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Cutting Edge: Itk-Dependent Signals Required for CD4⁺ T Cells to Exert, but Not Gain, Th2 Effector Function

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The TCR signals for the release of CD4 effector function are poorly understood. Itk plays an essential role in Th2, but not Th1, responses. However, when Itk is required during Th2 development is unclear. We followed the fate of Itk-deficient T cells during Th2 development in vitro and in vivo using an IL-4/eGFP reporter. Surprisingly, a similar frequency of itk⁻/⁻ CD4⁺ cells differentiated and committed to the Th2 lineage as wild-type cells. However, Itk-deficient Th2 cells failed to exert effector function upon TCR triggering. Loss of function was marked by defective transcriptional enhancement of Th2 cytokines and GATA3. IL-4 production in itk⁻/⁻ Th2cs could be rescued by the expression of kinase-active Itk. Thus, Itk is necessary for the release, but not gain, of Th2 function. We suggest that the liberation of effector function is tightly controlled through qualitative changes in TCR signals, facilitating postdifferentiation regulation of cytokine responses. The Journal of Immunology, 2006, 176:3895–3899.

Materials and Methods

Mice

Itk⁻/⁻ BALB/c mice (C.129S2(B6)-Itktm1Lky) (12) were crossed to BALB/4ger mice (C.B6-Tg[IL4tm1Lky]11) or DO11.10 TCR transgenics on a TCR-Cα⁻/⁻ (C.B6-Tg[IL4tm1Lky]11 or Tg[OVA10Dlo]) background. Mice were maintained in the pathogen-free animal care facility at the University of Rochester Medical Center (Rochester, NY).

Cell purification

CD4⁺ cells were enriched from the spleen and lymph node by Ab/complement-mediated lysis (4) and sorted (FACSAria; BD Biosciences) for naive CD4⁺ cells, >98.7% CD62LhighCD44low. Memory cells were enriched by depletion of CD62Lhigh cells (Miltenyi Biotec). T-depleted splenocytes (APCs) were isolated from WT mice by complement lysis (4) and irradiated (2000 rads). Naive DO11.10⁺ T cells were isolated by FACS sorting as for nontransgenic T cells.

T cell priming

A total of 10⁶ naive CD4⁺ T cells per well in 2 ml of complete RPMI 1640 medium with 10% heat-inactivated FCS was stimulated with plate-bound mAb H57.597 (0.5 µg/ml) and 37N51.1 (2 µg/ml) (37°C). Th1 priming: 10 U/ml IL-2, 2 µg/ml P3T cell peptide, 10 U/ml IL-12, and 60 U/ml TNF-α. Th2 priming: 10 U/ml IL-4, 2 µg/ml OVA257-264, 1 µg/ml IL-12, and 100 ng/ml GM-CSF.

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1 Abbreviation used in this paper: WT, wild type; KD, kinase dead; eGFP, enhanced GFP.
human IL-2 (rhIL-2; National Institutes of Health Research and Reference Reagent Program, Frederick, MD), 10 ng/ml rhIL-12 (Peprotech), and 40 μg/ml anti-IL-4 mAb (11B11). Th2 priming: 10 U/ml rhIL-2, 50 ng/ml murine rIL-4, and 50 μg/ml anti-IFN-γ mAb (XMG 1.2). After 5–6 days, cells were washed and restimulated at 1 × 10^5 cells in 200 μl in HS7-coated plates. Where designated, rhIL-2, rIL-4, anti-IL-4Rα mAb (M1, 50 μg/ml), anti-CD28 (2 μg/ml), anti-CD70 (1 μg/ml; Calbiochem), PMA (50 ng/ml), or cyclosporin A (2 μg/ml; Sigma-Aldrich) were added. Naive DO11.10 TCR-γδ CD4^+ T cells were electroporated with 1 μM OVA (543–559) peptide (pOVA) and APC.

Real-time RT-PCR

RNA was extracted (TRIzol; Invitrogen Life Technologies) and reverse-transcribed (RT for PCR kit; BD Clontech). Real-time PCR used Assays-on-Demand TaqMan primer/probe sets with an ABI prism 7900 sequence detection system (Applied Biosystems). Target levels were normalized to CD36.

Cytokine measurements

Cytokines from 48-h culture supernatants were measured by ELISA. The Cytokine Capture Assay (Miltenyi Biotec) was performed essentially as described (13). A total of 1 × 10^6 CD4^+ T cells were stimulated with plate-bound anti-TCRβ or PMA/ionomycin, harvested, and labeled with the bifunctional Ab “catch” reagent for 5 min on ice and warmed to 37°C with HS7 or PMA/ionomycin for 45 min for cytokine secretion. Cytokine was detected by FACS using a second anti-cytokine mAb. Gates were drawn on cells labeled without the catch reagent (“no catch”). Intracellular cytokine staining was performed using a BD Pharmingen kit. Brefeldin A was added to cultures 4 h before harvest.

Leishmania major infections

Mice were infected intradermally in one ear with 2 × 10^4 L. major promastigotes (4) in 10 μl of PBS and PBS in the contralateral ear. Four weeks post-infection, cells were isolated from the ear tissue with 1 mg/ml collagenase/dispase (Roche) for 30 min at 37°C. L. major-specific cytokine production was determined by ELISPOT (4).

T cell transfections

WT Itk or kinase-dead (KD, K390R) Itk, GATA3, and constitutively nuclear NFATc1 (14) cDNA were cloned into a bicistronic internal ribosomal entry site-GFP expression vector. On day 4 of Th2 priming, itk^−/− CD4^+ cells were electroporated with 4 μg of plasmid DNA using the Amaxa Mouse T cell nucleofector kit (Amaxa Biosystems). Transfected cells were incubated at 37°C overnight before restimulation and cytokine secretion analysis.

Results and Discussion

Th2 differentiation independent of Itk signals

CD4^+lowCD62L^high naive CD4^+ T cells from IL-4/eGFP reporter mice (4get) were primed under Th2 conditions, and the emergence of eGFP^+ cells was analyzed by flow cytometry (Fig. 1A). Surprisingly, the kinetics, frequency, and magnitude of early GFP expression were identical in Th2-primed WT 4get and itk^−/− 4get cells. Real-time PCR analysis confirmed the FACS data showing normal Th differentiation under strong polarizing conditions in the absence of Itk (10). Under Th2 priming, GATA3 and IL-4 were induced and Tbet was repressed, whereas Th1 priming induced Tbet and IFN-γ expression and GATA3 repression in itk^−/− cells (Fig. 1B). IL-5 and IL-13 also were induced in itk^−/− cells similarly to WT (Fig. 2B). WT and

FIGURE 1. ITK-independent Th1 and Th2 differentiation. A, Naive CD4^+ T cells from 4get mice were stimulated under Th2 priming conditions and analyzed by FACS for early eGFP expression. B, Naive CD4^+ T cells were stimulated under Th2 or Th1 priming conditions and mRNA purified for quantitative RT-PCR. Data are expressed as fold change over unstimulated naive CD4^+ T cells. C, Cells in B were restimulated with anti-TCRβ and cytokine secretion measured by ELISA. D, Naive cells were Th2-primed for 72 h and cytokine secretion measured by cytokine capture (Miltenyi). E, Cells as in A were primed for 5 days, and eGFP^+ cells were assayed for viability by FACS before (0 h) and after restimulation with anti-TCRβ (24 h). Numbers in quadrants are the percentage of CD4^+ T cells expressing the given marker. Results represent one of four comparable experiments.
cells also looked similar with respect to the low frequency of eGFP cells observed after neutral priming (1.00% and 0.93% eGFP, respectively). Despite induction of both Th1 and Th2 programs in \( \textit{itk} \) cells, only Th1 cells were capable of secreting effector cytokines on restimulation (Fig. 1C) (4). In contrast, although at very low frequency, both WT and \( \textit{itk} \) cells secreted IL-4 during the early Th2-priming period (Fig. 1D). We found no evidence for preferential death of \( \textit{itk} \) cells after either 5-day priming or following restimulation (24 h) (Fig. 1E) using 7-aminoactinomycin D exclusion as a marker of cell viability. Indeed, fewer activated \( \textit{itk} \) cells than WT cells were 7AAD positive on restimulation, consistent with \( \textit{itk} \) defects in activation-induced cell death (15).

Itk-deficient signals fail to support transcriptional enhancement of Th2 cytokine genes

Secondary stimulation of WT Th2-primed cells is accompanied by a 100- to 1000-fold transcriptional enhancement of Th2 cytokine gene expression over primary stimulation (16) (Fig. 2B). Single-cell analysis of WT 4get cells showed that a significant population of Th2-primed cells became at least 10-fold brighter for eGFP (eGFPhigh) on restimulation (Fig. 2A), suggesting...
mRNA differences were, in part, due to enhancement of transcription in individual cells. However, the absence of Itk led to a striking block in transcriptional enhancement of Th2 cytokines on restimulation as measured by a failure to up-regulate eGFP expression by FACS (Fig. 2A) and transcripts by RT-PCR (Fig. 2B). In addition, itk−/− Th2-primed cells were unable to sustain GATA3 expression on restimulation in contrast to other transcription factors such as NFATc1 (Fig. 2B). This does not appear to be due to aberrant Tbet expression (10) as Tbet mRNA expression was down-regulated in Th2-primed effectors (Fig. 2B).

Transcriptional enhancement and the release of Th2 effector function

To correlate transcripts with IL-4 protein production, we used cells from 4get mice and the cytokine capture assay. On restimulation, we found a strong positive correlation between enhanced transcripts, eGFPhigh cells, and the magnitude of IL-4 production in WT Th2 cells (Fig. 3A). Although the frequency of eGFP expressing itk−/− Th2-primed cells remained constant on restimulation (~65%), consistent with enhanced mRNA transcripts over naive cells (Fig. 2B), the failure to further enhance transcription led to a profound attenuation of IL-4 protein production (Fig. 3A). The absence of intracellular staining for IL-4 confirmed a defect in protein production and not secretion (Fig. 3B). Importantly, bypassing the TCR proximal signaling defects using PMA/ionomycin revealed the IL-4 potential of the Th2-primed itk−/− eGFP+ T cells: WT and itk−/− Th2-primed cells were identical in their ability to secrete IL-4, and both had lost the capacity to secrete IFN-γ (Fig. 3C) consistent with Th2 differentiation.

To directly address Th2 lineage commitment, WT and itk−/− DO11.10 TCR Tg+ T cells were primed under Th1 or Th2 conditions with pOVA/APC for 5 days, harvested, and re-stimulated for a further 5 days in the original or opposing polarizing conditions. Cells were stimulated for a third round in the absence of exogenous cytokine to determine their cytokine program (Fig. 3D). Itk-deficient CD4+ T cells failed to develop into IL-4 secreting cells even after multiple rounds of Th2 priming (Fig. 3D, middle panel) despite similar expansion as WT cells over three rounds of stimulation. Nevertheless, both Th2-primed WT and itk−/− cells were committed to the Th2 lineage as secondary priming under Th1 conditions failed to generate IFN-γ producing cells (Fig. 3D, right panel). Again, PMA/ionomycin restimulation of pOVA/APC Th2-primed itk−/− cells revealed their Th2 potential. Thus, in the absence of Itk, CD4+ cells differentiate into cells competent and committed to the Th2 lineage but secondary Itk-deficient TCR signals fail to support enhancement of cytokine mRNA levels critical for the production of Th2 cytokines.

Itk is required for optimal IL-2 and IL-4 responses (4, 10, 12, 17) and may be a component of CD28 costimulation (18, 19). Supplementation of secondary cultures with these missing components was unable to restore IL-4 secretion in the itk−/− effectors (Fig. 3E). In addition, coculture of itk−/− cells with Th2-primed WT cells also failed to restore Th2 effector function, suggesting the defect is not due to a missing Itk-dependent factor made by WT Th2s. As described (20), IL-4 transcriptional enhancement and IL-4 production in WT cells was highly dependent on calcium mobilization and was blocked by the addition of CsA (Fig. 3F). Indeed, ionomycin-supplemented TCR signals on restimulation (plus Iono 2°), but not at the time of initial priming only (plus Iono 1°), restored IL-4 secretion in itk−/− Th2 effectors (Fig. 3F). Our studies implicate the calcium flux as a critical regulator of Th2 effector function. Indeed, given that itk−/− cells have defects in calcium signaling both on initial priming and on restimulation, our data suggest that initial induction of IL-4 transcription is less sensitive to changes in the magnitude of the calcium flux than secondary transcriptional enhancement. Interestingly, the calcium flux of Th2 cells is poor, compared with that of Th1 cells, both in magnitude and duration (21). Thus, Th2 responses are likely to be highly susceptible to small changes in signals that regulate such a modest calcium response, as highlighted here in the absence of Itk.

To formally demonstrate Itk’s requirement for Th2 effector function, we introduced the Itk gene into Th2-primed itk−/− effectors. We compared WT-Itk with kinase-inactive (KD-Itk) given potential kinase-independent functions for Itk (22). Genes were introduced at the end of the Th2 priming conditions (4). WT-Itk (Fig. 3G) restored the frequency of IL-4-secreterors to that seen for WT Th2 cells (Fig. 3E), whereas the kinase-inactive Itk was less effective (Fig. 3G). Thus, Itk contributions to Th2 responses are necessary at the effector stage only. Moreover, full IL-4 activation appears to be Itk-kinase dependent.

Enhancement of IL-4 transcription in Th2 cells appears to be controlled by an inducible 3′ enhancer element in the IL-4 gene: DNase I hypersensitivity site Vα (16, 20). The Vα region is essential for IL-4 production in Th2 cells and may be a determinant of probabilistic IL-4 production in Th2 cells (16, 23). Both GATA3 and NFAT bind to this site and are regulated

![FIGURE 4](http://www.jimmunol.org/)  
*FIGURE 4.* Defects in release, but not gain, of Th2 effector function ex vivo. A, Ex vivo-purified CD4+ CD44highCD62Llow effectors/memory cells analyzed for eGFP expression before or after anti-TCRβ stimulation. Numbers below the boxes are the percentage of CD44highCD62Llow eGFP+ cells, and numbers in parentheses are the percentage of total CD4s. B, Effectors/memory cells were stimulated (anti-TCRβ) for 24 h and analyzed for IL-4 secretion. Quadrant numbers are the percentage of CD4+ T cells. C, eGFP expression of CD4+ cells isolated from the ear tissue of 4-wk L. major-infected (open overlay) or PBS-injected (filled overlay) mice. Numbers are the percentage of CD4+ cells in each gate. D, L. major-specific ELISPOT from the L. major-infected ear. Results represent one of three experiments.
by Itk, (NFATc1 (4) and GATA3; Fig. 2B) in Th2-primed effectors. Thus, Itk may play a role in the induction of this enhancer element and/or the transcription factors required for its activity. However, simple add-back of GATA3 or a constitutively nuclear NFATc1 to Th2-primed itk−/− cells failed to rescue IL-4 effector function (Fig. 3G). A recent report of Itk kinase activity in the phosphorylation of the transcription factor Tbet (24) suggests that Itk might act posttranslationally to enhance IL-4 gene expression.

**Itk-dependent release, but not gain, of Th2 effector function in vivo**

It is not known whether the impaired Th2 responses in vivo models are due to defects in differentiation, Th2 survival, homing or cytokine release (4–6). Th2 function was analyzed directly ex vivo in CD44highCD62Llow effector/memory CD4+ T cells from 4get mice. WT and Itk-deficient effector/memory populations contained similar frequencies of CD44+ eGFP+ cells (Fig. 4A), suggesting that Itk-independent Th2 differentiation can occur to physiological stimuli. Upon in vitro restimulation, eGFP expression was dramatically enhanced and marked by the release of IL-4 protein in WT cells but not in Itk-deficient effector/memory cells (Fig. 4, A and B). As with in vitro primed cells, PMA/ionomycin released their Th2 effector potential (data not shown). Similar defects in IL-4 were observed in memory cells from non-4get itk−/− mice.

We have previously described the requirement for Itk in Th2 responses to L. major infection (4). We used the 4get mice to determine whether a similar defect in release, but not gain, of Th2 effector function could explain the absence of Th2 activity in infected BALB/c itk−/− mice. Indeed, we were surprised to see a similar frequency of CD44+ eGFP+ cells at the infection site in both WT and itk−/− mice (Fig. 4C), suggesting Itk-independent generation and localization of a IL-4-competent T cell pool. However, the itk−/− eGFP+ population at the tissue site had a reduced frequency of eGFP+CD44+ T cells directly ex vivo (Fig. 4C) corresponding to defects in IL-4 production, but not IFN-γ, on restimulation to L. major Ags (Fig. 4D). Therefore, a similar defect in the ability to release Th2 effector function in the absence of Itk was observed ex vivo for both polyclonal and Ag-specific recall responses.

**The requirement for Itk in the Th2 effector T cell response, but not in Th2 differentiation, reveals that TCR signals for initiation and liberation of effector function are distinct. Indeed, the recent use of a dual reporter system for analysis of IL-4 transcription and IL-4 secretion supports the notion that IL-4 transcriptional competency and IL-4 production can be separated (25). These signal differences may provide a means for secondary control of effector function subsequent to primary differentiation. Given effector cells are likely to exert their effector function at an infected tissue site and not the priming lymph node, we propose that these distinct signals might also be spatially separated.**

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**Disclosures**

The authors have no financial conflict of interest.

**References**