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On the role of antigen in maintaining cytotoxic T-cell memory

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ABSTRACT **This study evaluated whether T-cell memory reflects increased precursor frequencies of specific long-lived T cells and/or a low-level immune response against some form of persistent antigen. Antivirally protective CD8$^+$ T-cell memory was analyzed mostly in the original vaccinated host to assess the role of antigen in its maintenance. T-cell mediated resistance against reinfection was measured in the spleen and in peripheral solid organs with protocols that excluded protection by antibodies. *In vivo* protection was compared with detectable cytotoxic T-lymphocyte precursor frequencies determined *in vitro*. In the spleen, *in vitro* detectable cytotoxic T-lymphocyte precursor frequencies remained stable independently of antigen, conferring resistance against viral replication in the spleen during reinfection. In contrast, T-cell mediated resistance against reinfection of peripheral solid organs faded away in an antigen-dependent fashion within a few days or weeks. We show that only memory T cells persistently or freshly activated with antigen efficiently extravasate into peripheral organs, where cytotoxic T lymphocytes must be able to exert effector function immediately; both the capacity to extravasate and to rapidly exert effector function critically depend on restimulation by antigen. Our experiments document that the duration of T-cell memory protective against peripheral reinfection depended on the antigen dose used for immunization, was prolonged when additional antigen was provided, and was abrogated after removal of antigen. We conclude that T-cell mediated protective immunity against the usual peripheral routes of reinfection is antigen-dependent.**

Immunological memory is usually long lived as experienced by life-long immunity to childhood diseases and the tremendous success of vaccination campaigns. This mostly reflects longevity of B-cell memory because protection is efficiently mediated by antibodies (1, 2). Far less is known about the duration and nature of T-cell memory. After primary exposure to antigen, elevated numbers of specific T cells remain in the host for years (3). There is strong evidence that B-cell memory is driven by antigen persisting within the host on follicular dendritic cells (4, 5). It is, however, open to debate as to whether T-cell memory is mediated by long-lived lymphocytes (6–10) or by a low-level immune response against persistent antigen (11–13). Furthermore, it is unclear whether the presence of elevated numbers of specific T cells as such are sufficient for protection against secondary infections. This study addresses both questions in viral model systems.

If T-cell memory were maintained by antigen, then (*i*) its duration should depend on the antigen-dose, (*ii*) short-lived T-cell memory should be prolonged by providing additional antigen, and (*iii*) removal of antigen should abrogate memory. We will demonstrate that *i*, *ii*, and *iii* are true for T-cell memory protective against peripheral nonmucosal reinfection. This study uses only functional assays and avoids the use of "memory markers," since the hope that reliable markers expressed specifically on memory T cells could be easily used to investigate the nature of T-cell memory has not been fulfilled yet (14). Similarly, studies using defined monoclonal memory T cells from T-cell receptor transgenic mice have been complicated because not all T cells can be synchronized (15). To compare our results with seemingly conflicting data from other groups (6–10), we use critical assays for protective T-cell memory *in vivo,* with the usual *in vitro* tests for T-cell memory.

This study focuses on CD8$^+$ T-cell memory. All *in vivo* assays for T-cell memory were done under conditions where contributions by neutralizing antibodies and CD4$^+$ T cells are excluded. Therefore, mice were vaccinated with one virus and then challenged with another recombinant virus, so that both of these viruses shared the CD8$^+$ T-cell epitopes, but were unable to induce crossreactive protective antibodies. This was achieved by vaccinating mice with vesicular stomatitis virus (VSV) and then challenging them with a vaccinia recombinant virus expressing the nucleoprotein of VSV (vacc-VSV-N). This nucleoprotein contains the dominant epitope for CD8$^+$ cytotoxic T lymphocytes (CTLs), but no epitope recognized by protective CD4$^+$ T cells in H-2^b mice (16). Because VSV-N is exclusively internal and not present on the vaccinia virus membrane, it cannot be accessed by neutralizing antibodies.

A situation, where T cells—and not antibodies—are predominantly protective, is reinfection of peripheral solid organs such as the skin, brain, kidney, or ovary (1, 2). In contrast, when reinfection starts primarily systemically, such as after i.v. infections or on mucous membranes, neutralizing antibodies are efficient and sufficient to protect (1, 2). Based on these considerations, T-cell memory in vaccinated mice was studied by avoiding the intravenous route of reinfection and by using a peripheral route. A physiological peripheral route of infection would be via the skin. For technical reasons (tropism of the viruses used and difficulties in quantifying infection), this physiological route had to be simulated, in most experiments, by infection of other peripheral solid organs, such as the brain or the ovaries.

An additional consideration in planning the present experiments was to demonstrate that the results were applicable to both main effector functions of CD8$^+$ T cells. Therefore, we analyzed one example where CD8$^+$ T-cell memory is mediated

Abbreviations: VSV, vesicular stomatitis virus; VSV-N, nucleoprotein of VSV; CTL, cytotoxic T lymphocytes; pfu, plaque-forming units; DTH, delayed type hypersensitivity; TCR, T-cell antigen receptor.

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by means of contact-dependent perforin induced cytotoxicity, and another example where $CD8^+$ memory T cells mediate their effect by means of cytokines. LCMV was chosen as a noncytopathic virus where protection against viral replication and subsequent immunopathology is mediated by perforin-dependent $CD8^+$ cytotoxic T cells (17, 18). Vaccinia virus was chosen as the model cytopathic virus, which is controlled by interferon-γ and tumor necrosis factor α (18); in this infection, the role of neutralizing antibodies was excluded as discussed above (16).

MATERIALS AND METHODS

Mice, Viruses, Peptides, and T-Cell Depletion *in Vivo*. Mice were between 8–12 weeks of age at the beginning of the experiment. The viruses used have been described (17, 19, 20). VSV-N-peptide (21) was purchased from Neosystem (Strasbourg, France). The monoclonal antibodies YTS191.1 and YTS169.7 were used for *in vivo* depletion of $CD4^+$ and $CD8^+$ T cells (22).

***In Vivo* Protection Assays for Functional $CD8^+$ T-Cell Memory.** The *in vivo* assay for the detection of CTL activity by challenge infections with vaccinia recombinant viruses has been described (16). Briefly, mice that had been immunized with either VSV or LCMV wild-type are challenged intracerebrally (i.c.) or intraperitoneally (i.p.) by infection with vaccinia recombinant viruses expressing VSV or LCMV proteins, respectively [5×10^3 plaque-forming units (pfu) in 30 μl i.c. and 5×10^6 pfu in 400 μl i.p.]. Five days after challenge infection titers of vaccinia recombinant viruses were determined in brains or ovaries (16). If VSV- or LCMV-specific CTL memory is functional, then the respective vaccinia recombinant virus is usually eliminated below detection levels at this time point.

Primary and Secondary *ex Vivo* Cytotoxicity Against VSV, Limiting Dilution Analysis. *Primary* ex vivo *cytotoxicity.* Mice were infected i.v. with 2×10^6 pfu of VSV. After 6 days, spleen cells were coincubated for 5 h with ^{51}Cr-labeled MC57 ($H\text{-}2^b$) target cells that were either uninfected or infected with VSV (15 pfu/cell, 2 h).

In vitro *generation of cytotoxicity.* Spleen cells (4×10^6) were restimulated in 24-well plates, either with VSV-infected-irradiated macrophages (2000 rad) or with irradiated (4000 rad) EL-4 ($H\text{-}2^b$) cells transfected with VSV-N ($EL\text{-}4_N$) (13, 23).

Limiting dilution analysis. Responder cells were restimulated with VSV-infected-irradiated (2000 rad) spleen cells in 96-well plates, using 24 duplicates per dilution step (24). Irradiated (2000 rad) spleen cells from unprimed mice were used as feeder cells for a total cell density of 5×10^5 cells per well. Bulk cultures were incubated for 5 days in the absence of rat ConA supernatant, whereas limiting dilution cultures were incubated for 5 days in the presence of rat ConA supernatant (10% vol/vol). Cultures were then tested for specific cytotoxicity by ^{51}Cr-release assays $EL\text{-}4_N$ target cells or on control EL-4 cells ($EL\text{-}4_{neo}$). In the limiting dilution analysis, wells were considered positive when specific lysis was higher than $\bar{x} + 3$ SD of values obtained in unprimed mice.

Peptide-Induced Delayed Type Hypersensitivity (DTH). VSV-N-peptide was injected into hind footpads of mice (aa49-62, 30 μl, 1 mg/ml in saline) (21, 25). The subsequent swelling was measured with a spring loaded caliper.

RESULTS

Duration of $CD8^+$ T-Cell Mediated Antiviral Immunity Depends on Antigen Dose and Form. Because VSV replicates only abortively extraneurally in mice (19), this allows vaccination with limiting antigen doses. To assess interleukin-dependent $CD8^+$ T-cell memory, mice were immunized with graded doses of VSV, and after a defined time, mice were challenged i.p. or i.c. with vacc-VSV-N to determine vaccinia virus titers in ovaries or choriomeninges 5 days later, respectively. If VSV-N-specific $CD8^+$ T-cell memory was protective, then vacc-VSV-N replication should be limited; such resistance is mediated exclusively by $CD8^+$ T cells in $H\text{-}2^b$ mice (16).

The influence of antigen dose was studied after immunization with VSV. After immunization with a high dose (2×10^6 pfu), anti-viral protection against growth of vacc-VSV-N in choriomeninges or ovaries disappeared within 90 days (Fig. 1 *A–C*). In contrast, $CD8^+$ T-cell frequencies remained elevated (Fig. 1*D*) and specific cytotoxicity could be restimulated *in vitro* in bulk cultures for more than 200 days (not shown). Immunization with a low VSV dose (2×10^3 pfu) generated protection against challenge infection with vacc-VSV-N for only 20 days (Fig. 1*F*). $CD8^+$ T-cell frequencies remained elevated and bulk cultures were positive up to 100 days (Fig. 1 *E* and *G*); thereafter, they decreased and bulk cultures became negative in two of five mice.

In a second approach, VSV antigen was used in a form with a short half-life. Mice were immunized with class I-binding VSV-N-peptide (21), which has a biological half-life of only a few hours (25). This immunization induced a primary $CD8^+$ T-cell response, which readily conferred antiviral protection for 1 day, but produced no long-term memory (Fig. 2*A*); to obtain memory, peptide had to be given in a depot form using complete or incomplete Freund's adjuvant (Fig. 2 *A* and *B*). These data suggest that protective immunity correlated either with the duration of antigen-persistence or with the extent of clonal expansion of T cells (clonal burst size) during the early immune response (8). The CTLp frequencies were low (around 2×10^{-6}; not shown) and could not be measured accurately enough to permit meaningful comparison. The fact, however, that similarly low $CD8^+$ T-cell frequencies were induced after peptide immunization, with or without adjuvant, suggested that adjuvant seemed not to enhance the initial response, but rather made the peptide persist and maintain a weak response. An attempt to disentangle the initial strength of primary responses from the duration of protective memory was made in the next set of experiments.

Maintenance of Protective T-Cell Memory *in Vivo* Requires Recent Restimulation with Antigen. Mice were immunized with a low VSV dose (2×10^3 pfu) so that antiviral protection was short lived (Fig. 1, Table 1, group A). Long-term antiviral protective immunity could, however, be restored by *in vivo* restimulation with additional antigen (Table 1, group B). A cell line transfected with VSV-N ($EL\text{-}4_N$) was used for this *in vivo* restimulation since it restimulates only primed—but not naive—$CD8^+$ T cells (23). Either repeated antigenic restimulation (group B1), or restimulation a few days before reinfection (group B2) could restore immunity. In contrast, even restimulation with large amounts of $EL\text{-}4_N$ early after immunization (group B3) did not maintain protection. Thus, the strength of the early immune response does not correlate with the duration of immunity. Rather, continued or recent antigenic restimulation is necessary to maintain protective immunity.

Removal of Antigen Abrogates the Duration of Immunity. The antigen dependence of T-cell memory is usually tested by adoptively transferring memory T cells into unprimed, i.e., antigen-free recipient mice, where transferred memory T cells have been shown to survive for a long time (6–10). These adoptive transfer protocols usually use irradiated animals as recipients. The following experiments investigated the influence of irradiation on survival and function of the transferred memory T cells (Fig. 3). Donor mice were immunized with LCMV, and 90 days later 2×10^7 memory spleen cells were adoptively transferred into untreated or irradiated syngeneic recipient mice. CTLp frequencies in untreated recipients were around 1/30,000. After multiplication with the number of spleen cells in the recipient (6×10^7), the absolute take of CTLp per spleen was around 2000 cells. Such recipients were

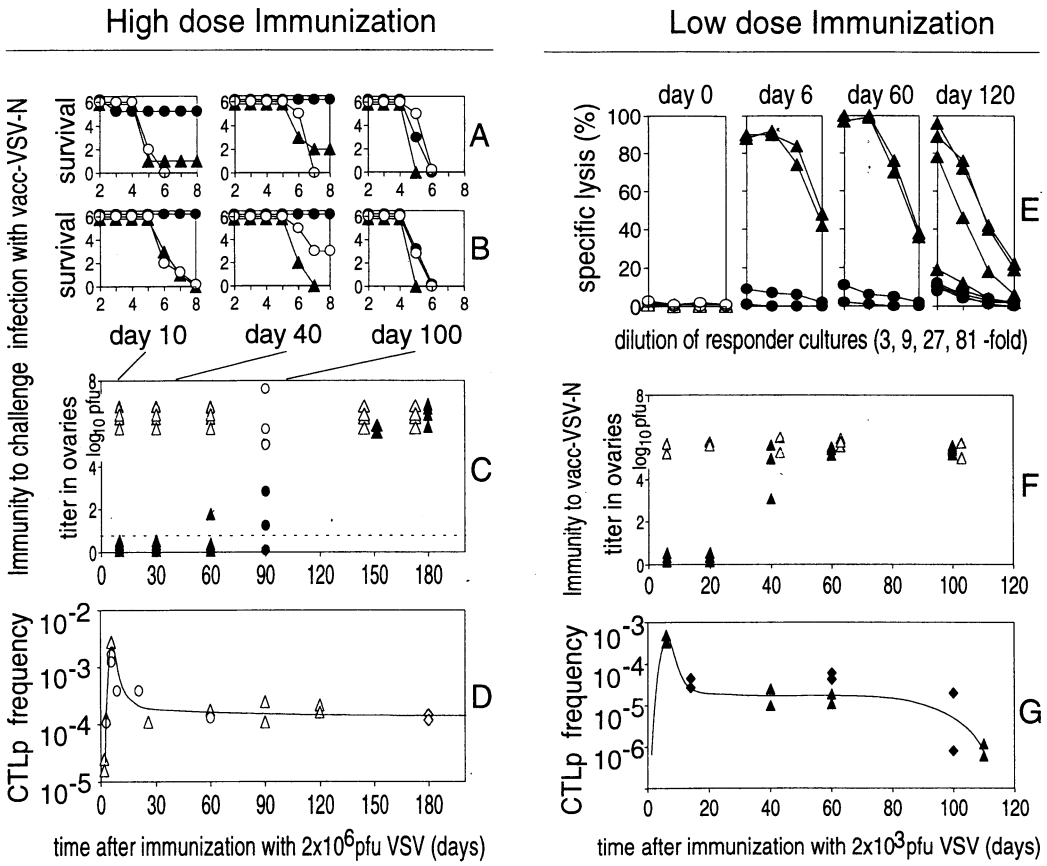

FIG. 1. Duration of antiviral protection depends on dose and form of immunizing antigen. C57BL/6 mice were immunized with VSV or VSV peptide and then challenged with a vaccinia recombinant virus expressing the nucleoprotein of VSV (vacc-VSV-N). If VSV-N-specific $CD8^+$ T-cell memory is functional, mice are resistant to vacc-VSV-N infection. (*A–D*) Immunization i.v. with 2×10^6 pfu VSV (high dose). (*A* and *B*) After 10, 40, and 100 days mice were challenged i.c. with vaccinia recombinant viruses: vacc-VSV-N (●), vacc-LCMV-N (▲). Unprimed control mice were challenged with vacc-VSV-N (○). BALB/c mice were used in *A* and in C57BL/6 mice were used in *B*. (*C*) After 10, 30, 60, 90, 150, and 180 days mice were challenged i.p. with vacc-VSV-N. Vacc-VSV-N titers in ovaries were determined 5 days later (detection level at 0.7 $\log_{10}$ pfu). VSV-primed C57BL/6 mice (solid symbols), unprimed control mice (open symbols). Triangles and circles represent independent experiments. (*D*) Limiting dilution analysis of frequencies of VSV-N-specific CTLp in spleens of C57BL/6 mice. Specificity of CTL for nucleoprotein was confirmed by split-well analysis (not shown). Triangles, circles, and diamonds represent independent experiments. (*E–G*) Immunization i.v. with 2×10^3 pfu of VSV (low dose). (*E*) After 6, 60, and 120 days spleen cells of mice were restimulated in bulk cultures and tested for VSV-N-specific cytotoxicity on EL-4 ($H\text{-}2^b$) target cells transfected with VSV-N (triangles) or on control EL-4_{neo} (circles). VSV-primed C57BL/6 mice (solid symbols), unprimed control mice (open symbols). (*F*) After 6, 20, 40, 60, and 100 days mice were challenged i.p. with vacc-VSV-N. Vacc-VSV-N titers were determined in ovaries 5 days after the challenge infections. VSV-primed C57BL/6 mice (solid triangles), unprimed controls (open triangles). (*G*) Limiting dilution analysis. Frequencies of VSV-N-specific CTLp in spleens of C57BL/6 mice. Triangles and diamonds represent independent experiments.

not protected against peripheral reinfection. In contrast, in irradiated recipients CTLp frequencies were only slightly higher (1/10,000), but due to irradiation spleen cell numbers were markedly diminished (5×10^6). Despite the markedly lower absolute take of CTLp (only 500 CTLp per spleen), mice were protected against peripheral challenge infection. The fact that irradiation of recipient mice lowered the absolute take of transferred $CD8^+$ T cells was confirmed by flow cytometric analysis, where irradiated and nonirradiated CD8α-deficient mice were used as recipients to facilitate the tracing of transferred $CD8^+$ T cells. Taken together, in nonirradiated recipient mice, memory $CD8^+$ T cells rapidly lost their capacity to protect against reinfection. However, irradiation of recipient mice induced a "qualitative change" of the transferred memory T cells, which restored their capability to protect against reinfection. We propose that, similar to persistent antigen, irradiation of recipient mice activates transferred memory T cells.

Antigenic Restimulation Enhances Extravasation of $CD8^+$ Memory T Cells. The above experiments revealed a striking discrepancy between clearly elevated frequencies of CTLp in the spleen, which need not be protective against peripheral viral challenge infection. We therefore assessed the distribution of VSV-specific $CD8^+$ T cells in spleen, lymph nodes, and blood after immunization with VSV (Fig. 4). Emigration to peripheral tissue was monitored using a $CD8^+$ T-cell-specific peptide-induced delayed type hypersensitivity assay (25). Two days after infection i.v. (Fig. 4*A*) VSV-specific T-cell responses were detectable exclusively in the spleen (top row), which filters out viruses from the blood (26). On day 6, at the peak of VSV-specific $CD8^+$ T-cell responses, $CD8^+$ T cells were distributed evenly throughout all lymphatic organs (second row); VSV-specific cytotoxic T-cell activity assessed directly *ex vivo* was comparable in spleen and lymph nodes (*Inset A1*). On day 6, specific $CD8^+$ T cells also recirculated through footpad tissue, as indicated by a strong DTH to peptide. After 40–90 days, specific $CD8^+$ T cells no longer efficiently recirculated from blood to lymph nodes; frequencies of VSV-specific $CD8^+$ T cells were 100 times lower in lymph nodes than in spleen and blood (third row, *Inset A2*). Recirculation of $CD8^+$ T cells through peripheral tissues was also inefficient, since peptide-induced DTH was absent. Boosting with antigen rapidly restimulated the recirculation of such "resting" $CD8^+$ T cells. Already 2 days after i.v. reinfection, they were found again in

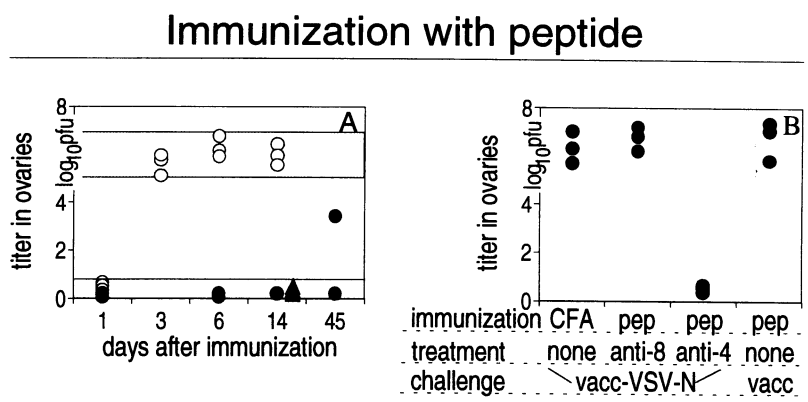

FIG. 2. Immunization with viral peptides: (*A*) C57BL/6 (H-2^b) mice were immunized i.p. with VSV-N peptide and dissolved in saline for 5 consecutive days (open circles) (2 mg per day). Alternatively, the peptide (2 mg) was given once, emulsified in complete Freund's adjuvant (solid circles) or incomplete Freund's adjuvant (solid triangles). One, 3, 6, 14, and 45 days after the last peptide injection, mice from both groups were challenged i.p. with vacc-VSV-N and 5 days after the challenge infection titers were determined in ovaries (16). The range of titers in unprimed control mice challenged with vacc-VSV-N is indicated by the shaded area. (*B*) C57BL/6 mice given complete Freund's adjuvant without peptide (CFA) were challenged i.p. with vacc-VSV-N on day 6. Mice given VSV-N peptide in saline for 5 consecutive days were *in vivo* depleted of CD4$^+$ or CD8$^+$ T cells ("anti-4," "anti-8") by injection of the respective monoclonal antibodies (1 mg) on days 4 and 5 before challenge infection with vacc-VSV-N on day 6. A fourth group of mice was immunized with VSV-N peptide in saline for 5 consecutive days and then challenged with antigenically unrelated vacc-LCMV-G ("vacc") on day 6 to test for VSV-N specificity.

lymph nodes and DTH was restored (bottom row, compared with top row). [A second VSV serotype (New Jersey), which crossreacts exclusively on the CD8$^+$ T-cell level, was used for reinfection (27)].

Memory T Cells Persist at the Site of Primary Infection. To determine where viral antigen might persist, we took advantage of the above finding that during the late memory phase specific CD8$^+$ T cells are no longer present in lymph nodes. It is an old observation that specific T cells preferentially accumulate at antigen-depots (28). Vice versa, the accumulation of VSV-specific T cells could be used to localize persistent antigen. Therefore, VSV was injected directly into mesenteric lymph nodes (Fig. 4*B*). T-cell induction took place in mesenteric lymph nodes (top row). At the peak of the response, on day 6, CD8$^+$ T cells recirculated through all lymphatic organs and through tissue, as indicated by strong DTH to peptide (not shown). CD8$^+$ T cells were concentrated in mesenteric lymph nodes for more than 30 days after infection with high VSV doses (2×10^6 pfu) (third row). After infection with a limiting VSV dose (2×10^3 pfu) mesenteric—but no other lymph nodes—were positive for 30 days, but no longer after 50 days. However, the spleen remained positive for up to 100 days (Fig. 1*G*). Taken together, specific CD8$^+$ T cells accumulated in lymph nodes draining the site of primary infection for weeks, suggesting that the draining lymph node was the site of antigen persistence. Interestingly, after infection with 2×10^3 pfu, VSV antigen persisted around 30 days, which is comparable to the duration of protective antiviral immunity observed after immunization with this dose (Fig. 1*F*).

Role of T-Cell Frequency vs. Activation for *in Vivo* vs. *in Vitro* T-Cell Memory. From the above experiments we concluded that elevated frequencies of specific CD8$^+$ T cells alone cannot mediate protection against reinfection of peripheral solid organs. This notion was strongly supported by additional experiments using T-cell antigen receptor-transgenic mice (TCR-tg$^+$) (29) (Table 2). These TCR-tg$^+$ mice have an extremely high frequency of CD8$^+$ T cells specific for LCMV-G (about 50–70% of all CD8$^+$ T cells are specific for GP, aa33-41, presented on D^b). Despite these high frequencies of specific CD8$^+$ T cells, unprimed, i.e. antigen-free transgenic

Table 1. CTL-mediated antiviral protection requires antigenic restimulation

Exp. group	Immunization, pfu VSV	Challenge infection after 14 days,* $\log_{10}$ pfu/ovaries	Additional antigenic restimulation	Challenge infection after 44 days,* $\log_{10}$ pfu/ovaries
A				
1	2×10^6	<0.7	None	<0.7
2	2×10^5	<0.7	None	<0.7
3	2×10^4	<0.7	None	<0.7
4	2×10^3	<0.7	None	6.20 ± 0.10
5	2×10^2	1.85 ± 1.62	None	5.97 ± 0.21
6	None	6.28 ± 0.14	None	6.03 ± 0.18
B				
1	2×10^3	ND	2×10^6 EL-4$_N$, on day 4, 8, 12, . . . , 40	2.00 ± 0.87
2	2×10^3	ND	2×10^6 EL-4$_N$, on day 40	1.50 ± 0.30
3	2×10^3	ND	2×10^7 EL-4$_N$, on day 4	6.14 ± 0.08
4	None	ND	2×10^6 EL-4$_N$, on day 4, 8, 12, . . . , 40	6.07 ± 0.16
5	2×10^3	ND	2×10^6 EL-4$_{neo}$, on day 4, 8, 12, . . . , 40	6.16 ± 0.16
6	None	ND	None	6.00 ± 0.22

*Fourteen and 44 days after immunization i.v. of C57BL/6 mice with VSV wild type, CTL-mediated protection against reinfection was assessed by challenge infection of mice i.p. with 1×10^7 pfu of vacc-VSV-N. Vacc-VSV-N titers in ovaries were measured 5 days after challenge infection. (Group A) Effect of immunizing virus dose on duration of CTL-mediated antiviral protection; in the absence of additional antigenic restimulation. (Group B) Effects of additional antigenic restimulation on duration of antiviral protection in mice primed with 2×10^3 pfu of VSV. The indicated number of EL-4$_N$ (irradiated with 4000 rad γ) were injected i.p. on the indicated days. ND, not determined.

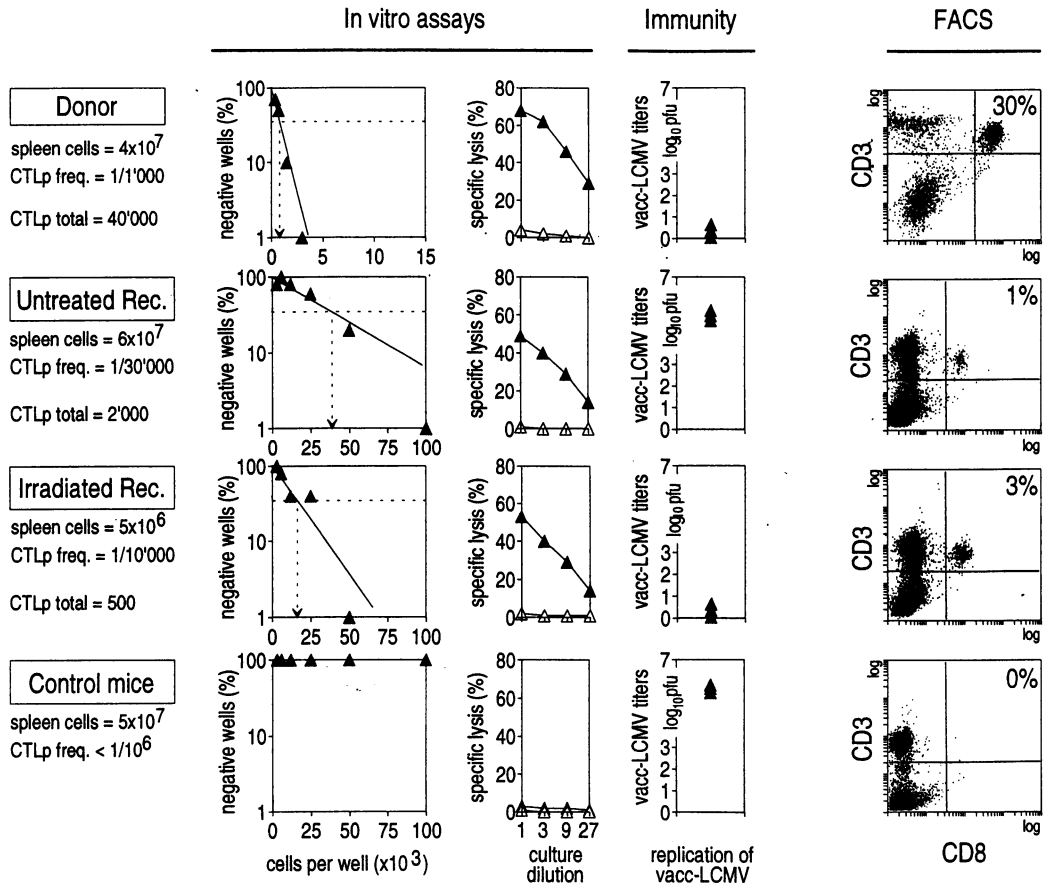

FIG. 3. Removal of antigen abrogates *in vivo* antiviral protection. Donor mice were infected with LCMV. After 2 months, 2×10^7 donor spleen cells were adoptively transferred into either untreated or irradiated recipient mice (450 rad γ 1 day before transfer). Control mice were not irradiated and adoptively transferred 2×10^7 spleen cells from unpaired mice. In all groups of mice limiting dilution analysis of spleen cells was performed (second column). The resulting frequencies are given in the first column: the absolute CTLp take per spleen ("CTLp total") = (CTLp-frequency) $\times$ (absolute cell count per spleen). Mice were also challenged i.p. with vacc-LCMV-G (1×10^7 pfu) and LCMV-G titers were determined in ovaries 5 days later (fourth column: "Immunity"). In a separate series of experiments 10^7 spleen cells from CD8$^+$ T-cell receptor transgenic mice specific for LCMV-G (29) were adoptively transferred into either untreated or irradiated CD8α-deficient mice. Control mice are CD8α-deficient mice not receiving any T cells.

mice were not protected against peripheral infection. They were not resistant against replication of vacc-LCMV-G in the brain (Table 2, groups A and B), demonstrating that high CD8$^+$ T-cell frequencies alone could not provide interleukin-dependent antiviral protection. Similarly, high frequencies of CD8$^+$ T cells in unprimed TCR-tg$^+$ mice could not confer perforin-dependent cytotoxicity against replication of LCMV in the choriomeninges after i.c. infection (groups G and H), or in footpads after intra-footpad infection (not shown). In contrast, after immunization with antigen, CD8$^+$ T cells in TCR-tg$^+$ mice readily gained the capacity to protect against such peripheral challenge infections (groups C and D, E and F, I and J, K and L). This demonstrated that for both functions, antiviral protection by interleukins and by perforin-dependent cytotoxicity, CD8$^+$ T cells had to be activated with antigen. Immunization with various forms of LCMV-G-antigen further illustrated that activated CD8$^+$ T cells, even at a frequency of only 10^{-3} (groups E and K), were more efficient in antiviral protection than nonactivated CD8$^+$ T cells at a frequency of 10^{-1} (groups B and H). Finally, immunization with antigen in the form of G-peptide demonstrated that nonactivated CD8$^+$ T cells could reach the activation status required for antiviral protection within 24 h after antigen contact (group D).

In contrast to these peripheral challenge infections, unprimed TCR-tg$^+$ mice were resistant against LCMV replication in lymphoid organs after challenge infection i.v. (Table 2, groups G and H), as had been shown (29). Obviously, recirculation of specific CD8$^+$ T cells is not necessary to protect against challenge infection i.v., since the main fraction of the virus is filtered out in the spleen (26); thus, it is directly delivered to the CD8$^+$ T cells, which can then be activated rapidly, i.e., within 24 h, as shown above. The above data illustrate a profound difference between on the one side peripheral challenge infection, where protection requires activated CD8$^+$ T cells to extravasate into these peripheral tissues, and on the other side i.v. infection of lymphoid organs, where also resting CD8$^+$ T cells are quickly activated and need not recirculate to protect. This may explain why studies using exclusively challenge infections i.v. for the *in vivo* readout of T-cell memory reached the conclusion that T-cell memory was antigen-independent (7).

DISCUSSION

After vaccination with an abortively replicating virus or with viral peptide, CTL-mediated protective antiviral T-cell memory was relatively short-lived and antigen-dependent. After disappearance of antigen, either in the original host or during adoptive-transfer experiments, frequencies of pathogen-specific CTLp remained high *in vitro*, but antiviral protective immunity *in vivo* disappeared. Similarly, high frequencies of virus specific T cells in unprimed TCR-transgenic mice failed to protect against infection; preactivation with viral antigen was necessary for protective immunity. Antigenic activation

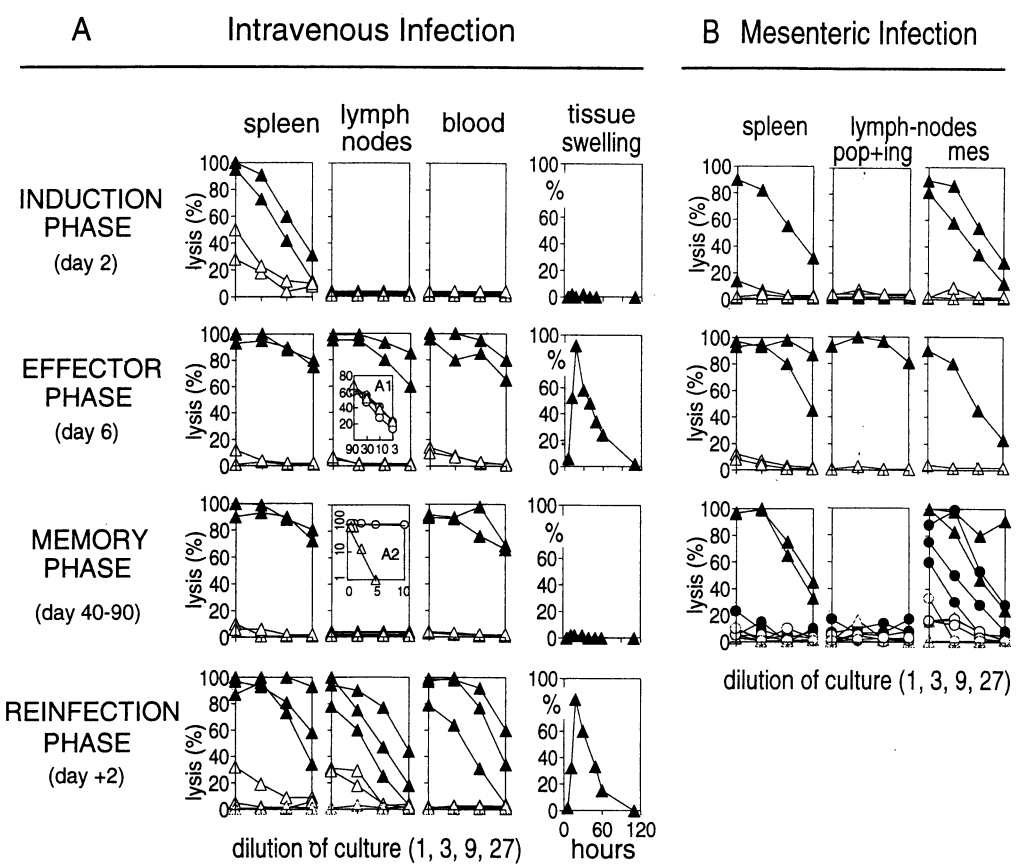

FIG. 4. Distribution of VSV-specific CTLp. (*A*) Intravenous infection: C57BL/6 mice were infected i.v. with VSV (serotype Indiana, 2×10^6 pfu). The localization of VSV-N-specific CTLp was assessed after 2 days (induction phase), 6 days (effector phase), and 40–90 days (memory phase). Fifty days after infection with VSV (serotype Indiana) some mice were reinfected with VSV (reinfection phase) (serotype New Jersey, 2×10^6 pfu i.v.). Spleen cells, lymph node cells (pooled from popliteal plus inguinal lymph nodes), and peripheral blood lymphocytes were restimulated *in vitro* with EL-4_N (23). After 5 days, VSV-N-specific cytotoxicity was tested on ^{51}Cr-labeled EL-4_N (solid triangles) or on EL-4_{neo} (open triangles). Similar results were obtained on VSV-infected or noninfected MC57 target cells (not shown). Spontaneous release was <20% for all assays. Migration of VSV-N-specific CTL to peripheral tissue was assessed by monitoring the footpad swelling reaction ("tissue swelling") after local injection of VSV-N-peptide (increase in footpad thickness as compared with thickness before injection. Average of 4 hind footpads, interindividual variation < 20%. (*Inset A1*) On day 6 primary *ex vivo* cytotoxicity was compared between spleen (circles) and mesenteric lymph nodes (triangles) on EL-4_N. The vertical axis indicates specific lysis (%), the horizontal axis indicates the effector/target ratio. Spontaneous ^{51}Cr-release was <15%, specific lysis of EL-4_{neo} was <5%. (*Inset A2*) On day 50 VSV-specific CTLp in spleens (triangles) were compared with lymph nodes (mesenteric pooled with inguinal) (circles) using limiting dilution analysis (24). The vertical axis indicates the percentage of negative wells, the horizontal axis indicates the number of responder cells per well ($\times 10^3$). Wells were considered positive when specific lysis was >3 SD above average specific lysis observed in unprimed mice. (*B*) Injection directly into mesenteric lymph nodes: C57BL/6 mice were infected into the mesenteric lymph nodes with VSV (2×10^6 pfu). After 2 days (induction phase), 6 days (effector phase), and 30 days (memory phase) spleen cells, cells pooled from popliteal and inguinal lymph nodes, or mesenteric lymph node cells were restimulated *in vitro* and tested for VSV-N-specific cytotoxicity on EL-4_N (solid symbols) or EL-4_{neo} (open symbols). Spontaneous release was <20% in all assays. (Circles and triangles: results from two independent assays are combined.)

enhanced the necessary migration of memory T cells from blood through solid tissues to lymph nodes. Antigenic activation also prepared T cells for immediate effector function.

In vitro, neither *recirculation* nor *activation* levels are measured because T cells are mechanically mixed together with their target cells and are given plenty of time to develop effector function (5–7 days). Thus, *in vitro* analysis determines pCTL frequency only.

Because CTLp, after removal or disappearance of antigen, do not extravasate into tissue and therefore accumulate in the spleen, they will be ineffective against infections occurring via nonmucosal peripheral routes, such as the skin or i.c. or i.p. challenge infections as used here. In contrast, for a challenge infection i.v., where virus is filtered out by the spleen and therefore directly delivered to memory cells, the situation is different, in that the memory CTLp are activated early and do not need to migrate through tissues.

The difference between the usually occurring peripheral route of infection and the i.v. route, which is found only rarely (e.g., arboviruses), can be dramatically illustrated in LCMV-specific TCR-transgenic mice (29). High frequencies of nonactivated pCTL that do not migrate through tissues did not protect such mice against i.c. challenge infection with vacc-LCMV-G (Table 2). These mice also died after i.c. LCMV infection and were not protected against LCMV infection into footpads (29). Thus, high frequencies of nonactivated CTLp do not protect against peripheral reinfection. In contrast, after i.v. challenge infection unprimed TCR-transgenic mice can control LCMV replication in the spleen more rapidly than unprimed nontransgenic mice (29).

The *in vivo* assay recently presented to detect antiviral immunity in antigen-free recipient mice where memory CTLp remained at high frequencies after transfer (7), is exceptional in the above discussed respects: recipient mice were sometimes irradiated before transfer and then challenged i.v. with LCMV to measure virus clearance from spleen after 8 days. (*i*) Because of the intravenous route of infection, CTLp recirculation was not necessary. (*ii*) CTLp were given an exceptionally

Table 2. Role of T-cell frequency and activation in peripheral and i.v. infection

Exp. group	Mice*	Immunization†	Status of specific CD8+cell: Frequency‡	Status of specific CD8+cell: Activation§	Vacc-LCMV-G titers in brains,¶ $\log_{10}$ pfu/g	LCMV infection: i.c. survival‖	LCMV infection: i.v. $\log_{10}$ pfu/spleen**
A	C57BL/6	None	($<10^{-6}$)	None	4.75 ± 0.3		
B	TCR-tg+	None	(10^{-1})	None	5.32 ± 0.2		
C	C57BL/6	Peptide-24h	($<10^{-6}$)	Yes	5.02 ± 0.2		
D	TCR-tg+	Peptide-24h	(10^{-1})	Yes	<1.7		
E	C57BL/6	LCMV-14d	(10^{-3})	Yes	<1.7		
F	TCR-tg+	LCMV-14d	(10^{-1})	Yes	<1.7		
G	C57BL/6	None	($<10^{-6}$)	None		0/5	4.23 ± 0.4
H	TCR-tg+	None	(10^{-1})	None		0/5	<0.7
I	C57BL/6	Vacc-LCMV-G	($<10^{-6}$)	Yes		0/5	ND
J	TCR-tg+	Vacc-LCMV-G	(10^{-1})	Yes		5/5	ND
K	C57BL/6	LCMV-14d	(10^{-3})	Yes		5/5	<0.7
L	TCR-tg+	LCMV-14d	(10^{-1})	Yes		5/5	<0.7

*Mice used were either C57BL/6 or TCR transgenic mice (TCR-tg+) recognizing the glycoprotein of LCMV in the context of D^b.

†LCMV-specific T cells were activated in experiment groups C and D by giving mice 500 μg of LCMV glycoprotein peptide (aa 33–41) in saline i.p. 24 h before challenge infection with vacc-LCMV-glycoprotein recombinant virus (vacc-LCMV-G). In experiment groups E, F, K, and I mice were immunized i.v. with LCMV (2000 pfu strain Armstrong) 14 days prior to the challenge infection with vacc-LCMV-G. In experiment groups I and J mice were immunized with vacc-LCMV-G (2×10^6 pfu i.v.) 2 days before challenge infection.

‡Frequency of LCMV-specific CD8+ T cells in spleens of mice. The detection level of limiting dilution analysis is 10^{-6}. Because ≈70% of all CD8+ T cells in the TCR-tg+ mice are LCMV specific, the frequency among spleen cells is around 10^{-1}.

§Activation status of LCMV-specific CD8+ T cells due to immunization of mice.

¶All mice were challenged i.c. with vacc-LCMV-G (1×10^3 pfu in 30 μl). Three days after this challenge infection brains were removed and vacc-LCMV-G titers determined in individual mice ($n = 4 - 6$, mean ± SD).

‖All mice were challenged i.c. with LCMV (100 pfu strain Armstrong). Within 10 days after challenge infection mice from groups G, H, and I had all succumbed to lethal choriomeningitis, whereas mice from groups J, K, and L survived without any clinical signs of disease.

**Mice were challenged i.v. with LCMV (2000 pfu strain Armstrong). Five days after this challenge infection LCMV titers were determined in spleens (5 mice per group, mean ± SD).

long time (8 days) to clear the virus, so that initial activation was not necessary. (*iii*) These experiments used a very high dose of virus (10^6 pfu) to be cleared by day 8, which is feasible only if a noncytopathic virus such as LCMV is used (17); a cytopathic virus would have to be cleared much earlier for the mouse to survive.

The notion that protective T-cell memory is antigen-dependent explains several earlier experimental and clinical observations. (*i*) Injection of tuberculin elicits a DTH reaction in vaccinated or tuberculosis-infected patients. Similar skin tests exist for many pathogens, such as brucellosis, leprosy, and histoplasmosis (1). Apparently DTH is found only for pathogens that induce granulomas, which are antigen depots remaining in the host for years. Similarly, protein–antigens induce only short-lived DTH (30), unless given in Freund's adjuvant (31), which also forms granulomas. (*ii*) After adoptive transfer, i.e., after removal of antigen, T-cell function disappears in the recipient. Therefore, recipient mice are usually irradiated (11). We hypothesize that irradiation causes activation of the transferred memory T cells similar to what persisting antigen usually does. (*iii*) Only CD4+ T cells isolated from lymph nodes draining the site of immunization—but not T cells from distant lymph nodes—proliferate to antigen (32). This accumulation of memory T cells lasts for months (33). (*iv*) Some studies have reported short-lived T-helper cell memory, e.g., against VSV (34), influenza (35), and Listeria (36, 37). Short-lived CTL memory was demonstrated in an early study where vaccination protected children against skin reinfection with vaccinia virus only for 4 weeks (38). Interestingly, vaccinia-specific CTLp frequencies remain high for years (39, 40). A similar situation with short-lived *in vivo* memory vs. long-lived *in vitro* memory may also exist in the influenza system (41, 42). (*v*) Peptides were so far reported to induce T cells (43, 44) only with adjuvant or coupled with a lipophilic tail (45). This probably reflects longer peptide persistence *in vivo*. Peptide in saline induces only short-lived responses that are easily missed (Fig. 2). (*vi*) Early memory B and T cells proliferate but, after several weeks, become resting cells (14, 46). Apparently persisting antigen drives this proliferation and keeps CTLp at the level of activation necessary for antiviral immunity. (*vii*) A study on anti-tumor immunity recently found that in mice immunized with tumor cells anti-tumor protection correlated with the persistence of dormant tumor cells in the bone marrow (47). Similar evidence has been introduced recently also for immunity against Leishmania in mice (48).

Migration Pathways of Memory T Cells. Naive T cells actively extravasate from blood to lymph nodes via high endothelial venules, whereas memory T cells are thought to extravasate into tissue and reach lymph nodes via afferent lymph (49). After disappearance of antigenic stimulation, CTLp appear to no longer extravasate into tissue nor reach the lymph nodes via high endothelial venules. Similar to Mel-14-deficient mice, T cells then accumulate in the spleen (50). Few other reports suggest accumulation of long-term memory T cells in the spleen (51), and an early study demonstrated that Listeria monocytogenes-specific T cells stop recirculating through the thoracic duct in antigen-free recipient mice (52). Earlier studies also demonstrated the existence of "residential" memory cells (53, 54).

How May Antigen Persist? In a search for persisting antigen we detected VSV-RNA only during the first 2 days after infection. Later, reverse transcription followed by nested PCR was negative in all organs tested (<40 RNA copies/100 μg organ). Because minute amounts of peptides are sufficient to stimulate T cells (55), a biochemical demonstration of persisting viral antigen in the form of peptide was technically not feasible. Antigen persistence could be assessed by using professional antigen presenting cells from memory mice as stimulators of primed, cloned, or of hybridoma T cells specific for the virus peptide under investigation. Although such tests were found to be positive with antigen-presenting cells from mice immunized up to 14 days before, antigen-presenting cells taken 3–4 weeks after immunization did not yield measurable restimulation (not shown). These findings suggest that either the amount of persisting antigen was too small and/or that the disruption of the anatomical structure of the lymphoid organ

had destroyed the niche where restimulation normally occurred *in vivo*.

The most likely sources of antigen-maintaining long-term antiviral immunity, observed after infections with many natural pathogens, are persistent infection, recurrent infections from hidden sites, or reinfection. In mice, LCMV tends to persist (17). In humans, several examples of persistent virus infections exist, such as Varicella–Zoster–Virus, herpes simplex virus, and Measles virus (56).

Because MHC-restricted T-cell recognition is paralleled by alloreactivity, T cells differentiate late during pregnancy and mature slowly after birth. Therefore, maternal memory T cells cannot be transmitted to offspring. From this point of view memory T cells are mostly important to the individual to control persistent virus infection, whereas vertically transmissible antibodies protect offspring during the early immunodeficiency phase. Interestingly, maintenance of protective antibody levels is also antigen-dependent (5, 57).

In conclusion, these experiments explain several controversies about the nature of cytotoxic T-cell memory, as discussed recently in a broader context (57–60). After immunization, persistence of memory T cells as determined *in vitro* appeared not to depend on antigen persistence. However, *in vivo* protective T-cell memory, which is relevant both in evolution and medicine, depended on persistent antigenic activation of these memory cells.

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