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Cutting Edge: CTLA-4 (CD152) Differentially Regulates Mitogen-Activated Protein Kinases (Extracellular Signal-Regulated Kinase and c-Jun N-Terminal Kinase) in CD4+ T Cells from Receptor/Ligand-Deficient Mice

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Although CTLA-4 (CD152) has potent inhibitory effects on T cell function, the signaling events affected by this coreceptor remain to be fully defined. Mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) act as crucial regulators of multiple aspects of cell function. Ab ligation studies have reported an inhibitory effect of CTLA-4 on TCR-induced ERK and JNK activation. In this study, we have re-examined the specificity of CTLA-4 inhibition of MAPKs by using natural ligand with ex vivo-purified CD4+ T cells deficient in CD80 and CD86 (double knockout), or CTLA-4, CD80, and CD86 (triple knockout). Under these conditions, CTLA-4 ligation was found to up-regulate and sustain JNK activation, while inhibiting ERK activity. At the same time, JNK activation could not account for CTLA-4 induction of TGF-β production. Our findings demonstrate that CTLA-4 cosignaling is more complex than previously appreciated, with an ability to differentially regulate members of the MAPK family in T cells. The Journal of Immunology, 2002, 169: 3475–3479.

Coreceptors CD28 and CTLA-4 (CD152) have opposing positive and negative effects on T cells, respectively (1–3). Both coreceptors bind to ligands B7-1 (CD80) and B7-2 (CD86), although the binding avidity of CTLA-4 is 20- to 50-fold higher, and may preferentially bind to CD80 (4). Abs to CTLA-4 can block T cell activation (5, 6), while CTLA-4-deficient mice show spontaneous lymphoproliferation (7, 8). By setting the threshold of TCR signaling, CTLA-4 has been implicated in autoimmunity, anti-tumor responses, and in the polarization of cells into Th1 cells (9, 10). CTLA-4 can also induce TGF-β production, an event that may account for aspects of CTLA-4 negative regulation (11–13).

A major question concerns the nature of cosignals responsible for CTLA-4 function. In this regard, CTLA-4 binds to lipid kinase phosphatidylinositol 3-kinase (PI3-K) (14), phosphatases PP2A and SHP-2 (15–17), and has been reported to inhibit serine/threonine kinases extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (18). Further, we have recently reported that CTLA-4 potently blocks surface expression of lipid microdomains (rafts, glycosphingolipid-enriched membranes) in T cells, thus limiting the availability of key proteins such as linker for activation of T cells required for TCR signaling (19, 20). To assess the influence of CTLA-4 on ERK/JNK pathways, purified CD4+ T cells from mice that are deficient in CD80 and CD86 (double knockout (DKO)), or CTLA-4, CD80, and CD86 (triple knockout (TKO)) were examined. Our findings demonstrate for the first time that CTLA-4 can differentially regulate the activation of members of the mitogen-activated protein kinase (MAPK) family.

Materials and Methods

Cells, reagents, and Abs

Mouse strains lacking CD80 and CD86 (DKO) or CD80/86 and CTLA-4 (DKO) have been described previously (21). CD4+ T cells from DKO and TKO mice were purified and cultured together with APCs from wild-type, CD86KO (CD80-expressing APCs), or CD80KO (CD86-expressing APCs) mice (21, 22). Anti-ERK1/2 and GST-c-jun were purchased from Upstate Biotechnology (Lake Placid, NY), anti-JNK-1, anti-trinitrophenol from BD PharMingen (San Diego, CA) and myelin basic protein (MBP) from Sigma-Aldrich (St. Louis, MO). Constitutively active JNK (JNK2-K c-Jun fusion protein) was a gift from Dr. A. Lin (University of Alabama, Birmingham, Alabama).

Cell preparation and cultures

Cell preparations from mouse spleens/lymph nodes and PBLs were prepared as described (21, 23). PBLs were stimulated with anti-CD3/CD28

Abbreviations used in this paper: PI3-K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; KO, knockout; DKO, double KO; TKO, triple KO; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; WTAPC, wild-type APC.
ERK1/2. Equal amounts of cell lysates were immunoblotted for JNK-1. Lower panel, cells; lanes 1–3, ERK activity in WTAPCs, DKO, and TKO T cells alone. Lower panel, Equal amounts of cell lysates were immunoblotted for ERK1/2. B, Proliferation of DKO and TKO T cells. Purified CD4+ T cells from DKO and TKO mice were cocultured with WTAPCs (ratio 1:10) and stimulated with anti-CD3 (1:1000) for the indicated periods of time. Cells were lysed, immunoprecipitated with anti-ERK1/2 mAbs, and subjected to an in vitro kinase assay.

Results and Discussion

To investigate the influence of CTLA-4 on signaling, CD4+ T cells were purified from mice lacking CD80 and CD86 (DKO), or CTLA-4, CD80, and CD86 (TKO) and were stimulated using CD80/CD86-positive APCs in the presence of anti-CD3 Ab. This provides a model for assessing signaling events in freshly isolated cells as mediated by CD80/CD86 interactions in the presence or absence of CTLA-4. Initial tyrosine and threonine phosphorylation studies failed to show a reproducible difference between the two sets of T cells (data not shown). We next assessed whether CTLA-4 could influence the status of MAPKs (ERK and JNK) in DKO and TKO T cells. Ab-mediated cross-linking of CTLA-4 has previously been reported to inhibit ERK and JNK (18). ERK1/2 activity was measured in an in vitro kinase assay using MBP as the substrate. DKO and TKO T cells were incubated with APCs for 5, 15, and 30 min (Fig. 1A). Under these conditions, T cells from TKO mice showed significantly higher levels of ERK activity than T cells from DKO mice (Fig. 1A, lanes 7–9 vs 4–6), indicating that the engagement of CTLA-4 by CD80/CD86 inhibited ERK activation. Therefore, this inhibition was similar to that observed using Ab to cross-link CTLA-4 (18). Although the overall level of activity between DKO and TKO T cells differed, the time course of activation was the same in both sets of cells. Anti-ERK immunoblotting confirmed that the same amount of protein was added at the indicated time points (Fig. 1A, lower panel). As an additional control, proliferation of TKO T cells was consistently higher than observed in DKO T cells (Fig. 1B).

Unlike ERKs, JNKs represent a subfamily of serine/threonine kinases that are activated in response to stress-related stimuli such as UV light, gamma radiation, and IL-1 (24). CD28 serves as a key coreceptor in TCR-mediated activation of JNK (25, 26). Therefore, purified DKO and TKO T cells were compared for TCR-induced

CD4+ T cells from DKO and TKO were cocultured with APCs (ratio 1:10), stimulated with anti-CD3 (1/1000 dilution) for 0, 5, 15, 30, and 60 min at 37°C. Cells were lysed, immunoprecipitated with ERK1/2 or JNK mAbs and subjected to an in vitro kinase assay as described (18). Sequential immunoprecipitations of ERK and JNK were conducted to compare kinase activities from the same cell cultures.

ERK and JNK assays

CD4+ T cells from DKO and TKO were cocultured with APCs (ratio 1:10), stimulated with anti-CD3 (1/1000 dilution) for 0, 5, 15, 30, and 60 min at 37°C. Cells were lysed, immunoprecipitated with anti-JNK-1 mAb, and subjected to an in vitro kinase assay. Lanes 4–6, ERK activity in DKO T cells; lanes 7–9, ERK activity in TKO T cells; lanes 1–3, ERK activity in WTAPCs, DKO, and TKO T cells alone. Lower panel, Equal amounts of cell lysates were immunoblotted for JNK-1.
JNK activation. DKO T cells showed significantly higher levels of JNK activity (average twice) than TKO cells (Fig. 2A, upper panel, lanes 4–7 vs 8–11). Importantly, JNK and ERK assays were conducted as sequential precipitations using the same cell lysates. Similar differences were noted with the preferential precipitation of JNK2 (data not shown). By contrast, resting T cells (i.e., the absence of APCs and anti-CD3, or cocultured with APCs plus anti-CD3 at time point zero) showed no activity (lanes 2 and 3). In addition to stimulation, CTLA-4 helped to sustain the heightened response. Although the activity in the TKO T cells returned to background levels by 30–60 min, the activity of JNK in DKO T cells was stable for 60 min (Fig. 2A, right panel). Anti-JNK immunoblotting of cell lysates showed the same amount of JNK protein loaded at each time point (Fig. 2A, lower panel). To rule out a possible contribution of JNK activity from APCs, APCs exposed to CTLA-4 Ig (i.e., to ligate CD80/86) were also examined. As seen in Fig. 2B, the low activity in these cells changed only marginally over 60 min in the presence of CTLA-4 Ig (lanes 1–5) and was much lower than seen with the combination of T cells and APCs (Fig. 2, B vs A). Therefore, our findings demonstrate that engagement of CTLA-4 by natural ligand potentiates and maintains JNK activation in the context of APCs and TCR ligation.

CD80 and CD86 have been reported to differentially bind to CTLA-4 (4). To assess whether CD80 and CD86 might differentially activate JNK, cells were cocultured with APCs expressing either CD86 (i.e., from CD80 KO mice) or CD80 (i.e., from CD86 KO mice). Under these conditions, both sets of APCs were found to potentiate JNK activity (Fig. 3, A and B, respectively). The main difference was in the time course of activation. Singly-expressing APCs showed a delayed onset of peak kinase activity (Fig. 3, A and B, right panel), a result likely related to the lower overall levels of CD80 and CD86 on these cells. In this instance, JNK activity in the DKO T cells usually peaked at 15–30 min, rather than 5 min (Fig. 3 vs Fig. 2A). However, over three experiments, no reproducible differences were observed between the different singly-expressing APCs. As a control, an anti-JNK immunoblot showed similar levels of JNK expression (Fig. 3, A and B, lower panel). Our finding indicates that CD80 and CD86 are each capable of activating JNK.

The potentiation of JNK activity suggests that CTLA-4 might differentially regulate transcription factors, an event that could contribute to the negative regulation of immune function. Of particular interest was TGF-β, a cytokine that is up-regulated by CTLA-4 (12, 13). To test whether CTLA-4 and JNK activity can alter TGF-β production, preactivated peripheral T cells were transfected with a constitutively active form of JNK (JNKK2-JNK1 fusion protein) and assessed for alterations in TGF-β production.

**FIGURE 3.** A, JNK activity in DKO and TKO T cells cocultured with CD86-expressing APCs. Cells were activated as described above for the indicated periods of time. Cells were lysed, immunoprecipitated with anti-JNK-1 mAb, and subjected to an in vitro kinase assay. Lanes 5–7, JNK activity in DKO T cells; lanes 8–10, JNK activity in TKO T cells; lanes 1–4, JNK activity in CD86, CD80 APCs, DKO, and TKO T cells alone. Lower panel, Equal amounts of cell lysates were immunoblotted for JNK-1. Right panel, Histogram depiction of the levels of JNK activity as detected by densitometric reading. B, JNK activity in DKO and TKO T cells cocultured with CD80-expressing APCs. Cells were lysed, immunoprecipitated with anti-JNK1 mAb, and subjected to an in vitro kinase assay. Lanes 1–3, JNK activity in DKO T cells; lanes 4–6, JNK activity in TKO T cells. Lower panel, Equal amounts of cell lysates were immunoblotted for JNK-1. Right panel, Histogram depiction of the levels of JNK activity as detected by densitometric reading.

**FIGURE 4.** Constitutively active JNK fails to influence TGF-β production in PBLs. PBLs transfected with mock (●) or constitutively active JNK (●) were stimulated with plate-bound anti-CD3, anti-CD3/CTLA-4, anti-CD3/CD28 and anti-CD3/CD28/CTLA-4 mAbs. After a 72-h stimulation, supernatant was taken and TGF-β production was measured by ELISA. HA, hemagglutinin.
Cells were stimulated with plate-bound combinations of anti-CD3, anti-CD3/CD28, anti-CD3/CTLA-4, and anti-CD3/CD28/CTLA-4 mAbs. Under these conditions, anti-CD3/CTLA-4 and anti-CD3/CD28/CTLA-4 ligation increased TGF-β production when compared with cells stimulated with anti-CD3 and anti-CD3/CD28 (Fig. 4). However, JNK/2-JNK/1 overexpression failed to potentiate TGF-β production when compared with vector-transfected cells. Expression of JNK/2-JNK/1 fusion protein was shown in an anti-hemagglutinin immunoblot (Fig. 4). Therefore, although JNK can phosphorylate and activate transcription factors such as c-jun (24), the pathway does not appear to account for CTLA-4 up-regulation of TGF-β.

In summary, our findings indicate that CTLA-4 cosignaling is more complex than previously appreciated with an ability to differentially regulate members of the MAPK family in T cells. The advantage of our system is the ability to use freshly purified CD4+ T cells that differ from each other only in the context of CTLA-4 expression. Further, with the absence of CD80/86, it is possible to present CD80 and/or CD86 on APCs de novo in the context of TCR-mediated stimulation. With this, DKO CD4+ cells (i.e., expressing CTLA-4) showed an activation of JNK concurrent with the inhibition of ERK when compared with cells lacking CTLA-4 (i.e., TKO). The same cell lysates were used in both ERK and JNK assays. With the caveat that T cells from TKO vs DKO mice have inherently different mechanisms of JNK regulation, our findings support a model where CTLA-4 engagement can differentially regulate ERK/JNK. In this context, thymic differentiation has been found to be comparable in CTLA-4-positive and -negative mice (27, 28).


