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Robert A. Rasmussen, *Dana Farber Cancer Institute*
Nagadenahalli B. Siddappa, *Dana Farber Cancer Institute*
Samir K. Lakhashe, *Dana Farber Cancer Institute*
Jennifer Watkins, *Dana Farber Cancer Institute*
Francois Villinger, *Emory University*
[Chris Ibegbu](#), *Emory University*
Ruth H. Florese, *National Cancer Institute*
Marjorie Robert-Guroff, *National Cancer Institute*
David C. Montefiori, *Duke University*
Donald N. Forthal, *University of California, Irvine*

Only first 10 authors above; see publication for full author list.

Journal Title: AIDS

Volume: Volume 26, Number 2

Publisher: Lippincott, Williams & Wilkins | 2012-01-14, Pages 149-155

Type of Work: Article | Post-print: After Peer Review

Publisher DOI: 10.1097/QAD.0b013e32834d3c4f

Permanent URL: <https://pid.emory.edu/ark:/25593/tr908>

Final published version: <http://dx.doi.org/10.1097/QAD.0b013e32834d3c4f>

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Accessed November 16, 2019 7:37 PM EST



Published in final edited form as:

AIDS. 2012 January 14; 26(2): 149–155. doi:10.1097/QAD.0b013e32834d3c4f.

High-level, lasting antiviral immunity induced by a bimodal AIDS vaccine and boosted by live-virus exposure: prevention of viremia

Robert A. RASMUSSEN^{a,b,*}, Nagadenahalli B. SIDDAPPA^{a,b,*}, Samir K. LAKHASHE^{a,b,*}, Jennifer WATKINS^{a,b}, François VILLINGER^{c,d}, Chris IBEGBU^d, Ruth H. FLORESE^e, Marjorie ROBERT-GUROFF^e, David C. MONTEFIORI^f, Donald N. FORTHAL^g, David O'CONNOR^h, and Ruth M. RUPRECHT^{a,b} for the Clade C Program Project Group

^aDana-Farber Cancer Institute, Boston, Massachusetts, USA

^bHarvard Medical School, Boston, Massachusetts, USA

^cDepartment of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, USA

^dYerkes National Primate Research Center, Emory University, Atlanta, Georgia, USA

^eVaccine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

^fDuke University Medical Center, Durham, North Carolina, USA

^gDivision of Infectious Disease, University of California Irvine, USA

^hDepartment of Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin, USA

Abstract

Objective—To characterize the correlates of protection from systemic infection in a vaccinated rhesus macaque (RM), RAT-9, which had been challenged sequentially with two related clade C simian-human immunodeficiency viruses (SHIV-Cs) yet remained aviremic for >5 years despite indirect evidence of cryptic infection.

Design—To measure long-term anti-SHIV-C immunity, host genetics and gene-expression patterns for protective correlates.

Methods—Long-term immune reactivity was evaluated and identification of virus in RAT-9 was attempted by RT-PCR analysis of concentrated plasma and blood transfer to CD8⁺ cell-depleted infant macaques. Full MHC genotyping of RAT-9, TRIM5 and KIR3DL allelic expression analysis of PBMC, and microarray gene expression analysis were performed.

Results—All attempts to detect/isolate virus, including blood transfer to CD8⁺ cell-depleted infant RM, were negative, and the animal maintained normal levels of memory CD4⁺ T cells in both peripheral blood and gut tissues. However, RAT-9 maintained high levels of anti-SHIV-C humoral and cellular immunity, including reactivity to non-vaccine neoantigens (Nef and Rev), up to 63 months post-initial challenge, suggesting chronic sub-threshold infection. RAT-9 expressed

Correspondence to Ruth M. Ruprecht, MD, PhD, Dana-Farber Cancer Institute, 450 Brookline Avenue, JFB-809, Boston MA 02215 USA. Tel.: +1 617 632 3719; fax: +1 617 632 3112; ruth_ruprecht@dfci.harvard.edu.

*equal contribution

Clade C Program Project Group: Girish HEMASHETTAR^a, Wendy WANG^a, Yin Ling WONG^a, Victor KRAMER^a, James G. ELSE^{c,d}, Elizabeth STROBERT^d, Karen STRAIT^{c,d}.

the Mamu A*001 allele but was B*008-B*017⁻, had a B13 serotype, and had increased expression of killer-cell immunoglobulin-like receptors (KIRs) previously linked to favorable outcomes of lentiviral infection. Elements of the gene expression profiling coincided with genotyping results. RAt-9 also displayed CD8⁺ cell noncytotoxic antiviral response (CNAR) activity.

Conclusions—RAt-9 is the first example of a virus-exposed, persistently aviremic animal that has maintained long-term, high-level cellular and humoral antiviral immunity in the absence of an identifiable cryptic reservoir.

Keywords

AIDS; vaccine; SHIV; clade C; long-term immunity; prevention of viremia

Introduction

Identifying correlates of protection against immunodeficiency virus infection remains a major goal and is important for rational AIDS vaccine design. Extensive efforts are underway to identify such correlates in individuals within the moderately successful RV144 trial [1]. Alternatively, vaccinated nonhuman primates that have resisted viral infection or had reduced pathogenesis after exposure to simian immunodeficiency viruses (SIVs) or chimeric simian/human immunodeficiency viruses (SHIVs) have also been used to examine potential correlates [2–4]. Understanding individual virus-host interactions is crucial to define protective parameters, including host genetics as well as innate and adaptive immunity.

We previously described a vaccinated rhesus macaque (RM), RAt-9, that remained aviremic following sequential mucosal challenges with two related yet distinct R5 clade C SHIVs (SHIV-Cs) [5]. RAt-9 was vaccinated with SIVmac239 Gag-Pol particles, HIV Tat and trimeric HIV clade C (HIV-C) 1084i [6] gp160, and orally challenged once with low-dose, tier 1 SHIV-1157ip. Env of the immunogen and first challenge virus were derived from primary HIV-Cs isolated from different infants within the same Zambian cohort of HIV-C⁺ mother/infant pairs [7]; the amino acid sequences of the two heterologous Envs diverged by 22.1%. Six months post-SHIV-1157ip challenge, RAt-9 was rechallenged intrarectally with high-dose SHIV-1157ipd3N4, a tier 2 strain (Fig. 1a).

Here we describe possible correlates of protection in RAt-9 that was followed prospectively for >5 years. Our results suggest that a combination of host genetics as well as innate and adaptive immune responses protected RAt-9 from the SHIV challenges and continued to contain virus despite indirect evidence of cryptic infection.

Methods

Animals

RM experiments were conducted according to the National Institute of Health guidelines on the care and use of laboratory animals at the AAALAC-accredited Yerkes National Primate Research Center (YNPRC, Emory University, Atlanta, GA), and approved by the Institutional Animal Care and Use Committees of YNPRC and Dana-Farber Cancer Institute.

Virus-specific humoral immune responses

Binding antibody (Ab) titers against viral proteins were determined by ELISA [5]; insect cell-produced HIV-C_{CN54} gp120 was used to avoid detecting anti-vaccinia Abs potentially

induced by immunogen impurities. Neutralizing antibody (nAb) titers were determined by TZM-bl assay [8]. Serum antibody-dependent cellular cytotoxicity (ADCC) titers were determined using CEM-NK^R cells coated with HIV-C gp120 [9]. For ADCVI, SHIV-1157ipEL-p-infected CEM-NKR-CCR5 cells were incubated with 1:100 diluted serum + rhesus PBMC effectors (effector:target = 10:1) and virus replication was monitored [10–11].

Virus-specific cellular immune responses

Interferon (IFN)- ELISPOT assays, intracellular cytokine staining and T-cell proliferative responses to indicated antigens were performed as described [5, 12].

Virus replication kinetics

PBMC were stimulated for 2d with Concanavalin A (5 µg/ml) in the presence of rIL-2 (10 U/ml). CD8⁺ cells or NK cells were depleted using CD8 Dynabeads (Invitrogen, CA) or CD16-MicroBeads (Miltenyi Biotech, Gladbach, Germany), respectively. Unfractionated, CD8⁺ cell-depleted or NK cell-depleted PBMC (2×10^6) were exposed to SHIV-1157ipEL-p (2.5×10^4 TCID₅₀) and p27 levels in culture supernatants measured by ELISA. Transwell CD8⁺ cell noncytotoxic antiviral response (CNAR) activity was measured as described [13].

MHC, TRIM5α and KIR3DL allele 13/14 genotyping, and gene expression analysis

MHC class I genotyping was performed using one PCR primer set to amplify full-length MHC class I alleles and another to amplify only the most polymorphic region of those alleles by Sanger sequencing [14]. RAt-9 TRIM5 genotype was determined as published [15]. Heterozygosity or homozygosity for the SNP which translates into the amino acid substitution 159H-Q unique to RM KIR3DL alleles 13 and 14, was determined using a Step-One Plus RT PCR System/OneStep Software v2.1 (Applied Biosystems) in duplicate [16]. DNA from 3 control monkeys for each H/H and H/Q genotype was also analyzed (Q/Q animals were unavailable). For gene expression analysis, total blood RNA was prepared and analyzed on RM Genome Arrays according to Affymetrix (Santa Clara, CA) instructions. Genes differentially expressed in RAt-9 (1.0 fold difference from mean baseline derived from the naive sibling, RE-m-6) were further evaluated using the David annotation tool (<http://apps1.niaid.nih.gov/david>) and Ingenuity Pathway Analysis (IPA).

In vivo anti-CD8 treatment and blood transfer

Four-month old infant RM were treated with anti-CD8 mAb (cM-T807) subcutaneously (10 mg/kg) on day -4 and i.v. (5 mg/kg) on days -1, 3 and 6. Blood (10 ml) was transferred from RAt-9 to each infant by slow i.v. infusion on d0.

Results

Long-term immune reactivity of RAt-9

Vaccinee RAt-9 remained aviremic after an initial low-dose SHIV-1157ip challenge. At that time, RAt-9 displayed peripheral blood T-cell reactivity to HIV Tat and SIV Gag [5], nAb activity against this first challenge virus and strong HIV-C Env-directed ADCC (Fig. 1b, c). Six months post-first challenge, RAt-9 resisted high-dose intrarectal SHIV-1157ipd3N4 rechallenge, a tier 2 late-stage SHIV-1157ip derivative; at the time of SHIV-1157ipd3N4 rechallenge, RAt-9 had maintained high nAb activity against SHIV-1157ip (but none against SHIV-1157ipd3N4) and showed strong ADCC and ADCVI activities. ELISPOT reactivity to SIV Gag and HIV Tat increased after the initial SHIV-1157ip challenge (Fig. 1c;[5]).

For >5 yrs, viral sequences were never seen (RT-PCR of plasma (detection limit, 50 copies/ml [17–18]) at 1–2 month intervals throughout; inguinal lymph node (LN) biopsy at month 8.5; DNA PCR of inguinal LN and rectal biopsies at month 52) - yet RAt-9 has maintained unusually high levels of both humoral and cellular virus-specific immunity (Fig. 1b, c, e, f), including nAbs against SHIV-1157ipEL-p (Env homologous to SHIV-1157ip [7]) and cross-clade reactivity to SHIV_{SF162P4}. RAt-9 never generated nAbs against the second virus, SHIV-1157ipd3N4 [19]. ADCC and ADCVI activities were also maintained.

Specific T-cell immunity remained high as shown by IFN- ELISPOTs after stimulation with SIV Gag or HIV Tat peptides, or with p11C, the *Mamu A*001* immunodominant Gag peptide (Fig. 1c); epitope mapping revealed five Gag regions recognized (Fig. 1g). At 51 months post-exposure, polyfunctional reactivity was evident in both central memory and effector memory CD8⁺ T cells (Fig. 1e), and both CD4⁺ and CD8⁺ T cells showed strong proliferative responses to SIV Gag (Fig. 1f).

Cryptic clade C SHIV infection with unknown viral reservoir

Long-term maintenance of virus-specific immunity in RAt-9 suggested chronic antigenic stimulation from undetectable reservoir(s). Moreover, RAt-9 showed cellular immunity to non-vaccine-related neoantigens, SIV Nef and HIV Rev (Fig. 1c, f). The latter is a regulatory protein required for late-stage structural protein expression that has never been detected in virions (Dr. Barbara Felber, NCI, Frederick MD, personal communication). Specific T-cell recognition of Rev could only have arisen from cryptic target-cell infection followed by MHC class I presentation of Rev peptides. Thus, Rev-targeted T-cell reactivity confirms cryptic infection in RAt-9.

To date, RAt-9 has maintained normal levels of both peripheral CD4⁺ memory T cells and gut CD4⁺ T cells (data not shown). Importantly, CD8⁺ cell-depleted PBMC of RAt-9 supported SHIV-1157ipEL-p replication (encoding *env* of SHIV-1157ip [7]) (Fig. 2a); detection of soluble CD8⁺ CNAR activity in separate experiments (Fig. 2b) suggest CNAR played a role in the CD8⁺-cell suppressive effects.

In efforts to reveal infectious virus, blood was transferred from RAt-9 into two infant macaques, whose immature immune systems make them highly susceptible to lentiviruses [19–20]. To enhance the recipients' susceptibility to infection, anti-CD8 mAb treatment was used to temporarily deplete CD8⁺ T and NK cells [21–24]; despite profound CD8⁺-cell depletion, no viremia ensued (Fig. 2c).

MHC Class I, TRIM5 α and KIR3DL allele 13/14 genotyping

Genotyping was performed to test for previously described favorable MHC class I alleles; RAt-9 was MHC Class I *Mamu-A1*001*⁺, *B*008*⁻, *B*017*⁻ and expressed a B13 serotype (*Mamu-B*041*⁺, *B*048*⁺ and *B*064*⁺; [25]) previously associated with delayed SIV disease progression [26]. Interestingly, RAt-9 also displayed a B11a serotype (allelic expression of *Mamu-B*012*, *B*030* and *B*038*), associated with rapid disease progression in SIVmac239-infected RM [26].

Expression of KIR3DL alleles 13 and/or 14 in NK cells, and identification of an associated SNP that results in a Q at AA position 159 instead of a consensus H in the other identified alleles, was shown to play a role in innate immunity-mediated protection against immunodeficiency virus infection in RM [16]. RAt-9 and her sibling (REm-6) showed homozygous KIR3DL H/H allele expression (Fig. 2d). RAt-9 also expressed homozygous TRIM5 TFP/TFP alleles; both allelic expression patterns of KIR3DL and TRIM5 have been correlated with protection from SIV infection [15–16, 27]. Peripheral blood gene expression profiling of RAt-9 (using REm-6 for baseline corrections) also showed

differential expression of genes involved in cell-mediated immunity, MHC-class I pathways, inflammatory responses, and the KIR3 family (Fig. 2e).

Discussion

RAt-9, vaccinated with recombinant protein immunogens, remained aviremic after sequential SHIV-C challenges, including high-dose SHIV-1157ipd3N4 challenge. Our earlier data suggested that the initial virus exposure boosted vaccine-induced cellular immunity via sub-threshold infection kept in check by vaccine-induced humoral immunity; boosted cellular responses then prevented infection with the more aggressive second virus. Here we report that: 1) strong nAb, ADCC and ADCVI activities likely contributed to overall protection, 2) MHC haplotype and innate immunity via enhanced KIR expression and CNAR activity were potential cofactors in viral containment, 3) RAt-9 maintained high-level SHIV-C-directed humoral and T-cell immunity for >5 years post-1st virus exposure and developed neo-antigen reactivity; 4) blood transfer from RAt-9 to CD8⁺ cell-depleted infant RM revealed no replicating virus. Together, the data suggest that RAt-9 harbors an undetectable SHIV-C infection resulting in continuous viral antigen exposure at levels sufficient to maintain cellular/humoral antiviral responses that, in turn, keep virus replication below detection levels.

We considered treating RAt-9 directly with anti-CD8 mAb to temporarily deplete CD8⁺ T cells and NK cells to allow resurgence of cryptic virus. However, anti-CD8 mAb treatment was contraindicated because RAt-9's high nAb and ADCC/ADCVI titers would have blocked viral spread. We also seek to assess the natural history of vaccine-induced, live-virus-boosted antiviral immunity and CNAR activity, which transient depletion of CD8⁺ cells in RAt-9 may alter [21, 24].

While the boost in vaccine-induced immunity after virus challenge may have been due to cross-presentation of antigen/antibody complexes in the absence of infection or to transient "hit-and-run" infection [28], the maintenance of high-level virus-specific immunity, including neoantigen reactivity, is indirect evidence of ongoing cryptic infection or at least viral protein production. However, the immune responses observed in RAt-9 are unlike those of HIV-infected, HAART-treated patients, where viremia levels become undetectable and cellular immunity drops despite persistent latent virus infection [29–30]. Furthermore, if low-level virus replication provides antigen stimulation that maintains virus-specific immunity in RAt-9, we found no evidence of SIV Gag p11c T-cell epitope escape that can occur with chronic immunodeficiency virus infection [31], as p11c reactivity was maintained. Of note, RAt-9 had no evidence of autoimmunity (data not shown).

Correlations between genetic factors and/or innate immunity, and protection from infection and/or disease progression have been identified in both HIV-exposed humans and lentivirus-exposed primates [32–39]; of those tested in RAt-9, we identified favorable TRIM5 and KIR3DL allelic expression, a B13 serotype and CNAR reactivity. The relative contribution of each to vaccine-induced protection in RAt-9 is unknown but the high levels of adaptive immune responses maintained in the animal suggest strong roles for both humoral and cellular immunity.

Acknowledgments

We thank Dr. Welkin Johnson and the Genetics Core of the New England Primate Research Center ((NEPRC) Southborough, Massachusetts) for TRIM5 genotyping, Drs. Reddy Gali, Center for Biomedical Informatics, Harvard Medical School, for help in microarray analyses, Susan Stephenson and Aftab Ansari for the determination of the KIR3DL alleles, and Mark Wilson for supplying REm-6 samples, and Gary Landucci and Candi Trac (UC Irvine) for technical assistance. Support is acknowledged by NIH grants PO1 AI048240 to R.A.R., S.L.H, J.G.E.

and R.M.R., RR-00165 to the YNPRC, RR00168 to the NEPRC, and by Center for AIDS Research Immunology Core grants, P30 AI060354 and P30 AI050409.

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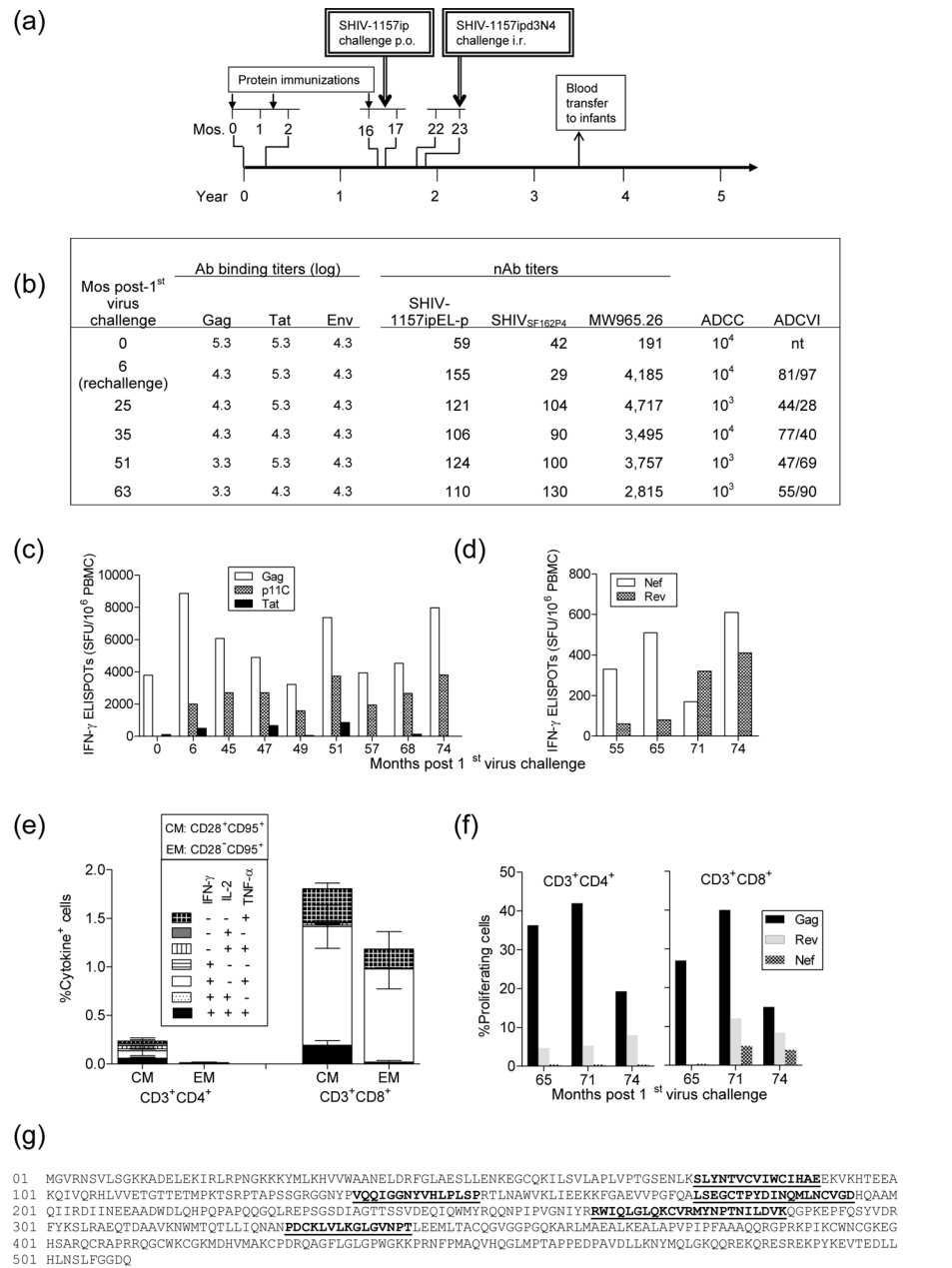


Fig. 1. Lasting virus-specific immune reactivity in Rat-9

(a) Timeline of immunizations, virus challenges and follow-up of Rat-9. (b) Sera from the time points indicated were tested by ELISA for Ab binding against SIV Gag, HIV Tat and HIV_{CN54} gp120; for nAb activity by TZM-bl assay against SHIV-1157ipEL-p, SHIV_{SF162P4} and Env-pseudotyped MW965.26; for ADCC activity against HIV-C gp120-coated target cells; and for ADCVI activity (the two values shown are % inhibition of virus replication using sera at 1:100 dilution and effector cells from different naïve RM; nt - not tested). (c, d) IFN- γ ELISPOTs after stimulation of PBMC collected at the time points indicated with (c): SIV Gag peptides, the Mamu A*001 immunodominant Gag peptide, p11C, or HIV Tat (p11C data are not available for time 0) and (d) peptides of neoantigens SIV Nef and HIV-1 Rev;. (e) Intracellular cytokine staining 51 months post-initial virus exposure showing the

frequency of central memory (CM) and effector memory (EM) T-cell subsets expressing the indicated cytokines singly or in combinations following SIV Gag-peptide exposure. (f) Antigen-specific T-cell proliferation in responses to SIV Gag and neoantigens (SIV Nef and HIV-1 Rev). (g) SIV Gag peptides (15 amino acids in length and overlapping by 11) recognized by IFN- ELISPOT assay 68 months post-first SHIV-C challenge are indicated by underlining. Peptides were tested individually.

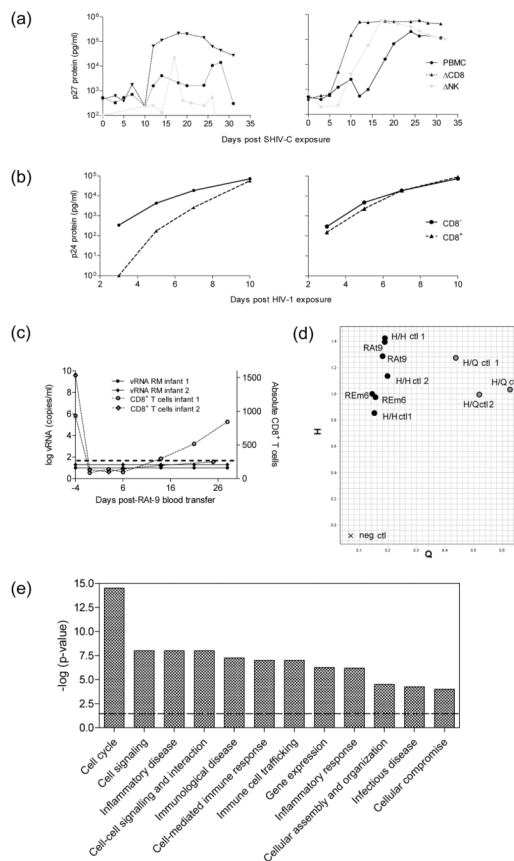


Fig. 2. In vitro and in vivo cell depletion studies, anti-HIV-1 activity of CD8⁺ cell soluble factors, KIR genotyping and gene expression analysis

(a) SHIV-1157ipEL-p replication in Rat-9 (left) PBMC with or without depletion of CD8⁺ T cells or NK cells. As control, PBMC of REM-6 (Rat-9's sister, right) were tested in parallel. (b) Mitogen stimulated, HIV-1 pNL4-3 (X4 strain)-exposed human CD4⁺ T cells from a healthy donor were incubated in the lower chamber of a Transwell plate insert (Corning) containing mitogen-stimulated CD8⁺ T cells from RAT-9 (left) or REM-6 (right) in the upper chamber of the plate. HIV-1 replication was monitored by measuring the concentration of HIV-1 p24 protein by ELISA. (c) Rat-9 blood transfer to anti-CD8 mAb (cM-T807)-treated infant RM. Naïve infant RM were treated with anti-CD8 mAb on days -4 and -1, and infused with 10 ml of whole blood from RAT-9 on d 0; anti-CD8 mAb was also given on days 3 and 6. Right Y-axis, open symbols: peripheral blood CD8⁺ T cells per μ l blood; left Y-axis, closed symbols: plasma viremia levels. (d) KIR3DL Allelic Discrimination Plot of RAT-9: 3 H/H homozygous controls (ctl) and 3 H/Q controls are shown as well as the expression plot for REM-6, RAT-9's sibling. (e) Functional pathway analysis of differentially expressed genes using Ingenuity pathway analysis (IPA) software. Dashed horizontal line indicates the significance threshold ($P=0.05$).