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## Quantitative differences in lipid raft components between murine CD4<sup>+</sup> and CD8<sup>+</sup> T cells

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### Abstract

**Background:** Lipid rafts have been shown to play a role in T cell maturation, activation as well as in the formation of immunological synapses in CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T cells. However, the differential expression of lipid raft components between CD4<sup>+</sup> and CD8<sup>+</sup> T cells is still poorly defined. To examine this question, we analyzed the expression of GM1 in T cells from young and aged mice as well as the expression of the glycosylphosphatidylinositol (GPI)-linked protein Thy-1 and cholesterol in murine CD4<sup>+</sup> and CD8<sup>+</sup> T cell subpopulations.

**Results:** We found that CD4<sup>+</sup>CD8<sup>-</sup> and CD8<sup>+</sup>CD4<sup>-</sup> thymocytes at different stages of maturation display distinct GM1 surface expression. This phenomenon did not change with progressive aging, as these findings were consistent over the lifespan of the mouse. In the periphery, CD8<sup>+</sup> T cells express significantly higher levels of GM1 than CD4<sup>+</sup> T cells. In addition, we observed that GM1 levels increase over aging on CD8<sup>+</sup> T cells but not in CD4<sup>+</sup> T cells. We also verified that naïve (CD44<sup>lo</sup>) and memory (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells as well as naïve and memory CD4<sup>+</sup> T cells express similar levels of GM1 on their surface. Furthermore, we found that CD8<sup>+</sup> T cells express higher levels of the GPI-anchored cell surface protein Thy-1 associated with lipid raft domains as compared to CD4<sup>+</sup> T cells. Finally, we observed higher levels of total cellular cholesterol in CD8<sup>+</sup> T cells than CD4<sup>+</sup> T cells.

**Conclusion:** These results demonstrate heterogeneity of lipid raft components between CD4<sup>+</sup> and CD8<sup>+</sup> T cells in young and aged mice. Such differences in lipid raft composition may contribute to the differential CD4 and CD8 molecule signaling pathways as well as possibly to the effector responses mediated by these T cell subsets following TCR activation.

### Background

Lipid rafts are characterized as organized plasma membrane domains enriched in sphingolipids and cholesterol, originally identified by their resistance to non-ionic detergent lysis at 4 °C [1,2]. These microdomains are enriched in GPI-linked proteins on the extracellular surface, such as

Thy-1 and CD59, and acylated signaling proteins on the cytoplasmic surface, including Src kinases, Ras proteins, G proteins, Vav, PKC, and LAT [1-4]. Lipid rafts play an integral role in synapse formation between antigen presenting cells and T cells due to their ability to serve as platforms for the recruitment of TCR and signaling molecules. To

identify lipid rafts on the surface of cells, GM1, a mono-sialoganglioside and glycosphingolipid, is a commonly used marker, which is detected using bacterial-derived cholera toxin B subunit (CTB) [5,6]. Other markers to lipid rafts include the GPI-linked proteins, which associate with sphingolipids, glycolipids and cholesterol in the cell membrane and with several cytoplasmic proteins possibly facilitating raft domains downstream signaling [reviewed in [7]].

Cholesterol is also essential to the formation and function of lipid rafts. Studies involving the extraction of membrane cholesterol by  $\beta$ -cyclodextrins, as well as membrane cholesterol sequestering by filipin and nystatin, implicate a critical role for cholesterol in lipid raft formation [reviewed in [8]]. The cholesterol molecule is believed to pack more tightly in the membrane with unsaturated fatty acid chains, increasing membrane order and conferring detergent resistance in these regions at low temperatures [9]. Thus, the overall concentration of cholesterol in cell membranes is believed to impact on cell function. Evidence from aging human immune cells suggests that an excess of membrane cholesterol may affect TCR signaling pathways, although the specific mechanisms involved are not completely understood [10,11].

During the process of T cell maturation in the thymus, the expression of CD4 and CD8 molecules changes on thymocyte subsets. Immature CD4<sup>-</sup>CD8<sup>-</sup> T cell progenitors, originating from the bone marrow, enter the thymus and undergo differentiation and selection to become immunocompetent mature T cells capable of emigrating to the peripheral lymphoid organs [12]. During this process, CD4<sup>-</sup>CD8<sup>-</sup> T cells become CD4<sup>+</sup>CD8<sup>+</sup> and then differentiate into mature CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>+</sup>CD4<sup>-</sup> T cells [12,13]. Interestingly, CD4 and CD8 molecules on fully differentiated mature T cells are palmitoylated and are constitutively associated with lipid raft microdomains [14].

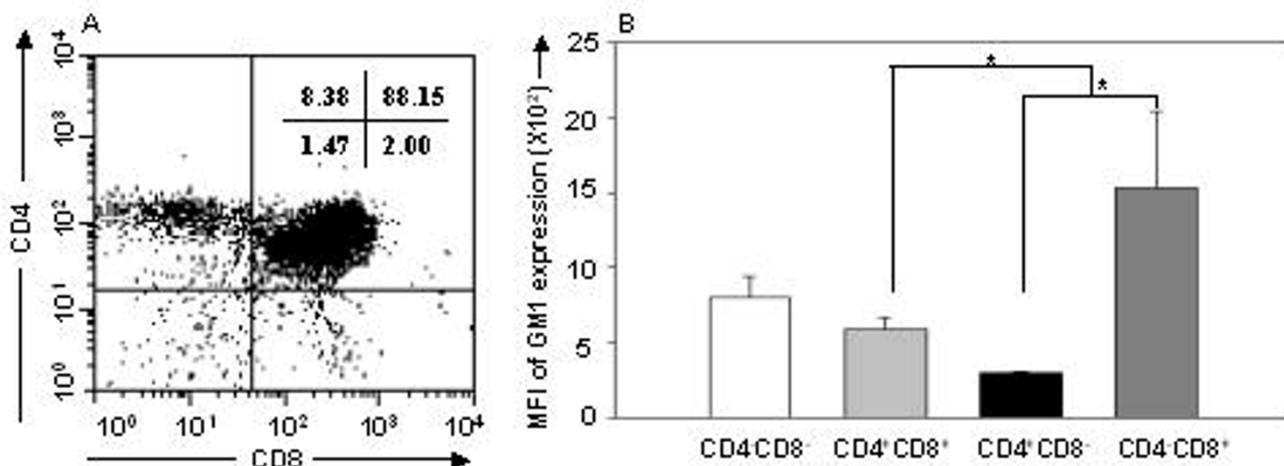
During antigen presentation, the CD8 and CD4 molecules in combination with the TCR bind to the peptide-MHC class I or II components, respectively, on antigen-presenting cells. This interaction favors the formation of immunological synapses where signaling, adhesion and cytoskeleton molecules are concentrated within lipid raft microdomains following TCR co-aggregation [15-18]. Although lipid rafts are important in all of these processes, an association between these effects and the quantitative levels of specific lipid rafts components, namely GM1, GPI-linked proteins and cholesterol, have not been described in thymic or peripheral T cell subsets. In addition, deficiencies in T cell signaling identified in aging human and murine cells could be potentially be explained by differences in lipid raft composition between cells from young versus old subjects. In the

present work, we have examined the GM1 expression on distinct CD4<sup>+</sup> and CD8<sup>+</sup> thymic and peripheral T cell subsets from young and aged mice as well as the levels of the GPI-anchored protein Thy-1 and cholesterol contents in these same cell populations.

## Results and discussion

Initial studies focused on the examination of the possible differential expression of the lipid raft component, GM1, within the cell membranes of murine thymic T cell subsets. The expression of GM1 was examined by flow cytometric analysis using FITC-conjugated CTB (Fig. 1). In these studies, we found that 100% of all thymic T cell subsets expressed GM1. These results performed in both Balb/c and Albino Swiss mice, between 2 and 18 months of age, are in accordance with previous observations demonstrating that the percentage of immature and mature fetal thymocytes expressing GM1 is quite similar [19]. Haks and colleagues have demonstrated that fetal CD4<sup>-</sup>CD8<sup>-</sup> cell subsets express higher levels of GM1 than fetal CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Although our current work involves thymocytes from adult mice, the lowering of GM1 levels as cells progress from the CD4<sup>-</sup>CD8<sup>-</sup> to the CD4<sup>+</sup>CD8<sup>+</sup> differentiation stage appears to be consistent with the Haks study and does not alter with age (Table 1). Furthermore, we did observe that GM1 expression was significantly increased on CD4<sup>-</sup>CD8<sup>+</sup> cells in comparison to CD4<sup>+</sup>CD8<sup>-</sup> mature thymocytes in young and aged mice (Fig. 1, Table 1). These findings were highly reproducible with repeated studies (n = 5). In addition, recent data using aged murine T cells have suggested that possible defects in lipid raft function and composition may occur with age possibly due to their inability to effectively recruit signaling molecules to the immunological synapse [20]. To examine possible alterations in lipid raft composition with age, we examined thymocyte subsets derived from 2-, 4-, 6-, 12- and 18-month-old mice for GM1 expression and failed to observe any significant age-associated differences in GM1 expression by any thymic subsets (Table 1).

Previous studies have demonstrated that lipid rafts may play a role in thymic T cell differentiation. In this context, the development of CD4<sup>-</sup>CD8<sup>-</sup> immature thymocytes to the CD4<sup>+</sup>CD8<sup>+</sup> stage requires the pre-TCR  $\alpha$  chain palmitoylation and recruitment to lipid raft domains [21]. Furthermore, the process of thymic selection of CD4<sup>+</sup>CD8<sup>+</sup> T cells to become CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>+</sup>CD4<sup>-</sup> mature thymocytes occurs after association of CD3 molecules to the TCR in the raft regions and interaction with the complex self peptide-MHC in the thymus [22-24]. Moreover, additional studies have demonstrated the polarization of lipid rafts to the sites of TCR-activation on mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells while CD4<sup>+</sup>CD8<sup>+</sup> thymocytes do not polarize lipid rafts in response to TCR-mediated signals [24]. Additionally, the commitment of thymocytes to the CD4<sup>+</sup>CD8<sup>-</sup>



**Figure 1**  
**GM1 expression on murine thymocyte subsets.** Total thymocytes were isolated and analyzed by flow cytometric analysis after staining with CTB-FITC, anti-CD4 Percp and anti-CD8-PE antibodies. **(A)** Percentage of CD4<sup>+</sup> and CD8<sup>+</sup> thymocyte subsets expressing GM1. **(B)** The median fluorescence intensity (MFI) of GM1 expression by distinct CD4 and CD8 expressing T cells (n = 5). A significant difference in the GM1 expression on the cell surface was observed between CD4<sup>+</sup>CD8<sup>+</sup> and CD8<sup>+</sup>CD4<sup>-</sup> or CD4<sup>+</sup>CD8<sup>-</sup> T cells (p ≤ 0.05) and between CD8<sup>+</sup>CD4<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup> T cells (p ≤ 0.05).

**Table 1: GM1 expression levels on distinct thymocyte subsets do not change with aging<sup>1</sup>**

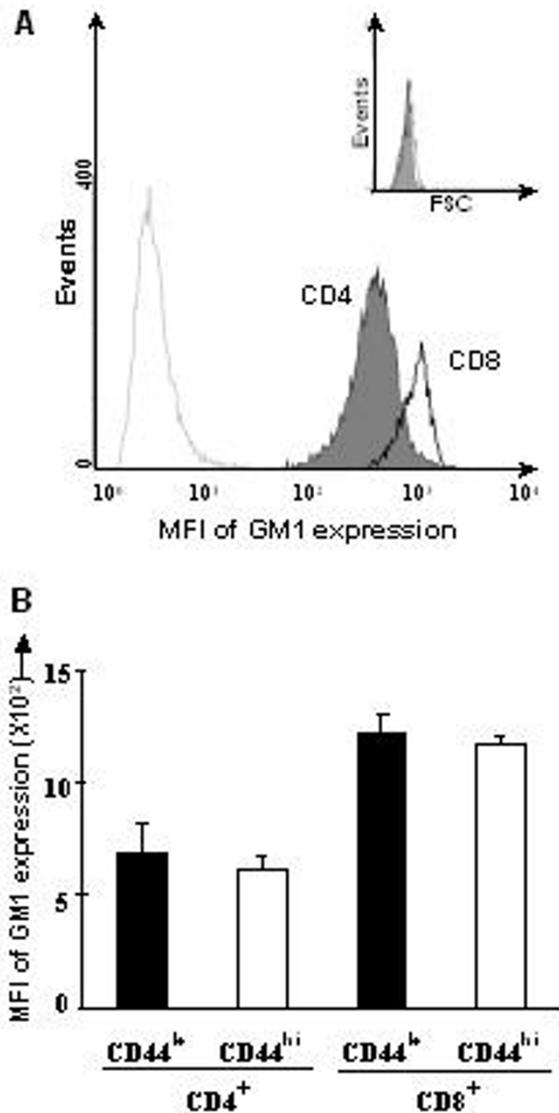
	2 months	4 months	6 months	12 months	18 months
<b>CD4<sup>+</sup>CD8<sup>-</sup></b>	797.8 ± 141.4	874.8 ± 45.1	1242.5 ± 343.0	1144.4 ± 426.3	947.5 ± 12.0
<b>CD4<sup>+</sup>CD8<sup>+</sup></b>	593.0 ± 71.4	622.14 ± 14.0	729.1 ± 129.1	672.6 ± 81.0	671.6 ± 63.9
<b>CD4<sup>-</sup>CD8<sup>-</sup></b>	302.3 ± 7.6	329.8 ± 18.3	360.7 ± 25.2	350.7 ± 6.6	387.1 ± 7.3
<b>CD8<sup>+</sup>CD4<sup>-</sup></b>	1534.7 ± 507.7	1316.6 ± 184.2	1554.0 ± 39.5	1472.3 ± 279.2	1561.4 ± 217.7

<sup>1</sup>Arbitrary values represent the mean ± SD of the mean fluorescence intensity of GM1 expression on the distinct thymocyte subsets from mice between 2 and 18 months of age. Experiments were performed utilizing at least two animals per time point.

lineage requires a significantly stronger stimulus and a prolonged MAPK signal compared to what was required for a CD8<sup>+</sup>CD4<sup>-</sup> lineage commitment [25]. Thus, it seems possible that differences in lipid raft components by thymic subsets may contribute to the process of T cell selection and differentiation. Given that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are derived from CD4<sup>+</sup>CD8<sup>+</sup> precursors, it may be possible that highly GM1-expressing CD4<sup>+</sup>CD8<sup>+</sup> cells are positively selected to become CD8<sup>+</sup> T cells, while lower GM1-expressing cells are selected to become CD4<sup>+</sup> T cells. This selection would not likely be occurring as a direct result of GM1 expression, but rather, through the effects of glycosphingolipid levels on TCR avidity and signaling during positive and negative selection in the thymus. Accordingly, Drake and Baciale have demonstrated that MHC class I tetramer binding to functional CD8<sup>+</sup> T cells requires lipid raft integrity [26]. It may be that the optimal

levels of GM1 for CD4<sup>+</sup> and CD8<sup>+</sup> T cells to survive negative and positive selection require distinct windows. Further studies will be necessary to answer this question.

Similar to our findings in the thymus, we found that 100% of peripheral splenic T cells express GM1 and that CD8<sup>+</sup> T cells expressed approximately 2–3 fold higher level of GM1 compared to CD4<sup>+</sup> T cells (Fig. 2A). We also verified by flow cytometry that there were no significant differences in cell size that might account for the differences in GM1 expression (insert in Fig. 2A). When we analyzed the expression of GM1 in T cell subsets derived from young and aged Balb/c mice, we observed the typical pattern of increased levels of GM1 on peripheral CD8<sup>+</sup> T cells when compared to CD4<sup>+</sup> T cells. However, in contrast to thymic T cell subsets, peripheral CD8<sup>+</sup> T cells derived from aged mice expressed significantly higher levels of



**Figure 2**  
**GM1 expression on splenic CD8<sup>+</sup> is significantly higher than CD4<sup>+</sup> T cells but not different between naïve and memory T cells.** (A) Splenic T cells were isolated and submitted to flow cytometric analysis using anti-CD4 or CD8 antibodies and anti-rat Alexa-594 as secondary antibody in combination with CTB-FITC (Left peak represents cells unstained with CTB) (n = 5). Insert shows overlay of CD4 (grey) and CD8 (unfilled, right peak) forward scatter histograms indicating their size. Unstained cells were used as control (unfilled, left peak). (B) Purified CD4 and CD8 T cells were stained with anti-CD4-PerCp or anti-CD8-Cy5 in combination with CTB-FITC and CD44-Pe. CD4<sup>+</sup> or CD8<sup>+</sup> cells expressing CD44<sup>lo</sup> or CD44<sup>hi</sup> were gated and MFI of GM1 expression in the distinct subpopulations were analyzed. Black bars represent naïve cells and white bars, memory cells. Each experiment was performed utilizing two to three mice.

GM1 when compared to young and aged CD4<sup>+</sup>T cells as well as young CD8<sup>+</sup>T cells (Table 2). CD4<sup>+</sup>T cells failed to demonstrate any significant differences in GM1 expression with age (Table 2).

Next, we examined whether there are any alterations in the expression of GM1 in peripheral CD44<sup>lo</sup> (naïve) and CD44<sup>hi</sup> (memory) CD4<sup>+</sup> and CD8<sup>+</sup> T cells [27], due to observations suggesting that these cell subsets have differential requirements for stimulation [28,29]. As shown in figure 2B, we failed to observe any significant differences in the expression of GM1 between CD44<sup>lo</sup>CD4<sup>+</sup> and CD44<sup>hi</sup>CD4<sup>+</sup> cells or CD44<sup>lo</sup>CD8<sup>+</sup> and CD44<sup>hi</sup>CD8<sup>+</sup> cells isolated from Balb/c mice spleens. These results are in contrast to studies utilizing total human T cells isolated from peripheral blood where the levels of GM1 were shown to be higher on memory T cells compared to naïve T cells [30].

To substantiate our findings showing differences in GM1 expression between CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, we examined GM1 expression in isolated lipid raft fractions of highly purified splenic T cell subsets from Swiss and Balb/c mice following sucrose gradient ultracentrifugation and immunodot analysis. In figure 3A, we observed that the quantity of GM1 was significantly higher in CD8<sup>+</sup> T cells compared to CD4<sup>+</sup> T cells given the same input of cells. Indeed, in both cell types, GM1 was predominantly expressed in the lipid raft fractions rather than the membrane/cytosolic fractions. Interestingly, the activation of T lymphocytes with GM1 has been shown to modulate the expression of T cell co-receptor molecules. In this context, it has been demonstrated that CD4 expression on the surface of human T cells is inhibited by GM1 treatment [16]. This GM1-induced CD4 down-modulation increases antigen-specific T cell responses [16]. These findings support our hypothesis that lipid rafts and their components, such as GM1, may differentially contribute to signaling and activation of both helper and cytotoxic T lymphocytes.

While GM1 is commonly utilized as a marker for lipid rafts on cellular surfaces, this marker may not be absolutely indicative of total lipid raft expression on cells as GPI-associated proteins and other glycolipids are also involved in raft formation. Thy-1, a GPI-linked protein, and cholesterol are enriched in lipid rafts and contribute to the formation of lipid raft membrane domains through the interaction with phospho- and sphingolipids, including GM1 [31,32]. In light of our GM1 findings, we next examined the expression of Thy-1 in lipid raft fractions isolated from CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 3B). Previously, we verified that the CD8<sup>+</sup> cell line, RF3370, expressed higher levels of GM1 than the CD4<sup>+</sup> T cell line, D0-11.10 (data not shown), at similar ratios and patterns as observed for primary peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells

**Table 2: Expression of GM1 on peripheral T cell subsets of young and aged mice**

	4 months	25 months
CD4 <sup>+</sup> CD8 <sup>-</sup>	997.15 ± 25.50	1298 ± 196.43
CD8 <sup>+</sup> CD4 <sup>-</sup>	2114.0 ± 292.92	3449.12 ± 54.17*

<sup>1</sup>Arbitrary values represent the mean ± SD of the mean fluorescence intensity of GM1 expression on T cell subsets from three young and aged Balb/c mice. \*Indicates significant differences in GM1 expression between CD8<sup>+</sup>CD4<sup>-</sup> cells from young and aged mice ( $p \leq 0.05$ ).

(Fig. 2A). Utilizing immunoprecipitation and immunoblot analyses, we analyzed the expression of Thy-1 in lipid raft fractions isolated from these cell lines. In accordance with our GM1 results, we found that the expression of Thy-1 was approximately 50 % higher in CD8<sup>+</sup> T cells as compared to CD4<sup>+</sup> T cells (Fig. 3B and 3C).

Finally, as cholesterol is a major component of lipid rafts, we also examined the possible differences in the levels of cholesterol within purified peripheral T cell subsets. As expected, the analysis of total cellular cholesterol levels revealed CD8<sup>+</sup> T cells also exhibit higher levels of total cholesterol compared to CD4<sup>+</sup> T cells, in a ratio of approximately 2:1 (Fig. 4).

These results support the concept that CD8<sup>+</sup> T cells do express greater numbers of lipid rafts and/or have a greater surface area of lipid rafts than CD4<sup>+</sup> T cells. Such differences may influence cellular activation and functional responses mediated by these cells following TCR activation. In fact, it has been demonstrated that changes in the cholesterol levels influence the interactive molecular stabilization and activity of CD4 among other molecules present in raft regions of the plasma membrane of T cells [20,32-34]. Polarization of membrane receptor molecules in lipid raft platforms is critical to immunological synapse formation. Differences in lipid raft content and/or numbers between T cell subsets may influence the intensity or threshold of signals required for T cell activation.

It is not unreasonable to propose that CD4<sup>+</sup> and CD8<sup>+</sup> T cells require differing levels of cell surface lipid rafts for optimal signaling. It is generally accepted that rafts are essential for function in both cell types, but the experimental approaches used often test for an "all-or-none" phenotype regarding lipid rafts. Our results suggest that the levels of lipid rafts in CD4<sup>+</sup> and CD8<sup>+</sup> T cells require some degree of fine tuning, seen in the relatively consistent GM1 expression in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 1A and ref. [18]). The recruitment of signaling molecules and lipid rafts to the immunological synapse is a hallmark of CD4<sup>+</sup> T cell activation. In contrast, it is interesting to note that CD8<sup>+</sup> T cells that do require lipid rafts for signaling do not polarize lipid rafts during signaling and acti-

vation [15]. Could this be due to the elevated levels of lipid rafts already present on these cells? If raft concentrations in CD8<sup>+</sup> T cells are maintained at a high level, no further capping may be necessary to mediate signaling at the site of cell-cell contact, whereas CD4<sup>+</sup> cells would require capping due to the relatively low lipid raft concentrations in these regions.

In summary, our results suggest that differences in lipid raft composition may contribute to the differential CD4 and CD8 molecule signaling pathways as well as possibly to the effector responses mediated by these T cell subsets following TCR activation.

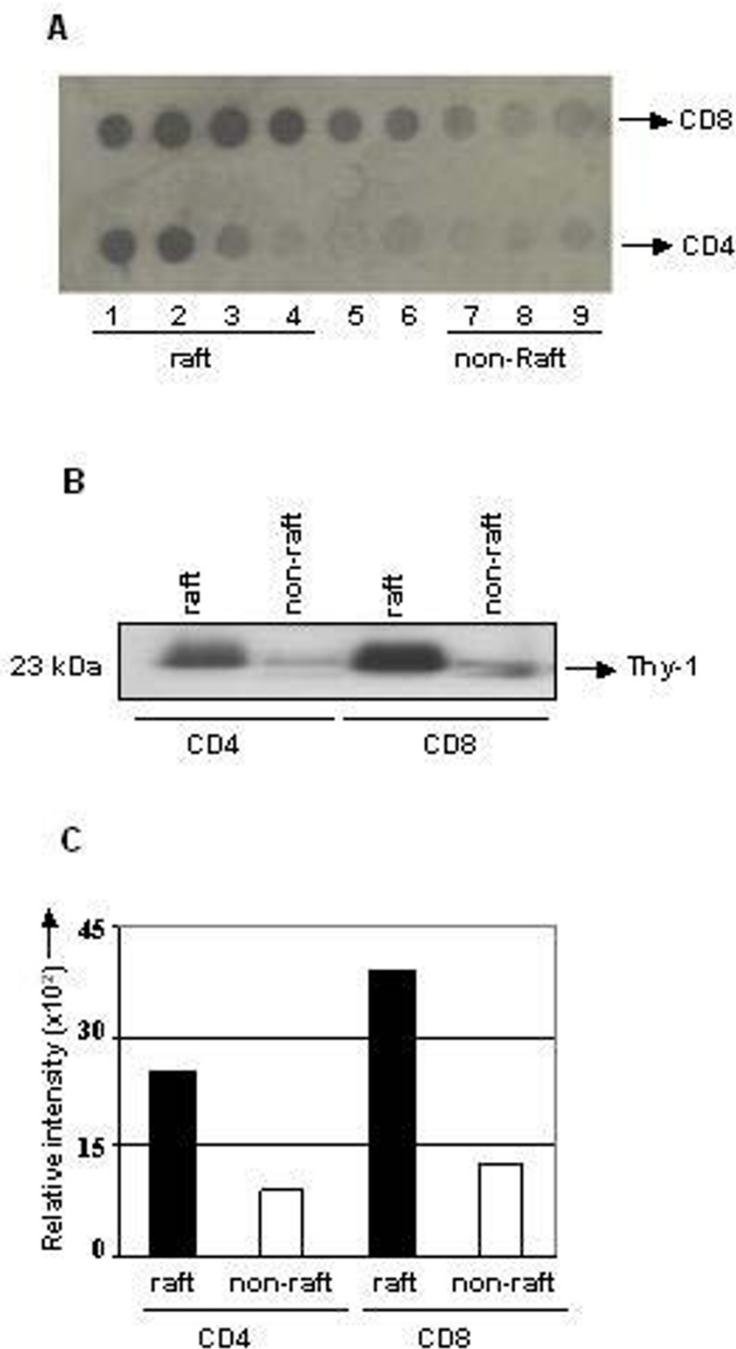
## Conclusion

Our results demonstrate heterogeneity of lipid raft components between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which might influence in distinct effector response of these cells. Based on these results, it would seem appropriate to investigate the activity of molecules associated with lipid rafts in T lymphocytes using purified CD4<sup>+</sup> and CD8<sup>+</sup> T cell subpopulations rather than total T cells to avoid variable and/or biased results.

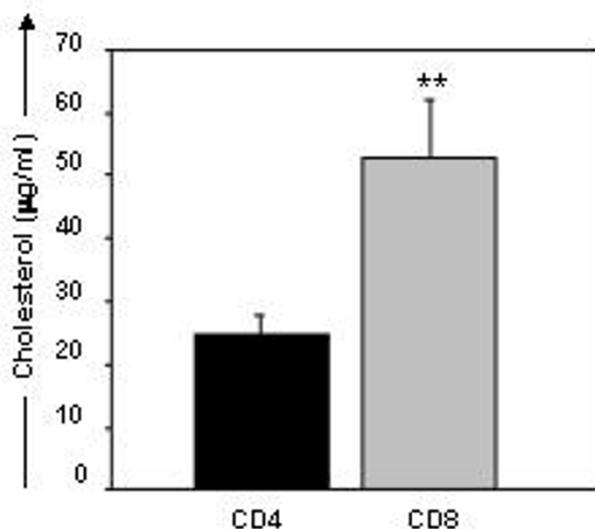
## Methods

### Thymocyte and T cell purification

Pooled thymi were homogenized in a glass potter within RPMI supplemented with 0.5% bovine serum albumin (BSA). Supernatant was collected, centrifuged and the cells were counted for subsequent analysis. Splenocytes were derived from the pooled spleens of albino Swiss or Balb/c mice and subsequently treated with ACK lysing buffer (150 mM NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub> and 0.001 mM EDTA) to remove erythrocytes. CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>+</sup>CD4<sup>-</sup> splenic T cells were purified via a negative selection technique utilizing mouse T cell subset columns (R&D system, Minneapolis, MN) following the manufacturer's instructions. Following isolation, T cells were ultracentrifuged at room temperature for 5 minutes and resuspended in PBS containing 0.5% BSA. Typically, fresh T cell subset selection yielded greater than 95% purity for CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cells as assessed by flow cytometric analysis using anti-CD3-FITC (clone 145-2C11) and anti-CD8-PE



**Figure 3**  
**GPI levels and Thy-1 GPI-anchored protein expression are higher in lipid raft domains from CD8<sup>+</sup> than CD4<sup>+</sup> T cells.** (A) Splenic T cells from young mice were treated with Triton X-100 and lysates were submitted to ultracentrifugation on a sucrose gradient for lipid rafts isolation. Equal volumes of fractions were analyzed by dot blot analysis using CTB subunit conjugated to HRP. Raft and non-raft fractions are indicated. (B) Raft or non-raft fractions of CD4 and CD8 cell lines were pooled together and immunoprecipitated with mAb specific for Thy1 and subsequently detected using anti-thy1 antibody. (C) The relative density of thy1 bands seen in (B) for CD4<sup>+</sup> and CD8<sup>+</sup> cells were calculated using the Image Quant software (Amersham Biosciences) and shown in bar diagram. Black bars represent raft-pooled fractions and white bars, non-raft pooled fractions.



**Figure 4**  
**Splenic CD8<sup>+</sup> T cells contain significantly higher levels of cholesterol than CD4<sup>+</sup> T cells.** Splenocytes were washed with PBS, lysed and cholesterol content was measured with a sensitive cholesterol oxidase-based fluorometric assay using the Amplex Red cholesterol kit (n = 3). A significant difference was observed between the CD4<sup>+</sup>CD8<sup>-</sup> and CD8<sup>+</sup>CD4<sup>-</sup> T cells (p ≤ 0.02).

(clone OKT8) and/or anti-CD4-PE (clone OKT4) antibodies (PharMingen/BD, San Diego, CA).

#### T cell lines

Subclones of the T cell hybridoma cell lines, D0-11.10 (CD4<sup>+</sup>, H2<sup>d</sup> restricted, OVA-specific) and RF3370 (CD8<sup>+</sup>, H2K<sup>b</sup> restricted, OVA specific) were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated bovine calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin.

#### Flow Cytometry analysis

Purified T cell subsets or T Cell lines were stained with FITC-conjugated Cholera Toxin B (CTB) (Calbiochem, San Diego, CA) in combination with either anti-CD4 or anti-CD8 and anti-CD44 antibodies conjugated with fluorochrome on ice for 15 minutes and subsequently analyzed on a FACScan flow cytometer (Becton Dickinson, Sand Diego, CA).

#### Lipid raft isolation

Murine primary CD4<sup>+</sup> and CD8<sup>+</sup> (5 × 10<sup>6</sup>) T cells or T cell lines (1 × 10<sup>8</sup> cells) were lysed in 0.4 ml of ice-cold MNE buffer (25 mM MES Ph 6.5, 150 mM NaCl, 2 Mm EDTA)

containing 1% Triton X-100, the E-64 protease inhibitors, and 1 mM sodium orthovanadate. These cell lysates were brought to 1 ml using 40% sucrose solution and then overlaid with 2 ml of sucrose 35% and 1 ml of sucrose 5% in MNE buffer. Lysates were then ultracentrifuged in an SW55Ti rotor (Beckman, Palo Alto, CA) at 100,000 g for 16 hours to separate lipid rafts from cytosol. Aliquots of 0.4 ml of gradient fractions were then collected to yield a total of 9 fractions, which were analyzed for low-density lipid raft components and high-density detergent soluble cytosolic and plasma membrane components by immunodot.

#### Immunodot analysis

One hundred µl of each fraction obtained from the ultracentrifugation for lipid raft isolation were blotted on nitrocellulose membranes, after which the membrane was blocked for 15 minutes using a 3% milk solution blocking buffer. After incubation, the membranes were incubated at 4 °C overnight with HRP-conjugated CTB diluted to 2 µg/ml. Membranes were subsequently washed twice in PBS and signal detection was performed by Hyperfilm ECL according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ).

#### Immunoprecipitation and western blot analysis

Lipid raft fractions 2, 3 & 4 and the non-raft fractions 7, 8 & 9 were prepared from CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines lysates were pooled and proteins were quantitated by Bradford reagents (Bio-Rad, Hercules, CA). Both pooled fractions were mixed with immunoprecipitation buffer (IP buffer) (10 mM Tris-Hcl pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub> 0.5% NP40, 1 mM Sodiumorthovanadate, leupeptine and pepstatin) with anti-thy1 mAb (Abcam, Cambridge, UK) along with protein agarose G beads (Calbiochem, San Diego, CA) overnight at 4 °C. Immunocomplexes with agarose-bound protein G were pelleted down by centrifugation at 10,000 rpm for 5 min. The pellets were washed three times with IP buffer and then subjected to SDS-PAGE, followed by immunoblotting. Pellets were solubilized in IP buffer containing 2-mercaptoethanol. After heating for 5 min at boiling water bath, proteins were separated by SDS-PAGE on 12% polyacrylamide gel and transferred onto 0.22 µm polyvinylidene (difluoride) membranes (Invitrogen). Immunoblot analysis was performed using anti-Thy-1 mAb at a dilution of 1:500. Signal detection was performed by Hyperfilm ECL according to the manufacturer's protocol (Amersham Biosciences).

#### Cholesterol Assays

Purified T cells were extensively washed with PBS prior to use and subsequently lysed in a buffer containing SDS 0.1%, Na<sub>2</sub>EDTA 1 mM and Tris-HCL 0.1 M, pH 7.4. These cells were subsequently examined for their cholesterol content using a sensitive cholesterol oxidase-based assay

using the Amplex Red cholesterol kit (Molecular Probes, Eugene, OR).

### Statistics Analysis

Significant statistical differences between groups were conducted using Student's *t* test and indicated as \* $p \leq 0.05$  or \*\* $p \leq 0.02$ .

### List of Abbreviations

GM1, monosialoganglioside; TCR, T cell receptor; CTB, Cholera Toxin B

### Authors' Contributions

VMC, DN, BG, AB and ES performed the experiments. VMC prepared the figures and co-wrote the paper with DN. DDT supervised the work and edited the manuscript.

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