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Aluminium Hydroxide Adjuvant Initiates Strong Antigen-Specific Th2 Responses in the Absence of IL-4- or IL-13-Mediated Signaling

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Previous studies demonstrate that aluminium hydroxide adjuvant (alum) produces increased Th1 responses in IL-4-deficient mice compared with wild-type animals, although the continued production of IL-5 by spleen cells from these mice also indicates that Th2 responses are induced. In the present study, we demonstrate that alum can induce Th2-associated IL-4 and IL-5 production in the absence of IL-4 signaling in mice deficient in either IL-4Rα or Stat6. The Th2 responses observed could not be due to IL-13 as IL-13 responses are also impaired in IL-4Rα- and Stat6-deficient mice. We also detected higher levels of IL-4 in IL-4Rα gene-deficient, though not Stat6-deficient, mice compared with their wild-type counterparts. The increased levels of IL-4 could be explained by the IL-4R being unavailable to neutralize this cytokine in IL-4Rα-deficient mice. While levels of IL-5 production in IL-4Rα- or Stat6-deficient mice were similar to IL-4-deficient and wild-type mice, other type 2-associated responses, which are largely or wholly IL-4 dependent, such as the production of IgG1 or IgE Abs, were either reduced or absent. We conclude that alum adjuvants can induce IL-4 and Th2 responses independently of IL-4 or IL-13, negating the requirement for an early source of IL-4 in the Th2 response induced by this adjuvant.


Materials and Methods

Adjuvant preparation

Alhydrogel (alum), purchased from Superfos Biosector (Vedbaek, Denmark), was mixed with a predetermined quantity of OVA (grade V; Sigma, Poole, U.K.) and incubated at room temperature for 20 min. A sample of the mixture was centrifuged at 14,000 × g for 10 min, and the supernatant was checked for unbound protein by bicinchoninic acid assay (Pierce, Rockford, IL).

Animals and inoculations

IL-4−/− and IL-4Rα−/− mice were generated as described previously (12, 15). These, and wild-type control animals (BALB/c), were bred at the Max Planck Institute for Immunobiology and maintained at the University of Strathclyde. Stat6−/− mice (14) were a gift from Professor J. Ihle; these and B6/129 control mice were bred and maintained at the University of Pennsylvania. Eight- to 10-wk-old female mice were inoculated s.c. with 0.1 ml of OVA (100 μg) adsorbed to alum and boosting inoculations administered in the same fashion 2 wk later.

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Determination of plasma Ab titers

Blood was sampled from mice for Ab determination 2 wk (IgG1 and IgG2a) and 4 wk (IgE) following boosting inoculations. ELISA were performed as described previously to detect Ag-specific IgG1 and IgG2a and total or specific IgE in plasma. Results are expressed as endpoint dilutions where the endpoint is determined as the final plasma dilution that yields a higher absorbance than a negative control plasma sample included in the assay. Comparisons between groups were performed using a Mann-Whitney *U* test.

Spleen cell responses

Spleens were aseptically removed 5 wk following the second inoculations with alum-adsorbed OVA, and cell suspensions were prepared by gently
teasing apart in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-ME, and 10% FCS (Life Technologies, Paisley, U.K.). RBCs were depleted by incubation in 0.17 M Tris and 0.16 M ammonium chloride (16) at 37°C for 5 min followed by washing. Viable cells were enumerated by trypan blue exclusion, and 100-μl aliquots of cell suspension containing 5 × 10⁵ cells were added to 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA). Triplicate aliquots (100 μl per well) of Con A (5 μg/ml) or OVA (from 0 to

FIGURE 2. IL-4 (a and b), IL-5 (c and d), and IFN-γ (e and f) production by cultured splenocytes removed from IL-4−/− (n = 5), IL-4Rα−/− (n = 5), Stat6−/− (n = 3), and wild-type mice (BALB/c, n = 5 or B6/129, n = 3) 5 wk after a second s.c. inoculation of OVA adsorbed to alum. Cytokine analysis was performed on cell cultures stimulated with either 1000 μg/ml OVA (a–d), 100 μg/ml OVA (e and f), or unstimulated cultures (Control) as described in Materials and Methods. Results are expressed as mean cytokine concentration as determined by ELISA ± SEM. Comparisons between groups were performed by Student’s t test.
1000 μg/ml) were added as appropriate. IL-4R neutralizing Ab (M1; kindly provided by Dr. M. Widmer, Immunex, Seattle, WA) or isotype-control IgG (PharMingen, San Diego, CA) were also added at this stage where required (17, 18). Following incubation for 60 h at 37°C, 5% CO₂, 150-μl aliquots of cell-culture supernatants were removed and stored at −70°C for cytokine assay.

**Cytokine assays**

IFN-γ, IL-4 (Genzyme, Cambridge, MA), and IL-5 (PharMingen) were detected by capture ELISA according to the manufacturer’s instructions. Cytokine concentrations in the cell cultures were determined from the standard curve (regression coefficient, r = 0.990 or better). Comparisons between groups were made using Student’s t test.

**Cell depletions**

Spleen cell suspensions were prepared from three IL-4Rα−/− mice inoculated with alum/OVA, pooled, and passed through sterile 100-μm monofilament filter cloth (Nitec; Cadisch Precision Meshes, London, U.K.). Cells were washed, resuspended in 1 ml of PBS, pH 7.2 (Life Technologies), with 0.5% BSA (Sigma), and T cell subset depletion was performed on 5 × 10⁷ spleen cells using magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Briefly, CD8⁺ cells were labeled with primary rat anti-CD8 Ab (YTS105.18), and magnetic depletion was conducted with rat IgG microbeads (Miltenyi Biotec). CD4⁺ cells were directly depleted with mouse CD4 (L3T4) microbeads. CD4⁺CD8⁺ depletions were conducted simultaneously on cells previously labeled with anti-CD8 (YTS105.18) as described above. Cells were

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**FIGURE 3.** Effect of anti-IL-4R Ab on IL-4 levels detected in splenocyte cultures prepared from Stat6−/− (n = 3) mice, IL-4Rα−/− mice (n = 5), and their respective control mice 5 wk after a second s.c. inoculation of OVA adsorbed to alum. a–d, IL-4 analysis was performed on cell cultures stimulated with 1000 μg/ml OVA or unstimulated cultures (Control) in the presence of either anti-IL-4R or an isotype control Ab as described in Materials and Methods. Results are expressed as mean IL-4 concentration as determined by ELISA ± SEM. Comparisons between groups were performed by Student’s t test.
Results

OVA-specific Ab production

As demonstrated previously, inoculation of BALB/c or B6/129 mice with Ag prepared in alum produced high titers of Ag-specific IgG1 (Fig. 1, a and b), but negligible amounts of IgG2a (Fig. 1, c and d), a pattern indicative of a polarized Th2 response. Also as previously demonstrated (Fig. 1, a and c), IL-4-deficient mice (IL-4−/−) produced levels of IgG1 that were slightly, though significantly, reduced (p ≤ 0.025) and IgG2a titers that were greatly increased compared with wild-type mice (p ≤ 0.01). In the present study, IL-4Ra-deficient mice (IL-4Ra−/−) also produced a shift in Ab subclass responses, and IgG1 and IgG2a titers were similar to those observed in IL-4−/− mice (Fig. 1, a and c), again demonstrating increased Th1 responses compared with wild-type mice. Likewise, Stat6-deficient mice (Stat6−/−) had reduced OVA-specific IgG1 titers (p ≤ 0.01; Fig. 1b) and increased IgG2a levels (p ≤ 0.01; Fig. 1d) compared with control B6/129 mice. Four weeks following secondary inoculations with alum/OVA, significant levels of total or OVA-specific IgE could be detected in BALB/c or B6/129 mice, respectively (Fig. 1e, p ≤ 0.01; Fig. 1f, p ≤ 0.01). However, this Ab isotype was undetectable in plasma prepared from similarly treated Stat6−/−, IL-4Ra−/−, or IL-4−/− mice.

Cytokine responses

When splenocytes from wild-type, IL-4−/−, and IL-4Ra−/− mice inoculated with alum/OVA were restimulated with Ag in vitro, no significant differences in the proliferative responses were detected between the groups (data not shown). While levels of IL-4 were undetectable in Ag-stimulated spleen cells from IL-4−/− mice (Fig. 2a), spleen cells from IL-4Ra−/− mice produced significant levels of this cytokine compared with unstimulated control cells (p ≤ 0.01). Spleen cells from alum-inoculated BALB/c control mice also produced significant amounts of IL-4, although these levels were significantly less than those detected in IL-4Ra−/− mice (p ≤ 0.01). Although spleen cells from alum/OVA-inoculated Stat6−/− mice also produced significant levels of IL-4 (Fig. 2b, p ≤ 0.01), unlike IL-4Ra−/− mice, the levels of IL-4 detected in Ag-stimulated spleen cell cultures were not significantly greater than those observed in spleen cells from wild-type mice.

Spleen cells from alum/OVA-inoculated IL-4Ra−/− as well as IL-4−/− mice could also be stimulated in vitro with OVA to produce significant levels of IL-5 (p ≤ 0.01), which were equivalent to those found using splenocytes from wild-type mice (Fig. 2c). Similar levels of IL-5 were also found in Ag-stimulated spleen cell cultures from Stat6−/− and wild-type B6/129 mice (Fig. 2d).

As demonstrated previously, spleen cells from alum/OVA-inoculated BALB/c mice did not produce significant levels of IFN-γ; however, cells from IL-4−/− and IL-4Ra−/− mice did produce significant levels of this cytokine on Ag restimulation in vitro (p ≤ 0.01 in both cases). Similarly, Stat6−/− mice produced significantly higher levels of IFN-γ compared with wild-type control mice (p ≤ 0.05).
Neutralization of soluble IL-4R in vitro

Spleen cells from alum/OVA-inoculated IL-4Rα-deficient mice were restimulated with Ag in the presence of an anti-IL-4R-neutralizing Ab or isotype-matched control Ab (Fig. 3). While the addition of the anti-IL-4R Ab produced significant increases in detectable levels of IL-4 in spleen cells from both B6/129 mice (Fig. 3a, p ≤ 0.05) and Stat6-/- mice (Fig. 3b, p ≤ 0.05) this was not the case with IL-4Rα-/- cells. The addition of IL-4R-neutralizing Abs to spleen cells from IL-4Rα-/- mice did not affect Ag-induced IL-4 production, whereas similar treatment of spleen cells from control BALB/c mice significantly increased detectable levels of IL-4 (p ≤ 0.01; Fig. 3, c and d). In fact, in the presence of anti-IL-4R Ab, levels of detectable IL-4 were similar in Ag-stimulated spleen cells from both wild-type and IL-4Rα-/- mice.

Identification of IL-4-producing cells

To determine the source of IL-4 production in alum/OVA-inoculated IL-4Rα-deficient mice, mixed spleen cell preparations were depleted of either CD4+ or CD4+ and CD8+ T cells (Fig. 4). As described above, Ag restimulation of IL-4Rα-/- spleen cells resulted in IL-4 production. However, depletion of CD4+ T cells resulted in complete ablation of IL-4 production, clearly demonstrating that CD4+ T cells were the source of IL-4 production in mixed cultures (Fig. 4a). Analysis of the cell suspensions by flow cytometry demonstrated that depletion using magnetic beads was ~99% effective (Fig. 4, b-d).

Discussion

In our previous studies, we have demonstrated that IL-4-deficient mice inoculated with Ag prepared in alum could not only produce Th1 responses as characterized by in vivo IgG2a production and in vitro IFN-γ production, but could also continue to induce a Th2 response characterized by the production of IL-5 by Ag-restimulated splenocytes (1). In other models of Th2 induction, such as the inductive phase of schistosome egg-induced granuloma, IL-5 has been thought to play an important, though indirect, role in Th2 induction via the recruitment of eosinophils and the subsequent production of IL-4 (18). However, we did not observe any reduction in the Th2 response following administration of alum/OVA to IL-5-deficient mice compared with wild-type control mice (data not shown). Similarly, more recent data has shown that there is no alteration in Th2 induction in the schistosome egg model in IL-5-/- mice (19). Thus, we hypothesized that this residual Th2 response, produced in the absence of IL-4, may be mediated by the compensatory action of IL-13, a cytokine that has been associated with mediating some of the effects of Th2 cells (20, 21). Nonetheless, analyses of alum-induced Th2 responses in IL-4-deficient mice were clearly limited due to the inability to analyze the production of IL-4, the key Th2 cytokine in these mice. Our current approach using IL-4Ra-deficient (12) and Stat6-deficient (14) mice has allowed analysis of IL-4 production by alum, not only in the absence of functional IL-4, but also in the absence of functional IL-13 signaling. This is due to the critical role that Stat6 plays in mediating signaling via IL-4Ra, a common component of both the IL-4 and IL-13 receptor complexes (12).

Most strikingly, these results demonstrate that in the absence of IL-4- or IL-13-mediated signaling, alum continues to prime splenocytes to produce IL-4 on Ag restimulation in vitro. This is intriguing as early studies of the growth of Th clones in vitro clearly describe IL-4 as a prerequisite autocrine growth factor for Th2 cells (22, 23). On this basis, a number of studies have established sources of this cytokine from cells of the innate and adaptive immune responses, which could act as prime movers in providing the initial source of IL-4 required to initiate Th2 responses in vivo. These cell types include NK1.1+ CD4+ T cells (24, 25), NK1.1+ CD4+ T cells (26, 27), eosinophils (18, 28), and cells of the mast cell/basophil lineage (29). However, in the present study we have demonstrated that using alum as an adjuvant can induce Th2 responses independently of IL-4, dispensing with the requirement for an initial source for IL-4 in the alum-induced Th2 response. The source of IL-4 in these present studies was clearly CD4+ T cells as depletion of this subset of lymphocytes was sufficient to completely ablate Ag-dependent IL-4 production in spleen cells from IL-4Ra-deficient mice. Furthermore, as IL-4Ra-deficient mice also lack IL-13-mediated signaling, the present study therefore produces a dilemma as to how Th2 responses are initiated in vivo and what effects of alum, independently of IL-4 or IL-13, mediate the development of Th2 responses. Previous studies in IL-4Ra- and Stat6-deficient mice have also described IL-4-independent pathways for IL-4 production. In studies of infection with the nematode parasite N. brasiliensis, IL-4Ra-deficient mice could produce reduced, though significant, levels of IL-4 compared with wild-type mice (30). CD4+ spleen cells from IL-4Ra-deficient mice could also be induced to produce IL-4 upon stimulation with anti-CD3 Ab, and this activity could subsequently be isolated to a population of CD62L+ , CD4+ T cells. Further analysis demonstrated that β2-microglobulin (β2m)-deficient mice, which lack NK1.1 cells, failed to produce IL-4 on stimulation with anti-CD3 in vivo, suggesting that the source of IL-4 in IL-4Ra-deficient mice was NK1.1+ CD4+ T cells. However, previous work has demonstrated that alum/OVA can induce IL-4 production and Th2 responses in β2m-deficient mice (31), indicating that NK T cell populations do not appear to be the source of the IL-4 response induced following in vivo inoculation with alum. A similar conclusion was made in studies where anti-IgD, another potent Th2-inducing stimulus, was administered to β2m-deficient mice and Stat6-deficient mice (32). As significant levels of IL-4 could be induced in both of these strains of mice, these studies indicate that an IL-4-independent mechanism of priming CD4+ T cells for IL-4 production must exist in vivo.

Despite the significant levels of IL-4 detected in vitro, restimulated spleen cells from OVA/alum-inoculated IL-4Ra-deficient mice, plasma IgG1 and IgE and spleen cell IL-5 production in these mice were similar to those found in IL-4-deficient mice. This would be consistent with the concept that in the absence of IL-4, IL-13 has no direct effect on these Th2 responses (12). Furthermore, as levels of IFN-γ in IL-4-deficient and IL-4Ra-deficient mice were similar, IL-13 does not appear to be able to mediate inhibition of IFN-γ production in the absence of IL-4. Thus, although it is clear that Th2 induction can continue independently of IL-4 production, there still remains the question of how alum inhibits Th1 responses in intact animals. Our previous studies (1), as well as those shown here, clearly demonstrate that this is dependent on the production of IL-4; however, the source of this cytokine remains to be defined. Therefore, further studies to address this question are being planned.

We were also intrigued why IL-4Ra-deficient mice should have greatly increased production of IL-4 compared with wild-type BALB/c mice. This was not the case in Stat6-deficient mice, where levels of IL-4 produced by in vitro-restimulated spleen cells were similar to levels found in spleen cells from OVA/alum-inoculated wild-type mice. One hypothesis that we tested was that IL-4Ra-deficient mice had increased levels of IL-4 due to the lack of neutralization of IL-4 by the IL-4Ra. An alternatively spliced variant of the IL-4Ra can also be transcribed and shed from the surface of lymphocytes as a soluble receptor. This soluble receptor also sequesters and neutralizes IL-4 (33), consequently preventing IL-4...
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