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Biochemical and structural analysis of hCEACAM1 and hTIM-3 interactions

We compared the hCEACAM1:TIM-3 structure obtained (Fig. 2e and Extended Data Table 2) with biochemical results derived from co-transfection studies performed with WT and mutant hCEACAM1 and hTIM-3 variants expressed in HEK293T cells (Extended Table 1a-b). Mutations of homologous amino acid residues of hTIM-3 predicted to be involved in mTIM-3 ligation independent of galectin-9 binding, including Glu62Ala, Arg69Ala, Arg111Trp (single nucleotide polymorphism, SNP, rs145478313) and Asp120Ala did not affect their expression in HEK293T cells (Extended Data Fig. 3f), but disrupted biochemical interactions with WT hCEACAM1 (Fig. 2c and Extended Data Fig. 3f-g). Insertion of a *TIM3* (also known as *HAVCR2*) SNP variant (rs201750016, Cys58Arg) into hTIM-3 and mutation of Cys109 (Cys109Ala), which forms a disulfide bond with Cys58, or mutation of hTIM-3 Cys110 (Cys110Ala), which forms a disulfide bond with Cys38[13], disrupted interactions with hCEACAM1 as assessed by co-IP (Extended Data Fig. 3h-j). Mutation of a third pair of disulfide bonds in hTIM-3 (Cys52Ala and Cys63Ala) did not affect interactions with hCEACAM1 (Extended Data Fig. 3j). In a corollary manner, hCEACAM1 mutants composed of natural allelic variants rs200708090 (Gln44Leu) and rs8111468 (Gln89His) as well as a Gly47Ala mutant of hCEACAM1, but not rs147100915 (Tyr34Cys), exhibited decreased biochemical association with WT hTIM-3 (Fig. 2d and Extended Data Fig. 3l-m). Supporting our biochemical studies structural models built from multiple X-ray crystallographic data sets confirmed an interaction between Gln89 of hCEACAM1 and Glu62 and Asp120 of hTIM-3 (Fig. 2f). Similarly, hCEACAM1 Gln44 exhibited structural interactions with Met118 and Asn119 (residues in the F-G loop) of hTIM-3 that may help to position hTIM-3 residue Asn119 and allow it to hydrogen bond with the main chain oxygen atoms of hCEACAM1 residues Thr56 and Pro57 (Fig. 2g). The biochemically important hCEACAM1 Gly47 residue (Extended Data Fig. 4g) also exhibited significant proximity to the F-G loop of hTIM-3 such that substitution of a residue with a side-chain (Ala) in this position would be predicted to displace the F-G loop away from hCEACAM1 (Extended Data Fig. 3n-p). Our structural analysis further revealed the critical importance of hCEACAM1 residue Asn42 interactions with Lys122 (F-G loop residue) of hTIM-3 (Extended Data Fig. 4f). Based upon this, we created hCEACAM1 mutants consisting of Asn42Ala and Arg43Ala which were observed to diminish the biochemical interactions between hCEACAM1 and hTIM-3 (Extended Data Fig. 3q-s). Together with surface plasmon resonance analysis that demonstrated binding of hCEACAM1:TIM-3 single chain protein to surface bound bacterially expressed N-terminal IgV-like domain of hTIM3 linked to glutathione-S-transferase (GST-TIM-3) fusion protein (Extended Data Fig. 4b), but not GST alone, which was inhibited by either a hTIM-3 peptide (aa. 58-77), but not scrambled peptide (Extended Data Fig. 4c and 4e), or an anti-hCEACAM1 monoclonal antibody (26H7) specific for the CEACAM1 N-domain[32,33] (Extended Data Fig. 4d-e), these studies provide evidence for direct protein interactions between the IgV-like domains of hCEACAM1 and hTIM-3.

We also compared WT hTIM-3 with a natural hTIM-3 variant (rs147827860 [Thr101Ile]), located in the E-F loop of hTIM-3 and disruptive of an N-glycosylation consensus sequence (Asn-Xaa-Ser/Thr) (Extended Data Fig. 2f and 3e). Although co-transfected hCEACAM1 could associate normally with the Thr101Ile hTIM-3 variant (Extended Data Fig. 5f), and augment biosynthesis of the core Thr101Ile protein (Fig. 2h), the Thr101Ile variant exhibited greatly diminished levels of immature (Endo-H sensitive) and mature (Endo-H resistant) glycoforms (Fig. 2h-i), increased intracellular retention (Extended Data Fig. 5a-b) and a near absence of this hypomorphic hTIM-3 variant on the cell surface even despite co-expression of WT hCEACAM1 (Extended Data Fig. 5a-b). Thus hTIM-3 expressed in the absence of hCEACAM1 behaves similarly to a hTIM-3 (Thr101Ile) variant lacking a site for a critical N-linked carbohydrate modification (Fig. 2j), further demonstrating the dependence of TIM-3 maturation and expression on CEACAM1.