assessment of iNKT cell function in mice previously infected *E. coli*, *S. aureus*, *S. typhimurium*, or *L. monocytogenes* but not other bacteria showed significant suppression of iNKT cell functions during recall response using α -GalCer as a surrogate secondary antigen. Specifically, when splenocytes from naïve and infected mice were cultured ex vivo in the presence of graded concentrations of α -GalCer, the iNKT cell response represented by proliferation, or IFN- γ and IL-4 secretion was defective in infected mice. Further, in vivo expansion of iNKT cells and transactivation of DC, B and NK cells normally observed following α -GalCer administration was also absent in bacteria infected mice.

iNKT cells are thought to be involved in various disease processes including autoimmune diseases and cancer. In particular, iNKT cells have been shown to play a detrimental role in ConA-induced hepatitis (170), a mouse model for human autoimmune hepatitis. As expected from hyporesponsiveness of iNKT cells, infected mice exhibited significantly reduced hepatitis as documented by decrease in ALT levels that correlates the extent of liver injury. The therapeutic activity of iNKT cells were also evaluated using B16 melanoma metastasis model and EAE, a mouse model for multiple sclerosis. Whereas loss of therapeutic efficacy of α -GalCer against melanoma metastasis was observed in infected mice, protective effect of α -GalCer in preventing EAE was retained.

In Chapter III, the mechanism of bacteria-induced iNKT cell hyporesponsiveness was explored guided by previous reports on α -GalCer-induced iNKT cell anergy. Known microbial glycolipid antigens specific for iNKT cells were absent in all the organisms

found to induce iNKT cell hyporesponsiveness in Chapter II. However, in the absence of cognate antigen, iNKT cells can be activated in a manner that depends on IL-12 secretion and endogenous ligand presentation by DCs activated by toll-like receptor (TLR) ligands (126). We therefore investigated whether TLR ligands can induce iNKT cell hyporesponsiveness. Our results showed that TLR4 and TLR7 ligands LPS and flagellin from *E. coli* and *Salmonella* were able to induce hyporesponsiveness in iNKT cells, but not with other TLR ligands. Further, IL-12 secretion by DCs induced by LPS and flagellin was required for the induction of iNKT cell hyporesponsiveness by bacteria.

Consistent with mechanisms observed with α -GalCer-induced iNKT cell anergy (25), bacteria-induced iNKT cell hyporesponsiveness was thymus-independent, which was indicated by suppressed iNKT cell response in infected thymectomized mice. Bacteria-induced hyporesponsiveness was also predominantly cell autonomous ruling out extrinsic factors such as tolerogenic APC to be responsible for the hyporesponsive phenotype. Likewise, a combination of PMA and ionomycin, which bypasses proximal TCR signaling events, could overcome the hyporesponsive phenotype of iNKT cells induced by bacteria, and exogenous addition of IL-2 also rescued hypoproliferation of iNKT cells probably due to defective IL-2 secretion in bacteria-infected mice.

Downregulation of activating receptors, including NK1.1, NKG2D, and possibly NKG2C/CD94 was observed in mice treated with bacteria, which might contribute to iNKT cell hyporesponsiveness in these animals. Notably, PD-1 molecule currently implicated in T cell exhaustion or anergy during chronic infection (195, 197, 210), was upregulated on hyporesponsive iNKT cells, at least during early phases of iNKT cell activation by bacteria, providing insight into possible molecular mechanisms of bacteria-induced hyporesponsiveness. Additionally, iNOS inhibitor, which rescues

hyporesponsive phenotype of iNKT cells in a cancer-bearing state (192), did not affect iNKT cell hyporesponsiveness. Absence of CD86 also did not affect iNKT cell hyporesponsiveness.

Collectively, results from Chapters II and III have shown that certain bacterial organisms can activate iNKT cells and render these cells functionally suppressed for a sustained time period, which was accompanied by sustained phenotypic changes indicative of anergic cells, and this hyporesponsiveness phenotype of iNKT cells was associated with decreased therapeutic activities.

A simple classic model of host defense against invading pathogens begins with phagocytosis of pathogens by APCs such as DCs and macrophages that are activated by recognition of pathogen associated molecular patterns including but not limited to various TLR ligands expressed by microbes, which is termed the danger signal (211-213). These APCs present microbial peptide antigens to CD4 T cells in conjunction with costimulation through CD80/CD86, CD40, CD70 and other molecules upregulated by the presence of the danger signal. The naïve helper CD4 T cells then differentiate into Th1 or Th2 cells to provide cytokine help for the other arms of the adaptive immune system including CD8 T cells and B cells that respond specifically to microbial antigens presented by DCs, or augment functions of macrophages, neutrophils and other cells of the innate immune system. iNKT cells function as the amplifier during early phases of the immune response by rapidly producing Th1 and/or Th2 cytokines in response to either direct stimulation of their TCRs by microbial glycolipid antigens in the case of *S. capsulata* (55, 130, 134) or *B. burgdorferi* (59), or indirect stimulation by activated DCs that present endogenous antigens along with proinflammatory cytokines in response to TLR activating signals (55, 63, 136, 190, 209). The explosive secretion of Th1 and/or

Th2 cytokines by iNKT cells transactivates DCs reciprocally as well as NK cells, T cells and B cells thereby modulating the rapidity of immune response against invading microbes (78).

When the same pathogen invades, a similar response is exhibited by the immune system with one key difference. While the cells of the innate immune system recapitulate the initial response, T and B cells, the two arms of adaptive immunity, exhibit a typical memory response, characterized by rapid clonal expansion and differentiation of the pathogen-specific memory cells to mediate immediate effector function against the pathogen (1). This rapid and strong immune response usually clears the pathogen even before development of symptoms and signs of infection.

Although iNKT cells are a subset of T cells expressing receptors that are characteristic of the adaptive immune system, their primary function is to bridge the innate and adaptive immunity. As a result, it was unclear whether iNKT cells would exhibit the features of innate or adaptive immunity. Surprisingly, it was neither, as the primary response of iNKT cells towards α -GalCer or certain species of bacteria resulted in functional inactivation of these cells for an extended period of time (Figure 34).

The induction of iNKT cell anergy by a pharmacological dose of α -GalCer, a potent purified synthetic ligand for iNKT cells, could be rationalized by excessive stimulation of TCR of iNKT cells in the absence of the danger signal, which in conventional T cells often results in anergy as well. However, a similar induction of iNKT cell hyporesponsiveness by bacteria that activates iNKT cells only indirectly through DCs that present autoreactive antigen and cytokines in the absence of specific microbial antigen may be a physiologically important reflection of in vivo response of iNKT cells. iNKT cells have the capacity to secrete explosive levels of both Th1 and Th2

cytokines, and repeated activation and functional response of these cells may result in a state of cytokine storm which may sometimes do more harm than good as tissue damage that occurs during infection is attributed to the toxicity of invading pathogens as well as excessive immune response. An uncontrolled response of iNKT cells will ensue without a mechanism to limit the function of these cells particularly because, unlike conventional T cells with restricted specificity, iNKT cells can respond to a wide variety of pathogens non-specifically. This functional promiscuity is critical for iNKT cells to bridge the innate and adaptive immune system with a severely restricted TCR repertoire in response to diverse pathogens, but these same beneficial characteristics can also have a detrimental effect. Furthermore, iNKT cells also have important immunomodulatory roles in cancer, autoimmunity, atherosclerosis and various other disease processes, and uncontrolled response by iNKT cells may have an adverse impact on the host health.

However, among multiple bacteria tested, a select few induced iNKT cell hyporesponsiveness. The apparent lack of iNKT cell hyporesponsiveness observed with *S. capsulata*, *E. faecalis*, and *S. pyogenes* may simply be attributed to the dose of treated bacteria. As it may be possible that the entire compartment of iNKT cells must acquire the hyporesponsive phenotype in order to exhibit an overall functional deficit, we cannot rule out that individual iNKT cells that were activated during treatment with these bacteria acquired the hyporesponsive phenotype while the remainder of iNKT cell population compensated to show normal response.

Otherwise, the variability in the capacity of bacteria to induce iNKT cell hyporesponsiveness is an actual physiological phenomenon and likely reflects the differences in the expression profile of PAMPs or TLR ligands of the particular species of bacteria. We have tested a wide variety of TLR ligands and have shown that LPS and

flagellin isolated from *E. coli* or *S. typhimurium* can induce iNKT cell hyporesponsiveness, but not CpG, imiquimod, lipoteichoic acid, polyinosinic acid-polycytidylic acid, indicating that the combination of PAMPs expressed on bacteria may determine their capacity to render iNKT cells hyporesponsive.

Interestingly, the induction of iNKT cell hyporesponsiveness by TLR ligands and whole bacteria required IL-12 secretion by DCs. It is certain that IL-12 is not the sole causative molecule for iNKT cell hyporesponsiveness as exogenous IL-12 administration does not induce iNKT cell hyporesponsiveness (data not shown). Further, other TLR ligands that were unable to induce hyporesponsiveness, in particular CpG, induce secretion of high levels of IL-12 by DCs (214). Other factors are therefore thought to be required in order to facilitate the induction of hyporesponsiveness. One example might be IL-18, another proinflammatory cytokine induced by LPS (136). An alternative mechanism that has not been explored thoroughly is that iNKT cells can be directly stimulated by TLR ligands. Prior reports of TLR-4 and TLR-9 expression by iNKT cells opens up this possibility (215, 216), although expression of TLR-4 has since been refuted (136) and TLR-9 seems to be dispensable in the induction of iNKT cell hyporesponsiveness (data not shown). The prominent candidate is TLR-5, and it is unknown whether this molecule has a direct role in iNKT cells. In addition to TLR ligands, some bacterial products may stimulate Type II NKT cells with diverse TCR repertoire. Sulfatide, one of the ligands specific for a subset of Type II NKT cells has been shown to induce iNKT cell anergy through indirect stimulation by DCs (198). Interestingly, this process was also dependent on IL-12. Involvement of DCs and IL-12 in the induction of iNKT cell anergy by sulfatide is reminiscent of bacteria-induced iNKT cell hyporesponsiveness. Also, downregulation of activating receptors, in particular

NKG2D, was observed in mice treated with bacteria. In humans, NKG2D ligands MICa and MICb have been shown to be upregulated in activated DCs in response to TLR ligands (217). Likewise, murine NKG2D ligands Rae-1 and H-60 might be upregulated in DC activated by bacterial products including TLR ligands. Therefore, downregulation of activating receptors might contribute to iNKT cell hyporesponsiveness in these animals.

The exact mechanism by which the hyporesponsive phenotype of iNKT cells is induced remains incompletely understood, but our current model of the bacteria- or α -GalCer-induced iNKT cell hyporesponsiveness is summarized in Figure 35. It should be noted that further understanding of the signaling pathways and outcome of TLR signaling in DCs and potentially in iNKT cells may provide a profound insight.

Several features of bacteria- and α -GalCer-induced iNKT cell hyporesponsiveness are reminiscent of features of T cell anergy from which we can draw insights into the molecular mechanism. T cell anergy is broadly categorized into clonal anergy and adaptive tolerance with distinct features (137), but interestingly, iNKT cell anergy seems to exhibit features of both types of T cell anergy. The activated phenotype of naïve iNKT cells characterized by CD69 and CD44 expression, the hypothesized absence of persistent antigen unless continuous DCs present the endogenous ligand for an extended period, and rescue of hypoproliferation by IL-2 are characteristics of clonal anergy, while anergy induction in naïve iNKT cells and suppression of IFN- γ and IL-4 production closely resemble adaptive tolerance.

It has long been known that calcium/calmodulin/calcineurin pathways are involved in the induction of clonal anergy as cyclosporine was able to block the induction (218). Specifically, it has recently been shown that NFAT1 activation is responsible for the induction of clonal anergy (142). On the other hand, during maintenance of clonal

anergy, activation of the calcium/calcineurin pathway in the cell is intact. The blockade instead appears to be in activation of the Ras/MAP kinase pathway and caused by constitutive activation of a GTP activating protein (GAP) (219, 220). Downstream ERK and JNK pathways are inhibited as a result of inactive Ras. The biochemical block in adaptive tolerance seems to be more proximal. It was shown in the staphylococcal enterotoxin B (SEB) model that adaptive tolerance inhibited activation-induced TCR zeta chain (p23) and ZAP-70 phosphorylation (221), and consequently intracellular calcium mobilization (222, 223). In cytochrome *c* double transgenic mice this effect was correlated with a block in phospholipase C- γ 1 phosphorylation, required for the generation of the IP3 that mediates calcium release from intracellular stores (137). In contrast, activation of the Ras/MAP kinase pathway does not seem to be involved. Additionally, biochemical block in signal transduction through the IL-2 receptor is also found in adaptive tolerance preventing exogenous IL-2 from restoring the proliferative capacity.

These findings in T cell anergy provide important insights into iNKT cell hyporesponsiveness. It would be extremely interesting to see whether Ras/MAP kinase pathway or proximal tyrosine kinase phosphorylation/PLC- γ 1 phosphorylation is affected in our model of bacteria- and α -GalCer-induced iNKT cell hyporesponsiveness. The possible involvement of NFAT1 would be also an interesting area of future research. In addition, we have preliminary findings suggesting the involvement of PD-1 in the induction of iNKT cell anergy. The molecular mechanism of T cell exhaustion during chronic infection mediated by PD-1 is unclear and further investigation into PD-1 signal transduction might be important.

Our results reporting iNKT cell hyporesponsiveness induced by bacteria have a

number of important implications for the therapeutic application of iNKT cell biology in disease models. It should be noted that infection by bacteria is a frequent occurrence in the human population, particularly in patients afflicted with various autoimmune diseases or cancer that are targets of iNKT-cell based therapies. In this setting, bacteria-induced hyporesponsiveness is extremely relevant. Indeed, during clinical trials, it has become apparent that iNKT cell prevalence and function in the human population is extremely variable (164, 178), which may be due to both genetic factors as well as environmental factors such as infection.

These findings may impose certain limitations on iNKT cell based therapies. In particular, significant progress has been made in utilizing α -GalCer or α -GalCer-pulsed DC for treatment of non-small cell lung cancer and various other metastatic malignancies (103-107). As efficacy of treatment will heavily depend on the function of iNKT cells, further investigation of the impact of infection on iNKT cell functions in human is warranted. If our murine studies indeed extend to human subjects, it may be critical to either modify treatment regimens to avoid use of α -GalCer therapy in patients previously exposed to anergy-inducing pathogens, or develop adjuvants that can break or overcome iNKT cell hyporesponsiveness.

In contrast, bacteria-induced hyporesponsiveness might actually benefit some patients suffering from diseases exacerbated by iNKT cell function such as human autoimmune hepatitis, atherosclerosis, and others. Indeed, as prior exposure to bacteria reduced disease severity during the ConA-induced hepatitis model, human autoimmune hepatitis might likewise be alleviated by infection. There has been growing scientific consensus that the exponential increase in the incidence of autoimmunity in developed countries may be a result of excessive hygiene and absence of beneficial infections that

may limit development of autoimmunity. While it may be absurd to deliberately infect people with pathogens to prevent autoimmune diseases, administration of certain microbial products such as flagellin with minimal toxicity that can induce iNKT cell hyporesponsiveness may prove useful in alleviating these diseases.

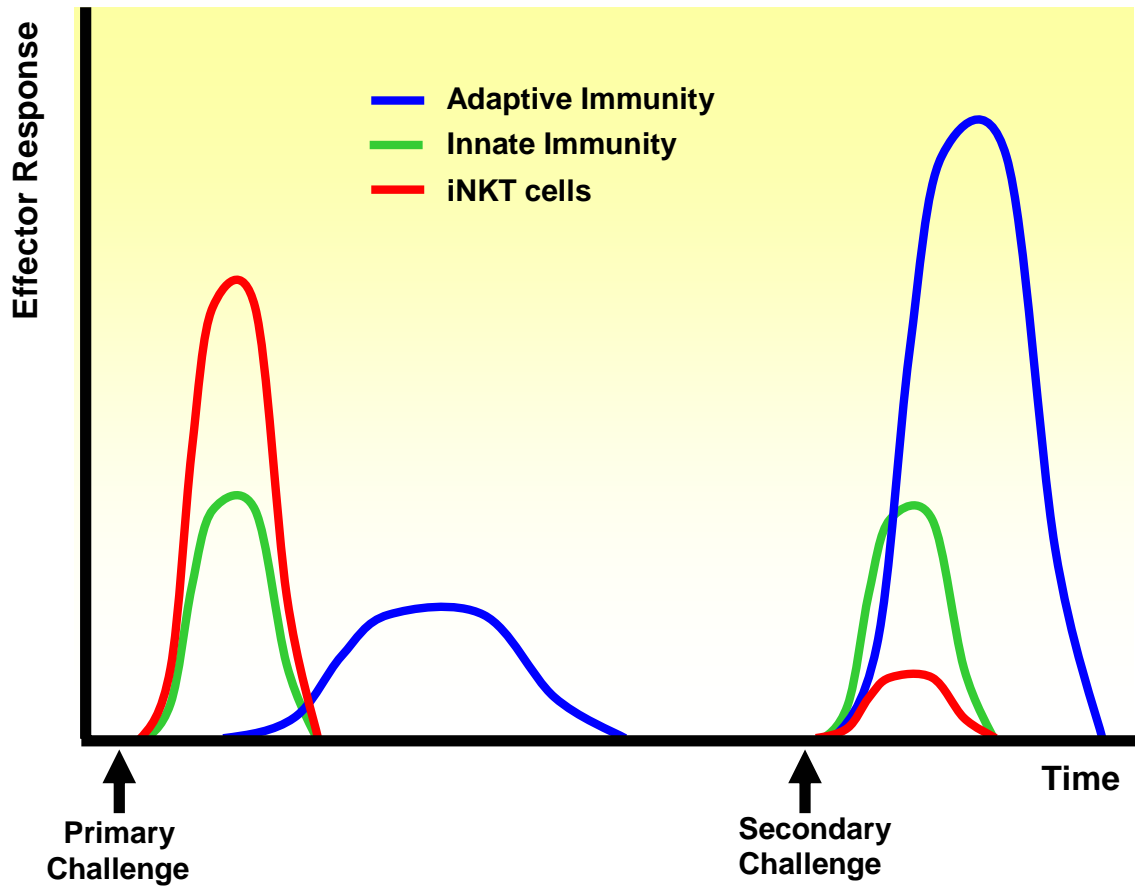


Figure 34. The primary and secondary responses of the innate immune system, the adaptive immune system, and iNKT cells. Innate immunity is characterized by an immediate response to the challenge. The primary and secondary responses are similar. Adaptive immunity is characterized by the latency during the primary response and a more rapid and stronger secondary response, which is termed a memory response. iNKT cells exhibit a rapid response during the primary response followed by hyporesponsiveness during the secondary response.

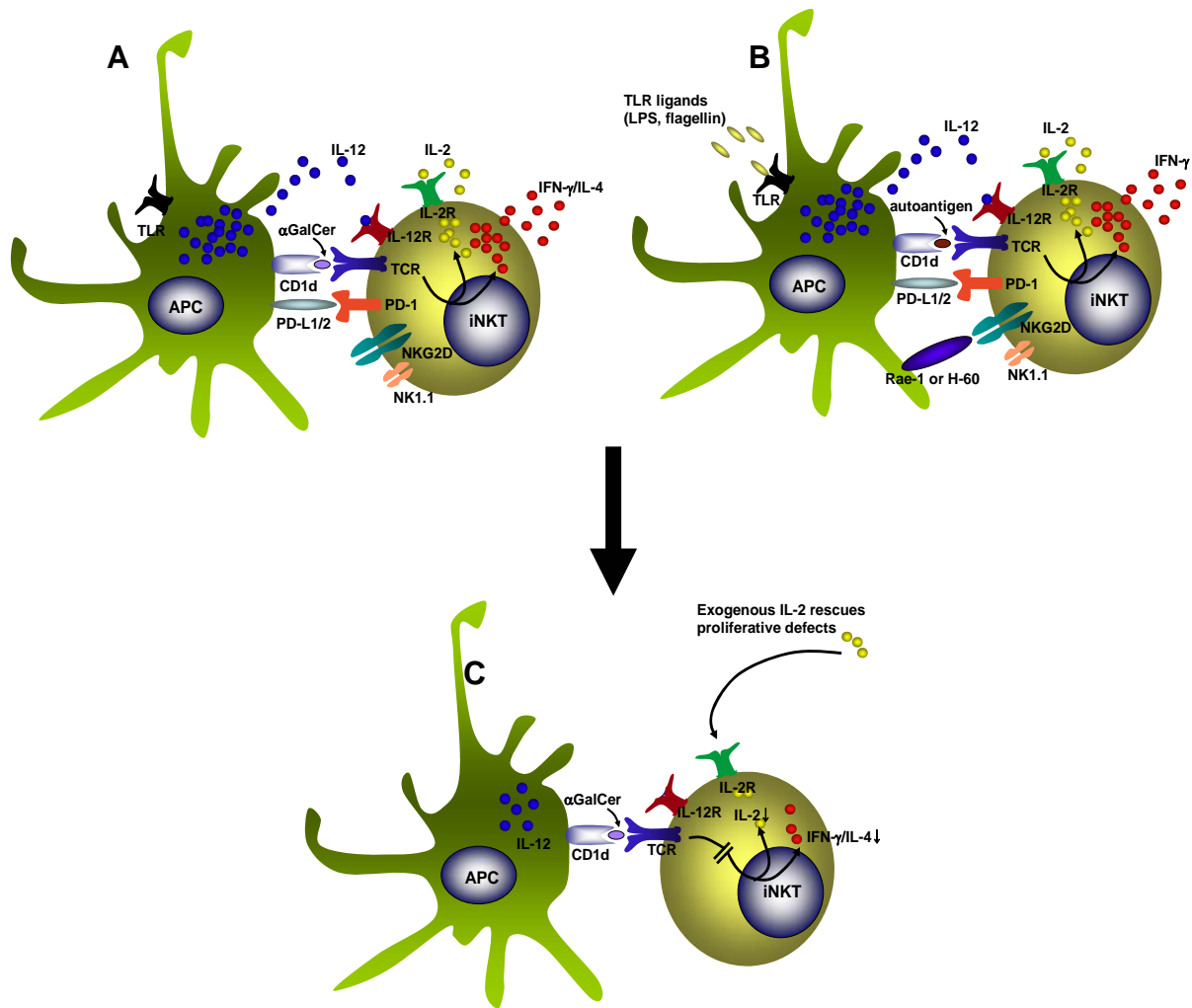


Figure 35. The model of α -GalCer- or bacteria-induced iNKT cell hyporesponsiveness. (A) α -GalCer is presented by CD1d expressed on APCs, which ligates the TCR of iNKT cells. In response iNKT cells produce IFN- γ and IL-4 which reciprocally activate APCs to secrete IL-12 that in turn further activates iNKT cells. Additionally, PD-L1/2 expressed on APCs engage PD-1 upregulated on iNKT cells. (B) Bacterial products including TLR ligands, for example LPS or flagellin, signal through TLR on APCs, likely DCs activating these cells. Activated DCs produce IL-12 and present an endogenous glycolipid ligand to iNKT cells. Activation of iNKT cells results in IFN- γ secretion. Expression of PD-L1/2 and induced expression of Rae-1 or H-60 engage with PD-1 or NKG2D expressed on iNKT cells possibly providing additional signals important for the induction of iNKT cell hyporesponsiveness. (C) During rechallenge with α -GalCer, iNKT cells from mice previously activated with a primary challenge with α -GalCer or bacteria show defects in TCR signaling pathway as well as secretion of IL-2, although IL-2 receptor signaling pathway is intact. This results in a suppressed response of iNKT cells to the rechallenge with α -GalCer.

APPENDIX

CELL-FATE MAPPING OF BONE MARROW STEM CELLS

Abstract

The hematopoietic stem cell (HSC) is defined as a self-renewing and multipotent cell that continuously repopulates the hematopoietic system throughout adult life. Among various tissue-derived stem cells, the HSC is one of the best characterized stem cells. It is also the only stem cell that is clinically applied in the treatment of diseases such as breast cancer, leukemia, and congenital immunodeficiency. At present, the scope of clinical application of these cells is largely limited to the hematopoietic system. However, recent developments in stem cell biology are opening up new possibilities. The prevailing concept has been that the self-renewal activity of HSCs maintains the HSC pool, and differentiation of HSCs provides a fresh supply of blood cells. By contrast, recently described bone marrow-derived multipotent adult progenitor cells (MAPC), which retain pluripotency, including the ability to repopulate the hematopoietic system, challenge the established idea that tissue-specific HSCs are maintained only through self-renewal activity. Furthermore, it is now thought that HSCs may be able to transdifferentiate into other tissue types such as neural, cardiac, or skeletal muscle tissue, although this capacity to transdifferentiate remains controversial. The use of adult stem cells has gained in popularity as the best means to bypass the ethical complication of

embryonic stem cell research. We hypothesized that HSCs derive from a precursor population, and can transdifferentiate into non-hematopoietic tissues as well as hematopoietic tissue *in vivo*. To test this hypothesis, I attempted to develop a transgenic mouse model that can be used for genetic tracing of the hematopoietic lineages. This mouse model system is designed to provide a definitive answer to the question of the origin of HSCs using an approach that mimics the natural *in vivo* system more closely than prior studies.

Background and Significance

Stem cells

Stem cells are a subset of cells in the body that maintain self-renewal activity and have the capacity to differentiate into multiple cell types. Stem cells may be categorized into two different types. One is pluripotent stem cells that differentiate into all three germ layers of endoderm, ectoderm and mesoderm. This cell type includes embryonic stem cells, embryonic germ cells, and multipotent adult progenitor cells (MAPC) (224). For instance, embryonic stem cells derived from the inner cell mass of the murine blastocyst *in vitro* have the ability to contribute to somatic and germline tissues when injected into the blastocyst of another mouse, which is exploited for generation of gene-targeted mice. Their use in humans is limited due to ethical, political and legal reasons. On the other hand, another type of stem cells is multipotent stem cells isolated from various tissues in fetal and adult animals. These cells differentiate into a limited number of lineages, usually restricted to the tissue these cells derive from. Somatic tissue-specific stem cells such as bone marrow hematopoietic stem cells (HSC), neuronal stem cells, hepatic stem cells and epidermal stem cells are in this category of cells. A number of different types of putative stem cells are found in the mouse bone marrow: HSC, MAPC, mesenchymal stem cell (MSC) and germline stem cell (GSC). However, the exact relationship among these stem cells as well as their true function *in vivo* remains elusive (Figure 36).

Hematopoietic stem cell (HSC)

HSCs are functionally defined by their unique capacity to self-renew and to give rise to all blood cell types. HSCs are the best characterized adult stem cells at present and

can be identified through surface staining. HSCs do not express mature blood lineage markers and express sca-1, c-kit and a low level of thy-1.1. Following the initial observation of stem cell activity in the sca-1⁺ fraction of the mouse bone marrow (225, 226), identification of c-kit expression allowed researchers to identify a highly enriched population of cells with hematopoietic stem cell activity within the mouse bone marrow (227-230). These cells are subdivided into long-term HSCs that have an extensive self-renewal activity and short-term HSCs that arise from long-term HSCs and have limited capacity to self-renew while retaining multipotency (231-233). These subsets of HSCs are distinguished by markers such as tie-2 and flt-3 (234, 235). It has been shown that a single HSC can give rise to long-term multilineage reconstitution and self-renewal in irradiated mice (236, 237). In addition, HSCs can be enriched based on the fact that they exclude Hoechst dye, a phenotype that represents an alternative description of HSCs (238).

The downstream progeny of HSCs that are lineage restricted oligopotent progenitor cells have also been identified (Figure 36). Common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), and early thymic precursor (ETP) are examples of progenitor cells (239-244).

Bone marrow multipotent adult progenitor cells (MAPC) copurifying with mesenchymal stem cells (MSC) are pluripotent stem cells.

Recent studies in stem cell research have revealed that bone marrow is a complex organ harboring a wide variety of stem cells aside from hematopoietic stem cells. MSCs and MAPCs are such examples. MSCs represent stromal cell precursors responsible for maintaining the structural integrity of bone marrow by generation of mesenchymal

tissues. Jiang *et al.* (224) reported the discovery of a putative pluripotent stem cell population in the bone marrow copurifying with MSCs. These cells were termed multipotent adult progenitor cells (MAPC) and are the focus of this research work. These cells were identified by limiting dilution of non-hematopoietic bone marrow cells and subsequent *in vitro* culture. FACS analysis of these cells indicate lack of CD34, CD44, CD45, c-kit, and major histocompatibility complex (MHC) class I and II expression, low levels of flk-1, sca-1 and thy-1 expression, and significant levels of CD13 and stage-specific antigen 1 (SSEA-1). It should be noted that MAPCs do not express any markers of the hematopoietic lineage, including the markers for HSCs (*e.g.* CD34, c-kit). A single MAPC is found to differentiate into cells of visceral mesoderm, neuroectoderm and endoderm *in vitro* and, when injected into an early blastocyst, a single MAPC contributes to most somatic cell types. Upon transplantation into a non-irradiated host, MAPCs engraft and differentiate to the hematopoietic lineage, in addition to the epithelium of liver, lung and gut. As MAPCs proliferate extensively without obvious senescence or loss of differentiation potential, these cells may substitute embryonic stem cells or cord blood cells as the cell source for therapy of inherited or degenerative diseases through autologous transplantation without complications from graft rejection (245-247).

Pluripotency of MAPCs is demonstrated by *in vitro* culture, by blastocyst injection, and by adoptive transfer experiments. In particular, MAPCs have been shown to give rise to hematopoietic tissue, suggesting that these cells may indeed be a precursor population that generates HSCs *in vivo*. On the other hand, the self-renewal activity of hematopoietic stem cells is considered the primary mechanism of maintaining the pool of HSCs at present, whereas the function and phenotype of MAPCs *in vivo* still remains elusive.

HSC tissue plasticity

Traditionally, adult stem cells have been viewed as cells restricted to the particular tissue of origin unable to differentiate into other tissues. Various reports in recent years challenge this idea by demonstrating that adult stem cells, under certain microenvironmental conditions, give rise to cell types in non-related tissues, possibly indicating that they can switch cell fate. In particular, HSCs, besides forming blood cells, have been reported to give rise to liver cells (248), skeletal muscle cells (249-255), pancreatic islet cells (256), and other cell types.

For instance, skeletal muscle is a site where significant tissue transdifferentiation has been observed. Bone marrow mononuclear cells transplanted into immunodeficient mice migrate to areas of muscle degeneration where they play a role in the regeneration of the damaged fibers (249). Subsequent studies showed that transplantation of enriched HSCs into irradiated mdx mice, a mouse model with increased muscle cell turnover, leads to a low level contribution to muscle and partial restoration of dystrophin expression (250). Whether this occurs via simple fusion of HSCs with the muscle fiber, or via transdifferentiation of HSCs into muscle satellite cells followed by fusion with muscle fibers is currently uncertain. One study provides evidence for a stepwise progression of bone marrow cells – which may be different from HSC as the cells were not purified – to satellite cells, mononucleated muscle stem cells, and then to multinucleated myofibers (252), whereas such a progression could not be reproduced in another study (255).

Bone marrow derived mononuclear cells have also been described to contribute to pancreatic islet cell regeneration. Following transplantation of bone marrow cells from mice that express green fluorescent protein (GFP) when the insulin gene is actively

transcribed, 1.7–3% donor derived insulin expressing cells could be detected in the pancreas of recipient mice (256). An appropriate control experiment ruled out cell fusion between bone marrow donor cells and recipient islet cells. Yet other studies suggested that, although donor cells can be detected in the pancreas, they might be endothelial and not endocrine cells. For instance, bone marrow transplantation led to normalization of glucose and insulin levels in streptozotocin-induced diabetic mice even before appearance of donor-derived cells in the pancreas thereby suggesting an indirect effect of bone marrow cells in insulin production of these mice (257).

The reports of tissue transdifferentiation have generated much confusion in the stem cell field amid excitement since the concept of tissue plasticity denies the principles in developmental biology that lineage restriction coincides with morphogenesis (258-262). Nevertheless, stem cell plasticity is an important phenomenon that deserves further attention as it can expand the scope of application for various tissue-specific stem cells. Previous reports on tissue transdifferentiation heavily relied on adoptive transfer experiments that have limitations in replicating the *in vivo* state of normal physiology.

Significance

The field of stem cell research is evolving rapidly and is currently receiving a strong focus in the scientific and political community. A wide spectrum of therapeutic applications in diseases such as neurodegenerative diseases, leukemia, myocardial infarction and diabetes await advancement in the stem cell field for a possible cure. Adult stem cells have a number of advantages to embryonic stem cells. Critical issues in ethics can be bypassed by using adult stem cells, and moreover, adult stem cells will continue to be a better source for histocompatible donor cells until human embryonic

cloning becomes a standard procedure and an extensive library of embryonic stem cell lines is available from which to choose histocompatible donors.

How various stem cell populations in the body are maintained, and what the relationships between the stem cells are, represent important questions that are not fully understood. Bone marrow harbors a heterogeneous set of stem cells that constitutes blood, bone, blood endothelium, and even germ tissues. Further, the possibility exists that a pluripotent stem cell population, MAPC, may be sustaining these tissue specific stem cells present in murine bone marrow as well as other stem cells localized in different tissues. Our understanding of bone marrow stem cells is still too primitive to provide a definite picture of lineage relationships, partly due to relative rarity of the stem cells as well as the extreme complexity and heterogeneity of cell types in the bone marrow unlike other tissues with limited types of parenchymal and stromal cells. The outcome of our proposed research will provide important clues to determine how various stem cell populations are maintained *in vivo*, and may help uncover unidentified stem cells in murine bone marrow that can be cultivated in stem cell research and therapy.

In addition, identification of GSCs brings new possibilities to the stem cell field and reproductive biology. Our investigation of the mechanism of menopause will include the complex interplay between bone marrow GSCs and the ovarian microenvironment and move away from a rather simplified model of declining number of oocytes in the female ovary. We may find that autologous transplantation of *in vitro* activated GSCs may prolong reproductive cycles in humans. *In vitro* development of oocytes from GSCs may become a less invasive source of human ova for *in vitro* fertilization compared to current ova extraction protocols.

It should be noted that the results obtained from our research proposal will be

applicable to human systems, as all the stem cell populations including HSCs described in mice have counterparts in human bone marrow with minor disparities in terms of surface phenotypes. Interestingly, MAPCs have also been isolated from rats and humans. Aside from the additional requirement of leukaemia inhibitory factor (LIF) by murine MAPCs for expansion, human and murine MAPCs share common features (224). There are striking similarities in the stem cell activity among different species due to highly conserved growth factors, cytokines and signaling pathways, which is speculated to be the natural consequence of the critical dependence of an organism on the activity of stem cells for survival throughout life.

The results from this research will help to resolve current scientific controversies regarding the fate of various stem cells and provide a way to expand the therapeutic applications of bone marrow stem cells to the treatment of cancer, diabetes, degenerative diseases, and congenital diseases.

Results

Overall strategy

The aim of this research work was to test the hypothesis that HSCs derive from a precursor population, and can transdifferentiate into non-hematopoietic tissues as well as hematopoietic tissues *in vivo*. To this endeavor, we have generated a transgenic mouse model to track bone marrow hematopoietic stem cells. The transgenic mouse model was meant to allow us to pulse-label HSCs *in vivo*, and to track the progeny from these cells. By determining whether the HSC pool is only comprised of HSCs derived from the previously pulse labeled HSCs, or is also comprised of unlabeled HSCs, we can infer the presence or absence of a precursor population (Figure 37). Specifically, MAPC represents the most likely candidate for this precursor population in light of its pluripotency. In addition, by tracking pulse labeled HSCs in other somatic tissues, we can study transdifferentiation of HSCs *in vivo*.

In detail, the bigenic transgenic system for cell-fate mapping of bone marrow stem cells that we proposed to develop consists of CreER, a fusion protein of cre recombinase and estrogen receptor, whose expression is driven by the c-kit promoter, and a reporter gene for CreER activity, LacZ/YFP (Figure 38). The c-kit promoter restricts CreER expression to c-kit⁺ stem cells, HSCs in particular (227, 230, 263), and avoids CreER expression in the putative precursor cells lacking c-kit expression (224). The c-kit promoter was deliberately chosen for the cell fate mapping of HSCs, as c-kit expression is currently the most reliable surface marker for identification of HSCs and pluripotent MAPCs are thought not to express c-kit. More importantly, an extensive promoter study confirms that the c-kit promoter used in our system with six Dnase hypersensitivity sites,

including important regulatory elements, is fully functional (264). The reporter gene driven by the β -actin promoter is actively transcribed ubiquitously throughout mouse development allowing us to track the stem cells differentiating into any cell types.

CreER is a fusion protein of Cre and a modified estrogen receptor that responds only to tamoxifen, an estrogen receptor agonist, and not to endogenous estrogen. In the absence of tamoxifen, CreER protein is normally excluded from the nucleus without access to floxed genes in the nucleus. In the presence of tamoxifen, however, CreER bound to tamoxifen translocalizes into the nucleus and its recombinase activity provided by the cre component of the fusion protein deletes the sequences encompassed by two loxP sites (265, 266). As the expression of CreER is restricted to c-kit⁺ stem cells, the recombination event will take place only in these stem cells and not any other cells.

The recombinase activity by CreER initiated by tamoxifen will result in the rapid deletion of the LacZ gene followed by *de novo* expression of yellow fluorescent protein (YFP) in the bigenic mice containing the LacZ/YFP construct. This genetic change is irreversible and permanent for the rest of the life of the cell that was expressing CreER at the time of tamoxifen injection. All future progeny of this cell will continue to express YFP, even in mature differentiated cell types that have lost c-kit expression, as the YFP gene is driven by the ubiquitously active β -actin promoter and not by the c-kit promoter. The ubiquitously active LacZ/YFP reporter gene, together with the stem cell-restricted CreER gene, allows us to pulse label stem cells and monitor self-renewal of these cells and/or differentiation of the cells into various lineages.

C-kit.CreER.ires.eGFP construct

C-kit is the tyrosine kinase surface receptor for stem cell factor (SCF). C-kit is

currently the single most important and functionally relevant surface marker for identification of HSCs and a number of other tissue-specific stem cells. The interaction between SCF and c-kit has been found critical for the maintenance of the HSC compartment by promoting self-renewal activity. Recently identified GSCs are also thought to be enriched among the c-kit⁺ population in murine bone marrow. GSCs are distinguished from HSCs by lack of sca1 expression. GSCs also express germline markers including mvh, Oct4, Dazl, Stella, Fragilis, and Nobox (263).

On the other hand, the putative precursor population to HSCs, MAPC, does not express c-kit. While the phenotype of MAPC in fresh bone marrow is unknown, MAPCs in culture do not display c-kit expression. This morphology and phenotype of MAPCs does not change after 30 to more than 120 population doublings (224).

The success of this research depends heavily on the quality of the promoter driving CreER expression. Mouse c-kit gene is over 80-kb long and includes at least 21 exons; the first 2 exons are separated by a large (about 20 kb) intron (267). Inherited c-kit expression defects due to distant upstream deletions suggest the existence of long-range-acting regulatory sequences (267-270). More importantly, the 5kb 5'UTR fragment of the c-kit gene is unable to drive the expression of a reporter gene in murine bone marrow (264, 271). In order to identify additional proximal regulatory elements Cairns *et al.* (264) cloned about 10 kb of 5' flanking sequences and the whole first exon and intron, and explored DNase I sensitivity of this DNA region in chromatin from c-kit-expressing hematopoietic, melanocytic, and embryonic stem (ES) cells as well as hematopoietic cells that do not express c-kit. Six DNase hypersensitivity regions were identified and a promoter construct including all the DNase hypersensitivity regions was generated and subsequently injected to study its function in the transgenic mouse system. The promoter

was able to drive GFP expression, the readout in this promoter study, in immature bone marrow cells and downregulate GFP expression upon maturation (264).

The construct was cloned using standard cloning techniques and the overall schematic of the construct is shown in Figure 39A.

Cell line transfection of c-kit.CreER.ires.eGFP construct

The P815 cell line was selected for testing the integrity of the construct, as P815 is a mastocytoma cell line expressing c-kit in which the construct should be active. Following transient transfection with Fugene lipofectamine, the expression of CreER was examined by staining with the primary rat-anti-Cre-Ab and the secondary rabbit-anti-ratIgG-PE-Ab 48 hours post transfection. CreER expression was observed in the transfected P815, but not in untransfected cells. Neither transfected nor untransfected cells showed staining with an isotype control for the anti-Cre-Ab (Figure 39B). The transfection result was reproducible in an independent experiment with P815 cells.

Pronuclear injection of c-kit.CreER.ires.eGFP construct and pups carrying the transgene

The construct was linearized by AatII and SalI sequential digestion and was provided to the Vanderbilt transgenic core facility for pronuclear injection. Two separate injections were performed and 26 pups were born. Two pups died soon after birth. 26 pups including the dead pups were genotyped by performing 300 bp PCR for detection of the CreER sequence. Four pups carried the transgene (Figure 39C).

CreER expression on bone marrow hematopoietic stem cells

The pups carrying the transgene were initially bred with C57BL/6 wild type mice.

The resulting pups carrying transgenes from founder 12, 20, and 22 were analyzed for expression of CreER using anti-cre antibody we received from Dr. Guoqiang Gu. When gated on large cells, a slight shift in cre staining was observed in pups from founder 12 whereas pups from 20 and 22 showed no staining with this antibody (Figure 40A). We also used anti-cre antibody obtained from Dr. Polk's laboratory, but non-specific staining prevented further evaluation of cre expression (data not shown).

Next, we tested the reporter activity of cre instead of relying on cre staining to analyze cre expression profile. The founder transgenic mice were bred with LacZ/YFP reporter mice. These mice express YFP protein upon recombination by cre. The double transgenic pups were genotyped and the first batch of pups from founder 18 was treated with tamoxifen-infused food for three weeks. Each mouse was fed with 2g of food that contained 2mg of tamoxifen on a daily basis. Following tamoxifen administration, we waited one month for mature hematopoietic cells to develop from YFP labeled hematopoietic stem cells and analyzed YFP expression with LSRII. These double transgenics showed no sign of YFP expression (Figure 40B).

Adoptively transferred bone marrow stem cells do not contribute to germline parasitism.

Germline parasitism by donor bone marrow cells in the recipient mice was examined by generating bone marrow chimeras of Ly5.1 and Ly5.2 male mice where the bone marrows of Ly5.2 recipient mice are replaced by the Ly5.1 bone marrow. The male recipient mouse was immediately bred with an Ly5.2 wild type female mouse and the progeny was analyzed for the expression of Ly5.1 and Ly5.2 with flow cytometry. The recipient mice were unable to reproduce for three months following lethal irradiation. These mice showed accelerated aging and started exhibiting white and gray coat color.

However, the mice regained reproductive capacity and began to breed. 13 pups were born from the chimeric male mice and wild type female mice. Surprisingly, all 13 pups from the breeding showed no expression of donor derived Ly5.1 indicating that germline parasitism did not occur (Figure 41). This result is in line with a recent publication by Eggan *et al* refuting extragonadal source of germline stem cells using a parabiotic system (272).

Discussion

Four transgenic founders were obtained from the pronuclear injection of the completed construct containing CreER gene under the control of the c-kit promoter. Progeny from three of the founders did not stain with cre antibodies obtained from various sources, but progeny from one of the founders showed minor staining. As cre antibodies in general have problems for use in fluorescent-activated cell sorting (FACS) and immunohistochemistry, we have decided to test the reporter activity of cre, instead of relying on cre staining to analyze cre expression profile. The founder transgenic mice were bred with LacZ/YFP reporter mice, which express YFP protein upon recombination by cre. The resulting double transgenic pups were genotyped and treated with tamoxifen-infused food for three weeks. However, these double transgenics showed no sign of YFP expression (Fig 40B).

One possibility was that p.o. administration of tamoxifen is not optimal for CreER activation of HSCs. We have subsequently administered tamoxifen i.p. when testing other founders, but have seen no recombination event. It was also a possibility that the YFP signal was not strong enough for detection. LSRII at the flow cytometry core facility did not have an excitation laser at 514nm that induces maximal excitation of YFP and used 532nm laser. While YFP does have a range of excitation wavelengths, the possibility remained that suboptimal excitation of YFP paired with low expression of the protein might have adversely affected the outcome of this experiment. Unfortunately, the antibodies available for YFP that can boost the signal are not optimal for FACS. Therefore, another cre reporter strain that expresses LacZ upon recombination that shows better sensitivity to cre activity was one alternative. We have however decided to pursue

another alternative, which was to use fluorescent microscopy using a specific filter for YFP. However, none of the progeny treated with tamoxifen showed YFP expression on fluorescent microscopy. As a result, we have decided that even the founder showing minor staining with anti-cre antibody is expressing CreER at levels too low to achieve optimum levels for efficient recombination of the reporter gene and decided another round of pronuclear injection of the construct might be necessary.

The transgenic mouse system that we generated was also to be used to test the hypothesis that the bone marrow stem cells may contribute to the generation of reproductive cells. The central dogma of reproductive biology has been that females of most mammalian species lose the capacity for oocyte production during fetal development, and only a finite number of oocytes present postnatally are responsible for the lifetime reproductive activity (273-277). For instance, in humans it has been estimated that, of 10^6 oocytes present at birth, less than 3×10^5 survive through puberty. The number of surviving oocytes decreases over time until menopause hits females as the remaining oocytes are unable to sustain the menstrual cycle. Similar observations have been made in female mice, which exhaust their pool of oocytes with age. A series of recent studies have, however, challenged this dogma by showing rapid turnover of murine oocytes during juvenile and adult life of mice (278). This high turnover rate of oocytes observed in adult mice was originally attributed to the non-follicle-enclosed germ cells present on the ovarian surface, but their number dropped precipitously following puberty and another source of oocytes was suspected. A landmark paper by Johnson *et al.* subsequently identified a putative germline stem cell (GSC) population in murine bone marrow and in peripheral blood that migrates to mouse ovary and generates bona fide oocytes (263). Germline contribution of these bone marrow stem cells, distinct from

hematopoietic stem cells by their lack of sca-1 expression but sharing c-kit expression, was shown by adoptive transfer of whole murine bone marrow into recipient mice whose ovaries were chemically ablated. Adoptive transfer of peripheral blood into recipient mice also supported oogenesis, implying continuous seeding of GSCs into ovary via blood circulation.

The question remains whether the oocytes developing from these transplanted GSCs are able to fully mature functionally and undergo fertilization with male sperms, or turn out to be an artificial non-functional development of stem cells misplaced from their original niche space. Therefore, while the transgenic mouse system was being optimized, I have also tested whether bone marrow stem cells can differentiate into ovarian follicles in an alternative method using the adoptive transfer system. As female mice subjected to lethal dose of irradiation undergo ovarian atresia which likely does not recover over time, germline parasitism by donor bone marrow cells in the recipient male mice was examined instead. The result showed that 13 pups were born from the chimeric male mice and wild type female mice showed no expression of donor derived Ly5.1 indicating that germline parasitism did not occur (Figure 41). This result is in line with a recent publication by Eggan et al refuting extragonadal source of germline stem cells using a parabiotic system (ref).

Once the transgenic mouse model system for cell-fate mapping of hematopoietic stem cells becomes available, we will test the hypothesis that HSCs derive from a precursor population, and can transdifferentiate into non-hematopoietic tissues as well as hematopoietic tissue *in vivo*. We will also address the contribution of bone marrow stem cells in gametogenesis. More specifically, we will test our hypothesis in two specific aims. In aim 1, we will determine the presence or absence of a precursor population to HSCs.

HSCs will be pulse-labeled with YFP and followed over time. A decreased number of YFP⁺ cells over time would demonstrate the presence of a precursor population to HSCs. Additionally, YFP-labeled HSCs will be examined with or without irradiation to determine the effect of bone marrow injury on the putative precursor population. We hypothesize that injury will activate a normally dormant precursor population, namely MAPC, to become activated and differentiate into HSCs. In aim 2, we will examine transdifferentiation of HSCs into non-hematopoietic tissues. Cell-fate mapping of HSCs will provide a definitive answer to the question of whether transdifferentiation occurs *in vivo*. The progeny of pulse-labeled HSCs will be followed in various tissues including pancreatic islets, heart, and skeletal muscle, to search for transdifferentiated HSCs. The putative bone marrow GSC that expresses c-kit will also be pulse-labeled using the same cell-fate mapping system in female mice. The contribution of GSCs in ovarian oogenesis will be observed in the presence or absence of injury *in vivo*.

These studies will enhance our understanding of the critical cellular events involved in the development of the hematopoietic system. The results from this research are designed to provide insight into the fate of stem cells and expand the therapeutic applications of stem cell biology.

Materials and Methods

Mice. C57BL/6 (B6) mice and Ly5.1 (B6SJL) mice were purchased from the Jackson Laboratory. LacZ/YFP mice were obtained from Dr. Mark Decaestecker's laboratory. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University (Nashville, TN).

Reagents. Restriction enzymes and DNA ligase were purchased from NEB. Anti-cre antibodies and rabbit-anti-rat-IgG-PE were kindly provided from Dr. Guoqiang Gu and Dr. Brent Polk. Anti-Ly5.2-FITC and anti-Ly5.1-PE were purchased from BD Biosciences-Pharmingen.

Cloning of of c-kit.CreER.ires.eGFP construct. The construct was cloned with standard cloning techniques. The c-kit promoter construct with eGFP was a kind donation from Dr. Sergio Ottolenghi at Universita Milano-Bicocca-Piazza delle Scienze, and CreER from Dr. Andrew McMahon at Harvard University. Ires sequence came from Stratagene. PBR322 was utilized as the plasmid backbone, to circumvent plasmid toxicity observed in initial cloning steps. GBE180, a pcnb deficient strain of DH5 α *E.coli* was used in the final steps of ligation in which ColE1-dependent plasmids such as pBR322 replicate in as low as one plasmid copy number per cell.

Genotyping of the transgenic mouse expressing c-kit.CreER.ires.eGFP construct. Mice were genotyped using standard PCR technique with tail DNA. The primers used to target the cre region of the construct were 5'-TGGAAGGCATGGTGGAGATCTTTG-3', and

5'-ATGAGAAGGAGCTGAGCTAGGCGG-3'.

Flow cytometry. Single-cell suspensions of P815 cells or primary bone marrow cells were prepared and stained with fluorescently-labeled mAbs as described previously (183). In all experiments, dead cells were excluded from the analysis by electronic gating. Flow cytometry was performed using a FACSCalibur instrument with CellQuest software (BD Immunocytometry Systems), or an LSRII instrument, and the acquired data were analyzed using FlowJo software (Tree Star Inc.).

Fluorescent microscopy. Single-cell suspensions of bone marrow cells were prepared and visualized with fluorescent microscopy using YFP filter. The cells were either fixed with 1% paraformaldehyde or not fixed prior to microscopy.

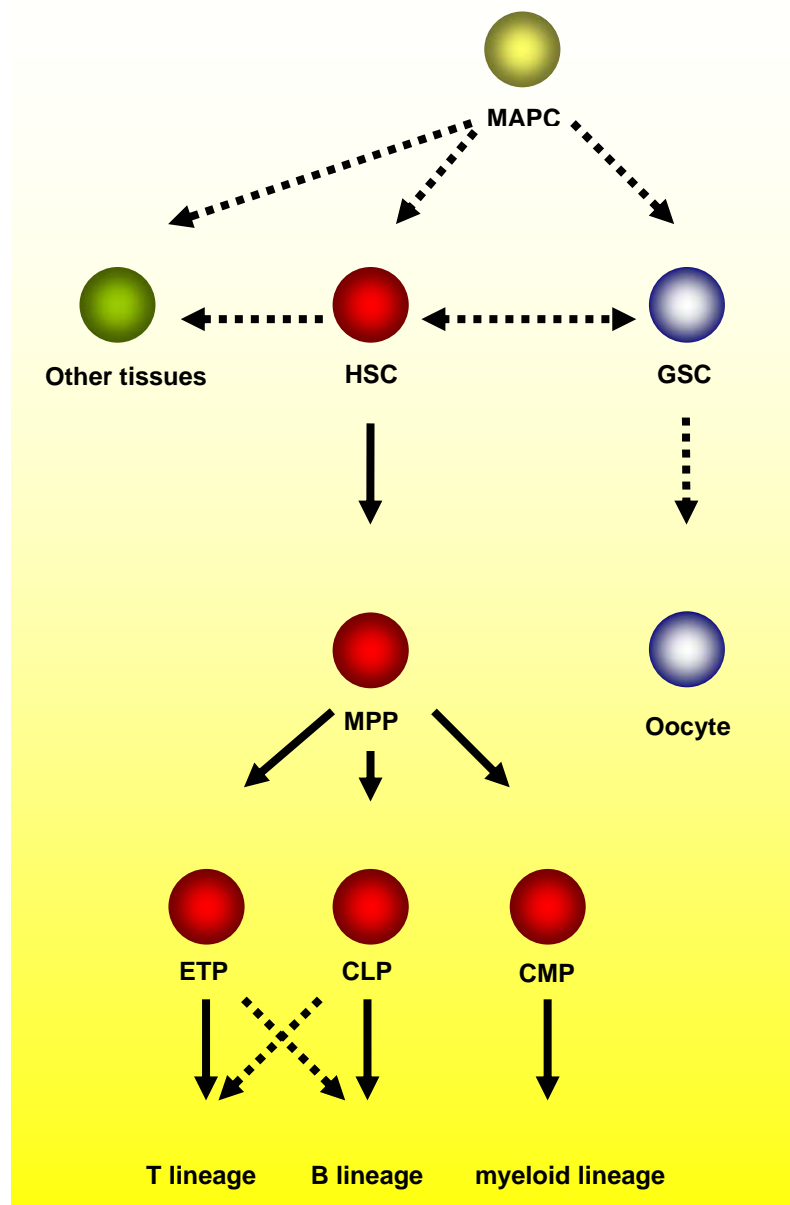


Figure 36. The lineage relationship among bone marrow stem cells and progenitor cells. Arrows indicate established relationship in vivo whereas dotted arrows indicate the capacity to differentiate but in vivo significance is uncertain. MAPC: multipotent adult progenitor cell, HSC: hematopoietic stem cell, GSC: germline stem cell, MPP: multipotent progenitor population, ETP: early thymic progenitor, CLP: common lymphoid progenitor, CMP: common myeloid progenitor.

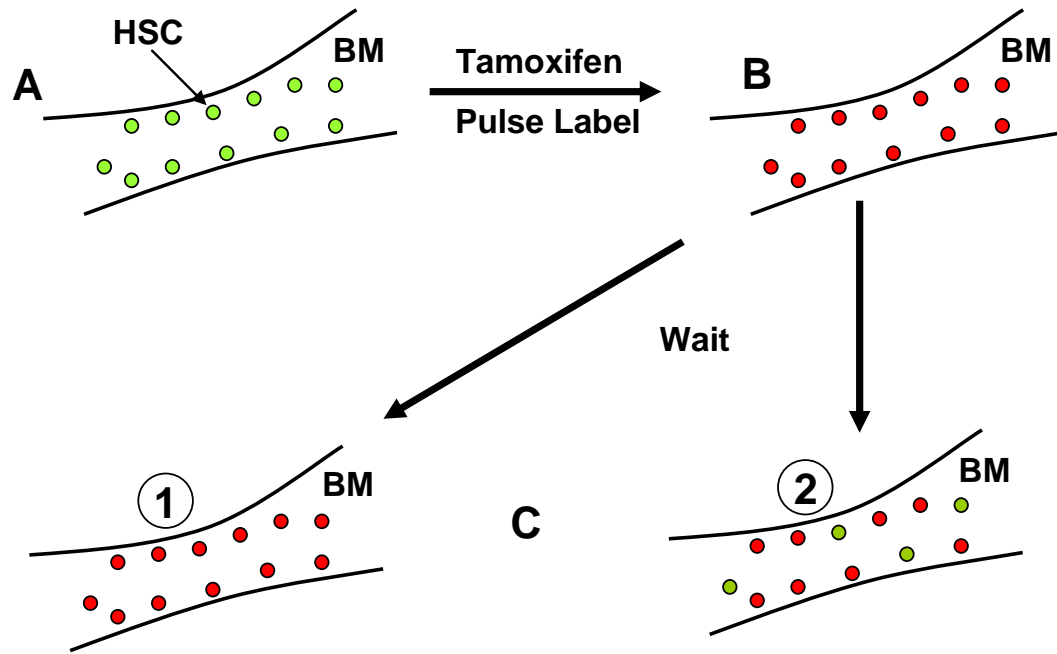


Figure 37. The overall experimental strategy for the cell-fate mapping of hematopoietic stem cells. **(A)** HSCs express CreER and GFP. **(B)** Tamoxifen treatment of HSCs results in RFP labeled HSCs. **(C)** Over time, labeled HSCs maintain the HSC compartment by themselves ① or a precursor population (MAPC) contributes to the HSC compartment ②.

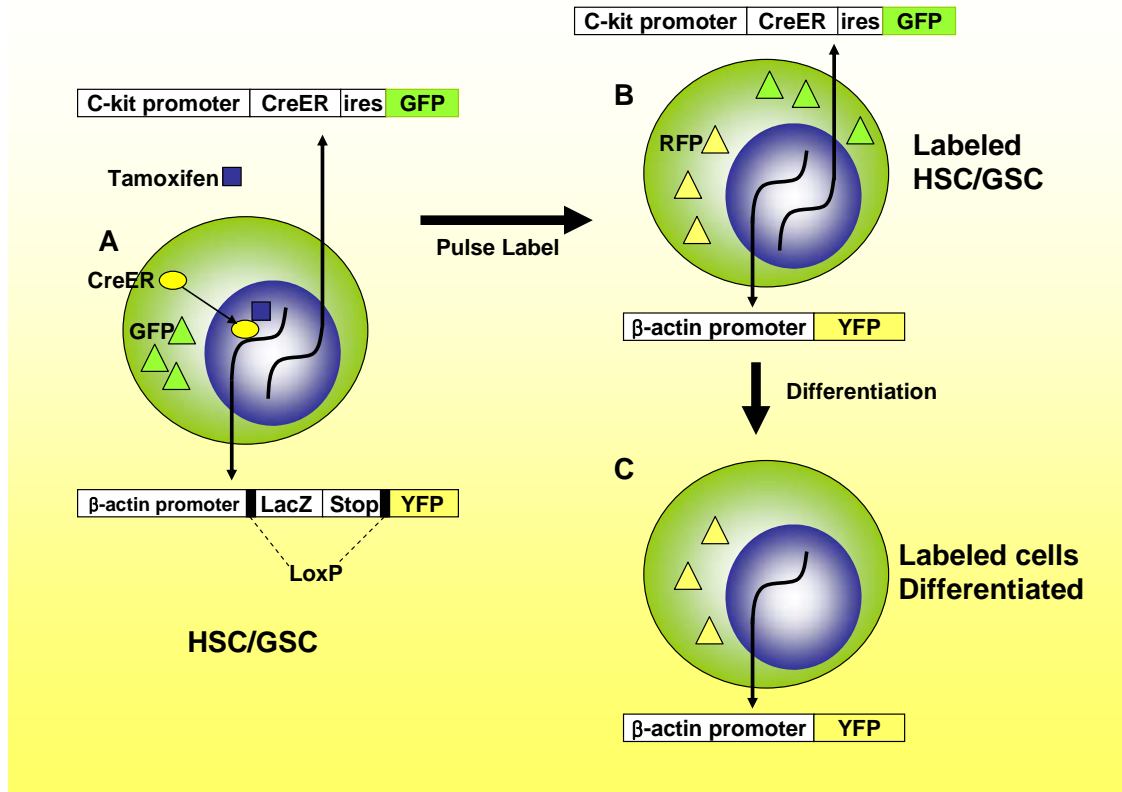


Figure 38. The schematic diagram for determining presence or absence of a precursor population to HSC. **(A)** HSCs express CreER and GFP. When tamoxifen is injected, CreER binds to tamoxifen, translocalizes into the nucleus, and recombines LoxP sites. **(B)** Upon recombination, the β -actin promoter begins to drive YFP expression in HSCs. **(C)** The progeny of these labeled stem cells will retain YFP expression following differentiation.

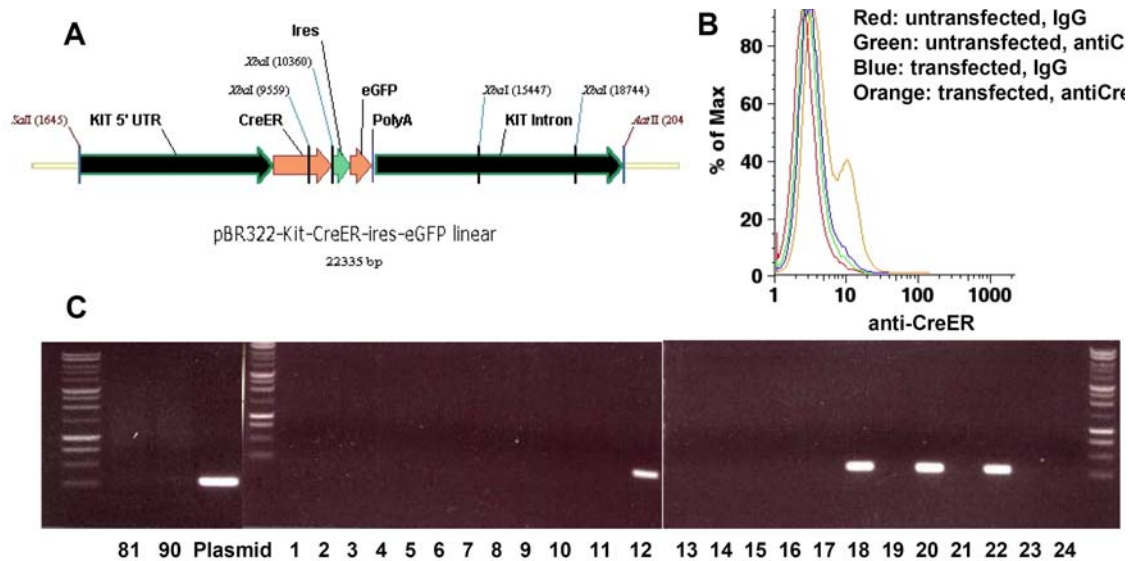


Figure 39. Generation of the transgenic mouse model for the cell-fate mapping of HSC. (A) Schematic representation of C-kit.CreER.ires.eGFP construct in pBR322 plasmid backbone. The plasmid was linearized by serial digestion with SalI and AatII and 19kb fragment containing 5'UTR of Kit promoter, CreER, ires, eGFP, polyadenylation signal, and Kit intron 1, was purified by gel electrophoresis in LMP agarose for pronuclear injection. (B) A fraction of purified construct from (A) was used for lipofectamine transfection of P815 mastocytoma cell line. The red peak represents untransfected cells with isotype control staining, the green peak untransfected cells with anti-CreER staining, the blue peak transfected cells with isotype control staining, and the orange peak transfected cells with anti-CreER staining. Data shown is representative of two independent experiments. (C) PCR analysis of pups from pronuclear injection of the purified construct into BDF1 background. 81 and 90 are dead pups. Plasmid is the positive control using the construct plasmid from (A). 1-24 are live pups that survived into adulthood. 12, 18, 20, and 22 are the pups carrying the transgene.

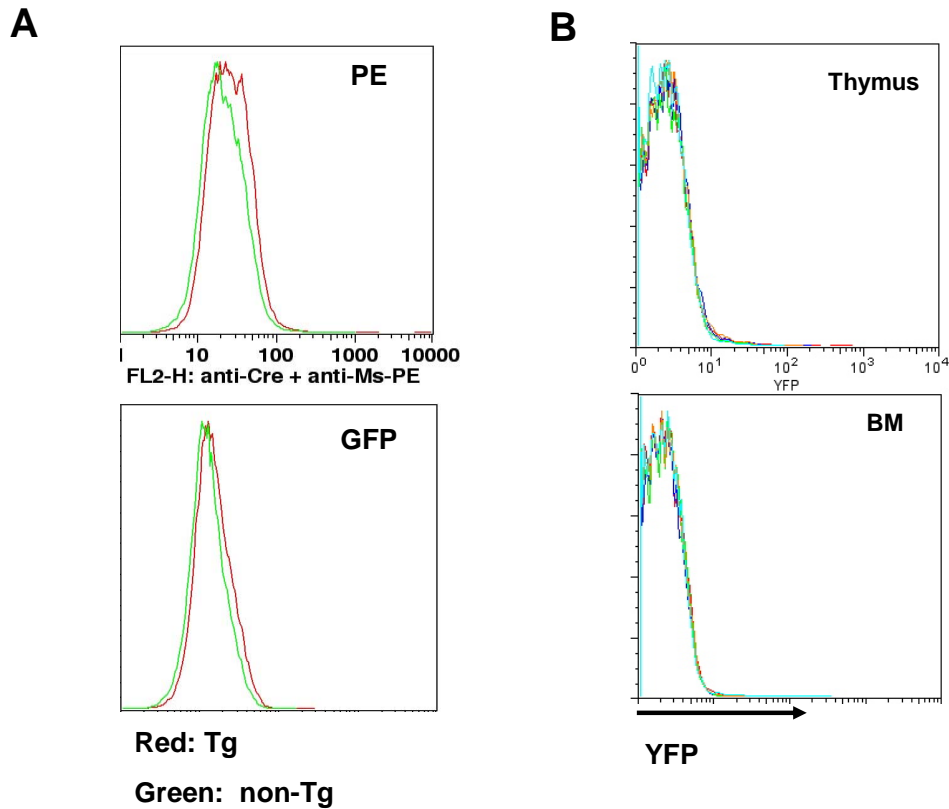


Figure 40. Expression of CreER by the transgenic mouse model. (A) Founder 12 stained with anti-cre IgG followed by secondary staining with anti-Ms-IgG-PE and gated on large cells from FSC and SSC profile. The upper plot shows the histogram on PE channel and the lower plot shows GFP channel. A minor shift is seen on founder 12. (B) Thymic and bone marrow cells were collected from three Founder 18 double transgenic pups treated with tamoxifen p.o. for three weeks and then sacrificed one month later and two LacZ/YFP transgenic without CreER. The cells were analyzed for YFP expression on LSRII. None of the mice showed YFP expression.

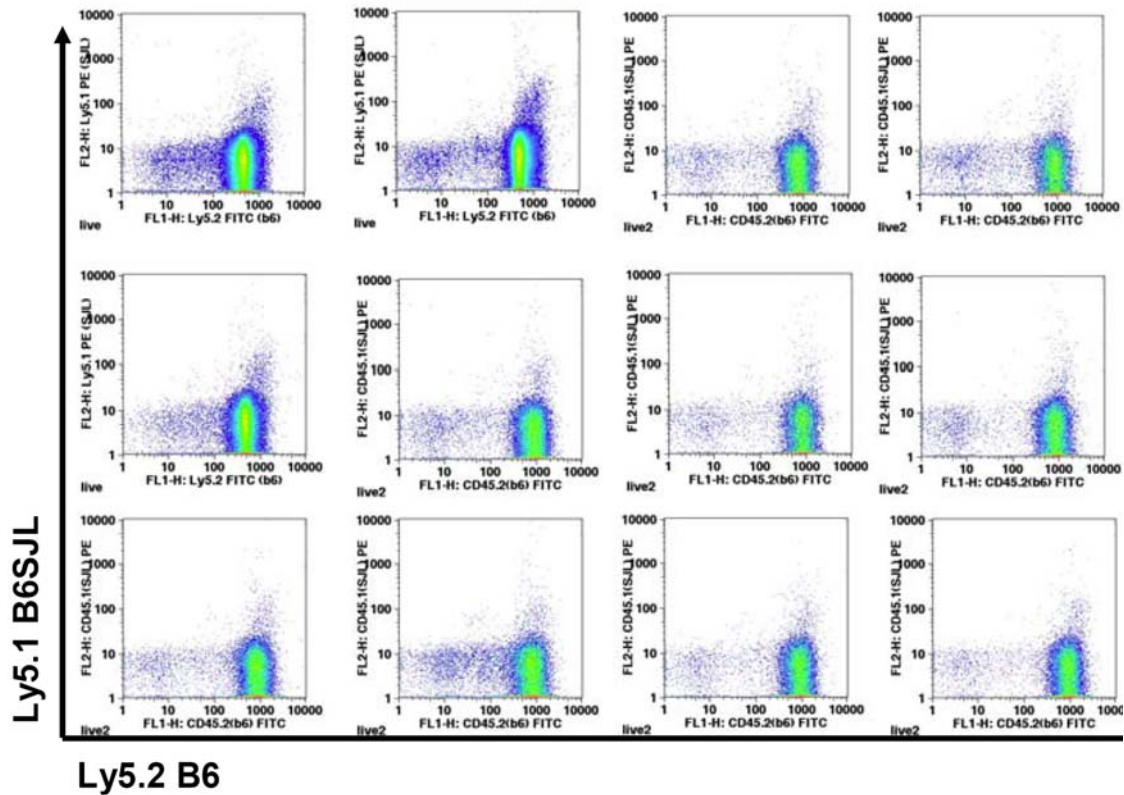


Figure 41. Adoptive transfer of bone marrow stem cells does not result in germline parasitism of the recipient mice. Germline parasitism by donor bone marrow cells in the recipient mice was examined by generating bone marrow chimeras of Ly5.1 and Ly5.2 male mice where the bone marrows of Ly5.2 recipient mice are replaced by the Ly5.1 bone marrow. The male recipient mice was immediately bred with Ly5.2 wild type female mice and the progeny was analyzed for the expression of Ly5.1 and Ly5.2 with flow cytometry. Twelve plots shown are Ly5.1 and Ly5.2 staining of the individual pupsprogeny. Transplanted Ly5.2 bone marrow did not contribute to reproduction of the recipient mice.

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