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Reduced Functional Capacity of CD8$^+$ T Cells Expanded by Post-Exposure Vaccination of γ-Herpesvirus-Infected CD4-Deficient Mice[1]

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Mice (I-A$^{b-/-}$) that lack CD4$^+$ T cells remain healthy for at least three months after respiratory exposure to the murine γ-herpesvirus 68 (γHV68), then succumb with symptoms of chronic wasting disease. Postexposure challenge of γHV68-infected I-A$^{b+/+}$ and I-A$^{b-/-}$ mice with a recombinant vaccinia virus (Vacc-p56) expressing an antigenic γHV68 peptide caused a massive increase in the numbers of D^{b}p56-specific CD8$^+$ T cells. Previous experiments showed that, despite the large numbers of potential CTL effectors, there was little effect on the long-term survival of the CD4-deficient group and no diminution in the level of persistent virus shedding and latency. Comparison of the expanded CD8$^+$D^{b}p56$^+$ sets in the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice indicated that these two T cell populations were not identical. More CD69^{high}CD8$^+$ D^{b}p56$^+$ T cells were found in the CD4-deficient mice, an effect that might be thought to reflect higher Ag load. By contrast, the mean fluorescence intensity of staining for the CD44 glycoprotein was diminished on CD8$^+$D^{b}p56$^+$ T cells from the I-A$^{b-/-}$ group, the level of CTL activity was lower on a per cell basis, and the relative prevalence of IFN-γ^+TNF-α^+ T cells detected after in vitro stimulation with the p56 peptide was decreased. Given that this experimental system provides an accessible model for evaluating postexposure vaccination protocols that might be used in diseases like HIV/AIDS, the further need is to clarify the underlying molecular mechanisms and the relative significance of lack of CD4$^+$ T help vs higher Ag load for these expanded CD8$^+$ effector populations. ***The Journal of Immunology,*** **2002, 168: 3477–3483.**

Infection with the γ-herpesviruses (γHV)[3] is characterized by an acute phase of virus replication infection in mucosal sites, followed by life-long latency in B lymphocytes and other cell types (1, 2). The progressive loss of CD4$^+$ T cells in HIV/AIDS is associated with enhanced shedding of EBV (the prototypic human γHV) from the oropharynx and greatly increased incidence of the γHV-associated tumors, lymphoma and Kaposi's sarcoma (1–5). This ultimately lethal escape of EBV and human herpesvirus 8-induced oncogenesis from immune control could reflect the loss of CD4$^+$ effectors and/or a progressive decline in CD4$^+$ T help for CD8$^+$ CTL populations (6). Much of the recent focus on the design of EBV vaccines has been on priming the CD8$^+$ T cell response, though it is likely that the incorporation of epitopes recognized by CD4$^+$ T cells will also be important (7).

The reactivation of lytic γHV replication in human AIDS can be modeled (8, 9) by the intranasal (i.n.) challenge of CD4-deficient I-A$^{b-/-}$ mice (10) with the closely related (11, 12) murine γ-herpesvirus 68 (γHV68). Though the level of productive γHV68 infection in lung epithelium (13) is significantly limited by the CD8$^+$ T cell response and the I-A$^{b-/-}$ mice remain clinically normal for ~100 days, all eventually succumb to a late-onset, fatal wasting disease (8). Evidence of persistent, though relatively low level, virus production is found in the respiratory tract, while substantial numbers of latently infected B cells and macrophages (14, 15) are detected consistently in the lymphoid compartment (8).

Both conventional I-A$^{b+/+}$ and Ig$^{-/-}$ μMT (16) mice deal with the lytic phase of γHV68 replication within 10 days of respiratory exposure, progressively limit the numbers of latently infected B cells and macrophages, and remain clinically normal (17–20). Long-term treatment of the Ig$^{-/-}$ mice with subset-specific mAbs has shown that either CD4$^+$ or CD8$^+$ populations can mediate partial, though not complete, control of this infection (21). The CD4$^+$ T cells were found to operate via an IFN-γ-dependent mechanism, while the CD8$^+$ T cells were not further compromised in CD4-depleted mice that were also treated with a mAb to IFN-γ. However, this demonstration of a likely role for IFN-γ-producing CD4$^+$ T cell effectors (21) does not preclude the possibility that CD4$^+$ T help is also important for the CD8$^+$ T cell response.

The two most prominent peptides recognized by responding CD8$^+$ T cells in H2^b mice infected with γHV68 are derived from a ssDNA-binding protein (p56) and a ribonucleotide reductase (p79), presented in the context of H2D^b and H2K^b, respectively (20, 22). Postexposure vaccination (23) of γHV68-infected I-A$^{b+/+}$ and I-A$^{b-/-}$ mice with a recombinant vaccinia virus (24) expressing p56 (Vacc-p56) massively expanded the CD8$^+$ T cell population specific for the D^{b}p56 epitope. However, the numbers of CD8$^+$ T cells that could be stained with a tetrameric complex of H2D^b and the p56 peptide (D^{b}p56 tetramer) declined progressively in both the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice (23). Furthermore, the infection was not controlled and the onset of the fatal γHV68-induced wasting disease was only slightly delayed in the boosted I-A$^{b-/-}$ group. Limited analysis of the CD8$^+$D^{b}p56$^+$ T cells in clinically compromised I-A$^{b-/-}$ mice indicated that at least some could still synthesize IFN-γ. The overall impression was that, unlike the situation (25–27) in CD4-deficient mice infected with the

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[3] Abbreviations used in this paper: γHV, γ-herpesvirus; i.n., intranasal; γHV68, murine γHV68; Vacc-p56, recombinant vaccinia virus expressing the p56 peptide; LCMV, lymphocytic choriomeningitis virus; BAL, bronchoalveolar lavage; MLN, mediastinal lymph node; HCV, hepatitis C virus.

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nonlytic lymphocytic choriomeningitis virus (LCMV), these γHV-68-specific CD8$^+$ T cells were not functionally impaired (23). We have now looked much more closely at the events following post-exposure challenge (23) of γHV68-infected I-A$^{b+/+}$ and I-A$^{b-/-}$ mice and have found that D^{b}p56-specific CD8$^+$ T cells expanded in the absence of concurrent CD4$^+$ T help show evidence of diminished activation.

Materials and Methods

Mice

Female C57BL/6J (B6, I-A$^{b+/+}$) mice (The Jackson Laboratory, Bar Harbor, ME) and MHC class II-deficient (I-A$^{b-/-}$) mice (Charles River Breeding Laboratories, Worcester, MA) were kept under specific pathogen-free conditions at St. Jude Children's Research Hospital (Memphis, TN). The genotype of the I-A$^{b-/-}$ mice (10) was verified by eye bleed and flow cytometry. Age-matched 8- to 12-wk-old mice were used at the initial priming phase of all experiments.

Virus infection and sampling

The mice were anesthetized with Avertin (2, 2, 2-tribromoethanol; Sigma-Aldrich, St. Louis, MO) and infected i.n. with 1×10^4 pfu of γHV68 (8). Some were then given 5×10^7 pfu of Vacc-p56 i.p. at least 6 wk after the primary infection. Both the construction of the Vacc-p56 recombinant incorporating the AGPHNDMEI (p56) epitope and the kinetics of the secondary response in persistently infected I-A$^{b+/+}$ and I-A$^{b-/-}$ mice have been described previously (23–24). At time of sampling, the mice were anesthetized with Avertin and bled from the right axillary artery (28). Blood was collected in tubes containing heparin (1000 U/ml; Elkins-Sinn, Cherry Hill, NJ), the inflammatory population was recovered from the lung by bronchoalveolar lavage (BAL) and single-cell suspensions were made from the mediastinal lymph nodes (MLN) and spleen (28). The PBL were separated on a 1-Step gradient (Accurate Chemical and Scientific, Westbury, NY) by centrifugation for 30 min at 2000 rpm. Lymphocytes were collected from the interface and washed, and residual erythrocytes were lysed with ammonium chloride. The BAL cells were adhered to plastic for 1 h at 37°C to remove macrophages and monocytes. The BAL, PBL, and MLN populations were pooled from five to six mice, while the spleens were analyzed from individuals.

Analysis of cell staining profiles

Spleen and MLN populations were enriched for the CD8$^+$ set by in vitro depletion with mAbs to I-A^b (M5/114.15.2) and CD4 (GK1.5), followed by sheep-anti-rat Ig and sheep-anti-mouse Ig-coated magnetic beads (Dynal Biotech, Oslo, Norway). Virus-specific CD8$^+$ T cells were stained with PE-labeled D^{b}p56 or K^{b}p79 tetrameric complexes (22) at room temperature, followed by anti-CD8α-Tricolor (Caltag Laboratories, South San Francisco, CA) and conjugated mAbs (BD PharMingen, San Diego, CA) specific for various phenotypic markers (CD69, LFA-1, CD44, CD62L) that are associated with lymphocyte activation (29, 30). Unenriched spleen and MLN populations were also stained with anti-CD8α-FITC and anti-CD4-PE, to verify the I-A$^{b-/-}$ mice and to allow the total counts for CD8$^+$D^{b}p56$^+$ T cells to be determined for the particular lymphoid organ. A limited analysis of perforin expression profiles used D^{b}p56$^+$ T cells that were fixed in 1% formalin, permeabilized with 0.1% saponin, then stained with anti-perforin (Kamiya Biomedical, Seattle, WA) and goat anti-rat Ig-FITC (BD PharMingen). All lymphocytes were stained and washed in ice-cold PBS containing BSA (0.1%) and azide (0.01%) and analyzed on a FACScan or FACSCaliber using CellQuest software (BD Biosciences, Mountain View, CA).

Flow cytometric analysis of cytokine production by p56-specific CD8$^+$ T cells

The various CD8$^+$ T cell populations were incubated with peptide (1 μM) for 5 h at 37°C in the presence of brefeldin A (10 μg/ml) and IL-2 (50 U/ml) before staining with anti-CD8α-FITC (22). The lymphocytes were then fixed with formalin (1%), permeabilized by saponin (0.5%), and stained for intracellular cytokine with anti-IFNγ-PE and anti-TNF-α allophycocyanin (BD PharMingen) for flow cytometric analysis.

Measurement of CTL activity

The level of CTL activity was determined using a standard 5 h ^{51}Cr release assay (31). The EL-4 (H2^b) targets were incubated with peptide (p56, 10 μg/ml) and ^{51}Cr (300 μCi) at 37°C for 1 h, washed and cocultured at 10^4/well with spleen cells at different E:T ratios. The supernatants were then harvested for gamma counting. Maximum release (*M*) was determined by adding Triton X-100 (1%) to the targets, while the level of spontaneous release (*S*) was for targets cultured in the absence of effector cells. The level of specific ^{51}Cr release was calculated as: the percentage of specific release = (counts − *S*)/(*M* − *S*), where counts are the experimental ^{51}Cr release in the presence of immune CD8$^+$ T cells.

Results

Quantitation of the primary response to γHV68 and the expansion by Vacc-p56 challenge

The profiles of clonal expansion and persistence for the CD8$^+$K^{b}p79$^+$ and CD8$^+$D^{b}p56$^+$ populations were essentially equivalent following primary i.n. exposure of naive I-A$^{b+/+}$ and I-A$^{b-/-}$ mice to γHV68 (Table I). The relative prevalence of the CD8$^+$ subset is, because of the absence of CD4$^+$ T cells, inevitably increased in the I-A$^{b-/-}$ lymphoid tissue. This is to some extent reflected (Table I) in the CD8$^+$K^{b}p79$^+$ (day 10) and CD8$^+$D^{b}p56$^+$ (day 40) frequencies. Even so, despite the fact that the γHV68-infected I-A$^{b-/-}$ mice show evidence of persistent virus replication in the respiratory tract and higher levels of latency in the spleen (8, 23), the numbers of γHV68-specific CD8$^+$ T cells present on day 40 in the spleen and blood were similar to those in the I-A$^{b+/+}$ controls.

Challenging γHV68-infected mice (Table I) with Vacc-p56 accentuated the tendency for the percentage of p56-specific CD8$^+$ T cells determined by both tetramer staining (Fig. 1, *top panels*) and restimulation with peptide (Fig. 1, *bottom panels*) to be higher in the I-A$^{b-/-}$ group, though this did not translate into significant

Table I. *Characteristics of the primary response*

Organ	Days After Infection[a]	% Virus-Specific CD8$^+$ T Cells				No. of Virus-Specific CD8$^+$ T Cells[b]			
		p79		p56		p79		p56	
		I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$
Spleen	10	0.2 ± 0.1[c]	0.6 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	0.2 ± −0.1[c]	1.0 ± 0.2	1.0 ± 0.2	1.7 ± 1.0
	20	2.7 ± 1.2	1.7 ± 1.2	0.7 ± 0.1	1.0 ± 0.3	2.9 ± 1.8	2.7 ± 0.9	0.8 ± 0.2	1.5 ± 0.9
	40	2.3 ± 0.6	2.0 ± 0.2	0.7 ± 0.3[c]	1.7 ± 0.7	3.4 ± 2.1	2.8 ± 0.5	1.4 ± 0.6	3.0 ± 2.1
PBL	10	1.6	1.3	11.6	10.2	0.2	0.5	1.6	3.7
	20	6.4	1.1	3.9	2.8	0.2	0.1	0.1	0.3
	40	4.6	2.9	1.1	2.2	1.1	0.4	1.3	0.9

[a] The B6 I-A$^{b+/+}$ and I-A$^{b-/-}$ mice were infected i.n. with 10^4 PFU of γHV68.
[b] The mean ± SD spleen cell counts (per mouse) are $\times 10^5$, while the values for the pooled PBL samples are $\times 10^3$ for Ficoll-separated lymphocytes from 1.0 to 1.5 ml of heparinized blood.
[c] Significantly different ($p < 0.05$).

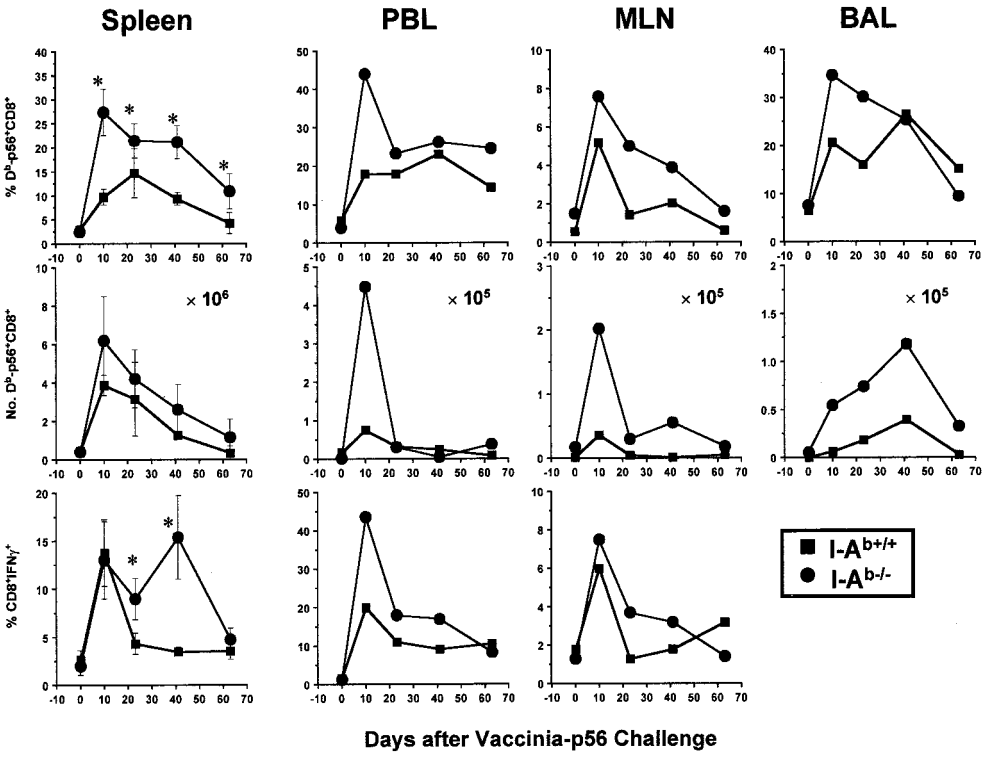

FIGURE 1. Quantitative analysis of the secondary D^bp56-specific $CD8^+$ T cell response. The B6 I-$A^{b+/+}$ and I-$A^{b-/-}$ mice were infected i.n. with 10^4 pfu γHV-68 1 mo before i.p. challenge with 5×10^7 pfu of Vacc-p56. The percentage of $D^b p56^+CD8^+$ cells was determined by tetramer staining while intracellular IFN-γ staining was measured after 5 h of in vitro stimulation with 1 μM of the p56 peptide. Spleen populations were enriched for the $CD8^+$ set and each data point shows the mean ± SD for five mice. The asterisk designates groups that were significantly different ($p < 0.05$). The MLN, PBL, and BAL samples were pooled.

differences in the numbers of $CD8^+D^bp56^+$ spleen cells (*middle panels*, Fig. 1). However, the disease status of these two groups of mice was, as time progressed, very different. By day 60 after Vacc-p56, which is >100 days after the primary exposure to γHV68, many of the boosted I-$A^{b-/-}$ mice were showing symptoms of the characteristic lethal wasting disease (8, 23). Also, despite the difference in disease status, the prevalence of virus-specific $CD8^+$ T cells fell equivalently by day 60 for the I-$A^{b-/-}$ and I-$A^{b+/+}$ groups (Fig. 1).

Differences in activation phenotype for the $CD8^+D^bp56^+$ T cells

The intensity of CD44 staining for $CD8^+D^bp56^+$ T cells from individual spleens and pooled BAL samples taken at various times after i.p. exposure to Vacc-p56 is illustrated in Fig. 2. The profiles (Fig. 2) show clearly that the $CD8^+D^bp56^+$ set from the I-$A^{b-/-}$ mice tends to express less CD44. Analyzing mean fluorescence intensity (MFI) for groups of five spleens at each time point established that this difference was significant at days 10, 20, and 60 after secondary challenge (Table II, column 1). The same effect can be seen when the results are compared as ratios (I-$A^{b+/+}$:I-$A^{b-/-}$) for several experiments, with the diminution in the I-$A^{b-/-}$ group being most obvious on days 10 and 20 for the spleen and day 10 for the BAL (Table II).

Lower levels of LFA-1 staining were also found for the $CD8^+D^bp56^+$ T cells recovered on day 10 from the spleens of the CD4-deficient mice, but this difference was not sustained in the longer term and the BAL population tended to express more LFA-1 (ratio, *final column*, Table II). It is to be expected that the larger BAL population (Fig. 1) from the I-$A^{b-/-}$ mice would be more activated, as there is continued productive infection in the lung (8, 23) in the absence of the $CD4^+$ subset. No significant differences were seen in the staining profiles for CD62L, the β_7 integrin, CTLA4, IL-2R, or CD43 (data not shown).

Unlike the situation for CD44 and LFA-1, well-defined high and low peaks were seen in the CD69 staining profiles for the $CD8^+D^bp56^+$ set (data not shown). Both the percentage frequencies and the numbers of $CD69^{high}CD8^+D^bp56^+$ T cells were consistently greater for spleen populations from the I-$A^{b-/-}$ group, and the numbers were also significantly higher for the BAL population at all time points (Table III). By this criterion, there are

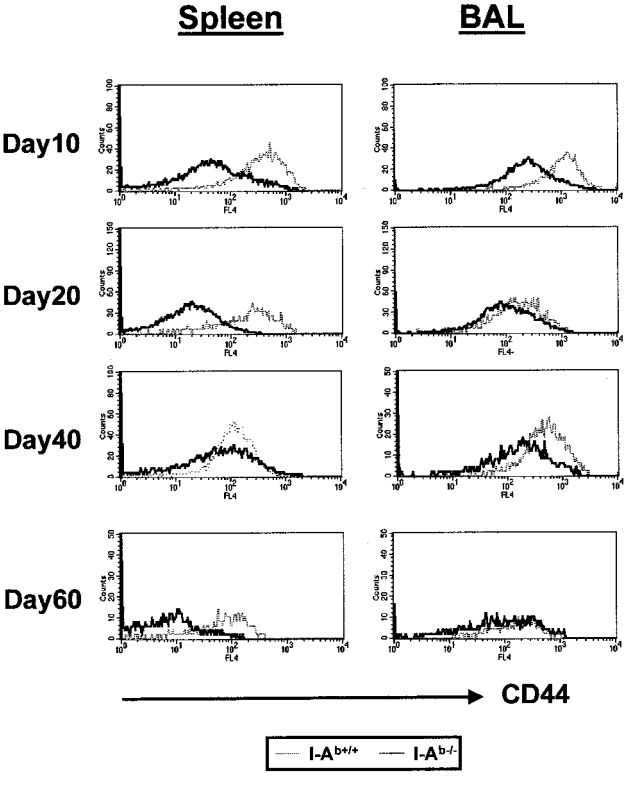

FIGURE 2. Profiles of CD44 staining for the secondarily stimulated $CD8^+D^b$ $p56^+$ set. The mice were primed and boosted as described in the footnote to Fig. 1. The quantitative analysis of MFI values is shown in Table II.

Table II. *Intensity of staining for secondarily stimulated CD8$^+$ D^{b}p56$^+$ T cells*[a]

Data set[a]	Days After Challenge	Mice/Organ	CD44	LFA-1
Individual spleens	10	I-A$^{b+/+}$	431.1 ± 48.7	313.1 ± 26.3
		I-A$^{b-/-}$	67.4 ± 7.8[b]	125.4 ± 9.0[b]
	20	I-A$^{b+/+}$	237.2 ± 27.5	119.0 ± 29.0
		I-A$^{b-/-}$	36.8 ± 6.6[b]	107.0 ± 15.9
	40	I-A$^{b+/+}$	144.8 ± 29.6	130.8 ± 7.7
		I-A$^{b-/-}$	154.8 ± 34.4	117.6 ± 16.1
	60	I-A$^{b+/+}$	88.6 ± 6.3	100.3 ± 7.8
		I-A$^{b-/-}$	36.1 ± 14.9[b]	95.7 ± 3.2
Ratio (I-A$^{b+/+}$:I-A$^{b-/-}$)	10	Spleen	5.3 ± 1.6	2.2 ± 0.5
		BAL	4.0 ± 2.1	0.5 ± 0.3
	20	Spleen	5.2 ± 1.7	1.1 ± 0.1
		BAL	1.5 ± 0.7	0.5 ± 0.2
	40	Spleen	1.2 ± 0.4	1.1 ± 0.1
		BAL	2.8 ± 1.1	0.2 ± 0.1
	60	Spleen	2.0 ± 0.8	1.1 ± 0.1
		BAL	1.4 ± 0.2	1.4 ± 0.4

[a] The mice were infected i.n. with 10^4 PFU of γHV68, then challenged i.p. with 5×10^7 PFU of Vacc-p56. The data set shows the mean ± SD MFI values for five individual spleens in one experiment, or the mean ± SD for the ratios for BAL and spleen for two to three experiments.
[b] Groups that were significantly different ($p < 0.05$).

relatively more activated virus-specific CD8$^+$ T cells in the CD4-deficient mice, as might be expected because of the greater prevalence of latently infected cells (8, 23).

Diminished prevalence of p56-specific CD8$^+$ T cells producing both IFN-γ and TNF-α

The initial analysis of this postexposure vaccination protocol in I-A$^{b+/+}$ and I-A$^{b-/-}$ mice showed little, if any, difference in the IFN-γ staining profiles for CD8$^+$ T cells stimulated for 5 h with 1 μM of the p56 peptide in the presence of brefeldin A (23). This observation has been confirmed and extended in the present study, which indicates that the frequencies of CD8$^+$IFN-γ$^+$ T cells are generally higher in the spleen, PBL, and MLN of the CD4-deficient group (Figure 1, *bottom panels*, and Table IV). However, the percentage of CD8$^+$IFN-γ$^+$ spleen cells (Fig. 1) does drop between days 40 and 60, when the I-A$^{b-/-}$ group starts to show evidence of clinical compromise.

Experiments over the past two years with virus systems in which all Ag is thought to be eliminated after the acute phase of the response have shown that the percentage of CD8$^+$ memory T cells that stain for both IFN-γ and TNF-α increases with time after challenge (32, 33). We found that this also seems to be the case following the Vacc-p56 challenge of the γHV68-infected I-A$^{b+/+}$ and I-A$^{b-/-}$ mice: the prevalence of CD8$^+$IFN-γ$^+$TNF-α$^+$ T cells was higher ($p < 0.05$ or less) in spleens sampled on days 40 or 60 rather than on day 10 (Table IV). However, the relative frequency of CD8$^+$ T cells that make both IFN-γ$^+$ and TNF-α$^+$ was significantly lower throughout in the CD4-deficient group (Fig. 3, Table IV). This was apparent for both the spleen (Fig. 3, Table III) and the BAL (Table IV) populations. Also, the MFI for both IFN-γ and TNF-α was significantly less on day 10 for CD8$^+$ T cells from the I-A$^{b-/-}$ mice, with this difference being also apparent on day 60 for TNF-α (Table IV, footnote c). In general, it seems that there is either a pattern of greater CD4-dependence for the production of TNF-α vs IFN-γ by CD8$^+$ T cells, or the IFN-γ$^+$TNF-α$^+$ cells are less prominent under conditions of greater Ag persistence.

Continued CTL activity after the Vacc-p56 challenge

Substantial p56-specific CTL activity was maintained in the spleens of both the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice for the 60 days subsequent to the Vacc-p56 challenge (Fig. 4, *a–d*). The levels were generally equivalent for the two groups of spleen populations, though the I-A$^{b-/-}$ set was significantly more potent on d10 (Fig. 4, *a* and *b*) at the lowest E:T ratio (7.3:1). However, when we corrected the percentage of ^{51}Cr release values for the CD8$^+$D^{b}p56$^+$ T cell frequencies determined by flow cytometry, we found that the CD8$^+$ effectors from the CD4-deficient mice were (on a per cell basis) significantly less active at the days 10, 20, and 60 timepoints (Fig. 4, *e–h*). Again, there was evidence of a decline between days 40 and 60, when the I-A$^{b-/-}$ mice show evidence of clinical compromise. Staining for cytoplasmic perforin at the day 10 timepoint also showed that the MFI was lower in CD8$^+$D^{b}p56$^+$ spleen cells from the I-A$^{b-/-}$ mice (8.1 ± 0.5 vs

Table III. *Prevalence of CD69^{high}CD8$^+$ D^{b+}p56 T cells*

Days After Challenge[a]	% CD69^{high}CD8$^+$ D^{b}p56 T Cells				No. of CD69^{high}CD8$^+$ D^{b+}p56 T Cells[b]			
	Spleen		BAL		Spleen		BAL	
	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$
10	1.8 ± 0.4	9.2 ± 4.1[c]	55.4 ± 6.0	52.9 ± 13.5	7.0 ± 1.0	57.1 ± 12.1[c]	3.6 ± 0.4	28.9 ± 7.4[c]
20	1.9 ± 0.5	10.1 ± 1.1[c]	19.0 ± 4.2	18.1 ± 9.7	6.0 ± 3.7	42.6 ± 15.2[c]	3.5 ± 0.8	13.4 ± 7.2[c]
40	6.0 ± 0.8	12.5 ± 3.5[c]	24.5 ± 9.2	38.7 ± 6.4	7.7 ± 0.9	32.7 ± 6.2[c]	9.7 ± 3.6	45.8 ± 7.6[c]
60	7.9 ± 3.7	13.3 ± 4.7[c]	18.6 ± 0.6	46.3 ± 1.0[c]	2.8 ± 2.7	15.5 ± 7.4[c]	0.6 ± 0.1	15.2 ± 0.3[c]

[a] These are the same experiments as those illustrated in Table II. The spleen data are for 10–15 individuals, while the BAL is for 2–3 pooled samples.
[b] The spleen cell counts (per mouse) are $\times 10^4$, while the BAL samples are $\times 10^3$.
[c] Significantly different ($p < 0.05$).

Table IV. *Relative distribution of IFN-γ^+TNF-α^+ and IFN-γ^+TNF-α^-CD8$^+$ T cells*

Days After Challenge[a]	% IFN-γ^+TNF-α^+ CD8$^+$ T Cells				% IFN-γ^+TNF-α^-CD8$^+$ T Cells				Ratio IFN-γ^+TNF-α^+:IFN-γ^+TNF-α^-			
	Spleen		BAL		Spleen		BAL		Spleen		BAL	
	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$	I-A$^{b+/+}$	I-A$^{b-/-}$
10	9.2 ± 2.2[b]	5.4 ± 2.6	14.6 ± 3.6	22.5 ± 3.9	4.7 ± 1.4[b]	7.7 ± 1.6	3.0 ± 0.3	11.2 ± 3.3	2.0 ± 0.4[b]	0.7 ± 0.2[c]	4.5 ± 0.6	1.9 ± 0.4
20	2.6 ± 0.7[b]	3.6 ± 0.8	14.5 ± 2.3	21.2 ± 4.5	2.1 ± 0.7[b]	5.4 ± 1.9	3.3 ± 0.2	10.3 ± 2.8	1.3 ± 0.3[b]	0.6 ± 0.2	4.5 ± 0.3	2.0 ± 0.2
40	2.8 ± 0.6[b]	7.7 ± 1.8	16.9 ± 3.2	18.9 ± 3.3	0.9 ± 0.1[b]	7.1 ± 1.8	3.6 ± 0.4	9.5 ± 2.5	3.0 ± 0.6[b]	1.2 ± 0.2	4.7 ± 0.6	2.3 ± 1.0
60	2.1 ± 1.0	2.5 ± 0.3	NT	NT	0.7 ± 0.3[b]	1.9 ± 0.6	NT	NT	3.4 ± 0.7[b]	1.2 ± 0.4	NT	NT

[a] The mice were primed with γHV68 and boosted with Vacc-p56 as described in Fig. 1. The T cells were sampled at the intervals shown after secondary challenge, stimulated for 5 h with 1.0 μM of the p56 peptide, fixed, and stained for IFN-γ and TNF-α. Typical FACS profiles are shown in Fig. 3. The results are mean ± SD of the ratios for 10–15 spleen cells (2 or 3 groups of 5) or 2–3 pooled BAL samples.

[b] Significantly different ($p < 0.05$) between the I-A$^{b+/+}$ and I-A$^{b-/-}$ group.

[c] The MFI values were significantly different ($p < 0.05$) for IFN-γ on day 10 (+/+, 1042 ± 96; −/−, 435 ± 91), and for TNF-α on day 10 (+/+, 146 ± 17; −/−, 69 ± 10) and day 60 (+/+, 80 ± 20; −/−, 46 ± 5).

13.2 ± 0.8). Thus, though a much higher Ag load is being maintained in the CD4-deficient group (8, 23), the individual T cells seem to be less potent CTL effectors.

Discussion

Postexposure vaccination has attractions as a potential clinical strategy for the treatment of both persistent virus infections and tumors (34–36). These (and previous) experiments (23) establish that a relatively modest immunization regime, a single dose of a recombinant vaccinia virus, can cause enormous expansion of virus-specific CD8$^+$ T cells in mice that have effectively (I-A$^{b+/+}$) or partially (I-A$^{b-/-}$) controlled γHV68 infection. However, what we have learned so far is that it is not sufficient just to increase CD8$^+$ T cell numbers. Despite the likelihood of continued antigenic stimulation in CD4-deficient mice (8, 9, 23), the CD8$^+$ T cell counts still decline to the point where wasting and death ensues (23). If this reflects that there is an absolute need for CD4$^+$ T

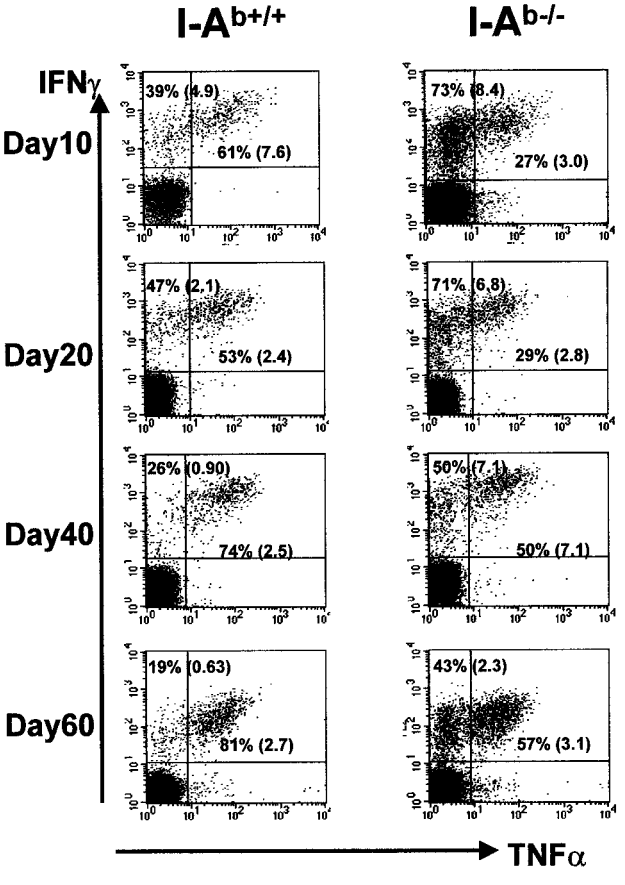

FIGURE 3. Characteristics of the IFN-γ and TNF-α staining profiles for p56 peptide-stimulated CD8$^+$ T cells. The mice were primed with γHV68 and boosted with Vacc-p56 as described in the footnote to Fig. 1. The CD8$^+$ T cells were enriched from the spleen, stimulated for 5 h with 1.0 μm p56 peptide in the presence of brefeldin A, then fixed and stained for TNF-α and IFN-γ. The relative prevalence of the IFN-γ$^+$TNF-α$^+$ cells from the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice is summarized in Table III.

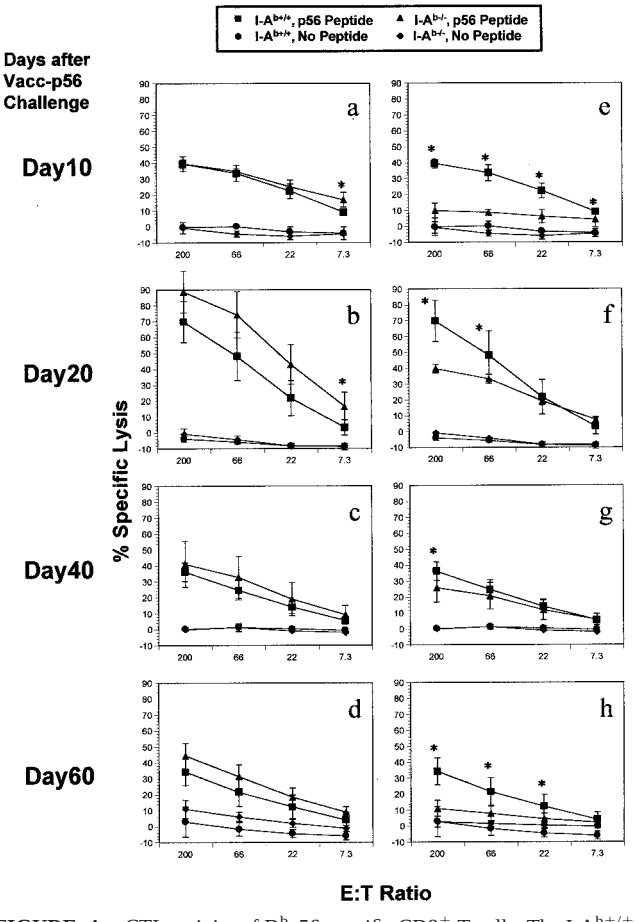

FIGURE 4. CTL activity of D^{b}p56-specific CD8$^+$ T cells. The I-A$^{b+/+}$ and I-A$^{b-/-}$ mice were primed and challenged as described in the footnote to Fig. 1. The spleen populations were tested directly ex vivo in a 5 h ^{51}Cr release assay with p56-peptide pulsed, or control, EL4 targets. *a–d*, The ^{51}Cr release data for whole spleen tested at the E:T ratios shown, while the ^{51}Cr release values in *e–h* were corrected for the percentage of CD8$^+$D^{b}p56-specific T cells.

cell effectors (21), there may be little that we can do with the γHV68 model to suggest approaches that could be useful in AIDS patients. Should, in contrast, the essential requirement be for continuing CD4$^+$ T help (6, 37), it may be possible to develop some therapeutic protocol for providing the necessary cytokines and/or activated DCs (38, 39).

The available evidence indicates that γHV68-specific CD8$^+$ T cells operate primarily by perforin or Fas-mediated cytotoxicity (40). This CTL effector mechanism is potentially subverted by the γHV68 K3 protein, which has been shown to reduce the half-life of nascent MHC class I glycoproteins and the diminished expression of γHV68 epitopes on the surface of infected fibroblasts (41). However, it is not known how important the K3 factor is in the epithelial cells that support the in vivo replication of γHV68. Also, no analysis is yet available of the possible effect of cytokines like TNF-α and IFN-γ on Ag presentation, either in DCs or on γHV68-infected lung cells.

The expanded CD8$^+$D^{b}p56$^+$ T cell populations that we recovered from the spleens of Vacc-p56-boosted, CD4$^+$ T cell-deficient I-A$^{b-/-}$ mice showed three measures of relative deficiency when compared with those from the I-A$^{b+/+}$ controls. These were: decreased CD44 expression, lower levels of CTL activity (on a per cell basis) for peptide-pulsed targets, and a decrease in the relative prevalence of lymphocytes producing both IFN-γ and TNF-α. In each case, the defect tended to be most apparent at the earliest (day 10) and the latest (day 60) timepoints. The difference between the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice on day 10 may be a measure of the need for CD4$^+$ T help to promote the initial phase of clonal expansion and differentiation. The diminished capacity of the CD8$^+$ effectors recovered on day 60 (day 100 after γHV68) could reflect a continuing need for CD4$^+$ T help and/or the consequences of persistent antigenic stimulation (8, 23).

It has long been recognized that CD44 is up-regulated on activated, effector, and memory CD8$^+$ T cells (42–44). Though all the CD8$^+$D^{b}p56$^+$ T cells recovered from the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice were CD44^{high}, the intensity of staining was generally lower for those from the CD4$^+$ T cell-deficient group. Recent cross-linking experiments indicate that CD44 is associated with CD4 and the CD3 complex on the surface of helper T cells, suggesting that there may be a functional relationship between the CD4-TCR and CD44 (45). In addition, CD44 is thought to be involved in cytoskeleton rearrangement and raft reorganization in T lymphocytes (46, 47). It is thus possible that the decreased expression of CD44 may affect both the character of TCR signaling and CTL function in CD8$^+$ T cells.

Virus-specific CD8$^+$ T cells recovered from mice persistently infected with LCMV, particularly under conditions of CD4$^+$ T cell deficiency, show evidence of much greater dysfunction than that found in this study (27). These LCMV-specific CD8$^+$tetramer$^+$ T cells neither produce IFN-γ nor mediate CTL activity. The difference is that naive mice depleted of both the CD4$^+$ and CD8$^+$ T cell subsets invariably die following infection with the cytopathic γHV68, while the essentially nonlytic LCMV induces a life-long carrier state. Thus, the LCMV Ag load can be enormous in mice that lack T cells, a situation that cannot be tolerated with γHV68 (21).

One study of HIV infection found that Ag-specific CD8$^+$ T showed normal profiles of IFN-γ production (48). However, there is other evidence that HIV-specific CD8$^+$ T cells may be functionally defective (49). This includes incomplete signaling and activation (50), reduced perforin expression (51), and inefficient trafficking to the site of infection (52). Similar findings have been made for primates infected with SIV (53).

Evidence of defective virus-specific CD8$^+$ T cell function was also found (54) in patients persistently infected with the hepatitis C virus (HCV). The HCV-specific CD8$^+$ T cells showed reduced synthesis of IFN-γ and TNF-α after stimulation with either mitogens or peptides. Even so, the results for the I-A$^{b+/+}$ mice in the present experiments suggest that, as there is no known CD4$^+$ T cell defect in HCV infection, it might be worth trying postexposure vaccination as a therapeutic protocol. The potential danger would, of course, be the induction of acute CD8$^+$ T cell-mediated immunopathology (55, 56).

Thus, the extent of the functional defect in virus-specific CD8$^+$ T cells from persistently infected mice may reflect the interaction between the magnitude and quality of TCR-mediated and associated signaling events. The analysis of both CD8$^+$ T cell turnover rates in normal and CD4-deficient mice indicates that the "memory" T cell pool is regularly stimulated by the reactivation of latent γHV68 to a lytic phase (57). The same is true for CD4$^+$ T cells in I-A$^{b+/+}$ mice that have effectively controlled the infection (58). The Ag load (55) in the I-A$^{b-/-}$ mice will be much higher as a consequence of the greater numbers of latently infected cells in the lymphoid tissue and the continued production of lytic virus in the respiratory tract (8, 23, 59). Though there is evidence of partial functional impairment, more CD8$^+$ T cells are CD69^{high} in the I-A$^{b-/-}$ mice. The much greater amounts of virus that can be tolerated in CD4-deficient mice infected with the essentially nonlytic LCMV lead to a complete functional paralysis, though not to T cell deletion. Further analysis with the γHV68 model needs to address the relative significance of continued antigenic stimulation vs absence of CD4$^+$ T cell help on the character of the CD8$^+$ T cell response.

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