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Deliberate removal of T cell help improves virus-neutralizing antibody production

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The B cell response to lymphocytic choriomeningitis virus is characterized by a CD4$^+$ T cell–dependent polyclonal hypergammaglobulinemia and delayed formation of virus-specific neutralizing antibodies. Here we provide evidence that, paradoxically, because of polyclonal B cell activation, virus-specific T cell help impairs the induction of neutralizing antibody responses. Experimental reduction in CD4$^+$ T cell help *in vivo* resulted in potent neutralizing antibody responses without impairment of CD8$^+$ T cell activity. These unexpected consequences of polyclonal B cell activation may affect vaccine strategies and the treatment of clinically relevant chronic bacterial, parasitic and viral infections in which hypergammaglobulinemia is regularly found.

Lymphocytic choriomeningitis virus (LCMV) is a noncytopathic RNA virus that is initially controlled by a strong CD8$^+$ T cell response in a perforin-dependent way[1,2]. In contrast, LCMV-neutralizing antibodies usually develop late in infection, after day 50, and remain at low titer[3]. This phenomenon is also found after infection with other poorly cytopathic or noncytopathic viruses such as human immunodeficiency virus (HIV)[4] and hepatitis C virus[5,6]. Neutralizing antibodies directed against low-cytopathic viruses increase in titer when CD8$^+$ T cell (cytotoxic T lymphocyte (CTL)) function is impaired or absent[3,7,8]. Possible mechanisms have been postulated to include CD8$^+$ T cell–dependent immunopathology[9,10], enhanced viral replication in the absence of CTLs[2,8] or the need for affinity or avidity maturation of specific antibodies. These observations also suggest a competitive relationship between cellular and humoral antiviral immune responses, as has been successfully modeled theoretically[11]. LCMV infection induces a strong CD4$^+$ T cell–dependent, virus-nonspecific, polyclonal hypergammaglobulinemia[12,13], a phenomenon often associated with 'persistence-prone', low-cytopathic viruses like HIV and hepatitis C virus or with chronic bacterial and parasitic infections. Yet a biological function for polyclonal B cell activation has remained elusive[14].

Here we have evaluated the function of T helper cells and found that paradoxically, because of polyclonal B cell activation, CD4$^+$ T cells impaired the formation of virus-neutralizing antibodies and favored virus persistence. This inverse causal relationship of T cell help and virus-specific antibody formation was modified only indirectly by CD8$^+$ T cells. Experimental deliberate suppression of T helper responses after LCMV infection resulted in enhanced neutralizing antibody formation while leaving CTL responses intact.

RESULTS

Polyclonal B cell activation *versus* neutralizing antibodies

Compared with results obtained with control C57BL/6 mice, infection of CD8$^+$ T cell–depleted C57BL/6 or CD8α-deficient (*Cd8a*$^{-/-}$) mice with high-dose (2×10^6 PFU) LCMV, strain WE (LCMV-WE) resulted in increased titers of neutralizing antibodies (**Fig. 1a**), correlating with increased viral replication (**Fig. 1b**). However, virus-induced polyclonal hypergammaglobulinemia was also modified by CTL function, as both CD8$^+$ T cell–depleted C57BL/6 mice and *Cd8a*$^{-/-}$ mice showed reduced polyclonal B cell activation early (day 12) after LCMV infection (**Fig. 1c**). CD4$^+$ T cells are required for hypergammaglobulinemia[13]. Thus, we examined whether CD4$^+$ T cell function was affected by functional CD8$^+$ T cell depletion. Indeed, CD4$^+$ T cells from CD8$^+$ T cell–depleted or *Cd8a*$^{-/-}$ mice showed a significant reduction in interferon-γ (IFN-γ) production after restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, compared with that of CD4$^+$ T cells from C57BL/6 mice (**Fig. 1d**). This impaired CD4$^+$ T cell function strictly correlated with the reduced peak in hypergammaglobulinemia in the absence of functional CD8$^+$ T cells (**Fig. 1c**), although restimulation by PMA and ionomycin also induces some IFN-γ production in naive mice, and absolute virus-specific CD4$^+$ T cell activation may therefore be slightly overestimated. Thus, early polyclonal B cell activation

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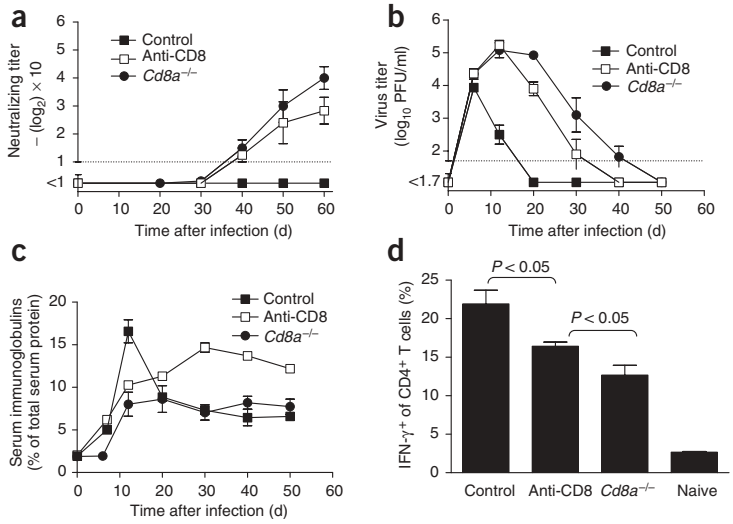

Figure 1 Influence of CD8$^+$ T cells on B cell and CD4$^+$ T cell responses. (**a**–**c**) C57BL/6 mice that were depleted *in vivo* of CD8 T cells on days –3 and –1 (Anti-CD8) or were left untreated (Control) and *Cd8a*$^{-/-}$ mice (*Cd8a*$^{-/-}$) were infected with 2 × 10^6 PFU LCMV-WE on day 0 (times, horizontal axes). (**a**) Quantification of LCMV-WE-specific neutralizing antibodies. (**b**) Virus plaque assay of virus titers in blood. (**c**) Analysis of total serum immunoglobulins by serum electrophoresis. (**d**) Splenocytes from *Cd8a*$^{-/-}$ mice or from CD8$^+$ T cell–depleted C57BL/6 mice (monoclonal antibody YTS 169.4) were infected with 2 × 10^6 PFU LCMV-WE. Then, 12 d later, cells were restimulated *in vitro* with PMA and ionomycin for 6 h and IFN-γ production by CD4$^+$ T cells was quantified by intracellular cytokine staining and flow cytometry. Splenocytes from naive mice served as a control. Data are expressed as mean ± s.e.m. of four to six mice per group and are representative of two to three experiments.

correlated with CD4$^+$ T cell function but was inversely correlated with neutralizing antibody responses.

Impairment of neutralizing antibody responses by CD4$^+$ T cells

To analyze whether the stated inverse relationship was due to a direct suppressive effect of virus-specific T cell help on neutralizing antibody formation, we infected mice with LCMV-WE in the presence or absence of adoptively transferred LCMV-specific CD4$^+$ T helper cells. We obtained these CD4$^+$ cells from Smarta1 mice, which carry a transgenic T cell receptor specific for the LCMV T helper epitope consisting of glycoprotein amino acids 61–80 (GP(61–80))[15]. We examined *Cd8a*$^{-/-}$ mice as recipients to exclude the possibility of a direct effect of T helper cells on CTL function. LCMV-infected *Cd8a*$^{-/-}$ mice that did not receive Smarta1 splenocytes developed a strong neutralizing antibody response (**Fig. 2a** and ref. 7). This result correlated with a transient reduction (>99%) in blood virus titers (**Fig. 2b** and ref. 7). In contrast, *Cd8a*$^{-/-}$ mice that received cells from Smarta1 mice failed to mount measurable neutralizing activity (**Fig. 2a**), and blood virus titers remained consistently high (**Fig. 2b**). This indicated that the addition of virus-specific CD4$^+$ T cell help inhibited the production of neutralizing antibodies. As expected, hypergammaglobulinemia was increased in the presence of additional virus-specific T cell help (**Fig. 2c**).

Next, we examined if the reduction in virus-specific T cell help was followed by enhanced neutralizing antibody formation. These experiments profited from knowledge of two characterized immunodominant LCMV-derived major histocompatibility complex (MHC) class II epitopes, the aforementioned GP(61–80) and nucleoprotein-derived NP(309–327)[16,17], which account for most of the LCMV-specific CD4$^+$ T cell response[18]. We took advantage of a newly isolated LCMV variant, LCMV-WE$_{del}$ (ref. 19), which contains an eight–amino acid deletion at positions 61–68 of the LCMV glycoprotein, spanning the immunodominant MHC class II H-2^b epitope GP(61–80) (ref. 16,17; **Table 1**). This epitope is recognized by Smarta1 T helper cells. Infection of *Cd8a*$^{-/-}$ mice with LCMV-WE$_{del}$ induced an early and strong neutralizing antibody response (**Fig. 2a**). This response was not altered by the presence of CD4$^+$ T cells from Smarta1 mice (**Fig. 2a**). This indicated that the inhibition of the neutralizing antibody response by virus-specific CD4$^+$ T cells occurred in an epitope-specific way. Consistent with the early induction of neutralizing antibodies, *Cd8a*$^{-/-}$ mice infected with LCMV-WE$_{del}$ showed rapid clearance of virus from peripheral blood (**Fig. 2b**) and had only moderate hypergammaglobulinemia (**Supplementary Fig. 1** online). These experiments demonstrated a causal, inverse correlation of CD4$^+$ T cell–dependent polyclonal B cell activation and neutralizing antibody responses. In addition, virus dosage influenced the degree of functional CD4$^+$ T cell anergy in the absence of CTLs. Infection of *Cd8a*$^{-/-}$ mice with low virus doses (for example, 200 PFU LCMV-WE) was followed by robust IFN-γ production by virus-specific CD4$^+$ T cells, whereas high virus doses (2 × 10^6 PFU LCMV-WE) led to impaired CD4$^+$ T cell function (**Supplementary Fig. 1** online). Accordingly, polyclonal B cell activation was suppressed and neutralizing antibodies were high after high-dose LCMV-WE infection, whereas hypergammaglobulinemia was enhanced and neutralizing antibody titers were low after low-dose LCMV-WE infection (**Supplementary Fig. 1** online). Low-dose (200 PFU) LCMV-WE$_{del}$ induced only minimal polyclonal B cell activation in *Cd8a*$^{-/-}$ mice. Again, the decreased hypergammaglobulinemia found after LCMV-WE$_{del}$ infection correlated inversely with neutralizing antibody production (**Supplementary Fig. 1** online).

Antibody formation in CD8$^+$ T cell–competent mice

Our data have provided evidence that virus-specific CD4$^+$ T cells may directly negatively influence neutralizing antibody formation. In

Table 1 Sequence of the LCMV glycoprotein CD4$^+$ T cell epitope

Amino acid position	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
WE	Tyr	Gly	Leu	Asn	Gly	Pro	Asp	Ile	Tyr	Lys	Gly	Val	Tyr	Gln	Phe	Lys	Ser	Val	Glu	Phe	Asp
WE$_{del}$	Tyr	–	–	–	–	–	–	–	–	Lys	Gly	Val	Tyr	Gln	Phe	Lys	Ser	Val	Glu	Phe	Asp

–, deleted amino acid.

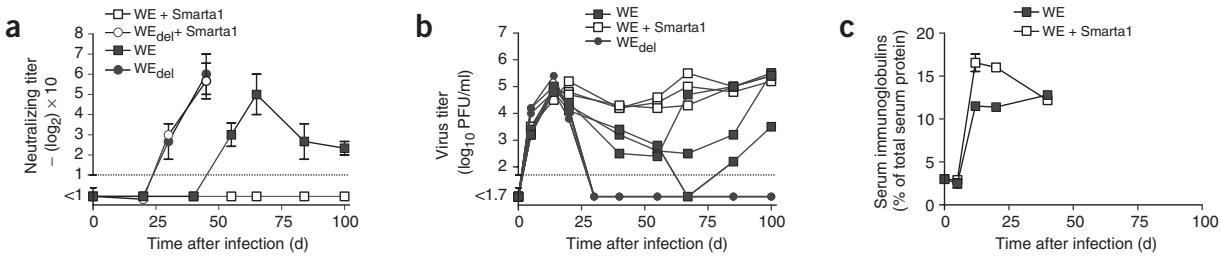

Figure 2 Inverse correlation between virus-specific T cell help and neutralizing antibody responses in *Cd8a*$^{-/-}$ mice. *Cd8a*$^{-/-}$ mice were infected with 5×10^3 PFU LCMV-WE (WE) or LCMV-WE$_{del}$ (WE$_{del}$). The mice also received 1×10^7 Smarta1 splenocytes intravenously on the day of infection (+ Smarta1) or were left untreated (times, horizontal axes). (**a**) Analysis of sera by neutralization assay against LCMV-WE. (**b**) Virus plaque assay of blood viral titers. (**c**) Analysis of total serum immunoglobulins by serum electrophoresis. Five individual mice were tested on day 12 after infection. Pooled serum of two to three mice was analyzed at days 20 and 30. Data are expressed as mean ± s.e.m. of three to five mice per group except in **b**, for which data from three individual mice are presented. All data are representative of two separate experiments (total $n = 5–8$).

contrast, CD8$^+$ T cells seemed to suppress antibody responses indirectly by reducing viral load and thereby maintaining T helper cell function. Thus, we postulated that experimentally reduced but not completely absent CD4$^+$ T cell help should impair hypergammaglobulinemia and promote the production of neutralizing antibodies in CTL-competent mice. For this, we infected wild-type C57BL/6 mice with LCMV-WE$_{del}$,. To confirm that the immunodominant GP(61–80) epitope was absent *in vivo* after infection with the variant virus, we infected C57BL/6 mice with 200 PFU LCMV-WE$_{del}$ or wild-type LCMV-WE. Splenocytes infected with wild-type but not variant LCMV demonstrated strong peptide-specific CD4$^+$ T cell IFN-γ production after *in vitro* restimulation 8 d later (**Fig. 3a**). In contrast, restimulation of the same splenocytes with the NP(309–327) MHC class II peptide produced comparable IFN-γ production (**Fig. 3a**). To determine the function of subdominant epitopes, we infected C57BL/6 mice with 200 PFU LCMV-WE or 200 PFU LCMV-WE$_{del}$ and restimulated splenocytes polyclonally with PMA and ionomycin. We detected approximately half the percentage of IFN-γ producing CD4$^+$ T cells in mice infected with LCMV-WE$_{del}$ compared with that in mice infected with LCMV-WE (**Fig. 3a**). Thus, the loss of the immunodominant MHC class II epitope resulted in weakened CD4$^+$ T cell responses.

To further confirm that LCMV-WE$_{del}$ had a complete absence of the GP(61–80) epitope *in vivo*, we infected Smarta1 mice with 200 PFU LCMV-WE or LCMV-WE$_{del}$. Whereas infection with LCMV-WE induced a notable downregulation of the activation marker CD62L on Smarta1 CD4$^+$ T cells at day 5 after infection, we found no indication of Smarta1 T cell activation after infection with LCMV-WE$_{del}$ (**Fig. 3b**). As noted for CD8$^+$ T cell–deficient mice, low-dose infection (200 PFU) of wild-type C57BL/6 mice with LCMV-WE$_{del}$ resulted in enhanced neutralizing antibody titers compared with LCMV-WE infection. Neutralizing serum activity became detectable by day 30 and remained increased until at least day 200 (**Fig. 3c**), whereas neutralizing antibodies remained low after LCMV-WE infection. Sera from LCMV-WE$_{del}$-infected mice neutralized both LCMV-WE$_{del}$ and wild-type LCMV-WE virus *in vitro*, whereas sera from LCMV-WE-infected mice could not neutralize either virus. These data indicated that the glycoprotein structure was conserved in both viruses and was equally accessible to neutralizing antibodies (**Fig. 3c**). This conclusion was supported by the comparable neutralization of LCMV-WE$_{del}$ and LCMV-WE *in vitro* by the monoclonal LCMV-WE-neutralizing antibody KL25 (ref. 20; **Fig. 3d**). LCMV glycoprotein–specific enzyme-linked immunosorbent assay (ELISA) IgG antibodies were also enhanced after infection with LCMV-WE$_{del}$ compared with antibodies present after infection with LCMV-WE (**Fig. 3e**). These ELISA IgG titers were induced early after infection (days 12–20), in contrast to neutralizing antibodies, which appeared after days 30–40 (**Fig. 3c**). In contrast, early total serum immunoglobulin concentrations (which represent mainly IgG isotypes[13]) were decreased in LCMV-WE$_{del}$-infected mice compared with LCMV-WE-infected mice (**Fig. 3f**). These results indicated that an intermediate amount of CD4$^+$ T cell help reduced polyclonal B cell activation and was associated with increased virus-specific IgG formation.

To rule out the possibility that a dominant function of the LCMV-WE$_{del}$ glycoprotein was itself responsible for enhanced neutralizing antibody responses, we co-infected C57BL/6 mice with 200 PFU LCMV-WE$_{del}$ and LCMV-WE. After co-infection, virus-specific IgG production was very low (**Fig. 3e**) and was associated with an enhanced hypergammaglobulinemia compared with that after infection with LCMV-WE$_{del}$ alone (**Fig. 3f**). This result suggested that infection with LCMV-WE$_{del}$ did not mediate an increased production of virus-specific antibodies itself, but that this increase was a result of the reduced polyclonal B cell activation. We next examined whether the different titers of LCMV-neutralizing antibodies found after infection with LCMV-WE or LCMV-WE$_{del}$ resulted in different protective capacities against a challenge infection. We collected and pooled sera from mice given primary infection with 200 PFU LCMV-WE or LCMV-WE$_{del}$ (days 40-80 after infection) and transferred this sera into naive C57BL/6 mice, which we then challenged intravenously with 200 PFU LCMV-WE. We also used control C57BL/6 mice that did not receive serum and infected them with LCMV-WE. On day 4 after challenge infection, virus titers in spleens in mice that had received serum from LCMV-WE$_{del}$-infected mice were 0.1% those of mice that received the same volume of serum from LCMV-WE-infected mice (**Fig. 3g**). Transfer of serum from LCMV-WE mice mediated only minor protection compared with that of control mice that had not received LCMV-immune serum (**Fig. 3g**).

Effects of partial CD4$^+$ T cell depletion

The hypothesis that CD4$^+$ T cell help may inhibit the formation of virus-neutralizing antibodies through the promotion of polyclonal B cell activation 'predicts' that experimental reduction but not complete elimination of functional T cell help should result in enhanced virus-specific antibody titers after wild-type LCMV-WE infection. We treated mice with titrated doses of the CD4$^+$ T cell–depleting monoclonal antibody YTS191.6 (ref. 21) before infecting the mice with 200 PFU LCMV-WE on day 0. The normal protocol of CD4$^+$ T cell depletion with two injections of YTS191.6, on days −3 and −1, resulted in complete disappearance of CD4$^+$ T cells from the blood up to day 12, as assessed by flow cytometry (**Fig. 4a**),

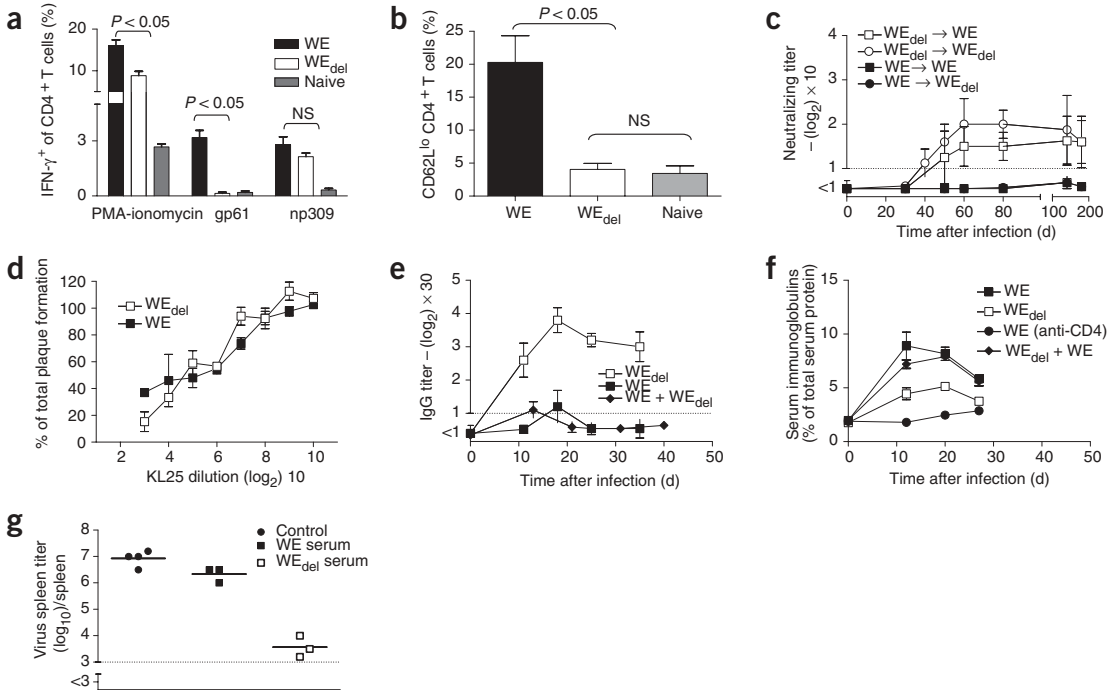

Figure 3 Infection with LCMV-WE_{del} results in moderate hypergammaglobulinemia but potent neutralizing antibody responses in immunocompetent C57BL/6 mice. (**a**) C57BL/6 mice were infected with 200 PFU LCMV-WE (WE) or LCMV-WE_{del} (WE_{del}) or were left uninfected (Naive). At 8 d after infection, splenocytes were re-stimulated with the immunodominant LCMV GP(61–80) or LCMV NP(309–327), or with PMA and ionomycin. IFN-γ production gated on $CD4^+$ T cells was assessed by flow cytometry. (**b**) Smarta1 mice were infected with 200 PFU LCMV-WE or LCMV-WE_{del} or were left uninfected. At 5 d after infection, activation of gated $CD4^+$ T cells was assessed by flow cytometry of CD62L expression. (**c**) C57BL/6 mice were infected with 200 PFU LCMV-WE or LCMV-WE_{del}, then sera were tested against LCMV-WE (→WE) or against LCMV-WE_{del} (→WE_{del}) by neutralization assay (times, horizontal axis). (**d**) The LCMV-neutralizing monoclonal antibody KL25 was serially diluted twofold (starting at 1 mg/ml) in a plaque-neutralization assay with LCMV-WE or LCMV-WE_{del}; samples were analyzed in triplicate. (**e**,**f**) C57BL/6 mice were infected with 200 PFU LCMV-WE or LCMV-WE_{del}. C57BL/6 mice depleted of $CD4^+$ T cells with monoclonal antibody YTS191.6 (anti-CD4) and C57BL/6 mice co-infected with 200 PFU LCMV-WE and LCMV-WE_{del} (WE + WE_{del}) served as controls. (**e**) ELISA of binding of serum IgG to recombinant LCMV glycoprotein. (**f**) Analysis of total serum immunoglobulin concentrations by serum electrophoresis. Data in **a**–**f** represent mean ± s.e.m. of four to eight individual mice and are representative of at least two separate experiments. (**g**) Pooled serum (100 μl) obtained 50–80 d after infection of C57BL/6 mice with 200 PFU LCMV-WE (WE serum) or LCMV-WE_{del} (WE_{del} serum) was injected intravenously into naive C57BL/6 hosts. Then, 2 h later, recipient mice were challenged with 200 PFU LCMV-WE and 4 d later, virus titers in the spleen were assessed by plaque assay. Control, infection of C57BL/6 mice without serum transfer. Data points represent results from individual mice.

associated with an absence of serum hypergammaglobulinemia (**Fig. 3f**). In contrast, treatment with a 1:100 dilution of the depleting antibody had a transient effect, such that $CD4^+$ T cells were initially depleted but had already regained normal numbers by day 12 after infection (**Fig. 4a**). Such transient and partial $CD4^+$ T cell depletion led to the development of increased LCMV-specific IgG antibody titers (**Fig. 4b**). As expected, complete depletion of $CD4^+$ T cells resulted in a failure to produce LCMV-specific IgG antibodies (**Fig. 4b**).

The positive effect of partial $CD4^+$ T cell depletion on the production of neutralizing antibodies was similarly demonstrated in $CD8^+$ T cell–deficient mice, which had already cleared the LCMV-WE infection from the peripheral blood by day 20 (**Fig. 4c**,**d**). Thus, the partial depletion of $CD4^+$ T cells provided another line of evidence supporting the proposal that limited T cell help results in the formation of an accelerated and improved neutralizing antibody response.

Coexistence of neutralizing antibody and CTL responses

Early $CD4^+$ T cell help is important in the generation of $CD8^+$ T cell memory[21–23]. Whereas the initial clonal burst and early $CD8^+$ T cell function is mostly $CD4^+$ T cell independent, memory $CD8^+$ T cell responses apparently require $CD4^+$ T cell help. Thus, the potential therapeutic use of virus variants lacking immunodominant MHC class II epitopes or partial $CD4^+$ T cell depletion *in vivo* may become a 'double-edged sword'. That is, enhanced neutralizing antibodies would have to compensate for a potentially impaired CTL memory response. We therefore examined acute and memory CTL responses induced by infection with LCMV-WE (with or without partial $CD4^+$ T cell depletion) or LCMV-WE_{del}. At early time points after infection (day 8), we found no significant differences in either LCMV-specific tetramer–positive $CD8^+$ T cell numbers (data not shown) or IFN-γ production after short-term (6 h) *in vitro* re-stimulation with MHC class I peptides derived from LCMV glycoprotein (GP(33–41) or GP(276–284)) or LCMV nucleoprotein (NP(396–404); **Fig. 5a**). Numbers of tetramer-positive memory $CD8^+$ T cells from mice infected with LCMV-WE (with or without partial $CD4^+$ T cell depletion) or LCMV-WE_{del} were also comparable at day 120 after infection (**Fig. 5b**,**c**). To test the functional capacity of these $CD8^+$ T cells, we partially depleted C57BL/6 mice of T helper cells and challenged these and nondepleted control mice, 130 days after infection with 200 PFU LCMV-WE, with CFSE-labeled splenocytes pulsed with the LCMV GP(33–41) peptide. We used flow cytometry to analyze the elimination of injected cells. Within 4 h, mice subjected to partial $CD4^+$ T cell depletion had

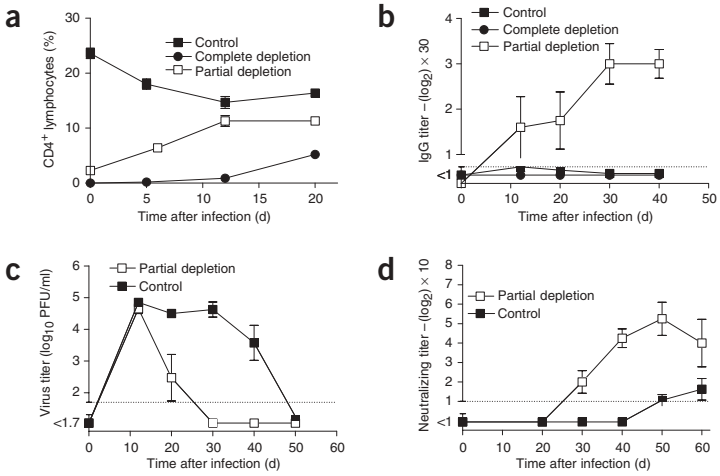

Figure 4 Enhanced neutralizing antibody response after partial CD4$^+$ T cell depletion *in vivo* in both immunocompetent C57BL/6 and *Cd8a*$^{-/-}$ mice. C57BL/6 mice (**a**,**b**) and *Cd8a*$^{-/-}$ mice (**c**,**d**) were infected with 200 PFU LCMV-WE (times, horizontal axes). Before infection, mice were depleted of CD4$^+$ T cells, completely (Complete depletion) or partially (Partial depletion), with titrated amounts of monoclonal antibody YTS191.6. Control, normal C57BL/6 mice (**a**,**b**) or normal *Cd8a*$^{-/-}$ mice (**c**,**d**) without depletion. (**a**) Degree of CD4$^+$ T cell depletion, monitored by flow cytometry. (**b**) ELISA of LCMV glycoprotein–specific IgG antibodies. (**c**) Plaque assay of virus titers in blood. (**d**) Neutralization assay of LCMV-WE-neutralizing antibodies. Data represent mean ± s.e.m. of four to five individual mice and are representative of two independent experiments.

eliminated 65.6% ± 4.97% of the peptide-pulsed cells, whereas LCMV-WE-infected control C57BL/6 mice showed a slightly reduced cytotoxicity *in vivo* (38.9% ± 2.54%; **Fig. 5d**). These experiments indicated that potent neutralizing antibody and CD8$^+$ T cell responses could potentially coexist *in vivo* and that partial depletion of CD4$^+$ T cells did not obviously impair CTL memory responses in our test conditions.

Influence of virus replication on antibody responses

The idea that T helper cell–dependent polyclonal B cell activation can impair production of virus-neutralizing antibodies was supported by experiments in both immunocompetent and CD8$^+$ T cell–deficient mice. Nevertheless, neutralizing antibody responses were usually shifted toward higher titers in experiments in which CD8$^+$ T cells were absent. This suggested an additional function for virus replication in the formation of neutralizing antibody responses. To directly determine this influence, we examined mice deficient for type I and type II interferon receptors (AGR mice), in which virus replication is enhanced[24–27].

Low-dose (200 PFU) LCMV-WE infection of AGR mice resulted in the development of an 'LCMV carrier status'. We did not detect neutralizing antibodies in AGR mice infected with LCMV-WE (**Fig. 6a**), despite the presence of high-titer virus replication (**Fig. 6b**). In contrast, infection of AGR-mice with 200 PFU LCMV-WE$_{del}$ resulted in sera that were able to neutralize sera even when diluted 1,000-fold. This neutralizing antibody response occurred in the presence of viral antigen concentrations that were comparable to those seen after wild-type LCMV-WE infection (**Fig. 6b**, days 8–20). However, by day 25 after infection, the appearance of neutralizing antibodies in mice infected with LCMV-WE$_{del}$ coincided with

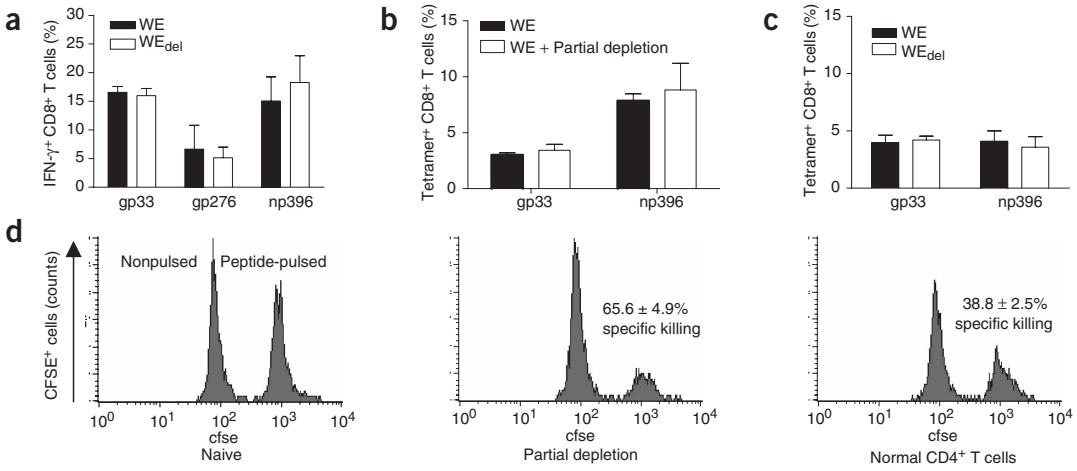

Figure 5 Normal early and memory CTL function can coexist with enhanced neutralizing antibody responses in immunocompetent C57BL/6 mice. (**a**) C57BL/6 mice were infected with 200 PFU LCMV-WE or LCMV-WE$_{del}$. Then, 8 d later splenocytes were re-stimulated *in vitro* for 6 h with the LCMV-derived immunodominant MHC class I peptides (GP(33–41) (gp33); GP(276–284) (gp276); NP(396–404) (np496)) and IFN-γ production by CD8$^+$ T cells was assessed by flow cytometry. Data are presented as mean ± s.e.m. of five individual mice per group and have been repeated twice with similar results. (**b**) C57BL/6 mice were infected with 200 PFU LCMV-WE (WE); some were partially depleted of CD4$^+$ T cell depleted on day −1 before infection on day 0 (WE + partial depletion). Then, 120 d later, LCMV-specific CD8$^+$ T cells numbers were determined by LCMV gp33– and LCMV np396–specific tetramer binding and flow cytometry. (**c**) C57BL/6 mice were infected with 200 PFU LCMV-WE or LCMV-WE$_{del}$. Then, 150 d later, LCMV-specific CD8$^+$ T cells numbers were measured by LCMV gp33– and LCMV np396–specific tetramer staining and flow cytometry. (**d**) *In vivo* cytotoxic capacity of memory CD8$^+$ T cells from C57BL/6 mice partially depleted of CD4$^+$ T cells or from nondepleted C57BL/6 mice, 130 d after infection, determined by challenge with CFSE^{hi} LCMV gp33 peptide–labeled splenocytes. Data in **b**–**d** represent mean ± s.e.m. of four to five individual mice per group and are representative of one experiment.

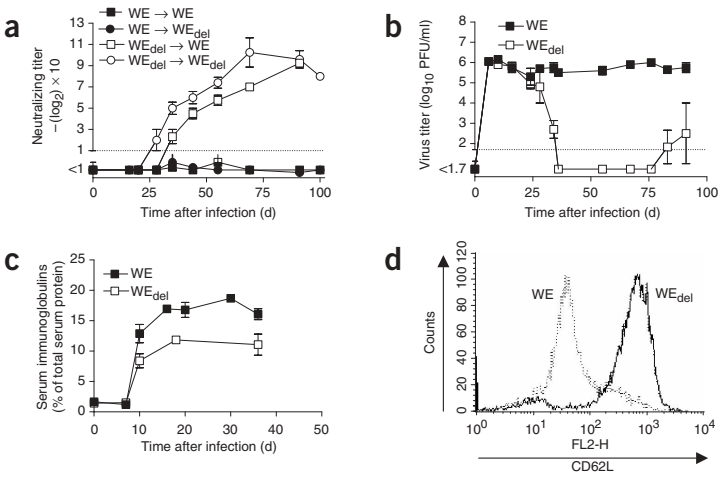

Figure 6 The efficiency of the neutralizing antibody response can be predicted largely by the extent of polyclonal B cell activation but is also modified by the extent of virus replication. (**a**–**c**) AGR mice were infected with 200 PFU LCMV-WE (filled symbols) or LCMV-WE$_{del}$ (open symbols). Times, horizontal axes. (**a**) Virus neutralization assay of neutralizing antibody titers to LCMV-WE (→WE) and LCMV-WE$_{del}$ (→WE$_{del}$). (**b**) Plaque-forming assay of blood virus titers. (**c**) Analysis of total serum immunoglobulins by serum electrophoresis. Data represent mean ± s.e.m. of two to five individual mice per time point. All data (**a**–**c**) are representative of two separate experiments. (**d**) Flow cytometry of CD62L expression on peripheral blood CD4$^+$ lymphocytes 7 d after infection with 200 PFU LCMV-WE (dotted line) or LCMV-WE$_{del}$ (solid line). Data are of one representative mouse per group (total, n = 5–6).

viral clearance to below the limit of detection in blood. As noted for CD8$^+$ T cell–deficient mice, neutralizing antibody 'escape variants' eventually arose and could be detected in the blood at late time points (>day 80; **Fig. 6b**).

LCMV-WE infection of AGR mice resulted in a sharp increase in total serum immunoglobulin concentrations (**Fig. 6c**), detectable by day 10 after infection and occurring in parallel with a massive downregulation of the activation marker CD62L on blood CD4$^+$ T cells (**Fig. 6d**). In contrast, only moderate hypergammaglobulinemia was measured in AGR mice infected with LCMV-WE$_{del}$ (**Fig. 6c**), associated with reduced CD4$^+$ T cell activation (**Fig. 6d**). Thus, here the induction of the neutralizing antibody response occurred independently of high viral load (apparent in mice lacking interferon receptors), but yet again correlated with reduced CD4$^+$ T cell–dependent early hypergammaglobulinemia. These experiments also indicated that a combination of high virus replication and limited early polyclonal B cell activation resulted in enhanced neutralizing antibody responses.

Virus-specific and nonspecific B cell competition

To elucidate the mechanisms by which CD4$^+$ T cell–dependent hypergammaglobulinemia impaired production of LCMV-specific neutralizing antibodies, we investigated whether this effect was limited to LCMV antigens or whether antibody responses to LCMV-unrelated antigens were also affected[30] (**Fig. 7**). We examined whether LCMV-induced polyclonal B cell activation correlated with decreased neutralizing antibody responses against vesicular stomatitis virus (VSV) after co-infection with both viruses. We infected C57BL/6 mice with 2×10^6 PFU VSV 6 d after LCMV-WE infection and determined the formation of neutralizing antibodies to VSV. We used this infection schedule

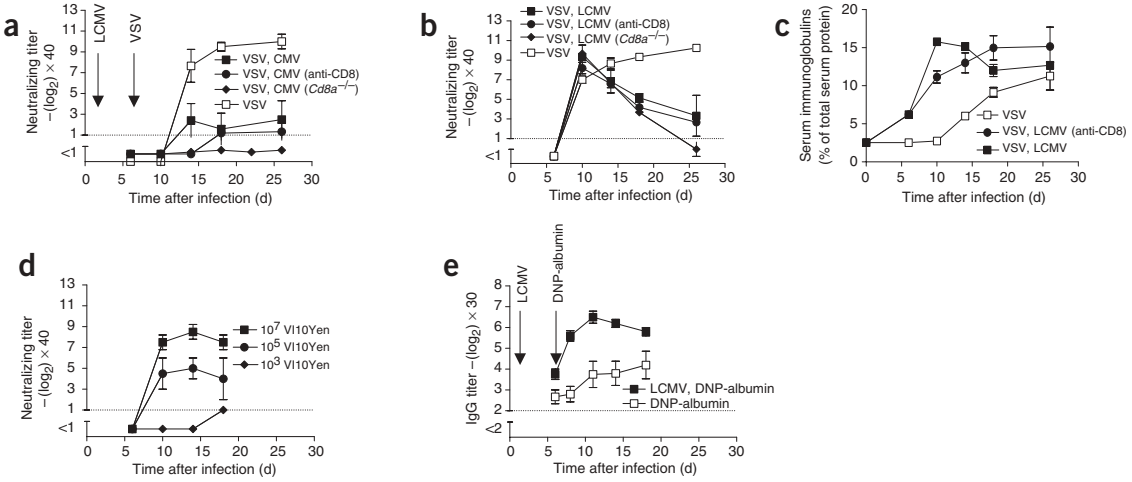

Figure 7 LCMV-induced polyclonal B cell competition acts in an antigen-nonspecific way and is dependent on B cell precursor frequency. (**a**–**c**) *Cd8a*$^{-/-}$ mice (VSV, LCMV (*Cd8a*$^{-/-}$)) or C57BL/6 mice (either depleted of CD8$^+$ T cells with monoclonal antibody YTS 169.4 on days −3 and −1 (VSV, LCMV (anti-CD8) or left untreated (VSV, LCMV)), were infected with 2×10^6 PFU LCMV-WE on day 0. Uninfected C57BL/6 mice served as controls (VSV). On day 6 after LCMV infection, mice were superinfected with 2×10^6 PFU VSV. Times, horizontal axes. (**a**,**b**) VSV-neutralization assay of VSV-neutralizing IgG (**a**) and total VSV-neutralizing immunoglobulins (IgM plus IgG; **b**). (**c**) Analysis of total serum immunglobulins by serum electrophoresis. (**d**) VSV-specific B cell receptor–transgenic VI10Yen splenocytes[28] (VI10Yen; 1×10^7, 1×10^5 or 1×10^3 cells) were adoptively transferred intravenously into naive C57BL/6 mice on day −1 before LCMV-WE-infection (2×10^6 PFU) on day 0 and VSV-neutralizing IgG-responses were measured after VSV infection (2×10^6 PFU) on day 6 with a VSV-neutralization assay (time, horizontal axis). (**e**) C57BL/6 mice were infected intravenously with 2×10^6 PFU LCMV-WE (LCMV, DNP-albumin) or were left uninfected (DNP-albumin). Then, 6 d later, 100 μg DNP-albumin was injected intravenously. Sera were tested by ELISA to detect of binding of IgG to DNP-albumin (time, horizontal axis). Data represent mean ± s.e.m. of three to six mice per time point. All data are representative of two separate experiments. Downward arrows (**a**,**e**) indicate time of immunization.

so that the peak of LCMV-induced hypergammaglobulinemia (day 12) would coincide with the time of IgG isotype switching in response to VSV infection (day 6). In C57BL/6 mice preinfected with LCMV-WE, we noted a reduction in neutralizing IgG antibodies to VSV (anti-VSV IgG) compared with that of control mice infected with VSV alone (**Fig. 7a**). This effect was also apparent in $CD8^+$ T cell–depleted or *Cd8a*$^{-/-}$ mice (**Fig. 7a**), indicating that LCMV-induced $CD8^+$ T cell–mediated immunopathology was not (or was not solely) responsible for the effect on neutralizing antibody responses noted. Although polyclonal B cell activation was reduced in $CD8^+$ T cell–deficient mice (**Fig. 7c**), it was obviously sufficient to suppress VSV-specific IgG production (**Fig. 7a**). In contrast, early neutralizing IgM antibodies to VSV were increased in mice co-infected with LCMV and VSV (**Fig. 7b**). Thus, LCMV-induced polyclonal B cell activation (**Fig. 7c**) was also able to inhibit IgG isotype switching of B cells 'encoding' antibodies specific for VSV. We next examined whether the LCMV-induced polyclonal B cell competition could be compensated by the presence of increased VSV-specific B cell precursor frequencies. We adoptively transferred splenocytes from a transgenic mouse expressing a 'switchable' neutralizing anti-VSV B cell receptor, VI10Yen[28], into naive C57BL/6 mice. We then infected recipient mice with 2×10^6 PFU LCMV-WE and coinfected the mice 6 d later with 2×10^6 PFU VSV, as described above. Inhibition of neutralizing anti-VSV IgG by LCMV coinfection was reversed by increased numbers of VI10Yen B cells in a dose-dependent way (**Fig. 7d**). This indicated that the inhibition was effective only at low B cell precursor frequencies. To confirm this finding with another experimental approach, we analyzed the ability of LCMV-induced polyclonal B cell activation to 'compete out' IgG responses specific for (2,4-dinitrophenyl)-albumin (DNP-albumin). High B cell precursor frequencies exist against DNP-albumin hapten carrier conjugates in naive C57BL/6 mice[29]. Anti-DNP-albumin IgG titers were not impaired but were even increased by LCMV preinfection, reflecting the polyclonal B cell stimulation (**Fig. 7e**). Thus, we noted the competitive effect of LCMV-induced polyclonal B cell activation against LCMV-related and LCMV-unrelated specificities, however, only if precursor frequencies of the relevant B cells were relatively low.

DISCUSSION

Delayed neutralizing antibody responses after infection with a persistent virus was, in our experiments, directly correlated with the activation kinetics and functional responsiveness of virus-specific $CD4^+$ T cells. A reduction in $CD4^+$ T cell function and/or reduction in $CD4^+$ T cell numbers reduced polyclonal B cell stimulation, apparently by 'concentration' of specific T cell help onto virus-specific B cells. In contrast, strong (and even normal) virus-specific $CD4^+$ T cell activation resulted in heightened early polyclonal B cell activation, which competed with virus-specific B cell activation and the formation of neutralizing antibodies. An absence of $CD8^+$ T cells indirectly affected neutralizing antibody production by enhancing virus replication, which in turn favored $CD4^+$ T cell anergy or unresponsiveness.

The inverse correlation between early hypergammaglobulinemia and neutralizing antibody responses affected also LCMV-unrelated protective antibody responses (for example, against VSV). This competition for antigen-specific B cells was seen only for B cells with physiologically low precursor frequencies.

Our experiments here cannot determine whether this biological consequences of polyclonal B cell activation results from a competition for B cell survival factors or for 'anatomical niches' that promote B cell isotype switching by rendering cognate $CD4^+$ T cell–B cell interactions more or less efficient. Should the competition involve anatomical structures, then $CD8^+$ T cell–induced immunopathological disruption of lymphoid tissue organization may further enhance this effect[9,10]. Additionally, increased virus replication may influence the kinetics of avidity and/or affinity maturation of virus-specific antibodies. The evidence presented here suggests that the latter effects are additional or secondary to the influence of polyclonal B cell activation.

Whereas high-affinity neutralizing antibodies are key effector mechanisms against acutely cytopathic virus infections, such as rabies (or VSV) and poliovirus[31], infections with poorly cytopathic or noncytopathic viruses, including LCMV, hepatitis C virus or HIV, typically result in delayed formation of protective neutralizing antibodies[3,31,32]. Instead, in these latter infections, virus replication is initially controlled by strong, although often transient, $CD8^+$ T cell responses[33]. For persistent virus-host interactions, a competitive coexistence of humoral and cellular immunity has been described experimentally[8] and theoretically[11]; thus, neutralizing antibodies may act as an additional line of defense when $CD8^+$ T cell responses become weakened. This competitive coexistence may be beneficial to the host by averting a combination of strong cytotoxicity and antibody responses that may favor immunopathology and autoimmune manifestations. However, virus persistence after HIV or LCMV infection could be substantially reduced if both CTLs and neutralizing antibodies were experimentally available at early time points after infection by exogenous addition[34,35]. In addition, the combination of neutralizing antibodies and $CD8^+$ T cells should suppress the risk of either CTL or neutralizing antibody escape variant formation[7,36]. We believe that coevolution of such noncytopathic or low cytopathic viruses and mammalian hosts has yielded mechanisms that avoid production of potent neutralizing antibody responses, thereby favoring virus persistence. With overwhelming virus infections such as LCMV, virus may persist not only as a result of $CD8^+$ T cell deletion and/or exhaustion but also as a consequence of increased polyclonal B cell activation[37].

Given the demonstration that $CD8^+$ T cells impair the formation of neutralizing antibodies partially indirectly by induction of $CD4^+$ T cell anergy, it might be difficult but not impossible to induce both a protective CTL and an accelerated neutralizing antibody response. In fact, we have demonstrated here that a reduction in $CD4^+$ T cell function early during LCMV infection can decrease polyclonal B cell activation and thus can improve neutralizing antibody responses in immunocompetent hosts while leaving CTL responses intact.

What may be learned from the LCMV model infections presented here to better elucidate of HIV pathogenesis? High HIV viremia can be associated with an anergic state of virus-specific $CD4^+$ T cells[38]. Antiviral therapy reduces HIV-induced polyclonal hypergammaglobulinemia[39] and in parallel increases neutralizing antibody responses against autologous virus[40]. These results are all consistent with our findings with LCMV. In addition, early hyperactivity of $CD4^+$ T cells represents an independent risk factor for disease progression after HIV infection[41]. As for LCMV, a delay in neutralizing antibody responses associated with accentuated T helper cell responses might enhance HIV replication and promote exhaustion or deletion or aging of protective T cells. HIV-induced hypergammaglobulinemia inversely correlates with HIV-unrelated IgG titers induced by vaccination against measles or diphteria[42], reminiscent of the LCMV-VSV B cell competition described here. Other factors may additionally account for the delayed appearance of HIV-neutralizing antibodies, including depletion of $CD4^+$ T cells, qualitative differences in $CD4^+$

T cell function, disruption of lymphatic architecture or glycan shields that conceal the neutralizing epitope on the HIV glycoprotein[43,44].

Our experiments may suggest potential strategies for experimentally and therapeutically influencing persistent virus infections by reducing virus-induced polyclonal B cell activation and thereby enhancing neutralizing antibody production. The deliberate impairment of T cell help during immunization either by the use of vaccines with mutated or deleted $CD4^+$ T cell epitopes or, alternatively, by partial depletion of $CD4^+$ T cell function *in vivo*, although counterintuitive and probably difficult to control, may be worth careful consideration.

METHODS

Mice. All mice were bred and maintained in specific pathogen–free conditions and experiments were done in accordance with institutional and Swiss guidelines. *Cd8a*$^{-/-}$, VI10Yen and Smarta1 mice were all on a C57BL/6 (H-2^b) background. AG129 mice were on a 129/Sv (H-2^b) background. All mice were obtained from the Institute for Labortierkunde, Faculty of Veterinary Medicine, University Zürich-Irchel, Switzerland.

Virus. The LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated on L929 and/or MC57 cells. Mice were infected intravenously with 2×10^2 PFU (low dose), 5×10^3 PFU (intermediate dose) or 2×10^6 PFU (high dose) LCMV-WE. LCMV-WE_{del} was previously isolated from LCMV-WE-infected DBA/2 mice[19]. VSV-Indiana (VSV-IND) and VSV-New Jersey (VSV-NJ) were originally obtained from D.Kolakofsky (University of Geneva, Switzerland) and were grown on BHK cells. Mice were infected intravenously.

Sequencing. The cDNA encoding LCMV-WE_{del} glycoprotein was sequenced as described[19].

Detection of virus and neutralizing antibody titers. LCMV virus titers were determined by plaque-forming assay on MC57 fibroblasts as described[45]. Detection of neutralizing activity against LCMV in mouse sera has been described[45]. Neutralizing activity against LCMV-WE or LCMV-WE_{del} was measured by plaque-reduction assay. The neutralizing titer was defined as the $\log_2$ dilution resulting in half-maximal reduction of plaques compared with the same amount of virus incubated with control sera from uninfected mice or medium alone. A titer of less than 1 indicates no detectable neutralization of a 1:10 predilution of serum incubated with an equal volume of virus for 90 min before addition of the mixture to MC57 fibroblasts. Neutralizing total (IgM plus IgG) and IgG antibodies to VSV were measured as described[46].

***In vivo* depletion of $CD4^+$ and $CD8^+$ T cells.** Depletion of cells used rat monoclonal antibodies specific for CD4 (YTS 191.6) or CD8 (YTS 169.4) as described[20] and were provided by H. Waldmann (Therapeutic Immunology Group, Oxford, UK). Antibody (200 µl) was injected intraperitoneally on days −3 and −1 (before infection at day 0). For partial $CD4^+$ T cell depletion, 200 µl of a 1:100 dilution (in PBS) was injected intraperitoneally on day −1. The efficiency of depletion was confirmed by flow cytometry of peripheral blood cells with fluorescein isothiocyanate– or phycoerythrin-labeled anti-CD4 or anti-CD8 (BD Pharmingen).

Serum protein electrophoresis to quantify hypergammaglobulinemia. Detection of serum immunoglobulin as a function of total serum protein was done by the Department of Clinical Chemistry, University Hospital, Zürich, Switzerland. Serum was separated by agarose gel electrophoresis (Sebia) and the fractions were quantified with a densitometer (Beckmann Coulter).

LCMV-specific tetramers. Tetramers specific for LCMV glycoprotein (GP(33–41) and GP(276–284)) and nucleoprotein (NP(396–404)) have been described[47]. Phycoerythrin-labeled tetramers were used.

Intracellular cytokine staining for IFN-γ and flow cytometry. Splenocytes were re-stimulated overnight with baculovirus-derived recombinant LCMV nucleoprotein in RPMI medium supplemented with FCS (10% final concentration) and β-mercaptoethanol (0.1 mM final concentration). For T cell re-stimulation, cells were cultured for 6 h in medium containing phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (500 ng/ml) or alternatively one of the following peptides (at a concentration of 50 µg/ml): GP(61–80), GP(33–41) and GP(276–286) or NP(309–328) and NP(396–404). Brefeldin A (10 µg/ml; Sigma-Aldrich) was added for the last 5 h of culture. Cells were then collected, washed once in PBS with 4% FCS and 12.5 mM EDTA and stained with phycoerythrin-conjugated anti-CD4 or anti-CD8 (BD Pharmingen). After being washed, cells were fixed with 4% paraformaldehyde (in PBS) for 10 min, then were permeabilized with PBS containing 4% FCS, 12.5 mM EDTA and 0.1% saponin (Sigma-Aldrich). For intracellular cytokine staining, cells were incubated for 45 min at 4 °C with fluorescein isothiocyanate– or allophycocyanin-conjugated anti-IFN-γ (BD PharMingen). After being washed twice with permeabilization buffer, cells were resuspended in PBS containing 4% FCS and 12.5 mM EDTA and were analyzed with a FACScan or FACSCalibur (BD).

Examination of $CD4^+$ T cell activation. Expression of the selectin CD62L on blood $CD4^+$ T cells was examined by staining with anti-mouse CD4–fluorescein isothiocyanate and anti-mouse CD62L–phycoerythrin (both from BD Pharmingen), followed by analysis with a FACSCalibur (BD).

***In vivo* cytotoxicity.** Assays for *in vivo* cytotoxicity were done with C57BL/6 splenocytes incubated for 1 h with or without LCMV-derived MHC class I GP(33–41) peptide and labeled for 10 min with carboxyfluorescein diacetate succinimidyl diester (CFSE) at a concentration of 5 µg/ml ($CFSE^{hi}$, peptide-labeled splenocytes) or 0.5 µg/ml ($CFSE^{lo}$, splenocytes not labeled with peptide; Molecular Probes). 'LCMV memory' or naive control mice were injected intravenously with 1×10^7 cells of each fraction. The number of $CFSE^+$ cells remaining in the blood after 4 h was determined by flow cytometry. Specific cytotoxicity was calculated with the following equation: cytotoxicity (%) = 1 – [($CFSE^{hi}$ (memory)/$CFSE^{lo}$ (memory))/($CFSE^{hi}$ (naive)/$CFSE^{lo}$ (naive))].

ELISA. For analysis of LCMV glycoprotein, 96-well plates were coated with 100 µl recombinant LCMV glycoprotein supernatant derived from HEK293 cells transfected with LCMV glycoprotein vector. Sera prediluted 1:30 were incubated for 90 min, washed with PBS containing 0.5% Tween 20, then incubated further with anti-mouse IgG–horseradish peroxidase (1:1,000 dilution; Sigma-Aldrich) for detection of virus-specific IgG responses. For the detection of IgG antibodies specific for DNP-albumin conjugate, plates were coated overnight with 10 µg/ml of DNP–serum albumin (Sigma-Aldrich). Plates were then blocked for 2 h at 25 °C with 2% BSA in PBS. Sera prediluted 1:30 were incubated for 90 min. Anti-mouse IgG–horseradish peroxidase (1:1,000 dilution; Sigma-Aldrich) was used to detect specific IgG responses. In all ELISAs, a green color reaction was produced with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) as a substrate (ABTS; Boehringer-Mannheim). The ELISA titer was defined as the $\log_2$ serum dilution resulting in an optical density at 450 nm twofold above background.

KL25 plaque-neutralization assay. For these assays, 30–50 PFU LCMV-WE or LCMV-WE_{del} were incubated in 96-well plates for 90 min with twofold dilutions (starting at 0.5 mg/ml) of the LCMV-neutralizing monoclonal antibody KL25 (ref. 20). Plaques were then evaluated on MC57 fibroblasts as described[45]. Foci were counted in triplicate and the percentage of plaque formation in the absence of KL25 (considered 100% PFU) was calculated and plotted against ($\log_2$) KL25 dilution.

Serum transfer. For measurement of the protective antibody capacity against a challenge LCMV infection, 100 µl of pooled serum after LCMV-WE or LCMV-WE_{del} infection of C57BL/6 mice (40–80 d after infection) was adoptively transferred intravenously into naive C57BL/6 mice. The mice were challenged 2 h later with 200 PFU LCMV-WE and spleen virus titers were measured 4 d later by plaque assay.

Statistical analysis. Data are expressed as mean and s.e.m. Where indicated, significance was assessed with the paired *t*-test. *P* values above 0.05 were considered not significant.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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