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ZAP70-Related SCID: Non-Redundant Dual Functions of the ZAP70 Catalytic and Scaffolding Regions

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Primary Severe Combined Immunodeficiency (SCID) is a form of heritable immunodeficiency, characterized by impaired adaptive immune responses [1]. It includes a group of genetic disorders originated by defects in one of several different genes that are critical for T lymphocyte production and/or function and involve defects in B lymphocytes as a primary or secondary cause [2].

The most common type of SCID is linked to the X chromosome (X-SCID), and therefore affects only males [3,4]. The X-SCID males possess mutations in the interleukin-2 (IL-2) receptor gamma chain gene (IL-2R) encoding a protein that is shared by at least six different receptors for interleukins, including IL-2 and IL-4. These receptors direct the development and maturation of T and B lymphocyte subtypes and help regulate the entire adaptive immune system.

Other forms of SCID follow an autosomal recessive inheritance pattern that affects males and females equally. Among them, a relatively rare genetic disorder is caused by mutations in a gene encoding the T cell antigen receptor (TCR)/CD3-zeta chain (CD3ζ) associated protein of 70 kDa (termed ZAP70) [5-7], which is reflected by abnormal TCR signaling.

ZAP70-related SCID was first described in a patient of Mennonite descent with $CD8^+$ lymphocytopenia and normal numbers of $CD4^+$ T cells that did not proliferate in response to mitogenic stimulation [5-8]. A variety of distinct mutations in *ZAP70* were identified in different patients, where the type of mutation determined the severity of the immunodeficiency [9,10]. Affected children present in the first year of life with recurrent bacterial, viral, and opportunistic infections, do not usually survive past their second year without hematopoietic stem cell transplantation.

ZAP70 is a non-receptor protein tyrosine kinase (PTK) that plays a critical role in TCR-linked signal transduction leading to T cell differentiation and maturation and acquisition of effector function. It was initially identified as a tyrosine phosphorylated protein with a molecular mass of 70 kDa that associates with the CD3ζ chain of activated T cells [11,12]. The transient binding of ZAP70 to CD3ζ involves the ZAP70 tandem SH2 domains, which directly interact with phosphorylated tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3ζ subunit [13].

The CD3ζ cytoplasmic tail possesses three copies of ITAMs, each containing two tyrosine residues critical for its function. TCR engagement by peptide-bound MHC molecules expressed on the surface of antigen-presenting cells (APC) stimulates Lck, a member of the Src family of PTKs, to phosphorylate the CD3ζ-ITAM-tyrosine residues. The phosphorylated ITAMs serve as high affinity binding sites for ZAP70, thereby enabling its recruitment to T cell-APC contact area, also termed the immunological synapse [14-17]. The tyrosine phosphorylated CD3ζ-bound ZAP70 undergoes phosphorylation by Lck as well as autophosphorylation on specific tyrosine residues, which alter its conformation and convert it into an active enzyme [18]. The activated ZAP70 then phosphorylates specific downstream molecules [19-22], leading to calcium mobilization, activation of Ras GTPase and rearrangement of the actin cytoskeleton. These transient intracellular signals permit the activation of selected transcription factors that promote the proliferation and differentiation of T cells.

Analysis of the phosphorylation sites of ZAP-70 and their impact on the function of the molecule demonstrated that phosphorylation of Tyr319, Tyr492 and Tyr493 serves to regulate the ZAP70 catalytic activity [23-25]. However, *in vivo* activation of T cells results in ZAP70 phosphorylation on additional tyrosine residues [26,27] which may also function as putative docking sites for SH2-containing proteins. ZAP70 Tyr315 was reported to serve as a binding site for members of the Crk family of adaptor proteins [27,28]. Furthermore, the CrkII-SH3 domain can interact with a variety of proteins that possess poly-proline rich regions [29], thereby recruiting additional effector molecules to the site of the engaged TCR. One CrkII-SH3 binding partner is the guanine nucleotide exchange factor, C3G, which among other molecules, regulates the GTPase Rap1 [30-32]. Activation of Rap1 increases the inside-out signaling pathway that relays signals to the integrin LFA-1, increasing its affinity to ICAM1, there by augmenting LFA-1/ICAM-1-mediated T cell adhesion.

The above information and additional published studies indicated that ZAP70 possesses a catalytic region and a separate protein-protein interaction (or scaffolding) region. Since distinct protein modules within a single molecule might operate in a cooperative manner, or independent of each other [33], it was of interest to decipher the relationships between these two regions within the ZAP70 molecule.

A recent study by Au-Yeung et al. [34] has solved this question by showing that under certain conditions, the ZAP70 protein exhibits biological functions in regulatory T cells (T_{reg}) that are independent of the ZAP70 catalytic activity.

To investigate the role of catalytically inactive ZAP70 in normal naïve and mature T cells, the authors have utilized a *ZAP70*-deficient mouse line in which they expressed a *ZAP70* mutant transgene that retains catalytic activity yet can also be inhibited by a PP1 inhibitor analog, termed 3-MB-PP1, a small-molecule kinase inhibitor [35]. The mutated analog-sensitive *ZAP70* protein, termed *ZAP70* (AS), possesses a methionine-to alanine substitution at position 414 in the ATP-binding domain, which allows it to accommodate the 3-MB-PP1 inhibitor. In addition, the 3-MB-PP1 molecule was found to specifically

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inhibit the ZAP70 (AS) catalytic activity, and did not affect wild-type ZAP70 or other Src- or Tec-family PTKs [36].

When testing early activation events in *ZAP70*(AS) and control *ZAP70*$^{+/-}$ CD4$^+$ T cells stimulated by TCR/CD3 crosslinking, both cell types responded by a robust increase in the intracellular free Ca^{2+} concentration [Ca^{2+}] and phosphorylation and activation of the Erk MAPK. However, when TCR/CD3 crosslinking antibodies were added to the cultured cells together with the 3-MB-PP1 inhibitor, a dose-dependent inhibition of Ca^{2+} mobilization and Erk activation was observed in *ZAP70*(AS), but not in *ZAP70*$^{+/-}$ CD4$^+$ T cells. In addition, *ZAP70*(AS) and control *ZAP70*$^{+/-}$ CD4$^+$ T cells responded to TCR/CD3 plus CD28 crosslinking by strong proliferation, while *ZAP70*(AS) but not *ZAP70*$^{+/-}$ CD4$^+$ T cell proliferation was inhibited by 3-MB-PP1. These results suggested that the catalytic activity of ZAP70 is required for both early and late activation of mature naïve CD4$^+$ T cells.

Similar studies performed in antigen-primed T cells demonstrated that interferon γ (IFN-) and IL-10 (IL-10) cytokine production by antigen-challenged T_H1 and T_H2 CD4$^+$ cells, respectively, require the catalytic activity of ZAP70. Furthermore, ZAP70 catalytic activity was required for the cytotoxic activity of primed alloreactive CD8$^+$ T cells, and for their ability to respond in tumor necrosis factor (TNF) production.

A surprise came when the requirement for the ZAP70 catalytic activity was tested in the suppressive response of CD4$^+$CD25$^+$ T_{reg} cells [34]. In this assay, Au-Yeung et al. [34] have shown that TCR/CD3-stimulated *Zap70*(AS) T_{reg} cells that are cocultured with *Zap70*$^{+/-}$ CD4$^+$ CD25$^-$ conventional T cells (T_{conv} cells) have led to suppression of the proliferative response of *Zap70*$^{+/-}$ T_{conv} cells. However, when the suppressive activity of *Zap70* (AS) T_{reg} cells was tested, the cells exhibited a similar inhibition of proliferation of *Zap70*$^{+/-}$ T_{conv} cells in the absence but also in the presence of 3-MB-PP1, even when using 3-MB-PP1 concentrations that inhibited the anti-TCR/CD3-induced proliferation of *Zap70* (AS) T_{conv} cells. Although the TCR/CD3-induced increase in [Ca^{2+}] and Erk activation in *Zap70*(AS) T_{reg} cells were sensitive to suppression by 3-MB-PP1, the activation-induced phosphorylation of Tyr319 and Tyr493 were not affected, apparently because they serve as substrates for phosphorylation by Lck, which is insensitive to 3-MB-PP1.

The results by Au-Yeung et al. [34] support previous findings showing that tyrosine phosphorylated ZAP70 can act as a scaffolding protein [27, 28, 37, 38], and suggest that this activity is independent of the ZAP70 catalytic activity.

Au-Yeung et al. [34] have further demonstrated that CrkII and C3G coimmunoprecipitate with activated ZAP70 from *Zap70* (AS) T_{reg} cells, even in the presence of 3-MB-PP1, and that activation of the downstream effector molecules, C3G and Rap1, was also independent of the catalytic activity of ZAP70 [38]. Furthermore, TCR/CD3-induced adhesion of *Zap70* (AS) CD4$^+$ T cells to ICAM-1-coated surface was not sensitive to inhibition by 3-MB-PP1, suggesting that the TCR/CD3-induced inside-out signaling that regulate T cell adhesion requires the scaffolding function of ZAP70, but is independent of the ZAP70 catalytic activity. The results indicate that ZAP70 is a candidate drug target in autoimmune diseases and allograft rejection, and suggest that different ZAP70-specific drugs may vary in their ability to modulate responses of distinct T cell subtypes.

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