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CD28 T cell costimulatory receptor function is negatively regulated by N-linked carbohydrates☆

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Abstract

CD28 is a cell surface glycoprotein expressed on T cells that modulates immune responses through its ability to transduce costimulatory signals. Even though nearly 50% of the molecular mass of CD28 is *N*-glycan, the physiological significance of CD28 glycosylation is at present unknown. In this report, we have investigated the function of hypoglycosylated wildtype CD28 and its splice variant, CD28i. When N-glycosylation was prevented through point mutations in N-glycosylation sites in CD28, or reduced by glycosidase inhibitors, the binding of CD28 to CD80 significantly increased. Stimulation of hypoglycosylated CD28 induced IL-2 promoter activity greater than that induced through the stimulation of wildtype CD28. Unlike hypoglycosylated wildtype CD28, hypoglycosylation of CD28i did not alter CD28i functions. Our data indicate that *N*-glycans of CD28 negatively regulate CD28/CD80 interactions, resulting in diminished CD28 signaling. It is also suggested that *N*-glycans regulate the density of CD28 clustering upon ligation with CD80/CD86. The results support the hypothesis that the N-glycosylation negatively regulates CD28-mediated T cell adhesion and costimulation.

Keywords: Cell surface molecules; Costimulation; T lymphocytes

Recent studies demonstrate that cell surface membrane receptors are profoundly affected by reduction of N- or O-glycosylation [1–6]. The reduced N-glycosylation of T cells from β1,6-*N*-acetylglucosaminyltransferase V deficient mice resulted in decreased T cell activation thresholds in response to TCR stimulation. These mice also showed an increase in susceptibility to autoimmune diseases [3]. O-glycosylation was demonstrated to be important for CD8αβ and class I major histocompatibility complex (MHC) Ag complex formation [4–6]. The *O*-glycan of CD8αβ enhanced the binding between CD8αβ and class I MHC and appeared to reinforce the weak binding of thymic immature $CD4^+CD8^+$ T cells' TCR to class I MHC.

The importance of CD28 costimulatory signaling for Ag-specific T cell responses has been amply documented in studies of activation-induced T cell differentiation to memory, regulatory, or effector T cells [7]. CD28 is a member of the immunoglobulin gene superfamily. The physiological ligands for CD28 are CD80 (B7-1) and CD86 (B7-2) [8]. Nearly 50% of the molecular mass of CD28 is modified through glycosylation [9]. We recently characterized a human CD28 splice variant, CD28i, in human T cells [10]. The CD28i splice variant is also N-glycosylated to greater than 40% of its molecular mass. The significance of N-glycosylation of CD28 has previously not been investigated.

In this report, we present data showing that N-glycosylation of human CD28 negatively regulates the

☆ *Abbreviations:* CD28i, a CD28 splice variant; CD28wt, wildtype CD28; CHO, Chinese hamster ovary cell; dMM, 1-deoxymannojirimycin; dNM, 1-deoxynojirimycin; HA, hemophilus influenza hemagglutinin; Swain, Swainsonine.

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interaction between CD28 and CD80. Mutation of all potential N-linked glycosylation sites resulted in significantly increased numbers of transfected cells expressing glycosylation defective CD28 adhering to CD80 expressing Chinese hamster ovary (CHO) cells. The stimulation of glycosylation mutant CD28 expressing cell lines with CD80 or anti-CD28 triggered increased costimulatory signaling activity compared with signaling activity following stimulation of cells expressing wildtype CD28. The treatment of Jurkat cells with inhibitors specific for N-glycosylation enzymes also enhanced the binding with CD80 and resulted in increased IL-2 promoter activity. Our data suggest that physiological N-glycosylation of CD28 reduces the adhesion/costimulation of T cells when stimulated by CD80 expressed on antigen presenting cells (APCs).

Experimental procedure

Abs and reagents. Anti-human CD28 (CD28.2) and FITC-goat anti-rabbit IgG were purchased from PharMingen (Mississauga, Canada). PE anti-human CD28 (YTH 931.12) was purchased from Serotec (Raleigh, NC). HRP-conjugated monoclonal rat anti-hemophilus influenza hemagglutinin antigen (HA) was purchased from Roche (Laval, Canada). Rabbit anti-HA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). N-glycosylation inhibitors, 1-deoxynojirimycin (dNM), 1-deoxymannojirimycin (dMM), and Swainsonine (Swain), were purchased from Calbiochem (La Jolla, CA).

Cell culture and the treatment with of N-glycosylation inhibitors. Jurkat human T cell line ($CD28^+$) and the murine T cell hybridoma, DC27 ($CD28^-$) [11], were grown in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 60 μg/ml penicillin-G, and 50 μg/ml streptomycin sulfate. CHO and CD80–CHO, which express transfected murine CD80 [12], were grown in DMEM supplemented with 5% FBS.

Fig. 1 shows the glycoprotein processing pathway and demonstrates the specific sites of action of inhibitors used in the study. Inhibitors were used at optimum concentrations, dNM, 1 mM; dMM, 1 mM; and Swain, 10 μM. The treatment with these inhibitors had no detectable effect on proliferation and protein synthesis as determined by incorporation of [^{3}H]thymidine and [^{3}H]leucine (data not shown). Jurkat cells or HA-tagged human CD28-transfected Jurkat cells [10] were cultured in the presence of inhibitors for 10 days prior to harvesting for cell–cell adhesion assay or protein assay by Western blotting.

Site-directed mutagenesis and construction of expression vectors. Site-directed mutagenesis was carried out based on PCR. Briefly, oligonucleotide primers between 25 and 40 bp long which contain the desired mutations were synthesized. These primers were used to generate CD28 or CD28i-fragments, which were then used to replace the wildtype fragment to generate the CD28 or CD28i mutants. The DNA sequences of the regions that contain the mutations were verified by DNA sequencing. To make the HA-tag inserts into CD28 or CD28i or their mutants, PCRs were performed using primers (sense

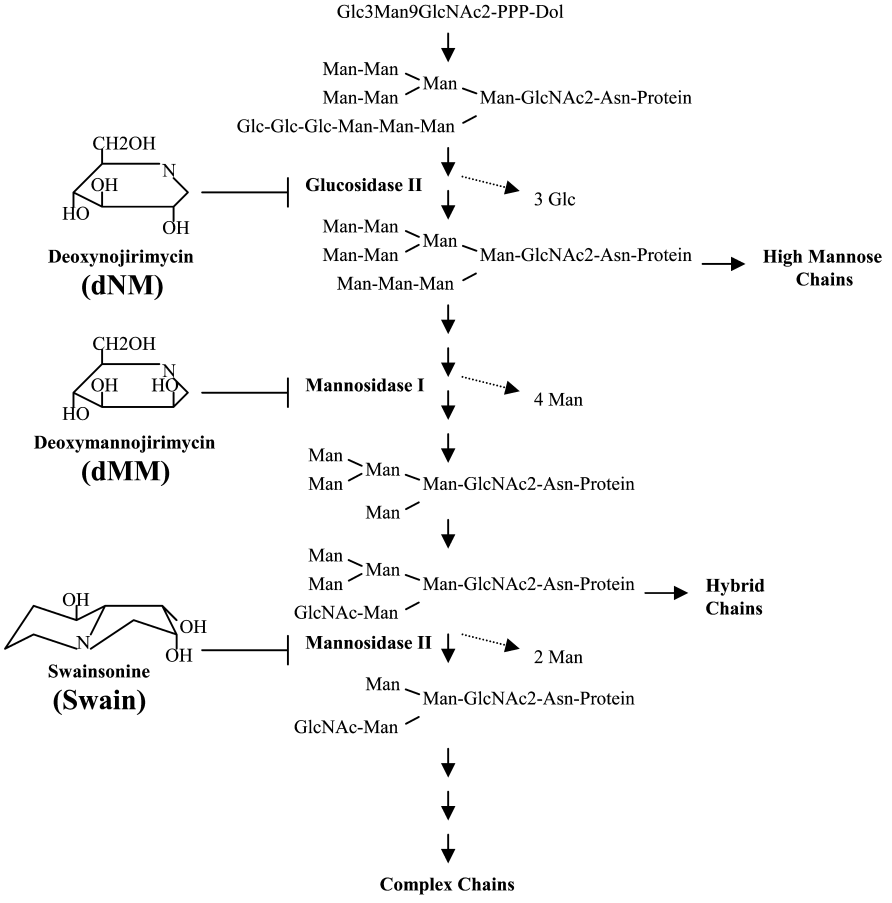

Fig. 1. Target mechanisms of the glycoprotein-processing inhibitors. Deoxynojirimycin (dNM) is an inhibitor of glucosidase I, deoxymannojirimycin (dMM) inhibits mannosidase I, and Swainsonine (Swain) inhibits mannosidase II. Dotted lines are the presumed pathways.

5′-AGGCTCGAGatgctcaggctgctcttggct-ctc-3′ and antisense 5′-CGTC TAGAtcaggagcgataggctgcgaagtc-3′) (capitals indicate the sequences of restriction digestion sites) that were ligated into *pcDNA3* (Invitrogen, Carlsbad, CA) using the *Xho*I and *Xba*I restriction sites, and subsequently sequenced to confirm the desired sequences.

Cell–cell adhesion interaction assay. The measurement of the adhesion between Jurkat cell and CD80–CHO or CHO cell was performed as described by Fargeas et al. [13]. Briefly, CD80–CHO or CHO were seeded in triplicate in flat-bottomed 96-well microtiter plates at 5×10^4 cells/well the day before the assay. Jurkat cells or DC27 transfectant cells were loaded with calcein-AM (Calbiochem) in PBS containing 0.1% BSA by passive diffusion for 1 h, washed three times with PBS, and added to the plates at 4×10^5 cells/well. After 30 min incubation at room temperature, nonadherent cells were removed by repeated washes by ice-cold Ca^{2+} Mg^{2+}-free PBS. The remaining cells were lysed in 2% Triton X-100 and fluorescence was determined at 485–538 nm using a Microplate Reader (Bio-Rad, Richmond, CA). For each sample, the percentage of cell binding was calculated as follows: percentage of binding = [amount of fluorescence/amount of fluorescence without washing (total input)] × 100.

Cell transfection and luciferase reporter gene assay. Jurkat cells or DC27 cells (2×10^6) were transfected using DEAE–dextran [10] with the indicated amounts of IL-2 promoter reporter plasmid, *pIL-2luc* [14], effector cDNA expression vectors or *pcDNA3*. Twenty-four hours after transfection, the recipient cells were either left untreated or incubated with anti-human CD28 (1 μg/ml), PHA (1 μg/ml) or Con A (5 μg/ml), and PMA (10 ng/ml) for 24 h, and then subjected to extract preparation by using a reporter lysis buffer (Promega) at 40 μl/10^6 cells. Luciferase activity was detected by mixing 20 μl extract with 100 μl luciferase substrate (Promega) and measured with a single photon channel of a scintillation counter (Beckman). In alternative studies, CD80–CHO and CHO were used as costimulator of PHA-activated Jurkat cells or Con A-activated DC27 transfectant cells. Each assay was performed in triplicate as follows: 5×10^4 Jurkat or DC27 transfectant cells were cocultured with 1×10^4 CHO or CD80–CHO in the presence of 1 μg/ml PHA (for Jurkat) and 5 μg/ml Con A (for DC27 transfectants).

Cell flow cytometry and immunoblotting. Cytometric analysis of CD28 expression and Western blotting specific for HA-tagged CD28 were performed as previously described [10].

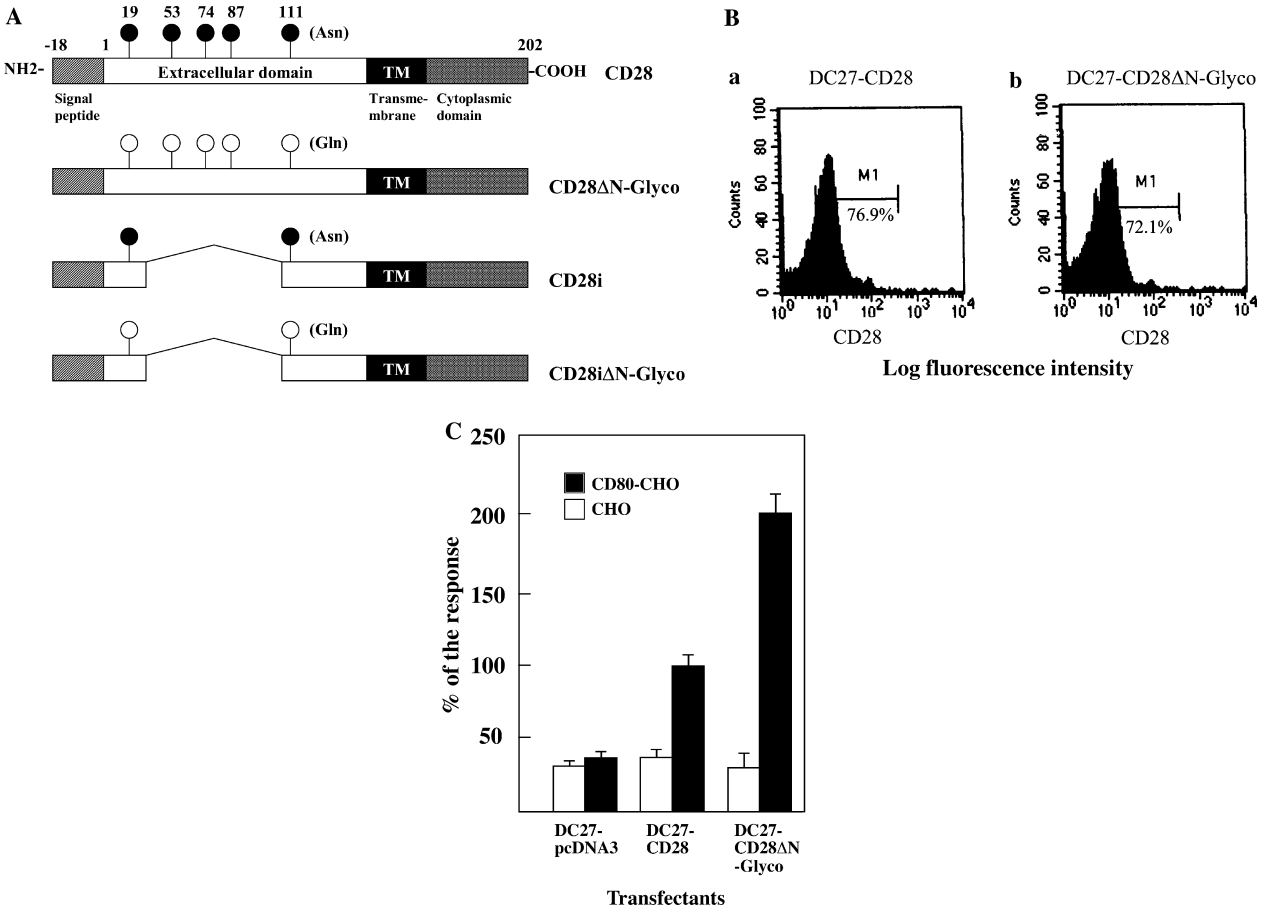

Fig. 2. Characterization of N-glycosylation deficient mutant CD28. (A) Schematic representation of human CD28, CD28i, and their point mutants. Locations of the potential sites for addition of N (Asn)-linked carbohydrate (●) are shown. Point mutants were designed to remove all potential N-linked glycosylation sites (Asn → Gln) shown as circles (○). (B) Flow cytometric analysis of non-N-glycosylated CD28 expressed on DC27 transfectant cell. DC27 cells were transfected with CD28wt or CD28ΔN-Glyco expressing plasmids. DC27-CD28 (a), and DC27-CD28ΔN-Glyco (b) were stained by PE anti-human CD28. M1 represents positive staining of CD28. (C) Adhesion interaction of non-N-glycosylated CD28 transfectants with CD80–CHO. Cell–cell adhesion assays were performed on CD28wt and CD28 mutant cell lines. The means ± SD of three independent experiments are presented. Percentage of the response = (percentage of binding of each transfectant against CHO or CD80–CHO/percentage of binding of DC27-CD28 against CD80–CHO) × 100. Open columns indicate adhesion interaction with CHO cells. Filled columns indicate adhesion interaction with CD80–CHO cells.

Statistical analysis. The mean, standard deviation of the mean, and paired or unpaired Student's t test results were used to analyze the data. When two groups were being compared, a P value of $\leqslant 0.05$ was considered to indicate a significant difference between the groups.

Results

N-glycosylation deficient CD28 enhances the adhesion of the transfectant cells to CD80–CHO

To investigate whether N-glycosylation of CD28 plays a significant role in CD28-mediated T cell functions, we prepared the human CD28 mutant (*CD28ΔN-Glyco*) that lacks all five N-glycosylation sites in the extracellular domain (Fig. 2A). *CD28ΔN-Glyco* and wildtype CD28 (CD28wt) expressing constructs were transfected into the CD28 deficient DC27 mouse T cell line [15]. Cell surface staining showed that both CD28ΔN-Glyco and CD28wt were expressed at similar levels in DC27 cells (Fig. 2B). To investigate whether the function of CD28ΔN-Glyco is different from that of CD28wt, we first investigated the binding of the CD28ΔN-Glyco transfectant cells with CD80 expressing CHO (CD80–CHO). As shown in Fig. 2C, we found that a greater number of CD28ΔN-Glyco transfectants bound to CD80–CHO than that of CD28wt-transfected control cells. The percent binding of CD28ΔN-Glyco cells was 2-fold higher than CD28wt cells. CHO cells lacking expression of CD80 did not bind either DC27-CD28 cell lines, indicating that binding was CD80-specific. Since both CD28wt and CD28ΔN-Glyco were expressed at similar levels we conclude that hypoglycosylation of CD28 results in increased adhesive properties of CD28ΔN-Glyco expressing cells.

N-glycosylation deficient CD28 enhances the costimulation of the transfectant cells by CD80–CHO or anti-CD28

The increase in the cellular binding may predict a stable CD28 ligation with CD80 and may result in stronger CD28 cytoplasmic signaling. We, therefore, investigated the CD28 costimulatory signaling activity in our transfected cell lines by measuring IL-2 gene promoter activity following stimulation by CD80–CHO. IL-2 promoter driven luciferase activity was increased in CD28ΔN-Glyco-transfected cells by 60% over CD28wt-transfected cells following stimulation with CD80 (Fig. 3A). Almost identical increases in IL-2 promoter activity were observed in CD28ΔN-Glyco-transfected cells compared to CD28wt-transfected cells when stimulated with PMA, Con A, and anti-CD28 (Fig. 3B). Together, we found that the costimulatory signaling activity in CD28ΔN-Glyco-transfected cells stimulated by CD80–CHO or PMA, Con A, and anti-CD28 was consistently greater than that induced in CD28wt-transfected cells. Accordingly, the

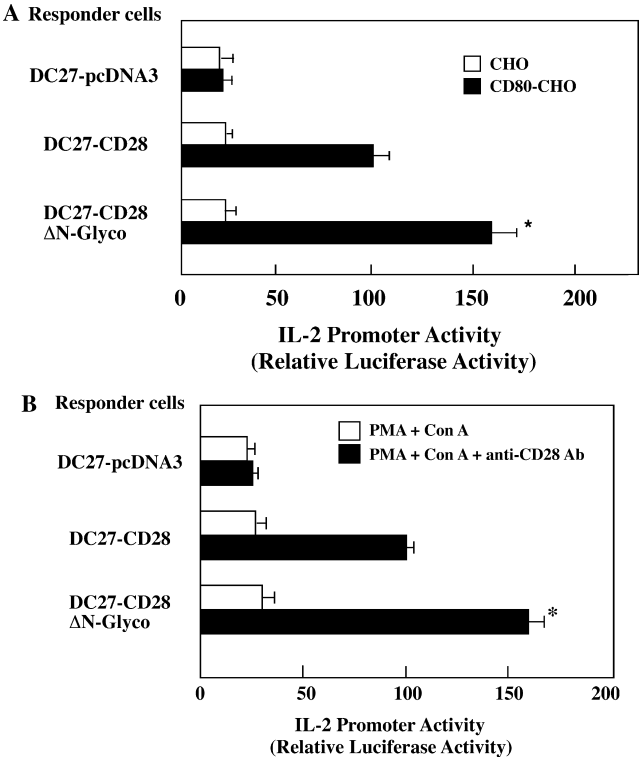

Fig. 3. Costimulatory signaling by N-glycosylation deficient mutant CD28. (A) Non-N-glycosylated CD28 triggers greater costimulatory signaling activity when activated by CD80–CHO cells. DC27 cells transfected with plasmids harboring different forms of human CD28 were further transfected with IL-2 luciferase reporter plasmids and then stimulated with Con A. The Con A-activated DC27 transfectants were cocultured with CD80–CHO for 24 h. Cell extracts were examined for luciferase activity. Open columns indicate cells stimulated with CHO cells. Filled columns indicate cells stimulated with CD80–CHO cells. *$p < 0.05$ (CD80–CHO-stimulated DC27-CD28 vs CD80–CHO-stimulated DC27-CD28ΔN-Glyco). (B) Non-N-glycosylated CD28 stimulates a greater costimulatory signaling in anti-CD28-stimulated transfectant cell. DC27 cells transfected with plasmids expressing different forms of human CD28 and were further transfected with the IL-2 luciferase reporter plasmid, and then stimulated with PMA + Con A. The activated DC27 transfectants were then stimulated with anti-CD28 for an additional 24 h. Cell extracts were examined for luciferase activity. Open columns indicate cells stimulated with PMA + Con A. Filled columns indicate cells stimulated with PMA + Con A + anti-CD28. *$p < 0.05$ (PMA + Con A + anti-CD28-stimulated DC27-CD28 vs PMA + Con A + anti-CD28-stimulated DC27-CD28ΔN-Glyco).

N-glycosylation of CD28 attenuates CD80 binding affinity and also causes reduced cytoplasmic signaling.

Hypo-N-glycosylation of CD28i does not alter CD28 signaling

Recently, we reported that CD28 signaling is amplified by the association of a splice variant of CD28, CD28i, in human T cells [10]. Since 40% of the molecular mass of CD28i is glycosylated we tested whether the function of CD28i is altered by hypo-N-glycosylation. We mutated the two potential N-glycosylation sites in CD28i and created DC27-CD28wt cell lines expressing

either the wildtype CD28i or the CD28iΔN-Glyco mutant forms (Fig. 2A). When CD28i or CD28iΔN-Glyco was expressed in the CD28wt transfectant cell line, we found that the presence of CD28i or CD28iΔN-Glyco did not significantly alter the binding of the transfectants to CD80–CHO (Fig. 4A). The data suggest that the adhesion of CD28 expressing cells to CD80 expressing cells is not affected by the glycosylation state of CD28i. It was also found that both wildtype CD28i and non-N-glycosylated CD28i equally increase CD80-stimulated IL-2 promoter activity in CD28 expressing cells (Fig. 4B). The data indicate that the N-glycosylation state of CD28i does not play a significant role in CD28i-mediated amplification of CD28 signaling.

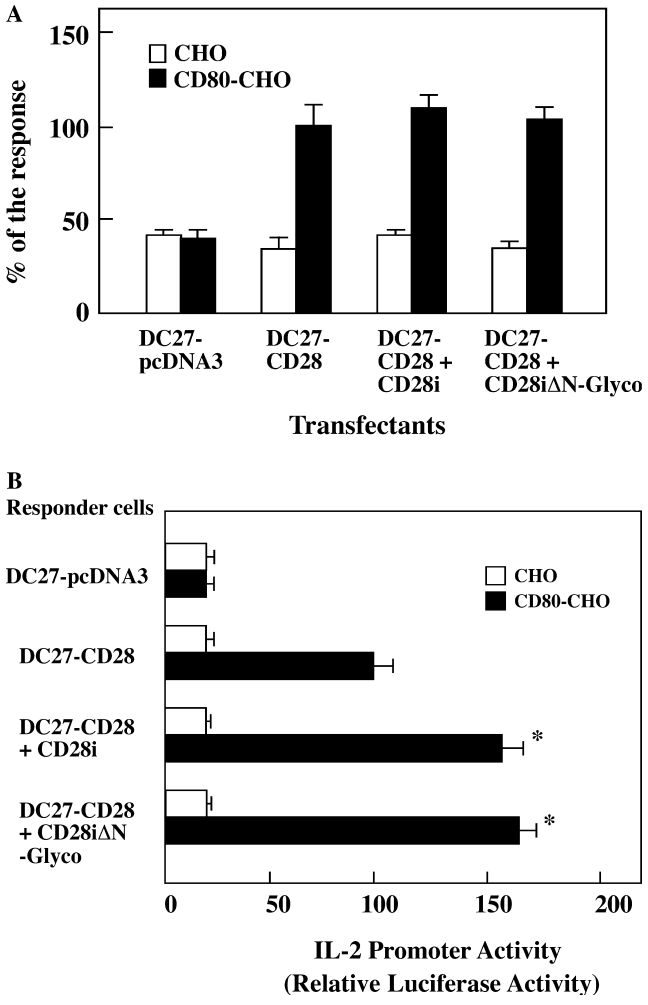

Fig. 4. Characterization of non-N-glycosylated CD28i expressed in CD28-transfected DC27 cells. (A) Adhesion of CD28-CD28i or CD28-CD28iΔN-Glyco transfectants to CD80–CHO cells. The CD28 expressing DC27 were transfected with CD28i or CD28iΔN-Glyco expression constructs. The adhesion to CD80–CHO cells was measured as in Fig. 1C. Open columns indicate adhesion interactions with CHO cells. Filled columns indicate adhesion interactions with CD80–CHO cells. (B) Costimulation activity induced by CD80–CHO cells. The CD28 expressing DC27 were transfected with CD28i or CD28iΔN-Glyco expression constructs. The costimulation activity induced by CD80–CHO cells was measured as described in Fig. 3A. Open columns indicate cells stimulated with CHO cells. Filled columns indicate cells stimulated with CD80–CHO cells. $*p < 0.05$ (CD80–CHO-stimulated DC27-CD28 plus CD28i or plus CD28iΔN-glyco vs CD80–CHO-stimulated DC27-CD28).

Inhibition of endogenous N-glycosylation enzymes increases Jurkat cell adhesion to CD80–CHO cells

To corroborate the physiological effect N-glycosylation has in regulating CD28 activity in T cells, we used various glycosylation inhibitors to attenuate endogenous glycosylation enzyme activities in the Jurkat cell line and measured CD28 induced adhesion and signaling.

The inhibitors 1-deoxynojirimycin (dNM), 1-deoxymannojirimycin (dMM), and Swainsonine (Swain) were utilized in blocking the biosynthesis of N-linked oligosaccharide structures in Jurkat cells. These inhibitors target different steps in the synthesis of different oligosaccharide structures (see Materials and methods). To assess the effect of these inhibitors on CD28 expression, Jurkat cells were cultured for 10 days in medium containing dNM, dMM or Swain at non-toxic doses. After 10 days of treatment we found that the level of cell surface CD28 was comparable between control cells and cells treated with different inhibitors (Fig. 5A). None of the inhibitors caused detectable cell death or growth inhibition of Jurkat cells throughout the 10 day culture (data not shown). In parallel cell cultures, HA-tagged CD28-expressing Jurkat cells were treated with the same inhibitors. HA-specific Western blotting of cell lysates from treated and untreated Jurkat cells showed that treatment with glycosylation inhibitors reduces the molecular mass of HA-tagged CD28 (Fig. 5B), indicating that inhibitor-treated cells have reduced levels of glycosylation. We next tested if inhibitor-treatment alters adhesion of Jurkat cells to CD80–CHO cells. All three inhibitor-treated Jurkat cell lines showed increased numbers of cells binding to CD80–CHO cells while the binding with wildtype CHO cells lacking CD80 expression remained unchanged (Fig. 5C). The above data support our findings with hypoglycosylated CD28ΔN-Glyco-transfected cells, and suggest that the lowered activity of endogenous N-glycosylation enzymes causes an increase in adhesion of $CD28^+$ T cells with $CD80^+$ cells.

Glycosylation inhibitors increase CD28 costimulatory signaling activity

We next investigated whether CD28 costimulatory signaling activity is altered in hypoglycosylated $CD28^+$ Jurkat cells. All three inhibitor-treated Jurkat cell cultures showed significant increases in IL-2 promoter activity over control cells when stimulated with CD80–CHO cells (Fig. 6A) or PMA and PHA and anti-CD28 (Fig. 6B). These results in total indicate that glycosylation plays a critical role in modulating CD28 activity.

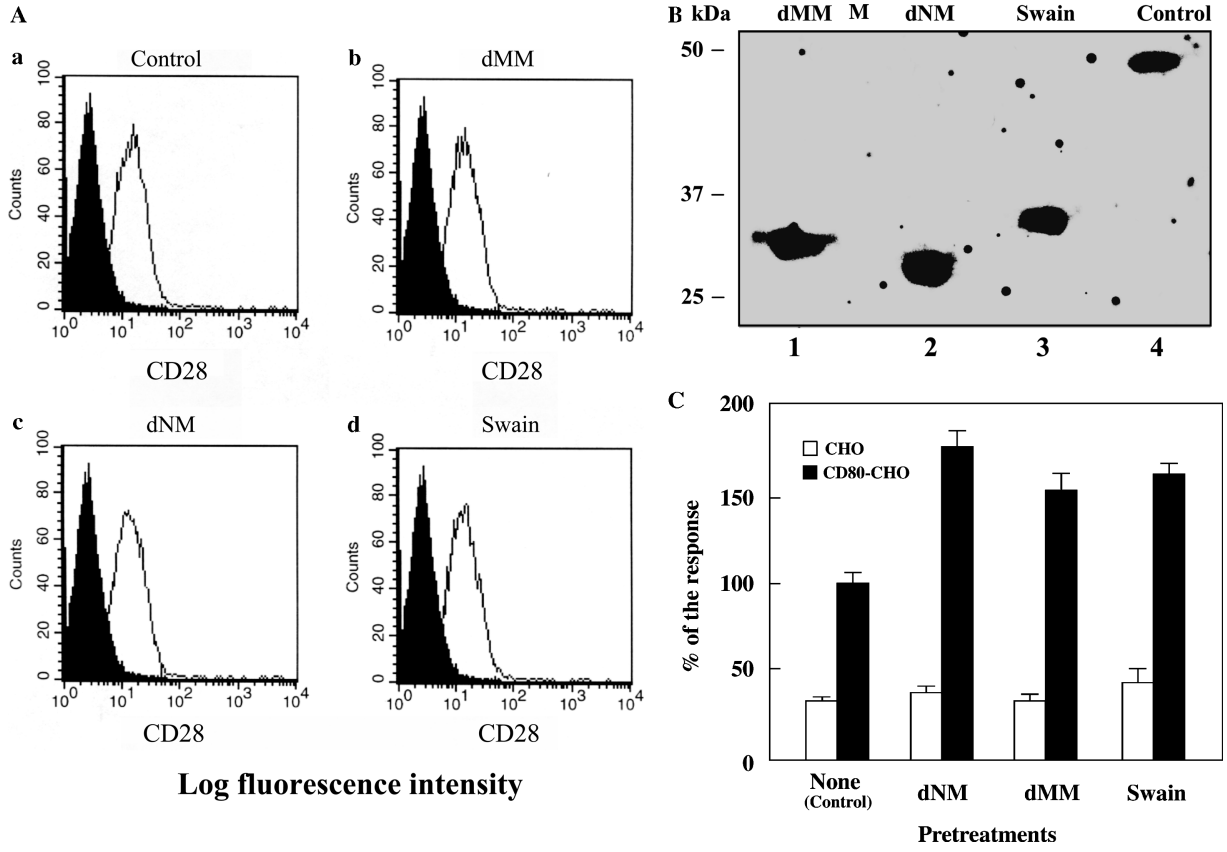

Fig. 5. Characterization of hypoglycosylated CD28 expressed on Jurkat cells. (A) Flow cytometric analysis of CD28 expression on Jurkat cells treated with N-glycosylation inhibitors. Jurkat cells treated with different N-glycosylation inhibitors were assessed for the expression of CD28. Shaded peaks represent unstained control, and unshaded peaks represent positive staining of CD28. Jurkat-Control, non-treated Jurkat cells; Jurkat-dMM, Jurkat cells were treated with dMM; Jurkat-dNM, Jurkat cells were treated with dNM; and Jurkat-Swain, Jurkat cells were treated with Swain. (B) Immunoblotting of CD28 expressed in Jurkat cells treated with N-glycosylation inhibitors. HA-tagged CD28-transfected Jurkat cells were treated with inhibitors (shown on the top of each lane) and whole cell lysates were run on SDS–PAGE and Western blotting performed using HRP-anti-HA. (C) Hypoglycosylation of Jurkat cells increases adhesion to CD80–CHO cells. Cell–cell adhesion interaction assays were performed as described in Fig. 2C. The means ± SD of three independent experiments are presented. Open columns indicate cells stimulated with CHO cells. Filled columns indicate cells stimulated with CD80–CHO cells.

Discussion

The glycosylation modulation of nascent proteins in the ER–Golgi system is an important mechanism for protein maturation and sorting to target cell organelles [2]. Furthermore, N-glycosylation of nascent proteins is essential for appropriate protein folding and oligomerization. Glycans also exert various influences on the functions of mature proteins to which they are attached [16]. A major function of glycans is to contribute to the stability of proteins by protecting them from proteases [2]. Moreover, the ligand binding structures of some receptors are dependent on glycans attached to the receptor structure [2,17–20]. It is also known that glycans regulate the alignment of the receptors within the immunological synapse, and the specific sugar modulation of receptors enhances ligand binding [1,4–6,17]. In addition to the above-mentioned functions of glycans, we have found that N-glycosylation of CD28 reduces CD28/CD80 interactions. The hypoglycosylated form of CD28 was also found to stimulate an increased costimulatory signaling activity in T cells. Our data indicate that N-glycosylation of CD28 negatively regulates the adhesion with $CD80^{+}$APCs and the costimulation of T cells.

In support of our observations on the negative role glycosylation plays in CD28/CD80 interactions, a previous study by van der Merwe showed that mutation of the potential N-glycosylation sites in CD80 resulted in increased binding of CD80 to CD28 through increased affinity [19].

A critical role for glycosylation in regulating immune functions was demonstrated in β1,6-*N*-acetylglucosaminyltransferase V deficient mice. These mice showed that T cells had hypoglycosylated TCRs and clustered more compactly upon TCR crosslinking [3]. The decreased volume of *N*-glycan attached to TCR complex may cause less hindrance to the clustering of receptors. These β1,6-*N*-acetylglucosaminyltransferase V deficient mice were also found to be prone to autoimmune diseases, indicating that glycosylation is critical to modulating

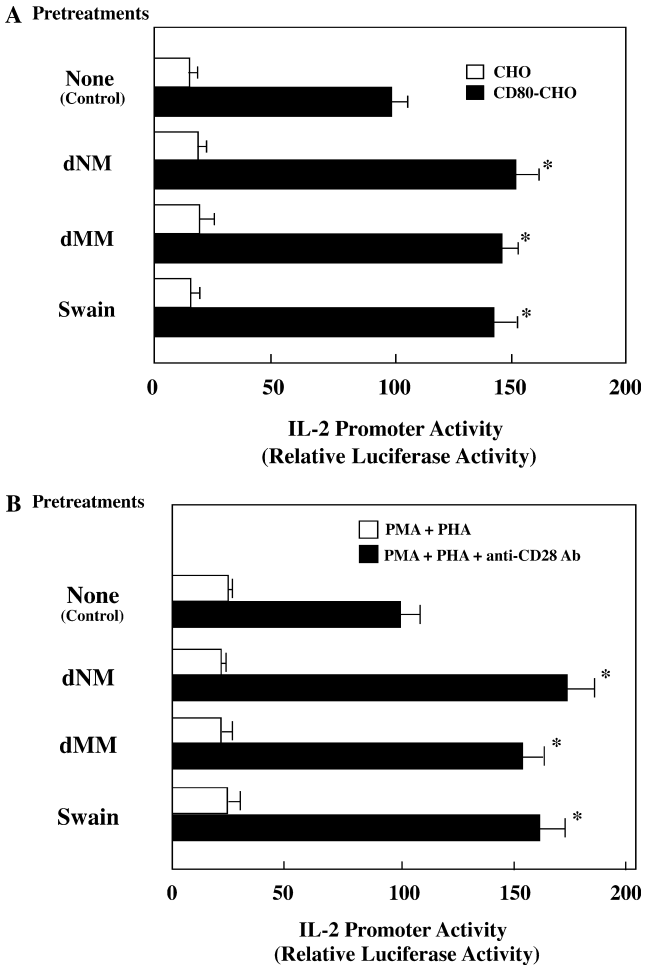

Fig. 6. Costimulatory signaling by hypoglycosylated CD28 expressed on Jurkat cells. (A) Hypoglycosylation of Jurkat cells increases CD28 costimulatory signaling activity in Jurkat cells upon CD80 ligation. IL-2 reporter plasmid was transfected into the inhibitor-treated Jurkat cells and then stimulated with PHA. The PHA-activated cells were also cocultured with CD80–CHO for 24 h. Cell extracts were examined for IL-2 promoter activity by luciferase assay. Open columns indicate cells stimulated CHO. Filled columns indicate cells stimulated with CD80–CHO. $*p < 0.05$ (CD80–CHO-stimulated inhibitor-treated Jurkat cells vs CD80–CHO-stimulated control Jurkat cells). (B) Hypoglycosylated CD28 stimulates a greater costimulatory signaling in Jurkat cells with anti-CD28 ligation. IL-2 reporter plasmid was transfected into inhibitor-treated Jurkat cells and then stimulated with PMA + PHA. The activated cells were then stimulated with anti-CD28 for an additional 24 h. Cell extracts were examined for luciferase activity. $*p < 0.05$ (PMA + PHA + anti-CD28-stimulated inhibitor-treated Jurkat cells vs PMA + PHA + anti-CD28-stimulated control Jurkat cells).

immune responses. It will be interesting to examine whether CD28 and CD80/CD86 adhesion are enhanced in these mice and play a causative role in increased immune responses and the development of autoimmune diseases. The regulation of glycosylation of immune receptors, especially CD28 and CD80, may be an important underlying mechanism for fine-tuning immune receptor/immune ligand interactions. This in turn may also influence the robustness of an immune response. Interestingly, aberrant protein glycosylation is frequently observed in various autoimmune diseases [20]. If glycosylation represents another level of regulation of immune responses, then therapeutic intervention using glycosylation modulators may prove to be a novel intervention strategy in regulating autoimmune and immunodeficient diseases.

Acknowledgments

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