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Enhanced Pulmonary Histopathology Induced by Respiratory Syncytial Virus (RSV) Challenge of Formalin-Inactivated RSV-Immunized BALB/c Mice Is Abrogated by Depletion of Interleukin-4 (IL-4) and IL-10

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In previous studies, children immunized with a formalin-inactivated respiratory syncytial virus vaccine (FI-RSV) developed severe pulmonary disease with greater frequency than did controls during subsequent natural RSV infection. In earlier efforts to develop an animal model for this phenomenon, extensive pulmonary histopathology developed in FI-RSV-immunized cotton rats and mice subsequently challenged with RSV. In mice, depletion of CD4$^+$ T cells at the time of RSV challenge completely abrogated this histopathology. Furthermore, the predominant cytokine mRNA present in lungs of FI-RSV-immunized mice during subsequent infection with RSV was that characteristically secreted by Th2 T cells, namely interleukin-4 (IL-4). In the present studies, we sought to determine the relative contributions of gamma interferon (IFN-γ), IL-2, IL-4, and IL-10 to the lymphocytic infiltration into the lungs observed following RSV challenge of mice previously immunized with FI-RSV. Mice previously immunized with FI-RSV or infected with RSV were depleted of IFN-γ, IL-2, IL-4, or IL-10 immediately before RSV challenge, and the magnitude of inflammatory cell infiltration around bronchioles and pulmonary blood vessels was quantified. The phenomenon of pulmonary-histopathology potentiation by FI-RSV was reproduced in the present study, thereby allowing us to investigate the effect of cytokine depletion on the process. Simultaneous depletion of both IL-4 and IL-10 completely abrogated pulmonary histopathology in FI-RSV-immunized mice. Depletion of IL-4 alone significantly reduced bronchiolar, though not perivascular, histopathology. Depletion of IL-10 alone had no effect. Depletion of IFN-γ, IL-2, or both together had no effect on the observed histopathology. These data indicate that FI-RSV immunization primes for a Th2-, IL-4-, and IL-10-dependent inflammatory response to subsequent RSV infection. It is possible that this process played a role in enhanced disease observed in infants and children immunized with FI-RSV.

Respiratory syncytial virus (RSV), a member of the *Pneumovirus* genus of the paramyxovirus family, is the most important cause of bronchiolitis and viral pneumonia in infants and children. Thus far, efforts to develop live or inactivated vaccines have been unsuccessful. In studies conducted in the 1960s, children previously immunized with a formalin-inactivated RSV vaccine (FI-RSV) developed severe pulmonary disease with greater frequency than did controls during subsequent natural RSV infection (13). Although enhanced disease in immunized subjects is a major concern in the development of a successful RSV vaccine, its immunological basis is not well understood. Previous studies of FI-RSV-immunized humans and more-recent studies of FI-RSV- or RSV subunit vaccine-immunized rodents have provided some insights into the possible immune mediators of this enhanced disease.

In previous studies in humans, as well as mice, inactivated RSV antigens efficiently stimulated CD4$^+$ T cells (2, 14). Peripheral blood lymphocytes from humans immunized with FI-RSV in the 1960s had increased virus-specific proliferative responses compared with lymphocytes of naive or RSV-infected controls, suggesting that FI-RSV induced an enhanced CD4$^+$ T-cell response (14). Recently, it was demonstrated that depletion of CD4$^+$ T cells at the time of RSV challenge abrogated the pulmonary histopathology observed in FI-RSV-immunized BALB/c mice (7). It is likely then that CD4$^+$ T cells stimulated by inactivated RSV antigens play a role in the increased levels of pulmonary histopathology observed in rodents previously immunized with FI-RSV or RSV subunit vaccines as well as in the enhanced disease following RSV infection of humans previously immunized with FI-RSV. Conversely, CD8$^+$ T cells did not appear to be implicated in this process, reflecting the weak RSV-specific CD8$^+$ T-cell response elicited by immunization with FI-RSV (7).

Recently, two distinct cytokine secretion patterns have been defined in murine CD4$^+$ T-cell clones (17). Th1 cells, but not Th2 cells, express gamma interferon (IFN-γ) and interleukin-2 (IL-2) and participate in classic delayed-type hypersensitivity responses. Th2 cells, but not Th1 cells, produce IL-4, IL-5, IL-6, and IL-10 and augment the humoral immune response. Both Th1 and Th2 CD4$^+$ T cells have been previously shown in other systems to mediate immunopathology in rodents and, more recently, disease in humans (12, 26, 27, 32). Further, it was recently demonstrated that the lungs of BALB/c mice primed with FI-RSV or F-subunit vaccine had higher levels of IL-4 mRNA transcripts than those of live-virus-primed mice following RSV challenge (10). Although the cellular origins of

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the cytokine mRNAs were not determined, these results suggested that the subsets of $CD4^+$ T cells infiltrating the lungs following RSV challenge of mice previously infected with RSV were different from the subsets infiltrating the lungs of those immunized with FI-RSV. It was suggested that mice previously infected with RSV had pulmonary inflammatory responses directed by Th1 cells, whereas those immunized with FI-RSV or a subunit vaccine had responses directed by Th2 cells. The observations that $CD4^+$ T-cell lines from mice immune to different RSV proteins exhibit distinct Th1- or Th2-like cytokine profiles on restimulation in vitro are consistent with this concept (1).

In the present study, we determined the relative contributions of IFN-γ, IL-2, IL-4, and IL-10 to the histopathology observed in FI-RSV-immunized BALB/c mice following subsequent challenge with RSV. Mice previously immunized with FI-RSV or infected with RSV were depleted of (i) IFN-γ, (ii) IL-2, (iii) IFN-γ and IL-2, (iv) IL-4, (v) IL-10, or (vi) IL-4 and IL-10 by administration of specific rat anti-cytokine monoclonal antibodies just prior to RSV challenge. The level of pulmonary histopathology following challenge was then quantified as described previously (7).

The Long (subgroup A) strain of RSV was used throughout. RSV and parainfluenza virus type 3 (PIV3) were grown in HEp-2 cells and titrated for infectivity by plaque formation in HEp-2 cell monolayer cultures as previously described (20, 25). FI-RSV, FI-PIV3, and RSV were purified from infected cell culture supernatants with an RK continuous-flow centrifuge, and RSV- and PIV3-rich fractions with an infectivity titer of 10^8 were inactivated with formalin as previously described (7, 24). Six- to eight-week old female BALB/c mice were obtained from Charles River Laboratories (Cambridge, Mass.) and maintained in microisolators during all phases of this study. Mice were anesthetized with methoxyflurane before virus inoculation and bled from the retroorbital venous plexus. A brief description of the immunizations is given in footnote *a* of Table 1. Control groups consisted of mice immunized with FI-PIV3 or infected with PIV3.

XMG-6 (anti-IFN-γ), S4B6 (anti-IL-2), and SFR3D5 (a rat immunoglobulin G [IgG] directed at a human HLA that served as a control) monoclonal antibodies were prepared as ascitic fluids of hybridoma-inoculated, pristane-primed *nu/nu* mice by Harlan Bioproducts for Science (Indianapolis, Ind.). Antibodies were partially purified by precipitation with 50% ammonium sulfate and dialyzed against phosphate-buffered saline to a final concentration of 1 mg of IgG per ml as determined by anti-rat IgG immunodiffusion plates (ICN, Costa Mesa, Calif.). 11B11 (anti-IL-4) and JES5-2A5 (anti-IL-10) monoclonal antibodies were purified cell culture supernatants purchased from Verax Inc. (Lebanon, N.H.). A brief description of the schedule of administration of the monoclonal antibodies is given in footnote *b* of Table 1.

Animals were bled for measurement of RSV-specific antibody levels 21 days following infection or immunization. Animals were then challenged on day 21 with 10^6 PFU (0.05 ml) of RSV intranasally. Animals were sacrificed by carbon dioxide asphyxiation on day 25, and the lungs were harvested for either virus quantification or evaluation of histopathology as previously described (18, 20, 25). The histopathology observed was similar to that previously described (8, 9). Enzyme-linked immunosorbent assay (ELISA) antibodies to the F protein were measured as previously described (24). The histopathology slides of lung tissue were read under code by Alex Sotnikov of Pathology Expertise and Services (Newton, Mass.) as previously described (18). A brief summary of the scoring system and statistical analysis is given in the footnotes to Table 2.

TABLE 1. Serum antibody responses and virus replication in FI-RSV-, FI-PIV3-, RSV-, and PIV3-immunized mice

Animals immunized or infected with[a]:	Animals treated with indicated monoclonal antibody prepn[b]	ELISA-F antibody titer (reciprocal mean $\log_2 \pm$ SE) on day 21[c]	Pulmonary replication of RSV challenge (mean $\log_{10}$ PFU/g $\pm$ SE)[d]
FI-RSV	Control	11.7 ± 2.6	≤1.6 ± 0.0
FI-PIV3	Control	4.5 ± 1.1	3.0 ± 0.7
RSV	Control	10.9 ± 1.7	≤1.6 ± 0.0
PIV3	Control	6.5 ± 1.0	3.4 ± 1.3
FI-RSV	Anti-IL-2 + anti-IFN-γ	11.7 ± 0.9	≤1.6 ± 0.0
FI-PIV3	Anti-IL-2 + anti-IFN-γ	6.9 ± 0.9	3.9 ± 0.7
RSV	Anti-IL-2 + anti-IFN-γ	11.3 ± 2.0	≤1.6 ± 0.0
PIV3	Anti-IL-2 + anti-IFN-γ	5.7 ± 1.7	3.3 ± 0.7
FI-RSV	Anti-IL-4 + anti-IL-10	12.5 ± 1.1	≤1.6 ± 0.0
FI-PIV3	Anti-IL-4 + anti-IL-10	6.9 ± 1.7	2.6 ± 0.9
RSV	Anti-IL-4 + anti-IL-10	12.9 ± 0.9	≤1.6 ± 0.0
PIV3	Anti-IL-4 + anti-IL-10	6.0 ± 2.1	2.9 ± 1.1

[a] Animals were immunized on day 0 with FI-RSV or FI-PIV3 (0.1 ml administered intramuscularly) or RSV or PIV3 (10^6 PFU in a 0.05-ml inoculum administered intranasally).

[b] 1 mg of each monoclonal antibody was administered intraperitoneally on day 19: SFR3D5 (a rat IgG directed at a human HLA antigen which served as a control), XMG-6 (to deplete IFN-γ), S4B6 (to deplete IL-2), 11B11 (to deplete IL-4), or JES5-2A5 (to deplete IL-10).

[c] The antigen used for the ELISA-F was purified RSV F glycoprotein. The first dilution of the ELISA was 1:40. Serum specimens without activity at this dilution were assigned a titer equivalent to one lower dilution (1:10).

[d] Mice were challenged with 10^6 PFU/0.05 ml intranasally on day 21, and 4 days later lungs were removed for virus quantification or, from separate animals, for histopathology.

The serum antibody responses and pulmonary virus replication are shown in Table 1. Immunization with FI-RSV and infection with RSV both induced moderate levels of ELISA-F antibodies similar to those previously observed (7). Consistent with previous observations, immunization with FI-RSV or live RSV induced complete protection in the lungs from subsequent RSV challenge (7). RSV replicated in the lungs of control mice to levels approximately 10-fold lower than those observed in previous studies (7). Pulmonary virus replication was not significantly affected by cytokine depletion at the time of challenge.

The pulmonary histopathology data and statistical analysis are summarized in Table 2. Previous studies in our laboratory demonstrated that BALB/c mice previously immunized with FI-RSV or previously infected with RSV develop lymphocytic infiltrates surrounding bronchioles and pulmonary blood vessels and lymphocytic and polymorphonuclear cell infiltration of alveoli following RSV challenge (7). In the present study, FI-RSV-immunized animals that received a control antibody preparation developed higher levels of bronchiolar and perivascular histopathology than previously RSV-infected or FI-PIV3-immunized, nondepleted animals. In contrast to our previous study (7), mice previously infected with RSV, which received a control antibody preparation, did not develop extensive pulmonary histopathology following RSV challenge. For reasons that are unclear, we have observed similar study-to-study variations in the extent of pulmonary histopathology observed following RSV challenge of rodents previously infected with RSV (6, 18, 20). It is important, therefore, that each study contain its own set of internal controls. It should be

TABLE 2. Pulmonary histopathology in FI-RSV-immunized animals is completely abrogated by depletion of IL-4 and IL-10

Animals immunized or infected with[a]:	Animals treated with indicated monoclonal antibody prepn[b]	Histopathology scores of the indicated pulmonary structure[c] following challenge with RSV[d]							
		Bronchioles				Vessels			
		No. scored	% with a score of 5 or 6	Ratio of geometric means[e]	Ratio of ratios[f]	No. scored	% with a score of 5 or 6	Ratio	Ratio of ratios
FI-RSV	Control	1,184	27.4	12.25	7.26	1,555	23.8	8.81	6.57
FI-PIV3	Control	1,208	2.2			1,323	3.6		
RSV	Control	1,312	1.7	1.69		1,297	1.7	1.34	
PIV3	Control	1,213	0.7			1,093	0.9		
FI-RSV	Anti-IFN-γ	999	20.9	3.46	1.52	1,215	24.3	2.65	0.53
FI-PIV3	Anti-IFN-γ	1,140	6.1			1,274	6.6		
RSV	Anti-IFN-γ	1,105	7.2	2.28		1,147	5.9	4.94	
PIV3	Anti-IFN-γ	1,225	3.3			1,330	0.2		
FI-RSV	Anti-IL-2	1,074	35.5	5.08	1.36	1,127	36.1	2.69	0.70
FI-PIV3	Anti-IL-2	1,169	6.4			1,018	13.3		
RSV	Anti-IL-2	1,138	8.3	3.75		1,363	4.0	3.85	
PIV3	Anti-IL-2	1,310	1.5			1,271	0.2		
FI-RSV	Anti-IL-2 + anti-IFN-γ	1,394	22.0	3.13	2.73	1,576	25.9	2.82	1.78
FI-PIV3	Anti-IL-2 + anti-IFN-γ	1,422	6.4			1,346	8.9		
RSV	Anti-IL-2 + anti-IFN-γ	1,384	2.9	1.15		1,532	2.2	1.59	
PIV3	Anti-IL-2 + anti-IFN-γ	1,293	2.0			1,314	0.5		
FI-RSV	Anti-IL-4	1,159	23.0	2.63	0.92[g]	1,275	23.1	4.24	1.01
FI-PIV3	Anti-IL-4	1,088	6.5			1,213	5.4		
RSV	Anti-IL-4	1,144	8.3	2.87		1,121	6.9	4.20	
PIV3	Anti-IL-4	1,309	1.9			1,173	0.3		
FI-RSV	Anti-IL-10	1,320	37.7	6.21	2.32	1,531	31.8	3.25	2.55
FI-PIV3	Anti-IL-10	1,305	10.0			1,565	16.6		
RSV	Anti-IL-10	1,486	2.3	2.68		1,562	1.1	1.28	
PIV3	Anti-IL-10	1,556	0.4			1,578	0.5		
FI-RSV	Anti-IL-4 + anti-IL-10	1,438	9.7	0.68	0.11[h]	1,503	8.4	0.60	0.08[i]
FI-PIV3	Anti-IL-4 + anti-IL-10	1,488	9.0			1,568	14.3		
RSV	Anti-IL-4 + anti-IL-10	1,486	14.4	6.12		1,540	16.4	7.45	
PIV3	Anti-IL-4 + anti-IL-10	1,331	1.3			1,343	0.6		

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] The percentage of bronchioles or vessels with an indicated score of an individual slide was determined, and a mean was determined for each experimental group. The mean percentage of bronchioles or vessels with a score of 5 or 6 is presented here. The scoring system of bronchiole or pulmonary blood vessel infiltration is as follows: 1, the surrounding space is free from infiltrating cells; 2, the surrounding space contains few infiltrating cells; 3, the surrounding space contains focal aggregates of infiltrating cells; 4, the structure is cuffed by one definite layer of infiltrating cells with or without focal aggregates; 5, the structure is cuffed by two definite layers of infiltrating cells with or without focal aggregates: and 6, the structure is cuffed by three or more definite layers of infiltrating cells with or without focal aggregates.
[d] See Table 1, footnote *d*.
[e] Because the data were positively skewed, comparisons were performed in log scale. The ratio of geometric mean scores, FI-RSV/FI-PIV3 or RSV/PIV3, is an estimate of the difference between the effects of RSV and PIV3 on pulmonary histopathology in the presence or absence of formalin inactivation, respectively.
[f] The ratio of ratios is an estimate of the influence of formalin inactivation on the difference between the effects of RSV and PIV3 on pulmonary histopathology.
[g] Significant decrease in bronchiolar histopathology at the 0.05 level compared with animals receiving the control antibody.
[h] Significant decrease in bronchiolar histopathology at the 0.01 level compared with animals receiving the control antibody or all remaining groups.
[i] Significant decrease in pulmonary vessel histopathology at the 0.05 level compared with animals receiving control antibody and at the 0.08 level when compared with all remaining groups.

noted that in each of our studies in mice, animals previously immunized with FI-RSV developed extensive pulmonary lymphocytic infiltration during subsequent RSV infection.

As seen in Table 2, depletion of IL-4 and IL-10 at the time of RSV challenge completely prevented the development of inflammatory cell infiltration around bronchioles of FI-RSV-immunized mice, and this effect was highly statistically significant in comparisons with mice receiving control antibody or with all remaining groups. A similar effect on perivascular histopathology in the lungs of these mice was seen in comparison with animals receiving control antibody. Depletion of IL-4 alone at the time of RSV challenge of FI-RSV-immunized mice prevented the development of peribronchial, though not perivascular, histopathology. Depletion of IL-10 alone, however, did not have an effect. Depletion of IFN-γ, IL-2, or both together at the time of RSV challenge did not alter the pulmonary histopathology of FI-RSV-immunized mice.

The results of the present study provide the first direct evidence for the roles of IL-4 and IL-10 in the pathogenesis of the pulmonary inflammatory response that occurs following RSV challenge of mice previously immunized with FI-RSV. Depletion of IL-4 and IL-10 at the time of RSV challenge of FI-RSV-immunized mice completely abrogated the enhanced pulmonary histopathology observed in nondepleted mice. De-

pletion of IL-4 alone had a smaller, though significant, effect on peribronchial histopathology. However, depletion of IL-10 alone did not diminish the level of pulmonary histopathology. These data indicate that the inflammatory response can proceed in the absence of IL-10. However, IL-10 depletion appeared to enhance the effect of depletion of IL-4 alone. It has been reported that IL-4 or IL-10 can act independently to inhibit IFN-γ production but that they synergize to optimally inhibit delayed-type hypersensitivity responses (23). It appears likely from results of the present study that IL-4 and IL-10 synergize in the pulmonary inflammatory response observed following RSV challenge of mice previously immunized with FI-RSV.

Our results complement previous findings of elevated levels of IL-4 mRNA in the lungs following RSV challenge of mice previously immunized with FI-RSV compared with mice previously infected with RSV (10). Importantly, they also extend these findings to demonstrate that IL-4 and IL-10 act together to mediate the immunopathology and that this response is completely abrogated when both cytokines are depleted. Results from the present study also support previous observations in mice that antibodies alone do not mediate pulmonary histopathology associated with prior FI-RSV immunization (6, 7). FI-RSV-immunized or RSV-infected mice had comparable levels of ELISA-F antibodies prior to challenge. Histopathology was completely abrogated in animals previously immunized with FI-RSV and depleted of both IL-4 and IL-10, although these animals had high levels of RSV-specific antibodies at the time of challenge. Thus, RSV-specific antibodies, in the absence of IL-4 and IL-10, are not sufficient to mediate pulmonary histopathology in the mouse.

The association of a predominant Th1 response with protection against microbiologic diseases has been documented in murine models for *Leishmania major*, *Schistosoma mansoni*, and *Candida albicans* (22, 29, 30) among others. In mice, a predominant Th2 response to immunization for these pathogens is associated not with protection but with disease progression. Immunopathology has also been associated with a Th2 response in the skin of atopic humans and airway inflammation in patients with allergic bronchial asthma (12, 26). Recently, it was shown that the majority of T-cell clones derived from bronchial biopsy of patients with allergic asthma can produce IL-4 and IL-5, though not IFN-γ (26). It has been postulated that Th2 cells, through local secretion of IL-4 and IL-5 that induces recruitment of inflammatory cells, mainly eosinophils, may orchestrate allergic airway inflammation (26, 28). It is important to note that increased numbers of eosinophils were noted in the lungs of two patients who died of severe pulmonary disease following immunization with FI-RSV in the vaccine trials in the 1960s (13). Furthermore, increased numbers of eosinophils were noted in the blood of FI-RSV vaccinees compared with controls during subsequent RSV infection (5). These observations of heightened eosinophilia in the lungs or blood of FI-RSV vaccinees during subsequent RSV infection are compatible with the suggestion that the human vaccinees, like their murine counterparts, had a predominant Th2 response to infection.

On the basis of the results of the present study and previous findings in rodents and humans, we offer a hypothesis for the enhanced disease that occurs in FI-RSV-immunized humans following natural infection with RSV that extends those previously suggested (3, 4, 7). Two major factors likely contributed to enhanced disease in FI-RSV vaccinees. First, FI-RSV failed to induce a high level of resistance to RSV replication. FI-RSV vaccinees developed high titers of serum antibodies to the F glycoprotein, but these antibodies had low levels of neutralizing activity (19). In a previous study, passive transfer of such antibodies failed to protect cotton rats from RSV infection, indicating that these antibodies had insufficient antiviral activity (6). In addition, it is unlikely that appreciable secretory IgA was induced in the respiratory tract, because the FI-RSV vaccine was given parenterally. It is also unlikely that a $CD8^+$ cytotoxic T-cell response developed in FI-RSV vaccinees, suggesting that this potent antiviral component of the cellular immune system was deficient in FI-RSV vaccinees (7, 21). Further, $CD4^+$ T cells were also unable to prevent the replication of RSV in the lungs of the vaccinees. Thus, with both local and systemic humoral immunity and protective cellular immunity to RSV largely deficient in FI-RSV vaccinees, pulmonary RSV replication was not restricted. RSV replication then provided the stimulus for other immune mediators to cause enhanced disease.

The second factor that contributed to enhanced disease in humans was a state of heightened as well as altered $CD4^+$ T-cell activity induced by FI-RSV. The heightened nature of the $CD4^+$ T-cell responses was suggested previously by the augmented proliferative response of peripheral blood lymphocytes of FI-RSV vaccinees (14), as well as studies in mice demonstrating that the pulmonary infiltration that occurs following RSV challenge of FI-RSV-immunized mice was completely dependent on $CD4^+$ T cells (7). The altered nature of the $CD4^+$ T-cell response can be inferred from the higher levels of IL-4 mRNA transcripts present in the lungs following RSV challenge of mice previously immunized with FI-RSV compared with mice previously infected with RSV (10), as well as by the present study, which demonstrates that this immunopathology is mediated by IL-4 and IL-10. We suggest that in humans, FI-RSV immunization primed for a biased $CD4^+$ T-cell response which occurred at the sites of RSV replication in the bronchioles and alveoli, resulting in local elaboration of the Th2 cytokines IL-4, IL-5, and IL-10, an increased number of infiltrating lymphocytes and eosinophils, and further local release of cytokines and other mediators of inflammation and bronchoconstriction. The more severe bronchiolitis and pneumonia illnesses were a reflection of augmented Th2-dependent infiltration of inflammatory cells into the bronchioles and alveoli occurring in pulmonary structures already damaged by replication of this cytopathic virus. It is reasonable to suggest from the existing data that the enhanced disease observed during RSV infection of FI-RSV vaccinees was a Th2-mediated pulmonary immunopathological reaction.

Results of the present study have important implications for RSV vaccine development and for development of vaccines for other viruses. Inactivated RSV vaccines, including FI-RSV, purified F glycoprotein, and a chimeric FG glycoprotein have each been shown to induce enhanced pulmonary histopathology in rodents (6, 20, 24, 31). Inactivated antigens in other viral systems have also been shown to induce altered cellular immune responses (16). Although some subunit or inactivated vaccines for viruses such as influenza A and B and hepatitis B are safe and efficacious, it is possible that vaccines being developed for other viruses may induce altered cellular immune responses, possibly resulting in enhanced disease. These results also suggest that therapeutic interventions that inhibit IL-4 and IL-10 or that enhance Th1 responses could be useful for the prevention or treatment of some specific Th2-mediated pulmonary immunopathologic processes. In addition, it is reasonable to suggest that either nonprotective or deleterious immune responses to vaccines for some diseases might be modulated at the time of immunization by interventions to minimize Th2 responses, such as depletion of IL-4 and IL-10, or to enhance Th1 responses by administration of IL-12 (11,

15). These interventions could result in protective immune responses without inducing disease enhancement.

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