CD8⁺ T Cells Complement Antibodies in Protecting against Yellow Fever Virus

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The attenuated yellow fever (YF) vaccine (YF-17D) was developed in the 1930s, yet little is known about the protective mechanisms underlying its efficiency. In this study, we analyzed the relative contribution of cell-mediated and humoral immunity to the vaccine-induced protection in a murine model of YF-17D infection. Using different strains of knockout mice, we found that CD4+ T cells, B cells, and Abs are required for full clinical protection of vaccinated mice, whereas CD8+ T cells are dispensable for long-term survival after intracerebral challenge. However, by analyzing the immune response inside the infected CNS, we observed an accelerated T cell influx into the brain after intracerebral challenge of vaccinated mice, and this T cell recruitment correlated with improved virus control in the brain. Using mice deficient in B cells we found that, in the absence of Abs, YF vaccination can still induce some antiviral protection, and in vivo depletion of CD8+ T cells from these animals revealed a pivotal role for CD8+ T cells in controlling virus replication in the absence of a humoral response. Finally, we demonstrated that effector CD8+ T cells also contribute to viral control in the presence of circulating YF-specific Abs. To our knowledge, this is the first time that YF-specific CD8+ T cells have been demonstrated to possess antiviral activity in vivo. The Journal of Immunology, 2015, 194: 000-000.
arm of the adaptive immune response when it comes to prevent-
ning a lethal outcome of YF infection, effector CD8+ T cells also
significantly contribute to viral control in the brain, which is the
decisive site for virus replication in this model system. Collect-
ively, our data demonstrate for the first time, to our knowledge,
that CD8+ T cells may play an important role in controlling in-
fec tion with YF virus.

Materials and Methods

Mice
Female C57BL/6 (wt B6) mice, as well as MHC class I- (MHC-I; β2
microglobulin−/−) and MHC-II (Aα−/−)–deficient mice on a B6 back-
ground, were obtained from Taconic Farms. B cell–deficient (µMT) mice were obtained directly from The Jackson Laboratory (Bar Harbor,
ME) or the local progeny of such animals. Perforin (Pfr)-deficient and IFN-
γ-deficient mice were originally obtained from The Jackson Laboratory,
and IFN-γ/Pfr double-deficient (IFN-γ/Pfr) mice were generated locally
by intercrossing of these strains, as previously described (15). Rag
−/−–deficient mice (B6.129S7-Rag1−/−[B6J]) and CD40L-deficient mice (B6.129P2-Cd40−/−[B6J]) and CXCR5-deficient mice (B6.129S2(Cg)-
Cxcr5tm10mpJ) came directly from The Jackson Laboratory. All mice used in
this study were 7–10 wk old and housed in a specific pathogen-free
facility. All experiments were approved by the national animal ethics
committee and performed in accordance with national guidelines.

Virus preparation and quantitation

YF-17D virus (Stamaril, Sanofi Pasteur; reconstituted as recommended
by the manufacturer) was propagated in Vero cells (ATCC CCL-81) grown
in DMEM containing 10% FCS, glutamine, and antibiotics (penicillin and
streptomycin). Cell monolayers were seeded 24 h earlier and infected with
virus at a multiplicity of infection of 0.001 in DMEM 2% FCS; infectious
supernatants were harvested when cytopathic effect was >60% (day 6
postinfection [p.i.], freeze-thawed, and clarified of cell debris at 2000 rpm
for 15 min at 4˚C. Viral stocks were stored as single-use aliquots at
−80˚C. An immune focus assay (IFA) was developed to quantitate infectious virus.

In brief, virus stocks were serially diluted (10-fold) in DMEM and ad-
sorbed for 1 h at 37˚C onto Vero cell monolayers in 24-well plates; cells
were then overlaid with media containing 0.9% methyl cellulose and in-
cubated at 37˚C for 3 d. After fixation and permeabilization, a mAb made
against NS3 from YF-17D virus was used for detection of virus Ags within
infected cells; foci of infection (analogous to plaques) were visualized
using a HRP-conjugated secondary Ab and counted. When virus titer in
the brain was determined, the IFA was performed on homogenized and
clarified 10% organ suspensions. The detection limit for virus in organs
was 200 PFU/g organ.

Neutralization of YF-17D virus by sera from vaccinated mice

Appropriately diluted YF-17D virus (resulting in ~70–100 PFU/well) was
incubated for 1 h at 37˚C with serial (2-fold) dilutions of mouse sera
obtained from individual YF-vaccinated mice. The virus-serum mixture
was subsequently added to Vero cell monolayers in 24-well plates and incubated for 2 h at 37˚C, after which the overlay media containing 0.9%
methyl cellulose was added. After 3 d of incubation at 37˚C, the overlay
media was removed and, after fixation and permeabilization, cells were
stained as described earlier (IFA). After counting, the neutralizing Ab titers
were defined as the highest serum dilution neutralizing >50% of the viral pla-
qued, was determined.

Vaccination and challenge

Mice were vaccinated by either i.v. or s.c. injection of YF-17D virus using
a dose of 105 PFU in 300 µL. For virus challenge, deeply anesthetized mice
were injected intracerebrally (i.c.) with 105 PFU YF-17D virus in a volume of
30 µL. In clinical protection studies, the mice were checked at least twice
a day for 2 wk after i.c. challenge and euthanized when irreversible signs of
illness (body weight drop, ruffled fur, twitching) together with neurologic
signs (hind-leg paralysis and failure to get into an upright position within 10 s
after being tilted) were observed. Mice developing sickness within the first 4
day postchallenge (p.c.) were excluded from the experiments.

Serum transfer and adoptive transfer of splenocytes

Serum was harvested from C57BL/6 mice vaccinated with 105 PFU YF-
17D virus i.v. 28 days earlier, and each recipient received a dose of 1 ml serum,
equally distributed between the i.v. and the i.p. route on day −3 and
additionally 0.3 ml i.v. on day −1 relative to virus challenge. Normal
mouse serum was used for control.

For splenocyte preparation, spleens were collected from donor mice
primed with 105 PFU YF-17D 28 d earlier and transferred to HBSS.
A single-cell suspension was obtained by gently pressing the spleens through
a nylon mesh (mesh size, 70 µm), followed by centrifugation and two washes
in HBSS after which 7 × 106 cells were injected into recipient mice i.v.; YF-
immune splenocytes (or naive splenocytes) were transferred to recipient
animals 2 d before i.c. challenge.

Flow cytometry

Single-cell suspensions of splenocytes were prepared as mentioned earlier.

Frequencies of Ag-specific CD8+ T cells in the spleen were determined by
intracellular cytokine staining performed after 5 h of incubation with
relevant peptides (0.1 µg/ml NS3, 268–275 or E, 4–12) (16) in the presence of
monensin (3 µM) at 37˚C in 5% CO2. After incubation, cells were stained
with Abs for cell-surface markers using PerCP-Cy5.5-CD8 and allophy-
cocyanin-Cy7-CD44. Subsequently, the cells were washed, permeabilized,
and stained for intracellular cytokine using allophycocyanin–IFN-γ.

FlowJo software (TreeStar).

Immunohistochemistry for CD4+ and CD8+ T cells

Brains were removed from deeply anesthetized and exsanguinated mice,
flushed in liquid nitrogen, and sectioned in a cryostat. Parallel brain sections
(20 µm thick) were fixed in 4% paraformaldehyde for 4 h and wash-
ed in PBS with 0.25% Triton X-100. After permeabilization, the sections
were incubated with biotinylated goat anti-rat IgG (50 µg/ml; BA9400; Vector Laborato-
ries) and HRP-conjugated streptavidin (1:300; RPN1231V; GE Health-
care), respectively, diluted in 10% FCS in PBS buffer. After washing, the
sections were counterstained with 1.9% (w/v) crystal violet. Immunohis-
cology for CD4+ and CD8+ T cells

Isolation of total RNA for quantitative PCR

Total RNA was extracted from the homogenized brains using the RNeasy midi kit
(Qiagen). For the qPCR components included Brilliant II qPCR Mastermix,
used according to the manufacturer’s instructions (Stratagene, AH Diag-
óstics). The qPCR components included Brilliant II qPCR Mastermix,
milliQ water, reverse-transcribed cDNA, and the forward and reverse
target gene primers (see Table I for primer sequences). Target gene ex-
pression was normalized to expression of the reference gene PBGD
(17). In brief, brains were removed from deeply anesthetized mice after
incubation overnight at 4˚C with purified rat anti-mouse IgG2a (2.5 µg/ml;
BD Pharmingen) or purified rat anti-mouse CD8a IgG (7 µg/ml; MCA1108GT;
AbD Serotec) diluted in 10% FCS in PBS buffer. After a rinse in PBS+
endogenous peroxidase activity was blocked with 1.9%

H2O2 in PBS, and sections were treated with 3,3-diaminobenzidine tetra-
ydrochloride and 0.003% H2O2 in PBS. Finally, sections were covered-
slipped with Depex.

Detection of mRNA in the brain by quantitative PCR

Brains from deeply anesthetized and exsanguinated mice were immediately
removed, snap-frozen in liquid nitrogen, and stored at −80˚C. Total RNA
was extracted from the homogenized brains using the RNasey midi kit
(Qiagen).
cycles of denaturation at 95°C (30 s), annealing at 60°C (60 s), and extension at 72°C (60 s). Each reaction was run in triplicate plus a no-template control and a no RT control. Results were analyzed using Mx3005P system software. The relative expression ratio (R) in each sample was calculated using a mathematical model based on the amplification efficiency (18):

\[
R = \left( \frac{E_{\text{target}}}{E_{\text{reference}}} \right)^\frac{\Delta C_P(\text{control-sample})}{\Delta C_P(\text{control-sample})}
\]

An amplification efficiency (E) of 100% corresponds to a doubling of the PCR product for every cycle. E is calculated from the slope of a standard curve based on 5-fold dilutions for each primer pair used (E = 10^(-slope)).

**Statistical evaluation**

A nonparametric Mann–Whitney U test was used to compare quantitative data (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 unless otherwise stated). GraphPad Prism (version 6) software was used for statistical analysis.

**Results**

*B cells are pivotal in the adaptive immune response to YF-17D vaccination, but other effector mechanisms mediate partial protection in B cell–deficient mice*

Mice are not susceptible to peripheral infection with YF-17D. However, it is possible to induce a lethal CNS disease by i.c. inoculation of the virus (20–22). In agreement with previous studies, we found that infection of wt B6 mice with 10^5 PFU YF-17D virus via the i.v. route or the s.c. route results in subclinical infection. In a preliminary evaluation of acute i.c. challenge of wt B6 mice with 10^4 PFU YF-17D virus, we noted initial signs of illness around days 6–7 p.c., progressing to severe lethal disease by days 8–9 p.c. in all the tested animals, demonstrating the stringency of this challenge model. Notably, when B6 mice were vaccinated with 10^7 PFU YF-17D virus i.v. or s.c. 4 wk before i.c. challenge, 100% protection was observed, demonstrating that YF-17D virus acts as a protective vaccine in mice and in humans (Fig. 1A). This allowed us to carry out vaccination-challenge experiments with the aim to investigate the requirement for specific cell subsets and effector molecules in mounting a protective immune response after YF-17D vaccination in mice.

To this end, we tested several strains of genetically modified mice with targeted immune defects. A complete vaccine failure (i.e., no survival when i.v. vaccinated animals were subsequently challenged i.c.) was observed in Rag1-deficient mice (Fig. 1B). This demonstrates not only the requirement for T cells and/or B cells in the vaccine-induced protection, but also suggests that, despite its attenuation, YF-17D is likely to be a cytolytic virus causing direct cell damage, although activation of innate immune mechanisms cannot be ruled out. Interestingly, even in the absence of B and T cells (Rag1 knockout [KO]), primary i.v. infection with YF-17D was still subclinical, which given the apparently cytolytic nature and replicative capacity of YF-17D virus, strongly supports the idea that virus control and clearance after peripheral infection/vaccination can be accomplished entirely by innate host mechanisms (23, 24).

When analyzing μMT mice lacking B cells, we found that, although B cell deficiency resulted in a high mortality, some vaccinated μMT mice could still mount a protective immune response and survive long term after i.c. viral challenge (23% survival; Fig. 1C). Given the pivotal role of effector CD8^+ T cells in the control of many other acute murine and human viral infections, we speculated that the protection observed in the absence of Ab responses might be mediated by this cell subset.

Several years ago, van der Most et al. (16) identified two YF virus–derived, MHC-I–restricted T cell epitopes in B6 mice: a dominant H-2K^b–restricted epitope mapped to the NS3 protein and an H-2D^b–restricted subdominant epitope found in the viral E protein. Consequently, we studied the CD8^+ T cell responses specific for these two murine epitopes in the spleen of B cell-deficient mice that had survived an i.c. viral challenge, and compared our findings with those in surviving wt B6 mice. Notably, B cell–deficient mice displayed an increased YF-specific CD8^+ T cell response compared with matched wt mice, as demonstrated by a higher frequency of IFN-γ-producing CD4^+ T cells responding to stimulation with both peptides (6.35 versus 2.16% for the NS3 epitope; 6.1 versus 1.69% for the E epitope) at day 15 p.c. (Fig. 1D). This was not due to an intrinsically increased capacity of B cell–deficient mice to generate an augmented CD8^+ T cell response, as we have previously observed that primary epitope-specific CD8^+ T cell responses in these mice tend to be of a lesser magnitude compared with those mounted in wt B6 mice (25, 26, and data not shown). These findings suggest that B cells are crucial for the YF-induced immune response in wt mice; however, in the absence of B cells and/or Abs, other effector mechanisms, likely involving CD8^+ T cells, may mediate a partial protection.

**CD8^+ T cell responses appear dispensable for long-term survival in vivo**

The importance of T cells in the immune response to viral infections is well documented; in particular, CD8^+ T cells synthesize a variety of effector molecules that may contribute to resistance against intracellular pathogens. Prf1 is a key effector molecule for CD8^+ T cell–mediated cytolysis of virus-infected cells; effector CD8^+ T cells can also release IFN-γ, among other cytokines, which can inhibit viral replication directly and can act as an immunomodulatory and immunostimulatory entity.
As a first approach to study the contribution of CD8⁺ T cell responses in the YF infection model, we tested MHC-I-deficient mice (β₂ microglobulin−/−) for their ability to mount a protective immune response after vaccination. We found that absence of CD8⁺ T cells did not impair vaccine-induced protection; thus, we observed no mortality after i.c. challenge of previously vaccinated MHC-I KO mice (Table II). Next, we analyzed vaccine-induced protection in mice with targeted deficiencies in IFN-γ and prf; similarly to MHC-I-deficient mice, both IFN-γ KO mice and prf KO mice displayed 100% survival rate after vaccination (Table II). Only the combined deficiency of these molecules (IFN-γ/prf double KO) resulted in a very modest impairment in vaccine-induced protection (20% mortality rate; Table II). These data suggest that, in otherwise immunologically intact mice, CD8⁺ T cell responses are not essential for clinical protection after vaccination with YF-17D in mice.

CD4⁺ T cells and CD40L are required for protection against a lethal outcome of YF-17D i.c. infection in previously vaccinated mice

CD4⁺ T cells can contribute to the immune response to a viral infection in very different ways. Ag-specific CD4⁺ T cells can migrate to sites of infection and carry out direct effector functions, or they can persist in secondary lymphoid organs and provide help for expansion and differentiation of other effector cells. The role of Th cells in the formation of high-affinity neutralizing Ab responses by B cells is well established, and follicular helper CD4⁺ T cells have
A pivotal role for Abs in the immune response to YF has been identified as the key CD4+ subset that mediates this function and promotes germinal center reactions (27). Expression of CXCR5 is one of the defining hallmarks of T follicular helper cells (28). CD40–CD40L interaction is also an essential signal for B cell activation, germinal center formation and differentiation, and isotype switching to IgG molecules. Having established a critical role for B cell responses in YF-17D–induced resistance to lethal outcome of i.c. challenge, we wanted to investigate the requirement for CD4+ T cell help in the observed protection.

Using mice deficient in MHC-II, we could demonstrate that CD4+ T cells were critical for the vaccine-induced protective response, as none of the vaccinated mice survived subsequent viral challenge (100% mortality rate; Table III). Similarly, lack of CD40L expression completely abolished vaccine-induced protection (Table III), suggesting that production of IgG is essential for long-term protection. We also sought to determine whether CXCR5+ T follicular helper cells would be critical for promoting YF-specific B cell responses. Surprisingly, lack of CXCR5 only very modestly reduced the overall survival after i.c. challenge of YF-vaccinated mice; mortality rate in CXCR5 KO mice was only 15% (Table III). Assuming a key role for Abs in vaccine-induced protection, this finding suggests that sufficient neutralizing Abs may be produced even without substantial germinal center formation (29).

Transfer of YF immune serum or YF-primed splenocytes confers protection from YF-17D challenge

The strikingly impaired protective immunity of YF-vaccinated B cell KO mice, CD40L KO mice, and MHC-II KO mice pointed toward a major role for humoral immunity in the vaccine-induced protective response. However, these findings did not unequivocally prove that Abs were the major effector molecules in mediating survival, because all of the earlier KO strains have been previously characterized as carrying defects in CD8+ T cells and thus were unable to clear virus (29). To address this issue, we performed adoptive transfer experiments in which YF-primed splenocytes were transferred to naive recipients before i.c. challenge. Spleen cells were removed from vaccinated donor mice at day 28 p.i. and injected i.v. into naive recipients (7 × 10^7/mouse); recipients were then challenged with 10^5 PFU YF-17D virus i.c. (Fig. 3B). Interestingly, we observed that transfer of primed splenocytes resulted in 70% protection from i.c. challenge, whereas no protection was observed in the groups that received either a sham injection (PBS) or naive splenocytes (Fig. 3B). Together with earlier observations in vaccinated B cell–deficient mice, which did not all succumb to i.c. challenge, these data could point to a role for cellular immunity in the protective immune response to YF-17D vaccine in mice.

Viral replication in the brains of nonimmunized and immunized wt B6 mice after i.c. challenge

Because only i.c. infected animals die of YF infection, virus replication in the brain appear to be essential, and for that reason we wanted to study virus titers in the brains of wt B6 mice at different time points after i.c. inoculation. This was done to address two issues: 1) whether the brain was a site of active viral replication after i.c. injection, and if so, what was its time course; and 2) whether prior vaccination would lead to virus control and clearance, as suggested from the lack of clinical disease in previously vaccinated animals. To this end, brains of naive mice and mice immunized 28 d before challenge were removed at days 3, 5, and 7 after infection.

### Table III. CD4+ T cell contribution to YF-17D virus–induced protection

<table>
<thead>
<tr>
<th>Strain</th>
<th>i.v. % Mortality (Dead/Total)^a</th>
<th>i.c. % Mortality (Dead/Total)^b</th>
<th>i.v. before i.c. % Mortality (Dead/Total)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (B6)</td>
<td>0% (0/15)</td>
<td>100% (10/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>MHC-II KO</td>
<td>0% (0/15)</td>
<td>100% (5/5)</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td>CD40L KO</td>
<td>0% (0/15)</td>
<td>100% (5/5)</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td>CXCR5 KO</td>
<td>0% (0/15)</td>
<td>ND</td>
<td>0% (0/15)</td>
</tr>
</tbody>
</table>

Percent mortality of WT B6 mice, MHC-II KO mice, CD40L KO mice, and CXCR5 KO mice p.i. with YF-17D virus via different routes.

^aMice were infected with 10^7 PFU YF-17D virus i.v.
^bMice were infected with 10^5 PFU YF-17D virus i.c.
^cMice were infected i.v. with 10^7 PFU YF-17D virus and 28 d later challenged i.c. with 10^5 PFU YF-17D virus.
after i.c. challenge (Fig. 4); day 7 represents the latest time point for an unbiased analysis of acutely challenged mice that would invariably succumb between days 8 and 9 p.c. In the unvaccinated group, increasing virus titers were observed over time, with all the animals uniformly displaying high viral loads (10^6–10^7 PFU/g organ) on day 7 p.c. (Fig. 4). In contrast, only transient replication was observed in vaccinated mice, and infectious virus was undetectable in the CNS by day 7 p.c. (Fig. 4). These results indicated that the brain is a site of active viral replication after i.c. challenge with YF-17D virus, and that vaccination leads to improved virus control in the brain. Notably, the presence of transient low-grade viral replication in the vaccinated group suggests that the i.c. viral inoculum is not completely neutralized by pre-existing YF-specific Abs. This observation is suggestive of a more complex mechanism of protection than the mere presence of YF-neutralizing Abs in the brain, requiring effectors capable of controlling virus in infected host cells.

**Detection of T cells in virus-infected brains: vaccination induces an accelerated recruitment of T cells into the CNS after i.c. challenge**

Infiltration of CD4^+ and CD8^+ T cells into the CNS has been observed in several models of flavivirus-mediated encephalitis in rodents (34–36). Furthermore, CD19^+ B cells can also traffic to the virus-infected CNS, where both IgM-producing extrafollicular plasmablasts and IgG-producing germinal center plasma cells can be found (37, 38).

To test the hypothesis that i.c. infection of mice with YF virus would result in T cell influx into the brain, we first performed a comparative histological analysis of sham-inoculated mice and mice infected i.c. with YF virus 7 d earlier (Fig. 5). As expected, cerebral injection of PBS did not result in migration of CD4^+ or CD8^+ cells into the brain (Fig. 5A, 5F), except at the injection site (data not shown). In contrast, multiple CD4^+ cells were observed in the meningeal membrane (Fig. 5B, 5C) and the vascular and perivascular spaces (Fig. 5B, 5D) at day 7 post YF infection. CD4^+ cells were also abundant in the brain parenchyma as shown for the hippocampal formation (Fig. 5B) and neocortex (Fig. 5B, 5E). Similarly, CD8^+ cells had also infiltrated the meningeal membrane (Fig. 5G, 5H), corpus callosum (Fig. 5G, 5I), external capsule (Fig. 5I), and fimbria (Fig. 5I, 5J) at day 7 post YF infection. The presence of both CD4^+ and CD8^+ T cells in the YF-infected brain was confirmed by flow-cytometric analysis of mononuclear cells isolated from the perfused brains of day 7 infected mice (Fig. 6).
To address the kinetics of lymphocyte infiltration and the role of prior immunization in this context, we used qPCR-based analysis of the CD4, CD8, and CD19 mRNA expression levels in the YF-infected brains (Fig. 7). WT B6 mice that were either naive or had previously received YF immunization were challenged i.c. with YF-17D virus, and on days 3, 5, and 7 p.c., brains were collected for analysis; mice receiving an i.c. injection with PBS served as controls. We observed a significantly higher expression of CD8 mRNA at days 3 and 5 p.c. in the brains of previously immunized mice compared with unvaccinated animals, whereas expression levels became similar in the two groups by day 7 p.c. (Fig. 7). The expression of mRNA for CD4 also significantly increased as the infection progressed, but no statistically significant difference between vaccinated and unvaccinated mice was observed at any time point. In contrast, within the time frame studied in this article, we found no evidence of B cell infiltration into the YF-infected CNS, because CD19 mRNA expression levels in both vaccinated and unvaccinated animals was similar to baseline as observed in the control group (data not shown).

Finally, to confirm that prior vaccination causes an accelerated recruitment of T cells to the CNS including Ag-specific CD8+ T cells, we studied the numbers and composition of mononuclear cells isolated from the perfused brains of day 5 infected mice (Fig. 8). As expected, our results revealed increased numbers of both CD4+ and CD8+ T cells in the CNS of vaccinated mice, and the infiltrating CD8+ T cells contained large numbers of YF-specific CD8+ T cells (Fig. 8).
YF-17D–induced protection in B cell KO mice is mediated by CD8+ T cells

The relative contribution of T cells to the YF-17D–induced protective response may be concealed by the presence of YF-specific Abs in vaccinated wt B6 mice. Therefore, we decided first to compare the vaccine-induced virus control in brains of immunized B cell–deficient mice that were or were not in vivo depleted of CD8+ or CD4+ T cells before i.c. viral challenge; nonimmunized animals were included for control (Fig. 9). Mice were vaccinated s.c. with 10^5 PFU YF-17D virus and challenged i.c. 28 d after priming; 1 d before challenge, the animals to be depleted were injected i.p. with anti-CD8 or anti-CD4 mAbs, and this treatment was repeated on days 1 and 4 p.c. At day 7 p.c., mice were euthanized, and virus titers in the brains were determined. Intact vaccinated mice displayed a significantly lower viral load compared with unvaccinated mice (Fig. 9). Interestingly, we also noticed that the vaccinated group contained animals displaying both low and high viral loads; this is consistent with our previous finding that vaccination of B cell–deficient mice resulted in survival rate of ∼25% of the animals tested (Fig. 1C). Strikingly, depletion of CD8+ T cells from immunized B cell–deficient mice uniformly abolished the vaccine-induced virus control, because viral loads in the CNS of these animals were similar to those of unvaccinated mice (Fig. 9). In contrast, when CD4+ T cells were depleted, vaccinated B cell–deficient mice were still able to control the infection, because viral titers in the brains of these animals

YF-17D–infected brains detected by flow cytometry. Lymphocytes were isolated from brains of naive mice and of YF-17D i.c. infected WT mice at day 7 p.i.; FACS analysis revealed the presence of CD4+ and CD8+ cells within the infiltrated population. Dot plots, gated on FSC/SSC as indicated, are representative of cells pooled from three mice. All CD4+ and CD8+ cells were confirmed to be CD3+CD45^highCD44^high (data not shown).

FIGURE 6. Infiltration of T cells into YF-17D–infected brains detected by flow cytometry. Lymphocytes were isolated from brains of naive mice and of YF-17D i.c. infected WT mice at day 7 p.i.; FACS analysis revealed the presence of CD4+ and CD8+ cells within the infiltrated population. Dot plots, gated on FSC/SSC as indicated, are representative of cells pooled from three mice. All CD4+ and CD8+ cells were confirmed to be CD3+CD45^highCD44^high (data not shown).

FIGURE 7. Expression levels of CD8 and CD4 mRNA in YF-17D–infected brains. mRNA expression levels in the brains of vaccinated (■) and nonvaccinated (○) WT B6 mice were compared at days 3, 5, and 7 after i.c. challenge. The mRNA level in the brain of an immunized mouse at day 7 p.c. is set to R = 1. mRNA levels in the brains of mice that had received a sham (PBS) i.c. injection (×) were used to determine the baseline mRNA expression for each molecule (dashed lines). Each dot represents an individual animal.
were significantly lower than those observed in unvaccinated animals and in vaccinated/CD8-depleted mice (Fig. 9). However, it should also be noted that none of the CD4-depleted mice matched the most efficient virus controllers found in the group of undepleted animals, suggesting that in the absence of Abs, not only CD8+ T cells, but also CD4+ T cells, albeit to a lesser extent, might contribute in controlling the virus spread in the CNS.

In vivo depletion of CD8+ T cells from vaccinated wt B6 mice results in impaired virus clearance from the CNS after challenge

The earlier results clearly showed that the YF vaccine–induced protection in B cell–deficient mice relied almost exclusively on CD8+ T cells. In contrast, in the presence of a humoral immune response, CD8+ T cells appeared redundant for long-term survival of immunized mice in vivo (cf. Table II). However, the faster recruitment of CD8+ T cells to the CNS of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs. To test this hypothesis, we analyzed the virus control in brains of immunized wt mice p.c. suggested to us that this subset of lymphocytes might also carry out antiviral effector functions in the presence of YF-specific Abs.

To address the antiviral effector mechanism(s) underlying cell-mediated immunity to YF, we analyzed the virus titers in brains of immunized wt, IFN-γ KO, prf KO, and IFN-γ/prf double-KO mice challenged i.c. 28 d after vaccination with 10^5 PFU YF-17D; strain-matched unvaccinated mice were included as controls (Fig. 10B). Expanding on the results regarding survival of vaccinated, i.c. challenged immunodeficient mice (see Table II), we observed that in the absence of IFN-γ or Prf, vaccination still resulted in highly efficient control of virus replication in the CNS. In contrast, in vaccinated mice lacking both effector molecules, we observed that all but one mouse contained high amounts of virus in the CNS after i.c. challenge (Fig. 10B). Taken together, these findings demonstrate that T cells expressing IFN-γ and/or Prf contribute significantly to protection against infection with YF virus in vivo.

Discussion

The YF-17D vaccine represents a good opportunity to study both the establishment of a protective response after vaccination and the host immune response to an acute viral infection, given the live attenuated nature of the YF-17D virus. The importance of neutralizing Abs in protection from YF has been suggested by earlier
studies (4). Similarly, numbers of circulating CD8+ T cells has also been found to increase as a result of YF vaccination, and for some time, this more or less represented what was known about the vaccine-induced immune response. In recent years, however, several studies have shown that the YF vaccine-induced immune response in humans is also characterized by the presence of a broad and polyfunctional CD8+ T cell response that can generate long-term immunological memory (5, 13, 14). However, until now the importance of this CD8+ T cell response in protection from YF infection has not been clear, and studies on this response in humans is also characterized by the presence of a broad and polyfunctional CD8+ T cell response that can generate long-term immunological memory (5, 13, 14). However, until now the importance of this CD8+ T cell response in protection from YF infection has not been clear, and studies on this

FIGURE 9. CD8+ T cells mediate protection in YF-17D–vaccinated B cell KO mice. Viral titers in the brains of unvaccinated (□) and vaccinated (■) B cell KO mice at day 7 after challenge. Some vaccinated animals were in vivo depleted of CD8+ (anti-CD8) or CD4+ (anti-CD4) T cells 1 d before i.c. challenge, and again at days 1 and 4 p.c. Each dot represents an individual animal. The detection limit for virus in organs was 200 PFU/g organ.

FIGURE 10. Impaired YF-17D virus clearance from the CNS of wt B6 mice in the absence of CD8+ effector T cells. (A) Viral titers in the brains of unvaccinated (□) and vaccinated (■) WT B6 mice at day 7 after challenge. Some vaccinated animals were in vivo depleted of CD8+ (anti-CD8) and/or CD4+ (anti-CD8/CD4; anti-CD4) T cells 1 d before i.c. challenge, and again at days 1 and 4 p.c. (B) WT B6 mice, IFN-γ KO, prf KO, and IFN-γ/prf double-KO mice that were either naive (□) or had been immunized s.c. with 10^5 PFU YF-17D virus 28 d earlier (■) were challenged i.c. with 10^4 PFU YF-17D virus. Viral loads in the brains at day 7 after challenge are compared for unvaccinated and YF-17D–vaccinated mice of each strain. Each dot represents an individual animal. The detection limit for virus in organs was 200 PFU/g organ. *p < 0.05, **p < 0.01, ****p < 0.0001.
question have been hampered by the lack of a small-animal model for YF infection.

In this report, we describe a murine model for YF-17D infection, which allows us to study in detail the generation of a protective immune response after vaccination. This was possible because, as in humans, peripheral infection with YF-17D virus in wt B6 mice resulted in immunity toward a subsequent viral challenge. Interestingly, we observed that, even in the absence of B and T cell responses (Rag1-deficient mice), mice did not succumb to peripheral infection with YF-17D virus, suggesting that effectors of the innate immune system may suffice to contain the limited peripheral infection resulting from even i.v. inoculation of the attenuated virus. This is in agreement with the finding that the vaccine-associated serious adverse events that can be observed in human vaccinees typically do not reflect virus reversion to virulence, but rather seem to be caused by host genetic factors (39, 40), possibly specific deficiencies in innate immune mechanisms.

In our model, we induce a uniformly lethal CNS disease by i.c. administration of YF-17D virus. The relevance of this challenge model compared with natural infection has been debated, and mice lacking both type I and type II IFN signaling pathways have been claimed to represent a better model for human YF infection because of their susceptibility to peripheral challenge with the 17D virus (23, 24). This may hold true if one aims to use YF-17D as a paradigm for wt YF virus, but this model is obviously not suitable for analysis of the vaccine-induced immunity, because no protective response can be mounted in those animals. Moreover, in that model, only a mild transient viral replication in visceral organs was observed, whereas the YF-17D viral burden in the brain increased steadily until the animals succumbed (24), suggesting that, in fact, mortality may be caused by viral replication inside the CNS, similarly to what is the case in our challenge model.

Using a panel of gene-targeted mice lacking selected genes relevant to the immune system, we unequivocally demonstrated the central role of humoral immunity in the vaccine-induced protection; moreover, we corroborated this finding by showing that immunity to YF can be passively transferred through transfusion with YF immune serum, as previously described (21, 41, 42). Notably, we also found that adoptively transferred YF-primed splenocytes could mediate protection, suggesting a functional role for cell-mediated immunity, although the possibility that activated B cells within the splenocyte pool may produce Abs in the recipient mice cannot be ruled out (43). Also supporting a role for T cell–mediated immunity, we observed an accelerated recruitment of both CD8+ T cells and CD4+ T cells to the brain in previously vaccinated mice. The appearance of infiltrating T cells in the CNS of vaccinated mice nicely matched the observed kinetics regarding the reduction in virus content. Thus, the presence of detectable virus in the CNS of most vaccinated mice on days 3 and 5 p.c. followed by complete clearance of infectious virus by day 7 p.c. demonstrate quite clearly that the i.e. inoculum is not completely neutralized by pre-existing Abs, and indicate that additional mechanisms are essential for optimal clearance of this virus.

More directly addressing the contribution of non-B cells to the in vivo protection, we observed that 23% of immunized B cell KO mice survived viral challenge. The presence of some resistance in the absence of B cells/antibodies suggested to us that effector T cells could mediate protection in this setting, and this hypothesis was confirmed by the impaired virus control noted in immunized B cell KO mice that were depleted of CD8+ T cells in vivo. When testing CD40L KO mice in vaccination challenge experiments, we found that protection is completely impaired, first of all suggesting a rather stringent requirement for isotype switching. However, the higher mortality observed in the absence of CD40L, as compared with the lack of B cells, may point to an additional direct role for this molecule in establishing an effective antiviral CD8+ T cell response to YF-17D. This has previously been demonstrated for other murine viral infections (30, 31, 44), as well as in the context of infection with West Nile virus, a related flavivirus, where CD8+ T cell trafficking to the CNS is markedly impaired in the absence of CD40–CD40L interaction (45). However, because CD40L is also expressed by macrophages and NK cells, among other cell subsets, we cannot formally rule out the contribution of additional cell types in influencing the outcome of YF-17D vaccination in CD40L KO mice.

Similar to CD40L-deficient mice, no YF-17D–vaccinated, MHC-II–deficient mice survived viral challenge. Again this could reflect a requirement for CD4+ T cells, either as antiviral effector cells (note that the virus infection causes a higher mortality in vaccinated Prf/IFN-γ virus double-KO mice than in CD8+ T cell–deficient mice) or as helper cells required for the generation and/or maintenance of memory CD8 T cells. Regarding these possibilities, it is of interest to note that depletion of CD4+ T cells immediately before challenge did not impact the vaccine-induced virus control, suggesting that these cells are not essential during the secondary response. In contrast, several studies have shown an accelerated decline of CD8+ T cell memory in the absence of CD4+ T cells (46–50).

Underscoring a role for CD8+ T cells as important mediators of virus clearance in the CNS, we observed that in vivo depletion of CD8+ T cells from vaccinated wt mice resulted in impaired and/or delayed viral clearance from the brain. Regarding the molecular mechanism(s) underlying the virus control exerted by CD8+ T cells, it is interesting to note that neither Prf nor IFN-γ–deficient mice appear to be seriously impaired in their ability to control YF infection of the CNS, at least when Abs are also produced. However, when both these effector mechanisms are deficient, virus control is significantly impaired, suggesting that the effector T cell–dependent virus control may be mediated by several internally redundant mechanisms. However, precisely how these molecular pathways interact and contribute in inhibiting YF virus replication remains to be determined. Indeed, it also remains to be determined precisely how Abs combat YF infection in the CNS of immunized mice after viral challenge; thus, an important question is whether these Abs can perform antiviral functions beyond neutralization of extracellular virions, to eliminate YF-17D virus present within CNS cells.

Interestingly, our observation on the role of CD8+ T cells in the control of virus replication in the YF-infected CNS seemingly contradicts the only previous report investigating the T cell response in the YF-infected murine brain. In the latter study, the authors isolated CD4+ and CD8+ T cells from the brains of immunized, challenged mice and, upon ex vivo stimulation with viral Ag, they found a significant increase in IFN-γ production only in the CD4+ subset (51). However, by using whole Ag for stimulation ex vivo, their analysis was strongly biased for the detection of MHC-II–restricted responses. Furthermore, that study was carried out using the neuroadapted Porterfield strain of YF-17D virus to immunize young mice (4–5 wk old), and that may also account for some discrepancies.

Importantly, our data reveal that the YF-induced CD8+ T cells not only function as a backup defense system when Abs are absent, but they also actively contribute to viral clearance from the CNS of wt mice with a normal humoral response. These findings are consistent with the existing knowledge on the contribution of CD8+ T cells to the recovery from related flaviviral infections in mice. For instance, CD8+ T cells have been demonstrated to have...
an important role in the murine host defense against dengue virus, and peptide immunization targeting dengue virus–specific CD8+ T cells was shown to accelerate viral clearance in infected mice (52). Furthermore, challenge of CD8+ T cell–deficient mice with West Nile virus resulted in increased mortality and virus persistence in the CNS of the surviving mice as compared with wt mice (53), and matching this observation, a recent study on vaccination against this virus revealed a complementary role for CD8+ T cells in protection against CNS disease (54). Moreover, in murine models of Japanese encephalitis virus infection, where humoral immunity has been shown to be sufficient in preventing virus spread to the CNS (55–57), an auxiliary contribution of CD8+ in protection against CNS disease (54). Moreover, in murine models of Japanese encephalitis virus infection, where humoral immunity has been shown to be sufficient in preventing virus spread to the CNS (55–57), an auxiliary contribution of CD8+ T cells has also been disclosed, and adoptive transfer of virus-specific cytotoxic T lymphocytes was shown to confer protection against lethal i.c. viral challenge (58).

In conclusion, our results for the first time, to our knowledge, formally demonstrate that YF-17D–induced CD8+ T cells are functional in vivo and may complement Abs in controlling viral infection. Our findings provide new insights regarding the mode of action of the YF vaccine, and open up the possibility that CD8+ T cell responses and protective immunity against dengue virus are critical for the protection of murine experimental solid tumors and pulmonary metastases. We are extremely grateful to P. Rasmussen, D. Bardenfleth, and B. Jensen for expert technical assistance throughout the study. Sussanne Petersen is acknowledged for technical assistance with regard to mouse brain sectioning. We thank Jason M. Millward for help in setting up the procedure for isolation of mononuclear cells from the CNS.

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References


