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Review

Doubting the TCR Coreceptor Function of CD8αα

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"The beginning of wisdom is found in doubting; by doubting we come to question, and by seeking we may come upon the truth." – Pierre Abélard

CD8 is a glycoprotein expressed on hematopoietic cells. Two isoforms of CD8, CD8αβ and CD8αα, have been identified that are distinct in their expression and function. Whereas CD8αβ serves as a T cell receptor (TCR) coreceptor to enhance the functional avidity and is constitutively expressed on MHC class I-restricted T cells, CD8αα marks T cells that are distinct from the conventional thymus-selected and MHC-restricted CD4$^+$ or CD8αβ$^+$ T cells. Inconsistent with a coreceptor function, CD8αα decreases antigen sensitivity of the TCR, and it can be transiently or permanently expressed on T cells, regardless of the MHC restriction of the TCR or the presence of conventional coreceptors. Together, these observations indicate that CD8αα on T cells marks a differentiation stage and that it likely functions as a TCR corepressor to negatively regulate T cell activation.

Introduction

The CD8 isoform, CD8αα, when expressed on T cells, is frequently described as an inefficient T cell receptor (TCR) coreceptor. In analogy to the conventional TCR coreceptors, CD4 and CD8αβ, that mark thymus-selected MHC class II- or class I-restricted TCRαβ$^+$ T cells, respectively, CD8αα also binds MHC ligands and is used as a lineage determinant to identify T cells that divert from the mainstream T cell subsets in terms of their origin, ontogeny, specificity, and function (Cheroutre, 2004). In mouse, CD8αα single-positive (SP) T cells predominate among the intraepithelial lymphocytes (IEL) of the small intestine and typically display an activated phenotype together with a characteristic innate-like signature (Cheroutre, 2004). In addition, CD8αα and CD8αβ are flexibly regulated on thymocytes depending on the developmental stage (Ellmeier et al., 1998; Garefalaki et al., 2002; Feik et al., 2005), and CD8αα can also be expressed together with CD4 (Paliard et al., 1988; Reimann and Rudolphi, 1995; Kenny et al., 2004) or CD8αβ (Terry et al., 1990; Moebius et al., 1991; Konno et al., 2002; Madakamutil et al., 2004) on activated mature TCRαβ$^+$ T cells. The latter implies that CD8αα may not serve as a conventional MHC class I-binding TCR coreceptor on these cells.

The activated status of CD8αα-expressing mature T cells suggests a relationship between CD8αα and the TCR-CD3 complex. The expression of CD8αα on TCRγδ$^+$ as well as on TCRαβ$^+$ cells or together with CD4 or CD8αβ on MHC class II- or MHC class I-restricted T cells indicates that this relationship is not limited by the nature of the TCR or coreceptor and that it is independent of MHC restriction. These observations also imply that the link between CD8αα and the TCR-CD3 activation complex is not directed by the adaptive TCR subunit but rather that they point toward a functional connection with the invariant signaling modules of the CD3 complex. The ability of the CD8α cytoplasmic tail to associate with both the early Src kinase p56^{lck} (Veillette et al., 1988), which serves to phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 components, and the linker of activation of T cells (LAT) (Bosselut et al., 1999), which mediates further downstream signaling, connects CD8αα to proximal and distal TCR-CD3 activation signaling cascades. Despite this link and the capacity of CD8αα to interact with MHC class I ligands, CD8αα neither supports positive selection of MHC class I-restricted thymocytes (Crooks and Littman, 1994; Fung-Leung et al., 1994; Nakayama et al., 1994) nor does it efficiently promote the productive activation of CD8-dependent MHC class I-restricted TCRs (Renard et al., 1996; McNicol et al., 2007). Furthermore, when expressed together with CD8αβ, CD8αα may downmodulate as opposed to enhance the functional avidity of the CD8αβ-TCR:Ag-MHC activation complex (Cawthon et al., 2001; Cawthon and Alexander-Miller, 2002), meaning that the contribution of CD8αα to the TCR-CD3 activation complex can be suppressive. The differential, independent, and highly regulated expression of CD8αα together with its unique biological properties indicate that CD8αα is not a functional homolog of CD8αβ and suggest instead that CD8αα may serve as an effective TCR corepressor rather than a functional TCR coreceptor.

This review will focus on the various aspects of CD8αα expression and function on T cells, encompassing the complex transcriptional organization of the *Cd8* locus, the inhibitory effects of CD8αα on TCR-CD3 activation, and the consequences of CD8αα expression on the fate of developing thymocytes and activated mature T cells.

Transcriptional Regulation of the *Cd8* Locus

In contrast to the CD8αβ expression, generated by the constitutive transcription of *Cd8a* together with *Cd8b*, transcription of *Cd8a* can also be upregulated alone, resulting in the expression of CD8αα homodimers (Ellmeier et al., 1998). On CD8αβ$^+$ T cells, the induced transcription of *Cd8a* results in the coexpression of CD8αα with CD8αβ (Madakamutil et al., 2004). The differential expression of CD8αα and CD8αβ dimers indicates that the transcription of the individual *Cd8* genes is coordinately as well as independently regulated.

The *Cd8a* and *Cd8b* genes are closely linked within a locus of 36 Kb in mice and 56 Kb in human (Kieffer et al., 2002). Numerous regulatory transcription elements have been identified distributed along this locus and in particular within the noncoding gene interval upstream of the *Cd8a* gene. Besides binding sites for several transcription factors, including Runx1 and 3, Ikaros, and GATA-3, various CD8 enhancer (E8) sites were identified that display different capacities to regulate *Cd8* transcription (Kioussis and Ellmeier, 2002). Enhancer-driven reporter gene transcription or individual and combined deletions indicated that specific enhancers ($E8_{I-V}$) control CD8 expression in various cell types and during development or under different conditions of activation (Ellmeier et al., 1998, 2002; Madakamutil et al., 2004; Feik et al., 2005). Enhancer $E8_I$, for example, is not required for CD8αβ or CD8αα expression in developing thymocytes, whereas constitutive expression of CD8αα on IEL or transient TCR-CD3 activation-induced expression of CD8αα on mature CD8αβ$^+$ T cells was abolished in the absence of a functional $E8_I$ enhancer (Ellmeier et al., 1997; Madakamutil et al., 2004). In contrast, $E8_{II}$ and $E8_{III}$ are required for expression of CD8αβ and CD8αα on immature thymocytes whereas $E8_{IV}$ drives expression of CD8αβ on single-positive (SP) thymocytes and mature T cells including a subset of CD4$^+$ T cells (Ellmeier et al., 1998, 2002; Feik et al., 2005). Several of the transcription factors such as Runx, Ikaros, and GATA-3 show strong binding preferences for particular enhancer sites or function in cohort with various chromatin-remodeling molecules that provide positive as well as negative regulation of the *Cd8* locus (Kioussis and Ellmeier, 2002). Further, the zinc finger protein MAZR (bound to nuclear receptor corepressor complexes) controls *Cd8* transcription in immature thymocytes by specifically targeting the $E8_{II}$ enhancer region, thus linking the enhancer function also with negative control of transcription (Bilic et al., 2006). Another nonredundant repressor mechanism involved in the plasticity of CD8 expression is the epigenetic control by DNA methylation. Initial demethylation of both *Cd8a* and *Cd8b* genes occurs during thymocyte maturation. The retention of this demethylated state in CD4$^+$ T cells provided the initial evidence that these lymphocytes had matured from CD8αβ-expressing precursor cells (Carbone et al., 1988a). The fully methylated state of *Cd8b* in immature DN thymocytes and TCRγδ$^+$ IEL indicates that these cells or their precursors never transcribed *Cd8b*. CD8αα$^+$ TCRαβ$^+$ T-IEL, in contrast, display a unique pattern of *Cd8b* methylation that differs from T cells that never expressed CD8αβ, including the CD8αα$^+$TCRγδ IEL (Hamerman et al., 1997). Because DNA methylation is a stable but not irreversible epigenetic signal that silences gene expression, it is possible that the unique *Cd8b* methylation resulted from remethylation of the *Cd8b* gene to terminate expression of CD8αβ on these CD8αα$^+$TCRαβ$^+$ T-IEL. Remethylation of the *Cd8a* gene has been detected in CD4$^+$ T cells and mature DN TCRαβ$^+$ thymocytes, indicating that remethylation of the *Cd8* genes might be another mechanism for epigenetic silencing of CD8 (Carbone et al., 1988a, 1988b; Wu et al., 1990). SP CD8αα$^+$TCRαβ$^+$ IEL in this case cannot control expression of CD8 at the level of remethylation of *Cd8a* and it is thus possible that the unique methylation pattern of *Cd8b* in CD8αα$^+$TCRαβ$^+$ T-IEL reflects epigenetic regulation to silence CD8αβ while permitting CD8αα expression.

CD8αα and CD8αβ Protein Expression

In addition to the complex transcriptional regulation of the *CD8* locus, differential expression of CD8αα and CD8αβ is also regulated at the protein level. Whereas CD8α protein can be expressed as disulphate-linked CD8αα homodimers or CD8αβ heterodimers, CD8β protein requires association with CD8α for its stable expression at the cell surface. This is not due to the inability of CD8β molecules to form homodimers, which can be formed intracellularly but are unstable and degrade rapidly. Cell-surface expression of human CD8ββ homodimers has been described upon transfection or on lymphocytes of human CD8β transgenic mice; however, the physiological significance of this is not known (Devine et al., 2000). CD8α and CD8β are both membrane-anchored glycoproteins belonging to the immunoglobulin-like super family. Despite their insignificant sequence homology, crystal structure analysis of their ectodomains indicated remarkable structural similarities in shape, size, and in the surface electrostatic potential of complementary determining regions (CDR) between the paired immunoglobulin variable region-like domains of CD8αα and CD8αβ (Devine and Kavathas, 1999; Chang et al., 2005). Surface plasmon resonance (SPR) analyses further indicated that the stalk regions of the CD8α and CD8β are both capable of presenting the Ig-like domain to interact with MHC class I ligands (Chang et al., 2005). BIAcore binding data indicated that at least for soluble CD8αα and CD8αβ, the interaction with classical MHC class I molecules occurs with comparable affinity (Kern et al., 1999; Gao and Jakobsen, 2000; Leishman et al., 2001). When expressed as cell-surface molecules, however, the coordinated binding of CD8αβ with TCR-engaged MHC class I is much stronger as compared to membrane-bound CD8αα (Witte et al., 1999; Bosselut et al., 2000). These observations suggest that the ectodomain and/or the transmembrane and cytoplasmic region of CD8β specifically contribute to enhance the quality of the CD8-MHC interaction. In sharp contrast, soluble or membrane-bound CD8αα interacts with much enhanced affinity with the mouse thymic leukemia antigen (TL), a nonclassical MHC class I molecule expressed in the thymus and on small intestine epithelium (Leishman et al., 2001). The differential affinity of CD8αα for TL and classical MHC class I molecules was largely mediated by changes in three contact residues in the exposed loops of the conserved α3 domain of TL (Attinger et al., 2005). Analysis of CD8αα-TL cocrystals further indicated that the enhanced ability of TL to interact preferentially with CD8αα concurred with the structural elimination of its antigen-presenting potential and that the cleft between the two helical domains, α1 and α2, which defines the antigen-binding groove for MHC class I molecules, is not existing in TL (Liu et al., 2003). This structural feature has important functional consequences and implies that TL is not an antigen-presenting molecule for TCRs and that when interacting with TL, CD8αα is not functioning as a TCR coreceptor.

Several mutational studies indicated that the individual alpha subunits of CD8αα homodimers contribute differentially to establish the CD8αα-MHC class I interaction. Structural analysis of human CD8αα-HLA-A2 (Gao et al., 1997) and mouse CD8αα-H2-Kb (Kern et al., 1998) cocrystals confirmed this remarkable asymmetrical interaction where one CD8α chain (CD8α1) provides approximately 75% of the contact interface, contacting the β2-microglobulin (β2 m) and the α2 but mostly the conserved

α3 domain of the MHC class I ligands, whereas the CD8α2 subunit contributes some contact points with the α3 domain only. The topology of the CD8β component of the heterodimer when binding to MHC class I is not known, but in analogy with the asymmetric interaction of the CD8αα subunits, two models have been proposed. Initial studies with electrostatics and specific point mutations suggested that in the human CD8αβ-MHC class I complex, CD8β might substitute the CD8α2 subunit that contributes the least to the CD8-MHC interaction (Gao et al., 1997; Devine and Kavathas, 1999). In contrast, site-directed mutagenesis of transfected mouse CD8 molecules showed that mutations in CD8α1 or both subunits rendered the asymmetric CD8αα merely nonfunctional as measured by IL-2 production, whereas the coreceptor function of mutated CD8α paired with wild-type CD8β was as efficient as wild-type CD8αβ (Chang et al., 2005). These data suggest that unlike human CD8αβ, in mouse, the CD8β subunit assumes the position of the CD8α1 of the CD8αα homodimers. In other studies, however, it was shown that mouse CD8α mutants expressed as a heterodimer with wild-type CD8β were less capable at restoring MHC binding compared to wild-type CD8α, whereas mouse CD8β variants unable to substitute for CD8α1 could efficiently replace CD8α2 in the CD8αβ-MHC class I complex (Chang et al., 2006; Devine et al., 2006). Furthermore, CD8α antibodies were able to block the CD8α1 site but not the CD8α2 position (Chang et al., 2006), suggesting that at least in mouse, CD8β can assume the proximal or distal position of the CD8 dimer when interacting with MHC class I complexes.

In addition to the coordinated interaction with the antigen-presenting MHC class I molecules, CD8 coreceptor also enhances intracellular TCR activation signaling events (Figure 1; Irie et al., 1998; Bosselut et al., 2000). CD8α physically associates with the Src kinase $p56^{lck}$ via conserved binding motifs in its cytoplasmic domain (Veillette et al., 1988), and when coengaged with a TCR-CD3:Ag-MHC activation complex, CD8α-associated $p56^{lck}$ phosphorylates CD3ζ-ITAMs, which in turn recruit and phospholylate ZAP-70 and other src homology domain 2 (SH2)-containing molecules. The binding of CD8α-associated $p56^{lck}$ to CD3ζ-associated ZAP-70 couples the TCR coreceptor function directly to TCR-CD3 activation. The CD8α subunit also associates with LAT (Bosselut et al., 1999, 2000). LAT on phosphorylation by ZAP-70 recruits a variety of adaptor and signaling molecules for further downstream signaling, hence linking the contribution of the TCR coreceptor also to distal signaling cascades (Zhang et al., 1998). Because only the CD8α subunit of the CD8αβ heterodimer associates with p56lck and LAT, it is likely that the differential topology of CD8β will affect the positioning of these signaling molecules in relation to the CD3-TCR complex. The physiological consequences of this flexibility of CD8αβ coreceptor interactions are not known but they might represent quanitative and/or qualitative differences for TCR activation.

CD8αβ Functions as an Effective TCR Coreceptor

The ability of CD8αβ to function as a superior TCR coreceptor as compared to CD8αα is in sharp contrast to the observations that soluble CD8αα and CD8αβ show similar MHC-binding affinity (Kern et al., 1999) and that the cytoplasmic tail of the CD8α but not the CD8β chain provides the physical association with the TCR-CD3 signaling components $p56^{lck}$ and LAT (Figure 1; Veillette et al., 1988; Bosselut et al., 1999). Nevertheless, the incapacity of CD8αα to efficiently substitute the CD8αβ coreceptor during thymic selection of CD8β-deficient MHC class I-restricted thymocytes (Crooks and Littman, 1994; Fung-Leung et al., 1994; Nakayama et al., 1994) or during activation of CD8-dependent primary T cells (McNicol et al., 2007) and the effect of CD8β that broadens the range of antigen recognition of T cells underscore the importance and absolute requirement of the CD8β contribution for efficient CD8 TCR coreceptor function (Karaki et al., 1992; Wheeler et al., 1992; Zamoyska, 1994; Renard et al., 1996; McNicol et al., 2007). The molecular mechanisms that render CD8β as the key subunit for CD8 TCR coreceptor function are still somewhat mysterious. Several CD8β chimeric constructs, specifically lacking the CD8β extracellular module or the intracellular domain or both, showed that the ectodomain of CD8β critically enhances the interaction of membrane-bound CD8 with MHC class I (Witte et al., 1999; Bosselut et al., 2000), whereas the cytoplasmic tail of CD8β augmented the association of CD8α with the intracellular signaling molecules $p56^{lck}$ and LAT (Bosselut et al., 2000). In addition, it was demonstrated that these two physically associated domains of CD8β could enhance CD8 coreceptor function independently of each other (Bosselut et al., 2000). It is not fully understood how the CD8β ectodomain can promote MHC class I ligation, but one possibility is that the shorter stalk of CD8β might play an important role (Wong et al., 2003). The stalk is heavily glycosylated and O-linked carbohydrates on the β but not the α stalk are heterogeneous, resulting from complex and flexible sialylations that differ during thymic development and upon TCR stimulation (Daniels et al., 2001; Moody et al., 2001, 2003; Merry et al., 2003; Wong et al., 2003). It has been postulated that the β stalk glycosylation modifications and glycan adducts might induce conformational changes that promote the CD8-MHC class I interaction (Moody et al., 2001). The intracellular domain of CD8β independently promotes CD8 coreceptor function by actively recruiting the TCR to lipid rafts and by enhancing the association of the CD8α subunit with lipid raft-localized $p56^{lck}$ and LAT (Figure 1; Arcaro et al., 2000, 2001; Pang et al., 2007). CD8αβ, but not CD8αα, physically interacts with TCR-CD3 via the CD3δ and hence substantially enhances raft association of TCR-CD3 (Doucey et al., 2003). The lipid raft association of CD8αβ is completely controlled by the presence of CD8β, and for mouse, this was shown to depend on CD8β palmitoylation (Arcaro et al., 2000), whereas human CD8αβ requires the CD8β ectodomain to pair with CD8α to evoke lipid raft localization and effective coreceptor function (Pang et al., 2007).

CD8αα Corepressor Function

Historically, CD8αα has been used as a convenient molecule to study the function of CD8. With CD8α transfectants, it was shown that CD8αα could function as an adhesion molecule able to strengthen the overall avidity by interacting with MHC class I ligands that were not engaged by the TCR (Miceli et al., 1991). The observation that tail-less CD8αα could augment reactivity to the same extent suggested that the adhesion role of CD8αα depended solely on the interaction of its extracellular part with MHC class I molecules (Miceli et al., 1991). Unlike the enhanced CD8αβ TCR coreceptor function, however, cell-cell

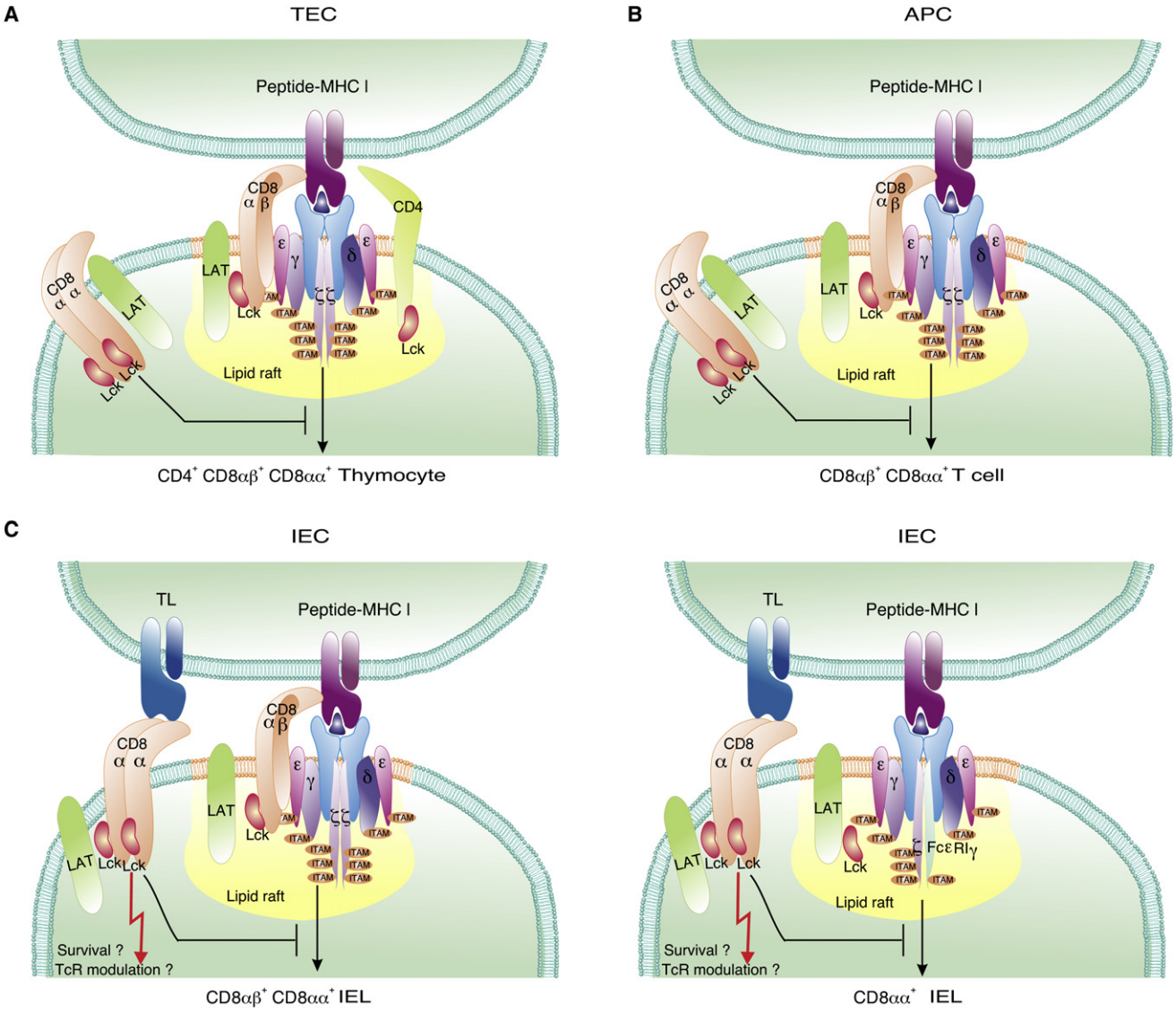

Figure 1. CD8αα Corepressor Function on Different Cell Types

Unlike the conventional coreceptors, CD8αβ and CD4, CD8αα repressor is excluded from lipid rafts that contain the TCR-CD3 (CD3ζ and/or FcεRγ+) activation complexes engaged with antigen-presenting MHC molecules. CD8αα corepressor negatively regulates TCR activation by disrupting lipid rafts and by sequestering signaling molecules required for TCR-initiated downstream signaling.

(A) CD4+CD8αβ+CD8αα+ TP thymocytes interact with MHC and self-antigens during agonist selection in the thymus. CD8αα repressor may sequester Lck and LAT, allowing for transient reduction in signal strength received through the agonist-selected TCRs.

(B) Some peripheral CD8αβ+ T cells transiently induce CD8αα upon primary activation with agonist ligands. CD8αα repressor on these activated T cells may temporarily sequester Lck and LAT and allow for transient reduction in signal strength received through the agonist-triggered TCRs.

(C) Left: Conventional coreceptor-dependent effector T cells reinduce CD8αα under the conditions of the gut microenvironment. The constitutive presence of CD8αα repressor may increase the threshold for activation on these antigen-experienced T cells that reside within the antigen-rich environment of the gut. CD8αα may interact with its ligand, TL, which is abundantly expressed by the gut epithelial cells and promote long-term survival of the antigen-experienced T cells. Right: DN TCRαβ+ thymocytes reinduce CD8αα upon migration to the gut. Constitutive expression of CD8αα repressor on these self-specific coreceptor-independent T cells might prevent aberrant self-reactivity. Interaction of CD8αα with TL ligand could promote long-term survival of these self-antigen-experienced T cells.

binding assays or SPR analysis of the soluble molecules indicated that CD8αβ did not increase cell adhesion or affinity for MHC class I ligands as compared to CD8αα (Garcia et al., 1996; Sun and Kavathas, 1997). The comparable adhesion mediated by CD8αα or CD8αβ is in strong contrast to the superior ability of membrane-bound CD8αβ to function as a TCR coreceptor. Therefore, the same characteristics that underscore the importance of CD8β as the hallmark of an efficient CD8 TCR coreceptor equally support the notion that CD8αα is not a functional homolog of CD8αβ TCR coreceptor. The requirement for effective CD8αβ coreceptor function as opposed to increased adhesion is most striking in conjunction with weak antigens and implies that the TCR coreceptor function is to enhance antigen sensitivity of low-affinity TCRs (Kerry et al., 2003; Maile

et al., 2005). As a consequence, T cells with low-affinity TCRs are coreceptor dependent for their activation and initial selection. With this in mind, a study with retroviral transfection of CD8-dependent and -independent TCRs into primary T cells isolated from wild-type or CD8β-deficient mice demonstrated that CD8αα cannot support activation of CD8-dependent TCRs, thus supporting the notion that CD8αα does not function as a TCR coreceptor (McNicol et al., 2007). In contrast, high-affinity TCRs, which function in the absence of coreceptors and appear on double-negative (DN) T cells, often express CD8αα (Levelt et al., 1999; Mixter et al., 1999; Wang et al., 2002; Gangadharan and Cheroutre, 2004). The expression of CD8αα in conjunction with high-affinity TCRs or the induction of CD8αα with increased TCR signal strength further cast doubts on CD8αα functions as a coreceptor to enhance antigen sensitivity of the TCR. Instead, the observations that enforced transgenic expression of CD8αα on DN thymocytes greatly impaired intracellular calcium responses and blocked efficient tyrosine phosphorylation of signaling components in response to TCR ligation suggest that CD8αα might function as a negative regulator of TCRs (van Oers et al., 1993). Consistent with this, with TCR transgenic T cell lines that express TCRs with identically affinity, it was demonstrated that coexpression of CD8αα together with CD8αβ specifically suppressed the CD8αβ-mediated increase in Ag sensitivity (Cawthon et al., 2001; Cawthon and Alexander-Miller, 2002).

Similar to other TCR corepressors, including CTLA-4 (Egen and Allison, 2002), CD8αα can be induced upon activation through the TCR-CD3 complex and the degree of induction increases proportionally to the signal strength (Barnden et al., 1997; Levelt et al., 1999; Cawthon et al., 2001; Wang et al., 2002; Madakamutil et al., 2004). Consequently, activation-induced CD8αα is directly related to the functional avidity of the activation complex and inversely related to the CD8αβ coreceptor dependency of the participating TCR. Because coexpression of CD8αα effectively decreases the functional avidity of TCRs and markedly diminishes or completely abolishes activation (van Oers et al., 1993; Cawthon et al., 2001), it can be concluded that CD8αα is not a redundant coreceptor but instead that CD8αα functions more likely as an effective TCR corepressor. Further, because CD8αα can be transiently induced on activated CD8αβ$^+$ T cells (Madakamutil et al., 2004) or constitutively expressed on IEL (Cheroutre, 2004), its inhibitory effect could either temporarily lower the functional avidity and attenuate an ongoing immune response (Figure 1B) or permanently increase the minimum signal strength required for restimulation of antigen-experienced T cells (Figure 1C). Although it is not fully understood how CD8αα functions as a corepressor, its ability to interact with MHC class I ligands as well as its capacity to associate with various signaling components of the TCR-CD3 complex indicate that CD8αα has the potential to interfere with TCR-mediated activation at different levels.

Unlike the activation-induced cointernalization of CD8αβ together with TCR-CD3, CD8αα expression increases with activation and reflects the disconnection between CD8αα and the TCR-CD3 activation complex consistent with its exclusion from the lipid rafts (Arcaro et al., 2000; Cawthon and Alexander-Miller, 2002; Pang et al., 2007). The increased presence of CD8αα outside the lipid raft compartment coincides with decreased functional avidity of the activation complex and indicates that the raft exclusion of CD8αα might be key to its suppressive effect (Cawthon and Alexander-Miller, 2002). It is thus possible that CD8αα binds non-lipid-raft-associated p56^{lck} and LAT and hence sequesters these signaling components from CD8αβ and TCR-CD3 activation complexes (Figure 1; Gangadharan and Cheroutre, 2004). Although this is a reasonable hypothesis, the enhanced capacity of CD8αβ coreceptor to effectively associate with p56^{lck} and LAT (Bosselut et al., 2000) would indicate that other mechanisms, in conjunction with the raft exclusion, might contribute to the repressor role for CD8αα. The efficient colocalization of CD8αβ together with TCR-CD3 activation complexes depends on the integrity of lipids rafts and the larger organization into membrane platforms (Horejsi, 2003). The observations that expression of CD8αα markedly reduces the colocalization and association of CD8αβ and TCR-CD3 could indicate that CD8αα actively disrupts the lipid raft integrity and thus abrogates the optimal association of CD8αβ with TCR (Cawthon and Alexander-Miller, 2002). Lipid raft disruption as a mechanism to interfere with TCR-CD3 activation has been described for other TCR repressors, including CTLA-4 (Rudd et al., 2002). Similarly to the CD8αα-CD8αβ coreceptor pair, CTLA-4 interacts with the same B7 ligands as its partner, CD28, but displays opposite functions: whereas CD28 serves as a TCR costimulatory receptor, CTLA-4 mediates TCR repressor activity (Alegre et al., 2001). CTLA-4 is also induced proportionally to the TCR signal strength (Egen and Allison, 2002), and together these observations suggest that lipid raft disruption and interference with colocalization of signaling molecules might be a general feature of repressors that are paired with TCR coreceptors. The repressor activity of CD8αα, however, is not limited to CD8αβ, and CD8αα-mediated suppression of DN thymocytes indicates that CD8αα can also directly serve to negatively regulate TCR-CD3 complexes, independently of conventional TCR coreceptors (Figure 1C; van Oers et al., 1993).

The CD8α cytoplasmic tail does not contain any immunoreceptor tyrosine-based inhibition motifs (ITIMs) typical of inhibitor receptors. In contrast, it is possible that CD8αα actively associates with inhibitory molecules as has been described for LAG-3, a TCR activation-induced repressor with close homology to the TCR coreceptor CD4 (Workman and Vignali, 2005). Similar to the CD8αα-CD8αβ receptor pair, LAG-3 shares MHC class II ligands with CD4 but serves to negatively regulate TCR-CD3 activation in part by actively recruiting intracellular inhibitory molecules (Iouzalen et al., 2001). Although no specific inhibitory molecules have been identified that directly bind to the cytoplasmic tail of CD8α, it is tempting to speculate that the linker for activation of B cells (LAB, NTAL, and recently renamed LAT2), a homolog of LAT, absent in naive T cells but transiently induced on activated T cells (Zhu et al., 2006) and abundantly expressed by CD8αα$^+$ IEL (Denning et al., 2007), might serve as an inhibitory adaptor for CD8αα. Although LAT2 also becomes phosphorylated in association with ITAM-containing activation receptors, including the FcεRIγ, it can actively compete with LAT and negatively regulate its activity in T cells (Zhu et al., 2006). It is interesting to note that the transiently induced CD8αα and LAT2 on activated T cells and the constitutive expression of these molecules on CD8αα$^+$ IEL also coincide with transiently or constitutively expressed FcεRIγ (Figure 1C). In T cells, FcεRIγ can participate in the CD3 complex, replacing CD3ζ dimers or forming

heterodimers with CD3ζ (Guy-Grand et al., 1994; Krishnan et al., 2003). For every three ITAM motifs present in each CD3ζ chain, there is only one ITAM per FcεRIγ unit, and hence it is possible that the reduced CD3 phosphorylation targets together with the counteracting effect of LAT2 on LAT and the presence of CD8αα are all part of various suppression mechanisms that function in concert to increase the threshold for productive T cell activation. Membrane-bound CD8αα negatively regulates coreceptor-dependent or coreceptor-independent TCR activation regardless of a productive interaction between its extracellular domain and the antigen-presenting MHC class I molecules (Cawthon et al., 2001). This is in sharp contrast to the potent capacity of soluble CD8αα (sCD8αα) molecules or CD8α-derived peptides to block the interaction between MHC and the CD8αβ coreceptor (Choksi et al., 1998; Kern et al., 1999; Sewell et al., 1999). The interference by sCD8αα results in the inhibition of CD3ζ tyrosine phosphorylation and indicates that the blocking by sCD8αα targets the earliest stage of activation consistent with inhibition of $p56^{lck}$ activation. The inhibition by sCD8αα does not affect high-affinity TCRs expressed on DN T cells and implies that the suppression mechanism of sCD8αα specifically targets low-affinity TCRs that require CD8αβ coreceptor function for their activation (Kwan-Lim et al., 1993; Sewell et al., 1999; Kerry et al., 2003). Similar suppressive effects have also been observed with CD8 antibodies that interrupt the interaction of CD8αβ with MHC ligand (Sewell et al., 1999). The inhibitory effect of sCD8αα is remarkably potent and only a minority of CD8αβ-MHC class I interactions need to be obstructed by sCD8αα to prevent T cell activation (Sewell et al., 1999). sCD8αα arises from alternative spliced mRNA in which the exon encoding the transmembrane domain has been deleted (Giblin et al., 1989; Norment et al., 1989). sCD8αα occurs naturally in human and correlates with advanced stages of various diseases including T cell leukemia, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and HIV infection. In mouse, an alternative spliced form of CD8α has been described as well, but in contrast to human sCD8αα, alternatively spliced mouse CD8α mRNA retains the transmembrane domain and results in membrane-bound tail-less CD8α' molecules (Zamoyska et al., 1985, 1989). Although all T cells transcribe and translate CD8α', only immature thymocytes express CD8α'β heterodimers at their cell surface, whereas mature T cells retain terminally sialylated CD8α'-containing complexes intracellularly (Zamoyska and Parnes, 1988). Membrane-bound CD8α' lacks the ability to associate with $p56^{lck}$, and therefore, even though it has an intact extracellular domain that can interact with MHC class I molecules, it is unable to function as a TCR coreceptor subunit (Zamoyska et al., 1989). It is interesting to note that an ancestral form of CD8α expressed in lower vertebrates has also retained the prototype CD8 Ig-like ectodomain as well as the hinge and transmembrane domain, whereas it lacks the $p56^{lck}$ consensus binding motif in the cytoplasmic portion, suggesting that these two physically linked functional units might have evolved separately (Hansen and Strassburger, 2000).

CD8αα and Thymic Differentiation of Agonist-Selected T Cells

Because of its structural homology and shared MHC class I ligands, CD8αα has unjustly been labeled as an alternative TCR coreceptor for CD8αβ. The observation that conventional mature MHC class I-restricted T cells are lacking in the periphery of CD8β-deficient mice indicated that CD8αα, abundantly expressed on *Cd8b*$^{-/-}$ DP thymocytes, are incapable of providing a coreceptor function during thymic selection (Crooks and Littman, 1994; Fung-Leung et al., 1994; Nakayama et al., 1994). In contrast, mature CD8αα$^+$TCRαβ$^+$ IEL develop normally in these *Cd8b*$^{-/-}$ mice, indicating that CD8αα$^+$SP IEL are independent of CD8αβ coreceptor for their development (Leishman et al., 2002). The absence of these CD8αα$^+$TCRαβ$^+$ T cells in *β2 m*$^{-/-}$ mice (Sydora et al., 1996) indicates that they either express a CD8 coreceptor-independent but MHC class I-dependent TCR, or that an interaction between the constitutively expressed CD8αα and MHC class I ligand maintains survival of the mature cells, perhaps by increasing the threshold for productive activation of their TCRs. The latter would be in agreement with the finding that these T cells express high-affinity TCRs that function typically in a coreceptor-independent fashion. Also consistent with this is the observation that the TCR repertoire of these CD8αα$^+$ TCRαβ$^+$ IEL is greatly enriched for high-affinity self-reactive TCRs that are otherwise deleted from the normal T cell repertoire during thymic-negative selection (Rocha et al., 1991). The presence of self-reactive T cells among the CD8αα$^+$TCRαβ$^+$ IEL has been used as evidence that these TCRαβ$^+$ T cells developed extrathymically (Rocha et al., 1991). Other data, however, indicate that self-specific CD8αα$^+$TCRαβ$^+$ IEL are thymus selected (Lambolez et al., 2007). We have shown that some preselected thymocytes express CD8αα at the immature DP stage (Gangadharan et al., 2006). By using TCR transgenic cells, we demonstrated that in contrast to DP thymocytes, these CD8αα-expressing triple-positive (TP) thymocytes survived and differentiated to DN or CD8αα$^+$ T cells when exposed to their cognate antigen in vitro (Figure 2A; Gangadharan et al., 2006). Intrathymic injection of TP thymocytes generated a substantial population of CD8αα$^+$ TCRαβ$^+$ IEL, whereas their DP counterparts generated exclusively conventional coreceptor-positive T cells in the periphery. These results provided direct evidence that TP thymocytes are precursors of agonist-selected T cells. Although it is not understood how these TP thymocytes can survive under agonist selection conditions, it is consistent with a repressor function for CD8αα that allows for a transient reduction of signal strength resulting in survival of these agonist-selected thymocytes. We identified the TCRαβ$^+$ DN thymocytes as the mature post agonist-selected thymic precursors and showed that upon transfer, these cells readily generated CD8αα$^+$TCRαβ$^+$ IEL in vivo (Gangadharan et al., 2006). The DN phenotype of agonist-selected IEL precursors is consistent with the accumulation of coreceptor-independent high-affinity TCRs among CD8αα$^+$TCRαβ$^+$ IEL. CD8αα expression on the TP thymocytes is also activation induced; however, unlike on mature T cells, CD8αα induction on these immature cells does not depend on a full TCRαβ or any MHC ligation (Gangadharan et al., 2006). Together with the observation that CD8αα is induced on anti-CD3-triggered *Rag1*$^{-/-}$ DN thymocytes, this indicates that the inducing signal for CD8αα on immature thymocytes might be given by the pre-TCR during TCRβ selection. The agonist selection pathway that allows for selective survival of precursor cells that express TCRs with high affinity for self also endows these cells with an activation-induced differentiation program and further underscores the

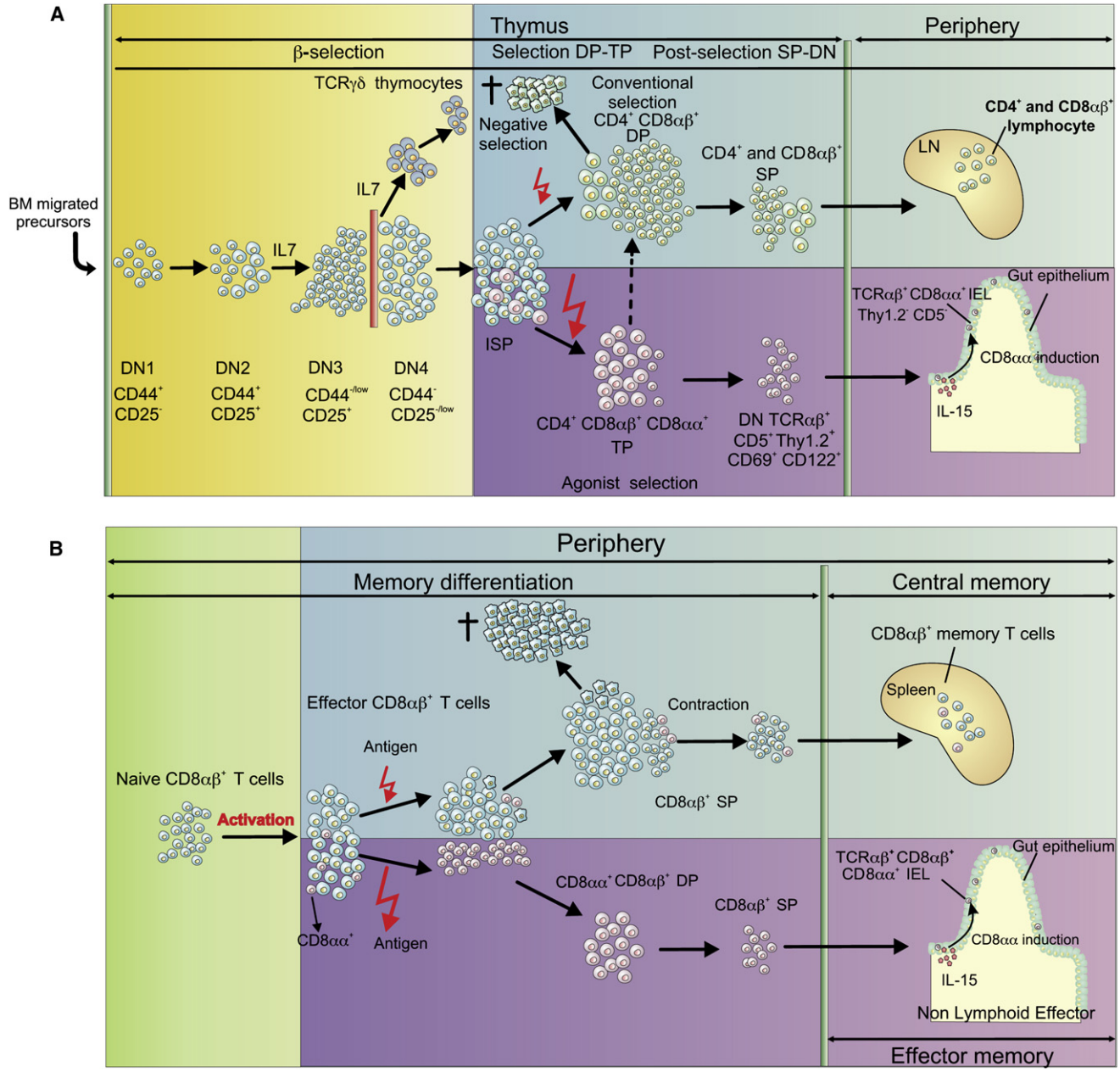

Figure 2. The Pathways of Agonist Selection in the Thymus and Effector Memory Differentiation in the Periphery Show Parallel Features for CD8αα-Expressing Cells

(A) In the thymus, CD8αα-expressing immature TP thymocytes may survive agonist selection conditions that otherwise delete CD8αα-negative DP counterparts. Positive-selected TP thymocytes differentiate to DN TCRαβ$^+$ mature cells that gain the capacity to migrate to the intestine and adapt the CD4$^-$CD8αβ$^-$CD8αα$^+$ TCRαβ$^+$ IEL phenotype in the IL-15-rich environment of the gut.

(B) In the periphery, CD8αα-expressing primary effector CD8αβ$^+$TCRαβ$^+$ T cells survive agonist-induced activation and differentiate to SP CD8αβ$^+$ TCRαβ primary effector T cells that gain the capacity to migrate to the intestine. Conventional CD8αβ$^+$TCRαβ$^+$ effector cells reinduce CD8αα in the presence of IL-15, locally in the gut, and reside there long-term as CD8αα$^+$CD8αβ$^+$ TCR IEL with an effector memory phenotype.

thymic CD8αα-dependent agonist selection process as a central drive for the unique differentiation and specialization of these self-specific mucosal memory CD8αα$^+$ T cells.

CD8αα and Peripheral Differentiation of Memory T Cells

Conventionally selected T cells differentiate to memory T cells in response to cognate nonself antigens encountered in the periphery (Sprent and Surh, 2002). The generation of immune memory provides the individual with enhanced protective immunity upon secondary encounter of the antigen (Sprent and Surh, 2002). Although it is now well established that memory T cells are direct descendants of primary effector T cells, it is still poorly understood why some effectors survive and differentiate to memory cells whereas the bulk of the effector population undergoes

full activation followed by activation-induced cell death. Interestingly, this process of memory differentiation has much in common with the selective survival and specific differentiation of CD8αα$^+$TCRαβ$^+$ IEL precursor cells during agonist selection in the thymus (Figure 2). Consistent with this, we showed that a subset of primary effector CD8αβ$^+$ T cells transiently induce CD8αα during early activation, and upon transfer, they showed increased capacity to survive and differentiate to memory T cells (Madakamutil et al., 2004). The transient induction of CD8αα on some of the responding CD8αβ$^+$ T cells is controlled by the enhancer E8$_I$, and E8$_I$-deficient CD8αβ$^+$ T cells are unable to induce CD8αα expression in response to anti-CD3 stimulation in vitro (Madakamutil et al., 2004). Consistent with an important role for CD8αα during CD8αβ$^+$ memory T cell differentiation, we showed that E8$_I$-deficient mice were greatly impaired in the generation of memory CD8αβ T cells (Madakamutil et al., 2004). The indication that membrane-bound CD8αα can be suppressive regardless of MHC ligation and the observation that memory T cells can be generated in the absence of TL expression (Williams and Bevan, 2005) would suggest that the critical role for CD8αα during memory differentiation is TL independent and that specific ligation of the CD8αα extracellular domain with TL or any other MHC class I molecule expressed by the APC is not required for survival and differentiation of memory precursor cells. It is thus possible that activation-induced CD8αα transiently abrogates ongoing activation by negatively intercepting TCR-CD3 complex-mediated signals via its cytoplasmic domain, alone (Figures 1B and 2B).

Not all memory precursors require CD8αα for their initial survival and differentiation, and other mechanism can lead to CD8αβ$^+$ memory T cells via CD8αα-independent mechanisms (Chandele and Kaech, 2005; Zhong and Reinherz, 2005). It is not known, however, whether the CD8αα-dependent memory cells differ in their phenotype, functional, and/or specific homing abilities from those generated via CD8αα-independent processes. Nevertheless, the induction of CD8αα at the initiation of an immune response, when the antigen dose is high, and the proportional relationship between the degree of CD8αα induction and TCR signal strength would imply that the CD8αα-dependent pathway of memory differentiation is an early activation event and selectively preserves effector T cells with high affinity or avidity for non-self antigens. Together with the observation that memory precursor cells that emerge under strong activation conditions preferentially differentiate to effector memory T (T_{EM}) cells, this would indicate that CD8αα-dependent memory differentiation selectively preserves the "fittest" effector cells to differentiate to T_{EM} cells that can provide protective immunity at peripheral sites that have the highest and most likely probability for re-entry of the pathogens.

CD8αα and Mucosal T Cells

Agonist-selected self-specific DN thymocytes or effector memory CD8αβ T cells that migrate to the intestine reinduce and maintain CD8αα expression, suggesting that continuous suppression by CD8αα on these antigen-experienced T cells might be part of the mucosal immune regulation to mediate immune quiescence in the antigen-rich environment of the gut. In addition, it is also possible that the interaction of CD8αα on the IEL with its ligand TL constitutively expressed by the intestinal epithelium (Hershberg et al., 1990) might regulate their homeostatic proliferation (Yamamoto et al., 1998) and promote long-term survival of these cells in the absence of IL-7R signals (Figure 1C; Masopust et al., 2006). Interaction with TL ligand can also stabilize prolonged expression of CD8αα and modify activation signals (Leishman et al., 2002; Madakamutil et al., 2004). The constitutive presence of CD8αα on these cells probably does not present an indefinite shut off of the activation potential, and it has been shown that increased antigen stimulation or crossligation of CD8 and TCR can override negative regulation by CD8αα (Sewell et al., 1999; Cawthon et al., 2001). The constitutive re-expression of CD8αα on agonist-selected self-specific DN IEL as well as on effector memory CD8αβ T cells indicates that CD8αα repressor induction is a general feature for mucosal T cells and serves as an active suppression mechanism to keep high-affinity and previously activated T cells in check by lowering their sensitivity for self or non-self antigens, but ready to go whenever the specific antigen load surpasses the increased threshold.

CD8αα on Non-T Cells

The expression and function of CD8αα is not exclusively in conjunction with TCR-CD3, and CD8αα can also be expressed on non-T cells, including NK cells and subsets of DCs, mast cells, and macrophages. Furthermore, human NK cells that express CD8αα are more cytotoxic than their CD8αα-negative counterparts (Srour et al., 1990). This is due to an indirect effect of CD8αα ligation on the NK cell that protects the NK effector cells from induced apoptosis. As a result, CD8αα$^+$ NK cells are capable of sequential lysis of multiple target cells (Addison et al., 2005). This ligation is through interaction with fellow NK cells and is independent of ligands expressed on the target cells. Protection from apoptosis is blocked by preincubation of the NK cells with MHC class I antibodies, suggesting that an interaction between CD8αα and MHC class I molecules expressed by the NK cells has antiapoptotic effects. It is not known how CD8αα mediates its effects on non-T cells, but it is tempting to speculate that the ability of CD8αα to modify activation signaling mediated by the invariant CD3 complex might extend its function to also control invariant ITAM-containing activation receptors such as FcRs expressed by these innate cells. The expression of CD8αα on different innate cell lineages indicates that in contrast to the MHC class I-restricted coreceptor function for CD8αβ on T cells, CD8αα might serve as a universal positive or negative modulator for a broad range of activating receptors that span adaptive and innate immune responses.

Conclusions

Looks are deceiving and much has been assumed about CD8αα because of its striking structural homology with the CD8αβ coreceptor. This misconception not only has led to incorrect interpretations for the function of CD8αα but it has also generated profound confusion and misunderstanding regarding the ontogeny and function of CD8αα-expressing T cells. The presence of CD8αα has been used as a marker for unconventional IEL and their thymic predecessors and for conventional CD8αβ$^+$ memory precursor cells. When assuming a redundant, ineffective coreceptor function for this molecule, the expression of CD8αα on T cells represents merely a marker of no functional

importance. However, when CD8$\alpha\alpha$ is considered a potent co-repressor induced on immature thymocytes or primary effector cells or re-expressed on mature antigen-experienced cells in the intestine, the importance of CD8$\alpha\alpha$ as a key regulator of activation and differentiation becomes unquestionable. It is therefore of utmost importance to consider the repressor function of CD8$\alpha\alpha$ for future analysis of this molecule and for reinterpretation of existing data considering the role of CD8$\alpha\alpha$ during T cell differentiation processes as well as the controversial data that surrounds the ontogeny and function of CD8$\alpha\alpha$-expressing T cells.

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