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Differential Impact of PD-1 and/or Interleukin-10 Blockade on HIV-1-Specific CD4 T Cell and Antigen-Presenting Cell Functions

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ABSTRACT
Antigen persistence in chronic infections and cancer upregulates inhibitory networks, such as the PD-1 and interleukin-10 (IL-10) pathways, that impair immunity and lead to disease progression. These pathways are attractive targets for immunotherapy, as demonstrated by recent clinical trials of PD-1/PD-L1 blockade in cancer patients. However, in HIV-1 infection not all subjects respond to inhibition of either pathway and the mechanistic interactions between these two networks remain to be better defined. Here we demonstrate that in vitro blockade of PD-L1 and/or IL-10Rα results in markedly different profiles of HIV-1-specific CD4 T cell restoration. Whereas PD-L1 blockade leads to balanced increase in gamma interferon (IFN-γ), IL-2, and IL-13 secretion, IL-10Rα blockade preferentially restores IFN-γ production. In viremic subjects, combined PD-L1/IL-10Rα blockade results in a striking 10-fold increase in IFN-γ secretion by HIV-1-specific CD4 T cells that is not observed in subjects with spontaneous (elite controllers) or therapy-induced control of viral replication. In contrast to the dramatic increase in IFN-γ production, concurrent blockade has a marginal additive effect on IL-2 production, IL-13 secretion, and HIV-1-specific CD4 T cell proliferation. IFN-γ produced by Thelper cells upregulates PD-L1, HLA I/II, and IL-12 expression by monocytes. The effect of combined blockade on IFN-γ was dependent on reciprocal reinforcement through IL-12. These studies provide crucial information on the different immunoregulatory qualities of PD-1 and IL-10 in progressive disease and link exhausted virus-specific CD4 T cells and monocytes in the regulation of IFN-γ and IL-12 secretion.

IMPORTANCE
Infection with HIV results in most people in uncontrolled viral replication and progressive weakening of the body defenses. In the absence of antiviral therapy, this process results in clinical disease, or AIDS. An important reason why HIV continues to multiply is that a population of white blood cells called CD4 T cells that targets the virus fails to work properly. At least part of this impairment is under the control of inhibitory mechanisms that can be blocked to improve the function of these CD4 T cells. In this report, we show that blocking one or two of the molecules involved, called PD-1 and IL-10, has different effects on the individual functions of these cells and that one is strongly improved. We investigate how these effects are caused by interactions between CD4 T cells and antigen-presenting cells. These observations can have implications for new therapeutic approaches in HIV infection.

Studies in animal models and humans have demonstrated that chronic viral infections lead to T cell exhaustion, which is defined as the progressive loss of functions in the setting of antigen persistence (reviewed in reference 1). In contrast to the significant progress made in understanding mechanisms of CD8 T cell exhaustion, CD4 T cell dysfunction remains much less explored. Data demonstrate both similarities and major differences in the exhaustion mechanisms mediating exhaustion of these cell subsets (2, 3). In HIV-1 infection, studies by our group (2, 3) and others (4, 5) have shown that PD-1 is upregulated on virus-specific CD4 T cells and mediates a dysfunction that is reversible upon PD-1 blockade in vitro. An increase in virus-specific CD4 T cells upon PD-1 blockade in simian immunodeficiency virus (SIV)-infected macaques demonstrated the relevance of these findings for immune interventions in vivo (6). Similarly, interleukin-10 (IL-10) is upregulated in progressive human immunodeficiency virus type 1 (HIV-1) disease and blockade of the IL-10 pathway enhances HIV-1-specific CD4 T cell function (7–10). Of note, most studies focused on proliferative responses and did not address potential qualitative differences in the functional profiles of HIV-1-specific CD4 T cells restored by these interventions, such as cytokine secreted by various differentiated Thelper subsets.

The multiplicity of mechanisms involved in T cell exhaustion limits the effectiveness of interventions when a single negative regulatory pathway is targeted in vivo and in vitro. A number of...
these factors are nonredundant, as illustrated by the synergistic improvement of virus-specific CD8 T cell responses and viral load control observed in the murine lymphocytic choriomeningitis virus (LCMV) model with PD-L1/IL-10 blockade (11) and PD-L1/LAG-3 blockade (12) and the recent clinical trial where combination of cytotoxic-T lymphocyte-associated antigen-4 (CTLA-4) and PD-1 antibodies resulted in marked tumor regression in a significant number of patients (13). The improved impact of a combined intervention has also been shown on HIV-1-specific CD8 T cells in vitro by simultaneous blockade of PD-1 and 2B4 (14). However, effects of combined interventions on virus-specific CD4 T cells remain essentially unexplored. Even more than for single blockade, the issue of a change in the quality of the Thelper response upon synergistic manipulations of exhaustion mechanisms will be critical to assessing their immunotherapeutic potential and the risk of side effects.

In this study, we addressed these issues with functional and phenotypic analyses of peripheral blood mononuclear cell (PBMC) subsets isolated from individuals at different stages of HIV-1 infection. We show that PD-1 and IL-10 pathways have different qualitative inhibitory impacts on the function of HIV-specific CD4 T cells and that combined blockade of both pathways in chronic viremic subjects resulted in a striking increase in gamma interferon (IFN-γ) secretion that appeared selective compared to other important Thelper cytokines. This cytokine had dual effects on monocytes, inducing IL-12p70 production while also upregulating PD-L1 expression after stimulation with the cognate antigen. These findings shed light on the impact that exhausted HIV-1-specific CD4 T cells have on antigen-presenting cell (APC) function and indicate a critical and nonredundant interplay among the PD-1, IL-10, IFN-γ, and IL-12 pathways to regulate HIV-1-specific CD4 T cell function and have implications for therapeutic interventions during chronic infection.

MATERIALS AND METHODS

Ethics statement. Peripheral blood was obtained from HIV-infected individuals at the Massachusetts General Hospital (MGH) in Boston. The study was approved by the MGH Institutional Review Board and written informed consent obtained from all study participants prior to enrollment in the study. All participants were adults (18 years old or older). All clinical investigations have been conducted according to the Declaration of Helsinki principles.

Clinical samples. PBMCs from (i) HIV controllers (viral load < 50 RNA copies/ml in the absence of antiretroviral therapy [ART]), (ii) chronic progressors (viral load > 2,000 RNA copies/ml), and (iii) ART-treated subjects (subjects on ART with viral load < 50 RNA copies/ml) were isolated by Ficoll density centrifugation. Freshly isolated PBMCs were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% human AB serum (Gemini Bioproducts).

Measurements of cytokine secretion. One million freshly isolated CD8 T cell-depleted PBMCs (RosetteSep CD8 depletion reagents; Stem-Cell) were incubated with an HIV Gag peptide pool (1 μg/ml/peptide) or left unstimulated in the presence of 10 μg/ml isotype control antibody or blocking antibodies against PD-L1 (clone 29E.2A3 [10 μg/ml]) and/or IL-10Rx (clone 37607; R&D). To investigate mechanisms of action of combined blockade, we also used anti-IFN-γ (clone b27; BD/Pharmin-gen) and anti-IL-12 (clone 24910; BD/Pharmin-gen) neutralizing antibodies. Cytokine secretion in supernatants was measured 48 h after stimulation based on our previous analyses of cytokine transcription and secretion (2). Measurements were performed using a Milliplex Map high-sensitivity human cytokine kit (Millipore) on a Bio-plex 200 array system (Bio-Rad Laboratories) per the instructions of the manufacturers.

Proliferation assays. Proliferation of HIV-1-specific CD4 cells was assessed as described previously (2, 3). CD8-depleted PBMCs were labeled with 1.25 μM carboxyfluorescein succinimidyl ester (CFSE) dye (Molec-ular Probes) and incubated with 10 μg/ml isotype control or blocking antibodies against PD-L1 (clone 29E.2A3 [10 μg/ml]) and/or IL-10Rx (clone 37607; R&D). After 7 days of incubation, cells were analyzed on an LSRII cytometer (BD Bioscience).

To investigate the differential impact of combined blockade on cytokine secretion and proliferation, CD8-depleted PBMCs were stimulated in the presence of blocking antibodies as described above. Half of the cell culture supernatant was collected at 48 h to measure cytokines and replenished with new medium. Proliferation of HIV-specific CD4 T cells on the same samples was measured at 7 days after stimulation.

Analysis of cell death after combined blockade of PD-L1 and IL-10Rx. Death of HIV-1-specific CD4 T cells was analyzed using annexin V per the manufacturer’s instructions. CD8-depleted PBMCs were stimulated in the presence of isotype control or anti-PD-L1 and anti-IL-10Rx antibodies as described above. Detection of HIV-1-specific CD4 T cells through upregulation of CD154 and CD69 was performed with a variant of a previously described protocol (15–17), using anti-CD40 (Miltenyi Biotec) to prevent CD40L downregulation, per the manufacturer's instructions. Cells were collected 48 h after stimulation and stained with CD3, CD4, CD8, CD69, CD154, 7AAD, CD19/CD14/CD56 (as an exclusion channel), and annexin V (BD/Pharmin-gen). Data from nonfixed cells were acquired on an Aria (BD Bioscience) fluorescence-activated cell sorter (FACS) equipped for biohazardous material.

Phenotypic analysis of PD-1 and its ligands. In order to investigate the effect of stimulated HIV-1-specific CD4 T cells on monocytes, we stimulated CD8-depleted PBMCs with HIV-1 Gag peptide pools in the presence of isotype control or blocking anti-IFN-γ. After 48 h, cells were stained with a dead cell dye (blue viability dye; Invitrogen) and fluorescent antibodies against CD3, CD4, CD8, CD69, CD154, 7AAD, CD19/CD14/CD56 (as an exclusion channel), and annexin V (BD/Pharmin-gen). Data from nonfixed cells were acquired on an Aria (BD Bioscience) fluorescence-activated cell sorter (FACS) equipped for biohazardous material.

Statistical analysis. Flow cytometry data were analyzed with FlowJo version 7.6.5 (TreeStar). Statistical analyses were performed using Prism 4.0 (GraphPad). Pairwise comparisons for cytokine secretion were performed using the Wilcoxon matched-pair test. For comparison of more than three groups with paired data, we used repeated measures of variants and Tukey posttests if the data followed a Gaussian distribution according to the Kolmogorov-Smirnov normality test. A Friedman test with Dunn posttest was used on data that did not follow a Gaussian distribution. To compare fold increases of cytokine secretion among subjects with different disease statuses, we used the Kruskal-Wallis and Dunn posttest. Correlation coefficients were calculated using the Spearman rank sum test.

RESULTS

Combined blockade of the PD-1 and IL-10 pathways has a strong additive effect on IFN-γ secretion by HIV-1-specific CD4 T cells in progressive disease. Previous studies have shown that both PD-1 and IL-10 blockade can restore some functions of exhausted HIV-1-specific CD4 T cells in vitro (2, 4, 7, 10, 18). Given the different mechanisms of action of these two pathways (19, 20), we reasoned that parallel assessment of cytokines corresponding to different Thelper cell subsets (IFN-γ for Th1, IL-2 as a cardinal cytokine of CD4 T cell function, and IL-13 as a Th2 cytokine) would allow us to investigate differences in the profiles of HIV-1-specific CD4 T cell responses restored by targeted inhibition of either pathway and to determine whether synergy can be achieved by combined blockade. We used an approach validated in previous studies (2, 10, 21) that is much more sensitive than standard intracellular cytokine staining (ICS) for detecting the impact of exhaustion pathways in vitro. In contrast to ICS assays, this method does not interfere with release of cytokines in the cul-
ture medium, which allows critical cytokine-mediated cross talk between immune cells to occur. Representative results are illustrated in Fig. 1A. Consistent with our previous reports (2, 10), blockade of either the PD-1 or IL-10 pathways in progressors resulted in a median 2.5-fold increase in IFN-\(\gamma\) secretion (Fig. 1B). However, closer consideration of individual responsiveness shows marked qualitative differences: anti-PD-L1 had a reproducible impact in the entire group with a relatively narrow range of responses, whereas the impact of anti-IL-10R\(\alpha\) varied widely. Combined PD-L1 and IL-10R\(\alpha\) blockade resulted in a dramatic median 9.8-fold increase in IFN-\(\gamma\) secretion (Fig. 1B), demonstrating striking restoration of this Th1-type HIV-1-specific CD4 T cell function.

In order to confirm that the increased secretion of IFN-\(\gamma\) observed was dependent on HIV-1-specific CD4 T cell help, we performed parallel experiments with CD3- or CD8-depleted PBMCs. We observed a complete abrogation of IFN-\(\gamma\) secretion under all blocking conditions when all T cells, rather than only CD8 T cells, were depleted (see Fig. S1A and B in the supplemental material). ICS assays performed during the first 12 h of stimulation showed that this early cytokine production was limited to the CD4 T cell subset (data not shown). We then used delayed ICS at 48 h which demonstrated a secondary IFN-\(\gamma\)-positive (IFN-\(\gamma\)/H11001/H9253) natural killer (NK) cell response that was CD4 T cell dependent (see Fig. S1C). In order to determine whether this NK response affected the profile of IFN-\(\gamma\) responses to PD-L1/IL-10R\(\alpha\) blockade, we compared the cytokine levels obtained with CD8 depletion alone versus combined removal of CD8 and CD56 cells (see Fig. S1D). NK depletion results in a general reduction in the IFN-\(\gamma\) levels in supernatants after stimulation by HIV-1 Gag but without altering the relative effects of PD-L1/IL-10R\(\alpha\) blockade compared to isotype control antibody results (see Fig. S1E). Thus, the strong additive effect of combined PD-L1/IL-10R\(\alpha\) blockade on IFN-\(\gamma\) production is due to HIV-1 CD4 T cells rather than to NK cell responses. Experiments repeated with pure live-sorted CD4 T cells (see Fig. S1F) or ICS assays at the 48 h time point after stimulation.
(see Fig. S2) further confirmed the augmented secretion of IFN-γ upon PD-L1/IL-10Rα blockade in this subset.

We next sought to define the impact of these in vitro interventions for subjects with spontaneous or therapy-induced control of viral replication. In ART-treated subjects and elite controllers, blockade of PD-1 or IL-10Rα led to significant increases of IFN-γ secretion by HIV-1-specific CD4 T cells, although the levels were lower than in untreated progressors (Fig. 1C; see also Fig. S3 in the supplemental material). In contrast to what we observed in untreated progressors, combined PD-L1/IL-10Rα blockade had only a modest impact in subjects with a controlled pathogen load (Fig. 1C). Our data thus show that whereas progressors were only moderately more responsive to blockade of individual pathways than aviremic subjects, they were characterized by a unique interplay among these three immunoregulatory networks in the presence of ongoing viremia. Finally, our data show that the combined blockade of different immunoregulatory networks involved in CD4 T cell exhaustion elicits distinct profiles of immune restoration and that the potential for synergy with combined PD-L1/IL-10Rα blockade is restricted to specific cytokines.

Intraindividual correlations show distinct patterns of responses to PD-1 and/or IL-10 blockade. As our data showed significant heterogeneity in the responsiveness to PD-L1 and/or IL-10Rα blockade among individuals, we wanted to define whether nonresponders to one blockade would respond to inhibition of the other pathway or to combined blockade. There was no correlation between the impact of anti-PD-L1 and that of anti-IL-10Rα on IFN-γ secretion by HIV-1-specific CD4 T cells (Fig. 3A), and subjects that responded better to manipulation of either pathway could be identified. We found a strong correlation between the

FIG 2 Concurrent blockade of PD-L1 and IL-10Rs produced minimal enhancement of IL-2 and IL-13 secretion compared to single blockade. The supernatants tested for IFN-γ in the experiments represented in Fig. 1 were assessed for IL-2 and IL-13 at 48 h after stimulation. (A and B) Representative examples of IL-2 (A) and IL-13 (B) production for one chronic progressor. (C and D) Statistical comparison of the impact (fold increase compared to isotypic control) of different blockade combinations on IL-2 (C) and IL-13 (D). Comparisons among groups were performed by Kruskal-Wallis followed by Dunn’s posttest. Short horizontal lines, median values; horizontal dotted lines, fold increase of 1 (no impact of blockade). Symbols for values: *, P = 0.05; **, P = 0.01; ***, P = 0.001.
The effect of combined PD-L1/IL-10R blockade and that of IL-10R blockade alone (Fig. 3B), whereas no correlation was present between the impact of the double blockade and that of PD-L1 blockade alone (Fig. 3C). Thus, for IFN-γ, simultaneous blockade appears to act as an amplifier of the response profile elicited by IL-10R blockade.

Similar analyses performed for IL-2 secretion also revealed weak correlations between the impact of PD-L1 and IL-10R blockades (Fig. 3D) and the impact of combined PD-L1/IL-10R and IL-10R blockades, respectively (Fig. 3E). In contrast, there was a strong correlation between the impacts of PD-L1/IL-10R and PD-L1 blockades (Fig. 3F). The addition of anti-IL-10R led to only a modest gain in IL-2 secretion compared to anti-PD-L1 alone, consistent with the fact that PD-L1, but not IL-10R, is a major regulator of IL-2 secretion.

**Simultaneous blockade of the PD-1 and IL-10 pathways has a modest impact on HIV-1-specific CD4 T cell proliferative responses.** Our previous work (2) showed that PD-L1 blockade affects cytokine secretion in both proliferating and nonproliferating HIV-1-specific CD4 T cells. We next sought to determine the impact of combined blockade on proliferation of HIV-1-specific CD4 T cells of progressors and how it compared to its effect on cytokine secretion. Consistent with previous studies (2, 7, 10), single blockade of the PD-1 or IL-10 pathways resulted in a significant but modest increase in proliferation as measured by 7-day CFSE assays (Fig. 4A and B). In sharp contrast to what we observed for IFN-γ secretion, concurrent blockade of the two pathways did not enhance proliferation of HIV-1-specific CD4 T cells more than did anti-PD-L1 alone (Fig. 4B). We confirmed that this discrepancy between the effects on proliferation and cytokine secretion was present within the same individuals (Fig. 4C to E). Although our data demonstrate that combined PD-L1 and IL-10R can differentially affect proliferation as well as the secretion of individual cytokines in the same subjects, further studies of the relationship between single and combined interventions on proliferation compared to effector functions are needed.

In order to determine whether the effect of combined PD-L1/IL-10R blockade on proliferation (as measured by CFSE assays) was limited by activation-induced cell death of HIV-1-specific CD4 T cells, we determined by annexin V staining the fraction of apoptotic cells within the HIV-1-specific CD4 T cell population identified by upregulation of CD69 and CD154 (15–17) after 48 h of stimulation with HIV-1 antigen (Fig. 4F). Data from three individuals (Fig. 4G) showed that in our in vitro system, PD-L1/IL-10R blockade did not increase cell death of HIV-1-specific CD4 T cells compared to isotype control conditions. These results suggest that simultaneous blockade of the PD-1 and IL-10 pathways has a preferential effect on IFN-γ secretion compared to proliferation and that the modest effect observed in CFSE assays is not the consequence of decreased survival offsetting the increase in proliferating cells.

IFN-γ produced by HIV-1-specific CD4 T cells in response to antigen induces upregulation of PD-L1 on monocytes. The dramatic increase in IFN-γ after combined PD-L1/IL-10R blockade suggests a specific interplay among these three pathways. Previous studies have shown that IFN-γ can upregulate PD-L1 on human endothelial cells (22), plasma cells in multiple myeloma (23) and blasts in acute myelogenous leukemia (AML) (24). However, the capacity of IFN-γ to modulate molecules of the PD-1 pathway on leukocyte subsets in the setting of HIV-1 infection remains to be
Combined PD-L1 and IL-10Rα blockade has a greater effect on IFN-γ secretion than on proliferation, with no significant impact on cell survival. CD8-depleted PBMCs were labeled with CFSE and stimulated with an HIV-1 Gag peptide pool in the presence of blocking antibody against PD-L1 and/or IL-10Rα or the isotype control. Proliferation of CD4 T cells was measured by flow cytometry at day 7 poststimulation. (A) Representative flow plots are shown for one CP individual. (B) Statistical analysis of the impact (fold increase compared to isotypic control) of PD-L1 and/or IL-10Rα blockade in 11 CP. (C to E) Impact of combined blockade compared to isotype antibody on proliferation and IFN-γ, IL-2, and IL-13 secretion in three CP. (F and G) Apoptosis measurements performed using annexin V staining on CD69+ CD154+ CD4 T cells 48 h after stimulation with an HIV-1 Gag peptide pool. (F and G) Representative example (F) and collective data (G) on nine CP. Short horizontal lines represent median values. Horizontal dotted lines represent a fold increase of 1 (no impact of blockade). N/S, not statistically significant.
determined. In order to address this issue, we stimulated CD8-depleted PBMCs with an HIV-1 Gag peptide pool in the presence of an isotype control or a neutralizing antibody against H9009/FN- H9253 and phenotyped cell subsets after a 48-h incubation. Stimulation of HIV-1-specific CD4 T cells resulted in a dramatic upregulation of PD-L1 on monocytes (Fig. 5) that was not observed in any other cell type analyzed (see Fig. S5 in the supplemental material). Antigenic stimulation of HIV-1-specific CD4 T cells also led to upregulation of HLA-DR and HLA class I but not CD86 (see Fig. S6 in the supplemental material), consistent with the known roles of CD4 T cells in monocyte stimulation (25, 26). Antibody neutralization of IFN-γ secreted by HIV-1-specific CD4 T cells almost completely abrogated the upregulation of PD-L1 on monocytes (Fig. 5). Blockade of IL-10R/H9251 did not appear to further increase the levels of PD-L1 on monocytes. Therefore, stimulation of HIV-specific CD4 T cells leads to IFN-γ secretion that induces upregulation of PD-L1 on monocytes—potentially acting as a negative-feedback control loop for T cell activation and cytokine production.

**Dual blockade of the PD-1 and IL-10 pathways restores IL-12 secretion by monocytes through an IFN-γ-dependent mechanism.** A critical aspect of helper functions is the reciprocal relationship between activation of antigen-specific CD4 T cells and modulation of APC activity. *In vitro* studies suggested that defective signals given by CD4 T cells could be detrimental to APC functions (27). We focused on the role of IL-12 because (i) this cytokine has a variety of critical immune functions, including promotion of IFN-γ production and differentiation of Th1 CD4 T cells (reviewed in reference 28), and (ii) this pathway is impaired in HIV-1 infection, with a decreased ability of APCs to produce IL-12 upon stimulation (29, 30). Stimulation of virus-specific CD4 T cells led to an increased secretion of IL-12 by CD8-depleted PBMCs (Fig. 6A and B; see also Fig. S7 in the supplemental material). ICS performed at 48 h further confirmed that monocytes were the main source of IL-12 in our assays (see Fig. S7). As illustrated in Fig. 6B, stimulation of HIV-1-specific T helpers cells in the presence of anti-PD-L1 resulted in a modest 1.7-fold increase in IL-12 secretion by monocytes. In contrast, IL-10Rα blockade led to a marked 6-fold increase in IL-12 production, consistent with the known role of IL-10 in inhibiting IL-12 production by APCs (reviewed in references 28 and 31). Concurrent blockade of both pathways induced a dramatic median 19-fold increase in IL-12 secretion (Fig. 6B). The effects observed with PD-L1 and/or IL-10Rα blockade on IL-12 production therefore closely mirrored our observations on IFN-γ secretion.

We next sought to define the interplay between IFN-γ and IL-12. We found a strong correlation between IFN-γ and IL-12 levels in the supernatants of HIV-1 Gag-stimulated CD8-depleted PBMCs (Fig. 5).

**FIG 5** Antigen-specific stimulation of HIV-1-specific CD4 T cells induces upregulation of PD-L1 on monocytes through an IFN-γ-dependent mechanism. CD8-depleted PBMCs were stimulated with HIV-1 Gag peptide pool in the presence of the isotype control or IL-10Rα and/or IFN-γ blocking antibody. Phenotypic analysis of PD-L1 expression on monocytes was performed 48 h after simulation. (A and B) Representative examples of the impact of antigen stimulation and IFN-γ neutralization on PD-L1 expression on monocytes. (C) Collective data comparing the fold increases of PD-L1 expression upon stimulation with HIV-1 Gag compared to “No Antigen” in the presence of isotypic control or neutralizing antibodies for IFN-γ (n = 10; Wilcoxon matched-pair test).
PBMCs (Fig. 6C). IFN-γ has been shown to induce IL-12 production in APCs and, in turn, IL-12 is needed for optimal IFN-γ production by CD4 T cells (reviewed in references 28 and 31). In order to test this reciprocal relationship in the setting of HIV-1 infection, we performed PD-L1 and/or IL-10Rβ blockade in the presence or absence of neutralizing anti-IFN-γ or anti-IL-12 antibodies. Our data showed that IFN-γ produced by HIV-1-specific CD4 T cells is a major regulator of IL-12 secretion by APCs (Fig. 6D). In contrast, blockade of the IL-12 pathway moderately reduced but did not abolish IFN-γ secretion, indicating that IL-12 is needed for optimal expression of IFN-γ but is not the primary stimulus (Fig. 6E). These data provide mechanistic insight into the mode of action of combined PD-L1/IL-10Rx blockade where, in the absence of inhibition by PD-1 and/or IL-10, IFN-γ secretion by HIV-1-specific CD4 T cells stimulates IL-12 expression in APCs, forming a positive-feedback loop to further increase expression of IFN-γ.

DISCUSSION
A major issue concerning T cell exhaustion in chronic infections is whether the different inhibitory pathways involved lead to the same type of immune impairment or differentially affect various T cell functions. Furthermore, little is known about which nonredundant inhibitory networks can be simultaneously targeted to revive immune responses. Here we address these issues by determining that the functional profiles of HIV-1-specific CD4 T cells restored by single and concurrent blockade of the PD-1 and IL-10 pathways present substantial differences. We identify a unique role for IFN-γ, a cytokine whose secretion by HIV-1-specific CD4 T cells is strongly enhanced by combined PD-L1/IL-10Rx blockade in progressive HIV-1 infection but not in the presence of spontaneous or therapy-induced control of viral replication. The resulting enhanced HIV-1-specific T cell function led to a secondarily CD4 T cell-dependent increase in natural killer cell function. IFN-γ secretion acts as a strong inducer of PD-L1 on monocytes, providing a negative-feedback control loop. The modulation of antigen-presenting cell function by HIV-1-specific CD4 T cells upon PD-L1 and/or IL-10Rx blockade is further illustrated by a largely IFN-γ-dependent restoration of IL-12p70 secretion by APCs. The strong additive effect of combined PD-L1 and IL-10Rx blockade in terms of HIV-1-specific Thelper cell functions restored appears to be specific to IFN-γ, as it is not observed for IL-2 or IL-13 or for proliferative capacity. Our results serve as an important proof of principle that manipulation of different mediators of exhaustion can be used to alter the “flavor” of the Thelper responses restored, which may have important implications for the development of novel immunotherapeutic strategies. It should be noted that this was a

FIG 6 Combined PD-L1/IL-10Rx blockade restores IL-12 secretion from antigen-presenting cells through an IFN-γ-mediated mechanism. (A and B) Measurements of IL-12 secretion by CD8-depleted PBMCs stimulated for 48 h with HIV-1 Gag peptide pools in the presence of isotypic control or PD-L1 and/or IL-10Rx blocking antibodies. (A) Representative examples of a chronic progressor. (B) Collective data showing the impact (fold increase compared to the isotypic control) of PD-L1 and/or IL-10Rx blockade on IL-12 secretion for 11 CP (Kruskal-Wallis test for comparison of the three groups followed by Dunn’s posttests for paired comparisons). Short horizontal lines, median values; horizontal dotted lines, fold increase of 1 (no impact of blockade). (C) Correlation analysis of the impact of various blockade combinations on IFN-γ and IL-12 secretion from the same individuals (Spearman’s rank correlation). (D) Collective data showing that blockade of IFN-γ reduced the secretion of IL-12 from APCs upon PD-L1 and/or IL-10Rx blockade (9 comparisons corresponding to 3 conditions in 3 subjects; Wilcoxon matched-pair test). (E) Collective data showing that neutralization of IL-12 reduced the levels of IFN-γ secretion in the presence of PD-L1 and/or IL-10Rx blockade (9 comparisons corresponding to 3 conditions in 3 subjects; Wilcoxon matched-pair test). Symbols for values: ****, P = 0.0001.
reduced system where CD8 T cells were deleted from the cell cultures. Further experiments addressing the impact of combined blockade on CD8 T cell function and how this impacts the interaction with other cell subsets need to be performed.

Over the past decade, there has been significant progress in the understanding of mechanisms of CD8 T cell exhaustion caused by antigen persistence. CD4 T cell impairment, in contrast, remains much less explored, especially in human chronic viral infections such as HIV-1. PD-1 and IL-10 are mediators of exhaustion shared by HIV-1-specific CD4 and CD8 T cells, whereas other inhibitory molecules play a preferential role in one or another of these subsets (e.g., CTLA-4 on CD4 T cells [3] and 2B4 and CD160 on CD8 T cells [2, 14]). PD-1 and IL-10 act through different mechanisms. Whereas PD-1 alters membrane-proximal signaling events in T cells when engaged along with the T cell receptor (TCR) and recruits phosphatases such as SHP-2 (32, 33), IL-10 is thought to act mostly indirectly through modulation of antigen-presenting cell function, including dendritic cells and monocyte/macrophages (34), but can also act directly on virus-specific CD4 T cells (35). These nonredundant modes of action likely contribute to the gain observed in vivo with simultaneous PD-L1/IL-10 blockade in the LCMV model (11). Interestingly, besides the cross talk between the PD-1 and IL-10 pathways that we describe here, previous studies have shown that bacterial products resulting from microbial translocation can upregulate PD-1 levels on monocytes (36), which in turn induce IL-10 production that inhibits HIV-1-specific CD4 T cell proliferation. Another study showed that recombinant IL-10 increased expression of PD-L1 but not PD-L2 on monocyte-derived macrophages (37). These data underline the importance of reciprocal regulation of the PD-1 and IL-10 pathways that occurs at different levels.

Consistent with our previous studies on single PD-1 blockade, we show here that combined blockade of PD-1 and IL-10 can also restore function of HIV-1-specific CD4 T cells in ART-treated subjects, although to a lesser extent than in viremic individuals. Therapeutic anti-PD1 and -PD-L1 antibodies have recently yielded remarkable results in metastatic human cancers in clinical trials (38, 39), with overall good tolerance, although rare serious adverse events were reported. In vitro data suggest that blockade of the PD-1 pathway can help purge viral reservoirs by reactivating latent virus (discussed in reference 40), and a phase 1 clinical trial testing the safety and efficacy of PD-1 blockade to reduce viral reservoirs and enhance T cell immunity in ART-treated HIV-1-infected subjects is currently under review by the AIDS Clinical Trials Group (ACTG). It will thus be important to determine whether combined PD-1/IL-10 blockade (or another combined intervention) will be more effective at restoring effective immune responses and purging reservoirs in ART-treated individuals without excessive toxicity.

Our report also sheds light on the interplay between exhausted HIV-1-specific CD4 T cells and antigen-presenting cells and indicates a potential of combined blockade for restoring antigen-presenting cell function through improved CD4 T cell help. We focused our efforts on IL-12, a pluripotent cytokine secreted mainly by activated APCs whose production is greatly impaired in HIV-1 infection. Dual blockade of PD-L1 and IL-10Rα induced a striking increase in IFN-γ secretion by HIV-specific CD4 T cells that led to a subsequent induction of IL-12p70 secretion by antigen-presenting cells. Administration of IL-12 during acute SIV infection led to lower plasma viral loads and lower proviral DNA loads in PBMCs and lymph nodes (41), whereas IL-12 treatment during chronic SIV infection did not reduce viremia but improved the cytotoxicity of NK and T cells (42). Further studies will be necessary to determine whether combined PD-1/IL-10 blockade has similar effects on dendritic cells and whether the APCs licensed under conditions of dual blockade subsequently display improved antigen-presenting cell capacity.

Our results suggest that the therapeutic potential of combined PD-1/IL-10 blockade may significantly depend on the role played by Th1-type CD4 T helper responses in protective immunity to a specific disease. It is important that whereas IFN-γ secretion by HIV-1-specific CD4 T cells has not been positively associated with viral control in established HIV-1 infection, IFN-γ-producing Th1 CD4 T cell responses may be critical for protective immunity at mucosal surfaces. Studies in a murine model of HSV infection showed that mobilization of effector CD8+ CTLs to infected peripheral tissue requires IFN-γ produced by CD4 T cells (43). Such responses may be critical for HIV-1 vaccines that, besides eliciting protective antibodies, should probably be able to generate cellular immune responses capable of controlling initial foci of infection at the mucosal surfaces. Thus, concurrent manipulation of the PD-1 and IL-10 pathways, in particular, as adjuvant strategies at the site of immunogen administration, may have applications for preventive vaccine development.

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