Clinical Immunology 252 (2023) 109632

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Single-cell immune profiling of Meniere Disease patients

Marisa Flook [a,b,c], Alba Escalera-Balsera [a,b,c], Paulina Rybakowska [d], Lidia Frejo [a,b,c], Angel Batuecas-Caletrio [e,f], Juan C. Amor-Dorado [g], Andres Soto-Varela [h,i], Marta Alarcón-Riquelme [d], Jose A. Lopez-Escamez [a,b,c,j,*]

[a] Otology & Neurotology Group CTS 495, Department of Genomic Medicine, GENYO, Centre for Genomics and Oncological Research Pfizer/University of Granada/ Andalusian Regional Government, PTS Granada, Spain
[b] Division of Otolaryngology, Department of Surgery, Instituto de Investigación Biosanitaria, ibs.GRANADA, Granada, Universidad de Granada, Granada, Spain
[c] Sensorineural Pathology Programme, Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Madrid, Spain
[d] Genetics of Complex Diseases Group, Department of Genomic Medicine, GENYO, Centre for Genomics and Oncological Research: Pfizer/University of Granada/ Andalusian Regional Government, Granada, Spain
[e] Department of Otolaryngology, Hospital Universitario Salamanca, Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain
[f] Division of Otolaryngology, Department of Surgery, Universidad de Salamanca, Salamanca, Spain
[g] Department of Otolaryngology, Hospital Can Misses, Ibiza, Spain
[h] Division of Otoneurology, Department of Otorhinolaryngology, Complexo Hospitalario Universitario, Santiago de Compostela, Spain
[i] Department of Surgery and Medical-Surgical Specialities, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[j] Meniere's Disease Neuroscience Research Program, Faculty of Medicine & Health, School of Medical Sciences, The Kolling Institute, University of Sydney, Sydney, New South Wales, Australia

ARTICLE INFO

Keywords:
Allergy
Inflammation
Cytokines
Mass cytometry
Meniere Disease

ABSTRACT

Background: Meniere Disease (MD) is an inner ear syndrome, characterized by episodes of vertigo, tinnitus and fluctuating sensorineural hearing loss. The pathological mechanism leading to sporadic MD is still poorly understood, however an allergic inflammatory response seems to be involved in some patients with MD.
Objective: Decipher an immune signature associated with the syndrome.
Methods: We performed mass cytometry immune profiling on peripheral blood from MD patients and controls. We analyzed differences in state and differences in abundance of the different cellular subsets. IgE levels were quantified through ELISA on supernatant of cultured whole blood.
Results: We have identified two clusters of individuals according to the single cell cytokine profile. These clusters presented differences in IgE levels, immune cell population abundance, including a reduction of $CD56^{dim}$ NK-cells, and changes in cytokine expression with a different response to bacterial and fungal antigens.
Conclusion: Our results support a systemic inflammatory response in some MD patients that show a type 2 response with allergic phenotype, which could benefit from personalized IL-4 blockers.

1. Introduction

Meniere Disease (MD, MIM 156000) is a rare and chronic inner ear syndrome, defined by sensorineural hearing loss (SNHL) associated with episodes of vertigo, tinnitus, and aural fullness [1]. MD is a heterogeneous disease with a well-established genetic contribution [2]. Various classifications of MD have been described, according to the associated comorbidities identified by cluster analysis [3,4], radiological findings in the angle of the vestibular aqueduct of the temporal bone [5] or cytokine profile in peripheral blood [6]. Histopathological studies on human temporal bones [7] and magnetic resonance studies [8] have confirmed an accumulation of endolymph in the cochlear duct, termed endolymphatic hydrops (EH). However, the pathological mechanism leading to the formation of EH in sporadic (non-familial) MD is poorly understood. In this context, there is enough evidence to support that genetic factors and autoimmunity/inflammation could be involved in the etiology of MD [9].

MD has been associated with various autoimmune or auto-inflammatory diseases, namely rheumatoid arthritis, thyroid diseases, systemic lupus erythematosus, psoriasis, and vitiligo [10–13]. Also, allelic variants in immune-related genes, such as *MICA, TLR10, NFKB1* have been associated with SNHL progression in MD [14–16]. Moreover,

* Corresponding author at: Otology & Neurotology Group CTS495, GENYO, PTS Granada, Avenida de la Ilustración, 114, 18016 Granada, Spain.
E-mail address: jalopezescamez@ugr.es (J.A. Lopez-Escamez).

https://doi.org/10.1016/j.clim.2023.109632
Received 15 February 2023; Accepted 29 April 2023
Available online 11 May 2023
1521-6616/© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

mutations in various immune-related genes have been detected in a case of MD with comorbid suspected autoinflammatory thyroid disease [17]. Furthermore, the allelic variant rs4947296, a quantitative trait locus that regulates the expression of various genes in the TWEAK/Fn14 pathway is associated with bilateral MD and it is present in up to 18% of patients with comorbid autoimmune diseases [18].

On the other hand, there are several reports of an over-expression of cytokines and IgE in MD patients [6,19–24]. Ma *et al* observed that high levels of IgE could be used as a predictor of acute low-tone SNHL recurrence and MD transformation, since patients with acute low-tone SNHL developed MD during follow-up [20]. Zhang *et al* have also found that MD patients had higher levels of IgE, IL-4, IL-5 IL-10 and IL-13 in serum samples [23]. Besides, they described the presence of IgE in the ampulla, macula, semicircular canal, and endolymphatic sac by immunohistochemistry and dense deposits of IgE in the utricle of MD patients. Frejo *et al* have reported a subset of MD patients with increased proinflammatory cytokine production [6]. Moreover, antigenic stimulation with allergenic mold extracts provoked an increased production of pro-inflammatory cytokines in MD patients. Still, it is not known if these triggers are specific for MD.

Since a subset of patients that present an elevated pro-inflammatory cytokine basal profile with an altered response after antigenic stimulation, we proposed to investigate the role of antigenic stimulation and the mechanism of proinflammatory response in MD.

2. Methods

2.1. Subject recruitment

This work was carried out according to the principles of the Declaration of Helsinki [25] revised in 2013 for investigation with humans and following the ethical standards recognized by the Spanish biomedical research law. The Human Ethics Review Board in all participating medical centers approved the experimental protocols of this study (PI17/1644; PI20-1126) and all donors signed a written informed consent.

This study included a total of 26 patients with definite MD, and 13 healthy controls, which were recruited from the Otoneurology Clinics of four academic hospitals in Spain. Individuals over the age of 18 years old who fulfilled the clinical diagnosis of definite MD, according to the diagnostic criteria of the Barany Society [1], that had signed an informed consent were included in the study. Individuals that were under immunosuppressor, or antihistaminic treatment were excluded from the study. Clinical information from each patient was gathered during recruitment, which can be found summarized in Supplementary Table 1.

2.2. Blood sampling and stimulation

Peripheral blood was collected into EDTA-coated vacutainers (BD Biosciences, #367525) between 8:00 and 13:00. Blood samples were sent to GENYO and processed within 24 h of sample collection.

Whole blood was cultured under three conditions: unstimulated[1] (UNS), stimulated with lipopolysaccharide (LPS) (InvivoGen, #tlrl-peklps), and stimulated with allergenic extract from Aspergillus niger (ASP[2]) (DST, #42020860). So, blood was mixed 1:1 with RPMI 1640 (Thermo Fisher Scientific, #61870–044) supplemented with 1% MEM (Thermo Fisher Scientific, #11140–035) and 1% Sodium Pyruvate (Biowest, #L0642–500). If stimulation was taking place either 50 ng/mL LPS, or 10 μg/mL ASP was added, and cells were incubated for 6 h at 37 °C, 7% CO_2 in sterile 5 mL polystyrene round-bottom tubes (Corning, #352054). Each sample was cultured in duplicate. To one tube, a protein transport inhibitor cocktail of Brefaldin A (eBioscience, #00-4506-51) and Monensin (eBioscience #00-4505-51) was added prior to incubation, and in the other tube no protein transport inhibitor was added.

After incubation, the blood cells in the tube with protein transport inhibitor were incubated for 5 min at room temperature in 6.25 μM Cisplatin (Sigma Aldrich, #479306), for live/dead staining. Before storing the samples at −80 °C, samples were incubated for 10 min at RT with Proteomic Stabilizer (Smarttube, #PROT1-1 L).

The tube with blood cells without protein transport inhibitor was centrifuged for 10 min at 1500 rpm, supernatant was collected and stored at −80 °C.

2.3. Mass cytometry immunophenotyping

For immunophenotyping, a panel of 29 metal-conjugated monoclonal antibodies was optimized and employed. All antibodies were validated, pre-titered and aliquoted into per-test amounts and stored at −80 °C. Antibodies were either purchased from Standard BioTools Inc. in pre-conjugated format or unlabelled antibodies were purchased in a carrier-protein-free format and conjugated with the indicated metal isotope using Maxpar X8 Antibody Labeling Kits (Standard BioTools Inc.), following the manufacturer's protocol. Antibodies against the following cytokines were used: IL-1β, IL-4, IL-6, IL-8, IL-10, TNFα, MCP-1/CCL2, and MIP-1β/CCL3. The complete list of antibodies and corresponding metal tags can be found in Supplementary Table 2.

Cells were stained and acquired by CyTOF as previously described [26]. Briefly, whole blood samples were thawed on a roller at 4 °C for 45 min and red blood cells are lysed with thaw-lyse buffer (Smarttube, #THAWLYSE1). Cell concentration was determined, and 1.5×10^6 cells were barcoded using Cell-ID 20-plex Pd Barcoding Kit (Standard BioTools Inc., #FLU201060-K). Differentially Pd-tagged samples were combined and incubated with extracellular targeted antibodies (Supplementary Table 2) for 30 min at 4 °C. This was followed with cell permeabilization with Perm-S buffer (Standard BioTools Inc., #FLU201066) and staining with intracellular antibodies (Supplementary Table 2) for 30 min at 4 °C. Cells were then washed and stained with a DNA intercalator, 0.25 μM 191Ir/193Ir (Standard BioTools Inc., #201192B) for 1 h at room temperature. After this, cells were left in 2% formaldehyde (Thermo Fisher Scientific, #28906) overnight at 4 °C. Cells in the fixative solution were washed with cell staining buffer (Standard BioTools Inc., #FLU201068) and MiliQ water, cells were diluted to 5×10^5 cells/mL in MiliQ water containing 1:10 diluted EQ beads (Standard BioTools Inc.) and filtered through a 100 μm mesh (Miltenyi Biotec, #130–110-917). Cells were acquired at a 250 events/s using a CyTOF 2 Helios upgraded mass cytometer (Standard BioTools Inc.). Machine tuning was performed during start-up and after 5 h. Samples were stained and run in 40 batches. Each batch consisted of the sample conditions per patient: unstimulated, stimulated with LPS, or ASP and a batch control consisting of an unstimulated blood sample from a healthy donor, that underwent the same viability staining, fixation and storing as the study samples, and was thawed and stained in parallel with the samples in study.

2.4. Analysis of mass cytometry data

The .fcs files obtained from mass cytometry analysis were normalized using the processing function within the CyTOF acquisition software (version 6.7.1016) based on the run EQ four element beads.

Signal cleaning, outlier detection, file debarcoding, file aggregation, and normalization using a reference sample were carried out following the default parameters in the methods previously described [27]. Only files with over 65% recovery after debarcoding were used for analysis and visualization of the generated data. This was carried out with the CyTOF workflow [28] and TreekoR [29] R packages, which perform dimensionality reduction and unsupervised clustering, manual

[1] UNS - unstimulated / basal level

[2] ASP - *Aspergillus niger* allergenic extract

annotation, and differential testing.

The CyTOF workflow transforms the markers intensities using an arcsinh (inverse hyperbolic sine) with cofactor 5, making the distributions more symmetric. For visualization purposes only, the data is further transformed in a 0 to 1 scale, using the 1% low percentile and the 99% high percentile as boundaries. Cell clustering, using the surface markers, was performed with FlowSOM [30] and ConsensusClusterPlus [31], which are fast methods that allow high and low frequency population identification. This was followed by a manual merging of 20 metaclusters, based on the heatmap of marker characteristics across metaclusters with dendrograms and dimensionality reduction plots (tSNE and UMAP).

Prior to the differential analysis, unknown/unassigned cells to cell populations were filtered out, using the filterSCE function from the catalyst package [32].

In the CyTOF workflow, the differential analyses use the diffcyt package [33]. Various differential analyses were performed in the different categories: (a) controls against MD, (b) individuals with low cytokines (LC) against individuals with high cytokines (HC), (c) unstimulated against stimulated with LPS, and (d) unstimulated against stimulated with *Aspergillus niger*.

With CyTOF workflow, differential abundance[3] (DA) testing was performed using a generalized linear mixed model (GLMM). With GLMM, the response variable was the cell counts per cell type and sample. The fixed effect was defined by the condition variable (disease group, treatment, or cluster). The random effect was defined by the sample ID, to model the overdispersion in proportions. A second model was used from untreated against treated comparisons, which included a random effect defined by the patient ID to account for experiment pairing.

With CyTOF workflow, for differential state[4] (DS), the median expression of the 9 cytokines was calculated in each cell population and sample, which were used as a response variable in the linear mixed model (LMM). For absent cell populations in a sample, *NAs* were introduced. A filter to remove clusters with very low counts was applied. Markers with expression below 2 in at least one third of the samples were discarded from analysis.

In the analysis performed with CyTOF workflow, the *p*-values were corrected with a Benjamini-Hochberg adjustment using a false discovery rate (FDR) cutoff of 0.05. For DA, correction was performed for cell population and for DS it was corrected for state marker per cell population.

TreekoR was used to perform DA analysis with some modifications. FlowSOM clustering performed for the CyTOF workflow was used for TreekoR analysis. A hierarchical tree was constructed from the scaled median marker expression for each cluster, using the HOPACH method. The default value of 5 maximum children per parent node was used. For each patient, two proportions were calculated: %total and %parent. % Total refers to the proportions of cells from a cluster in each node of the tree relative to the total number of cells in the sample: (number of cells in a cluster) ÷ (number of cells in the sample). %Parent refers to the proportion of cells in each node of the tree relative to the cluster in the direct parent node of the tree (number of cells in a cluster) ÷ (number of cells in a cluster + number of cells in sibling clusters). To test if there was a significant difference between both groups, the count model EdgeR, adapted for differential abundance was used for each node in the hierarchical tree on the clusters, using %total and %parent. The cell proportions per sample were used for graphical representation of the differences of %total between groups.

Analyses were performed under R version 4.1.2.

[3] DA - Differential abundance

[4] DS – Differential state

2.5. Patient clustering

Mclust [34], a Gaussian Mixture modelling was used to obtain model-based clusters of cytokine expression. This package provides mclustBIC and Mclust functions to create clusters and find the one with the best BIC score. The decision regarding the number of clusters was based on the Bayesian Information criteria. The best model found was an EVE model (ellipsoidal, equal volume and orientation), the means and variance of the clusters can be seen in Supplementary Table 3.

2.6. Sandwich-ELISA

Frozen supernatant samples were thawed immediately prior to analysis. IgE was measured using the commercially available IgE Human Uncoated ELISA Kit with Plates (ThermoFisher, # 88–50,610-22), following the kit-specific protocols provided by the manufacturer. The absorbance was measured at 450 nm with a 570 nm correction, using the infinite m200 Nanoquant (Tecan).

2.7. Statistical analysis and visualizations

Clinical data were analyzed by R, using the stats package [35]. We applied a Fisher exact test or *t*-test for quantitative variables and Wilcoxon test for qualitative variables. Two-way repeated measure ANOVA tests were performed on Sandwich-ELISA data, followed by paired *t*-tests using RStudio. Mann-Whitney *U* tests were performed on granulocyte to lymphocyte ratio data using the stats package [35]. *P*-values below 0.05 were considered significant.

The following R packages were used for the visualizations of mass cytometry results: cytofWorkflow [28], tidyr [36], ggprism [37], ggplot2 [38], ggpubr [39] and plotrix [40].

3. Results

3.1. There is a difference in abundance of granulocytes between unstimulated Meniere Disease patients and controls

Mass cytometry was performed on whole blood from 26 MD patients and 13 controls (Supplementary Table 4). After quality control, we analyzed 24 MD and 11 healthy control unstimulated samples, which allowed us to identify 13 cell populations by manual gating of unsupervised clustering identified according to the marker expression (Fig. 1A-D).

The CyTOF workflow allows testing for differences in the abundance of the defined cell populations - differential abundance (DA) – and on the differences on the marker expression within each cluster – differential state (DS). With CyTOF workflow, no differences were observed in either DA or DS, when comparing unstimulated samples of MD against controls.

TreekoR package evaluates not only the total DA of a given cluster or cell population, but also the difference in proportion to the previous node [29]. TreekoR analysis showed differences in the %parent between 3 nodes/parents, which corresponded to $CD4^+$ T-cells (adjusted *p*-value = 0.001) and granulocytes (adjusted p-value = 0.009). Additionally, it revealed differences in the %total in 13 nodes, of which the most significant per cell population can be observed on Fig. 1E (Supplementary Table 4).

Granulocytes, in the %total, showed 5 clusters with increased abundance and 5 clusters with decreased abundance, and in the %parent showed 4 clusters with decreased abundance in MD patients, compared to controls. In $CD4^+$ T-cells, we found a cluster with increased abundance in the %total, and 5 clusters with increased abundance and 3 with decreased abundance in the %parent.

For unstimulated samples, we observed that some cytokine values showed a frequency distribution that could represent two groups (Fig. 2).

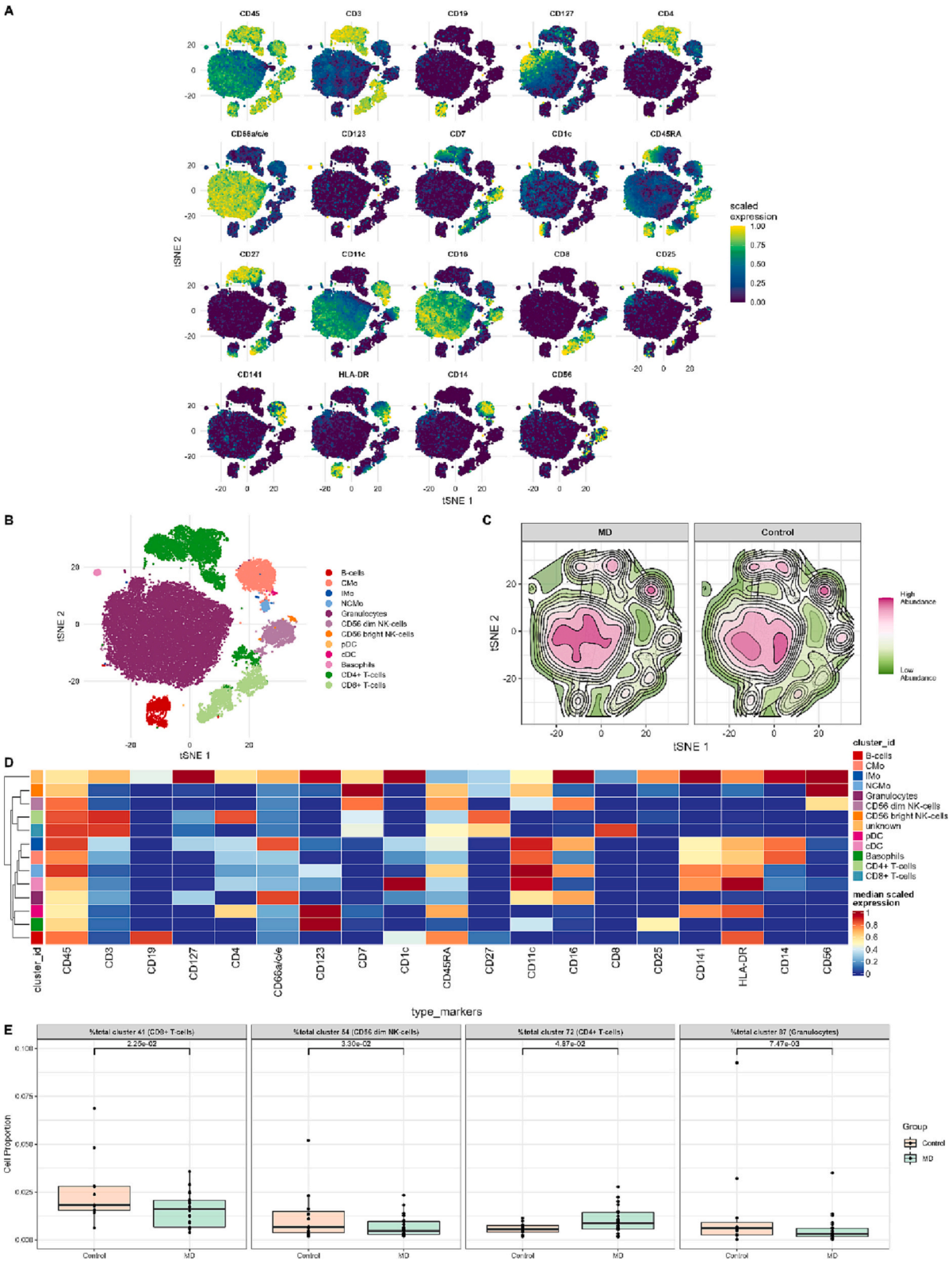

Fig. 1. – Phenotypic differences between non-stimulated Meniere disease (MD) patients compared to controls. (A) tSNE plots of the arcsinh-transformed expression of the type (extracellular) markers in a subset of 1000 cells per sample (24 MD and 11 Controls). (B) tSNE of a subset of 1000 cells per sample (24 MD and 11 Controls) colored according to the manually annotated cell clusters. (C) tSNE of a subset of 5000 cells per group, colors vary according to cell abundance density. (D) Median scaled expression of the type markers across the thirteen annotated whole blood cell clusters. (E) Boxplot of the relative frequency of cells (cell proportion) in the identified clusters and corresponding manually annotated clusters. *P*-value represents differences in the %total of cells between controls and MD patients found by TreekoR analysis. CMo – classical monocytes, IMo – intermediate monocytes, NCMo – non-classical monocytes, pDC – plasmacytoid dendritic cells, cDC – classical dendritic cells.

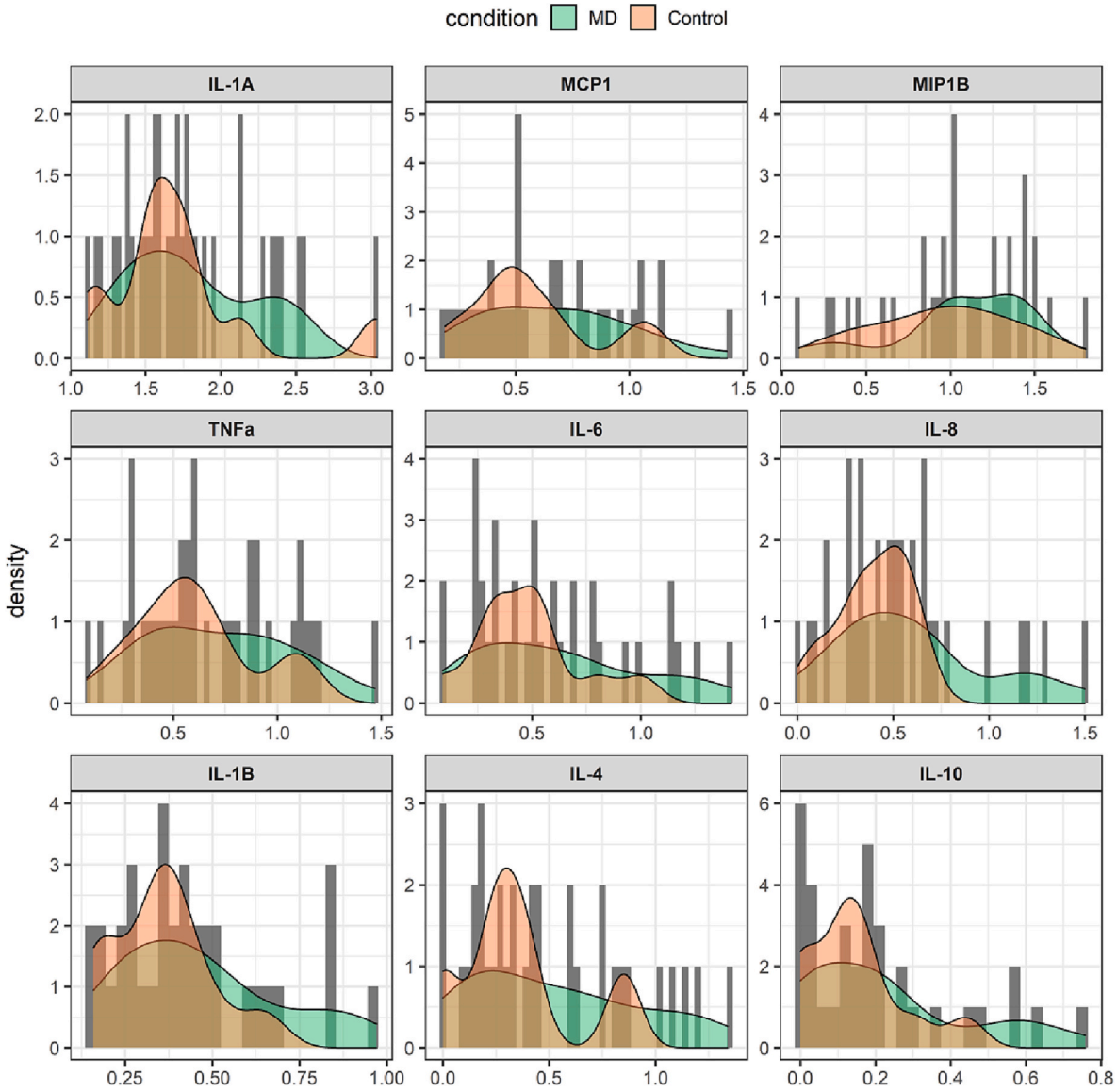

Fig. 2. Frequency histogram and density plot of the cytokine median expression of unstimulated whole blood by the individual, colored by Meniere Disease (MD) or control group.

Mclust package was used to obtain model-based clusters of cytokine expression (Fig. 2, Supplementary Fig. 1) [34]. Thus, we obtained a cluster of 25 individuals (10 controls and 15 MD) – cluster Low, and a cluster of 10 individuals (1 control and 9 MD) – cluster High. Statistical analysis of the clinical and demographic variables revealed no differences between the clusters (Supplementary Table 5). None of the patients had any known respiratory or skin allergies (Supplementary Table 1).

3.2. Clustering individuals according to their unstimulated levels of cytokines revealed differences in expression of various cytokines and cell population abundance

We compared cluster High to cluster Low, to identify what immune populations provoked the differential immune response between these individuals. When comparing these clusters with CyTOF workflow, we found significant differences in abundance of several cell populations, being $CD56^{dim}$ NK-cells the immune population where the differences were most significant (adjusted p-value = 0.0015) (Fig. 3D). It was observable that all differential abundant cell population were higher in individuals with lower levels of cytokines, except for granulocytes (Fig. 3D), which translates into higher granulocyte/lymphocyte ratios in cluster High individuals (Supplementary Table 6). These results are further supported by the analysis carried out with TreekoR, where we found differences in the %parent between 10 nodes/parents, these matched mostly to $CD56^{dim}$ NK-cells (adjusted p-value >0.04). Also, it revealed differences in the %total of 42 nodes, that corresponded mostly to granulocytes (Supplementary Table 7, Supplementary Fig. 2).

When testing for DS by CyTOF workflow, we found that there were differences in 3 cell populations, most noticeably in intermediate monocytes (Imo), which present significant differences in the production of various cytokines (adjusted p-value <0.032) (Fig. 3E).

The MD patients included in cluster Low[5] (MDL) and MD patients in cluster High[6] (MDH) were compared to the controls present in cluster Low. Differences were only found by TreekoR in 11 parent/nodes associated with granulocytes and one parent/node associated with $CD4^{+}$

[5] MDL - Meniere Disease patients included in cluster Low
[6] MDH - Meniere Disease patients included in cluster High

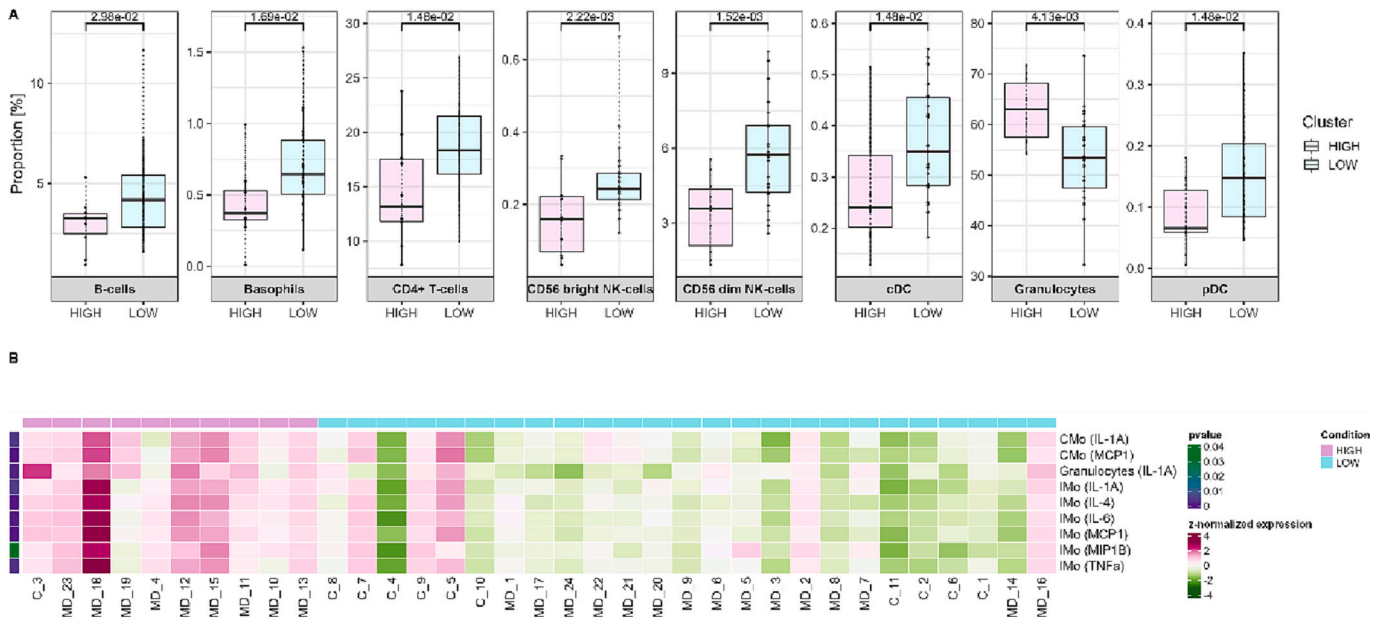

Fig. 3. Phenotypic differences between non-stimulated individuals in cluster High compared to individuals in cluster Low. (A) Boxplot of the relative frequency of cells (cell proportion) in the identified manually annotated cluster. *P*-value represents differences in abundance between Cluster Low and Cluster High, identified by CyTOF workflow. (B) Heatmap of the z-normalized expression of state (cytokine) markers in different manually annotated cell populations, the top 50 differences are represented. CMo – classical monocytes, IMo – intermediate monocytes, NCMo – non-classical monocytes, pDC – plasmacytoid dendritic cells, cDC – classical dendritic cells.

T-cells (Supplementary Table 9) when comparing MDL to the controls. Contrastingly, when comparing MDH to controls, we found differences in NK-cells by CyTOF workflow and TreekoR (Supplementary Table 8). TreekoR found additional differences in granulocytes, classical monocytes (CMo), B-cells, basophils, and T-cells (Supplementary Table 8). Furthermore, differences were found in the expression of IL-1α by granulocytes (adjusted p-value = 0.035).

The response to LPS and ASP stimulation was also compared between individuals in clusters High and Low. DS analysis found significant differences in the expression of IL-1α from granulocytes and CMo, and IL-4, IL-6 and MCP1 by CMo (Supplementary Fig. 3) after ASP stimulation. Similarly, after LPS stimulation, we observed differences in the expression of IL-4 and MCP1 by CMo and IL-1α by granulocytes (Supplementary Fig. 4).

3.3. Differences in the response to Aspergillus niger was found between cluster High and cluster Low

To investigate the role of antigenic stimulation in the proinflammatory response in MD, we cultured unstimulated peripheral blood and stimulated peripheral blood with *Aspergillus niger* allergenic extract (ASP) and with Lipopolysaccharide (LPS).

Regarding DS analysis, no differences were found after ASP nor LPS stimulation.

3.4. Cluster High individuals show increased levels of IgE in unstimulated conditions

The differences in the immune response between cluster High and cluster Low, suggest a possible allergic response, therefore we quantified IgE levels in these individuals. When comparing MD to controls, we observed a significant interaction between group and stimulation on IgE levels ($p = 1.5 \times 10^{-2}$) (Fig. 4A). unstimulated condition, cluster High showed significantly increased levels of IgE when compared to cluster

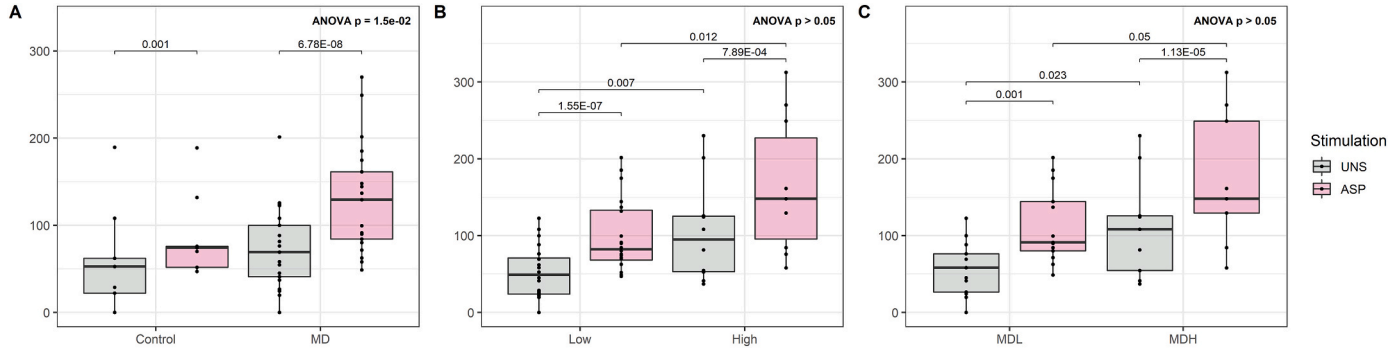

Fig. 4. IgE levels in supernatant without stimulation and after antigenic stimulation with Aspergillus niger allergenic extract. A two-way repeated measures ANOVA was performed to account for differences between groups and within stimulated samples. Paired *t*-test were performed to identify differences between groups and after stimulation. (A) IgE levels in MD ($N = 27$) and Controls ($N = 12$) without stimulation, and in MD ($N = 26$) and Controls ($N = 14$) after Aspergillus niger allergenic extract stimulation. (B) IgE levels in cluster High ($N = 10$) and cluster Low ($N = 22$) without stimulation, and in cluster High (N = 10) and cluster Low ($N = 24$) after ASP stimulation. (C) IgE levels in MDH ($N = 9$) and MDL (N = 14) without stimulation, and in MDH (N = 9) and MDL (N = 14) after Aspergillus niger allergenic extract stimulation. Use color.

Low ($p = 0.007$) (Fig. 4B), and the same was observed between MDH and MDL ($p = 0.023$) (Fig. 4C). After ASP stimulation, we found differences between cluster High and cluster Low ($p = 0.012$) (Fig. 4B).

4. Discussion

In this study, we used mass cytometry on peripheral blood from MD patients and controls to search for an immune signature related to the syndrome.

Our results illustrate that there are two clusters (endophenotypes) of patients with MD according to the cellular abundance and cytokine profile. A cluster with low levels of cytokines, composed of controls and MD patients, with a similar pattern of cell abundance and cytokine expression and a second cluster composed mostly of MD patients with high levels of cytokines that show a particular single cell profile.

Individuals in Cluster High show an increased abundance of granulocytes and lower abundance of B-cells, NK-cells, $CD4^+$ T-cells, basophils, and dendritic cells (DC) when compared to cluster Low. Regarding DS, we observed that individuals from cluster High show increased levels of cytokine expression mainly in granulocytes and monocytes, with the most pronounced differences being found in IL-4 and MCP1 expression in monocytes. MDH patients showed increased abundance of granulocytes and double negative (DN) T-cells and a lower abundance of all other cell types, except classical DC (cDC) and non-classical monocytes (NCMo), for which no differences were found, when comparing to cluster Low.. MDL patients when compared to controls revealed solely differences in some granulocyte clusters and one $CD4^+$ T-cell cluster by TreekoR. So, we confirmed that there is a cluster of patients MDL with a similar immune profile to controls and a cluster of patients MDH that show higher levels of cytokines, accompanied by increased granulocyte population. This finding confirms previous studies showing two subgroups of patients according to IL-1β and TNFα levels in cultured PBMC supernatant [6].

MD patients showed differences in the abundance of granulocytes, $CD4^+$ T-cells, $CD8^+$ T-cells and $CD56^{dim}$ NK-cells, when comparing to controls. Neutrophil to lymphocyte ratio (NLR) is a parameter that allows the evaluation the inflammatory state of an individual. Some studies on peripheral vestibular vertigo [41], namely vestibular neuritis [42], and MD [43] described a higher NLR in these patients than in controls. We attempted an approximation to this calculation through our findings, where we calculated the ratio between granulocytes (neutrophils and eosinophils) and lymphocytes, and in fact it was higher in MD than controls, despite not statistically significant, nevertheless we did observe that this value was higher in cluster High than in cluster Low. Moreover, it has been described an increased level of neutrophils and leukocytes in MD patients when compared to controls [43], in line with our findings of increased granulocytes in MD patients' unstimulated samples. So, NLR could have potential clinical value when classifying MD patients.

An early study described an increased activity of NK cells in MD [44]. Contrastingly, our results have found an increase in $CD56^{dim}$ NK-cells in controls when compared to MD patients. Likewise, we have observed that MDH patients have a reduced number of NK-cells when compared to controls, as do cluster High individuals compared to cluster Low. NK-cell reduction in peripheral blood has been also reported in autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis and type 1 diabetes [45]. As no differences were found in the clinical history or demographics of individuals in cluster High and cluster Low, which could be due to sample size, it is not possible to speculate if these differences lead to a protective or pathogenic role in the disease, as NK cells are necessary to the balance of innate and adaptive response.

A flow cytometry study with 46 quiescent MD patients and 46 controls [46] found an increase in $CD4^+$ cells and decrease in $CD8^+$ cells in MD patients. For our study, solely patients in quiescent phase were recruited, therefore the differences in our findings may be due to a higher resolution level from our experiments, as differences were not found in all clusters of $CD4^+$ and $CD8^+$ cells.

Our group has evaluated the levels of several cytokines in the supernatant of cultured PBMCs from MD patients and controls [6,19,21]. We reported increased levels of IL-8, TNFα, IL-4, IL-1α, MCP-1, and MIP1β in MD patients and IL-10, IL-1β, IL-6 in MDH patients. A study using endolymphatic sac luminal fluid (ELF) and serum from MD patients and acoustic neuroma patients found significant differences in the expression of IL-6 and TNFα in ELF, but no differences were found in the serum [47]. Moreover, increased levels of TNFα, IL-1α, IL-8, CTACK, MIP1α, MIP1β, G-CSF, HGF, IL-4 and IL-10 have been found in the serum of MD patients when compared to controls [23,24,46]. In this study, we did not identify any differences in cytokine expression between MD patients and controls. However, when comparing cluster Low (14 MD and 11 controls) to cluster High (9 MD and 1 control) we observed differences in the levels of TNFα, IL-4, IL-6, IL-1α, MCP-1, and MIP1β in granulocytes and monocytes. A recent study by Zou *et al* [24] have described the possible role of NET formation in the MD pathological process, which could be related to our findings in granulocyte abundance and cytokine expression. IL-4 has a pivotal role in type 2 immune response, such as allergic inflammation, which is majorly produced by polarized Th_2 cells, along with IL-5, Il-6, IL-13 and IgE [46]. MCP1/CCL2 is a regulator of migration and infiltration of monocytes, NK-cells and memory T-cells [48]. This chemokine has been described as a polarizing factor from Th_0 to Th_2 cell phenotype, as it can directly activate the IL-4 promotor, leading to IL-4 production [49]. Yet, it is important to mention that studies have shown that CCL2 can also promote Th_1 response depending on factors such as CCL2 induction timing, tissue site and type of pathogen [50]. Furthermore, we also observed a difference in expression of TNFα and MIP-1β/CCL4, which have been linked to type 1 immune response [51]. Nevertheless, several studies have also reported the role of TNFα [52,53], IL-6 [54], IL-1α [55], and MIP1β [56] in allergic reactions. With this in mind, we decided to determine the levels of IgE in the supernatant of unstimulated cultured whole blood. As with the previously mentioned cytokines, we observed no differences when comparing MD to controls, but observed an increase in IgE levels in cluster High when compared to cluster Low. These results suggest that patients in cluster High may have a MD phenotype of autoinflammation and type 2 immunity, as it has been described that they can modulate each other [57] (Fig. 5).

To study the role of antigenic stimulation in the proinflammatory response in MD, we stimulated peripheral blood from MD patients and controls with LPS or *Aspergillus niger* allergenic extract (m33). LPS has been used to study inflammation in MD, as it mimics many inflammatory effects of cytokines, such as TNFα, IL-1β and IL-6. When comparing MD patients to controls we identified no differences in cytokine expression to this stimulus, suggesting that they do not have a different response. Nevertheless, when comparing cluster Low to cluster High, we observed differences in the expression of various cytokines in monocytes.

Aspergillus niger has been described to induce IgE-mediate hypersensitivity in susceptible individuals [58] and is recognized by TLR2 and TLR4 [59]. Our data indicates that ASP did not produce a different response in MD and controls, as such we cannot assume that this allergen could trigger MD. However, we observed differences in the response to ASP between cluster high and cluster Low. Interestingly, we found significant differences in the production of IL-4 and MCP1/CCL2 by classical monocytes, cytokines that have been linked to a type 2 immune response. Still, through the measurement of IgE in supernatant of whole blood stimulated with ASP, we found no differences between MD and controls, and as such could not determine a sensitization to this allergen and define it as a trigger of the disease.

We found differences in cluster High and cluster Low in cytokines associated with type 2 immune response and in IgE levels, therefore it is possible that they represent an allergic and a nonallergic form of the disease. Several atopic diseases present allergic and nonallergic forms, such as dermatitis, asthma, and rhinitis, being the major difference between both types the presence of elevated total and specific IgE levels

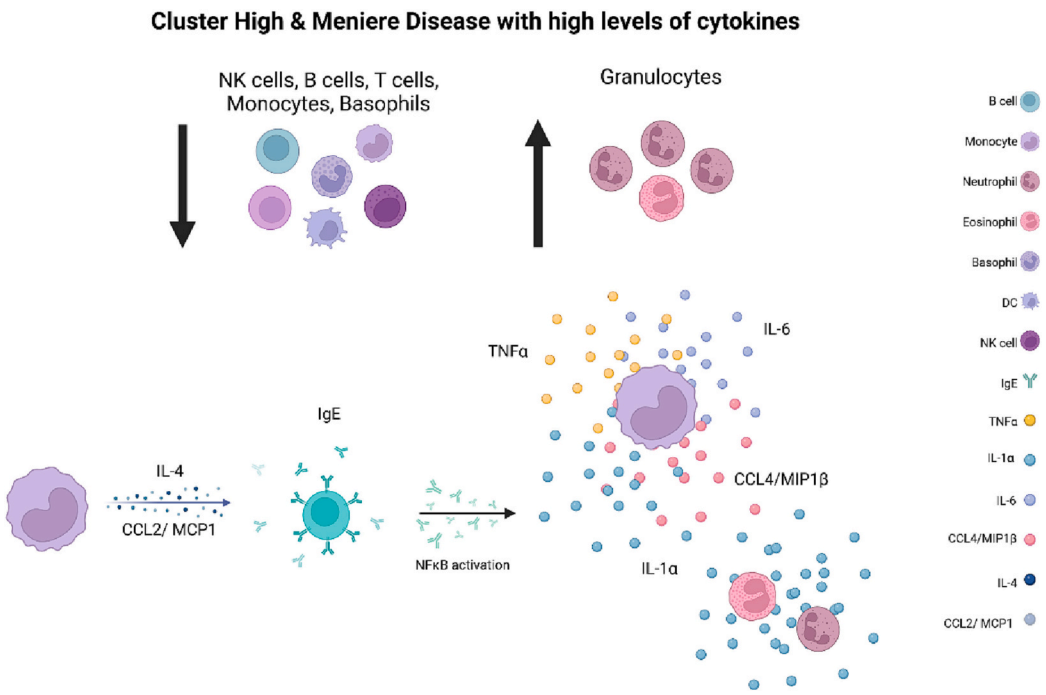

Fig. 5. IL-4 and IgE mediated inflammatory response in individuals from cluster High. Schematic representation of major findings in differential abundance analysis comparing cluster High to cluster Low: decreased abundance of NK-cells, B-cells, T-cells, monocytes and basophils, and increased abundance of granulocytes in cluster High. Schematic representation of possible mechanism in cluster High: monocyte increased production of CCL2 and IL-4, lead to an increase of IgE, which induces a proinflammatory response with increased production of TNFα, IL-6 and CCL4 from monocytes and IL-1α from monocytes and granulocytes.

[60].

A recent study described increased levels of IgE, IL-4, IL-5, IL-10, and IL-13 in serum samples from 103 MD patients compared to 72 controls [23]. They also reported 27.2% MD patients with high basal levels of IgE when compared to controls. However, no significant differences were found regarding sex, age, and other clinical features, when comparing MD patients with high levels of IgE to patients with low levels of IgE, in line with our findings [23]. Further corroborating our results, they also found increased levels of IL-4 in patients with high levels of IgE, but no differences in IL-10 between the two groups [23]. So it is possible that elevated IL-4 leads to IgE deposition in MD, causing proinflammatory cytokines induction, resulting in local inflammation [23]. Additionally, we identified no subjects with diagnosed autoimmune diseases in cluster High, suggesting that these patients do not have an autoimmune background, but rather an autoinflammatory or allergic background. Taking this into account, patients from cluster High could possibly benefit from treatment with IL-4 blockers, such as dupilumab, which has been described as effective treatment for diseases such as severe asthma, chronic rhinosinusitis, and allergic dermatitis [61].

The major limitations of our study are the sample size and the window of time in which samples were collected. Blood samples should be collected in a tighter timeframe and from a higher number of patients to validate our findings.

In conclusion, patients with MD include at least two endophenotypes, according to the distribution of the immune cell subpopulations and cytokine profile. We identified a subgroup of patients that seems to have a type 2 immune response involving IgE and IL4 leading to persistent inflammatory status. Thus, the levels of IgE and cytokines should be measured and tracked in patients with sporadic MD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2023.109632.

Funding

This work was supported by B-CTS-68-UGR20 Grant by FEDER Funds, PI17/1644 and PI20-1126 grants from ISCIII by FEDER Funds from the EU, CLINMON-2 from the Meniere's Society UK, and Impact Data Science (IMP0001). MF is funded by F18/00228 grant from ISCIII by FEDER Funds from the EU. AEB is funded by the EU's Horizon 2020 Research and Innovation Programme, Grant Agreement Number 848261. LF is funded by CD20/0153 grant from ISCIII by FEDER Funds from the EU. Funding for open access charge: Universidad de Granada / CBUA.

Authors' contributions

Conceptualization – JALE.
Data curation – MF.
Formal Analysis – MF.
Funding acquisition – JALE, LF, MF.
Investigation – MF.
Methodology – MF, AEB, PR.
Project administration – JALE.
Resources – ABC, JCAM, ASV.
Software – MF, AEB, PR.
Supervision – JALE, LF, MAR.
Visualization – MF.
Writing – original draft – MF. JALE
Writing – review & editing – JALE, PR, AEB, LF, ABC, JCAM, ASV, MAR.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

The datasets generated produced in this study are available in the following database: -
Mass Cytometry: FlowRepository, FR-FCM-Z5R8

Acknowledgements

The authors would like to acknowledge the technical support from Olivia Santiago and Jose Diaz Cuellar in the Cytometry Core facility of Genyo research center.

References

[1] J.A. Lopez-Escamez, J. Carey, W.-H. Chung, J.A. Goebel, M. Magnusson, M. Mandalà, D.E. Newman-Toker, M. Strupp, M. Suzuki, F. Trabalzini, A. Bisdorff, Diagnostic criteria for Menière's disease, J. Vestib. Res. 25 (2015) 1–7, https://doi.org/10.3233/VES-150549.

[2] A. Gallego-Martinez, J.A. Lopez-Escamez, Genetic architecture of Meniere's disease, Hear. Res. 397 (2020), 107872, https://doi.org/10.1016/j.heares.2019.107872.

[3] L. Frejo, A. Soto-Varela, S. Santos-Perez, I. Aran, A. Batuecas-Caletrio, V. Perez-Guillen, H. Perez-Garrigues, J. Fraile, E. Martin-Sanz, M.C. Tapia, G. Trinidad, A. M. García-Arumi, R. González-Aguado, J.M. Espinosa-Sanchez, P. Marques, P. Perez, J. Benitez, J.A. Lopez-Escamez, Clinical subgroups in bilateral Meniere Disease, Front. Neurol. 7 (2016) 182, https://doi.org/10.3389/fneur.2016.00182.

[4] L. Frejo, E. Martin-Sanz, R. Teggi, G. Trinidad, A. Soto-Varela, S. Santos-Perez, R. Manrique, N. Perez, I. Aran, M.S. Almeida-Branco, A. Batuecas-Caletrio, J. Fraile, J.M. Espinosa-Sanchez, V. Perez-Guillen, H. Perez-Garrigues, M. Oliva-Dominguez, O. Aleman, J. Benitez, P. Perez, J.A. Lopez-Escamez, Extended phenotype and clinical subgroups in unilateral Meniere disease: A cross-sectional study with cluster analysis, Clin. Otolaryngol. 42 (2017) 1172–1180, https://doi.org/10.1111/coa.12844.

[5] D. Bächinger, C. Brühlmann, T. Honegger, E. Michalopoulou, A. Monge Naldi, V. G. Wettstein, S. Muff, B. Schuknecht, A.H. Eckhard, Endotype-phenotype patterns in Meniere's Disease based on gadolinium-enhanced MRI of the vestibular aqueduct, Front. Neurol. 10 (2019) 303, https://doi.org/10.3389/fneur.2019.00303.

[6] L. Frejo, A. Gallego-Martinez, T. Requena, E. Martin-Sanz, J.C. Amor-Dorado, A. Soto-Varela, S. Santos-Perez, J.M. Espinosa-Sanchez, A. Batuecas-Caletrio, I. Aran, J. Fraile, M. Rossi-Izquierdo, J.A. Lopez-Escamez, Proinflammatory cytokines and response to molds in mononuclear cells of patients with Meniere disease, Sci. Rep. 8 (2018) 5974, https://doi.org/10.1038/s41598-018-23911-4.

[7] C.S. Hallpike, H. Cairns, Observations on the pathology of Ménière's syndrome, J. Laryngol. Otol. 53 (1938) 625–655, https://doi.org/10.1017/S0022215100003947.

[8] I. Pyykkö, J. Zou, D. Poe, T. Nakashima, S. Naganawa, Magnetic resonance imaging of the inner ear in Meniere's disease, Otolaryngol. Clin. N. Am. 43 (2010) 1059–1080, https://doi.org/10.1016/j.otc.2010.06.001.

[9] L. Frejo, J.A. Lopez-Escamez, Cytokines and inflammation in Meniere disease, Clin. Exp. Otorhinolaryngol. 15 (2022) 49–59, https://doi.org/10.21053/ceo.2021.00920.

[10] I. Gazquez, A. Soto-Varela, I. Aran, S. Santos, A. Batuecas, G. Trinidad, H. Perez-Garrigues, C. Gonzalez-Oller, L. Acosta, J.A. Lopez-Escamez, High prevalence of systemic autoimmune diseases in patients with Menière's disease, PLoS One 6 (2011), e26759, https://doi.org/10.1371/journal.pone.0026759.

[11] H.J. Hahn, S.G. Kwak, D.-K. Kim, J.-Y. Kim, A. Nationwide, Population-based cohort study on potential autoimmune association of Ménière Disease to atopy and vitiligo, Sci. Rep. 9 (2019) 4406, https://doi.org/10.1038/s41598-019-40658-8.

[12] S.Y. Kim, Y.S. Song, J.H. Wee, C. Min, D.M. Yoo, H.G. Choi, Association between Ménière's disease and thyroid diseases: a nested case-control study, Sci. Rep. 10 (2020) 18224, https://doi.org/10.1038/s41598-020-75404-y.

[13] J.S. Tyrrell, D.J.D. Whinney, O.C. Ukoumunne, L.E. Fleming, N.J. Osborne, Prevalence, associated factors, and comorbid conditions for Ménière's disease, Ear Hear. 35 (2014) e162–e169, https://doi.org/10.1097/AUD.0000000000000041.

[14] S. Cabrera, E. Sanchez, T. Requena, M. Martinez-Bueno, J. Benitez, N. Perez, G. Trinidad, A. Soto-Varela, S. Santos-Perez, E. Martin-Sanz, J. Fraile, P. Perez, M. E. Alarcon-Riquelme, A. Batuecas, J.M. Espinosa-Sanchez, I. Aran, J.A. Lopez-Escamez, Intronic variants in the NFKB1 gene may influence hearing forecast in patients with unilateral sensorineural hearing loss in Meniere's disease, PLoS One 9 (2014), e112171, https://doi.org/10.1371/journal.pone.0112171.

[15] I. Gazquez, A. Moreno, I. Aran, A. Soto-Varela, S. Santos, H. Perez-Garrigues, A. Lopez-Nevot, T. Requena, M.A. Lopez-Nevot, J.A. Lopez-Escamez, MICA-STR A.4 is associated with slower hearing loss progression in patients with Ménière's disease, Otol. Neurotol. 33 (2012) 223–229, https://doi.org/10.1097/MAO.0b013e31824296c8.

[16] T. Requena, I. Gazquez, A. Moreno, A. Batuecas, I. Aran, A. Soto-Varela, S. Santos-Perez, N. Perez, H. Perez-Garrigues, A. Lopez-Nevot, E. Martin, R. Sanz, P. Perez, G. Trinidad, M.E. Alarcon-Riquelme, R. Teggi, L. Zagato, M.A. Lopez-Nevot, J. A. Lopez-Escamez, Allelic variants in TLR10 gene may influence bilateral affectation and clinical course of Meniere's disease, Immunogenetics. 65 (2013) 345–355, https://doi.org/10.1007/s00251-013-0683-z.

[17] J. Zou, Autoinflammatory characteristics and short-term effects of delivering high-dose steroids to the surface of the intact endolymphatic sac and incus in refractory Ménière's disease, J. Otolaryngol. 14 (2019) 40–50, https://doi.org/10.1016/j.joto.2019.01.001.

[18] L. Frejo, T. Requena, S. Okawa, A. Gallego-Martinez, M. Martinez-Bueno, I. Aran, A. Batuecas-Caletrio, J. Benitez-Rosario, J.M. Espinosa-Sanchez, J.J. Fraile-Rodrigo, A.M. García-Arumi, R. González-Aguado, P. Marques, E. Martin-Sanz, N. Perez-Fernandez, P. Pérez-Vázquez, H. Perez-Garrigues, S. Santos-Perez, A. Soto-Varela, M.C. Tapia, G. Trinidad-Ruiz, A. del Sol, M.E. Alarcon Riquelme, J. A. Lopez-Escamez, Regulation of Fn14 receptor and NF-κB underlies inflammation in Meniere's disease, Front. Immunol. 8 (2017) 1739, https://doi.org/10.3389/fimmu.2017.01739.

[19] M. Flook, L. Frejo, A. Gallego-Martinez, E. Martin-Sanz, M. Rossi-Izquierdo, J. C. Amor-Dorado, A. Soto-Varela, S. Santos-Perez, A. Batuecas-Caletrio, J. M. Espinosa-Sanchez, P. Pérez-Carpena, M. Martinez-Martinez, I. Aran, J.A. Lopez-Escamez, Differential proinflammatory signature in vestibular migraine and Meniere disease, Front. Immunol. 10 (2019) 1229, https://doi.org/10.3389/fimmu.2019.01229.

[20] Y. Ma, Q. Sun, K. Zhang, L. Bai, L. Du, High level of IgE in acute low-tone sensorineural hearing loss: A predictor for recurrence and Meniere Disease transformation, Am. J. Otolaryngol. 42 (2021), 102856, https://doi.org/10.1016/j.amjoto.2020.102856.

[21] M.-D.-C. Moleon, E. Martinez-Gomez, M. Flook, A. Peralta-Leal, J.A. Gallego, H. Sanchez-Gomez, M.A. Montilla-Ibañez, E. Dominguez-Durán, A. Soto-Varela, I. Aran, L. Frejo, J.A. Lopez-Escamez, Clinical and cytokine profile in patients with early and late onset Meniere Disease, J. Clin. Med. 10 (2021) 4052, https://doi.org/10.3390/jcm10184052.

[22] M. Roomiani, F. Dehghani Firouzabadi, A.-A. Delbandi, B. Ghalehbaghi, A. Daneshi, N. Yazdani, B. Fazeli Delshad, A. Asghari, Evaluation of serum immunoreactivity to common indigenous iranian inhalation and food allergens in patients with Meniere's Disease, Null (2021) 1–10, https://doi.org/10.1080/08820139.2020.1869252.

[23] N. Zhang, Y. Lyu, J. Guo, J. Liu, Y. Song, Z. Fan, X. Li, N. Li, D. Zhang, H. Wang, Bidirectional transport of IgE by CD23 in the inner ear of patients with Meniere's disease, J. Immunol. 208 (2022) 827–838, https://doi.org/10.4049/jimmunol.2100745.

[24] J. Zou, Z. Zhao, X. Song, G. Zhang, H. Li, Q. Zhang, I. Pyykkö, Elevated G-CSF, IL8, and HGF in patients with definite Meniere's disease may indicate the role of NET formation in triggering autoimmunity and autoinflammation, Sci. Rep. 12 (2022) 16309, https://doi.org/10.1038/s41598-022-20774-8.

[25] World Medical Association, World medical association declaration of Helsinki: ethical principles for medical research involving human subjects, JAMA. 310 (2013) 2191–2194, https://doi.org/10.1001/jama.2013.281053.

[26] P. Rybakowska, S. Van Gassen, K. Quintelier, Y. Saeys, M.E. Alarcón-Riquelme, C. Marañón, Data processing workflow for large-scale immune monitoring studies by mass cytometry, Comput Struct, Biotechnol. J. 19 (2021) 3160–3175, https://doi.org/10.1016/j.csbj.2021.05.032.

[27] P. Rybakowska, M.E. Alarcón-Riquelme, C. Marañón, Key steps and methods in the experimental design and data analysis of highly multi-parametric flow and mass cytometry, Comp. Struct. Biotechnol. J. 18 (2020) 874–886, https://doi.org/10.1016/j.csbj.2020.03.024.

[28] M. Nowicka, C. Krieg, H. Crowell, L. Weber, F. Hartmann, S. Guglietta, B. Becher, M. Levesque, M. Robinson, CyTOF workflow: differential discovery in high-throughput high-dimensional cytometry datasets [version 4; peer review: 2 approved], F1000Research. 6 (2019), https://doi.org/10.12688/f1000research.11622.4.

[29] A. Chan, W. Jiang, E. Blyth, J. Yang, E. Patrick, treekoR: identifying cellular-to-phenotype associations by elucidating hierarchical relationships in high-dimensional cytometry data, Genome Biol. 22 (2021) 324, https://doi.org/10.1186/s13059-021-02526-5.

[30] S. Van Gassen, B. Callebaut, M.J. Van Helden, B.N. Lambrecht, P. Demeester, T. Dhaene, Y. Saeys, FlowSOM: using self-organizing maps for visualization and interpretation of cytometry data, Cytometry Part A. 87 (2015) 636–645, https://doi.org/10.1002/cyto.a.22625.

[31] M.D. Wilkerson, D.N. Hayes, ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking, Bioinformatics. 26 (2010) 1572–1573, https://doi.org/10.1093/bioinformatics/btq170.

[32] H. Crowell, V. Zanotelli, S. Chevrier, M. Robinson, CATALYST: Cytometry dATa anALYSis Tools. https://github.com/HelenaLC/CATALYST, 2022.

[33] L.M. Weber, M. Nowicka, C. Soneson, M.D. Robinson, Diffcyt: differential discovery in high-dimensional cytometry via high-resolution clustering, Commun. Biol. 2 (2019) 1–11, https://doi.org/10.1038/s42003-019-0415-5.

[34] L. Scrucca, M. Fop, T.B. Murphy, A.E. Raftery, mclust 5: clustering, Classification and Density Estimation Using Gaussian Finite Mixture Models, R J 8, 2016, pp. 289–317.

[35] R: A Language and Environment for Statistical Computing. https://www.R-project.org/, 2021.

[36] H. Wickham, M. Girlich, tidyr: Tidy Messy Data. https://github.com/tidyverse/tidyr, 2022.

[37] C. Dawson, ggprism. https://github.com/csdaw/ggprism, 2021.

[38] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag, New York, 2016. https://ggplot2.tidyverse.org.

[39] A. Kassambara, ggpubr: "ggplot2" Based Publication Ready Plots. https://CRAN.R-project.org/package=ggpubr, 2020.

[40] J. Lemon, Plotrix: A package in the red light district of R, R-News 6, 2006, pp. 8–12.

[41] A.K. Bintang, E.I. Magasingan, Severity-dependent variations of the neutrophil to lymphocyte ratio (NLR) in peripheral vestibular vertigo, Egypt. J. Neurol. Psychiatry Neurosurg. 57 (2021) 169, https://doi.org/10.1186/s41983-021-00425-x.

[42] J.H. Chung, J. Lim, J.H. Jeong, K.R. Kim, C.W. Park, S.H. Lee, The significance of neutrophil to lymphocyte ratio and platelet to lymphocyte ratio in vestibular neuritis, Laryngoscope 125 (2015) E257–E261, https://doi.org/10.1002/lary.25204.

[43] N.A.A. Quaranta, A.E. Salzo, V. Pontillo, E. Scarano, B. Sergi, P.M. Picciotti, Neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) in Ménière's disease and vestibular neuritis, Hear. Balance Commun. 19 (2021) 235–239, https://doi.org/10.1080/21695717.2021.1943779.

[44] T. Fuse, T. Hayashi, N. Oota, S. Fukase, S. Asano, T. Kato, M. Aoyagi, Immunological responses in acute low-tone sensorineural hearing loss and Ménière's disease, Acta Otolaryngol. 123 (2003) 26–31, https://doi.org/10.1080/0036554021000028074.

[45] U.C. Kucuksezer, E. Aktas Cetin, F. Esen, I. Tahrali, N. Akdeniz, M.Y. Gelmez, G. Deniz, The role of natural killer cells in autoimmune diseases, Front. Immunol. 12 (2021), https://doi.org/10.3389/fimmu.2021.622306 (accessed June 30, 2022).
[46] E. Keles, A. Gödekmerdan, T. Kalidağ, I. Kaygusuz, S. Yalçin, H. Cengiz Alpay, M. Aral, Meniere's disease and allergy: allergens and cytokines, J. Laryngol. Otol. 118 (2004) 688–693, https://doi.org/10.1258/0022215042244822.
[47] C. Huang, Q. Wang, X. Pan, W. Li, W. Liu, W. Jiang, L. Huang, A. Peng, Z. Zhang, Up-regulated expression of interferon-gamma, interleukin-6 and tumor necrosis factor-alpha in the endolymphatic sac of Meniere's disease suggesting the local inflammatory response underlies the mechanism of this disease, Front. Neurol. 13 (2022), 781031, https://doi.org/10.3389/fneur.2022.781031.
[48] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant Protein-1 (MCP-1): an overview, J. Interf. Cytokine Res. 29 (2009) 313–326, https://doi.org/10.1089/jir.2008.0027.
[49] S. Singh, D. Anshita, V. Ravichandiran, MCP-1: function, regulation, and involvement in disease, Int. Immunopharmacol. 101 (2021), 107598, https://doi.org/10.1016/j.intimp.2021.107598.
[50] T.R. Traynor, A.C. Herring, M.E. Dorf, W.A. Kuziel, G.B. Toews, G.B. Huffnagle, Differential roles of CC chemokine ligand 2/monocyte chemotactic protein-1 and CCR2 in the development of T1 immunity, J. Immunol. 168 (2002) 4659–4666, https://doi.org/10.4049/jimmunol.168.9.4659.
[51] F. Annunziato, C. Romagnani, S. Romagnani, The 3 major types of innate and adaptive cell-mediated effector immunity, J. Allergy Clin. Immunol. 135 (2015) 626–635, https://doi.org/10.1016/j.jaci.2014.11.001.
[52] L.Y. Liu, M.E. Bates, N.N. Jarjour, W.W. Busse, P.J. Bertics, E.A.B. Kelly, Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha, J. Immunol. 179 (2007) 4840–4848, https://doi.org/10.4049/jimmunol.179.7.4840.
[53] G.S. Whitehead, S.Y. Thomas, K.H. Shalaby, K. Nakano, T.P. Moran, J.M. Ward, G. P. Flake, H. Nakano, D.N. Cook, TNF is required for TLR ligand-mediated but not protease-mediated allergic airway inflammation, J. Clin. Invest. 127 (2017) 3313–3326, https://doi.org/10.1172/JCI90890.
[54] A. Doganci, K. Sauer, R. Karwot, S. Finotto, Pathological role of IL-6 in the experimental allergic bronchial asthma in mice, Clin. Rev. Allergy Immunol. 28 (2005) 257–270, https://doi.org/10.1385/CRIAI:28:3:257.
[55] M.A.M. Willart, K. Deswarte, P. Pouliot, H. Braun, R. Beyaert, B.N. Lambrecht, H. Hammad, Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33, J. Exp. Med. 209 (2012) 1505–1517, https://doi.org/10.1084/jem.20112691.
[56] Y. Kobayashi, H.H. Chu, A. Kanda, Y. Yun, M. Shimono, L.M. Nguyen, A. Mitani, K. Suzuki, M. Asako, H. Iwai, CCL4 functions as a biomarker of type 2 airway inflammation, Biomedicines. 10 (2022) 1779, https://doi.org/10.3390/biomedicines10081779.
[57] M. Sylvester, A. Son, D.M. Schwartz, The interactions between autoinflammation and type 2 immunity: from mechanistic studies to epidemiologic associations, Front. Immunol. 13 (2022), 818039, https://doi.org/10.3389/fimmu.2022.818039.
[58] W.-R. Lin, Y.-H. Chen, M.-F. Lee, L.-Y. Hsu, C.-J. Tien, F.-M. Shih, S.-C. Hsiao, P.-H. Wang, Does spore count matter in fungal allergy?: the role of allergenic fungal species, allergy asthma, Immunol. Res. 8 (2016) 404–411, https://doi.org/10.4168/aair.2016.8.5.404.
[59] A. Meier, C.J. Kirschning, T. Nikolaus, H. Wagner, J. Heesemann, F. Ebel, Toll-like receptor (TLR) 2 and TLR4 are essential for aspergillus-induced activation of murine macrophages, Cell. Microbiol. 5 (2003) 561–570, https://doi.org/10.1046/j.1462-5822.2003.00301.x.
[60] N. Novak, T. Bieber, Allergic and nonallergic forms of atopic diseases, J. Allergy Clin. Immunol. 112 (2003) 252–262, https://doi.org/10.1067/mai.2003.1595.
[61] H. Morita, K. Matsumoto, H. Saito, Review of biologics in allergy and immunology, J. Allergy Clin. Immunol. (2022), https://doi.org/10.1016/j.jaci.2022.08.009.