Research paper

Persistent lentivirus infection induces early myeloid suppressor cells expansion to subvert protective memory CD8 T cell response

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ABSTRACT

Background: Memory CD8\textsuperscript{+} T cell responses play an essential role in protection against persistent infection. However, HIV-1 evades vaccine-induced memory CD8\textsuperscript{+} T cell response by mechanisms that are not fully understood.

Methods: We analyzed the temporal dynamics of CD8\textsuperscript{+} T cell recall activity and function during EcoHIV infection in a potent PD\textsubscript{1}-based vaccine immunized immunocompetent mice.

Findings: Upon intraperitoneal EcoHIV infection, high levels of HIV-1 GAG-specific CD8\textsuperscript{+} T lymphocytes recall response reduced EcoHIV-infected cells significantly. However, this protective effect diminished quickly after seven days, followed by a rapid reduction of GAG-specific CD8\textsuperscript{+} T cell number and activity, and viral persistence. Mechanistically, EcoHIV activated dendritic cells (DCs) and myeloid cells. Myeloid cells were infected and rapidly expanded, exhibiting elevated PD-L1/L2 expression and T cell suppressive function before day 7, and were resistant to CD8\textsuperscript{+} T cell-mediated apoptosis. Depletion of myeloid-derived suppressor cells (MDSCs) reduced EcoHIV infection and boosted T cell responses.

Interpretation: This study provides an overview of the temporal interplay of persistent virus, DCs, MDSCs and antigen-specific CD8\textsuperscript{+} T cells during acute infection. We identify MDSCs as critical gatekeepers that restrain antiviral T cell memory responses, and highlight MDSCs as an important target for developing effective vaccines against chronic human infections.

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1. Introduction (7406)

Human immunodeficiency virus type one (HIV-1) is one of the most devastating infectious agents existing worldwide for the past 37 years. There were approximately 25.7 million people living with HIV at the end of 2018 with 1.1 million people becoming newly infected in 2017 globally. The induction of protective T cell immunity is a prerequisite for the durable control of HIV-1 [1,2]. However, host immunosuppression is a hallmark of HIV-1 and other persistent viral infections. Despite initial antiviral immune activity, persistent viruses eventually evade host immune responses [1, 3] and induce expansion of immune regulatory cells in the host that suppress antiviral T cell immunity [4, 5], facilitating persistent chronic infection [6–8].

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immunosuppression is also seen as a host adaptation that enables long-term survival and coexistence with the pathogen, because individuals with genetic ablation of core immunosuppressors often die after infection [9]. The dynamics of the immunosuppressive response and how this host adaptation affects memory T cell recall responses and function driven by prior vaccination remains largely unclear. However, understanding these mechanisms will be critical for the design of an effective vaccine or immunotherapy against HIV-1 and other chronic diseases.

CD8+ T cells play a critical role in vaccine-mediated protection against a number of viral and bacterial pathogens [10,11]. After vaccination, naive CD8+ T cells are primed and undergo a rapid expansion phase to generate large numbers of effector cells for pathogen elimination. Subsequently, a contraction period takes place in which most effector cells are eliminated, leaving a small, long-lived memory cell pool [12]. When individuals encounter the vaccine-related pathogen, antigen-specific memory T cells can respond swiftly with robust proliferation and upregulation of effector function. Analysis of cellular requirements for generating a memory CD8+ T cell recall response during acute viral infection has suggested a critical role for dendritic cells (DCs) and CD4+ helper T cells. Activation of memory T cells in response to systemic or localized infection is predominantly dependent on DCs, and the number of responding memory CD8+ T cells is profoundly decreased during the recall response to various acute infections in DC-depleted mice [13]. However, the dependence of CD8+ T cell recall response on CD4+ T cells remains controversial. In some cases, CD4+ T cells assist proliferative CD8+ T cell recall responses, whereas in other situations, CD4+ T cells appear to be dispensable for the secondary response [14-16].

During chronic lentiviral infection, both CD4+ and CD8+ T cell responses are suppressed by various mechanisms, and these cells subsequently acquire an exhausted phenotype characterized by upregulation of inhibitory molecules such as PD-1, Tim3 or vista, and reduced production of effector molecules such as IFN-γ, TNF, granulymes, and perforin [5,8,17]. Myeloid-derived suppressor cells (MDSCs) have recently emerged as a major suppressor of immune responses in chronic infection and tumors [18-22]. MDSCs are immature myeloid cells that are induced and accumulated during persistent viral infection [23,24], and suppress both innate and adaptive immune responses through many mechanisms. For example, they produce arginase 1 (ARG1), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (NOS2), NADPH oxidase and immunosuppressive cytokines to suppress DC, CTLs and natural killer (NK) cell function, deplete nutrients required by lymphocytes, which causes proliferative arrest of antigen-activated T cells, and expands CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) [19,25-28].

We recently developed a potent sPD1-p24fc/EP DNA DC-targeting vaccine, which consistently induces a high frequency of durable HIV-1 GAG-specific CD8+ T lymphocytes [29]. Following vaccination, we detected up to 21% of p24 tetramer-positive CD8+ T cells in the mouse spleen, and these exhibited broad reactivity, polyfunctionality and cytotoxicity. This model vaccine was subsequently employed to achieve complete and long-lasting protection against lethal mesothelioma challenge by CD8+ T cells in immunocompetent BALB/c mice [18]. We have now utilized this vaccine to analyze the secondary T cell immune response to a persistent lentivirus, and the correlation of the recall responses in prevention and curing of persistent infection. We adopted the lentiviral model of EcoHIV, which establishes persistent infection in immunocompetent BALB/c mice [30,31]. Considering that immunosuppression was typically observed during chronic infection [20,24,32], we reasoned that efficient reactivation of large numbers of memory CD8+ T cells with broad reactivity and polyfunctionality might be sufficient to rapidly eliminate infected cells before the induction of immunosuppressive cells, thus preventing persistent infection. However, our results indicated that despite effective initial memory CD8+ T cell reactivation, there was a rapid expansion of MDSCs within 7 days post-infection (dpi), which profoundly subverted the memory CD8+ T cell response both in terms of number and function at a very early stage of infection, resulting in viral persistence.

2. Materials and methods

2.1. Mice and viruses

6- to 8-week- old female BALB/c (Charles River Laboratory), OT-1 (Rag1/2+/+) mice were maintained under specific-pathogen-free conditions in the Laboratory Animal Unit. EcoHIV strains from David Volsky of Columbia University were grown and quantified, as described previously [30].

2.2. Mouse immunization and challenge

Construction of the DNA vaccines sPD1-p24fc and p24fc and the immunization procedure were previously described [18,29]. Mice received three DNA immunizations by intramuscular injection with EP given every three weeks at a dose of 100 μg per mouse. After the final immunization, the mice were challenged with EcoHIV via i.p. route and subsequently sacrificed at 1, 2, 3, or 12 weeks, after which sera and splenocytes were collected for analysis.

2.3. qPCR quantitation of proviral load

qPCR quantitation of total HIV DNA in EcoHIV-infected mice and DNA standardization was conducted as previously described [33] with forward primer 5’-CCCTCAATGTCGATGATAAGC-3’ and reverse primer 5’-GACTCAAGCGAAGCTTATGT-3’ for LTR. DNA content was standardized by amplification of murine β globin with forward
prime 5'-GGTTTCTCCCGGCTTGAT-3' and reverse primer 5'-CGCTCCCCCTTTCTCTG-3'.

2.4. Evaluation of gag p24-specific T cell responses

Evaluation of IFNγ-producing T cells were conducted by ELISPOT assay (Millipore) as previously described [18,29]. Briefly, splenocytes were stimulated with 10 μg/ml of HIV-1 p24 peptide or peptide pools in vitro (NIH, catalog no. 8117). The peptide pools were generated by dividing 59 GAG p24 peptides into three pools. Each pool contains 19–20 peptides spanning amino acids 1–87 (pool 1), 77–167 (pool 2), and 157–231 (pool 3). The final concentration of each peptide in three pools is 2 μg/ml. Peptide GAG A-I (AMQMLKDTI) is specific to CD8+ T cells, whereas peptide GAG 26 (TSNPPIPVGDIYKRWILGL) is specific to CD4+ T cells. Cells stimulated with 500 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) plus 1 μg/ml calcium ionomycin or left in media only served as positive and negative controls, respectively. Cells were incubated at 37 °C, and 5% CO2, for 20 h. ImmunoSpot analyzer (Thermo Fisher Scientific) was used to detect the spots. Tetramer-positive CD8+ T cells were detected using MHC class I tetramer H2d-Kd-AMQMLKDTI (Beckman Coulter).

2.5. T cell proliferation assay

Splenocytes isolated from BALB/c mice were labeled with 1 μM CFSE (Invitrogen) at 10⁶ cells/ml in PBS for 10 min in a 37 °C water bath, and cultured in the presence of HIV-1 GAG p24 peptide pools (2 μg/ml), or with anti-CD28 (2 μg/ml) and anti-CD3 (0.1 μg/ml) antibodies. Cells were collected after five days of coculture, and stained with fluorochrome-conjugated antibodies. Data were acquired using a FACSaria III flow cytometer (BD Biosciences), and analyzed with FlowJo software v10 (TreeStar Inc.).

2.6. Antigen-presenting cell functional assays

Total DCs and CD8+ T cells were purified from the mice's spleens by anti-CD11c microbeads (Biolegend) and immunomagnetic beads (Invitrogen), respectively. Total DC and CD8+ T cell populations were determined as ~95% pure by flow cytometry. Purified DCs were cultured with 10⁵ CFSE-labeled CD8+ OT-I T cells at a ratio of 1:1 with or without the presence of SIINFEKL peptide (GenScript) for 5 days, and then were stained with fluorochrome-conjugated antibodies and analyzed on a BD Aria III flow cytometer (BD Biosciences).

2.7. T cell suppression assay

Total MDSCs were enriched from the spleens of day 14 EcoHIV-infected mice via MACS beads. The average purity of monocyte populations from three experiments was ~90%. Myeloid cells were added to 10⁵ CFSE-labeled splenocytes from sPD1-p24Gc/EP DNA vaccinated and unchallenged mice, and cultured for 5 days with p24 peptide pools or CD28/CD3 antibodies before evaluating proliferation.

2.8. MDSC isolation and culture

MDSCs were isolated from spleens of EcoHIV-infected BALB/c mice using the Miltenyi Biotech MDSC Isolation Kit, according to the manufacturer's protocols. The purity of MDSCs was greater than 95% as determined by flow cytometry. To assess CD8+ T cell-mediated apoptosis, MDSCs and freshly isolated CD8+ T cells were co-cultured at MDSC-to-CD8+ T cell ratios of 1:1, 1:2, and 1:5 in a 37 °C incubator for 24 hrs before being subjected to analysis of active caspase-3.

2.9. Myeloid cell depletion in vivo

Mice immunized and infected with EcoHIV were given intraperitoneal injections of 100 μg of anti-Gr1 (RB6–8C5) or a rat IgG2b isotype control (LTF-2) (Bio X Cell), at 7, 9, and 11 dpi. Blood, peritoneum macrophages and spleen were collected on day 14.

2.10. Double immunofluorescence staining on paraffin sections

The samples were de-paraffinized and rehydrated, as previously described [34]. After 30 min block with normal goat serum at room temperature, samples were incubated with primary antibody at 4 °C overnight, followed by an Alexa448/568/647-conjugated secondary antibody for 1 h at room temperature. Primary antibodies used in this study include: p24 (DAKO, M085701–1; 1/5 dilution); CD3 (DAKO, A0452; 1/50 dilution); Gr1 (Abcam); F4/80 (Biolegend, 123,102; 1/50 dilution). To differentiate between individual cells, samples were incubated with 1 μg/ml Hoechst 33,258 (Thermo Fisher Scientific) for 5 min at room temperature, followed by rinsing with water.

2.11. Confocal microscopy

A Carl Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) was used to perform Confocal microscopy. ZEN (Zeiss) was used to assign colors, which were collected simultaneously: Alexa 488 (Thermo Fisher Scientific) and HNPP/Fast Red, which fluoresce red when excited by a 568-nm laser; Alexa 488 (Thermo Fisher Scientific), which fluoresces green when excited at 488 nm; Alexa 647 (Thermo Fisher Scientific), which fluoresces cyan when excited at 647 nm; and the differential interference contrast (DIC) image, which is in grayscale.

2.12. Statistical analysis

A two-tailed Student t-test was performed to determine statistical significance between different groups. An additional test using the Bonferroni-Holms method was performed when multiple comparisons being made. P-value of less than 0.05 was considered to be statistically significant. Data are presented as the mean values ± standard error (s.e.m.).

2.13. Study approval

All experimental procedures were approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR 3638–15).

3. Results

3.1. EcoHIV established persistent infection and induced immune suppression in immune competent mice

EcoHIV is a chimeric HIV strain that have been modified to infect mice by replacing HIV env (1405 bp) with the coding region of the ecotropic envelope gp80 of ecotropic murine leukemia virus (MLV) in the backbone of HIV-1 clade B (Fig. 1a). HIV-1 cis-regulatory elements were preserved, and expression of the entire construct was driven by the HIV-1 LTR. It is capable to infect, replicate and persist in peritoneum macrophages and CD4+ T cells of infected mice [30,31], and has been used in several HIV-1 vaccine and therapeutic studies [30,33,35,36]. To study if EcoHIV infection leads to an immunosuppressive environment, we injected a group of BALB/c mice with 400 ng p24 EcoHIV intraperitoneally (i.p.), performed kinetic analysis of proviral load in blood for up to 12 weeks, and measured expression of the inhibitory molecules PD-1 and Tim3 on T cells, as well as numbers of Treg cells and MDSCs in the lymph node or spleen at 12 weeks post infection (wpi). Successful establishment of persistent infection was
consistently confirmed by the presence of provirus in PBMCs, measured every 1 or 2 weeks for 12 weeks (Fig. 1b and 1c). At week 12, mice were sacrificed, and provirus copies were detectable in spleen and peritoneal macrophages from all mice tested (Fig. 1d). Viral replication was determined by detection of viral protein p24 in spleens of EcoHIV-infected mice by immunohistochemistry (IHC) staining using an anti-p24 antibody (Fig. 1e). Persistent infection induced a p24-specific CD8+ T cell response, and up to 1.06% and 1.53% of CD8+ T cells in lymph nodes and spleens, respectively, were positive for H2-Kd-GAG A-I tetramer binding (Fig. 1f and Fig. S1).
Treg cells (CD4+CD25+Foxp3+) and MDSCs (CD11b+Gr-1+) and expression of PD-1 and Tim-3 on CD8+ T cells in mouse lymph nodes and spleens. We did not observe significant decrease of frequencies of the CD4+ or CD8+ T cells. However, EcoHIV infection led to up-regulation of PD-1 and Tim-3 on CD8+ T cells (Fig. 1g), and expansion of MDSCs in the spleen and Treg cells in the lymph nodes (Fig. 1h and Fig. S1). Lymphoid architecture is integral to induction and maintenance of immune responses, and EcoHIV infection resulted in slight splenic disorganization at 12 wpi, with a higher proportion of F4/80+ myeloid cells and a corresponding loss of T cells (Fig. 1i), consistent with previous reports of other chronic infection, such as lymphocytic choriomeningitis virus (LCMV) [24]. These results suggest that although EcoHIV does not induce canonical immunosuppression as shown in patients with AIDS with significant loss of CD4 T-cells, it establishes persistent infection and induces immune suppression in chronically infected mice.

3.2. Prophylactic vaccination-mediated virus clearance was restrained by day 7

After establishing the EcoHIV model, we aimed to study the role of memory CD8+ T cell recall responses in prevention of persistent infection. For this purpose, we used the sPD1-p24fc/EP DNA vaccine [29]. This vaccine exploits the binding of PD-1 to ligands expressed on DCs by fusing soluble PD-1 with HIV-1 GAG p24 antigen. In previous studies, this vaccine consistently elicited up to 21.2% of p24 tetramer-positive CD8+ T cells in the spleen, with broad reactivity, polyfunctionality and cytotoxicity [29]. We also included a non-DC-targeting vaccine, p24fc/EP, as a control. Using the same immunization schedule from that work, we immunized groups of BALB/c mice (n = 5) with 100 μg DNA intramuscularly via electroporation (EP) three times at 3-week intervals (Fig. 2a). T cell responses to vaccination were measured at 2 weeks after the final vaccination. Subsequently, mice were challenged
i.p. with EcoHIV (400 ng p24/mouse) 6 weeks after the last vaccination. Proviral load were measured at 7, 14 and 21 dpi. We found that for all groups of mice, levels of H2-Kd-GAG A-I tetramer+ CD8+ T cells in the blood correlated with the potency of the functional CD8+ T cell response elicited by the vaccine [18, 29], sPD1-p24/EP vaccination consistently elicited a strong antigen-specific CD8+ T cell response. Up to 19.7% of CD8+ T cells in blood were positive for H2-Kd-GAG A-I tetramer at 2 wpi (Fig 2b). Three non-overlapping peptide pools spanning the entire p24 protein were also used to stimulate splenocytes of three sacrificed mice from each group, and 200–1000 ELISpots per 10^6 splenocytes were detected with each of these pools in sPD1-p24c-vaccinated mice (Fig 2c), indicating substantial breadth of the elicited T cell response. In contrast, considerably fewer than 200 ELISpots per 10^6 splenocytes were detected with each of these pools in the control vaccine group (Fig 2c).

After the EcoHIV challenge, proviruses were readily detected in spleen, peritoneal macrophages and PBMC in all mice (Fig 2d). In p24c-vaccinated mice, we observed a significant reduction of proviral load in peritoneal macrophages at 7 dpi relative to controls, but not in splenocytes or PBMCs(Fig 2d). In contrast, there was ~80% reduction of infected cells in the spleen and peritoneal macrophages of sPD1-p24c-vaccinated mice relative to controls at 7 dpi, demonstrating the efficacy of sPD1-p24c/EP in eliminating infected cells (Fig 2d and 2e). However, we saw no significant reduction in infected cells in peripheral blood. Moreover, we were still able to detect a proviral load in the weeks that followed, suggesting that vaccine-induced CD8+ T cells failed to prevent the establishment of persistent infection (Fig 2f). It is critical to note that we observed less reduction of proviral load in spleens and peritoneal macrophages from sPD1-p24c-vaccinated mice relative to controls at 14 and 21 dpi (Fig 2f and 2g), suggesting that accelerated elimination of infected cells by vaccine-induced immunity was abrogated after 7 dpi.

3.3. EcoHIV subverted virus-specific T cell immunity by day 7

To better understand this abrogation, we performed a longitudinal analysis of T cell responses. Virus-specific CD8+ T cells were measured by H2-Kd-GAG A-I tetramer staining of splenocytes at 7, 14 and 21 dpi [18, 29]. We found that high levels of CD8+ T cells were elicited in the spleens of vaccinated mice after viral challenge, with 18.2–41.6% of CD8+ T cells positive for H2-Kd-GAG A-I tetramer at 7 dpi (Fig 3a). In contrast, we observed less than 1% H2-Kd-GAG A-I tetramer+ CD8+ T cells in control mice, suggesting that infection induces rapid expansion of vaccine-induced virus-specific CD8+ T cells (Fig 3a). However, despite ongoing viral replication, we found a significant decline in virus-specific CD8+ T cells in the spleens of vaccinated mice at 2 and 3 wpi, coinciding with impaired viral clearance. ~4.53–21.20% and 4.01–8.99% of CD8+ T cells in the spleens of vaccinated mice were positive for H2-Kd-GAG A-I tetramer at 14 and 21 dpi, respectively (Fig 3a and Fig 3S), suggesting rapid suppression of the virus-specific CD8+ T cell response after 7 dpi.

During chronic infection, virus-specific CD8+ T cells persist in a nonfunctional exhausted state and become unresponsive to viral antigens (1). These exhausted CD8+ T cells are characterized by the expression of PD-1 and Tim3 and an inability to proliferate (1). To understand the functional states of virus-specific T cells in EcoHIV-infected mice, we analyzed PD-1 and Tim3 expression, as well as the proliferative capacity of CD4+ and CD8+ T cells in response to ex vivo antigen stimulation. We observed significantly elevated PD-1 and Tim3 expression on CD4+ and CD8+ T cells in the spleens of vaccinated mice at 2 and 3 wpi (Fig 3b). Significantly increased PD-1 expression was also observed on T cells that are critical for long-lasting protection against virus, CD8+ effector memory (Tem) and CD8+ central memory T cells (Tcm) (Fig 3c and Fig 3S). We next assessed the proliferative capacity of the T cells. P24 peptide pools that gave a positive cell-mediated immune response by ELISPOT were used to stimulate splenocytes in an ex vivo cyt fluorometric proliferation assay based on CFSE staining [29]. To validate this assay and set a threshold for a positive response, we analyzed splenocytes from vaccinated and unchallenged mice stimulated with p24 peptide pools or anti-CD3/CD28 antibodies. We found that T cells from all tested mice showed proliferative responses to CD3/CD28 stimulation. However, only T cells from vaccinated and unchallenged mice proliferated effectively in response to the p24 peptide pools, whereas CD4+ and CD8+ T cells from challenged mice failed to demonstrate proliferation response to p24 peptide (Fig 3d and 3e). We observed more than 80% inhibition of antigen-specific CD4+ and CD8+ T cell proliferation at 7 dpi, demonstrating the unresponsiveness of those T cells upon viral antigen reencounter within the first week (Fig 3f). Moreover, we observed increased levels of Treg cells in spleens of vaccinated mice at 2 and 3 wpi (Fig 3g). These results suggest that despite an initially efficient T cell recall response, virus-specific T cells become unresponsive to viral antigens and nonfunctional within 7 days after viral challenge, with a notable increase in Treg cell production.

3.4. Therapeutic vaccination fails to accelerate elimination of infected cells

In tumor-bearing mice, repeated sPD1-p24c/EP DNA vaccination produces an active immune state that prevents the emergence of exhausted PD-1+ and Tim-3+ CD8+ T cells and Treg cells, leading to therapeutic cure of pre-existing mesothelioma [18]. We hypothesized that a similar repeated vaccination regimen of sPD1-p24c/EP DNA could boost virus-specific memory CD8+ T cell response in mice and induce further clearance of infected cells. To test this hypothesis, 20 BALB/c mice were immunized intramuscularly three times: 10 animals with sPD1-p24c/EP DNA, and 10 with PBS as a control (Fig 4a). The sPD1-p24c DNA vaccine induced a strong virus-specific CD8+ T cell response, with H2-Kd-GAG A-I tetramer+ CD8+ T cells representing 5–14% of PBMCs (Fig 4b). Subsequently, all 20 animals were challenged i.p. with EcoHIV at a dose of 400 ng p24 at 6 weeks after the last vaccination; five mice in each group (vaccine or PBS) were subsequently immunized intramuscularly with 100 μg sPD1-p24c/EP DNA three times at 3-week intervals, starting at 1 dpi. We sacrificed all mice at 12 wpi, with the exception of one control mouse and two prophylactically vaccinated mice that died before week 12. Vaccine efficacy was investigated by measuring the extent of EcoHIV infection in splenocytes and peritoneal macrophages by qPCR. Virus-specific T cell response was determined by H2-Kd-GAG A-I tetramer staining of splenocytes at week 12. Consistent with previous results, prophylactic vaccination significantly lowered proviral load in both spleen and peritoneal macrophages (Fig 4c). The proportion of H2-Kd-GAG A-I tetramer+ CD8+ T cells was significantly higher in mice that received both prophylactic and therapeutic vaccination than mice that received prophylactic vaccination alone, suggesting that therapeutic vaccination boosted virus-specific CD8+ T cell reactivation (Fig 4d). However, we saw no further reduction of proviral load in splenocytes or peritoneal macrophages in these mice (Fig 4c), indicating that these T cells very likely become exhausted soon after reactivation, similar to the fate of reactivated virus-specific T cells following viral challenge in the prophylactic vaccine group (Fig 3). We consistently observed high expression of Tim3 and PD-1 in CD8+ T cells in the spleens of both prophylactic-only and prophylactic plus therapeutically vaccinated mice (Fig 4e). Increased frequency of Treg and MDSC was observed in all challenged mice (Fig 4f and 4g). Moreover, repeated therapeutic vaccination elicited only low levels of p24-specific CD8+ T cell response in unvaccinated and challenged mice: only 1.87–3.08% of CD8+ T cells in their spleens were H2-Kd-GAG A-I tetramer+ after the third vaccination (Fig 4d). We conclude that therapeutic vaccination can enhance virus-specific T cell reactivation during early infection, but cannot prevent the rise of exhausted PD-1+ and Tim-3+ CD8+ T cell population. Treg cells, preventing effective cure of EcoHIV infection.
3.5. DCs are activated in ecohiv-infected mice and primed CD8+ T cell ex vivo

DCs play a critical role in CD8+ T cell priming and reactivation in vivo [12,13]. To understand the cause of CD8+ T cell non-responsive-ness to the therapeutic vaccine and decreased CD8+ T cell number and function during EcoHIV infection, we performed a kinetic analysis of various subpopulations of DCs over 3 weeks after i.p. inoculation of EcoHIV at a dose of 400 ng p24. By flow cytometry, we identified multiple DC populations (Fig. S3), including the two major murine splenic conventional DC (cDC) populations, CD8- and CD8+; monocyte-derived DCs (Mo-DCs) that are recruited from blood monocytes into lymphatic tissues during infection; and plasmacytoid DCs (pDCs). These cDCs were identified from total non-lymphocytes...
by high expression of CD11c and lack of Ly6C. Mo-DCs were identified by high expression of CD11c, CD11b, and Ly6C. pDCs can be identified based on CD11c<sub>lo</sub>, B220<sup>+</sup>, Ly6C<sup>+</sup>, and CD11b/C0<sup>+</sup>. CD8<sup>+</sup> DCs and Mo-DCs cross-present antigens phagocytosed from the environment via MHC class I, and are potent initiators of CD8<sup>+</sup> T cell responses, whereas CD8<sup>+</sup>C0<sup>+</sup> DCs primarily initiate a Th2 cell response [37,38].

pDCs are the most potent type I interferon (IFN-I)-producing cells in response to viral infection in humans and mice, and provide critical resistance to infection by limiting viral spread and by activating multiple innate and adaptive immune cells [39].

We found that the overall proportion of DCs increased in the spleen over the course of three weeks of infection, whilst CD4<sup>+</sup> and CD8<sup>+</sup> T cells progressively decreased (Fig. 5a). Among the various DC populations, the proportion of CD8<sup>+</sup> cDCs and Mo-DCs cross-present antigens phagocytosed from the environment via MHC class I, and are potent initiators of CD8<sup>+</sup> T cell responses, whereas CD8<sup>+</sup>C0<sup>+</sup> DCs primarily initiate a Th2 cell response [37,38]. pDCs are the most potent type I interferon (IFN-I)-producing cells in response to viral infection in humans and mice, and provide critical resistance to infection by limiting viral spread and by activating multiple innate and adaptive immune cells [39].

3.6. EcoHIV induces rapid expansion of MDSCs that inhibited T cells proliferation

MDSCs have emerged as a major suppressor of immune response in chronic infection and cancer [18,19,24]. To understand the rapidly suppressed T cell response in EcoHIV-infected mice, we evaluated myeloid cell populations over three weeks of infection. The proportion of MDSCs, identified as Gr1<sup>+</sup> and CD11b<sup>+</sup> in Figure S3, increased at day 7, reaching about 2-fold expansion relative to uninfected mice and PD-L2, compared to unchallenged mice (Fig. S4). These results suggest expansion and activation of these DC subsets by EcoHIV. We therefore conducted a longitudinal analysis of the stimulatory capacity of DCs from EcoHIV-infected spleens. CD11c<sup>+</sup> DCs isolated at 1, 2, 3, 5, 7, 14, or 21 dpi were cultured with ovalbumin (OVA)-specific CD8<sup>+</sup> cells from OT-I mice, those with transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes designed to recognize ovalbumin residues, and the OVA-SIINFEKL peptide for 5 days. Our **ex vivo** data showed that EcoHIV infection resulted in increased DC stimulatory capacity at day 1, and these DCs remained functional in priming CD8<sup>+</sup> T cells ex vivo during the following weeks (Fig. 5c and 5d).
(Fig. 6a). These cells continued to increase, peaking at day 14 with nearly 3-fold more MDSCs than in naive mice. We then determined the cellular composition of this population. Total MDSCs were subdivided into three main populations: Gr1$^{hi}$Ly6C$^{lo}$F4/80$^{SSC_{hi}}$ eosinophils, Gr1$^{hi}$Ly6C$^{lo}$F4/80$^{SSC_{int}}$ monocytes, and Gr1$^{hi}$Ly6C$^{lo}$F4/80$^{SSC_{int}}$ neutrophilic cells (Fig. S4) [24]. The number of neutrophilic cells substantially decreased during the first two weeks of infection by about 10-fold before rebounding by week 3 (Fig. 6b). Both monocytes and eosinophils expanded during the first two weeks of infection, peaking at day 14. Eosinophils subsequently contracted to naive levels by day 21, while monocyte numbers remained high: nearly three-fold higher than naïve mice at day 21. All three populations showed increased expression of inhibitory receptors PD-L1 and PD-L2 after day 14 (Fig. S5a).

The increase in MDSCs during EcoHIV infection coincided with the functional exhaustion of virus-specific CD8$^+$ T cells (Fig. 3 and 6a), raising the possibility that MDSCs suppress the EcoHIV-specific CD8$^+$ T cell response. To assess whether these cells were immunosuppressive, we co-cultured Gr1$^+$ MDSCs isolated from EcoHIV-infected mice with total splenocytes from vaccinated but unchallenged mice at different ratios ranging from 1:5 to 1:135, and stimulated them with p24 peptide pools. Gr1$^+$ MDSCs from naive mice were tested as a control. We found that Gr1$^+$ MDSCs from EcoHIV-infected mice suppressed CD8$^+$ T cell proliferation in a cell number-dependent manner (Fig. 6c). Both p24+monocytic and neutrophilic cell populations based on flow cytometry. Data is shown in a column graph as mean ± SEM. A two-tailed Student’s t-test and Bonferroni-Holms method was used.

**3.7. MDSCs are infected and resistant to CD8$^+$ T cell-induced apoptosis**

To characterize the infected cells that escape from vaccine-induced CTL killing, we measured the phenotype of p24$^+$ cells in the spleen at 2 and 3 wpi by flow cytometry. Besides a small population of p24$^+$ CD4$^+$ T cells, by 2 wpi more than 80% of p24$^+$ cells in the spleens were CD11b$^+$Gr1$^+$ MDSCs (Fig. S6a). At week 3, there was a significant decline of p24$^+$CD4$^+$ T cells in the spleen, but not p24$^+$Gr1$^+$ cells (Fig. 7a). We then determined the cellular composition of p24$^+$MDSCs by subdividing the MDSCs into monocytes, neutrophils, and eosinophils (as defined above) over the course of three weeks. We found that both p24$^+$monocytic and neutrophilic cell populations expanded during the first two weeks of infection (Fig. 7b and Fig. S6b). Expansion of neutrophilic cells peaked at day 14 and remained high at day 21, while monocytic cells continued to increase at day 21, reaching ~3-fold expansion relative to day 14 (Fig. 7f), suggesting enrichment of EcoHIV-infected monocytic cells. Consistently with this hypothesis, IHC staining showed that almost all p24$^+$ cells in the spleen were Gr1$^+$ at 12 wpi (Fig. 7c), suggesting that infected MDSCs likely represent a cell type that escapes host immunity.

We have previously reported that sPD1-p24Fc elicited functional antigen-specific CD8$^+$ T cells can eliminate MDSCs through apoptosis in a malignant mesothelioma mouse model [18,40]. We therefore compared the interaction between vaccine-induced CD8$^+$ T cells and
MDSCs derived either from EcoHIV-infected mice at day 14 or MDSCs from the malignant mesothelioma model. Virus-specific CD8+ T cells from vaccinated and unchallenged mice were co-cultured with MDSCs from either EcoHIV-infected mice or malignant mesothelioma mice for analysis of active caspase-3 via flow cytometry as described previously [18, 40]. Consistent with previous findings, virus-specific CD8+ T cells caused apoptosis in MDSCs from both mouse models in a cell number-dependent manner (Fig. 7d). However, compared with MDSCs from the cancer model and EcoHIV-infected mice was measured based on expression of caspase-3 after co-culturing with CD8+ T cells from vaccinated mice for 24 hrs. Data is shown in a column graph as mean ± SEM. e. MDSCs do not induce significant apoptosis of CD8+ T cells, with data shown as in a column graph as mean ± SEM. Data represent three independent experiments with five mice per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. A two-tailed Student t-test and Bonferroni-Holms method was used.

3.8. Antibody depletion of mdsc enhances clearance of eohiv-infected cells in vivo

To test whether depletion of MDSCs will enhance clearance of EcoHIV-infected cells in vivo through direct depletion of infected myeloid cells and boost of T cell function, we monitored the abundance of Gr1+ cells in the blood of three naive mice for 5 days after a single injection of 100 μg anti-Gr1 as previously described [24] (Fig. S7). Treatment with anti-Gr1 significantly reduced the abundance of Gr1+ myeloid cells to less than 1% in the blood after 1 day, and there was no significant increase in Gr1+ cells in the blood after 5 days of treatment (Fig. S7a and 7b). It was previously reported that anti-Gr1 does not completely deplete Gr1+ cells. A small population with the antibody on the surface will remain for more than 4 days, and will not be detected by fluorophore-conjugated RB6–8C5 (Ly6C/G) and 1A8 (anti-Ly6G) antibody staining [24]. Therefore, we measured total blood myeloid cells with an antibody against CD11b. We found that treatment with anti-Gr1 reduced total myeloid cells by more than 80% after 1 day, from ~35% to <7%. Abundance of myeloid cells did not increase significantly at day 2 and 3, and by day 5, increased to ~10% in treated mice (Fig. S7a and 7c).

We next immunized groups of BALB/c mice (n = 5) with 100 μg sPD1-p24fc DNA intramuscularly via EP three times at 3-week intervals (Fig. 8a). Subsequently, mice were challenged intraperitoneally with EcoHIV (400 ng p24/mouse) at 6 weeks after the last vaccination. 100 μg of anti-Gr1 or control antibody (rat IgG2b) was given on days 7, 9, and 11. At day 1 after the 1st antibody injection, we found reduction of total CD11b+ and Gr1+ myeloid cells in the blood (Fig. 8b). Significant reduction of DCs subpopulations that express Ly6c, Mo-DC and pDC, was also observed (Fig. 8c). At 14 dpi, mice were sacrificed, and frequency of MDSCs and DCs and provirus copies were tested in blood, spleen and peritoneal macrophages from all mice. In the blood, we found significant reduction of MDSC and Mo-DC in depleted mice (Fig. 8d). Moreover,
depleted mice have lower frequency of p24+ blood MDSC, and a ~600-fold reduction of proviral loads in peritoneum macrophages (Fig. 8e and 8f). These results suggest that depletion of MDSC enhanced clearance of EcoHIV infected cells. The number of p24+ cDC, a population that do not express ly6c, were also significantly reduced in the blood of depleted mice (Fig. 8e), suggesting an enhanced clearance of viral infection by host immunity. Further analysis revealed that expression level of PD-1 on CD8+ Tem in blood was significantly decreased in these mice (Fig. 8g), suggesting a boost of T cell function. Moreover, statistical analysis showed that expression of
PD1 on blood Tem is positively correlated with frequency of p24+ MDSC and CD11b+ myeloid cells (Fig. 8h). In the spleen, only Mo-DC were significantly reduced in anti-Gr1 treated mice (Fig. S8A). Moreover, depleted mice tended to have an increase in interferon γ-producing p24-specific T cells (Fig. S8b, 8c and 8d), reduced proviral loads in the spleen (Fig. 8f). However, these differences were not statistically significant, suggesting a limited effect of anti-Gr1 antibody on depletion of MDSCs and reduction of EcoHIV infection there. Our data suggest that by reducing the numbers of ly6c+ cells, we were able to enhance elimination of infected cells in vivo, and likely boosted CD8+ T cell function to clear infected ly6c negative cells.

4. Discussion

Here we have identified an unexpected role of myeloid cells in facilitating viral evasion of memory CD8+ T cell response at very early stage of viral infection. We present an extensive analysis of innate cell activation and memory CD8+ T cell response following virus challenge of vaccinated, immunocompetent BALB/c mice. Our results indicate that EcoHIV activates DCs and myeloid cells during the first week of infection, and initiates both effective CD8+ T cell priming in naive mice and recall of memory T cells response and effector function in vaccinated mice. However, myeloid cells serve as shelters to provide a mechanism for the virus to escape CTL-mediated apoptosis, and infection led to rapid expansion and accumulation of myeloid cells with T cell-suppressive function, resulting in diminished virus-specific CD8+ T cell numbers and function, and reduced vaccine-mediated virus clearance within 7 dpi. These data provide not only a detailed picture of DC, myeloid cell, and memory CD8+ T cell reactivation in response to persistent viral infection, but also new insight into the mechanisms that undermine lentiviral evasion of vaccine-induced T cell immunity to establish persistent infection.

Thus far, it has remained unclear when and to what extent persistent infection suppresses CD8+ memory T cell reactivation during recall response. Data are currently only available regarding the role of MDSCs in suppressing CD8+ T cell function during primary infection [20,24,32]. We recently developed a sPD1-p24c/EP DNA DC-targeting vaccine, which induces a strong and durable HIV-1 GAG-specific CD8+ T lymphocyte response, with broad reactivity, polyfunctionality and cytotoxicity [29]. We have now utilized this model vaccine to investigate its potential to confer complete protection against persistent lentiviral infection. We have analyzed the secondary T cell immune response to EcoHIV, a persistent lentivirus that induces immunosuppression during the infection, and the role of the recall responses in preventing persistent infection. We found that after EcoHIV challenge, there was robust proliferation of antigen-specific CD8+ T cells, which reduced the number of virus-infected cells in spleen and Peritoneum macrophages before day 7. However, EcoHIV also infected MDSCs, which are resistant to CD8+ T cell-mediated killing, while also inducing a rapid expansion of this cell population (Fig. 6 and Fig. 7). The expanded MDSCs profoundly subverted the memory CD8+ T cell response in terms of both number and function at a very early stage of infection, resulting in persistent viral infection. Our data have revealed the time-course of this process, and the important role of myeloid cells in subverting memory recall responses to virus and thereby facilitating viral persistence. We did not observe significant reduction of proviral load in PBMC in sPD1-p24c vaccinated and challenged mice, which might be due to the higher frequency of MDSCs in the blood compared to spleen (Fig. 8b and Fig. 1h), and the resistance of infected MDSC to CD8+ T cell-mediated killing.

Our description of the expansion of Gr1+ myeloid cells following EcoHIV challenge in vaccinated mice is similar to the phenomenon observed in chronic LCMV [24,41]. Previous studies using models of acute and chronic LCMV infection, the ARM and C13 strains [42,43], respectively, induced accumulation of Ly6G+ monocytes and Gr1+ neutrophils in lymphoid organs and blood during the first three days [24]. However, the expansion of myeloid cells was only enhanced and sustained over time after peaking at 14 dpi in persistent C13 virus infection, but not in the ARM strain [24]. In our study, accumulation of MDSCs in vaccinated and EcoHIV-challenged mice started before 7 dpi and likewise peaked at 14 dpi. In C13 infection, both DCs and myeloid cells are capable of stimulating T cell proliferation during the first few days of infection, and CD11b+ myeloid cells only begin actively suppressing the priming of T cells at 14 dpi [44,45]. But in EcoHIV-infected mice, only DCs are functional in T cell priming (Fig. 5). Myeloid cells from EcoHIV-infected mice were not able to stimulate T cell proliferation before 7 dpi (data not shown). Instead, Gr1+ cells suppressed memory CD8+ T cells proliferation during this time period in vaccinated and EcoHIV-challenged mice (Fig. 6), suggesting earlier induction of MDSCs during secondary response. This might be because memory T cells response employ different mechanisms from those involved in the priming of naïve T cells. It is also possible that a robust memory CD8+ T cell response during the first few days of EcoHIV infection accelerated MDSC induction. Consistent with this, it has previously been reported that interferon γ produced by effector T cells upon recognition of viral antigens can induce iNOS production in monocyctic cells, which in turn inhibits T cell function [46]. Interestingly, there is a slight recovery of CD4+ T cell proliferation at 3 wpi, coincide with the slightly drop of MDSC frequency (Fig. 3).

Of note, persistent LCMV infection has been linked to increased susceptibility of DCs and myeloid derived cells to infection [47,48]. Their faster replication in DCs and myeloid cells outpaces the ability of the adaptive immune response to contain the infection, while also driving excessive immune activation, and inducing MDSC expansion [43,48]. MDSCs repress T cell function, and consequently hinder viral clearance [46]. Although the frequency of EcoHIV infected cells is lower in mice overall, it also infects DCs and myeloid cells (Fig. 6 and Fig. 8), and induces MDSC expansion unlike C13-infected mice, in which the majority of MDSCs are neutrophils. EcoHIV induced ly6c+ monocyctic myeloid cell expansion, with continuous reduction of Gr1+ neutrophils. Further investigation revealed that both monocytes and neutrophils are infected. However, MDSCs from EcoHIV-infected mice were more resistant to CTL-mediated apoptosis compared to MDSCs from our malignant mesothelioma model (Fig. 7), in which most MDSCs are Gr1+ [18], suggesting that EcoHIV induced monocytes might be more resistant to CTL-mediated apoptosis, while infected neutrophils were eliminated by virus-specific CD8+ T cells.

Similarly, induction of myeloid cells expansion was demonstrated in blood of SIV infected macaques after two weeks of virus inoculation [32]. Expansion of MDSC in blood of HIV patients have also been observed in recent years [49]. However, infection of MDSCs during HIV/SIV infection has not been reported. Moreover, the impact of MDSC on HIV vaccine strategies, in particular on HIV therapeutic vaccine remains unknown. Our data suggests that MDSCs was induced and suppressed T cell recall response as early as 7 dpi in vaccinated and EcoHIV-challenged mice. In EcoHIV-challenged naïve mice, repeated therapeutic vaccination elicited only low levels of p24-specific CD8+ T cell response (Fig. 4d). It is unclear whether EcoHIV infection suppressed CD8+ T cell priming by therapeutic vaccination, or first immunization-primed, antigen-specific CD8+ T cells quickly became exhausted in infected mice. Testing earlier timepoints post vaccination would reveal whether these T cells quickly became exhausted or were never elicited. The role of MDSCs on antiviral T cell memory recall responses in HIV vaccine clinical trial or macaque model of SIV/HIV infection might be important to investigate for developing effective vaccines.

We depleted myeloid cells during EcoHIV infection using an antibody (RB6–8C5) that recognizes Gr1. Gr1 is a marker predominantly expressed by MDSCs, and has been used in many studies to deplete neutrophils, monocytes, and MDSCs [24,50]. We tested the dose of...
antibody delivery that could reduce MDSCs in the blood by > 90% (Fig. S7), and subsequently used the same dose and regimen of antibody delivery to deplete MDSCs in vaccinated and infected mice. We found significant decrease of total myeloid cells, MDSCs and ly6c+ pDCs and Mo-DCs at day 1 after antibody injection. Moreover, the loss of myeloid cells and pDCs correlated with decreased PD1 expression on CD8+ Tem cells, and significantly lowered frequency of p24+ cells and proviral load in peritoneal macrophages (Fig. 8). Surprisingly, we did not observe a significant impact on proviral loads in the spleen. The failure to reduce provirus in spleen significantly may be due to multiple factors. Although myeloid cells are infected by EcoHIV, the frequency of these cells in the spleen is relatively low (Fig. 1), and were not effectively depleted (Fig. S8). Moreover, they are but one of many cell types infected by EcoHIV at 2 wpi, including DCs (Fig. 8) and CD4+ T cells (Fig. 7). We did not observe a significant increase in memory CD8+ T cell response, which is likely because MDSCs are just one of several factors involved in the suppression of T cell responses during chronic infection. Other factors, such as Treg cells, and PD1- PDL1 pathway, IL-10, and TGF-β, might suppress T cell response in MDSCs depleted mice. Moreover, Mo-DCs and pDCs also express ly6c, and were depleted by anti-G1 antibody. The depletion of Mo- DCs and pDC might compromise the T cell response in vivo. In summary, we demonstrated that depletion of MDSC can enhance viral clearance in vaccinated mice. We believe that strategies that counteract the effects of MDSCs during acute infection could be highly valuable for the development of effective vaccines against chronic human infections like HIV and other persistent virus.

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Author contributions

ZC, HW and LL are the principal investigator and co-principal investigators with overall responsibility for the design of the study. LL, QL and JP analyzed the data. LL, ZC, HW and KY contributed to drafting the manuscript. LL, QL, PJ, ZD and JS extracted plasmid DNA and/or generated virus. LQ, PJ, LL, TZ, TH, KL and FJ conducted T cell proliferation, ELISpot and FACS analyses. All authors were involved in the interpretation of the data.

Declarations of interests

The authors declare no financial or commercial conflicts of interest.

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Supplementary materials

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