Evidence suggests that both host and viral factors influence disease severity after infection with respiratory syncytial virus (RSV). To characterize the effects of pertussis toxin (PT) sensitization on subsequent RSV infection, BALB/c mice were treated with PT parenterally before RSV challenge. Priming with purified and detoxified PT before RSV challenge increased postchallenge weight loss and mortality. PT priming changed the kinetics, location, and composition of the cellular infiltrate in the lung but altered neither antibody responses nor virus titers. Passive transfer of PT-sensitized splenocytes produced similar responses. Priming with purified, but not genetically detoxified, PT propagated a modest type 2 cytokine response to RSV antigens. However, anti–interleukin-4 treatment before RSV challenge failed to abrogate the effects of PT priming. These data confirm that the preexisting immune environment can change virus-specific immunity and provide both a model for study of RSV disease and evidence that noninfectious immunomodulators may impact pathogen-specific immunity.

Respiratory syncytial virus (RSV) causes symptoms that range from mild coryza to bronchiolitis and pneumonia during seasonal epidemics. Although RSV infection remains restricted to the upper respiratory tract in most healthy adults and children, ~40% of RSV-infected infants develop lower respiratory tract illness (LRTI) [1]. Estimates based on US hospital discharge data suggest that RSV-related bronchiolitis alone necessitates 51,240–81,985 annual hospitalizations among children <1 year old [2]. The mechanisms by which some infants limit RSV infection to the upper respiratory tract, whereas others experience mild-to-severe LRTI, are unclear. Factors known to predispose children to severe RSV disease, such as chronic cardiopulmonary conditions, prematurity, and severe combined immunodeficiency [3–6], fail to explain the variable disease severity in otherwise healthy children.

Evidence from RSV disease models suggests that variations in immunopathology may influence the severity of RSV illness. Studies in the BALB/c mouse model of inactivated RSV vaccine–enhanced disease suggest that selective activation of interleukin (IL)–4-secreting T helper (Th) cell subsets during primary exposure to viral antigens may alter the cytokine environment and thus influence the severity of disease upon subsequent RSV challenge [7–9]. Transgenic mice, which constitutively overexpress IL-4, have delayed viral clearance, reduced cytotoxic lymphocyte (CTL) activity, and increased lung pathology after RSV challenge in contrast to normal or IL-4–deficient control animals [10]. Together, this evidence suggests that RSV illness may be enhanced when infection occurs in the context of non–RSV-specific cytokine synthesis.

Conditions correlated with systemically dysregulated cytokine production include helminthic infections, atopic disease, and exposure to immunomodulating bacterial proteins such as pertussis toxin (PT) [11]. PT consists of 2 associated subunits: the enzymatically active A protomer, which modiﬁes the α-subunit of G/Go transmembrane signaling proteins, and the B oligomer, which mediates binding to eukaryotic cells and possesses mitogenic activity for lymphocytes [12, 13]. PT exerts multiple effects on the immune system and can augment IgE synthesis, hypersensitivity reactions, and IL-4 responses to coadministered antigens in mice [14–16]. Studies in mice suggest that these effects stem from PT’s ability to potentiate T cell proliferation and secretion of both type 1 (interferon [IFN]–γ and IL-2) and type 2 (IL-4 and IL-5) cytokines in response to coinjected antigens, largely through receptor binding activity, rather than enzymatic activity [17]. Both whole-cell and acellular pertussis vaccines, typically administered to children between ages 2 and 6 months, include PT. Studies show that immunization of children and mice with whole cell vaccines induces type 1 cytokine secretion by Th cells, whereas the less reactogenic acellular diphtheria–tetanus toxoids–pertussis vaccine (DTP) induces a type 2 or mixed cytokine profile [18]. Although the age at pertussis vaccination and the highest in-
cidence of severe RSV disease in infants coincide temporally, no connection between PT sensitization and altered responses to subsequent infections has been drawn.

We demonstrated elsewhere that simultaneous exposure to RSV and pertussis antigens alters the immune response to RSV in a BALB/c mouse model [19]. In these studies, we used this complex but clinically relevant model to determine whether preexisting immune activation by PT or pertussis vaccines alters the outcome of RSV disease. To evaluate the effects of PT sensitization on the immune response to RSV, mice were injected parenterally with purified PT, chemically detoxified PT, or genetically detoxified PT 1 week before RSV challenge. The genetically detoxified 9K-129G mutant toxin, generated by introduction of 2 amino acid substitutions within the A protomer, demonstrates physical properties and adjuvanticity indistinguishable from wild-type PT but induces neither leukocytosis nor histamine sensitivity in mice [20]. By including this, as well as chemically detoxified PT in the form of the DTP vaccine administered to infants as part of a regular immunization schedule, we examined whether enzymatically inactivated PT antigens mediate the same effects as purified PT.

Materials and Methods

Reagents. Acer-Imune (diphtheria and tetanus toxoids and acellular pertussis vaccine adsorbed) DTP vaccine, tetanus and diphtheria toxoids for adult use purogenated DT vaccine, and diphtheria and tetanus toxoids for pediatric use purogenated DT vaccine were obtained from Lederle-Praxis Laboratories (American Cyanamid, Pearl River, NY). The DTP preparation (lot 386-910) contained ~1.5 limits of flocculation (Lf) U of diphtheria toxoids (Dtxo), 1 Lf U of (tetanus toxoids [Ttox]), and 60 hemagglutinin U of PT per 100-μL dose (estimated quantity of PT based on published analysis, 2 μg/dose [21]). Adult and pediatric DT formulations (lots 372-500 and 359-906) were mixed 1:1 for a final concentration of 1.5 Lf U of Dtxo and 1 Lf U of Ttox. All 3 preparations contain alum and were administered as 0.2 human doses. This dosage induces DTP-specific IL-4 production in the lung [22]. Purified PT (List Biologicals, Campbell, CA) and genetically detoxified PT mutant 9K-129G (provided by Rino Rappuoli, Sclavo Research Center, Siena, Italy) were diluted in sterile PBS to a final concentration of 2 ng/μL. Each dose of PT and 9K129G contained 200 ng of PT protein in 100 μL of PBS, given intramuscularly (im). The anti-IL-4 neutralizing antibody 11.B11 [23] and isotype control antibody R187, a rat IgG1 specific for the p30 gag protein of murine leukemia virus (American Type Culture Collection, Rockville, MD), were diluted in sterile PBS and administered intraperitoneally (ip) at 200 μg per dose.

Mice and virus. Pathogen-free female 8-week-old BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC) or Harlan-Sprague-Dawley (Indianapolis). Mice were cared for as described elsewhere [24]. RSV challenge stock was derived from the A2 strain of RSV, as described elsewhere [25].

Experimental design. Mice received 2 doses of DTP (n = 55 mice), DT (n = 30 mice), purified PT (n = 42 mice), 9K-129G (n = 30 mice), or PBS (n = 55 mice) im at 0 and 4 weeks. One week after the second immunization, mice were anesthetized in with ketamine (40 μg/g body weight) and xylazine (6 μg/g body weight) before intranasal inoculation with 10⁵ pfu live RSV in 100 μL of Eagle MEM (EMEM). For IL-4 depletion, 11.B11 or isotype control R187 was administered ip on 3 successive days, starting 1 day before RSV challenge. Lungs from a subset of mice in each group were harvested 4 days after RSV challenge, to evaluate RSV titers and cytokine mRNA expression, 8 days after challenge for RSV titers, and 6–10 days after challenge for histopathology. Body weight and mortality were evaluated daily after challenge for a subset of 18 mice per group for PT-, DTP-, and PBS-primed animals and for 12 mice per group for DT- and 9K-129G-prime animals. Data represent combined observations from 3 independent experiments. Data significance was determined by Student’s 2-tailed t test or analysis of variance (ANOVA) for analysis of normal variable distribution between paired data points using StatView software (Abacus Concepts, Berkeley, CA). Significance of differences in mortality and weight loss between groups was determined by nonparametric methods by Wilcoxon rank sum test and Kruskal-Wallis test, respectively.

Plaque-forming unit assays. Lung tissue was removed, weighed, and immediately quick frozen in EMEM supplemented with 10% fetal bovine serum (FBS). Tissues were maintained at 4°C while they were individually ground. Clarified lung supernatants were diluted and inoculated onto subconfluent HEp-2 cell monolayers in 12-well plates (Costar, Cambridge, MA). After 1 h, plates were covered with 0.75% methylcellulose in EMEM/10% FBS and incubated for 4 days at 37°C [27]. Monolayers were then fixed with 10% buffered formalin and stained with hematoxylin-eosin (HE). Plaques were counted with the aid of a dissecting microscope.

mRNA isolation and detection by Northern blot. Right lungs removed 4 days after RSV challenge and quick frozen in liquid
Figure 2. Pertussis toxin (PT) priming increased mortality after respiratory syncytial virus (RSV) challenge. Data are percentage of surviving mice/group (n = 18 for diphtheria-tetanus [DT] toxoids–pertussis [DTP], PT, and PBS; n = 12 for DT and 9K-129G) daily after RSV challenge. One of 18 PBS-primed animals died, resulting in a 94% overall survival rate. DT priming did not increase mortality over that of controls, but DTP priming decreased survival, and PT and 9K-129G priming equally reduced survival even more dramatically (P < .05 at day 12; Wilcoxon rank sum test).

Pathology. Lungs were inflated via the left main bronchus and fixed in 10% phosphate-buffered formalin. Sections stained with HE were examined for density, composition, and location of inflammatory infiltrates from 5 mice per time point per group. Representative results are shown.

In vitro splenocyte stimulation and cytokine detection. Spleens harvested from 6 mice per group 6 weeks after RSV challenge were disrupted with a sterile blunt syringe end in RPMI/10% FBS. Pooled splenocytes were isolated by centrifugation at 1500 g on a 4-mL cushion of FicoLite for mouse lymphocytes (Atlanta Biologicals, Norcross, GA). The lymphocyte band was aspirated, washed, resuspended in RPMI/10% FBS at a density of 2 × 10^6 cells/mL, and distributed at 100 µL/well into 96-well plates (Costar). Splenocytes from each group were stimulated in triplicate with PT or 9K-129G at 1 µg/mL, immunofluorescence-purified RSV fusion (F) protein at 100 ng/mL (gift of Connaught Laboratories, Toronto), phytohemagglutinin (PHA) at 10 µg/mL (Pharmacia Biotech, Uppsala, Sweden), or medium alone. Cells were incubated at 37°C with 5% CO₂ for 48 h. Supernatants from each well were removed and assayed immediately for secreted IL-4 and IFN-γ by using murine cytokine ELISA kits (Endogen, Woburn, MA) according to the manufacturer’s instructions.

Results

PT priming and weight loss after RSV challenge. Mice were primed twice with purified PT, chemically inactivated PT in

<table>
<thead>
<tr>
<th>Priming regimen</th>
<th>Day 4</th>
<th>Day 8</th>
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<tbody>
<tr>
<td></td>
<td>(n = 18/group)</td>
<td>(n = 6/group)</td>
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<tr>
<td>PT</td>
<td>6.7 ± 0.4^a</td>
<td>2.0 ± 0.8 (3/6)</td>
</tr>
<tr>
<td>9K-129G</td>
<td>6.1 ± 0.3</td>
<td>0.73 ± 1.1 (2/6)</td>
</tr>
<tr>
<td>DTP</td>
<td>6.6 ± 0.8</td>
<td>1.8 ± 0.2 (2/6)</td>
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<tr>
<td>Diphtheria–tetanus toxoids</td>
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<td>&lt;1.8</td>
</tr>
<tr>
<td>PBS</td>
<td>6.4 ± 0.4</td>
<td>&lt;1.8</td>
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^a Data are geometric mean ± SE (no. of mice with detectable titers). PT, pertussis toxin; DTP, acellular diphtheria–tetanus toxoids–pertussis vaccine.

| Image 57x484 to 297x690 |
Figure 3. Pertussis toxin (PT) priming altered composition, kinetics, localization, and density of cellular infiltrates after respiratory syncytial virus (RSV) challenge. Lungs from control, PT-, and 9K-129G–primed mice were harvested on days 5–10 after RSV challenge and fixed immediately. Sections stained with hematoxylin–eosin were evaluated for density and location of cellular infiltrates. Sections from unprimed controls are shown at days 6 (A), 8 (B), and 10 (C). Lungs from PT-primed mice at days 6 (D), 8 (E), and 10 (F) show increased early interstitial inflammation followed by profound edema. Priming with 9K-129G on days 6 (G), 8 (H), and 10 (I) produced intermediate pattern. Sections include representative results from 5 mice/time point/group. Original magnification, ×62.5.
DTP vaccine, genetically detoxified 9K-129G PT, DT vaccine that lacked pertussis antigens, or PBS. One week after the second priming, mice were challenged intranasally with RSV and observed daily for weight loss and mortality. We show weight change as an arithmetic mean ± SE; daily values include only surviving mice. PT-primed mice had the greatest peak weight loss (figure 1); each lost an average of ~7 g or ~30% of pre-challenge body weight by day 8 after challenge. PT-primed mice showed significant weight change and delayed recovery of body weight, compared with PBS-primed control animals (P < .05, Kruskal-Wallis test). Mice primed with the genetically detoxified 9K-129G protein demonstrated weight loss and recovery intervals indistinguishable from mice primed with wild-type PT. Neither DTP nor DT priming altered weight loss significantly, compared with PBS-primed controls (P > .10).

PT, 9K-129G, and DTP priming significantly increased mortality after RSV challenge. In the BALB/c mouse model, primary RSV infection without prior immune manipulation rarely results in mortality; therefore, no LD<sub>50</sub> for primary infection was defined in this system. In these experiments, 1 of 18 PBS-primed mice died after RSV challenge, resulting in a survival rate of 94% (figure 2). In contrast, PT priming significantly increased mortality after RSV infection, reducing survival to 55% by day 12 after challenge (P < .05, Wilcoxon rank sum test). Priming with 9K-129G produced results indistinguishable from wild-type PT exposure, resulting in 58% survival (figure 2). DTP priming also increased mortality significantly over controls, reducing day 12 after-challenge survival to 78%, but affected mortality to a lesser degree than did priming with PT and 9K-129G (P < .05). Priming with DT did not increase mortality: 12 of 12 mice recovered from RSV challenge. Because the only difference in the DTP and DT vaccines is the inclusion of pertussis antigens (PT, filamentous hemagglutinin, and pertactin), this suggests that both chemically inactivated and genetically detoxified PT may increase RSV-induced mortality following challenge. Repeated immunization with DTP, 9K-129G, or PT in the absence of viral challenge produced neither detectable illness nor mortality (n = 10 mice/treatment, data not shown).

Virus titers in primed and unprimed mice. To determine whether increased or prolonged viral replication induced the observed increases in illness and mortality, RSV titers in the
lungs were evaluated 4 \((n = 18/\text{group})\) and 8 days \((n = 6/\text{group})\) after challenge. Day 4 was established elsewhere as the peak of viral replication in the BALB/c mouse, and virus is generally undetectable \(< 1.8 \log_{10} \text{pfu/g}\) by day 8 [25]. No significant differences in peak virus titers were determined among primed and control groups (table 1). Although PT-primed \((3 \text{ of } 6)\) and DTP-primed \((2 \text{ of } 6)\) mice had low but detectable virus titers on day 8, this difference was not significant \((P > .10, \text{Student’s } t\text{-test}).\)

**PT priming and RSV-induced pathology.** To identify mechanisms associated with increased mortality, sections from right lungs harvested on days 5–10 after RSV challenge were stained with HE and evaluated for density and location of cellular infiltrates. Six days after RSV challenge, lung sections from PBS-primed control animals showed a sparse, predominantly lymphocytic interstitial infiltrate typical of primary RSV infection (figure 3A). By day 8, the lymphocytic infiltrate was seen in periarterial and perivenous regions (figure 3B), and by 10 days after challenge, the infiltrate was concentrated in the perivenous spaces with rare small and discrete regions of alveolar edema (figure 3C). In contrast, PT priming resulted in dense interstitial infiltrate at day 6 after RSV challenge, characterized by the presence of large mononuclear cells and lymphocytes in the interstitial spaces (figure 3D). Interstitial inflammation peaked on day 7, and by day 8 after challenge, in lungs from PT-primed mice, there was a reduction in the density of the interstitial infiltrate along with areas of localized alveolar edema (figure 3E). Ten days after challenge, this edema was profound, with fluid in alveolar spaces and in bronchioles throughout the lung (figure 3F). Although priming with 9K-129G increased weight loss and mortality in a pattern indistinguishable from wild-type PT priming, lungs from 9K-129G-primed mice had slightly less generalized edema and lacked the large interstitial leukocytes seen in PT-primed mice. By day 6 after challenge, 9K-129G-primed mice had increased density in interstitial infiltrates (figure 3G), compared with controls, with continued dense periarterial and perivenous infiltrate evident by day 8 (figure 3H). However, 10 days after challenge, 9K-129G-primed mice had lymphocytic infiltrates with many foci of alveolar edema that was more extensive than in controls (figure 3I). DTP priming produced an intermediate response, with less edema than PT priming but more than in controls, whereas DT-primed mice were similar to controls (data not shown). These data indicate that PT priming alters the composition, density, kinetics, and localization of the cellular infiltrate in the mouse lung after RSV infection.

Lung weight may serve as a general index of edema. In order to assess this, the left lungs from the 5 animals in each treatment group described above were weighed immediately after harvest. Data shown are the means of these lung weights in grams ± SE. At day 6, no differences could be detected between PT- or 9K-129G-primed and control animals \((0.168 ± 0.02, 0.158 ± 0.01, \text{and } 0.162 ± 0.01; P > .10, \text{ANOVA}).\) However, by day 8 after infection, PT-treated animals had significantly greater mean lung weights than did either 9K-129G-primed or control animals \((0.216 ± 0.02, \text{PT}; 0.152 ± 0.02, 9K-129G; 0.126 ± 0.01, \text{control}; P < .05 \text{ for PT vs. either group}).\) This increased weight persisted at day 10 after infection \((0.244 ± 0.02, \text{PT}; 0.148 ± 0.01, 9K-129G; 0.124 ± 0.01, \text{control}; P < .05). Priming with 9K-129G increased the mean lung weight slightly, compared with controls, but did not reach statistical significance.

**PT priming increased IL-4 message levels after RSV challenge.** To determine whether PT priming altered the cytokine environment in the lung at the time of challenge, mRNA was isolated from lungs of PT-, DTP-, DT-, and PBS-primed mice 4 days after RSV challenge and was evaluated for IFN-γ and IL-4 message levels. Previous studies in the RSV-BALB/c model demonstrated predominantly IFN-γ response after primary challenge. Mice primed with diphtheria-tetanus toxoids–pertussis (DTP) and DT before RSV challenge show some increase in relative quantity of IL-4 mRNA \((0.456 \text{ and } 0.727 \text{ IL-4:IFN-γ ratios, respectively}); PT-primed mice had even greater increase in IL-4 message, compared with unprimed controls \((0.844 \text{ IL-4:IFN-γ ratio}).\)

**Figure 4.** Pertussis toxin (PT) priming increased interleukin (IL)-4 mRNA expression after respiratory syncytial virus (RSV) challenge. Poly A+ RNA was isolated from lungs 4 days after RSV challenge and examined by Northern blot for expression of IL-4 and interferon (IFN)-γ message. Each lane represents mRNA pooled from 2 mice. Unprimed mice \((\text{right 2 lanes})\) demonstrate predominantly IFN-γ response after primary challenge. Mice primed with diphtheria-tetanus toxoids–pertussis (DTP) and DT before RSV challenge show some increase in relative quantity of IL-4 mRNA \((0.456 \text{ and } 0.727 \text{ IL-4:IFN-γ ratios, respectively}); PT-primed mice had even greater increase in IL-4 message, compared with unprimed controls \((0.844 \text{ IL-4:IFN-γ ratio}).\)

<table>
<thead>
<tr>
<th>Cytokine response, pg/mL</th>
<th>In vitro recall antigen</th>
<th>Interferon-γ</th>
<th>Interleukin-4</th>
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<td><strong>PT</strong></td>
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<tr>
<td>RSV F protein</td>
<td>5193.5</td>
<td>437.4</td>
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<tr>
<td>PHA</td>
<td>8415.1</td>
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<tr>
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<td>RSV F protein</td>
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<tr>
<td>PHA</td>
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**NOTE.** Data are results of 6 pooled mouse spleens per in vivo treatment group and are means of quadruplicate wells minus background. PT, pertussis toxin; F, fusion; PHA, phytohemagglutinin.
priming alters RSV disease

Figure 5. Depletion of interleukin (IL)-4 failed to eliminate pertussis toxin (PT)-enhanced mortality and weight change after respiratory syncytial virus (RSV) challenge. PT-primed or control unprimed mice were treated with anti-IL-4 neutralizing antibody 11.B11 or R187 isotype control before RSV challenge. PT-primed mice had significantly greater peak weight loss (A) than did unprimed controls, regardless of antibody treatment (P < .05, Student’s t test).

PT and 9K-129G priming resulted in inverse patterns of RSV-specific cytokine secretion. To determine whether PT and 9K-129G affect the recall response to RSV, splenocytes were isolated 6 weeks after RSV challenge from mice primed with PT or 9K-129G before challenge. Isolated splenocytes were stimulated in vitro with purified PT, purified RSV F protein, PHA, or medium. Levels of secreted IL-4 and IFN-γ in the culture supernatant were determined by ELISA at 96 h. Table 2 shows IL-4 and IFN-γ production in response to the in vitro stimuli. Splenocytes from mice primed in vivo with PT in vivo produced a dominant IL-4 response to stimulation with both PT and RSV antigens. In contrast, 9K-129G priming in vivo led to a predominantly IFN-γ response to both PT and RSV antigens on subsequent restimulation. These data suggest that PT and 9K-129G may influence immune responses to RSV through more than one mechanism.

PT priming before RSV challenge and antibody isotype selection. PT priming alters antibody isotype selection in favor of the IgG1 isotype to simultaneously administered antigens, including coadministered RSV [19]. Serum from animals primed with PT, DTP, DT, or PBS was collected 6 weeks after RSV challenge and was assayed for RSV-specific antibody levels by ELISA. Neither PT, DTP, nor DT priming altered the ratio of IgG1 to IgG2a, compared with the PBS-primed controls (0.926, 0.923, 1.021, and 0.926, respectively). Despite elevated lung IL-4 mRNA levels in PT-primed mice, PT priming 1 week before RSV challenge did not increase IgG1 production.

IL-4 depletion at RSV challenge and effects of PT priming. To determine whether elevated IL-4 mRNA levels in the lung after RSV challenge mediated or were merely associated with PT-enhanced RSV disease, the anti–IL-4 neutralizing antibody 11.B11 [23] was administered to PT-primed mice before RSV challenge. PT-primed and unprimed control mice were treated with 11.B11 or isotype control R187 monoclonal antibodies for 3 successive days starting 1 day before RSV challenge, to determine the effect of IL-4 depletion on weight change and survival. PT-primed mice treated with anti–IL-4 antibodies still showed more weight loss than control mice (figure 5A; P < .05, Kruskal-Wallis test). Unprimed mice given control or 11.B11 antibody demonstrated 92% and 100% survival, respectively, whereas PT priming reduced survival to 62% in isotype control-treated mice and 50% in anti–IL-4–treated animals by day 12 after challenge (figure 5B; P < .05, Wilcoxon rank sum test). Virus titers in the lung at days 4 and 8 after challenge did not differ in any of the 4 treatment groups (data not shown).

Thus, IL-4 depletion at the time of RSV challenge did not eliminate the effects of PT priming on illness outcome.

Passively transferred PT-treated effector cells and RSV disease. To confirm that the effects of PT priming on RSV pathogenesis stem from altered immune responses, rather than from systemic changes in epithelial or endothelial integrity, immune effector cell populations from naive mice were sensitized in vitro with PT and transferred to naive recipient mice before RSV challenge. Splenocytes from pathogen-free BALB/c mice
were isolated and stimulated in vitro with PT or PBS for 24 h, thoroughly washed, and selectively depleted of CD4 or CD8 cells or of neither cell population before transfer into naive recipient BALB/c mice. Recipient mice then were challenged with RSV and evaluated for weight change and alterations in pathology. Mice that received PT-treated splenocytes had increased weight loss and delayed recovery after RSV challenge, compared with mice that received PBS-treated splenocytes (figure 6; $P < .05$, Kruskal-Wallis test). Depletion of either CD4 or CD8 T lymphocyte subsets from the transferred cell population had no impact on this effect, suggesting that cells other than $T$ lymphocytes might be responsible for mediating altered pathology. Alternatively, we cannot exclude the possibility that both subsets contributed independently to the sensitizing activity. Transfer of PT-treated splenocytes in the absence of RSV challenge caused no observable weight change.

To determine whether passively transferred PT-sensitized cells induce pathology similar to that seen after in vivo PT priming, lung sections from recipient mice were examined 8 days after RSV challenge. The lungs of animals given PT-treated splenocytes showed severe intra-alveolar and intra-bronchiolar edema without admixed inflammatory cells (data not shown). Bronchovascular and perivenous regions were infiltrated with mononuclear cells, similar to lungs of control animals that received PBS-treated splenocytes. Although the edema induced by PT-treated splenocyte transfer did not appear as generalized as that in mice primed with PT in vivo (seefigure 3), the morphology and location of cellular infiltrates and the presence of edema suggested that similar pathogenic mechanisms may be involved in both settings.

Discussion

Increased understanding of the roles played by cytokine-secreting effector cells during the initial response to infection has led to a growing body of work on dual antigen exposure. Evidence from in vitro studies suggests that genetic background plays some role in determining the “default” phenotype of activated Th cell precursors [27], but that the cytokine milieu present during antigenic stimulation also influences CD4 lymphocyte development [28]. Primary activation of a naive Th cell in the presence of type 2 cytokines might lead to subsequent Th2-dominated recall responses. Evidence from animal models suggests that persistent infection with pathogens that promote IL-4 expression may depress the cell-mediated immunity required to clear subsequent viral or bacterial infections [29–31]. Cytokine production and lymphocyte activation induced by an immune response to one pathogen or by constitutive immune disorders, such as atopic disease, may play an important role in influencing immunity to concurrent or subsequent infections.

We previously investigated the association between preexisting polarized immune responses induced through transgenic or vaccine-driven cytokine expression and severe RSV disease in the BALB/c mouse model of RSV infection [9, 10]. The experiments described here examined whether PT sensitization, which alters immune responses to coadministered antigens [17, 19], could influence the immune response to subsequent intranasal RSV infection. PT priming increased weight loss and mortality after RSV challenge. This enhanced RSV disease corresponded with altered pulmonary pathology marked by profound intra-alveolar edema. Although PT-primed mice also demonstrated increased IL-4 mRNA levels in the lung after RSV challenge, PT priming induced neither the prolonged viral replication nor the persistent interstitial cellular infiltrate seen in the context of constitutive overexpression of IL-4 [10]. Treatment with an IL-4–neutralizing antibody before RSV challenge failed to ameliorate the effects of PT priming on weight loss and mortality. A genetically detoxified PT molecule, 9K-129G, also increased weight loss and mortality when administered before RSV challenge, reinforcing observations that the immunomodulatory effects of PT stem from the receptor-binding moiety rather than the ADP-ribosyltransferase activity [13, 17, 20, 32].

Although 9K-129G mutant toxin increased illness and mortality in a pattern similar to that of wild-type PT, 9K-129G priming did not result in the same degree of alveolar and bronchiolar edema, nor did it recruit the prominent large mononuclear population into the interstitium. Although PT priming induced an IL-4–dominated memory response to both PT and RSV antigens on restimulation in vitro, 9K-129G priming induced an IFN-$\gamma$–dominated response to both. This suggests...
that only wild-type PT priming would result in IL-4 up-regulation on secondary stimulation in vivo, although whether this would enhance RSV disease after reinfection in a susceptible host cannot be determined in this model.

The development of profound edema after the immigration of an atypical cell population into the lungs of PT-primed mice suggests that the effector cells promote increased capillary endothelial permeability through secreted factors or other signaling mechanisms. The ability to alter the immune response to RSV challenge through passive transfer of PT-sensitized splenocytes demonstrates that PT-sensitized cells can exert a profound effect on bystander cells in vivo and that cells other than CD4 and CD8 lymphocytes appear to play a key role. Although the non–T cell activity was not identified in these studies, candidate cells included in the splenocyte fraction include NK cells, monocytes, neutrophils, or other non–T cell effector cells. Further studies are required to identify the effector population and to determine whether this influence proceeds from skewed cytokine, chemokine, or growth factor secretion by sensitized cells; selective effector cell activation; altered lymphocyte trafficking by sensitized cells; or a combination of these.

The differences between RSV-specific immunity in PT-primed and IL-4 overexpressing mice, the failure of anti–IL-4 treatment to abrogate PT effects, and the lack of IL-4 responses after 9K-129G priming suggest that IL-4 induction is not required for severe illness in this context. Elevated IL-4 levels here may play a complementary, rather than a direct, role in disease pathogenesis. Recent studies in mice demonstrate that IL-4 has multiple effects on the response to pertussis vaccines, regulating inflammatory cell recruitment into the lung and inducing a cascade of anti-inflammatory cytokines such as IL-6 and IL-1 receptor agonist [22]. These effects may contribute to the altered pathology in PT-primed mice, but other undefined mechanisms clearly play some role in enhancing disease in the absence of IL-4. Complex immunomodulating agents that induce the elevation of IL-4 might also mediate other changes simultaneously through distinct effector mechanisms. These results also do not eliminate the possibility that other type 2 cytokines, such as IL-13, might mediate the described effects; further experiments will be required to explore this possibility. However, the effects of PT sensitization on RSV disease did not conform to a canonical type 1/type 2 paradigm and may depend on the intersection of multiple immune pathways. Together, these data show that nonviral antigens can evoke an immunologic milieu that alters the RSV-specific immune response.

Although infection with Bordetella pertussis is linked to an increased incidence of bronchial asthma, DTP vaccination does not appear to increase wheezing episodes in children [33]. Thus, clinical implications of the studies described here are unclear. However, these studies and others cited emphasize the importance of understanding how antigen-specific responses may cross-regulate responses to concomitant or subsequent infections. The ability of bacterial antigens to alter the immune response to a live virus has implications that should be considered in the rational design of vaccines and immunization schedules, as well as in the analysis of the pathogenesis of natural infections.

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References


