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Regulation of invariant NKT cell development and function by a 0.14 Mbp locus on chromosome 1: a possible role for *Fcgr3*

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Abstract

Invariant NKT (iNKT) cells are tissue-resident innate-like T cells critical to the host immune response. We previously identified a 6.6 Mbp region on chromosome 1 as a major regulator of iNKT cell number and function in C57BL/6 and 129X1/SvJ mice. Here, we fine-mapped this locus by assessing the iNKT cell response to alpha-galactosylceramide (αGalCer) in a series of B6.129 congenic lines. This analysis revealed the presence of at least two genetic elements that regulate iNKT cell cytokine production in response to αGalCer. While one of these genetic elements mapped to the B6.129c6 interval containing *Slam* genes, the dominant regulator in this region mapped to the 0.14 Mbp B6.129c3 interval. In addition, we found that numbers of thymic iNKT cells and DP thymocytes were significantly lower in B6.129c3 mice, indicating that this interval also regulates iNKT cell development. Candidate gene analysis revealed a 5-fold increase in *Fcgr3* expression in B6.129c3 iNKT cells, and we observed increased expression of FcγR3 protein on B6.129c3 iNKT cells, NK cells, and neutrophils. These data identify the B6.129c3 interval as a novel locus regulating the response of iNKT cells to glycosphingolipid, revealing a link between this phenotype and a polymorphism that regulates *Fcgr3* expression.

Introduction

Semi-invariant iNKT cells comprise an unusual innate-like T cell subset that plays significant roles in the host immune response to bacterial and viral pathogens (1–3). iNKT cells recognize glycolipids and glycosphingolipids presented by the MHC class I-like molecule CD1d (4–6). The prototypical glycosphingolipid agonist alpha-galactosylceramide (αGalCer) is structurally similar to glycosphingolipids from *Bacteroides fragilis* (7) and is a

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Conflict of Interest

The others declare no conflicts of interest for this work.

potent activator of iNKT cells (6, 8–11). Upon activation by α GalCer presented by CD1d, iNKT cells rapidly produce large amounts of chemokines and cytokines (12–14) and contribute to an orchestrated activation of both innate and adaptive immune cells including dendritic cells, macrophages, and natural killer (NK) cells (15–19). The iNKT cell subset, therefore, is uniquely poised to shape the quality and magnitude of the developing host immune response.

Invariant NKT cell number and function varies dramatically among mice of different genetic backgrounds. Wild-derived inbred strains (e.g., PWD/PhJ, Cast/EiJ) have barely detectable numbers of iNKT cells (20, 21), and there is significant strain-dependent variability even among common laboratory inbred strains (21–25). Accumulating evidence suggests that genetic background has a significant influence on the role of iNKT cells in the host immune response. For example, iNKT cells are critical in the clearance of the opportunistic pathogen *Pseudomonas aeruginosa* from the lung in BALB/cJ mice, but are dispensable in C57BL/6J mice (26). Similarly, pathology in iNKT cell-deficient mice infected with *Borrelia burgdorferi* manifests as joint inflammation in BALB/c mice (27) and as myocarditis in C57BL/6J mice (28). Therefore, a thorough understanding of the genetic determinants that regulate iNKT cell development and function is necessary to understand the role of iNKT cells in the host immune response.

Numerous reports have described polymorphic genetic loci that regulate iNKT cell number and function (20, 29–35). We and others have identified a region on chromosome 1 that regulates iNKT cell development and the response to α GalCer (25, 29, 31, 36). We previously demonstrated that iNKT cells in 129X1/SvJ mice produced significantly lower amounts of cytokine after α GalCer challenge than did iNKT cells in C57BL/6J mice. Using B6.129 congenic mice, we identified the genetic interval spanning from rs222297065 to D1MIT115 (Chr1: 171.03 - 179.60 Mbp) as a regulator of the response of iNKT cells to *in vivo* α GalCer challenge (31). This ~6.6 Mbp locus is densely populated with numerous immunologically relevant genes, including signaling lymphocyte activation markers (SLAMs) that modulate iNKT cell development and function (37). Interestingly, this locus overlaps extensively with several autoimmune susceptibility loci (38–40) and there are numerous reports of an association between iNKT cell numbers and autoimmunity (25, 41–43).

To refine this interval and identify candidate genes that regulated the responsiveness of iNKT cells to α GalCer, we generated additional B6.129 subcongenic lines with overlapping intervals. Here, we report the mapping of the iNKT cell response to α GalCer to a minimal 0.14 Mbp interval (Chr1: 171.032-171.170) containing 4 genes and 2 microRNAs. In addition, we found that this interval regulates total thymocyte numbers and total iNKT cell number. Finally, we identify *Fcgr3* as a possible candidate iNKT cell regulatory gene due to the association of increased iNKT cell Fc γ R3 expression and the impaired response of iNKT cells to α GalCer stimulation observed in B6.129c3 mice.

Results

Refinement of the 129X1/SvJ interval on chromosome 1

We previously reported that a 6.6 Mbp genetic region on chromosome 1 containing the *Slam* genes regulated iNKT cell function (31). Given previous reports that SLAMF1 and SLAMF6 are required for iNKT cell development and the *Slam* genes have been reported to regulate thymic iNKT cell numbers (31, 44), we hypothesized that polymorphisms in one or more of the *Slam* genes are responsible for the differences in the iNKT cell response to α GalCer between C57BL/6 and 129X1/SvJ mice. To test this hypothesis, we generated 4 subcongenic strains: B6.129c2, B6.129c3, B6.129c4, and B6.129c6 with overlapping 129X1/SvJ intervals ranging from 0.14 Mbp to 1.1 Mbp that spanned the centromeric region of the B6.129c1 interval containing *Slam* family genes (Figure 1a). Mapping of the 129 derived-interval boundaries (Figure 1b) revealed that B6.129c2 and B6.129c6 strains possess *Slam* family genes derived from 129X1/SvJ, while B6.129c3 and B6.129c4 strains possess *Slam* family genes derived from C57BL/6J.

Invariant NKT cell cytokine production in response to α GalCer maps to a 0.14Mbp region on chromosome 1

To determine which 129X1/SvJ intervals regulated the iNKT cell response to glycolipid, we assessed serum IFN γ and IL-4 cytokine levels after administration of α GalCer to C57BL/6 and B6.129 congenic strains. This analysis revealed that each of the tested B6.129 congenic strains exhibited significantly reduced levels of serum cytokines in comparison to the B6 control (Figure 2a). To determine whether this decrease in α GalCer-induced cytokine was due to reduced production by iNKT cells, we evaluated IFN γ and IL-4 production by CD1dtetramer/PBS57-gated cells in response to α GalCer using intracellular flow cytometry staining (Figure 2b). Consistent with the reduced serum cytokine production in the B6.129 congenic strains in response to α GalCer administration, we observed significantly lower levels of iNKT IFN- γ and IL-4 production in B6.129c2, B6.129c3, and B6.129c4 strains as compared to the B6 parental strain (Figure 2b). These data indicated that the dominant regulator of iNKT cell cytokine production in response to α GalCer mapped to the minimal 0.14 Mbp B6.129c3 interval.

Interestingly, examination of the cytokine production in B6.129c6 mice revealed an intermediate phenotype. iNKT IFN- γ and IL-4 production in B6.129c6 mice were significantly higher in comparison to B6.129c3 mice, while B6.129c6 IFN- γ was significantly lower in comparison to B6 parental mice (Fig. 2b). B6.129c6 IL-4 was also lower than B6 mice, although this reduction did not reach statistical significance ($p=0.0880$) (Figure 2b). Together, these data indicated the presence of at least two genetic elements in the B6.129c2 region that regulate iNKT cell cytokine production in response to α GalCer: a dominant regulator mapping to the 0.14 Mbp B6.129c3 interval that did not contain *Slam* genes, and a second subdominant regulator mapping to the B6.129c6 interval containing *Slam* genes.

Differences in *in vivo* iNKT cell cytokine production could be the result of an iNKT cell-intrinsic phenomenon, or from differences in antigen processing and presentation. To

determine whether the difference in iNKT cell cytokine production was an iNKT-intrinsic phenomenon, we directly compared cytokine production from purified B6 and B6.129c3 iNKT cells after stimulation. Equal numbers of sorted splenic iNKT cells from B6 WT and B6.129c3 mice were stimulated with anti-CD3 and anti-CD28, after which supernatants were analyzed for cytokine and chemokine production. We observed a significant reduction in cytokines and chemokine secretion from B6.129c3 iNKT cells compared to their B6 counterparts (Figure 3). Taken together, these data indicate that one or more of the genes contained within the B6.129c3 interval regulates iNKT cell cytokine production through an iNKT-intrinsic process.

Reduced numbers of thymic iNKT cells in B6.129c3 mice

Since it has been previously reported that the *Nkt1* locus which encompasses the B6.129c3 congenic interval, regulates thymic iNKT cell number (29), we asked whether this phenotype could be mapped to the B6.129c3 interval. A comparison of thymic iNKT cell numbers revealed a significant reduction in B6.129c3 thymic iNKT cells compared to their B6 counterparts (Figure 4a). Further analysis revealed that there was no significant difference in the overall frequency of thymic iNKT cells (data not shown), and that the decrease in thymic iNKT cell number in B6.129c3 mice was associated with a decrease in total thymocyte number (Figure 4a). Specifically, we observed a specific decrease in the number of DP, but not DN, CD4 or CD8 SP, B6.129c3 thymocytes. These data suggest that the B6.129c3 interval indirectly regulates iNKT cell development through its influence on the DP stage of thymocyte development.

Accumulating data suggest that there is significant strain-dependent variation in the distribution of iNKT cell subsets (23, 45). Therefore, we considered the possibility that the iNKT cell-intrinsic differences in cytokine production was due to regulation of the distribution of iNKT cell subsets by the B6.129c3 congenic interval. iNKT1, iNKT2, and iNKT17 subsets were identified according to their expression of PLZF and ROR γ t (45) (Figure 4b, left). Examination of the frequency and number of thymic iNKT cell subsets in C57BL/6 and B6.129c3 mice revealed a decrease in the numbers of iNKT1 and iNKT2 cells, consistent with the decrease in total iNKT cell numbers (Figure 4b). The iNKT17 subset also showed a trend toward lower numbers in the B6.129c3 mouse, albeit not significantly lower. Taken together, these results suggest that the B6.129c3 interval regulates the number of thymic iNKT cells but does not significantly affect the distribution of iNKT cell subsets.

Increased expression of Fc γ R3 on B6.129c3 iNKT cells

The B6.129c3 congenic interval contains 4 genes and two micro-RNAs (Table 1). Examination of gene expression data using the Immgen database (46) suggested that one of the genes, *Mpz* (myelin protein zero) is not expressed in leukocytes, and we confirmed this data using PCR and gene-specific primers (data not shown).

We next investigated whether one or more of the remaining three genes, *Fcgr3* (Fc fragment of IgG receptor III), *Cfap126* (cilia and flagella associated protein), or *Sdhc* (succinate dehydrogenase complex c), were differentially expressed in B6.129c3 naive mice compared to C57BL/6 controls. We sorted splenic iNKT cells from C57BL/6 and B6.129c3 mice and

used quantitative PCR to assess gene expression. Interestingly, we found that *Fcgr3* gene expression was significantly higher (5.5-fold change) on B6.129c3 iNKT cells compared to their B6 counterparts (Figure 5a). In contrast, no significant differences in *Cfap126* or *Sdhc* expression were observed between the two strains.

We next determined whether the increase in *Fcgr3* gene expression correlated with an increase in protein expression on the surface of B6.129c3 iNKT cells. Similar to a previous report (47), we observed a very low, but detectable level of Fc γ R3 expression on naïve iNKT cells from B6.129c3 mice as well as from parental strain 129X1/SvJ mice (Figure 5b). To confirm the increased expression of Fc γ R3 in B6.129c3 mice, we also assessed expression on neutrophils which express significant levels of this protein. This analysis similarly revealed increased Fc γ R3 expression on neutrophils from both B6.129c3 and 129X1/SvJ parental mice (Fig. 5b). We then compared iNKT Fc γ R3 expression between α GalCer-treated B6 and B6.129c3 mice to determine if activation altered the relative expression. While this analysis revealed significantly greater Fc γ R3 on iNKT cells and NK cells, the relative expression difference was similar to that seen in naïve mice (Fig. 5c). Together, these data suggest that one or more polymorphisms in the B6.129c3 interval regulates *Fcgr3* gene and cell surface expression, and identifies *Fcgr3* as a candidate gene responsible for regulation of the iNKT cell responsiveness to α GalCer.

Discussion

The ability of iNKT cells to rapidly produce large amounts of cytokines and chemokines in response to pathogens puts them in a unique position to shape the developing host immune response. In this context, natural genetic variants that modulate iNKT cell function could have significant downstream effects on the host immune response to pathogens. We recently demonstrated that two seemingly conflicting reports regarding the role of iNKT cells in the clearance of *Pseudomonas aeruginosa* from the lung (48, 49) could be reconciled when genetic background was taken into consideration (26). For reasons that are still unclear, BALB/c iNKT cells make a significant contribution to the host immune response after *P. aeruginosa* infection, but B6 iNKT cells do not. The strain-dependent role of iNKT cells in bacterial clearance was associated with strain-dependent differences in the response of lung iNKT cells to α GalCer. Elucidation of genetic variants that regulate iNKT cell function is necessary to understand their role in the host immune response.

Invariant NKT cell number, function, and tissue-specific homing varies widely among inbred strains of mice (22, 23, 31, 45) and several polymorphic loci that regulate iNKT cell number have been identified (20, 25, 29, 31, 32, 36, 43, 50). In an investigation into the role of iNKT cells in type 1 diabetes, it was reported that autoimmune-prone non-obese diabetic (NOD) mice possessed very low numbers of iNKT cells (25, 41, 51). A genetic locus, *Nkt1*, regulating the number of thymic iNKT cells was identified and mapped to a distal region on chromosome 1 (D1MIT15 - D1MIT155). The *Nkt1* locus contained several immunologically relevant genes including, *CD247*, *Fc γ R2b*, *Fc γ R3*, and the *Slam* family of receptors including *Slamf1* and *Slamf6* (30, 36), which are required for iNKT cell development in the thymus (37). A subsequent report implicated *Slamf1* in the regulation of thymic iNKT cell numbers as well as iNKT IL-4, but not IFN γ , production (29). Here, we

have refined this locus further and have found evidence suggesting the presence of at least two regulators of NKT cell number and function. Our data suggest that *Slam* genes may not be the major regulators of the iNKT cell response to α GalCer in this region. Although B6.129c6 mice that possess the *Slam*¹²⁹ and *Fcgr*^{B6} alleles exhibited reduced IFN- γ and IL-4 production in comparison to B6 mice, the dominant effect was observed in B6.129c3 and B6.129c4 mice that possess *Slam*^{B6} and *Fcgr*¹²⁹ alleles. Conversely, the observation that B6.129c6 iNKT cell cytokine production was significantly greater than their B6.129c3 counterparts in both cytokines measured is consistent with the presence of an additional gene or genetic modifier regulating this phenotype. Together, these data suggest the possibility that there are multiple genetic elements in the larger B6.129c2 interval that regulate the function of iNKT cells: a novel genetic element that we identified in the B6.129c3 interval, and one or more genes (most likely *Slam* genes) or genetic modifiers in the B6.129c6 interval.

We identified *Fcgr3* as a candidate gene in regulating the iNKT cell response. Fc γ R3 (CD16) is an activating receptor that binds IgG and signals through an immune-tyrosine activation domain (ITAM) and is highly expressed on monocytes, macrophages, neutrophils, and NK cells (47, 52). We found that Fc γ R3 gene and protein expression was significantly increased on iNKT cells, NK cells, and neutrophils from B6.129c3 and 129X1/SvJ parental mice compared to C57BL/6 controls. The underlying basis for this differential expression is still unclear. A comparison of *Fcgr3* promoter sequence (as defined by the Eukaryotic promoter database, (53)) between C57BL/6 and 129S1/SvImJ strains revealed no nucleotide differences. However, over 100 upstream variants were identified, suggesting the possibility that a substitution in an enhancer element could affect expression (data not shown).

Here, we confirm previous reports that iNKT cells express low, but detectable levels of Fc γ R3 (47, 54, 55). These previous reports demonstrate that engagement of Fc receptors can significantly modulate iNKT cell function. In one study, intravenous Ig (IVIG) treatment was found to exert some of its effect through its action on iNKT cells (54), and IVIG administration to C57BL/6 mice resulted in diminished iNKT cell activation. Co-administration of α GalCer and IVIG resulted in severely reduced IL-4 and IFN γ production compared to mice treated with α GalCer alone, and this inhibition was dependent on Fc γ R3 (55). Our finding that increased expression of Fc γ R3 on B6.129c3 iNKT cells is associated with decreased cytokine production is consistent with these data, although it remains unclear whether there is increased Fc γ R3 signaling in B6.129c3 iNKT cells.

Given the association of increased *Fcgr3* in B6.129c3 mice and alterations in thymocyte number, it is possible that the NKT cell-intrinsic changes in function stem from differences in development. Fc γ R3 is expressed on developing thymocytes (56, 57) and *Fcer1 γ* , which encodes the signaling chain of Fc γ R3 contributes to TCR signaling (58, 59). Interestingly, *Fcer1 γ* was recently demonstrated to be over-expressed in NKT1 cells, and deletion of *Fcer1 γ* resulted in altered frequencies of NKT1 and NKT2 subsets (60). Whether the impaired iNKT cell function observed in B6.129c3 mice is associated altered Fc γ R3 and/or *Fcer1 γ* signaling will require further study.

Although our results suggest a role for *Fcgr3* in the regulation of iNKT cells, we cannot rule out the other genetic elements that lie within the B6.129c3 region. We did not see differences in gene expression of *Sdhc* or *Cfap126* genes on sorted splenic iNKT cells. Mutations in the *Sdha*, *b*, and *c* genes have been linked to susceptibility to mitochondrial disorders (61). *Cfap126* has been shown to have a function on pancreatic β cells (62), but studies of this gene on iNKT cells have not been performed. Several microRNAs have had reported roles in regulating iNKT cell development, including the Let-7 family of microRNAs (63–65). The microRNA Mir6546 present in the B6.129c3 interval appears to be rare (66, 67) and its function has not been studied in immune cells. Therefore, although this interval contains multiple genes and genetic elements, the most likely iNKT cell regulator within our interval is *Fcgr3*.

In summary, we have fine-mapped a locus that regulates the iNKT cell response to the agonist glycolipid α GalCer. Our findings suggest that the previously described *Nkt1* locus contains at least two genetic elements that regulate NKT cell number and function, and that the dominant regulator in the region maps to a congenic interval containing Fc γ R3, identifying it as a probable candidate gene for these phenotypes. Further characterization of this genetic locus and of the Fc receptor signaling pathway will aid in our understanding of the role of iNKT cells in host immunity.

Materials and Methods

Mice and Reagents

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred at the University of Vermont. B6.129c1 mice were described previously (38). B6.129c2, B6.129c3, B6.129c4 and B6.129c6 strains were generated by backcrossing B6.129c1 mice to C57BL/6J mice, and intercrossing the heterozygous progeny. Offspring in which recombinants were identified were backcrossed to B6 to allow for the generation of homozygous congenic lines. The congenic interval boundaries were determined using primer sets designed to amplify across informative insertions/deletions and SNPs (Table 2). PCR products were sequenced to confirm boundaries. All mice were age and sex matched as indicated in the figure legends. Mice were housed in the specific pathogen-free barrier facility at the University of Vermont. The alpha-galactosylceramide (Avanti Polar Lipids, Alabaster, Alabama) was prepared as described previously (31) and administered i.p. at indicated doses in a 100 μ L volume. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee.

Serum cytokine analysis

Mice were bled via cardiac puncture, after which serum was collected and frozen at -20°C until analysis by ELISA according to the manufacturer's instructions (Biolegend, San Diego, CA, BD Biosciences, Franklin Lakes, NJ).

Flow cytometry

Splenocytes and thymocytes were obtained by gently pressing through a 70 μm nylon mesh. RBC's were lysed using Gey's solution. Cells were stained at 4°C in PBS + 2% FBS

containing 0.2% sodium azide for 30 min., after which they were washed and resuspended in PBS + 1% PFA. Abs used in these experiments were anti-CD4 (RM4-5), CD45 (30-F11), TCR β (H57-597), NK1.1 (PK136), CD8 (53-6.7) (Biolegend), and CD11b (M1/70), CD16/32 (2.4G2) and CD19 (6D5) (BD Biosciences). UV Live Dead staining reagent was included in all experiments (Life technologies, ThermoFisher, Grand Island, NY). CD1d tetramer loaded with PBS-57 was provided by the National Institutes of Health (NIH) tetramer facility (Emory University Vaccine Center, Atlanta, GA).

For intracellular cytokine staining (ICS), cells were isolated from the spleen as described above and stained with Ab to surface markers. In every ICS experiment, cells were analyzed directly *ex vivo* without further treatment. After washing in staining buffer, cells were fixed with 4% PFA for 30 min at 4°C. After washing in permeabilization buffer (0.1% saponin, 1% FCS, 0.1% sodium azide in PBS), cells were stained with anti-IL-4 (11B11) and anti-IFN γ (XMG1.2) or isotype-matched control antibodies. All intracellular antibodies were from Biolegend. Data was collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Purified CD16/32 (Clone 93) (Biolegend) was used in all samples prior to the addition of Abs to block nonspecific Ab binding, except for samples in which Fc γ R3 expression was being assessed. In those cases, Fc block was not performed, and conjugated anti-CD16/CD32 (2.4G2) (BD Biosciences, San Jose, CA) was added to the surface stain master mix. As controls for Fc γ R3 staining and to control for the addition of conjugated 2.4G2 on all Fc receptors, cells were stained with unconjugated 2.4G2 Ab in order to block receptors and subsequently stained with conjugated 2.4G2. As a separate control, cells were stained with unconjugated 2.4G2 Ab and subsequently stained with an isotype matched control (IgG2a, Biolegend).

For nuclear transcription factor staining, cells were surface stained as above, after which they were fixed overnight using the Foxp3 transcription factor staining buffer set (eBiosciences/ThermoFisher, San Diego, CA). The next day cells were washed, blocked with rat IgG, and stained with anti-PLZF (9E12) and ROR γ t (Q31-378) (BD). After staining, cells were washed, resuspended, and data were immediately collected on a LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (FlowJo LLC).

Cell isolation and culture for ex vivo cytokine analysis

Splenocytes were enriched for CD4 T cells by depletion of CD8 (53-6.7), CD11b (M1/70), MHCII (M5-114) (BioXcell, West Lebanon, NH) with anti-rat and anti-mouse IgG magnetic beads (Qiagen, Hilden, Germany). After enrichment, cells were stained with anti-TCR β , CD1dtetramer/PBS57, and DAPI, and iNKT cells were purified by fluorescence activated cell sorting (FACS Aria III). Cell purity for all sorts was between 83% and 97%. After sorting, cells were placed in modified Click's culture medium (5% FBS, 2mM glutamine, Pen/Strep, 50 μ M 2-mercaptoethanol, 20 μ g/mL gentamycin) and incubated on anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml)-coated plates at 37 °C and 5% CO₂. 72 h later, supernatants were harvested and analyzed via Milliplex assay, according to the manufacturer's instructions (EMD Millipore, Darmstadt, Germany). The milliplex kits used were the Mouse

cytokine/chemokine magnetic bead panel followed by a custom kit containing GM-CSF, IFN γ , IL-2, IL-4, IL-5, IL-10, IL-13, and IL-17.

Quantitative real-time PCR

Sorted iNKT cells were washed in cold PBS, after which cell pellets were snap frozen and kept at -80°C until RNA was isolated. RNA isolation was performed using the RNeasy Micro kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was synthesized using Superscript III (ThermoFisher, San Diego, CA). *Mpz* and *Pcp4l-1* expression was evaluated by PCR using the gene specific primers: *Mpz* forward 5'-CGGACAGGGAAATCTATGGTGC-3', reverse 5'-TGGTAGCGCCAGGTAAGAG-3'. *Pcp4l-1* forward 5'-ATGAGCGAGCTTAACACCAAA-3', reverse 5'-CTGCCAGGCTTCCCTTTTTC-3' using cDNA derived from central nervous system tissue (a gift from D. Kremontsov, University of Vermont). Expression of *Fcgr3*, *Sdhc*, and *Cfap126* was evaluated using Assay on Demand (AOD) Taqman probes (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. qPCR assay on demand (AOD) was run using the Perfecta qPCR SuperMix (Quanta Biosciences, Beverly MA). Samples were incubated with UNG SuperMIX at 45°C for 5m, then denatured initially at 95°C for 3m, followed by: 95°C denaturation for 15 s, and a combined 60°C annealing and extension step for 45 s for 40 cycles. Fold change was calculated by determining $2^{-\text{CT}}$ using mouse β -Actin as the endogenous control. Data was collected on an ABI Prism 7900HT Sequence Detection System.

Statistics

All animal experiments were conducted in a non-randomized and non-blinded fashion. One way-ANOVA, two-way ANOVA, or unpaired T tests were used where appropriate. ANOVA post-hoc analysis comparisons were made using Dunnett's test, Sidak's multiple comparisons test, or Holm-Sidak's multiple comparisons test where appropriate. In all cases, tests were considered significant when $p < 0.05$. All groups were shown to have similar variance in distribution. Sample size estimates were based on previous experience. In Figure 2B, 3 mice were excluded from the analysis due to poor α GalCer injections. When data were combined from multiple different experiments, data were first normalized to the C57BL/6 reference strain in each experiment. Normalization was accomplished by dividing each data point in an experiment by the average of the C57BL/6 response in that experiment. All statistical analysis was performed using Graph Pad Prism (GraphPad Software, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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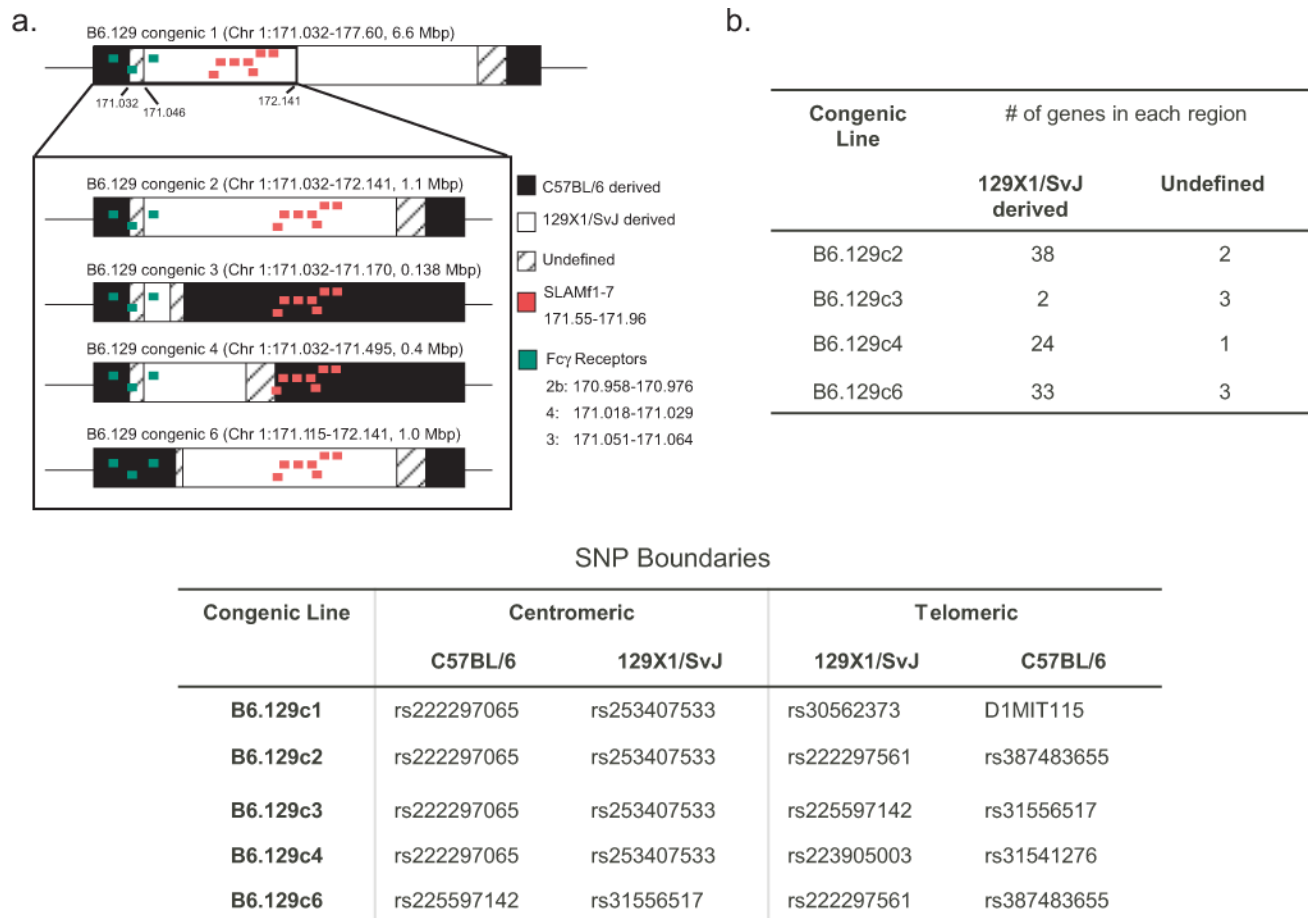
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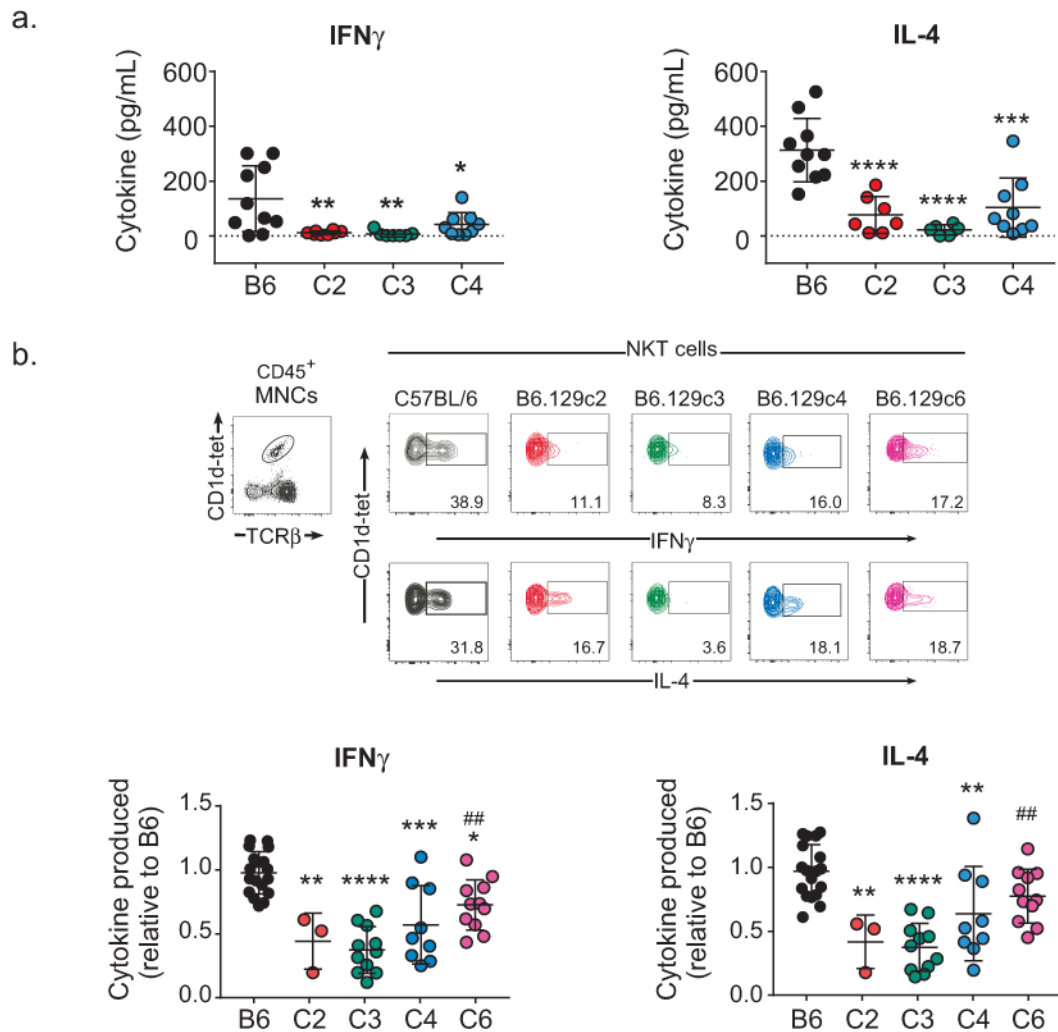
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**FIGURE 1.**

Fine-mapping of the B6.129c1 congenic interval. A). Schematic of the chromosome 1 congenic intervals used in this study. Black regions denote C57BL/6 derived regions, white denotes 129X1/SvJ derived regions, and the cross-hatched region is either C57BL/6 or 129X1/SvJ derived (undefined). For reference, the Slam family genes are denoted in red and the Fc receptor gene family is denoted in green. B). (Top) Number of protein-coding genes predicted in each subcongenic interval. (Bottom) Reference SNPs that define boundaries for each congenic line on the centromeric and telomeric ends.

**FIGURE 2.**

Regulation of α GalCer-induced iNKT cell cytokine production maps to a 0.14 Mbp region on chromosome 1. A). Decreased serum cytokine in B6.129 congenic mice in response to α GalCer. Blood was collected from B6 or B6.129 congenic mice 2 h after α GalCer administration. Serum cytokine levels were assessed using ELISA. Statistical analysis was performed using 1-way ANOVA followed by Sidak's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. B). *Top*: Representative intracellular staining of cytokine-producing iNKT cells in response to α GalCer. Splenocytes were isolated 2 h after α GalCer administration. iNKT cells were identified using CD1d-tetramer/PBS57 and TCR β . The percentages of iNKT cells expressing cytokines were determined using an isotype control for each mouse. *Lower*: Decreased iNKT cell cytokine production in B6.129 congenic mice in response to α GalCer. The data represent the relative level of iNKT cell cytokine production. Data are the combined data from 5 separate experiments using female mice 8-14wks of age and are presented as the mean \pm s.d. All mice were age-matched to controls in each experiment. Statistical analysis was performed using 2-way ANOVA followed by Tukey's multiple comparison test. * = comparison of B6 to B6.129 congenics. #

= comparison of B6.129c3 to B6.129 congenics. *p 0.05, **, ##p 0.01, ***p 0.001, ****p 0.0001.

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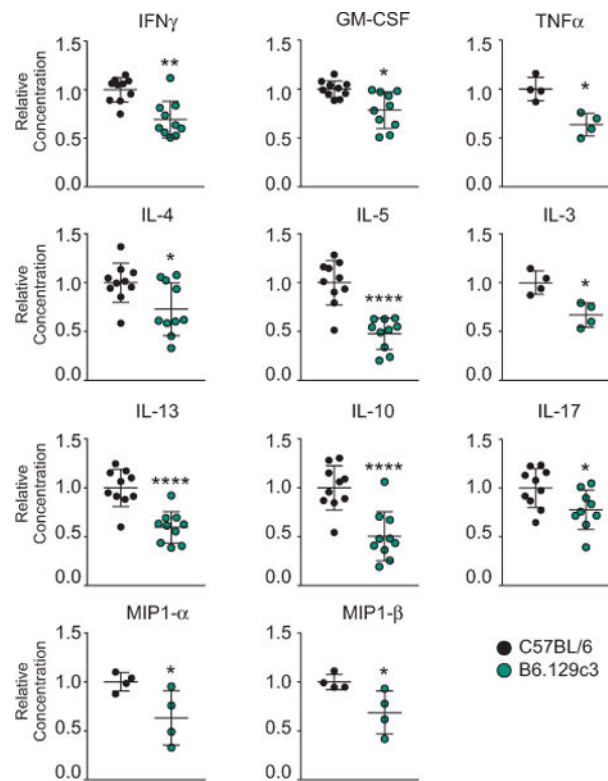


FIGURE 3.

Reduced cytokine production in B6.129c3 mice is iNKT cell-intrinsic. iNKT cells were sorted from C57BL/6 or B6.129c3 splenocytes. Cells were stimulated using anti-CD3 and anti-CD28 for 72 h, after which supernatants were analyzed using Milliplex. Data represent the cumulative results from 3 separate experiments using 8 - 11 week old female mice. Mice were age-matched in each experiment. Statistical analysis was performed using a 2-way ANOVA followed by the Holm-Sidak multiple comparison test and is reported as the mean normalized concentration \pm s.d. *p 0.05, **p 0.01, ****p 0.0001.

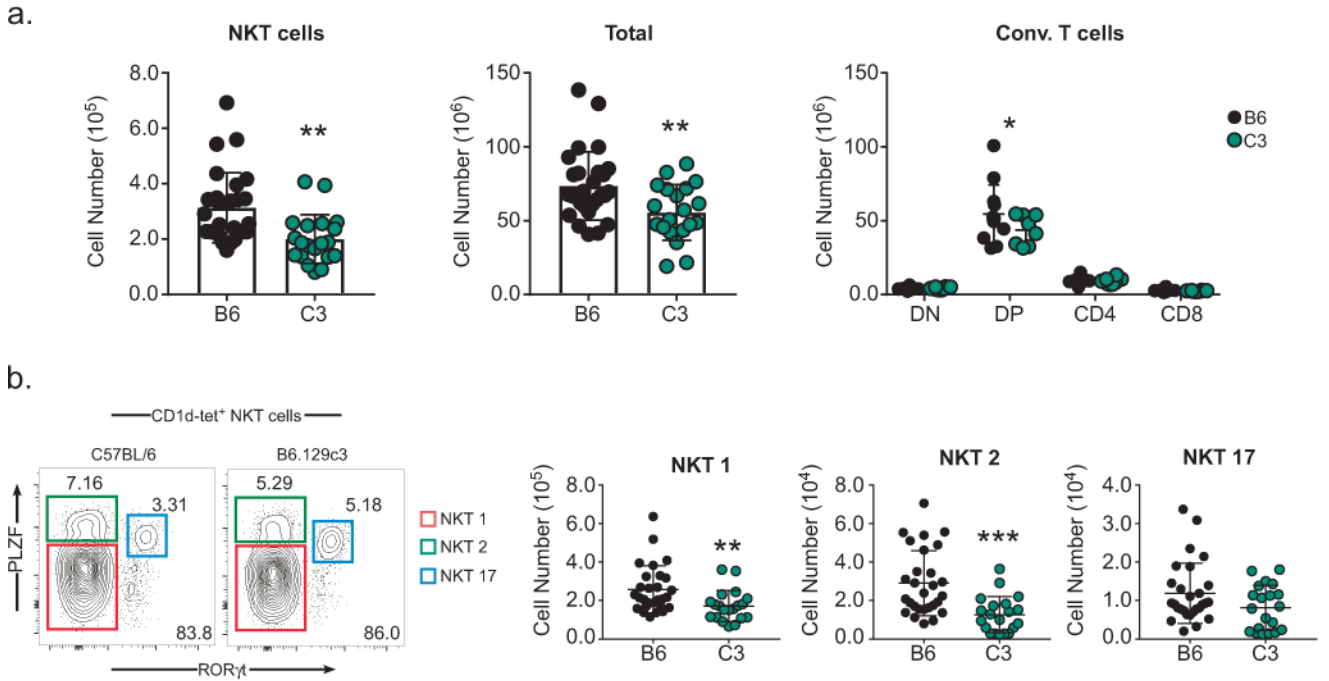
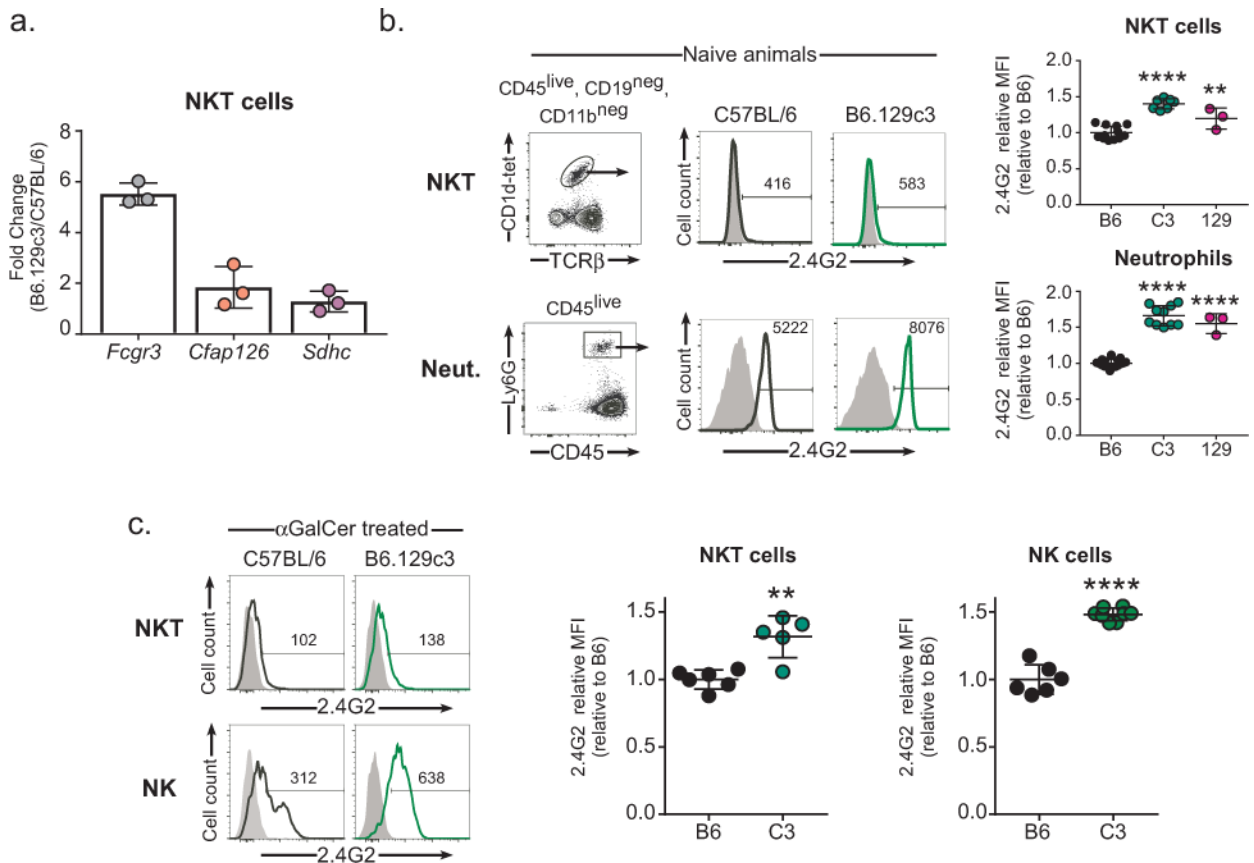


FIGURE 4. iNKT cell numbers in the thymus are regulated by the B6.129c3 interval. A). iNKT cell (*left*), total thymocyte (*middle*), thymocyte subset (*right*) numbers were compared between C57BL/6 and B6.129c3 mice. B). No significant difference in the distribution of thymic iNKT subsets between B6 and B6.129c3 mice. iNKT cell subsets were identified based on PLZF and ROR γ t expression as indicated by colored boxes (*left*). Cumulative numbers of iNKT cell subsets are shown (*right*). Data represent the cumulative results of 4 separate experiments using 9 – 13 week old female mice. Mice were age matched in each experiment. Statistical analysis was performed using an unpaired T-test. *p 0.05, **p 0.01, ***p .001

**FIGURE 5.**

Increased *Fcgr3* gene and protein expression on B6.129c3 iNKT cells. A) Increased *Fcgr3* gene expression in B6.129c3 splenic iNKT cells. Spleen iNKT cells were sorted using CD1d tetramer/PBS-57 for RNA isolation. Gene expression was determined using quantitative PCR. Data is reported as the fold change relative to B6. B) Increased cell surface protein expression of FcγR3 on naive B6.129c3 iNKT and neutrophils. Representative flow cytometry histograms show expression levels of FcγR3 on B6.129c3 iNKT cells (upper) and neutrophils (lower). Shaded dark histograms represent cells blocked with unconjugated 2.4G2 Ab and subsequently stained with conjugated isotype control. Heavy lined histograms are cells stained with conjugated 2.4G2 Ab. The numbers represent the median fluorescence intensity (MFI). Graphs depict the normalized MFI of 2.4G2 relative to the B6 control. Data represents the mean ± s.d. and is the cumulative data from 2 separate experiments of female mice aged 6-8wks. C) Increased cell surface protein expression of FcγR3 on αGalCer treated B6.129c3 iNKT and NK cells. Shaded dark histograms represent cells blocked with unconjugated 2.4G2 Ab and subsequently stained with conjugated isotype control. Heavy lined histograms are cells stained with conjugated 2.4G2 Ab. The numbers represent the median fluorescence intensity (MFI). Graphs depict normalized MFI of 2.4G2 relative to the B6 control. Data represents the mean ± s.d. and is representative data from 2 independent experiments using 10 or 14 week old female mice. Statistics were performed using a 1-way

ANOVA and followed by a post-hoc comparison to B6 controls using Dunnett's test. **p < 0.01, ****p < 0.0001

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Table 1

Genetic elements within the B6.129c3 interval.

Gene	Name	Position	Non-synonymous SNPs
<i>Fcgr3</i>	Fc receptor IgG, low affinity III	171.051-171.064	12
<i>Cfap126</i>	Cilia and flagella associated protein	171.113-171.126	1
<i>Mpz</i>	Myelin Protein Zero	171.150-171.161	1
<i>Sdhc</i>	Succinate Dehydrogenase Complex Subunit C	171.127-171.150	2
<i>Mir6546</i> <i>GM27844</i>	microRNA	171.064 171.079	n.a.

Gene positions were determined according to Ensembl release 90. Structural variants, missense variants, and splice variants were determined using the Mouse Phenome Database, Sanger 4 and NCBI dbSNP databases. SNP values are those that result in amino acid changes between C57BL/6 and 129S1/SvImJ. The B6.129c3 interval boundaries are on chromosome 1 location 171.032 Mbp to 171.170 Mbp.

Table 2

Primers for each SNP used in genotyping B6.129 congenic mice

SNP	Forward Primer 5'-3'	Reverse Primer 5'-3'
rs222297065	ACCGGACCATCTAGTGCATT	TCAGCACCTACTGGCACATT
rs253407533	ACTGGAGGTGAGGACACAGACA	ATGGTCCTTGGCTCTCCTTATG
rs222297561	CGCTCTGTTGGTTACTCTGAAG	AGAAGAGGCACACTGAGTAGGA
rs387483655	CCAGCCAAGTTATTAGGTTTCAG	CAGGTGGTTCAGATCGATTACA
rs239276905	ACCATTGGCTGAAGAGTCTG	GAAGCTGCCAAGTTCTGCTA
rs8240241	CAGAAGACAACCAGCAGAAG	CGAATAGGACTCTCGCTGTA
rs223905003	TGGTGGCCATTAGTCACTCTTC	GAGCAATGACCATCAGACCTAT
rs31541276	AACACATGGTGGCTGACAAC	CAGTCTAGCAGGACTTAAGG
rs237951232	CCATCCTCTCACCTTCACT	GGGCTTTCTAACTGCTCCTC
rs31556517	CTTGTCTCTCCTGCCTACAC	TGTCTGCCTGGAACACTCTT

All SNPs were identified using the Ensembl Gene annotation system, release 90 (68).