Late Dominance of the Inflammatory Process in Murine Influenza by γ/δ^+ T Cells

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Summary

The inflammatory response in the lungs of mice infected with an influenza A virus consists largely of macrophages and $CD3^+$ T cells. Most T lymphocytes recovered before day 7 after infection express mRNA for the T cell receptor α/β (TCR-α/β), while TCR-γ/δ mRNA$^+$ cells are found at much higher frequency over the next 7 d. The predominant surface phenotype for the TCR-γ/δ mRNA$^+$ population is $CD3^+4^-8^-$ TCR-α/β^-. Some lymphocytes expressing all the known Vγ genes are found in the inflammatory exudate, but V$\gamma2^+$/V$\gamma1^+$ and V$\gamma4^+$ T cells are present at highest frequency. The response is staged, with maximal numbers of V$\gamma4^+$ cells occurring on day 10 after infection, while the predominant phenotype on day 13 is V$\gamma2$/V$\gamma1^+$. The emerging peak in numbers of V$\gamma4^+$ lymphocytes is paralleled by increasing numbers of macrophages expressing hsp mRNA. The later maxima found for the V$\gamma2^+$/V$\gamma1^+$ T cells is consistent with the possibility that at least some of these lymphocytes are responding to the hsp$^+$ cells and are functioning to resolve the inflammatory process.

Lymphocytes bearing the TCR-α/β heterodimer provide help for influenza-specific B cells and clear infectious influenza virus from mouse lung within 7–10 d of respiratory exposure (1, 2). In contrast, the part played by T cells expressing the TCR-γ/δ in the host response to this virus, or to any other pathogen, is not yet understood. Much speculation has concentrated on the possibility that the TCR-γ/δ lymphocytes represent a first line of defence against, in particular, bacterial infection (3–7). This idea is based on the following sets of observations. Most of the γ/δ T cells found in adult mice are located in surface epithelia (8–12). The repertoire of Vγ genes is very constrained, with characteristic Vγ phenotypes (9, 10) dominating the skin (V$\gamma5$), gut (V$\gamma7$), and reproductive tract (V$\gamma6$). A range of T cell hybridomas with reactivity to mycobacterial heat shock proteins (hsp)[1] are all V$\gamma1^+$ (6, 13), a proportion of the expanded T cell population found in lymph nodes after stimulation with mycobacteria is V$\gamma2^+$, (Pardoll, D., personal communication), and TCR-γ/δ^+ lymphocytes recovered from the lungs of mice exposed to mycobacterial antigens proliferate selectively after heat shock (14). The concept that many of the γ/δ T cells utilize a relatively limited repertoire to recognize ubiquitous ligands, such as hsp, induced by invading pathogens has thus gained ascendance.

We report here that cells expressing TCR-γ/δ mRNA are prevalent at the site of inflammation late in the course of influenza pneumonia. Although this appears to contradict the "early response, self-surveillance" (3) hypothesis for the γ/δ T cells, many of the macrophages in the inflammatory exudate that can be lavaged from the lungs of such mice express hsp mRNA. Maximal numbers of hsp$^+$ cells are found when the γ/δ^+ T lymphocytes are also present at high frequency, after the stage that infectious virus has been cleared. γ/δ T cells may therefore have a part to play in the resolution of the inflammatory process.

Materials and Methods

Mice. The 8–10-wk-old female C57Bl/6J mice used in these experiments were purchased from The Jackson Laboratory, (Bar Harbor, ME) and were maintained under specific pathogen-free (SPF) conditions.

Priming. The mice were inoculated intranasally (i.n.) with 30 μl of PBS containing a 1:5 dilution of either chick-embryo grown A/HKx31 (H3N2) influenza virus or normal chick allantoic fluid (2), following parenteral anesthesia with Avertin (2,2,2 tribromoethanol). This induces a severe, but generally nonfatal, pneumonia. The dose of virus was equivalent to 600 hemagglutinating units (HAU) and the inocula were free of bacteria and endotoxin.

[1] *Abbreviations used in this paper:* HAU, hemagglutinating units; hsp, heat shock protein; LIE, lung inflammatory exudate; SPF, specific pathogen free.

Lung Lavage Cells. Inflammatory cells were lavaged from the lungs of mice that had first been anesthetized and exsanguinated, as described previously (2). In some experiments the phagocytic cells in this lung lavage population were identified by incubation for 1 h in the presence of 2.051 μm latex beads (Dow Chemical Co., Midland, MI). Macrophages were removed by adhering cells (10^6/ml) to plastic petri dishes for 1 h at 37°C.

Hybridization Probes. ^{35}S-labeled RNA probes were synthesized by transcription in vitro of linearized pGEM™ plasmids (Promega Biotec, Madison, WI) containing TCR gene fragments. The Cγ and Vγ2-Jγ2-Cγ probes, which detect Cγ1 and Cγ2, consisted of a 460-bp XbaI-PstI fragment and a 550-bp KpnI-PstI fragment respectively, of the cDNA clone 4/203 (15). The Vγ2 probe, which detects Vγ1 and Vγ2, consisted of a 202-bp PstI-XbaI fragment derived from the clone 4/203 (15). A 158-bp HindIII-ClaI fragment, obtained from the cDNA clone TC-17 (16), was used as a Vγ4-specific probe. The Vγ5 probe consisted of a 213-bp EcoRV-KpnI fragment obtained from the clone TC-13 (16). A 182-bp KpnI-SphI fragment, derived from the clone TC-11 (16), was used as a Vγ6-probe. The Vγ7 probe consisted of a 350-bp EcoRI-BglI cDNA obtained from Dr. Yohtaroh Takagaki (Boston, MA). The Cβ probe was a 650-bp EcoRV-ScaI fragment derived from pHDS11 (17) and detects Cβ1 and Cβ2. The Cα probe was derived from the clone pHDS58 (18). The CD3 probe, which detects the δ chain of the CD3 complex, was isolated from the clone pPgBC9 provided by Dr. Cox Terhorst (Boston, MA). The Cδ probe was a 300-bp EcoRI-HindIII fragment derived from a T cell clone cDNA library (Hayday, A., unpublished observations).

Analysis for mRNA$^+$ Cells. Paraformaldehyde-fixed, cytocentrifuge preparations of lung inflammatory cells (pooled from three to six mice) were analyzed by in situ hybridization as described previously (19) using ^{35}S-labeled, TCR-specific, antisense mRNA probes. For histological analysis, intact lungs from infected B6 mice were snap-frozen, and 5-μm sections were placed on silane-treated microscope slides (20) and post-fixed in 4% paraformaldehyde at 20°C for 20 min before in situ hybridization. Counts for at least 1,000 positive cells from each coded slide were used to determine the frequency of cells expressing a particular phenotype, the negative control being the values determined from control slides exposed to probes transcribed in the sense orientation. The hsp probe was derived from mRNA isolated from a TCR-γ/δ$^+$ hybridoma (13), and has been shown by sequence analysis to be the murine homologue of the bacterial hsp65 (Kyes, S., R. L. O'Brien, W. K. Born, and A. Hayday, unpublished observations).

Lymphocyte Separation. Inflammatory cell populations were first depleted of macrophages by plastic adherence and then stained with T cell–specific antibodies for single color cell sorting (21) using a FACStar Plus (Becton Dickinson, Mountain View, CA). The mAb reagents used were the rat anti-CD3-1, YCD3 (22), and the hamster anti-TCR-α/β, H57.597 (23). Lymphocytes were stained with the primary reagent for 30 min, washed, stained with mouse anti–rat FITC (Jackson Immunoresearch, West Grove, PA) or goat anti–hamster FITC (Southern Biotechnology Associates, Birmingham, AL) for a further 30 min and then pelleted through FCS.

Results

The number of cells that can be lavaged from the respiratory tract of B6 mice infected intranasally with the HKx31 virus increases by a factor of >20-fold, with maximum leukocyte counts occurring 7 d after infection (Fig. 1 *a*). This lung inflammatory exudate (LIE) contains substantial numbers of T cells and plastic-adherent macrophages (Fig. 1 *a*), but few B cells (2). The CD4/CD8 T cell ratio is ~1:3 and ~1:500 of the nonadherent population can be shown to be influenza-specific, class I MHC-restricted CTL precursors (2).

Localization of γ/δ T Cells to the Lung. Cells expressing TCR-γ/δ RNA were readily identified by in situ hybridization analysis (15) of frozen lung sections from influenza-infected mice (Fig. 2 *a*). Very few γ/δ RNA$^+$ cells were found in sections from noninfected mice, or from controls that were dosed intranasally with allantoic fluid (data not shown). Comparable analysis of the lung lavage cells with TCR-specific probes indicated that the majority of the nonadherent T cells present at 5–7 d after infection were TCR-α/β$^+$ (Fig. 1 *b*). However, the lymphocyte component of the LIE obtained at 10–15 d after infection was dominated by the TCR-γ/δ$^+$ population (Figs. 1 *b* and 3 *a*), which represented between 15 and 30% of nonadherent LIE cells in more than 10 independent experiments. The total number of LIE T cells (CD3 mRNA$^+$) cells expressing TCR-γ/δ genes increased dramatically between 5 and 13 d after infection (Fig. 1 *c*). In addition, many of the γ/δ$^+$ lymphocytes appeared to be dividing (Fig. 2 *b*), indicating that this population is not simply comprised of intra-epithelial lymphocytes that are shed passively as a consequence of virus-induced damage to the respiratory mucosa.

The nonadherent LIE population was sorted for lymphocytes expressing cell surface TCR-α/β receptors or CD3. The percentage of cells positive for Cα or Cβ mRNA was maximal in the TCR-α/β$^+$ set, while the frequency of cells positive for Cγ and V-J-Cγ mRNA was high in the TCR-α/β$^-$ set (Exps. 1 and 2, Table 1). Also, virtually all of αβ and γ TCR mRNA$^+$ cells were CD3$^+$. The expression of cell-surface CD3 on most of the γ mRNA$^+$ population and the enrichment of γmRNA into the TCR-α/β$^-$ sorted set

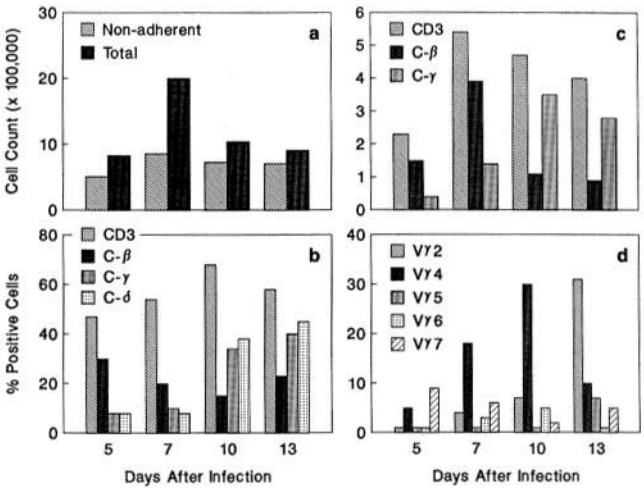

Figure 1. The composition of pooled lung-lavage cells from B6 mice that were infected intranasally with a nonlethal dose of the HKx31 (H3N2) influenza A virus showing: (*a*) The total number of lung-lavage cells per mouse recovered before and after adherence on plastic (10^6 cells/ml) for 1 h at 30°C; (*b*) the frequency of nonadherent LIE cells expressing CD3- and TCR-encoded genes; (*c*) the number of nonadherent LIE cells that express mRNA for CD3, TCR-β, TCR-γ, or TCRδ; and (*d*) Vγ gene segment expression in nonadherent LIE cells.

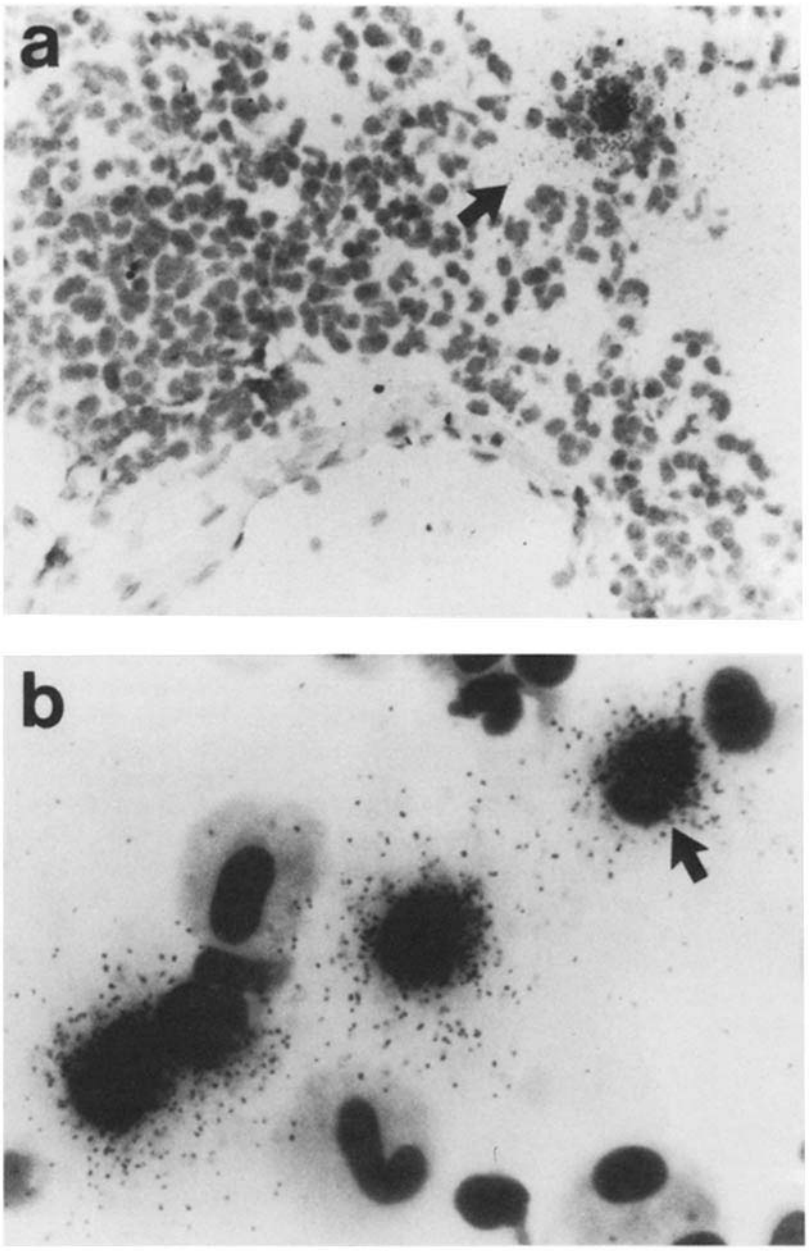

Figure 2. Cells expressing TCR-γ mRNA were detected in sections of lung (*a*) and in cytocentrifuge preparations of lung-lavage cells (*b*) 10 d after infection with the HKx31 virus. The arrows show a positive cell in situ (*a*) and an example of a cell expressing TCR-γ mRNA that appears to be dividing (*b*).

of cells indicates that these lymphocytes are very likely expressing the TCR-γ/δ heterodimer.

Relatively few of the γ mRNA$^+$ cells express either CD4 or CD8, while the converse is true for lymphocytes positive for Cα or Cβ (Table 2). Complement-mediated lysis of nonadherent LIE cells with a mAb to CD8 eliminated most of the lymphocytes with TCR-α/β mRNA, but greatly enriched for the γ/δ set (Table 2). This effect was less marked after CD4 depletion, reflecting that the inflammatory exudate contains fewer CD4$^+$ T cells (2).

Distribution of Vγ Phenotypes. The spectrum of known Vγ gene segments (26) could be detected in the LIE cells. The probe used here for Vγ2 does not distinguish between Vγ1 and Vγ2, so any Vγ1$^+$ cells that are present are included in the set labeled Vγ2. The most prevalent phenotype was Vγ4 until 13 d after infection, when lymphocytes utilizing the Vγ2 (or Vγ1) gene were present at high frequency (Figs. 1 *d* and 3 *b*). This pattern in the distribution profiles of Vγ2$^+$ (or Vγ1$^+$) and Vγ4$^+$ T cells was seen consistently for at least seven, independently derived pools of LIE cells from infected mice.

Lack of Secondary Infection and Expression of hsp mRNA. The dominance of the lymphocyte component of the inflammatory exudate by TCR-γ/δ mRNA$^+$ cells from day 10, which is after the time that infectious virus is normally cleared from the lung (1, 2), indicates that this set of lymphocytes could be responding to something other than viral proteins/peptides. Viral mRNA could not be detected by PCR

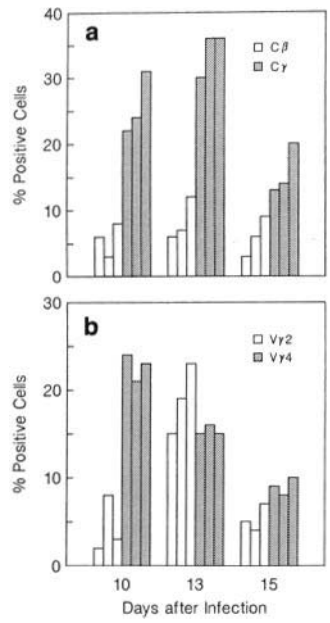

Figure 3. Patterns of TCR Cβ and Cγ (*a*), and Vγ2 (or Vγ1) and Vγ4-gene (*b*) segment expression are shown for nonadherent LIE cells from three separate pools (each from three B6 mice) infected with HKx31 virus 10–15 d previously.

analysis in the lungs of mice beyond 10 d of infection (Eichelberg, M., M. Wang, W. Allan, R. G. Webster, and P. C. Doherty, manuscript in preparation). Lungs from each of 5 mice sampled at 0, 5, 7, 10, and 13 d after infection with the HKx31 influenza virus were thus checked for the presence of bacteria (including mycoplasma) and fungi. No evidence of significant secondary infection was found at any time point (data not shown).

Possibly the γ/δ T cells are specific for endogenous ligands induced as a consequence of the infectious process, the obvious candidate being an hsp (6, 13, 14). Lung lavage cells from mice infected 13 d previously were thus probed for expression of the murine homologue of mycobacterial hsp65. Analysis of a day 13 LIE population established that the majority of the hsp mRNA$^+$ cells are removed by plastic adherence, while the frequency of lymphocytes expressing TCR genes is enriched by this procedure (Table 3). The impression that most of the hsp is in macrophages was confirmed in a time course experiment utilizing engulfment of latex particles as a marker for LIE populations that had not been adsorbed on plastic (hsp$^+$latex$^+$, Fig. 4 *a*). However, it was also apparent that not all the hsp$^+$ cells are phagocytic (Total hsp$^+$, Fig. 4 *a*).

Table 1. *Distribution of TCR mRNA in Sorted LIE Populations*

		Percent positive cells				
Exp.	Probe	Unseparated	α/β^-	α/β^+	CD3$^-$	CD3$^+$
1	Cβ	5	2	38	2	10
	Cγ	24	44	3	4	39
2	CD3		48	51		
	Cβ		5	43		
	Cα		4	47		
	Cγ		49	2		
	V-J-Cγ		44	2		

Lung lavage cells from B6 mice infected intranasally 10 d previously were depleted of macrophages by plastic adherence and stained for single-color flow cytometric separation, as described in Materials and Methods.

Table 2. *Consequences of Depleting CD4$^+$ or CD8$^+$ T Cells from the LIE Population*

	Percent cells positive 10 d after infection*		
Probe	Intact	CD4$^-$	CD8$^-$
Cα	9	19	3
Cβ	15	21	7
Cδ	18	29	57
Cγ	17	24	50
CD3	27	47	71

* The inflammatory cells were depleted of macrophages by plastic adherence and then exposed to either the RL172 (24) mAb to CD4 or the 31M (25) mAb to CD8 before incubation in rabbit complement.

Table 3. *Expression of a Murine Heat-Shock Protein (hsp65) Gene in Adherent Lung-Lavage Cells*

	Percent positive cells	
Probe	Total cells	Nonadherent cells
HSP65*		
Antisense	27.6	2.1
Sense	1.4	1.5
TCR		
CD3	9.3	48.9
Cγ1	6.8	31.3
Cβ	4.1	16.4

LIE cells were obtained from B6 mice 13 d after infection with HKx31 virus and analyzed for hsp and TCR gene expression, with (nonadherent) or without (total) prior adherence to plastic.

* A cDNA encoding the murine homologue of the bacterial hsp 65 gene was derived from mRNA isolated from a γ/δ$^+$ hybridoma (13) with reverse transcriptase before amplification in the PCR. Products were ligated into pGEM4Z™ plasmid, sequenced to verify the identity of the cDNA, and used to generate ^{35}S-labeled sense and antisense mRNA probes for hybridization in situ with LIE cell populations.

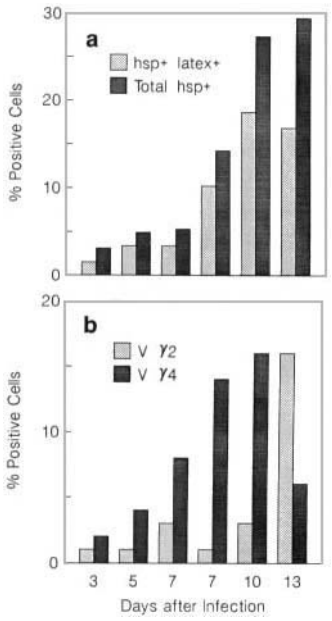

Figure 4. The percentages of hsp mRNA$^+$ LIE cells, some of which had phagocytosed latex particles, are shown for two experiments in *a*. The female B6 mice were infected intranasally with the HKx31 influenza virus and sampled on days 3–7 or days 7–10. The distribution of Vγ2$^+$ (or Vγ1) and Vγ4$^+$ cells is illustrated in *b*.

The frequency of hsp$^+$ cells (Fig. 4 *a*) early in the course of the infectious process is no greater than that found for control mice given chick allantoic fluid intranasally (day 3, 7.8%; day 5, 2.4%; day 7, 4.0%). Even so, the total number of inflammatory cells that can be lavaged from the lung is 10 to 20 times greater following influenza infection so there are many more hsp$^+$ cells present. The kinetics of the increased prevalence of hsp$^+$ latent macrophages parallels the rise in frequency for the Vγ4$^+$ T lymphocytes, while the peak for the Vγ2$^+$ (or Vγ1$^+$) subset occurs later (Fig. 4, *a* and *b*).

Discussion

The prevalence of TCR-α/β mRNA$^+$ cells in the LIE declines with virus clearance, which is already apparent by day 7 after infection (2). This is consistent with findings from cell transfer experiments, using both immune lymphocyte populations and T cell clones, that infected cells are eliminated by CD8$^+$ effectors that recognize influenza virus peptides presented in the context of class I MHC glycoproteins (1, 27, 28). The acute-phase α/β T cell response in murine influenza is thus a classical example of T cell–mediated immunity (CMI) in a virus infection, taking ~6 d to develop in the regional lymph node after exposure to large amounts of antigen (self MHC+viral peptide), and resolving quickly in the lung following the termination of the infectious process. The CD3$^+$ component of the LIE then becomes dominated by γ/δ mRNA$^+$ cells, the numbers of which remain high until at least 13 d after infection. The increase of these lymphocytes in the virus-infected lung is paralleled by the induction of the genes encoding the 65-kD heat shock protein in the inflammatory macrophages. Though α/β T cells can be specific for bacterial hsp (29), there was no correlation between the appearance of high numbers of hsp$^+$ macrophages and TCR-α/β mRNA$^+$ lymphocytes in the LIE.

Early after the induction of inflammation, some lymphocytes representing all the known Vγ phenotypes are present in the lung lavage population. This initial phase might reflect random recruitment of γ/δ T cells from the available phenotypes in blood, together with involvement of any γ/δ T cells that are already present in the lung. Influenza A viruses can cause severe damage to epithelia and blood/tissue barrier systems (30) in the absence of a specific cell-mediated immune (CMI) or antibody response. Analysis with other acute virus infections (31) would suggest that this nonspecific inflammatory process then gives way (from about day 6 after infection) to a process of cellular recruitment mediated essentially by virus-specific, CD8$^+$ α/β^+ lymphocytes. These T cells promote the localization of large numbers of monocyte/macrophages and "resting" T lymphocytes from the recirculating pool. Hence, a central question concerning the cells in the LIE that express γ/δ mRNA is whether they are responding specifically to the events occurring in the virus-infected lung, or are simply bystanders drawn in during the course of the conventional α/β T cell–induced CMI. The magnitude, kinetics, and selectivity of the γ/δ T cell response in influenza pneumonia indicate that the former may be the case.

Although cells expressing a variety of Vγ mRNA can be detected, the response is dominated by cells expressing either Vγ4 or Vγ2/Vγ1. Many of these lymphocytes are dividing (Fig. 2), implying that such cells may be playing an active part in the disease process. The present experiments raise the possibility that the kinetics of the late response by the Vγ2$^+$/Vγ1$^+$ lymphocytes is in some sense comparable to that found for the conventional α/β T cells. The difference may be in the timing of the expression of high concentrations of the putative target antigens, hsp for the Vγ2$^+$/Vγ1$^+$ T cells and viral peptide+class I MHC glycoprotein for the TCR-α/β^+ effectors, respectively. The numbers of hsp mRNA$^+$ macrophages in the LIE increase steadily between days 7 and 10 after infection, while the counts for Vγ2$^+$/Vγ1$^+$ TCR mRNA$^+$ cells peak on day 13. Thereafter the inflammatory process in the lung resolves, suggesting that the Vγ2$^+$/Vγ1$^+$ T cells may eliminate hsp$^+$ macrophages.

The earlier dominance (particularly on day 10) of the γ/δ T cell component in the inflammatory process by the Vγ4$^+$ set also indicates that these lymphocytes are specifically responding. Perhaps, together with the α/β T cells, the Vγ4$^+$ population is secreting cytokines (32, 33) that maintain macrophage activation and help to minimize possible secondary infection. The induction of hsp in the majority of the adherent cells, which does not obviously correlate with the presence of infectious virus, could reflect the operation of this mechanism.

An important difference between the α/β and γ/δ T cells in virus-specific cell-mediated immunity may be that the target antigen is completely absent before an encounter with the invading pathogen in one case (α/β) but not in the other (γ/δ). Laboratory mice do not normally encounter H3N2 influenza A viruses, while mammalian hsp is likely to be in-

duced in a great variety of situations (34). The slower kinetics of response could indicate that the threshold for lymphocyte proliferation, which can be demonstrated for both α/β and γ/δ T cells in mice with influenza (2), is different for the two sets of lymphocytes. Because of the frequency with which autologous ligands such as hsp might be expected to occur, there may be a strong evolutionary selection against a capacity for γ/δ T cells to mount a potent response before encountering substantial numbers of hsp^+ targets. The same is probably true for the induction of effector function.

Given the sparse distribution of γ/δ T cells in normal lung sections from our SPF mice, it seems possible that the large numbers of $V\gamma2^+/V\gamma1^+$ and $V\gamma4^+$ cells that are localizing to lung following influenza infection are being generated in lymphoid tissue (32, 33, 35) and are, like any other T cell, entering the site of pathology from the circulation (29). Whatever its origin or basis, it does seem apparent that high levels of γ/δ expression are a preeminent feature late in this viral pneumonia.

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