Systemic and Neuronal Inflammatory Markers in a Mouse Model of Mevalonate Kinase Deficiency: a Strain-comparative Study

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Abstract. *Background/Aim: There is a lack of reliable animal models for the study of the rare auto-inflammatory disease mevalonate kinase deficiency (MKD). The one most frequently used is a biochemical model, obtained by treating BALB/c mice in order to block the mevalonate pathway, thus attempting to reproduce the inflammatory pattern presented in patients. This study aims to assess the role played in pathology by the inflammasome and the reliability of this model. Materials and Methods: We mimicked MKD using two different mice strains (BALB/c and C57BL/6), evaluating typical inflammatory markers of MKD and inflammasome modulation. Results: Without significant differences, both strains exhibited a general MKD-like inflammation, including the modulation of the molecular platform inflammasome, mimicking the characteristics observed in human patients. Conclusion: Although with some limitations, the mouse model appears robust and suitable for studying MKD. Results do not seem to vary with the mouse strain used, and appear to be treatment-dependent. Finally, in vivo inflammasome activation was assessed for the first time here.*

Mevalonate kinase deficiency (MKD, OMIM #260920) is a rare autosomal disorder caused by a dysfunction of the mevalonate kinase gene coding for the enzyme mevalonate kinase in the cholesterol pathway (1). Besides recurrent episodes of fever and inflammation characterizing MKD, patients suffering from the most severe form, mevalonic aciduria, also exhibit neurological involvement, including developmental delay, ataxia, dysmorphic features, failure to thrive and often death during infancy or early childhood (2, 3).

Mevalonate kinase is essential for isoprenoid biosynthesis, a pathway producing cholesterol and non-sterol intermediate compounds farnesyl pyrophosphate (FPP), and geranyl-geranyl pyrophosphate (GGPP), known to be involved in the control of several cell functions through protein prenylation (farnesylation and geranyl-geranylation, respectively) (Figure 1). In particular, the lack of GGPP results in augmented caspase-1-dependent interleukin-1β (IL-1β) secretion, which is the major cytokine responsible for the inflammatory systemic effects observed in patients with MKD (4, 5).

Recently, in a mouse model of Alzheimer's disease, a key molecule responsible for the activation of neuroinflammation was reported to be a domain found in NAIP, CIITA, HET-E, and TP-1 (NACHT), leucine rich repeats (LRR) and pyrine domain (PYD) domains-containing protein 3 (NALP3) (6). NALP3-driven inflammasome is necessary to activate caspase-1 which causes IL-1β production, therefore it is believed to play a crucial role in MKD pathogenesis (7). Moreover, NALP3 was found to be involved in other forms of neurodegeneration (8, 9). These data make assessing the role of NALP3 in this model intriguing.

However, although the knowledge of MKD pathogenesis has increased in the past decade, no targeted therapy is available. 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 biochemical mouse model of MKD (10) showing that the inhibition of the mevalonate pathway, through the use of amino*bis*phosphonates and statins, leads to a moderate inflammatory phenotype that could be amplified by the subsequent injection of a bacterial compound, such as

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Key Words: Mevalonate kinase deficiency, BALB/c, C57BL/6, alendronate, muramyl dipeptide, inflammasome.

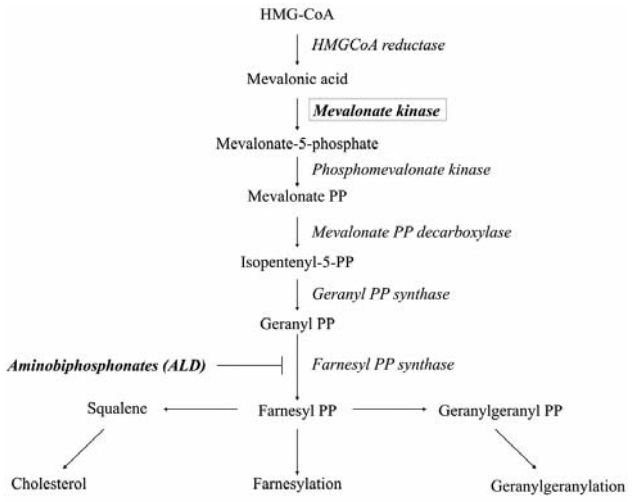

Figure 1. *Schematic representation of the mevalonate pathway. Aminobisphosphonate (ALD, alendronate) used in the experiments is indicated in bold characters and the red square indicates the enzyme (mevalonate kinase) mutated in mevalonate kinase deficiency. HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; PP: Pyrophosaphate.*

muramyl dipeptide (MDP) or lipopolysaccharide (LPS) (11, 12). The aminobisphosphonate alendronate (ALD) inhibits the mevalonate pathway and should restore the physiological activation of a dose-dependent inflammatory process. However, the acute phase of the inflammation is not caused by ALD alone but by the presence of ALD together with a pro-inflammatory agent, *i.e.* MDP or LPS (11). To date, for this *in vivo* model, only BALB/c mice have been used. Thus, in order to establish if this model is reliable and consistent, or if it might be strain-dependent, we tested the same experimental design using two of the most commonly used mouse strains: BALB/c and C57BL/6, both treated with ALD and MDP. To verify the effects of treatments, both general and MKD-specific markers have been assessed, including spleen leukocyte infiltration; serum release of pro-inflammatory cytokines, chemokines and serum amyloid-A (SAA); and the production of NALP3 in the cerebellum and its concentration in serum.

Materials and Methods

Reagents. Bacterial MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine hydrate) and LPS-free alendronate (ALD), were purchased from Sigma Chemical Co. Aldrich (St Louis, MO, USA).

Animals. Twenty BALB/cOlaHsd (BALB/c) male mice (Harlan, Udine, Italy), and twenty C57BL/6 male mice (kindly provided by the ICGEB animal factory, Trieste, Italy) aged 6-8 weeks and weighing between 20-25 g, were used in this study. Mice were housed in standard cages with a 12-h light/dark cycle, with free access to tap water and pellet food. The environmental temperature was constantly maintained at 21˚C and the mice were kept under pathogen-free conditions. Experiments were carried out in accordance with institutional guidelines in compliance with international and Italian laws (EEC Council Directive 86/609, OJL 358, December 1987 and Italian Ministry of Health registration number 62/2000-B, October 6, 2000), upon approval by the institutional Ethical Committee (permit number: 7-2.10.2006).

The experimental design was previously described (10). Briefly, each strain was randomly divided into four groups of five animals each: control group (saline injections); ALD group, treated with ALD at 13 mg/kg at time 0 h; MDP group, MDP given at 100 μg/kg at 72 h; ALD+MDP group, ALD at 13 mg/kg at time 0 h and MDP at 100 μg/kg at 72 h (Table I). All solutions were administered by the intraperitoneal route.

Every day the body weight of mice was measured with an UWE SC-300 scale (Technical Advantages, Brisbane, QLD, Australia).

Sample collection. Blood was collected directly into test tubes by submandibular (13) puncture at different time points of the experiment (Table I) and immediately before sacrifice. Serum was recovered by centrifugation at 2000 ×*g* at 4˚C, and then stored at −80˚C until used. Two hours after the last injection (MDP or saline) mice were sacrificed by decapitation.

Immediately, brains were extracted and collected for subsequent immunohistochemical analyses. Vials were stored in dry ice and then at −80˚C until used.

Spleens were extracted, weighed on JW-250 scales (Technical Advantages) and stored first in dry ice and then at −80˚C for histochemical staining.

Determination of cytokines and chemokines release. The analyses of cytokines and chemokines including interleukin-6 (IL-6), granulocyte colony stimulating factor (G-CSF), keratinocyte chemoattractant (KC), IL-1β, monocyte chemoattractant protein-1 (MCP-1), interleukin 12(p40) (IL-12(p40)) and tumor necrosis factor α (TNF-α), were performed on animal serum using magnetic bead-based multiplex immunoassays (Bio-Plex®; BIO-RAD Laboratories, Milan, Italy) following the manufacturer's instructions. Data from the reactions were acquired using a Bio-Plex 200 reader, while a digital processor managed data output and Bio-Plex Manager® 6.0 software (BIO-RAD Laboratories) presented data as median fluorescence intensity (MFI) and concentration (pg/ml).

Determination of serum amyloid A (SAA) and NALP3 concentration. Specific enzyme-linked immunosorbent assay (ELISA) (Cusabio, Wuhan, P.R. China) kits were performed in triplicate to assess serum concentration of SAA and NALP3. The experimental procedures were performed according to the manufacturer's protocols.

Histo- and immunohistochemistry. Spleens were embedded in Cryobloc (Diapath, Bergamo, Italy), cut at 8 μm in a cryostat (Slee Cryostat; Emme 3 Biotecnologie, Milan, Italy) and sections were stained with hematoxylin and eosin. Spleen leukocyte infiltration was evaluated using a Leica DC100 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

After sacrifice, brains were extracted and frozen in dry ice before further processing. For immunohistochemistry, brains were embedded in Cryobloc and cut at 8 μm thickness. Subsequently, the longitudinal slices were fixed in 4% paraformaldehyde (PFA; Sigma Chemical Co. Aldrich, St. Louis, MO, USA) dissolved in phosphate

Table I. *Experimental design*.

Strain		Group	T1	T2	T3	T4	→ 74 h
				24 h	48 h	72 h	
BALB/c (n)	C57/black (n)			Measurement of body weight			
5	5	Control (Ctrl)	Blood collection + saline			Saline	
5	5	ALD	Alendronate (13 mg/kg)	Blood collection		Saline	Blood collection
5	5	MDP	Saline			Muramyl dipeptide (100 μg/kg)	and sacrifice
5	5	ALD + MDP	Alendronate (13 mg/kg)		Blood collection	Muramyl dipeptide (100 μg/kg)	

n: Number of animals per group; Ctrl: control; ALD: alendronate; MDP: muramyl dipeptide.

buffered saline (PBS) and processed for immunohistochemistry. Briefly, sections were blocked for 1 h at room temperature with 10% normal goat serum (NGS; Euroclone, Milan, Italy) plus 0.1% Triton-X-100, then incubated overnight at 4˚C with primary antibodies dissolved in 5% NGS plus 0.1% Triton-X-100. Immunostaining was revealed the following day with fluorescent secondary antibodies (Alexa Fluor® 555 Conjugate and Alexa Fluor® 488 Conjugate; Cell Signaling Technology, Beverly, MA, USA). Glass coverslips were mounted using Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and examined with an Axioplan 2 optical microscope (Carl Zeiss, Oberkochen, Germany). Primary antibodies used in this study were mouse monoclonal antibodies ionized calcium-binding adapter molecule 1 (IBA1; Abcam, Cambridge, UK) and rabbit polyclonal NALP3 antibodies (ThermoScientific, Waltham, MA, USA).

Data analysis. All results are expressed as the mean±standard deviation (SD), except for SAA, which is reported as a percentage compared to that of the double treatment (100%). Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test. Statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA, USA).

Results

Mice did not exhibit any particular behavioural change ascribable to experimental procedures and the weight of treated mice increased without significant differences compared to control animals.

Blood was also collected from control groups at baseline, and from ALD groups at 24 and 48 h, in order to evaluate the effect of ALD on pro-inflammatory markers (IL-1β, NALP3 and SAA) at different times (Table I). However, no differences were noticed among ALD-treated groups at any of the time points, thus only results of 72 h are shown.

Double treatment induces an inflammatory phenotype. The spleen weights of BALB/c and C57BL/6 mice have been standardized taking into account the total weight of the animals (Figure 2a), and no differences were noticed between different strains.

The pro-inflammatory effect of the combination of ALD and MDP was evident in histological analysis of the animals' spleens. Compared to the physiological condition (Figure 2b), the spleen of mice treated with both ALD and MDP clearly exhibited leukocyte infiltration (Figure 2c, red circles).

Increased concentration of inflammatory cytokines and chemokines in mouse serum. The serum cytokine and chemokine concentrations in BALB/c and C57BL/6 control and treated mice were assessed using magnetic bead-based multiplex immunoassays (Figure 3). IL-6, KC, G-CSF and IL-1β values were significantly higher both in BALB/c and in C57BL/6 mice treated with ALD plus MDP compared to the respective controls. At the same time for both strains, single treatments with ALD or MDP did not significantly affect the production of these cytokines. MCP-1, IL-12(p40) and TNF-α were also affected only by the double treatment, even if in a non-significant manner. Indeed, their serum concentrations did not change after single treatments, and only a slight increase was noticeable after the administration of ALD-plus-MDP (data not shown).

SAA increases after double treatment. An ELISA assay was carried out to evaluate the serum concentration of SAA. We set the serum level exhibited by the double-treated groups as 100% of SAA production (the maximum level of inflammation), and reported the values of other groups as a percentage compared to that of double-treated mice (Figure 4).

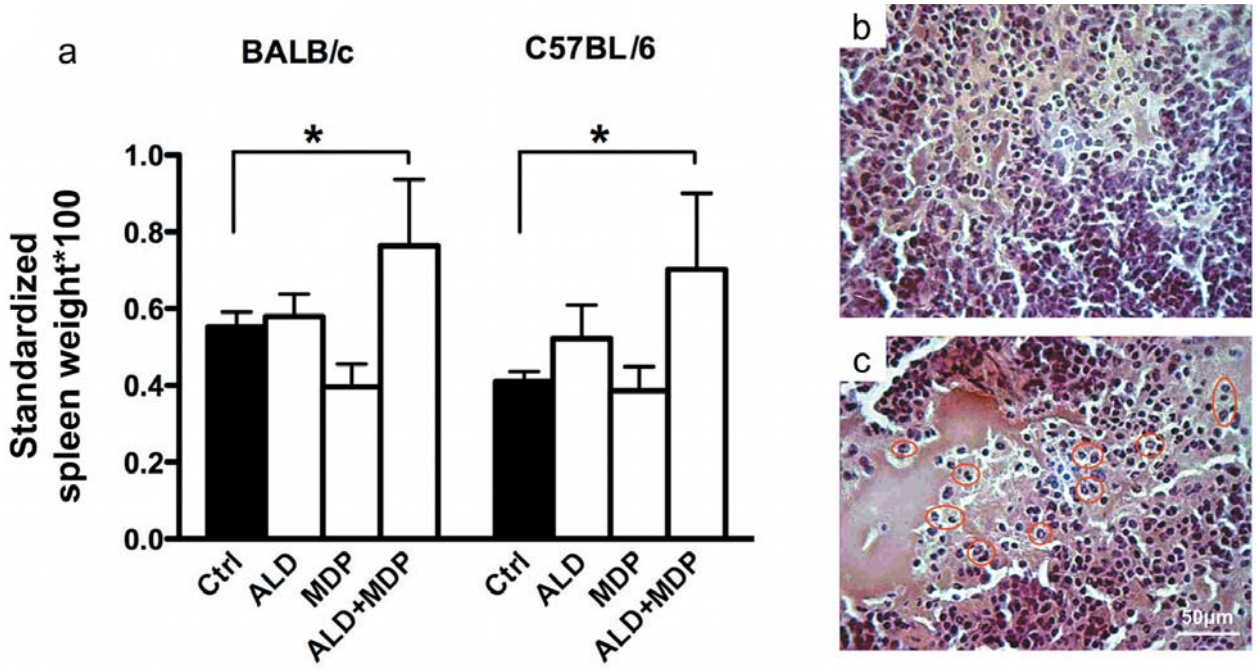

Figure 2. *Effects of treatments on spleen weight and morphology. Mice from each strain (BALB/c, n=20; C57BL/6, n=20) were divided into four groups: Ctrl (control animal, saline), alendronate (ALD, 13 mg/kg), muramyl dipeptide (MDP, 100 μg/kg), and ALD plus MDP. Compounds were intraperitoneally injected. a: Spleen weights measured at 72 h (T4) have been standardized taking into account the total weight of the animals. *p<0.05 after one way ANOVA test followed by the Bonferroni correction for multiple comparisons. Haematoxylin and eosin-stained BALB/c spleens: spleen parenchyma of a control animal under physiological conditions (b), compared to a double-treated mouse (c), in which the infiltration of lymphomonocytic and polymorphonuclear cells is evident. Microscope magnification: x40; scale bar: 50 μm; no differences were noticed between spleens from BALB/c and C57BL/6 mice (data not shown).*

For both strains, ALD-plus-MDP treatment led to a significant increase of SAA values compared to the respective control groups. At the same time, ALD or MDP given alone, led to intermediate values of SAA in both strains, in between those of controls and groups with double treatment.

Response of NALP3-driven inflammasome to the treatments. The production of serum NALP3 was assessed in control and treated BALB/c and C57BL/6 mice by performing ELISA (Figure 5). NALP3 concentration was markedly higher in the BALB/c group treated with ALD-plus-MDP compared to the control group; in C57BL/6 mice the difference between these two groups was much more significantly. Single treatments with ALD or MDP did not increase NALP3 in either strain.

Moreover, immunohistochemical analysis in brain sections revealed that activation of microglial cells in the cerebellar area of double-treated animals was markedly increased compared to controls, as revealed by an increase in IBA1 staining. NALP3 staining was also increased, showing that, as in the systemic compartment, the NALP3 inflammasome component is activated by combine ALD and MDP treatment even in the brain (Figure 6).

Discussion

MKD still lacks a genetic animal model (*i.e.* knockout mouse). Animal models developed to reproduce the homozygous form of MKD are not compatible with life. The only genetic mouse model of MKD is a heterozygote, obtained by the deletion of one *MVK* allele (14); however, that model only partially reproduces the MKD-like inflammatory phenotype; moreover, it does not exhibit the features of neurological dysfunction.

The biochemical block used to reproduce the inflammatory phenotype of MKD, albeit limited, represents a helpful tool to study the main markers of inflammation which characterize the human disease. In fact, we proposed an MKD animal model treating BALB/c mice with the amino*bis*phosphonate alendronate, blocking the pathway and increasing susceptibility to pro-inflammatory compounds, such as LPS or MDP (10). The inflammatory phenotype developed in this model resembles in many aspects the MKD characteristics observed in patients. Thus, this model aims to represent a feasible tool for testing potential anti-inflammatory drugs. However, prior to assessing the action of these compounds, it is necessary to verify the reliability of the model. To do this,

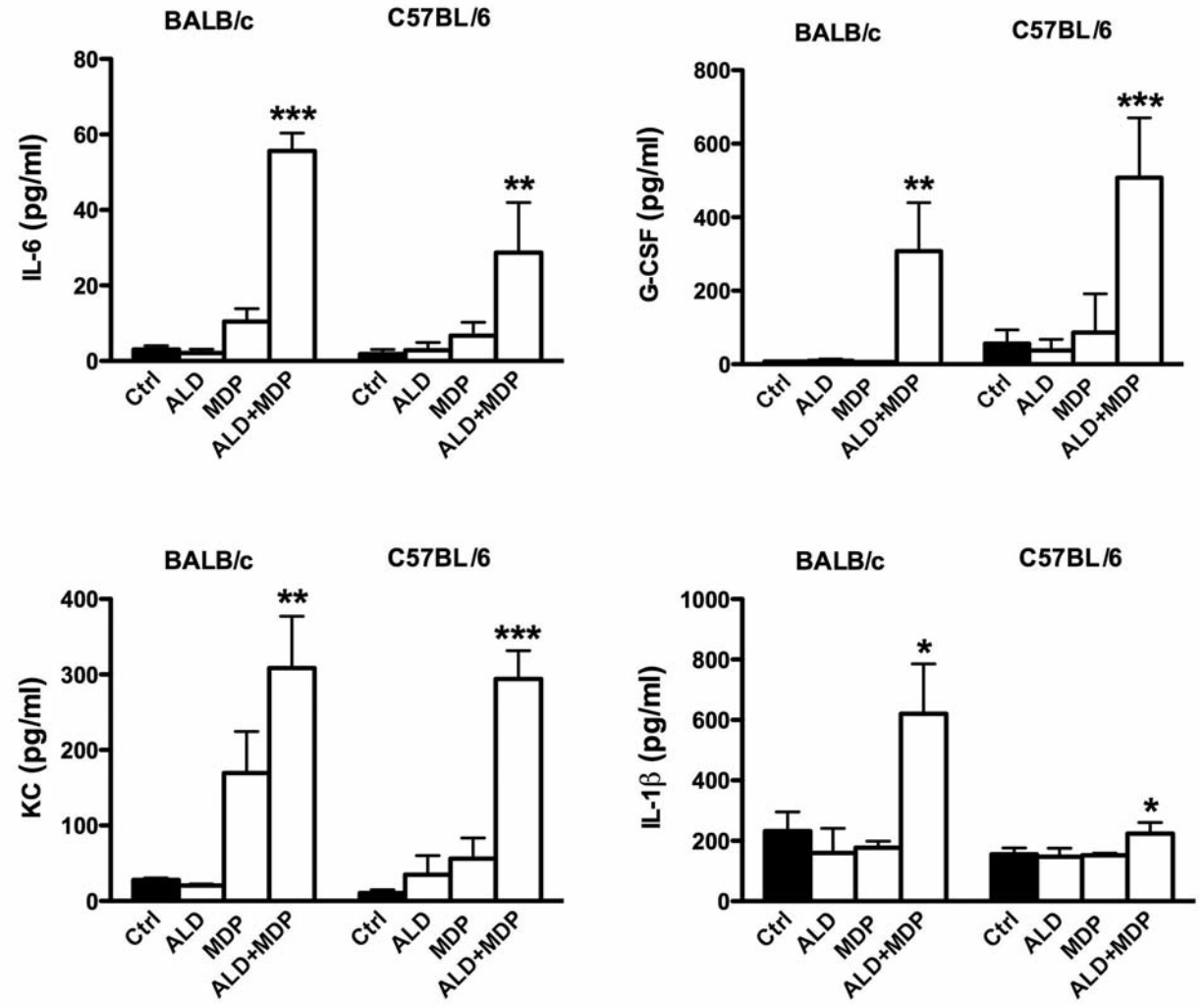

Figure 3. *Cytokines and chemokines dysregulated after treatments. Mice from each strain (BALB/c, n=20; C57BL/6, n=20) were divided into four groups: Ctrl (control animal, saline), alendronate (ALD, 13 mg/kg), muramyl dipeptide (MDP, 100 μg/kg), and ALD plus MDP. Compounds were intraperitoneally injected. Interleukin-6 (IL-6), granulocyte colony stimulating factor (G-CSF), keratinocyte chemoattractant (KC) and interleukin-1β (IL-1β) were found to be significantly up-regulated in the ALD-plus-MDP group when compared to the control group. This trend was consistent for all these molecules in both strains. *p<0.05; **p<0.01; ***p<0.001 after one way ANOVA test followed by the Bonferroni correction for multiple comparisons.*

in the present study we tested the biochemical block using two different mouse strains, BALB/c and C57BL/6. There were no remarkable differences in the inflammatory markers between the two different strains: ALD and MDP together were able to increase the inflammatory phenotype in BALB/c and C57BL/6 mice.

Indeed, general inflammatory markers, such as KC, G-CSF and SAA, spleen weight and leukocyte infiltration, as well as parameters linked more to MKD, such as IL-6, IL-1β and NALP3 activation, were affected in both strains in the same way. Different chemokines (KC and G-CSF), able to recruit and guide the migration and proliferation of immune cells (15), rose in concentration, even if only after the double stimulus of ALD with LPS. Indeed, it should be noted that LPS-alone is a necessary although not sufficient factor, since it acts only as a trigger for inflammation in a pathological environment (16), in this case the blockade of the mevalonate pathway.

Furthermore, even IL-1β and IL-6 were affected in a consistent way in both BALB/c and C57BL/6 mice. These two cytokines are thought to be strictly involved in MKD (5, 17), and in particular the IL-1 family; accordingly, several biological therapies are successfully targeted at these molecules (18, 19).

To our knowledge, this is the first time that ALD was shown to be effective in the brain as well. Our immunohistochemical

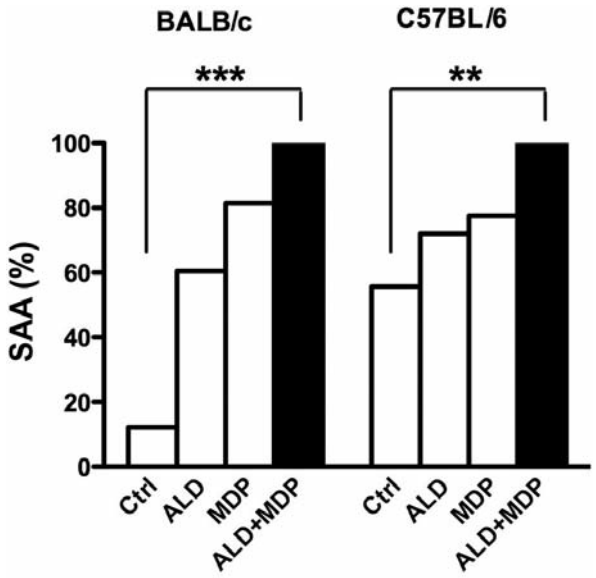

Figure 4. *Effects of treatments on serum amyloid A levels. Mice from each strain (BALB/c, n=20; C57BL/6, n=20) were divided into four groups: Ctrl (control animal, saline), alendronate (ALD, 13 mg/kg), muramyl dipeptide (MDP, 100 μg/kg), and ALD plus MDP. Compounds were intraperitoneally injected. Data are reported as the percentage compared to that of the double treatment, considered as 100% of SAA production. **p<0.01; ***p<0.001 after one way ANOVA test followed by the Bonferroni correction for multiple comparisons.*

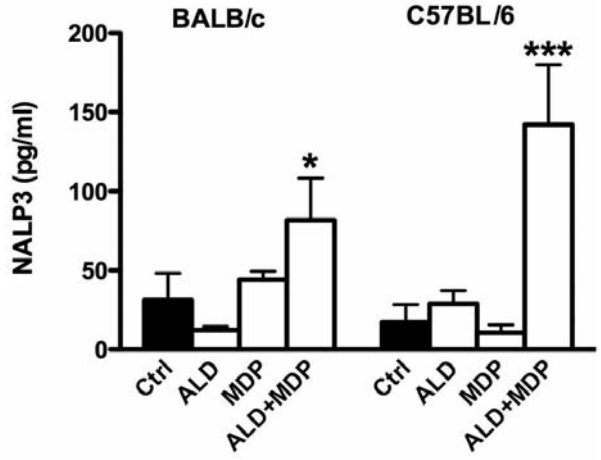

Figure 5. *Effects of treatments on serum domain found in NAIP, CIITA, HET-E, and TP-1 (NACHT), leucine rich repeats (LRR) and pyrine domain (PYD) domains-containing protein 3 (NALP3) levels. Mice from each strain (BALB/c, n=20; C57BL/6, n=20) were divided into four groups: Ctrl (control animal, saline), alendronate (ALD, 13 mg/kg), muramyl dipeptide (MDP, 100 μg/kg), and ALD plus MDP. Compounds were intraperitoneally injected. Double treatment significantly increased levels of NALP3 in both strains compared to the respective control groups. *p<0.05; ***p<0.001 after one way ANOVA test followed by the Bonferroni correction for multiple comparisons.*

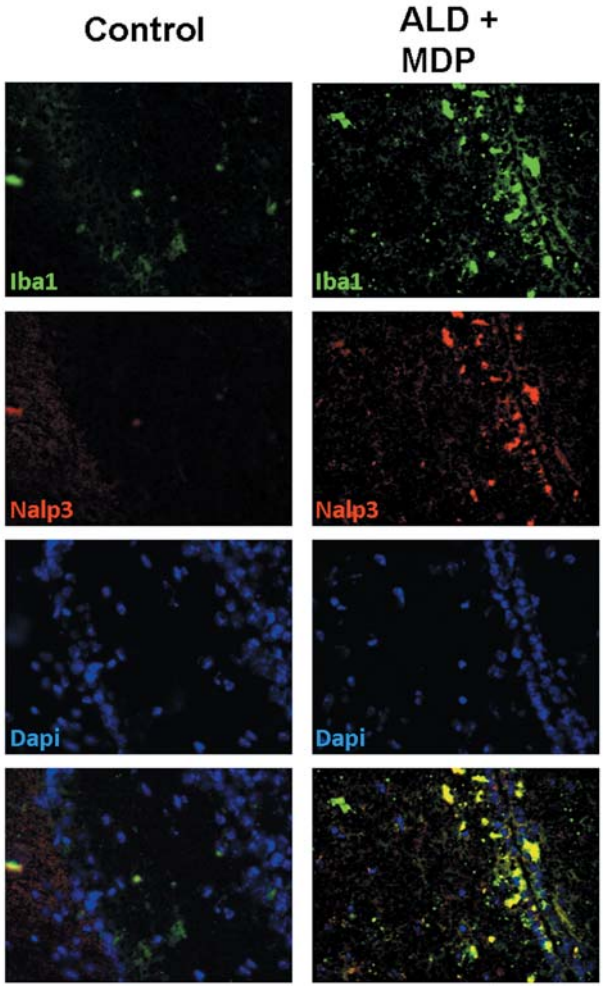

Figure 6. *Immunostaining reporting cerebellar portions of both a control and a double-treated BALB/c mice (left and right panel, respectively). Slices were treated in order to reveal the microglial protein ionized calcium-binding adapter molecule-1 (IBA1; green), the protein of the inflammasome domain found in NAIP, CIITA, HET-E, and TP-1 (NACHT), leucine rich repeats (LRR) and pyrine domain (PYD) domains-containing protein 3 (NALP3; red) and nuclei (4',6-diamidino-2-phenylindole, DAPI; blue). After the double stimulus, microglial cells are activated and start to produce NALP3, in contrast to what happens in the controls. No differences were noticed between brains in BALB/c and C57BL/6 (data not shown).*

analysis demonstrates that NALP3 is present in microglial cells in the cerebellum, while the presence of NALP3 has been previously demonstrated in the whole brain (20), and we found that its expression could be modulated by ALD and MDP. For the first time we showed that NALP3 expression increases after the biochemical blockade of the mevalonate pathway in neuronal tissue, highlighting its importance not only at the systemic level (monocytes) but also in the central nervous system. Microglial cells are from the same lineage as

monocytes (21) and from our results we conclude that in our experimental setting they behave as their circulating counterpart. Moreover we did not find any difference between the two strains examined, thus it is possible that this represents a common mechanism in neuroinflammation. Furthermore, KC and G-CSF, besides their classical role, seem to be active in the nervous system, since several studies reported that both play a role in neuroprotection (22-24).

In conclusion, all the evaluated parameters were comparable between BALB/c and C57BL/6 mice: no differences were observed in markers previously evaluated only in BALB/c mice (SAA, Splenic morphology, cytokines and chemokines) (10, 25), nor in the modulation of a new molecular target potentially involved in MKD, NALP3. Thus, we can reasonably assume that the effects seen in our MKD animal model are caused by the treatments given to the mice, regardless of their different strains.

These findings could, therefore, be the starting point to assess the effect of different drugs for the treatment of MKD using a consistent mouse model, characterized by the activation of inflammasome in the central nervous system, a parameter that is crucial in order to evaluate the efficacy of treatments in the most severe form of MKD.

Conflicts of Interest

The Authors declare that they have no competing interests.

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