

Prevention of Allergen-Specific, Th2-Biased Immune Responses In Vivo: Role of Increased IL-12 and IL-18 Responsiveness¹

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The factors that control development of adaptive responses to exogenous Ag remain incompletely understood. An ability to selectively direct immunity toward a specific phenotype would be of clinical benefit in numerous immunological disorders. Administration of chemically modified allergen glutaraldehyde-polymerized OVA (OA-POL) leads to >90% reductions in murine IgE and >500-fold increases in IgG2c responses that develop upon subsequent immunization with native Ag. In the present study, we examine the mechanisms underlying this reorientation of the type 2 dominant response that would normally develop. Lack of endogenous IL-12 or IFN- γ results in markedly reduced induction of IgG2c responses following OA-POL treatment, but only IFN- $\gamma^{-/-}$ mice demonstrate reduced capacity to prevent IgE induction. This indicates that while both IL-12 and IFN- γ are critical promoters of type 1 immunity, only IFN- γ is required to maximally inhibit development of type 2 immune responses. Compared with OVA-immunized mice, CD69⁺ T cells from OA-POL-immunized mice demonstrate elevated IL-12R β_2 , IL-18R α , and IL-18R β mRNA levels, as well as increased IFN- γ production in response to rIL-12 or rIL-18 stimulation. Collectively, these data indicate that preventing induction of type 2 immune responses is critically dependent on altered T cell responsiveness to these cytokines. The finding that targeted, Ag-specific manipulation of IL-12 and IL-18 responsiveness can be used to shape the phenotype of the dominant immune response that develops suggests that specifically targeting IL-12 and IL-18 receptor expression may offer clinical options for clinical prophylaxis or intervention. *The Journal of Immunology*, 2005, 175: 4956–4962.

In recent decades, there has been a marked increase in both allergen sensitization and the expression of immediate hypersensitivity-driven allergic diseases in the developed world. Consequently, there is a need to develop strategies, both for immunization programs and for therapeutic purposes, which would allow selective promotion of specific forms of immunity. IL-12, IL-18, and IFN- γ are important endogenous promoters of type 1 responses and represent potentially valuable therapeutic tools. IL-12 was initially identified as a principal cytokine required for the development of IFN- γ producing Th1-like cells (1). Production of, or responsiveness to, IL-12 is clearly reduced in atopic individuals (2–4), suggesting that deficiencies in the IL-12 axis play an important role in the development of some allergic diseases. IL-18 has been shown both to suppress type 2 responses, particularly in the presence of IL-12 (5, 6), or to exhibit proallergic activities (7–9), depending on the conditions studied. However, we (10) and others (11, 12) have demonstrated that IL-18 has a potent capacity to limit induction of type 2 cytokine responses in the absence of IFN- γ , suggesting that it may play an important role in limiting the severity of allergic disease in vivo, an environment which is inherently deficient in IFN- γ production.

Glutaraldehyde-polymerized OVA (OA-POL)⁴ is a homogeneous preparation of polymeric OVA with an average molecular mass of 3.5×10^7 Da. In vivo administration of OA-POL virtually prevents induction of murine OVA-specific IgE responses in response to immunization with unmodified allergen (13, 14) and abrogates existing IgE responses (15, 16) despite repeated OVA immunization with strong, type 2 immunity-inducing adjuvants. Concomitant with the dramatic decreases in IgE levels seen following OA-POL administration, 500- to 1000-fold increases in OVA-specific IgG2a/2c production and marked increases in the IFN- γ :IL-4 ratio are observed. As such, these chemically polymerized allergens provide a useful tool for better defining the mechanisms that control induction of exogenous Ag-specific immunity toward Th1- vs Th2-biased responses.

In the present study, we examine the mechanisms that underlie the capacity of this family of chemically modified Ags to shift a default, strongly biased type 2 response toward balanced immunity. We find that the capacity of OA-POL to inhibit development of type 2 immunity upon allergen-specific immunization is virtually identical in wild-type, in B cell-deficient, and in IL-12 p40^{-/-} mice, suggesting that B cells and IL-12/IL-23 are not required to limit induction of type 2 immunity. In contrast, the capacity of OA-POL to inhibit development of IgE responses is markedly reduced in IFN- γ -deficient mice, demonstrating that the capacity to mount effective IFN- γ responses is important for limiting the intensity of IgE production. Following OA-POL treatment of normal C57BL/6 (B6) mice, we find no evidence for increased IL-12 or IL-18 production but rather demonstrate increased T cell IL-12R β_2 and IL-18R mRNA expression. These increases in receptor expression are associated with stronger Ag-dependent IFN- γ responses to IL-12 and IL-18 in vitro. Collectively, the data argue that altering responsiveness to endogenously produced IL-12 and

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⁴ Abbreviation used in this paper: OA-POL, glutaraldehyde-polymerized OVA.

IL-18 (hence enhancing the capacity to produce IFN- γ) is a powerful technique for promoting more balanced allergen-specific immune responses.

Materials and Methods

Mice

B6, BALB/c mice and Sprague-Dawley rats were bred at the University of Manitoba breeding facility. IL-12 p40^{-/-}, IFN- γ ^{-/-}, and μ MT (B cell-deficient) mice, all on B6 backgrounds, were purchased from The Jackson Laboratory and bred at the University of Manitoba under specific pathogen-free conditions. All mice were used in accordance with guidelines of the Canadian Council on Animal Care.

Preparation of polymerized OVA

Polymerized OVA (OA-POL) was prepared as described previously (17). Briefly, glutaraldehyde (6% in saline) (Sigma-Aldrich) was added dropwise to a 25-mg/ml solution of OVA (five times recrystallized; ICN Biochemicals) in 0.1 M acetate buffer (pH 5.5) to obtain a final molar ratio of 200:1. After dialysis, the product was gel filtered on a Bio-Gel A-50m column (Bio-Rad), and OA-POL was recovered as a single, sharp peak with an average molecular mass of 3.5×10^7 (~800 times that of native OVA). This method results in a homogeneous preparation of polymerized OVA rather than a mixture of variably polymerized products that are generated via other approaches (18–22).

Immunizations and cell culture

To induce Ab responses, mice were administered saline or nothing or 100 μ g of OA-POL (in saline) i.p. on days -14, -12, and -10 and immunized i.p. on day 0 with 2.0 μ g of OVA adsorbed onto 2.0 mg of Al(OH)₃ adjuvant (alum). Mice were bled 10 and 14 days after OVA (alum) immunization to measure primary Ab responses. To examine *in vitro* cytokine production, mice were treated with nothing or OA-POL as above, immunized with 2.0 μ g OVA (alum) on day 0, and sacrificed on day 5. Alternatively, mice were treated with 100 μ g of OA-POL or native OVA on days 0, 2, and 4 in saline and sacrificed on day 8. In these experiments, some mice were treated with anti-NK1.1 (grown and purified from supernatants of hybridoma PK136, obtained from American Type Culture Collection) on days 6 and 7, a regimen previously shown to deplete NK cells *in vivo* (23). After sacrifice, spleens were removed aseptically and cultured at 7.5 million cells/ml (1 ml/well) in 48-well tissue culture plates (Corning Science Products). Where indicated, rIL-12 (a gift from Dr. M. Gately, Hoffmann-LaRoche, Nutley, NJ) or rIL-18 (R&D Systems) was added to culture at 100 ng/ml. Tissue culture supernatant was harvested at 24 h for analysis of IL-4, 48 h for analysis of IFN- γ , and 72 h for IL-5 and IL-13, times previously determined to reflect peak responses.

Determination of Ab responses

OVA-specific IgE titers were determined by passive cutaneous anaphylaxis while OVA-specific IgG1 and IgG2a/2c titers were determined by ELISA as described previously (24). Total IgE, IgG1, IgG2c (in B6 mice), and IgG2a (in BALB/c mice) were measured by ELISA using rat anti-mouse IgE or sheep anti-mouse IgG (for all IgG isotypes) (Southern Biotechnology Associates) as a capture Ab, followed by biotinylated rat anti-mouse IgE H chain mAb (Serotec), biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates), biotinylated goat anti-mouse IgG2c (Southern Biotechnology Associates), or biotinylated goat anti-mouse IgG2a (Southern Biotechnology Associates) and streptavidin-conjugated alkaline phosphatase. Color was developed following addition of *p*-nitrophenyl phosphate (Sigma-Aldrich). Purified anti-DNP mouse IgE prepared from hybridoma 26.82 (a gift from Dr. A. Froese, University of Manitoba, Winnipeg, Manitoba, Canada) was used as a standard for IgE, and purified IgG_{2c} (BD Pharmingen) was used as a standard for IgG_{2c}. Internal standards of monoclonal mouse IgG₁ (MAB2) and IgG_{2a} (PK136) culture supernatants were calibrated against purified IgG1 mAb generated by Dr. G. Lang (University of Manitoba) and IgG_{2a} (UPC 10; Sigma-Aldrich). The sensitivity of these assays was typically 0.5 ng/ml for IgE, 0.5 ng/ml for IgG₁, 0.1 ng/ml for IgG_{2a}, and 0.1 ng/ml for IgG_{2c}.

Cytokine ELISAs

Cytokine concentrations in tissue culture supernatants were determined by ELISA using the following Ab pairs for coating/development: IL-4, clone 11B1/clone BVD6-24G2 (BD Pharmingen); IL-5, clone TRFK 4/clone TRFK 5 (BD Pharmingen); and IL-13, clone 38213.11/polyclonal goat anti-mouse IL-13 (R&D Systems). IFN- γ concentrations were determined

by ELISA as described previously (25). Detection limits were 0.15 U/ml for IFN- γ , 1.2 U/ml for IL-4, 10 pg/ml for IL-5, and 75 pg/ml for IL-13. The concentration of each sample was calculated from a minimum of two points that fell on the linear portion of the standard curve established using recombinant cytokine standards serially diluted on every assay plate. Intra-assay variability was between 5 and 10% in most circumstances.

Flow cytometry and cell sorting

To enrich for OVA-activated T cells, spleen cells from OVA- or OA-POL-immunized mice were cultured with OVA (1.0 mg/ml for 48 h) and stained with PE-Cy5-labeled rat anti-mouse CD4 (BD Pharmingen) and FITC-labeled hamster anti-mouse CD69 (BD Pharmingen). For detection of NK cells, cells were stained with PE-labeled rat anti-mouse NK1.1 or PE-labeled rat anti-mouse CD49b (BD Pharmingen). Isotype controls were conjugated with PE, FITC, or PE-Cy5 and had irrelevant specificities. Samples were run on an EPICS Altra High Speed FACS (Beckman Coulter) controlled by EXPO 32 Multi Comp Software (Beckman Coulter).

Real-time PCR

mRNA samples were harvested from spleen cell samples or 48-h spleen cell cultures using an RNeasy mini kit (Qiagen), as per manufacturer's instructions. cDNA was prepared using either Oligo dT (IL-12R β_2) or random hexamers (IL-18R α or IL-18R β) and Superscript II RNase H Reverse Transcriptase (Invitrogen Life Technologies). Real-time PCR was conducted using a LightCycler (Roche) using the following primers: IL-12R β_2 , 5'-AATTCAGTACCGACGCTCTCA and 3'-ATCAGGGGCTCAGGCTCTTCA; IL-18R α , 5'-GTGCACAGGAATGAAACAGC and 3'-ATTAAAGGTCCAATTGCGACGA; and IL-18R β , 5'-GGAGTGGGAAATGTCAGTAT and 3'-CCGTGCCGAGAAGGATGTAT. PCR products from selected experiments were run on agarose gels to confirm they were the predicted size.

Statistical analysis

Ag-specific IgE levels were log 2 transformed and are presented as geometric means. Data for all other parameters are expressed as the mean + SEM. Comparison of the effectiveness of OA-POL treatment between strains was done using a two-way ANOVA using SAS software. Values were considered statistically significant when $p < 0.05$.

Results

OA-POL pretreatment dramatically decreases IgE production

To assess the impact of exposing mice to polymerized allergen before OVA immunization, B6 mice were treated with nothing, or OA-POL (three injections of 100 μ g in saline), then immunized with OVA (alum). OVA (alum) immunized mice mounted typical type 2-biased immune responses, characterized by high levels of OVA-specific and total IgE with weak OVA-specific and total IgG2c responses. Mice treated with OA-POL exhibited reductions of 90–95% in their development of both specific and total IgE responses (Fig. 1A). Consistent with this type 1 dominated immune response, OVA-specific IgG2c levels were 500- to 800-fold greater in mice receiving OA-POL treatment (Fig. 1A).

B6 mice are often considered to be more readily biased toward development of type 1 dominated immunity. Therefore, we also assessed the capacity of OA-POL to inhibit generation of IgE responses in BALB/c mice, a strain that displays a tendency toward enhanced type 2 immunity. OVA-specific IgE responses in OA-POL-treated BALB/c mice were also dramatically reduced upon OA-POL treatment, typically exhibiting 70–90% inhibition (Fig. 1B). Enhancement of type 1 Ab production (IgG2a) was marked in BALB/c mice, with OVA-specific IgG2a responses several thousand fold higher in OA-POL-treated BALB/c mice than in controls (Fig. 1B). Thus, pretreatment with OA-POL results in a shift from development of the default type 2-biased response characteristic of OVA (alum)-immunized mice to a type 1 dominated immune response, independent of the genetic propensity of that strain of mice toward type 1- or type 2-biased immunity.

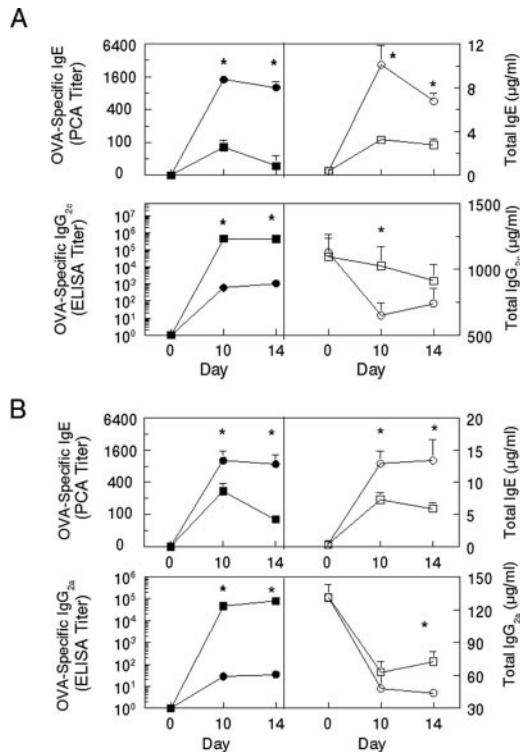


FIGURE 1. Pretreatment with chemically modified allergen results in marked inhibition of IgE production. B6 (A) or BALB/c (B) mice were treated with nothing (circles) or 100 μg of OA-POL in saline (squares) on days -14, -12, and -10. All mice were immunized with 2.0 μg of OVA (alum) on day 0 and bled on days 10 and 14 for analysis of Ab levels. Closed symbols indicate OVA-specific Ig levels, and open symbols indicate total Ig levels. Mean + SEM shown ($n = 24$ mice from six independent experiments for B6 mice, $n = 4$ mice from one experiment for BALB/c mice). Significant differences between untreated and OA-POL-treated mice are indicated by * (p values in the range of $p < 0.05$ to $p < 1 \times 10^{-11}$).

Reduction in type 2 cytokine production following OA-POL treatment is equally efficient in B cell-deficient mice

Given that OVA-specific Abs have dramatically lower affinity for OA-POL than unmodified OVA (18), we hypothesized that B cells are not essential for OA-POL-mediated reduction of type 2 immunity. However, as B cells are crucial in the abrogation of type 2 immune responses following intranasal allergen administration (26), we directly examined the role of B cells in the capacity of this chemically modified Ag to shape developing immune responses. B cell-deficient (μ MT) mice were immunized with OVA (alum) following treatment with nothing or OA-POL, then OVA-driven spleen cell cytokine production was assessed 5 days later in culture. As shown in Fig. 2A, treatment of B6 mice with OA-POL resulted in substantial (~60%) decreases in production of type 2 cytokines IL-4, IL-5, and IL-13. Inhibition seen in μ MT mice treated with OA-POL was virtually identical to that which developed in wild-type mice (Table I). IL-10 production was unchanged in both strains following OA-POL treatment (data not shown). Thus, while B6 mice consistently produced stronger IFN- γ responses than did μ MT mice, their response to OA-POL administration was not detectably different in the presence or absence of B cells.

As a complementary approach to assess the overall balance of type 1 vs type 2 bias in cytokine production following OA-POL pretreatment, we examined the ratio of IFN- γ to IL-4 produced in response to allergen-specific restimulation. OVA (alum) immuni-

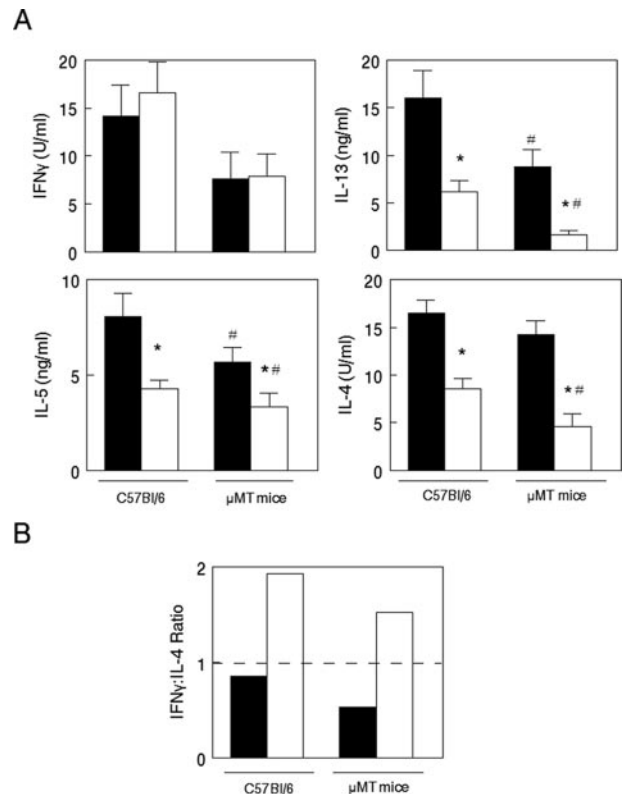


FIGURE 2. B cell-deficient and wild-type C57BL/6 mice exhibit identical reductions in the development of type 2 cytokine production following treatment with chemically modified allergen. A, C57BL/6 and μ MT mice were treated with nothing (■) or OA-POL (□), immunized with OVA (alum), and sacrificed on day 5. Spleen cells were cultured in the presence of OVA, and production of IFN- γ , IL-4, IL-5, and IL-13 was measured. B, Ratio of mean IFN- γ to IL-4 production. Mean + SEM shown ($n = 16$ mice in three individual experiments). Significant differences between untreated and OA-POL-treated mice of the same strain are indicated by * and between C57BL/6 and μ MT mice are indicated by # (p values in the range of $p < 0.05$ to $p < 0.001$).

zation in the absence of OA-POL treatment results in an IFN- γ :IL-4 ratio < 1.0 (Fig. 2B) as would be anticipated for type 2 dominated immune responses. In contrast, in OA-POL-treated, OVA (alum)-immunized B6 and μ MT animals, this ratio is > 1 , indicating a marked shift toward a type 1 dominated response (Fig. 2B). Notably, the magnitude of this increase is similar in both B6 and μ MT mice (Table I). Collectively, these data argue that the absence of B cells, while significantly reducing the overall intensity of some cytokine responses to exogenous protein Ags (Fig. 2A), does not affect the capacity of different forms of Ag to elicit inherently type 1- or type 2-biased response against the same Ag.

Endogenous IFN- γ , but not IL-12, is critical for IgE inhibition

Induction of type 1 immunity is strongly associated with elevated IL-12 production and responsiveness in many systems. Therefore, we examined the role played by endogenous IL-12 in the reduction of type 2 immunity and concomitant increases in type 1 responses that result from OA-POL administration. Mice lacking the capacity to produce IL-12 ($p40^{-/-}$) or IFN- γ ($IFN-\gamma^{-/-}$) were pretreated with nothing or OA-POL before OVA (alum) immunization. As expected, control B6 mice exhibited marked inhibition of OVA-specific IgE and simultaneous increases in OVA-specific IgG2c production following OA-POL treatment (Table II). In the absence of endogenous p40 production, the capacity of OA-POL to inhibit

Table I. The balance of type 1: type 2 cytokine production is shifted by OA-POL treatment

Strain ^b	No. of Mice/Expts	Fold Decrease in OVA-Specific Cytokine Responses ^a				Net Increase in IFN- γ :IL-4 Ratio
		IL-4	IL-5	IL-13	IFN- γ	
B6	22/4	1.92×	1.19×	2.57×	0.974×	225%
μ MT	18/3	2.76×	1.71×	5.21×	0.965×	286%

^a Mice were OVA(alum) immunized 10 days after OA-POL or saline pretreatment. OVA-specific cytokine responses were assessed by ELISA after 5 days primary culture as described in *Materials and Methods*. The values given describe the fold decrease in OVA-specific responses in OA-POL vs control-treated mice.

^b μ MT mice are on a B6 genetic background.

development of OVA-specific IgE responses was unaffected, with ~12-fold reductions (~93% inhibition) in both strains. The magnitude of increased OVA-specific IgG2a levels was substantially lower in the absence of p40, revealing the dissociation of the two responses and suggesting that IL-12/IL-23 is important in promoting type 1 immunity but is dispensable for inhibiting development of type 2 responses.

In contrast, in IFN- γ ^{-/-} mice, both the magnitude of the decrease in OVA-specific IgE and the increase in OVA-specific IgG2c were substantially reduced. Changes in total Ig levels following administration of OA-POL were similar in all strains examined (Table II). Thus, the absence of endogenous IL-12 (and IL-23) has no detectable impact on the ability of this chemically modified protein allergen to inhibit development of IgE production but is clearly required for optimal induction of type 1 immunity. This strongly suggests that IL-12 is not an essential negative regulator of the development of type 2 immunity. In contrast, endogenous IFN- γ production is an important contributor to both promoting type 1 immunity and inhibiting IgE production.

Increased responsiveness to IL-12 following treatment with chemically modified allergen

Consistent with the results described above, in vitro restimulation with OVA or OA-POL under a wide array of experimental conditions consistently failed to demonstrate increased production of IL-12/IL-23 component p40 or IL-12 p70 in wild-type B6 mice, following priming with either OVA or OA-POL (data not shown). However, while p40-deficient mice lack the capacity to produce IL-12, they have the same ability to respond to IL-12 as wild-type mice (27, 28). Therefore, we investigated responsiveness to Th1-promoting cytokines to test our hypothesis that modified allergen administration increases responsiveness to IL-12 and IL-18, thereby inducing the IFN- γ that is required for strong suppression of allergen-specific type 2 immunity.

T and NK cells are the principal IFN- γ -producing cells in the spleen in response to IL-12 or IL-18 (29). To focus on the response of Ag-stimulated T cells, mice were treated with 100 μ g of OVA

(saline) or 100 μ g of OA-POL (saline) on days 0, 2, and 4 along with purified rat anti-mouse NK1.1 mAb PK136 on days 6 and 7 to deplete NK 1.1⁺ cells. Following sacrifice on day 8, spleen cells from these mice were cultured in the presence of exogenous IL-12 or IL-18 $\pm$ OVA. Depletion efficiency ranged from 60 to 90% as assessed by both NK1.1 and CD49b staining (data not shown). As shown in Fig. 3, NK1.1-depleted mice treated with chemically modified Ag produced significantly more IFN- γ in response to OVA + IL-12 at 48 h ($p = 0.006$) and in response to IL-18 ($p = 0.02, 0.004$) than did mice receiving unmodified OVA.

Increased responsiveness to IL-12 and IL-18 results from increased T cell expression of IL-12 and IL-18 receptors

To determine whether increased responsiveness to IL-12 and IL-18 was a result of increased receptor expression, we directly examined mRNA levels for IL-12R β_2 , the subunit mediating IL-12 responsiveness (30), and both subunits of the IL-18R (IL-18R α and IL-18R β) in OA-POL- and OVA-treated, anti-NK1.1-treated animals. Receptor mRNA levels were examined immediately at sacrifice to gauge the level of receptor expression in vivo and after 48 h in vitro stimulation with OVA, as that was the time point

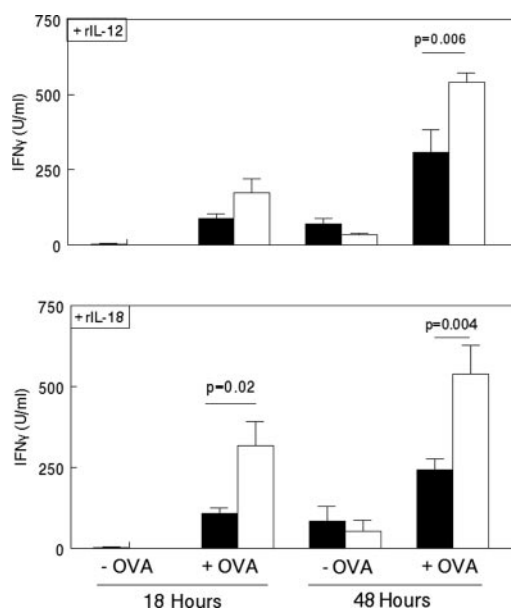


FIGURE 3. In vivo administration of OA-POL increases T cell responsiveness to IL-12 or IL-18. Mice were immunized with 100 μ g of OVA (saline) (■) or 100 μ g of OA-POL (□) on days 0, 2, and 4. All mice were then treated i.p. with anti-NK1.1 mAb PK136 on days 6 and 7, then sacrificed on day 8. IFN- γ production by spleen cells cultured in the presence of 100 ng/ml rIL-12 or rIL-18 was examined in the presence or absence of OVA. Mean $\pm$ SEM shown ($n = 10$ mice from three independent experiments). Significant differences between OVA- and OA-POL-treated mice are indicated.

Table II. Alterations in Ab production resulting from OA-POL treatment

Strain	No. of Mice/Expts	Fold Decrease in IgE ^a		Fold Increase in IgG _{2c}	
		OVA specific	Total	OVA specific	Total
B6	24/6	11.8	3.12	393	1.23
IL-12 p40 ^{-/-}	15/3	12.7	2.58	94.3	0.97
IFN- γ ^{-/-}	16/4	4.73	2.08	33.7	1.12

^a Mice were OVA(alum) immunized 10 days following OA-POL or saline pretreatment, then bled 10 and 14 days later to assess OVA-specific and total IgE and IgG_{2a/2c} levels as described in *Materials and Methods*. Values provided are mean fold decreases in Ab production in OA-POL vs control mice.

where elevated IL-12 and IL-18 responsiveness was most evident. Significantly more mRNA for both IL-18R α and IL-18R β was evident in NK1.1-depleted spleen cells from mice exposed to OA-POL compared with OVA *in vivo* (Fig. 4A). As found for the enhanced IFN- γ production in Fig. 3, such increases were dependent upon the presence of Ag.

In contrast, increased IL-12R β_2 mRNA levels were not consistently seen. Because the effects of OA-POL are Ag dependent (Fig. 3) and OVA specific (16), we hypothesized that the principal effect of OA-POL treatment was on OVA-specific T cells. Because this is a heterogeneous population that in total is likely to comprise <1% of the total T cell population in whole spleen cell cultures, changes in IL-12R β_2 mRNA expression among OVA-specific T cell populations could be masked by stable receptor expression on the majority of T cells. Thus, to examine T cell populations enriched for OVA specificity, we cultured spleen cells from OVA (saline)- or OA-POL (saline)-immunized mice with OVA for 48 h and used flow cytometry to select recently activated T cells on the basis of CD4 and CD69 expression. As shown in Fig. 4B, OVA-activated CD4/CD69 $^+$ T cells from OA-POL (saline)-immunized mice contained ~3-fold more IL-12R β_2 mRNA than CD4/CD69 $^+$ T cells from OVA (saline)-immunized mice.

Collectively, these data argue that treatment of mice with a homogeneous, chemically polymerized allergen increases expression of both IL-18 and IL-12 receptors on allergen-specific T cells. It indicates that the resulting elevated responsiveness to endogenously produced IL-12/IL-18 plays a key role in the capacity of this chemically modified Ag to steer developing immune responses.

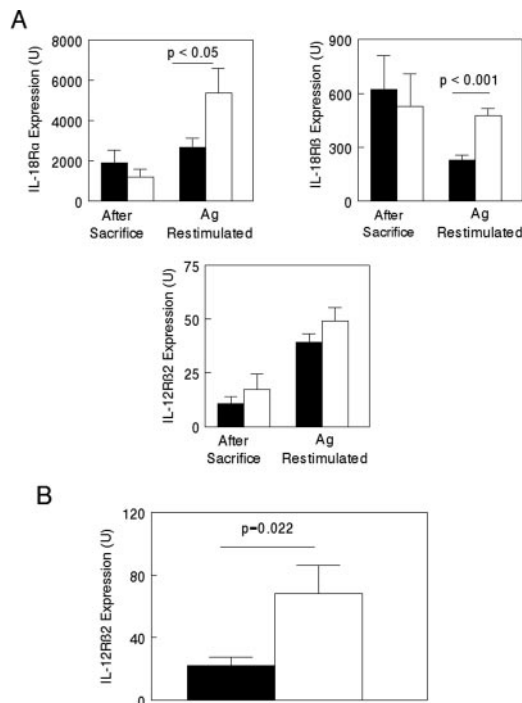


FIGURE 4. Increased T cell responsiveness to IL-12 and IL-18 reflects increased IL-12 and IL-18 receptor expression. Mice were immunized with 100 μ g of OVA (saline) (■) or 100 μ g of OA-POL (□) on days 0, 2, and 4 and sacrificed on day 8. A, IL-12 and IL-18 receptor mRNA expression was determined in spleen cells from mice treated with anti-NK1.1 on days 6 and 7. B, IL-12R β_2 mRNA levels in purified CD4 $^+$ CD69 $^+$ populations isolated from OVA-activated cultures of spleen cells. Mean $\pm$ SEM shown (n = 8 mice from two experiments). Significant differences between OVA and OA-POL mice are indicated.

Discussion

The key findings of this article, that targeted, Ag-specific manipulation of IL-12 and IL-18 responsiveness can be used to shape the phenotype of the dominant immune response that develops, suggest that specifically targeting IL-12 and IL-18 receptor expression may offer clinical options for clinical prophylaxis or intervention. Several human and murine studies attest to the regulatory role of overall IL-12 and IL-18 responsiveness in determining the intensity and dominance of type 2 immunity. BALB/c mice (widely considered as an "allergic" or type 2-biased mouse strain) have lower basal IL-12 responsiveness than B6 mice (considered a "nonallergic" strain) (29). NC/Nga mice, which spontaneously develop atopic dermatitis and high IgE levels, exhibit substantially impaired IFN- γ production even following administration of IL-12 (31). The presence of high levels of IL-12 p40 homodimer, a specific antagonist of IL-12 activity, in lung biopsies from asthmatic individuals, but not biopsies from control individuals, suggests that inhibition of local IL-12 activity in the lungs may exacerbate allergic responses by limiting responsiveness to endogenously produced IL-12 (32). In examining IFN- γ responses from PBMCs of allergic and normal controls stimulated with IL-12 $\pm$ IL-2 (2, 33, 34) or IL-18 (34, 35), allergic individuals frequently display lower IFN- γ production than nonatopic controls, also consistent with apparently decreased responsiveness to these cytokines. Finally, genetic studies examining IL-12 and IL-18 receptor subunits in allergic individuals reveals an increased prevalence of mutations in the IL-12R β_2 and IL-18R α subunits, the latter of which confers IL-18 hyporesponsiveness (35, 36). Although the studies cited here suggest that naturally occurring or environmentally triggered decreases in IL-12/IL-18 responsiveness can predispose one to enhanced development of type 2 dominated immune responses, our data extend this understanding by demonstrating that deliberate manipulation of the capacity to respond to type 1-inducing cytokines can inhibit development of excessive type 2 immunity.

It is interesting to note that data presented in this study and in our previous studies (10, 28) demonstrate that production of IFN- γ , but not IL-12, is critical for efficient inhibition of the development of type 2 immunity. Thus, the demonstration that the principal mechanism behind inhibition of type 2 immune responses comes as a result of enhanced responsiveness to endogenously produced IL-12 and/or IL-18 seems, superficially, to be somewhat inconsistent. However, these data are wholly consistent with a model where the principal limitation allowing the development of Th2 immune responses is not a reduced availability of IL-12 or IL-18, but rather, a limited capacity to respond to endogenously produced IL-12 and IL-18. Increasing biological responsiveness to these Th1-promoting cytokines results in enhanced IFN- γ production, the availability of which is crucial for preventing the manifestation of Th2-dominated immediate hypersensitivity. This suggests that manipulations, which alter biological responsiveness to endogenously produced cytokines and not necessarily changing expression of the cytokines themselves, may prove to be useful tools for redirecting the "default" immune response that would normally be induced by exposure to the unmodified Ag.

OA-POL treatment limits the intensity of both *de novo* type 2 immune responses (Fig. 1) and abrogates existing type 2 immunity (15, 16) while increasing the intensity of type 1 immune responses. We demonstrate in the present study that this is associated with a significant increase in expression of receptors for type 1-inducing cytokines and, consequently, increased IFN- γ production. Other studies using murine models have suggested additional mechanisms for redirecting immune responses. These include a shift in

the balance of type 1 vs type 2 cytokines produced by mucosal or peripheral T cells, the induction of "blocking" IgG4 Abs, and the formation of IL-10 producing CD4⁺CD25⁺ regulatory T cells. In contrast to animal models of immune redirection, which argue that inhibition of IgE synthesis and airway hyperreactivity following mucosal administration of allergen is mediated by IL-10 (37–42), IL-10 production was not enhanced following OA-POL treatment (data not shown). Furthermore, concomitant with the decreased IgE production were increased levels of OVA-specific IgG2c, a finding that differs strikingly from the global down-regulation of allergen-specific type 1 and type 2 immune responses usually seen when the allergen is administered directly to mucosal surfaces (37–39, 41, 42). An alternative explanation for the observed redirection of the immune response, the induction of high titers of "blocking" Ab, possibly IgG2c and IgG1, is not supported by the undiminished effectiveness of OA-POL treatment in B cell-deficient μ MT mice. Rather, following the administration of chemically polymerized allergen, it appears that the principal mechanism mediating the observed shift from type 2 to type 1 dominance of the immune response is the development of naive, OVA-specific T cells into Th1-like effector cells.

The specific mechanism through which glutaraldehyde-polymerized allergens increase IL-12 and IL-18 receptor expression is presently unclear, but a number of potential mechanisms are being examined: 1) the larger size of OA-POL and the reduced affinity of OVA-specific Abs for OA-POL (18) may target OA-POL to a population of "inhibitory" DCs distinct from that targeted by native OVA, (i.e., plasmacytoid DCs (43)); 2) in contrast to soluble OVA, OA-POL triggers DC maturation in a way that facilitates the preferential development of IFN- γ -producing effector T cells; and 3) the higher concentration of T cell epitopes in a single OA-POL molecule results in a greater density of MHC:OVA peptide complexes on the surface of the APCs that provides a qualitatively different signal to the T cells, which directs them toward increased IL-12/IL-18R expression and IFN- γ production. Work is presently underway to explore these possibilities.

In the present study, IL-12^{-/-} mice demonstrate no decrease in their ability to inhibit IgE production following OA-POL treatment (Table II). This is likely due to both residual IFN- γ production observed in these mice and increased IL-18 production in IL-12-deficient mice as reported previously (10, 44). In contrast, OA-POL was markedly less effective in limiting the induction of OVA-specific IgE synthesis in IFN- γ ^{-/-} mice compared with IL-12^{-/-} or B6 controls (Table II). However, despite reduction in the efficacy of OA-POL treatment in IFN- γ ^{-/-} mice, there remained significant inhibition of type 2 Ab production, suggesting the existence of other IFN- γ -independent inhibitors of type 2 immunity. Although there is growing recognition that IL-18 is not exclusively a promoter of type 1 responses (9, 45), we (10) and others (11, 12) have demonstrated that IL-18 can act in an IFN- γ -independent manner to directly inhibit generation of type 2 immunity. Thus, it is possible that IL-18 is responsible for the residual inhibition of IgE synthesis that is seen following administration of chemically modified allergen to IFN- γ ^{-/-} mice, especially in light of the observation that OA-POL enhances T cell IL-18R mRNA expression (Fig. 4A).

The recent identification of IL-23 (comprised of p19 and IL-12 p40), as another member of the IL-12 "family" of heterodimeric cytokines, adds additional complexity to our understanding of the regulation of type 1 responses. IL-23 is similar to IL-12 in many of its biologic activities (inducing T cell proliferation and IFN- γ production) but acts principally on memory rather than naive T cells (46). As the effectiveness of OA-POL in reducing IgE synthesis is equivalent in control B6 and p40^{-/-} mice, this also sug-

gests that IL-23 does not play an essential role in limiting induction of IgE production.

In summary, OA-POL treatment results in profound redirection of the response that is normally elicited upon allergen exposure by shifting it from type 2 dominated immunity toward balanced immunity characterized by 90–97% inhibition of IgE and concomitant 500- to 1000-fold increases in IgG2c responses that develop upon subsequent exposure to unmodified Ag. B cells are not required for this reshaping of immune capacity. Although both IFN- γ and IL-12 are required for optimal induction of type 1 immunity (i.e., increases in OVA-specific IgG2c titers), IFN- γ , but not IL-12, is a critical negative regulator of the development of type 2 immune responses. OA-POL administration in vivo results in significantly increased IL-12 and IL-18 responsiveness by OVA-specific T cell populations as a result of increased IL-12R β_2 and IL-18R mRNA expression. Collectively, these data indicate that responsiveness to endogenously produced type 1 cytokines is a critical checkpoint in the initiation of type 2 immune responses and suggest that targeted manipulation of IL-12/IL-18R expression may represent a potentially useful method of immune manipulation.

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Disclosures

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