

An Ovalbumin-IL-12 Fusion Protein Is More Effective than Ovalbumin Plus Free Recombinant IL-12 in Inducing a T Helper Cell Type 1-Dominated Immune Response and Inhibiting Antigen-Specific IgE Production¹

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Optimal treatment of allergic diseases requires that the cytokine profile of allergen-specific T cells be redirected, with the conversion of Th2 profiles into Th1 cytokine profiles. This conversion, however, is difficult, since Th2 effector cells have relatively fixed cytokine profiles. To more effectively redirect the cytokine profiles of T cells, we constructed a cytokine fusion protein that contained the Ag OVA, fused to IL-12. Immunization with the OVA-IL-12 fusion protein induced anti-OVA IgG2a Ab and large quantities of OVA-specific IFN- γ production. The Ag specificity of this response was dependent upon covalent linkage of Ag and IL-12, since immunization of mice with OVA alone induced little or no IFN- γ , while immunization with OVA and free rIL-12 enhanced T cell production of IFN- γ , but the IFN- γ production was not OVA specific. To examine the effects of OVA-IL-12 in reversing ongoing Th2-dominated immune responses, BALB/c mice previously primed with OVA in alum to induce a Th2-dominated response, were vaccinated with the OVA-IL-12 protein. In such mice, OVA-IL-12 was much more effective than OVA plus free rIL-12 in significantly increasing Ag-specific IFN- γ production and significantly decreasing Ag-specific IL-4 production. Moreover, OVA-IL-12 increased serum anti-OVA IgG2a and decreased anti-OVA IgE. These studies indicate that OVA-IL-12 can convert immune responses characterized by high IL-4 and high IgE synthesis into Th1-dominated responses in an Ag-specific manner. *The Journal of Immunology*, 1997, 158: 4137–4144.

Allergic diseases are caused by the overproduction in allergic individuals of Th2 cytokines by allergen-specific CD4⁺ T cells. Optimal treatment of allergic diseases requires that the cytokine profile of allergen-specific cells be redirected, with the conversion of Th2 cytokine profiles into Th1 cytokine profiles. This conversion, however, is difficult, since Th2 effector cells have relatively fixed cytokine profiles (1).

IL-12 is a heterodimeric cytokine secreted by macrophages and dendritic cells that is potent in driving the differentiation of naive CD4⁺ T cells toward the Th1 phenotype, by enhancing the production of IFN- γ , but not that of IL-4 (2, 3). Recent studies have shown that IL-12 has enormous potential as an immunomodulator and adjuvant in models of allergy and of infectious diseases and cancer therapy (4–7). Although we and others have previously shown that IL-12 greatly enhances IFN- γ production and inhibits IL-4 production in memory CD4⁺ T cells during in vitro responses (8, 9), the capacity of IL-12 to enhance IFN- γ synthesis and inhibit

IL-4 synthesis during ongoing in vivo immune responses is controversial. Several in vivo studies have demonstrated that rIL-12 as an adjuvant, while enhancing IFN- γ synthesis, may in some situations paradoxically also increase IL-4 synthesis in Ag-primed CD4⁺ T cells (10–13).

The paradoxical increase in IL-4 synthesis driven by rIL-12 during in vivo immune responses may result from Ag-nonspecific effects of IL-12 that may cause a rebound increase in the production of IL-10. To minimize the Ag-nonspecific effects of IL-12 and to drive immune responses toward Ag-specific Th1 responses, we constructed a cytokine fusion protein that contained the T cell-dependent Ag OVA fused to murine IL-12. We hypothesized that the covalent linkage of the IL-12 molecule to the Ag would limit the in vivo IL-12 effects to Ag-specific cells. Our results show that the OVA-IL-12 fusion protein enhanced IFN- γ production in lymph node and splenic CD4⁺ T cells in an Ag-specific fashion and promoted the production of Ag-specific IgG2a. Moreover, the OVA-IL-12 fusion protein was effective in reversing the cytokine profile of an immune response dominated by high IL-4 production, and effectively decreased OVA-specific IgE synthesis in OVA-primed mice.

Materials and Methods

Mice

Seven- to nine-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the laboratory animal facility at Stanford University. All animal protocols used in this study were approved by the Stanford University committee on animal welfare.

Cytokines and Ags

Murine rIL-4, rIFN- γ , and rIL-12 were generously provided by DNAX Research Institute (Palo Alto, CA), Genentech Institute (South San Francisco, CA), and Genetics Institute (Cambridge, MA), respectively. OVA

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was obtained from ICN Biomedicals (Montreal, Canada) and Sigma Chemical Co. (St. Louis, MO), and keyhole limpet hemocyanin (KLH)⁴ was purchased from Calbiochem Co. (San Diego, CA).

Antibodies

Anti-mIL-4 mAb BVD4-1D11 and BVD6-24G2 were obtained from M. Howard, DNAX Research Institute. Anti-mIFN- γ mAb R46A2 (HB 170; from American Type Culture Collection, Rockville, MD) and XMGI.2 (from T. Mosmann, University of Alberta, Edmonton, Canada) were purified from ascitic fluids by ammonium sulfate precipitation and DEAE ion exchange chromatography. Anti-mIL-12 p40 mAb C15.6 (rat IgG1) and C17.8 (rat IgG2a; from G. Trinchieri, Wistar Institute, Philadelphia, PA) were purified from ascites by affinity chromatography after ammonium sulfate precipitation. Anti-p21 mAb (rat IgG2a, CRL 1741), obtained from American Type Culture Collection (ATCC), was purified from ascites fluid and used as a control. Anti-Ly 2.2 mAb (CD8; HB 130) and anti-L3T4 mAb (CD4; hybridoma GK1.5, ATCC, TIB 207) were purified from ascites fluids by ammonium sulfate precipitation.

Anti-OVA mAbs were produced by immunization of BALB/c mice with OVA twice weekly in the footpads; the initial immunization was in CFA, and subsequent immunizations were with OVA in PBS or IFA. The popliteal and axillary lymph nodes were harvested and fused with SP-2/0-Ag14 as previously described (14). Wells that demonstrated growth were screened for binding to OVA by ELISA and were subcloned at 0.3 cells/well to assure clonality. Two anti-OVA mAb generated from this fusion, 6C1 (IgG1) and 3A11 (IgG2a), were used as standards for isotype-specific ELISA. Rabbit anti-OVA serum was purchased from Cappel Co. (Durham, NC). Polyclonal mouse anti-OVA serum was prepared from serum of immunized mice after repetitive injections of OVA in CFA followed by OVA in IFA.

Horseradish peroxidase (HRP)-labeled goat Abs to mouse IgG, IgG1, and IgG2a were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). A rat anti-mouse IgE mAb, EM95, was provided by R. Coffman (DNAX Research Institute, Palo Alto, CA) and was purified from hybridoma supernatants by ammonium sulfate precipitation followed by chromatography on DEAE-Sephacel (Pharmacia, Piscataway, NJ). HRP-labeled goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and HRP-labeled anti-rat IgG was purchased from Southern Biotechnology Associates.

Cell line and medium

High Five (*Trichoplusia ni*) insect cells (Invitrogen Co., San Diego, CA) were maintained in a T-75 flask as a monolayer or in a culture flask as a suspension using EX-CELL 401 medium (JRH Biosciences, Lenexa, KS) at 27°C. All cultures of lymph node cells and splenic cells from BALB/c mice were maintained in DMEM (Sigma Chemical Co.) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), L-glutamine, β -ME, and gentamicin.

Construction of a baculoviral vector for expression of OVA-IL-12 fusion protein in insect cells

A baculovirus vector encoding the OVA-IL-12 gene for expression in insect cells was generated as follows. The gene for OVA was cloned by PCR from the OVA-containing plasmid pAc-Neo-OVA (a gift from Mark Moore, Genentech, and Mike Bevan, University of Washington, Seattle, WA) (15), using primers containing the desired restriction sites.⁵ The IL-12 p40 gene was inserted downstream of the OVA gene, separated by a spacer encoding six glycine residues in a mammalian expression plasmid (16) containing an SV40 origin of replication. The combined OVA-IL-12 p40 genes were then transferred into the baculovirus vector, pAcUW51 (PharMingen Co., San Diego, CA), at the *Bam*HI site downstream of the polyhedrin promoter. The IL-12 p35 gene, obtained from the plasmid pEDp35 (a gift from Stanley Wolf) (17), was ligated into the same baculovirus vector, pAcUW51, at the *Eco*RI site downstream of the p10 promoter.

Expression and purification of the OVA-IL-12 fusion protein

A single recombinant pAcUW51 plasmid containing the genes for the OVA-mIL-12 fusion protein was isolated by plaque purification and co-transfected with BaculoGold DNA into Sf9 insect cells (BaculoGold system, PharMingen). The recombinant baculovirus was amplified and then transfected in High Five insect cells. One liter of baculovirus High Five insect cell supernatant containing the OVA-IL-12-expressed protein was applied to an immunoaffinity chromatography resin prepared by coupling the anti-OVA mAb, 3A11.2, to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotechnology, Inc., Piscataway, NJ). The column was washed with 10 vol of PBS, and the adsorbed fusion protein was eluted with 50 mM glycine buffer containing 0.15 M NaCl (pH 3.0). Fractions were immediately neutralized in 0.05 vol of 1 M sodium phosphate (pH 8.0), and fractions containing the protein were pooled and dialyzed against PBS. Fractions containing the OVA-IL-12 fusion protein were quantitated by SDS-PAGE, Western blot, and ELISAs using mAb specific for OVA and for the IL-12 p40 subunit.

SDS-PAGE and immunoblot analysis

After SDS-PAGE, proteins were transferred onto nitrocellulose by semidry electroblotting, performed as previously described (18). Blots were probed either with mouse anti-OVA serum at 1 μ g/ml or with rat anti-IL-12 p40 Ab (C17.8), washed, and exposed to HRP-labeled anti-mouse IgG or HRP-labeled anti-rat IgG. The blots were then developed with the ECL system (Amersham, Arlington Heights, IL) according to the manufacturer's directions.

OVA ELISA

Ninety-six-well plates were coated with 2 μ g/ml of anti-OVA mAb 3A11.2. The plates were incubated overnight, then washed and blocked. Samples of OVA-IL-12 fusion proteins were added and incubated overnight at 4°C. OVA (ICN) was used as standard. Plates were subsequently developed with polyclonal rabbit anti-OVA Ab, followed by HRP-labeled goat anti-rabbit Ig.

Immunization

BALB/c mice were injected i.p. or in the footpads with rOVA or OVA-IL-12 fusion protein. In preliminary studies, we immunized mice with 2, 10, 25, 50, and 100 pM OVA-12 fusion protein and found that 50 pM induced the optimal OVA-specific immune response in terms of T cell proliferation and Ab production. Subsequent experiments were performed by immunizing mice with 50 pM OVA-IL-12 fusion protein. For OVA sensitization before vaccination with OVA-IL-12, footpads of mice were injected with 10 μ g of OVA adsorbed onto 2 mg of Al(OH)₃ adjuvant.

In vitro stimulation of lymph node and spleen cells

Spleens or lymph nodes were obtained at various times from immunized mice as indicated in the figure legends, and single cell suspensions were prepared as previously described (9).

Cytokine assays

The quantities of IL-4, IL-12, and IFN- γ in culture supernatants were determined by a sandwich ELISA using mAbs specific for each cytokine. The mAbs for coating the plates and the biotinylated second mAbs were as follows: for IL-4, rat anti-mouse IL-4 (BVD4-1D11) and biotinylated anti-mouse IL-4 (BVD6); for IFN- γ , rat anti-mouse IFN- γ (HB170) and biotinylated rat anti-mouse IFN- γ (XMGI.2); and for IL-12, rat anti-mouse IL-12 p40 (C17.8) and biotinylated rat anti-mouse IL-12 p40 (C15.6). Standard curves were generated using recombinant cytokines. The lower limit of detection was 3 pg/ml for IL-4, 125 pg/ml for IFN- γ , and 30 pg/ml for IL-12 p40.

The biological activity of IL-12 in the OVA-IL-12 fusion protein was determined by the ability to stimulate the proliferation of PHA-activated human lymphocytes. Mouse rIL-12 (generously provided by Stanley Wolf, Genetics Institute) was used as a standard.

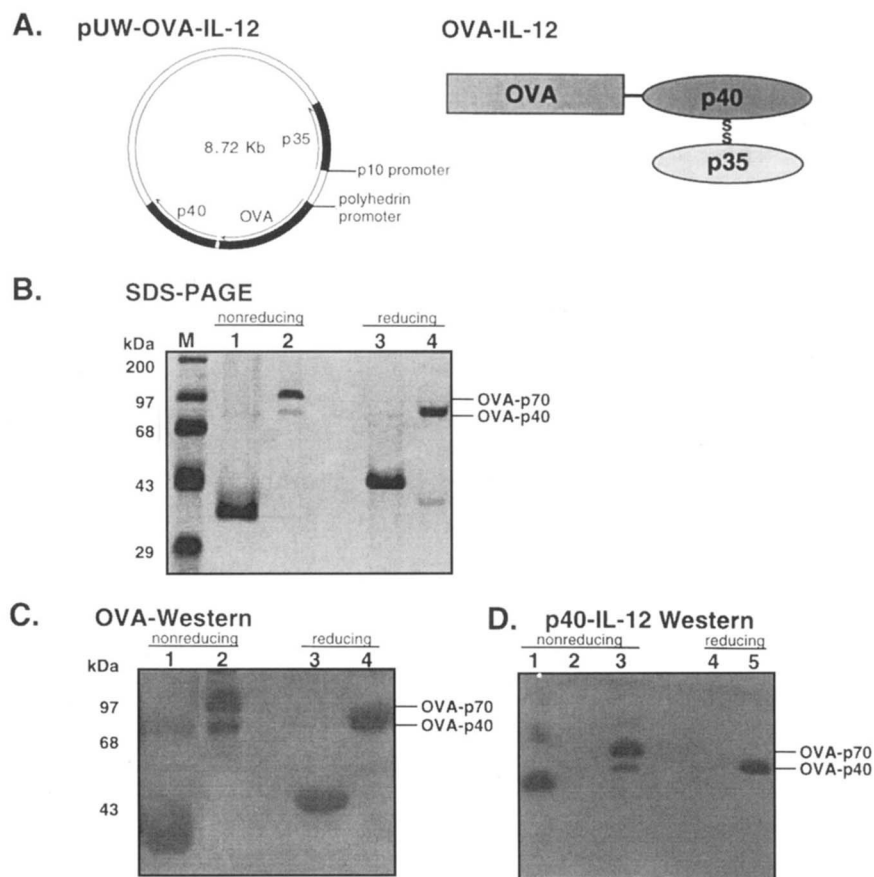
Determination of anti-OVA Ab isotypes

Mice were bled during the course of experiments, and the amount of OVA-specific Ab in the sera was measured by ELISA using mAbs specific for each isotype. Briefly, ELISA plates were coated with 5 μ g/ml OVA. After coating, serial diluted sera were added to the plates and incubated overnight at 4°C. Anti-OVA IgG1 mAb (6C1), anti-OVA IgG2a mAb (3A11), and anti-OVA IgG from hyperimmunized mice were used as standards for

⁴ Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; HRP, horseradish peroxidase.

⁵ H. T. Maecker, D. T. Umetsu, R. H. DeKruyff, and S. Levy. 1997. Polarized immune response induced by DNA vaccination with ovalbumin-cytokine fusion constructs. *Vaccine*. In press.

FIGURE 1. Characterization of the rOVA-IL-12 fusion protein. *A*, Schematic diagram of pUW-OVA-IL-12, the baculoviral vector, and of the expressed OVA-IL-12 fusion protein. *B*, SDS-PAGE of the OVA-IL-12 fusion protein. Molecular weight markers were run in lane *M*. Lane 1, rOVA, nonreducing conditions; lane 2, OVA-IL-12, nonreducing conditions; lane 3, rOVA, reducing conditions; lane 4, OVA-IL-12, reducing conditions. *C*, Western blot with anti-OVA serum. Lane 1, rOVA, nonreducing conditions; lane 2, OVA-IL-12, nonreducing conditions; lane 3, rOVA, reducing conditions; lane 4, OVA-IL-12, reducing conditions. Nitrocellulose blots were incubated with anti-OVA serum followed by HRP-labeled anti-mouse IgG. *D*, Western blot with anti-IL-12 mAb. Lane 1, rIL-12; lane 2, rOVA, nonreducing conditions; lane 3, OVA-IL-12, nonreducing conditions; lane 4, rOVA, reducing conditions; lane 5, OVA-IL-12, reducing conditions.



quantitation of each IgG subclass and total IgG, respectively. After washing, HRP-labeled anti-mouse IgG1, IgG2a, and IgG were added and incubated for 2 h at room temperature. After additional washing, OPD substrate was added and developed, and the OD was determined at 492 nm. Determination of OVA-specific IgE was performed by ELISA, using rat anti-mouse IgE (0.5 μ g/ml) to coat the plates. After the samples were applied, the plates were washed, and biotinylated OVA (5 μ g/ml) was added. The plates were again washed and developed with streptavidin-peroxidase in PBS.

Proliferation assay

The lymph node or splenic cells (5×10^5 cells/well) were incubated in 96-well microtiter plates with varying concentrations of Ag (OVA or KLH) for 4 days in a total volume of 200 μ l. The cells were pulsed with 1 μ Ci [^{3} H]TdR during the last 18 h of the culture. The cells were collected onto glass-fiber filters with a PHD cell harvester (Cambridge Technology, Inc., Watertown, MA), and the amount of radioactivity was measured in a beta counter (Beckman Instruments, Fullerton, CA).

Statistics

Student's *t* test was used to determine the statistical differences between the various experimental and control groups.

Results

Expression and characterization of an OVA-IL-12 fusion protein

We generated an OVA-IL-12 fusion protein in a baculoviral expression system by molecularly linking OVA to the IL-12 p40 subunit of IL-12. The p35 subunit of IL-12 was attached by a single disulfide linkage (Fig. 1*A*). An rOVA protein was also produced in the baculoviral virus expression system and was used as a control. Purified OVA-mIL-12 fusion protein, analyzed by SDS-PAGE and by immunoblotting using mAb against OVA or the p40 subunit of IL-12 (IL-12 p40), is shown in Figure 1, *B* and *C*. Most

of the purified protein contained both the p40 and p35 subunits of IL-12, and only a small fraction appeared to lack the p35 subunit.

The purified fusion protein was tested for its ability to support proliferation of PHA-activated human lymphocytes. OVA-IL-12 protein stimulated vigorous proliferation of the PHA-activated lymphocytes in a dose-dependent manner, while rOVA induced background proliferation (Fig. 2*A*). The OVA-IL-12 fusion protein was about 50-fold less active than rIL-12 on a mole for mole basis. The activity was specific and could be inhibited by a neutralizing anti-p40 mAb (C17.8, rat IgG2a), but not by a control mAb (anti-p21, rat IgG2a; Fig. 2*B*). The decreased activity of OVA-IL-12 compared with that of rIL-12 may be partially due to the an imbalance in the production of the fusion protein subunits, resulting in an inactive OVA-p40 chain lacking the IL-12 p35 subunit. Alternatively, the OVA component of the OVA-IL-12 protein might sterically hinder binding of the IL-12 component to its receptor, although our construct contained a six-glycine linker between the carboxyl terminus of the OVA and the amino terminus of the IL-12 p40. Decreased activity of cytokine fusion proteins has also been reported by Gillies et al. (19) and Chen et al. (20) with granulocyte-macrophage CSF and IL-4.

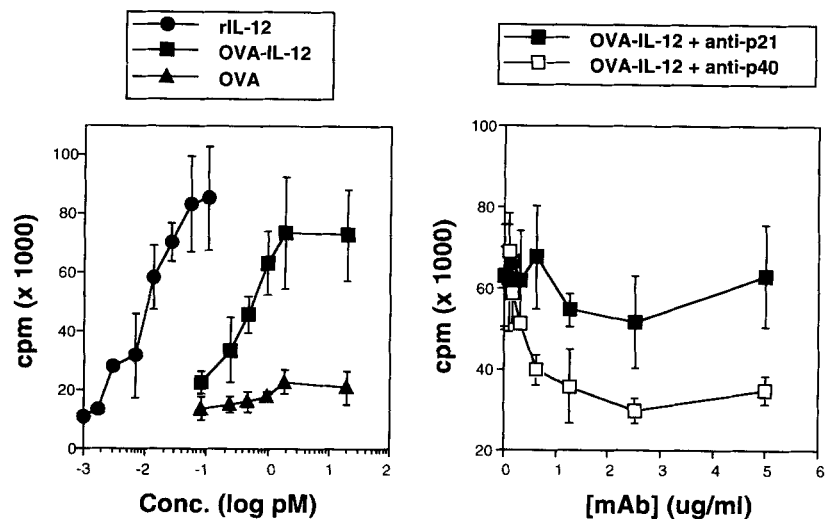
Immunization with OVA-IL-12 fusion protein increases OVA-specific proliferation and IFN- γ production

To examine the immunogenicity of the OVA-IL-12 fusion protein, BALB/c mice were immunized with the soluble OVA-IL-12 fusion protein (50 pM or 5.8 μ g) or rOVA (50 pM or 2.5 μ g). Seven days later, the draining lymph node cells were stimulated in vitro with OVA or KLH for 4 days to assess the proliferative response and the cytokine profile of OVA-specific cells. Lymph node cells

A. Proliferation of PHA-activated lymphocytes

B. Treatment with anti p40 mAb

FIGURE 2. Biological activity of the OVA-IL-12 fusion protein. **A**, PHA-activated human lymphocytes were incubated with increasing concentrations of the OVA-IL-12, rOVA, or rIL-12. Proliferation was measured by [^{3} H]thymidine uptake 16 h later. The results are expressed as the mean counts per minute $\pm$ SD of triplicate determinations. **B**, PHA-activated lymphocytes were incubated in increasing concentrations of a neutralizing mAb, anti-P40 (C17.8; rat IgG2a) or a control isotype, anti-p21 (rat IgG2a), along with 1.3 μ g OVA-IL-12 fusion protein.



	in vivo	in vitro
●	OVA-IL-12	OVA
○	OVA-IL-12	KLH
■	OVA	OVA
□	OVA	KLH

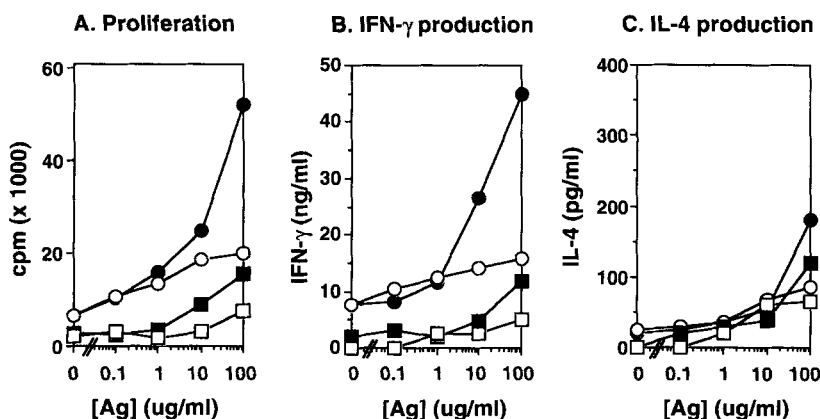


FIGURE 3. OVA-specific immune response induced with the OVA-IL-12 fusion protein. BALB/c mice were immunized in the footpads with the OVA-IL-12 fusion protein (50 pM or 5.8 μ g), rOVA (50 pM or 2.5 μ g), or PBS alone. Seven days later, the lymph node cells were incubated in vitro with increasing amounts of OVA or KLH for 3 days and pulsed with 1 μ Ci [^{3} H]thymidine from 16 h (A). The lymph node cells were stimulated in vitro for 4 days, and the supernatants were analyzed by ELISA for IFN- γ (B) or IL-4 (C). The values represent the mean of triplicate determinations of two separate experiments.

primed by immunization with the OVA-IL-12 fusion protein proliferated vigorously and produced high levels of IFN- γ when stimulated in vitro with OVA (Fig. 3, A and B). The extent of proliferation and the amount of IFN- γ produced by the cells taken from OVA-IL-12-primed mice were much greater than those of cells taken from mice primed with OVA alone. The cells from mice primed with OVA-IL-12 proliferated poorly and produced minimal IFN- γ when stimulated with an irrelevant Ag, KLH, indicating that the immune responses induced with the OVA-IL-12 fusion protein was OVA specific (Fig. 3, A and B). The level of IL-4 production in the immunized mice was relatively low in all groups and not significantly different between groups (Fig. 3C). Cytokine production was dependent on the presence of CD4 $^{+}$ T cells in mice immunized with the OVA-IL-12 fusion protein, since depletion of CD4 $^{+}$, but not that of CD8 $^{+}$, T cells greatly reduced IFN- γ and IL-4 production (Fig. 4). Similar results were observed using spleen cells taken from mice immunized in the peritoneum 7 to 10 days previously (data not shown).

Immunization with OVA and free rIL-12 induces proliferation and IFN- γ production, which is not OVA specific

To investigate the importance of the physical linkage between OVA and IL-12 in the OVA-IL-12 fusion protein, mice were injected twice with the OVA-IL-12 protein (50 pM or 5.8 μ g), a mixture of rOVA (50 pM or 2.5 μ g) and free rIL-12 (1 pM or 70 ng), or rOVA (50 pM). The low amount of free rIL-12 (1 pM) was chosen because the biological activity of the OVA-IL-12 fusion protein was 50-fold less active than an equivalent molar amount of free rIL-12. Cells from mice immunized with the OVA-IL-12 protein proliferated (Fig. 5A) and produced IFN- γ (Fig. 5B) when stimulated in vitro with OVA. In contrast, cells from mice immunized with a mixture of OVA and free rIL-12 proliferated and produced IFN- γ independent of OVA, indicating that immunization with free rIL-12 resulted in the development of non-Ag-specific cells that spontaneously proliferated and spontaneously produced cytokines. These non-Ag-specific effects were also

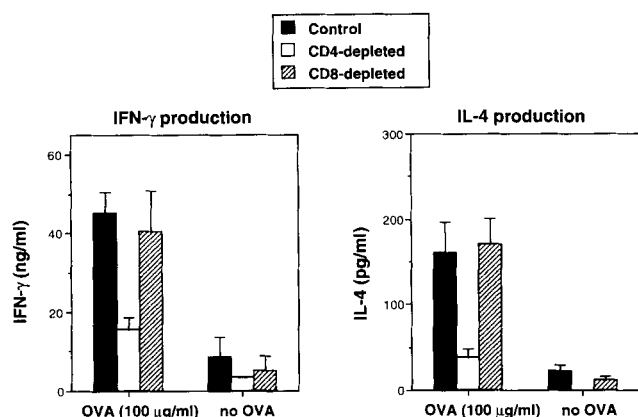


FIGURE 4. CD4⁺ T cells are the major cell type for cytokine production induced with the OVA-IL-12 fusion protein. BALB/c mice were immunized as described in Figure 3. Seven days after immunization, the lymph node cells were incubated in vitro with anti-Ly2 or anti-L3T4 on ice for 10 min, followed by incubation with complement at 37°C for 45 min. After washing, the cells from each treatment group were stimulated with 100 μg of OVA or with medium alone for 4 days, after which supernatants were analyzed for cytokine content by ELISA. The data represent the mean $\pm$ SD of triplicate determinations.

examined in mice immunized with a mixture of KLH and OVA-IL-12 or with a mixture of KLH and free rIL-12. Significantly higher levels of spontaneous IFN- γ production were noted in cells taken from mice receiving KLH and rIL-12 than in those taken from mice receiving KLH and OVA-IL-12 (data not shown). Furthermore, the use of larger amounts of rIL-12 (5 pM (350 ng) or 10 pM (700 ng)) mixed with the OVA increased these nonspecific effects even further (data not shown). Thus, physical linkage between OVA and IL-12 greatly enhanced the Ag-specific effects of IL-12.

Immunization with the OVA-IL-12 fusion protein promotes the production of OVA-specific IgG2a

To examine the effect of the OVA-IL-12 fusion protein on the production of anti-OVA-specific Ab, BALB/c mice were immunized twice with the OVA-IL-12 (50 pM or 5.8 μg), rOVA (50 pM or 2.5 μg), or a mixture of rOVA (50 pM) and free rIL-12 (1 pM or 70 ng). Mice were bled weekly for 6 wk, and Ab responses in the sera were determined by isotype-specific ELISA. Immunization with the OVA-IL-12 protein gave the highest titers of anti-OVA IgG and IgG2a, which peaked at 4 wk (Fig. 6). Injection with a mixture of OVA and rIL-12 gave intermediate levels of anti-OVA IgG and IgG2a production. Immunization with the OVA-IL-12 protein or with a mixture of OVA and rIL-12 gave higher titers of anti-OVA IgG1 production than immunization with OVA alone. Importantly, the OVA-IL-12 fusion protein induced the highest IgG2a/IgG1 ratios, which were severalfold higher than those induced with OVA alone or a mixture of OVA and rIL-12.

The OVA-IL-12 fusion protein causes a switch from a Th2- to a Th1-dominant immune response

To determine the effect of the OVA-IL-12 fusion protein on an established Th2 dominated immune response, mice were injected with OVA (10 μg) in alum, which is known to induce a Th2-dominated immune response, characterized by enhanced IL-4 production and elevated IgE (21, 22). The level of IL-4 produced by lymph node cells from such mice 9 to 10 days after a single immunization ranges from 1000 to 5000 pg/ml. Three weeks after priming, mice were vaccinated with OVA-IL-12, OVA alone, or a

mixture of OVA and rIL-12 (four times, every other day). Ten days after the last vaccination, mice were bled, and anti-OVA Ab isotypes were determined. The draining lymph nodes from the mice were removed, and the lymph node cells were stimulated in vitro with OVA.

As shown in Figure 7, the OVA-IL-12 vaccination significantly increased the anti-OVA IgG2a Ab titer (from 0.806 to 3.349 μg/ml) and decreased anti-OVA IgE production (from OD₄₉₂ = 1.444 to 0.625), compared with the OVA vaccination. The IgG titer increased slightly in the groups immunized with OVA-IL-12 or with a mixture of OVA and rIL-12 compared with that in groups given the OVA vaccination. The levels of IgG1 formation were not significantly different between groups. Vaccination with a mixture of OVA and rIL-12 was less effective at increasing anti-OVA IgG2a production and decreasing anti-OVA IgE formation. The OVA-IL-12 vaccination also increased IFN- γ production and decreased IL-4 production compared with the OVA vaccination (Fig. 8; IFN- γ , from 15 to 82 ng/ml; IL-4, from 1818 to 845 pg/ml). Vaccination with a mixture of OVA and free rIL-12 was less effective in increasing IFN- γ production and decreasing IL-4 production compared with the OVA-IL-12 vaccination. As with primary immunization with free rIL-12, significant IFN- γ production occurred in the absence of OVA stimulation, indicating that free rIL-12 had significant Ag-nonspecific effects. Thus, these results suggested that the OVA-IL-12 fusion protein could convert a Th2-dominant immune response into Th1-dominated response in an OVA-specific manner. In this regard, the OVA-IL-12 fusion protein was significantly more effective than the mixture of free rIL-12 and OVA.

Discussion

In this report we showed that an OVA-IL-12 fusion protein induced a Th1-dominated immune response in an OVA-specific manner. The response was characterized by high OVA-specific IgG2a Ab titers and production of high levels of IFN- γ from OVA-specific CD4⁺ T cells. Moreover, in mice primed with OVA in alum to generate a Th2-dominated OVA response, the OVA-IL-12 fusion protein decreased OVA-specific IL-4 production by CD4⁺ T cells and significantly decreased OVA-specific IgE formation, while it increased OVA-specific IgG2a Ab and increased OVA-specific IFN- γ secretion. These results indicate that the OVA-IL-12 fusion protein was effective in converting an immune response characterized by high IL-4 and high IgE production into a Th1-dominated immune response in an Ag-specific fashion.

We undertook this project because of our interest in altering the Th2-dominated cytokine profiles of allergen-specific T cells in allergic individuals, employing the activity of IL-12 to enhance IFN- γ and inhibit IL-4 synthesis. We wished to establish an Ag-specific therapy by improving or modifying conventional allergen immunotherapy for allergic disease. Conventional allergen immunotherapy, performed by administration of the highest tolerated dose of allergen, appears to affect uncommitted but Ag-specific memory T cells by reducing allergen-specific IL-4 production and increasing allergen-specific IFN- γ production (23–25). Although conventional allergen immunotherapy is highly effective and is Ag specific, such therapy requires many years of injections, is not always successful, and carries the risk of anaphylaxis. Using IL-12 covalently linked to the Ag OVA (an Ag that is responsible for egg allergy in humans), we found that Ag-driven “immunotherapy” with OVA-IL-12 could reduce allergen-specific production of IL-4, enhance allergen-specific IFN- γ production, and generate such changes for OVA much more effectively and rapidly than with Ag alone.

FIGURE 5. Immunization with a mixture of OVA and free rIL-12 induced nonspecific immune responses. BALB/c mice were first immunized in the footpads (on day 0 and again on day 7) with OVA-IL-12 (50 pM or 5.8 μ g), rOVA (50 pM or 2.5 μ g), or a mixture of rOVA (50 pM) and free rIL-12 (1 pM or 70 ng, bioequivalent in terms of IL-12 to OVA-IL-12). Seven days after the last immunization, the lymph node cells were stimulated *in vitro* with increasing amounts of OVA for 4 days, and the levels of cytokines in the supernatants were determined by ELISA. The proliferative response was measured after a 3-day incubation followed by pulsing with [^{3} H]thymidine for 16 h.

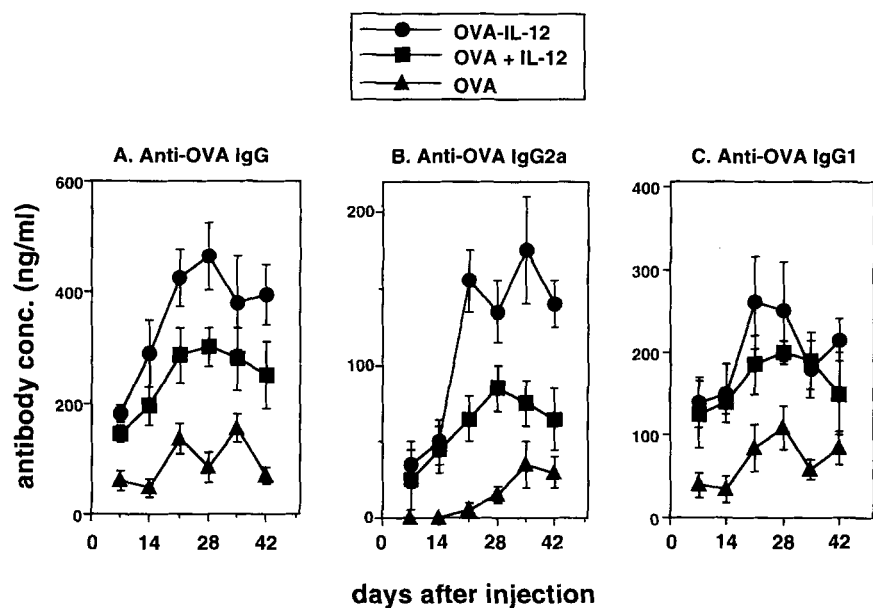
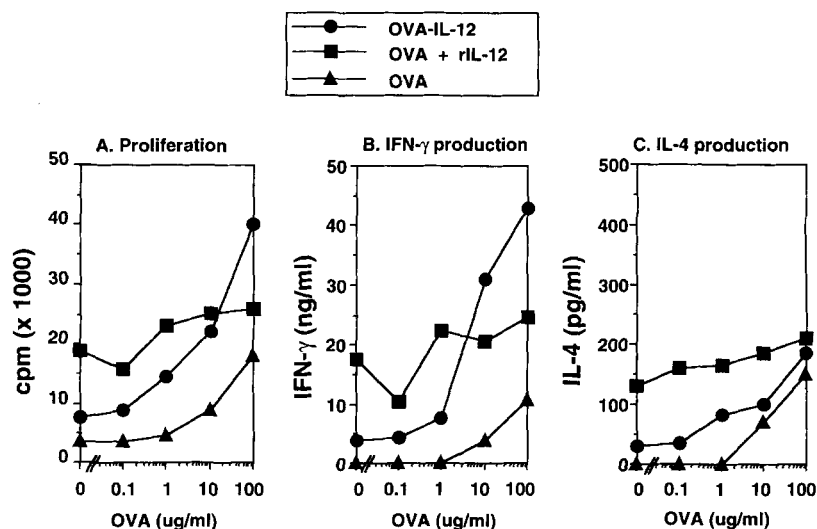


FIGURE 6. Production of anti-OVA Abs in mice immunized with the OVA-IL-12 fusion protein. BALB/c mice were vaccinated in the footpads (on day 0 and again on day 7) with OVA-IL-12 (50 pM or 5.8 μ g), rOVA (50 pM or 2.5 μ g), or a mixture of rOVA (50 pM) and free rIL-12 (1 pM or 70 ng, bioequivalent in terms of IL-12 to OVA-IL-12). The mice were bled every week for 6 wk, and the levels of anti-OVA Ab isotypes in the sera were quantitated by isotype-specific ELISA as described in *Materials and Methods*.

It is known that IL-12 is extremely potent in enhancing IFN- γ synthesis and, in some cases, in inhibiting IL-4 synthesis in CD4 $^{+}$ T cells, particularly in primary immune responses, as demonstrated in both *in vitro* and *in vivo* experimental models (4–7, 26). However, the capacity of IL-12 to affect memory T cells is controversial, in that IL-12, while enhancing IFN- γ and inhibiting IL-4 and IL-10 synthesis in some systems (8, 9), had no effect on or enhanced IL-4 synthesis in other models (10–13, 27). The activation state of the T cell can affect its ability to respond to IL-12, since resting T cells, which are able to express receptors for IL-12 on activation, respond to IL-12, whereas activated effector T cells, particularly Th2 cells, lose their IL-12 receptors and become unresponsive to IL-12 (2, 28, 29). Responsiveness to IL-12 is also affected by the presence of large numbers of Ag-specific T cells that remain uncommitted with respect to differentiation and commitment to a Th1 or Th2 phenotype during Ag-driven immune responses (30). Such uncommitted cells appear to maintain responsiveness to IL-12 and can be driven by IL-12 toward the Th1 phenotype on subsequent exposure to Ag.

A most surprising characteristic of IL-12 when covalently linked to OVA was that the effects of IL-12 appeared to be con-

fined to OVA-specific cells. Whereas the presence of free rIL-12 induced a great increase in Ag-nonspecific IFN- γ production as seen by the *in vitro* production by lymph node cells of IFN- γ in the absence of antigenic stimulation, immunization with the OVA-IL-12 fusion protein, in both naive and Ag-primed mice, resulted in enhanced *in vitro* IFN- γ production, which was elicited only on stimulation with Ag. By contrast, immunization of mice with a mixture of free rIL-12 and OVA resulted in the development of cells spontaneously proliferating and spontaneously secreting IFN- γ , even when very small amounts of rIL-12 (e.g., 70 ng) were used. In addition, the OVA-IL-12 fusion protein was much more effective than free rIL-12 in reducing Ag-specific IL-4 synthesis and in reducing Ag-specific IgE synthesis. Thus, Ag-nonspecific effects of IL-12 were greatly reduced by covalent linkage of IL-12 to OVA, but such linkage enhanced the Ag-specific effects of IL-12.

The precise reason for the efficiency with which the OVA-IL-12 fusion protein reduces Ag-specific IL-4 and IgE synthesis with minimal Ag-nonspecific effects compared with free rIL-12 is not clear. One possibility is that the linkage between OVA and IL-12 allows the IL-12 activity to localize to sites in immune organs

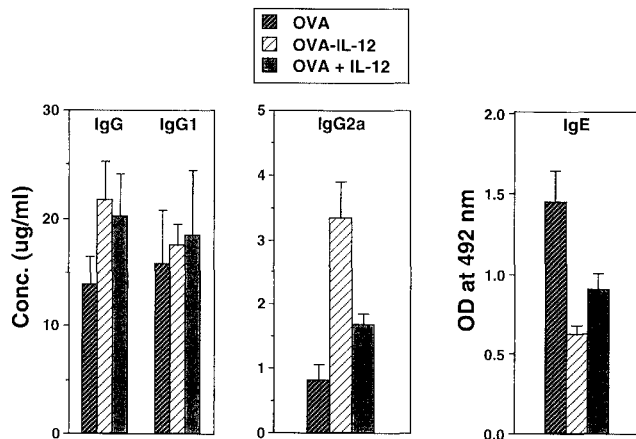


FIGURE 7. Production of anti-OVA Ab isotypes and IgG subclasses in OVA (alum)-primed BALB/c mice immunized with the OVA-IL-12 fusion protein. BALB/c mice were injected in the footpads with commercial OVA (10 μ g) in alum. Three weeks later, the mice were immunized in the footpads with OVA-IL-12 (50 pM or 5.8 μ g), rOVA (50 pM or 2.5 μ g), or a mixture of rOVA (50 pM) and free rIL-12 (1 pM or 70 ng, bioequivalent in terms of IL-12 to OVA-IL-12; four times every other day). The mice were bled 10 days after the last injection, and serum Ab levels were determined by ELISA as described in *Materials and Methods*. The values represent the mean $\pm$ SD of triplicate determinations.

where OVA-specific cells are being activated, minimizing nonspecific effects, for example, on NK cells. The presence of cytokines in the local microenvironment of Ag-specific immune responses is thought to be advantageous in T cell immunobiology and in tumor therapy, since direct injection of cytokine gene-containing vectors or injections of cells secreting specific cytokines resulted in tumor regression and acquisition of systemic antitumor immunity (31). In addition, the fusion protein may efficiently induce OVA-specific Th1 immune responses by increasing the in vivo half-life of injected IL-12 activity compared with that of free rIL-12. Similar prolongation of cytokine half-lives has been reported with IL-2 or GM-CSF fused to mAbs (19, 20).

Few therapies, other than therapy with OVA-IL-12 in mice and conventional allergen immunotherapy in humans, have been successful in altering ongoing Th2-dominated immune responses. Topical therapy with IL-12 as an adjuvant in the lungs of mice has been shown to be effective in reducing airway hyper-reactivity and in reducing pulmonary eosinophilia, but the effectiveness of topical therapy is significantly reduced when IL-12 is administered after Ag exposure in the lungs, and it is unclear whether cytokine synthesis in the lungs is altered in such secondary responses (4). In experimental leishmania infection, Nobors et al. (28) reported that a switch from a Th2 to a Th1 response could be induced by treatment with IL-12. However, in that instance, treatment in combination with the antimony-based anti-leishmanicidal drug Pentostam to decrease the Ag levels was required for IL-12 to inhibit the Th2 response. Finally, it has been reported that immunization with plasmid DNA containing cDNA for Ag could induce a Th1 response that dominates over an ongoing protein-induced Th2 response in an Ag-specific manner (32). However, the long term implications of vaccination with naked DNA is unclear, and in addition, since short immunostimulatory DNA sequences that contain CpG dinucleotides may enhance IFN- α and IFN- β synthesis (33), the specific mechanisms by which plasmid DNA containing cDNA for Ag enhances Th1 cell type bias is not clear.

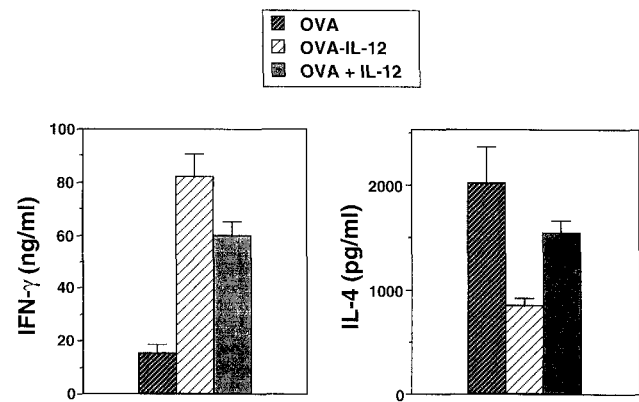


FIGURE 8. Cytokine production from the lymph node cells in OVA (alum)-primed BALB/c mice immunized with the OVA-IL-12 fusion protein. BALB/c mice were immunized as described in Figure 7. Ten days after the last injection, cells from the draining lymph nodes were prepared and incubated in vitro with OVA (100 μ g) for 4 days. Cytokine levels were determined by ELISA as described in *Materials and Methods*. The values represent the mean $\pm$ SD of triplicate determinations.

In summary, we have shown that an OVA-IL-12 fusion protein induced an OVA-specific, Th1-dominated immune response characterized by enhanced OVA-specific IFN- γ production. Furthermore, in a setting of pre-existing or ongoing immunity characterized by high IL-4 and IgE synthesis, the OVA-IL-12 fusion protein greatly reduced OVA-specific IgE production, while increasing OVA-specific IgG2a production, and greatly enhanced Th1-dominant cytokine synthesis in an Ag-specific fashion. These results suggest that Ag-IL-12 fusion proteins may be beneficial in the treatment of diseases caused by undesired Th2-dominated responses, including allergic disorders and certain parasitic infections (34), especially when Ag-specific effects of vaccination are desired. This may be particularly relevant to allergic diseases, in which the major allergens (and, in many instances, the major allergenic proteins) have been identified and for which clinical experience (over >50 yr) with immunotherapy using unmodified allergens is extensive.

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