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Neither interleukin-6 nor signalling via tumour necrosis factor receptor-1 contribute to the adjuvant activity of Alum and Freund's adjuvant

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SUMMARY

The potential contribution made by the inflammatory cytokines, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) to the adjuvant activity of aluminium hydroxide gels (Alum) or Freund's complete adjuvant (FCA) was studied by comparing the immunological responses of IL-6- or TNF receptor 1- (p55; TNFR-1) deficient mice with immunocompetent control mice. While both TNFR-1- and IL-6-deficient mice primed with ovalbumin (OVA) prepared in either Alum or FCA produced similar IgG1 responses in comparison to control mice, the pattern of T-helper type 1- (Th1) dependent IgG2a production was significantly altered. In TNFR-1-deficient mice, IgG2a responses were greater than in control mice when FCA, but not when Alum, was used as an adjuvant. Correspondingly, spleen cells from FCA-inoculated TNFR-1-deficient mice restimulated with antigen *in vitro* produced higher Th1 cytokine (interferon-γ; IFN-γ) levels with no alteration in Th2 cytokine (IL-4, IL-5, IL-6 and IL-10) production in comparison with wild-type mice. Higher levels of IgG2a were also detected in IL-6-deficient mice compared with wild-type mice following inoculation with OVA prepared in either FCA or in Alum. Furthermore, analysis of cytokine production by spleen cells revealed that both Th1 and Th2 cytokine production was higher in IL-6-deficient mice compared with control mice. As the majority of the effects of TNF-α are mediated via TNFR-1, we conclude that this cytokine inhibits the adjuvant activities of FCA. Furthermore, the results also imply that immunopotentiating effects of FCA or Alum adjuvant are both inhibited by IL-6.

INTRODUCTION

Freund's adjuvant and the aluminium-based compounds have been employed as immunological adjuvants for some 50 and 70 years, respectively. Although a vast number of agents with similar activity has been subsequently described,[1] aluminium hydroxide (Alum) is unique in its continuous use in human vaccines. Despite this, Alum is not an ideal adjuvant for all applications due to its inability to induce the T helper type 1 (Th1) immune responses required for the induction of protective immunity to many intracellular pathogens.[2] In contrast, while Freund's complete adjuvant (FCA) has been demonstrated to stimulate Th1 responses,[3–6] its use is entirely restricted to experimental situations as it produces unacceptable toxicity reactions. However, given our present vastly increased understanding of immunological pathways, it is surprising that little is yet understood about how traditional adjuvants work, knowledge of which may allow rational design of novel adjuvants. Current theories have extensively implicated the macrophage as playing a central role in the mechanisms of actions of adjuvants. First, it has been suggested that some adjuvants may function by targeting antigen to macrophages, thus increasing antigen presentation. Second, it is thought that local inflammatory reactions induced by many adjuvants serve to increase macrophage infiltration to the site of injection[7] and in fact, it is considered that local inflammation and formation of a small granuloma are essential for adjuvant activity.[7] Amongst the proinflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in particular have been closely linked with the induction of local and systemic inflammation to a number of agents.[8,9] As most of the proinflammatory effects of TNF-α are mediated through TNF receptor-1 (TNFR-1; p55;[10,11]) the recent availability of mice deficient for IL-6 and TNFR-1, has allowed further characterization of the role of these cytokines in inflammation. While both IL-6- and TNFR-1-deficient mice have previously been described as having normal lymphocyte development, they both have defective macrophage inflammatory responses and consequently display increased susceptibility to *Listeria monocytogenes* infection.[12–14] This would indicate that these cytokines should have an important role in the immunopotentiating activity of adjuvants such as Alum and FCA. To

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Abbreviations: Alum, aluminium hydroxide; FCA, Freund's complete adjuvant; FDC, follicular dendritic cell; FIA, Freund's incomplete adjuvant; TNFR-1, tumour necrosis factor receptor-1.

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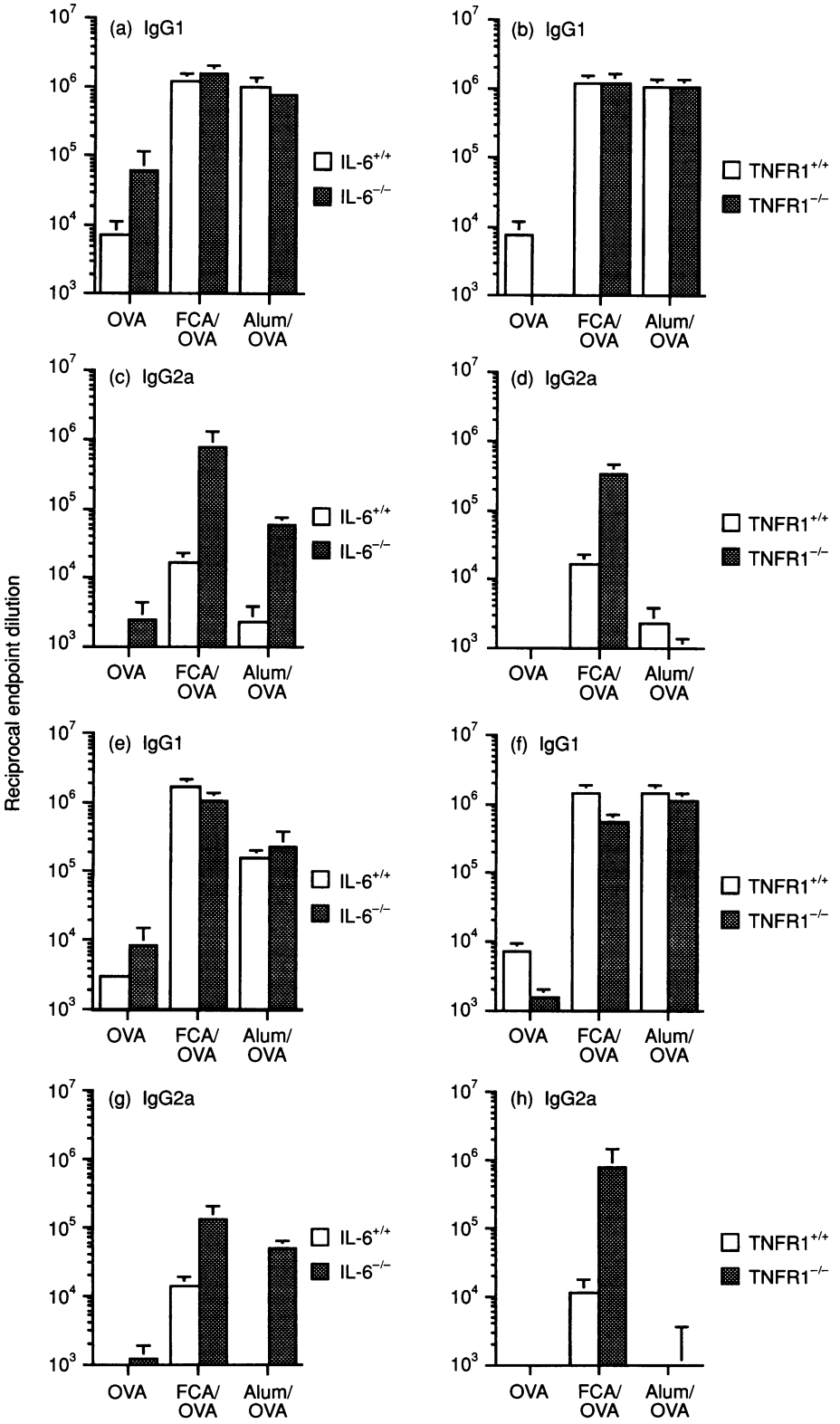

Figure 1. Analysis of plasma antibody titres at 2 weeks (a–d) and 4 weeks (e–h) after the second s.c. inoculation of wild-type (+/+) or gene-disrupted (−/−) mice with OVA in PBS, adsorbed to Alum or emulsified in FCA. IgG1 (a, b, e and f) and IgG2a (c, d, g and h) antibody titres were estimated by end-point analysis and values represent mean end-point dilutions ± SEM. Comparisons between groups were made using a Mann–Whitney *U* test.

address this possibility, we have therefore analysed the immunological responses of IL-6- and TNFR-1-deficient mice inoculated with antigen prepared in Alum or FCA and compared these with similarly treated wild-type control mice.

MATERIALS AND METHODS

Adjuvant preparation

Alhydrogel (Alum; purchased from Superfos Biosector a/s, Vedbaek, Denmark) was mixed with a predetermined quantity of ovalbumin (OVA; Grade V, Sigma, Poole, Dorset, UK) 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 (BCA) assay (Pierce, Rockford, IL). OVA was emulsified in FCA/Freund's incomplete adjuvant (FIA) by repeated passage through a double-hubbed emulsifying needle until a stable emulsion was formed.

Animals and inoculations

TNFR-1$^{-/-}$ and IL-6$^{-/-}$ mice were generated as described previously.[12,13] These, and wild-type control animals of the same strain (129/SvJ) were bred and maintained at the University of Strathclyde. Groups of five, 8–10-week-old female mice were immunized subcutaneously (s.c.) with 0·1 ml of OVA (100 μg) in phosphate-buffered saline (PBS), adsorbed to Alum or emulsified in FCA. Boosting inoculations were performed in the same fashion 2 weeks later, but FIA was used as adjuvant in mice previously treated with OVA in FCA.

Determination of plasma antibody titres

Blood was sampled for antibody determination 2 and 4 weeks following boosting inoculations with OVA alone or prepared in Alum or Freund's adjuvants. Enzyme-linked immunosorbent assays (ELISA) were performed as described previously[15] to detect antigen-specific IgG, IgG1 and IgG2a in plasma. Results are expressed as end-point dilutions where the end-point is determined as the final plasma dilution which 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.

Splenocyte responses

Groups of five mice were inoculated twice s.c. with 100 μg of OVA (100 μl) in PBS, Alum, or emulsified in FCA/FIA as described above. Spleens were aseptically removed 7 weeks following the second inoculations and proliferation and cytokine production were assessed as described previously.[5]

Cytokine assays

Cytokines [IL-4, IL-5, IL-6, IL-10 and interferon-γ (IFN-γ)] were detected by capture ELISA as previously described.[5] Optimum concentrations of cytokine reagents were determined prior to use and are shown in Table 1. Cytokine concentrations in the cell cultures were determined from the standard curve

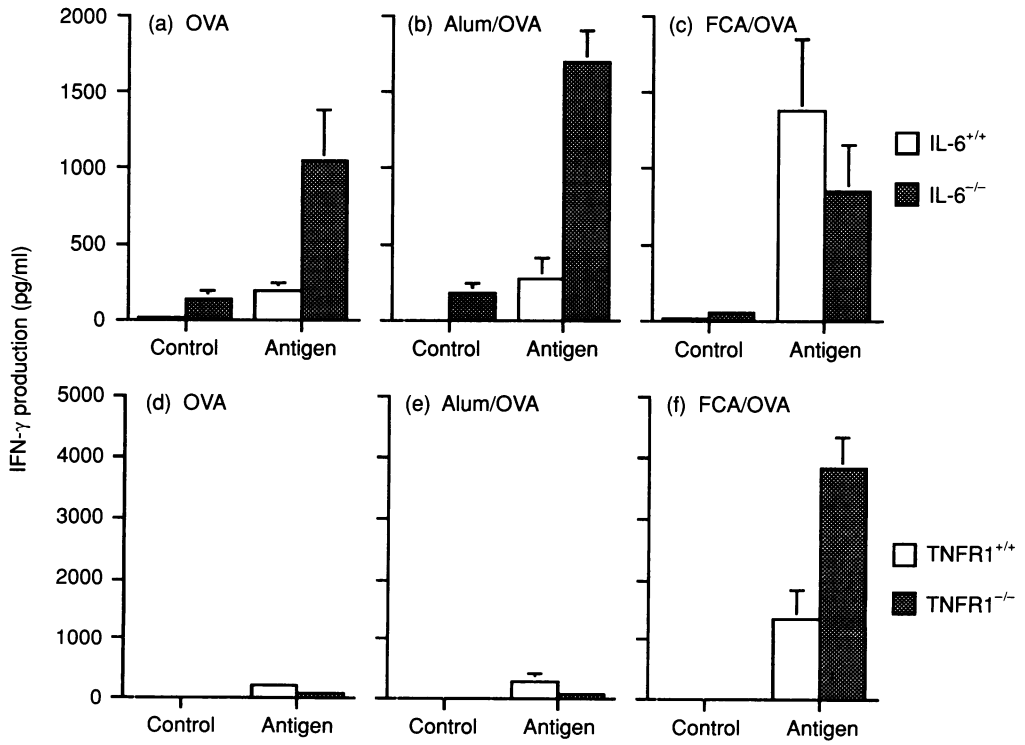

Figure 2. IFN-γ production by cultured splenocytes removed from wild-type (+/+) and gene-disrupted (−/−) mice 7 weeks after a second s.c. inoculation of OVA in PBS (a and d), adsorbed to Alum (b and e), or emulsified in FCA (c and f). Cytokine analysis was performed on cell cultures stimulated with 1000 μg/ml OVA (Antigen) or unstimulated cultures (Control). Results are expressed as mean IFN-γ concentration as determined by ELISA ± SEM. Comparisons between groups were performed using Student's *t*-test.

Table 1. Description of cytokine reagents and concentrations used in capture ELISAs

Cytokine	Capture antibody (concentration)	Standards (ng/ml)	Detecting antibody (concentration)
IL-4	11B11 (2 μg/ml)	0–1	Goat polyclonal (1 μg/ml)
IL-5	TRFK5 (2 μg/ml)	0–1·6	TRFK4 (1 μg/ml)
IL-6	MP5-20F3 (2 μg/ml)	0–10	MP5-32C11 (0·5 μg/ml)
IL-10	JES5-2A5 (2 μg/ml)	0–25	SXC-1 (1 μg/ml)
IFN-γ	R4-6A2 (2 μg/ml)	0–7	XMG1.2 (1 μg/ml)

Antibodies and standards were purchased from Pharmingen, San Diego, CA except for the reagents for the IL-4 ELISA which were purchased from Genzyme. Optimum concentrations for each reagent are given below as determined prior to use.

(regression coefficient, $r=0{\cdot}990$ or better). Comparisons between groups were made using Student's t-test.

RESULTS

OVA-specific antibody production

Analysis of IgG subclasses 2 weeks after the second inoculation demonstrated that while both Alum and FCA significantly enhanced OVA-specific IgG1 antibody production by wild-type mice (Fig. 1a, b; $P=0{\cdot}014$ and $P=0{\cdot}019$, respectively), increased titres of IgG2a antibodies were only observed in wild-type mice when FCA was used as adjuvant (Fig. 1c, d; $P<0{\cdot}01$). Similar results were observed 4 weeks after boosting inoculations, with both Alum and FCA significantly enhancing OVA-specific IgG1 responses (Fig. 1e, f; $P<0{\cdot}025$) while only FCA boosted OVA-specific IgG2a responses (Fig. 1g, h; $P=0{\cdot}026$). Although deletion of either the TNFR-1 gene or the IL-6 gene had no significant effect on IgG1 production at any time-point in any of the groups inoculated with antigen prepared in adjuvant (Fig. 1a, b, e, f), this was not the case when IgG2a production was analysed. Thus, while IL-$6^{-/-}$ mice inoculated with either Alum/OVA or FCA/OVA produced significantly increased levels of IgG2a compared with wild-type mice at week 2 (Fig. 1c; $P=0{\cdot}025$ and $P=0{\cdot}043$, respectively), TNFR-$1^{-/-}$ mice only had significantly increased titres of IgG2a following inoculation with FCA/OVA but not Alum/OVA (Fig. 1d; $P=0{\cdot}017$). Although at week 4 levels of IgG2a were again higher in TNFR-$1^{-/-}$ compared with wild-type mice following inoculation with FCA/OVA (Fig. 1hr; $P=0{\cdot}032$) at this time-point, IL-$6^{-/-}$ mice only produced greater IgG2a titres than wild-type mice if Alum and not FCA was used as adjuvant (Fig. 1g; $P=0{\cdot}013$). Analysis of antibody responses to OVA prepared in the absence of adjuvants demonstrated that OVA-specific IgG1 titres were only significantly lower in TNFR-$1^{-/-}$ compared with wild-type mice and only at the later time-point following inoculation (Fig. 1f; $P=0{\cdot}04$).

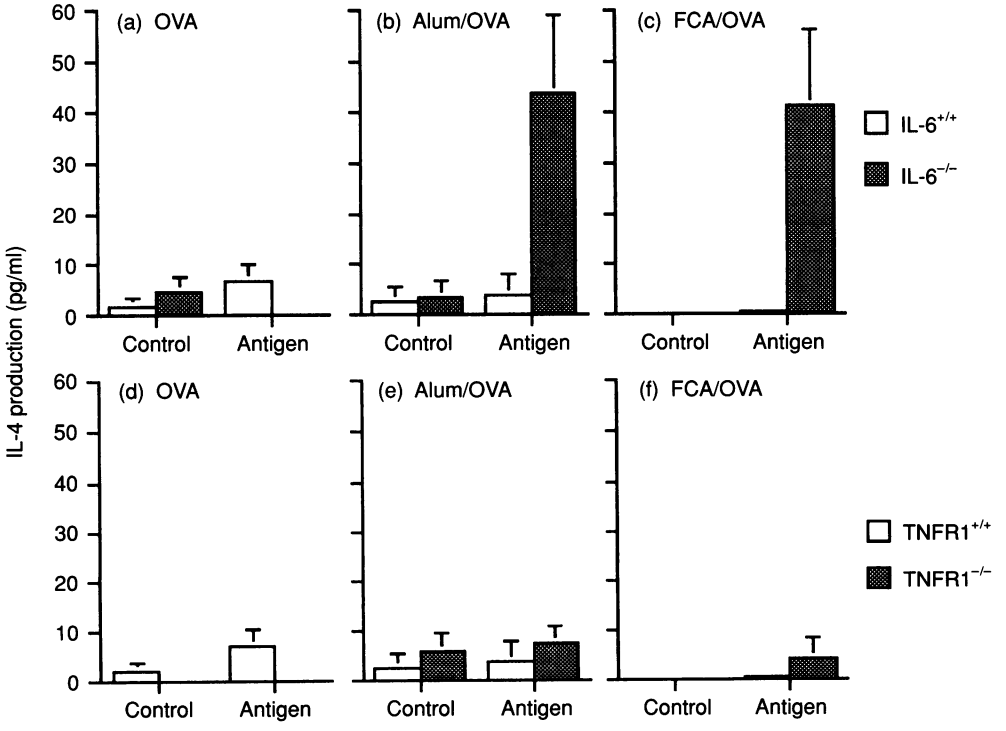

Figure 3. IL-4 production by cultured splenocytes removed from wild-type (+/+) and gene-disrupted (−/−) mice 7 weeks after a second s.c. inoculation of OVA in PBS (a and d), adsorbed to Alum (b and e), or emulsified in FCA (c and f). Cytokine analysis was performed on cell cultures stimulated with 1000 μg/ml OVA (Antigen) or unstimulated cultures (Control). Results are expressed as mean IL-4 concentration as determined by ELISA ± SEM. Comparisons between groups were performed using Student's t-test.

Spleen cell cytokine production

Analysis of IFN-γ production in spleen cell supernatants from wild-type mice following antigen stimulation revealed, as described previously,[5,6] that significant production of this cytokine by spleen cells was dependent on inoculation with antigen prepared in FCA ($P=0{\cdot}03$). Neither splenocytes from wild-type mice inoculated with Alum/OVA nor with OVA alone produced IFN-γ following *in vitro* antigen stimulation. However, in comparison with wild-type mice, IL-6-deficient mice produced increased Th1 responses, as determined by IFN-γ production, when OVA (Fig. 2a; $P=0{\cdot}05$) or Alum/OVA (Fig. 2b; $P=0{\cdot}0007$), was used for *in vivo* priming, but not when FCA/OVA was used (Fig. 2c). Although no significant differences in IFN-γ production could be observed in TNFR-1$^{-/-}$ mice compared with control mice following *in vivo* sensitization with OVA alone (Fig. 2d) or Alum-adsorbed OVA (Fig. 2e), higher levels of IFN-γ were observed in TNFR-1$^{-/-}$ mice inoculated with OVA emulsified in FCA (Fig. 2f; $P=0{\cdot}009$).

IL-6-deficient mice inoculated with OVA/PBS did not produce significantly greater levels of the Th2-associated cytokine, IL-4, than wild-type mice when inoculated with antigen in PBS (Fig. 3a). However, production of this cytokine was markedly increased when antigen was prepared in either Alum (Fig. 3b; $P=0{\cdot}024$) or FCA (Fig. 3c; $P=0{\cdot}035$). TNFR-1$^{-/-}$ and TNFR-1$^{+/+}$ mice did not appear to produce significantly different levels of this cytokine irrespective of the presence or absence of adjuvant during *in vivo* priming (Fig. 3d–f).

Although significant production of IL-5 over background levels could be observed in spleen cells from OVA-treated IL-6-deficient mice (Fig. 4a; $P=0{\cdot}004$), this was not significantly greater than levels observed in the wild-type mouse strain. However, as with IL-4, significantly greater levels of IL-5 were observed in spleen cell supernatants from IL-6-deficient mice inoculated with OVA prepared in either Alum adjuvant (Fig. 4b; $P=0{\cdot}003$) or FCA (Fig. 4c; $P=0{\cdot}05$) in comparison with similarly treated wild-type mice. As with IL-4 production, TNFR-1 deficiency did not appear to affect IL-5 production (Fig. 4d–f) despite a small, though insignificant increase in IL-5 in spleen cells from TNFR-1$^{-/-}$ mice which had been primed with antigen prepared in FCA (Fig. 4f).

While IL-10 production by IL-6$^{-/-}$ spleen cells was not significantly higher than wild-type mice following inoculation of OVA in PBS (Fig. 5a), significantly higher levels of this cytokine could be detected following priming with OVA adsorbed to Alum (Fig. 5b; $P=0{\cdot}0003$) or emulsified in FCA (Fig. 5c; $P=0{\cdot}047$). Consistent with the data presented on IL-4 and IL-5, production of IL-10 in TNFR-1$^{-/-}$ and TNFR-1$^{+/+}$ was not significantly different irrespective of the nature of the priming inoculation given (Fig. 5d–f).

Similar levels of IL-6 were produced by splenocytes from wild-type mice in response to *in vitro* restimulation with OVA,

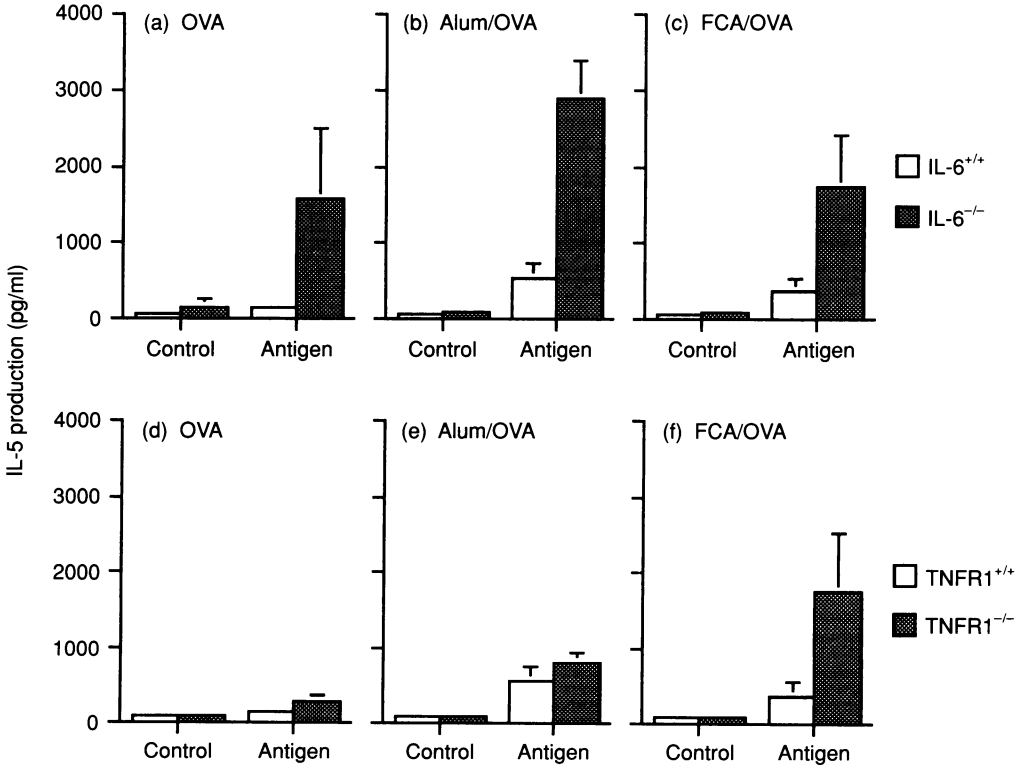

Figure 4. IL-5 production by cultured splenocytes removed from wild-type (+/+) and gene-disrupted (−/−) mice 7 weeks after a second s.c. inoculation of OVA in PBS (a and d), adsorbed to Alum (b and e), or emulsified in FCA (c and f). Cytokine analysis was performed on cell cultures stimulated with 1000 μg/ml OVA (Antigen) or unstimulated cultures (Control). Results are expressed as mean IL-5 concentration as determined by ELISA ± SEM. Comparisons between groups were performed using Student's *t*-test.

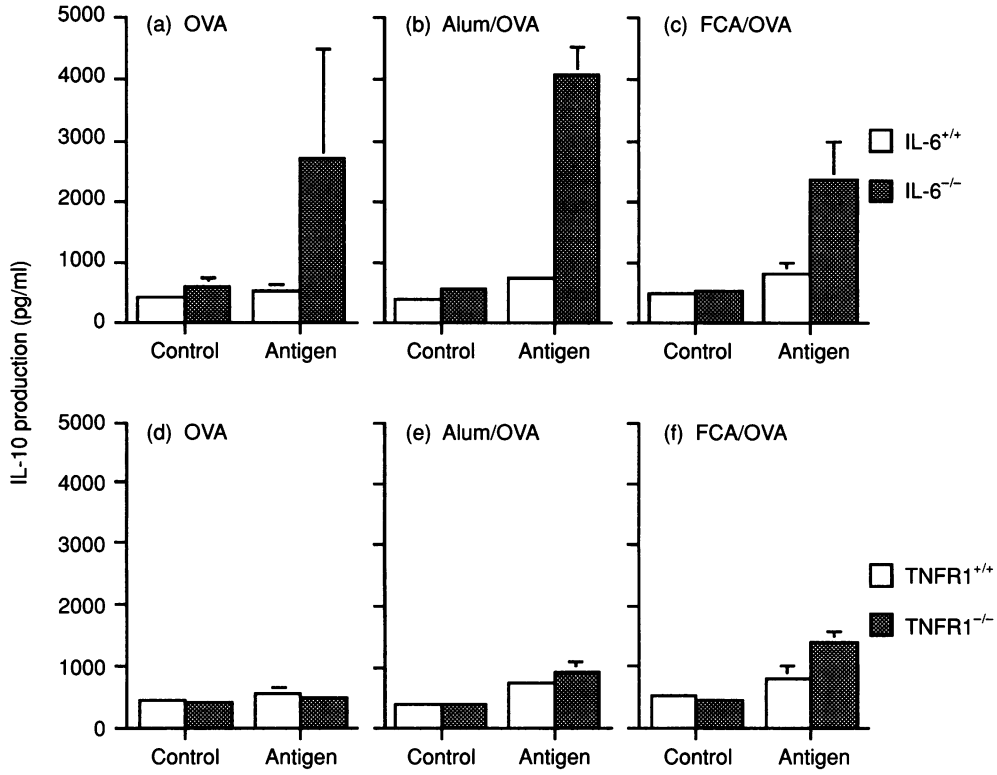

Figure 5. IL-10 production by cultured splenocytes removed from wild-type (+/+) and gene-disrupted (−/−) mice 7 weeks after a second s.c. inoculation of OVA in PBS (a and d), adsorbed to Alum (b and e), or emulsified in FCA (c and f). Cytokine analysis was performed on cell cultures stimulated with 1000 μg/ml OVA (Antigen) or unstimulated cultures (Control). Results are expressed as mean IL-10 concentration as determined by ELISA ± SEM. Comparisons between groups were performed using Student's *t*-test.

irrespective of the presence or absence of adjuvants. As expected, no significant production of IL-6 could be measured in the IL-6-deficient mice (Fig. 6a–c). The only difference in IL-6 production detected between TNFR-1$^{-/-}$ and TNFR-1$^{+/+}$ mice was following administration of OVA adsorbed to Alum (Fig. 6b) which resulted in the production of lower levels of IL-6 by the TNFR-1-deficient group.

DISCUSSION

Previous studies using antigens administered in the absence of adjuvants have demonstrated that both IL-6-and TNFR-1-deficient mice produced significantly lower IgG responses in comparison with wild-type mice.[16,17] Within the germinal centre environment, follicular dendritic cells (FDC) are an important source of IL-6 which has been implicated in the maturation of B cells into plasma blast cells.[17] Furthermore, TNFR-1 signalling has recently been demonstrated to be essential for FDC differentiation,[16] suggesting that either a lack or impairment of germinal centre formation or failure in FDC function or differentiation as a result of these deficiencies may be responsible for reduced IgG production.[16,17] Consistent with the proposed role for FDC in the maintenance of antibody production,[18] the data presented here show that following administration of antigen in the absence of adjuvant, the lower titres of IgG1 observed in TNFR-1$^{-/-}$ mice compared with wild-type control mice was only significant at week 4 and not at week 2 of the experiment. However, our present study also demonstrates that no differences in IgG (data not shown) or IgG1 production could be demonstrated between TNFR-1-deficient or IL-6-deficient and wild-type control mice when antigen was formulated in adjuvant. This indicates that the adjuvant activities of FCA and Alum, as demonstrated by their ability to enhance IgG or IgG1, is independent of IL-6 and signalling via TNFR-1 and furthermore suggests that fully functional FDC are not required for this activity of adjuvants.

When adjuvant-induced, antigen-specific IgG2a production was considered in comparison with wild-type mice, IL-6$^{-/-}$ mice produced higher IgG2a responses to OVA formulated with either adjuvant and TNFR-1$^{-/-}$ mice produced increased IgG2a responses when antigen was inoculated with FCA, though not with Alum. As B-cell IgG2a production is known to be IFN-γ dependent,[19,20] this indicates that IL-6 and signalling through TNFR-1 actually have an inhibitory role in the generation of Th1 responses by adjuvants. Consistent with these antibody data, spleen cell supernatants from IL-6-deficient mice contained increased levels of IFN-γ in comparison with wild-type controls. Interestingly, although production of the IgG1 antibody subclass has been previously demonstrated to be dependent on IL-4 and IL-5,[21,22] the observed increase in spleen cell Th2 cytokine production in IL-6-deficient mice was not accompanied by higher IgG1 levels compared with control mice. Thus while the antibody subclass data presented here indicate a greater Th1 response in IL-6$^{-/-}$ mice than in control mice, the cytokine data suggest that IL-6 deficiency results in a general increase in both Th1 and Th2 cytokine production.

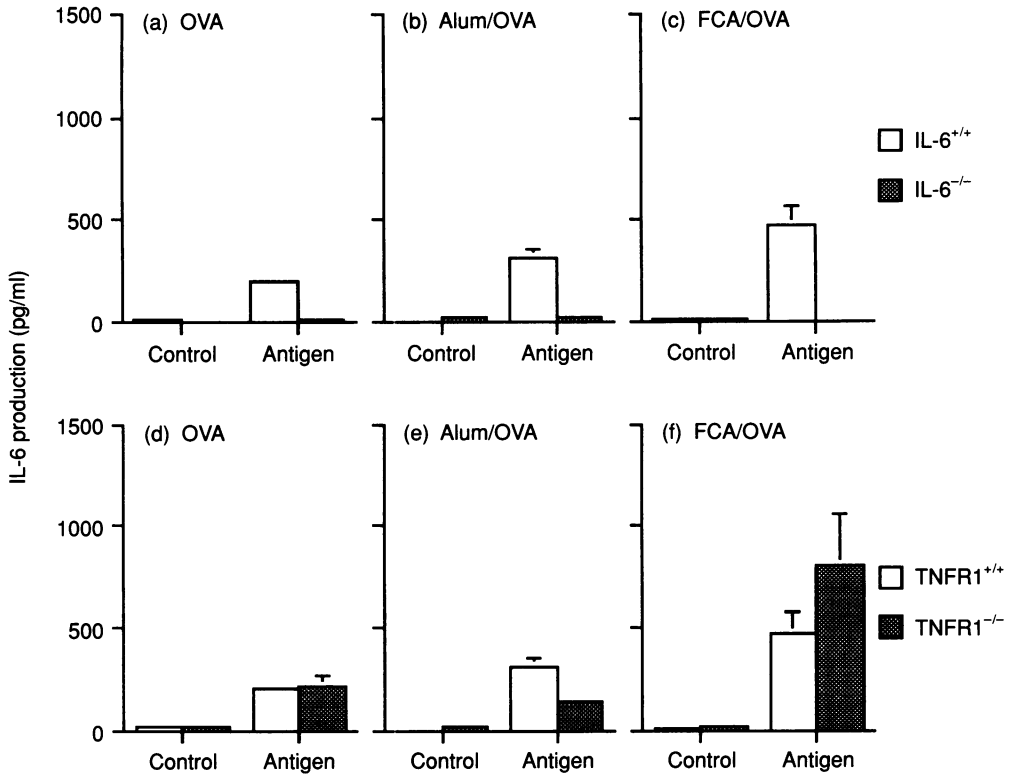

Figure 6. IL-6 production by cultured splenocytes removed from wild-type (+/+) and gene-disrupted (−/−) mice 7 weeks after a second s.c. inoculation of OVA in PBS (a and d), adsorbed to Alum (b and e), or emulsified in FCA (c and f). Cytokine analysis was performed on cell cultures stimulated with 1000 μg/ml OVA (Antigen) or unstimulated cultures (Control). Results are expressed as mean IL-6 concentration as determined by ELISA ± SEM. Comparisons between groups were performed using Student's *t*-test.

IL-6-deficient mice are highly susceptible to *Listeria* infection compared with their immunocompetent counterparts,[13] and recent studies have suggested that this increased susceptibility may be due to inefficient neutrophilia.[23] Similarly, IL-6-deficient mice are also more susceptible to infection with *Candida albicans* than wild-type mice. However, although a defective neutrophil response was also observed in this disease model, IL-6 deficiency resulted in increased IL-10 production and consequently a reduced Th1 response.[24,25] In contrast, we have observed that compared with control mice, IL-6$^{-/-}$ mice had increased Th1 responses as determined by IgG2a and IFN-γ production irrespective of which adjuvant was used. However, higher levels of the Th2 cytokines, IL-4, IL-5 and IL-10 were also detected in spleen cell supernatants from IL-6-deficient mice inoculated with OVA prepared in either FCA or Alum. This increase in Th2 cytokine production would probably more than counterbalance any increase in IFN-γ production. Thus the increased susceptibility observed in both of these infectious disease models may not be due to the presence or absence of a Th1 type response but the presence of Th2-associated cytokines, such as IL-10. This cytokine has been shown to be a major factor associated with susceptibility not only to *C. albicans*[26] but also to *Listeria* infection.[27] Interestingly, we have recently demonstrated that IL-6-deficient mice are also more susceptible to *Toxoplasma gondii* infection than are wild-type control mice,[28] despite their increased production of the potentially protective cytokine, IFN-γ.[29]

Unlike IL-6-deficient mice where IFN-γ production was enhanced with either adjuvant, spleen cells from TNFR-1-deficient mice, produced more IFN-γ following *in vitro* stimulation with OVA only when FCA had been used as an adjuvant for *in vivo* priming. Furthermore, neither splenocyte IL-4, IL-5, nor IL-10 production was altered in TNFR-1$^{-/-}$ mice suggesting that only Th1 responses can be affected by signalling through this receptor. Previous studies,[4,5,30] as well as this one, have demonstrated that the use of FCA as an adjuvant in immunocompetent mice produces a predominantly Th1-type response while Alum, on the other hand, produces a polarized Th2-type response. We have previously demonstrated that this effect is due to the ability of Alum to suppress Th1 development via an IL-4-dependent mechanism.[6] We conclude from the present study that while signalling via TNFR-1 is not involved in inhibition of the generation of a Th1 response by Alum adjuvant, it appears to down-regulate the continued development of this response in FCA-treated mice. Although it has been demonstrated that TNF-α is in fact necessary for IFN-γ production by natural killer cells,[31] these data would suggest that TNF-α may also act via TNFR-1 to down-regulate production of IFN-γ.

In conclusion, the studies presented above indicate that IL-6 or signalling via TNFR-1 do not appear to be necessary for adjuvant activity and may in fact, be involved in the inhibition of certain aspects of adjuvant function. As IL-6 and TNF-α are extensively involved in the induction of undesirable

local and systemic inflammatory effects, our data also suggest that, in the context of rational adjuvant design, failure to stimulate the production of these mediators would be a beneficial feature of an adjuvant.

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