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Genetic Regulation of NKT Cell Function

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Abstract:

NKT cells are specialized T cells that play important roles in the host immune response to bacteria and viruses. NKT cells produce a wide variety of cytokines and chemokines after being activated by glycolipids such as α-galactosylceramide (αGalCer). Previous work suggested that the ability of NKT cells to be activated by αGalCer mapped to a genetic region encompassing a gene family (*Slam* genes) that is known to be important in NKT cell development, but the exact gene in this region which regulates NKT cells is unknown. This study utilizes a panel of C57BL/6 (B6) mice containing different regions of chromosome 1 derived from 129X1/SvJ mice (B6.129 congenics) to identify candidate genes regulating NKT cell function by positionally mapping the genes within this locus. We assessed NKT cell function in B6.129c2 (C2), B6.129c3 (C3), and B6.129c4 (C4) mice, which contain 129 intervals ranging from 0.1-1 megabase pairs (Mbp). To assess NKT cell function, we injected mice with αGalCer, which specifically activates NKT cells. Flow cytometry was utilized to determine NKT cell IL-4, TNF, and IFN-γ expression on a per cell basis and ELISA assays were conducted to observe the overall magnitude of the NKT cell response. There was a significant reduction in the TNF, IL-4, and IFNγ production in all congenic mice as compared to B6 controls. These data suggested that the NKT cell response to αGalCer mapped to a 0.1 Mbp region on chromosome 1 (the C3 interval), which excluded *Slam* genes as potential genes regulating these NKT cell functions. Possible candidate genes of interest in this locus are *ApoA2*, which encodes a protein involved in lipid transport, and *Fcer1g*, which encodes a protein that has recently been implicated in the development of different NKT cell subsets.

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Cell Types:
APC – Antigen presenting cell
DC – Dendritic cell
NK cell – Natural killer cell
NKT cell – Natural killer T cell

Mice:
129 – 129X1/SvJ
B6.129c1 – congenic 1
B6.129c2 – congenic 2
B6.129c3 – congenic 3
B6.129c4 – congenic 4
B6 – C57BL/6J
NOD – Non-obese diabetic mice

Other Terminology:
αGalCer – α-galactosylceramide
CD1 – glycoprotein expressed on APCs
CD1d – glycoprotein expressed on APCs, part of the CD1 family
FACS – Fluorescence activated cell sorting
FBS – Fetal bovine serum
IFN – Interferon (cytokine)
IL – Interleukin (cytokine)
Ly108 – *Slamf6*
Mbp- megabase pair
MHC – Major histocompatibility complex
Mtb – *Mycobacterium tuberculosis*
PBS – Phosphate buffered saline
SAP – SLAM-associated protein
SLAM- Signaling lymphocytic activation molecule-family of genes
TCR – T cell receptor
TNF – Tumor necrosis factor (cytokine)

Literature Review:

Background

Why do some people get extremely sick after they are infected with a pathogen, but other people only experience mild illness? Part of the explanation is that there are differences in our genetic makeup, which influence the way our immune systems respond to the same pathogen. This genetic variation in part has been attributed to an innate-like T cell subset called NKT cells. NKT cell genetic variation has been implicated in the susceptibility of both mice and humans to various autoimmune diseases, such as autoimmune diabetes and systemic lupus erythematosus [1, 2]. NKT cells have either harmful or protective roles in many pathogenic states such as in microbial infection [3, 4], autoimmune disease [5], allergies [6], and cancer [7]. What is not understood however, is how genetic variation affects NKT cell responses. We investigated the NKT cell response in genetically variant congenic mice in the hopes of understanding how natural genetic variation affects cytokine production of the immune response.

NKT Cells and their involvement in the immune system

NKT cells are important regulatory lymphocytes that serve a multitude of functions such as promoting immunity to tumors, bacteria, and viruses, and in suppressing cell-mediated autoimmunity [8]. NKT cells mediate these functions by secreting large amounts of cytokines (small soluble proteins that are involved in cell communication). NKT cell activation leads to the activation of other leukocyte subsets involved in both the adaptive and innate immunity [9]. This suggests that NKT cells are involved early on in the developing immune response and form a bridge between the innate and adaptive immune system [9]. NKT cells are thymus-derived lymphocytes that are regulated by self and non-self ligands, such as those presented by

CD1d molecules [10]. The difference between regular T cells and NKT cells is that the NKT T cell receptor (TCR) does not interact with peptide antigen, but rather recognizes glycolipids presented by CD1d, an antigen–presenting molecule [11]. Antigen-presenting cells such as dendritic cells (DCs) constitutively express CD1d and assist in NKT cell activation following presentation of lipid antigen [12]. In the lungs, DCs are capable of capturing lipid antigens and presenting them to NKT cells [13].

CD1d

CD1d is highly conserved throughout evolution and is very similar in mice and humans [14]. CD1d is a non-polymorphic MHC-1 class like glycoprotein that belongs to the CD1 family [15]. The CD1 family contains two groups. Group one consists of CD1a, CD1b, and CD1c, while group two consists of CD1d, and CD1e [16]. Mouse models have been shown to have only CD1d, and lack Group 1 and CD1e. This is believed to be due to a deletion of the region surrounding Group 1 genes that occurred via chromosomal translocation [17]. Group one presents lipids instead of peptides as antigens to non-MHC-restricted T cells [18]. CD1 molecules are transmembrane proteins that are related to MHC molecules expressed on APCs [19]. CD1 molecules are distinguishable from MHC class I and class II molecules due to their lack of polymorphism [19]. CD1d presents its antigen to NKT cells [15]. Some of the lipids presented by CD1 include lipids found in the cell walls of mycobacteria [31]. CD1d is expressed by many cells including dendritic cells (DCs), macrophages, B cells and granulocytes [20]. CD1d assembles with phospholipids and sphingolipids [10]. The majority of CD1d-restricted T cells recognize α-galactosylceramide (αGalCer), a glycosphingolipid [21]. αGalCer is a marine sponge derived glycosphingolipid and is the most potent stimulator of NKT cells [22]. A structurally related α-galactosylceramide has also been found to be

produced by *Bacteroides fragilis* in the human microbiota [23]. This suggests that αGalCer is present in human microbiota. Through the use of their TCRα-invariant TCRs, NKT cells are able to recognize αGalCer bound to CD1d [21]. αGalCer is a NKT cell activator and has previously been utilized to drive NKT cell responses [24]. NKT cells are able to recognize both self and foreign lipid antigens that are presented by CD1d. NKT cell activation triggers many immune responses such as cytokine secretion [18]. Murine CD1d localizes to the plasma membrane and MHC II containing compartments [25]. CD1d recycling between the plasma membrane and the MHC II compartments is needed for presentation of CD1d antigens. This recycling of CD1d is believed to facilitate exchange of self and foreign CD1d-bound antigens [26].

CD1d Structure

CD1d is a heterodimer and is made up of a light chain and a heavy chain that are non-covalently interacting. The heavy chain folds into five domains. All CD1 molecules have: extracellular α1, α2 and α3 domains, a transmembrane domain, and cytoplasmic tail [27].. These domains are membrane-anchored by the transmembrane region and end in a short cytoplasmic tail. The heavy chain α1 and α2 domains fold into a super domain in order to form an antigen binding groove. The α3 light chain domain serves as a support for the complex. The antigen binding groove is confined by α-helices that are anti-parallel and supported by an antiparallel β-sheet platform. CD1d structure is similar to that of MHC class I molecules [10], which is why CD1d is able to mimic antigen presentation in ways similar to MHC I complexes.

NKT cell and CD1d Interaction

The α in αGalCer references the orientation of the carbohydrate head group and the lipid backbone [18]. αGalCer has a fatty acyl chain and sphingosine base. αGalCer has two hydrophobic regions in its binding groove and via hydrophobic interaction it is able to bind to CD1d [21]. Previous studies have observed αGalCer activates only NKT cells, and there is minimal bystander activation in mice lacking CD1d [24]. αGalCer is highly used due to its strong binding to the NKT cell TCR [28]. The NKT TCR has a positive docking pocket containing three complementary determining regions (CDRs) on the α-chain and the β-chain [22]. The interaction of NKT TCR and CD1d is facilitated primarily by CDR2β and CDR3α loops, whereas the interaction between NKT TCR and αGalCer is between CDR1α and CDR3α [22]. Crystal structure of CD1 shows a deep binding groove that has two hydrophobic sites that interact with hydrocarbon chains, such as those of αGalCer [29].

Structural studies surrounding the presentation of αGalCer by CD1d show that the Cermide component of α-galactosylceramide fits snugly in the cleft of the CD1d, while the galactosyl head group pokes upward and is available for TCR recognition [30]. αGalCer binds to CD1d and then the complex of glycolipid plus CD1d binds the NKT cell, eliciting its activation [31]. CD1d presents lipid antigens such as glycolipids which are captured in its hydrophobic groove to NKT cells [32]. NKT cells recognize lipids on bacteria and other disease causing agents [33]. TCR invariant CD1d T cells are highly specific. They are able to recognize glucose and galactose, but are not able to recognize mannose (a stereoisomer of both glucose and galactose) as part of a glycosphingolipid. Several other sugar head groups are also unrecognizable by TCR invariant CD1d T cells [21]. It was shown that if αGalCer contained an extra galactose [αGal(1–2)GalCer] it would not be recognized by NKT cells, unless it was cleaved in lysosomes in order to generate αGalCer [34]. This suggest that the TCR of CD1d

restricted T cells is very specific when distinguishing between hydrophilic groups of lipid antigens [16].

NKT Cells and Cytokine Production

NKT cells express markers of both NK (natural killer) cells as well as T cells, thus making them unique from either subset. Activation of NKT cells with a specific glycolipid agonist α-galactosylceramide, leads to a activation of different types of other cells such as dendritic cells, NK cells, T, and B lymphocytes [35]. After activation, NKT cells rapidly produce many cytokines that are responsible for a communication cascade between the innate and adaptive immune systems [36]. The types of cytokines and amounts produced are essential in determining how the immune system will respond to an infection [37]. NKT cell activation leads to proinflammatory and regulatory cytokine release [24]. NKT cells release Th1-type (type 1 T helper) cytokines such as IFNγ and tumor necrosis factor (TNF), as well as Th2-type (type 2 T helper) cytokines such as IL-4 [38]. IFNγ is an activator of macrophages and is an inducer of Class II MHC complexes. IL-4 is involved in B cell activation, controls class II MHC expression, and is involved in differentiation fate T cell subsets. TNF is a cell signaling protein and is involved with inflammation. In the absence of TNF mice show increased infection susceptibility and reduced contact hypersensitivity [39]. Th1-type cells interact with macrophages and T cells, while Th2-type cells interact with B cells, eosinophils, and mast cells. Cytokines that are proinflammatory lead to downstream activation of other cells types. NK activation is dependent on cytokines secreted during NKT cell activation. In mice, this is vital for αGalCer–mediated tumor clearance [24]. Thus, NKT cells are an early player in the immune response, and have the potential to regulate disease susceptibility [4]. Activation of CD1d T

cells may possibly bridge the temporal gap between the onset of innate immunity and the adaptive responses of MHC-restricted T cells [40].

NKT Cells and Disease

It is believed that NKT cells evolved primarily to respond to microbial pathogens [41]. NKT cells are involved in a variety of infections ranging from bacterial infection and viral infection to autoimmunity. NKT cells are involved directly in the cellular immunity to *Mycobacterium tuberculosis* (Mtb), which requires a type 1 cytokine response in order to control infection. The innate control of bacterial replication of Mtb is associated with CD1d-restricted invariant NKT cells [42]. NKT cell activation is dependent on CD1d expression by infected macrophages [42]. NKT cells even when in limited quantity were sufficient to restrict the replication of Mtb. NKT cells were observed to have a direct bactericidal effect even when synthetic ligands were absent [42].

NKT cells and CD1d-expressing antigen presenting cells have been implicated in heart disease. NKT cells and CD1d APCs have previously been detected in atherosclerotic lesions of humans and mice [43]. Atherosclerosis is the hardening and thickening of the arteries and is the main cause of heart disease. In order to study the role of NKT cells in atherosclerosis, ApoE$^{-/-}$ mice were treated with LPS and showed that plaque size increased with increasing numbers of NKT cells producing IL-4. It was believed that NKT cells were responsible for increased autoantibodies and in turn this lead to increased development of atherosclerotic lesions [44]. CD1d is present on DCs in atherosclerotic lesions in humans [45]. NKT cells, which are present in human carotid arteries with atherosclerotic lesions co-localize with CD1d, which is expressed by dendritic cells in the plaque, confirming that NKT cell activation occurs inside the lesion and affects plaque instability [46]. Apolipoprotein E–deficient (ApoE$^{-/-}$) mice that

were treated with αGalCer resulted in accelerated atherosclerosis and showed recruitment of NKT cells to the atherosclerotic lesions [47]. $CD1d^{-/-}$ $ApoE^{-/-}$ mice that were treated with αGalCer had no impact on disease progression, suggesting that atherosclerosis progression is CD1d-dependent [47].

Slam Genes and the SAP Adapter

Slam (Signaling lymphocytic activation molecule) genes are important in NKT cell development. *Slam* genes are involved in the modulation of the immune response by adjusting the signals received by white blood cells and are critical for the development of antibody responses [48]. It has been observed that *Slam* genes have modulatory effects during T cell activation [48]. Slam genes have also been shown to be polymorphic, or have several variations (alleles) of the genes in the population [49]. It has been shown that a genetic region that encompasses *Slam* genes affects NKT number and function [9]. *Slam* genes have been implicated in regulating NKT cell number in the congenic mouse [50]. The *Slam* locus on chromosome 1 has been implicated in controlling thymic NKT cell number in NOD mice [51]. When members of the SLAM family, SlamF1 and SlamF6 had blocked signaling it was observed that developmental arrest of NKT cells occurred [52]. This suggests an interaction of the *Slam* locus and NKT cell development and that haplotypes or abnormalities in the *Slam* locus could affect NKT cell development. SLAM receptors and the SLAM-associated protein (SAP) are expressed in immune cells and interact physically via their cytoplasmic domain [53].

SAP is a 128 long amino acid cytoplasmic protein that binds to the conserved tyrosine containing motif in the intracellular domain of SLAM receptors [54, 55]. SAP is expressed in T cells and NK cells and regulates cytokine production and cytotoxicity [56]. SAP contributes to cytokine production via the regulation of TCR mediated induction of GATA-3 (transcription

factor) and Th2 cytokines [55]. Mutations in SAP lead to X-linked lymphoproliferative disease and other immunodeficiency diseases [57]. The absence of SAP-family adaptors causes the SLAM family to undergo a "switch-of-function," which affects inhibitory signals that suppress immune cell functions [58]. This means that SLAM proteins modulate immune responses through their signaling via SAP.

Genetic variation in NKT cells and Disease

Previous work has suggested a link between genetic loci associated with NKT cell function and immune-mediated disease. A number of studies link NKT cells and autoimmunity. Non-obese diabetic (NOD) mice, a model of spontaneous autoimmune T-cell mediated diabetes, showed decreased NKT cell numbers [59]. Regions of chromosome 1, which is involved with lupus susceptibility, have been identified in controlling NKT cell number in the NOD mouse [60]. Chromosome 1 has also been identified in being linked to myocarditis susceptibility [61].

B6.129c1 are mice with a 4.5 Mbp portion of 129 genome overlaid onto B6 background on chromosome 1. B6.129c1 mice that were infected with Coxsackivirus B3 showed reduced severity of myocarditis (inflammation of the heart), but showed increased pathology in the liver, suggesting that NKT cells may be protective for myocarditis, but pathogenic in the liver [49]. Susceptibility to murine lupus is related to polymorphisms in *Slam/CD2* genes. The strongest candidate gene for regulating this response is Ly108 (*Slamf6*) [62]. Haplotype variability is believed to influence autoimmunity and in turn leads to the development of lupus in mice [62]. B6.129 congenic mice were observed to exhibit autoimmunity, while C57BL/6J 129/SvJ mice did not. The haplotype variations between the strains was believed to play a

direct role in lupus development. This suggests a deeper involvement of SAP and SLAM in NKT cell development and immune response.

Overview:

The Boyson lab has previously demonstrated that NKT cell function was highly dependent on the mouse genetic background [9]. Using a congenic mouse model in which a portion of 129 genome was overlaid onto a B6 background, the lab investigated NKT cell number and function in congenic mice as compared to B6. The Boyson lab demonstrated that natural genetic variation in a 4.5 megabase (Mbp) pair region on chromosome 1 in the B6.129c1 congenic mouse regulates NKT cell number and function [9]. They reported that this interval regulates the number of liver NKT cells, and the cytokine production of splenic NKT cells. The c1 congenic interval contained over 80 genes, making it difficult to pinpoint which gene(s) are critical in regulation of NKT cells.

My specific project was to fine-map the genetic locus of the congenic mouse in order to bring us closer to identifying which gene or genes control a reduced immune response in congenic mice as compared to the B6 control. By evaluating NKT response following stimulation we are able to map the modulating response to a specific congenic locus and in turn decrease the number of possible candidate genes.

Results:

Congenic Mice

Congenic mice models were obtained through crossing purebred B6 and 129 mice. The obtained heterozygote was then backcrossed to a purebred B6 mouse. The mice were screened and a B6.129c1 mouse was obtained (Fig. 1). Further backcrossing to a purebred B6 allowed for the creation of other congenic mice such as the B6.129c2, B6.129c3, and B6.129c4 (Fig 2). The B6.129 congenic mouse has a genome in which a portion of 129 genome was overlaid onto a B6 background. The Boyson lab has previously shown that this interval regulates cytokine production of splenic NKT cells. The c1 congenic interval contains over 80 genes in the 129 derived/undetermined region, while the c3 contains only 14 genes within this interval (Fig 2).

Titration of αGalCer

A titration of αGalCer was conducted in order to determine the proper dose needed to stimulate NKT cells, but also not hyper stimulate them. Congenic and B6 mice were initially injected with 2μg of αGalCer, but there was no observed cytokine response difference between any of the injected strains (data not shown). Previous studies have shown that αGalCer causes a dose dependent response in cytokine production [63]. These data suggested that a titration of αGalCer was needed to determine the correct amount to be administered. Therefore, we administered varying doses of αGalCer to B6 mice and assessed NKT cell cytokine production using intracellular staining followed by flow cytometry. The EC_{50} of the titration suggested that a working dose of 0.1μg of αGalCer was needed to stimulate, but not over stimulate the NKT cell response (Fig. 3). Upon finding the proper dose we injected B6,

B6.129c2, B6.129c3, and B6.129c4 mice with αGalcer and collected serum cytokine and intracellular cytokine data. The titration of αGalCer showed that 0.1μg of αGalCer was needed for NKT cell activation.

Serum Cytokine Response

In order to assess the total *in vivo* response of NKT cells to αGalCer in the mouse we measured serum cytokine levels. ELISA assays were utilized to measure serum cytokine response following αGalCer administration. We injected B6 and B6.129 congenic mice with 0.1μg αGalCer. Two hours later cardiac punctures were performed and blood collected. The blood was spun and the serum was separated. Serum obtained was frozen until it could be analyzed via ELISA. All three congenic mice showed significantly lower amounts of IFNγ, IL-4, and TNFα production (Fig. 4). IFNγ serum cytokine production was significantly lower in B6.129c2 ($p=.0044$), B6.129c3 ($p=.0052$), B6.129c4 ($p=.0015$), as compared to B6 mice. IL-4 serum cytokine production was significantly lower in B6.129c2 ($p=.0011$), B6.129c3 ($p=.0018$), B6.129c4 ($p=.0022$), as compared to B6 mice. TNFα serum cytokine production was significantly lower in B6.129c2 ($p=.0003$), B6.129c3 ($p=.0008$), B6.129c4 ($p<.0001$), as compared to B6 mice. These data indicated that serum cytokine production in response to the NKT cell-specific agonist αGalCer was reduced in all congenic mice as compared to the control, B6.

Intracellular Cytokine Response

Serum cytokine levels reflect both the total number of responding NKT cells and the amount of cytokine produced by each strain's NKT cells. To investigate the cell-intrinsic

response of NKT cells to αGalCer, we assessed NKT cell intracellular cytokine production after αGalCer administration. B6 and congenic mice were injected with αGalCer and 2 hrs later splenocytes were isolated and stained. Intracellular cytokine production by NKT cells was measured via flow cytometry and analyzed in FlowJO in order to measure per cell cytokine secretion. All three congenic mice showed significantly lower amounts IFNγ and IL-4. B6.129c2 and B6.129c3 showed significantly less TNFα production as compared to B6, while B6.129c4 TNFα cytokine production was not significantly different than B6 (Figs. 5 and 6). IFNγ intracellular cytokine production was significantly lower in B6.129c2 (p=.0077), B6.129c3 (p=.0011), B6.129c4 (p=.0130), as compared to B6 mice. IL-4 intracellular cytokine production was significantly lower in B6.129c2 (p=.0096), B6.129c3 (p=.0021), and B6.129c4 (p=.0287), as compared to B6 mice. TNFα intracellular cytokine production was significantly lower in B6.129c2 (p=.0316), and B6.129c3 mice (p=.0013), but was not significantly lower in B6.129c4 (p=.1276), as compared to B6 mice. Overall intracellular cytokine production was reduced in all three congenic strains as compared to the control, B6 (Fig 6).

Mapping to B6.129c3 interval has led to the identification of new candidate genes.

These data suggest that the modulated NKT cell response observed in congenic mice following activation by αGalCer maps to a 0.1 Mbp region on chromosome 1 (Fig 2.) (the c3 interval 171.046: 171.197). The B6.129c3 interval is the smallest interval of the current congenic strains tested. This interval contains 14 candidate genes, 4 of which are in the determined region and 10 which are in the undetermined region (Table 1). Further analysis is needed in order to determine which gene or genes are responsible for the NKT cytokine

response. NKT cell numbers have yet to be enumerated following αGalCer injection, our focus was strictly on cytokine function in these experiments.

Discussion:

Our data suggest that the modulated NKT cell response observed in congenic mice following activation by αGalCer maps to a 0.1 Mbp region on chromosome 1 (the c3 interval 171.046: 171.197) (Fig. 2). In these experiments, αGalCer was used as an agonist because of its ability to bind CD1d and stimulate NKT cells. It is presumed that this interaction mimics the recognition of bacteria-derived glycolipids and glycosphingolipids by NKT cells. It has previously been demonstrated that NKT cells recognize glycolipids from Gram-negative bacteria, including *Borrelia* and *Sphingomonas*, as well as Gram-positive *Streptococci*, which is pathogenic [64],[65]. Therefore, one implication of our results is that genetic polymorphisms that map to the c3 interval could affect how well NKT cells respond to certain bacterial pathogens. Previous studies implicated chromosome 1 in autoimmunity and pointed to the *Slam* locus as being responsible for disease susceptibility [9]. *Slam* genes, such as *Slamf1* and *Slamf6*, have be identified as potential candidate genes in the genetic control of NKT cell number in the thymus [51]. My data suggest that the genetic region modulating NKT cell function is located within the B6.129c3 interval, which does not contain *Slam* genes. While suggesting that *Slam* genes are not causing the modulation of NKT cell function in the spleen, my data do not however rule out other roles of *Slam* genes in regulating NKT cell development. *Slam* genes could very well impact NKT cell number which has not been looked at by my data. It could be reasoned that *Slam* genes control NKT cell number, while other genes within the smaller C3 interval regulate NKT cell responses to stimulation.

Studies surrounding human systemic lupus erythematosus (SLE) and murine congenic mice suggested that genes on chromosome 1 left individuals at a genetic predisposition to contracting SLE [66]. SLE is a chronic autoimmune disease in which autoantibodies are produced against self-antigens. *Slam* genes were believed to be the primary genes of interest in the development of autoimmunity. NKT cells have been implicated in the development of

autoimmunity [67]. NKT cell development is dependent on SLAM receptor signaling. Mice lacking SAP, which is needed for SLAM signaling, also lack NKT cells [68]. This information as well as other studies led us to initially suspect the *Slam* locus as responsible for a decreased immune response in congenic mice. After analysis of the congenic strains it was observed that the data maps to the B6.129c3 interval. *Slam* genes are not within the B6.129c3 interval, which leads us to believe that another gene or genes are modulating the NKT cell response to αGalCer. In the B6.129c3 interval there are 4 genes which we are certain are contained in the interval and 10 genes which are in an undetermined region meaning that they could be either B6 or 129 derived. When taking all 14 genes into consideration we have now selected new genes as possible candidate genes of interest.

The data suggested that *Slam* genes, known immunomodulatory genes, are no longer candidate genes for the responsiveness of NKT cells. The B6.129c3 interval contains no *Slam* genes, but still displays a decreased NKT serum cytokine and intracellular cytokine response. These data suggest that polymorphisms in *Slam* genes do not appear to play a role in NKT cell cytokine production in response to αGalCer. This finding does not support our hypothesis and rather leads us to examine new candidate genes of interest. New candidate genes of interest are those encoding *ApoA2* and *Fcer1g*.

Our finding that *ApoA2* is in the c3 interval suggests that it is a possible candidate gene that regulates NKT cell function. Previously, it was shown that another apolipoprotein, ApoE, was important in lipid antigen presentation by CD1d [69]. ApoE works by binding to the LDL receptor [43]. Decreased lipid presentation on CD1d molecules was observed in ApoE -/- mice [43], suggesting that its presence is directly involved in CD1d presentation and NKT cell immune response. This suggests that ApoE could also be involved in antigen presentation to NKT cells and could interact with αGalCer. ApoA2 is in the same way an

apolipoprotein and could theoretically have similar functions, thus why we have decided to explore *ApoA2* as a candidate gene. Apolipoprotein E (ApoE) is a polymorphic glycoprotein that is synthesized mostly in the liver. It is a plasma protein that is involved in lipid transport and metabolism. It is involved in VLDL and is a ligand for a LDL receptor. High levels of ApoE are found in cerebrospinal fluid and it is speculated that it plays a role in lipid redistribution in the brain [70]. Previous studies have shown that NKT cell activation accelerates atherosclerosis in mice that are ApoE deficient [43]. In atherosclerosis, low-density lipoprotein (LDL) accumulation and modification in the vessel wall causes an immune response [43]. ApoA2 is a lipoprotein that is similar in nature to ApoE. Both ApoA2 and ApoE are protein coding genes and had been linked to LDL metabolism. We speculate that ApoE and ApoA2 serve similar purposes and therefore genetic variation in ApoA2 could be affecting CD1d binding and in turn affecting NKT cell function.

Fcer1g is involved in generation of iNKT1 cells. Strong mRNA signal for the Fcer1g chain correlates with a low expression of CD3ζ (T cell coreceptor). Fcer1g is part of the high affinity IgE receptor. It also interacts with the CD3ζ chain in the TCR complex [71]. The complex then triggers TCR signaling. Mice that are Fcer1g deficient show decreased levels of NKT cells [71]. This gene's involvement in NKT cell development leads us to draw the conclusion that it might also be responsible for the decreased cytokine production observed in congenic strains of mice. The B6.129c3 mouse has all B6 genes with the exception of 14 genes. One of these genes is *Fcer1g*, which has been linked to NKT cells before, thus leading us to believe that it may be responsible for the decreased cytokine response by NKT cells in congenic strains of mice.

Our data are suggestive of a gene or genes in the B6.129c3 region modulating the decreased NKT cell response to αGalCer, but in order to further determine which gene(s) are

responsible for the decreased NKT cytokine secretion in the congenic strains further mapping of the B6.129c3 interval is needed. This mapping is currently ongoing and should bring us closer to identifying what gene or genes modulate the NKT immune response in congenic mice.

Methodology:

Experimental Design

Mice: C57BL/6, B6.129c2, B6.129c3 and B6.129c4 mice housed in the HSRF animal care facility were injected with either αGalCer or vehicle (PBS + 0.05% Tween-20) intraperitoneally (i.p.). Mice were euthanized and organs harvested 2 hours after administration of αGalCer. Blood was collected by cardiac puncture. Blood vials were spun on a Legend Micro 17R centrifuge at 10,000g for 10 minutes (Thermo Scientific). The top layer containing serum was removed and frozen at 4°C. All procedures and experiments were conducted in accordance with standards set by the University of Vermont Institutional Animal Care and Use Committee (IACUC- #16-016).

Reagents: αGalCer was prepared by re-suspending stock in sterile phosphate-buffered saline (PBS), 0.5% Tween-20, followed by diluting in sterile PBS with a pH of 7.4 to a final concentration of 0.05% Tween-20. Vehicles contained PBS, 0.05% Tween-20.

Flow Cytometry/Antibodies: The antibodies used were: anti-TCR-β; PE-Cy7 (H57-597), anti-CD11b; APC-Cy7, anti-IFNγ; Alexa 647 (XMG1.2), anti-IL-4; Alexa 647 (11B11), anti-TNF; Alexa 647 (MP6-XT3). CD1d tetramer loaded with PBS57 (an αGalCer-like compound) was obtained from the NIH tetramer facility (Emory University Vaccine Center, Atlanta, GA). These antibodies are used for surface and intracellular cytokine staining. The cells were isolated from the organs by pressing through 70µm nylon mesh screen and homogenized with a 3cc syringe plunger. The cells were transferred to a 15mL tube, spun at 500g at 4°C for five minutes. The supernatant was then discarded and the pellet re-suspended in 0.5mL of cold 1X

PBS pH 7.4. Cells were lysed with Gey's solution, washed, and counted. Cells were then stained with surface markers conjugated to antibodies. After the cells were washed with staining buffer, they were fixed, and permeabilized using a commercially available fixative (FixPerm; BD Biosciences) and then stained with Alexa647-conjugated anti-cytokine antibody. The data was collected on the LSRII flow cytometer in the College of Medicine Flow Cytometry facility, and analyzed via FlowJo software (FlowJo, LLC).

ELISA: ELISA assays were conducted using commercially available kits to measure serum cytokines IFNγ, IL-4, and TNFα. ELISA was performed according to manufacturer's instructions. 96-well plates were coated with coating buffer (0.1M sodium carbonate pH 9.5) and capture antibody (BioLegend) and left at 4°C overnight. Following incubation, the plate was washed and the protein binding sites blocked in assay diluent (10%FBS in PBS) for at least one hour at room temperature. Standards were serially diluted and added to plate for a minimum of 2 hours at room temperature. Samples were also added to the plate for a minimum of 2 hours at room temperature. The plate was washed in a plate-washer and biotinylated antibody was added for one hour at room temperature. The plate was washed and HRP Streptavidin was diluted 1:1000 in assay diluent and added for 30 minutes. The plate was washed and a 1:1 dilution of BD TMB substrate regent set was added until reaction occurred. Sulfuric acid was utilized to stop the reaction. The plate was read at 450nm on a ELX800 plate reader (BIO-TEK Instruments).

Statistics: Differences between B6 and congenic mouse IFNγ, TNFα, and IL-4 production were evaluated using 1-way ANOVA analyses in GraphPad PRISM. The EC_{50} of αGalCer was calculated by fitting dose-response curves of NKT cell cytokine production vs. αGalCer using

non-linear regression. Dunnett's multiple comparison test at $p<0.05$ was utilized to determine significance.

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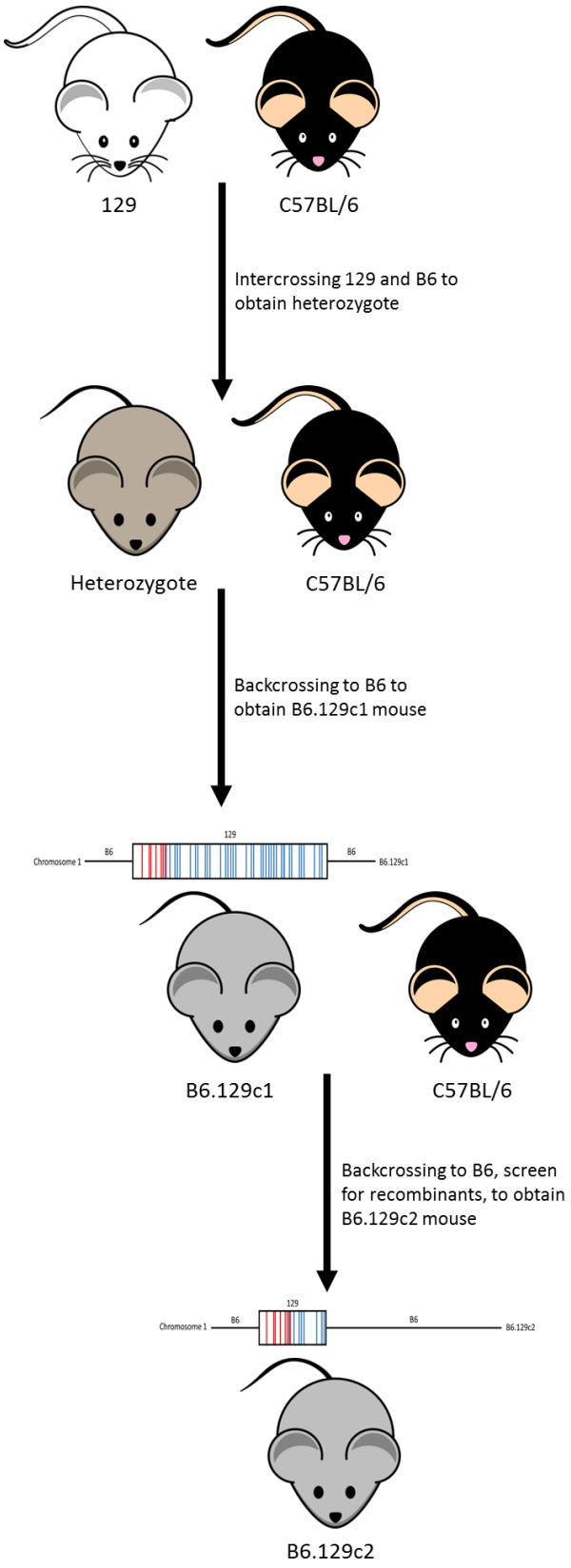


Figure 1. Intercrossing of a 129 mouse to B6 mouse to obtain a congenic animal. A 129 mouse was crossed to a C57BL/6 mouse in order to obtain a heterozygous mouse. The heterozygous mouse was then backcrossed to a B6 mouse and they were screened for recombinants. This resulted in a B6.129c1 mouse.

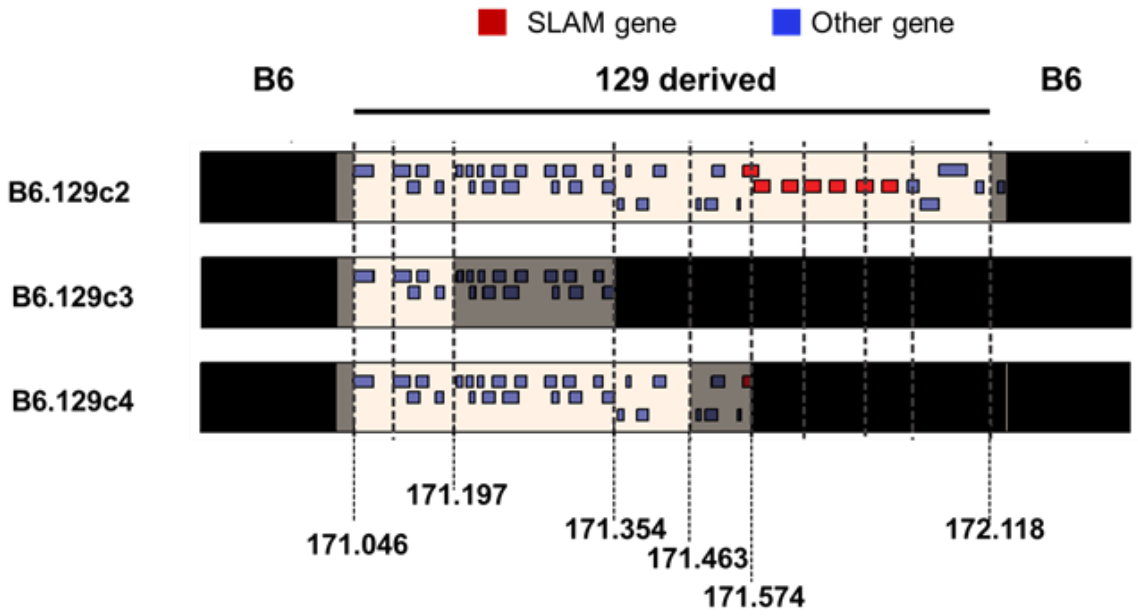
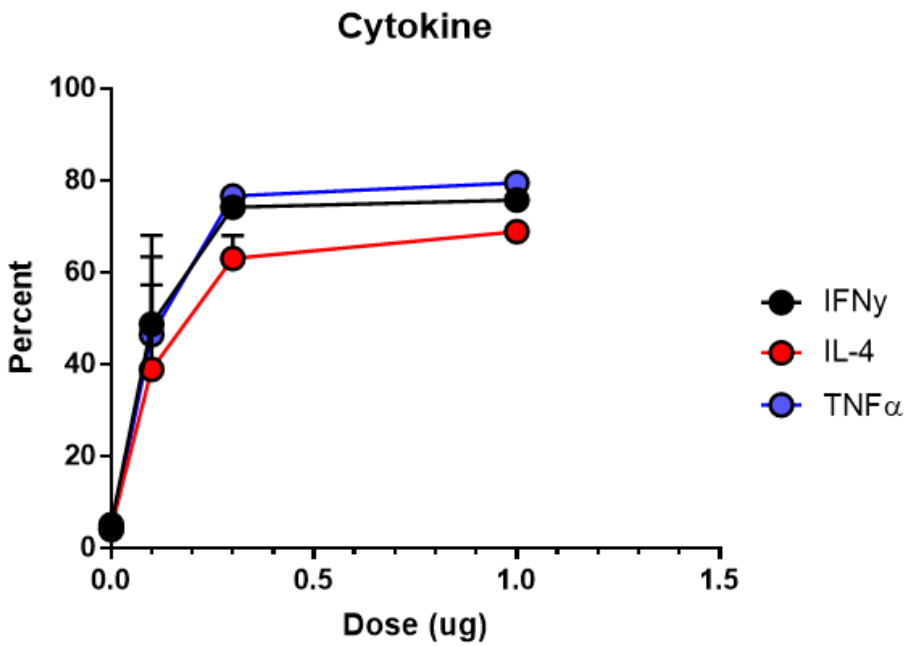


Figure 2. Congenic Mouse Strains. B6.129c2, B6.129c3 and B6.129c4 mouse strain intervals with base pair locations highlight the varying amount of 129 mouse genome embedded on top of B6 background. Black shading is representative of B6 derived genome, yellow shading is representative of 129 derived genome, and grey shading is representative of an undetermined region in which it is unknown if the genome is B6 or 129 derived.

A.



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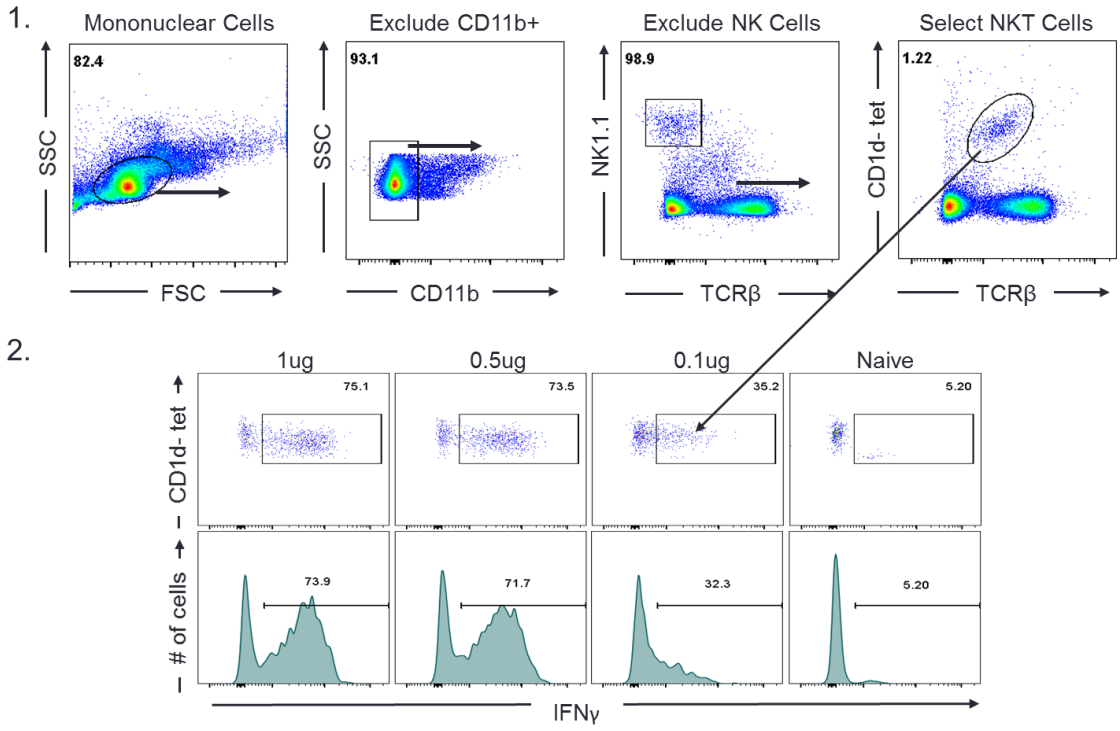

Figure 3. αGalCer Titration for dosing and gating scheme. A. αGalCer titration indicating the proper amount needed to be injected into mice to obtain activation of NKT cells. Data showed that 0.1µg αGalCer was needed to stimulate NKT cells.
B. Gating scheme of NKT cells obtained from splenocytes. Cells were stained with fluorophore-conjugated antibodies, data were collected on a flow cytometer, and then examined using FlowJo software. 1) Pseudoplot graphs show NKT cells are CD1d-tetramer and TCRβ positive. 2). Pseudoplots and histograms show the amount of cytokine produced 2h after αGalCer challenge in B6 mice.

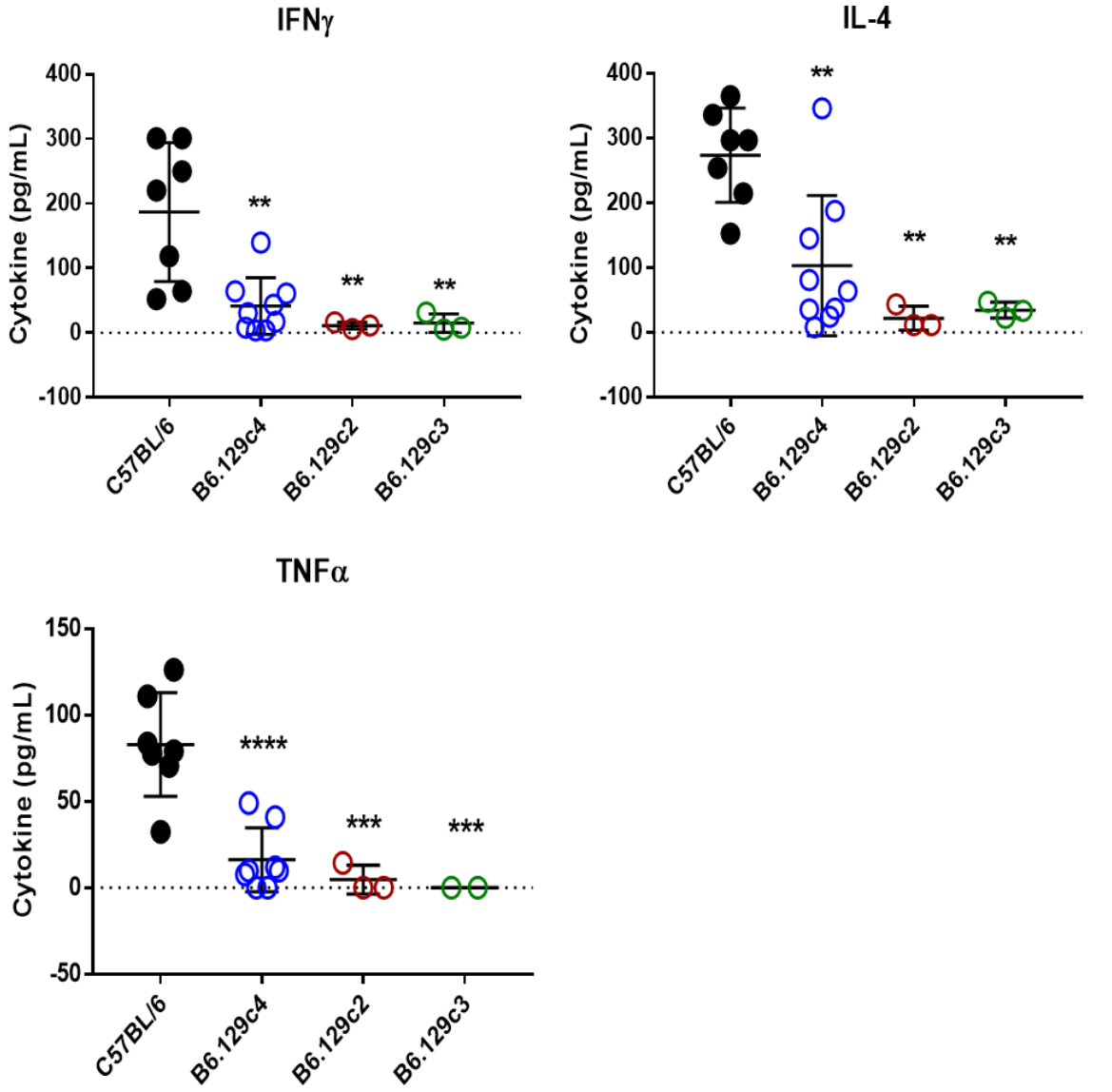

Figure 4. Serum cytokine production 2hr after αGalCer administration. Measurement of serum cytokines after 0.1μg αGalCer administration. All three B6.129 congenic strains exhibited significantly lower responses to αGalCer compared to the B6 strain control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

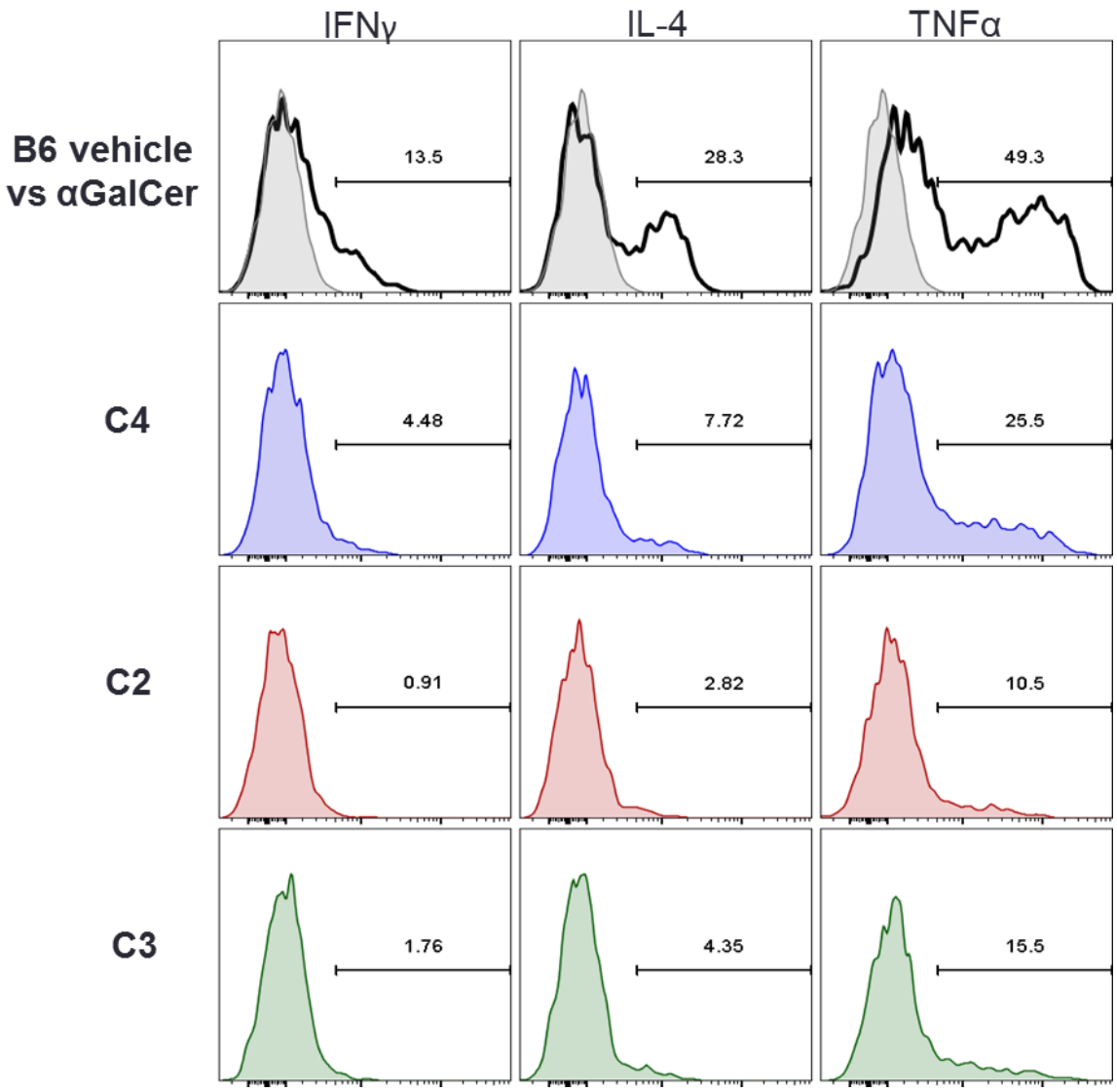

Figure 5.
Intracellular NKT cytokine production histograms. Histogram representations of measurement of intracellular NKT cytokines after 0.1µg αGalCer administration. B6.129 congenic strains exhibited lower responses to αGalCer compared to the B6 strain control. Light grey graph denotes B6 injected with vehicle. Black line denotes B6 injected with 0.1µg αGalCer.

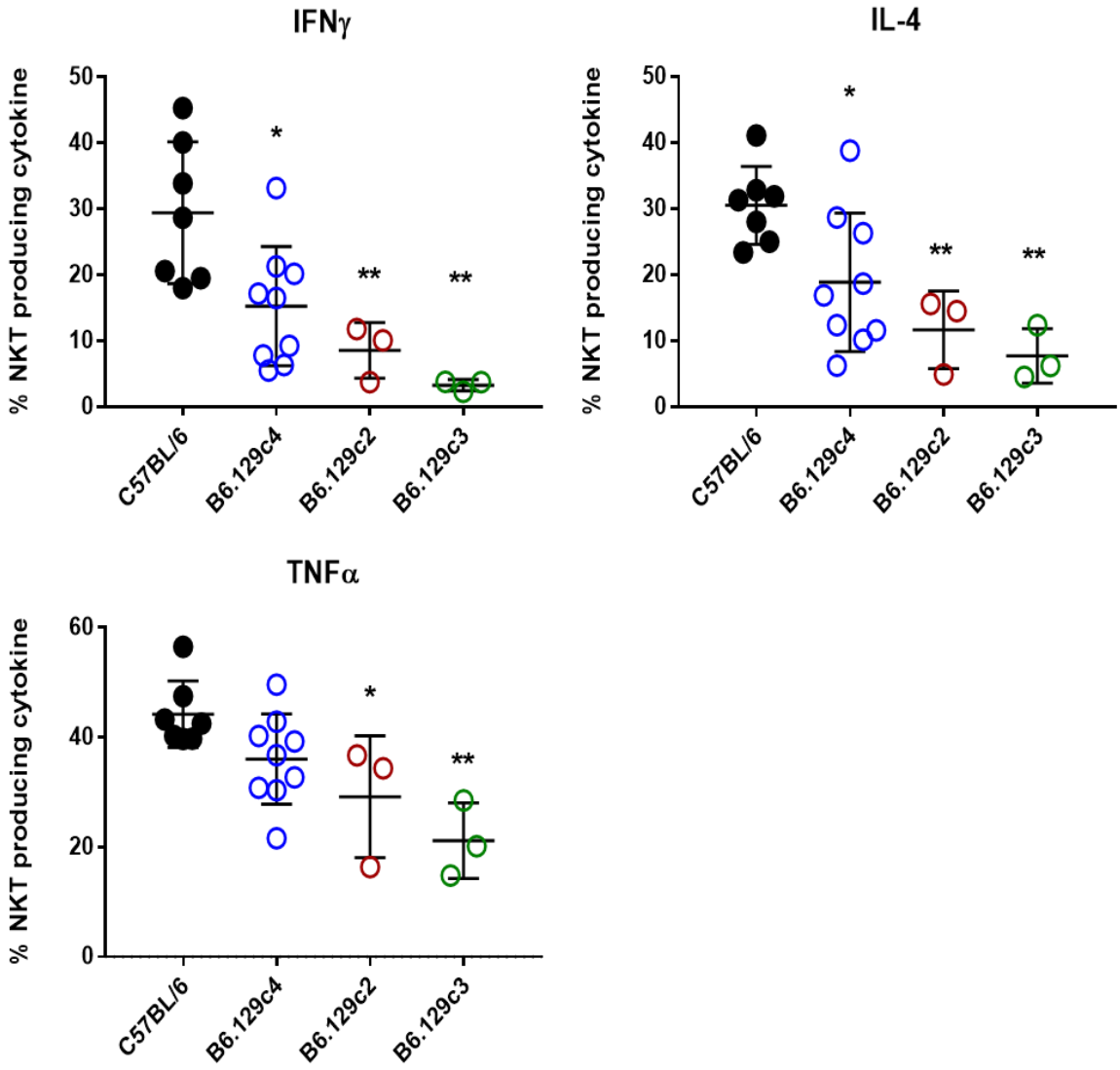

Figure 6. NKT intracellular cytokine production 2hrs after αGalCer administration. Intracellular cytokine production by NKT cells after 0.1μg αGalCer challenge. All three B6.129 congenic strains exhibited significantly lower responses to aGalCer compared to the B6 strain control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Genes in defined region	Genes in undefined region	
Cfap126 171,113,918-171,126,967	**Nr1i3** 171,213,970-171,220,701	**Adamts4** 171,250,421-171,260,637
Sdhc 171,127,165-171,150,603	**Tomm40l** 171,216,011-171,222,514	**B4galT3** 171,270,328-171,276,896
Mpz 171,150,711-171,161,130	**ApoA2** 171,225,054-171,226,379	**Dedd** 171,329,145-171,342,331
Pcp4l1 171,173,262-171,196,268	**Fcer1g** 171,229,572-171,234,365	**Nit1** 171,338,008-171,345,646
	Ndufs2 171,234,853-171,251,388	**Pfdn2** 171,345,670-171,359,254

Table 1. Genes located in B6.129c3 interval. There are four genes that are in the defined interval of the B6.129c3 mouse and 10 genes which are undefined. Genes in the undefined region could be from either parental strain, B6 or 129. Numbers listed below gene names are representative of gene position.