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*J Immunol* 2007; 178:1645-1653; doi: 10.4049/jimmunol.178.3.1645
http://www.jimmunol.org/content/178/3/1645

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Role of CD40-Dependent Down-Regulation of CD154 in Impaired Induction of CD154 in CD4^+ T Cells from HIV-1-Infected Patients

Carlos S. Subauste,† Angela Subauste,‡ and Matthew Wessendarp*

CD40-CD154 interaction is pivotal for cell-mediated immunity. There are contradictory reports on whether HIV-1 infection impairs CD154 induction. The interaction between CD40 and CD154 is important not only because it results in activation of APCs but also because it controls CD154 by diminishing expression of this molecule. Compared with healthy controls, CD4^+ T cells from HIV-1^+ patients had impaired induction of CD154 when T cell activation was mediated by CD40^+ APCs. In contrast, T cell activation in the absence of these cells resulted in normal CD154 expression. CD154 induction in HIV-1^+ patients and controls were similar upon blockade of CD40-CD154 binding. Defective regulation of CD154 appeared to occur downstream of the control of mRNA levels because up-regulation of CD154 mRNA was not impaired by HIV-1 infection. This work identifies CD40 as a mediator of impaired CD154 induction in HIV-1 infection and explains why this defect was not detected by studies where T cell activation was triggered independently of CD40^+ APCs. In addition, dysregulation of CD154 in HIV-1 infection likely contributes to immunodeficiency because diminished expression of CD154 induced by CD40 is of functional relevance, resulting in decreased dendritic cell maturation. The Journal of Immunology, 2007, 178: 1645–1653.

The level of CD154 expression has profound effects on the generation of immune responses (7). This finding together with the broad range of effects of CD154 indicates that expression of this molecule must be tightly regulated. One mechanism of regulation is dependent on the binding of CD40 to CD154 because this interaction causes down-regulation of CD154 (8–10). Thus, T cell-APC interaction is key for two events: induction of CD154 and regulation of the level of expression of CD154.

In addition to a decrease in the number of CD4^+ T cells, functional defects in cell-mediated immunity play an important role in the pathogenesis of immunodeficiency associated with HIV-1 infection (11, 12). The clinical relevance of CD154 as well as its central role as a regulator of cell-mediated and humoral immunity led to studies that examined whether induction of CD154 is defective in CD4^+ T cells from HIV-1^+ patients. Although there are reports that HIV-1 infection is accompanied by defective CD154 induction upon T cell activation (13–18), other studies did not find evidence of this defect (19, 20). A common feature of the studies that reported a normal induction of CD154 in CD4^+ T cells from HIV-1^+ patients is that T cell activation was induced in the absence of CD40^+ APC (19, 20). We conducted experiments to examine the hypothesis that CD40-dependent regulation of CD154 expression is involved in the defective induction of CD154 on CD4^+ T cells from HIV-1^+ patients. Work aimed at solving the conundrum on whether HIV-1 impairs CD154 expression is important because of the likely implications in the understanding of mechanisms of immune evasion by HIV-1 and in the pathogenesis of immunodeficiency caused by the virus.

Materials and Methods

HIV-1^+ patients and control subjects

Blood samples were collected from 52 HIV-1-infected patients followed at the Infectious Diseases Center of the University of Cincinnati. Plasma viral load (VL) was measured by RT-PCR (Amplificor; Roche). Blood samples were collected from 14 healthy volunteers and used as control subjects.

Induction of CD154 expression

Purified CD4^+ T cells and monocytes were obtained from PBMC by negative depletion using immunomagnetic beads as previously described (21). Populations of CD4^+ T cells were on average: 92.2 % CD3^+ CD4^+ when obtained from HIV-infected patients and 96.8 % CD3^+ CD4^+ when obtained from control subjects. Monocyte populations were >92%
CD14⁺ in both groups of individuals. Cells were cultured in complete medium consisting of RPMI 1640 plus 10% human AB serum (Gemini Bio-Products). Serum was prescreened and found to be devoid of mitogenic activity. Several conditions were used to induce CD154 expression. CD4⁺ T cells (5 x 10⁶/ml) were stimulated with autologous monocytes (5 x 10⁶/ml) with or without staphylococcal enterotoxin B (SEB; 0.1 μg/ml; Toxin Technology) or anti-CD3 mAb (OKT3; 10 μg/ml; American Type Culture Collection (ATCC)). In certain experiments, monocytes were cultured in RPMI 1640 plus 10% FBS (HyClone) with or without GM-CSF (5 ng/ml; PeproTech) for 24 h to up-regulate CD40 expression.

For stimulation with *Toxoplasma gondii*, CD4⁺ T cells (5 x 10⁶/ml) were incubated either with monocytes (1.25 x 10⁶/ml) that had been challenged with *T. gondii* tachyzoites at a multiplicity of infection of four tachyzoites per monocyte (21). Anti-CD28 and anti-CD40 mAbs (BD Biosciences) were added at a concentration of 1 μg/ml each. CD4⁺ T cells were also incubated with THP-1 cells (5 x 10⁶/ml; ATCC) plus either SEB (1 μg/ml) or anti-CD3 mAb (20 μg/ml). In certain experiments, THP-1 cells were fixed with paraformaldehyde as described (21) before addition of CD4⁺ T cells and anti-CD3 mAb. T cell activation was also conducted by incubating CD4⁺ T cells with U937 cells (5 x 10⁶/ml; ATCC) plus anti-CD3 mAb (5 μg/ml) to stimulate CD4⁺ T cells with immobilized anti-CD3 mAb. 96-well plates were incubated overnight at 4°C with anti-CD3 mAb (OKT3 10 μg/ml in PBS) followed by addition of purified CD4⁺ T cells. Doses of SEB and anti-CD3 chosen were predetermined to be optimal for induction of CD154 expression in each experimental condition. CD154 induction was assessed at optimal time points: 6 h in the case of SEB and anti-CD3-induced T cell stimulation, and at 18 h in the case of *T. gondii*-induced T cell stimulation. No difference in the kinetics of CD154 induction was noted between healthy controls and HIV-1⁺ patients. When indicated, an anti-CD40 mAb that inhibits CD40-CD154 binding (mAb 89; 5 μg/ml; Beckman Coulter) was added to CD4⁺ T cells and either monocytes or THP-1 cells. Daudi cells (ATCC) were added to activated CD4⁺ T cells as indicated.

**Induction of dendritic cell maturation**

Monocyte-derived dendritic cells were obtained from healthy controls as previously described (22). Autologous CD4⁺ T cells were incubated with adherent monocytes with or without SEB and anti-CD40 mAb as described above. CD4⁺ T cells were fixed with paraformaldehyde and extensively washed (21) followed by addition to dendritic cells at a ratio of two CD4⁺ T cells to a dendritic cell. Expression of CD83 was analyzed by flow cytometry after 24 h.

**Flow cytometry**

Cells were stained with anti-CD4 FITC, anti-CD4 PerCP, anti-CD69 allo- phycocyanin, anti-CD154 PE, and appropriate isotype control mAbs (BD Biosciences). Cells were fixed with 1% paraformaldehyde and were analyzed using a FACSCalibur (BD Biosciences). Expression of CD69 and CD154 was analyzed on 2.5 x 10⁶ CD3⁺ CD4⁺ T cells/well. Serial dilution of *T. gondii*-stimulated CD4⁺ T cells by addition of nonstimulated CD4⁺ T cells revealed that the lower limit of detection of *T. gondii*-specific CD69⁺ CD154⁺ CD4⁺ T cells is 0.035%.

For detection of intracellular and membrane CD154, CD14⁺ cells were first stained with anti-CD3-allophycocyanin, anti-CD4 PerCP, and anti-CD69 allophycocyanin mAbs. Cells were fixed and permeabilized by using IntraPrep permeabilization reagent (Beckman Coulter) following manufacturer’s instructions. Thereafter, cells were stained with anti-CD154-PE or appropriate isotype control mAbs (BD Biosciences). After fixation with 1% paraformaldehyde, cells were analyzed using a FACSCalibur. Expression of CD154 membrane fluorescence intensity (MFI) was analyzed in 5 x 10⁵ CD3⁺ CD4⁺ T cells. Expression of CD40 on monocytes was examined by incu- bating cells with anti-CD40-PE mAb (BD Biosciences) and anti-CD14 FITC (BD Biosciences) followed by analysis using FACSCalibur. Anti-CD3-PE mAb (Beckman Coulter) was used to examine maturation of monocyte-derived dendritic cells.

**Immunofluorescence and confocal microscopy**

CD4⁺ T cells were fixed and permeabilized as described (23). Cells were incubated with blocking buffer followed by incubation with anti-CD154 mAb or isotype control mAb for 1 h. After washing with blocking buffer, cells were incubated with Alexa 568-conjugated secondary Ab (Molecular Probes, 1 μg/ml) in the presence of a concentration of 1 μg/ml each. CD4⁺ T cells were then analyzed by confocal microscopy using a LSM 510 laser scanning confocal microscope (Carl Zeiss).

**Real-time quantitative RT-PCR**

CD4⁺ T cells and monocytes were incubated with or without SEB for 4 h (predetermined optimal time point for up-regulation of CD154 mRNA) followed by isolation of total cellular RNA using the RNeasy kit (Qiagen). After treatment with DNase (Ambion), 0.5 μg of RNA was used to gen- erate cDNA using oligo(dT)₁₈, random hexamer primers and Superscript III reverse transcrip- tase (Invitrogen Life Technologies). cDNA was subjected to RT-PCR using the LightCycler SYBR green fluorophore. Primer sequences for CD69 and CD154 were previously described (18, 24). Expression of these genes was assessed by real-time quantitative RT-PCR using a DNA Engine Opticon System (MJ Research). Two microtiters of cDNA was used as template for RT-PCR using the Quantitect SybrGreen kit (Qiagen) and 20 PM (each) primers in a final volume of 25 μl. Each measurement was performed in triplicate and the threshold cycle number at which the amplification of target sequence becomes detectable by fluorescence was determined. Serial dilutions of cDNA were run to ensure linearity and reliability of the assay. Analysis by agarose gel electrophoresis was performed to confirm product length and purity of the amplicon. Real-time quantitative RT-PCR of 18s rRNA was also performed for every sample as an internal control. Primer sequences for 18s rRNA were as follows: forward, ACTCAAACGCGAAAACTCTACCC; reverse, CCAGACAAATGCTCAACCAAC. Each sample was normalized according to the content of 18s rRNA to compare levels of CD154 mRNA in unstimulated and stimulated CD4⁺ T cells from controls and HIV-1⁺ patients. CD154 is induced in the subpopulation of CD4⁺ T cells that becomes CD69⁺ upon stimulation. Thus, CD154 mRNA levels were also expressed as the ratio of CD154 mRNA to CD69 mRNA or were corrected by the percentage of stimulated CD4⁺ T cells that acquired CD69 expression by flow cytometry.

**Statistical analysis**

Statistical significance was assessed by Student’s t test and ANOVA using InStat version 3.0 (GraphPad).

**Results**

**Induction of CD154 in the presence of CD40⁺ APC is impaired in CD4⁺ T cells from HIV-1⁺ patients**

The interaction between CD40⁺ APC and T cells affects the levels of CD154 expression. Thus, to compare induction of CD154 on CD4⁺ T cells from HIV-1⁻ patients and controls, we conducted experiments using purified CD4⁺ T cells and monocytes to main- tain the ratio between these cells constant. Purified CD4⁺ T cells were incubated with autologous monocytes in the presence or absence of SEB. Flow cytometry analysis of CD4⁺ T cells from both controls and HIV-1⁻ patients revealed that stimulation with SEB up-regulated expression of the activation marker CD69 (Fig. 1A). In addition, CD154 was induced on CD69⁺ CD4⁺ T cells. Thus, comparison of the levels of CD154 expression in controls and HIV-1⁻ patients was conducted on gated CD69⁺ CD4⁺ T cells. Fig. 1B shows that HIV-1⁺ patients with CD4 counts <200/μl exhibited a significantly lower induction of CD154 in response to SEB compared with controls (p < 0.0001). In contrast, induction of CD69 was not significantly different in any of the groups of HIV-1⁻ patients compared with controls (p = 0.3) (Fig. 1C). Analysis of plasma VL indicated that patients with >10,000 viral copies/ml had a significantly lower induction of CD154 compared with controls (p < 0.0001) (Fig. 1D). A total of 88.9% of patients with VL >10,000 copies/ml compared with 14.3% of those with VL <10,000 viral copies/ml exhibited levels of CD154 that were <2 SD from the mean of healthy controls. Thus, using a superan- tigen in the presence of CD40⁺ APC and constant ratio of CD4⁺ T cells to APC, these experiments confirm that CD4⁺ T cells from HIV-1⁻ patients have impaired induction of CD154.

Next, we activated CD4⁺ T cells using THP-1 mononuclear cells. This cell line expresses MHC class II molecules, FeR, as well as CD40-enabling superantigen- and anti-CD3 mAb-induced T cell activation in the presence of CD40-CD154 interaction. These studies were performed focusing on CD4⁺ T cells from HIV-1⁻ pa- tients that had a high likelihood of defective CD154 induction.
Plasma VL >10,000 viral copies/ml rather than CD4+ T cell count <200/μl was used as selection criteria to avoid targeting samples that may not provide sufficient CD4+ T cells for the studies. Fig. 2, A and B, shows that the induction of CD154 in response to SEB or anti-CD3 mAb was impaired on CD4+ T cells from the HIV-1+ patients (p < 0.0002). In contrast, up-regulation of CD69 was not affected in CD4+ T cells from these patients (p > 0.1) (Fig. 2, C and D). CD154 can also exist in an intracellular compartment. We evaluated expression of both membrane and intracellular CD154 (total CD154). Preliminary studies using confocal microscopy confirmed that anti-CD154 mAb effectively detects intracellular protein. CD4+ T cells from controls and HIV-1+ patients with VL >10,000 viral copies/ml were incubated with THP-1 cells plus SEB or anti-CD3 mAb. Expression of CD154 was examined by flow cytometry in permeabilized CD4+ T cells. Similar to our studies of membrane CD154 (Fig. 2), CD4+ T cells from HIV-1+ patients have a significantly decreased expression of total CD154 (MFI) in response to SEB (controls = 53.8 ± 5.8 vs HIV = 20.2 ± 3.2) and anti-CD3 mAb (controls = 37 ± 2.5 vs HIV = 14.5 ± 1.7) (n = 4–5/group; p < 0.007). Taken together, these results revealed that induction of CD154 is impaired in CD4+ T cells from HIV-1+ patients activated in the presence of CD40+ APC.

**Induction of CD154 in the absence of CD40 is normal in HIV-1+ patients**

The results presented above as well as our prior work reveal a defect in induction of CD154 on CD4+ T cells from HIV-1+ patients. In contrast to our findings, other investigators have reported that induction of CD154 is unimpaired in CD4+ T cells from HIV-1+ patients. Those studies were performed by inducing T cell activation in the absence of CD40+ APC. We conducted experiments in parallel to those described in Fig. 1 to examine CD154 induction when CD4+ T cells are stimulated without CD40+ APC. Purified CD4+ T cells from HIV-1+ and healthy controls were incubated with immobilized anti-CD3 mAb. Testing of CD4+ T cells that included HIV-1+ patients described in Fig. 1 revealed no defect in CD154 induction (Fig. 3A) (p = 0.1). This was the case even in patients with CD4 counts of <200/μl or those with VL >10,000 viral copies/ml (Fig. 3A and data not shown).

To further explore the possibility that the presence of CD40 is critical for defective CD154 expression associated with HIV-1+, CD4+ T cells from HIV-1+ patients and controls were incubated with U937 cells. In contrast to THP-1 cells, U937 cells lack CD40 expression. CD4+ T cells from controls and HIV-1+ patients were incubated with U937 cells with or without anti-CD3 mAb (U937 cells are FeR+). Similar to the studies conducted with immobilized anti-CD3, no defect in CD154 expression was detected even in patients with CD4+ T cell counts <200/μl or VL >10,000 viral copies/ml (p = 0.6) (Fig. 3B and data not shown). Induction of CD69 by immobilized anti-CD3 mAb or U937 plus anti-CD3 mAb was similar in the groups of HIV-1+ patients and controls (p > 0.3).

We took an additional approach to examine the role of CD40 in impaired induction of CD154 associated with HIV-1 infection. CD4+ T cells from controls and HIV-1+ patients were incubated
with anti-CD3 mAb and either mock-sorted THP-1 cells (contain CD40^+ cells) and THP-1 cells sorted for CD40^+ cells. Although stimulation by THP-1 cell preparations that contained CD40^+ cells resulted in diminished CD154 induction on CD4^+ T cells from HIV-1^+ patients (p = 0.02), CD154 induction was similar in controls and HIV-1^+ patients when stimulated with CD40^- THP-1 cells (p = 0.5) (Fig. 4). Taken together, induction of CD154 in the absence of CD40^- APC is normal in CD4^+ T cells from HIV-1^+ patients.

**Stimulation of CD4^+ T cells from HIV-1^+ patients by CD40^- APC results in decreased protein but not mRNA levels of CD154**

CD154 can have both transcriptional and posttranscriptional regulation (25). Thus, we examined whether diminished levels of CD154 mRNA can explain defective protein expression of CD154 associated with HIV-1 infection. CD4^+ T cells from controls and HIV-1^+ patients with VL >10,000 viral copies/ml were incubated with autologous monocytes with or without SEB. Examination of CD69 and CD154 protein expression by flow cytometry revealed that the levels of CD154 were markedly diminished on CD4^+ CD69^- T cells from HIV-1^+ patients compared with controls (Fig. 5A) (p = 0.002). Next, we measured CD69 and CD154 mRNA levels using real-time quantitative RT-PCR. CD154 mRNA in these conditions arose overwhelmingly from CD4^+ T cells because preliminary studies indicated that CD154 mRNA levels in purified monocytes were either undetectable or 1 x 10^4 times lower than those in purified CD4^+ T cells (data not shown). CD154 is induced in activated CD4^+ T cells that become CD69^- (see Fig. 1A) (15, 17). Indeed, the analysis of CD154 protein expression was conducted by gating on CD4^+ CD69^- T cells. However, in a population of whole CD4^+ T cells, the degree of CD154

**FIGURE 2.** Polyclonally activated CD4^+ T cells from HIV-1^+ patients have defective induction of CD154 in the presence of CD40^- APC. Purified CD4^+ T cells from controls or HIV-1^+ patients with plasma VL >10,000 viral copies/ml were incubated with THP-1 cells plus either SEB (A and C) or anti-CD3 mAb (B and D). Expression of CD154 (A and B) and CD69 (C and D) were assessed by flow cytometry. *, p < 0.0002. Circles are superimposed when values are similar.

**FIGURE 3.** Polyclonally activated CD4^+ T cells from HIV-1^+ patients have normal induction of CD154 in the absence of CD40^- APC. CD4^+ T cells from healthy controls and HIV-1^+ patients were cultured with immobilized anti-CD3 mAb (A) or with U937 cells plus anti-CD3 mAb (B). Expression of CD154 was assessed by flow cytometry. Circles are superimposed when values are similar.
up-regulation is dependent on the fraction of T cells that become CD69$. Although there is no significant difference in the induction of CD69 between controls and HIV-1$ patients, the percentage of CD69$ cells varies among individuals (Fig. 1C). Comparison of CD154 mRNA levels between controls and HIV-1$ patients that exhibited a similar percentage of CD69$ T cells revealed comparable degree of CD154 mRNA up-regulation in response to superantigen stimulation despite impaired CD154 protein expression in CD4$ T cells from HIV-1$ patients (Fig. 5B). Next, to allow comparison among all individuals examined, CD154 mRNA levels were normalized against CD69 mRNA. CD154 mRNA levels were not significantly different from those of controls (Fig. 5C) ($p = 0.5$). Finally, normalization of CD154 mRNA against the percentage of CD69$ T cells revealed similar ratios in both controls and HIV-1$ patients (controls $= 5.0 \pm 2.0$; HIV-1$ = 6.6 \pm 3.0$; $p = 0.7$). These results suggest that HIV-1 infection can impair protein expression of CD154 without affecting mRNA levels.

CD154 induction in CD4$ T cells from HIV-1$ patients is no longer defective when CD40-CD154 interaction is blocked

The studies described so far revealed that CD154 protein expression on CD4$ T cells from HIV-1$ patients is impaired when activation is triggered by CD40$ APC. These findings raised the possibility that impaired CD154 expression associated with HIV-1 occurs at the level of CD40-dependent regulation of CD154. Interestingly, this is a mechanism that can control CD154 protein expression without affecting mRNA levels (10). Thus, we conducted experiments to explore whether CD40 was responsible for defective CD154 induction associated with HIV-1 infection. First, we examined the possibility that CD154 was not being detected by flow cytometry because of capping induced by soluble CD40 secreted by APC (9). CD40$ cells (THP-1) were fixed with paraformaldehyde followed by extensive washing to prevent the potential presence of soluble CD40. CD4$ T cells from controls and HIV-1$ patients with VL $> 10,000$ viral copies/ml were stimulated with paraformaldehyde-fixed THP-1 cells plus either SEB or anti-CD3 mAb. The MFI of CD154 on CD4$ T cells from HIV-1$ patients remained impaired despite fixation of THP-1 cells (SEB: controls $= 102.0 \pm 10.0$ vs HIV $= 32.7 \pm 6.1$; anti-CD3 mAb: controls $= 114.3 \pm 17.3$ vs HIV $= 38.9 \pm 5.9$; $n = 3$ to $5$/group; $p < 0.02$). Thus, it is highly unlikely that secretion of soluble CD40 explains impaired CD154 expression in HIV-1$ infection.

To further explore whether CD40 was responsible for defective CD154 induction associated with HIV-1 infection, we first...
confirmed that CD40 down-regulates CD154 expression. We examined the effects of increased CD40 expression on induction of CD154. GM-CSF controls CD40 expression on monocytes (26). Indeed, stimulation of monocytes with GM-CSF caused a marked up-regulation of CD40 (CD40 MFI: control = 5.2 ± 0.6; GM-CSF = 32.7 ± 3.3; n = 3). Next, we incubated CD4+ T cells from healthy subjects with anti-CD3 mAb plus control or GM-CSF-treated (CD40high) monocytes. As shown in Fig. 6A, activation of CD4+ T cells using CD40high monocytes was accompanied by a significantly lower expression of CD154 (p = 0.001; n = 3). Decreased CD154 expression was not caused by impaired T cell activation because it took place despite a significantly higher percentage of activated CD69+ cells in CD4+ T cells incubated with CD40high monocytes (p = 0.02; n = 3) (Fig. 6B). This last result is likely explained by the increase in FcR expression induced by GM-CSF (27). Next, we examined CD154 induction when CD40-CD154 interaction is blocked by an anti-CD40 mAb. Activation of CD4+ T cells in the presence of control monocytes and anti-CD40 mAb caused on average a 1.8-fold increase in CD154 expression (n = 3). Importantly, blockade of CD40-CD154 binding caused on average a 7.9-fold increase in CD154 expression in CD4+ T cells activated with CD40high monocytes. As a result, CD154 induction was no longer diminished in CD4+ T cells stimulated with CD40high monocytes. Anti-CD40 mAb had no effect on T cell activation as assessed by CD69 induction (p = 0.6). Blockade of CD40-CD154 binding also caused an increase in CD154 expression in CD4+ T cells activated by either monocytes plus SEB (average 1.8-fold increase; see Fig. 7A), THP-1 plus SEB (average 3.1-fold increase; see Fig. 7C) or THP-1 cells plus anti-CD3 mAb (average 2.8-fold increase; data not shown) (from 7 to 9).

Next, we determined whether the changes in CD154 expression induced by CD40 are of functional relevance. CD4+ T cells were activated by CD40+ APC in the presence or absence of blocking anti-CD40 mAb followed by fixation with parafomaldehyde. These CD4+ T cells were added to immature dendritic cells and CD83 expression was examined to assess dendritic cell maturation. CD4+ T cells activated when CD40-CD154 interaction is unpaired (control mAb) caused diminished dendritic cell maturation compared with CD4+ T cells activated when CD40-CD154 interaction is blocked (anti-CD40 mAb) (Fig. 6C) (p = 0.01). Taken together, the interaction between CD40 and CD154 during T cell stimulation reduces expression of functional CD154.

We examined the effects of blocking CD40-CD154 binding on CD154 expression on CD4+ T cells from HIV-1+ patients and controls. This was conducted by using different models to induce T cell activation. CD4+ T cells from HIV-1+ patients with defective induction of CD154 in response to SEB were stimulated with this superantigen in the presence of either anti-CD40 or control mAb. In marked contrast to stimulation in the presence of control mAb, CD154 expression was no longer significantly different between controls and HIV-1+ patients when CD40-CD154 binding was prevented by the anti-CD40 mAb (p = 0.1) (Fig. 7A).

We previously reported that Ag-induced CD154 induction is defective on CD4+ T cells from most HIV-1+ patients (17). Using the opportunistic pathogen T. gondii as a model, we observed that CD154 levels were no longer different between HIV-1+ patients and controls when CD4+ T cells were stimulated with T. gondii-infected monocytes in the presence of the anti-CD40 mAb (p = 0.2) (Fig. 7B). Finally, using THP-1 cells as accessory cells, blockade of CD40-CD154 binding restored expression of CD154 to levels similar to those of CD4+ T cells from controls in response to stimulation with SEB or anti-CD3 mAb (p > 0.1) (Fig. 7C and data not shown). Taken together, using multiple models of T cell stimulation, our results indicate that CD154 expression is no longer defective on CD4+ T cells from HIV-1+ patients when the binding of CD40 to CD154 is blocked.
A mechanism by which CD40 can regulate CD154 takes place after CD154 is expressed on the cell surface. Membrane CD154 binds CD40 resulting in endocytosis of CD154 (8). Using a model previously described (8), we determined whether impaired expression of CD154 on CD4+ T cells from HIV-1+ patients is explained by increased CD40-induced internalization of CD154. CD4+ T cells were incubated with U937 plus anti-CD3 mAb to induce CD154 expression. This was followed by exposure to CD40+ cells (Daudi cells). Fig. 8 shows that the expression of CD154 is significantly diminished when activated CD4+ T cells that already express CD154 are exposed to CD40+ cells (p < 0.0001). However, the decrease in CD154 expression induced by CD40+ cells was indistinguishable between CD4+ T cells from controls and HIV-1+ patients (p > 0.4). Taken together, CD40-dependent down-regulation of CD154 plays a key role in explaining impaired induction of CD154 in HIV-1 infection and this phenomenon likely takes place before expression of surface CD154.

**Discussion**

The pivotal role of CD154 in regulation of numerous aspects of the immune response prompted studies that explored whether induction of this molecule is defective in HIV-1 infection. We considered important to use physiologically relevant models of T cell activation. Thus, we have explored this question by activating CD4+ T cells via APC because CD154 induction occurs through TCR signals provided by these cells and CD40 expressed on APC regulates CD154 expression (15, 17). Using multiple models of T cell activation mediated by CD40+ APC (superantigen, CD3 ligation, antigenic stimulation), we report that CD4+ T cells from HIV-1+ patients have defective CD154 induction compared with cells from healthy controls. In parallel studies, activation in the absence of CD40+ APC resulted in normal CD154 expression on CD4+ T cells from HIV-1+ patients, even those with low CD4+ T cell counts and high plasma VLs. Impaired expression of CD154 on CD4+ T cells from HIV-1+ patients was secondary to CD40-induced down-modulation of CD154 because binding to CD40 decreased CD154 expression leading to diminished CD154-dependent cellular responses, and blockade of CD40-CD154 binding normalized the induction of CD154 in CD4+ T cells from HIV-1+ patients. Defective CD154 did not arise because of abnormalities in APC from HIV-1+ patients because this defect was noted when CD4+ T cells were stimulated with a CD40+ monocyctic cell line. In addition, CD4+ T cells from HIV-1+ patients exhibit impaired CD154 induction even if they are stimulated with APC from healthy controls (15). The findings that we report herein are important not only because they uncovered a mechanism that explains impaired CD154 induction in HIV-1 infection but also because they explain why CD154 expression on CD4+ T cells from HIV-1+ patients was reported to be normal in studies that used T cell activation in the absence of APC.

Although CD154 induction is impaired in HIV-1 infection, other work has revealed that some degree of CD40-CD154 signaling must still be operative because, for example, this signaling pathway enhances HIV-1 replication during the cross-talk of T cells and dendritic cells (28). A likely explanation for this apparent inconsistency is that activation of cellular responses by engagement of members of the TNFR superfamily including CD40 have different requirements for receptor multimerization and adapter protein recruitment (29, 30). Thus, these findings raise the possibility that diminished CD40 engagement as a result of a impaired CD154
expression may still allow signals that modulate HIV-1 replication while may not provide sufficient CD40 stimulation for induction of CD40-dependent cell-mediated immune responses. In support of this contention is the fact that the level of CD154 expression on CD4+ T cells directly influences type-1 cytokine production, the generation of CTL and correlates with protection during viral infection (31). These findings together with the central role of CD154 as a signal that induces dendritic cell activation and maturation suggest that impaired CD154 expression results in an ineffective cell-mediated immune response that is incapable of eradicating HIV-1. Indeed, addition of exogenous CD154 enhances HIV-1-specific CD4+ T lymphocyte proliferation, IFN-γ production, and cytotoxic activity in infected individuals (32, 33).

The data on the functional relevance of defective CD154 expression is particularly strong in the case of opportunistic pathogens. CD4+ T cells from most HIV-1+ patients not only have defective induction of CD154 in response to opportunistic pathogens but the diminished levels of CD154 expression correlate strongly with impaired secretion of IL-12 (17). Moreover, addition of rCD154 restored IL-12 production in response to the opportunistic pathogen T. gondii (17). These findings as well as our previous work led us to propose that impaired CD154 induction explains, at least in part, defective type 1 cytokine response associated with HIV-1 (15, 17, 34). Taken together, an impaired induction of CD154 is likely to play an important role in explaining fundamental aspects of HIV-1 infection: an inadequate immune response against the virus and susceptibility to opportunistic infections.

Activated T cells can secrete soluble CD154 (10). However, the release of soluble CD154 is not considered an important mechanism of regulation of CD154 expression during T cell-APC interaction because the expression of CD154 on the membrane of activated T cells and the secretion of soluble CD154 are independently regulated (10) and engagement of CD40 is not required for CD154 secretion (35). CD154 is regulated a multiple levels that include transcription and mRNA stability (25). Although CD40 can down-regulate CD154 mRNA levels, this is a late phenomenon (9). Indeed, a decrease in CD154 protein expression induced by CD40 takes place when CD154 mRNA levels are unchanged (10). In this regard, our studies indicate that the interaction between CD40 and CD154 contributes to impaired CD154 expression in CD4+ T cells from HIV-1+ patients and this defect appears unlikely to be caused by diminished steady-state levels of CD154 mRNA. CD40 can regulate CD154 expression at a posttranslational level. The binding of CD40 to CD154 causes receptor-mediated endocytosis of CD154 followed by degradation (8). However, as stated by the authors of this work, this study did not exclude other mechanisms by which CD40 regulates CD154 expression. Indeed, we find no evidence that internalization of CD154 once it becomes expressed on the surface of T cells and binds CD40 explains impaired CD154 induction in CD4+ T cells from HIV-1+ patients. Additional levels of control of CD154 are likely to exist because regulation of CD154 is similar to that of TNF-α and expression of this cytokine can be controlled at the level of translation (36).

Defects in the CD40-CD154 pathway are relevant not only to X-HIM and HIV-1 infection but also to other diseases such as chronic lymphocytic leukemia. Pertinent to our studies is the report of defective CD154 induction in CD4+ T cells from patients with chronic lymphocytic leukemia (37). Moreover, impaired CD154 induction associated with this disease has been reported to be caused by CD40-mediated down-regulation of CD154 protein expression without affecting CD154 mRNA levels (37). Thus, these findings together with the present work argue for the importance of alterations in CD40-dependent regulation of CD154 in the pathogenesis of immunodeficiency.

It has been proposed that gp120 explains impaired induction of CD154 in HIV-1 infection (18). This phenomenon requires exposure of T cells to high concentrations of virions and impairs CD154 protein expression as well as CD154 mRNA levels. However, the normal expression of CD154 on CD4+ T cells stimulated by immobilized anti-CD3 mAb or anti-CD3 mAb plus CD40+ accessory cells (even in patients with plasma VL 200,000 copies/ml) (this study, Ref. 20) argue for an alternative mechanism to explain impaired CD154 induction in HIV-1 infection. Indeed, using APC-driven T cell activation, we find that CD40-dependent down-regulation of CD154 is responsible for diminished CD154 induction in CD4+ T cells from HIV-1+ patients. In addition, defective protein expression of CD154 in HIV-1 infection can occur despite no apparent decrease in CD154 mRNA levels. These results are in agreement with the demonstration that CD40 lowers CD154 expression without affecting CD154 mRNA levels (10). Taken together, these findings point toward dysregulation of CD154 in HIV-1 infection at a level downstream of control of CD154 mRNA levels.

In a previous study, we reported that CD154 induction in response to polyclonal T cells activation is impaired even in HIV-1+ patients with CD4+ T cell counts >500/μl (15). However, the fact that those experiments were conducted primarily using PBMC instead of purified CD4+ T cells suggested that a diminishing CD4+ T cell to APC ratio in HIV-1+ patients contributed to the defect (15). Indeed, using a constant CD4+ T cell-APC ratio, the current work indicates that defective CD154 induction in response to polyclonal T cell stimulation is common in HIV-1+ patients with CD4+ T cell counts <200/μl or in patients with plasma VL of >10,000 copies/ml. Of particular relevance to the immune response against opportunistic pathogens is our previous demonstration that induction of CD154 in response to Candida albicans, T. gondii, and CMV is defective in CD4+ T cells from most HIV-infected patients, including those with CD4+ T cell counts higher than 500/μl if they have plasma VLs > 500 copies/ml (17). Thus, these findings are in agreement with the contention that impaired CD154 contributes to defective type 1 cytokine response in HIV-1 infection, an abnormality that is detected before a decrease in CD4+ T cell counts (38).

In summary, our studies indicate that rather than having an intrinsic defect in the capacity to express CD154 upon T cell activation, CD4+ T cells form HIV-1+ patients exhibit a defect in the control of CD154 expression in the presence of CD40+ APC. Our results support a model whereby CD4+ T cells from these patients are more susceptible to CD40-dependent down-regulation of CD154. Given that the decrease in CD154 expression caused by CD40 is of functional relevance because it diminishes CD154-dependent cellular responses (dendritic cell maturation), this defect in regulation of CD154 expression is predicted to contribute to immune evasion and immunosuppression associated with HIV-1 infection.

Acknowledgments
We thank Cecilia Subauste for critical review of the manuscript. We thank Lisa Haglund, Peter Frame, and Peter Grubbs for providing blood samples from HIV-1+ patients.

Disclosures
The authors have no financial conflict of interest.

References


