

Aluminium hydroxide down-regulates T helper 2 responses by allergen-stimulated human peripheral blood mononuclear cells

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Summary

Background Aluminium hydroxide (alum) is a commonly used adjuvant for specific immunotherapy of allergic diseases. While alum is traditionally associated with murine Th2 sensitization, little is known about its effects on secondary allergic responses in humans.

Methods We investigated the *in vitro* effects of alum on peripheral blood mononuclear cells (PBMC) from atopic donors. PBMC from 18 grass pollen-sensitive rhinitic subjects were stimulated with *Phleum pratense* (Phl p) in the presence or absence of alum. After 6 days culture, cytokine production was measured by ELISA and T cell proliferation by radiolabelled thymidine incorporation. The effect of alum on the expression of human leucocyte antigen and CD80/CD86 on cultured antigen-presenting cells was assessed by flow cytometry.

Results PBMC cultured with Phl p and alum showed a significant decrease in both IL-5 and IL-13 production compared with allergen alone ($P < 0.005$ and $P < 0.001$, respectively), but no change in IFN- γ or IL-12 production or proliferative responses. These alum-induced changes in T helper (Th)2 cytokine production were unaffected by the addition of neutralizing antibodies to IL-4 or IL-12. Culture of PBMC with alum induced increased expression of CD86 ($P = 0.004$) and HLA ($P = 0.01$) on monocytes while the expression of CD80 was decreased ($P = 0.02$).

Summary Alum down-regulates allergen-driven Th2 cytokine responses while Th1 cytokines are unaffected. These data confirm that alum is a useful adjuvant for inclusion in allergen immunotherapy vaccines.

Keywords adjuvants, allergy, aluminium hydroxide, human, immunotherapy, T lymphocytes

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Introduction

Aluminium hydroxide (alum) has been widely used as an adjuvant in human and veterinary vaccines for over 70 years [1]. Alum has been administered to hundreds of millions of people, has a good safety profile, and provides adjuvanticity with a variety of antigens. Despite this, the mechanisms that underlie the effect of aluminium salts on the immune system are poorly understood. One feature of alum is to act as a depot, involving the retention of antigen and its slow release either from the site of injection or local draining lymph nodes [2], thus enhancing immune responses by prolonged antigen presentation. Secondly, immunization with aluminium-adsorbed vaccines has been shown to induce high titred and long-lasting antibody responses in humans [3]. However, these adjuvants are ineffective in inducing cell-mediated and cytotoxic T cell responses and can augment immunoglobulin (Ig)E antibody responses [4].

Allergic inflammation is associated with raised levels of allergen-specific IgE and infiltration and activation of eosinophils influenced by the T helper (Th)2-derived cytokines, IL-4 and IL-5 [5]. Allergen-specific immunotherapy is an effective treatment for atopic disease [6] and has well-documented clinical efficacy [7–9]. Standard clinical protocols for allergen immunotherapy involve the subcutaneous injection of allergen extracts adsorbed to alum. Treatment involves gradually increasing the dose of vaccine, leading to a maintenance phase of monthly injections which are continued, in general, for 3–5 years. The mechanisms that underlie this treatment are thought to either involve deviation of the immune response towards a protective Th1 response [10] or the induction of a subset of CD4⁺ CD25⁺ regulatory cells [11, 12]. Findings based on primary responses in mice, that demonstrate that alum elicits preferential Th2 responses, suggest that the use of alum as an adjuvant for pollen immunotherapy in man may possibly be detrimental.

In this study, we aimed to determine the influence of alum on allergen-stimulated human peripheral blood mononuclear cells (PBMC) isolated from atopic individuals suffering from seasonal hayfever. We measured both Th1- and Th2-type cytokines, and proliferative responses to grass pollen extract and examined the effects of neutralizing antibodies to IL-4

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and IL-12. Furthermore, we studied the influence of alum on human leucocyte antigen (HLA) and CD80/CD86 (B7-1/B7-2) expression by antigen-presenting cells (APCs) derived from these subjects.

Materials and methods

Subject characterization

The study was approved by the Ethics Committee of the Royal Brompton and Harefield NHS trust and performed with the written consent of all participants. All donors (seven males, 11 females; age 33 years (24, 39); (median; lower quartile, upper quartile)) suffered from hayfever and were characterized as sensitive to grass pollen by positive (9 mm; 8, 13) skin prick tests to *Phleum pratense* (Phl p) (Soluprick, ALK Abelló, Horsholm, Denmark) of at least 5 mm greater than the negative diluent control (0 mm; 0, 0). All donors had raised serum allergen-specific IgE tests to grass pollen (RAST: 28 IU/mL; 19, 66) [13] and median total IgE measurements of 135 IU/mL (62, 190).

Peripheral blood mononuclear cells isolation and culture

Heparinized blood was collected by venopuncture and PBMC were separated by Histopaque (Sigma, Poole, UK) density-gradient centrifugation. Cells were washed twice with RPMI-1640 (Invitrogen, Paisley, UK) and resuspended in RPMI-1640 supplemented with 5% human AB⁺ sera (Sigma), 2 mmol/L L-glutamine (Invitrogen) and 100 U/mL penicillin-streptomycin (Invitrogen). Previous studies have identified that a range of 0.2–20 µg/mL of *P. pratense* can elicit cytokine production and proliferative responses from PBMC cultures (12). In order to detect both increases and decreases in these profiles induced by alum, we chose to stimulate cells with 5 and 0.5 µg/mL of Phl p. Concentrations of alum used were based on the ratio of alum to Phl p used in the standard immunotherapy vaccine (Alutard, ALK Abelló). 1×10^6 cells were cultured in 48-well plates for 6 days in the presence of 5.6 or 56 µg/mL of alum (pH, 8.0–8.5) (ALK Abelló) and/or unadsorbed 0.5 or 5 µg/mL of *P. pratense* (ALK Abelló) at 37 °C with 5% CO₂. In addition, PBMC from the same donors were incubated with the standard grass pollen vaccine comprising of 5000 SQU of Phl p pre-adsorbed to alum (equivalent to 5 µg/mL Phl p and 56 µg/mL of alum; ALK Abelló) under the same conditions. Cellular proliferation was assessed by the addition of 0.5 µCi of tritiated methylthymidine (Amersham, Aylesbury, UK) to 100 µL of mixed-cell suspension in a round-bottomed 96-well plate, in triplicate, for the final 8 h of culture. For neutralisation experiments we used 2 µg/mL anti-IL-4 (BD Pharmingen, Cowley, U K) or 2 µg/mL anti-IL-12 (R&D systems) as previously described [14].

Cytokine enzyme-linked immunosorbent assay

After 6 days of culture, production of IFN-γ, IL-5, IL-10 and IL-13 was measured by ELISA using BD Pharmingen matched antibody pairs and human recombinant cytokines as standards (PeproTech, London, UK). IL-12 was measured using matched antibody pairs from PeproTech. The limits of the assay detection were 4–10 pg/mL.

Flow cytometry of cell surface marker expression

After 72 h cultured PBMC were washed twice in FACS staining buffer (phosphate-buffered solution (PBS) plus 0.1% bovine serum albumin (BSA) and 0.09% sodium azide) and stained with labelled antibodies to CD80, CD86, HLA-DR/DP/DQ (Dako Cytomation, Ely, UK), CD14 (Cymbus Biotechnology, Chandlers Ford, Hants) and CD19 (Sigma) for 30 min on ice. After a further wash cells were analysed with a FACScan flow cytometer (Beckton Dickinson, Mountainview, CA, USA) and the results analysed using Win MDI (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA).

Statistical analysis

Data were compared using the two-tailed Wilcoxon matched-pairs signed-ranks test using the GraphPad Instat program (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered a significant difference. Cytokine and proliferative values are expressed with the background (media only) levels subtracted from allergen-stimulated values. The average ($\pm$ SD) amount of cytokine detected in unstimulated cultures were: IL-5, 20 ± 52 pg/mL; IL-10, 9 ± 9 pg/mL; IL-12, 101 ± 46 pg/mL; IL-13, 113 ± 296 pg/mL; IFN-γ, 15 ± 9 pg/mL and average ($\pm$ SD) proliferation was 544 ± 287 cpm.

Results

Effects of alum on allergen driven cytokine production by peripheral blood mononuclear cells

Initial experiments investigated the *in vitro* effects of alum on allergen-stimulated PBMC derived from atopic subjects sensitive to grass pollen. PBMC were incubated with grass pollen extract (0.5 and 5 µg/mL) in the presence or absence of alum at either 5.6 or 56 µg/mL for 6 days. In order to test different doses of grass pollen extract and alum, the allergen and adjuvant were not pre-adsorbed in these experiments. Stimulation of PBMC from atopic subjects sensitive to grass pollen with allergen alone resulted in a significant increase in Th2 cytokines which is characteristic of a Th2-biased allergic response. Cells stimulated with 0.5 µg/mL allergen in the presence of either 5.6 or 56 µg/mL of alum produced significantly less IL-5 compared with allergen alone ($P < 0.001$, $P < 0.001$; Table 1). Little allergen-driven IFN-γ or IL-12 production was detectable at this lower allergen concentration. Allergen-stimulated IL-10 production was unaffected by the presence of alum at 0.5 µg/mL of Phl p. A significant reduction in IL-5 production was also observed when cells were stimulated with 5 µg/mL of grass pollen in the presence of alum (5 µg/mL allergen, 56 µg/mL alum; $P < 0.005$; Fig. 1). In addition, IL-13 production was significantly decreased when PBMC were stimulated with 5 µg/mL of allergen and 56 µg/mL of alum ($P < 0.001$). No differences in the production of IL-10, IFN-γ and IL-12 were observed under these conditions. Culture of cells with the higher allergen dose (5 µg/mL) and a lower alum concentration (5.6 µg/mL) resulted in increased IL-10 production ($P < 0.03$). PBMC proliferated in a dose-dependent fashion in response to allergen but proliferative responses were unaffected by the presence of alum (Phl p, 5 µg/mL,

Table 1. Effects of alum on allergen-driven cytokine production and proliferative responses

Phl p	0.5 µg/mL			5 µg/mL		
	None	5.6 µg/mL	56 µg/mL	None	5.6 µg/mL	56 µg/mL
IL-5 (Δpg/mL)	45.5 (22.8, 671.0)	41.4 (2.1, 486.9)	5.2 (0.8, 215.6)	370.6 (194.3, 1295.9)	450.0 (136.0, 750.0)	90.9 (35.9, 390.2)
IL-10 (Δpg/mL)	10.3 (5.9, 17.7)	16.9 (6.7, 34.0)	10.6 (4.6, 26.5)	58.1 (27.2, 88.6)	121.9 (64.0, 137.6)	71.3 (43.0, 131.5)
IL-12 (Δpg/mL)	0 (0, 6.6)	2.4 (0, 9.6)	0 (0, 0)	0 (0, 38.6)	19.9 (5.4, 34.3)	0 (0, 34.6)
IFN-γ (Δpg/mL)	0 (0, 3.9)	0.6 (0, 0.2)	0.6 (0, 1.9)	22.5 (6.0, 34.8)	22.6 (3.7, 69.1)	25.4 (2.3, 155.6)
Proliferation (Δcpm)	3020 (1704, 3862)	2150 (194, 5149)	1654 (698, 3106)	13 466 (4556, 22 809)	24 200 (8035, 28 429)	5932 (2969, 34 677)

$n = 18$ except proliferation where $n = 9$ and IL-12 where $n = 15$. Data are expressed as medians (lower quartile, upper quartile). Numbers shown in bold represent significant differences between parameters measured in the presence or absence of alum.

13 466 cpm (4556, 22 809); Phl p, 5 µg/mL + alum, 56 µg/mL, 5932 (2969, 34 677)).

In order to assess the overall effect of alum on Th responses we calculated the ratio of IFN-γ to IL-5 production. Results shown in Fig. 2 demonstrate that the ratio of IFN-γ to IL-5 was significantly increased upon the addition of alum at both 5.6 and 56 µg/mL ($P < 0.01$, $P < 0.001$). Mean values ($\pm$ SE) were: Phl p, 0.31 ± 0.19 ; Phl p + 5.6 µg/mL alum (0.93 ± 0.41) and Phl p + 56 µg/mL alum (2.15 ± 1.04). This suggests that alum can promote skewing of allergen-driven responses towards a Th1 profile.

Alum (56 µg/mL) had no effect upon cell viability as measured by trypan blue exclusion after 6 days of culture (media, 0.60 ± 0.12 , media + alum, 0.50 ± 0.05 , $n = 6$; $P = 0.43$).

Effects of standard alum-adsorbed vaccine on ratio of interferon-γ to interleukin-5

The controlled adsorption of antigens on preformed alum gels is critical to produce standardized vaccines. Therefore, other experiments investigated the effects on PBMC responses of an antigen–alum preparation used for grass pollen immunotherapy injections compared with the same amount

of allergen, independent of alum. Results show that the standard alum-adsorbed vaccine affects *in vitro* cytokine production in a manner similar to alum and *P. pratense* added independently to cell cultures (Fig. 3). Again, a significant ($P < 0.001$) decrease in IL-5 production is observed in cultures containing the adsorbed allergen/alum preparation compared with allergen alone. Also, alum has no effect on IFN-γ levels and the overall ratio of IFN-γ to IL-5 remains in favour of a Th1 or Th0 response.

Effect of neutralization of interleukin-4 and interleukin-12 on peripheral blood mononuclear cells cultured with alum and Phleum pratense

PBMCs from six subjects were incubated with 56 µg/mL of alum and/or 5 µg/mL of Phl p. Similar to previous results, alum induced a significant decrease in allergen-driven IL-5 ($P = 0.03$) but no effect upon IFN-γ production (Fig. 4). The inclusion of neutralizing antibodies to either IL-12 or IL-4 had no effect on the relative changes in IL-5 or IFN-γ production. Overall IL-5 production was reduced by the inclusion of anti-IL-4 but the difference in this change with respect to the inclusion of alum was not significant.

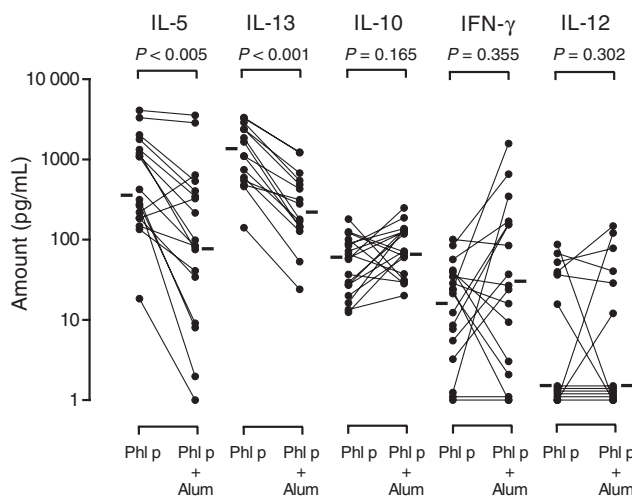


Fig. 1. Effect of alum on allergen-driven cytokine production. Human peripheral blood mononuclear cells ($n = 15$ – 18) were cultured for 6 days in the presence of *Phleum pratense* (Phl p) (5 µg/mL) and/or alum (56 µg/mL). IFN-γ, IL-5, IL-10, IL-12 and IL-13 production was measured by ELISA and values are shown with values of unstimulated cultures subtracted from stimulated cultures.

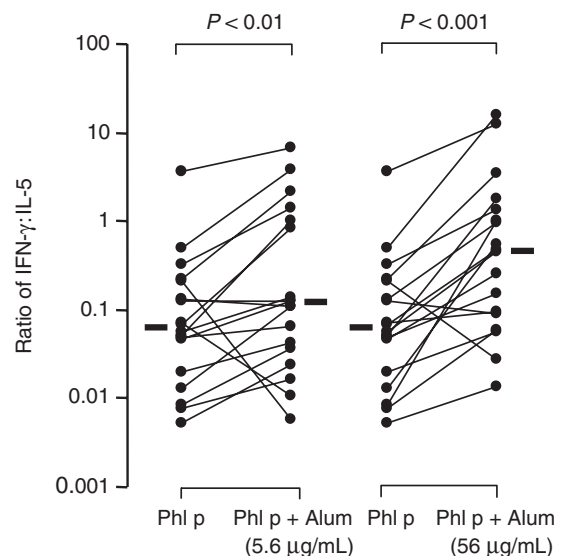


Fig. 2. Alum increases the ratio of IFN-γ to IL-5. Human peripheral blood mononuclear cells ($n = 18$) were cultured for 6 days in the presence of alum (5.6, 56 µg/mL) and *Phleum pratense* (Phl p) (5 µg/mL). IFN-γ and IL-5 production were measured by ELISA.

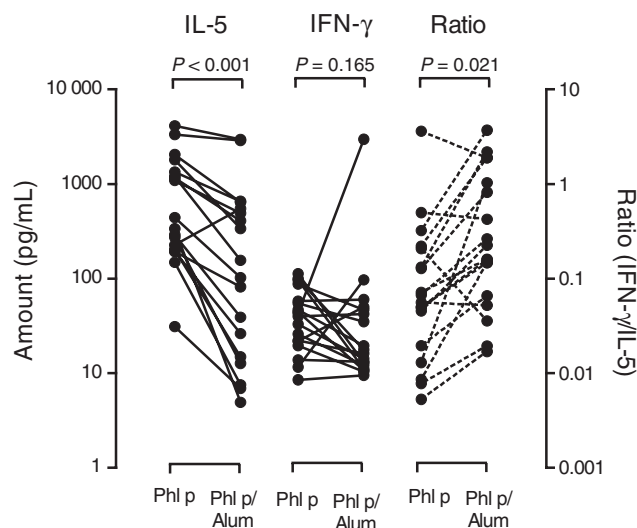


Fig. 3. Effects of standard alum-adsorbed grass pollen vaccine on cytokine production. Human peripheral blood mononuclear cells ($n = 18$) were cultured with vaccine (containing 5000 SQU *Phleum pratense* (Phl p) and 56 $\mu\text{g/mL}$ alum) or 5000 SQU Phl p (equivalent to 5 $\mu\text{g/mL}$ Phl p) for 6 days. Cytokine production was measured by ELISA.

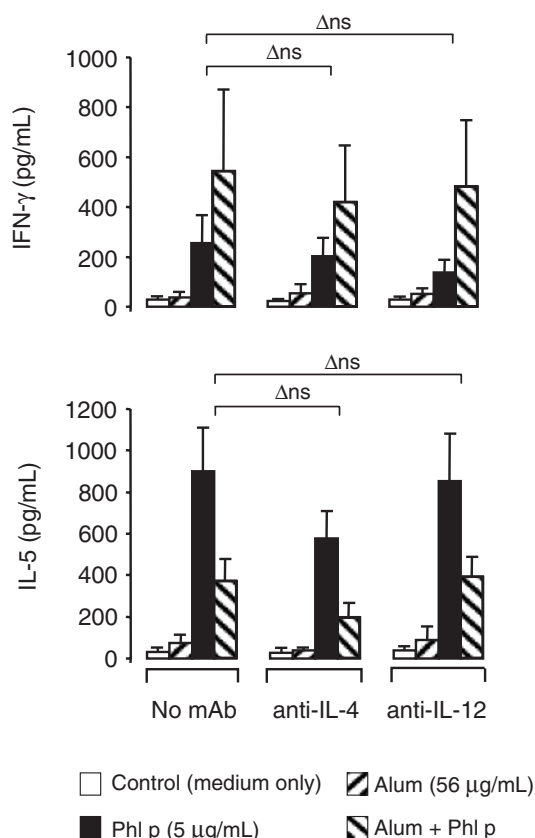


Fig. 4. Changes in IFN- γ and IL-5 production are not dependent on either IL-4 or IL-12. Human peripheral blood mononuclear cells ($n = 6$) were cultured for 6 days in the presence of *Phleum pratense* (Phl p) (5 $\mu\text{g/mL}$) and/or alum (56 $\mu\text{g/mL}$) with the addition of neutralizing antibodies to either IL-4 or IL-12. The figure displays the mean ($\pm$ SE) of the relative change in cytokine production induced by alum calculated by dividing the value induced by Phl p by the value of Phl p with alum.

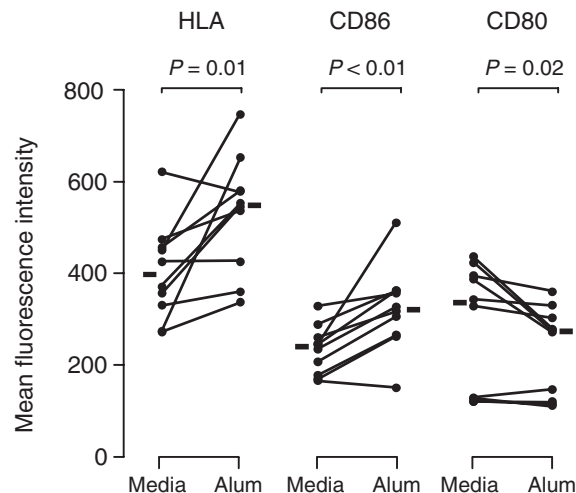


Fig. 5. Effect of alum on monocyte expression of cell surface markers. Human peripheral blood mononuclear cells ($n = 10$) were cultured for 72 h in the presence of alum (5.6 $\mu\text{g/mL}$) or medium alone. Cell surface marker expression was analysed by flow cytometry. Results show the mean fluorescence intensity of cells gated on the CD14 $^{+}$ monocyte population.

Effects of alum on human leucocyte antigen and CD80/CD86 expression by antigen presenting cells

PBMC were cultured in the presence of 5.6 $\mu\text{g/mL}$ alum and after 72 h the cells were analysed by flow cytometry for expression of CD80, CD86 and HLA. CD14 was used as a marker for monocytes and CD19 as a marker of B cells. The results shown in Fig. 5 give values of mean fluorescence intensity which represents the levels of cell surface expression of these molecules. Alum induced a significant decrease in CD80 expression ($P < 0.05$) and a significant increase in CD86 expression ($P < 0.005$) on monocytes. Alum significantly increased the expression of HLA ($P = 0.01$). Alum had no effect on the expression of CD80 or CD86 on CD19 $^{+}$ B-cells and inhibited monocyte cell marker detection at ten-fold higher concentrations (data not shown). Cultures stimulated with allergen in the presence of alum showed a significant decrease in the monocyte-expression of CD80 (Phl p, 320 ± 62 , Phl p + alum, 223 ± 33 ; $P = 0.026$) but no difference in the expression of CD86 (226 ± 25 , 267 ± 15) or HLA (394 ± 30 , 342 ± 32).

Discussion

The aim of this study was to determine the effects of alum on allergen-stimulated PBMC from atopic patients. The results show that alum is able to significantly down-regulate Th2 cytokine production when cultured in the presence of PBMC and grass pollen allergen. No changes in Th1-type cytokines were observed when cells were stimulated in the presence of alum. Furthermore, we have shown that changes in cytokine production induced by alum are not dependent on IL-12 or IL-4. Alum enhanced cell surface expression of HLA and CD86 and reduced CD80 on monocytes but had no effect upon B cell surface marker expression.

Alum is frequently used as an adjuvant in murine models of allergic inflammation. Typical protocols involve the

sensitization of mice by repeated subcutaneous injections of an antigen adsorbed to alum [15–17]. The subsequent Th2-associated responses, including IL-4 production and generation of IgG1 antibodies upon antigen re-challenge, first led to the conclusion that alum is a Th2 adjuvant [17]. Numerous other studies have confirmed that alum selectively promotes Th2 responses in mice [18–20]. In human allergic disease, the normal process of sensitization is very different from that used in animal models. For aeroallergens, sensitization occurs by natural exposure of mucosal surfaces in the upper airways to low doses of allergens over many years. Recipients of allergen immunotherapy receive subcutaneous injections of allergen extracts adsorbed to aluminium hydroxide in increasing doses, up to a maintenance phase, overall receiving very high doses of allergen. Thus, any immunomodulatory properties of aluminium adjuvants will have an effect on an established immune response. This represents an entirely different system to the use of alum to promote primary immune responses in naïve mice. Results presented here clearly demonstrate that the Th2-inducing effects of alum in naïve mice are not relevant to its effects in human allergic subjects.

The mechanisms by which alum acts as an adjuvant in humans are not fully understood. However, the immunogenicity of alum is largely attributed to its depot effect [2, 21]. The retention of antigen followed by its slow release over time results in the continued capture and presentation of antigen by APCs, which in combination with the particulate nature of alum allows enhanced uptake by phagocytic cells [22]. Alum also promotes inflammatory responses by inducing the production of the pyrogenic cytokine IL-1 [21, 23].

Results presented here demonstrate the effect of alum on allergen-stimulated PBMC *in vitro* on Th2 cell-associated cytokines IL-5 and IL-13. *In vivo* alum may influence many different cell types such as B cells and dendritic cells. For example, alum may influence B cell antibody production and class switching. In addition, the depot effect of alum may dramatically effect antigen presentation of the adsorbed allergen. *In vivo* studies investigating the effects of alum are of considerable interest, however, as alum has dramatically divergent effects on antigen-stimulated cytokine production by human cells compared with mice. *In vivo* investigation in a murine model may not be representative of the human system. Potential *in vivo* experiments in humans would be complex as alum is likely to exert effects both at the site of injection and at local lymph nodes.

Our data suggest that alum does not act via an IL-12 or IL-4 dependent mechanism as addition of neutralizing antibodies to either IL-12 or IL-4 had no effect on the immunomodulatory properties of alum in our system. Although the effects of alum in mice seems to differ significantly from that in humans, this finding is in agreement with studies in mice that demonstrate that deletion of either the IL-4 or IL-4R α gene has no effect on the differentiation of Th2 cells induced by alum [16, 24].

One of the few reports on the effects of alum in humans reported that alum up-regulated monocyte accessory molecules in an IL-4-dependent manner [23], in turn concluding that alum activates Th2 cells to produce IL-4 which then stimulates the changes observed on the monocytes. Our observations show up-regulation of CD86 expression and down-regulation of CD80 expression by monocytes exposed

to alum. CD86 co-stimulation by engagement with CD28 has been linked to adjuvant-mediated activation of APCs [25] whereas CD80 has been reported to preferentially bind to CD152 (CTLA-4) [26] which is involved in the down-regulation of immune responses. This profile was not observed on allergen-stimulated cells, which may reflect receptor internalization after T cell receptor cross linking [27]. Thus, our results support the view that alum promotes the activation of APCs. It would also be of interest to assess the effects of alum on antigen presentation by dendritic cells and antibody production by B cells.

Alum continues to be used successfully as an adjuvant in the specific immunotherapy of allergic disease [9, 28, 29]. Mechanisms underlying successful immunotherapy involve the modulation of allergen-specific T cell responses, either from Th2 to Th1 [10, 30, 31], or via the induction of the regulatory cytokines, IL-10 or TGF- β [11, 12]. Results presented here demonstrate that alum skews allergen-stimulated PBMC responses *in vitro* away from a Th2 profile. Moreover, under certain conditions, alum can also increase IL-10 production by PBMC although the significance of this finding should be addressed with caution. Thus, in the context of allergen immunotherapy, use of alum as an adjuvant provides excellent support for the deviation of allergen-driven cytokines away from a disease-associated Th2 phenotype and, potentially, in the production of regulatory cytokines.

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