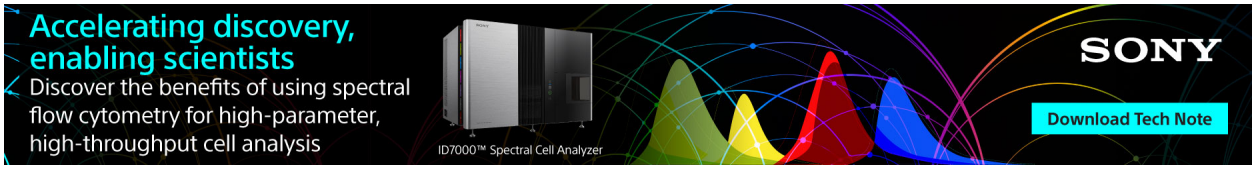

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Latent Murine γ-Herpesvirus Infection Is Established in Activated B Cells, Dendritic Cells, and Macrophages[1]

Emilio Flaño,[2*‡] S. Mazher Husain,[†‡] Jeffery T. Sample,[†‡§] David L. Woodland,[2*‡§] and Marcia A. Blackman[2,3*‡§]

Intranasal infection of mice with the murine γ-herpesvirus MHV-68 results in an acute lytic infection in the lung, followed by the establishment of lifelong latency. Development of an infectious mononucleosis-like syndrome correlates with the establishment of latency and is characterized by splenomegaly and the appearance of activated CD8$^+$ T cells in the peripheral blood. Interestingly, a large population of activated CD8$^+$ T cells in the peripheral blood expresses the Vβ4$^+$ element in their TCR. In this report we show that MHV-68 latency in the spleen after intranasal infection is harbored in three APC types: B cells, macrophages, and dendritic cells. Surprisingly, since latency has not previously been described in dendritic cells, these cells harbored the highest frequency of latent virus. Among B cells, latency was preferentially associated with activated B cells expressing the phenotype of germinal center B cells, thus formally linking the previously reported association of latency gene expression and germinal centers to germinal center B cells. Germinal center formation, however, was not required for the establishment of latency. Significantly, although three cell types were latently infected, the ability to stimulate Vβ4$^+$CD8$^+$ T cell hybridomas was limited to latently infected, activated B cells. *The Journal of Immunology,* **2000, 165: 1074–1081.**

The γ-herpesviruses establish a delicate balance between life-long latency in the host and immune control of the infection. Murine γ-herpesvirus-68 (MHV-68)[4] is a γ_2-herpesvirus that shares biological features and sequence homology with EBV and human herpesvirus-8 (1, 2). MHV-68 provides an excellent mouse model in which host-γ-herpesvirus interactions can be studied. Intranasal infection of mice with MHV-68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of latency. Levels of latent virus in the spleen peak around 14 days after infection, drop quickly, and remain stable for life in an immunologically competent and genetically unmanipulated animal (3).

Subsequent to the acute phase of the response, MHV-68 infection produces a syndrome similar to EBV-induced infectious mononucleosis in humans. This syndrome is characterized by splenomegaly (1, 4), Ag nonspecific B cell activation (5), and lymphocytosis of the peripheral blood (6). The splenomegaly is a consequence of increased numbers of cycling CD4$^+$ T cells, CD8$^+$ T cells, and B cells, and the blood lymphocytosis largely reflects increased numbers of activated CD8$^+$ T cells (4, 6). A striking feature of MHV-68 infectious mononucleosis-like syndrome is the pronounced expansion of CD8$^+$ T cells bearing Vβ4$^+$TCR (6). This expansion is not MHC restricted (6, 7) and appears to be independent of classical MHC class I or II molecules (7). The identity of the stimulatory ligand for the Vβ4$^+$CD8$^+$ T cell expansion remains elusive, but its expression correlates with peak levels of splenic latency at 2 wk after infection (7, 8).

Analogous to EBV infection, B cells are a principal reservoir of latent MHV-68 after intranasal infection (9–11), and transfer RNA gene expression associated with latency has been localized to splenic germinal centers (12–14). Latent MHV-68 has also been described in lung epithelial cells (15) and peritoneal macrophages and B cells (16). Recently, we have described lytic Ag presentation by splenic macrophages, dendritic cells, and B cells at time points after clearance of lytic infection from the lung, but the analysis did not distinguish between persistent lytic virus and latent virus in these cell types (17). The current experiments were initiated to identify the predominant hemopoietic cell types harboring latent virus at the peak of latency and to examine their participation in the activation of Vβ4$^+$CD8$^+$ T cells associated with the establishment of the infectious mononucleosis-like syndrome.

Materials and Methods

Mice

C57BL/6J (B6) and C57BL/6-CD28$^{tm1\ Mak}$ (18) (CD28$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions before MHV-68 infection at 8–16 wk of age and in biolevel 3 containment after infection. All animal procedures in these experiments were approved by the institutional animal care and use committee at St. Jude Children's Research Hospital (Memphis, TN).

Virus stocks, infection, and sampling of mice

The stock of MHV-68 (clone G2.4) was obtained from Prof. A. A. Nash (Edinburgh, U.K.), propagated in OMK cells (ATCC 1566CRL, American Type Culture Collection, Manassas, VA), and titrated on NIH-3T3 fibroblast s(ATCC CRL1568, American Type Culture Collection) monolayers, as previously described (3). Mice were anesthetized with 2,2,2-tribromoethanol and infected intranasally with 600 PFU of MHV-68 in a total

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[4] Abbreviations used in this paper: MHV-68, murine γ-herpesvirus-68; i.n., intranasal; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PNA, peanut agglutinin; MEF, murine embryonic fibroblast; LDA, limiting dilution analysis; CD40L, CD40 ligand.

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volume of 40 μl PBS. Splenocytes and/or peripheral blood were analyzed at various times after infection.

The lacZ *hybridoma assay*

Characterization of $V\beta4^+CD8^+$ *lacZ*-inducible T cell hybridomas that specifically respond to MHV-68-infected spleen cells 14 days postinfection has been previously described (7). Ag presentation and development of the histochemical reaction were performed as previously described (7, 8). Briefly, T cell-depleted spleen cells were used as stimulator cells, titrated in 2-fold dilutions, and plated. Representative *lacZ*-inducible T cell hybridomas 4BH-98 or 5BH-11 were added, incubation proceeded overnight, and β-galactosidase activity was assessed in individual wells. The background stimulation was determined by using naive APCs as stimulators.

Flow cytometry

After erythrocyte lysis in hemolytic Gey's solution, cells were stained for FACS analysis using combinations of the following Abs and lectins: CD1d (CD1.1, Ly-38), CD11b (Mac-1α), CD11c, CD16/CD32 (FcBlock), CD19, CD23 (IgE FcR), CD25 (IL-2 Rα), CD38, CD40, CD43 (Ly-48), CD44, CD45R (B220), CD62L, CD69, CD80 (B-7), CD95 (Fas), CD138 (Syndecan-1), TCR Vβ4, CD8α, IgD^b, IgM, $I\text{-}A^b$, and peanut agglutinin (PNA). All reagents were purchased from PharMingen (San Diego, CA), except CD8α (Caltag, Burlingame, CA) and PNA (Sigma, St. Louis, MO). At least 20,000 live cells were gated and acquired on a FACScan flow cytometer, and the data were analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Infective center assay

Infective center assays were performed as previously described (8). Briefly, serial dilutions (in triplicate) of T cell-depleted splenocytes were plated onto monolayers of NIH-3T3 cells and overlaid with carboxymethylcellulose; after 6 days of culture, plaques were quantitated. As this assay also measures lytic virus, the possible contribution of lytic virus to the titers was determined by parallel analysis of cell lysates. Thus, duplicate samples were subjected to a single cycle of freeze/thawing, resuspended, and directly analyzed (without centrifugation) for the development of plaques.

Virus limiting dilution analysis (LDA)

LDA of virus titer was performed as previously described (11, 17), with modifications. Murine embryonic fibroblasts (MEF) were harvested from BALB/c embryos and grown in DMEM supplemented with tumor cocktail (19) and 10% FCS. Serial 2-fold dilutions of splenocytes starting at 5×10^4 cells/well were plated onto MEF monolayers (1.5×10^4/well) in flat-bottom 96-well plates. Twenty-four wells were plated for each dilution. The numbers of wells exhibiting cytopathic effect were counted after 3 wk of culture. The presence of lytic virus was determined as described above by simultaneous analysis of the total cell lysate obtained after a single cycle of freeze/thawing.

Cell purification

FACS sorting was performed on a MoFlo (Cytomation, Fort Collins, CO) or on a FACStar Plus (Becton Dickinson, Mountain View, CA) equipped with a high speed sorting module. Pooled splenocytes from five to seven mice at 14 days after infection were used in all experiments. For B cell sorting, an aliquot of cells was first incubated with FcBlock and then stained with FITC-conjugated anti-CD11c, PE-conjugated anti-CD11b, and Cy-Chrome-conjugated anti-B220. B cells ($B220^+$, $CD11b^-$, $CD11c^-$) were sorted. To control for loss of virus titer or stimulatory activity that might result from the binding of Abs and/or from the physical stress of the sorting, cells were stained and mock sorted as single viable cells. At the same time and to avoid B cell cross-contamination, the rest of the sample was B cell-depleted using anti-B220 and a mixture of anti-rat and anti-mouse Ig Dynabeads (Dynal, Oslo, Norway). The remaining population was stained as described above and sorted as macrophages ($B220^-$, $CD11b^+$, $CD11c^-$) or dendritic cells ($B220^-$, $CD11b^-$, $CD11c^+$). After magnetic depletion the quantity of B cells in the remaining population was <0.01%, and the purity of the sorted populations was >99%. For activated/resting B cell sorting, spleen cells were incubated with FcBlock and then stained with FITC-conjugated PNA and Cy-Chrome-conjugated anti-B220. Activated B cells were sorted as $PNA^{high}B220^+$, and resting B cells were sorted as $PNA^{low}B220^+$. The purities of the sorted populations were >95%.

Spleen cells were depleted of T and B cells according to the following protocol. B cells were depleted by magnetic sorting as described above, followed by incubation with the IgM anti-Thy1 mAb AT83 (20) and a mixture of rabbit and guinea pig complement (Cedarlane, Ontario, Canada) to deplete T cells. The percentage of B cells in the final population was <0.5%.

Germinal center B cells were enriched as previously described (21) with modifications. Splenocytes were Fc-blocked and stained with a cocktail of biotinylated Abs to CD11b, CD11c, Ly-6G (GR-1), Ter119 (Ly-76), CD3, CD4, CD8, DX5, IgD, and CD138 (PharMingen). After washing, cells were depleted using streptavidin-conjugated Dynabeads (Dynal). The resulting cells were enriched 2- to 5-fold for germinal center B cells as judged by staining with PNA and anti-B220.

Results

A high frequency of splenic dendritic cells is latently infected

In a previous report we described the presentation of MHV-68 lytic cycle protein epitopes by splenic B cells, macrophages, and dendritic cells at a time after infection corresponding to the peak of splenic latency (17). An infective center assay confirmed that this was due to the presence of cell-associated virus rather than persistence of Ag-loaded APC, but we did not distinguish whether the virus was produced as a consequence of reactivation from latency or persistent lytic infection. It has previously been shown that B cells and peritoneal macrophages harbor latent virus (9–11, 16), but latency has not been described in splenic macrophages or dendritic cells. Therefore, the present studies were initiated to determine whether splenic macrophages and dendritic cells harbor latent MHV-68 and, if so, to determine the frequency of latency in each of these cell types.

To analyze the presence of latent virus in the three different cell populations, splenocytes isolated from MHV-68-infected mice at 14 days after infection were FACS-sorted into purified populations of B cells ($B220^+$, $CD11b^-$, $CD11c^-$), macrophages ($B220^-$, $CD11b^+$, $CD11c^-$), and dendritic cells ($B220^-$, $CD11b^-$, $CD11c^+$). Analysis was performed at 14 days postinfection because at this time point latent virus is at peak levels, and lytic virus is largely cleared (3, 22, 23). Latent virus in these purified populations was quantitated by LDA of in vitro virus reactivation (11). Thus, sorted cell populations were plated onto monolayers of MEF to allow in vitro reactivation of MHV-68, and the resulting virus-induced cytopathic effect was quantitated 3 wk later. Data were plotted as the percentage of wells positive for cytopathic effect at each cell concentration per well (Fig. 1), and linear regression analysis was used to determine the frequency of latently infected cells (Table I). As expected from previous reports (9–11, 16), latent reactivatable virus was detected in macrophages and B cells, but, unexpectedly, the highest frequency of latent virus was found in the dendritic cell population (Table I). In fact, the frequency of latent infection in dendritic cells was 7-fold greater than that in B cells. Taking into account the relative representation of each of the three cell types, approximately equivalent numbers of latently infected B cells and dendritic cells exist in the spleen (Table I).

A parallel series of LDA plates was set up in each experiment with duplicate samples that had been frozen and thawed once to destroy viable cells, thereby allowing pre-existing infectious virus to be distinguished from reactivation of latent virus. Previous studies established that one cycle of freeze/thawing does not substantially reduce the titer of cell-associated lytic virus (8). The LDA viral reactivation assay is approximately 10-fold more sensitive than plaque assays for detecting preformed virus, as it allows prolonged time for reactivation or outgrowth of preformed infectious virus (Ref. 24 and our unpublished observations). The results showed that there were low or undetectable levels of lytic virus (Fig. 1), consistent with the fact that lytic virus is largely cleared by day 14 (3, 22, 23) and supporting the conclusion that the majority of the virus detected in the LDA is attributed to latent virus. Specifically, the data in Fig. 1 show that there was a very low, but measurable, frequency of lytic virus in unmanipulated spleen cells

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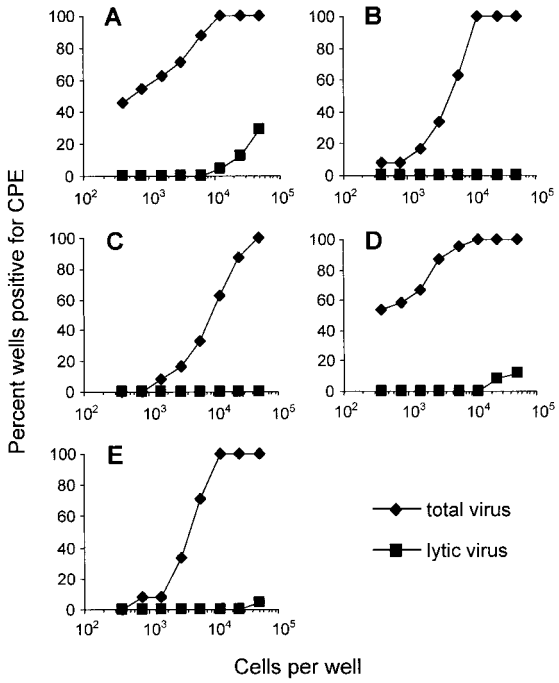

FIGURE 1. B cells, macrophages, and dendritic cells in the spleen harbor latent MHV-68. An LDA to detect reactivation from latency was performed on spleen cells 14 days after infection. Data are presented as the percentage of wells that scored positive for viral cytopathic effect as a function of the number of cells plated per well, 3 wk after plating. Spleen cells were analyzed directly (*A*), FACS-stained and mock-sorted (*B*), or sorted as B cells ($B220^+CD11b^-CD11c^-$; *C*), dendritic cells ($B220^-CD11b^-CD11c^+$; *D*), or macrophages ($B220^-$ $CD11b^+CD11c^-$; *E*). Lytic (infectious) virus results were obtained from duplicate samples when the cells were lysed by freeze/thawing before plating. The data presented are the average of two independent experiments, representative of four experiments with similar results, each analyzing pooled spleens from seven mice.

(1/132,000; Fig. 1*A*) and dendritic cells (1/346,000; Fig. 1*B*). Lytic virus was also detected in the macrophages (Fig. 1*E*), although the frequency was too low to be calculated. In mock sorted cells (Fig. 1*B*) and B cells (Fig. 1*C*), the lytic MHV-68 titers were below the limit of detection of the assay. Therefore, it can be concluded that virus reactivation measured at this time after infection in the spleen predominantly reflects latent virus, and that the quantity of preformed infectious virus is insignificant.

In the course of these studies we discovered that the sorting procedure lowered the reactivation frequency of latently infected cells. Mock sorting controls in which cells were stained and then sorted as single viable cells showed an approximately 5-fold decrease in virus reactivation compared with unsorted cells (Fig. 1, *A* and *B*), although the cell viability of the sorted populations was 99% at the time of plating. Apparently, stress induced by sorting has a dramatic effect on the ability to detect latently MHV-68-infected cells, at least as measured by this assay. Therefore, the frequencies obtained for FACS-sorted cells should be considered the lower limit of the true reactivatable frequency. Importantly, this control rules out the possibility that Ab binding has caused increased levels of reactivation.

Activated B cells are the major B cell reservoir of MHV-68 at the peak of latency

The high levels of expression of the MHV-68 transfer RNA-like genes (a marker of latency) associated with germinal centers and primary follicles during MHV-68 latency (12–14) raised the possibility that latency may be associated preferentially with germinal center B cells, but this has not been formally shown. Therefore, we also analyzed latency among different subpopulations of B cells. PNA was used as a marker for activated/germinal center B cells (25). To confirm the germinal center phenotype of the isolated $B220^+PNA^{high}$ cells, the expression of different activation markers and B cell Ags in spleen cells analyzed at the same time after infection was determined. As is shown in Fig. 2, $B220^+PNA^{high}$ B cells were blasted, $CD19^+$, $CD80^+$, $CD44^{high}$, $CD23^{low}$, $CD95^+$ cells, expressing high levels of $I\text{-}A^b$ and low levels of IgD, and showed heterogeneous down-regulation of CD38, all hallmarks of germinal center B cells (26, 27). Moreover, they expressed markers of activated/cycling B cells ($CD25^{high}$, $CD43^{high}$, $CD62L^{low}$, $CD69^{high}$), and they were negative for expression of Syndecan-1 (CD138; data not shown). Note that these cells exhibited high levels of expression of CD40, a phenotype related to B cell survival and rescue from apoptosis (28, 29).

Table I. *Quantitation of MHV-68 latency within subsets of spleen cells 14 days after infection*

Cells	MHV-68 Reciprocal Frequency (95% confidence limits)[a]	% of Cells[b]	Total No. of Cells[c]	Latently Infected Cells[d]
Expt. 1				
Spleen	1,521 (0–4,966)	100	2×10^8	1.3×10^5
Mock sorting[e]	7,596 (4,251–9,747)	100	2×10^8	2.6×10^4
B cells[f]	12,555 (10,642–18,673)	59.7	1.2×10^8	9.5×10^3
Dendritic cells[g]	1,297 (377–1,971)	6.7	1.3×10^7	1×10^4
Macrophages[h]	3,571 (955–6,451)	5.6	1.1×10^7	3×10^3
Expt. 2				
Spleen	1,955 (239–3,731)	100	2×10^8	1×10^5
Activated B cells[i]	890 (255–1,592)	5.2	1×10^7	1.1×10^4
Resting B cells[j]	35,208 (23,995–225,236)	45.4	9.1×10^7	2.5×10^3

[a] Frequency ± 95% confidence limits were determined by linear regression analysis of LDA data, as determined in Figs. 1 and 3.
[b] Percentage of each subset of total spleen cells was determined by FACS analysis.
[c] Total number of cells were estimated from the percentage of total spleen, based on an estimate of 2×10^8 cells/spleen at 14 days after infection.
[d] Number of latently infected cells based on the frequency of latency within each cell type and the estimated total number of cells.
[e] Mock sorted cells were stained and sorted for live, single events.
[f] B cells were sorted as $B220^+CD11b^-CD11c^-$. The sorted populations were 99.24 ± 0.28% B cells, 0.15 ± 0.01% macrophages, and 0.73 ± 0.73% dendritic cells.
[g] Dendritic cells were sorted as $B220^-CD11b^-CD11c^+$. The sorted populations were 95.60 ± 0.26% dendritic cells, 1.26 ± 0.55% macrophages, and 0.00 ± 0.00% B cells.
[h] Macrophages were sorted as $B220^-CD11b^+CD11c^-$. The sorted populations were 95.59 ± 0.45% macrophages, 0.45 ± 0.35% dendritic cells, and 0.00 ± 0.00% B cells.
[i] Activated B cells were sorted as $B220^+PNA^{high}$.
[j] Resting B cells were $B220^+PNA^{low}$.

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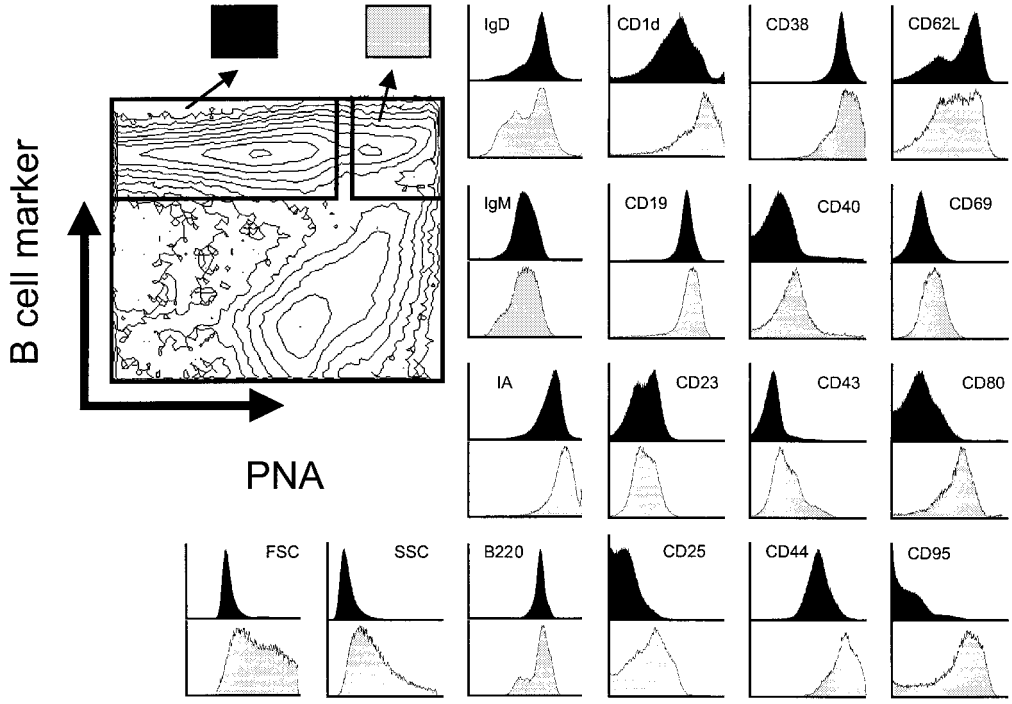

FIGURE 2. Phenotypic analysis of splenic B cells 14 days after infection. B cells expressing PNA at high or low density were analyzed for the expression of the following cell surface molecules and characteristics: I-A^b, IgD, B220, CD1d, CD19, CD23, CD25, CD38, CD40, CD43, CD44, CD62L, CD69, CD80, CD95, forward scatter, and side scatter.

The in vitro reactivation results revealed that after FACS sorting, PNA^{high} B cells ($B220^+PNA^{high}$) had 20-fold higher frequency of latent infection than PNA^{low} ($B220^+PNA^{low}$) B cells (Fig. 3 and Table I). Although resting B cells contain latent virus at a low frequency, latency at 14 days after infection is harbored preferentially in activated B cells with a germinal center phenotype.

Expression of the latency-associated ligand that stimulates $V\beta4^+CD8^+$ T cell hybridomas is restricted to activated B cells

The ligand driving the expansion of $V\beta4^+CD8^+$ T cells, a key feature of the infectious mononucleosis-like phase of MHV-68 infection, has not been identified. However, we are able to assess ligand expression by the ability to stimulate a panel of $V\beta4^+CD8^+$ T cell hybridomas generated from latently infected mice during the infectious mononucleosis-like phase of infection. Using this approach, we have previously shown that the $V\beta4^+CD8^+$ T cell stimulatory activity was expressed by T cell-depleted spleen cells isolated from MHV-68-infected mice at the peak of latency (7). Furthermore, FACS-sorted $B220^+$ cells expressed the stimulatory activity. The observation here that B cell latency was preferentially associated with activated B cells made it important to test whether the $V\beta4^+CD8^+$-stimulatory activity was expressed by activated, but not resting, B cells. First, using representative $V\beta4^+CD8^+$ T cell hybridomas, we tested the stimulatory capacity of B cells that had been enriched for germinal center cells by a non-flow cytometric-based negative depletion protocol compared with T cell-depleted spleen cells from day 14-infected mice. The data indicated that there was an approximately 2-fold increase in stimulation of a representative T cell hybridoma (Fig. 4*A*) that correlated with the increase in latency as measured by the infective center assay (Fig. 4*B*) and was consistent with the approximately 2-fold increase in germinal center B cells attained in this experiment. Second, we directly compared the stimulatory ability of sorted populations of PNA^{high} and PNA^{low} B cells. The data (Fig. 5) show that despite the fact that latent virus is associated both with PNA^{high} and PNA^{low} B cells, albeit at different frequencies (Table I), the $V\beta4$ hybridoma stimulatory activity resided exclusively in the activated, PNA^{high} B cell population.

We then addressed whether latently infected macrophages and dendritic cells expressed the stimulatory ligand as well. We had previously shown that splenic $Mac\text{-}1^+$ cells from latently infected mice did not stimulate the hybridomas, but that T- and B-depleted cells had strong stimulatory capacity (7), raising the possibility that latently infected dendritic cells expressed the stimulatory ligand. Again using representative $V\beta4^+CD8^+$ T cell hybridomas, we directly tested the stimulatory ability of the FACS-sorted B cells, macrophages, and dendritic cells from the spleens of day 14-infected mice. The initial analysis of FACS-sorted cells (data not shown)

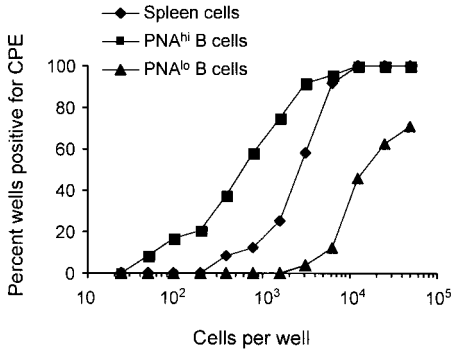

FIGURE 3. Activated, PNA^{high} B cells contain most of the latent virus in the B cell population. Latently infected cells were assessed in FACS-sorted PNA^{high} (■) and PNA^{low} (▲) subsets of splenic B cells (◆) 14 days after infection by LDA, as described in Fig. 1. The data presented are from a single experiment representative of three independent experiments with similar results, each analyzing pooled spleens from seven mice.

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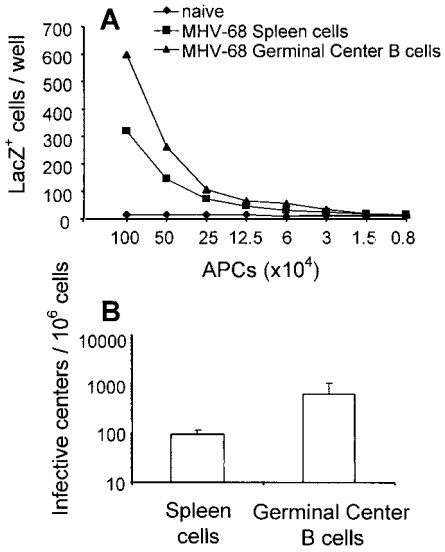

FIGURE 4. Germinal center B cells have increased Vβ4$^+$CD8$^+$ T cell stimulatory capacity, which correlates with an increase in MHV-68 latency. *A*, The ability of T cell-depleted spleen cells (■) and germinal center-enriched B cells (▲; see *Materials and Methods*) 14 days after infection to stimulate a representative Vβ4$^+$CD8$^+$ T cell hybridoma (5BH-11) is shown. Naive T cell-depleted spleen cells (♦) were used as negative controls. *B*, Latent viral titers in T cell-depleted spleen cells and germinal center-enriched B cells at 14 days after infection were estimated by an infective center assay. The infective center numbers are the mean values of triplicate wells. Error bars represent the SD.

indicated that the B cells, but not the macrophages or dendritic cells, stimulated the hybridomas. To confirm that the inability of the macrophages and dendritic cells to stimulate the hybridomas was not the consequence of a loss of stimulatory capacity associated with the lower titers of reactivatable virus after FACS sorting (Fig. 1, *A* and *B*, Table I), a non-flow cytometric-based negative depletion was used to enrich for non-T and non-B spleen cells. The residual population contained significant percentages of macrophages (~40%) and dendritic (~15%) cells. Importantly, there was no Vβ4 stimulation by this population (Fig. 6), reinforcing the conclusion based on analysis of the FACS-sorted populations that the Vβ4 stimulatory capacity was exclusively expressed by latently infected B cells. Furthermore, there was enhanced hybridoma stimulation in the same experiments by a population of spleen cells that had been enriched for germinal center B cells, reinforcing the earlier conclusion that expression of the Vβ4-

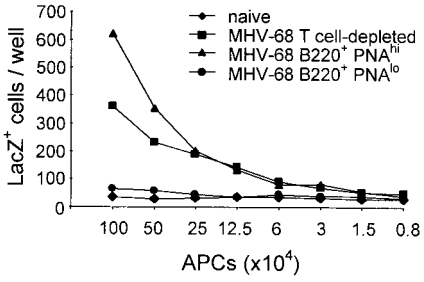

FIGURE 5. Activated B cells present the ligand that stimulates Vβ4$^+$CD8$^+$ T cell hybridomas. Stimulation of a representative MHV-68-specific Vβ4$^+$CD8$^+$ hybridoma (4BH-98) by T-depleted spleen cells (■) 14 days after infection. B cell subsets were FACS sorted as germinal center B cells (B220$^+$PNA^{high}; ▲) or follicular B cells (B220$^+$PNA^{low}; ●). Naive T cell-depleted spleen cells (♦) were used as negative controls.

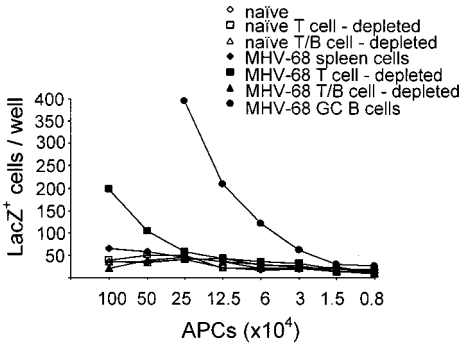

FIGURE 6. T cell- and B cell-depleted splenocytes do not express the ligand that stimulates Vβ4$^+$CD8$^+$ T cell hybridomas. The ability of spleen cells 14 days after infection to stimulate a representative Vβ4$^+$CD8$^+$ T cell hybridoma (4BH-98) was assessed. Spleen cells from MHV-68-infected mice were tested directly (♦) or following T cell depletion (■), T cell and B cell depletion (▲), or germinal center cell enrichment (●) as described in *Materials and Methods*. The percentage of B cells in the T/B-depleted population was <0.5%. Naive splenocytes that were unseparated (◇), T cell depleted (□), or T cell and B cell depleted (△) were used as controls.

stimulatory ligand was preferentially expressed in latently infected, activated B cells.

The inability of latently infected macrophages and dendritic cells to stimulate the hybridomas appeared to contradict our previous report that a non-B, non-T population contained Vβ4 stimulatory activity (7). However, retrospective analysis of the phenotype of those cells indicated the presence of a small residual population of blasted B220^{low} B cells (data not shown). As our current results indicated that activated B cells exclusively express Vβ4 stimulatory activity, we had apparently inadvertently enriched for a subset of highly stimulatory, activated B cells. In the current experiments our protocol for T/B cell depletion had been modified to eliminate this residual population of activated B cells. Taken together, our current data demonstrate that B cells, macrophages, and dendritic cells contain latent virus, but only B cells express the latency-associated stimulatory ligand for Vβ4$^+$CD8$^+$ T cell activation. Furthermore, the stimulatory ligand resides exclusively in the activated subset of latently infected B cells.

Germinal center formation is not required for the establishment of latency or Vβ4$^+$CD8$^+$ T cell stimulation

The high levels of latency in activated B cells and the association of latent gene expression with germinal centers (12–14) raised the possibility that MHV-68 uses the normal germinal center reaction for the establishment/maintenance of latency, as has been proposed for EBV (30). To test the requirement for germinal center formation in establishing latency and in the generation of Vβ4$^+$CD8$^+$ T cell-stimulatory activity, we analyzed MHV-68-infected CD28$^{-/-}$ mice. CD28$^{-/-}$ mice have been shown to be deficient in germinal center formation and to have defective T cell activation (31). Comparative analysis of infected B6 and CD28$^{-/-}$ mice showed that although the percentages of B cells in the spleen are similar in both mouse models (Fig. 7*A*), the CD28$^{-/-}$ mice do not show a corresponding increase in the number of PNA^{high} B cells (Fig. 7*B*). Thus, MHV-68 causes the expected increase in numbers of B cells in the spleen characteristic of MHV-68-induced splenomegaly, but the activated B cells do not differentiate into germinal center B cells. Analysis of T cell activation in the two strains showed that the lymphocytosis of the peripheral blood was not dependent on CD28, as the numbers of CD8$^+$ T cells increased comparably (Fig. 8*A*). In addition, there was no difference in expression of the Vβ4

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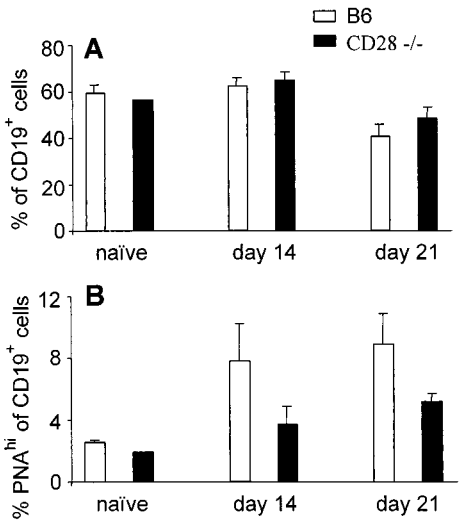

FIGURE 7. Comparison of B cell activation in MHV-68-infected B6 and CD28$^{-/-}$ mice. B6 and CD28$^{-/-}$ mice were assessed for the percentage of CD19^{+} splenic B cells (*A*) and levels of activated, PNA^{high} B cells (*B*) before infection (naive) and at 14 and 21 days after MHV-68 infection. Error bars correspond to the SD. The *p* values for the differences in the percentage of activated cells between B6 and CD28$^{-/-}$ mice at 14 and 21 days postinfection, determined by the Mann-Whitney test, are 0.01 and 0.05, respectively.

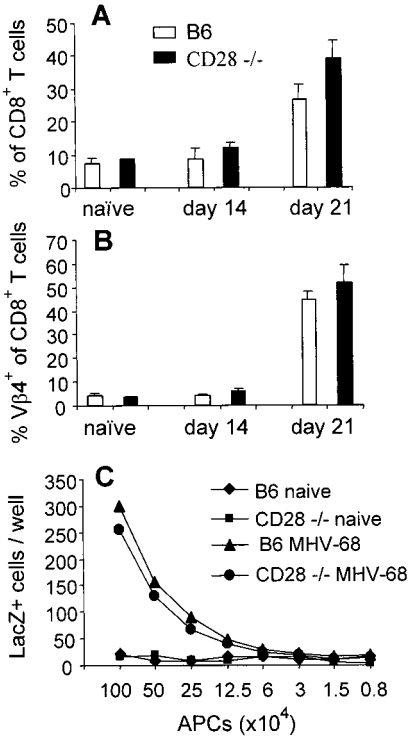

FIGURE 8. MHV-68-induced Vβ4^{+}CD8^{+} T cell activation/expansion are not dependent on CD28. B6 and CD28$^{-/-}$ mice were assessed for the percentage of CD8^{+} T cells among total blood lymphocytes (*A*) and the percentage of Vβ4^{+} T cells among CD8^{+} T cells (*B*) before (naive) and 14 and 21 days after MHV-68 infection. *C*, The ability of T cell-depleted spleen cells from B6 (▲) and CD28$^{-/-}$ (●) mice at 14 days after MHV-68 infection to stimulate a representative Vβ4^{+} T cell hybridoma, 4BH-98, was assessed. T cell-depleted splenocytes from naive B6 (◆) and CD28$^{-/-}$ (■) mice were used as controls. Error bars represent the SD.

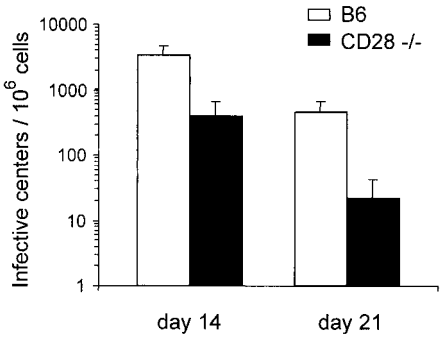

FIGURE 9. MHV-68 splenic latency in B6 and CD28$^{-/-}$ mice. Latent viral titers in T cell-depleted spleen cells at 14 and 21 days after infection were estimated by an infective center assay. The infective center numbers are the mean values of triplicate wells assessed in at least two separate experiments. Error bars represent the SD. The *p* values for the differences between B6 and CD28$^{-/-}$ mice at 14 and 21 days postinfection, determined by the Mann-Whitney test, are both 0.001.

stimulatory ligand, as there were comparable levels of Vβ4^{+}CD8^{+} T cell expansion in the peripheral blood (Fig. 8*B*) and spleen (data not shown), and T cell-depleted spleen cells from day 14-infected mice stimulated a representative Vβ4^{+}CD8^{+} T cell hybridoma to comparable levels (Fig. 8*C*). Finally, the absence of CD28 did not prevent the establishment of latency on day 14 (Fig. 9). The decreased levels of infective centers in the CD28$^{-/-}$ mice may reflect the reduced pool of activated B cells in these mice (Fig. 7*B*), although the observation that the levels in CD28$^{-/-}$ mice are progressively lower at 14 and 21 days after infection is intriguing and may indicate that there is a defect in the maintenance of latency. Long term experiments are in progress to address this possibility. Thus, the data show that germinal center formation per se is not required for B cell proliferation, stimulation of Vβ4^{+}CD8^{+} T cells, or establishment of latency.

Discussion

Analysis of splenic latency has shown that three types of APC harbor latent MHV-68: B cells, macrophages, and dendritic cells. Despite this, only latently infected, activated B cells express the ligand that drives the characteristic expansion of Vβ4^{+}CD8^{+} T cells associated with the infectious mononucleosis stage of MHV-68 infection. Furthermore, although activated B cells with a germinal center phenotype harbor the highest level of reactivatable latent virus and Vβ4^{+} T cell hybridoma stimulatory capacity, analysis of CD28$^{-/-}$ mice, which fail to form germinal centers, showed that germinal center formation per se was not required for establishment of latency or stimulation of Vβ4^{+}CD8^{+} T cells.

The identification of dendritic cells and splenic macrophages as reservoirs of latent MHV-68 virus after intranasal infection is an exciting and novel finding. Therefore, it is important to formally demonstrate that the virus is truly latent and not due to slow, chronic lytic infection. Unfortunately, latency gene expression is in the early stages of characterization, and it is not yet possible to use expression of diagnostic latency genes to define latency. Recent evidence suggests that MHV-68 may have a complex program of latency gene expression, analogous to EBV (32), as different patterns of expression of putative latency genes were shown in different organs (33). Although we have recently reported that the expression of M2 is latency associated, nothing is known about the cell type specificity of M2 expression (34). In the absence of a molecular definition of latency, the LDA viral reactivation assay has been carefully developed and used to distinguish lytic from

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latent virus (11, 16, 23). Importantly, this assay is approximately 10-fold more sensitive for detecting lytic virus in disrupted cells than the standard plaque assay, as the prolonged incubation time allows sufficient time for the outgrowth of preformed infectious virus (11, 24). Therefore, in the absence of a molecular definition of latency, it is reasonable to conclude that the virus detected in dendritic cells and macrophages is latent, although we acknowledge that it is formally possible that the assay fails to detect slow, chronic viral replication as lytic virus.

Macrophages, dendritic cells, and activated B cells constitute the APC of the immune system, which are responsible for initiation of Ag-specific responses. The observation here that all three types of APC harbor latent virus may have important implications for the ability of host immune responses to control reactivation from latency. Latently infected macrophages, dendritic cells, and B cells have the potential to be targets of the immune response, and latent infection might interfere with cytokine synthesis or cell surface Ag expression, resulting in modulation of the host immune response and favoring viral persistence.

The current studies show that a high frequency of dendritic cells harbors latent virus. Taking into account the absolute numbers of B cells and dendritic cells, the two cell types constitute approximately equal pools of splenic latency in intranasally infected mice, although we cannot rule out the possibility that the flow cytometry-associated reduction in latency levels detected by in vitro reactivation assays is different for the two cell types. The high frequency of latency in dendritic cells in this study makes it somewhat surprising that latency had not previously been identified in dendritic cells. Whereas early studies suggested the presence of a latently infected adherent cell population (9), subsequent studies in B cell-deficient μMT mice were consistent with the conclusion that B cells were the exclusive latently infected cell type in the spleens of intranasally infected mice (35). However, the observation that B cells are required for the establishment of splenic infection (after intranasal infection) provides a likely explanation for the absence of other latently infected cell types in the spleens of μMT mice (11, 15, 35).

The observation that dendritic cells harbor latent virus raises the question of whether these cells are also targets for lytic infection. Dendritic cells are extremely efficient at stimulating primary T cell responses, because of their ability to secrete chemokines that attract naive T cells; their high density expression of MHC molecules, MHC/peptide complexes, and costimulatory molecules; and their ability to secrete high levels of IL-12 (36). Thus, viral Ag presentation by infected dendritic cells might have important implications for the initiation of the acute anti-viral response. This possibility warrants further investigation.

Despite the fact that B cells are not the only latently infected cell type, they clearly play a unique role in latent MHV-68 infection. First, they appear to be required for trafficking of MHV-68 to the spleen after intranasal infection (15). Second, they appear to control virus reactivation and chronic infection (23). Third, the current results show that they are the only latently infected cell type capable of activating Vβ4$^+$CD8$^+$ T cell hybridomas. Identification of potential specific roles for latently infected macrophages and dendritic cells requires further investigation.

The finding that activated B cells are unique in their expression of the Vβ4$^+$CD8$^+$ T cell stimulatory ligand is consistent with previous reports that in vivo Vβ4 expansion is dependent on the presence of CD4$^+$ T cells, B cells, and CD40L (8, 37). It is somewhat surprising that Vβ4 expansion is not also dependent on CD28 expression, as both CD28/B7 and CD40L/CD40 signaling pathways play critical roles in B cell responses, and early studies suggested that CD28 ligation was required for induction of CD40L expression (38). However, more recently it has been shown that TCR triggering alone can induce CD40L expression (39, 40). Clearly, Vβ4 expansion occurs in CD28$^{-/-}$ mice, and although germinal center B cells are not formed, B cells have been activated in the sense that they expand in number comparably to B6 mice. The data indicate that although Vβ4 stimulatory activity is preferentially associated with latently infected PNA^{high} B cells in B6 mice, full progression to germinal center formation is not required for expression of the stimulatory ligand in activated B cells.

The exclusive ability of activated B cells to stimulate Vβ4$^+$CD8$^+$ T cell hybridomas also has significant implications for understanding the nature of the stimulatory ligand. Previous studies showing that presentation of the ligand appears to be independent of MHC molecules (7) indicate that the ligand is unusual. There are several possible explanations for the selective ligand expression in activated B cells. First, it is possible that the exclusive expression of the ligand by B cells reflects cell-specific patterns of latent gene expression (33), analogous to the multiple forms of latency in EBV (32). A more complete understanding of the genes expressed during MHV-68 latency will be required to test this idea. Second, there may be differential Ag processing of the uncharacterized Vβ4 ligand in B cells. For example, B cells preferentially present Ags internalized by the B cell Ig receptor (41, 42). Third, it is possible that the Vβ4 ligand is a self molecule, such as a B cell activation marker, that is up-regulated to sufficient levels during infection to exceed established tolerance.

The association between activated B cells and latency described in this report is consistent with the possibility that the establishment of MHV-68 latency in mice is similar to the process described for EBV, in that establishment/maintenance of viral latency may exploit the normal germinal center reaction. Although our data show that latency can be established in the absence of the germinal centers in CD28$^{-/-}$ mice, an important question is whether long term MHV-68 latency requires the generation of memory cells, a germinal center-dependent function, as it has been shown that EBV persists in memory B cells (30). The pool of resting B cells that harbor latent virus identified in the current study could represent memory B cells, in which long term latency may be maintained. Therefore, it will be important to examine long term latency in CD28$^{-/-}$ and other mice incapable of germinal center formation and/or incapable of generating memory B cells. Another interesting possibility that warrants further investigation is that MHV-68 infection allows formation of germinal centers in CD28$^{-/-}$ mice by bypassing the normal requirements for T cell help in the establishment of germinal centers. Although it has been shown in VSV-infected CD28$^{-/-}$ mice that germinal centers are not formed in the absence of T cell help (18), this has not been examined directly in MHV-68-infected mice.

CD28$^{-/-}$ mice have also been reported to be deficient in T cell activation, although the absence of T cell costimulation can be compensated by the strength of the signal, which is affected by Ag density, or by alternative costimulatory signals (reviewed in Ref. 38). Although the current studies did not directly assess the requirement for CD28 in the initial stages of T cell activation in response to acute viral infection, the T cell profile during the infectious mononucleosis phase of the response in terms of activation/expansion of both CD8$^+$ T cells and Vβ4$^+$CD8$^+$ T cells was comparable in B6 and CD28$^{-/-}$ mice. It is likely that the acute T cell response is initiated normally, particularly in light of the possibility that dendritic cells may be lytically infected, as dendritic cells are potent APC and would probably bypass the requirement for CD28 in initiating the anti-viral T cell response.

In conclusion, the finding that MHV-68 establishes latency in multiple cell types, including B cells, macrophages, dendritic cells, and lung epithelial cells, is in accordance with similar findings for

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human herpesviruses such as EBV, CMV, and Kaposi sarcoma-associated herpesvirus (32, 43–47). Despite this, B cells appear to play the pivotal role in both the establishment and maintenance of latency and in triggering the expansion of $V\beta4^+CD8^+$ T cells during the infectious mononucleosis stage of infection. MHV-68 infection of mice provides an excellent experimental model system for determining the significance of discrete reservoirs of latency in cells other than B cells.

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