Supplementary figure 1
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A

Promotes cell cycle progression

Marker	Gene name	αGC-pre/B6
Ki67	Mki67	7.75
Aurora kinase B	Aurkb	8.09
budding uninhibited by benzimidazoles 1 homolog (yeast)	Bub1	6.56
Cyclin A2	Ccna2	8.8
Cyclin B1	Ccnb1	9.7
Cyclin B2	Ccnb2	9.92
Cyclin E1	Ccne1	4.29
Cyclin F	Ccnf	8.26
Coiled coil domain containing 28B	Ccdc28b	3.02
Coiled-coil domain containing 109B	Ccdc109b	38.48
Cyclin-dependent kinase 1	Cdk1	8.01
Cell division cycle 6	Cdc6	5.88
Cell division cycle 20	Cdc20	3.51
Cell division cycle 25b	Cdc25b	5
CDC42 effector protein 4	Cdc42ep4	6.02
Cell division cycle 45	Cdc45	3.34
Cell division cycle associated 2	Cdca2	6.19
Cell division cycle associated 3	Cdca3	7.38
Cell division cycle associated 5	Cdca5	5
Cell division cycle associated 8	Cdca8	6.35
Claspin	Clspn	7.06
establishment of cohesion 1 homolog 2 (S. cerevisiae)	Esco2	7.79
RAD51 associated protein 1	Rad51ap1	6.54
TPX2, microtubule-associated, homolog (Xenopus laevis)	Tpx2	7.68

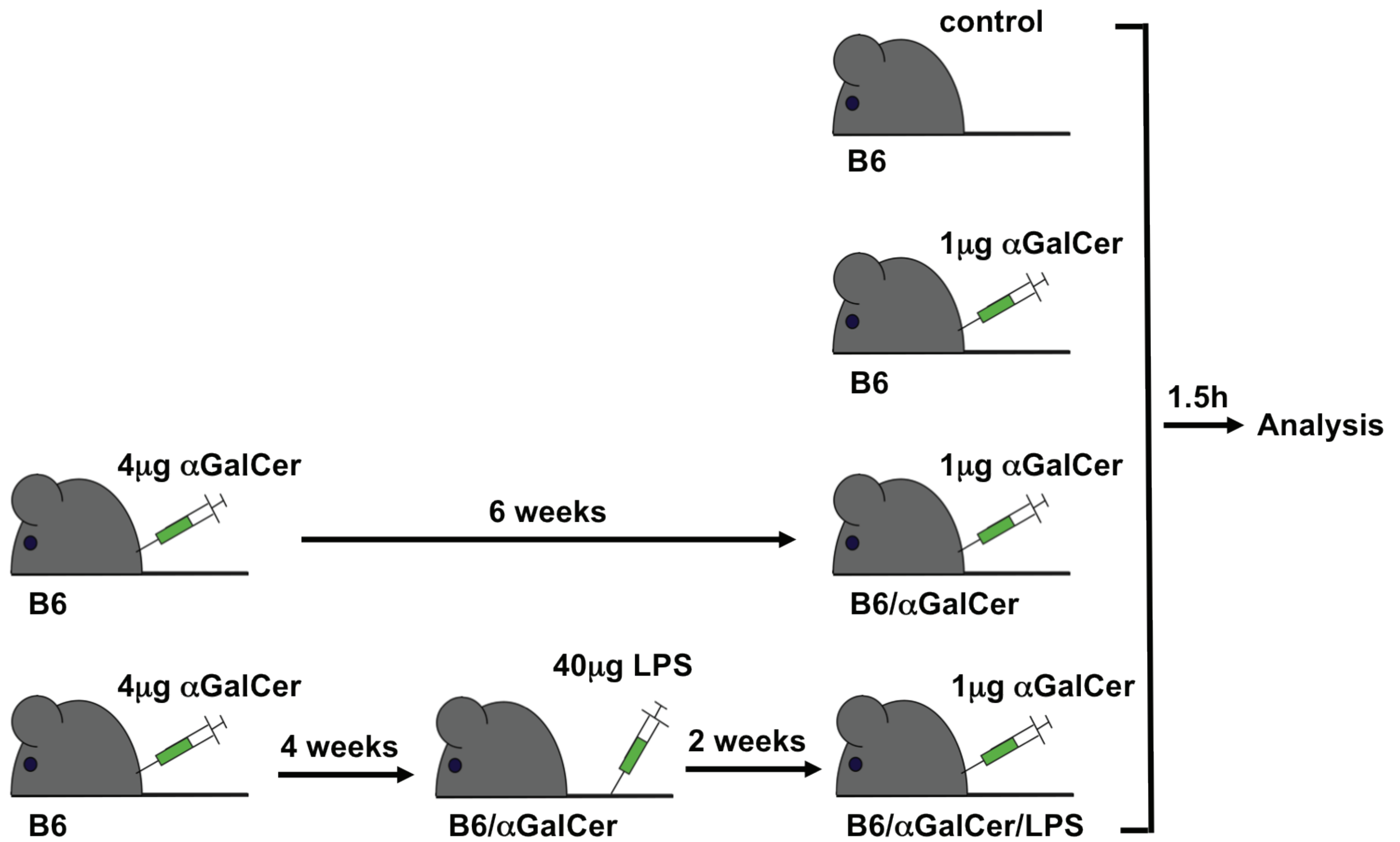
B

Inhibits cell cycle progression

Marker	Gene name	αGC-pre/B6
p21Cip1 (cyclin-dependent kinase inhibitor 1A (P21))	Cdkn1a	-1.81
p27Kip1 (cyclin-dependent kinase inhibitor 1B)	Cdkn1b	-1.13
p57Kip (cyclin-dependent kinase inhibitor 1C (P57))	Cdkn1c	n.ex.
p16Ink4a (cyclin-dependent kinase inhibitor 2A)	Cdkn2a	n.ex.
p15Ink4b (cyclin-dependent kinase inhibitor 2B (p15)	Cdkn2b	n.ex.
p18Ink4c (cyclin-dependent kinase inhibitor 2C (p18)	Cdkn2c	2.34
p19Ink4d (cyclin-dependent kinase inhibitor 2D (p19)	Cdkn2d	2.23

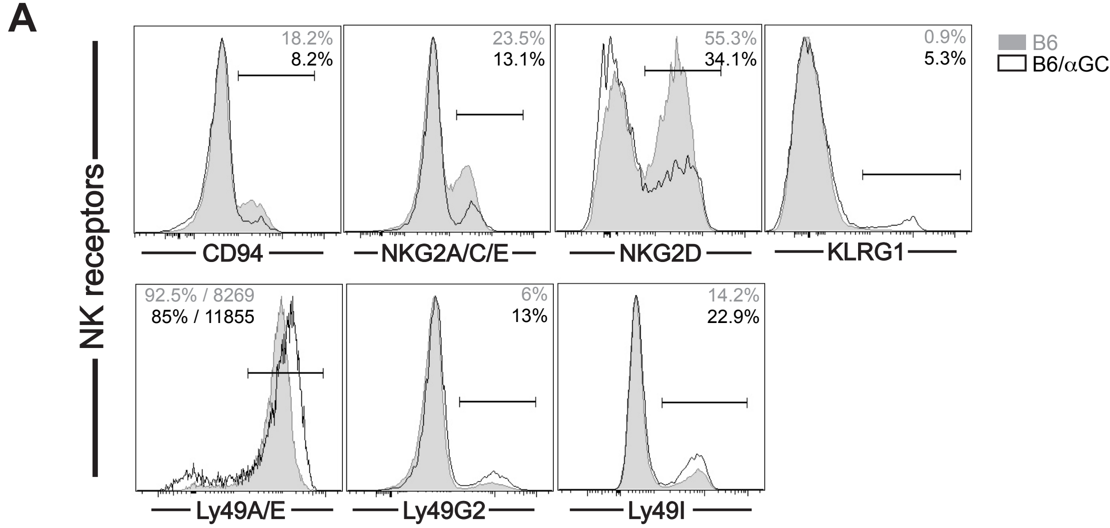
Supplementary figure 1. mRNA expression of cell cycle related genes by αGalCer pretreated *i*NKT cells: Tables show the calculated ratios of mRNA expression levels for the indicated markers in splenic *i*NKT cells from C57BL/6 mice injected one month earlier with 4µg αGalCer (αGC-pre) divided by the amount from *i*NKT cells from C57BL/6 control (B6) mice. Expression of genes indicated in red was up-regulated more than two-fold in αGalCer pretreated *i*NKT cells compared to control *i*NKT cells. n.ex. = not expressed. mRNA values were averaged from two independent experiments.

Supplementary figure 2
Sag et al.

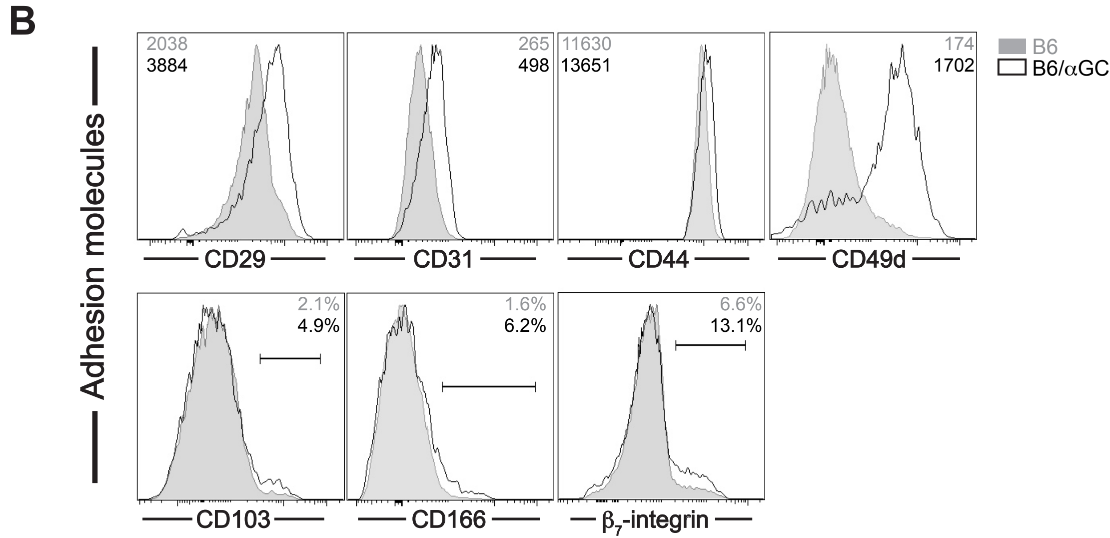


Supplementary figure 2. LPS does not overcome the TCR hypo-responsiveness of αGalCer pretreated *i*NKT cells:
Outline for the experiment depicted in Fig. 4C. 1µg αGalCer was injected i.v. into wild type mice (B6, 2nd row), mice i.v. injected six weeks earlier with 4µg αGalCer (B6/αGC, 3rd row) or mice i.v. injected six weeks earlier with 4µg α GalCer and two weeks earlier with 40µg LPS i.v. (B6/αGC/LPS, 4th row). Splenic *i*NKT cells were analyzed 90 min later for production of the indicated cytokines.

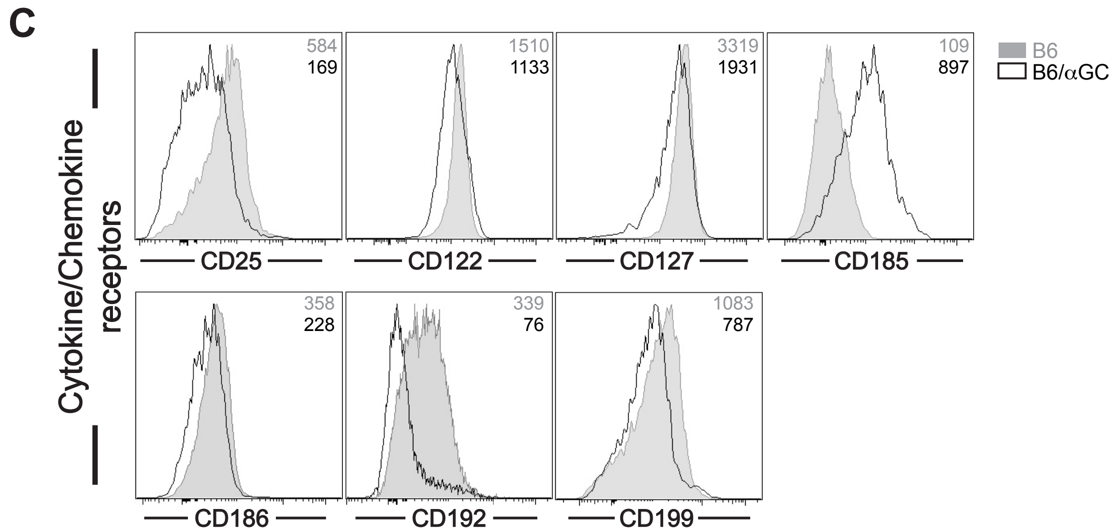
Supplementary figure 3
Sag et al.



Marker	Gene name	αGC-pre/B6
NK1.1 (CD161)	Klrb1c	-2.14
CD94	Klrd1	-1.47
NKG2A (CD159a)	Klrc1	-3.95
NKG2C	Klrc2	-1.92
NKG2E	Klrc3	n.ex.
NKp46 (CD335)	Ncr1	n.ex.
CD314 (NKG2D)	Klrk1	-2.25
NKR-P1A	Klrb1a	3.88
KLRG1	Klrg1	4.4
Ly49a	Klra1	n.ex.
Ly49e	Klra5	6.51
Ly49g	Klra7	6
Ly49h	Klra8	7.22
Ly49i	Klra9	2.35



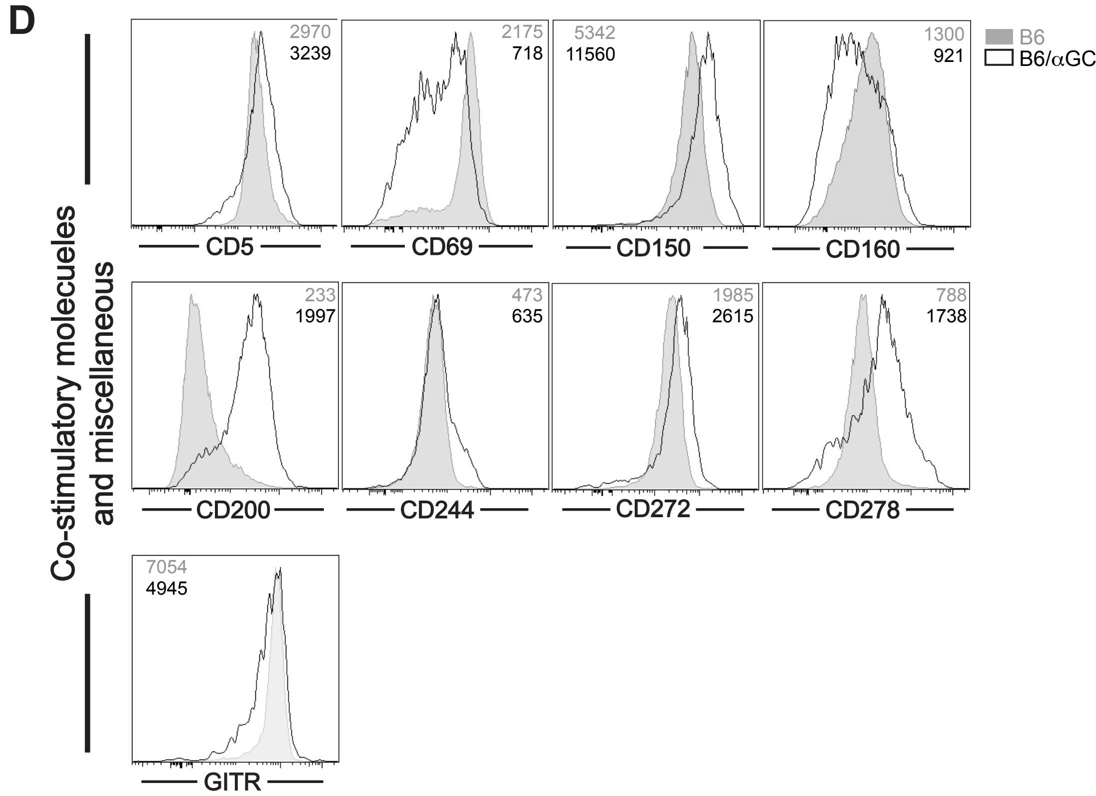
Marker	Gene name	αGC-pre/B6
CD29	Itgb1	2.3
CD44	CD44	-1.13
CD49d	Itga4	12.72
CD51	Itgav	3.1
CD61	Itgb3	-2.78
CD103	Itgae	1.74
CD166	Alcam	41.44
b7-integrin	Itgb7	1.15



Marker	Gene name	αGC-pre/B6
CD25 (IL-2Ra)	Il2ra	-5.55
CD119 (IFNgR1)	Ifngr1	-2.39
CD122 (IL-2/15Rb)	Il2rb	-2.37
CD123 (IL-3Ra)	Il3ra	1.7
CD124 (IL-4/13Ra)	Il4ra	n.ex.
CD125 (IL-5Ra)	Il5ra	n.ex.
CD126 (IL-6Ra)	Il6ra	n.ex.
CD127 (IL-7Ra)	Il7ra	-1.65
CD210 (IL-10Ra)	Il10ra	1.51
IL-12Rb1 (CD212)	Il12rb1	-1.15
IL-12Rb2	Il12rb2	-2.21
IL-18R1	Il18r1	-1.68
CXCR2 (CD182)	Cxcr2	n.ex.
CXCR3 (CD183)	Cxcr3	-1.19
CXCR4 (CD184)	Cxcr4	1.16
CXCR5 (CD185)	Cxcr5	505.89
CXCR6 (CD186)	Cxcr6	-3.16
CXCR7 (CD187)	Cxcr7	n.ex.
CCR1 (CD191)	Ccr1	n.ex.
CCR2 (CD192)	Ccr2	-3.45
CCR3 (CD193)	Ccr3	n.ex.
CCR4 (CD194)	Ccr4	n.ex.
CCR5 (CD195)	Ccr5	-4.36
CCR6 (CD196)	Ccr6	n.ex.
CCR7 (CD197)	Ccr7	3.73
CCR8 (CD198)	Ccr8	n.ex.
CCR9 (CD199)	Ccr9	-7.66
CCR10	Ccr10	1.12

Supplementary figure 3 cont.
Sag et al.

D



Marker	Gene name	αGC-pre/B6
CD4	Cd4	1.66
CD5	Cd5	1.81
CD69	Cd69	-1.52
CD150 (SLAM)	Slamf1	-1.21
CD152 (CTLA)	Ctla4	2.23
CD153 (CD30L)	Tnfsf8	2.86
CD160	Cd160	-1.84
CD200	Cd200	14.9
CD223 (LAG3)	Lag3	30.02
CD244 (2B4)	Cd244	6.66
CD272 (BTLA)	Btla	1.81
CD278 (ICOS)	Icos	1.1
CD279 (PD-1)	Pdcd1	14.44
CD355 (CRTAM)	Crtam	7.93
BATF	Batf	1.9
Bcl6	Bcl6	2.66
Blimp-1	Prdm1	-2.51
FR4	Folr4	3.34
GITR	Tnfrsf18	-1.6
Gp49b	Lilrb4	-1.27
LIGHT	Tnfsf14	-2.37
Ly108/SLAM6	Slamf6	3.63
SLAM7	Slamf7	-2.31
SLAM8	Slamf8	n.ex.
SLAM9	Slamf9	n.ex.
TIM-3	Havcr2	n.ex.

E

Marker	Gene name	αGC-pre/B6
4-1BBL	Tnfsf9	1.81
Caspase 3	Casp3	n.ex.
Cbl-b	Cblb	-1.21
CD98	Slc3a2	-1.33
CD178/FasL	Fasl	-6.06
DAGK alpha	Dgka	1.54
Egr2	Egr2	6.45
Egr3	Egr3	-13.47
GRAIL	Rnf128	1.36
GRG-4	Tle4	-1.13
Ikaros	Ikzf1	n.ex.
Itch	Itch	-1.01
Jumonji	Jarid2	1.76
LDHA alpha	Ldha	1.16
RGS2	Rgs2	-2.39
RPTPsigma	Ptprs	n.ex.
RPTPkappa	Ptprk	n.ex.
SOCS2	Socs2	-6.17

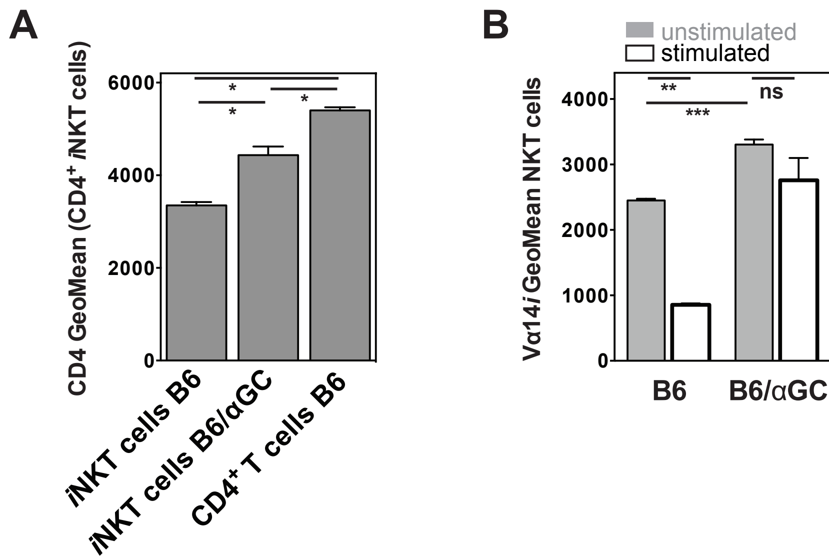
F

Marker	Gene name	αGC-pre/B6
IL-2	Il2	-7.75
IL-4	Il4	-1.79
IL-10	Il10	10.17
IL-13	Il13	-9.95
IL-17A	Il17a	-1.48
IL-21	Il21	7.38
IFNg	Ifng	-1.13
TNF	Tnf	-3.11
GM-CSF	Csf2	-8.98
CCRL2	Ccrl2	-3.56
TGFb3	Tgfb3	3.05

changes after αGalCer

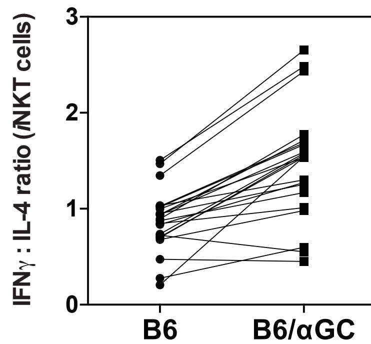
Supplementary figure 3. Surface marker and mRNA expression by αGalCer pretreated *i*NKT cells: Splenic *i*NKT cells from C57BL/6 control (B6) or C57BL/6 mice injected one month earlier with 4µg αGalCer (B6/αGC) were stained for the indicated surface proteins (A-D). The numbers in the histograms denote either the geometric mean values on *i*NKT cells or the percentage of positive cells within the depicted gate from the indicated mice. Where both values are given the geometric mean refers to the cells inside of the depicted gate, with grey numbers indicating B6 and black numbers indicating B6/αGC. Tables on the right (A-D) and in (E) and (F) show the calculated ratios of mRNA expression levels for the indicated markers in *i*NKT cells from C57BL/6 mice injected one month earlier with 4µ g αGalCer (αGC-pre) divided by the amount from *i*NKT cells from C57BL/6 control (B6) mice. For data presented in (F) *i*NKT cells were analyzed 90 min after a secondary i.v. injection of 1µg αGalCer. Expression of genes indicated in green was down-regulated more than two-fold in αGalCer pretreated *i*NKT cells compared to control *i*NKT cells. Genes indicated in red were up-regulated more than two-fold in αGalCer pretreated *i*NKT cells. n.ex. = not expressed. For the histograms representative data from at least two independent experiments are shown for each panel. mRNA values were averaged from two independent experiments.

Supplementary figure 4
Sag et al.



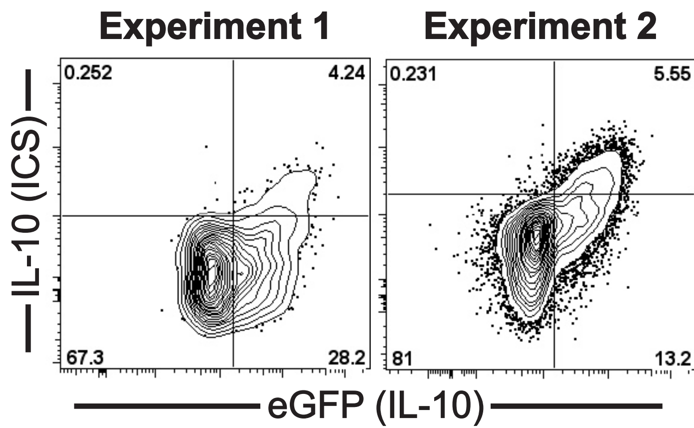
Supplementary figure 4. Expression of CD4 and TCR on αGalCer pretreated *i*NKT cells:
(A) Expression of CD4 on *i*NKT cells and CD4⁺ T cells from spleen from C57BL/6 control (B6) or C57BL/6 mice injected one month earlier with 4µg αGalCer (B6/αGC). **(B)** Control B6 or B6/αGC mice were either left untreated (unstimulated) or injected i.v. with 1µg αGalCer (stimulated), and the expression of Vα14*i* TCR was measured on splenic *i*NKT cells with CD1d/αGalCer-tetramers 16h later. ns = not significant. $p_{(B6\ vs\ B6/\alpha GC)} = 0.017$. These data represent the summary data for the representative data shown in Fig. 5C and 5D respectively. Representative data from at least three independent experiments are shown for each panel.

Supplementary figure 5
Sag et al.



Supplementary figure 5. αGalCer pretreated *i*NKT cells display a T_h1 - cytokine bias:
Splenic *i*NKT cells from C57BL/6 control (B6) or C57BL/6 mice injected 4 - 6 weeks earlier with 4μg αGalCer (B6/αGC) were re-challenged with 1μg αGalCer injected i.v. and 90 min later the expression levels of indicated cytokines in splenic *i*NKT cells were determined by intracellular staining. Expression levels of IFNγ and IL-4 were determined and the IFNγ : IL-4 ratio was calculated. The graph summarized the medium results from 22 independent experiments (2-5 mice/group) with lines connecting values from identical experiments. $p_{(B6 \text{ vs } B6/\alpha GC)} < 0.001$. No influence of the fluorochrome conjugated to the respective cytokine antibody used in different experiments was noticed on the resulting IFNγ : IL-4 ratio (data not shown).

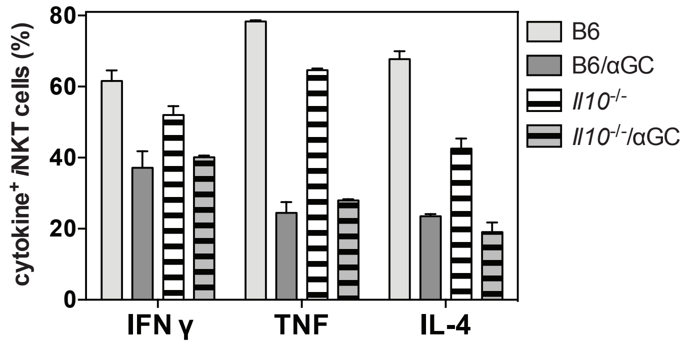
Supplementary figure 6
Sag et al.



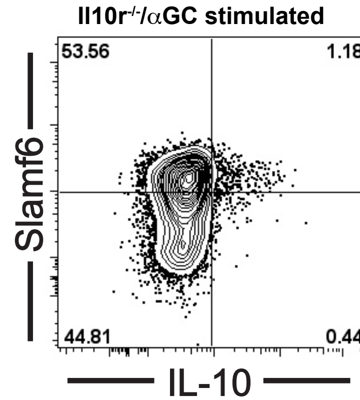
Supplementary figure 6. eGFP expression in *Il10*^{GFP} reporter mice labels cells expressing IL-10:
Il10^{GFP} reporter mice were injected with 4μg αGalCer and were one month later re-challenged with 1μg αGalCer. IL-10 expression of *i*NKT cells was analyzed 16h later by eGFP expression and intracellular staining for IL-10 protein. The numbers in the dot-plots denote the percentage of cells in the respective quadrants. Quadrant settings are based on *Il10*^{GFP} control mice. Representative data from at least three independent experiments are shown.

Supplementary figure 7
Sag et al.

A

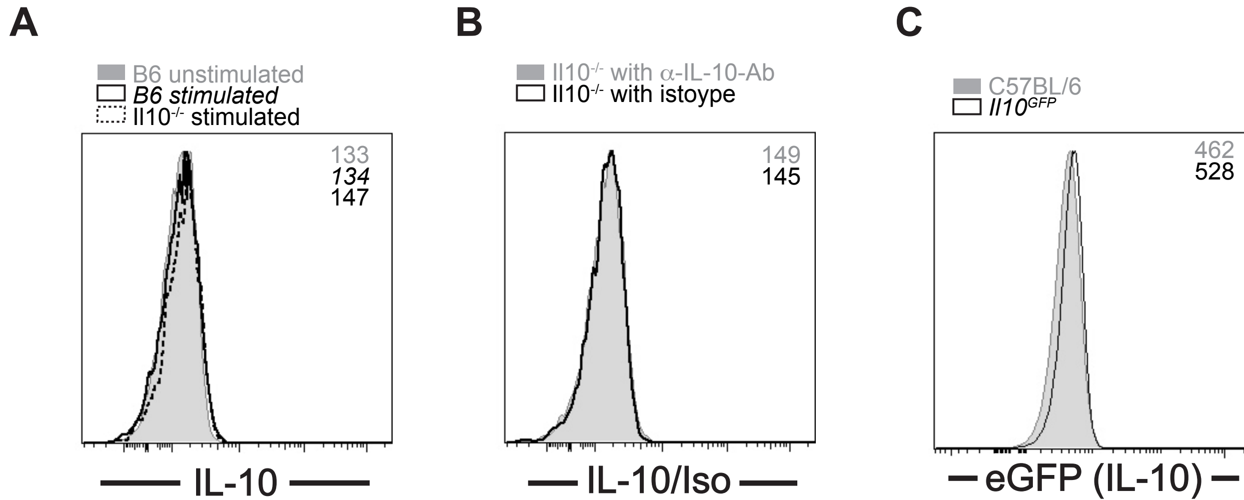


B



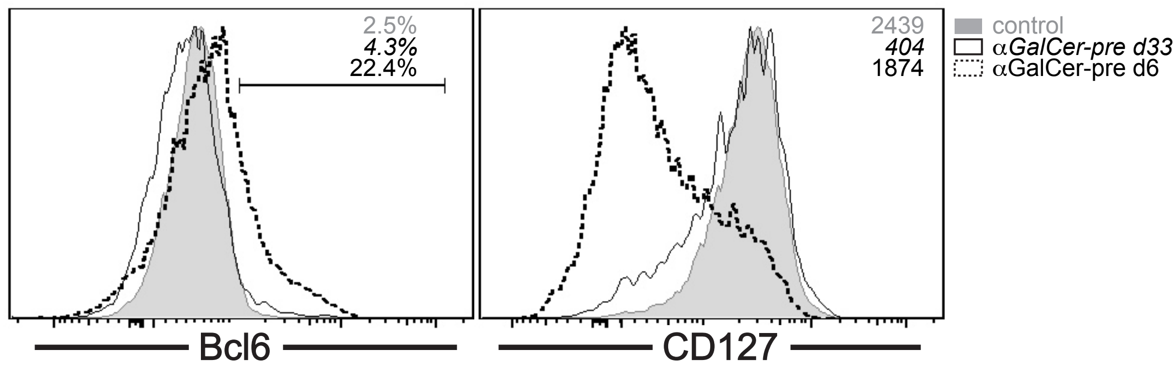
Supplementary figure 7. IL-10 is not required for induction of NKT10 cells:
(A) 1µg αGalCer was injected i.v. into wild type (B6) or *Il10*$^{-/-}$ mice or the same strains injected one month earlier with 4µg αGalCer as indicated (B6/αGC and *Il10*$^{-/-}$/αGC). *i*NKT cells were analyzed 90 min later for the expression of the indicated cytokines ($p_{(B6\ vs\ Il10-/-)} > 0.5$ for all three cytokines). **(B)** Splenocytes from IL-10 receptor deficient (*Il10r*$^{-/-}$) mice injected one month earlier with 4µg αGalCer were stimulated with PMA and ionomycin *in vitro* for 4h in the presence of protein transport inhibitors and then stained with αSlamf6 and αIL-10 antibody. Representative data from two independent experiments are shown.

Supplementary figure 8
Sag et al.



Supplementary figure 8. Background staining of IL-10 and IL10^{GFP}:
(A) *i*NKT cells from IL-10 deficient (*Il10$^{-/-}$*) or C57BL/6 (B6) mice were left untreated (unstimulated) or were stimulated with PMA and ionomycin *in vitro* for 4h in the presence of protein transport inhibitors (stimulated) and then stained with anti-IL-10 antibody. Because IL-10 producing cells are rare in recently stimulated splenic *i*NKT cells, they are not visible in a histogram. **(B)** *i*NKT cells from IL-10 deficient mice (*Il10$^{-/-}$*) were stimulated with PMA and ionomycin *in vitro* for 4h in the presence of protein transport inhibitors and then stained either with anti-IL-10 antibody (α-IL-10-Ab) or with an isotype control antibody (isotype). The short-term stimulation (1-4h) of *i*NKT cells did not cause changes in the FSC/SSC parameters or in the background staining and/or autofluorescence of markers not expressed by *i*NKT cells (data not shown). As the IL-10 background levels were identical under our *in vitro* conditions, *i*NKT cells from unstimulated C57BL/6 mice were used as negative control throughout. **(C)** Overlay of *i*NKT cells from untreated *Il10^{GFP}* and C57BL/6 mice. The numbers in the histograms denote the geometric mean values. As the IL10^{GFP} background levels were higher in *i*NKT cells from *Il10^{GFP}* than from C57BL/6 mice, *i*NKT cells from unstimulated from *Il10^{GFP}* mice were used as negative control throughout. Representative data from at least two independent experiments are shown.

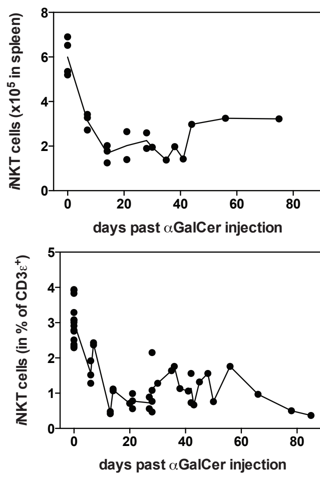
Supplementary figure 9:
Sag et al.



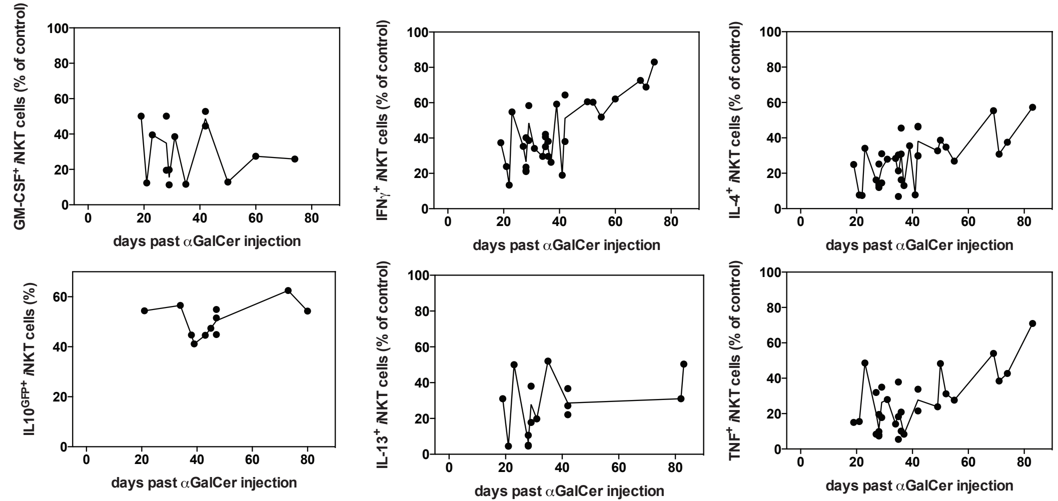
Supplementary figure 9. NKT10 cells are distinct from NKT_{FH} cells:
Expression of Bcl6 in (left panel) or CD127 on (right panel) splenic *i*NKT cells from C57BL/6 control (control, tinted) or C57BL/6 mice injected six days (αGalCer-pre d6, dotted line) or 33 days (αGalCer-pre d33, solid line) earlier. The numbers in the histograms denote the percentage of $Bcl6^{+}$ cells in the cells within the gate depicted in the histogram or the geometric mean values for CD127 on *i*NKT cells. The numbers in the dot-plots denote the relative percentage of cells in the respective quadrants. These data are representative data for the summary data shown in Fig. 7B.

Supplementary figure 10:
Sag et al.

A



C

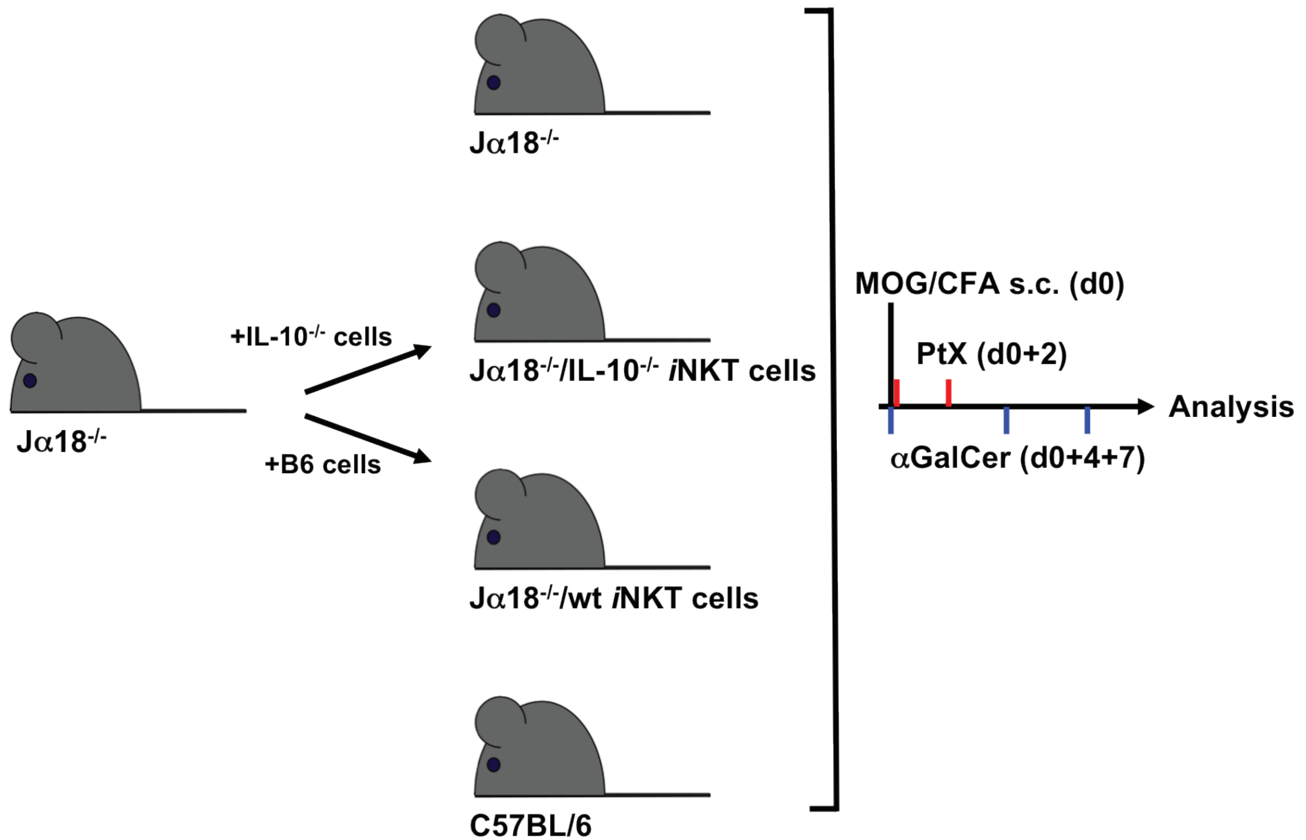


B

Bcl6+ iNKT cells (%)
BrdU+ iNKT cells (%)
CD4+ iNKT cells (%)
CD49d+ iNKT cells (%)
CD69+ iNKT cells (%)
CD127+ iNKT cells (%)
CD150+ iNKT cells (%)
CD200+ iNKT cells (%)
CD278+ iNKT cells (%)
CD279+ iNKT cells (%)
CD314+ iNKT cells (%)
FR4+ iNKT cells (%)
Ki67+ iNKT cells (%)
NK1.1+ iNKT cells (%)
NRP1+ iNKT cells (%)
Slamf6+ iNKT cells (%)
days past αGalCer injection

Supplementary figure 10. Development of the αGalCer-induced changes over time:
(A, B) Splenic *i*NKT cells from C57BL/6 control (d0) or C57BL/6 mice or *Il10*^{GFP} mice injected previously with 4µg α GalCer (days indicated) were stained for the indicated surface and intracellular proteins. **(C)** Alternatively, mice were re-challenged with 1µg αGalCer i.v. and the cytokine response by *i*NKT cells was determined 90 min later. For cytokines detected by intracellular cytokine staining the intensity of the staining depended on the fluorochrome utilized. Therefore, to make the values comparable, the values are presented as percentage of the cytokine response in the control mice (d0) (% of control). The graphs in (A-C) summarize data from independent experiments with each data point representing the mean of one experiment.

Supplementary figure 11
Sag et al.



Supplementary figure 11. NKT10 cells regulate EAE in an IL-10 dependent manner:
Outline for the experiment depicted in Fig. 7B. *Jα18*$^{-/-}$ mice left untreated (Jα18$^{-/-}$ control) or injected with 5 x 10^6 splenic *i*NKT cells enriched from either C57BL/6 (Jα18$^{-/-}$/wt *i*NKT cells) or *Il10*$^{-/-}$ mice (Jα18$^{-/-}$/IL-10$^{-/-}$ *i*NKT cells). These mice and C57BL/6 control mice were then challenged with MOG/CFA (s.c. d0) and pertussis toxin (i.v. d0 and i.p. d2) to induce EAE. Furthermore, all mice were treated three times i.v. with αGalCer (d0, d4, d7) and disease progression was scored daily from day eight onwards.