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CORRESPONDENCE

Controversies about the subcellular localization and mechanisms of action of the Alzheimer's disease-protective CD33 splice variant

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Genome-wide association studies (GWAS) identified an allele of the microglial CD33 (Siglec-3) cell surface receptor that protects against Alzheimer's disease (AD). Protected individuals express less full-length CD33 and more alternatively spliced D2-CD33 lacking the amino-terminal sialoglycan-ligand-binding domain. The mechanism by which D2-CD33 reduces AD risk is unknown. A recent excellent review [1] concluded that the protective allele may act via gain of function, with excess cell surface D2-CD33 reducing risk via "a higher propensity toward microglial activation through mechanisms similar to the well-recognized functions of TREM2 and its co-receptor, DAP12". However, our earlier study [8] showed that D2-CD33 (a.k.a. CD33m or CD33ΔV-Ig) is diverted to intracellular peroxisomes (consistent with a conserved peroxisomal targeting signal in the cytosolic tail).

This evidence supports an alternate protection mechanism—increasing D2-CD33 decreases full-length CD33 at the cell surface, thus reducing its sialoglycan-recognizing inhibitory capacity [7]. This adaptive loss of function—is distinct from, but not necessarily incompatible with possible gains of function suggested by Estus et al. However, our data suggest that new functions for D2-CD33 arise in the peroxisome. Given the prognostic and potential therapeutic significance of this disease-protective allele and the critical importance of understanding its mechanism, it is important to reverify the intracellular localization of D2-CD33, and address specificity of antibodies used to indicate this diversion.

Initial discovery of the intracellular form in myelomonocytic lineage cells [8] was based on studies with and without permeabilization, using well-known anti-CD33 antibodies WM53 and HIM3-4 [6], which recognize the first and second extracellular domains of CD33, respectively. To further explore this intracellular pool, we generated a new antibody A16121H by immunization against the predicted amino-terminal peptide (DLTHRP) of the D2 domain [4]. While we cannot rule out variation in signal peptidase cleavage sites, this approach ensured elimination of cross-reactivity with full-length CD33, in which this sequence is embedded within the protein. Here we reconfirm specificity of the A16121H antibody using recombinant soluble proteins (Fig. 1a) and repeat flow cytometry studies using A16121H on THP-1 and U-937 monocytic cell lines, affirming the cytosolic localization of the epitope (Fig. 1b). The amino-terminally exposed A16121H antibody epitope appears highly susceptible to intracellular proteases, as several methods of cell lysis could not detect the epitope in a Western blot [8]. The only way to consistently detect this epitope is under specific conditions of gentle permeabilization of healthy, intact myelomonocytic cells. Notably, all published reports of D2-CD33 cell surface expression used artificial cell systems over-expressing transfected constructs, and differential binding of pre-existing CD33 antibodies. It remains to be determined if any D2-CD33 is expressed on cell surfaces of non-transfected non-malignant myelomonocytic cells in general, and microglia in particular.

Notably these two hypotheses (gain vs. loss of function) for D2-CD33 protection against AD are not mutually exclusive. Increase in alternate splicing and consequent reduction

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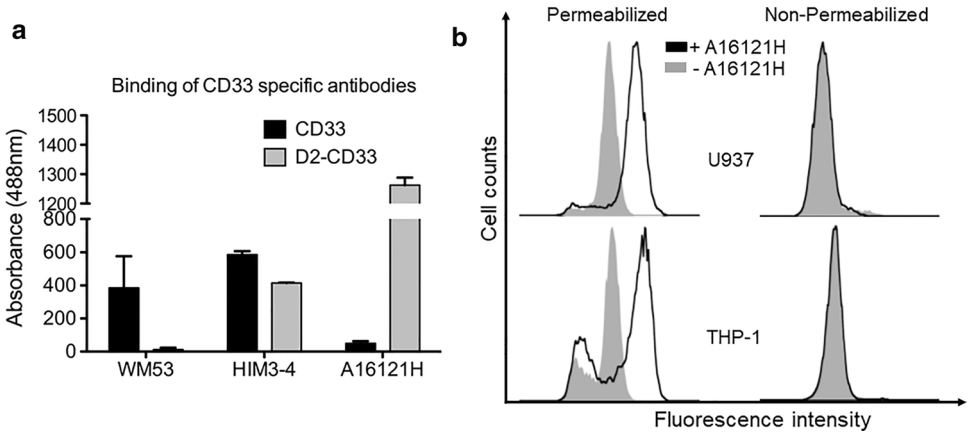

Fig. 1 Confirming A16121H antibody specificity and intracellular localization of D2-CD33. **a** Antibody specificity. This panel repeats data mentioned but not shown in our original paper [8]. Protein A coated plates incubated with affinity-purified full-length or truncated extracellular domains of human CD33 as Fc-chimeras were washed with 0.1% Tween20 in TBS (TBS-T), blocked with 1%BSA in TBS-T and probed with antibodies against domain 1 (clone WM53; BD Pharmingen, Cat: 555449; 1:1000 dilution), domain 2 (clone HIM3-4; BioLegend, Cat: 303302; 1:1000 dilution) or the D2-CD33 amino-terminus (A16121H; BioLegend; 10 µg/ml). Fluorophore-conjugated secondary antibodies against mouse IgG or rat IgG2 provided visualization. **b** Flow cytometry analysis of non-permeabilized or permeabilized U-937 or THP-1 cells with A16121H antibody. All incubations and washes were on ice or at 4 °C. Freshly confluent cells were washed with 10 mM EDTA in PBS, and permeabilized with Cytoperm/Cytofix solution (BD Pharmingen, Cat: 554714) for 20 min on ice. Non-permeabilized controls were maintained in 1%BSA in PBS and 10 mM EDTA. Cells were washed with 10 mM EDTA in PBS and probed with antibody A16121H (10 µg/ml). Fluorophore-conjugated secondary antibody against rat IgG2 provided visualization with a BD FACSCalibur

of inhibitory full-length CD33 may indeed enhance microglial activity, clearing amyloid and/or other toxic products as suggested by others [3]. This could be due to a gain of function suggested in the review [1], but our data suggest that this function should be sought in peroxisomes [5], not the cell surface [8]. Direct mechanistic studies of microglia naturally expressing different forms must decipher whether loss of cell surface function is sufficient to explain protection, or if a further gain of function in peroxisomes or elsewhere can explain how some CD33 variants protect. Unfortunately, recapitulating native microglial phenotypes outside the brain appears difficult, because an environment-dependent transcriptional network specifies human microglial identity [2]. Regardless, the Estus et al. review addresses many other important issues regarding CD33 and AD, and we look forward to continued evolution of research in this area.

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