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Al adjuvants can be tracked in viable cells by lumogallion staining

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Abstract

The mechanism behind the adjuvant effect of aluminium salts is poorly understood notwithstanding that aluminium salts have been used for decades in clinical vaccines. In an aqueous environment and at a nearly neutral pH, the aluminium salts form particulate aggregates and one plausible explanation of the lack of information regarding the mechanisms could be the absence of an efficient method of tracking phagocytosed aluminium adjuvants and thereby the intracellular location of the adjuvant.

In this paper we want to report upon the use of lumogallion staining enabling the detection of phagocytosed aluminium adjuvants inside viable cells. Including micro molar concentrations of lumogallion in the culture medium resulted in a strong fluorescence signal from cells that had phagocytosed the aluminium adjuvant. The fluorescence appeared as spots in the cytoplasm and by confocal microscopy and co-staining with probes presenting fluorescence in the far-red region of the spectrum, aluminium adjuvants could to a certain extent be identified as localized in acidic vesicles i.e. lysosomes.

Staining and detection of intracellular aluminium adjuvants was not only achieved by diffusion of lumogallion into the cytoplasm and thereby highlighting the presence of the adjuvant, but could also be achieved by pre-staining the aluminium adjuvant prior to incubation with cells. Pre-staining of aluminium adjuvants resulted in bright fluorescent particulate aggregates that remained fluorescent for weeks and with only a minor reduction of fluorescence upon extensive washing or incubation with cells. Both aluminium oxyhydroxide and aluminium hydroxyphosphate, two of the most commonly used aluminium adjuvants in clinical vaccines, could be pre-stained with lumogallion and were easily tracked intracellularly after incubation with phagocytosing cells.

Staining of viable cells using lumogallion will be a useful method in investigations of the mechanisms behind aluminium adjuvants' differentiation of antigen-presenting cells into inflammatory cells. Information will be gained regarding the phagosomal pathways and the events inside the phagosomes, and thereby the ultimate fate of phagocytosed aluminium adjuvants could be resolved.

Keywords

Aluminium adjuvant, lumogallion, flow cytometry, phagolysosome.

1. Introduction

Aluminium based adjuvants in the form of aluminium salts have been used in pharmaceutical vaccine formulations for many decades. The aluminium adjuvants are dispersed in aqueous solvents at circumneutral pH where the aluminium salts form particles and aggregates in the nm to μm range. In spite of their extensive use a clear mechanistic understanding of how aluminium salts potentiate the induction of an immune response is still missing (Exley et al., 2010). Adsorption of antigens onto aggregates of the adjuvant and their subsequent release at the inoculation site has long been recognized as a possible *modus operandi* of the adjuvant (Lindblad, 2004). One alternative mechanism could be the induction of an inflammatory response by aluminium salts and indeed injection of aluminium adjuvants results in cell injury and a sterile inflammation (Rock et al., 2010). Cell injury at the injection site triggers the release of danger associated molecular patterns (DAMPs) and an infiltration of inflammatory cells together with increased levels of relevant chemokines has been shown at the inoculation site (Lu et al., 2013). Furthermore, an inflammatory response may also be induced by the assembly and activation of the Nalp3 inflammasome by antigen presenting cells (APCs) after phagocytosis of aluminium adjuvants, leading to the release of IL-1β and IL-18 (Hornung et al., 2008, Eisenbarth et al., 2008, Marrack et al., 2009). Considering the efficiency and continued use of aluminium adjuvants, experimental information concerning the mechanisms behind the induction of an immune response by aluminium adjuvants is surprisingly limited.

To be able to verify the distribution and action of aluminium salts after injection at immunization sites, a robust method allowing for their traceability is warranted. Lumogallion has been used as a histological stain to detect the presence of aluminium in both plant and mammalian tissues (Silva et al., 2000, Uchumi et al., 1998) and it has recently been used to demonstrate the unequivocal presence of aluminium adjuvant in a monocytic cell line (Mold et al., 2014). In this paper we intend to report upon the use of lumogallion in the staining of living cells after phagocytosis of aluminium adjuvants. For this purpose we utilized a monocytic cell line with known phagocytizing capacity and a mouse glioma cell line. Staining of living cells by lumogallion will open up new possibilities of tracking intracellular aluminium adjuvants in viable cells thereby revealing the intracellular fate and pathway(s) of aluminium adjuvants after endocytosis.

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2. Materials and Methods

2.1. Cell lines

THP-1, (ATCC TIB-202), was obtained from LGC Standards, UK and the GL261 mouse glioma cells of C57BL/6 origin was kindly provided by Dr. G Safrany, Hungary.

2.2. Materials

The aluminium adjuvant preparations used herein were Alhydrogel AlO(OH) and Adju-Phos $AlPO_4$ purchased from Brenntag Biosector (Frederikssund, Denmark). Al_2O_3 60 nm nanoparticles coated with Aluminic Ester and Aluminium Hydroxide Nanopowder/Nanoparticles $Al(OH)_3$ 10-20 nm were obtained from US Research Nanomaterials (Houston Tx, USA).

Lumogallion (CAS 4386-25-8) came from TCI Europe N.V., Antwerp, Belgium and morin hydrate (CAS 654055-01-3) was obtained as a powder from Sigma-Aldrich, St. Louis, MO, USA.

All other reagents were of analytical grade.

2.3. Cell culture

THP-1 and GL261 cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum of EU grade, (PAA laboratories, Linz, Austria), and 100 μg/ml of gentamicin (PAA laboratories, Linz, Austria). This medium will be referred to as R10. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and the cells were maintained by sub-culturing once every third day.

2.4. Co-culture with aluminium adjuvants and staining of the cells

Triplicates of THP-1 cells 0.5 x 10^6 cells per ml were co-cultured in 96 well plates with Alhydrogel AlO(OH) adjuvant corresponding to final concentrations of aluminium content ranging from 0.8 to 50 μg/mL in a total volume of 200 μl R10 culture medium during 4 to 16 h (over night) at 37°C. Controls consisted of cells cultured in R10 medium in the absence of aluminium adjuvant. Lumogallion was then added from a stock solution in RPMI 1640 medium to final concentrations of 1.6 to 20 μM and the cells were further incubated at 37°C for various time intervals (30 minutes up to over night). Controls consisted of cells cultured with and without any aluminium adjuvant and without any addition of lumogallion. Specified

concentrations of aluminium, lumogallion and incubation periods of each experiment are described in the figure legends. After staining, the triplicates from the incubations were pooled and centrifuged at 1,000 x g before the cells were re-suspended in 300 µl 1% (w/v) paraformaldehyde (PFA). Finally the cells were analysed by flow cytometry using an Accuri C6 flow cytometer and standard settings.

In experiments using morin, co-culture with aluminium adjuvant was performed as previously described before morin was added to the cells at a final concentration of 160 µM using a saturated solution of morin in RPMI 1640 medium (800 µM morin) as stock solution. Co-culture, washing, fixation and analysis by flow cytometry was performed as previously described.

2.5. Pre-staining of aluminium formulations

2.5.1. Staining of aluminium formulations with lumogallion:

Various formulations of aluminium adjuvants corresponding to 500, 250, 125 or 62.5 µg aluminium / ml were incubated with 50 µM lumogallion in a total volume of 1 ml R10 medium. The suspensions were incubated at room temperature over night on a rocking table and the next day the aluminium particles were collected by centrifugation for 10 minutes at 13,000 x g. Finally the particles were re-suspended in 1 ml R10 medium and the fluorescence of the suspensions was measured by spectrofluorometry using excitation 490 nm, emission 580 nm and a cut off filter of 530 nm.

2.5.2. Pre-staining of Alhydrogel AlO(OH) adjuvant:

Alhydrogel AlO(OH) corresponding to 4 mg aluminium / ml was incubated over night at room temperature on a rocking table with 50 µM lumogallion in a total volume of 1 ml R10 medium. The next day the adjuvant was collected by centrifugation for 10 minutes at 13,000 x g. The collected adjuvant was re-suspended in 1 ml R10 medium and stored in the refrigerator until further use.

2.5.3. Release of fluorescence from pre-stained Alhydrogel AlO(OH) upon washing:

Alhydrogel AlO(OH) corresponding to 4 mg aluminium / ml was incubated over night at room temperature on a rocking table with 50 µM lumogallion in a total volume of 1 ml R10 medium. The next day the suspension was diluted 20 times with R10 medium and 1 ml of the diluted suspension was withdrawn and centrifuged for 10 minutes at 13,000 x g. The supernatant was collected and the pellet was re-suspended in a new 1 ml portion of R10 medium. After 5 minutes on a rocking table

the sample was once again centrifuged for 10 minutes at 13,000 x g. The supernatant was collected again and the pellet re-suspended in a new 1 ml portion of R10 medium and placed on a rocking table for 5 minutes before the sample was centrifuged, the supernatant collected and the pellet re-suspended in R10 medium. This procedure was repeated until 10 supernatants were collected. Finally the fluorescence from all of the collected supernatants and a sample corresponding to the suspension obtained after the first re-suspension of stained aluminium adjuvant was measured by spectrofluorometry using excitation 490 nm, emission 580 nm and a cut off filter of 530 nm.

2.5.4. Pre-staining with morin:

Alhydrogel AlO(OH) corresponding to 0.5 mg aluminium / ml was incubated over night at room temperature on a rocking table with 250 μM morin in a total volume of 1 ml PBS. The next day the adjuvant was collected by centrifugation for 10 minutes at 13,000 x g. The collected adjuvant was re-suspended in 0.5 ml R10 medium and stored in the refrigerator until further use.

2.5.5. Zeta potential analysis:

Zeta potential analysis was performed using a Zeta Potential/Particle Sizer NICOMP 380 ZLS, Particle Sizing System, Santa Barbara, CA, USA. Alhydrogel and pre-stained Alhydrogel were suspended in PBS at a concentration of 2 mg/ml and analysed using ZPW388 Application Version 2.00 with the settings; temperature 20 °C, liquid visco: 1.002, liquid Index of Ref: 1.333, dielectric constant: 78.5 and electrode spacing: 0.4 cm.

2.6. Co-culture with pre-stained Alhydrogel AlO(OH).

Triplicates of THP-1 cells 0.5 x 10^6 cells per ml were co-cultured in 96 well plates with lumogallion or morin pre-stained Alhydrogel AlO(OH) adjuvant corresponding to final concentrations of aluminium ranging from 0.8 to 50 μg/ml in a total volume of 200 μl R10 culture medium from 4 to 24 h at 37°C. Controls were composed of cells cultured in R10 medium and cells cultured in the presence of un-stained aluminium adjuvant. Specified concentrations of pre-stained aluminium adjuvant and incubation periods of each experiment are described in the figure legends. After incubation with aluminium adjuvant (pre-stained and un-stained aluminium adjuvant)

the triplicates from the incubations were pooled and centrifuged for 5 minutes at 1,000 x g before the cells were re-suspended in 300 µl 1% (w/v) PFA. Finally the cells were analysed by flow cytometry using an Accuri C6 flow cytometer and standard settings.

2.7. Microscopy.

THP-1 cells were co-cultured with aluminium adjuvants or pre-stained adjuvant as earlier described and after re-suspension in 1% (w/v) PFA the cell suspensions were incubated for 15 minutes at room temperature. The cells were then collected by centrifugation for 5 minutes at 1,000 x g and washed twice with 500 µl PBS. Finally the cells were re-suspended in a small volume of PBS and mounted on microscope slides using ProLong® Gold Antifade Mountant with DAPI (Life Technologies, ThermoFisher Scientific, MA USA). After mounting the samples were analysed on a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). DAPI was excited with the 405 nm laser and emitted light was detected between 401-529 nm. Lumogallion was either excited with the 561 nm laser and emitted light was collected between 585-734 nm or when using dual staining with LysoTracker Deep Red excited with the 488 nm laser and emitted light was collected between 553-695 nm. LysoTracker Deep Red was excited with the 633 nm laser and emitted light was collected between 638-759 nm. Z-stack images were obtained at 63x magnification and analysed with ZEN 2012 (Carl Zeiss Microscopy GmbH). The samples were also analysed by fluorescence microscopy using a light microscope (BX-53, Olympus LRI Instruments AB, Lund, Sweden) equipped with a mercury lamp and filters for fluorescence (U-MWG, U-MWB, U-MWU). Images were taken in 40x magnification using an Olympus Color View digital camera and captured using cellSens Dimension imaging software (Olympus).

GL261 cells were seeded into culture chambers (BD Falcon CultureSlides, uncoated from BD Biosciences, MA USA) using 200 µl, 1 x 10^5 cells/ml in each chamber and the cells were allowed to adhere by incubation over night at 37°C. The next day 200 µl aluminium adjuvant ranging from 0.3 to 10 µg/ml was added to the chambers and the cells were incubated at 37°C during 4 to 16 h (over night). Lumogallion was then added to a final concentration of 5 µM and incubated over night at 37°C. Controls consisted of cells cultured with and without any aluminium adjuvant and without any

addition of lumogallion. The cells were then washed with 2 x 400 µl PBS, fixed in 100 µl 1% (w/v) PFA during 15 minutes at room temperature and washed again with 2 x 400 µl PBS. Finally the cells were mounted using ProLong® Gold Antifade Mountant with DAPI and analysed by confocal or fluorescence microscopy.

GL261 cells co-cultured with lumogallion pre-stained aluminium adjuvant were after incubation washed with 2 x 400 µl PBS, fixed and analysed as described above.

Specified concentrations of aluminium, lumogallion and incubation periods of each experiment are described in the figure legends.

2.8. Flow cytometry and staining with lumogallion and a second fluorochrome

2.8.1. Staining with lumogallion and a FITC labelled antibody

Triplicates of THP-1 cells, 0.5 x 10^6 cells per ml, were co-cultured in a 96 well plate with Alhydrogel AlO(OH) adjuvant corresponding to a final concentration of 12.5 µg aluminium / ml in a total volume of 200 µl R10 culture medium during 4 hours at 37°C. Lumogallion was added to a final concentration of 5 µM from a stock solution in RPMI 1640 medium and the cells were further incubated at 37°C over night. Controls consisted of cells cultured with and without any aluminium adjuvant and without any addition of lumogallion together with addition of a FITC labelled isotype control. After staining the triplicates from the incubations were pooled and centrifuged at 1,000 x g before the cells were re-suspended in 300 µl PBS containing 0.1 % (w/v) BSA and 0.1 % (w/v) human IgG. Each pooled sample was then sub-divided into two samples and incubated on ice for 30 min with FITC-labelled anti CD45 or a FITC labelled isotype control (BD Bioscience, San Jose, CA, USA). Finally the cells were washed with 500 µl PBS, re-suspended in 400 µl 1% PFA (w/v) and analysed by flow cytometry using an Accuri C6 flow cytometer with 70 % compensation due to over-flow of lumogallion fluorescence into the green fluorescence channel.

2.8.2. Staining with lumogallion and MitoTracker Deep Red

Triplicates of THP-1 cells 0.5 x 10^6 cells per ml were co-cultured in a 96 well plate with lumogallion pre-stained Alhydrogel AlO(OH) adjuvant (prepared as described in section 2.5.2.) corresponding to a final concentration of 12.5 µg aluminium / ml in a total volume of 200 µl R10 culture medium over night at 37°C. The next day the triplicates from the incubations were pooled and centrifuged for 5 minutes at 1,000 x

g before the cells were re-suspended in 300 µl R10 medium containing 5 nM MitoTracker Deep Red (Molecular Probes, Life Technologies, ThermoFisher Scientific, MA USA). The samples were incubated for 30 minutes at 37°C before the cells were collected by centrifugation for 5 minutes at 1,000 x g, washed with 400 µl PBS and fixed in 400 µl 1% (w/v) PFA. Controls consisted of cells cultured in R10 medium with and without staining with MitoTracker Deep Red. Finally the cells were analysed by flow cytometry using an Accuri C6 flow cytometer with standard settings without any compensation of fluorescence over-flow into neither of the FL3 nor FL4 channels.

2.8.3. Staining with lumogallion and LysoTracker Deep Red

GL261 cells, 0.5 x 10^5 cells per ml, were co-cultured in culture chambers with 2.5 µg/ml of AlO(OH) during 6 h at 37°C in a total volume of 400 µl R10 medium. Lumogallion at a concentration of 10 µM was added to the culture and the next day the cells were washed with PBS, and further stained with 200 nM LysoTracker Deep Red in R10 medium (Molecular Probes, Life Technologies, ThermoFisher Scientific, MA USA) for 1 h at 37°C. The cells were washed with PBS, fixed in PFA, washed, mounted using ProLong® Gold Antifade Mountant with DAPI and analysed by confocal microscopy. Controls consisted of cells cultured with and without AlO(OH) and with and without staining with lumogallion and MitoTracker Deep Red.

3. Results

3.1. Staining of viable cells

Fluorescence achieved due to aluminium adjuvants phagocytosed by cells can easily be detected by flow cytometry (Fig. 1) or microscopy (Fig. 2) after staining living cells with lumogallion. Several hours of incubation with lumogallion was necessary to reach a staining equilibrium (Fig. 1C), and importantly, no effect on the viability of THP-1 cells was observed by estimating the cell number by counting or by the MTT assay after incubation in the presence of 50 μM lumogallion during 24 h (higher concentrations or longer incubation times were not investigated). Based on three determinations after over night incubation in R10 medium 0.91 x 10^6 +/- 0.03 x 10^6 cells /ml were counted, and after co-culture with 50 μM lumogallion a cell concentration of 0.86 x 10^6 +/- 0.03 x 10^6 cells /ml was recorded, whereas the MTT assay resulted in an absorbance at 600 nm of 1.07 +/- 0.04 in R10 medium and 1.14 +/- 0.04 in the presence of lumogallion.

Staining of living cells with lumogallion revealed the internalization of aluminium adjuvant particles by phagocytosing cells growing both as suspension cells and as adherent cells (Fig. 2). Confocal images clearly showed the aluminium particles to be intracellular and that staining was localized in the cytoplasm (Fig. 2A).

3.2. Pre-staining of Aluminium adjuvants

Aluminium adjuvants can also be pre-labelled with lumogallion before incubation with cells. Lumogallion strongly binds to aluminium adjuvant particles although some lumogallion was released from the particles upon prolonged washing. The release of lumogallion was relatively modest as shown in figure 3A, in which less than 20 % of the particle associated fluorescence was dissipated during the second to the tenth re-suspension. Pre-staining of Alhydrogel affected the z-potential of the aluminium aggregates from +13 +/- 4 mV to +3 +/- 2 mV after pre-staining (n=7) and possibly also a slight increase in aggregate size. By the use of polystyrene particle size standards 3.8 +/- 0.2 % of the Alhydrogel aggregates were larger than 3 μm as showed by flow cytometry and forward scatter, whereas in that three independent preparations of pre-stained Alhydrogel showed 4.6 +/- 0.8 % of the aggregates larger than 3 μm.

Pre-staining of the aluminium particles using lumogallion can be done prior to co-culture with cells in order to detect and trace endocytosed aluminium adjuvant particles within the cells (Fig. 4). However, not all aluminium formulations can be stained by lumogallion and figure 5 shows the fluorescence obtained from some clinical as well as experimental aluminium adjuvants after incubation with lumogallion. After incubation and staining, the particles were collected by centrifugation and no investigations were done regarding the recovery of aluminium adjuvant after centrifugation, although a clearly visible pellet was obtained with all of the aluminium preparations. A non-fluorescent white pellet was obtained using Al_2O_3 nanoparticles whereas AlO(OH) nanoparticles pellets were clearly yellow as observed by direct vision.

3.3. Lumogallion fluorescence

The lumogallion emission spectrum is extremely broad, ranging from 520 to 650 nm with a peak around 580 nm, after excitation at 490 nm. The broad emission spectrum makes it difficult to combine lumogallion with other fluorochromes, although by using the compensation setting in the flow cytometer software, green fluorescence from fluorochromes such as FITC can be analysed in combination with lumogallion (Fig. 6). By appropriate use of software compensation (70 – 80 %), the over-flow of lumogallion fluorescence into the green fluorescence channel can be attenuated to an acceptable level. However, with fluorochromes emitting light in the far-red no over-flow of lumogallion fluorescence was obtained. Figure 6 shows two examples of combining lumogallion with other fluorescent probes; one example shows the combination of lumogallion and surface staining using FITC-labelled antibodies (Fig. 6A). The other example shows the combination with intracellular staining achieved by MitoTracker Deep Red (Fig. 6B), a probe that upon diffusion into the cells, accumulates in mitochondria and shows fluorescence in the far-red region of the spectrum.

The combination of lumogallion with other fluorescent probes is of utmost importance in studies using microscopy to localize intracellular aluminium adjuvant. LysoTracker Deep Red is a probe that upon accumulation in acidic compartments shows fluorescence in the far-red region of the spectrum and figure 7 clearly shows the co-localisation of aluminium particles in lysosomes. No over-flow of lumogallion

or LysoTracker Deep Red fluorescence was detected when the cells were stained with only one of the probes as controls.

3.4. Staining with morin

Morin is another commonly used histological stain of aluminium (Lu et al., 2013, Eticha et al., 2005, Wen and Wisniewski, 1985) that has a much lower water solubility compared to lumogallion. The fluorescence signal from cells containing phagocytosed aluminium adjuvant particles was several magnitudes lower when using morin, compared to analogous cells stained with lumogallion (Fig. 8). Alhydrogel AlO(OH) adjuvant particles can also be pre-stained with morin similarly to lumogallion pre-staining, however, compared to staining with free morin, pre-staining did not result in any increased fluorescence intensity from the cells (Fig. 8A and B).

4. Discussion

Histological staining using lumogallion was recently described as a method of intracellular identification of aluminium adjuvants (Mold et al., 2014). Herein we have demonstrated the use of lumogallion in the detection and tracing of aluminium adjuvant that is not only restricted to histological sections but can also be used on viable cells. In this paper we want to describe the use of lumogallion as a probe for staining intracellular aluminium adjuvants of viable cells. Lumogallion can either be added directly to the cells and after diffusion stain intracellular aluminium adjuvants, or prior to incubation with cells, fluorescently label the aluminium adjuvants and thereby creating traceable aluminium formulation in a cellular framework.

The fluorescence signal obtained after staining viable cells containing phagocytosed aluminium adjuvant particles was extremely intense. Already at μM concentrations of lumogallion during the staining process, a clear signal was obtained by flow cytometry (Fig. 1) whereas the diffusion of lumogallion and binding to intracellular aluminium needed several hours to reach equilibrium (Fig. 1C). In a staining protocol with lumogallion, over-nigh incubation is suggested although a clear and unambiguous signal was observed already after less than an hour. In experiments where relative fluorescence intensities do not have to be considered, staining can therefore be performed in less than an hour.

Lumogallion is strongly adsorbed on particles/aggregates of both AlO(OH) and $AlPO_4$, two aluminium salts used in clinical vaccines (Fig 4 and 5), and the particles/aggregates can be pre-stained with lumogallion before co-culture with cells in order to trace the phagocytosed particles (Fig. 4). Compared to non-stained Alhydrogel, no increased cytotoxicity was observed after pre-staining based on counting of cells after co-culture of THP-1 with Alhydrogel and pre-stained Alhydrogel respectively.

Some lumogallion was released from the particles upon prolonged washing and the release can be hypothesised to correspond to the amount of solubilized Al^{3+} ions during the washing procedure. The release of lumogallion was relatively low and less than 20 % of the fluorescence was lost as a result of an intense washing procedure, as shown in figure 3.

Lumogallion has been reported to bind to Al^{3+} ions (Wu et al., 1995). However, based on the shown pre-labelling of the adjuvant particles further binding mechanisms as deposition of the lumogallion-Al^{3+} complex onto the adjuvant particles has to be considered. Nanoparticles often form μm-sized aggregates and these particles, as well as particles/aggregates formed by AlO(OH) and $AlPO_4$ used in pharmaceutical adjuvants, can be easily collected by centrifugation. However, the size distribution of the particles/aggregates will not be the same in the different aluminium formulations and this will affect the recovery of particles/aggregates after centrifugation. No measures were taken to investigate the recovery of particles/aggregates after pre-staining with lumogallion and this may to some extent reflect the difference in fluorescence intensity between nanoparticles as well as conventional preparations of AlO(OH) and $AlPO_4$ shown in figure 5. Nanoparticles of Al_2O_3 on the other hand showed almost no fluorescence after pre-staining although a clearly visible pellet was seen after harvesting the particles by centrifugation, indicating the unfeasibility of staining Al_2O_3 particles with lumogallion. The Al_2O_3 nanoparticles used were coated with "Aluminic Ester" by the manufacturer, and further experiments will establish whether the coating of the particles affects the possibility to pre-label the Al_2O_3 particles or whether it is aluminium oxide as such that has a low interaction with lumogallion.

Dual staining with other fluorescent probes is a necessity to ensure appropriate intracellular tracking of aluminium adjuvants using lumogallion. The broad emission of lumogallion makes it difficult to use other probes emitting fluorescence in the 520 to 620 nm wavelength range. Only flow cytometers or other instruments where the over-flow of emitted light in the different PM-tubes can be compensated provide useful information if the secondary emitter overlaps with the lumogallion emission, as exemplified by dual staining with a FITC labelled antibody (green fluorescent probes) shown in figure 6A. However, no interference was observed in the far-red region of the spectrum (Fig. 6B), and probes labelled with far-red emitting fluorochromes are well suited to perform dual staining experiments using lumogallion as the aluminium probe and this is clearly visualised by the use of the LysoTracker Deep Red probe (Fig. 7).

After phagocytosis, phagosomes containing aluminium adjuvant will mature into phagolysosomes having a low pH. Lumogallion and LysoTracker staining will co-

localise in phagolysosomes containing aluminium adjuvant and co-localisation of lumogallion and LysoTracker fluorescence was clearly shown in GL261 cells co-cultured with aluminium adjuvant (Fig. 7). Not all lumogallion staining co-localised with the LysoTracker probe and this will occur in early phagosomes in which the pH has not yet decreased. A pH decrease during maturation into phagolysosomes will increase the solubility of the aluminium adjuvant, releasing Al^{3+} ions and hydroxide ions. Formation of hydroxide will attenuate the pH decrease and lack of co-localisation with the LysoTracker probe can especially be seen in phagosomes with a strong lumogallion staining reflecting phagosomes with a high content of aluminium adjuvant hampering the pH decrease.

Morin is another histological stain used to detect aluminium that also can be used to detect intracellular aluminium adjuvants in viable cells. Similarly to lumogallion, aluminium adjuvants can be detected both by spontaneous diffusion of morin into cells or by pre-staining of the adjuvant particles/aggregates before co-culture with the cells (Fig. 8). Compared to lumogallion, the morin emission spectrum has a narrow bandwidth and it is much easier to combine with other fluorochromes in multiple staining of a sample. However, the morin fluorescence is several magnitudes weaker than lumogallion, and when using morin to detect intracellular aluminium adjuvants, care has to be taken as regards the autofluorescence caused by the cells themselves.

This report has focused on the tracing of aluminium adjuvants in viable cells using probes commonly used as histological stains. The staining methods described in this paper will make it easy to detect and trace the presence of intracellular aluminium adjuvants and will make it possible to elucidate the intracellular pathways and fate of endocytosed aluminium adjuvants in the future.

The rationale to use a phagocytizing cell line in this work is obvious as aluminium is used in several vaccines and little is known of the fate of added adjuvant aluminium. The GL261 mouse glioma cell line was used to demonstrate that also cells with an origin from non-phagocytic cells can take up aluminium and lumogallion. Additionally aluminium/lumogallion could be used for labelling of tumour cells and *in vivo* tracking either after isografting or after use of tumour cells in vaccines.

The accessibility of instruments such as confocal microscopes and image cytometry, integrating flow cytometry and fluorescence microscopy with image analysis, just to mention some technologies, have enhanced the information that can be gathered after staining viable cells with fluorescent probes. In this paper, we highlight two histological stains that also can be used to stain viable cells. Today a diversity of histological stains with all kinds of specificities is used as research tools and in clinical diagnosis (Bancroft and Gamble, 2007). Several of these stains may also have the potential of staining viable cells and thereby make it possible to introduce new experimental approaches in cell biology.

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Legends

Fig. 1 Staining of THP-1 with lumogallion.

A. THP-1 cells were co-cultured with various amounts of Alhydrogel AlO(OH) during 24 h and then stained by continuous culture in the presence of 10 μM lumogallion during 16 h. The fluorescence from the cells was analysed by flow cytometry and the fluorescence intensity was determined as the mean fluorescence channel of the cells.

B. THP-1 cells were stained with various concentrations of lumogallion after co-culture with (■) or without (●) AlO(OH), 12.5 μg/ml, during 4 h. After an additional 16 h in the presence of various concentrations of lumogallion, the fluorescence intensity of the cells was determined by flow cytometry.

C. THP-1 cells were stained for various incubation times with lumogallion. Cells were co-cultured during 24 h in the presence (■) or absence (●) of AlO(OH), 12.5 μg/ml. After addition of 10 μM lumogallion, cells were withdrawn at various time intervals and analysed by flow cytometry.

Fig.2 Cells co-cultured with Alhydrogel AlO(OH) and stained with lumogallion.

Cells were co-cultured with either 25 μg/ml (THP-1 cells) or 5 μg/ml (GL261 cells) of AlO(OH) during 7 h. Lumogallion at a concentration of 5 μM was added to the culture and the next day the cells were washed with PBS, fixed in PFA and mounted using ProLong® Gold Antifade Reagent.

A. Orthogonal projection of a THP-1 cell co-cultured with Alhydrogel AlO(OH). The cell was stained with DAPI (blue) and lumogallion (red) to visualize intracellular aluminium particles. The image is based on a confocal z-stack containing 45 sequential 0.2 μm thick optical sections and shows section 23 in the centre of the cell.

B. Fluorescent image of the cell line GL261 co-cultured with Alhydrogel AlO(OH).

Fig. 3 Release of lumogallion from pre-stained Alhydrogel AlO(OH) particles.

AlO(OH) was incubated over night with lumogallion and collected by centrifugation. Collected AlO(OH) particles were re-suspended in R10 medium and after 5 min re-centrifuged. Re-suspension and centrifugation

were repeated 10 times, each time the supernatants were collected and their fluorescence was determined. The figure shows the fluorescence in the supernatant after clearance of particles/aggregates after each washing step (■). The figure also shows the fluorescence associated with dispersed adjuvant particles after the first re-suspension corresponding to 476 RFU (●).

Fig.4 Cells co-cultured with pre-stained Alhydrogel AlO(OH).

A. THP-1 cells were co-cultured during 4 h (●) or 24 h (■) with various concentrations of AlO(OH) pre-stained with lumogallion. The cells were harvested and the mean fluorescence intensity of the cells was determined by flow cytometry.

B. Fluorescence microscope image of GL261 cells co-cultured with 5 μg/ml pre-stained AlO(OH) over night before the cells were washed with PBS, fixed in PFA and mounted using ProLong® Gold Antifade Reagent. To the right; cells cultured in R10 medium.

Fig.5 Pre-staining of aluminium formulations with lumogallion.

Aluminium preparations ranging from 62.5 to 500 μg/ml were incubated over night with 50 μM lumogallion in R10 medium. The next day the particles were harvested, the pellets were re-suspended in 1 ml R10 medium and their fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength of 580 nm. Alhydrogel AlO(OH) light grey, $AlPO_4$ medium grey, AlO(OH) nanoparticles dark grey and Al_2O_3 nanoparticles black.

Fig. 6 Flow cytometry of cells stained with both lumogallion and a second fluorescent probe.

A. THP-1 cells were co-cultured with Alhydrogel AlO(OH), 12.5 μg/ml during 4 h and stained over night with 5 μM lumogallion. The next day the cells were harvested, washed, re-suspended in PBS containing 0.1 % BSA, 0.1 % h-IgG, stained with FITC labelled anti CD45 and analysed by flow cytometry using 70 % compensation of overflow of lumogallion fluorescence into the green PM-tube.

a). Cells co-cultured with AlO(OH), and stained with lumogallion.

b). Cells co-cultured with AlO(OH), and stained with lumogallion and anti CD45-FITC.

c). Cells co-cultured with AlO(OH), and stained with a FITC labelled isotype control antibody.

d). Cells co-cultured with AlO(OH), and stained with anti CD45-FITC

B. THP-1 cells were co-cultured with Alhydrogel AlO(OH) particles (12.5 μg/ml) pre-stained with lumogallion over night. The next day 5 nM of the mitochondrial probe MitoTracker Deep Red was added to the cells. After 30 min the cells were harvested, washed with PBS, fixed in 0.1 % PFA and analysed by flow cytometry using no compensation on the PM-tubes.

a). Cells cultured in R10 medium.

b). Cells cultured in R10 medium and stained with MitoTracker Deep Red.

c). Cells co-cultured with pre-stained Alhydrogel AlO(OH) particles and stained with MitoTracker Deep Red

Fig. 7 GL261 cells co-cultured with Alhydrogel AlO(OH) and stained with lumogallion and LysoTracker Deep Red.

Cells were co-cultured with 2.5 μg/ml of AlO(OH) during 7 h. Lumogallion at a concentration of 5 μM was added to the culture and the next day the cells were washed and stained with 100 nM LysoTracker Deep Red. Finally the cells were washed with PBS, fixed in PFA and mounted using ProLong® Gold Antifade Reagent.

Orthogonal projection of a GL261 cell co-cultured with Alhydrogel AlO(OH). The cell was stained with DAPI (blue), lumogallion (green) and LysoTracker Deep Red (red) to visualize nucleus, intracellular aluminium particles and lysosomes, respectively. The images are based on a confocal z-stack containing 20 sequential 0.45 μm thick optical sections and shows section 10 in the centre of the cell.

Fig. 8 Flow cytometry of THP-1 cells stained with morin or lumogallion after co-culture with or without Alhydrogel AlO(OH).

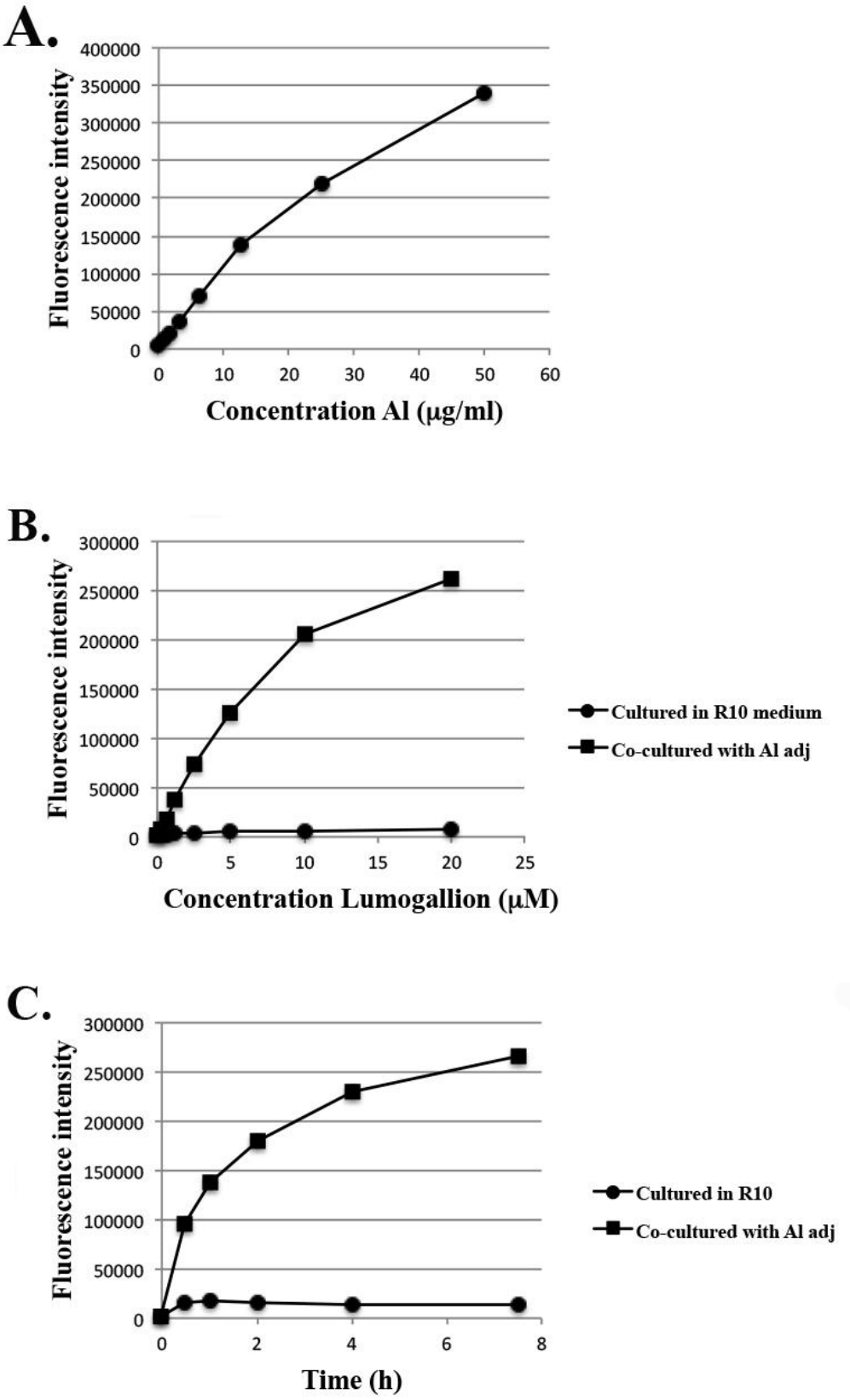
A. Cells co-cultured with Alhydrogel AlO(OH), 12.5 μg/ml, (grey histogram) and stained with 160 μM morin, cells cultured in R10 and stained with 160

μM morin (light grey histogram) and non-stained cells co-cultured with AlO(OH) (black histogram).

B. Cells co-cultured with Alhydrogel AlO(OH) particles pre-stained with morin, 50 μg/ml, (grey histogram) and cells cultured in R10 medium (black histogram).

C. Cells co-cultured with Alhydrogel AlO(OH), 12.5 μg/ml, (grey histogram) and stained with 5 μM lumogallion, cells cultured in R10 and stained with 5 μM lumogallion (light grey histogram) and non-stained cells co-cultured with AlO(OH) (black histogram).

Fig. 1



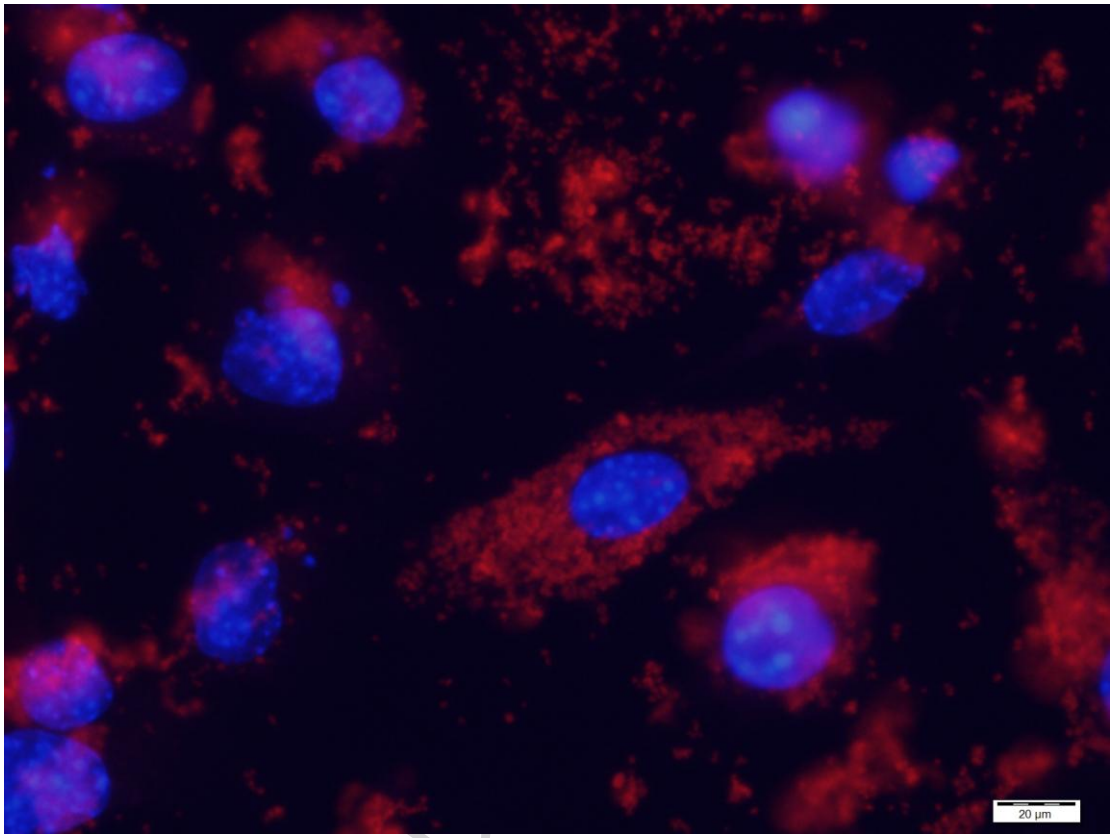


Figure 2A

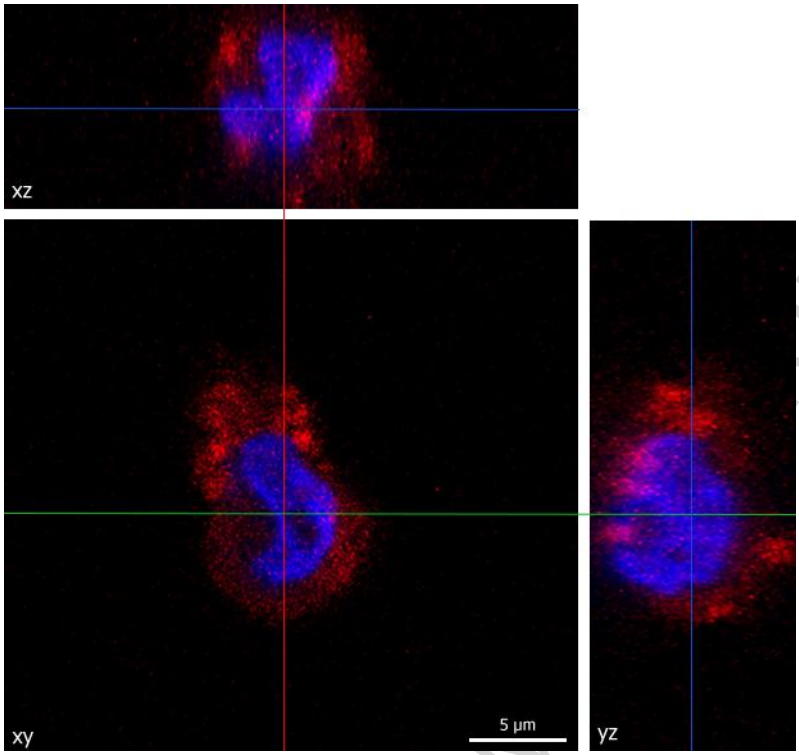


Figure 2b

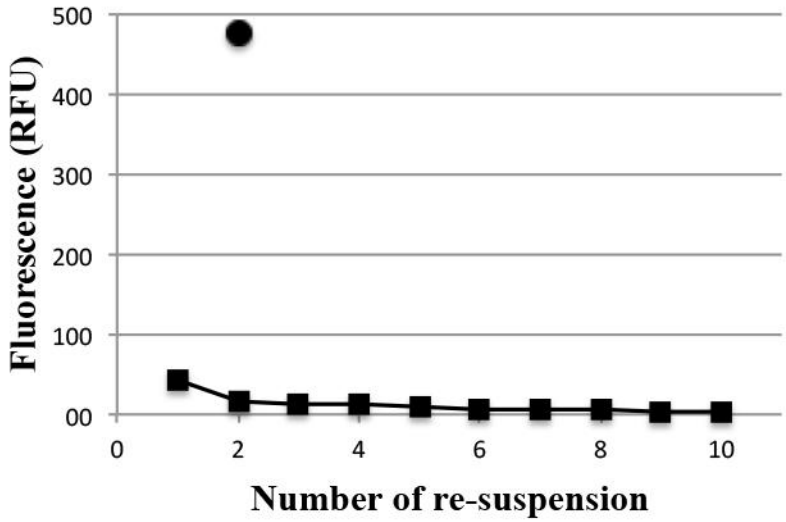
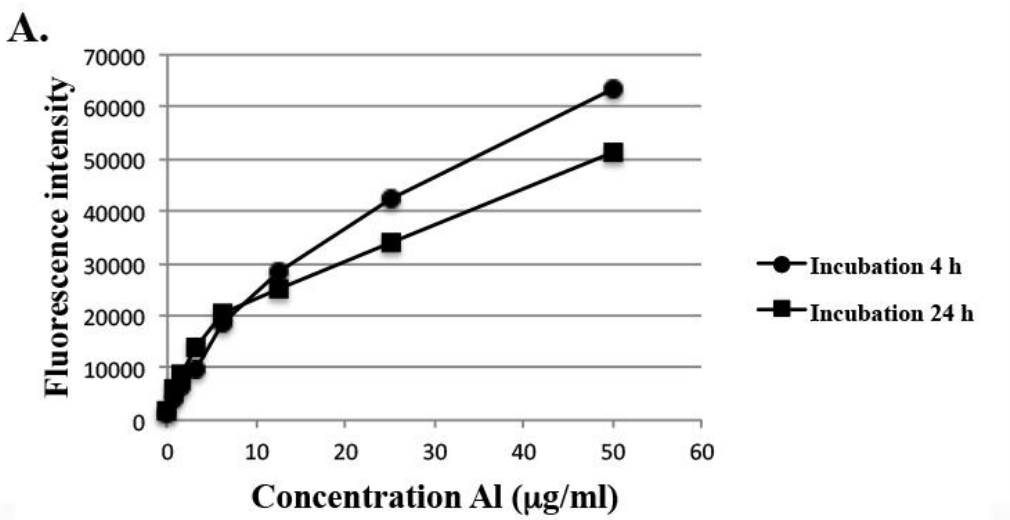


Figure 3

Fig. 4



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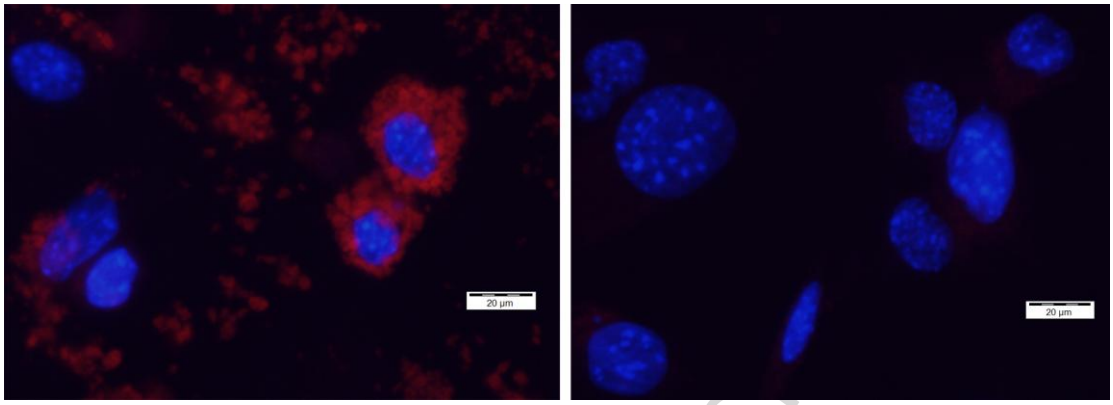
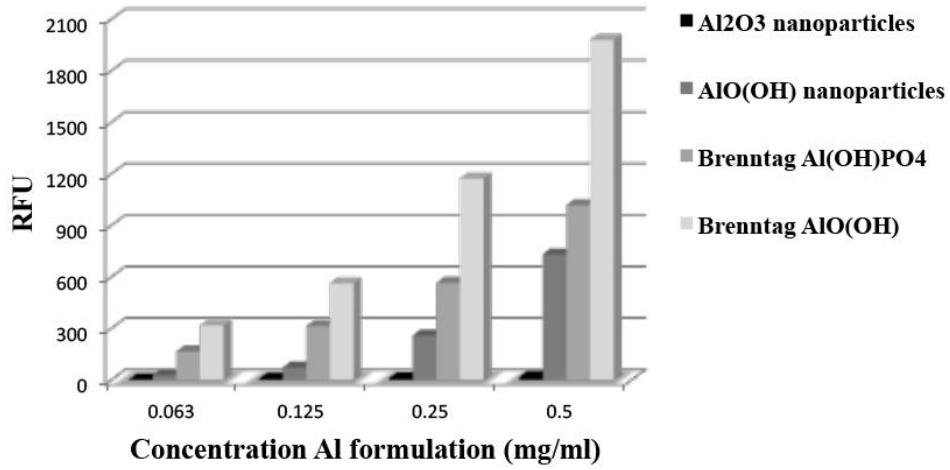


Figure 4b

Fig. 5



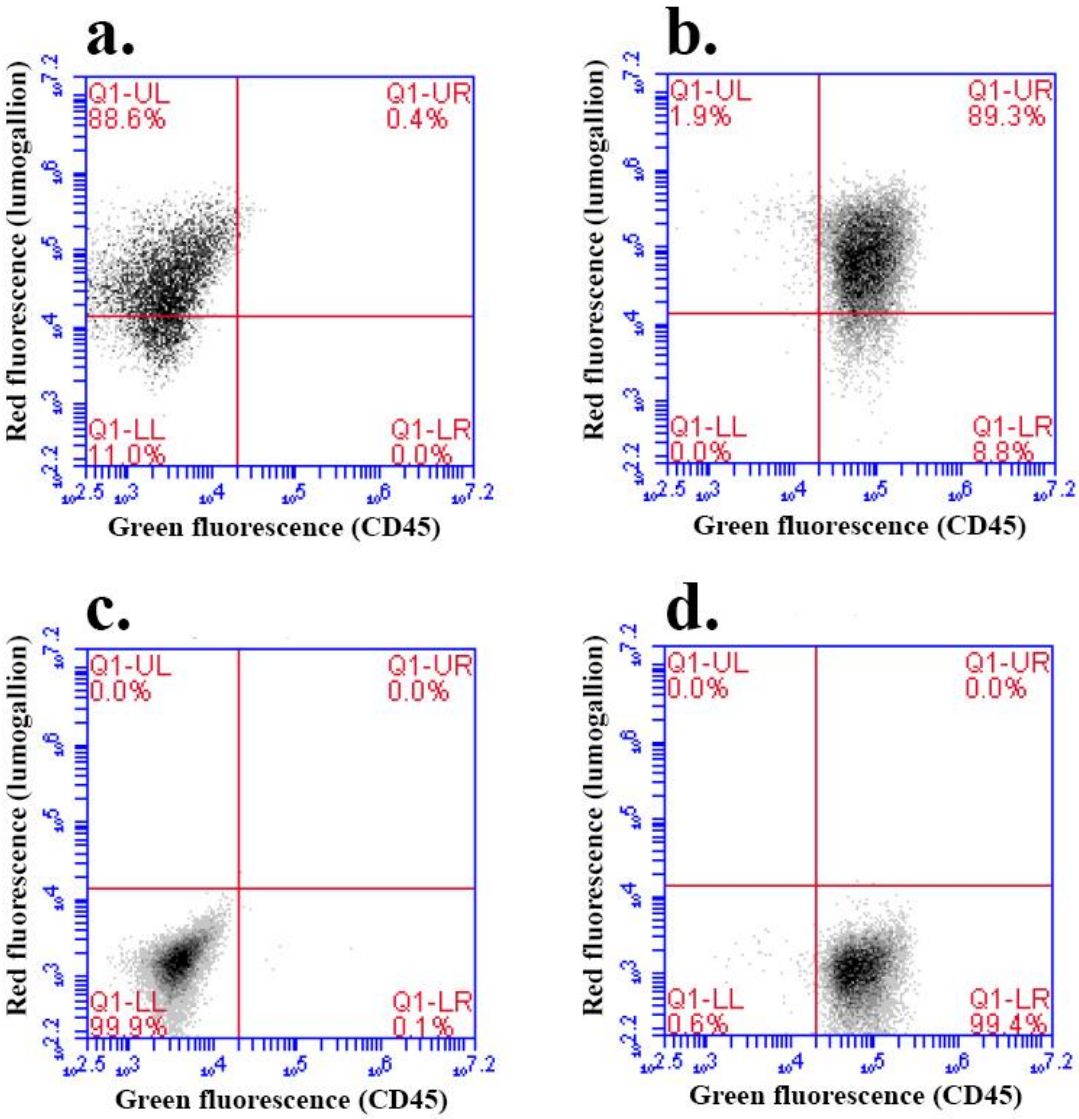
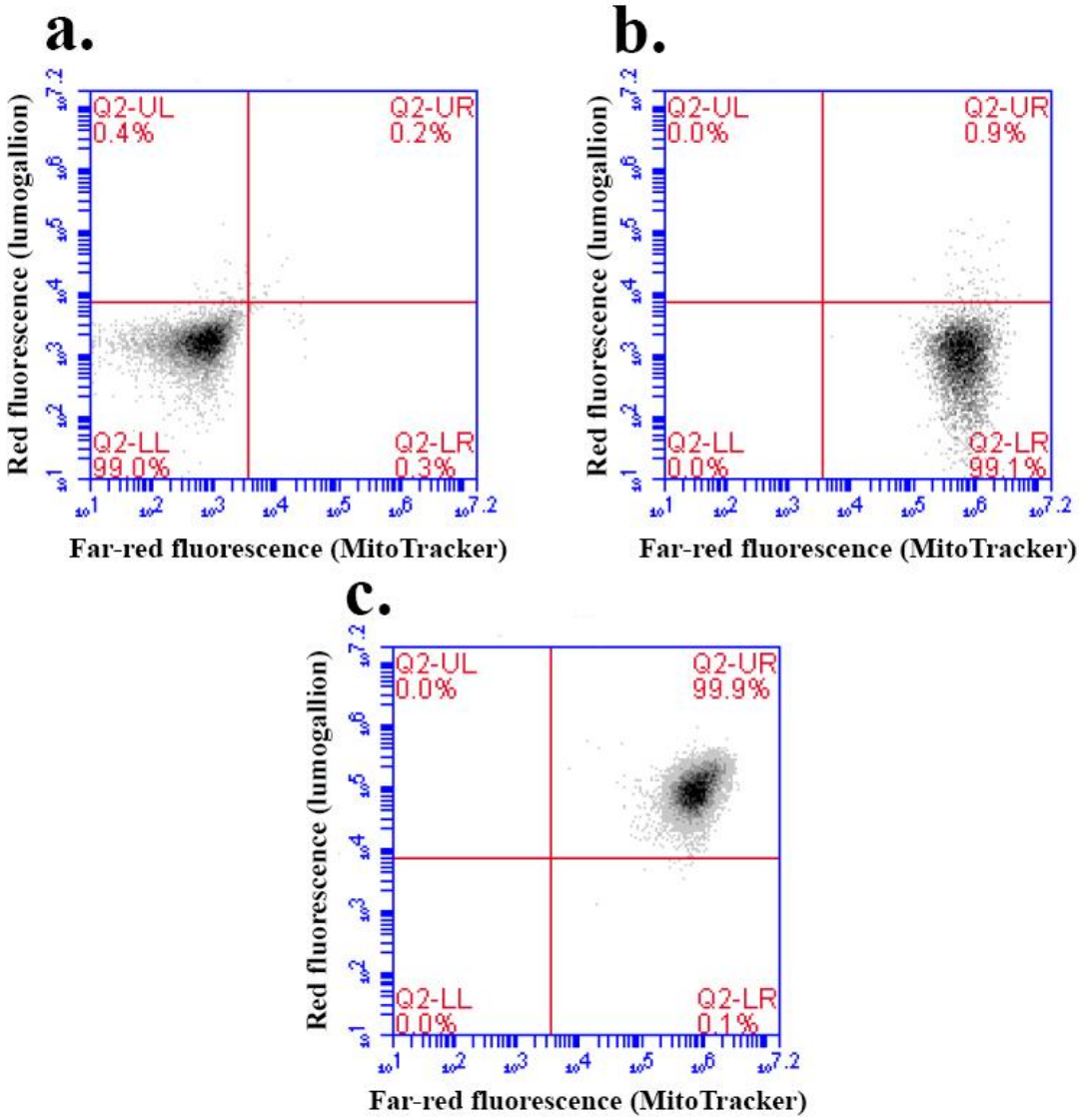


Figure 6a



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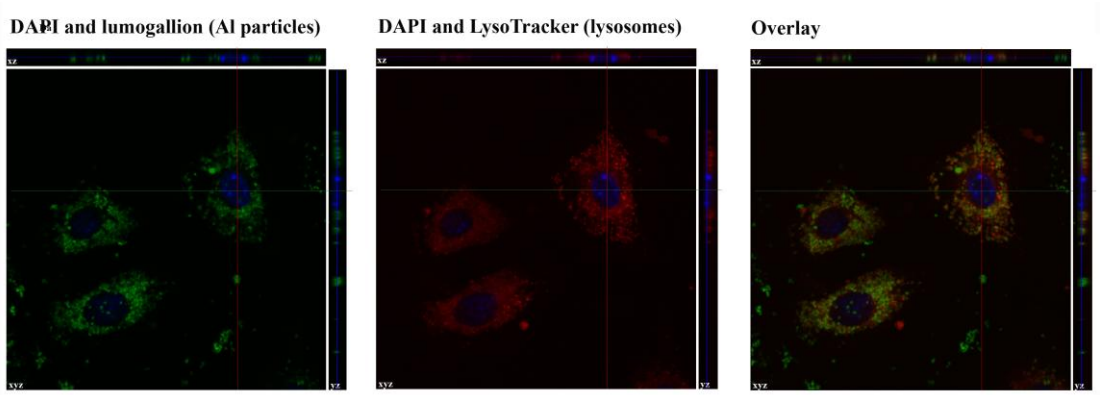

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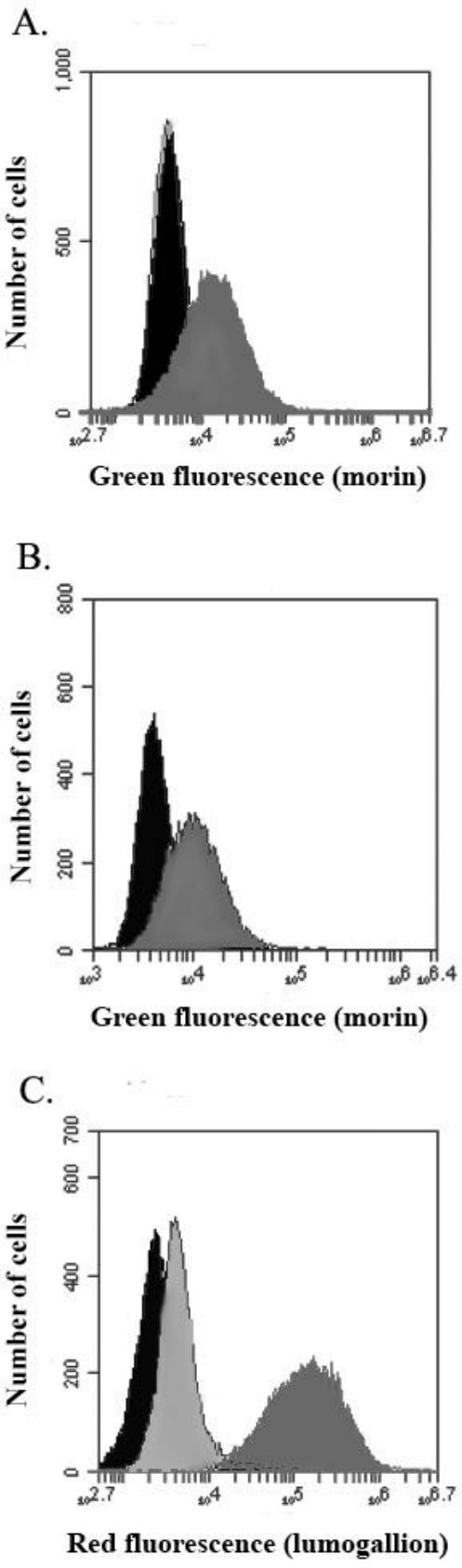
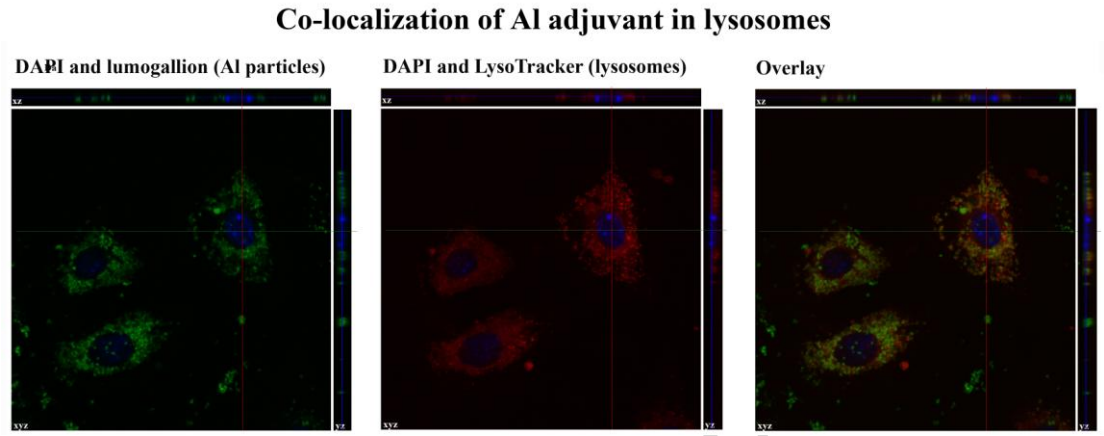


Figure



Graphical abstract

Highlights

- Fluorescent staining of aluminium adjuvants by lumogallion.
- Tracking of phagocytosed and intracellular identification of aluminium adjuvant in viable cells.
- Fluorescent labelling of aluminium adjuvants used in clinical vaccines.