

Supplementary Materials for The Membrane-Bound Enzyme CD38 Exists in Two Opposing Orientations

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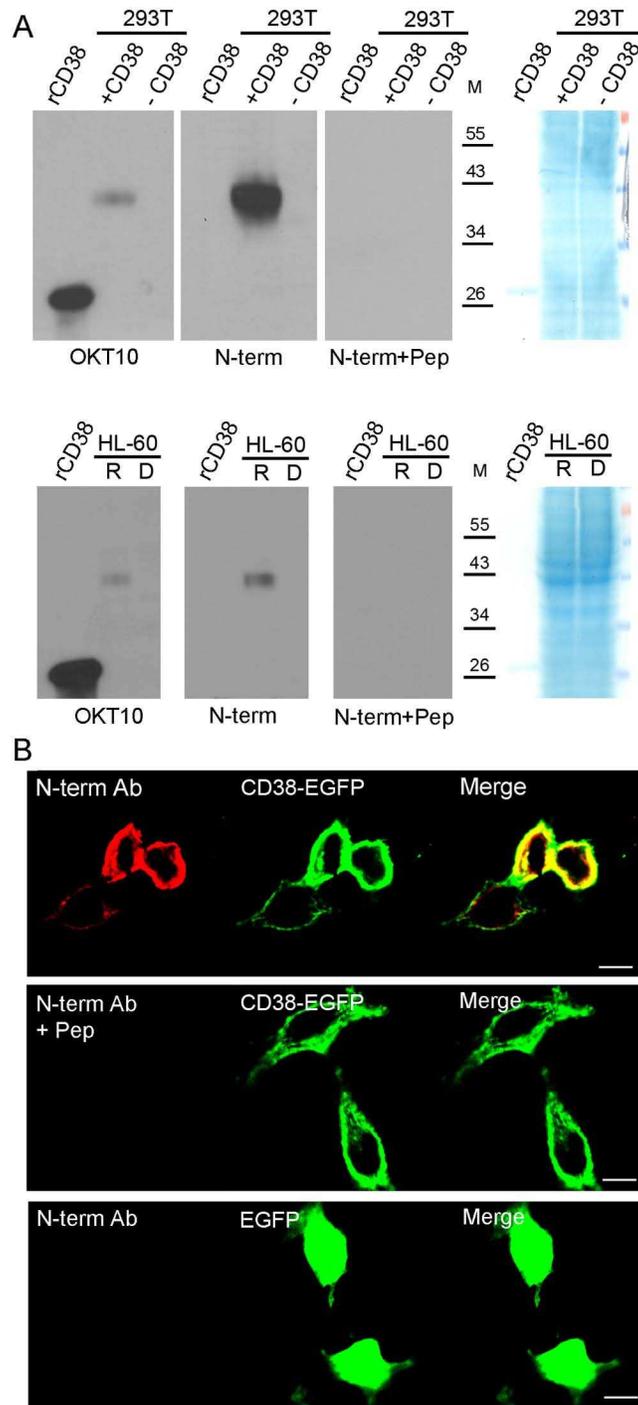


Fig. S1. Validation of the monoclonal N-term antibody. (A) Cell extracts were prepared from transfected HEK 293T cells expressing CD38 (+CD38). The upper panels show that the N-term antibody recognized a protein of about 43 kD, which corresponded to CD38. The protein band was absent in the control, non-transfected cells (-CD38). The N-term antibody did not recognize recombinant CD38 (rCD38) lacking the N-terminal tail and the transmembrane segment. Pre-incubation of the N-term antibody with the tail peptide (N-term + Pep) blocked recognition of CD38. OKT10, a commonly used monoclonal antibody against the C-terminal domain of CD38,

recognized rCD38 as well as CD38 expressed by the transfected cells. A Coomassie-stained blot shows equal loading in each lane. The lower panels are similar to the upper panels, except that the extracts were prepared from differentiated HL-60 cells induced by treatment with RA (R) for 4 days to express CD38, or from control cells treated with only the vehicle, DMSO (D). (B) Immunostaining using the N-term antibody. HeLa cells were transfected with a plasmid encoding CD38 fused to EGFP tag at its C-terminus (35). Cells were fixed and incubated with the N-term antibody (red). The staining colocalized with EGFP fluorescence (green) in the merged image (top panels). N-term antibody staining was blocked when the antibody was pre-incubated with the N-terminal peptide (middle panels). Control cells were transfected with the pEGFP plasmid alone. Thus, no N-term staining was observed (bottom panels). Scale bar: 20 μ m. The data shown are representative of three independent experiments.

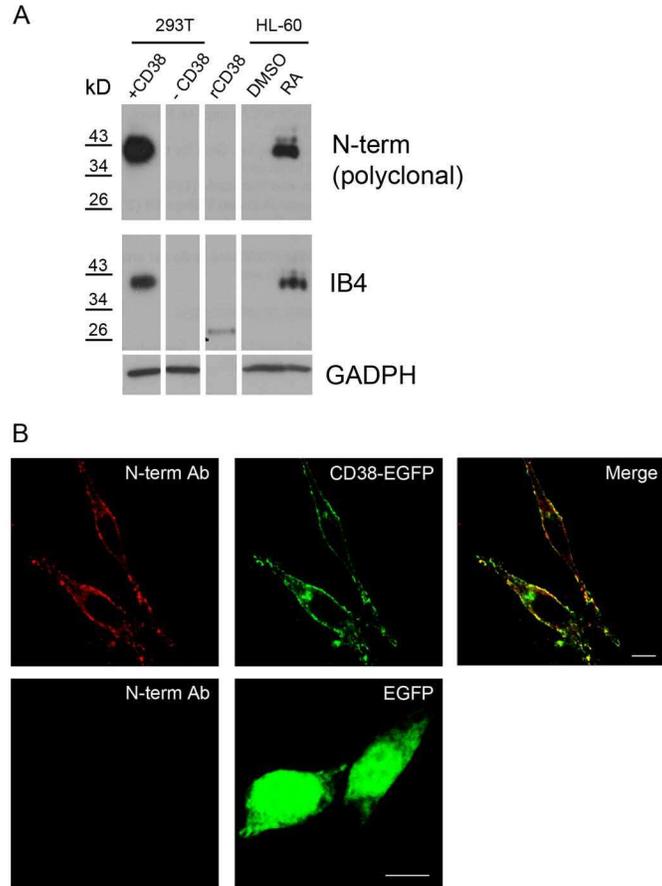


Fig. S2. Validation of the polyclonal N-term antibody. The experimental conditions used were similar to those described in fig. S1 except that a polyclonal N-term antibody was used. The antibody recognized CD38 in transfected HEK 293T cells (left panels) and in HL-60 cells treated for 4 days with RA, but not in the control DMSO-treated HL-60 cells (D) (right panels). IB4, an antibody against the C-terminal domain of CD38, recognized both the recombinant CD38 (rCD38) and the CD38 expressed in cells. The polyclonal N-term antibody (N-term) recognized the CD38 expressed in cells, but not the rCD38 lacking the tail segment. Equal protein loading was confirmed by Western blotting with an antibody against GADPH. Because non-reducing conditions was used during protein preparation, the sizes of CD38 and rCD38 would be roughly 43 and 26 kD, respectively. **(B)** Recognition of CD38 by immunofluorescent staining of HeLa cells expressing CD38-EGFP. Positive staining of polyclonal N-term antibody (red) was colocalized with the fluorescence of CD38-EGFP (upper panels). Controls were cells transfected with pEGFP plasmid alone (lower panels). Scale bar: 20 μ m. The data shown are representative of three independent experiments.

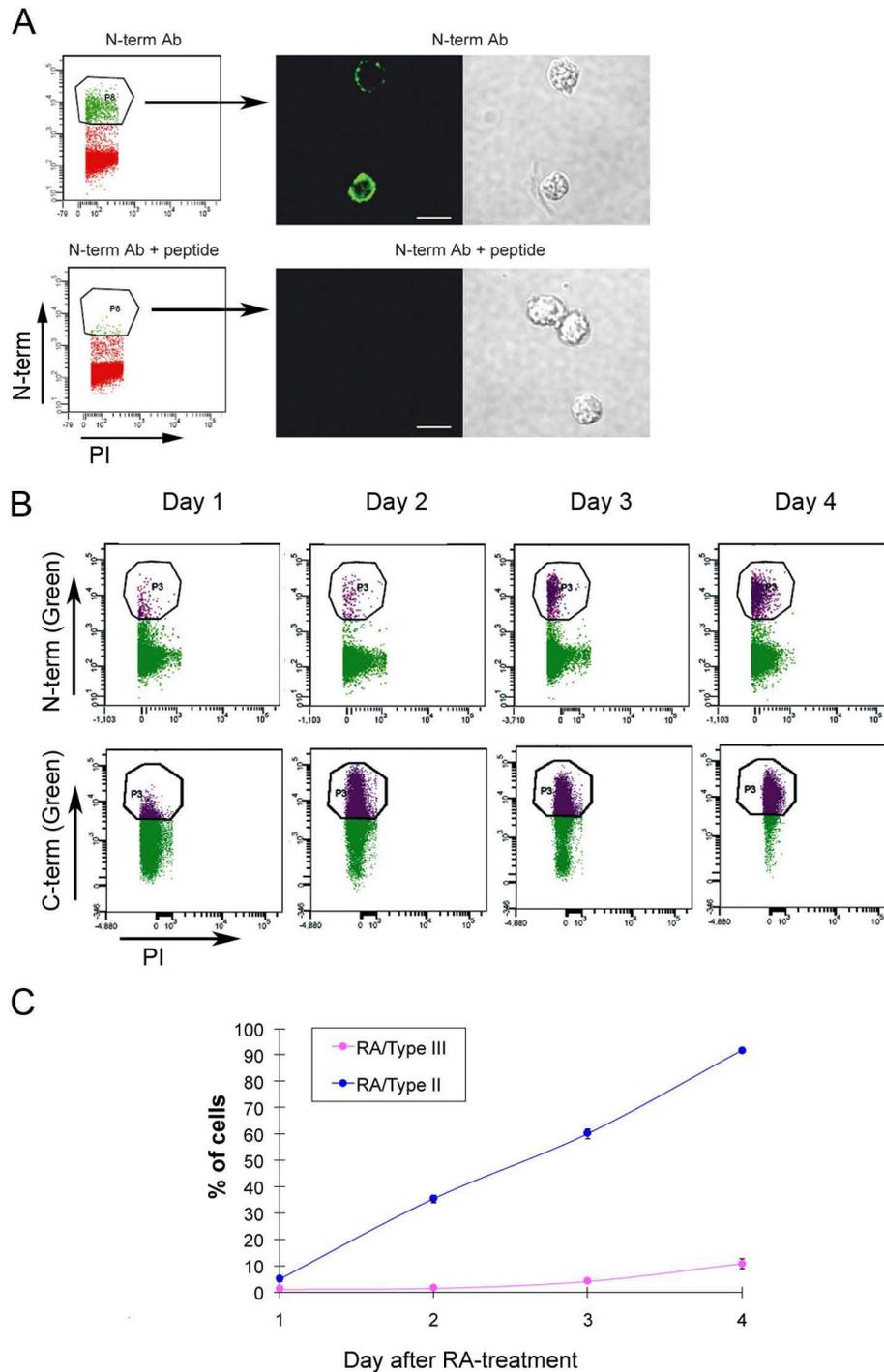


Fig. S3. The polyclonal N-term antibody demonstrated the presence of type III CD38 in RA-treated HL-60 cells. The experimental conditions were the same as those described in the Fig. 1. (A) Flow cytometric analysis showed positive staining of type III CD38 with the polyclonal N-term antibody that was blocked by pre-incubation with the N-terminal peptide (left panel). The immunofluorescence micrographs show that the sorted intact HL-60 cells after 4 days of treatment with RA stained positively with the polyclonal N-term antibody. The staining was

blocked by pre-incubation of the N-term antibody with the N-terminal peptide. Scale bar: 10 μm . **(B)** Flow cytometric analysis showed that during treatment with RA, the amount of the type III form of CD38 increased concomitantly with that of the type II form. Black polygon indicates gated cells that were positively stained for type II or type III CD38. **(C)** Time courses of the increased amounts of type II and type III CD38 during treatment with RA. The data shown are representative of three independent experiments.

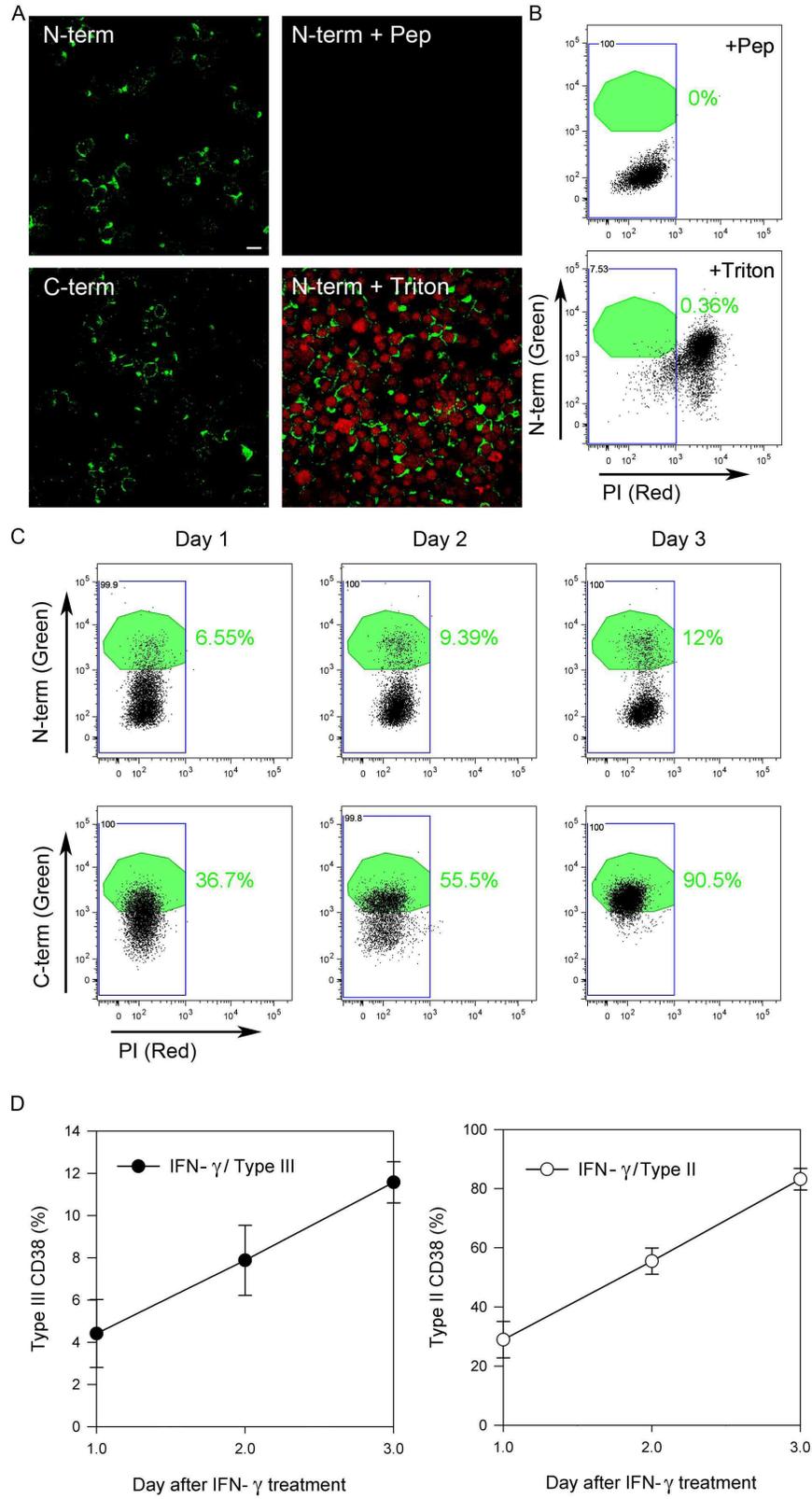


Fig. S4.

Fig. S4. Detection of both forms of CD38 on the surface of IFN- γ -treated monocytes. U937 cells were activated with IFN- γ (500 U/ml). After 3 days, cells were immunostained with a monoclonal N-terminal antibody (N-term) to reveal the presence of type III CD38 on the cell surface. Cell integrity during staining was monitored by co-staining with the cell-impermeant DNA stain PI. Only cells that showed immunofluorescence for CD38 but did not have nuclear staining were considered truly positive and were collected by flow cytometry. **(A)** Micrographs of IFN- γ -treated U937 cells showing surface immunofluorescence (green) indicating the presence of type III CD38. Preincubation of the monoclonal antibody with the N-terminal peptide (N-term + Pep) blocked the immunostaining, indicating antibody specificity. Cells also exhibited the type II isoform of CD38, as shown by positive staining with IB4, a monoclonal antibody against the C-terminal domain (C-term) of CD38. Cells treated with 0.1 % Triton X-100 (N-term + Triton) to deliberately compromise membrane integrity showed both immunofluorescence for CD38 and nuclear staining (red). Scale bar: 10 μ m. **(B)** Flow cytometry plots of two control treatments. Pre-incubation of the N-term antibody with the N-terminal peptide (+ Pep) blocked the immunostaining (upper panel). Cell permeabilization with Triton X-100 (+ Triton) rendered all cells stained positively by the nuclear stain PI and also by N-term antibody (lower panel). The blue square indicates gated live cells and the green polygon indicates gated cells that were positively stained for type II or type III CD38. **(C)** Flow cytometric analysis of the surface expression of type III CD38 (upper panels) and type II CD38 (lower panels). Intact U937 cells were harvested during the time course of treatment with IFN- γ . At the indicated times, live cells were stained as described in the Materials and Methods and were analyzed by flow cytometry. The blue square indicates gated live cells and the green polygon indicates gated cells that were positively stained for type II or type III CD38. **(D)** Time courses of the production of type II and type III forms CD38 during the treatment of U937 cells with IFN- γ . The data shown are representative of three independent experiments.

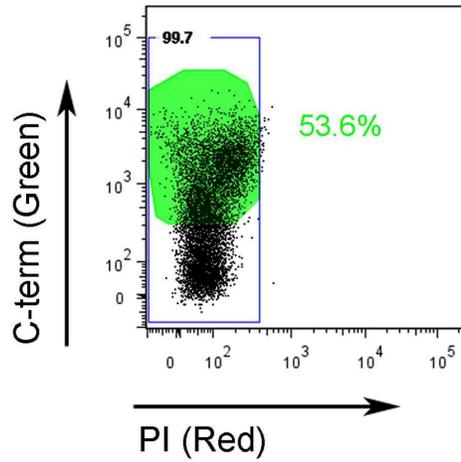


Fig. S5. Immunostaining of human PBMCs with the C-term antibody against CD38. Human PBMCs were incubated with a C-term antibody against CD38 and an Alexa Fluor 488-conjugated donkey anti-rabbit IgG in the presence of PI to monitor for cell integrity. Cells were then sorted by flow cytometry, and the result showed that about 53% of the cells had type II CD38 on the cell surface. The data shown are representative of three independent experiments.

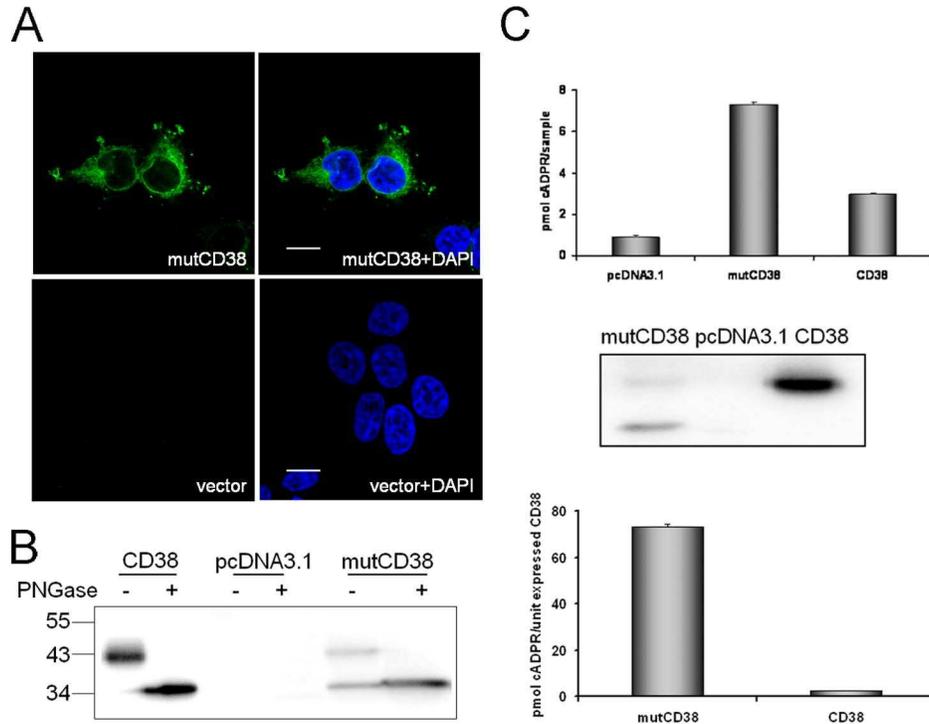


Fig. S6. The mutCD38 protein is biologically active. **(A)** HEK 293T cells were transiently transfected with pcDNA3.1 (lower panels) or pcDNA3.1-mutCD38 (upper panels) and were incubated with the Ab77 antibody against CD38 together with the nuclear stain, DAPI. Scale bar: 10 μ m. **(B)** Western blots of wild-type CD38 and mutCD38. Deglycosylation of proteins with PNGase resulted in a reduction in the size of the band corresponding to wild-type CD38. Extracts of cells transfected with plasmid encoding mutCD38 showed two bands, one of which was similar in size to that of wild-type CD38, which was also sensitive to PNGase, and another smaller band that was not sensitive to PNGase. The results showed that most of the mutCD38 was not glycosylated (smallest band), an indication of the type III orientation. **(C)** The intracellular cADPR concentrations in HEK 293T cells transfected with plasmids encoding either mutCD38 or CD38 were increased compared to those of control cells. The upper chart shows data presented as pmol cADPR per sample without normalization to the amounts of the CD38 proteins in the cells. The lower chart presents the same results normalized to the amount of expressed CD38 protein, as determined by Western blotting analysis (middle panel). MutCD38 was 20 to 30-times more effective than wild-type CD38 in increasing intracellular cADPR concentrations. The difference is statistically significant with $P < 0.05$ by t -test. The data shown are representative of three independent experiments.