T Cell Source of Type 1 Cytokines Determines Illness Patterns in Respiratory Syncytial Virus–infected Mice

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Abstract

Manipulation of the cytokine microenvironment at the time of vaccination can influence immune responses to remote challenge, providing a strategy to study the molecular pathogenesis of respiratory syncytial virus (RSV) vaccine-enhanced disease in the mouse model. Although treatment with antibody against IL-4 or recombinant IL-12 (rIL-12) at the time of formalin-inactivated RSV vaccination induced a similar shift in the pattern of cytokine mRNA expression upon live virus challenge, anti–IL-4 treated mice had increased $CD8^+$ cytotoxic T lymphocyte activity and reduced illness compared with rIL-12–treated mice. To define effector mechanisms responsible for these patterns, $CD4^+$ and/or $CD8^+$ T lymphocytes were selectively depleted in vivo at the time of RSV challenge. In rIL-12–treated mice, $CD4^+$ lymphocytes made the largest contribution to IFN-γ mRNA, RSV clearance, and illness, while in anti–IL-4 treated mice, $CD8^+$ lymphocytes were the major effector. The effector responsible for virus clearance also mediated illness, suggesting that efficiency of virus clearance determined disease expression. These results demonstrate that the phenotype of effector cells involved in the immune response to virus challenge may be a more important determinant of disease than patterns of cytokine expression classically assigned to Th1 and Th2 lymphocytes. (*J. Clin. Invest.* 1997. 99:2183–2191.)
Key words: vaccination • cytotoxic T lymphocytes • IL-4 • IFN-γ • IL-12

Introduction

Respiratory syncytial virus (RSV)[1] is the major respiratory pathogen of infants and young children, causing an estimated 91,000 hospitalizations and 4,500 deaths annually in the United States alone (1). Only partial immunity is achieved despite repeated infection. Clinical trials of a formalin-inactivated alum-precipitated (FI) RSV vaccine (FI-RSV) in the 1960s showed that the vaccine elicited complement-binding antibodies but failed to protect against infection, and that the severity of subsequent disease was frequently enhanced (2–5). Live, attenuated RSV vaccines did not result in enhanced pulmonary disease upon natural infection, but were in other respects equally unsuccessful (6–8). On the basis of these and other observations, it has been hypothesized that the immune response induced by RSV may contribute to the development of disease (1, 9).

The regulation of immune responses induced by pathogens is better understood since the identification of distinct $CD4^+$ Th lymphocyte subsets in mice (10). These cell types are categorized mainly by the constellation of cytokines they produce. The signature cytokine of Th1 cells is IFN-γ. Th1-associated cytokines promote isotype switching from IgM to IgG2a, and are primarily associated with cell-mediated immunity. In contrast, the signature cytokine of Th2 cells is IL-4. Th2-associated cytokines promote isotype switching from IgM to IgG1, IgE, or IgA, and are mainly involved in humoral immunity (11). In addition to Th cells, the cytokine environment is produced and influenced by other cell types. $CD8^+$ cytotoxic T lymphocytes (12, 13) can be divided into functionally distinct subsets that secrete different patterns of cytokines and play unique regulatory roles. Moreover, differences in the activation of γ/δ T lymphocytes (14), NK cells (15), and antigen-presenting cells such as macrophages (16) and B lymphocytes (17, 18) may influence the cytokine milieu and thereby disease outcomes.

For most infectious diseases caused by intracellular pathogens, a dominant type 2 cytokine response is associated with disease progression, while a dominant type 1 cytokine production is protective (11, 19–26). In the murine model of RSV, it has been shown previously that the vaccine formulation used for priming can induce distinct patterns of cytokine mRNA expression in mice challenged with live RSV (27). Immunization with FI-RSV primes mice for an increase in IL-4 mRNA expression relative to IFN-γ mRNA expression upon RSV challenge. In contrast, priming with live RSV leads to a relative decrease in IL-4 mRNA expression compared with IFN-γ mRNA levels.

While the cytokine expression pattern is important, other properties of the effector cells producing the cytokines must also be considered. Previous study has shown that interfering with IL-4 activity at the time of RSV immunization by neutralizing monoclonal antibody induces a type 2 to type 1 shift in pattern of cytokine expression after RSV challenge. This was accompanied by an improved clinical outcome and increased $CD8^+$ CTL activity (28). Using recombinant IL-12 (rIL-12) treatment at the time of immunization also shifts the cytokine response pattern from type 2 to 1 at the time of subsequent

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1. *Abbreviations used in this paper:* FI, formalin-inactivated alum-precipitated; LCMV, lymphocytic choriomeningitis virus; pfu, plaque forming units; rIL-12, recombinant IL-12; RSV, respiratory syncytial virus.

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live virus challenge, and results in diminished RSV replication. However, rIL-12 did not diminish illness or augment $CD8^+$ CTL responses when given in concert with FI-RSV (29). The complexity of the immune response to infectious pathogens and importance of viewing cytokine-associated events with a broader view than just the Th1/Th2 paradigm was also demonstrated in a report examining the pathogenesis of leishmaniasis in IL-4–knockout mice (30). To determine whether the phenotype of the cytokine-producing cell population impacts the outcome of RSV-induced disease, $CD4^+$ and/or $CD8^+$ lymphocytes were selectively depleted in vivo before RSV challenge of mice previously immunized with FI-RSV. While treatment with anti–IL-4 and rIL-12 at the time of FI-RSV immunization caused similar shifts in the cytokine profiles, rIL-12 promoted a type 1 $CD4^+$ response, and anti–IL-4 promoted a type 1 $CD8^+$ lymphocyte response.

Methods

Mice, RSV immunogen, and virus. Pathogen-free female BALB/c mice, 8–10 wk old, were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and cared for according to the Guide for the Care and Use of Laboratory Animals as previously described (31). Preparations of FI-RSV and preparation of stock of RSV have been previously reported (27). Both the vaccine preparation and the challenge stock were derived from the A2 strain.

Cytokine and antibodies. Murine rIL-12 was expressed from cloned cDNAs (32). The lot used in this study was MRB021693-1.2 (Genetics Institute, Cambridge, MA) with a specific activity of 5.6×10^6 U/mg as determined by phytohemagglutinin blast assay (33). 11B.11, a monoclonal antibody against murine IL-4 (34), was kindly provided by the Biological Response Modifiers Program, National Cancer Institute (Frederick, MD). Monoclonal antibodies GK1.5 (35), 2.43 (36), and HB151 (37) were prepared as ascitic fluid of hybridoma-inoculated, pristane-primed BALB/c nu/nu mice. GK1.5 and 2.43 make monoclonal antibodies against murine L3T4 (CD4), and Lyt2 (CD8), respectively. Hybridoma HB151 makes a monoclonal antibody against human HLA-Dr5, and was used as an irrelevant antibody control. Total protein and albumin concentrations were quantitated using a Multistat III Microcentrifugal Analyzer (Instrumentation Laboratory Inc., Lexington, MA). Protein electrophoresis was performed with a Titan Gel high-resolution REP SP-30 kit and the gamma globulin fraction was determined by densitometry with an Electrophoresis Data Center (Helena Laboratories, Beaumont, TX). Ascites fluid was diluted in PBS to 1 μg immunoglobulin/μl before injection.

Plaque assays and neutralization tests. Dilutions of the clarified supernatant were inoculated on 2-d-old HEp-2 monolayers, 80% confluent in 12-well plates (Costar Corp., Cambridge, MA) under 0.75% methyl cellulose in 10% EMEM (31). After incubation for 4 d at 37°C, the monolayers were fixed, stained with hematoxylin-eosin, and plaques were counted under a dissecting microscope. Plaque-reduction neutralization assays were performed by mixing dilutions of heat-inactivated serum starting at 1:40 with equal volumes of titered virus stock for 1 h at room temperature with the complement. The serum dilution producing 60% plaque reduction was calculated by linear regression and considered the neutralization titer (31).

RSV-specific immunoglobulin isotype ELISA. BCH4, a persistently RSV-infected BALB/c fibroblast cell line, as well as BC, its uninfected parent cell line, were bound to the solid phase on Immulon II 96-well plates (Nunc, Inc., Roskilde, Denmark). Serially diluted mouse serum samples, starting at dilutions of 1:80, were added to each well. Plates were incubated, washed, and goat anti-murine IgG1 or IgG2a conjugated to alkaline phosphatase (PharMingen, San Diego, CA) diluted 1:1,000 was added. After another incubation, plates were washed, substrate was added for 30 min at room temperature, and OD_{405} was determined (28). A serum dilution was considered positive if the mean optical density of two BCH4 cell wells was greater than twice that of BC-coated wells and > 0.1.

Cytotoxic T lymphocyte assay. Whole lung lymphocytes were isolated by Ficoll-Hypaque (1.09 specific gravity) cushion centrifugation. BCH4 and BC target cells labeled with ^{51}Cr (DuPont- New England Nuclear, Boston, MA) were incubated with effector cells for 4 h at 37°C in 96-well microtiter plates as described (28). Spontaneous and total releases were obtained by treating the target cells with 10% RPMI and 5% Triton X-100 detergent, respectively. Each point is the mean from three replicate wells using lymphocytes pooled from six mice in each group. The specific release of ^{51}Cr from target cells is defined as $100 \times (\textit{sample cpm} - \textit{background cpm})/(\textit{total cpm} - \textit{background cpm})$.

mRNA extraction and Northern blotting. The total RNA from whole lungs was extracted, and polyA RNA was isolated, electrophoretically separated, and transferred to membranes as previously described (27). Hybridization with ^{32}P oligonucleotide probes was performed as previously described (27). After washing, membranes were exposed to Kodak X-omat film at −70°C (Eastman Kodak Co., Rochester, NY). Laser densitometry was performed with an LKB UltroScan XL using GelScan XL software (Pharmacia Fine Chemicals, Piscataway, NJ). Oligonucleotide probes for murine IL-4, IL-10, IFN-γ, TNF-α, and β-actin were purchased from R & D Systems, Inc. (Minneapolis, MN) or Clontech Laboratories Inc. (Palo Alto, CA). A cocktail of oligonucleotides designed for IL-12 p40 and p35 components was previously described (29).

In vivo T cell subset depletion. $CD4^+$, $CD8^+$, and both, or no lymphocyte subsets were depleted in mice by treatment with GK1.5, 2.43, both, or the irrelevant control HB151. Each mouse was injected with 100 μg of the appropriate monoclonal antibody intraperitoneally on three successive days, starting 2 d before the live virus challenge. Thereafter, maintenance injections of 250 μg of monoclonal antibody were given weekly (37). Efficacy of these treatments was determined by FACS® analysis of heparinized whole peripheral blood using FITC or phycoerythrin-conjugated rat anti–mouse antibody to CD8 or CD4, respectively (Becton Dickinson, Mountain View, CA). Each antibody conjugate was incubated with 200 μl of whole blood for 30 min at room temperature. The red blood cells were lysed with a buffered ammonium chloride solution and the remaining lymphocytes were washed twice and then resuspended in PBS containing 5% FBS before analysis on an Epic 753 FACS® (Coulter Corp., Miami, FL).

Experimental design. Each mouse was immunized with FI-RSV containing 2.2×10^6 plaque forming unit (pfu) equivalents of virus antigen intramuscularly, and challenged with 10^7 pfu of live RSV intranasally 4 wk later as previously described (27, 28). For anti–IL-4 treatment, each mouse was given 200 μg 11B.11 monoclonal antibody intraperitoneally for three successive days, starting at 1 d before immunization. For rIL-12 treatment, rIL-12 was administered 1 μg/mouse intramuscularly in the same syringe with the RSV immunogen. The lymphocyte subset in vivo depletion at the time of challenge was executed as described above. Mouse serum samples were collected on the day of live RSV challenge and again 2 wk later. Cytokine mRNA measurements and virus plaque assays were performed on lungs harvested on day 4 after challenge. Lymphocytes isolated from lung tissues harvested 6 d after challenge were assayed for CTL activity. Lung histologic samples 6 d after challenge were prepared. Illness assessments, including weight loss and clinical scores, were performed as previously described (28, 29). Data were derived from six individual mice from each group at each time point. All treatments, sample processing time points, and immunologic assays were performed on the same day for all experimental groups, and the entire study was performed as a single experiment.

Statistics. Plaque assay results, weights, antibody titers, and densitometry measurements between groups were evaluated by one-way analysis of variance. Significant differences between data from specific groups were defined as those with a P value ≤ 0.05. The relationship of rIL-12 dose to subsequent cytokine responses in lung was ana-

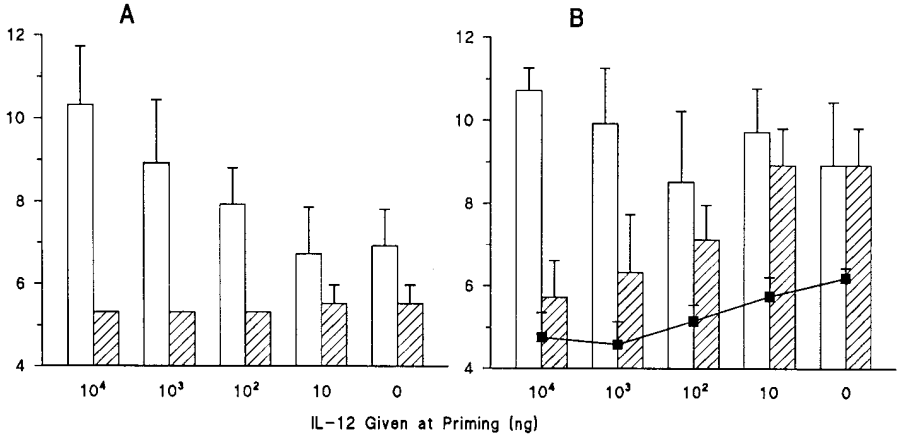

Figure 1. Dose-effect relationship between rIL-12 and RSV-specific immunoglobulin isotype antibody response and viral titer reduction. Mouse serum samples were collected 1 d before (*A*, eight per group) and 2 wk after (*B*, five to six per group) challenge. The virus replication levels in lungs 4 d after challenge (six mice per group) are measured as mean $\log_{10}$pfu/g±SD. Antibody titers are expressed as mean $\log_2$ reciprocal serum dilution±SD. Values < 1:80 were assigned the value 1:40 for statistical calculations. Log_2 serum IgG2a, □; $\log_2$ serum IgG1, ▨; $\log_{10}$ pfu/g lung, ■.

lyzed by linear regression modeling the cytokine to the dose of rIL-12, and asking whether the slope was significantly different than zero. The analysis was done using SAS software (SAS Institute, Inc., Cary, NC).

Results

Dose-response effect of rIL-12. Previously we have evaluated the adjuvant effects of rIL-12 on induction of immune responses against RSV infection in mice (29). Despite reduction in RSV replication and shift toward a type 1 cytokine expression pattern, rIL-12 treatment did not increase CTL activity or decrease illness after challenge. In this study, we evaluated the dose-response effect of rIL-12 on RSV-induced immune responses. Giving 10 ng–10 μg rIL-12 treatment at the time of immunization induced a dose-related reduction in RSV replication, an increase in IgG2a, and a decrease in IgG1 isotype antibody titers (Fig. 1). There was also a dose-related shift in the cytokine mRNA expression pattern. IL-4 mRNA in lung after RSV challenge was inversely correlated with the dose of rIL-12 given at the time of immunization ($P = 0.04$) (Fig. 2). However, rIL-12 treatment did not improve illness outcome (measured by weight loss and illness score) or increase $CD8^+$ CTL activity after challenge regardless of dose (data not shown). These data combined with previous results showed that a simple shift in the pattern of cytokine expression does not determine illness outcome, suggesting that the cell populations responsible for cytokine production, not the cytokines themselves, may be key determinants of illness.

Illness pattern. We first sought to determine which T cell populations were responsible for cytokine production and illness outcome. The selective depletion of $CD4^+$ and/or $CD8^+$ T lymphocytes was performed at the time of challenge. Peripheral blood FACS® analysis verified that 98% of $CD4^+$ and 100% of $CD8^+$ T cells were depleted when mice were injected with GK1.5 and 2.43 monoclonal antibodies, respectively (data not shown). Control mice primed only with killed virus antigen exhibited a typical 20% weight loss after live RSV challenge,

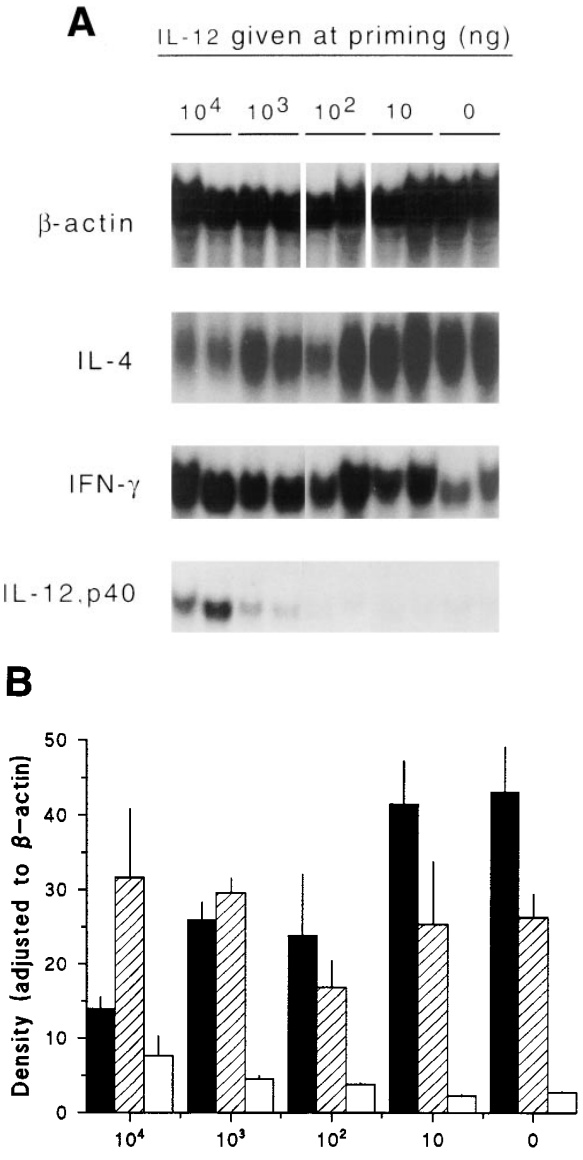

Figure 2. Dose-effect relationship between rIL-12 and cytokine mRNA expression. Mice were immunized with FI-RSV with rIL-12 ranging from 0 to 10 μg and 4 wk later were challenged with live virus. Each cytokine mRNA was detected from whole lungs dissected 4 d after challenge (two lungs per sample, two samples per group). Cytokine mRNA Northern blot (*A*) shows two representative lanes from each group. Below, the corresponding densitometry data are shown as the mean and standard deviation of three samples per group. IL-4, ■; IFN-γ, ▨; IL-12, p40, □.

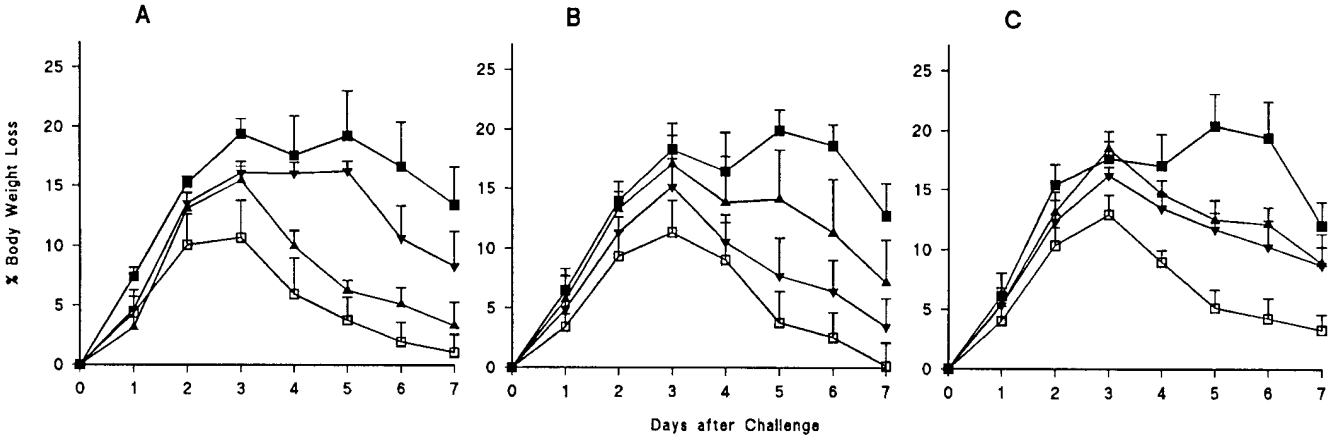

Figure 3. T lymphocyte subsets responsible for weight loss after RSV challenge. Mice (six per group) were immunized with FI-RSV plus rIL-12 (*A*), anti–IL-4 (*B*), or nothing (*C*), and 4 wk later were challenged with live virus. They were treated with GK 1.5 (anti–murine CD4), 2.43 (anti-murine CD8), both, or isotype control at the time of challenge. Mean percentages of body weight loss±SD are shown. No depletion, ■; CD^4 depletion, ▲; CD^8 depletion, ▼; both depleted, ⊟.

while depletion of both $CD4^+$ and $CD8^+$ lymphocytes resulted in almost no weight loss. Mice depleted of either $CD4^+$ or $CD8^+$ lymphocytes had intermediate weight loss after challenge (Fig. 3 *C*).

In the rIL-12–treated group, depletion of $CD4^+$ lymphocytes significantly reduced the amount of weight loss compared with the nontreated group ($P = 0.01$ at day 5). In contrast, depletion of $CD8^+$ lymphocytes had significantly less effect on weight in the rIL-12–treated group than in the nontreated group ($P = 0.03$ at day 5; Fig. 3 *A*). In the anti–IL-4 treated group, weight loss was reduced more by $CD8^+$ lymphocyte depletion than by $CD4^+$ depletion, similar to the nontreated group (Fig. 3 *B*). The illness scores assigned by a blinded observer showed the same patterns as the weight loss curves (data not shown). These data suggest that the immune responses responsible for illness in mice treated with rIL-12 and anti–IL-4 at the time of immunization are dominated by a different composition of effector populations. $CD4^+$ lymphocytes are the major effectors in rIL-12–treated mice, while $CD8^+$ lymphocytes play the major role in anti–IL-4–treated mice.

RSV replication in lung. Virus replication in lungs was evaluated at day 4 after challenge. In untreated mice primed only with the FI-RSV immunogen, the virus titer was 4.41±0.28 $\log_{10}$pfu/g at day 4 after live RSV challenge. When both $CD4^+$ and $CD8^+$ lymphocyte subsets were depleted, the viral titer was 5.36±0.41 $\log_{10}$pfu/g. Depletion of the $CD4^+$ lymphocyte subset alone had a greater effect on virus clearance than $CD8^+$ depletion suggesting that most virus clearance was mediated by $CD4^+$ lymphocyte effector mechanisms (Fig. 4). As previously shown, rIL-12 treatment at the time of FI-RSV priming resulted in a significant reduction in virus titer ($P < 0.0001$ comparing nondepleted groups). Mice treated with rIL-12 during FI-RSV priming in which both $CD4^+$ and $CD8^+$ lymphocytes were depleted before challenge had a virus titer of 3.99±0.42 $\log_{10}$pfu/g that was significantly lower than in double-depleted mice primed with FI-RSV antigen only (0.0004), indicating that other effector mechanisms than $CD4^+$ and $CD8^+$ T lymphocytes were involved in virus clearance. Depletion of $CD4^+$ lymphocytes alone eliminated the reduction of virus titer in this group, while depletion of $CD8^+$ lymphocytes had no effect on virus titer reduction. This suggests that $CD4^+$ lymphocytes are major effectors responsible for viral clearance in FI-RSV–primed mice treated with rIL-12. In contrast, anti–IL-4-treated FI-RSV–primed mice showed the opposite pattern. Mice depleted of $CD4^+$ lymphocytes had reduction in virus titers similar to the undepleted control mice, while mice depleted of $CD8^+$ lymphocytes were no longer able to effectively reduce virus titers. This suggests that $CD8^+$ lymphocytes are major effectors for virus clearance in anti–IL-4-treated, FI-RSV–primed mice. These data show that the reduction in virus titer seen in mice treated with rIL-12 versus anti–IL-4 at the

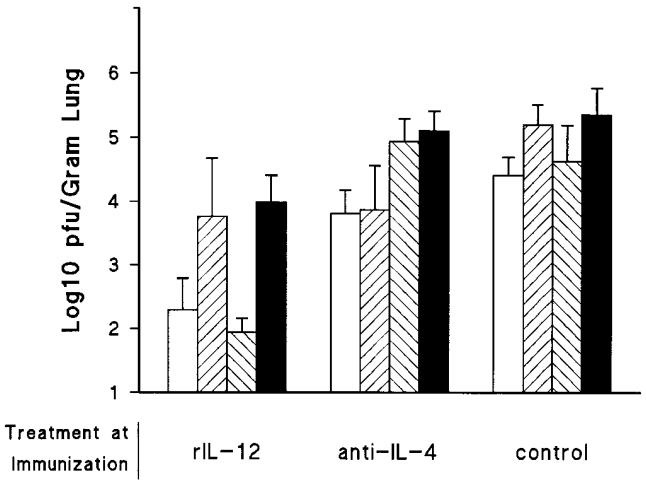

Figure 4. T lymphocyte subsets responsible for the viral titers in lungs after RSV challenge. Mice (six per group) were immunized with FI-RSV plus rIL-12, anti–IL-4, or isotype control, and 4 wk later were challenged with live virus. They were treated with GK 1.5, 2.43, both, or isotype control at the time of challenge. Mean $\log_{10}$ pfu/g lung±SD are shown. No depletion, □; CD^4 depletion, ▨; CD^8 depletion, ▧; both depleted, ■.

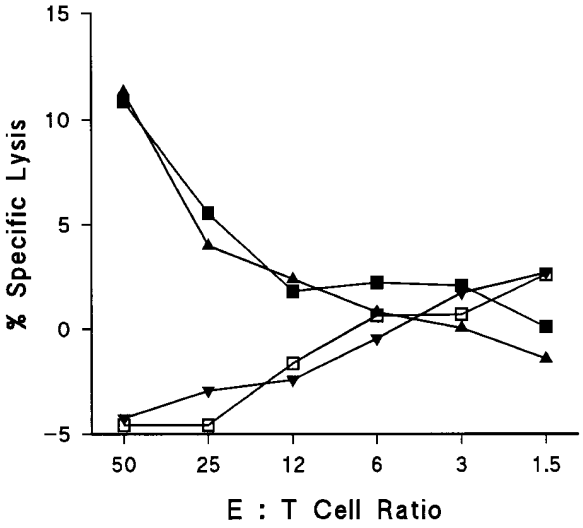

Figure 5. Phenotype of RSV-specific cytotoxic T cell activity in anti–IL-4–treated mice. Lymphocytes from lungs of six mice in each group on day 6 after challenge were pooled. ^{51}Cr-labeled BCH4 target cells were used in a direct measure of RSV-specific cytotoxic activity that did not include in vitro stimulation of effectors. Each point represents the mean from three replicate wells. No depletion, ■; CD^4 depletion, ▲; CD^8 depletion, ▼; both depleted, ⊞.

time of FI-RSV immunization is mediated by different effector populations.

CTL activity. It was demonstrated in the previous study that interference with IL-4 activity at the time of immunization by neutralizing monoclonal antibody induces a type 2 to type 1 immune response shift, accompanied by an improved clinical outcome and increased $CD8^+$ CTL activity (28). Here, the primary lung CTL activity at day 6 after challenge was measured in mice that had different cytokine manipulations at the time of immunization. CTL activity was not detectable after challenge in mice primed with FI-RSV intramuscularly alone or when treated with rIL-12 (data not shown). In anti–IL-4–treated mice, increased CTL activity was detected, and was abrogated by selective $CD8^+$ lymphocyte depletion in vivo. Depletion of $CD4^+$ lymphocytes alone had no effect on CTL activity (Fig. 5). These data were consistent with in vitro depletion results, suggesting that anti–IL-4, but not rIL-12, treatment at the time of immunization resulted in augmented CTL activity mediated by $CD8^+$ effectors after RSV challenge.

Cytokine mRNA expression pattern. Next we asked how $CD4^+$ and $CD8^+$ lymphocyte subpopulations contribute to cytokine mRNA production after live virus challenge in mice treated with anti–IL-4 or rIL-12 at the time of FI-RSV immunization. $CD4^+$ lymphocyte depletion at the time of live virus challenge virtually eliminated IL-4 and IL-10 mRNA production regardless of treatment, suggesting that both IL-4 and IL-10 were produced predominantly by $CD4^+$ T lymphocytes (Fig. 6). TNF-α mRNA increased in mice treated with either anti–IL-4 or rIL-12 at the time of immunization (Fig. 6). In rIL-12–treated mice, combined $CD4^+$ and $CD8^+$ depletion reduced, but did not eradicate, TNF-α expression, suggesting that TNF-α was produced by CD4−/CD8− cells in this setting.

IFN-γ mRNA production levels varied in mice depleted of different T lymphocyte subsets. In mice primed only with FI-RSV antigen, both $CD4^+$ and $CD8^+$ lymphocytes contributed to IFN-γ mRNA expression. In rIL-12–treated mice, $CD4^+$ depletion eliminated 59.5% of IFN-γ mRNA production, while $CD8^+$ depletion eliminated only 24.5% (Fig. 6; Table I). Both $CD4^+$ and $CD8^+$ depletion failed to eradicate IFN-γ mRNA expression completely, suggesting that other effector cells contribute to IFN-γ mRNA synthesis in rIL-12–treated mice. In contrast, while $CD4^+$ depletion in anti–IL-4 treated mice eliminated only 19.9% of IFN-γ mRNA secretion, $CD8^+$ depletion eliminated 75.2% of IFN-γ mRNA expression, suggesting that IFN-γ in anti–IL-4–treated mice was produced mainly by $CD8^+$ cells (Fig. 6; Table I). Taken together, these

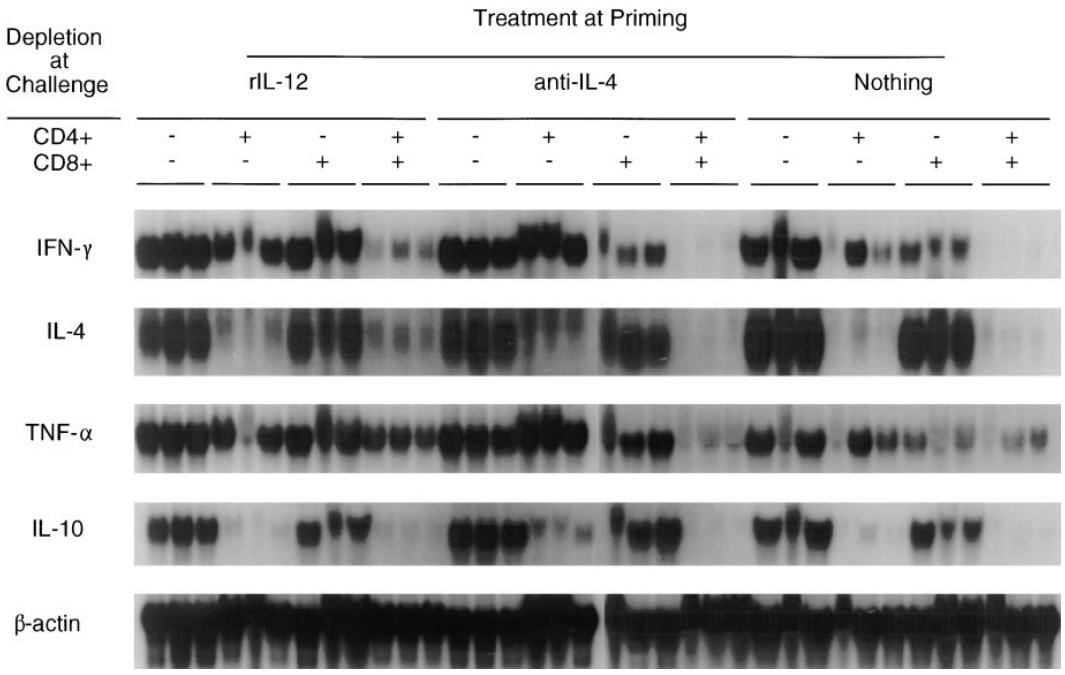

Figure 6. T lymphocyte subset depletion alters cytokine mRNA expression pattern in lungs after RSV challenge. Mice were immunized with FI-RSV plus rIL-12, anti–IL-4, or isotype control, and 4 wk later were challenged with live virus. They were in vivo depleted of $CD4^+$ or/and $CD8^+$ cell subsets at the time of challenge. Cytokine mRNA Northern blot (two lungs per sample, three samples per group) is shown.

Table I. Effect of In Vivo T Lymphocyte Subset Depletion on Reduction of Whole Lung IFN-γ mRNA in Mice Primed with FI-RSV and Challenged with RSV

T subset depletion at challenge	Treatment at immunization		
	Control	Anti–IL-4	rIL-12
$CD4^+$	70.2*	19.9	59.5
$CD8^+$	74.2	75.2	24.5
$CD4^+$ and $CD8^+$	99.1	98.6	88.2

*Mean percent reduction compared with nondepleted group.

data suggest that the responding cytokine patterns after challenge were due to phenotypically different lymphocyte effectors. In the case of anti–IL-4–treated mice, it appears that IFN-γ (or type 1 cytokine pattern) is primarily a product of $CD8^+$ T cells, whereas in rIL-12–treated mice, $CD4^+$ T cells are the dominant source.

RSV-specific immunoglobulin isotype and neutralization antibody responses. There were no detectable neutralizing antibody titers in serum samples collected on the day of challenge which is expected after a single immunization with FI-RSV intramuscularly (Table II). Mice primed with FI-RSV immunogen alone produced relatively high IgG1 and low IgG2a antibody titers (35, 36; Table II). Treatment with rIL-12 at the time of immunization results in greater induction of IgG2a isotype and less production of IgG1. Treatment with anti–IL-4 augments the prechallenge IgG2a response, but has little effect on IgG1 induction. In nondepleted mice after challenge there was a neutralizing antibody response in all groups of similar magnitude. $CD4^+$ cell depletion before challenge prevented the appearance of neutralizing antibody and boosting of the isotype response in all groups, while $CD8^+$ cell depletion had no effect on antibody production (Table II). These results suggest that, regardless of the different effector populations being activated in the context of cytokine manipulation, $CD4^+$ T lymphocytes still play a significant role in inducing humoral immunity to RSV.

Discussion

In vivo T lymphocyte subset depletions were performed to define the subpopulations responsible for cytokine production, virus clearance, and illness in RSV-infected mice. Treatment with rIL-12 or anti–IL-4 at the time of killed RSV immunization resulted in a similar shift toward a type 1 pattern of cytokine expression. However, anti–IL-4 treatment resulted in improved illness outcome, and increased cytotoxic T cell activity in lung after challenge; rIL-12 treatment did not induce these effects (28, 29). We have shown that in anti–IL-4 treated mice, $CD8^+$ lymphocytes were the dominant effector cell, while in rIL-12–treated mice $CD4^+$ lymphocytes were dominant. In addition to their roles in cytokine production, the key effector cells in each treatment group had the greatest impact on both viral clearance and illness expression. Thus, the price for viral clearance is immunopathology potentially leading to illness. Optimizing the efficiency of the effector population mediating viral clearance should minimize RSV-induced disease, and should be a primary goal of vaccine development.

The vaccine-enhanced illness caused by formalin-inactivated RSV vaccines in the 1960s is a major impediment to advancing new RSV candidate vaccines to clinical trials, and was associated with altered lung pathology (2–5) and an enhanced lymphoproliferative response in vitro (38). Enhanced pathology has also been induced after live RSV challenge in mice immunized with FI-RSV vaccine (27, 39, 40). The pathology is dependent on the $CD4^+$ T lymphocyte population (37, 40), and appears to be related to selective activation of lymphocyte subpopulations that have different cytokine secretion profiles (27, 41). Upon RSV challenge, mice previously immunized intramuscularly with inactivated virus produced a dominant type 2 pattern of cytokine expression. In contrast, challenge of mice immunized with live RSV by either mucosal or parenteral routes produced a type 1 pattern (27). These findings have suggested a working model for the pathogenesis of RSV vaccine-enhanced illness: RSV immunization can selectively activate subpopulations of lymphocytes that, upon rechallenge, result in different levels of illness expression based on cytokine secretion patterns.

*Table II. Effect of In Vivo T Lymphocyte Depletion on RSV-specific Immunoglobulin Isotype and Neutralizing Antibody Response in Serum**

			2 wk after challenge			
	Immunogen/treatment	Day of challenge	No depletion	CD4 depletion	CD8 depletion	Both depleted
IgG1	FI-RSV	7.32±2.51 (4/8)	9.12±2.17 (4/5)	7.82±1.97 (4/6)	9.32±2.24 (4/5)	8.32±2.10 (5/6)
	FI-RSV + anti–IL-4	6.86±1.77 (4/8)	9.92±0.55 (5/5)	6.99±2.58 (2/6)‡	9.32±2.00 (5/6)	6.65±2.07 (2/6)‡
	FI-RSV + rIL-12	< 6.32 (0/8)	6.15±2.04 (1/6)	5.82±1.22 (1/6)	6.32±1.74 (1/5)	5.99±1.63 (1/6)
IgG2a	FI-RSV	7.57±2.05 (5/8)	10.12±0.45 (5/5)	6.65±1.03 (5/6)	11.12±0.45 (5/5)	8.32±1.67 (5/6)
	FI-RSV + anti–IL-4	9.45±1.81 (7/8)	10.92±0.89 (5/5)	8.49±1.47 (6/6)§	10.32±2.45 (5/6)	7.65±1.63 (5/6)§
	FI-RSV + rIL-12	10.07±0.74 (8/8)	10.65±0.52 (6/6)	10.65±0.82 (6/6)	10.92±0.55 (5/5)	10.92±0.52 (6/6)
Neutralizing antibody‖	FI-RSV	< 4.32 (0/8)	6.18±0.97 (5/5)	< 4.32 (0/5)	6.77±1.47 (5/5)	< 4.32 (0/5)
	FI-RSV + anti–IL-4	< 4.32 (0/8)	6.48±0.78 (5/5)	< 4.32 (0/5)	6.76±0.87 (5/5)	< 4.43 (0/5)
	FI-RSV + rIL-12	< 4.32 (0/8)	5.02±1.67 (4/5)	< 4.32 (0/5)	5.07±1.14 (5/5)	< 4.32 (0/5)

*Data are shown as $\log_2$ mean titer±SD or $\log_2$ mean titer (No. responders/No. tested). Negative samples were assigned $\log_2$ 40 for immunoglobulin isotype and $\log_2$ 10 for neutralizing antibody in statistical calculations. ‡$P < 0.05$ vs. no depletion group in IgG1 treated with anti–IL-4. §$P < 0.05$ vs. no depletion group in IgG2a treated with anti–IL-4. ‖$P < 0.05$ among all nondepleted and $CD8^+$ depletion groups in neutralizing antibody titers by one-way variance analysis.

The potential importance of the composition of immune responses to RSV has made the choice of adjuvant a critical factor in the ultimate safety and efficacy of candidate RSV vaccines. The nature of the primary immune response to vaccine can determine the composition of the response to subsequent challenge by the pathogen. Manipulation of the cytokine milieu at the time of vaccination has the potential to increase vaccine immunogenicity and safety (42). For intracellular pathogens, including RSV, one goal of vaccination should be to induce a type 1 cytokine environment. Certain type 1 cytokines, such as IFN-γ, have been given in conjunction with immunization to help enhance immunogenicity and promote cell-mediated responses (43). IL-12, a macrophage-derived heterodimeric cytokine (44, 45), has been shown to have potential as a vaccine adjuvant. Vaccination of BALB/c mice with leishmania antigens and IL-12 promotes the development of leishmania-specific $CD4^+$ Th1 cells. These mice are resistant to subsequent infection with wild-type parasites, suggesting that IL-12 may be a key component to vaccine efficacy when IFN-γ–dependent cell-mediated immunity is required (46). In schistosomiasis, vaccination with antigen plus IL-12 reduces subsequent schistosome egg–induced pathology, including granuloma formation and tissue fibrosis (47, 48). We have demonstrated that rIL-12 treatment at the time of immunization shifts a type 2 to a type 1 cytokine response pattern at the time of subsequent live virus challenge and results in diminished RSV replication (29), and others have reported that IL-12 injected at the time of vaccination increases immunity against a neurotropic herpes virus infection in mice (49).

CTLs play a major role in host defenses in most viral infections. CTLs control acute infection directly by destroying virus-producing cells and possibly by releasing cytokines with antiviral activity, such as IFN-γ and TNF-α. Passively transferred $CD8^+$ CTLs were shown to clear RSV rapidly, but enhance pulmonary pathology, when given to mice at high doses (50). It was also reported that the natural occurrence of the $CD8^+$ T cell response after intranasal RSV infection of calves correlated with virus clearance and recovery (51). RSV-specific CTLs have also been shown to develop in response to natural infection of humans, and their detection is associated with relative protection from disease (52–54). While previous studies have shown that both $CD4^+$ and $CD8^+$ T lymphocytes are involved in clearance of RSV (37), $CD8^+$ CTL clones appear to be more efficient at clearing RSV than $CD4^+$ T cell clones (39, 50).

This study has confirmed the results of previous studies (28, 29) showing that anti–IL-4 and rIL-12 treatment at the time of immunization with FI-RSV promotes the development of a type 1 cytokine environment in RSV-challenged mice. Anti–IL-4 treatment was again shown to augment $CD8^+$ CTL activity after RSV challenge. We have now shown that the type 1 cytokine environment in mice treated with anti–IL-4 during immunization is produced mainly by $CD8^+$ lymphocytes, while the type 1 cytokine response in mice treated with rIL-12 during immunization is produced mainly by $CD4^+$ lymphocytes. In the anti–IL-4 treated mice, $CD8^+$ lymphocytes are therefore playing a role as cytolytic effectors to clear virus-infected cells, and may also serve a regulatory function by contributing to the cytokine milieu and promoting Th1 $CD4^+$ lymphocyte differentiation. The concept of $CD8^+$ lymphocytes as immune regulators has also been suggested in other experimental systems (12, 13). Whether the effect of $CD8^+$ lymphocytes on illness depends only on efficient virus clearance or involves modulation of $CD4^+$ Th differentiation and reduction of IL-4–mediated disease mechanisms remains unclear.

In mice treated with rIL-12 at the time of immunization with FI-RSV, there was no increase in $CD8^+$ CTL activity or improvement in clinical outcome as there was with anti–IL-4 treatment. However, a recent study evaluating rIL-12 treatment of lymphocytic choriomeningitis virus (LCMV) infection showed that low doses of IL-12 improved immunity to LCMV infection and increased splenic $CD8^+$ T cell numbers. In contrast, high doses of IL-12 impaired resistance to LCMV infection and reduced virus-specific CTL activity (55). The postulated mechanism for IL-12–mediated immunotoxicity was that high concentrations of IL-12 induced TNF-α expression, which could be detrimental to protective $CD8^+$ T cell responses (56). Therefore, we evaluated a range of rIL-12 doses on RSV-induced immune responses. Treating with 10 ng–10 μg rIL-12 at the time of immunization produced a dose-effect on RSV replication, immunoglobulin isotype antibody responses, and cytokine mRNA expression pattern. However, rIL-12 treatment did not improve illness outcome or increase $CD8^+$ CTL activity after challenge regardless of dose (Fig. 1). In our system, cytokine manipulation, including anti–IL-4 and rIL-12 treatment, was performed at the time of immunization and TNF-α mRNA expression level was detected at the time of remote virus challenge. There was no correlation of TNF-α mRNA expression and the level of CTL activity. Therefore, reducing the dose of rIL-12 used at immunization did not promote $CD8^+$ CTL activity as it did when used as treatment in the LCMV system, and $CD8^+$ CTL activity was not correlated with TNF-α expression in RSV-infected mice.

In rIL-12–treated mice, virus titers in lungs after challenge were significantly reduced despite depletion of both $CD4^+$ and $CD8^+$ T lymphocytes ($P = 0.0004$). rIL-12–treated mice depleted of both $CD4^+$ and $CD8^+$ T lymphocytes also retained some degree of mRNA expression of IFN-γ and TNF-α (Fig. 6). While the reduced virus titers may be related to the slightly higher prechallenge IgG2a antibody titer it is possible that, vaccine-inducible cell populations, other than $CD4^+$ or $CD8^+$ T lymphocytes, contributed to RSV clearance. Candidates for this activity include NK cells and γ/δ T lymphocytes. The γ/δ T lymphocytes are present at sites of infection in lungs and other mucosal surfaces (57–59), and have been shown to influence the course of microbial infection (60–63). The γ/δ T cells have the potential to secrete cytokines, including IFN-γ and TNF-α (64). IL-12 not only stimulates proliferation of γ/δ T cells, but also strongly induces IFN-γ production by these cells (65, 66). It has been recently reported that γ/δ T cells in vivo secrete IFN-γ and IL-4 differentially in response to intracellular bacteria or extracellular parasites (14). Therefore, the viral titer reduction may be attributable to IFN-γ and TNF-α secreted by expanded γ/δ T cells induced by rIL-12 treatment at the time of killed RSV antigen immunization. However, like NK cells, we know of no example in which the γ/δ T cell population has been shown to be vaccine inducible.

Vaccine manipulation of the microenvironment to favor the development of desired immune responses by introducing cytokines or cytokine antagonists into vaccine formulations promises a rational approach for future RSV vaccine development. Administration of IL-12 with a vaccine antigen that could be processed and presented by class I major histocompatibility molecules might be more likely to promote a $CD8^+$

effector population upon subsequent viral challenge. Learning how to modulate the immune response to viral pathogens by selectively activating T cell populations at the time of immunization has the potential to increase vaccine efficacy and improve vaccine safety.

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