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Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type

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Abstract

Aluminum hydroxide (AlOOH) has been used for many years as a vaccine adjuvant, but little is known about its mechanism of action. We investigated in this study the in vitro effect of aluminum hydroxide adjuvant on isolated macrophages. We showed that AlOOH-stimulated macrophages contain large and persistent intracellular crystalline inclusions, a characteristic property of muscle infiltrated macrophages described in animal models of vaccine injection, as well as in the recently described macrophagic myofasciitis (MMF) histological reaction in humans. AlOOH-loaded macrophages exhibited phenotypical and functional modifications, as they expressed the classical markers of myeloid dendritic cells (HLA-DR^{high}/CD86^{high}/CD83^{+}/CD1a^{-}/CD14^{-}) and displayed potent ability to induce MHC-II-restricted antigen specific memory responses, but kept a macrophage morphology. This suggests a key role of macrophages, in the reaction to AlOOH-adjuvanted vaccines and these mature antigen-presenting macrophages may therefore be of particular importance in the establishment of memory responses and in vaccination mechanisms leading to long-lasting protection.

Keywords: Macrophage; Aluminum hydroxide; CD83

1. Introduction

Vaccine adjuvants have been used for many years to induce strong and sustained humoral immune responses, but little is known about their mechanisms of action. The most frequently used adjuvant in commercial vaccines is aluminum hydroxide (AlOOH). The adjuvant activity of these molecules seems to result partly from a deposition effect and from antigen adsorption, as they can retain small amounts of antigen at the injection site for a prolonged period [1]. AlOOH immunoadjuvant property is also associated with the induction of Th2-type responses in animal models [2]. Aluminum salts also exert immunomodulatory activities on isolated cells, especially those of the monocyte-macrophage lineage. Indeed, the adsorption of tetanus toxoid onto aluminum hydroxide leads to a significant increase of in vitro antigen-induced T-cell proliferation, correlated with an increase in tetanus toxoid uptake and IL-1 secretion by monocytes [3]. Exposure of human peripheral blood mononuclear cells (PBMC) to aluminum hydroxide induces monocyte differentiation into mature CD83^{+} dendritic cells (DC), with a typical DC morphology, which strictly requires paracrine secretion of IL-4 by helper T lymphocytes [4]. Aluminum hydroxide also increases the survival and proliferation of murine bone marrow-derived macrophages in vitro [5].

Intramuscular injection of aluminum adjuvants causes tissue reactions and may lead to the local secretion of cell recruitment and differentiation factors. Several studies have been performed in laboratory animals to evaluate the safety of vaccines containing aluminum hydroxide adjuvants, as required by the EMEA guidelines (EMEA (1997); note for guidance on preclinical pharmacological and toxicological

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testing of vaccines; CPMP/SWP/465/95) [8]. These studies included the histological examination of the injection site after intramuscular administration, which showed a homogeneous pattern of macrophage infiltration within 1–2 weeks of vaccine injection (EMEA (2002); Hexavac: European Public Assessment Report (EPAR), scientific discussion). Follow-up study of the histological reaction has also been carried out in rabbits and cynomolgus monkeys after the intramuscular injection of diphtheria, tetanus, pertussis, and polio and the combined polio and diphteria vaccines, respectively (Verdier et al., manuscript in preparation). Both studies showed that aggregates of macrophages persisted for several months between muscle fibers. These observations are consistent with those of Gherardi et al., obtained after intramuscular injection of aluminum hydroxide-containing Hepatitis B vaccine in the rat [6]. There was a clear predominance of macrophages following the injection of aluminum hydroxide-containing vaccines in animals, and the data obtained illustrate the key role of this cell type in the physiological reaction to aluminum hydroxide containing vaccines.

Muscle reaction to vaccine injection thus consists in a granuloma with striking muscle fascia infiltration by aluminum-loaded macrophage, a histological entity called macrophagic myofasciitis (MMF) that is also found in some human vaccinees. Altogether, these results strongly suggest that macrophages may play a critical role in vaccine-induced immune responses.

This led us to investigate the effect of aluminum adjuvants on isolated macrophages. As reported here, we found that aluminum-loaded macrophages differentiate into mature, specialized antigen-presenting cells different from monocyte-derived DC.

2. Material and methods

2.1. Human monocyte isolation and differentiation

PBMC were isolated from the blood of healthy HIV-seronegative donors by Ficoll-hypaque density gradient centrifugation. Monocytes were separated from PBMC by incubation for 1 h in 75 cm^2 culture flasks to allow adhesion. They were cultured in DMEM-glutamax medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated (+56 °C for 30 min) FCS (Roche Diagnostics, Mannheim, Germany), 1% antibiotic mixture (penicillin, streptomycin, neomycin, Life Technologies) and 15 ng/ml GM-CSF (Leucomax, Schering-Plough, Kenilworth, NJ, USA), at +37 °C in a humidified 5% CO_2 in air atmosphere for 3 days. The monocytes were then harvested, washed with PBS and distributed in 48-well plates (0.2×10^6 cells per well), 96-well plates ($10–30 \times 10^3$ cells per well), or in 25 cm^2 culture flasks (5×10^6 cells), in 10% FCS culture medium, supplemented with 30 ng/ml GM-CSF and 2 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA). All experiments were performed on days 7–10 of culture, at the time of morphological and phenotypic differentiation, in DMEM or RPMI containing 1% normal human AB serum (DMEM-NHS or RPMI-NHS). Macrophages contained less than 10% residual lymphocytes. In these conditions, differentiated macrophages are $CD14^{high}$, $CD71^{high}$, $HLA\text{-}DR^{high}$, $CD86^{high}$, $CD163^{high}$, $CD32^{dull}$, $CD16^{dull}$, $CD80^{dull}$, $CD64^{dull}$, $CD11b^{dull}$, $CD1a^{-}$, $CD83^{-}$ (see Figs. 3 and 4) [11].

2.2. Adjuvants and antigens

Aluminum hydroxide gel (AlOOH, 10 mg/ml of Al, Reheis, Berkeley Heights, NJ, USA; provided by Aventis Pasteur vaccine production service, Marcy l'Etoile, France) was incubated overnight at a final concentration of 100 μg/ml in DMEM-NHS at 4 °C. In these conditions, the concentration of protein in the culture medium was 400 μg/ml. This protein/adjuvant ratio results in the saturation of negative charges on aluminum hydroxide [12]. Diphtheria and tetanus toxoid were from Aventis-Pasteur vaccine production service. Adjuvant and antigens contained no preservatives and no endotoxins.

2.3. Electron microscopy

Macrophages were incubated with or without 2 μg/ml AlOOH for 2 days. At day 2 or 9, the cells were scraped, centrifuged, and pellets were fixed in 2.5% glutaraldehyde. Pellets were then placed in fresh fixative for an additional 2 h. Samples were trimmed to 1 mm cubes and postfixed in 1% osmium tetroxide for 2 h prior to dehydration and infiltration with Epon resin. Ultrathin sections (80 nm) were cut from each block with an ultramicrotome and collected on mesh hexagonal grids. The grids were subsequently stained with uranyl acetate and lead citrate and examined using a Philips CM120 electron microscope.

2.4. Measurement of macrophage viability and immunoassays for cytokines

Macrophage viability was evaluated by the MTT assay (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. IL-1Ra and TNFα ELISA kits were obtained from R&D Systems.

2.5. Quantitative flow cytometric analysis of cell surface molecule expression

Macrophages were incubated with various concentrations of AlOOH, and in some experiments with a neutralizing anti-IL-4 mAb (2 μg/ml, clone B-S4, Diaclone, Besançon, France), or an isotype-matched irrelevant mAb. Macrophages were detached from the plastic, incubated for 30 min at 4 °C with 1 mg/ml purified human IgG (Tegeline, LFB, Les Ulis, France) and then with fluorochrome-conjugated mAb for 30 min at 4 °C. The cells were washed twice, fixed in 1% paraformaldehyde (w/v),

and analyzed for fluorescence using an LSR flow cytometer (BD Bioscience, San Jose, CA, USA). Viable cells were gated on the forward- and side-scatter dual parameter dot plot. Mean equivalent fluorochrome bound per cell (MEF) values were obtained for FITC and PE stainings by using fluorosphere calibration beads (Dako, Glostrup, Denmark) as a standard.

We used FITC-, R-PE- or PC5-conjugated mouse mAbs directed to HLA-DR (Immu-357, IgG1, Immunotech, Marseilles, France), CD1a (NA1/34, IgG2a, Dako), CD14 (M5E2, IgG2a, Pharmingen, San Diego, CA, USA), CD16 (3G8, IgG1, Pharmingen), CD32 (FL18.26), IgG2b (Pharmingen), CD3 (UCHT1, IgG1, Immunotech) and CD71 (Ber-T9, IgG1, Dako), CD80 (L307.4, IgG1, Pharmingen), CD83 (HB15.a, IgG2b, Immunotech), CD86 (FUN-1, IgG1, Pharmingen), CD163 (GHI/61, IgG1, Pharmingen), CD4 (13B8.2, IgG1, Immunotech). The corresponding isotype-matched irrelevant controls were used for each sample.

2.6. Endocytosis assay

Macrophages, cultured in the presence or absence of AlOOH, were incubated with 1 mg/ml FITC-dextran (M_r = 70,000; 60 Å, Sigma) for 30 min at either 4 °C (negative control) or 37 °C in DMEM supplemented with 10% FCS. Macrophages were then washed five times in cold PBS, directly scraped off the plastic with a rubber policeman and transferred into tubes for flow cytometric analysis. To exclude dead cells from analysis, 2 μg/ml 4′,6′-diamidino-2-phenylindole, dihydrochloride (DAPI), were added to each tube, and live, DAPI-blue fluorescence negative cells were gated (UV laser excitation).

2.7. Antigen presentation assay

Macrophages (10, 20 or 30 × 10^3 cells per well in 96-well plates) were incubated with diphtheria (0.15 Lf/ml) plus tetanus toxoid (0.05 Lf/ml), AlOOH (3 μg/ml) or with antigens plus AlOOH for 2 days in RPMI-NHS. Autologous or heterologous frozen PBMC were then thawed, and depleted of residual monocytes using anti-CD14-coated magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. CD14-depleted cells contained less than 1% residual monocytes, as confirmed by flow cytometric analysis. Macrophages were washed with PBS; and 10^5 CD14-depleted cells were added to each well. After 5 days of coculture, we assessed ^{3}H-thymidine incorporation (1 μCi per well) overnight.

We used a similar coculture model to assess proliferation with the membrane fluorescent dye PKH26. CD14-depleted PBMC were stained with the fluorescent dye PKH26 (Sigma), according to the manufacture's instructions, before coculture with macrophages. On day 7, non-adherent cells were harvested, and labeled for the flow cytometric detection of CD3 and CD4. The PKH26 fluorescent signal was recorded for gated live cells in the FL2 channel (560–579 nm). A minimum of 10,000 $CD4^+$ T-cells were analyzed per sample.

3. Results

3.1. Morphological and phenotypic changes induced by AlOOH

We first assessed the toxicity of AlOOH by the MTT assay, by incubating macrophages cultures for 3 days with AlOOH concentrations ranging from 0.1 to 100 μg/ml. The

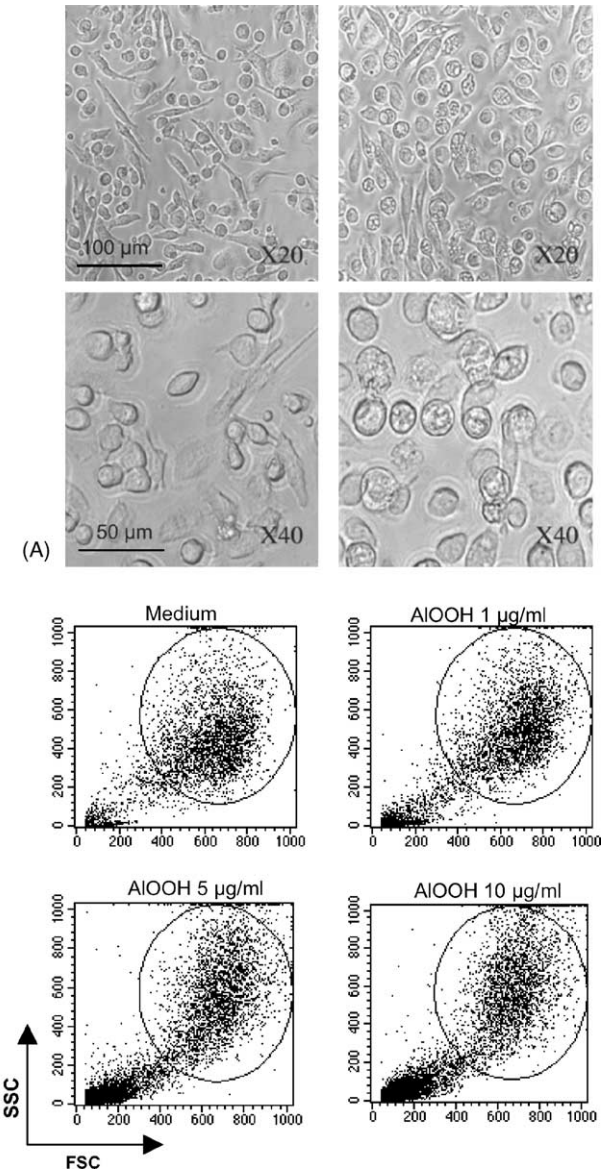

Fig. 1. Morphological and light scatter characteristics of AlOOH-treated macrophages. (A) Morphological aspect of control (left) and AlOOH-treated macrophages (right). (B) Light scatter plots of control and AlOOH-treated macrophages. The round region indicates which cell population was gated for analysis. Results are from 1 representative experiment out of 20.

dose of AlOOH used for vaccination is about 0.8 mg per dose of vaccine. The AlOOH concentration resulting in 50% mortality was 11±1.7 μg/ml ($n = 13$) (data not shown). AlOOH concentrations ranging from 1 to 5 μg/ml did not induce more than 30% toxicity. Treatment with 2 μg/ml AlOOH for 2 days induced morphological changes in macrophages: cells remained tightly adherent, but increased their size and granularity, and intracytosolic vacuoles appeared (Fig. 1A). This was confirmed by changes in flow cytometric forward/side light scatter parameters (Fig. 1B).

Following the injection of AlOOH-containing vaccine in vivo, muscle infiltrated macrophages bear crystalline inclusions of aluminum hydroxide. We assessed the presence of such inclusions in AlOOH-treated macrophages in vitro. By electron microscopy, we observed numerous, large crystalline inclusions in macrophages treated for 2 days with AlOOH (Fig. 2B and C), very similar to those observed in vivo. These crystalline inclusions were still observed in macrophages 7 days after the removal of AlOOH (Fig. 2D).

We investigated the effect of AlOOH on macrophage surface molecule expression pattern (Fig. 3 and Table 1). Phenotypic changes were observed for AlOOH concentrations ranging from 1 to 10 μg/ml, and were maximal at the dose of 5 μg/ml. HLA-DR, and more importantly CD86 and CD71, expression levels were significantly upregulated by AlOOH. In our hands, CD80 was rarely expressed by macrophages and we showed that for macrophages expressing CD80, AlOOH decreased its level of expression. Twenty-four percent and 44% of macrophages constitutively expressed the IgG-Fc-receptors CD16 and CD32, respectively, and 80% of macrophages were positive for the scavenger receptor CD163, with a high MEF. We found that AlOOH downregulated both FcγR as well as the expression of CD163. AlOOH also decreased CD14 expression, characterized by a progressive inhibition of MEF, leading to the absence of positive cells at concentrations of AlOOH above 5 μg/ml. As for crystalline inclusions, these modulations of markers expression were still observed in macrophages 7 days after the removal of AlOOH (data not shown). Furthermore, we investigated the consequences of CD14 loss in macrophages in long-term culture by stimulating these cells with LPS and measuring TNFα and IL-1Ra concentrations in the supernatant. As shown in Table 2, AlOOH-treated macrophages did not produce significant amounts of these cytokines, demonstrating that the loss of CD14 was indeed associated with an impaired response to LPS. This suggests that AlOOH induces macrophage differentiation rather than a transient activation, and that the persistance of crystalline inclusions may be linked to that of the AlOOH-induced phenotype modifications.

Table 1
Effect of AlOOH on surface molecule expression pattern

Membrane phenotype	MEF ($\times 10^3$)[a]		Modulation[b] (%)
	Medium	AlOOH (5 μg/ml)	
HLA-DR	272 ± 73	498 ± 155	92 ± 24** ($n = 12$)
CD71	62 ± 14	71 ± 21	37 ± 40 ($n = 5$)
CD80	14 ± 5	3.6 ± 1.4	−69 ± 10*** ($n = 6$)
CD86	82 ± 17	145 ± 31	78 ± 15*** ($n = 8$)
CD16	18 ± 6.3	5 ± 2.3	−64 ± 20* ($n = 3$)
CD32	74 ± 1.7	34 ± 13	−55 ± 12* ($n = 3$)
CD14	190 ± 46	9.8 ± 3.6	−88 ± 5*** ($n = 9$)
CD163	115 ± 41	3.2 ± 0.4	−95 ± 2.6*** ($n = 4$)

Macrophages were cultured with medium alone or with AlOOH for 2 days, and cells were labeled with fluorescent mAbs and analyzed by flow cytometry.

[a] Mean equivalent fluorochrome bound per cell (MEF ± S.E.M.).

[b] MEF modulation induced by AlOOH, as a percent of untreated cell MEF value. n is the number of experiments.

* $P < 0.05$ (unpaired Student's t test).

** $P < 0.01$ (unpaired Student's t test).

*** $P < 0.001$ (unpaired Student's t test).

3.2. Dendritic cell markers and functions

As AlOOH-treated macrophages expressed surface molecules similar to those described for cultured dendritic cells (HLA-DR^{high}, CD86^{high}, and CD14^{-}), we further investigated the expression of dendritic cell-specific markers. CD1a is a marker of immature dendritic cells, the expression of which is lost during the maturation process, whereas

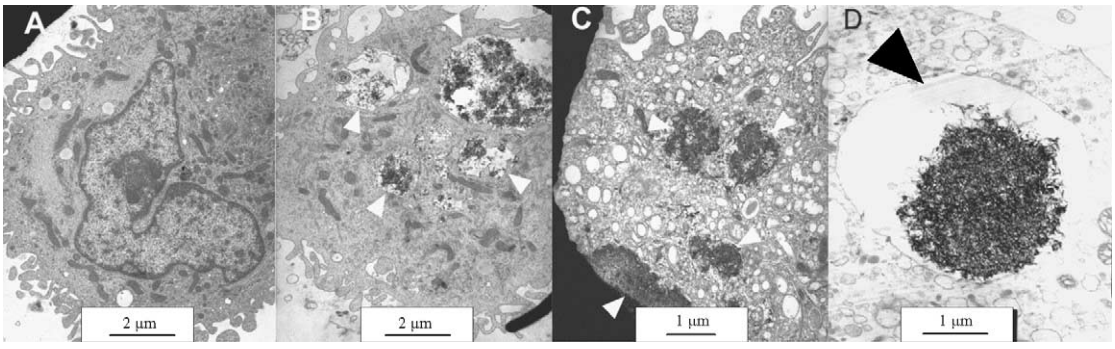

Fig. 2. Crystalline inclusions in AlOOH-loaded macrophages (electron microscopy). (A) Untreated macrophages, with a typical macrophage nucleus morphology. (B) Macrophages were treated with AlOOH for 2 days. (C) Macrophages were treated with AlOOH for 2 days, (D) thoroughly washed with PBS and cultured for an additional 7 days. Arrowhead indicate crystalline inclusions.

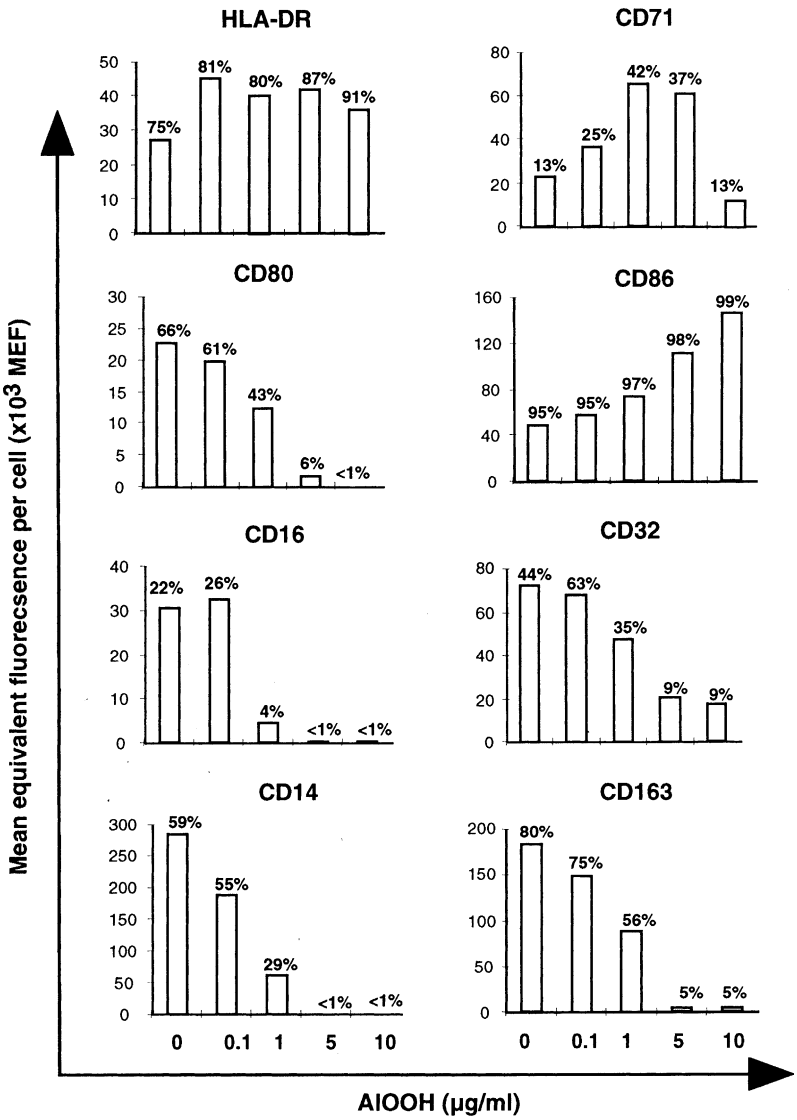

Fig. 3. Dose–response effect of AlOOH on surface molecule expression pattern. Macrophages were cultured with medium alone or with AlOOH for 2 days, and cells were labeled with fluorescent mAbs and analyzed by flow cytometry. Bars indicate mean equivalent fluorochrome bound per cell values. Numerical values are the percentages of positive cells. Data are from one representative experiment. Statistical analysis of the whole data obtained at the dose of 5 μg/ml AlOOH is given in Table 1.

Table 2
AlOOH effect on macrophage response to LPS

LPS dose (μg/ml)	Control cells			AlOOH-treated cells		
	0	1	10	0	1	10
TNFα	<10	214 ± 11	565 ± 10	<10	34 ± 4	65 ± 0.5
IL-1Ra	238 ± 23	1032 ± 52	1320 ± 30	143 ± 36	250 ± 11	239 ± 10

Macrophages were cultured with or without 2 μg/ml AlOOH for 3 days, thoroughly rinsed with PBS and further cultured without AlOOH for 7 days and then stimulated for another 2 days with LPS before assessment of cytokine production by ELISA. Data are from one of three independent experiments. Cytokine data are means ± S.D. of duplicate determinations (pg/ml).

the level of expression of the mature dendritic cell marker CD83 rises. Less than 3% of macrophages constitutively expressed low levels of CD1a and CD83. Incubation of macrophages with 5 μg/ml AlOOH for 2 days induced a readily detectable CD83 expression, with no detectable CD1a expression (Fig. 4A). This induction of CD83 was dose-dependent: it was detected at concentrations greater than 1 μg/ml (7.5×10^3 MEF and 16% positive cells) and reached 37×10^3 MEF and 90% positivity at 10 μg/ml (Fig. 4B). Kinetic analysis showed that after 15 h of

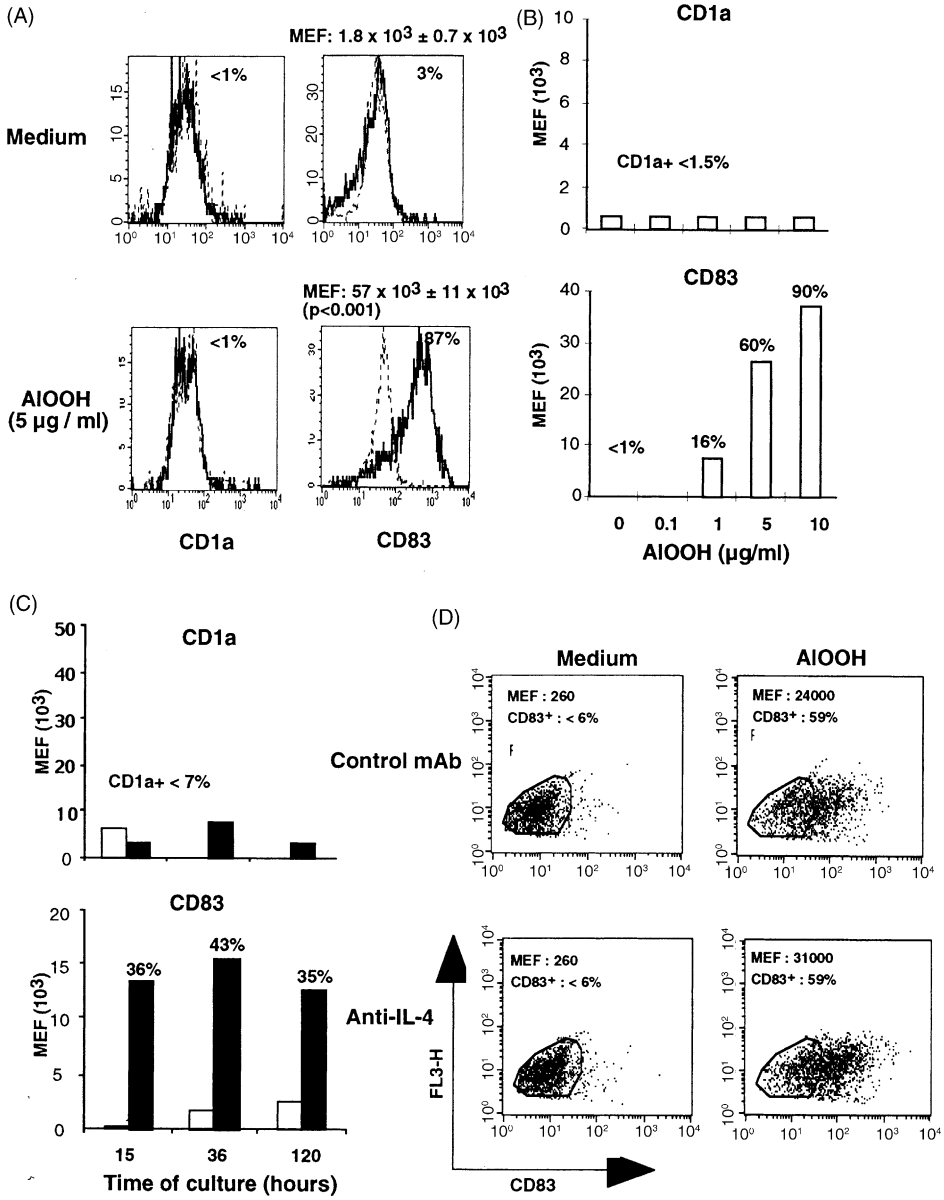

Fig. 4. Effect of AlOOH on the expression of CD1a and CD83. (A) CD1a and CD83 expression (thick lines) by macrophages after a 2-day AlOOH stimulation. Dotted lines indicate fluorescence distribution of cells stained with isotype-matched irrelevant controls. Numerical values are the percentage of positive cells. Data are from 1 of 5 experiments. Values represent MEF of CD83 (mean ± S.E.M. of 10 experiments). (B) Dose–response effect of AlOOH on CD1a and CD83 expression (2-day stimulation). Bars indicate mean equivalent fluorochrome bound per cell values. Numerical values are the percentage of positive cells. Data are from one of two experiments. (C) Kinetic of AlOOH effect on CD1a and CD83 expression. (□): medium, (■): AlOOH. Bars indicate mean equivalent fluorochrome bound per cell values (MEF). Numerical values are the percentage of positive cells. Data are from one of two experiments. (D) CD83 induction by AlOOH in the presence of a neutralizing anti-IL-4 mAb or an irrelevant isotype matched mAb. Horizontal axis is the CD83-specific fluorescence intensity.

incubation with 2 μg/ml AlOOH, 36% of macrophages were $CD83^+$ (13.6×10^3 MEF), this level remained stable for 5 days (Fig. 4C). No CD1a expression was detected on macrophages, regardless of the concentration of AlOOH and the time of culture (Fig. 4). This dissociation of CD1a and CD83 expression suggests that AlOOH-treated macrophages, although morphologicaly clearly different from monocyte-derived dendritic cells, acquired a mature dendritic cell-like phenotype.

Although no IL-4 could be detected in supernatant by means of ELISA (data not shown), we investigated whether AlOOH-induced CD83 expression on macrophages was an IL-4-dependent phenomenon, as described by Ulanova et al. [4] for monocytes in AlOOH-treated PBMC cultures. We incubated macrophages for 3 days with AlOOH in the presence of neutralizing anti-IL-4 antibody, and CD83 expression was still induced by AlOOH, showing that this maturation mechanism is

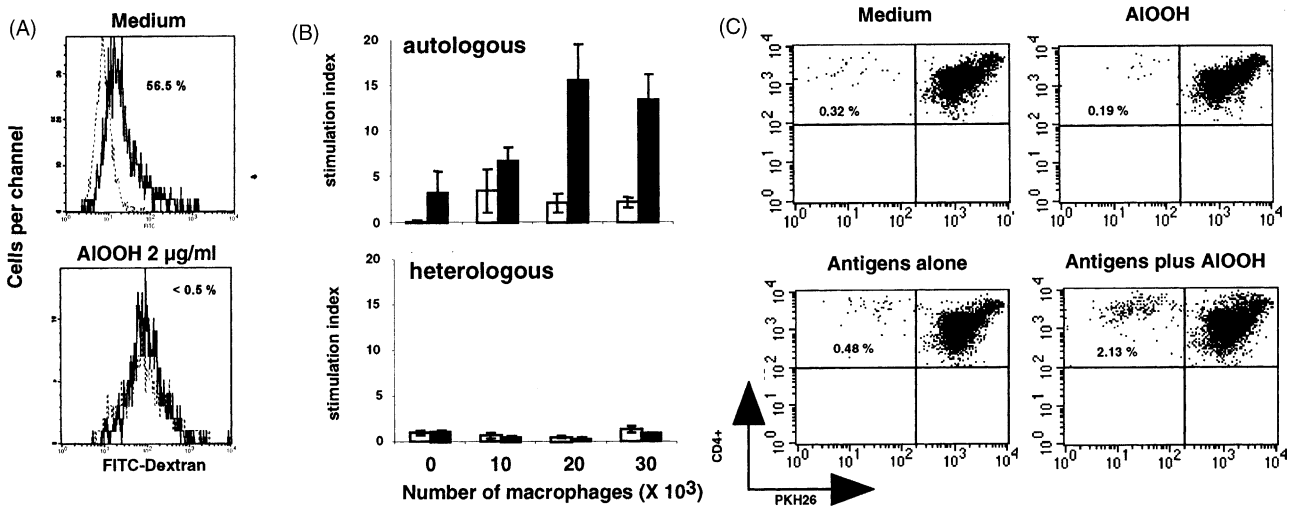

Fig. 5. Endocytosis and antigen-presenting capacities of AlOOH-treated macrophages. (A) FITC-dextran specific fluorescence after uptake at 4 °C (dotted lines, negative control) or 37 °C (thick lines). Numerical values are the percentage of live cells that took up FITC-dextran. Data are from one of two experiments. (B) Lymphocyte proliferation induced by AlOOH- and antigen-loaded macrophages. Stimulation index of thymidine incorporation in lymphocytes cocultured with macrophages previously pulsed with diphtheria + tetanus toxoids (Ags) with (■) or without (□) AlOOH. Results are expressed as stimulation index. Data are from one of three experiments and are expressed as means ± S.D. of triplicate determinations. (C) $CD4^+$ T-cell proliferation induced by AlOOH- and antigen-loaded macrophages. Macrophages were pulsed in medium alone, or in the presence of AlOOH, Ags, or Ags and AlOOH. PKH26-stained autologous lymphocytes were cocultured with macrophages and analyzed by flow cytometry for CD3, CD4, and cell proliferation (PKH26 membrane dye). Plots show PKH26-specific (horizontal axis) and CD4-specific (vertical axis) fluorescence on gated $CD3^+/CD4^+$ T-cells. Numerical values are the percentage of cells that underwent proliferation. Data are from one of two experiments.

different from the one described by Ulanova et al. [4] (Fig. 4D).

We investigated the capacity of other stimulating molecules such as IL-4, IL-4 + GM-CSF, IL-13, Dexamethasone and LPS, as well as that of latex beads, to induce CD83 expression on macrophages. No induction of CD83 expression was observed on macrophages (data not shown).

As dendritic cells lose their endocytosis capacity during the maturation process [13], we investigated this cell function in AlOOH-stimulated macrophages. We found that 56.5% of unstimulated macrophages took up detectable amounts of FITC-dextran particles, whereas less than 0.5% of AlOOH-stimulated macrophages did (Fig. 5A), showing that the treatment of macrophages with AlOOH also leads to the complete loss of endocytic capacity.

As dendritic cells are efficient, specialized antigen-presenting cells, whereas macrophages are less efficient in antigen presentation, we also investigated the ability of AlOOH-treated macrophages to present antigens. As expected, macrophages incubated with low doses of antigens alone (diphteria and tetanus toxoids) displayed a weak capacity to induce autologous lymphocyte proliferation. On the other hand, macrophages incubated with the same dose of Ag in the presence of AlOOH were potent inducers. Heterologous lymphocytes did not proliferate in the same conditions (Fig. 5B). Moreover, macrophages incubated with AlOOH alone did not induce proliferation in either autologous or heterologous T-cells (data not shown). To identify the lymphocyte subpopulation that proliferated in response to AlOOH plus Ag-treated macrophages, we performed the same experiments with PKH26-stained lymphocytes. Cells that proliferated in response to AlOOH plus Ags were $CD3^+/CD4^+$ T-cells (Fig. 5C). Overall, these data suggest that AlOOH increases the capacity of macrophages to initiate an MHC-II-restricted antigen-specific response.

4. Discussion

Overall, data from animal models have shown that in vivo, macrophage recruitment is a normal response to the intramuscular injection of AlOOH-containing vaccines [6]. However, it has also been suggested that the presence of a MMF in muscle would be associated with a diffuse arthromyalgia and fatigue syndrome [6,7,9,10], raising questions about the physiological or pathophysiological role of macrophages infiltrating muscle fascia. Although the interaction between cell types that can occur in vivo are far too complex to be reproduced in vitro, isolated cell culture has for long been used and may provide data that make sense when brought together with the in vivo situation. In a first step, we therefore focused on macrophagic response to aluminum gels and showed that macrophages incubated with aluminum hydroxide exhibit a mature dendritic cell-like phenotype, with enhanced ability to present antigen to helper T-cells.

Among phenotypical modifications induced by AlOOH, the induction of CD83 expression on macrophages is striking. This member of the immunoglobulin superfamily is a marker of mature dendritic cells [13,14], and is believed to play a role in antigen presentation or cell–cell

interactions [15]. Immature DC ($CD1a^+/CD83^-$) can be obtained from blood monocytes by stimulation with GM-CSF and IL-4. Further activation with LPS, TNF or CD40L induces monocyte-derived DC maturation, which typically involves the loss of CD1a and the acquisition of CD83 [13]. Upon stimulation with GM-CSF and IL-4, in vitro differentiated macrophages can also acquire a $CD1a^+/CD14^{dim}$ DC phenotype, and can also mature leading to heterogeneous CD83 expression [16,17]. The phenotype of AlOOH-stimulated macrophages clearly differed from monocyte-derived DC and from cells described by Palucka et al. [16] and Zou et al. [17], since they remained tightly adherent, without dendrites, did not express CD1a at any time of culture, and differentiated independently of IL-4. This suggests a different process of differentiation that does not require the immature macrophage-derived dendritic cell intermediate for completion.

Our functional data strongly suggest that AlOOH-loaded macrophages are specialized antigen-presenting cells. Indeed, pulsing macrophages with low doses of recall antigens in the presence of AlOOH significantly increased their capacity to induce autologous $CD4^+$ T lymphocyte proliferation. Thus, AlOOH may increase macrophage ability to induce an MHC-II-restricted antigen-specific response. These functional modifications are consistent with the increased expression of CD83, CD86 and HLA-DR induced by AlOOH on macrophages. Moreover, AlOOH-treated macrophages rapidly lost their endocytic ability suggesting that they may not further take up antigens after treatment, a common feature of mature DC [18]. They also lost their expression of Fcγ receptors, CD14 and showed a persistent unresponsiveness to LPS, evocative of a decrease of innate immunity functions.

Ulanova et al., recently showed that a 2-day exposure of whole PMBC to aluminum hydroxide induces monocytes to acquire CD83 expression, a typical DC morphology, and an increased expression of MHC class II molecules and CD86 [4]. This monocyte differentiation is independent of GM-CSF but requires paracrine IL-4 production by T lymphocytes [4]. AlOOH-treated macrophages cannot have undergone this type of differentiation for the following reasons: (i) we tested the effect of AlOOH on macrophages, i.e. on already differentiated cells, but not on monocytes. (ii) We did not detect IL-4 production by AlOOH-stimulated macrophages (data not shown), and the anti-IL-4 neutralizing mAb did not prevent AlOOH-induced CD83 expression. (iii) The morphology of adherent aluminum-loaded macrophages clearly differed from that of the aluminum-induced, monocyte-derived DC described by Ulanova et al. [4]. Taken together, our results and those of Ulanova et al. are complementary, and show that AlOOH can induce monocyte differentiation into myeloid dendritic cells via an IL-4-dependent pathway, but can also act directly on already differentiated macrophages, inducing IL-4-independent differentiation into mature, specialized antigen-presenting macrophages (MAPM).

Molecular signaling pathway(s) involved in AlOOH-induced macrophage maturation should be investigated. We indeed detected no production of TNFα, IL-1β, IL-12, IL-10 or GM-CSF in the supernatants of AlOOH-treated macrophages (data not shown). We may hypothesize that intracellular signaling pathways could be activated directly, leading to gene activation, for example through the NF-κB system. On another hand, receptors of the TLR family might be involved, keeping in mind that TLR4 and TLR9 mediate activity of other molecules with adjuvant activity such as LPS and CpG-DNA [19,20]. Furthermore, in our hands, CD83 expression was not induced on macrophages, upon stimulation with cytokines such as IL-4, IL-13 or GM-SCF, or with dexamethasone, or LPS, suggesting that the mechanism involved in AlOOH-induced CD83 expression was not restricted by these molecules. The absence of effect of LPS underlines that endotoxin contamination of AlOOH is not responsible for this observation. Incubation of macrophages with two types of latex beads with sizes similar to AlOOH particles (0.45 and 3 μm), did not induce CD83 expression on macrophages and did not modulate CD86 and HLA-DR expression (data not shown) suggesting that the effect of AlOOH is not a consequence of particle phagocytosis. However, it would be of interest to investigate whether the uptake of necrotic or apoptotic cells by macrophages is involved in these modifications.

Aluminum-loaded macrophages bear crystalline inclusions that can be observed by electron microscopy for at least 7 days after AlOOH removal. These inclusions are very similar to those observed after vaccine injection in vivo. This raises the question of whether macrophages differentiation is a response to these inclusions or not. For this purpose, it will be necessary to determine whether other aluminic or non-aluminic adjuvants can induce a similar macrophage differentiation in the absence of inclusions (e.g. particulate and non-particulate adjuvants). In conclusion, aluminum hydroxide induces macrophage differentiation in vitro into a new type of mature, specialized antigen-presenting cell, distinct from the already known dendritic cell types. The putative involvement of this particular cell type in the establishment of memory responses and the mechanisms through which vaccination establishes long-lasting protection should thus be investigated. In particular, the question of whether AlOOH-treated macrophages may exhibit a particular resistance to apoptosis, and/or transport antigen to the lymph node and activate naive antigen-specific T-cells should be addressed.

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