Journal of Inorganic Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

# Aluminum induces inflammatory and proteolytic alterations in human monocytic cell line

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ARTICLE INFO

*Article history:*
Received 11 May 2015
Received in revised form 18 September 2015
Accepted 18 September 2015
Available online xxxx

*Keywords:*
Aluminum chloride
Imject Alum
Cytokines
Chemokines
Matrix metalloproteinases
Multiplex immunoassay

ABSTRACT

The increasing exposure to aluminum has been linked with the development of different human pathologies (e.g., breast cancer, myofasciitis, neurodegenerative diseases), probably due to the consistent presence of aluminum salts in widely diffused cosmetic products and vaccines. However, the mechanisms underlying immunologic and proliferative alterations still remain unknown.

In the present study we investigated the ability of different aluminum compounds (i.e., aluminum chloride vs Imject® Alum, a mixture of aluminum and magnesium hydroxide) to trigger both inflammatory and proteolytic responses in U-937 human monocytic cell line. We demonstrated, by multiplex immunoassay analyses, that monocytic cells treated with both Imject Alum and aluminum chloride showed different and peculiar expression profiles of 27 inflammatory mediators and 5 matrix metalloproteinases, with respect to untreated control cells. In particular, we found dose-dependent significantly increased levels of pro-inflammatory cytokines, growth factors, and chemoattractant chemokines; whereas among metalloproteinases, only collagenolytic protease showed a significant dose-dependent increase in Imject-treated cells with respect to controls and Al-chloride treated cells. Noteworthy, we found only in Imject Alum-treated cells the significant positive correlations among collagenolytic metalloproteinase and increased expression of pro-inflammatory chemokines, suggesting a possible involvement of aluminum in regulating the acute inflammatory responses.

In agreement to emerging evidences, for the first time we demonstrated that the treatment of monocyte cells with aluminum-based adjuvant is able to induce an inflammatory status and a proteolytic cascade activation. In fact, the cell treatment with Imject Alum induced increased levels of several cytokines and proteinases, suggesting these monocyte mediators as possible biomarkers for aluminum-linked diseases. The identification of the biochemical pathways involved in Al-induced cell injury pave the way for improving the knowledge on the potential impact of aluminum in human physio-pathology.



## 1. Introduction

Aluminum represents a potential toxic element which has been linked to a plethora of human diseases, mainly through wide and continuous exposure by water, food, cosmetics and drugs containing significant amount of this metal. However, no physiological function has been associated with its intake and exposure. On the other hand, evidence supports the association between aluminum accumulation in human body and the development of a variety of pathological conditions, such as dialysis dementia, Alzheimer and Parkinson diseases, breast cancer, myofasciitis, osteomalacia and microcytic anemia [1].

Aluminum compounds and aluminum-based adjuvants have been widely investigated in vivo and in vitro to identify their possible involvement (role and mechanisms of action) in several human pathologies.

Aluminum compounds (such as Al chloride, Al nitrate, Al sulfate, Al hydroxide) are usually found in cosmetic formulations (e.g., deodorants, antiperspirants), antacid drugs, vaccine adjuvants, water and food treated or cooked with aluminum utensils [2,3]. The exposure to aluminum compounds has been related to some biochemical and metabolic alterations. In fact, several studies described pro-oxidant effects [4], modifications of the essential metal homeostasis [5], DNA double strand breaks [6], and altered release of some cytokines related to the main inflammatory pathways [7,8].

Aluminum-based adjuvants, commonly named "Alum", are the most common immune-stimulants found in widespread formulations of human vaccinations [9]. In particular, Alum has been used in human

*Abbreviations:* BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; IFN-γ, interferon gamma; IP-10, interferon gamma-induced protein 10; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PDGF, platelet derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factors alpha; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.jinorgbio.2015.09.009
0162-0134/

vaccines against diphtheria, hepatitis A and B and papilloma virus [10,11], in order to potentiate both the efficacy of weak antigens and shape the immune response [10].

Alum is aluminum compounds dispersed in water to form heterogeneous suspensions or gels of hydrated colloid particles that consist of micron-sized aggregates [12–14].

The term "Alum" includes several formulations of aluminum-based adjuvants [15]. One of them, Imject® Alum, is commonly used in animals as a new alternative adjuvant [16–18]. Although it has been tested for immunogenicity [19–21], and adjuvanticity [15,22–25], it is not licensed for human immunization.

Even if Imject Alum, a combination of aluminum and magnesium hydroxide, is not used in commercially available vaccines, this study aimed to compare the specific cellular and biochemical effects of aluminum chloride vs Imject Alum, on the basis of some preclinical immunological studies, highlighting the strong capacity of aluminum salts to induce inflammatory responses [3,9,25].

Although there is a paucity of data about the mechanism promoting humoral responses [9], the uptake and cellular internalization of aluminum-based adjuvants by THP-1 monocytic cells have been recently demonstrated [26,27].

Furthermore, the presence of aluminum has been confirmed in the skin [28], in subcutaneous nodules of children after injection of vaccines containing aluminum hydroxide [29]. Intramuscular administration in cynomolgus monkeys of a Diphtheria–tetanus vaccine containing aluminum oxyhydroxide or aluminum hydroxyphosphate-based adjuvants provoked an increased aluminum concentration in the injection site [30]. Finally, it has also been demonstrated the presence of Alum crystalline inclusions in macrophages [31], suggesting their intracellular uptake via phagocytic mechanisms [27,32,33].

The altered metabolic pathways induced by aluminum have not been discovered and/or completely understood; although this metal is recognized as biochemically highly reactive, it has been suggested that aluminum is critical for activating and driving the inflammosome, as well as apoptosis and modifications of nucleic acids in different cellular models [15,34,35].

On the basis of these evidences, the aim of this study was to investigate if Imject Alum and $AlCl_3$ could modulate both the inflammatory and proteolytic profiles of U-937 human monocytic cell line, evaluating the release in culture media of 32 biomolecules, including cytokines, chemokines, growth factors and matrix metalloproteinases (MMP).

## 2. Methods

### 2.1. Cell culture and treatments

U-937 cells (ATCC® CRL 1593.2™) were cultured in a complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% antibiotics). The monocyte cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. To detect the biomarkers released from monocytes treated with aluminum salts, we performed experiments in serum free media. Monocytic cells were cultured until confluence, and subsequently exposed to increasing concentrations of $AlCl_3$ (25, 50, 100, 200 μM) or Imject® Alum (25, 50, 100, 200 μg/ml) for 24 h. In particular, we selected the μM range for aluminum chloride and μg/ml range for the Imject Alum, in full agreement to previous studies demonstrating that at these concentrations aluminum salts are able to induce cellular and biochemical alterations [6,15].

Untreated U-937 monocytic cells, maintained for 24 h in serum free culture media, were used as control. Each of the cell treatments was performed in duplicates in 96 well plates, with a final volume of 200 μl per well. Each experiment was carried out twice, and intra- and inter-experiment variabilities were evaluated for the statistical analyses. The number of intact cells after co-culture with and without aluminum compounds was counted through the trypan blue exclusion test (commonly used method to enumerate the amount of viable cells).

To correctly evaluate the proteolytic and inflammatory pathways in U-937 monocytic cell line after the treatment with aluminum chloride and Imject Alum adjuvant, we treated $0.8 \times 10^6$ cells with 1 μg/ml of lipopolysaccharide (LPS) in a serum-free medium at 37 °C during 24 h in culture dishes [36].

### 2.2. Biochemical determinations

Total protein concentrations in serum-free media were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Milan, Italy).

Cytokine and MMP concentrations have been analyzed by BioPlex multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with a monoclonal antibody specific for the target proteins, according to the manufacturer's instructions (Bio-Rad Lab, Hercules, USA) [8].

Serum free culture media from U-937 cells have been analyzed for the cytokine content using the 27-plex panel of Pro™ Human Cytokines, including IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α and VEGF.

The evaluation of the MMP in serum free culture media has been performed by using the 5-plex panel of Human MMP Panel 2 (including MMP-1, MMP-2, MMP-7, MMP-9, MMP-10).

Levels of cytokines and MMPs were determined using a Bio-Plex 200 array reader, based on Luminex X-Map Technology (BioRad, Hercules, USA) that detects and quantifies multiple targets in a 96-well plate within a single small fluid volume (~50 μl).

Analysis of each sample was performed in duplicate. Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v.6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve; the lower detection limit was 0.6 pg/ml for the cytokines and 2.0 pg/ml for the MMPs, according to the manufacturer's indications (Bio-Rad, Hercules, USA) [8].

### 2.3. Statistical analysis

Each variable was expressed as the mean ± SD, unless otherwise specified. The differences were analyzed and compared by the chi-squared test or 2way-ANOVA, Student t-test or Mann–Whitney test, according to variable parametric or non-parametric characteristics, respectively. Pearson's or Spearman's correlations explored the relationships of variables. All statistical tests were two-tailed, and the significance was set at $p < 0.05$. Data were analyzed with Prism software for Windows-7, version 3.1 (Graph-Pad, San Diego, USA).

## 3. Results and discussions

### 3.1. Assay controls

According to the manufacturer's protocols and indications, the intra- and inter-assay CVs of serum-free medium samples containing aluminum compounds were always below 10%. The curve generated using spiked samples paralleled the standard curve (data not shown). All these data suggest that the "matrix" of serum-free media containing aluminum compounds did not affect the metalloproteinases and cytokines immunoassay performance, even if originally developed for plasma/serum specimens.

Aluminum compounds induce different cell viabilities.

The aim of the resent study was to investigate the possibility of using the human monocytic cell line U-937 as a relevant in vitro model to evaluate the effects of $AlCl_3$ and Imject Alum on the release in serum-

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free culture media of 32 biomolecules related to inflammatory and proteolytic pathways.

The human U-937 monocytic cell line has been utilized in several studies investigating immune function and inflammosome activation by Al compounds [37,38], even though some previous studies on Al adjuvants have been performed mainly in monocytic THP-1 cell line and peripheral blood mononuclear cells [15,33].

As shown in Fig. 1, the co-culture of U-937 monocytic cell line with increased concentrations of $AlCl_3$ did not significantly alter the cell viability, whereas the treatment of cells with Imject Alum resulted in a dose-dependent reduction of the amount of growing cells (cell number based on the results of the trypan blue exclusion test). Moreover, we did not observe any evident variation of pH indicator in RPMI 1640 culture media after the aluminum salt addition up to final concentration of 200 μg/ml, neither in controls nor in tests.

Our data are in agreement with previous fluorescence microscopy studies demonstrating that cell cytoplasm of THP-1 monocytic cell line co-cultured in the presence of the Imject Alum adjuvant (200 μg/ml) showed a significantly higher concentration of intracellular aluminum and a consistent loss of cell integrity in comparison to native untreated cells [27].

### 3.2. Aluminum-linked interleukins release

#### 3.2.1. Pro-inflammatory cytokines

Serum free culture media collected from U-937 cells treated with $AlCl_3$ and Imject Alum adjuvant have been analyzed for the cytokine content by using the Bio-Plex 27-plex panel of Pro™ Human Interleukins, including pro- and anti-inflammatory cytokines, chemokines, and growth factors. For what concerns the 18 biomolecules differently involved in the common pro-inflammatory pathway, no significant difference between aluminum chloride and aluminum Imject adjuvant was found for the cytokine profile involving pro-inflammatory cytokines (IL-2, IL-6, IL-15, IL-7 and TNF-α), colony stimulating factors (G-CSF, GM-CSF) and chemokines (MIP-1β, Eotaxin).

On the contrary, the co-culture of U-937 monocytic cell line with Al compounds revealed an increased secretion of peculiar pro-inflammatory cytokines and chemokines in serum-free media with respect to untreated control cells (Fig. 2). In particular, Imject Alum adjuvant treatment induced in a dose-dependent manner significantly increased levels of the pro-inflammatory cytokines IL-1β, IL-12p70 and IFN-γ. Noteworthy, at the highest Al concentration (200 μg/ml), IFN-γ was statistically different from control cells ($p < 0.05$). Serum free media from Al chloride-treated cells showed dose-dependent increased levels of cytokines with respect to untreated controls, even though did not reach statistical significance level (Fig. 2a).

The co-culture of U-937 cells with Imject Alum adjuvant resulted in an increased level of C-C and CXC chemokines, such as IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2 ($p < 0.05$), MIP-1α/CCL3 and RANTES/CCL5 (Fig. 2b). In particular, RANTES/CCL5, MIP-1α/CCL3 and IP-10/CXCL10 chemokines resulted up to 1.5-fold higher in cells treated with 200 μg/ml of Imject Alum adjuvant with respect to untreated control cells; whereas, chemokines IL-8/CXCL8 and MCP-1/CCL2 showed up to three-fold higher levels of secretion of these pro-inflammatory molecules with respect to the untreated control cells ($p < 0.05$) (Fig. 2B).

Our present observations are in full agreement with literature data describing significantly increased expression and release of MCP-1/CCL2 in an alum neurotoxicity model [39]. Interestingly, Al chloride treated cells did not secrete significantly different amounts of chemokines with respect to control untreated cells, except for the chemokine RANTES, and pro-inflammatory cytokine IL-1β and IFN-γ.

Our data are in agreement with the paucity of observations on cytokines obtained with Al compound treatments, in particular for IL-1β, IL-6 and IL-8 [15,33,37,38].

#### 3.2.2. Anti-inflammatory cytokines and growth factors

Although the interleukins IL-5 and IL-7 with immuno-modulatory functions, and the anti-inflammatory cytokines IL-1ra, IL-4 and IL-9 were found not significantly modified by both aluminum compounds; in serum free media we detected also dose-dependent increased levels of both anti- inflammatory IL-10 and IL-13, with levels up to 1.5-fold higher with respect to untreated control cells at the highest aluminum dose of 200 μg/ml (Fig. 3a).

Among growth factors examined in our study by BioPlex technology, only the secretion of VEGF resulted at increased levels in serum free media of U-937 cells treated with Imject Al adjuvant, with dose-dependent concentrations up to 1.5-fold higher with respect to control cells ($p < 0.05$) (Fig. 3b). Levels of VEGF measured in serum free media from Al chloride treated cells were not statistically different from control cells (Fig. 3b). No significant differences were also found for the other growth factors present in the 27-plex immunoassay kit (bFGF and PDGF-bb) (data not shown).

### 3.3. Extracellular secretion of matrix metalloproteinases (MMP) by aluminum compounds

The evaluation of the MMP release in serum free culture media collected after the treatment of U-937 with and without aluminum chloride and Imject Alum adjuvant has been performed using the 5-plex panel of Human MMP Panel 2 (including MMP-1, MMP-2, MMP-7, MMP-9, MMP-10).

The secretion of MMP-2 (Gelatinase A; EC 3.4.24.24), MMP-9 (Gelatinase B; EC 3.4.24.35), and MMP-10 (Stromelysin-2; EC 3.4.24.22) was found not significantly affected by Al compounds with respect to control cells (data not shown).

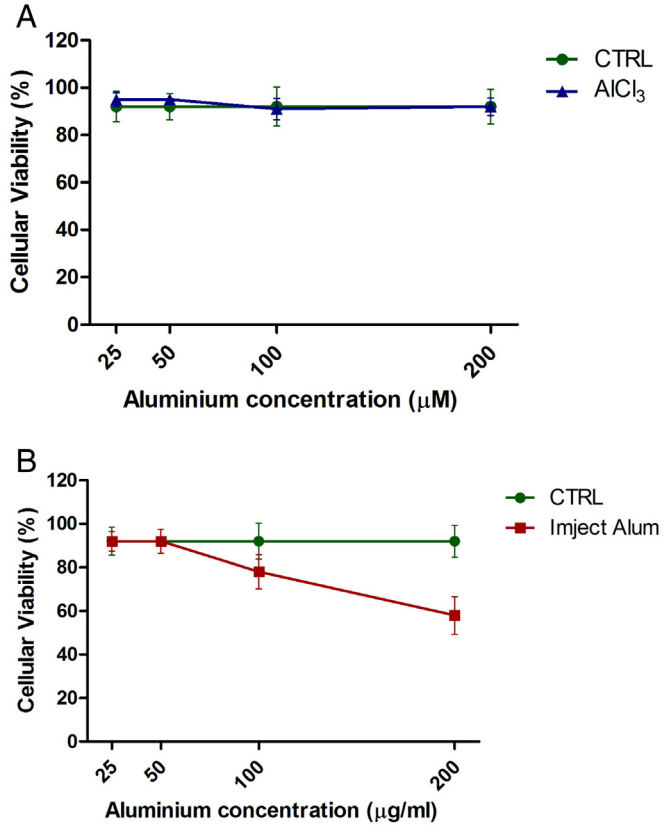


**Fig. 1.** Percentage of viable U-937 cells after a co-culture with Al-chloride (A) and Imject Alum adjuvant (B), with respect to the untreated control cells.

On the other hand, the treatment of monocytic cells with aluminum chloride did not significantly alter the release of MMP-1 (Collagenase 1; EC 3.4.24.7) with respect to control cells, whereas the LPS induced an increased secretion of this collagenolytic protease in the culture media, up to two-fold the amount found in untreated cells (Fig. 4). Noteworthy, the treatment of monocytic U-937 cells with Imject Alum adjuvant showed a significant dose-dependent increase of MMP-1 protease release in the extracellular milieu, up to 4.5-fold higher than that found in control cells ($p < 0.05$) and two-fold higher the amount observed after LPS treatment (Fig. 4). In addition, MMP-1 levels detected in culture media of Imject Alum-treated U-937 cells were found also significantly increased with respect to $AlCl_3$ treated cells ($p < 0.05$), whose levels were found similar to control cells (Fig. 4a).

Moreover, at lower concentrations of both Al compounds a similar dose-dependent increase of extracellular secretion of MMP-7 (Matrilysin 1; EC 3.4.24.23) (Fig. 4b) was found. In fact, whereas LPS treatment did not alter the ability to secrete MMP-7 in U-937 cells, both Al compounds are capable to induce specific release of this extracellular matrix degradative protease with up to 1.3-fold higher level with respect to untreated control cells.

The only literature data linking aluminum and MMPs demonstrated that alumina ceramics induced MMP-2 mRNA expression osteoblast cells [40]; and that the aluminum endoprostheses stimulated the specific secretion of MMP-2 and MMP-9 in the interface tissues between bone and hip implants [41].

Up to now, our study is the first evidence that Imject Alum was able to stimulate in human monocytes the extracellular release of peculiar MMPs.

### *3.4. Correlation between matrix metalloproteinases and cytokines induced by aluminum compounds*

The statistical analyses of our data, allowed us to detect two main statistically significant correlations. As depicted in Fig. 5, we found that increasing concentrations of Imject Alum adjuvant were significantly correlated to increased levels of the chemokine IL-8/CXCL8

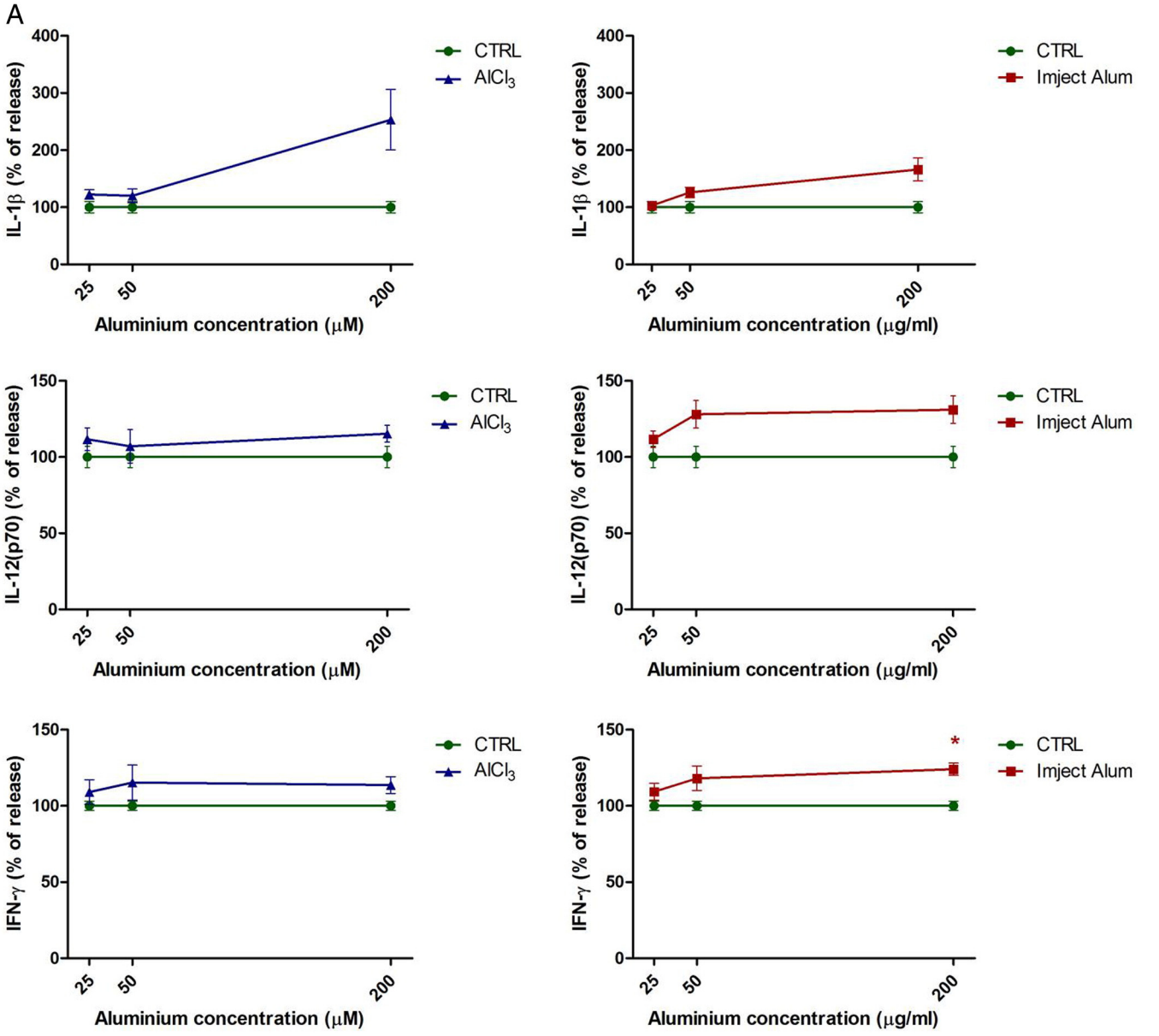


**Fig. 2.** Dose-dependent release of pro-inflammatory cytokine (A) and chemokines (B) from monocyte cells treated with different aluminum compounds vs untreated control cells. The values are expressed as percentage with respect to control (* = $p < 0.05$).

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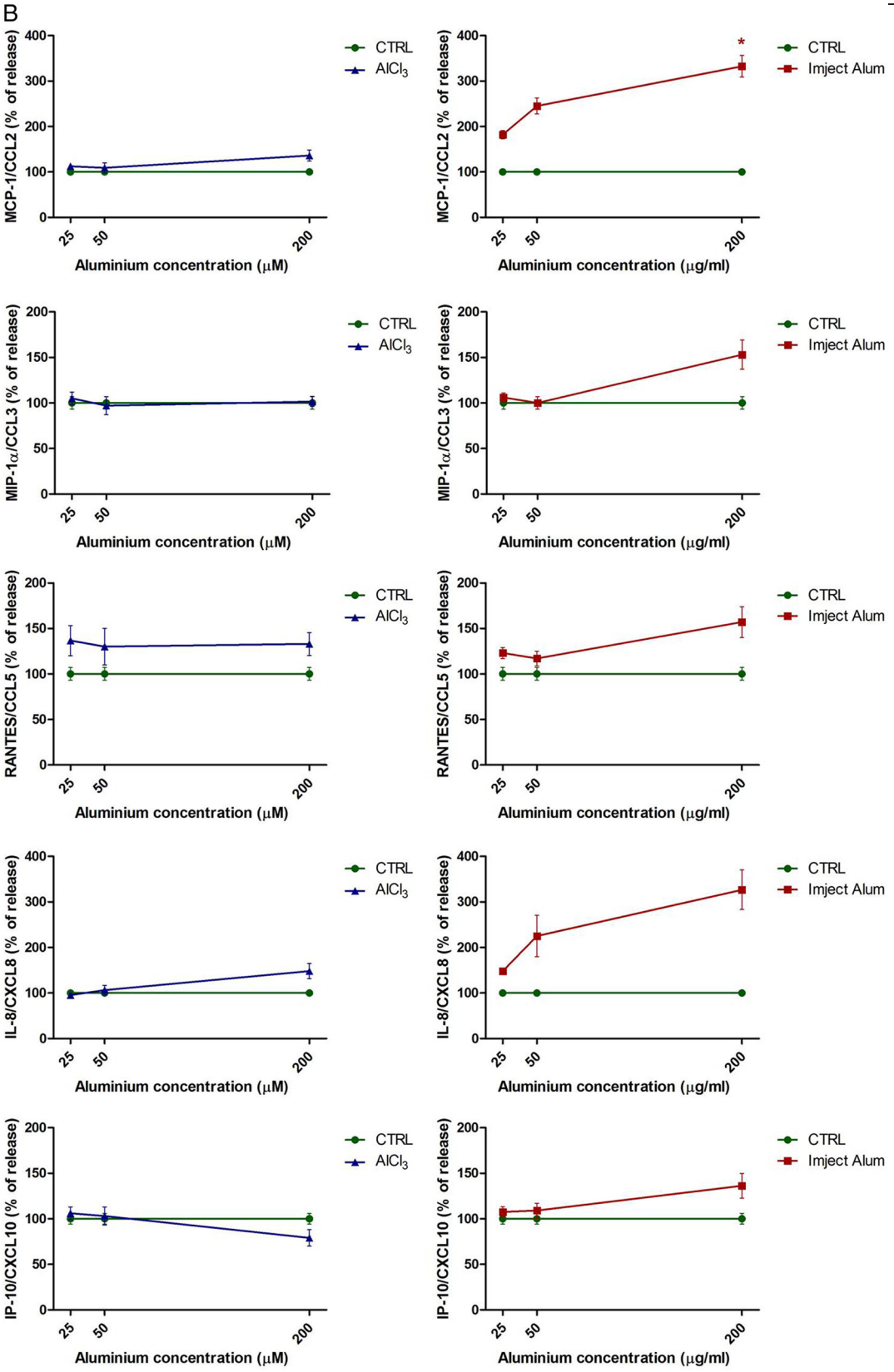


**Fig. 2** (*continued*).

(with pro-inflammatory function) and collagenase MMP-1 ($r^2 = 0.9937$). In the same way, we found a significant positive correlation between MMP-1 levels and the amount of chemokine MCP-1/CCL2 ($r^2 = 0.9958$). Our data suggest that aluminum Imject adjuvant is able to significantly induce both proteolytic and inflammatory mechanisms, releasing extracellularly crucial signaling biomolecules involved in a synergistic manner in inflammosome and protease network.

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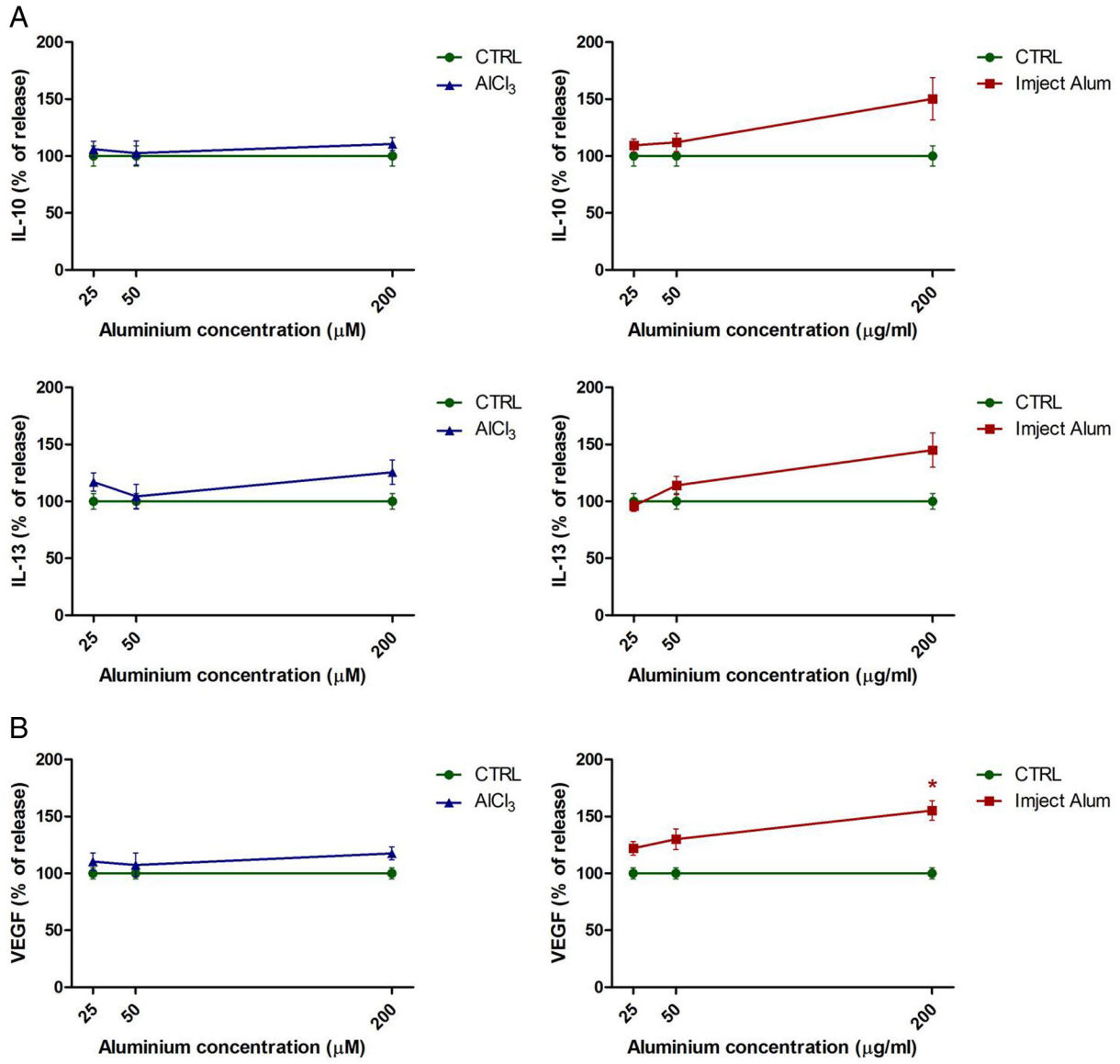


**Fig. 3.** Percentage of extracellular secretion of anti-inflammatory cytokines (A) and growth factor VEGF (B) after treatment of U-937 monocyte cells with increased concentrations of Al-chloride and Imject Alum adjuvant (* = $p < 0.05$).

## 4. Conclusions

Our results revealed that human monocytic cells treated with aluminum-based adjuvant release into the extracellular microenvironment both proteolytic enzymes and inflammatory factors, at significantly higher levels than that found in untreated and LPS-stimulated monocytic cells.

Although some studies have highlighted that the in vitro treatment of both human monocytic cell lines and peripheral blood mononuclear cells with aluminum-based adjuvants may be able to induce the intracellular accumulation of aluminum [27], as well as the extracellular release of some pro-inflammatory cytokines (e.g., IL-1β, IL-18,IL-10) [15,33,37,38], the main biological and biomolecular implications of aluminum accumulation within monocytes remain to be elucidated.

For the first time, our study demonstrated that the treatment with aluminum-based adjuvant induced monocytes to significantly release in a dose-dependent manner higher amounts of interleukins and matrix metalloproteinases in extracellular microenvironment with respect to untreated cells, with peculiar profiles of interleukins (in particular, pro-inflammatory cytokines and CC/CXC chemokines) which significantly correlated to higher levels of specific extracellular matrix proteases.

Although our results do not support an "absolute" cause-effect relationship among inflammatory/proteolytic pathways and aluminum Imject adjuvant treatment, our data are in agreement with the previous demonstration that aluminum compounds (in particular, Imject aluminum-based adjuvant) may accumulate within the monocyte cells [27]. Here we demonstrated that Imject Alum was able to induce in a dose-dependent manner the release of both inflammatory signal molecules (i.e., cytokines, chemokines and growth factors) and proteolytic enzymes (members belonging to the matrix metalloproteinases family).

Literature data described that tissue injection or the cell treatment with aluminum-based adjuvants may be able to accumulate aluminum within the monocyte cells [27], activate the complement cascade and immune functions [42], then inducing the secretion of some pro-inflammatory interleukins [15,33], and the translocation of aluminum particles in distant organs by MCP-1/CCL2 mechanism [39].

So, our results suggest a panel of biomolecular markers useful for a deeper understanding of the mechanisms regulating immunostimulatory properties of aluminum-based adjuvants, highlighting these signal

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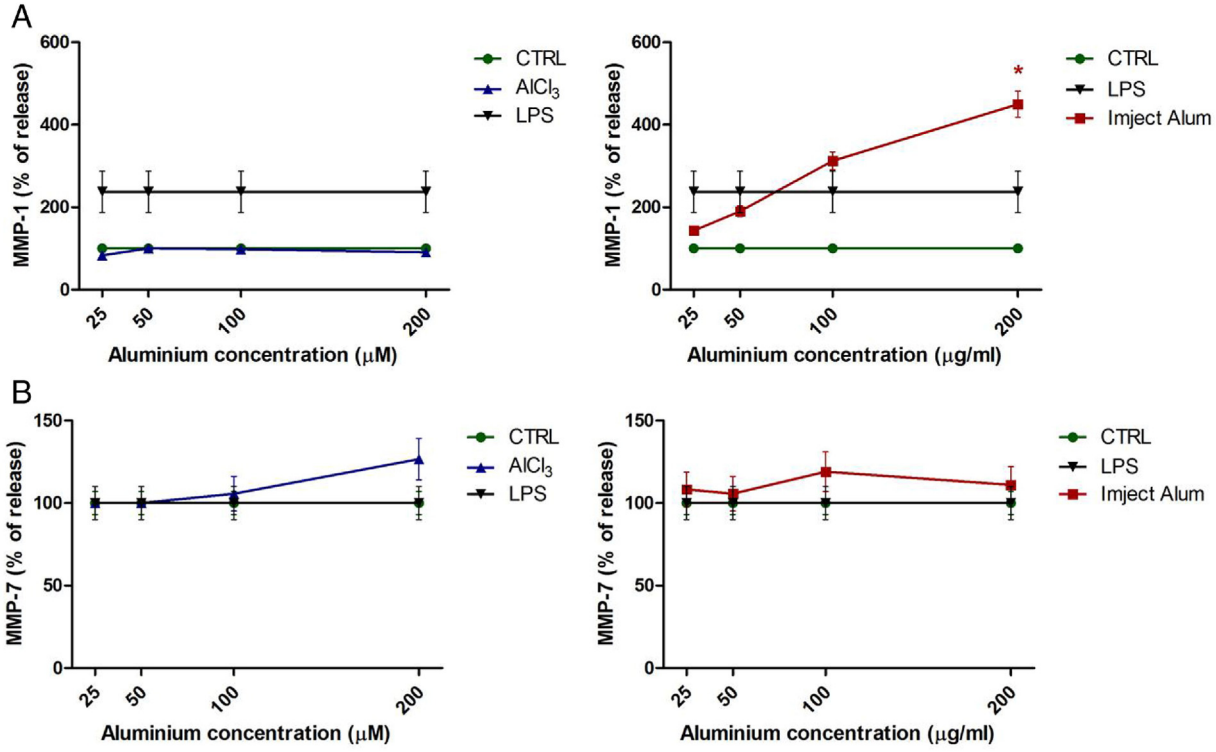


**Fig. 4.** Release in percentage of matrix metalloproteinase-1 (A) and MMP-7 (B) in human U-937 monocyte cells treated with increased concentrations of aluminum compounds (co-culture with Al-chloride and Imject Alum adjuvant, with respect to the untreated control cells. (Imject Alum vs Ctrl * = $p < 0.05$).

biomolecules as possible coordinated risk factors hypothesizing the rational design of safer vaccines.

The previous unequivocal identification of monocyte intracellular aluminum-based adjuvant [27] and our present results about the significant Imject Alum-related secretion of cytokines and proteases, add novel pieces of the puzzle for understanding the adjuvanticity of aluminum-based adjuvants administered in human vaccinations.

Noteworthy, it has been previously demonstrated that inflammatory mediators (like pro-inflammatory cytokines and chemokines) secreted after aluminum-based adjuvant treatment of monocyte/macrophage were identified and involved in several human diseases (e.g., myalgia, chronic fatigue and cognitive dysfunctions [3,39]. Our novel findings of peculiar matrix metalloproteinases secretion after aluminum-based adjuvant treatment of monocyte/macrophage and its significant positive correlations with pro-inflammatory chemokines, provide insights on biomolecular pathways linked to the aluminum adjuvant adverse effects.

Finally, it is noteworthy that only after aluminum-based adjuvant treatment of U-937 monocyte cells we were able to reveal the macrophage chemoattractant chemokines (i.e., MCP-1/CCL2), highly released in monocyte microenvironment in correlation with collagenolytic extracellular matrix proteases, and not after aluminum chloride powder resuspended in PBS. The aluminum-based adjuvant accumulation in monocyte microenvironment may be possibly related to increased protein oxidative stress, proteolytic processes and inflammatory status, suggesting a complex network predisposing tissues toward inflammatory promotion, initiation and progression of tissue proteolytic degeneration.

In this respect, a possible explanation for the divergent response intensity observed in our study for the two tested aluminum compounds may be linked to the physico-chemical properties of aluminum salts. The particulate nature of Imject Alum (respect to the more soluble nanoparticle composition of aluminum chloride solution) may be responsible for the previously described phagocytosis stimulation and intracellular accumulation of aluminum [15,27,33,39]. In particular, it

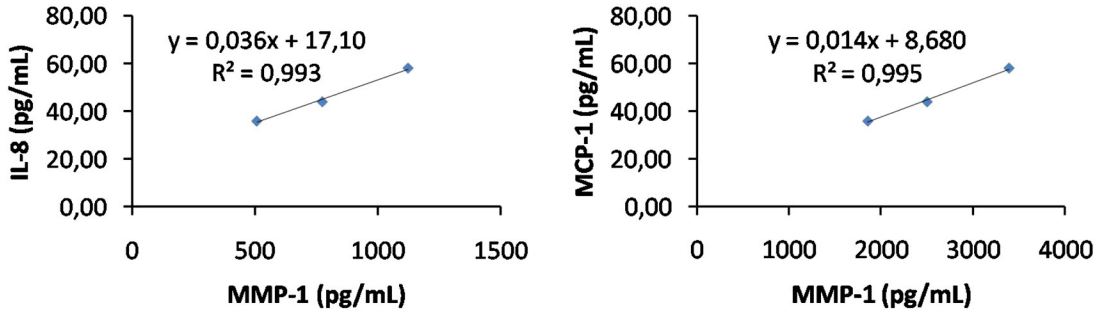


**Fig. 5.** Linear regression analyses of the correlations among levels of matrix metalloproteinase-1 (MMP-1) and pro-inflammatory chemokine IL-8/CXCL8 ($r^2 = 0.9937$, $Y = 0.036 \times +17.104$), and monocyte chemo-attractant MCP-1/CCL2 ($r^2 = 0.9958$, $Y = 0.0144 \times +8.6809$) in U-937 human monocyte cell line treated with increased concentrations of Al-chloride and Imject Alum adjuvant.

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is well known that several physicochemical parameters of Imject alum (such as particle size distribution, electrical charge, and the hydrated colloid nature of the precipitate), affect the adjuvanticity of alum preparation [15].

Our data, supported also by other recent evidences [3,39], suggest crucial roles of macrophage recruitment, inflammatory cytokine and chemokine release in patients with systemic/neurologic manifestations or autoimmune inflammatory syndrome induced by adjuvants [39], highlighting that exogenous factors (like tissue injury by aluminum injection) may induce ROS-linked interleukins, protease release and macrophage activation, predisposing tissue to "inflammatory phenotype" with a higher risk of subsequent disease evolution [3].

According to possible limitations of our study, we have not the "exact" significance for the observed positive correlation between MMP-1 and chemokine CCL-2/MCP-1; but we may provide only some hypotheses. It is well known that, after inflammatory stimuli, monocyte/macrophage cells are able to actively secrete CCL-2/MCP-1 chemokine and also to release extracellularly several MMPs, including MMP-1 [43,44]. In our study, we described for the first time that Al salts may be able to induce the secretion of several metalloproteinases and cytokines, and that the extracellular release of both MMP-1 and macrophage chemoattractant chemokine may be significantly and positively correlated. A possible explanation is that during inflammation stimuli macrophage may secrete matrix metalloproteinase-1 with extracellular matrix degradative potential, and also capable to release peculiar chemokines useful to recruit further monocytes, likely assisting in orchestrating the regulation of acute inflammatory responses [43].

Noteworthy, it has been previously demonstrated that adjuvants alum salts may be able through CCL-2 and CXCL8 involvement to induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells, suggesting that during vaccination, adjuvants may increase recruitment of immune cells into the injection site, accelerating monocyte differentiation into dendritic cells, increasing antigen uptake, and enhancing adaptive immune responses [45].

Although a cause–effect relationship between Al-based adjuvant and systemic/neurologic/autoimmune/inflammatory syndrome has not been completely clarified and understood [3], our findings suggest that aluminum-based adjuvant may play a biomolecular role in the development of immune-related human diseases. Further experimental and clinical investigations are needed to discern the possible effects of aluminum-based adjuvant intracellular accumulation.

## Conflict of interest statement

The authors have no conflict of interest.

## Acknowledgments

We would like to thank Prof. C. Exley (The Birchall Centre, Lennard-Jones Laboratorie, Keele University, Keele, Stafford-Shire, ST5 5BG, UK) for the willingness and courtesy to supply the Imject Alum compounds.

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Please cite this article as: D. Ligi, et al., Aluminum induces inflammatory and proteolytic alterations in human monocytic cell line, J. Inorg. Biochem. (2015), http://dx.doi.org/10.1016/j.jinorgbio.2015.09.009

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Please cite this article as: D. Ligi, et al., Aluminum induces inflammatory and proteolytic alterations in human monocytic cell line, J. Inorg. Biochem. (2015), http://dx.doi.org/10.1016/j.jinorgbio.2015.09.009