Virus-Specific and Bystander CD8+ T-Cell Proliferation in the Acute and Persistent Phases of a Gammaherpesvirus Infection

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The cycling characteristics of CD8+ T cells specific for two lytic-phase epitopes of murine gammaherpesvirus 68 (γHV68) have been analyzed for mice with high or low levels of virus persistence. The extent of cell division is generally reflective of the antigen load and suggests that γHV68 may be regularly reactivating from latency for some months after the resolution of the acute phase of the infectious process. Although γHV68 infection is also associated with massive proliferation of lymphocytes that are not obviously specific for the virus, the level of "bystander-induced" cycling in a population of influenza virus-specific CD8+ T cells was generally fourfold lower than the extent of cell division seen for the antigen-driven, γHV68-specific response. The overall conclusion is that turnover rates substantially in excess of 5 to 10% over 6 days for CD8+ "memory" T-cell populations are likely to be reflective of continued antigenic exposure.

Respiratory challenge with murine gammaherpesvirus 68 (γHV68) induces massive turnover in the CD8+ T-cell compartment (13, 24). This pattern of sustained lymphocyte proliferation persists long after both γHV68 replicative infection is controlled in epithelial sites and peak numbers of latently infected B lymphocytes are detected in the spleen (4). These cycling CD8+ T cells comprise at least two distinct populations. The first consists of multiple sets of γHV68-specific CD8+ T cells that are, in C57BL/6J (B6) mice, responding to at least six different viral peptides derived from proteins expressed during the lytic phase of the infectious process (18). The second is an unusual CD8+ T-cell population that expresses a Vβ4 T-cell receptor chain and expands greatly in numbers after the end of the initial, lytic phase of the infectious process (24). These CD8+ Vβ4+ T cells show some evidence of oligoclonality (14), although they do not seem to be recognizing viral peptides or major histocompatibility complex class I or II glycoproteins (6). Furthermore, the dramatic increase in the size of the CD8+ Vβ4+ set is totally dependent on concurrent, CD40 ligand-mediated CD4+ T-cell help (3, 9). This effect is not seen in major histocompatibility complex class II$^{-/-}$ (I-A$^{b-/-}$) mice.

In the present analysis, we used these CD4+ T-cell-deficient I-A$^{b-/-}$ mice (12) and conventional B6 (I-A$^{b+/+}$) congenic mice to characterize the proliferation of virus-specific CD8+ T cells under conditions of differential, continuing γHV68 challenge. The I-A$^{b+/+}$ group effectively controls lytic γHV68 infection within 12 days of the initial intranasal (i.n.) exposure, while I-A$^{b-/-}$ mice show persistent evidence of γHV68 replication in the respiratory tract and succumb to a late-onset wasting disease that develops after 80 to 100 days (2, 4). Latently infected B cells and macrophages can be demonstrated

in both groups over the very long term by an infectious-center assay and by limiting-dilution analysis.

CD8+ T cells were obtained from the spleen and from the infected lungs (1) by bronchoalveolar lavage (BAL). The experiments focus on the two most prominent γHV68 epitopes, H2D^{b}p56 (D^{b}p56) and H2K^{b}p79 (K^{b}p79). The γHV68 p56 peptide (AGPHNDMEI) is derived from a single-stranded DNA binding protein, while the p79 peptide (TSINFKVI) is derived from the large ribonucleotide reductase (18). The possible contribution of "bystander" activation (8, 16, 22, 25, 26) to the total pool of cycling CD8+ T cells was also analyzed for a "memory" population specific for the prominent influenza virus D^{b}NP$_{366}$ epitope (10). Influenza-immune I-A$^{b+/+}$ mice were first infected intraperitoneally with H1N1 influenza A virus and then boosted at least 6 weeks prior to γHV68 challenge by respiratory infection with an H3N2 virus that shares the same nucleoprotein gene (10, 15).

The bromodeoxyuridine (BrdU)-positive (BrdU+) CD8+ population showed maximal prevalence in both the spleen and BAL populations recovered from the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice at day 20 after the initial exposure to γHV68 (Fig. 1A to D). The percentages of BrdU+ T cells in the splenic CD8+ D^{b}p56+ and CD8+ K^{b}p79+ sets were also remarkably concordant for the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice until day 60 after the i.n. γHV68 challenge (Fig. 1B and C), with the values ranging from >80% on day 20 to about 30% on day 60. The same was true for the BAL populations until day 40, although there was evidence of more cycling in the I-A$^{b-/-}$ mice by day 50 (Fig. 1E and F). This result presumably reflects the persistent, low-level production of lytic virus in the I-A$^{b-/-}$ mouse respiratory tract (4).

Analysis at later time points (days 70 to 90) showed a continuing profile of greater cycling for both the total and the virus-specific CD8+ T-cell populations in the BAL populations from the I-A$^{b-/-}$ mice (Fig. 2D to F). This difference between the I-A$^{b+/+}$ and I-A$^{b-/-}$ groups was also apparent for the virus-specific CD8+ T cells recovered on day 90 from the spleen (Fig. 2B and C), although the effect was not evident in the total CD8+ T-cell population (Fig. 2A). A further pulse-

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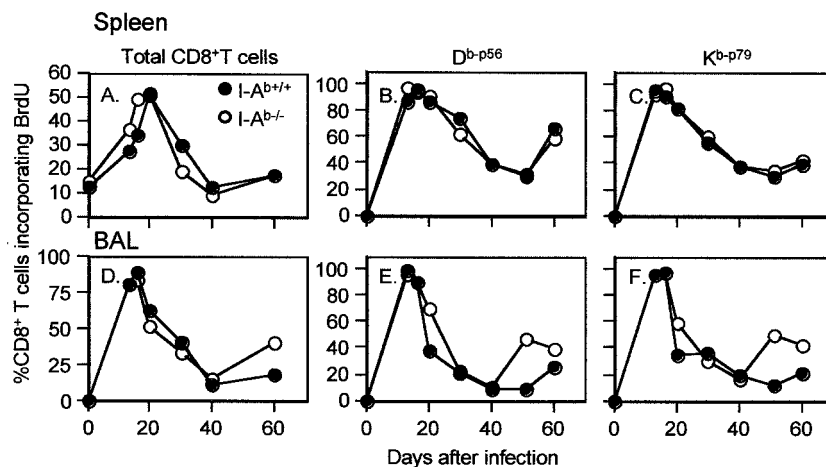
FIG. 1. Kinetic analysis of BrdU incorporation in the CD8$^+$ K^{b}p79$^+$ and CD8$^+$ D^{b}p56$^+$ sets during the first 60 days after γHV68 infection (13, 19, 23). I-A$^{b+/+}$ and I-A$^{b-/-}$ mice were infected i.n. with 2×10^3 PFU of γHV68 and given 0.8 mg of BrdU/ml in their drinking water for 6 days prior to sampling. The results for pooled spleen (A, B, and C) and BAL (D, E, and F) populations from four or five mice at each time point show the percentages of BrdU$^+$ cells for all gated CD8$^+$ lymphocytes (A and D) and for the virus-specific D^{b}p56$^+$ (B and E) and K^{b}p79$^+$ (C and F) sets. The lymphocytes were stained with anti-CD8–tricolor and phycoerythrin-labeled K^{b}p79 or D^{b}p56 tetramers, fixed, and stained with anti-BrdU–fluorescein isothiocyanate (13, 19, 23). The cells were then analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson, Mountain View, Calif.). A minimum of 2,000 CD8$^+$ tetramer-positive events were analyzed for each sample.

chase analysis (12) also indicated that both the CD8$^+$ D^{b}p56$^+$ (Fig. 3A and B) and the CD8$^+$ K^{b}p79$^+$ (Fig. 3C and D) sets were proliferating at a higher rate in the I-A$^{b-/-}$ mice between day 50 and day 70 after infection.

Some evidence of bystander activation for the influenza virus CD8$^+$ D^{b}NP$_{366}^+$ set was found in both the spleen and BAL populations recovered during the first 18 days after i.n. γHV68

challenge (Table 1). Although we refer to this effect throughout as bystander activation, a description that implies T-cell receptor-independent stimulation by cytokines (8, 19, 26), it is also formally possible that there is some unidentified cross-reactivity between a γHV68-encoded epitope and D^{b}NP$_{366}$
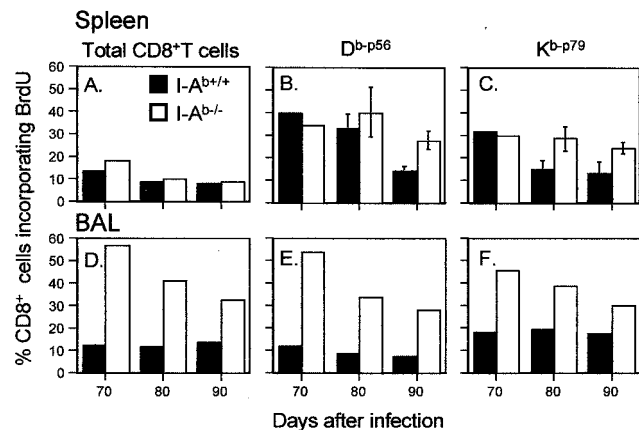

FIG. 2. Prevalence of BrdU$^+$ CD8$^+$ K^{b}p79$^+$ and CD8$^+$ D^{b}p56$^+$ T cells in I-A$^{b+/+}$ and I-A$^{b-/-}$ mice at 70 to 90 days after γHV68 infection. The spleen results (A, B, and C) are presented as means and standard deviations for five mice per group, while the BAL samples (D, E, and F) were pooled from the same mice. The values for the I-A$^{b+/+}$ and I-A$^{b-/-}$ spleens were significantly different ($P < 0.05$) on day 90 for both epitopes (B and C). The other differences between the I-A$^{b+/+}$ and I-A$^{b-/-}$ mice in panels A to C were not significant. The BrdU incorporation profiles are shown for total, gated CD8$^+$ lymphocytes (A and D) and for the D^{b}p56$^+$ (B and E) and K^{b}p79$^+$ (C and F) populations. The mice were infected i.n. with 2×10^3 PFU of γHV68 and given BrdU in their drinking water for 6 days prior to sampling. The γHV68-specific CD8$^+$ T-cell populations were analyzed as described in the legend to Fig. 1.
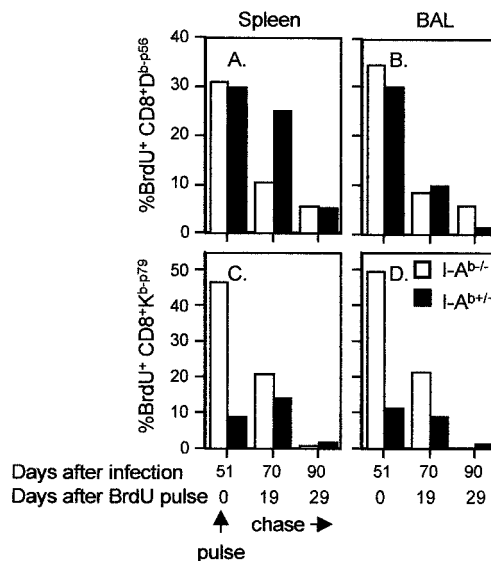

FIG. 3. Pulse-chase analysis of γHV68-specific CD8$^+$ T cells isolated after the peak of the protracted CD8$^+$ Vβ4$^+$ T-cell proliferation (24) in I-A$^{b+/+}$ mice. The mice were given drinking water containing BrdU for 6 days, commencing 45 days after i.n. infection with γHV68. They were assayed at the end of the pulse period (day 51) and again 19 and 29 days later (chase). Spleens (A and C) and BAL populations (B and D) were pooled from three to five animals for each time point, enriched for CD8$^+$ T cells, stained with the D^{b}p56 and K^{b}p79 tetramers, and analyzed for BrdU incorporation as described in the legend to Fig. 1.

TABLE 1. Cycling characteristics of influenza virus-specific and γHV68-specific CD8$^+$ T cells following γHV68 infection

Site sampled[a]	Day	% CD8$^+$ tetramer-positive BrdU$^+$ cells specific for[a]:		
		Influenza virus D^{b}NP$_{366}$[a]	γHV68	
			D^{b}p56	K^{b}p79
Spleen	0	6.2 ± 1.5		
	12	18.9 ± 4.4	84.5 ± 3.8	92.5 ± 1.2
	16	19.2 ± 4.3	62.5 ± 8.9	79.9 ± 10.2
	18	11.7 ± 3.9	47.0 ± 5.1	70.6 ± 11.1
BAL	0	1.5		
	12	8.7	46.7	52.4
	16	9.5	66.5	95.5
	18	4.5	18.6	53.5

[a] The spleen results are expressed as means and standard deviations for five mice, while the BAL samples were pooled.

[b] I-A$^{b+/+}$ mice were infected intraperitoneally with $10^{8.5}$ 50% embryo-infective doses (EID$_{50}$) of the PR8 (H1N1) influenza A virus, boosted i.n. 1 month later with $10^{6.5}$ EID$_{50}$ of the HKx31 (H3N2) influenza A virus (10, 11), and then challenged i.n. after another 6 weeks with γHV68. They were given drinking water containing BrdU for 6 days prior to sampling. The BrdU incorporation profiles were then analyzed for γHV68-specific (D^{b}p56$^+$ and K^{b}p79$^+$) and influenza virus nucleoprotein-specific (D^{b}NP$_{366}$) CD8$^+$ T-cell populations.

(17). The percentages of BrdU$^+$ CD8$^+$ D^{b}NP$_{366}$$^+$ T cells in the spleen were significantly increased ($P < 0.05$) over background values (day 0) at 12 and 16 days after i.n. exposure to γHV68, although the prevalence of cycling CD8$^+$ D^{b}NP$_{366}$$^+$ T cells was three- to fourfold lower than that for the CD8$^+$ D^{b}p56$^+$ and CD8$^+$ K^{b}p79$^+$ sets (Table 1). The results are very comparable to those found previously during the acute phase of infection with influenza B virus (5) and suggest that γHV68 has no particular propensity to drive the proliferation of "irrelevant" CD8$^+$ memory T cells.

In general, this analysis of cycling for the CD8$^+$ D^{b}p56$^+$ and CD8$^+$ K^{b}p79$^+$ T-cell populations in I-A$^{b+/+}$ and I-A$^{b-/-}$ mice indicates that the extent of cell division above background levels is correlated with the antigen load. Although every CD8$^+$ D^{b}NP$_{366}$$^+$ T cell divides multiple times during the acute, antigen-driven phase of the host response to the readily eliminated influenza A viruses (11), the background turnover rates for influenza virus-specific CD8$^+$ D^{b}NP$_{366}$$^+$ memory T cells in the spleen average about 5% (5, 10) (Table 1, day 0). Particularly in the secondary H3N2→H1N1 response used to prime the mice analyzed for bystander activation in Table 1, the extent of BrdU incorporation drops to the level characteristic of long-term memory as soon as the virus is eliminated from the lungs (11).

Much higher levels of cycling (20 to 40%) (Fig. 1 and 2) were detected for the CD8$^+$ D^{b}p56$^+$ and CD8$^+$ K^{b}p79$^+$ T-cell populations recovered from γHV68-infected I-A$^{b+/+}$ mice for at least 2 months after evidence of virus replication (4) could no longer be found in the respiratory tract (day 12). This result suggests that, although infectious virus cannot be recovered directly from the spleen by a conventional plaque assay, there may be a continuing process of reactivation from latency in the in vivo situation. Virus reactivation could in turn lead to rapid CD8$^+$ T-cell-mediated elimination of the now productively infected B cells and macrophages and, as a consequence, a progressive reduction in the extent of γHV68 latency. By 80 to 90 days after the initial γHV68 challenge, the numbers of cycling CD8$^+$ D^{b}p56$^+$ and CD8$^+$ K^{b}p79$^+$ T cells in I-A$^{b+/+}$ mice had fallen (Fig. 2) to levels more characteristic of those associated with "resting" memory for other viruses. The counts continued to be high in the I-A$^{b-/-}$ congenic mice, where evidence of continuing lytic infection was found in the lung epithelium (4).

The results of these experiments thus support the idea that any evidence of CD8$^+$ T-cell proliferation that is substantially above a "homeostatic background" (7, 21, 23) of 5 to 10% over 6 days is reflective of continued exposure to antigen (20). Evidence of bystander activation can be found at the acute phase of the host response to an unrelated pathogen, but any bystander proliferation is at a much lower level than that associated with the antigen-driven effect.

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