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Research Article

CD1e Genotype Pilot Study of Cytokine Production and T-Cell Proliferation: A Novel Relationship Between *in Vitro* Immune Function and CD1e Polymorphism in Brazilians

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Abstract

The MHC class I-like CD1a-e molecules have a processing and presentation role for lipid and glycolipid antigens of self and micro-organisms such as mycobacteria. In this pilot study, CD1e polymorphisms were determined by sequencing exons 2 and 3, for eight Brazilians (BR), five black Africans (AF) and eight Australians of European descent (AU/EU). Three *CD1e* exon 2 genotypes were identified (**01/*01*, **02/*02* and **01/*02*); more AF individuals having *CD1e*02/*02* than the other ethnic groups. Statistical analysis revealed significantly greater T-cell proliferation in cultured PBMC of AF versus AU/EU individuals, with BR at intermediate levels; for stimulation with phytohaemagglutinin (PHA) relative to lipopolysaccharide (LPS), Freund's complete adjuvant (FCA) and unstimulated control cells ($p<0.01$). Additionally, PBMC activation with PHA, LPS and FCA produced statistically significant differences between AF and AU/EU, and AF and BR groups for cytokine levels ($p<0.05$). Individual cytokines with significant differences were IL-2, IL-4, IL-10 and TNFα, with IL-6 levels displaying the least variation. Cultures of PBMC from *CD1e* homozygotes (**01/*01* and **02/*02*) contained significantly more TNFα than those from *CD1e* heterozygotes (**01/*02*) ($p<0.05$). To our knowledge, this pilot study is the first to show a relationship of CD1e polymorphisms with immune function *in vitro*.

Keywords: CD1e Polymorphism; Non-Classical MHC; Brazilian Genome; Mycobacterial Disease; Tuberculosis; Leprosy

Abbreviations

IFN - Interferon; IL - Interleukin; MHC - Major Histocompatibility Complex; PBMC - Peripheral Blood Mononuclear Cells; PCR - Polymerase Chain Reaction; TNFα - Tumour Necrosis Factor alpha

Introduction

CD1 is a non-classical MHC molecule occurring in five glycosylated isoforms, CD1a-e [1-3]. It is found on antigen presenting cells, notably dendritic cells, which are the most potent stimulators of immune responses (for reviews please see [4,5]). CD1 resembles MHC class I, having three α domains (α_1-α_3) associated with β_2-microglobulin [1,2,6], with a binding pocket for CD1b, -c and -d, analogous to the peptide binding groove of MHC classes I and II [7]. However, instead of presenting peptides, CD1 molecules present lipid and glycolipid antigens to responder cells. The lipid tails are positioned in the deep hydrophobic pocket of CD1 isoforms [8], suited to binding long alkyl chains of molecules such as mycolic acid, an antigen for recognition of pathogenic mycobacteria [9].

Unlike other CD1 isoforms, CD1e is not present on the cell surface [1]. It is involved in processing of self and microbial lipid and glycolipid antigens, being required for antigen presentation to CD1b-restricted T cells [10]. Located mostly in late endosomes and lysosomes, CD1e associates with MHC class II (HLA-DR) or CD1b in lysosomes [1,11], where it loads antigen onto CD1b of dendritic cells [11]. Separate pathways exist for antigen presentation via CD1 and MHC class II in dendritic cells [12]. Importantly, mycolic-acid specific CD1b-restricted memory T cells are found in the blood of tuberculosis patients, hence relevant for immune defence against mycobacteria [13]. Glycolipids presented by other CD1 isoforms include α-galactosylceramide to NKT cells on CD1d [14], which is well known and provides protection against Gram-negative bacteria [15].

Although CD1 was not initially known to be polymorphic [16,17], polymorphisms of the five CD1 isoforms have be demonstrated

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[18-21]. However, they depend on the ethnic group studied [22-24]. CD1e may be more polymorphic than the other CD1 isoforms, prompting further investigations. Polymorphisms in exons 2 and 3 of CD1e have been documented in black African individuals [21].

Both Africa and Brazil have relatively high rates of infection for the mycobacterial diseases of leprosy and tuberculosis [25-27], leprosy being difficult to eliminate from certain areas of Brazil, such as the Amazon region [25]. Indeed, already in 1913 the then Head of the Public Health Division in Brazil, Oswaldo Cruz, reported a high frequency of leprosy cases among native Amerindians, who live in the Amazon region [28]. Furthermore, leprosy may have originated in East Africa or in the Asian region close to Africa [29]. As the slave trade facilitated African gene flow into the Brazilian population [30-32], a study on CD1e polymorphisms in Brazilians is needed to assess similarity to those in black Africans and if there is any relevance to immune function. We have analysed CD1e polymorphisms of Brazilians, black Africans and Australians of European descent by DNA sequencing; and conducted tests of immune function on cultured PBMC of individuals. CD1e polymorphisms and immune function in the different ethnic groups were assessed. The purpose of this pilot study was to investigate whether *in vitro* T-cell proliferation and cytokine production correlate with CD1e genotype.

Materials and Methods

Subjects

This study was conducted in accordance with the Declaration of Helsinki of 1964, as revised in 2008, with approval from the Human Research Ethics Committee of the University of Western Australia. None of the authors had a conflict of interest in this research. Participants were recruited from the Brazilian, African and Australian communities in Perth, Western Australia. Both the Brazilian ethnic group (BR) and that of Australians of European descent (AU/EU) consisted of eight individuals, each with four males and four females. The AU/EU group served as a control. Five negroid Africans were recruited (AF group), two males and three females. For study inclusion, participants had to be healthy and aged from 20-60 years. They were asked to complete a short questionnaire on their ethnic background and stating any previous contact with tuberculosis or leprosy in their family.

PBMC Separation and Culture

PBMC were separated from heparinised blood samples (20 ml) after underlaying with 15 ml of Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) per tube, by gradient-based centrifugation, as described previously [33]. Afterwards, 1/3 of the PBMC preparation was used for genomic DNA isolation and the remaining 2/3 used for cell culture experiments.

PBMC were cultured in RPMI 1640 GlutaMax (Invitrogen, Carlsbad, CA, USA) with the addition of 5% human serum and 1% antibiotics (Invitrogen, Carlsbad, CA, USA). The cells were cultured in sets of 4 wells ($2x10^6$ cells per 3 ml well) in Costar® 6-well cell plates (Corning Incorporated, New York, NY, USA). A control group and the following three conditions were used to induce cytokine expression: 1) 10 µl/ml of Freund's Complete Adjuvant (FCA) (Sigma, St. Louis, MO, USA); 2) 10 µl/ml of Lipopolysaccharide (LPS-K12, InvivoGen, San Diego, CA, USA); and 3) 25 µl/ml Phytohemagglutinin (PHA, Sigma, St. Louis, MO, USA). FCA consists of heat-killed and dried *Mycobacterium tuberculosis* emulsified in mineral oil. Once injected, it attracts antigen presenting cells and enhances the immune response. Its lipid antigens are processed and presented by the CD1e pathway [34]. LPS is part of the cell wall of Gram-negative bacteria and activates antigen presenting cells through Toll-like receptor 4 (TLR4) [35]. PHA cross-links surface molecules on T cells and is useful in a positive control for T-cell activation. The conditions were chosen to provide different pathways of T-cell activation. Cultures were incubated for approximately 24 hr in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Thereafter, the cells were used for RNA extraction and measurement of CD1e expression using quantitative real-time PCR, whereas the culture supernatants were collected for measurement of cytokine levels.

Genomic DNA Isolation

PBMC were homogenized gently in 0.5 ml DNA homogenization buffer containing 0.1 M sodium chloride, 0.01 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.3 M Tris-HCl (pH 8.0) and 0.2 M sucrose. After transfer of the homogenate to a 1.5 ml tube, 31 µl of 10% sodium dodecyl sulphate (SDS) was added, then mixing and incubating at 65°C for 30 min. Following addition of 87 µl of 8 M potassium acetate and mixing, the tube was incubated on ice for 1 hr and centrifuged at 19,000 g for 10 min at 4°C. To the supernatant was added 2 µl RNase (500 µg/ml), incubating at 37°C for 1 hr. After adding an equal volume of a 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1) and mixing thoroughly, the tube was centrifuged at 3,500 g for 5 min at 4°C. To the upper (aqueous) phase was added 0.1 volume of 3 M sodium acetate and 2.5 volumes of cold 100% ethanol. DNA was precipitated overnight at -70°C. After centrifuging at 19,000 g for 30 min, the supernatant was discarded. The pellet was washed twice with 70% ethanol, air dried and resuspended in deionised water or Tris-EDTA buffer (pH 8.0). Genomic DNA was used for amplification of exon 2 and exon 3 of CD1e by PCR and then DNA sequencing.

CD1e PCR and DNA Sequencing

Using genomic DNA from PBMC, regions of CD1e exon 2 and exon 3 were amplified for sequencing to identify CD1e polymorphisms, according to a previous study [19]. Primers are provided in Table 1. Briefly, PCR was performed with 1 µl of DNA template in 25 µl reaction volumes using a programmable thermal cycler, PTC-100™ (MJ Research, Inc., Watertown, MA, USA). Thermocycling consisted of 5 min denaturation at 95°C, then a "hot start" with 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 65°C and 1 min extension at 72°C; followed by 5 min extension at 72°C. Agarose gel electrophoresis was conducted to confirm that PCR products were of the expected sizes, 310 and 297 bp for CD1e exons 2 and 3 [21].

The Ultraclean PCR Clean-up DNA purification kit (MOBIO, Carlsbad, CA, USA) was used to purify samples. Then they were sent to Macrogen (Seoul, Korea) for sequencing. On receipt of the data, sequences were edited using 4Peaks for Mac V1.7 (Nucleobytes, Amsterdam, The Netherlands). The method of naming genotypes as either *CD1e*01/*01*, *CD1e*01/*02* or *CD1e*02/*02* [18,21] is given in Figure 1.

Cytokine Measurements

After an incubation of 24 hr, supernatants of each sample from the three activation conditions and unstimulated controls were collected. IL-2, IL-4, IL-6, IL-10, IL-17A, TNFα and IFNγ were measured by Cytometric Bead Assay (BD BioSciences, San Jose, CA, USA), according to the protocol of the manufacturer.

BrdU proliferation Assays

BrdU proliferation assays were performed in 96-well U-bottom plates (Sarstedt, Newton, NC, USA) using a colorimetric kit (Roche Diagnostics, Basel, Switzerland), following the company protocol. Absorbance was measured at 405 nm with an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland).

RNA Isolation and Reverse Transcription PCR

Total RNA was prepared from PBMC using RNAzol® RT (Molecular Research Center, Cincinnati, OH, USA), according to the instructions of the manufacturer. After extraction and drying, RNA was suspended in 30 µl of nuclease-free water. RNA was free of contaminating DNA and proteins, as confirmed by values within 1.6-1.9 for the 260 nm / 280 nm spectrophotometric ratio. RNA concentrations were low, so RNA isolated was used at maximal volumes (7 µl per sample) for reverse transcription PCR.

Prior to the reverse transcription step, RNA was routinely treated with Promega DNase (Promega Corporation, WI, USA), as a precautionary measure. After treatment, the protocol included incubation at 65°C for 10 min to inactivate the DNase. The Reverse Transcription System protocol (Promega Corporation, WI, USA) was used to obtain complementary DNA (cDNA) by PCR. Tubes containing the reagents were placed on a programmable thermal cycler, PTC-100™ (MJ Research, Inc., Watertown, MA, USA). Following PCR, an UltraClean® PCR clean-up kit (MO BIO, Carlsbad, CA, USA) was used for cDNA purification and samples were stored at 4°C.

Investigation of CD1e Expression By Real-Time PCR

Real time PCR amplification was performed in 10 µl volumes using reagents from a Promega kit (Promega Corporation, WI, USA) and a Corbett Research Rotor-Gene 3000 (Corbett Research, Sydney, Australia) to quantify mRNA expression of CD1e. The fluorescent dye, SYBR green (Invitrogen, Carlsbad, CA, USA), was used to

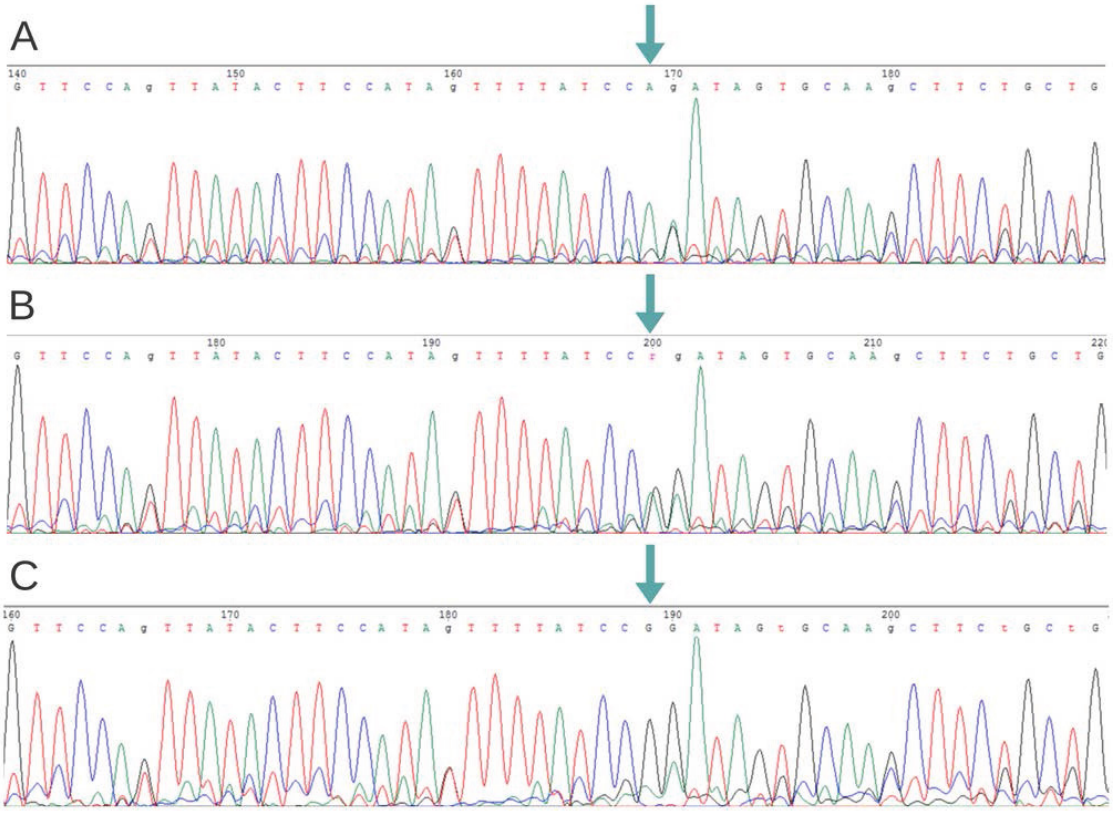

Figure 1: Genotype naming method according to the nucleotide marked with an arrow in the sequence shown. A: *CD1e*01/*01* genotype (Adenine); B: *CD1e*01/*02* genotype (Adenine → Adenine or Guanine); C: *CD1e*02/*02* genotype (Adenine → Guanine).

quantify PCR products due to binding of CD1e primers (Table 1). Following PCR amplification, a melt curve was constructed to determine the quality of the PCR reaction using fluorescence data from 70°C to 99°C.

Statistical Analysis

Proliferation measures were found to be non-Normally distributed, so a Friedman's (non-parametric) Analysis of Variance was used to compare the values between activations, after taking into account the repeated measures on each sample, for each ethnic group separately. An alternative to the non-parametric test was to analyse a transformation of the measures, and this was performed and compared with the non-parametric approach. The Box-Cox transformation was applied to the measurements to give a variable whose distribution was as close to Normal as possible. The cytokine concentrations were also non-Normally distributed, so were treated in a similar manner. The parametric analyses were implemented using a random-effects regression model. In addition to analysing the cytokine concentrations separately for each cytokine and repeated for each ethnic group, a single model was also used to assess the independent contributions of each of the 4 variables concurrently (multivariate model). Because this was beyond the scope of the non-parametric analysis, this could only per performed using the parametric approach. The SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) software was used for data analysis, and a p-value <0.05 was taken to indicate a statistically significant association in all tests.

Results

Demographics from Questionnaire Data

From 21 completed questionnaires received via e-mail, there were 10 male and 11 female participants, with ages from 22-50 years. In the Brazilian group (BR), all had at least one European ancestor, with one having Lebanese background. The origin of most BR participants were southeastern states: Sao Paulo, Rio de Janeiro and Minas Gerais. However, two BR group members with parents from Minas Gerais were born outside Brazil, one with an Australian parent. All black African group (AF) members reported Bantu ancestry and had parents from southern or central Africa: South Africa, Zimbabwe and Zambia. Four AF participants were from Zimbabwe and one from South Africa. The Australian/European (AU/EU) group members were born in Australia with ancestry from the United Kingdom, except one not born in Australia who was from Spain.

Most AF and BR group members confirmed receiving previous tuberculosis vaccinations, whereas approximately 80% of AU/EU participants were unvaccinated for tuberculosis or were unsure of this. Overall, only three questionnaire respondents (1 AF, 2 AU/EU) reported relatives having had tuberculosis infections but none in contact with leprosy.

CD1e DNA Polymorphisms Identified

DNA sequencing results from PCR products of the 21 participants yielded only two alleles, *CD1e*01* and *CD1e*02*, with frequencies of 48.3% and 51.7%. The genotype frequencies for *CD1e*01/*01*, *CD1e*01/*02* and *CD1e*02/*02* were 38%, 28% and 33%, respectively. Table 2 shows genotype frequencies according to ethnic group. Although sample numbers are small, limiting the ability to draw conclusions, differences between ethnic groups are visible. A proportion of heterozygous individuals exists in each ethnic group, with *CD1e*01/*01* homozygotes predominating in the BR and AU/EU groups and *CD1e*02/*02* homozygotes in the majority in the AF group.

T-Cell Proliferation Assay Results

Under all activation and control conditions, T-cell proliferation was highest for the AF group, lowest for the AU/EU group, with the BR group falling between them (Figure 2). By statistical analysis as described earlier, variation of T-cell proliferation results within the AF group but not BR or AU/EU groups was highly significant (p=0.0001). T-cell proliferation within the AF group due to PHA stimulation was significant compared to LPS, FCS and control activation (p<0.0001 in all cases). LPS stimulation was significant to a lesser extent when compared to FCA (p=0.0338). LPS and FCA stimulation versus control activation were not significant. One of the eight AU/EU individuals had notably higher T-cell proliferation, unstimulated (control) and for each activation condition (data not shown).

Table 2. CD1e genotype frequencies (%) within ethnic groups

CD1e allele assignment			
Ethnic group	**01/*01*	**01/*02*	**02/*02*
AU/EU	50	50	0
BR	50	25	25
AF	0	20	80

Table 1: PCR primer sequences* and Tm values

CD1e exon 2	Sense	GGC TCT ACA ATC CTA TCA TCT AGC AGC	58°C
	Anti-sense	GCT TAG AGC AAC GAA CTT ACA TTC AAG	
CD1e exon 3	Sense	CAG ACC CCT TCG AGA TCC AGA TAT TAG C	62°C
	Anti-sense	GGG GAG AGA GAG AGT TGG GCT CAC C	

* Previously published [19].

Cytokine Expression by Cytometric Bead Array

Reflecting results of the T-cell proliferation assays, cytokine concentrations in supernatants of activated PBMC were generally greatest for the AF group, least in the AU/EU group and intermediate for the BR group (Figure 3). Significant differences were seen for AF versus AU/EU group and AF versus BR, for stimulation with PHA, LPS or FCS ($p<0.05$). Results shown represent an average of different activation conditions. Individual cytokines with significant differences were IL-2, IL-10, IL-4 and TNFα. No significant differences were seen for IFNγ, IL-6 and IL-17A. Remarkably, IL-6 levels varied little between groups.

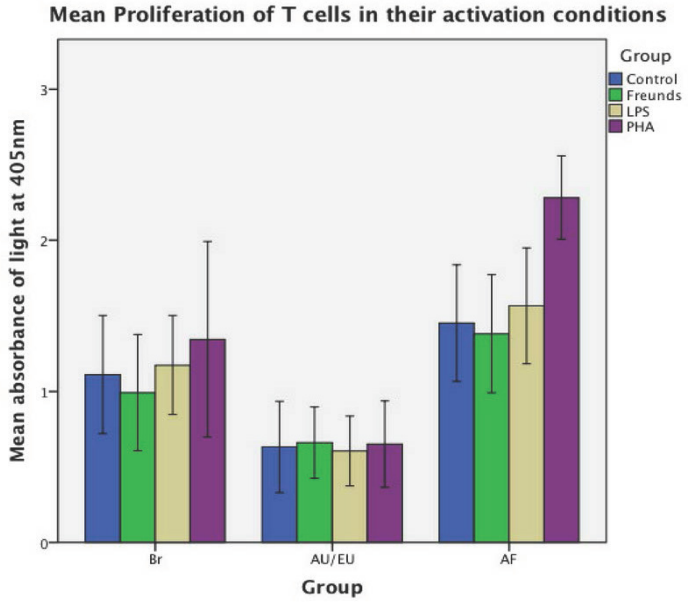

Figure 2: Proliferation of T cells according to group and activation condition. Error bars represent standard deviation.

There were pairwise differences in cytokines, when variation due to ethnic group and activation method were adjusted for by parametric analysis using a multivariate model. IL-17A versus TNFα as well as IL-17A versus IFNγ and IL-2 versus IL-4 were not significantly different after accounting for other variables. TNFα versus IFNγ expression was significantly different ($p=0.0496$) to a lesser extent than the remaining significant combinations; namely, TNFα versus IL-6 ($p=0.001$), IFNγ versus IL-10 ($p=0.0002$), IL-2 versus IL-10 ($p=0.0004$), IL-6 versus IL-17A ($p=0.0001$) and the others ($p<0.0001$).

Cytokine Expression for Different CD1e Genotypes

The three genotypes identified were *CD1e*01/*01*, *CD1e*01/*02* and *CD1e*02/*02*, although the AU/EU group had no *CD1e*02/*02* individuals and nobody in the AF group had *CD1e*01/*01* genotype. Figure 4 shows that cytokine concentrations in cultured PBMC supernatants were less for *CD1e*01/*02* heterozygotes than homozygous individuals of either genotype. However, this was only significant for TNFα. Results shown represent an average of different activation conditions. Interestingly, both homozygous

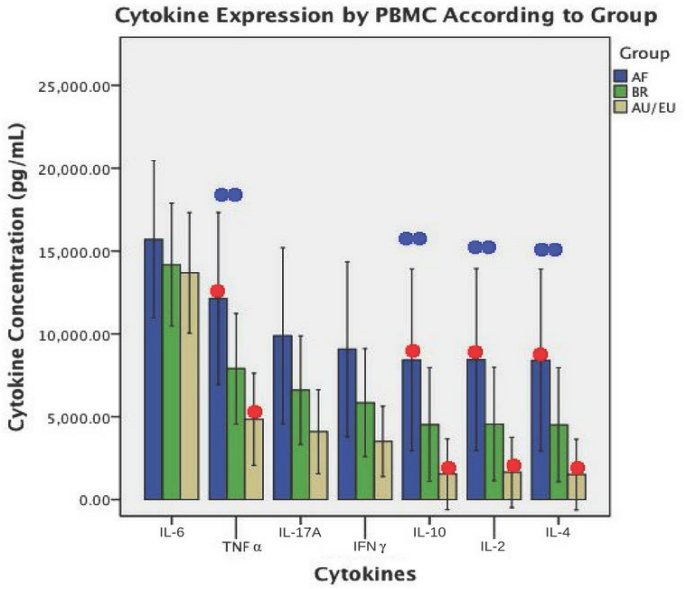

Figure 3: Mean cytokine concentration of seven cytokines by PBMC according to group. Significant differences were seen for IL-2, IL-4, IL-10 and TNFα (double blue dots) ($p<0.05$). Then significant differences between participant groups were analysed. Significant differences were only found between AF and AU/EU, and AF and BR groups for the four previously mentioned cytokines (red dots) ($p<0.05$). Error bars represent standard deviation.

genotypes had significantly more TNFα expression than heterozygotes ($p<0.05$).

The Relative Contributions of Ethnic Group, Activation Method and Cytokine

According to parametric analysis with a multivariate model, the contribution of ethnic group alone was only significant for AF versus AU/EU groups ($p=0.0139$). Ethnic group had a less significant effect overall ($p=0.0389$) than either activation method or cytokine (both $p<0.0001$). Of course, there were variations in pairwise combinations of cytokines, which have been described.

Comparison of the Influences of Activation Method and Cytokine Expression for Each Genotype

Parametric testing in a multivariate model was performed for all three genotypes, *CD1e*01/*01*, *CD1e*01/*02* and *CD1e*02/*02*. For the samples of each genotype, highly significant contributions were made by the activation method used and the cytokine measured ($p<0.0001$ in each case). The effects of activation methods and expression of individual cytokines varied within each genotype, with differing levels of statistical significance.

Effects of Activation Method on Cytokine Expression in Samples of Each Genotype

By parametric analysis with a multivariate model to assess contributions of variables independently, cytokine expression varied with both activation method and genotype. Hence, FCA

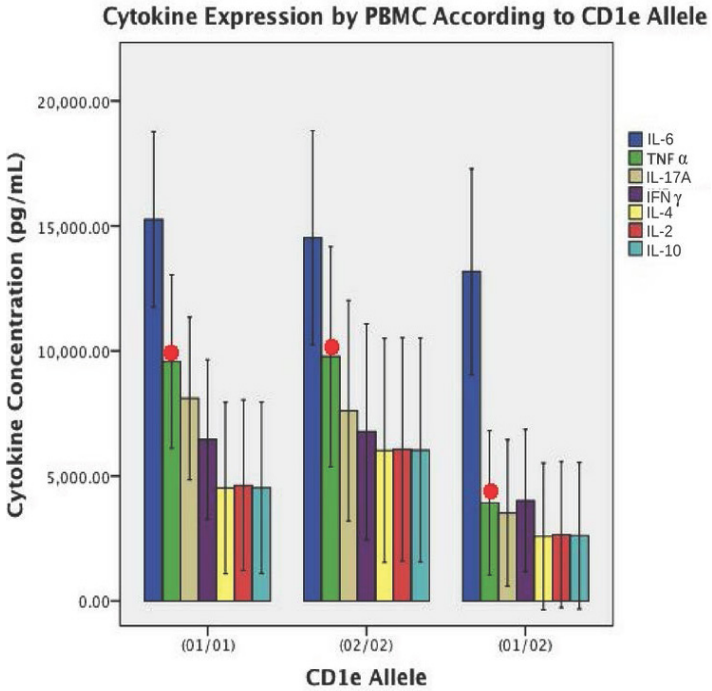

Figure 4: Mean cytokine expression of seven cytokines by PBMC according to CD1e genotype. A significant difference was only found for TNFα (red spots) when comparing both homozygous (*CD1e*01/*01* and *CD1e*02/*02*) versus heterozygous (*CD1e*01/*02*) participants ($p<0.05$). Heterozygous participants showed significantly lower total TNFα expression. Error bars represent standard deviation.

activation was only significant in samples from *CD1e*01/*01* ($p<0.0001$) but not *CD1e*01/*02* or *CD1e*02/*02* individuals. Conversely, PHA activation was not significant in *CD1e*01/*01* samples, whereas it was significant with those from *CD1e*01/*02* and *CD1e*02/*02* individuals ($p<0.0001$ in both cases). LPS activated all three genotypes significantly and control samples were not activated significantly, as expected.

Cytokines were compared for differences in the relative contributions of ethnic group and activation method to their expression. Of all cytokines tested besides IL-10, the activation method was significant ($p<0.0001$, except IL-4 with $p=0.0017$). Ethnic group was only significant with IL-2 ($p<0.0436$), 4 ($p=0.0173$), 10 ($p=0.0178$) and 17A ($p=0.0398$). Furthermore, the parametric test and multivariate analysis revealed that significance was mainly for differences between the AF and AU/EU groups (IL-2, 4, 10 and 17A) but also between BR and AU/EU groups (IL-10 and 17A). Differences were not significant between the AF and BR ethnic groups for any of the cytokines tested in the multivariate model with a parametric test.

Differences in Cytokine Expression According to Genotype

When all cytokines were compared in a pair wise manner, for each of the three genotypes by parametric analysis using the multivariate model, the data was not easy to interpret. Expression of TNFα, IFNγ and IL-17A versus IL-2 and IL-4 was highly significant for each genotype ($p<0.0001$). IL-6 expression was also highly significant for all three genotypes when compared to that of IL-2, 4 and 10 ($p<0.0001$). Other cytokine pairs which had significant differences for all three genotypes, albeit to a lesser extent, were IL-10 versus TNFα and IL-17A. Interestingly, cytokines in which the only non-significant difference was for the *CD1e*01/*01* genotype were IL-2 versus IL-10, as well as IL-6 versus TNFα and IL-17A; and conversely for IFNγ versus IL-10, the only non-significant difference in cytokine expression was for *CD1e*02/*02* individuals. When considering cytokines only, thus not genotype or ethnic group, differences in expression were significant for most pairs of cytokines except IL-2 versus IL-4, as well as IL-17A versus TNFα and IFNγ.

Discussion

We found a statistically significant reduction in TNFα expression by PBMC of heterozygous individuals with *CD1e*01/*02* alleles, as opposed to homozygotes. To our knowledge, this is the first study to demonstrate a difference in immune function *in vitro*, associated with CD1e polymorphisms. Tourne et al. [34] suggested that homozygotes with *CD1e*04* alleles might respond abnormally to *Mycobacterium tuberculosis*, based on *in vitro* studies involving site-directed mutagenesis. CD1e is not expressed on the cell surface [1] and as Tourne et al. [34] had found a decrease in antigen presentation of the *M. tuberculosis* antigen, hexamannosylated phosphatidyl-myo-inositol (PIM_6), alongside reduced endosomal processing and transport of *CD1e*04* protein, we have found yet another mechanism of preventing a normal immune response to lipid and glycolipid antigens.

Of six CD1e alleles known so far [18-21], we only observed two, *CD1e*01* and *CD1e*02*, with allele frequencies of 48.3% and 51.7%. Although this is only a pilot study with small sample numbers, our result is not surprising, as allele frequencies of 49% and 51% have been reported for the same alleles in people of diverse ethnic backgrounds [18]. We had clear DNA sequencing results for all 21 participants for CD1e exon 2 but only 17 of 21 for exon 3. Thus *CD1e*03*, *CD1e*04* and *CD1e*06* alleles can be excluded in 17 participants, as these alleles have single point mutations in exon 3. Without ruling them out in four participants, *CD1e*03* and *CD1e*04* may occur at low frequency [19]; and *CD1e*06* has only been found in black Africans [21], whereas the four participants were in the BR and AU/EU ethnic groups. Lacking participants clearly *CD1e*04*, it is possible that T-cell proliferation in PBMC treated with FCA (containing PIM_6) was not significantly different between groups in our pilot study because CD1e protein processing and transport were normal [34].

To date, a search of the literature reveals no mRNA study on CD1e polymorphisms. However, we were unable to rule out transcription of polymorphic variants of CD1e after conducting real-time PCR, using cDNA after reverse transcription with CD1e exon 2 and exon 3 primers shown in Table 1. Melting curve analysis of samples from different ethnic groups under different *in vitro* PBMC activation conditions reflected CD1e genotypes, as expected (data not shown). To exclude transcription of polymorphic variants,

further downstream analysis of the PCR product was needed for confirmation but was not done.

An implication of decreased TNFα expression in *CD1e*01/*02* heterozygotes versus homozygotes is increased susceptibility to mycobacterial infections such as tuberculosis and leprosy. In support of this, TNFα is needed for macrophage recruitment to granulomas via chemokines [36] and for control of various mycobacterial infections [37,38]. Autoimmune diseases such as systemic lupus erythematosus (SLE) occur at low frequencies in tropical Africa. An explanation suggested long ago in the Lancet [39], is maintenance of a TNFα allele in the African but not African-American population by selective pressure to reduce malarial pathology. Such selective pressures could explain the differences in CD1e polymorphisms observed in our small pilot study, representing alternative benefits of both homozygous and heterozygous allele distributions. Of course, another implication of our findings is greater risk of autoimmunity in homozygotes versus *CD1e*01/*02* heterozygotes (for a treatment-related review of the role of TNFα in autoimmunity see Chen and Oppenheim [40]).

Differences we observed in T-cell proliferation and cytokine production dependent on ethnic group could also indicate selective forces at work. AF and AU/EU ethnic groups were at opposite poles of the spectrum of T-cell proliferation due to PHA stimulation (a potent T-cell mitogen) and production of IL-2, IL-4, IL-10 and TNFα. However, only a larger study could demonstrate gene flow of CD1e alleles into the Brazilian population from African and European ancestors [30-32]; and any role of the *CD1e*02/*02* genotype in increased T-cell responsiveness of black African individuals. However, parametric testing in a multivariate model with our data revealed that activation by PHA was only significant for *CD1e*01/*02* and *CD1e*02/*02* genotypes ($p<0.0001$ for both) but not *CD1e*01/*01* individuals. Increased expression of costimulatory molecules and capacity for T-cell stimulation by PHA in African-Americans has been documented [41]. Interestingly, increased T-cell responsiveness to LPS by AF group members could potentially be important for immune function due to lower levels of circulating LPS in Africans compared to Caucasians [42]. However, in our small study the observed greater responsiveness of T cells to LPS within the AF group compared to control stimulation was not statistically significant. Toll-like receptor 4 (TLR4) is a receptor for LPS [35] but in our study TLR polymorphisms were not examined, although they are important to mycobacterial disease susceptibility, notably TLR2 polymorphisms [43-47]. Relatively similar induction of IL-6 expression for AF, BR and AU/EU groups was noteworthy but as per our other findings, confirming the data and determining the evolutionary significance would require a larger study.

A thorough multivariate analysis of data using a parametric test, non-parametric methods not being practical that were used to confirm other data, supports the concept of selective forces on CD1e polymorphisms. Significant activation of PBMC by FCA was found with *CD1e*01/*01* ($p<0.0001$) but not *CD1e*01/*02* or *CD1e*02/*02* genotypes. Possible explanations are an alteration of function or that activation requires a "second signal" (soluble or via cell contact) in certain people. In a recent study using blood samples from individuals of the Chinese Han ethnic group, the autoimmune peripheral nerve disease Guillain-Barré syndrome was associated with *CD1a*02/*02* genotype ($p<0.001$) but not CD1e polymorphisms [48]. In contrast, *CD1a*01/*02* was associated with reduced disease susceptibility ($p<0.001$). The involvement of cytokines was not assessed. Such disadvantages of CD1 polymorphisms can provide negative selective pressure.

Modulation of the immune response due to *Trypanosoma cruzi* infection was found by antibody-mediated blocking of CD1d on cells in blood samples from Brazilian individuals with Chagas disease of the heart [49]. Reduced expression of IFNγ by $CD4^{-}8^{-}$ gamma delta (γδ) T cells exposed to parasites *in vitro*, from patients with disease, occurred in blood samples treated with antibody before exposure to parasites. Such effects on immune reactions in a disease state can be of advantage even when infection cannot be eliminated. Hence, any polymorphisms affecting CD1 function and subsequent immune responses beneficially could provide positive selective pressure.

In conclusion, we have extended work on the role of CD1 isoforms in immune responses to lipid and glycolipid antigens by analysing polymorphisms of CD1 in three small ethnic groups; namely, black African, Brazilian and Australian (of European descent). Although small sample numbers are involved, we have found reduced TNFα expression in PBMC of heterozygotes with a *CD1e*01/*02* genotype versus homozygous individuals. We believe this is the first *in vitro* demonstration of an effect of CD1e polymorphisms on immune function. Therefore, we suggest this warrants a larger study to confirm our findings, statistically significant in our pilot study.

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