Highlights

- Alum increases the specific antibody titers to KLH in gilthead seabream and European seabass.
- Alum and Freund's adjuvants induce the expression of gene coding for pro-inflammatory interleukin-1β.
- Alum fails to promote interleukin-1β release by seabream leukocytes.
- Alum increases NADPH oxidase-dependent reactive oxygen species (ROS) production and cell death.
- Damage-associated molecular patterns release from dying cells may mediate Alum adjuvant activity.

Aluminum is a powerful adjuvant in teleost fish despite failing to induce interleukin-1β release

Diego Angosto[1], Azucena López-Muñoz[1], Alicia García-Alcazar[2], José Meseguer[1], María P. Sepulcre[1], Victoriano Mulero[1]

[1]Departamento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, IMIB-Arrixaca, 30100 Murcia, Spain.

[2]Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n, Puerto de Mazarrón, 30860 Murcia, Spain

Address correspondence and reprint requests to Prof. Victoriano Mulero, Departamento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, 30100 Murcia, Spain. E-mail address: vmulero@um.es

Running title: Aluminum is a powerful adjuvant in teleost fish

Abstract

Although Aluminum salts (Alum) have been extensively used in human vaccination for decades, its mechanism of action is controversial. In fish, the use of Alum as a vaccine adjuvant is scarce and there are no studies aimed at identifying its mechanism of action. In the present study we report that Alum is a powerful adjuvant in the gilthead seabream (*Sparus aurata* L.*,* Sparidae) and the European seabass (*Dicentrarchus labrax* L., Moronidae). Thus, Alum increased the specific antibody titers to the model antigen keyhole limpet hemocyanin as the commonly used Freund's adjuvant did in both species. In addition, both adjuvants were able to increase the transcript levels of the gene encoding the major pro-inflammatory mediators interleukin-1β (Il1b). Strikingly, however, Alum failed to promote Il1b release by seabream leukocytes and even impaired Il1b induction, processing and release in macrophages. However, it increased NADPH oxidase-dependent reactive oxygen species (ROS) production in gilthead seabream leukocytes and purified granulocytes. In addition, Alum promoted gilthead seabream leukocyte death independently of ROS production and caspases, suggesting that damage-associated molecular patterns release from dying cells mediate Alum adjuvant activity. Our results pave the way for future studies aimed at investigating the relevance of danger signals generated by Alum *in vivo* on its adjuvant activity in order to increase our understanding of the mechanisms of action of Alum in fish vaccines and to help in the design of new adjuvants for aquaculture.

Keywords: Alum, adjuvants, vaccines, reactive oxygen species, cell death, teleost, fish.

1. INTRODUCTION

Over more than 80 years the aluminum salts, such as aluminum hydroxide, here referred as Alum, have been successfully used in vaccines to enhance the antigen-specific immune responses in combination with a specific antigen (Lindblad, 2004). However, the definitive mechanism by which Alum acts is unclear and several mechanisms have been proposed. The antigen depots is considered one of the principal mechanism of the Alum vaccines action (Mannhalter et al., 1985) but this hypothesis has been questioned since other studies have demonstrated that the depot formation by Alum is not required for its adjuvanticity (Hutchison et al., 2011). Alum interaction with dendritic cell membrane lipids mechanism has also been proposed (Flach et al., 2011) but not confirmed by other studies. It is also known that Alum is able to activate caspase-1 within the inflammasomes leading to the release of interleukin-1β (IL-1β) and IL-18 (Li et al., 2007; Sokolovska et al., 2007). Several studies confirmed the ability of Alum to activate the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome through phagosomal destabilization (Hornung et al., 2008) and the indispensability of this activation for Alum-mediated adjuvanticity (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008). In addition, other studies reported that although the NLRP3 inflammasome is crucial for Alum-mediated IL-1β secretion, is dispensable for alum adjuvant activity (Franchi and Nunez, 2008; Kuroda et al., 2011; McKee et al., 2009). Finally, the release of endogenous danger signals, such as urid acid (Kool et al., 2008) and DNA (Marichal et al., 2011; McKee et al., 2013), in response to alum crystals has also been proposed as the mechanism responsible for its adjuvanticity by NLRP3 inflammasome-dependent and independent mechanisms.

The evolutionary aspects of IL-1β secretion and inflammasome activation has received little attention in fish and the results obtained so far are controversial. Thus, none of the non-mammalian vertebrate IL-1β genes show conserved caspase-1 processing site (Bird et al.,

2002). In addition, although caspase-1 is not involved in the processing of IL-1β from gilthead seabream (*Sparus aurata*) and the classical activators of the NLRP3 inflammasome, including Alum, fail to activate caspase-1 (Angosto et al., 2012; Compan et al., 2012), it has recently been found that recombinant zebrafish (*Danio rerio*) caspase A and B and European seabass (*Dicentrarchus labrax*) caspase-1 can process *in vitro* zebrafish (Vojtech et al., 2012) and European seabass (Vojtech et al., 2012) IL-1β, respectively.

Although Alum adjuvants have not extensively been used in fish, an interesting study showed that Alum was able to induce higher specific antibody titers to the *Edwarsiella tarda* Et49 antigen than Freund's incomplete adjuvant (FIA) but lower side effects and protection (Jiao et al., 2009). Another study has reported Alum adjuvants produce less internal damage, such as the formation of granulomas, than the classic FIA in turbot *Scophtalmus maximus* (Noia et al., 2014). Therefore, additional studies aimed at evaluating the mechanisms involved in the adjuvanticity of Alum in teleost fish and its efficacy as an adjuvant are required. Here, we show that Imject Alum strongly increased the specific antibody titers to the model antigen keyhole limpet hemocyanin (KLH) in gilthead seabream and European seabass to similar levels as FIA. Mechanistically, Alum failed to promote Il1b release by seabream leukocytes but increased NADPH oxidase-dependent reactive oxygen species (ROS) production and cell death.

2. Materials and Methods

2.1 Animals, vaccination and sample collection

Healthy specimens (150 g mean weight) of gilthead seabream and European seabass were maintained at the Oceanographic Centre of Murcia (Spain) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/hour) with natural

temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Burgos, Spain). Fish were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Bioethical Committees of the University of Murcia (approval number #537/2011).

In order to evaluate the effect of Alum on adaptive immunity, the animals were injected intraperitoneally three times in a two months period (Fig. 1) with 0.5 ml PBS (control fish) alone, 200 µg KLH in 0.5 ml PBS (Sigma-Aldrich), 200 µg KLH in 0.5 ml PBS emulsified with 0.5 ml FIA, or 1 ml PBS containing 200 µg KLH and 4 mg Imject Alum Adjuvant (2 mg aluminum hydroxide and 2 mg magnesium hydroxide, Thermo Fisher Scientific). FIA was emulsified with KLH solution by forcing the mixture back and forth through a needle for approximately 10-15 minutes until emulsion did not disperse on the surface of water. Imject Alum was mixed with KLH following the manufacturer's recommendations. Briefly, Alum was added dropwise with constant mixing to KLH solution and mixed for 30 minutes to effectively adsorb antigen. The samplings were collected seven days after the last booster. For that, specimens (n=5 fish/group/time) were anesthetized with 40 ppm of clove oil, decapitated, weighed and the head-kidneys removed and processed for gene expression analysis, as described below. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80ºC until use.

2.2 Cell cultures

Seabream head kidney leukocytes obtained as described elsewhere (Sepulcre et al., 2002) were maintained in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5 % FCS (Gibco) and 100 I.U./ml penicillin and 100 µg/ml streptomycin (P/S, Biochrom). Some

116 experiments were conducted using purified cell fractions of macrophages (MØ) and
117 acidophilic granulocytes (AG), the two professional phagocytic cell types of this species
118 (Roca et al., 2006; Sepulcre et al., 2002). Briefly, AGs were isolated by MACS using a
119 monoclonal antibody specific to gilthead seabream AGs (G7) (Sepulcre et al., 2002). MØ
120 monolayers were obtained after overnight culture of $G7^-$ fractions in FCS-free medium and
121 their identity was confirmed by the expression of the M-CSFR (Roca et al., 2006).
122 Seabream MØ, AGs and total leukocytes from seabream head kidney were stimulated
123 for 16 h at 23°C with 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum*
124 ATCC19264 cells (*Va*DNA) in sRPMI supplemented with 0.1% FCS and P/S (Sepulcre et
125 al., 2007). In some experiments, leukocytes were pre-treated for 1 h with 50 μM of the pan-
126 caspase inhibitor Z-VAD-FMK (Calbiochem-Merck), 10 μM of the NADP oxidase inhibitor
127 dibenziodolium chloride (DPI) and then incubated for 1h with 40 μg/ml Imject Alum. In
128 some experiments, cell supernatants from control and stimulated leukocytes were collected,
129 clarified with a 0.45 μm filter and concentrated by precipitation with 20% trichloroacetic
130 acid (Sigma-Aldrich).
131
132 2.3 Cell viability
133 Aliquots of cell suspensions were diluted in 200 μl PBS containing 40 μg/ml propidium
134 iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was
135 analyzed by using flow cytometry (BD Biosciences).
136
137 2.4 Western Blot
138 Cells were lysed at 4°C in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1%
139 Triton X-100, 0.5% NP-40, and a 1:20 dilution of the protease inhibitor cocktail P8340 from
140 Sigma-Aldrich).The protein concentrations of cell lysates were estimated by the BCA

protein assay reagent (Pierce) using BSA as a standard. Cell extracts and concentrated supernatants were analyzed on 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed using a 1:5000 dilution of a rabbit monospecific antibody to gilthead seabream IL-1β (Angosto et al., 2012; Lopez-Castejon et al., 2007) and enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol. Membranes were then reprobed with a 1:5000 dilution of an affinity purified rabbit polyclonal to histone H3 (#ab1791, Abcam).

2.5 ROS production assays

ROS derived from NADPH oxidase were measured as the luminol-dependent chemiluminescence produced by 0.2 $x10^6$ AGs or by $0.5x10^6$ leukocytes as described elsewhere (Mulero et al., 2001). This was achieved by adding 100µM luminol (Sigma) and 1µg/ml phorbol myristate acetate (PMA, Sigma-Aldrich), while the chemiluminescence was recorded every 127s for 1h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of triple readings from 6 different samples, expressed as the maximum and slope of the reaction curve from 127 to 1016s, from which the apparatus background was subtracted.

Mitocondrial ROS production was measured using the Mitosox™ Red mitochondrial superoxide indicator (Thermo Fisher Scientific) and the accompanying manufacturer's instructions. As a positive control, cells were incubated for 6 h with 40 µg/ml of the mitochondrial electron transport chain complex III inhibitor Antimycin A. Data were collected in the form of side scatter (SSC) dot plot, green (FL1) vs. red (FL2) fluorescence dot plot and red (FL2) fluorescence histograms using a flow cytometer (BD Biosciences).

166 2.6 Analysis of gene expression

167 Total RNA was extracted from tissues or cell pellets with TRIzol Reagent (Thermo
168 Fisher Scientific), following the manufacturer's instructions, and quantified with a
169 spectrophotometer (NanoDrop, ND-1000). The RNA obtained from tissue (*in vivo*
170 experiments, n=5) or cell pellets (*in vitro* experiments, n=3) was treated with DNase I,
171 amplification grade (1 unit/µg RNA, Thermo Fisher Scientific) to remove genomic DNA
172 traces that might interfere with the PCR reactions and the SuperScript III RNase H−Reverse
173 Transcriptase (Thermo Fisher Scientific) was used to synthesize first strand cDNA with
174 oligo-dT_{18} primer from 0.5-1µg of total RNA, at 50ºC for 50 min. The β-actin (*actb*) gene
175 was analyzed by semi-quantitative PCR performed with an Eppendorf Mastercycle Gradient
176 Instrument (Eppendorf). Reaction mixtures were incubated for 2 min at 95°C, followed by
177 35 cycles of 45 s at 95°C, 45 s at the specific annealing temperature, 1 min at 72°C, and
178 finally 10 min at 72°C.

179 The expression of the genes coding for IL-1β was analyzed by real-time PCR performed
180 with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core
181 Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95˚C,
182 followed by 40 cycles of 15 s at 95˚C, 1 min at 60˚C, and finally 15 s at 95˚C, 1 min at 60˚C,
183 and 15 s at 95˚C. For each mRNA, gene expression was corrected by the ribosomal protein
184 S18 gene (*rps18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$)(Pfaffl,
185 2001). The specific primers used were: 5'-GGGCTGAACAACAGCACTCTC-3' and 5'-
186 TTAACACTCTCCACCCTCCA-3' for il1b (accession number **AJ277166**) and for 5'-
187 AGGGTGTTGGCAGACGTTAC-3' and 5'-CTTCTGCCTGTTGAGGAACC-3' *rps18*
188 (accession number **AY587263**). In all cases, each PCR was performed with triplicate
189 samples and repeated in at least three independent samples.

190

191 2.7 Determination of IgM antibody titer

192 The KLH specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic, Ltd.)
193 following the manufacturer's instructions. In short, serial dilutions of serum from control or
194 KLH-vaccinated fish were added to KLH pre-coated 96-well ELISA plates, followed by a
195 monoclonal antibody specific to gilthead seabream or European seabass IgM, and then an
196 anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich).
197 Finally, the chromogen tetramethylbenzidine (TMB) was added and the absorbance was read
198 at 450 nm using a FLUOstart luminometer (BGM, LabTechnologies).

199

200 2.8 Statistical analysis

201

202 ANOVA and a Tukey multiple range tests were applied to determine differences among
203 groups. A Student *t*-test was used to determine differences between two groups. The critical
204 value for statistical significance was taken as $p \leq 0.05$. The asterisks *, ** and *** refer to p
205 <0.05, $p<0.01$ and $p<0.001$, respectively. All statistical analyses were carried out using the
206 GraphPad Prism 5 program.

207

208 **3. Results**

209 *3.1 Alum increases specific antibody titer and the expression of genes encoding*
210 *proinflammatory mediators in gilthead seabream and European seabass*

211 The impact of Alum adjuvant on the humoral adaptive immune response was measured
212 as the presence of KLH-specific IgM in the serum of vaccinated fish. As expected, the
213 animals vaccinated with KLH emulsified with FIA showed a much higher antibody titer than
214 non-vaccinated and KLH-vaccinated animals (Figs. 2a and 3a). In addition, animals
215 vaccinated with KLH and Alum showed rather similar antibody titers as their KLH/FIA-

vaccinated counterparts in both species (Figs. 2a and 3a). Notably, both adyuvants increased the mRNA levels of gene encoding the key pro-inflammatory molecule Il1b (Figs. 2b and 3b). Similarly, Alum also had a powerful adjuvant effect in European seabass (Fig. 3a) and increased head-kidney *il1b* mRNA levels (Fig. 3b).

3.2 Alum fails to induce the release of IL-1β by seabream leukocytes and purified macrophages

To evaluate the impact of Alum in the production, release and processing of IL-1β, seabream head kidney leukocytes and macrophages were pre-stimulated for 16 h with bacterial DNA (VaDNA), a powerful PAMP for this species that is able to induce *il1b* mRNA and the intracellular accumulation, processing and release of Il1b (Angosto et al., 2012; Olavarria et al., 2010; Sepulcre et al., 2007), and then incubated for 1 h with Alum. The results show that stimulation of both cell populations with VaDNA resulted in the accumulation of mainly pro-Il1b, while Alum alone failed to do so (Figs. 4 and 4b). Strikingly, Alum was unable to promote the processing and release of Il1b in VaDNA-primed leukocytes (Fig. 4a), while it inhibited its processing/release (Fig. 4b) and transcript levels (Fig. 4c) in macrophages.

3.3 Alum induces ROS production by gilthead seabream leukocytes

As particulate adjuvants are able to activate ROS that in turn are required for the activation of the NLRP3 inflammasome (Harijith et al., 2014), we analyzed the production of ROS triggered by PMA, a NADPH oxidase activator, by Alum-primed seabream leukocytes. The results show that Alum was indeed able to prime seabream leukocytes for both basal and PMA-triggered ROS production with higher potency than the TLR9 agonist VaDNA (Fig. 5a). To evaluate the cell source of ROS in response to Alum, ROS production

241 was measured in AG-enriched ($G7^+$) and depleted- ($G7^-$) cell fractions. Although VaDNA
242 was able to primed AGs for the production of ROS, Alum failed to do so (Fig. 5b). As
243 expected, ROS production was not detectable in AG-depleted cell fractions (Fig. 5b), since
244 they mainly contain lymphocytes and precursor cells. These results suggest an indirect effect
245 of Alum in AGs. To test this hypothesis, AGs were treated with cell-conditioned media from
246 total head kidney leukocytes stimulated with VaDNA or Alum and then their ROS
247 production triggered by PMA was analyzed. The results showed that conditioned medium
248 from VaDNA-stimulated leukocytes were able to prime ROS production by AGs, probably
249 to the presence of VaDNA, whereas conditioned medium from untreated and Alum-treated
250 leukocytes failed to prime ROS production by these cells (Fig. 5c). Collectively, these
251 results suggest that Alum indirectly increases ROS production by AGs through acting and
252 promoting an interaction with another cell type.
253
254 3.4 NADPH oxidase is the source of ROS generated by AGs in response to Alum
255 As the source of ROS in response to particulate inflammasome activators is
256 controversial (Harijith et al., 2014), we next examined whether the mitochondria were the
257 cellular source of ROS generated by seabream AGs in response to Alum by using
258 MitoSOX™, a red mitochondrial superoxide indicator for live cells. The results showed no
259 differences between untreated, and VaDNA- and Alum- treated AGs (Fig. 6a). As expected,
260 AGs treated with Antimycin A, which inhibits the mitochondrial electron transport chain
261 complex III, increased mitochondrium-derived ROS (Fig. 6a).
262 We next study whether NADPH oxidase was the source of ROS in response to Alum by
263 using the NADPH oxidase inhibitor DPI in the presence or absence of its activator PMA.
264 The results showed that pharmacological inhibition of NADPH robustly inhibited ROS
265 production by VaDNA and Alum-stimulated AGs either in the presence or absence of PMA

(Fig. 6b). Collectively, these results indicate that NADPH oxidase is the source of ROS in Alum-treated AGs.

3.5 Alum is able to induce ROS and caspase-independent cell death in AGs

We found that around a 20% of AGs died in the presence of 40 µg/ml of Alum (Fig. 7a), while viability slightly increased when cells are treated with the bacterial DNA, as previously shown (Sepulcre et al., 2011). Unexpectedly, pharmacological inhibition of either ROS production with DPI or apoptosis with the pan-caspase inhibitor Z-VAD-FMK failed to rescue AG viability, suggesting than neither ROS nor apoptosis are involved in the cell death of AGs induced by Alum.

4. Discussion

Although Alum has been extensively used in human vaccination for decades, its mechanism of action is controversial. Its use in fish vaccination is more limited and there are no studies aimed at identifying its mechanism of action. We have found here that Alum is a powerful adjuvant in both gilthead seabream and European seabass, where similar KLH-specific antibody titers were obtained with Alum and FIA, the classical adjuvant used in fish vaccination. Although both adjuvants were able to increase pro-inflammatory *il1b*, future studies aimed at revealing the type of adaptive immune response elicited by each adjuvant to different antigens are necessary.

In mammals, Alum crystals were found to activate the NLPR3 inflammasome through phagosomal destabilization (Hornung et al., 2008) which results in the release of high amount of IL-1β and IL-18 (Li et al., 2007; Sokolovska et al., 2007). However, the relevance of NLRP3 inflammasome activation by Alum on its adjuvant activity is very controversial: while earlier studies reported that Alum-mediated NLRP3 inflammasome activation is indispensable for Alum adjuvanticity (Eisenbarth et al., 2008; Kool et al., 2008; Li et al.,

2008), other studies found it completely dispensable (Franchi and Nunez, 2008; Kuroda et al., 2011; McKee et al., 2009). Our results in gilthead seabream show that Alum is unable to induce Il1b release; in fact, it was able to inhibit Il1b release in macrophages. This observation, together with the lack of the conserved caspase-1 processing site in non-mammalian IL-1βs (Bird et al., 2002), and the inability of caspase-1 to process Il1b and the failure of the classical activators of the NLRP3 inflammasome, including Alum, to activate caspase-1 in gilthead seabream (Angosto et al., 2012; Compan et al., 2012), suggest that the adjuvant activity of Alum in fish is independent of the inflammasome.

Several studies have found that Alum crystals promote the release of endogenous danger signals, such as uric acid (Kool et al., 2008) and DNA (Marichal et al., 2011; McKee et al., 2013). We also observed that Alum is able to induce NADPH oxidase-dependent ROS production and cell death of AGs *in vitro*. Notably, however, ROS are not responsible for AG death, which is also caspase-independent. This is not surprising, since gilthead seabream AGs are extremely resistant to ROS production (Sepulcre et al., 2011). Therefore, it is tempting to speculate that Alum crystals causes host DNA released from dying cells that will then act as a damage-associated molecular pattern to mediate Alum adjuvant activity by preferentially boosting T_H2-dependent humoral responses, as occurs in mammals (Marichal et al., 2011; McKee et al., 2013). Future studies should investigate the relevance of danger signals generated by Alum *in vivo* on its adjuvant activity as well as the pattern-recognition receptors involved in order to increase our understanding of the mechanisms of action of Alum in fish vaccines and to help in the design of new adjuvants for aquaculture.

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415

416

Figure Legends

Fig. 1. Schematic drawing of the experimental design for seabream and seabass immunization. The animals were intraperitoneally (i.p) injected with PBS, KLH, KLH+FIA and KLH+Alum for 3 times and samples collected at the indicated times. PBS: Phosphate-Buffered Saline, KLH: Keyhole Limpet Hemocyanin, Alum: Aluminium hydroxide hydrate, FIA: Freund's Incomplete Adjuvan

Figure 2. FIA and Alum are both powerful adjuvants in gilthead seabream. (a) The KLH-specific antibody titers in the serum of gilthead seabream specimens vaccinated with PBS, KLH (200 µg/fish), KLH+FIA and KLH+Alum (4 mg/fish) were determined by ELISA. The data are shown as the mean ± SEM in triplicate pooled sera from 5 fish at the indicated serum dilutions. **(b)** The transcript levels of *il1b* were determined by RT-qPCR in the head-kidney of 5 gilthead seabream. Gene expression levels were normalized to *rps18* mRNA levels and data represent mean ± SEM. The asterisks denote statistically significant differences according to a Student *t* test. *p<0.05 and **p<0.01. n.s, not significant.

Figure 3. FIA and Alum are both powerful adjuvants in European seabass. (a) The KLH-specific antibody titers in the serum of European seabass specimens vaccinated with PBS, KLH (200 µg/fish), KLH+FIA and KLH+Alum (4 mg/fish) were determined by ELISA. The data are shown as the mean ± SEM in triplicate pooled sera from 5 fish at the indicated serum dilutions. **(b)** The transcript levels of *il1b* were determined by RT-qPCR in the head-kidney of 5 European seabass. Gene expression levels were normalized to *rps18* mRNA levels and data represent mean ± SEM. The asterisks denote statistically significant differences according to a Student *t* test. **p<0.01 and ***p<0.001. n.s, not significant.

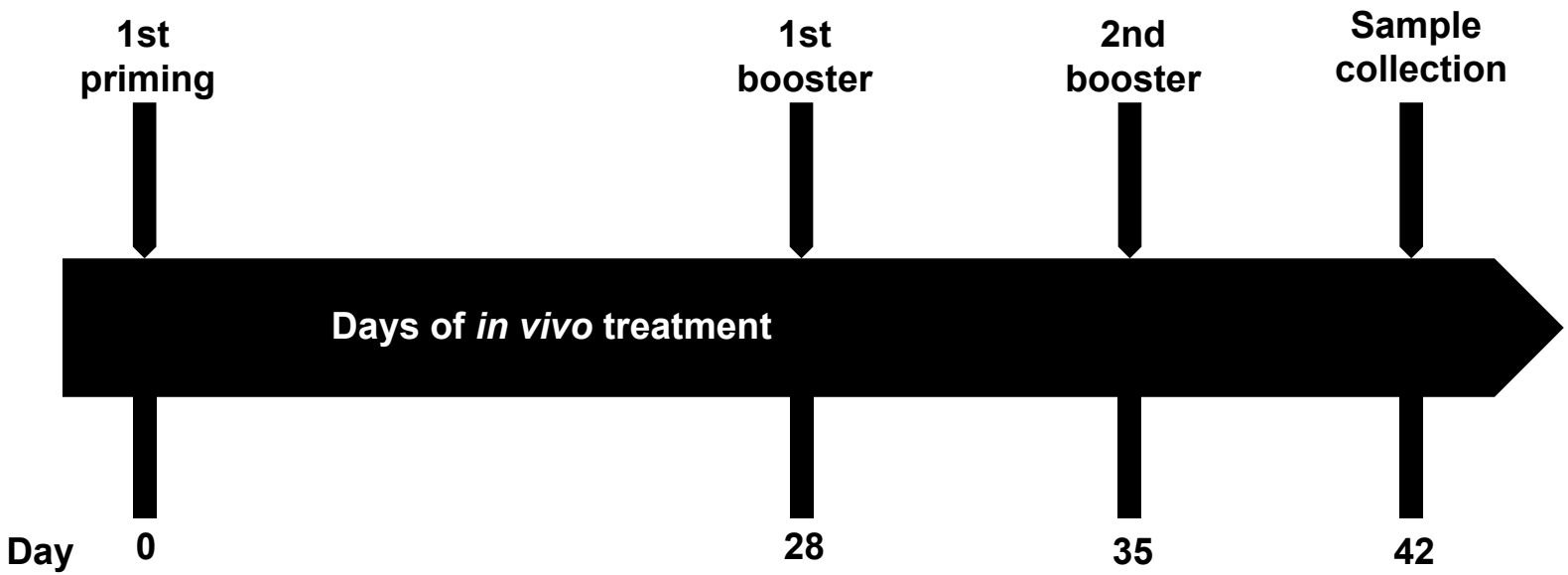
Figure 4. Alum fails to promote Il1b release in seabream leukocytes. Seabream head kidney leukocytes **(a)** and macrophages **(b)** were stimulated for 16 h with 50 μg/ml bacterial genomic DNA (VaDNA), 40 μg/ml Alum and the combination of both. Cell lysates (5×10^6) and concentrated supernatants obtained from 5×10^6 cells and the macrophage extract (40 μg) and the macrophages concentrated supernatants (from 400 μg cell extract) were probed with a monospecific polyclonal antibody to seabream Il1b. Migration positions of the mature and pro-cytokine forms are indicated. Results are representative of two independent experiments. **(c)** Modulation of *il1b* gene expression in seabream macrophages treated with 50 μg/ml VaDNA, 40 μg/ml Alum and the combination of both. Gene expression levels were normalized to *rps18* mRNA levels and are expressed as relative fold change compared with the control macrophages. The asterisks denote statistically significant differences among the treatments according the Student *t* test. *p<0.05 and ***p<0.001.

Figure 5. Alum induces ROS production in gilthead seabream AGs. (a, b) Head-kidney leukocytes (a) and AGs ($G7^+$ cells) and the rest of the cells ($G7^-$ cells) were incubated with PBS (control), 50 μg/ml bacterial genomic DNA (VaDNA) and 40 μg/ml Alum for 16 h in the presence or absence of PMA. **(c)** AGs ($G7^+$ cells) were incubated with conditioned medium (CM) obtained from head-kidney leukocytes treated for 16 h with PBS, 50 μg/ml VaDNA and 40 μg/ml Alum. The results are presented as mean ± SEM of triplicates from 3 independent samples. The asterisks denote statistically significant differences according to a Student *t* test. *p<0.05, ***p<0.001 and ****p<0.001. n.s, not significant. a.u.f., arbitrary units of fluorescence.

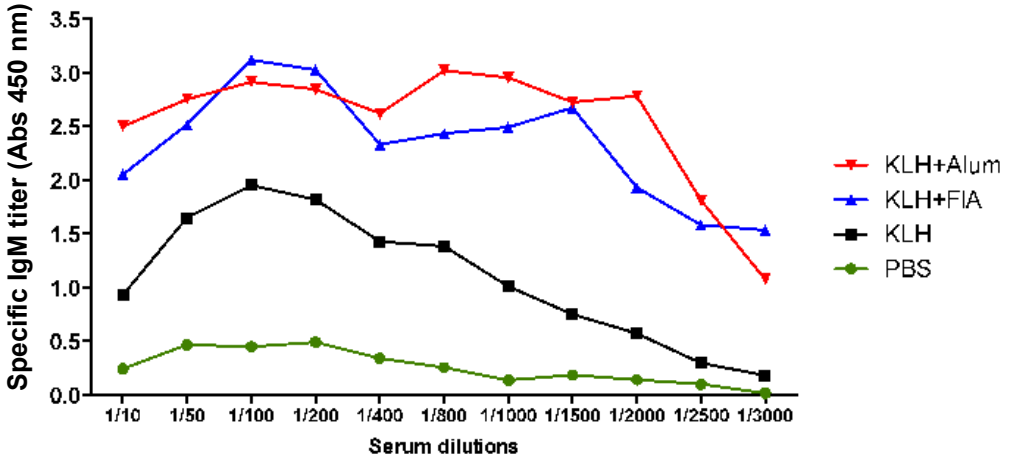
Figure 6. NADPH oxidase is the source of ROS generated by AGs in response to Alum. **(a)** Seabream head kidney leukocytes were incubated with PBS (control), 50 μg/ml bacterial genomic DNA (VaDNA) and 40 μg/ml Alum for 16 h or with 40 ug/ml antimycin A (AA) for 6 h. Afterwards, the cells were incubated for 30 min with MitosoxTM Red mitochondrial superoxide indicator and analyzed by flow cytometry in gated AGs (FSC^{high}/SSC^{high}). The data are shown as mean ± SEM of triplicates from 3 independent samples. Representative dot plots are also shown. **(b)** ROS production triggered or not by PMA of AGs ($G7^+$ cells) primed for 16 h with PBS (control), 50 μg/ml bacterial genomic DNA or 40 μg/ml Alum. The NADPH oxidase inhibitor DPI (1 μM) was also added to half of the samples together with luminol. The results are presented as mean ± SEM of triplicates from 3 independent samples. The asterisks denote statistically significant differences according to a Student *t* test. *p<0.05, **p<0.01 and ****p<0.0001. n.s, not significant. a.u.f., arbitrary units of fluorescence.

Figure 7. Alum induces AG cell death. Seabream head kidney leukocytes were incubated for 16 h with PBS (control), 50 μg/ml bacterial genomic DNA (VaDNA) and 40 μg/ml Alum in the presence or absence of 1 μM of the NADPH oxidase inhibitor DPI or 50 μM of the pan-caspase inhibitor Z-VAD-FMK. Afterwards, the percentage of dead cells (PI^+ cells) was determined by flow cytometry. The results are shown as the mean ± SEM of three replicates from 3 independent samples. The asterisks denote statistically significant differences according to a Student *t* test. ****p<0.0001. n.s, not significant.

i.p injection:
PBS
KLH
KLH+Alum
KLH+FIA



a)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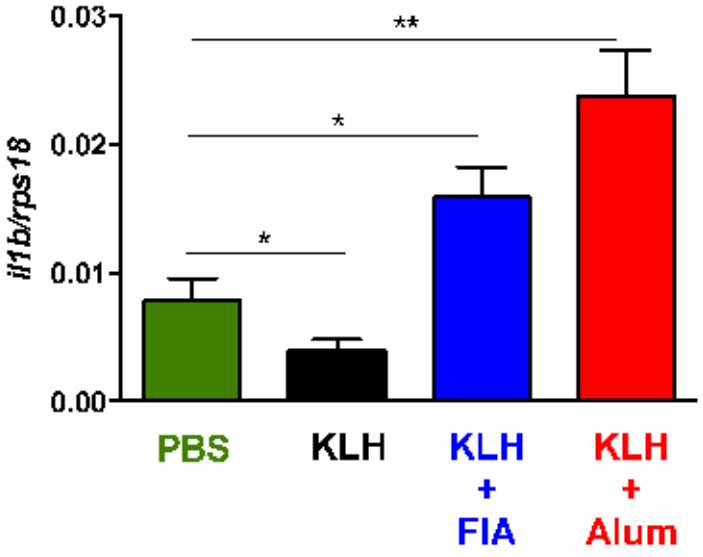
2.0
1.5
1.0
0.5
0.0
1/10
1/50
1/100
1/200
1/400
1/800
1/1000
1/1500
1/2000
1/2500
1/3000
Serum dilutions
KLH+Alum
KLH+FIA
KLH
PBS

b)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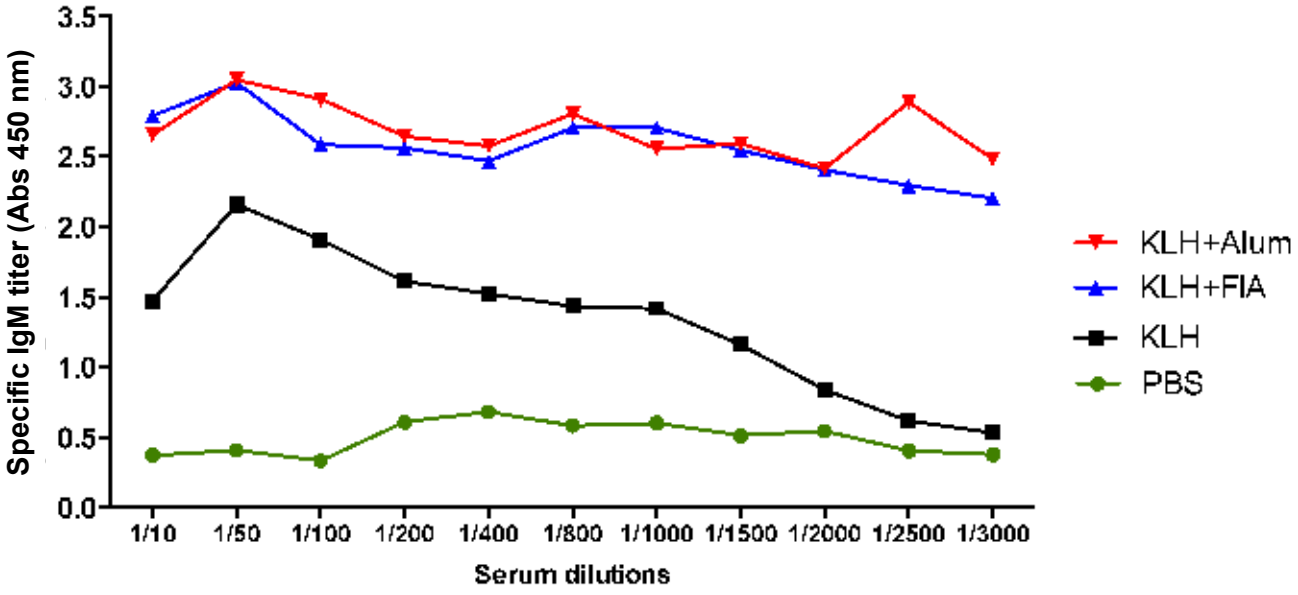
0.02
0.01
0.00
**
*
*
PBS
KLH
KLH + FIA
KLH + Alum

a)


Specific IgM titer (Abs 450 nm)
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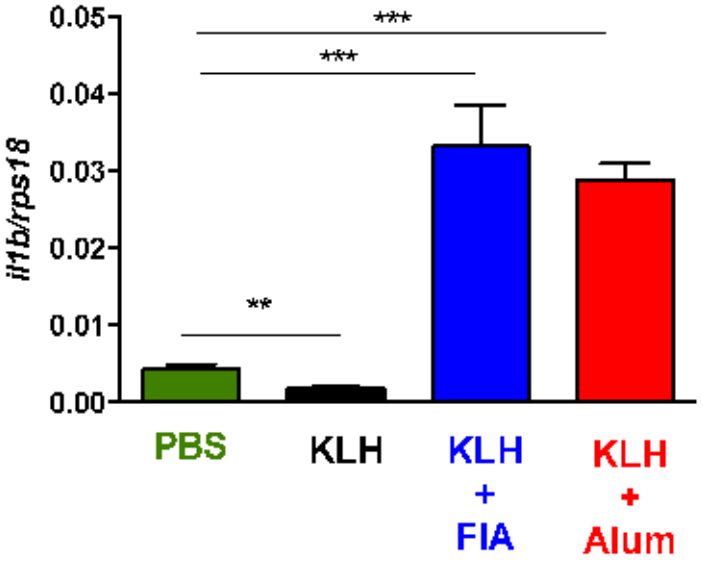
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1/10
1/50
1/100
1/200
1/400
1/800
1/1000
1/1500
1/2000
1/2500
1/3000
Serum dilutions
KLH+Alum
KLH+FIA
KLH
PBS

b)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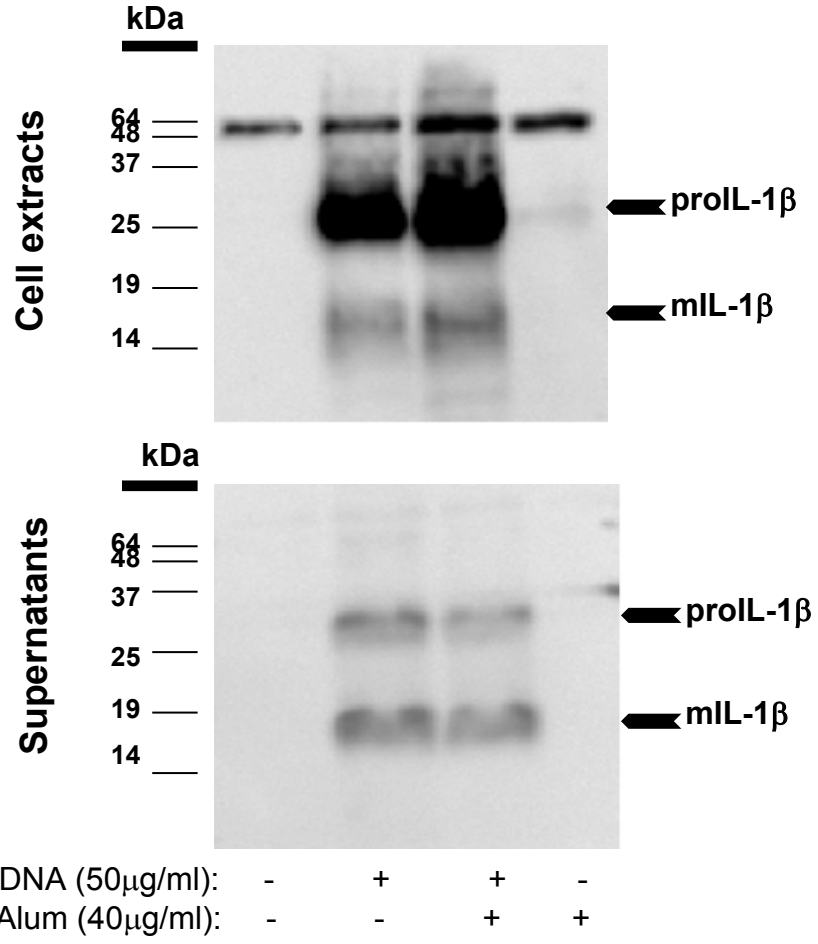
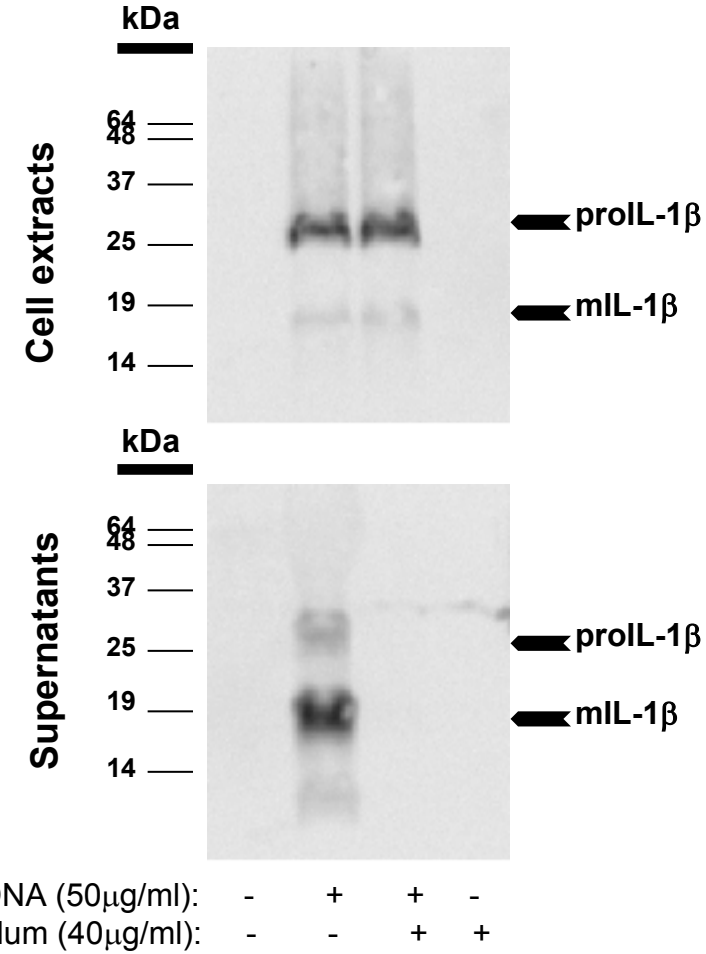
0.04
0.03
0.02
0.01
0.00
***
***
**
PBS
KLH
KLH + FIA
KLH + Alum

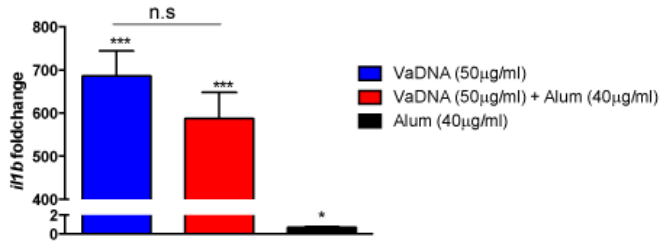
a) Seabream leukocytes



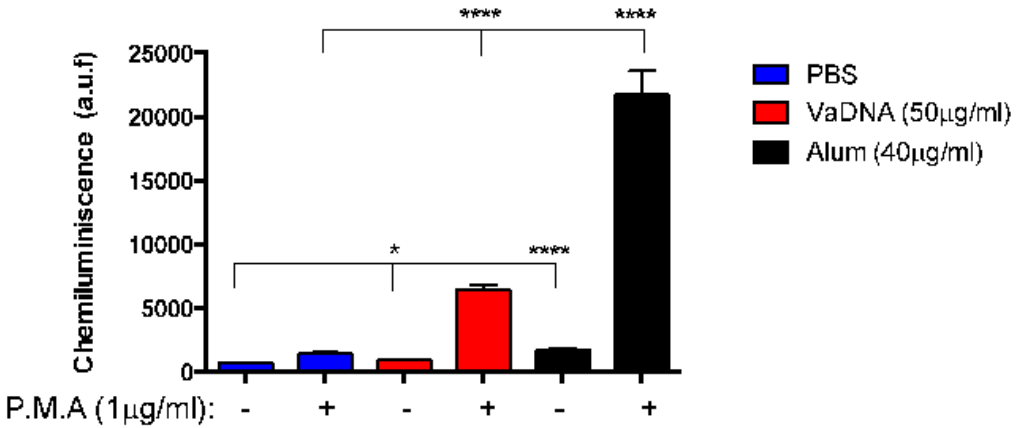
b) Seabream macrophages



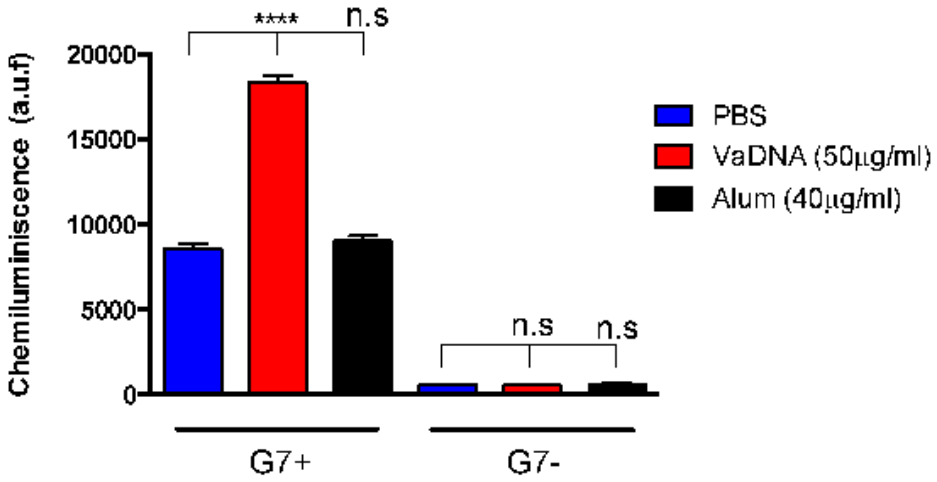
c) Seabream macrophages



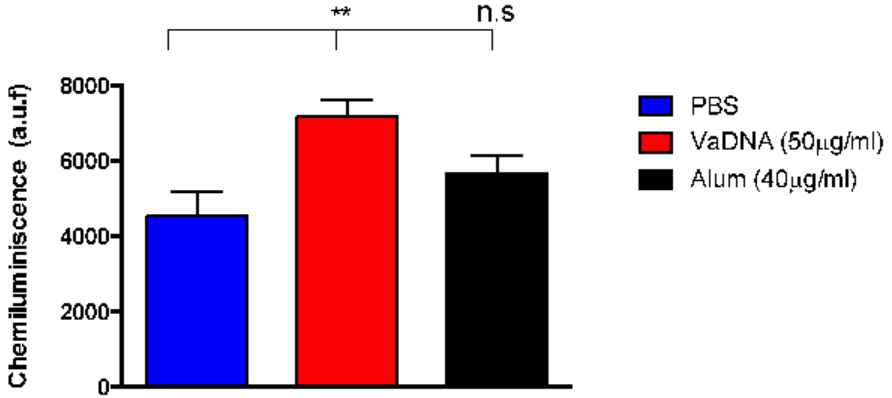
a) Head-kidney leukocytes



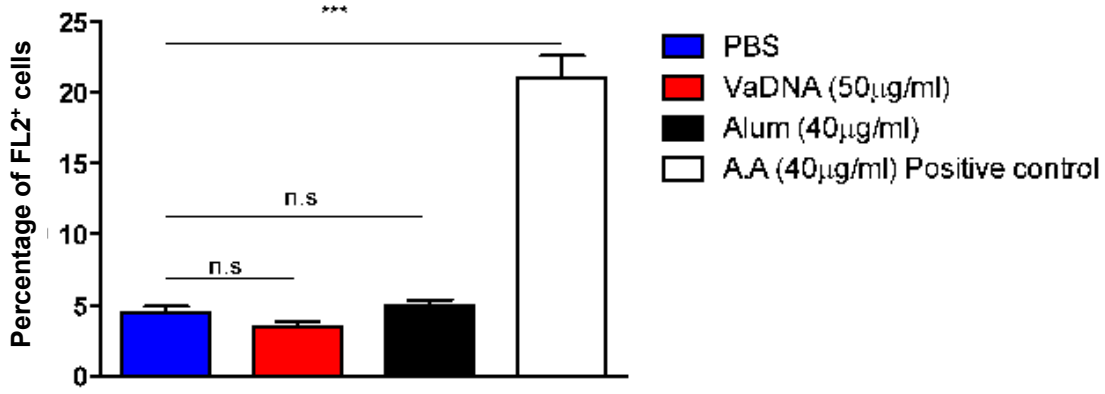
b) AGs-enriched (G7⁺) vs. depleted (G7⁻) cell fractions

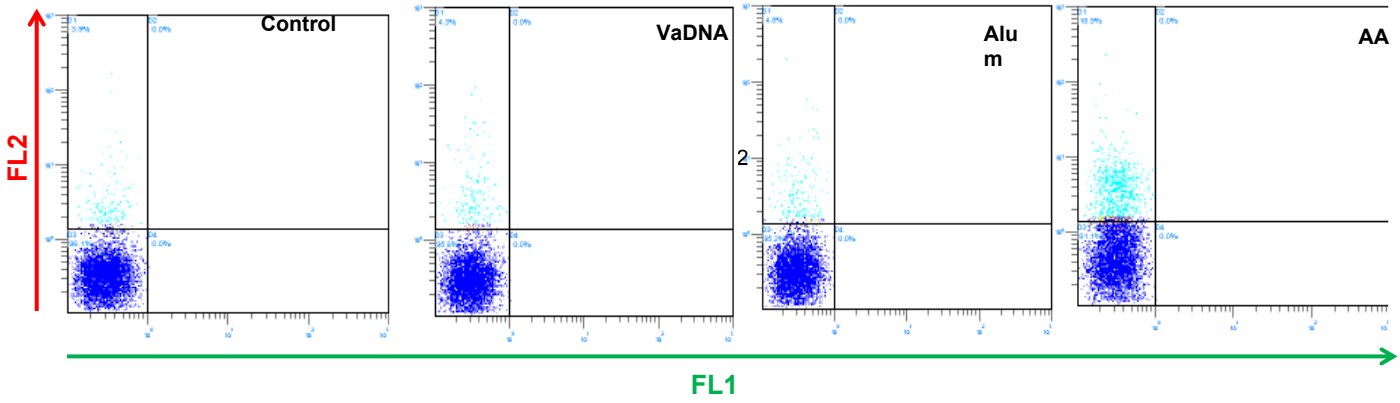


c) AGs treated with conditioned medium

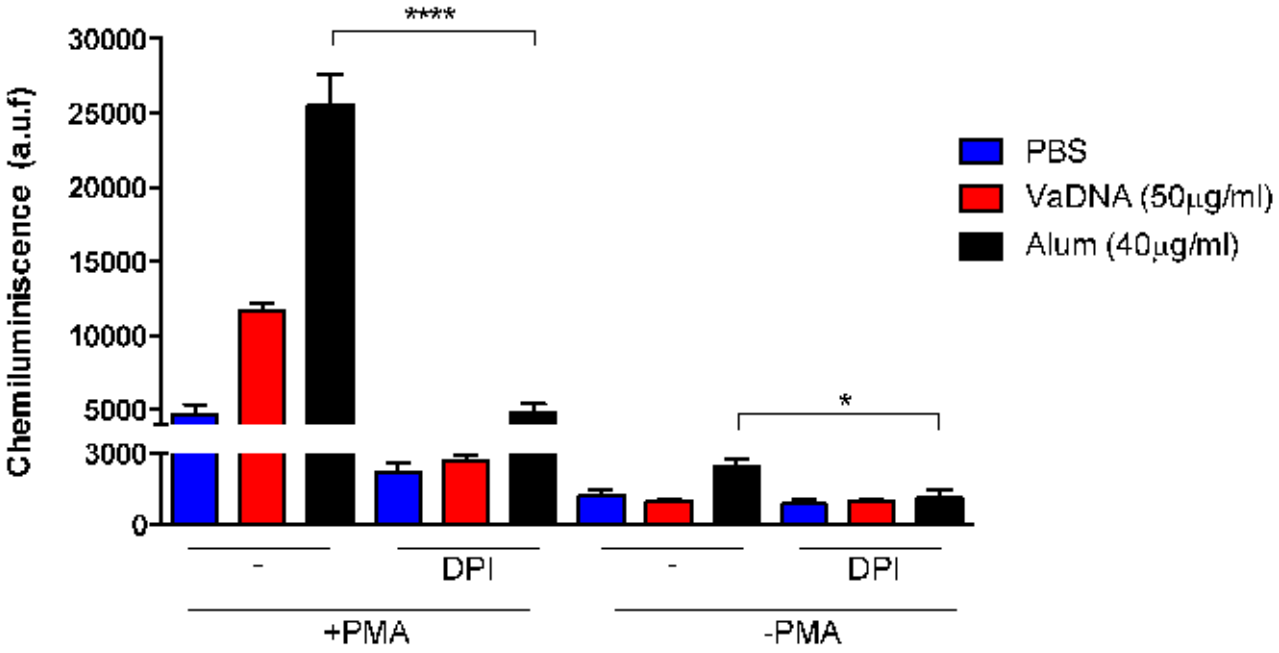


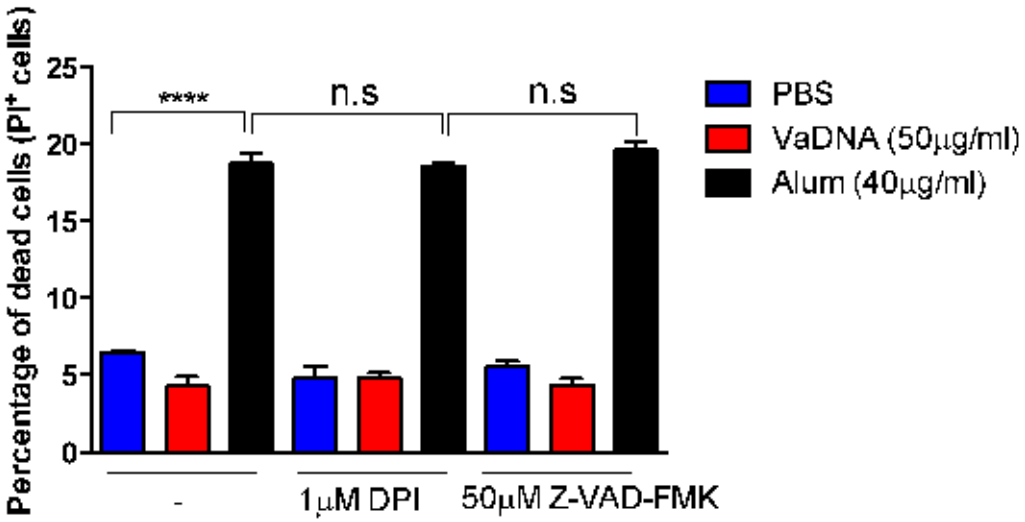
a) Mitochondrial ROS production by AGs





b) Respiratory burst of leukocytes




Percentage of dead cells (PI+ cells)
25
20
15
10
5
0
****
n.s
n.s
PBS
VaDNA (50μg/ml)
Alum (40μg/ml)
-
1μM DPI
50μM Z-VAD-FMK