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Inducible costimulator facilitates T-dependent B cell activation by augmenting IL-4 translation

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ABSTRACT

The inducible costimulator (ICOS) is highly expressed in follicular helper T (Tfh) cells, a subset of CD4 T cells that migrate into the B cell zone and facilitate germinal center reactions. Although ICOS is known to play a critical role in forming the Tfh cell population during immune responses, its contribution to the effector functions of Tfh cells remains unclear. Using activated mouse splenic CD4 T cells we demonstrate that ICOS assists TCR-mediated signal transduction by potentiating the PI3K-AKT-mTOR signaling cascade that leads to hyper-phosphorylation of p70S6K and 4E-BP1, events that are known to augment cap-dependent mRNA translation. Consequently, ICOS costimulation promotes the formation of polysomes on IL-4 mRNA in a PI3K-dependent manner. Furthermore, we show that the supply of IL-4 becomes a limiting factor for T-dependent B cell activation during *in vitro* co-culture when the ICOS-PI3K signaling axis is disrupted in T cells. This ICOS costimulation-dependent translational control may ensure targeted delivery of IL-4 to cognate B cells during T–B collaborations in the germinal center.

1. Introduction

ICOS is a member of the CD28 family of T cell costimulatory receptors (Hutloff et al., 1999; Greenwald et al., 2005). Unlike CD28, however, ICOS is induced in CD4 and CD8 T cells after T cell activation, and is highly enriched in effector/memory T cells. ICOS binds to ICOS ligand (ICOSL), which is expressed in APCs, including B cells, as well as in nonlymphoid cells (Swallow et al., 1999). *In vitro* experiments have shown that, in general, ICOS plays similar costimulatory functions to CD28 – enhancement of T cell proliferation, cytokine production, and survival of activated T cells (Watanabe et al., 2005; Bonhagen et al., 2003; Hutloff et al., 1999). Nonetheless, ICOS has a crucial, non-redundant role *in vivo*, as one of its most important contributions is the ability to support the generation of follicular helper T (Tfh) cells, a subset of CD4 cells that migrate to the B cell zone and facilitate germinal center (GC) reactions in peripheral lymphoid organs (Vinuesa and Cyster, 2011). Thus, in both humans and mice, ICOS-deficiency causes defects in Tfh cell generation, GC reactions, and antibody production (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Warnatz et al., 2006).

Although ICOS can potentiate T cell receptor (TCR)-mediated phosphoinositide 3-kinase (PI3K) activation and Ca^{2+} mobilization, the molecular mechanisms and the *in vivo* roles of each signaling component are not fully understood. To address these issues, we generated a knock-in strain of mice that possesses a Tyr-to-Phe mutation at Tyr181 in the context of the YMFM PI3K signaling motif in the cytoplasmic tail of ICOS, termed ICOS-YF mice (Gigoux et al., 2009). This mutation abrogates ICOS's ability to bind to PI3K, without affecting cell surface expression patterns and its ability to induce Ca^{2+} flux. Importantly, mice expressing ICOS-YF had defects in Tfh cell generation, GC reactions, antibody class switching, and antibody affinity maturation, similar to mice that are deficient in ICOS (ICOS-KO). Congruent to our results, T cell-specific deletion of the PI3K catalytic subunit p110δ also abrogated Tfh cell generation (Rolf et al., 2010). Thus, ICOS-mediated PI3K signaling plays a critical role in facilitating Tfh cell generation. We and others have found that ICOS costimulation enhances TCR-mediated expression of key cytokines that facilitate T cell growth (e.g., IL-21) and B cell expansion (e.g., IL-4) through PI3K *in vitro* (Gigoux et al., 2009; Rolf

Abbreviations: AID, activation induced cytosine deaminase; BiP, binding immunoglobulin protein; eIF4E, eukaryotic initiation factor 4E; 4E-BP, eIF4E-binding protein; IRES, internal ribosome entry site; mTOR, mammalian target of rapamycin; S6K, ribosomal protein S6 kinase; Tfh, follicular B helper T cells.

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et al., 2010). Recent work also shows that TCR-independent ICOS-PI3K signaling during T cell interactions with non-cognate B cells in the T–B border facilitates T cell migration into the B cell follicle *in vivo* (Xu et al., 2013). Therefore, ICOS is thought to promote Tfh cell generation by guiding Tfh precursor cells into the B cell area and fostering T–B collaboration once cognate T–B conjugates are formed. However, if and how ICOS promotes the effector function of mature Tfh cells are yet to be tested.

Recent evidence indicates that the transcription and translation of the IL-4 gene is segregated by time and anatomical location of helper T cells (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). CD4 T cells possessing IL-4 mRNA, without secreting IL-4, appear early after T cell priming in the T cell zone of the secondary lymphoid organ. However, CD4 T cells that gain the capacity to secrete IL-4 proteins appear only at the later phase of an immune response in the B cell zone, particularly in GCs. These IL-4 secreting cells have been shown to be Tfh cells based on molecular markers and the fact that they are often found in conjugation with cognate B cells with signs of Ab class switching (Reinhardt et al., 2009). Presumably, this tight regulation of IL-4 secretion might allow targeted delivery of IL-4 from Tfh cells to cognate B cells during T cell–B cell interactions. These data also suggest that there might be a T cell–B cell communication mechanism that acutely elevates IL-4 protein synthesis in Tfh cells. Although restimulation of primed CD4 T cells with TCR ligation alone can augment IL-4 protein synthesis *in vitro* (Reinhardt et al., 2009), whether or not there is an additional requirement for costimulatory signals *in vivo* is unknown.

The translation of eukaryotic mRNA is predominantly regulated at the level of initiation (Sonenberg and Hinnebusch, 2009). Mature mammalian mRNA species have post-transcriptional modifications at both ends: the 5′-cap (7-methyl guanylation) and the 3′ polyadenylation tail. For the initiation of translation, the mRNA should be activated by eukaryotic initiation factors (eIFs) and then subsequently loaded with the 40S ribosome subunit charged with methionyl tRNA (Met-tRNAi). Both the mRNA activation step and the formation of Met-tRNAi pools are highly regulated such that they can be suppressed by nutrient starvation or stress. However, cellular mRNA species possessing internal ribosome entry site (IRES) are able to maintain their translation in the face of general translational inhibition (Johannes and Sarnow, 1998). Importantly, in resting T cells that have been previously activated, translational initiation machineries are predominantly suppressed (Scheu et al., 2006). These suppression mechanisms can be released when primed T cells receive strong TCR signals, forming the molecular basis of spatio-temporal segregation of cytokine transcription and translation (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009).

The Ser/Thr kinase mTOR plays a central role in the regulation of mRNA translation in mammalian cells (Ma and Blenis, 2009; Livingstone et al., 2010). Normally, mTOR is activated when cells are exposed to sufficient nutrients (especially amino acids), energy (ATP), and growth factors/mitogens. Activated mTOR phosphorylates downstream translational regulators including p70S6K (S6K) and eIF4 binding proteins (4E-BPs). The S6K is activated by phosphorylation and in turn phosphorylates multiple downstream targets involved in translation initiation such as eIF4B (Ma and Blenis, 2009). On the other hand, 4E-BPs are inactivated by a series of phosphorylation events to allow its target, eIF4E, to recruit eIF4G and other translational initiation factors to the m^7G 5′-cap structure of mRNA (Ma and Blenis, 2009). These biochemical events overcome translational repression typically imposed by the 5′ untranslated region (5′ UTR) of the mRNAs leading to augmented translational initiation and accumulation of polysomes, mRNA species actively translated by ribosomes. Importantly, one of the key upstream stimulators of mTOR is the PI3K-AKT signaling pathway that is activated by growth factors and, in activated T cells, by ICOS.

We therefore hypothesize that ICOS may promote IL-4 synthesis in Tfh cells during T cell–B cell interactions by activating the PI3K-Akt-mTOR signaling cascade. Here, we provide biochemical evidence that the ICOS-PI3K signaling axis potentiates the TCR-mediated mTOR signaling pathway, culminating in the increase of polysome formation on IL-4 mRNA. Consistent with this, IL-4 is a limiting factor in T-dependent B cell responses when ICOS-PI3K signaling is impaired. These results support the importance of mTOR in ICOS-mediated translational control of IL-4 expression in the context of T–B collaborations during GC reactions.

2. Materials and methods

2.1. Animals

ICOS-YF knock-in ($Icos^{yf/yf}$) or ICOS-KO ($Icos^{-/-}$) mouse strains were previously described (Tafuri et al., 2001; Gigoux et al., 2009). C57BL/6 and OT-II transgenic mice were purchased from Jackson Laboratory. The BAC transgenic AID-GFP reporter mice were provided by Dr. R. Casellas (NCI, Bethesda, USA) (Crouch et al., 2007). All the mice were in C57BL/6 background (minimum N10) and were housed in the IRCM Animal Facility under specific pathogen-free conditions. Animal experiments were performed according to animal use protocols approved by the IRCM Animal Care Committee.

2.2. Antibodies

Functional grade purified anti-CD3 (145.2C11), CD28 (37.51), ICOS (C398.4A) and control Armenian hamster IgG, as well as staining anti-CD4-PECy7 and CD19-APC were purchased from eBioscience. Goat anti-Armenian hamster antibody was from Jackson Immunoresearch. 7-AAD was purchased from BD Pharmingen. Immunoblot antibodies against AKT, pAKT (S473), 4E-BP1, p4E-BP1 (S65), pS6K (T389), and S6K were purchased from Cell Signaling. HRP-conjugated goat anti-rabbit antibody was purchased from Bio-rad.

2.3. In vitro CD4 T cells activation and restimulation

CD4 T cells were isolated by negative selection from splenocytes using CD4 T cell Isolation Kit II (Miltenyi) according to the manufacturer's instructions. T cells were cultured in RPMI1640 medium supplemented with 10% FBS, glutamine, penicillin, streptomycin, β-mercaptoethanol and HEPES. Purified CD4 T cells were activated by culturing with plate-bound anti-CD3 (3 μg/mL) and soluble anti-CD28 (2 μg/mL) for 2 days and were subsequently expanded in media containing 100 U/mL IL-2 (Peprotech) for 3 days. For restimulation experiments, CD4 T cell blasts were harvested and incubated for 1 min at room temperature with primary antibodies: anti-CD3 (1 μg/mL) plus anti-ICOS (2 μg/mL) or anti-CD3 plus hamster IgG (2 μg/mL) as control. Immediately after addition of secondary Ab (goat anti-hamster IgG, 20 μg/mL), the cells were transferred to a water bath at 37 °C and incubated up to 20 min depending on experimental settings.

2.4. Immunoblot analysis

Restimulation was stopped by adding ice-cold PBS with 10% FBS, 1 mM Na_3VO_4, 1 mM EDTA pH 8.0. Cells were lysed in TNE lysis buffer (1% NP-40 in 50 mM Tris, pH 7.5, 2 mM EDTA, pH 8.0, 5 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 5 mM NaF, 150 mM NaCl, and protease inhibitor cocktail (Sigma)) for 20 min on ice. Cell debris was removed by centrifugation at 16,000 × *g* for 30 min and the