Published in final edited form as:

Vet Immunol Immunopathol. 2008 January 15; 121(1-2): 8–22. doi:10.1016/j.vetimm.2007.07.011.

Failure of low-dose recombinant human IL-2 to support the survival of virus-specific CTL clones infused into severe combined immunodeficient foals: lack of correlation between in vitro activity and in vivo efficacy

Robert H. Mealey^{*}, Matt H. Littke, Steven R. Leib, William C. Davis, and Travis C. McGuire
Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

Abstract

Although CTL are important for control of lentiviruses, including equine infectious anemia virus (EIAV), it is not known if CTL can limit lentiviral replication in the absence of CD4 help and neutralizing antibody. Adoptive transfer of EIAV-specific CTL clones into severe combined immunodeficient (SCID) foals could resolve this issue, but it is not known whether exogenous IL-2 administration is sufficient to support the engraftment and proliferation of CTL clones infused into immunodeficient horses. To address this question we adoptively transferred EIAV Rev-specific CTL clones into four EIAV-challenged SCID foals, concurrent with low-dose aldesleukin (180,000 U/m²), a modified recombinant human IL-2 (rhuIL-2) product. The dose was calculated based on the specific activity on equine PBMC in vitro, and resulted in plasma concentrations considered sufficient to saturate high affinity IL-2 receptors in humans. Despite specific activity on equine PBMC that was equivalent to recombinant equine IL-2 and another form of rhuIL-2, aldesleukin did not support the engraftment and expansion of infused CTL clones, and control of viral load and clinical disease did not occur. It was concluded that survival of Rev-specific CTL clones infused into EIAV-challenged SCID foals was not enhanced by aldesleukin at the doses used in this study, and that in vitro specific activity did not correlate with in vivo efficacy. Successful adoptive immunotherapy with CTL clones in immunodeficient horses will likely require higher doses of rhuIL-2, co-infusion of CD4⁺ T lymphocytes, or administration of equine IL-2.

Keywords

EIAV; horse; SCID; CTL; adoptive transfer; IL-2

1. Introduction

Significant progress has been made toward defining the mechanisms of immune control of lentiviral infections, and it is evident that viral specific CTL and CD4⁺ helper T lymphocytes are critically important in limiting lentiviral replication. Nonetheless lentiviruses persist, and

^{*}Corresponding author: Robert H. Mealey, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040. Telephone: (509) 335-6672. FAX: (509) 335-8529. rhm@vetmed.wsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

in HIV-1 infection, T cell responses ultimately fail to control virus replication and progression to AIDS results. Protective vaccines will undoubtedly need to induce CTL and CD4+ T cell responses, but development of such vaccines is hampered because the correlates of T cell-mediated protection are still not known. Specific knowledge gaps include how to induce and support a protective CTL response in the face of CD4+ T lymphocyte deficiency, the specific epitopes that must be recognized by CTL to control lentivirus replication, and the qualitative characteristics (i.e. functional avidity) of protective CTL.

A growing body of evidence indicates that CD8+ CTL are critical in containing lentiviral replication. The appearance of virus-specific CTL in the peripheral blood is temporally associated with the decline of primary viremia in acutely HIV-1-infected patients, and occurs well before serum neutralizing antibody activity is detected (Borrow et al., 1994; Koup et al., 1994). High levels of HIV-1-specific CTL are detected in HIV-1-infected clinical long-term nonprogressors (Rinaldo et al., 1995), and CTL activity is inversely correlated with viral load (Betts et al., 1999). Moreover, loss of HIV-1-specific CTL activity is associated with rapid clinical progression to AIDS (Klein et al., 1995). In addition, high numbers of tetramer-positive CD8+ T lymphocytes coincide with control of primary viremia in acutely HIV-1-infected patients (Wilson et al., 2000), and an inverse association between circulating tetramer-positive CD8+ T lymphocytes and viral load has been reported (Ogg et al., 1998). In the SIV system, emergence of CTL in rhesus monkeys coincides with virus clearance during primary infection (Kuroda et al., 1999). Moreover, depletion of CD8+ T lymphocytes in infected monkeys is associated with a rapid increase in viremia (Jin et al., 1999; Schmitz et al., 1999). Vaccines that induce high frequency SHIV-specific CTL responses in rhesus monkeys result in reduced viral loads and prevention of clinical AIDS following homologous SHIV challenge (Barouch et al., 2000; Barouch et al., 2001a; Barouch et al., 2001b).

Deficient CD4+ T lymphocyte help in HIV-1-infected individuals is likely the main determinant of the functional impairment and decline of virus-specific CD8+ CTL, leading to loss of immune control of viremia and clinical progression to AIDS. One mechanism by which CD4+ T cells support CTL proliferation and survival is by secretion of IL-2 (Altfeld and Rosenberg, 2000; Cheng et al., 2002; Lanzavecchia and Sallusto, 2000). In an effort to enhance T lymphocyte responses in HIV-1-infected patients, recombinant human IL-2 (rhuIL-2) administration has been used. In these patients, rhuIL-2 can augment immune responses and increase CD4+ T lymphocyte counts (Aladdin et al., 2000; Davey et al., 1997; Jacobson et al., 1996). Recombinant huIL-2 has also been administered to HIV-1-infected individuals to support adoptively transferred HIV-1-specific lymphocytes and CTL (Klimas et al., 1994; Koenig et al., 1995b; Tsoukas et al., 2001). In addition, low-dose aldesleukin, a modified form of rhuIL-2, has been used successfully to support adoptively transferred MART-1 specific CTL clones in metastatic melanoma patients (Yee et al., 2002).

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus that causes persistent infection in horses. Specific immune responses are required for EIAV control, since severe combined immunodeficient (SCID) foals fail to eliminate the initial viremia following challenge with EIAV, as compared to normal foals (Perryman et al., 1988). SCID in Arabian foals is caused by a frame-shift mutation in the gene encoding the catalytic subunit of DNA-dependent protein kinase (Shin et al., 1997; Wiler et al., 1995), and has an autosomal recessive mode of inheritance (Perryman and Torbeck, 1980). The equine SCID defect is more severe than its murine counterpart in that SCID foals are incapable of forming either coding or signal joints (Shin et al., 1997). Adoptive transfer of EIAV-specific T and B lymphocytes to a SCID foal results in functional CTL and neutralizing antibody activity, and is protective against EIAV challenge (Mealey et al., 2001). The specific contribution of infused CTL to the protective effects observed in this study was difficult to determine due to the neutralizing antibody response (Mealey et al., 2001).

As in HIV-1 and SIV, virus-specific CTL are critically important in EIAV control. The initial plasma viremia in acute EIAV infection is terminated prior to the appearance of neutralizing antibody, but concurrent with the appearance of CTL (Hammond et al., 1997; McGuire et al., 1994). CTL epitopes have been identified in Gag, Pol, Env, Rev, and in the protein encoded by the S2 open reading frame (Lonning et al., 1999; McGuire et al., 2000; Mealey et al., 2003; Zhang et al., 1998). Indirect evidence for CTL-mediated control of EIAV replication was provided by the observation that plasma viral variants escaped recognition of Env-specific CTL in an infected horse (Mealey et al., 2003).

The functional avidity of the interaction between CTL and their target cells determines the efficiency with which CTL recognize and kill their specific targets (Gallimore et al., 1998). In comparison to low avidity CTL, high avidity CTL of the same specificity recognize target cells expressing lower antigen density, and initiate lysis of targets more rapidly at any given antigen density (Derby et al., 2001). Adoptive transfer experiments in mice infected with LCMV as well as in mice infected with vaccinia virus constructs expressing HIV-1 proteins indicate that the in vivo protective effects of CTL correlate with their avidity (Alexander-Miller et al., 1996; Derby et al., 2001; Gallimore et al., 1998), but such studies have not been performed in a naturally occurring lentiviral infection. We have identified high avidity EIAV Gag-specific CTL in horses with attenuated clinical disease (Chung et al., 2005), and have shown that moderate to high avidity Rev-specific CTL are associated with control of viral load in EIAV-infected horses (Mealey et al., 2003). Significantly, no viral escape from high avidity CTL recognizing the Rev-QW11 epitope was observed. The region of Rev containing this epitope is highly conserved among strains of EIAV, and independent studies by other investigators show that this sequence does not change during long-term EIAV infection (Belshan et al., 2001; Leroux et al., 1997).

The goals of the current study were to determine whether low-dose aldesleukin was sufficient to support the engraftment and proliferation of CTL clones adoptively transferred into SCID foals, and ultimately, whether CTL could protect against lentiviral challenge in the absence of CD4⁺ T cell help and neutralizing antibody. Since SCID foals lack functional T and B lymphocytes, this unique model system allows assessment of the protective effects of lentiviral-specific CTL in the absence of other adaptive immune responses. Therefore, we infused high to moderate avidity Rev-QW11-specific CTL clones into four EIAV-challenged SCID foals, and administered aldesleukin subcutaneously at a dose of 180,000 U/m² (based on the specific activity on equine PBMC), once or twice daily.

2. Materials and Methods

2.1. Horses

Four SCID foals (A2193, A2199, A2202, and A2205) were obtained by selective breeding of Arabian mares and a stallion heterozygous for the SCID trait (Crawford et al., 1996; Mealey et al., 2001; Perryman et al., 1988; Perryman and Torbeck, 1980). Initial diagnosis of SCID was based on persistent lymphopenia (McGuire et al., 1974; Mealey et al., 2001), and confirmed by identification of the homozygous mutation in the DNA-PK(CS) gene sequence (Shin et al., 1997). For the first two days of life, SCID foals were housed in individual box stalls with their dams to allow ingestion of colostrum, and adequate passive transfer of maternal IgG was confirmed. Within three days of birth, a kidney biopsy was performed and equine kidney (EK) cells were established in cell culture for use as targets in CTL assays (McGuire et al., 1994; McGuire et al., 2000; Mealey et al., 2003). Foals were then removed from their dams, housed in isolation stalls, and fed a commercial mare's milk replacer. In an effort to prevent bacterial infection as well as opportunistic lung infection by *Pneumocystis carinii* (Perryman et al., 1978), SCID foals were administered systemic antibiotics, including one or more of the following: trimethoprim-sulfamethoxazole (20 mg/kg, PO, q 12 hrs), azithromycin

(10 mg/kg, PO, q 24 hrs), ceftiofur (2 mg/kg, IM or IV, q 12 hrs), and cefpodoxime (10 mg/kg, PO, q 12 hrs). Foals A2202 and A2205 also received weekly infusions of normal horse plasma containing antibodies against adenovirus (Perryman et al., 1978).

Horse A2150 is an eight-year-old Arabian mare that has been infected with EIAV_{WSU5} for seven years and has the equine lymphocyte antigen (ELA)-A1 haplotype (Mealey et al., 2003). In addition, A2150 has CTL directed against the conserved Rev-QW11 epitope (Mealey et al., 2003) that is presented by the ELA-A1-associated 7-6 and 141 MHC class I molecules (McGuire et al., 2003; Mealey et al., 2006). Because the sire (stallion A2152) of SCID foals has the 7-6 allele and is capable of presenting the Rev-QW11 epitope to A2150 CTL (Mealey et al., 2006), offspring inheriting the 7-6 allele from stallion A2152 would also have target cells recognized by A2150 Rev-QW11-specific CTL. In addition, the ELA-A1 haplotype is well-represented in our breeding herd of SCID-carrying Arabian mares. For these reasons, A2150 was chosen as the source of Rev-QW11-specific CTL for cloning and subsequent infusion into SCID foals A2193, A2199, A2202, and A2205. All experiments involving horses and foals were approved by the Washington State University Institutional Animal Care and Use Committee.

2.2. In vitro biologic activity of recombinant IL-2 (rIL-2) on equine PBMC

The ability of different forms of rIL-2 to cause proliferation of stimulated equine PBMC was determined as described (Gately et al., 1995) with modifications. Normal horse PBMC were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) and seeded into 175 cm² flasks at 1×10^8 cells per flask in 20 ml RPMI 1640 with 10% autologous serum, 10 µg/ml gentamicin, 50 µM 2-mercaptoethanol (culture media), and 10 µg/ml phytohemagglutinin-P (PHA-P). Cells were incubated at 37°C with 5% CO₂ for three days. The cells (now PHA-activated lymphoblasts) were then harvested, washed, and re-seeded into 175 cm² flasks at 6×10^5 /ml in 50 ml of culture media without PHA-P, but with 20 IU/ml rhuIL-2 (Roche Diagnostics, Indianapolis, IN). After an additional four days of incubation, the cells were again harvested and washed three times with HBSS to remove IL-2. The cells were suspended in culture media at 2×10^6 /ml and seeded into 96-well round bottom plates at 100 µl per well. Dilutions of different forms of rIL-2, including aldesleukin (Proleukin®, Chiron, Emeryville, CA), regular rhuIL-2 (Roche Diagnostics), and recombinant equine IL-2 (Pierce Endogen, Rockford, IL), were then added to the wells in triplicate, and the plates were incubated for two days. The cells were then labeled with 0.25 µCi ³H thymidine per well and incubated for an additional 16 to 20 h. The cells were then harvested and counts per minute determined by liquid scintillation. For each form of rIL-2, one unit of specific activity was defined as the concentration (ng/ml) that resulted in 50% maximal proliferation of equine PBMC, and was calculated by fitting the curve with nonlinear regression using GraphPad Prism version 3.03 (GraphPad Software, San Diego, CA).

2.3. Aldesleukin kinetics in SCID foals

Plasma concentrations of aldesleukin following SQ administration of 180,000 U/m² (one unit = concentration in ng/ml resulting in 50% maximal proliferation of equine PBMC as calculated above) to SCID foals were determined in duplicate at frequent intervals during a 24-hour period using a Human IL-2 ELISA Kit (Pierce Endogen, Rockford, IL) according to the manufacturer's instructions. Body surface area for dosing was calculated using the following formula: $1.09 + 0.008 \times \text{body weight (kg)}$ (Hodgson et al., 1993).

2.4. Generation and infusion of Rev-QW11-specific CTL clones

Rev-QW11-specific CTL clones were generated as described (Mealey et al., 2007). Briefly, PBMC were isolated from horse A2150 and initially stimulated with the Rev-QW11 peptide for one week. Viable cells were then isolated and CD3⁺/CD8⁺ cells were sorted into sterile

tubes by fluorescence-activated cell sorting (FACS) using anti-equine CD3 mAb F6G (Blanchard-Channell et al., 1994; Lunn et al., 1998), anti-equine CD8 mAb ETC91A (Tschetter et al., 1998), and a FACS Vantage Cell Sorter (Becton Dickinson, San Jose, CA). The sorted CD8⁺ T lymphocytes were counted and plated at limiting dilution (0.3 cells/well) into anti-CD3 (F6G) -coated U-bottom 96-well plates in 50 μ l culture medium containing 50 IU/ml rhuIL-2 (Roche Diagnostics) and 10³ irradiated allogeneic PBMC (irradiated with 3,000 rad using the cobalt-60 irradiation unit at the Washington State University Nuclear Radiation Center). Cloning plates were incubated for 7–14 days without manipulation, depending on rate of growth. Wells positive for clonal growth were further expanded and transferred into 24-well flat-bottom plates, then 6-well flat-bottom plates. After sufficient numbers of cells were generated, flow cytometry and CTL assays were performed to confirm phenotype and CTL activity as described (Mealey et al., 2005; Mealey et al., 2006; Mealey et al., 2007). Briefly, direct CTL activity of cloned CTL was measured using a ⁵¹Cr release assay with a 17-h incubation period using autologous (A2150) EK target cells. Target cells were pulsed with various amounts of Rev-QW11 peptide as indicated in the figures. The formula, % specific lysis = [(E-S)/(M - S)] $\times$ 100, was used, where E is the mean of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and M is the mean maximal release from three target cell wells with 2% Triton X-100 in distilled water. The E:T cell ratio was 20:1, and each well contained ~30,000 target cells. Only assays with a spontaneous target cell lysis of <30% were used. The SE of percent specific lysis was calculated using a formula that accounts for the variability of E, S, and M (Siliciano et al., 1985). Significant lysis was defined as the percent specific lysis of peptide-pulsed target cells that was >10% and also >3 SE above the nonpulsed target cells. Functional avidity of CTL clones was determined as described (Chung et al., 2005; Mealey et al., 2003), and was defined as the Rev-QW11 peptide concentration that resulted in 50% maximal specific lysis (EC₅₀) of A2150 EK target cells. The EC₅₀ was calculated after transforming the % specific lysis data to % maximal lysis (with the lowest % specific lysis value set to 0% and the highest % specific lysis value set to 100%) and fitting the curve with nonlinear regression using GraphPad Prism version 3.03 (GraphPad Software). A CTL clone was considered to have high functional avidity if the EC₅₀ was 11 nM or less (Chung et al., 2005). Expanded cells of each CTL clone were then aliquotted and cryopreserved in liquid nitrogen until SCID foals were available.

After an adequate number of EK cells were available following the birth of a SCID foal, a CTL assay was performed using these EK targets incubated (pulsed) with the Rev-QW11 peptide, and Rev-QW11-stimulated effectors from A2150 (the source of the CTL clones). If significant specific lysis was observed, then the experiment could proceed, and cryopreserved cloned CTL were thawed and expanded as before (Mealey et al., 2007). When adequate numbers of cloned CTL became available, lysis of Rev-QW11-pulsed recipient SCID foal EK targets was confirmed. Prior to infusion into a SCID foal, cloned CTL were harvested from the culture plates, layered over Ficoll-Paque Plus to remove dead cells, and washed in Hanks balanced salt solution (HBSS). After washing, the cells were resuspended in 100 ml sterile HBSS and infused IV over 10 minutes. Foals were inoculated IV with 10⁶ TCID₅₀ EIAV_{WSU5} (Mealey et al., 2001; O'Rourke et al., 1989; Perryman et al., 1988) either prior to CTL clone infusion, or on the day of CTL clone infusion (Table 1).

2.5. Immunologic and virologic monitoring

For each SCID foal, rectal temperatures and clinical status were recorded daily, and CBC with platelet counts were performed on whole blood collected every 2 days. Platelet counts less than 100,000 / μ l were considered thrombocytopenic. For determination of viral load, plasma was collected and stored at -80°C every 2 days. Whole blood was collected weekly for isolation of PBMC for flow cytometry and CTL assays. Plasma viral load was determined by extracting plasma viral RNA and performing real-time quantitative RT-PCR as described (Mealey et al.,

2005). To determine if Rev-QW11 escape variants occurred, the EIAV *rev* gene was amplified from extracted plasma viral RNA by RT-PCR, then cloned and sequenced as described (Mealey et al., 2003).

3. Results

3.1. Specific activities of aldesleukin, regular rhuIL-2, and recombinant equine IL-2 (reqIL-2) on equine PBMC

Aldesleukin differs from native huIL-2 in that it is not glycosylated, it has no N-terminal alanine, and serine is substituted for cysteine at amino acid position 125 (rhuIL-2₁₂₅) (Baigent, 2002). The lack of glycosylation is common to other forms of recombinant IL-2 and is due to production in *E. coli*. The amino acid changes were introduced to create a more homogeneous population of molecules that fold only in the biologically active conformation (Baigent, 2002; Doyle et al., 1985; Wang et al., 1984). The ability of rhuIL-2₁₂₅ to support PBMC proliferation is equivalent to that of native huIL-2 (Doyle et al., 1985), but the serine substitution results in a greater specific activity (IU/mg) than that of unmodified rhuIL-2 (Wang et al., 1984). In addition, rhuIL-2₁₂₅ supports the proliferation of equine PBMC in a dose-dependent manner, as well as the long-term (up to six months) proliferation of equine PBMC (Doyle et al., 1985). Aldesleukin was used in the current study for the reasons above, and because it is readily available for clinical use. Moreover, aldesleukin was not cost-prohibitive (like other forms of rhuIL-2 and reqIL-2) at the dosage and duration of administration used.

To confirm that aldesleukin was effective in causing proliferation of equine cells as reported, and to compare its effects to other forms of recombinant IL-2, the specific activities of aldesleukin, regular rhuIL-2 (Roche Diagnostics), and reqIL-2 (Pierce Endogen) were determined on PHA-stimulated equine PBMC. One unit of specific activity was defined as the concentration in ng/ml required to induce 50% maximal proliferation, and was calculated by nonlinear regression analysis of the proliferation curves. Although aldesleukin resulted in a lower peak level of proliferation than regular rhuIL-2 and reqIL-2 (Fig. 1a), its calculated specific activity (0.7 ng/ml) on equine PBMC was not significantly different than that of reqIL-2 (0.9 ng/ml), or regular rhuIL-2 (0.5 ng/ml) (Fig. 1b). Although the specific activity of regular rhuIL-2 on equine PBMC was the same as that reported in the product specifications as determined on mouse CTLL-2 cells (1 IU = 0.5 ng/ml), the specific activity of reqIL-2 on equine PBMC was over 2,000-fold greater than that reported in the product specifications as determined on mouse CTLL-2 cells (1 IU = 2 µg/ml). However, the labeled specific activity of aldesleukin (18 million IU = 1.1 mg/ml, or 1 IU = 0.06 ng/ml) was 11.7 times greater than that determined on equine PBMC (Table 2). Therefore, instead of its labeled specific activity, the specific activity of aldesleukin measured on equine PBMC (0.7 ng/ml) was used to calculate the 180,000 U/m² dose administered to the foals in this study.

3.2. Subcutaneous administration of aldesleukin in SCID foals is well-tolerated and results in plasma concentrations comparable to those considered therapeutic in humans

Since the kinetics of aldesleukin in Arabian SCID foals was not known, plasma concentrations were determined following aldesleukin administration (180,000 U/m², SQ) in two one-month-old Arabian SCID foals. This dose was chosen based on human pharmacokinetic data (Jacobson et al., 1996; Sundin and Wolin, 1998), and was near the low end of the maximum non-toxic dose (Jacobson et al., 1996). One of the foals (A2193) was subsequently used in the adoptive transfer study, while the other foal (A2184) was not used due to MHC class I incompatibility with the CTL clone donor. The plasma concentrations of aldesleukin peaked between two and four hours after SQ administration, and ranged from 21 to 57 pM (equivalent to 0.3 to 0.9 ng/ml) (Fig. 2). These peak concentrations and time to peak concentration were similar to those

obtained in human patients receiving SQ rhuIL-2 doses of 250,000 to 500,000 IU/m² (Jacobson et al., 1996). Antigen activated human T and B lymphocytes express high affinity IL-2 receptors and bind IL-2 at very low concentrations, within the range of 1–100 pM (Jacobson et al., 1996; Smith, 1989). Thus, the plasma aldesleukin concentrations achieved following SQ administration in SCID foals were compatible with those capable of saturating high affinity IL-2 receptors on human T lymphocytes (Jacobson et al., 1996).

3.3. Effects of Rev-QW11-specific CTL clone infusions and daily or twice daily SQ administration of aldesleukin in SCID foals

Four SCID foals with EK target cells that were recognized by A2150 Rev-QW11-specific CTL effectors were available for this study. These SCID foals received infusions of 2.4×10^8 to 1.8×10^9 cloned Rev-QW11-specific CTL, were administered 180,000 U/m² aldesleukin (based on specific activity on equine PBMC) SQ once or twice daily, and were challenged with 1×10^6 TCID₅₀ EIAV_{WSU5} at various time points (Table 1). The CTL clones used for infusion were chosen based on their functional avidity (Mealey et al., 2003), which was determined previously on A2150 EK targets for four of the five infused clones. These CTL clones had high (EC₅₀ ≤ 11 nM) to moderate functional avidity (Fig. 3).

SCID foal A2193 received 1.0×10^9 cells of the high avidity Rev-QW11-specific CTL clone R8-0603 (Fig. 3), concurrent with EIAV inoculation. Prior to infusion, the expanded cells of CTL clone R8-0603 were 96.4% CD3+/CD8+, and resulted in 36% specific lysis of Rev-QW11-pulsed A2193 EK target cells (Fig. 4a). After CTL clone infusion, aldesleukin was administered once daily for the duration of the experiment. Based on CBC analysis, no significant increase in peripheral blood lymphocyte (PBL) numbers occurred following CTL clone infusion (Fig. 5a). Plasma viral RNA was first detected seven days post EIAV inoculation (DPI), and peaked at 4.9×10^6 copies/ml on 29 DPI (Fig. 5a). Concurrent with increasing viral load, platelet counts began to decrease 14 DPI (from a mean of 305,000 /μl), reaching a low of 44,000 /μl on DPI 29 (Fig. 5a). Although no increase in peripheral blood CD3+/CD8+ cells was detected by flow cytometry, a transient increase in peripheral blood CD2+/CD8+ cells occurred (Fig. 6a). These were likely NK cells (Lunn et al., 1995; Magnuson et al., 1987), which could have proliferated in response to aldesleukin administration, or in response to the CTL infusion. However, the number of detected CD2+/CD8+ cells in A2193 was much lower than that detected in a normal horse control (Fig. 6a). The normal horse control values were consistent with those previously published (Hines et al., 1996). At no time was CTL activity detected in A2193 PBMC (data not shown). Due to EIAV-induced clinical disease, A2193 was euthanized 29 DPI. Cloned viral RNA sequences from A2193 plasma obtained at DPI 9, 16, and 28 indicated that the predominant plasma viral species at each time point did not contain amino acid changes within or flanking the Rev-QW11 epitope (data not shown). Thus, selection of CTL escape variants did not contribute to the lack of viral control, as has been observed following adoptive transfer of an HIV-1 Nef-specific CTL clone (Koenig et al., 1995a). In contrast to our current results, we previously identified significant amino acid variation within an EIAV envelope CTL epitope (Env-RW12) in plasma viral RNA obtained from an EIAV-challenged SCID foal that was infused with EIAV-specific lymphocytes, including CTL directed against the epitope (Mealey et al., 2004).

SCID foal A2199 was inoculated with EIAV six days prior to infusion with CTL clones R5.1-0404 (1.2×10^8 cells) and R9-0603 (1.2×10^8 cells). Clone R9-0603 had high functional avidity (Fig. 3), while the avidity of clone R5.1-0404 was not determined due to an insufficient number of cells. The timing of virus inoculation and CTL infusion was chosen so that the infused CTL would be more likely to encounter antigen early and thus proliferate. This was a possible contributing factor in the apparent failure of CTL engraftment in foal A2193. Because neither clone R5.1-0404 nor R9-0603 expanded in culture to the desired number of cells (at

least 1.0×10^9) in the time-frame needed, both clones were infused together into A2199 so that the greatest number of cells possible was infused. Both clones had a CD3+/CD8+ phenotype, and both displayed Rev-QW11-specific CTL activity against A2199 EK target cells (Fig. 4b). To further enhance the probability of CTL engraftment and expansion after infusion, A2199 received twice daily aldesleukin administration. However, no increase in total PBL, CD3+/CD8+ cells, or CD2+/CD8+ cells was observed, and viral load and platelet count measurements were similar to those in A2193 (Figs. 5b and 6b). No CTL activity was detected at any time point (data not shown). The experiment was terminated early due to secondary adenoviral pneumonia (confirmed at necropsy), which necessitated euthanasia of foal A2199 on DPI 14. Although adenoviral pneumonia is a common complication in SCID foals (Perryman et al., 1978), neither A2193 nor A2199 were administered adenoviral immune plasma due to the possibility of introducing anti-lymphocyte alloantibodies that could result in lysis of the infused CTL. However, based on the experience with A2199, subsequent SCID foals received weekly infusions of adenoviral immune plasma (Perryman et al., 1978). Prior to use, this plasma was determined to be free of anti-lymphocyte alloantibodies capable of lysing A2150 PBMC (the source of the CTL clones) in a lymphocytotoxicity assay (Bailey, 1980; Terasaki et al., 1978) (data not shown).

SCID foal A2202 also was inoculated with EIAV six days prior to CTL clone infusion. This foal received 1.8×10^9 cells of moderately high avidity CTL clone R5-0603 (Fig. 3), which had a CD3+/CD8+ phenotype and Rev-QW11-specific CTL activity against A2202 EK target cells (Fig. 4c). Similar to the previous two foals, viral load increased while platelet counts decreased, and A2202 was euthanized due to EIAV-induced clinical disease on DPI 30 (Fig. 5c). Although there were transient increases in platelet count (DPI 12) and total PBL count (DPI 14), there was no corresponding decrease in viral load at these time points. Despite a transient and slight increase in CD3+/CD8+ and CD2+/CD8+ cells the day after infusion, cells with these phenotypes were not detected thereafter (Fig. 6c), and CTL activity was not detected at any time (data not shown).

The last SCID foal, A2205, received 1.6×10^9 cells of moderate avidity CTL clone R10-0603 (Fig. 3), 10 days after EIAV inoculation. Clone R10-0603 had a CD3+/CD8+ phenotype, and Rev-QW11-specific CTL activity against A2205 EK target cells (Fig. 4d). Since twice daily administration of aldesleukin offered no advantage over once daily administration in the previous two foals, aldesleukin was administered once daily to A2205. No increases in PBL, CD3+/CD8+ cells or CD2+/CD8+ cells occurred, and the outcome was similar to that of the previous three foals (Figs. 5d and 6d). At no time was CTL activity detected in A2205 PBMC (data not shown).

4. Discussion

This study was designed to test the hypothesis that high to moderate avidity Rev-QW11-specific CTL clones could protect SCID foals against EIAV challenge. To promote survival and proliferation of the infused CTL clones in the absence of CD4+ T cell help, aldesleukin, a modified form of rhuIL-2, was administered at a dose of 180,000 U/m² (based on specific activity on equine PBMC) SQ to each foal, either once or twice daily. Despite the ability of aldesleukin to cause proliferation of equine lymphocytes in vitro, and the ability to achieve plasma levels that would be considered efficacious in humans, engraftment and proliferation of infused CTL clones was not detected in the foals of this study. No protective effects associated with CTL clone infusion or aldesleukin administration were observed.

A transient increase in CD2+/CD8+ cells, but not CD3+/CD8+ cells, was detected in the peripheral blood of two foals after CTL clone infusion. These cells were most likely NK cells, which occur in SCID foals (Lunn et al., 1995; Wyatt et al., 1987), and can proliferate and

develop cytotoxicity against murine YAC-1 lymphoma or human K562 erythroleukemia cell lines following treatment with IL-2 (Magnuson et al., 1984; Magnuson et al., 1987). These cells could have briefly proliferated in the two foals of the current study in response to aldesleukin treatment, as subcutaneous administration of rhuIL-2 results in expansion of peripheral blood NK cells in humans (Jacobson et al., 1996). Alternatively, these cells could have proliferated in response to heterologous MHC class I molecules present on the infused CTL clones. Although CTL assays confirmed that the SCID foal recipients shared ELA-A1-associated MHC class I molecules with the CTL clone donor (A2150), A2150 had a different dam and sire than did each of the SCID foals. Thus, the CTL clones most likely expressed MHC class I molecules not shared by the SCID foal recipients, and these heterologous molecules could have resulted in NK cell activation. It is therefore possible that cytotoxicity elicited by NK cells contributed to elimination of infused CTL clones. Due to this possibility, pretreatment with immunosuppressive drugs to decrease NK cell activity prior to CTL infusion might have been beneficial. NK cell activity was not measured in the current study, and further work is needed to confirm any role NK cells might have in the elimination of CTL clones infused into SCID foals.

Other adoptive transfer studies in animals and humans have evaluated single or multiple infusions using on the order of 10^7 to 10^{11} lentivirus or melanoma-specific T-cells, with or without IL-2 administration (Brodie et al., 1999; Brodie et al., 2000; Dudley et al., 2002; Flynn et al., 2005; Ho et al., 1993; Klimas et al., 1994; Mitsuyasu et al., 2000; Pu et al., 1999; Villinger et al., 2002; Walker et al., 2000; Yee et al., 2002). Studies in mice indicate that immune CD4⁺ T cells are required for survival of infused virus-specific CD8⁺ T cells (Berger et al., 2000; Hunziker et al., 2002). In HIV-1-infected patients, co-infusion of autologous or syngeneic CD4⁺ T cells, or treatment with rhuIL-2, enhances the long term survival of infused autologous or syngeneic CD8⁺ T cells (Mitsuyasu et al., 2000; Walker et al., 2000). Similarly, the median survival of melanoma-specific autologous CTL clones infused into human patients increases from 6.7 days to 16.9 days with rhuIL-2 treatment (Yee et al., 2002). In other studies, adoptively transferred HIV-1-specific autologous CTL clones traffic to lymphoid tissues and elicit anti-viral effects, without rhuIL-2 treatment (Brodie et al., 1999; Brodie et al., 2000). The number of infused autologous CD8⁺ T cells in the peripheral blood of human patients peaks by 48 hours post infusion (Brodie et al., 2000; Walker et al., 2000), and the majority of infused cells are cleared from the circulation shortly thereafter (Brodie et al., 2000; Ho et al., 1993; Walker et al., 2000), accumulating in the lungs and secondary lymphoid organs (Ho et al., 1993; Walker et al., 2000). Autologous HIV-1-specific CTL clones can be rapidly eliminated within hours of infusion into human patients not receiving rhuIL-2 or CD4⁺ T cell co-infusions, presumably due to apoptosis as a result of antigen-specific cell death (Tan et al., 1999). Conversely, large numbers ($\sim 10^{11}$) of tumor-reactive autologous T lymphocytes infused into lymphodepleted human melanoma patients, concurrent with high dose rhuIL-2 therapy, can result in persistent clonal lymphocyte repopulation and tumor regression (Dudley et al., 2002).

Given the large variability in T cell adoptive transfer protocols, the numbers of infused CD8⁺ T cell clones in the present study were similar to that used by some investigators, and less than that used by others. A previous infusion of 4.5×10^9 EIAV-stimulated CD8⁺ T cells together with 9.2×10^9 EIAV-stimulated CD4⁺ cells resulted in engraftment and prolonged expansion in a SCID foal, with protective effects against EIAV challenge (Mealey et al., 2001). The number of EIAV-specific CTL in the infused population was estimated to be 6.1×10^6 by limiting dilution analysis. The infused lymphocytes (which were derived from a half-sibling donor and were homologous at the detectable MHC class I and II loci), were first detected in the peripheral blood five days post-infusion, and were readily identified thereafter in the circulation by flow cytometry and CTL assays. Because the infused lymphocytes were derived from EIAV-stimulated bulk PBMC, they also contained EIAV-specific B cells. As a result, the SCID foal recipient developed EIAV-specific neutralizing antibody, and the protective

effects of the infused CTL could not be specifically dissected (Mealey et al., 2001). Thus, the present study was designed to determine the role of CTL alone in protection against EIAV. Although the total number of CD3+/CD8+ cells infused into each foal in the current study was somewhat less than the total number of CD3+/CD8+ cells infused in the previous successful study, the number of EIAV-specific CTL infused into each foal in the current study was far greater. Since CD8+ T cells infused into humans can rapidly clear the circulation and traffic to the lungs and lymphoid organs, it is possible that this occurred in the present study, contributing to the inability to detect the infused CTL clones in the peripheral blood with the methods used. Accumulation of CD8+ T cells in the lungs and lymphoid organs was not assessed in the current study. Alternatively, the infused Rev-specific CTL clones could have undergone rapid apoptosis upon encounter with antigen, as has been reported with HIV-1-specific CTL clones infused into human patients (Tan et al., 1999).

While the lower number of infused cells could have been a factor in the failure to detect engraftment and proliferation in the present study, it is likely that inadequate levels of in vivo IL-2 activity and/or the lack of CD4+ T cell help were more significant factors. Given our finding that aldesleukin was 11.7 times less effective in causing equine PBMC proliferation than the labeled specific activity (IU) would indicate, we calculated the administered dose of 180,000 U/m² based on our measured specific activity on PHA-activated equine lymphoblasts. This is considered a low dose in humans, effective in stimulating immune reactivity but less than the maximum non-toxic dose (Jacobson et al., 1996). Although the concentration of IL-2 required to saturate high affinity receptors on equine T cells is not known, it is probable that our aldesleukin dose was either not high enough to support the survival of the infused IL-2-dependent CTL clones, or that equine IL-2 will be required for in vivo efficacy in the horse. Even though the labeled activities (IU) for each product were different (labeled potency of aldesleukin >> labeled potency of regular rhuIL-2 >> labeled potency of reqIL-2), all three had equivalent specific activities on equine PBMC on a ng/ml basis. Given this equivalency, and the fact that aldesleukin is by far the least expensive per ng of the three, aldesleukin remains an attractive choice for administration to equine foals. However, further investigation of the effects of increased dosages is warranted, especially since aldesleukin resulted in a lower level of maximal proliferation than did regular rhuIL-2 and reqIL-2. Alternatively, administration of reqIL-2, or co-infusion of EIAV-specific CD4+ T cells, may be required for successful engraftment and survival of EIAV-specific CTL clones infused into SCID foals.

In summary, SCID foals provide a means to dissect the correlates of lentivirus immune control that is unavailable in any other model system. Adoptive transfer of EIAV-specific CTL clones to EIAV-challenged SCID foals provides a rigorous way to test the hypothesis that CTL can control a lentivirus in the absence of other mechanisms of adaptive immunity. Future studies should focus on methods to generate and infuse greater numbers of homologous cloned CTL with high levels of CTL activity and functional avidity, as well as methods to support the prolonged survival of infused CTL by co-infusion of homologous immune CD4+ T cells, administration of higher doses of rhuIL-2, or administration equine IL-2.

Acknowledgments

This work was supported in part by U.S. Public Health Service grants AI058787, AI067125, and AI060395 from the National Institute of Allergy and Infectious Disease.

The important technical assistance of Emma Karel and Lori Fuller is acknowledged.

References

Aladdin H, Larsen CS, Moller BK, Ullum H, Buhl MR, Gerstoft J, Skinhoj P, Pedersen BK. Effects of subcutaneous interleukin-2 therapy on phenotype and function of peripheral blood mononuclear cells

- in human immunodeficiency virus infected patients. *Scand J Immunol* 2000;51:168–175. [PubMed: 10722371]
- Alexander-Miller MA, Leggatt GR, Berzofsky JA. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc Natl Acad Sci USA* 1996;93:4102–4107. [PubMed: 8633023]
- Altfeld M, Rosenberg ES. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr Opin Immunol* 2000;12:375–380. [PubMed: 10899028]
- Baigent G. Recombinant Interleukin-2 (rIL-2), aldesleukin. *J Biotechnol* 2002;95:277–280. [PubMed: 12007868]
- Bailey E. Identification and genetics of horse lymphocyte alloantigens. *Immunogenetics* 1980;11:499–506. [PubMed: 6242885]
- Barouch DH, Craiu A, Santra S, Egan MA, Schmitz JE, Kuroda MJ, Fu TM, Nam JH, Wyatt LS, Lifton MA, Krivulka GR, Nickerson CE, Lord CI, Moss B, Lewis MG, Hirsch VM, Shiver JW, Letvin NL. Elicitation of high-frequency cytotoxic T-lymphocyte responses against both dominant and subdominant simian-human immunodeficiency virus epitopes by DNA vaccination of rhesus monkeys. *J Virol* 2001a;75:2462–2467. [PubMed: 11160750]
- Barouch DH, Santra S, Kuroda MJ, Schmitz JE, Plishka R, Buckler-White A, Gaitan AE, Zin R, Nam JH, Wyatt LS, Lifton MA, Nickerson CE, Moss B, Montefiori DC, Hirsch VM, Letvin NL. Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J Virol* 2001b;75:5151–5158. [PubMed: 11333896]
- Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W, Biliska M, Craiu A, Zheng XX, Krivulka GR, Beaudry K, Lifton MA, Nickerson CE, Triglona WL, Punt K, Freed DC, Guan L, Dubey S, Casimiro D, Simon A, Davies ME, Chastain M, Strom TB, Gelman RS, Montefiori DC, Lewis MG, Emini EA, Shiver JW, Letvin NL. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 2000;290:486–492. [PubMed: 11039923]
- Belshan M, Baccam P, Oaks JL, Sponseller BA, Murphy SC, Cornette J, Carpenter S. Genetic and biological variation in equine infectious anemia virus Rev correlates with variable stages of clinical disease in an experimentally infected pony. *Virology* 2001;279:185–200. [PubMed: 11145901]
- Berger DP, Homann D, Oldstone MB. Defining parameters for successful immunocytotherapy of persistent viral infection. *Virology* 2000;266:257–263. [PubMed: 10639312]
- Betts MR, Krowka JF, Kepler TB, Davidian M, Christopherson C, Kwok S, Louie L, Eron J, Sheppard H, Frelinger JA. Human immunodeficiency virus type 1-specific cytotoxic T lymphocyte activity is inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS Res Hum Retroviruses* 1999;15:1219–1228. [PubMed: 10480635]
- Blanchard-Channell M, Moore PF, Stott JL. Characterization of monoclonal antibodies specific for equine homologues of CD3 and CD5. *Immunology* 1994;82:548–554. [PubMed: 7530685]
- Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 1994;68:6103–6110. [PubMed: 8057491]
- Brodie SJ, Lewinsohn DA, Patterson BK, Jiyamapa D, Krieger J, Corey L, Greenberg PD, Riddell SR. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat Med* 1999;5:34–41. [PubMed: 9883837]
- Brodie SJ, Patterson BK, Lewinsohn DA, Diem K, Spach D, Greenberg PD, Riddell SR, Corey L. HIV-specific cytotoxic T lymphocytes traffic to lymph nodes and localize at sites of HIV replication and cell death. *J Clin Invest* 2000;105:1407–1417. [PubMed: 10811848]
- Cheng LE, Ohlen C, Nelson BH, Greenberg PD. Enhanced signaling through the IL-2 receptor in CD8+ T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8+ T cells rather than promotion of cell death. *Proc Natl Acad Sci USA* 2002;99:3001–3006. [PubMed: 11867736]
- Chung C, Mealey RH, McGuire TC. Evaluation of high functional avidity CTL to Gag epitope clusters in EIAV carrier horses. *Virology* 2005;342:228–239. [PubMed: 16139857]

- Crawford TB, Wardrop KJ, Tornquist SJ, Reilich E, Meyers KM, McGuire TC. A primary production deficit in the thrombocytopenia of equine infectious anemia. *J Virol* 1996;70:7842–7850. [PubMed: 8892906]
- Davey RTJ, Chaitt DG, Piscitelli SC, Wells M, Kovacs JA, Walker RE, Falloon J, Polis MA, Metcalf JA, Masur H, Fyfe G, Lane HC. Subcutaneous administration of interleukin-2 in human immunodeficiency virus type 1-infected persons. *J Infect Dis* 1997;175:781–789. [PubMed: 9086130]
- Derby M, Alexander-Miller M, Tse R, Berzofsky J. High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. *J Immunol* 2001;166:1690–1697. [PubMed: 11160212]
- Doyle MV, Lee MT, Fong S. Comparison of the biological activities of human recombinant interleukin-2 (125) and native interleukin-2. *J Biol Response Mod* 1985;4:96–109. [PubMed: 3920358]
- Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–854. [PubMed: 12242449]
- Flynn JN, Pistello M, Isola P, Zaccaro L, Del Santo B, Ricci E, Matteucci D, Bendinelli M. Adoptive immunotherapy of feline immunodeficiency virus with autologous ex vivo-stimulated lymphoid cells modulates virus and T-cell subsets in blood. *Clin Diagn Lab Immunol* 2005;12:736–745. [PubMed: 15939748]
- Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM, Rammensee HG. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J Exp Med* 1998;187:1647–1657. [PubMed: 9584143]
- Gately, MK.; Chizzonite, R.; Presky, DH. *Current Protocols in Immunology*. John Wiley & Sons, Inc; 1995. Measurement of human and mouse interleukin-12; p. 6.16.1-6.16.15. Ref Type: Serial (Book, Monograph)
- Hammond SA, Cook SJ, Lichtenstein DL, Issel CJ, Montelaro RC. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. *J Virol* 1997;71:3840–3852. [PubMed: 9094660]
- Hines MT, Palmer GH, Byrne KM, Brassfield AL, McGuire TC. Quantitative characterization of lymphocyte populations in bronchoalveolar lavage fluid and peripheral blood of normal adult Arabian horses. *Vet Immunol Immunopathol* 1996;51:29–37. [PubMed: 8797274]
- Ho M, Armstrong J, McMahon D, Pazin G, Huang XL, Rinaldo C, Whiteside T, Tripoli C, Levine G, Moody D, et al. A phase 1 study of adoptive transfer of autologous CD8+ T lymphocytes in patients with acquired immunodeficiency syndrome (AIDS)-related complex or AIDS. *Blood* 1993;81:2093–2101. [PubMed: 8471768]
- Hodgson DR, McCutcheon LJ, Byrd SK, Brown WS, Bayly WM, Brengelmann GL, Gollnick PD. Dissipation of metabolic heat in the horse during exercise. *J Appl Physiol* 1993;74:1161–1170. [PubMed: 8482654]
- Hunziker L, Klennerman P, Zinkernagel RM, Ehl S. Exhaustion of cytotoxic T cells during adoptive immunotherapy of virus carrier mice can be prevented by B cells or CD4+ T cells. *Eur J Immunol* 2002;32:374–382. [PubMed: 11813156]
- Jacobson EL, Pilaro F, Smith KA. Rational interleukin 2 therapy for HIV positive individuals: daily low doses enhance immune function without toxicity. *Proc Natl Acad Sci USA* 1996;93:10405–10410. [PubMed: 8816813]
- Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 1999;189:991–998. [PubMed: 10075982]
- Klein MR, Van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, Eeftink Schattenkerk JK, Osterhaus AD, Schuitemaker H, Miedema F. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* 1995;181:1365–1372. [PubMed: 7699324]

- Klimas N, Patarca R, Walling J, Garcia R, Mayer V, Moody D, Okarma T, Fletcher MA. Clinical and immunological changes in AIDS patients following adoptive therapy with activated autologous CD8 T cells and interleukin-2 infusion. *AIDS* 1994;8:1073–1081. [PubMed: 7986402]
- Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, Boots LJ, Davey V, Pantaleo G, Demarest JF, Carter C, et al. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat Med* 1995a;1:330–336. [PubMed: 7585062]
- Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, Boots LJ, Davey V, Pantaleo G, Demarest JF, Carter C, et al. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat Med* 1995b;1:330–336. [PubMed: 7585062]
- Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994;68:4650–4655. [PubMed: 8207839]
- Kuroda MJ, Schmitz JE, Charini WA, Nickerson CE, Lifton MA, Lord CI, Forman MA, Letvin NL. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J Immunol* 1999;162:5127–5133. [PubMed: 10227983]
- Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells [In Process Citation]. *Science* 2000;290:92–97. [PubMed: 11021806]
- Leroux C, Issel CJ, Montelaro RC. Novel and dynamic evolution of equine infectious anemia virus genomic quasispecies associated with sequential disease cycles in an experimentally infected pony. *J Virol* 1997;71:9627–9639. [PubMed: 9371627]
- Lonning SM, Zhang W, Leib SR, McGuire TC. Detection and induction of equine infectious anemia virus-specific cytotoxic T-lymphocyte responses by use of recombinant retroviral vectors. *J Virol* 1999;73:2762–2769. [PubMed: 10074123]
- Lunn DP, Holmes MA, Antczak DF, Agerwal N, Baker J, Bendali-Ahcene S, Blanchard-Channell M, Byrne KM, Cannizzo K, Davis W, Hamilton MJ, Hannant D, Kondo T, Kydd JH, Monier MC, Moore PF, O'Neil T, Schram BR, Sheoran A, Stott JL, Sugiura T, Vagnoni KE. Report of the Second Equine Leucocyte Antigen Workshop, Squaw valley, California, July 1995. *Vet Immunol Immunopathol* 1998;62:101–143. [PubMed: 9638857]
- Lunn DP, McClure JT, Schobert CS, Holmes MA. Abnormal patterns of equine leucocyte differentiation antigen expression in severe combined immunodeficiency foals suggests the phenotype of normal equine natural killer cells. *Immunology* 1995;84:495–499. [PubMed: 7751035]
- Magnuson NS, Perryman LE, Wyatt CR, Ishizaka T, Mason PH, Namen AE, Banks KL, Magnuson JA. Continuous cultivation of equine lymphocytes: evidence for occasional T cell-like maturation events in horses with hereditary severe combined immunodeficiency. *J Immunol* 1984;133:2518–2524. [PubMed: 6207234]
- Magnuson NS, Perryman LE, Wyatt CR, Mason PH, Talmadge JE. Large granular lymphocytes from SCID horses develop potent cytotoxic activity after treatment with human recombinant interleukin 2. *J Immunol* 1987;139:61–67. [PubMed: 3108403]
- McGuire TC, Leib SR, Lonning SM, Zhang W, Byrne KM, Mealey RH. Equine infectious anaemia virus proteins with epitopes most frequently recognized by cytotoxic T lymphocytes from infected horses. *J Gen Virol* 2000;81:2735–2739. [PubMed: 11038386]
- McGuire TC, Leib SR, Mealey RH, Fraser DG, Prieur DJ. Presentation and binding affinity of equine infectious anemia virus CTL envelope and matrix protein epitopes by an expressed equine classical MHC class I molecule. *J Immunol* 2003;171:1984–1993. [PubMed: 12902502]
- McGuire TC, Poppie MJ, Banks KL. Combined (B- and T-lymphocyte) immunodeficiency: a fatal genetic disease in Arabian foals. *Journal Of The American Veterinary Medical Association* 1974;164:70–76. [PubMed: 4358832]
- McGuire TC, Tumas DB, Byrne KM, Hines MT, Leib SR, Brassfield AL, O'Rourke KI, Perryman LE. Major histocompatibility complex-restricted CD8+ cytotoxic T lymphocytes from horses with equine infectious anemia virus recognize Env and Gag/PR proteins. *J Virol* 1994;68:1459–1467. [PubMed: 8107209]

- Mealey RH, Fraser DG, Oaks JL, Cantor GH, McGuire TC. Immune reconstitution prevents continuous equine infectious anemia virus replication in an arabian foal with severe combined immunodeficiency: lessons for control of lentiviruses. *Clin Immunol* 2001;101:237–247. [PubMed: 11683583]
- Mealey RH, Lee JH, Leib SR, Littke MH, McGuire TC. A single amino acid difference within the alpha-2 domain of two naturally occurring equine MHC class I molecules alters the recognition of Gag and Rev epitopes by equine infectious anemia virus-specific CTL. *J Immunol* 2006;177:7377–7390. [PubMed: 17082657]
- Mealey RH, Leib SR, Pownder SL, McGuire TC. Adaptive immunity is the primary force driving selection of equine infectious anemia virus envelope SU variants during acute infection. *J Virol* 2004;78:9295–9305. [PubMed: 15308724]
- Mealey RH, et al. Cloning and large-scale expansion of epitope-specific cytotoxic T lymphocytes using an anti-equine CD3 monoclonal antibody and human recombinant IL-2. *Vet Immunol Immunopathol*. 2007;10.1016/j.vetimm.2007.04.001
- Mealey RH, Sharif A, Ellis SA, Littke MH, Leib SR, McGuire TC. Early detection of dominant Env-specific and subdominant Gag-specific CD8+ lymphocytes in equine infectious anemia virus-infected horses using major histocompatibility complex class I/peptide tetrameric complexes. *Virology* 2005;339:110–126. [PubMed: 15979679]
- Mealey RH, Zhang B, Leib SR, Littke MH, McGuire TC. Epitope specificity is critical for high and moderate avidity cytotoxic T lymphocytes associated with control of viral load and clinical disease in horses with equine infectious anemia virus. *Virology* 2003;313:537–552. [PubMed: 12954220]
- Mitsuyasu RT, Anton PA, Deeks SG, Scadden DT, Connick E, Downs MT, Bakker A, Roberts MR, June CH, Jalali S, Lin AA, Pennathur-Das R, Hege KM. Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 2000;96:785–793. [PubMed: 10910888]
- O'Rourke KI, Perryman LE, McGuire TC. Cross-neutralizing and subclass characteristics of antibody from horses with equine infectious anemia virus. *Vet Immunol Immunopathol* 1989;23:41–49. [PubMed: 2559537]
- Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 1998;279:2103–2106. [PubMed: 9516110]
- Perryman LE, McGuire TC, Crawford TB. Maintenance of foals with combined immunodeficiency: causes and control of secondary infections. *Am J Vet Res* 1978;39:1043–1047. [PubMed: 208432]
- Perryman LE, O'Rourke KI, McGuire TC. Immune responses are required to terminate viremia in equine infectious anemia lentivirus infection. *J Virol* 1988;62:3073–3076. [PubMed: 2839723]
- Perryman LE, Torbeck RL. Combined immunodeficiency of Arabian horses: confirmation of autosomal recessive mode of inheritance. *Journal Of The American Veterinary Medical Association* 1980;176:1250–1251. [PubMed: 7429919]
- Pu R, Omori M, Okada S, Rine SL, Lewis BA, Lipton E, Yamamoto JK. MHC-restricted protection of cats against FIV infection by adoptive transfer of immune cells from FIV-vaccinated donors. *Cell Immunol* 1999;198:30–43. [PubMed: 10612649]
- Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, Panicali D, Mazzara G, Liebmann J, Cottrill M, et al. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol* 1995;69:5838–5842. [PubMed: 7637030]
- Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallion BJ, Ghayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999;283:857–860. [PubMed: 9933172]
- Shin EK, Perryman LE, Meek K. A kinase-negative mutation of DNA-PK(CS) in equine SCID results in defective coding and signal joint formation. *J Immunol* 1997;158:3565–3569. [PubMed: 9103416]

- Siliciano RF, Keegan AD, Dintzis RZ, Dintzis HM, Shin HS. The interaction of nominal antigen with T cell antigen receptors. I. Specific binding of multivalent nominal antigen to cytolytic T cell clones. *J Immunol* 1985;135:906–914. [PubMed: 2409158]
- Smith KA. The interleukin 2 receptor. *Annu Rev Cell Biol* 1989;5:397–425. [PubMed: 2688708]
- Sundin DJ, Wolin MJ. Aldesleukin therapy in HIV-infected patients. *Am J Health Syst Pharm* 1998;55:1520–1523. [PubMed: 9676300]
- Tan R, Xu X, Ogg GS, Hansasuta P, Dong T, Rostron T, Luzzi G, Conlon CP, Screaton GR, McMichael AJ, Rowland-Jones S. Rapid death of adoptively transferred T cells in acquired immunodeficiency syndrome. *Blood* 1999;93:1506–1510. [PubMed: 10029578]
- Terasaki PI, Bernoco D, Park MS, Ozturk G, Iwaki Y. Microdroplet testing for HLA-A, -B, -C, and -D antigens. The Phillip Levine Award Lecture. *Am J Clin Pathol* 1978;69:103–120. [PubMed: 75690]
- Tschetter JR, Davis WC, Perryman LE, McGuire TC. CD8 dimer usage on alpha beta and gamma delta T lymphocytes from equine lymphoid tissues. *Immunobiology* 1998;198:424–438. [PubMed: 9562867]
- Tsoukas CM, Turner HM, Hatzakis GE, Blake GP, Goodhew JE, Kilby DL, Kovacs CM, Luetkehoelter JR, Routy JP, Walmsley SL, Bernard NF. Improvement of HIV-specific immunity in HIV-infected twins treated with highly active antiretroviral therapy, interleukin 2, and syngeneic adoptively transferred cells. *AIDS Res Hum Retroviruses* 2001;17:887–900. [PubMed: 11461675]
- Villinger F, Brice GT, Mayne AE, Bostik P, Mori K, June CH, Ansari AA. Adoptive transfer of simian immunodeficiency virus (SIV) naive autologous CD4(+) cells to macaques chronically infected with SIV is sufficient to induce long-term nonprogressor status. *Blood* 2002;99:590–599. [PubMed: 11781243]
- Walker RE, Bechtel CM, Natarajan V, Baseler M, Hege KM, Metcalf JA, Stevens R, Hazen A, Blaese RM, Chen CC, Leitman SF, Palensky J, Wittes J, Davey RT Jr, Falloon J, Polis MA, Kovacs JA, Broad DF, Levine BL, Roberts MR, Masur H, Lane HC. Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection. *Blood* 2000;96:467–474. [PubMed: 10887107]
- Wang A, Lu SD, Mark DF. Site-specific mutagenesis of the human interleukin-2 gene: structure-function analysis of the cysteine residues. *Science* 1984;224:1431–1433. [PubMed: 6427925]
- Wiler R, Leber R, Moore BB, VanDyk LF, Perryman LE, Meek K. Equine severe combined immunodeficiency: a defect in V(D)J recombination and DNA-dependent protein kinase activity. *Proc Natl Acad Sci USA* 1995;92:11485–11489. [PubMed: 8524788]
- Wilson JD, Ogg GS, Allen RL, Davis C, Shaunak S, Downie J, Dyer W, Workman C, Sullivan S, McMichael AJ, Rowland-Jones SL. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* 2000;14:225–233. [PubMed: 10716497]
- Wyatt CR, Magnuson NS, Perryman LE. Defective thymocyte maturation in horses with severe combined immunodeficiency. *J Immunol* 1987;139:4072–4076. [PubMed: 3500980]
- Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci USA* 2002;99:16168–16173. [PubMed: 12427970]
- Zhang W, Lonning SM, McGuire TC. Gag protein epitopes recognized by ELA-A-restricted cytotoxic T lymphocytes from horses with long-term equine infectious anemia virus infection. *J Virol* 1998;72:9612–9620. [PubMed: 9811694]

Figure 1.

a. Equine biologic activities of human recombinant IL-2 (rhuIL-2; Roche), equine recombinant IL-2 (reqIL-2; Endogen), and aldesleukin (Chiron). Equivalent concentrations of each were added to 7-day cultures of PHA-stimulated equine PBMC in triplicate, and ^3H thymidine uptake was determined after 2 days. CPM, counts per minute. Error bars are SD. **b.** Regression curves of biologic activities for rhuIL-2, eqIL-2, and aldesleukin, normalized to % maximal proliferation. Specific activity equivalent to one unit was defined as the rIL-2 concentration (ng/ml) required to induce 50% maximal proliferation (dotted lines; values shown in parentheses) of PHA-stimulated equine PBMC.

Figure 2.

Plasma aldesleukin concentrations in two one-month-old Arabian SCID foals following subcutaneous administration of $180,000 \text{ U/m}^2$ (based on specific activity on equine PBMC) aldesleukin. Plasma aldesleukin concentrations were measured in duplicate using a Human IL-2 ELISA Kit (Pierce Endogen). Error bars are SD.

Functional Avidities of Rev-QW11-specific CTL clones on A2150 targets

Figure 3.

Functional avidities of infused Rev-QW11-specific CTL clones. Percent specific lysis was determined on A2150 EK target cells pulsed with increasing concentrations of Rev-QW11 peptide. E:T cell ratios were 20:1. EC₅₀, peptide concentration resulting in 50% maximal specific lysis (dotted lines). Actual minimum and maximum % specific lysis for clones R9-0603, R8-0603, R5-0603, and R10-0603 were 1.0 and 32.3, 0 and 33.3, 0.5 and 34.4, and 3.0 and 61.7, respectively.

Figure 4.

CD3/CD8 phenotype and CTL activity of Rev-QW11-specific CTL clones immediately prior to infusion into SCID foals. **a.** Clone R8-0603 prior to infusion into A2193. **b.** Clones R5.1-0404 and R9-0603 prior to infusion into A2199. **c.** Clone R5-0603 prior to infusion into A2202. **d.** Clone R10-0603 prior to infusion into A2205. For CTL assays, SCID foal recipient EK target cells were pulsed with 10^5 nM Rev-QW11 peptide. E:T ratio, 20:1. Three well replicates were used, and error bars are SE.

Figure 5. Peripheral blood platelet counts (red lines), lymphocyte counts (green lines), and plasma viral loads (blue lines) for SCID foals A2193 (a.), A2199 (b.), A2202 (c.), and A2205 (d.). Days that CTL clones were infused are indicated.

Figure 6.

Phenotype of mononuclear cells in peripheral blood before and after infusion of CTL clones into SCID foal A2193 (**a.**), A2199 (**b.**), A2202 (**c.**), and A2205 (**d.**). Absolute numbers of monocytes (blue bars) and lymphocytes / μ l of peripheral blood were determined by CBC, and numbers of CD2+/CD8+ (red bars) and CD3+/CD8+ (green bars) cells were calculated based on flow cytometric analysis. Values for an immunocompetent EIAV-infected inapparent carrier horse are provided for comparison.

Table 1

SCID foal	Rev-QW11-specific CTL clone(s) infused	Total number of cells infused	Age of foal at CTL infusion	Frequency and route of aldesleukin administration	Day of EIA V challenge*	Outcome
A2193	R8-0603	1.0×10^9	4 wks	once daily, SQ	0	Euthanized 29 DPI**
A2199	R5.1-0404, R9-0603***	2.4×10^8	4 wks	twice daily, SQ	-6	Euthanized 14 DPI
A2202	R5-0603	1.8×10^9	4 wks	twice daily, SQ	-6	Euthanized 30 DPI
A2205	R10-0603	1.6×10^9	5 wks	once daily, SQ	-10	Euthanized 24 DPI

* All foals were challenged with 10^6 TCID₅₀ EIAV WSU5. The day of EIAV challenge is relative to the day of CTL clone infusion.

** DPI, days post EIAV inoculation.

*** Foal A2199 received an infusion of CTL clone R5.1-0404 (1.2×10^8 cells) and CTL clone R9-0603 (1.2×10^8 cells) on the same day.

Table 2

	Product Labeled Specific Activity (one unit)*	Equine PBMC Specific Activity (one unit)**	Fold Difference***
aldesleukin	0.06 ng/ml	0.7 ng/ml	11.7 (less)
reqlL-2	2.0 ug/ml	0.9 ng/ml	2222 (greater)
rhIL-2	0.5 ng/ml	0.5 ng/ml	none

* Determined by manufacturer with standard mouse CTLL-2 cell assay.

** Determined on PHA-stimulated equine PBMC as described in Material and Methods. These values were not significantly different from each other based on regression analysis.

*** Fold difference in specific activity on equine cells relative to product labeled specific activity.