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Short communication

Dynamics of CD3γε and CD3δε dimer expression during murine T cell development

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ABSTRACT

The preTCR, γδTCR, and αβTCR are the three isoforms of the T cell antigen receptor that are expressed during thymocyte development. Signaling by these isoforms is required at different stages of T cell development for lineage commitment, thymocyte maturation, and repertoire selection. All three isoforms are multimeric complexes, which are dependent on invariant CD3 dimers (CD3γε and CD3δε) and TCRζζ dimers for their assembly, stable surface expression and signal transduction. Notably, differences have been reported regarding the requirement for CD3δ in the assembly, surface expression and signaling abilities of the three TCR isoforms. Specifically, it has been shown that both the preTCR and γδTCR do not require CD3δ to transduce signals, whereas the αβTCR does. The differences noted between the murine αβ- and γδTCRs in their requirement for CD3δ can be easily explained by the fact that CD3δ is a component of the αβTCR but not the γδTCR. However, it is not clear why the preTCR does not require CD3δ, considering that CD3δ has been reported to be a subunit of the preTCR. Because the preTCR can be expressed on thymocytes at the immature $CD4^-CD8^-$ stage and, to a lesser extent, at the later $CD4^+CD8^+$ stage, it is conceivable that CD3δε dimer expression is developmentally regulated during early T cell development such that preTCRs expressed on immature $CD4^-CD8^-$ thymocytes contain primarily CD3γε dimers while those expressed on $CD4^+CD8^+$ thymocytes express both CD3δε and CD3γε dimers. To investigate this, we determined whether the expression of CD3δ and CD3γ are developmentally regulated and whether there are differences in the availability and/or stability of CD3δε and CD3γε dimers during early T cell development. We report that even though both CD3δ and CD3γ were expressed at relatively high levels in immature $CD4^-CD8^-$ thymocytes, CD3γε dimers predominated over CD3δε dimers at this early stage. However, expression of CD3δε dimers was rescued when pTα, TCRβ and TCRα chains were also expressed at the $CD4^-CD8^-$ stage, indicating that the relative amounts of pTα, TCRβ and TCRα chains during early thymocyte development control the stability and, therefore, availability of CD3δε dimers.

1. Introduction

The development of T cells is dependent on the signaling capacity of a fully assembled antigen receptor. There are three isoforms of the T cell antigen receptor (TCR) that are expressed during thymocyte development. Two of the three isoforms are mature TCRs, in that they are also expressed on functional T cells residing in the periphery. Both of these isoforms, the αβTCR and the γδTCR, are comprised of an antigen-binding clonotypic heterodimer (TCRαβ or TCRγδ) and the signal-transducing invariant CD3ε, CD3γ, CD3δ and TCRζ chains, and it is the expression of the αβTCR and γδTCR that defines the αβ and γδ T cell lineages, respectively. The third isoform, which is known as the preTCR, is similar to the αβTCR in composition except that it expresses the invariant preTα (pTα) chain in place of the TCRα chain. The expression of this isoform on immature thymocytes serves to test whether the TCRβ chain is productively rearranged (termed β selection), promotes transition of thymocytes from the $CD4^-CD8^-$ (double negative; DN) to the $CD4^+CD8^+$ (double positive; DP) stage, and is believed to denote cells choosing the αβ lineage.

The invariant CD3 and TCRζ chains play a role not only in TCR signal transduction but also in TCR assembly and surface expression (reviewed in Hayes et al., 2003). TCR assembly starts in the endoplasmic reticulum (ER), where all TCR subunits are synthesized and CD3γε, CD3δε and TCRζζ dimers are formed (Alarcon et al., 1988; Bonifacino et al., 1988; Klausner et al., 1990). The CD3 dimers then pair with individual clonotypic TCR chains (i.e., TCRα, -β, -γ, -δ) or pTα via oppositely charged residues in their respective transmembrane domains prior to formation of the disulfide linkage between the two appropriate TCR chains (Alarcon et al., 1988; Klausner et al., 1990; Saint-Ruf et al., 1994; Kearse et al., 2005). TCRζζ is added as the final component of the TCR complex in the ER before the

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entire complex is transported to the Golgi compartment. It is in the Golgi where the carbohydrate moieties on the glycosylated subunits are processed before transportation of the fully assembled TCR complex to the cell surface (reviewed in Klausner et al., 1990). Studies using cell lines, as well as mice with null mutations in the genes encoding the invariant TCR subunits, demonstrate that loss of any one of these subunits results in either undetectable or unstable TCR isoform expression (reviewed in Hayes et al., 2003). Without stable expression *in vivo*, TCR signaling is impaired and profound effects on T cell development ensue (Love et al., 1993; Liu et al., 1993; Malissen et al., 1993, 1995; Dave et al., 1997; DeJarnette et al., 1998; Haks et al., 1998).

There are notable differences among the TCR isoforms in their requirement for CD3δ for assembly, surface expression and signaling ability. It has been shown that αβ T cell development but not γδ T cell development is defective in CD3δ-deficient mice (Dave et al., 1997). This difference between murine αβ and γδ T cells in their requirement for CD3δ is due to the fact that αβTCRs contain CD3δε dimers while γδTCRs do not (Hayes and Love, 2002). As a consequence, the αβTCR fails to be stably expressed on CD3δ$^{-/-}$ thymocytes, resulting in a block in αβ T cell development at the DP stage (Dave et al., 1997). Interestingly, CD3δ-deficient mice have wild-type numbers of DP thymocytes, indicating that the DN to DP transition is not compromised in these mice (Dave et al., 1997; Berger et al., 1997). As assembly, surface expression and signaling by the preTCR are required for the DN to DP transition, these findings suggest that CD3δ may not be a component of the preTCR. Biochemical analyses of the subunit composition of preTCR complexes expressed on thymic lymphomas, which were transformed at different developmental stages, do not provide a solution to this issue, as their results are conflicting regarding whether CD3δ is indeed a component of the preTCR (Groettrup et al., 1993; Mombaerts et al., 1995; Jacobs et al., 1996). However, in the one study that analyzed the subunit composition of preTCR complexes expressed on *ex vivo* TCRα$^{-/-}$ thymocytes, CD3δ is in fact found to be physically associated with the preTCR (Berger et al., 1997). It is important to note that this latter study used unfractionated TCRα$^{-/-}$ thymocytes as the source of preTCR^{+} cells. Because >95% of TCRα$^{-/-}$ thymocytes are DP, it is conceivable that the preTCRs containing CD3δε dimers represent those expressed on DP thymocytes rather than those expressed on DN thymocytes. If CD3δε dimers are limiting at the DN stage but not the DP stage, then this could provide an explanation for not only the contradictory reports concerning the subunit composition of the preTCR expressed on the various thymic lymphomas but also why CD3δ appears to be dispensable for preTCR assembly, surface expression and function.

In the current study, we sought to determine whether the expression of CD3δ and CD3γ are developmentally regulated and/or whether there are differences in the availability and/or stability of CD3δε and CD3γε dimers during early T cell development. Here, we report that all CD3 subunits were expressed in high levels at the DN stage compared to their levels at the DP stage. Despite the high level of expression of both CD3δ and CD3γ in DN thymocytes, CD3γε dimers predominated over CD3δε dimers at this early stage. Rescue of CD3δε dimers, however, was observed when the preTCR and/or αβTCR were expressed, indicating that CD3δε dimers are unstable unless they are incorporated into a fully assembled preTCR and/or αβTCR. This finding is consistent with those from *in vitro* studies demonstrating the selective degradation of CD3δ in the absence of one or more of the other TCR subunits (Lippincott-Schwartz et al., 1988; Chen et al., 1988; Bonifacino et al., 1989). Taken together, the data show that it is the expression levels of pTα, TCRβ and TCRα chains during early thymocyte development that control CD3δε dimer stability and availability.

2. Materials and methods

2.1. Mice

C57BL/6J (B6), B6; SJL-Tg(TcrAND)53Hed/J (AND TCR Tg) and B6.129S2-*Tcra*^{tm1Mom}/J (TCRα$^{-/-}$) mice were purchased from the Jackson Laboratory. B6.129S6-*Rag2*^{tm1Fwa} (RAG$^{-/-}$) and B6.129-*H2-Ab1*^{tm1Gru}*B2m*^{tm1Jae} (MHC$^{-/-}$) mice were purchased from Taconic Farms. C57BL/6-*Cd3d*^{tm1} (CD3δ$^{-/-}$) mice were provided by D. Kappes (Fox Chase Cancer Center, Philadelphia, PA) and 129-*Cd3g*^{tm1} (CD3γ$^{-/-}$) mice were provided by D. Wiest (Fox Chase Cancer Center, Philadelphia, PA). B6.129-*Cd3e*^{tm1} (CD3ε$^{-/-}$) and B6.129-*Lat*^{tm1} (LAT$^{-/-}$) mice were provided by P. Love (NIH, Bethesda, MD). All mice used in this study were bred and maintained in the Department of Laboratory Animal Resources at SUNY Upstate Medical University in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. Mouse protocols were approved by the SUNY Upstate Medical University Committee on the Humane Use of Animals. Mice were sacrificed at 6–8 weeks of age, except RAG$^{-/-}$ mice, which were sacrificed at 4 weeks of age.

2.2. Antibodies and reagents

Monoclonal antibodies (mAbs) used for flow cytometric analysis, cell sorting and immunoprecipitations included anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-TCRγδ (UC7-13D5), anti-TCRβ (H57-597), anti-CD3γε/δε (145-2C11), anti-CD19 (1D3), anti-CD25 (PC61), anti-CD44 (IM7), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-Vα11 (RR8-1), and anti-Vβ3 (KJ25), which were purchased from BioLegend, eBiosciences and BD Pharmingen. Antibodies (Abs) used for Western blot analysis were goat anti-CD3ε (Santa Cruz Biotechnology, Inc.), rabbit anti-CD3δ (R9) (provided by L. Samelson, NIH, Bethesda, MD), HRP-conjugated Protein A (Transduction Laboratories), and HRP-conjugated donkey anti-goat IgG (H + L) (Jackson ImmunoResearch Laboratories). The anti-CD3γε hybridoma (7D6) (Coulie et al., 1991) was obtained from D. Wiest and A. Singer (NIH, Bethesda, MD) and was used to produce ascites. Purified anti-CD3γε mAb was conjugated to the fluorochrome Alexa Fluor 488 (Invitrogen) according to the manufacturer's instructions. Alexa Fluor 488-conjugated streptavidin was purchased from Invitrogen. Lastly, Protein A-Sepharose, which was used for all immunoprecipitations, was purchased from Sigma.

2.3. Immunoprecipitations and Western blot analysis

Thymocytes were lysed in a buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, protease inhibitors (Roche Laboratories), and 1% Brij97 (Aldrich). Lysates were then subjected to three rounds of immunoprecipitation with the anti-CD3γε (7D6) mAb followed by one round of immunoprecipitation with the anti-CD3γε/δε (145-2C11) mAb. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes and blotted with Abs against CD3ε and CD3δ.

2.4. Flow cytometric analysis

Flow cytometric analysis for surface antigens was performed as previously described (Laird and Hayes, 2009). Intracellular staining for CD3 dimers was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Surface staining for CD3 dimers on immature DN thymocytes from TCRα$^{-/-}$ mice was performed using an enzymatic amplification staining kit (Flow-Amp Systems) according to the manufacturer's instructions. Surface staining for CD3 dimers on thymocytes from