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Fatal Pancreatitis in Simian Immunodeficiency Virus SIV_{mac251}-Infected Macaques Treated with 2′,3′-Dideoxyinosine and Stavudine following Cytotoxic-T-Lymphocyte-Associated Antigen 4 and Indoleamine 2,3-Dioxygenase Blockade

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Human immunodeficiency virus (HIV) infection is associated with immune activation, $CD4^+$-T-cell loss, and a progressive decline of immune functions. Antiretroviral therapy (ART) only partially reverses HIV-associated immune dysfunction, suggesting that approaches that target immune activation and improve virus-specific immune responses may be needed. We performed a preclinical study in rhesus macaques infected with the pathogenic simian immunodeficiency virus SIV_{mac251} and treated with ART. We tested whether vaccination administered together with cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) blockade and treatment with the indoleamine 2,3-dioxygenase (IDO) inhibitor 1-methyl-D-tryptophan (D-1mT), decreased immune activation and improved vaccine efficacy. The treatment did not augment vaccine immunogenicity; rather, it dramatically increased ART-related toxicity, causing all treated animals to succumb to acute pancreatitis and hyperglycemic coma. The onset of fulminant diabetes was associated with severe lymphocyte infiltration of the pancreas and complete loss of the islets of Langerhans. Thus, caution should be used when considering approaches aimed at targeting immune activation during ART.

The chronic phase of human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) infection is characterized by persistent immune activation and progressive immune exhaustion (12). Successful antiretroviral therapy (ART), only partially reverses HIV-associated immunologic defects (9). T regulatory cells (T_{reg} cells) are $CD4^+$ T cells that maintain the physiologic equilibrium of the immune system, regulating immune responses against pathogens and preventing autoimmune conditions (26). T_{reg} cells have been proposed to suppress protective anti-HIV cell-mediated immunity (16, 31) and to inhibit the development of HIV-specific $CD8^+$ T-cell responses following therapeutic vaccination (18). T_{reg} cells constitutively express the forkhead family transcription factor Foxp3, high levels of the interleukin-2 (IL-2) receptor α-chain CD25, and cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 is a negative regulatory molecule and a target for immunomodulatory therapy for cancer treatment (33) and HIV-1/SIV infection (7). CTLA-4/B7 interaction activates the expression of the inducible enzyme indoleamine 2,3-dioxygenase 1 (IDO1) in antigen-presenting cells (10, 21). IDO1 catalyzes the rate-limiting step of the catabolism of the essential amino acid tryptophan (Trp) into the kynurenine (Kyn) pathway (20), favoring the development and differentiation of T_{reg} cells (22). Increased IDO1 expression and activity are observed during HIV/SIV infection and may contribute to virus persistence by suppressing antiviral T-cell responses (2, 4, 6). IDO2 is a homolog of IDO1, which also exerts immunoregulatory function but has less potent enzymatic activity (21). IDO inhibitors such as 1-methyl-D-tryptophan (D-1mT) have been used in SIV-infected macaques (5) and in clinical trials with the aim to restore specific responses to the virus (trials NCT00617422, NCT00567931, and NCT00739609). Although the D isomer of 1mT has been suggested to preferentially inhibit IDO2 activity, in contrast to the L isomer, which blocks IDO1 (21), D-1mT has been shown to efficiently inhibit HIV-induced Trp catabolism *in vitro*, which is likely mediated by IDO1 (2).

We previously targeted T_{reg} cell activity SIV-infected macaques by administering MDX-010, an antibody that blocks CTLA-4, with or without ART treatment and/or vaccination (8, 14). During acute infection, MDX-010 resulted in increased T-cell activation and viral replication, decreased the number of T_{reg} cells in systemic and mucosal sites, and, surprisingly, increased IDO1 expression and activity (Kyn/Trp ratio) (8). Similarly, administration of MDX-010 to chronically infected ART-treated macaques decreased ART responsiveness (8) and had no effect on IDO1 expression. In contrast, treatment of chronically SIV_{mac251}-infected animals with ART and D-1mT decreased virus levels in plasma and lymph nodes (5) but only transiently inhibited IDO activity. These data suggest the activation of complex compensatory counterregulatory mechanisms (5, 29). Thus, we tested whether the effect of simultaneous CTLA-4 and IDO blockade in SIV_{mac251}-infected, ART-treated macaques could overcome these negative regulatory feedbacks and increase specific T-cell re-

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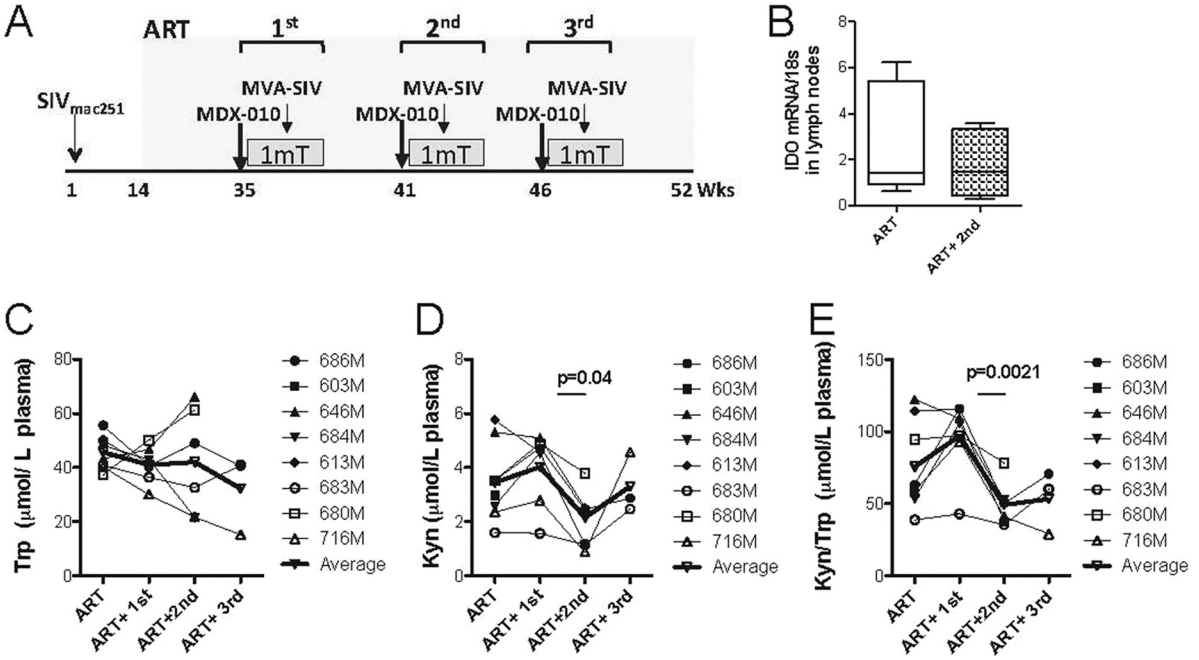

FIG 1 Study design and effect of CTLA-4 and IDO blockade on Kyn/Trp ratio. (A) Study design. Details are given in Materials and Methods. (B) IDO mRNA/18S rRNA ratio in the lymph nodes during ART only (week 32) and at 1 week from the second treatment. (C to E) Trp (C) and Kyn (D) levels and Kyn/Trp ratio (E) measured at 1 week after treatment with 1mT. Each animal is represented by a symbol, as indicated. In all graphs the thick lines represent the mean values among all animals at each time point.

sponses after vaccination with MVA-SIV. We show that while the treatment did not increase SIV-specific responses, it unexpectedly resulted in fulminant diabetes in all the animals in the study.

MATERIALS AND METHODS

Animals and study design. Eight animals (*Macaca mulatta*; Covance Research Products, Alice, TX) were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. The care and use of the animals were in compliance with all relevant institutional (NIH) guidelines. Macaques were screened for the presence of the Mamu-A*01 allele. Antech Diagnostic measured lipase and glucose levels in the plasma and creatinine and blood urea nitrogen levels. Eight macaques were simultaneously infected intrarectally with SIV_{mac251} (29). ART was given daily to all of the eight macaques under conditions of mild sedation starting at week 14 until the end of the study (week 52) and consisted of 10 mg/kg 2,3′-dideoxyinosine (ddI) (Videx), 1.2 mg/kg 2′-didehydro-3′-deoxythymidine (d4T) (stavudine) twice a day, and subcutaneous administration of 20 mg/kg (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) (tenofovir). All macaques received 3 doses of 10 mg/kg of the CTLA-4-blocking monoclonal antibody MDX-010 (Medarex, Bloomsbury NJ) intravenously at weeks 35, 41, and 46 (8). Starting one day after each MDX-010 injection, all macaques were given 45 mg/kg D-1mT for 11 days by intraesophageal feeding (Fig. 1A) (5). In addition, all macaques received recombinant modified vaccinia virus Ankara (MVA)-based vaccines expressing the structural and regulatory genes of SIV (29) 6 days after each MDX-010 inoculation. All animals were euthanized when moribund.

Quantitation of IDO1, TGF-β, and SIV RNA in tissues and of Kyn/Trp ratio in plasma. Total RNA was extracted from whole tissue with RNeasy Plus (Qiagen) and reverse transcribed with the QuanTitect reverse transcription kit (Qiagen). cDNA quantification for the 18S rRNA, IL-10, IDO1, transforming growth factor β (TGF-β), SIV *gag* genes was performed by real-time PCR (ABI 7000) using a SYBR GreenEr PCR mix (Invitrogen). The primers for the 18S rRNA gene were 5′-GCCCGAAGC GTTTACTTTGA-3′ (forward) and 5′-TCCATTATTCCTAGCTGCGGT ATC-3′ (reverse); the IDO1, TGF-β, and SIV *gag* gene primers were described elsewhere (21). A real-time nucleic acid sequence-based amplification (NASBA) assay was used to quantitative SIV RNA in plasma (25). Detection of Trp and Kyn in plasma was performed by high-performance liquid chromatography (HPLC), as previously described (32).

T-cell number, phenotype, and immunohistochemistry. Mononuclear cells from blood and lymph nodes were isolated as described elsewhere (29). $CD4^+$ and $CD8^+$ counts were periodically determined with the FACS/Lyse kit (BD Immunocytometry Systems, San Jose, CA). The antibodies used for the fluorescence-activated cell sorter (FACS) analysis were for CD8β (2ST8.5H7; Beckman Coulter), CD28 (CD28.2), CD4 (L200), CD3ε (SP34), Ki67 (B56), CD95 (DX2), CD25 (M-A251), IL-2 (clone MQ1-17H12), tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (4SB3 and B27; BD Pharmingen), FoxP3 (PCH101; eBioscience), and $Gag_{181-189}$ CM9 (p11C) (CTPYDINQM)- or Tat_{28-35} SL8 (STPESANL)-Mamu-A*01 tetrameric complexes (Beckman Coulter). Protocols for the staining are described elsewhere (29). Samples were run on a FACSCalibur or LSRII (BD). Data were analyzed with FlowJo. Tissues were collected and fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. A BH2 Olympus light microscope with 10× and 40× objectives was used for evaluation.

Statistical analysis. Differences between values for ART alone and ART plus treatment were assessed by the nonparametric Mann-Whitney test.

RESULTS AND DISCUSSION

CTLA-4 and IDO blockade transiently reduces the Kyn/Trp ratio. Eight SIV_{mac251}-infected macaques treated with ddI, d4T, and PMPA were administered 10 mg/kg MDX-010 intravenously, followed by oral administration of D-1mT 1 day after MDX-010 for 11 consecutive days (Fig. 1A). To expand immune responses to-

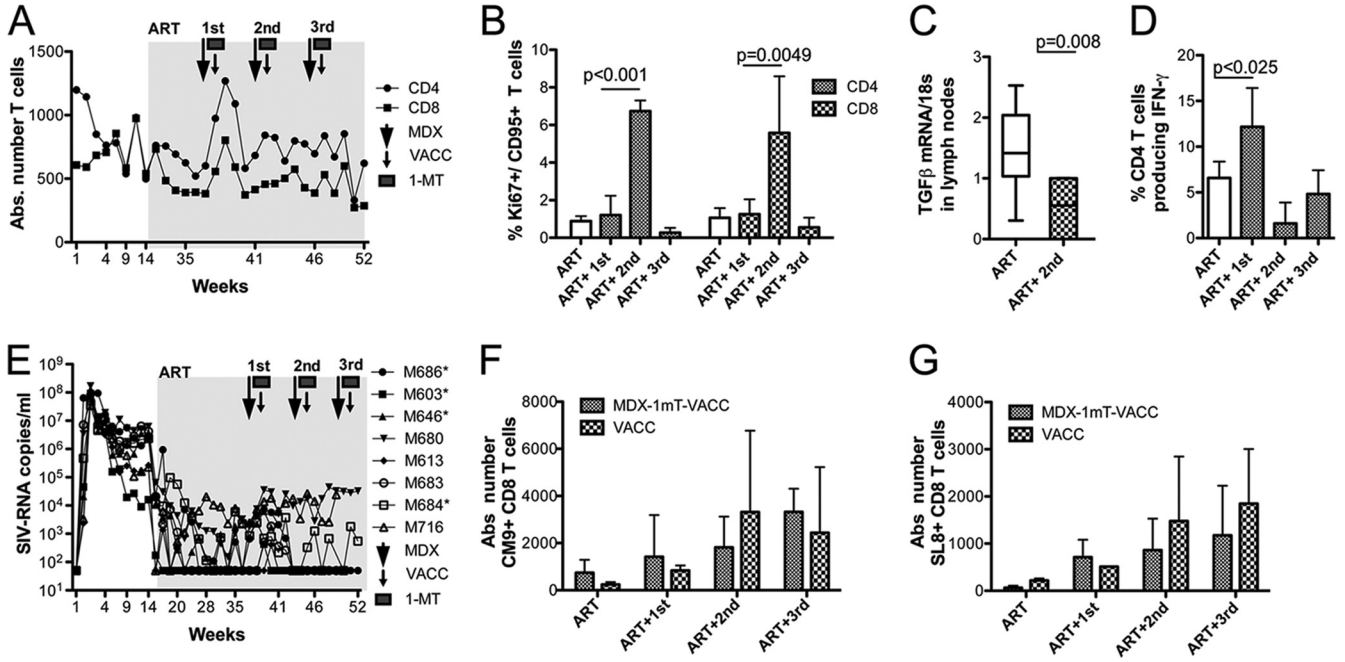

FIG 2 Effect of CTLA-4 and IDO blockade on T-cell numbers and vaccine immunogenicity. (A) Average values of the absolute numbers of $CD4^+$ and $CD8^+$ T cells in the blood over time. The arrows and the dark squares represent each MDX-010 and D-1mT administration. The gray area represents ART. (B) Averages ± standard deviations of the frequencies of $Ki67^+$ $CD95^+$ $CD4^+$ and $CD8^+$ T cells during ART only (week 32) and at 1 week after each treatment (weeks 38, 42, and 46). (C) TGF-β mRNA normalized on 18S rRNA in the lymph nodes during ART only (week 32) and at 1 week from the second treatment. (D) Frequency of T_{h1} cells, defined as $CD4^+$ T cells producing IFN-γ, after 6 h of stimulation with PMA-ionomycin. (E) SIV_{mac251} viral RNA in plasma over time. (F and G) Absolute numbers of tetramer$^+$ $CD95^+$ $CD8^+$ T cells specific for SIV *gag* (CM9) (F) and for SIV *tat* (SL8) (G) in blood. The bars represent the averages ± standard deviations for 4 MamuA*01^+ macaques used in the current study and 3 MamuA*01^+ macaques used in previous studies, vaccinated with the same vaccine and treated with the same antiretroviral treatment (8), at 1 week from each vaccination.

ward SIV epitopes, we immunized the macaques with intramuscular MVA-SIV *gag*, *pol*, *env ret*, *tat*, and *nef* (29) at 6 days after MDX-010 was given, during D-1mT administration. The treatment with MDX-010, D-1mT, and vaccination was repeated three times during ART (Fig. 1A). No significant changes were observed in the IDO1 expression measured as levels of IDO1 mRNA in lymph nodes after the second treatment (Fig. 1B). Thus, we assessed the effects on IDO activity, measuring plasma levels of Trp and Kyn during ART only (week 32) and 1 week after each treatment (weeks 38, 43, and 48) by HPLC (Fig. 1C to E). We observed no significant changes in the levels of Trp, whereas Kyn was significantly decreased after the second treatment (week 38 versus week 43, $P = 0.04$) (see Fig. S1A and S1B in the supplemental material). Accordingly, the Kyn/Trp ratio was significantly lower after the second treatment than after the first treatment ($P = 0.0021$) (Fig. 1E). Thus, the concurrent blockade of CTLA-4 and IDO was associated with a transient effect on the Kyn/Trp ratio, which was, however, more profound than what was previously observed with either treatment alone (5, 8).

CTLA-4 and IDO blockade increases T_{h1} cell proliferation while blocking T_{reg} cell functions. We previously showed that treatment with CTLA-4 and IDO blockade was associated with an increase in blood $CD4^+$ T cells and CD4 mRNA expression, respectively (8). In the current study, the blood $CD4^+$-T-cell count (Fig. 2A) significantly increased 1 week after the first treatment (week 38) ($P = 0.031$) compared to week 32 (ART alone), but this increase was not durable. After the second administration, there was a trend of an increase in the number of $CD8^+$ T cells (Fig. 2A). Interestingly, the frequencies of Ki67-positive $CD4^+$ and $CD8^+$ T cells were significantly, but transiently, increased after the second treatment (Fig. 2B). Accordingly, the level of transforming growth factor β (TGF-β), a cytokine that blocks proliferation and lymphocyte activation and is involved in the generation and expansion of T_{reg} cells (11), was significantly reduced in lymph nodes ($P = 0.008$) following the second treatment (Fig. 2C). Treatment with MDX-010 and D-1mT was intended to increase T_{h1} while decreasing T_{reg} cell frequency and/or function. We measured changes in the frequencies of peripheral $CD4^+$ T cells that produced IFN-γ (T_{h1}) upon stimulation with phorbol myristate acetate (PMA)-ionomycin and of $Foxp3^+$ $CD25^+$ $CD4^+$ T cells (T_{reg} cells) (Fig. 2D; see Fig. S1C in the supplemental material). No changes in the frequency of T_{reg} cells were observed, in agreement with human studies on CTLA-4-blockade *in vivo* (28). Importantly, the number of T_{h1} cells was significantly increased after the first treatment compared to week 32 (ART only) ($P < 0.025$).

CTLA-4 and IDO blockade does not affect virus level or augment vaccine-elicited SIV-specific responses. The increase of $Ki67^+$ $CD4^+$ T cells could adversely affect ART-dependent control of viral replication. However, no changes were observed in the viral load during ART or after each treatment (Fig. 2E). To assess whether MDX-010 and D-1mT treatment increased vaccine-elicited SIV-specific immune responses, we measured $CD8^+$ T-cell responses against SIV *gag* and *tat* by tetramer staining in the 4 MamuA*01^+ macaques in the study (Fig. 2F and G, respectively) and in 3 MamuA*01^+ macaques treated with ART only and vaccinated with the same modality. We observed a progressive in-

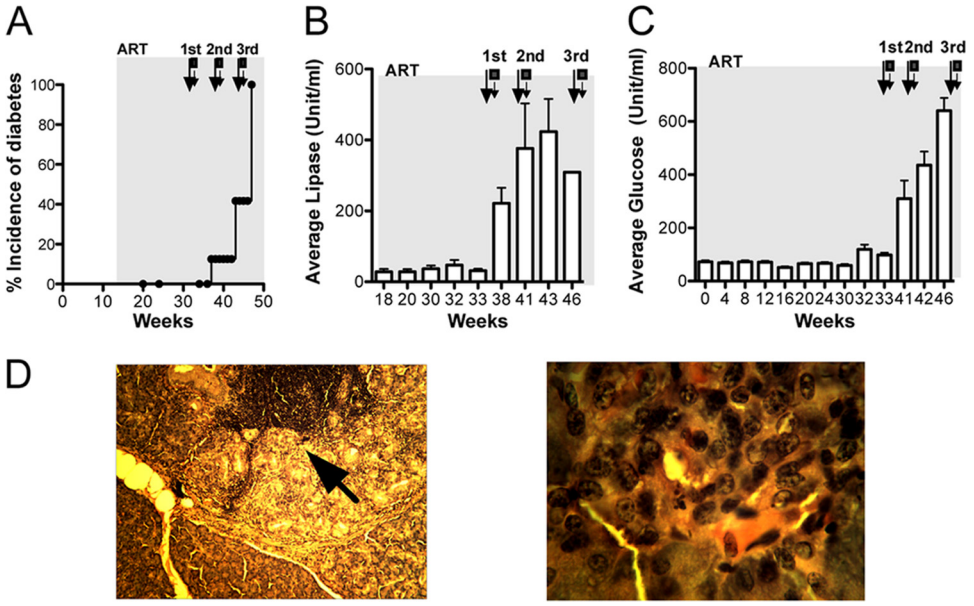

FIG 3 Treatment related-metabolic dysfunctions and onset of fulminant diabetes. (A) Incidence of diabetes after each treatment. (B and C) Effect of the treatment on plasma lipase (B) and glucose (C) levels. (D) Microscopic examination of pancreatic tissues collected at necropsy from animal M683, revealing tissue destruction (left panel) and lymphocytic infiltration (left panel, arrow, and right panel).

crease in the frequency of tetramer-positive *gag*- and *tat*-specific $CD8^+$ T cells after vaccination. However, this increase did not significantly differ between animals treated with ART alone and animals receiving MDX-010 and D-1mT in addition to ART (Fig. 2F and G). Thus, treatment with CTLA-4 and IDO blockade did not increase the immunogenicity of the vaccine administered during ART.

CTLA-4 and IDO blockade in ddI-, PMPA-, and d4T-treated macaques is associated with a high frequency of acute pancreatitis. ART-related toxicity can result in pancreatitis and diabetes in both humans and macaques (1, 15, 17, 19). Previously, in studies where the same antiretroviral regimen was given to the same animal model starting at 14 weeks postinfection, we have observed an incidence of diabetes of 18% staring at 25 to 30 weeks from ART initiation (see Table S1 in the supplemental material). The onset of diabetes was not augmented by CTLA-4 blockade given to SIV-infected animals undergoing the same ART treatment for the same amount of time (16, 17), while it was increased 33% in animals treated with D-1mT and ART (see Table S1 in the supplemental material) (8, 14). Of note, in this study, ART was initiated at a later stage of chronic infection, possibly explaining why in two of the three animals that developed diabetes this occurred very early on. Strikingly, all eight animals in the present study developed acute pancreatitis. Two animals were diagnosed with diabetes after the first treatment (week 21 to 23 after ART initiation), three animals after the second treatment (week 27 to 28 after ART initiation), and the remaining three animals at the end of the second treatment and during the third treatment (week 28 to 32 after ART initiation) (Fig. 3A; see Table S1 in the supplemental material). Accordingly, the levels of glucose and lipase increased following treatment (Fig. 3B and C). Microscopic examination of pancreatic tissues collected at necropsy revealed complete loss of islets of Langerhans and lymphocytic infiltrates (Fig. 3D shows results for animal M683). Lymphocytic aggregates were also present in the lung, gallbladder, kidney, and heart but were not greater in number, distribution, or size than those routinely observed in nondiabetic SIV-infected primates (data not shown). Animals did not present other signs of immune-related toxicity such as skin rashes or colitis, as observed in cancer patients treated with MDX-010 alone (33). Finally, the combination of the treatments did not affect the renal function as suggested by the measurement of creatinine and blood urea nitrogen levels, which were normal or slightly elevated only in 2/8 animals at the time of the sacrifice (see Table S1 in the supplemental material).

In summary, we demonstrate here that simultaneous CTLA-4 and IDO blockade in conjunction with ddI, PMPA, and 4dT treatment resulted in a modest and transient reduction of the plasma Kyn/Trp ratio and no significant changes in IDO1 mRNA expression in lymph nodes. Although the D isomer of 1-mT does not affect IDO1 but efficiently inhibits IDO2 in cell-free assays (21), D-1mT inhibits HIV-induced tryptophan catabolism *in vitro* (2) and enhances antitumor responses in mice (13). The selective interference of D-1mT with one of these partially redundant enzymes may also account for the transient nature of the decrease in the plasma Kyn/Trp ratio, which was also observed in our previous study in which D-1mT alone was administered (3). It is also possible that the decrease in Kyn/Trp ratio observed during the second treatment is an indirect consequence of repeated CTLA-4 blockade rather than a direct effect of D-1mT.

This strategy transiently increased $CD4^+$ T-cell counts but did not interfere with ART-mediated control of viral replication or augment SIV-specific immune responses.

Both CTLA-4 and IDO are involved in the maintenance of immune tolerance *in vivo* in humans and mice (23, 27). We observed a decrease in TGF-β expression in the lymph nodes and increased lymphocyte proliferation. These drug-induced changes in homeostasis were transient, suggesting the existence of multiple regulatory feedback pathways. Also, our aim was to increase

vaccine-induced immune responses following vaccination with MVA-SIV, but IDO and CTLA-4 blockade did not augment the immunogenicity of this vaccine modality.

Pancreatic toxicity caused by the nucleoside reverse transcriptase inhibitor ddI and 4dT is well established (1). Also, the maximum plasma concentration of ddI can be increased when it is administered in combination with PMPA (tenofovir) (15), thus increasing ddI-related toxicity, including pancreatitis and lactic acidosis (24). A portion of ART-treated HIV-infected individuals develop pancreatitis accompanied by increased plasma lipase and diabetes, but ART-related pancreatitis is normally lower in incidence and symptoms are milder than what was observed here (17, 19). In our study, all the animals developed pancreatitis and fulminant diabetes soon after CTLA-4 and IDO inhibitors were administered, which were not resolved by insulin administration. All macaques presented a complete loss of the islets of Langerhans together with lymphocytic infiltrates (Fig. 3D) and high plasma levels of lipase and glucose. Although the combination of MDX-010 and D-1mT has not been tested in the absence of ART, we did not observe such severe and rapid toxicity when the drugs were given alone to SIV-infected macaques or in combination with the same cocktail/dose of ART, suggesting that all the components could have participated in the toxicity.

Since we observed an increase in T_{h1} responses and a decreased T_{reg} function, we hypothesize that the specific pancreatic damage induced by ART, combined with the effect that CTLA-4 and IDO blockade may have on immune homeostasis, might have exacerbated autoimmunity to pancreatic antigens. This hypothesis will need to be tested using a combination of MDX-010 and D-1mT in the presence or absence of ART in studies specifically aimed at investigating pancreatic toxicity and autoimmune diabetes.

If confirmed, the hypothesis that the pancreatic damage is due to autoimmune or inflammatory events might have important repercussions for the management of pancreatic cancer. Thus, T_{reg} cells are increased in the peripheral blood and in the tumor microenvironment of patients with invasive cancer. These T_{reg} cells may mitigate the immune response against cancer and may partly account for the poor immune response against tumor antigens (28). Elevated IDO activity in tumor-draining lymph nodes has been associated with suppression of antitumor immunity and poor prognosis in humans (22). Strategies incorporating T_{reg} cell depletion improved the efficacy of cancer vaccines in mice with pancreatic cancer (30). The induction of pancreatic tissue damage by ddI and d4T may expose sufficient amounts of self antigens under conditions where tolerance is interrupted by the simultaneous blockade of CTLA-4 and IDO. Thus, the simultaneous blockade of CTLA-4 and IDO is not likely to be an effective immune restoration treatment in HIV infection; we suggest that this strategy may be tested in the treatment of human pancreatic cancer.

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