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T Regulatory Cell Depletion Reactivates Latent SIV in Controller Macaques while Boosting SIV-specific T Lymphocytes

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Abstract

T regulatory cells (Tregs) are critical in shaping the latent HIV/SIV reservoir, as they are preferentially infected, reverse $CD4^+$ T cell activation status and suppress CTL responses. To reactivate latent virus and boost cell-mediated immune responses, we performed *in vivo* Treg depletion with Ontak (Denileukin diftitox) in two SIVsab-infected controller macaques. Ontak induced significant (>75%) Treg depletion, major $CD4^+$ T cell activation and only minimally depleted $CD8^+$ T cells. The overall ability of Tregs to control immune responses was significantly impaired in spite of their incomplete depletion, resulting in both reactivation of latent virus (virus rebound to 10^3 vRNA copies/ml of plasma in the absence of antiretroviral therapy) and a significant boost of SIV-specific $CD8^+$ T cell frequency, with rapid clearance of reactivated virus. As none of the latency reversing agents in development have such dual activity, our strategy holds great promise for cure research.

Keywords

Simian immunodeficiency virus; rhesus macaques; viral replication; single copy assay; T regulatory cells; Ontak; SIVsab

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Introduction

The burden of the HIV epidemic, which spreads unabated such that for every HIV-infected person that starts antiretroviral therapy (ART) two new people become infected, calls for a cure (1). The “Berlin patient” (2) demonstrated that a cure for HIV infection is feasible, but the mechanisms responsible for achieving success in this case are complex and poorly understood. Further, virus relapse in the “Mississippi baby” (3, 4) and nonhuman primate (NHP) studies documenting seeding of the viral reservoir prior to detectable viremia (5) suggest that virus eradication strategies should involve therapeutic approaches that go beyond ART.

Multiple strategies for an HIV cure are currently being investigated. One of the most advanced strategies is the “shock and kill” approach, consisting of induction of viral expression in latently infected cells that could trigger immune-mediated clearance of the infected cells through cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells or immunotoxins (6, 7). The most critical limitation of this strategy is the lack of effective latency reversing agents (LRAs). Available LRAs activate only a minor fraction of resting cells, the most critical component of the reservoirs (1, 8). On the other hand, even if such effective compounds become available, reactivated virus cannot be effectively cleared due to HIV/SIV-specific CTL impairment and exhaustion (4). Therefore, there is intense research aimed at both improving LRA efficacy, as well as restoring the impairment of SIV-specific T cells.

There is strong evidence that Tregs can become latently infected with HIV and may represent a potentially important HIV reservoir: (i) Tregs expand in blood and tissues in chronically HIV-infected patients and SIV-infected macaques (9); (ii) the fraction of Tregs containing HIV/SIV DNA is higher than that of non-Tregs in HIV-infected patients on ART (10) and in SIV-infected rhesus macaques (RMs) (11); (iii) Tregs are less susceptible to cell death than conventional T cells (9). As such, therapeutic interventions aiming at Treg depletion may directly contribute to the reduction of the size of virus reservoir.

Furthermore, through their regulatory function, Tregs can indirectly shape the reservoir. During the acute HIV/SIV infection, Tregs may decisively contribute to the rapid establishment of the HIV reservoir by reversing the immune activation status of $CD4^+$ T cells (9). During chronic HIV/SIV infection, Tregs contribute to the impairment of CTL responses, as suggested by the following observations: Treg expansion correlates with loss of CTL function (12–14); *ex vivo* Treg depletion from blood and lymph nodes (LNs) enhances T cell responses to HIV or SIV antigens (9); HIV nonprogressors have a high perforin/Fox-P3 ratio; and HLA $B27^+$ and $B57^+$ HIV-specific $CD8^+$ T cells from elite controllers are able to evade Treg suppression (15, 16). Inhibition of cellular immune responses by Tregs was also reported in other infectious and noninfectious conditions, such as hepatitis C and several types of cancer (9). Furthermore, Treg depletion resulted in a significant improvement of cellular immune responses and prolonged survival in cancer patients with T lymphomas (9). Altogether, these observations support a major involvement of Tregs in suppressing the protective effector immune responses against HIV. It is thus conceivable that Treg depletion in HIV/SIV-subjects may improve cell-mediated immunity

and increase HIV/SIV-infected cell clearance. This effect of the Treg depletion may be all the more critical for the “shock and kill” strategies, which require increased killing of the reactivated virus.

Here, we assessed Treg contribution to shaping the SIV reservoir by depleting Tregs *in vivo* in two spontaneous-controller RMs infected with SIVsab (17, 18). RM treatment with Ontak (recombinant IL-2 coupled with diphtheria toxin) resulted in significant Treg depletion, induced T cell activation and virus reactivation. The combined effect of Treg depletion and antigenic stimulation by the reactivated virus boosted SIV-specific T lymphocytes. As such, Treg depletion, alone or in combination with other LRAs, appears to be a promising approach for virus eradication.

Materials and Methods

Animals, infection, treatments, and samples

Two RMs (*Macacca mulatta*) received plasma equivalent to 300 50% tissue culture-infective doses ($TCID_{50}$) of the SIVsab92018 (18). Animals were housed and handled in accordance with guidelines for the care and use of laboratory animals from the U.S. Public Health Service, the American Association for Accreditation of Laboratory Animal Care, and the Animal Welfare Act (19). The University of Pittsburgh Institutional Animal Care and Use Committee approved all protocols and procedures.

SIVsab infection follow-up was performed as described (17, 18). After one and a half year postinfection, when the animals had completely controlled the virus, they were treated twice with Ontak (Ligand Pharmaceutical, La Jolla CA) intravenously (15 μg/kg) for 5 consecutive days at 21 days interval, as reported (20). Blood samples were collected prior to Ontak administration and then at 1, 3, 7, 10, 14, 17, 21 days posttreatment (dpt) initiation. Plasma and mononuclear cells were isolated as described (17, 18) for viral load (VL) quantification and flow cytometry.

Viral quantification

Plasma VLs were quantified with an ultrasensitive qPCR assay, as described (17). This assay has a sensitivity of 1 vRNA copies/ml; however, due to frequent sampling which limited the volumes of plasma, assay sensitivity was 5 copies/ml.

Antibodies and flow cytometry

Whole blood was stained for flow cytometry with multiple combinations of the following mAbs: CD3-FITC (SP34) ; CD20-PE (3G8); CD8-PerCP (SK1); CD4-APC (L200); CD25-FITC (2A3); HLA-DR-PerCP (l243); Ki-67-FITC (B56) (all from BD Biosciences) and FoxP3-APC (PCH101) (EBiosciences), as described (17, 18). Data were acquired with a FACSCalibur flow cytometer (BD Immunocytometry Systems) and analyzed with CellQuest software (BD Biosciences). $CD4^+$ and $CD8^+$ T cell percentages were obtained by first gating on lymphocytes and then on $CD3^+$ T cells. Immune activation, and proliferation markers were determined by gating on lymphocytes, then on $CD3^+$ T cells, and finally on $CD4^+CD3^+$ or $CD8^+CD3^+$ T cells.

Intracellular staining for SIV-specific T cells

Env and Gag SIVsab-specific CD4 and CD8 responses (IFNγ, TNFα, IL-2, MIP-1β and CD107a) were measured by intracellular staining (ICS) and assessed by flow cytometry, as described (8), using SIVsab-specific peptide pools: Env (52 peptides) and Gag (pool 1: 1–68, pool 2: 69–136 peptides). SIV-specific cells were acquired the same day on a custom four-laser BD LSR-II instrument (BD Bioscience). Only singlet events were gated and a minimum of 250,000 live CD3 cells were acquired with FACSDIVA 8.0. Populations were analyzed using FlowJo software version 7.6.5 (Tree Star Inc. Ashland, OR) and the graphs were generated with GraphPad Prism 6.04.

Granzyme and perforin measurements

The measurements were performed using anti-Granzyme B PE (eBioscience, San Diego, CA) and anti-human perforin antibodies from BD Biosciences (δG9). Standard intracellular cytokine stimulation and staining procedures were performed as described (21). Monensin (1 μg/mL) and concanamycin A (CMA; 0.2 μM; Sigma-Aldrich) were used for inhibition of cytotoxic granule acidification. For each tube, between 500,000 and 1,000,000 total events were acquired with an LSRII flow cytometer (BD Immunocytometry Systems, San Jose, CA). Antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each antibody. Data analysis was performed using FlowJo (version 8.5.2; TreeStar, Ashland, OR). Reported data have been corrected for background, when appropriate.

Statistical analyses

The limited number of included RMs did not permit us to perform statistical calculations. However, the various parameters were compared within the same animal prior and after reactivation with Ontak, to assess the biological effects of this treatment. For the increases in perforin expression by $CD4^+$ and $CD8^+$ T cells, results of both treatments were grouped together and paired t test was performed using Prism Graph Pad.

Results and Discussion

Treg depletion in SIVsab-infected RMs superelite controllers

Two RMs were experimentally infected with SIVsab and followed for 500 days. Similar to our previous results (17, 18), after very active virus replication during the acute infection (Supplemental Fig. 1a), with significant depletion of $CD4^+$ T cells (Supplemental Fig. 1b), SIVsab was completely controlled in RMs during chronic infection (Supplemental Fig. 1a). This allowed for control of chronic immune activation (Supplemental Fig. 1 c and d) and restoration of the $CD4^+$ T cells in the gut (Supplemental Fig. 1b). As suggested by previous results, control was likely due to effective cellular immune responses (18).

Effective Treg depletion with Ontak

Ontak administration to SIVsab-infected controller RMs after 500 days of complete control of viral replication resulted in significant depletion of the $CD25^+$ $CD4^+$ T cells (Figure 1a). Depletion was rapid and massive, with up to >50% of the $CD25^+$ $CD4^+$ T cells being

depleted by 14 dpt. However, after the second administration of Ontak, there was a very robust increase in the number of $CD25^+$ $CD4^+$ T cells (Figure 1a). The number of $CD8^+$ T cells expressing CD25 showed a similar pattern of initial depletion followed by a robust rebound (Figure 1a).

We further monitored the dynamics of Fox-$P3^+$ $CD25^+$ $CD4^+$ T cells and report that Ontak administration induces a rapid and massive (75–85%) depletion of the Fox-$P3^+$ $CD25^+$ $CD4^+$ T cells (Figure 2b). A trend of Fox-$P3^+$ $CD25^+$ $CD4^+$ T cell rebound was observed prior to the second administration of Ontak (Figure 1a). The impact on the $CD8^+$ T cells was only moderate, as the frequency of $CD8^+$ T cells expressing Fox-P3 is very low (Figure 1b).

Experimental Treg depletion with Ontak in spontaneous controller RMs boosts T cell immune activation

A major increase of $CD4^+$ T cell activation was observed after administration of Ontak. The frequency of the circulating $CD4^+$ and $CD8^+$ T cells expressing Ki-67 dramatically increased (on an average of 8–10 fold) after each Ontak administration (Figure 1c). These robust increases of $CD4^+$ and $CD8^+$ T cell activation persisted beyond the five-day course of each Ontak administration.

One may argue that these Ontak-induced increases in the levels of immune activation may have inadvertently boosted the size of the $CD25^+$ $CD4^+$ T cell fraction, which is associated with cell activation. This is not necessary a weakness of our approach. Ontak targets the cells that express CD25 and depletes them through the immunotoxin component of the drug. As such, increases in the pool of CD25-expressing $CD4^+$ T cells will only result in an increase in the pool of the Ontak-depleted $CD4^+$ cells, thus improving the drug ability to directly target the reservoir.

Experimental Treg depletion with Ontak reactivated the latent SIVsab in controller RMs

Prior to Ontak administration, the levels of SIVsab in plasma were below 5 vRNA copies/ml (Figure S1). After treatment, SIVsab rebounded up to 10^3 copies/ml (Figure 1d). This virus rebound, albeit relatively low, is not negligible and strongly suggests that Ontak could be used either alone or in combination with other conventional LRAs for virus reactivation. Unlike LRAs currently tested, which require the presence of effective CTLs to clear the cells expressing the reactivated virus, Ontak has the advantage that the cells targeted by this drug are destroyed by the diphtheria toxin that is coupled to the IL-2. Furthermore, through Treg depletion, Ontak has also the ability to boost the cell mediated immune responses necessary for effective clearance of the reactivated virus.

Experimental Treg depletion with Ontak boosted SIV-specific T cells in SIVsab-infected controller RMs

One of the most notable effects of Treg depletion with Ontak was a significant boost of SIV-specific $CD8^+$ T cells (Figure 2a). This increase occurred in every test performed, with every tested peptide pool, and thus the absolute numbers (/ml) of the aggregated SIV-specific $CD4^+$ and $CD8^+$ T cells at different time points following different rounds of Ontak administration, as determined by intracellular cytokine staining and flow cytometry, were

significantly boosted (p=0.0001 and p=0.0029 for $CD4^+$ and $CD8^+$ T cell, respectively) in Ontak-treated RM (Figure 2b). SIV-specific $CD4^+$ T cells increased in RM1-10 from 6,430/ml prior to Ontak administration to 23,437/ml at 7 dpt, while in RM2-10 they increased from 7,456/ml prior to Ontak administration to 19,864/ml at 7 dpt. Similarly, SIV-specific $CD8^+$ T cells increased in RM1-10 from 10,346/ml prior to Ontak administration to 47,899/ml at 7 dpt, while in RM2-10 they increased from 5,678/ml prior to Ontak administration to 45,673/ml at 7 dpt. Furthermore, measurements of granzyme and perforin as surrogate markers of cytotoxic responses revealed a massive boost of their expression on both $CD4^+$ and $CD8^+$ T cells. Thus, perforin expression increased 4–8 folds on $CD4^+$ T cells (p=0.0342) and 2–7 folds on the $CD8^+$ T cells (p=0.0313) at 7 dpt (Figure 2c).

Altogether, our results demonstrate the feasibility of Treg depletion with Ontak in chronically SIV-infected RMs, also pointing to the efficacy of the proposed approach on both reactivating the latent virus and boosting CTL responses.

Other approaches targeting Tregs have also been reported to have potential impact for cure research. Treg blockade with the anti-CTLA-4 drug ipilimumab in an HIV-infected patient on ART had significant effects on the total number and phenotype of $CD4^+$ T cells. Furthermore, the drug induced a profound increase in cell-associated unspliced HIV RNA, resulting in a subsequent decline in plasma HIV RNA. (22). Similarly, Treg blockade with the anti-CTLA-4 human antibody MDX-010 in SIVmac-infected RMs receiving ART (23) resulted in decreased expression of IDO and TGF-β in tissues and was associated with decreased viral RNA levels in the lymph nodes and increased effector function of both SIV-specific $CD4^+$ and $CD8^+$ T cells. Dampening Treg function in SIV-infected RMs did not have detrimental virologic effects and was shown to provide a valuable approach to complement ART and therapeutic vaccination during treatment of HIV-1 infection (23). These results further validate our approach as a promising cure strategy.

As such, our results provide strong proof of concept data that supports approaches aimed at altering the number and function of Tregs as a strategy for cure research. Treg depletion has a dual effect, leading to both reactivation of the latent virus and improved clearing of the reactivated virus. As none of the LRAs in development has been reported to have such a dual activity, Treg depletion, alone or in combination with other LRAs, holds great promises for cure research, and has a real potential to provide both improved “shock (through reactivation by LRA AND Treg-depleting agent) and kill (through CTL boosting after Treg depletion)” effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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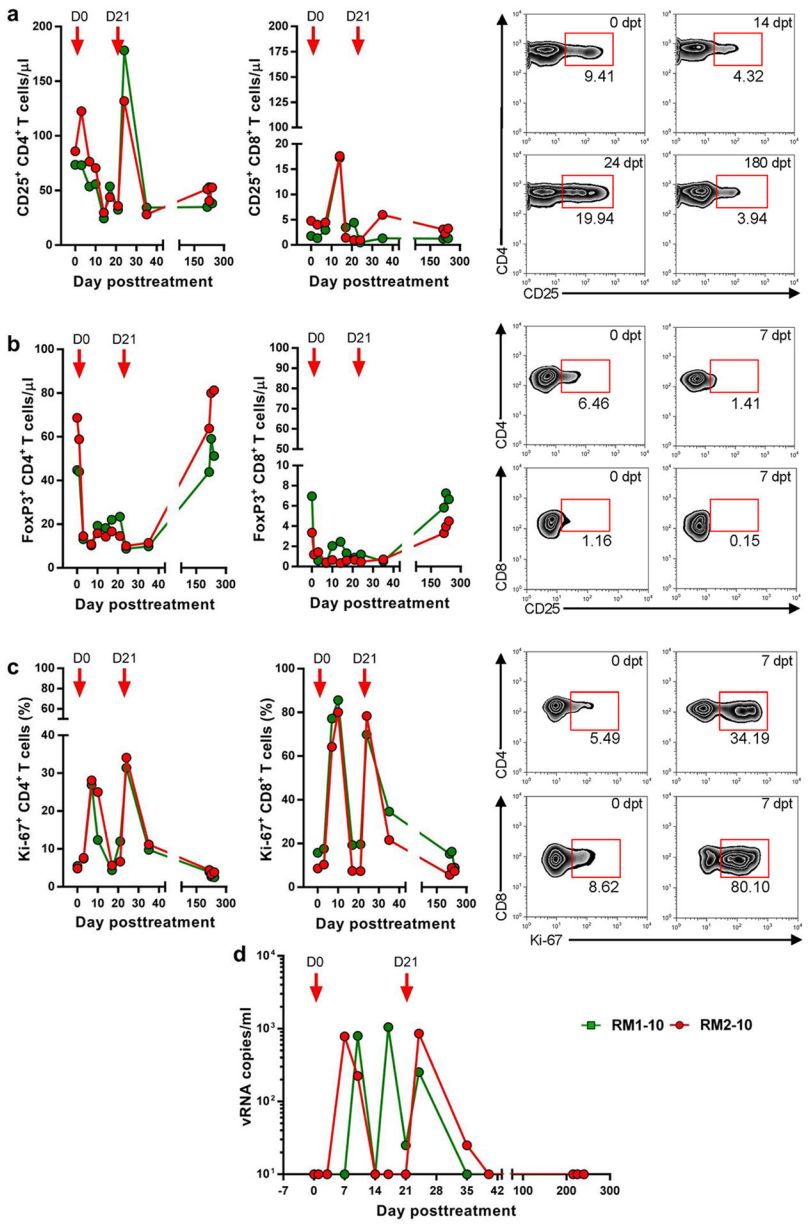

Figure 1. Treg depletion with Ontak results in increased T cell proliferation and virus reactivation in SIVsab-infected RM controllers

(a) Depletion of T cells expressing CD25 is transient and is followed by robust increases coincidental with increases in the levels of T cell activation; (b) Efficient depletion of Tregs ($FoxP3^+$ $CD4^+$ T cells) and minimal changes in the levels of $FoxP3^+$ $CD8^+$ T cells; (c) Increases in the immune activation/proliferation levels (Ki-67) of $CD4^+$ and $CD8^+$ T cells. Representative flow cytometry plots illustrate CD25-expressing $CD4^+$ T cell and Treg depletion and the increases in immune activation; (d) Plasma virus rebounds after Ontak administration. Times of Ontak administration are depicted as red arrows. dpt-day posttreatment.

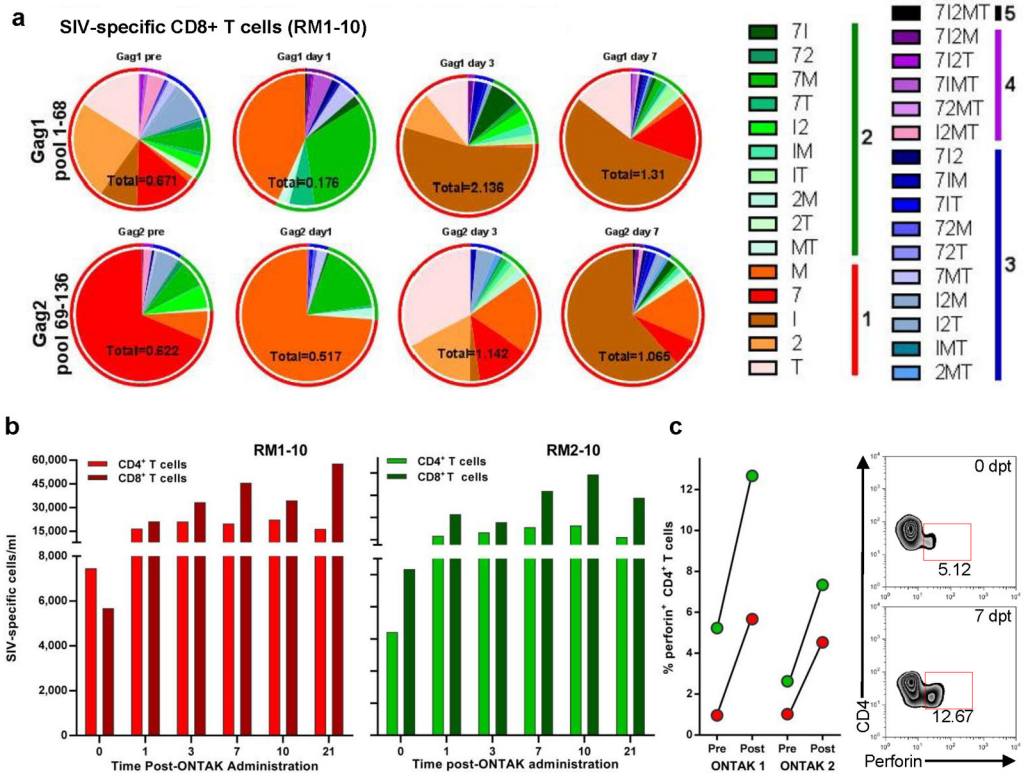

Figure 2. Ontak administration boosted the overall CTL responses in SIVsab-infected controller RMs

(a) Serial monitoring of SIV-specific T cell polyfunctionality after the first round of Ontak administration was achieved by stimulating PBMCs with Gag SIVsab92018 peptide pools followed by intracellular cytokine staining. Cytokines tested for include: TNF-α (T); IL-2 (2); IFN-γ (I); CD107α (7); and MIP-1β (M). Data are representative of both RMs. Absolute numbers of $CD8^+$ T cells/ml for each time point are marked beneath each pie graph. The pie charts depict functionality based on the combination of cytokines expressed. The color scheme represents the number of cytokines produced by the SIV-specific T cells and their proportion is illustrated as color-coded ring surrounding the pie charts. (b) Absolute numbers (per ml) of the aggregated Gag- and Env-specific $CD4^+$ and $CD8^+$ T cells at different time points following the first round of Ontak administration. The x-axis numbers represent days post-Ontak administration. (c) Significant boosting of the frequency of $CD4^+$ T cells expressing perforin after Ontak administration. Representative flow cytometry plots illustrate increase in perforin expression by the $CD4^+$ T cells after Treg depletion.