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Development of an *In Vitro*, Human, Cell-based Assay to Investigate the Role of Peanut Lipids and Invariant NKT cells in Allergic Sensitisation

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1) Introduction

IgE-mediated allergies are increasing in prevalence, with IgE-mediated food allergies affecting up to 10% [1] of children and 6% [2] of adults worldwide. However, the mechanisms underpinning the first phase of IgE-mediated allergy, allergic sensitisation, are unclear. Recently, the involvement of lipids in allergic sensitisation has been proposed. Food allergen sources, such as peanuts, are composed of allergenic proteins accompanied by other compounds, including lipids. Current research suggests lipid-allergen interactions can influence a Th2 shift towards allergic sensitisation through the presentation of lipids via CD1d on dendritic cells (DCs), activating iNKT cells [3] **(Fig. 1)**. However, there is limited research in this area, especially utilising human cells and in vitro assays. Thus, the aim of this research is to develop an *in vitro* method to study the role of peanut-allergen associated lipids and invariant natural killer T (iNKT) cells, in allergic sensitisation.

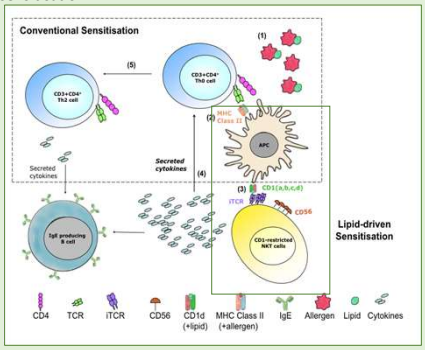

Figure 1. Conventional and lipid-driven sensitisation. (1) Lipids associated with allergens are taken up by antigen presenting cells (APCs). (2) Conventional protein antigen presentation by MHC Class II to naive CD4 Th0 cells. (3) Lipid presentation by CD1 to NKT cells. (4) leading to cytokine secretion (primarily IL-4). (5) Subsequent differentiation to CD4 Th2 cells.

2) Methods

Due to low abundance of iNKT cells in human peripheral blood, iNKT cells were expanded over 14 days by stimulation with the glycolipid, α-Galactosylceramide (α-GalCer), which is a potent activator of iNKT cells. The expanded iNKT cells were then co-cultured with autologous dendritic cells. Prior to co-culture, the DCs were stimulated for 24 hours with either peanut oil (total lipid fraction isolated from peanuts – purchased from Handa Fine Chemicals), Ara h 8 (peanut allergen), or peanut oil with Ara h 8. DMSO was used as a negative control, and α-GalCer used as a positive control. The co-culture allowed DCs to present the lipids to iNKT cells, and subsequent Th1 or Th2 cytokine production measured by flow cytometry. The developed assay, presented in **Figure 2**, was performed utilising blood donated from non-allergic and peanut-allergic subjects (The University of Nottingham's Medical School Ethics Committee (232-1902) and the NHS Health Research Authority (21/SC/0183), respectively).

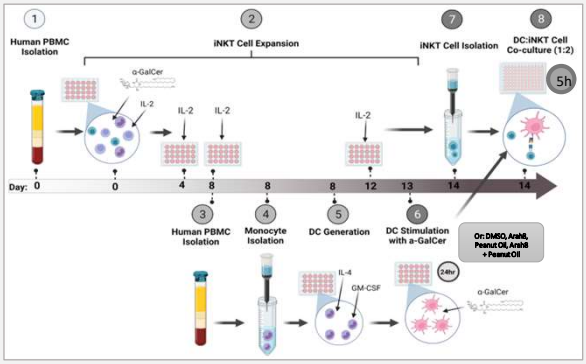

Figure 2. A method for *In vitro* expansion of iNKT cells and subsequent co-culture with lipid-pulsed DCs. (1) PBMCs were isolated from whole human blood and (2) stimulated with a-GalCer and IL-2 to induce iNKT cell expansion. The cells were incubated for up to 14 days, (3) re-stimulating with IL-2 every 4 days. (4) At Day 8 of iNKT expansion, blood was obtained from the same donor and PBMCs were isolated. (5) Monocytes were isolated using CD14+ immunomagnetic isolation and stimulated for 5 days to generate immature DCs (iDCs). (6) iDCs were pulsed with a-GalCer, DMSO, peanut oil, or Ara h 8 and incubated for 24 hours. (7) At Day 14 of iNKT cell expansion, iNKT cells were isolated from the PBMC culture by immunomagnetic isolation (Miltenyi Biotech. isolation beads). (8) The isolated iNKT cells and the stimulated DCs were then co-cultured together at a ratio of 1:2 (DC:iNKT) for 5 hours. Th1 and Th2 cytokine release was then measured and analysed by flow cytometry.

3) Results

iNKT Expansion

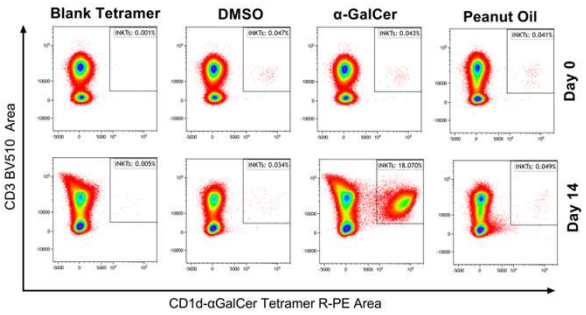

Figure 3. iNKT cell expansion utilising peanut-allergic PBMCs. Representative dot plots indicating percentage of iNKT cells stained with the blank tetramer control, or cultured with either α-GalCer, DMSO control, or peanut oil at days 0 and 14 of culture. All events were gated on live, CD19 negative lymphocytes.

Exemplar flow cytometry data showing the addition of α-GalCer induced a clear expansion of iNKT cells by Day 14 in this peanut-allergic donor (Figure 3). There was no expansion seen in the Blank tetramer or the DMSO controls. There was also no iNKT expansion after peanut oil stimulation.

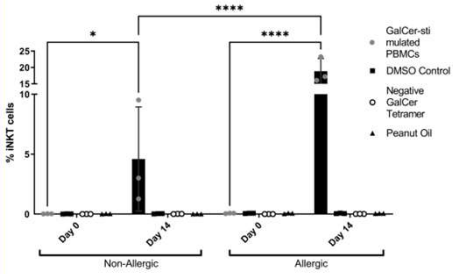

Figure 4.. Average iNKT cell percentages at Day 0 and Day 14 of expansion iNKTs were stimulated with α-GalCer, DMSO, peanut oil, or stained with the negative tetramer control, and cultured for 14 days (n=6).

iNKT expansion was repeated 3 times in both non-allergic and peanut-allergic individuals. Here, we show α-GalCer stimulation resulted in significantly higher iNKT cells compared to Day 0 of culture, in both non-allergic (n=3, $p<0.05$) and peanut-allergic subjects (n=3, $p<0.0005$) (Figure 5). The percentage of iNKT cells was also significantly higher in peanut-allergic subjects compared to non-allergic controls ($p<0.0005$) at Day 14 of culture. Stimulation with peanut oil did not induce iNKT expansion after 14 days in either subject group.

iNKT:DC Co-culture

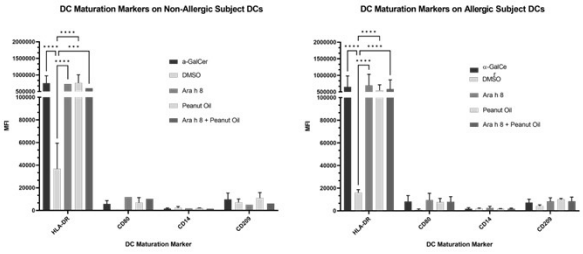

Figure 5. Expression of DC maturation markers. A bar chart to show the Median Fluorescent Intensity (MFI) of DC expression of maturation markers, after stimulation with α-GalCer, peanut oil, Ara h8, and the DMSO control.

The DC analysis highlights in both non-allergic and peanut-allergic subjects, expression of the maturation marker, HLA-DR, was significantly upregulated by peanut oil, α-Galcer, and Ara h 8 stimulation, compared to the DMSO control (Figure 5).

The iNKTs co-cultured with autologous α-GalCer-pulsed DCs displayed increased IL-4, IL-10, and IFN-γ secretion within 5 hours of co-culture, in both non-allergic and peanut-allergic subjects. The iNKT co-cultures with peanut oil-pulsed DCs displayed some increases in IFN-γ production, but decreases in IL-4 production, in both subject groups (Figure 6).

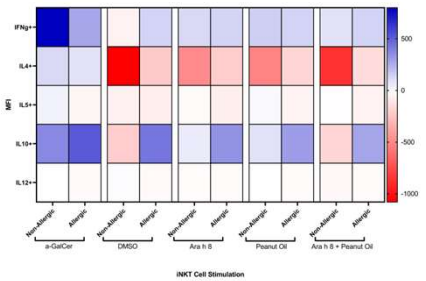

Figure 6. Heat Map to show changes in iNKT cell cytokine production during DC:iNKT co-culture. Results shown are changes in cytokine MFI from Day 14 of iNKT cell expansion, after 5 hours of co-culture with stimulated DCs (n=6). (Data points are the average MFI during co-culture minus the average MFI at Day 14 of expansion).

4) Conclusion

Overall, an in vitro, human, cell-based assay was established where allergen-associated lipids, such as peanut lipids, can be used in substitute of α-GalCer, to determine whether lipids enhance iNKT cell Th2 cytokine secretion, shifting towards a state of allergic sensitisation. The results indicate the total lipid fraction from peanuts affects DC maturation and iNKT cell cytokine secretion, which suggests they do play a role in allergic sensitisation. However, these results need to be repeated in more participants.

References:
[1] Prescott, S., Pawankar, R., Allen, K. urden in children. *World Allergy Organization Journal*, 6:21.
[2] Lee, S. (2017). IgE-mediated food allergies in children: prevalence, triggers, and management. *Korean Journal of Pediatrics*, 60(4), p.99.
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