Influenza Virus-specific CD4+ T Helper Type 2 T Lymphocytes Do Not Promote Recovery from Experimental Virus Infection

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Summary

T lymphocytes play a primary role in recovery from viral infections and in antiviral immunity. Although viral-specific CD8+ and CD4+ T cells have been shown to be able to lyse virally infected targets in vitro and promote recovery from lethal infection in vivo, the role of CD4+ T lymphocytes and their mechanism(s) of action in viral immunity are not well understood. The ability to further dissect the role that CD4+ T cells play in the immune response to a number of pathogens has been greatly enhanced by evidence for more extensive heterogeneity among the CD4+ T lymphocytes. To further examine the role of CD4+ T cells in the immune response to influenza infection, we have generated influenza virus-specific CD4+ T cell clones from influenza-primed BALB/c mice with differential cytokine secretion profiles that are defined as T helper type 1 (Th1) clones by the production of interleukin 2 (IL-2) and interferon γ (IFN-γ), or as Th2 clones by the production of IL-4, IL-5, and IL-10. Our studies have revealed that Th1 clones are cytolytic in vitro and protective against lethal challenge with virus in vivo, whereas Th2 clones are noncytolytic and not protective. Upon further evaluation of these clonal populations we have shown that not only are the Th2 clones nonprotective, but that pulmonary pathology is exacerbated as compared with control mice as evidenced by delayed viral clearance and massive pulmonary eosinophilia. These data suggest that virus-specific CD4+ T cells of the Th2 subset may not play a primary role in virus clearance and recovery and may lead to immune mediated potentiation of injury.

Both antigen-nonspecific effector mechanisms, e.g., natural killer cells, (1, 2) and antigen-specific effector mechanisms, e.g., T lymphocytes (3), play a crucial role in the host response to virus infection. Many studies over the past 20 yr have added to the understanding of the function and heterogeneity of T lymphocytes responding during virus infection. Early studies suggested that the CD4-8+ CTL is the crucial antigen-specific effector cell in the host immune response to viral infection (4). In the influenza model, the role that CD8+ CTLs play in the host immune response to this virus has been extensively examined (5–10). In early studies it was shown that CD8+ CTLs, which are restricted by class I MHC molecules, can lyse virally infected cells and promote recovery from viral infection in vivo. In addition, IFN-γ and other cytokines with antiviral activity, e.g., TNF-β, have been suggested to play a role in viral clearance mediated by CD8+ CTLs (10, 11).

A number of laboratories have examined the role of the CD4+8- T cell in host immune response to virus infection in general (3) and influenza infection in particular (12–16). Results from this laboratory demonstrated that influenza-specific CD4+ T lymphocytes can lyse virally infected targets in vitro and eliminate infectious virus and promote recovery from lethal experimental infection when clones of these CD4+ T cells are adoptively transferred in vivo (12). Studies involving transgenic mice homozygous for the β_2-microglobulin (β_2-m) gene disruption that lack functional class I MHC and CD8+ T cells, have demonstrated that influenza A virus can be cleared from the respiratory tract of these mice, thus suggesting the role of CD4+ T cells and natural killer cells as in vivo antiviral effectors (14). Additionally, work using athymic (nude) mice has added further evidence that influenza-specific class II–restricted T cells can promote B cell responses in vivo, and that antihemagglutinin (HA)[1] antibodies alone can mediate clearance of influenza virus (16).

The ability to further dissect the role that CD4+ T cells

[1] *Abbreviations used in this paper:* HA, hemagglutinin; HAU, HA units; HIFCS, heat-inactivated FCS; HPF, high powered fields; huR, human recombinant; RSV, respiratory syncytial virus.

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play in the immune response to a number of pathogens has been greatly enhanced by evidence for more extensive heterogeneity among the $CD4^+$ T lymphocytes (17). Stable long-term $CD4^+$ clones that secrete IL-2 and IFN-γ upon antigenic stimulation are classified as belonging to the Th1 subset, whereas those secreting IL-4, IL-5, and IL-10 belong to the Th2 subset. The functions of Th1 and Th2 clonal populations are markedly different. Although both Th1 and Th2 cells can provide help for B cell (antibody) responses, Th2 cells are more effective (18). Th1 clones preferentially induce delayed-type hypersensitivity and macrophage activation (19). For each of these functions, the profile of the cytokines secreted by Th1 and Th2 cells are the primary predictors of the differences in effector functions of these $CD4^+$ T cell subsets in vivo.

A number of infectious disease models have examined the differential functional role of Th1 vs. Th2 T cells. In the well described *Leishmania* model, Th1 and Th2 cells, and/or their respective cytokines, have different effects on the clearance of the microorganism during experimental infection (20–23). Recent studies on experimentally induced murine acquired immunodeficiency syndrome (MAIDS) have suggested that Th2 cytokines play a central role in the progression of that disease (24). In addition, work by Clerici et al. (25) has suggested that production of Th2 cytokines by HIV positive individuals may be a sensitive marker for progression to AIDS. However, at present there is very little information on the in vivo antiviral effector activity of $CD4^+$ T cells of the Th2 subset (26).

In the present study we have examined a panel of influenza-specific $CD4^+$ clones with cytokine secretion profiles that define them as belonging to either the Th1 or Th2 subset. We have found that clonal populations of influenza-specific Th1 T cells are cytolytic in vitro and protective in vivo, and that Th2 clones are noncytolytic in vitro and do not promote recovery in vivo. In addition, adoptive transfer of Th2 clones lead to massive pulmonary eosinophilia in the recipients and delayed viral clearance as evidenced by elevated viral titers as compared with control infected animals. These data demonstrate in a model of acute progressive viral infection that $CD4^+$ Th2 cells do not play a primary role in virus clearance in vivo, and that the induction of this in vivo effector activity may be detrimental to the host recovery process.

Materials and Methods

Animals. Murine pathogen-free male and female BALB/c (H-2^d), C57Bl/6 (H-2^b), and C3H (H-2^k) mice 4–6 wk of age were purchased from Taconic Farms, Inc., Germantown, NY and used at 6–9 wk of age.

Viruses. Influenza virus strains A/JAP/57 (A/JAPAN/305/57 [H2N2]), A/JAP/62 (A/JAPAN/170/62 [H2N2]), A/AA/67 (A/Ann Arbor/7/67 [H2N2]), A/TAI/64 (A/Taiwan/64 [H2N2]), A/BEL/42 (A/Bellamy/42 [H1N1]), A/MEM/71 (A/Memphis/1/71 [H3N2]), A/PR/8 (A/Puerto Rico/8/34 [H1N1]), and B/Lee (B/Lee/40) were grown in the allantoic cavity of 10-d-old embryonated hen's eggs and stored as infectious allantoic fluid as previously described (27). Recombinant influenza strains A/JAP/BEL (A/Japan/305/57 × A/Bellamy/42 [H2N1]) and A/X-7F1 (A/NWS/42 × A/RI/5/57 [H0N2]) were propagated in the same manner. Determination of virus titer, expressed as hemagglutinating units (HAU), was done as previously described (27).

Cell Lines. The A20-1.11 (H-2^d) B cell lymphoma (28) was maintained in 55% DMEM high glucose, (GIBCO BRL, Gaithersburg, MD) and 35% DMEM low glucose, (GIBCO BRL) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (HIFCS; Hyclone Laboratories, Logan, UT), 1% glutamine (GIBCO BRL), 1% nonessential amino acids (GIBCO BRL), 5×10^{-5} M 2-ME, and antibiotics (10 U/ml penicillin G and 10 μg/ml streptomycin sulfate [GIBCO BRL]). The P815 (H-2^d) Ia^- mastocytoma and MDCK cell line (10) were maintained in DMEM low glucose (GIBCO BRL), 10% HIFCS, 1% glutamine, and antibiotics.

Immunization of Mice for the Generation of Th2 and Th0 Clones. Details of the immunization protocol have been described elsewhere (29). Briefly, 6–8-wk-old female BALB/c mice were immunized in the hind foot pads with a 1:1 suspension of UV-inactivated influenza A/JAP/57 (50 HAU) in phosphate-buffered saline (PBS, pH 7.3) and incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). After 10–14 d, the animals were killed, and draining inguinal and popliteal lymph nodes were removed and processed through a tissue sieve (Bellco Glass Inc., Vineland, NJ). For in vitro secondary bulk cultures, processed lymph node cells were washed once in serum-free medium, treated with 1,000 HAU UV-inactivated virus, and plated in 6-well plates at a density of 25×10^6 cells per well in IMDM (GIBCO BRL), 10% HIFCS, 1% glutamine, 5×10^{-5} M 2-ME, and antibiotics (complete media).

Cloned T Lymphocyte Lines. The procedures developed to establish and maintain cytolytic influenza specific bulk cultures and clones from spleens of immunized mice are described in detail elsewhere (30, 31). The procedure to isolate clones from lymph node bulks is as previously described (29). Briefly, clones were derived by limiting dilution in 96-well flat-bottomed plates from day 7 in vitro secondary bulk cultures. Specifically, 0.5, 1, or 5 bulk culture cells were plated per well with 10^6 irradiated (2,000 rad), virally infected (1,000 HAU), syngeneic splenocytes in complete media, plus 10 U/ml human recombinant IL-2 (huRIL-2) (Biosource International, Inc., Camarillo, CA) as a source of growth factor. The clones derived using this method were restimulated every 10–14 d with irradiated, virally infected, syngeneic splenocytes in complete media plus 10 U/ml huRIL-2 and expanded to 6-well plates (No. 3506; Costar Corp., Cambridge, MA) containing 10^5/ml clone cells, 4×10^6/ml infected, irradiated, syngeneic spleen cells, and 10 U/ml huRIL-2 in complete media.

Cytokine Production. Supernatants from clones were harvested 48 h after stimulation in the absence of huRIL-2, and frozen at −20°C. Production of IFN-γ was assayed by ELISA as previously described with reagents provided by R. Schreiber (Washington University, St. Louis, MO) (32). Production of IL-4, IL-5, and IL-10 was determined using a previously described mouse cytokine ELISA protocol (33, Sehy, D. W. [PharMingen], personal communication). Briefly, the appropriate primary mAb (anti-mouse IL-4, IL-5, or IL-10; PharMingen, San Diego, CA) was diluted to 0.5–4 μg/ml in 0.1 M $NaHCO_3$ (pH 8.2) coating buffer, 50 μl was added to wells of an enhanced protein binding ELISA plate (Cat. no. 25805-96, Corning Glass Inc., Corning, NY), and the plates were sealed and incubated overnight at 4°C. The plates were then washed with PBS + 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) twice and blocked with PBS + 10% FCS for 2 h at room temperature. After washing the plate with PBS/Tween twice, standards

(IL-4, IL-5, and IL-10; PharMingen) and samples were added at 200 μl/well and incubated overnight at 4°C. The plates were washed four times with PBS/Tween, and then 100 μl of the appropriate diluted biotinylated anticytokine detecting mAb was added (0.5–4 μg/ml in PBS + 10% FCS) and allowed to incubate at room temperature for 45 min. The plates were washed six times with PBS/Tween and 100 μl avidin-peroxidase (Sigma Chemical Co.) diluted 1:400 in PBS + 10% FCS was added and allowed to incubate at room temperature for 30 min, after which the plates were washed eight times with PBS/Tween. Within 5 min of use, ABTS substrate (150 mg 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [from Sigma Chemical Co.] in 500 ml 0.1 M citric acid in ddH_2O, pH 4.35) was mixed with 10 μl H_2O_2 per 10 ml of substrate and plated 100 μl/well and allowed to develop at room temperature. ELISA plates were read at OD 405 nm using an automated microplate reader (model EL 340; Bio Tek Instruments, Inc., Winooski, VT). Quantities of each cytokine (IFN-γ, IL-4, IL-5, and IL-10) were extrapolated from standard curves generated for each ELISA assay.

Lung Virus Titration by MDCK Cell Plaque Assay. The MDCK cell plaque assay for titration of lung viral titers has been described in detail elsewhere (10). The following modifications to that protocol were made. After the lungs were homogenized and prepared according to protocol, serial dilutions from 10^{-1} to 10^{-8} were made in cold PBS supplemented with 0.75% BSA, 0.1 g/l $MgCl_2$, 0.1 g/l $CaCl_2$, and antibiotics. 50 μl of each dilution was added to the MDCK monolayer in duplicate and incubated at room temperature for 30 min. The remainder of the procedure is as previously described. Results are expressed as the mean plaque-forming units determined by the calculation [(Number of plaques per well)/0.05 ml] × 1/dilution factor.

Assays for Cell-mediated Cytotoxicity. The ^{51}Cr release cytotoxicity assay was carried out as previously described (27). Briefly, 10^6 target cells, either A20-1.11 or P815, were either left uninfected, infected with influenza A/JAP/57 or B/Lee in the presence of ^{51}Cr for 1.5 h at 37°C. Targets were then washed three times and plated 5×10^3 cells/well in 96-well, flat-bottomed, microtiter tissue culture plates in 0.1 ml vol of RPMI + 10% HIFCS. Effector cells were added in a 0.1 ml vol of RPMI + 10% HIFCS to appropriate wells in quadruplicate. E/T ratios ranged from 1:1 to 50:1 depending on assay. The plates were incubated at 37°C in 10% CO_2 for 6 h. From each well, 0.1 ml of supernatant was removed and counted on an Isomedic Gamma Counter (ICN Biomedicals, Inc., Costa Mesa, CA). The percent specific lysis was determined as previously described (10). SEM were always <5% of the mean value and are omitted.

Assay of Cellular Proliferation. The proliferative response of cloned T cell lines was assessed by [^3H]TdR incorporation, as previously described (34). Results are expressed as the mean cpm of quadruplicate cultures at the peak of the proliferative response (day 3 of culture). SEM were always <10% of the mean value and are omitted.

Intranasal Influenza Virus Inoculation. Intranasal inoculation of mice was performed as previously described (10). The procedure was modified from that described by using light anesthesia from methoxyflurane (Pitman-Moore, Mundelein, IL). To evaluate a dose response to intranasal virus, animals received serial 10-fold dilutions of allantoic fluid in cold PBS ranging from 10^{-2} to 10^{-6} and animals were watched daily for morbidity and/or mortality. LD_{50} values were calculated according to a modified Spearman and Karber method (35).

Adoptive Transfer Procedure. Adoptive transfer of day 7 viable cloned cells was performed as previously described (10). 8-wk-old male BALB/c mice were intranasally inoculated with 10 LD_{50} influenza A/JAP/57 virus and within 30 min, 10^7 clone cells in 0.5 ml IMDM (GIBCO BRL) were injected intravenously. Control mice were injected with 0.5 ml IMDM i.v. alone. Mice were watched daily for 21 d for morbidity and/or mortality. For determination of lung viral titers and histology, mice were killed on day 4 or 5 post adoptive transfer. The lungs were then aseptically removed at the bronchi, snap-frozen in liquid nitrogen, and frozen at −70°C until used.

Histologic Examination of Lungs. Frozen lungs harvested and described above were thawed in 10% buffered formalin and sent to American HistoLabs, Inc. (Gaithersburg, MD) where they were paraffin embedded and sectioned. Each lung specimen was stained with hemotoxylin and eosin or an eosinophil-specific stain, specifically Lennert Giemsa or Luna. Morphometry was performed to quantitate the number of eosinophils present. Sections of right lung from each group were examined under high power oil immersion (100×) with a total of 400 high powered fields (HPF) counted per mouse recipient.

Results

Generation and Characterization of CD4+ Th2 Clones. BALB/c mice were inoculated in the hind footpad with noninfectious A/JAPAN/57 virus in incomplete Freund's adjuvant as described (Materials and Methods). Draining lymph nodes from four mice were harvested and individual lymph node cell suspensions prepared. The lymph node cells were stimulated in vitro with noninfectious influenza virus. After 10 d, viable lymphoid cells were cloned under limiting dilutions conditions in the presence of A/JAPAN/57-infected, irradiated splenocyte stimulators in medium supplemented with 10 U/ml huRIL-2. From this cloning, thirteen stable T lymphocyte clones were isolated and characterized. All clones were $CD4^+$ and represented a heterogeneous array of $CD4^+$ T lymphocyte subsets including Th1, Th2, and Th0 phenotype clones (17, 36). Clones were maintained in continuous culture as described (Material and Methods).

From this panel, two $CD4^+$ clones, 5B4 and T5C8, were expanded for further characterization. These two clones displayed the pattern of antigen dependent lymphokine production characteristic of $CD4^+$ Th2 T cells. Both 5B4 and T5C8 secreted IL-4, IL-5, and IL-10, but no detectable IFN-γ in response to influenza virus (Table 1). Another $CD4^+$ T cell clone, G1, showed a typical Th1 pattern with secretion of IFN-γ in response to A/JAPAN/57 virus as previously reported (12). Neither 5B4 or T5C8 produced detectable IL-2 as described in a bioassay using a CTLL variant which is sensitive to both IL-2 and IL-4 (not shown).

Both clone 5B4 and T5C8 were MHC restricted in their proliferative response to the A/JAPAN/57 virus–treated splenocyte stimulators (Fig. 1 *a*) as has been previously reported for the Th1 clone G1 (12). These two Th2 clones did not proliferate in response to the antigenically unrelated type B influenza strain B/LEE (Fig. 1 *a*). To determine the target influenza polypeptides recognized by these Th2 clones, the proliferative response of these clones to type A influenza virus field strains and reassortant viruses was evaluated. The Th2

Table 1. *Cytokine Production by Th1 and Th2 Clones*

Cytokine produced	G1 (Th1)	5B4 (Th2)	T5C8 (Th2)
IFN-γ (U/ml)*	254	<1	<1
IL-4 (pg/ml)	<1	390	450
IL-5 (pg/ml)	<1	1,367	1,789
IL-10 (pg/ml)	<1	66	34

* 48-h culture supernatants from clones stimulated with irradiated, A/JAPAN/57-infected BALB/c splenocytes in the absence of huRIL-2 were assayed on at least two separate occasions for cytokine production by ELISA (Materials and Methods).

clone T5C8 and the Th1 clone G1 recognized virus strains expressing either the A/JAPAN/305/57 HA or strains of the H2N2 subtype possessing structurally related HA protein. This suggests that the Th1 clone G1 and the Th2 clone T5C8 recognize sites on the A/JAPAN/57 HA (Fig. 1 *b*).

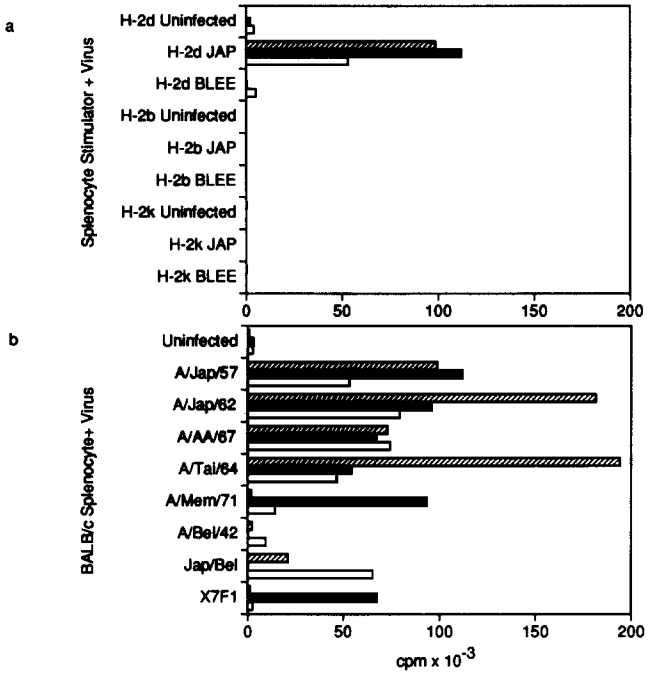


Figure 1. MHC restriction and fine antigen specificity of Th1 and Th2 clones. (*a*) 10^6 irradiated BALB/c (H-2^d), C57Bl/6 (H-2^b), and C3H (H-2^k) splenocytes were mock infected or infected with influenza A/JAPAN/57 or BLEE and cocultured with 2.5 × 10^4 clone cells for 72 h. Proliferation of clones G1 (*hatched bar*), 5B4 (*solid bar*), and T5C8 (*open bar*) as measured by [^{3}H]TdR incorporation from quadruplicate cultures is shown. Data is representative of four independent assays. (*b*) Clones G1 (*hatched bar*), 5B4 (*solid bar*), and T5C8 (*open bar*) were cocultivated with BALB/c (H-2^d) splenocytes infected with different strains of influenza A virus (see Materials and Methods). Proliferation as measured by [^{3}H]TdR incorporation from quadruplicate cultures is shown. Data is representative of three independent assays.

In contrast, clone 5B4 recognizes only virus strains possessing the N2 neuraminidase, suggesting that this clone recognizes a conserved site on the A/JAPAN/57 neuraminidase (Fig. 1 *b*).

Both Th2 clones were tested for cytolytic activity on A/JAPAN/57-infected A20-1.11 lymphoma cells that express I-A^d and I-E^d molecules. Neither clone lysed infected A20-1.11 cells, but as previously reported by us (12), the G1 clone did show specific cytolytic activity on A/JAPAN/57 infected, but not uninfected A20-1.11 cells (Fig. 2). To further test the cytolytic potential of T5C8 and 5B4, the two Th2 clones along with G1 were examined for lectin dependent cytolytic activity (37) by coincubation of cloned T cells with target cells in the presence of concanavalin A (5 μg/ml final in the assay) or phytohemagglutinin-P (50 μg/ml final in the assay). Only the cytolytic CD4$^+$ clone G1 showed lectin-dependent cytolytic activity on uninfected target cells (data not shown).

In Vivo Antiviral Effector Activity of CD4$^+$ Th2 Clones. To evaluate the antiviral activity of cloned Th2 T cells in vivo, clones 5B4 and T5C8 and the Th1 clone G1 were adoptively transferred into lethally infected syngeneic BALB/c mice. As reported previously (12), the CD4$^+$ Th1 clone G1 promoted recovery from lethal A/JAPAN/57 virus infection (Fig. 3). In contrast, neither 5B4 nor T5C8 were able to promote recovery from lethal infection after adoptive transfer (Fig. 3). This failure of the Th2 clones to promote recovery was repeatedly observed in four separate experiments carried out over 1 yr with cloned CD4$^+$ T cell populations maintained in continuous in vitro culture over that period.

In Vivo Effector Activity and Pulmonary Virus Titers. The recovery from lethal pulmonary influenza infection mediated by adoptively transferred CD8$^+$ and CD4$^+$ Th1 T cells is associated with reduction in pulmonary virus titers (10, 12). To assess the effect of the Th2 clones on virus clearance, lethally infected recipients of clones 5B4, T5C8, and the Th1 clone G1 were killed at days 4 and 5 post infection and T cell transfer,

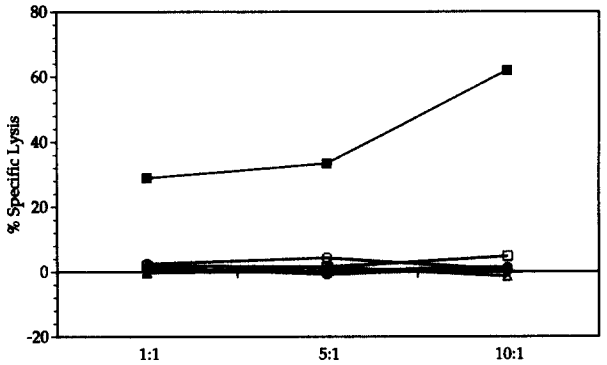


Figure 2. Cytolytic potential of Th1 and Th2 clones. Clones G1 (■), 5B4 (▲), and T5C8 (●) were tested for reactivity on uninfected (*open symbols*) or A/JAPAN/57-infected (*solid symbols*) A20-1.11 target cells as described (Materials and Methods). Spontaneous release from uninfected and infected target cells was <10%. Data is representative of at least three separate experiments.

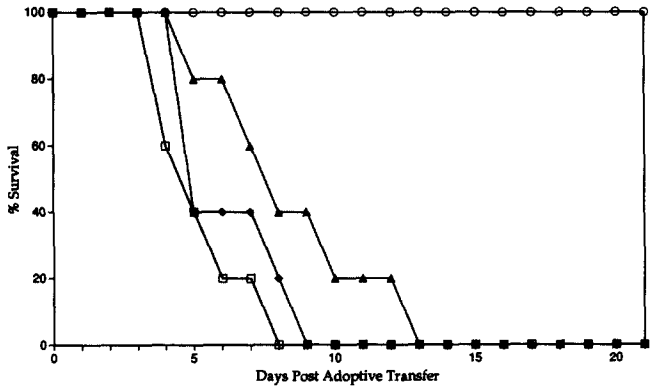


Figure 3. Adoptive transfer of Th1 and Th2 clones into lethally challenged BALB/c mice. Within 30 min of intranasal inoculation of a lethal dose of influenza A/JAPAN/57 into BALB/c mice, 10^7 clone cells (day 7 post stimulation) were injected intravenously. Mice were observed for 21 d for morbidity and mortality. Clones G1 (○), 5B4 (▲), and T5C8 (◆) were injected into groups of five animals each. Five control animals (□) received no clone. Results are representative of four experiments.

and pulmonary virus titers determined. As Table 2 shows, lung virus titers were decreased over 100-fold relative to controls in recipients of the $CD4^+$ Th1 clone G1. In contrast, the recipients of the Th2 clones 5B4 and T5C8 showed no decrease in pulmonary virus titers compared with control infected animals which received no cells. It is noteworthy that for recipients of either Th2 clone, lung virus titers were elevated relative to controls. This slight enhancement in virus levels at day 4 post infection and transfer was observed for each Th2 clone in two independent experiments.

In Vivo Morphologic Correlates of Th2 T Cell Activity. Lungs of control infected mice and recipients of the $CD4^+$ T cell clones were subjected to gross and microscopic pathologic analysis. Grossly, at day 4 post infection, the lung of control infected mice and infected G1 recipients were well aerated with focal areas of hemorrhage and collapse. In contrast, the lungs of the Th2 clone recipients showed extensive congestion and diffuse collapse (not shown).

The most striking difference in the response pattern of the Th2 clone recipients was evident at the microscopic level (Fig. 4). At day 4 post infection, lungs of control mice had histologic changes typical of early influenza pneumonitis with patchy involvement of medium and small airways, focal necrosis of respiratory epithelium, and peribronchiolar and intraalveolar infiltrate consisting of mononuclear and polymorphonuclear leukocytes (Fig. 4 *a*). Recipients of clone G1 showed few foci of inflammation with modest evidence of perivascular accumulation of lymphoblasts in focal areas of pneumonia (Fig. 4 *b*). In contrast, the lungs of 5B4 and T5C8 recipients were more diffusely involved with a dense accumulation of polymorphonuclear leukocytes around vessels and airway walls (Fig. 4 *c*) which was not evident in sections from control or G1 recipients.

When sections of lungs from infected control and clone recipients were stained with the Lennart's Giemsa or Luna stain to highlight eosinophils, extensive infiltration of the lungs of Th2 recipients by eosinophils was evident (Fig. 4 *d*). Fig. 5 shows quantitative morphometry data on the frequency and density of eosinophils in 400 microscopic fields taken from sections of control and clone recipients. More than 20% of fields from the lungs of Th2 recipients showed two or greater eosinophils per HPF. Sections from control or G1 recipients had <1% of fields showing significant eosinophil accumulation.

Table 2. *Pulmonary Virus Titers in Recipients of Th1 or Th2 Clones*

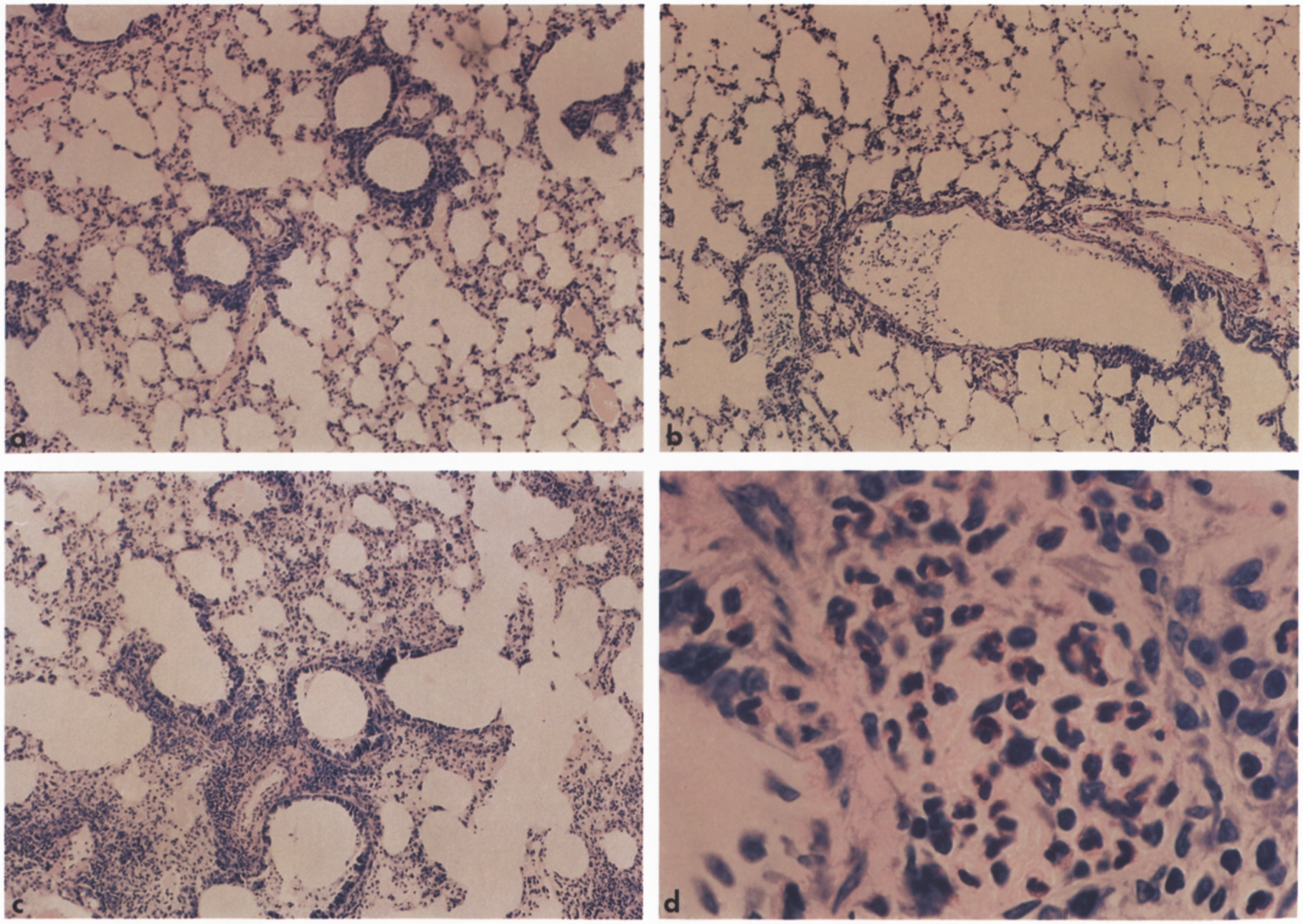
	Experiment 1*	Experiment 2	
Clone*	day 4	day 4	day 5
G1 (Th1)	2.0×10^{2}‡	5.1×10^{3}	ND§
5B4 (Th2)	3.9×10^{5}	4.0×10^{5}	1.9×10^{5}
T5C8 (Th2)	4.2×10^{5}	4.0×10^{5}	3.0×10^{5}
No clone	7.3×10^{4}	7.3×10^{4}	2.1×10^{4}

* 10^7 cloned T cells were injected intravenously into BALB/c mice infected with 10 LD_{50} of A/JAPAN/57 virus. Control mice were injected with 0.5 ml of serum-free media. On the indicated day, mice were killed and lung homogenates prepared for virus titration.
‡ Values are the plaque forming units of virus/ml of lung extract from individual mice at the indicated day post infection. Data are representative of results in four separate experiments.
§ Not done.

Discussion

In this report we demonstrate that clonal populations of $CD4^+$ T lymphocytes with different cytokine secretion profiles have distinctly different in vivo effector activities in response to pulmonary influenza infection. Specifically, two $CD4^+$ clones with a Th2 cytokine profile failed to promote recovery from lethal type A influenza infection after adoptive transfer, whereas a $CD4^+$ Th1 clone promoted recovery. Recipients of these two Th2 clones, however, did show a marked eosinophil infiltration in sections of their lungs. Also, whereas the Th1 clone G1 reduced pulmonary virus titers in infected recipients, the recipients of the two Th2 clones had slightly elevated pulmonary virus titers relative to control infected animals that received no T cells. These findings raise the possibility that in this model of experimental pulmonary virus infection, induction of a $CD4^+$ Th2 response may lead not to recovery from infection, but rather to an immunopotentiation of viral infection and injury.

The role of $CD4^+$ T lymphocytes in virus elimination and recovery from experimental virus infection is not completely understood (3). In the murine influenza model, early evidence suggested that induction of a $CD4^+$ T cell response during pulmonary infection leads to an increase in pulmonary inflammation and enhanced morbidity (38). At least one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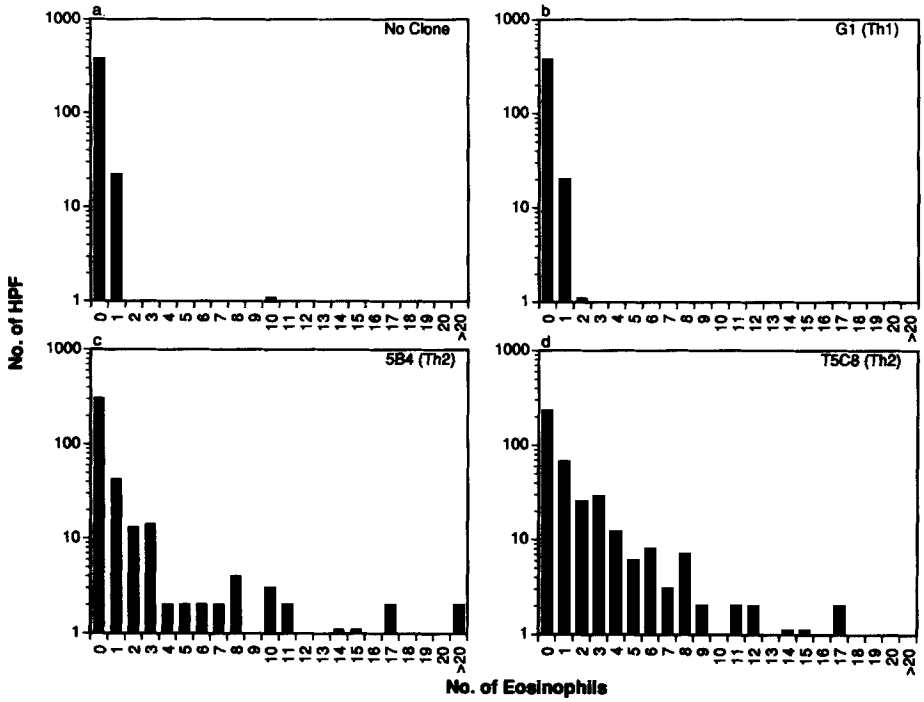

Figure 5. Quantitative morphometry on lung sections from mice adoptively transferred with Th1 or Th2 clones. 8-wk-old BALB/c mice were intranasally infected with a 10 LD_{50} dose of A/JAPAN/57 and injected with 10^7 clone cells or media alone within 30 min as described (Materials and Methods). Mice were killed and their lungs were aseptically removed on day 4 and subsequently sent for histologic sections and staining with the eosinophil specific Lennert Giemsa stain. Sections were examined by a blinded observer and 400 individual 100× HPF were evaluated per group. Data from each is group is shown separately, (*a*) No Clone, (*b*) G1, (*c*) 5B4, and (*d*) T5C8. The number of eosinophils counted per HPF is indicated on the x-axis and the number of HPF with that number of eosinophils is indicated on the y-axis. Data is representative of two separate experiments.

subsequent report is in agreement with this view (13). On the other hand, studies with clonal populations of activated influenza-specific $CD4^+$ T lymphocytes suggest that $CD4^+$ T cells can upon adoptive transfer into infected recipients, promote virus elimination from the lungs and recovery (12, 39). The findings reported here and our earlier studies (12) on the in vivo effector activity of the $CD4^+$ Th1 clone G1 are consistent with this view. Also, virus elimination and recovery from experimental pulmonary influenza infection has been reported in mice lacking functional $CD8^+$ T lymphocytes (14). This latter finding suggests that $CD4^+$ T lymphocytes can orchestrate recovery from influenza infection in the absence of $CD8^+$ T lymphocytes although the requirement for $CD8^+$ T cells in recovery may be dependent on the virulence of the challenge virus (40).

The mechanism(s) by which $CD4^+$ Th1 T cells like the G1 clone promote virus clearance and recovery from pulmonary virus infection are not well understood. Scherle et al. (39) have shown that adoptively transferred $CD4^+$ T cells can provide help for the production of neutralizing antiinfluenza virus antibody. This antibody response in turn leads to virus clearance from the lungs of infected T cell–deficient (nude) mice. Furthermore, these investigators have shown that transfer of neutralizing anti-HA antibody alone into infected SCID mice leads to influenza virus clearance from the lungs of these immunodeficient mice (41). To date, we have been unable to demonstrate enhanced antibody production after transfer of either the Th1 clone G1 or the Th2 clones, 5B4 or T5C8 into A/JAPAN/57-immunized nude mice (Graham, M. B., and T. J. Braciale, unpublished observations). The reasons for this are not clear. Differences in the B cell response to different influenza virus strains is one possible explanation for the apparent lack of B cell help observed with these clones. In the studies of Scherle et al. (39, 41) influenza viruses of the H1N1 and H3N2 subtype were used, whereas the A/JAPAN/57 virus used in our transfer study is of the H2N2 subtype. Influenza viruses of the H2N2 subtype have been reported to be mitogenic for B cells of the $H\text{-}2^d$ haplotype (42). Whether virus strain differences can account for the lack of $CD4^+$ T cell help and/or alteration in B cell function under our experimental conditions awaits to be determined.

In addition to help for antibody responses, $CD4^+$ T cells could promote recovery from infection by direct cytolysis of MHC class II positive virus–infected cells and/or by antigen dependent release of cytokines with antiviral activity. Recent evidence in the influenza model (43) suggests that an important inflammatory cytokine produced by $CD4^+$ Th1 T cells,

Figure 4. Adoptive transfer of Th2 clones leads to massive pulmonary eosinophilia. 4 d post intranasal challenge with a lethal dose of A/JAPAN/57 and adoptive transfer of 10^7 T cell clones, mice were killed and their lungs were removed, snap frozen, and stored at −70°C until examination (Materials and Methods). Lung sections were stained with the eosinophil specific Lennart's Giemsa stain. Representative 10× magnification (original) are shown in *a*, *b*, and *c*: (*a*) Control infected recipient, (*b*) Recipient of Th1 clone G1. (*c*) Recipient of Th2 clone 5B4. *D* shows a high power (100×) original magnification of an area with extensive infiltration in recipient of Th2 clone 5B4.

IFN-γ, may not be essential for the antiviral activity of CD4+ T cells in vivo in experimental influenza infection. The contribution of other T cell cytokines and contact dependent cytolytic activity is currently being examined.

The failure of the Th2 T cell clones to promote virus clearance and recovery was unexpected in view of the evidence that Th2 T cells augment antibody responses (18, 44). That the two Th2 clones were functional in vivo was evidenced by the eosinophil rich inflammatory response that they evoked in the recipient mice. Perhaps more surprising was the finding that Th2 recipients had slightly higher pulmonary virus titers than controls. This raises the possibility that the induction of a strong Th2 response during viral infection could suppress other host defense mechanisms. In view of the inhibitory role of the Th2 cytokine IL-10 on CD4+ Th1 T cell induction, (45), IL-10–mediated suppression of a CD4+ Th1 response could be one mechanism to account for elevated virus titers in the recipients of Th2 clones.

Our findings on the in vivo effect of virus-specific Th2 T cells are similar in some respects to recent results of Alwan et al. (26). In an experimental murine model of respiratory syncytial virus (RSV) infection, these investigators showed an eosinophil-rich exudate in bronchial lavage from RSV-infected recipients of RSV-specific CD4+ T cells enriched for the Th2 like cytokine activity. In that model, Th2 cells both enhanced morbidity and inflammation and enhanced viral clearance after adoptive transfer (26). Since RSV infection in mice is mild and self limited (46), it is possible that the antiviral effect as well as the immunopathologic effect of transferred Th2 T cells may reflect the same T cell effector mechanisms operating in an otherwise self limiting disease. It is also noteworthy that in the MAIDS murine model of immunodeficiency virus infection, the production of the Th2 cytokine IL-4 may play a central role in disease pathogenesis and progression (24). Additionally, induction of a Th2 T cell response has also been proposed to play an important role in the pathogenesis of human HIV infection (25).

In the current report we have used activated clonal populations of virus-specific CD4+ Th2 T cells to address the in vivo role this T cell subset may play in recovery from overwhelming viral infection. In our hands Th2 T cells represent the minority of the CD4+ T cell response definable in vitro at the clonal level (Graham, M. B., unpublished observations). Recent observations by Carding et al. (47) suggest a more complicated pattern of T cell responses to virus infection in vivo. These investigators reported that during experimental pulmonary influenza infection, both Th1 and Th2 cytokines are activated in the lung as determined by cytokine gene transcription. It is likely that the CD4+ T cell response during viral infection reflects a balance between antiviral effector mechanisms and inhibitory or regulatory mechanisms with no direct antiviral activity.

In conclusion our data suggest that during acute infection with a lytic virus, such as type A influenza, CD4+ T cells of the Th2 subset may not play a primary role in virus clearance and recovery. Furthermore, these data raise the possibility that the induction of a CD4+ T cell response against a lytic virus like influenza could lead to immune-mediated potentiation of injury. It will be important to determine the extent to which CD4+ Th2 T cells contribute to immune mediated pathology during viral infection and the mechanisms by which Th2 T cells produce injury.

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