

Distinct Types of Lung Disease Caused by Functional Subsets of Antiviral T Cells

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Summary

T cells appear to play a central role in viral bronchiolitis, but the effects of different functional and phenotypic subgroups of T cells have not been defined. To test the activities of T cells recognizing individual proteins of respiratory syncytial (RS) virus, virus-specific T cell lines were produced from mice primed by scarification with recombinant vaccinia viruses expressing the major surface glycoprotein (G), fusion protein (F) or second matrix (22K) protein of RS virus. As previously reported, the *in vitro* characteristics of these cells are predetermined by the choice of RS virus protein: 22K-specific cells are predominantly class I-restricted cytolytic CD8⁺ cells; F-specific cells, a mixture of cytolytic CD8⁺ cells and CD4⁺ cells with a T helper 1 cell (Th1) cytokine secretion profile, whereas those from G-sensitized mice are almost exclusively CD4⁺, with Th2 characteristics. Mice infected intranasally with RS virus showed mild illness and recovered fully, but developed respiratory distress after intravenous injections of T cells. Dose-for-dose, infected mice receiving G-specific cells suffered the most severe (sometimes fatal) illness, characterized by lung hemorrhage, pulmonary neutrophil recruitment (shock lung) and intense pulmonary eosinophilia. This disease was further enhanced by coinjection of 22K-specific cells, which alone caused mild shock lung without eosinophilia. F-specific cells caused minimal enhancement of pathology and had little or no effect on the disease caused by G-specific cells. Each cell line reduced lung virus titer and combined injections of G- and 22K-specific cells eliminated infection completely. The *in vitro* characteristics of these antiviral T cell lines therefore predict the pathological effects *in vivo*. Moreover, different forms of viral bronchiolitis can be caused by functionally distinct types of activated T cell.

Viral bronchiolitis is the single most common cause of hospitalization of infants in the Western world, but the development of effective preventative or therapeutic stratagems has been hampered by lack of information about its pathogenesis. The majority of cases are caused by respiratory syncytial (RS)¹ virus, the annual hospitalization costs of which were estimated to be \$300,000,000 in 1988 in the United States alone, with 91,000 children admitted (1). Antiviral immunity appears not only to protect against infection but also to contribute to lung pathology. The first evidence that specific immunity could be harmful came in the 1960s, when children were vaccinated with formalin-inactivated RS virus. Vaccine recipients developed strong serological responses, but were not protected against infection. Moreover, the majority of vaccinees who subsequently became infected with RS virus developed severe lower respiratory tract disease, with significant mortality. The reasons for vaccine-augmented disease have

been studied (2–5), but no safe, effective vaccine has yet been produced.

Animal studies suggest that specific activated T cells are probably responsible for this phenomenon of disease augmentation, and may also be responsible for many of the pathological effects seen during primary and secondary RS virus infections. Passive transfer of specific antiviral Ab does not enhance disease, either protecting against infection and disease or being neutral in effect. Mice infected with RS virus develop a lymphocytosis in the bronchoalveolar lavage (BAL) fluid, dominated by CD8⁺ α/β T cells at the time of virus elimination (6). Treatment of such mice by injection of T cell-depleting anti-CD4 and/or anti-CD8 mAbs reduces or abolishes disease, while enhancing virus replication (7). Adoptive transfer of CD8⁺ CTL lines or clones can eliminate infection *in vivo*, while also causing hemorrhagic, neutrophilic, and sometimes fatal pneumonitis (8). CD4⁺ Th cells also have distinct antiviral and pathogenic effects, and may be more potent on a cell-for-cell basis than CD8⁺ T cells (9). During reinfection, both T cell subsets have to be depleted to completely abolish the enhanced pathological response (7). Cotton rats vaccinated with formalin-inactivated RS virus (3, 10, 11)

¹ Abbreviations used in this paper: BAL, bronchoalveolar lavage; CS, culture supernatant; F, fusion protein; G, major surface glycoprotein; RS, respiratory syncytial; rVV, recombinant vaccinia virus; 22K, 22kDa second matrix protein of RS virus.

and mice vaccinated with recombinant vaccinia viruses (rVV) encoding some single proteins of RS virus (12, 13) show a reduction in lung virus titer after challenge with RS virus. In both these situations, there can also be a paradoxical increase in the severity of lung pathology (3, 5, 10, 11, 14, 15). In mice given formalin-inactivated RS virus intramuscularly, vaccine-enhanced disease is abolished by CD4⁺ cell depletion *in vivo* but is little affected by depletion of CD8⁺ cells (4). The immune mechanisms responsible for enhanced disease in mice primed with recombinant vaccinia viruses have not been defined.

In RS virus-infected mice previously sensitized with rVV-expressing individual RS virus proteins, BAL has shown the patterns of immunopathology to vary depending on the RS virus protein to which the mice are sensitized. Remarkably, mice sensitized to the major surface glycoprotein (G) develop RS virus-specific Th cells which release IL-4 and IL-5 in response to antigen (16), and after infectious challenge with RS virus, suffer disease characterized by a striking increase in BAL eosinophils (14–25%, from <3% in controls) (15). Recently, virus-specific T cell lines were derived from mice primed by scarification with rVV-expressing single RS virus proteins (17). For the present studies, we selected three cell lines as being of special interest: the G-specific line, because it is dominated by CD4⁺ T cells with Th2 characteristics; the fusion protein (F)-specific line, because it contains CD4⁺ cells with Th1 characteristics, as well as class I-restricted CTL; and the 22K-specific line, because it is dominated by CTL alone. Immune responses to these three proteins are also of special interest in terms of vaccine development. F and G are the main surface glycoproteins against which Ab responses occur, and 22K is the main target for CTL in the mouse. We now report the *in vivo* effects of T cell lines specific to these three RS virus proteins, and show that these T cells can transfer the antiviral and pathogenic effects seen in mice sensitized to single viral proteins to naive syngeneic recipients.

Materials and Methods

Viruses and Mice

The human A2 strain of RS virus was grown and assayed for infectivity in HEP2 cells, as previously described (9). Mock-infected HEP2 cells were treated in the same way to derive control antigen. Infectious units of RS virus (i.e., each of which is able to induce a single immunoperoxidase-positive cell) were regarded as equivalent to PFU. All rVV were the kind gifts of Drs. Wertz, Ball, and Anderson (University of Alabama, Birmingham, AL) and were produced by insertion of the cDNA transcript of the RNA sequence for a specific protein from the A2 strain of RS virus according to the protocol described by Ball et al. (18). All rVV were grown in HEP2 cells, and infectivity was measured by plaque assay in 24-well plates (Costar Corp., Cambridge, MA). Recombinant VV-F (VF 317), G (VAG 301), and 22K have been described previously (12, 13, 19). Similar rVV expressing β Gal was used as a control. All virus stocks and cells were free of mycoplasma infection by DNA hybridization (Gen-probe Inc., San Diego, CA). Allantoic fluid containing influenza A X31 (H3N2) was provided by Dr. A. Douglas (National Institute for Medical Research, Mill Hill, London, UK)

at 4,096 hemagglutination units (HAU) ml⁻¹. Female BALB/c mice 3–4-mo-old (Harlan Olac, Bicester, Oxon, UK) were infected intranasally with 2×10^5 PFU of RS virus, 7 HAU of influenza, or scarified over the rump and tail base with rVV stock diluted to contain 3×10^6 PFU in 10 μ l/mouse (19). rVV-infected mice were checked for formation of typical infective lesions on days 3 to 5.

T Cell Lines

Spleen cells from primed mice were suspended at 10^6 cells/ml with 2.5×10^5 cells/ml RS virus-infected autologous splenocytes (stimulator cells) at a multiplicity of infection (m.o.i.) of 0.1 PFU/cell, in 15 ml RPMI 1640 medium (GIBCO BRL, Paisley, Scotland) supplemented with 10% FCS, antibiotics, 2-ME, and glutamine (RPMI/10). After 5 d, cells were washed and suspended at 2×10^5 cells/ml with normal syngeneic irradiated spleen cells (2×10^6 cells/ml). After 5–6 d of antigenic “rest,” cells were washed and stimulated with antigen presented by normal syngeneic irradiated cells infected with RS virus at similar cell densities. This stimulation/rest cycle was repeated up to seven times. It was necessary to add 5% Con A-stimulated rat spleen cell culture supernatant (Con A supernatant) to the predominantly CD8⁺ T cell line derived from mice primed with rVV-22K after the second cycle, in order to maintain growth (see Results). As controls, spleen cells from unprimed mice and mice inoculated with rVV- β Gal (by scarification) or HEP2 cell material (transnasally) were cultured as described for RS virus-specific T cell lines. Cell transfers were performed by tail vein injection within 4 h of intranasal challenge.

Fluorescent Staining for Lymphocyte Surface Markers

For three-color analysis, cell pellets were incubated first with Abs to TCR- α/β (affinity purified H57–597 at a final concentration of 1.15 μ g/ml), TCR-C γ (Uc7–13D5 bioreactor supernatant kindly given by Dr. J. Bluestone (University of Chicago, Chicago, IL), used at 1 in 100), CD3 (145–2c11 supernatant, final concentration 1 in 100), CD45RB (16A, rat mAb kindly given by Dr. K. Bottomly (Yale University, New Haven, CT), final concentration 1 in 1,000), or left without first layer Ab. Bound Ig was detected by FITC-conjugated goat anti-hamster Ab (Cappel Organon Teknika Corp., West Chester, PA) which also reacts with rat Ig. After blocking with 1 mg/ml affinity-purified rat Ig (Sigma Chemical Co., St. Louis, MO), appropriate dilutions of coupled Ab to CD4 (GK 1.5-PE; Becton Dickinson & Co., Mountain View, CA) and CD8 (53–6.7-biotin, Becton Dickinson & Co.) were added; the CD8 stain was developed with streptavidin-duochrome (Becton Dickinson & Co.). For two-color stains, appropriate dilutions of GK 1.5-PE and 53–6.7-FITC (Becton Dickinson & Co.) were used. All procedures were performed on ice with 0.1% sodium azide and 1% BSA. Cells were analyzed on a FACScan[®] flow cytometer (Becton Dickinson & Co.).

Cytotoxicity Assay

P815 cells were infected overnight with RS virus at an moi of 2 PFU/cell or rVV at 10 PFU/cell. Uninfected or rVV- β Gal-infected P815 cells served as control targets. A chromium release assay was performed as previously described (19). Briefly, targets were labeled with Na₂⁵¹CrO₄ for 50 min at 37°C, washed, and diluted to 10⁵/ml. Effector cells were mixed with targets and incubated for 3 h in 96-well plates. The percent lysis was calculated by measuring ⁵¹Cr release into the supernatant: $100 \times [(sample\ cpm - background\ cpm)/(total\ cpm - background\ cpm)]$, where total cpm is the radioactivity released from targets treated with Triton X-100.

Cytokine Assays

Cell lines were cultured with irradiated syngeneic spleen cells that were either uninfected or infected with the RS virus. Culture supernatants (CS) were tested for cytokines on day 1 to 3 after antigenic stimulation.

IL-2 Assay. IL-2 dependent CT.EV cells (20) were maintained in RPMI/10 and 5% IL-2 (CS of X6310 line expressing mRNA for IL-2 [21]). These were washed four times and suspended at 10^5 cells/ml. 5×10^3 cells were incubated with $50 \mu\text{l}$ of each test sample in 96-well round-bottomed microtiter plates for 42 h before addition of $0.5 \mu\text{Ci/well}$ of [^3H]TdR for 6 h. The cellular DNA was harvested onto filters, dried, and assayed by liquid scintillation counting. The results were calculated as the mean cpm of triplicates after subtraction of counts obtained when cells were incubated with medium alone (typically 200–400 cpm). In each assay, wells containing rIL-2 acted as positive controls. Selected CS were also tested in the presence or absence of 11B11 anti-IL-4 or S4B6 anti-IL-2 monoclonals, which confirmed that the action on CT.EV cells could be fully ascribed to IL-2. Alternatively, CTLL cells were used to measure IL-2/4 levels. In this case, cells were pulsed with [^3H]TdR at 18–24 h, and inhibition of proliferation by anti-IL-2 was used to identify stimulation caused by IL-2. In the presence of anti-IL-2, residual proliferation is caused by IL-4 and is fully inhibitable by anti-IL-4 (see below). Although multiple dilutions and time intervals up to day 4 were tested, here we report the results of assays on 1:2 dilutions of CS collected on day 1 or 2, as optimal levels were found at this time. Essentially similar methods were used to measure IL-3, -4, and -5, with the following modifications.

IL-3 Assay. The B13 cell line (LyH7-B13) was maintained with 10 U/ml rIL-5 (CS of X6310 cell line expressing mRNA for IL-5 [21]). This line responds to both IL-5 and IL-3, and in the presence of anti-IL-5 Ab (TRFK.5 hybridoma CS at 1:20 dilution [22]), behaves in an IL-3-responsive manner. Under these conditions, proliferation is proportional to that of IL-3-responsive 32D cells (16 and our unpublished results).

IL-4 Assay. IL-4 dependent cell line CT.4S (20) was maintained in medium supplemented with 5% rIL-4 (CS of X6310 cell line expressing mRNA for IL-4 [21]). Results from this assay are concordant with results obtained from CTLL cells stimulated by CS in the presence of anti-IL-2 Ab (see above).

IL-5 Assay. B13 cells were maintained with rIL-5 produced from transfected X6310 cells as above. The proliferation inhibited by TRFK.5 Ab was taken to represent IL-5 activity (16). Residual proliferation in the presence of anti-IL-5 is probably caused by IL-3 (see above).

In Vivo Testing of Cell Lines

The cell lines were injected into mice intravenously within 4 h of intranasal infection. Mice were inspected daily for signs of illness, and weighed before and at intervals after challenge as a quantitative index of disease. All surviving mice were killed on day 4 and subjected to BAL, and lungs were homogenized for virus titration.

In experiment 1, 13 mice were infected with RS virus and another 12 were given control material intranasally (mock infection). Of the RS virus-infected mice, four were injected intravenously with 3×10^6 G-specific cells, two with similar numbers of 22K-specific cells, and four with both cell lines (total, 6×10^6 cells). Of the mock-infected mice, four were injected with G- and four with 22K-specific cells. The remaining mice were left as controls.

All cells were injected 3 d after the third cycle of antigen stimulation in vitro.

In experiment 2, 24 mice were infected intranasally with RS virus, 8 with influenza A, and 4 with mock antigen. Of the RS virus-infected mice, four were injected with 3×10^6 G-specific cells, four with a mixture of G and 22K cells at a 3:1 ratio (total, 4×10^6 cells), four with a 3:3 mixture (total, 6×10^6), four with a 1:3 mixture, and four with 3×10^6 22K-specific cells alone. All the mock-infected mice, four of the eight influenza-infected, and four uninfected mice were injected with a 3:3 mixture of cells. The remaining virus-infected mice were left as controls, without injection. In this experiment, all cells were injected 3 d after the fifth cycle of antigen stimulation in vitro.

In experiment 3, the in vivo effects of F- and G-specific cell lines were compared on day 3 after the third cycle of antigen stimulation. Groups of mice were infected intranasally with RS virus ($n = 15$), influenza ($n = 6$), given mock HEp2 antigen ($n = 6$), or left uninoculated ($n = 3$). Of the RS virus-infected mice, four were given 3×10^6 F-specific cells, a further four were given 3×10^6 G-specific cells, and four given both G- and F-specific cells (total, 6×10^6 cells per mouse). The remaining three mice were left without cell transfers. Half of the influenza or mock-infected mice were injected with both G- and F-specific cells, which were also injected into all three uninoculated mice.

Bronchoalveolar Lavage

Mice were given 3 mg pentobarbitone intraperitoneally and exsanguinated via the femoral vessels. The thorax was opened, and 1.2 mm portex tubing introduced into the trachea at the cricothyroid membrane. 1 ml of 12 mM lignocaine in PBS was washed in and out six times over a 1–2-min period to promote elution of adherent cells. 2 ml of hemolytic HBSS was added to the cell suspension for 5 min, and $150 \mu\text{l}$ was removed for spectrophotometric estimation of hemoglobin content (8). The remaining cells were washed and cytocentrifuge preparations made, which were fixed and stained with Giemsa's reagent. 300–500 cells per slide were identified and counted by oil immersion light microscopy.

Virus Titration in the Lung Tissues

After BAL, both lungs from each mouse were homogenized together in 1 ml of virus stabilization buffer as previously described (8). Homogenates were centrifuged at $10,000 g$ for 1 min and supernatants stored in liquid nitrogen before determination of virus titer in HEp2 cells were cultured in square, flat-bottomed 25-well plates. Wells were infected with dilutions of standards or homogenates in $500 \mu\text{l/well}$ of PBS for 1.5 h, and stained for RS virus at 18–24 h by immunoperoxidase. The theoretical limit of detection for this assay is ~ 5 PFU/mouse, or 25 PFU per gram of lung tissue.

Results

Phenotype of T Cell Lines. As in previous studies (17), splenic T cell cultures from 22K primed mice rapidly became dominated by CD4^+8^+ cells. A typical FACS[®] analysis on day 3 after the third cycle is shown in Fig. 1 C. These cells required added Con A supernatant to grow. The G-specific line was comprised mainly of CD4^+8^- T cells (Fig. 1 A), and continued to grow without addition of exogenous cytokines. The F-specific line also grew without added cytokines and on day 3 after the second cycle, contained 28%

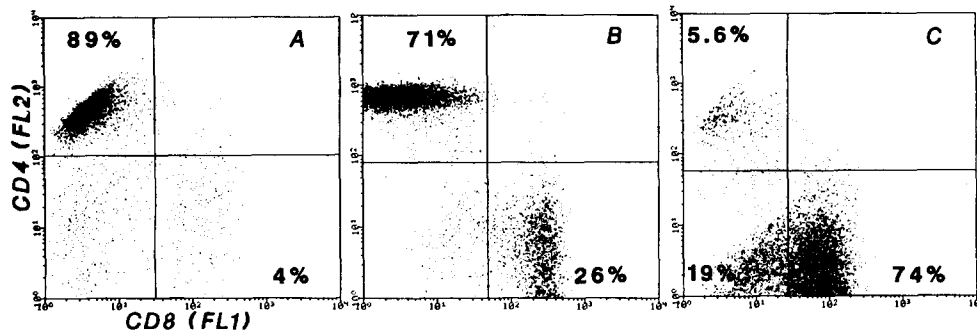


Figure 1. FACS[®] analysis of G-, F-, and 22K-specific cell lines. Cell lines from mice primed with rVV-G, -F, or -22K were expanded by cycles of antigen stimulation. Methods of derivation differed only in the RS virus protein to which the mice were sensitized, except that exogenous cytokines were added to maintain growth of 22K-specific cells as the level of endogenous CD4⁺ cells (and T cell help) declined in these cultures. Lines were stained with anti-CD4 coupled to GK1.5

PE and anti-CD8 coupled to 53-6.7 FITC. In these examples, the G line (A) is 89% CD4⁺8⁻ and 4% CD4⁺8⁺, whereas the F line (B) is 71% CD4⁺8⁻ and 26% CD4⁺8⁺ and the 22K line (C) is 5.6% CD4⁺8⁻ and 74% CD4⁺8⁺, with a further 19% in the CD4⁺8⁻ "tail" of cells, which appears to be continuous with the main CD8⁺ cell population.

CD4⁺8⁻ and 41% CD4⁺8⁺ cells. At this same time, a G line was 50% CD4⁺8⁻ and 26% CD4⁺8⁺. By the third cycle, their ratios had changed to 48 and 34% for F cells and 71 and 13% for G cells, respectively. A typical CD4/8 analysis of an F line after the fourth stimulation is shown in Fig. 1 B.

Functional Characteristics of Cell Lines In Vitro. From the third cycle of stimulation, cytokine production and cytotoxicity against P815 (H-2K^d) target cells showed stable characteristics. The 22K-specific line produced no detectable IL-2, IL-4, or IL-5 in response to antigen, but supernatants of antigen-stimulated cultures were rich in IL-3. By contrast, the G-specific line produced IL-5, IL-4, and IL-3 but not IL-2 in response to antigen (Table 1). The F-specific line released excess IL-2 but not IL-5 (Table 2).

In cytotoxicity assays against P815 cells, the 22K-specific line showed virus- and protein-specific cytotoxicity against cells infected with RS virus or rVV-22K. F-specific cells lysed P815 cells infected with RS virus or rVV-F (51 and 49% specific lysis, respectively, at killer/target ratio [K/T] 50:1), but did not cause significant lysis of targets infected with rVV-G (15%) rVV-βGal (8%) or uninfected targets (13%)

to a significant degree. The G-specific line cause <15% lysis of any of these targets at K/T ratios ≤50:1.

Signs of Illness After Cell Transfers. In all cases, mock-infected mice appeared well, with stable or slightly increasing body weights and were not affected by cell transfers. Influenza-infected mice appeared mildly ill, lost 3-5% of body weight by day 4 but were not affected by cell transfers. RS virus-infected mice generally appeared well unless injected with T cells.

In experiment 1, RS virus-infected mice not injected with cells showed a decline in weight to 93.2 ± 0.5% of starting weights by day 2, recovering thereafter to 96.4 ± 0.5 by day 4. By contrast, RS virus-infected mice injected with G, 22K, or both cell lines became rapidly unwell with lethargy, ruffled fur, tachypnoea, cyanosis, and progressive weight loss, which was most severe among mice injected with both cell lines (83.5 ± 4.2% of starting weight on day 4), followed by G recipients (87.9 ± 8%), and was least in 22K recipients (90 ± 3.8%).

In experiment 2, RS virus-infected mice did not lose weight except when injected with T cells. Those given G or 22K cells alone, and those given a 1:3 or 3:1 mixture of cells lost

Table 1. Cytokine Production by 22K- and G Protein-Specific T Cell Lines

	22K line		G line	
	Without Ag	With Ag	Without Ag	With Ag
	<i>cpm × 10⁻³</i>			
IL-2	0.2 ± 0.02	0.16 ± 0.02	0.29 ± 0.02	0.26 ± 0.05
IL-3	4.89 ± 1.16	14.67 ± 1.11	4.37 ± 0.79	14.5 ± 2.02
IL-4	0.16 ± 0.04	0.17 ± 0.02	0.80 ± 0.02	5.50 ± 0.54
IL-5	-0.25 ± 0.04	0.013 ± 0.001	0.35 ± 0.47	10.82 ± 0.59

Production of IL-2, -3, -4, and -5 by 22K- and G protein-specific T cell lines, expressed as thousands of cpm. Background proliferation of IL-2-responsive CT.EV cells (135 ± 38 cpm), IL-4 responsive CT.4S (84 ± 14), and IL-3/5-responsive B13 (478 ± 150) cells have been subtracted. Positive controls for each of these cell lines (5% CS from × 6310 cell lines producing IL-2, -4, or -5) were 11,601 ± 213; 2,939 ± 111; and 9,477 ± 787, respectively. The IL-5 readout is the inhibition of B13 [³H]TdR incorporation by anti IL-5 mAb TRFK-5, which leaves residual incorporation here ascribed to IL-3. The 22K line generates good levels of IL-3, but not IL-2, IL-4, or IL-5 (typical of CTL), whereas the G line generates IL-4 and IL-5, but not IL-2 (typical of Th2).

Table 2. IL-2 and IL-5 Production by F and G Cell Lines

	CTLL proliferation		B13 proliferation	
	No anti-IL-2	With anti-IL-2	No anti-IL-5	With anti-IL-5
	<i>cpm</i> × 10 ⁻³			
F line	5.65 ± 0.6	0.41 ± 0.55	8.84 ± 0.5	8.4 ± 1.3
G line	3.13 ± 0.85	2.87 ± 1.15	13.00 ± 1.6	4.6 ± 1.0

Cytokine production by F and G cell lines, expressed in thousands of cpm. Background proliferation of CTLL (480 cpm) and B13 (630 cpm) cells was not affected by respective anticytokines, and has been subtracted. Ab inhibition shows that the F line generates good levels of IL-2 but not IL-5, whereas the G line generates IL-5 but not significant IL-2. CS from the F line contains IL-3, which causes B13 cells to proliferate regardless of anti-IL-5 Ab. CS from the G line also contains IL-3 (accounting for nearly one third of the total B13 proliferation) and IL-4, which appears responsible for most of the CTLL proliferation (see 16 and 17 for further discussion).

10–20% of body weight by day 4. Those given 3:3 mixtures (total 6 × 10⁶ cells) lost 25.5 ± 2.0% of starting weight by day 4 (Fig. 2). Deaths due to the severity of augmented lung disease occurred between days 3 and 4 in one mouse given G-specific cells, and two mice given G and 22K cells at a 3:1 ratio. No deaths due to lung disease occurred in other groups or in other experiments, although three mice failed to recover from anesthesia.

In experiment 3, RS virus-infected mice maintained static weight unless simultaneously injected with T cells, in which

case they became ill by day 2 after transfers. Infected recipients of F-specific cells lost 6.7% and G-specific recipients 13.3% of starting weights by day 4. Simultaneous injection of both cell lines produced effects similar to G-specific cells alone up to day 3 (Fig. 3).

Effects of Transfer of Individual Cell Lines on BAL Parameters. In all cases, no significant lung hemorrhage, PMN efflux, eosinophilia, or lymphocytosis occurred in mock-infected mice regardless of cell transfers. Influenza-infected mice developed lymphocytosis and PMN efflux, but were again unaffected by cell transfers (e.g., experiment 3, influenza only: 9.1 ± 1.1% PMN; influenza with cell transfers: 6.1 ± 1.4% PMN,

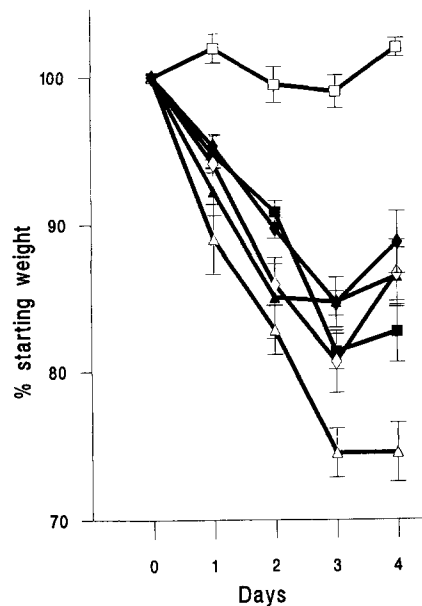


Figure 2. Weight loss caused by G- and 22K-specific T cell lines in RS virus-infected mice. Mean group weights ± SEM of RS virus infected mice in experiment 2, with or without cell transfers, calculated as percent of starting weight for each mouse. Groups are: (□) RS virus infection, no cells; (■) RS virus and 3 × 10⁶ 22K cells; (◆) RS virus and mixed cells (10⁶ G and 3 × 10⁶ 22K); (△) RS virus and mixed cells (3 × 10⁶ of each); (◇) RS virus and mixed cells (3 × 10⁶ G and 10⁶ 22K); (▲) RS virus and 3 × 10⁶ G cells. Control groups not infected with RS virus (i.e., infected with influenza or mock infected) were not affected by cell transfers and are not shown in the figure.

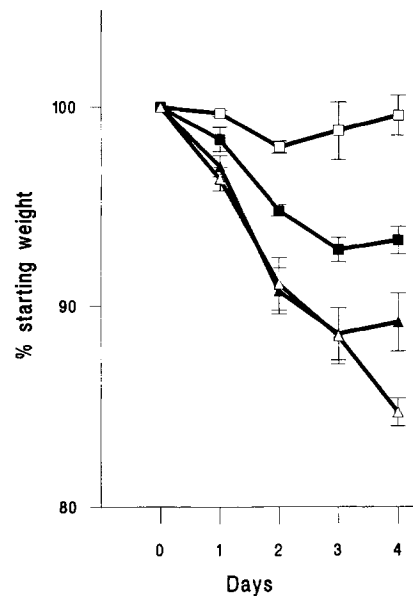


Figure 3. Weight loss caused by G- and F-specific T cell lines in RS virus-infected mice. Mean group weights ± SEM of RS virus-infected mice, with or without cell transfers in experiment 3, calculated as percent starting weight for each mouse. Groups are: (□) RS virus infection, no cells; (▲) RS virus and 3 × 10⁶ G cells; (■) RS virus and 3 × 10⁶ F cells; (△) RS virus and mixed cells (6 × 10⁶ total). Control groups not infected with RS virus (i.e., infected with influenza or mock infected) were not affected by cell transfers and are not shown in the figure.

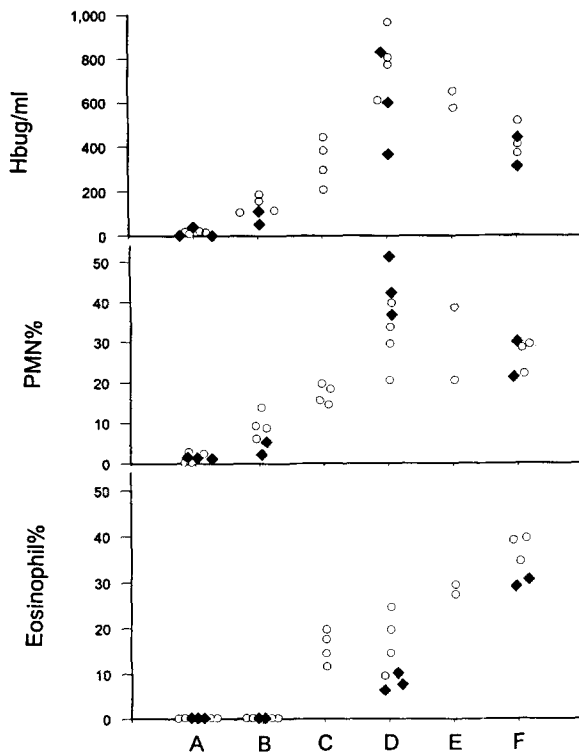


Figure 4. Pathological effects of G- and 22K-specific cell lines. Groups of RS virus-infected mice: (A) left without i.v. injection; (B) injected with 3×10^6 22K-specific cells; (C) 3×10^6 22K and 10^6 G-specific cells; (D) 3×10^6 of each line; (E) 10^6 22K and 3×10^6 G-specific cells; and (F) 3×10^6 G-specific cells. Cell transfers were done at the same time as RS virus infection, either after the third (experiment 1, \blacklozenge) or after the fifth (experiment 2, \circ) cycle of in vitro growth. Either cell line caused lung hemorrhage and PMN efflux into BAL fluid (shock lung), and the effects of combined cell transfers were additive in this regard. By contrast, only G-specific cells cause eosinophilic pneumonitis, the severity of which was reduced by the addition of 22K-specific cells. Neither cell line enhanced disease in influenza- or mock-infected mice (data not shown, see text).

$p = 0.2$; hemorrhage: $67 \pm 7 \mu\text{g/ml}$ without, $70 \pm 5.5 \mu\text{g/ml}$ with cells, $p = \text{NS}$). RS virus-infected mice developed mild pulmonary lymphocytosis as previously described (23), but no other pathological effects unless T cells were injected.

T cell injections only affected disease in RS virus-infected mice. In all experiments, such mice given G-specific cells showed brisk lung hemorrhage, PMN and eosinophil efflux. Those given 22K cells showed mild lung hemorrhage, occasional slight PMN efflux (5.7% in one mouse in experiment 1, Fig. 4), but not eosinophilia (Figs. 4 and 5). Injecting F-specific cells into such mice caused slight but significant lung hemorrhage (Fig. 5, top; 29 ± 3 in A vs $109 \pm 20 \mu\text{g/ml}$ in B, $p = 0.03$, Student's *t* test) but no PMN or eosinophil efflux.

Effects of Combined Transfer Different Cell Lines. Equal mixtures of G and 22K cells caused an increase in lung hemorrhage and PMN efflux, compared with the effects of either cell line alone. Combining the results of experiments 1 and 2, addition of 3×10^6 22K to 3×10^6 G cells enhanced PMN efflux from 26.8 ± 2 to $36.7 \pm 3.7\%$ ($p = 0.045$, Student's *t* test). By contrast, the eosinophilia seen in recipients of G-specific cells was reduced by coadministration of 22K

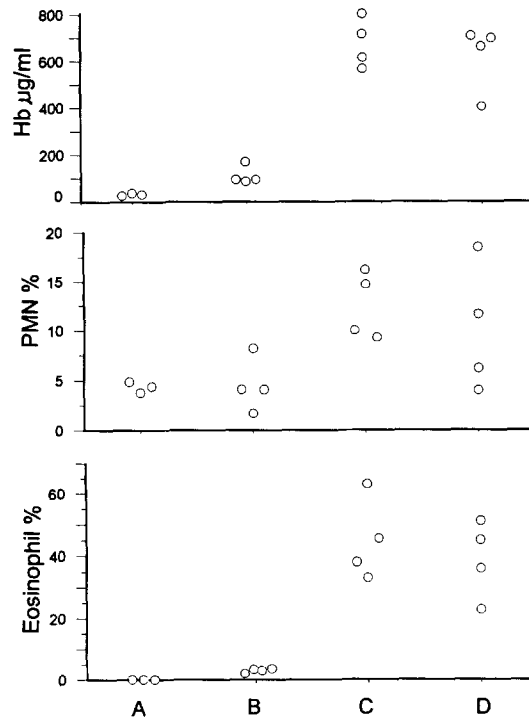


Figure 5. Pathological effects of G- and F-specific T cell lines. Groups of RS virus-infected mice: (A) no i.v. injection; (B) injected with 3×10^6 F protein-specific T cells; (C) injected with both cell lines (6×10^6 total); and (D) injected with 3×10^6 attachment protein G-specific T cells. On day 4, all mice were subjected to BAL for quantification of lung hemorrhage (BAL Hb, $\mu\text{g/ml}$, top) and differential cell counts (e.g., PMN% and eosinophil%, middle and bottom). The F-specific cells caused slight but significant lung hemorrhage (29 ± 3 in A vs $109 \pm 20 \mu\text{g/ml}$ in B, $p = 0.03$, Student's *t* test) but not PMN or eosinophil efflux. The G-specific cells caused severe lung hemorrhage ($618 \pm 72 \mu\text{g/ml}$), eosinophil, and PMN efflux. F- and G-specific cells together caused pathological effects similar to G cells alone. No additional pathological effects were caused by injection of T cells into uninfected, mock-, or influenza-infected mice (see text).

cells (e.g., 30.4 ± 0.3 vs $8.3 \pm 1.15\%$, $p = 0.003$ in experiment 2). Unequal mixtures produced immediate results (Fig. 4). Combined injections of F and G cells caused pathological effects similar to G cells alone (Fig. 5).

Effects of Cell Transfer on RS Virus Replication. In experiment 1, either the 22K or G cell line caused a significant ($p \leq 0.05$, Student's *t* test) reduction in lung virus titer compared with those not given cells, whereas coinjection of both cell lines eliminated virus completely (Fig. 6 A). In experiment 2, the same pattern was seen (Fig. 6 B; data from asymmetrical transfers not shown, but where no lung virus was detected). In experiment 3, RS virus-infected mice injected with F-specific cells had greatly reduced lung virus titers, whereas G-specific cells or combinations of F- and G-specific cells virtually eliminated virus from the lungs (Fig. 6 C).

Discussion

These studies show that injection of viral protein-specific T cell lines into naive mice coinfecting with RS virus can reduce virus replication in the lungs, but that each also produces

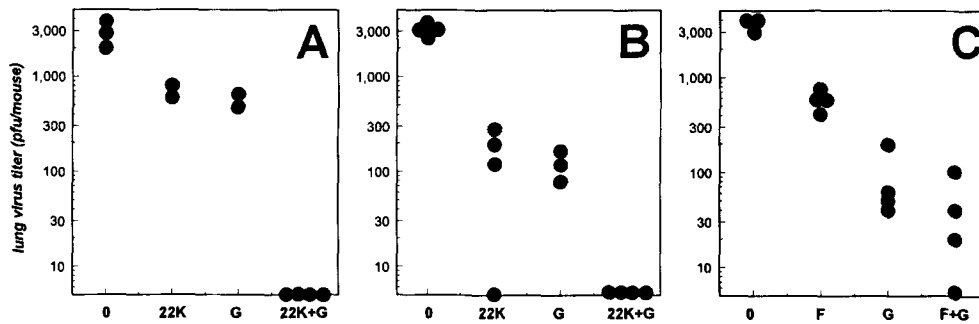


Figure 6. Antiviral effects of injection of T cell lines. Mice were subjected to BAL on day 4 after intranasal infection with RS virus, and lungs homogenized for determination of virus titer. The performance of BAL before lung virus recovery reduces lung virus titers (23), but does not invalidate the comparisons shown here. Results for individual mice are shown in infectious units (pfu) per mouse (y-axis). The theoretical limit of detection for this assay is ~ 5 PFU/mouse. Results

have not been corrected for lung weight, since acute changes in weight result from infiltration of fluid and not from changes in true pulmonary mass. Such a correction would falsely lower virus titers in inflamed lungs that were unaffected in their ability to support virus replication. In experiment 1 (A) 22K- and G-specific cell lines were tested by intravenous transfer after three cycles in vitro stimulation, and in experiment 2 (B) after five cycles. In C, the effects of F- and G-specific cells are shown. No virus could be isolated after coinjection of G- and 22K-specific cells, either with equal numbers (i.e., 3×10^6 of each, 22K+G or F+G) or unequal numbers (i.e., ratios of 3:1 or 1:3) of cells (data not shown).

a characteristic pattern of disease enhancement. The immunopathology in mice given specific T cells largely reproduces that seen in mice sensitized to the RS virus protein which the cells recognize, and correlates with the phenotypic and functional properties of the T cell lines in vitro.

First, RS virus-infected mice injected with T cells from mice primed with rVV-F develop mild disease augmentation of a similar pattern to that seen in mice sensitized by rVV-F infection (15). This recombinant induces strong RS virus-specific CTL (24) and Th cell (25) responses. The T cell line contained both CD4⁺ cells (which produced IL-2 but not IL-5) and CD8⁺ CTL.

Second, priming with rVV-22K (which expresses the principal target for K^d-restricted CTL and induces CTL in vivo [19]) leads to a cell line that is almost exclusively CD8⁺, has CTL (but not Th) activity, and induces more marked disease augmentation, with lung hemorrhage PMN efflux. This pattern of disease may be caused by CTL recognition of infected pulmonary epithelial cells, thereby resulting in a breakdown of the alveolar/capillary membrane.

Third, the G-specific cell line induces a more severe and quite distinct pattern of disease with severe eosinophilic pneumonitis similar to that seen in RS virus-infected mice sensitized with rVV-G (15). In vitro, the G-specific line releases IL-3, IL-4, and IL-5, but little IL-2. On the basis of results obtained from Th cell clones, Street et al. (26) and others have proposed that Th cells can be divided into Th1 (producing IFN- γ , IL-2, and IL-3) and Th2 (producing IL-3, IL-4, and IL-5, among other factors). One explanation for the present results is that the RS virus-specific Th cells induced by rVV-G are predominantly Th2 cells which produce cytokines that induce the differentiation, maturation, migration, proliferation, and survival of eosinophils. In murine leishmaniasis, cells that behave as Th1 cells protect against infection, whereas those that exhibit Th2 properties enhance DTH and the severity of the disease and are associated with chronicity (27, 28). These Th subsets may also be relevant to in vivo responses to other infectious agents such as schistosomes (29), listeria (30), and *Brucella abortus* and *Nippostrongylus* (26), but the studies described herein are the first to indicate that such Th

subsets might also explain pathological responses to viral infections.

These findings are largely consistent with our previous studies, in which polyclonal RS virus-specific T cell lines were separated into CD4⁺ and CD8⁺ T cell-enriched fractions by immunomagnetic adhesion. Transfer of CD4⁺, CD8⁺, or both cell fractions caused RS virus-infected mice to become ill and lose weight, whereas infection alone caused no overt illness. Either fraction also caused an increase in the severity of lung pathology (as monitored by BAL) with the appearance of lung hemorrhage and PMN efflux. In addition, recipients of CD4⁺ cells developed pulmonary eosinophilia. There is some evidence from these studies that coinjection of CD4⁺ and CD8⁺ cells may reduce the severity of pathology, compared with the effects of either subset alone (9). In these studies, mice given both G and 22K lines showed enhanced PMN efflux and reduced eosinophilia compared with those given 22K or G cells alone. F-specific cells alone caused minimal enhancement of disease, and did not much influence the disease caused by G-specific cells either in terms of weight loss or lung pathology. They did, however, exhibit antiviral effects.

In other experimental situations, sequential actions of CD8 and CD4 subsets may be required for antiviral DTH responses (31), and cooperation of both subsets may be required to protect against infection (32). Different subsets may have different roles at different sites (33, 34), and in different genetic strains of animal. For example, protective T cells that recognize the nucleoprotein of vesicular stomatitis virus are CD4⁺ in H-2^k mice, but CD8⁺ in H-2^b strains (35).

Although these studies highlight the potentially pathogenic role of Th cells that recognize the attachment protein G, they should not be taken to indicate that all subunit or recombinant vaccines containing G will be pathogenic, or that the same applies to other strains of mice or to other species. Mice seem to be more prone to pathogenic responses than cotton rats (10), and BALB/c mice may be particularly susceptible to eosinophilia (36) and perhaps to the development of CTL-induced immunopathology (37), thereby making them the best available model in which to define mechanisms

of disease augmentation. A second important issue is the timing and strength of the T cell response. These studies show what can happen if highly activated T cells are present at the start of infection. A less exuberant T cell response would be less likely to be pathogenic, and is probably an essential component of the protective immune response to any effective vaccine.

In addition to possible implications for vaccine development, the appearance of eosinophils in the lungs of sensitized RS virus-infected mice may be relevant to the pathogenesis of asthma in humans. Eosinophilia in the lung and blood is a frequent finding in asthmatics, in whom the bronchial epithelium is infiltrated with Th2-like lymphocytes (38). Wheezing is an important clinical sign of bronchiolitis, and respiratory viral infections are common precipitants of attacks

of asthma (39, 40). Children with a history of bronchiolitis often suffer respiratory symptoms typical of asthma in later childhood (41, 42) and respiratory morbidity in adult life may also be affected (43). The mechanisms by which viral infections might cause delayed effects recently have been reviewed (44).

In conclusion, different components of the immunopathological response to RS virus infection are associated with T cell recognition of particular viral proteins. A vaccine that induced moderate, balanced T cell responses and high levels of neutralizing, fusion-inhibiting Ab at the mucosal surface would appear ideal. Further studies of the immune and pathological responses to modified recombinant proteins may show how protective immunity can be induced without causing harmful reactions to subsequent RS virus infections.

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