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CD8 Co-receptor promotes susceptibility of CD8⁺ T cells to transforming growth factor- β (TGF- β)-mediated suppression

Andrew Zloza,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Michael C. Jagoda,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Gretchen E. Lyons,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Michael C. Graves,

Division of General Surgery, Department of Surgery, Medical University of South Carolina, Charleston, SC 29425, USA

Frederick J. Kohlhapp,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Jeremy A. O'Sullivan,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Andrew T. Lacey,

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

Michael I. Nishimura, and

Division of General Surgery, Department of Surgery, Medical University of South Carolina, Charleston, SC 29425, USA

José A. Guevara-Patiño

Department of Surgery, Committee on Immunology, The University of Chicago, Surgery Brain Research Building, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

José A. Guevara-Patiño: jguevara@uchicago.edu

Abstract

Correspondence to: José A. Guevara-Patiño, jguevara@uchicago.edu.

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CD8⁺ T cell function depends on a finely orchestrated balance of activation/suppression signals. While the stimulatory role of the CD8 co-receptor and pleiotropic capabilities of TGF- β have been studied individually, the influence of CD8 co-receptor on TGF- β function in CD8⁺ T cells is unknown. Here, we show that while CD8 enhances T cell activation, it also enhances susceptibility to TGF- β -mediated immune suppression. Using Jurkat cells expressing a full-length, truncated or no $\alpha\beta$ CD8 molecule, we demonstrate that cells expressing full-length $\alpha\beta$ CD8 were highly susceptible, $\alpha\beta$ CD8-truncated cells were partially susceptible, and CD8-deficient cells were completely resistant to suppression by TGF- β . Additionally, we determined that inhibition of Lck rendered mouse CD8⁺ T cells highly resistant to TGF- β suppression. Resistance was not associated with TGF- β receptor expression but did correlate with decreased Smad3 and increased Smad7 levels. These findings highlight a previously unrecognized third role for CD8 co-receptor which appears to prepare activated CD8⁺ T cells for response to TGF- β . Based on the important role which TGF- β -mediated suppression plays in tumor immunology, these findings unveil necessary considerations in formulation of CD8⁺ T cell-related cancer immunotherapy strategies.

Keywords

CD8 T cells; CD8 co-receptor; TGF- β ; Tumor-induced suppression; Lck; SMADs

Introduction

CD8 is a surface glycoprotein on CD8⁺ T cells (CTLs) which participates as a co-receptor in the CTL:antigen presenting cell (APC) interaction by binding the $\alpha 3$ domain of class I major histocompatibility complex (MHC-I) [1]. CD8 co-receptor in CTLs has been ascribed dual function (1) increasing CTL:APC adhesion and T cell receptor (TCR):peptide-loaded MHC-I (TCR:pMHC-I) affinity [2]; and (2) signaling through Src-family tyrosine kinase Lck promoting its recruitment to the TCR/CD3 complex [3, 4], leading to second messenger signaling and gene transcription-induced cytokine production, cell activation, and proliferation [5].

Transforming growth factor- β (TGF- β) is a pleiotropic cytokine with regulatory activity affecting T cell proliferation, differentiation, and survival [6, 7]. Its activity is associated with self-tolerance, inflammation resolution, regulatory T cell/Th17 differentiation, and tumor-induced immuno-suppression, while its absence in mice results in lethal autoimmune disease [8–10]. TGF- β function is initiated by its binding to cell-surface TGF- β receptor II (TGF- β -RII) which phosphorylates TGF- β receptor I (TGF- β -RI) [11]. This complex phosphorylates transcription factors SMAD2/3, which bind SMAD4, translocate into the nucleus, and regulate gene transcription [12, 13]. TGF- β signaling inhibition occurs through (1) SMAD7 inhibition of SMAD2/3 and (2) SMAD7-associated SMURF2 degradation of TGF- β receptors [14].

While CD8 co-receptor and TGF- β have been individually studied, information on the influence of CD8 signaling on the effects of TGF- β on CD8⁺ T cells is lacking. We show here that while CD8-Lck signaling enhances T cell activation, it also enhances susceptibility to TGF- β -mediated suppression in human and mouse. These findings reveal an

unappreciated role for CD8 co-receptor in balancing activation\suppression of CD8⁺ T cell function and highlight a TGF- β -mediated mechanism for cell contraction relevant to cancer immunology.

Methods

Cell lines and mice

The Jurkat-FHCRC cell line (clone E6-1) was obtained from ATCC (Manassas, VA). Jurkat cells are human T cell lymphoma established from the peripheral blood of a 14-year-old boy [15]. Jurkat cells require two signals for activation, TCR signaling and a phorbol ester (i.e. phorbol myristate acetate, PMA), for maximal IL-2 production. Jurkat cells expressing tyrosinase-specific TCR and $\alpha\beta$ CD8, $\alpha\beta'$ CD8 or no CD8 were prepared as described [16]. T2 cells are a Tap-1^{-/-} human B/T cell lymphoma. Cells were maintained in RPMI (Mediatech, Inc., Manassas, VA) with 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), 2% penicillin/streptomycin and 2 mM L-glutamine (Mediatech). Experiments using eight-week-old, specific-pathogen-free, C57BL/6 and C57BL/6-Tg(Tcr α Tcr β)1100Mjb/J (OT-I) mice (Jackson Laboratories, Bar Harbor, ME) were conducted according to Institutional Animal Care and Use Committee (IACUC) guidelines.

Human IL-2 ELISA

ELISA was performed as per manufacturer protocols (eBioscience, San Diego, CA). T2 cells were peptide-loaded (2 h) with tyrosinase_{368–376} or MART-1_{27–35}, washed twice, and cocultured with Jurkat cells and 50 ng/ml PMA (18–24 h, 1:1 ratio). Indicated cultures were treated with 50 ng/ml TGF- β (full-length, recombinant human TGF- β 2 expressed in *E. coli* and reactive in human and mouse cells; EMD Chemicals, Inc., Gibbstown, NJ).

Mouse cell in vitro culture

Splenocytes were obtained after mechanical dissolution and red blood cell lysis and treated $\pm$ anti-CD3/CD28 antibodies (6–24 h). Some cells were pre-treated with Lck Inhibitor (4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3, 2-d]pyrimidin-7-yl-cyclopentane; 50 ng/ml or 1 μ g/ml, EMD Chemicals). Indicated cultures were treated with 50 ng/ml TGF- β (full-length, recombinant human TGF- β 2 expressed in *E. coli* and reactive in human and mouse cells; EMD Chemicals, Inc., Gibbstown, NJ).

Antibodies and flow cytometry

Human (h)CD8-AF700,APC (OKT-8), hCD3-FITC,PerCPCy5.5 (OKT3), h $\alpha\beta$ TCR-APC (IP26), mouse (m)CD8-FITC,PE-Cy7 (53-6.7)] were purchased from eBioscience; mCD3-APC-Cy7 (17A2) from BD Biosciences (San Diego, CA); m/hGranzyme B-APC (3002) from Invitrogen (Carlsbad, CA); hLck-FITC (LCK-01), mLck-FITC (3A5), hTGF- β -RI (MM0016-7B09), mTGF- β -RI (RM0016-3A11), m/hSmad3 (C-8) and m/hSmad7 (N-19) from Santa Cruz Biotechnology (Santa Cruz, CA); and hTGF- β -RIIFITC and mTGF- β -RII-PE from R&D Systems (Minneapolis, MN). Cells were pre-incubated with FcBlock (BD), stained with Yellow LIVE/DEAD (Invitrogen) and extracellular antibodies (30 min, 4°C), washed and fixed (2% formaldehyde). For intracellular staining, GolgiStop-treated (BD) cells were additionally fixed/permeabilized (Cytofix/Cytoperm, BD), stained with

intracellular marker antibodies (30 min, 4°C), and fixed (2% formaldehyde-Perm/Wash (BD)). Fluorescence was measured using an LSR-II flow cytometer (BD), and data analyzed using FlowJo software (Tree Star, Ashland, OR). For CD8 blocking experiments, CD8 mAb (clone 2.43.1, 1 µg/ml) was used (The Fitch Monoclonal Facility, The University of Chicago, Chicago, IL).

Statistical analyses

Student's *t* test (two-tailed) was used to calculate the *P* value. *P* < 0.05 was considered statistically significant.

Results

CD8 expression impacts the susceptibility of Jurkat T cells to TGF-β-mediated suppression

It is unknown how the adhesion and signaling functions of CD8 impact TGF-β action on CD8⁺ T cells [2]. Using antibodies to block CD8 can lead to either stimulation or suppression [17]; therefore, to determine the role of CD8 in conferring susceptibility to TGF-β-mediated suppression, we employed the use of the human Jurkat cell line. Jurkat cells are amenable to gene modification [18–20], and the similarity of their signaling characteristics to primary human T cells is well described [21]. Specifically, in our study, Jurkat cells were transduced to express the tyrosinase_{368–376}-specific TCR and (1) complete αβCD8 (“αβCD8”); (2) truncated αβCD8 (“α'β'CD8”) with full-length extracellular, but deleted intracellular portion; and (3) no CD8 (“CD8[−]”); as described [16]. Both α'β'CD8 and “α'β'CD8” Jurkat cells expressed similar CD8 levels (Supplemental Fig. 1A). Jurkat cells were cocultured (18 h) with tyrosinase_{368–376} or irrelevant (MART-1_{27–35}) peptide-loaded T2 cells and PMA (50 ng/ml). To indicated cultures 50 ng/ml TGF-β, a concentration similar to that found in healthy donor serum was added after 1 h of incubation. Because Jurkat cells secrete IL-2 following antigen stimulation [21, 22], supernatants were assayed for IL-2 by ELISA. Expression of α'β'CD8 on Jurkat cells resulted in slight reduction in IL-2 compared with “αβCD8” Jurkat cells and significant reduction by CD8[−] Jurkat cells (Fig. 1a). Addition of TGF-β induced strong reduction in IL-2 by CD8-expressing Jurkat cells. Greatest suppression was consistently observed in αβCD8 versus α'β'CD8 Jurkat cells (64.4% versus 30.8% suppression, respectively; Fig. 1b). Even at highest suppression levels, IL-2 was above background (Fig. 1a, dashed line) and above IL-2 from Jurkat cells not expressing tyrosinase-specific TCR or cocultured with MART-1_{27–35} peptide-loaded targets (data not shown). Interestingly, CD8[−] Jurkat cells were resistant to TGF-β-mediated suppression (Fig. 1a–b). To determine whether inhibition of CD8 downstream signaling-associated molecule, Lck, would result in similar resistance, αβCD8 Jurkat cells were cocultured with tyrosinase_{368–376}-loaded T2 cells, PMA (50 ng/ml), and 50 ng/ml TGF-β (added after 1 h of coculture) ± Lck inhibitor (50 ng/ml, added 30 min prior to coculture). Addition of the Lck inhibitor decreased TGFβ-mediated suppression (88–61%) (Supplemental Fig. 1B). These data demonstrate that CD8 inversely regulates Jurkat T cell responsiveness to TGF-β.

Lck inhibition reduces mouse primary CD8 T cell susceptibility to suppression by TGF- β

To determine whether the effect of CD8 signaling on TGF β suppression observed using Jurkat cells translated to primary CD8⁺ T cells, mouse spleen CD8⁺ T cells were activated in vitro with α CD3/ α CD28 (1 μ g/ml) antibodies $\pm$ Lck inhibitor (50 ng/ml or 1 μ g/ml, present 30 min prior to and throughout culture) $\pm$ TGF- β (50 ng/ml, added 30 min into and throughout culture). Similarly, to test resistance in a peptide-stimulated mouse model, OT-I mouse splenocytes (OVA_{257–264} transgenic) were stimulated with OVA_{257–264} (1 μ g/ml) $\pm$ Lck inhibitor $\pm$ TGF- β . Since CTL granzyme B is inhibited by TGF- β at the mRNA level [23], we measured its production by intracellular flow cytometry (Fig. 1c) after 4-h culture. Similarly as in Supplemental Fig. 1B, CD8⁺ T cells incubated with Lck inhibitor were highly resistant to TGF β -mediated suppression (Fig. 1d–e and Supplemental Fig. 2A–B). While granzyme B production by CD8⁺ T cells not treated with Lck inhibitor was significantly reduced by TGF- β , Lck inhibitor-treated CD8⁺ T cells showed less resistance to TGF- β -mediated suppression of granzyme B. CD8⁺ T cells treated with 1 μ g/ml Lck inhibitor were completely resistant to TGF- β -mediated suppression. Analogous to the lack of CD8 co-receptor in CD8- Jurkat cells, blocking of CD8 signaling via administration of a CD8-blocking monoclonal antibody (clone 2.43.1) showed decreased granzyme B production in mouse CD8⁺ T cells and such blocking of CD8 signaling decreased susceptibility of such CD8⁺ T cells to TGF- β -mediated suppression (data not shown). Together these data support the findings that CD8-Lck signaling inversely regulates T cell responsiveness to TGF- β -mediated suppression.

CD8 T cell susceptibility to TGF- β correlates with Smad levels but not TGF- β receptor I, TGF- β receptor II, $\alpha\beta$ TCR or Lck expression

TGF- β -mediated suppression is dependent on T cell detection of TGF- β through TGF- β receptors I and II [7], which are induced by $\alpha\beta$ TCR activation. To determine whether resistance to TGF- β -mediated suppression observed was mediated by the level of receptor expression, TGF- β receptors I/II and $\alpha\beta$ TCR expression was determined. Receptors levels were similar among activated CD8⁺, $\alpha\beta$ CD8, and $\alpha'\beta'$ CD8 Jurkat cells (Fig. 2a) and likewise among activated mouse CD8⁺ T cells $\pm$ Lck inhibitor (Fig. 2b and Supplemental Fig. 2C–D). Additionally, CD8⁺ T cells expressed similar Lck levels $\pm$ TGF- β treatment, while inhibition of Lck via a pharmacological inhibitor decreased Lck signaling but not Lck expression in mouse CD8⁺ T cells (Supplemental Fig. 2E) and Jurkat cells (data not shown). Therefore, the observed CD8-mediated resistance to suppression by TGF β was not due to variability in TGF- β -RI, TGF- β -RII, $\alpha\beta$ TCR or Lck expression.

To determine whether CD8 alters TGF- β signaling pathways, we examined the impact of stimulation on Smad3 (TGF- β signal propagator) and Smad7 (TGF- β signal inhibitor) in Jurkat cells and Lck-inhibited OT-I CD8⁺ T cells after activation. CD8⁺ Jurkat cells had the greatest expression of Smad7 and the least expression of Smad3 levels; $\alpha'\beta'$ CD8 Jurkat cells had moderate expression of Smad7 and moderate expression in Smad3 levels; and $\alpha\beta$ CD8 Jurkat cells had the lowest expression of Smad7 and highest expression of Smad3 levels (Fig. 2c–d). Similarly, OT-I CD8⁺ T cells with 1 μ g/ml Lck inhibition had had the greatest expression of Smad7 and the least expression of Smad3 levels; OT-I CD8⁺ T cells with 50 ng/ml Lck inhibition had moderate expression of Smad7 and moderate expression of Smad3

levels; and OT-I CD8⁺ T cells without Lck inhibition had the lowest expression Smad7 and the greatest expression of Smad3 levels (Supplemental Figs. 2F–G). These results suggest that the signaling function of CD8 (through Lck) impacts TGF- β -mediated suppression by altering the TGF- β signaling pathway.

Discussion

We describe here a previously unappreciated role of the CD8 co-receptor in regulating CD8⁺ T cell susceptibility to TGF- β . While TGF- β is released from suppression-inducing cells (e.g. Treg cells), its action upon target cells involves the target's susceptibility to TGF- β . Our study assigns such susceptibility mediation role to the CD8 co-receptor and its intracellular signaling partner, Lck. Specifically, we demonstrate that elimination of CD8–Lck signaling (by CD8 truncation/elimination or Lck inhibition) decreases susceptibility of human and mouse CD8⁺ T cells to TGF- β -mediated suppression of effector cytokine production. Interestingly, in the absence of exogenous TGF- β treatment, intracellular CD8 truncation itself does not significantly decrease IL-2 but does significantly reduce Jurkat cell susceptibility to TGF- β . Though in these cells CD8 truncation lead to a disconnection between Lck and the CD8 co-receptor, Lck may have been activated via an alternative pathway, including signaling through alternative molecules on Jurkat cells, including CD45 [24, 25]. This finding highlights an important role for adhesion of CD8 to MHC-I, where such adhesion increases IL-2 production but also contributes to CD8⁺ T cell susceptibility to TGF- β -mediated suppression. Additionally, complete elimination of CD8 significantly decreases IL-2 as well as further reduces Jurkat cell susceptibility to TGF- β . IL-2 production by CD8⁺ Jurkat cells was still considerably above background, TCR and antigen control conditions, as well as above TGF- β -treated α CD8 Jurkat IL-2 production levels, indicating that increased suppression would be possible if these cells were further susceptible to TGF- β -mediated suppression. While there is some evidence that human and mouse CD8 co-receptor is dispensable in T cell:APC adhesion [26, 27], our findings add evidence to studies [28, 29] demonstrating a significant adhesion role for CD8 co-receptor in CD8⁺ T cells. Our findings also indicate that TCR–pMHC interaction itself is vital and in the context of high affinity TCR–pMHC interaction can overcome deletion of CD8–Lck interaction, but only partially compensate for complete deletion of CD8.

Recently, by pre-treating cells with TGF- β , Giroux et al. demonstrated that PKC θ signaling (downstream of Lck) allows naïve CD4⁺ T cells to overcome a TGF- β -induced unresponsiveness threshold and become activated [30]. Essentially, this study mimics the ability of cells to overcome TGF- β suppression after stimulation (i.e. like a naïve cell overcomes TGF- β upon specific/sufficient activation). In our hands, CD8:Lck has similar affects when TGF- β is added prior to activation (personal observation); however, our study expands on the understanding of Lck signaling effects by showing a reciprocal function for Lck signaling after the addition of TGF- β in cells that have already been activated (i.e., like an effector cell reacts to an activation-induced increase in TGF- β). Therefore, in testing two different scenarios, both findings accurately describe T cell responses to temporally separated combinations of activation and inhibition signals.

Our findings demonstrate that CD8-Lck-associated suppression by TGF- β is not a result of the level of TGF- β receptor I/II or $\alpha\beta$ TCR expression. Additionally, our findings show correlations between Smad3 and Smad7 level changes and resistance to TGF- β -mediated suppression. The combination of such changes may in part explain the complete resistance of CD8⁻ Jurkat cells (and high Lck inhibitor-treated CD8⁺ T cells) and the partial resistance of $\alpha\beta$ TCR Jurkat cells (and low Lck inhibitor-treated CD8⁺ T cells) compared to $\alpha\beta$ TCR Jurkat cells (and CD8⁺ T cells not treated with Lck inhibitor).

While our proposed third role for CD8 (conferring susceptibility to TGF- β) may seem to contrast with widely accepted activation-inducing CD8 functions, such action by CD8-Lck may in essence define a negative-feedback mechanism by which CD8⁺ T cells control the magnitude/timing of effector response. During CD8⁺ T cell expansion, there is a parallel, but delayed, increase in TGF β which after surpassing a threshold leads to contraction in non-resistant cells. Based on our findings here, further study may be warranted into the effect of TGF- β in high versus low affinity/avidity CD8:APC interactions and the role of CD8:Lck signaling in modulating the response of these cells to TGF- β -mediated suppression. Likewise, based on the direct role which tumor-induced TGF- β mediates suppression of tumor antigen-specific CD8⁺ T cell function, these findings unveil necessary considerations in formulation of CD8⁺ T cell-related cancer immunotherapy strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

CD8-Lck impacts the susceptibility of CD8⁺ T cells to TGF- β -mediated suppression. **a** Cumulative figure of ELISA mean IL-2 production by respective Jurkat cells after 18-h 1:1 culture with T2 cells (preloaded with 1 μ g/ml tyrosinase₃₆₈₋₃₇₆ peptide), 50 ng/ml PMA and $\pm$ 50 ng/ml TGF- β . **b** Graphical representation of relative % suppression by TGF- β from data in A. **c** Flow cytometry gating schematic on mouse splenocytes. **d** Cumulative figure of mean granzyme B production by mouse CD8⁺ T cells after 4-h culture with anti-CD3/CD28 mAbs (1 μ g/ml) $\pm$ 50 ng/ml TGF- β $\pm$ Lck inhibitor (50 or 1 μ g/ml, as indicated). **e** Graphical

representation of relative % suppression by TGF- β from data in D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Dashed line represents background staining. Figures represent one set of experiments done in triplicate and repeated at least two additional times with similar results

Fig. 2.

CD8 T cell susceptibility to TGF- β correlates with Smad levels but not TGF- β receptor I, TGF- β receptor II, or $\alpha\beta$ TCR expression. **a** Expression of TGF- β -RI, TGF- β -RII, and $\alpha\beta$ TCR by respective Jurkat cells after 18-h 1:1 culture with T2 cells (pre-loaded with 1 μ g/ml tyrosinase_{368–376} peptide) and 50 ng/ml PMA. **b** Expression of TGF- β -RI, TGF- β -RII, and $\alpha\beta$ TCR by mouse CD8⁺ T cells after 4-h culture with anti-CD3/CD28 mAbs (1 μ g/ml) $\pm$ Lck inhibitor (1 μ g/ml). **c** Smad3 levels after 30-min coculture (as described in a). **D**, Smad7

levels after coculture (as described in c).* $P<0.05$, ** $P<0.01$. Figures represent one set of experiments done in triplicate and repeated at least two additional times with similar results

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