

Mouse model of mevalonate kinase deficiency: comparison of cytokine and chemokine profile with that of human patients

Annalisa Marcuzzi¹, Valentina Zanin¹, Giulio Kleiner¹, Lorenzo Monasta² and Sergio Crovella^{1,3}

BACKGROUND: Mevalonate kinase deficiency (MKD) is a rare genetic autoinflammatory disease caused by blocking of the enzyme mevalonate kinase in the pathway of cholesterol and isoprenoids. The pathogenic mechanism originating an immune response in MKD patients has not been clearly understood.

METHODS: We investigated the dysregulation of expression of selected cytokines and chemokines in the serum of MKD patients. The results have been compared with those observed in an MKD mouse model obtained by treating the mice with aminobisphosphonate, a molecule that is able to inhibit the cholesterol pathway, mimicking the genetic block characteristic of the disease.

RESULTS: Interleukin (IL)-1 β , IL-5, IL-6, IL-9, IL-17, granulocyte colony-stimulating factor, monocyte chemoattractant protein-1, tumor necrosis factor- α , and IL-4 expression were dysregulated in sera from MKD patients and mice. Moreover, geraniol, an exogenous isoprenoid, when administered to MKD mice, restored cytokines and chemokines levels with values similar to those of untreated mice.

CONCLUSION: Our findings, which were obtained in patients and a mouse model mimicking the human disease, suggest that these cytokines and chemokines could be MKD specific and that isoprenoids could be considered as potential therapeutic molecules. The mouse model, even if with some limitations, was robust and suitable for routine testing of potential MKD drugs.

Mevalonate kinase deficiency (MKD; OMIM no. 251170), is a rare autoinflammatory disease caused by mutations within the second enzyme of the mevalonate pathway (mevalonate kinase (MK), encoded by *MVK*) (1). MK is an essential enzyme in isoprenoid biosynthesis; this pathway produces cholesterol and nonsterol intermediate compounds (farnesyl pyrophosphate and geranyl-geranyl pyrophosphate), which are known to be involved in the control of several cell functions through protein prenylation (farnesylation and geranyl-geranylation, respectively) (2) (Figure 1). The shortage of geranyl-geranyl pyrophosphate, and the consequent decreased rate of protein geranyl-geranylation, leads to increased interleukin (IL)-1 β secretion (3–6); this observation gives new insights into MKD pathogenesis, such as the

causal link between the cholesterol pathway dysfunction and the patient's phenotype.

Although in the past decade the knowledge of MKD pathogenesis has increased, an etiologic therapy for this orphan drug disease is still unavailable.

Different treatments, including steroids, statins, anti-tumor necrosis factor- α (TNF- α), and anti-IL-1 therapy, have been used with variable success to prevent and cure MKD inflammatory symptoms (7,8), whereas only supportive therapies are available for mevalonic aciduria, the more severe form of MKD.

The search for new drugs and therapies for MKD could take advantage of an animal model mimicking the characteristics of the human disease. For this reason, we developed a mouse model of MKD (9), showing that the chemical inhibition of the mevalonate pathway through the use of an aminobisphosphonate (alendronate (Ald) or pamidronate) and statins (lovastatin) leads to a moderate inflammatory phenotype that could be amplified by bacterial compounds such as muramyl dipeptide (MDP) or lipopolysaccharide (LPS) (10,11). Ald inhibits the mevalonate pathway and should regenerate the physiologic activation of a dose-dependent inflammatory process. However, the acute phase of the inflammation is not caused by Ald alone but by Ald together with a proinflammatory agent, i.e., MDP or LPS.

We, and other authors, have recently proposed the use of natural exogenous isoprenoids, such as geraniol (GOH), as a potential therapeutic approach for MKD (10–12). Owing to their isoprenoid structure, these compounds are supposed to enter the mevalonate pathway and bypass the biochemical block, thus reconstructing the pathway and limiting the shortage of geranyl-geranyl pyrophosphate (4,5,9). Moreover, the isoprenoids can rescue inflammation in the MKD mouse model (9) as well as in the Ald/lovastatin-LPS-treated Raw 264.7 cell lines (10), and in human monocytes (13). Based on the previous partial success of biological drugs, we decided to evaluate the modulation of cytokines and chemokines in our MKD animal model in the presence or in the absence of GOH.

The objective of our study is to compare the inflammation profile of the MKD mouse model with the profile of MKD patients, with the aim of finding analogies useful for the search of new anti-inflammatory drugs and treatments in an established and robust animal model of the disease.

¹Health Genetics Unit, Institute for Maternal and Child Health—IRCCS “Burlo Garofolo”, Trieste, Italy; ²Epidemiology and Biostatistics Unit, Institute for Maternal and Child Health—IRCCS “Burlo Garofolo”, Trieste, Italy; ³Department of Medicine and Surgery and Health Sciences, University of Trieste, Trieste, Italy. Correspondence: Annalisa Marcuzzi (annalisa.marcuzzi@burlo.trieste.it)

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RESULTS

Serum Cytokine–Chemokine Levels in MKD Patients

Even if patients showed eight different mutations, expression of IL-1β, IL-5, IL-6, IL-9, and granulocyte colony–stimulating factor (G-CSF) was significantly upregulated, independently of genetic profile, as compared with controls, whereas expression of IL-17, monocyte chemotactic protein-1 (MCP-1), and IL-4 was downregulated in MKD patients as compared with controls (Table 1 and Figure 2).

Serum Cytokine–Chemokine Levels in Ald-Treated Mice

Experimental timing and dosages of Ald were chosen according to the literature: Ald reached the maximum effect 3 d after injection and concentrations <6.5 mg/kg had no effect on the animal, whereas this compound was toxic at concentrations >13 mg/kg (14).

Serum levels of the cytokines and chemokines IL-1β, IL-5, IL-6, IL-9, IL-17, G-CSF, MCP-1, TNF-α, and IL-4 in control mice and mice treated with different concentrations of Ald are shown in Figure 3. Blocking the mevalonate pathway resulted in increased values of these cytokine–chemokine levels, with the exception of IL-6, in mice treated with aminobisphosphonate. Moreover, the response to this treatment seemed to be dose-dependent because higher doses of Ald led to higher values of analyzed molecules.

Similarly to MKD patients, IL-4 was downregulated in Ald-treated mice as compared with untreated mice (Table 2).

GOH Reduces Inflammatory Cytokine Levels in MKD Mouse Model

We induced an inflammatory state in Ald-treated animals using MDP to mimic the acute phase of the human disease. GOH reduced the MDP-induced inflammatory response in Ald/MDP-treated mice by entering the pathway and significantly

normalizing the expression of the analyzed cytokines and chemokines (Figure 4). GOH treatment had no significant effect on the tested cytokines if compared with untreated animals (data not shown).

DISCUSSION

In our study we analyzed selected cytokines and chemokines, which are known to increase in human autoinflammatory diseases and are believed to play a role in the pathogenesis of MKD, in patients, controls, and a well-established mouse model (15). We also analyzed cytokine and chemokine down- or upregulation in MKD patients as compared with controls. Our findings are comparable to those observed in our animal model. When considering Ald treatment, the expression levels of IL-1β, IL-5, IL-6, IL-9, IL-17, G-CSF, and MCP-1 were dysregulated as compared with those of controls both in MKD patients and in animals treated with Ald.

TNF-α values in MKD patients did not differ with respect to the controls, whereas they increased in animals treated with Ald. This discrepancy can be explained by the different timing of blood collection. The human samples were collected during a remission period, which can bias the results because TNF-α expression increases during the acute phase and then quickly decreases (half-life of 6 min) (15). Instead, animal samples were collected under the effect of Ald, which is able to induce a moderate inflammatory response that accounts for the increase of TNF-α expression.

IL-4 is well known to have a strong anti-inflammatory role (16): we observed that a blocked mevalonate pathway downregulates this cytokine in MKD patients and in animals treated with Ald. Moreover, we detected an increase of IL-4 levels in animals after the injection of the proinflammatory molecule MDP (17) and, inversely, a decrease after the administration of GOH, which has anti-inflammatory

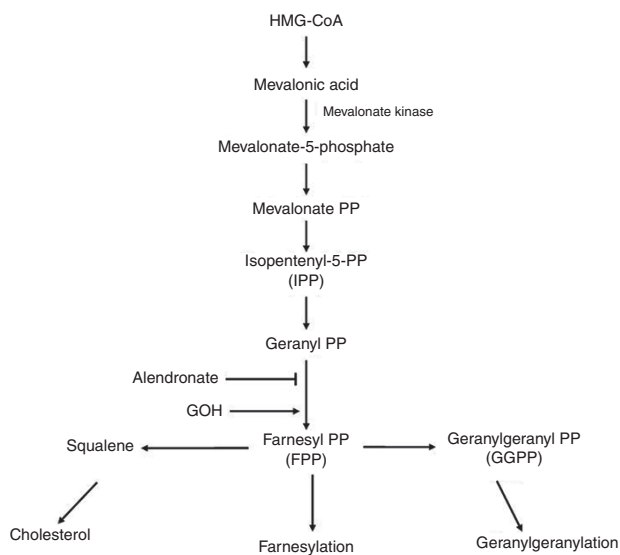


Figure 1. Schematic representation of the mevalonate pathway. Compounds used in the experiments (alendronate and geraniol (GOH)) are indicated along the pathway. HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PP, pyrophosphate.

Table 1. Serum cytokine–chemokine levels in MKD patients

Official symbol	Controls	MKD patients	P value
IL-1β	5.37 ± 5.75	145.1 ± 104.2	0.0014**
IL-5	nd <3.1	11.55 ± 3.62	0.0104*
IL-6	13.28 ± 6.3	47.85 ± 54.72	0.0032**
IL-9	24.33 ± 10.93	39.17 ± 20.82	0.0004**
IL-17	102.9 ± 17.83	85.34 ± 36.24	0.0377*
G-CSF	43.52 ± 10.71	88.9 ± 84.14	0.0022**
MCP-1	51.52 ± 34.05	48.26 ± 51.49	0.0426*
TNF-α	32.67 ± 9.75	41.93 ± 25.61	0.3345, NS
IL-4	7.99 ± 1.26	6.05 ± 2.74	0.015*
IL-10	12.15 ± 4.9	16.35 ± 6.96	0.0055**
IL-13	11.99 ± 4.63	14.79 ± 4.95	0.0037**

Cytokine and chemokine values in controls and MKD patients. Values are shown as means ± SD (pg/ml).

G-CSF, granulocyte colony–stimulating factor; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MKD, mevalonate kinase deficiency; nd, not detectable (<detection limit); NS, not significant; TNF, tumor necrosis factor.

*P < 0.05; Mann–Whitney test; **P < 0.01; Mann–Whitney test.

properties (18,19). No theory has been proposed yet to link IL-4 secretion with a dysregulation of the mevalonate pathway. We hypothesize that the observed trend could be linked to the anti-inflammatory role of IL-4.

When considering our results, we are aware that the interquartile ranges of cytokine and chemokine expression values in MKD patients are quite wide. This interindividual variability could be explained by hypothesizing the interaction between the *MVK* genetic defects and the residual MK activity. Indeed, residual MK activity varies from <0.5 to 7%, depending upon the type of *MVK* mutation (20,21). Unfortunately, an evaluation of the quantitative residual MK activity was not available for our patients. Our hypothesis is supported by the fact that, even in mice, the detected amount of cytokines and chemokines seems to be Ald dependent, as different concentrations of Ald led to different responses (22); the genetic background could account for these differences.

The consistent variation of cytokine expression in humans and mice suggests that the dysregulation of this particular panel of cytokines could be MKD specific or peculiar to the blocked biosynthesis of cholesterol and/or its intermediates. Indeed, when administered to animals, exogenous GOH was

able to bypass the aminobisphosphonate biochemical block, reconstituting the mevalonate pathway and thus normalizing cytokine and chemokine values (23).

Despite being encouraging, our results need further and deeper investigations aimed at establishing whether GOH could be a potential therapeutic molecule for MKD. We are also aware that the main limits of our study are represented by the different levels of inflammation between the patients with an endogenous block and the mice treated with Ald, and by the fact that the biochemical block of the pathway in the model is substantially different from the genetic defects of patients. To partly overcome these limitations, we are developing a new protocol that extends the times of Ald administration to better mimic the progression of the inflammatory condition of the patient.

In conclusion, even if our findings are preliminary owing to the small number of MKD patients analyzed and the limits related to our animal model, they should be considered as a first step in research aimed at better disclosing the pathogenesis of MKD: the fact that some cytokines and chemokines appear to have a similar dysregulation in MKD patients and in the animal model could be useful for the development of novel therapeutic strategies for this orphan drug disease. The animal

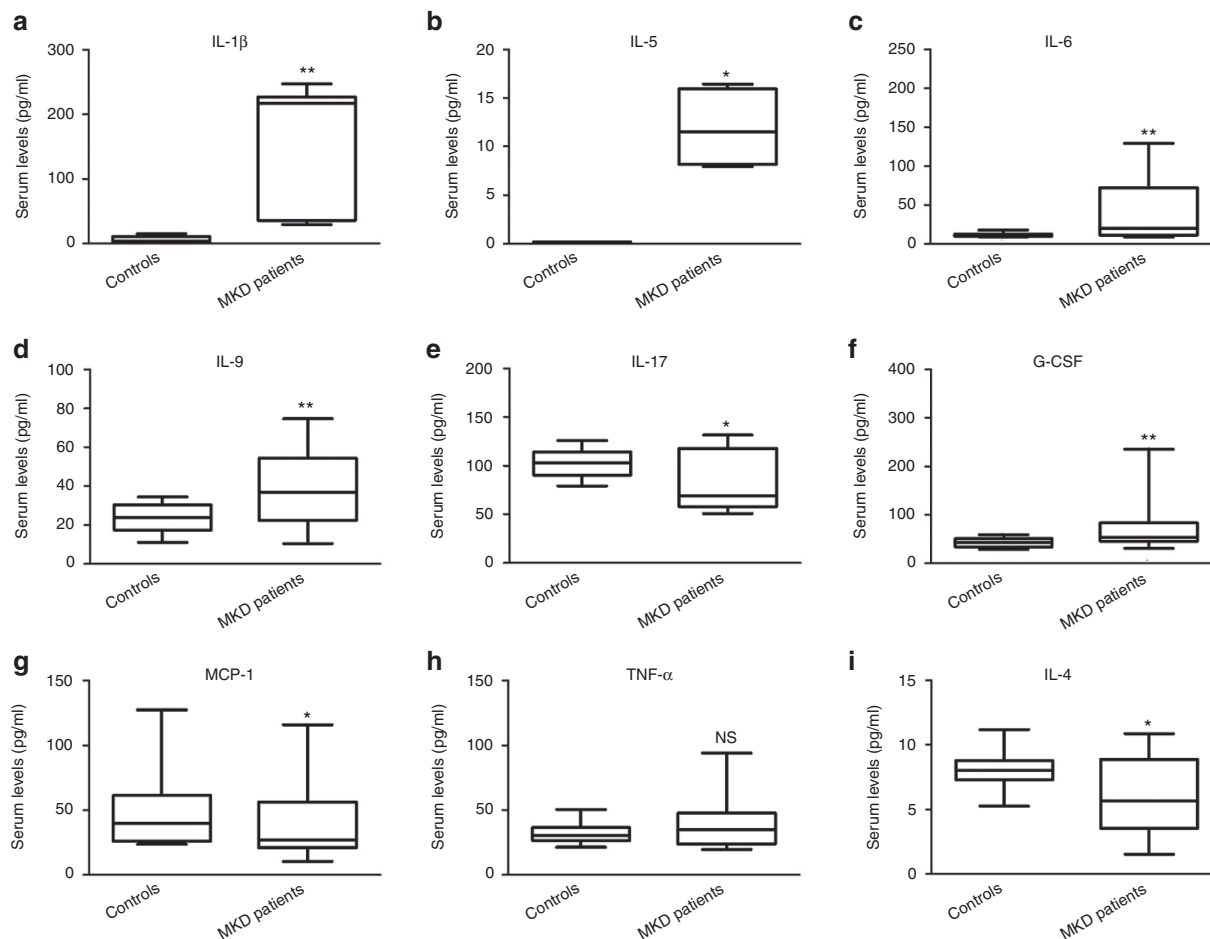


Figure 2. Box plots of serum levels of cytokines and chemokines: (a) interleukin (IL)-1β, (b) IL-5, (c) IL-6, (d) IL-9, (e) IL-17, (f) granulocyte colony-stimulating factor (G-CSF), (g) monocyte chemoattractant protein-1 (MCP-1), (h) tumor necrosis factor-α (TNF-α), and (i) IL-4 for eight mevalonate kinase deficiency (MKD) patients not in the acute phase, and 35 controls. Ends of the whiskers represent the 10th and the 90th percentiles. Outliers not shown. NS, not significant. **P* < 0.05; Mann-Whitney test; ***P* < 0.01; Mann-Whitney test.

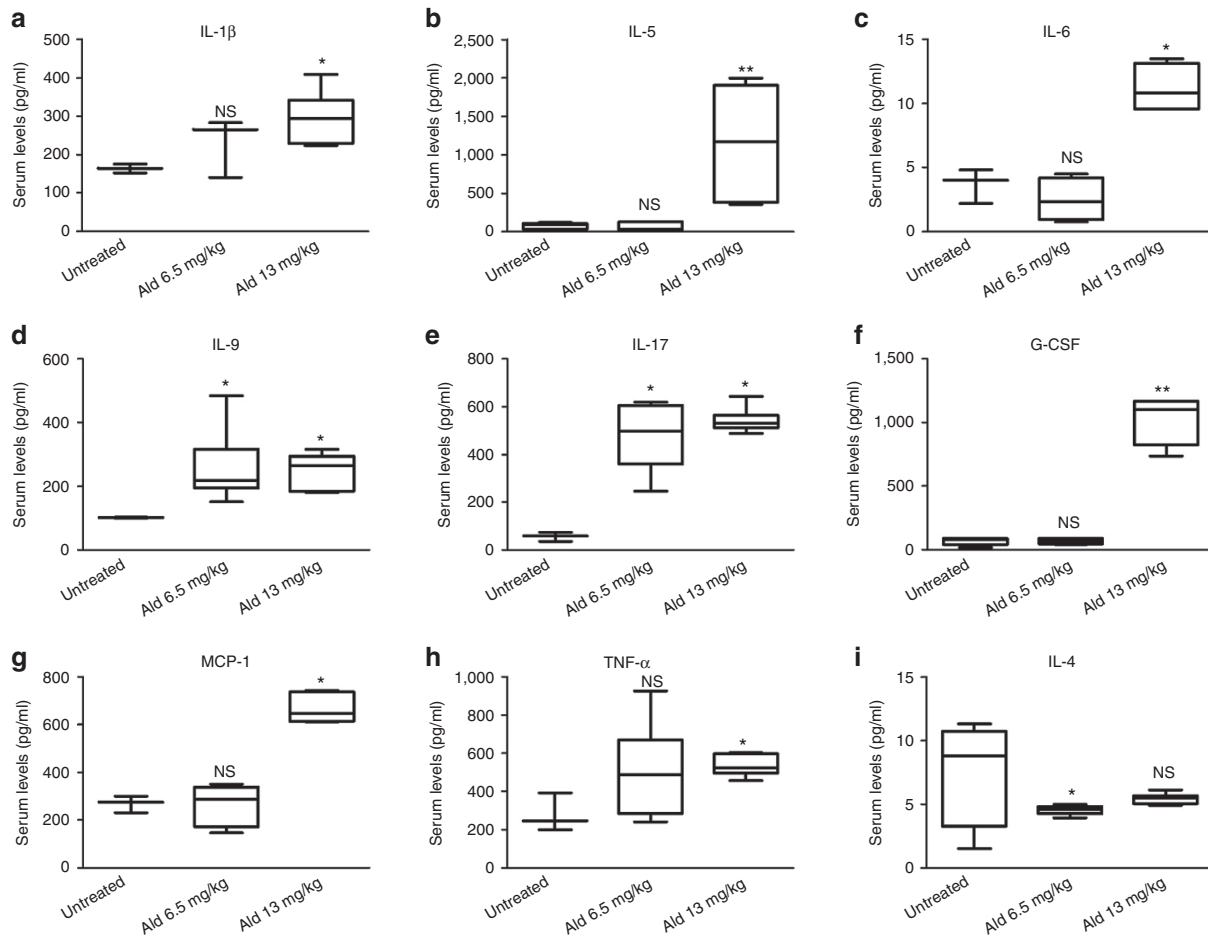


Figure 3. Box plots on serum levels of cytokines and chemokines: (a) interleukin (IL)-1 β , (b) IL-5, (c) IL-6, (d) IL-9, (e) IL-17, (f) granulocyte colony-stimulating factor (G-CSF), (g) monocyte chemoattractant protein-1 (MCP-1), (h) tumor necrosis factor- α (TNF- α), and (i) IL-4 were analyzed in BALB/c mice: untreated; 3 d after alendronate (Ald) 6.5 mg/kg; 3 d after Ald 13 mg/kg (left, middle, and right box plots, respectively). Ends of the whiskers represent the 10th and the 90th percentiles. Outliers not shown. Significance was measured using group of untreated as reference. NS, not significant. * $P < 0.05$; Mann-Whitney test; ** $P < 0.01$; Mann-Whitney test.

Table 2. Serum cytokine-chemokine levels in Ald-treated mice

Official symbol	Untreated	Ald 6.5 mg/kg	<i>P</i> value, untreated vs. Ald 6.5 mg/kg	Ald 13 mg/kg	<i>P</i> value, untreated vs. Ald 13 mg/kg
IL-1 β	163.9 \pm 11.34	229.4 \pm 77.57	0.7, NS	295.4 \pm 67.66	0.0238*
IL-5	76.56 \pm 39.39	59.05 \pm 53.37	0.8357, NS	1,161 \pm 698.5	0.0012**
IL-6	6.71 \pm 1.28	2.89 \pm 1.74	0.4, NS	6.07 \pm 3.19	0.05, NS
IL-9	101 \pm 1	257 \pm 116.9	0.023*	250.1 \pm 56.2	0.024*
IL-17	56.31 \pm 18.91	477 \pm 151.5	0.0238*	541.5 \pm 53.24	0.0238*
G-CSF	66.54 \pm 29.96	66.62 \pm 22.85	1, NS	1,023 \pm 185.3	0.0043**
MCP-1	268.3 \pm 35.3	262.2 \pm 86.6	1, NS	665.6 \pm 59.16	0.0238
TNF- α	279.5 \pm 101.7	504 \pm 250.8	0.26, NS	535.2 \pm 55.32	0.0238*
IL-4	7.61 \pm 4.22	4.53 \pm 0.37	0.0144*	5.44 \pm 0.41	0.05, NS
IL-10	87.5 \pm 14.77	47.35 \pm 24.61	0.0173*	78.22 \pm 45.74	0.42, NS
IL-13	51.17 \pm 17.20	112.3 \pm 37.96	0.095, NS	149.6 \pm 34.26	0.0238*

Cytokine and chemokine values in Ald-treated mice. Values are shown as means \pm SD (pg/ml).

Ald, alendronate; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NS, not significant; TNF, tumor necrosis factor.

* $P < 0.05$; Mann-Whitney test; ** $P < 0.01$; Mann-Whitney test.

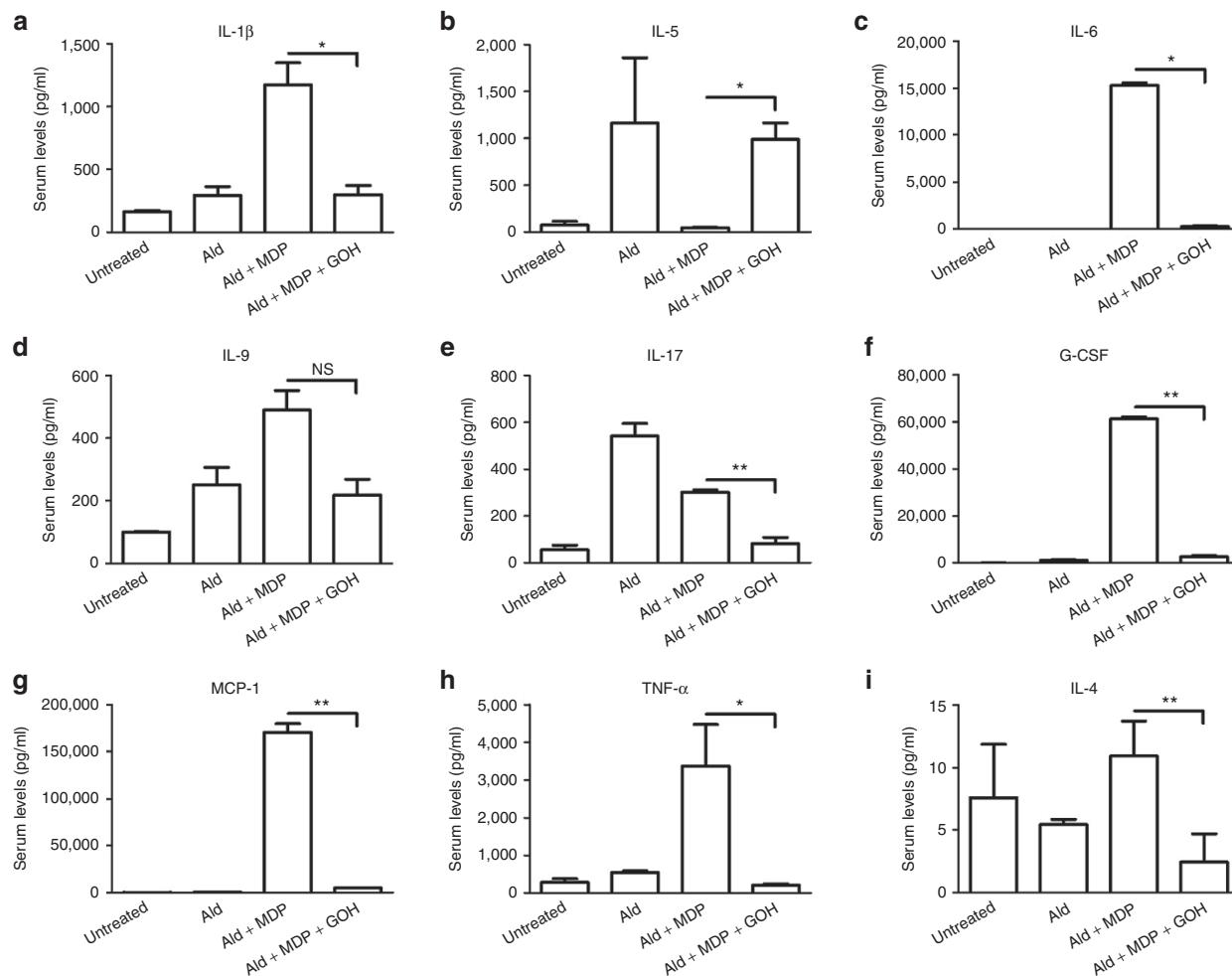


Figure 4. Cytokines and chemokines: (a) interleukin (IL)-1 β , (b) IL-5, (c) IL-6, (d) IL-9, (e) IL-17, (f) granulocyte colony-stimulating factor (G-CSF), (g) monocyte chemoattractant protein-1 (MCP-1), (h) tumor necrosis factor- α (TNF- α), and (i) IL-4 values in untreated mice and mice treated with alendronate (Ald), alendronate/muramyl dipeptide (Ald+MDP), and alendronate/muramyl dipeptide/geraniol (Ald+MDP+GOH). Values are shown as mean \pm SD. Mann-Whitney test was used to calculate the difference between Ald+MDP and Ald+MDP+GOH groups. NS, not significant. * P < 0.05; Mann-Whitney test; ** P < 0.01; Mann-Whitney test. P values: IL-1 β : P = 0.0211; IL-5: P = 0.0199; IL-6: P = 0.0001; IL-9: P = 0.0543; IL-17: P = 0.0068; G-CSF: P = 0.0002; MCP-1: P = 0.0011; TNF- α : P = 0.0391; and IL-4: P = 0.0046.

model, even with some limitations, was robust enough to be suitable for searching for and testing new drugs for MKD.

METHODS

Reagents

Bacterial MDP and Ald were supplied by Sigma (St Louis, MO). GOH was supplied by Euphar group (Piacenza, Italy).

Blood Samples

After approval by the Independent Bioethics Committee of the IRCCS “Burlo Garofolo” and after written informed consent obtained from parents or caregivers, blood was collected by venipuncture from 35 controls and eight MKD patients; both of the groups were aged between 2 and 9 y (Table 3). Controls were individuals without concurrent infections and/or autoimmune diseases. All MKD patients had no concurrent infection and were not in the acute phase of the disease.

Animals

BALB/c male mice (Harlan, Udine, Italy), aged 6–8 wk and weighing between 25 and 30 g were used in this study, which was carried out in accordance with the Italian Ministry of Health registration no. 62/2000-B, 6 October 2000, and approved by the University of Trieste

Ethical Committee. The experimental design has been previously described (9). Briefly, mice were randomly divided into groups of six animals each: group 1, untreated controls; group 2, Ald (6.5 or 13 mg/kg) on day 0 and MDP 100 μ g/kg on day 3; group 3, Ald and MDP as in group 2 plus GOH 250 mg/kg on day 2. All solutions were administered by intraperitoneal route (Table 4). After 2 h of MDP stimulation, blood was collected directly into test tubes following decapitation. Serum was obtained by centrifugation at 2,000g at 4 $^{\circ}$ C and then stored at -80 $^{\circ}$ C.

Cytokine Release Evaluation

The analysis of 22 cytokines and chemokines (including IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40-p70), IL-13, IL-17, eotaxin, G-CSF, granulocyte-macrophage colony-stimulating factor, interferon- γ , MCP-1, macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , regulated on activation, normal T cell expressed and secreted, and TNF- α) was performed on serum samples using a magnetic bead-based multiplex immunoassay (Bio-Plex; BIO-RAD Laboratories, Milano, Italy) following the manufacturer’s instructions. Data from the reactions were acquired using the Bio-Plex 200 reader, whereas a digital processor managed data output and the Bio-Plex Manager software returned data as median fluorescence intensity and concentration (pg/ml) (BIO-RAD Laboratories).

Table 3. Background information of patients and controls

	Controls	MKD patients
Number	35	8
Male/female	15/20	4/4
Age (mean ± SD)	8.8 ± 4.8	8.57 ± 5.96
MKD mutations	—	V377I/I268T V377I/V310M S135L/V377I V377I/V377I V377I/V377I G336S/G336S I268T/V377I V377I/H20P

MKD, mevalonate kinase deficiency.

Table 4. Schematic representation of the experimental design mouse model

Experimental design	Saline solution	Ald	MDP	GOH
Day 0	Group 1	Groups 2/3		
Day 1				
Day 2				Group 3
Day 3	Group 1		Groups 2/3	

Group 1, untreated controls. Group 2, Ald (6.5 or 13 mg/kg) on day 0 and MDP 100 µg/kg on day 3. Group 3, Ald and MDP as in group 2 plus GOH 250 mg/kg on day 2. All of the solutions were administered by the intraperitoneal route.

Ald, alendronate; GOH, geraniol; MDP, muramyl dipeptide.

Data Analysis

For each set of experiments, values were analyzed calculating medians, interquartile ranges, means, and SDs. Box plots were used to represent data distribution. The nonparametric Mann-Whitney test was used when appropriate. *P* values were calculated on the basis of two-tailed tests. Statistical analysis was performed using GraphPad Prism V.5.0 for Windows (GraphPad Software, San Diego, CA). A *P* value of <0.05 was considered for statistical significance (<http://www.graphpad.com/prism/p5.htm>).

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