Comparison of CD8+ T Cell Responses to Cytomegalovirus between Human Fetuses and Their Transmitter Mothers

Béatrice Pédron, Valérie Guérin, François Jacquemard, Aline Munier, Fernand Daffos, Philippe Thulliez, Yannick Aujard, Dominique Luton, and Ghislaine Sterkers

Laboratory of Immunology, Department of Neonatology, and Department of Maternity, Robert Debré Hospital, and Department of Maternity, Beaujon Hospital, Assistance Publique–Hôpitaux de Paris, and Department of Maternity andLaboratoire de la Toxoplasmose, Institut de Puericulture, Paris, France

Background. The mechanisms responsible for the increased susceptibility of fetuses to cytomegalovirus (CMV) were studied by comparing CD8+ T cell responses to the virus in susceptible fetuses to those in their comparatively more resistant mothers.

Methods. Included in the study were 16 transmitter mothers who underwent seroconversion during the first trimester of pregnancy as well as their fetuses, who were positive for CMV in amniotic fluid by polymerase chain reaction at 17–19 weeks of gestation. Fetal and maternal blood samples were collected between the 22nd and 39th week of gestation. Cytotoxic T lymphocytes (CTLs) that had activated (HLA-DR+), effector/memory (CD28+/H11002), and memory (CD18 high) phenotypes; that stained with the HLA-A2/pp65 or the HLA-B7/pp65 multimer; and that secreted interferon (IFN)–γ were enumerated by flow cytometry. Viral loads were determined simultaneously.

Results. The results showed (1) similar levels of activated, effector/memory, and memory CTLs in fetuses and mothers but a smaller pp65-specific CTL pool in fetuses (median, 0.015% vs. 0.99%; \(p < 0.003\)); (2) similar percentages of CTLs secreting IFN-γ after stimulation with ionomycin/phorbol myristate acetate in fetuses and mothers but lower percentages of CTLs secreting IFN-γ after stimulation with a CD3 monoclonal antibody in fetuses (median, 1% vs. 14%; \(p = 0.01\)); and (3) higher viral loads (mean, 17,290 vs. <250 genome equivalents/mL) in fetuses.

Conclusion. Impaired viral clearance might be related to a defective expansion of the pp65-specific CTL pool and/or to the immaturity of IFN-γ-secreting cells in fetuses.
inal in young children [5]. Yet, human neonates seem competent to mount mature CD8+ T cell responses to CMV and to other intracellular pathogens [6, 7]. These cytotoxic T lymphocyte (CTL) type 1 responses are believed to depend on mature Th1 responses. Thus, the skewing toward Th2 responses observed in animal models might be less obvious in humans. Other studies have concluded that immunodeficiency related to immaturity is the culprit [8–10]. For human CMV specifically, one recent report demonstrated that otherwise immunocompetent young children with postnatally acquired primary CMV infection accumulated markedly fewer CMV-specific CD4+ T cells that produced interferon (IFN)–γ than did adults [10]. In contrast to a limitation in the development of a Th1 response, the CD8+ T cell compartment seemed to be unaltered in this series of infants.

Symptoms are more frequent in congenital CMV infection than in postnatally acquired primary infection, which is usually a clinically silent event. Moreover, sequelae are particularly frequent in fetuses if the virus is transmitted as a result of primary infection during the first trimester of pregnancy. Here, we speculate that the high severity of disease after early CMV transmission occurs because of a pronounced defect in the host response. Two prerequisites are needed for testing this hypothesis. First, a quantitative analysis of immune responses is required. New tools, such as the use of various phenotypic markers, multimeric major histocompatibility complex/peptide staining, and intracellular cytokine assays, now make this possible. Second, this pathological situation must be compared with a physiological one. Congenital infection with CMV, because it is acquired after primary infection of the comparatively resistant mother, provides a unique opportunity to make this comparison. In the present study, therefore, we comparatively examined the magnitude of immune responses to early primary CMV infection in fetuses and their mothers.

Although CD4+ T cell immunity and NK cells play an important role, the CD8+ T cell response appears to be the most important component mediating the elimination of CMV-infected cells [11–13]. On encountering viruses, CD8+ T cells are activated and differentiate from naïve T cells into effector T cells that control virus replication through cytolysis and secretion of specific cytokines and into memory cells that provide enhanced immunity after renewed contact with the same pathogen. To study the development of these CTLs, various phenotypic markers can be used [14, 15]. Here, we used HLA-DR expression on CD8+ T cells as a marker of activation, CD28 down-regulation as a marker of differentiation into effector/memory cells, and CD18high expression as a marker of memory cells. In addition, we used the HLA-A2/NLVPMVATV and HLA-B7/TPRVTGGGAM multimers to quantify the CMV-specific CTL pool. These peptides in the tetrameric protein pp65 of cytomegalovirus are indeed the predominant targets of CTL responses to CMV in both adults [16–21] and children [22]. Finally, we used an intracellular cytokine flow cytometry assay for the enumeration of IFN-γ-secreting CD8+ T cells.

**METHODS**

**Case patients and control subjects.** Case patients consisted of 16 fetuses with congenital CMV infection and their mothers. Inclusion criteria included maternal primary CMV infection before the 12th week of gestation with subsequent primary CMV infection of the fetus. Diagnosis of primary CMV infection in the mother was based on either the demonstration of CMV-specific seroconversion or the presence of CMV-specific IgM and/or low IgG avidity on routine follow-up of CMV-negative pregnant women. Diagnosis of primary CMV infection in the fetus was based on positivity for CMV in amniotic fluid by polymerase chain reaction (PCR) at 17–19 weeks of gestation. Immunological studies were performed on residual blood samples originally obtained for hematological, biochemical, and virological analyses. Samples were collected by cordocentesis (for fetuses) or veinopuncture (for mothers) either between the 24th and 33rd weeks of gestation (n = 12), at the time of termination of pregnancy under medical assistance (at 22 weeks of gestation; n = 1), or at birth in a full-term infant (n = 3). Because no blood samples were available from a time point before primary CMV infection in the case patients, as controls we used cord blood samples from full-term infants (the control population for the case fetuses), as detailed elsewhere [23], and CMV-negative blood samples from adult volunteer donors <40 years old (the control population for the case mothers).

The study was approved by the local ethics committee of the Robert Debré Hospital perinatal department. For use of residual blood samples obtained by cordocentesis that were originally collected for hematological, biochemical, and virological analysis in fetuses; for peripheral blood samples from their mothers; and for cord blood control samples, oral information was given to the parents but written consent was not obtained. For blood sampling of adult control subjects, which was specifically performed for the purpose of the study, all individuals provided written, informed consent.

Blood samples were collected in acid-citrate-dextrose solution to prevent coagulation. All tests were performed on the day of sampling. HLA typing of the case mothers and fetuses was performed by the sequence specific–primer low-resolution technique (Olerup SSP HLA Low Resolution; GenoVision) as recommended by the manufacturer.

**Measurement of CMV load.** DNA was extracted from 200 μL of whole blood that had been collected in EDTA by use of the High Pure PCR Template Preparation Kit (Roche Biomédicals) in accordance with the manufacturer’s instructions, except that absorbed DNA was eluted from the column with 50 μL of elution buffer. Real-time quantification of CMV DNA
was performed using the LightCycler instrument (Roche Bio-
medicals) in accordance with a method that has been described
elsewhere [24].

**Immunophenotyping.** Analysis was performed on a 2-laser
FACSCalibur instrument (BD Biosciences) using CellQuest Pro
software (version 4.0.2; BD Biosciences). Absolute values of T
cells (CD8⁺, CD4⁺, and CD3⁺CD45⁺) were determined using a
TruCount-based assay (BD Biosciences).

For analysis of expression of surface markers, the following
fluorescent-labeled conjugated monoclonal antibodies (MAbs)
were used in different combinations: CD3–fluorescein isothio-
cyanate (FITC; Immunotech), CD8–PECy5 (Immunotech),
HLA-DR–phycoerythrin (PE; BD Biosciences), CD28–PE (BD
Biosciences), and CD18–FITC (Dako). In brief, 100 μL of whole
blood was added to a premixed solution of MAbs at the ap-
propriate dilution. After incubation for 15 min at room tem-
perature in the dark, red blood cells were lysed. Then, samples
were washed twice and resuspended in FACSFlow (BD Bio-
sciences). For the analysis of each sample, viable cells were gated
into the lymphocyte population according to forward and side
scatter characteristics, and 10,000 events were acquired in this
gate. Absolute values were ascertained by multiplying the per-
centages of each subpopulation with the absolute values for
CD8⁺ T cells as determined by the TruCount-based assay.

**Enumeration of HLA-A2/pp65– or HLA-B7/pp65–specific
CD8⁺ T cells.** Peripheral blood mononuclear cells (PBMCs)
were isolated from whole blood using density-gradient sepa-
ration of lymphocytes in UNI-SEPmaxi tubes (Novamed). On
the day of collection, PBMCs were incubated with 10 μL of
HLA-A2/pp65–PE (NLVPVMATV) or HLA-B7/pp65–PE
(TRPVTGGGAM) multimeric complex (Proimmune or Dako)
for 30 min at 4°C in the dark. Then, CD8–PECy5 MAb and
CD3–FITC MAb were added. After a 20-min incubation at 4°C
and washing with FACSFlow, cells were fixed with CellFix (BD
Biosciences) and then analyzed on a FACSCalibur instrument
using CellQuest Pro software. Gates were established on the
viable lymphocyte population by use of forward and side scatter
parameters. A minimum of 0.5 × 10⁶ cells were acquired within
the lymphocyte gate to ensure that a sufficient number of CD8⁺
cells were available for analysis. Specificity of the HLA-A2/pp65
or HLA-B7/pp65 multimers was confirmed by the lack of stain-
ing of PBMCs from HLA-A2– and HLA-B7–negative or CMV-
seronegative kidney transplant recipients (data not shown). The
cutoff for positivity, based on the results found in control sam-
ple, was 0.01%.

**Quantification of IFN-γ–secreting cells.** PBMCs (1 × 10⁶/
ml) were diluted with RPMI 1640 medium (Gibco) with 10%
heat-inactivated fetal calf serum (BioWest) and either (1) 550
ng/mL ionomycin (Sigma-Aldrich) and 55 ng/mL phorbol myr-
istate acetate (PMA; Sigma-Aldrich), (2) 2 μg/mL CD3 MAb
(Janssen-Cilag) in the presence of 2 μg/mL CD28 MAb (Im-
munotech), or (3) 5 μg/mL CMV (inactive form; Biogenesis)
with 10 IU/mL recombinant interleukin (IL)–2 (Roche), 2 μg/
ml CD49d (BD Biosciences), and 2 μg/mL CD28. Unstimu-
lated cultures were used a negative controls. These cultures
were incubated at 37°C in a humidified 5% CO₂ atmosphere for 18
h, with the final 17 h including the secretion inhibitor brefeldin
A at 10 μg/mL (Sigma-Aldrich).

### Table 1. Main clinical features of the fetuses.

<table>
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<tr>
<th>Fetus</th>
<th>Gestational age, a weeks</th>
<th>HLA type</th>
<th>ALT, IU/L</th>
<th>Platelet count, 10⁵ platelets/mm³</th>
<th>IgM level, mg/dL</th>
<th>Viral load, GE/mL</th>
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</table>

**NOTE.** ALT, alanine aminotransferase; CNS, central nervous system; GE, genome equivalent; IUGR, intrauterine growth retardation; ND, not determined.

a Gestational age at the time of immunological and virological investigations.

b Pregnancy was terminated under medical assistance.
Figure 1. Similar levels of activated (HLA-DR+) and effector/memory (CD28+/H11002+) cytotoxic T lymphocytes in fetuses and mothers. Results are expressed as individual values (white circles) of CD3+CD8+ cell subsets. Cord blood samples from full-term infants were used as controls for the fetuses. Control mothers are healthy, age-matched volunteer blood donors. Horizontal bars indicate group medians. Similar values were found in infected fetuses and their infected mothers. P<.05 was considered to indicate statistical significance. NS, not significant.

Intracytoplasmic staining was performed in accordance with the manufacturer’s recommendation. After fixation and permeabilization by use of the IntraStain Kit (Dako), CD3-FITC, CD8-PECy5 (Immunotech), and IFN-γ-PE (BD Biosciences) MAbs were incubated for 15 min at room temperature, washed, and fixed for 30 min before analysis.

Analysis was performed on a FACSCalibur instrument. List-mode multiparameter data files (each file with forward scatter, side scatter, and 4-fluorescent parameters) were analyzed using CellQuest Pro software. Files were gated on small CD3+ lymphocytes. A minimum of 20,000 gated events was acquired.

Statistical analysis. Nonparametric Wilcoxon tests were used to compare values between different groups. P<.05 was considered to indicate statistical significance.

RESULTS

Clinical features and viral loads in fetuses and their mothers. The 16 fetuses infected with CMV and their 16 transmitter mothers who were included in the study were recruited between November 2003 and June 2006. The primary infection in the mothers was diagnosed on routine follow-up as described in Methods. No clinical symptoms were noticed in the 16 mothers, and CMV DNA was at an undetectable level (<250 genome equivalents [GE]/mL) whenever tested (n = 13) at the time of immunological investigations. Only 1 mother had received specific antiviral chemotherapy before the immunological investigations.

Diagnosis of intrauterine transmission to the fetus was based on the detection of viral DNA in amniotic fluids at 17–19 weeks of gestation. As shown in table 1, high viral titers were found in all but 2 fetuses (mean [range], 17,290 [250 to 75,500] GE/mL). No symptoms were noticed in 14 of the 16 fetuses. One presented with intrauterine growth retardation, and another presented with developmental abnormalities of the central nervous system (CNS) and thrombocytopenia.

Similar levels of activated and differentiated circulating CTLs in infected fetuses and their transmitter mothers. As shown in figure 1, and in agreement with previous results [23], higher levels of HLA-DR+ (P<.0001) and CD28− (P=.0001) CTLs were found in infected case fetuses, compared with those in control fetuses. A higher (P=.005) level of the memory (CD18high) CTL subset, which was not previously investigated, was also observed in the 6 fetuses tested. Up-regulation of HLA-DR, down-regulation of CD28, and an increase in the percentages of CD18high CTLs were also observed in the case mothers relative to the control mothers. More importantly, the levels of circulating HLA-DR+, CD28−, and CD18high CTLs in the case fetuses were similar to or even higher than those in their transmitter mothers. This observation supports the concept that CMV induces an at least equivalent and possibly even more intense increase in these subsets in fetuses relative to that in mothers. Representative flow cytometry images for 1 fetus and his mother are shown in figure 2.

High levels of CD28− CTLs but low levels of pp65-specific CTLs in fetuses. We used the HLA-A2/NLVPMVATV and HLA-B7/TPRVTGGMG multimers to analyze the frequencies of circulating CTLs specific for these immunodominant epitopes in the pp65 protein. Results for 3 transmitter mothers and their 3 fetuses who shared HLA-A2 and for 2 transmitter mothers and their 2 fetuses who shared HLA-B7 are shown in
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Figure 2. Representative data on HLA-DR, CD28, and CD18 expression among cytotoxic T lymphocytes in 1 fetus-mother pair. Percentages of HLA-DR+, CD28+, and CD18high cells within the CD3+CD8+ cell population are indicated on the relevant quadrant. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Figure 3. In addition to HLA-A2– or HLA-B7–positive transmitter mothers, 2 HLA-A2–positive mothers who did not transmit HLA-A2 to their fetuses and 1 fetus who inherited HLA-A2 from his father were studied. Results for the entire HLA-A2 and HLA-B7 populations are shown in figure 4. A dramatically lower median percentage of pp65-specific CTLs was observed in the fetuses; in contrast, similar percentages of CD8+CD28+ T cells were observed in these fetuses and their transmitter mothers.

Low frequency of CTLs secreting IFN-γ after in vitro stimulation via the T cell receptor (TCR) in fetuses. The capacity to secrete effector cytokines after short-term stimulation in vitro is a typical feature of fully differentiated cells in an antigen-experienced individual. In the present study, the low level of pp65-specific T cells and the small amount of blood available from the fetuses precluded the use of CMV peptides or active virus to enumerate antigen-specific CTLs. We therefore used a CD3 MAb that activates T cells via the TCR to compare IFN-γ responses in the fetuses and their mothers.

As shown in figures 5 and 6, the magnitude of the IFN-γ response to CD3 MAb was strikingly lower in fetuses. In contrast, IFN-γ secretion after stimulation with ionomycin/PMA, which bypasses the TCR, was similar in fetuses and mothers. Also, the percentages of CD8+CD28+ T cells were similar in the fetuses and mothers tested for IFN-γ secretion.

As also illustrated in figure 5, CD8+ T cells secreting IFN-γ...
Figure 3. Nos. of cytotoxic T lymphocytes specific for the NLVPMVATV and TPRVTGGGAM peptides in HLA-A2–positive or HLA-B7–positive fetus-mother pairs. Peripheral blood mononuclear cells from fetuses and their respective mothers were stained with CD8 monoclonal antibody (MAb), CD3 MAb, and tetramers and were subsequently analyzed by flow cytometry. The percentages of CD3⁺CD8⁺tetramer⁺ cells among the total CD3⁺CD8⁺ cell population are given for each subject. PE, phycoerythrin.
after stimulation with an inactive form of CMV could be found in some mothers, a result that likely involved cross-presentation. There were no detectable IFN-γ–secreting CD8+ T cells in fetuses. More precisely, IFN-γ–secreting CD8+ T cells could be detected in 5 of 8 mothers who were tested (median [range], 0.095% [0%–15.43%]) and in 0 of 8 fetuses who were tested.

**DISCUSSION**

To better define the capacity to respond to CMV during early life, we conducted what is to the best of our knowledge the first study comparing the immune status in susceptible fetuses and their comparatively more resistant transmitter mothers. Several important conclusions emerged from this study.

The first conclusion is that transmitter mothers can serve as physiological control subjects, because pregnancy does not seem to influence the CD8+ T cell response to CMV. Specifically, transmitter mothers with primary CMV infection have percentages of CD28+/H11002 CTLs that do not differ quantitatively from values recently documented in adults with recent primary infection [26]. Furthermore, the range of percentages—from 0.29% to 3.3% of pp65-specific CTLs in HLA-A2– and HLA-B7–positive mothers—reaches a magnitude similar to that found in immunocompetent nonpregnant individuals who are CMV positive [27, 28]. In line with the present data, a previous study has shown that CMV-specific immune responses, as determined by the enumeration of TNF-α–producing CD4+ T cells in blood, showed no difference between pregnant and nonpregnant individuals [25].

The second conclusion is that fetuses with primary CMV infection accumulate markedly fewer pp65-specific CTLs than do their transmitter mothers. Discrepant with this interpretation, 2 recent studies of CMV infection during early life have concluded that CTLs might be mature at birth. In one study, Tu et al. [10] did not observe any quantitative difference in the levels of circulating HLA-A2/pp65–specific CTLs between children with postnataally acquired CMV infection and adults with primary infection. The reason for the discrepancy between their results and ours might be the age of the case patients. Indeed, CMV was transmitted during fetal life as a consequence of a primary infection occurring during the first trimester of pregnancy in our study, whereas infection occurred postnatally in Tu et al.'s study. In the second study, the conclusion made by Marchant et al. [6] was based on the observation that primary infection had the potential to elicit expansion of the pp65-specific CTL population in fetuses. It appears, however, that there was a considerable range in the magnitude of the responses in Marchant et al.'s study, given that 2 of 7 fetuses had circulating pp65-specific CTL levels similar to those found by others in older infants [10] and in adults [28]. Yet, in 5 of 7 fetuses, circulating pp65-specific CTL levels were well below 0.25%, reminiscent of the low levels found in our series of fetuses. Further studies are needed to specifically address the relationship between the level of pp65-specific circulating CTLs and gestational age.

One trivial explanation for the higher level of pp65-specific CTLs observed in mothers might be the entry of these cells into the circulation from infected tissue only after the acute infection has resolved, whereas CMV-specific cells should remain trapped in the target organs and/or draining lymph nodes in fetuses with viremia. This hypothesis is, however, unlikely, given that CMV-specific T cells seem to be preferentially found in the peripheral blood and not extravascular tissues [29]. Instead, aging should strongly influence the hierarchy of CMV protein recognition by CD8+ T cells. We chose to focus our analysis on the CMV pp65 protein because of its well-characterized role as an immunodominant target of the CTL re-
Figure 5. Typical results of flow cytometry analysis comparing interferon (IFN)–γ expression in 1 infected fetus and his mother. Cells positive for both CD8 and IFN-γ within the CD3 gate after stimulation with (1) ionomycin/phorbol myristate acetate (iono/PMA), (2) a combination of CD28 and CD3 monoclonal antibody (CD3), or (3) a combination of CD28, CD49d, recombinant interleukin-2, and an inactive form of the cytomegalovirus (CMV) are shown. Their percentages among the CD3+CD8+ cell population are indicated in the corresponding quadrants.

Response to CMV [30]. It is clear from recent studies that CTL-mediated immune responses to CMV in both healthy and immunocompromised individuals are directed toward a much wider range of antigens than was initially appreciated [31, 32]. How these antigens contribute to protection is still a matter of debate. Interestingly, in the present study, the detection of sizable pp65-specific CTL responses was associated with the control of CMV replication in resistant mothers, whereas low levels
of circulating pp65-specific CTLs and high viral loads were observed in fetuses. This observation is in line with several reports showing that the appearance of pp65-specific populations is temporally associated with a decrease in peripheral blood CMV load [15–17]. It further suggests that the pp65-specific CTL pool should be the main effector arm of the adaptive immune response in the control of viral disease.

In contrast to the poor expansion of the pp65-specific CTL pool in fetuses, the expansions of the activated, effector-memory, and memory CTL pools were essentially equivalent or even higher in fetuses. The finding that fetuses have limited pp65-specific CD8+ T cell responses but normal levels of effector/memory CTLs associated with persistent CMV infection is, to the best of our knowledge, unprecedented. This observation reinforces the idea that the CTLs induced by CMV in fetuses may in fact be different in specificity or binding avidity from the most potent CTLs generated during CMV infection in immunocompetent individuals. The mechanisms responsible for the development of a distinct target antigen during early life are totally unknown. It is possible that the virus elicits distinct specificity via a different cell tropism and/or host-pathogen relationship. Alternatively, the immaturity of the immune system might be primarily involved. Further studies are required to shed light on this matter.

Finally, the last conclusion from the present study is that fetal CD8+ T cells have defective IFN-γ production after in vitro stimulation via the TCR, whereas IFN-γ secretion after ionomycin/PMA stimulation reaches similar levels in fetuses and mothers. This conclusion supports the concept of a less mature functional phenotype in fetuses. The underlying mechanism of this reduced functionality is not clear. An impaired ability of dendritic cells to acquire a mature phenotype [33], to produce IL-12 [34], and to express CD40L [35] and a deficiency of CMV-specific CD4+ T cells [10] have been reported during early life. All of these functions play a key role in the generation of virus-specific IFN-γ-secreting CD8+ T cells. They might therefore contribute to the impairment of CTL function. Another potential immunological mechanism might relate to the immaturity of the NK cell compartment. The main function of NK cells is to kill target cells. All data on human umbilical
cord blood NK cells have pointed toward an immaturity of the NK cell compartment, on the basis of low cytotoxic activity. NK cell immaturity might therefore contribute to higher viral loads in congenital CMV infection. NK cells also indirectly shape the nature of the adaptive immune response, mainly by producing profound amounts of cytokines, resulting in the activation of dendritic cells [36, 37]. The immaturity of NK cells might therefore contribute to the attenuation of the adaptive immune response in fetuses. Alternatively, functional differences between mothers and fetuses should be attributed to differences in CTL specificities. Early studies of the immune response to CMV focused on a restricted group of antigens that were considered to be immunodominant, notably pp65 and IE1 [12, 16, 17, 38]. This view has now given way to an understanding that the CD8+ T cell response to CMV is much broader than anticipated [31, 39]. The importance of the T cell immune responses to all of these CMV antigens for protection against CMV replication and disease is still undetermined. The individual role played by various CMV peptides will need to be further addressed in both immunocompetent and immunocompromised individuals to enlighten this point.

Analysis of the relationship between clinical symptoms, viral load, and immunological features was not feasible in this limited series of patients. Note, however, that the fetus with CNS abnormalities had the highest viral load (75,500 GE/mL) in juxtaposition with a relatively low CD28- CTL level (262 cells/mm³) and a percentage of pp65-specific CTLs at the limit of detection (0.01%). Further studies in a larger series of patients to determine whether viral load and the magnitude of the immune response are inversely correlated would be of great interest.

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References


