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, Hnrpll deficiency effectively increases the NKT:T cell ratio in the periphery. However, Hnrpll 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, Hnrpll 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 a chain (Vα14Jα18 in mice and Vα2-Jα18 in humans) that is paired with a limited repertoire of Vjβ chains (Vβ8.2, Vβ7, and Vβ2 in mice and Vβ11 in humans) [2,3]. The antigen receptor of NKT cells recognizes glycolipid antigens, such as α-galactosylceramide (α-GalCer), 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 NK1.1lo (stage 1). These differentiate to become CD44hi NK1.1hi 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 [2,3].

The heterogeneous nuclear ribonucleoprotein L-Like (hnRNPLL), is a member of the hnRN 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 (Hnrplltlu/thu) [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 Ptpc [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 Hnrplhua mutation does not affect conventional 3β T cell differentiation in the thymus but it greatly shortens the survival of naïve and memory 2β T cells in the peripheral lymphoid tissues [15]. This is independent of the change CD45/Ptpc splicing, and must be explained by alternative splicing among the hundreds of other mRNAs that are regulated directly or indirectly by Hnrpl [15,18]. NKT cells express the tyrosine phosphatase CD45, similar to conventional 3β T cells which can express up to eight different isoforms at the cell surface due to mRNA alternative splicing of three variable exons on the Ptpc mRNA [19]. The expression of the CD45 isoforms on conventional T cells is regulated in a development- and activation-dependant manner that is regulated by exon silencing by Hnrpl [13,14,15]. Here we examine the requirement for Hnrpl in NKT cells and show that although hnRNPL is required for the splicing of CD45 isoforms in both 3β T cells and NKT cell lineages it does not affect NKT cell accumulation and survival, although it appears to play a role in maintaining basal expression of the differentiation marker NK1.1. Our studies highlight a fundamental difference between the action of hnRNPL in conventional T cells and NKT cells.

Results

hnRNPL 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 Hnrplhua/thu mice with α-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 Hnrpl-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 Ptpc 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 3β 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 Hnrplhua/thu mice (Figure 1B). This result demonstrates that hnRNPL is critical for silencing Ptpc exons 4, 5 and 6 within NKT cells, and Hnrpl mutation has a comparable effect on this process in NKT and conventional T cells.

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

Given that NKT cells and conventional T cells develop in the thymus from the same precursor, we wanted to examine if the Hnrpl mutation affected NKT cell development, function and homeostasis. We stained the thymus, spleen and liver of wild type and Hnrplhua/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 Hnrplhua/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, Hnrpl mutation did not affect the number of NKT cells in the thymus, spleen or liver (Figure 2B&2C). However, we observed that NKT cells from Hnrplhua/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 Hnrplhua/thu mice suggesting an important role for hnRNPL 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 Hnrplhua/thu mice in the thymus, spleen or liver (Figure 2E&2F). Thus, while NKT and conventional T cells share a requirement of hnRNPL to control their CD45 isoforms, they differ in their hnRNPL requirement for accumulating in normal numbers.

Decreased NK1.1 during NKT cell development in Hnrplhua/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 Hnrplhua/thu mice and observed a slight increase in the mean frequency and absolute number of CD44hi NK1.1+ (Stage 1) and CD44hi NK1.1+ (Stage 2) NKT cells and a reduction in the mean frequency and number of mature CD44hi NK1.1+ (Stage 3) NKT cells in the thymus of Hnrplhua/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 Hnrplhua/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 Hnrplhua/thu NKT cells in the thymus and spleen had significantly lower NK1.1 expression (Figure 3E&F). NK cells in Hnrplhua/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 3β 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 Hnrpl mutation does not affect the expression of CD1d on DP cells (Figure 3I). 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 Hnrplhua/thu NKT cells compared to wild type cells (Figure 3J). These results support the notion that hnRNPL is not required for the development of NKT lineage in the thymus or periphery but is required for full induction of NK1.1.

Hnrpl 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 hnRNPL in NKT cell development, mixed bone marrow chimeras (BMC) were used to investigate the importance of hnRNPL 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 Hnrpl+/−
C57BL/6 bone marrow and CD45.2+ Hnrpllthu/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 Hnrpllthu/thu CD45.2+ hemopoietic stem cells was gauged by the percentage of B cells that were CD45.2+, since this lineage

Figure 2. Normal numbers of NKT cell in the thymus and periphery of Hnrpllthu/thu mice despite of reduced number of conventional T cells in the periphery. (A) Graphs show absolute number of CD4 and CD8 conventional T cells in wild type (+/+) and Hnrpllthu/thu (thu/thu) mice (n = 9 for wild type and n = 8 for Hnrpllthu/thu analysed in two independent experiments). Bars represent the mean ± s.d. values. (B) Representative flow cytometric dot plots of NKT cells (α-GalCer-loaded CD1d tetramer+ TCRβ+) in the thymus (Thy) and spleen (Spl) in wild type (+/+) and Hnrpllthu/thu (thu/thu) mice. (C) Graph shows absolute number of NKT cells in the different tissues of wild type (+/+) and Hnrpllthu/thu (thu/thu) mice (n = 12 for wild type and n = 11 for Hnrpllthu/thu analysed in three independent experiments). Bars represent the mean ± s.d. values. (D) Graph shows the ratio of absolute number of NKT cells to absolute number of TCRβ+ conventional T cells (n = 12 for wild type (+/+) and n = 11 for Hnrpllthu/thu (thu/thu) analysed in three independent experiments). Bars represent the mean ± s.d. values. (E) Representative flow cytometric dot plots comparing the expression of CD4 versus TCRβ+ on CD1d-tetramer+ TCRβ+ NKT cells in the thymus, and spleen of wild type (+/+) and Hnrpllthu/thu (thu/thu) mice. (F) Graph shows absolute number of CD4+ and CD4− NKT cell subsets in the thymus, spleen and liver of individual wild type (+/+) and Hnrpllthu/thu (thu/thu) mice (n = 9 for wild type and n = 8 for Hnrpllthu/thu analysed in two independent experiments). Bars represent the mean ± s.d. values.

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expresses very little Hnrpll and is not affected by the Hnrpll 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 Hnrpll
thu/thu derived conventional TCRβ+ 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 Hnrpll
thu/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 Hnrpll
thu/thu versus

Figure 3. Decreased NK1.1 during NKT cell development in Hnrpll
thu/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 Hnrpll
thu/thu (thu/thu) mice based on expression of CD44 and NK1.1 that were gated on α-Galer-Cd1d tetramer+ TCRβ+ 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 Hnrpll
thu/thu analysed in two independent experiments). Bars represent the mean ± s.d. values. (E, F) Graphs show the geometric mean florescence 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+ TCRβ+ NKT 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. (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 Hnrpll
thu/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 Hnrpll\textsuperscript{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 Hnrpll\textsuperscript{thu/thu} mice can survive normally in the presence of IL-15

The maturation of NKT cells from the NK1.1\textsuperscript{th} to NK1.1\textsuperscript{hi} 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 Hnrpll\textsuperscript{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 Hnrpll mutation did not affect the expression of CD122 as determined by flow cytometric staining of NKT cells between wild type and Hnrpll\textsuperscript{thu/thu} mutant cells (Figure 5A–C). Next we examined the survival of wild type and Hnrpll\textsuperscript{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 Hnrpll\textsuperscript{thu/thu} NKT cells to IL-15 in vitro at each concentration of IL-15 tested (Figure 5D).

Hnrpll 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 Hnrpll\textsuperscript{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-\(\gamma\), IL-4, TNF, IL-17 and IL-2 (Figure 6). There was little or no difference in the patterns of IFN-\(\gamma\), IL-4, TNF, IL-17 and IL-2 secretion between the Hnrpll\textsuperscript{thu/thu} and wild type thymic NKT cells (Figure 6A&B). In addition wild type and Hnrpll\textsuperscript{thu/thu} derived NKT cells secreted comparable levels of IFN-\(\gamma\), IL-4, TNF, IL-17 and IL-2 in the spleen and liver (Figure 6C&D). Overall the results indicate that the Hnrpll mutation does not affect cytokine production by in vitro activated NKT cells.
Discussion

hnRNPLL has previously been shown to control the survival of naive CD4\(^+\) and CD8\(^+\) 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 \textit{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\(^+\) and CD8\(^+\) TCR\(\alpha\)\(\beta\) cells we did not observe any effect of the \textit{HNRPPL}\textsuperscript{thu/thu} allele on NKT cell development, effector cell responses or the maintenance of stable NKT cell numbers in the lymphoid and non-lymphoid tissues. However, \textit{HNRPPL}\textsuperscript{thu/thu} 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 \textit{HNRPPL}\textsuperscript{thu/thu} mutation disrupts binding to ARS sequences found in \textit{Ptprc} exons 4–6, leads to inclusion of these exons and a dramatic shift to CD45\(\text{RA}^+\), \(\text{RB}^+\) and \(\text{RC}^+\) 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 \textit{HNRPPL} 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 \textit{HNRPPL}\textsuperscript{thu/thu} 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


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Figure 5. \textit{HNRPPL} mutation does not affect the up-regulation of CD122 in the developmental stages of NKT cell. (A) Representative overlay histograms showing the expression of CD122 on the surface of NKT cells from the thymus of a wild type and \textit{HNRPPL}\textsuperscript{thu/thu} mouse. (B) Representative overlay histograms comparing CD122 expression on stage 1 (CD44\(\text{lo}\) NK1.1\(\text{lo}\)), stage 2 (CD44\(\text{hi}\) NK1.1\(\text{lo}\)) and stage 3 (CD44\(\text{hi}\) NK1.1\(\text{hi}\)) NKT cells in the thymus in wild type (left) and \textit{HNRPPL}\textsuperscript{thu/thu} mice (right). (C) Graph shows the geometric mean fluorescence intensity (MFI) of CD122 expression on stage 2 and 3 NKT cells and DP thymocytes in the thymus and NKT cells and TCR\(\beta\) cells in the spleen. Bars represent the mean value of each group ± s.d. from one of two independent experiments with a group of n = 3–5 mice per group. (D) In vitro survival of NKT cells culture in the presence of varying concentrations of IL-15. Data shows the percentage of viable cells by 7-AAD exclusion after 3 days of culture for wild type (+/+) and \textit{HNRPPL}\textsuperscript{thu/thu} (thu/thu) thymic NKT cells. Graph from one of three independent experiments represents the average of viable cells recovered from duplicate cultures with 2 or 3 mice per group.

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 \textit{Ptprc} mRNA in CD4\(^+\) and CD8\(^+\) 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 \textit{Ptprc} mRNA of the three variable exons. The \textit{HNRPPL} mutation disrupts binding to ARS sequences found in \textit{Ptprc} exons 4–6, leads to inclusion of these exons and a dramatic shift to CD45RA\(^+\), RB\(^+\) and RC\(^+\) 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 \textit{HNRPPL} mutant mice was also not responsible for the disruption to T cell homeostasis observed in these mice [18].

Role of hnRNPLL in NKT Cells

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A population of mature NK1.1\(^+\) NKT 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-\(\gamma\) [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 Hnrpll\(^{thu/thu}\) 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 (Sapphire 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 Biologend, APC, FTC or PE-Cy7 labelled anti-NK1.1 (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 Biologend, Pacific Blue labelled anti-CD44 (clone IM7) from Biologend, Alexa Fluor 700 labelled anti-CD45.1 (clone A20) from Biologend, 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 eBio4B10) from eBioscience, FITC labelled rat anti-mouse CD122 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-CD45RC (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 at the dark for 30 mins. Cells then washed with flow cytometry buffer containing 2% Bovine Serum and 0.1% NaN₃ in PBS. Flow cytometry was performed on a LSR II (BD Biosciences) and analysed using FlowJo (Tree Star) software.

References


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

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.