# ViroMag increases, assists and controls infection of Primary T cells

## Gag-Specific CD8+ T Lymphocytes Recognize Infected Cells before AIDS-Virus Integration and Viral Protein Expression1

*Jonah B. Sacha, Chungwon Chung, Eva G. Rakasz, et al*
*The Journal of Immunology,* 2007, 178: 2746–2754

**Application:** ***ViroMag*** was successfully used to **synchronize adsorption** of SIVmac239 on PBMC-derived CD4+ T lymphocytes.


**Abstract**
CD8+ T cells are a key focus of vaccine development efforts for HIV. However, there is no clear consensus as to which of the nine HIV proteins should be used for vaccination. The early proteins Tat, Rev, and Nef may be better CD8+ T cell targets than the late-expressed structural proteins Gag, Pol, and Env. In this study, we show that Gag-specific CD8+ T cells recognize infected CD4+ T lymphocytes as early as 2 h postinfection, before proviral DNA integration, viral protein synthesis, and Nef-mediated MHC class I down-regulation. Additionally, the number of Gag epitopes recognized by CD8+ T cells was significantly associated with lower viremia ($p$ = 0.0017) in SIV-infected rhesus macaques. These results suggest that HIV vaccines should focus CD8+ T cell responses on Gag.


**Material and Methods**

*Cell isolation and culture*

Target cells were generated from freshly isolated PBMC obtained from SIV-naive Indian rhesus macaques using Ficoll-Paque PLUS density centrifugation. CD4+ T cells were isolated using CD4 microbeads and LS columns. Targets were activated by incubating overnight with staphylococcal enterotoxin B (2.5 µg/ml) and Abs to CD28 (2.5 µg/ml), CD3 (2.5 µg/ml), and CD49d (2.5 µg/ml) and cultivated in the presence of IL-2 (100 U/ml). CD4+ targets were consistently >95% CD3 and CD4 positive. Cells were cultured in R15-100 (RPMI 1640 medium containing 15% FCS and 100 U/ml IL-2).

*Generation of SIVmac239 and synchronized infections*

SIVmac239 was generated (16) and purified (17) as described previously. Briefly, Vero cells were transfected with plasmid DNA encoding proviral sequences. CEMx174 cells were added to the Vero cultures, and the virus was subsequently expanded on CEMx174 cells. Cell-free supernatant was collected 2 days after peak syncytium formation. The virus was subsequently purified through a 20% sucrose cushion immediately before use in the kinetic ICS (KICS) assay. ***ViroMag*** beads were obtained from OZ Biosciences. SIVmac239 was magnetized with ***ViroMag*** beads and cells synchronously infected according to the manufacturer's instructions with a multiplicity of infection $\leq 1$. Briefly, target cells were incubated with the magnetized virus for 15 min in the presence of a magnetic field, washed once in PBS, washed once with 0.25 µg/ml trypsin EDTA and then washed twice with PBS. Infected targets were then incubated in R15-100 (RPMI 1640 medium containing 15% FCS and 100 U/ml IL-2). Cells treated with tenofovir were incubated with 400 µM tenofovir for at least 2 h before infection and throughout the experiment.

## Pol-Specific CD8+ T Cells Recognize Simian Immunodeficiency Virus-Infected Cells Prior to Nef-Mediated Major Histocompatibility Complex Class I Downregulation

*Jonah B. Sacha, Chungwon Chung, Jason Reed, et al.*
*Journal of Virology*, 2007, 81: 11703–11712

**Application:** ***ViroMag*** was successfully used to **synchronize adsorption** of SIVmac239 on PBMC-derived CD4+ T lymphocytes and study infection kinetics development. ViroMag results in an approximate **10-fold increase** in TCID50 levels compared to a standard 4-h infection.

**Abstract**
Effective, vaccine-induced CD8+ T-cell responses should recognize infected cells early enough to prevent production of progeny virions. We have recently shown that Gag-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells at 2 h postinfection, whereas Env-specific CD8+ T cells do not recognize infected cells until much later in infection. However, it remains unknown when other proteins present in the viral particle are presented to CD8+ T cells after infection. To address this issue, we explored CD8+ T-cell recognition of epitopes derived from two other relatively large virion proteins, Pol and Nef. Surprisingly, infected cells efficiently presented CD8+ T-cell epitopes from virion-derived Pol proteins within 2 h of infection. In contrast, Nef-specific CD8+ T cells did not recognize infected cells until 12 h postinfection. Additionally, we show that SIVmac239 Nef downregulated surface major histocompatibility complex class I (MHC-I) molecules beginning at 12 h postinfection, concomitant with presentation of Nef-derived CD8+ T-cell epitopes. Finally, Pol-specific CD8+ T cells eliminated infected cells as early as 6 h postinfection, well before MHC-I downregulation, suggesting a previously underappreciated antiviral role for Pol-specific CD8+ T cells.

**Material and Methods**
*Cell isolation and culture***.** Target cells were generated from freshly isolated peripheral blood mononuclear cells (PBMC) obtained from SIV-naive Indian rhesus macaques using Ficoll-Paque PLUS density centrifugation. CD4+ T cells were isolated using CD4 microbeads and LS columns. Targets were activated by incubation overnight with staphylococcal enterotoxin B (2.5 µg/ml) and antibodies to CD28 (2.5 µg/ml), CD3 (2.5 µg/ml), and CD49d (2.5 µg/ml) and cultivated in the presence of 100 U interleukin-2 (IL-2)/ml. CD4+ targets were consistently >95% CD3 and CD4 positive. For MHC-I kinetic experiments, CD4+ targets were activated with concanavalin A (10 µg/ml) up to 72 h prior to infection. Cells were cultured in R15-100 (RPMI 1640 containing 15% fetal calf serum and 100 U/ml IL-2).

**Generation of SIVmac239 and synchronized infections.** SIVmac239 was generated (11) and purified (32) as previously described. Briefly, Vero cells were transfected with plasmid DNA encoding proviral sequences. CEMx174 cells were added to the Vero cultures, and the virus was subsequently expanded on CEMx174 cells. Cell-free supernatant was collected 2 days after peak syncytium formation. The virus was subsequently purified through a 20% sucrose cushion immediately prior to use in the kinetic ICS (KICS) assay. ***ViroMag*** beads were obtained from OZ Biosciences (Marseille, France). SIVmac239 was magnetized with ***ViroMag*** beads, and cells were synchronously infected according to the manufacturer's instructions at a multiplicity of infection (MOI) of 1. Briefly, target cells were incubated with the magnetized virus for 15 min in the presence of a magnetic field, washed once in phosphate-buffered saline (PBS), washed once with 0.25 µg/ml trypsin-EDTA to remove surface-bound virions, and then washed two more times with PBS. Identical results were obtained in all assays when the trypsin-EDTA wash was excluded (data not shown). Infected targets were then incubated in R15-100.

To measure 50% tissue culture infectious dose (TCID50), activated, primary CD4+ T cells were infected with 10-fold dilutions of sucrose-purified virus using the ***Magnetofection*** technique as described above. Titer was determined from the lowest virus concentration that gave rise to infected cultures 8 days after infection, as determined by Gag p27 enzyme-linked immunosorbent assay. **The magnetic-infection technique resulted in an approximate 10-fold increase in TCID50 levels compared to a standard 4-h infection (data not shown).**

## AIDS virus – specifi c CD8 + T lymphocytes against an immunodominant cryptic epitope select for viral escape

*Nicholas J. Maness, Laura E. Valentine, Gemma E. May, et al.*
*The Journal of Experimental Medicine* 2007, 204: 2505-2512

**Application:** Magnetofection (***ViroMag***) was used to infect target cells derived from SIV-naive macaques that were either *Mamu-B*17+* or *Mamu-B*17-* to study the effect of site-directed mutagenesis with the T6913C mutation. 20 – 30% of the target cells were infected after 24 h with either the wild-type SIVmac239 or the SIV-T6913C escape mutant virus.

**Abstract**
Cryptic major histocompatibility complex class I epitopes have been detected in several pathogens, but their importance in the immune response to AIDS viruses remains unknown. Here, we show that Mamu-B*17+ simian immunodefi ciency virus (SIV)mac239-infected rhesus macaques that spontaneously controlled viral replication consistently made strong CD8+ T lymphocyte (CD8-TL) responses against a cryptic epitope, RHLAFKCLW (cRW9). Importantly, cRW9-specific CD8-TL selected for viral variation in vivo and effectively suppressed SIV replication in vitro, suggesting that they might play a key role in the SIV specific response. The discovery of an immunodominant CD8-TL response in elite controller macaques against a cryptic epitope suggests that the AIDS virus – specific cellular immune response is likely far more complex than is generally assumed.

**Material and Methods**
Cells were infected with SIV (either wild-type SIVmac239 or mutant SIV-T6913C) using the Magnetofection technique, and recognition was measured by ICS after 24 h as described previously (Jonah B. Sacha, et al; The Journal of Immunology, 2007, 178: 2746–2754.

## Efficient inhibition of SIV replication in rhesus CD4+ T-cell clones by autologous immortalized SIV-specific CD8+ T-cell clones

*Jacob T. Minang, Eugene V. Barsov, Fang Yuan, et al.*
*Virology*, 2008, 372: 430–441

**Application:** ***ViroMag*** was used to study the kinetics of viral replication in immortalized rhesus macaque CD4+ T-cell clones in vitro and to assist in characterizing the immunological mechanisms of CTL-mediated viral control. An hTERT transduced rhesus macaque CD4+ T-cell clone was infected with molecularly cloned SIVmac239 using magnetic beads and then cultured in 24-well tissue culture plates at 1×106 cells/well. The frequency of SIV Gag p27 expressing CD4+ T cells was determined 2, 5, 8 and 12 days post infection (PI) by flow cytometry.

**Abstract**
CD8+ cytotoxic T lymphocyte (CTL) responses play an important role in controlling the replication of primate lentiviruses. Induction of these responses is a key objective for most current AIDS vaccine approaches. Despite a variety of approaches for measuring properties and activities of CTL, the functions responsible for controlling viral replication in vivo have not been clearly identified. Assays measuring CTL-mediated suppression of viral replication in vitro are beginning to be used as possible correlates of in vivo virus suppressive activity, but the utility and interpretive value of these assays are typically limited by properties of the cells that have been used. We investigated the capacity of SIV-specific CTL clones (effectors), immortalized by transduction with human telomerase reverse transcriptase (hTERT), to suppress SIV replication in autologous hTERT immortalized CD4+ T-cell clones (targets). Immortalized and non-immortalized SIV-specific effector cells showed IFN-γ production and degranulation in response to viral antigen specific stimulation and significantly inhibited SIVmac239 replication (2 to 4 log decrease in viral RNA or cell-associated proviral DNA) (pb0.0005). Our in vitro assays of inhibition of viral replication, using T-cell clones as effectors and targets, provide a well-defined approach for evaluating possible mechanisms of CTL-mediated control of viral production which may involve direct killing of infected target cells and/or release of proinflammatory cytokines such as IFN-γ and TNF-α. The use of hTERT immortalized effector and target cells for such assays preserves relevant functional properties while providing a convenient, reproducible means of conducting studies over time.

**Material and Methods**
CD4+ T-cell clones were generated from Indian rhesus macaque PBMC and splenocytes. Briefly, highly enriched CD4+ T cells were isolated from PBMC and splenocytes by negative selection using Miltenyi LD columns and anti-CD8 microbeads followed by positive selection using MS columns and anti-CD4 microbeads. CD4+ T-cell clones were obtained after 2-week expansion in limiting dilution cultures containing irradiated human PBMC, IL-2 and anti-CD3 mAb and maintained as described above for CTL clones.

Infection of CD4+ T-cell clones with SIVmac239 CD4+ T-cell clones were activated with anti-CD3 mAb (T-25 flask-bound: 5 µg/mL) and IL-2 (50 IU/mL) for 48 h and then incubated with SIVmac239. Infections were carried out either by incubating CD4+ T cells with aliquots of the virus stock overnight (12–18 h) with 2 µg/mL polybrene or for 2–3 h using the ***ViroMag*** Magnetofection reagents and procedures, according to manufacturer's recommendation (OZ Biosciences, Marseille, France). Approximately 0.25 mL virus stock with 1.2×109 viral RNA copies Eq/mL was added per 1×106 CD4+ T cells (~ 3×102 viral RNA copies/target cell). Virus exposed CD4+ T cells were washed twice with sterile PBS to remove any residual non-incorporated viral material, prior to use in assays.

## The Mamu B*17-restricted SIV Nef IW9 to TW9 mutation abrogates correct epitope processing and presentation without loss of replicative fitness

*Jacob T. Minang, Matthew T. Trivett, Lori V. Coren, et al.*
*Virology* (2008) Mar 5 [Epub ahead of print]

**Application:** ***ViroMag*** was used to study the kinetics of viral replication in immortalized rhesus macaque CD4+ T-cell clones in vitro and to assist in characterizing the immunological mechanisms of CTL-mediated viral control.


**Abstract**

CD8+ cytotoxic T lymphocytes (CTL) play an important role in controlling virus replication in HIV- and SIV-infected humans and monkeys, respectively. Three well-studied SIV CTL determinants are the two Mamu A□01-restricted epitopes Gag CM9 and Tat SL8, and the Mamu B□17-restricted epitope Nef IW9. Point mutations leading to amino acid replacements in these epitopes have been reported to mediate SIV escape from CTL control. We found that synthetic peptides containing mutations in SIV Gag CM9 and Tat SL8 were no longer recognized by the respective CTL. On the other hand, the described I-to-T replacement at the N-terminal amino acid residue of the SIV Nef IW9 epitope only moderately affected CTL recognition of the variant peptide, TW9. In an attempt to dissect the mechanism of escape of the Nef TW9 mutation, we investigated the effect of this mutation on CTL recognition of CD4+T cells infected with an engineered SIVmac239 that contained the TW9 mutation in Nef. Although, the wild type and mutant virus both infected and efficiently replicated in rhesus macaque CD4+T cells, the TW9 mutant virus failed to induce IFN-γ expression in an SIV Nef IW9-specific CTL clone. Thus, unlike escape from Gag CM9- or Tat SL8-specfic CTL control presumably by loss of epitope binding, these results point to a defect at the level of processing and/or presentation of the variant TW9 epitope with resultant loss of triggering of the cognate TCR on CTL generated against the wild type peptide. Our data highlight the value of functional assays using virus-infected target cells as opposed to peptide-pulsed APC when assessing relevant escape mutations in CTL epitopes.


**Material and Methods**

A clonal population of resting CD4+Tcells was activated with anti-CD3 mAb (T-25 flask-bound: 5 µg/mL) and IL-2 (50 IU/mL) for 48 h and then incubated with aliquots of freshly prepared virus stocks using the ***ViroMag*** Magnetofection reagents and procedures according to manufacturer's recommendation (OZ Biosciences, Marseille, France). Approximately 0.25 mL virus stock containing 1.2×109 viral RNA copies Eq/mL were added per 1×106 CD4+T cells. Incubations were carried out on a magnetic plate at 37 °C in a humidified atmosphere of 5% CO2 for 2 h and the virus-exposed CD4+Tcells washed twice with PBS to remove residual non-incorporated viral material prior to use in assays.