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GITR Triggering Protects HIV-specific CD4+ T cells from Apoptosis

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ABSTRACT

Background. Infection and death of HIV-specific CD4+ T cells is dramatic and ongoing during HIV infection, and contributes weak HIV-specific CD4+ T cell responses. Triggering the lymphocyte costimulatory molecule glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) potentiates effector T cell responses in part by protecting T cells from apoptosis, but role of triggering GITR in HIV disease is unstudied.

Methods. We compared CD4+ T cell expression of GITR by flow cytometry in HIV infected and uninfected subjects with the Mann Whitney test. In addition, we examined the impact of agonistic monoclonal antibody triggering of GITR on the percentage of CD4+ and CD8+ T cells demonstrating intracellular expression of TNF-α and IFN-γ to HIV p55 and control antigens in twelve HIV-infected subjects using the Wilcoxon test. We then related the magnitude of the response to GITR triggering to the peripheral CD4+ count using Pearson correlations. Lastly, we examined the impact of GITR triggering on CD4+ T cell apoptosis by assessing intracellular expression of activated caspase-3 on CD4+ T cells expressing TNF-α to HIV p55 and other antigens using a Mann-Whitney test.

Results. The percentage of CD4+ T cells expressing GITR at baseline was greater in HIV-infected subjects (34.70% vs. 10.59%, p=0.035), but, in contrast to healthy controls, PHA failed to induce additional GITR expression in HIV-infected subjects (34.70% vs. 45.03%, p=0.402). Antibody triggering of GITR increased the percentage of HIV p55-specific CD4+ T cells secreting TNF-α (0.51% vs. 1.00%, p=0.008) and IFN-γ (0.37% vs. 0.51%, p=0.034). CD4+ T cell cytokine secretion to pooled peptides to CMV, EBV and influenza (CEF) was unaffected by GITR triggering, as were CD8+ T cell responses to all antigens. The magnitude of the HIV-specific TNF-α response to GITR triggering correlated directly with the absolute peripheral CD4+ T cell count (p=0.009, Pearson R=0.714). Lastly, expression of the intracellular apoptosis marker activated caspase-3 was reduced preferentially in HIV-specific CD4+ T cells after GITR triggering (12.15 vs 10.45, p<0.001).

Conclusions. Despite HIV-related impairments in GITR expression on CD4+ T cells, GITR triggering enhances HIV-specific CD4+ T cell cytokine secretion, and protects HIV-specific CD4+ T cells from apoptosis. GITR triggering is thus a novel and modifiable host response that protects CD4+ T cells from apoptosis during HIV infection.

INTRODUCTION

- Apoptosis of CD4+ T cells is central to HIV pathogenesis.
- Preventing CD4+ T cell apoptosis may preserve HIV-specific cellular immune responses, and even forestall the development of AIDS.
- Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a member of the TNF receptor family of molecules that is expressed on activated and Ag-specific lymphocytes.
- Triggering GITR with its natural ligand, GITR ligand, or with agonistic antibodies, enhances antigen-specific effector T cell responses, in part by making T cells resistant to apoptosis.
- Triggering other members of the TNF receptor family has been explored as a means of heightening immune responses to HIV, and GITR triggering enhances murine immune responses in retroviral infection and retroviral vaccination
- GITR triggering has not been explored in the setting of HIV infection

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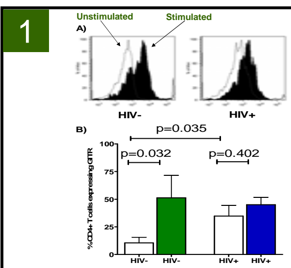

In HIV infection, baseline CD4+ T cell expression of GITR is increased, and PHA induction of GITR expression is impaired.

CD4+ T cells were stained for GITR before and after PHA stimulation in PBMC from eight HIV-infected subjects and six HIV-uninfected subjects (average age 42.5 and 45.2, respectively, P=0.647).

The percentage of CD4+ T cells expressing surface GITR was greater at baseline in HIV-infected subjects (34.70% vs. 10.59%, P=0.035; Figure 1A). PHA stimulation increased surface expression of GITR in uninfected subjects (10.59% to 51.30%, P=0.032), but not in HIV-infected subjects (34.70% vs. 45.03%, P=0.402; Figure 1B).

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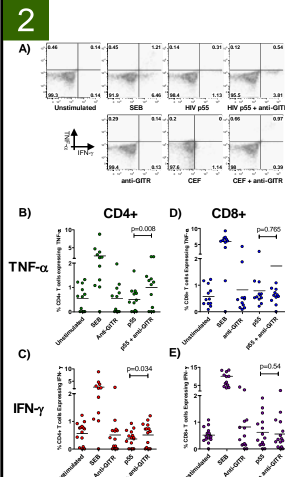

GITR triggering enhances HIV p55-specific CD4+ T cell cytokine secretion.

We tested the hypothesis that antibody triggering of GITR would enhance HIV-specific cytokine secretion by comparing the percentage of CD4+ and CD8+ T cells expressing intracellular TNF-α and IFN-γ to HIV p55 and other antigens via intracellular cytokine staining.

HIV p55-specific CD4+ T cell expression of both TNF-α and IFN-γ was enhanced by GITR triggering, whereas GITR triggering alone or with pooled CEF peptides induced little additional expression of TNF-α and IFN-γ (A).

In twelve subjects with chronic HIV infection (Table 1, below), GITR triggering significantly increased the mean percentage of CD4+ T cells expressing HIV p55-specific TNF-α 0.51% vs. 1.00%, P=0.008; Figure 2B) and IFN-γ expression (0.37% vs. 0.51%, P=0.034; Figure 2C). Antibody triggering of GITR did not alter HIV p55-specific CD8+ T cell expression of TNF-α (0.79% vs. 0.59%, P=0.765; Figure 2D) or IFN-γ (0.63% vs. 0.56%, P=0.542; Figure 2E). CEF-specific T cell responses were unaffected by GITR triggering (data not shown).

The increase in HIV-specific CD4+ T cell expression of TNF-a after GITR triggering correlates with the peripheral CD4 count.

To determine whether clinical progression of HIV infection impacts CD4+ T cell responsiveness to GITR triggering, we assessed the correlation between the peripheral CD4 count with the magnitude of the increase in the percentage of CD4+ T cells expressing TNF-a after GITR triggering in PBMC from twelve HIV-infected subjects. The increase in the percentage of HIV p55-specific CD4+ T cells expressing intracellular TNF-a after GITR triggering correlated directly with the peripheral absolute CD4+ T cell count (P=0.009, Pearson R=0.714; Figure 2F, at right) but not with the plasma viral load (P=0.13, Pearson R=-0.463) or antiretroviral treatment status (P=0.13, Pearson R=-0.466)

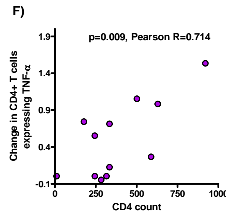

Table 1. Characteristics of HIV+ Study Subjects					
Age (years)	Sex	Year of diagnosis	CD4 Count (cells/ mm³)	HIV viral load (copies/ mL)	Antiretroviral regimen
50	M	1993	922	<75	TDF, ABC, ATV/r
53	M	1989	631	<75	TDF, FTC, ATV/r
39	M	1992	245	4,340	TDF, 3TC, AZT, LPV/r
40	M	1991	317	88	TDF, ABC, 3TC, ATV/r
43	F	1986	335	<75	TDF, ABC, 3TC, LPV/r, SQV
41	M	2003	285	254,182	None
48	M	1988	503	<75	TDF, FTC, EFV
38	M	1995	245	<75	d4T, 3TC, LPV/r
55	M	1988	179	<75	ABC, 3TC, ATV/r
42	F	2003	12	293,739	None
65	M	1991	590	<75	TDF, ABC, 3TC, ATV/r
39	M	1992	8	61,886	None

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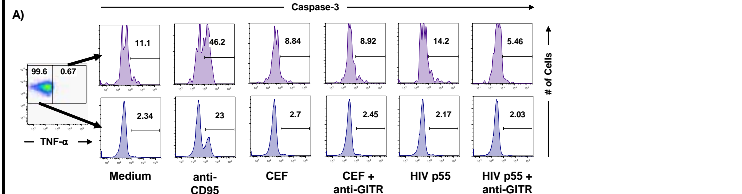
GITR triggering reduces apoptosis of HIV-specific CD4+ T cells.
Because GITR triggering augmented CD4+ T cell responses to HIV p55 but not to CEF, and since GITR triggering has been shown in murine models to protect T cells from apoptosis, we tested the hypothesis that GITR triggering enhanced HIV-specific CD4+ T cell cytokine secretion by protecting HIV-specific CD4+ T cells from apoptosis. To avoid confounding, we studied the impact of GITR triggering on apoptosis in HIV-specific CD4+ T cells from patients not on antiretroviral therapy.



B)

% CD4+ T cells expressing activated intracellular caspase-3

HIV p55 / HIV p55 + anti-GITR / HIV p55 + isotype control

TNF+ / TNF-

Simultaneous CD4+ T cell intracellular expression of TNF-α and activated caspase-3 was assessed in unfractionated PBMC from viremic HIV-infected subjects after stimulation with control medium, HIV p55, or pooled CEF peptides with and without GITR triggering. Intracellular expression of activated caspase-3 was greater in TNF-α expressing CD4+ T cells after stimulation with HIV p55 as compared to unstimulated cells (median 12.15% vs. 7.41%, P<0.001; Figure 3A). Intracellular expression of activated caspase-3 was not, however, increased in CD4+ T cells responding to CEF (10.22% vs 7.41%, P=0.200).

Further, activated caspase-3 expression was significantly reduced after GITR triggering in CD4+ T cells expressing TNF-α to HIV p55 (12.15% vs. 10.45%, P<0.001; Figure 3B), but not in CEF-specific CD4+ T cells (10.22% vs. 9.76%, P=1.000). Lastly, intracellular expression of activated caspase-3 was not impacted in CD4+ T cells not expressing TNF-a to antigenic stimulation, nor when using an isotype control antibody.

CONCLUSIONS

- GITR expression on CD4+ T cells is abnormal
- GITR triggering protects HIV-specific CD4+ T cells from apoptosis
- GITR triggering restores HIV-specific CD4+ T cell expression of TNF-α and IFN-γ
- No impact on CD8/non-HIV responses
- GITR triggering merits further testing as an adjunct to optimization of the HIV immune response

METHODS

Subjects and cell isolation. HIV-infected adults, and uninfected controls, gave informed consent to donate whole blood in a research protocol approved by the Dartmouth College Committee for the Protection of Human Subjects. PBMC were isolated by ficoll density gradient centrifugation, and cultured in RPMI 1640 supplemented with penicillin, streptomycin, HEPES buffer, L-glutamine and 10% FCS.

Antibodies and cell subsets. PBMC were stained with fluorochrome-conjugated monoclonal antibodies directed against CD3 and CD4 or CD8 (BD Biosciences). T cells were defined as CD3+ cells within the lymphocyte cloud on a forward scatter-side scatter plot. All analyses were conducted on T cells expressing CD4 or CD8.

Inducible GITR expression on CD4+ T cells. PBMC were incubated for two hours at 37C in 5% CO2 in medium alone or medium plus PHA (Sigma), and the percentage of CD4+ T cells expressing surface GITR was characterized using a fluorochrome-conjugated monoclonal antibody (R&D Systems). Specificity of staining was confirmed using an isotype control.

Antigens. In assays of T cell cytokine responses and CD4+ T cell apoptosis, PBMC were stimulated with the HIV protein p55 (NIH AIDS Research and Reference Reagent Program/BioMolecular Technologies). Responses to pooled peptides of CMV, EBV and influenza were assessed in parallel (CEF; NIH AIDS Research and Reference Reagent Program). Staphylococcal enterotoxin B (SEB; Sigma) was used as a positive control in assays of intracellular cytokine expression.

GITR triggering. Prior to intracellular cytokine staining, cells were incubated overnight with monoclonal antibodies against GITR at 10 mcg/ml, or with nonspecific antibodies, in culture medium. A control condition in which cells were incubated with monoclonal antibody against GITR without antigenic stimulation was included for all experiments.

Intracellular caspase-3 staining. Freshly-isolated PBMC from HIV-infected subjects not on antiretroviral therapy were stimulated for two hours with HIV p55 and CEF with and without GITR triggering. After a two hour incubation with experimental and control antigens, plus five hours of incubation with brefeldin A and an overnight refrigeration, cells were stained for surface expression of CD3 and CD4 and intracellular expression of TNF-a and activated caspase-3 (BD Biosciences) using a standard fixation, permeabilization and staining protocol (Caltag). SEB (Sigma) was used as a positive control for intracellular TNF-a staining, and monoclonal antibodies against CD95 (Upstate) were used as a positive control for the detection of apoptosis. An isotype control antibody was used to confirm the GITR-specificity of the effects of anti-GITR antibodies.

Statistical analysis. CD4+ T cell expression of GITR was compared between conditions using a Mann-Whitney test, as was the percentage of CD4+ T cells expressing intracellular activated casepase-3. The percentage of CD4+ or CD8+ T cells expressing intracellular cytokines in multiple subjects was compared in pooled samples using the Wilcoxon test. The correlation between CD4+ counts and serum HIV viral loads was measured using a Pearson chi-squared test. All analyses were conducted on Prism 4 software (GraphPad, SanDiego, CA).