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Differential Requirement for the CD45 Splicing Regulator hnRNPLL for Accumulation of NKT and Conventional T Cells

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Abstract

Natural killer T (NKT) cells represent an important regulatory T cell subset that develops in the thymus and contains immature (NK1.1lo) and mature (NK1.1hi) cell subsets. Here we show in mice that an inherited mutation in heterogeneous ribonucleoprotein L-like protein (hnRNPLLthunder), that shortens the survival of conventional T cells, has no discernible effect on NKT cell development, homeostasis or effector function. Thus, Hnrpl deficiency effectively increases the NKT:T cell ratio in the periphery. However, Hnrpl mutation disrupts CD45RA, RB and RC exon silencing of the Ptprc mRNA in both NKT and conventional T cells, and leads to a comparably dramatic shift to high molecular weight CD45 isoforms. In addition, Hnrpl mutation has a cell intrinsic effect on the expression of the developmentally regulated cell surface marker NK1.1 on NKT cells in the thymus and periphery but does not affect cell numbers. Therefore our results highlight both overlapping and divergent roles for hnRNPLL between conventional T cells and NKT cells. In both cell subsets it is required as a trans-acting factor to regulate alternative splicing of the Ptprc mRNA, but it is only required for survival of conventional T cells.

Introduction

Natural Killer T (NKT) cells are a specialized subset of T lymphocytes that have the ability to regulate the immune response in a range of diseases [1,2]. Unlike conventional T cells, NKT cells express an invariant T-cell receptor α chain (Vα14Jα18 in mice and Vα2[4][Jα18 in humans) that is paired with a limited repertoire of Vβ chains (Vβ8.2, Vβ7, and Vβ8 in mice and Vβ11 in humans) [2,3]. The antigen receptor of NKT cells recognizes glycolipid antigens, such as α-galactosylceramide (αGalCer), presented by the nonclassical MHC-I like molecule CD1d [2,4,5]. NKT cells are positively selected from CD4+ CD8+ double positive (DP) thymocytes in a CD1d dependent manner in the thymus [6] and pass through four developmental stages that can be distinguished on the basis of CD24, CD44 and NK1.1 expression [2,3]. The most immature cells are CD24+ but lack expression of CD44 and NK1.1 (stage 0) [7], and these give rise to CD24+ cells that are CD44hi and NK1.1hi (stage 1). These differentiate to become CD44hi NK1.1lo cells (stage 2) at which point they can be either exported to the periphery or continue to mature in the thymus [8,9]. The final stage of maturation coincides with up-regulation of NK1.1 to become CD44hi NK1.1hi (stage 3) cells [8,9]. The development and the maintenance of stable numbers of mature NKT cells in the thymus and periphery is determined by a wide range of factors including transcription factors (e.g. c-Myc, Egr2, PLZF, T-Bet) and cytokine signaling via IL-15 and TGF-β [2,5]. The development and function of NKT cells can also be influenced by costimulatory molecules such as SLAM/SAP, CD80/CD86 and ICOS [2,3]. Another important feature of NKT cells is that upon activation they are able to rapidly produce a diverse range of cytokines and this gives rise to an array of functionally distinct NKT cell subsets in both mice and humans that can be distinguished on the basis of the patterns of cytokine secretion [10,11,12]. Because NKT cell numbers are critical to the outcome of many diseases [1], and that the numbers vary widely between individuals [3], it is crucial that we understand how the development and function of NKT cells differs from that of other T cell subsets.

The heterogeneous nuclear ribonucleoprotein L-Like, hnRNPLL, is a member of hnRNP protein family that is essential for mRNA alternative splicing in T cells [13,14,15]. The role of hnRNPLL was revealed by a recessive loss of function mouse mutation isolated in an N-ethyl-N-nitrosourea (ENU) mutagenesis screen, thunder (Hnrplthunder) [15]. This mutation disrupts the first of three RNA-recognition motif domains in the hnRNPLL protein that binds to activation-responsive silencing (ARS) elements in the
variably expressed exons 4, 5 and 6 of Ptprc [16,17]. As a result, there is a failure to silence the inclusion of these exons in naïve and memory T cells so that cell surface CD45 protein shifts from the normal isoforms on T cells, CD45RB and RO, to forms such as CD45RA and CD45RC that are normally not found on T cells [15]. The Hnrlplmutation does not affect conventional 3b T cell differentiation in the thymus but it greatly shortens the survival of naïve and memory 3b T cells in the peripheral lymphoid tissues [15]. This is independent of the change CD45/ptprc splicing, and must be explained by alternative splicing among the hundreds of other mRNAs that are regulated directly or indirectly by Hnrlpl [15,18]. NKT cells express the tyrosine phosphatase CD45, similar to other mRNAs that are regulated directly or indirectly by Hnrlpl. NKT cells express the tyrosine phosphatase CD45, similar to other mRNAs that are regulated directly or indirectly by Hnrlpl.

Results

hnRNPLL is required for the splicing of CD45 isoforms in NKT cells

Flow cytometric analysis of CD45 isoform expression on NKT cells was performed by staining thymus and spleen cells from wild type and Hnrlplthu/thu mice with a -GalCer-loaded CD1d tetramers and antibodies to TCRβ [20,21]. This revealed that there was high expression of CD45 and no difference in total CD45 staining intensity at the cell surface in Hnrlpl-mutant NKT cells (Figure 1A). NKT cells from wild type mice expressed very little CD45RA or RC isoforms, which include the variable segments encoded in Ptprc exons 4 and 6 respectively, and intermediate levels of the CD45RB isoform derived from exon 5. This pattern of CD45 isoform expression is similar to that observed on conventional 3b T cells (CD4 cells in Figure 1B). By contrast, CD45RA, CD45RB and CD45RC isoforms were increased to between 10 and 100 times higher levels on NKT cells from Hnrlplthu/thu mice (Figure 1B). This result demonstrates that hnRNPLL is critical for silencing Ptprc exons 4, 5 and 6 within NKT cells, and Hnrlpl mutation has a comparable effect on this process in NKT and conventional T cells.

Normal NKT cell numbers in the thymus and periphery of Hnrlplthu/thu mice

Given that NKT cells and conventional T cells develop in the thymus from the same precursor, we wanted to examine if the Hnrlpl mutation affected NKT cell development, function and homeostasis. We stained the thymus, spleen and liver of wild type and Hnrlplthu/thu mice for conventional and NKT cells. Conventional T cells in the thymus were not significantly different in frequency or absolute number between wild type and Hnrlplthu/thu mice, but the mutation reduced the number of peripheral CD4+ and CD8+ T cells (Figure 2A) which we have previously shown is caused by loss of naïve CD4+ and CD8+ cells [15]. By comparison, Hnrlpl mutation did not affect the number of NKT cells in the thymus, spleen or liver (Figure 2B&C). However, we observed that NKT cells from Hnrlplthu/thu mice have a reproducibly lower level of TCRβ expression as measured by intensity of tetramer staining compared to wild type cells. Thus, in the spleen the ratio of absolute number of NKT cells to absolute number of TCRβ+ conventional T cells was significantly increased in Hnrlplthu/thu mice suggesting an important role for hnRNPLL in maintaining a normal NKT:T cell ratio in the periphery (Figure 2D). Two subsets of NKT cells can be distinguished based on CD4 expression, namely CD4+ and CD4− [3]. There was no significant difference in the number of CD4+ or CD4− NKT cell subsets between wild type and Hnrlplthu/thu mice in the thymus, spleen or liver (Figure 2E&F). Thus, while NKT and conventional T cells share a requirement of hnRNPLL to control their CD45 isoforms, they differ in their hnRNPLL requirement for accumulating in normal numbers.

Decreased NK1.1 during NKT cell development in Hnrlplthu/thu mice

NKT cells differentiate in the thymus and progress through well-defined stages that can be distinguished on the basis of expression of CD44 and NK1.1 [3]. We examined the developmental stages of NKT differentiation in the thymus and peripheral tissues of wild type and Hnrlplthu/thu mice and observed a slight increase in the mean frequency and absolute number of CD44+ NK1.1+ (Stage 1) and CD44+ NK1.1+ (Stage 2) NKT cells and a reduction in the mean frequency and number of mature CD44− NK1.1+ (Stage 3) NKT cells in the thymus of Hnrlplthu/thu mice compared to wild type controls (Figure 3A&B). Stage 2 NKT cells can undergo maturation to stage 3 cells either in thymus or peripheral tissues. We also observed a similar trend with an increase in NK1.1+ NKT cells in the spleen of Hnrlplthu/thu mice with a corresponding reduction of NK1.1+ NKT cells compared to wild type animals (Figure 3C&D). However the change in cell numbers within the different cell subsets was not statistically significant, and rather than representing a defect in NKT cell maturation, they may simply be secondary to the fact that Hnrlplthu/thu NKT cells in the thymus and spleen had significantly lower NK1.1 expression (Figure 3E&F). NK cells in Hnrlplthu/thu mice also displayed a lower cell surface NK1.1 expression but, similar to the NKT cell population, no difference in the accumulation of these cells in the peripheral lymphoid tissues compared to conventional 3b T cells was detected (Figure 3G&H).

The positive selection of NKT cells occurs in a CD1d-dependent manner from DP thymocytes [6]. Analysis of CD1d expression in the thymus showed that the Hnrlpl mutation does not affect the expression of CD1d on DP cells (Figure 3F). T-Bet (Tbx21) is another important factor in the transition from Stage 2 to Stage 3 cells by up-regulating CD122 expression [22]. Intracellular flow cytometric staining of thymic NKT cells revealed normal T-Bet expression in Hnrlplthu/thu NKT cells compared to wild type cells (Figure 3F). These results support the notion that hnRNPLL is not required for the development of NKT lineage in the thymus or periphery but is required for full induction of NK1.1.

Hnrlpl mutant NKT cells exhibit a cell autonomous decrease in NK1.1 in mixed bone marrow chimeras

To investigate the possibility that homeostatic effects were masking a role for hnRNPLL in NKT cell development, mixed bone marrow chimeras (BMC) were used to investigate the importance of hnRNPLL on NKT and T cell development in a competitive situation. Irradiated C57BL/6 mice were reconstituted with an equal mixture of CD45.1-marked wild-type Hnrlpl+/+...
C57BL/6 bone marrow and CD45.2 \textit{Hnrpll}^{thu/thu} C57BL/6 bone marrow cells. Recipient mice were analysed 12–14 weeks after injection by examining donor derived B cells, conventional T and NKT cells. The relative reconstitution of each chimeric mouse by \textit{Hnrpll}^{thu/thu} CD45.2\textsuperscript{+} hemopoietic stem cells was gauged by the percentage of B cells that were CD45.2\textsuperscript{+}, since this lineage...
expresses very little Hnplll and is not affected by the Hnplll mutation [15]. The results showed that comparing the ratio of T cells to B cells in the recipient mice, there was a significant decrease in the proportion of conventional T cells in the spleen compared to wild type animals (Figure 4A). The competitive disadvantage of Hnplllthu/thu derived conventional TCR$\alpha$$\beta^{+}$ cells in the periphery is consistent with previous studies in mixed chimeras [13]. In contrast there was no significant difference in the ratio of wild type or Hnplllthu/thu derived NKT cells to B cells in the spleen of recipient mice (Figure 4B), establishing that the loss of function of hnRNPLL did not affect the differentiation or long-term survival of mature NKT cells. Detailed analysis of Hnplllthu/thu versus

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**Figure 3. Decreased NK1.1 during NKT cell development in Hnplllthu/thu mice.** (A, C) Representative flow cytometric dot plots showing the development stages of NKT cell in the (a) thymus and (c) spleen in intact wild type (+/+ and Hnplllthu/thu (thu/thu) mice based on expression of CD44 and NK1.1 that were gated on $\alpha$-Galcer-Cd1d tetramer$^+$ TCR$\beta^{+}$ NKT cells. (B, D) Graphs show the absolute number of the three development stages of NKT cell in the (b) thymus and (d) spleen and liver respectively (n = 9 for wild type and n = 8 for Hnplllthu/thu analysed in two independent experiments). Bars represent the mean ± s.d. values. (E, F) Graphs show the geometric mean fluorescence intensity (MFI) of NK1.1 in the thymic and splenic NKT cells respectively (Data are representative of three independent experiments with n = 3–5 mice per group in each). Bars represent the mean ± s.d. values. (G) Graph shows the absolute number of NK1.1$^{+}$ TCR$\beta^{+}$ NK cell in the spleen (Data are representative of three independent experiments with n = 3–5 mice per group in each). Bars represent the mean ± s.d. values. (H) Graph shows the geometric mean fluorescence intensity (MFI) of NK1.1 in the splenic NK cells (Data are representative of three independent experiments with n = 3–5 mice per group in each). Bars represent the mean ± s.d. values. (I) Representative overlay histograms comparing the expression of CD1d on DP thymocytes and B220$^+$ cells in the spleen. (J) Representative overlay histograms showing the intracellular expression of T-Bet in NKT cells from the thymus of a wild type (dotted line) and Hnplllthu/thu mouse (solid line) (Data are representative of two independent experiments with n = 3–5 mice per group in each).

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wild-type NKT cells in the mixed chimeras revealed that, consistent with the results shown above, the mean fluorescence intensity of NK1.1 expression was significantly lower on 

\[ HnrpL \]

thu/thu-derived NKT cells in the thymus (Figure 4C) and spleen (Figure 4D). Taken together, these data show that hnRNPLL is not required for the development or persistence of NKT cells in the immune system, in contrast to its role in conventional T cells, but it functions cell intrinsically to regulate NK1.1 expression in NKT cells.

Mature NKT cells in 

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thu/thu mice can survive normally in the presence of IL-15

The maturation of NKT cells from the NK1.1hi to NK1.1lo stage coincides with expression of the CD122 that confers responsiveness to IL-15 and leads to increased expression of NK1.1 and their long term survival in vivo [23,24]. Based on our hypothesis that hnRNPLL is required to regulate NK1.1 expression but does not influence NKT cell maturation, a prediction is that the 

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thu/thu NKT cells should show comparable expression of CD122 and survival in the presence of IL-15 compared to wild type cells. Indeed, the 

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thu/thu mutation did not affect the expression of CD122 as determined by flow cytometric staining of NKT cells between wild type and 

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thu/thu mutant cells (Figure 5A–C). Next we examined the survival of wild type and 

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thu/thu thymic NKT cells in the presence or absence of IL-15 for 3 days. The viability of the cells was analysed by staining with 7-AAD and quantitated by flow cytometry. We found no difference in the survival between wild type or 

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thu/thu NKT cells to IL-15 in vitro at each concentration of IL-15 tested (Figure 5D).

HnrpL mutation does not disrupt cytokine by NKT cells

Immature and mature NKT cells are able to rapidly secrete cytokines following activation [2,25]. We examined the cytokine production by wild type and 

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thu/thu NKT cells following short-term activation of freshly isolated cells in the thymus, spleen and liver. NKT cells were stimulated in vitro in the presence of PMA and ionomycin for 3–4 h and intracellular cytokine analysis was examined for IFN-γ, IL-4, TNF, IL-17 and IL-2 (Figure 6). There was little or no difference in the patterns of IFN-γ, IL-4, TNF, IL-17 and IL-2 secretion between the 

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thu/thu and wild type thymic NKT cells (Figure 6A&B). In addition wild type and 

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thu/thu derived NKT cells secreted comparable levels of IFN-γ, IL-4, TNF, IL-17 and IL-2 in the spleen and liver (Figure 6C&D). Overall the results indicate that the 

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mutation does not affect cytokine production by in vitro activated NKT cells.
hnRNPLL has previously been shown to control the survival of naive CD4<sup>+</sup> and CD8<sup>+</sup> conventional T cells in the peripheral circulation [15]. In this report we show that hnRNPLL is required for exon silencing of the variable exons of the Ptprc nascent mRNA in NKT cells and this function is conserved across multiple cell lineages in the immune system [15]. In contrast to conventional CD4<sup>+</sup> and CD8<sup>+</sup> TCR<sup>+</sup> cells we did not observe any effect of the hnRNPLL<sup>thu/thu</sup> allele on NKT cell development, effector cell responses or the maintenance of stable NKT cell numbers in the lymphoid and non-lymphoid tissues. However, Hnrpll<sup>thu/thu</sup> derived NKT and NK cells displayed lower expression of the developmental marker NK1.1 that appears to be independent of IL-15 and TCR signaling. This study reveals a divergent role for hnRNPLL within different T cell lineages, which presumably reflects a difference in their dependence on alternative mRNA splicing.

The NK1.1<sup>hi</sup> to NK1.1<sup>lo</sup> transition represents an important developmental checkpoint in NKT cell maturation that relies on both TCR and IL-15 signaling [23,24,26,27], but the homeostatic proliferation and maintenance of mature NK1.1<sup>hi</sup> cells in the immune system is independent of TCR-CD1d signaling and relies instead on the availability of IL-15 and signaling through the IL-15R on NKT cells [24]. Importantly NKT cells are not the only cells in the immune system that rely on IL-15 for their homeostasis, memory CD8<sup>+</sup> CD44<sup>hi</sup> T cells and NK cells proliferate strongly in response to IL-15 [11,28].

CD45 is an abundant tyrosine phosphatase expressed by leukocytes and undergoes alternative splicing of the three variable exons 4, 5 and 6 to yield multiple isoforms. The two related proteins hnRNPL and hnRNPLL function as trans acting factors that regulate exon silencing of the variable exons of Ptprc mRNA in CD4<sup>+</sup> and CD8<sup>+</sup> T cells [13,14,15,17,29]. As shown here wild type NKT cells normally express only low levels of the high molecular weight CD45 isoforms CD45RA and CD45RC at the cell surface as they rely on a functional hnRNPLL to mediate the exon silencing of Ptprc mRNA of the three variable exons. The Hnrpll<sup>mut</sup> mutation disrupts binding to ARS sequences found in Ptprc exons 4–6, leads to inclusion of these exons and a dramatic shift to CD45RA<sup>+</sup>R<sup>B</sup> and RC<sup>+</sup> isoforms in NKT cells. Despite the inappropriate expression of the high molecular CD45 isoforms, this did not affect NKT cell development or effector responses following activation. This is similar to our recent observation that constitutive expression of CD45 isoforms in conventional T cells of hnRNPLL mutant mice was also not responsible for the disruption to T cell homeostasis observed in these mice [18].

The mechanism responsible for the lower NK1.1 expression in Hnrpll<sup>thu/thu</sup> NKT cells is unclear, but our data would suggest that it is independent of T-Bet and CD122 as the expression of both proteins was comparable between wild type and mutant cells. A focus of future studies would be to identify the specific cis- or trans acting factor(s) that directs NK1.1 gene expression, to identify if they are targets of mRNA splicing controlled by hnRNPLL. The biological significance of NK1.1 expression itself by NKT cells is
unclear. A population of mature NK1.1− NK cells exists in the periphery of normal mice and these cells are capable of producing similar amounts of cytokines to their NK1.1+ counterparts [30]. However, antibody mediated NK1.1 cross-linking is capable of activating NKT cells to produce IFN-γ [31] and NK1.1 expression levels transiently decrease following TCR mediated NKT cell activation [32,33]. The human counterpart of this molecule (CD161c) is also a known maturation marker for human NKT cells [3], but again, the functional significance of this molecule in humans is unknown. Thus, whether the NK1.1 cell surface receptor is important for the function of NKT cells remains an interesting question that needs to be resolved.

In conclusion, our studies have demonstrated that hnRNPLL is necessary for CD45 alternative splicing in both conventional T and NKT cells. The studies presented here also reinforce that we still have a lot to learn about mRNA splicing in the immune system. In naïve conventional T cells it controls their long-term survival and homeostasis, but in NKT cells it does not affect lineage development or effector cell differentiation but has an effect on regulation of an important developmental marker NK1.1.

**Materials and Methods**

**Mice**

The Hnrpllthu/thu mouse strain has been previously described and was generated and maintained on a C57BL/6 background [15]. The C57BL/6 (B6) inbred mouse strain was originally obtained from Stanford University. All mice were housed and maintained in specific pathogen free conditions at the Australian Phenomics Facility and all animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee. The following is the ethics number approval for the proposal at the time: Proposal Nos: J.IG.31.04.

**Cell Preparation and Flow Cytometry**

Single cell suspensions from thymus, spleen and liver were labelled with mixtures made of the following antibodies: PE...
labelled CD1d tetramer loaded with α-galactosylceramide (Saphe- 
phore Bioscience) was produced in house using a construct 
provided by M. Kronenberg (La Jolla Institute for Allergy and 
Immunology, San Diego, CA). FITC labelled anti-TCRβ (clone 
H57-597) from BD Pharmingen or APC-Cy7 labelled anti-TCRβ 
(clone H57-597) from Biologent, APC, FITC or PE-Cy7 labelled 
antigen-K1.1 (clone PK136) from BD Pharmingen, Alexa Fluor 700 
labelled anti-CD4 (clone RM4-5) or APC-Cy7 labelled anti-CD4 
(clone GK1.5) from BD Pharmingen, PE-Cy7 labelled anti-CD8 
(clone 53-6.7) from Biologent, Pacific Blue labelled anti-CD44 
(clone IM7) from Biologent, Alexa Fluor 700 labelled anti-CD45.1 
(clone A20) from Biologent, PerCP-Cy5.5 labelled anti-CD45.2 
(clone 104) from BD Pharmingen, FITC labelled anti-CD45 (clone 
30-F11) from BD Pharmingen, PerCP-Cy5.5 labelled anti-mouse/ 
human T-Bet (clone eBioB10) from eBioscience, FITC labelled 
rat anti-mouse CD1d22 from BD Pharmingen, PerCP labelled anti- 
CD45R/B220 (clone RA3-6B2) from BD Pharmingen, Biotin 
anti-CD45RA (clone 14.8) from BD Pharmingen, FITC labelled 
anti-CD45RB (clone 16A) from BD Pharmingen, PE labelled anti- 
CD5RC (clone DNL-1.9) from BD Pharmingen, Qdot 605 
 streptavidin conjugate from Invitrogen, PE labelled anti-CD1d 
(clone 1B1) from BD Pharmingen.

Cell suspensions were incubated with appropriate mixture of 
antibodies at 4°C in the dark for 30 mins. Cells then washed with 
flow cytometry buffer containing 2% Bovine Serum and 0.1% 
NaCl in PBS. Flow cytometry was performed on a LSR II (BD 
Biosciences) and analysed using FlowJo (Tree Star) software.

Generation of Bone Marrow Chimeras

Bone marrow chimeric mice were generated by the injection of 
C57BL/6 (CD45.1 or CD45.2) and Hnrpl2m/+m (CD45.2) donor 
bone marrow cells into irradiated C57BL/6 (CD45.1) recipients. 
The irradiation dose was 2 x 10^5 rad and mixed at a 1:1 ratio of 
donor cells injected intravenously at a 200 µL/mouse into the tail 
veins of recipients. Mice were sacrificed 12–14 weeks after the 
transplantation for ex vivo analysis of the immune system.

Survival assay

Thymic NKT cells were cultured in vitro in the presence or 
absence of IL-15 (R&D Systems) for 3 days at 37°C and 5% CO2. 
The cells were harvested from the plate and stained with antibodies 
to cell surface markers and 7-aminomucycin (7-AAD) (Invitrogen) 
and the cell viability was analysed by flow cytometry as described 
above.

Cell culture and intracellular cytokine staining

For in vitro stimulation assay, single cell suspensions were made 
from thymus, spleen and liver and cells were cultured in RPMI 
1640 medium (Gibco) supplemented with penicillin, streptomycin, 
Glutamax, 1 mM sodium pyruvate (Sigma Aldrich), 0.1 mM 
nonessential amino acids (Sigma Aldrich), 10 mM Hepes (Sigma 
Aldrich), 55 µM 2-ME (Gibco) and 10% FCS in a 24-well plate in 
the presence or absence of 50 ng/ml PMA (Sigma), 500 ng/ml 
imonomycin (Sigma), and Golgi Stop (BD Pharmingen) for 3–4 h 
at 37°C and 5% CO2. After culture, surface markers were stained 
as detailed above prior to fixation of the cells. Cells were washed and 
stained intracellularly with FITC labelled anti-IL-4 (clone 11B11) 
from BD Pharmingen, Alexa Fluor 700 labelled anti-IL-2 (clone 
JE66-5H4) from Biologent, APC labelled anti-TNF (clone 
MP6-XT22) from eBioscience, Alexa Fluor 700 labelled anti-IL-17A 
(clone TC11-18H10.1) from Biologent and APC labelled anti- 
IFN-γ (clone XMG1.2) using the eBioscience Fixation/Permeabil- 
ization kit according to the manufacturer’s instructions. Flow 
cytometry analysis of samples was performed on a LSR II (BD 
Biosciences) and analysed using FlowJo (Tree Star) software.

Statistical analyses

Statistical analysis was performed with the Mann-Whitney 
Rank-Sum U-test using GraphPad Prism software.

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Author Contributions

Conceived and designed the experiments: GH CG DG. Performed the 
experiments: MY. Analyzed the data: MY. Contributed reagents/ 
materials/analysis tools: MY GH CG DG. Wrote the paper: GH CG DG.

References

Science 269: 185–186.
to the NK T cell lineage. Science 296: 553–555.
a natural killer T (NKT) cell developmental pathway involving a 
thymus-dependent NK1.1+/CD4+CD8+CD1d-dependent precursor stage. J Exp Med 
Diverse cytokine production by NKT cell subsets and identification of 
subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer 
Regulation of CD1d alternative splicing by heterogeneous ribonucleoprotein, 
splicing regulators identifies hnRNP LA as a distinct signal-induced repressor of 
arrangement programmed by heterogeneous nuclear ribonucleoprotein 
response sequence mediates activation-induced alternative splicing of CD45. 
via a regulated exonic splicing silencer. EMBO J 24: 2792–2802.
CD45RA and RC isoforms for TCR signaling and peripheral T cell deficiency 
resulting from heterogeneous nuclear ribonucleoprotein L-like mutation. 


