Lymphotoxin- α -Deficient Mice Can Clear a Productive Infection with Murine Gammaherpesvirus 68 but Fail To Develop Splenomegaly or Lymphocytosis†

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Respiratory challenge with murine gammaherpesvirus 68 (MHV-68) leads to an acute productive infection of the lung and a persistent latent infection in B lymphocytes, epithelia, and macrophages. The virus also induces splenomegaly and an increase in the number of activated CD8 T cells in the circulation. Lymphotoxin- α -deficient (LT $\alpha^{-/-}$) mice have no lymph nodes and have disrupted splenic architecture. Surprisingly, in spite of the severe defect in secondary lymphoid tissue, LT $\alpha^{-/-}$ mice could clear a productive MHV-68 infection, although with delayed kinetics compared to wild-type mice, and could control latent infection. Cytotoxic T-cell activity was comparable in the lungs and spleens of LT $\alpha^{-/-}$ and wild-type mice. However, splenic gamma interferon responses were substantially reduced in LT $\alpha^{-/-}$ mice. Furthermore, LT $\alpha^{-/-}$ mice failed to develop splenomegaly or lymphocytosis. Although germinal centers were absent, LT $\alpha^{-/-}$ mice were able to class switch and showed significant virus-specific antibody titers. This work demonstrates that organized secondary lymphoid tissue is not an absolute requirement for the generation of immune responses to viral infections.

Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring rodent pathogen (6) which is closely related to *Epstein-Barr virus* (EBV), the Kaposi's sarcoma-associated *human herpesvirus 8*, and *Herpesvirus saimiri* (9, 28). Intranasal administration of MHV-68 results in acute productive infection of lung alveolar epithelial cells and a latent infection in several cell types, including B lymphocytes and macrophages (3, 10, 26, 31). Infectious virus is cleared from the lungs 10 to 13 days after infection by a T-cell-mediated process (7, 10). The antibody response develops several weeks after infection (25). Control of latent virus, once established, appears to involve the redundant action of either T- or B-cell-mediated pathways (26). Mechanisms which control latent virus do not develop efficiently in the absence of CD4 T cells, leading to viral reactivation in the lungs (7).

MHV-68 induces an inflammatory infiltrate in the lungs, enlargement of the lymph nodes, splenomegaly, and a lymphocytosis comprised mainly of activated CD8 T cells (20). The latter resembles the mononucleosis induced during EBV infection in humans, although the epitopes recognized by the CD8 T cells and the mechanism by which they become activated during MHV-68 infection have not been defined (7, 27). Splenomegaly and lymphocytosis are dependent on both CD4 T cells and B cells (6, 20, 26). Based on studies using lymphocytic choriomeningitis virus (LCMV), it has been proposed that organized secondary lymphoid tissue is essential for antiviral immunity (16). Cytokines of the tumor necrosis family (TNF) superfamily such as lymphotoxin- α (LT α) are required for the development of organized secondary lymphoid tissue. Thus, LT $\alpha^{-/-}$ mice lack lymph nodes and have disrupted

splenic architecture (4). LT α exists in both homo- and heterotrimeric forms (29). The predominant heterotrimeric form $\alpha_1\beta_2$ binds to the LT β receptor (LT β R) and mice genetically deficient in this receptor also lack lymph nodes and have disrupted splenic architecture, indicating that secondary lymphoid tissue architecture may depend on interactions between LT $\alpha_1\beta_2$ and the LT β R (13, 21). However, the finding that LT $\beta^{-/-}$ mice have some lymph nodes and less disorganized spleens (2, 18) and that complementation of LT $\alpha^{-/-}$ mice with TNF transgenes rectifies defective splenic architecture suggests a more complex model (1, 17). Initial reports on the phenotype of LT $\alpha^{-/-}$ mice showed that antibody responses to various antigens were greatly diminished and that germinal centers did not form following antigen challenge (4, 12). However, Matsumoto et al. (19) later showed that administration of high doses of protein antigen in adjuvant could induce class switching and affinity maturation in the absence of germinal centers. In addition, dendritic, NK, and NK T cells are present in reduced numbers in the spleens of LT $\alpha^{-/-}$ mice (14, 15, 32).

In addition to long-term or developmental effects, LT α could also play a major role in the acute response to viral infections by killing virus-infected cells, by costimulation and up-regulation of surface molecules, or by induction of other cytokines and chemokines (29). In the present study, we examined the importance of both acute and long-term effects of LT α in the immune response to a murine gammaherpesvirus.

MATERIALS AND METHODS

Mice. Breeding pairs of LT $\alpha^{-/-}$ mice (8) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Wild-type 129/B6 mice were obtained from a breeding colony maintained at the La Jolla Institute for Allergy and Immunology. Mice were bred and housed under specific-pathogen-free conditions in the animal resource center at the institute. The genotypes of LT $\alpha^{+/+}$ or LT $\alpha^{-/-}$ mice were verified on sacrifice of the animals by visual inspection for lymph nodes. Age- and sex-matched 6- to 20-week-old LT $\alpha^{+/+}$ and LT $\alpha^{-/-}$ mice were used in all experiments.

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FIG. 1. Clearance of lytic MHV-68 from the lungs of LT α ^{-/-} mice is delayed. LT α ^{-/-} and LT α ^{+/+} mice were infected intranasally with 2×10^5 PFU of MHV-68. At various times after infection, lungs were harvested and virus titers determined in lung homogenates by plaque assay. Data are expressed as log₁₀ PFU/0.1 g of lung tissue for individual mice.

Viral infection and sampling. MHV-68 (clone G2.4) was obtained from A. A. Nash, Edinburgh, United Kingdom, and stocks were grown in owl monkey kidney cells (ATCC CRL 1556). Mice were anesthetized with Avertin (2,2,2-tribromoethanol) and infected intranasally with 2×10^5 PFU of the virus in phosphate-buffered saline per mouse. At various times after infection, the mice were terminally anesthetized with Avertin and bled from the right axilla or vena cava. Blood was collected in tubes containing heparin (1 U/ml). The inflammatory cells infiltrating the lung were harvested by bronchoalveolar lavage (BAL) via the trachea, and single-cell suspensions were prepared from the spleen, as previously described (3). Cell viability was determined by trypan blue exclusion. Following BAL, the lungs were removed and stored frozen at -80°C prior to virus titration.

Virus titration and infectious centers assay. Titers of replicating virus were determined by plaque assay on NIH 3T3 cells (ATCC CRL1658) as described previously (7). Briefly, dilutions of stock virus, homogenized mouse tissues, or homogenized splenocytes were adsorbed onto NIH 3T3 monolayers for 1 h at 37°C and overlaid with carboxymethyl cellulose (CMC). After 6 days, the CMC overlay was removed, and the monolayers were fixed with methanol and stained with Giemsa to facilitate determination of the number of plaques. The detection limit of this assay is 10 PFU/0.1 g of lung tissue or 8 PFU/ 10^7 splenocytes, based on plaques recovered from homogenates of uninfected lung or splenocytes spiked with known amounts of virus.

The frequency of latently infected lymphocytes was determined using an infectious center assay. Leukocyte suspensions prepared from lymph nodes or spleen were plated at various cell densities on monolayers of NIH 3T3 cells, incubated overnight, and then overlaid with CMC. The cells were cocultured for 6 days, after which the overlay was removed and the number of plaques was determined as described above.

Cytotoxicity assays. Cytotoxic T-cell activity was determined using a redirected assay on suspensions of lymph node, spleen, or BAL cells. Cell suspensions were incubated with ^{51}Cr -labeled P815 cells in the presence of the monoclonal antibody (MAb) 2C11 to CD3 (2 $\mu\text{g}/\text{ml}$) for 6 to 8 h at 37°C as previously described (7). The level of specific ^{51}Cr release is a measure of the total (virus-specific and nonspecific) cytotoxicity.

Flow cytometric analysis. Cells were stained with phycoerythrin- or fluorescein-conjugated MAbs as previously described (22). All antibodies were purchased from PharMingen (San Diego, Calif.). Isotype controls were included in each assay.

Cytokine ELISAs. Gamma interferon (IFN- γ) levels in culture supernatants from cells that had been restimulated in vitro with virus-infected splenic antigen-presenting cells were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (22). Uninfected antigen-presenting cells or cultures containing infected antigen-presenting cells alone were used as controls. All reagents were obtained from PharMingen.

ELISA for virus-specific antibody. Serum antibody titers were determined by ELISA. Nunc Maxisorp plates were coated overnight at 4°C with a 1/100 dilution of sucrose gradient-purified MHV-68 in 0.1 M sodium bicarbonate (pH 9.0). Plates blocked with phosphate-buffered saline containing 1% bovine serum albumin were incubated for 1 h at room temperature with various dilutions of serum from animals sampled 50 days after infection with MHV-68. Sera from uninfected mice and positive control sera were included in each assay. Bound

antibody was detected using peroxidase-conjugated anti-mouse antisera from Southern Biotechnology (Birmingham, Ala.) and ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] substrate. The absorbance was measured at 405 nm. The titer of a serum sample was taken as the $-\log_{10}$ of the highest dilution which gave a reading of >0.1 .

Statistical analysis. Data were analyzed with SigmaStat software (Jandel Scientific, St. Rafael, Calif.) using Student's *t* test or the Mann-Whitney rank sum test, depending on whether the data were normally distributed.

RESULTS

LT α ^{-/-} mice can clear replicating MHV-68 from their lungs. Mice homozygous for a targeted disruption of the LT α gene had no detectable lymph nodes, although lymph nodes were clearly visible in all $+/+$ mice examined. As secondary lymphoid tissue is thought to be important in generating immune responses, we expected these mice to be profoundly immunodeficient. However, 15 days after an intranasal challenge with 2×10^5 PFU of MHV-68, five of six LT α ^{-/-} mice had cleared replicating virus from their lungs (Fig. 1). At days 5 and 7 after infection, the lung virus titers of the LT α ^{-/-} mice were not significantly different from those of wild-type mice. However, analysis of lung virus titers at day 10 after infection showed that viral clearance was delayed in LT α ^{-/-} mice: at this time point all of the wild-type mice but none of the LT α ^{-/-} mice had cleared virus (Fig. 1). The lungs of the LT α ^{-/-} mice remained clear of replicating virus at days 30 and day 55 after infection (Fig. 1). These data suggest that LT α is not required for clearance of a primary challenge with MHV-68 or for long-term control of the virus. Furthermore, the results show that lymph nodes and organized lymphoid tissue in the spleen are not essential for clearance of replicating virus or for preventing viral reactivation in the lungs. However, the immune response is more effective in LT α ^{+/+} mice.

Latent virus was assessed by an infectious center assay. Although most replicating virus in the lungs had been cleared in both LT α ^{-/-} and LT α ^{+/+} mice, at day 15 after infection, the frequency of infectious centers in splenocytes from LT α ^{-/-} mice ($[2,086 \pm 1,726]/10^7$ splenocytes) was significantly higher ($P = 0.00794$, Mann-Whitney rank sum test) than in splenocytes from $+/+$ mice ($[286 \pm 137]/10^7$ splenocytes), indicating a higher load of latent virus (Fig. 2). However, at day 30 after

FIG. 2. LT α ^{-/-} mice show a transient increase in latent MHV-68 in the spleen. LT α ^{-/-} and LT α ^{+/+} mice were infected with MHV-68 as described above, and spleens were harvested at day 15 or 30 after infection. The frequency of infectious centers was determined by plaque assay after overnight incubation of splenocytes on NIH 3T3 cell monolayers. Data are expressed as plaques/ 10^7 splenocytes for individual mice.

FIG. 3. $LT\alpha^{-/-}$ mice develop more severe inflammation in their lungs than wild-type mice following infection with MHV-68. $LT\alpha^{-/-}$ or $LT\alpha^{+/+}$ mice were infected intranasally with MHV-68, and lungs were harvested 15 days later. Five-micrometer hematoxylin-and-eosin-stained sections of paraffin-embedded lung tissue are shown. Sections of the lungs of uninfected $LT\alpha^{-/-}$ and $LT\alpha^{+/+}$ mice are also shown for comparison. Original magnification, $\times 20$.

infection, the frequency of infectious centers was $<100/10^7$ for both $LT\alpha^{-/-}$ and $LT\alpha^{+/+}$ mice, indicating that the increase was transient (Fig. 2). Little or no replicating virus (0 to 3 PFU/ 10^7 splenocytes) was detected in the spleens of either $LT\alpha^{-/-}$ or $LT\alpha^{+/+}$ mice.

$LT\alpha^{-/-}$ mice develop more severe inflammation in the lungs. Although $LT\alpha^{-/-}$ mice were able to clear replicating MHV-68 from their lungs, histological examination at day 15 after infection showed more severe inflammatory infiltrates in the lungs of $LT\alpha^{-/-}$ than in lungs of $LT\alpha^{+/+}$ mice (Fig. 3). Much

TABLE 1. LT $\alpha^{-/-}$ mice develop more severe inflammation in their lungs than wild-type mice^a

Day	Severity of inflammation in lungs (mean $\pm$ SD)	
	LT $\alpha^{+/+}$	LT $\alpha^{-/-}$
15	1.8 $\pm$ 0.4	3.5 $\pm$ 0.5 ^b
30	1.7 $\pm$ 0.5	3.3 $\pm$ 0.6 ^b

^a Mice were infected intranasally with 2×10^5 PFU of MHV-68. At days 15 and 30 after infection, mice were sacrificed and lungs were harvested and fixed in formalin; 5- μ m paraffin sections were stained with hematoxylin and eosin, and the severity of inflammation was scored as 1 (none), 2 (slight), 3 (significant), 4 (severe), or 5 (very severe).

^b Statistically significant difference in histological scores for lung sections from LT $\alpha^{-/-}$ mice and wild-type mice at both day 15 ($P < 0.0001$) and day 30 ($P < 0.0001$) postinfection (Mann-Whitney rank sum test).

of the inflammation in the lungs of LT $\alpha^{-/-}$ mice appeared in the form of perivascular cuffs (Fig. 3). Lung pathology in hematoxylin-and-eosin-stained sections of lung tissue from MHV-68-infected mice was scored by three independent observers in a blinded fashion (Table 1). There was a highly significant difference in the scores for the degree of inflammation in slides of LT $\alpha^{-/-}$ lung tissue compared with that from wild-type mice at both days 15 ($P < 0.0001$) and 30 ($P < 0.0001$) after infection with MHV-68 (Fig. 3; Table 1). Higher numbers of cells were also recovered by BAL from the lungs of LT $\alpha^{-/-}$ than from the lungs of LT $\alpha^{+/+}$ mice (Fig. 4), although this difference was not statistically significant ($P = 0.065$ at day 15; $P = 0.1$ at day 12). The increased inflammation may reflect the delayed viral clearance in the LT $\alpha^{-/-}$ mice. Alternatively, LT α could be required for terminating immune responses, as previously suggested for TNF and FasL (23, 30). Another possibility is that leukocyte trafficking is altered in LT $\alpha^{-/-}$ mice. Very few cells were recovered by BAL from the lungs of uninfected LT $\alpha^{-/-}$ ($[6.0 \pm 4.0] \times 10^4$ cells/mouse) or LT $\alpha^{+/+}$ ($[4.5 \pm 4.5] \times 10^4$ cells/mouse) (Fig. 4). Although no extensive inflammation was observed in histological sections of any of the uninfected mice, the lungs of LT $\alpha^{-/-}$ mice did appear to have small inflammatory infiltrates around some of the vessels

FIG. 4. Cell numbers in the BAL of LT $\alpha^{+/+}$ and LT $\alpha^{-/-}$ mice. Numbers of cells recovered in the BAL were determined at intervals after intranasal infection of LT $\alpha^{+/+}$ and LT $\alpha^{-/-}$ mice with MHV-68. Data are means $\pm$ SDs of cell counts for three to six mice at each time point. Viable cell counts were determined by trypan blue exclusion.

FIG. 5. LT $\alpha^{-/-}$ mice do not develop splenomegaly during MHV-68 infection. Cell numbers in the spleen were determined at intervals after intranasal infection of LT $\alpha^{+/+}$ and LT $\alpha^{-/-}$ mice with MHV-68. Single-cell suspensions were prepared from individual mouse spleens, and viable cell counts were determined by trypan blue exclusion. Data are means $\pm$ SDs of cell counts from two separate experiments at days 15 and 30 and a single experiment at day 7. Groups of three mice were used in each experiment. Asterisks denote that the difference in spleen cell counts between LT $\alpha^{-/-}$ and LT $\alpha^{+/+}$ mice at day 15 after infection was statistically significant.

which were not observed in the lungs of the wild-type mice (Fig. 3).

LT $\alpha^{-/-}$ mice fail to develop splenomegaly or lymphocytosis. MHV-68 infection of wild-type mice is characterized by splenomegaly and an accumulation of activated CD8 T cells in the blood which resembles the mononucleosis seen in EBV infection of humans. Both are dependent on both T and B cells. However, LT $\alpha^{-/-}$ mice fail to develop splenomegaly or lymphocytosis following infection with MHV-68 (Fig. 5 and 6). The number of cells in the spleens of wild-type mice had increased considerably by day 15 after infection, whereas there was no increase in cellularity of the spleens of LT $\alpha^{-/-}$ mice (Fig. 5). Similarly, there was a large increase in the proportion of activated T cells (detected by the increased expression of CD44) in the blood of wild-type but not LT $\alpha^{-/-}$ mice at day 30 after infection (Fig. 6). The increase in the wild-type mice was predominantly due to an increase in activated CD8 T cells. However, there was also a modest increase in the proportion of CD4 cells expressing high levels of CD44 in $+/+$ but not $-/-$ mice. The difference in percentages of alpha/beta T-cell receptor ($\alpha\beta$ TCR) cells, CD8 T cells, and CD8 CD44^{hi} T cells in LT $\alpha^{-/-}$ and LT $\alpha^{+/+}$ mice 30 days after infection was highly significant ($P < 0.0001$, Student's *t* test). The increase in activated T cells in the wild-type mice persisted for at least 50 days after infection. There was also an increase in the percentage of activated CD8 T cells in the spleens of wild-type but not LT $\alpha^{-/-}$ mice, although the increase was not as dramatic as that in the blood (data not shown). There was little, if any, increase in the percentage of B cells in the blood of LT $\alpha^{-/-}$ mice (indicated by the percentage of CD19-positive cells). Thus, the reduced number of activated T cells in the blood of these mice did not result in uncontrolled proliferation of B cells.

Virus-induced IFN- γ responses are attenuated in LT $\alpha^{-/-}$ mice, although CTL activity is unimpaired. Cytotoxic T-lymphocyte (CTL) activity was decreased only slightly if at all in the lungs or spleens of MHV-68-infected LT $\alpha^{-/-}$ mice (Fig. 7A). It is very likely that viral clearance in these mice is mediated by CTL and that the spleen can act as an alternative to lymph nodes in providing an environment for

FIG. 6. LTα^{-/-} mice do not develop lymphocytosis following infection with MHV-68. LTα^{-/-} and LTα^{+/+} mice were infected with MHV-68. At 0, 30, or 50 days after infection, mice were killed by Avertin overdose and blood was collected from the superior vena cava. Following lysis of red blood cells, peripheral blood leukocytes were stained with phycoerythrin- or fluorescein-conjugated MAbs as previously described (23). The detection limit was less than 1% based on staining with isotype-matched control antibodies. The resulting populations were analyzed by flow cytometry. (A) Fluorescence-activated cell sorting profiles showing the percentage and activation status of CD8 T cells in the blood of representative LTα^{-/-} or LTα^{+/+} mice 30 days after infection with MHV-68. CD44 up-regulation is used as a marker of T-cell activation. (B) Mean data from two separate experiments at day 30 and single experiments at days 0 and 50. Groups of three mice were used in each experiment. Asterisks denote that the difference in percentages of αβTCR, CD8, and activated (CD44^{hi}) CD8 T cells in the blood of LTα^{+/+} and LTα^{-/-} mice at day 30 after infection was statistically significant ($P < 0.001$ for each cell subset). There was also a significant difference between the two groups of mice in the percentages of activated (CD44^{hi}) CD4 T cells at day 30 after infection ($P < 0.05$).

the development of CTL. In contrast, IFN-γ production, which has previously been shown to be both virus specific and T cell dependent in this model, was significantly attenuated in LTα^{-/-} mice (Table 2): at day 15 after infection, splenocytes from LTα^{-/-} mice produced less than one-third of the amount of IFN-γ produced by splenocytes from LTα^{+/+} mice following *in vitro* restimulation with virus-infected antigen-presenting cells. This difference was statistically significant ($P = 0.0028$, Mann-Whitney rank sum test). A similar difference between the groups was seen at day 30 after infection (Table 2).

Virus-specific antibody responses and class switching in LTα^{-/-} mice. Serum antibody titers were determined by ELISA 50 days after infection with MHV-68. Although there was a trend toward reduced virus-specific total immunoglobulin (Ig), IgG2a, and IgG2b responses and increased IgM titers in LTα^{-/-} mice, the differences were not statistically significant (Fig. 7B). IgG1 levels were similar in LTα^{-/-} and LTα^{+/+} mice. IgG3 levels were either undetectable or close to the limit of detection (Fig. 7B), and IgA was undetectable (data not shown) in both LTα^{+/+} and LTα^{-/-} mice. These data show that LTα^{-/-} mice can still mount significant humoral responses to MHV-68 and that class switching still occurs, despite the absence of germinal centers in these mice.

DISCUSSION

The experiments described in this study clearly show that organized secondary lymphoid tissue is not a prerequisite for the development of effective immune responses to viral infections. In this respect, our conclusions differ from those of Karrer et al. (16), who found that LCMV could not be cleared in mutant *aly/aly* (alymphoplasia) mice which also lack lymph nodes and have disorganized splenic architecture. However, in a separate study, mice deficient in both TNF and LTα were shown to be able to clear LCMV in experiments similar to those described by Karrer et al., although various immune responses were decreased in these mice (11). These data are in agreement with our own, which show that immune responses are suboptimal in LTα^{-/-} mice, although such responses are still effective in mediating viral clearance (Fig. 1). A further study, on LCMV infection in LTβ^{-/-} and LTα^{-/-} mice, showed that both groups of mice were able to clear the virus from the blood (5), unlike the *aly/aly* mice (16). However, in contrast to the earlier report (11), there was a significant delay in viral clearance. Berger et al. (5) used a strain and dose of virus and a route of inoculation different from those used by Eugster et al. (11), which may explain the difference in results. In general, the defect in the *aly/aly* mice appear to have more profound effects on the immune system than the absence of LTα, as determined by a number of different immune param-

FIG. 7. Cell-mediated and humoral immune responses to MHV-68 in LT $\alpha^{-/-}$ mice. (A) LT $\alpha^{-/-}$ and LT $\alpha^{+/+}$ mice show comparable CTL activity in the lungs and spleen. Single-cell suspensions were prepared from the spleens of individual mice, while BAL cells were pooled from groups of three mice at day 12 after infection with MHV-68. CTL activity was determined in a 6-h redirected ^{51}Cr release assay. Mean percent specific lysis for spleen CTL is shown. Similar results were obtained in two separate experiments. Data for one experiment are shown. (B) MHV-68-specific antibody responses in LT $\alpha^{-/-}$ mice. Serum was collected from LT $\alpha^{+/+}$ and LT $\alpha^{-/-}$ mice 50 days after infection with MHV-68. Virus-specific antibody responses were determined by ELISA. Data are expressed as mean serum antibody titers $\pm$ SD for three individual mice.

eters, suggesting that this is not due to the lack of secondary lymphoid tissue alone. The aly/aly mice were recently shown to have a point mutation in the gene encoding NF- κ B-inducing kinase (24). The fact that this kinase is an intermediate in the signaling pathways of several different members of the TNF receptor (TNFR) superfamily explains why the effect is more severe than deficiency in LT α alone.

In this study, LT $\alpha^{-/-}$ mice showed delayed viral clearance (Fig. 1) and a transient increase in latent virus in the spleen (Fig. 2). However, once the virus was cleared, the LT $\alpha^{-/-}$ mice were able to maintain long-term control of latent virus, unlike mice lacking CD4 T cells, which show viral reactivation in the lungs at around day 25 after infection (7). The increased inflammation observed in the lungs of LT $\alpha^{-/-}$ mice could reflect the increased viral load. Alternatively, LT α might be required for down-regulating the immune response by initiating activation-induced cell death. Both TNF and lymphotoxin have been reported to trigger apoptotic death of T lymphocyte blasts in vitro (23). The LT α 3 homodimer would be the most likely form of LT α to mediate activation-induced cell death since it interacts with the TNFR60 and TNFR75, which have been implicated in this process. A similar role has been proposed for Fas (which is also a member of the TNF superfamily) (30). Another possibility is that leukocyte trafficking is altered in the absence of LT α . This might affect both virus-infected and uninfected mice. One of the initial publications describing LT $\alpha^{-/-}$ mice reported abnormal lymphocyte clusters in the perivascular regions of the lungs of unmanipulated mice (4). We also observed small inflammatory infiltrates around some of the vessels of the lungs of uninfected LT $\alpha^{-/-}$ mice (Fig. 3).

These infiltrates were absent in the lungs of wild-type mice. While these infiltrates might reflect some type of low-grade infectious process, they could also result from a defect in leukocyte trafficking, as proposed by Banks et al. (4).

Since the LT $\alpha^{-/-}$ mice can clear infectious virus from their lungs, although the draining (mediastinal) lymph node is absent, the questions of where the immune response develops and how viral clearance is mediated arise. Chromium release assays showed that there was a strong CTL response both in the lung and in the spleen (Fig. 7A). Thus, the most likely explanation is that virus is cleared by CTL which develop in the spleen. Davis et al. also concluded that the spleen played an important role in the immune response to an attenuated strain of *Salmonella enterica* serovar Typhimurium in LT $\alpha^{-/-}$ mice (which lack both gut-associated lymphoid tissue and peripheral lymph nodes) (8). In the latter study, splenectomy substantially decreased antigen-specific IgA responses in LT $\alpha^{-/-}$ but not wild-type mice. However, it is intriguing that the IgA response to *Salmonella* serovar Typhimurium in LT $\alpha^{-/-}$ mice was not completely abolished after splenectomy. In the present study, in addition to CTL activity, recall IFN- γ responses were detected in the spleens of LT $\alpha^{-/-}$ mice. However, unlike the CTL activity, the recall IFN- γ responses in the spleens of LT $\alpha^{-/-}$ mice were substantially reduced compared to those in wild-type mice (Table 2).

Splenomegaly is dependent on both CD4 T cells and B cells, and it is likely that interactions between these cell types are facilitated by organized splenic architecture. Both splenomegaly and lymphocytosis were completely absent in LT $\alpha^{-/-}$ mice, although only a modest reduction in the ability to mediate viral clearance was observed. This represents a clear separation between these effects and viral clearance: the absence of lymphocytosis and splenomegaly does not lead to persistence of replicating virus or more than transient increases in the load of latent virus.

Initial reports on the phenotype of LT $\alpha^{-/-}$ mice showed greatly diminished antibody responses to various antigens, including UV light-inactivated herpes simplex virus (4, 12). Splenic architecture was disorganized, and germinal centers did not form following antigen challenge. However, Matsu-moto et al. (19) later showed that administration of high doses of protein antigen in adjuvant could induce class switching and affinity maturation in the absence of germinal centers. Our current data on virus-specific antibody responses in MHV-68-infected LT $\alpha^{-/-}$ mice support the conclusions of the latter study. Significant MHV-68-specific antibody titers and class switching were observed; LT $\alpha^{-/-}$ mice can produce humoral responses to a strong challenge, such as a high dose of a

TABLE 2. LT $\alpha^{-/-}$ mice show decreased splenic recall IFN- γ responses to MHV-68^a

Mouse group	Mean IFN- γ concn (ng/ml) $\pm$ SD	
	Day 15	Day 30
LT $\alpha^{+/+}$	9.8 $\pm$ 2.5	7.2 $\pm$ 3.0
LT $\alpha^{-/-}$	3.2 $\pm$ 3.2	2.0 $\pm$ 1.1

^a Mice were infected intranasally with 2×10^5 PFU of MHV-68, and spleens were harvested 15 or 30 days after infection. Splenocytes were restimulated with MHV-68-infected antigen-presenting cells, and supernatants were collected 24, 72, and 96 h later for analysis of IFN- γ levels by ELISA. Peak values (which usually occurred after 72 h in culture) are reported. Two separate experiments were performed at day 15, and one experiment was done at day 30. Splenocyte cultures from three individual mice were tested in each experiment. There was a statistically significant difference between recall IFN- γ responses for LT $\alpha^{-/-}$ and LT $\alpha^{+/+}$ and at day 15 ($P = 0.0028$, Mann-Whitney rank sum test).

protein antigen or live replicating virus. Lower doses of antigen or inactivated virus, although effective in wild-type mice, appear to be insufficient to provoke a response in $LT\alpha^{-/-}$ mice (4, 12). The antibody response in MHV-68 infection develops rather late and therefore is unlikely to be involved in primary viral clearance (25). However, antibody may play a role in long-term control of latent virus or in resistance to secondary challenge.

In summary, $LT\alpha$ and organized secondary lymphoid tissue are not essential for primary clearance of MHV-68 or for long-term control of latent virus. Immune responses which develop in the absence of $LT\alpha$ are generally reduced but still effective. Splenomegaly and lymphocytosis, which are observed during MHV-68 infection of wild-type mice, are completely absent in $LT\alpha^{-/-}$ mice, suggesting that these responses do not play an essential role in the control of viral replication.

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