Proximal signaling control of human effector CD4 T cell function

Francesca I. Okoye [a,b], Sandeep Krishnan [c], Meena R. Chandok [a],
George C. Tsokos [d], Donna L. Farber [a,*]

[a] *Division of Transplantation, Department of Surgery, University of Maryland School of Medicine, MSTF Building,
Room 400, 685 W. Baltimore Street, Baltimore, MD 21201, USA*
[b] *Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[c] *Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, USA*
[d] *Rheumatology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA*

Abstract   The functional coupling of T cell receptor (TCR)-mediated signaling events in primary human T cells remains undefined. We demonstrate here that alterations in the expression of proximal TCR-coupled signaling subunits are associated with distinct effector capacities in differentiated human CD4 T cells. Analysis of proximal signaling profiles using biochemical and single cell approaches reveals decreased CD3ζ and ZAP-70 expression correlating with functional anergy, with increased CD3ζ/ ZAP-70 expression and phosphorylation connoting acquisition of effector capacity. By contrast, the FcRγ signaling subunit known to be expressed in human effector cells and in T cells from the autoimmune disease SLE is up-regulated upon activation, yet does not correlate with functional capacity in effector cells, and does not alter signaling or function in primary FcRγ transfectants. Our results have implications for targeting signaling molecules in immunotherapy and evaluating the functional consequence of signaling alterations associated with autoimmunity and chronic diseases.

Introduction

Activation of T lymphocytes through the T cell antigen receptor (TCR) triggers a series of intracellular signaling events culminating in IL-2 gene transcription in the nucleus (for reviews see [1,2]), commencing a differentiation process leading to the generation of effector T cells. Effector T cells can be distinguished from naive or unprimed T cells by the expression of specific cell surface activation and differentiation markers [3], by enhanced activation kinetics and lower activation threshold [4,5], by the acquisition of effector function including the ability to rapidly produce effector cytokines [6,7], and by an increased propensity for activation-induced cell death [8]. While the biochemical pathways coupled to TCR engagement in resting T cells and T cell lines have been extensively characterized [9], little is known regarding how TCR-mediated signals are transduced in effector T cells. Moreover, the TCR-coupled signaling processes that control activation and function in effector T cells remain largely unknown, and are critical parameters to design strategies to modulate T cell function in autoimmunity and chronic infection where effector T cells can predominate.

* Corresponding author. Fax: +1 410 706 0311.
  E-mail address: dfarber@smail.umaryland.edu (D.L. Farber).

Proximal TCR-mediated signaling events coupled directly to TCR engagement involve the phosphorylation on tyrosine residues of the TCR-associated CD3ε and CD3ζ subunits by the p56^{lck} tyrosine kinase, resulting in the recruitment, phosphorylation and activation of the 70-kDa SH2-containing ZAP-70 tyrosine kinase [10,11]. Activated ZAP-70 subsequently phosphorylates linker adapter molecules such as SLP-76 and LAT leading to activation of distal MAP kinases and ultimately to the activation and mobilization of nuclear transcription factors for IL-2 gene transcription [1]. While these proximal and distal signaling processes are operable in resting T cells, we previously identified differences in TCR-coupled proximal signaling in primary human effector CD4 T cells marked by decreased CD3ζ protein expression and upregulation of the related ITAM-containing signaling subunit FcRγ [12,13], typically associated with the high affinity IgE FcR [14]. In effector cells, the FcRγ subunit formed a new TCR/CD3ε/FcRγ complex contrasting the conventional TCR/CD3ε/CD3ζ complex expressed by resting T cells and T cell lines [15]. Effector cell-associated signaling changes including decreased CD3ζ expression have been found in T cells in cancer, autoimmunity and chronic viral infections [12,16,17] and upregulation of FcRγ expression has been also found in the peripheral T cells of patients with systemic lupus erythematosus (SLE) [18]. It is not known whether these signaling differences common to effector cells and disease-associated T cells reflect alterations in functional capacity or represent changes occurring during the course of T cell differentiation.

In this study, we investigated how the expression of the proximal signaling molecules, CD3ζ and FcRγ, in human effector CD4 T cells was coupled to functional regulation using biochemical and single cell analyses of primary human CD4 T cells and transfectants. We found that sustained activation of human CD4$^+$CD25$^-$ T cells by anti-CD3/anti-CD28 antibodies generated effector cells producing IFN-γ and IL-2 whereas stimulation with anti-CD3 and autologous monocytes resulted in functionally hyporesponsive effector cells, with only a small proportion producing cytokines. Analysis of proximal signaling profiles using biochemical and single cell approaches reveals decreased CD3ζ and ZAP-70 expression correlating with functional anergy, with increased CD3ζ/ZAP-70 expression and phosphorylation connoting acquisition of effector capacity. By contrast, the FcRγ signaling subunit known to be expressed in human effector cells and in T cells from the autoimmune disease SLE is upregulated upon activation in both effector types, yet does not correlate with functional capacity, nor does it drive signaling or functional alterations in FcRγ transfectants. Our results have implications for targeting signaling molecules in immunotherapy and for identifying the functional consequence of signaling alterations associated with autoimmunity and chronic diseases.

Materials and methods

Human cells

Heparinized peripheral venous blood was obtained from consenting healthy adult volunteers, or as Leukopacs purchased from BRT Laboratories (Baltimore, MD).

Antibodies

IgM anti-CD3 (2Ad$_2$A$_2$) was generously provided by Dr. Robert Siliciano (Johns Hopkins University, Baltimore, MD). Anti-FcRγ and anti-phosphotyrosine (4G10) were purchased from Upstate Biotechnology (Charlottesburg, VA). PE-conjugated anti-TCRζ (2H2D9) was purchased from Immunotech (Marseille, France), and anti-human/mouse ZAP-70 from Invitrogen (Carlsbad, CA). Anti-CD3 (UCHT1) and the following fluorochrome-conjugated antibodies to CD14 (M5E2), CD69 (FN50), CD25 (M-A251), CD152 (BN13), CD86 (FUN-1), IL-2, IFN-γ, and CD4 (RPA-T4) were purchased from BD-Pharmingen (San Jose, CA). For confocal analysis, anti-CD3 (UCHT1; Sigma), isotype control antibodies, FITC- and TRITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell isolation and stimulation

Human CD4 T cells were purified from peripheral blood mononuclear cells (PBMC) by negative selection using the CD4 T cell isolation kit and autoMACS™ as per the manufacturer's recommendations (Miltenyi Biotec, Auburn, CA), and subsequently depleted of CD4$^+$CD25$^+$ T cells using anti-CD25 conjugated microbeads (Miltenyi Biotec), yielding >98% pure CD4$^+$CD25$^-$ T cells. For antigen presenting cells (APC), peripheral blood monocytes were purified by positive selection with anti-CD14 magnetic microbeads (Miltenyi Biotec) and were found to uniformly express CD86 (B7-2).

CD4$^+$CD25$^-$ T cells (1×10^6/ well in a 24-well plate) were activated either with Dynabeads$^®$ CD3/CD28 T cell expander (Invitrogen, Carlsbad, CA) at a 1:1 cell:bead ratio, or as previously described [13,15] with soluble anti-CD3 antibody (4 μg/ml UCHT1) and APC (2×10^6/well) for 48 h at 37 °C in complete RPMI medium supplemented with 50 U/ml of recombinant human IL-2 (hIL-2) (Peprotech, Rocky Hill, NJ). The resultant activated CD4 T cells were purified by centrifugation through Ficoll (LSM, ICN/Cappel, Aurora, OH), and residual monocytes depleted using anti-CD14-coupled magnetic Dynabeads (Invitrogen), yielding 99% purity [15].

Western blotting

For western blot analyses, T cells (2×10^6) were left untreated or activated by anti-CD3 IgM antibody for 2 min at 37 °C before lysing in 1% NP40 lysis buffer with protease/phosphatase inhibitors as previously described [13]. Lysates were resolved using 10 or 12% NuPAGE$^®$ Bis-Tris gels (Invitrogen), transferred to nitrocellulose and blots incubated with antibodies to phosphotyrosine and actin followed by HRP-conjugated goat anti-mouse (Biorad, Hercules, CA) as described [13].

Primary T cell transfection

CD4$^+$CD25$^-$ T cells were stimulated with Dynabeads$^®$ CD3/CD28 and 50 U/ml hIL-2 for 24 h, washed and transfected by nucleofection (Human T cell Kit; Amaxa, Gaithersburg, MD) with 20 μg of pMG-FcRγ [19] or a control CMV expression vector and cultured for 18 h in complete RPMI media, prior to biochemical and functional analyses.