Single cell profiling reveals a reduction of CD56dim NK-cells and other immune cells in patients with Meniere disease and high levels of cytokines

Marisa Flook (marisa.flook@genyo.es)  
Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research  
https://orcid.org/0000-0002-2695-0739

Alba Escalera-Balsera  
GENYO: Centro Pfizer - Universidad de Granada - Junta de Andalucia de Genomica e Investigacion Oncologica

Paulina Rybakowska  
GENYO: Centro Pfizer - Universidad de Granada - Junta de Andalucia de Genomica e Investigacion Oncologica

Lidia Frejo  
GENYO: Centro Pfizer - Universidad de Granada - Junta de Andalucia de Genomica e Investigacion Oncologica

Angel Batuecas-Caletrio  
Universidad de Salamanca

Juan Carlos Amor-Dorado  
Hospital Can Misses

Andres Soto-Varela  
Universidade de Santiago de Compostela

Marta Alarcón-Riquelme  
GENYO: Centro Pfizer - Universidad de Granada - Junta de Andalucia de Genomica e Investigacion Oncologica

Jose Antonio Lopez-Escamez  
GENYO: Centro Pfizer - Universidad de Granada - Junta de Andalucia de Genomica e Investigacion Oncologica  
https://orcid.org/0000-0002-8583-1430

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Abstract

Background

Meniere Disease (MD) is an inner ear syndrome, characterized by sensorineural hearing loss associated with episodes of vertigo, tinnitus, and aural fullness. The pathological mechanism leading to sporadic MD is still poorly understood, however an inflammatory response seems to be involved in some patients with MD.

Methods

We performed mass cytometry immunoprofiling on peripheral blood from MD patients and controls to decipher an immune signature associated with the syndrome.

Results

We have identified two clusters of individuals according to the single cell cytokine profile. These clusters presented differences in immune cell population abundance, including a reduction of CD56\textsuperscript{dim} NK-cells, and changes in cytokine expression with a different response to bacterial and fungal antigens. Moreover, we observe a difference in the levels of IgE between these two clusters.

Conclusions

Our results support a systemic inflammatory response in some MD patients that show an allergic phenotype, which could benefit from personalized IL-4 blockers. Immunoprofiling of patients with MD may lead to a better understanding of endotypes of the disease.

Background

Meniere Disease (MD, MIM 156000) is a rare and progressive inner ear syndrome, characterized 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 (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 autoinflammatory diseases, namely rheumatoid arthritis, autoimmune thyroid disease, 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). 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 (17).

On the other hand, there are several reports of an over-expression of cytokines and IgE in MD patients (6, 18–22). 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 (19). 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 (22). 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, it was described that antigenic stimulation with allergenic mold extracts from Aspergillus and Penicillium provoked a significantly increased production of IL-1β, TNFα, IL-6 and IL-1RA in MD patients. Still, it is not known if these triggers are specific for MD or if any bacterial or viral antigen may also trigger an abnormal immune response in 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.

Methods

Subject recruitment

This work was carried out according to the principles of the Declaration of Helsinki (23) revised in 2013 for investigation with humans and following the ethical standards recognized by the Spanish biomedical research law. The Institutional Review Board in all participating medical centers approved the experimental protocol of this study 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. Over-aged individuals 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. Patients who suffered from another associated otological disease, such as paroxysmal positional vertigo, vestibular neuritis, vestibular schwannoma, or any other cause that could mimic MD or 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 5.

Blood sampling and stimulation

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

Whole blood was cultured under three conditions: unstimulated, stimulated with lipopolysaccharide (LPS) (InvivoGen, #tlrl-peklps), and stimulated with allergenic extract from Aspergillus niger (ASP) (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 6h at 37°C, 7% CO₂ 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, #PROT1-1L).

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

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. A list of antibodies and corresponding metal tags can be seen in Table 1.

**Table 1 - Mass Cytometry panel.**
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<th>Lanthanide</th>
<th>Marker</th>
<th>Clone</th>
<th>Provider</th>
<th>Reference</th>
<th>RRID</th>
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<td></td>
<td>Sigma</td>
<td>479306</td>
<td>-</td>
<td>live/dead</td>
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The panel was designed to phenotypically characterize blood cell populations, including markers for lineage, function, differentiation, and cytokines. Antibodies were divided in extra- and intracellular staining cocktails. Monoclonal antibodies that were not provided by Standard BioTools Inc. were in-house conjugated with the corresponding metal. BC- barcoding; IC – intracellular; EX – extracellular; cis-Pt - cis-Diamineplatinum(II)dichloride

Cells were stained and acquired by CyTOF as previously described (24). 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,
Cell concentration was determined, and $1.5 \times 10^6$ 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 (table 4) 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 (table 4) 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 1h 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 MilliQ water, cells were diluted to $5 \times 10^5$ cells/mL in MilliQ 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/second using a CyTOF 2 Helios upgraded mass cytometer (Standard BioTools Inc.). Machine tuning was performed during start-up and after 5 hours. 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.

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 (25). 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 (26) and TreekoR (27) R packages, which perform dimensionality reduction and unsupervised clustering, manual 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 (28) and ConsensusClusterPlus (29), 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 (30).

In the CyTOF workflow, the differential analyses use the diffcyt package (31). Various differential analyses were performed in the different categories – controls against cases and reference against treatment: (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, 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 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.

Patient clustering

Mclust (32), a Gaussian Mixture modelling was used to obtain model-based clusters of cytokine expression. The decision regarding the number of clusters was based on the Bayesian Information criteria.

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, # 88-50610-22), 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).

Statistical analysis and visualizations

Clinical data were analysed by R, using the stats package (33). We applied a Fisher exact test for quantitative variables and Wilcoxon test for qualitative variables. Mann-Whitney U tests were performed on Sandwich-ELISA data using GraphPad Prism version 5.00 for Windows. Mann-Whitney U tests were performed on granulocyte to lymphocyte ratio data using the stats package (33). P-values below 0.05 were considered significant.
The following R packages were used for the visualizations of mass cytometry results: cytofWorkflow (26), tidyr (34), ggprism (35), ggplot2 (36), ggpubr (37) and plotrix (38).

**Results**

There is a difference in abundance of granulocytes between Meniere Disease patients and controls at basal levels

Manual gating of unsupervised clustering identified 13 cell populations according to the marker expression, of which a population with positive staining for most type (lineage) markers, labeled as unknown was excluded (Fig. 1A-C).

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). No differences were observed in either DA or DS, when comparing unstimulated samples of MD against controls at baseline.

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 (27). 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. 1D (additional file 1).

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.

At basal levels, we observed that some of the cytokine values showed a frequency distribution that could represent two groups (Fig. 2), which would be individuals with high and low basal levels of cytokines, respectively.

Mclust package was used to obtain model-based clusters of cytokine expression (32). The decision regarding the number of clusters was based on the Bayesian Information Criteria (additional file 2). 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 (Table 2). None of the patients had any known respiratory or skin allergies, namely only one patient from cluster High had a known allergy to pregabalin.
Table 2
Clinical and demographic variables assessed in individuals from cluster High and individuals from cluster Low.

<table>
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<th>Variable</th>
<th>Cluster High</th>
<th>Cluster Low</th>
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<td>46.00</td>
<td>1.0000</td>
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Cluster High & Cluster Low & p-value

| Response to corticosteroids (%yes) | 43.00 | 40.00 | 1.0000 |

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

Manual gating identified 13 cell populations. A population with positive staining for most type (lineage) markers, labelled as unknown was excluded (Fig. 3A-B).

When comparing cluster High to cluster Low with CyTOF workflow, we found significant differences in abundance of several cell populations, being CD56\textsuperscript{dim} NK-cells the immune population where the differences were the most significant (adjusted p-value = 0.0015) (Fig. 3C). It was observable that of the cell population with differential abundance, all were higher in individuals with lower levels of cytokines, except for granulocytes (Fig. 3C), which translates into higher granulocyte/lymphocyte ratios in cluster High individuals (additional file 3). These results are further supported by the analysis carried out with TreekoR, where we found differences in the %parent between 10 nodes/parents, of which 5 corresponded to tree tips, these matched mostly to CD56\textsuperscript{dim} NK-cells (adjusted p-value > 0.04). Also, it revealed differences in the %total of 42 nodes, of which 20 correspond to tree tips, that corresponded mostly to granulocytes (additional file 4).

When testing for DS by cell subpopulation 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 IL-1\textalpha, IL-4, IL-6, MCP1, MIP-1\beta and TNF\alpha (adjusted p-value < 0.032) (Fig. 3D).

The MD patients included in cluster Low (MDL) and MD patients in cluster High (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\textsuperscript{+} T-cells (additional file 5) when comparing MDL to the controls. Contrastingly, when comparing MDH to controls, we found differences in NK-cells by CyTOF workflow and TreekoR (additional file 6). TreekoR found additional differences in granulocytes, classical monocytes (CMo), B-cells, basophils, and T-cells (additional file 7). Furthermore, differences were found in the expression of IL-1\textalpha by granulocytes (adjusted p-value = 0.035).

Differences in the response to \textit{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 \textit{Aspergillus niger} allergenic extract (ASP) and with Lipopolysaccharide (LPS).

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

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-1\textalpha from granulocytes and CMo, and IL-4, IL-6 and MCP1 by CMo (additional file 8) after ASP stimulation. Similarly, after LPS stimulation, we observed differences in the expression of IL-4 and MCP1 by CMo and IL-1\textalpha by granulocytes (additional file 9).

Cluster High individuals show increased levels of IgE at basal levels
The levels of IgE by sandwich-ELISA in the supernatant of cultured whole blood were quantified to evaluate the possibility of type 2 hypersensitivity in these individuals. No significant differences were found in the levels of IgE at basal levels nor after ASP stimulation when comparing MD to controls (Fig. 4A and 4D). At basal levels, cluster High showed significantly increased levels of IgE when compared to cluster Low ($p = 0.0327$) (Fig. 4B), and the same was observed between MDH and MDL ($p = 0.0404$) (Fig. 4C). After ASP stimulation, no significant results were found, however, the differences between cluster High and cluster Low were borderline significant ($p = 0.0524$) (Fig. 4E-F).

**Discussion**

In this present 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. MD 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 controls. 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-1$\beta$ and TNF$\alpha$ levels in cultured PBMC supernatant (6).

MD patients showed differences in the abundance of granulocytes and CD4$^+$ T-cells, when comparing the %total and %parent to controls, and differences in CD8$^+$ T-cells and CD56$^{dim}$ NK-cells, when comparing the %total to controls. For CD8$^+$ T-cells and CD56$^{dim}$ NK-cells, a cluster for each was observed with lower abundance in MD patients. Granulocytes, in the %total, showed 5 clusters with increased abundance and 5 clusters with decreased abundance, compared to controls. These differences in abundance could correspond to different granulocytic subpopulations that have not been further discriminated, as we could not distinguish neutrophils from eosinophils. In CD4$^+$ T-cells, we found a cluster with increased abundance in the %total.

Neutrophil to lymphocyte ratio (NLR) is a parameter that allows the evaluation the inflammatory state of an individual. Some studies on peripheral vestibular vertigo (39), namely vestibular neuritis (40), and MD (41) described a higher NLR in these patients than in controls. When 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 (41), in line with our findings of increased granulocytes in MD patients at basal levels. So, NLR could have potential clinical value when classifying MD patients.

An early study described an increased activity of NK cells in MD (42). Contrastingly, our results have found an increase in CD56\textsuperscript{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 (43). As no differences were found in the clinical history or demographics of individuals in cluster High and cluster Low 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.

Another early flow cytometry study carried out in a series of 16 MD patients and 9 controls (44), described that the percentage of CD4\textsuperscript{+} T cells was higher in the acute phase of MD. However, no differences were found for CD4 and CD8 percentages between quiescent MD patients and controls. Yet, a different flow cytometry study with 46 quiescent MD patients and 46 controls (45) found an increase in CD4\textsuperscript{+} cells and decrease in CD8\textsuperscript{+} 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\textsuperscript{+} and CD8\textsuperscript{+} cells.

DN T-cells were found increased in MDH patients. These cells are believed to have a pathogenic role, as they are found expanded in patients with rheumatic disorders (46). These cells can produce inflammatory cytokines, such as IL-2 and TNF\textalpha, can help B-cells enhance its autoantibody production, and have been found in inflamed tissues (46). The higher abundance of DN T-cells and granulocytes could reflect a persistent inflammatory state in MDH patients.

Several studies have been conducted evaluating the cytokine levels of MD patients. Our group has evaluated the levels of several cytokines in the supernatant of cultured PBMCs from MD patients and controls (6, 18, 20). From the cytokines quantified in this study, we had observed increased levels of IL-8, TNF\textalpha, IL-4, IL-1\alpha, MCP-1, and MIP1\beta in MD patients and IL-10, IL-1\beta, 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 TNF\textalpha in ELF, but no differences were found in the serum (47). Moreover, increased levels of IL-4 and IL-10 have been found in the serum of MD patients when compared to controls (22, 45). 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 TNF\textalpha, IL-4, IL-6, IL-1\alpha, MCP-1, and MIP1\beta in granulocytes and monocytes. IL-4 has a pivotal role in type 2 immune response, such as allergic inflammation, which is majorly produced by polarized Th\textsubscript{2} cells, along with IL-5, IL-6, IL-13 and IgE (45). 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\textsubscript{0} to Th\textsubscript{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\textsubscript{1} response depending on factors such as CCL2 induction timing, tissue site and type of pathogen. Furthermore, we also observed a difference in expression of TNF\textalpha and
MIP-1β/CCL4, which have been linked to type 1 immune response (50). Nevertheless, several studies have also reported the role of TNFα (51, 52), IL-6 (53), IL-1α (54), and MIP1β (55) in allergic reactions. With this in mind, we decided to determine the levels of IgE in the supernatant of the cultured whole blood at basal levels. 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 controls. 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 (56) (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 and Aspergillus niger allergenic extract. LPS is an outer membrane component of gram-negative bacteria that induces a characteristic pattern of cytokine release that regulates inflammation, through TLR4 stimulation, which activates NADPH oxidase and NFκB (57). Therefore, it has been used to study inflammation in MD, as it mimics many inflammatory effects of cytokines, such as TNFα, IL-1β and IL-6, which have been previously described to have increased expression in MD (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. However, these had already been identified at basal levels, so may not represent a difference in the response to LPS, but a difference in the individuals.

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 neither groups, 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 (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 (22). 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 (22). 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 (22). Moreover, it has been described that increased levels of IgE could be a predictor of MD transformation in patients with acute low-tone sensorineural hearing loss (19). CD23, a low affinity receptor for IgE, can induce the secretion of IL-4, leading to local IgE production (22). An increased deposition of IgE and CD23 expression in the vestibular end organs of MD patients (22) has been observed, so it is possible that elevated IL-4 leads to IgE deposition in MD, causing proinflammatory cytokines induction, resulting in local inflammation. 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).

**Conclusions**

1. Patients with MD include at least two endophenotypes, according to the distribution of the immune cell subpopulations and cytokine profile.

2. A subgroup of patients seems to have a type 2 immune response involving IgE and IL4 leading to persistent inflammatory status.

3. The levels of IgE and cytokines should be measured and tracked in patients with sporadic MD.

**Abbreviations**

AAO-HNS - American Academy of Otolaryngology–Head and Neck Surgery

ASP – *Aspergillus niger* allergenic extract

CD (-4, -8, -56) – Cluster of differentiation

cDC – Classical dendritic cells

CMo – Classical monocytes

CyTOF - Cytometry by time of flight

DA – Differential abundance

DS – Differential state

EDTA - Ethylenediaminetetraacetic acid

EH – Endolymphatic hydrops

FDR – False discovery rate

GLMM – Generalized linear mixed model

IgE - Immunoglobulin E

IL (-1α, -1β, -1RA, -4, -5, -6, -10, -13) – Interleukin (-1 alpha, -1 beta, -1 receptor antagonist, -4, -5, -6, -10, 13)

IMo – Intermediate monocytes

LMM - Linear mixed model
LPS – lipopolysaccharide

MCP1 - Monocyte chemoattractant protein-1

MD – Meniere Disease

MDH – Meniere Disease patients with high levels of cytokines

MDL – Meniere Disease patients with low levels of cytokines

MICA – Major histocompatibility complex class I polypeptide–related sequence A

MIP-1β - Macrophage inflammatory protein-1β

NA – Non-applicable

NCMo – Non-classical monocytes

NFKB1 - Nuclear Factor Kappa B Subunit 1

NK-cells – Natural Killer cells

NLR – Neutrophil to lymphocyte ratio

pDC – Plasmacytoid dendritic cells

rpm – rotations per minute

RT – room temperature

SNHL – Sensorineural hearing loss

TLR (-2, -4, -10) – Toll-like receptor (-2, -4, -10)

TNFα - Tumor necrosis factor alpha

tSNE - t-distributed stochastic neighbor embedding

TWEAK - TNF-related weak inducer of apoptosis

UMAP - Uniform manifold approximation and projection

UNS – unstimulated / basal level

Declarations

Ethics approval and consent to participate

The Institutional Review Board in all participating medical centers approved the experimental protocol of this study and all donors signed a written informed consent.
Consent for publication
Not applicable

Availability of data and materials
The datasets generated and analysed during the current study are available in the FlowRepository, under the project ID FR-FCM-Z5R8 and can be accessed via following link:
https://owrepository.org/id/RvFrwXR05VnnqP4kangHqDRT5puZBkW3jW3a4Cx26pL4zYv8125jH24tLwYaaqBT.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
Concept and design: JALE
Patient recruitment: ABC, JCAM, ASV
Panel design and optimization: MFP, PR
Sample processing and staining: MFP
Bioinformatic analysis: MFP, AEB
Statistical analysis: MF
Interpretation of data: MFP, PR, LF
Drafting of the manuscript: MF
Critical revision of the manuscript for important intellectual content: JALE, PR, MAR
Supervision: JALE

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References


Table 4

Table 4 is not available with this version.

Figures
**Figure 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.
Figure 2

*Frequency histogram and density plot of the cytokine median expression of unstimulated whole blood by individual.*
Figure 3

**Phenotypic differences between non-stimulated individuals in cluster High compared to individuals in cluster Low.** (A) Median scaled expression of the type markers across the 13 annotated whole blood cell clusters. (B) tSNE of a subset of 1000 cells per sample (25 Cluster Low and 10 Cluster High) 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) 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. (E) 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

Figure 4

**IgE levels in supernatant at basal levels and after antigenic stimulation with Aspergillus niger allergenic extract.**

A - IgE levels in MD (N = 27) and Controls (N = 12) at basal levels. B - IgE levels in cluster High (N = 10) and cluster Low (N = 22) at basal levels. C - IgE levels in MDH (N = 9) and MDL (N = 14) at basal levels. D - IgE levels in MD (N = 26) and Controls (N = 14) after Aspergillus niger allergenic extract stimulation. E - IgE levels in cluster
High (N = 10) and cluster Low (N = 24) after ASP stimulation. F - IgE levels in MDH (N = 9) and MDL (N = 14) after Aspergillus niger allergenic extract stimulation.

**Figure 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.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- additionalfile1.xlsx
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- additionalfile3.xlsx
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• adfile7boxplottreekoRHvLnew.png
• adfile8DHeatmaphlaspnew.tif
• adfile9DHeatmaphlLPSnew.tif