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Supporting Information

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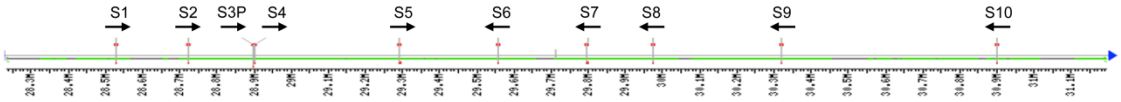
Direct identification of rat iNKT cells reveals remarkable similarities to human iNKT cells and a profound deficiency in LEW rats

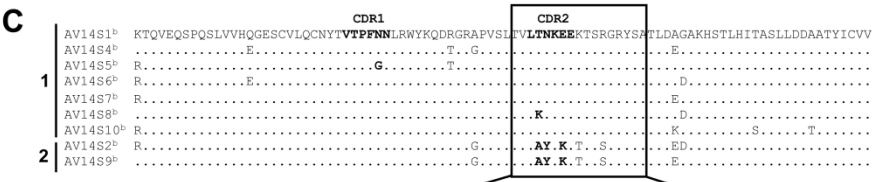
A BN *TRAV14* gene segments, chromosome 15



B

	BN actual genome		BN obsolete sequence NW_042969		F344 *AV14* gene segments identified up to date		
CDR2 Type	Gene Name	Positions at NW_047454.2	Gene Name (based on IMGT) (21)	Homology to actual genome	New name	Previous name	Reference
1	*AV14S1*	390863-391393					
2	*AV14S2*[b]	583401-583932	*TRAV11-10*	99%	*AV14S2*[f]	*AV14S3*	[9] and this study
(1)	*AV14S3P*[b]	759841-760370					
1	*AV14S4*[b]	762278-762809	*TRAV11-3*	100%	*AV14S4P*[f]	*AV14S4*	[9]
1	*AV14S5*[b]	1154998-1155529	*TRAV11-7*	100%	*AV14S5*[f]		Identified in this study
1	*AV14S6*[b]	1418612-1419143	*TRAV11-1*	100%	*AV14S6*[f]	*AV14S1*	[9]
1	*AV14S7*[b]	1659363-1659894	*TRAV11-5* *TRAV11-6*	100% 100%	*AV14S7*[f]	*AV14S2*	[9]
1	*AV14S8*[b]	1834544-1835071	*TRAV11-2* *TRAV11-8*	100% 100%	*AV14S8*[f]	*AV14S8*	[12] and this study
2	*AV14S9*[b]	2181139-2181670	*TRAV11-4*	100%			
1	*AV14S10*[b]	2760694-2761225	*TRAV11-9*	100%			

C



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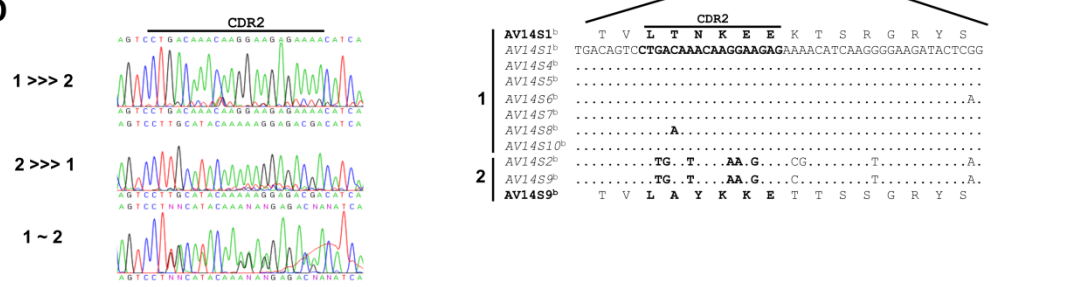

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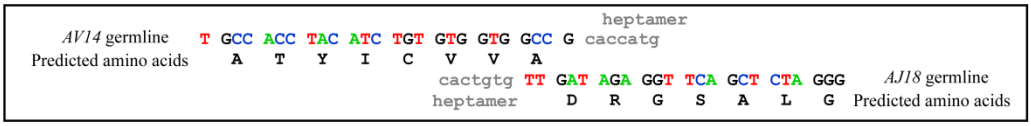
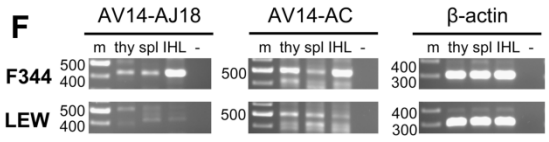
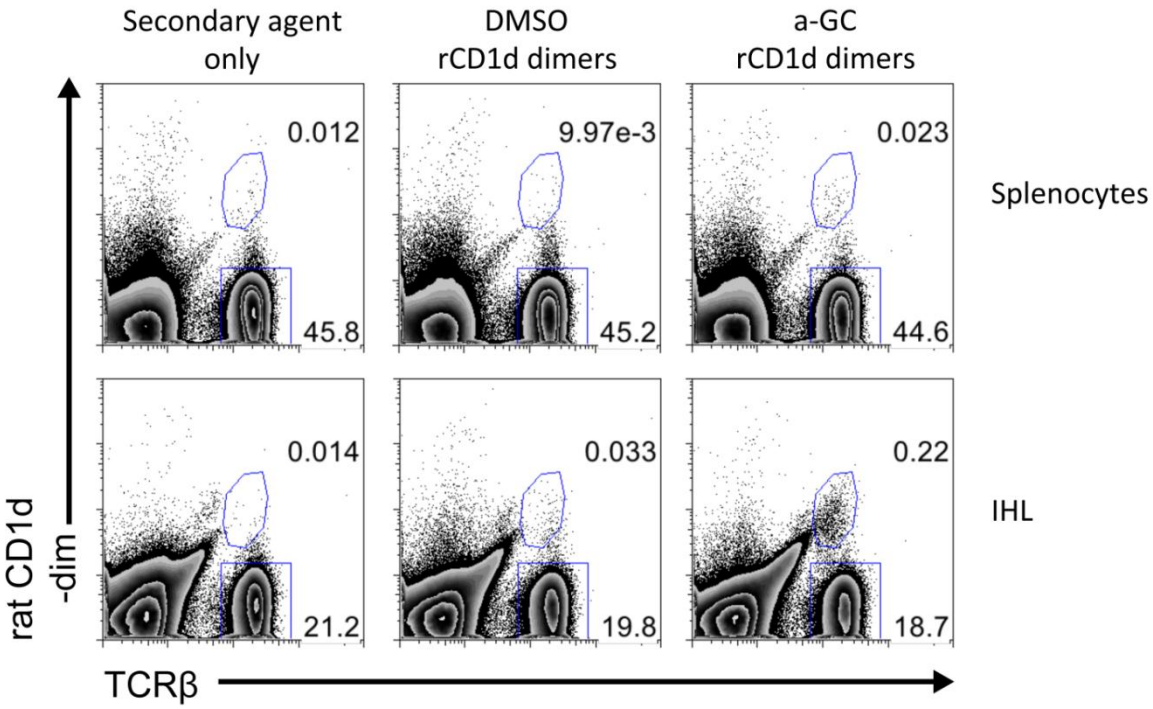


F



Supporting Figure 1. (A) Rat *AV14* gene segments in BN genome. Arrows indicate transcription orientation. (B) New nomenclature of rat *AV14* gene segments and comparison of all sequences reported to date of BN and F344 inbred rat strains. In addition to some of the published F344 *AV14* gene segments, in this study we also identified a homologous for *AV14S5* after cloning AV14-TCRα chain cDNAs derived from F344 splenocytes. (C) AV14 predicted amino acid sequences and CDR2α nucleotide sequences. The upper alignment shows the amino acid sequences predicted for the BN *AV14* gene segments, without the signal peptide. The lower alignment shows the nucleotide sequences of the BN *AV14* gene segments from which the amino acid sequences were predicted. In addition, this alignment also shows representative predicted amino acid sequences of one type 1 (above) and one type 2 (below) *AV14* gene segments. The CDR2α region is highlighted with bold letters. (D) Representative examples which illustrate the evaluation of *AV14* gene usage after RT-PCR and sequencing. (E) Partial germline sequences of *AV14* and *AJ18* gene segments and their predicted amino acid sequences. The sequence shown for *AV14* is the same as in all 10 *AV14* gene segments of BN inbred rats (from which the rat genome has been sequenced). (F) RT-PCR analysis of TCRα chains. Expected PCR sizes: AV14-AJ18, 421 bp and AV14-AC, 502 bp. RNA was prepared from thymocytes (thy), splenocytes (spl) and intrahepatic lymphocytes (IHLs). Data shown are from one of two similar experiments performed.



Supporting Figure 2. Splenocytes and IHLs derived from F344 inbred rats were stained with or without rat CD1d dimers (loaded with α-GalCer or vehicle control (DMSO) and a the secondary reagent used to visualize the dimers (PE-labeled donkey F(ab')2 fragment anti-mouse IgG (H+L) with minimal cross-reactivity to rat and other species serum proteins (Dianova)). Anti-TCRβ mAb (R73-FITC, BD Biosciences) was included in the stainings.

Supporting Table 1. iNKT cell characterization [a)]

		F344		LEW	
		Spleen	IHLs	Spleen	IHLs
iNKT cells among total lymphocytes		0.013 ± 0.007[b)]	0.24 ± 0.12 [b)]	0.005 ± 0.009[c)]	0.006 ± 0.008[c)]
iNKT cells among αβ T cells		0.03 ± 0.018 [b)]	1.05 ± 0.52 [b)]	0.012 ± 0.020[c)]	0.024 ± 0.033[c)]
iNKT cells expressing NKR-P1A/B		19.76 ± 17.23	79.66 ± 11.54	n.d.[e)]	n.d.
αβ T cells expressing NKR-P1A/B		6.78 ± 1.10	12.97 ± 1.28	8.29 ± 1.44[c)]	11.46 ± 1.06[c)]
iNKT cells among all NKR-P1A/B$^+$ αβ T cells		0.38 ± 0.31	9.79 ± 3.7	n.d.	n.d.
iNKT cells	DN (CD4$^-$ and CD8β$^-$)	63.83 ± 18.99[c)]	73.46 ± 5.57[c)]	n.d.	n.d.
	CD4$^+$ (CD8β$^-$)	31.59 ± 17.05[c)]	23.58 ± 6.08[c)]	n.d.	n.d.
	CD8β$^+$ (CD4$^-$)	1.78 ± 3.7[c)]	2.39 ± 2.53[c)]	n.d.	n.d.
	CD8β$^+$ and CD4$^+$	2.78 ± 8.36[c)]	0.55 ± 0.87[c)]	n.d.	n.d.
	CD8α$^+$ (CD4$^-$)	12.22 ± 5.09	10.79 ± 3.34[d)]	n.d.	n.d.
	CD8α$^+$ and CD4$^+$	4.38 ± 3.51	3.25 ± 3.15[d)]	n.d.	n.d.
iNKT cells expressing BV8S4A2$^+$ TCRs		24 ± 20.73	41.61 ± 9.32	n.d.	n.d.
αβ T cells expressing BV8S4A2$^+$/BV8S2A1$^+$ TCRs		6.80 ± 0.06	7.70 ± 1.23	8.19 ± 1.46[c)]	4.99 ± 0.33[c)]

[a)] Figure 2A and B depict for most of the cases how the data were analyzed. In all cases where applicable the final percentages were obtained after subtraction of cells stained with vehicle-CD1d dimers. The mAb R78 binds to BV8S4A2 and BV8S2A1-positive TCRs in F344 and LEW inbred rats, respectively [10]. The data are shown as mean ± SD of the indicated cell populations as analyzed by flow cytometry, of three individual animals (n = 3) except when indicated otherwise.

As an example the final iNKT cell numbers specifically stained with α-GalCer-CD1d dimers among 100,000 F344 IHLs expressing CD4$^+$, CD8β$^+$ or being DN or CD4$^+$CD8β$^+$ (Figure 2*A*) are 48, -3.3, 139.9 and 1.25 respectively, which leads to the following proportions of iNKT cell subsets: 25.83% CD4$^+$, 0% CD8β$^+$, 74.14% DN and 0.026 CD4$^+$CD8β$^+$. The data shown in this Table were calculated in this manner.

[b)] Data shown are mean ± SD of n = 10 animals.

[c)] Data shown are mean ± SD of n = 9 animals.

[d)] Data shown are mean ± SD of n = 6 animals.

[e)] n.d. Not determined.

Supporting Table 2. *AV14* gene segment usage in F344 and LEW inbred rat strains [a)]

		F344				LEW		
	Experiment	1	2	3	4	1	2	3
AV14-AJ18	Thymus	n.d.[b)]	2 >> 1	2 >>> 1	2 >>> 1	1 >>> 2	n.o.[c)]	n.o.
	Spleen	2 >>> 1	1 ~ 2	2 >>> 1	2 >>> 1	1 ~ 2	1 >> 2	n.o.
	IHLs	2 >> 1	1 ~ 2	2 >>> 1	2 >>> 1	n.o.	n.o.	n.o.
AV14-AC	Thymus	n.d.	2 > 1	2 >>> 1	2 >>> 1	1 >>> 2	1 >> 2	1 >> 2
	Spleen	2 >> 1	2 > 1	2 >>> 1	2 >>> 1	1 >>> 2	1 >> 2	1 >> 2
	IHLs	2 >> 1	1 ~ 2	2 >>> 1	2 >>> 1	n.o.	1 ~ 2	1 >>> 2

[a)] Usage of type 1 and 2 *AV14* gene segments was addressed by RT-PCR and sequencing. RT-PCR products were obtained as described in the Supporting Methods section and in Supporting Figure 1F. The method of data evaluation to show the abundance of either AV14 type with “>” symbols is illustrated in Supporting Figure 1D.

[b)] n.d. Not determined.

[c)] n.o. Not obtained appears when no RT-PCR product or sequences were obtained.

Supporting table 3. Phenotypic characterization of expanded iNKT cells [a)]

	Spleen				IHLs
	d 7		d14		d7
	PLZF	Dimer	PLZF	Dimer	Dimer
% of iNKT cells	2.96 ± 1.29	1.29 ± 1.05	48.13 ± 30.23	17.91 ± 10.29[c)]	37.44 ± 12.15
Fold expansion	44.30 ± 19.31	16.71 ± 6.19	397 ± 295.31	165 ± 180.54[c)]	80.63 ± 106.89
DN ($CD4^-$ and $CD8\beta^-$)	87.91 ± 4.30	84.35 ± 7.96	95.82 ± 1.17	93.37 ± 1.62[c)]	93.96 ± 1.98
$CD4^+$ ($CD8\beta^-$)	11.48 ± 4.22	13.05 ± 8.26	3.97 ± 1.13	6.23 ± 1.46[c)]	4.04 ± 0.57
$CD8\beta^+$ ($CD4^-$)	0.53 ± 0.65	1.91 ± 0.99	0.17 ± 0.08	0.32 ± 0.36[c)]	1.71 ± 1.88
$CD8\beta^+$ and $CD4^+$	0.07 ± 0.12	0.67 ± 0.69	0.03 ± 0.05	0.06 ± 0.06[c)]	0.28 ± 0.34
$CD8\alpha^+$ ($CD4^-$)	10.23 ± 0.58	10.61 ± 3.04	2.14 ± 0.56	2.88 ± 1.46[c)]	n.d. [e)]
$CD8\alpha^+$ and $CD4^+$	1.76 ± 1.22	4.09 ± 3.05	0.14 ± 0.07	0.76 ± 0.60[c)]	n.d.
NKR-P1A/B	22.37 ± 7.09[d)]	22.9 ± 3.02[b)]	2.1 ± 2.08[d)]	2.93 ± 0.57[b)]	63.02 ± 20.01
iNKT cells expressing $BV8S2A2^+$ TCRs	53.00 ± 3.95[d)]	36.09 ± 3.7[b)]	46.26 ± 2.52[d)]	31.72 ± 9.42[b)]	43.26 ± 2.78[d)]
iNKT cells expressing $BV16^+$ TCRs	0.30 ± 0.05[d)]	0.84 ± 0.72[b)]	0.17 ± 0.01 [d)]	0.46 ± 0.33[b)]	3.32 ± 0.22[d)]

[a)] The table shows the mean percentages ± SD of the indicated cell populations (analyzed by flow cytometry) of four individual cultures (n=4) except when indicated otherwise. iNKT cells were identified as PLZF+ cells or as cells stained with α-GalCer-CD1d dimers. The final percentages were obtained after subtraction of cells stained with vehicle-CD1d dimers, for cells stained with α-GalCer-CD1d dimers or control isotype staining for cells stained with anti-PLZF mAb.

[b)] Data shown are mean ± SD of n = 5 animals ‡ (n=5)

[c)] Data shown are mean ± SD of n = 3 animals † (n=3)

[c)] Data shown are mean ± SD of n = 2 animals * (n=2)

[e)] n.d. Not determined.

Supporting Methods

RT-PCR and sequencing

RNA was extracted from primary cells prepared as described in the supporting information or from cells obtained after 14 days of culture with α-GalCer (splenocytes) using the RNeasy Mini kit from Qiagen. cDNA was prepared from 500 ng RNA with a First Strand Synthesis kit (Fermentas). Semi-quantitative RT-PCR was performed with Tac polymerase (Fermentas) whereas for sequencing experiments Phusion polymerase (Finnzymes) was used. AV14-AJ18 PCRs were carried out with the *rVa14lead f* (CTTCTGCAGAAAAACCATGGGGAAG) and the *rJa18lead r* (AGGTGTGACAGTCAGCTGAGTTCC) primers. Based on sequence comparison of all *AV14* gene segments detected in the BN rat genome, the *rVa14lead f* primer is expected to amplify all AV14 gene segments regardless of their CDR2 type. AV14-AC PCRs were performed with the *rVa14lead f* and the *rCa_seq2nd* (AGTCGGTGAACAGGCAGAGGG) primers. The primers used for amplification of rat β-actin were: *RNBetaActFwd* (CACCACCACAGCTGAGAGG) and *RNBetaActRev* (AGACAGCACTGTGTTGGCATAG). All PCRs were conducted with an annealing temperature of 66ºC and 35 amplification cycles, except for β-actin where only 28 cycles were carried out and for some sequencing experiments of F344 splenocytes and LEW-derived RNA where amplification was performed during 40 cycles. In sequencing experiments PCR products of the expected size were purified after gel electrophoresis. Sequencing was carried out with BigDye 3.1 (AB Applied Biosystems) and the *rVa14lead f* primer if the CDR2α region was analyzed or the *rAV14int primer* (CCTTCAATGCAATTACACTGTG) when AJ usage was addressed. Sequences were analyzed in an ABI 3100 sequencer.

Cloning of AV14-TCRα chains

AV14-TCRα chain cDNAs were cloned from F344 splenocytes derived cDNA into the EcoRI and BamHI restriction sites of the retroviral expression vector pczCFG5 IZ. AV14-TCRα chain cDNAs were amplified by RT-PCR using the primers *rVa14EcoRI-Fow* (GGGCTAGAATTCTGCAGAAAAACCATGGGGAAG) and *Ca end BamHI* antisense (ATGCGGATCCTCAACTGGACCACAGCCTTAGCGTCATGAG), which contain the restriction sites (underlined) which were used for insertion into the expression vector. Clones obtained containing an insert of the expected size were sequenced. One novel F344 *AV14* gene segment was identified. The sequence can be found in GenBank under JQ074230 (Accession number).